Hollow Fiber System of Tuberculosis

(HFS-TB)

A Laboratory Manual to Guide System Engineering, Study Design and Execution

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List of Abbreviations

%T	Percentage of time
AUC	Area under the curve
BSC	Biological safety cabinet
BSL	Biosafety level
CFU	Colony Forming Units
CLSI	Clinical and Laboratory Standards Institute
DMSO	Dimethyl sulfoxide
EMA	European Medicines Agency
FBS	Fetal bovine serum
FDA	Food and Drug Administration
HFS-TB	Hollow Fiber System of Tuberculosis
ID	Internal diameter
MHA	Muller Hinton agar
MIC	Minimal Inhibitory Concentration
MOI	Multiplicity of infection
OD	Optical Density
PAPR	Powered air purifying respirators
PI	Principal Investigator
PK/PD	Pharmacokinetic/Pharmacodynamics
PPE	Personal protective equipment
RLU	Relative luminescence units
SDA	Sabouraud Dextrose agar
$T_{1/2}$	Half-life
XDR-TB	Extensively drug resistant tuberculosis

1 Introduction

Tuberculosis continues to be a top killer of mankind, and is responsible for 3% of all deaths in some low and middle-income countries.[1,2] Short course chemotherapy is the preferred approach for drug susceptible tuberculosis and lasts for 6 months in the simplest pulmonary tuberculosis cases but up to 9 months in diseases such as tuberculous meningitis.[3,4] Treatment of multidrug resistant tuberculosis can be long, and last up to two years. It has now been demonstrated that currently used treatment regimens employ non-optimized doses, and concentrations that are not optimized for synergy or additivity.[5–16] Therefore, when developing new treatment regimens, it will be necessary to apply rigorous pharmacokinetic/pharmacodynamics (PK/PD) principles.[17] In recent years, the development of virtually incurable tuberculosis has left some patients therapeutically destitute.[18] Therefore, there has been a recent commitment to develop shorter duration treatment regimens for drug susceptible and drug resistant tuberculosis, and newer therapies for incurable tuberculosis.

Although animals models have traditionally been used to study the effects of anti-tuberculosis drugs, more PK/PD studies have been performed with the hollow fiber system model of tuberculosis (HFS-TB).[5–9,13–15,19–31] The HFS-TB was developed in 2001 and results of experiments were first presented for rifampin PK/PD studies in 2003.[32]

The intended use of the HFS-TB is to study the effect of anti-tuberculosis drugs on *Mycobacterium tuberculosis*. It is not intended as a tool to study the immunology of the disease, nor the molecular nuances of different mycobacterial physiological states. Therefore, its utility is primarily the study of the effects of different doses of antibiotics on *M. tuberculosis* and the interaction of these antibiotic doses with each other.

The advantages of the HFS-TB include, but are not limited to:

1. The ability to mimic the concentration-time profile of drugs as observed in tuberculosis patients, both in plasma and at the site of infection. Bacterial responses to dynamic concentrations of drug differ from responses to the static concentrations used in test tubes, tissue culture plates or solid agar plates.

2. The ability to mimic different metabolic or physiological populations of *M. tuberculosis,* and the effect of dynamic drug concentrations on these bacteria. While the exact physiological populations encountered in tuberculosis lesions in humans is unknown, it is presumed that some of the bacteria are in log-phase growth, some are semi-dormant, and some are non-replicating persisters. These approximations emerged as part of inductive generalizations to effects of individual drugs in the sputum of patients with pulmonary tuberculosis, but are concordant with interpretations of some limited observations in human sputum and even more limited observations in human lungs.[32]

3. The ability to quantify drug susceptible and drug resistant subpopulations. For example, a distinct advantage of the HFS-TB over rodent tuberculosis models is the ability to optimize inoculum burden to enable study of the potential emergence of drug resistance.

4. The ability for repetitive sampling from the same HFS-TB system to assess the quantitative changes of drug susceptible and drug resistant bacterial burden in relation to drug concentration-time profiles. In contrast, a limitation of animal models is that sampling for quantifying drug

susceptible and/or drug resistant isolates is a terminal procedure. Repetitive sampling in the HFS-TB vastly improves statistical power, time-to-event analysis and repeated event analysis.

5. The ability to culture the entire contents (approximately 20 mL) of the peripheral compartment of the HFS-TB at the end of an experiment to enable the assessment of a given compound's potential to completely eradicate *M. tuberculosi*s at early time points (i.e., one or two months). The data from the HFS-TB can inform the likely time point in a clinical trial setting that can be considered for proof of efficacy rather than relying on relapse rates (as is current practice) from *in vivo* models of infection.

6. The ability to test two and three drug combinations, at different doses for each drug, in the HFS-TB. Antibiotics may exhibit efficacy linked to either peak or area under the concentration-time curve (AUC) or percentage of time (%T) that the concentration persists above the minimal inhibitory concentration (MIC). In combination therapy, the antibiotics may exhibit concentration-dependent synergy, additivity or antagonism, which may be linked to peak, AUC, and $\%T_{MIC}$ exposures. Thus, the ability to test two and three drug combinations, at different doses for each drug, in the HFS-TB, allows identification of dose and concentration-dependent synergy or antagonism in a more tractable fashion than in the thousands of animals that would be required to identify the full exposure-response surfaces to drug combinations.

In 2014, the forecasting accuracy of the HFS-TB underwent formal examination by the Food and Drug Administration (FDA) and European Medicine Agency (EMA).[33,34] In this assessment, the HFS-TB model was found to have an accuracy of 94% in predicting optimal doses for the clinic, optimal PK/PD drug exposures, susceptibility breakpoints, and emergence of drug resistance.[35–39] FDA has inserted specific language on the use and value of the Hollow Fiber System model in its Guidance to Industry on Pulmonary Tuberculosis: Developing Drugs for Treatment. [40] In 2015, EMA qualified this model as a validated tool for drug development use, a distinction that has not yet been conferred on animal models of tuberculosis.

The EMA Qualification Opinion for the HFS-TB can be found at:
http://www.ema.europa.eu/docs/en_GB/document_library/Regulatory_and_procedural_guideline/2015/02/WC500181899.pdf

1.1 Model description

A schematic of the HFS-TB is shown in Figure 1-1 and a representative set up is shown in Figure 1-2. The HFS-TB model consists of (a) a peripheral or pharmacodynamic compartment for the *M. tuberculosis* inoculum and (b) a central drug containing or pharmacokinetic compartment. The hollow fibers are semipermeable fiber bundles with pores of different molecular-weight cutoffs (typically 40 kDa, but can be different based on experimental requirements) that are encased in a plastic cartridge. Several different types of fibers are used, with the most common being cellulosic and polysulfone fibers. The specific choice is based on the physicochemical properties of the drugs under study (e.g., charge, how easily the drug sticks to fibers, etc.). The cartridge space outside the capillary lumina (or the space surrounding the hollow fibers) is termed the "peripheral compartment", and generally represents a volume of 20 mL. The study isolate, e.g., extracellular or intracellular *M. tuberculosis,* is inoculated into the peripheral compartment, and the culture suspension is maintained therein for the duration of the experiment. Fresh media is circulated within the central compartment via a computer controlled peristaltic pump. Nutrients, which

are small molecules, diffuse from the central into the peripheral compartment. Used media is removed from the system at a rate equal to the inflow of fresh media, into a sealed container that has bleach to make up a final concentration of ≥ 10%. The media inflow and outflow rates also dictate the half-life of the drugs used in the treatment. Drugs are administered to the central compartment via a syringe pump which is programmed (based on experimental needs) to mimic desired peak concentration and time to peak concentration encountered in patients. The drug(s) diffuse across the hollow fibers into and out of the peripheral compartment via first-order kinetic principles. The HFS-TB can be used to mimic a single drug profile or combination drug profile in the central compartment with a half-life that mimics that encountered in lungs of patients. *M. tuberculosis* cells are too big to pass into the central compartment and thus are confined to the peripheral compartment.

