Development of an Alamar Blue™ Viability Assay in 384-Well Format for High Throughput Whole Cell Screening of *Trypanosoma brucei brucei* Bloodstream Form Strain 427

Melissa L. Sykes and Vicky M. Avery*

Eskitis Institute for Cell and Molecular Therapies, Griffith University, Nathan, Queensland, Australia

Abstract. There is an urgent need for new compounds for the drug development pipeline for treatment of patients with African sleeping sickness. One approach for identifying such compounds is by high throughput screening (HTS) of compound collections. For time and cost considerations, there is a need for the development of an assay that uses at least 384-well formats. To our knowledge, there are currently no viability assays for whole cell screening of trypanosomes in the 384-well plate format. We have developed and optimized an Alamar Blue viability assay in a 384-well format for *Trypanosoma brucei brucei* bloodstream form strain 427 (BS427). The assay had a $Z' > 0.5$ and tolerated a final dimethylsulfoxide concentration of 0.42%. Drug sensitivity was compared with those reported from previously developed 96-well methods and was found to be comparable. The sensitivity and cost benefit of the Alamar Blue assay make it an excellent candidate for HTS application.

INTRODUCTION

Human African trypanosomiasis (HAT) is caused by two species of trypanosomes, *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*. The incidence of HAT annually was recently estimated as 17,500 new cases, which occur in some 36 countries in sub-Saharan Africa.¹ *T. b. rhodesiense* is mainly prevalent in eastern and southern Africa and *T. b. gambiense* is predominantly in western and central Africa. The disease has two distinct stages. The first stage has few, if any specific symptoms. The second stage, once the parasites have crossed the blood-brain barrier, causes severe neurologic symptoms and eventually a somnolent state, giving the disease its name. Ultimately, if left untreated, the disease is fatal.

Few advances have been made in the treatment of HAT and most of the current drugs available were discovered before the 1950s.² The combination of drugs used to treat HAT are generally either toxic or have difficult administration regimens attached to them. The second stage of HAT (resulting in neurologic symptoms) is only treatable with the drugs melarsoprol and eflornithine. Melarsoprol is an arsenic derivative that has been associated with up to a 10% mortality rate, and data has been documented to suggest there may be resistance to this drug.³ Eflornithine results in a lower mortality rate,⁴ however, it requires multiple intravenous infusions per day, which involves additional costs. Implementation and compliance in poorly equipped areas where the disease is prevalent is difficult. All of these factors highlight the urgent need for new HAT drugs to be developed.

One approach taken for early drug discovery, instigated for some areas in which this disease has been neglected, has been high throughput screening (HTS) of diverse compound collections for identification of active hits by whole cell screening.⁵ However, in general, there have been few reports based on HTS of whole cell assays for HAT parasites. A luciferase viability assay adapted to a 96-well format has most recently been used for HTS of a small compound library against *Trypanosoma brucei brucei*.⁶ However, this 96-well method is

not suitable for undertaking HTS of larger libraries because of the intensive labor requirements and expense. HTS of known HAT targets, such as trypanothione reductase⁷ in a 384-well format are documented, but there are no reports of 384-well assays against the whole parasite. The development of a whole cell viability assay in a 384-well format would thus be highly valuable for drug discovery.

In this report, we describe an Alamar Blue assay for detection of viability in a 384-well assay format. Alamar Blue is a REDOX indicator that responds to chemical reduction of the growth medium by viable cells by exhibiting a fluorescent signal. Detection of growth by this chemical reduction is caused by metabolism within cells and although the site is not known, it is thought to occur in the cytoplasm or mitochondria of mammalian cells.⁸ It has been postulated that the reduction of Alamar Blue in bloodstream trypanosomes is by the alternative oxidase pathway.⁹ The Alamar Blue assay has been extensively used for the detection of drug susceptibility in whole cell cultures of trypanosomes for a number of years and was first published by Raz and others¹⁰ and also reported in other publications.^{11–13} The Alamar Blue assay has been readily used because of many benefits it has compared with viability assays used previously. The benefits reported are its simplicity, lack of radioactive materials, transferability to the field if necessary, lower cost, and non-toxicity to trypanosomes.¹⁰

To the best of our knowledge this is the first report of a 384-well assay used for HTS of *T. b. brucei* whole cell screening. *T. b. brucei* has been routinely used in screening for initial identification of anti-trypanosomal compounds for potential anti-HAT drugs.⁵ *T. b. brucei* is not infective to humans because it is lysed by a high density lipoprotein in the bloodstream.¹⁴ This species is the cause of trypanomiasis in cattle, which also act as a reservoir for the parasite. Different sensitivities to known drugs have been documented between *T. b. brucei* and the human-infective species *T. b. gambiense* and *T. b. rhodesiense*.^{15–17} However, for identification of leads from HTS, confirmed active compounds are further screened against HAT infective strains to determine selective activity.

The assay we describe was established in accordance with HTS standards by measuring statistical parameters including the Z' factor (Z'), % coefficient of variation (% CV), and signal-to-background assay window to reflect reproducibility. In addition, optimization of the assay incorporated such parameters as cell concentration, compound solvent assay

* Address correspondence to Vicky M. Avery, Eskitis Institute for Cell and Molecular Therapies, Griffith University, Eskitis Building N27, Brisbane Innovation Park, Don Young Road, Nathan, Queensland 4111, Australia. E-mail: v.avery@griffith.edu.au

sensitivity, and compound dilution medium. The Z' factor, a parameter for determining assay performance in the absence of test compounds, should ideally be as close to 1 for the assay to be considered highly reproducible.¹⁸ A negative Z' factor or a value approaching 0 is considered to signify an assay not being reproducible and therefore not amenable to HTS and requiring further optimization. The % CV is the variation of measurements relative to the mean and is a statistical measure used to estimate assay performance.¹⁹ A Z' factor > 0.5 is often interpreted as an indicator of acceptable assay quality.²⁰ For any assay, if CV is > 0.17 (17%), the Z' factor will not reach 0.5.²⁰ Thus, an assay will become less amenable to HTS. A CV value closer to 0 will reflect a more reproducible assay for application to HTS.

