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Determination of the Cytostatic and Cytocidal Activities of Antimalarial Compounds and their Combination Interactions

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Abstract

Determining the antiplasmodial activity of candidate antimalarial drugs *in vitro* identifies new therapies for drug-resistant malaria. Importantly though, activity can be either growth-inhibitory (cytostatic) or parasite-kill (cytotoxic), or both. The simple methods described here can allow for distinction between these, as well as definition of drug interactions between two or more compounds. The latter is important in the definition of novel drug combination therapy for malaria. These methods involve live malarial parasite red blood cell culture, routine pharmacology, high-throughput detection of parasite DNA with fluorescent reporters, and routine mathematical analysis of dose – response curves. The techniques and approaches are accessible to most laboratories and require minimal special equipment beyond a fluorescent plate reader and tissue culture facilities.

Keywords

Plasmodium falciparum; Antiplasmodial Activity; Cytostatic; Cytotoxic; Drug Combination Analysis

Introduction

The rise of antimalarial multidrug resistance necessitates identification of new drugs and drug combination therapies. Notwithstanding the difficulties inherent in live malarial parasite cell culture, which typically requires co-culture with human erythrocytes or hepatocytes, this protocol describes simple and inexpensive assays that can be used to investigate the activity of potential antimalarials. Use of fluorescent reporters such as SYBR Green I take advantage of the lack of nuclei in erythrocytes. Use of fluorescent reporters improves upon earlier assays that required expensive reagents such as radioactively labeled hypoxanthine (Desjardins *et al.* 1979), monoclonal antibodies (Druilhe *et al.* 2001), or extensive washing to prevent quenching due to hemoglobin (Quashie *et al.* 2006). SYBR Green I in particular minimizes background due to an increase in fluorescence intensity upon intercalation with double-stranded DNA (Bennet *et al.* 2004).

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The assays described here can be tailored to study cytostatic growth inhibition (Basic Protocol 1; Bennet *et al.* 2004), higher-dose cytotoxic parasite kill (Basic Protocol 2; Paguio *et al.* 2011), as well as the effects of drug combinations following the concepts described by Chou and Talalay (Basic Protocol 4; Chou and Talalay, 1984; Suberu *et al.*, 2013). Differences between cytostatic and cytotoxic assays include constant vs. bolus dosing, altered drug concentrations and initial parasitemias, as well as the addition of washing steps. Where necessary, quantification of cytotoxic potency (determination of LD₅₀) can be confirmed using the described limiting dilution assay (Basic Protocol 3), adapted from a recently described assay for determining the rate of cell-killing (Sanz *et al.*, 2012). Adapting any of these assays for drug combination analysis would involve the same general approach but would also require the generation of serial dilutions at multiple fixed drug ratios near the pharmacologically relevant doses for each drug in the combination.

Materials

Plasmodium falciparum culture at 2% hematocrit (volume packed RBC/volume media). If needed, stock parasite culture can be obtained from MR4 [<http://www.mr4.org/>] or other suppliers.

Complete Media (see “Reagents and Solutions”)

Type O⁺ human serum (off-the-clot, heat-inactivated). Many sources for serum and whole blood exist, we obtain our supply from Biochemed (<http://www.biochemed.com/>)

Fresh O⁺ human whole blood, washed to isolate the erythrocytes, and stored in Incomplete Media (see “Reagents and Solutions”).

10% Giemsa Dye (Sigma)

Drug stock solutions (in DMSO, deionized water, or 50% ethanol)

96-well plate, clear well bottom with opaque well sides to prevent fluorescence interference

10,000x SYBR Green I stock from the vendor (Invitrogen), or synthesized (see Bennett *et al.*, 2004)

5% CO₂, 5% O₂, and 90% N₂ gas mix. There are multiple ways to produce balanced gas mixture, in our laboratory custom blend is procured from Roberts Oxygen (<http://www.robertsoxygen.com/>)

Specialized Equipment

Air-tight chamber for storing properly gassed 96 – well plates

37°C Incubator Eppendorf 5804 centrifuge with A-2-DWP rotor

Spectra Max GeminiTM EM Microplate Reader, or similar bottom-read fluorescence plate-reader

