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Marseille, 11th of December 2013.

Dear Dr Buntrock,

**RE: Submission JoVE51464R2**

Please find below our responses to the peer-review for our manuscript JoVE51464R2 entitled “High throughput quantitative expression screening and purification applied to recombinant disulfide-rich venom proteins produced in *E. coli*”.

We thank the reviewers for their constructive comments and we have incorporated them into the manuscript where suitable. Each of the reviewer comments are addressed in further detail below (our responses are shown in blue).

**Reviewer #1:**

Major Concerns:

My major criticism is that it took me too much effort trying to piece together the different steps of the described protocols. For clarity, at each step, please indicate the source and destination plate names and/or positions on the robot worktable. For example, the transformation mix indicated in step 1.3 of the "Precultures and plating" protocol has never been mentioned before (does it correspond to DW96 at position 11 ?). Also the role of the transformation plate at position 13 in step 1.1.3 is not defined.

The positions on the robot worktable for the source and destination plates at each step have been defined to avoid confusion in the protocols. The transformation mix comes from the manual transformation protocol. The origin of the transformation mixes and their role in the protocol are now more clearly stated.

Minor Concerns:

1. Page 3 paragraph 2 - "high throughput manual approach". When it comes to manual I would be more cautious not to use the high-throughput qualifier.

The phrase high throughput manual approach could be removed and replaced by “can also be used manually to improve efficiency’ but as stated in the same paragraph one can achieve 96/384 points/week with manual protocols which can be considered HTP by many labs so we left it as it is for the time being. If the editor wants the substitution of text can be done.

Page 4 paragraph 1 - "in all constructs proteins are cloned" please correct!

This has been fixed.

1. Last sentence of paragraph 6 need to be rephrased

The sentence has been rephrased.

1. Page 5 intro - manually instead of "in a manual way"

This has been adjusted.

1. Page 5 - section 1.1.3 needs to be rephrased for clarity. A section 1.1.4 ?

Step 1.1.3 has been better defined for clarity. The authors do not believe that a Step 1.1.4 is required.

1. Page 8 section 6.4 - please rephrase for clarity

This has been rephrased for clarity.

1. Page 9 section 7.3 - "after which stage" please rephrase

This has been changed to ‘at this point’.

1. Page 12 section 8.2.5 - Please indicate the temperature of incubation. Also either 700 or 1200 ul according to steps 8.1.14 & 15

The temperature of incubation has been included in Step 8.2.4.

For the nickel affinity purification, there are two options for the protocol. Either the use of 200 ul final volume of resin (which is Section 8.1 – Protocol A) or the use of 50 ul final volume of resin (which is Section 8.2 – Protocol B). The protocols are independent of each other, and only one of the two protocols is performed in any one experiment. This is detailed in the introduction. Accordingly, for Step 8.2.5, the volume is 1200 ul (1000 ul of lysate + 200 ul of resin slurry). Step 8.1.14 and 8.1.15 have no influence over the volume at Step 8.2.5.

1. What does "normal 200 ul tips" mean ?

The ‘normal’ has been removed from the text. In this case, it just meant ‘not wide-bore tips’, but it is redundant and therefore was removed.

1. Page 13 section 9.4 - Please rephrase

Step 9.4 has been rephrased for clarity.

1. Page 13 section 10.1.1 - Not clear. Please rephrase

Steps 10.1.1- 10.1.4 have been clarified.

1. Page 16 Discussion paragraph 1 - most laboratories

This change has been made.

1. Page 16 Discussion paragraph 1 - "Using this approach,…" Reformulate this sentence and be more precise concerning the additional variables. A reference ?

The additional variables to be optimized have already been discussed in the introduction, therefore we have directed the reader to the introduction regarding the precise list of variables and their relevant references. The sentence “Using this approach….” has been reformulated. It is now: ‘Using DsbC fusions and high throughput methods, within a week the soluble expression of multiple targets can be observed11 and then additional variables, such as those discussed in the introduction, can be screened in subsequent rounds on those targets that require further optimization.’

**Reviewer #2:**

Minor Concerns:

1. Figure 4 is missing from the revised ms.

To the best of our knowledge, Figure 4 was included in the manuscript. Other reviewers had comments on Figure 4, so we believe the figure was included.

1. Although the article is entitled " High throughput quantitative expression…." The exemplification of the method is limited to 2 proteins only which would not require HTP/automation to produce. It would be helpful for results for a full 96 well plate experiment to be shown so that the quality of data from the protocol could be seen.

We have significantly changed our representative results to incorporate the results seen from a screen on a full set of 96 samples.

1. The mass spec analysis should be shown for the examples since in one case no cleaved product is seen on the SDS-PAGE.

We have now provided an example of a mass spec analysis as part of the representative results.