The Alamar Blue whole cell assay for estimation of the viability of *T. b. brucei* we describe fully satisfies HTS standards with respect to sensitivity to a known panel of drugs similar to those reported in previously published protocols using 96-well formats.^{11–13}

To investigate the effects on assay parameters such as cell growth, linearity, and sensitivity, the conditions for the incubation step with Alamar Blue were varied to include different incubation temperatures and time periods. The ability to successfully culture trypanosomes in a 384-well format accurately and reproducibly detect growth inhibition is demonstrated. The parameters that influenced these outcomes and the suitability of the resultant assay for HTS are discussed.

MATERIALS AND METHODS

Parasites and *in vitro* culture. *T. b. brucei* BS427 cells were kindly supplied by Dr. Achim Schnauffer (University of Edinburgh, Edinburgh, Scotland), while at the Seattle Biomedical Research Institute (Seattle, WA). The trypanosomes were maintained in log-phase growth in 25-cm² vented tissue culture flasks (Corning, Lowell, MA) by sub-culturing at either 24-hour or 48-hour intervals. Cells were grown in complete HMI-9 medium²¹ supplemented with 10% fetal calf serum (FCS) and 100 IU/mL of penicillin/streptomycin (Invitrogen, Carlsbad, CA). Cells were grown by incubating in a humidified atmosphere of 5% CO₂ at 37°C.

Statistical analysis of samples. For optimization of cell density and reagent usage, eight-well sample sets per condition were used. For initial experiments, these combinations of conditions were analyzed by comparing the signal-to-background ratio (cell concentrations versus addition of the same volume of HMI-9 medium alone) and % CV.

For larger well sample sets, the signal-to-background ratio of control plates (1) and Z' (3) were calculated according to Zang and others,¹⁸ where (1) S/B = mean signal/ mean background, (2) SD = standard deviation, and (3) Z' factor = $1 - [(3 \times SD_A + 3SD_B)/(\text{mean } A - \text{mean } B)]$ where A is the mean end point signal of each assay (cell growth detected in relative volumes and concentrations of *T. b. brucei* in HMI-9 medium in a 384-well plate after growth for 72 hours, then detection); and B is the background signal for HMI-9 incubated for 72 hours with no cell addition, or with addition of 2.3 μM pentamidine to the cells to cause 100% culture death. Sample set sizes were 192 wells each as a minimum.

Final optimized HTS assay conditions. The HTS assay was determined by initial set up and variation of conditions as outlined in the Materials and Methods. For optimized HTS

assay conditions, reagent and cell additions were made with a Multidrop™ liquid handler (Thermo Scientific, Newington, NH) under sterile conditions. Fifty-five microliters of 2,000 cells/mL of *T. b. brucei* in HMI-9 medium were added to a black, clear-bottomed 384-well lidded plate (BD Biosciences, Franklin Lakes, NJ). Cells were incubated for 24-hours at 37°C in an atmosphere of 5% CO₂ before addition of 5 μL of compounds/dimethylsulfoxide (DMSO) for control wells. Compounds suspended in 100% DMSO or 100% DMSO as controls were pre-diluted 1:20 in HMI-9 medium without FCS by using a Minitrack™ robotic liquid handler (PerkinElmer, Waltham, MA). Five microliters of diluted sample was added to the plate to give a final DMSO concentration of 0.417% in the assay. Cells were incubated for an additional 48 hours at 37°C. Ten microliters of 70% Alamar Blue (Biosource, Bethesda, MD) was added to each well (diluted in HMI-9 medium supplemented with 10% FCS) to a final concentration of 10% in the assay. The plate was then incubated for two hours in an incubator under the same conditions, removed from the incubator, and incubated for 22 hours in the dark at room temperature. Wells were read at 535 nm (excitation) and 590 nm (emission) on a Victor II™ Wallac plate reader (PerkinElmer).

Maximum cell density and doubling time of *T. b. brucei* in flask culture. A growth curve was plotted to estimate the maximum number of cells attainable in flask culture before cell death occurred. This growth gave an indication of the potential of the maximum cell numbers that could be used for assay development. An initial cell density of 400 cells/mL was inoculated into a 25-cm² flask (in a total of 10 mL of HMI-9 medium). A 100- μL sample of the culture was taken at 24-hour intervals over a 5-day period. Cell counts were determined by taking three 100- μL samples and combining them with an equal volume of fixing solution (10% formaldehyde). A sample of this was counted on a haemocytometer to estimate cells per milliliter at each interval.

Doubling time was estimated for cells in culture to determine the approximate cell inoculum that would ensure a viable cell count at the time of Alamar Blue reagent addition. Doubling time was calculated by counting cells in the log phase of growth ($1\text{--}3 \times 10^6$ cells/mL) and substituted into the equation $T_d = (t_2 - t_1) \times \log(2)/\log(q_2/q_1)$ where t_d = doubling time. Two measurements were made, the initial count quantity (q_1) at time 0 (t_1) and 24-hours after the initial count (q_2) at time 1 (t_2).

Detection of trypanosomes inoculated from a grown culture in to a 384-well plate. For detection of trypanosomes fresh from 25-cm² flask culture, 55, 45, and 35 μL of trypanosomes at $3, 2$ and 1×10^6 cells/mL concentrations were transferred from a flask to 384-well plates and either 10 μL or 15 μL of Alamar Blue reagent at a final concentration of 10% was added to each volume of trypanosomes. One plate was incubated at 37°C in an atmosphere of 5% CO₂ for 2 hours, followed by incubation at room temperature for 22 hours. The other plate was incubated for 2 hours at 37°C before measuring the signal. The % CV and signal-to-background ratio between well samples was used to determine reagent and trypanosome volumes that could be used for further assay development for optimal growth in 384-well plates. The sample set for each combination were eight wells each, which enabled determination of the best conditions that satisfied the statistical parameters used to define an acceptable HTS assay.

Growth and detection of trypanosomes in a 384-well plate.

For growth and detection of trypanosomes in a 384-well, plates were inoculated at a variety of cell concentrations from 0 to 4,000 trypanosomes/mL in 55 μ L of HMI-9 medium and incubated at 37°C in an atmosphere of 5% CO₂ for 72 hours. Three wells were harvested and counted with a hemocytometer to estimate growth. Alamar Blue was added to each cell concentration (either 10- μ L or 15- μ L additions) for comparison. The signal-to-background ratio and SD of sample wells for each of the assay and trypanosome/reagent volume combinations were calculated to measure assay performance. Each treatment had a total of eight wells.

