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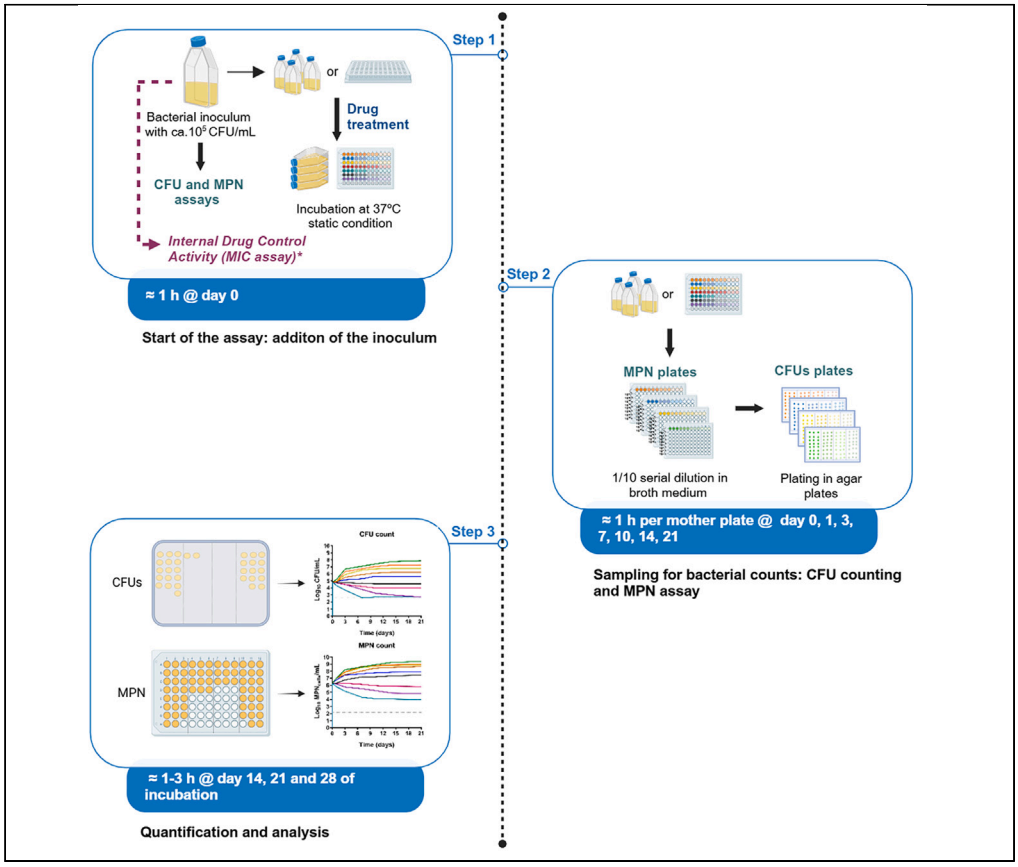
Protocol to quantify bacterial burden in time-kill assays using colony-forming units and most probable number readouts for *Mycobacterium tuberculosis*



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Highlights

The protocol describes inoculum preparation and conditions for time-kill assays

MPN in a 96-well plate and 2.5 µL CFU plating techniques used as simultaneous readouts

Detailed procedures for quantifying and analyzing MPN and CFU data

Here, we present a protocol to perform a time-kill assay (TKA) to quantify bacterial burden at multiple time points using colony-forming units and most probable number readouts simultaneously. We describe steps for preparing inoculum, experimental conditions, and sampling bacterial counts. We then detail procedures for quantification and analysis. TKAs provide longitudinal data reflecting the dynamics of the antibiotic effect over time against a planktonic culture and quantify the concentration-effect relationship.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Protocol to quantify bacterial burden in time-kill assays using colony-forming units and most probable number readouts for *Mycobacterium tuberculosis*

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SUMMARY

Here, we present a protocol to perform a time-kill assay (TKA) to quantify bacterial burden at multiple time points using colony-forming units and most probable number readouts simultaneously. We describe steps for preparing inoculum, experimental conditions, and sampling bacterial counts. We then detail procedures for quantification and analysis. TKAs provide longitudinal data reflecting the dynamics of the antibiotic effect over time against a planktonic culture and quantify the concentration-effect relationship.

For complete details on the use and execution of this protocol, please refer to Van Wijk et al.[1]

BEFORE YOU BEGIN

This protocol is designed for *Mycobacterium tuberculosis* (*Mtb*). Growth times and broth media need to be adapted if other bacteria are used.

This protocol outlines the execution of time-kill assays (TKA) that can be carried out either in tissue culture flasks or in 96-well plates. The choice of format will impact on the throughput capacity of the assay and the possibility of performing subsequent studies that might require larger volumes (i.e., drug quantification or RNA seq, etc.)

This protocol describes two complementary methods to determine the bacterial count in an *in vitro* culture: CFU and MPN assays. While Colony Forming Unit (CFU) enumeration is able to detect viable

cells that form CFU in agar, the Most Probable Number (MPN) assay is an outgrowth liquid method able to additionally detect a sub-population of viable but not culturable bacteria.[2]

Different CFU plating methods have been reported by van Wijk et al. (2023).[1] However, only the 2.5 μL CFU plating method is described in this protocol. This method is preferred as it substantially increases assay throughput by reducing the amount of material required and the time of manipulation. It allows for seeding bacteria directly on agar plates with a multichannel micropipette, thereby facilitating handling.

Furthermore, this protocol describes an optimization of the published MPN test[3] to be used as readout of the bacterial burden in *in vitro* preclinical evaluation of drug/regimen against *Mtb*. The method is performed in a 96-well plate format and consists of: (i) making dilutions of a sample in a liquid growth medium, (ii) incubating at 37°C and (iii) determining the viable cell numbers (MPN_{cells}/ml) using a well-established method,[4] as described in the sections "sampling for bacterial counts: CFU counting and MPN assay" and "CFU and MPN calculations" below. These 96-well plates will be named "MPN plates" from now on.

