IMI2 GA853989 - ERA4TB
European Regimen Accelerator For Tuberculosis

WP2 – WP In vitro profiling

D2.1. Implementation of the HFS-TB in a BSL-3 setting in Europe

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Definitions

Partners of ERA4TB are referred to herein according to the following codes:

Grant Agreement. The agreement signed between the beneficiaries and the IMI JU for the undertaking of the ERA4TB project.

Project. The sum of all activities carried out in the framework of the Grant Agreement.

Work plan. Schedule of tasks, deliverables, efforts, dates and responsibilities corresponding to the work to be carried out, as specified in Annex I to the Grant Agreement.

Consortium. The ERA4TB Consortium, comprising the legal entities signatories of the Grant Agreement.
Consortium Agreement. Agreement concluded amongst ERA4TB participants for the implementation of the Grant Agreement. Such an agreement shall not affect the parties' obligations to the Community and/or to one another arising from the Grant Agreement.

Table of contents

1. Abstract

The Hollow Fiber System for tuberculosis (HFS-TB) is a preclinical tool for drug evaluation qualified by the European Medicines Agency in 2015. The system allows the reproduction of selected drug exposures to infer pharmacokinetic (PK) and pharmacodynamic (PD) parameters, which can feed *in silico* models able to inform the design of Phase 2/3 clinical trials.

UNIZAR was tasked with the implementation of the HFS-TB in BSL-2 and BSL-3 settings within the ERA4TB consortium. The implementation was divided into two main steps: (i) assessment of experimental needs and internal capacity, and (ii) standardization of experimental operational procedures.

First, we tested and selected the more suitable equipment and materials in terms of compatibility, size, and ease to handle, to perform mono and combinatorial assays in BSL-2 and BSL-3 facilities with *Mycobacterium tuberculosis*. Internal experimental capacity assessments were performed to define economic cost and personnel efforts needed for HFS-TB experiments. Timeframe estimates revealed "protocol design" and "troubleshooting anticipation" as key steps for a successful experimental outcome.

The second step involved optimization and standardization of experimental operational procedures. This included characterization of bacterial growth dynamics after varying inoculum sized in different media, and assessment of drug-cartridge suitability; we used moxifloxacin as a tester drug. For this, we developed suitable sampling methods both for PK profile verification (including decontamination and shipment procedures to ERA4TB partner) and for PD measurements.

The two implementation steps allowed us to establish the HFS-TB workflow at UNIZAR: (i) an initial phase of drug-fibers compatibility, (ii) mimicking of PK profiles of the drug of interest, (iii) drug PK/PD assessment under a broad set of conditions in BSL-2 facilities (using the avirulent *M. tuberculosis* H37Ra strain) and, (iv) drug PK/PD assessment of a narrow set of conditions in BSL-3 facilities (using the virulent *M. tuberculosis* H37Rv strain). Building efficient partnerships with ERA4TB members was key on the implementation. This workflow was closely interlinked with partners 4 (IPL) and 12 (UU), which provided analytical and modelling & simulation capacities, respectively.

In summary, we have successfully implemented HFS-TB at UNIZAR in BSL-2 and BSL-3 facilities. Our implementation studies have contributed to the standardization of the HFS-TB and should be considered when designing protocols to allow reproducibility across laboratories.

2. Introduction

The Hollow Fiber System for tuberculosis (HFS-TB) is a preclinical tool for *in vitro* drug evaluation approved and qualified by the European Medicines Agency in 2015 (EMA/CHMP/SAWP/47290/2015 Corr.). The HFS-TB mimics drug concentration-time profiles within a cartridge, inside which *M. tuberculosis* can be continuously culture (**Figure 1**). After *M. tuberculosis* is exposed to a wide range of dose-responses, the integration of PD biomarkers (i.e., CFU counts) with mathematical modelling allows for the forecast of optimal dosing schedules of clinical outcomes with high accuracy and, can also, determines dose breakpoints associated with drug resistance. Moreover, it does not raise ethical concerns associated with *in vivo* experimentation (in fact it can reduce the number of preclinical *in vivo* experiment needed), limits doses tested for both single and combinatorial regimen studies, allows for multi-sampling, and experiments are not time-limited, so they can last long enough to quantify the emergence of resistance.

Methodological guidelines for HFS-TB can be found in the literature, however, there are no standard procedures or mandatory quality control steps, and most publications provide insufficient data for intra-laboratory reproducibility. In this context of scarce information, UNIZAR has successfully implemented the HFS-TB in BSL-2 and BSL-3 laboratories within the ERA4TB consortium.

