Instructions for Use

Malaria Ab ELISA

IVD

CE

REF **EIA-5048**

Σ **192**

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Please use only the valid version of the Instructions for Use provided with the kit.
Verwenden Sie nur die jeweils gültige, im Testkit enthaltene, Gebrauchsanweisung.
Si prega di usare la versione valida dell'inserto del pacco a disposizione con il kit.
Por favor, se usa solo la version valida de la metodico técnico incluido aqui en el kit.

Table of Contents

1 INTENDED USE

3rd Generation Enzyme Immunoassay (ELISA) for the determination of antibodies to Plasmodium species in human sera and plasma.

The kit is intended for the screening of blood units and the identification of people that came into contact with the protozoa and developed an immunological response.

The kit is for in vitro diagnostic use only and the test has to be carried out by professional people, opportunely trained.

2 INTRODUCTION

Plasmodium species are obligate intracellular protozoa related to Babesia and Toxoplasma. Plasmodium species reproduce sexually in mosquitoes; mosquitoes transmit the resulting sporozoites into humans where the organisms reproduce asexually. The sporozoites multiply within the liver; resulting merozoites invade erythrocytes where the merozoites multiply or mature into male and female gametocytes which eventually will be taken up by a mosquito during a blood meal.

P.falciparum and P.vivax cause approximately 80% and 15%, respectively, of all cases of Malaria.

Malaria is the most severe infectious disease of tropical and subtropical areas of the world that still is heavily affecting millions of people and generating millions of casualties.

The detection of anti P. species antibodies can identify in suspected individuals a case of recent or past malaria.

3 PRINCIPLE OF THE TEST

Recombinant proteins representing immunodominant epitopes of Plasmodium species, are coated onto wells of a microplate.

Recombinant proteins have been carefully selected to ensure the screening of all antibodies to P. species. Serum or plasma samples are added to these wells and, if antibodies specific to P. species (IgG, IgM or IgA) are present in the sample, they will form stable complexes with the recombinant antigens in the well.

Antigen-antibody complexes are then identified through the successive addition of: (1) same biotinylated recombinant proteins specific to P. species and; (2) horseradish peroxidase HRP Streptavidin conjugate.

The hydrolytic activity of horseradish peroxidase allows for the quantification of these antibody-antigen complexes.

Peroxidase substrate solution is then added.

During incubation, a blue colour will develop in proportion to the amount of anti P. species antibodies bound to the well, thus establishing their presence or absence in the sample. Wells containing samples negative for anti-P. species antibody remain colourless.

A stop solution is added to each well and the resulting yellow colour is read on a microplate reader at 450 nm.

4 COMPONENTS

The following describes the composition of the 192 tests/kit format.

1. **Microplate (MICROPLATE)** n° 2 microplates.
   12 strips of 8 breakable wells coated with Plasmodium species specific recombinant antigens. Plates are sealed into a bag with desiccant.
2. **Negative Control (CONTROL −)** 1 x 4.0 mL/vial.
   Ready to use control.
   Polypropylene vial with plastic screw cap, white color coded.
   It contains human serum negative for P. species antibodies and 0.1% Kathon GC as preservatives. The negative control is pale yellow colored.
3. **Positive Control (CONTROL +)** 1 x 4.0 mL/vial.
   Ready to use control.
   Polypropylene vial with plastic screw cap, blue color code
   It contains human serum positive for P. species antibodies and 0.1% Kathon GC as preservatives. The Positive Control is light green coloured.
   Important Note: *Even if this component has been treated with chemicals able to inactivate P. species, this does not fully ensure the absence of viable pathogens, and therefore, the control should be handled as potentially bio hazardous, in accordance with good laboratory practices.*
4. **Calibrator**: **(CAL)** 2 vials.
   Borosilicate glass vial with under cap and plastic screw cup, yellow color coded.
   Dissolve carefully the content of the lyophilized vial with the volume of EIA grade water reported on its label. Mix well on vortex before use.
   Important Notes:
   1) When dissolved the Calibrator is not stable. Store in aliquots at -20 °C.
   2) Even if this component has been treated with chemicals able to inactivate P. species, this does not fully ensure the absence of viable pathogens, and therefore, the control should be handled as potentially bio hazardous, in accordance with good laboratory practices.
5. **Wash buffer concentrate 20X (WASHBUF 20X)** 2 x 60 mL/bottle.
   20x concentrated solution.
   Polypropylene bottle with plastic screw cap, white color coded.
   It contains 0.1% Kathon GC. Once diluted, the wash solution contains 10 mM phosphate buffer saline pH 7.0±0.2 and 0.05% Tween 20.
6. **Conjugate # 1 (CONJ 1)** 8 vials.
   Borosilicate glass vial with under cap and plastic screw cup, white color coded.
   The vial contains lyophilized biotinylated P. species recombinant antigens.
   Vials are to be fully dissolved with 6 mL of the conjugate # 2.
7. **Conjugate # 2 (CONJ 2)** 1 x 60 mL/bottle.
   Polypropylene bottle with plastic screw cap, red color coded.
   The solution contains HRP conjugated with streptavidin in Tris saline Buffer supplemented with 0.05% Kathon GC, Tween 20 and BSA. This component is red coloured.
8. **Chromogen/Substrate (SUBS TMB)** 1 x 50 mL/bottle.
   Polypropylene amber bottle with plastic screw cap, white color coded.
   Ready-to-use component. It contains 50 mM citrate buffer pH 3.5-3.8, 4% dimethylsulphoxide, 0.03% tetra-methyl-benzidine or TMB and 0.02% hydrogen peroxide or H_2O_2.
   Note: *To be stored protected from light as sensitive to strong illumination.*
9. **Stop Solution (Sulphuric Acid) (H_2SO_4 0.3M)** 1 x 32 mL/vial.
   Polypropylene vial with plastic screw cap, white color coded.
   It contains 0.3 M H_2SO_4 solution.
   Attention: Irritant (H315, H319; P280, P302+P352, P332+P313, P305+P351+P338, P337+P313, P362+P363)
10. **Sample Diluent**: **(DILSPE)** 1 x 25 mL/vial.
    Polypropylene vial with plastic screw cap green color coded.
    Contains Tris buffer supplemented with 0.05% Kathon GC and Tween 20; used for specimen dilution. This component is green coloured.
11. Plate sealing foils n° 4
12. Package insert n° 1

