We addressed the points that were raised by editors and reviewer with details as below.

**Editorial comments:**  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.  
  
2. Please provide an email address for each author on the first page.  
  
3. Please define all abbreviations before use.  
  
4. Please use SI units, e.g. please use “µL” instead of “µl”.  
  
5. 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. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.  
  
6. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. 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.  
  
7. 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.  
  
8. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. 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: ECM-630 electroporation.  
  
9. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.  
  
10. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.  
  
11. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.  
  
12. Protocol: 1.1: “Streak out”? How? Using what? How is the plate warmed? At which temperature? If it should be kept for one day, what is the keeping conditions? Please describe the step clearly or refer to an appropriate protocol or reference.  
  
13. Protocol: 1.2: How to pick a single colony? Using what? “inoculate”, How?  
  
14. Protocol: 1.3: Shake for how long? How long is needed to reach mid-log phase? Please provide a quantitative measure.  
  
15. Protocol: 1.4: How to prepare? In which container? Please describe clearly or refer to an appropriate protocol or reference.  
  
16. Protocol: 1.5: How is mixing done? In which container? Incubate at which temperature?  
  
17. Protocol: 1.6: How to mix? Incubate for how long?  
  
18. Protocol: 1.7: “Pour”, How? Using what?  
  
19. Protocol: 1.8: Please revise the step to make it clear that there is only one plaques in each tube.  
  
20. Protocol: 1.10: How that is done? For how long is that incubated?  
  
21. Protocol: 1.11: Swirling by hand?  
  
22. Protocol: 1.12: How is the transfer done?  
  
23. Protocol: 1.14: How? In which container?  
  
24. Protocol: 1:15: “gently stir” by hand? Otherwise, what is the speed?  
  
25. Protocol: 1.17: “without combining the pellets”, how?  
  
26. Protocol: 1.18: How to remove the stir bar?  
  
27. Protocol: 1.19: Are all accumulated in one bottle at the end of this step?  
  
28. Protocol: 1.20: Using pipette?  
  
29. Protocol: 1.21: How?  
  
30. Protocol: 1.22: Please use proper numbering if next steps are sub-part of this step, i.e. 1.22.1, 1.22.2, etc.  
  
31. Protocol: 1.23: How the step is done?  
  
32. Protocol: 1.25: Please avoid using commercial language, please use generic terms instead.  
  
33. Protocol: 1.26: Please define pre-warmed. What is the temperature? “Rinse”, how?  
  
34. Protocol: 1.27: Incubate what?  
  
35. Protocol: 1.28: Please use Jove style for numbering the sub-steps.  
  
36. Protocol: 2: If the text is a Note, please indicate it as a Note. Otherwise, please move it to Discussion.  
  
37. Protocol: 2.1: Please revise the step and make it clear of how to do the step.  
  
38. Protocol: 2.3: How? In what?  
  
39. Protocol: 2.4: Please describe the step clearly.  
  
40. Protocol: 2.5: How? Please describe the step clearly.  
  
41. Protocol: 2.11: “centrifuge briefly”, please provide a quantitative measure.  
  
42. Protocol: 2.13: Please use Jove style for numbering the sub-steps. Please ensure you answer the “how” question, i.e., how is the step performed?  
  
43. Protocol: Please revise the numbering in 2.  
  
44. Protocol: 2.13 (second 13): “Add”, “mix”, How?  
  
45. Protocol: 2.11 (second 11): Please avoid using commercial language, please use generic terms instead.  
Centrifuge at which temperature? “Discard”, how?  
  
46. Protocol: 2.12 (second 12): How? Please describe the step clearly.  
  
47. Protocol: 3: Please move the discussion to the Discussion section.  
  
48. Protocol: 3.1.1: Please use the imperative tense.  
  
49. Protocol: 3.1.2: Incubate what?  
  
50. Protocol: 3.2.1: Please use the imperative tense. Steps should include an action.  
  
51. Protocol: 3.2.2: Please describe the step clearly.  
  
52. Protocol: 3.3.1: Please revise the step. If referring to a Table, please clearly mentioned the Table number. Please do not embed any Table in the Manuscript. Please follow JoVE policy.  
  
