METHODS MANUAL FOR PRODUCT TESTING OF MALARIA RAPID DIAGNOSTIC TESTS

Manual of Standard Operating Procedures for Assessment of Malaria Rapid Diagnostic Tests within the Product Testing Programme of the World Health Organization

At the US Centers for Disease Control and Prevention, Atlanta

Version Seven
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For internal use

Foundation for Innovative New Diagnostics (FIND)
Geneva, Switzerland

WHO Global Malaria Programme (GMP)
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Acknowledgements

This manual was developed from the recommendations of WHO informal consultations on malaria rapid diagnostic test quality assurance in Manila 2003 & 2004, Geneva 2006, Kisumu 2006, Atlanta 2006, Philadelphia 2007[1], subsequent meetings of the WHO-FIND malaria RDT evaluation programme steering committee, and the WHO *Methods Manual for Laboratory Control Testing of Malaria Rapid Diagnostic Tests*, with which it is designed to be used.

Version One of this manual was compiled by WHO-Regional Office for the Western Pacific, WHO-Special Programme for Research and Training in Tropical Diseases (TDR), the Foundation for Innovative New Diagnostics (FIND), US Centers for Disease Control and Prevention (CDC), Australian Army Malaria Institute (AMI), Research Institute for Tropical Medicine (Philippines), Hospital for Tropical Disease UK (HTD), and the Kenya Medical Research Institute.

Version Two modifications by FIND, in consultation with WHO/TDR
Version Three modifications by FIND, WHO/TDR, WHO/GMP and US CDC
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Version Six modifications by FIND, WHO/GMP, CDC, HTD
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[1] Malaria rapid diagnosis: making it work. Meeting report 20–23 January 2003. Manila: World Health Organization; 2003; Towards quality testing of malaria rapid diagnostic tests: evidence and methods. Manila: World Health Organization; 2006.

MALARIA RDT PRODUCT TESTING METHODS MANUAL
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Chapter 1: INTRODUCTION

1.1 List of Abbreviations

Term	Explanation
AMI	Army Malaria Institute
CDC	US Centers for Disease Control and Prevention
ELISA	Enzyme-linked immunosorbent assay
FIND	Foundation for Innovative New Diagnostics
GMP	Global Malaria Programme
FP	False positive
HRP2	Histidine rich protein 2
HTD	Hospital for Tropical Disease
IFU	Instructions for Use
IR	Invalid rate
pLDH	parasite lactate dehydrogenase
p/μL	Parasites per microlitre
PDS	Panel Detection Score: the percentage of parasite-positive panel samples of a defined parasite density against which all RDTs of both lots showed a positive result.
QA	Quality Assurance
QC	Quality Control
RDT	Rapid Diagnostic Test. For the purposes of this manual, this refers to immunochromatographic lateral flow devices for the detection of malaria parasite antigens
SOP	Standard Operating Procedure
TDR	WHO-World Bank-UNICEF-UNDP Special Programme for Research and Training in Tropical Diseases
WPRO	Western Pacific Regional Office
WHO	World Health Organization

1.2 Objectives and Scope of this Methods Manual

This procedure manual details a protocol for the product testing of antigen-detecting malaria rapid diagnostic tests (RDTs) using samples of frozen parasites and recombinant malaria antigen panels prepared for this purpose.

The manual describes a protocol to:

- assess accuracy of RDTs against a specimen bank consisting of recombinant antigen, culture-derived parasites and wild-type parasites, and parasite-negative blood samples;
- assess the stability of RDTs at various temperatures;
- describe aspects of RDTs affecting ease of use in the field;
- assess the labelling of RDT kits and the instructions of use; and
- record results in a database.

The manual also describes a protocol for stability testing by the manufacturer intended as an optional part of the WHO product testing programme.

This manual refers to standard operating procedures (SOPs) in the *Methods Manual for Laboratory Quality Control Testing of Malaria Rapid Diagnostic Tests, Version 8*, where collection and preparation of the wild-type samples is also described.

The manual is intended only for use in laboratories conducting product testing for the WHO.

1.3 Introduction

Malaria Rapid Diagnostic Tests (RDTs) are used increasingly for diagnosis of malaria, particularly in remote tropical areas where good microscopy-based diagnosis is impractical. RDTs must therefore be robust, simple and safe to use, and reliably demonstrate when malaria parasitaemia is, and is not, present. This manual details methods for product testing to allow comparison of the aspects of commercially-available tests that are relevant to procurement, particularly for use in the periphery of health services.

TECHNICAL ASPECTS OF TESTING MALARIA RDTs

Malaria RDTs, as referred to in this manual, are immunochromatographic lateral flow devices that detect parasite antigen. Capture of dye-labelled 'signal' antibody-antigen complex by a fixed 'capture' antibody produces a visible line on a nitrocellulose strip, signifying a positive test result. Different products target various antigens specific to *plasmodia*. Blood, product reagent and labelled antibody-antigen complex are drawn along the nitrocellulose-fiber strip by capillary action and flushing with a reagent /buffer solution.

Sensitivity of malaria RDTs is therefore dependent on several factors, including the rate of flow of blood up the nitrocellulose strip, the adherence of capture antibody (Ab) to the strip, ability of the Ab to bind antigen (Ag), and the integrity of the signal Ab-dye conjugate. All these factors are subject to deterioration in adverse transport and storage conditions, and rates of deterioration and their effect on outcomes can vary between products.

The relationship between antigen concentration and parasite density can vary with the degree of sequestration of parasites, the stage of parasite growth, and the persistence of antigen after reduction or elimination of the parasite population. Testing described in this manual is performed against a bank of recombinant antigen, culture-derived parasites, wild-type parasites and parasite-negative blood samples. Preparation of the wild-type samples is described elsewhere [1].

DEVELOPMENT OF THIS METHODS MANUAL

This methods manual is developed from recommendations of WHO informal consultations on quality assurance of malaria rapid diagnostic tests in Manila in 2003 [2], and Manila in 2004, Kisumu in 2006 [3], and Philadelphia in 2007. Subsequent changes have been guided by subsequent WHO informal consultants of the steering committee. Some procedures are referred to the WHO methods manual for quality control testing of RDTs [1], and the numbering of chapters and SOPs reflects the numbering in the same RDT quality control methods manual.

The control copy is based with the WHO-Global Malaria Programme and the Foundation for Innovative New Diagnostics. Correspondence should be addressed to malaria_rdt@who.int; cunninghamj@who.int (WHO/GMP);
or sandra.incardona@finddx.org (FIND).

1. Methods manual for laboratory quality control testing of malaria rapid diagnostic tests. Version 8, June 2016. Geneva: FIND / WHO-GMP; 2016 [http://www.who.int/malaria/publications/rdt-lab-quality-manual/en/].
2. Malaria rapid diagnosis: making it work. Meeting report 20–23 January 2003. Manila: World Health Organization;2003.
3. Towards quality testing of malaria rapid diagnostic tests: Evidence and methods. Manila: World Health Organization: 2006.

1.4 Specimen Bank Terms of Reference

Criteria for specimen banks / product testing sites for malaria rapid diagnostic test product evaluation (to be read in conjunction with *Towards quality testing of malaria rapid diagnostic tests: Evidence and methods*. Manila: World Health Organization; 2008.

BACKGROUND

Product testing will take place under coordination of WHO in one laboratory. This laboratory will receive and store samples from collecting sites contracted by WHO (or FIND, in collaboration with WHO), perform part of sample characterization, and test products submitted by manufacturers. They will maintain a database of samples, provide technical advice to regional RDT laboratories performing post-purchase testing, and dispatch specimens where requested to these laboratories.

TECHNICAL COMPETENCE REQUIREMENTS

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

EQUIPMENT AND SPACE REQUIREMENTS

- Freezers (-80^{0}C) with alarm and ensured power supply.
- Refrigeration (RDTs)
- Incubators with humidity control dedicated to project
- Bench space
- Storage space for RDTs (temperature-controlled)
- ELISA readers and related devices
- Computer access

TERMS OF REFERENCE

- Receive specimens, maintain a specimen bank, and associated sample database
- Characterize samples with quantitative ELISA, and further characterization as agreed with WHO
- Perform testing of malaria RDT on request from WHO, according to an agreed protocol, including:
    - Panel detection score, false positive rate, invalid rate against an agreed panel
    - Temperature stability testing
    - Ease-of-use assessment
    - Description of problems in RDT preparation and/or interpretation
    - Assessment of product labelling and IFU
- Collaborate in establishing a protocol for product testing

- Be overseen by an established IRB system, and obtain timely IRB approval for activities
- Work in collaborative way with regional laboratories and (other) specimen bank - product testing laboratory
- Be involved in an external quality assurance programme
- Collaborate with WHO in publication of results
- Provide technical advice to sub-regional RDT QA laboratories.

1.5 WHO-FIND RDT Evaluation Programme Steering Committee

Outline of role and function of the WHO-FIND Malaria RDT Evaluation Programme Steering Committee (formerly Specimen Bank Steering Committee), constituted to oversee technical aspects of the specimen bank and product testing programme.

BACKGROUND

The development of the specimen bank and product testing programme was the subject of WHO expert technical consultations in Manila in 2003 and 2004, Geneva, Kisumu and Atlanta in 2006. This expert group has been formalized by WHO oversee technical aspects of the programme.

SPECIMEN BANK STEERING COMMITTEE

The WHO-FIND Malaria RDT Evaluation Programme Steering Committee will oversee technical and logistical aspects of programme development, functioning by teleconference and face-to-face meetings at least once per year.

The Group will oversee the use of samples to ensure access is within the terms of reference and the ethical approval of sample collection.

TERMS OF REFERENCE

- The Malaria Diagnostic Specimen Bank and Evaluation Steering Committee will provide recommendations to WHO on:
- Development and modifications of SOPs for specimen collection and use
- Accumulation and content of specimen bank, and characterization and maintenance
- Policy on access to specimen bank
- Protocols for laboratory-based testing of the accuracy and stability of malaria RDTs, including product testing and lot testing
- Review and approve results of product testing, prior to publication.

MEMBERSHIP

- WHO/GMP (2)
- FIND (2)
- Specimen Bank(s): CDC (1)
- Collection sites (rotating)
    - 1 African, (1)
    - 1 non-African (1)
- Medicines Sans Frontieres (1)
- Hospital for Tropical Disease (UK) (1)
- Army Malaria Institute (Australia) (1)

Additional expertise may be added as needed at each meeting.

DURATION OF MEMBERSHIP

- Membership shall terminate if the institution ceases to have a contract with WHO, or FIND, with the exception of AMI and MSF.

- Contracted collection and lot-testing sites will rotate annually.
- Membership shall be reviewed (renewed or replaced) every two years.

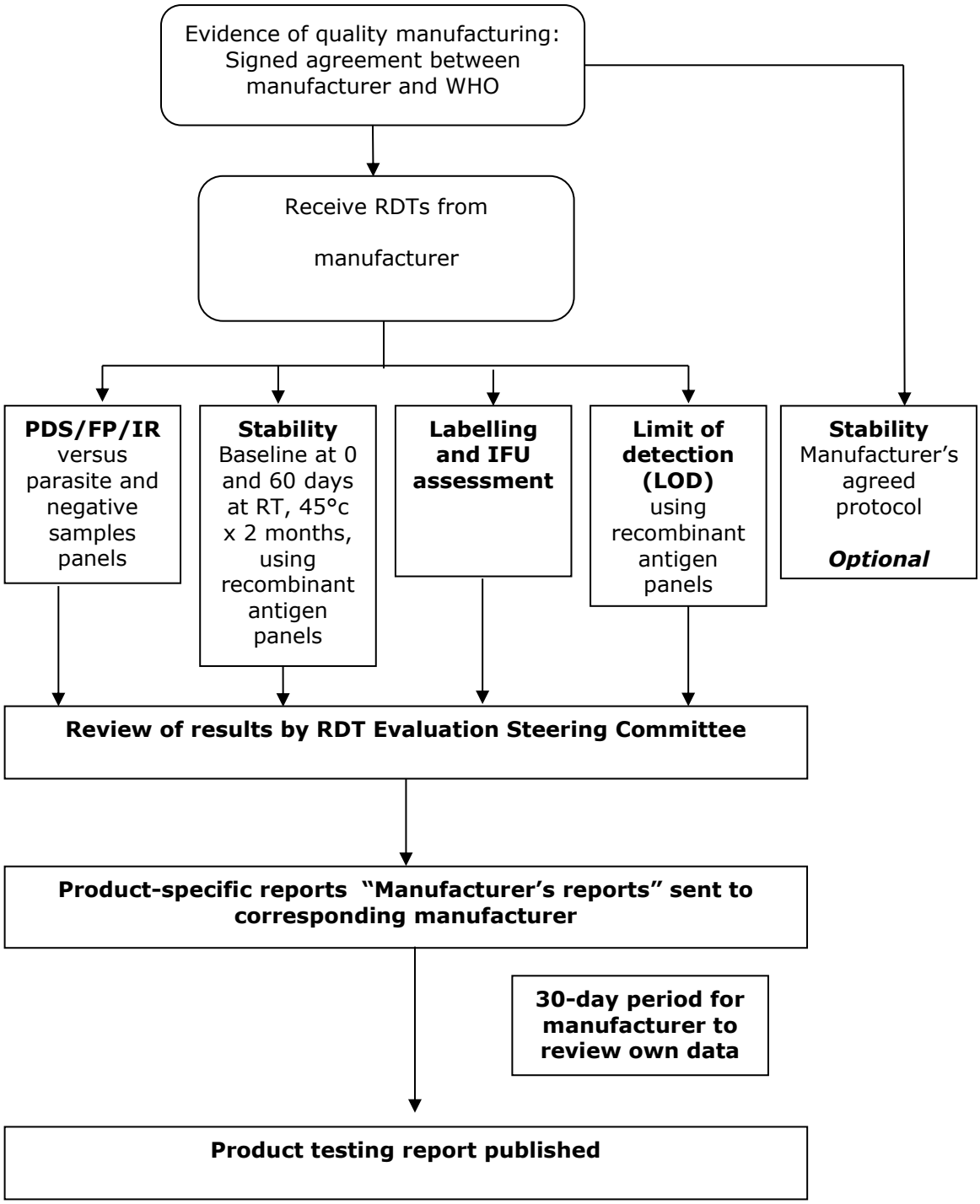
MODE OF OPERATION

The Malaria Diagnostic Specimen Bank and Evaluation Steering Committee shall meet at least annually. Between meetings, decisions on access to the bank and approval of results not affecting policy decisions may be made by a sub-group meeting by teleconference or email. This shall consist of a minimum of:

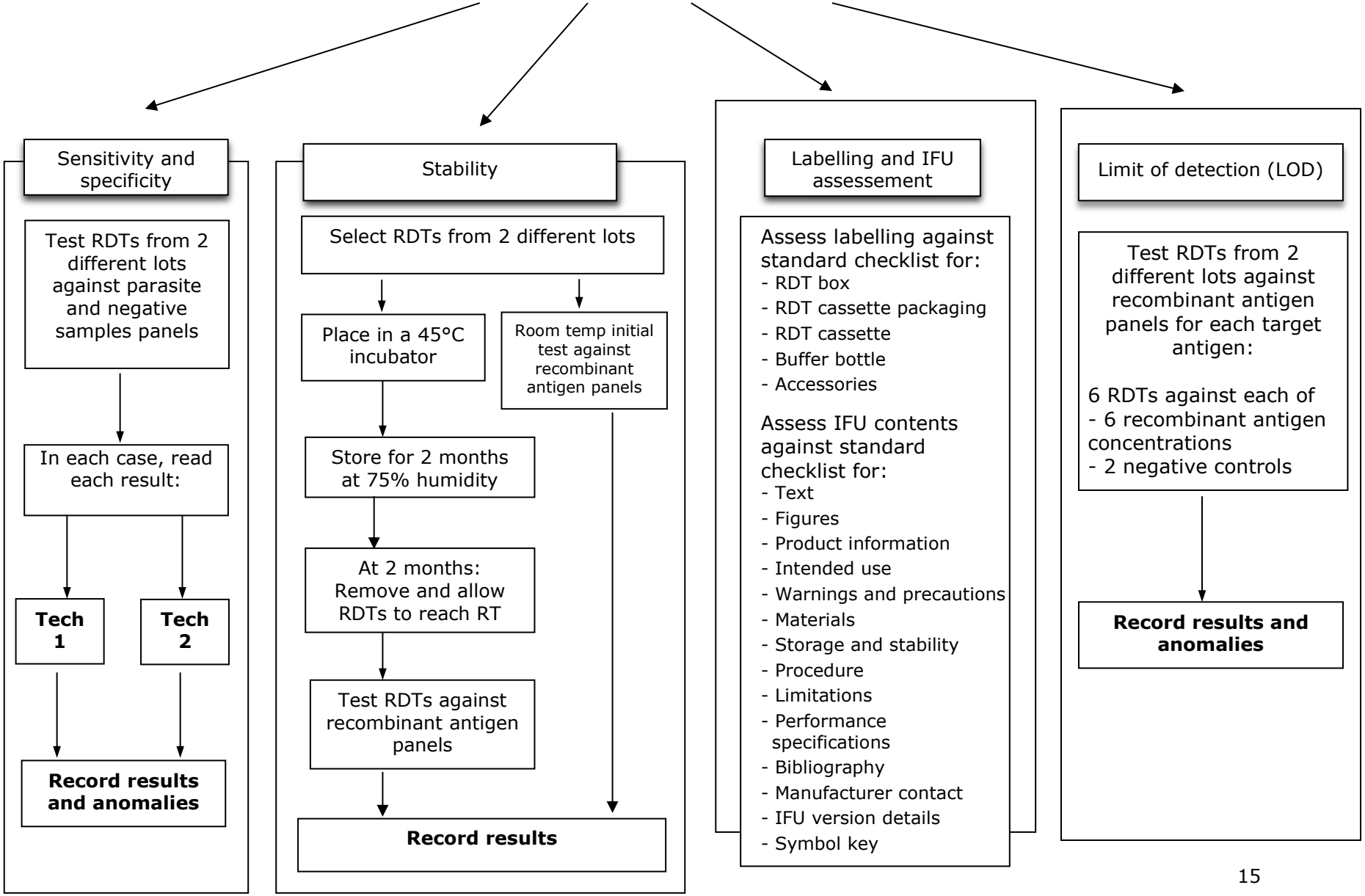
- WHO/GMP (1)
- FIND (1)
- CDC (1)

Two other participants of the most recent steering committee annual meetings.

1.6 Outline of Product Testing Protocol



1.7 RDT Product Testing Flow Chart Expanded



1.8 Selection of Evaluation (Challenge) Panel

The panel is outlined in Table in SOP 3.3.

All samples in the testing panel must have defined:
- Species (PCR)
- Geographical origin
- Parasite density
- Antigen concentration, determined by quantitative ELISA, for
    - HRP2 (for *P.falciparum* only)
    - pLDH
    - aldolase

The panel should have minimum turnover per year.

Prior to testing, it must be ensured that sufficient samples from a particular case are available to complete that round of testing on both lots of all products submitted. This can be determined as follows:

Number of aliquots required for parasite samples at 200 parasite/uL and negative samples, allowing for duplicate testing:

$$\frac{([\#\text{ products at } 5\mu L] \times 5) + ([\#\text{ products at } 10\mu L] \times 10)) \times 2 \times 2}{50} \times 1.25$$

Both equations allow 25% more blood than required.
B allows duplicate testing

CRITERIA FOR SELECTION

Culture-based *P. falciparum* samples:
HRP2: Should include a mixture of HRP2 type A, B and C types. Geographical origin: Should ideally include Asian, African and American parasites, preferably with a predominance of African.

Wild-type P. vivax samples:
Geographical origin depending on availability.

Both culture-based and wild-type samples:

i. Antigen concentration must be within the following range:

	HRP2	Pf LDH	Pf aldolase	Pv LDH	Pv aldolase
Minimum	0.6 ng/mL	0.2 ng/mL	0 ng/mL	1.6 ng/mL	1.7 ng/mL
Maximum	74 ng/mL	53.5 ng/mL	9.9 ng/mL	47.9 ng/mL	15 ng/mL

ii. These ranges have been determined by selecting the mid-90th percentile range of antigen concentrations based on the bank available for previous rounds of Product Testing Median.
iii. Concentrations for all target antigens must not significantly differ from panels of prior Testing Rounds.
iv. Geographical origin: Depending on availability. Should ideally include Asian, African and American parasites, preferably with a predominance of African origin for *P. falciparum*.

Parasite-negative samples:

i. 'Clean-negative' samples should be from afebrile patients, with no known infectious disease, blood dyscrasia or immunological abnormality. Samples from endemic countries should be confirmed as parasite-negative by PCR for *P. falciparum, P. vivax, P. ovale, P. malariae*, and *P. knowlesi.*
ii. Ratio of clean negative samples from endemic and non-endemic countries should be at least 1:1, with preference for endemic country specimens, if availability allows.
iii. 'Dirty negative' samples are prepared from blood samples from individuals presenting with the following blood factors or pathogens, which have been determined in previous Product Testing Rounds to present the highest risks of false positive test results: human anti-mouse antibodies (HAMA), anti-nuclear antibodies (ANA), rheumatoid factor (RhF), dengue, schistosomiasis. If possible, the 'dirty-negative' panel should be composed with equal numbers of samples for each of these sample types.
iv. The ratio of 'clean-negative' versus 'dirty-negative' samples should ideally be 1:1, depending on availability.

1.9 Noting and Varying Procedures in this Manual

Where procedures for product testing, such as preparation of RDTs, are covered by the Methods Manual for Laboratory Quality Control Testing of Malaria Rapid Diagnostic tests, this is noted in the text. In these cases, the introduction to the manual should be consulted regarding varying of operating procedures for product testing.

Further variations to procedures should be discussed within The WHO-FIND Malaria RDT Evaluation Programme Steering Committee, prior to modification.

For quality assurance purposes, a reference copy of the Methods Manual should be kept and procedures signed and noted by responsible officer/technicians overseeing the procedures to confirm it is understood (in the table at the end of each chapter). A further copy should be available in the laboratory /specimen collection site.

Hard copies of all forms should be retained, in addition to electronic archiving.

Malaria RDT Product Testing Methods Manual

Chapter 2: PRODUCT TESTING

SOP 2.1 RDT Register

PURPOSE

This Standard Operating Procedure (SOP) describes the process for receipt and dispatch of Rapid Diagnostic Tests (RDTs).

SCOPE

This procedure applies to the malaria RDT product testing programme of WHO and FIND with the US Centers for Disease Control and Prevention.

PROCEDURE

1. Front Desk Receipt (if samples are not received directly into laboratory)

a) Complete the RDT Front Desk Register (Form 002).
b) Contact the responsible officer.
c) If the responsible officer is not available or the consignment is received outside office hours, place the box in a climate controlled area with temperatures <25°C and contact the person as soon as possible.

2. Lab Receipt

a) Complete the RDT Register (Form 005).
b) File any accompanying transport documentation in the RDT register folder.
c) File the RDT company instructions for use in the designated folder.
d) Store the RDTs at ≤25°C immediately.
e) Label temperature monitor with date of receipt, for return to institution where it is to be read.

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials
24/02/14	6	Change reference to company methods sheet to instructions for use and indication for front desk receipt storage from refrigerator to ≤25°C	JC
11/12/17	7	Revised form 005	JC, SI

SOP 2.2 Rules for Evaluating RDTs

PURPOSE

This SOP outlines the overall principles for evaluating rapid diagnostic tests in the product testing programme.

SCOPE

This procedure applies to the malaria RDT product testing programme of WHO and FIND with the US Centers for Disease Control and Prevention.

OVERVIEW OF PROCEDURES

1. Selection of RDTs - Testing of separate lots

a) Evaluation of performance against the challenge panel:
Each lot of RDTs should be evaluated independently. Testing sets of Lot 1 and Lot 2 of each product are tested alternately, completing a testing set of Lot 1 of all products, then a testing set of Lot 2 etc, until both lots of all products are tested against all panel samples.

b) Stability assessment:
Where more than one incubator is required for a round of testing, lots should be stored in separate incubators.

Where RDT workload requires consecutive testing (insufficient incubator space available for all products in the testing round), evaluation of separate lots of the same product should be separated temporally.

2. Overall flow of testing against challenge panels

a) RDTs of both lots are tested against the *P. falciparum* culture and parasite-negative panel (*Phase 1*), and those reaching adequate performance criteria (see below) are tested against the full panel of wild-type and parasite-negative samples (*Phase 2*), and undergo stability testing.
b) Products that have previously reached WHO performance criteria in previous Rounds of product testing, are excluded from Phase 1 and pass direct to Phase 2 evaluation.
c) Products that are not intended to detect *P. falciparum* are only tested against the Phase 1 negative panel, and progress to Phase 2 based on false positive rate only.

3. Eligibility for testing against full challenge panel

If pass criteria achieved with culture-derived ***P. falciparum*** and parasite-negative samples (phase 1), then testing can proceed to challenge panel for that product. Pass for phase 1 (culture-derived ***P. falciparum*** and parasite-negative samples) requires:

- ≥80% overall Panel Detection Score (PDS)[2] for RDTs of both lots combined against 2000 parasites/μL samples.
- ≤50% false positive rate against 20 clean-negative samples tested on 2 RDTs of each lot (i.e. ≤40 of 80 FP across both lots)

*Note: Re-submitted products that have previously achieved WHO procurement criteria are **excluded from Phase 1**.*

4. Organization of testing, and interpretation and recording of RDT results

Over the entire testing round, all RDT products should be tested against all samples in the panel.

Organization of testing session

a) At beginning of a testing session, enter products to be evaluated and panel samples and densities (sample set) to be used.
b) Generate record sheets for the selected products and sample set, for each technician (2 sheets in total, Form 33). Separate results sheets are generated and completed for each technician.
c) A similar number of low density *P. falciparum,* high density *P. falciparum* and negative samples should be selected together, in a proportion calculated to ensure a similar proportion is maintained throughout the testing process, allowing RDTs to be tested against each low density and negative samples in duplicate, and high density sample once. *P. vivax* samples are included in a quantity to ensure to constant proportion throughout.
d) (Record sheets will include the required number of rows for low and high density samples, and random numbers for all samples).
e) A single technician should then re-label low-density and negative sample tubes with random number labels (Form 32). Where possible, this technician should not be involved in further testing of samples.
f) Perform testing and record results on Form 33:
   - First technician records visual result.
   - Second technician records visual result within 30 minutes and is blinded to first results.
   - The order of reading between technicians should be varied frequently over the testing period.
g) All result sheets and reporting should include the Product code (Catalogue Number) of the product.

5. Selection of samples from panel

See individual SOPs.

Number of aliquots required:
A: 2000 parasite/μL

$$\frac{([\#\text{ products at } 5\mu L] \times 5) + ([\#\text{ products at } 10\mu L] \times 10)}{50} \times 1.25$$

B: 200 parasite/μL and negative samples

[2] Panel Detection Score (PDS) is defined as the percentage of parasite-positive panel samples of a defined parasite density against which all RDTs of both lots showed a positive result.

$$\frac{(([\#\text{ products at } 5\mu L] \times 5) + ([\#\text{ products at } 10\mu L] \times 10)) \times 2}{50} \times 1.25$$

Both equations allow 25% more blood than required.
B allows duplicate testing.

The above is calculated by the programme at the start of each day.

Samples should be thawed for 30 minutes, and stored at 4^0C.
Samples should be discarded 8 hours after thawing.

6. Interpretation of results

a) Results of control and test lines are recorded by each technician by noting the test line intensities with a rating of 0 to 4, with 0 being noted in case of absence of a test line, and 1, 2, 3 or 4 being noted for visible test lines of varying intensities (from lowest to highest), using a standardized color chart provided by FIND.
b) Marked abnormalities or issues affecting interpretation, such as poor blood clearance, should be recorded in the comments section, using the standard abbreviations listed below.
c) Absent Control Lines: If control line is recorded as absent by either technician ('Invalid test result'), the test is recorded as invalid by that technician. (In such cases, the result is not included in calculation of detection rates during later analysis).

Notes recorded during RDT testing[3]:

Description	Short ID
Red background	RB
Red background obscuring test line(s)	ORB
Incomplete clearing	IC
Incomplete migration	IM
Failed migration	FM
Strip misplaced in cassette (shift)	SM
Specimen pad not seen in sample window	PAD
Ghost test lines	GL
Diffuse test lines	DL
Patchy broken test line	PL
Buffer remains pooled in buffer well	BP
Other (opens a box for typing free text)	OT

Abnormalities associated with buffers should be indicated and dated in the ease of use description form.

[3] *See document 'Malaria RDT Functional Anomalies' at https://www.finddx.org/wp-content/uploads/2016/02/malaria_rdt_guide_for_observations_30jul13.pdf*

Where blood clearance or flow is frequently poor or other anomalies are noted, digital photographs of at least one example should be obtained and saved electronically in a folder specific for the product name and code.

