HHS Public Access

Author manuscript

Methods Mol Biol. Author manuscript; available in PMC 2023 September 14.

Published in final edited form as:

Methods Mol Biol. 2021 ; 2314: 595–609. doi:10.1007/978-1-0716-1460-0_26.

Minimum Inhibiting Concentration Determination in Liquid Cultures and on Solid Medium

Qinglan Wang,

Helena I. M. Boshoff

Tuberculosis Research Section, Laboratory of Clinical Immunology and Microbiology, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD, USA

Abstract

Antimicrobial susceptibility testing is the mainstay of tuberculosis drug development programs. In this chapter we describe methods for determination of the minimum inhibitory concentration of compounds against *Mycobacterium tuberculosis* growing in liquid media as a function of carbon source, detergent and environmental stress imposed by acidic pH as well as reactive nitrogen intermediates. Methods for determining the effect of bovine serum albumin in the growth medium on antimicrobial susceptibility are also described. Finally, we provide a method for antimicrobial susceptibility testing on agar medium.

Keywords

Minimum inhibitory concentration (MIC); agar MIC; carbon source; detergent; liquid MIC; nitrosative stress; acidified medium

1. Introduction

The goal of current drug discovery efforts for tuberculosis is to accelerate the decline in global incidence of this disease by developing regimens that are superior to frontline and second line drug combinations in terms of safety and duration of treatment while remaining affordable and less prone to the development of drug resistance in the face of non-adherence. Whole cell screening against *Mycobacterium tuberculosis* has been key to the discovery of all drug candidates in the tuberculosis drug discovery pipeline over the last few decades. Several drug discovery efforts have attempted to recapitulate aspects of *in vivo* pathogenesis of the organism that were deemed as critical for the replication or survival of the pathogen in humans including growth in macrophages [1–3], survival at acidic pH [4], hypoxia [5], biofilm formation [6], nutrient starvation [7], growth on fatty acids [8–10], glucose, cholesterol [1], multi-stress conditions [11], as well as growth under nutrient rich conditions [12,13]. The importance of recapitulating *in vivo* relevant growth conditions was emphasized by the discovery of several hits that lacked *in vivo* efficacy due to the dependence of the mechanism of activity on glycerol metabolism [13,14], a carbon source only available to the organism in the form of glycerol-based host lipids. In all these

corresponding author: helena.boshoff@nih.gov.

studies, compounds were screened under the *in vivo* relevant conditions, hits validated by dose titration with subsequent determination of minimum inhibitory concentrations (MIC) of those hits that inhibited growth of the bacillus.

Determination of MIC values is critical in developing structure-activity relationships (SAR) of scaffolds discovered in such screens, to guide further biological evaluation of the compounds and to understand mechanism of action. Interpretation of whole cell SAR is driven not only by target-based SAR but also differences in uptake, efflux, metabolism and protein binding. Biological evaluations of growth inhibitors to understand mechanism of action are in turn, always interpreted as a function of MIC values. In this respect, the MIC value is dependent on the growth condition, for example, the concentration that inhibits full growth of the bacillus can depend on nutrient sources as well as the physical state of the medium (liquid versus agar). The determination of MIC values on solid media is often overlooked in drug discovery studies. However, this is an important consideration since agar MIC values, important information for generation of resistant mutants on solid media, do not always correspond to MIC values observed during growth in liquid media [15].

This chapter describes methods for the determination of MIC values during growth on different *in vivo* relevant carbon sources as well as restriction of growth imposed by acidic pH combined with nitrosative stress. *In vivo* relevant carbon sources include glucose, cholesterol and fatty acids. The nitrosative stress used in our methods is derived from reactive nitrogen intermediates formed by acidified nitrite [16]. The methods in this chapter describe a limited set of growth readouts but these assays can readily be adapted to other established measurements of mycobacterial growth [17]. In addition, methods to explore the role of detergent effects are described since the MIC values of several compounds including rifampicin and QcrB-targeting imidazopyridines, are significantly affected by the presence and type of detergent in the medium. The relevance of detergent effects in tuberculosis drug discovery are outside the scope of this discussion. In this chapter, we provide methods for understanding the effects of the non-hydrolyzable detergent, Tyloxapol, as opposed to Tween 80 which is hydrolyzed by the mycobacterium to generate the carbon source, oleic acid. Growth of *M. tuberculosis* to yield suspensions amenable for growth inhibition assays necessarily require use of detergent to generate homogenous cell suspension. However, it should be noted that a detergent-free MIC assay has been described [18]. Our experience is also that the presence of bovine serum albumin (BSA) in the medium may dramatically affect MIC values. Although the effect of protein binding is complex and difficult to interpret in drug discovery with examples of highly protein-bound compounds being *in vivo* efficacious compared to analogs with lower protein binding [14], we simply provide a method to explore the effect of BSA binding on MIC since the SAR can often be accentuated in the absence of BSA in the medium. Synthesis of analogs that overcome the protein binding to such an extent as to allow sufficient target engagement remains empirical. Finally, we include methods for the determination of MIC values of compounds on agar medium. The reader is however, encouraged to read previous descriptions of MIC determination on solid medium [17].