53. Protocol: 3.3.2: Incubate what? In which container?  
  
54. Protocol: 3.3.3: Please avoid using commercial language, please use generic terms instead.  
  
55. Protocol: 3.3.4: How? Please describe the step clearly.  
  
56. Protocol: 3.3.5: Please avoid using commercial language, please use generic terms instead. Please describe the step clearly.  
  
57. Protocol: 3.3.8: How is the transfer is done? Using what?  
  
58. Protocol: 4: Please revise the numbering.  
  
59. Protocol: 4.1: How?  
  
60. Protocol: 4.2: How?  
  
61. Protocol: 4.2 (second 2): How?  
  
62. Protocol: 4.6: Please use JoVE style for numbering the sub-steps. Please describe the steps clearly.  
  
63. Protocol: 4.14: How?  
  
64. Protocol: 4:15: Please describe the step clearly.  
  
65. Protocol: 4:15 (second 15): How?  
  
66. Protocol: 5: Please move the discussion to the Discussion section.  
  
67. Protocol: 5:3: Add with what? Pipette?  
  
68. Protocol: 5.4: Please describe the step clearly.  
  
69. Protocol: 5.6: Please describe the step clearly.  
  
70. Protocol: 5.7: How?  
  
71. Protocol: 5.8: “Remove”, How?  
  
72. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.  
  
73. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”  
  
74. Please upload each Figure individually to your Editorial Manager account as a .png or a .tiff file. Please combine all panels of one figure into a single image file.  
  
75. Please revise the Figures numbering. Currently, Figure 4 is mentioned after Figure 6.  
  
76. Figure 2: Please describe clearly the Figure in the legend. What is each panel? Please describe each row. What each circle is presenting? Please add a scale bar.  
  
77. Figure 5: Please describe clearly the Figure in the legend. Please add a scale bar.  
  
78. Figure 7: Please describe clearly the Figure in the legend. What is each row? Please add a scale bar.  
  
79. Figure 8: Please remove panel (B) and change it to a Table and number it accordingly.  
  
80. Please revise the Representative Results as well as Discussion. Some parts of current Discussion section can be presented in the Representative Results.  
  
81. Representative Results: Please discuss all figures in the Representative Results. However, for figures showing the experimental set-up, please reference them in the Protocol.  
  
82. Representative Results: Please explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.  
  
83. Discussion: 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  
84. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.  
  
**Response:**

**Based on editor’s valuable comments, we modified our manuscript to address each point as listed above in the revised manuscript.**

**Reviewers' comments:**  
**Reviewer #1:**  
Manuscript Summary:  
This provides an overview protocol for the creation of synthetic phage antibody libraries.  
  
Major Concerns:  
This protocol paper reiterates much that has been previously published while missing essential details that are to be found in other papers. In particular, nothing is said about the antibody scaffold used, the strategy behind antibody library creation (e.g. which sites are to be mutated, with what diversity and why) and the sequences of mutagenic oligonucleotides. As it is this publication is very superficial.

**Response:**

**Based on the valuable comments, we added a lot of essential details of experiment. Sites to be mutated and sequences of mutagenic oligonucleotides are provided in Table 3.**

**Reviewer #2:**  
Manuscript Summary:  
The most part of this manuscript is very clear, the protocol is reasonably easy to read. There are a few things to be fixed.  
  
Minor Concerns:  
1. The steps numbering in the section 2 is problematic, it is 11 again after 13

**Response:**

**Corrected.**

2. This is an experiment protocol type of paper, it would be better if the authors can list the expected yield of each step. For instance, normally how much ss DNA from section 2, how much ccc DNA from section 3.

**Response:**

**Yields of ss DNA, ccc DNA and titer of expected electro-competent cells are provided in corresponding sections.**

3. In section 3, the authors use "trimer codon phosphoramidite-based oligonucleotides". However, it is not clear that how this is designed. It would be helpful to illustrate this design to justify the "advantage" over NNK.

**Response:**

**Sites to be mutated and sequences of mutagenic oligonucleotides are provided in Table 3. Design of diversity is a combination of our experience and published literature. The advantage of trimer codon phosphoramidite-based oligonucleotides over NNK is cited in the text.**

4. Again in section 4, what is the normal efficiency to expect? To generate a reasonable sized library, how many transformations are needed? Are they all going to inoculated for shaking overnight?