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials
14/04/2009	2	Invalid test result procedure changed	DB
11/05/2009	2a	Updated phase 1 pass criteria in line with Steering Committee and manufacturers letters	DB
12/03/2010	3	Clarification of selection of number of aliquots, and proportions of different samples, and test anomalies description	DB, SJ
10/6/2011	4	Minor clarification of technician reading order	DB
Aug-Sept 2012	5	Alternate lots 1 and 2 during testing (rather than sequential testing). Introduction of negative samples into phase 1. Standardization of reporting of anomalies.	DB, NC, JG, JC
24/02/14	6	Revision to number of negative samples in Phase 1 and criteria for moving to Phase 2	JC
14/04/14	6	Revised list of RDT anomalies and cataloguing of photos of anomalies	JC
19/11/15	7	Revision to conditions for exclusion of products previously meeting WHO Procurement criteria from phase 1.	JC
20/12/17	7	Updated forms 32 and 33, included rating of test line intensities, updated reference to standard list of RDT abnormalities.	JC, SI, JG

SOP 2.2a Evaluating Performance Against the Challenge Panel (Panel detection score/false positive rate)

PURPOSE

This SOP describes the procedure for positivity testing of a Rapid Diagnostic Test against parasite-positive and parasite-negative samples.

SCOPE

This procedure applies to the malaria RDT product testing programme of WHO and FIND with the US Centers for Disease Control and Prevention.

PROCEDURE (Fig. 1)

Follow the procedure for receipt of RDTs (SOP 2.1).
Store RDTs at ≤25°C.

1. Selection of RDTs

a) Select up to 5 products, preferably of similar format.
b) Select RDTs from one lot of each product (See SOP 2.2).
c) Determine number from required blood volume: use 50µL aliquot allowing 10µL margin. (See SOP 2.2 for equations.)
d) Keep RDTs and buffer with labelled product box on workbench, with clear workspace between different RDT products.
e) Keep RDTs in their unopened protective envelopes: do not open RDT envelopes until immediately before use.

2. Selection of panel samples (Table A)

PHASE 1 AND 2

Over the total testing round, all products should be tested against all samples of the panel, (in Phase 1: excluding re-submitted products that have previously achieved WHO product testing criteria, and are excluded from Phase 1, and non-*P. falciparum* products, that are tested against negative samples only).

Lot 1 and Lot 2 of each product are tested alternately, completing a testing set of Lot 1 of all products, then a testing set of Lot 2 etc., until both lots of all products are tested against all panel samples.

a. Calculate number of 50µL aliquots that will be required for each sample, for number of RDTs to be tested that day (from volume required for those products). See SOP 2.2.

PHASE 1

b. Select from the 20 Pf culture-derived panel and 20 clean-negative (≈10 endemic, 10 non-endemic origin (ratio of at least 1:1, if possible) samples:

- Select # high-density samples per strain
  and

- Select # low-density samples per strain
  and
- Select # clean negative samples
- Refer to SOP 2.2 for quantity of aliquots

Follow randomization and re-labelling procedure (Section 2.2a, 9)

- Record RDT product and samples withdrawn for testing on randomization chart (Form 32)

PHASE 2

c. Select from 100 wild-type HRP2 positive *P. falciparum*, 100 wild-type or culture-derived HRP2 negative *P. falciparum*, 35 *P. vivax* wild-type panel and 80 parasite-negative samples (as 20 clean negative samples have been used already during Phase 1):

- Select # high-density samples per strain
  and
- Select # low-density samples per strain
  and
- Select # negative samples
- Refer to SOP 2.2 for quantity of aliquots.
  Schedule of samples should be set-up beforehand to ensure all testing groups have similar ratios of high and low density, species, and negative samples across Phase 1.

Follow re-labelling procedure (Section 2.2a, 9)

- Record RDT product and samples withdrawn for testing on randomization chart (Form 32)

RANDOMIZATION PROCEDURE
- Generate labels of sequential numbers from 1 upward, for number of samples
- Remove aliquots required for testing session from freezer
- Mix aliquots randomly in container / or bench, then remove one by one
- Place label on each tube as they are removed, and note correlation between ID and label number in Randomization Chart (Form 032)

PHASE 1 AND 2

3. Allow aliquots to thaw, then store at 4°C when not in use.

4. All samples withdrawn must be used within 8 hours of thaw. Do not re-freeze.

5. Follow RDT preparation procedure for each product, using calibrated micropipette to obtain correct blood volume

6. Reading the RDTs

a) Read first result by technician 1 at near minimum time recommended by manufacturer.
b) Read second result (technician 2) within 30 minutes of minimum time.

7. Invalid test results

Where control line is not visible, record result as 'invalid'. Do not repeat test.

8. Record results on separate visual reading record sheets

Record test line intensities for each test line of each RDT tested, as described in SOP 2.2, using Form 033. Each technician records his/her results on a separate paper copy of form 033. Then transcribe results in dedicated database with double data entry.

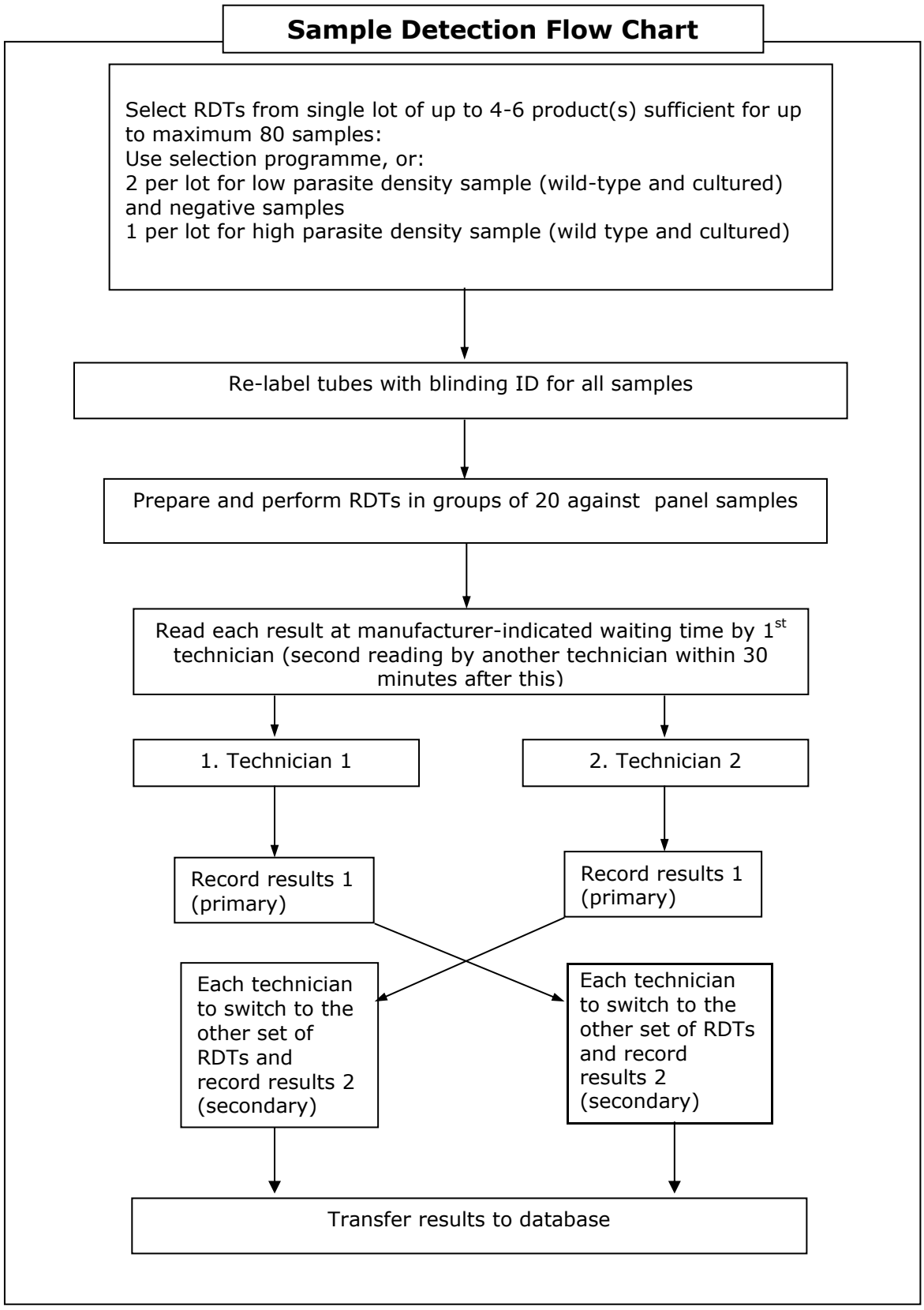
Table A. Number of RDTs (per lot) required for testing (Not including heat stability assessment)

Panel	Panel samples	RDTs at each dilution	All tests per lot
Pf culture panel	20 lines	2 low, 1 high	60
Phase 1 negative panel	20 samples	2	40
Recombinant HRP2	*8 dilutions*	*6 per dilution*	*Optional, not published*
Recombinant pfLDH	*8 dilutions*	*6 per dilution*	
Recombinant PvLDH	*8 dilutions*	*6 per dilution*	
Recombinant aldolase	*8 dilutions*	*6 per dilution*	
Wild type Pf, HRP2 positive	100	2 low, 1 high	300
Wild type or culture-derived Pf, HRP2 negative	40	2 low, 1 high	120
Pv	35	2 low, 1 high	105
Po	0		0
Pm	0		0
Phase 2 Negative panel	80	2	160
Total tested			**785**
Spare ~15%			115
Total (excl. recombinant testing)			**900**

Table B. Pass criteria for sensitivity/specificity testing against Phase 1 panel

Sample	Pass Criteria	Notes
Culture-derived *P. falciparum* samples	Detection of 16 of 20 samples (80%) at 2000 parasites/µL equivalence, by both lots	Failures may go through limited further testing to elucidate performance against antigen variants
Clean-negative samples	Negative results on at least 40 of 80 RDTs tested (2 RDTs on 20 samples x 2 lots)	

Fig. 1. Sample Detection Flowchart



9. Transfer results to database – data entry 1 and 2

For each test conducted with each sample, transcribe all results (i.e. test line intensities for each test line, including the control line, and any observations of abnormalities) in the dedicated database, by using double data entry. In case of discrepancies highlighted by the database at the second data entry, cross-check results against the paper form 033 and discuss with the relevant technicians, as required.

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials
14/04/2009	2	Densitometer removed, panel modifications, invalid result procedure, other minor changes	DB
Aug-Sept 2012	5	Alternate lots 1 and 2 during testing (rather than sequential testing). Introduction of negative samples into phase 1. Standardization of reporting of anomalies. Update of randomization procedure	DB, JG, JC
Feb 2014	6	Increase of negative samples in Phase 1 from 10 to 20 and corresponding changes to Phase 2, Table A and Table B	JC
Dec 2017	7	Added panel of HRP2 negative Pf samples, updated number of tests for recombinant panels, updated Table A, provided more detail on how to record results and transcribe into database, some editing of text and Figure for more clarity	JC, SI

SOP 2.2b RDT Temperature Stability Assessment

PURPOSE

This SOP describes the procedure for stability testing of a Rapid Diagnostic Test.

SCOPE

This procedure applies to the malaria RDT product testing programme of WHO and FIND with the US Centers for Disease Control and Prevention.

PROCEDURE (Refer to Fig. 1)

1. General

a) RDTs are tested at time = 0, and at 2 months. Between time=0 and 60 days (2 months); RDTs are stored at room temperature (21-25 °C) and in incubators programmed at 35 °C and 45 °C and 75% humidity.
b) At time = 0, from each lot to be tested at that time, the required number of RDTs are stored each at room temperature, at 35°C and 45°C
   - 4 to be tested against a negative sample,
   - 19 to be tested against 200 parasite/μL *P.falciparum* and *P.vivax* samples (15 against Pf and 4 against Pv),
   - 48 to be tested against each recombinant protein panel HRP2, Pf LDH, Pv pLDH and/or aldolase (allowing for 6 RDTs tested against each of 8 dilutions), depending on which antigen(s) is/are targeted by the RDT's test line(s),
   - 7 to be tested against 2000 parasite/μL *P.falciparum* samples and *P.vivax* (5 against Pf and 2 against Pv),
   - 5 spare RDTs.
c) Allow a minimum of 3 days to calibrate incubators prior to conducting baseline testing, and record temperature and humidity daily throughout the incubation period.
d) All dates for all stability tests should be clearly marked on a wall calendar and/or laboratory diary in the laboratory.
e) Where incubator capacity is insufficient for all RDTs, lots should be tested sequentially.
f) Where more than one incubator is used, each lot of a product should be in a separate incubator.
g) Required number of RDTs for each temperature assessment should be made up from RDTs from at least 4 boxes (kits) of each RDT production lot (to reduce chance of non-random intra-RDT variability within the lot). Buffer will be taken from 1 box (kit) only, unless provided with each test envelope.
h) RDTs should be stacked in incubators to allow air circulation against at least 2 sides of box, and not in direct contact with walls or floor of incubator.
i) Boxes may be placed in open plastic containers if there is concern about deterioration of cardboard packaging during incubation
j) Remove RDTs from incubators on day = 60.
   If it is not possible to test RDTs on day = 60, they should be placed at 4°C and must be tested within 7 days.

2. Preparation of product and sample

a) Select products according to selection protocol (SOP 2.2) and print record sheets.

b) Where lots are incubated in parallel, test lots of the same product separately.[4]
c) Test in air-conditioned (low humidity) environment, at ≤25°C with good lighting.
d) Thaw aliquots designated for RDT stability testing
e) Store samples aliquots at 4°C when not in use
f) All samples must be used within 8 hours of thaw. Do not re-freeze.
g) Note that blinding is not possible, as a varying numbers of RDTs are tested against different samples.
h) Select sufficient RDTs for a small set of aliquots.
i) Follow RDT preparation procedure for each product, using calibrated micropipette to obtain correct blood volume.

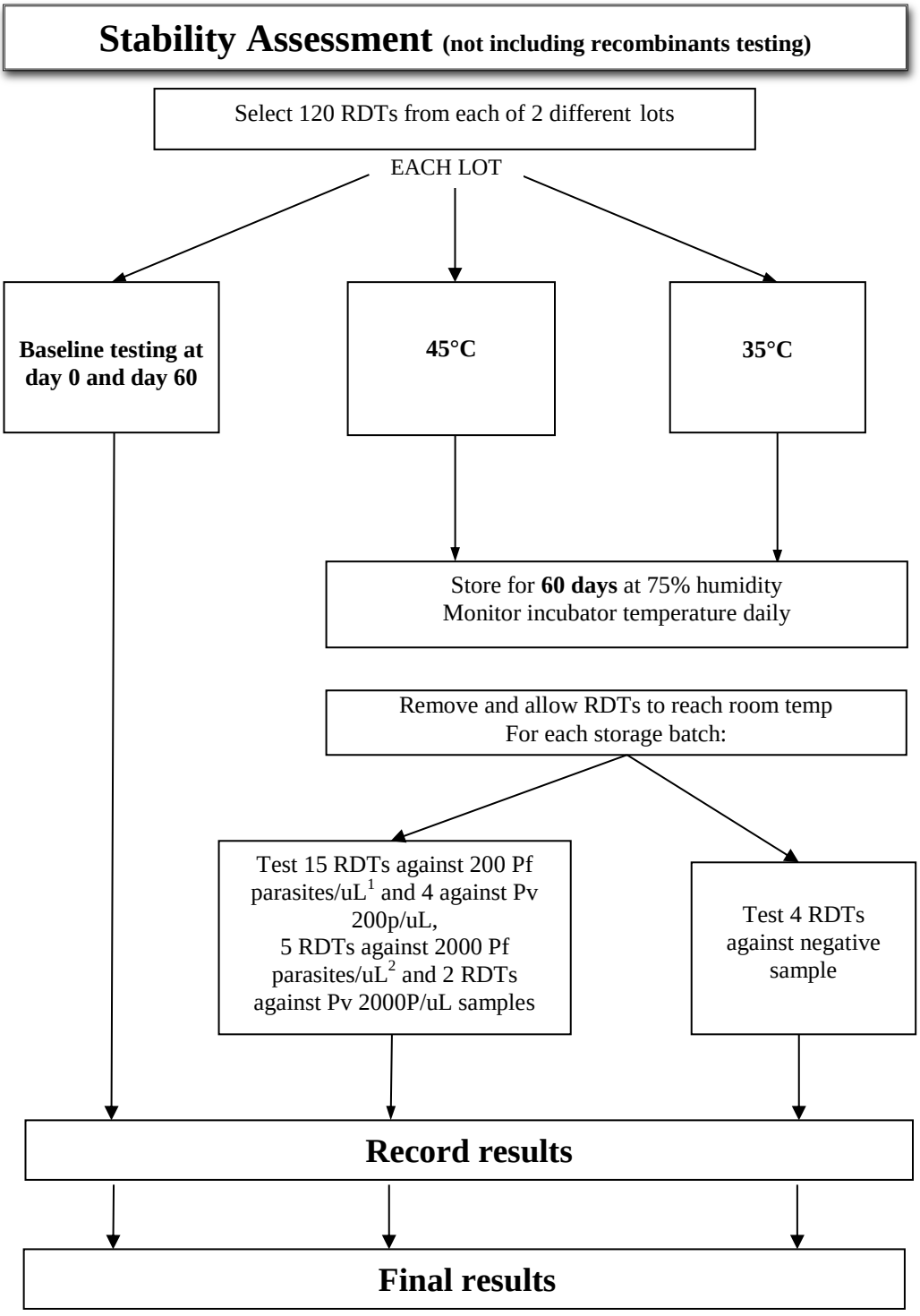
3. Reading results

a) Read first result by first technician at near minimum time recommended by manufacturer.
b) Read second result (technician 2) within 30 minutes of first reading.
c) Invalid test results: Where control line is not visible, record result as 'invalid'. Do not repeat test.
d) Record on separate record sheets (Forms 035) the test line intensities for each test line (including control line) and any observations of abnormalities, as described in SOP 2.2
e) Then transcribe results into dedicated database, with double data entry

Table A. Number of RDTs required per lot for STABILITY TESTING

Test	**RDT required for Pf, Pv and negative samples**	**Recombinant HRP2, PfLDH, PvLDH and/or aldolase, depending on antigen(s) targeted by the RDT test line(s)**
Baseline	30	48 per antigen
Baseline x 2mth	30	48 per antigen
45°C x 2 mth	30	48 per antigen
35°C x 2 mth	30	48 per antigen
Total tested	120	192 per antigen

[4] Testing single product at a time reduces chance of error in preparation technique.


Stability Assessment (not including recombinants testing)
Select 120 RDTs from each of 2 different lots
EACH LOT
Baseline testing at day 0 and day 60
45°C
35°C
Store for 60 days at 75% humidity
Monitor incubator temperature daily
Remove and allow RDTs to reach room temp
For each storage batch:
Test 15 RDTs against 200 Pf parasites/uL[1] and 4 against Pv 200p/uL,
5 RDTs against 2000 Pf parasites/uL[2] and 2 RDTs against Pv 2000P/uL samples
Test 4 RDTs against negative sample
Record results
Final results

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials
14/04/2009	2	Invalid test result procedure added. Densitometer removed. RDTs from number of kit-boxes at each temperature.	DB
12/03/2010		Revised numbers of tests at each temperature, per SC decision	DB
21/6/2011		Addition of PvpLDH panels	DB
13/7/2011		Procedure for recombinant panel preparation	JG, DB
Aug-Sept 2012	5	Addition of recombinant antigen to stability testing panel.	DB, RDT-SC
Feb 2014	6	Addition of primate Pv samples as option; corrected totals for required RDTs and parasite density in flow chart; revised source of recombinant and deleted concentrations	JC, JB, JG, IG
Dec 2017	7	Added wild-type Pv samples and recombinant panels; updated RDT totals in the text, table and flowchart, some more detail on how to record results	JC, SI

SOP 2.2c Ease of Use Description

PURPOSE

This document outlines the protocol for assessing the ease of use and safety of a rapid diagnostic test. Itis a description of the test device itself as well it may include description of device design details, packaging, labelling and ancillary items.

SCOPE

This procedure applies to the malaria RDT product testing programme of WHO and FIND with the US Centers for Disease Control and Prevention.

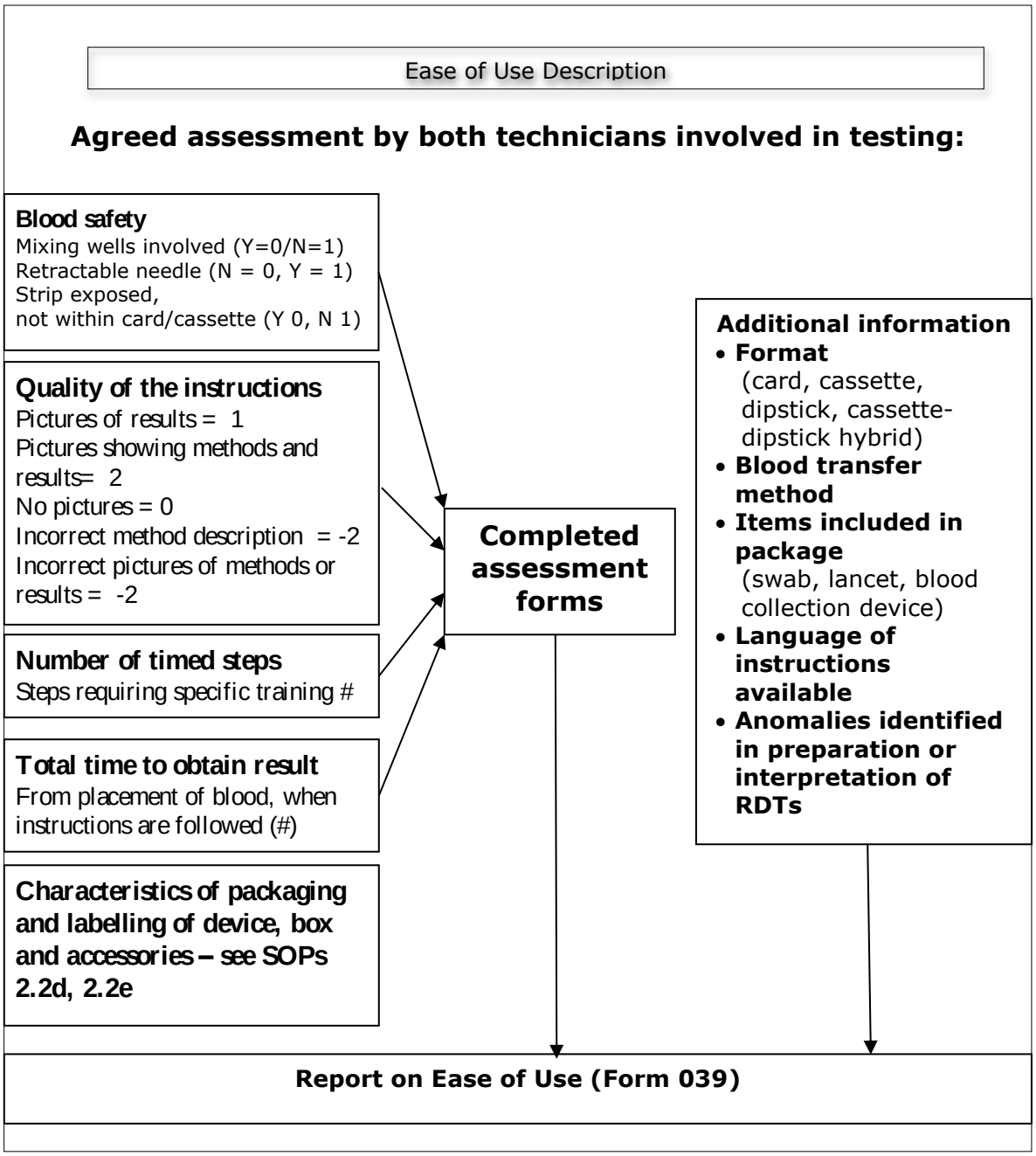
PROCEDURE

1. The Ease of Use Description should be completed after performance testing is completed, and technicians are experienced in the use of the device.

2. Two technicians should jointly assess the test, accompanying materials, including instructions for use, accessories +/- packaging and labelling according to Figure 1 (below).

3. The assessment should be entered on Form 039.

4. Any problems noted with the test preparation and/or interpretation during the challenge and stability studies should be noted under the comments section of Form 039.



PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials
Feb 2014	6	Expansion to include i) description of device and kit packaging and labelling, as well as accessories; ii) addition of negative points for incorrect methods or pictures of methods or results; iii) mention of anomalies being reported	JC
2018	7	Added reference to SOPs 2.2d and 2.2e for IFU and labelling assessments	SI

SOP 2.2d Assessment of RDT Product Labelling

PURPOSE

This SOP outlines the overall principles for assessing manufacturer's adherence to international standards for labelling malaria RDT products including the packaging, the device, components, and accessories.

SCOPE

This procedure applies to the malaria RDT product testing programme of WHO and FIND with the US Centers for Disease Control and Prevention.

PROCEDURE

Complete checklist: HarTchecklist: best practices labeling and IFU found here:

http://www.who.int/malaria/publications/atoz/round-7-malaria-rdt-checklist-best-practices-labelling-IFU.pdf?ua=1

http://www.who.int/malaria/publications/atoz/round-7-malaria-rdt-annex-aid-labeling-IFU.pdf?ua=1

SOP 2.2e Assessment of Instructions for Use

PURPOSE

This SOP outlines the overall principles for assessing manufacturer's adherence to international standards and WHO recommendations and preferences for malaria RDT instructions for use.

SCOPE

This procedure applies to the malaria RDT product testing programme of WHO and FIND with the US Centers for Disease Control and Prevention

PROCEDURE

Complete checklist: HarTchecklist: best practices labeling and IFU found here:

http://www.who.int/malaria/publications/atoz/round-7-malaria-rdt-checklist-best-practices-labelling-IFU.pdf?ua=1

http://www.who.int/malaria/publications/atoz/round-7-malaria-rdt-annex-aid-labeling-IFU.pdf?ua=1

SOP 2.2f Stability Assessment at Manufacturing Site

This SOP was required for Rounds 1 and 2 of Product Testing, and becomes optional for manufacturers from Round 3 onward.

PURPOSE

This SOP describes the procedure for stability testing of a Rapid Diagnostic Test at the manufacturing site, using standards provided by the WHO-FIND malaria rapid diagnostic test evaluation programme.

SCOPE

This procedure applies to manufacturers participating in the malaria RDT product testing programme of WHO and FIND with the US Centers for Disease Control and Prevention.

TESTING PROCEDURE (Refer to Fig. 1)

1. General

a) Testing should occur at Day = 0, using 24 RDTs from each of 2 lots (total 48).
   - Test 8 RDTs against 200 parasites/µL
   - Test 8 RDTs against 2000 parasites/µL
   - Test 8 RDTs against negative sample

b) Store sufficient RDTs in the incubator/environmental chamber to allow testing of 40 RDTs (20 per lot) every 3 months for the remaining duration of the shelf-life designated for that product by the manufacturer, and sufficient RDTs at 4°C (2–8°C) to allow testing of RDTs (12 per lot) every 3 months for the remaining duration of the shelf-life.

c) Every three months, test 20 RDTs from each lot stored in the incubator and 12 from each lot stored at 4°C:
   From 4°C storage:
   - Test 4 RDTs against 200 parasites/µL
   - Test 4 RDTs against 2000 parasites/µL
   - Test 4 RDTs against negative sample

   From incubator:
   - Test 8 RDTs against 200 parasites/µL
   - Test 8 RDTs against 2000 parasites/µL
   - Test 4 RDTs against negative sample

d) Incubated RDTs should be stored at the maximum storage temperature recommended by the manufacturer.
e) Allow a minimum 3 days to calibrate incubators prior to conducting baseline testing.
f) Incubator temperatures and refrigerator temperatures should be recorded daily on a chart attached to the incubator or closely accessible
g) Mark days when testing is due at 3-month intervals for remainder of shelf life.
h) RDTs should be stacked in incubators in their normal packaging (boxes / kits), allowing air circulation against at least 2 sides of box, and not in direct contact with walls or floor of incubator.

i) All documentation should be readily accessible if manufacturer site inspections occur.

Table A. Number of RDTs required <u>per lot</u> for Stability Testing

Test	**RDT required *per lot at storage temperature***	**RDT required *per lot at 4℃***	**Total RDTs (both lots)**
Baseline	24		48
Each 3 months	20	12	64
Expiry date	20	12	64
Example for 2 year shelf-life, 3 month old lot when testing commences:			
Baseline	*24*		*48*
6 x 3 months	*120*	*72*	*384*
Expiry date	*20*	*12*	*64*
Spare	*20*	*12*	*64*
Total per lot	***184***	***96***	***560***

2. Preparation of product and sample

a) Blood samples to be used s the positive standard for testing are supplied from WHO / CDC (manufacturer must arrange and fund courier), in aliquots of 200 parasites/μL and 2000 parasites/μL, and should be stored at ≤-20°C. Test in air-conditioned (low humidity) environment, at ≤25°C with good lighting.
b) Thaw sufficient aliquots of blood samples designated for RDT stability testing for 30 minutes at room temperature (<30°C) (Each aliquot is approximately 50 μL, so for RDTs requiring 5 μL blood, it is recommended to thaw 3 samples)
c) Store samples aliquots at 4°C after thawing
d) All blood samples must be used within 8 hours of thaw. Do not re-freeze.
e) Withdraw the correct number of RDTs of each lot from storage (incubator and 4°C – Fig. 1) and allow to reach room temperature before opening envelope.
f) Test the correct number of RDTs from each lot (diagram below), against aliquots of 200 parasites/microL and 2000 parasites/μL and parasite-negative samples. (If all RDTs have failed at 200 parasite/μL at a previous testing interval, this aliquot may be removed from the testing procedure at future testing intervals. Follow manufacturer's RDT preparation procedure for each product, using pipette or manufacturer's blood transfer device to obtain correct blood volume.
g) If both lots of a product fail to detect the 200 parasites/μL sample at the initial test, the 200 parasite/μL sample may be excluded from future testing and subsequent tests at 3-month intervals conducted using the 2000 parasites/μL sample only.