The MIC value in our methods is taken as the concentration of compound that leads to complete growth inhibition. However, for the assays that measure fluorescence or

absorbance readouts, the concentration that leads to 50, 90, and 99 % growth inhibition can be calculated as previously described [17]. The frontline anti-tubercular drugs show little variation in MIC as a function of carbon source. However, rifampicin is affected by detergent composition of the medium showing a 10-fold lower MIC value when using Tween 80 as detergent compared to Tyloxapol (*see* Table 1). Rifampicin also has a lower MIC value in the absence of BSA. The stability of compounds in the medium over the duration of the assay as well as to metabolism by the bacillus will affect interpretation and the understanding of the MIC values. A discussion of this is outside the scope of this chapter but the measurement of parent compound concentrations remaining in the assay will greatly facilitate the understanding of the SAR of analogs with inexplicable absence of whole cell activity.

2. Materials

All aqueous solutions are made with distilled deionized water. Media for cultivation of cells need to be filter sterilized or autoclaved as described. Dimethyl sulfoxide (DMSO) does not need to be filter sterilized although DMSO source bottles are reserved for cell culture purposes only and preferably dispensed in a biosafety cabinet. All work with *M. tuberculosis* will be done in a biosafety cabinet according to institutional guidelines.

2.1 Preparation of inoculum

1. *Mycobacterium tuberculosis* H37Rv (ATCC 27294), *M. tuberculosis* pMSP12::GFP (Addgene) or other strain (*see* Note 1).
2. 7H9/ADC/Tween 80 broth: Dissolve 4.7 g Middlebrook 7H9 broth base (Difco), 2 mL glycerol (add with a 3-mL sterile syringe) and 0.5 mL Tween 80 (add with a 1-mL sterile syringe) in 900 mL water (*see* Note 2). Aseptically add 100 mL albumin-dextrose complex (ADC) and filter sterilize (0.2 μm filter) (*see* Note 3). ADC is made by mixing 50 g BSA fraction V (low heavy metals), 20 g glucose, 8.1 g NaCl in 1 L water. The ADC is filter sterilized (0.2 μm filter) and stored at 4 °C.
3. 7H9/Glucose/BSA/Tyloxapol broth: Mix 4.7 g Middlebrook 7H9 broth base, 4 g Glucose, 5 g BSA fraction V (low heavy metals), 0.81 g NaCl, and 0.5 mL Tyloxapol (add with a 1-mL sterile syringe) (*see* Note 2) in 1000 mL water. The medium is filter sterilized (0.2 μm filter) and can be stored at room temperature for 2 weeks.
4. 7H9/dipalmitoyl phosphatidylcholine (DPPC)/cholesterol/BSA/Tyloxapol broth: Mix 4.7 g Middlebrook 7H9 broth base, 5 g BSA fraction V (low heavy metals), 0.81 g NaCl in 1000 mL water. Make a 10 mg/ml stock of DPPC in ethanol. It will dissolve at room temperature upon vortexing. Add 500 μL of this solution to the broth base giving a final DPPC concentration of 5 μg/mL. To 1 mL ethanol add 500 μL Tyloxapol, heat at 70 °C. Then add 24 mg cholesterol and heat again until completely dissolved. Add this solution to the 1 L medium described above and filter sterilize (0.2 μm filter). This carbon source medium needs to be kept at 37 °C and used within 24 hours.

5. 7H9/Glucose/BSA/Tween 80 broth: Mix 4.7 g Middlebrook 7H9 broth base, 4 g Glucose, 5 g BSA fraction V (low heavy metals), 0.81 g NaCl, and 0.5 mL Tween 80 (add with a 1-mL sterile syringe)(*see* Note 2) in 1000 mL water. The medium is filter-sterilized (0.2 μm filter) and can be stored at room temperature for 2 weeks.

6. 7H9/cholesterol/BSA/Tyloxapol broth: Mix 4.7 g Middlebrook 7H9 broth base, 5 g BSA fraction V (low heavy metals) and 0.81 g NaCl in 1000 mL water. Gently warm this medium to 37 °C. To 1 mL ethanol add 500 μL Tyloxapol, heat at 70 °C. Then add 96 mg cholesterol and heat again until completely dissolved. Add this solution to the 1 L prepared medium and filter sterilize. This carbon source medium needs to be kept at 37 °C and used within 24 hours.

7. 7H9/DPPC/casitone/Tyloxapol: Mix 4.7 g Middlebrook 7H9 broth base, 0.3 g Bacto casitone, 0.81 g NaCl, and 0.5 mL Tyloxapol (add with a 1-mL sterile syringe) (*see* Note 2) in 1000 mL water. Make a 14 mg/mL stock of DPPC in ethanol. It will dissolve at room temperature upon vortexing. Add 1000 μL of this solution to the broth giving a final DPPC concentration of 14 μg/mL. Filter sterilize (0.2 μm filter) and, although less susceptible to precipitation than cholesterol media, should be used within a week.

8. 7H9/cholesterol/BSA/Tween 80 broth: Mix 4.7 g Middlebrook 7H9 broth base, 5 g BSA fraction V (low heavy metals) and 0.81 g NaCl in 1000 mL water. Gently warm this medium to 37 °C. To 1 mL ethanol add 500 μL Tween 80 (*see* Note 2), heat at 70 °C. Then add 96 mg cholesterol and heat again until completely dissolved. Add this solution to the 1 L medium and filter sterilize. This carbon source medium needs to be kept at 37 °C and used within 24 hours.

