**Piperaquine Survival Assays (PSA) to evaluate the**

***in-vitro* and *ex-vivo* susceptibility of *Plasmodium falciparum* to piperaquine**

**Procedure**

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# 1. Purpose

This procedure describes two assays to evaluate the susceptibility of *Plasmodium falciparum* to piperaquine. The ***in-vitro* Piperaquine Survival Assay (PSA)** is performed on 0-3 hour post-invasion rings obtained from culture-adapted parasites. The ***ex-vivo* Piperaquine Survival Assay** (***ex-vivo* PSA**) is performed on parasite isolates freshly collected from patients with malaria. In both assays, parasites are exposed to 200 nM piperaquine (PPQ) for 48 hours – approximating their drug exposure in patients treated with piperaquine based ACT – and their survival is assessed 72 hours later as described in Duru et al. 2015 (1).

# 2. Scope

This procedure details the steps required to determine the percentage of viable parasites at 72 hours, following a 200-nM, 48-hour exposure of PPQ. Samples suitable for testing are either adapted parasite lines (synchronized at the 0-3 hour post-invasion ring stage) in the *in-vitro* PSA or clinical parasite isolates (freshly obtained from patients with *P. falciparum* monoinfection) in the *ex-vivo* PSA.

# 3. Abbreviations

ACD acid citrate dextrose

PPQ piperaquine

HEPES 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

PBS phosphate-buffered saline

QA quality assurance

QC quality control

RBC red blood cell

RPMI Roswell Park Memorial Institute

PPQ Piperaquine tetraphosphate tetrahydrate

PSA Piperaquine survival assay

RT room temperature

WBC white blood cell

L liter

mL milliliter

µL microliter

µg microgram

ng nanogram

mM millimolar

µM micromolar

nM nanomolar

µm micrometer

MW molecular weight

U unit

# 4. Duties and Responsibilities

This procedure is designed for use by appropriately-equipped laboratories that perform *in-vitro* and *ex-vivo* drug susceptibility tests on *P. falciparum*. To perform the procedure successfully, training is required and participation in an appropriate QA/QC proficiency scheme is recommended. Strains with known survival rates expressed by the in-vitro PSA will be available soon at ATCC (www.atcc.org).

# 5. Materials and Equipment

## 5.1. Materials

***General***

* Sterile pipettes (2 mL, 5 mL, 10 mL, 25 mL)
* Sterile tips (10 µL, 20 µL, 200 µL, 1000 µL)
* Sterile transfer pipettes
* Cell culture flasks with vented cap (25 cm2)
* Cell culture flasks with vented cap (75 cm2)
* 48-well culture plates
* Microscope slides
* Microscope slide staining jars
* Centrifuge tubes (15 mL, 50 mL)
* Microcentrifuge tubes (1.5 mL)
* Polystyrene round-bottom tubes (5 mL)
* Disposable sterilization filter units, 0.22 µm, 1 L capacity
* 70% ethanol

***Blood sample collection***

* ACD Vacutainers® (Becton-Dickinson, #364816)
* BD Vacutainer® Safety-LokTM Blood Collection Set (Becton-Dickinson, #364815)
* BD Vacutainer® One Use Holder (Becton-Dickinson, #367281)

***Parasite culture***

* RPMI-1640 + GlutaMAXTM-1 (Gibco, #61870-044) stored at 4°C
* Albumax II (Invitrogen, #11021-037) stored at 4°C
* Sterile human serum (ABO/Rh blood group 0+) stored at –20°C
* RBCs (ABO/Rh blood group 0+) stored at 4°C
* HEPES Buffer solution 1 M (Gibco, #1563-056, 100 mL) stored at 4°C
* Gentamicin solution, 10 mg/mL (Invitrogen, #15710-072) stored at RT
* Hypoxanthine 10 mM (c·c·pro, #Z-41-M, 100 mL) stored at 4°C
* Distilled water for cell culture (Gibco, #10977-035, 500 mL) stored at RT
* Methanol stored at RT
* Giemsa stain (Merck, #1.09204.0500, 500 mL) stored at RT
* Glycerolyte 57 (Baxter, #4A7833) stored at RT