3. Reading and reporting results

a) Read result within the time period specified by manufacturer, rating line intensity 0-4 against colour intensity chart provided (Use colour closest to colour of positive line).
b) Record on the record sheet provided in hard copy and electronic copy.
c) Submission of results may cease once all RDTs have failed at any testing interval.

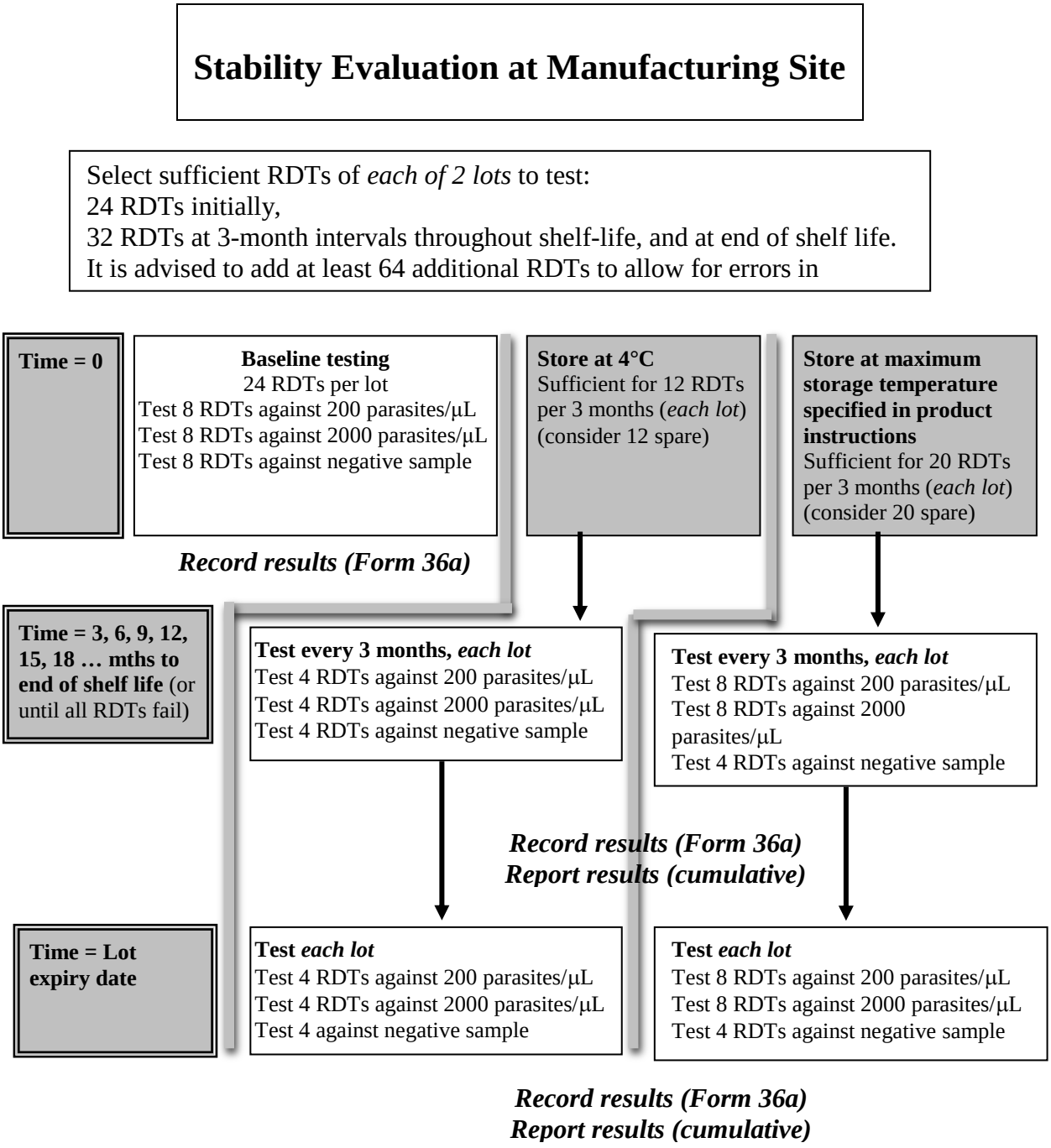


Fig. 1. Stability Test Flowchart

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials
25/02/10	3	SOP becomes optional	DB
2018	7	Re-numbered the SOP; removed mention about submission of results to WHO	JC

SOP 2.3 Performing a Rapid Diagnostic Test (RDT)

PURPOSE

This SOP describes the procedure for performing a Rapid Diagnostic Test.

SCOPE

This procedure applies to the malaria RDT product testing programme of WHO and FIND with the US Centers for Disease Control and Prevention.

PROCEDURE

1. RDTs must be brought to room temperature first prior to use (<30°C).
2. Remove the RDT packaging.
3. Check desiccant for any color changes (i.e. blue to white etc, if relevant). If present, discard RDT and use another kit for testing.
4. Test the RDT as per manufacturer's instructions. Use calibrated micropipettes (note: most RDT kits have blood collecting tubes or sample loops) when dispensing blood onto the cassette.
5. Results of control and test lines are recorded with a rating of 0 to 4, with 0 being noted in case of absence of a test line, and 1, 2, 3 or 4 being noted for visible test lines of varying intensities (from lowest to highest), using a standardized color chart provided by FIND.
   Marked abnormalities or issues affecting interpretation, such as poor blood clearance, should also be recorded as comments, using the standard abbreviations listed below.
   If control line is absent, the test is recorded as invalid.

Notes recorded during RDT testing[5]:

Description	Short ID
Red background	RB
Red background obscuring test line(s)	ORB
Incomplete clearing	IC
Incomplete migration	IM
Failed migration	FM
Strip misplaced in cassette (shift)	SM
Specimen pad not seen in sample window	PAD
Ghost test lines	GL
Diffuse test lines	DL
Patchy broken test line	PL
Buffer remains pooled in buffer well	BP
Other (opens a box for typing free text)	OT

[5] *See document 'Malaria RDT Functional Anomalies' at https://www.finddx.org/wp-content/uploads/2016/02/malaria_rdt_guide_for_observations_30jul13.pdf*

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials
2018	7	Added calibrated micropoipettes, and more details about reading and recording of results.	SI

SOP 2.4 Result Communication Pathway

AIM

To provide guidelines for the communication of results of RDT product testing between relevant stakeholders

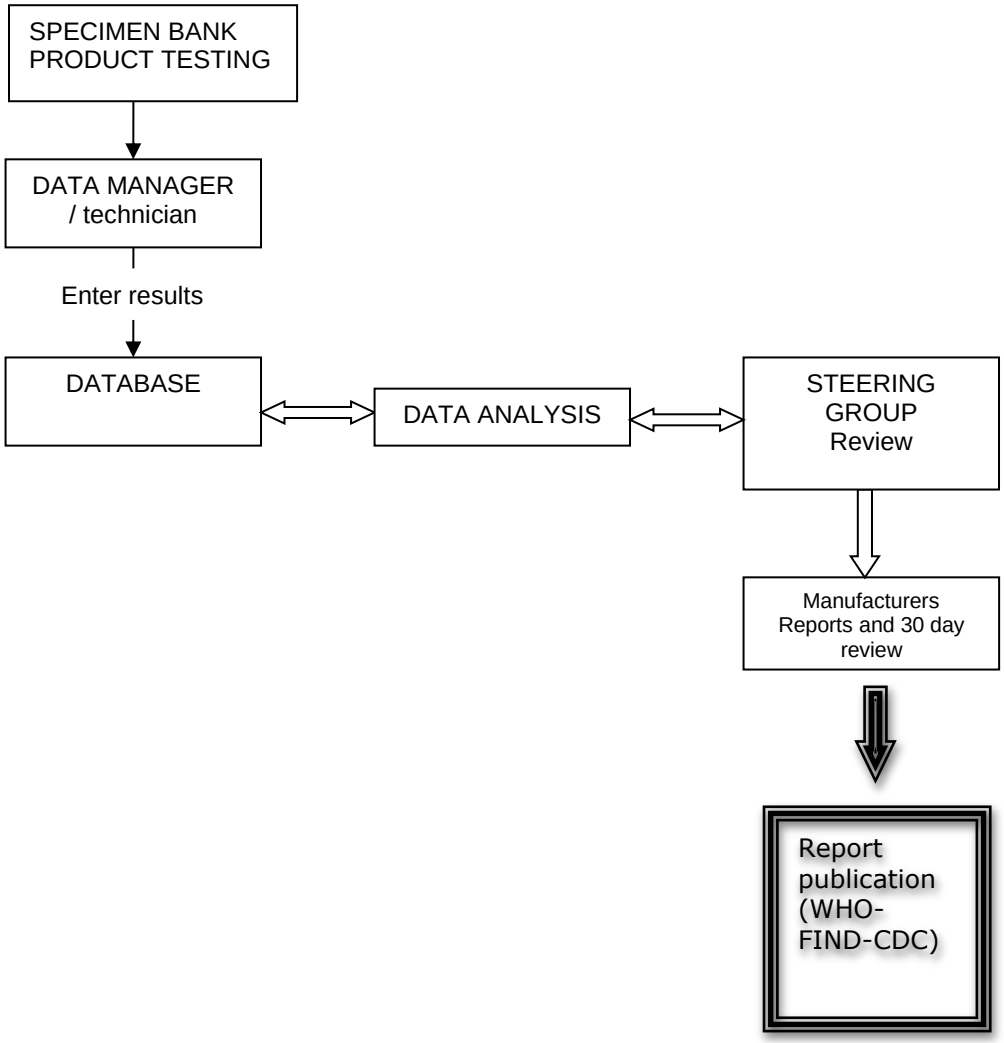
SCOPE

This procedure applies to the malaria RDT product testing programme of WHO and FIND with the US Centers for Disease Control and Prevention.

PURPOSE

This document specifically relates to the communication of results obtained through the process of Product Testing (immediate and long term) of malaria RDTs.

TEST RESULTS DATABASE FLOW



PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials
12/03/2010	3	Data analysis added to diagram	DB
14/08/2014	6	Manufacturers reports and report publication added to diagram	JC

SOP 2.5 Proper Storage of RDTs

AIM

To maintain quality of (and blood-borne virus) RDTs while transporting, storing and using in the field

BACKGROUND

Malaria rapid diagnostic tests and rapid tests for other diseases including HIV and Hepatitis B and C are biological tests that deteriorate on exposure to high temperature, and deteriorate rapidly on exposure to high humidity. They may also deteriorate through freeze-thawing. To maintain sensitivity, it is important to store in as close as possible to the conditions specified by the manufacturer.

PURPOSE

This SOP describes the procedure for storing and using malaria (and blood–born virus) RDTs.

SCOPE

This procedure applies to the malaria RDT product testing programme of WHO and FIND with the US Centers for Disease Control and Prevention.

EQUIPMENT

Electronic temperature monitors

Thermometers

PROCEDURE

1. Keep RDTs in controlled temperature storage (21-25°C) and maintain all RDT products at same temperature and conditions. Record temperature daily.

2. Refrigerated storage is required for RDTs used for comparison in heat stability trial (4°C). Do not freeze.

3. Do not use RDTs if moisture-proof envelope is damaged, or the desiccant color indicates exposure to moisture.

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials

SOP 2.6 RDT Interpretation Standardization

PURPOSE

This SOP describes quality assurance procedures for RT interpretation by technican readers during product testing

SCOPE

This procedure applies to the malaria RDT product testing programme of WHO and FIND with the US Centers for Disease Control and Prevention.

INTRODUCTION

RDTs in the product testing programme are rated on the basis of visual detection of a test line on an RDT, read in good light at the recommended reading time of the manufacturer. This best reflects the conditions of intended product use. Technician readers are rotated throughout product testing process to minimize the effects of inter-reader test interpretation.

Line intensity is assessed relative to a standard colour chart produced by the Programme. As testing is performed against many samples with relatively low antigen concentration, test lines are frequently very faint, and some variation in interpretation of very faint lines (present, versus non present) is inevitable. This SOP is aimed at minimizing variation between readers and standardizing interpretation.

PROCEDURE

1. Prior to commencing reading in product testing programme:
2. Achieve normal level vision on Snellen reading chart, using visual supports to be used in routine reading.
3. Complete Ishihara reading chart.
4. Complete correct reading of 3 full test results charts for Pf-specific and combination tests.
5. Results of Ishihara reading chart to be discussed within programme if abnormal, and more detailed testing of ability to interpret tests undertaken (e.g. with red background).
6. Prior to commencement of RDT testing round, and at least every month during testing:
7. Assemble at least used 20 RDTs recently tested, including at least 5 with very faint test lines.
8. Technician readers to read assembled tests blinded to results of other readers.
9. Compare and document comparative results. Discuss discrepant results and interpretation.

MATERIALS

Snellen Chart.pdf
Use of Snellen Chart.pdf
Ishihara chart.pdf
Generic Pf Quiz1.pdf
Generic Pf Quiz2.pdf

Generic Pf Quiz3.pdf
Generic Pf Quiz Answer keys.pdf
Generic Pf Pan Quiz1.pdf
Generic Pf Pan Quiz2.pdf
Generic Pf Pan Quiz3.pdf
Generic Pf Pan Answer Keys.pdf

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials
Aug-Sept 2012	5	This section introduced	DB, JG

Malaria RDT Product Testing Methods Manual

Chapter 3: PANEL

SOP 3.1 Receipt and Storage of Panel Samples

PURPOSE

This Standard Operating Procedure (SOP) describes and references the process for receipt and Storage of Specimen Bank Panel samples.

SCOPE

This procedure applies to the malaria RDT product testing and lot testing programmes of WHO and FIND with the US Centers for Disease Control and Prevention.

PROCEDURE

1. Front Desk Receipt (If Applicable)

a) Responsible officer should be contacted immediately to arrange appropriate storage of panel samples.

2. Lab Receipt

a) Note full contents of sample transport containers while transferring to -80°C storage
b) Reconcile list of samples received with the list shared prior to shipment transmitted from consigner
c) Update electronic sample database with new samples received.
d) Transmit updated list to consignor, and responsible officer, indicating any discrepancies with transport list previously received from consigner.
e) File any accompanying transport documentation in the shipment register folder.

3. Lab Dispatch – See SOP 3.19 of the Methods Manual for laboratory quality control testing of malaria RDTs version 8 (June 2016)

a) Complete the standard MS Excel file ("Blank Sample Information Form_Standard File") with all sample information, complete the consigner form with numbers of aliquots to be dispatched, and transmit to the consignee and responsible officer.
b) Confirm date of delivery will be a business day, when consignee is able to receive and process appropriately. Await confirmation that shipment can proceed.
c) Having received confirmation from consignee that shipment can proceed; follow procedure for shipment of samples (SOP 3.5) and further documentation requirements (SOP 3.6). Usually, packaging and transport will be undertaken by a contracted courier. The consigner will be expected to provide a full inventory of contents, matching the consignee database, and assist the contracted courier in completion of other required documentation and packaging of samples.

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials
14/08/14	6	Reference to SOP 3.19 MM for lab QC v.7 added	JC
2018	7	Reference updated to MM for lab QC of RDTs version 8. Edited text for more clarity, and included reference to standard Excel file for sample information.	SI, JC

SOP 3.2 Panel Sample Characterization

PURPOSE

This document specifically relates the details of each sample type that will be used for product testing of malaria rapid diagnostic tests (RDTs).

SCOPE

This procedure applies to the malaria RDT product testing programme of WHO and FIND with the US Centers for Disease Control and Prevention.

AIM

To provide details referring to number of isolates and parasite density for sample types used for product testing of RDTs.

PROCEDURE

1. For all isolates of cultured *P. falciparum:*

a) HRP2, aldolase, and pLDH content will be quantitated by ELISA (See SOPs 5.1–5.4).

2. For all isolates of wild-type *P. falciparum* and *P. vivax*:

a) HRP2 (for *P. falciparum* only), aldolase, and pLDH content will be quantitated by ELISA (See SOPs 5.1–5.4).
b) Nested PCR assay will be performed for species identification (See SOP 5.8).
c) HRP2 structure may be characterized by PCR amplification and sequencing (see SOP 5.9), in case of *P. falciparum* only.

3. For all parasite-negative samples:

a) Nested PCR assay to confirm absence of *Plasmodium* DNA
b) Confirmatory test for pathology (e.g. Rh F positive)

Sample type	Details	P	L	LM
Cultured *P. falciparum*	20 isolates (mixture of HRP2 A, B, C)	X		X
	200 parasite/uL equivalence, 2000			
Recombinant antigen[b] To determine LOD	6 Serial dilutions of HRP2, PvLDH, PfLDH and aldolase	X	X	X
Wild-type *P. falciparum* Total 100 common *P.falciparum*,	200 parasite/µL and 2000[a] parasite/µL dilution	X	X	
Optional addition of further HRP2 variants	Sites: Asia/Pacific Africa South and Central America			
	10-15 isolates per site, if availability allows			
	Characterized by ELISA, PCR (species)			
	Optional - characterize HRP2 structure			
Wild-type *P. vivax*	35 isolates 200 and 2000[a] parasite/µL	X	X	
Parasite-negative human blood Total 100, with numbers for each sample type depending on availability	Anti-nuclear antibody (ANA) 5-10	X		
	RPR positive 5-10			
	Rheumatoid factor positive 5-10			
	Heterophile antibody positive 5-10			
	Anti-mouse antibody positive 5-10			
	Clean negatives (none of above) 50		X	
	Other tropical diseases[e], including: Chagas disease dengue typhoid leishmaniasis schistosomiasis			

a rarely 5000 parasite/µL dilutions are used for some samples rather than 2000 parasite/µL dilutions
b Optional, not used for formal product assessment to date
P: Product testing panel. L: Lot-testing panel. LM: Manufacturers panel.
Shaded sections are not currently in use.

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials
14/04/2009	2	Panel table updated	DB
14/08/2014	6	HRP2 structure ID is not done systematically. Therefore changes made to reflect this.	JC
2018	7	Added recombinant panels, removed the not applicable ones (e.g. primate parasites; in text added P. vivax	SI, JC

SOP 3.3 Use of Panel Samples

PURPOSE

This Standard Operating Procedure (SOP) describes the method for the use of panel samples.

SCOPE

This procedure applies to the malaria RDT product testing programme of WHO and FIND with the US Centers for Disease Control and Prevention.

PROCEDURE

1. Take out required panel samples from the freezer and place on a rack.
2. Leave on the bench and let stand at room temperature for 30 minutes.
3. Randomize samples according to SOP 2.2a.
4. Once thawed, panel samples may be utilized.
5. Store inside the refrigerator at 4°C if not for immediate use.
6. Vortex for 10 seconds prior to opening.
7. Samples should be used within 8 hours of thawing.

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials
2018	7	Included randomization of samples and vortexing prior use.	JC

SOP 3.4 Packaging of Quality Control Samples for Transport

PURPOSE

This Standard Operating Procedure (SOP) describes methods for proper packaging prior to transport of QC samples.

SCOPE

This procedure applies to the malaria RDT product testing and lot testing programme (SOP 3.17 MM laboratory quality control manual version 7) of WHO and FIND with the US Centers for Disease Control and Prevention.

BACKGROUND

Proper packaging and labelling of the material being shipped is vital to maintaining the integrity of the specimens, preventing accidents, and ensuring that there are no delays due to violations of regulations. The packaging requirements for various types of laboratory materials are subject to international and national regulations. There are a number of licensed agencies worldwide that provide training for personnel who need to know how to package materials in compliance with international regulations.

The international regulations for the transport of infectious materials by any mode of transport are based upon the Recommendations of the United Nations Committee of Experts on the Transport of Dangerous Goods (UN). International organizations such as the Universal Postal Union (UPU), the International Civil Aviation Organization (ICAO), and the International Air Transport Association (IATA) have incorporated these recommendations into their respective regulations. The World Health Organization serves in an advisory capacity to these bodies.

The regulations specify five types of materials that must meet the requirements for safe transport. The requirements differ depending on which category of material is being shipped:

a) **Infectious substances:** Those substances known or reasonably expected to contain pathogens. Pathogens are defined as microorganisms (including bacteria, viruses, rickettsiae, parasites, fungi) or recombinant microorganisms (hybrid or mutant), that are known or reasonably expected to cause infectious disease in animals or humans.

b) **Diagnostic specimens:** Any human or animal material including, but not limited to, excreta, secreta, blood and its components, tissue and tissue fluids being transported for diagnostic and investigation purposes, but excluding live infected animals.

c) **Biological products:** Those products derived from living organisms, which are manufactured and distributed in accordance with the requirements of national governmental authorities which may have special licensing requirements, and are used either for prevention, treatment, or diagnosis of disease in humans or animals, or for related development, experimental or investigational purposes. They include, but are not limited to, finished or unfinished products such as vaccines and diagnostic products.

d) **Genetically modified micro-organisms and organisms:** Micro-organisms and organisms in which genetic material has been purposely altered through genetic engineering in a way that does not occur naturally.

e) **Clinical waste and medical waste:** Clinical waste and medical waste are wastes derived from the medical treatment of humans or animals or from bioresearch, where there is a relatively low probability that infectious substances are present.

f) **Carbon dioxide, solid (dry ice):** Dry ice is classified as a dangerous good by IATA. The product does not contain oxygen and may cause asphyxiation. Exposure may cause nausea and respiratory problems, and contact may cause frostbite.

g) **Other dangerous goods:** Under this classification are cryogenic liquids, ethanol solutions, methanol, pyridine, strong formaldehyde solutions, hypochlorite solutions, aviation regulated liquids, and iodine.

In general, all of the above categories of materials should be shipped using the basic triple packaging system, in addition to the specific requirements necessary for that category (see sections below for category specific instructions). Packaging materials for this system should be manufactured in compliance with the Dangerous Good Regulations. There are a number of manufacturers who can provide containers manufactured to these specifications. The triple packaging system is (Fig. 1):

a) **Primary receptacle:** A labelled primary watertight, leak-proof receptacle containing the specimen.

b) **Secondary receptacle:** A second durable, watertight, leak-proof receptacle (e.g. plastic bag) to enclose and protect the primary receptacle(s). Several wrapped primary receptacles may be placed in one secondary receptacle. Sufficient additional absorbent material must be used to cushion multiple primary receptacles. Specimen data forms, letters, and information to identify the specimen, the sender, and the receiver should be placed in a waterproof bag and taped to the outside of the secondary receptacle.

c) **Outer shipping package:** The secondary receptacle is placed in an outer shipping package that protects it and its contents from outside influences such as physical damage and water while in transit.

PROCEDURE

Preliminary note on applicable instructions:

For the purpose of transport, malaria RDT quality control (QC) samples are treated as Biological substance Category B. Packing instruction therefore fall under IATA dangerous goods regulations packing instructions 650: Infectious substances in category B.

The use of dry ice requires a declaration of Dangerous Goods class 9, UN1845, and must comply with packing instruction 904. The instructions given here comply with all these rules.

Packaging and transport is usually overseen by a professional courier company when shipping between countries. In such cases, the courier's instructions on packaging should be followed. These should comply fully with the relevant IATA regulations, and regulations of the countries of the consigner and the destination and through which the

package is transiting. Laboratory personnel involved in the process should familiarize themselves with this SOP prior to the courier's arrival, to facilitate rapid packaging and transfer. IN cases of in-country transport by the specimen collecting institution, staff should fully familiarize themselves with the SOP and with national regulations, and liaise beforehand with the airline concerned.

1. All quality control (QC) sample aliquots must be packaged in sealed cryovials (e.g. screw-cap tubes with O-ring) supplied by the WHO-FIND programme, and labelled with the complete ID code (e.g. PH01 F04 2000).

2. During manipulation and transfer of the QC sample aliquots, extreme care must be taken to avoid thawing. Prepare foam boxes with ice packs for quick transfers and handle the aliquots as quickly as possible. If possible, work in a cool air-conditioned room.

3. The sealed tubes must be placed in a cryboxes and then in suitably sized plastic bag together with a small amount of absorbent material, for example cotton wool. The bag must be sealed, either using a bag heat-sealer or waterproof adhesive tape.

4. The cryoboxes must be labelled with the ID code of the QC sample aliquots and with the Biological Substances Category B.

5. The plastic bags may then be placed in sealable paper bags, labelled with the relevant ID codes and the UN 3373.

6. To ensure samples remain frozen during transport, the bags must be placed in a container (foam box) with cooling material (dry ice). Ensure that all bags are well covered with dry ice, and that the amount if dry ice is sufficient for the expected transport time.

7. The foam box must then be placed in an outer packaging. The outer packaging must conform to *IATA Dangerous Goods Regulations Packaging Instruction 650*. The box must have the appropriate markings on the outside.

   An extra label is required on the outside of the over pack stating:

   "INNER PACKAGES COMPLY WITH PACKING INSTRUCTIONS 650"

8. The outer packaging must be labelled with the following information (Fig. 2):
   - The sender's name, address and contact telephone/fax numbers.
   - The UN Classification numbers and proper shipping names:

UN 3373
BIOLOGICAL SUBSTANCE CATORGORY B
[MALARIA Vol. X mL]
UN 1845 DRY ICE

   - The total volume X of QC sample aliquots contained in the package.
   - The weight of dry ice included in the package at commencement of shipment.
   - The receiver's name, address and contact telephone/fax numbers.
   - ***UN 3373 label and Biological Substance Category B***
   - ***Miscellaneous label class 9*** (if dry ice is being used).

9. It may be of benefit to include an additional label requesting: "Keep package at -70°C ". The box should be sealed using wide sealing tape, taking care not to obscure the labels with the tape and leaving a gap for venting of the dry ice.