To estimate if there was any cell death during the 24-hour period of Alamar Blue incubation using the final HTS conditions, three wells were harvested and counted at 48, 72 (time of Alamar Blue addition), and 96 (the end point read time of the assay) hours.

Optimization of assay DMSO concentration. Fifty-five microliters of *T. b. brucei* (2,000 cells/mL) was inoculated into a 384-well plate and incubated for 24-hours. Five microliters of various concentrations of DMSO diluted in HMI-9 (containing FCS) were added to each plate. Plates were incubated for an additional 48 hours and the HTS-optimized protocol was used. One 384-well plate was used to calculate the Z' for each treatment.

Determination of reference compound 50% inhibitory concentration (IC₅₀). Reference compounds used for estimation of drug sensitivity for this assay were pentamidine (Sigma, St. Louis, MO) and diminazene aceturate (Sigma). Pentamidine and diminazene are used in the treatment of disease caused by *T. brucei*. Pentamidine is used to treat persons with HAT and diminazene is a veterinary drug used against *T. b. brucei* to combat infections in cattle.

The IC₅₀ of each reference compound was calculated by plotting % inhibition (100% inhibition = 2.3 μ M pentamidine) against log (reference compound) in PRISM 4 software (GraphPad Software Inc., San Diego, CA) (denoted IC₅₀ by PRISM). The IC₅₀ was the concentration of compound that was estimated to lead to 50% of growth with minimum 0% growth (cells co-incubated with 2.3 μ M pentamidine) and maximum 100% growth (no compound addition). Each concentration dose was screened in triplicate.

Optimization of assay compound dilution medium. Comparisons of HMI-9 medium with and without FCS, water, and phosphate-buffered saline (PBS) were used to determine the most effective dilution medium for compounds that exhibited minimal effect on assay performance. The final optimized HTS assay conditions were used in this experiment varied only in the dilution medium used.

Assessment of cell inocula for linearity in the HTS assay. Based on the assay described earlier in this report, increasing increments of inoculum from 0.0625 $\times$ to 2 $\times$ were used. Replicates of 42 wells for each condition were used. Cells were incubated for 72 hours and 10 μ L of Alamar Blue was then added as per the optimized HTS assay. Cell counts were compared for inocula from pre-incubation and post-incubation with Alamar Blue (2 hours at 37°C in an atmosphere of 5% CO₂ and 22 hours at room temperature). The doubling time of cells from the end of a 72-hour incubation to after incubation with Alamar Blue was calculated.

Assessment of cell inocula for linearity in a variation of the HTS assay. For a variation of the HTS assay described earlier

in this report, increasing increments of inoculum from 0.0625 $\times$ to 2 $\times$ were used. Replicates of 42 wells for each condition were used. Cells were grown at 37°C in an atmosphere of 5% CO₂ for 48 hours and 10 μ L of Alamar Blue was added to each well. After an additional incubation for 24-hours at 37°C in an atmosphere of 5% CO₂, cell numbers were estimated by counting four well replicates. The remainder of the wells were read at 535 nm (excitation) and emission (590 nm).

To determine the maximum number of cells attainable in Alamar Blue under standard growth conditions for 24-hours, cells were added to a flask and adjusted by using the doubling time equation to an inoculum that would result in an estimated 3×10^6 cells/mL under 24-hours of normal growth conditions (in the absence of Alamar Blue). A 10% final concentration of Alamar Blue was added to the flask and three aliquots counted after incubation.

Sensitivity of the HTS assay and variation of the HTS assay to diminazene and pentamidine. The IC₅₀ values of reference compounds diminazene and pentamidine were estimated in the HTS and variation of the HTS Alamar Blue assays under the experimental conditions described in this report. All doses were assayed in three replicates and experiments were repeated twice. The IC₅₀ values of each reference compound were calculated by plotting the % inhibition (100% inhibition = 2.3 μ M pentamidine) against log (reference compound) in PRISM 4 software.

The IC₅₀ values of the reference compounds were also calculated by counting cells at each dose point in each assay format using PRISM4 software. Four wells were counted per dose of reference compound.

RESULTS

Maximum cell density and doubling time of *T. b. brucei* in flask culture. The maximum number of cells that could be grown in a 25-cm² flask was estimated to be 3×10^6 cells/mL (Figure 1). The doubling time in the logarithmic phase of growth ($1-3 \times 10^6$ cells/mL) was estimated to be 6.8 hours in 10 experiments. Using the doubling time, we estimated that 2,000 cells/mL of inoculum would give a final cell count in the flask of 3×10^6 cells/mL at growth for 72 hours, using a mean doubling time of 6.8 hours and assuming a constant growth rate.

Detection of trypanosomes in a 384-well plate inoculated directly from culture. Direct transfer of 55, 45, and 35 μ L of cell

FIGURE 1. Growth curve of *Trypanosoma brucei brucei* in a 25-cm² flask over five days. The inoculum density at day 1 was at 400 cells/mL. Each point was estimated from 3 pooled wells from a 384-well plate.

concentrations of $1\text{--}3 \times 10^6$ cells/mL from a culture to 384-well plates could be detected with various signal-to-background ratios. The variability of measurements were determined as % CV, which was calculated as the SD of the sample/mean of the sample $\times 100$. For all cell concentrations, 55 μL of trypanosomes gave the greatest signal-to-background ratio for each reagent addition. After a 2-hour incubation with Alamar Blue, the best signal-to-background ratio obtained was 2.45, which was achieved with 15 μL of Alamar Blue and an inoculum of 3×10^6 cells/mL. However, the signal window was not greater than $3\times$ the SD of the maximum signal as recommended for HTS.²² No treatment gave an acceptable window and SD of samples for an estimation of reproducibility.

With a 24-hour incubation with Alamar Blue, the signal window was increased for all treatments. For an addition of 3×10^6 cells/mL, the lowest % CV at a 24-hour incubation obtained was with the addition of 55 μL of trypanosomes and 10 μL addition of Alamar Blue (4.46%), which also produced the greatest signal (Figure 2) with a signal window of 7.24. By reducing the parasite inoculum to 2×10^6 cells/mL, the % CV was further reduced to 1.18%. There was still a high signal in comparison to no cells (Figure 2) and a signal window of 5.24. For 55- μL additions over a range of cell densities, the signal from Alamar Blue after a 24-hour incubation at room temperature was linear (Figure 3).

