TECHNICAL SERIES

Malaria RDT's

Performance Evaluation and Quality Assurance - Perspectives

...Setting trends

Background

Early rapid diagnosis of malaria is crucial to the healthcare programmes in endemic countries. Their increasing importance is in response to increasing drug costs and recognition of the importance of early and correct treatment to attain reduction in malaria morbidity and mortality.

Malaria RDT's in the last decade have played a vital role in this regard in reaching objective treatment to affected populations even in resource poor settings, where the traditional microscopic diagnosis is either impractical or impossible.

In the last decade there has been an increase in the operational use of RDT's as well as an increase in number of companies manufacturing or dealing in malaria RDT's.

Wide range of field and laboratory trials have been conducted in the last decade to assess the accuracy and effectiveness of various products to establish their performance, quality and appropriateness for use in the endemic areas.

These trials have given the users and decision makers a huge database to review. In addition, local testing in country of use works to further supplement and validate this performance data for decision making.

Published field trials of Malaria RDT's have however shown high variability in performance due to the following reasons:

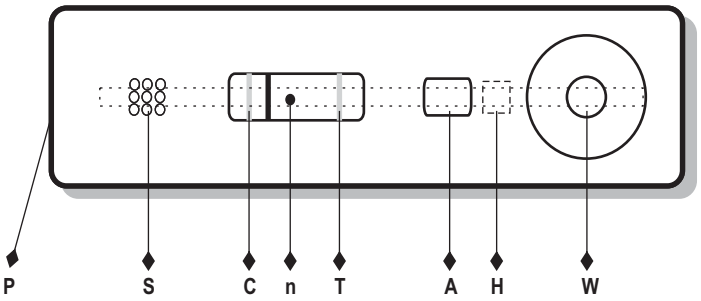
- Poor study methods, analysis & reporting.
- Inexperienced manpower.
- Incorrect handling.
- Poor preparation and interpretation.

Attempts have been made to undertake larger centralized trials to evaluate the performance of Malaria RDT's.

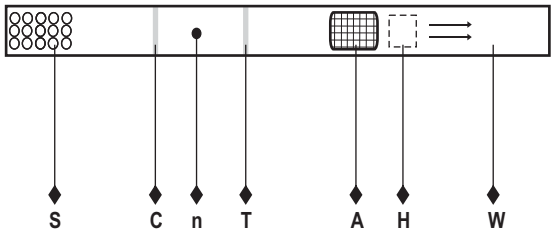
Before discussing the alternative approaches to the performance evaluation of Malaria RDT's, it would be useful to understand the architecture and functioning of Malaria RDT's.

Typical Architecture and Design of Malaria RDT's

Device



Dipstick



The Essential Components of A Malaria RDT:

W = Wicking area for sample / buffer.
A = Conjugate pad containing the gold conjugate to target antigen specific antibody.
C = Control line striped with relevant antibody to give a test run validation.
S = Soak pad that absorbs the unreacted sample post test conclusion.
H = HAMA Blocking Reagents embedded on sample pad.
T = Test line striped to target antigen specific antibody.
n = Nitrocellulose membrane.
P = Plastic housing (for device).

Components to be Used with RDTs:


Desiccant with indicator to ensure moisture free pack.

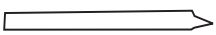

Buffer bottle for test run.


Alcohol swab.


Glass / plastic test tube (for dipstick).

Sample dispensing loop.

Sample dispensing straw.


Lancet for obtaining finger prick blood.

