

Initiative for Quality Assurance of Malaria Rapid Diagnostic Tests

*Outline of
product testing
and associated protocols*

World Health Organization (WHO) – Regional Office for the Western Pacific

UNICEF / UNDP / World Bank / WHO Special Programme for Research and Training
in Tropical Disease (TDR)

Foundation for New Innovative Diagnostics (FIND)



Aims and Scope

This document provides an overview of the product testing scheme for antigen-detecting malaria rapid diagnostic tests under the WHO – FIND Malaria RDT Evaluation Programme. It describes the composition, characterization and management of the specimen bank, the protocols for product testing and use of product testing results, as well as the evidence and methods on which the specimen bank and protocols are based. The evaluation described provides performance data against a panel designed to mimic natural infections as closely as possible while fulfilling the necessity for standardization of testing. It is not, therefore, intended to fully replace field testing of RDTs.

This paper aims to provide manufacturers and procurers of malaria RDTs with information to guide submission of products to the programme, and to guide interpretation of results.

Acknowledgements

The malaria RDT evaluation programme is a partnership of the World Health Organization - Regional Office for the Western Pacific (WHO/WPRO), the Foundation for Innovative New Diagnostics (FIND) and the UNICEF / UNDP / World Bank / WHO Special Programme for Research and Training in Tropical Disease (TDR).

The specimen bank collection and characterization is performed by RITM, Philippines; CNM, Cambodia; Inst. Pasteur, Cambodia; DMR, Myanmar; University of Lagos, Nigeria; Inst. Pasteur, Central African Republic; IHRDC, Tanzania; KEMRI Kisumu, Kenya; Inst. Pasteur, Madagascar; CIDEIM, Colombia; IMT, Peru; US CDC, Atlanta, USA; Hosp. Trop. Disease, UK; AMI/QIMR, Australia, NBI, South Africa;.

The WHO and FIND acknowledge the contribution in time and expertise of the above institutions, MSF Holland, and the technical advisers of the WHO informal consultations on malaria RDTs at Manila 2003, Manila 2004, Geneva 2006, Kisumu 2006, Atlanta 2006 and Philadelphia 2007 in protocol development and oversight of the project.

Funding has been provided predominantly by The Bill and Melinda Gates Foundation (through FIND), AusAID (through WHO/WPRO), and WHO/TDR. This document was compiled by Sandra Incardona-Mazerand, under contract to FIND.

The designations employed and the presentation of the material in this publication do not imply the expression of any opinion whatsoever on the part of the World Health Organization concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of the frontiers or boundaries. The mention of specific companies or of certain manufacturers' products does not imply that they are endorsed or recommended by the World Health Organization in preference to others of a similar nature that are not mentioned. Errors and omissions excepted, the names of proprietary products are distinguished by initial capital letters.

The World Health Organization does not warrant that the information contained in this publication is complete and correct and shall not be liable for any damages incurred as a result of its use.

	0
<hr/>	
<u>I. INTRODUCTION.....</u>	5
<u>II. PRODUCT TESTING OF RAPID DIAGNOSTIC TESTS.....</u>	8
A. TESTING SITE.....	8
B. CONTENTS OF THE SPECIMEN BANK.....	8
1. SUB-PANELS OF THE SPECIMEN BANK.....	8
2. TECHNICAL ISSUES AND CHOICES.....	9
C. OUTLINE OF THE PRODUCT TESTING PROTOCOL.....	10
1. 'PHASE 1' PANEL TEST.....	11
2. 'PHASE 2' PANEL TEST.....	11
3. STABILITY TESTING.....	12
4. EASE OF USE ASSESSMENT.....	12
5. REVIEW AND PUBLICATION OF RESULTS.....	12
D. QUALITY ASSURANCE OF THE RDT PRODUCT TESTING.....	13
<u>III. SPECIMEN BANK CONTENTS AND SAMPLES PREPARATION.....</u>	18
A. SUB-PANEL 1: RECOMBINANT ANTIGENS.....	18
1. CONTENTS.....	18
2. SAMPLES PREPARATION.....	19
B. SUB-PANEL 2: P. FALCIPARUM CULTURE LINES.....	19
1. CONTENTS.....	19
C. SUB-PANEL 3: WILD PARASITE SAMPLES.....	20
1. CONTENTS.....	20
2. COLLECTION SITES AND LABORATORIES.....	21
3. SAMPLE PREPARATION.....	22
4. QUALITY ASSURANCE OF THE SAMPLE PREPARATION.....	23
5. CHARACTERIZATION OF THE SAMPLES.....	24
D. SUB-PANEL 4: NEGATIVE CONTROL SAMPLES.....	27
1. CONTENTS.....	27
2. SAMPLES PREPARATION.....	28
E. MAINTENANCE OF THE SPECIMEN BANK.....	28
<u>IV. MANAGEMENT OF THE SPECIMEN BANK AND RDT PRODUCT TESTING.....</u>	30
A. THE MALARIA DIAGNOSTICS SPECIMEN BANK AND EVALUATION STEERING GROUP.....	30
B. OUTLINE OF MANAGEMENT POLICIES.....	30
1. SPECIMEN BANK MANAGEMENT AND POLICY OF ACCESS.....	30
2. MANAGEMENT, RULES AND DEFINITIONS OF THE PRODUCT TESTING.....	31
<u>V. EVIDENCE AND METHODS FOR RDT PRODUCT TESTING.....</u>	33
A. ANTIGEN CONTENTS.....	33
1. QUANTIFICATION OF ANTIGENS BY ELISA.....	33
2. RELATIONSHIP BETWEEN ANTIGEN CONTENT AND PARASITE DENSITY.....	33

3.	STUDY-BASED CHOICES FOR THE SPECIMEN BANK	34
B.	DNA EXTRACTION AND MOLECULAR DIAGNOSIS OF PARASITE SPECIES.....	35
C.	ANTIGEN VARIATION.....	35
1.	<i>PLASMODIUM</i> LDH AND ALDOLASE VARIATION	35
2.	<i>P. FALCIPARUM</i> HRP2 VARIATION AND ITS IMPACT ON RDT RESULTS.....	36
3.	STUDY-BASED CHOICES FOR THE SPECIMEN BANK	37

List of abbreviations

ACT	Artemisinin-based combination therapy
AMI	Army Malaria Institute (Queensland, Australia)
ANA	Anti-nuclear antibody
BET	Ethidium Bromide
CDC	Centers for Disease Control and Prevention (Atlanta, United States of America)
CIDEIM	Centro Internacional de Entrenamiento y Investigaciones Médicas (Cali, Colombia)
CNM	National Center for Parasitology, Entomology and Malaria Control (Phnom Penh, Cambodia)
DMR	Experimental Medicine Research Division (Department of Medical Research, Yangon, Myanmar)
DNA	Desoxyribonucleic Acid
EDTA	Ethylene Diamine Tetraacetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
FIND	Foundation for Innovative New Diagnostics
GMP	Good Manufacturing Practices
HAMA	Human Anti-mouse Antibody
HIV	Human Immunodeficiency Virus
HRP2	Histidine-rich Protein 2
HTD	Hospital for Tropical Diseases (London, United Kingdom of Great Britain and Ireland)
ID	Identification number
IHRDC	Ifakara Health Research and Development Center (Bagamoyo, Tanzania)
IMT	Instituto de Medicina Tropical (Universidad Peruana Cayetano Heredia, Lima, Peru)
IPB	Institut Pasteur de Bangui (Bangui, Central African Republic)
IPC	Institut Pasteur du Cambodge (Phnom Penh, Cambodia)
IPM	Institut Pasteur de Madagascar (Antananarivo, Madagascar)
KEMRI	Kenya Medical Research Institute (Kisumu, Kenya)
LDH	Lactate Dehydrogenase
MABs	Monoclonal Antibodies
MB	Molecular Biology
NBI	National Bioproducts Institute (xxx, South Africa)
Non-Pf	'Non <i>Plasmodium falciparum</i> species' (<i>P. vivax</i> , <i>P. malariae</i> , <i>P. ovale</i>)
Pan	<i>Plasmodium</i>
PCR	Polymerase Chain Reaction
Pf	<i>Plasmodium falciparum</i>
Pm	<i>Plasmodium malariae</i>
Po	<i>Plasmodium ovale</i>
Pv	<i>Plasmodium vivax</i>
QA	Quality Assurance
QC	Quality Control
RDT	Rapid Diagnostic Test
RITM	Research Institute of Tropical Medicine (Manila, Philippines)
RPR	Rapid Plasma Reagin
SD	Standard Diagnostics (Seoul, South Korea)
SOP	Standard Operating Procedure
TDR	UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases
UL	University of Lagos (Lagos, Nigeria)
WHO	World Health Organization
WPRO	Western Pacific Regional Office of the World Health Organization

I. Introduction

Malaria rapid diagnostic tests (RDTs) are filling a valuable and growing role in providing parasite-based diagnosis of malaria in areas where good quality microscopy cannot be sustained. In this role, they can bring great improvements in the early recognition and management of both malarial and non-malarial fever, through demonstration (or exclusion) of antigens specific for malaria parasites in host (patient) blood. Parasite-based 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. The term 'RDT' in this document refers specifically to antigen-detecting lateral flow (immunochromatographic) tests.

While RDT-based diagnosis is applicable in a range of management conditions, the predominant role is in malaria diagnosis away from major health centres in tropical and sub-tropical (malaria-endemic) areas. In these areas, transport, storage, re-supply, and supervision are difficult and often limited. In such conditions, health workers must consistently provide an accurate diagnosis to guide the management of malarial and non-malarial fevers that may be rapidly fatal. Therefore, it is essential that malaria RDTs are sensitive and specific, simple to use, and stable in ambient conditions of storage and transport, retaining their accuracy for long periods.[1]

Manufacturers of malaria RDTs are faced with a number of challenges in producing high quality products. Among these has been a lack of established standards for sensitivity, specificity, and stability, and a lack of access to good quality reference material for use in assessing compliance with such standards (an unpublished World Health Organization (WHO) review of 26 manufacturer stability test protocols shows a high variability in both protocols and reference standards). National regulatory authorities in endemic countries face similar problems in designing and implementing appropriate regulatory standards to ensure the tests used in national programmes are of appropriate quality. Procuring agencies require high quality comparative performance data to determine appropriate products for the intended area of use.

The published literature contains evidence of highly variable sensitivity of RDTs in field use (reviewed in [2-6]). While this may partly reflect study design, it is clear that significant problems arise with the accuracy of malaria RDTs in the hands of their intended users, and this is supported by unpublished data from national programmes. Deficiencies in manufacture are likely to account for some shortfalls in quality, but variability in thermal stability,[7] variability of the target antigen (Pf HRP2),[8, 9] and technical requirements for RDT preparation and interpretation also play a role in reducing diagnostic accuracy.

The WHO, in cooperation with a number of research institutions (Annex 1), has been developing standardized methods to test RDT performance, guided by a number of technical consultations since 2002. This programme is currently overseen by the WHO-Regional Office for the Western Pacific (WHO/WPRO) and the UNICEF / UNDP / World Bank / WHO Special Programme for Research and Training in Tropical Disease (TDR) in partnership with the Foundation for Innovative New Diagnostics (FIND), and aims to ensure and demonstrate RDT quality relevant to their predominant area of use.[2, 10] This programme is developing three tiers of quality control (Figure I-1):

- Product testing (comparative performance data of available products) and provision of reference standards for developers and manufacturers
- Lot-testing (testing of product conformity to expected standards at time of purchase)
- Testing at the level of the end-user (to demonstrate accuracy to both health workers and patients).

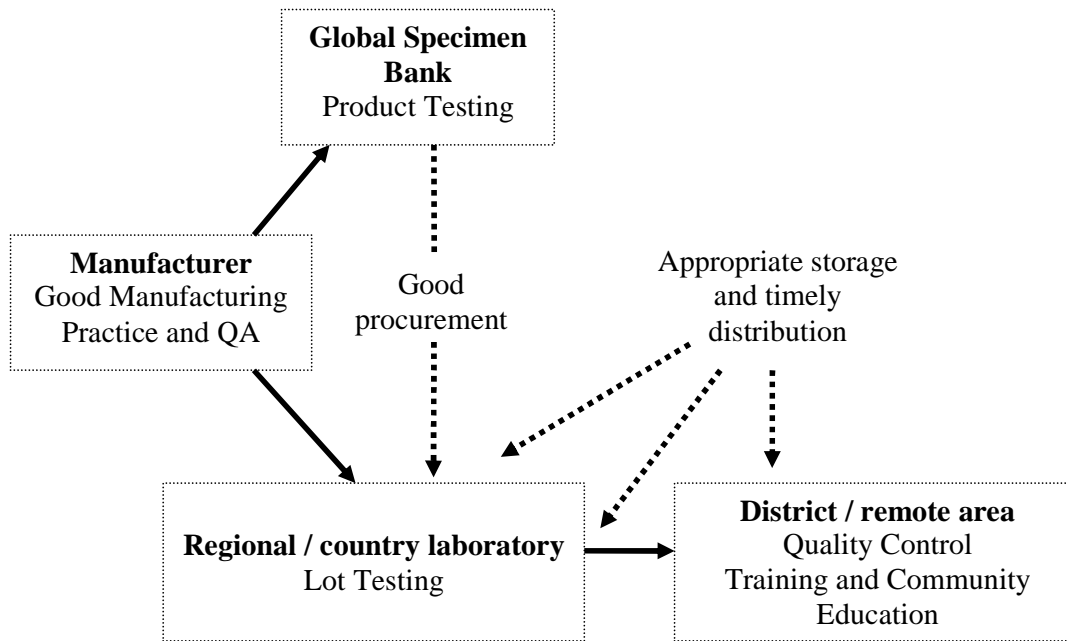


Figure I-1: Schematic representation of the Malaria RDT quality assurance programme

While there are other essential elements to RDT use, including appropriate training and instructions (job-aids),[11, 12] and the development and implementation of appropriate algorithms for the use of positive and negative results,[13] providing demonstrably good quality RDTs is a precondition to addressing these other aspects of use.