9. 7H9/glucose/casitone/Tyloxapol medium: Mix 4.7 g Middlebrook 7H9 broth base, 4 g glucose, 0.3 g Bacto casitone, 0.81 g NaCl, and 0.5 mL Tyloxapol (add with a 1-mL sterile syringe) (*see* Note 2) in 1000 mL water. The medium is filter-sterilized (0.2 μm filter) and can be stored at room temperature for 2 weeks.

10. 7H9/butyrate/Tyloxapol (pH = 6) medium: Dissolve 4.7 g Middlebrook 7H9 broth base, 230 μL butyric acid (giving a final butyrate concentration of 2.5 mM), 5 g BSA fraction V (low heavy metals), 0.81 g NaCl and 0.5 mL Tyloxapol in 1000 mL water. The pH of the medium should be 5.95. If the pH is not around 6, either 6 M HCl or 6 M NaOH is added to adjust the pH to 6.0. The medium is sterilized by filtration through a 0.2 μm filter and can be stored at room temperature for 2 weeks.

11. 7H9/butyrate/nitrite/Tyloxapol (pH = 6) medium: Dissolve 4.7 g Middlebrook 7H9 broth base, 230 μL butyric acid (giving a final butyrate concentration of 2.5 mM), 5 g BSA fraction V (low heavy metals), 0.81 g NaCl, and 0.5 mL Tyloxapol in 1000 mL water. The pH of the medium should be 5.95. If the pH is not around 6, either 6 M HCl or 6 M NaOH is added to adjust the pH to 6.0. The medium is sterilized by filtration through a 0.2 μm filter. On day of MIC set-up, sodium nitrite is added to a final concentration of 0.1 mM. Sodium nitrite

prepared fresh as a 1 M stock in water, filter sterilized through a 0.2 μm filter and diluted 1:10,000 in the 7H9/butyrate/Tyloxapol (pH = 6) medium.

12. Sterile 60 mL square polycarbonate bottles (Nalgene ™)
13. 25 mg/mL kanamycin prepared in water and filter sterilized. Store at −20 °C as aliquots.
14. Incubator shaker.

2.2 Preparation of plates for liquid MIC determination

1. 96-well V-bottom polypropylene plates
2. DMSO
3. Drug controls made as DMSO stocks as appropriate (*see* Note 4) including 10 mM isoniazid stock, 1 mM rifampicin stock, 0.2 M pyrazinamide stock and/or 10 mM linezolid stock (*see* Note 5).
4. 2–20 μl Multichannel Pipette.
5. 20μl and 200 μl sterile filter tips (RAININ).

2.3 Preparation of plates for solid medium MIC determination

1. Same materials described in Section of 2.1.
2. 96-well, U-bottom, microtiter plates with lids
3. 1000 μl Multichannel Pipette.
4. 1ml sterile filter tips (RAININ).
5. Sterile basins.
6. 7H11/OADC medium: Add 21 g Middlebrook 7H11 agar base (Difco), 5 mL glycerol (add with a 6-mL sterile syringe) with 900 mL water in a clean glass bottle containing a magnetic stir bar. Autoclave at 15 pounds pressure at 121 °C for 15 minutes. Cool to 60 °C in a warm water bath. Aseptically add 100 mL oleic acid-dextrose-albumin complex (OADC). OADC consists of 8.1 g NaCl, 50 g BSA fraction V (low heavy metals), 20 g glucose, 30 mL sodium oleate solution and water to a total volume of 1000 mL. Sodium oleate solution is made by warming 30mL water containing 0.19 mL 10 M NaOH to 56 °C, adding 0.6 mL oleic acid and swirling until clear. The OADC is filter-sterilized (0.2 μm sterile filters) and stored at 4 °C
7. Heating block.

2.4 Measuring growth using an inverted mirror

1. 96-well, U-bottom, sterile microtiter plates with lids
2. Sterile basins
3. Enlarging inverted mirror

4. Ziplock bags

2.5 Measuring growth using Alamar Blue

1. 96-well clear flat-bottom, sterile black microtiter plates with lids (*see* Note 6)
2. Sterile basins
3. Ready-to-use Alamar Blue solution (DAL1100, ThermoFisher) (*see* Note 7)
4. Colorimeter/Fluorimeter (eg. CLARIOstar Plus, BMG LABTECH)
5. Ziplock bags

2.6 Measuring growth using fluorescence of *M. tuberculosis* pMSP12::GFP

1. 96-well, flat-bottom, sterile black microtiter plates with lids
2. Sterile basins
3. Fluorimeter (eg. CLARIOstar Plus, BMG LABTECH)
4. Ziplock bags

2.7 Measuring growth on solid medium

1. Sterile basins
2. Ready-to-use Alamar Blue solution (DAL1100, ThermoFisher)
3. Ziplock bags
4. 1–10μl or 2–20 μl Multichannel Pipette
5. 20μl sterile filter tips (RAININ)

