1 **TITLE:**
2 Micro-Colony Forming Unit Assay for Efficacy Evaluation of Vaccines against Tuberculosis
3
4 **AUTHORS AND AFFILIATIONS:**
5
6 David Pires[1,2,3], Paulo J. G Bettencourt[1,2]
7
8 [1] Universidade Católica Portuguesa, Faculty of Medicine, 2635-631 Rio de Mouro, Portugal
9 [2] Universidade Católica Portuguesa, Center for Interdisciplinary Research in Health, 1649-023
10 Lisboa, Portugal
11 [3] Host-Pathogen Interactions Unit, Research Institute for Medicines, iMed-ULisboa, Faculty of
12 Pharmacy, Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-003 Lisboa, Portugal
13
14 Email address of the co-author:
15 David Pires (dpires@ucp.pt)
16
17 Corresponding author:
18 Paulo J. G. Bettencourt (pbettencourt@ucp.pt)
19
20 **SUMMARY:**[A1][A2]
21 The determination of colony-forming units is the gold-standard technique for quantifying most
22 bacteria, including the slow-growing *Mycobacterium tuberculosis* that can take weeks to form
23 visible colonies. Here we describe a streamlined approach to CFU determination called micro-
24 CFU, with increased time-efficiency, reduces lab space and reagent cost, and is easily scalable to
25 medium and high throughput experiments.
26
27 **ABSTRACT:**
28 Tuberculosis (TB), the leading cause of death worldwide by an infectious agent, killed 1.6 million
29 people in 2022 and was surpassed by COVID-19 during the 2019-2021 pandemic. The disease is
30 caused by the bacterium *Mycobacterium tuberculosis* (*M.tb*). The *M. bovis* strain Bacillus
31 Calmette-Guérin (BCG), the only TB vaccine, is the oldest licensed vaccine in the world, still in
32 use. Currently, there are 12 vaccines in clinical trials and dozens of vaccines under pre-clinical
33 development. The method of choice used to assess the efficacy of TB vaccines in pre-clinical
34 studies is the enumeration of bacterial colonies by the colony-forming units (CFU) assay. This
35 time-consuming assay takes 4 to 6 weeks to conclude, requires substantial laboratory and
36 incubator space, has high reagent costs, and is prone to contamination. Here we describe an
37 optimized method for colony enumeration, the micro-CFU (mCFU), that offers a simple and rapid
38 solution to analyze *M.tb* vaccine efficacy results. The mCFU assay requires tenfold fewer
39 reagents, reduces the incubation period threefold, taking 1 to 2 weeks to conclude, reduces lab
40 space and reagent cost, and minimizes the health and safety risks associated with working with
41 large numbers of *M.tb*. Moreover, to evaluate the efficacy of a TB vaccine, samples may be
42 obtained from a variety of sources, including tissues from vaccinated animals infected with
43 Mycobacteria. We also describe an optimized method to produce a unicellular, uniform, and
44 high-quality mycobacterial culture for infection studies. Finally, we propose that these methods

45 should be universally adopted for pre-clinical studies of vaccine efficacy determination,
46 ultimately leading to time reduction in the development of vaccines against TB.
47

48 **INTRODUCTION:** [A3]

49 Tuberculosis (TB) is the leading cause of death worldwide by a single infectious agent, bacterium
50 *Mycobacterium tuberculosis* (*M.tb*), killing more people than any other pathogen. In 2021, TB
51 was responsible for 1.6 million deaths and was surpassed by COVID-19 during the 2019-2021
52 pandemic [1]. Moreover, according to the World Health Organization´s Global TB Report of 2022,
53 the COVID-19 pandemic was responsible for an increase in new TB cases. The WHO also reports
54 large drops in the number of people diagnosed with TB during this period, which could increase
55 further the number of TB cases [1].
56

