Dear Dr. Ricca,  
  
Your manuscript, JoVE59102 "Spore adsorption: a non-recombinant display system," has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.  
  
After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). If submitting as a .tif or .psd, please ensure that the image is 1920 x 1080 pixels or 300 dpi.  
  
Your revision is due by **Oct 24, 2018**.  
  
To submit a revision, go to the [JoVE submission site](http://www.editorialmanager.com/jove" \t "_blank) and log in as an author. You will find your submission under the heading "Submission Needing Revision".  
  
Best,  
  
Peer Review,  
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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.  
2. Please revise lines 32-34, 74-77, 94-95, 170-171, 200-202 to avoid previously published text.

The lines have been changed with the sentences in red  
3. Please revise the title to avoid punctuation and to represent the content included in the protocol.

we changed the title following also the suggestionof reviewer 4

4. Please define all abbreviations before use. Done

5. 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, dashes, or indentations.

Done  
6. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). Done

7. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. 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. See examples below:  
Line 107: Please specify the proteins that will be used in the video and specify incubation temperature. Done  
Line 115: Please provide the composition of binding buffer. Is it the same as acid buffer? What volume of buffer is used to wash? Done  
Line 123: Please specify the centrifugation parameters (force and time) and antibodies used. Done  
Line 192: Please describe how this step is actually performed. Done

Lines 239-251: We cannot film a generalized protocol; we need specific details of a specific experiment. For instance, please specify the enzymes, optimal buffer, enzyme reaction conditions used in these steps. Done

Line 251: Please specify the fresh buffer and new substrate added. Done

8. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step. Done

9. Please include single-line space s between all paragraphs, headings, steps, etc. Done

10. After you have made all the recommended changes to your protocol (listed above), 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

Done

11. 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

Done

12. 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.

Done

13. Are any figures reprinted from previous publications? If yes, 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].”

The figures are original and never published before

14. Figure 3: The yellow circle can hardly be seen. Please revise to make it more visible.

The images of figure 3 are from ImageJ software, and the yellow circle comes automatically from the program. We have now added a hard yellow circle to the original one to make it more visible.

15. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.  
Other comments have been added in the discussion (lines 334-335 and 337-338)

16. References: Please do not abbreviate journal titles.

References have been corrected  
  
  
  
**Reviewers' comments:**  
  
Reviewer #1:  
  
Manuscript Summary:  
This is a nice description of use of bacterial spores to adsorb proteins, to assess the levels of adsorbed proteins, and then use of these spores with adsorbed proteins.  
  
Major Concerns:  
The English usage needs much work, as there are many examples of use of inappropriate words or grammar. However, the meaning of everything is clear. Three examples in the summary are: 1) line 21 change "expose" to "adsorb"; 2) line 22, change "activity" to "activities"; and line 23 - change "have been shown" to "are also."  
Done

Minor Concerns:  
1) line 53 and elsewhere - I am not sure that they spore structure is "peculiar" - perhaps "novel" is a better word. The spore is a “peculiar” cell form due to its quiescent metabolism, its dehydrated cytoplasm and surrounding layers.

2) line 96 and elsewhere - make it clear that it is the spores with adsorbed enzymes that are not recovered, not the enzymes themselves.

The Introduction has been modified clarifying this point.

3) lines 123-126 - what about analyzing the supernatant from step 4 in the Adsorbtion reaction?

This is the analysis described at point 4 and named “indirect evaluation of the efficiency of adsorption”. A sentence has been added at lines 112-113 to clarify this point.

4) lines 139, 143 - define DIC and FACS - and other abbreviations as well.

Done

5) line 187 - which supernatant?

The point has been clarified (lines 194-195)

6) line 192 - perhaps say something here about the "membrane".

Done

7) line 195 and elsewhere - "Dot blot apparatus", not "Dot apparatus".

Done

8) line 225 - software such as ImageJ?

two examples of possible software have been reported.

9) line 243 - if the pH must be maintained at ~4.0 to keep proteins adsorbed, how does this influence enzyme activity assays?

when possible, it is recommended to perform the enzymatic reaction at a pH lower than the isoelectric point of the adsorbed enzyme. If not possible, a bit release of the adsorbed protein could occur (less than 15%, ref 26). A comment about this point has been added in the discussion (lanes 337-338)

10) line 273 - spore-free supernatant, not cell-free.

Corrected

11) Legends to Fig 2-5, make clear that *B. subtilis* spores (I presume) are used in these experiments.

We have specified in the text that spores of *Bacillus subtilis* have been used in the described protocols.

12) Fig 2 legend - what is the fluorescent protein in panel B, and the enzyme in panel C? If the assay is at pH 7, do most protein deadsorb? Would this preclude reuse of these spores?

Figure 2 has been modified and now it reports the results obtained with the adsorption of the monomeric red fluorescent protein mRFP on the *B. subtilis* spores. Also the text (paragraph “representative results”) has been corrected

13) line 289 and elsewhere - fluorescent here should be "fluorescence".

Corrected

14) line 310 - recycled should be reused - any data on what % of adsorbed protein is lost after one use of the spores, especially if for a reaction at pH 7?

A sentence about the loss of product in the second reaction has been added in the introduction (lines 88-90)

15) References 12 and 13 - titles of articles are in all capitals.

Done.

16) Fig. 3 - I am not sure that panel B is needed - these numbers could be added to the inset in panel A. Perhaps note in text about the inset in panel A, that the adsorbed protein is in spores' outer layers.