Growth and detection of trypanosomes in a 384-well plate.

Initial studies indicated that $2\text{--}3 \times 10^6$ cells/mL provided the best reproducibility as determined by estimation of % CV and signal:window ratio from direct inoculation from the flask to a 384-well plate. To determine the most suitable inoculum density to obtain optimal parasite growth over a 72-hour period that would not exceed approximately 3×10^6 cells/mL and with the best signal:noise ratio, the cell number used as the inoculum per well for 384-well plates was titrated as follows: 0, 250, 500, 1,000, 1,500, 2,000, 3,000, and 4,000 cells/mL. Growth of the cells after a 72-hour incubation are shown in Figure 4. Approximately 3×10^6 cells/mL was the maximal attainable number using an initial inoculum of 2,000 cells/mL. An increase in inoculum to 3,000 cells/mL resulted in a decrease in cell number, suggesting that the cells were reaching the death stage of a typical growth curve (Figure 4; Figure 1 shows growth curve in flask) at this concentration. The maximum inoculum selected for use for assay development was therefore 2,000 cells/mL.

Detection of Alamar Blue was found to be optimal with 10- μL reagent additions, with a slightly higher signal window

FIGURE 2. Combination of cell concentrations and reagent volumes for signal detection when a 384-well plate was inoculated directly from flask culture and detected by the Alamar Blue assay. All results are for 55- μL additions of *Trypanosoma brucei brucei*. Results for each cell concentration and Alamar Blue volume were averaged over eight replicate wells.

FIGURE 3. Linearity of detection of cell number from inoculation of *Trypanosoma brucei brucei* cells directly from flask and with a 10- μL addition of Alamar Blue. $R^2 = 0.99$. Counts were made at the time of addition of Alamar Blue and were averaged over three wells for each cell density.

across all inocula (Figure 5). The lowest % CV and highest signal window combination with a 10- μL detection volume were observed with 2,000 cells/mL (Figure 5; 2.71% and 10.61%, respectively).

When we took into consideration the % CV and signal window of the Alamar Blue assay, acceptable conditions were obtained using an inoculum of 1,000–4,000 cells/mL (the signal window $>$ than $3\times$ the SD of the maximum signal with a % CV $< 10\%$ as estimated then to have a potential $Z' > 0.5$). However, when we collectively took into consideration the best attainable % CV, signal window, and maximal cell number, the 384-well data obtained suggested the best conditions for an HTS Alamar Blue assay were 55 μL of trypanosomes, with a 2,000 cell/well inoculum, and using 10 μL of detection reagent (Figures 2–5).

When we used an inoculum of 2,000 cells/mL and following the final HTS conditions, we observed a negligible change in cell number between the point of addition of Alamar Blue (72 hours) and the 24-hour incubation with Alamar Blue at room temperature (Figure 6).

Optimization of DMSO concentration. Final DMSO concentrations ranging from 0% to 8.5% were tested in the 384-well assay. Stock 100% DMSO was pre-diluted 1:20 into medium, and 5 μL of this dilution was added to the assay mixture after growth of 2,000 cells/mL for 24-hours in 55 μL of media. Cells were grown for an additional 48 hour before

FIGURE 4. Cell counts of *Trypanosoma brucei brucei* after growth for 72 hours in a 384-well plate with a 55- μL inoculum. Cell counts were made from three pooled wells.

FIGURE 5. Signal window and % coefficient of variation of samples from varying inocula of *Trypanosoma brucei brucei* after growth for 72 hours and detection with the Alamar Blue viability assay. Ten-microliter and 15-µL additions of the Alamar Blue reagent were compared. The signal windows were calculated over eight replicate wells per treatment variation.

detection with Alamar Blue. The incubation before addition was used to ensure that trypanosomes were established before addition of reference drugs in DMSO. Our results show that a final concentration of 0.42% DMSO was well tolerated in the Alamar Blue assay and demonstrated no significant decrease in signal (Figure 7) or visible death of trypanosomes. Increasing the DMSO concentration to 0.83% significantly reduced the Z' to -0.3 , which was unsuitable for HTS. The Z' obtained was 0.71 when using 0.42% DMSO.

Comparisons were also made between manual and automated additions of reagents. No difference in the Z' was observed between the manual and automated dispensing. Thus, use of the Multidrop liquid handling system was implemented.

Reference compound IC_{50} estimation and cell inoculation density. Reference compounds were screened over a dose range of inoculum densities of 2,000, 1,500, 1,000, 500, and 250 cells/mL with the optimized HTS cell concentrations, incubation times, Alamar Blue volume, and maximum DMSO concentration. Estimations of reference compound IC_{50} values were initially undertaken by the serial dilution of compound stocks in 100% DMSO into HMI-9 medium. A 5-µL volume of

FIGURE 6. Growth of *Trypanosoma brucei brucei* cells in a 384-well plate using the optimized Alamar Blue assay format. Alamar Blue was added after growth for 72 hours. The plate was then incubated for 2 hours at 37°C in an atmosphere of 5% CO_2 then for 22 hours at room temperature. There was a negligible difference in the cell number from the Alamar Blue addition to the read point at 96 hours. Cell counts were made from triplicate pooled wells.

FIGURE 7. Dimethylsulfoxide (DMSO) dose and assay signal in the 384-well assay format. The signal at each DMSO dose was averaged from triplicate samples.

these dilutions was added to each well after an initial 24-hour incubation of cells in the absence of the compound. The final DMSO concentration at the highest dose used in the assay was 0.27% for diminazene and 0.08% for pentamidine. The low concentration of DMSO did not affect assay performance. Reference IC_{50} values were compared with those previously published from 96-well methods.^{11–13} In the 384-well Alamar Blue assay, 2,000 cells/mL gave an IC_{50} of 4.4 nM for pentamidine and 65.2 nM for diminazene. A reduction in the cell inoculum resulted in an increase in sensitivity to the compounds. At an inoculum of 250 cells/mL, pentamidine had an IC_{50} of 1.7 nM and diminazene had an IC_{50} of 25.0 nM (Figure 8).