RDT-lot testing for control programmes and manufacturers has been conducted through this programme since 2003 at the Research Institute for Tropical Medicine (RITM) in the Philippines and the Institute Pasteur Cambodge (IPC) and National Malaria Control Centre (CNM) in Cambodia, with support from the Hospital for Tropical Disease (HTD), London, UK, the Centres for Disease Control and prevention (CDC), Atlanta, USA, and the Army Malaria Institute (AMI), Brisbane, Australia.[14, 15] This network is now expanding to include other institutions in Asia, Africa and South America. Development of improved positive control wells suitable for use in remote clinics has been proceeding through a partnership of these institutions and the National Bioproducts Institute in South Africa.[16]

This document describes the first tier of the RDT evaluation programme, Product Testing, and the specimen bank that will support this process. It provides an overview of the protocols used for product testing, composition and management of the specimen bank and of the product testing results, and the evidence and methods on which the specimen bank and protocols are based. The performance data derived from product testing will guide WHO procurement as

well as procurement recommendations to member states, and form the performance data set for future WHO prequalification of malaria RDTs. This will complement the current WHO requirement for certification of conformity with ISO13485:2003, and for provision of a heat stability protocol and specific product information.

II. Product testing of Rapid Diagnostic Tests

The RDT product testing consists in a laboratory-based assessment of RDTs in terms of sensitivity, specificity, stability and ease of use, against a large specimen panel of well-characterized antigen and parasite samples, using standardized protocols, controlled manipulations and a unique testing location.

The composition of the product testing panel and the testing procedures are designed to ensure that parameters critical to RDT performance remain essentially constant while individual samples in the bank are replaced as it becomes depleted. The results of product testing must be interpreted in light of this, and the assessment is not intended to completely replace field trials in real conditions of use.[3]

A. Testing Site

The rapid diagnostic tests will be evaluated at the Malaria Branch, Division of Parasitic Diseases at the Centers of Disease Control and Prevention (CDC, Atlanta, USA), one of the major operating components of the Department of Health and Human Services (HHS) of the United States of America. This laboratory houses the global specimen bank against which performance is assessed. Part of the specimen bank samples are prepared at the CDC itself, others are shipped from distant production facilities and/or collection laboratories to the CDC for characterization and inclusion in the specimen bank.

B. Contents of the specimen bank

1. Sub-panels of the specimen bank

The specimen bank is intended to be a repository for a comprehensive global reference standard against which the performance of malaria rapid diagnostic tests can be assessed. To supply a reference for tests that detect the targeted *Plasmodium* antigens, the bank must:

- Provide a range of clinically-relevant antigen concentrations (detection sensitivity)
- Provide a representative variety of antigens, both in terms of polymorphism of the antigen itself (known antigen variants), and in terms of geographic origin of the parasites from which these antigens are derived
- Provide a clinically-relevant standard for stability testing
- Provide a panel of parasite-negative samples that include probable causes of false-positive results and likely differential diagnoses of malarial fever

To achieve these aims, and to allow comparisons between large numbers of RDTs and between RDTs over time with well-characterized and stable testing parameters, the specimen bank has been designed to include:

- Serial dilutions of recombinant antigens, at precise concentrations, for which the equivalence to parasite densities has previously been studied (sub-panel 1)
- Culture-derived parasite samples, diluted to precise parasite densities (sub-panel 2)
- Wild parasite samples derived from infected hosts and diluted to the same precise parasite densities (sub-panel 3)

- Negative control samples, partly with blood factors possibly causing false-positive results, partly corresponding to so-called “clean negative samples” (sub-panel 4)

The contents of each sub-panel and the sample preparation protocols are described in detail in Chapter III and Annex 2.

2. Technical issues and choices

Variations from malaria RDT testing in field trials

While RDTs are designed to test fresh human blood, the requirement for a large and stable panel necessitates the use of cryo-preserved samples. These are prepared in conditions designed to minimize loss of antigen content, and to provide a sample that mimics fresh blood infected with naturally-occurring parasites as much as possible, while fulfilling the requirement for standardized and repeatable testing.[14] However, some differences may occur, both through the effects of preparation and storage on the target antigens, and on the effect of freeze-thaw induced lysis of cells on flow on the RDT. As RDTs lyse cells in fresh blood as an initial step, lysis of the frozen sample is of limited significance, and is essential for standardized internal quality assurance in the manufacturing process.[7]

A further variation from field equivalence is the use of a micro-pipette to supply blood to the RDT device rather than the blood transfer device provided by the manufacturer. This is necessary as blood is obtained from a cryo-tube rather than a finger-prick. It also ensures consistency of testing by reducing the likelihood of operator error (page 13).

Predominance of *P. falciparum* in the parasite sample panels

The four major human malaria species are represented in the specimen bank, with a predominance of *P. falciparum* reflecting the higher importance of this species in terms of disease severity and case numbers. Furthermore, *P. falciparum* is expected to present higher variations between parasite samples in antigen content, due to parasite sequestration and structural variation of the *P. falciparum* specific antigen HRP2 (Page 33).

Antigen levels and parasite densities used for malaria RDT testing

For RDT product testing with recombinant antigens, relevant antigen concentration ranges have been selected on the basis of previous studies of the relationship between the parasite density and the antigen content in the blood. The purpose is to allow comparison of the RDTs lower detection limits, usually situated around 100 parasites per microlitre of blood [17, 18]

Culture-derived and wild parasite samples are diluted to well-calibrated parasite densities, chosen according to the following rationales:

- (i) High parasite density = 2000 or 5000 parasite// μ L = clinically relevant parasite density which is significantly above the density at which RDT sensitivity variations have been reported. Specimen bank samples at this dilution level are expected to produce strongly positive RDT results, and intend to reveal major dysfunctions of malaria RDTs.

- (ii) Low parasite density = 200 parasite// μL = a parasite density close to the lower limit of clinical-relevance,¹ and close to the limit of sensitivity of RDTs reported in field evaluations and of standard field microscopy. Specimen bank samples at this dilution level are expected to be detected, possibly with a faint RDT result, and are likely to detect clinically-significant product and lot-to-lot variations.
- (iii) Medium parasite density = 500 parasite// μL = intermediate parasite density prepared for non-*P. falciparum* species only (*P. vivax*, *P. malariae* and *P. ovale*), for which detection of very low parasite densities is not clinically so critical. Furthermore, various malaria RDTs have been reported to be less sensitive for detection of non-*P. falciparum* species, compared with *P. falciparum*. [4, 19, 20] Specimen bank samples at this dilution level should be detected even when the 200 parasite// μL samples produce negative RDT results.

C. Outline of the product testing protocol

The general principle of the testing algorithm is shown in

Figure II-1. The RDT manufacturer must:

- Provide evidence of good manufacturing practice in the form of accreditation of full conformity with ISO13485:2003 (quality manufacturing of medical devices). The close US FDA equivalent US FDA 21 CFR part 820 can be used if a clear indication of full compliance is provided by the US FDA,
- Undertake an in-house real-time stability test and provide results for publication,
- Agree to publication of all performance data once the product is submitted to the programme.

The manufacturer is provided with a short panel of calibrated parasite samples from five *P. falciparum* culture lines at high and low parasite densities (high: 2000 or 5000 parasites/ μL , low: 200 parasites/ μL). This ‘manufacturer panel’ is a subset of the sub-panel 2 of the Malaria Specimen Bank, described into more detail in Chapter III and Annex 2. It allows quality control testing at the manufacturing site prior to product submission, and one of the culture lines is later used for stability testing by the manufacturer. Manufacturers will be required to cover courier costs for delivery of these panel samples.

The testing procedure is performed on two different lots of RDTs provided by the manufacturer. The testing algorithm includes four main steps:

- Phase 1 challenge against recombinant antigens and culture-derived parasites,
- Phase 2 challenge against samples derived from wild parasites and the parasite-negative panel,

¹ NOTE ON USE OF 200 parasites/ μL AS LOWER LIMIT FOR RDT EVALUATION: The WHO informal consultations in 1999 and 2003 recommended that RDTs should detect 100 parasites/ μL with 95% sensitivity.[2] This is considered a parasite density likely to be indicative of current or impending significant malarial illness in a host with low immunity, and consistent with the lower limit of good quality field microscopy. The relevance of detection of parasites below this density is controversial. The RDT evaluation described here does not indicate a departure from that standard. The variation in the relationship between parasite density and antigen concentration (Page 33), the inherent inaccuracy of microscopy due to chance variation, human error and parasite loss from thick blood films, and the potential for antigen loss during harvesting and cryo-preservation, necessitate the use of a higher parasite density for the product testing.

- Heat thermal stability test (at CDC, and at the site of manufacture),
- A descriptive Ease-of-use assessment.

A product must perform satisfactorily against the Phase 1 panel to proceed to further evaluation. This performance will be determined by the specimen bank steering group and communicated to manufacturers before product submission.

1. 'Phase 1' Panel Test

A first screening step allows the selection of RDT products meeting certain minimal quality requirements. This phase 1 panel test uses samples from sub-panels 2 and 3, for which critical characteristics and production conditions remain unchanged over the years (page 18). RDTs are tested against serial dilutions of recombinant antigens and well-characterized culture-derived *P. falciparum* samples at high and low parasite densities. Only those RDTs with adequate performance will be included in further testing. The minimal requirements for further testing are less stringent, to prevent exclusion of products that may perform adequately on wild parasite samples but not in culture-derived parasite samples: positive RDT results must be obtained with at least one recombinant antigens at the concentration equivalent to 200 parasites/ μ L, or with a minimal proportion of samples at high parasite density (90% detected samples at 2000 or 5000 parasites/ μ L) and at low parasite density (50% detected samples at 200 parasites/ μ L).

2. 'Phase 2' Panel Test

Performance of both RDT lots is further assessed against a panel with a larger number of wild parasite samples from the blood of infected hosts, and with negative control samples (*Figure II-2*). This phase 2 panel test is based on the sub-panel 3, intended to better reflect the variety of parasites originating from different endemic areas of the world, and sub-panel 4 for evaluation of the RDTs specificity using a carefully chosen series of parasite-negative samples.

Sub-panel 3 comprises 100 *P. falciparum*, 20 *P. vivax*, 5 *P. malariae* and 5 *P. ovale* isolates. Due to the difficulty in obtaining mono-species infections of non-*P. falciparum* parasites, *P. vivax*, *P. ovale* and *P. malariae* from non-human primates may be substituted. *P. falciparum* samples are included at low (200 parasites/ μ L) and high (2000 or 5000 parasites/ μ L) parasite densities, and additional 500 parasites/ μ L dilutions are included for non-*P. falciparum* samples. The rationales for deciding the sample numbers and dilution levels for the four species have been previously described (page 9). For blinded reading by technicians, low parasite density samples and *Plasmodium*-negative samples are re-labeled and batches combined before testing. A summary of such working rules ensuring the quality of the whole testing process is given later (page 13).

The sub-panel 4 consists of “clean negative” samples from the blood of healthy individuals, and samples containing blood factors or components known to be associated with RDT cross-reactions, and likely differential diagnoses of clinical malaria.

The results of the phase 2 panel test are expressed in terms of percentage of detected samples at high and low parasite densities (sensitivity), and of true negative results obtained with the

different sets of *Plasmodium*-negative control samples (specificity). Detailed performance results against individual components of the panel will also be available.

3. Stability testing

RDTs heat stability is assessed on 2 different RDT lots against a reference *P. falciparum* culture line (Nigeria XII strain), at two parasite densities (including 200 parasites/ μ L). The choice of a reference culture line allows more standardization of stability tests over the years. The reference parasite strain originates from Africa, and expresses the Pf HRP2 structural group B with a typical antigen concentration (Page 36).

The CDCs stability test algorithm is schematically shown in *Figure II-3*. After baseline testing, the RDTs are incubated for two months at 4°C, 35°C and 45°C (at 75% humidity), and re-tested. If the results indicate a good stability of the product (more than 80% positive results with RDTs stored two months at 45°C), testing may be repeated with RDTs stored 6 months at 4°C and 45°C.

Manufacturers will be required to perform real-time testing on the same 2 RDT lots at the upper limit of their recommended storage temperature and until their specified expiry date, using Nigeria XII strain provided by the specimen bank.

4. Ease of use assessment

The ease of use assessment consists in a descriptive evaluation of the RDT product (*Figure II-4*). Assessment focuses on the following critical aspects:

- RDT format
- Total preparation time
- The necessity of time control
- Blood safety
- The quality of the instructions and the languages in which the instructions are provided
- The blood transfer device
- The items included in the RDT package

The description will be accompanied by recommendations on the appropriateness of various parameters for different conditions of use.

5. Review and publication of results

All results are reviewed by the Malaria Diagnostics Specimen Bank and Evaluation Steering Group (page 30), which may recommend repeating certain evaluation steps or performance of complementary tests if contradictory results were obtained. A performance report will be sent to the RDT manufacturer, and all results published. Results of the real-time stability assessment by the manufacturer will be published as they become available.

Data on product performance obtained through the WHO Malaria Diagnostics Evaluation Programme may be used as a reference for future procurement of RDTs by WHO, other UN agencies and national health authorities. Manufacturers will be informed of all performance

results, but these results can not be used as evidence of WHO endorsement or 'certification' of a product.

D. Quality assurance of the RDT product testing

Product testing will be conducted according to Standard Operating Procedures (SOPs) developed through prior testing experience and based on recommendations of expert consultations on this subject. A dry run using a limited number of tests and abbreviated panel has been conducted to refine these procedures. The quality of critical steps is controlled, as follows:

- **Quality of the malaria RDTs and their use:**
All RDTs are stored in a controlled environment at $\leq 25^{\circ}\text{C}$; the pouch is opened and desiccant checked immediately before use; manufacturer instructions are followed with the exception of use of the blood transfer device provided by the manufacturer (a micropipette is used to ensure correct blood volume).
A temperature-monitoring device will be included with the RDTs for shipment to the testing site.
- **Quality and objectivity of the RDT reading results:**
Three different readings are performed: two independent readings by two technicians, and a third reading with a densitometer to give a quantitative determination of intensity for weak positive reaction.
All wild parasite samples at low parasite density are first randomized with a similar number of negative control samples and re-labeled for blinded reading of the RDT results.
- **Quality of the specimen bank samples:**
SOPs have been established for the preparation of all specimen bank samples [21]. Recombinant antigens, culture lines of parasites, and wild-type samples are selected taking into account previous evidence and data from specifically conducted studies.. All diluted parasite samples are stored and transported at -70°C , and are used only once within 8 hours of thawing (REF Ag Stability Studies if available).