10. All infectious substances must be accompanied by a **Sender's Declaration for Dangerous Goods**, indicating shipment of infectious substances and the use of dry ice in the shipment.
    A list of quality control samples contained in the package should be included in an envelope within the outer container, and taped on the outside of the outer packaging. For more detail on transport documentation, see SOP 3.6

11. Commercial couriers may elect to transport samples under a higher IATA classification.

Fig. 1. Packaging of quality control sample aliquots

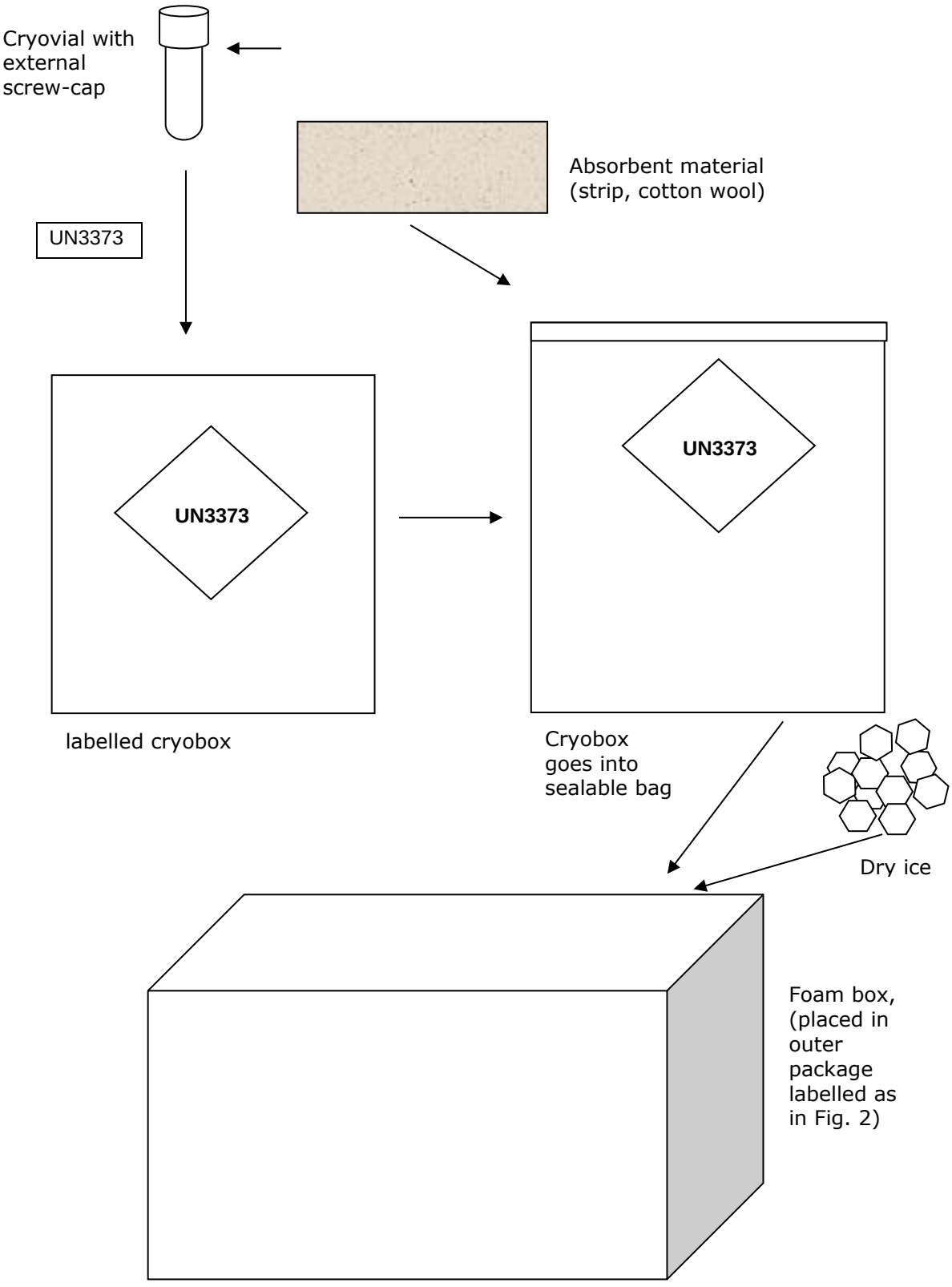
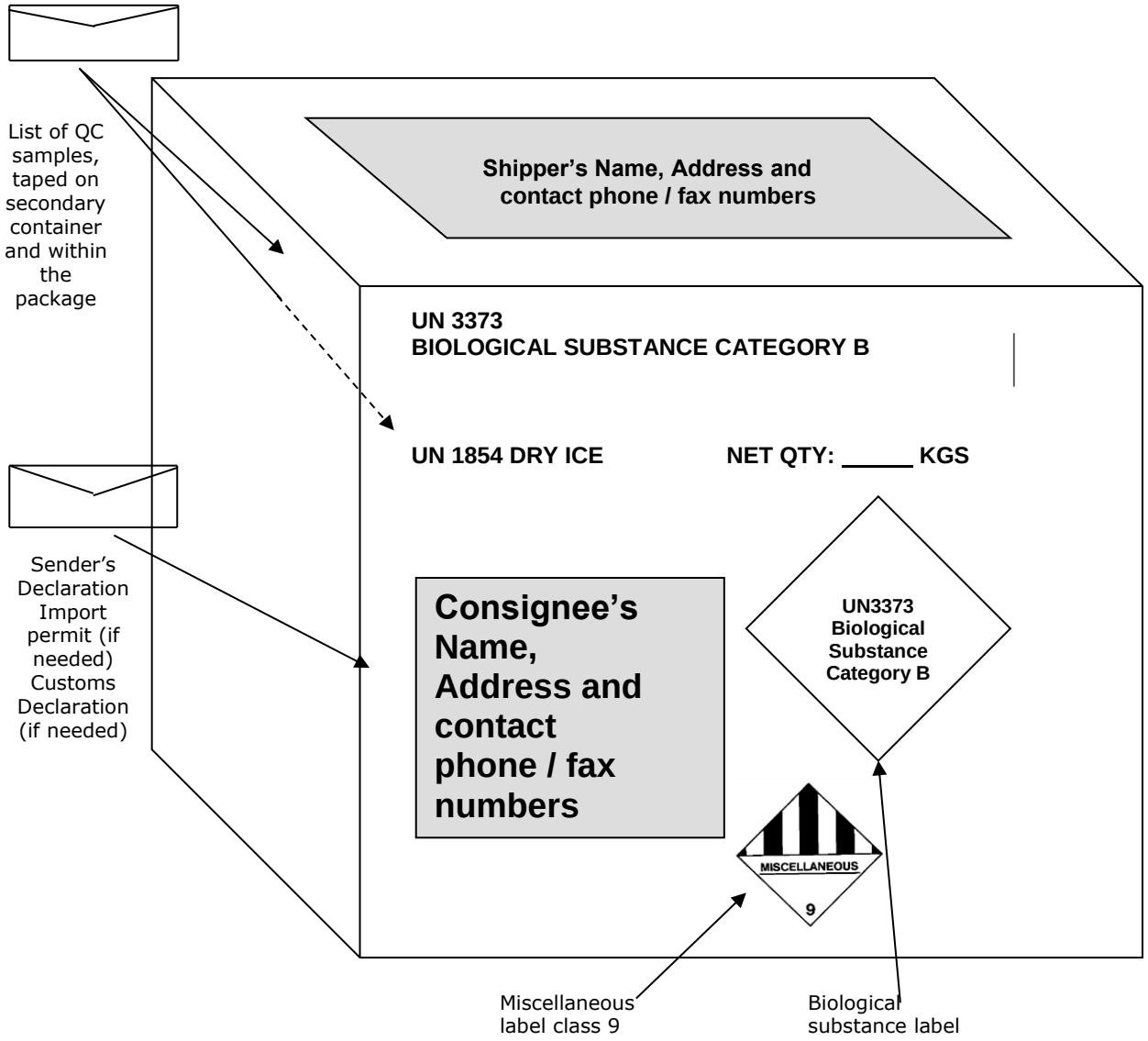


Fig. 2. Example of correct labelling for a shipment of infectious substances (QC samples, dangerous goods label class 6) chilled with dry ice (dangerous goods label class 9)



REFERENCES

1. Requirements and guidance for external quality assessment for health laboratories. Geneva: World Health Organization; 1989 (WHO/DIL/LAB/99.2).
2. Standard operating procedure manual for WHO Polio Laboratory – Chapter 9: Specimen and isolate transport. Melbourne: Victoria Infectious Diseases Reference Laboratory; 2001,
3. Infectious substances shipping guidelines: the complete reference guide for pharmaceutical and health professionals, 7th edition. Montreal: International Air Transport Association (IATA); 2006.

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials

SOP 3.5 Documentation of Transported Panel Samples

PURPOSE

This Standard Operating Procedure (SOP) describes the essential documents required when transporting panel samples, in addition to documentation required by consignee and consignor countries for transport of human blood products.

SCOPE

This procedure applies to the malaria RDT product testing and lot testing programme (SOP 3.18 Methods Manual Laboratory Quality Control Version 7, 2014), of WHO and FIND with the US Centers for Disease Control and Prevention.

PROCEDURE

For the transport of product testing panel samples, the following documents need to be prepared:

1. Documents completed within Institution:

a) Specimen Referral and Receipt Logs: The institution should keep a standard record detailing dispatch and receipt of Product Testing of specimen bank panel samples. The record should contain a description of specimen including volume, destination and source, mode of transport, date sent and received, and designated lab personnel (consignee and consignor). Problems encountered during transport or receipt should be documented. The record should be kept in the laboratory at all times.

2. Documents to attach to package for transport:

a) Sender's Declaration of Dangerous Goods: It is recommended to include 6 copies for international shipments and 2 copies for domestic packages. See Fig. 1 for an example.

b) A packing list: which includes shipping name, the receivers address, the number of packages, detail of contents (UN 3373 - Biological Substance Category B) source, weight, value (required for international shipping only – Fig. 1)

c) Customs declaration with appropriate information for national authorities including UN 3373 - Biological Substance Category B declaration.

d) Instruction sheet: This document describes the nature of the specimens, prescribed manner of handling, and the purpose for which the material will be used. Appropriate background on the material, such as screening tests done, potential hazards, and storage conditions are also included.

e) Airway bill (Fig. 2): The airway bill should be marked with the following information:
- Name, address, telephone/fax of receiver
- Number of specimens
- "Highly perishable"
- "Contact receiver upon arrival" (include telephone number)

- Airway bill handling information
- **"URGENT: DO NOT DELAY: Biological specimens -- highly perishable -- store at -70°C"**

f) Export/import documentation e.g. waiver letter. These vary with national government regulations, and the onus is on consignee and consignor to ensure regulations are fulfilled prior to transport.

Fig. 1. Example of packing list for attachment to outside of shipment

1 November 2003

TO WHOM IT MAY CONCERN:

This shipment contains Biological Substance Category B in accordance with IATA packing instruction 650. These samples are to be used for research and laboratory testing purposes only. These samples have no commercial value and are not for resale. For customs purposes only we place a nominal value of US $10.

Contents:

Full scientific name: Human blood containing dead malaria parasites
Volume per vial: 0.05 mL
Number of vials: 2000
Country of origin: The Philippines

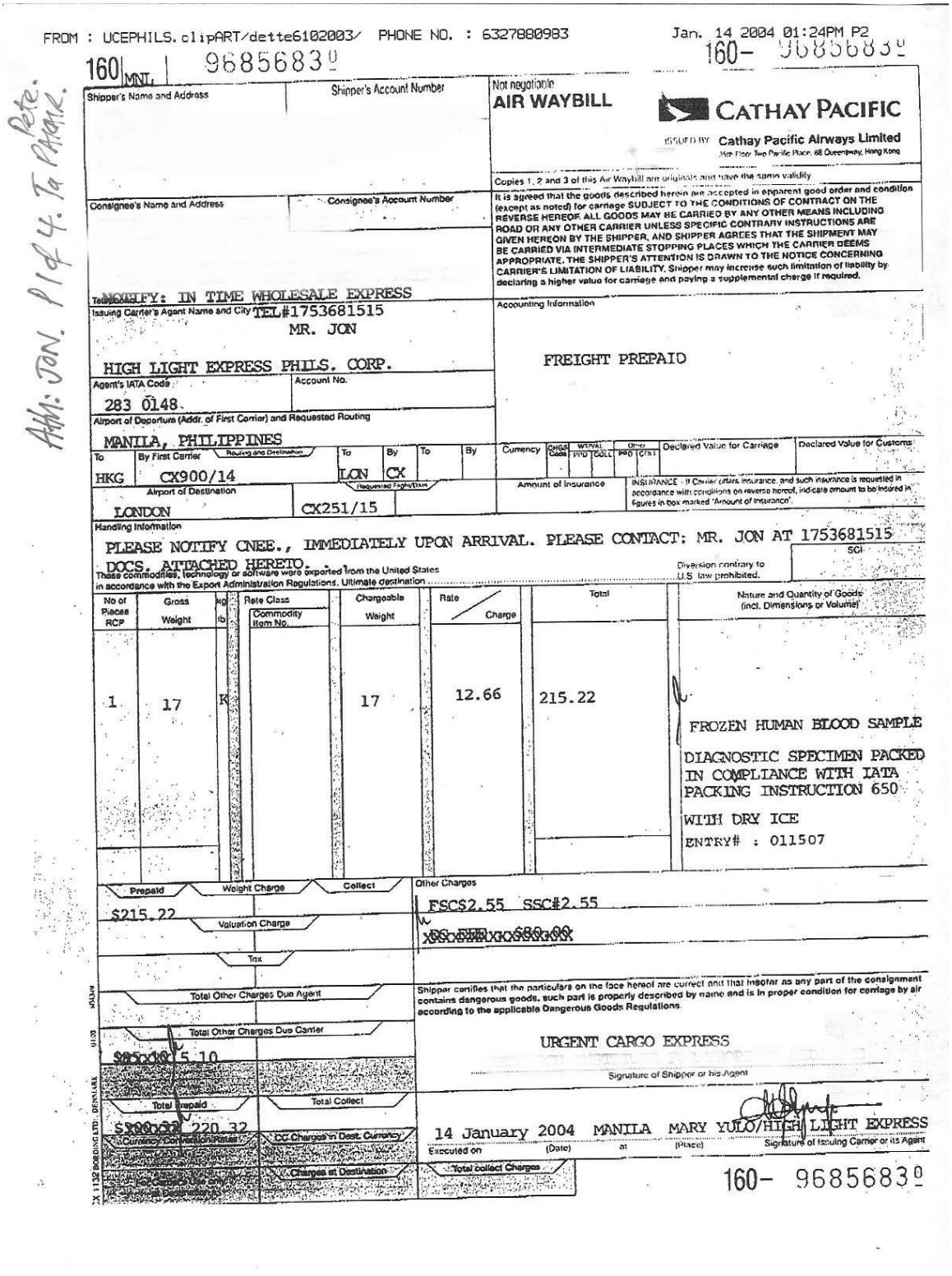
From: <Consigner>
<Address>

To: <Consignee>
<Address>

Value - US$10.00

e.g.
World Health Organization
Regional Office for the Western Pacific
UN Avenue
1000 Manila
PHILIPPINES

Fig. 2: Example of a completed dangerous goods form for the transportation of an infectious substance on dry ice. The statement "over pack used" need only be included if such a packaging system was used.



REFERENCES

1. Requirements and guidance for external quality assessment for health laboratories. Geneva: World Health Organization; 1989 (WHO/DIL/LAB/99.2).
2. Standard operating procedure manual for WHO Polio Laboratory – Chapter 9: Specimen and isolate transport. Melbourne: Victoria Infectious Diseases Reference Laboratory; 2001.
3. Infectious substances shipping guidelines: the complete reference guide for pharmaceutical and health professionals, 7th edition. Montreal: International Air Transport Association (IATA); 2006.

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials

SOP 3.6 Coordination of Transport of Panel Samples

PURPOSE

This Standard Operating Procedure (SOP) describes guidelines for coordination of shipment of quality control samples for testing malaria RDTs. The transport of quality control samples requires careful planning and coordination between the consignor, the carrier and the consignee.

SCOPE

This procedure applies to the malaria RDT product testing and lot testing (SOP 3.19 Methods manual laboratory quality control version 7, 2014) programmes of WHO and FIND with the US Centers for Disease Control and Prevention.

PROCEDURE

1. Transport planning

a) It is the responsibility of the sender to ensure the correct designation, packaging, labelling and documentation of all materials sent from the laboratory.

b) The efficient transport of infectious materials requires good co-ordination between the sender (the shipper), the carrier, and the receiver (the consignee or receiving laboratory), to ensure that the material is transported safely and arrives on time and in good condition. Such co-ordination depends upon well-established communication and a partner relationship among the three parties.

2. Advance arrangements with the consignee

a) Once it has been decided that materials need to be shipped from the laboratory, the receiver should be contacted and informed of the nature of the materials to be sent.
b) The consignee (receiving party) must be notified beforehand of specimens to be sent for shipping, and acknowledgement of preparedness for receipt sent to the consignor.
c) The sender should inquire about any import permits or other documents required by the receiving laboratory's national government. If permits are needed, the receiving laboratory will need to obtain the CURRENT permit and send it (usually a faxed copy) to the shipping laboratory so that the permit can be given to the carrier.
d) The sender and receiver (consignee) should then make advance arrangements for a mutually convenient time for shipment to ensure that the appropriate staff is available to receive the shipment. It is recommended to plan to avoid weekend arrivals.

3. Advance arrangements with the carrier

a) Once a shipment is necessary, the laboratory personnel should contact a carrier familiar with handling infectious substances and diagnostic specimens and make arrangements to ensure that:

- The shipment will be accepted.

- The shipment is undertaken by the most direct routing, avoiding weekend arrival.
- Archives and documentation of the shipment progress will be kept.
- The conditions of the shipment while in transit will be monitored.
- The sender and consignee will be notified of any delays.

b) The sender should request any necessary shipping documents that the carrier may require or any specific instructions necessary to ensure safe arrival of the shipment. The carrier may also provide advice on packaging.
c) In cases of delays, the consignor must arrange for both the consignee and consignor to be notified immediately by the carrier and advised on expected arrival arrangements.

4. Notification of the consignee of departure

IATA guidelines require that once the package has been sent, the receiver (consignee) should be immediately notified of the following:

- Number of specimens (nature and quantity)
- Flight details (airline, flight number, arrival date and time)
- Airway bill number
- "Please inform if not received"

5. Notification of the consignor

Once the package has been received, the receiver should immediately notify the sender of the receipt and condition of the shipment (including temperature) and any problems encountered. This can be facilitated by the sender including a 'fax back' form in the shipment that the receiver can then return.

REFERENCES

1. Requirements and guidance for external quality assessment for health laboratories. Geneva: World Health Organization; 1989 (WHO/DIL/LAB/99.2).
2. Standard operating procedure manual for WHO Polio Laboratory – Chapter 9: Specimen and isolate transport. Melbourne: Victoria Infectious Diseases Reference Laboratory; 2001.
3. IATA regulations handbook. Montreal: International Air Transport Association (IATA); 2003.

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials

SOP 3.7 Preparation of Panel Samples from Cultured Parasites

AIM

To prepare panel samples from cultured parasites to be used in product testing (PT) of malaria rapid diagnostic tests (RDTs). The sample should simulate fresh clinical specimens of parasites in blood, but have a parasite density close to the lower limit of detection by RDTs (200 parasites/μL) and at a moderate level of parasitaemia (2000 parasites/μL). The samples must be stored under conditions permitting minimal deterioration, allowing qualitative and quantitative detection of loss of sensitivity.

PURPOSE

This Standard Operating Procedure (SOP) describes the process for preparing dilutions (panel samples) of cultured parasites for product testing of malaria RDTs.

SCOPE

This procedure applies to the malaria RDT product testing programme of WHO and FIND with the US Centers for Disease Control and Prevention.

PROCEDURE

1. Prepare Samples to Simulate Fresh Clinical Specimens

a) Begin preparations only after parasitaemia of culture contents reach > 1.0%.
b) Synchronize parasite cultures to ring-stage (SOP 4.7).
c) Pool culture flask contents of the same strain into a 50 mL centrifuge tube.
d) Centrifuge at 2200 rpm for 10 minutes and remove culture supernatant.
e) Add 5-10 mL of fresh media, resuspend RBC pellet, and transfer to 15 mL centrifuge tube.
f) Centrifuge again at 2200 rpm for 10 minutes and remove supernatant.
g) Depending on culture conditions, add enough AB+ or O+ serum to give a 40% haematocrit.
h) Mix thoroughly by rotation for 15–30 minutes.

2. Determine Initial Parasite Density

a) Make and Giemsa stain both a thin and thick film smear of 40% HCT solution (SOP 4.5).
b) Count total number of parasites per an amount equal or greater than 2000 RBCs.
c) Counts should be performed by two microscopists.
d) Determine initial parasitaemia (SOP 4.6) by averaging two counts.
e) If counts are off by more than 20%, each technician count an additional 1000 RBCs. If still off by more than 20%, incorporate a third microscopist to count 3000 cells and average counts most similar to each other.
f) Determine total number of RBCs per microlitre of infected blood using a Coulter Counter or other means of quantification.
g) Calculate number of parasites per microlitre of blood using the following equation:

$$\frac{\text{\# of Parasites}}{\mu\text{L Infected Blood}} = \text{Parasitaemia} \times \text{Total Number RBCs}$$

e.g. if a culture has a parasitaemia of 1.4%, with a red blood cell count of 4.19 X 10^6/µL, then the parasite density would equal 58 660p/µL.

(0.014) X (4 190 000) = 58 660p/µL

3. Preparing Dilutions

a) Prepare dilutions of "parasitized blood" in "parasite free" donor blood.
b) Donor blood must be blood group O+ or O- and be screened prior to dilutions for HIV, HBV, and HCV.
c) The total volume of donor blood per culture is based on the following assumptions:
d) Samples will be dispensed in tubes of 50µL aliquots.
e) 300 tubes minimum are needed at 200p/µL.
f) 150 tubes minimum are needed at 2000p/µL.
g) 15 tubes of each dilution at 250µL for ELISA testing.
h) Serial dilutions are used when going from a higher to a lower parasite density.
i) When preparing dilutions, rotate freshly mixed blood for a minimum of 30 minutes before aliquoting.
j) Make three thin and thick smears of each dilution for quantitative analysis
k) Test lower dilutions (200p/µL and 2000p/µL) on quality-assured RDT, and compare with 200p/µL control sample.
l) Calculate volume of undiluted parasitized blood added to parasite-free donor blood needed to make a total of 10mL at a dilution of 5000p/µL. Use the equation:

$$C_1V_1 = C_2V_2$$

e.g.: If the undiluted parasite density is 58 660p/µL, then 0.85mL of parasitized blood must be added to 9.15mL of donor blood in order to lower the parasite concentration to 5000p/µL.

(58 660)(X) = (5000)(10mL)
X = 50 000/58 660
X = 0.85mL
10mL – 0.85mL = 9.15mL

m) A total of 6mL of 5000p/µL dilution will be used for aliquots.
n) Combine the remaining 4mL with 6mL of negative blood to make 10mL 2000p/µL dilution.
o) Combine 1.8mL of 2000p/µL dilution with 16.2mL negative blood to make 18mL 200p/µL dilution.
p) Dispense dilutions in 50µL aliquots.
q) Send 200µL samples for ELISA confirmation.
r) Store at -80 Celsius.

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials

SOP 3.8 Labelling of Bank (Panel) Samples

PURPOSE

This Standard Operating Procedure (SOP) describes methods for proper packaging prior to transport of QC samples for testing malaria RDTs.

SCOPE

This procedure applies to the malaria RDT product testing programme of WHO and FIND with the US Centers for Disease Control and Prevention.

BACKGROUND

Labelling should identify samples to the individual source or case without giving personal information that would allow linking to a named person, and should identify the specific nature of the material relevant to testing results (e.g. parasite density, type of parasite). Labelling should be of a type that allows blinding by over labelling, and that is simple enough to minimize personnel time spent on the labelling process. Labels should be clearly legible, and stable in the conditions of storage and use of the sample container.

PROCEDURE

1. Cryovial Labelling

- All panel sample isolates must be packaged in sterilized screw-cap tubes with an external thread, such as 1.8mL cryovials (currently this is the method of storage).
- Using a multi-dispense pipette, prepare **50µL** aliquots of all dilutions needed for each sample, as well as for negative control samples. Undiluted samples can be stored in larger volumes, e.g. 1mL.
- Label the cryovials with the following information:

a) Positive samples:

ISO country code - number (round) of collection* - species - case number - parasite density

e.g. **KH04V09-2000** would be Cambodia, 4th collection, *P. vivax*, 9th case (of that species on that collection), 2000 parasites/µL

* "number (round) of collection" indicates a field trip to collect samples. Where sample collection extends continuously over a longer period, the lab needs to determine an appropriate way of distinguishing collections (e.g. numbering by transmission season).

b) Negative samples:

ISO country code - lot number - negative - sample number (There is no collection number)

e.g. **KH01N04** would be the 4th negative sample contributed by the Cambodian collection site to the bank.

e.g. **US01N23** would be the 23rd sample contributed by USCDC to the bank. This could be procured by TDR, FIND or a laboratory. However, it was prepared (aliquoted out) at USCDC, and CDC will hold all the other relevant details that can be readily traced from this code number through the database.

c) Culture samples:

US* - culture round - species - strain – parasite density (Collection number is replaced by the culture round, and sample number is replaced by the strain name)

e.g. **US01F3D7-200** would be cultured *P. falciparum* prepared during the 1st culture round, strain 3D7, 200 parasites/µL

* All culture samples for the manufacturer's panel will be grown at the CDC. There is no need to include other country codes.

d) Undiluted samples:

For undiluted samples, the ID follows the same structure as above, except that the parasite density extension is replaced by the extension: - undiluted

e.g. **KH04V09-undiluted** would be Cambodia, 4th collection, *P. vivax*, 9th case (of that species on that collection), undiluted blood from the patient

e) Samples diluted according to antigen concentrations:

When undiluted samples are diluted according to their antigen concentration, then the sample ID starts the same as the undiluted one, and the extension is replaced by an identifying number (3 digits, e.g. -001, -002, -003) which identifies the particular dilution done on a particular day.

e.g. **KH04V09-001** would be the first dilution prepared from sample KH04V09-undiluted.

AA = **ISO** Country code, e.g. NG for Nigeria
# = round of collection
A = species (i.e. F for *P. falciparum*) F, V, O, M
= unique specimen identifier
= dilution (e.g. 200)

Example:	**NG 05 F 14 2000**	Fifth collection round from Nigeria *P. falciparum* from case 14, diluted to 2000 parasties/µL

ISO country codes are given for each laboratory in the table below.

RDT laboratory codes

Country	ISO country code
ASIA	
Philippines	PH
Cambodia	KH
Myanmar	MM
AFRICA	
Tanzania	TZ
Ethiopia	ET
Kenya	KE
Nigeria	NG
Central African Republic	CF
Senegal	SN
Madagascar	MG
AMERICAS	
Colombia	CO
Suriname	SR
Peru	PE
OTHER	
Australia	AU
USA	US
United Kingdom	GB

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials
14/04/2009	2	Labelling revised	DB, AA
2018	7	Added labelling for undiluted samples and those diluted according to antigen concentrations	SI

Malaria RDT Product Testing Methods Manual

Chapter 4: CDC MALARIA CULTURE PROCEDURE

SOP 4.1 Preparation of Reagents and Media for Culture of Malaria Parasites

PURPOSE

This SOP outlines the preparation of all necessary media for laboratory culture of glycerolyte- cryopreserved *Plasmodium* spp.

SCOPE

This procedure applies to the malaria RDT product testing programme of WHO and FIND with the US Centers for Disease Control and Prevention.

INTRODUCTION

- Perform all work in a biological safety cabinet (BSC), using aseptic technique.
- Use universal precautions in handling blood and blood products.
- Prepare media and reagents before starting procedure.

MATERIALS AND REAGENTS

1. Reagents (used for thawing and freezing parasites)

- 12% NaCl, sterile

NaCl	6.0 gm
Deionized water	50.0 mL

- 1.6% NaCl, sterile

NaCl	0.8 gm
Deionized water	50.0 mL

- 0.9% NaCl / 0.2% Dextrose, sterile

NaCl	0.45 gm
Dextrose	0.10 gm
Deionized water	50.00 mL

- Glycerolyte 57 Solution (Fenwal #4A7833)

2. Medium (used for culturing parasites)

a) **Incomplete RPMI** from powder (with double glucose & hypoxanthine)

Purified H_2O (tested for cell culture)	850.0 mL
RPMI 1640 (Life Technologies #31800)	1 package

(with Phenol Red, with L-Glutamine; without HEPES, without $NaHCO_3$)

HEPES	7.15 gm
Dextrose	2.0 gm
Hypoxanthine [25 mg/mL in 2N NaOH]	2.0 mL
Gentamycin	0.5 mL

i. Measure the purified water into a 1000 mL beaker with a magnetic stirring rod.
ii. Dissolve the above ingredients using a magnetic stirrer. Check pH. Adjust to pH 7.2 if necessary by adding 1N NaOH or 1N HCl.
iii. Transfer solution to 1L volumetric flask and add enough purified water to complete 1000 mL.
iv. Filter-sterilize in Nalgene 450 mL, 0.2 um filter. Stable at 2-8°C for 30 days.

b) **Complete RPMI** (Prepare in the BSC using aseptic technique)

Incomplete RPMI (prepared above)	180.0 mL	or:	45.0 mL
Human O+ serum* (heat inactivated)	20.0 mL		5.0 mL
7.5% $NaHCO_3$	6.4 mL		1.6 mL

i. Warm the ingredients to 37°C.
ii. Add the ingredients in the order listed; mix.
iii. Filter-sterilize using Millipore 0.22 um, GP Express (PES membrane) Stericup™.
iv. Stable at 2–8°C for 7 days.

* Serum donors should not have been in a malarious area for at least two years and should not be on any antibiotic therapy. For best results, collect non-fasting blood and pool 12 to 20 units of serum. Dispense in 40 mL aliquots. Heat-inactivate at 56°C for 45 minutes. Test quality of new lots by parallel culture for 3–4 subcultures (2 weeks).

* When culturing parasites from patients of unknown blood type, use human AB+ serum. To culture parasites from animals, ideally use serum from a non-immune donor of the same species or use human AB+ serum.

3. Human O+ erythrocytes (RBCs)

a) Collect blood* in Vacutainer® tubes containing ACD; mix well; store at 2–8°C for up to 4 weeks before processing. Blood donors should not have been in a malarious area for at least two years and should not be on any antibiotic or antimalarial therapy. When culturing parasites from animals, ideally use erythrocytes from a non-immune, non-infected donor of the same species or use human O+ RBCs.
b) In BSC, using a sterile pipette, transfer whole blood to sterile 50 mL conical tubes.
c) Centrifuge 10 minutes at 500 x g.
d) Remove plasma and buffy coat (containing WBCs), aspirating into a vacuum flask containing bleach.
e) Using 25 mL pipette, add incomplete RPMI 1640 to each tube — q.s. to 40 mL;
f) Mix well by pipetting up and down twice.
g) Centrifuge 10 minutes at 500 x g.
h) Remove supernatant by aspirating into vacuum flask.
i) Repeat steps e–g.
j) Add an equal volume of cRPMI with 10% O+ serum to give a 50% haematocrit.
k) The cells are stable at 2–8°C for up to 2 weeks.

4. Supplies and equipment

a) Laminar flow biological safety cabinet (BSC)
b) Water bath, 37°C
c) Incubator, 37°C, with turbo blower — CO_2 not needed
d) Slide warmer, 37°C, covered with white lab-top paper, plastic side up (or

Styrofoam racks)

e) Vacuum line, with a 4 litre side-arm flask (trap containing 2 inches of bleach) and a Vacuset® by Inotech for good control of aspiration
f) Low rpm Vortex
g) Bunsen burner
h) Centrifuge, with swinging carriers to accommodate 50 mL and 15 mL tubes
i) Refrigerator, 2–8°C
j) Freezers: -20°C, -80°C, -180°C (vapor phase of liquid nitrogen) with appropriate racks, towers, cryoboxes, etc.)
k) Gas tank containing special gas mixture:
    - 5% CO_2
    - 5% O_2
    - 90% N_2
l) Sterile syringes: 1, 3, 5, 10, and 20 cc
m) Sterile needles: 19g, 20g, and 25g
n) Sterile pipettes: 1, 2, 5, 10, and 25 mL
o) Sterile centrifuge tubes: 15 and 50 mL
p) Sterile, cotton-plugged Pasteur pipettes, 9"
q) Sterile, canted neck, tissue culture flasks for cultures:

Volume 10% RBCs in complete RPMI	Flask size	Corning catalog #
5.0 mL	T-25 - 25 cm^2	430168
15.0 mL	T-75 - 75 cm^2	430720
25.0 mL	T-150 - 150 cm^2	430823

r) Clean, dry microscope slides, 1" x 3", frosted on one end
s) Laboratory timer
t) Equipment thermometers
u) Cryovials: 2.0 mL with O-ring screw-cap (Sarstedt 72.694.006)
v) Slow freezing apparatus — "Mr. Frosty" (Nalgene #5100-0001) (with 250 mL 100% isopropyl alcohol)

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials

SOP 4.2 Thawing and Inoculation of Glycerolyte-Cryopreserved Parasites

PURPOSE

This SOP describes the procedure for thawing preserved *Plasmodium* spp. in a manner that ensures optimal parasite survival, as well as describes the protocol for inoculation into fresh media.