Figure 1-1 HFS-TB Schematic

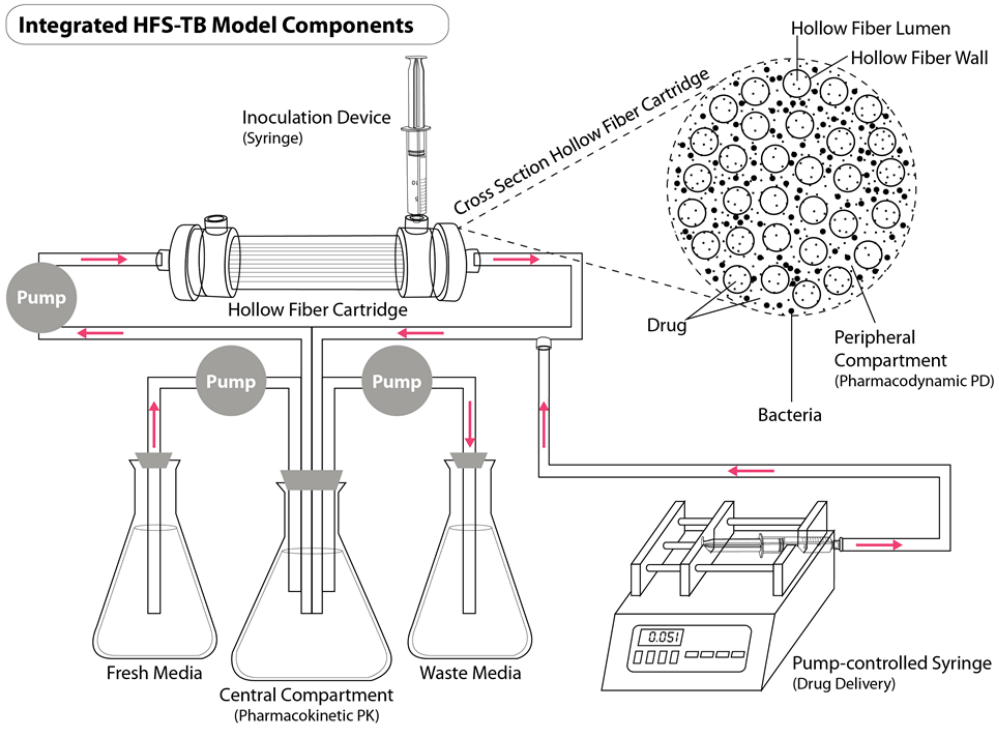
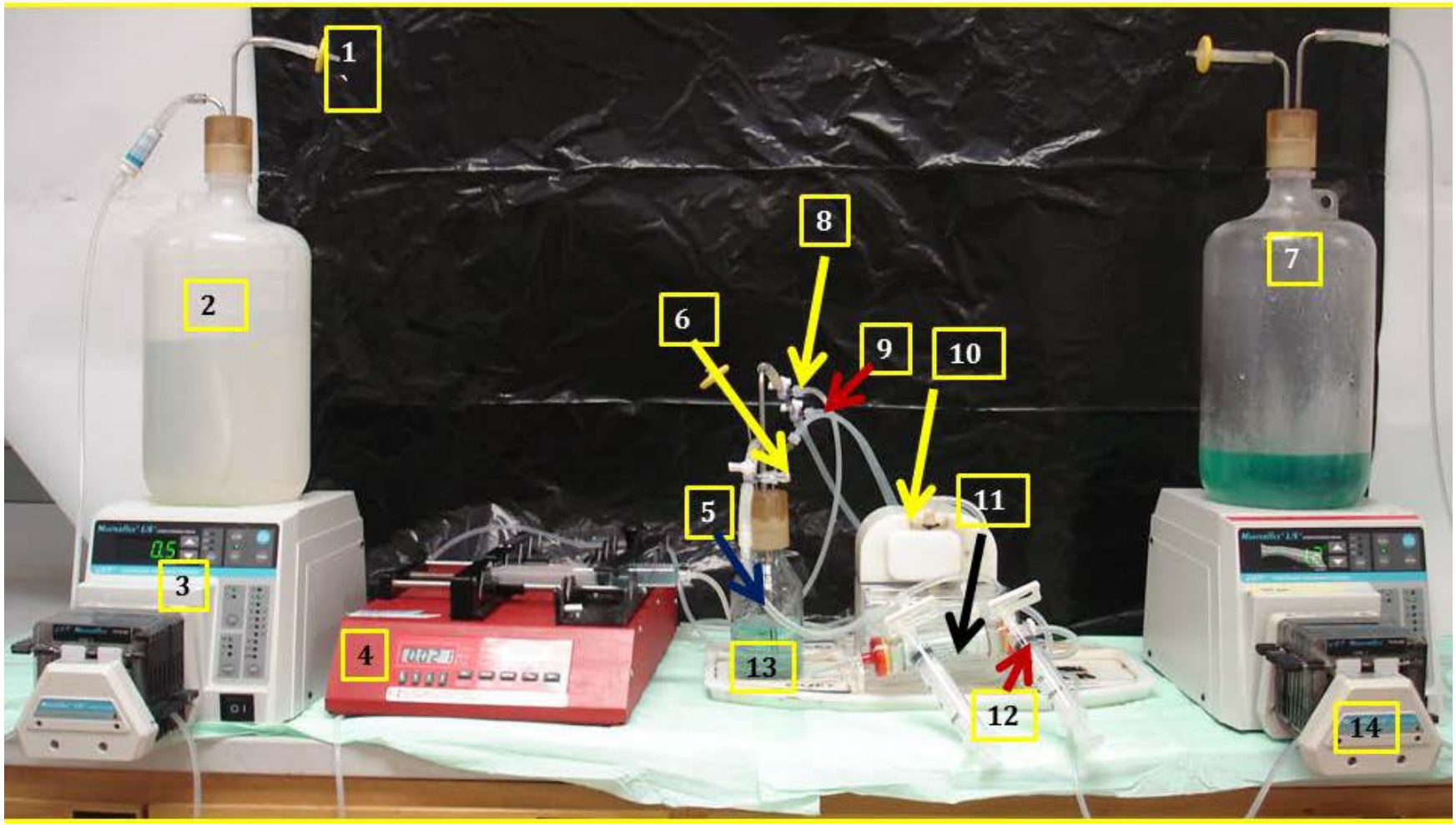


Figure 1-2 Representative Assembly of the HFS-TB



A representative assembly of the HFS-TB describing the apparatus used to put together one complete system: 1) Air filter; 2) Fresh media reservoir; 3) Pump for fresh media inflow; 4) Syringe pump for drug infusion; 5) Central reservoir outflow port connected to the inflow of the hollow fiber cartridge inflow for fresh media and drug supply to the cartridge; 6) Drug pharmacokinetics sampling port; 7) Used media reservoir; 8) Port to infuse fresh media to the central reservoir; 9) Ports to infuse the drugs to the central reservoir; 10) Duet pump; 11) Hollow fiber cartridge; 12) Syringes for peripheral compartment sampling; 13) Central reservoir; 14) Pumps for used media out flow.

