Chapter 12

Mycobacterium tuberculosis High-Throughput Screening

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Abstract

High-throughput screening is a valuable way to identify hit compounds that combined with a robust medicinal chemistry program could lead to the identification of new antibiotics. Here, we discuss our method for screening large compound libraries with virulent *Mycobacterium tuberculosis*, possibly one of the more difficult bacteria to use because of its slow growth and assignment to Biosafety Level-3 by the CDC and NIH. The principles illuminated here, however, are relevant to the execution of most bacteria high-throughput screens.

Key words *Mycobacterium tuberculosis*, Mtb, H37Rv, Tuberculosis, TB, High-throughput screening, HTS, Antitubercular, Antibacterial, Antimicrobial, Antibiotics

1 Introduction

Notwithstanding recent improvements in either the prevention or treatment of tuberculosis (TB), its resurgence has led to an intensified effort in the development of treatments for *Mycobacterium tuberculosis (*Mtb). The global burden for TBTuberculosis (TB), according to the World Health Organization (WHO) in 2010, accounted for 8.5–9.2 million new cases and 1.2–1.5 million deaths (including deaths from TB among HIV-positive people) [1]. thus making TB the second leading cause of death from an infectious disease worldwide (after HIV, which caused an estimated 1.8 million deaths in 2008) [2]. The renewed interest in this disease has impacted not only the diagnostics and therapeutics areas but also the tools and technologies used to develop therapeutics. Aided by technologies such as high-throughput screening (HTS) and medicinal chemistry, rapid evaluation of potential antimicrobials has increased dramatically. It is especially desirable to identify new types of TB drugs acting on novel drug targets with no cross-resistance to existing drugs or interfering interactions with antiretrovirals.

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The Southern Research High Throughput Screening Center team has screened >1.2 million compounds in single-dose screens and 70,000 compounds in dose-response in an in vitro assay against Mtb H37Rv adapted from the microdilution alamarBlue (AB) broth assay originally reported by Collins and Franzblau [3]. This method has been established as our standard operating procedure for end point determination and enumeration of potential inhibitory compounds against Mtb H37Rv since 2004 [4–6]. Recently, we have improved the assay by using Promega's BacTiter-Glo™ Microbial Cell Viability (BTG) end point reagent.

Laboratory manipulations with Mtb require meticulous attention to detail and all the parameters involved due to the pathogenicity of the organism (*see* **Note 1**) but also due to the sensitivity in growth of the organism. The screening assay as described here is robust and reproduces consistently if variables in the assay are accounted for and controlled. Subheading 3 contains three main parts, i.e., preparation of the components, execution of the Mtb assay, and execution of a counter-screen in mammalian cells. The discussion in Subheading 4 details some of the problems encountered and solutions.

2 Materials (*See* Note 2)

2.1 Media/Broth and Agar Plates (See Note 3)

1. 10× ADC (albumin, dextrose, catalase) (*see* **Note 4**): Weigh out 25 g BSA, 10 g glucose, and 0.015 g catalase. Bring the total volume up to 500 mL with Milli-Q water and dissolve dry components. Filter sterilize through a 0.2 μm 500 mL filter flask. Store at 4 °C up to 1 week.
2. 5 % Tween 80 solution: In a 100 mL glass flask with a stir bar, weigh out 0.5 g Tween 80. Bring total volume to 10 mL with Milli-Q water and dissolve, with heat if necessary.
3. 20 % Glycerol solution: In a 100 mL glass flask with a stir bar, weigh out 2 g of glycerol. Bring total volume to 10 mL with Milli-Q water and dissolve, with heat if necessary.
4. 7H9 Media/broth: Weigh 4.7 g Middlebrook 7H9 broth base into a 2 L glass flask with a stir bar. Add 880 mL of Milli-Q water and stir on a stir plate to dissolve. Add 10 mL of Tween 80 solution and 10 mL of glycerol solution. Autoclave the 7H9 solution for 20 min at 121 °C. The media will appear slightly cloudy at this time but will clear up as it cools. Allow the 7H9 solution to cool until it has reached room temperature.
5. H9 Media/broth: Add 100 mL of the 10× ADC solution (**item 1**) to the 7H9 solution. Filter through a 0.2 μm 1 L filter flask.