Optimization of compound dilution medium. Reference compounds when diluted in water resulted in a decrease in sensitivity when compared with results obtained after dilution of compounds with medium (Table 1). Dilutions made with medium without FCS and PBS had a negligible effect on the estimated reference IC_{50} values attained using the Alamar Blue 384-well assay format (Table 1). The cell number after growth for 72 hours was slightly less with pre-dilution with water (2.6×10^6 cells/mL compared with 3.0×10^6 cells/mL with medium containing FCS).

Assessment of cell inocula for linearity in the HTS assay. After incubation of cells for 72 hours under standard HTS assay conditions, the cell concentration had reached $3.16 \times 10^6 \pm 2.12 \times 10^4$ cells/mL (Figure 9A). After an additional incubation for 24-hours in the presence of Alamar Blue ($t = 96$ hours), this concentration was at a similar level of $2.92 \times 10^6 \pm 2.8 \times 10^4$ cells/mL. Reducing the standard HTS inoculum by half resulted in a cell count of $2.45 \times 10^6 \pm 3.25 \times 10^4$ cells/mL at 72 hours, with a final cell count of $3.12 \times 10^6 \pm 8.46 \times 10^4$ cells/mL at the 96-hour time point (Figure 9A).

The doubling time of cells during the incubation step with Alamar Blue for 2 hours at 37°C followed by 22 hours at room temperature was estimated initially using 3 concentrations of cell inocula (0.625, 0.125, and 0.25× the HTS inoculum) and found to be 40 hours. Using 0.25× HTS inoculum, which was estimated to produce less than 3×10^6 cells/mL under these conditions, we found that the doubling time of cells incubated in Alamar Blue at 2 hours at 37°C and 22 hours at room temperature was 42.01 ± 6.3 hours. Maximum cell number for this inoculum was therefore not reached at 96 hours at a final concentration of $2.66 \times 10^6 \pm 1.77 \times 10^5$ cells/mL.

FIGURE 8. Diminazene and pentamidine 50% inhibitory concentration estimations from the *Trypanosoma brucei* Alamar Blue viability assay and various concentrations of trypanosome inocula. Percent activity was relative to that of a medium addition to the plate (no cells) versus maximum signal of no compound addition for each cell inoculum dilution. Dilution of compounds were made in HMI-9 medium. Each dilution was made in triplicate over the experiment.

The Alamar Blue signal was determined over a range of cell inocula. It was estimated to be within the linear range for inocula up to $0.5 \times$ the standard HTS cell inoculum at the 96-hour end point of the assay (Figure 9B and C). Using the doubling time equation for $0.5 \times$ the inoculum, 3×10^6 cells/mL density would have been reached at approximately 14 hours of the 24-hours incubation with Alamar Blue.

Assessment of cell inocula for linearity in a variation of the HTS assay. The cell number plateaued from $0.5 \times$ to $2 \times$ the standard HTS cell inoculum at the 72-hour end point of the assay (Figure 9A). This finding was also observed for the Alamar Blue signal in which the signal began to plateau at cell inocula greater than $0.5 \times$ the standard HTS cell inoculum (Figure 9B). It was estimated in the Alamar Blue assay that the signal was linear to $0.5 \times$ that of the HTS inoculum (Figure 9C).

From the cell number plateau, the maximum number attained was approximately 2×10^6 cells/mL. The maximum cell number in Alamar Blue after the 24-hour incubation was determined to be $2.23 \times 10^6 \pm 2.12 \times 10^4$ cells/mL in a flask culture.

Sensitivity of the HTS assay and variation of the HTS assay to diminazene and pentamidine. In an assay incorporating incubation with Alamar Blue under growth conditions for 24-hour post-incubation with compound of interest, the reference compound diminazene had an IC_{50} value of 135.2 ± 8.5 nM. Under standard HTS assay conditions, the IC_{50} value was 64.0 ± 7.1 nM (Figure 10A). There was no significant variation in the IC_{50} values for pentamidine in the two assay formats (ratio = 0.92 ± 0.03 nM). No difference was observed between the two assay formats when comparing the actual cell counts used to estimate the IC_{50} values of the reference compound diminazene (IC_{50} value = 81.5 ± 8.0 nM for the standard HTS format and 93.2 ± 17.9 nM for the modified method (Figure 10B).

TABLE 1

Estimation of reference compound activity with the Alamar Blue 384-well assay using water, HMI-9 medium (with and without FCS), and PBS to pre-dilute compounds*

IC_{50} (nM)	PBS	Water	FCS	No FCS
Pentamidine	40.0 ± 1.3	163.2 ± 3.6	17.0 ± 1.1	29.8 ± 2.3
Diminazene	87.8 ± 6.0	254.9 ± 4.4	72.6 ± 1.2	91.8 ± 2.3

* Values are 50% inhibitory concentrations (IC_{50}) $\pm$ SD. The IC_{50} values were calculated from triplicate doses over an experiment. PBS = phosphate-buffered saline; FCS = fetal calf serum.

At the 72-hour interval of the standard HTS assay format, pentamidine had an IC_{50} of 3.9 ± 0.3 nM and diminazene had an IC_{50} of 84.4 ± 6.7 nM (Figure 11). After an additional incubation of cells in the presence of the respective compounds and Alamar Blue, pentamidine had an IC_{50} of 4.1 ± 0.2 nM and diminazene had an IC_{50} of 110.8 ± 12.2 nM.

DISCUSSION

We report the successful development and optimization of a cell viability assay in a 384-well format for use in HTS of *T. b. brucei* that exhibits many benefits compared with previously published assay methods. Notably, the assay is reproducible, fits statistical parameters, has similar sensitivities to known drugs as described in previously published results for 96-well plate protocols, and is less expensive than currently used 96-well assays. Importantly, the assay is biologically relevant. Statistically, the assay has performed well, accommodating the Z' factor criteria (cutoff = 0.5, but closest to 1 as possible). To our knowledge, this is the first report of a 384-well cell viability assay for *T. b. brucei*.