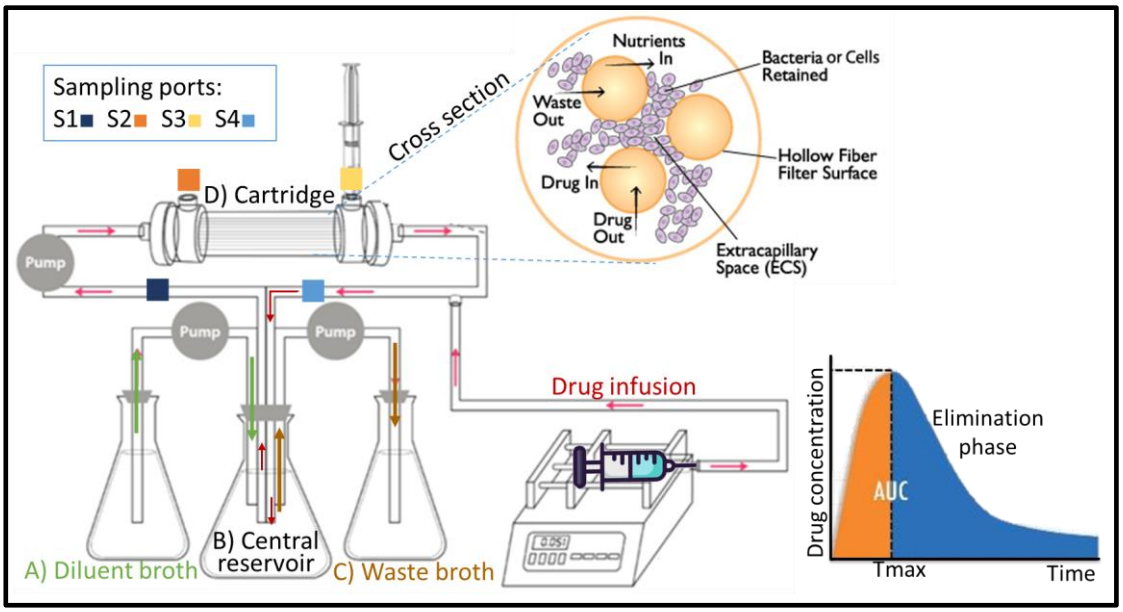


Figure 1. Schematic representation of the Hollow Fiber System for Tuberculosis. A basic HFS-TB unit is depicted. A, B, and C are compartments in which broth medium is recirculated and extracted from the system using peristaltic pumps. A pump-controlled syringe administers the drug into the tubing mimicking the PK profile of the selected drug (bottom-right inset). Compartment D is the cartridge with thousands of hollow fibers through which the medium is circulated. The scheme shows a cross-section of a cartridge. Cartridge fibers are membrane filters with 0.03-0.1 µm diameter pores. Bacteria are inoculated and grow outside the fibers, in the extra-capillary space (ECS), preventing them from crossing into the inner side. The system has four sampling ports: S1 and S4 ports for medium sampling, and S2 and S3 for bacterial sampling. The figure was adapted from Gumbo *et al.*, 2015. DOI: 10.1093/infdis/jiv183.

3. Experimental needs, internal capacity assessment and HFS-TB implementation at UNIZAR

An initial assessment of the experimental needs and internal capacity was performed to identify requirements for the HFS-TB implementation in BSL-2 and BSL-3 laboratory settings at UNIZAR. These included: (i) remodelling of laboratory facilities, (ii) defining optimal materials and equipment needed for HFS-TB system assembly, (iii) user training, (iv) establishing timing and experimental workflows and, (v) creating synergistic partnerships within the ERA4TB consortium.

a. Facilities

Performing HFS-TB experiments require specific equipment and spatial disposition of such that are organized into modules. Each one consists of a biosafety cabinet, a four shelves incubator able to hold up to 8 cartridges (or units, see **Figure 1**), and a stainless-steel shelf with capacity for the necessary equipment (i.e., pump, bottles, etc.) (**Figure 2**). In order to fit this optimized disposition, two BSL-2 laboratories in the Department of Microbiology, Faculty of Medicine, University of Zaragoza were refurbished during the summer of 2021. Biosecurity BSL-2 accreditations were also obtained from the Biosecurity Committee at the University of Zaragoza. These allowed to create capacity for 4 HFS-TB modules in BSL-2, leading to a total of 32 cartridges.

BSL-3 capacity was created at the "*Centro de Encefalopatías y Enfermedades Transmisibles Emergentes*", Faculty of Veterinary, University of Zaragoza. A dedicated room in the centre, a state-of-the-art facility for prion research, was adapted for airborne pathogens. One extended module has been created in this BSL-3 laboratory, able to run up to 12 cartridges in parallel.

Figure 2. HFS-TB basic equipment module. The stainless-steel shelf is placed in between the incubator and the biosafety cabinet (where cartridges are sampled) to allow for easiness of manipulation.

b. Equipment and Materials for HFS assembling

There is no single HFS-TB experiment similar to the next one. Every time an experiment is performed, a new HFS-TB unit must be assembled in order to account for the drug-specific requirements. As such, the

most suitable equipment and materials in terms of compatibility, size, time set up and user friendly, and reusability were tested to assemble the systems.

Four different types of pumps were tested including one syringe pump for drug infusion, one duet pump for broth recirculation between the cartridge and the central compartment, and two peristaltic pumps for drug clearance. A specific pump control software was acquired for the syringe and peristaltic pumps.

Due to the different physicochemical properties of the drugs to be tested, specially related to their hydrophilic and hydrophobic properties, the composition of the materials used is critical for a successful experimental outcome. For this, several types of cartridges were tested including small and medium size; cellulose, polysulfone (PS), and hydrophilic PVDF fiber materials; and high and low flux fiber type cartridges. Similarly, tubing features were selected based on the length availability, inner diameter, and material type (silicone, and polypropylene). Likewise, other items were chosen to assemble the system as different materials of hose barb fittings, hydrophobic filters, polystyrene, and polycarbonate bottles, and glass and polypropylene syringes with different volumes 1, 3, 10, 20, and 50 mL **(Figure 3)**.

Figure 3. Equipment and materials for HFS assembling. (Left to right, first row) The following are depicted: a syringe pump, a clearance pump, a smaller clearance pump, and a duet pump with fittings and items for assembling. **(Second row)** a syringe, silicone tubing, central reservoir bottle, a cap for waste and diluent reservoirs, and bottles for the diluent and wasted broth. **(Third row)** different type of cartridges.

