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Antimicrobial Synergy Testing by the Inkjet Printer-Assisted Automated Checkerboard Array and the Manual Time-Kill Method

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Cover Letter

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November 16, 2018

Re: Revised Manuscript, JoVE58636

Dear Editors,

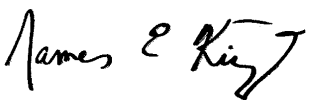
We would like to submit for your consideration a revised methods article entitled "Antimicrobial synergy testing by inkjet printer-assisted automated checkerboard array and time-kill."

Our science editor contact with whom we have been working is **Dr. Jaydev Upponi**. He indicated that we should request our article be considered in the JoVE **Immunology and Infection Section**.

In the manuscript, we describe the methodology for two techniques used to determine the combinatorial effects of antimicrobials on bacterial pathogens. These are specifically the checkerboard synergy test and the time-kill assay. We believe that a JoVE article will be of significant interest to the field as these two techniques are not well described in available materials in the public domain. Further we believe that a JoVE type video would be immensely helpful for scientists trying to successfully implement these techniques in their laboratories. We recently described in the literature a new technique for performing checkerboard synergy testing that makes use of inkjet printing technology to facilely set up orthogonal antibiotic titrations. This allows the testing to be set up in a minute rather than an hour, and we believe represents a tremendous advance in the field. We present the details of the technique in the JoVE article and believe the technique is best illustrated in the combination JoVE text/video format. Importantly, based on our prior work, the US Centers for Disease Control and Prevention (CDC) has now deployed this inkjet printing methodology in their ARLN (Antimicrobial Resistance Laboratory Network) to enable these centers to test experimental and combinations of antimicrobials at will against pan-drug resistant pathogens. This capability will be used to identify new treatment options for infections that would otherwise be untreatable. We expect therefore there to be considerable interest in the JoVE article, and we hope that you consider it for publication in your journal.

The manuscript submission has not been previously published nor is under consideration for publication elsewhere. The authors have read and agree upon the contents of the submission. All authors have contributed substantially to the work.

Sincerely yours,



James E. Kirby, MD

TITLE:

Antimicrobial Synergy Testing by the Inkjet Printer-Assisted Automated Checkerboard Array and the Manual Time-Kill Method

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Antimicrobial synergy, antibiotic synergy, synergy, antimicrobial resistance, colistin resistance, carbapenem-resistant Enterobacteriaceae, time-kill synergy, checkerboard array, automation, inkjet printing

SUMMARY:

Antimicrobial synergy testing is used to evaluate the effect of two or more antibiotics used in combination and is typically performed by one of two methods: the checkerboard array or the time-kill assay. Here, we present an automated, inkjet printer-assisted checkerboard array synergy technique and a classic time-kill synergy study.

ABSTRACT:

As rates of multidrug-resistant (MDR) pathogens continue to rise, outpacing the development of new antimicrobials, novel approaches to treatment of MDR bacteria are increasingly becoming a necessity. One such approach is combination therapy, in which two or more antibiotics are used together to treat an infection against which one or both of the drugs may be ineffective alone. When two drugs, in combination, exert a greater than additive effect, they are considered synergistic. In vitro investigation of synergistic activity is an important first step in evaluating the possible efficacy of drug combinations. Two main in vitro synergy testing methods have been developed: the checkerboard array and the time-kill study. In this paper, we present an automated checkerboard array method that makes use of inkjet printing technology to increase the efficiency and accuracy of this technique, as well as a standard manual time-kill synergy method. The automated checkerboard array can serve as a high-throughput screening assay, while the manual time-kill study provides additional, complementary data on synergistic activity and killing.

The checkerboard array is a modification of standard minimum inhibitory concentration (MIC) testing, in which bacteria are incubated with antibiotics at different concentration combinations and evaluated for growth inhibition after overnight incubation. Manual performance of the checkerboard array requires a laborious and error-prone series of calculations and dilutions. In the automated method presented here, the calculation and dispensing of required antibiotic stock solution volumes are automated through the use of inkjet printer technology. In the time-kill synergy assay, bacteria are incubated with the antibiotics of interest, both together and individually, and sampled at intervals over the course of 24 hours for quantitative culture. The results can determine whether a combination is synergistic and whether it is bactericidal, and provide data on inhibition and killing of bacteria over time.

INTRODUCTION:

The spread of multidrug-resistant (MDR) bacterial pathogens, particularly MDR Gram-negative bacteria such as carbapenem-resistant Enterobacteriaceae (CRE), has left clinicians with increasingly limited options for successful anti-infective therapy[1], a problem exacerbated by the sluggish pace of novel antibacterial drug discovery[2,3]. Antimicrobial synergy, in which two drugs used in combination exert a greater-than-additive effect, offers the possibility of salvaging existing antibiotics for use in treatment of MDR bacteria, even when these bacteria are resistant to one or both of the antibiotics individually. The techniques described in this paper provide two complementary methods of in vitro synergy testing that, when used together, allow investigators to efficiently screen antimicrobial combinations of interest for evidence of synergistic activity (the automated checkerboard array method) and then to further evaluate the kinetics of inhibition and killing demonstrated by promising combinations identified in the screening stage (the manual time-kill method).

One of the most commonly used methods of in vitro synergy testing is the checkerboard array assay, a modification of minimum inhibitory concentration (MIC) testing in which the inhibitory activity of two different antibiotics against a bacterial isolate are tested over a range of concentration combinations[4,5]. If the two drugs exert greater than additive activity when used together, the combination is considered synergistic[6]. However, setting up a checkerboard array manually involves a series of calculations and diluting and pipetting steps that are laborious and vulnerable to human error. These constraints have had the effect of limiting the use of synergy testing primarily to the retrospective evaluation of small numbers of antibiotic combinations and bacterial isolates, and results have not always been consistent among studies[7–11]. Furthermore, the complexity of synergy testing has contributed to its unavailability in the clinical microbiology laboratory and to the virtual absence of in vitro synergy testing data from clinical studies of combination therapy[12,13].

In order to increase the efficiency and throughput of the checkerboard array method, we made use of an automated MIC testing technique previously developed in our laboratory that uses inkjet printing technology to precisely and consistently dispense small volumes of antibiotic stock solution into wells in a microtiter plate[14]. The platform obviates the need for complex calculations and multiple pipetting steps. The associated software calculates and dispenses

appropriate volumes of antibiotics to create a two-dimensional checkerboard array if the user simply inputs the desired concentration range and stock solution concentration of the antibiotics. We initially tested this method against a collection of CRE isolates[15] and subsequently have focused on testing colistin-containing combinations for activity against colistin-resistant isolates[16]. Colistin is a drug of last resort generally reserved for use in the treatment of MDR Gram-negative pathogens[17,18], and colistin resistance renders already MDR bacteria nearly pan-resistant[19], making them ideal candidates for the development of novel therapeutic strategies using drugs to which they are insensitive individually. We found that the combination of colistin and the protein synthesis inhibitor antibiotic minocycline had a very high rate of synergy, even against strains that were resistant to each of these drugs individually, presumably because colistin exerts a subinhibitory permeabilizing effect on even colistin-resistant bacteria. We have chosen this combination to use as an example in this paper. Of note, synergy testing can also be used to evaluate for enhanced efficacy of two drugs which are both effective individually.

The automated checkerboard array method facilitates rapid, high-throughput synergy testing. However, the checkerboard array method does have limitations. As a modified MIC assay, it provides data only on inhibition of bacterial growth and not on killing, and it does not provide data on antibiotic effects over time. By contrast, manual performance of time-kill synergy assays is more labor intensive but provides information on both inhibition and killing over a 24-hour time course[20,21]. We used time-kill analysis on a smaller number of isolates to confirm our checkerboard array results and to determine whether the synergistic combinations we identified were also bactericidal.

Both checkerboard array and time-kill synergy methods provide valuable information on the activity of drug combinations, and are particularly useful in evaluating potential novel therapeutic options for highly resistant bacterial pathogens. The methods also have inherent limitations. The standard microbroth dilution MIC method has a known expected error range of $\pm$1 two-fold dilution[22], which is increased when two drugs are tested together in a checkerboard array. The standard definition of synergy, which considers a combination synergistic only if the drugs are active together at one-fourth their respective MICs[6], takes into account this expected variability, but such variability (which is thought to result from a combination of biological and technical fluctuations[23]) inevitably generates uncertainly about the reliability of synergy results. The lack of established quality-control standards for synergy testing is also a current limitation. Perhaps the most significant limitation of all synergy testing methods is the lack of established correlations between in vitro results and clinical outcomes when combinations are used to treat patients[24]. Simpler and more rapid synergy testing methods, such as the automated checkerboard array method described here, may facilitate the integration of in vitro synergy testing within clinical trials or other evaluations of patient outcomes in order to better characterize the relationship between in vitro and in vivo effects in the future.

The automated checkerboard array method that we present here offers an option for high-throughput screening of a variety of combinations and allows for quick evaluation of unusual,

"high risk-high reward" combinations without a major investment of time and resources. The time-kill method, which we subsequently demonstrate, can provide additional supportive information on the synergistic activity of the combination and can help to characterize its bactericidal activity and antibacterial kinetics.

PROTOCOL:

CAUTION: Use appropriate safety procedures when working with bacteria. Wear gloves and a lab coat at all times. Perform work in a biosafety cabinet if aerosols will be generated or working with high risk pathogens.

NOTE: Twenty to 24 hours before starting experiments, streak out the bacterial isolate(s) to be tested (from a colony-purified, minimally passaged stock frozen at -80 °C in tryptic soy broth with 50% glycerol stock) onto a blood agar plate. Incubate the plate at 35 °C in ambient air.

1. Inkjet printer-assisted automated checkerboard array synergy

1.1. Make antimicrobial stock solutions (colistin and minocycline).

1.1.1. Determine antibiotic stock solution concentrations based on solubility of antibiotics and desired final concentrations in checkerboard array. Make 10 mg/mL stocks of colistin and minocycline for this example. Use the CLSI M100 document to determine appropriate solvents for each antibiotic[25]. Both colistin and minocycline are water-soluble; because the D300 inkjet printer requires the addition of surfactant for proper aqueous fluid handling, dissolve the antibiotics in ultrapure deionized water with 0.3% polysorbate 20.

1.1.2. Weigh out antibiotic powder using an analytical balance and calculate volume of solvent needed to obtain goal stock concentration.

1.1.2.1. If the antibiotic is supplied as a salt (e.g. colistin sulfate, minocycline hydrochloride) or in hydrated form (e.g. meropenem trihydrate), or if it is reported by the manufacturer to have less than 100% purity, perform a potency calculation[26] to determine the quantity of solvent required.

1.1.2.2. Follow this example of minocycline hydrochloride with a stated purity of 900 μg/mg:
Assay purity: 900 μg/mg
Water content: None
Active fraction: 0.926 (obtained by dividing the molecular weight of minocycline (457.48 Da) by the molecular weight of minocycline hydrochloride (493.94 Da)).
Potency = (Assay purity) * (1 – water content) * (active fraction)
= (900 μg/mg) * (1) * (0.926) = 833.4 μg/mg or 83.34%
Then determine the volume of solvent required as follows:
Volume (mL) = [Weight (mg) * Potency (μg/mg)] ÷ [Concentration (μg/mL)]
So, for example, if 34.7 mg of minocycline hydrochloride powder is weighed out, use the

following calculation to determine the volume of solvent required to make a 10 mg/mL solution:

$$\text{Volume} = \frac{(34.7\ \text{mg}) * (833.4\ \mu\text{g/mg})}{10{,}000\ \mu\text{g/mL}} = 2.89\ \text{mL}$$

1.1.3. Pour antibiotic powder into a 15 mL conical tube and add the appropriate volume of water plus 0.3% polysorbate 20. Vortex until dissolved.

1.1.4. Aliquot antibiotic stock solution into 0.5 mL microcentrifuge tubes and store at -80 °C until ready for use.

1.2. Perform quality control (QC) of antimicrobial stocks for use in checkerboard array experiments at least one day prior to synergy testing so that QC results can be evaluated before using the stock for synergy testing.