6. 7H11 agar plates: In a 2 L flask, dissolve 21 g of 7H11 agar base in 890 mL Milli-Q water. In a 100 mL glass flask, weigh out 2 g of glycerol. Add approximately 10 mL Milli-Q water and dissolve, with heat if necessary. Add back to broth base. Autoclave for 20 min at 121 °C. Allow the 7H11 to cool to 55 °C and add 100 mL Middlebrook OADC (oleic, albumin, dextrose, catalase) and mix. Aliquot 30 mL of 7H11 agar into sterile Petri plates carefully to prevent air bubbles from forming. Let the plates cool to room temperature and allow the plates to remain at room temperature overnight to dry and to check for contamination. Store plates for long term at 4 °C inverted in a sealed plastic bag up to 1 month.
7. Tryptic soy agar (TSA) plates: Weigh 40 g of dehydrated media (TSA) and bring up to 1 L with Milli-Q water. Autoclave at 121 °C for 20 min. Allow to cool to 45–50 °C. Dispense 30 mL into sterile Petri dishes carefully to prevent air bubbles from forming. Let the plates cool to room temperature. Allow the plates to remain at room temperature overnight to dry and to check for contamination. Store plates for long term at 4 °C inverted in a sealed plastic bag for up to 1 month.

2.2 alamarBlue Assay

1. 18.2 % Tween 80 solution: Weigh 18.17 g Tween 80 into a 200 mL glass flask. Dilute the Tween 80 with 100 mL of Milli-Q water. Autoclave at 121 °C for 20 min. 18.2 % Tween 80 may be kept for up to 2 months.

2.3 Mammalian Cell Cytotoxicity Assay

1. Cryopreservation media: 95 % Heat-inactivated FBS and 5 % DMSO.

2.4 Equipment

Where two pieces of equipment are indicated, one is located in the BSL-2 laboratory and one in the BSL-3.

1. Beckman Coulter Biomek FX.
2. Two—Thermo Matrix WellMates, with standard bore cassette.
3. Two—Incubators in which the humidity level can be controlled to 95 % or higher.
4. Two—PerkinElmer EnVision Multilabel Plate Readers.

3 Methods

Carry out all procedures at room temperature unless specified otherwise. In order to maintain sterility, all preparations should be carried out in a biological safety cabinet (BSC).

3.1 Stock Mtb H37Rv Preparation

All work with Mtb H37Rv occurs in a certified biological safety cabinet (BSC) within a BSL-3 laboratory (*see* **Note 5**).

3.1.1 Subculture Growth

1. Inoculate 5 mL of 7H9 media in a screw cap 50 mL conical tube with 0.2 mL of thawed Mtb H37Rv (ATCC 27294) from American Type Culture Collection. Loosely screw conical cap back on and cover cap with parafilm to allow for adequate gas exchange while preventing evaporation during incubation.
2. Incubate the subculture in a ~90 % humidified incubator at 37 °C without CO_2. Gently swirl the culture daily to mix the media.
3. Monitor the growth for turbidity. Take OD_{615} of subculture (pipette 66 μL of culture into Corning 384-well black clear bottom plate and read absorbance on EnVision using 615 nm filter.)
4. After about 7–8 days, the OD_{615} should be approximately 0.2 OD or a #1 on the McFarland turbidity standard.

