P.falciparum drug sensitivity assay using **SYBR® Green I V1.0**

Procedure

In vitro Module

WorldWide Antimalarial Resistance Network (WWARN)

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Version History

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1. Purpose

This procedure describes the SYBR®Green I-based antimalarial drug susceptibility fluoroassay for laboratory/field use which is optimized for minimal infrastructure and technical equipment requirements while achieving appropriate sensitivity levels. The assay measures the DNA content of malaria-infected erythrocytes by fluorochrome staining the parasite DNA.

2. Scope

This procedure details the steps required to determine the effects of a range of antimalarial drugs on the growth of all *Plasmodium* species/*P. falciparum* in fresh clinical (ex vivo) and/or culture-adapted parasites using the SYBR® Green I method.

The methods for culture of *P. falciparum* erythrocytic stages (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/learning/procedures/invitro).

3. Abbreviations

CMS	Complete Media with Serum
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
HCl	Hydrochloric acid
IC50	Half maximal inhibitory concentration
M	molar (mol/L)
mg	milligram
mL	milliliter
ng/mL	nanograms per milliliter
Pf	*Plasmodium falciparum*
QA	Quality Assurance
QC	Quality Control
RT	Room temperature (18 – 30 ^{o}C)
µ	micron
µL	microliter
µM	micrometer

4. Duties and Responsibilities

This procedure is designed for use by appropriately-equipped laboratories working on *in vitro* drug susceptibility testing of *P. falciparum*. Training is required to perform the procedure successfully. 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

- Amber 1.5 mL microtubes, Eppendorf
- Clear, cell culture-treated 96-well microtiter plates (suggested supplier Nunc)
- Centrifuge tube
- Conical Tubes, 15 mL
- Conical Tubes, 50 mL, Flat top, Polypropylene
- Cylinder
- Gloves: non-powdered
- Micropipetor tips, sterile
- Nalgene MF75 Series 0.2 µM Disposable Sterilization Filter Units: 500mL and 1 L
- Nalgene MF75 Series Filter Unit Receivers, 250 mL
- Needles
- Parafilm® sealing film
- Pasteur pipettes
- 1 mL, 2mL, 5mL, 10mL and 25mL serological pipettes
- Sterile plugged and unplugged glass 9 inch Pasteur pipettes
- Super frost Disposable Microscope Slides, 75 x 25mm
- Transfer pipettes
- Vacuum pump
- Volumetric flasks
- Weighing boat

5.2 Equipment

- Centrifuge with swinging bucket rotor e.g. Eppendorf 5804R
- Class II Bio safety cabinet
- Fluorescence reader
- Freezers
- Liquid handler
- Medical grade gas bottles and manometer
- Refrigerator
- Vacuum trap
- Water bath
- Water-jacketed incubator set at 36 – 38°C e.g. NAPCO CO2 7000
- Weighing balance

5.3 Reagents

- 0.5 M EDTA pH 8.0
- 10% CMS

- 70% ethanol
- Giemsa Modified Azure Blend, EM
- Human erythrocytes, compatible with plasma, stored at 2–8°C
- Medical grade gas: 90% N_2, 5% CO_2, 5%O_2
- Methanol
- Saponin
- SYBR Green I supplied by invitrogen as 10000x concentrate in DMSO; stored at –20°C.
- Triton X-100
- Water for cell culture applications
- Antimalarial drugs – for example quinine sulphate, desethylamodiaquine, piperaquine, pyronaridine, mefloquine, lumefantrine, chloroquine diphosphate, dihydroartemisinin, and primaquine phosphate, atovaquone, artemisinin, artesunic acid. Standard reference drugs supplied by the WWARN QA/QC programme are recommended.

5.4 Sample

- Parasitized whole blood from *Pf*-infected subjects
- *P. falciparum* culture

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 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 at least every 3 months.

6.2 Preparation of lysis buffer (1 L)

- Dissolve 15.76 g Tris-HCl completely in about 700 mL cell culture water using a magnetic stirrer.
- Adjust pH to 7.5 using concentrated hydrochloric acid.
- Add 20 mL 0.5 M EDTA to give a final concentration of 10mM (2% w/v).
- Add 160 mg saponin (0.016 % w/v final).
- Add 16.0 mL Triton X-100 (1.6 % v/v final).
- Add cell culture water to bring the final volume to 1 Litre.
- Mix the solution thoroughly, avoiding the creation of bubbles.
- Vacuum filter the solution using 0.2µ pore to remove any particulate matter and store indefinitely at RT.

6.3 SYBR Green I stock solution

- Thaw 10000x SYBR Green I concentrate (invitrogen) at RT in laminar flow hood in a darkened room.
- Aliquot 30 µL into amber-colored Eppendorf tubes, label with the day's

date and store at -20°C for up to 6 months.

6.4 Lysis buffer containing SYBR Green I (15 mL)

- This solution should be made fresh in a darkened room.
- Thaw one 30 µL aliquot of SYBR Green I (Section 6.5).
- Add 30 µL SYBR Green I to 15 mL lysis buffer (20x final SYBR Green concentration). 15 mL lysis buffer is adequate for one 96 plate.
- Pipette to mix, avoiding the creation of bubbles.

6.5 Preparation of malaria cultures and sensitivity assay

- Determine % parasitaemia of malarial culture.
- For fresh field isolates ≤ 0.3%, run the assay at 2% hematocrit in complete medium (preparation of medium is described in WWARN procedure INV02) without reducing the parasitaemia.
- If parasitaemia of culture-adapted samples or fresh field isolates are >0.3, dilute to 0.3% or 0.15% parasitaemia using complete culture medium for 72 or 96hr incubations respectively at 2% hematocrit in complete medium. A 72h assay is adequate for most drugs; 96h incubation can be used for slow acting drugs like antibiotics.
- Fresh field isolate are not washed prior to the assay
- Using automated liquid handler or manually, add 100 µL malaria-infected erythrocytes to each well on a pre-dosed drug plate (for preparation see WWARN procedure INV03).
- Incubate cultures for 72hrs or 96hrs at 37°C in a humidified chamber, under a gas mixture of 90% N_2, 5% O_2, and 5% CO_2 , or in a candle jar.
- After the 72hr or 96 hr incubation, add 100 µL lysis buffer containing 20x SYBR Green I (see section 6.6) to each well, in a dark room.
- Incubate the plates at RT in the dark for 24 hrs.
- Read fluorescence on a fluorescence plate reader with excitation and emission wavelength bands centered at 485 and 530 nm, respectively.
- Determine the IC_{50} using an appropriate analysis programme.

7. References

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