SCOPE

This procedure applies to the malaria RDT product testing programme of WHO and FIND with the US Centers for Disease Control and Prevention.

INTRODUCTION

- Perform all work in a biological safety cabinet (BSC), using aseptic technique.
- Use universal precautions in handling blood and blood products.
- Prepare and assemble all necessary reagents and supplies before beginning procedure.
- Work quickly, with no delays at any stage of the thawing process.

MATERIALS AND REAGENTS

(For preparation of reagents and list of supplies, see SOP 4.1)

1. Reagents

- 12.0% NaCl, filter-sterilized
- 1.6% NaCl, filter-sterilized
- 0.9% NaCl / 0.2% dextrose, filter-sterilized

2. Medium--Complete RPMI with human O+ serum (or AB+ serum if from original patient or monkey material)

3. Human O+ erythrocytes, washed

4. Supplies

- Syringes (1cc, 10cc), needles (25g, 19g)
- Tube (50 mL centrifuge tube or round bottom tube: 10 mL Vacutainer or 30 mL Oak Ridge)
- Low speed Vortex

PROCEDURE

1. Thaw the cryovial containing the parasites quickly.

a) Remove the frozen parasites from -180^{0}C or -70^{0}C and transport, on dry ice, to the laboratory.
b) Place vial in 37^{o}C water bath for 1–2 minutes to thaw, shaking gently. Take care to keep the screw cap top of the vial above the water surface. Carefully wipe the exterior of the vial with 70% alcohol then place the vial in the BSC.

c) Use a sterile 2 mL pipette to remove the thawed parasites from the cryovial. Note the volume and transfer to a sterile 50 mL centrifuge tube for immediate processing. *(Or use a sterile red-top Vacutainer tube vented with a 19g x 1 inch needle.)*

2. To prevent haemolysis, the RBCs containing parasites must be treated with a series of decreasing concentrations of salt.

a) Using a 1 cc syringe with a 25 gauge needle, measure 12% NaCl, 0.2 mL per mL of thawed culture. (For example, if the volume of thawed culture is 0.5 mL, measure 0.1 mL of 12% NaCl.)
b) While gently shaking (or use a low-rpm vortex: 800–1000 rpm) the thawed culture, add the 12% NaCl to the thawed culture at the rate of 1–2 drops per second.
c) Cap the tube, vortex gently (800–1000 rpm), and allow it to stand without further shaking for 3 minutes. Set a timer.
d) If using a Vacutainer tube, insert a 19g needle to relieve pressure.
e) Using a 10 cc syringe with a 19 or 20 gauge needle, measure enough 1.6% NaCl to give the proportion of 10 mL of 1.6% NaCl per mL of thawed culture. (For example, if the original volume of thawed is 0.5 mL, measure 5 mL of 1.6% NaCl.)
f) As soon as the timer sounds, add this 1.6% NaCl using the same drop-wise method as in step 2 above.
g) Cap the tube and vortex gently (800–1000 rpm). (Remove vent needle.)
h) Immediately centrifuge at 500 x g for 10 minutes.
i) Remove all but 0.5 mL of the supernatant using a sterile Pasteur pipette, aspirating into a vacuum trap containing bleach. Tap gently, vortex gently (800–1000 rpm) to re-suspend the pellet.
j) If using a Vacutainer tube, insert a 19g needle to relieve pressure
k) Using a 10 cc syringe with a 19 or 20 gauge needle, measure 0.9% NaCl / 0.2%
    - dextrose (in an amount equal to that of the 1.6% NaCl used in step 4.)
    - For example, if the original volume of the thawed culture is 0.5 mL, the amount of 0.9% NaCl / 0.2% dextrose needed is 5 mL.
l) Add this reagent to the packed cells, in the same drop-wise fashion as in step 2 above. Cap tube and vortex gently (800–1000 rpm). (Remove vent needle.)
m) Immediately centrifuge at 500 x g for 10 minutes.
n) Remove all but 0.5 mL of the supernatant as in step i. above. The parasites are now prepared for inoculation.

3. Inoculation

a) Prepare tissue culture flask (T-25 with a non-vented cap, Corning catalog # 430168). Add 4.5 mL of complete RPMI with 10% human O+ serum*, warmed to 37°C. *Note: If blood type of vial contents is not known to be O, use AB+ serum.
b) Label the flask with the species, strain and date inoculated; for example, Pf FC27, 3/14. Place the labelled flask on the 37°C slide warmer or on a styrofoam tube rack.
c) With a sterile 2 mL pipette, transfer 2.0 mL of the medium from the flask to the centrifuge tube and re-suspend the packed cells containing parasites. Mix well by pipetting up and down and transfer the suspension to the flask. Note the total volume. Pellet volume = total volume - 2.0 mL medium.
d) Subtract pellet volume from 0.5 mL. Add that volume of washed human O+ erythrocytes to the flask (so that total RBC volume in the flask is 0.5 mL. Cap the flask and rock gently to mix.
e) Flame both ends of a sterile, cotton-plugged, Pasteur pipette. Attach it to the tubing from the tank containing a special gas mixture: 5% CO_2, 5% O_2, and 90% N_2. Flush the culture flask with a gentle flow of this gas for 30-45 seconds.
f) Cap the flask tightly, and carefully place the culture in the 37°C incubator,

placing it flat on the shelf. Note that a CO_2 incubator is not needed because the necessary gas mixture is inside the flask.

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials

SOP 4.3 Daily Maintenance of Malaria Parasite Cultures

PURPOSE

This SOP describes the procedure for maintaining a healthy malaria culture following initial parasite inoculation.

SCOPE

This procedure applies to the malaria RDT product testing programme of WHO and FIND with the US Centers for Disease Control and Prevention.

INTRODUCTION

- Perform all work in a biological safety cabinet (BSC), using aseptic technique.
- Use universal precautions in handling blood and blood products.
- Prepare media and reagents before starting procedure.

MATERIALS AND REAGENTS

(For preparation of media and reagents and a list of needed equipment and supplies see General Procedures: Culture of Malaria Parasites.)

1. **Medium—Complete RPMI with 10% human O+* serum, warmed to 37°C**
2. **Human O+* erythrocytes (RBCs), washed 3 times in incomplete RPMI 1640**
   *Note: When adapting a new field isolate (or parasites from animal blood), use Human AB+ serum (or animal species-specific serum and RBCs) for the first few subcultures.

PROCEDURE

1. Change the medium daily.

a) Warm the complete RPMI to 37°C for about 30 minutes before use.
b) Carefully transfer the culture flasks from the 37°C incubator to the 37°C slide warmer (or Styrofoam tube rack) in the BSC, keeping them as level as possible. Try not to disturb the erythrocytes that have settled on the bottom.
c) Allow the flasks to settle for about 10–15 minutes.
d) To one side of the BSC, label one microscope slide per culture with the strain, flask number, date inoculated and today's date.
e) Aseptically remove a sterile 9" Pasteur pipette from the can. Using a Bunsen burner, make a 90° bend about 10 mm above the end of the pipette.
f) Remove the cotton plug and attach the pipette to the vacuum line, equipped with a trap flask containing bleach. (Vacuset® by Inotech allows for good control of aspiration.)
g) Carefully insert the pipette into the culture flask so that the tip is just above the layer of RBCs. (Using canted-neck tissue culture flasks simplifies this procedure.)
h) Slowly aspirate the medium from the culture. (Tip the flask slightly toward the Pasteur tip to remove as much medium as possible without losing RBCs.)
i) Remove the Pasteur pipette from the flask and disconnect the vacuum line.
j) Briefly stand the flask up, allowing any remaining medium to drain.

k) Quickly insert the same Pasteur pipet into the flask and scrape the RBC layer until the capillary is filled about 1–2 mm.
l) Cap the flask and quickly put the drop of RBCs on the slide.
m) Discard the Pasteur pipette and quickly make a thin smear on the slide, using a second slide (at a 40–45^{o} angle) to spread the drop of blood out to a feathered edge. See procedure: Preparation of blood smears for diagnosis of malaria.
n) Quickly dry the smear by placing it at the front of the laminar flow BSC, where the air flows down.
o) Using a sterile pipette, add the appropriate amount of fresh, warmed medium to the culture, allowing the medium to flow down to the bottom of the flask, washing off the RBCs. Discard the pipette.
p) Cap the flask securely and lay the flask flat on the slide warmer. Gently lift the cap end of the flask up and down several times to mix. Loosen the cap.
q) Flame both ends of a sterile, cotton-plugged, 9" Pasteur pipette and attach it to the gassing apparatus — tubing from a tank containing a special gas mixture: 5% CO_2, 5% O_2, and 90% N_2. Open the valve slightly to introduce a gentle flow of gas so that you do not allow the culture to splash.
r) Insert the Pasteur pipette into the culture flask and flush with the gas mixture.
    - 25 cm^2 flask — 30 seconds
    - 75 cm^2 flask — 45 seconds
    - 150 cm^2 flask — 60 seconds
s) Remove the Pasteur pipette and quickly cap the flask. Discard Pasteur pipette.
t) Turn the valve to stop the flow of gas mixture.
u) Keeping the flask as level as possible, carefully transfer it to the 37^{o}C incubator. Place the flask flat on the incubator shelf.
v) Incubate at 37^{o}C for 24 hours. Medium must be changed daily. Note that a CO_2 incubator is not needed because the necessary gas mixture is inside the flask.
w) Repeat steps e–v for each culture flask.

2. Check the parasitaemia of the culture to monitor culture conditions and to determine the amount of inoculum to use when seeding a new culture.

a) Process the blood smears prepared in steps III.A.12 to 14 of CDC laboratory manual*. (Laboratory manual, Malaria Branch, Division of Parasitic Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA)*
b) Fix each smear by dipping for 5 seconds in absolute methanol, acetone-free. Allow the fixed smears to dry.
c) Stain the smears in fresh "working Giemsa stain". See procedure: Staining malaria parasites.
d) Allow the smears to dry. (They can be placed on end in a test tube rack in the incubator, or dried quickly with a hair drier.)
e) Examine the feathered edge of the smear, using the 100x oil immersion objective, and an ocular equipped with a grid for counting cells.
f) Using a laboratory cell counter, count the total number of RBCs inside the grid. Record this number on the worksheet.
g) Count the number of infected RBCs in the grid area. Record the number of infected RBCs with rings, trophozoites, schizonts, and "other" forms in separate columns on the worksheet. (Do not include sick or dead-looking parasites in the count.)
h) Repeat steps a and b for enough fields so that at least 5000 RBCs have been counted.
i) Calculate the percentage of parasitaemia by dividing the number of infected RBCs by the total number of RBCs. Record this result on the worksheet.

*Caution: If the parasitaemia rises above 3%, the culture may "crash" unless the medium is changed at least twice daily.

3. Inoculation (seeding or subculturing) of new cultures biweekly
See SOP 4.4

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials

SOP 4.4 Inoculation of New Malaria Culture Flasks

PURPOSE

This SOP outlines the protocol for "seeding" malaria culture into new flasks in order to control parasitaemia and avoid colony death due to lack of fresh red blood cells.

BACKGROUND

To maintain a *Plasmodium* species in culture, this procedure must be done at least twice weekly for most strains (usually done on Tuesdays and Fridays).

SCOPE

This procedure applies to the malaria RDT product testing programme of WHO and FIND with the US Centers for Disease Control and Prevention.

PROCEDURE

1. Determine the amount of subculture inoculum needed to approximate 0.1% parasitaemia in the "daughter" flask.

a) The amount of inoculum from the "mother" flask depends on both the % parasitaemia of the "mother" flask and the size of the new flask. Determine the amount of inoculum needed for 5.0 mL cultures by using the table on the following page.
b) Established "laboratory" strains are usually subbed to 0.05% or 0.1%.
c) New patient isolates, etc. are subbed to 0.2% to 0.5%.
   - For 15 mL cultures, multiply this amount by 3.
   - For 25 mL cultures, multiply this amount by 5.

2. Determine the amount of RBC/medium suspension needed for subculture.

a) Prepare the appropriate amount of 10% O+ RBCs in warmed cRPMI for the new culture flask.

Medium amount	RBC amount	Flask size
4.5 mL	0.5 mL	25 cm^2 (T25)
13.5 mL	1.5 mL	75 cm^2 (T75)
22.5 mL	2.5 mL	150 cm^2 (T150)

b) Remove the inoculum amount determined in (1) from the suspension amount determined in (2).

3. Inoculation procedure

a) Carefully transfer the "mother" flask to the 37°C slide warmer in the BSC.
b) Mix the contents of the "mother" flask well and transfer the appropriate volume (see table below) to the new (warmed) culture flask. Cap both flasks.
c) Label the newly inoculated flask with species, strain, identifying letter, and date inoculated; for example: Pf FC27-A 3/14.
d) Mix the new culture by gently lifting the cap end up and down.
e) Flush each culture flask with the special gas mixture as described in **III.A.17-20** of Daily Maintenance of Malaria Parasite Cultures. (*Laboratory manual, Malaria Branch, Division of Parasitic Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA)*
f) Cap the flask tightly and, keeping the flask flat, transfer to the 37°C incubator for 24 hours.
g) Change the medium and check the parasitaemia daily as described in Daily Maintenance of Malaria Parasite Cultures. Note that the parasitaemia should be approximately 0.1% after one day.

INOCULATION OF NEW MALARIA CULTURE FLASKS

% parasitaemia of "mother" flask	Amount of inoculum needed (mL) to seed 5.0 mL flask at 10% haematocrit		
	0.05%	0.1%	0.2%
8.0	0.03	0.06	0.12
7.0	0.035	0.07	0.14
6.0	0.04	0.08	0.16
5.0	0.05	0.10	0.20
4.0	0.065	0.13	0.26
3.8	0.065	0.13	0.26
3.6	0.07	0.14	0.28
3.4	0.075	0.15	0.30
3.2	0.08	0.16	0.32
3.0	0.085	0.17	0.34
2.8	0.095	0.19	0.38
2.6	0.105	0.21	0.42
2.4	0.115	0.23	0.46
2.2	0.125	0.25	0.5
2.0	0.125	0.25	0.50
1.9	0.13	0.26	0.52
1.8	0.14	0.28	0.56
1.7	0.145	0.29	0.58
1.6	0.155	0.31	0.62
1.5	0.165	0.33	0.66
1.4	0.18	0.36	0.72
1.3	0.19	0.38	0.76
1.2	0.21	0.42	0.84
1.1	0.225	0.45	0.90
1.0	0.25	0.50	1.00
0.9	0.28	0.56	1.12
0.8	0.315	0.63	1.26
0.7	0.355	0.71	1.42
0.6	0.415	0.83	1.66
0.5	0.5	1.00	2.00

Parasitaemia of "Mother" Flask (%)	Desired Parasitaemia after Subculture for 5 mL Cultures									
	0.05%		0.10%		0.20%		0.25%		0.50%	
	Inoculum	cRPMI/RBC*	Inoculum	cRPMI/RBC*	Inoculum	cRPMI/RBC*	Inoculum	cRPMI/RBC*	Inoculum	cRPMI/RBC*
0.5	0.50	4.50	1.00	4.00	2.00	3.00	2.50	2.50	5.00	0.00
0.6	0.42	4.58	0.83	4.17	1.67	3.33	2.08	2.92	4.17	0.83
0.7	0.36	4.64	0.71	4.29	1.43	3.57	1.79	3.21	3.57	1.43
0.8	0.31	4.69	0.63	4.38	1.25	3.75	1.56	3.44	3.13	1.88
0.9	0.28	4.72	0.56	4.44	1.11	3.89	1.39	3.61	2.78	2.22
1.0	0.25	4.75	0.50	4.50	1.00	4.00	1.25	3.75	2.50	2.50
1.1	0.23	4.77	0.45	4.55	0.91	4.09	1.14	3.86	2.27	2.73
1.2	0.21	4.79	0.42	4.58	0.83	4.17	1.04	3.96	2.08	2.92
1.3	0.19	4.81	0.38	4.62	0.77	4.23	0.96	4.04	1.92	3.08
1.4	0.18	4.82	0.36	4.64	0.71	4.29	0.89	4.11	1.79	3.21
1.5	0.17	4.83	0.33	4.67	0.67	4.33	0.83	4.17	1.67	3.33
1.6	0.16	4.84	0.31	4.69	0.63	4.38	0.78	4.22	1.56	3.44
1.7	0.15	4.85	0.29	4.71	0.59	4.41	0.74	4.26	1.47	3.53
1.8	0.14	4.86	0.28	4.72	0.56	4.44	0.69	4.31	1.39	3.61
1.9	0.13	4.87	0.26	4.74	0.53	4.47	0.66	4.34	1.32	3.68
2.0	0.13	4.88	0.25	4.75	0.50	4.50	0.63	4.38	1.25	3.75
3.0	0.08	4.92	0.17	4.83	0.33	4.67	0.42	4.58	0.83	4.17
4.0	0.06	4.94	0.13	4.88	0.25	4.75	0.31	4.69	0.63	4.38
5.0	0.05	4.95	0.10	4.90	0.20	4.80	0.25	4.75	0.50	4.50
6.0	0.04	4.96	0.08	4.92	0.17	4.83	0.21	4.79	0.42	4.58
7.0	0.04	4.96	0.07	4.93	0.14	4.86	0.18	4.82	0.36	4.64
8.0	0.03	4.97	0.06	4.94	0.13	4.88	0.16	4.84	0.31	4.69
9.0	0.03	4.97	0.06	4.94	0.11	4.89	0.14	4.86	0.28	4.72
10.0	0.03	4.98	0.05	4.95	0.10	4.90	0.13	4.88	0.25	4.75

*RBCs are added at the same haematocrit as the culture inoculum, e.g. 10% Hct

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials

SOP 4.5 Malaria Microscopy: Blood Film Preparation, Staining and Reading

PURPOSE

This SOP describes the procedure for preparing thin and thick blood smears on a microscope slide in order to diagnose infection and assess parasitaemia.

SCOPE

The procedure is outlined in SOP 4.01 Methods Manual for laboratory quality control of malaria RDTs, version 8, 2016, applies to the malaria RDT product testing and lot testing programme of WHO and FIND with the US Centers for Disease Control and Prevention.

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials
14/08/14	6	Combine SOPs 4.5, 4.6 and make reference to MM v.7 SOPs	JC
2018	7	Updated reference to MM v8	SI

SOP 4.6 Synchronization Methods for Plasmodium Species

PURPOSE

This SOP outlines the methods used to obtain a malaria culture in which all parasites are at the same developmental stage.

SCOPE

This procedure applies to the malaria RDT product testing programme of WHO and FIND with the US Centers for Disease Control and Prevention.

PROCEDURE

1. Selecting for rings

a) Sorbitol – based on the fact that RBCs containing older forms have a surface transporter for sorbitol, thus will take it in, inducing a sudden change in osmotic pressure, causing RBCs containing mature forms to haemolyse.
   - Prepare 5% Sorbitol: 5 g sorbitol in 100 mL dH2O; filter sterilize; store at 2–8^{0}C.
   - Centrifuge the RBCs; remove supernatant; resuspend the pellet in 5 x volume of 5% sorbitol. Allow to stand for 5 minutes.
   - Centrifuge; discard supernatant; wash pellet in medium, e.g. RPMI. This should contain a high percentage of younger forms, but also may contain debris from ruptured schizonts, etc. If so, treat with 52% Percoll.

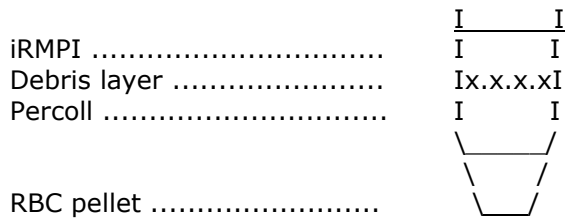
b) Percoll treatment – cleans up the debris from burst schizonts.
   - Prepare 52% Percoll.
   - 40 mL of 100% Percoll (Amersham Biosciences catalog # 17-0891-01)

+10 mL of 5x iRPMI 50 mL of 80% Percoll

32.5 ml of 80% Percoll

+17.5 mL 1x iRPMI 50 mL of 52% Percoll

   - Resuspend a 0.5 pellet from Sorbitol-treated culture in 2–3 mL cRPMI. *(Resuspend a 3 mL pellet in 6 mL cRPMI.)*
   - Transfer 5 mL 52% Percoll to a 15 mL centrifuge tube.
   - (Use three tubes with 5 mL 52% Percoll for 3 mL pellet.)
   - Gently layer the resuspended pellet over the 52% Percoll.
   - Centrifuge at 500 x g for 15 minutes.
   - Resulting layers:



- With a sterile Pasteur pipette, carefully aspirate the debris layer, then the liquid layers.
- Resuspend the pellet with cRPMI; mix.
- Centrifuge 5 minutes at 500 x g. Discard supernatant.
- Add medium (or plasma) to the desired haematocrit.

2. Selecting for schizonts, based on the fact that knobs on erythrocytes containing mature trophs and schizonts prevent rouleaux formation, thus will be less dense than uninfected or ring-infected RBCs.

a) Plasmagel

i. Centrifuge RBCs, wash once in Hanks, resuspend in 4x volume Hanks.
ii. Add equal volume of Plasmagel; mix. Allow to stand 30 minutes at 37°C.
iii. Supernatant contains mostly mature forms–spin down to make DNA, slides, etc.
iv. Pellet contains mostly younger forms–use for freezing, restarting culture, etc.

b) Gelatin flotation

- Reagent preparation
  i. Use gelatin–porcine skin approx 300 bloom (Sigma G-2500)
  ii. Prepare a 1% solution of gelatin in RPMI 1640 (with HEPES).
  iii. Heat at 50–55^{0}C for 15 min. with stirring. (Just make sure it dissolves properly and the protein doesn't burn.)
  iv. Let cool to about 40^{0}C, add bicarbonate, divide and freeze. Sterilize with a 0.22 filter.

- Separation
  i. Pre-warm all solutions to 37^{0}C.
  ii. Wash once with wash media (RPMI-1640, HEPES, $NaHCO_3$).
  iii. Wash once with 1:1 gelatin/wash media (at least 3 mL for 1 mL of blood).
  iv. Calculate the amount of gelatin needed.
  v. Formula:

Volume V= [(V cells/0.17) - V cells]/2.

  vi. Add the same volume (V) of gelatin solution and wash media and mix.
  vii. Add to a clear tube at 37^{0}C. The height of the solution should be between half and 2/3 of the tube. Position the tube straight in a 37^{0}C bath. Make sure it stands straight!
  viii. Allow the cells to settle until a very distinct separation line is observed.
  ix. Remove the upper portion containing knobby parasitized erythrocytes.

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials

SOP 4.7 Freezing Parasites in Glycerolyte

PURPOSE

This SOP outlines the proper technique for freezing and storage of *Plasmodium* spp., for use at a later time.

SCOPE

This procedure applies to the malaria RDT product testing programme of WHO and FIND with the US Centers for Disease Control and Prevention.

INTRODUCTION

- Perform all work in a biological safety cabinet (BSC), using aseptic technique.
- Use universal precautions in handling blood and blood products.
- Prepare and assemble all necessary reagents and supplies before beginning procedure.
- Work quickly, with no delays at any stage of the freezing process.
- Parasites survive best when very young ring stages are frozen in glycerolyte. (We have had poor results with DMSO.)

MATERIALS AND REAGENTS

- Glycerolyte 57 Solution (Fenwal #4A7833)
- For other supplies and equipment, see General procedures: culture of malaria parasites.

PROCEDURE

1. Prepare the parasite to be frozen.

a) Ring stage parasites can be frozen from culture flasks or from fresh blood in anticoagulant: EDTA, ACD or heparin.
b) Transfer the culture or blood to a sterile 50 mL centrifuge tube *(or sterile red-top Vacutainer tube)*.
c) Centrifuge at 500 x g for 10 minutes.
d) Remove the supernatant by aspirating into a vacuum flask containing bleach. Take care not to remove the "brown layer" at the top of the RBCs.
e) Estimate the volume of the packed cells, e.g. 1 mL.
f) If desired, adjust the parasitaemia to 1% by adding the appropriate amount of washed O+ RBCs, 50% haematocrit. You must spin again and remove supernatant. Note the adjusted volume of packed cells.

2. To prevent the RBCs containing parasites from lysing during the freezing process, they must be treated by gradually increasing the salt concentration.

a) In a sterile syringe, with a 25g needle, measure glycerolyte in an amount equal to 1/3 the volume of the packed cells, e.g. 0.33 mL.
b) While gently shaking (or using a low-rpm vortex) the tube of packed cells, at the rate of 1–2 drops per second, add this glycerolyte to the tube. Cap the tube and vortex gently.

c) Allow to stand for 5 minutes without further shaking. Set a timer.
d) In a sterile syringe, with a 20g needle, measure glycerolyte in an amount equal to 4/3 the original volume of the packed cells, for example 1.33 mL.
e) As soon as the timer sounds, add this glycerolyte to the parasites, in the same drop-wise fashion as in step 2 above.
f) Mix well, using gentle vortex.
g) Dispense 1.0 to 1.5 mL per cryovial. Label these vials with the species, strain, glycerolyte, % rings, volume, and date frozen. If sample is original patient blood or monkey blood, add that comment.
h) Place the vials in the slow freezing apparatus, Mr. Frosty (Nalgene #5100-0001); put Mr. Frosty in a -70^{0}C to -80^{o}C freezer. The parasites will freeze at a rate of -1^{o}C per minute.
i) After 18 to 24 hours, transfer the cryovials to pre-chilled cryoboxes and transport on dry ice to the -180^{o}C (vapor phase of liquid nitrogen) freezer for long-term storage.

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials

Malaria RDT Product Testing Methods Manual

Chapter 5: SAMPLE CHARACTERIZATION

SOP 5.1 Cellabs Pty HRP2 ELISA Kit Procedure

PURPOSE

This SOP describes the materials, equipment, and procedures required to correctly and safely use the Cellabs malaria antigen HRP2 ELISA kit to diagnose malaria using blood samples. Protocol includes:

- Setting up dilutions of recombinant HRP2 antigen for a standard calibration curve
- Preparation of blood samples for use in the assay
- Running of the ELISA in a 96 well plate format and interpretation of results.

SCOPE

This SOP has been developed for the training of laboratory personnel using the Cellabs malaria antigen HRP2 ELISA kit for malaria diagnosis in clinical and research settings. For the WHO-FIND malaria RDT evaluation programme, this SOP describes the use of this assay in the assessment of HRP2 antigen content within patient blood samples that form part of the global specimen bank.

PRINCIPLE OF TEST

Cellabs malaria antigen ELISA is suitable for the detection, in a blood sample, of the antigen HRP2 solely expressed in *Plasmodium falciparum*. HRP2 contained within the test specimen is bound to wells of an anti-HRP2 plate by monoclonal antibodies directed against the HRP2 protein. Antibodies conjugated with horseradish peroxidase enzyme then bind the HRP2 antigen at a different epitope. Unbound material is removed with a wash step, a substrate solution of TMB is added to the wells and the reaction product is subsequently quenched using an acid stop solution. The colour intensity of the resulting product is directly proportional to the HRP2 concentration and is measured as ΔOD 450/620 nm.

ASSAY AND SPECIMEN REQUIREMENTS

NB: All reagents should equilibrate to room temperature for at least 15 min before use.

- Sarstedt tubes
- Vortex
- Anti-HRP2 coated test plate*
- Recombinant PfHRP2 (lyophilized, manufactured by Microcoat GmbH, Germany)
- Positive kit control containing 110ng/mL PfHRP2*
- Enzyme conjugate 200x (MAPO)*
- Conjugate diluent (MACD)*
- Substrate chromagen 20x (TMB) (MASC)*
- Substrate buffer (MASB)*
- Stopping solution (MASS)*
- 1 X PBST (1 X PBS; 0.1% (v/v) Tween 20)
- Plate lids
- Micropipettes (50 – 200 μL and 100-1000 μL)
- Multichannel micropipette (50 – 250 μL)
- Automated plate washer
- 37°C incubator
- Spectrophotometer

*Contained within Cellabs HRP2 kit boxes.

INSTRUCTIONS FOR PERFORMING THE ASSAY

1. Preparation of standards and test samples

a) Eight standards are used as a reference positive and prepared in serial dilution for this assay. Human blood that has been frozen and thawed is used as a diluent for the standards and as a negative control. The positive kit control containing PfHRP2 is used to produce the standards that are diluted in human blood, see Table A.

b) Human blood is used to dilute the positive kit control and is pipetted into sarstedt tubes. The positive kit control should then be added to the first tube at an appropriate dilution to provide a starting concentration of 30 ng/mL. Doubling dilutions made thereafter form the 8 reference points that will generate the standard curve. Between transfers from one tube to another, blood containing HRP2 should be pipetted up and down several times and a vortex used to mix each tube. A fresh pipette tip should be used between each transfer.

c) Depending on the concentration of HRP2 added to the specimen being tested, dilution may be appropriate for the test samples to fit within range of the calibration curve. If necessary, this should be done using human blood and a conversion factor applied during data analysis.