Microsoft Excel, Sigma Plot, or other data-analysis program that allows non-linear curve fitting

Basic Protocol 1: Cytostatic (IC₅₀) or Growth-Inhibition Assay- (adapted from Bennet et al. 2004)

This protocol is used to quantify the ability of a compound to slow the proliferation of bulk intraerythrocytic *P. falciparum* culture. Where possible, this should be measured over the course of more than 1 parasite life cycle (more than 48 hours). The compound, in various concentrations, is continuously incubated with low parasitemia culture, and growth is assessed relative to no-drug control growth through the use of a fluorescent DNA-intercalating dye, SYBR Green I. The 50% growth inhibitory concentration (IC₅₀) is determined through non-linear curve fitting of the data. The use of multiwell-plates and a fluorescence plate reader allows for high-throughput screening, and the assay has been successfully adapted to 384 and even 1536-well plate formats.

1. Using a Giemsa smear, calculate *P. falciparum* culture parasitemia and adjust the culture to 4% hematocrit and 1% parasitemia in complete culture media (% parasitemia is the percentage of red blood cells that are infected, hematocrit is the volume percent of packed red blood cells).
2. Prepare drug solutions at 2x the desired target concentrations in complete media. Aliquot 100 μ L of each concentration into each of three wells in a 96-well plate. We recommend 3 wells for each drug concentration such that each assay is done in triplicate, and we recommend 3 independent assays (9 determinations in total) for each drug to compute reliable IC₅₀. Include no-drug controls for each strain of *P. falciparum* that is being analyzed, as shown schematically in Table 1.
3. Add 100 μ L of the 4% hematocrit/1% parasitemia culture to each of the wells, producing a sample at 1x drug concentration and 2% hematocrit/1% parasitemia, mixing well to ensure the RBCs are fully suspended in the media.
4. Place the plate in an air-tight incubator chamber and flush with a 5% CO₂, 5% O₂, and 90% N₂ gaseous mix for 3 minutes to ensure a proper atmosphere. Such an atmosphere is necessary for proper parasite growth, unless the strain has previously been conditioned on reduced oxygen.
5. Incubate at 37°C for 72 hours, flushing the chamber once a day with new gas.
6. Prepare a parasite-standard curve by counting a Giemsa smear visually using a light microscope and setting up small cultures at 2% hematocrit and a range of predetermined parasitemia values and add to plate wells in triplicate (Table 2). Include 0% parasitemia wells (uninfected RBCs in complete media) to account for background fluorescence, as shown in Table 2.
7. Prepare a solution of 50x SYBR Green I in culture media. Add 50 μ L of the SYBR Green I solution to each well (final concentration 10x), mixing well to fully suspend the RBCs, while also protecting from direct light.

8. Place the plate back into the air-tight chamber, gas, and store at 37°C for 1 hour, protecting from light.
9. Measure fluorescence for each well (λ_{ex} 490 nm; λ_{em} 538 nm with 530 nm band-pass and 538 nm long-pass filters) using the Spectra Max Gemini™ EM Microplate Reader, or similar bottom-read fluorescence plate-reader, while also setting the internal temperature to 37°C (note some plate readers require 5–10 min pre-equilibration to correct temperature).
10. Transfer the raw fluorescence data to Microsoft Excel or a similar data analysis program.
11. Calculate fluorescence background by averaging the three values for the RBC-only wells, and subtract this value from values for all other wells on the plate.
12. Generate a standard curve for each parasite strain by plotting the average fluorescence vs. predetermined parasitemia, and fit to a linear equation.
13. Average the fluorescence for each drug concentration sample (and the no-drug control), and use the respective standard curve to convert these values to % parasitemia.
14. Divide the % parasitemia for each drug-treated sample by the final % parasitemia of the no-drug control and plot % growth vs. [drug], using a log scale for drug concentration.
15. These growth curves can be fit to a sigmoidal function, which is then used to calculate the drug concentration that would yield 50% growth. This is the IC₅₀. See (Bennet et al., 2004 for additional details and representative plots).