***Parasite synchronization***

* 10X PBS (Gibco, #70013-016, 500 mL) stored at RT
* Heparin sodium 5,000 U/mL (Rotexmedica, #3862340) stored at 4°C
* Percoll (Sigma, #P4937) stored at 4°C
* D-sorbitol (Sigma, #S1876) stored at RT

***Piperaquine Survival Assay (PSA)***

* Piperaquine tetraphosphate tetrahydrate (provided by WWARN) stored at 4°C
* Lactic acid (Sigma #L1750,) stored at RT
* Distilled water for cell culture (Gibco, #10977-035, 500 mL) stored at RT

## 5.2. Equipment

* Pipettor
* Micropipettes
* Water-jacketed incubator at 37°C with tri-gas mixture (5% CO2, 5% O2, 90% N2)
* Class II biosafety cabinet
* Vortexer
* Centrifuge with swinging bucket rotor (e.g., Eppendorf 5702)
* Microcentrifuge
* Oil-immersion microscope
* Freezer (–20°C or below)
* Refrigerator
* Water bath
* Weighing balance

# 

# 6. Procedure

## 6.1. Aseptic procedures

* All procedures (except centrifugation, vortexing, 37°C incubations, and preparing stained blood smears) are performed in a Class II biosafety cabinet, which is wiped down with aseptic solution at the start and end of each day.
* The incubator and storage surfaces are cleaned with aseptic solution at least every 3 months.

## 6.2. Reagent preparation

### *6.2.1. Preparation of culture medium*

* Prepare the culture medium as described in Table 1.
* Use a magnetic stirrer to ensure that components are completely dissolved.
* Heat-inactivate the human serum at 56°C for 30 minutes prior to use.
* After preparation, filter the medium using a 0.22 µm pore size, 1 L filter.
* Store at 4°C for up to 2 weeks.

***Table 1.*** *Volumes of reagents for 1 L of culture medium*

|  |  |  |
| --- | --- | --- |
| Reagent | Stock solution | Volume/weight |
| RPMI-1640 medium |  | 928 mL |
| HEPES | 1 M | 25 mL |
| Gentamicin | 10 mg/mL | 2 mL |
| Hypoxanthine | 10 mM | 20 mL |
| Albumax II |  | 5 g |
| Heat-inactivated human serum |  | 25 mL |

### *6.2.2. Preparation of PPQ solution*

1. Resuspend the PPQ (provided by WWARN as a pre-weighed ~1 mg aliquot) in 0.5% lactic acid to obtain a 200µ/mL stock solution. Vortex to dissolve completely.
2. Make 50 µL aliquots in sterile 1.5 mL microcentrifuge tubes.
3. Store at –20°C for up to 6 months.

### *6.2.3. Preparation of uninfected red blood cells*

* RBCs are used to lower parasitemia and adjust hematocrit as needed in parasite cultures and PSA.
* RBCs should test negative for hemoglobinopathies and G6PD deficiency before use.
* Collect whole blood in a 250 mL pouch containing ACD anticoagulant.

1. Spray the blood pouch with 70% ethanol and let it dry in the biosafety cabinet.
2. Open carefully by cutting the pouch’s tube with sterile scissors.
3. Transfer the blood into 50 mL centrifuge tubes.
4. Store at 4°C for up to 3 weeks.

* Before using RBCs in parasite cultures and PSA:

1. Rule out hemoglobinopathies and G6PD deficiency.
2. Centrifuge 25 mL of whole blood at 1000 g for 10 minutes.
3. Remove the plasma and buffy coat.
4. Add cold (4°C) RPMI to a final volume of 50 mL.
5. Centrifuge at 1000 g for 10 minutes.
6. Remove the supernatant.
7. Repeat steps 4-6 twice.
8. Add 3 mL of RPMI to RBC pellet.
9. Centrifuge at 1000 g for 10 minutes.
10. Store (without mixing the cells) at 4°C up to 4 days.