57 The Bacillus Calmette-Guérin (BCG) is a live-attenuated strain of the pathogenic *Mycobacterium*
58 *bovis*, used for the first time as a vaccine more than 100 years ago. This is the only vaccine against
59 TB and is the oldest licensed vaccine in the world still in use [2, 3]. Currently, there are 12 vaccines
60 in different phases of clinical trials [4], and dozens of vaccines are under pre-clinical development
61 [5, 6]. Pre-clinical assessment of vaccines against TB includes the evaluation of the safety and
62 immunogenicity [7], which can be obtained in diverse animal models such as zebrafish, mice,
63 guinea pigs, rabbits, cattle, and non-human primates [8–10]. Additionally, assessing the capacity of
64 a vaccine to induce protection against *M.tb* infection and/or transmission, i.e., the vaccine
65 efficacy, requires an *M.tb* challenge *in vivo* [5, 11]. Interestingly, BCG vaccination induces
66 non-specific effects that affect the survival of other bacterial and viral pathogens [12, 13] through
67 the mechanism of trained immunity [14]. To quantify the viable bacterial burden in an infected
68 animal, the method of choice is the enumeration of bacterial colonies through the colony-
69 forming units (CFU) assay [5, 15]. CFU is a unit that estimates the number of microorganisms
70 (bacteria or fungi) that form colonies under specific growth conditions. CFUs originate from viable
71 and replicative microorganisms, and the absolute number of living microorganisms within each
72 colony is difficult to estimate. It is uncertain whether a colony has originated from one or more
73 microorganisms. The CFU unit reflects this uncertainty, hence a great variability can be observed
74 in replicates of the same sample. This is a time-consuming assay, requires specialized technicians
75 trained to work in a Biosafety Level 3 (BSL3) facility, substantial laboratory and incubator space,
76 takes from 4 to 6 weeks to conclude, and is prone to contamination.
77

78 In this study, we describe an optimized method for colony enumeration, the micro-CFU (mCFU),
79 and offer a simple and rapid solution to analyze the results [15–20]. The mCFU assay requires tenfold
80 fewer reagents, reduces the incubation period threefold, taking 1 to 2 weeks to conclude,
81 reduces lab space and reagent cost, and minimizes the health and safety risks associated with
82 working with large numbers of *M.tb*. We propose that this method should be universally adopted
83 for pre-clinical studies of vaccine efficacy determination, ultimately leading to time reduction in
84 the development of vaccines against TB. Finally, this optimized method of CFU enumeration has
85 been used to quantify not only Mycobacteria but also other bacteria, such as *Escherichia coli* and
86 *Ralstonia solanacearum* [21].
87

88 **PROTOCOL:** [A4]

89
90 NOTE: The protocol described here is for BCG but can be applied to any Mycobacteria. BCG can
91 be used as a surrogate bacterium for TB experiments when BSL3 facilities are not available [22]. The
92 following procedures using BCG should be performed under a biosafety level 2 (BSL2) laboratory
93 and follow the appropriate biosafety guidelines and good laboratory practices for the
94 manipulation of hazard group 2 microorganisms.
95
96 **1. Culture media preparation**
97
98 1.1. Prepare Middlebrook 7H9 broth supplemented with 10% (v/v) OADC enrichment (oleic
99 acid, albumin, dextrose, catalase), according to the supplier's instructions. Supplement the
100 broth with 0.05% (v/v) of Tyloxapol.
101
102 NOTE: Tyloxapol is a non-ionic liquid polymer that has been used as a surfactant to prevent
103 bacterial clump formation [16].
104
105 1.2. Prepare Middlebrook 7H10 solid medium supplemented with 10% (v/v) OADC
106 enrichment (oleic acid, albumin, dextrose, catalase) according to the supplier's instructions.
107
108 1.3. Distribute 40 mL of medium per square Petri dish (120 mm x 120 mm). Allow the plates
109 to dry to minimize condensation at the surface of the agar.
110
111 NOTE: This specific size of petri dish is fundamental to allow direct transposal of at least 96
112 droplets from a 96-well plate. Effective drying of the plates will later facilitate the plating of small
113 droplets of bacterial suspension and prevent the droplets from spreading.
114
115 1.4. Prepare either Roswell Park Memorial Institute Medium (RPMI 1640) or Dulbecco's
116 Modified Eagle Medium (DMEM) to produce the infection medium. In either case, supplement
117 the medium with 10% fetal calf serum, 1% L-glutamine, and 1 mM sodium pyruvate. Do not add
118 penicillin and streptomycin to the medium.
119
120 **2. Sample preparation**
121
122 2.1. Obtain samples from a variety of sources. Typically, to quantify CFU to evaluate the
123 efficacy of a TB vaccine, acquire samples come from tissues from vaccinated and unvaccinated
124 animals. For example, mouse lung and spleen [11] or macaque lung, thoracic and peripheral
125 lymph nodes, spleen, liver, skin, blood, bone marrow, and bronchoalveolar lavage wash [23].
126 Alternatively, obtain samples from *in vitro* cultures of macrophages/dendritic cells/neutrophils
127 infected with BCG [18–20, 24–26].
128
129 **3. Production of BCG culture**
130
131 NOTE: For *in vivo* studies of TB vaccines, the aim is to improve the efficacy of BCG. Therefore,
132 BCG-vaccinated groups are usually used as control. BCG strains used for human vaccination are