Basic Protocol 2: Cytocidal (LD₅₀) or Parasite-Kill Assay- (Paguio et al. 2011)

This protocol uses a similar approach Basic Protocol 1 to determine the concentration of a bolus dose of drug that kills 50% of parasites (i.e., the LD₅₀). The duration of the exposure of the *P. falciparum* culture to drug is 6 hr in the protocol below, but can be varied to reflect drug half-life in media or plasma, as well as to probe any parasite stage specificity for the activity of the drug. The parasites are incubated with the compound for less than one life-cycle (typically 6 hours as a starting point, see Paguio *et al.* 2011) and then the drug is washed away. The surviving parasites are allowed to grow through one complete life cycle before staining with the same SYBR Green I dye. Comparison to no-drug control allows for determination of the 50% lethal dose (LD₅₀). The LD₅₀ that is calculated often (but not always) depends on the bolus incubation time and can be parasite stage – specific (Paguio *et al.* 2011). Also, additional control experiments that calculate LD₅₀ at different endpoints should be done for drugs that might potentially induce extended quiescence phenomena along with parasite death, if distinction between these is desired.

1. Using a Giemsa smear, count the *P. falciparum* and set up a culture at 4% hematocrit and 2% parasitemia in culture media

2. As in the cytostatic assay, prepare a series of drug concentrations at 2x the desired target concentration in complete media and aliquot 100 μ L into each of three wells in the 96-well plate, as well as no-drug controls for each strain.
3. Add 100 μ L of the 4% hematocrit/2% parasitemia culture to each of the assigned wells, producing a sample at 1x drug concentration and 2% hematocrit/1% parasitemia, mixing well to ensure the RBCs are suspended in the media.
4. Place the plate in an air-tight incubator chamber and flush with a 5% CO₂, 5% O₂, and 90% N₂ gaseous mix for 3 minutes to ensure a proper atmosphere.
5. Incubate at 37°C for 6 hours.
6. Wash the plate by spinning in an Eppendorf 5804 centrifuge with A-2-DWP rotor at 700g for 3 min, removing the supernatant from each well carefully to avoid disturbing the pellet (using a multichannel pipette), and resuspending in 200 μ L of fresh media. Repeat twice more, for a total of 3 washes.
7. Return the plate to the air-tight chamber, flush with gas, and incubate at 37°C for 48 hours, flushing the chamber with new gas each day.
8. Perform steps 6–15 from the cytostatic assay (Basic Protocol 1). Plot as % survival vs. drug dose to compute LD₅₀. See (Paguio et al., 2011) for representative plots and additional details. Note that for drugs not previously examined (Paguio et al., 2011) control washes should be done to verify that the drug being examined is completely removed in step 6.

Basic Protocol 3: Limiting Serial-Dilution Assay To Determine Efficacy of Parasite Kill. Adapted from (Sanz et al. 2012)

Warning: this assay requires extensive parasite culturing and 30 days to complete. It is more expensive and (much) more tedious than the above two assays.

To rigorously test conclusions from the LD₅₀ assay described in Basic Protocol 2, this assay determines the parasite-kill ability of a compound through a limiting dilution method. Comparison between parasite-free controls and cultures diluted down to (in theory) 1 parasite per well can be used to back-calculate the number of viable parasites that survived a given bolus drug dose. Plotting this survival vs. concentration of bolus drug treatment allows calculation of the LD₅₀. The caveats related to duration of drug exposure mentioned above also apply to this assay.

1. Using a Giemsa smear, count the *P. falciparum* and set up a culture at 4% hematocrit and 2% parasitemia in culture media
2. As in the cytostatic and cytotoxic assays (Basic Protocol 1 and 2), prepare a series of drug concentrations at 2x the desired target concentration in complete media and aliquot 100 μ L into each well in the 96-well plate.
3. Add parasites as in the above two assays, to generate final 2% hematocrit/0.5% parasitemia. Note that parasitemia can be adjusted, but in general a lower initial

parasitemia is used because these plates will develop for a much longer time (30 days) and cultures often die spontaneously (often referred to as “culture crash”) if parasitemia becomes too high.

