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	surface of CARA microplate wells permits semi-quantitative assessment of bacterial numbers via fluorometry. The CARA detects approximately a 2-3 log10 difference in bacterial numbers and predicts a minimal bactericidal concentration leading to ≥ 99% bacterial kill (MBC≥99). The CARA helps determine whether a molecule is active on bacilli that are replicating, non-replicating, or both. Pilot experiments using the CARA facilitate the identification of which concentration of test agent, and time of compound exposure, require further evaluated by colony forming unit (CFU) assays. In addition, the CARA can predict if replicating actives are bactericidal or bacteriostatic.
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Visualization of the charcoal agar resazurin assay (CARA) for semi-quantitative, medium-throughput enumeration of mycobacteria

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SHORT ABSTRACT:

The **c**harcoal **a**gar **r**esazurin **a**ssay (CARA) is a semi-quantitative, medium-throughput method to assess activity of test agents against mycobacteria that are replicating, non-replicating, or both. The CARA permits rapid evaluation of time- and concentration-dependent activity and identifies parameters to pursue by colony forming unit (CFU) assays.

LONG ABSTRACT:

There is an urgent need to discover and progress anti-infectives that shorten the duration of tuberculosis (TB) treatment. *Mycobacterium tuberculosis*, the etiological agent of TB, is refractory to rapid and lasting chemotherapy due to the presence of bacilli exhibiting phenotypic drug resistance. The **c**harcoal **a**gar **r**esazurin **a**ssay (CARA) was developed as a tool to characterize active molecules discovered by high-throughput screening campaigns against replicating and non-replicating *M. tuberculosis*. Inclusion of activated charcoal in bacteriologic agar medium helps mitigate the impact of compound carry-over, and eliminates the requirement to pre-dilute cells prior to spotting on CARA microplates. After a 7-10 day incubation period at 37 °C, the reduction of resazurin by mycobacterial microcolonies growing on the surface of CARA microplate wells permits semi-quantitative assessment of bacterial numbers via fluorometry. The CARA detects approximately a 2-3 $\log_{10}$ difference in bacterial numbers and predicts a minimal bactericidal concentration leading to $\geq$ 99% bacterial kill ($MBC_{\geq 99}$). The CARA helps determine whether a molecule is active on bacilli that are replicating, non-replicating, or both. Pilot experiments using the CARA facilitate the identification of which concentration of test agent and time of compound exposure require further evaluation by colony forming unit (CFU) assays. In addition, the CARA can predict if replicating actives are bactericidal or bacteriostatic.

INTRODUCTION:

Mycobacterium tuberculosis, the etiological agent of tuberculosis, can survive in a host in a latent state that is refractory to antibiotic eradication. Phenotypic (non-genetic) resistance of *M. tuberculosis* during infection is believed to be due, in part, to populations of non-replicating bacilli[1,2]. Class I persisters displaying phenotypic drug resistance arise via rare stochastic mechanisms amongst large populations of drug sensitive cells[3,4]. Class II persisters are rendered non-replicating by factors of host immunity, including external stresses in microenvironments encountered during infection. We recently developed an *in vitro* model of Class II non-replicating persistence to mimic conditions confronted by *M. tuberculosis* in activated macrophages and granulomas. The multi-stress model of Class II persistence includes conditions that slow growth, such as a fatty acid carbon source, and completely halt growth, such as mild acidity (pH 5.0), hypoxia (1% O_2), and nitric oxide and other reactive nitrogen intermediates [5-9]. Large-scale screening employing this multi-stress model of non-replication, and other Class II non-replicating models, has yielded diverse molecules whose activity is directed against the non-replicating state[5-7,9-18]. The same non-replicating screens also revealed a category of molecules that possess activity against both replicating and non-replicating bacilli, termed "dual actives"[7,10,12,19,20].

Antibacterial drug discovery in the hit-to-lead phase entails extensive characterization of candidate molecules to choose leads for hit expansion, preliminary pharmacologic characterization, target identification, and preliminary *in vivo* efficacy studies. As an early step, anti-infectives are classified by their bacteriostatic or bactericidal mechanism of action and, if bactericidal, whether bacterial kill is time- and/or concentration-dependent. The colony forming unit (CFU) assay is the classical, gold standard method to address these questions. In the CFU assay, bacteria are exposed to a test agent, after which aliquots are removed, serially diluted, and aliquots of dilutions are spread on solid bacteriologic medium and incubated to permit growth of surviving cells. Finally, bacterial colonies are enumerated. The CFU assay requires large numbers of microtiter plates to dilute cells and agar-containing Petri-plates to enumerate surviving colonies. The CFU assay for slow growing mycobacteria is hampered by their slow generation time (18-24 hours), which

requires approximately 3 weeks for colonies to appear on plates. Furthermore, incubator space is often limited in specialized biosafety-level-3 facilities.

While cumbersome, CFU assays are the gold standard to characterize the impact of non-replicating and dual-active molecules on mycobacteria. Non-replicating assays are subject to high false-positive rates as many are coupled to replicating assays to assess cellular viability[6-8]. For example, a compound that has potent activity against replicating *M. tuberculosis* (a "replicating active") may fail to kill during the non-replicating assay, yet can still kill by virtue of carry-over from the non-replicating phase of the assay to the recovery phase of the assay, which is conducted under conditions that support replication ("replicating conditions"). Compound carry over further complicates analysis of dual active molecules, making it difficult to distinguish whether activity was replicating, non-replicating, or dual.

To address the issues described above, we developed the **c**harcoal **a**gar **r**esazurin **a**ssay (CARA) for rapid, semi-quantitative enumeration of mycobacterial species such as *M. tuberculosis*, *M. bovis* BCG, and *M. smegmatis*[7] (**Figures 1** and **2**). In buffer solutions *in vitro*, activated charcoal rapidly sequesters most of the standard drugs used to treat tuberculosis[7]. Activated charcoal in CARA microplates binds compounds that may carry over from an assay microplate, and this feature of the CARA eliminates the requirement to serially dilute assay well contents prior to enumeration [7,21,22]. The number of mycobacterial microcolonies on the agar surface of CARA microplates is estimated by the addition of resazurin, a blue dye, whose reduced form, resorufin, is a pink molecule whose fluorescence is measured by a spectrophotometry[23]. CARA microplates are developed at 1-2 days for *M. smegmatis* and 7-10 days for slow growing mycobacteria such as *M. tuberculosis* and *M. bovis* BCG. The CARA has a narrow dynamic range of ~ 2-3 $\log_{10}$ difference in CFU. When used instead of a CFU assay, a single CARA microplate replaces approximately 5 96-well plates used for serial dilutions and 120 tri-style agar plates used for plating. Interpretation of CARA data helps guide subsequent studies by determining which incubation times and compound concentrations to test in more laborious CFU-based assays.