133 ideal for testing in animal models. In this case, a culture of BCG must be reconstituted according
134 to the supplier's instructions [27]. However, a BCG culture for *in vivo* studies can also be produced
135 in-house [11]. The production of unicellular, uniform, and high-quality BCG culture for *in vitro*
136 infection protocols has been produced very successfully in several studies [11, 16, 18–20, 26, 28, 29], using
137 the following protocol, which can also be used for animal challenge studies.
138
139 3.1. Culture 50 mL of BCG in 7H9 broth[A5][A6], at 37 °C, with agitation at 200 rpm.
140
141 NOTE: Vary the volume according to the needs of the experiment.
142
143 3.2. Every day, for 8-10 days, collect 100 µL of the culture and dilute it by adding 900 µL of
144 PBS in a 1 mL cuvette. Then proceed by measuring the optical density[A7][A8] of bacteria (OD at
145 λ=600 nm) in a spectrophotometer. Draw a growth curve from those values. Identify the mid-
146 log phase of the culture (when the OD is doubling consistently per unit of time).
147
148 3.3. Prepare a subsequent culture and incubate until reaching the mid/late log growth phase
149 as in steps 3.1 and 3.2. Use the values obtained in the previous step as guidance. [A9][A10]
150 Ensure that the culture does not reach the stationary growth phase (when the OD starts
151 stabilizing) to maintain a good quality culture of viable bacteria.
152
153 3.4. Collect the culture at the mid/late log growth phase. Centrifuge at 3000 x *g* for 10 min.
154 Remove the supernatant.
155
156 3.5. Add 10 mL of PBS to wash the bacteria. Centrifuge at 3000 x *g* for 10 min. Remove the
157 supernatant.
158
159 3.6. Resuspend the bacteria with 5 mL of infection media. Place the tube in an ultrasound
160 bath for 15 min, full power at 80 Hz.
161
162 3.7. Centrifuge at 1000 x *g* for 10 min. Collect the supernatant avoiding the pellet as it is rich
163 in bacterial clumps that should be avoided in a high-quality BCG culture and discard it.
164
165 3.8. Measure the OD of the supernatant. Here, cultures at the exponential growth phase,
166 with an OD_{600} of 0.1 are equivalent to 1 x 10^7 CFU/mL.
167
168 NOTE: Each laboratory should produce their own BCG growth curves before starting experiments
169 to establish a linear regression between OD at λ=600 nm and CFU using the spectrophotometer.
170 Please note that spectrophotometers have different light path distances, which can vary the
171 readings obtained for the same sample.
172
173 3.9. Carry out simple calculations to establish the number of bacteria to add to each host cell
174 culture. The number of bacteria per host cell is the Multiplicity of Infection (MOI). Use an MOI
175 of 10 bacteria per host cell which is the most common MOI used for BCG infection experiments.
176