### *6.2.4. Preparation of 5% sorbitol solution*

1. Add 50 g of D-sorbitol to 1 L of culture water.
2. Stir until completely dissolved.
3. Sterilize the solution using a 0.22 μM pore size, 1 L filter.
4. Store at 4°C up to 1 year.

### *6.2.5. Preparation of Percoll® solution*

These steps are done at RT, in a Class II biosafety cabinet, and using sterile reagents.

1. Mix 9 volumes of Percoll with 1 volume of 10X PBS to make Percoll 90% solution. Mix thoroughly. Store at 4°C up to 2 months.
2. Freshly prepare heparinized RPMI by adding 15 µL heparin (5,000 U/mL) to 10 mL RPMI.
3. Freshly prepare Percoll 75% solution by adding 1.5 volumes of heparinized RPMI to 7.5 volumes of Percoll 90% solution.

### *Preparation of Giemsa stain*

* Giemsa (Merck) – Add 1 volume of Giemsa to 9 volumes of 0.4% NaCl solution. Stain smears for 30 minutes and rinse.

***Table 2****. Information regarding the storage and shelf-life of reagents*

|  |  |  |
| --- | --- | --- |
| Reagent | Storage temperature | Shelf-life |
| Culture medium | 4°C | 2 weeks |
| Sorbitol 5% solution | 4°C | 1 year |
| PPQ solution | –20°C | 6 months |
| Percoll® 90% solution | 4°C | 2 months |
| Heparinized RPMI | RT | Freshly made |
| Percoll® 75% solution | RT | Freshly made |
| Uninfected RBCs in ACD | 4°C | 3 weeks |
| Uninfected RBCs for culture | 4°C | 4 days |
| Giemsa solution | RT | Freshly made |

## 6.3. Collection and preparation of blood from malaria patients

All steps are performed at RT unless otherwise stated. All procedures following blood draw should be performed in a biosafety cabinet to avoid bacterial contamination.

### *6.3.1. Sample collection*

* Aseptically draw venous blood into an ACD Vacutainer® using the BD Vacutainer® Safety-LokTM Blood Collection Set and holder.
* Mix by gently inverting tube 5 times and store the blood at 4°C up to 24 hours.

### *6.3.2. Sample preparation*

1. Prepare a thin blood smear using a drop of blood. Stain with Giemsa (section 6.2.6).
2. Evaluate the parasitemia.
3. Centrifuge the blood tube at 800 g for 5 minutes.
4. Remove the plasma and the buffy coat.
5. Transfer the RBC pellet to a 50 mL centrifuge tube.
6. Add 20 mL of RPMI (pre-warmed to RT).
7. Centrifuge at 800 g for 5 minutes.
8. Remove the supernatant.
9. Repeat steps 6-8 twice.

The infected RBCs are now ready to be processed for *ex-vivo* PSA, long-term storage (section 6.3.3), or culture-adaptation (section 6.4).

### *6.3.3. Preparation of P. falciparum samples for long-term storage*

This step is optional but provides backup aliquots that can be used years later.

1. In a 50 mL centrifuge tube, add Glycerolyte (3X volume of RBC pellet) drop-wise at RT to the RBC pellet obtained in section 6.3.2.
2. Mix gently with a pipette.
3. Let it stand for 1 minute at RT.
4. Distribute 500 µL of the preparation into sterile cryotubes.
5. Freeze at –80°C for at least 18 hours.
6. Transfer vials to liquid nitrogen for long-term storage.