Materials and equipment are thus assembled for each HFS-TB experiment as illustrated in **Figure 4**.

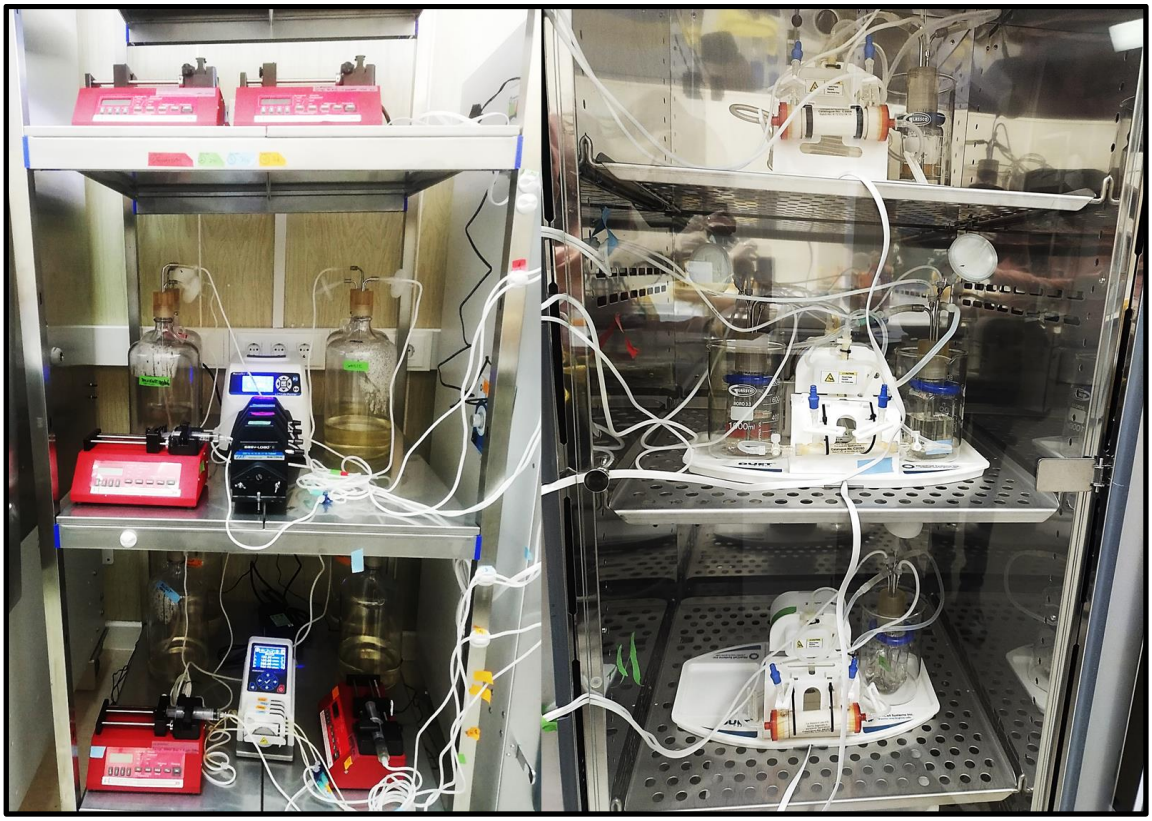


Figure 4. The HFS-TB at UNIZAR. (Left) a shelve with five red syringe pumps, two peristaltic pumps for drug clearance, and four diluent and waste bottles with broth media. (Right) Silicone tubing connects bottles with central reservoirs placed inside beaker glasses besides duet pumps inside the incubator, which holds three white duet pumps with cartridges attached.

Current capacity allows to simultaneously test 32 cartridges for monotherapy and combination studies with two drugs in a BSL-2 setting. When three drugs are tested in combination, due to the complexity of the setup, capacity is reduced up to 24 cartridges in BSL-2. In a BSL-3 setup, capacity gets further reduced based on the number of drugs in the combination (**Table 1**).

Table 1. Cartridge capacity of the HFS-TB at UNIZAR. The maximum number of cartridges to be tested simultaneously is dependent on the biosafety level and the number of drugs in a single combination.

Biosafety Level	One or two drugs	Three drug combinations
BSL-2	32 cartridges	24 cartridges
BSL-3	12 cartridges	8 cartridges