177 **4. Micro-Colony Forming Unit Assay**
178
179 NOTE: After an *in vivo* or *in vitro* infection experiment is completed, the enumeration of bacteria
180 can be performed by mCFU. For *in vivo* studies, samples must be first homogenized in a bead
181 beater or another tissue homogenizer. For *in vitro* cultures of macrophages/dendritic
182 cells/neutrophils infected with BCG, samples must be lysed using a non-ionic detergent (e.g.,
183 0.05% solution of non-ionic, non-denaturing detergent). [A11]
184
185 4.1. Serial dilutions using a 96-well plate: perform serial 10-fold dilutions of the lysates, in a
186 sterile 96-well plate according to the scheme in **Figure 1A**. Distribute the lysates [A12]on rows A
187 and E. For each plate, the maximum number of samples and/or replicates is 24.
188
189 [Place **Figure 1** here]
190
191 4.2. Add 180 µL of dH_2O to the remaining wells to perform the serial dilution.
192
193 4.3. Using a 12-channel pipette resuspend the lysates in row A and transfer 20 µL to row B (20
194 µL lysate + 180 µL dH_2O). Homogenize well. Sequentially repeat this step for rows B and C until
195 reaching the last dilution in row D.
196
197 NOTE: We usually perform three dilutions (10^0, 10^{-1}, 10^{-2}, 10^{-3}) thus using 4 rows of the plate (A-
198 D or E-H) for each set of 12 samples and/or replicates.
199
200 4.4. Micro droplet plating: use a 0.5-10 µL (thin tips are preferred) multichannel pipette to
201 transfer 5 µL from each row of the 96-well plate to the solid medium square plate, according to
202 **Figure 1B**.
203
204 4.5. While slowly pipetting the 5 µL droplets, allow them to slightly touch the agar. This will
205 help to take off the droplet from the tip towards the agar and reduce the possibility of retention
206 of the liquid inside the tip.
207
208 4.6. Allow the droplets to dry, close the agar plate, and incubate it at 37 °C while monitoring
209 bacterial growth. Optionally, incubate the agar plates in a sealed plastic bag to prevent the
210 plates from drying.
211
212 4.7. Micro-colony counting. Following approximately 6-10 days of incubation, check for
213 individual colonies, visible to the naked eye (**Figure 2**).
214
215 [Place **Figure 2** here]
216
217 4.8. Count the colonies using the lowest magnification objective (4x or lower) of an inverted
218 optical microscope or magnifying glass. Counts should be performed in the dilutions where the
219 number of colonies is lower than 300 and higher than 30. Alternatively, use a camera to take a

220 picture of the droplet to manually count colonies on the computer or use software such as
221 ImageJ to automate colony counting.
222
223 4.9. To express cell numbers in CFU/mL, use the following equation:

224 $$\mathrm{CFU/mL} = \mathrm{C} \times \frac{1000}{\mathrm{V}} \times \frac{1}{\mathrm{Dil}}$$

225 Where C = number of colonies counted, V = volume plated in µL, and Dil = dilution where the
226 colonies were counted (10^0, 10^{-1}, 10^{-2}, 10^{-3}). For example, if 30 colonies were counted in a 5 µL
227 droplet in dilution 10^{-2}, then:

228 $$30 \times \frac{1000}{5} \times \frac{1}{10^{-2}} = 6 \times 10^5 \ CFU/mL$$

229
230 **5. Micro-Colony Forming Unit Counting in Fiji (ImageJ)**
231
232 NOTE: The mCFU method allows CFU quantification of large sets of samples. Pictures of the
233 droplets may be recorded for posterior analysis to facilitate colony counting. Several
234 photographic devices can produce images with sufficient quality for this purpose. These include
235 digital cameras, webcams, camera-attached microscopes and magnifying glasses, and cell
236 phones. Free image analysis software such as ImageJ offer the possibility of manual or automated
237 colony counting in those images. To demonstrate both methods, Fiji will be used, which is a
238 distribution of ImageJ that packages several tools for scientific image analysis [30]. Fiji can be
239 downloaded from https://fiji.sc/.
240
241 5.1. Manual counting method.
242
243 5.1.1. Open the image containing the mCFU in Fiji. Select **Plugins > Analyze > Cell Counter**.
244
245 5.1.2. On the Cell Counter menu, select **Initialize** and then select a counter (e.g., Type 1).
246
247 5.1.3. Proceed by clicking on each colony. Each click will be shown on the picture and will update
248 the counter (**Figure 3**). To undo accidental clicks, select **Delete**.
249
250 [Place **Figure 3** here]
251
252 5.1.4. Register the value displayed on the counter. Click the **Reset** button to reset the count and
253 open a new image to count additional samples.
254
255 NOTE: Further instructions on this plugin can be found at https://imagej.net/plugins/cell-
256 counter.
257
258 5.2. Automated counting method.
259
260 5.2.1. Open the image containing the mCFU in Fiji. Select **Image > Type > 8-bit**. This will convert
261 the image to an 8-bit gray-scale image.

