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# Rapid Screening of Inhibitors of *Mycobacterium tuberculosis* Growth Using Tetrazolium Salts

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## Abstract

With the increased need for novel antimicrobials to improve the existing treatment for tuberculosis, to combat multidrug-resistant tuberculosis, and to address the presence of latent bacilli in a large population throughout the world, which can reactivate and cause active disease, there is a need for rapid, low-cost, high-throughput assays for screening new drug candidates. A micro-plate-based Alamar blue assay meets these requirements. In addition to the identification of the antimicrobial activities of compounds, determination of their toxicities is important. The high costs involved in testing compounds in whole animal models has led to the development of *in vitro* cytotoxicity assays using human and animal cell lines. Microplate-based Alamar blue and cytotoxicity assays have been applied to search for novel antimicrobials to treat tuberculosis. These methods are described in detail herein.

### Keywords

Alamar blue; cytotoxicity; MIC; *Mycobacterium tuberculosis*; tetrazolium

## 12.1 Introduction

Tuberculosis (TB) is the major cause of death due to an infectious agent worldwide, and treatment of this ancient disease now faces new challenges because of the increase in multidrug-resistant tuberculosis (MDR-TB) [1]. Most of the TB treatment drugs, as we know them today, were developed some decades ago. The current treatment for the disease spans a period of 6 to 8 months with a combination four-drug regimen of isoniazid (INH), rifampin (RIF), ethambutol (EMB), and pyrazinamide (PZA), which when followed as recommended is highly effective. However, because of unpleasant side effects of the treatment, many patients find it difficult to continue treatment for such a prolonged period of time. This results in treatment failure and development of drug resistance. The second-line drugs used to treat patients who do not respond to treatment with first-line drugs are less effective and very expensive [2]. In addition, treatment of the disease is complicated by the presence of latent, nonreplicating bacilli harbored by a large portion of the population throughout the world. These bacilli have the potential to reactivate and cause active disease [3, 4], but current TB therapy is mainly effective against replicating and metabolically active

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bacteria [5, 6]. Therefore, to improve the existing treatment for TB, there is a need to discover novel drugs that would attack the disease-causing bacilli in new ways, overcoming the increasing problems of MDR strains, duration of treatment, and latent infections. According to the guidelines set by Global Alliance for TB drug development [www.tballiance.org (Scientific Blueprint)], selection of a lead compound generally starts with an initial evaluation of its preliminary characteristics. These include *in vitro* determination of the minimum inhibitory concentration (MIC) against *Mycobacterium tuberculosis* and assessment of toxicity using a eukaryotic cell line followed by evaluation of bioavailability and efficacy in animal models. Substantial progress has been made in the past decade in understanding the molecular basis of drug resistance in *M. tuberculosis* [7], but understanding the mechanisms of action of antimicrobial agents is important in designing novel antibiotics that are active against the resistant strains. Many factors are involved in understanding the mode of action of antimicrobial agents, but the first and foremost is the susceptibility of microorganisms to these agents [8].

### 12.1.1 Minimum Inhibitory Concentration

MICs are considered the gold standard for determining the susceptibility of organisms to antimicrobials and are usually defined as the lowest concentrations of antimicrobial agents that inhibit more than 99% of bacterial growth. MICs are often used to confirm susceptibility or resistance to drugs but can also be used as a research tool to determine the *in vitro* activity of new antimicrobials. For the purpose of this chapter, MIC is defined as the lowest concentration of the antimicrobial that inhibits the visible growth of a microorganism using a microplate-based Alamar blue assay (MABA) system.

Drug-susceptibility testing or MIC determination using solid culture systems such as Lowenstein-Jensen (LJ) medium or Middlebrook agar take about 3 weeks to get results [9, 10]. Various alternative methods have been developed that have dramatically reduced the time required for susceptibility testing from weeks to days [11, 12, 13]. However, two methods of choice for determination of MIC using *M. tuberculosis* have emerged: (1) the radiometric BACTEC 460 TB method using BACTEC 12B vials [14, 15] and (2) the colorimetric microtiter plate–based method using Alamar blue [16, 17]. Both methods can be used to evaluate new compounds reducing the time to complete a test. The BACTEC 460 system is a radiometric assay used to determine susceptibility to an antimicrobial very rapidly and has long been the system of choice, but it is less useful for high-throughput screening because of the high cost and the generation of radioactive waste. The MABA system is a simple, rapid, low-cost, high-throughput system that does not require expensive instrumentation as the growth of bacteria can be measured by a visual color change [16, 17], and this is the method described in this protocol.