**Reviewer #3:**

Major concerns:

The paper does not contain much new Information that has not been published: The most helpful tag (i.e. the DsbC gene product) is described in other studies and the same holds true for the expressed targets. Gateway cloning (Invitrogen) is a well-established but expensive technique. This submission contains no results with exception of two of the "representative" proteins (Figure 6). I am aware that JoVe is a methods journal, but I feel that at least some data supporting the validity of the statements about the techniques have to be included in any method paper. A few general comments are just not enough. It is the substantial lack of data confirming the usefulness of the techniques which is the serious shortcoming of the paper. A wider readership would also appreciate some data to give a better feeling of the value of the DsbC tag in conjunction with larger more commonly used proteins. A laboratory with a good reputation for high-throughput work (and the lab in Marseille is well known for its HTP-achievements) can and should provide direct evidence to allow a serious judgment of the claims inside the publication. Thus, without addition of quite a few more data (at least a table giving a considerable number of purifications with the ratio of successful/failed plus the pI and lengths of the targets), I consider this paper not suitable for publication. Right now it looks more like a copy of a set of detailed lab protocols.

*Editorial Comment: Considering JoVE is a methods journal, we do not require that you add any additional data to your manuscript. However, if you believe the addition of the data suggested by the above reviewer will significantly improve your manuscript, please add it to your manuscript.*

Our representative results section and figure have been reworked to include the analytical expression results for a full 96 targets. We hope that the data provided now demonstrate more thoroughly the effectiveness of the technique. Additionally, we have also provided the number of disulfides, number of residues and isoelectric point for each target and included these in our analysis of results.

For the wider readership not interested by the expression of animal venom peptides, we have also included a sentence regarding our previous publication that states that the disulfide rich proteins screened previously not only came from venomous sources, but also included disulfide-rich enzyme inhibitors from a wide range of species including plants, pigs, cows and humans.

Minor Concerns:

See Manual remarks in attached or mailed PDF file; pages without comments are not shown.

*Editorial Comment: Please see the attached PDF "PR#3comments to JoVE51464R2."*

Typographical errors as well as suggestions highlighting a lack of clarity or for improved delivery have been adjusted accordingly.

The sections highlighted in yellow are required by JoVE as a guide to which parts of the protocol we wish to demonstrate in the associated video.

With regards to the Table of Materials/Equipment, this was submitted to JoVE as an excel file and the formatting problems seem to have appeared upon conversion to pdf format, as they are not present in the original excel document.

Additional Comments to Authors:

In all other aspects the method is well presented and written. I think it can be reproduced in a laboratory with HTP equipment and even in a lab with less high-tech apparatus. Some of the description may be too detailed, especially Figure 5 (transfer from 96- to 24-well plates). I suggest dropping this figure. The autoinduction media are published in much detail in reference 27; the Table 1 (taking 2 pages) describing this technique is probably not necessary in large format. The less important details are given in the accompanying pdf file with my hand-written comments. Pages without comment are not included. Some are just language or typos while others are minor suggestions to the authors.

*Editorial Comment: You may maintain the current level of detail in your manuscript as the purpose of a JoVE article is to provide a detailed account of your methods to both specialists and non-specialists.*

For the purposes of making the protocols accessible to people outside the field of analytical protein expression, and to have all the relevant information available in one ‘go-to guide’, we have decided to leave the current level of detail in the manuscript including all figures and tables. Table 1 (submitted as an excel file to JoVE) was not intended to take 2 pages, and this will need to be adjusted for the final publication.

**Reviewer #4:**

Minor Concerns:

1. The second paragraph of the abstract is difficult to read and deserves some polishing. Similarly, the penultimate paragraph on p.4 ("As a general rule … on the protein tested") is unclear and should be rewritten.

Both paragraphs have been re-written for improved delivery and clarity.

1. In the first paragraph p.3, the authors mention published work (ref.11), but the main conclusions of this work are not summarized. These conclusions should be briefly provided to ease the reading of the rest of the paragraph without reading the manuscript cited.

We have briefly summarized our results by including the sentence: "We demonstrated that using DsbC as a fusion partner for production in the strain BL21 (DE3) pLysS vastly outproduced (in both yield and number of soluble proteins obtained) any other combination of strain and fusion tested11. "

1. End of 2nd paragraph p.3, the text refers to a protocol detailed in Fig.3. It is difficult to understand why this particular protocol has been chosen, this should be clarified.

We have added the sentence: "The parameters in the pipeline were selected based on extensive screening experiments11,22, which allowed us to choose the most useful conditions for protein production."

1. Detailed protocol 3.3 (p.7). The authors mentioned that glucose is depleted after 4 hours in the auto-induction media. Does this information come from their experience and/or from published work? This should be mentioned.

