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Using Phage Display to Develop Ubiquitin Variant Modulators for E3 ligases

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TITLE:

Using Phage Display to Develop Ubiquitin Variant Modulators for E3 ligases

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SUMMARY:

Ubiquitination is a critical protein post-translational modification, dysregulation of which has been implicated in numerous human diseases. This protocol details how phage display can be utilized to isolate novel ubiquitin variants that can bind and modulate the activity of E3 ligases that control the specificity, efficiency, and patterns of ubiquitination.

ABSTRACT:

Ubiquitin is a small 8.6 kDa protein that is a core component of the ubiquitin-proteasome system. Consequently, it can bind to a diverse array of proteins with high specificity but low affinity. Through phage display, ubiquitin variants (UbVs) can be engineered such that they exhibit improved affinity over wildtype ubiquitin and maintain binding specificity to target proteins. Phage display utilizes a phagemid library, whereby the pIII coat protein of a filamentous M13 bacteriophage (chosen because it is displayed externally on the phage surface) is fused with UbVs. Specific residues of human wildtype ubiquitin are soft and randomized (i.e., there is a bias towards to native wildtype sequence) to generate UbVs so that deleterious changes in protein conformation are avoided while introducing the diversity necessary for promoting novel interactions with the target protein. During the phage display process, these UbVs are expressed and displayed on phage coat proteins and panned against a protein of interest. UbVs that exhibit favorable binding interactions with the target protein are retained, whereas poor binders are washed away and removed from the library pool. The retained UbVs, which are attached to the phage particle containing the UbV's corresponding phagemid, are eluted, amplified, and concentrated so that they can be panned against the same target protein in another round of phage display. Typically, up to five rounds of phage display are performed, during which a strong selection pressure is imposed against UbVs that bind weakly and/or promiscuously so that those with higher affinities are concentrated and enriched. Ultimately, UbVs that demonstrate higher specificity and/or affinity for the target protein than their wildtype counterparts are isolated and can be characterized through further experiments.

INTRODUCTION:

Understanding the molecular details of protein-protein interactions is critical for delineating the signal transduction mechanisms of biological processes, particularly those that contribute to clinically important diseases. In recent years, phage display has been utilized as a practical and accessible method to isolate proteins/peptides with much improved binding to a desired target protein[1–4], which in turn can be used as intracellular probes of protein-protein interactions.

Ubiquitination is a cascade of enzymatic activities (E1 activating enzyme→E2 conjugating enzyme →E3 ligases) that covalently conjugate ubiquitin (Ub) to protein substrates to target them for degradation or to mediate cell signaling changes. In addition, deubiquitinases catalyze the removal of ubiquitin from proteins. Therefore, in cells, there are thousands of Ub-dependent protein-protein interactions, the vast majority of which recognize a common surface with low affinity but high specificity to allow weak interactions through large and diverse surfaces.

Ernst et al. introduced mutations into known binding regions of Ub in order to see if they could enhance binding affinity for a protein of interest while still maintaining high selectivity[5]. A combinatorial library of over 10 billion (7.5×10^{10}) Ub variants (UbVs) with mutations at positions across the Ub surface that mediate the known Ub-protein interactions was developed. This library consisted of phagemids that express the M13 bacteriophage pIII coat protein fused to diversified UbVs. Therefore, individual UbVs can be displayed on the phage surface via the coat protein upon expression. During the selection process, phage that display UbVs with considerable binding interactions with the target protein will be retained and enriched in subsequent rounds of phage display, whereas phage displaying UbVs that bind poorly to the target protein are washed away and removed from the phage pool. The retained phage particles contain the phagemid corresponding to their displayed UbV, allowing them to be sequenced and further characterized once isolated.

Using this protein engineering strategy, UbV inhibitors were developed for human deubiquitinases[5] and viral proteases[6]. Importantly, we have generated inhibitory UbVs for human HECT-family E3 ligases through hijacking the E2-binding site and activating UbVs that occupy a Ub-binding exosite on the HECT domain[7]. We can also inhibit monomeric RING-family E3s by targeting the E2 binding site and induce UbV dimerization to activate homodimeric RING E3s[8]. For multi-subunit RING E3s, UbV can achieve inhibition by targeting the RING subunit (e.g., for APC/C complex[9]) or disrupting complex formation (e.g., for SCF E3s[10]). Collectively, UbVs can be leveraged to systematically interrogate protein-protein interactions in the Ub-proteasome system (UPS) so that we can better decipher biochemical mechanisms of UPS enzymes and to identify and validate functional sites for therapeutic intervention.