NOTE: The QC technique described here is identical to the technique that would be used for minimum inhibitory concentration (MIC) testing of individual drugs and can be used as such with any strains of interest to the investigator.

1.2.1. Prepare bacterial suspension.

1.2.1.1. Take an aliquot of each antibiotic stock out of the -80 °C freezer to start thawing while preparing bacterial suspension. Vortex once thawed to ensure that the antibiotic is in solution.

1.2.1.2. Select an appropriate QC strain and determine the acceptable MIC range for drugs being tested based on **Table 5A-1** in CLSI M100[25]. For the drugs here, use *E. coli* ATCC 25922; the MIC ranges for this strain are 0.25-1 μg/mL for minocycline and 0.25-2 μg/mL for colistin.

1.2.1.3. Select a range of antibiotic concentrations to test that will include the entire QC range. Use the range of 0.0156 μg/mL to 8 μg/mL for minocycline and colistin for ATCC 25922.

1.2.1.4. Add 1 mL of 0.9% sodium chloride to a 12 mm x 75 mm round bottom glass culture tube. Select one or two colonies from an overnight plate of ATCC 25922 and vortex gently to suspend.

1.2.1.5. Check the concentration of bacteria using a McFarland reader. Adjust as needed by adding more 0.9% sodium chloride or more bacteria to achieve a 0.5 McFarland turbidity reading.

1.2.1.6. Make a 1:300 dilution of the 0.5 McFarland suspension by adding 100 μL of the suspension to 30 mL of cation-adjusted Mueller-Hinton broth (CAMHB) in a 50 mL conical tube to reach a final cell density of 5×10^5 CFU/mL, as recommended by CLSI[27].

1.2.1.7. Using a sterile inoculating loop, isolation streak a drop of the starting inoculum onto a blood agar plate to confirm inoculum purity, and incubate at 35 °C in ambient air.

1.2.2. Add antimicrobials to a flat-bottom, square-well, clear, untreated 384-well plate using the D300. Perform this step immediately after preparing bacterial suspension so that suspension can be added to the plates within 15 minutes of preparation[26].

1.2.2.1. Turn on the D300 inkjet printer and the associated computer. Open the software program.

1.2.2.2. Start a new file. Above the image of the plate grid, right-click on **Plate 1** and choose **Edit plate**. Select the appropriate plate type (**384 well**) and additional volume (50 μL).

1.2.2.3. Add fluids (i.e., antibiotic stocks) to the protocol by clicking the plus sign next to **Fluids** on the left-hand panel. Add two fluids (colistin and minocycline).

1.2.2.3.1. Hover over the panel that appeared for Fluid 1 and click the pencil to edit. Name the fluid "Colistin", change **Class** to "Aqueous + Tween 20", change **Concentration** to 10,000, and change concentration units to μg/mL (note that stock concentration is 10 mg/mL, i.e., 10,000 μg/mL). Leave **Dispense b**y at **Concentration** and leave the remainder of the fields at their default settings. Click **OK**.

1.2.2.3.2. Repeat the procedure above for Fluid 2 (minocycline).

1.2.2.3.3. Click the **Current Protocol** tab at the top of the screen and change **Concentration (mass)** to **μg/mL** to determine the units used for final well antibiotic concentrations.

1.2.2.4. Select 10 wells in the grid by clicking and dragging, then click **Titration** at the top of the screen. For **Specify titration using** select **Highest concentration**, for **Fluid** choose **Colistin**, for **Highest Concentration** enter **8** (make sure units are μg/mL), and for **Distribution** select **1:2 (50%)**. Leave default values in place for the rest of the window and click **OK**.

1.2.2.5. Repeat the procedure above for minocycline to generate the minocycline titration.

1.2.2.6. Save the protocol, and then click the **Run** button at the top left.

1.2.2.7. Click the **Start** button. Load a 384-well plate (with lid removed) into the plate holder and press **Loaded** under the "Load Plate 1 – Synergy" prompt.

NOTE: This prompt is chosen by the software and does not indicate that synergy testing is being performed. Place a T8+ cassette into the cassette slot and press **Loaded** under the **Load a T8+ cassette** prompt.

1.2.2.8. When prompted, add antibiotic stock solution to the indicated reservoirs on the cassette. Follow instructions on the screen for proper loading and dispense carefully to avoid getting any bubbles in the solution. After each solution is added, press the **Filled** button.

1.2.2.9. Once the inkjet printer has added antibiotic stock in appropriate volumes to each well and the **Run completed** box appears, click **Exit**, remove the plate, and turn off the D300.

1.2.3. Add bacterial suspensions to 384-well plate and incubate plate.

1.2.3.1. Pour the previously prepared bacterial suspension into a sterile reagent reservoir.

1.2.3.2. Use a multichannel pipette to add 50 µL of bacterial suspension to all antibiotic-containing wells. Add 50 µL of CAMHB without bacteria to an empty well; this will be the negative control well to confirm sterility of the media.

1.2.3.3. Place plate in a 35 °C ambient air incubator and incubate for 16-20 hours[26].

NOTE: A different duration of incubation may be required if organisms other than *Enterobacteriaceae* are being tested; consult CLSI M100[25] for organism-specific recommendations.

1.2.4. Read plate on a microplate reader at an optical density of 600 (OD_{600}) and analyze results.

1.2.4.1. Using a spreadsheet program, shade cells with an OD_{600} value of ≥0.07 green, indicating growth, and cells with a value of <0.07 red, indicating no growth.

NOTE: These values were determined based on visual inspection of growth vs. no growth and correlation with OD readings for these experiments; OD_{600} readings from wells containing media alone were consistently below 0.07. Appropriate cutoffs may differ with different plate readers and bacteria.

1.2.4.2. Determine the MIC for each drug. The MIC is the lowest concentration of drug at which bacterial growth is inhibited. If the MIC is within the expected QC range according to the CLSI M100 document[25], the stock solution is appropriate for use.

1.3. Prepare bacterial suspension for checkerboard array.

1.3.1. Take an aliquot of each antibiotic stock out of the -80 °C freezer to start thawing while preparing bacterial suspension. Vortex once thawed to ensure antibiotic is in solution.

1.3.2. Add ~1 mL of 0.9% sodium chloride to a 12 mm x 75 mm round bottom glass culture tube. Select one or two colonies from an overnight plate of bacteria (in this case, *E. coli* strain FDA-CDC 0494) and vortex gently to suspend these in the 0.9% sodium chloride.

1.3.3. Check the concentration of bacteria using a McFarland reader. Adjust as needed by adding more 0.9% sodium chloride or more bacteria to achieve a 0.5 McFarland turbidity reading.

1.3.4. Make a 1:300 dilution of the 0.5 McFarland suspension by adding 100 µL of the suspension to 30 mL of CAMHB in a 50 mL conical tube to reach a final cell density of 5 x 10^5 CFU/mL[27].

1.4. Add antimicrobials to a flat-bottom, square-well, clear, untreated 384-well plate using the D300.

NOTE: Perform this step immediately after preparing bacterial suspension so that suspension can be added to the plates within 15 minutes of preparation[26].

1.4.1. Turn on the inkjet printer, start a new file, and add fluids to the protocol as in steps 1.2.2.1-1.2.2.3.

1.4.2. Generate the synergy grid.

1.4.2.1. Click the **Synergy** icon at the top of the screen and proceed through the steps. For **Type** select **Two or more fluids factored together**. For **Plate**, it is not necessary to exclude any wells; click **Next** on this step without making changes.

1.4.2.2. On the **Titration** tab, enter the antibiotic concentrations and placement.

1.4.2.2.1. In order to add minocycline in decreasing doubling dilutions from 32 to 0.031 µg/mL, in addition to a negative well with no antibiotic, down the *y* axis, enter 12 for **Titration levels** on the left panel. For **Specify titration using**, select Highest concentration; for **Fluid,** select Minocycline; for **Highest concentration**, enter 32 (make sure the unit is set at µg/mL; if it is not, close the Synergy dialog box and change under the **Current Protocol** tab). Make sure that the **Include 0** value box is checked. Change **Distribution** to 1:2 (50%).

1.4.2.2.2. Repeat these steps for colistin on the right-hand panel, using 12 titration levels and a highest concentration of 16. Click **Next**.

1.4.2.3. On the **Layout** tab, choose **Titration levels of first 2 fluids determine the number of rows and columns in a layout grid**. Click **Next**. If the grid appears as expected, click **Finish**.

1.4.3. Save the protocol, then click the **Run** button at the top left.

1.4.4. Click the **Start** button. Load a 384-well plate (with the lid removed) into the plate holder and press **Loaded** under the **Load Plate 1 – Synergy** prompt. Place a T8+ cassette into the cassette slot and press **Loaded** under the **Load a T8+ cassette** prompt.

1.4.5. When prompted, add antibiotic stock solution to the indicated reservoirs on the cassette. Follow instructions on the screen for proper loading and dispense carefully to avoid getting any bubbles in the solution. After each solution is added, press the **Filled** button.

1.4.6. Once the D300 dispenser has added antibiotic stock in appropriate volumes to each well and the **Run completed** box appears, click **Exit**, remove the plate, and turn off the D300.

1.5. Add bacterial suspensions to 384-well plate and incubate plate.

1.5.1. Pour the previously prepared bacterial suspension into a sterile reagent reservoir.

1.5.2. Use a multichannel pipette to add 50 μL of the suspension to all wells in the checkerboard array. Add 50 μL of CAMHB without bacteria to an empty well; this will be the negative control well to confirm sterility of the media. Incubate in a 35°C ambient air incubator for 16-20 hours[26].

1.6. Read plate on a microplate reader at OD_{600} and analyze checkerboard array results.

1.6.1. First, check the purity plate and ensure that the isolated colonies are of a single morphology that is consistent with the expected morphology of the organism being tested.

1.6.2. Using a spreadsheet program, shade cells to indicate growth and no growth as in step 1.2.4.1.

1.6.3. Determine the MIC for each drug. For a drug that does not inhibit bacterial growth at the highest concentration tested, the MIC is considered to be off-scale.

1.6.4. For each well in which growth is inhibited, determine the fractional inhibitory concentration (FIC) for each antibiotic based on that antibiotic's MIC (see **Figure 1B and Figure 2B**).

NOTE: The FIC is the ratio of the concentration of antibiotic in a well in which growth is inhibited to its MIC; so for a drug with an MIC of 8 μg/mL, a well containing 8 μg/mL of that drug has an FIC of 1, while a well containing 4 μg/mL has an FIC of 0.5.

1.6.5. Calculate the fractional inhibitory concentration index (FIC_I) value for each well in which growth is inhibited as the sum of the FICs of each of the drugs in that well.

1.6.6. Determine the lowest FIC_I at which growth is inhibited (minimum FIC_I). If the minimum FIC_I is ≤ 0.5, consider the combination synergistic; if 0.5-4, consider the combination indifferent; and if >4, consider the combination antagonistic. If the combination is synergistic at some concentration combinations but antagonistic at others, note this result but consider the

combination overall antagonistic.

2. Time-kill synergy testing

2.1. Make antimicrobial stock solutions. If this step is performed ahead of the experiment, freeze stocks at -80 °C until ready for use.

2.1.1. Determine antibiotic stock solution concentrations based on solubility of antibiotics and desired final concentrations in time-kill studies. In this example, make colistin and minocycline stocks at a concentration of 1 mg/mL. Use the CLSI M100 document to determine appropriate solvents for each antibiotic[25]. Use water, as recommended, for both colistin and minocycline.

2.1.2. Weigh out antibiotic powder using analytical balance and calculate volume of solvent needed to obtain goal stock concentration. If needed, perform a potency calculation prior to determining the quantity of solvent required, as described in step 1.1.2.1 above.

2.1.3. Pour antibiotic powder into 15 mL conical tubes and add the appropriate volume of water. Vortex until dissolved.

2.2. Using the manual broth microdilution method described in CLSI M07[26], perform QC of antimicrobial stocks for use in time kill synergy experiments. Perform this step at least one day prior to synergy testing so that QC results can be reviewed before using the stock.

