Estimation of *Plasmodium falciparum* drug susceptibility by the ^{3}H-hypoxanthine uptake inhibition assay

Procedure

In vitro Module

WorldWide Antimalarial Resistance Network (WWARN)

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Version History

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1.0	Creation of procedure	20 Feb 2012

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1. Purpose

This procedure describes the standard 42-hour ^{3}H-hypoxanthine uptake inhibition method for evaluation of *ex vivo Plasmodium falciparum* isolates susceptibility to antimalarial drugs. This sensitive method measures the incorporation of ^{3}H-hypoxanthine into parasite DNA as a measure of parasitic replication in red blood cells. Special facilities and procedures for handling and disposal of radioactive substances are required.

2. Scope

This procedure details the steps required to determine the effects of a range of antimalarial drugs on the growth of P. falciparum in fresh clinical (ex vivo) and/or culture-adapted parasites using the standard 42-hour ^{3}H-hypoxanthine uptake inhibition method. Samples suitable for testing are obtained from patients with P. falciparum monoinfections with parasite densities of 0.05 % or greater.

The methods for culture of *P.falciparum* erythrocytic stages (WWARN procedure INV01), preparation of appropriate parasite culture medium (INV02), preparation of pre-dosed plates (INV03) 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

P. falciparum	*Plasmodium falciparum*
IC_{50}	Half maximal inhibitory concentration
RPMI	Roswell Park Memorial Institute 1640 series of Cell Culture Medium
RBC	Red blood cells
RT	Room temperature (18–30^{o}C)

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 is required to perform the procedure successfully and to be aware of the additional regulations governing the storage, use and disposal of radioactive substances mandated by each country. Competency may be assessed observationally by an approved trainer. Participation in an appropriate QA/QC proficiency scheme is recommended.

5. Materials and Equipment

5.1 Materials

- 96-well microculture plates (suggested supplier Nunc or Falcon)
- Sterile Vacutainer® ACD or EDTA tubes
- Sterile graduated pipettes
- 100 mL and 1 L volumetric flasks
- Nalgene MF75 Series 0.2 µM Disposable Sterilization Filter Units: 500mL and 1 L or equivalent
- Nalgene MF75 Series Filter Unit Receivers, 250 mL or equivalent
- Sterile vials
- Vials for liquid scintillation counter and Whatman 3M glass-filter paper
- Filter plate (Unifilter GF/B, Perkin-Elmer)
- Scintillation cocktail (Microscint O, Perkin-Elmer)
- Monohydrochloride tritiated hypoxanthine, stored according to manufacturer's instructions

5.2 Equipment

- Water jacketed Incubator set at 36–38°C
- Candle jar or incubation chamber with gas mixture
- Freezer (-20°C or below)
- Refrigerator
- Cell harvester
- Liquid scintillation counter
- Multichannel pipette (20–200 µL)
- One set of adjustable pipettes
- Class II Biological safety cabinet
- Water-bath or heater block
- Weighing balance
- Magnetic stirring plate
- Device for tube agitation
- Centrifuge with swinging bucket rotor e.g. Eppendorf 5804R

5.2 Reagents

- RPMI 1640, powdered medium, stored at 4°C
- Sodium bicarbonate, $NaHCO_3$, FW 84.01, stored at RT
- HEPES, $C_8H_{18}N_2O_4S$, FW 238.1, stored at RT
- L-glutamine, $C_5H_{10}N_2O_3$, FW 146.1, stored at RT
- Glucose, $C_6H_{12}O_6$, FW 180.16, stored at RT
- Sterile human serum stored at -20°C
- Hydrochloric acid 1N
- Sodium hydroxide 1N.
- Water for cell culture applications
- Giemsa modified Azure Blend

- Washing Medium = complete medium (INV02) without human serum or Albumax
- Complete medium for isotopic test = complete medium (INVO2) without 50mg/L hypoxanthine
- RBCs (blood group 0 or A+)
- Medical grade gases: 85-90% N_2, 5-10% O_2, 5% CO_2

6. Procedure

6.1 Aseptic procedures

- All procedures (except centrifugation) are performed in a Level II biosafety cabinet.
- The biosafety cabinet surface is wiped down with aseptic solution (Anios or equivalent) at the beginning and the end of every day.
- Close the valves on gas cylinders at the end of each day.
- The incubator and the storage surfaces are cleaned with aseptic solution at least every 3 months.

6.2 Preparation of washing and complete medium

1. The washing medium is identical to complete medium for malaria culture of *P. falciparum* (INV02) without human serum or Albumax (INV02-1).
2. The complete medium for isotopic test is identical to complete medium for malaria culture of *P. falciparum* (INV02) without 50 mg/L hypoxanthine (INV02-2).

6.3 Preparation of ^{3}H-hypoxanthine solution

- Holding and use of radioisotopic compounds requires government authorization.
- Stock solutions should be handled in a biological safety cabinet.
- ^{3}H-hypoxanthine is generally available as the monohydrochloride salt solubilized in 5 mL methanol, with a specific activity of 185 MBq (5.0 mCi).
- Prepare a solution of ^{3}H-hypoxanthine at 40 µCi/ml (1.48 MBq/ml) by adding 5 mL ^{3}H-hypoxanthine in methanol to 120 ml of complete medium for isotopic test (see 6.2).
- Aliquot the diluted ^{3}H -hypoxanthine solution into 10 propyethylene tubes.
- Store the diluted ^{3}H-hypoxanthine tubes at +4°C or -20°C according to manufacturer specifications.