262
263 5.2.2. Select the **Oval** tool in the Tool Bar and draw an oval around the area with the colonies
264 (**Figure 4A**). The oval may be adjusted after being drawn.
265
266 5.2.3. Select **Edit > Clear Outside**, to remove any interference from the outside area (**Figure 4B**).
267 Select **Image > Adjust > Threshold**.
268
269 5.2.4. Move the sliders in the Threshold menu until the colonies appear in red and background
270 noise is minimized (**Figure 4C**).
271
272 5.2.5. Select **Apply** and exit the Threshold window. A black-and-white image is generated
273 (**Figure 4D**).
274
275 5.2.6. Select **Analyze > Analyze Particles**. In the Analyze Particles window, specify the range for
276 colony area (between 1 and infinity, measured in squared pixels) and circularity (between 0 and
277 1, where 1 is a perfect circle; **Figure 4E**).
278
279 5.2.7. Select **Outlines** in the Show popup menu. Check **Display Results** for detailed
280 measurements for each colony in the results window. Check **Clear Results** to erase any previous
281 measurements. Check the **Summarize** box to display the summarized results of the
282 measurements (**Figure 4E**).
283
284 5.2.8. Initiate the analyzer by selecting **OK**. A new window appears displaying all the outlined
285 colonies that were detected and counted. The results window displays the details for each
286 colony, and the summarized results window shows the total colonies counted (**Figure 4F**).
287
288 NOTE: The settings for size and circularity will vary with the image's resolution and magnification
289 and the colonies' size and shape. Repeat the process several times until the best settings are
290 found that detect all colonies. Further instructions on the Analyze Particles plugin can be found
291 at https://imagej.nih.gov/ij/docs/menus/analyze.html#ap.
292
293 [Place **Figure 4** here]
294
295 **REPRESENTATIVE RESULTS:**
296 The mCFU assay described here increases the amount of information that can be retrieved from
297 a single Petri dish to at least 96-fold. **Figure 5** depicts a comparison of two drug-delivery methods
298 for the repurposed use of saquinavir (SQV) [31, 32] as a host-directed drug to treat
299 tuberculosis[A13][A14]. In this assay, four different strains of *Mycobacterium tuberculosis* were used
300 to infect primary human macrophages. *M. tuberculosis* H37Rv laboratory strain and three clinical
301 strains isolated from patients with active TB by the Portuguese National Institute of Health's Dr.
302 Ricardo Jorge (INSA): a drug-susceptible strain (INSA code 33427) a multiple drug-resistant (MDR)
303 strain (INSA code 34192) and an extensively drug-resistant (XDR) strain (INSA code 163761). Each
304 infection by each strain was further multiplied into eight different treatment conditions
305 comparing three different concentrations of free-drug or liposome-loaded drug to the respective