Upon request:

Calibration Curve: (CAL N° …)

5 x 2.0 mL/vial. Ready to use standard curve ranging: 0 - 0.5 - 1 - 2.5 - 5 WHO IU/mL.

(CAL1 = 0 IU/mL, CAL2 = 0.5 IU/mL, CAL3 = 1 IU/mL, CAL4 = 2.5 IU/mL, CAL5 = 5 IU/mL)

Contains serum proteins, 0.3 mg/mL gentamicin sulphate and 0.1% Kathon GC as preservatives.

Standards are colored with decreasing intensity of alimentary blue dye.

5 MATERIALS REQUIRED BUT NOT PROVIDED

1. Calibrated Micropipettes (200 µL and 10 µL) and disposable plastic tips.
2. EIA grade water (bidistilled or deionised, charcoal treated to remove oxidizing chemicals used as disinfectants).
3. Timer with 60 minute range or higher.
4. Absorbent paper tissues.
5. Calibrated ELISA microplate thermostatic incubator capable to provide a temperature of +37 °C.
6. Calibrated ELISA microwell reader with 450 nm (reading) and possibly with 620-630 nm (blanking) filters.
7. Calibrated ELISA microplate washer.
8. Vortex or similar mixing tools.

6 WARNINGS AND PRECAUTIONS

1. The kit has to be used by skilled and properly trained technical personnel only, under the supervision of a medical doctor responsible of the laboratory.
2. When the kit is used for the screening of blood units and blood components, it has to be used in a laboratory certified and qualified by the national authority in that field (Ministry of Health or similar entity) to carry out this type of analysis.
3. All the personnel involved in performing the assay have to wear protective laboratory clothes, talc-free gloves and glasses. The use of any sharp (needles) or cutting (blades) devices should be avoided. All the personnel involved should be trained in biosafety procedures, as recommended by the Center for Disease Control, Atlanta, U.S. and reported in the National Institute of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.
4. All the personnel involved in sample handling should be vaccinated for HBV and HAV, for which vaccines are available, safe and effective.
5. The laboratory environment should be controlled so as to avoid contaminants such as dust or air-born microbial agents, when opening kit vials and microplates and when performing the test. Protect the Chromogen (TMB) from strong light and avoid vibration of the bench surface where the test is undertaken.
6. Upon receipt, store the kit at 2 °C - 8 °C into a temperature controlled refrigerator or cold room.
7. Do not interchange components between different lots of the kits. It is recommended that components between two kits of the same lot should not be interchanged.
8. Check that the reagents are clear and do not contain visible heavy particles or aggregates. If not, advise the laboratory supervisor to initiate the necessary procedures for kit replacement.
9. Avoid cross-contamination between serum/plasma samples by using disposable tips and changing them after each sample.
10. Avoid cross-contamination between kit reagents by using disposable tips and changing them between the use of each one.
11. Do not use the kit after the expiration date stated on the external container and internal (vials) labels.
12. Treat all specimens as potentially infective. All human serum specimens should be handled at Biosafety Level 2, as recommended by the Center for Disease Control, Atlanta, U.S. in compliance with what reported in the Institutes of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.
13. The use of disposable plastic-ware is recommended in the preparation of the liquid components or in transferring components into automated workstations, in order to avoid cross contamination.
14. Waste produced during the use of the kit has to be discarded in compliance with national directives and laws concerning laboratory waste of chemical and biological substances. In particular, liquid waste generated from the washing procedure, from residuals of controls and from samples has to be treated as potentially infective material and inactivated before waste. Suggested procedures of inactivation are treatment with a 10% final concentration of household bleach for 16-18 hours or heat inactivation by autoclave at 121 °C for 20 min.
