Estimation of *Plasmodium falciparum* drug susceptibility *ex vivo* by HRP2 ELISA v1.0

Procedure

In vitro Module

WorldWide Antimalarial Resistance Network (WWARN)

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Version History

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1.0	Creation of procedure	31 Aug2011

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1. Purpose

This procedure describes a simplified ELISA-based HRP2 drug susceptibility assay for field use which is optimized for minimal infrastructure and technical equipment requirements while achieving appropriate sensitivity levels. This procedure is designed for *ex vivo* testing (i.e. use with fresh P. falciparum isolates) only.

All currently available malaria drug susceptibility assays are based on the assessment of parasite growth and development under drug exposure. The amount of histidine-rich protein 2 (HRP2) produced during parasite development reflects parasite growth, development, and multiplication. This assay uses HRP2 as a surrogate for parasite growth and its inhibition by antimalarial drugs. The amount of HRP2 is measured using a simple enzyme-linked immunosorbent assay (ELISA). The HRP2 ELISA measures the quantity of HRP2 produced by *P. falciparum* during a 72 hour incubation and its inhibition by antimalarial drugs.

2. Scope

This procedure details the steps required to determine the effects of a range of antimalarial drugs on the growth of fresh *P.falciparum* clinical isolates using the HRP2-ELISA method.

The methods for culture of *P.falciparum* erythrocytic stages (INV01), preparation of appropriate parasite culture medium (INV02) and the synchronisation of culture adapted *P. falciparum* clones into the ring stage (INV04), necessary for certain drug sensitivity assays, may be downloaded from the WWARN website (http://www.wwarn.org/research/tools/procedures).

3. Abbreviations

BSA	Bovine serum albumen
ELISA	Enzyme-linked immunosorbent assay
HRP2	Histidine-rich protein 2
P. falciparum	*Plasmodium falciparum*
IC 50	Half maximal inhibitory concentration
PBS	Phosphate-buffered saline
TMB	Tetramethylbenzemidine

4. Duties and Responsibilities

This procedure is designed for use by appropriately-equipped laboratories working on *in vitro/ex vivo* drug susceptibility testing of *P. falciparum*. Training, including familiarization with the principles and practice of ELISA testing, is required to perform the procedure successfully. Competency may be assessed observationally by an approved trainer. Participation in an appropriate QA/QC proficiency scheme, such as that run by WWARN, is recommended – see https://www.wwarn.org/research/tools/qaqc.

5. Materials and Equipment

5.1 Materials

- 96-well microculture plates e.g. Costar 3599 or Falcon 3070 pre-coated with antimalarial drugs. See WWARN procedure INV03: preparation of fresh and dried pre-dosed plates.
- Complete RPMI 1640 Medium (10.43g RPMI 1640 powder + 6 g HEPES + 25 mg gentamycin + plus 0.5% w/v of Albumax I + distilled water to 1L). Add NAHCO3 (2.8 ml of 7.5% NAHCO3 per 100ml medium before use). See WWARN procedure INV02for details of medium preparation and storage.
- RBCs (blood group 0 or same as patient). See WWARN procedure INV01.
- Sterile disposable phlebotomy tool
- Sterile trays
- PBS pH 7.4
- Tween® 20 (e.g. Sigma-Aldrich, P1379)
- BSA (e.g. Sigma-Aldrich, A9647)
- TMB
- 96-well, high protein-binding capacity, ELISA plates
- Sulphuric acid
- Antibody 1: MPFM-55A (e.g. Immunology Consultants Laboratories, Inc, Newberg, OR, USA or equivalent)
- Antibody 2: MPFG-55P (e.g. Immunology Consultants Laboratories, Inc, Newberg, OR, USA or equivalent)

5.2 Equipment

- Incubator
- Candle jar or incubation chamber with gas mixture
- Freezer (-20°C or below)
- ELISA plate reader (capable of reading at 450nM)
- Multichannel pipette (20-200 µL)
- One set of adjustable pipettes (20-200 µL; 100-1000 µL)

6. Procedure

6.1 Sample collection:

- Samples from patients with *P. falciparum* mono-infections and parasite densities of **0.002 % or greater** (approx. 100 parasites per µL or more). Samples with parasite densities of 1% or more may be used but should be diluted before culture with uninfected RBCs to obtain a density of approximately 0.2% to limit the inoculum effect (i.e. the influence of high parasite densities on test outcome).