The detailed SOPs will be published prior to commencement of product testing.

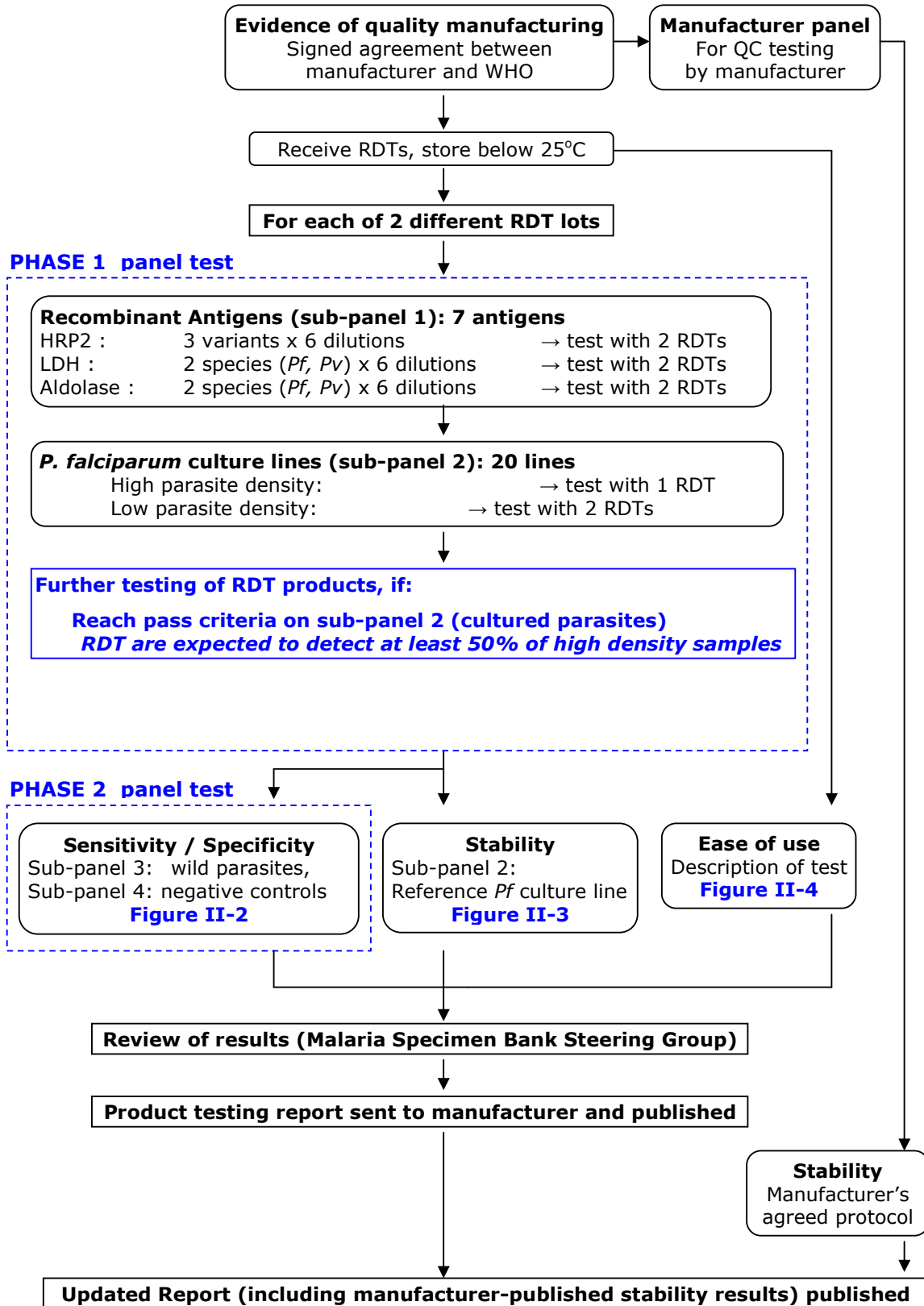
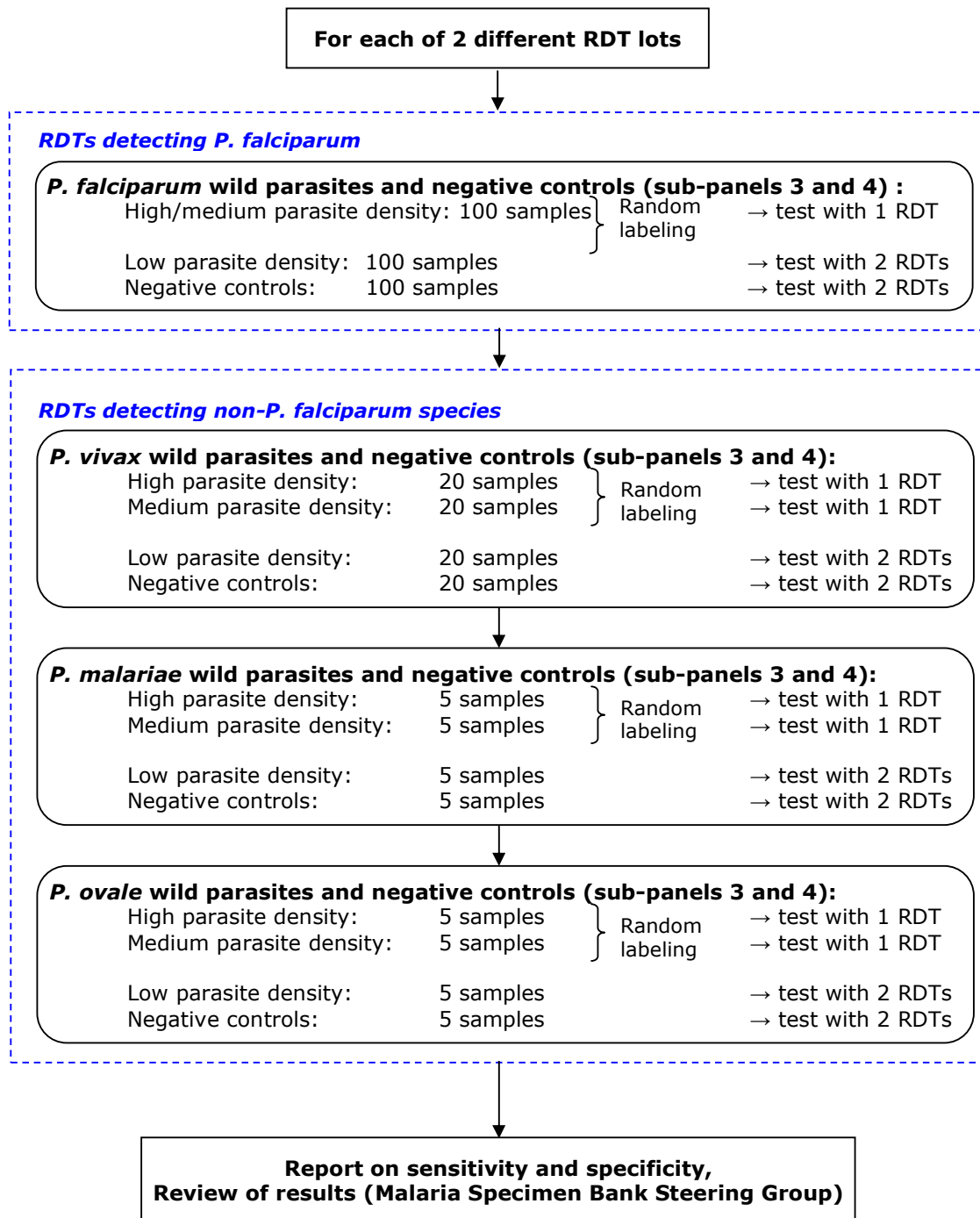


Figure II-1: Overview of RDT product testing algorithm



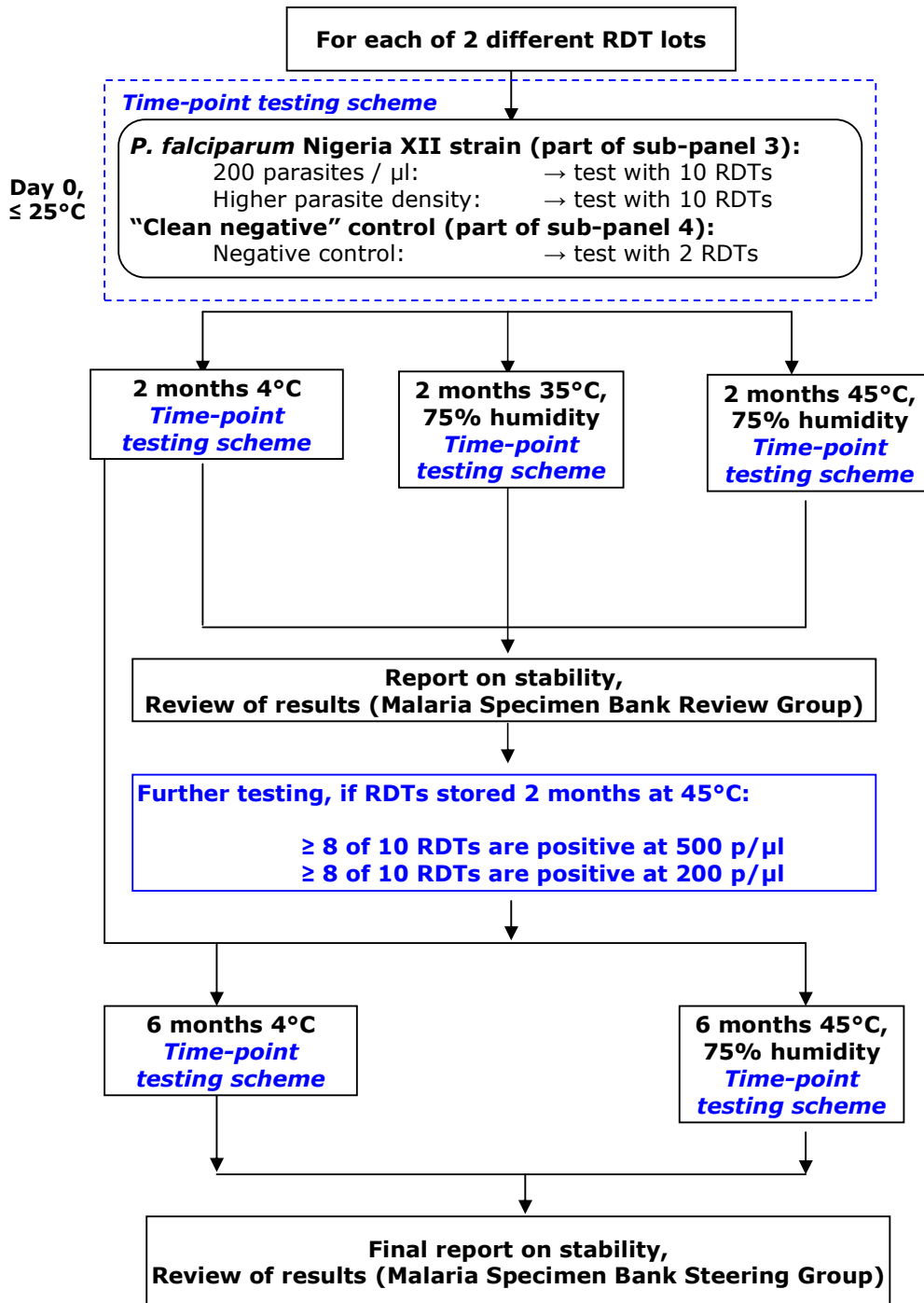


Figure II-3: Stability testing of malaria RDTs²

² An additional stability test is performed by the manufacturer using samples of the same *P. falciparum* culture line (product stability at the maximum recommended storage temperature for the recommended shelf-life).

