**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. **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 OD600 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; OD600 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 hours26”** **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 FICI may in reality be lower than the FICI calculated by this method (i.e. it may actually be more synergistic than suggested by the calculated FICI), 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 5x105 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 M10025 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 FICI was discussed in section 1.6.6, described as “the lowest FICI at which growth is inhibited”. The term “minimum FICI” 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 dilution25 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.)**