15. Accidental spills from samples and operations have to be adsorbed with paper tissues soaked with household bleach and then with water. Tissues should then be discarded in proper containers designated for laboratory/hospital waste.
16. The Stop Solution is an irritant. In case of spills, wash the surface with plenty of water
17. Other waste materials generated from the use of the kit (example: tips used for samples and controls, used microplates) should be handled as potentially infective and disposed according to national directives and laws concerning laboratory wastes.

7 SPECIMEN: PREPARATION AND WARNINGS

1. Blood is drawn aseptically by venipuncture and plasma or serum is prepared using standard techniques of preparation of samples for clinical laboratory analysis. No influence has been observed in the preparation of the sample with citrate, EDTA and heparin.
2. Avoid any addition of preservatives to samples; especially sodium azide as this chemical would affect the enzymatic activity of the conjugate, generating false negative results.
3. Samples have to be clearly identified with codes or names in order to avoid misinterpretation of results. When the kit is used for the screening of blood units, bar code labeling and electronic reading is strongly recommended.
4. Haemolysed (red) and lipemic ("milky") samples have to be discarded as they could generate false results. Samples containing residues of fibrin or heavy particles or microbial filaments and bodies should be discarded as well as they could give rise to false positive results.
5. Sera and plasma can be stored at + 2 °C - 8 °C for up to five days after collection. For longer storage periods, samples can be stored frozen at -20 °C for several months. Any frozen sample should not be frozen/thawed more than once as this may generate particles that could affect the test result.
6. If particles are present filter using 0.2 µm - 0.8 µm filters to clean up the sample for testing.
7. Do not use heat inactivated samples as they could give origin to false reactivity.

8 PREPARATION OF COMPONENTS AND WARNINGS

A study conducted on an opened kit has not pointed out any relevant loss of activity up to 2 months.

Microplates:
Allow the microplate to reach room temperature (about 1 hour) before opening the container. Check that the pouch is not broken or that some defect is present indicating a problem of storage. In this case call the customer service. Unused strips have to be placed back into the aluminum pouch, in presence of desiccant supplied, firmly zipped and stored at +2 °C - 8 °C. When opened the first time, residual strips are stable up to two months.

Negative Control:
Ready to use. Mix well on vortex before use.

Positive Control:
Ready to use. Mix well on vortex before use. Handle this component as potentially infective, even if a potential infectious agent, if present in the control, has been chemically inactivated.

Wash buffer concentrate:
The 20x concentrated solution has to be diluted with EIA grade water up to 1200 mL and mixed gently end-over-end before use. As some salt crystals may be present into the vial, take care to dissolve all the content when preparing the solution. In the preparation avoid foaming as the presence of bubbles could give origin to a bad washing efficiency.
Note: *Once diluted, the wash solution is stable for 1 week at +2 °C - 8 °C.*

Conjugate # 1 & Conjugate #2 mix solution:
The Conjugate # 1 & Conjugate #2 mix solution must be prepared immediately before the dispensation of the Sample Diluent.
Add 6 mL Conjugate # 2 directly to one vial of Conjugate # 1 and mix gently by inversion to dissolve the lyophilized powder. This preparation is sufficient for 24 tests, or 3 complete strips.
***Important Note: Any unused portion of this reconstituted Conjugate # 1 Solution may be stored at 2 °C - 8 °C for no more than 12 hours*.**

Conjugate # 2:
Ready to use reagent. Mix well on vortex before use.