The following protocol describes how to employ a previously generated phage displayed UbV library to target a protein of interest and how to enrich the UbV binders that interact with the target protein through successive rounds of phage display.

PROTOCOL:

1. Reagent preparation

1.1. PBS (phosphate buffered saline): Mix 50 mL of 10x PBS solution with 450 mL of ultrapure H_2O. Sterilize by filtration and store at 4 °C or room temperature (~20-25 °C).

1.2. 10% BSA (bovine serum albumin): Slowly add 1 g of BSA to 7 mL of ultrapure H_2O and mix until fully dissolved (no clumps). Top up with ultrapure H_2O until the final volume is 10 mL. Sterilize by filtration and store at 4 °C.

1.3. PB buffer (PBS supplemented with 1% BSA): Slowly add 5 g of BSA to 400 mL of ultrapure H_2O and 50 mL of PBS and mix until fully dissolved (no clumps). Top up with ultrapure H_2O until the final volume is 500 mL. Sterilize by filtration and store at 4 °C.

1.4. PBT buffer (PBS supplemented with 1% BSA and 0.05% Tween 20): Slowly add 5 g of BSA to 400 mL of ultrapure H_2O and 50 mL of PBS and mix until fully dissolved (no clumps). Add 250 µL of Tween 20. Top up with ultrapure H_2O until the final volume is 500 mL. Sterilize by filtration and store at 4 °C.

1.5. PT buffer (PBS supplemented with 0.05% Tween 20): Mix 1 mL of Tween 20 with 400 mL of PBS. Top up with ultrapure H_2O until the final volume is 2 L. Sterilize by filtration and store at 4 °C or room temperature (~20-25 °C).

1.6. 2YT broth: Add 16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl to 800 mL of ultrapure H_2O and mix until fully dissolved (no clumps). Top up with ultrapure H_2O until the final volume is 1 L. Sterilize by autoclaving and store at room temperature (~20-25 °C).

1.7. LB/carb plates: Add 12.5 g of pre-made LB mixture and 7.5 g of agar to 400 mL of H_2O. Top up with ultrapure H_2O until the final volume is 500 mL and sterilize by autoclaving. Make sure agar is fully dissolved and wait for it to cool to below 60 °C. Add 500 µL of 100 mg mL carbenicillin, mix well, and pour into plate. Store at 4 °C.

1.8. LB/tet plates: Add 12.5 g of pre-made LB mixture and 7.5 g of agar to 400 mL of ultrapure H_2O. Top up with ultrapure H_2O until the final volume is 500 mL and sterilize by autoclaving. Ensure agar is fully dissolved and wait for it to cool to below 60 °C. Add 500 µL of 10 mg mL tetracycline, mix well, and pour into plate. Store at 4 °C.

1.9. 20% PEG (polyethylene glycol)/2.5 M NaCl: Add 50 g of PEG-8000 and 36.5 g of NaCl to 200 mL of ultrapure H_2O. Mix until fully dissolved.

NOTE: This may take a while; heating can help. Top up with ultrapure H_2O until the final volume is 250 mL. Sterilize by filtration or autoclaving.

1.10. 0.1 M HCl: Mix 20 mL of 1 M HCl with 180 mL of ultrapure H_2O. Sterilize by filtration and store at room temperature (~20-25 °C).

1.11. 1 M Tris (pH 11.0): Add 6.1 g of Tris base to 40 mL of ultrapure H_2O. Adjust pH to 11.0 with HCl and mix until Tris is fully dissolved. Top up with ultrapure H_2O until the final volume is 50 mL. Sterilize by filtration and store at room temperature (~20-25 °C).

1.12. 100 mg/mL (1000x) carbenicillin: Add 2 g of the carbenicillin disodium salt to 20 mL of ultrapure H_2O. Mix until fully dissolved. Sterilize by filtration and store at -20 °C.

1.13. 50 mg/mL (1000x) kanamycin: Add 1 g of kanamycin sulfate to 20 mL of ultrapure H_2O. Mix until fully dissolved. Sterilize by filtration and store at -20 °C.

1.14. 10 mg/mL (1000x) tetracycline: Add 0.2 g of tetracycline hydrochloride to 20 mL of 70% ethanol. Mix until fully dissolved. Sterilize by filtration and store at -20 °C.

2. Protein preparation

2.1. Determine the concentration of the target protein stock in µM. The most convenient way to assess protein concentration is to measure the absorbance of the protein stock at 280 nm. If the concentration is known in mg/mL, convert it to µM using the molecular weight of the target protein. A range of methods can be used depending on the protein of interest; see the Discussion section for details.