### 12.1.2 Bacterial Growth Inhibition Assay

MABA is used for measuring cell proliferation and viability by monitoring the oxidation-reduction state of the environment of cellular growth. As with the tetrazolium salts, Alamar blue is a soluble redox dye that is stable in culture medium and nontoxic [18]. The oxidized dye is blue and nonfluorescent; upon reduction it turns pink and fluorescent, therefore, growth can be determined by a visual color change or by using a fluorometer. Alamar blue

has been successfully used to assess the susceptibility of *M. tuberculosis* to various antimicrobials in several laboratories [16, 17, 19].

### 12.1.3 Toxicology

The design of new therapeutic regimens relies on preclinical data to choose promising drugs and dosage schedules to be evaluated further in clinical trials. The identification of compounds with potential toxic activity is an important aspect in the testing of new antimicrobials. The relatively high costs, low throughput, and animal distress involved in testing compounds in whole animal models has led to the development of *in vitro* assays making use of human and animal cell lines that can be used for the cytotoxicity determination assays.

### 12.1.4 Eukaryotic Cytotoxicity Assay

Cytotoxicity may be defined simply as the cell-killing property of a chemical compound and is independent of the mechanism of death. Most cytotoxicity assays measure the amount of cell death that occurs in culture. When cell membranes are compromised, they become porous and allow macromolecules to leak out; these molecules can then be quantitated and used to estimate viability. For example, a typical assay might measure the presence of intracellular enzymes, such as lactate dehydrogenase in the culture supernatant [20]. The reduction of tetrazolium salts is now widely used as a reliable way to determine cytotoxicity. The yellow tetrazolium compound ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 H-tetrazolium, inner salt; MTS) and an electron coupling reagent (phenazine ethosulfate; PES), with an enhanced chemical stability, combined with tetrazolium salt makes a stable solution. The tetrazolium compound is reduced by metabolically active cells to a purple formazan product, in part by the action of dehydrogenase enzymes (which generate reducing equivalents such as NADH and NADPH) [21, 22]. The quantity of formazan is measured by a 96-well plate reader at 490 nm absorbance and is directly proportional to the number of viable cells in the culture medium [23].

Two commonly used cell lines for testing cytotoxicity are African green monkey kidney cells (Vero) and human hepatoma (HepG2) cells. Vero cells do not generally modify compounds that are added to them [24], whereas HepG2 cells are capable of modifying compounds [25, 26]. Both cell lines are grown as monolayers, and in order to keep the cell cultures healthy and actively growing, it is necessary to subculture them at regular intervals. The most common method of subcultivation is the use of proteolytic enzymes such as trypsin or collagenase to break the cell to substrate and intercellular connections. After the proteolytic disassociation of cells into a single cell suspension, the cells are diluted with fresh media and transferred into new culture flasks. The cells attach to the surface of the flask and begin to grow and divide and reach near confluency when they are again ready to be subcultured or used for testing purposes.

In this chapter, we describe a rapid *in vitro* method using the MABA to determine MIC values for novel mycobacterial growth inhibitors. In addition, we describe the use of spectrophotometric method for determining the cytotoxic effects of these growth inhibitors.

We also describe how to maintain the eukaryotic cell line and determine the concentration of the test compound causing 50% loss of cell viability (also referred to as $IC_{50}$).