Chromogen/Substrate:
Ready to use. Mix well on vortex before use.
Be careful not to contaminate the liquid with oxidizing chemicals, air-driven dust or microbes. Do not expose to strong illumination, oxidizing agents and metallic surfaces. If this component has to be transferred use only plastic, possible sterile disposable container.

Sulphuric Acid:
Ready to use. Mix well on vortex before use.
Attention: Irritant (H315, H319; P280, P302+P352, P332+P313, P305+P351+P338, P337+P313, P362+P363)

Legend:

Warning H statements:

H315 – Causes skin irritation.
H319 – Causes serious eye irritation.

Precautionary P statements:

P280 – Wear protective gloves/protective clothing/eye protection/face protection.
P302 + P352 – IF ON SKIN: Wash with plenty of soap and water.
P332 + P313 – If skin irritation occurs: Get medical advice/attention.
P305 + P351 + P338 – IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P337 + P313 – If eye irritation persists: Get medical advice/attention.
P362 + P363 – Take off contaminated clothing and wash it before reuse.

Sample Diluent:
Ready to use. Mix well on vortex before use.

Calibrator:
Dissolve carefully the content of the lyophilized vial with the volume of EIA grade water reported on its label. Mix well on vortex before use.

9 INSTRUMENTS AND TOOLS USED IN COMBINATION WITH THE KIT

1. Micropipettes have to be calibrated to deliver the correct volume required by the assay and must be submitted to regular decontamination (household alcohol, 10% solution of bleach, hospital grade disinfectants) of those parts that could accidentally come in contact with the sample. They should also be regularly maintained in order to show a precision of 1% and a trueness of ±2%. Decontamination of spills or residues of kit components should also be carried out regularly.
2. The ELISA incubator has to be set at +37 °C (tolerance of ±0.5 °C) and regularly checked to ensure the correct temperature is maintained. Both dry incubators and water baths are suitable for the incubations, provided that the instrument is validated for the incubation of ELISA tests.
3. The ELISA washer is extremely important to the overall performances of the assay. The washer must be carefully validated and correctly optimized using the kit controls and reference panels, before using the kit for routine laboratory tests. Usually 4-5 washing cycles (aspiration + dispensation of 350 µL/well of washing solution = 1 cycle) are sufficient to ensure that the assay performs as expected. A soaking time of 20-30 seconds between cycles is suggested. In order to set correctly their number, it is recommended to run an assay with the kit controls and well characterized negative and positive reference samples, and check to match the values reported below in the section “Internal Quality control”. Regular calibration of the volumes delivered by, and maintenance (decontamination and cleaning of needles) of the washer has to be carried out according to the instructions of the manufacturer.
4. Incubation times have a tolerance of +5%.
5. The ELISA reader has to be equipped with a reading filter of 450 nm and ideally with a second filter (620-630 nm, strongly recommended) for blanking purposes. Its standard performances should be (a) bandwidth < 10 nm; (b) absorbance range from 0 to > 2.0; (c) linearity to > 2.0; repeatability > 1%. Blanking is carried out on the well identified in the section “Assay Procedure”. The optical system of the reader has to be calibrated regularly to ensure that the correct optical density is measured. It should be regularly maintained according to the manufacturer ‘s instructions.
6. When using an ELISA automated work station, all critical steps (dispensation, incubation, washing, reading, data handling) have to be carefully set, calibrated, controlled and regularly serviced in order to match the values reported in the section “Internal Quality Control”. The assay protocol has to be installed in the operating system of the unit and validated as for the washer and the reader. In addition, the liquid handling part of the station (dispensation and washing) has to be validated and correctly set. Particular attention must be paid to avoid carry over by the needles used for dispensing and for washing. This must be studied and controlled to minimize the possibility of contamination of adjacent wells. The use of ELISA automated work station is recommended for blood screening when the number of samples to be tested exceed 20-30 units per run.
7. When using automatic devices, in case the vial holder of the instrument does not fit with the vials supplied in the kit, transfer the solution into appropriate containers and label them with the same label peeled out from the original vial. This operation is important in order to avoid mismatching contents of vials, when transferring them. When the test is over, return the secondary labelled containers to 2 °C - 8 °C, firmly capped, if not contaminated by the use (in case of an instrument with a fixed needle, without disposable tips).