2 HFS-TB Study Design

2.1 General considerations

The following describes the general study design for a typical set of HFS-TB experiments. However, differences in laboratory setup and considerations regarding drug(s) being tested and analyses to be performed will necessitate customization and modification of the design in the manual. This will include, for example, preliminary experiments to determine optimal conditions for each drug to be studied within the HFS-TB to ensure that proper concentration-time profiles can be achieved. Details of preliminary experiments for customizing design are not included in the manual.

2.2 The number of HFS-TB units used

The number of HFS-TB (i.e., individual systems) used depends on the study design. The basic designs used to examine the effect of drug include dose-ranging (viz., dose-effect) studies, dose-fractionation studies, and optimal design of combination regimens. Each experimental data point (i.e., dose, frequency, etc.) is repeated in triplicate in separate HFS-TB units.

2.3 Performance of dose-ranging studies

Principles of PK/PD design of anti-tuberculosis agents have been published.[35] The 4 parameter Hill-model, i.e., the inhibitory sigmoid E_{max} model for dose versus microbial kill, mandates that at least 5 doses be tested.[41,42] For increased precision we recommend at least 8 doses, each performed in triplicate. Thus, at a minimum 24 HFS-TB units need to be set up for dose ranging studies. The HFS-TB is set up as described in Section 5.

Specific considerations are as follows:

1. Dose-ranging studies are performed under all growth conditions (e.g., extracellular, intracellular log phase; semi-dormant), each one iteratively optimizing the next, while dose fractionation is performed with one growth condition.
2. The design supports full parameter estimation for the inverted "U" curve model of Tam et al., and the Gumbo quadratic function for drug resistance emergence.[19,43]
3. Dose-ranging studies for microbial kill should be performed at the same time as studies for resistance emergence. This is mandatory for proper dose selection, and forestalls emergence of acquired drug resistance such as extensively drug-resistant tuberculosis (XDR-TB), foretold by HFS-TB studies in the past.[6,7,44]
4. Total bacterial burden in the HFS-TB can be determined by sampling the peripheral compartment and performing quantitative culture on solid media (CFU/ml determination) and/or liquid culture (i.e., Time to positivity [TTP] via Mycobacteria Growth Indicator Tube [MGIT] assay).
5. For quantifying the drug resistant subpopulation, the same serial dilutions for the quantitative culture are placed on Middlebrook 7H10 agar, supplemented with antibiotic concentrations of the drugs under study. The antibiotic concentration is often the critical concentration but may be any concentration (selected by investigators) based on the mechanism of resistance for that drug.
6. Actual concentrations of investigational drug(s) in serial samples collected across the dosing interval from the HFS-TB central compartment are determined via an appropriately qualified

bioanalytical method (typically LC-MS/MS). In case of the intracellular TB experiments, both intracellular and extracellular drug concentrations are measured.

7. The CFU/mL are analyzed in (a) inhibitory sigmoid E_{max} models, (b) quadratic function for drug resistance, and (c) inhomogeneous differential equation systems pharmacology models (see Gumbo et al.[6] for full list of equations and parameters), together with the actual drug concentrations achieved in the systems (not the intended concentrations).

2.4 Performance of dose-fractionation studies

The purpose of these hollow fiber studies is to identify the PK/PD index linked to optimal microbial kill or resistance suppression. Microbial kill parameters are based on total microbial burden identified, while resistance emergence and suppression is defined by the size of the bacterial burden on antibiotic supplemented agar.

The dose-fractionation experiment is intended to use different dosing frequencies to separate exposure and time-dependent parameters. Without dose fractionation, each of these factors would increase proportionately with dose in a linear fashion. For example, different frequencies of administration can result in identical exposure parameters (e.g., equal AUC with once daily dosing versus splitting the dose into two doses) but have very different concentration-time profiles. Thus, by using multiple different dose and frequency combinations, the co-linearity between peak concentration, AUC and % time concentration persists above MIC is disrupted.

Specific considerations are as follows:

1. The drug exposures to be used for dose fractionation must not be arbitrarily chosen or chosen for convenience. Since optimal Fisher information is expected to occur at EC_{20}, EC_{50}, and EC_{80}, these drug exposures should be identified in dose-effect in Section 2.3, and then used in dose-fractionation.
2. The drug's half-life must be taken into consideration in designing the dosing interval for dose fractionation studies.
3. The most optimal PK/PD index (e.g., AUC/MIC, Cmax/MIC, %T above MIC) is then chosen through standard PK/PD analyses.
    a. Briefly, each parameter is used as a response variable in an inhibitory sigmoid E_{max} model (total CFU/mL versus PK/PD index), or quadratic function for resistance (drug resistant CFU/mL versus PK/PD index).
    b. The index with the lowest Akaike information criterion or Bayesian information criterion is selected as the optimal index.
    c. A full description of the supportive mathematical analyses is beyond the scope of the manual, please refer to the citations for further information.

3 Biosafety considerations for working with *Mycobacterium tuberculosis*

Assembly of the HFS-TB systems and execution of all HFS-TB experiments must take place inside a biosafety cabinet (Class II, BSC) that resides in a biosafety level 3 (BSL3) laboratory. During incubation of peripheral compartment cultures, the HFS-TB systems are placed in incubators (with glass door or window in door), which are also in the BSL3 laboratory. Entering, exiting, and working within the BSL3 laboratory is in accordance with the standard biosafety policies and procedures established by the institution's BSL3 Lab Committee or Environmental Health and Safety Office and are consistent with the WHO TB Laboratory Biosafety Manual (2012). [45]

3.1 Aseptic techniques pertaining to HFS-TB

3.1.1 Adding culture suspension to the cartridge

1. Each HFS-TB cartridge mounted on its duet pump is brought into the biological safety cabinet (BSC).
2. All surfaces of the HFS-TB setup, as shown in Figure 1-2, are wiped with 10% bleach (prepared fresh daily).
3. *M. tuberculosis* cultures are loaded into the peripheral compartment with a loading syringe using the following steps:
   a) First, 20 mL of the culture is aspirated into a sterile needleless syringe with Luer lock.
   b) The loading syringe is then Luer locked onto the loading port of the HFS cartridge, to form a sealed connection. The *M. tuberculosis* culture is then slowly loaded into the peripheral compartment. After that, the loading port is locked, and syringe removed.
   c) After removal, the loading syringe is then dipped into 10% bleach, 20 mL of bleach gently aspirated, and the 20 mL then slowly dispensed into a waste container of bleach. The syringe is then discarded into a biohazardous waste container.
   d) A new sterile 20 mL syringe is then added to the loading port, which stays locked at all times.
   e) The cartridge and pump base are then wiped with 10% bleach. Wait 10 minutes then wipe the cartridge with 70% ethanol. The system is then taken out of the BSC and set in the incubator.
   f) In the event of a spill, the affected areas are disinfected with 10% bleach. Each and every spill event should be reported to the PI and the biosafety officer.