- After thorough disinfection of the skin, collect a minimum 1 mL of blood by venipuncture using a sterile disposable phlebotomy tool and a heparinized container.
- Prepare thick and thin blood films, thoroughly dry the slides, fix thin films with methanol, and stain with Giemsa (3%, 20 minutes), microscopically examine the slides (oil, 1000x magnification), assess and record parasite density, and the proportion of rings and trophozoites.

6.2 Sample preparation and culture:

- The HRP2 drug sensitivity field test uses 72 hours of incubation at 37°C.
- For assays requiring one complete 96 well plate, prepare 25 mL of parasitized blood diluted in culture medium (cell-medium mixture, CMM). Dispense **24.06 mL** of complete RPMI 1640 into a sterile, disposable tube (note: medium filled tubes may be prepared in advance and stored at 4°C for several days). Add **0.94 mL** of parasitized blood per tube to obtain a CMM with approximately 1.5% hematocrit (assuming a 40% hematocrit in the parasitized blood sample).
- Add 200 µL of the resulting CMM to each well of the pre-dosed plates using a multichannel pipette (start with row A and proceed to higher drug concentrations).
- Cover the plates and incubate for 72 hours at 37°C in a candle jar or in an incubator with CO_2-enriched atmosphere using a gas mixture of 5% CO_2, 5% O_2, and 90% N_2 or candle jar. If possible, re-gas and gently agitate the plates every 24 hrs.
- After 72 hours incubation, prepare another thick and thin blood film to determine adequate parasite growth, defined as a 4 to 10 fold increase in parasite density over the time period. If there was little or no increase in parasite density, discard sample and re-check culture conditions, if possible.

6.3 Sample haemolysis:

- After the 72-hour incubation period, plates are removed from the incubator and stored at or below –20°C until all the wells are completely frozen. A simple household freezer may be also be used. Freezing may take from 60 minutes to 24 hours, depending on the equipment used. A minimum overnight period is recommended.
- Once completely frozen, plates may either be processed immediately (if an ELISA plate reader is available), or stored and transported frozen to laboratory facilities equipped to perform ELISA tests.
- Thaw the plates when ready to perform the ELISA assay. If the RBCs are not completely haemolysed i.e. all wells look completely clear on visual inspection, repeat the freezing and thawing cycle as above at least once until complete hemolysis is achieved.

6.4 HRP2 ELISA

The HRP2 ELISA measures the quantity of histidine-rich protein 2 (HRP2) produced by *P. falciparum* during the 72 hour incubation and its inhibition by antimalarial drugs.

6.4.1 Step 1: Preparation of antibody-coated ELISA plates

- Antibody-coated, 96-well, high-binding ELISA plates are ideally prepared in batches. They may be stored at -20°C for up to 12 months.
- Dilute primary IgM antibody (MPFM-55A, Immunology Consultants Laboratories, Inc, Newberg, OR, USA or equivalent) to a final concentration of **1.0 µg/mL in PBS.**
- Add 100 µl of diluted antibody to each well of an ELISA plate, using a multichannel pipette.
- Seal the plate and incubate at 4°C overnight. Using a pipette, remove and discard the diluted antibody from the ELISA plate, inverting and rapping the plate onto a paper towel to remove the remaining liquid (bang dry).
- Freshly prepare a blocking solution of 2% bovine serum albumin (BSA) solution in PBS. For each plate dissolve 0.4 g of BSA in 19.6 mL PBS.
- Add 200 µl of blocking solution to each well, cover and incubate at room temperature for 2 hours. Discard the blocking solution and bang dry as before.
- Prepare 0.05% Tween® 20 washing solution by adding 0.5 ml Tween® 20 to 999.5 mL PBS.
- Wash each well by adding and discarding 200µl 0.05% Tween® 20 washing solution. Bang dry as before. Repeat the wash step one more time. Seal each plate into an airtight plastic bag and store frozen at -20°C for up to 12 months.

