Evaluation of rapid diagnostic tests: malaria

WHO–Regional Office for the Western Pacific/TDR

Parasite-based diagnosis of malaria has gained increasing importance as the rising cost of effective drug therapy has made expenditure on diagnostics, which can help to avoid unnecessary treatment, more attractive to malaria control programmes. Malaria is a severe and often rapidly fatal disease, with a high potential cost for health if diagnosis fails and an infection is missed. Accurate diagnosis has further potential benefits for case management by alerting health workers to the probability of non-malarial causes of fever, while demonstration of parasitaemia can improve adherence to anti-malaria therapy.

Malaria rapid diagnostic tests (RDTs) are a relatively new and evolving technology, which can provide parasite-based diagnosis in remote areas where microscopy is difficult to support and where there is limited control of test storage conditions and supervision of users. It is imperative that RDTs used in such conditions are simple, reliable and stable, and that this can be demonstrated in order to guide procurement and appropriate use.

I. TYPES OF MALARIA RDTs

Malaria RDTs, sometimes called dipsticks or malaria rapid diagnostic devices (MRDDs), detect specific antigens (proteins) produced by malaria parasites. These antigens are present in the blood of chronically infected or recently infected individuals. The presence of antigen is indicated by a colour change on an absorbent nitrocellulose strip. Some RDTs can detect only one species (*Plasmodium falciparum*), usually by detecting either histidine-rich protein 2 (HRP2) or parasite-specific lactate dehydrogenase (pLDH). Other RDTs detect one or more of the other three species of malaria parasite that infect humans (*Plasmodium vivax, Plasmodium ovale and Plasmodium malariae*), by detecting various other antigens[1].

RDTs commonly come in three formats. The simplest form is a dipstick, in which the absorbent nitrocellulose strip is placed in wells containing blood and/or buffer. The nitrocellulose strip can also be placed in a plastic cassette or on a card, which tend to be more expensive but are simpler to use.

When in good condition, some products can achieve a sensitivity similar to that commonly achieved by good field microscopy (~100 parasites μl^{-1}). However, the sensitivity can vary between products; the recommended sensitivity is ≥95% at ≥100 parasites μl^{-1} for *P. falciparum*[2,3].

II. THE NEED FOR FIELD EVALUATIONS OF MALARIA RDTs

Like other biological tests, malaria RDTs are prone to deterioration through exposure to heat and humidity, and through manufacturing faults. Laboratory and field trials have a major role in determining the suitability for use of the rapidly expanding range of tests available. However, malaria RDT trials face inherent difficulties in design and execution. The apparent accuracy of any RDT in detecting malaria parasites will depend on various factors, including: the concentration of the target antigen in host blood; the mechanics of antigen and antibody flow along the nitrocellulose strip; the physical condition of the RDT, including the integrity of antibodies and conjugate; the availability of target epitopes to bind antibodies in the tests (that is, variation in antigen structure); the quality of test preparation and interpretation; and the accuracy of the reference standard.

The trial design and the interpretation of trial results must take into account the likely conditions of intended use, the limitations of comparative standards and specific characteristics of malaria epidemiology (BOX 1).

III. GENERAL ISSUES IN STUDY DESIGN

1. Reference standards

Most studies use thick- and thin-film microscopy as a 'gold standard'. PCR is usually more sensitive for detection and species identification[4–10], but is subject to various technical limitations, is expensive, and is not generally accepted as a primary means of malaria diagnosis. Microscopic examination of a single blood sample can miss a parasitaemia that is fluctuating, whereas PCR and an RDT that targets persistent antigen might detect this[11]. Microscopy, PCR and RDTs that detect rapidly cleared antigens will more accurately reflect treatment-related parasite clearance (in the absence of high gametocyte density)[12–18]. PCR is therefore useful to assess whether discordant results are parasite-positive or parasite-negative, distinguishing true false-positive results from apparent false-positive results. If PCR is used for discordant cases, it should also be used for a sample of concordant negative cases to determine the false-negative rate of both slides and RDTs.

Microscopy is highly technician-dependent, requiring blinded confirmatory readings[19–21]. Pre-qualification of microscopists, blinded reading of blood films by more than one microscopist and a planned system to resolve discordant microscopy results are all essential if microscopy is used as a gold standard. However, it should be borne in mind when interpreting RDT trials that the standard of microscopy available as an alternative to RDTs in normal operational conditions might be much lower than the standard achieved under trial conditions.