3.1.2 Removing culture samples from the cartridge

Since this is a potential aerosol generating maneuver, this step takes place in the BSC.

1. Sampling times for peripheral compartment depend on the experiment being performed and are also chosen based on optimal experimental design derived from statistical criteria. A commonly used schedule is once every 7 days.
2. For sampling, each system is disconnected from electricity, so that media stops circulating.
3. The hollow fiber system is then moved to the BSC, and the base of assembly as well as the cartridges and tubing wiped with 10% bleach.

4. Contents of the peripheral compartment are then mixed using the two syringes attached permanently to the sampling ports. If there is air in the peripheral compartment, it is removed by simply pulling back on one of the syringes and then locking; **but the syringe should NOT be removed, and air purged out because this is a high aerosolization risk with high burden *M. tuberculosis***. At this stage, the peripheral compartment has not yet been opened and the culture is still contained within the cartridge.
5. The sampling ports are then locked, and one of the 20 mL syringes removed while the sampling ports are still locked.
6. A 3 mL Luer lock syringe (no needles) is attached; the port is unlocked, and *M. tuberculosis* culture slowly withdrawn into the syringe. The exact sample volume to be collected depends on required quantitative output, and assays to be performed on the cultures. In general, sampling should not exceed 10% of the total volume in the peripheral compartment.
7. The culture is then dispersed into aerosol tight leak-proof screw cap tubes for further testing, e.g., bacterial load determination, and, if required by study design, extraction of nucleic acid and proteins.
8. Then 10% bleach is aspirated into the 5 mL syringe used for sampling and discarded into a biohazardous waste container with bleach.
9. The 20 mL syringe is then returned to the locked sampling port and Luer locked.
10. The hollow fiber system cartridge and pump base as well as the locked sampling port are then wiped with 10% bleach and taken back to the incubator.
11. In the event of a spill, the affected areas are disinfected with 10% bleach. Each and every spill event should be reported to the PI and the biosafety officer.

3.1.3 Handling the cartridge at the end of the experiment

1. At the end of the HFS-TB study, each system is first disconnected from electricity, so that media stops circulating.
2. The HFS-TB is then moved to the BSC, and the base of assembly as well as the cartridges and tubing wiped with 10% bleach.
3. Next, 20 mL of concentrated bleach is added (via a syringe) to the peripheral compartment to make a final concentration of 10% bleach based on the total volume in the system.
4. All surfaces of the HFS-TB setup, are wiped with 10% bleach and taken out of the BSC.
5. The system is then allowed to circulate for a day, but with no fresh media or removal of media (i.e., no dilution).
6. After 24 hours, the hollow fiber cartridge and its tubing are then placed in autoclave bags and autoclaved.

3.2 Specific biosafety response to HFS-TB cartridge failure in BSL3 incubator

1. After inoculation of mycobacteria, the HFS-TB is maintained within the incubator. Any cartridge spill during this time would occur within the incubator.
2. Each day, after inspecting the tubing, the laboratory staff inspects the HFS-TB through the

window of the incubator. **In the event that a leak of a *M. tuberculosis* culture from the peripheral compartment is observed, DO NOT OPEN THE INCUBATOR DOOR. Inform EVERYBODY within the BSL3 lab to exit**.

3. The power to the HFS-TB experiments within the affected incubator would be turned off to stop fluid circulating and stop any further leaks. Note, the electrical outlet would be outside the incubator.
4. The staff in the BSL3 lab should exit the BSL3 lab immediately, correctly removing and disposing of their protective apparel (gown, respirator, shoe covers, head cover, and gloves).
5. Post signs so that others will not enter the contaminated area.
6. Call the principal investigator (PI) and biosafety officer.
7. The PI and biosafety officer will douse towels in bleach solution. These individuals will then inspect the HFS-TB cartridges through the incubator window. After satisfying themselves as to the location of the leak, they will come up with a disinfectant plan. The general plan is as follows:
   a) Open the incubator door slowly.
   b) Place absorbent material on top of the spill, making sure material is in contact with spill. DO NOT RUB the absorbent material across the spill. If the area of the spill is greater than the area of the absorbent pad, use more pads.
   c) Allow absorbent material to absorb the spill. Visually inspect the other items in the incubator for splashes and douse with 10% bleach if appropriate.
   d) Wipe the outside of the HFS-TB cartridge with towels doused in 10% bleach. A second person will disconnect cartridge away from the leakage areas without touching leakage areas, and the cartridge will be placed in a biohazardous waste bag.
   e) Disinfect gloves. Then, using forceps, pick-up and place the absorbent material in the leakage area in the incubator into the small biohazardous waste bag.
   f) Next, flood the area of the leak in the incubator with 10% bleach, and then wait for 15 minutes before wiping off with paper towels doused in alcohol. Then, place these paper towels in small biohazardous waste bag.
   g) Disinfect gloves, remove them and place in biohazard bag.
   h) Put on new gloves.
   i) Spray the outside of the bag with disinfectant.
   j) Seal the biohazards waste bag with autoclave tape.
   k) Immediately proceed to autoclave.
   l) The PI will then notify the biosafety officer.

3.3 Personnel training on HFS-TB use in the BSL3 laboratory

Personnel working with the HFS-TB MUST be trained on both the HFS-TB system and BSL3 laboratory procedures. Thus, they must be proficient in both sets of procedures before carrying out experiments with *M. tuberculosis* in the BSL3 laboratory.

3.3.1 HFS-TB engineering training

Personnel are first trained in basic laboratory aseptic techniques and how to work inside a biosafety cabinet. Personnel are then trained on HFS-TB assembly, HFS-TB maintenance, HFS-TB sampling, changing of media bottles and syringes, programing of syringe pumps, and trouble-shooting. This hands-on training initially takes place in a BSL2 laboratory with Level 2 pathogens, such as *M. tuberculosis* H37Ra or a slow-growing mycobacterium strain, and standard TB drugs. Replicates should be setup within the experiment, as well as replicate experiments performed. Expected experiment and quality control results must be consistently observed. Training may take up to one year. Competency in aseptic techniques and HFS-TB engineering must be demonstrated to (observed by) the PI.

3.3.2 BSL3 laboratory training

Training includes the institution's biosafety policies and procedures and the procedures for working with *M. tuberculosis*. The training must be supervised, documented and signed by someone competent in both BSL3 laboratory procedures and HFS-TB construction. Re-training will occur every 2 years for each laboratory member. Training includes the following topics:

1. Potential risks to health, the symptoms of tuberculosis, *M. tuberculosis* disease transmission, methods used to monitor infection, and potential treatment of tuberculosis.
2. Precautions to be taken to minimize aerosol formation and prevent exposure.
3. Wearing and use of protective equipment and clothing, specifically powered air purifying respirators (PAPR).
4. Handling of *M. tuberculosis* cultures.
5. Laboratory design, biological safety cabinet levels and inspection, and airflow conditions.
6. Prevention of incidents and steps to be taken by workers in the case of incidents (biohazard incidents, chemical, electrical and fire hazards).
7. Waste management.
8. Use of anemometer (operation, identification of malfunctions, maintenance).