3. Methods

3.1 Preparation of inoculum

1. In a 60 mL square bottle seed 10 mL of the growth medium to be used for the MIC determination (*see* Notes 8 and 9) with 200 μl freezer stocks of *M. tuberculosis* (*see* Note 1).
2. Incubate at 37 °C at 15 rpm for approximately 7 days or 10–12 days for growth in 7H9/butyrate//Tyloxapol (pH = 6) medium until the culture reaches an OD_{650} of 0.2–0.4. The *M. tuberculosis* pMSP12::GFP strain is grown with 25 μg/mL kanamycin.
3. Dilute the cells 1:100 in the respective medium (*see* Note 10) and incubate as before for 4–5 days until the culture reaches an OD_{650} of approximately 0.2. The *M. tuberculosis* pMSP12::GFP strain is grown with 25 μg/mL kanamycin.
4. Dilute the cells 1:1000 in the respective medium for liquid MIC and 1:200 for agar MIC, with the following exceptions: dilute cells 1:10 for liquid MIC in 7H9/butyrate/Tyloxapol (pH = 6) medium; and 1:10 for liquid MIC in 7H9/butyrate/nitrite/Tyloxapol (pH = 6) medium (*see* Note 10). The *M. tuberculosis*

pMSP12::GFP strain is diluted in media with 25 μg/mL kanamycin. Transfer the cell suspension to a sterile basin (*see* Note 11).

3.2 Preparation of plates for liquid MIC determination

1. Dispense 40 μL stock solution of compound in column 1 of the 96-well polypropylene plate (*see* Notes 12, 13, 14 and 15).
2. Dispense 20 μL of DMSO to columns 2–12 in the plate.
3. Transfer 20 μL of compound from column 1 into column 2. Mix by pipetting up and down 6 times. Continue to make a 2-fold serial dilution by transferring 20 μL of the dilution from column 2 into 3 and then into subsequent columns ending with column 11.
4. Prepare similar drug dilutions with the control drugs (*see* Notes 4 and 16).
5. Using a 20 μL multichannel pipette with clean sterile tips for each row, transfer 2 μL of the drug dilution to the microtiter plate to be used for MIC determination.

3.3 Preparation of plates for solid medium MIC determination

1. Prepare drug dilutions as described in **steps 1–5** of Section 3.2.
2. Using a 1000 μL multichannel set at 200 μL, transfer warm 7H11/OADC into the wells of the microtiter plate taking care to avoid formation of bubbles (*see* Note 17).
3. Dry the plates with lids on for 24 hours inside the biosafety cabinet.

3.4 Measuring growth using an inverted mirror

1. Using a multichannel pipette with clean sterile tips for each row, add 200 μL of cells prepared in **step 4** of Section 3.1 to the 96-well U-bottom microtiter plates containing the 2 μL of compound (*see* **step 5** in Section 3.2). Mix the cells and compound and transfer 100 μL of the cell/compound mix to a clean sterile microtiter plate as a technical repeat (*see* Notes 11 and 15).
2. Replace the plate lids and place the duplicate microtiter plates in ziplock bags and incubate for 1 weeks at 37 °C.
3. Record growth at the end of week 1 by placing the plates above an enlarging inverted mirror (*see* Note 18).
4. Return the plates to the ziplock bags and incubate at 37 °C for an additional week.
5. Record growth at the end of week 2 (*see* **step 3** in Section 3.4)
6. The MIC is taken as the lowest concentration of compound that results in full inhibition of growth (*see* Notes 19 and 20).

3.5 Measuring growth using Alamar Blue

1. The set-up for MIC is performed as in the **Step 1** of section 3.4 using 96-well clear flat-bottom black microtiter plates with the exception that an additional duplicate set of copies of the microtiter plates will be required for readouts at week 2 (*see* Note 20). One duplicate set of plates are labeled as “week 1”, the other duplicate set of plates are labeled as “week 2”.
2. After one week of incubation, add 10 μL of Alamar Blue solution to each well of “week 1” plates. Replace the plates in the ziplock bags and incubate for 24 hours at 37 °C.
3. Growth can be recorded as a visual MIC with the MIC value taken as the lowest concentration of compound that inhibits the reduction of the dye as seen by inhibition of blue to pink conversion. Alternatively, the MIC can be recorded in plate reader by absorbance at 570nm and 600nm or fluorescence using excitation between 530–560nm with emission recorded at 590–595nm (*see* Note 6).
4. At week 2, add 10 μL of Alamar Blue solution to each well of “week 2” plates. Replace the plates in the ziplock bags and incubate for 24 hours at 37 °C.
5. Repeat step 3 above for reading the results at week 2.

3.6 Measuring growth using fluorescence of *M. tuberculosis* pMSP12::GFP

The *M. tuberculosis* pMSP12::GFP strain is used for measuring the MIC under conditions of highly restricted growth as occurs at low pH in the presence or absence of nitrite. Pyrazinamide is active against *M. tuberculosis* at low pH and is an essential control for MIC determination in 7H9/butyrate/Tyloxapol (pH = 6) and 7H9/butyrate/nitrite/Tyloxapol (pH = 6) media (*see* Note 16). The *M. tuberculosis* pMSP12::GFP strain can also be used for MIC determination under any of the above growth conditions in order to generate growth inhibition graphs for compounds.