2.2.1. Select an appropriate QC strain and determine the acceptable MIC range for drugs being tested based on **Table 5A-1** in CLSI M100[25]. For the drugs here, use *E. coli* ATCC 25922; the MIC ranges for this strain are 0.25-1 μg/mL for minocycline and 0.25-2 μg/mL for colistin.

2.2.2. Prepare antibiotic-containing broth microdilution plates.

2.2.2.1. Select the highest concentration of antibiotic to be tested so the entire QC range can be included. Use a range of 0.016 to 8 μg/mL for minocycline and colistin for ATCC 25922.

2.2.2.2. Dilute the antibiotic stocks to a working solution in CAMHB at **two times** the highest concentration needed (because it will be diluted 1:1 with the suspension of bacteria). In this example, dilute both stocks from 10 mg/mL to 16 μg/mL.

2.2.2.3. Using a multi-channel pipette, add 100 μL of each of the 2x antibiotic suspensions to a well in the first column of a clear, round-bottom, untreated 96-well plate and add 50 μL of plain broth (i.e., without antibiotic) to each well of the subsequent columns.

2.2.2.4. Remove 50 μL of antibiotic-containing broth from each well in the first column and add to the wells in the second column. Pipette up and down several times to mix the contents, generating an antibiotic concentration half that of the concentration in the first column.

2.2.2.5. Repeat step 2.2.2.4 with each column, so that a series of serial two-fold dilutions, each with a volume of 50 μL, is prepared. Change pipette tips between each dilution step if desired to eliminate the possibility of antibiotic carryover. Note that the resultant concentrations are all still 2x the final concentrations, as they will subsequently be diluted 1:1 with bacterial suspension.

2.2.2.6. Do not add any antibiotic to the final two columns, as these will be the negative and growth control columns.

2.2.3. Prepare bacterial suspension.

2.2.3.1. Prepare a 0.5 McFarland suspension from an overnight plate of *E. coli* ATCC 25922 in 0.9% sodium chloride as described in steps 1.2.1.5-1.2.1.6.

2.2.3.2. Make a 1:150 dilution of the 0.5 McFarland suspension by adding 50 μL of the suspension to 7.5 mL of CAMHB.

NOTE: The final bacterial suspension will be diluted 1:300 once it is mixed 1:1 with the antibiotic solution, reaching the CLSI-recommended cell density of 5×10^5 CFU/mL[27]).

2.2.4. Add bacteria to the microplate and incubate.

2.2.4.1. Add 50 μL of the bacterial suspension to each well, except in the 11th column. Add 50 μL of CAMHB to the 11th column (negative control column).

2.2.4.2. Incubate plate at 35 °C for 16-20 hours.

NOTE: A different duration of incubation may be required if organisms other than *Enterobacteriaceae* are being tested; consult CLSI M100[25] for organism-specific recommendations.

2.2.4.3. Read plate for growth visually using transmitted light and, for each antibiotic, determine the lowest concentration in which there is no growth; this is the MIC. Consult the CLSI M07 document for additional details on visual interpretation of MIC[26]. If the MIC is within the expected QC range, the stock solution is appropriate for use.

2.3. Start initial culture.

2.3.1. Make a 0.5 McFarland suspension of test organism in sterile 0.9% NaCl as described above.

2.3.2. Add 100 µL of the 0.5 McFarland suspension to 5 mL of CAMHB in a 25 mm x 150 mm glass round bottom culture tube with stainless steel closure and vortex gently to mix.

2.3.3. Using a sterile inoculating loop, isolation streak a drop of the diluted suspension onto a blood agar plate to confirm inoculum purity and incubate at 35 °C in ambient air.

2.3.4. Replace closure on tube and incubate in a test tube rack on a shaker at 35 °C in ambient air for at least 3 hours, until logarithmic-phase growth is reached (see step 2.6.1). Proceed to step 2.4 while the culture is in the incubator.

2.4. Prepare antimicrobial solutions in 25 mm x 150 mm glass culture tubes.

2.4.1. Take out antimicrobial stock aliquots from -80 °C freezer to thaw. Vortex once thawed to ensure antibiotic is in solution.

2.4.2. While initial culture is incubating, add 10 mL of CAMHB to five autoclaved 25 mm x 150 mm glass culture tubes and add antimicrobial stock solutions as follows.

NOTE: For a synergy study, at least one drug should be at a concentration that does not affect the growth curve individually[28]; this can be determined by evaluating the effects of individual drug concentrations prior to the synergy study.

2.4.2.1. Tube 1: Add appropriate quantity of antibiotic #1 to obtain target final antibiotic concentration. In this case, add 10 µL of 1 mg/mL colistin stock to obtain a final colistin concentration of 1 µg/mL, as this is a concentration that is ineffective against the strain used in this example.

2.4.2.2. Tube 2: Add appropriate quantity of antibiotic #2 to obtain final antibiotic concentration to be tested. In this case, add 10 µL of 1 mg/mL minocycline stock to obtain a final concentration of 1 µg/mL, a concentration that is ineffective against the strain being used in this example.

2.4.2.3. Tube 3: Add the same quantity of antibiotic #1 **and** antibiotic #2 as used in tubes 1 and 2. In this case, add 10 µL of 1 mg/mL minocycline stock and 10 µL of 1 mg/mL colistin stock.

2.4.2.4. Tube 4: Add no antibiotics; this will be the growth control tube.

2.4.2.5. Tube 5: Add no antibiotics; this will be the negative control tube.

2.5. Prepare 96 deep well polypropylene plates with 2 mL wells for serial dilutions by adding 900 µL of sterile 0.9% sodium chloride to rows B-H of columns 1-5 with a multichannel pipette.

2.6. Prepare starting inoculum and add to tubes.

2.6.1. Once the initial culture has reached logarithmic growth phase (~3 hours for *Klebsiella pneumoniae*, the organism used in this example), remove the culture tube from the shaker, vortex gently, transfer ~1 mL of suspension to a 12 mm x 75 mm glass culture tube, and check density with a McFarland reader.

2.6.1.1. If it is less than 1.0 McFarland, return tube to the shaker and incubate longer. If it is greater than 1.0 McFarland, add CAMHB to the tube, vortex gently, and re-sample, repeating the process until the suspension is at 1.0 McFarland.

2.6.2. Add 100 µL of the 1.0 McFarland suspension to tubes 1-4 and vortex gently.

2.7. Sample aliquots from each culture and perform serial ten-fold dilutions.

2.7.1. At time 0 (immediately after adding bacteria to the tubes) and at 1, 2, 4, 6, and 24 hours, remove a 150 µL aliquot from each culture tube by tilting the tube so that only the sterile pipette tip enters the tube and not the unsterile pipettor shaft during aliquot withdrawal. Add aliquots, respectively, to consecutive wells in the first row of the previously prepared 96 deep well plate. Return tubes to a test tube rack on a shaker in a 35 °C ambient air incubator immediately after removing aliquots at each time point.

2.7.2. Using a multichannel pipette, remove 100 µL from row A, add to row B (which contains 900 µL of 0.9% sodium chloride), and pipette up and down 4-5 times to mix, creating a 1:10 dilution. Discard tips following each dilution step to prevent carryover of bacteria, which can lead to falsely elevated colony counts.

2.7.3. Repeat step 2.7.2 for rows B-H with new pipette tips for each row.

2.8. Plate diluted samples for colony counts using the drop plate method[29,30].

2.8.1. Label Mueller-Hinton agar plates with the antibiotic conditions and dilution to be plated.

2.8.2. Using a multichannel pipette and extra-long tips (to ensure that tips reach into suspension), remove 10 µL from each well in column one and dispense carefully in a row onto the appropriately labeled plate. If small (100 mm diameter) plates are used, dispense 3 rows (each consisting of drops from rows A-H of a single column) per plate; on large (150 mm diameter) plates, dispense 8 rows per plate.

2.8.3. Allow drops to dry completely (~15 minutes).

2.8.4. At 24 hours, place a 10 µL drop taken directly from the negative control tube in an indicated area of one of the plates to test for sterility. Invert plates and incubate overnight at 35 °C in ambient air.

2.9. Count colonies and calculate cell density. Mark colonies with a fine-tip permanent marker on the reverse of the plate to avoid double-counting or missing colonies.

2.9.1. First, check the purity plate and ensure that the isolated colonies are of a single morphology that is consistent with the expected morphology of the organism being tested.

2.9.2. For each dilution series, identify drops with 3-30 colonies (typically one drop per dilution series). Count the colonies in these drops and record the count along with the dilution factor.

2.9.2.1. If there are no drops in a dilution series with 3-30 colonies, count the colonies in the last drop with >30 colonies and the first drop with <3 colonies (these should be adjacent drops).

2.9.3. For each dilution series, calculate number of colony forming units per milliliter (CFU/mL) in the sample based on the number of colonies in the drop using the following formula: CFU/mL = $n(1/d)(100)$ where n is the number of colonies, d is the dilution factor (1 for undiluted sample (row A), 0.1 or 10^{-1} for the first 1:10 dilution (row B), 0.01 or 10^{-2} for the second 1:10 dilution (row C), and so on, and the constant 100 accounts for the fact that the total volume of the drop is 10 μL, while the final value is expressed in CFU/mL, i.e., CFU/1000 μL. Use a spreadsheet containing formulas that calculate CFU/mL from colony count to simplify this process.

2.9.3.1. For dilution series where more than one drop was countable (or where two drops had to be counted because no drop fell in range), average the final CFU/mL counts for all counted drops.

2.9.3.2. Because the lower limit of detection is 300 CFU/mL (3 colonies in the undiluted drop), record and plot colony count as ≤300 CFU/mL for dilution series in which there are <3 colonies in the undiluted drop.

2.9.4. Inspect the sterility control drop from time 24; if any growth is observed in this drop, the results of the experiment should not be used.

2.10. Graph and analyze results.

2.10.1. Plot growth curves from the three antibiotic-containing cultures and the growth control on the same graph. Plot time on the *x* axis and CFU/mL, using a logarithmic scale, on the *y* axis.

2.10.2. Calculate the difference in CFU/mL between the combination tube at time 24 and the most active single agent at time 24. If the difference is ≥2 $\log_{10}$, consider the combination synergistic. Then calculate the difference in CFU/mL between the combination tube at time 24 and at time 0. If the difference is ≥3 $\log_{10}$, consider the combination bactericidal.

REPRESENTATIVE RESULTS:

Figure 1A presents a grid from a checkerboard array synergy experiment in which minocycline

in concentrations of 0-32 μg/mL was combined with colistin at concentrations of 0-16 μg/mL and tested against *E. coli* strain FDA-CDC 0494. The values represent spectrophotometric readings at optical density 600 nm (OD_{600}). Wells with OD_{600} values below 0.07 (which corresponds to no growth by visual inspection) are shaded red, while wells with OD_{600} values ≥0.07 (which corresponds to growth by visual inspection) are shaded green. For each drug, the minimum inhibitory concentration (MIC; bolded) is the lowest concentration of drug that inhibits bacterial growth. For minocycline, this is 32 μg/mL, and for colistin, it is 8 μg/mL. The shading is retained in **Figure 1B**, but values within the wells in which growth is inhibited are replaced by fractional inhibitory concentration index (FIC_I) values. These are determined as follows: in each well, the fractional inhibitory concentration index (FIC) of each drug is calculated by dividing the concentration of antibiotic in that well by the drug's MIC, and the FIC_I is calculated by summing the two FICs. Wells with an FIC_I value of ≤0.5, which is considered the cutoff for synergy, are indicated with a broken-line border, and the well with the lowest FIC_I value (0.094) is bolded. Because the minimum FIC_I value is in the synergistic range, the combination is considered synergistic.

Figure 2A and **Figure 2B** show grids analogous to those in **Figure 1A** and **Figure 1B**, but in this case the combination does not demonstrate synergy against the isolate tested (*K. pneumoniae* isolate BIDMC 4), because the minimum FIC_I at which growth is inhibited is 1, which is >0.5.