## 6.4. *In-vitro* culture adaptation

All steps are performed at RT unless otherwise stated. All procedures are performed in a level II biosafety cabinet to avoid bacterial contamination. **All *P. falciparum* samples are suitable for *in-vitro* culture adaptation, regardless of parasitemia.**

### *6.4.1. Freshly-collected samples*

1. Warm culture medium to 37°C in a water bath.
2. Add 5 mL of culture medium to a 25 cm2 culture flask.
3. Add 150 µL of the infected RBC pellet (section 6.3.2) and 50 µL of uninfected RBCs (section 6.2.3) to achieve a 4% hematocrit.
4. Mix gently.
5. Maintain the culture in an incubator under the following conditions: 37°C, humid atmosphere, 5% O2 and 5% CO2, 90% N2.
6. Replace 5 mL of culture medium (pre-warmed to 37°C) daily. Check the parasitemia daily by making Giemsa-stained thin blood smears.
   1. If no or few parasites are detected (< 0.1% of the initial parasitemia), add 50 µL of uninfected RBCs (section 6.2.3) weekly in addition to replacing medium daily. Stop the culture if no parasites are observed in 60 days.
   2. If parasites are detected and are growing normally (parasitemia increases 1.5- to 10-fold every 48 hours), maintain the parasitemia under 3%. For assays, dilute the pellet with uninfected RBCs to achieve a 0.5-1% parasitemia and add culture medium to obtain a 4% hematocrit.
7. Adaptation is considered successful after 2 weeks of uninterrupted parasite culture.

### *6.4.2. Cryopreserved samples*

1. Remove a tube containing cryopreserved *P. falciparum* parasites (section 6.3.3) from liquid nitrogen.
2. Thaw the vial in a 37°C water bath. Wear eye-protection. Avoid contact between water and the cap of the tube.
3. After thawing, transfer the solution to a 15 mL centrifuge tube.
4. Centrifuge at 800 g for 5 minutes.
5. Remove the supernatant.
6. Add 1 volume (equal to the initial volume in the cryotube) of 3.5% NaCl solution drop-wise while shaking the tube gently.
7. Let it stand for 1 minute at RT.
8. Add 12 mL of RPMI (pre-warmed to 37°C) and mix gently.
9. Centrifuge at 800 g for 5 minutes.
10. Remove the supernatant.
11. Resuspend the RBCs in 10 mL of culture medium (pre-warmed to 37°C).
12. Transfer to a 25 cm2 culture flask and add uninfected RBCs to obtain a 2% hematocrit.
13. Maintain the culture in an incubator under these conditions: 37°C, humid atmosphere, 5% O2, 5% CO2 and 90% N2, and follow the procedure in section 6.4.1.

## 6.5. *Ex-vivo* PSA

All steps are performed at RT unless otherwise stated. All procedures are performed in a sterile biosafety cabinet to avoid bacterial contamination. ***P. falciparum* samples with parasitemia ≥ 0.1% are suitable for this assay.**

### *6.5.1. Preparation of P. falciparum sample and drug solution*

* Prepare a parasite solution by adding 50 µl of infected RBCs (section 6.3.2) to 2.5 mL of culture medium.
* If the parasitemia is > 1%, dilute the pellet with uninfected RBCs to obtain a 0.5-1% parasitemia and with culture medium to obtain a 2% hematocrit.
* Make a thin smear (= initial parasitemia, “INI”).
* Prepare the vehicle control solution by adding 20 µL of 0.5% lactic acid to 2 mL of culture medium. Prepare the PPQ test solution by adding 20 µL of PPQ stock solution (section 6.2.2) to 2 mL of culture medium. Mix both solutions by vortexting.
* Do not re-use the thawed aliquot of PPQ. Always use a fresh aliquot.