**Response:**

**The normal efficiency per one electroporation is from 1-10 x 109. Depending on the expected library size and theoretical library diversity, time of electroporation can be increased and thus library size will be increased correspondingly. For phage library generation, in our experience, it is needed to be shaking overnight.**

5. The phagemid backbone seems to have only a regular promoter. In section 4, shaking the transformant directly overnight may cause certain bias. How do the authors address this issue?  
  
**Response:**

**The normal efficiency per one electroporation is from 1-10 X 109. Depending on the theoretical library diversity and expected library size, time of electroporation can be increased and thus library size will be increased correspondingly. For phage library generation, in our experience, it is needed to be shaking overnight, otherwise phage cannot be generated efficiently. It is possible to cause bias from overnight culture. However, sometimes, the bias may favor the selection of Fab phage clones that have good expression, good solubility and correct folding.**

**Reviewer #3:**  
Comments and Suggestions  
1. The first report of use of an oligonucleotide to direct mutagenesis of a residue in a single-stranded bacteriophage virus is Hutchison CA, Phillips S, Edgell MH, Gillam S, Jahnke P & Smith M (1978) Mutagenesis at a specific position in a DNA sequence. J Biol Chem 253, 6551-6560.

**Response:**

**The citation is added in introduction section.**

2. The first report of using more than one oligonucleotide to direct mutagenesis of multiple DNA sequences in bacteriophage is Weiss GA, Watanabe CK, Zhong A, Goddard A & Sidhu SS (2000) Rapid mapping of protein functional epitopes by combinatorial alanine scanning. Proc Natl Acad Sci U S A 97, 8950-8954.

**Response:**

**The citation is added in introduction section.**

3. High-efficiency electrocompetent SS320 cells are now commercially available (http:/ /www.lucigen.com/Phage-Display-Competent-Cells/#subcat-tabs1).

**Response:**

**Yes, the high-efficiency electro-competent SS320 cells are commercially available. However, in this protocol, we prepared M13KO7 pre-infected electro-competent SS320, which is not commercially available.**

4. Please list the genotype of SS320 bacteria.

**Response:**

**The genotypes of SS320 and CJ236 are added in Table 1.**

5. Why start 8 cultures and only use one (i.e.,112-115)?

**Response:**

**Corrected and addressed in the text (See lines 145-147).**

6. Line 115: "in to" should be "into"

**Response:**

**Corrected (See line 149).**

7. Why grow 115 mL of cells (line 115), when only 15 mL will be used in the next step (line 117)?

**Response:**

**Corrected (See lines 149-152).**

8. We find that DNA that has been spot dialyzed gives more transformants in electroporation.

**Response:**

**Thanks for sharing the experience. In a similar way, we found that the yield and transformants in electroporation can be increased by using centrifugal filter device for dialysis and extraction of Kunkel reaction products (See lines 345-350).**

9. Is uracil added to the culture medium for CJ236 cells?

**Response:**

**Yes, uracil is added to the culture medium of CJ236 for generation of dU-ssDNA.**

10.What are the recipes of MP (line 208) and PBS (line 337)?

**Response:**

**The recipe of MP is unknown and is from a commercial kit. The recipe of PBS is added in Table 2.**

11. Do the authors encourage users to bias CDRs in favor of certain amino acids?

**Response:**

**Yes, we prefer to bias CDRs in favor of certain amino acids. This may increase the Fab solubility and thermostability. This also depends on the purpose of synthetic phage library.**

12.We monitor conversion of single-stranded into CCC DNA during in vitro replication by agarose gel electrophoresis.

**Response:**

**Yes, conversion of single-stranded into CCC DNA during in vitro replication is monitored by agarose gel electrophoresis (See Figure 6 and Line 303 and 354).**

13. One can also use template DNA than contains stop codons in the CDR coding regions to eliminate expression of non-recombinants in the library.

**Response:**

**Yes, stop codons in the CDR coding regions can avoid the wild-type display in the library. But we found that the template without stop codon can keep the clones with 1, 2, or 3 CDRs replaced by the mutagenesis primers, which may be also functional against some antigens in phage panning.**

14.What is the benefits of creating an immunosandwich with proteins A and L, versus using just one and anti-Flag Ab (assuming the Flag epitope is part of the Fab)?