## 12.2 Materials

### 12.2.1 Inhibition of Mycobacterial Growth

#### 12.2.1.1 Preparation of *Mycobacterium tuberculosis* Working Stocks

1. *M. tuberculosis* $H_{37}$Rv reference strain ATCC 25618 (American Type Culture Collection).
2. Middlebrook OADC (oleic acid, albumin, dextrose, catalase) enrichment: 8.5 g/L sodium chloride, 50.0 g/L bovine albumin (fraction V), 0.04 g/L catalase, 0.3 mL/L oleic acid, and 20.0 g/L dextrose (Sigma) in deionized water. Filter sterilize (*see* **Note 1**).
3. Tween-80 (Sigma): Prepare a 20% (w/v) stock. Filter sterilize through 0.2-μm membrane and store at 4°C.
4. Middlebrook 7 H9 broth (Difco): Dissolve in deionized water at 4.7 g per 900 mL, add 0.2% (v/v) glycerol and autoclave. Supplement with 10% (v/v) OADC and 0.05% (v/v) Tween 80 (*see* **Note 2**).
5. Middlebrook 7 H11 agar (Difco): Dissolve 21.0 g in 900 mL deionized water, add 0.2% (v/v) glycerol, and autoclave. Supplement with 10% (v/v) OADC. Pour 25 mL in each of 100 × 15 mm Petri dishes.
6. Tryptic soy agar (Difco) (*see* **Note 3**).
7. 16 × 150 mm glass culture tubes with rubber-lined screw caps (Kimble).
8. 25 × 8 mm disposable stir bars (VWR International).
9. 1 μL sterile disposable inoculating loops (Nunc).
10. Lowenstein=Jensen medium (LJ) slants (BD Diagnostic).
11. 250 mL and 1000 mL polycarbonate Erlenmeyer flasks with polypropylene screw caps (Nalgene).
12. Polypropylene 2.0 mL cryogenic vials (Corning).

#### 12.2.1.2 Alamar Blue Susceptibility Assay

1. 96-well round-bottom tissue culture plates with low evaporation lid (Becton Dickinson).

[1]Gently warm the oleic acid solution until it melts, do not microwave or heat above 55°C. Albumin should be added gradually to avoid clumping. Store OADC at 4°C, protected from light. The OADC supplement is extremely heat-labile and should not be added to hot media.

[2]Mycobacteria, particularly *M. tuberculosis*, have a tendency to clump in culture because of the presence of a thick waxy outer cell wall. The addition of a non-ionic detergent to the media reduces the amount of clumping and provides a more homogenous suspension of cells.

[3]Tryptic soy agar is used as an initial growth medium to observe colony morphology and develop a pure culture. If the culture has contaminating bacteria in it, they will become apparent in 24 to 48 h.

2. Sterile deionized water.
3. Middlebrook 7 H9 broth (Difco): Dissolve in deionized water at 4.7 g per 900 mL, add 0.2% (v/v) glycerol, and autoclave. Supplement with 10% (v/v) OADC and 0.05% (v/v) Tween 80 (*see* **Note 2**).
4. Middlebrook OADC (oleic acid, albumin, dextrose, catalase) enrichment: 8.5 g/L sodium chloride, 50.0 g/L bovine albumin (fraction V), 0.04 g/L catalase, 0.3 mL/L oleic acid, and 20.0 g/L dextrose (Sigma) in deionized water. Filter sterilize (*see* **Note 1**).
5. Stock cultures containing $2 \times 10^7$ CFU/mL of **M. tuberculosis** $H_{37}Rv$ strain. Store frozen at −80°C.
6. Dimethyl sulfoxide (DMSO) (Sigma).
7. 100% ethanol.
8. Test compounds: Prepare as 20 mg/mL stocks in appropriate solvents such as deionized water, DMSO, or ethanol, depending on the solubility of the compound.
9. Isoniazid (INH) (Sigma): 10 mg/mL stock solution in deionized sterile water. Dilute to 25 μg/mL for the working stock.
10. Parafilm.
11. 10X Alamar blue dye solution (Biosource International). Use at 1X final concentration.

### 12.2.2 Growth of Eukaryotic Cells and Toxicity Test

#### 12.2.2.1 Maintenance of Eukaryotic Cell Lines

1. African green monkey kidney cell line (Vero) ATCC CCL-81 (American Type Culture Collection).
2. Human hepatocellular liver carcinoma cell line (HepG2) ATCC HB-8065 (American Type Culture Collection).
3. RPMI 1640 medium with L-glutamine (Invitrogen) (*see* **Note 4**).
4. RPMI complete medium. Supplement RPMI 1640 with 1.5 g/L sodium bicarbonate (Sigma), 10 mL/L 100 mM sodium pyruvate (Mediatech), 140 mL/L 100X nonessential amino acids (Mediatech), 100 mL/L 100X penicillin/streptomycin solution (10,000 I.U/10,000 μg/mL) (Mediatech), and 10% (v/v) bovine calf serum (BCS) (Hyclone) (*see* **Note 5**).

[4]The medium supplements are added to a number of different incomplete media along with 10% (v/v) bovine calf serum to make complete medium. The pH of the complete media should be 7.0. The addition of nonessential amino acids may reduce the pH, and this can be adjusted by adding potassium hydroxide (KOH) solution to the media.