Malaria RDT's: Immunological Considerations

RDT Combinations	Possible Detection Systems	Detects
Pf only	Monoclonal Anti Pf HRP-II specific	*P. falciparum* infection
Pf only	Monoclonal Anti Pf pLDH specific	*P. falciparum* infection
Pf and Pan	Monoclonal Anti Pf HRP-II specific + Monoclonal Anti Pan pLDH specific (co-specific to *P. falciparum, P. vivax, P. ovale* and *P. malariae)*	*P. falciparum* infection & Differentiate *P. falciparum* infection and non *P. falciparum* infection
Pf and Pan	Monoclonal Anti Pf HRP-II specific + Monoclonal Anti Pan Aldolase specific (co-specific to *P. falciparum, P. vivax, P. ovale* and *P. malariae)*	*P. falciparum* infection & Differentiate *P. falciparum* infection and non *P. falciparum* infection
Pf and Pan	Monoclonal Anti Pf pLDH specific + Monoclonal Anti Pan Aldolase specific (co-specific to *P. falciparum, P. vivax, P. ovale* and *P. malariae)*	*P. falciparum* infection & Differentiate *P. falciparum* infection and non *P. falciparum* infection
Pf and Pan	Monoclonal Anti Pf pLDH specific + Monoclonal Anti pan pLDH specific (co-specific to *P. falciparum, P. vivax, P. ovale* and *P. malariae)*	*P. falciparum* infection & Differentiate *P. falciparum* infection and non *P. falciparum* infection
Pf and Pv	Monoclonal Anti Pf HRP-II specific + Monoclonal Anti *P. vivax* pLDH specific	*Speciates P. falciparum* infection & *P. vivax* infection
Pan only	Monoclonal Anti pan pLDH specific (co-specific to *P. falciparum,* P. vivax, P. ovale and *P. malariae)*	All malaria parasite infection
Pf, Pv and Pan	Monoclonal Anti Pf HRP-II specific + Monoclonal Anti *P. vivax* pLDH specific + Monoclonal Anti pan pLDH specific (co-specific to *P. falciparum, P. vivax, P. ovale* and *P. malariae)*	*Speciates P. falciparum* infection, P. vivax infection, Detects non *P. falciparum* and *P. vivax* infections

General Comments:

- All HRP-II detection tests, have a better analytical sensitivity and a lower limit of detection for *P. falciparum* malaria as compared to pLDH and Aldolase based assays.
- Since HRP-II persists in the blood circulation 2-3 weeks even after successful treatment testing within the said "hang over"period will give "false positive results". Hangover period of pLDH assays is much shorter than for HRP-II based assays.
- HRP-II detection systems by and large do display better thermal stability over other detection systems based on pLDH and aldolase. However, product to product the stability may vary.
- pLDH based detection assays have lower analytical sensitivity as compared to HRP-II based assays for detection of *P. falciparum* infections.
- pLDH and Aldolase based assays also have lower thermal stability as compared to HRP-II assays.

Similarities and Differences in Malaria RDTs

Though the various RDT's appear to be similar, they vary considerably in their functioning due to the intrinsic character of the critical components employed. The performance of each RDT is based on the optimization of all these functional components, which vary from manufacturer to manufacturer in terms of their basic characteristics and processing methods employed during manufacture.

Component	Typical function	Impact on RDT
Sample Pad	Usually contains HAMA blocking agents.	Improves specificity by blocking RF and heterophilic antibodies.
Buffer	Brings about lysis of the red cells, especially of the sequestered parasite and release of target antigens.	The lysing efficiency of the buffer • dictates the revelation of the target antigen from the parasitized red cells. • Formulation effectiveness impacts sensitivity of test.
Gold Conjugate	Binds to the Target antigen as the lysed blood flows through the conjugate pad.	Primary binding agent to the target antigen, it influences sensitivity, specificity and binding affinity to target antigen.
Nitrocellulose Membrane	Serves as a migration template /platform for the reaction and directs reactants and reaction kinetics of each test.	**Large porosity membranes:** reactants move fast. Better background clearance, however, leads to loss of test sensitivity since accords less time for reagents to react and bind. **Small porosity membranes:** Reactants move slowly, slower background clearance, improves test sensitivity as reagents have more time to react and bind.
Test lines	Capture antibody directed towards the specific malaria antigen in the sample.	Secondary binding agent in tandem with gold conjugate; drives sensitivity, specificity and affinity of binding and signal intensity.
Control line	Reacts with excess / unreacted conjugate to validate test run.	Ideally should be the weakest visible intensity to serve as a practical comparator for test intensity.
Soak pad	Absorbs excess reactants at test culmination.	Absorbance capacity impacts back flow of reactants post test completion.

The final optimization of each product will then have an impact on its functioning and the resultant

- Migration and Reaction Kinetics
- Sensitivity
- Specificity
- Thermal stability
- Ease of use

Problems associated with the usage of Malaria RDT's

- RDT's retrieved from cold storage (2-8°C must be allowed to come to room temperature (ambient temperature, in case of field conditions) before the pouches are opened and the test used. When specimen is added to a cold device, it attracts a 'moisture rush' thereby altering the migration properties of the membrane. If specimen is added to 'cold' devices the blood flow is usually slowed down affecting the background clearance and visualization of test results: especially in specimen containing low target antigen concentration the test result affected. At lower temperatures the antigen-antibody binding is less than optimum, leading to loss in sensitivity and resultant signals.
- Use of specimen transfer devices such as loops and straws are extremely simple to use. However, to ensure accuracy and precision of sample delivery, user training has to be imparted to build usage competence with actual users.
- The end point of a Malaria RDT reaction is qualitative. Its interpretation has an element of personal subjectivity, that introduces variability. What appears as a weak positive to one reader may well be negative for another. Usually “tie break”method or “best of 3” is the best way to resolve interpretative subjectivity. User training, hands on experience in reading and interpreting RDT end points must be assessed for consistency as a prerequisite. This is especially relevant when samples used during evaluation are low analyte concentrations, nearing the detection limits of RDT's are being used that generate very low signal intensities.