4. Place the plate in an air-tight incubator chamber and flush with a 5% CO₂, 5% O₂, and 90% N₂ gaseous mix for 3 minutes to ensure a proper atmosphere.
5. Incubate at 37°C for 6 hours.

This exposure time can be varied depending on what concepts are being explored, see Paguio et al. 2011).

6. while the parasites are incubating with the drug, prepare 2% hematocrit RBCs in complete media (40 mL per strain being analyzed).

This large amount of culture will be needed for making serial dilutions for the limiting dilution approach

7. Wash the drug treatment plate by spinning in an Eppendorf 5804 centrifuge with A-2-DWP rotor at 700g for 3 min, removing the supernatant from each well carefully (using a multichannel pipette), and resuspending in 200 µL of culture media. Repeat two more times, for a total of 3 washes.

This step should remove drug; note that some antimalarial drugs (e.g., artemether, artemisinin and artesunate) covalently modify their target(s) and cannot be completely washed away. This adds some level of additional complexity to interpretation of the results.

8. Dilute 40 µL of the drug treated wells into 160 µL of fresh RBCs in complete media, serially, to reduce parasite load per well from 400,000 (starting parasitemia) to 16,000, see Figure 1.
9. Dilute 35 µL of the 16,000 parasite/well culture into 165 µL fresh RBCs in the first column of a new plate, reducing the parasite load per well to approximately 1,400, see Figure 1.
10. Serially dilute this mixture by diluting 100 µL of this mixture into 100 µL of fresh RBCs for the well in the next column, continue for each of the remaining columns. Discard 100 µL from the last (11th) column, and reserve the 12th column for a background measurement (RBCs only; no parasites), see Figure 1.

Each plate is a single assay. In a standard 96-well plate the additional rows can be used for replicates, additional strains, or additional culture treated with different bolus drug dose. Note that to calculate LD₅₀ 6 to 10 different drug doses will be needed. As for the Bennett and Paguio assays, we suggest 3 independent assays, each done in triplicate, for 9 determinations in total. If each drug dose is analyzed in triplicate, the assay requires 4 plates per strain per drug (e.g., 4 plates are required to calculate CQ LD₅₀ for one strain of *P. falciparum*).

Ideally, the number of parasites per well should (in theory) now be approximately as shown in Table 3.

11. Place the plate in an air-tight incubator chamber and flush with a 5% CO₂, 5% O₂, and 90% N₂ gaseous mix for 3 minutes to ensure a proper atmosphere.
12. Incubate at 37°C for 4 weeks, with maintenance (see below), flushing the chamber with balanced gas every day.
13. After 1 week of incubation, make a new suspension of 2% hematocrit fresh RBCs in complete media, and add 100 µL to each well to feed the culture. Put the plate(s) back in the chamber, gas, and return to 37°C incubator.
14. On week 3, centrifuge the plates to pellet the cells, and replace 50 µL of the supernatant with 50 µL of fresh culture media. Put back in the chamber, gas, and return to 37°C incubator.
15. During week 4 develop the plates as described in steps 7–10 of the cytostatic assay (Basic Protocol 1) protocol, but using a 10x SYBR Green I solution instead of 50x.
16. Average the values for each drug concentration at each dilution factor.
17. Count the wells that show fluorescence signal above the RBC-alone background (the 12th column).
18. Back-calculate the number of starting parasites that have survived (P) using the following formula, where X is the dilution factor of the culture (2 if done as described above), and n is the number of wells that showed parasite growth:

$$P = X^{n-1} \quad (1)$$

19. Plot the calculated number of parasites that have survived vs. the drug concentration. It can be helpful to display the concentration axis in log scale.
20. Fit the data to a sigmoidal curve and determine the LD₅₀ by identifying the concentration that produces 50 % effect, relative to complete parasite kill. See (Paguio et al., 2011) for representative curve fits and LD₅₀ calculations.