[5]The serum should be stored frozen. Before adding it to the RPMI 1640 media, remove the serum bottle from the freezer and allow it to acclimate to the room temperature for about 10 min and place in a 37°C water bath. Excessive temperature can degrade the heat-labile nutrients in the serum. Thawing of serum is crucial to its performance. It is recommended to periodically swirl the serum container while thawing, otherwise cryoprecipitates are formed, which are often insoluble. Filtering serum to remove cryoprecipitates is not recommended as it could result in loss of nutrients.

5. 1 M KOH in deionized water, filter sterilize and store at 4°C (*see* **Note 4**).
6. Hank's balanced salt solution: without sodium bicarbonate, calcium, and magnesium (Mediatech).
7. Trypsin EDTA 1X solution: 0.05% trypsin, 0.53 mM EDTA in Hank's balanced salt solution.
8. Phosphate-buffered saline (PBS) solution: 0.210 g/L monobasic potassium phosphate, 9 g/L NaCl, 0.726 g/L dibasic potassium phosphate in deionized water, pH 7.2 (Invitrogen).
9. 250 mL 75 $cm^2$ sterile tissue culture flasks with standard cap.
10. Bright-Line hemocytometer (Hausser Scientific).
11. Compound research microscope (Olympus).
12. Incubator at 37°C with 5% $CO_2$ and 75% humidity.
13. Basic inverted microscope (Zeiss).

#### 12.2.2.2 Cytotoxicity Assay

1. African green monkey kidney cell line (Vero) ATCC CCL-81 (American Type Culture Collection).
2. Human hepatocellular liver carcinoma cell line (HepG2) ATCC HB-8065 (American Type Culture Collection).
3. RPMI 1640 medium with L-glutamine (Invitrogen) (*see* **Note 4**).
4. RPMI complete medium. Supplement RPMI 1640 with 1.5 g/L sodium bicarbonate (Sigma), 10 mL/L 100 mM sodium pyruvate (Mediatech), 140 mL/L 100X nonessential amino acids (Mediatech), 100 mL/L 100X penicillin/streptomycin solution (10,000 I.U/10,000 μg/mL) (Mediatech), and 10% (v/v) bovine calf serum (BCS) (Hyclone) (*see* **Note 5**).
5. 1 M KOH in deionized water, filter sterilize and store at 4°C (*see* **Note 4**).
6. Hank's balanced salt solution: without sodium bicarbonate, calcium, and magnesium (Mediatech).
7. Trypsin EDTA 1X solution: 0.05% trypsin, 0.53 mM EDTA in Hank's balanced salt solution.
8. PBS solution: 0.210 g/L monobasic potassium phosphate, 9 g/L NaCl, 0.726 g/L dibasic potassium phosphate in deionized water, pH 7.2 (Invitrogen).
9. Sterile water.
10. 250 mL 75 $cm^2$ sterile tissue culture flasks with standard cap.
11. Bright-Line hemocytometer (Hausser Scientific).
12. Compound research microscope (Olympus).

13. Basic inverted microscope (Zeiss).
14. Incubator at 37°C with 5% $CO_2$ and 75% humidity.
15. DMSO.
16. Test compounds: Prepare as 20 mg/mL stocks in DMSO. Based on the MIC of the compound to be tested for cytotoxicity, make further dilutions. Make five serial dilutions of each test compound in 1640 RPMI complete medium (*see* **Note 6**), resulting in the final concentration ranging from 1X MIC to 25X MIC.
17. 96-well flat-bottom tissue culture plates with low evaporation lid (Becton-Dickinson).
18. Promega Cell Titer 96 Aqueous One Solution Cell Proliferation Kit.
19. Microplate reader with 490-nm wavelength filter.

## 12.3 Methods

### 12.3.1 Inhibition of Mycobacterial Growth

#### 12.3.1.1 Preparation of *Mycobacterium tuberculosis* Working Stocks

1. Starting from the ATCC stock of *M. tuberculosis* H37Rv, streak a loopful using a 1-μL inoculating loop onto 7 H11 agar plate.
2. Incubate at 37°C for 3 to 4 weeks to obtain isolated colonies.
3. Pick a single colony from the plate and inoculate into 10 mL of 7 H9 broth in a sterile glass culture tube with a stir bar (*see* **Note 7**).
4. Incubate at 37°C with slow stirring.
5. Spread 0.5 mL from the newly made tube culture onto an LJ slant or 7 H11 agar plate as a low-passage backup.
6. Take periodic readings of the culture at $OD_{600}$ until mid log phase ($OD_{600}$ = 0.6 to 0.9).
7. Transfer 1 mL culture into 10 mL 7 H9 medium.
8. Incubate with slow stirring at 37°C for future use (*see* **Note 8**). Simultaneously, take 10 mL from the 10 μL culture and spread onto a Tryptic soy agar plate (*see* **Note 3**).