### *6.5.2. Ex-vivo PSA*

1. In a 48-well culture plate, add:
   * 100 µL of vehicle control solution in the ‘’non-exposed’’ well and
   * 100 µL of PPQ solution in ‘’PPQ-exposed’’ well.
2. Add 900 µL of infected RBC suspension to each well.
3. Mix gently with pipette while avoiding contact between wells.
4. Maintain the 48-well culture plate in an incubator under these conditions: 37°C, humid atmosphere, and 5% O2, 5% CO2 and 90% N2 for 48 hours.
5. After 48 hours, transfer the contents of the wells into 15 mL centrifuge tubes (non-exposed and PPQ-exposed).
6. Centrifuge at 800 g for 2 minutes.
7. Remove the supernatant.
8. Add 12 mL of RPMI (pre-warmed at 37°C) and resuspend the RBC pellet.
9. Centrifuge at 800 g for 5 minutes.
10. Remove the supernatant.
11. Add 1 mL of culture medium (pre-warmed to 37°C) and resuspend the RBCs.
12. Transfer the suspension into 2 new wells in the 48-well culture plate.
13. Maintain the 48-well culture plate in an incubator under these conditions: 37°C, humid atmosphere, and 5% O2 and 5% CO2 and 90% N2 for 24 hours.
14. Mix and transfer the culture from each well into a 1.5 mL microcentrifuge tube.
15. Centrifuge briefly (2000 rpm for 30 seconds).
16. Use 2 µL of the pellet and make a thin blood smear from both samples.
17. Fix the smears in methanol for 2 seconds and stain using Giemsa (section 6.2.6).

## 6.6. *In-vitro* PSA

All steps are performed at RT unless otherwise stated. All procedures are performed in a level II biosafety cabinet to avoid bacterial contamination. **All *P. falciparum* samples are suitable for this assay, if the culture adaptation is successful.**

### *6.6.1. Preparation of 0-3 hour post-invasion rings*

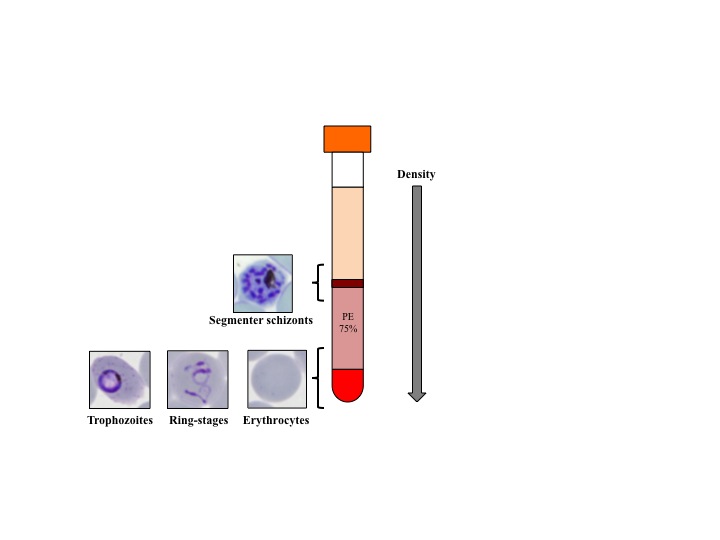
1. Use culture-adapted parasites (section 6.4) in a 25 cm2 culture flask.
2. Allow the parasitemia to increase by changing the culture medium daily without adding uninfected RBCs.
3. Check the parasitemia daily; when it increases to 2-5%, transfer the culture to a 15 mL centrifuge tube.
4. Centrifuge at 800 g for 5 minutes.
5. Remove the supernatant.
6. Transfer the RBC pellet (~200 µL) to a 75 cm2 culture flask containing 35 mL of culture medium (pre-warmed to 37oC) and 500 µL of uninfected RBCs.
7. Increase the parasitemia up to 4% by changing the culture medium daily.
8. When the proportion of rings is up to 50%, transfer the culture to a 50 mL centrifuge tube.
9. Centrifuge at 800 g for 5 minutes.
10. Remove the supernatant.
11. Add 10 volumes of 5% sorbitol (pre-warmed to 37°C) to the RBC pellet (1 volume).
12. Incubate 10 minutes at 37°C and vortex for 5 seconds.
13. Centrifuge at 800 g for 5 minutes.
14. Remove the supernatant.
15. Transfer the RBC pellet to a 75 cm2 culture flask containing 35 mL of culture medium (pre-warmed to 37°C).
16. Repeat steps 9-15, 30-48 hours later (depending on parasite development and stages at the time of sorbitol treatment).
17. After 30 hours of culture, make a thin blood smear and stain with Giemsa.