Figure 3 illustrates the optical density readings from a checkerboard synergy grid in which several skipped wells occurred (*Enterobacter cloacae* complex isolate BIDMC 27). Skipped wells are wells in which bacterial growth is inhibited despite the presence of bacterial growth in adjacent wells with higher concentrations of antibiotic. This phenomenon, which is known to occur in standard MIC testing as well, is likely due to biological variability in bacterial growth characteristics from well to well and to the sensitivity of some antibiotics to small differences in bacterial inoculum[23,31,32]. If more than one skipped well occurred in a checkerboard array, we discarded the results and repeated the assay.

Figure 4 presents examples of time-kill synergy results of three combinations tested against *K. pneumoniae* isolate BIDMC 32. Colony counts are indicated in a logarithmic scale on the *y*-axis and time, in hours, on the *x*-axis. The difference between the starting inoculum in the tube containing the drug combination and the concentration of bacteria in that tube at 24 hours is illustrated by the red bar and number, while the difference between the concentration of bacteria at 24 hours between the tube containing the combination and the tube containing the most active single agent alone is illustrated by the blue bar and number. **Figure 4A** shows results from the combination of colistin and minocycline; this combination was synergistic (difference between concentrations of bacteria exposed to combination and to most active agent alone ≥2 $\log_{10}$ CFU/mL at 24 hours) and bactericidal (decline from starting inoculum to concentration at 24 hours ≥3 $\log_{10}$ CFU/mL). **Figure 4B** shows results from the combination of colistin and clindamycin, a combination that was synergistic but was not bactericidal. This combination inhibited growth of the bacteria, which neither drug did alone, but did not kill them. **Figure 4C** shows results from the combination of colistin and erythromycin, which was neither bactericidal nor synergistic.

FIGURE AND TABLE LEGENDS:

Figure 1. Checkerboard array results demonstrating synergy (minocycline + colistin tested against *E. coli* strain FDA-CDC 0494**). (A)** Spectrophotometric readout and growth interpretation of a checkerboard array. Values in cells are optical density readings at 600 nm (OD_{600}). Cells with OD_{600} values below 0.07 (corresponding to no growth by visual inspection) are shaded red, while cells with OD_{600} values $\geq$0.07 (corresponding to growth by visual inspection) are shaded green. **(B)** Fractional inhibitory concentration index (FIC_I) calculation. Shading indicating growth or no growth has been retained. Values for colistin and minocycline along *x*- and *y*-axes, respectively, now represent the fractional inhibitory concentration (FIC), or the ratio of the concentration of the drug in that column or row to the minimum inhibitory concentration (MIC) of that drug alone. The value in each cell is the FIC_I, or the sum of the FICs of the two drugs in that well. The large broken line-bordered box encloses wells with an FIC_I of $\leq$0.5. The thick-bordered cell indicates the well with the lowest FIC_I in which growth is inhibited, or the minimum FIC_I. Because the minimum FIC_I is $\leq$0.5, the combination is considered synergistic.

Figure 2. Checkerboard array results of a combination that does not demonstrate synergy (minocycline + colistin tested against *K. pneumoniae* isolate BIDMC 4). (A) Optical density values at 600 nm and growth interpretation of checkerboard array results as described for **Figure 1A**. **(B)** Fractional inhibitory concentration index (FIC_I) calculation as described for **Figure 1A**. Because the minimum FIC_I is >0.5, the combination is not considered synergistic.

Figure 3. Checkerboard array results that are uninterpretable due to skipped wells (minocycline + colistin tested against *Enterobacter cloacae* complex isolate BIDMC 27). Optical density values at 600 nm and growth interpretation of checkerboard array results as described for Figure 1A. Several skipped wells, in which bacterial growth is inhibited despite the presence of growth in adjacent wells with higher concentrations of antibiotic, are demonstrated. Results are not interpretable, and experiment needs to be repeated.

Figure 4. Time-kill synergy results of three combinations tested against *K. pneumoniae* isolate BIDMC 32. Colony counts are indicated in a logarithmic scale on the *y*-axis and time, in hours, on the *x*-axis. The difference between the concentration of bacteria in the combination at 24 hours and the starting inoculum in the tube is illustrated by the red bar and number. If the decline from starting inoculum to concentration at 24 hours is ≥3 $\log_{10}$ CFU/mL, the combination is considered bactericidal. The difference between the concentration of bacteria at 24 hours between the tube containing the combination and the tube containing the most active single agent alone is illustrated by the blue bar and number; if there is ≥2 $\log_{10}$ CFU/mL reduction, the combination is considered synergistic. **(A)** Colistin (CST) + minocycline (MIN), a combination that is both synergistic and bactericidal. **(B)** Colistin + clindamycin (CLI), a combination that is synergistic but not bactericidal. **(C)** Colistin + erythromycin (ERY), a combination that is neither synergistic nor bactericidal. These results were initially published as part of a study of the synergistic activity of colistin-containing combinations against colistin-resistant *Enterobacteriaceae*, in which we demonstrated that colistin was synergistic with a

number of antibiotics that are active individually only (e.g. clindamycin) or primarily (e.g. erythromycin) against Gram-positive bacteria[16]. (Note that erythromycin was synergistic by checkerboard array against the strain shown, but not by time-kill, so it has been selected here as an example of a non-synergistic combination.) We hypothesized that colistin, which is known to act by permeabilization of the Gram-negative outer membrane, exerts a sub-inhibitory permeabilizing effect on colistin-resistant Gram-negative bacteria, allowing entry of drugs such as clindamycin that normally cannot enter the Gram-negative cell. Panel (A) of this figure has been modified from Brennan-Krohn, Pironti, and Kirby 2018[16], copyright © American Society for Microbiology, Antimicrobial Agents and Chemotherapy, 62(10), 2018, pii: e00873-18, doi: 10.1128/AAC.00873-18.

DISCUSSION:

The two methods described here both provide information about the activity of antimicrobials used in combination compared to their individual activity. The automated, inkjet printer-assisted digital dispensing method is an adaption of the method described in the Clinical Microbiology Procedures Handbook[33], while the time-kill method more closely follows the corresponding protocol from the same reference[34].

In the checkerboard array method, calculations to determine the necessary volume of antimicrobial stock to add to each well as well as the dispensing of these volumes is automated, thus eliminating some of the major potential sources of error encountered in a manual checkerboard array. It is still essential, however, that the investigator determines that original stocks are made at the intended concentration and that goal final concentrations are entered into the D300 software correctly. Adding the antimicrobial suspension to wells in a 384-well plate can be challenging at first and requires care to ensure that pipette tips enter the appropriate wells and that liquid does not splash up the edges of the wells. An automated liquid handler can be used in place of a hand-held multichannel pipette to increase the speed and accuracy with which the bacterial suspension is added to wells. As described in the protocol, the D300 requires the addition of the surfactant, polysorbate 20 (P-20), for proper liquid handling. A different surfactant, polysorbate 80, at a concentration of 0.002%, has been noted to lower colistin MICs for organisms with colistin MICs of <2 µg/mL in standard broth microdilution assays.[35, 36] Our laboratory previously demonstrated that P-20 at concentrations up to 0.0015% had no effect on D300-assisted MIC results in comparison with reference BMD[14]. In the assay example presented here, the maximum P-20 is concentration is 0.0014%.

One problem we encountered with some checkerboard array assays was a large number of skipped wells. This occurred at a disproportionate rate with certain antibiotics. Specifically, in a screen of combinations against a collection of carbapenem-resistant *Enterobacteriaceae*, we found that while 49 of 521 trials (9.4%) were unusable due to multiple skipped wells, 2 of the 12 antibiotics tested (fosfomycin and cefepime) accounted for 46 of these trials (94%). Such increased rates may be more likely in drugs that are particularly susceptible to the inoculum effect[31,32,37]. Of note, CLSI does not recommend testing fosfomycin in broth dilution[25] due to concerns about the reliability of results with this method, which may explain the unreliable results seen with this drug. Some modifications can be made to automated checkerboard

method according to investigator preference. Antimicrobials can be dispensed into plates already containing bacterial suspension, rather than into empty wells, if this is preferable for reasons of workflow within the laboratory. While 384-well plates were used here, the method can also be carried out in 96-well plate assays with appropriate modification of well volume. The use of a 96-well plate format may help in reducing skipped wells for antibiotics that are particularly sensitive to small changes in inoculum. When calculating FIC_I, there may be situations where the MIC is off-scale (i.e., higher than tested), including situations where the drug being tested has no activity individually against the type of organism being tested. In these cases, the FIC can be calculated based on assuming the MIC is one dilution higher than the highest concentration tested. This is the most conservative strategy, as it assumes the maximal possible FIC value for any dilution where inhibition is observed during synergy testing. For example, if the actual MIC were instead two doubling dilutions above the highest concentration tested, then the corresponding FICs would be two-fold lower than the conservative assignments, and so on.

In order to accurately assess the bactericidal activity of drugs in a time-kill assay, it is essential that cultures be in logarithmic-phase growth, particularly when cell-wall active antibiotics are being tested[28]. For the rapidly-growing bacteria used in this example (*K. pneumoniae*), 3 hours of incubation with shaking was appropriate to reach this growth phase, but different amounts of time may be necessary for different organisms. In general, the culture should appear visibly but not heavily turbid. The appropriate amount of time can be determined by constructing a growth curve with colony counts taken at serial time points (e.g., every 30 minutes for 4-6 hours)[38]. The intended starting inoculum in the time-kill study is also important. The target concentration of the starting inoculum is approximately 5×10^5 to 1×10^6 CFU/mL. The dilution described here (100 μL of a 1.0 McFarland suspension in 10 mL of media) generates this inoculum for *Klebsiella pneumoniae* and other *Enterobacteriaceae* species on which we have tested it. If the density of the starting inocula in an experiment using different organisms is significantly higher or lower than this, then a different dilution may be needed. (The appropriate dilution required for a given species can be determined by performing a plate count of a 0.5 or 1.0 McFarland suspension to determine how many organisms this turbidity represents, then calculating the amount by which the initial suspension must be diluted to reach the appropriate final concentration.) If, on review of plate counts from the synergy study, the starting inoculum of any of the antibiotic-containing tubes is found to have been significantly lower than the starting inoculum of the growth control, this may indicate either antibiotic carryover or very rapid killing of bacteria in the brief time between addition of bacteria to the antibiotic-containing tube and removal of the aliquot for plating. If the actual number of colonies in the undiluted drop in a series is *lower* than the number of colonies in subsequent dilutions, this suggests antibiotic carryover effect. Different options have been described for preventing this effect, including spreading a single aliquot over an entire plate[38] or spinning down the sample, removing the supernatant, and re-suspending in sterile saline prior to plating[39]. At each time point in the time-kill method, it is also critical for the investigator to efficiently but accurately remove an aliquot from each culture tube and perform serial dilutions. Delays during this process, particularly during early time points that occur in close succession, can lead to prolonged periods during which cultures are not been incubated and

shaken, whereas careless dispensing and serial dilutions can lead to inaccurate plate counts. Compared to the spread plate method of plate counting, in which 100 μL of each dilution is spread over an entire agar plate, the drop plate method described is far more rapid, requires a much smaller number of agar plates, and allows for faster counting, as the maximum countable number of colonies for each drop is 30, whereas up to 300 colonies can typically be counted from a spread plate. However, the spread plate method is also an option if investigators are more comfortable with this technique. If drops spread into each other after dispensing with a multichannel pipette, individual application of more widely spaced drops with a single-channel pipette can be performed instead. In our experience, cooling plates at 4 °C prior to dispensing drops seemed to reduce excessive spreading.

One limitation of the techniques described here is that the results of the two types of synergy assay (checkerboard array and time-kill) are not always concordant, and since most published synergy articles use one method or the other rather than both together, it can be difficult to know how to integrate data from the two types of assays. Because the automated checkerboard array method we developed is simple and high-throughput, we have used it in effect as a kind of screen to test combinations against a larger number of isolates and to determine which concentration combinations were synergistic. We then performed a smaller number of time-kill studies, selecting combinations and concentrations that had been effective in the checkerboard array. Of note, because the checkerboard assay is typically performed on a microbroth dilution scale, while the time-kill assay uses larger volumes (similar to a macrobroth dilution), we found that FICs were sometimes different between the two methods, with higher concentrations generally required in the time-kill assay to demonstrate activity. This phenomenon has been noted previously when macrobroth and microbroth dilution MIC assay results are compared for Gram-negative bacilli[26] and when larger inocula (as used in time-kill studies) are compared with the standard inoculum used in microbroth dilution and checkerboard array assays[32]. A specific limitation of the checkerboard array is the inherent variability in microbroth dilution MIC testing[22]. While FIC_I cutoffs for synergy account for this variability mathematically[6] such variability inevitably raises concern about the reliability and consistency of checkerboard array results.