PROTOCOL:

1. Preparation of CARA microplates:

1.1 Autoclave 900 mL of Middlebrook 7H11 agar containing 0.2% glycerol and 0.4% activated charcoal in a 2-liter Erlenmeyer flask, or alternatively, 450 mL in a 1-liter glass beaker. Include a large autoclavable stir bar in the flask or beaker. Cover the opening of the flask or beaker with aluminum foil and attach to glass with autoclave tape.

1.2 Cool to touch (approximately 55-65 °C) on a magnetic stir plate set at a low speed to maintain the charcoal in suspension.

1.3 Perform all subsequent steps aseptically in a biosafety hood that has a magnetic stir plate.

1.4 Remove foil. Add 100 mL OADC supplement (OADC supplement, when used at 10%, yields final media concentrations of 0.2% dextrose, 0.5% albumin, 0.085% NaCl, 0.0005% oleic acid, and 0.4 mg/mL catalase) to the 2-liter Erlenmeyer flask, or 50 mL OADC to the 1-liter beaker, and continue mixing.

1.5 If using an Erlenmeyer flask, pour approximately 25-40 mL of 7H11-OADC-charcoal into a sterile reagent reservoir. If using the beaker, it is not necessary to use a reagent reservoir.

1.6 Fill a 96-well microplate with 200 µL/well of 7H11-OADC-charcoal from the reagent reservoir or beaker. Work quickly to avoid agar solidification and introduction of bubbles. Avoid splashing solid medium outside of the wells, as that can be a source of fungal contamination. Using a multichannel pipette, use one set of 12 filter tips for the transfer of 7H11-OADC-charcoal to the 8 rows (A-H) of the 96-well plate.

Note: The medium solidifies quickly. To avoid clogging pipette tips, change them frequently.

1.7 Alternatively, pour CARA microplates using a p1000 electronic multichannel pipette with filter tips to assist in preparing numerous plates.

Note: Due to the low volume of microplate wells, the agar in CARA microplates solidifies within minutes of pouring.

1.8 Place stacks of CARA microplates in resealable plastic bags to avoid drying out.

1.9 Store CARA microplates at 4 °C.

2. Setting up replicating and non-replicating MIC_{90} assays:

2.1 Inoculate *M. tuberculosis*, *M. bovis* BCG or *M. smegmatis* at an OD_{580} of 0.01 - 0.1 and expand to mid-log phase (OD_{580} ~ 0.5) in Middlebrook 7H9-ADN (Middlebrook 7H9 containing 0.2% glycerol, 0.2% dextrose, 0.5% albumin, and 0.085% NaCl) or 7H9-OADC (Middlebrook 7H9 containing 0.2% glycerol and 10% OADC supplement).

2.2 Grow *M. tuberculosis* and *M. bovis* BCG as ~ 20 mL standing cultures in cell culture flasks and *M. smegmatis* with shaking in polypropylene round-bottom (4 mL culture) or 50 mL conical centrifuge tubes (10-20 mL culture). Incubate pathogenic mycobacteria at 37 °C with 20% O_2 and 5% CO_2, and *M. smegmatis* at 37 °C with 20% O_2.

2.3 Set up a minimal-inhibitory concentration (MIC_{90})-style experiment under replicating and non-replicating conditions (**Figure 2**).

NOTE: Test agents are usually assayed in duplicates or quadruplicates to permit testing 4, or 2 compounds, respectively, per 96-well microplate. For example, in a 96-well plate, one test agent can be assayed in rows A-D and another in rows E-H. The DMSO (vehicle) is in columns 1, 2, and 12, and the test agent dilution series runs from column 3 (lowest concentration) to column 11 (highest concentration). MIC_{90}-style assays typically employ 2-fold dilution series. There are numerous non-replicating models available for mycobacteria [14-16,18,24,25] and for illustrative purposes, we are using a multi-stress model of non-replication [6,8,9].

2.3.1 For the replicating assay, distribute 200 µL cells in 7H9-ADN at an OD_{580} of 0.01 into all wells of a clear-bottomed, tissue culture treated 96-well plate.

2.3.2 For the non-replicating assay, wash cells twice in phosphate buffered saline (PBS) containing 0.02% tyloxapol, and resuspend cells in non-replicating medium (0.05% KH_2PO_4, 0.05% $MgSO_4$, 0.005% ferric ammonium citrate, 0.0001% $ZnCl_2$, 0.1% NH_4Cl, 0.5% BSA, 0.085% NaCl, 0.02% tyloxapol, 0.05% butyrate; pH adjusted to 5.0 with 2N NaOH).

2.3.2.1 Dilute cells to an OD_{580} of 0.1 in non-replicating medium and add $NaNO_2$ from a freshly prepared 1 M stock to a final concentration of 0.5 mM.

2.3.2.2 Distribute 200 µL cells at an OD_{580} of 0.1 into all wells of a clear-bottomed, tissue culture treated 96-well plate.

NOTE: Prepare compound dilutions as 100-fold stock solutions in DMSO. Thus, a typical MIC plate testing the impact of a molecule at a final concentration 0.4 - 100 µg/mL would require stock solutions of 0.04 – 10 mg/mL in DMSO.

2.3.3 Add 2 µL of dilutions of test agent 1 into rows A-E and 2 µL of dilutions of test agent 2 into rows E-H. Mix thoroughly.

2.3.4 Add 2 µL vehicle control (usually DMSO) into control wells, columns 1, 2, 12 (rows A-H). Mix thoroughly.

2.3.5 For each experiment, include at least one positive control such as rifampicin from 0.004 to 1 µg/mL (replicating assay) and/or 0.08 to 20 µg/mL (non-replicating assay).

Note: The use of 6-bromo-1H-indazol-3-amine [9] at 0.1 to 25 µg/mL is recommended as a control compound that has selective, $NaNO_2$-dependent activity in the multi-stress model of non-replication.

2.3.6 For replicating assays, incubate microplates at 37 °C at 20% O_2 and 5% CO_2 for 7 days (*M. tuberculosis* and *M. bovis* BCG) or 1-48 hours (*M. smegmatis*). For the multi-stress model of non-replication, incubate microplates for 7 days at 37 °C at 1% O_2 and 5% CO_2 (*M. tuberculosis* and *M. bovis* BCG).

3. Inoculation of CARA microplates:

3.1 At time points in which the CARA will be used as a read-out, carefully resuspend well contents of the MIC_{90}-style assay plate using a p200 multichannel pipette set at 50 - 75 µL. Pipette up and down at least 5-10 times and gently swirl the well contents in a circular motion using the pipette tips.

3.2 Transfer 10 µL of assay well contents to the CARA microplate. Ensure that the order of the well contents on the assay plate matches the order of the well contents of the CARA microplate. Avoid splashing during the transfers and make sure the 10 µL are spotted into the middle of the CARA microplate wells. Confirm the 10 µL absorbs into the CARA microplate.