**Response:**

**Protein A and protein L can bind to the correctly folded light chain and heavy chain of the antibody template we used. Therefore, using proteins A/L ELISA can ensure the folding of light chain and heavy chain of Fab displayed. Anti-Flag can also be used as Flag epitope is part of the Fab vector. However, Flag epitope is located at the C-terminal of light chain and it is possible that the Fab is not well folded but it still be recognized by anti-Flag antibody and may cause false positive.**

15.Why not have the box for electrocompetent cell preparation on the side of Figure 1?

**Response:**

**Corrected in Figure 1.**

16.Figures 2, 5, and 7 are not obvious without cartoons or text accompanying them.

**Corrected in Figure 4, 5 and 7. Legends are provided in the text.**

17. What is meant by "strand displaced DNA" in Figure 6?

**Response:**

**Strand displaced DNA is an inherent activity of T7 DNA polymerase and citation is added in text (lines 612-614).**

18.It is hard to see what components are in what buffer without separating lines.

**Response:**

**Corrected in Table 2.**

19.Please provide more information regarding the mutagenic oligonucleotides (i.e., length, sequence, T m , purity, etc.).

**Response:**

**Sequences of mutagenic oligonucleotides are added in Table 3.**

**Reviewer #4:**  
This manuscript entitled "Construction of synthetic phage displayed Fab library with tailored diversity", Huang et al. described a protocol for preparing phage-displayed synthetic antibody library with tailored diversity. The authors achieved Fab diversity of 5x109.  
Overall, the manuscript is well written and the experiments were carefully designed. A good amount of work was done in describing the protocol in depth. The manuscript fits within the scope of the journal. However, a minor revision is necessary to improve the overall quality of the manuscript. Specific comments and suggestions are enumerated below:  
1. Lane 104, indicate estimated time to reach OD=0.8, for example - 5-7 hrs, etc.

**Response:**

**Added in line 117.**

2. Lane 115, did you mean 2YT/kan/tet medium?

**Response:**

**Corrected in line 149.**

3. In the 'prepared solution' section 'superbroth medium salt' is redundant and can be deleted.

**Response:**

**Corrected in Table 2.**

4. Lane 115, would you estimate 'fastest-growing culture' by OD or eye-balling?

**Response:**

**Corrected in line 149. Growing is visualized by eye that the culture will become turbid. OD monitor is not used in this step.**

5. Starting lane 141, add a subheading (such as evaluation of electrocompetent cells)

**Response:**

**Based on the journal format, modification is made from lines 207-221.**

6. Electrocompetent cell preparation: A high aeration is preferred (~ 250 rpm) whereas the authors used 200 rpm. Secondly, single use aliquots of cells (in 50-100ul) are preferred than what was used in the study.

**Response:**

**200 rpm is routinely used in our preparation. Thanks for sharing the experience. We found that 250 rpm sometimes can cause noise within the shaker. To get good aeration, baffled flasks in our experience have to be used. 350 µL per aliquot of competent cell are used to for electroporation within 0.2-cm gap electroporation cuvette. Small volume of electroporation cells (50-100 µL) can be used within 0.1-cm gap electroporation cuvette.**

7. Lane 147, indicate the volume of cell-DNA mixture to be transferred to cuvette.

**Response:**

**The information is added in line 197.**

8. Lane 185. The centrifugation speed of 28880xg seems too high to pellet only the bacterial cells.

**Response:**

**Corrected in line 257 to 12,000 × g.**

9. It was not clear how the oligonucleotides were designed.

**Response:**

**Sequences of mutagenic oligonucleotides are added in Table 3.**

10. Lane 318. Centrifugation speed of 27000xg seems too high to pellet insoluble matter.

**Response:**

**Corrected in line 393 to 12,000 × g.**

11. The authors used the highest dilution plate for counting CFU which may not yield representative colonies. Ideally, from the serial dilution plates, the one should be chosen that has countable colonies (generally 30-300 colonies) and used for CFU calculation.

**Response:**

**Corrected in corresponding position of text.**

12. A small number of clones were sequenced to determine the diversity which was then linked to 'functional' diversity. A citation is needed.

**Response:**

**We increased sequencing clone number. Meanwhile, relevant citation is added in discussion section.**

13. For sequence analysis, was the whole phagemid DNA sequenced?

**Response:**

**The variable heavy and light chains were sequenced.**