Because of the limitations inherent to all in vitro synergy testing methods (including cultivation of bacteria in an artificial growth medium, static antibiotic concentrations, and a limited time course), results obtained by these methods must be confirmed and further evaluated using supplemental techniques. Such methods include in vitro pharmacokinetic/pharmacodynamic (PK/PD) studies (e.g., the hollow fiber infection model[40]), animal models, and, ultimately, human PK/PD and efficacy studies. The automated checkerboard array method described here, by providing a rapid method with which to screen combinations for potential synergistic activity, allows for more targeted utilization of these techniques. Further automation of all of these methods, as well as more systematic investigation of the relationship between in vitro parameters and clinical outcomes, will be important in scaling up the use of synergy testing and increasing its clinical applicability.

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Concentration Minocycline (µg/mL)

	0	0.016	0.031	0.063	0.125	0.25	0.50	1	2	4	8	16
32	0.045	0.044	0.043	0.044	0.043	0.043	0.042	0.043	0.043	0.044	0.045	0.047
16	0.075	0.040	0.038	0.038	0.038	0.038	0.039	0.039	0.039	0.038	0.039	0.040
8	0.206	0.225	0.077	0.037	0.038	0.039	0.039	0.039	0.038	0.038	0.038	0.040
4	0.319	0.319	0.275	0.257	0.075	0.039	0.040	0.040	0.039	0.039	0.040	0.042
2	0.341	0.326	0.322	0.311	0.261	0.042	0.041	0.041	0.040	0.040	0.040	0.042
1	0.322	0.323	0.311	0.327	0.309	0.235	0.215	0.044	0.108	0.043	0.040	0.042
0.50	0.315	0.320	0.311	0.324	0.327	0.310	0.277	0.250	0.262	0.089	0.041	0.042
0.25	0.313	0.312	0.316	0.320	0.318	0.319	0.333	0.315	0.335	0.255	0.042	0.044
0.125	0.310	0.306	0.306	0.304	0.321	0.320	0.318	0.311	0.303	0.194	0.069	0.043
0.063	0.301	0.287	0.297	0.291	0.297	0.291	0.282	0.278	0.287	0.197	0.040	0.039
0.031	0.300	0.282	0.291	0.294	0.298	0.291	0.294	0.282	0.276	0.160	0.039	0.039
0	0.313	0.311	0.306	0.307	0.312	0.309	0.312	0.304	0.292	0.177	0.040	0.041

Growth (+) Growth (-)

Concentration Colistin (µg/mL)

(a)

FIC Minocycline (concentration/MIC)

	0	0.002	0.004	0.008	0.016	0.031	0.063	0.125	0.250	0.500	1.000	2.000
1	-	1.002	1.004	1.008	1.016	1.031	1.063	1.125	1.250	1.500	2.000	3.000
0.50	-	0.502	0.504	0.508	0.516	0.531	0.563	0.625	0.750	1.000	1.500	2.500
0.25	-			0.258	0.266	0.281	0.313	0.375	0.500	0.750	1.250	2.250
0.125	-					0.156	0.188	0.250	0.375	0.625	1.125	2.125
0.063	-					0.094	0.125	0.188	0.313	0.563	1.063	2.063
0.031	-							0.156		0.531	1.031	2.031
0.016	-										1.016	2.016
0.008	-										1.008	2.008
0.004	-										1.004	2.004
0.002	-										1.002	2.002
0.001	-										1.001	2.001
0	-	-	-	-	-	-	-	-	-	-	-	-

Growth (+) Growth (-)

FIC Colistin (concentration/MIC)

(b)

Concentration Minocycline (µg/mL)												
32	0.041	0.041	0.042	0.042	0.042	0.042	0.042	0.041	0.040	0.041	0.041	0.052
16	0.041	0.041	0.041	0.040	0.039	0.039	0.039	0.039	0.039	0.039	0.041	0.053
8	0.043	0.041	0.040	0.039	0.039	0.039	0.039	0.039	0.039	0.039	0.041	0.054
4	0.140	0.119	0.106	0.094	0.038	0.046	0.043	0.046	0.043	0.043	0.040	0.053
2	0.200	0.200	0.215	0.194	0.139	0.039	0.039	0.039	0.039	0.039	0.040	0.052
1	0.236	0.220	0.249	0.238	0.247	0.040	0.040	0.040	0.040	0.039	0.040	0.057
0.50	0.247	0.221	0.252	0.250	0.259	0.040	0.040	0.040	0.041	0.041	0.041	0.054
0.25	0.246	0.242	0.257	0.262	0.276	0.042	0.042	0.042	0.042	0.041	0.041	0.049
0.125	0.266	0.228	0.258	0.252	0.276	0.040	0.040	0.040	0.041	0.041	0.041	0.046
0.063	0.279	0.241	0.263	0.265	0.290	0.041	0.041	0.041	0.041	0.040	0.041	0.042
0.031	0.270	0.225	0.258	0.257	0.289	0.040	0.039	0.039	0.039	0.039	0.040	0.044
0	0.363	0.244	0.283	0.266	0.321	0.039	0.039	0.039	0.045	0.039	0.039	0.041
	0	0.016	0.031	0.063	0.125	0.25	0.50	1	2	4	8	16

Growth (+) Growth (-)

Concentration Colistin (µg/mL)

(a)

FIC Minocycline (concentration/MIC)												
4	-	4.063	4.125	4.25	4.5	5	6	8	12	20	36	68
2	-	2.063	2.125	2.25	2.5	3	4	6	10	18	34	66
1	-	1.063	1.125	1.25	1.5	2	3	5	9	17	33	65
0.50	-				1	1.5	2.5	4.5	8.5	16.5	32.5	64.5
0.25	-					1.25	2.25	4.25	8.25	16.25	32.25	64.25
0.125	-					1.125	2.125	4.125	8.125	16.125	32.125	64.125
0.063	-					1.063	2.063	4.063	8.063	16.063	32.063	64.063
0.031	-					1.031	2.031	4.031	8.031	16.031	32.031	64.031
0.016	-					1.016	2.016	4.016	8.016	16.016	32.016	64.016
0.008	-					1.008	2.008	4.008	8.008	16.008	32.008	64.008
0.004	-					1.004	2.004	4.004	8.004	16.004	32.004	64.004
0	-	-	-	-	-	-	-	-	-	-	-	-
	0	0.063	0.125	0.25	0.50	1	2	4	8	16	32	64

Growth (+) Growth (-)

FIC Colistin (concentration/MIC)

(b)

Figure 3

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Concentration Minocycline (µg/mL)	0	0.016	0.031	0.063	0.125	0.25	0.50	1	2	4	8	16
32	0.046	0.049	0.047	0.047	0.045	0.045	0.045	0.047	0.045	0.048	0.047	0.046
16	0.046	0.048	0.048	0.043	0.042	0.045	0.042	0.045	0.044	0.047	0.045	0.049
8	0.039	0.040	0.039	0.038	0.037	0.038	0.042	0.038	0.038	0.038	0.039	0.040
4	0.039	0.039	0.038	0.038	0.038	0.038	0.038	0.038	0.038	0.038	0.038	0.040
2	0.043	0.043	0.042	0.040	0.040	0.040	0.040	0.040	0.040	0.040	0.041	0.043
1	0.153	0.140	0.132	0.046	0.041	0.041	0.041	0.042	0.042	0.045	0.041	0.043
0.50	0.259	0.288	0.276	0.272	0.040	0.113	0.041	0.043	0.042	0.043	0.039	0.041
0.25	0.352	0.402	0.371	0.324	0.043	0.097	0.044	0.044	0.045	0.046	0.041	0.042
0.125	0.563	0.553	0.540	0.475	0.044	0.047	0.044	0.048	0.051	0.048	0.045	0.046
0.063	0.594	0.578	0.572	0.518	0.048	0.190	0.051	0.045	0.051	0.055	0.048	0.047
0.031	0.543	0.524	0.524	0.452	0.037	0.038	0.038	0.038	0.037	0.038	0.039	0.039
0.016	0.558	0.534	0.538	0.473	0.367	0.038	0.039	0.037	0.038	0.038	0.039	0.039
0.008	0.583	0.558	0.555	0.474	0.366	0.042	0.043	0.042	0.041	0.043	0.042	0.043
0	0.587	0.550	0.556	0.474	0.044	0.042	0.048	0.041	0.045	0.042	0.043	0.043

Growth (+)

Growth (-)

Concentration Colistin (µg/mL)