10 PRE ASSAY CONTROLS AND OPERATIONS

1. Check the expiration date of the kit printed on the external label of the kit box. Do not use if expired.
2. Check that the liquid components are not contaminated by naked-eye visible particles or aggregates. Check that the Chromogen/Substrate is colourless or pale blue by aspirating a small volume of it with a sterile transparent plastic pipette. Check that no breakage occurred in transportation and no spillage of liquid is present inside the box. Check that the aluminium pouch, containing the microplate, is not punctured or damaged.
3. Dilute all the content of the 20x concentrated Wash Solution as described above.
4. Dissolve the Conjugate # 1 as described in the proper section.
5. Allow all the other components to reach room temperature (about 1 hour) and then mix as described.
6. Set the ELISA incubator at +37 °C and prepare the ELISA washer by priming with the diluted washing solution, according to the manufacturer's instructions. Set the right number of washing cycles as found in the validation of the instrument for its use with the kit.
7. Check that the ELISA reader has been turned on at least 20 minutes before reading.
8. If using an automated workstation, turn it on, check settings and be sure to use the right assay protocol.
9. Check that the micropipettes are set to the required volume.
10. Check that all the other equipment is available and ready to use.
11. In case of problems, do not proceed further with the test and advise the supervisor.

11 ASSAY PROCEDURE

The assay has to be carried out according to what reported below, taking care to maintain the same incubation time for all the samples in testing.

Manual assay:

1. Resuspend the content of the correct number of Conjugate # 1 vials with 6 mL of Conjugate # 2 before starting to dispense samples and controls.
   One vial of such mixed conjugate is largely sufficient for 3 strips. It is recommended to dissolve only the vials strictly necessary for the run.
2. Place the required number of wells in the microplate holder. Leave the 1st well empty for the operation of blanking.
3. Dispense 50 µL Sample Diluent in all the wells, except A1 used for blanking.
4. Then dispense 150 µL of Negative Control in triplicate, 150 µL Positive Control in single and then 150 µL of Calibrator in duplicate in proper wells.
5. Add 150 µL of Samples in each properly identified well. Mix gently the plate on the work surface, avoiding overflowing and contaminating adjacent wells, in order to fully disperse the sample into the diluent.
6. Incubate the microplate for **60 min at +37 °C.**

   Important note: *Strips have to be sealed with the adhesive sealing foil, supplied, only when the test is carried out manually. Do not cover strips when using ELISA automatic instruments.*

7. Wash the microplate with an automatic washer by delivering and aspirating 350 µL/well of diluted washing solution as reported previously (section 9.3).
8. Pipette 200 µL Conjugate # 1 and Conjugate #2 mix, prepared as described before, into each well, except the 1st blanking well, and cover with the sealer

   Important note: *Be careful not to touch the plastic inner surface of the well with the tip filled with the Conjugate. Contamination might occur.*

9. Incubate the microplate sealed for **60 min at +37 °C.**
10. Wash as in section 7.
11. Dispense 200 µL of Chromogen/Substrate mixture into each well, the blank well included. Then incubate the microplate **at room temperature (18 °C - 25 °C) for 30 minutes**. Start the timing immediately after addition of this component to the first well.

    Important note: *Do not expose to strong direct illumination. High background might be generated.*

12. Pipette 100 µL Sulphuric Acid into all the wells using the same pipetting sequence as in step 13 to stop the enzymatic reaction. Addition of acid will turn the positive controls and positive samples from blue to yellow.
13. Measure the colour intensity of the solution in each well, as described in section 9.5, at 450 nm filter (reading) and at 620-630 nm (background subtraction, strongly recommended), blanking the instrument on A1.

Important notes:

1. *If the second filter is not available ensure that no finger prints are present on the bottom of the microwell before reading at 450 nm. Finger prints could generate false positive results on reading.*
2. *Reading has to be carried out just after the addition of the Stop Solution and anyway not any longer than 30 minutes after its addition. Some self oxidation of the chromogen can occur leading to high background.*

Automated assay:

1. Resuspend the content of the correct number of Conjugate # 1 vials with 6 mL Conjugate # 2 before the assay is started. Once the lyophilized powders are dissolved and mixed well, they have to be mixed together into a plastic container and the assay may begin.

   Note: *One vial of such mixed conjugates is largely sufficient for 3 strips. It is recommended to dissolve only the vials strictly necessary for the run.*

2. In case the test is carried out automatically with an ELISA system, we suggest to make the instrument dispense 50 µL Sample Diluent first and then 150 µL controls and samples, shaking the microplate to homogenize the dilution. Before the next sample is aspirated, needles (when fixed) have to be duly washed to avoid any cross-contamination among samples or disposable tips have to be changed.
3. For the next operations follow the operative instructions reported before for the Manual Assay.
4. It is strongly recommended to check that the time lap between the dispensation of the first and the last sample will be calculated by the instrument and taken into consideration by delaying the first washing operation accordingly.