2. Local epidemiology, choice of study population and sampling

The sensitivity of a malaria RDT is dependent on the parasite density, and the positive predictive value, calculated from a 2×2 table, is dependent on the underlying parasite prevalence in the group recruited to the study (for a fuller discussion, see *Evaluation*

of diagnostic tests for selected infectious diseases: general principles in this supplement). The parasite density in the study population therefore needs to be carefully defined (that is, should be part of the recruitment criteria). The study design will depend on the overall aims of the study, and could include all patients fitting certain clinical criteria or could be restricted to known parasitaemic patients and non-parasitaemic controls. It is preferable to measure the parasite density, if this can be done accurately, and to stratify the results accordingly. As a minimum, a summary of previous surveys, local anti-malaria treatment practices and the epidemiology of the study area must be stated.

IV. FACTORS AFFECTING TEST PERFORMANCE

See Table 1 for a full list.

1. Specimen sampling and preparation

Malaria RDTs are designed primarily for testing fresh capillary blood obtained though a finger-prick, but in comparative trials of several products obtaining sufficient blood required a venous puncture, transferred by a micropipette. The use of archived frozen specimens of blood from parasitaemic patients allows the testing of RDTs in a controlled environment, and standardization of samples temporally and between laboratories. Standardized, archived specimens are essential for longitudinal stability studies (for a fuller discussion, see Section IV in *Evaluation of diagnostic tests for selected infectious diseases: general principles* in this supplement). However, the performance of RDTs prepared using such specimens can differ from the performance obtained using fresh, finger-prick blood (see section IV.2 below).

2. Transport and storage of RDTs

Like other biological tests that rely on antibody–antigen interactions, current malaria RDTs deteriorate more quickly on exposure to moisture (humidity) and high temperature. The product instructions commonly specify storage between 2°C and ~30°C, but this can be difficult to achieve in a field trial and might be impossible in operational use in remote areas. In the tropics, RDTs that remain in non-air-conditioned vehicles or under tin roofs for long periods can rapidly lose sensitivity. It is therefore essential that storage and transport are carefully controlled and documented, to determine whether temperature exposure has influenced the results and whether the trial conditions are equivalent to the expected conditions of operational use.

Protecting packaging from mechanical damage and minimizing the time from opening the test envelope to preparation of the RDT will reduce exposure to humidity. Retaining a sample of trial RDTs under controlled temperature (for example, at 4°C) will allow a comparison to be made with unused RDTs returned from the field at the end of the trial, indicating whether significant deterioration in RDT quality occurred in the field during the trial period.

3. Training and choice of technicians, test preparation and interpretation

End-users of malaria RDTs are likely to be remote health workers, often volunteers with limited training. The technicians performing evaluations are commonly highly trained, experienced malaria control personnel. The performance of technicians in a trial environment can therefore differ significantly — positively or negatively — from that of the intended end-users. Preparation and interpretation can also be affected by manual dexterity, visual acuity and available lighting. To perform the tests in a realistic test environment, trialists should document the training and previous experience of technicians, using either the intended end-users of the tests, or noting differences between the technicians and the end-users.

Various studies have documented significant variation between technicians in both RDT preparation and interpretation[24–33]. The accuracy of RDTs can be affected by incorrect blood volume and reagent (buffer) volume. Therefore, using separate technicians for different products in comparative RDT studies can bias the results. Multiple blinded readings and/or rotation of technicians will reduce the likelihood of bias. The duration of time between preparation and reading must be documented. Comment on late changes in RDT line intensity can be useful, although late readings should not be used in analysis.

Various blood-transfer devices are provided with malaria RDTs. As the accuracy and consistency of performance of these devices can vary, the mode of blood transfer must be documented. Either the transfer device provided with the tests should be used, usually to collect a blood sample from a finger-prick, or a standardized method should be used with all tests. The use of the device provided with the product will allow a truer comparison of the accuracy of the whole test package. The use of a micropipette or other standardized method for blood transfer allows truer comparison of the actual lateral flow devices that draw the blood along the nitrocellulose strip, independent of the quality of the transfer device. In a comparative study of three or more test devices, it is difficult to obtain adequate blood from a finger-prick sample and venous blood is preferable. In such cases, a micropipette is preferable to the transfer devices designed for finger-prick sampling.