Note: There are no dilutions required prior to spotting cells on the CARA microplates.

3.3 Bind stacks of CARA microplates with plate tape and then place into a resealable plastic bag. Incubate CARA microplates at 37 °C with 20% O_2 (*M. smegmatis*) or 1% O_2 and 5% CO_2 (*M. tuberculosis* and *M. bovis* BCG).

3.4 For *M. tuberculosis* and *M. bovis* BCG, replicating assays: incubate for 7 days; for *M. tuberculosis* and *M. bovis* BCG non-replicating assays, incubate for 10 days; for *M. smegmatis*, replicating assays, incubate for 1-2 days; for *M. smegmatis*, non-replicating assays, incubate 2-3 days.

Note: Times are estimates and may be modified accordingly for different replicating, non-replicating, and stress conditions.

4. Developing CARA microplates:

4.1 Develop the CARA microplates when a film of bacterial growth, or larger, macroscopic colonies, are visible on the negative (vehicle) control wells.

Note: After prolonged incubation, CARA microplate wells often appear dry and we recommend pre-wetting well contents with sterile PBS. This serves to prevent the charcoal from absorbing resazurin, which can lead to low fluorescence or well-to-well variation.

4.2 Using a single set of 12 p200 tips with a multichannel pipette, dispense 40 µL of sterile PBS along the side of the wells and allow the PBS to distribute across the top of the agar/bacterial microcolonies.

4.3 Prepare CARA developing reagent by mixing 5 mg resazurin (0.01% final) and 50 mL of 5% Tween80 in PBS. Vortex and sterile filter.

Note: An alternative CARA developing reagent can be prepared by mixing commercially prepared resazurin liquid solution at 1:1 (vol/vol) with 10% Tween80 in PBS.

4.4 Add 50 µL of freshly prepared CARA developing reagent to each well of the CARA microplate using a 12-channel pipette. Rock plates back and forth a few times to help distribute reagent across the agar and bacterial mat in each well.

4.5 Place the plates in a resealable plastic bag and incubate at 37 °C for at least 30 minutes for *M. smegmatis* and 45-60 minutes for *M. tuberculosis* or *M. bovis* BCG.

Note: If the vehicle control wells fail to turn pink within the first hour, the plates can be re-bagged and incubated for longer periods of time.

4.6 Prior to reading fluorescence, place CARA microplates in a biosafety hood for 15 minutes at room temperature with their lids removed. When using BSL3 spectrophotometers outside of a biosafety cabinet, adhere an optical quality PCR sticker over the plate and seal tightly by pressing gently on the sticker surface with a soft paper towel.

4.7 Determine fluorescence via top read with excitation at 530 nm and emission at 590 nm. It is not necessary to blank the plate.

5. Data analysis:

5.1 Plot inhibitor concentration on the X-axis on a $\log_{10}$ scale and fluorescence on the Y-axis on a linear scale. Use a scatter plot employing a curve fit such as "log inhibitor versus response-variable slope (4 parameters)". Plot data points as means +/- standard error.

REPRESENTATIVE RESULTS:

Anticipated CARA results are described in **Figure 2** and summarized in **Table 1**. For replicating cells, the CARA is run in parallel with a standard MIC_{90} assays, and for non-replicating assays, the CARA is run in parallel with an adapted MIC_{90} assay that is coupled to an outgrowth phase. The concentration of test agent that results in failure of CARA-fluorescence to rise above background levels is the CARA-$MBC_{\geq 99}$ (**Figure 3a**). The "≥99" subscript indicates that the CARA-MBC provides an estimated concentration of test agent that gives rise to ≥2 $\log_{10}$ bacterial kill (≥99% kill).

The liquid broth MIC_{90} assay is unable to discriminate between bactericidal and bacteriostatic activity and this distinction has to be resolved by a CFU assay. By convention, the threshold that distinguishes bactericidal from bacteriostatic activity for slow-growing mycobacteria is approximately 2-3 $\log_{10}$ kill over 7 days [7,26]. Since the dynamic range of the CARA is also 2-3 $\log_{10}$ kill, the CARA can provide an estimate of bactericidal or bacteriostatic activity. The CARA easily identifies some replicating actives as bacteriostatic due to failure of these compounds to decrease CARA fluorescence to background levels (**Figure 2**). However, some compounds with bacteriostatic activity against replicating *M. tuberculosis* have a potent post-antibiotic effect, meaning that they continue to inhibit regrowth of bacteria during the recovery phase even in the absence of compound carry-over. This effect can be difficult to recognize in the CARA assay. Compounds are suspected of exerting a post-antibiotic effect if they display a "static window", defined as a > 4-fold shift to the right between the MIC and CARA curves (**Figure 3b**). The static window indicates that a molecule active against replicating *M. tuberculosis* may be bacteriostatic instead of bactericidal. In some cases, the static windows are apparent only after inspection of an expanded Y-axis for CARA fluorescence (**Figure 3c and 3d**).

CARA and MIC_{90} data are usually plotted together (**Figure 4**). Representative data for replicating- and non-replicating-active molecules tested by MIC_{90} and CARA are shown in **Figure 4**. There are 4 major activity classes for molecules: 1) replicating bactericidal (demonstrated with isoniazid, **Figure 4a**); 2) replicating bacteriostatic (demonstrated with linezolid, **Figure 4b**); 3) non-replicating bactericidal (demonstrated with oxyphenbutazone, **Figure 4c**); and, 4) replicating-active (bacteriostatic or bactericidal) and non-replicating bactericidal (demonstrated with PA-824, **Figure 4d**). Importantly, **Figures 4a** and **4b** demonstrate that while isoniazid and linezolid appear to have activity against non-replicating bacteria by the MIC_{90} assay, the CARA suggests they are inactive under the non-replicating conditions tested. To test the utility of the CARA in predicting a molecule's time- and concentration-dependent impact, replicating *M. smegmatis* was exposed to increasing concentrations of rifampicin (**Figures 5a-d**), and at various times between 1-24 hours, aliquots were spotted onto CARA plates. These data indicated that rifampicin exerted an impact

as early as 1 hour (**Figure 5a**), displayed increasing bactericidal activity between 3-24 hours (**Figures 5b-d**), and killed ≥2-3 $\log_{10}$ at ~10 μg/mL by 24 hours (**Figure 5d**). A similar experiment testing quadruplicates of a vehicle control and 9 drug concentrations, and at 4 time points, would be prohibitive by a standard CFU-based assay.

Thus, the CARA has a role in drug discovery as a medium-throughput, rapid mechanism to identify a molecule's activity profile. CARA predictions should be rigorously evaluated using a standard CFU assay. The addition of 0.4% activated charcoal to Petri plates for CFU analysis may help improve correlation to CARA data, and may result in more accurate CFU counts, the magnitude of the correction generally being proportional to the compound's potency [21,22].