Figure 4

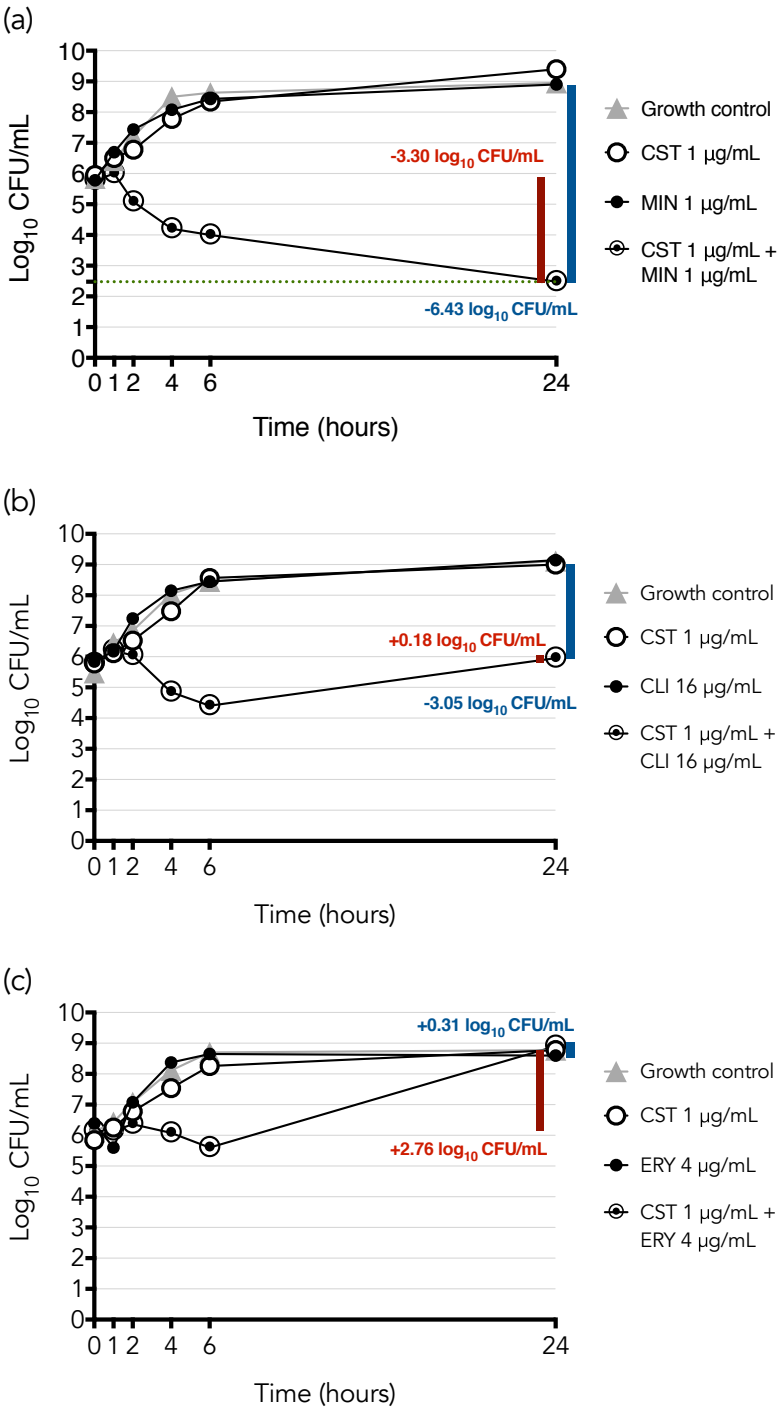

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number
Escherichia coli strain ATCC 25922	ATCC	25922
0.5 mL microcentrifuge tubes	USA Scientific	1605-0000
1 L 0.22 µm bottle-top filter	Thermo Scientific Nalgen	597-4520
12 mm x 75 mm borosilicate glass round bottom culture tubes	Fisherbrand	14-961-26
15 mL conical tubes	Phenix	SS-PH15
15 x 100 mm or 15 x 150 mm Mueller Hinton agar plates	Thermo Scientific	R01620 or R04050
25 mm stainless steel closures for 25 x 150 mm glass culture tubes	Bellco	2005-02512
25 x 150 mm borosilicate glass round bottom culture tubes	Bellco	2011-25150
348-well sterile clear, flat-bottom, untreated microplates with lids	Greiner Bio-One	781186
50 mL conical tubes	Phenix	SS-PH50
50 mL sterile reagent reservoirs	Corning	4870
96 deep well polypropylene microplate with 2 mL wells	Fisherbrand	12-566-612
96-well sterile clear, round-bottom, untreated microplates with lids	Evergreen	222-8032-01R
Cation adjusted Mueller Hinton broth	BD Diagnostics	212322
Colistin sulfate	Alfa Aesar	J60915
D300e Control Software	HP/Tecan	
DensiCHEK Plus McFarland reader	bioMérieux	21250
Excel spreadsheet software	Microsoft	
Extra long SHARP 10 µL Precision Barrier Tips	Denville Scientific	P1096-FR
HP D300 digital dispenser	HP/Tecan	
HP D300 T8+ cassettes	HP/Tecan	30097370
Minocycline hydrochloride	Chem-Impex	14302
Picus 12-channel 10-300 µL pipette	Sartorius	735461
Polysorbate 20	Fisher Bioreagents	BP-337
Sodium chloride	Fisher Chemical	S271
Spectrophotometer	Tecan	Infinite M1000 PRO
Xplorer 12-channel 50-1200 µL pipette	Eppendorf	2231000328

Comments/Description

QC strain

Brand name: Tween 20

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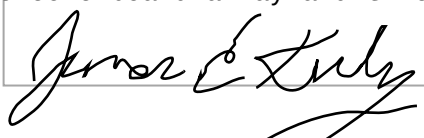
Article Title:	Antimicrobial synergy testing by inkjet printer-assisted automated checkerboard array and time-kill
Signature:	
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Editorial comments:

Changes to be made by the Author(s) regarding the written manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
The manuscript has been proofread and all identified spelling and grammar issues have been corrected.

2. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: HP D300, etc.
"HP- Hewlett Packard" commercial language removed from D300 citations except in Table of Materials and Reagents). In general we now refer to the D300 as an inkjet printer generically per the editor's suggestion. However, in some instances the attributes of the D300 software, disposables, and mechanics are platform specific are referred to specifically as necessary for clarity. However, the commercial manufacturer is no longer cited except in the materials and methods section.

3. Please rephrase the Introduction to include a clear statement of the overall goal of this method.
The following statement has been added to the first paragraph of the introduction (lines 63-68) to more clearly lay out the goals of the method: "The techniques described in this paper provide two complementary methods of *in vitro* synergy testing that, when used together, allow investigators to efficiently screen antimicrobial combinations of interest for evidence of synergistic activity (the automated checkerboard array method) and then to further evaluate the kinetics of inhibition and killing demonstrated by promising combinations identified in the screening stage (the manual time-kill method).

4. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. Please move the discussion about the protocol to the Discussion.
Instructions in the protocol have been changed to the imperative mood where they were not already.

The following note has been added to the beginning of the protocol (lines 133-135): "NOTE #1: Use appropriate safety procedures when working with bacteria. Wear gloves and a lab coat at all times. Perform work in a biosafety cabinet if aerosols will be generated or working with high risk pathogens."

The final two steps of the protocol, which involved discussion, have been moved to the discussion section.

5. Please revise the Protocol steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.
This revision has been carried out.

6. 1.6.2: Please describe how the minimum inhibitory concentration is determined.
The following text has been added to define the minimum inhibitory concentration (lines 241-242): "The MIC is the lowest concentration of drug at which bacterial growth is inhibited."

7. 2.3.2: How long is the culture incubated?
This is now specified as a minimum of 3 hours (now step 2.3.4), with a reference to step 2.6.1, where the possible need for further incubation, depending on the turbidity reading at 3 hours, is discussed.

8. 2.8.3: Does time 24 mean 24 hours?
This does mean 24 hours, and now reads "At 24 hours" instead of "At time 24" (line 423).

9. After you have made all the recommended changes to your protocol (listed above), please re-evaluate your protocol length. There is a 10 page limit for the Protocol, and a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
The revised protocol is less than 10 pages, and the highlighted text is less than 2.75 pages.

10. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.
All highlighted areas are now complete sentences.

11. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
All details for performing the highlighted steps have been included in highlighting.

12. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
The discussion section has been edited significantly to focus on these areas.

13. References: Please do not abbreviate journal titles.
The journal title that was abbreviated is now written out (reference 15).

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript describes in vitro synergy testing methods using both checkboard and time kill assays. However, the abstract and introduction do not concisely explain the goals of this paper. The primary goal would seem to be the use of the D300 for preparation of checkerboard synergy panels. Including the manual time kill assays seems to dilute the value of using the D300 for checkerboard assays. As authors mention, detailed procedures for synergy testing by checkerboard (manual preparation of panels) and time kill assay are discussed (more thoroughly) in ASM's Clinical Microbiology Procedures Handbook.

The abstract and introduction have been edited to clearly lay out the goals of the paper and to describe the rationale for including both the novel automated checkerboard array method as well as the more established time-kill technique. A representative statement added to the introduction is as follows (lines 63-68): "The techniques described in this paper provide two complementary methods of *in vitro* synergy testing that, when used together, allow investigators to efficiently screen antimicrobial combinations of interest for evidence of synergistic activity (the automated checkerboard array method) and then to further evaluate the kinetics of inhibition and killing demonstrated by promising combinations identified in the screening stage (the manual time-kill method)."

Major Concerns:

1. It is unclear if the reader is expected to have previous knowledge with the D300 for performing MIC tests before they embark on a more complicated two drug assay. I could not find a JOVE article for using the D300 for MIC testing of single agents. But it would be very beneficial to have such a stand alone procedure that could provide details of using this instrument in addition to providing details for preparation and QC of stock solutions, preparation of test inocula, determining MIC endpoints, etc. It would also be helpful if procedural steps were consistent with standard recommendations for susceptibility testing such as those described by CLSI, EUCAST or ISO, where appropriate. There are many places in this paper that such details are lacking or inconsistent with standard recommendations. Inclusion of a "supplemental notes" type of section where any benefits or limitations/pitfalls of using the D300 for MIC panel preparation would be useful. Appropriate references to any additional technical details of MIC testing (reading endpoints) would be useful.

The quality control technique (1.2) described is identical to the technique that would be used for testing the MIC of single drug. A note indicating such has now been added to the start of this section (lines 173-175): "Note: the QC technique described here is identical to the technique that would be used for minimum inhibitory concentration (MIC) testing of individual drugs and can be used as such with any strains of interest to the investigator."

As suggested, additional references to relevant CLSI procedures have been added; such references are now included in sections 1.1.1, 1.1.2.1, 1.2.1.2, 1.2.1.6, 1.2.2, 1.2.3.3, 1.2.4.2, 1.3.4, 1.4, 1.5.2, 2.1.1, 2.2, 2.2.1, 2.2.3.2, and 2.2.4.2.

2. Subsequently, a second companion paper could describe use of D300 for preparation of checkerboard panels. This would be used in conjunction with the original single drug MIC paper and would not repeat recommendations in the initial paper; it would not be a stand alone. It is somewhat more complex to control variables (and perform quality control) when preparing panels for testing combinations of drugs and looking for subtle reductions in MICs to define synergy. Pitfalls and limitations of preparation of checkerboard panels and checkerboard synergy testing should be clearly addressed.

As noted above, the technique for performing a single-drug MIC assay with the D300 is already included within this paper as part of the essential step of quality control testing.

Additional discussion of the pitfalls and limitations of checkerboard array testing is now included in the introduction and discussion sections.

3. When I first read the title I thought in some way the D300 could assist with time kill testing. The fact that authors will provide recommendations for the time kill assay are not clearly stated in the abstract. Authors might consider a third paper for time kill assays not just for synergy testing but for examining activity of single agents as well. Testing single agents are obviously part of the time kill synergy procedure but a dedicated procedure to kill curves would be useful. Again, pitfalls and limitations of this procedure should be thoroughly discussed.

The title has been changed to, "Antimicrobial synergy testing by inkjet printer-assisted automated checkerboard array and manual time-kill methods" to avoid the implication that the D300 is used in the time-kill technique.

As now described in more detail, we included the time-kill assay in this paper because we have found the combination of the automated checkerboard array screen with subsequent synergy testing by time-kill to be a valuable integrated approach to synergy testing. For investigators who have not previously used either method, we expect that a protocol that includes both components of this approach would be useful.

Additional discussion of the pitfalls and limitations of checkerboard array testing is now described in the introduction and discussion sections.

4. The background and the discussion sections are quite long and contain considerable information that is not "procedural". Anyone that attempts to use the D300 for preparing checkerboard synergy panels or time kill assays should have considerable knowledge in synergy concepts, synergy test methods and multidrug resistant organisms. Once again, clear goals would help the reader relate to any non-procedural content provided. Authors should consider removing/consolidating some of the information in the introduction and discussion or expand it if a goal is to describe the rationale, interpretation, limitations, clinical and/or research use etc. of synergy tests.

Explanations of standard checkerboard array and time-kill methods in the background and discussion section have been minimized, as suggested by the reviewer. As noted above, the discussion section has been changed to focus primarily on procedural content.

5. Separating out much of the content in this manuscript related to use of the D300 into two papers and another paper for kill curves could streamline all procedures, reduce redundancy and in my mind be easier to follow and would likely get more use.

As noted above, the difference in methodology between single-drug MIC testing and synergy testing with the D300 is very small, and splitting these techniques into two separate methods papers would result in almost entirely redundant procedures. Because the checkerboard array and time-kill methods provide distinct but complementary types of data on synergistic activity, they are frequently used together in synergy investigations, and many publications include results of both methods. We believe, therefore, that many readers would be interested in a procedural article that includes both methods, while those who are interested only in one method or the other can choose to watch only the relevant section of the video.

6. The yellow shaded areas appear to be redundant instructions. It is unclear how these will play out in a video.

The main redundant step is preparation of a 0.5 McFarland suspension of bacteria at [lines 187-192 and 252-255] and of a 1.0 McFarland suspension at [lines 398-403]. Leaving these steps un-highlighted would result in leaving out necessary sub-steps (see Editorial Comment 11 above), but I anticipate that, when filming, a brief, abbreviated sequence can be included for subsequent preparations of the starting suspensions.

7. Since I have not used the D300, I cannot comment on the instructions for use of this instrument although the stepwise approach is easy to follow and seems thorough.

We agree that these instructions should be straightforward to follow, as they are quite detailed and the D300 software is generally user-friendly.

8. The checkerboard example provided, minocycline and colistin, raises some concerns: 1) CLSI EUCAST Joint recommendations for colistin MIC testing include broth microdilution specifically without surfactant as it has been demonstrated that surfactant (artificially) enhances colistin activity. Authors should address this fact since Tween is recommended when preparing MIC panels using the D300; and 2) it would be clearer if the example had an MIC endpoint for colistin (Figure 1A).