Table A. Dilution of the positive kit control

Conc required (ng/mL)	30	15	7.5	3.75	1.88	0.938	0.469	0.234
Working stock (ng/mL)	110	30	15	7.5	3.75	1.88	0.938	0.469
Volume stock (μL)	272	500	500	500	500	500	500	500
Volume diluent (μL)	728	500	500	500	500	500	500	500
Total volume (μL)	1000	1000	1000	1000	1000	1000	1000	1000

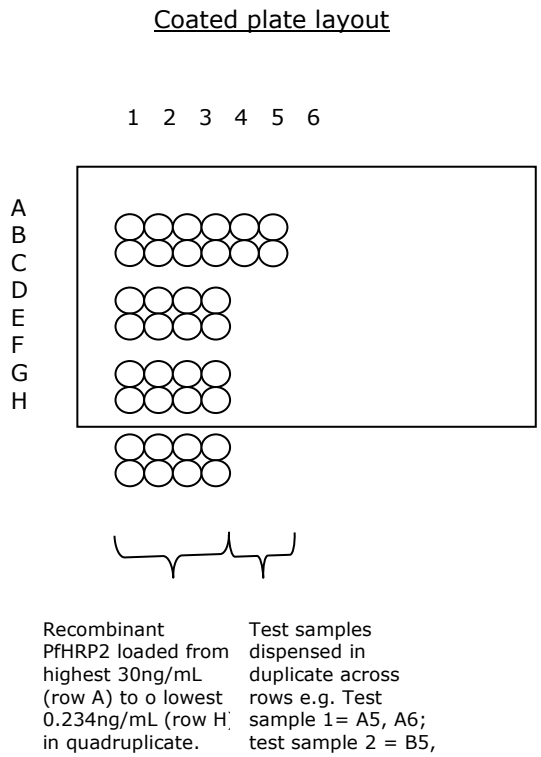
2. Preparation of the coated plate

a) With a standard micropipette, 100 μL of each of the 8 pre-prepared standards (30-0.234 ng/mL) should be added to the wells of column one to four (A-H) in parallel.

b) Test samples should then be added to each of the wells consecutively from A5 as far as G12. Each test sample should be duplicated in the adjacent row i.e. test sample 1 will be dispensed into wells A5 and A6. H11 and H12 should contain 100 μL of human blood used as the negative control (see Fig. 1). For one plate, 31 specimens can be tested. When all samples have been added to

the coated plate, cover the plate with a plastic lid and leave for 1 hour at 37ºC, without agitation (i.e. static).

Fig. 1. Loading format for coated plates



3. Wash steps and preparation of enzyme conjugate

a) The wash solution contained within the assay kit is 20 x PBST. A 1 Litre stock of 1 X PBST should be made up and used to wash the wells of the coated plate 3x with an automatic plate washer set to fill the wells with 300 μL solution.

b) Working strength enzyme conjugate should be made up fresh. Per plate, 55 μL enzyme conjugate 200x should be diluted in 11 mL conjugate diluent and mixed thoroughly. Using a multichannel pipette, 100 μL working strength enzyme conjugate should be dispensed to all test wells. The plate should then be covered with a plastic lid and incubated for 1 hour at 37ºC, without agitation (i.e. static).

4. Wash steps and development of substrate

a) Plates are washed as before, with 3x with 300 μL wash solution. Working strength substrate should be made up fresh. Per plate, 550 μL substrate chromagen should be diluted in 11 mL substrate buffer. Using a multichannel pipette, 100 μL of working substrate should then be dispensed into all test wells. The plate should then be covered with a plastic lid and incubated at room temperature for approx. 5 to 15 mins in the dark. Judgment should be used when to proceed to the next stop based on the saturation of the wells corresponding to the highest / most concentrated points on the standard curve.

b) The assay should then be quenched by dispensing 50μL of stopping solution into all test wells. The endpoint absorbance of the wells should be read at 450 nm with a reference wavelength 620 nm. The plates should be read as soon as possible (within 10 mins) of adding stop solution.

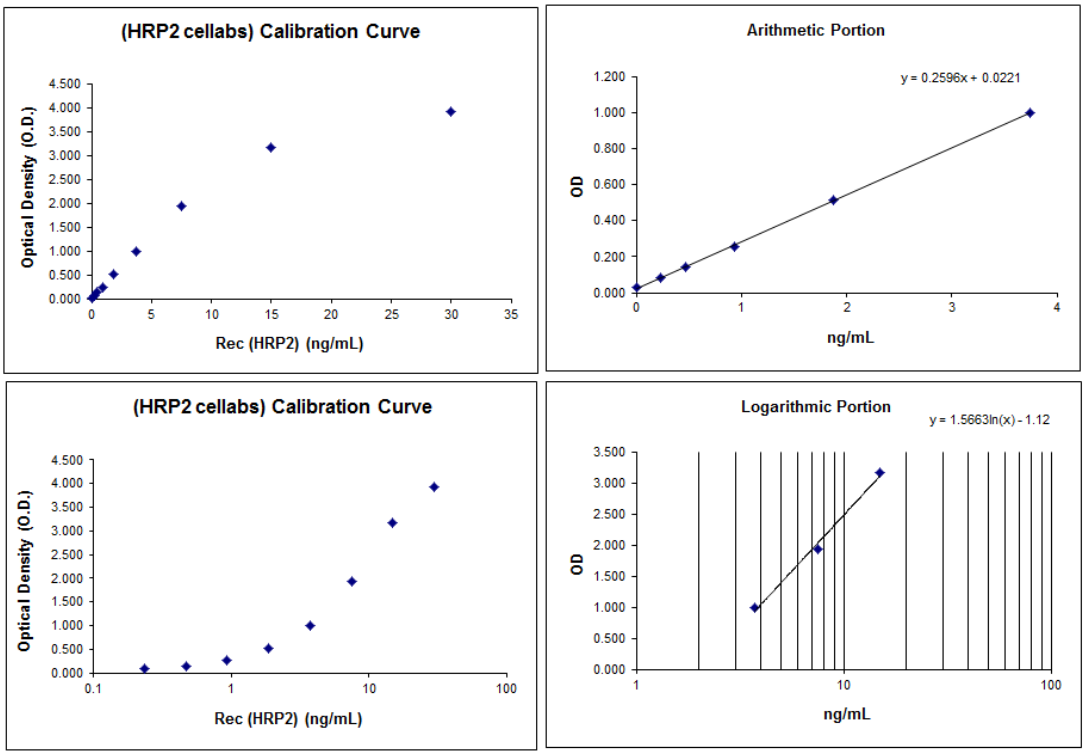
5. Interpretation of results

a) The spectrophotometer will make a print out of ΔOD 450/620 nm results and these should then be transcribed to another PC. Each point comprising the standard curve has been replicated 4 times therefore a mean OD for each point should be calculated in EXCEL. The specimens are in duplicate, thus need to be averaged. Mean ODs for the standards, specimens and negative control should all be entered into the EXCEL data file to make a scatter graph (calibration curve). Both arithmetic and logarithmic curves are plotted.

b) The arithmetic calibration curve will be parabolic in shape. The logarithmic calibration curve should be sigmoidal in shape. ODs that are > 30 ng/mL standard on the logarithmic curve will begin to plateau on the curve. Test samples with ODs higher than the 30 ng/mL standard should not be interpreted because the assay begins to saturate with HRP2 antigen at this point. The negative control should have an OD < 0.100.

c) Separate plots for selected points from the logarithmic and arithmetic curves are used for fitting trend-lines and obtaining equations which are used to calculate concentrations from OD for each test sample. The calculation uses a re-arrangement of the algebraic expression, y = mx + c. ODs are converted to HRP2 concentration in ng/mL. The result on a test specimen is adjusted for any pre-dilution of the specimen.

Fig. 2. Generation of logarithmic and arithmetic trend lines for data interpretation



HEALTH AND SAFETY

1. Hazardous reagents

Table B. Hazardous chemicals used in the Cellabs malaria antigen HRP2 ELISA

Product	Fire hazard	Health hazard	Toxicity	Storage requirements
Hydrogen peroxide (TMB)	Explosive under heat	Irritant to eyes/skin/nasal passage	Moderately toxic	Easily decomposes 2–8 ^{0}C
Hydrochloric acid (stop solution)	Flammable	Irritating to eyes/skin. Burns. Harmful by ingestion	Toxic	Keep in a locked store
TMB	Flammable	Harmful swallowed/inhaled/absorbed by skin	Toxic	Store solutions in light proof container at 4–8 °C

2. Safety precautions

- Disposable latex or nitrile gloves must be worn while handling clinical specimens and reagents. All clinical material, i.e. all components containing blood, must be autoclaved before disposal. The assay stop solution contains hydrochloric acid a corrosive and hazardous substance. Avoid eye and skin contact by wearing protective clothing and eye protection.

- Hands must be washed once work has been completed.

3. Technical precautions

- Components must not be used after their expiry date.
- Different batches/lots of reagents should never be interchanged.
- Storage of reagents must be at the recommended conditions.
- Contamination of reagents should be avoided by changing pipette tips where necessary.

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials
2018	7	Updated reagent list Updated technical details	RO

SOP 5.2 Qualisa pLDH ELISA for Malaria Antigen in Blood

PURPOSE

This SOP describes the materials, equipment, and procedures required to correctly and safely use the Qualpro Diagnostics Qualisa malaria antigen pLDH ELISA kit to detect pLDH malaria antigen using blood samples. Protocol includes:

- Setting up dilutions of recombinant pLDH antigen for a standard calibration curve
- Preparation of blood samples for use in the assay
- Running of the ELISA in a 96 well plate format and interpretation of results.

SCOPE

This SOP has been developed for the training of laboratory personnel using the Qualisa malaria antigen pLDH ELISA kit for malaria in research settings. For the WHO-FIND malaria RDT evaluation programme, this SOP describes the use of this assay in the assessment of pLDH antigen content within patient blood samples that form part of the global specimen bank.

PRINCIPLE OF TEST

Qualisa malaria antigen ELISA is suitable for the detection, in a blood sample, of the four species of malaria infecting humans. After whole blood is lysed, pLDH in the blood specimen is bound to immobilized Pan-specific monoclonal antibodies. Unbound material is then removed with a rigorous wash step and bound pLDH recognized by addition of a biotinylated antibody Pan-specific for pLDH. After removal of unbound biotinylated antibody with additional rigorous washing, streptavidin-peroxidase is added. Peroxidase activity is measured using a substrate solution of TMB and the reaction product is subsequently quenched using an acid stop solution. The colour intensity of the resulting product is directly proportional to the pLDH concentration and is measured as ΔOD 450/620 nm.

ASSAY AND SPECIMEN REQUIREMENTS

NB: All reagents should equilibrate to room temperature for at least 15 min before use.

- Sarstedt tubes
- Vortex
- Anti-pLDH coated test plate*
- Recombinant pLDH (Microcoat GmbH)
- Sample diluent*
- Antibody reagent*
- Enzyme conjugate*
- Conjugate diluent*
- TMB substrate solution*
- Stop solution*
- 1 X PBST (1 X PBS; 0.01% Tween-20)
- Plate lid
- Micropipettes (50–200 μL and 100–1000 μL)
- Multichannel micropipette (50–250 μL)
- Automated plate washer
- 37°C incubator

- Spectrophotometer

*Contained within individual Qualisa pLDH kit boxes.

INSTRUCTIONS FOR PERFORMING THE ASSAY

1. Preparation of standards and test samples

a) Eight standards are used as a reference positive and prepared in serial dilution for this assay. Uninfected human blood that has been frozen and thawed is used as a diluent for the standards and as a negative control. A purified recombinant form of *Plasmodium vivax* (Pv) LDH expressed in *E. coli* (Microcoat GmbH) is used to produce the standards that are diluted in human blood, see Table A. Blood used to dilute the recombinant is pipetted into Sarstedt tubes. Stock antigen is added to the first tube at an appropriate dilution to provide a starting concentration of 30 ng/mL. Doubling dilutions made thereafter form the 8 reference points that will generate the standard curve. Between transfers from one tube to another, blood should be thoroughly mixed using a vortex and the samples pipetted up and down once or twice prior to making the dilutions in order to "coat" the tips sufficiently. A fresh pipette tip should be used between each transfer.

b) Depending on the concentration of pLDH in the specimen being tested, dilution may be appropriate for the test samples to fit within range of the calibration curve. If necessary, this should be done using malaria-free human blood and a conversion factor applied during data analysis.

Table A. Preparation of the dilution set for creating the calibration curve

*Microcoat GmbH recombinant pLDH protein (x ng/mL)**

Conc required (ng/mL)	30	15	7.5	3.75	1.88	0.938	0.469	0.234
Working stock (ng/mL)	x	30	15	7.5	3.75	1.88	0.938	0.469
Volume stock (μL)	x	150	150	150	150	150	150	150
Volume diluent (μL)	x	150	150	150	150	150	150	150
Total volume (μL)	300	300	300	300	300	300	300	300

*****Please note that the initial dilution required for the starting point of the assay (30 ng/mL) will depend on the concentration of the stock being used.

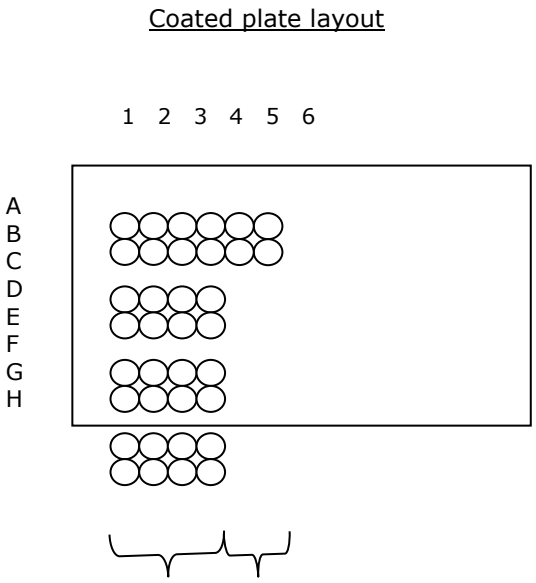
2. Preparation of the coated plate

a) Take out the required number of well strips from packaging and fix into the plate carcass.
b) Using a multichannel pipette, 100 μL sample diluent should be added to all wells of the anti-pLDH coated microplate.

c) With a standard micropipette, 25 μL of each of the 8 pre-prepared standards (30-0.234 ng/mL) should be added to the wells of column one to four (A-H) in parallel.

d) Test samples should then be added to each of the wells consecutively from A5 as far as G12. Each test sample should be duplicated in the adjacent row, i.e. test sample 1 will be dispensed into wells A5 and A6. H11 and H12 should contain 25 μL of human blood used as the negative control (see Fig. 1). For one plate, 31 specimens can be tested unless additional controls are included. When all samples have been added to the coated plate, cover the plate with a plastic lid/sealer and incubate at 37°C with agitation at 580 rpm for 50 mins.

Fig. 1. Loading format for coated plates



3. Wash steps

The wash solution contained within the assay kit is 20x PBST. A 1 Litre stock of 1x PBST should be made up and used to wash the wells of the coated plate x 6 with an automatic plate washer set to fill the wells with 350 μL solution.

4. Addition of detection antibody

a) Working strength antibody reagent should be made up fresh. Per plate, 220 μL antibody reagent stock solution (50x) should be diluted in 11 mL conjugate diluent and mixed thoroughly. Using a multichannel pipette, 100 μL working strength antibody reagent should be dispensed to all test wells. The plate should then be covered with a plastic lid and incubated with agitation at 580rpm for 30 mins at 37 ºC.

b) Plates should then be washed rigorously again as described in 7.3.

5. Addition of enzyme conjugate

a) Working strength enzyme conjugate should be made up fresh. Per plate, 220 μL enzyme conjugate stock solution (50x) should be diluted in 11 mL conjugate diluent and mixed thoroughly. Using a multichannel pipette, 100 μL working strength antibody reagent should be dispensed to all test wells. The plate should then be covered with a plastic lid/sealer and incubated for 30 mins at room temperature (on the bench).

b) Plates should then be washed rigorously again as described in 7.3.

6. Development of substrate

a) The TMB substrate contained within the kits is already at working strength. 100 μL of working substrate should be dispensed into each well using the multichannel pipette.

b) The plate should then be covered with a plastic lid and incubated at room temperature for approx. 5 to 15 mins in the dark depending on the local environment (i.e ambient temperature of the lab). Judgment should be used when to proceed to the next stop based on the saturation of the wells corresponding to the highest / most concentrated points on the standard curve.

c) The acid stop solution is contained within the assay kit and 100μL should be then dispensed in all wells. The endpoint absorbance of the wells should be read as soon as possible (within 10 min of adding the stop solution) at 450 nm with a reference wavelength 620 nm.

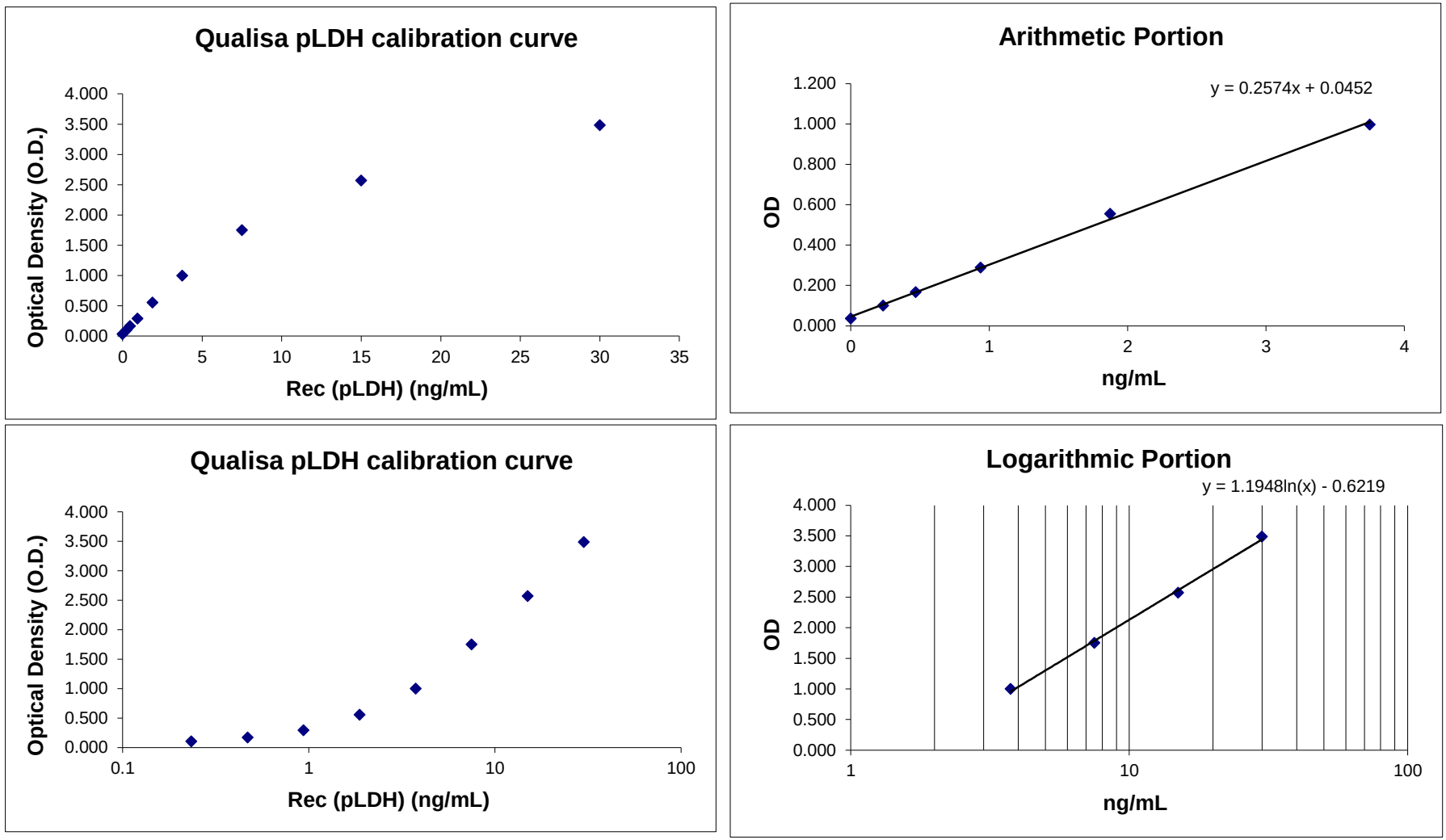
7. Interpretation of results

a) The spectrophotometer will make a print out of ΔOD 450/620 nm results and these should then be transcribed to another PC. Each point comprising the standard curve has been replicated 4 times therefore a mean OD for each point should be calculated in EXCEL. The specimens are in duplicate, thus need to be averaged. Mean ODs for the standards, specimens and negative control should all be entered into the EXCEL data file to make a scatter graph (calibration curve). Both arithmetic and logarithmic curves are plotted (Fig. 2).

b) The arithmetic calibration curve will be parabolic in shape. The logarithmic calibration curve should be sigmoidal in shape. ODs that are > 30 ng/mL standard on the logarithmic curve will begin to plateau on the curve. Test samples with ODs higher than the 30 ng/mL standard should not be interpreted because the assay begins to saturate with pLDH antigen at this point. The negative control should have an OD < 0.100.

c) Separate plots for selected points from the logarithmic and arithmetic curves are used for fitting trend-lines and obtaining equations which are used to calculate concentrations from OD for each test sample. The calculation uses a re-arrangement of the algebraic expression, $y = mx + c$. ODs are converted to pLDH concentration in ng/mL. The result on a test specimen is adjusted for any pre-dilution of the specimen.

Fig. 2. Generation of logarithmic and arithmetic trend lines for data interpretation



HEALTH AND SAFETY

1. Hazardous reagents

Table B. Hazardous chemicals used in the Qualisa malaria antigen pLDH ELISA

Product	Fire hazard	Health hazard	Toxicity	Storage requirements
Hydrogen peroxide (TMB)	Explosive under heat	Irritant to eyes/skin/nasal passage	Moderately toxic	Easily decomposes 2–8 ^{0}C
Sulphuric acid (stop solution)	Flammable	Irritating to eyes/skin. Burns. Harmful by ingestion.	Toxic	Keep in a locked store
TMB	Flammable	Harmful swallowed/inhaled/absorbed by skin	Toxic	Store solutions in light proof container at 4–8 °C

2. Safety precautions

- Recombinant pLDH used as a standard has been shown to be non-infectious in an *E. coli* recombinant expression system.
- Disposable latex or nitrile gloves must be worn while handling clinical specimens and reagents. All clinical material i.e. all components containing blood must be autoclaved before disposal. The assay stop solution contains sulphuric acid a corrosive and hazardous substance. Avoid eye and skin contact by wearing protective clothing and eye protection.
- Hands must be washed once work has been completed.

3. Technical precautions

- Components must not be used after their expiry date.
- Different batches/lots of reagents should never be interchanged.
- Storage of reagents must be at the recommended conditions.
- Contamination of reagents should be avoided by changing pipette tips where necessary.

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials
16/05/11	4	Edited to bring up to date	RRC
14/08/14	6	Edited to bring up to date	RRC, IV, JC
2018	7	Updated with Qualisa kit instead of Standard Diagnostics.	RO

SOP 5.3 Biotinylation of Monoclonal Antibodies for Aldolase ELISA Procedure

PURPOSE

This Standard Operating Procedure (SOP) describes procedures outlined in the EZ-Link® Sulfo-NHS-LC-Biotinylation Kit for biotinylation of monoclonal antibodies (MAbs) prior to Malaria Antigen Detection – Capture ELISA with anti-aldolase MAb.

BACKGROUND

Biotin is a small naturally occurring vitamin that binds with high affinity to avidin and streptavidin proteins. Because it is so small (244 Da), biotin can be conjugated easily to many proteins without altering their biological activities. The labelled protein or other molecule may then be detected easily in ELISA, dotblot or Western blot application using streptavidin or avidin probes. The following procedure usually yields incorporation of 8–12 biotins per molecule of IgG when labelling antibodies.

AIM

To improve sensitivity of Malaria Antigen Detection – Capture ELISA with anti-aldolase MAb

SCOPE

This procedure applies to the malaria RDT product testing programme of WHO and FIND with the US Centers for Disease Control and Prevention.

REAGENTS, SUPPLIES, AND EQUIPMENT

1. Reagents

- Anti-aldolase monoclonal antibodies (National Bioproducts Institute) – Store at -20°C
  - MAb M/B 7–20, 10 mg in PBS pH 7.2 [4.4 mg/mL]
  - MAb C/D 11–4, 10 mg in PBS pH 7.2 [5.7 mg/mL]

- Biotinylation Kit – Pierce catalog # 21430
  - EZ-Link® Sulfo-NHS-LC-Biotin, 25 mg (Store at -20°C with desiccant)
  - HABL, 1 ml, 10 mM in 0.01 N NaOH (Store at 2–8°C)
  - Avidin (Affinity Purified), 10 mg (Store at -20°C)

- Ultrapure water (Mediatech Cellgro catalog # 25-055-CM)
- PBS, 0.01 M, pH 7.2 (CDC BIOS catalog # CP0636)

2. Supplies

- Screw-cap centrifuge tubes, 2 mL and 15 mL polypropylene
- Micropipette tips, 20 µL and 200 µL
- Slide-A-Lyzer® Dialysis Cassette Kit, 10K MWCO, 0.5-3 mL – Pierce catalog # 66382
- Cuvettes for spectrophotometer

3. Equipment

- Vortex Mixer
- Spectrophotometer – Pharmacia Biotech Ultrospec 3000
- Pipettors
- Timer

PROCEDURE

1. General Safety

a) Wear gloves, lab coat, and safety glasses while handling all human or animal blood products.
b) Dispose of all pipettes, etc. into a covered pan; autoclave for 60 minutes.
c) Wipe work surfaces with disinfectant (e.g. 0.8% Vesphene).

2. Biotinylation Procedure

a) Calculations

By using the appropriate molar ratio of biotin to protein, the extent of labelling can be controlled. (With dilute protein solutions, a greater fold molar excess of biotin is necessary compared to more concentrated protein solutions.) Generally, use 12-fold molar excess of biotin for a 10 mg/mL protein solution or 20-fold molar excess of biotin for a 2 mg/mL protein solution.

i. Calculate amount of biotin to use
20-fold molar excess of biotin for a 2 mg/mL protein sample.

$$\text{mL protein} \times \frac{\text{mg protein}}{\text{mL protein}} \times \frac{\text{mmol protein}}{\text{mg protein}} \times \frac{\text{20 mmol biotin}}{\text{mmol protein}} = \text{mmol biotin}$$

ii. Calculate the volume of 10 mM Sulfo-NHS-LC-Biotin (biotin) (prepared in step B.3.) to add to the reaction
557 = MW of Sulfo-NHS-LC-Biotin.
400 = # µL of water in which 2.2 mg of biotin is dissolved to make a 10 mM solution.

$$\text{mmol biotin} \times \frac{\text{557 mg}}{\text{mmol biotin}} \times \frac{\text{400 µL}}{\text{2.2 mg}} = \text{µL biotin}$$

Example: for 1 mL of a [2.0 mg/mL] MAb (assume 150 000 MW) solution,
~27 µL of 10 mM biotin will be added.

$$\text{1 mL MAb} \times \frac{\text{2 mg MAb}}{\text{1 mL MAb}} \times \frac{\text{1 mmol MAb}}{\text{150 000 mg MAb}} \times \frac{\text{20 mmol biotin}}{\text{1 mmol MAb}} = \text{0.000266 mmol biotin}$$

$$\text{0.000266 mmol biotin} \times \frac{\text{557 mg}}{\text{mmol biotin}} \times \frac{\text{400 µL}}{\text{2.2 mg}} = \text{26.9 µL biotin reagent}$$

b) Biotin labelling reaction

i. Remove vial of biotin from freezer and allow to come to room temperature before opening in step 3.
ii. Prepare [2.0 mg/ml] stock solutions of monoclonal antibodies.

- M/B 7-20, 10 mg in PBS pH 7.2 [4.4 mg/mL]
  455 µL of [4.4 mg/mL] + 545 µL of PBS (0.1M, pH 7.2) = 1.0 mL of [2.0 mg/mL]

- C/D 11-4, 10 mg in PBS pH 7.2 [5.7 mg/mL]
  351 µL of [5.7 mg/mL] + 649 µL of PBS (0.1M, pH 7.2) = 1.0 mL of [2.0 mg/mL]

iii. Immediately before use, prepare a 10 mM biotin solution by adding 2.2 mg to 400 µL of ultra pure water.
iv. Based on calculations (see section a), add the appropriate volume of the biotin solution to the protein solution.
v. Incubate reaction for 30 minutes at room temperature (or 2 hours on crushed ice).
vi. Protein labelling is complete at this point.

c) Purification of the protein by removing excess (unbound) biotin for optimal stability and performance.