Basic Protocol 4: Modifying the Cytostatic and Cytocidal Assays for Assessing Synergy of Drug Combinations-Adapted from (Chou and Talalay 1984; Suberu et al. 2013)

In order to investigate potential drug combination therapies, the cytostatic and cytocidal assays (Basic Protocols 1 and 2) can be adapted to determine whether drug interactions are synergistic, additive, or antagonistic. Typically this starts with two different drugs mixed at a defined molar ratio, but multiple drug combinations can also be analyzed in an iterative approach. Using serial dilutions of a fixed ratio of the drugs near their individual pharmacologically relevant doses, a growth or survival curve is generated vs. the concentration of each compound and the fractional inhibitory concentration/fractional lethal dose of each compound is determined. In the “1 point” Chou–Talalay approach. Summation of these FIC values allows for determination of the combination index and designation of the type of interaction.

1. In order to analyze a combination, the individual (monotherapy) activity of each compound must first be determined as described above.
2. Prepare a stock solution of each drug at 16x IC₅₀ (or LD₅₀) in complete culture media. Mix these solutions to generate a fixed volume ratio (each drug now at 8 x effective dose).

The ratio may be expressed in terms of absolute concentration (molar ratio of each drug) or in terms of relative IC₅₀/LD₅₀ (i.e. we often express a ratio that contains IC₅₀ for drug A and IC₅₀ for drug B as a “1:1 IC₅₀” ratio).
3. Serially dilute the mixture two-fold 7 times, to yield a set of 8 concentrations.
4. Add 100 µL of each dilution to a fresh 96-well plate in triplicate; also include no-drug control wells.
5. Add 4% hematocrit culture to the plate (suggested 2% parasitemia for cytocidal, 1% parasitemia for cytostatic), 100 µL in each well, including the no-drug control.
6. Follow steps 4–15 of the cytostatic assay (Basic Protocol 1), or 4–8 of the cytocidal assay (Basic Protocol 2). However, when plotting the growth/survival data, two plots are required; data are plotted versus the absolute concentration of either drug in the mixture, yielding two curves.

The curve fits generate not an IC₅₀ or LD₅₀, but what is often termed the “pseudo IC₅₀” or “pseudo LD₅₀”. Pseudo IC₅₀ and LD₅₀ are specific for the drug ratio that is being examined. Different drug ratios can produce different pseudo IC₅₀ or LD₅₀, revealing different drug interactions (i.e., in theory a drug combination can show synergy at one ratio but antagonism at another). Ideally, ratios that span the drug combination molar ratio that is likely to be present in vivo should be examined.

7. Convert the pseudo IC₅₀/pseudo LD₅₀ into a fractional inhibitory concentration (FIC)/fractional lethal dose (FLD) for each compound using the following equations:

$$FIC = \frac{\text{pseudoIC}_{50}}{\text{monotherapy IC}_{50}} \quad (2)$$

$$FLD = \frac{\text{pseudoLD}_{50}}{\text{monotherapy LD}_{50}} \quad (3)$$

8. Calculate the combination index (CI) by summing the two FIC or FLD values for the two compounds:

$$CI_{AB} = FIC_A + FIC_B \quad (4)$$

$$CI_{AB} = FLD_A + FLD_B \quad (5)$$

9. Use the CI to assign synergy ($CI = 1$), additivity ($1 < CI \leq 2$), or antagonism ($CI > 2$) (Bhattacharya *et al.* 2009; Matthews *et al.* 2013).

Note that the choice of cutoff values for determining additivity vs. antagonism is somewhat arbitrary in the literature. We recommend Chou & Talalay (1994) and references within for additional discussion on these interaction definitions.

Reagents and Solutions

Complete Media- store at 4°C for up to one week

Incomplete Media plus
10% type O⁺ human serum
25 mM HEPES
20 µg/L gentamicin
Adjust pH to 7.4 when at 37°C