1) The following explanation has been added to the Discussion section (lines 573-577) to explain why the Tween concentrations used in these experiments are unlikely to affect the results: "As described in the protocol, the D300 requires the addition of the surfactant, polysorbate 20 (P-20), for proper liquid handling. A different surfactant, polysorbate 80, at a concentration of 0.002%, has been noted to lower colistin MICs for organisms with colistin MICs of <2 µg/mL in standard broth microdilution assays.[35, 36] Our laboratory previously demonstrated that P-20 at concentrations up to 0.0015% had no effect on D300-assisted MIC results in comparison with reference BMD.[14] In the assay example presented here, the maximum P-20 is concentration is 0.0014%."

2) The example in Figure 1 has been changed to an isolate with an on-scale colistin MIC as recommended.

9. Some of the time kill assay examples are peculiar as clindamycin and erythromycin are generally considered as drugs for gram-positive bacteria and colistin is considered a drug for gram-negative bacteria. Authors should explain their selection of drugs for the examples.
Our previous work (PMID 30061285) demonstrated *in vitro* synergistic activity of colistin combined with a range of antibiotics, including protein synthesis inhibitors that are active individually only (e.g. clindamycin) or primarily (e.g. erythromycin) against Gram-positive bacteria. Examples from this work are shown in the present study. (Note that erythromycin was synergistic by checkerboard array against the strain shown, but not by time-kill, so was selected for Figure 4 as an example of a non-synergistic combination.) We hypothesized that colistin, which is known to act by permeabilization of the Gram-negative outer membrane, exerts a sub-inhibitory permeabilizing effect on colistin-resistant Gram-negative bacteria, allowing entry of drugs such as clindamycin that normally cannot enter the Gram-negative cell. This is now explained in the introduction (lines 93-97, for the combination of colistin plus minocycline) and in the Figure 4 legend (lines 545-554, for the three combinations shown in this figure).

10. Based on goals of this paper, it may or may not be helpful for authors to emphasize that there are few studies that demonstrate the clinical utility of synergy studies for predicting clinical outcomes. This in part, is a reason why synergy testing is rarely performed in the clinical setting and may be more useful in research settings (e.g., pharmaceutical studies, retrospective studies). Nevertheless, due to the difficulties in controlling all test variables, there are limitations to using both checkerboard and time kill assays in non-clinical settings as well. Authors do nicely address the need for alternative methods for assessing synergism in the last paragraph in the discussion.
Additional discussion of this important limitation has been added to the introduction (lines 118-124) as follows: "Perhaps the most significant limitation of all synergy testing methods is the lack of established correlations between *in vitro* results and clinical outcomes when combinations are used to treat patients.[24] Simpler and more rapid synergy testing methods, such as the automated checkerboard array method described here, may facilitate the integration of *in vitro* synergy testing within clinical trials or other evaluations of patient outcomes in order to better characterize the relationship between *in vitro* and *in vivo* effects in the future."

Minor Concerns:
1. Line 63 - aren't checkerboard assays sometimes examined visually?
The line containing this description has been removed as part of edits to the introduction, including reduction of unnecessarily basic descriptions of synergy testing.

2. Line 87 - sometimes synergy assays may be of interest to see if the combination has enhanced activity even if both drugs are susceptible. This reflects some therapy approaches (e.g. b-lactam plus aminoglycoside for gram-negatives)
The following line has been added (now lines 97-99): "Of note, synergy testing can also be used to evaluate for enhanced efficacy of two drugs which are both effective individually."

3. Line 94 - it would be helpful to indicate "manual performance of time kill assays".
This phrase (now line 103) now reads, “manual performance of time-kill synergy assays”.

4. Line 121 - investigators have shown the polysorbate interferes with activity of colistin (please see general comments above)
As noted in the response to comment 8 above (and now in the Discussion section (lines 573-577)), we do not expect the concentrations of polysorbate-20 used in this assay to affect colistin MICs, based on data that includes previous work in our lab.

5. Line 136 - suggest adding another step to clearly demonstrate an example of actual weight of powder and volume of solution used and/or (preferably) describe stock solution preparation as commonly recommended in widely used standards for susceptibility testing (CLSI, EUCAST, ISO).
The description of how to determine the volume of solvent has been changed to be clearer and to more closely resemble the description provided in CLSI M07 (now lines 149-166). An example with actual values has also been used.

6. Line 141 - how long for storage? -20 C not acceptable for all antibiotics
The option for storage at -20°C has been removed (now line 169).

7. Line 146 - how subculture bacteria to obtain colonies for inoculum preparation (for all tests described in the paper)
The following note has been added to the beginning of the protocol (lines 136-138): “NOTE #2: Twenty to 24 hours before starting experiments, streak out the bacterial isolate(s) to be tested (from a colony-purified, minimally passaged stock frozen at -80°C in tryptic soy broth with 50% glycerol stock) onto a blood agar plate. Incubate the plate at 35°C in ambient air.”

8. Line 149 - CLSI (M100-S28) range is 0.25-1 for minocycline?
This has been corrected to read “0.25-1” instead of “0.2-1” (now line 181).

9. Line 153 - it is unclear why a solution with drug and not broth only is used for a negative control? Could not even this very low concentration inhibit some contaminants? Usually the negative control confirms sterility of the system and is used as a "clear" broth for comparative purposes when reading/measuring MICs.
The purpose of including this low concentration was to assess sterility of both media and antibiotic solution at a concentration lower than those being used in the assay (with the rationale that any potential contaminants that could not grow at this concentration of antibiotic would likewise be inhibited at the higher concentrations used in testing). For simplicity, however, the negative control row contents have been changed in the manuscript, as suggested by the reviewer, to contain broth alone (lines 231-232).

10. Line 158 - standard procedures describe addition of colonies to saline to prepare a homogeneous suspension…bacteria do not "dissolve" in inocula media.
The word “dissolve” has been change to the phrase “vortex gently to suspend” (now line 185).

11. Line 193 - how does one "confirm fluids are correct" and what does this mean?
This was simply a suggestion to the user check over the protocol before proceeding with the experiment to make sure that it all of the parameters had been entered appropriately, but it has been removed to avoid confusion.

12. Line 205 - according to this protocol, the bacterial suspension is prepared before the drug is removed from the freezer and the checkerboard panel is prepared. It is assumed that the drug panel can be prepared very quickly so the standardized bacterial suspension is used within 15 minutes of preparation, as is generally recommended. At the beginning of this protocol, it might be helpful to review the approximate times the major steps would take. Presentation of an organizational diagram/chart of some sort for steps performed on Day 1, Day 2, Day 3 would be useful.
1.1. The step of removing drug from the freezer has been moved earlier in the protocol (now steps 1.2.1.1 and 1.3.1) to ensure that thawing will not delay time between preparation of the suspension and addition to plates. Dispensing of the plates can indeed be completed within the recommended 15 minute time interval. The following comment has been added in steps 1.2.2 and 1.4 to emphasize the importance of adding the bacterial suspension within 15 minutes of preparation: "Important: perform this step immediately after preparing bacterial suspension so that suspension can be added to the plates within 15 minutes of preparation.[26]"

I defer to the editors about whether an organizational timeline would be appropriate; if so I can prepare this or simply additional insert day and time notations into the protocol as desired.

13. Line 212 - can plate be read manually also?
While it is technically possible to read MICs manually from a 384-well plate, noting and indicating all relevant wells for a synergy grid and transferring these results manually to a spreadsheet is not practical.

14. Line 217 - It would be helpful to indicate if no growth is absolute clarity (or not) since subtle hazes might be encountered. How would discordance between any manual and OD reading be handled? If a goal of this paper is to provide a thorough description of checkerboard testing, notes related to complications that might be encountered should be addressed. This (dependent on goals) might be for colistin/minocycline only or in more general terms. Since the outcome of checkerboard testing is very dependent on slight differences in MICs between single drugs and the combination, knowing how to read endpoints is probably more important than reading individual drug MICs.
The description of the selection of OD_{600} cutoffs for growth vs no growth is further clarified (lines 238-242): "Note: these values were determined based on visual inspection of growth vs. no growth and correlation with OD readings for these experiments; OD_{600} readings from wells containing media alone were consistently below 0.07. Appropriate cutoffs may differ with different plate readers and bacteria."

15. Line 218 - where does one find acceptable QC ranges?
As noted in section 1.2.1.2, the QC ranges are found in the Clinical and Laboratory Standards Institute (CLSI) document M100. The reference is now repeated in lines 244-245 for added clarify.

16. Line 257 - here the negative well has no antibiotic; can a similar negative well be used for QC tests (e.g., Line 153)?
A similar negative well is now recommended for QC (lines 231-232; see response to comment 9).

17. Line 267 - this step could use more explanation since it seems not all wells will be used. (Figure 1A uses 168 wells/isolate and plate contains 384 wells)
This step has been removed as it was not necessary for the protocol described here and added confusion.

18. Line 284 - line 257 states there will be a negative control? Where is the uninoculated well(s)?
The sentence "Add 50 μL of CAMHB without bacteria to an empty well; this will be the negative control well to confirm sterility of the media. Incubate in a 35°C ambient air incubator for 16-20 hours[26]" has been added (lines 290-292).

19. Line 295 - calculation and interpretation of FICs needs further clarification and examples. Isn't FIC the ratio of MIC of agent #1 in the combination well to the MIC of agent #1 alone?
This is correct, but in a synergy grid there are multiple concentration combinations at which growth is inhibited, so there are different FICs depending on which well is considered. The explanation of the FIC and FIC index has been clarified (lines 298-311).

20. Line 300 - need rationale for this recommendation. Many would argue that if the MIC for agent #1 is off scale, one cannot interpret checkerboard synergy results reliably.
The strategy described is a maximally conservative strategy for ensuring that a combination will only be called synergistic if it would be synergistic even if the off-scale drug's MIC is only one doubling dilution above the highest concentration tested. The true FIC_I may in reality be lower than the FIC_I calculated by this method (i.e. it may actually be more synergistic than suggested by the calculated FIC_I), but this technique prevents overcalling synergy. This is now clarified in the discussion section (lines 592-600). Many situations where synergy testing is of particular interest are those in which the MICs for one or both drugs are off-scale, so not interpreting any of these results would significantly limit the scenarios in which synergy testing could be used.

21. Line 302 - need further clarification and examples for FICI. Also, what to do if both synergism and antagonism are encountered within the same combination for an isolate?

Examples and further details of FICI calculation are included in the legends to Figures 1B and 2B, which are referenced in step 1.6.4. Because the examples and figures were changed to an isolate with an on-scale colistin MIC, they should be clearer.

The following sentence has been added to clarify the approach when both synergy and antagonism are found within the same experiment (lines 309-311): "If the combination is synergistic at some concentration combinations but antagonistic at others, note this result but consider the combination overall antagonistic."

22. Line 341 - why not dispense 50 ul broth to columns 2 through 12 and then add 50 ul stock to wells in columns 1 and 2 and serially dilute from columns 2 through 11? Only one pipettor volume (50 ul) would be needed.
We have described a standard serial dilution procedure (now lines 336-338). Users may adjust the procedure as indicated if they prefer to avoid changing pipettor volumes.

23. Line 348 - from experience, it I not necessary to change tips when performing serial dilutions from columns 2 through 11 (must use separate tips for dispensing initial drug to columns 1 and 2). Also, what about mixing contents of well before each transfer step?
It is true that changing tips with each dilution is not necessary for many antibiotics, but we have worked with some for which carryover was problematic. We phrased the sentence ("Pipette tips can be changed between each dilution step to eliminate the possibility of antibiotic carryover", lines 343-344) to indicate that the user may choose whether or not to change tips with each dilution.

The phrase, "Pipette up and down several times to mix the contents" has been added (line 340).

24. Line 361 - what is the targeted final concentration of bacteria/ml in each well?
The target final concentration is $5x10^5$ CFU/mL, as recommended by CLSI; this is now stated in lines 354-355.

25. Line 366 - is 16-20 hours incubation appropriate for all organisms that might be tested against combinations of colistin and minocycline? (again, goals of this paper)
The following sentence has been added (lines 359-361; also added to section 1.2.3.3): "(Note: a different duration of incubation may be required if organisms other than *Enterobacteriaceae* are being tested; consult CLSI M100[25] for organism-specific recommendations.)"

