Plasmodium falciparum Growth or Invasion Inhibition Assays

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Method Article

**Keywords:** malaria, inhibition, growth, antibodies, drugs

**Posted Date:** January 5th, 2024

**DOI:** https://doi.org/10.21203/rs.3.pex-2507/v1

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Abstract

Growth inhibition assays are a well established method to quantify the ability of antibodies, drugs, and other compounds to inhibit the in vitro growth of asexual blood stages of P. falciparum. The assays are widely used to quantify the activity of acquired and vaccine-induced antibodies to P. falciparum, to quantify the activity of antimalarial drugs and evaluate drug resistance. The assay is performed in vitro using human red blood cells.

Introduction

Reagents

See procedure for details

Equipment

See procedure for details

Procedure

Protocol for growth inhibition assays performed over one or two cycles of parasite replication:

1. **Repeatedly synchronise parasite cultures in the 1-2 weeks prior to performing the assay.**
   
   Recommended methods for synchronisation:
   
   1. Sterile 5% sorbitol (in water): performed when parasites are mainly ring forms by resuspending parasite culture pellet in sorbitol solution, incubating for 5 mins, centrifuge at 1500 rpm for 5 mins, then resuspend pellet back into culture medium
   2. Gelatin enrichment of pigmented trophozoites: involves floatation of mature-stage parasites in 0.75% gelatin
   3. Magnet purification of mature pigmented parasitized RBCs

2. **Use highly synchronous parasite culture in the assays** (this is critical). Most PRBCs must be at the late pigmented trophozoite or preferably schizont stage. Do not use if cultures have many ring-stage parasites (very important)

Setting up the assay:
3. **Starting parasitemia should be around 0.5 – 0.8% for a one-cycle assay, and around 0.1-0.3% for a two-cycle assay.** Reduce the parasitaemia of starting culture by adding fresh RBCs as appropriate.

4. **Prepare parasites:** resuspend parasites at 1% haematocrit (eg 50ul of cell pellet in 5ml of medium) in RPMI-HEPES with 5% pooled serum and 0.25% Albumax II (0.25% equals 2.5g in 1 litre). RPMI-HEPES must be properly supplemented with glutamine (2mM) and hypoxanthine (50mg/ml). Good to also include gentamicin to help prevent bacterial contamination.

5. **Setting up the plates:** Use U-bottom 96 well tissue culture plate (Falcon 3077). Test all samples in duplicate. Include positive and negative controls in every assay run (Eg. inhibitory antibodies, samples from non-exposed donors). Include negative controls on every plate if setting up multiple plates (can use method A or B).

   **Method A):**
   
   1. Aliquot samples to be tested in inhibition assays into U-bottom 96 well plates. Keep plates on ice to avoid samples evaporating
   
   2. Add parasite suspension to each well of the 96 well culture plate. Final volume should be 25 or 50 ml. For consistency, use a multi-pipettor (Eg. Eppendorf) or multi-channel pipettor to add parasite suspension to wells. Cover wells with plate lid.

   **Method B):**
   
   a. Add parasite suspension (as above) to plates first
   
   b. Add samples to each well

6. **Prepare a smear** from the remaining parasite sample used for the assay – use this smear later to determine the parasitemia and stage of culture (then store these smears for later reference)

7. **Put plate into humidified chamber,** gas (with mix of 95% nitrogen, 4% CO\(_2\), 1%O\(_2\)), seal chamber and incubate at 37°C. For incubation chamber, best to use specialised culture chambers. Can also use glass or plastic dessicators or candle jars sealed with silicon grease. Include wet paper towels or tissues inside the chamber to create a humid environment. Be sure to sit the test plates on a blank or empty plate to avoid direct contact of the test plates with wet paper towels (to avoid possible contamination of cultures with yeast or bacteria). Be sure to gas the incubation chamber fully, from both sides if there are two ports. It may be preferable to repeat this after 30 mins.

8. For **two cycle assays:** at 48 hours, add fresh medium to each well (do not remove existing medium). For 25 ml cultures, add 5 ml. For 50ml cultures, add 10ml.

9. **Agitation:** Twice daily gentle agitation for 1 minute (or until pellet is resuspended) of the culture chamber may help redistribute parasites and create more even growth in the wells. Our results suggest
this slightly increases the sensitivity of the assay, but it is not essential.

10. Keep a sample culture in incubator to monitor developmental stage of parasites and guide the timing of harvesting parasites

11. **Measure parasitemia** (by flow cytometry, microscopy, or pLDH quantification)

   1. **One-cycle assay**: measure parasitemia at 36-48 hours (or microscopy at 24 hours) by flow cytometry. Best to measure parasitemia when parasites are at late ring or early trophozoite stage if measuring parasitemia by flow cytometry.

   2. **Two-cycle assay**: measure parasitemia at 80-96 hours (this varies with the parasite line used)

### Troubleshooting

**General Notes:**

- Culture volumes in 96-well plates can be 25-100 ml. We routinely use 50 ml volumes. Growth rates at 50ml are generally a little higher than 25 ml cultures. U-bottom plates are more practical, and growth rates are slightly better for U bottom plates

- Use only sterile equipment, aliquot samples and parasites in class II hood

- Always include non-immune controls (and positive controls if possible)

- Clean the glass or plastic incubation chamber before and after use to avoid the build-up of yeast that may contaminate cultures.

- Add extra glutamine (2mM) to culture medium to ensure good growth of parasites (do use Albumax alone as culture medium)

- Always use fresh RBCs in the starting culture. Store RBCs in CPD buffer, not PBS or RPMI-HEPES

**Notes on testing human serum or plasma samples:**

- Test samples at 1/10 dilution or less. Testing samples at a 1/5 dilution leads to problems with non-specific inhibition

- Ideally, all samples should be dialysed to equilibrate pH and remove non-specific inhibitors (Eg. antimalarial drugs and antibiotics)

- Avoid repeated freeze-thaw cycles with serum/plasma samples

### Time Taken

Usually conducted over a 24-72 hour period, depending on the purpose of the assay
Anticipated Results

References


Acknowledgements

Thanks to Kristina Persson, Danny Wilson, Michelle Boyle, and Fiona McCallum who worked on optimising the protocol.

There are no competing interests or conflict of interests.