1. The inoculum is prepared as described in **steps 1– 4** of section 3.1.
2. Using a multichannel pipette, add 200 μL of cell suspension to black microtiter plates containing the 2 μL of compound from the compound dilution plates (*see* **step 5** in section 3.2). Mix the cells and compound and transfer 100 μL of the cell/compound mix to a clean sterile black microtiter plate to generate a technical repeat (*see* Notes 11 and 15).
3. Replace the plate lids, place the microtiter plates in ziplock bags and incubate at 37 °C (*see* Note 21).
4. At 10-days and 20-days time points, measure fluorescence at an excitation wavelength of 485 nm and emission recorded at 535 nm.
5. The fluorescence readout values can be plotted as growth inhibition curve using the values from the DMSO control for 0% growth inhibition and 5 μM rifampicin as 100% growth inhibition. From this, the IC50 (half maximal inhibitory concentration) and pIC50 (pIC50 = $-\log_{10}$ (IC50) can be determined.

In addition, the compound concentrations that result in 90% and 99% reduction in fluorescence signal should be recorded.

3.7 Measuring growth on solid medium

1. The inoculum is prepared as described in section 3.1.
2. Using a 10 μL or 20 μL multichannel pipette, add 2 μL of cell suspension to the center of the agar in each well.
3. Replace the plate lids, place the microtiter plates in ziplock bags and incubate for 24 h at 37 °C before inverting the plates for further incubation (*see* Note 22).
4. Record visual growth after one week of incubation and again after two weeks (*see* Note 18).
5. After recording visual growth at week 2, add 10 μL of Alamar Blue solution to each well.
6. Once Alamar Blue reagent has been added, the plates should not be inverted. Replace the plates in the ziplock bags and incubate for 24 hours at 37 °C.
7. The MIC is recorded as the concentration of compound that results in no visible growth on top of the agar. This value typically corresponds to the concentration of compound that inhibits the reduction of Alamar Blue to its pink reduction product.

Notes

1. Widely used strains of *M. tuberculosis* including H37Rv (ATCC 27294), Erdman, HN878, and CDC1551 all show reproducible robust growth in the respective media. At times, clinical strains of *M. tuberculosis* including drug resistant isolates may need to be tested. Clinical isolates tend to have more variable growth rates and nutrient requirements depending on the genetic background of the strain and need to be grown accordingly. In addition, many clinical isolates do not grow well in minimal media preventing their reproducible assessment under these conditions. The *M. tuberculosis* pMSP12::GFP is cultured with 25 μg/mL kanamycin.
2. Glycerol and detergents such as Tween 80 and Tyloxapol are highly viscous and cannot be dispensed by pipetting unless diluted solutions are prepared. A syringe with plunger allows sufficiently accurate dispensing of these for larger volumes of media. For the preparation of volumes of growth media less than 1 L, it is preferable to use stocks of 25 % glycerol and 25 % Tyloxapol or Tween 80 prepared in water. These stocks should be filter-sterilized (0.2 μm filter) and a calculated volume is dispensed accordingly.
3. 7H9 Middlebrook broth base containing glycerol and detergent can be autoclaved prior to addition of ADC.

4. *M. tuberculosis* including H37Rv (ATCC 27294), Erdman, HN878 and CDC1551 all have similar MIC values to standard drugs such as isoniazid and rifampicin. Drug-resistant clinical isolates have to be tested with the appropriate drug controls with linezolid (stock concentration of 10 mM) being the most widely applicable control drug for drug-resistant isolates except for resistant strains that have been isolated from patients treated with this oxazolidinone.

5. Drug stocks should be stored frozen in aliquots. Although drugs such as isoniazid, rifampicin and linezolid can be frozen and thawed several times without noticeable changes in MIC values, it is good practice to aliquot drugs as needed. Pyrazinamide is only active against *M. tuberculosis* grown at pH 6.

6. The Alamar Blue assay is based on reduction of the resazurin dye to a pink product. Recording of absorbance or fluorescence using a plate reader enables inhibition curves to be graphed with determination of pIC50 values. For absorbance data, the percentage inhibition (PI) can be calculated using the equation as follows:

$$\mathrm{PI} = \left(1 - \frac{\mathrm{O2} * \mathrm{A2} - \mathrm{O1} * \mathrm{A1}}{\mathrm{O2} * \mathrm{P1} - \mathrm{O1} * \mathrm{P2}}\right) * 100\ \%$$

where: O1 = 80586, molar extinction coefficient (E) of oxidized resazurin at 570 nm, O2 = 117216, E of oxidized resazurin at 600 nm, A1 = absorbance of test well (containing drug) at 570 nm, A2 = absorbance of the same test well at 600 nm, P1 = absorbance of control well (no drug) at 570 nm, P2 = absorbance of control well at 600 nm. For fluorescence data, the PI is calculated as follows:

$$\mathrm{PI} = \left(1 - \frac{\mathrm{FI\,590\,of\,test\,well}}{\mathrm{FI\,590\,of\,control\,well}}\right) * 100\ \%$$

where: FI 590 = fluorescent intensity at 590 nm emission (560 nm excitation). IC 50 is calculated based on the curve generated from plotting compound concentration vs PI, and pIC50 is calculated using the equation: $\mathrm{pIC50} = -\log_{10}(\mathrm{IC50})$.

