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

Procedure

In vitro Module
WorldWide Antimalarial Resistance Network (WWARN)

Procedure ID: INV08

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**Version History**

<table>
<thead>
<tr>
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</tr>
</tbody>
</table>

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 Contents

1. Purpose ...........................................................................................................................................4
2. Scope ................................................................................................................................................4
3. Abbreviations ...................................................................................................................................4
4. Duties and Responsibilities ..............................................................................................................4
5. Materials and Equipment ..................................................................................................................5
   5.1 Materials .......................................................................................................................................5
   5.2 Equipment ....................................................................................................................................5
6. Procedure ..........................................................................................................................................6
   6.1 Aseptic procedures .........................................................................................................................6
   6.2 Preparation of lysis buffer (1 L) .....................................................................................................6
   6.3 SYBR Green I stock solution .........................................................................................................6
   6.4 Lysis buffer containing SYBR Green I (15 mL) ...........................................................................7
   6.5 Preparation of malaria cultures and sensitivity assay .................................................................7
7. References ..........................................................................................................................................7
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°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% N₂, 5% CO₂, 5%O₂
• 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% N₂, 5% O₂, and 5% CO₂, 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 IC₅₀ using an appropriate analysis programme.

7. References

Testing and Application to Clinical Isolates. Antimicrobial Agents And Chemotherapy; Vol. 51(4) p. 1172–1178