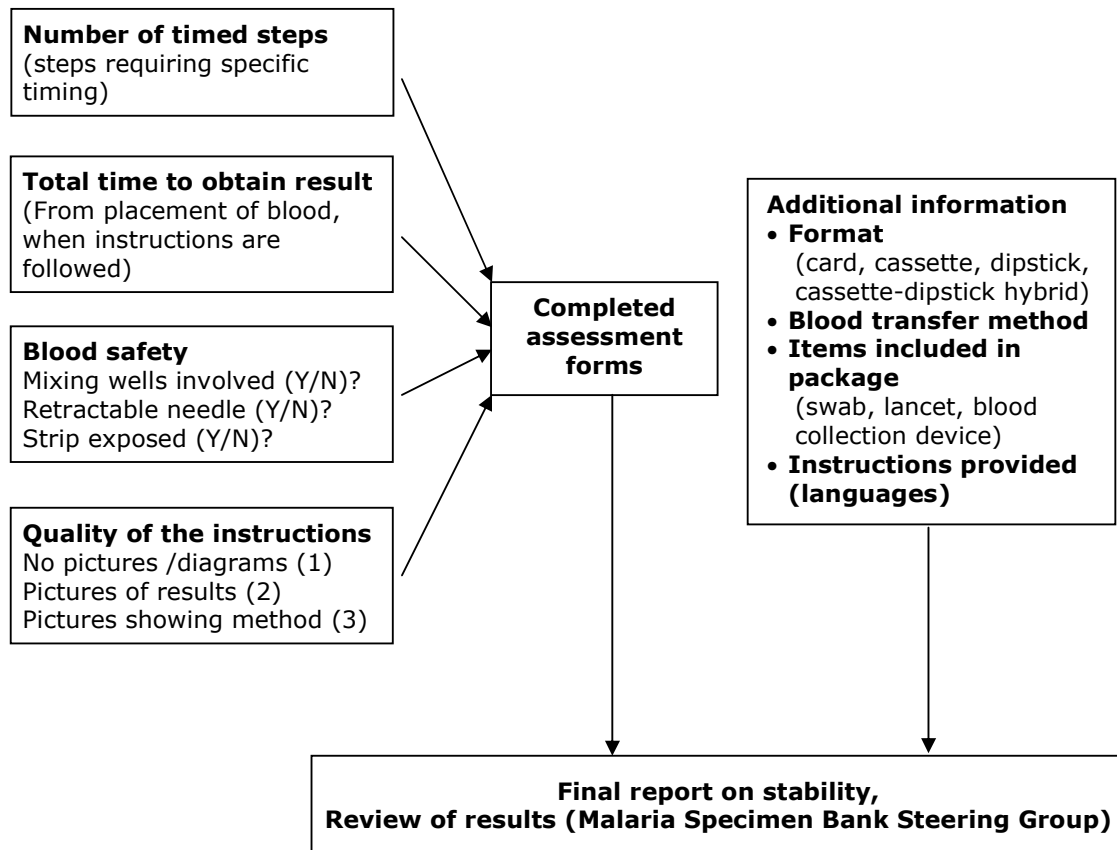


Figure II-4: 'Ease of use' testing of malaria RDTs

III. Specimen bank contents and samples preparation

The malaria specimen bank, hosted at the Malaria Branch, Division of Parasitic Diseases, (CDC, Atlanta, USA), comprises four sub-panels (Annex 2). Sub-panel 1 consists in dilution series of recombinant proteins, which are the three major antigens used for malaria diagnosis by RDTs: *P. falciparum* histidine-rich protein 2 (Pf HRP2), *Plasmodium* lactate dehydrogenase (pLDH) and *Plasmodium* aldolase. Sub-panel 2 contains a series of cultured *P. falciparum* strains, while sub-panel 3 consists in wild parasite samples prepared with the blood of naturally infected individuals or, in some cases, of experimentally infected primates. Sub-panel 4 provides a range of negative control samples. All samples are prepared according to Standard Operating Procedures (SOPs) which are regularly reviewed, based on the experience of the collection laboratories and on recommendations of the Malaria Diagnostics Specimen Bank and Evaluation Steering Group (page 30).

A. Sub-panel 1: Recombinant antigens

This set allows performance assessment of malaria RDTs against precisely known concentrations of recombinant target antigens of known amino-acid sequence and manufacturing quality.

1. Contents

In the case of pLDH and *Plasmodium* aldolase, species-specific detection sensitivity is assessed with recombinant proteins of both *P. falciparum* and *P. vivax*. Both antigens are structurally well conserved within these species (page 35).[22, 23] One recombinant protein will be chosen for each of pLDH and aldolase antigens for *P. falciparum* and *P. vivax*. Selection is based on comparative data of relative detection of available recombinant antigens against multiple available RDTs, and ELISA, to minimize the potential of structural differences between recombinant and natural antigens introducing bias into RDT performance data. Available recombinant antigens have been compared against RDT and ELISA and further recombinant antigens are currently being cloned and expressed for assessment of suitability.

In the case of the *P. falciparum* specific antigen HRP2, high structural variability occurs and this affects the lower limit of detection of HRP2-detecting RDTs (page 36) [8, 9] HRP2 has been classified into three structural variant groups for the purpose of the testing programme (Type A – high repeat frequency, B – intermediate repeat frequency and C/Borderline – low repeat frequency), on the basis of the frequency of repeats of major target epitopes. A recombinant antigen from each structural group is included³.

Six dilutions of each recombinant antigen are used, designed to cover the lower range of antigen detection of commonly-used RDTs, providing a comparison of detection at very low antigen concentration .

³ HRP2 has been divided by the Programme into three structural groups based on the frequency of repeats of common target epitopes (discussed in more detail later).

2. Samples preparation

Currently, some recombinant proteins are obtained from commercial suppliers who produce well-characterized, recombinant proteins using standard good manufacturing practices (GMPs), such as CTK Biotech (California, USA), the National Bioproducts Institute (South Africa) and Standard Diagnostics (South Korea).

An independent production of recombinant proteins in laboratories of the Malaria RDT Evaluation Programme network is also being developed, so that the entire process can be controlled and remain unchanged over years. Proteins are derived from *P. falciparum* laboratory reference strains and from recently collected, culture-adapted parasites at the Army Malaria Institute (Australia). The gene coding for the antigen is cloned into JM109 cells, then protein expression and purification is performed at the National Bioproducts Institute using their GMP-labelled facilities and manufacturing process. Determination of the gene sequence at different cloning stages and characterization of the protein end-product ensure that no sequence change has occurred during the entire process.

The recombinant proteins are diluted to the six target concentrations in venous donor blood, which has previously been tested by microscopy and malaria RDTs to ensure absence of malaria infection. The dilutions are then aliquoted in 50 μ L volumes and stored at -70°C . The quality of the dilutions is ensured by strict working rules (manipulation of the samples at 4°C , reverse pipetting of blood, etc., page 23) and by subsequent characterization of the samples by ELISA quantification of the antigen contents.

B. Sub-panel 2: P. falciparum culture lines

Twenty culture-adapted strains of *P. falciparum* of widely varied geographic origin have been selected to constitute a stable source of well-characterized parasites, continuously available over years. Parasite culture-derived samples allow better control of the effects of parasite stage and accumulation of stable antigens on RDT performance.

1. Contents

Twenty (20) *P. falciparum* culture lines have been selected among strains that had been adapted to culture and cryostabilized after only a few culture cycles in order to minimize culture-induced changes of the parasites. The selection of the parasite strains would be based on three main criteria:

- The concentration of the three antigens HRP2, pLDH and aldolase, measured by ELISA in parasite sample dilutions at 200 parasites per microliter (parasite// μL).
 - Previous studies have determined the natural variation of antigen concentration at this parasite density in geographically widely distributed wild parasite samples. For inclusion into the sub-panel 2, the parasite strain must display an antigen content in the middle 90% of the observed range of antigen concentrations at 200 parasite// μL .
- The structural group of the Pf HRP2 antigen, to ensure the natural variation of this antigen in parasites of reflected in the panel.
 - Previous studies have described the natural variation of the Pf HRP2 antigen, the impact of this variation on RDT results (classification into the three

structural groups A, B and C/borderline), and the relative proportions of HRP2 variants in different endemic areas. Similar proportions have then been applied to the sub-panel 2 composition (inclusion of 3, 14 and 3 culture lines possessing an HRP2 antigen of type A, B and C/borderline, respectively,(page 36).

- Diversity of geographic origin is maintained, with inclusion of parasites originating from Africa, Asia and the Pacific, and the Americas.

For each culture line, dilutions at high (2000 or 5000 parasite// μ L) and low (200 parasite// μ L) parasite densities are prepared for product testing (choice of parasite densities, page 9). A summary of the culture lines, their geographic origins and their HRP2 variant groups is shown in Annex 2.

A subset of these 20 culture lines has been selected for the so-called ‘manufacturer panel’(page10). This consists in dilutions of 5 *P. falciparum* culture lines that are provided to the manufacturer for in-house quality control testing before actually starting the product testing. Selection of these 5 culture lines follows the same rationale as described above: the strains originate from 3 different continents (Africa, the Americas, and the Western Pacific), and representatives from each Pf HRP2 structural groups are chosen with the same proportions as above (1, 3 and 1 culture lines with Pf HRP2 type A, B and C, respectively).
Samples preparation

The continuous culture of the parasite strains is based on a previously published standard protocol.[24] Before dilution to the target parasitaemias, the cultures are synchronized at the young trophozoite stage with a standard protocol based on sorbitol treatment.[25] After resuspension in a 40% hematocrit mixture of O+ blood cells and AB+ plasma, the parasite density is determined by two independent microscopists, based on a red cell count. Dilutions at 5000 parasite// μ L, 2000 parasite// μ L and 200 parasite// μ L are then prepared, using venous donor blood uninfected by *Plasmodium* parasites (screened by microscopy and malaria RDTs). The dilutions precision and stability of antigen during the dilution process are ensured by the same working rules as for dilutions of wild parasite samples (page 23).

All samples are additionally characterized by PCR to ensure single-strain cultures, and antigen is quantitated by ELISA to ensure antigen concentration is within the mid-90th percentile of the expected range.

C. Sub-panel 3: Wild parasite samples

Wild parasite samples of the four human *Plasmodium* species are prepared from venous blood of naturally infected patients in the Western Pacific Region, South-East Asia, Africa and South-America. In the case of *P. vivax*, *P. malariae* and *P. ovale*, single-species infections are relatively uncommon in most endemic areas. Some samples are therefore derived from primates experimentally infected with parasite strains which have been initially isolated from human patients. This sub-panel approaches most closely the real conditions of use of a malaria RDT, since the samples contain naturally infecting parasites.

1. Contents

Sub-panel 4 comprises 100 *P. falciparum*, 20 *P. vivax*, 5 *P. malariae* and 5 *P. ovale* samples, with each one being derived from a single infected source case. Samples are prepared from the venous blood of the infected host by dilution to precisely calibrated parasite densities: high (2000 or 5000 parasites/ μ l) and low (200 parasites/ μ L), with additional dilutions at a medium parasite density (500 parasites/ μ L) for non-*P. falciparum* species only. The selection criteria of the parasite densities have been described above (page 9). A summary of the samples, their geographic origin and their Pf HRP2 structural group is shown in Annex 2. The selected collection sites and preparation protocols are detailed in the following paragraphs. The final dilutions are characterized by microscopy, molecular species typing, Pf HRP2 sequencing, antigen quantitation and screening of viral infections (page 24). Recurrent collections will ensure availability of wild parasite samples over coming years. Replacement is planned to ensure that the composition of the wild parasite sample set remains stable in terms of antigen variant group and geographic origin (page 28).

2. Collection sites and laboratories

Laboratories are contracted by WHO and FIND in different endemic areas of the Western Pacific Region, of the African continent and of South-America in order to ensure a broadly scattered geographic origin of the wild parasite samples (Figure III-1). Selection of these laboratories is based on a series of criteria:

- Geographic area
- Quality of infrastructures and staffing
- Quality of previous / ongoing work in the field of malaria, particularly on malaria diagnostics
- Ease of access to malaria endemic recruitment sites
- Experience in field work logistics

Malaria patient recruitment is conducted in health facilities with skilled local health staff, good working conditions, availability of HIV counselling services, and within easy reach of the laboratory where the samples are processed.

All collection laboratories have obtained authorization from the respective National and WHO Ethics Committees before recruiting patients and preparing specimen bank samples. All initial collections have been attended by a consultant experienced in the use of the collection and preparation protocols, and staff have been specifically trained. The collection laboratories will have an annual external quality assessment of infrastructure, working conditions, equipment, staff supervision and management, quality assurance documentation, internal and external quality control, and safety. The preparation of wild parasite samples will be regularly reviewed.

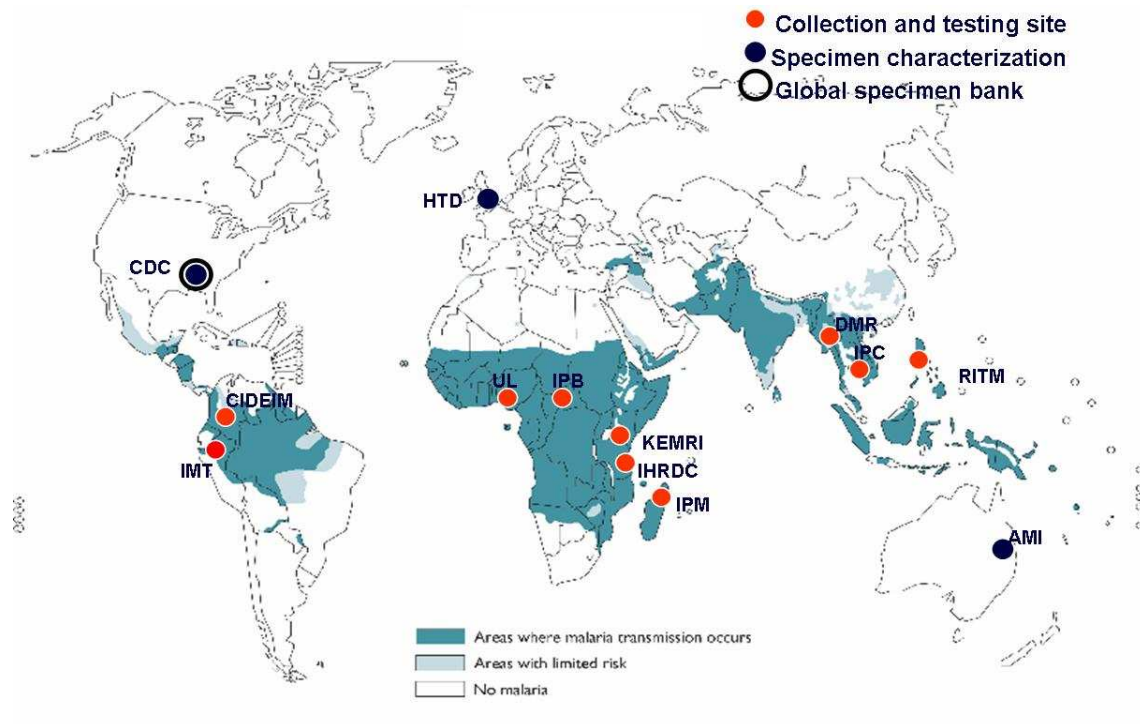


Figure III-1: Laboratories doing sample collection, characterization and hosting the malaria specimen bank

3. Sample preparation

The preparation of wild parasite samples can be divided into three main steps: i) recruitment of *Plasmodium*-infected patients in the field and collection of venous blood ('parasitized blood'), ii) procurement of blood from non-infected donors ('parasite-free blood'), and iii) dilution of the 'parasitized blood' with the 'parasite-free blood' to high, medium and low level parasite densities (high level: 5000 or 2000 parasites per microliter of blood, medium level: 500 parasite// μ L, low level: 200 parasite// μ L), aliquoting and freezing. Inclusion of samples in the specimen bank is based on final characterization results. The whole process is schematically presented in Figure III-2 and Figure III-3.