7. Alamar Blue solution can be made from resazurin powder (e.g. R7017, Sigma) in water at a concentration of 0.04%.

8. *M. tuberculosis* cultures should not be propagated for more than 2–3 sub-cultures since *in vitro* growth conditions tend to favor the emergence of strains that have lost phthiocerol dimycoserosate (PDIM) [19]. Freezer stocks are made by freezing sufficient numbers of aliquots of a confirmed PDIM-positive strain [20] to allow reproducible testing over 2–3 years, depending on the estimated throughput and duration of the laboratory or project.

9. For growth in BSA-free conditions and in low pH medium (7H9/butyrate/Tyloxapol (pH = 6)), it is recommended to start cultures from freezer stocks in the respective BSA-containing carbon source medium (pH = 6.8) to an OD_{600}

of 0.2–0.4, then dilute cells 1:100 in the respective BSA-free or 7H9/butyrate/Tyloxapol (pH = 6) medium (*see* **step 3** of Section 3.1).

10. For MIC testing in 7H9/butyrate/nitrite/Tyloxapol (pH = 6) medium, cells are cultured in the absence of nitrite (in **step 3** of Section 3.1). When cells are ready, dilute cells 1:10 in 7H9/butyrate/nitrite/Tyloxapol (pH = 6) medium for MIC set-up.

11. For 7H9/cholesterol/BSA/Tyloxapol medium, it is important to keep the medium warm during set-up for MIC since cholesterol rapidly precipitates as the medium cools down.

12. It is important to ensure that pipettes are calibrated. For most pipettes there is a 10% pipetting error implying that the error is larger on smaller volumes. Thus, it is recommended not to pipette a lower volume than 2 μL using a 10 μL or 20 μL pipette.

13. Compound stock solutions in DMSO need to be fully dissolved. In some instances, sonication in a water bath or gentle heating (depending on compound stability to heat) may be necessary to ensure full dissolution. Compounds that do not fully dissolve at the initial compound concentration need to be further diluted with DMSO until fully dissolved. In the latter case, the highest concentration in the MIC set-up will be lower. There are certain chemical series that are not stable or soluble in DMSO. These compounds may be soluble in solvents such as ethanol or water. In the latter case, stock solutions need to be filter sterilized and the serial dilutions should be performed with respective solvent.

14. Stock concentrations depend on the desired highest concentration in the MIC set-up. As a rule, the DMSO concentration in the MIC set-up should not be higher than 1% after addition of cells. In the procedure outlined in the chapter, a stock concentration of 10 mM will give a final concentration of 100 μM.

15. Dilution of compound in DMSO is important when the solubility in aqueous medium is unknown. However, for compounds whose aqueous solubility is not limiting, the method can be accelerated by performing drug dilutions in aqueous medium. In this case, 2 μL of drug is added directly to the first column of the sterile microtiter plate (plate 1) containing 200 μL of growth medium in column 1 and 50 μL of growth medium in all other wells. Dilutions are performed by transferring 50 μL from column 1 to column 2 and serially to the next columns ending with column 11 after which the last 50 μL is discarded. A duplicate plate (plate 2) containing 50 μL of medium in columns 2–12 is made by transferring 100 μL from column 1 in plate 1 to this plate and serially diluting as for plate 1. After this, an equal volume of cells (50 μL) in the respective growth medium prepared as in section 3.3 is added. The final drug concentration is 50 μM if the drug stock concentration is 10 mM.

16. Pyrazinamide results in 90% growth inhibition at 500 −1000 μM at pH = 6.

17. The 7H11/OADC agar medium rapidly solidifies during preparation of the plates. To overcome this, it is advisable to keep the basin with agar medium on a hot plate and to work rapidly. In addition, the tips need to be replaced after each agar addition. It is harder to accurately dispense warm agar medium and as a result, at least 4 copies are required for each plate.

18. It is recommended to record MIC data by photographing the plates.

19. Compound precipitation can confound interpretation of growth. In addition, cholesterol may precipitate out of plates containing 7H9/cholesterol/BSA/Tyloxapol medium if the plates cool down at any time point. In these cases, an alternative method of MIC determination using Alamar Blue (section 3.5) or fluorescence of *M. tuberculosis* pMSP12::GFP (section 3.6) as a readout is necessary.

20. The standard time period for recording growth inhibition of compounds to obtain a full MIC value, is 1 week. However, some compounds may act to slow down growth without resulting in complete growth inhibition at any time point. For these compounds, the level of growth inhibition may be time-dependent in which case a 2-week readout is beneficial. Note that interpretation of growth inhibition of compounds during extended periods of incubation is affected by compound stability in the medium and to *M. tuberculosis* metabolism. Compound stability can be determined by measuring compound concentrations in the wells by, for example, extraction into acetonitrile followed by liquid chromatography-mass spectrometry analysis.

21. Time points for growth readout under restrictive growth conditions including 7H9/butyrate/Tyloxapol (pH = 6) and 7H9/butyrate/nitrite/Tyloxapol (pH = 6) are longer than growth conditions that support florid growth. The time points for these slower growth conditions are typically 10 days as first time point and 20 days as second time point.

22. Plates are initially incubated upright to ensure that the liquid from the cell suspension is drawn into the agar. After this, plates are inverted to prevent condensation from accumulating in the lids which can fall onto the agar.