Trials aimed at assessing the local suitability of products must address the suitability of the instructions and the technical requirements of the products to the proposed end-users (for example, volunteer health workers or householders) and to patients. As test sensitivity is dependent on user technique[25–31], this information is important in assessing RDT suitability. The quality of product instructions and training can be documented both quantitatively (sensitivity, specificity and proportion of observed mistakes) and qualitatively (preferences of end-users).

4. Recent treatment

Recent treatment with antimalarial therapy can have a variable effect on the sensitivity and specificity of RDTs. Tests detecting persistent antigens such as HRP2 can remain positive several days after the eradication of parasites[4,16,22,23,34], resulting in lower specificity for current infection. Relatively higher sensitivity will be present if treatment has reduced but not eradicated parasites compared with tests that detect transient antigens such as pLDH or aldolase[11,12,15,35,36]. Additionally, parasites observed by microscopy after treatment might not be viable. Recent anti-malarial treatment must be noted with the results, or these cases excluded from the study. In many situations an accurate treatment history might not be available, in which case a note on treatment practices and the availability of anti-malarial drugs at the study site will assist interpretation of results.

5. Laboratory facilities and testing site

The interpretation and performance of malaria RDTs is affected by ambient lighting and humidity, respectively. Microscopy used as a comparative standard is highly dependent on the performance of the technician, the quality of the microscope and quality of slide preparation and staining.

Table 1 | **Significant factors influencing the results of malaria RDT-based diagnosis**

Component	Significance or vulnerability
Test devices	
Transport and storage	
Packaging (humidity)	Must exclude moisture. Humidity rapidly degrades RDTs
Temperature (and duration)	Higher temperatures accelerate degradation through: deconjugation of signal antibody–dye complex; detachment of capture antibody from wick; unfolding of binding sites of antibodies; and change in nitrocellulose wick flow characteristics Freeze-thawing during transport can have a similar effect
*Preparation and interpretation**	
Buffer volume	Controls flow, and sometimes lysis
Blood volume	Inadequate volume reduces available antigen Excess volume inhibits clearance of blood stain, reducing clarity of result
Age and storage of blood sample	Stored blood can lose antigen activity, and early lysis and protein coagulation can inhibit flow
Visual acuity of technician	Test lines can be faint at low parasite density
Patient and parasite	Parasite density in peripheral blood, and total parasite load (including sequestered parasites) determines available antigen Antigen production varies with parasite life cycle, and between parasites Antigen structure varies between parasites Presence of substances prone to cause false-positive reactions can vary between patients
Reference standard	
Microscopy or PCR	Poor sensitivity reduces apparent RDT specificity Poor specificity reduces apparent RDT sensitivity
Condition of sample (laboratory-based trials)	
Age /storage of blood	Rate of loss of antigenic activity varies between antigens Lysis of cells can occur during mixing or storage
Preparation of dilutions	Cell lysis and aggregation of parasitized cells can reduce uniformity and affect flow
Study population and parasites (especially field trials)	
Population/hosts	Parasite density affects sensitivity Parasite prevalence can affect predictive values Treatment history and effectiveness of treatment varies between patients
Parasite age, source	Antigen activity varies between wild and cultured parasites Wild and cultured parasites can be sequestered in host, and stage of parasite growth affects antigen concentration : parasite density ratio

*Interpretation of the final result also requires training. RDT, rapid diagnostic test.

6. Co-morbidities

Certain conditions such as non-specific fever associated with heterophile antibodies and the presence of rheumatoid factor or anti-mouse antibodies can result in false-positive results on some RDTs, although this is probably not common. If a high rate of false-positive results is encountered, further characterization of blood samples should be considered, together with detection of parasite DNA. Although a note of main common co-morbidities should be mentioned in the description of the population, it is not usually necessary to include detailed detection of co-morbidities in the study design.

V. QUALITY ASSURANCE

Quality-assurance issues common to diagnostic field trials are discussed in Section III of *Evaluation of diagnostic tests for selected infectious diseases: general principles* in this supplement. Blinding of RDT technicians and microscopists to clinical data and to each other's results and pre-qualification of microscopists are particularly important, and often require considerable pre-planning. Quality assurance of microscopy results is crucial, and should also include a pre-determined policy on discrepant results between microscopists. As with other aspects of the trial, one individual should be designated to oversee this process.