Alternative approach to Laboratory Quality Assurance of Malaria RDT's

For large scale comparative trials of malaria RDT's, availability of standardized, fresh and well characterized clinical specimen is essential. However, achieving this has posed significant challenges due to variability linked to specimen collection, preservation and logistics involved.

To overcome these challenges, centralized evaluations with regards to the performance evaluation of malaria RDT's have used alternative methods. These methods have involved alternate materials such as cultured *P. falciparum* parasites at different parasitic densities, wild type *P. falciparum* and *P. vivax* specimen etc. These samples have been diluted and cryo-preserved before use. During evaluation the panels so prepared have been thawed and used to assess the performance of the malaria RDT's with regards to their performance especially towards sensitivity, specificity and other characteristics.

Effect of Clinical Specimen Quality on Performance of Malaria RDT's

Malaria RDT's are designed and standardized primarily for testing specimen obtained from fresh capillary whole blood in field conditions, and blood correctly collected through veni puncture in laboratory settings. Venous blood collected in appropriate anticoagulants, when stored at 2-8^{0} C may be stable for up to 72 hours provided they are not contaminated.

Unhindered sample flow is at the heart of the performance of a malaria RDT. When the sample does not flow on the Malaria RDT, blood clearance on the nitrocellulose membrane is affected. Poor clearance affects visualization of the test results and also the sensitivity and specificity of the RDT.

The following aspects of sample quality affect the functioning of malaria RDT's.

- Cold blood samples can flow differently from those stored at R.T.
- Stored blood can loose target antigen activity.
- Early lysis, protein coagulation, presence of artefacts inhibit the flow of blood specimen on RDT's.
- Freeze thaw accelerates target antigen denaturation and alters blood flow properties on malaria RDT's, hampering their performance.
- In some instances, for some products improvement in RDT sensitivity has been reported, owing to target antigen release through parasite lysis.

Performance of RDT's using venous blood specimen and archived blood specimen can differ from performance obtained with fresh finger prick blood.

Effect of Prepared whole blood controls on performance of Malaria RDT's

Though Malaria RDT's are essentially designed and optimized to work with capillary finger prick whole blood samples, for large scale laboratory based trials it may become necessary to procure well characterized veni puncture blood and use diluted or archived whole blood for longitudinal studies.

Preparation of serial parasite dilution necessitates mixing parasitized blood into well characterized parasite negative whole blood. The preparation of serial dilution can affect quality of the diluted blood due to:

- Inadequate mixing.
- Blood type incompatibility.
- Freeze thawing during storage and use.
- Cell lysis due to use of small bore pipettes for dispensing samples.

The method of preparation of whole blood controls can affect the blood flow on the RDT's, greatly influencing on the thresh holds of sensitivity, specificity and the validity of the Malaria RDT's.

Effect of Artificial panels and reference materials on the performance of RDT's

It is well known that the production of target antigen varies with the stage of the parasite development. Yet at present there is insufficient information on the relationship of target antigen concentration and parasite density. In vivo, during the malarial infection as the parasitaemia increases, there is a sustained build up of the target antigens in the blood. On the other hand in vitro, when the parasite is grown in culture systems the dynamics of the production of the target antigen is completely different. This is probably due to the dissimilar environment for growth between the cultured and the wild parasites.
Thus, the relationship between parasite density and target antigen activity varies significantly between wild type parasites and cultured parasites.
This fact introduces a disconnect between "parasitaemia" and "Target antigen concentration". When reference material from these two systems are used for assessing performance of RDT's, the reference material prepared from the "cultured parasites"would not accurately reflect the target antigen concentrations as may be present in the wild type parasites, even for synchronous stage of parasitaemia or stage of development.

Importance of Clinical Samples

Target antigen sequences vary from region to region for the Malaria parasites. Especially for Pf HRP-II, wide diversity in Pf HRP-II sequences both within and between countries have been reported. Variation in the number and combination of repeats within the Pf HRP II sequences have been shown to affect the sensitivity of Malaria RDT's. Additionally various isolates also differ in terms of:

- Antigen expression.
- Structural variations.
- Variations in parasite density versus target antigen concentration.