The MPN assay with three technical replicates per sample is described in this protocol. However, up to 10 replicates can be used depending on the laboratory capacities. The correct standard replicates commonly used and cited in regulatory guidelines, including the FDA, are typically 3, 5, 8 or 10 replicates; standard MPN tables are usually built around the cited replicates.[4,5]

Depending on the purpose of the experiment, the MPN assay can be performed in 7H9 broth medium: 7H9 base supplemented with 10% OADC (oleic, albumin, dextrose and catalase Middlebrook enrichment), 0.5% glycerol (Gly) and 0.05% tyloxapol (Tx) (7H9-OADC-Gly-Tx) or in 7H9 broth medium supplemented with 50% *Mtb* culture filtrate or supernatant (SN) (7H9-OADC-Gly-Tx+SN) containing resuscitation-promoting factors (RPF).[6]

Importantly, bacterial frozen stocks need to be prepared and characterized (CFU enumerated) before launching TKA experiments to limit inter-assay variability.

When conducting both readouts at the same time, CFU counting can be performed directly from the diluted samples in MPN plate (step-by-step method details, sampling for bacterial counts: CFU counting and MPN assay). This reduces the time of manipulation, an important aspect since experiments with *Mtb* are performed in a Biosafety Level 3 (BSL-3) laboratory.

Institutional permissions

Experiments described in this protocol have been performed with Mtb in a BSL-3 facility. The studies were approved by the following committees at each involved institution: the Biosafety Committee of the University of Zaragoza, the "Comité d'Evaluation des Risques Biologiques" (CERB) from the Institut Pasteur in Lille, the "Struttura di Raccordo del Servizio di Prevenzione e Protezione "from the Università degli Studi di Pavia, the "Ausschuss für Biologische Sicherheit" from the Research Center Borstel Leibniz Lung Center, and the Biosafety Officer of the District Government of the City of Cologne.

Preparation of characterized bacterial stocks

Timing: ≈45 days

Times are approximate since they depend on the rate of bacterial growth to reach a certain optical density. Here an optical density at 600 nm (OD_{600}) was used.

1. Prepare main culture stocks of bacteria at OD_{600} = 0.4–0.6 in 7H9 broth (7H9-OADC-Gly-Tx).

2. Make 1 mL aliquots of bacterial culture stocks and store at −80°C for at least 1 week.
3. Determine the bacterial concentration of the stocks.
   a. Select and thaw three random vials.
   b. Dilute and plate to perform CFU enumeration.

   Note: The starting inoculum of the TKA is prepared based on the actual CFU/mL of the bacterial stocks.

4. Log the OD_{600} information of the stocks.

   Note: Tyloxapol (Tx) 0.025% is added to the standard broth medium for routine liquid culture to generate homogenous cell suspension, which helps to prevent clumping of bacterial aggregates. In contrast to Tween 80, tyloxapol is not metabolized by *Mtb*, which makes it more stable and efficient at preventing clumping and does not add a confounding factor when assessing the natural growth of the bacteria in a specified carbon source medium. Do not include Tx in media used for TKA (see below).

Preparation of buffers, media, and drug solutions

Timing: ≈2 h, 5–7 days before the start of the experiment

5. Prepare 7H9 broth medium without Tx for culturing of *Mtb* (7H9 broth + 10% OADC + 0.5% Gly) as the routine protocol (then named 7H9-OADC-Gly, from now on).
   a. Prepare the required volume of 7H9 base medium supplemented with 0.5% Gly and autoclave according to manufacture instructions on the bottle of the media.
   b. Add OADC to a final concentration of 10% (v/v).
   c. Store the broth at 4°C, use within 2 weeks.

   Note: For TKA experiments, Tx is not added to the standard broth medium to avoid any potential effects on the activity of test compounds since it affects the integrity of the cell wall. The absence of Tx in the TKA culture does not impact bacterial growth, but it may affect CFU counting due to the suspension not being well homogeneous. To prevent this, Tx is added to the broth used for serial dilutions of the culture before plating, ensuring proper homogenization and accurate CFU counts.

   Note: Different broth media can be used depending on the purpose of the experiment.

6. Prepare plates with solid agar 7H10 medium supplemented with 10% OADC, 0.5% Gly and 0.1% Tx (7H10-OADC-Gly-Tx).
   a. Prepare the required volume of 7H10 base medium supplemented with 0.5% Gly and 0.1% Tx.
   b. Autoclave at 121°C for 10 min according to manufacture instructions on the bottle of the media.
   c. Add OADC to a final concentration of 10% (v/v).
   d. Fill each rectangular plate (86 × 128 mm, ref. 264728) with 35–40 mL of medium.

   Note: Add 50–55 mL of medium when using square Petri dishes (120 × 120, ref BP124-05).

   e. Allow plates to dry open for 40–45 min in a biosafety cabinet under sterile conditions.
   f. Store agar plates at 4°C and use them within 2–4 weeks.

   Note: Later, during the 2.5 μL drop seeding procedure, this step will help the drop to correctly embed onto the agar.

Note: The addition of Tx to 7H10 medium prevents colony roughness, providing a smooth colony morphology, making colony counting easier. In the presence of Tx, mycobacterial cells will grow on the solid medium with a colony morphology like that of *E. coli*.

7. Prepare drug stock solutions by dissolving compounds in its preferred solvent as recommended by the manufacturer.
   a. Aliquot the stock solution into 0.2 mL tubes, light protected them when required.
   b. Store at −20°C.

   Note: Thawed vials should not be reused, unless an analysis confirming stability after freeze/thaw cycles has been previously performed.

8. Prepare the required drug working solution concentration in 7H9-OADC-Gly broth from an aliquot of a stock solution by making dilutions steps.

   ⚠ CRITICAL: Step 6.e. is a critical step to ensure proper CFU enumeration. The right moisture on the agar plate plays a crucial role. If agar plates are too wet, the 2.5 μL drop will spread over the agar surface and collapse with nearby drops, thus compromising CFU enumeration. If agar plates are too dry, the 2.5 μL drop will not disperse enough making it challenging to accurately enumerate bacterial counts. We found that allowing agar plates to dry open in the biosafety cabinet for 40–45 min gives the right moistures conditions for proper drop plating and accurate CFU enumeration.