Assay considerations that have been applied to the assay for HTS include the statistical fit to standards, which reflect reproducibility (taking in to consideration cell number), DMSO sensitivity, and the type of pre-dilution that may be required for compounds before HTS. Selection of the cell concentration used was an important factor in the experiment for assay reproducibility. We report that the concentration of trypanosome inoculum required to obtain conditions that adhered to the HTS criteria was critical. To determine the appropriate cell inoculum to use in this 384-well assay system, we first needed to ascertain the maximum cell number per milliliter. The maximum cell number in culture (Figure 1) and in the plate (Figure 4) was 3×10^6 cells/mL, which is consistent with previous estimations made in HMI-9 medium of other blood-stream species.²³

The doubling time was calculated to be an average of 6.8 hours, and a maximum cell inoculum of approximately 2,000 cells/mL was calculated to give a final cell density of approximately 3.0×10^6 cells/mL after incubation for 72 hour in a total volume of 55 μ L. This cell inoculum was also fortuitously the most applicable in terms of % CV and signal-to-background

FIGURE 9. Cell counts at varying inocula using the high throughput screening (HTS) protocol at (i) $t = 72$ hours growth, (ii) $t = 96$ hr assay end point after addition of Alamar Blue, and a variation of the HTS protocol (iii) with $t = 72$ hours consisting of 48 hours of cell growth, followed by 24-hours of growth in the presence of Alamar Blue at 37°C and in an atmosphere of $5\% \text{ CO}_2$. **A**, From 0.0625–2X the HTS inocula to show the plateau of cell number from inocula for each condition (i–iii). **B**, From 0–1X the HTS inocula as output at Ex535/Em590 of the assay signal to show the plateau of the Alamar Blue signal at 0.5–1X the inoculum in conditions ii and iii. **C**, Linearity from 0 to 0.5X the inoculum in conditions ii and iii.

ratio for the assay (Figure 5). Using these conditions, we also demonstrated IC_{50} values for reference compounds that were comparable with reported values in the literature (Figure 8 and Table 2). Detection of 3×10^6 cells/mL of trypanosomes (with a 72-hour incubation of an initial 2,000 cells/mL inoculum) with Alamar Blue was showed to be within the linear range with an r^2 of 0.99 (Figure 3). Collectively, these factors supported the cell concentration used for the initial inoculum and final cell number detected. This finding has not previously been reported in such detail for an Alamar Blue assay for detection of viability of *T. b. brucei* BS427.

Determining the DMSO tolerability of an assay system that is designed for HTS is critical because compound library collections are principally diluted in DMSO. Tolerability to DMSO has not been widely reported in the literature for *T. b. brucei* Alamar Blue assays. Merschjohann and Steverding¹³ used final DMSO concentrations up to 1% in an assay with no effect reported, whereas we demonstrate that the maximum final DMSO concentration that resulted in greatest reproducibility was 0.42% (Figure 7). The cell density at the time of drug addition in the reported 96-well assay (2×10^3 cells/well) would be similar to that in the 384-well assay (1.3×10^3 cells/well), with an estimated doubling time of 6.8 hours. One factor that may have resulted in different published observations of DMSO sensitivity could be the different medium used in each of the protocols. Differences in culture medium may effect doubling times of the trypanosomes, which could result

in an increase in the sensitivity of the 384-well assay. It is not possible to determine the cause of the difference from these experiments but it does highlight the need to explore such parameters as compound solvent sensitivity when establishing or varying assay methods and conditions.

Because pre-dilution of compounds in DMSO (caused by DMSO sensitivity) was necessary before addition to the assay mixture, we explored potential diluents. During a HTS campaign, it is not uncommon for the screening library to be pre-diluted before beginning HTS. Exclusion of FCS from such a dilution medium may reduce the risk of certain contaminating microorganisms,²⁴ which may originate as there is a brief exposure of plates to the atmosphere while the dilution is being carried out. Fetal calf serum has also been found to influence cellular toxicity because of various components binding to inhibitor compounds.²⁵ Therefore, dilutions and storage of compounds in a medium containing FCS has the potential to influence compound activity. Thus, for these reasons, FCS was not included in the preparation of compound samples for HTS.

The use of water as a diluent had a significant effect on the IC_{50} value of the reference compounds diminazene and pentamidine, reducing sensitivity 2.8-fold and 5.5-fold, respectively, compared with media without FCS (Table 1). There was also a decrease in the cell number observed with the addition of water. This finding most likely was either the result of a slight change in the buffering capacity of the medium or a consequence of an osmotic effect of water on the cells, potentially causing cell lysis. For optimal 384-well assay perfor-

FIGURE 10. 50% inhibitory concentration values for diminazene (i) high throughput screening (HTS) $t = 96$ hours assay with 72 hours of growth followed by 24-hours of growth in the presence of Alamar Blue (2 hours at 37°C in an atmosphere of $5\% \text{ CO}_2$ and 22 hours at room temperature) and a variation of the HTS protocol (ii) with 48 hours of cell growth followed by 24-hours of growth in the presence of Alamar Blue at 37°C in an atmosphere of $5\% \text{ CO}_2$ for 24-hours. **A** is estimated using the assay fluorescent signal and **B** is estimated using cell counts at each compound dilution. Controls were $2.3 \mu\text{M}$ pentamidine for the negative control and 0.42 dimethylsulfoxide for the positive control.

FIGURE 11. 50% inhibitory concentration values for pentamidine (**A**) and diminazene (**B**) estimated using cell counts of reference compound dilutions from the high throughput screening assay (i) at 72 hours of cell growth and (ii) after incubation with Alamar Blue for 2 hours at 37°C in an atmosphere of $5\% \text{ CO}_2$ and 22-hours at room temperature. Controls were $2.3 \mu\text{M}$ pentamidine for the negative control and 0.42 dimethylsulfoxide for the positive control.

TABLE 2

Examples of IC₅₀ estimations with the Alamar Blue 96-well assay formats currently found in the literature for *Trypanosoma brucei brucei* bloodstream forms*

Compound	Reference	IC ₅₀ (nM)	Incubation time (hours)	Inoculum cells/mL (cells/well)
Diminazene	Lanteri and others ¹¹	90	48 (+24 with AB)	N.S. (5 × 10 ⁴)
Diminazene	Merschjohann and Steverding ¹³	500	24 (+24 with AB)	1 × 10 ⁴ (2 × 10 ³)
Diminazene	Stewart and others ¹²	990	70 (+2 with AB)	2 × 10 ³ (200)**
Pentamidine	Lanteri and others ¹¹	10	48 (+24 with AB)	2.6 × 10 ³
Pentamidine	Stewart and others ¹²	40	70 (+2 with AB)	2 × 10 ³ (200**)

* IC₅₀ = 50% inhibitory concentration, ** = authors have referenced Raz and others¹⁰ for protocol, assuming they have used the inoculum for *T. b. rhodesiense*. AB = Alamar Blue; N.S. = not specified.

mance, HMI-9 medium without FCS was the final diluent of choice. However, as shown in Table 1, PBS did not significantly affect the IC₅₀ values and thus could also be suggested as an alternative.