Incomplete Media- store at 4°C for up to one month

RPMI 1640 plus
23 mM NaHCO₃
11 mM glucose
0.75 mM hypoxanthine

Commentary

Background Information

The rise of multidrug resistance in *P. falciparum* malaria has renewed interest in the identification of novel candidate drugs and novel drug combination therapies. In order to screen for new drugs, various *in vitro* assays have been developed that utilize radioactive hypoxanthine incorporation (Desjardins *et al.* 1979), ELISA detection of parasite lactate dehydrogenase (Druilhe *et al.* 2001), or various DNA-intercalating dyes which take advantage of the lack of nuclei in red blood cells (RBCs) (e.g. Bennet *et al.* 2004; Quashie *et al.* 2006). In order to simplify these growth-inhibition assays, and to avoid the cost of radioactively labeled compounds, lysing and extensive washing of cells, or dyes that require removal of hemoglobin or the use of UV illumination, 10 years ago we developed an assay that used SYBR Green I added directly to the infected RBCs (no washing steps required), with measurement via a fluorescence-capable well-plate spectrophotometer (Bennet *et al.* 2004). The combination of ease of use and the multiwell plate format allows for rapid and much less expensive screening of multiple compounds and multiple parasite strains.

Recent molecular studies of drug resistance phenomena have generated considerable interest in more rapidly defining the ability of antimalarial compounds to kill *Plasmodium* parasites, not merely inhibit their growth. The classical mode of action of chloroquine that is typically invoked involves inhibiting the crystallization of toxic free heme that is released upon catabolism of host RBC hemoglobin by the intraerythrocytic parasite. However, a number of

recent studies show that this activity does not correlate with the ability of the drug to kill parasites (Gligorijevic *et al.* 2008; Cabrera *et al.* 2009; Paguio *et al.* 2011; Gaviria *et al.* 2013; Gorka *et al.* 2013a; Gorka *et al.* 2013b). In order to fully define the activity of an antimalarial drug, quantification of both cytostatic and cytocidal activity is required, leading to the development of newer assays that probe cytocidal potency (Paguio *et al.* 2011; Hasenkamp *et al.* 2013), or the rate at which a defined concentration of a drug will kill parasites *in vitro* (Sanz *et al.* 2012). Adaptations of the SYBR Green I assay have been used to probe the differences between cytostatic (lower dose, constant long-term drug exposure growth inhibition) and cytocidal (higher dose, short-term exposure parasite kill) activities of common antimalarials and the cellular biochemistry that may be related to the resistance to either activity (Paguio *et al.* 2011; Gaviria *et al.* 2013).

Since the advent of artemisinin combination therapies (ACTs), it has become necessary to more thoroughly investigate drug combinations *in vitro*. Much of the work in developing drug combinations is (quite justly) focused on defining complementary pharmacokinetics and clinical efficacy of the individual drugs, but it is also imperative to understand possible interactions the two compounds might have within the parasite. These can be essential in analyzing potential cross-resistance for drug combinations (Burrows *et al.* 2013). Thorough investigation can also lead to identification of combinations where each compound selects against resistance to the other (Lukens *et al.* 2014). Determination of the type of interaction between potential combinations, (synergistic vs. additive vs. antagonistic), is another key layer to *in vitro* screening. Mathematical derivations performed by Chou and Talalay years ago are equally applicable to antimalarial combination drug screening. These have lead to hybrid techniques summarized here that entail assigning interactions based on fixed-ratio combinations (Chou and Talalay 1984). The abbreviated “1 point” Chou - Talalay Method allows for investigation of possible synergy at a minimum of one combination ratio, as opposed to the generation of a full isobologram, which requires analysis of several ratios (Berenbaum 1978). Importantly, the combination ratios inspected should include those expected within human blood, which can be estimated from individual drug PK/PD curves.

Critical Parameters

In order to obtain a full growth or survival curve, it may be necessary to run a 3- or 4-point titration across several orders of magnitude of drug concentration to determine the approximate drug dilution range for a complete 8-point assay. For reliable quantification, typically the 8-point range will need to span about 3 orders of magnitude in order to report the full parasite response. To account for error intrinsic to the use of parasite cell culture, the assay should be run independently at least 3 times with 3 different culture preparations. The triplicate wells run in a single assay account only for in-assay variations (SYBR Green loading, plate-reader instrumental error, aliquoting of culture into the plate, etc.).