Inoculum preparation

Timing: ≈1 h, 3 days before the start of the experiments

9. Start a *Mtb* culture in the required volume of 7H9-OADC-Gly medium.

   Note: The total volume needed will be dependent on the number of conditions to be tested and, normally, considers 10 mL of bacterial culture per flask or 25 mL per 96-well plate, 250 μL/well.

   a. Thaw one aliquot vial of the characterized bacterial frozen stock prepared as described above.
   b. Prepare a bacterial suspension of ca. 10^4 CFU/mL (pre-inoculum).

   Note: Calculations are based on the actual bacterial concentration of frozen stocks.

   c. Incubate the pre-inoculum at 37°C, without CO_2, for 3 days in static conditions.

   Note: This step allows bacteria to recover and reach a concentration ca. 10^5 CFU/mL.

10. Plate serial dilutions of the pre-inoculum for CFU enumeration at day −3 using the CFU counting method, as described below in section "sampling for bacterial counts: CFU counting and MPN assay".

Preparation of the "mother plates": Addition of drugs

Timing: ≈2 h, 0–2 days before the start of the experiments

This step involves dispensing drugs into the 96-well plate before adding the bacterial culture. Plates with test conditions will be referred to as "mother plates" from now on.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Neg. control	Neg. control	Neg. control	Neg. control	Neg. control	Neg. control	Neg. control	Neg. control	Neg. control	Neg. control	Neg. control	Neg. control
B	Comp. 1 1/10x MIC	Comp. 1 1/10x MIC	Comp. 1 1/10x MIC	Comp. 1 1/4x MIC	Comp. 1 1/4x MIC	Comp. 1 1/4x MIC	Comp. 1 1/2x MIC	Comp. 1 1/2x MIC	Comp. 1 1/2x MIC	Comp. 1 1x MIC	Comp. 1 1x MIC	Comp. 1 1x MIC
C	Comp. 1 2x MIC	Comp. 1 2x MIC	Comp. 1 2x MIC	Comp. 1 4x MIC	Comp. 1 4x MIC	Comp. 1 4x MIC	Comp. 1 10x MIC	Comp. 1 10x MIC	Comp. 1 10x MIC	Comp. 1 100x	Comp. 1 100x	Comp. 1 100x
D	Comp. 2 1/10x MIC	Comp. 2 1/10x MIC	Comp. 2 1/10x MIC	Comp. 2 1/4x MIC	Comp. 2 1/4x MIC	Comp. 2 1/4x MIC	Comp. 2 1/2x MIC	Comp. 2 1/2x MIC	Comp. 2 1/2x MIC	Comp. 2 1x MIC	Comp. 2 1x MIC	Comp. 2 1x MIC
E	Comp. 2 2x MIC	Comp. 2 2x MIC	Comp. 2 2x MIC	Comp. 2 4x MIC	Comp. 2 4x MIC	Comp. 2 4x MIC	Comp. 2 10x MIC	Comp. 2 10x MIC	Comp. 2 10x MIC	Comp. 2 100x	Comp. 2 100x	Comp. 2 100x
F	Comp. 3 1/10x MIC	Comp. 3 1/10x MIC	Comp. 3 1/10x MIC	Comp. 3 1/4x MIC	Comp. 3 1/4x MIC	Comp. 3 1/4x MIC	Comp. 3 1/2x MIC	Comp. 3 1/2x MIC	Comp. 3 1/2x MIC	Comp. 3 1x MIC	Comp. 3 1x MIC	Comp. 3 1x MIC
G	Comp. 3 2x MIC	Comp. 3 2x MIC	Comp. 3 2x MIC	Comp. 3 4x MIC	Comp. 3 4x MIC	Comp. 3 4x MIC	Comp. 3 10x MIC	Comp. 3 10x MIC	Comp. 3 10x MIC	Comp. 3 100x	Comp. 3 100x	Comp. 3 100x
H	Pos. Control	Pos. control	Pos. control	Pos. control	Pos. control	Pos. control	Pos. control	Pos. control	Pos. control	Pos. control	Pos. control	Pos. control

Figure 1. Example of a "mother plate" layout for TKA

The following steps describe the preparation of mother plates, either by manually adding the compound to each well or by using an automatic digital dispenser (e.g., Tecan HP D300e digital dispenser). When possible, we recommend using an automatic dispenser to ensure accurate drug delivery into the plates.

Dose-range concentrations tested in TKA typically include sub-MIC and over-MIC values, such as 1/10x, 1/4x, 1/2x, 1x, 2x, 4x, 10x and 100x MIC. This can be adapted based on specific assay requirements.

11. Design the layout of the mother plate (Figure 1).
    a. Set the position of each condition/concentration within the plate.

Note: Each concentration is tested in triplicate.

    b. Always include negative (no bacteria, no drug) and positive (bacteria, no drug) growth controls and indicate clearly their position in the plate.

Note: When testing drugs dissolved in DMSO, consider including positive controls of growth with the corresponding final DMSO concentrations in the plate to ensure DMSO is not affecting the growth of the bacteria.

Note: When testing new drugs or resistant strains, it is recommended to include a drug with a well-known effect at a specific concentration as internal assay control. This helps to validate the assay and provides a basis for comparing the efficacy of tested drugs.

Note: Depending on the total volume to be sampled per well at each time point (for CFU counting, MPN assay, bioanalysis or other relevant readouts based on the purpose and the design of the experiment), replicates of the mother plate should be prepared to cover the total volume needed for the whole experiment. For instance, it may be necessary to prepare one mother plate per time point, or alternatively, one mother plate for every two time points if less volume is needed per time-point. The type of the 96-well plate described here allows a maximum volume per well of 250 μL.

12. Add the required amount of drug to each well:
    a. When adding the drug manually in each well.
        i. Prepare the drug working solutions in broth medium at 10x the final concentration to be tested in the TKA,
        ii. Following the plate layout shown in Figure 1, manually add 25 μL of the 10x drug working solution to each well to achieve the final desired drug concentration in every well.

Note: 225 μL of bacterial culture will then be added to a final volume of 250 μL/well.

Note: This is just an example of preparation. Drug working stock concentration and bacterial culture volume can be adjusted to maintain a final volume of 250 μL/well and achieve the desired final drug concentration.

    b. Or, add the required amount of drug using an automatic dispenser (the use of Tecan HP D300e digital dispenser is described here as an example):
        i. Introduce the designed plate layout (Figure 1) to the dispenser machine through the corresponding software.
        ii. Set the final volume of culture to 250 μL/well and the maximum final dimethyl sulfoxide (DMSO) concentration allowed per well to 2%.