3. Dialysis method

a) For each protein sample to be purified, label a beaker and add 1000 mL PBS.
b) For each 0.5–2.0 mL sample, remove one dialysis membrane cassette from pouch. Handle the cassette only on the frame. Do not touch the membrane.
c) Mark an "X" on the top corner port that will be used to inject sample.
d) Slip the top edge of the cassette into the groove of the appropriate size buoy.
e) Float this assembly in the beaker of PBS for 30 seconds to hydrate the membrane.
f) Remove the cassette from the buffer and tap bottom edge gently on paper towels to remove excess liquid. DO NOT BLOT MEMBRANE.
g) Fill a 5 mL syringe with sample, leaving a small amount of air in the syringe.
h) Taking care not to pierce the membrane, with the bevel sideways, insert the needle tip through the port marked with an "X".
i) Inject the sample slowly; inject the remaining air to flush any remaining sample.
j) With the needle still inserted in the cassette cavity, remove almost all of the air compressing the membrane windows so that the sample solution contacts the greatest window surface area. (Leave a small amount of air so that the needle does not pierce the membrane.)
k) Remove needle from cassette. The gasket will reseal so that the sample will not leak.
l) Slip top edge of cassette back into the groove of the buoy. Return the to the same, labelled beaker of PBS. Add a small magnetic stir bar.
m) Place on a magnetic stirrer (set to a slow speed). Allow to dialyse for 2 hours at room temperature.
n) Change the PBS. Allow to dialyse for 2 hours at room temperature, with slow stirring.
o) Again change the PBS. Allow to dialyse overnight at 2–8^0 C, with slow stirring.
p) To remove the sample after dialysis, fill a syringe with a volume of air at least equal to the sample volume.

q) With the needle bevel sideways, insert only the tip of the needle through the port. Using the other (previously unused) top port, inject air into the cassette cavity. (Air is used to further separate the membrane so reduce risk of penetration by the needle.)
r) Rotate the cassette until the port with the syringe is on the bottom.
s) Slowly remove the dialysed sample.
t) Remove the syringe needle from the cassette. Discard the membrane cassette.
u) Transfer contents to a 2 mL screw cap tube, labelled with the protein, biotin-labelled, dialysed, concentration, and date. (Draw a ✱ on the cap.)
v) Store at 2–8^{0}C.

4. HABA assay for measuring level of biotin incorporation

a) Reagent preparation

- Remove reagents from -20^{o}C or 2–8^{o}C and allow to come to room temperature.
- HABA/avidin solution – 1 mg avidin
  60 µL 10 mM HABA in 1 N NaOH
  1.94 mL PBS
- The A_{500} of this solution should be about 0.9 to 1.3.
- If a precipitate forms in the solution, it can be filtered and then used.
- Stable if stored at 2–8^{o}C for up to 2 weeks.

b) Procedure

- Set the spectrophotometer absorbance at 500 nm. Use PBS as a blank.
- Pipette 90 µL of HABA/avidin solution into a 1 cm cuvette.
- Measure the absorbance and record the value as **A_{500} HABA/avidin**.
- Add 10 µL of biotinylated protein to this cuvette. Mix well.
- Once the value remains constant for 15 seconds, measure the absorbance.
- Record this value as A500 HABA/avidin/biotin sample.
- If this reading is ≤0.3, dilute the sample in PBS and repeat the assay (but remember to account for the dilution during calculations.)

c) Calculation of moles of biotin per mole of protein

- These calculations are based on the Beer Lambert Law (Beer's Law):

$$A_\lambda = \varepsilon_\lambda bC$$

A = the absorbance of a sample at a particular wavelength (λ).
λ = 500 nm for the HABA assay.
ε = absorptivity or extinction coefficient at the wavelength (λ). For HABA/avidin samples at 500 nm, pH 7.0 extinction coefficient = 34 000 mL / (M^{-1} cm^{-1})
b = cell path length expressed in centimeters (cm).
A 10 mm square cuvette has a path length of 1 cm.
C = the concentration of the sample expressed in mmoles/mL.

- The following values are needed for calculating the number of moles of biotin per mole of protein or sample:
  - Concentration of the protein or sample used expressed as mg/mL
  - Molecular weight (MW) of the protein or sample used expressed as Daltons

- Absorbance at 500 nm for HABA/avidin solution (A500 HABA/avidin)
- Absorbance at 500 nm for HABA/avidin/biotin sample mixture (A500 HABA/avidin/biotin)
- Dilution factor (if the sample was diluted before addition to the HABA/avidin solution)

- Calculation #1 – biotinylated sample concentration (mmoles/mL)

$$\text{biotinylated sample (mmoles/mL)} = \frac{\text{protein concentration (mg/mL)}}{\text{MW of protein (Daltons)}} = \text{Calc \#1}$$

- Calculation #2 – change in absorbance at 500 nm

$$\Delta \text{ A500} = (0.9 \times \text{A500 HABA/avidin}) - (\text{A500 HABA/avidin/biotin}) = \text{Calc \#2}$$

- Calculation #3 – concentration of biotin (mmoles /mL):

$$\frac{\text{mmoles biotin}}{\text{mL reaction mixture}} = \frac{\Delta \text{ A500}}{(34\,000 \times b)} = \frac{\text{Calc \#2}}{(34\,000 \times b)} = \text{Calc \#3}$$

- Calculation #4 – the mmoles of biotin per mmole of protein

$$\frac{\text{mmoles biotin}}{\text{mmoles protein}} = \frac{(\text{Calc \#3}) \times 10^{*} \times \text{dilution factor}^{**}}{\text{Calc \#1}}$$

* Since 90% of the HABA/avidin/biotin sample mixture is HABA/avidin solution and 10% is sample, a factor of 10 is used here.
** Use additional dilution factor only if sample was diluted before performing HABA assay.

- MAb samples

Calculation #1 = mmoles biotinylated protein per mL =

$$\frac{\text{protein concentration (mg/mL)}}{\text{MW of protein (Daltons)}} = \frac{2.0 \text{ mg/mL}}{150\,000} = 1.33 \times 10^{-5} \quad \textit{Calc \#1}$$

Calc #2 for MAb M/B 7-20 $\Delta A_{500} = (0.9 \times 0.946) - 0.64 = 0.2114$
Calc #2 for MAb C/D 11-4 $\Delta A_{500} = (0.9 \times 0.943) - 0.63 = 0.2187$

$$\frac{\text{mmoles biotin}}{\text{mL reaction mixture}} = \frac{\Delta A_{500}}{(\varepsilon \times b)} = \frac{\textit{Calc \#2}}{(34\,000 \times b)} = \textit{Calc \#3}$$

Calc #3 for MAb M/B 7-20 $\quad \frac{0.2114}{34\,000} = 6.2 \times 10^{-6}$

Calc #3 for MAb C/D 11-4 $\quad \frac{0.2187}{34\,000} = 6.4 \times 10^{-6}$

$$\frac{\text{mmoles biotin}}{\text{mmoles protein}} = \frac{\textit{Calc \#3} \times 10 \times \text{dilution factor}}{\textit{Calc \#1}} = \textit{Calc \#4}$$

Calc #4 for MAb M/B 7-20 $\quad \frac{6.2 \times 10^{-6} \times 10 \times 1}{1.33 \times 10^{-5}} =$ 4.66 average # biotin molecules per MAb molecule

#4 for MAb C/D 11- 4 $\quad \frac{6.4 \times 10^{-6} \times 10 \times 1}{1.33 \times 10^{-5}} =$ 4.81 average # biotin molecules per MAb molecule

REFERENCES

1. Pierce – Instructions EZ-Link® Sulfo-NHS-LC-Biotinylation Kit

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials

SOP 5.4 CDC Aldolase ELISA for Malaria Antigen in Blood

PURPOSE

This SOP describes the materials, equipment, and procedures required to correctly and safely use the CDC in-house aldolase ELISA to diagnose malaria using blood samples. Protocol includes:

- Setting up dilutions of recombinant *Plasmodium falciparum* (Pf) aldolase antigen for a standard calibration curve
- Preparation of blood samples for use in the assay
- Running of the ELISA in a 96 well plate format and interpretation of results.

SCOPE

This SOP has been developed for the training of laboratory personnel using the CDC in-house aldolase ELISA for malaria diagnosis in clinical and research settings. For the WHO-FIND malaria RDT evaluation programme, this SOP describes the use of this assay in the assessment of aldolase antigen content within patient blood samples that form part of the global specimen bank.

PRINCIPLE OF TEST

The CDC aldolase ELISA is suitable for the detection, in a blood sample, of the four species of malaria infecting humans. Uncoated plates with a high affinity to protein/peptide (Nunc Maxisorp) are coated with monoclonal antibodies raised against aldolase protein. Lysed blood test samples are then added to the plate such that Pf aldolase can bind to antibodies. A biotinylated detection monoclonal antibody is then added, which binds to a different epitope on the antigen. The addition of Neutravidin HRP (Pierce, lyophilized powder) conjugated to substrate solution TMB will then cause a colour change in the substrate solution. After addition of an acid stop solution, the colour will become stabilized. The colour intensity of the resulting product is directly proportional to the aldolase concentration and is measured as ΔOD 450/620 nm.

ASSAY AND SPECIMEN REQUIREMENTS

NB: All reagents should equilibrate to room temperature for at least 15 min before use.

- Sarstedt tubes
- Vortex
- Plate rocker/orbital shaker that is able to rotate at 600 rpm.
- Preparation microtitre plate (uncoated)
- High protein/peptide affinity binding plate (Nunc Maxisorp)
- Recombinant Pf aldolase (lyophilized, manufactured by Microcoat GmbH, Germany)
- Capture monoclonal antibody: Unlabelled mAb M/B7-20.
- Biotinylated detection monoclonal antibody: C/D 11-4
- Coating buffer: 0.1M Carbonate/bicarbonate buffer pH 9.6
- Wash buffer: 1 X PBST (1 X PBST: 0.1% Tween 20)
- Blocking buffer: 1% (w/v) BSA in 1 X PBST
- Lysis buffer: 1% (w/v) BSA in 1 X PBST containing 0.5% (v/v) nonidet-P 40 (NP-40)
- Neutravidin HRP (Pierce, lyophilized powder)

- TMB substrate solution (Millipore)
- Stop solution: 1M H_3PO_4
- Plate lids (including aluminium ones required for cryostorage of blocked, coated plates)
- Micropipettes (50–200 μL and 100–1000 μL)
- Multichannel micropipette (50–250 μL)
- Automated plate washer
- Spectrophotometer

NeutrAvidin lyo preparation: 1 mg/mL
400 µL distilled water into glass vial, allow lyo to resuspend
1600 µL 1 X PBS into glass vial
Dispense 15 µL into multiple single-use tubes, cap, and freeze at -20°C

1M Sodium Hydroxide: 1M = 40 g into 1000 mL ddH_20. Prepare 250 mL.

Coating buffer: For 50 mL (10 mL per plate required). Make on day required. Do not keep.
1.465 g Sodium Hydrogen Carbonate
2.146 g Sodium Carbonate Decahydrate
Dissolve into 46.5 mL ddH_20
Add 3.5 mL 1M NaOH
pH 9.6

1 X PBST: 1 X PBST, 0.1% (v/v) TWEEN 20
1 mL TWEEN 20 into 1000 mL of 1 X PBS

Blocking buffer: 1% (w/v) BSA in 1 X PBST
1 g BSA into 100 mL 1 X PBST

Lysis buffer: Blocking buffer with 0.5 % (v/v) nonident-P40 (NP-40)
125 µL NP-40 into 25 mL blocking buffer

STOP solution:
1M H_3PO_4
Mr = 98

INSTRUCTIONS FOR PERFORMING THE ASSAY

1. Plate coating

Coating buffer should be made up fresh before each set of assays. Capture monoclonal mAb M/B 7-20 should be diluted in coating buffer to a final concentration of 2 μg/mL and 100 μL dispensed into all 96 wells of the uncoated Nunc Maxisorp plate. A standard plate lid/sealer should then be added and the plate left static overnight at 4ºC in preparation for performing the next steps of the assay the following day.

2. Wash steps and blocking

Freshly coated plates should be washed for 3 cycles with 250 μL 1 X PBST per well using an ELISA plate washer. To prevent non-specific binding of proteins to the antibodies, 250 μL blocking buffer should then be dispensed into all 96 wells of the plate. A standard plate lid/sealer should then be added and the plate incubated at room temperature on a plate rocker/orbital shaker set to 580 rpm for 1 hour. Prior to loading of the plate with test samples, the plate should then be washed again for 3 cycles with 250 μL 1 X PBST. If plates are not to be used further that same day, they should be

patted to ensure wells are completely dry and stored with sealed aluminium cryo-lids/sealers at -80°C. When frozen plates are removed to use another day, they must be allowed to thaw completely on the bench before re-washing for 3 cycles with 250 μL1 X PBST and patting dry prior to addition of samples for testing.

3. Preparation of standards and test samples

a) Eight standards are used as a reference positive and prepared in serial dilution for this assay. Malaria-negative human blood that has been frozen and thawed is used as a diluent for the standards and as a negative control. A purified recombinant form of Pf aldolase expressed in *E. coli* is used to produce the standards that are diluted in human blood, see Table A. The protein is manufactured by Microcoat GmbH, Germany and is HIS-tagged for purification. Negative blood used to dilute the recombinant is pipetted into Sarstedt tubes. Stock antigen is added to the first tube at an appropriate dilution to provide a starting concentration of 250 ng/mL. Doubling dilutions made thereafter form the 8 reference points that will generate the standard curve. Between transfers from one tube to another, blood should be thoroughly mixed using a vortex and the samples pipetted up and down once or twice prior to making the dilutions in order to "coat" the tips sufficiently. A fresh pipette tip should be used between each transfer.

b) Depending on the concentration of aldolase in the specimen being tested, dilution may be appropriate in order for the test samples to fit within range of the calibration curve. If necessary, this should be done using negative human blood and a conversion factor applied during data analysis.

Table A. Preparation of the dilution set for creating the calibration curve

(Pf aldolase (x ng/mL)* varies depending on aliquots being used)

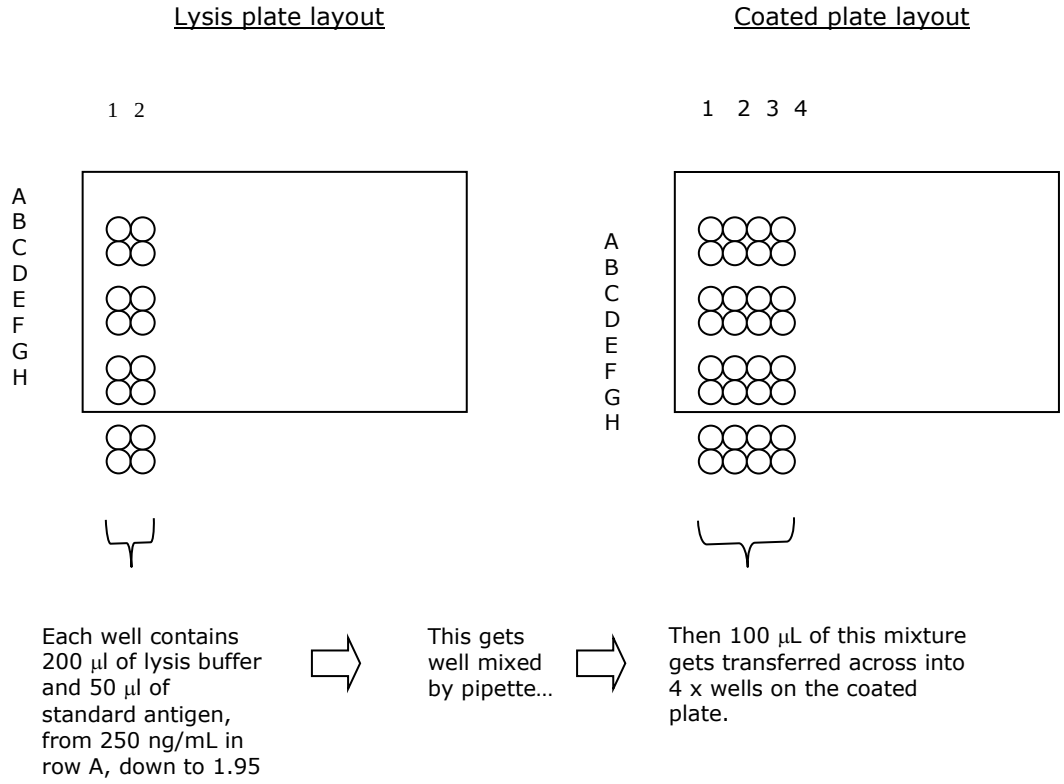
Conc required (ng/mL)	250	125	62.5	31.25	15.62	7.8	3.9	1.95
Working stock (ng/mL)	x	250	125	62.5	31.25	15.62	7.8	3.9
Volume stock (μL)	x	150	150	150	150	150	150	150
Volume diluent (μL)	x	150	150	150	150	150	150	150
Total volume (μL)	1000	300	300	300	300	300	300	300

4. Preparation of the lysis plate

a) To begin, 200 μL lysis buffer should be dispensed into wells of an uncoated/lysis plate that will contain blood samples. Subsequently, 50 μL of each of the 8 pre-prepared standards (250-1.95 ng/mL) should then be added to the wells of column one (A-H) and two (A-H) in parallel. NOTE: ONLY HALF A PLATE IS REQUIRED. Each well on this uncoated lysis plate prepares enough material of each standard/sample for DUPLICATES on the actual coated plate. Thus only columns 1-6 of each uncoated lysis plate are used per full coated ELISA plate.

b) The test samples should then be added to each of the wells consecutively from A3 as far as F6. G6 should contain 50 μL of human blood used as the negative control. H6 should contain 50 μL blocking buffer as this will be used as a "blank" to control for any significant background buffer may give rise to. For one plate, 30 specimens can be tested as all wells from the lysis plate will then be duplicated in the coated plate.

Fig. 1. Loading format for lysis and coated plates



5. Transfer of blood to the coated plate

With a multi-channel pipette, 100 μL should then be transferred from each well containing lysed blood to wells of the anti-aldolase coated test plate so that each column of wells is tested in duplicate filling the 96 well microtitre plate. The plate should then be covered with a lid and incubated at room temperature on a plate rocker/orbital shaker set to 580 rpm for 1 hour.

6. Detection and development of substrate

a) Prior to detection steps, the coated plate should then be washed again for 3 cycles with 250 μL 1 X PBST. Biotinylated detection mAb C/D 11-4 should then be diluted in blocking buffer to a working concentration of 1 μg/mL and 100 μL of the solution dispensed into all 96 wells of the coated plate. A plate lid should be added and the plate incubated again at room temperature on a plate rocker/orbital shaker set to 580 rpm for 1 hour.

b) The plate should then be washed again for 3 cycles with 250 μL 1 X PBST. Working strength enzyme conjugate should then be prepared by diluting 1:1000

Neutravidin HRP in 1 X PBST (11 μL in 11 mL if a 1 mg/mL stock was prepared from the NeutrAvidin lyo stock) and 100 μL of the solution dispensed into all but wells H11 and H12 (the "blank" wells). For the blank wells, 100 μL X PBST should be added. A plate lid should then be added and the plate left to stand on the bench at room temperature for 30 mins.

c) The TMB substrate is already at working strength but should be equilibrated to room temperature prior to use. The coated plate should be washed for another 3 cycles with 250 μL 1 X PBST after which, 100 μL of TMB should be dispensed into all 96 wells. The plate should then be covered with a plastic lid and incubated at room temperature for approx. 5 to 15 mins in the dark. Judgment should be used when to proceed to the next stop based on the saturation of the wells corresponding to the highest / most concentrated points on the standard curve.

d) When the assay has reached saturation, 100μL STOP solution should be then dispensed in all wells. The endpoint absorbance of the wells should be read at 450 nm with a reference wavelength 620 nm as soon as possible (within 10 min of adding the STOP solution).

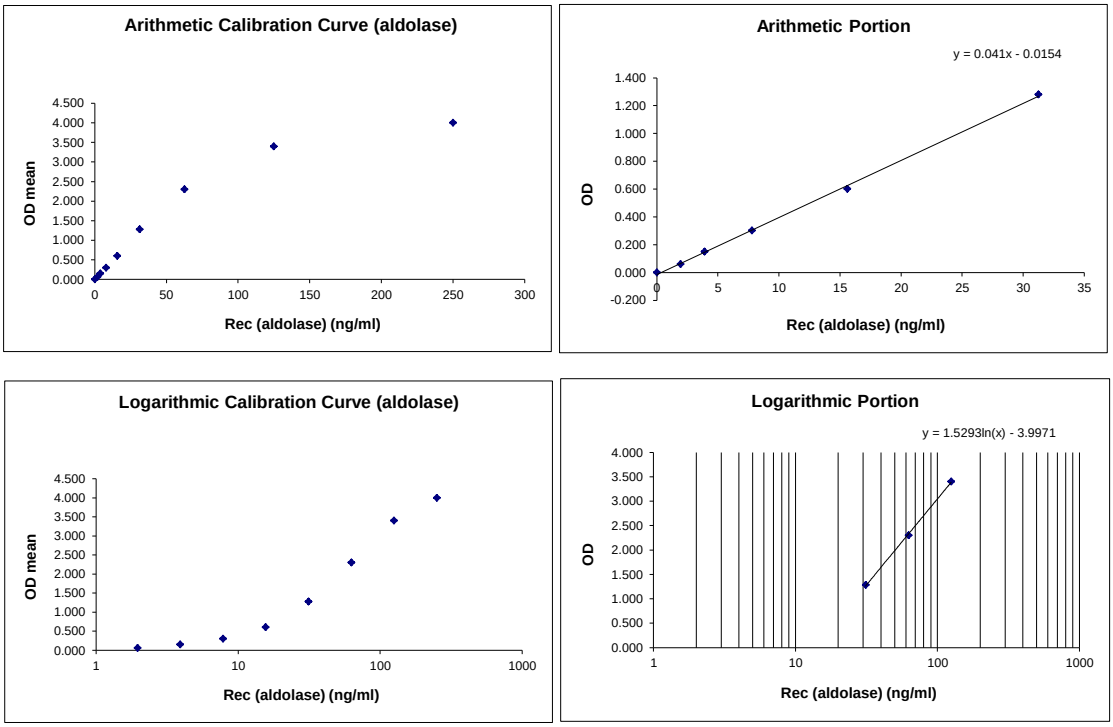
7. Interpretation of results

a) The spectrophotometer will make a print out of ΔOD 450/620 nm results and these should then be transcribed to another PC. Each point comprising the standard curve has been replicated 4 times therefore a mean OD for each point should be calculated in EXCEL. The specimens are in duplicate thus need to be averaged. Mean ODs for the standards, specimens and negative control should all be entered into the EXCEL data file to make a scatter graph (calibration curve). Both arithmetic and logarithmic curves are plotted.

b) The arithmetic calibration curve will be parabolic in shape. The logarithmic calibration curve should be sigmoidal in shape. ODs that are > 250 ng/mL standard on the logarithmic curve will begin to plateau on the curve. Test samples with ODs higher than the 250 ng/mL standard should not be interpreted because the assay begins to saturate with aldolase antigen at this point. The negative specimen should have an OD < 0.100 but it is not uncommon that a higher than usual background may be observed in this assay owing to the use of a biotinylated detection antibody.

c) Separate plots for selected points from the logarithmic and arithmetic curves are used for fitting trend-lines and obtaining equations which are used to calculate concentrations from OD for each test sample. The calculation uses a re-arrangement of the algebraic expression, $y = mx + c$. ODs are converted to aldolase concentration in ng/mL. The result on a test specimen is adjusted for any pre-dilution of the specimen.

Fig. 2. Generation of logarithmic and arithmetic trend lines for data interpretation



HEALTH AND SAFETY

1. Hazardous reagents

Table B. Hazardous chemicals used in the CDC aldolase ELISA

Product	Fire hazard	Health hazard	Toxicity	Storage requirements
Hydrogen peroxide (TMB)	Explosive under heat	Irritant to eyes/skin/nasal passage	Moderately toxic	Easily decomposes 2–8^{0}C
Orthophosphoric acid (stop solution)	Flammable	Irritating to eyes/skin. Burns. Harmful by ingestion	Toxic	Keep in a locked store
TMB	Flammable	Harmful swallowed/inhaled/absorbed by skin	Toxic	Store solutions in light proof container at 4–8°C

2. Safety precautions

- Recombinant *Plasmodium falciparum* aldolase used as a standard has been shown to be non-infectious in an *E. coli* recombinant expression system.
- Disposable latex or nitile gloves must be worn while handling clinical specimens and reagents. All clinical material i.e. all components containing blood must be

autoclaved before disposal. The assay stop solution contains orthophosphoric acid; a corrosive and hazardous substance. Avoid eye and skin contact by wearing protective clothing and eye protection.

- Hands must be washed once work has been completed.

3. Technical precautions

- Components must not be used after their expiry date.
- Different batches/lots of reagents should never be interchanged.
- Storage of reagents must be at the recommended conditions.
- Contamination of reagents should be avoided by changing pipette tips where necessary.

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials
24/05/11	4	Edited to bring up to date	RRC
14/08/14	6	Edited to bring up to date	RRC, IG, JC
2018	7	Updated technical details	RO

SOP 5.5 Dilution Protocol for Recombinant pLDH, HRP2 reagents and blood samples (ELISA)

PURPOSE

This Standard Operating Procedure (SOP) describes the procedure for calculating dilutions of reagents or samples used in the HRP2, pLDH, and aldolase ELISA procedures.

SCOPE

This procedure applies to the WHO malaria rapid diagnostic test quality assurance initiative. The SOP may be adopted by the head of the department to be compatible with pre-existing SOPs and local conditions retaining the elements of this SOP.

PROTOCOL

1. Use the following calculation to perform any dilutions of reagents or samples:

$$\frac{\text{(Concentration required)}}{\text{Stock concentration}} \times \text{Total volume required} = \text{Volume of stock required}$$

2. Record the volumes used and relevant information in Form 029.
3. Do not discard any record associated with any quality assurance scheme.
4. Always use reverse pipetting when diluting blood or other viscous substances.
5. Do not pipette a volume less than 20µL.
6. Use a separate disposable tip for each transfer to avoid cross contamination.

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials

SOP 5.6 Protocol for Recording ELISA Results

PURPOSE

This Standard Operating Procedure (SOP) describes the procedure for recording results generated from ELISA work.

SCOPE

This procedure applies to the malaria RDT product testing programme of WHO and FIND with the US Centers for Disease Control and Prevention.

PROTOCOL

1. All laboratory work is to be recorded in a hardcover laboratory book. This will serve as a hard copy record for the results saved on computer and will also serve as a daily record and audit trail of the work carried out.
2. Record the date of the test, lot number of kit and reagents used, any deviations from the standard operating procedure and any problems encountered.
3. Record all dilutions on the dilution audit form as well (See SOP 5.5 "Dilution Protocol" and Form 029 "ELISA Dilution Form")
4. Record all results onto the computer.
5. Record all raw data onto the ELISA reporting form (Form 028).
6. The data may be entered manually or transferred onto the computer; it is preferable that two people check the results to avoid transcription errors.
7. All computer data must be backed up every day in case of a computer mishap.
8. Laboratory notebooks must be photocopied on a weekly basis and the photocopies stored in a folder away from the laboratory. This is to prevent loss of data in case of a mishap.

Table A. Checklist of information to be recorded in the hardcover laboratory book

Date of test
Lot number of kit
Lot number of reagents (e.g. positive control, etc.)
Diluents used
How reagents were made up
Reason for deviation from SOP
Problems encountered
Record ambient temperature

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials

SOP 5.7 Extraction of Genomic DNA from Whole Blood Using QIAamp Protocol

PURPOSE

This SOP describes how to extract genomic DNA from whole blood samples, eventually to be used for *Plasmodium* species identification

SCOPE

This procedure applies to the malaria RDT product testing programme of WHO and FIND with the US Centers for Disease Control and Prevention.

REAGENTS

- QIAGEN Protease (Proteinase K)
- QIAGEN Buffer AL
- QIAGEN Buffer AW1
- QIAGEN Buffer AW2
- ddH2O
- Ethanol

PROCEDURE

NOTE: Heat a water bath or heating block to 56°C for use in step 4.

1. Pipette 20 µL QIAGEN protease (or Proteinase K) into the bottom of a 1.5 mL microcentrifuge tube.
2. Add 200 µL whole blood sample to the microcentrifuge tube. If the volume is less than 200 µL add the appropriate amount of PBS.
3. Add 200 µL Buffer AL to the sample. Mix by pulse-vortexing for 15 seconds.
4. Incubate at 56°C for 10 minutes.
5. Briefly centrifuge the 1.5 mL tube to remove drops from inside of the lid.
6. Add 200 µL ethanol (96–100%) to the sample, and mix again by pulse-vortexing for 15 seconds. After mixing, briefly centrifuge the tube to remove any residue from the lid.
7. Carefully apply the mixture from step 6 to a QIAamp Spin Column (in a 2 mL collection tube) without wetting the rim, close the cap, and centrifuge at 8000 rpm for 1 minute.
8. Place the Spin Column in a clean 2 mL collection tube and discard the tube containing the filtrate.
9. Carefully open the Spin Column and add 500 µL Buffer AW1 without wetting the rim, close the cap, and centrifuge at 8000 rpm for 1 minute.
10. Place the Spin Column in a clean 2 mL collection tube and discard the tube containing the filtrate.
11. Carefully open the Spin Column and add 500 µL Buffer AW2 without wetting the rim, close the cap, and centrifuge at 13000 rpm for 3 minutes.
12. Place the Spin Column in a clean 1.5 mL microcentrifuge tube and discard the collection tube containing the filtrate.
13. Carefully open the Spin Column and add 200 µL ddH2O.
14. Incubate at room temperature for 1 minute, and then centrifuge at 8000 rpm for 1 minute.
15. Store isolated DNA at -20°C for future use.

REFERENCES

1. Blood and Body Fluid Spin Protocol. QIAamp® DNA Mini Kit and QIAamp DNA Blood Mini Kit Handbook. QIAGEN. February 2003.