Acknowledgement

Funding for this work was provided in part by the Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does the mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

References

1. VanderVen BC, Fahey RJ, Lee W, Liu Y, Abramovitch RB, Memmott C, Crowe AM, Eltis LD, Perola E, Deininger DD, Wang T, Locher CP, Russell DG (2015) Novel inhibitors of cholesterol degradation in Mycobacterium tuberculosis reveal how the bacterium's metabolism is constrained by the intracellular environment. PLoS Pathog 11 (2):e1004679. doi:10.1371/journal.ppat.1004679 [PubMed: 25675247]

2. Stanley SA, Barczak AK, Silvis MR, Luo SS, Sogi K, Vokes M, Bray MA, Carpenter AE, Moore CB, Siddiqi N, Rubin EJ, Hung DT (2014) Identification of host-targeted small molecules that restrict intracellular Mycobacterium tuberculosis growth. PLoS Pathog 10 (2):e1003946. doi:10.1371/journal.ppat.1003946 [PubMed: 24586159]

3. Christophe T, Ewann F, Jeon HK, Cechetto J, Brodin P (2010) High-content imaging of Mycobacterium tuberculosis-infected macrophages: an in vitro model for tuberculosis drug discovery. Future Med Chem 2 (8):1283–1293. doi:10.4155/fmc.10.223 [PubMed: 21426019]

4. Darby CM, Ingolfsson HI, Jiang X, Shen C, Sun M, Zhao N, Burns K, Liu G, Ehrt S, Warren JD, Andersen OS, Brickner SJ, Nathan C (2013) Whole cell screen for inhibitors of pH homeostasis in Mycobacterium tuberculosis. PLoS One 8 (7):e68942. doi:10.1371/journal.pone.0068942 [PubMed: 23935911]

5. Mak PA, Rao SP, Ping Tan M, Lin X, Chyba J, Tay J, Ng SH, Tan BH, Cherian J, Duraiswamy J, Bifani P, Lim V, Lee BH, Ling Ma N, Beer D, Thayalan P, Kuhen K, Chatterjee A, Supek F, Glynne R, Zheng J, Boshoff HI, Barry CE 3rd, Dick T, Pethe K, Camacho LR (2012) A high-throughput screen to identify inhibitors of ATP homeostasis in non-replicating Mycobacterium tuberculosis. ACS Chem Biol 7 (7):1190–1197. doi:10.1021/cb2004884 [PubMed: 22500615]

6. Wang F, Sambandan D, Halder R, Wang J, Batt SM, Weinrick B, Ahmad I, Yang P, Zhang Y, Kim J, Hassani M, Huszar S, Trefzer C, Ma Z, Kaneko T, Mdluli KE, Franzblau S, Chatterjee AK, Johnsson K, Mikusova K, Besra GS, Futterer K, Robbins SH, Barnes SW, Walker JR, Jacobs WR Jr., Schultz PG (2013) Identification of a small molecule with activity against drug-resistant and persistent tuberculosis. Proc Natl Acad Sci U S A 110 (27):E2510–2517. doi:10.1073/pnas.1309171110 [PubMed: 23776209]

7. Grant SS, Kawate T, Nag PP, Silvis MR, Gordon K, Stanley SA, Kazyanskaya E, Nietupski R, Golas A, Fitzgerald M, Cho S, Franzblau SG, Hung DT (2013) Identification of novel inhibitors of nonreplicating Mycobacterium tuberculosis using a carbon starvation model. ACS Chem Biol 8 (10):2224–2234. doi:10.1021/cb4004817 [PubMed: 23898841]

8. Ananthan S, Faaleolea ER, Goldman RC, Hobrath JV, Kwong CD, Laughon BE, Maddry JA, Mehta A, Rasmussen L, Reynolds RC, Secrist JA 3rd, Shindo N, Showe DN, Sosa MI, Suling WJ, White EL (2009) High-throughput screening for inhibitors of Mycobacterium tuberculosis H37Rv. Tuberculosis (Edinb) 89 (5):334–353. doi:10.1016/j.tube.2009.05.008 [PubMed: 19758845]

9. Maddry JA, Ananthan S, Goldman RC, Hobrath JV, Kwong CD, Maddox C, Rasmussen L, Reynolds RC, Secrist JA 3rd, Sosa MI, White EL, Zhang W (2009) Antituberculosis activity of the molecular libraries screening center network library. Tuberculosis (Edinb) 89 (5):354–363. doi:10.1016/j.tube.2009.07.006 [PubMed: 19783214]

10. Early JV, Casey A, Martinez-Grau MA, Gonzalez Valcarcel IC, Vieth M, Ollinger J, Bailey MA, Alling T, Files M, Ovechkina Y, Parish T (2016) Oxadiazoles Have Butyrate-Specific Conditional Activity against Mycobacterium tuberculosis. Antimicrob Agents Chemother 60 (6):3608–3616. doi:10.1128/AAC.02896-15 [PubMed: 27044545]

11. Gold B, Roberts J, Ling Y, Quezada LL, Glasheen J, Ballinger E, Somersan-Karakaya S, Warrier T, Warren JD, Nathan C (2015) Rapid, Semiquantitative Assay To Discriminate among Compounds with Activity against Replicating or Nonreplicating Mycobacterium tuberculosis. Antimicrob Agents Chemother 59 (10):6521–6538. doi:10.1128/AAC.00803-15 [PubMed: 26239979]