306 non-treated controls. Finally, each condition was analyzed at four different time points post-
307 infection. [A15][A16]To quantify intracellular bacterial growth, the total amount of lysates extracted
308 from each time point was 32. Having performed three serial dilutions to each lysate increased
309 the number to 128 samples. Multiplied by the four time points analyzed results in 512 samples.
310 Since the experiment was repeated using macrophages from at least three different blood
311 donors, the total number of analyzed samples increased to 1536. Using the mCFU method
312 described here, this experiment accounted for only 16 square Petri dishes versus 1536 that would
313 be necessary using the standard CFU protocol. As shown in **Figure 5**, the results obtained with
314 this method can demonstrate statistically significant differences between treatments.
315
316 **FIGURE AND TABLE LEGENDS:**
317 **Figure 1.** [A17][A18]**Schematic representation of the mCFU protocol.** (**A**) Serial 10-fold dilutions of
318 the BCG-containing lysates in a 96-well plate. (**B**) Square Petri dish containing solid culture
319 medium and overlayed by ninety-six 5 µL droplets. Droplets are pipetted directly from the 96-
320 well plate using a multichannel pipette. Created with BioRender.com.
321
322 **Figure 2. Micro-colony forming units of BCG following 10 days of incubation.** On the left, a photo
323 of a square Petri dish overlayed by ninety-six 5 µL droplets as previously represented in **Figure**
324 **1B**. On the right, individual photos of 3 droplets correspond to an original lysate (10^0) and two
325 dilutions (10^{-1}, 10^{-2}). Photos were taken using a DSRL camera equipped with an 18-55 mm zoom
326 lens (plate) or a 105 mm macro lens (droplets).
327
328 **Figure 3. A manual method for counting mCFU using the Cell counter plugin on Fiji software.**
329 The blue dots indicate colonies already clicked on by the user. The menu on the right displays the
330 number of colonies counted so far (41).
331
332 **Figure 4. An automated method for counting mCFU using Fiji software.** (**A, B**) The region of
333 interest with the colonies is selected using the oval selection tool and the outside area is removed
334 using the clear outside command. (**C, D**) A black-and-white image of the colonies is generated
335 using the Threshold tool. (**E, F**) The number of colonies is quantified using the analyze particles
336 tool.
337
338 **Figure 5. The mCFU method can produce high amounts of data from a small number of agar**
339 **plates.** This set of experiments tested the efficacy of the drug saquinavir (SQV) to induce
340 intracellular killing of *M.tb* laboratory and clinical strains in human macrophages. SQV was
341 administered to the macrophage cultures in its free form or loaded in liposomes (LipSQV). The
342 treatments were performed in three different concentrations: 50, 20, and 10 µg/mL.
343 Macrophages were infected with different *M.tb* strains for 3 h and then treated with selected
344 concentrations of LipSQV and free SQV. LipUnloaded (liposomes without drug) and DMSO were
345 used as controls. To evaluate bacterial intracellular survival, at discrete time points, macrophages
346 were lysed, and serial dilutions of the bacterial suspension were plated on 7H10 agar plates.
347 mCFU units were counted following 2–3 weeks. Lines depict the average mCFU per sample from
348 at least 3 independent experiments. Bars represent the average mCFU percentage calculated
349 relative to the respective controls at day 7 post-infection. Symbols represent each experiment

350 with macrophages from a different donor. Error bars represent the standard error of the mean.
351 Multiple group comparisons were performed using one-way ANOVA followed by a Holm-Sidak
352 post-hoc test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. [A19][A20]This figure has been modified from[33].
353

354 **DISCUSSION:**

355 TB is an important public health problem with increasing importance, particularly in low and
356 middle-income countries. The disruption of healthcare settings to diagnose and treat TB during
357 the COVID-19 pandemic caused a negative impact on the incidence of new cases [1]. In addition,
358 the multi-drug and extensively-drug resistant *M.tb* strains, and the co-infection of *M.tb* and HIV
359 must be urgently addressed to control this epidemic [1, 34]. New vaccines to replace or improve
360 BCG are currently in clinical trials, and new candidates could hit the market in the coming years
361 [35]. [A21][A22]The development of new vaccine candidates against TB relies on pre-clinical studies
362 using diverse animal models. The vaccine efficacy determination in animal models requires
363 bacterial burden quantification in the infected organs to demonstrate the protective effect of the
364 vaccine [5, 36]. [A23][A24]Currently, there are direct and indirect methods to quantify the bacterial
365 burden in infected mice and non-human primates. The direct methods include histological
366 preparations and bacterial staining using fluorescent auramine-o and Ziehl-Neelsen [37], the
367 quantification of mycobacterial load in samples based on RT-qPCR [38], and others.
368