[6]The concentration of the solvent carrier (DMSO) for the test compound in the medium with cells should not exceed 1%; beyond this, the cells start to die because of the toxic effect of the solvent. If a higher concentration of the compound is required, make dilutions using the RPMI 1640 complete medium instead of DMSO. It is recommended to always set up a separate plate to determine that the solvent (DMSO) is at an acceptable (nontoxic) concentration.

[7]It is always recommended to start cultures on a solid medium by taking a loopful of the thawed stock and streaking on an appropriate agar plate. After growth, pick a colony from the plate and add to liquid medium as it is sometimes difficult to start the bacterial growth in a liquid medium from the frozen state.

[8]The purpose of having a staggered series of backup cultures lagging behind the primary cultures is if the primary culture is contaminated, there is no need to start a 10 mL culture again from the seed stock.

9. Divide the rest of the inoculum from the10 mL 7 H9 mid log-phase culture between two 250-mL Erlenmeyer flasks containing 50 mL 7 H9 medium each. This is passage 1 (*see* **Note 9**). Incubate the flask cultures on a rotary shaker at 125 rpm at 37°C until they reach an $OD_{600}$ of 0.6 to 0.9.
10. For a second scale-up, divide the 50 mL culture between two 1-L Erlenmeyer flasks each containing 500 mL 7 H9 broth. This is passage 2. Spread 10 μL of this culture on a Tryptic soy agar plate to check for contamination (*see* **Note 3**).
11. The 500 mL culture is the working stock and is ready to be frozen in aliquots when it reaches an $OD_{600}$ of 0.6 to 0.9.
12. Add 1.5 mL aliquots of the 500 mL culture to 2.0-mL cryovials, swirling the culture flask frequently to keep the bacteria in suspension.
13. Place the vials in appropriately labeled freezer boxes in the order in which they were filled. Store the culture stocks frozen at −80°C.
14. Take one cryovial from each freezer box and thaw on ice.
15. Make eight 10-fold serial dilutions of each stock culture vial taken from the freezer box.
16. Plate 0.1 mL on 7 H11 agar plates and incubate at 37°C for 2 to 3 weeks.
17. Identify a plate with about 100 to 200 colonies, count the colonies, and calculate CFU/mL, which should be approximately $2 \times 10^7$ CFU/mL (*see* **Note 10**).

#### 12.3.1.2 Alamar Blue Susceptibility Assay

1. Take a sterile 96-well round-bottom microplate and add 200 μL sterile deionized water to all of the wells on the outer perimeter (Fig. 12.1) (*see* **Note 11**).
2. Add 98 μL 7 H9 broth in the wells in column 2, rows B to G. Add 50 μL 7 H9 broth to the rest of the wells from B3 to G11 (Fig. 12.1).
3. Add 2 μL of the test compound from the 20 mg/mL stock concentration to the wells in column 2, rows B to F, and 2 μL of INH from the 25 μg/mL working stock concentration to well G2 (Fig. 12.1).
4. Take 50 μL from column wells B2 to G2 and transfer to column wells B3 to G3. Make identical serial dilutions (1:2) up to B10 to G10. Discard the excess 50 μL medium from the wells B10 to G10 so that each well has only 50 μL volume remaining. The wells B11 to G11 serve as a compound free control.
5. Thaw the frozen stock cryovial and adjust to $2 \times 10^7$ CFU/mL of *M. tuberculosis* $H_{37}Rv$ on ice.

[9]Passage number is important, the working stocks should be no greater than passage 6, the lower the better. Every time a culture is subcultured or plated onto a solid media, it is considered a passage.
[10]To determine the CFU/mL, take one vial from each freezer box to plate out serial dilutions, keeping track of the boxes the vials come from. This is done in order to avoid any variations in the CFU/mL from cryovials filled at different times.
[11]The water is added to the wells on the outer perimeter of the microplate in order to minimize the evaporation of the medium in the test wells during incubation.