The sensitivity of the 384-well Alamar Blue assay was compared with that of the 96-well assay reported in the literature by evaluating the IC₅₀ values of the reference compounds diminazene and pentamidine. These compounds were selected because they were the most commonly used control compounds in recent studies, which assayed *T. b. brucei* bloodstream forms. The IC₅₀ values of 10 nM–40 nM for pentamidine and 90 nM–990 nM for diminazene were obtained using the final 384-well HTS protocol. These results reflect the published results for the 96-well assay format. Figure 8 shows the IC₅₀ values for diminazene and pentamidine at various cell concentrations. An inoculum of 2,000 cells/mL was used as the cell concentration in the assay because the IC₅₀ values obtained for this concentration of cells were the closest to previously published results (65.2 nM diminazene and 4.4 nM pentamidine; Figure 8). After modification of the assay protocol to take into account sensitivity to DMSO, namely predilution of compounds in 100% DMSO into HMI-9 medium without FCS, the IC₅₀ values obtained were 29.8 nM for pentamidine and 91.8 nM for diminazene (Table 1). Both of these values are within an acceptable range of the published data.

The linearity of the reaction and the resultant assay sensitivity are important considerations. The cell number in the standard HTS assay format described was approximately 3 × 10⁶ cells/mL at the time of addition of Alamar Blue (Figure 6), which was shown to be the approximate maximum attainable cell number in culture (Figure 4). This cell number was also within the linear range of detection for the reagent Alamar Blue (Figure 3). In the HTS assay, after addition of Alamar Blue and incubation of the cells for 2 hours at 37°C, followed by incubation for 22 hours at room temperature, there was a significantly reduced growth rate of the cells, estimated at a doubling time of approximately 42 hours. The corresponding change in the cell number over this 24-hour period resulted in inocula > 0.5× that was not within the log phase of cell growth (Figure 9A). This finding would correspond to a representative 1× HTS inoculum that was not in the log phase of growth at the assay end point (as demonstrated by a plateau in cell number from the 0.5× HTS inoculum; Figure 9B). Similarly, the Alamar Blue signal in the assay was also linear to 0.5× the HTS inoculum.

To determine if the non-linearity of the 1× inoculum at 96 hours in the HTS assay effected assay sensitivity compared with the 72-hour cell growth before addition of Alamar Blue, cell counts were made at these intervals to estimate the IC₅₀ for the reference compounds diminazene and pentamidine (Figure 11). There was no significant difference in pentami-

dine sensitivity and only a 1.3-fold difference in the diminazene IC₅₀ for these intervals.

Separately, to determine if an assay could be performed in which all potential cell concentrations had not reached or just reached the maximum of 3 × 10⁶ cells/mL at the end point of the assay, an alternative assay was investigated. This assay was a variation of the HTS optimized assay. The variation involved primarily altering the incubation of cells in the presence of the Alamar Blue to 24 hr at 37°C in an atmosphere of 5% CO₂, rather than 2 hours at 37°C in an atmosphere of 5% CO₂ followed by incubation for 22 hours at room temperature (and after an incubation time of 48 hours rather than 72 hours of the cells). Incubation with Alamar Blue at 37°C in an atmosphere of 5% CO₂ has been commonly used (Table 2). It has been reported that this incubation has no effect upon the doubling time of *T. b. rhodesiense* and *T. b. gambiense* over a period of several days.¹⁰

However, we found that Alamar Blue in standard growth conditions effects the growth of *T. b. brucei* over a 24-hour period by reducing the doubling time on average to 30 minutes less than that of the doubling time in media alone. The maximum number of cells attainable in Alamar Blue under these growth conditions was 2.23 × 10⁶ cells/mL compared with 3.24 × 10⁶ cells/mL in the absence of Alamar Blue (demonstrated by the plateau of cell number in Figure 9A). Therefore, Alamar Blue under these conditions and concentration (10% in the assay as commonly used in protocols in Table 2) has an inhibitory effect on cell growth and metabolism. This finding resulted in the end point of this assay not being within the estimated linear range of cell growth for cell inocula between the 0.5× and 1× HTS assay inocula (Figure 9A). This finding also corresponded to a non-linearity of the assay over the 0.5× inoculum in the Alamar Blue assay readout (Figure 9B and C).

The two variant incubations with Alamar Blue were compared with determine the assay sensitivities using the reference compounds diminazene and pentamidine. There was no difference observed between the IC₅₀ value of pentamidine, with a ratio (of 24-hours of Alamar Blue incubation in standard growth conditions compared with the HTS assay developed) of 0.92, although a window of activity was observed for diminazene at a ratio of 2.11×, with an IC₅₀ value of 135.2 nM in the variant assay and 64.0 nM in the HTS assay (Figure 10A). To determine if this difference in activity for diminazene was caused by changes in maximum cell counts reached during the 24-hour incubation with Alamar Blue under growth conditions, cell counts were made for each dilution of compound in each assay and the IC₅₀ values of the reference compounds were determined (Figure 10B). This comparative analysis demonstrated that there was no difference in the IC₅₀ values estimated using both assay methods (at a ratio of 0.87 of the variant assay to the HTS assay) or for the same

estimations of the pentamidine IC_{50} . There may have been an interaction with Alamar Blue and diminazene that caused lower IC_{50} values in the variant assay, although this effect was not observed in an Alamar Blue artifact assay, whereby diminazene at the same dose-response concentrations was incubated with converted Alamar Blue with no cells. Another potential explanation is that there may be a reduced metabolic activity of cells incubated under growth conditions in Alamar Blue, which in concert with the mode of diminazene action could cause a reduction of the metabolic effects of diminazene, causing an apparent loss of activity (such metabolic effects would not be accounted for with cell counts). The mode of action of diminazene and pentamidine are not well known, although they are both diamidine structures; the mode of transport for diminazene is by P2 transporters, and the mode of transport for pentamidine is by P2, high-affinity pentamidine transporter, and low-affinity pentamidine transporter.¹¹

To improve the linearity of the standard HTS assay described, the cell number and, consequently, the DMSO concentration would need to be reduced. Because there was no significant difference between the HTS 72-hour and 96-hour assay intervals in terms of sensitivity to the two reference drugs, and assay sensitivity was not increased by incubation with Alamar Blue as a part of a 72-hour end point assay, the reduction of the cell inoculum and potential library concentration as a consequence would not be necessary for improvement of assay performance. Furthermore, the negative effect of Alamar Blue upon cell growth while co-incubating with cells under growth conditions may affect the activity of compounds.