Patient recruitment and blood collection (Figure III-2)

Febrile patients aged 5 years or more (age limits are higher in some sites, according to country-specific ethics requirements) and not having taken any known anti-malaria treatment in the past month are screened for malaria by RDT and/or thick blood film. If a strongly positive RDT result and/or a sufficient parasite density are detected, the patient is informed and his consent for venous blood collection and screening for viral infections (hepatitis B and C, HIV I and II) is requested. If positive, patients are informed of viral screening results, through the locally-appropriate health service mechanism.

A unique identification number (ID) is assigned to the patient, venous blood is collected (10 mL in EDTA tubes, 5 mL in a plain tube), and two slides with thick and thin blood films and two blood spots on filter paper are prepared. All samples are immediately transported to the laboratory in appropriate storage conditions.

Preparation of parasite-free blood (Figure III-3)

For dilution of the *Plasmodium*-infected patient blood, “parasite-free” blood is prepared by centrifugation of O- or O+ whole blood and replacement of the O- or O+ plasma by AB+ plasma (ensures compatibility with all patient blood groups). The whole blood and the plasma are obtained from informed and consented volunteer donors or from accredited blood banks (mostly National Blood Transfusion Centres). Blood donors are tested for malaria (microscopy, RDT) and viral infections (hepatitis B and C, HIV I and II, by ELISA). Screening of these infections by the blood banks is verified and completed if necessary. Blood is only used if all tests are negative. The quality of the “parasite-free” blood mixture is ensured by rapid preparation at 4°C, good homogenization and use within 36 hours.

Dilution of the parasitized blood (Figure III-3)

The patient’s infection is characterized for species and parasite density by thin/thick film analysis by two experienced, prequalified microscopists, using a discrepancy limit of 20% and a white cell count of the patient’s blood for calculations (see below). The mean parasite density is used for calculating the dilution steps down to a high-level (2000 or 5000 parasite// μ L), a medium-level (500 parasite// μ L, for non-*P. falciparum* species only) and a low-level parasite density (200 parasite// μ L). The dilutions are first prepared in a small test volume of 1 mL, to check for fulfillment of two criteria: i) no agglutination of red blood cells, ii) at least a faint positive RDT result at the low-level (for *P. falciparum* infections) or at the medium-level (for non-*P. falciparum* infections) parasite densities. Larger volumes of dilutions are then prepared and checked again for the same criteria. If satisfactory, these dilutions are aliquoted in 50 μ L volumes in pre-labeled cryotubes and immediately frozen at -70°C.

4. Quality assurance of the sample preparation

Quality of the malaria microscopy

The species and parasite density of the patient’s infection are determined by blinded thin/thick film analysis by two prequalified microscopists. If at least one microscopist identifies a mixed species infection, the sample is not processed further. Parasite densities are calculated against an accurate white cell count of the patient blood, or the Earl-Perez method is used for parasite quantitation⁴. The two calculated parasite densities must have a maximal discrepancy of $\leq 20\%$. If not, the two counts are repeated and, if discrepancy remains higher than 20%, the sample is discarded.

Quality of the malaria RDTs used for screening

Malaria RDTs can be used for the initial patient screening prior to microscopy. Two different RDT brands are used, usually to detect Pf HRP2 and Pf LDH, with pan-specific LDH and/or pan-specific aldolase. RDTs are stored at a controlled temperature. If locally prepared specimen bank samples are available, the quality of the RDTs is assessed prior to use.

⁴ Previous studies have shown that thick film parasite quantitation using the Earl-Perez method and against an accurate white cell count are equivalent.

Quality of blood dilution

All dilutions are performed with successive dilution steps and factors between 2 and 10. Calibrated micropipettes and the reverse pipetting method are used (large volumes of blood are slowly dispensed with sterile plastic pipettes), and the blood is homogenized on slowly rotating hematology wheels and a minimal mixing time depending on the volume (≤ 1 mL: at least 15 min, > 1 mL: at least 1 hour). To avoid degradation of the parasite antigens, the maximal delay between blood collection and freezing of the final dilution aliquots is 24 hours. During this time, all blood samples and dilutions are maintained at 2°C to 8°C.

5. Characterization of the samples

Summary of characterization of wild parasite samples from venipuncture to inclusion in the specimen bank:

- Parasite densities of the patient's infection are determined by expert microscopy analysis of a thick film (see above).
- Parasite species are initially identified by microscopy, but the definitive species determination is performed with molecular biology tools (nested PCR, page 35). Samples with mixed species infections are excluded from the specimen bank.
- The parasite antigen activity of dilutions is tested with malaria RDTs. If the low-level (for Pf infections) or medium-level (for non-Pf infections) dilutions produce negative results with all test RDTs, the samples may be excluded from the specimen bank. (In all cases, samples are tested by quantitative ELISA before being include in a testing panel).
- Antigen contents of dilutions are quantified with ELISA, against established standard curves. Only samples having an antigen content within the middle 90% of previously observed variations are included in the specimen bank (page 34).
- HRP2 variation is determined by sequencing of the Pf HRP2 gene and classification in type A, type B and type C/borderline structural groups. Multi-clone infections producing indeterminate sequence results are considered to be in the HRP2 structural group B. Precise proportions of type A, B, C/borderline samples are included in the specimen bank, consistent with previously observed proportions in geographically widely distributed parasite samples (page 37).
- Infections by hepatitis B, hepatitis C, and HIV I and II viruses are eventually screened with rapid diagnostic tests upon patient recruitment, but a definitive and systematic screening is done with ELISA (commercial reference kits, according to the national guidelines of the respective countries). Positive samples are excluded from the specimen bank.
- Some aliquots of the initial patient blood (whole blood, serum and cell pellet) are retained at -70°C for additional tests in the future if warranted (e.g. additional molecular analyses).
- The diagram in *Figure III-4* shows a summary of all tests.

Conditions for patient screening :

- 1) Febrile patient
- 2) 5 years or older (higher age limits are used in some sites)
- 3) No recent intake of anti-malarials (time limit 14–30 days)
- 4) *Not anaemic (in some sites only)*