6. Add 100 μL thawed stock to 10 mL fresh 7 H9 medium and mix well. Add 50 μL of this mixture to wells B2 to G11 so the final concentration is $1 \times 10^5$ CFU/mL in each well. The final concentration will range from 200 μg/mL to 0.78 μg/mL for the test compounds (*see* **Note 12**) and 0.25 μg/mL to 0.0009 μg/mL for INH. Negative control wells (B11 to G11) have only the solvent (Section 2.1.2, step 8) and bacterial culture added.
7. Seal the plate with Parafilm and incubate at 37°C for 5 days (*see* **Note 13**).
8. Add 10 μL Alamar blue reagent to well B11. Reincubate the plate at 37°C for 24 h. If well B11 turns pink, add the Alamar blue reagent mixture to all the remaining wells in the microplate (*see* **Note 14**).
9. Reseal the plate and incubate for an additional 24 h at 37°C. Record the color change in all of the wells at the end of incubation. Blue indicates no growth, whereas pink indicates growth (*see* **Note 15**).

### 12.3.2 Growth of Eukaryotic Cells and Toxicity Test

#### 12.3.2.1 Maintenance of Eukaryotic Cell Lines

1. Grow cells in 75 $cm^2$ tissue culture flasks in 30 mL RPMI 1640 complete media (*see* **Note 16**) at 37°C in a $CO_2$ incubator.
2. To harvest a cell monolayer, remove and discard the culture medium. Rinse the cell layer with 5 mL PBS. (*see* **Notes 16** and **17**).
3. Add 2 to 3 mL of trypsin solution to the cell layer and check the progress of the enzyme treatment every 5 min for a maximum of 15 min. Mono-layers that are particularly difficult to detach can be placed at 37°C (*see* **Note 18**).
4. Add 8 to 10 mL of RPMI 1640 complete medium to the cell suspension and wash any remaining cells from the bottom of the culture flask. Pipette the cell suspension up and down several times to ensure a single cell dispersion.
5. Count the cells using a hemocytometer as follows: add 10 to 20 μL of medium containing cells to the hemocytometer, count the cells in four large corner squares

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[12]The concentration of the test compounds used here are representative values and can be changed according to individual requirements and the nature of the compound being tested.
[13]Alternately, the plates can be sealed in a Ziplock bag and a damp paper towel placed on top of the plate inside the bag in order to prevent evaporation during incubation.
[14]If the B11 well remains blue, the Alamar blue reagent should be added to another control well C11, reincubated for additional 24 h, and checked for color change. This is done to make sure the cells are growing normally in the control wells. If there is no color change even after 48 h, the plates should be discarded and the assay repeated.
[15]Wells may appear violet after 24 h incubation but invariably change to pink after extended incubation at 37°C indicating growth. The MIC is defined as the lowest concentration of compound in which the dye remains blue. A standard scanner can be used to generate a permanent record of the plate (Fig. 12.1).
[16]PBS and RPMI 1640 complete media are stored at 4°C and should be brought to room temperature before use.
[17]Trypsin is the most common disassociating solution, and its action is inhibited by serum. Residual amounts of serum are often responsible for the failure of the trypsin solution to detach the cells from the substrate. To avoid this, the cells are washed with PBS before treatment with trypsin.
[18]Cells vary greatly in how fast they come off the flask. After adding trypsin to the cells in the tissue culture flask and incubating for 10 to 15 min, a quick screening of the cell suspension under an inverted microscope is recommended in order to ensure complete detachment of cells from the surface of the tissue culture flask and also to get a suspension of at least 95% single cells. Cells should be trypsinized until they come off the flask, but not longer.

(these are 1 mm × 1 mm in area) using a microscope at low magnification (10×) (*see* **Note 19**). Take the average of the four counts and multiply by $10^4$ to obtain the number of cells per milliliter.

6. Seed a new 75 $cm^2$ tissue culture flask containing 30 mL RPMI 1640 complete media with $5 \times 10^4$ cells.
7. Repeat steps 1 to 4 as the cells near confluence (2 to 3 days).