3.1.2 Preparation of Stock Mtb H37Rv

1. Inoculate 30 mL of 7H9 with 1.5 mL (5 %) of the subculture in a 250 mL vented cap flask. The total volume may be adjusted depending on the amount of the stock desired considering that only 1/2 to 2/3 of the final volume will be collected.
2. Incubate flask in humidified incubator at 37 °C without CO_2. Swirl the culture daily to mix. Monitor the growth for turbidity.
3. Take OD_{615} of the subculture (pipette 66 μL of culture into Corning 384-well black clear bottom plate and read absorbance on EnVision using 615 nm filter).
4. After about 2–3 weeks, the OD_{615} should be approximately 0.6–0.8 (4–8 × 10^7 CFU/mL) or a #3–4 on the McFarland turbidity scale.
5. Streak out the culture from each flask onto a TSA plate to check for bacterial contaminants (*see* **Note 6**) before the cultures are combined.
6. Incubate overnight at 37 °C without CO_2 and check the plates for any growth.
7. If the cultures do not show any sign of contamination based on the TSA plates, combine the cultures and allow them to sit at ambient temperature for an hour.
8. Large clumps of bacteria will settle to the bottom of the flask. Remove the top 1/2 to 2/3 of the culture into a fresh flask or conical tube. Aliquot 1 mL of the culture into 2 mL cryovials and freeze at −80 °C.
9. Streak the pooled culture that remains onto TSA plates and incubate overnight at 37 °C without CO_2 to check for contamination.

3.1.3 Stock Titration

1. After at least 24 h, thaw three of the frozen aliquots. Do not combine aliquots.
2. Create a 1:10 dilution of each aliquot in 7H9 media.
3. Continue 1:2 serial dilution for ten dilutions.
4. Plate 100 μL of each dilution onto the 7H11 agar plates.
5. Place plates in polyethylene plastic bags (2 plates/bag).
6. Secure loosely and incubate inverted in a humidified incubator at 37 °C without CO_2 for ~21 days and monitor for colony growth and contamination.
7. Count the colonies after 21–28 days. Accurate counts can be made in the range of 30–300 colonies per plate.
8. Determine the average CFU/mL of the triplicate aliquots.

3.2 Mtb Microtiter Plate Assay

3.2.1 Media Preparation

1. Prepare 7H9 media at least 24 h prior to compound and Mtb H37Rv addition. (Media may be prepared up to a week in advance and stored at 4 °C.) The amount of 7H9 media needed is ~36 mL per microtiter plate (*see* **Note** 7), i.e., 12 mL for the addition of the bacteria and 24 mL for preparation of the compounds.
2. Place 10 mL of prepared media in a 50 mL conical and incubate in a humidified incubator overnight at 37 °C without CO_2. Check for contamination in the media by turbidity.

3.2.2 Compound Addition to Assay and Control Microtiter Plates

1. Compound addition occurs in a BSL-2 laboratory in a BSC. Barcoded assay plates containing the compounds are prepared the day that the bacteria will be added to the plates.
2. Test compounds which are in 100 % DMSO are diluted with 7H9 media at twice the screening concentration. DMSO is maintained at a constant 2 % concentration. The screening concentration is dependent upon the composition of the compound library and the resources to follow up on hits. For chemically diverse libraries, we have used 10 μg/mL and 10 μM.
3. Control compound stocks are 2.5 mg/mL amikacin and 10 mg/mL ethambutol dissolved in sterile Milli-Q water. Add DMSO to a final concentration of 2 %. Aliquots are frozen at −80 °C for single use (*see* **Note 8**).
4. The Biomek FX is used to add 25 μL of test compound, control compound, or media to each well of the 384-microtiter plate (Fig. 1). It usually takes 4–41/2 h to add compounds and controls to 100 microtiter plates.

3.2.3 Control Plates

1. Each assay run contains one plate of uninoculated medium, two plates of Mtb inoculated medium, and two other plates containing ethambutol or amikacin at their approximate MIC and 20 times the MIC.