FIGURE LEGENDS:

Figure 1. The CARA is a predictive tool in drug discovery. This diagram summarizes the utility of the CARA as an intermediate stage between drug screening (single point screening, cherry-picking, and dose-response assays) and time-consuming hit-to-lead assays (CFU assays and target identification). [Adapted with permission from *Gold et al., Antimicrobial Agents and Chemotherapy, 2015* [7].]

Figure 2. Schematic of the broth MIC_{90} assay and CARA with anticipated results. Both MIC_{90} and CARA results are presented for 8 possible activities. The color-coding for MIC_{90} microplate wells is white (no growth) and brown (growth), and for CARA microplates is black (no fluorescence) and pink (resorufin fluorescence). Data are hypothetical. [Adapted with permission from *Gold et al., Antimicrobial Agents and Chemotherapy, 2015* [7].]

Figure 3. Specialized CARA terms and definitions. The minimal bactericidal concentration of a molecule resulting in background levels of CARA fluorescence is the CARA-$MBC_{\geq 99}$ (**a**). Under replicating conditions, a ≥4-fold shift of the CARA-$MBC_{\geq 99}$ to the right of the MIC_{90} often indicates bacteriostatic activity and is called a "static window" (**b**). For molecules with a potent post-antibiotic effect, static windows may be difficult to observe (**c**) and require expansion of the Y-axis (CARA fluorescence) to visualize (**d**). Data are hypothetical. [Adapted with permission from *Gold et al., Antimicrobial Agents and Chemotherapy, 2015* [7].]

Figure 4. Illustrative MIC_{90} and CARA results for select compounds. Data for MIC_{90} (red) and CARA (blue) are demonstrated for (**a**) isoniazid (INH), (**b**) linezolid, (**c**) oxyphenbutazone, and (**d**) PA-824. Wild-type *M. tuberculosis* H37Rv was exposed to compounds for 7 days under standard replicating conditions or the multi-stress model of non-replication [7-9]. MIC_{90} assays and CARA were performed as shown in **Figure 2**. [Adapted with permission from *Gold et al., Antimicrobial Agents and Chemotherapy, 2015* [7].]

Figure 5. Dose- and time-dependent activity of rifampicin. The non-pathogenic, fast-growing *M. smegmatis* was exposed to increasing concentrations of rifampicin under replicating conditions and aliquots were sampled for CARA at 1 (**a**), 3 (**b**), 6 (**c**) and 24 (**d**) hours. [Adapted with permission from *Gold et al., Antimicrobial Agents and Chemotherapy, 2015* [7].]

Table 1. Summary of anticipated results in **Figure 2**. [Adapted with permission from *Gold et al., Antimicrobial Agents and Chemotherapy, 2015* [7].]

DISCUSSION:

The CARA was originally developed to alleviate a bottleneck in progressing non-replicating- or dual-active molecules[7]. The CARA serves as an intermediate step between concentration-response confirmation of primary screening hits and CFU assays (**Figure 1**). Since a single CARA plate can replace numerous microtiter plates required to prepare serial dilutions, and agar-containing Petri plates used to enumerate surviving bacteria, the CARA provides a simple means to rapidly assess a molecule's activity and test multiple variables at once, including compound concentration and time of exposure to the compound.

In a CFU assay, in addition to serial dilutions that often range up to 10^6-fold, the agar plate typically dilutes molecules by an additional ~ 800-fold (10 μL onto 8 mL in a tri-style Petri plate). Our two-stage, multi-stress screening assay for compounds active on non-replicating *M. tuberculosis* has a carry-over factor of 5-fold, that is, a compound present in the non-replicating stage of the assay is present at one-fifth the original concentration in the outgrowth (replicating) phase of the assay [6-9]. The CARA mitigates carry-over effects by sequestering small molecules with activated charcoal. The majority of tuberculosis drugs and clinical candidates bind activated charcoal rapidly and completely, with the exception of the aminoglycoside streptomycin[7]. Inclusion of activated charcoal in agar plates for CFU assays prevented bacterial growth inhibition, or bacterial kill, by carried-over TMC207 and PA-824[7,21,22]. Thus, by virtue of incorporating activated charcoal in the bacteriologic agar, the CARA is not dependent on serial dilutions of cells and test agent.

The CARA can help predict bacteriostatic or bactericidal impact of replicating actives and bactericidal impact of non-replicating actives. In general, the CARA is used in parallel with standard MIC_{90} assays. The predictive power of the CARA comes from comparing MIC_{90} and CARA results (**Figure 2** and **Table 1**). When used to study anti-mycobacterial agents, the CARA can accurately predict activity that is replicating bactericidal (**Figure 4a**), replicating bacteriostatic (**Figure 4b**), non-replicating bactericidal (**Figure 4c**), or dual active (**Figure 4d**). The CARA also permits simple evaluation of a compound's activity across both dose and time. CFU assays alone require a lot of effort and material to assess activity of a compound over a wide range of doses and times, but the task becomes manageable when CARA results narrow the range of conditions to those in which the compound is demonstrably active (**Figure 5**).

While the CARA has utility in studying the action of anti-infectives on mycobacteria, the assay has limitations. The CARA has a narrow dynamic range (2-3 $\log_{10}$) and may not be appropriate for conditions in which one anticipates there may not be more than 2 to 3 $\log_{10}$ bacterial kill. CARA predictions require further study using a more stringent and accurate method to enumerate bacterial numbers, such as a CFU assay. The post-antibiotic effect of some anti-infectives, such as PAS, can confound the CARA as a predictive tool to distinguish between compounds with bactericidal and bacteriostatic activity against replicating *M. tuberculosis*[7].

There are two recommendations to improve the quality of the CARA. First, one must pour CARA microplates rapidly before the agar solidifies, while maintaining volumetric accuracy and avoiding splashing outside of microwells. Regardless of the number of plates required, we recommend making 0.5- to 1-liter batches of medium to help maintain the medium in liquid form for the duration of the time required to pour plates. Changing tips frequently while filling microplates with medium avoids using partially clogged tips. Second, one must minimize artifactual variability in fluorescence between replicates. Mycobacterial microcolonies, in particular for pathogenic mycobacteria such as *M. tuberculosis*, often grow erratically. For example, microcolonies growing on the agar surface may vary in size, shape, height, or may extend up to the interior walls of microplate wells. One challenge is to cover all bacilli uniformly with the developing reagent. Another hurdle is that after prolonged incubation at 37 °C, the solid bacteriologic medium of the CARA microplates may become dry and prone to absorbing the developing reagent. Since activated charcoal can bind resazurin and quench fluorescence[7], there may be well-to-well variation due to dry wells absorbing the resazurin developing solution and the activated charcoal quenching resazurin fluorescence. Pre-wetting the surface of all CARA microplate wells with PBS immediately before adding the developing reagent mitigates both of these problems – the developing reagent will reach all mycobacterial colonies equally, and the resazurin remains safely above the activated charcoal. The CARA may have applications in identifying and characterizing phenotypes of mycobacterial mutants, or in medium-throughput drug discovery assays for other bacterial species.