Troubleshooting

To obtain reliable curve fits, every attempt should be made to include as many concentrations as possible that fall between 0% and 100% growth/survival. If the initial 8-point assay does not encompass the full range of activity, these assays should be repeated since the fit may not accurately quantify IC₅₀ or LD₅₀. The largest source of inconsistency

in these assays can be the variable growth of a culture. In order to maximize signal-to-noise in the fluorescence readings, additional no-drug controls can be included on a separate plate and developed with SYBR Green at different times (e.g., 48 hrs vs. 54 hrs). If the dynamic range between the background RBC fluorescence and the control culture fluorescence is not at least 4- or 5-fold (near 10-fold is optimal), it may be necessary to let the plates incubate for up to 24 hours longer to allow for additional growth and development of a higher signal-to-noise ratio. Alternatively, starting parasitemia can be adjusted to fine tune dynamic range. We find it useful to include well-characterized reference strains (e.g., CQS HB3 and CQR Dd2) and a well-characterized reference drug (e.g., CQ) in all our assays as an “internal control” for these and other variables.

Anticipated Results

Typically, the growth or survival curves will show plateaus at 100 and 0%, respectively, with negative slope of the curves spanning 1 – 2 orders of magnitude in drug concentration. However, it is possible for these plateaus to occur slightly above 100%, or to not fully reach 0%. These deviations from theory can be due to culture conditions, or to more complex drug pharmacology/drug interactions than space permits description of here. Reference data for common antimalarial drugs such as chloroquine (CQ), amodiaquine (AQ) and quinine (QN) can be found in (Bennett et al., 2004) and (Paguio et al., 2011).

Time Considerations

Setting up a cytostatic assay should only require about 1–2 hours, depending on the number of compounds and strains tested and the time required to make serial dilutions. Similarly, setting up the Paguio cytotoxic assay will require 2–3 hours, depending on the number of compounds and strains assayed. Following the 6 hour incubation, washing of the plates will require another 1–2 hours. Incubation of the plates to allow for full growth requires about 72 hours for cytostatic and at least 48 hours for cytotoxic assays.

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Figure 1. Plating of the Limiting Dilutions. 400,000 drug-treated (and washed) parasites are diluted to achieve a final concentration of 1 parasite per well utilizing the original plate (Drug Treatment Plate) as well as a second plate (Parasite Dilution Plate).

Table 1

Diagram of a 96 – Well Plate Setup for a Cytostatic Assay vs chloroquine (CQ), for three strains of *P. falciparum*.

	HB3 (CQS)			7G8 (CQR)			Dd2 (CQR)					
	1	2	3	4	5	6	7	8	9	10	11	12
A	1 μ M CQ			1 μ M CQ			10 μ M CQ			No drug Dd2		
B	500 nM CQ			500 nM CQ			5 μ M CQ			No drug HB3		
C	250 nM CQ			250 nM CQ			2.5 μ M CQ			No drug 7G8		
D	100 nM CQ			100 nM CQ			1 μ M CQ					
E	50 nM CQ			50 nM CQ			500 nM CQ					
F	10 nM CQ			10 nM CQ			100 nM CQ					
G	5 nM CQ			5 nM CQ			50 nM CQ					
H	1 nM CQ			1 nM CQ			10 nM CQ					

Table 2

Diagram of a 96 – Well Cytostatic Assay Plate with Standard Curve Cultures.

	HB3 (CQS)						Dd2 (CQR)					
	1	2	3	4	5	6	7	8	9	10	11	12
A	1 μ M CQ			No drug HB3			10 μ M CQ			No drug Dd2		
B	500 nM CQ			1% p HB3			5 μ M CQ			1% p Dd2		
C	250 nM CQ			2% p HB3			2.5 μ M CQ			2% p Dd2		
D	100 nM CQ			4% p HB3			1 μ M CQ			4% p Dd2		
E	50 nM CQ			6% p HB3			500 nM CQ			6% p Dd2		
F	10 nM CQ			8% p HB3			100 nM CQ			8% p Dd2		
G	5 nM CQ			10 % p HB3			50 nM CQ			10 % p Dd2		
H	1 nM CQ			RBC's			10 nM CQ					

Table 3

Hypothetical Number of Parasites in Each Dilution of the Limiting Dilution Assay in the Parasite Dilution Plate.

Column	# of Parasites per well
1	1,400
2	700
3	350
4	175
5	87
6	43
7	21
8	10
9	5
10	2
11	1
12	0