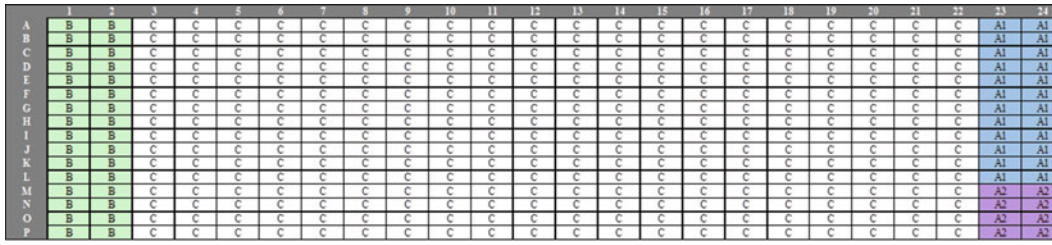

Fig. 1 The layout of the 384-well microtiter plate used in the *Mycobacterium tuberculosis* assay. B contains only media, 1 % DMSO, and Mtb H37Rv and serves as the 100 % growth control. Amikacin is included in the positive control wells in every assay plate at two concentrations. The high concentration, 2.5 μg/mL, completely inhibits Mtb growth and is used instead of uninoculated medium as background (A1). The low concentration of amikacin, 0.13 μg/mL, is the approximate MIC (A2) and is used to monitor assay performance. The test compounds (C) are in the remaining 320 wells

2. Media-only microtiter plate contains media used in the compound addition and plating of the bacteria (uninoculated). This plate is used to ensure media sterility at all stages of the assay. Read the absorbance at 615 nm on the Perkin Elmer EnVision.
3. The growth control/coefficient of variation (CV) plates contain Mtb H37Rv in media plus 1 % DMSO in all 384 wells.
4. The ethambutol control plate is included to confirm that a contaminating organism is not present after incubation since mycobacteria are susceptible to ethambutol but most other organisms are not. Columns 1 and 2 contain 1 % DMSO in 7H9 media and columns 23 and 24 contain Mtb H37Rv in media plus 1 % DMSO. The remaining 320 wells are equally divided between 0.5 and 10 μg/mL ethambutol.
5. The amikacin control plate is included to measure the consistent response of the mycobacterium to a known inhibitor throughout the multiple runs of a screening campaign. Columns 1 and 2 contain 1 % DMSO in 7H9 media and columns 23 and 24 contain Mtb H37Rv in media plus 1 % DMSO. The remaining 320 wells are equally divided between 0.13 and 2.5 μg/mL amikacin in media plus 1 % DMSO.

3.2.4 Mtb H37Rv Addition

1. Transport assay plates into the BSL-3 facility for addition of the mycobacterium.
2. Inside a BSC, thaw frozen stocks of H37Rv, pipette to mix, and inoculate the 7H9 media with a final in well dilution of 1–2 $\times$ 10^5 CFU/mL (*see* **Note 9**).
3. Place the inoculated media on a stir plate and allow the media to mix.
4. Carefully place the sterile WellMate cassette on the WellMate Dispenser. Make sure to keep the tips and tubing ends sterile.

Prime the WellMate by pumping uninoculated 7H9 media through the WellMate cassette and allow to sit in the lines for 1–2 min. Dispense an additional 5–10 mL through the lines.

5. Dispense 50 μL of uninoculated 7H9 media to one blank 384-well plate. This will be the control plate to monitor contamination and a negative end point control.
6. Dispense 25 μL of uninoculated 7H9 media to one empty 384-well plate. Observe any tips that may not be dispensing accurately.
7. Dispense 25 μL of diluted Mtb H37Rv (Subheading 3.2.4, **step 2**) to the CV plate. This will act as your CV plate to check WellMate dispensing for plating the bacteria and for the end point addition at the beginning of the run. Visually check that the WellMate dispensed into the center of each well.
8. Continue to dispense the diluted Mtb H37Rv to every well of the assay plates (test compounds plus ethambutol and amikacin control plates) using the Matrix WellMate.
9. After dispensing to ten plates (*see* **Note 10**), tap the sides of the plates to flatten the meniscus and knock any droplets of media on the sides into the assay wells and transfer the plates to the incubator.
10. Dispense 25 μL of uninoculated 7H9 media to a second empty 384-well plate. Observe any tips that may not be dispensing accurately.
11. Dispense 25 μL of diluted Mtb H37Rv (Subheading 3.2.4, **step 2**) to the CV plate. This will act as your CV plate to check that the WellMate is still dispensing accurately at the end of the run.
12. Incubate plates, stacked 2–3 high, in humidified incubator at 37 °C without CO_2 for 7 days.