Done

17) Fig. 5 - in panel B, "Surnatant" should be "Supernatant".

Done

18) Table - some of the names of material/Equipment have been cut off I presume the Superwhite uncharged microscope should be followed by slides, and something is also missing after "blotting".  
Corrected  
  
  
Reviewer #2:  
  
Manuscript Summary:  
The authors present several broadly-applicable protocols for adsorbing proteins onto Bacillus spores and characterising 'loaded' spores. The protocols are straightforward and clearly described, and would be of use to researchers who would like to try this approach for the first time. The MS does require copy-editing for language issues particularly in the protocol section itself.  
  
Major Concerns:  
Some references in the introduction are quite dated, especially in regard to spores as vaccine delivery vehicles. Recent references include: Copland et al Front Immunol. 2018 Mar 12;9:346., Zhou et al J Med Microbiol. 2017 Jan;66(1):83-89., Sun H et al Parasit Vectors. 2018 Mar 7;11(1):156.

The original paper (ref. 18) and recent reviews (ref. 8 and 9) on the spore systems have been cited. The suggested references describe specific examples of spore display and we believe are not appropriate.  
  
Emphasis is placed on the use of spores as a molecular display system akin to phage display. This can be achieved using recombinant spores but it is less clear how this would work with the in vitro adsorption techniques described here. This should be clarified.

The Introduction has been modified and it has been clarified that the non-recombinant spore display is proposed as a delivery system and not to replace phages in the screening of peptide libraries.

Although preparation of the spores is outside of the scope of these protocols, some specification of the spores used here would be helpful to allow researchers to gauge the appropriateness of the protocols for their particular application. For example, is this suitable for spores that have been autoclave sterilised, or those from all Bacillus species?

In the text (line 107) we specified that *Bacillus subtilis* spores are used in the described experiments and that autoclaved spores can also be used (lines 78-80).

Minor Concerns:  
  
The software used for densitometric analysis should be stated. Is there appropriate open-source or freely available software that could be recommended?

Examples of open-source and freely available software have been added in the text.  
  
Figure 2A - lane labels are misaligned.

Corrected  
  
A cytometry dot plot of physical characteristics of the spores (FSC/SSC) and any recommended gating approaches would add to Figure 2B

The graphs of figure 2C (in the new version of figure 2) were obtained analyzing the entire spore population (10,000 events, ungated) and were intended to be only an example of the methodology.

Figure resolution is poor throughout review pdf

The resolution has been increased  
  
l240 - 'paragraph 1' should read 'protocol 1'

Done  
  
  
  
  
Reviewer #3:  
  
Manuscript Summary:  
The technical quality is fair. This topic and experimental protocol could be very interesting for readers working in the specific field. The manuscript can be acceptable for publication in JoVE. However, in its present form there are some parts to be supplemented or revised.  
  
Major Concerns:  
1. A recent study entitled "Display of native proteins on Bacillus subtilis spores" described a method of native protein display on the Bacillus spore surface that obviates the need to construct fusion proteins to display a motif. Does this display system be classified as recombinant or non-recombinant display system? In my opinion, It is necessary to add comments on this display system in the manuscript.

A comment has been added to clarify the point (lines 92-99).

2. The introduction may be shortened to the information most relevant to the work.

Done

3. All micrographs in figures 2B, 3 & 4 are of poor quality. New and improved micrographs are required. The micrographs have been replaced.

Minor Concerns:

1. Line 115: Is the binding buffer same as an acid buffer(line 107)? If not, please provide the recipe for binding buffer. The binding buffer is the acid buffer used in the adsorption reaction, as clarified in the text (lines 112-115)

2. Line 131: Replace "slides" by "slide".

Done

3. Line 137: The first letter of "Fluoresscent" written in lowercase.

Done

4. Line 321-323: Please provide references for the following statement "with small proteins or peptides adsorbed more efficiently than large".

The sentence has been deleted.

5. Line 333: What does "ad hoc" mean? Please describe in more detail

The word “ ad hoc” has been replaced with “ specific” (line 345)

6. In section "Adsorption reaction": some details is lost. For example, "in a shaker bath": how many is rotation rate? ; "Wash the pellet with the binding buffer": how many the volume?

More details have been added in the text

7. In figure 5: How to determine the adsorbed protein molecules per spore. A detailed calculation procedure would be helpful to illustrate the adsorption efficiency.

The amount of adsorbed protein is indirectly calculated determining by dot-blot the amount of unbound protein. We added the indication of the calibration curve to extrapolate the concentration of heterologous protein in each sample dots and calculate the average of the significative values (line 239)

8. References list:  
Reference should be given in a reference list according to the Journal's style. So writing style of references must be checked again.  
Line 383-384: please check the article and journal name. Done  
Line 392, 395, 397, 400, 406: the journal name is not abbreviated. Done  
  
  
  
  
Reviewer #4:  
  
Manuscript Summary:  
The manuscript is well written and describes in detail protocols for adsortion and recycling of B. subtilis spores as a platform for adsorption of enzymes and antigens without the need to generate genetically modified strains. The protocol will find interest for those interested in the use of the platform as an alternative for the display of proteins for different purposes.  
  
Major Concerns:  
No major concern.  
  
Minor Concerns:  
Alternative title: "Spore adsorption: a non-recombinant display system for enzymes and antigens"

the title has been changed