c. Training of HFS-TB personnel at UNIZAR

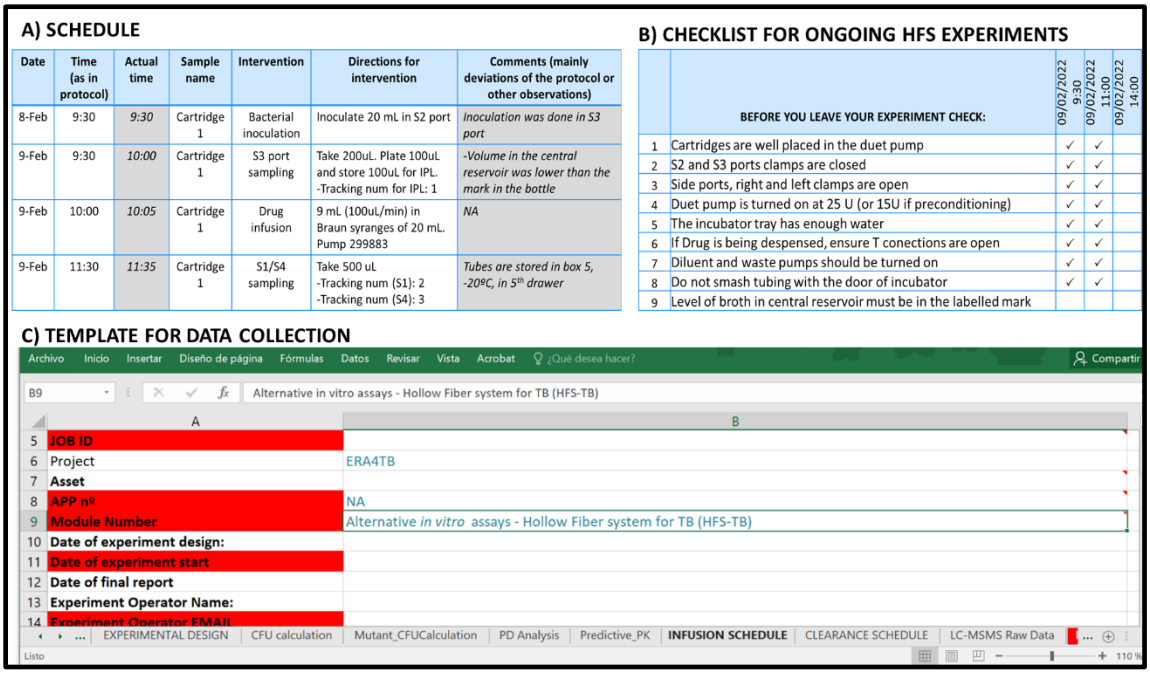
Theoretical and experimental workshops were organized at UNIZAR to train four postdoctoral researchers and three technicians (from now on, “the users”) according to internally developed standardized

protocols (**Figure 5**). When planning an HFS-TB experiment, it is important to define the experience of the users involved in such experiment in order to assess for cartridge handling capacity, since skilled users are able to handle more cartridges at the same time. After the standardization of procedures, UNIZAR recommends handling of only one cartridge for beginners, such experiments might involve determination of bacterial growth dynamics or the simulation of single-drug PK profiles. Experienced users are able to manage up to four cartridges simultaneously in more complicated HFS-TB experimental designs.

Figure 5. Theoretical and experimental training at UNIZAR. Hands-on training was provided to four postdoctoral scientist and three lab technicians. Currently, eight UNIZAR team members are trained to perform HFS-TB experiments.

User's training involved directions for experimental planning, material preparation, system manipulation, pump calibration, monitoring of ongoing experiments, data collection, and data analysis. Schedule and checklist templates were created for the strict monitoring of HFS procedures (**Figure 6**). In order to minimized user error, an experimental protocol for every HFS experiment is produced with detailed information regarding timelines, materials, procedures, and settings. The experimental schedule is organized and printed before assembling the system; such schedule gathers information pertaining to date, time, samples ID, general directions and a section to record protocol deviations (**Figure 6A**). A printed checklist with settings and warnings to follow whenever a pump is turned on is also placed next to the ongoing HFS (**Figure 6B**). A template for raw data collection was created and adapted to the Excel format designed by the standardization workgroup within ERA4TB and in collaboration with WP1; it

includes tabs to gather information from three types of HFS-TB experiments: drug-fiber compatibility test and PK/PD evaluation of both monotherapy and combinatorial studies. The output of this template allows for R-script-based data analysis (**Figure 6C**).



A) SCHEDULE

Date	Time (as in protocol)	Actual time	Sample name	Intervention	Directions for intervention	Comments (mainly deviations of the protocol or other observations)
8-Feb	9:30	*9:30*	Cartridge 1	Bacterial inoculation	Inoculate 20 mL in S2 port	*Inoculation was done in S3 port*
9-Feb	9:30	*10:00*	Cartridge 1	S3 port sampling	Take 200uL. Plate 100uL and store 100uL for IPL. -Tracking num for IPL: 1	*-Volume in the central reservoir was lower than the mark in the bottle*
9-Feb	10:00	*10:05*	Cartridge 1	Drug infusion	9 mL (100uL/min) in Braun syranges of 20 mL. Pump 299883	*NA*
9-Feb	11:30	*11:35*	Cartridge 1	S1/S4 sampling	Take 500 uL -Tracking num (S1): 2 -Tracking num (S4): 3	*Tubes are stored in box 5, -20ºC, in 5th drawer*

B) CHECKLIST FOR ONGOING HFS EXPERIMENTS

	BEFORE YOU LEAVE YOUR EXPERIMENT CHECK:	09/02/2022 9:30	09/02/2022 11:00	09/02/2022 14:00
1	Cartridges are well placed in the duet pump	✓	✓	
2	S2 and S3 ports clamps are closed	✓	✓	
3	Side ports, right and left clamps are open	✓	✓	
4	Duet pump is turned on at 25 U (or 15U if preconditioning)	✓	✓	
5	The incubator tray has enough water	✓	✓	
6	If Drug is being despensed, ensure T conections are open	✓	✓	
7	Diluent and waste pumps should be turned on	✓	✓	
8	Do not smash tubing with the door of incubator	✓	✓	
9	Level of broth in central reservoir must be in the labelled mark			

C) TEMPLATE FOR DATA COLLECTION

Figure 6. HFS-TB monitoring procedures. (A) Example of an experimental schedule, **(B)** checklist, and **(C)** data collection template.

d. Time and effort monitoring

An integral and critical part of the HFS-TB implementation at UNIZAR was to determine the actual capacity of the group to absorb a large number of experimental requests from the EFPIA and Consortium members. For this, understanding actual time and personnel effort commitments at every experimental step is of utmost importance (**Figure 7**). In this line, users registered the actual time spent in each activity related to the execution of five experiments for, at least, two users per HFS-TB experiment (**Figure 7A**). Activities were classified into five categories: (i) planning, (ii) material and equipment preparation, (iii) experimental hands-on work, (iv) results and reporting and, (v) troubleshooting. Most demanding categories were the planning of activities and the experimental hand-on work, with two thirds of the actual allocated time (**Figure 7B**). When looking at individual experiments, in one case, users faced troubleshooting during the experimental execution, which represented 12% of the total time invested. (**Figure 7C**). It is expected that as users gain experience such setbacks will be minimized. The analysis revealed that planning and troubleshooting anticipation are key aspects to shorten timelines for a successful experimental outcome.