26. Line 368 - here growth is read visually. Line 13 suggests use of an OD reader. Why the difference?
It is unclear which line the reviewer is referring to (line 13 is on the title page; presumably this was a typo). However, the method being described here (now line 362) is broth microdilution in a 96-well plate, whereas the OD reader was used for 384-well plates, where, as noted above, manual reading is challenging.

27. Line 373 - need to explain that log phase is critical when assessing cidal activity.
We now state in sections 2.3.4 and 2.6.1 that the culture must be grown to log phase. In addition, the following text emphasizing importance of this factor is now included in the discussion section (lines 601-608): "In order to accurately assess the bactericidal activity of drugs in a time-kill assay, it is essential that cultures be in logarithmic-phase growth, particularly when cell-wall active antibiotics are being tested.[28] For the rapidly-growing bacteria used in this example (*K. pneumoniae*), 3 hours of incubation with shaking was appropriate to reach this growth phase, but different amounts of time may be necessary for different organisms. In general, the culture should appear visibly but not heavily turbid. The appropriate amount of time can be determined by constructing a growth curve with colony counts taken at serial time points (e.g. every 30 minutes for 4-6 hours).[38]"

28. Line 374 - could screw capped tubes be used if only horizontal shaker is available?
The procedure described here is for a horizontal shaker – the tubes are placed in a test tube rack. The use of a test tube rack is now explicitly stated (lines 373 and 410). Tubes with slide on caps (metal or plastic) are far more efficient to use for multiple samples as they do not fall off as screw on caps tend to do with rotary motion (requiring them to be taped to the tube) and do not have to be unscrewed for sampling, which increases the manipulation required and chance for contamination; hence, our recommendation.

29. Line 378 - do tubes need any special washing/treatment?
The tubes need to be sterile; the word "autoclaved" has been added (now line 379).

30. Line 382 & 386 - it is unclear why authors are saying 1 ug/ml colistin and minocycline are "ineffective individually"? More thorough recommendations for selection of drug content is needed.
The text has been changed (lines 382-34) to explain how individually ineffective concentrations should be determined: (Note: For a synergy study, at least one drug should be a concentration that does not affect the growth curve individually;[30] this can be determined by evaluating the effects of individual drug concentrations prior to the synergy study.)" In addition, the choice of concentrations for this experiment has been clarified with the following phrase (lines 385-386, 389-390): "…as this is a concentration that is ineffective against the strain being used in this example."

31. Line 391 - here authors discuss "sterility" control and above "negative" control.
The term "sterility control" has been changed to "negative control" here (now line 394) to follow the pattern of the previous wording.

32. Line 393 - is it necessary to describe how to make sterile normal saline? If yes, shouldn't reagents be prepared during initial organizational steps?
We agree that it is likely not necessary to describe how to make sterile normal saline, and have removed these instructional steps.

33. Line 400 - would the 3 hours differ for different species? (goals of this paper) Is there any visual indicator that culture is at log phase? If tube has a stainless steel closure (line 374), can this tube be safely vortexed vigorously?
The sentence (now lines 398-399) now reads "Once the initial culture has reached logarithmic growth phase (~3 hours *for Klebsiella pneumoniae*, the organism used in this example)…". In addition, the following text has been added in the discussion section (lines 601-608) to explain how to determine the appropriate time for incubating different organisms: "In order to accurately assess the bactericidal activity of drugs in a time-kill assay, it is essential that cultures be in logarithmic-phase growth, particularly when cell-wall active antibiotics are being tested.[28] For the rapidly-growing bacteria used in this example (*K. pneumoniae*), 3 hours of incubation with shaking was appropriate to reach this growth phase, but different amounts of time may be necessary for different organisms. In general, the culture should appear visibly but not heavily turbid. The appropriate amount of time can be determined by constructing a growth curve with colony counts taken at serial time points (e.g. every 30 minutes for 4-6 hours).[38]"

Vigorous vortexing is not recommended; as stated throughout the procedure, glass culture tubes should be vortexed gently.

34. Line 406 - Does this suspension require mixing before colony counts?
The phrase "and vortex gently" has been added to this line (now line 404).

35. Line 409 - - how do you suggest removing 100 ul from 10 ml broth in a 25 x 150 mm tube? What kind of pipettor/tips since these are very long tubes?
We remove the aliquot by tilting the tube to the side so that only the sterile pipette tip and not the non-sterile portion of the pipettor shaft enters the tube; this is now stated in lines 407-408 ("…remove a 150 μL aliquot from each culture tube by tilting the tube so that only the sterile pipette tip enters the tube and not the unsterile pipettor shaft during aliquot withdrawal.") The appropriate technique will be demonstrated in the video to make this technique extremely clear.

36. Line 415 - would it be more efficient to transfer aliquots to agar plate during preparation of next serial dilution (saves tips and time)? E.g., remove aliquot to prepare next dilution. Then remove another aliquot from same well to inoculate agar. Need separate tips for each dilution.
[Now line 416] The technique described by the reviewer would not be feasible, as the volume transferred for dilution is 100 μL, whereas the volume used for plate count drops is 10 μL. As described in our methods currently, the technique is efficient because a multichannel pipettor can be used for making dilutions, and a separate multichannel pipettor can then be used to dispense an entire column of dilutions onto the agar plate at once.

37. Line 419 - is there a reason "Mueller Hinton" is the recommended agar? It is often easier to count colonies on opaque media (e.g., BAP).
[Now line 418] Mueller-Hinton agar is translucent, so plate counts can easily be marked directly on the reverse of the plate. The instruction "Mark colonies with a fine-tip permanent

marker on the reverse of the plate if desired to avoid double-counting or missing colonies" has been added to section 2.9 to indicate how the Mueller-Hinton plate is used in counting.

38. Line 420 - are 3 rows of wells A-H or 24 spots dispensed to each small MHA plate? Should spots be plated in duplicate since this method of colony counting is rather crude?
The contents of the 3 rows are now defined more clearly in line 422 ("each consisting of drops from rows A-H of a single column"). We have seen almost no variability between replicates taken immediately in succession from the same wells using this method, so do not typically perform the drop counts in duplicate.

39. Line 446 - would it be helpful to subculture initial inocula to BAP where it is easier to observe a potential mixed culture? Also, what about inoculum sterility checks for checkerboard assay? So much is invested in these procedures (and so many steps cannot be controlled) an additional step to more thoroughly control inoculum purity would be wise.
We agree that a starting inoculum purity check is reasonable and have added this to the time-kill assay procedure in steps 2.3.3 (inoculation) and 2.9.1 (plate check), and also to the checkerboard assay procedure in steps 1.2.1.7 (inoculation) and 1.6.1 (plate check).

40. 463 - how does one determine if a different dilution is needed?
A sentence explaining how the appropriate dilution can be determined has been added as follows (lines 614-617): "The appropriate dilution required for a given species can be determined by performing a plate count of a 0.5 or 1.0 McFarland suspension to determine how many organisms this turbidity represents, then calculating the amount by which the initial suspension must be diluted to reach the appropriate final concentration."

41. Line 521 - Figures. Could growth be red and no growth green (seems more intuitive that red would be growth)? It would be helpful to describe the important MIC values/wells (single drugs and critical combinations of concentrations) and highlight them individually during the video.
Because the intuitive sense of which color represents growth will likely vary among readers (for example, some may think of red as stop (no growth) and green as go (growth), based on common usage with traffic signals), we do not feel there is a specific reason to change the color key; the key is clearly presented at the bottom of the figure. The MIC wells are now highlighted in bold, as suggested.

42. Line 530 - see suggestions for description of FIC above
This example is now of an organism with an on-scale MIC, as recommended above, and only wells with growth inhibition are included within the bordered box, as suggested below. We anticipate that these changes will make the explanation of the FIC clearer.

43. Line 536 - it would be helpful to discuss minimum FICI above
The minimum FIC_I was discussed in section 1.6.6, described as "the lowest FIC_I at which growth is inhibited". The term "minimum FIC_I" has now been added to this section.

44. Line 534 - it is confusing to have both growth and no growth wells included in the large thick-bordered box.
This box (now bordered in a broken line) has been changed to include only no-growth wells.

45. Line 552 - Combinations selected in this example are peculiar (see above) and I assume these are real results that were obtained? It would be helpful to explain rationale for these choices of drug combinations.
These are real results from published work (PMID 30061285), as noted in response to "Major Concerns" question #9 above. The specific example was chosen because it presented a particularly clear example of synergy. The rationale for the selection and our hypothesis as to its mechanism of action are now explained in the introduction (for the combination of colistin plus minocycline; lines 93-97) and in the Figure 4 legend (for the three combinations shown in this figure; lines 545-554).

46. Line 568 - CLSI does NOT have any procedure for checkerboard or time-kill synergy studies
This was an error in the text – the references were correct but the text described the Clinical Microbiology Procedures Handbook as being published by CLSI rather than by the American Society for Microbiology. This has been corrected (lines 558-561). (Of note, in its M26-A publication, "Methods for Determining Bactericidal Activity of Antimicrobial Agents," CLSI does provide definitions for the interpretation time-kill synergy studies).

47. Line 615 - it is generally not recommended for fosfomycin to be tested in a broth medium. There are multiple problems testing fosfomycin by itself which may be confounded further when testing this agent in combination. Enterobacter species often demonstrate heteroresistance to colistin and this could account for multiple skips.
The following comment has been added (lines 584-586) to make note of the issue with testing colistin in broth dilution: "Of note, CLSI does not recommend testing fosfomycin in broth dilution[25] due to concerns about the reliability of results with this method, which may explain the unreliable results seen with this drug." Because multiple skips were rare with colistin, we have not added discussion of heteroresistance in this context.

48. Reference list - may require modification based on goals of this paper
References have been added/removed in accordance with text that has been added/removed. As the emphasis in the introduction and discussion sections is now more focused on procedures and on limitations of the methods described, the references have changed accordingly.

49. Materials - add multichannel pipettor; other drugs used in time kill
The multichannel pipettor has been added to the materials list. The drugs used in the time-kill study are the same as those used in the checkerboard array.

Reviewer #2:
Manuscript Summary:

Although it is an interesting article about new robust, reproducible and automatable antimicrobial activity methods is very important include clsi guidelines for assay validation data

Major Concerns:
The following information about this new method consigned in the CLSI C60 protocol is required: Assay Validation & Post-Validation Monitoring as follow
Limits of Quantitation:
* Linearity and Dilution
* Imprecision
* Assay Interferences
* Accuracy
Also is necessary include the parameters consigned in protocol CLSI C24, Statistical Quality Control for Quantitative Measurement Procedures: Principles and Definitions, for calculate the systematic error

Likewise, the parameters recorded in the CLSI User Protocol for Evaluation of Qualitative Test Performance; Approved Guideline-SecondEdition CLSI document EP12-A2. for this method are not known.
The CLSI C60 document ("*Blood Alcohol Testing in the Clinical Laboratory; Approved Guideline*") is not applicable to the procedures presented in this article. More generally, however, the various CLSI documents for test validation are designed for evaluation of tests and procedures being used on patient samples in a clinical microbiology laboratory. The techniques in our article are research methods and are in no way presented as validated to the level needed for direct introduction into a clinical laboratory. Furthermore, we are describing methods in the article, not presenting a test to be approved for clinical use. Should any viewer or lab ever wish to attempt to adapt the tests presented here for any type of clinical use, they would have to perform full validations, establish and verify appropriate clinical parameters, etc within their lab. That is not the purpose of the methods article presented here.

Minor Concerns:
-Please include the comparison and validation of this method against CLSI M26 and checkerboard methods using the different quality control strains
CLSI M26 does not provide quality control minimum bactericidal concentration (MBC) values for antibiotics presented here or for any antibiotics in their respective classes (tetracyclines and polymyxins) and does not provide any quality control reference ranges for synergy testing, either by time-kill or checkerboard array. Indeed, there are no established quality control guidelines for synergy testing. As now noted in the introduction, this is an acknowledged limitation of synergy testing methods at present. (Quality control testing of antimicrobial stocks, however, is presented in detail in our paper.)