12. Ballell L, Bates RH, Young RJ, Alvarez-Gomez D, Alvarez-Ruiz E, Barroso V, Blanco D, Crespo B, Escribano J, Gonzalez R, Lozano S, Huss S, Santos-Villarejo A, Martin-Plaza JJ, Mendoza A, Rebollo-Lopez MJ, Remuinan-Blanco M, Lavandera JL, Perez-Herran E, Gamo-Benito FJ, Garcia-Bustos JF, Barros D, Castro JP, Cammack N (2013) Fueling open-source drug discovery: 177 small-molecule leads against tuberculosis. ChemMedChem 8 (2):313–321. doi:10.1002/cmdc.201200428 [PubMed: 23307663]

13. Pethe K, Sequeira PC, Agarwalla S, Rhee K, Kuhen K, Phong WY, Patel V, Beer D, Walker JR, Duraiswamy J, Jiricek J, Keller TH, Chatterjee A, Tan MP, Ujjini M, Rao SP, Camacho L, Bifani P, Mak PA, Ma I, Barnes SW, Chen Z, Plouffe D, Thayalan P, Ng SH, Au M, Lee BH, Tan BH, Ravindran S, Nanjundappa M, Lin X, Goh A, Lakshminarayana SB, Shoen C, Cynamon M, Kreiswirth B, Dartois V, Peters EC, Glynne R, Brenner S, Dick T (2010) A chemical genetic screen in Mycobacterium tuberculosis identifies carbon-source-dependent growth inhibitors devoid of in vivo efficacy. Nat Commun 1:57. doi:10.1038/ncomms1060 [PubMed: 20975714]

14. Manjunatha UH, Smith PW (2015) Perspective: Challenges and opportunities in TB drug discovery from phenotypic screening. Bioorg Med Chem 23 (16):5087–5097. doi:10.1016/j.bmc.2014.12.031 [PubMed: 25577708]

15. Ioerger TR, O'Malley T, Liao R, Guinn KM, Hickey MJ, Mohaideen N, Murphy KC, Boshoff HI, Mizrahi V, Rubin EJ, Sassetti CM, Barry CE 3rd, Sherman DR, Parish T, Sacchettini JC (2013) Identification of new drug targets and resistance mechanisms in Mycobacterium tuberculosis. PLoS One 8 (9):e75245. doi:10.1371/journal.pone.0075245 [PubMed: 24086479]

16. Stuehr DJ, Marletta MA (1985) Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to Escherichia coli lipopolysaccharide. Proc Natl Acad Sci U S A 82 (22):7738–7742. doi:10.1073/pnas.82.22.7738 [PubMed: 3906650]

17. Blanco-Ruano D, Roberts DM, Gonzalez-Del-Rio R, Alvarez D, Rebollo MJ, Perez-Herran E, Mendoza A (2015) Antimicrobial susceptibility testing for Mycobacterium sp. Methods Mol Biol 1285:257–268. doi:10.1007/978-1-4939-2450-9_15 [PubMed: 25779321]

18. Cho S, Lee HS, Franzblau S (2015) Microplate Alamar Blue Assay (MABA) and Low Oxygen Recovery Assay (LORA) for Mycobacterium tuberculosis. Methods Mol Biol 1285:281–292. doi:10.1007/978-1-4939-2450-9_17 [PubMed: 25779323]

19. Domenech P, Reed MB (2009) Rapid and spontaneous loss of phthiocerol dimycocerosate (PDIM) from Mycobacterium tuberculosis grown in vitro: implications for virulence studies. Microbiology 155 (Pt 11):3532–3543. doi:10.1099/mic.0.029199-0 [PubMed: 19661177]

20. Slayden RA, Barry CE 3rd (2001) Analysis of the Lipids of Mycobacterium tuberculosis. Methods Mol Med 54:229–245. doi:10.1385/1-59259-147-7:229 [PubMed: 21341079]

Table 1.

MIC in μM of drugs in series of media.

Compounds	Media						
	7H9/ADC/ Tw	7H9/ Glucose/BSA/T w	7H9/ Glucose/BSA/ Tx	7H9/Glucose/ Casitone/Tx	7H9/ DPPC/BSA/ Tw	7H9/DPPC/ Casitone/Tx	7H11/OADC Agar
EMB	1	2	2	1	1	1	3.5
SM	0.25	0.5	0.5	0.25	0.5	0.25	0.5
LNZ	1.56	3.1	1.56	1.56	3.1	3.1	1.56
BDQ	1.5	5	1.5	1.5	5	1.5	1.5
MXF	0.1	0.2	0.2	0.2	0.2	0.2	0.2
INH	0.28	0.28	0.28	0.28	0.13	0.13	0.28
PA824	0.1	0.3–0.8	0.3	0.2	0.3	0.2	0.2
RIF	0.005	0.01	0.08	0.08	0.01	0.05	0.16

EMB: ethambutol; SM: streptomycin; LNZ: linezolid; BDQ: bedaquiline; MXF: moxifloxacin; INH: isoniazid; RIF: rifampicin; Tw: tween 80; Tx: tyloxapol