Note: For *Mtb*, the maximum allowable concentration of DMSO in the culture should ideally not exceed 2%; however, this limit may vary for other bacteria.

Note: The use of DMSO as a solvent is recommended when using the Tecan digital dispenser because, unlike water-based buffers or other solvents, it ensures consistent dispensing performance, particularly at small volumes (picoliters to microliters).

        iii. Add the compound directly from the stock solution into each well by following the digital dispenser software instructions.
    c. Keep drug-containing plates in the fridge (4°C) wrapped in aluminum foil until inoculation.

Note: In case of TKA performed in culture flasks, drugs are added manually into the flask from the working drug stock solutions and according to the final concentration set per flask. Thus, drug stock solution dilutions might be needed to prepare the working solutions.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Mycobacterium tuberculosis H37Rv strain		GenBank: NC_000962.3
Chemicals, peptides, and recombinant proteins		
Glycerol	Sigma	15523-1L-M
Tyloxapol	Merck-Sigma	T8761-50G

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
DMSO	Merck-Sigma	D5879-1L
Difco Middlebrook 7H10 Agar 500 g	Becton Dickinson	262710
Difco Middlebrook 7H9 Broth 500 g	Becton Dickinson	271310
OADC Middlebrook Enrichment 500 mL	Becton Dickinson	212351
Resazurin	Merck-Sigma	R7017-1G
Activated charcoal	Sigma	C5510
Software and algorithms		
D300e Control Software (version 3.4.2)	Tecan Life Sciences	
Other		
Polystyrene 96-well plates	TPP; Nunc	92096; 167008
Tissue culture flask (25 cm^2)	TPP	90025
Polystyrene rectangular plates (86 × 128 mm)	Thermo Scientific	264728;
Polystyrene square plates (120.5 × 120.5 mm)	Corning	BP124-05
Nunc Edge 96-well, non-treated, flat-bottom microplates	Thermo Fisher Scientific	267427
TECAN D300e Digital dispenser machine	Tecan Life Sciences	HP Model F0L56A

MATERIALS AND EQUIPMENT

7H9-OADC-Gly		
Reagent	Final concentration	Amount
7H9 base	N/A	1.88 g
Glycerol (Gly)	0.5%	2 mL
OADC	10%	40 mL
dH_2O	N/A	360 mL
Total	N/A	**400 mL**
Store at −4°C for up to 2–4 weeks.		

7H9-OADC-Gly-Tx		
Reagent	Final concentration	Amount
7H9 base	N/A	1.88 g
Glycerol (Gly)	0.5%	2 mL
Tyloxapol (Tx)	0.05%	0.2 mL
OADC	10%	40 mL
dH_2O	N/A	360 mL
Total	N/A	**400 mL**
Store at −4°C for up to 2–4 weeks.		

7H10-OADC-Gly-Tx		
Reagent	Final concentration	Amount
7H10 base	N/A	9.5 g
Glycerol (Gly)	0.5%	2.5 mL
Tyloxapol (Tx)	0.1%	0.5 mL
OADC	10%	50 mL
dH_2O	N/A	450 mL
Total	N/A	**500 mL**
Store at −4°C for up to 2–4 weeks.		

STEP-BY-STEP METHOD DETAILS

Start of the assay: Addition of the inoculum

Timing: ≈1 h, timing may vary depending on the number of flasks/mother plates; day 0

This section describes Day 0, which is the start of the TKA experiment. It includes transferring the bacterial inoculum into the flasks/mother plates containing the drug at the tested concentration range and performing CFU and MPN assays on the sample at that time point.

1. After 3 days of inoculum incubation, transfer the culture to the appropriate number of 25 cm^2 tissue culture flasks (10 mL per flask) or into the mother plates (250 μL/well), depending on the type of experiment.

   Note: Drugs are already added to each well of the mother plates at the desired concentration prior the bacterial culture addition step using the digital dispenser or manually. The corresponding steps are described in step 12 under "before you begin" and "preparation of the 'mother plates': addition of drugs" section.

   Note: In the case of TKA performed in flasks, manually add drugs from the working stocks to each flask at the desired concentrations after adding inoculum.

   Note: Do not forget to always include a non-treated bacterial growth control.

Sampling for bacterial counts: CFU counting and MPN assay

Timing: 1 h per mother plate (as in Figure 1), at every selected time point

This section describes how to take samples from the culture, perform serial dilutions to generate MPN plates, and plate serial dilution samples in 7H10-OADC-Gly-Tx medium at designated experimental time points (Figure 2). Standard time points for *Mtb* are 0, 1, 3, 7, 10, 14, and 21 days, but these can be adapted to experimental needs.

2. Fill all wells from rows B to H of a Nunc Edge 96-Well Flat-Bottom microplate (MPN plate) with 180 μL of standard 7H9-OADC-Gly-Tx or 7H9-OADC-Gly-Tx+SN.

   Note: Tx is used to disperse *Mtb* cultures and prevent clump formation within the microplate, facilitating MPN readings.

   ⚠ CRITICAL: Specific 96-well plates having large reservoirs filled with sterile water surrounding the edge of the plate should be used to prepare the serial dilution, as these plates will also serve as the MPN plates. This is important to prevent well evaporation due to the long incubation times. It also reduces the edge effect. It is highly recommended to use the same 96-well plate described in this protocol and indicated in the key resources table (Nunc Edge 96-Well Flat-Bottom, Thermo Fisher, ref. 267427).

3. Considering the plate layout in Figure 1, where row A of the mother plate is designated for the negative control, transfer 50 μL of each sample from row B of the mother plate into row A of an MPN plate.
   a. Repeat this process by transferring samples from row C to row A of a different MPN plate.
   b. Continue similarly for rows D to H of the mother plate.

   Note: This may change according to the specific layout of your plate and where you put the controls.