3.3.3 HFS-TB work inside the BSL3 laboratory

This training specifically emphasizes study design, HFS-TB construction and upkeep, sampling procedures, and HFS-TB spill procedures inside the BSL3. Training must be supervised, documented and signed by someone competent in BOTH BSL3 and HFS-TB construction. The institution's biosafety officer then certifies personnel to perform work with high burden cultures of *M. tuberculosis*, after observing the personnel demonstrate safety when working on the HFS-TB setup with *M. tuberculosis*.

4 Materials

The following Table 4-1 lists some specific materials that are required for the HFS-TB experiments and should be viewed with the following considerations:

- All items are available from several vendors and manufacturers, with generally no specific preference for the materials; indeed, they are used interchangeably.
- Ancillary supplies such as bleach, 70% ethanol, etc. will be required and should be identified in advance by each laboratory.
- Exact tubing length required depends on the physical setup of the lab, size of the BSL3 suite, the physical distances between where fresh media and outflow bottles sit relative to incubator, the volume of drug to be used, and the internal diameter of the tubing used.

Table 4-1 Materials required for HFS-TB system

Item
Middlebrook 7H9 broth
Middlebrook 7H10 or 7H11 agar
OADC supplement
RPMI-1640 medium
Fetal bovine serum (heat inactivated)
Dextrose
Tween-80
Citric acid, anhydrous
Hollow fiber cartridges
4 L size plastic autoclavable bottles
Central reservoir bottles
Central reservoir caps (level adjusted)
Media bottles (1 gallon)
Media bottle caps
14 gauge silicone tubing
13 gauge silicone tubing
Male Luer lock ring to barb
Female Luer lock ring to barb
3-way stopcock
4-way stopcock
20 mL syringes
3 mL syringes
1 mL syringes
Syringe pump
Peristaltic pump (recommended: Masterflex series, Cole-Palmer)
Peristaltic pump head (recommended: Masterflex series,Cole-Palmer)
Peristaltic pump cassette (recommended: Masterflex series, Cole-Palmer)
Hollow fiber base pumps
0.22 μM filters
Calibrated digital pH meter
Dissolved-O_2 sensor
O_2 4,100-ppb transmitter
Study Drugs

4.1 Media preparation

Preparation of solid and liquid media takes place in a BSL2 laboratory. This can be accomplished with automated plate and tube dispensers (there a several commercial ones on the market), or manually using pipettes. After preparation, the media is stored or moved to the BSL3 laboratory for HFS-TB experiments.

4.1.1 Preparation of Middlebrook 7H9 liquid medium

1. Dissolve 4.7 g of Middlebrook 7H9 powder in 900 mL dH_2O, per powder manufacturer's instructions.

2. Add 2 mL glycerol and mix well.
3. Autoclave at 121^{0}C for 15 min and let cool completely (4 L size plastic autoclavable bottles are utilized).
4. After cooling, place media in an incubator at 37^{o}C for 2 days and check all containers for contamination.
5. Store medium at room temperature.

   ***Note**: Do not use medium that has been stored for longer than a month.*
6. Right before use, add 2.5 mL 20% Tween-80 (filter sterilized) and 100 mL of OADC supplement.

   ***Note**: If the OADC is added to the sterile medium while the medium is > 55^{o}C, the components of the OADC, such as dextrose, will degrade.*
7. At this point the media can be filter sterilized or used directly.
8. Media to which the supplement and Tween-80 have been added should be stored at 4^{o}C and used within two weeks.

4.1.2 Preparation of Middlebrook 7H10 agar plates

1. Dissolve 19 g of 7H11 powder in 900 mL dH_2O, per manufacturer's instructions.
2. Add 5 mL glycerol and swirl to obtain a smooth suspension.

   ***Note**: Boil if necessary to completely dissolve the powder.*
3. Autoclave at 121°C for 15 min.
4. Add 100 mL Middlebrook OADC Enrichment and 2.5 mL 20% Tween-80 (filter sterilized) to the medium when cooled to 50-55^{o}C.
5. Mix well and pour approximately 23 mL into each petri dish/plate. This volume is a general guide and can be varied depending on the size of the plates in use.
6. Agar plates should be stored at 4^{o}C in aseptic conditions (in clean plastic bags or boxes) and used within two weeks.

4.1.3 Preparation of antibiotic containing agar

The protocol to prepare the liquid or solid agar plate is the same as described in the previous section.

1. Prepare the drug to be added to the medium to 1000x concentration, filter sterilize and then add 1 mL of the drug per 1 L medium. As an example, to make 1.0 mg/L rifampin, make 1,000 µg/mL, sterile filter, and then add 1 mL to the 1L of media.

   ***Note**: The drug should be added to the agar medium when cooled to 55^{0}C. Additional considerations for stability and light sensitivity etc. depend on the particular drug being tested.*

2. Selection of drug concentration is based on the following:

   The MIC of the strain used by each laboratory should be identified using methods specified by Clinical and Laboratory standards,[46] and used to derive the laboratory specific 3x MIC concentration (Concentration #1 in Table 4.2) . The 3x MIC value is used to capture efflux pump

induced resistance in the absence of chromosomal mutations.[9,47] Concentration #2 in Table 4.2 is the critical concentration used by the CLSI for those drugs tested[46]. Concentration #3 in Table 4.2 is defined as an exposure-response based metric, the MIC breakpoint, as identified using PK/PD studies and computer-aided clinical trial simulations as those MICs above which patients fail therapy.[48–50] These have been validated in prospective clinical trials.[50–53] For new drugs, the initial critical concentrations will be those derived using PK/PD studies, per examples published in the literature.[51] The concentrations chosen in Table 4.2 are examples used in past studies, and not prescriptive. Investigators will choose their own concentrations as justified by the specific aims of their experiments.

Table 4-2 Final concentrations and storage conditions of routinely used drugs

Drug[a]	Concentration #1	Concentration #2 (mg/L)	Concentration #3 (mg/L)	Storage conditions
Rifampin[51–55]	3x MIC	1.0	0.125	0°C; maximum 24 hour storage
Isoniazid[51,52]	3x MIC	0.2	0.0312	0°C; maximum 72 hour storage
Pyrazinamide[50,51]	3x MIC	100	50	0°C; maximum 72 hour storage
Ethambutol[51,56]	3x MIC	5	4	0°C; maximum 72 hour storage
Moxifloxacin	3x MIC	2	-	Room temperature; 7 days

[a]Superscript refers to references for Concentration #3.

4.1.4 Preparation of acidified medium

The protocol for preparation of the acidified medium is the same as in Section 4.1.1., except that anhydrous citric acid is used to lower the pH of the medium. The medium should be pH 5.8.

1. Add 720 mg/L citric acid before autoclaving the media.
2. Measure the pH using a sensitive and appropriately calibrated pH meter. Add more citric acid if required via titration and re-assess pH. Autoclave the medium.
3. For liquid medium add 20 g/L dextrose and filter sterile the medium before use.
4. For solid agar plates, add 100 mL of 10% OADC supplement when the medium is cooled to 55°C.
5. For drug-supplemented agar, add as instructed in Section 4.1.3.