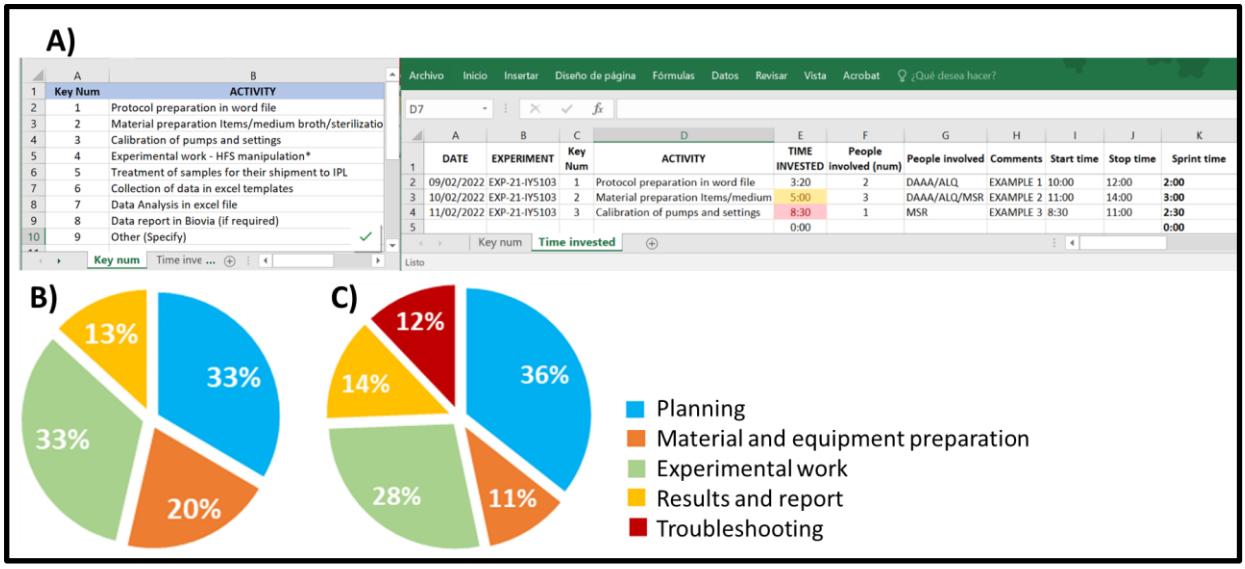


Figure 7. Effort monitoring in HFS-TB experiments. (A) Templates for time monitoring in HFS-TB activities. **(B)** Percentage of time dedicated to every category of HFS-TB. The plot represents the mean of five independent experiments. **(C)** Percentage of time dedicated in a single experiment, which involved troubleshooting while the experiment was ongoing.

e. The HFS-TB experimental workflow at UNIZAR

Working in BLS-3 conditions is more tedious, cumbersome and limiting than in BSL-2 conditions. This is due to the pathogenic nature of the infectious agent (virulent airborne *M. tuberculosis* in BSL-3 conditions) and the strict biosafety measures that needed such as working in negative pressure laboratories and wearing specific personal protective equipment (i.e., N95 facemasks), with strict operational procedures (**Figure 8**).

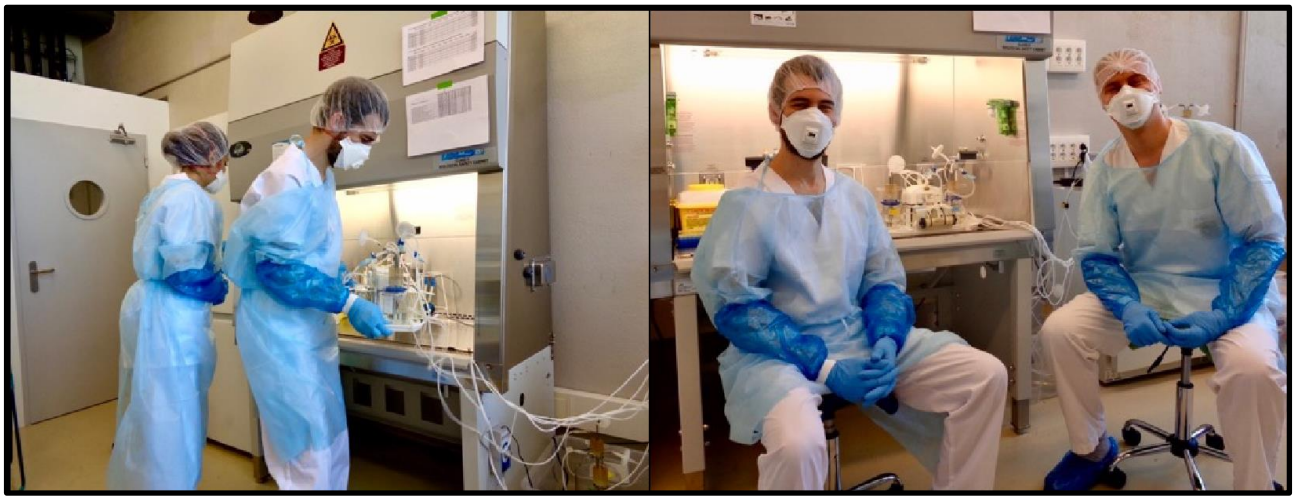


Figure 8. The HFS-TB in a BSL-3 setting at UNIZAR. **(Left)** Handling of the HFS-TB. **(Right)** Training session in the BSL-3 with visiting scientist from UU (right).