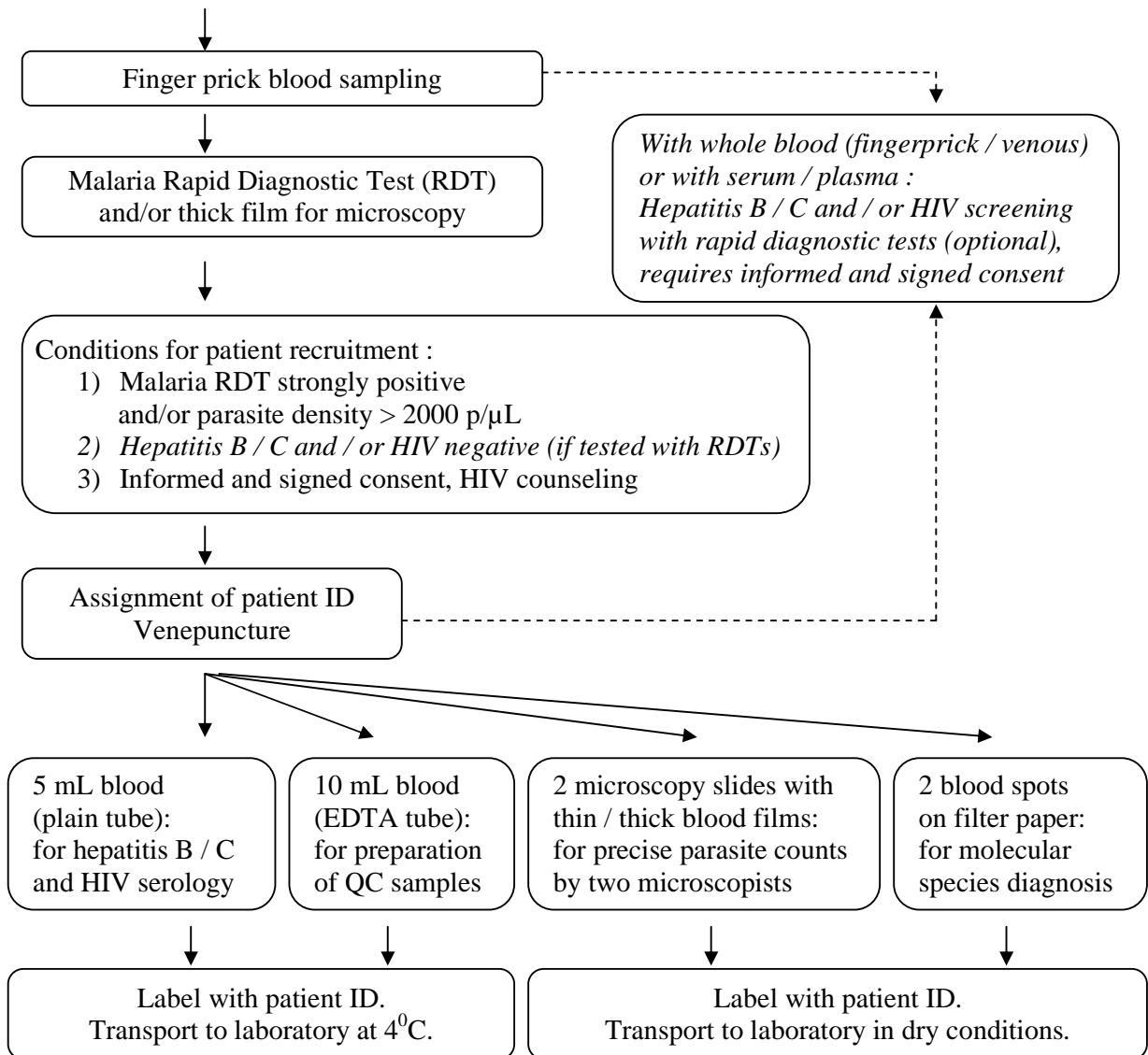


Figure III-2: Patient recruitment and blood collection in the field

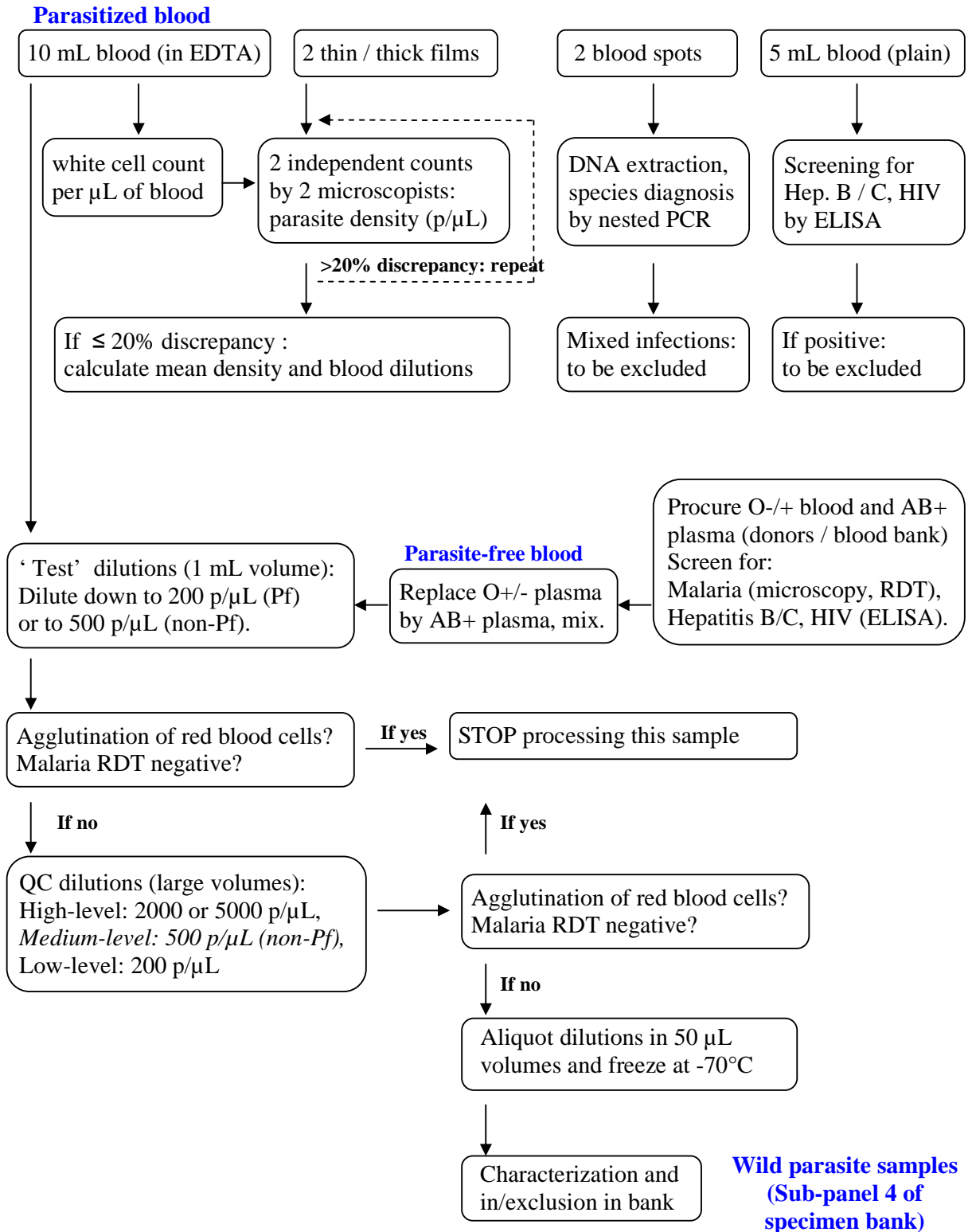


Figure III-3: Preparation of wild parasite samples in the laboratory

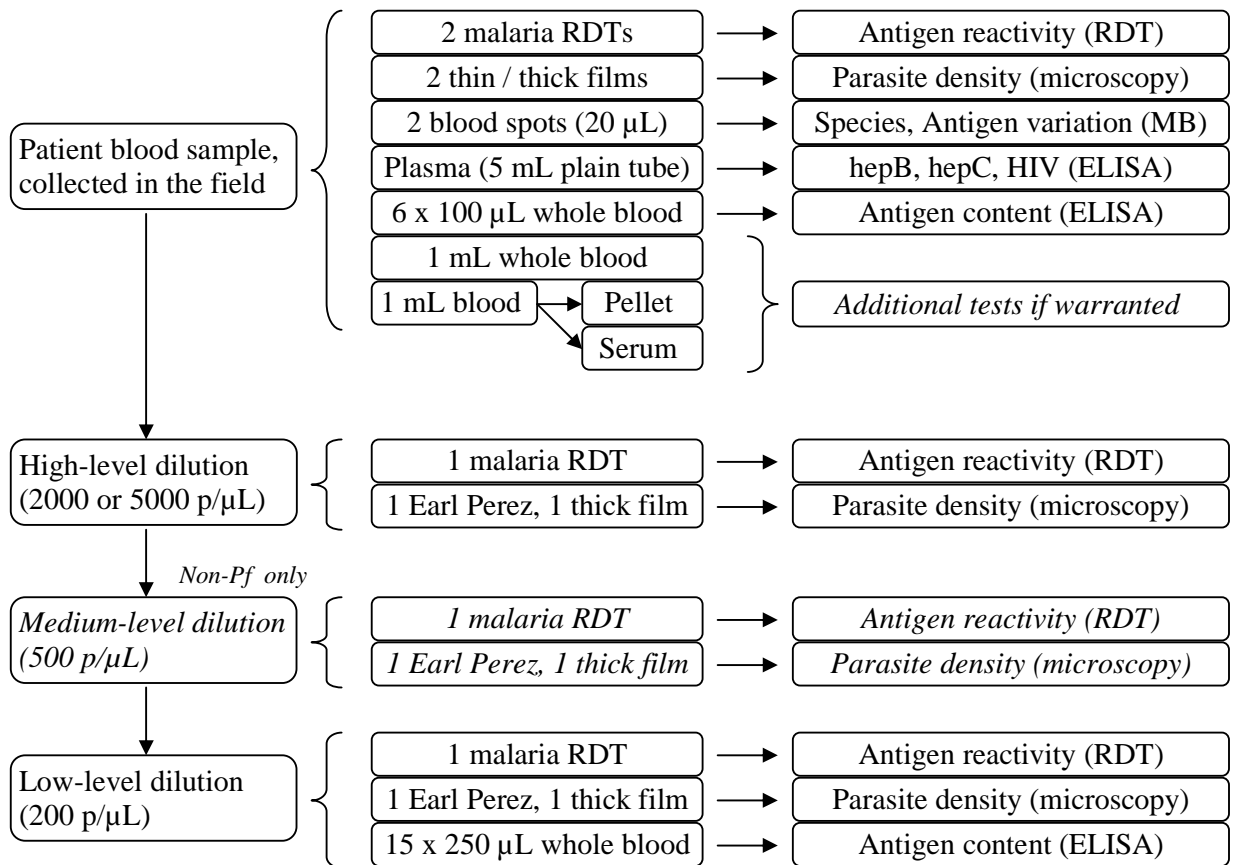


Figure III-4: Characterization of patient blood samples and dilutions

D. Sub-panel 4: Negative control samples

The parasite-negative challenge panel is designed to include:

- Samples from cases with characteristics previously recorded to cause, or suspected to cause, false-positive RDT results
- Samples from cases with possible differential diagnoses of malarial fever
- Samples with no known disease or cause of false-positive results.

1. Contents

It has been reported that blood factors unrelated to malaria, mostly antibodies, can produce a non-specific positive result with some malaria RDTs. Published studies have demonstrated or strongly suggested that the rheumatoid factor and heterophile antibodies (HAMA) have been responsible for the occurrence of false-positive RDT results.[26-28] In the case of other blood factors, like anti-nuclear antibodies (ANA), anti-mouse antibodies and the RPR positivity, their eventual cross-reaction with the monoclonal antibodies in malaria RDTs is rather suspected.

It is similarly important to evaluate the risk of false-positive malaria RDT results in patients suffering from a disease with malaria-like symptoms that is commonly occurring in malaria endemic areas. Selected diseases for the RDT testing programme are dengue fever, typhoid

fever, schistosomiasis, leishmaniasis and Chagas disease. Samples from HIV-infected individuals are also included in this sub-panel, because of commonly occurring co-infection of malaria and HIV, especially in some African countries.

For each of these selected characteristics, blood, serum or plasma samples from at least five different individuals are prepared with a low and a high titer of the relevant antibodies.

Finally, sub-panel 4 also comprises a set of “clean negative control samples”, consisting in blood samples from 50 healthy volunteers in which none of the previously listed blood factors or infections have been detected.

2. Samples preparation

Whole blood samples are obtained from accredited blood banks or collected from volunteer donors. The protocols for recruitment and sample preparation are based on those described for the wild parasite sample preparation (page 20), with the following three main differences:

- Malaria infection is excluded by microscopy and RDT during recruitment, and subsequent confirmatory diagnosis by nested PCR (page 35),
- Specific tests for detecting relevant blood factors or non-malaria infections are performed during the patient recruitment, according to relevant national or international guidelines or protocols.
- Collected venous blood is distributed in 50 μ L aliquots, without previous dilutions, within a timeframe of 24 h during which the blood is kept at 4°C.

Serum or plasma samples are derived from archived samples at the Malaria Branch, Division of Parasitic Diseases (CDC, Atlanta, USA), or from commercial sources that have performed any relevant tests of blood factors or non-malaria infections. All samples are subjected to screening of malaria by nested PCR, then aliquoted in 50 μ L volumes.

E. Maintenance of the specimen bank

Consistency over the years will be ensured for each sub-panel by the following mechanisms:

- It is verified that the recombinant antigens (sub-panel 1) manufacturing process does not undergo major changes, and batch-to-batch consistency is tested against comparative ELISA standard curves. In production laboratories of the QA-RDT network, expression clones are cryopreserved, and regular sequencing of the HRP2, pLDH and aldolase genes ensures consistency of all sequences.
- The *P. falciparum* culture lines (sub-panel 2) are cryopreserved between each sample preparation period. Each batch of diluted samples is tested for parasite density, consistent antigen amounts and unchanged Pf HRP2 sequence.
- For wild parasite samples (sub-panel 3), a steady replacement cycle ensures that there is no large panel change from year to year. Newly prepared dilutions undergo the usual characterization process (page 24). Antigen contents must be in the middle 90% of previously studied antigen levels (page 34) at least for two of the three antigens Pf HRP2, LDH and aldolase. Inclusion of replacement samples in the specimen bank is then decided according the structural group of Pf HRP2 (if applicable), by maintaining the relative proportions of groups A, B and C in the panel (page 37), and according to the geographical origin, by ensuring that each structural group has representatives from Africa, Asia / Western Pacific, and the Americas (in this order of emphasis).

- For replacement of negative control samples (sub-panel 4), the collection sites or commercial sources are maintained as much as possible, and the samples are tested for consistent levels of antibodies / blood factors (if applicable).

IV. Management of the specimen bank and RDT product testing

A. The Malaria Diagnostics Specimen Bank and Evaluation Steering Group

The technical and logistical aspects of the malaria specimen bank and the malaria RDT evaluation programme is mainly driven by the recommendations of the Malaria Diagnostics Specimen Bank and Evaluation Steering Group (subsequently referred to as the Steering Group). This group is constituted by representatives of the following Organizations or Institutions (number of representatives in brackets), and meets at least one time per year:

- WHO/TDR (Geneva, Switzerland) (2)⁵
- WHO/WPRO (Manila, Philippines) (1)
- FIND (Geneva, Switzerland) (2)
- CDC (Atlanta, USA) (1)
- Collection laboratories (annual rotation)
- 1 laboratory in the African Region (1)
- 1 non-African laboratory (Asia, Western Pacific, South-America) (1)
- Médecins Sans Frontières (Holland) (1)
- Hospital for Tropical Diseases (London, UK) (1)
- Army Malaria Institute (Queensland, Australia) (1)
- External expertise from consultants may temporarily be included

Between full meetings of the Steering Group, decisions may be made by a subgroup consisting of TDR (1), WPRO (1), FIND (1), CDC (1) and other members as expertise requires.

The Terms of Reference of the Steering Group consist in providing recommendations on:

- The management of the malaria specimen bank, including:
 - Content, characterization and maintenance of the specimen bank
 - Policy on access to bank samples
- Development and modification of protocols for specimen collection and use
- including Product Testing and Lot Testing of malaria lateral flow RDTs
- Development and modification of protocols for laboratory-based testing of other antigen-detecting diagnostics
- Review product testing results prior to publication

B. Outline of management policies

1. Specimen bank management and policy of access

The decisions concerning the inclusion, exclusion and characterization of samples for the specimen bank are based on criteria of published and unpublished evidence, discussed and developed by the Steering Group. These criteria are described in detail in specific paragraphs

⁵ Figures in brackets indicate the number of representatives.

concerning the different specimen bank sub-panels (page 18), their characterization (page 24), as well as contents and variation of the antigens (page 33 and 35).

The protocols for collection and/or preparation of specimen bank samples (recombinant proteins, parasite culture lines, wild parasite samples and parasite-negative samples) have been developed in collaboration with the laboratories included in the QA-RDT network, and are agreed by the Steering Group. SOP modifications require the approval of the responsible WHO Officer, with eventual consultation of the Steering Group.

Inclusion of the collection laboratories of the QA-RDT network is based on a series of criteria detailed elsewhere (page 21) and is decided within the WHO – TDR – FIND partnership, with consultation of the Steering Group.

Access to the specimen bank samples and associated information is at the final discretion of WHO, in collaboration with FIND, and on the advice of the Steering Group. In general, wild parasite samples are only available for testing of RDTs within the network supported and coordinated by WHO and FIND. Culture-derived parasite samples may be accessed by diagnostics manufacturers and developers, through the approval mechanism described above, and at a cost decided by WHO (aimed at covering costs incurred in providing the sample).

2. Management, rules and definitions of the product testing

Main rules

The criteria for inclusion of RDT submitted products in the malaria RDT Evaluation Programme have been outlined previously (page 10), and access to a more detailed list of criteria is available online.[29] The manufacturer can withdraw products from the Evaluation Programme up to 2 months after having received the manufacturer panel samples for in-house testing, but not after commencement of the product testing. Submission of the physical product indicates agreement to test the product. RDT products first undergo a phase 1 evaluation, and are then subjected to the complete phase 2 evaluation if the phase 1 criteria for adequate test performance are fulfilled (page 11). The Steering Group will give recommendations on equivocal results.

The phase 1 and phase 2 testing results are systematically reviewed by the Steering Group. All product testing results will be published and made available to the manufacturer, but cannot be used for promotional purposes or as evidence for product certification (page 12).

WHO will list all evaluated products and performance data on a dedicated page of the WHO website and in a hard copy publication. WHO may remove a product from the website list (de-listing) or require its re-submission if changes of the RDT product justify its re-definition as a new product, or if data obtained from field testing are considered to be consistently outside those of the product testing programme.

Joint-submission and joint-listing of identical RDT products with different names is possible if they are produced on the same production line, and if the concerned companies provide all required documentations demonstrating that these RDT products are the same. In this case, only one product can be submitted for testing, and the identical RDT products will be jointly listed with the performance results.

Main definitions

A RDT product is considered to be different or to be a new product if one of the following specifications have changed:

- (i) Monoclonal antibodies (MABs): change of the target epitope, or of the species from which the target antigen for MAB development is derived
- (ii) Dye conjugate: change of specifications or type of label
- (iii) RDT format: change in the assay presentation (dipstick, cassette, card, etc.)
- (iv) RDT manufacturer: production of similar RDTs by different manufacturers, except if joint listing of RDTs has been justified and accepted (see below)

Other changes, which could have the potential to significantly affect RDT performance, such as changes in raw materials or components (MABs, buffers, nitrocellulose membranes, etc.), do not require the re-definition of a RDT as a new product, but the manufacturer should provide WHO with data demonstrating the equivalence of performance of the changed RDT product.

The definition of a RDT lot is the responsibility of the RDT manufacturer, and must be compatible with the ISO13485:2003 or US FDA 21 CFR part 820 certification.

A more detailed description of the Malaria Diagnostics Specimen Bank and Evaluation Steering Group, policies, rules and definitions is available on the WHO-WPRO Malaria Rapid Diagnostics website.[29]

V. Evidence and methods for RDT product testing

A. Antigen contents

The detection sensitivity of malaria RDTs is generally mentioned in terms of lowest detectable parasite density (lower limit of detection). However, a positive or negative RDT result rather depends on the amount of antigen contained in the blood sample. The antigen content in infected venous blood can be quantified by ELISA, by establishing a reference standard curve with known amounts of recombinant antigens. ELISA protocols and standard curves have been set up for the three antigens HRP2 (*P. falciparum*), pLDH and aldolase, the relationship between parasite density and antigen content in blood samples is being assessed. The results of this study will provide the basis for deciding about the recombinant proteins and their concentrations in dilution series to be used for RDT product testing.

1. Quantification of antigens by ELISA

After analysis of standard curves and range of antigen detection, the CELISA kit (Cellabs, Australia) and SD Malaria Antigen ELISA kit (Standard Diagnostics, South Korea) were selected for quantitation of HRP2 and pLDH respectively.

Protocols follow the manufacturers' recommendations, but are optimized to improve quantitation. Samples are diluted to operate within the linear range of the standard curves of the respective ELISAs.

A quantitative ELISA has been developed for the programme by US CDC, using recombinant aldolase and anti-aldolase monoclonal antibodies provided through WHO, to assess aldolase content.

2. Relationship between antigen content and parasite density

Using the above described protocols and standard curves, antigen concentrations have been determined in culture line and wild parasite samples at calibrated parasite densities. The preparation of blood samples at 200, 500, 2000 and / or 5000 parasites per microliter of blood was previously described, for parasite culture lines in this document.