VI. ARCHIVING OF USED RDTs AND REFERENCE STANDARDS

All malaria blood films should be retained for later review. Blood spots and samples for PCR examination should similarly be retained in appropriate storage conditions where possible. As with paper records, samples should be clearly identified and stored securely with strict conditions for access (for a fuller discussion, see *Evaluation of diagnostic tests for selected infectious diseases: general principles* in this supplement).

Archiving of RDTs is useful to confirm certain aspects of performance such as blood clearance. However, archived RDTs have limited value for confirming results as the intensity of test and control lines can change after the specified reading time owing to back-flow of blood and other factors. RDT results used for analysis must always be recorded within the reading time specified by the manufacturer.

VII. ACTION ON RESULTS

As with all diagnostic trials, case management should be based on the principle of treatment according to the result of the reference sample or the normal (for example, clinical) method of diagnosis, rather than the test under evaluation. Where the reference standard (for example, microscopy) is known to be imperfectly sensitive, consideration can be given to treating cases that are RDT-positive but microscopy-negative, if treatment is safe, while investigating for other aetiologies. These procedures must be included in ethics submissions, and clearly stated in the study protocol.

VIII. SPECIAL CONSIDERATIONS FOR LABORATORY-BASED TRIALS

Studies comparing several devices generally require larger volumes of venous blood, and trials to establish sensitivity thresholds or to compare tests separated temporally as in stability studies, require blood to be stored and often diluted. Consequently, RDTs in these trials are no longer being tested in their design environment, and the possible effects of changes in samples during storage on the performance of the tests must be understood.

1. Effects of storage of samples on test performance

Antigen activity can be lost when blood is stored at room temperature and can still deteriorate slowly at low temperatures, although some antigens (for example, HRP2) are relatively stable. Repeated freeze–thawing can accelerate antigen denaturation and change

Box 1 | **Minimum standards for field component of sensitivity/specificity trials of malaria RDTs**

All trials should follow the general criteria for diagnostics trials outlined in *Evaluation of diagnostic tests for selected infectious diseases: general principles* in this supplement. The points listed below should be considered in the design of all comparative field trials of malaria RDTs, and should be documented. Each section should be recorded in published trials. The design of laboratory-based trials should be similar, but with special emphasis on the integrity of parasite samples used for testing.

Checklist for study design and analysis of results

Record details of RDTs used:
- ❑ Manufacturer (company name, site of manufacture)
- ❑ Batch number (includes strip, reagents, wells)
- ❑ Date of manufacture
- ❑ Date of expiry
- ❑ Whether product is under trial or commercially available

Record general description of test kits:
- ❑ Packaging type (sealed individually, multiple strips in same canister and so on)
- ❑ State and type of packaging, and whether canisters of test strips have been opened prior to the first patient being seen (RDTs in damaged packaging should not be used)
- ❑ Inclusion of desiccant with strips
- ❑ Inclusion of lancets/capillary tubes needed to perform the test (or otherwise note the items used)

Describe storage/transport conditions since manufacture:
- ❑ Duration of storage
- ❑ General temperature and humidity at storage (monitoring of temperature and humidity if available). RDTs should be stored away from direct sunlight
- ❑ Time to complete use from opening of canister (when dipsticks with this type of packaging are used)

Describe the trial site:
- ❑ Climatic conditions (mean local temperature and humidity)
- ❑ Workplace conditions (type of facility, lighting used for reading RDTs)
- ❑ Local malaria situation

Describe the trial subjects:
- ❑ Criteria for patient selection (symptoms and signs, relation to normal selection for treatment, exclusion criteria)
- ❑ Demographics (age, sex)
- ❑ Recent anti-malarial therapy

Describe the technique used:
- ❑ Time of test package opening to time of use
- ❑ Blood extraction (venous or capillary)
- ❑ Blood transfer to test (device provided by manufacturer, or pipette)
- ❑ Time from blood extraction to placing sample on RDT
- ❑ Time taken to obtain reading (according to manufacturer guidelines, or reason if longer)

Record each line on strip separately, including control. Record of intensity is not necessary, but useful in some trials.