#### 12.3.2.2 Cytotoxicity Assay

1. Using a near confluent (75% to 95%) 75 $cm^2$ tissue culture flask, remove the media and rinse the cells with 5 mL PBS buffer without disturbing the monolayer (*see* **Note 17**).
2. To detach cells from the surface of the flask, add 2 to 3 mL trypsin solution to the cell layer and incubate at 37°C. Check the progress of the enzyme treatment every 5 min for a maximum of 15 min (*see* **Note 18**).
3. Add 5 mL RPMI 1640 complete media and pipette up and down to form a single cell suspension.
4. Count 10 μL aliquot of the cells on a hemocytometer as described in Section 12.3.2.1, step 4.
5. Calculate and adjust cell suspension to $5 \times 10^4$ cells/mL by adding fresh RPMI 1640 complete medium.
6. Dispense 100 μL/well in a sterile 96-well flat-bottom plate in wells B2 to G11 (Fig. 12.2).
7. Add 200 μL sterile water to wells A1 to A12, H1 to H12, and B1 to G1 on the outer perimeter of the 96-well plate to limit evaporation (*see* **Note 11**). Add 200 μL RPMI 1640 complete medium in wells from B12 to G12 as a media only (negative) control.
8. Incubate plate for 2 h in the $CO_2$ incubator to allow the cells to attach to the surface of the plate.
9. Add the test compounds to the wells B3 to G11 starting at 1X MIC and increasing to 25X MIC (Fig. 12.2). Each concentration of the test compound should be done in triplicate. Three compounds can be tested in each 96-well plate. Bring up the total volume of wells B2 to G11 to 200 μL by adding an appropriate amount of fresh RPMI 1640 complete medium. The wells B2 to G2 serve as the cells only control.
10. Incubate the plates for 72 h at 37°C in the $CO_2$ incubator. Visually inspect under the inverted microscope every 24 h to check for contamination or cell lysis.
11. After 72 h remove the media from all wells and wash the cells with 200 μL PBS buffer.

[19]It is easy to double-count cells that lie on the lines between individual small squares. For cells that touch the lines defining the squares, only count those touching the top or left side of an individual square.

12. Add 100 μL RPMI 1640 complete medium to each well from B2 to G12 (*see* **Note 16**).
13. Add 10 μL thawed Cell Titer 96 Aqueous One Solution Reagent to wells B2 to G12 and incubate at 37°C in $CO_2$ incubator for 4 h.
14. Read the absorbance at 490-nm wavelength using a microplate reader (*see* **Note 20**).
15. Calculate the $IC_{50}$ value for each test compound. Background absorbance, which is due solely to the reaction of the reagents, should be deducted from the absorbance values of the treated and untreated cells. The mean absorbance of the medium-only control is $IC_{0.}$ The mean absorbance obtained from the cells-only control is $IC_{100}$ and for the test compounds is $IC_{100T.}$ To obtain the corrected absorbance, $IC_0$ is subtracted from the $IC_{100}$ and $IC_{100T}$ absorbance values. To determine $IC_{50}$ for each compound, plot the corrected absorbance at 490 nm (y axis) versus concentration of the compound tested (x axis), and calculate the $IC_{50}$ value by determining the x-axis value corresponding with half the difference between the corrected $IC_{100}$ and corrected $IC_{100T}$ absorbance values [27] (Figs. 12.2 and 12.3).

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[20]Absorbance values lower than the control cells indicate a reduction in the rate of cell growth. Conversely, a higher absorbance indicates an increase in cell proliferation

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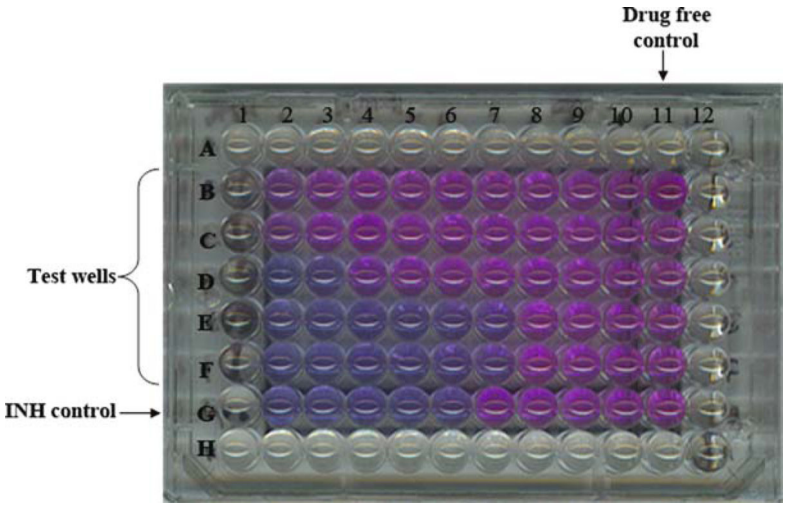