DISCLOSURES: There are no conflicts of interest to disclose.

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Figure

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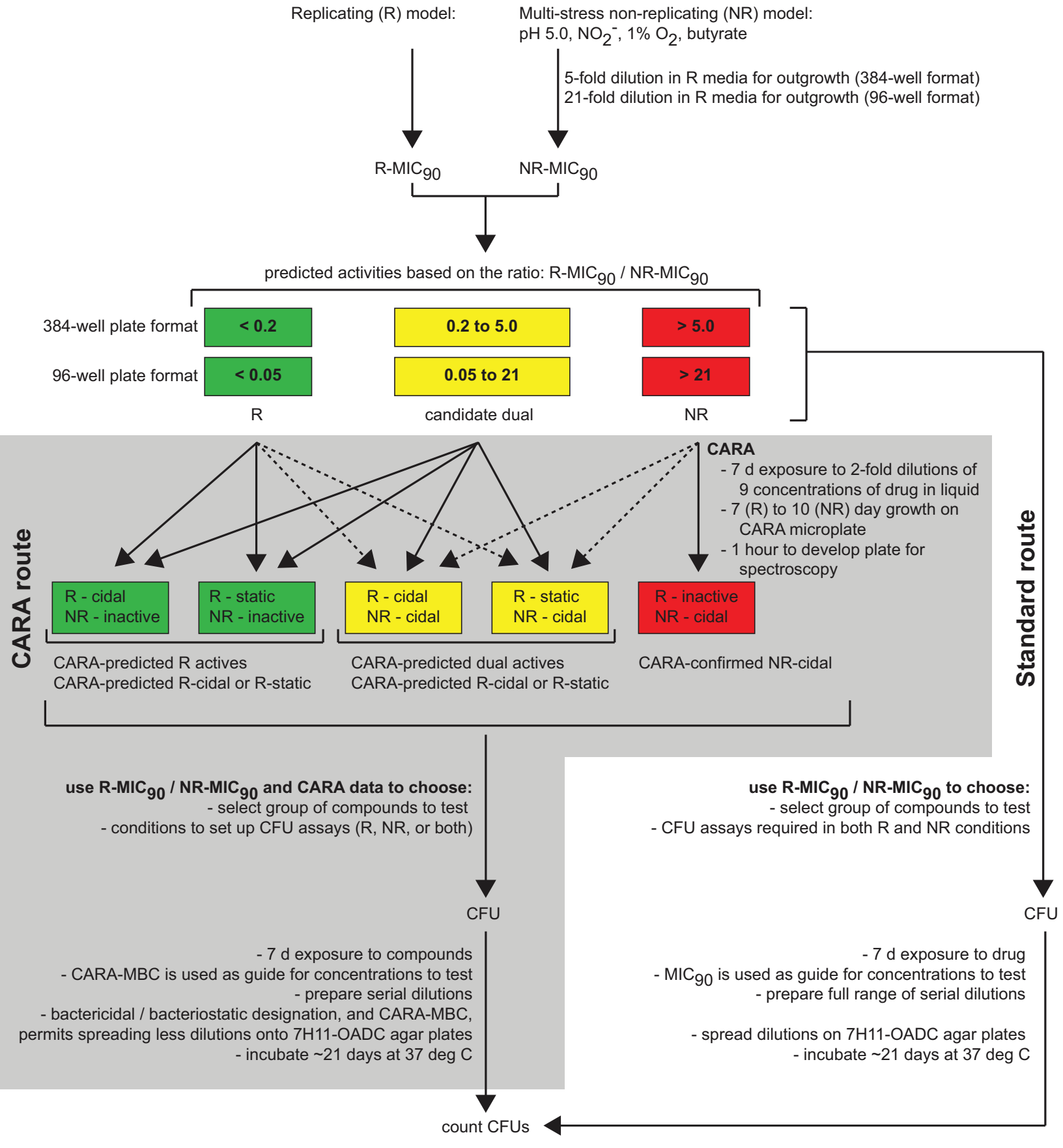

Figure

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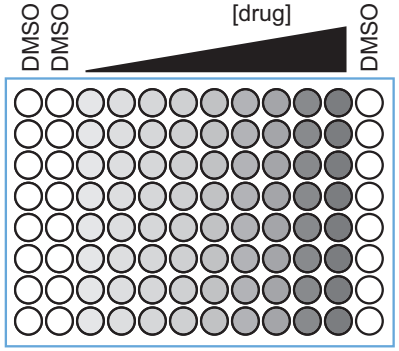
activity of compounds against bacteria that are:

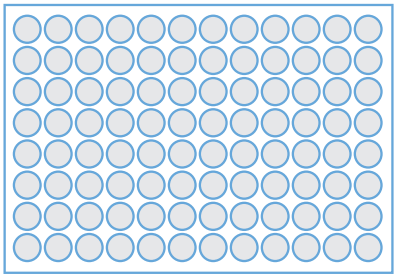
Example	NR	R
1	inactive	weak cidal
2	inactive	potent cidal
3	inactive	static
4	inactive	static + PAE*
5	cidal	cidal
6	cidal	static
7	cidal	inactive
8	inactive	inactive



7 day drug exposure in R or NR conditions

non-replicating

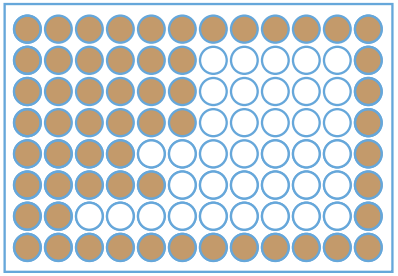
Transfer 10 µL directly to outgrowth plate



NR outgrowth plate
each well contains 200 µL 7H9-OADC liquid broth medium

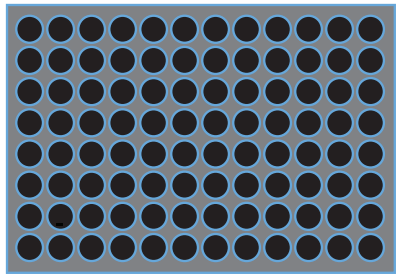
Incubate 37 deg C for 10 days

Ex.	NR
1	inactive
2	inactive
3	inactive
4	inactive
5	cidal
6	cidal
7	cidal
8	inactive



read OD_{580}

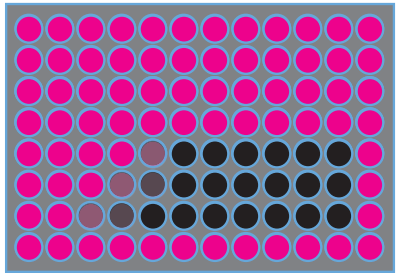
Transfer 10 µL directly to CARA plate - no serial dilutions