UNIZAR has created different HSF-TB capacity both in BSL-2 and BSL-3 biosafety levels. In order to maximized the output of such capacity, we have developed an HFS-TB workflow of HFS-TB divided into several phases encompassing both BSL-2 and BSL-3 activities (**Figure 9**):

(i) Phase I: drug-fiber compatibility test and PK profile assessment in BSL-2
(ii) Phase II: PKPD experiments in BSL-2 with a large set of experimental conditions
(iii) Phase III: confirmatory PKPD experiments in BSL-3 with a reduced set of experimental conditions

After definition of the study objectives and experimental design in collaboration with the asset owners and other ERA4TB partners (see below), the objective of Phase I is to ensure the compatibility of the drug(s) of interest with the HFS materials, including cartridge fibers, and standardize drug concentration-time profiles in the system. Phase II aims to test a wide range of dose-response assays and conditions in BSL-2 facilities with the avirulent *M. tuberculosis* H37Ra strain. Finally, Phase III is used to confirm with the pathogenic *M. tuberculosis* H37Rv strain the PKPD profiles obtained in Phase II. For this, just a small subset of selected doses and conditions are tested in BSL-3 conditions (**Figure 9, left**).

A critical aspect for EFPIA in the preclinical development of their assets is to be able to anticipate milestone achievement. For this, at UNIZAR, we performed an assessment of the timelines needed for every HFS-TB phase in our workflow (**Figure 9, right**). On average, Phase 1 can lasts up to two months. Tasks in here involve experimental plan design, material preparation, sterility tests, pump calibration, assembling and preconditioning of the system, desired PK profile execution, and drug quantification studies. Results obtained in the first phase allow to establish Go/NoGo decision checkpoints before embarking onto the next phase. Four months is the estimated experimental timeline for Phase II, which includes the same tasks as in Phase 1 plus three additional ones, i.e., (i) a step for inoculum adaptation to the system, (ii) bacterial culture and dose administration, and (iii) bacterial survival readout. Phase I and Phase II are performed in BSL-2 facilities and up to 32 cartridges can be run simultaneously. Phase 3 is performed in BSL-3 facilities and it comprises the same tasks and timelines as in Phase II, although with fewer experimental conditions than Phase II (up to 12 cartridges) and the use of *M. tuberculosis* H37Rv.

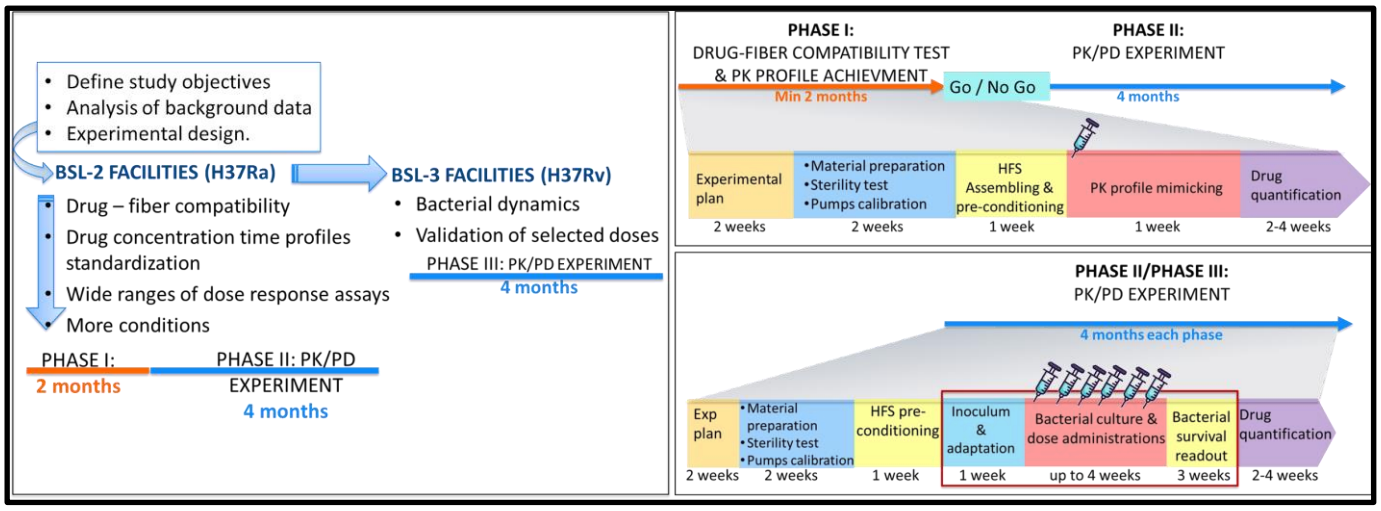


Figure 9. The HFS-TB workflow at UNIZAR. (Left) Specific activities to be performed at different phases and according to the biosecurity level and, **(right)** tasks and time required for every phase of the HFS-TB workflow in BSL-2 (Phase I & II) and BSL-3 (Phase III).