It takes a single experienced operator ~1.5 h to execute a 100 plate run.

3.2.5 alamarBlue End Point Addition

1. Calculate the amount of alamarBlue needed for the run (4 mL for 384-well test plates).
2. Combine two parts of alamarBlue to 1.5 parts of 18.2 % Tween 80 (*see* **Note 11**).
3. Set up a new sterile WellMate cassette head on the dispenser and prime it with the alamarBlue/18.2 % Tween 80 solution.
4. Remove the assay plates from the incubator.
5. Starting with one of the Mtb CV plates, add 9 μL of the alamarBlue/18.2 % Tween 80 solution to every well.

6. Visually check the CV plate for dispensing to the center of each well.
7. Continue to add the solution to the assay plates. If all of the dispense steps have gone smoothly, then alamarBlue is added to the second CV plate at the end. If there has been a dispense issue either when the bacteria or end point reagent was added, then the second CV plate is moved to that place in the line. Tap the sides of the plates to flatten the meniscus and knock any droplets on the sides of the wells into the assay wells.
8. Incubate plates (stacked 2–3 high) in humidified incubator for 1 day at 37 °C without CO_2.
9. After 24 h, check that the bacteria control wells have turned pink (*see* **Note 12**).
10. Seal the plates with the aluminum foil seals. Bottom read the plates on the Perkin Elmer EnVision plate reader using the fluorescence setting with a 535 nm excitation and a 590 nm emission. If the bottom read is not an available option, then seal the plates with clear seals and top read (*see* **Note 13**).

3.2.6 Alternate End Point—BacTiter Glo™ End Point Addition (See Note 14)

1. Calculate the amount of BacTiter Glo™ needed for run (12 mL for 384-well plate) (*see* **Note 15**).
2. Equilibrate the two BacTiter Glo™ reagents to room temperature (*see* **Note 16**).
3. Mix the BacTiter reagent bottles as instructed by the manufacturer and combine in a flask. Protect the container from light by wrapping the container with foil.
4. Remove the media only plate and check that there is no turbidity in the media or indication of contamination.
5. Remove one Mtb CV plate from the incubator and allow it to equilibrate to room temperature.
6. Add 25 µL of BacTiter Glo™ to the Mtb CV plate.
7. After 10 min, seal the plate with a clear top seal.
8. Monitor the signal of the CV plate using the Perkin Elmer EnVision on the standard luminescence setting at 0.1-s-integration time. Read the plate every 5 min until the maximum signal is achieved and the signal begins to drop.
9. Based on the time to reach the maximum signal, calculate the timing from the WellMate dispense to the EnVision read to determine the number of plates that may be batched together in one dispense before the plates need to be read to capture the maximum signal.

10. Equilibrate the compound plates to room temperature and dispense 25 μL of BacTiter Glo™ to the assay plates, seal, incubate at room temperature, and read as in **step 8**.

3.2.7 Dose-Response Assay

Hits are picked from the original compound plates and screened in a dose-response assay using a stacked-plate method wherein each compound dilution is inter-plate rather than intra-plate [4]. Compounds screened in dose-response are tested in 10 twofold dilutions.

3.2.8 Preparation of Mammalian Cell Stocks

Using mammalian cells from frozen stocks facilitates the mammalian cell cytotoxicity assay by dissociating compound preparation from cell production.

1. Harvest Vero cells using TrypLE Express.
2. Collect the Vero cells in a 50 mL conical tube and centrifuge at 340*g* for 10 min.
3. Resuspend the cells in 20 mL c-MEM and determine cell concentration.
4. Centrifuge the cells at 340*g* for 10 min.
5. Resuspend the cells in cryopreservation media at 1.2×10^7 cells/mL.
6. Aliquot 1 mL of cells into the 2 mL cryovials.
7. Rate freeze the cells at 1 °C/min and then transfer to liquid N_2 for long-term storage.

