Editorial and production comments:
NOTE: Please include a line-by-line response to each of the editorial and reviewer comments in the form of **a letter** along with the resubmission.

Editorial Comments:

• **Title:** Please remove the superfluous words "Protocol for the" from the title.

Reply: We changed the title accordingly.

• **Text Overlap:** Please re-write lines 75-78, 109-115, 140-157, 178-183, 185-189, 302-307 to avoid overlap with previous publications.

Reply: We thank the editor for the note and modified the text accordingly.

• **Protocol Detail:** Please ensure homogeneity between the video and text. Make sure all details mentioned in the video are present in the text.

Reply: We ensured that all described steps in the video match the text in the protocol.

• **Protocol Numbering:** Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. Please add a one-line space after each protocol step.

Reply: Done.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Reply: The discussion was modified according the Jove instructions.

• **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are (PS-DNase) (Epicentre), ChemiDoc Gel
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

Reply: Done.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/**website**) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Reply: No particular permission needed see provided link:
https://www.nature.com/nature-research/reprints-and-permissions/permissions-requests#permission-requests-from-authors

• **Video Comments:** 11:28-11:49 has a thin black border at the top and needs to be adjusted slightly to fill the frame. Please upload any new versions of the video here and be sure to include the article number in the filename:
https://www.dropbox.com/request/5LvqaE4ZOCiHwyLda6ey?oref=e

Reply: We removed the black border in the movie.

Comments from Peer-Reviewers:

Reviewer #1:
Manuscript Summary:
Wein et al. present a protocol for constructing a model plasmid carrying an antibiotic resistance gene, performing an evolution experiment during which the antibiotic resistance of cells in the population is monitored, and characterizing the plasmid population by gel electrophoresis. There is a lack of knowledge generally about how plasmids, particularly those that mediate multidrug resistance, persist within bacterial populations. We could learn a great deal if the approach illustrated here was applied to other systems by users of this protocol. Monitoring of changes in multimerization and supercoiling are, in particular, not included in many such studies.

Major Concerns:
1. Abstract: The abstract should mention the overall purpose and importance of what other scientists could learn from employing this protocol. Right now the abstract and the rest of the manuscript seem too narrowly focused on the authors' specific observations in their Nature Communications paper. Those are worth mentioning, for sure, but there are many other potential outcomes that could be monitored with this protocol. For example, it could be used on a newly isolated plasmid that is giving multidrug resistance, in which case one could skip Step 1.

Reply: We thank the referee for this valuable comment and modified the abstract accordingly (lines 53-54). We now included the information about studying natural antibiotic plasmids (omitting step1) in the introduction (lines 98, 127) and discussion (lines 388-390). Indeed, we mention that the protocol can be applied for the study of plasmid evolution and persistence in general, including other plasmids or other mobile genetic elements (lines 42-43).

2. Bottleneck size and duration of experiment. Lines 177-188: There is no explanation for why three different bottleneck sizes are employed. Why these? Are they necessary? I would want someone using this protocol to understand how increasing or decreasing the dilution factor would be expected to change the evolutionary dynamics (e.g., increasing the dilution factor will favor chance in evolution and be expected to give a wider variety of mutant plasmid outcomes but they may be less optimal). There should be probably be a bacterial experimental evolution review cited for this point. Setting 98 transfers as the length of the experiment also seems highly arbitrary. Wouldn't one want to potentially adjust that depending on how evolution progresses?

Reply: We agree with the referee and modified the protocol (lines 117-118, 199-200,) to explain the bottleneck treatment. The transfers are not arbitrary as after 98 transfers, one bottleneck treatment reaches 1000 generation. We decided to not include this information in this manuscript/protocol yet note that the number of transfers depends on the experimental design (line 223-224). Indeed, a reference to a detailed review on evolution experiments is in place and we added that in that point.

3. Lines 98-103: More explanation of what plasmid multimers are and the effect of nicking on supercoiling and gel mobility is likely going to be needed for most readers to understand what is going on here. The authors might illustrate the different plasmid forms next to the gel figure or find one or more citations to go along with a more detailed explanation they add in the text.

Reply: We thank the referee for this comment. We now included a more detailed explanation in the introduction (lines 106-115). Thanks also for the suggestion to include plasmid symbols for the explanation of plasmid multimers. We now added the symbols in Figure 5 (and in the movie).

4. It is my understanding that representative results section should explain in much more detail the example data that are being shown in Figures 4 and 5 so that someone using this protocol would be able to interpret a curve of antibiotic resistance over time or patterns of plasmid bands on the gels. The current version only comments at a very high level on the meaning of the data. This reads as more of a discussion of the results.

Reply: We thank the referee for that comment. In the current version we modified the text to include more information about the representative results of our study (lines 313-315) and furthermore point the reader to our study for more details.

5. Figure 4: What is the transfer dilution factor for the results shown here? The pulse of antibiotic shown in this figure and why one would add that treatment is not explained in the description of the method, so it may be confusing to readers.

Reply: We thank the referee for this comment, this information was indeed lacking. We now included a sentence in the results to explain the antibiotics treatment (see lines above).

6. Figure 5: Results are shown for the pCON plasmid given the enzymatic treatments shown in the text. The bands are not labeled in terms of what molecular species they represent. I think it's essential in this figure that an evolved plasmid with known changes in multimer state, etc., is shown alongside the pCON plasmid, so that someone using this protocol would know better how to interpret changes in the banding patterns they observed.

Reply: Right. The symbols have been added to that figure. We note that our protocol is focused on the execution of steps in the experiment while for details on the outcome of our specific experiment we point the reader to our Nat Comms publication.

Minor Concerns:
Line 150: Probably should be edited to "remove supernatant, and resuspend in" instead of "dilute in" for clarity of what is happening.

Reply: Done.

Line 235: Abbreviating Open Circles as OC here seems unnecessary. The term "Open Circles" is not used in Figure 5, but should be to connect the text better with the expected results.

Reply: Done.

Reviewer #2:
Manuscript Summary:
This is a neat succinct article describing several useful protocols applied to studying plasmid evolution - Introducing marker genes into a plasmid, Experimental evolution, and a method for visualising plasmid architecture. These are all really useful methods that it will be helpful for people to have to hand, and also very relatively low cost approaches which I imagine is perfect for this kind of format.

Major Concerns:
I have no major concerns.

Reply: Thank you!

Minor Concerns:
I have a few minor comments:
- it should be noted that numerous people have demonstrated plasmid persistence though evolution in the absence of selection. These should be cited.

Reply: We agree with the referee and added references work by others in the introduction (line 85-86).

- In section 1 it would be good to describe the selective media that the electroporated bacteria were plated onto to make sure this process is successful. Obviously be not necessarily for someone trying this for the first time.

Reply: We agree with the referee. Indeed, we mention the plating on selective media following electroporation in lines 157-160.

- The link with the visualization of plasmid conformation isn't followed through that well. Can the authors share some results from their evolution experiment to show this changed over time. I think it would be more cohesive and more interesting if figure 5 included some results in the manor of figure 4.

Reply: We modified the text accordingly. Please see our reply to R1's comments.