- If the proportion of mature schizonts (10-12 nuclei) is > 0.5%, go to step 18.

- If the proportion of mature schizonts (10-12 nuclei) is < 0.5%, repeat step 16.

1. Transfer the culture suspension to a 50 mL centrifuge tube.
2. Centrifuge the culture suspension at 800 g for 5 minutes.
3. Remove the supernatant.
4. Transfer 400 µL of the pellet to a 15 mL centrifuge tube and return the remaining pellet to culture conditions.
5. Add 4 mL of heparinised RPMI (pre-warmed to 37°C) (section 6.2.5) to the 400 µL pellet and mix gently.
6. Incubate for 15 minutes at 37°C.
7. In a 15 mL centrifuge tube, add 4 mL of 75% Percoll solution at RT.
8. Carefully layer the 4 mL RBC suspension in heparinised RPMI on top of the 75% Percoll solution (slant the 15 mL centrifuge tube at a very low angle and slowly add the RBC suspension drop-wise along the wall of the tube using a transfer pipette). If this step is correctly performed, you must observe two separated phases: the infected RBC suspension on the top, and the Percoll 75% solution on the bottom.
9. Centrifuge at 1000 g for 15 minutes. You must observe an intermediate phase containing the mature schizonts (**see Figure 1**).
10. Collect this phase carefully and transfer it to a 15 mL tube.
11. Add up to 13 mL of RPMI (pre-warmed to 37°C). Mix by inverting the tube.
12. Centrifuge at 800 g for 5 minutes.
13. Remove the supernatant.
14. Using 0.5 µL of the pellet, make a thin smear, fix it with methanol, and stain it with Giemsa (section 6.2.6). Schizonts must be concentrated (> 10%). Ring stages must be rare (< 10%). While the slide is staining, proceed to steps 33-36.
15. Add 10 mL of culture medium (pre-warmed to 37°C) to the infected RBCs.
16. Add 200 µL of uninfected RBCs.
17. Transfer the RBC suspension to a 25 cm2 flask.
18. Place in an incubator under these conditions: 37°C, humid atmosphere, 5% O2, 5% CO2 and 90% N2 for exactly 3 hours.

**Figure 1:** *Differential density of parasites of different stages enabling segmenting schizont selection on 75% Percoll.*



**The following steps must be performed within 1 hour maximum.**

1. Mix the culture and transfer 0.5 mL into a 1.5 mL microcentrifuge tube.
2. Centrifuge 2000 rpm for 30 seconds.
3. Using 2 µL of the pellet make a thin smear, fix it with methanol, and stain it with Giemsa (Rapid stain RAL555 is preferred since it will take < 1 minute).
4. Quickly evaluate the proportion of ring-stages (must be > 0.5%).
5. Transfer the culture suspension to a 15 mL centrifuge tube.
6. Centrifuge at 800 g for 5 minutes. Remove the supernatant.
7. Add 10 volumes of 5% sorbitol (pre-warmed to 37°C) to the RBC pellet (1 volume).
8. Incubate for 10 minutes at 37°C and vortex for 5 seconds.
9. Centrifuge at 800 g for 5 minutes. Remove the supernatant.