The results showed a correlation between antigen concentration and parasite density. Relatively weak correlation factors were observed, especially in the case of Pf HRP2, because of varying antigen contents in different samples despite identical parasite densities, and probably due to variation in binding of antigen by monoclonal antibodies:

Various factors can cause antigen content variation at a given parasite density. Some are of a technical nature, e.g. eventual effects of blood sample components (donor blood used for dilution, anticoagulants) or inaccuracies during sample preparation (microscopy, pipetting, mixing). Antigen content is also influenced by the parasite stage (particularly in the case of pLDH), the duration of the infection, the inherent antigen expression level of the parasite and antigen variant type (in the case of Pf HRP2, page 36), variations in the parasite load (parasite

sequestration in the case of *P. falciparum*), and persistence of antigen after parasite elimination (particularly of Pf HRP2).

The SOPs of the sample preparation process have been designed to minimize the effect of these factors (page 20).

A published study has shown that the amount of secreted Pf HRP2 protein per parasite indeed varies from one parasite stage to another.[30] It is similarly possible that the level of HRP2 transcription and of Pf HRP2 protein production varies from one parasite strain to another. However, the significance of this to clinical sensitivity is not clear, as HRP2 persists and therefore accumulates in the circulation over subsequent cycles of parasite development. The relationship between antigen concentration and parasite density would be further clarified by future studies .

3. Study-based choices for the specimen bank

The results of the above described study have driven decisions concerning the different sub-panels of the product testing specimen bank.

Sub-panel 1

This sub-panel consists of dilution series of recombinant Pf HRP2 (three variant types, according to page 35), Pf LDH, Pv LDH, Pf Aldolase and Pv Aldolase, with the purpose to compare the lower limits of RDT detection. The range of recombinant antigen concentrations is therefore situated around the equivalent of a parasite density of 200 parasite// μ L, which has been previously determined for each recombinant protein. In the case of Pf HRP2, where different protein variants produce different ELISA results, the equivalent of 200 parasite// μ L has been specifically assessed for each variant of sub-panel 1.

Sub-panels 2 and 3

Parasite culture lines for sub-panel 2 and wild parasite samples for sub-panel 3 were chosen and prepared according to the criteria described in the corresponding paragraphs. Subsequently, the contents of HRP2 (*P. falciparum* only), LDH and aldolase in all 200 parasite// μ L dilutions were determined by ELISA. Based on the range of antigen content observed at 200 parasite// μ L in all collected wild parasite samples, those with atypical antigen concentrations, (i.e. being situated within the 5% upper and 5% lower range of antigen), were excluded.

In the case of Pf HRP2, quantitation by ELISA is affected by the variation of this antigen. ELISA results can therefore not be regarded as an accurate assessment of antigen content, but they ensure that the specimen bank samples have antigen contents within a wide acceptable range. By excluding only the “far outliers” (i.e. samples with antigen content in the 5% upper and lower ranges), and by including parasites with different HRP2 variants in given proportions (page 12), the introduction of any bias is limited and the specimen bank mimics as much as possible the wide diversity occurring in wild parasites from different malaria endemic areas.

B. DNA extraction and molecular diagnosis of parasite species

The infecting species present in the wild parasite samples of the specimen bank has to be precisely identified, and more particularly mixed infections have to be excluded. The sample preparation procedure includes a high quality microscopy analysis, with species diagnosis on both a thick and a thin film by two experienced microscopists. Nevertheless, it has amply been reported that more sensitive molecular diagnosis methods detect higher rates of mixed infections, because minor species at very low parasite density are frequently overlooked by microscopy.[31-34] Whole blood in EDTA or blood-spots on filter paper are therefore collected from the patient, subjected to DNA extraction, and the infecting species is diagnosed by nested PCR analysis.

Commercial kits are used for extraction of DNA from venous blood samples (QIAamp DNA Blood BioRobot MDx Kit) and from filter paper blood spots (QIAamp Media MDx, QIAamp DNA Mini, or QIAamp DNA Blood Mini Kits). A robot is used for automated high-throughput extraction, minimizing the risk of contamination and manipulation errors (BioRobot MDx). The two kits procedures are based on the same DNA extraction principle: after lysis and protease digestion, the sample is mixed with ethanol and filtered through silica columns fixing the genomic DNA. After several wash steps, the DNA is eluted and stored at -20°C until PCR analysis.

The infecting species are identified by species-specific nested PCR amplification, using a published protocol which is currently regarded as the reference and has been used in numerous clinical and field studies since its publication.[32-37] This protocol is based on amplification of the 18S ribosomal RNA gene of the parasite. Two *Plasmodium*-specific primers are used for the primary amplification step, then four different species-specific primer pairs are used for four separate and specific amplification reactions of *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. Additionally, the primate parasite *P. knowlesi* is detected by using a previously published protocol, since this parasite has been shown to infect humans in various Asian countries.[38, 39] The presence or absence of each of these species is assessed by standard agarose gel electrophoresis and BET staining of the PCR products.

C. Antigen variation

The robustness of malaria RDTs is based, among others, on their capacity to recognize epitopes in the target antigen, whatever the geographical origin and the genetic background of the malaria parasite. It is therefore essential to assess possible sequence variations which may alter the number and binding affinity of the epitopes in the parasite antigen targeted by the monoclonal antibodies (MABs) in the RDTs, as well as the eventual impact of such variations on the detection performance of the RDTs. The targeted epitopes of the HRP2, pLDH, and aldolase antigens of commercial RDTs are not publicly known. The genetic diversity of the antigens has therefore been assessed by sequencing the entire coding region of the pLDH, aldolase and HRP2 genes of geographically widely distributed parasites by collaborating institutions.

1. *Plasmodium* LDH and aldolase variation

The genetic diversity of LDH has recently been studied in wild isolates of the four *Plasmodium* parasite species infecting human (*P. falciparum*: 49 isolates from Africa, the

Americas, Asia, the Western Pacific, and the Middle East ; *P. vivax* and *P. malariae*: 10 and 17 isolates, respectively, from Africa, Asia and the Americas ; *P. ovale*: 13 isolates from Africa and South-East Asia).[23] The resulting amino acid sequences of *P. falciparum*, *P. vivax* and *P. malariae* LDH were 100% identical within each species, with only a few cases of synonymous nucleotide variations. Only one non-synonymous mutation was noted in a published *P. falciparum* laboratory strain sequence. In the case of *P. ovale*, three different LDH protein sequences were observed because of non-synonymous mutations in three positions.

Even though larger confirmatory studies would be useful, these findings suggest that LDH variation is very limited. It appears unlikely that LDH variation is the reason for the varying RDT detection sensitivities of *P. falciparum*, *P. vivax* and *P. malariae*. It could possibly account for the rather poor detection of *P. ovale* by LDH-based RDTs, but this hypothesis would need to be confirmed by further investigations.[4, 20]

Another recently published study of the entire aldolase gene sequence in *P. falciparum* and *P. vivax* isolates has similarly shown very little sequence variation within each species.[21] The study was based on 36 *P. falciparum* isolates originating from Africa, the Western Pacific Region and South-East Asia, and on 18 *P. vivax* isolates from the Western Pacific and South-East Asia. A small number of synonymous mutations were found in the wild parasite isolates, and only one non-synonymous mutation was found in a laboratory strain of each species.

As for LDH, it seems highly unlikely that antigen diversity could be responsible for different performances of aldolase-based malaria RDTs, at least for the detection of *P. falciparum* and *P. vivax*.

2. *P. falciparum* HRP2 variation and its impact on RDT results

Published studies

A recently published study based on 75 *P. falciparum* culture lines and isolates from 19 different malaria endemic countries has shown an extensive diversity of Pf HRP2 nucleotide sequences.[8] The translated Pf HRP2 protein sequences consisted of various amino acid repeats which vary in composition, number and order. Detection of a subset of these parasite isolates with two different RDTs revealed that the detection of relatively low parasitaemias (≤ 250 parasites per microliter of blood, parasite// μL) was highly dependent on the Pf HRP2 sequences, while all isolates were well detected at parasitaemias higher than 1000 parasite// μL . More particularly, the RDT detection sensitivity could be predicted with a regression model, establishing a threshold for highly probable sensitive test results ($p > 0,5$) based on the multiplied numbers of type 2 and type 7 repeats (number of type 2 x type 7 repeats > 43).

The observation that HRP2 sequence variation affects RDT performances was further supported by a study demonstrating variable reactivity of HRP2-specific monoclonal antibodies (MABs) with geographically distinct *P. falciparum* isolates and different MABs recognizing different epitopes.[9]

Further studies in the context of the RDT product testing scheme (unpublished data)

The two published reports justified the need of a more extensive study of the worldwide Pf HRP2 sequence variation in *P. falciparum* parasites in the context of this RDT product testing scheme. Pf HRP2 sequences have been determined in a total number of 373 parasite samples (as at 2 November 2007) originating from Africa, Asia, South-East Asia, the Western Pacific, South America and the Caribbean. The resulting Pf HRP2 sequences have been classified according to their predicted detection sensitivity by Pf HRP2-based RDTs (Figure V-1), based on the previously described regression model,[8] as well as further statistical analysis with a larger number of samples: types A and B are predicted to be well detected at parasitaemias below 250 parasite// μ L, while the probability of detection of types C and borderline below this parasitaemia is predicted to be low.

In all investigated geographical areas, the major HRP2 variant was of type B. type C and borderline HRP2 variants represented at least 30% of the parasite samples in Asian countries and the Western Pacific, while they were much less prevalent in Africa (15%) and were not detected in South and Central America in this sample.

Area	n	A > 100	B 50 - 100	C < 43	Borderline 44 - 49
Africa	135	14 (10%)	101 (75%)	12 (9%)	8 (6%)
China	10	2 (20%)	5 (50%)	3 (30%)	0
Pacific	84	9 (11%)	50 (77%)	9 (11%)	16 (19%)
S. America	54	9 (16%)	45 (83%)	0	0
S.E. Asia	90	8 (9%)	47 (52%)	31 (34%)	4 (4%)
Total	373	42 (11%)	248 (66%)	55 (15%)	28 (8%)

Figure V-1: Distribution of HRP2 variant types in malaria endemic areas

P. falciparum parasite samples were obtained from Africa (Benin, Burkina Faso, Cameroon, Gambia, Ghana, Guinea, Kenya, Liberia, Malawi, Nigeria, Niger, Sierra Leone, Sudan, Tanzania, Uganda, Zambia), China, the Western Pacific Region (East Timor, Papua New Guinea, Solomon Islands, Vanuatu), South America and the Caribbean (Brazil, Colombia, Ecuador, Haiti, Honduras, Peru, Santa Lucia, Suriname, Columbia), as well as South-East Asia (Cambodia, Indonesia, Malaysia, Philippines, Thailand, Vietnam).

HRP2 variants were classified according to the multiplied number of type 2 and type 7 amino acid repeats⁶, with 50 being the threshold for sensitive detection by HRP2-based malaria RDTs (below 50: type C and borderline, 50 and above: type A and B).[8]

3. Study-based choices for the specimen bank

The results of this study were used to decide the composition of the product testing specimen bank, in terms of HRP2 variants. The rationale was to test malaria RDT products against all three categories of HRP2 variants (type A with high repeat number, type B with intermediate repeat number, and type C and borderline, with low repeat numbers), and to apply the relative

⁶ AMI working classification

proportions of the investigated sample set (11% 'Type A', 66% 'Type B', 23% 'Type C and borderline') to all sub-panels made of *P. falciparum* parasite samples.

- Sub-panel 1 (recombinant antigen panel) contains three recombinant HRP2 preparations, with one for each HRP2 variant category,
- Sub-panel 2 (parasite culture lines) is made of 20 *P. falciparum* culture lines, with 3, 14 and 3 lines harboring a type A, type B a type C / borderline HRP2 sequence, respectively,
- Sub-panel 3 (wild parasite samples) contains 100 *P. falciparum* parasite samples prepared from infected individuals, with HRP2 sequences belonging to 'Type A' in 11 samples, to type B in 66 samples and to type C / borderline in 23 samples,
- The 'manufacturer panel' consists of 5 *P. falciparum* culture lines, with 1, 3 and 1 lines expressing the Pf HRP2 structural groups type A, B and C / borderline, respectively,
- For stability testing, one *P. falciparum* culture line with the major type B Pf HRP2 structural group has been selected.

