DRG® Malaria Ab Elisa (EIA-5048)

Revised 16 Sept. 2010 rm (Vers. 2.1) **USA: RUO**

Please use only the valid version of the package insert provided with the kit.

1 INTENDED USE

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

In the United States, this kit is intended for Research Use Only.

2 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.

3 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. It contains human serum negative for P. species antibodies and 0.1% Kathon GC as preservatives. The negative control is pale yellow color coded.
3. **Positive Control (CONTROL +)** 1 x 4.0 ml/vial.
   Ready to use control. It contains human serum positive for P. species antibodies and 0.1% Kathon GC as preservatives. The Positive Control is light green color coded.
   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.
   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. 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.
   The vial contains lyophilized biotinylated P. species recombinant antigens.
   Vials are to be fully dissolved with 6 ml of the conjugate 1 diluent.
7. **Conjugate 1 Diluent (CONJ 1 DIL)** 1 x 60 ml/bottle.
   Used to dissolve the lyophilized powder of Conjugate # 1, it contains Tris saline Buffer supplemented with 0.05% Kathon GC, Tween 20 and BSA.
8. **Conjugate # 2 (CONJ 2)** 1 x 30 ml/bottle.
   The solution contains HRP conjugated with streptavidin in Tris saline Buffer supplemented with 0.05% Kathon GC, Tween 20 and BSA. This component is colour coded in red.
9. **Chromogen/Substrate (SUBS TMB)** 1 x 50 ml/bottle.
   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.*
10. **Stop Solution (Sulphuric Acid) (H_2SO_4 0.3M)** 1 x 32 ml/vial.
    It contains 0.3 M H_2SO_4 solution.
    Attention: Irritant (Xi R36/38; S2/26/30).
11. **Sample Diluent**: **(DILSPE)** 1 x 14 ml/vial.
    Contains Tris buffer supplemented with 0.05% Kathon GC and Tween 20; used for specimen dilution. This component is colour coded in light blue.
12. Plate sealing foils n° 4
13. Package insert n° 1

4 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 450nm (reading) and possibly with 620-630nm (blanking) filters.
7. Calibrated ELISA microplate washer.
8. Vortex or similar mixing tools.

5 SPECIMEN: PREPARATION AND WARNINGS

1. Blood is drawn aseptically by venepuncture 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°-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 0.2-0.8 µ filters to clean up the sample for testing.
7. Do not use heat inactivated samples as they could give origin to false reactivity.

6 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 hr) 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:
The Conjugate # 1 mix solution must be prepared immediately before the dispensation of the samples.
Add 6 ml Conjugate # 1 diluent directly to one vial of Conjugate # 1 to dissolve the lyophilized powder. This preparation is sufficient for 32 tests, or 4 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 (Xi R36/38; S2/26/30)
Legenda: R 36/38 = Irritating to eyes and skin.
S 2/26/30 = In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.

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.

7 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 450nm and ideally with a second filter (620-630 nm) 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 labeled containers to 2°C - 8°C, firmly capped.

8 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 colorless 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 aluminum 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 hr) 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 manufacturers 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.

9 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.

Automated assay:
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. Before the next sample is aspirated, needles have to be duly washed to avoid any cross-contamination among samples or tips have to be changed. For the next operations follow the operative instructions reported below for the Manual Assay. 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.

Manual assay:
1. Resuspend the content of the correct number of Conjugate # 1 vials with Conjugate # 1 Diluent before starting to dispense samples and controls.
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 150 µl Conjugate # 1 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 for **30 min at +37°C.**

10. Pipette 100 µl of Conjugate # 2 in all the wells, except A1, and gently agitate the microplate to mix the conjugates.

    Important Note: *This solution must be added to the bottom of each well to ensure proper performance. Inadequate mixing of the two solutions (Conjugate # 1 and Conjugate # 2) may reduce the binding of Streptavidin HRP (Conjugate # 2) to the biotinylated reagents and consequently affect the performance of the assay. Be sure to provide an adequate mixing when the Conjugate # 2 is added, both in the manual and in the automated procedures.*

11. Incubate the microplate sealed for **30 min at +37°C**.
12. Wash as in section 7.
13. Dispense 200 µl of Chromogen/Substrate mixture into each well, the blank well included. Then incubate the microplate **at room temperature (18-25°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.*

14. 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.
15. Measure the color intensity of the solution in each well, as described in section 9.5, at 450 nm filter (reading) and possibly at 620-630 nm (background subtraction), 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 450nm. 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.*

10 ASSAY SCHEME

Method	Operations
Sample Diluent	50 µl
Controls	150 µl
Samples	150 µl
1st incubation	**60 min**
Temperature	+37°C
Wash step	4-5 cycles
Conjugate # 1	150 µl
2nd incubation	**30 min**
Temperature	+37°C
Conjugate # 2	100 µl
3rd incubation	**30 min**
Temperature	+37°C
Wash step	4-5 cycles
TMB/H2O2	200 µl
4th incubation	**30 min**
Temperature	r.t.
Sulphuric Acid	100 µl
Reading OD	450 nm

An example of dispensation scheme 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 CAL = Calibrator
POS = Positive Control S = Sample

11 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 450nm 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.*

12 REFERENCES / LITERATURE

1. Garcia LS et al. Update on Malaria.
   Clin Microbiol News Lett; 1992, 14:65-9
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.