Record organization and experience of RDT readers /technicians:
- ❑ One or multiple readers
- ❑ Same technician/reader per RDT type, or alternating
- ❑ Blinding to microscopy results, results of other RDT readers, and preferably to clinical presentation (latter might not be possible in some circumstances)
- ❑ Identity of technicians/readers for later comparison (can be coded)
- ❑ Training/experience in use of (this) RDT (including date of training, validation of quality of training), and comparison with intended end-users

Record significant difficulties encountered with the tests:
- ❑ Significant or recurrent problems encountered in kit preparation (including opening of packaging and obtaining blood)
- ❑ How the exact RDT preparation technique used compared with that detailed in the manufacturers insert
- ❑ Consider a formal independent qualitative appraisal of 'ease of use' of product by each technician

Ensure microscopy is of high quality:
- ❑ Reagents used
- ❑ Time from preparation to staining
- ❑ Staining method
- ❑ Pre-qualification and training of microscopists
- ❑ Blinding
- ❑ Criteria for counting parasites and assessment of slide negativity, parasite density

Consider collecting blood dried on filter paper or in EDTA to allow for later clarification through PCR or ELISA . The criteria for settling discordant results (for example, PCR, ELISA or independent microscopist) should be formulated beforehand and clearly stated.

As conditions of humidity and temperature can vary considerably in some endemic areas, a record of the ambient temperature and weather condition (rain, sunshine, cloud cover (or if possible humidity)) on each day of the trial might be useful.

Issues concerning ethics and patient consent are detailed in *Evaluation of diagnostic tests for selected infectious diseases: general principles* in this supplement. See APPENDICES 1 and 2 for sample data-collection and consent forms.

blood-flow properties, but can also improve RDT sensitivity in some cases, possibly owing to antigen release through parasite lysis. Cold blood samples can also flow differently than samples at room temperature. Prolonged storage of unfrozen blood can increase the deposition of some target antigens on the wall of containers and low adsorbence containers are therefore preferable.

Preparing serial parasite dilutions necessitates mixing parasitized blood into parasite-negative blood. Premature cell lysis can be exacerbated by the use of small-bore pipettes for measuring and mixing. Inadequate mixing and blood-type incompatibility can also result in clumping of parasitized cells, which can affect subsequent blood flow. Therefore, the method of preparing dilutions can have a greater influence on the relative thresholds of sensitivity between different tests than the quality of the products themselves. Methods for preparation and storage of archived blood samples are available elsewhere[37].

2. The use of cultured parasites

The relationship between parasite density and antigen activity will vary in samples of both cultured and wild parasites. Antigen production varies with the stage of the parasite[35,38] and this is crucial for the test sensitivity of synchronous parasite samples such as those derived from culture. The differing growth conditions of cultured and wild parasites can also influence antigen production. Antigen can be detected from sequestrated parasites *in vivo* although few parasites might be present in peripheral blood samples[39–41].

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FURTHER INFORMATION

WHO Malaria RDT website:
http://www.wpro.who.int/sites/rolt

APPENDIX 1 | OUTLINE OF DATA COLLECTION FORM

ID No. _____ **Date:** ___/___/__ __ __ __ **Time:** ……
dd /mm/ yyyy

PATIENT DETAILS

NAME _____ AGE _____ (in yrs) SEX M ❑ F ❑
Address _____

For tracing where necessary. Address and identifying information will be separated from test results for analysis.

CURRENT SYMPTOMS
Duration of current illness _____days

Symptoms *(current)*

	No	Yes
Fever	❑	❑
Chills	❑	❑
Sweating	❑	❑
Headache	❑	❑
Others	❑	❑

Specify_____

Temperature _____°C

RECENT CLINICAL HISTORY

Symptoms (*e.g. past week, month*)

	No	Yes
Fever	❑	❑
Chills	❑	❑
Sweating	❑	❑
Headache	❑	❑
Others	❑	❑

Specify_____

TREATMENT
(*In the past month, what type of medicines have you taken?*)

	No	Yes
Chloroquine	❑	❑
Sulfadoxine/Pyrimethamine	❑	❑
Primaquine	❑	❑
Others	❑	❑

Specify_____

(*Do you keep any malaria medicine at home?*)
Access to medication ❑ ❑

Adjust according to local needs. Can include 12 month fever/malaria history, including anti-malaria medication.
Detailed clinical history can be valuable in guiding interpretation of results, but reliability will vary.