f. ERA4TB partnerships for the HFS-TB

UNIZAR is leading the implementation of the HFS-TB model within the ERA4TB consortium. However, additional key expertise is needed to complement the HFS-TB model and extract its full potential. This expertise is provided by two ERA4TB partners:

- 4- Institut Pasteur de Lille (IPL)
- 12- Uppsala University (UU)

Phase I of the HFS-TB work flow test drug-fiber compatibility and PK profiles achievements (see **Figure 9**). For this, experimental samples are taken at designated time points, processed according to decontamination protocols, shipped to IPL where the amount of drug is quantified by LC-MSMS. Thus, IPL contributes to the quantification of the study compounds in the HFS-TB samples.

Output data of HFS-TB experiments can inform the design of future clinical trials or provide information on the PD driver governing the activity of the drugs under study. As such, clear experimental designs need to be defined before starting each experiment with the end goal in mind. WP5 (UU) provides modelling and simulation (M&S) capacities needed for accurate protocol design, and data interpretation and translation.

All these activities (UNIZAR, experimental, IPL, analytical, UU, M&S) are integrated in a Plan-Do-Study-Act (PDSA) strategy which allows for improvement of methods and interaction with partners (**Figure 10**). Briefly, protocols and progression plans for individual assets and combos are first subjected to a detailed plan design coordinated by UNIZAR and WP5 (UU), together with the asset owner. Execution of the plan involves the experimental *in vitro* contribution by UNIZAR and IPL, as well as the *in silico* M&S by UU. Experiments are executed, revised and improved, if possible. How every partner is engaged in a feed-back loop in this process is shown in **Figure 10A**. It is noticeable that the involvement of all partners is needed in every Phase. After experimental execution, results are subjected to analysis by UNIZAR and WP5 as shown in the PDSA cycle (**Figure 10B**). As a result of this process, the main standardized protocols for HFS-TB have been successfully implemented in BSL-2 and BSL-3 laboratories.

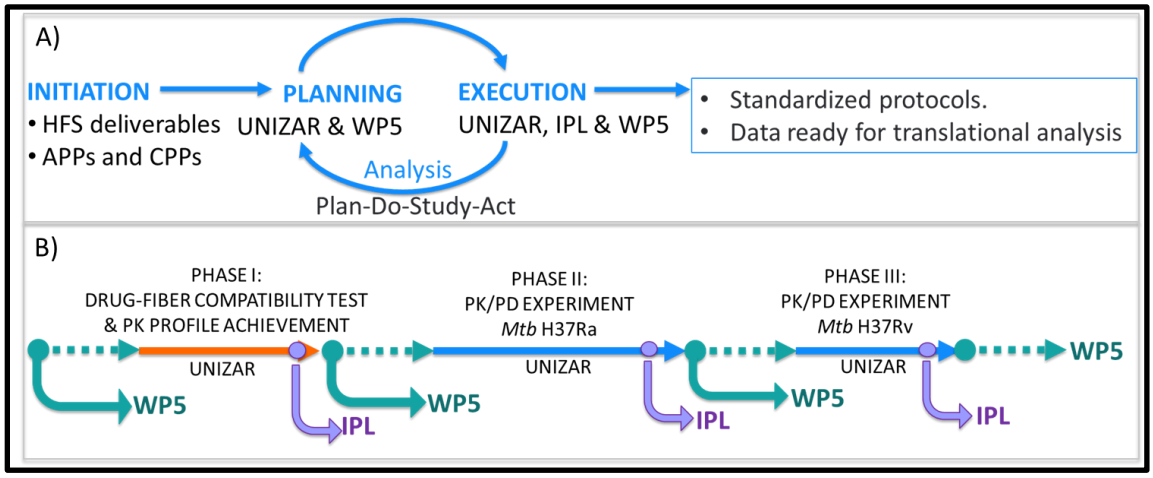


Figure 10. **Partnership engagement in the ERA4TB HFS-TB. (A)** Plan-Do-Study-Act strategy for HFS-TB implementation. **(B)** Workflow of experimental execution between partners.

4. Probing the system: moxifloxacin studies

Moxifloxacin (MXF), a well-known anti-TB drug currently recommended by the WHO, was chosen as the tester drug for our HFS-TB at UNIZAR. Standardization of operational procedures included bacterial growth dynamics assessment in different medium content, different types of cartridges, and different inoculum sizes. We also assessed sampling methods for optimizing PD measurement as well as for PK

profile verification. Tested conditions were evaluated in BSL-2 facilities and procedures were finally implemented in BSL-3 facilities.

Human plasma concentrations of MXF after an oral dose of 400 mg were mimicked for 10 days against both *M. tuberculosis* H37Ra (BSL-2) and *M. tuberculosis* H37Rv (BSL-3) strains. Two types of PS cartridges were sampled from the ICS (intracapillary space) and ECS (extra-capillary space) ports to verify the expected drug profile over time, which were well replicated in both conditions (**Figure 11**).