Note: *When the sample is added the color of the well turns from green to blue. The color change is visible to the naked-eye and its intensity can be read according to the procedure of verification of dispensation (chapter 12).*

12 ASSAY SCHEME

Method	Operations
Sample Diluent	50 µL
Controls	150 µL
Calibrator(*)	150 µL
Samples	150 µL
1st incubation	**60 min**
Temperature	+37 °C
Wash step	4-5 cycles
Conjugate # 1&2 mix	200 µL
2nd incubation	**60 min**
Temperature	+37 °C
Wash step	4-5 cycles
TMB/H_2O_2	200 µL
3rd incubation	**30 min**
Temperature	r.t.
Sulphuric Acid	100 µL
Reading OD	450 nm

Procedure of Verification of Dispensation:

The addition of Sample Diluent, Samples, Conjugate#1+2 mix solution, Chromogen/Substrate, is verified by reading wells at 405nm, according to what defined in the following table:

Steps (addition of)	Volumes do dispense	Verification (reading)
Empty well	/////	$OD_{405\ nm} < 0.050$
Sample Diluent	50 µL	$OD_{405\ nm} \geq 0.130$
After sample addition	150 µL	$OD_{405\ nm} \geq 0.350$
Mixed Conjugate 1+2	200 µL	$OD_{405\ nm} \geq 0.150$
Chromogen/Substrate	200 µL	$OD_{405\ nm} \geq 0.090$

(*) Important Notes:

The Calibrator (CAL) does not enter into the Cut Off calculation; therefore its use is not mandatory, but anyway recommended for internal quality control procedure

An example of dispensation scheme (manual assay) is reported below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	S2										
B	NC	S3										
C	NC	S4										
D	NC	S5										
E	CAL (*)	S6										
F	CAL (*)	S7										
G	POS	S8										
H	S1	S9										

Legenda: BLK = Blank NC = Negative Control POS = Positive Control S = Sample
CAL (*) = Calibrator – Not Mandatory

13 INTERNAL QUALITY CONTROL

A check is performed on the controls and the calibrator any time the kit is used in order to verify whether their OD 450 nm values are as expected and reported in the table below.

Ensure that the following results are met:

Check	Requirements
Blank well	≤ 0.100 $OD_{450\ nm}$ value
Negative Control (NC)	≤ 0.200 mean $OD_{450\ nm}$ value after blanking Absorbance of individual negative control values must be less than or equal to 0.200. If one value is outside this range, discard this value and recalculate mean. If two values are outside this range the run should be repeated.
Positive Control	Mean $OD_{450\ nm}$ ≥ 0.500

If the results of the test match the requirements stated above, proceed to the next section.

If they do not, do not proceed any further and perform the following checks:

Problem	Check
Blank well > 0.100 $OD_{450\ nm}$	1. that the Chromogen/Substrate solution has not become contaminated during the assay
Negative Control (NC) > 0.200 $OD_{450\ nm}$ after blanking	1. that the washing procedure and the washer settings are as validated in the pre-qualification study; 2. that the proper washing solution has been used and the washer has been primed with it before use; 3. that no mistake has been done in the assay procedure (dispensation of positive control instead of the negative one); 4. that no contamination of the negative control or of the wells where the control was dispensed has occurred due to spills of positive samples or of the enzyme conjugate; 5. that micropipettes have not become contaminated with positive samples or with the enzyme conjugate 6. that the washer needles are not blocked or partially obstructed.
Positive Control < 0.500 $OD_{450\ nm}$	1. that the procedure has been correctly executed; 2. that no mistake has been done in the distribution of the controls (dispensation of negative control instead of positive control. In this case, the negative control will have an $OD_{450\ nm}$ value > 0.150 too.) 3. that the washing procedure and the washer settings are as validated in the pre-qualification study; 4. that no external contamination of the positive control has occurred.

Should these problems happen, after checking, report any residual problem to the supervisor for further actions.

Important Note:

If the Calibrator has used, verify the following data:

Check	Requirements
Calibrator	S/Co > 1.0

If the results of the test don't match the requirements stated above, operate as follows:

Problem	Check
Calibrator S/Co < 1	1. that the procedure has been correctly executed; 2. that no mistake has been done in its distribution (ex.: dispensation of negative control instead of calibrator) 3. that the washing procedure and the washer settings are as validated in the pre-qualification study; 4. that no external contamination of the calibrator has occurred.

Anyway, if all other parameters (Blank, Negative Control, Positive Control), match the established requirements, the test may be considered valid.