The above differences introduce variations in apparent RDT sensitivities from region to region. These factors also make setting lower detection limits for Malaria RDT's in terms of Parasite density practically and biologically incredible. In fact it makes strong case for well designed and executed local level performance evaluation of products even stronger.

Commutability of Reference Materials & Standards

The fundamental goal of laboratory medicine remains that "results for patient samples should be comparable independent of the medical laboratory that produced the results" Cummutability of a Control material is its ability to have inter assay properties comparable to properties demonstrated by authentic clinical samples. Commutability must be validated and demonstrated amongst all the methods that will use the material including the reference test procedure.

Until commutability of a reference material is not established, results from various methods cannot be legitimately compared.

Non commutability of reference materials can be caused by Matrix alteration or a on native analyte. The Matrix effect or Matrix bias is caused by the differences in sample Matrix of the reference material and the native clinical samples.

Non native forms of the target analytes, can produce a different measurement signal than expected from the native forms of analyte.

Ideally the sample matrix includes all components of a material system except the analyte itself.

Test calibration with reference materials that are not commutable can cause poorer rather then improved agreement of results among methods for native samples. Poor commutability of reference materials cause grave difference in apparent product performance and introduce bias in measurement of analytes, compromising comparability of product performance.

Setting Standards for Sensitivity and Performance

Since Malaria RDT's detect the appropriate 'target antigens' and not the 'parasite' per se, "parasite antigen concentrations" could be a more appropriate benchmark to be used to set the lower limits of detection for Malaria RDT's. Determination of the relationship between target antigen concentration to parasite density would be a critical step towards this goal.

There is an urgent need to make available commutable reference panels that are well characterized in terms of target antigen concentration and their antigenic structure for the objective evaluation of the performance of malaria RDT's and for their unbiased comparability.

Summary

Various methods have been devised and are in the process of evolution with regards to validating the sensitivity, specificity and performance of Malaria RDT's. Each method has the potential to significantly alter the specimen characteristics in ways that is detrimental to the performance characteristics of malaria RDT's in unpredictable ways.

Preparation and availability of commutable Reference material and their wide availability to manufacturers, regulators and users, by setting a common and consistent "reference Benchmark" could lead to the desired outcome for improvement in quality, consistency, performance and deliverance of "better Malaria RDT's" to the users.

Till such time:

- Well designed and executed field / laboratory evaluations of Malaria RDT's.
- Performed by well trained personnel.
- Using clinical / well characterized specimen bank.
- At the user level.

perhaps remains the most appropriate, effective and practical method for assessing the performance of Malaria RDT's and their appropriateness for use in each setting.

Do's & Don'ts of Malaria RDT Assay

Do's	Don'ts
1. Once opened use the device immediately.	1. Do not use blood specimen, stored for more than 72 hours.
2. Always check the colour of desiccant immediately upon opening the pouch. It should be blue in colour. If the desiccant has turned colourless or pink, discard the test device and use another device.	2. In case the test device pouches have been stored at 4-8°C, do not open the pouches immediately after retrieving from 4-8°C storage. Cold devices will attract 'moisture rush' and thereby alter migration properties of the membrane leading to erroneous results.
3. In case of anti-coagulated venous blood, ratio of blood & anticoagulant should be accurate as recommended.	3. Do not use cold whole blood specimen (stored at < 25°C) as test specimen. Cold blood can flow differently and may effect the clearance of test window besides, the sensitivity and specificity of the Malaria RDT's.
4. In case the test device pouches have been stored at 4-8°C, they must be allowed to come to ambient temperature before opening/testing.	4. Do not use clotted, partially clotted, lysed, or contaminated whole blood specimen for testing.
5. Always evenly mix anti-coagulated blood specimen gently before testing.	5. Do not reuse the sample applicator/ loop.
6. Always make sure that the blood from specimen applicator/ loop has been completely transferred to the sample pad.	6. Do not intermix the buffer and devices of different lots.
7. Stored cold whole blood specimen must be allowed to come to ambient temperature before testing.	7. Do not dispense less / excess number of buffer drops in the buffer port other than those recommended in test procedure. This could effect the reaction kinetics between target antigen and capture elements of the test system.
8. Read the result only at the end of recommended reading time.	8. Do not move the device during assay. It could effect the buffer flow.
9. Always lay the test device on a flat surface horizontally before testing.	9. Do not compare test band intensity with control band intensity.
10. Users interpreting the test result must be well-trained to read the test band signals especially with low analyte concentration specimen.	10. Do not repeatedly freeze and thaw test specimen. Repeated freeze-thaw cycles accelerate target antigen denaturation and alter blood flow properties on the RDT's, hampering their performance.

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