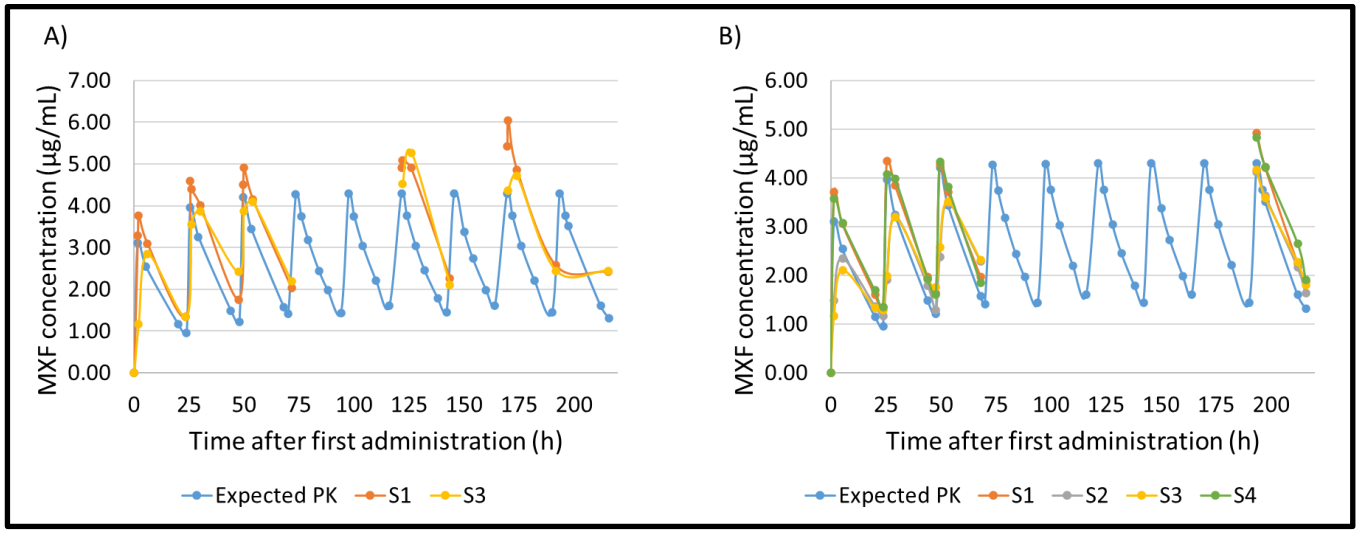


Figure 11. PK profiles of MXF in two types of PS cartridge for 10 days. **(A)** Small PS cartridge inoculated with *M. tuberculosis* H37Ra. **(B)** Medium PS cartridge inoculated with *M. tuberculosis* H37Rv.

At the same time, samples were collected for PD analysis (CFU/mL) throughout 10 days and plated on agar from both control and treatment cartridges. As shown in **Figure 12**, at the administered dose, MXF was highly effective at reducing the bacterial burden below the limit of quantification (LOQ). In addition, no major differences were found in MXF bacterial killing between *M. tuberculosis* H37Ra and H37Rv strains, supporting our dual BSL-2/BSL-3 strategy.

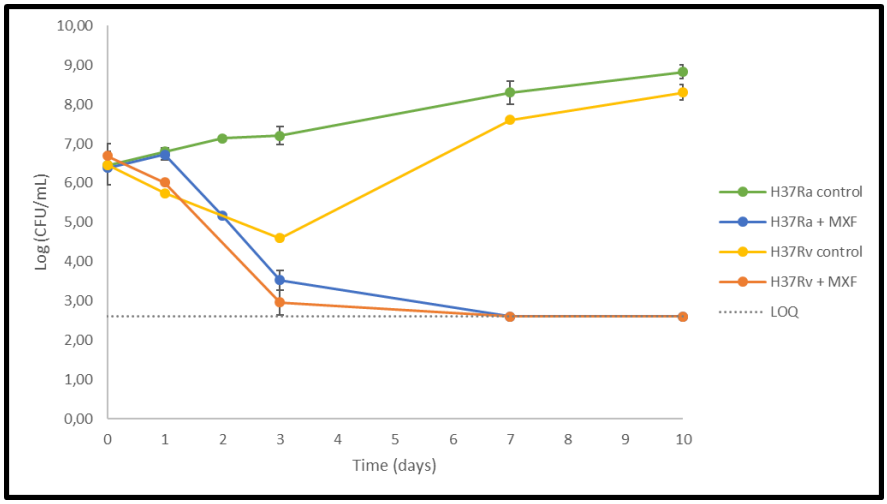


Figure 12. Moxifloxacin in the HFS-TB. *M. tuberculosis* H37Ra and H37Rv was treated with a mimicked dose of 400 mg of MXF for 10 days. Untreated controls were included. The experiment was performed in PS cartridges. LOQ, Limit of Quantification.

5. Conclusions

Within the ERA4TB consortium, the HFS-TB model has been successfully implemented in both BSL-2 and BSL-3 facilities at UNIZAR. This is the only such facilities available within the European Union (and one of the very few in the world).

New molecules are entering in the ERA4TB pipeline and several APPs have been already approved involving HFS-TB studies. The large capacity of up to 32 cartridges in BSL-2 and 12 cartridges in BSL-3 makes UNIZAR a hub for HFS-TB experimentation and ensures meeting all the experimental requirements needed by EFPIA and other interested partners within ERA4TB.

Future actions during the life of the ERA4TB project will focus on using the HFS-TB model to evaluate new promising TB drug candidates. Sustainability of the system after ERA4TB will be in the core of UNIZAR strategies.

6. Supporting materials

The supporting multimedia material can be accessed through the following links:

List of supporting videos:

1- Example of HFS-TB manipulation and sampling (1).Link

2- Example of HFS-TB manipulation and sampling (2).Link

3- HFS-TB module composed of incubator, shelf and biosafety cabinet. Link