CARA plate
each well contains 200 µL 7H11-OADC agar + 0.4% activated charcoal

Incubate 37 deg C for 10 days

add mixture of AB:Tw80 directly on top of Mtb growth



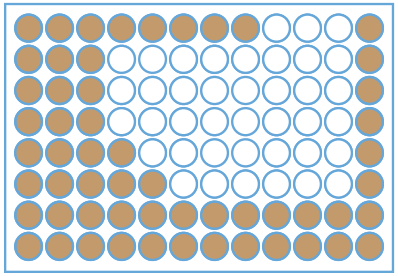
CARA plate
incubate 1 hour at 37 degrees C and then read fluorescence

non-replicating

replicating

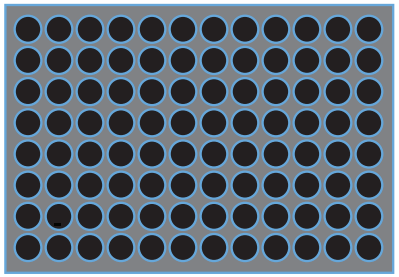
Incubate 37 deg C for 7 days

Ex.	R
1	weak cidal
2	potent cidal
3	static
4	static + PAE*
5	cidal
6	static
7	inactive
8	inactive



read OD_{580}

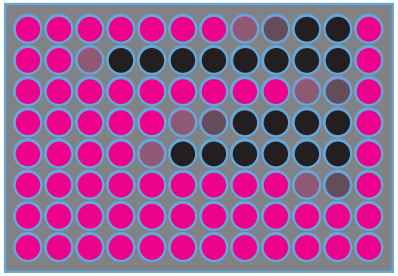
Transfer 10 µL directly to CARA plate - no serial dilutions



CARA plate
each well contains 200 µL 7H11-OADC agar + 0.4% activated charcoal

Incubate 37 deg C for 7 days

add mixture of AB:Tw80 directly on top of Mtb growth



CARA plate
incubate 1 hour at 37 degrees C and then read fluorescence

replicating

Figure

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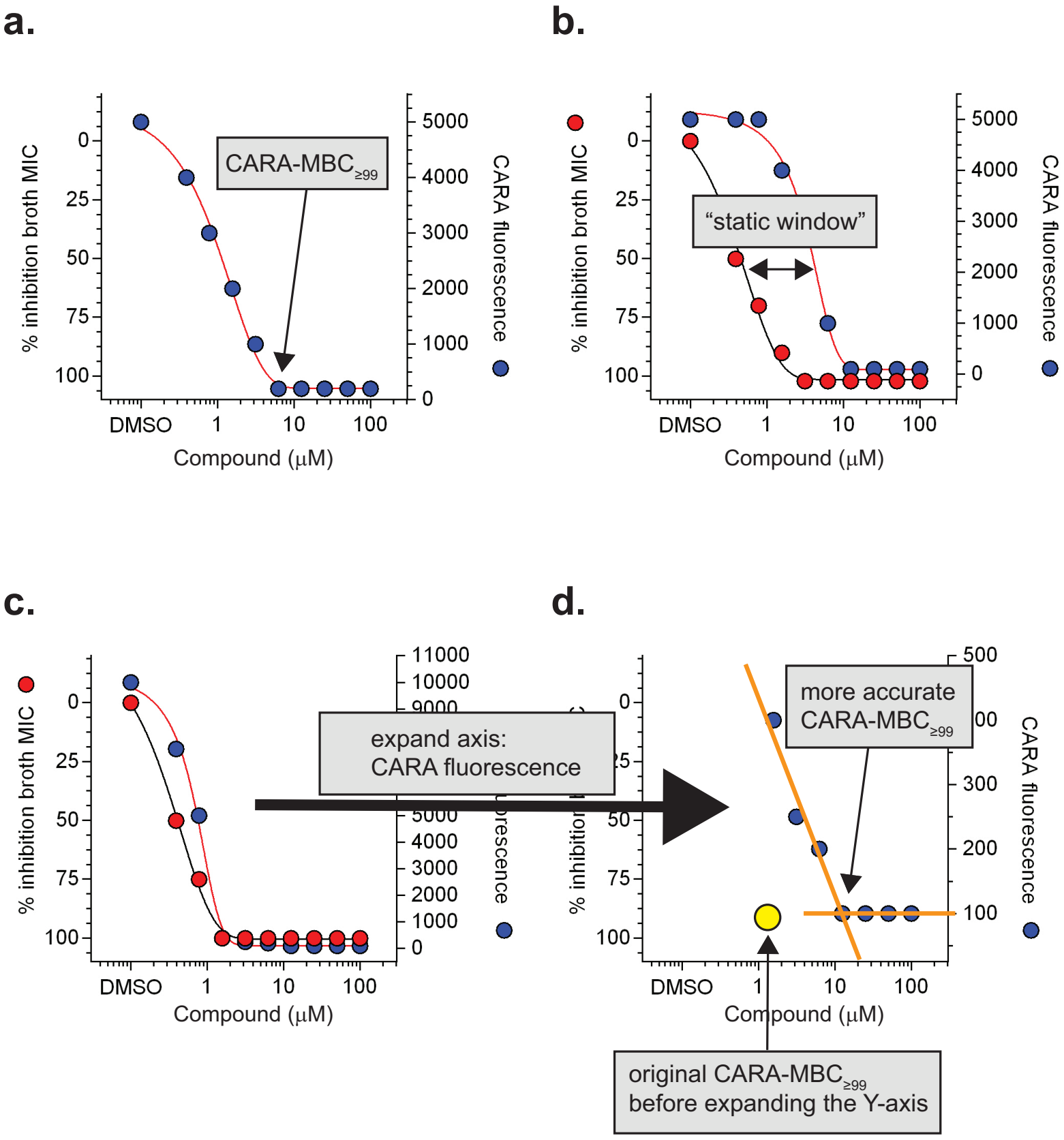

