August 9, 2016

**Re:** Revisions to JoVE Manuscript 54854\_R1\_050416

To Whom It May Concern:

Thank you for your consideration of our manuscript, “Conjugative Mating Assays for Sequence-Specific Analysis of Transfer Proteins Involved in Bacterial Conjugation”, for publication in the *Journal of Visualized Experiments* (JoVE). We would also like to thank the editor and reviewers for their excellent comments and insights. We have extensively revised our manuscript as per the reviewer comments, which are outlined below. In the process of revising our manuscript, we have also carefully reviewed and revised any grammatical errors. Several points in particular were noted by the reviewers:

**Editor**

1. The revised document has been revised using track changes as requested.
2. Grammar and Spelling – we have taken the opportunity to carefully review the spelling and grammar during our revisions as noted by the editor. Thank you.
3. We have removed the italics for “gene” as requested, confirmed that all degrees are denoted by the “degree” symbol and included DOI for references where possible.
4. We have changed 3.1.8 to a note as it is more appropriate that way and changed instances of “your/you”
5. We have removed highlights from the sections/lines noted and included in highlight the counting and calculation steps.
6. Additional details requested – plasmids have been cited, both in the text and Table 2; we have include example thermocycler settings as a note under protocol 1.2.7.3; we have included antibiotic concentrations where appropriate in the text of the protocol.
7. We have included our copyright permissions as requested.
8. Please note that we have numbered out tables as follows: Table 1 – Materials, Table 2 – *E. coli* strains and plasmids used in this study, Table 3 - A list of primers used in this study, and Table 4 - Abolished mating efficiency by TraF deletion construct. Please advise if this is not as the tables would be viewed in the final publication as we can renumber accordingly.

**Reviewer 1**

* We thank the reviewer for their excellent comments and insights. We have rewritten the introduction to be more general in terms of being more “big picture” as requested while introducing the F plasmid as an example of a larger conjugative plasmid (thank you indeed for this comment, it makes the framing of the protocol more approachable). As we note in our response to Reviewer 2 below, this manuscript has been written to make these methods more approachable for researchers who are coming to conjugative systems from a more structural standpoint and may not have the extensive background of plasmid biology as other groups.
* Regarding Reviewer 1’s specific comments:

1. We have amended the statement on line 119 as requested.
2. The strain used is DY330R – We apologize for the confusion and included strain DY300 for completeness in the original submission. We have clarified this in the text and Table 2 as requested.
3. The recombineering protocols as noted for DY330R – We apologize for the misprint of 37 °C temperature versus 32 °C as noted by the reviewer. We have also included a note after step 2.1.7 as requested regarding growth conditions of DY330R.
4. Selective markers - We thank the reviewer for highlighting this issue. We agree with the reviewer that inclusion of extra selective pressure is unduly challenging to the cells, have rechecked all our experiments to confirm that the extra selective pressures are not required and revised our complete protocol accordingly.
5. Figure 1 – We have revised the figure as requested to be less confusing to the reader.

**Reviewer 2**

* We thank the reviewer for their time and efforts in review of our manuscript. In response to their concern regarding existing protocols, we do agree that many of the protocols are available. However, the purpose of our manuscript was to provide a description of the conjugative mating assay for coupling to structural studies such as X-ray crystallography, which is the major focus of our lab. The conjugative mating assay may be more routine for microbiology groups, in particular those that are well-versed in plasmid biology. However we have found that a detailed protocol, such as a JoVE article, would be of significant benefit for labs such as ours, whose main focus is structural studies of conjugative systems and wish to couple these analyses with functional studies of the component proteins. Accordingly we believe that our manuscript provides benefit for the scientific community.

**Reviewer 3**

* We thank the reviewer for their efforts and excellent comments, and have addressed them as noted below:

1. We have revised the text noted for lines 59, 64, 71 and 627 as requested.
2. Regarding the text referring to “connecting the cytoplasms of donor and recipient cells”. We agree that it is indeed still unclear if the mating pair formation complex assembled by the conjugative T4SS does indeed physically connect the respective cytoplasms via a lumen of the pilus. Although Silverman’s group noted during the cryo-EM analysis of the F-pilus (Wang et al. J. Mol. Biol. (2009) 35, 22-29) that the transfer of DNA was likely through the lumen of the pilus based on the calculated size of the pilus lumen, which is where our original statement originated, a clear connection of the donor and recipient cytoplasms has yet to be established. We have therefore revised our language accordingly to remove this statement/inference.
3. Lines 83-84 – we have revised our text as noted by the reviewer regarding high mutation rates and conjugative mediated evolution.
4. We have revised our introduction and discussion to clearly indicate that the methods described in the protocol are for larger conjugative plasmids. We agree with the reviewer that the Smillie et al. (2010) paper provided an excellent discussion of mobile plasmids and that they identified a large range of sizes in mobile plasmids. We include this in our introduction and discussion, and more clearly articulate that the protocol we describe, in particular the homologous recombination portion, are for these larger conjugative plasmids that are less amenable to standard recombination methods as applied for smaller plasmids.

Thank you again for your consideration of our manuscript for publication in *JoVE*. If there are any questions or concerns with the manuscript, please do not hesitate to contact me by email at audette@yorku.ca.

Sincerely,

Gerald F. Audette