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials

SOP 5.8 Identification of Plasmodium Species by PCR Assay

PURPOSE

This SOP describes how to perform a nested polymerase chain reaction (PCR)-based assay for the detection and identification of malaria parasites.

BACKGROUND

This assay will be performed on whole blood known or believed to be infected with *Plasmodium* spp. The results will be used to identify and differentiate between the four main human malaria species. This is a nested Polymerase Chain Reaction, amplifying a portion of the *Plasmodium* SSU rRNA gene, in which both genus and species specific primers are used.

SCOPE

This procedure applies to the malaria RDT product testing programme of WHO and FIND with the US Centers for Disease Control and Prevention.

REAGENTS

- Expand High Fidelity Enzyme Mix (Taq DNA polymerase and Tgo DNA polymerase)
- Expand High Fidelity Buffer (10X) with 15 mM Mg Cl2
- 2mM dNTPs
- ddH2O
- Genus and species-specific primers
- Template DNA

PROCEDURE

1. General

a) Always record the date the assay was performed and note any changes to the SOP during the run in the hardcover laboratory book.
b) Bring buffer solution, DNA template, and primers well to room temperature (20–30ºC) before use.
c) Keep enzyme mix at -20ºC until needed.
d) Optimal incubation times and temperatures for thermal cycling depend on the system used and are determined individually.
e) Positive controls for nest 1 will come from *P. falciparum* SSU rRNA gene.
f) Prior to use, ensure species-specific primers are working properly by testing against positive and negative controls.
g) Perform all mixing of reagents in a sterile environment.
h) Use a separate disposable tip for each transfer to avoid cross contamination.

2. Extraction of genomic DNA from whole blood (Refer to SOP 5.7)

3. Preparation for Nest 1 PCR

a) Briefly vortex and centrifuge all reagents before starting.

b) Prepare a master mix prior to addition of template DNA (Table A).
c) Use a 1.5 mL microfuge tube when making master mix.
d) Add 1.0 µL of forward and 1.0 µL reverse genus-specific primers (Table 5) for each reaction at a concentration of 100 ng/µL or 15 mM.
e) Upon completion, pipette up and down to mix reagents.
f) A total of 3 PCR reactions will be performed; the sample in question, a positive, and a negative control.
g) Add 18 µL of master mix to three 0.2 µL thin-walled PCR tube, and make note which tube will have sample DNA as well as positive and negative controls.
h) Add 2 µL template DNA to sample tube and positive control, and 2 µL H2O to negative control to give **20 µL** total volume per PCR tube.

Table A. Nest 1 PCR master mix

REAGENTS	VOLUME NEEDED	NUMBER OF PCR REACTIONS	TOTAL VOLUME
dd H2O	11.8 µL	3	35.4 µL
10X Buffer	2.0 µL	3	6.0 µL
dNTP's	2.0 µL	3	6.0 µL
Primers	2.0 µL	3	6.0 µL
Polymerase	0.2 µL	3	0.6 µL
Total:	**18 µL**		**54 µL**

4. Thermal cycling of Nest 1

a) Place samples in a thermal block cylinder, and start cycling using the thermal profile for Nest 1 (Table B).
b) Run for 30 cycles.
c) Store PCR product at 4ºC when not in use.

Table B. Nest 1 thermal profile

STAGE	TEMPERATURE	TIME
Initial Denaturation	94ºC	5 min
Denaturation	95ºC	30 sec
Annealing	53ºC	30 sec
Elongation	68ºC	1 min 30 sec
Final Elongation	68ºC	5 min
Cooling	4ºC	Unlimited

Desired BP Size: 1.05 Kb

5. Preparation for Nest 2 PCR (Species Identification)

a) Briefly vortex and centrifuge all reagents before starting.
b) Prepare a second master mix in a 1.5 µL microfuge tube prior to the addition of template DNA and primers (Table C).
c) Upon completion, pipette up and down to mix reagents.
d) A total of 6 PCR reactions will be performed; 4 using each species-specific set of primers, 1 negative control, and 1 positive control.
e) Add 1.0 µL of forward and 1.0 µL reverse species-specific primers (Table E) for each reaction at a concentration of 100 ng/µL or 15 mM.
f) Add 1.0 µL of template DNA (PCR product from nest 1 reaction) to each of the seven PCR tubes to give **20 µL** total volume per tube.

Table C. Nest 2 PCR master mix

REAGENTS	VOLUME NEEDED	NUMBER OF PCR REACTIONS	TOTAL VOLUME
dd H2O	12.8 µL	6	76.8 µL
10X Buffer	2.0 µL	6	12.0 µL
dNTP's	2.0 µL	6	12.0 µL
Forward Primer	1.0 µL	6	6.0 µL
Reverse Primer	1.0 µL	6	6.0 µL
Polymerase	0.2 µL	6	1.2 µL
Total:	**19 µL**		**114 µL**

6. Thermal cycling of Nest 2

a) Place samples in a thermal block cylinder, and start cycling using the thermal profile for Nest 2 (Table D).
b) Run for 30 cycles.
c) Store PCR product at 4ºC when not in use.

Table 4: Nest 2 thermal profile

STAGE	TEMPERATURE	TIME
Initial Denaturation	94º C	5 min
Denaturation	95º C	30 sec
Annealing	55º C	30 sec
Elongation	68º C	1 min
Final Elongation	68º C	5 min
Cooling	4º C	Unlimited

7. Species Identification

a) Run PCR products from Nest 2 on a 1.5% agarose gel.
b) Only two bands should fluoresce; the positive control, and one species-specific PCR product.
c) Match the band to proper species-specific primer, and identify, if any, which *Plasmodium* parasite the sample is infected with.

Table E. Genus and species-specific primer pairs for Nest 1 and Nest 2 PCR reactions

Nest 1: Genus Specific	
rPLU6 (forward)	5'-TTA AAA TTG TTG CAG TTA AAA CG-3'
rPLU5 (reverse)	5'-CCT GTT GTT GCC TTA AAC TTC-3'
Nest 2: *P. falciparum* specific	
rFAL1 (forward)	5'-TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT-3'
rFAL2 (reverse)	5'-ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC-3'
Nest 2: *P. malariae* specific	
rMAL1 (forward)	5'-ATA ACA TAG TTG TAC GTT AAG AAT AAC CGC-3'
rMAL2 (reverse)	5'-AAA ATT CCC ATG CAT AAA AAA TTA TAC AAA-3'
Nest 2: *P. ovale* specific	
rOVA1 (forward)	5'-ATC TCT TTT GCT ATT TTT TAG TAT TGG AGA-3'
rOVA2 (reverse)	5'-GGA AAA GGA CAC ATT AAT TGT ATC CTA GTG-3'

Nest 2: *P. vivax* specific

rVIV1 (forward)	5'-CGC TTC TAG CTT AAT CCA CAT AAC TGA TAC-3'
rVIV2 (reverse)	5'-ACT TCC AAG CCG AAG CAA AGA AAG TCC TTA-3'

Desired BP sizes:

P. falciparum	205 bp
P. Malariae	144 bp
P. Ovale	787 bp
P. Vivax	117 bp

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials

SOP 5.9 Amplification and Sequencing of P. Falciparum Histidine-Rich Protein 2 Gene pfHRP2

PURPOSE

This SOP describes the method for polymerase chain reaction amplification and sequencing of the exon 2 fragment for the histidine-rich protein 2 gene (pfhrp2) on chromosome 8 of the *Plasmodium falciparum* genome. This method can be applied to fresh guanidine-preserved whole blood samples of *P. falciparum*, and to dried blood filter-paper samples of *P. falciparum*.

SCOPE

This procedure applies to the malaria RDT product testing programme of WHO and FIND with the US Centers for Disease Control and Prevention.

PROCEDURE

Extract parasite DNA from your blood samples and store at -20^{o}C until required.

1. Primer sequences

Round One: hrp2F1: CAAAAGGACTTAATTTAAATAAGAG
hrp2R1: AATAAATTTAATGGCGTAGGCA

Round Two: hrp2F2: ATTATTACACGAAACTCAAGCAC
hrp2R1: AATAAATTTAATGGCGTAGGCA

2. Make Primer stocks

a) Primer storage stock: Reconstitute lyophilized primers with nuclease-free water to 500 µg/mL (500 ng/µL)

b) Primer working stock: Dilute the primer storage stock to 50ng/µL (add 50 µL storage stock into 450 µL nuclease-free water).

c) Make up 1.25mM dNTPs stock: 12.5 µL of each dNTP (Promega, 100mM) is added to 950 µL nuclease-free water, total volume 1mL.

3. Set up PCR reactions

a) (A total of 50 µL per reaction) Add the following reagents into a Master Mix in an Eppendorf tube:

Round one:

Ingredient	**Amt for one reaction**	**Amt for total reactions plus one reaction**
10 x buffer	5 µL	
1.25 mM dNTPs	8 µL	
25mM $MgCl_2$	5 µL	
AmpliTaq-Gold	0.25 µL	
Primer (hrp2 F1)	1.5 µL	
Primer (hrp2 R1)	1.5 µL	
Genomic DNA	2 µL (add after aliquoting)	
Water	26.75 µL	

b) Label PCR plates/ tubes with sample name, date and operator name. Mix master mix and aliquot 48 µL in to PCR wells/tubes.
c) Add 2 µL gDNA in to each well/tube.
d) Press to close the lids.
e) Place in thermal cycler and cycle under the following conditions:

- 96 degrees for 10 min
- 96 degrees for 30 seconds
- 55 degrees for 30 seconds
- 70 degrees for 1 minute

f) Lid 105^{o}C. Repeat steps 2 to 4 for a total of 40 cycles.

4. Visualizing PCR product

Run 5 µL of the PCR product on a 2% agarose gel in 1 x TAE buffer (100 volts for ~30 min) after Rd 1. If a clean band between 800 to 1200 bp can be seen on the gel, then no need to do the second round PCR for that sample. If no band was seen, proceed to the 2nd round PCR using the same condition as the 1st round, but use primers hrp2F2 and hrp2R1 and 1 µL of the first round product as the template.

5. Clean up the PCR product

Use a Machery-Nagel (NucleoSpin Extract II Catalogue Number 740 609.50) or QIAgen DNA Clean-up kit (QIAquick PCR Purification Kit Catalogue Number 28104) to clean the PCR product from the buffers and dyes, following the manufactuer's instructions exactly. Repeat Step 4.4 to estimate the concentration of the eluted PCR product using 2 Log DNA ladder (New England Biolabs) as standard. Store the eluted clean PCR product at -20^{o}C.

6. Sequencing

a) Make a Master Mix of the following reagents; calculate for each sample to sequence plus one: (For variable amounts, this is calculated by optimizing the experiment. Start with the highest amount)
   - 2.4 µL Dye Terminator Matrix Version 3.1
   - 2.4 µL 5 x Sequencing Buffer
   - 0.25–1 µL of the forward primer from the PCR reaction (50 ng/ µL)
   - 2.0–2.75 µL nuclease-free water

b) Aliquot 7.8 µL of the Master Mix into labelled PCR tubes (strips), then add PCR product containing 10 to 15 ng DNA as estimated in Step 4.5. Make up to 12 µL with nuclease-free water. Total is 12 µL per well.

c) Cycle in a PCR Thermal Machine under the following conditions:

   - *Step 1*: 96 degrees C for 1 minute
   - *Step 2*: 96 degrees C for 10 seconds
   - *Step 3*: 50 degrees C for 5 seconds
   - *Step 4*: 60 degrees C for 4 minutes

d) Repeat Steps 2–4, for 25 cycles. Ensure there is a 4^{o}C hold at the end of this reaction.

7. Clean up after sequencing reaction.

a) Add 72 µL of 70% Isopropanol to each samples, pipette gently to mix, then transfer to a clean labelled Eppendorf tube. Vortex each tube and allow to stand on the bench for 15 minutes.
b) Place in a centrifuge to spin at 14 000 rpm at room temperature for 30 minutes. Keep the hinge of the Eppendorf tube to the outside of the spin so that you will know where the pellet can be expected to be. (Can use a refrigerated centrifuge, but may end up with more salt in the pellet than you would get with a non-refrigerated centrifuge.)
c) Carefully remove the tubes with all the hinges to one direction, tip off the liquid and use a pipette to extract the last remnants of the liquid.
d) Then add 300 µL of 70% isopropanol to each tube, vortex and spin again at 14 000 rpm for 10 minutes.
e) Carefully remove the tubes again from the centrifuge with all the hinges facing one direction, tip off the liquid and stand the tube on its edge upside down to dry at room temperature for 1 hour.
f) Place all samples in an Eppendorf tube freezer box and store at -20^{o}C until sequencing.

Contamination avoidance: Prepare buffers and primers in a separate room from amplified PCR product. Use pipettes, gowns, tips and gloves specifically for that area to avoid contamination, do not take them in to areas where PCR product has been amplified.

a) Use nuclease-free water.
b) Ensure that work areas are kept clean and wipe down after use.
c) Optimization may be required under certain conditions such as:
   - the amount of DNA template from the sample.
   - differing the amounts of Mg
   - raising the primer amount to 2.5 uL (as sometimes this can work better)
   - varying the cycle length of either round or the number of cycles.

REFERENCES

1. Baker J, McCarthy J, Gatton M, Kyle DE, Belizario V, Luchavez J, et al. Genetic diversity of Plasmodium falciparum histidine-rich protein 2 (Pf HRP2) and its effect on the performance of PfHRP2-based rapid diagnostic tests. J Infect Dis. 2005;92:870–7.

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials

Malaria RDT Product Testing Methods Manual

Chapter 6: EQA

SOP 6.1 Equipment Temperature Monitoring

PURPOSE

Regular temperature monitoring of incubators, refrigerators, and freezers is necessary to ensure accuracy of temperature settings. Routine general maintenance of all equipment, meanwhile, is essential to keep them in good condition.

Hence, this SOP describes the procedure for temperature checks, as well as maintenance on all appropriate equipment.

SCOPE

This procedure applies to the malaria RDT product testing programme of WHO and FIND with the US Centers for Disease Control and Prevention.

PROCEDURE

1. Thermometers with appropriate temperature ranges are used for each incubator, refrigerator, and freezer in the laboratory.
2. Daily temperature readings are recorded in daily temperature monitoring sheets posted in front of the equipment.
3. Temperature checks are done at a set time every day by designated lab personnel.
4. Personnel should make arrangements with other staff to perform the temperature monitoring if they are away on annual or sick leave.
5. At the end of each month daily temperature sheets are placed in a folder and arranged in convenient order.
6. Relevant personnel must be notified in case of temperature deviation outside the acceptable ranges.

REFERENCES

1. Unit temperature and maintenance records Standard Operating Procedures. Brisbane: Australian Army Malaria Institute; 2000 (unpublished report).

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials

SOP 6.2 Document Control

PURPOSE

To detail the Quality Control of WHO-coordinated malaria rapid diagnostic test laboratory testing.

SCOPE

This procedure applies to the malaria RDT product testing programme of WHO and FIND with the US Centers for Disease Control and Prevention.

PROCEDURE

1. General

a) WHO controls the issue, approval and updating of all quality related documents and data. The Quality Manager's role is to maintain a Document Master List and ensure that laboratory personnel perform their own internal checks of their documents and data (Ref A) and that the internal audits adequately address the issue document control in their Internal Audits Checklists.

b) The SOP applies to:
   - Quality Policy
   - Methods Manual (Standard Operating Procedures, Ref A)
   - Forms
   - Standards, Acts, Regulations and Codes
   - Electronic Data

2. Registers

a) This Methods Manual (SOP) acts as a register for quality documentation such as Forms, SOPs and Work Instructions. Templates are maintained in the Methods Manual as registered forms.

b) A distribution list is maintained by WHO showing where the copies of the Methods Manual are located.

3. Issue status

All quality documentation is to have an issue status in order that obsolete documents can be identified. This can be found in the header of the document together with the "file name". The file path can be found in the footer.

4. Amendments/raising

Amendments are made only by authorized WHO and designated laboratory officers agreed with WHO. Suggested amendments should be communicated to the officer.

5. Obsolete documents

Obsolete documents are to be removed from the active documentation system and placed in the archive folder. Hard copies are to be removed from the department and destroyed or placed in archive folder where their retention is important.

6. Authorization

Some Quality Documents require "authorization". These Quality Documents, for example, Duty Statements and SOPs, have specified areas in the "Headers" identify who has authorized the document.

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials

SOP 6.3 Document Storage

PURPOSE

This SOP describes the process for storage of documents as part of RDT QA.

SCOPE

This procedure applies to the malaria RDT product testing programme of WHO and FIND with the US Centers for Disease Control and Prevention.

PROCEDURE

1. Documents

a) All documents generated as part of the QA are to be archived for 5 years.
b) Records must be legible.
c) If paper-based records are kept, they are to be filed in an organized manner.

2. Computer

a) The computer must be password protected.
b) Records stored electronically are to be well organized.
c) Data stored on the computer must be backed-up regularly and the back-up ideally stored in separate building.
d) An electronic copy should be sent to responsible WHO officer for archiving.

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials

SOP 6.4 Corrective Action

PURPOSE

This Standard Operating Procedure describes the system for recording problems and creating solutions.

SCOPE

This procedure applies to the malaria RDT product testing programme of WHO and FIND with the US Centers for Disease Control and Prevention.

PROCEDURE

1. The Corrective Actions Register (CAR) (Form 030) is to record all incidents that impact on the normal operation of the RDT QA laboratory, both administrative and technical. The register is to be used to record suggestions as to how systems may be improved.

2. When an incident occurs (e.g. tests fails to work, results reported incorrectly, incubator temperature out of range, freezer alarms) the incidence is to be noted in the register and the head of the department or scientist is to be notified.

3. All staff are authorized to record incidence in the register. Entries are to include a brief description of the incidence, action taken to address the e issue and staff initials and date.

4. The head of the department is responsible to review the register in their department to familiarize themselves with what has been occurring and then initial the register to indicate that the register to indicate that the entries has been sighted and they are familiar with the action taken (this should occur weekly).

5. Where action taken is incorrect or inadequate, the head of the department should provide feedback to the staff member/departmental staff on further action.

6. Review of the register should an agenda item for all staff meetings as it promotes the culture of continuous improvement and is a useful training tool.

7. The assumption with CARS is that any action initially taken to address the system is in most cases of a temporary nature and the problem required an investigation of all aspects of the problem, consultation with external parties and the identification of the "cause of the problem" once identified, preventative action is put in place.

8. Preventative action often requires significant efforts such as changing procedures/forms, raising additional administrative paper work, development and delivery of training.

PROCEDURE HISTORY

Date dd/mm/yy	Version	Comments	Initials

Malaria RDT Product Testing Methods Manual

Chapter 7: FORMS

Form 002: RDT Front Desk Register (Optional)

For boxes addressed to RDT QC laboratory

Received by (sign)	**Date received (dd/mm/yy)**	**Label on box**

Form 005: RDT Register

RDTs SHOULD be stored BELOW 25°C, AND CONDITIONS NOTED WHEN THIS IS NOT POSSIBLE

RECEIPT								DESCRIPTION				
Date Received	Received From	Source Manufacturer	Name of RDT	Source Manufacturer	Catalog Number	Lot numbers	Expiry	Quantity Received	Type of Packaging	Condition	Temp. Monitor	Other Comments
dd/mm/yy	**Courier**	**Company Name**	**Product Name**	**Country**			**dd/mm/yy**	**boxes/test per box**	**box,cooler, ect…**		**yes or no**	

Form 022: Incubator Calibration Sheet (unless computerized tracking is monitored)

Name and Temperature Range of Incubator: _____

Name and Serial Number of Reference Thermometer _____

DATE	TIME	DIAL TEMPERATURE (^{0}C)	SIGNATURE	DATE	TIME	ACTUAL TEMPERATURE (^{0}C)*	SIGNATURE

a) Measure actual temperature using reference thermometer

Form 023: Temperature Monitoring Form

Equipment: _____ Location: _____ Month: _____

T(^{0}C)																														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Sign:																														

Date: _____ Comments: _____

_____ _____

_____ _____

_____ _____

Form 028: ELISA Reporting Form

RDT Kit	Manufacturer	Diluent Used (for recombinant Ag)	Lot Number	Expiry date

Date of Testing _ _ / _ _ / _ _ _ _ (dd/mm/yyyy) Operator __ __ __ __ __ __ __

96 well template (enter sample number)

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

96 well OD readings result template (enter OD reading)

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Form 028: ELISA Reporting Form

Result table (may need to include RDT results)

Sample number	Sample ID (e.g. blank, standards, controls, tests, PCW)	OD reading	Extrapolated Concentration ng/ml	RDT result (optional)
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
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40				
41				
42				
43				
44				
45				
46				
47				

Form 028: ELISA Reporting Form (contd.)

Sample number	Sample ID (e.g. blank, standards, controls, tests, PCW)	OD reading	Extrapolated Concentration ng/ml	RDT result
48				
49				
50				
51				
52				
53				
54				
55				
56				
57				
58				
59				
60				
61				
62				
63				
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Form 029: ELISA Dilution Form

Date of Testing _ _ / _ _ / _ _ _ _ (dd/mm/yyyy) Technician _____

Name of stock to be diluted e.g. Recombinant HRP2 _____

Conc required	Stock Conc	Total volume required	Size of automatic pipette used	Volume of Stock in total volume	Size of automatic pipette used	Volume of diluent in total volume

Form 030: Corrective Action Register

Description of Problem/Incident Signature Date _ _ / _ _ / _ _ _ _ (dd/mm/yyyy)
Action Taken to Resolve Problem Signature Date _ _ / _ _ / _ _ _ _ (dd/mm/yyyy)
Cause of Problem
Preventative Action Taken Signature Date _ _ / _ _ / _ _ _ _ (dd/mm/yyyy)
Verification of Effectiveness Signature Date _ _ / _ _ / _ _ _ _ (dd/mm/yyyy)

Form 032: Randomization Chart

Date: dd/mm/yyyy	**Set:**
ID	**Random number allocated**
	1
	2
	3
	4
	5
	6
	7
	8
	9
	10
	11
	12
	13
	14
	15
	16
	17
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	35
	36
	37
	38
	39
	40
	41
	42
	43
	44
	45

Date: dd/mm/yyyy	**Set:**
ID	**Random number allocated**
	46
	47
	48
	49
	50
	51
	52
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	56
	57
	58
	59
	60
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	90

Form 033: Panel Testing – Technician Result Sheet

PANEL TESTING - TECHNICIAN RESULT SHEET								(Form 33)
		Date (dd/mm/yyyy)						
		Manufacturer						
Set (A or B)		Product						
LOT (1 or 2)		Catalog						
		Lot						
		Expiry						
		Test Procedure:				Specimen(µl)	BufferDrops	Read Time
1st Read		Technician (Name .....)						

Random ID #	Time of test	Time of reading	Result					
			Cont	Line 1	Line 2	Line 3	Comments	
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
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14								
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36								
37								
38								
39								
40								

Technician Signature: _____

PANEL TESTING - TECHNICIAN RESULT SHEET (Form 33)

Date (dd/mm/yyyy)

Manufacturer

Set (A or B) **Product**

LOT (1 or 2) **Catalog**

Lot

Expiry

Test Procedure: Specimen(µl) | BufferDrops | Read Time

2nd Read **Technician** (Name .....)

Random ID #	Time of test	Time of reading	Result					
			Cont	Line 1	Line 2	Line 3	Comments	
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
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12								
13								
14								
15								
16								
17								
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37								
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39								
40								

Technician Signature: _____

Form 034: Panel Testing – Densitometer Result Sheet

Not in use after Round 1.

PANEL TESTING DENSITOMETER RESULT SHEET

Date (dd/mm/yyyy)**:**
Manufacturer:
Product:
Cat. Number
Lot:

Technician (Name .....)

Densitometer					
Random ID #	Cont	Line 1	Line 2	Line 3	Comment

Signed: .....

Form 035: Stability Test – Technician Result Sheet

STABILITY TEST:TECHNICIAN RESULT SHEET								(Form 35)
Date (dd/mm/yyyy)								
Manufacturer								
Product								
Catalog								
Lot			**Temperature Storage(RT,35°,45°):**					
Expiry			**Time of Read (Interval in Days):**					
						Specimen(µl)	BufferDrops	Read Time
1st Read		**Technician** (Name .....)						

Random ID#	Time of test	Time of Reading	Result					
			Cont	Line 1	Line 2	Line 3	Comments	
Negative								
Negative								
Negative								
Negative								
Pf -200								
Pf -200								
Pf -200								
Pf -200								
Pf -200								
Pf -200								
Pf -200								
Pf -200								
Pf -200								
Pf -200								
Pf -200								
Pf -200								
Pf -200								
Pf -200								
Pf -200								
Pf -2000								
Pf -2000								
Pf -2000								
Pf -2000								
Pf -2000								
Pv- 200								
Pv- 200								
Pv- 200								
Pv- 200								
Pv-2000								
Pv-2000								
Technician Signature:								

STABILITY TEST:TECHNICIAN RESULT SHEET								(Form 35)
Date (dd/mm/yyyy)								
Manufacturer								
Product								
Catalog								
Lot			**Temperature Storage(RT,35°,45°):**					
Expiry			**Time of Read (Interval in Days):**					
						Specimen(µl)	BufferDrops	Read Time
2nd Read		**Technician** (Name .....)						
Random ID#	Time of test	Time of Reading	Result					
			Cont	Line 1	Line 2	Line 3	Comments	
Negative								
Negative								
Negative								
Negative								
Pf -200								
Pf -200								
Pf -200								
Pf -200								
Pf -200								
Pf -200								
Pf -200								
Pf -200								
Pf -200								
Pf -200								
Pf -200								
Pf -200								
Pf -200								
Pf -200								
Pf -200								
Pf -200								
Pf -2000								
Pf -2000								
Pf -2000								
Pf -2000								
Pf -2000								
Pv- 200								
Pv- 200								
Pv- 200								
Pv- 200								
Pv-2000								
Pv-2000								
Technician Signature:								

Form 036a: Stability Test – Manufacturer's Result Sheet

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Manufacturer:
Product:
Lot:
Catalogue Number:
TIME OF READING (INTERVAL IN MONTHS) ('0', or '3', '6' etc):
Technician (Name .....) **Colour chart used:**

Parasite density: Negative, 200, 2000 para/uL	**Time of preparation**	**Time of reading**	**Result:** Rate colour intensity 0-4 using colour chart provided)					
			Control	Line 1	Line 2	Line 3	Comment	Interpretation (species)
Negative								
Negative								
Negative								
Negative								
Negative								
Negative								
Negative								
Negative								
200 para/µL								
200 para/µL								
200 para/µL								
200 para/µL								
200 para/µL								
200 para/µL								
200 para/µL								
200 para/µL								
2000 para/µL								
2000 para/µL								
2000 para/µL								
2000 para/µL								
2000 para/µL								
2000 para/µL								
2000 para/µL								
2000 para/µL								

Result should be recorded according to intensity of line, referenced against colour rating chart provided.
'Control': Control line. 'Line 1 -3': Test result lines, as appropriate for the product.

Signed: .....

Form 037: Stability Test – Densitometer Result Sheet

Not in use after Round 1

STABILITY TESTING DEMSITOMETER RESULT SHEET							
Date (dd/mm/yyyy)**:**							
Manufacturer:			**Blood sample ID:**				
Product:			**Temperature of Storage:**				
Cat. Number:							
Lot:							
TIME OF READING (INTERVAL IN DAYS) ('0', or '60')							
Technician Name:							
Parasite density: parasite/uL	**Time of preparation**	**Time of reading**	**Result**				
			Control	Line 1	Line 2	Line 3	Comment
Neg							
Neg							
Neg							
Neg							
Neg							
200							
200							
200							
200							
200							
200							
200							
200							
200							
200							
200							
200							
200							
200							
200							
200							
2000							
2000							
2000							
2000							
2000							

Signed: …….

Form 038: Stability Test – Master Result Sheet

STABILITY TESTING MASTER RESULT SHEET										**Test interval:**						**Storage temperture:**	
Date: (dd/mm/yy):		**Manufacturer:**					**Product:**					**Cat. Number:**			**Lot:**	**Blood sample ID:**	
Sample /Dilution	Technician 1						Technician 2						Densitometer (Not in use after Round 1)					
	Result 1					Comment	Result 2					Comment	Densitometer Result				Comments	Comment
	Cont	Line 1	Line 2	Line 3	Interpretation		Cont	Line 1	Line 2	Line 3	Interpretation		Cont	Line 1	Line 2	Line 3		
Neg																		
Neg																		
Neg																		
Neg																		
Neg																		
200																		
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Signed: .....

Form 039: Ease of Use Assessment Sheet

Part 1 (left hand side)

				Form 039: EASE OF USE ASSESSMENT RESULTS							
Product	**Manufacturer**	**Lot Numbers**	**Catalogue number**	**Blood safety**[†]				**Instruction quality**[‡]			
				Mixing wells involved (y=0/n=1)	Retractable needle (needle=0/retract=1)	Strip Exposed (exposed=0/covered=1)	Score (max. 3)	No diagram/picture	Diagram/picture of result (1)	Diagram of result & method (2)	Score (max.2)
Signed:______________________________											

Part 2 (right hand side)

						Items included in package*		**Buffer**					
Combined score (max. 5)	Number of timed steps	Total time to result	Blood transfer device	Format	Language of instruction		Dessicant Color Change (y/n)	Buffer Conatiner does not puncture	Buffer does not flow freely	insufficient buffer in bottle or vial	empty buffer bottle or vial	discoloured buffer	Comments