We have added a reference to this step, and also at Step 3.4 added, "…. providing the optimal growth conditions for BL21 (DE3) pLysS or Rosetta 2 (DE3) pLysS, in our hands22."

1. Detailed protocol 8 (p.9-12). The imidazole concentrations indicated in the various buffers are tailored for nickel affinity purification. This should be clearly mentioned, indicating that these concentrations should be adapted when using other ions (e.g. cobalt).

In the note at the beginning of Section 8 we have inserted the sentence: "For purification, the specified imidazole concentrations are applicable to nickel affinity resin. If alternative ions (eg. cobalt) are used, then the concentrations should be adjusted accordingly."

1. Detailed protocol 10 (p.13-14). The authors forget to mention that proteins might stick to or precipitate on the affinity beads upon imidazole elution, a phenomenon that is not uncommon. The authors should address this point and suggest alternative strategies (e.g. resuspend affinity resin with Laemmli buffer followed by SDS-PAGE analysis). This should help identify expression/purification conditions that could be improved by possibly slightly changing expression/purification parameters, rather than discarding the clone used (false negative).

This point is now covered in Step 10.1.3.

1. Detailed protocol 10.1.2 (p.13). Relating to the above comment (6), analysis of whole cell lysates may not be good at observing poorly expressed proteins that will only be detected upon concentration on affinity resin. Once again, sticking/precipitation of this protein to/on the affinity beads will lead to a false negative.

This point is now covered in Step 10.1.3.

1. The word "Unfortunately" should be removed at the beginning of the discussion.

This has been done.

1. In the discussion or earlier in the detailed protocol, alternatives to imidazole elution such as cleavage of the affinity tag directly on the beads should be mentioned. The purified protein obtained using this approach is often purer than the one obtained by increasing imidazole concentration, and is possibly also better suited for subsequent biochemical and biophysical characterizations.

In the introduction text, p4, paragraph 1, when discussing the options for construct design and cleavage, we have mentioned that cleavage can be tested on the elution fraction or ‘on column’. As cleavage is only an optional step in the protocol, we have refrained from an in-depth discussion of the issue, so as to not bombard and deter the reader with too many options.

1. Analysis of protein/protein interactions by co-expression in E. coli could be performed using the same protocol. This should be mentioned/detailed in the discussion.

The use of the protocol for protein-protein interactions by incorporating co-expression has been included in the discussion with the reference:

Vincentelli, R. & Romier, C. Expression in Escherichia coli: becoming faster and more complex. *Current Opinion in Structural Biology* **23**, 326-334 (2013).

More references have also been included for protein-protein and protein-DNA interactions:

Charbonnier, S. *et al.* High-throughput determination of domain-ligand affinities by automated holdup assay allows quantification of linear motif specificities. (under revision).

Jolma, A. *et al.* DNA-binding specificities of human transcription factors. *Cell* **152**, 327-339 (2013).

1. The authors mention several times in their manuscript that scale-up is normally straightforward from their small-scale analysis. This is not what is often observed in different laboratories. The authors should discuss whether this is based on their own experience with the protocol described.

We have further clarified this in the introduction, saying that ‘In our experience, the strategy also gives good reproducibility upon scale-up using the same culture (temperature, media, aeration etc.) and purification conditions (same resin, buffers etc.).

1. In Fig.2 and its legend, the method used to go from the entry to the final expression clones is not described; is this Gateway LR reaction? In addition, does clonase means BP Gateway reaction?

JoVE discourages the use of commercial language, therefore we have had to keep mention of the Gateway system to a minimum. We do use the Gateway system, and this is now mentioned in the figure legend for Figure 2. Rather than referring to Gateway cloning directly throughout the text, we refer to recombinational cloning instead, however this is a general moniker, which Gateway cloning falls under. Figure 2 has now been modified so that it says ‘enzyme’ instead of ‘clonase’. In terms of Gateway cloning, the inset in the top left shows the BP reaction, where the enzyme is BP clonase, then the main reaction shown (His-DsbC vector + enzyme + entry clone) is the LR reaction, where the enzyme is the LR clonase. Given that there are various methods for recombinational cloning, we have omitted these specifics in the figure.

1. In Fig.3 the word "again" is not self-explanatory. Please clarify.

The word again has been removed and replaced by ‘after trying other strains and temperatures’.

1. Fig.4 and its legend are also not self-explanatory. What is described here? Please clarify.

Figure 4 is a schematic representation of the work table layout for our liquid handling robot. The legend has been adjusted to give a more thorough explanation of what is shown and the function of each module shown. The numbers displayed in the figure correspond to the positions we refer to throughout the protocols.

Thank you for your time and consideration of our manuscript.

Sincerely,

Natalie Saez and Renaud Vincentelli