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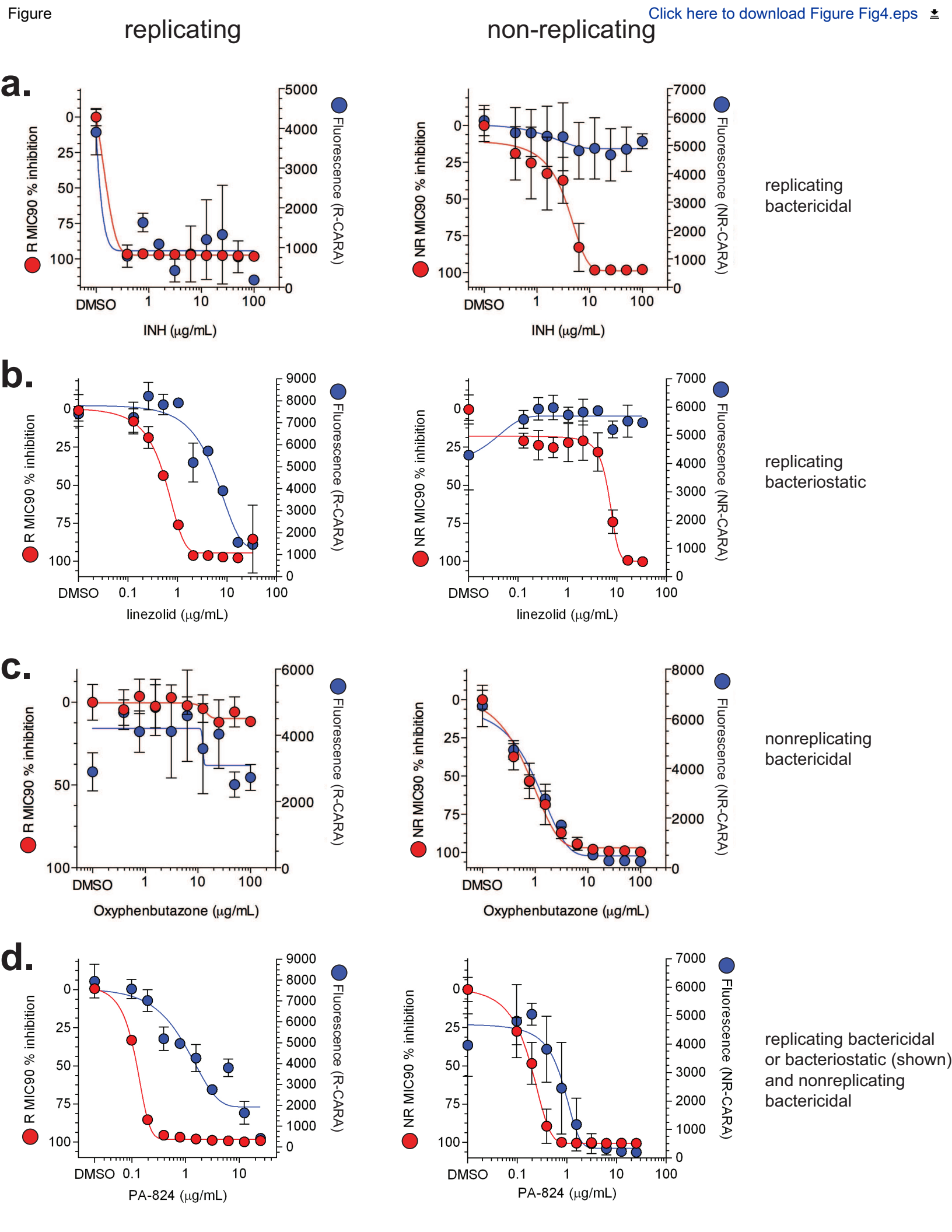

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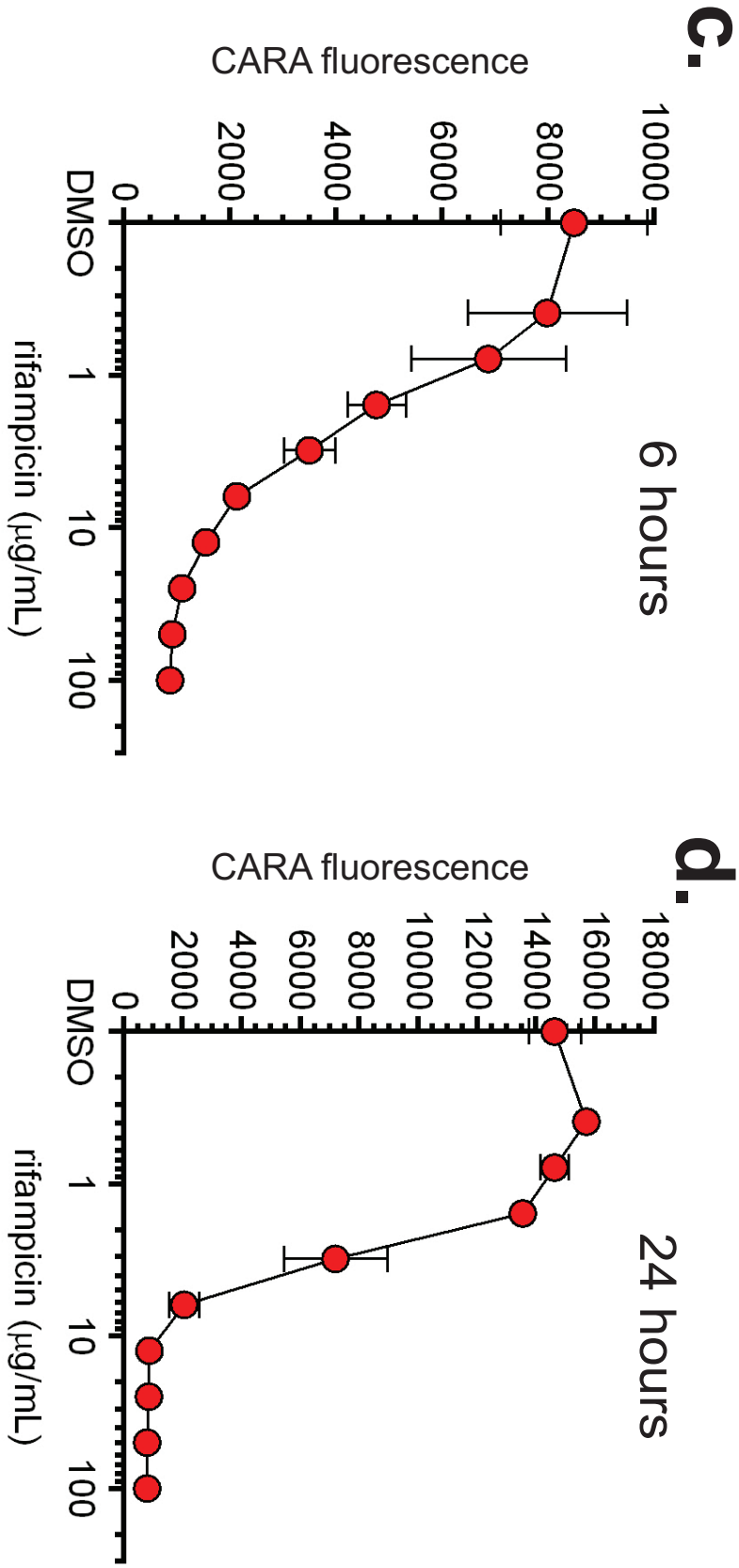