3.2.9 Mammalian Cell Cytotoxicity Assay (See Note 17)

1. Hit compounds from the single-dose Mtb screen are screened using a stacked-plate method using the same 10 twofold dilutions as the Mtb assay.
2. 5 μL of compounds in c-MEM + 1.25 % DMSO are added at 5× concentration to columns 3–22 of each Corning 384-well assay plate.
3. Columns 1 and 2 contain 5 μL of c-MEM + 1.25 % DMSO and columns 23 and 24 contain 5 μL of 500 μM hyamine in c-MEM + 1.25 % DMSO.
4. Determine the amount of media (c-MEM) required for the assay assuming 36 mL per plate.
5. To the required volume of MEM add heat-inactivated FBS (final concentration 10 %) and P/S (final concentration 1 %). Mix.
6. Thaw the required number of aliquots of Vero cells rapidly in warm water.
7. Wash the cells by diluting in c-MEM and centrifuge at 340*g* for 10 min. Remove the supernatant.

8. Resuspend the cells in c-MEM and count using Trypan blue exclusion to determine cell viability.
9. Adjust to 125,000 cells/mL with c-MEM in a flask.
10. Stir on a stir plate for 5 min.
11. Prime the Wellmate with c-MEM + 1.25 % DMSO.
12. Plate 5 μL/well into two CV plates. Carefully place the cassette lines into the stirring cells. Prime the lines with cells and plate 20 μL/well into all columns of the CV plates. Ensure that there are no dispense problems. Continue dispensing 20 μL of cells to the assay plates containing 5 μL of compound.
13. Incubate at 37 °C and 5 % CO_2 with ~90 % humidity for 72 h.
14. Calculate the amount of CellTiter Glo™ needed for the run (12 mL for one 384-well plate).
15. Equilibrate the two CellTiter Glo™ reagents to room temperature.
16. Follow the manufacturer's instructions for mixing the CellTiter Glo™ reagents and combine into a flask (this method follows the manufacturer's instructions using a 1:1 end point addition). Protect the container from light by wrapping the container with foil.
17. Bring the CV plates to room temperature.
18. Add 25 μL of CellTiter Glo to the plates.
19. After 10 min, read the plates on the Perkin Elmer EnVision using the standard luminescence setting at 0.1-s integration time.
20. Ensure that there is not a dispense issue with the cassette head. If there is not, proceed with the compound assay plates.
21. Equilibrate the assay plates to room temperature.
22. Dispense 25 μL of CellTiter Glo to 10 of the assay plates.
23. Incubate at room temperature for 10 min.
24. Read on the Perkin Elmer EnVision as above.
25. Continue the process in groups of ten plates.

3.3 Data Analysis

1. Data from the microtiter plates is exported outside of the BSL-3 from the Perkin Elmer EnVision as individual .csv files named by the plate barcode. The data files are imported into IDBS Activity Base for data analysis and association with compound ID.
2. Results for each well are expressed as percent inhibition (% Inhibition) calculated as: 100 × ((Median Cell Ctrl-High Dose Ctrl Drug) − (Test well-High Dose Ctrl Drug))/(Median Cell Ctrl-High Dose Ctrl Drug).

3. Thirty-two control wells containing Mtb only and 24 wells containing Mtb and 2.5 μg/mL amikacin are included on each assay plate and are used to calculate Z' value [7] for each plate.
4. Any compound with an inhibition of ≥90 % is defined as a hit in the single-dose screen.
5. The dose-response data is analyzed using a four-parameter logistic fit (Excel Fit equation 205) with the maximum and minimum locked at 100 and 0. From these curves, Mtb IC_{90} and IC_{50} values can be calculated and CC_{50} values for Vero cell cytotoxicity.

During the course of a screening campaign, the median values for the Mtb control wells, 2.5 μg/mL amikacin, 0.13 μg/mL amikacin, and Z' value are graphed for each individual plate. This information on plate-to-plate and batch-to-batch variability is used to identify problems in the assay.