### *6.6.2. Preparation of P. falciparum sample and drug solution*

* If parasitemia from step 39 is > 1%, add uninfected RBCs and culture medium (pre-warmed to 37oC) to obtain a 0.5-1% parasitemia and 2% hematocrit.
* Make a thin smear (= initial parasitemia, “INI”).
* Prepare the vehicle control solution by adding 20 µL of lactic acid 0.5% to 2 mL of culture medium. Prepare the PPQ test solution by adding 20 µL of PPQ stock solution (section 6.2.2) to 2 mL of culture medium. Mix both solutions by vortexting.
* Do not re-use the thawed aliquot of PPQ. Always use a fresh aliquot.

### *6.6.3. In-vitro PSA*

1. In a 48-well culture plate, add:
   * 100 µL of vehicle control solution in the ‘’non-exposed’’ well and
   * 100 µL of PPQ solution in ‘’PPQ-exposed’’ well.
2. Add 900 µL of infected RBC suspension to each well.
3. Mix gently with a pipette while avoiding contact between wells.
4. Maintain the 48-well culture plate in an incubator under these conditions: 37°C, humid atmosphere, 5% O2, 5% CO2 and 90% N2 for 48 hours.
5. After 48 hours, transfer the contents of the wells to 15 mL centrifuge tubes (non-exposed and PPQ-exposed).
6. Centrifuge at 800 g for 2 minutes.
7. Remove the supernatant.
8. Add 9 mL of RPMI (pre-warmed to 37°C). Resuspend the RBCs by inverting the tube.
9. Centrifuge at 800 g for 5 minutes.
10. Remove the supernatant.
11. Add 1 mL of culture medium (pre-warmed to 37°C) and mix to resuspend the RBCs.
12. Transfer the suspension into 2 new wells in the 48-well culture plate.
13. Maintain the 48-well culture plate in an incubator under the following conditions: 37°C, humid atmosphere, 5% O2 and 5% CO2 and 90% N2 for 24 hours.
14. At 72h Mix and transfer the culture from each well into a 1.5 mL microcentrifuge tube.
15. Centrifuge at 2000 rpm for 30 seconds.
16. Use 2 µL of the pellet and make a thin blood smear from both samples.
17. Fix the smears in methanol for 2 seconds and stain with Giemsa (section 6.2.6).

## 6.7. Microscopic examination to determine parasite survival rates

Three different thin blood smears must be stained and read per assay:

* “INI” smear to define the initial parasitemia at 0 hours
* “NE” smear to define the non-exposed parasitemia at 72 hours
* “PPQ” smear to define the PPQ-exposed parasitemia at 72 hours

1. At 100X magnification under immersion oil, count the number of infected RBCs containing viable parasites in a total of 10,000 RBCs (by estimating the number of RBCs per field or per grid). The smear must be homogenous: count areas on the slide containing 200-400 RBCs per field. Do not count areas at the edge of the smear since parasites will concentrate in these areas. Only viable parasites are scored; pyknotic and dead trophozoites forms are not scored.
2. Determine the proportion of viable parasites (% survival) in NE and PPQ slides.
3. Calculate:

* Growth rate = NE/INI
* Percentage survival (%) = (PPQ/NE) x 100

1. Percentage survival values are interpretable if growth rate:

* ≥ 1 for *ex-vivo* PSA
* ≥ 1.5 for *in-vitro* PSA

**7. References**

**Plasmodium falciparum dihydroartemisinin-piperaquine failures in Cambodia are associated with mutant K13 parasites presenting high survival rates in novel piperaquine in vitro assays: retrospective and prospective investigations**. Duru V, Khim N, Leang R, Kim S, Domergue A, Kloeung N, Ke S, Chy S, Eam R, Khean C, Loch K, Ken M, Lek D, Beghain J, Ariey F, Guerin PJ, Huy R, Mercereau-Puijalon O, Witkowski B, Menard D. BMC Med. 2015 Dec 22;13:305. doi: 10.1186/s12916-015-0539-5.

**Appendix A. Blood sample management for PSA**



**Appendix B. Schematic representation of PSA**