RDT RESULTS (*On separate form*)

ID No. _____ **Date:** ___/___/__ __ __ __ **Time:** ……
dd /mm/ yyyy

Time blood drawn: ……

Line 1	Line 2	Control line	Result

0 (no band), 1+ (weak band), 2+(medium band), 3+ (strong band)

It is useful to use a standard chart of line intensity

Comments _____
(e.g. flow problems, background staining)

Microscopy and repeat RDT results will be on separate forms.
Microscopy form should include species, and parasite count of sexual and asexual forms.

A form should also be developed for calculating parasite density (e.g. from white cell count and agreed parasite count):

$$\frac{\text{Asexual parasites counted}}{\text{WBC counted}} \times \text{WBC } \mu l^{-1} = \text{parasites } \mu l^{-1}$$

All data forms should be signed by technician entering data, and data encoders.

APPENDIX 2 | SAMPLE INFORMED CONSENT FORM

(A separate patient information sheet containing this information should also be provided)

A | PURPOSE OF THE STUDY

To diagnose malaria precisely, we need to do laboratory tests. Rapid tests to diagnose malaria within 30 minutes (RDTs) are now available but we do not know if they are accurate or reliable.

The main purpose of this study is to evaluate two rapid tests for the diagnosis of malaria. We would like to compare the result of these rapid tests with the results of a laboratory-based test to see if they are as accurate as laboratory tests.

B | STUDY PROCEDURES

If you agree to participate in the study, you will be assigned a study number. We will prick your finger and take a drop of blood from you. Your name will not appear on any specimens or study forms.

C | VOLUNTARY PARTICIPATION

Your decision not to participate or to withdraw from participation will not affect the care you will receive at the clinic in any way. Even if you do agree to become a study participant, you can withdraw from the study at any time (verbally).

D | DISCOMFORT AND RISKS

You might feel a small amount of discomfort or have a small amount of bruising on your finger where the blood was taken.

E | BENEFITS

There will be no immediate benefits from your participation in the study. When the study results are known and if the rapid tests are acceptable in terms of accuracy, everyone who comes to the clinic could benefit from having this test available to diagnose malaria and receive the right treatment the same day.

F | COMPENSATION

There will be no monetary compensation for this study, but routine medical consultation and appropriate referral services are available.

G | CONFIDENTIALITY STATEMENT

The records concerning your participation are to be used only for the purpose of this research project. Your name will not be used on any study forms or labels on laboratory specimens or in any report resulting from this study. At the beginning of the study, we will give you a study identification number and this number will be used on the forms and on the laboratory specimens. Any information obtained in connection with this study will be kept strictly confidential. Only members of the study team will have access to information linking your name with your study number.

H | QUESTIONS AND FREEDOM TO WITHDRAW FROM THE STUDY

You can withdraw from the study at any time without affecting your present or future medical care at the clinic. You can contact any of the study personnel if you have questions about the research. (Please give the contact name, telephone number and address of the contact person for each site).

I | RESULTS PUBLICATION

When the researchers have analysed the data, the results and the explanation of its implications will be posted at the clinic for everyone's information.

J | PARTICIPANT STATEMENT

I have been informed verbally and in writing about this study and understand what is involved. I also know whom to contact if I need more information. I understand that confidentiality will be preserved. I understand that I am free to withdraw from the study at any time without affecting the care I normally receive at the clinic. I agree to participate in this study as a volunteer subject and will be given a copy of this information sheet to keep.

_____ _____

Date Name of participant

_____

Signature (or thumb print or cross) of participant

_____ _____

Date Name of witness

_____

Signature of witness

_____ _____

Date Name of guardian

_____

Signature (or thumbprint or cross) of guardian and relationship to participant

K | INVESTIGATOR'S STATEMENT

I, the undersigned, have defined and explained to the volunteer in a language he/she understands, the procedures of this study, its aims and the risks and benefits associated with his/her participation. I have informed the volunteer that confidentiality will be preserved, that he/she is free to withdraw from the trial at any time without affecting the care he/she will receive at the clinic. Following my definitions and explanations the volunteer agrees to participate in this study.

_____ _____

Date Name of investigator who gave the information about the study

Signature: _____