4 Notes

1. Mtb has been classified as a biosafety level 3 (BSL-3) agent by the Centers for Disease Control (CDC) and should be handled accordingly. We recently published a paper on the general safety procedures we use for HTS in containment [8]. Although the CDC has established some general rules for the handling of BSL-3 agents, each institution's biosafety committee will establish its own rules under these guidelines for handling a specific agent in its BSL-3 facility. Consequently, we have not provided details related to the regulatory and safety handling of this pathogen and refer the reader to this publication for general details and their institutional biosafety committee for detailed guidance.

   For efficiency, all work such as preparing the end point reagents that does not involve Mtb is performed at BSL2.
2. Table 1 contains information on the sourcing of materials that we have used successfully in HTS campaigns since, in our experience, the source for all of these components may be critical for a reproducible and robust HTS. If other sources are used they should be evaluated for their effect on the growth of H37Rv and their effect on the efficacy of know Mtb inhibitors. In either case, reagents should be purchased from one lot in sufficient quantity to execute the entire HTS campaign.
3. Other media/broths can be used to grow mycobacteria. Another common broth is 7H12 which is 7H9 broth supplemented with 0.1 % casitone, 5.6 μg/mL palmitate, 0.5 % bovine serum albumin, and 4 μg/mL catalase. These media

Table 1
Sources for materials used in successful HTS campaigns

Product	Manufacturer	Catalog number
7H11 agar base	Difco	283810
alamarBlue	Life Technologies	DAL1100
Amikacin sulfate	Sigma-Aldrich	A1774
BacTiter Glo	Promega	G8233
Bovine serum albumin	Sigma-Aldrich	A7638
CellTiter Glo	Promega	G7573
DMSO	Sigma-Aldrich	8418
Eagle's minimal essential media (MEM)	American Type Culture Collection (ATCC)	30-2003
Ethambutol dihydrochloride	Sigma-Aldrich	E4630
Fetal bovine serum (FBS)	Life Technologies	16140-063
Glucose	Sigma-Aldrich	G7528
Glycerol	Fisher Scientific	BP229-4
Middlebrook 7H9 broth base	Difco	271310
Middlebrook OADC	Fisher Scientific	211886
Mycobacterium tuberculosis H37Rv	ATCC	27294
Penicillin/streptomycin	Life Technologies	15140-122
Sodium chloride	Fisher Scientific	S271
TrypleE Express	Life Technologies	12604013
Tryptic soy agar (TSA)	Sigma-Aldrich	22091
Tween 80	Sigma-Aldrich	P1754
Vero cells	ATCC	CCL-81

formulations along with others were empirically determined to be best suited to growing mycobacterium in vitro many decades before current drug discovery initiatives began [9, 10]. Unfortunately, their composition can have a devastating effect on the drug discovery process [11].

4. Alternatively, Middlebrook 10 % ADC enrichment can be purchased form Becton Dickinson. The quality of the bacteria stock and the assay both rely on consistency in the media and the components that make up the media. The 10× ADC solution is a critical component in the media and dramatic variation in assay performance may be attributed to this component. There are differences from lot to lot in commercially purchased ADC, so our preference was to control this up front by

generating our own. In this reagent, the BSA has proven to be the most critical feature. Begin by testing a lot of BSA prior to purchasing and upon confirmation of consistent data then purchase sufficient amount of that lot of BSA for all future studies. The 10× ADC and media may be made up to a week in advance but fluctuations in performance have been noted after storage for a week. Additionally, it should be noted that Tween 80 will produce inconsistent results as the component ages. Therefore, replace the Tween 80 if a colorimetric change from pale yellow to dark yellow/orange is detected. Autoclaving the various media solutions in a container at least two times the volume of the media is also recommended not only to ensure that media is not lost to "boiling over" but also to ensure that media reaches a high enough temperature throughout to ensure a sterile culture.