Table 1_revised as Excel format

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Assay type	Parameter	Example A	Example B	Example C	Example D	Example E
	Example(s) in Figure 2	1,2	3	4	4	5
	non-replicating assay	inactive	inactive	inactive	inactive	cidal
	replicating assay	cidal	static + no PAE	static + weak PAE	static + potent PAE	cidal
	activity classification	R-cidal	R-static	R-static	R-static	dual-active
	Ratio of MIC90's [a]: R / NR	< 0.2	< 0.2	< 0.2	< 0.2	0.2 to 5
CARA: non-replicating	no fluorescence decrease	yes	yes	yes	yes	–
	dose-dpt decrease to background at MIC_{90}	–	–	–	–	maybe
	dose-dpt decrease to background at ≥ 4X MIC_{90}	–	–	–	maybe	maybe
CARA: replicating	static window	–	yes	yes	maybe	–
	no fluorescence decrease	–	yes	–	–	–
	dose-dpt decrease to background at MIC_{90}	yes	–	–	maybe	yes
	dose-dpt decrease to background at ≥ 4X MIC_{90}	–	–	yes	maybe	–
	expand axis to view SW?	–	–	–	yes	–
	alternative explanation	–	–	charcoal may not bind drug effectively	charcoal may not bind drug effectively	–

Example F	Example G	Example H	Example I
6	5	6	7
cidal	potent cidal	potent cidal	cidal
static (+/- PAE)	cidal	static (+/- PAE)	inactive
dual active	dual-active	dual-active	NR-active
0.2 to 5	> 5	> 5	> 5
–	–	–	–
maybe	yes	yes	yes
maybe	–	–	–
yes	–	yes	–
maybe	–	maybe	yes
maybe	yes	maybe	–
maybe	–	maybe	–
maybe	–	maybe	–
charcoal may not bind drug effectively	–	charcoal may not bind drug effectively	–

Name of Material / Equipment	Company	Catalog number
Middlebrook 7H9	Beckton Dickinson	271310
Middlebrook 7H11	Beckton Dickinson	298810
Middlebrook OADC	Beckton Dickinson	212351
BSA, heat shock	Roche	3118958001
activated charcoal	Sigma	C5510
PBS, Dulbecco's Ca^{2+} and Mg^{2+} free	Life Technologies / Invitrogen	14190-144
tween 80	Sigma	P8074
tyloxapol	Sigma	T8761
sodium nitrite	Sigma	2252
rifampicin	Sigma	R3501
6-bromo-1H-indazol-3-amine	Alfa Aesar	H34095
potassium phosphate monobasic	Sigma	P0662
magnesium sulfate, heptahydrate	Sigma	M1880
ferric ammonium citrate	Sigma	F5879
zinc sulfate, heptahydrate	Sigma	Z0251
ammonium chloride	Sigma	A9434
butyric acid, liquid	Sigma	B103500
resazurin powder	Sigma	R7017
sodium chloride	J.T. Baker	4058-01
prepared resazurin solution	Invitrogen	DAL1100
PCR stickers	Denville	B1212-5
spectrophotometer	Molecular Devices	M5
96-well, tissue culture treated microplates	Corning	3595
reagent reservoirs	VWR	89094-678
resealable plastic bags	VWR	395-94602
14 mL Polypropylene round-bottom tubes	Corning	352059
50 mL conical centrifuge tube	Corning	352070
75 cm^2 Cell culture flask	Corning	431464U
clear, flat bottom tissue culture treated 96-well microplate	Costar	3595
Prism 6 for OS X	GraphPad	http://www.graphpad.com/scientific-software/prism/

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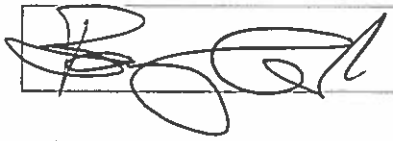
Signature:	 Date: 2-29-16

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03-22-16

We are grateful to the editors for constructive suggestions. Please find point-by-point replies (indented blue text) and modified/added text has been changed to red in the main article.

Editorial comments:

1. Editor modified the formatting of the manuscript and made minor copy-edits. In keeping with the JoVE format, a few of your protocol steps may have been revised to the imperative tense and any step that could not be written in the imperative tense may have been added as a "Note". Please maintain the current formatting throughout the manuscript. You can find the updated manuscript attached to this e-mail.

Relevant text has been modified accordingly.

2. In the Author Affiliations section of the manuscript, please provide an email address for each author.

We added email addresses for each author on Page 1.

3. Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1) and then 1.1.1) if necessary. Please make sure that all steps are lined up at the left margin with no indentations and there is a one line space between each protocol step (done for you). Please note that in the remainder of this letter, steps are referred to using the JoVE format, for e.g. in section 1, step 4 is referred to as step 1.4.

We tried to use your recommended format. Please see the new section "setting up replicating and non-replicating MIC90 assays", with subheadings 4, 4.1, and 4.2.

4. Please define all abbreviations when they are first used, e.g. OADC.

To our knowledge, all abbreviations have been corrected to indicate what they mean the first time they appear in the text.

5. Please add more details to the following protocol steps

a) 1.7 – it is not clear what is meant by "repetitive injuries". Please revise.

Repetitive injuries such as carpal tunnel and intense thumb pain are very common when performing high-throughput style assays in the laboratory. To simplify the protocol, we simply described using the p1000 electronic pipettor and removed text about repetitive injuries.

b) 2.1 – What are the concentrations of albumin, dextrose, NaCl, oleic acid and catalase? How is the inoculation done and what is the volume of the culture? What are the culture conditions (temperature, agitation etc.)?

All concentrations have been added to the text.

c) 2.2 – how is the experiment set up? Please provide step-wise details written in the imperative tense, as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.). Please also simplify this step into sub-steps (e.g. 2.2.1, 2.2.2 etc.) so that individual steps contain only 2-3 related actions per step.

Relevant text has been modified accordingly.

6. After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages OR if there steps in protocol that will NOT be filmed, please highlight 2.75 pages or less of text (which includes headings and spaces) to identify which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVE's instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

Our protocol falls within the recommended 3 page limit.

7. JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Please remove all commercial sounding language from your manuscript. Examples of commercial sounding language in your manuscript are "alamarBlue™", "Ziploc" etc. All commercial products should be sufficiently referenced in the table of materials/reagents.

We have removed text containing "AlamarBlue", "Ziploc", and "Kimwipe".

8. Please revise the legend for figure 5 so that all parts of the figure (A-D) are mentioned.

The Fig5 legend has been revised to include parts A-D.

9. Please expand your discussion to cover: 1) modifications and troubleshooting, 2) critical steps in the protocol.

We have added a final paragraph in the discussion to explore modifications, troubleshooting and critical steps in our protocol.

10. Please upload Table 1 as an Excel file.

We have uploaded the original Excel file of Table 1.

11. Please ensure that all materials used in the Protocol appear in the Table of Materials, e.g. OADC.

The Table of Materials has been significantly expanded to include all reagents.

12. Please remove the trademark (™) symbol(s) from the table of reagents/materials.

All trademark symbols have been removed.