369 The mycobacterial growth inhibition assay (MGIA) *in vitro* assay based on the BACTEC MGIT
370 system, is a sensitive method used to quantify live mycobacterial burden in vaccinated and
371 unvaccinated animals. The BACTEC MGIT system is a fully automated instrument that can test for
372 the presence of mycobacteria in specifically designed test tubes. The vial contains a fluorescent
373 reporter that reacts to the concentration of oxygen in the sample. The fluorescence is
374 proportional to the amount of oxygen consumed, giving an indication of the number of live
375 bacteria present in the sample, every hour [39]. In the MGIA method, PBMCs and autologous serum
376 samples are collected from animals and co-cultured with mycobacteria for 96 h. Then, adherent
377 monocytes are lysed to release intracellular mycobacteria. The BACTEC MGIT system will quantify
378 the bacterial burden indirectly by measuring oxygen consumption [40]. This method is typically
379 used for drug resistance studies but has been applied recently to the TB vaccine efficacy
380 evaluation [40].
381

382 These methods can be complemented with the standard CFU to determine the total number of
383 viable mycobacteria. For example, the evaluation of vaccine candidates in a murine aerosol *M.tb*
384 challenge experiment is achieved by enumerating colonies obtained from tissues by CFU [11].
385

386 The mCFU method described here can be a substantial improvement over the classic CFU
387 method, as is more rapid to perform and inexpensive in terms of reagents used and in terms of
388 laboratory space required. This method allows the simultaneous comparison of a large set of
389 samples in a single experiment, thus decreasing the effects of assay-to-assay variations. For
390 example, as shown in the experiment described in **Figure 5**, the CFU were determined for 1536
391 samples using the mCFU method in 16 square Petri dishes, which represents a 96-fold reduction
392 in the number of plates used. This is more relevant for experiments using primary biological and
393 clinical samples where availability may be an issue. The compact form in which the colonies are

displayed on an agar plate conveniently allows the use of specialized or common image-recording equipment. This, in combination with automated colony counting methods such as the one described here using the Fiji software, helps reduce the reliance on the user's ability to identify and quantify the CFU. Even so, like the standard CFU technique, this method suffers from the inability to distinguish merged colonies leading to a potential under-representation of the CFU. However, this can be minimized by selecting a higher dilution to perform the quantification or by counting the colonies under the microscope before their overgrowth results in colony merger. This is more relevant if the sample produces colonies with heterogeneous growth rates. Researchers should validate mCFU implementation for their specific experimental conditions in those cases.

The critical steps within the protocol are the following points: Step 4.1 The serial dilutions should be performed using a 96-well plate and the dilutions must be thoroughly mixed; Step 4.5 The micro droplet plating should be performed with a 0.5-10 µL multichannel pipette to transfer 5 µL from each row of the 96-well plate to the solid medium square plate, without touching and damaging the medium; Step 4.7 The micro-colony counting must be performed between 6-10 days of incubation. However, at the time of counting, care must be taken to ensure the colonies are not excessively large or too small.

The modifications and troubleshooting of the technique include the utilization of different bacterial strains. Therefore, for each particular strain the optimal growth medium should be used. The limitations of the technique are identical to the limitations of the conventional CFU method, which include the difficulty to enumerate bacteria in low dilutions. Additionally, specific limitations of this method include the necessity of using a microscope to enumerate the colonies.

Finally, this method can be used to quantify the bacterial burden in TB vaccination studies and also for test drug resistance/susceptibility studies and host-pathogen interaction studies. For example, **Figure 5** shows the application of mCFU for the determination of intracellular killing of *M.tb* strains including clinical strains, multi-drug resistant and extensively-drug resistant strains treated with different drug formulations, in human macrophages. Importantly, this method can also be applied to any microorganism, provided that the culturing conditions for each organism are met.

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DISCLOSURES:
DP and PJGB declare that the study was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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