4.2 Media to support anaerobic culture experiments

Middlebrook 7H9 broth is prepared as described above. The media is placed in large, custom built anaerobic chambers, and shaken every 12 hours for up to 4 days prior to use. The anaerobic chambers are custom equipment, which are very large at up to 24 feet in size. Consequently, execution of anaerobic culture experiments is highly specialized and dependent upon specific institutional infrastructure. HFS-TB setups are assembled inside the anaerobic chambers as described in previous sections and with appropriate modifications as required for each specific lab.

4.3 Drug solution preparation for drug delivery pumps

Drug dissolution is dependent on the physicochemical properties of the drug being utilized. Drugs that are purchased from commercial sources (e.g., Sigma-Aldrich) are supplied with information on solvents and storage will be pre-specified by the vendors.

The standard drugs – isoniazid, rifampin, ethambutol, and pyrazinamide – are prepared as follows:

1. Isoniazid, ethambutol, and pyrazinamide are dissolved in water and then filter sterilized using 0.22uM filters.
2. Rifampin is first dissolved in 100% DMSO then filter sterilized using 0.22 μM nylon filters and back diluted in media or water in such a way that the final concentration of DMSO becomes less than 1%, which is non-toxic to *M. tuberculosis*.

When a drug is first introduced to the laboratory, it should be tested for stability once reconstituted in room air and at 37°C. The drug is dissolved and kept under incubation conditions like those which will be used during the experiment. During the incubation, the drug concentration is measured at multiple time points overs several days using a suitable assay such as LC-MS/MS. The stability of the drug will determine how often syringe pumps are loaded with drug. For example, stable drugs such as isoniazid would have the drug containing syringes changed once every 7 days.

5 HFS-TB engineering and assembly

5.1 *M. tuberculosis* inoculum preparation

5.1.1 Log-phase growth in 7H9 culture medium

1. Pipet 10 mL Middlebrook 7H9 broth supplemented with 10% OADC and 0.0025% Tween-80 (v/v), as described in Section 4.1.1, into a 15 mL screw cap culture tube.
2. Thaw the frozen vial of *M. tuberculosis* stock culture in a 37°C water bath. The bacterial burden in the expanded stock culture depends on the final bacterial density achieved when the stock culture was originally expanded. Typical densities used include 6.0, 7.0, or 8.0 $\log_{10}$ CFU/mL. The specific density is chosen based on HFS-TB experimental conditions for that particular study.
3. Wipe clean the outside of each stock vial with 70% ethanol and aseptically transfer the entire contents (~1 mL) into the 15 mL culture tube containing the media from Step 1.
4. Incubate the culture with gentle shaking or rolling in the incubator at 37°C until desired optical density at a wavelength of 600 nm (OD_{A600}) is achieved. This varies from 0.08 to 1.0 depending on the type of experiment designed. The culture can now be expanded generate the larger volume needed for a hollow fiber study, depending on the number of HFS-TB units in the study.
5. Check for any bacterial or fungal contamination in the inoculum by plating a portion of the culture on Muller Hinton agar (MHA) and Sabouraud Dextrose agar (SDA), respectively. SDA and MHA are then incubated at 35°C for 48 hours. Other types of agar, such as blood agar can be used, depending on the laboratory. Although longer incubation durations may be more appropriate for detection of fungal contamination, the 48-hour duration is utilized to support the real-time decision-making requirements in the presence of suspected contamination.

6. To expand the culture to experimental volume, use 500 mL sterile, aerosol-tight culture flasks. Do not fill more than half the capacity of the flask. Dilute the culture 10-fold in the culture medium and incubate at 37°C with gentle shaking or rolling in the incubator for 4 days.
7. Before using the culture, as prepared in Step 4, check the OD again and check for contamination on MHA and SDA.
8. Dilute to the required OD for each experiment. This will depend on the experiment being performed. Check the final bacterial density of the culture by measuring the OD_{A600}. Also, determine the proportion of viable and non-viable *M. tuberculosis* in the culture from Step 4 using the LIVE/DEAD bacterial viability assay (see Section 5.1.4). In general, an OD_{A600} of ~0.01 easily yields a starting inoculum of 6-7 $\log_{10}$ CFU/mL. However, the relationship between OD_{A600} and *M. tuberculosis* burden should be established by each laboratory using their own spectrophotometers, and local techniques. If the OD is low, then incubate the culture for 4 more days**.** If the OD is higher than desired, dilute in Middlebrook broth to the desired OD, and recheck using OD method and LIVE/DEAD bacterial assay.

5.1.2 Semi-dormant growth in acidic conditions (pH 5.8)

Semi-dormant growth in acidic conditions is performed as described in Gumbo et al., 2009.[19]

1. Begin with a contaminant-free log phase growth culture and follow Steps 1-7 in Section 5.1.1.
2. Inoculate the acidified Middlebrook broth, as prepared in 4.1.3, with *M. tuberculosis* from Step 1. Then incubate at 37°C with gentle shaking or rolling in the incubator for 4 days.
3. Check for any bacterial or fungal contamination in the inoculum by plating a portion of the culture on MHA and SDA, respectively, as described above.
4. Dilute to the required OD for each experiment using acidified Middlebrook 7H9. The final inoculum will depend on the experiment being performed. Verify the final bacterial burden using OD and the LIVE/DEAD bacterial viability assay, as described above.

5.1.3 Growth in THP-1 cell culture

Intracellular growth within the THP-1 human monocyte cell line is performed as described in previous publications.[22–24,28,29,47,57–62]

1. Prepare RPMI-1640 medium with 10% FBS and keep at 37°C with 5% CO_2.
2. THP-1 cells are propagated in RPMI-1640 medium with 10% FBS at 37^{o}C to log phase growth and checked for mycoplasma contamination using a standard method. There are several mycoplasma commercial kits on the market, and each comes with its own specifications and methods.
3. Following Steps 1-8 in Section 5.1.1, prepare a log-phase culture of *M. tuberculosis* in RPMI-1640 medium with an OD_{A600} of 0.6, that should yield approximately 10^6 CFU/mL. The bacterial burden of the culture, used for the inoculum, depends on the type of experiment to be performed, e.g., may be different for pediatric TB models.
4. Dilute the THP-1 cultures in trypan blue (a ratio of 1:10) and using a hemocytometer as well as an automated cell counter to count the viable THP-1 cell number. Adjust the final cell number to $1.5x10^6$ THP-1 cells/mL.

5. Use log-phase growth *M. tuberculosis* culture (Step 3 above) to infect the THP-1 cells, incubate over 4 hours at 37°C with 5% CO_2. The standard multiplicity of infection (MOI) is 1:10 (bacteria to macrophage ratio) but can be varied depending on the experiment being performed, e.g., MOI of 1:1 is used for HFS-TB experiments ≥ 4 weeks.
6. Once the desired MOI is achieved, transfer the cells to 50 mL centrifuge tubes and spin at 1,000 rpm for 5 min at room temperature. Remove the supernatant to get rid of the extracellular bacteria. Wash one more time with warm RPMI-1640 and re-suspend the pellet.
7. Inoculate 20 mL of the infected cells into the peripheral compartment of each HFS-TB.
8. Count the number of THP-1 cells inoculated into each system as described in Step #4.
9. Spin remaining inoculum, remove supernatant and add 0.5% Triton X-100 in phosphate buffered saline. Vortex the reconstituted suspension to obtain the macrophage lysate, and then perform serial dilutions for *M. tuberculosis* cultures in order to quantify the CFU/mL of the bacteria (see Section 5.4).