14 CALCULATION OF THE CUT-OFF

The tests results are calculated by means of a cut-off value determined with the following formula on the mean $OD_{450\ nm}$ value of the Negative Control (NC):

NC + 0.300 = Cut-Off (Co)

The value found for the test is used for the interpretation of results as described in the next paragraph.

Important note: *When the calculation of results is performed by the operating system of an ELISA automated work station, ensure that the proper formulation is used to calculate the cut-off value and generate the correct interpretation of results.*

15 INTERPRETATION OF RESULTS

Test results are interpreted as ratio of the sample $OD_{450\ nm}$ and the Cut-Off value (or S/Co) according to the following table:

S/Co	Interpretation
< 0.9	Negative
0.9 – 1.1	Indeterminate
> 1.1	Positive

Samples showing a value of S/Co < 0.9 are considered negative and this result indicates that the patient has not been infected by Plasmodium species.

Samples showing a value of S/Co > 1.1 are considered positive and this result is indicative of a recent or past Plasmodium species infection.

Samples showing a S/Co value in the grey-zone 0.9 – 1.1 have to be retested after 2-3 weeks to verify whether or not the result has become positive.

Important notes:

1. *Interpretation of results should be done under the supervision of the responsible of the laboratory to reduce the risk of judgment errors and misinterpretations.*
2. *According to US NIH's directive, any positive result in blood screening should be confirmed by a Confirmatory method capable to detect antibodies to Malaria antigens before a diagnosis of infection is formulated.*
3. *Nucleic Acid Tests (NATs) for Malaria ssp are not intended to confirm an antibody assay by definition. However they may be used by the responsible of the laboratory to decide whether or not the blood unit can be transfused, even in presence of antibodies.*
4. *As proved in the Performance Evaluation of the product, the assay is able to detect anti-Malaria ssp antibodies earlier than some other commercial kit. Therefore a positive result, not confirmed with these less sensitive commercial kits, cannot be considered a false positive result, unless other evidences are present. The sample should be submitted to a Confirmation assay.*
5. *When test results are transmitted from the laboratory to an informatics centre, attention has to be done to avoid erroneous data transfer.*
6. *Diagnosis of Malaria infection has to be done and released to the patient only by a qualified medical doctor. The presence of antibodies does not mean, anyway, that the patient is undergoing an infection at the moment of analysis. Antibodies can last for the life of the patient even in absence of Malaria ssp live organisms in blood. The diagnosis of Malaria ssp infection should be done only in presence of other clinical and diagnostic evidences (presence of Malaria antigen in blood by PCR or other methods).*

An example of calculation is reported below:

The following data must not be used instead or real figures obtained by the user.

Negative Control: 0.048 – 0.050 – 0.052 $OD_{450\ nm}$
Mean Value: 0.050 $OD_{450\ nm}$
Lower than 0.200 – Accepted
Cut-Off = 0.050 + 0.300 = 0.350

Positive Control: 1.000 $OD_{450\ nm}$ mean value
Higher than 0.500 – Accepted

Calibrator: 0.810 $OD_{450\ nm}$ mean value
S/Co > 1 – Accepted

Sample 1: 0.070 $OD_{450\ nm}$
Sample 2: 1.690 $OD_{450\ nm}$
Sample 1 S/Co < 1 = negative
Sample 2 S/Co > 1 = positive

16 PERFORMANCES

16.1 Sensitivity

The **Analytical Sensitivity** of the assay, has been defined by means of First WHO Reference Reagent for Anti-malaria (Plasmodium Falciparum) human serum, NIBSC code: 10/198

Results of limiting dilution of the reference standard into a negative specimen (Neg Control) are shown in the table below, reporting the mean values obtained in the analysis of three different lots of the device

WHO IU/mL	OD450 nm	Co/S
10	3.767	10.3
5	2.485	6.8
2.5	1.310	3.6
1	0.547	1.5
0.5	0.276	0.8
0.25	0.161	0.4
Neg. Control	0.060	0.2

The sensitivity shown by the assay is better than 1 WHO IU/mL.

In addition the sensitivity of the system was also assessed on the panel supplied by **NIBSC**, UK, for antibodies to Plasmodium species.