6.4 Sample collection

- Collect a minimum of 1 ml of blood by venipuncture (WWARN procedure CLI08) in Vacutainer® ACD or EDTA tubes.
- Following collection, samples must be stored and transported at 4°C for

ex-vivo testing within 48 hours.

- Assess and record the parasite density and confirm monoinfection and absence of altered parasites (WWARN procedure CLI10).
- Samples suitable for testing are obtained from patients with *P. falciparum* monoinfections with **0.05 % or greater** parasite densities (parasitaemia).
- Blood samples with >1% parasitaemia may be used but should be diluted with uninfected RBCs (human blood type O or A+) to obtain an approximate parasitaemia of 0.5 %– 0.8% before testing to limit the inoculum effect.

6.5 Sample preparation

- Transfer the whole blood sample into a 10–15 mL centrifuge tube.
- Centrifuge the tube at 1000 x *g* (see Appendix A for conversion between RCF and RPM) at 4°C or at room temperature) for 5–10 minutes until the red blood cells pellet and there is a clear separation of pellet from supernatant.
- Using a serological or transfer pipette, carefully remove and discard as much the plasma (clear supernatant) as possible (about 0.5–1 mL in a 5 mL blood sample) without disrupting the red blood cells and store at -20°C.
- Remove the white blood cells layer immediately above the red cell pellet using a serological or transfer pipette and throw.
- Fill the tube with washing medium (see 6.2.1) and gently invert to resuspend and wash the red cell pellet.
- Centrifuge the tube at 4°C or room temperature at 1000 x *g* for 5–10 minutes.
- Discard the washing medium.
- Wash the red cell pellet a further three times as above.
- Discard the final wash. Continue immediately to step 6.6.

6.6 Preparation of the parasitized erythrocyte suspension

One 96-well plate requires 20 mL of red cell suspension at hematocrit 1.5%. To prepare, to a clean test tube:

- Add 18.8 mL of complete medium for isotopic test (see 6.2.2).
- Add 0.8 mL of 40 µCi/ml ^{3}H-hypoxanthine solution.
- Add 0.40 mL of washed parasitized erythrocytes (the pellet hematocrit is about 75 – 80%).
- If parasitaemia > 1%, dilute parasitized erythrocytes with uninfected erythrocytes (human blood type O or A+) to obtain a final parasitaemia between 0.5 and 0.8%.
- Shake the mixture carefully to resuspend the red blood cell pellet

evenly.

- Pipette 200 µL of the suspension into each well of a 96-well plate pre-dosed with antimalarial drugs (INV03). Alternatively, 100 µL of suspension may be used: in this case, reduce the drug concentrations in the pre-dosed plates by 50%.
- Cover the plate and shake carefully.
- Incubate the plate for 42 hours at 37°C in an incubator gassed by a mixture of 5% CO_2, 5–10% O_2, and 85–90% N_2 at 95% humidity. If an incubator is not available, a candle jar can be used.
- Immediately after the end of the incubation time, freeze the plate below -20°C.

6.7 Determination of incorporated ^{3}H hypoxanthine

- Thaw the plate at 37–40°C to lyse the red blood cells.
- Collect and wash the contents of each well onto a pre-marked standard filter or a filter plate using a cell harvester.
- Dry the filter or the filter plate at 37°C for 1 hour.
- Either place each dried standard filter paper into a separate scintillation vial and add 2 ml of scintillation cocktail or add 25 µl of scintillation cocktail into each well of the dried filter plate.
- Measure the radioactivity incorporated into parasite nucleotides using a liquid scintillation counter set up for vial or plates for 2 minutes.
- Record results as counts per minute (cpm).
- Radioactive waste must be stored in designated containers and disposed according to regulatory guidelines.
- Determine the IC_{50} using an appropriate analysis program.

7. References

Basco LK. *Field application of in vitro assays for the sensitivity of human malaria parasites to antimalarial drugs*. World Health Organization. Available from: http://www.who.int/malaria/publications/atoz/9789241595155/en/index.html (Accessed: 29 November 2010).

Briolant S, Baragatti M, Parola P, Simon F, Tall A, Sokhna C, Hovette P, Mabika Mamfoumbi M, Koeck J-L, Delmont J, Spiegel A, Castello J, Gardair J-P, Trape J-F, Kombila M, Minodier P, Fusai T, Rogier C, Pradines B. *Multinormal in vitro distribution model suitable for distribution of Plasmodium falciparum chemosusceptibility to doxycycline.* Antimicrobial Agents Chemotherapy, 2009, 53:688-695.

Appendix A: Conversion between RCF (x g) and RPM

Relative centrifugal force, or RCF (measured in times gravity or x *g*), depends on both revolutions per minute (RPM) and the radius of the centrifuge's rotor. The relationship between RCF (x *g*) and RPM is as follows:

$$g = (1.118 \times 10^{-5}) * (\text{radius of rotor in cm}) * (\text{RPM})^2$$

A conversion calculator can be found at:
http://www.beckmancoulter.com/resourcecenter/labresources/centrifuges/rotorcalc.asp

A conversion table can be found at:
http://www.piercenet.com/files/TR0040-Centrifuge-speed.pdf