5. The quality of the stock of Mtb is critical to ensuring reproducible and consistent results during screening. Again media preparation is key to this process. Due to the length of time required to generate the bulk culture, incubation in an upright humidified incubator is preferential to a water bath shaker due to the increased potential for contamination from a heated water bath. Furthermore, the propensity for Mtb to aggregate increases even with slow rotation in a shaker. Setting up the cultures in vented flasks substantially larger than the volume of the bulk culture allows adequate aeration and gently swirling the culture daily allows for adequate mixing of the media. Once the stock culture has been created and frozen, randomly selected vials are used to test for sterility and to calculate the stock Mtb concentration.
6. Mtb grows poorly on TSA which supports the growth of most potential contaminating microorganisms.
7. An experienced operator can easily run 100 plates in one run. However, we suggest that smaller batches, i.e., 50 plate batches, should be run until everyone is comfortable with work in the BSL-3.
8. These stocks are not stable for extended lengths of time. New stocks should be made periodically.
9. Due to the length of time for Mtb culturing and potential variability from one culture to the next, frozen stocks are used in the screen. This decreases the potential for any variants in batches of a large screening campaign which may not be evident until the end of the screen. The total 384-well assay volume for each well is 50 μL which provides adequate media to sustain the culture. The volume also decreases the edge effect from dehydration of the outer wells over the 7–8-day incubation period.

10. Handling the plates in batches of ten is somewhat arbitrary and depends on whether there is a single operator or additional people to move plates from the hood to the incubator. Groups of plates are moved into the incubator as the run proceeds in order to make space in the BSC. Media and bacteria are at room temperature but are not affected the same way as mammalian cells by exposure to room temperature and no added CO_2.
11. The 18.2 % Tween 80 solution and alamarBlue, 1.5:2 solution, are critical. As previously noted, fluctuations may occur due to the Tween 80 over time and alter the permeability of the bacteria to the alamarBlue.
12. The conversion from blue to pink may fluctuate depending on the length of time the plates are outside of the incubator during the addition of the alamarBlue mixture.
13. The Perkin Elmer EnVision will read fluorescence intensity, produced from alamarBlue, either from the top or bottom of the plate. However, the signal is more robust if the plates are sealed with an aluminum foil seal and read from the bottom.
14. BacTiter Glo™ end point reagent provides a simple mix, plate, and read luminescence end point. This reagent has several advantages over the alternative alamarBlue end point. The first is a shortened assay length, 7 days versus 8 days. The second is the decreased number of steps that the plates are handled by the screener. This decreases the potential to introduce contamination into an already lengthy assay and increases the safety of the procedure for the screener. Table 2 contains a comparison of EC_{50}, EC_{90}, and MIC values of known inhibitors of Mtb.

Table 2
Comparison of EC_{50}, EC_{90}, and MIC values of know inhibitors of TB

	AlamarBlue (AB)			Bac Titer-Glo (BTG)		
Compound	EC_{50} (μg/mL)	EC_{90} (μg/mL)	MIC (μg/mL)	EC_{50} (μg/mL)	EC_{90} (μg/mL)	MIC (μg/mL)
Rifampicin	0.02	0.02	0.16	0.02	0.03	0.04
Pyrimethamine	25.09	28.00	100.00	24.27	46.37	100.00
Isoniazid	0.19	>5.00	NA	0.13	0.20	0.31
Ethambutol	3.45	>200.00	NA	1.50	1.64	6.25
Cycloserine	24.76	28.01	100.00	23.55	26.38	100.00
Amikacin	0.12	0.14	0.16	0.07	0.12	0.16

15. This method will add 25 μL/well of BacTiter Glo™ across the plate. This is a modification from the manufacturer's instructions to accommodate the allowable volume per well in a 384-well plate.
16. It is important for this reagent that both the plates and the BacTiter Glo™ reagent are at room temperature to decrease temperature-based fluctuations in the data.
17. Normally, hits from the antimicrobial HTS are also evaluated in a mammalian cell cytotoxicity screen to ensure that the compounds are not cytotoxic to all cells. Historically, the cell line (Vero) most frequently used has been a kidney epithelial cell line derived from an African green monkey. We have also used THP and HepG-2 cells which may be more physiologically relevant.

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