A standardized Alamar Blue 96-well viability assay protocol for *T. b. brucei* cells has not been reported in the literature. Thus, to enable comparison between the 96-well and 384-well assay formats, we summarized protocols from the literature that used comparable methods (Table 2). It is not possible to make a direct comparison of the 96-well and 384-well formats because of different volumes, inocula, incubation times, incubation temperature with Alamar Blue, media, and doubling time of cells. However, estimations can be made to determine the similarities of the assays in terms of initial and final cell densities and how this may impact sensitivity/reproducibility.

In the 384-well HTS assay, the final cell concentration after the 72-hour incubation was approximately 3×10^6 cells per/mL. Cell growth was estimated to be minimal during the final 24-hour period of incubation after addition of Alamar Blue (Figure 6). From the published data for the 96-well assay, the incubation time with Alamar Blue was generally at 37°C in an atmosphere of 5% CO_2 . Raz and others found that Alamar Blue had no effect upon the doubling time of *T. b. rhodesiense* and *T. b. gambiense* over a period of several days at 37°C in an atmosphere of 5% CO_2 .¹⁰ However, we found that incubation with Alamar Blue at 37°C in an atmosphere of 5% CO_2 had an effect upon the doubling time of the cells over a 24-hour period and resulted in a maximum cell number of 2.23×10^6 cells/mL in culture under these conditions. It is not possible to determine final cell numbers from assays in Table 2 using a 24-hour incubation with Alamar Blue, due to unknown cell densities¹¹ and differing media used.¹³ Based on the data in Table 2 and using an estimated doubling time of 6.8 hours, use of the protocol reported by Stewart and others¹¹ would result in an approximated maximal cell number (3×10^6 cells/mL) after a 72-hour incubation, which is equivalent to 3.3×10^5 cells per well in the final assay volume, including Alamar Blue.

The 384-well assay protocol we report results in an approximate final cell density of 2.1×10^5 cells/well in the final assay volume (Figure 6). Final cell numbers are thus comparable. Importantly, the assay reported is within the linear range of detection (Figure 3), and the cell population did not reach a decline phase at detection, (as demonstrated in Figure 6), which could result in loss of sensitivity/reproducibility.

Cell concentrations at the time of addition of reference compound indicates that cell number in the 384-well assay is less (1.3×10^3 estimated cells/well with a constant doubling time of 6.8 hours) than for the 96-well protocol used by Lanteri and others.¹¹ This approximately 40-fold variability in initial concentration did not result in a significant difference in assay sensitivity between the two assay protocols. We found a reduction in the IC_{50} of diminazene was relatively low, at approximately 2.5× with an 8-fold reduction in the 2,000 cell/mL inoculum (Figure 8). This finding may partially account for the negligible difference in sensitivity observed between the 96-well and 384-well formats, however incubation times and density of cells are other important factors to consider which are not directly comparable.

The optimized 384-well HTS assay we report has a Z' of 0.71, which indicates that the assay performance is suitable for HTS. There are no reported Z' estimations for the Alamar Blue assay in an HTS format for *T. b. brucei* whole cells in the literature. The assay detection system has proven reproducible for other cell types; e.g., a Z' of 0.85 was reported for HEPG2 mammalian cells in a 96-well assay format.²⁶

Our results show that the 384-well optimized Alamar Blue HTS assay developed for estimation of *T. b. brucei* whole cell viability is reproducible, with comparable pentamidine and diminazene sensitivities previously reported for Alamar Blue 96-well protocols.^{11–13} A significant benefit of the HTS assay is that incubation with Alamar Blue for 2 hours at 37°C in an atmosphere of 5% CO_2 , followed by 22 hours at room temperature only effects assay linearity at high concentrations, and this does not appear to effect the sensitivity of the assay. Cell numbers do not increase significantly in terms of doublings compared with the Alamar Blue incubation period; this step is mainly for development of the assay signal rather than quantification of the effect of compounds on cells. Because an assay incorporating incubation with Alamar Blue at 37°C in an atmosphere of 5% CO_2 for 24-hours as part of a 72-hour cycle of cell growth hampers cell division, compound activity may be affected. Such an alternative assay format may not be as sensitive as the HTS assay we describe.

The HTS assay is a more cost-effective assay than some of the alternative viability detection methods. For example, an ATP detection assay that has been used in a 96-well format by Mackey and others,⁶ when converted to a 384-well format and assuming a volume of 25 μ L/well as suggested by the manufacturer, would result in a reagent cost per well 3.8× greater than that given in this report. Although the HTS Alamar Blue assay we report has a total incubation time of 96 hours, batches of plates could potentially be run in relatively large numbers, which is based on a standard incubator capacity that would be approximately 80–100 × 384-well plates in a 5-day cycle. The assay also has a 48-hour exposure to potential compounds, which is important when considering the current need to treat patients with drugs for up to 14 days.⁴ A 24-hour incubation would reduce the longer acting potential of some compounds.

The assay described in this report is amenable to automated liquid handler use and fulfills the necessary and desirable criteria for a HTS compatible assay because it is cost-effective, time-effective, simple, robust, and reproducible. It is therefore recommended for future use in HTS applications.

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Authors' address: Melissa L. Sykes and Vicky M. Avery, Eskitis Institute for Cell and Molecular Therapies, Griffith University, Eskitis Building N27, Brisbane Innovation Park, Don Young Road, Nathan, Queensland 4111, Australia, E-mails: m.sykes@griffith.edu.au and v.avery@griffith.edu.au.

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