5.1.4 Viability Methods

1. Examine OD during the growth of *M. tuberculosis* and inoculum preparation as described above in Section 5.1.1. Growth of *M. tuberculosis* cultures is monitored/verified by change in OD. Inocula are prepared from the cultures at specific ODs.
2. Each laboratory should perform work that quantifies the relationship between OD and CFU/mL with their specific spectrophotometer, thus identifying a formula that relates CFU/mL to OD in their laboratory. In this assessment the CFU/ml is determined by the quantitative culture method (see Section 5.4 for details).
3. A second viability method should be employed for verification, such as the LIVE/DEAD assay which can be performed using commercially available kits. Briefly, this test consists of two fluorescent dyes that bind to nucleic acids: (i) propidium iodide which has red fluorescence but only enters "dead" cells with damaged cytoplasm, and (ii) green fluorescing SYTO9 which enters all cells, both live and dead. The emission properties of the mixture change due to quenching and the displacement of dyes from DNA by one another.[63]
4. While the exact steps and mixtures are specified by the manufacturer, prior to use each laboratory should determine the optimal operating conditions for the LIVE/DEAD assay. Notably, appropriate fluorescent dye mixtures for use in *M. tuberculosis* staining across a range of SYTO9 and propidium iodide concentrations should be determined, as well as the laboratory-specific relationship between relative luminescence units (RLU) for live versus dead *M. tuberculosis* cells vs. CFU/mL and the corresponding formula for calculation of CFU/mL based on RLU with their specific luminometer.
5. The OD and the LIVE/DEAD assay should be in agreement for inoculum preparation. The inoculum used for the HFS-TB is also serially diluted and cultured on Middlebrook 7H10 agar plates, with and without antibiotic, to determine the exact CFU/mL used in the study, as well as the pre-existent drug-resistant sub-population. Repeated measures of CFU/mL also monitor the consistency of the inoculum preparation, thus serving as a quality control.

5.2 Assembly and use of HFS-TB in the BSL3 laboratory

5.2.1 General considerations

Given the customized nature of the HFS-TB system, the assembly order of the HFS-TB components varies according to person even within each team, and to the type of experiment being designed. There are multiple steps required to setup the system, which vary according to how many drugs are being examined in the system.

Therefore, this section is intended to provide a general description of the assembly process for each HFS-TB unit; however, the assembly process will need to be customized according to the type of HFS-TB experiments, physical layout, and preferred practices within each laboratory.

5.2.2 Assembly Steps

All HFS-TB construction steps follow aseptic technique and should all take place within the BSC.

1. Use 1, 4 L clear plastic autoclavable bottle to collect the HFS outflow/waste media. Add 400 mL bleach to each of the outflow media bottles, so that the final bleach concentration is 10-15% (v/v).
2. The central reservoir and hollow fiber cartridge are placed in the BSC, and the cartridge inflow and outflow tubing is connected to the central reservoir outflow and inflow ports.
3. After assembly, still within the BSC, each unit is manually primed (pumped) to test for correct direction of flow, circulation of media, and leaks.
4. Connect the fresh media reservoir bottle and the waste media bottle using 14-gauge tubing to the respective ports on of the hollow fiber system.
5. The units are then taken out of the BSC, placed in the incubators and preconditioned for at least 72 hours. Conditioning involves inflow of fresh media and outflow of used media at the same rates that will be used throughout the experiment (see Section 5.3). For standard therapy (isoniazid, rifampin, pyrazinamide, and ethambutol), the conditioning is for 72 hours with the same media that will be used during experiments.
6. During the 72 hours of conditioning the systems are also visually inspected for leaks and any possible contamination.
7. On the day of *M. tuberculosis* inoculation, materials required for this step are gathered in the BSC including 10% bleach, 70% ethanol, a waste receptacle containing 10% bleach, autoclave bags, and 20 mL needless syringes.
8. The peripheral compartment is removed from the incubator and taken to the BSC.
9. Flasks of *M. tuberculosis* culture (log-phase growth bacteria, or semi-dormant *M. tuberculosis* under acidic conditions; prepared in Sections 5.1.1 and 5.1.3) are removed from the incubator and taken to the BSC. The flask surfaces are wiped with 10% bleach, and then opened within the BSC.
10. For intracellular *M. tuberculosis* cultures, the *M. tuberculosis*-infected THP-1 cell culture prepared in Section 5.1.3 is removed from the incubator and taken to the BSC. The flask surfaces are wiped with 10% bleach, and then opened within the BSC.

11. *M. tuberculosis* cultures are loaded into the peripheral compartment (see Section 3.1.1) and is then taken out of the BSC to the incubator.
12. The computerized syringe pumps are then programmed according to the infusion schedule and volumes required per the experimental design. After loading the drug syringes into the pumps and purging the infusion tubing of air, the tubing is connected to drug infusion port of the hollow fiber system. Before starting the syringe pump to infuse the drugs, ensure the ports are open by turning the locks to open position.
13. Each time HFS-TB units are inspected during a study the staff will inspect the HFS-TB through the window of the incubator. In the event of a leak, DO NOT OPEN THE INCUBATOR DOOR, but exit and call PI and biosafety officer and initiate spill procedures (see Section 3.2).
14. Sampling of the central compartment for pharmacokinetic analysis is performed via a self-sealing septum connected the central reservoir via a 3-way stop cock. Sampling is performed inside the incubator since the central compartment contains sterile media and not *M. tuberculosis* cultures. This is described in detail in Section 5.3.5
15. Sampling of the peripheral compartments for culture is a potential aerosol generating maneuver. The times for sampling the peripheral compartment are communicated to all other users of the BSL3 laboratory. Sampling of the peripheral compartment is performed at various time points (usually once a week, but this will depend on study design and the anticipated rate of kill by the drug under study) and is ALWAYS performed within the BSC (see Section 3.1.2).
16. At the end of the experiment, the HFS-TB units are taken into the BSC following procedures in Section 3.1.3.

5.3 Pharmacokinetic considerations for HFS-TB

5.3.1 Pump calibration

The different pumps are calibrated according to manufacturer's instructions and differ by specific type of pump. These are supplied by manufacturer at time of purchase.

5.3.2 Setting HFS-TB volume

The volume used in the HFS-TB will vary depending on factors that will need to be optimized for each specific experiment. These include, for example:

- Target half-life of the drug(s)
- Target peak concentrations of the drug(s)
- Various media requirements (e.g., amount of FBS or BSA) that are dictated by the drug physicochemical properties
- Extent of adsorption of drugs to the hollow fiber tubing and cartridge material
- Available drug quantities for study

Once a suitable volume is determined for the experimental conditions, HFS-TB volume is kept constant throughout the duration of the experiment by ensuring that the inflow and outflow rates are equal. This is accomplished by setting the height of the outflow dip tube within the central compartment reservoir.

The dip tube height sets the level of the meniscus and is calibrated to allow outflow from the system only when the central compartment reservoir exceeds a pre-specified volume, which corresponds to the target volume of distribution of the drug within the HFS-TB. By setting the outflow pump to a rate equal to or higher than the inflow pump, the volume of the system remains constant. Any potential increase in system volume due to infusion of drug and/or fresh media raises the central compartment reservoir height above the outflow dip-tube opening allowing the rapid removal of excess volume by the outflow pump.