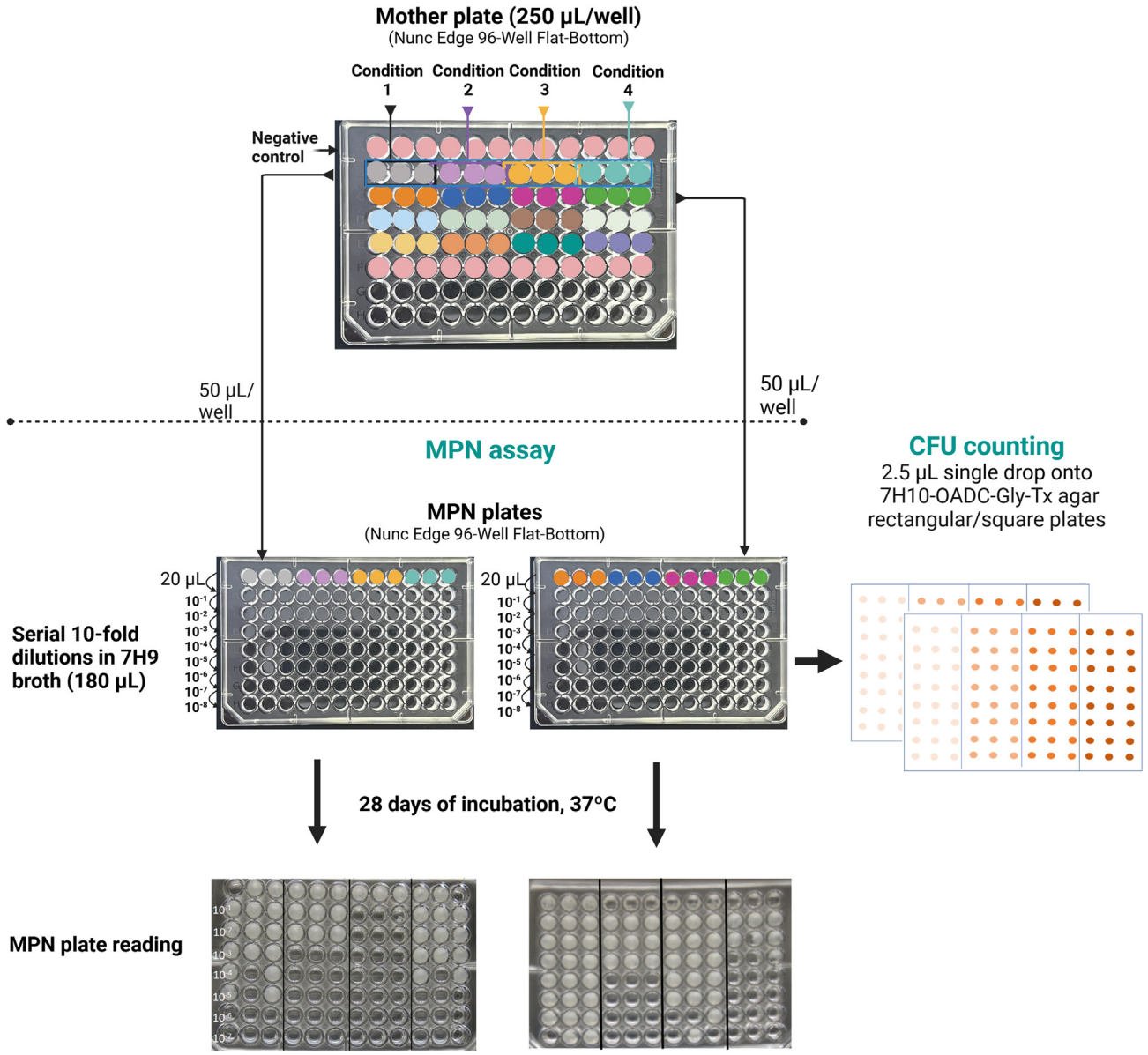

Figure 2. Example of a TKA plate processing workflow

Colors within the mother plate represent varying drug concentrations or conditions. Samples from row B of the mother plate are transferred to row A of an MPN plate, samples from row C are transferred to row A of a different MPN plate and this process continues for each row. After transfer, samples are serially diluted and then, CFU enumeration is performed directly from the MPN plates. This process is repeated for every TKA time point.

4. Perform 10-fold serial dilution in your MPN plates by pipetting 20 µL from Row A to Row B, then from Row B to Row C and so on, by using a multichannel micropipette.
   a. Mix carefully the sample by pipetting up and down before taking and transferring the volume.

   Note: Tips must be changed after every dilution step.

5. Transfer 2.5 µL of the serial dilution samples to a rectangular plate filled with 7H10-OADC-Gly-Tx agar using a multichannel pipette for bacterial CFU enumeration.
6. Allow CFU plates to dry in the biosafety cabinet before incubation.

7. Add 1.8 mL of sterile distilled water in each reservoir surrounding the edge of the 96-well plate.
8. Incubate the CFU and MPN plates at 37°C wrapped in aluminum foil in closed plastic Tupperware-like boxes (to prevent evaporation), in static conditions and without CO_2.
9. Count colonies after 14 and 21 days of incubation.

Note: Both counts will be reported in the raw data template, although only final numbers at day 21 will be considered to calculate CFU/mL. CFU/mL will be calculated as the average of three replicates, and results will be reported in $\log_{10}$ CFU/mL.

10. Read MPN plates after 28 days of incubation by visual observation using an inverted mirror to identify positive/turbid and negative growth in the wells.
    a. Note the number of the positive growth per each dilution.
    b. Report the MPN in the raw data template to calculate the MPN_{cells}/mL.
    c. Calculate single MPN_{cells}/mL value as described in the section "CFU and MPN calculations", step 12, below.

Note: To double-check the positivity of the growth in wells, 30 μL of Resazurin 0.01% (w/v) can be added to each well after visual observation.

Note: In step 3, sampling 50 μL of culture from the mother plate increases throughput and provides the flexibility to perform additional assays from the same well, if required. However, this might have an impact on the limit of detection of the method. To further lower the limit of detection, it is recommended to increase the sample volume up to 200 μL. However, this would decrease throughput, as more mother plate replicates per time point would be needed if additional assays or readouts are performed alongside the CFU and MPN assays.

Note: Plate serial dilutions on Middlebrook 7H10-OADC-Gly-Tx plates without antibiotic. Activated charcoal (0.4% w/v) can be used for high drug concentration conditions to avoid drug carry over, which could mask the actual CFU counts.