6.4.2 Sample dilution

- The HRP2 ELISA method described in this procedure is extremely sensitive and able to assay reproducibly the drug sensitivity of fresh clinical blood samples at pre-culture parasite densities as low as **0.002% (c. 100 parasites/µL)**.
- Samples from patients with pre-culture parasite densities of **≤0.1% (approx. 5000 parasites/µL)** may be assayed directly following culture (i.e. the culture samples can directly be transferred to the ELISA plates).
- If the parasite density in the patient's blood was **>0.1% (approx. 5000 parasites/µL),** and the parasite density was **not** adjusted by dilution with uninfected RBCs prior to culture, the test samples should be diluted **to a final volume of 100 µL per well and the equivalent of 0.05%** pre-culture parasite density after culture directly on the ELISA plate as follows.
    - **Example:** a patient blood sample with a pre-culture parasite density of 0.25% should be diluted 1 in 5 (0.25%/0.05%) to obtain the equivalent of a 0.05% pre-culture parasite density. To do this, add 80µL of distilled water to each well of the ELISA plate. Using a multichannel pipette, and after thorough mixing, transfer 20µL sample from each well of the culture plate to the equivalent well of the ELISA plate and mix again. Start transferring

samples from the wells containing the highest drug concentration. This way a single set of pipette tips can be used for an entire plate.

- Add at least one well of an external positive control to each plate. A positive control could be, for example, a culture sample with known, very high parasitaemia. This sample will provide information on the maximum 450 nm absorbance – see 6.4.4 - that can be obtained in the ELISA. The positive control should always give a higher absorbance than the drug-free controls.

6.4.3 Step 3: Adding samples to ELISA plates and incubation

- Transfer 100 µL of sample, diluted if necessary (see 6.4.2), from the culture plate to an antibody-coated ELISA plate, previously warmed to room temperature.
- Incubate for 1 hour at room temperature in a humidified chamber.
- Wash 2 times in PBS/Tween (200 µl/well) and bang dry.

6.4.4 Step 4: Dilute the second antibody conjugate and add to ELISA plate

- Prepare the second antibody conjugate diluent, 2% BSA/1% Tween®20 in PBS, by adding 2 gm BSA and 1 mL Tween®20 to 97 mL PBS and adjusting to pH 7.4 if possible. 10 mL of diluent is needed for every test plate.
- Prepare a 200x stock solution (10 – 40 µg/mL) of second antibody conjugate (MPFG-55P or equivalent) using 2% BSA/1% Tween®20 in PBS (as above). Store the stock solution at 4^{o}C for a maximum four weeks.

  Note: The required second antibody concentration, which determines the ELISA sensitivity, may vary in the range from **0.05 to 0.2 µg/mL** because the conjugate activity will vary from batch to batch and will decrease if the antibody is stored unfrozen for extended periods of time. Each new batch of second antibody should be titrated to determine the appropriate conjugate concentration to use with test samples. This is done by carrying out a preliminary test using serial dilutions of a culture sample with known parasite density titrated against a range of second antibody concentrations from 0.05 to 0.2µg/mL.
- For each test plate, freshly prepare 1x antibody conjugate by adding 50 µL of 200x antibody stock solution to 10 mL of diluent. Mix carefully.
- Using a multichannel pipette, add100 µl of 1x antibody conjugate to each well of the test plate.
- Cover the plate and incubate for 1 hour at room temperature in a humidified chamber.
- Wash the plate two times using 200 µl 0.05% Tween®20 in PBS for each well, banging dry after each wash (see 6.4.1).

6.4.5 Step 5: Add substrate and read sample absorbance

- Add 100 µl per well of **TMB chromogen** and incubate for **2 to 10 minutes** at room temperature in the dark.
    - The duration of exposure to TMB depends on the activity of the TMB as well as the parasite density. Stop the reaction as soon as the controls (in which the HRP2 concentration is highest) have turned bright blue and the wells with the highest drug concentration are very pale blue.
- Stop the reaction by adding 50 µl of 1 M sulphuric acid to each well.
    - Prepare the stop solution by adding 10 mL of sulphuric acid to 90 mL of distilled water. SAFETY NOTE: always add sulphuric acid to water, never water to sulphuric acid.
- Using an ELISA plate reader, measure the absorbance **at 450 nm.**
- **Note:** if all wells turn dark blue then either:
    a) the parasite density may be too high
    Action: dilute the culture samples to lower density and repeat; or
    b) the ELISA is too sensitive.
    Action: reduce the concentration of the second antibody; or
    c) the incubation with TMB was too long
    Action: reduce the TMB incubation time

7. References

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