**Fig. 12.1.**

Representative 96-well microplate format for screening compounds to determine their MICs on *M. tuberculosis* $H_{37}$Rv using MABA

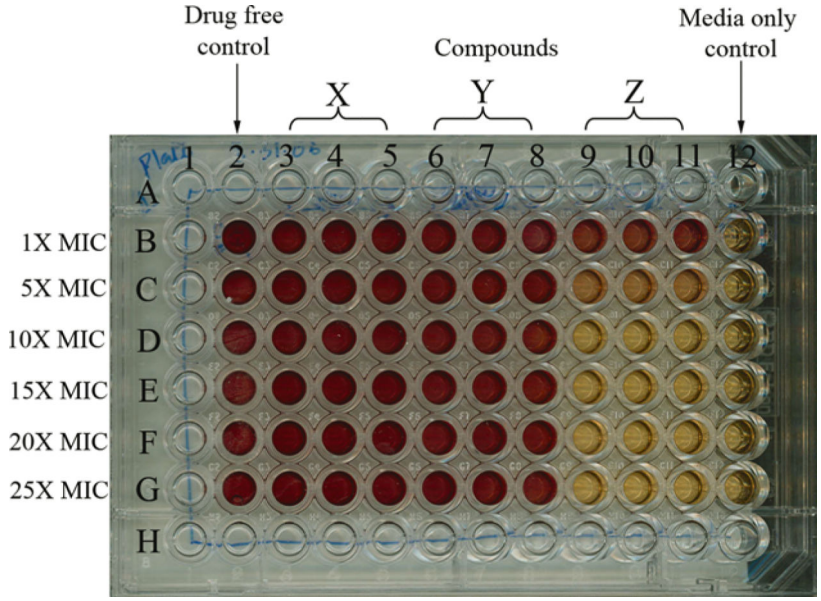


**Fig. 12.2.**

Representative 96-well plate format for screening compounds to determine their effect on the viability of a eukaryotic cell line

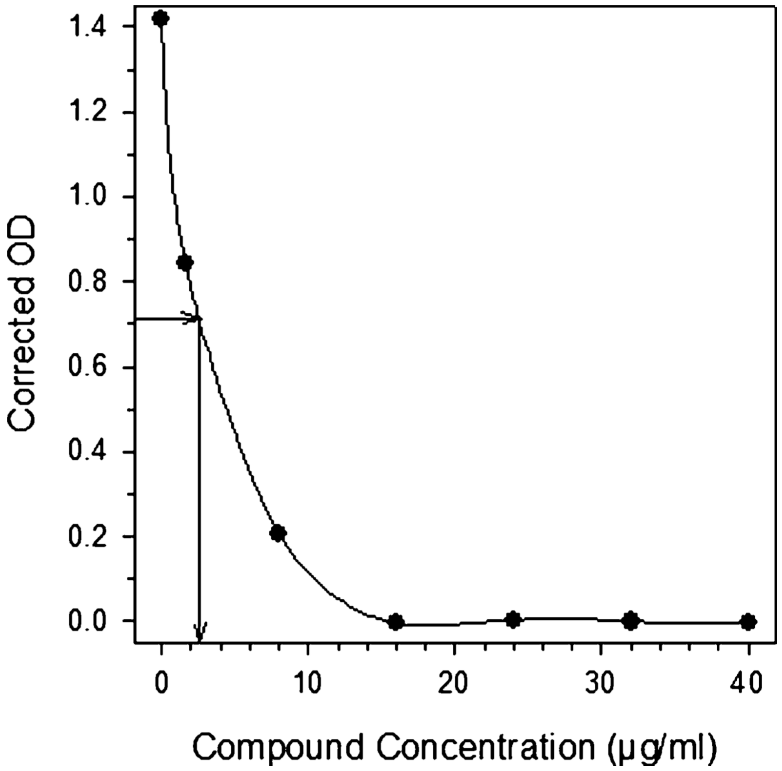


**Fig. 12.3.**

Representative graph showing cytotoxic effect of a compound and determination of its $IC_{50}$ value (~ 2.5 µg/mL)