Note: Determine the number of MPN plates needed for the assay by considering the total number of rows with conditions across all mother plates. As described in Step 3, each row from the mother plate is transferred to an individual 96-well plate (Nunc Edge 96-Well Flat-Bottom) to perform serial dilutions, which will be used as the MPN plate. For example, if the mother plate has 7 rows (B to H) with conditions that include the positive control in triplicates, as shown in Figure 1, a total of seven MPN plates would be needed, assuming that row A of the mother plate is designated for negative controls.

⚠ CRITICAL: Steps 7 and 8 of this section are critical to prevent evaporation during the incubation period.

CFU and MPN calculations

11. Enumerate and calculate CFU/mL.
    a. Count and record the number of CFU for each dilution of all individual replicates after 14 and 21 days of incubation of the plates.

Note: When plating 2.5 μL drops, the ideal countable range for calculating a CFU/mL value is approximately 5–50 colonies. Choose the lowest dilution that provides you with this range for CFU enumeration.

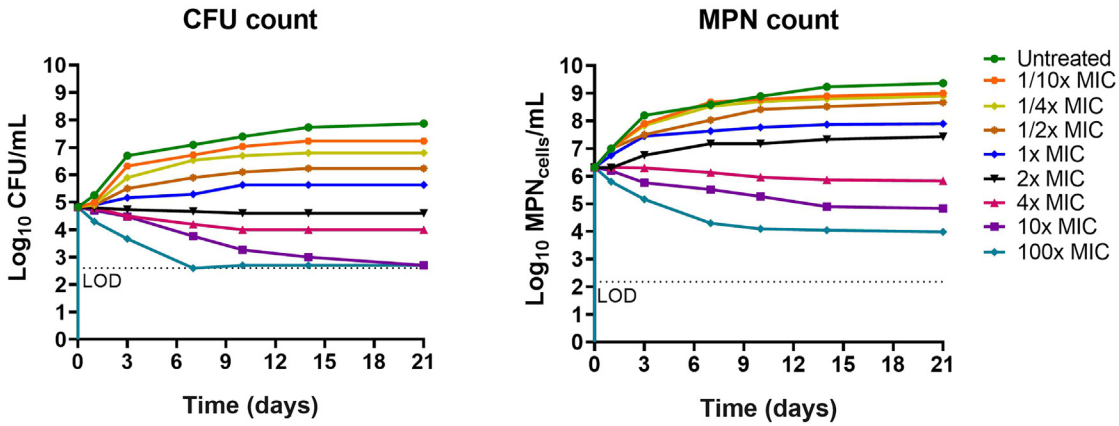

Figure 3. Mock time-kill kinetics curve

CFU (left) and MPN (right) readouts. Drug concentrations correspond to times the MIC value of the antibiotic of choice in the TKA.

b. Report results that fall outside the Reliable Observation Range (ROR, 5–50 colonies) as Above the Quantification Limit (AQL, uncountable) and Below Quantification Limit (BQL zero colonies).

Note: ROR is established based on the experimental setup and represent values that have been observed and mutually agreed upon by the authors.

Note: When plating a 2.5 μL drop, the limit of detection (LOD) is > 2.6 log CFU/mL. If a lower LOD is needed, the plated volume needs to be increased.

c. Select dilution with the ROR to be considered for final calculation for each individual replicate.
d. Calculate CFU/mL by taking the number of colonies from the counts on day 21, factoring in the dilution factor and the volume plated.

Note: Calculate the mean CFU/mL and the standard deviation (SD) from all CFU/mL values of the three replicates.

e. Convert to Log_{10} CFU/mL the mean CFU/mL.
f. Graphically represent Log_{10} CFU/mL *vs.* time for visual inspection using a GraphPad Prism software, or similar (Figure 3).
g. Calculate the effective concentration values (ECs: EC_{20}, EC_{50} or EC_{80}) of the drug of study using GraphPad Prism software, or similar.[7]

Note: Select a specific time point (typically Day 7 or Day 10) where clear separation between the effects of different drug concentrations on bacterial killing is observed, and the maximum and minimum effects are well-defined.

12. Calculate MPN/mL.
    a. Indicate the number of wells/replicates with visible growth per dilution.
    b. Select three consecutive dilutions following the instructions of the US Food and Drugs administration (https://www.fda.gov/food/laboratory-methods-food/bam-appendix-2-most-probable-number-serial-dilutions#tab1) under section "Selecting Three Dilutions for Table Reference".[4]

Examples	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	Obtained combination
A	3	3	3	1	0	0	0	3,1,0
B	3	3	3	3	2	0	0	3,2,0
C	3	3	0	0	0	0	0	3,0,0

Figure 4. Examples of MPN readout

The table indicates how to record the number of positive wells per each dilution and how to select the three consecutive dilutions for analysis. Numbers in the table correspond to the number of replicates with visible growth of each dilution. Yellow highlighted boxes are selected, and values are noted in the "obtained combination" column, for further analysis.

Note: To increase accuracy, start by selecting the highest dilution with positive growth in all three wells/replicates (meaning having all the three wells (n = 3) positive) as your first selected dilution. Then, select the next two higher dilutions to form a set of three consecutive dilutions. For example, as shown in Figure 4, in Case A, dilution 10^{-3} is first selected, followed by dilutions 10^{-4} and 10^{-5} to complete the set. In Case B, dilution 10^{-4} is selected first, followed by 10^{-5} and 10^{-6}. In Case C, dilution 10^{-2} is chosen first, followed by the next two dilutions.

c. Record the combination of three numbers from the three selected dilutions.

Note: This corresponds to the pattern of growth. Referring to Figure 4, this combination is 3, 1, 0 for Case A, 3, 3, 0 for Case B, and 3, 0, 0 for Case C.

d. Determine the MPN value corresponding to this combination using Table 1, from De Man et al., 1983.[5]

Note: This value corresponds to the probable number of cells in the lowest selected dilution among the three selected ones.

e. Calculate the viable cells in the initial bacterial suspension per mL (MPN_{cells}/mL) using the following formula:

$$MPN_{cells}/mL = \text{MPN value from "table 1"} \times \text{dilution factor of the lowest selected dilution} \times (1000\ \mu L/\text{volume of sample})$$

Dilution factor corresponds to the first most concentrated dilution (not very clear) among the three selected ones.

f. Compare the treatment group data to the control data at the corresponding time-points to quantify the drug effect.