Annex 1: Laboratories of the Malaria RDT Evaluation Programme Network

Global Specimen Bank / Samples Characterization

Division of Parasitic Diseases
National Centers for Disease Control and Prevention (CDC)
4770 Buford Highway
MS F36 Bldg 109, Room 1121
NE Atlanta GA 30341
USA

Samples Characterization

Australian Army Malaria Institute (AMI)
Weary Dunlop Drive
Gallipoli Barracks
Enoggera QLD 4051
AUSTRALIA

Department of Clinical Parasitology
Hospital for Tropical Diseases (HTD)
Mortimer Market, Capper Street
London WC1E 6AU
UK

Production of Recombinant Proteins

National Bioproducts Institute (NBI)
Private Bag X9043
Pinetown 3610, Natal
SOUTH AFRICA

Collection laboratories

Research Institute of Tropical Medicine (RITM)
Filinvest Compound
Alabang, Muntinlupa City
PHILIPPINES

Laboratory of Molecular Epidemiology
Institut Pasteur du Cambodge (IPC)
#5, Monivong Blvd, P.O. Box 983
Phnom Penh
CAMBODIA

Experimental Medicine Research Division,
Department of Medical Research (DMR)
No. 5, Ziwaka Road, Dagon P.O., Yangon 11191
MYANMAR

Department of Medical Microbiology and Parasitology
College of Medicine (RM 308) of the University of Lagos (UL)
University of Lagos
Idiaraba, Lagos
NIGERIA

Institut Pasteur de Bangui (IPB)
BP 923 Bangui
CENTRAL AFRICAN REPUBLIC

Centre for Clinical Research
Kenya Medical Research Institute (KEMRI)
Po Box 54
Kisumu
KENYA

Ifakara Health Research and Development Centre (IHRDC)
360 Kiko Avenue
Mikocheni, Dar-es-Salaam
UNITED REPUBLIC OF TANZANIA

Institut Pasteur de Madagascar (IPM)
Unité du Paludisme - Malaria Unit
Institut Pasteur de Madagascar
BP 1274 - Antananarivo 101
MADAGASCAR

Centro de Entrenamiento y Investigaciones Médicas (CIDEIM)
Avenida 1-N 3-03
Cali
COLOMBIA

Instituto de Medicina Tropical Alexander von Humboldt
Universidad Peruana Cayetano Heredia
Av. Honorio Delgado 430
Urb. Ingenieria, San Martin de Porres
AP 4314 Lima
PERU

Annex 2: Summary of the Malaria Specimen Bank

Sample type	n	Antigen ^a	n	Origin ^b	n	Testing conditions
PHASE ONE PANEL						
Recombinant antigen (sub-panel 1)	7	Pf HRP2 type A	1		1	6 serial dilutions, range of antigen concentrations: equivalent to 500 p/μL down to below 100 p/μL
		Pf HRP2 type B	1		1	
		Pf HRP2 type C	1		1	
		Pf LDH	1	To be finalized	1	
		Pv LDH	1		1	
		Pf aldolase	1		1	
<i>P. falciparum</i> culture lines (sub-panel 2)	20	Pf HRP2 type A	3	SE Asia / WP	xx	dilutions at 200 p/μL (low) and 2000 or 5000 p/μL (high)
		Pf HRP2 type B	14	Africa	xx	
		Pf HRP2 type C	3	S America	xx	
PHASE TWO PANEL						
Wild parasites, derived from human (sub-panel 3)						
<i>P. falciparum</i>	100	Pf HRP2 type A	10	SE Asia / WP	xx	dilutions at 200 p/μL (low) and 2000 or 5000 p/μL (high)
		Pf HRP2 type B	60	Africa	xx	
		Pf HRP2 type C	30	S America	xx	
Wild parasites, derived from human or primates (sub-panel 3)						
<i>P. vivax</i>	20					dilutions at 200 p/μL (low), 500 p/μL (medium), 2000 or 5000 p/μL (high)
<i>P. malariae</i>	5					
<i>P. ovale</i>	5					
Plasmodium-negative samples (sub-panel 4)						
Anti-nuclear Ab positive	5-10					dilutions at high and low titres of relevant antibodies / blood factors
RPR positive	5-10					
Rheumatoid factor positive	5-10					
Heterophile Ab positive	5-10					
Anti-mouse Ab positive	5-10					
Chagas	5-10					
Leishmaniasis	5-10					
Schistosomiasis	5-10					
Typhoid	5-10					
HIV	5-10					
Clean negative (none of above)	5-10					

Note : all *Plasmodium*-positive samples are characterized by microscopy, molecular species diagnosis, Pf HRP2 sequencing (if applicable), and antigen quantitation by ELISA (paragraph xxx).

^a Where relevant, the parasite antigen is indicated (Pf HRP2 types A, B, C : see paragraph xxx).

^b Recombinant proteins are either commercially available or are produced in laboratories of the QA-RDT network, The origins of the parasite strains are grouped into : South-East Asia / Western Pacific, Africa, South America. n = number of samples, p/μL = parasites per microlitre of blood.

Sample type	n	Antigen ^a	Origin ^b	Testing conditions
MANUFACTURERS PANEL (subset of the sub-panel 2 of the specimen bank)				
<i>P. falciparum</i> culture lines	5			
Name of the Pf strains :				
Benin I		Pf HRP2 type A	Benin, Africa	
Santa Lucia		Pf HRP2 type B (low)	El Salvador, C America	dilutions at 200 p/μL (low) and 2000 or 5000 p/μL (high)
Nigeria XII *		Pf HRP2 type B (med.)	Nigeria, Africa	
FC27/A3		Pf HRP2 type B (high)	Papua New Guinea, WP	
PH1		Pf HRP2 type C	Philippines, WP	

Note : samples characterized by microscopy, molecular species diagnosis, Pf HRP2 sequencing, and antigen ELISA.

^a Indication of the parasite antigen (Pf HRP2 types A / B / C and antigen content high / medium / low).

^b Geographical origins of parasite strains : Africa, Central America, Western Pacific.

* Pf culture line used as the reference standard for RDT stability testing at the CDC and by the manufacturer.

n = number of samples, p/μL = parasites per microlitre of blood.

References

1. Jorgensen P, Chanthap L, Rebuena A, Tsuyuoka R, Bell D: **Malaria rapid diagnostic tests in tropical climates: the need for a cool chain.** *The American Journal of Tropical Medicine and Hygiene* 2006, **74**(5):750-754.
2. Kumar A, Sharma VP, Thavaselvam D, Sumodan PK: **Clinical trials of a new immunochromatographic test for diagnosis of Plasmodium falciparum malaria in Goa.** *Indian Journal of Malariology* 1996, **33**(4):166-172.
3. Bell D, Wongsrichanalai C, Barnwell JW: **Ensuring quality and access for malaria diagnosis: how can it be achieved?** *Nat Rev Microbiol* 2006, **4**(9):682-695.
4. Moody A: **Rapid diagnostic tests for malaria parasites.** *Clin Microbiol Rev* 2002, **15**(1):66-78.
5. Murray CK, Bell D, Gasser RA, Wongsrichanalai C: **Rapid diagnostic testing for malaria.** *Trop Med Int Health* 2003, **8**(10):876-883.
6. Wongsrichanalai C: **Rapid diagnostic techniques for malaria control.** *Trends Parasitol* 2001, **17**(7):307-309.
7. Chiodini PL, Bowers K, Jorgensen P, Barnwell JW, Grady KK, Luchavez J, Moody AH, Cenizal A, Bell D: **The heat stability of Plasmodium lactate dehydrogenase-based and histidine-rich protein 2-based malaria rapid diagnostic tests.** *Trans R Soc Trop Med Hyg* 2007, **101**(4):331-337.
8. Baker J, McCarthy J, Gatton M, Kyle DE, Belizario V, Luchavez J, Bell D, Cheng Q: **Genetic diversity of Plasmodium falciparum histidine-rich protein 2 (PfHRP2) and its effect on the performance of PfHRP2-based rapid diagnostic tests.** *J Infect Dis* 2005, **192**(5):870-877.
9. Lee N, Baker J, Andrews KT, Gatton ML, Bell D, Cheng Q, McCarthy J: **Effect of sequence variation in Plasmodium falciparum histidine-rich protein 2 on binding of specific monoclonal antibodies: Implications for rapid diagnostic tests for malaria.** *J Clin Microbiol* 2006, **44**(8):2773-2778.
10. De Monbrison F, Gerome P, Chaulet JF, Wallon M, Picot S, Peyron F: **Comparative diagnostic performance of two commercial rapid tests for malaria in a non-endemic area.** *Eur J Clin Microbiol Infect Dis* 2004, **23**(10):784-786.
11. Lee MA, Aw LT, Singh M: **A comparison of antigen dipstick assays with polymerase chain reaction (PCR) technique and blood film examination in the rapid diagnosis of malaria.** *Annals of the Academy of Medicine, Singapore* 1999, **28**(4):498-501.
12. Rennie W, Phetsouvanh R, Lupisan S, Vanisaveth V, Hongvanthong B, Phompida S, Alday P, Fulache M, Lumagui R, Jorgensen P *et al*: **Minimising human error in malaria rapid diagnosis: clarity of written instructions and health worker performance.** *Trans R Soc Trop Med Hyg* 2007, **101**(1):9-18.
13. Kolaczinski J, Mohammed N, Ali I, Ali M, Khan N, Ezard N, Rowland M: **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.** *Annals of Tropical Medicine and Parasitology* 2004, **98**(1):15-20.
14. Cooke AH, Chiodini PL, Doherty T, Moody AH, Ries J, Pinder M: **Comparison of a parasite lactate dehydrogenase-based immunochromatographic antigen detection assay (OptiMAL) with microscopy for the detection of malaria parasites in human blood samples.** *The American Journal of Tropical Medicine and Hygiene* 1999, **60**(2):173-176.

15. Congpuong K, Bualombai P, Jitchamroen S, Konchom S: **Comparison of the OptiMAL rapid test with routine microscopic examination of Giemsa-Stained Thick Blood Film for diagnosis of malaria.** *Journal of the Medical Association of Thailand = Chotmai het thangphaet* 2001, **84**(3):357-363.
16. Lon CT, Alcantara S, Luchavez J, Tsuyuoka R, Bell D: **Positive control wells: a potential answer to remote-area quality assurance of malaria rapid diagnostic tests.** *Trans R Soc Trop Med Hyg* 2005, **99**(7):493-498.
17. WHO (2000). **New Perspectives: Malaria Diagnosis.** Report of a joint WHO/USAID informal consultation 25-27 October 1999. Geneva, World Health Organization.
18. WHO (2003). **Malaria Rapid Diagnosis: Making it Work.** Meeting report 20-23 January 2003. Manila, World Health Organization.
19. Dyer ME, Tjitra E, Currie BJ, Anstey NM: **Failure of the 'pan-malarial' antibody of the ICT Malaria P.f/P.v immunochromatographic test to detect symptomatic Plasmodium malariae infection.** *Trans R Soc Trop Med Hyg* 2000, **94**(5):518.
20. Bigaillon C, Fontan E, Cavallo JD, Hernandez E, Spiegel A: **Ineffectiveness of the Binax NOW malaria test for diagnosis of Plasmodium ovale malaria.** *J Clin Microbiol* 2005, **43**(2):1011.
21. WHO (2006). **Rapid Diagnostic Tests for Malaria: Methods Manual for Laboratory Quality Control Testing. Version 4.** Manila, World Health Organization - Regional Office for the Western Pacific.
22. Lee N, Baker J, Bell D, McCarthy J, Cheng Q: **Assessing the genetic diversity of the aldolase genes of Plasmodium falciparum and Plasmodium vivax and its potential effect on performance of aldolase-detecting rapid diagnostic tests.** *J Clin Microbiol* 2006, **44**(12):4547-4549.
23. Talman AM, Duval L, Legrand E, Hubert V, Yen S, Bell D, Le Bras J, Ariey F, Houze S: **Evaluation of the intra- and inter-specific genetic variability of Plasmodium lactate dehydrogenase.** *Malar J* 2007, **6**(1):140.
24. Trager W, Jensen JB: **Human malaria parasites in continuous culture.** *Science* 1976, **193**(4254):673-675.
25. Lambros C, Vanderberg JP: **Synchronization of Plasmodium falciparum erythrocytic stages in culture.** *J Parasitol* 1979, **65**(3):418-420.
26. Mishra B, Samantaray JC, Kumar A, Mirdha BR: **Study of false positivity of two rapid antigen detection tests for diagnosis of Plasmodium falciparum malaria.** *J Clin Microbiol* 1999, **37**(4):1233.
27. Iqbal J, Sher A, Rab A: **Plasmodium falciparum histidine-rich protein 2-based immunocapture diagnostic assay for malaria: cross-reactivity with rheumatoid factors.** *J Clin Microbiol* 2000, **38**(3):1184-1186.
28. Moody AH, Chiodini PL: **Non-microscopic method for malaria diagnosis using OptiMAL IT, a second-generation dipstick for malaria pLDH antigen detection.** *British Journal of Biomedical Science* 2002, **59**(4):228-231.
29. Bell D: **Malaria rapid diagnostic tests: one size may not fit all.** *Clinical Microbiology Reviews* 2002, **15**(4):771; discussion 771-772.
30. Desakorn V, Dondorp AM, Silamut K, Pongtavornpinyo W, Sahassananda D, Chotivanich K, Pitisuttithum P, Smithyman AM, Day NP, White NJ: **Stage-dependent production and release of histidine-rich protein 2 by Plasmodium falciparum.** *Trans R Soc Trop Med Hyg* 2005, **99**(7):517-524.
31. Brown AE, Kain KC, Pipithkul J, Webster HK: **Demonstration by the polymerase chain reaction of mixed Plasmodium falciparum and P. vivax infections undetected by conventional microscopy.** *Trans R Soc Trop Med Hyg* 1992, **86**(6):609-612.

32. Snounou G, Viriyakosol S, Zhu XP, Jarra W, Pinheiro L, do Rosario VE, Thaithong S, Brown KN: **High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction.** *Mol Biochem Parasitol* 1993, **61**(2):315-320.
33. May J, Mockenhaupt FP, Ademowo OG, Falusi AG, Olumese PE, Bienzle U, Meyer CG: **High rate of mixed and subpatent malarial infections in southwest Nigeria.** *Am J Trop Med Hyg* 1999, **61**(2):339-343.
34. Siripoon N, Snounou G, Yamogkul P, Na-Bangchang K, Thaithong S: **Cryptic Plasmodium falciparum parasites in clinical P. vivax blood samples from Thailand.** *Trans R Soc Trop Med Hyg* 2002, **96**(1):70-71.
35. Singh B, Bobogare A, Cox-Singh J, Snounou G, Abdullah MS, Rahman HA: **A genus- and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies.** *Am J Trop Med Hyg* 1999, **60**(4):687-692.
36. Toma H, Kobayashi J, Vannachone B, Arakawa T, Sato Y, Nambanya S, Manivong K, Inthakone S: **A field study on malaria prevalence in southeastern Laos by polymerase chain reaction assay.** *Am J Trop Med Hyg* 2001, **64**(5-6):257-261.
37. Alves FP, Durlacher RR, Menezes MJ, Krieger H, Silva LH, Camargo EP: **High prevalence of asymptomatic Plasmodium vivax and Plasmodium falciparum infections in native Amazonian populations.** *Am J Trop Med Hyg* 2002, **66**(6):641-648.
38. Jongwutiwes S, Putaporntip C, Iwasaki T, Sata T, Kanbara H: **Naturally acquired Plasmodium knowlesi malaria in human, Thailand.** *Emerg Infect Dis* 2004, **10**(12):2211-2213.
39. Singh B, Kim Sung L, Matusop A, Radhakrishnan A, Shamsul SS, Cox-Singh J, Thomas A, Conway DJ: **A large focus of naturally acquired Plasmodium knowlesi infections in human beings.** *Lancet* 2004, **363**(9414):1017-1024.