Results of three lots of product, expressed as S/Co values, are summarized in the table below:

Member ID	Plasmodium species	S/Co
71/281 Version 3.0, dated 14/04/2008	p.Vivax	> 5.0
72/348 Version 3.0, dated 14/04/2008	p.Vivax	> 2.0
72/092 Version 3.0, dated 04/06/2015	p. Falciparum	> 1.1
71/326 Version 3.0, dated 14/04/2008	p.Vivax	> 5.0
72/096 Version 1.0, dated 08/07/2015	p. Malariae	> 5.0
72/345 Version 3.0, dated 14/04/2008	p. Falciparum	> 5.0
72/341 Version 3.0, dated 14/04/2008	p. Falciparum	> 5.0

Moreover, the sensitivity of the Product Malaria Ab ELISA was assessed on the Panel **EFS Ac anti-PALUDEEN**, lot n° 07.191026 (DQ030), supplied by the Etablissement Francais Du Sang (EFS), France, with the following results:

Sample	ID	S/Co
Ac PALU n° 1	6914478877	> 2.5
Ac PALU n° 2	69081724786	> 2.0
Ac PALU n° 3	4406095555	> 4.0
Ac PALU n° 4	Diluent	< 0.9

Finally, the sensitivity has been examined testing the **Control Sample CRS PAL lot 2701163** (Contrôle interne monoparamétrique Ac anti–plasmodium) supplied by the Etablissement Francais Du Sang (EFS) with three lots of the device. The control has been found always positive obtaining an index value higher than 1.2 (S/Co > 1.2)

The **Diagnostic Sensitivity** of the assay has been calculated on a panel of samples positive for antibodies to Plasmodium species, previously classified positive by a reference method.

The test shows a sensitivity > 95% on plasma and sera.

16.2 Diagnostic Specificity

It has been calculated on panels of negative blood donors, previously determined negative by the reference method (Diamed/Biorad).

The assay shows a specificity > 98% on plasma and sera.

16.3 Reproducibility

It has been evaluate by examining the negative control, the calibrator and the positive control in 16 replicates in three different runs carried out with the device lot # 0407.

Results are reported in the tables below:

Negative Sample (N = 16)				
Mean values	*1st run*	*2nd run*	*3rd run*	*Average value*
$OD_{450\ nm}$	0.136	0.146	0.153	0.145
Std. Deviation	0.009	0.014	0.011	0.011
CV %	6.4	9.3	7.5	7.7
Calibrator (N = 16)				
Mean values	*1st run*	*2nd run*	*3rd run*	*Average value*
$OD_{450\ nm}$	0.889	0.860	0.844	0.864
Std. Deviation	0.051	0.048	0.094	0.064
CV %	4.3	3.8	7.0	5.0
Positive Sample (N = 16)				
Mean values	*1st run*	*2nd run*	*3rd run*	*Average*
$OD_{450\ nm}$	3.191	3.300	3.175	3.222
Std. Deviation	0.062	0.098	0.103	0.088
CV %	1.9	3.0	3.2	2.7

From the data above the following statistical values have been derived:

Mean values N = 48	**Negative Sample**	**Calibrator**	**Positive Sample**
$OD_{450\ nm}$	0.145	0.864	3.222
Std. Deviation	0.011	0.064	0.088
CV %	7.7	5.0	2.7

17 REFERENCES / LITERATURE

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2. Krogstad DJ et al. Plasmodium species (Malaria) principles and practice of infectious diseases,
   4th ed, Mandell GL et al., 1995, 2415-27.
3. Smith JH et al. Malaria: clinical laboratory features.
   Clin Microbiol News Lett, 1995, 17(24): 185-8.

SYMBOLS USED

Symbol	English	Deutsch	Italiano	Español	Français
CE	European Conformity	CE-Konformitäts-kennzeichnung	Conformità europea	Conformidad europea	Conformité normes européennes
	Consult instructions for use *	Gebrauchsanweisung beachten *	Consultare le istruzioni per l'uso	Consulte las instrucciones de uso	Consulter les instructions d'utilisation
IVD	*In vitro* diagnostic medical device *	*In-vitro*-Diagnostikum *	Diagnostica in vitro	Diagnóstico in vitro	Diagnostic in vitro
REF	Catalogue number *	Artikelnummer *	No. di Cat.	No de catálogo	Référence
LOT	Batch code *	Chargencode *	Lotto no	Número de lote	No. de lot
	Contains sufficient for <n> tests *	Ausreichend für <n> Prüfungen *	Contenuto sufficiente per "n" saggi	Contenido suficiente para <n> ensayos	Contenu suffisant pour "n" tests
	Temperature limit *	Temperaturbegrenzung *	Temperatura di conservazione	Temperatura de conservacion	Température de conservation
	Use-by date *	Verwendbar bis *	Data di scadenza	Fecha de caducidad	Date limite d'utilisation
	Manufacturer *	Hersteller *	Fabbricante	Fabricante	Fabricant
	Caution *	Achtung *			
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