5.3.3 Flow rates to mimic half-life

The HFS-TB uses a dilution rate to drive a specific desired half-life ($t_{1/2}$). Therefore, details on setting up specific dilution rates will depend on the pharmacokinetics of drug under study. Here we give a general overview, including an example of isoniazid from a published study.

1. Fresh media is pumped into the system via an inlet tube that also features the infusion pump (drug) inlets. Thus, the fresh media carries the infused drug into the central compartment.
2. The target time to maximum concentration (T_{max}) is equal to the infusion duration and is based on the known or anticipated time to peak concentration in humans for the particular drug. This is programmed by matching time to maximum concentration with duration of infusion of the drug, which is controlled by the syringe pump.
3. The elimination rate of the drug from the HFS-TB system is determined on the basis of the media inflow rate. This is because used medium is removed from the system at a rate equal to the inflow of fresh medium and will carry drug with it (see Section 5.3.1). The required inflow rate can be determined using the following equation:

   $$\text{Inflow rate (mL/h)} = \text{central compartment volume (mL)} \times \ln(2)/t_{1/2}\ \text{(h)}$$

   where $t_{1/2}$ is the target (mono-exponential) half-life for the investigational drug and the inflow rate can be considered to represent the “clearance” for the HFS-TB system.

5.3.4 Drug concentrations and syringe pump

Drug concentrations in syringes depend on the volume in the central compartment and the inflow/outflow rate so that there is continuous dilution of drug even during its own infusion, and the desired or targeted peak concentration. It also depends on the rate of drug infusion chosen.

The siliconized tubing length utilized to carry drug from the syringe pump will vary and depend on (a) the diameter of tubing (gauge), (b) the spatial arrangement of the incubators and the stand where the syringe pump is located and (c) the volume of drug to be infused. Spatial arrangement depends on the configuration of the lab and where incubators are in relationship to syringe pump, fresh media containers, and used media containers. Based on those considerations each lab should determine the optimal length of tubing. There are no maximum or minimum lengths of tubing specified.

5.3.5 Sampling of the central compartment for pharmacokinetic analysis

Note, sampling of the central compartment for pharmacokinetic (PK) samples is performed inside the incubator since the central compartment contains sterile media and not *M. tuberculosis* cultures.

Specific considerations regarding PK sample collection are as follows:

1. The sampling schedule is selected based on the half-life (-lives) of the drug(s) under study. It is recommended that sampling times be selected based on optimal sampling theory, as this limits the required number of samples as compared to empiric or convenience-based sampling schedules while maximizing information gained from each sample.
2. The central compartment fluid sample is removed from each HFS-TB unit at each of the predetermined time points via the drug sampling self-sealing port. The required sample volume depends on the drug concentration assays to be used for each study, the sensitivity of the assay and other characteristics of drug and assay.
3. The samples are then placed in suitable storage conditions (i.e., frozen) prior to batch analysis. **Note that storage conditions should be tailored depending on the physicochemical characteristics and stability of the drug or drug combination**.
4. The drugs are assayed using either published or newly developed methods. Analytical methods should be sufficiently qualified by the individual lab for each experimental condition (i.e., media type) prior to use for drug concentration analysis.

5.4 Verification of *M. tuberculosis* populations in HFS-TB

It is vital to continuously verify that the intended bacterial population (metabolic phenotype) is maintained throughout the study; this is accomplished through comparison of observed and expected growth rates. The data are generally not used to make decisions in real time, but rather are employed to help assess experimental validity.

The growth rates of the bacteria in the HFS-TB are measured and calculated for each HFS-TB using appropriate methods such as CFU/mL, OD_{600}, LIVE/DEAD assay,[63] and ribosomes/CFU.

For CFU/ml, cultures from the peripheral compartment are serially diluted 10-fold in normal saline (? PBS with glycerol) in test tubes. From each dilution, 0.2 mL is spread on Middlebrook 7H10 agar with 10% OADC, sealed in CO_2 impermeable bags, incubated in CO_2 at 37°C for 21 days, and then colonies are counted.

For OD_{600} and LIVE/DEAD assays see Sections 5.1.1 and 5.1.4 for details.

For ribosomes/CFU, RNA is extracted from the cultures removed from the peripheral compartment using an appropriate RNA extraction kit (e.g., RNeasy minikit, Qiagen) and the proportion of RNA that is ribosomes is then calculated using standard, published methods.[64]

The observed growth rates are then compared with typical growth rates from the equivalent assays as reported in published literature (Table 3).[20]

Table 3 Reported growth rates of the three *M. tuberculosis* metabolic population

Assay (unit)	Log-phase growth bacteria (95% CI)	Semidormant bacteria (95% CI)	Non-replicating persisters (95% CI)
Live/dead slope (RFU/h)	60.72 (33.76 to 87.68)	7.48 (3.06 to 11.90)	4.72 (−0.56 to 10.01)
OD_{600} ($\times 10^{-4}$units/h)	20.79 (12.03 to 29.55)	1.98 (0.35 to 3.62)	1.12 (−0.48 to 2.73)
Log_{10} CFU/mL/day (slope)	0.069 (0.059 to 0.079)	0.013 (0.007 to 0.018)	0.005 (0.001 to 0.009)
Ribosomes/CFU (10^{-5})	4.021 (3.204 to 4.838)	2.555 (1.340 to 3.770)	1.723 (1.294 to 2.153)

Given the dependence on experimental pH to ensure bacteria remain in the proper growth condition, for semi-dormant bacteria the pH is monitored each time the peripheral compartment is sampled (the pH in the media is buffered). RNA sequencing may also be performed on the bacterial samples to validate that the metabolic signature for semi-dormant bacteria under acidic conditions is present.

For non-replicating persisters under hypoxia, oxygen tension in the liquid medium is monitored at least 5 days of the week over the entire treatment period, to ensure that it is 10 parts per billion (ppb) or lower.[65] The bacterial cultures are also examined for α-crystalline protein via 2D gel by some laboratories. While we prefer the RNA sequencing signature, α-crystalline protein plus Nile red staining is adequate as well.

5.5 Quality control

For all HFS-TB studies, the following quality control steps should be taken:

1. Possible contamination of inoculum is excluded with cultures 24-48 hours prior to inoculation.
2. The CFU/mL of the inoculum is examined at the beginning of each study by the standard quantitative culture method. It should not deviate from the intended bacterial burden more than 20%.
3. Contamination of THP-1 macrophages with mycoplasma is checked for at least 48 hours before the macrophages are infected with *M. tuberculosis.*
4. Contamination rates of HFS-TB units and agar and reasons for contamination in the HFS-TB are addressed at the end of each study.
5. The intended concentration-time profiles of drugs should be compared to those actually measured and used to calculate the % bias for each sample at each time point. Such bias should also be explained and minimized in the next set of experiments.
6. The %CV between HFS-TB replicates is identified for (a) pharmacokinetic parameters, (b) CFU/mL, and (c) proportion of resistant *M. tuberculosis*. The %CV should be no more than 20%. The reasons for deviations should be explored at end of study in order to minimize them in the next study.

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