Note: Example of MPN calculation. The observed growth patterns per dilution in triplicate from one condition are as follows: in the dilution of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, all 3 wells of each dilution are positive. In the 10^{-5} dilution, two wells are positive and 1 one well is negative. All wells are negative in 10^{-6} and 10^{-7} dilutions. Based on these observations, the selected dilutions are 10^{-4}, 10^{-5} and 10^{-6} dilutions giving a combination number of 3, 2, 0 (the number of positive wells in 10^{-4}, 10^{-5} and 10^{-6} dilutions, respectively). The MPN value from Table 1 corresponding to this combination is thus 9.3 and this number corresponds to the probable number of cells in 10^{-4} dilution. In this case the dilution factor applied is

Table 1. MPN calculation table

Number of turbid wells observed in each selected dilution			MPN (into the first dilution)	Confidence limits (≥ 95%)	
				Low	High
0	0	0	<0.30	0.00	0.94
0	0	1	0,30	0.01	0.95
0	1	0	0,30	0.01	1.00
0	1	1	0,61	0.12	1.70
0	2	0	0,62	0.12	1.70
0	3	0	0,94	0.35	3.5
1	0	0	0,36	0.02	1.70
1	0	1	0,72	0.12	1.7
1	0	2	1,1	0.4	3.5
1	1	0	0,74	0.13	2.00
1	1	1	1,1	0.4	3.5
1	2	0	1,1	0.4	3.5
1	2	1	1,5	0.5	3.8
1	3	0	1,6	0.5	3.8
2	0	0	0,92	0.15	3.50
2	0	1	1,4	0.4	3.5
2	0	2	2.0	0.5	3.8
2	1	0	1,5	0.4	3.8
2	1	1	2.0	0.5	3.8
2	1	2	2,7	0.9	9.4
2	2	0	2,1	0.5	4.0
2	2	1	2,8	0.9	9.4
2	2	2	3,5	0.9	9.4
2	3	0	2,9	0.9	9.4
2	3	1	3,6	0.9	9.4
3	0	0	2,3	0.5	9.4
3	0	1	3,8	0.9	10.4
3	0	2	6,4	1.6	18.1
3	1	0	4,3	0.9	18.1
3	1	1	7,5	1.7	19.9
3	1	2	12	3	36
3	1	3	16	3	38
3	2	0	9,3	1.8	36.0
3	2	1	15	3	38
3	2	2	21	3	40
3	2	3	29	9	99
3	3	0	24	4	99
3	3	1	46	9	198
3	3	2	110	20	400
3	3	3	>110		

For triplicate wells inoculated from each of three successive 10-fold dilutions.[5]

10,000 (or 10^4) and 20 μL were inoculated for each dilution. By considering those parameters and using the formula to calculate the MPN_{cells}/mL, the MPN_{cells} of this condition is 4.65×10^6. MPN_{cells}/mL = 9.3×10000 x (1000 μL/20 μL) = 4.65×10^6.

Note: Alternatively, an MPN calculator in Excel, MPN_ver6.xls, published by Jarvis et al., 2010[8] can be used as well. This Excel file can be downloaded at: www.wiwiss.fu-berlin.de/fachbereich/vwl/iso/ehemalige/wilrich/index.html.

EXPECTED OUTCOMES

A graphical representation of log_{10} CFU/mL and log_{10} MPN_{cells}/mL *vs.* time for the different conditions, i.e., the different antibiotic concentrations tested (Figure 3).

TKA experiments are designed to provide detailed longitudinal curves that illustrate the efficacy of the tested drug over time at different concentrations. These curves are expected to reveal a concentration-effect relationship, although this relationship may vary depending on the nature of the drug being tested. For example, at the highest concentrations, a killing effect may be observed, characterized by a significant reduction in bacterial counts over time. At concentrations near the MIC, a static effect might occur, where bacterial growth is inhibited but not completely eliminated. Below MIC values, no significant inhibitory effect is typically expected, allowing normal bacterial growth. However, this pattern is not consistent across all drugs and can differ depending on the drug's mode of action and its interaction with the bacterial population.

In addition, the MPN assay, being more sensitive than CFU enumeration at detecting residual bacterial loads not detected by CFU enumeration, is anticipated to reveal higher bacterial counts in most conditions, providing additional information on the activity of tested drugs against non-culturable bacteria. For instance, under circumstances where CFU results suggest sterilization (no detectable growth), MPN may detect viable but non-culturable (VBNC) bacteria, reflecting residual bacterial populations. Similarly, MPN may reveal evidence of bacterial regrowth in conditions where this is not observed by CFU enumeration. These differences underscore the importance of using multiple readouts to fully capture bacterial responses to drug treatment.

LIMITATIONS

For CFU determinations, plating a small volume of 2.5 μL increases assay throughput facilitating the analysis of more conditions at a time with respect to the 100 μL standard plating. This is achieved by reducing operator manipulations, materials needed and incubator space in the BSL-3 laboratory. However, the quantification limit is higher (>2.6 log CFU/mL), decreasing the sensitivity of the technique. To increase sensitivity, 10, 100 μL or even higher volumes can be plated in regular petri dishes.

For MPN quantification, the positivity of the growth at each well per dilution is identified by visual observation making the method more prone to reader's bias. However, this can be overcome by doing a secondary reading validation by another operator. It is recommended to perform mock training with different operators. Alternatively, it can be used as a growth indicator like resazurin. Although this extra step might make the method more laborious, it increases the accuracy of the measurement (Figure 5). Additionally, cultures in 96-well plates are more susceptible to evaporation and condensation issues due to extended incubation times, as in the case of MPN plates, potentially compromising the validity of the readout. This, can be avoided by using appropriate 96-well plates with edge reservoirs that can be filled with sterile distilled water, and adequate incubation conditions to keep the environment moist, as indicated in section "sampling for bacterial counts: CFU counting and MPN assay", steps 7 and 8.

TROUBLESHOOTING

Problem 1

For the 2.5 μL CFU plating method (step 5, sampling for bacterial counts: CFU counting and MPN assay), the drop may spread on top of the agar and mix with others. This might happen if the plates are not sufficiently dry.

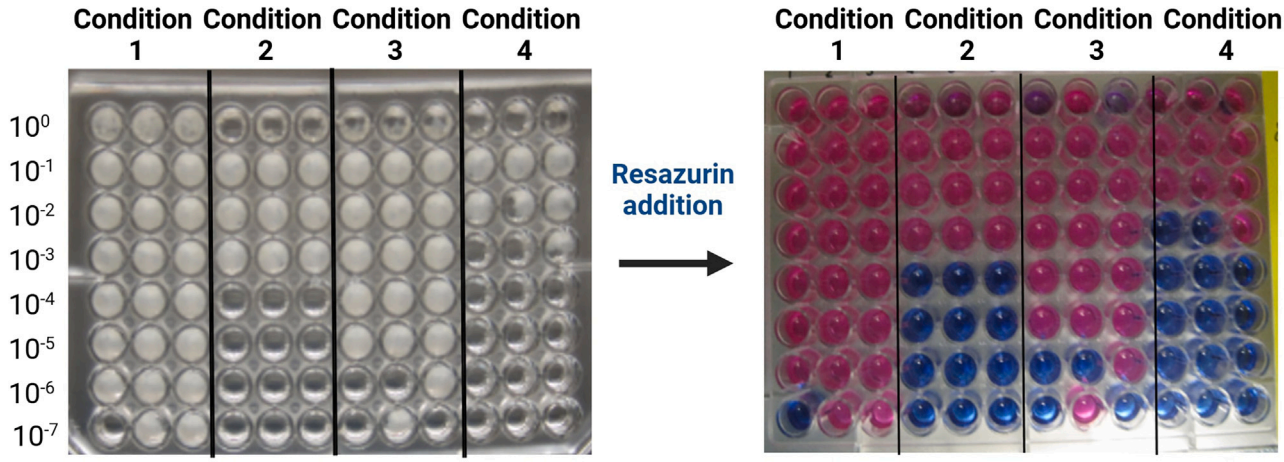

Figure 5. Representative images of *Mtb* growth from an MPN plate after 28 days of incubation

Positive/turbid and negative growth in wells per dilution are shown from each condition. The presence or absence of growth is confirmed after incubation in the presence of resazurin addition.

Potential solution

When preparing 7H10 agar plates (step 6, Preparation of buffers, media, and drug solutions), let them dry open in the biosafety cabinet for 40–45 min before use. This step ensures plates have the right moisture conditions for proper drop plating and accurate CFU enumeration.

Problem 2

For the MPN assay, in case of using broth, reagents or compounds that precipitate, it is difficult to differentiate by visual observation between the positive growth and the precipitant (step 10, sampling for bacterial counts: CFU counting and MPN assay).

Potential solution

During MPN reading, after visual observation, we recommend using a resazurin solution to confirm whether the turbidity is due to bacterial growth (positive result) or drug precipitation (false positive). Resazurin is a redox indicator that changes color from blue to pink in the presence of viable bacterial growth. This method helps differentiate between true bacterial growth and drug precipitates, ensuring more accurate MPN readings.

Problem 3

Suspicion of contamination in some wells of the MPN assay, such as the presence of non-homogeneous growth within the plates (step 10, sampling for bacterial counts: CFU counting and MPN assay).

Potential solution

Plate samples from wells with visible growth, including suspicious ones, onto blood agar or another rich agar medium such as Muller-Hinton to confirm the presence of contamination. If contamination is present, colonies of contaminants will appear on the agar. This step helps determine whether the turbidity observed is due to bacterial growth of the target organism or contamination, which could affect the validity of the results. Additionally, ensure that all sterile techniques are strictly followed during the process to minimize the risk of contamination.

Problem 4

False activity due to drug carryover when high drug concentration tested (sampling for bacterial counts: CFU counting and MPN assay).

Potential solution

This issue can be addressed by incorporating activated charcoal (0.4% w/v) into the 7H10 agar plates to adsorb any residual drug from the samples, reducing the risk of carryover. This process prevents the residual drug from interacting with the bacteria and ensures that any observed effect on bacterial growth is due to the drug being tested, and not the leftover drug that may still be present on the plate, which could unintentionally affect the bacteria.

Problem 5

Evaporation of the MPN plates and/or mother plates during incubation (step 8, sampling for bacterial counts: CFU counting and MPN assay).

Potential solution

This may happen depending on the incubator type, highlighting the importance of using specific 96-well plates with edge-reservoirs to allow the addition of sterile distilled water to maintain a humid environment, reducing evaporation from the wells during incubation. For long experiments, always check water level and add it frequently, if needed. Additionally, place a recipient or platter tray containing sterile distilled water inside the incubator to further minimize evaporation and condensation. These measures ensure that the volume inside each well remains consistent throughout the incubation period, reducing the risk of results being affected by drying out.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ainhoa Lucía (ainhoalq@unizar.es).

Technical contact

Questions about technical specifics on executing the protocol should be directed to and will be answered by the technical contacts, Marie Sylvianne Rabodoarivelo (mrabodoarivelo@unizar.es) and Ainhoa Lucía (ainhoalq@unizar.es).

Materials availability

This study did not generate new unique materials of reagents.

Data and code availability

Original/source data for the 2.5 μL CFU- plating method described in the paper is from the published article in ref.[1]

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This work reflects only the author's views, and the JU is not responsible for any use that may be made of the information it contains.

The graphical abstract and Figure 2 were created using Biorender.com (Rabodoarivelo, M. [2025] https://BioRender.com/r25n183).

AUTHOR CONTRIBUTIONS

Conceptualization, M.S.R., S.R.-G., and A.L.; methodology, M.S.R., E.H., C.G., D.A.A.-A., J.G., L.S., M.D.M., L.C.-M., G.D., and D.R.; visualization, M.S.R.; supervision, J.R., R.M., M.R.P., S.R.-G., and A.L.; project administration, J.R., R.M., M.R.P., S.R.-G., and A.L.; funding acquisition, J.R., R.M., M.R.P., S.R.-G., and A.L.; writing – original draft, M.S.R., S.R.-G., and A.L.; review and editing, all authors. All authors read and approved the final version of the article.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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