NOTE: If the protein is truncated (specific protein domain/motif) or tagged, remember to account for these changes in molecular weight when converting from mg/mL to µM.

2.2. Prepare three microcentrifuge tubes labeled “round 1”, “round 2/3”, and “round 4/5”.

2.2.1. Calculate the volume of protein necessary to dilute it to 1 µM in 800 µL PBS and aliquot this volume into the tubes without adding PBS.

NOTE: If the amount of target protein available is low, the concentration can be lowered to as little as 0.25 µM.

3. Preparation for round one of selection

3.1. Prepare a seed culture for culturing the phage input for round two of selection.

3.1.1. Inoculate 5 mL of 2YT/tet with a well-isolated *Escherichia coli* colony and incubate overnight at 37 °C with 200 rpm orbital shaking.

3.2. Coat the plate for the first round of selection.

3.2.1. Dilute the target protein in the “round 1” tube with the appropriate amount of PBS and aliquot 100 µL into eight wells of a 96-well binding plate (i.e., the target plate).

NOTE: Phage display can be done for four different proteins simultaneously on a single plate by coating the wells in the corners of the plate.

3.2.2. Optional control: If the target protein is tagged, such as with GST or MBP (maltose binding protein), coat eight wells in another 96-well binding plate with 100 µL of a 1 µM solution containing the appropriate epitope tag. This will be used to remove undesired phage that bind to the tag non-specifically.

3.2.3. Shake plate(s) overnight at 4 °C with 200 rpm orbital shaking.

4. Round one of selection

4.1. Prepare the round two input.

4.1.1. Inoculate 30 mL of 2YT/tet with 200 µL of the seed culture from step 3.1.

4.1.2. Incubate at 37 °C with 200 rpm orbital shaking until the bacteria are in mid-log phase (OD_{600} ≅ 0.6 – 0.8). This takes approximately three hours.

4.2. Block target plate.

4.2.1. Remove the coating solution from the plate, or both plates if a control plate has been made, by inverting and shaking over a sink. Pat dry on paper towels.

4.2.2. Add 300 µL of PB buffer to each coated well.

4.2.3. Remove the PB buffer as in step 4.2.1 and add another 200 µL of PB buffer.

4.2.4. Incubate at room temperature (~20-25 °C) for 1 h with 300 rpm orbital shaking.

4.3. Prepare phage library.

4.3.1. Thaw the phage library on ice and dilute it to 100x the library diversity in PBS. For example, if the library diversity is 1×10^{10} and the library concentration is 1×10^{13}, dilute the library so that the concentration is 1×10^{12}. For the libraries used here, dilute them 10-fold in PBS by combining 1 mL of the library with 9 mL of PBS.

NOTE: Library diversity should have been determined during library creation and cannot be easily assessed otherwise. Library concentration can be determined by inoculating *E. coli* cells in mid-log phase (OD_{600} ≅ 0.6 – 0.8) and plating on LB/carb.

4.3.2. Add 1/5 volume of PEG/NaCl. For example, for 10 mL of the previously diluted library, add 2 mL of PEG/NaCl.

4.3.3. Incubate on ice for 30 min.

4.3.4. Centrifuge at 11,000 x *g* for 30 min at 4 °C, discard supernatant, and recentrifuge for 2 min to pull down the remaining supernatant.

NOTE: Put the tube in the rotor in the same orientation the second time as it was the first to keep the pellet in the same place, therefore, making it easier to see.

4.3.5. Gently resuspend the phage pellet in 1 mL of PBT per protein. For four proteins, resuspend the pellet in 4 mL of PBT.

NOTE: Try not to touch the pellet and do not introduce air bubbles.

4.4. Display phage to the target protein(s).

4.4.1. Optional control: Add 100 µL of phage library to each coated well in the control plate. Incubate at room temperature (~20-25 °C) for 1 h with 300 rpm orbital shaking and transfer the library from the control plate to the target plate. Skip step 4.4.2.

4.4.2. Remove the PB buffer from the target plate as in step 4.2.1 and add 100 µL of the phage library to each coated well.

3.4.3. Incubate at room temperature (~20-25 °C) for 1 h with 300 rpm orbital shaking.

4.5. Elute round one phage.

4.5.1. Remove phage library and wash the coated wells four times with PT buffer. Invert the plate and tap on a paper towel to remove the last drops.

NOTE: If multiple target proteins are coated on one plate, try to minimize the flow of all subsequent solutions between the wells.

4.5.2. Add 100 µL of 0.1 M HCl to each coated well and incubate at room temperature for 5 min with 300 rpm orbital shaking.

4.5.3. Neutralize the pH by adding 12.5 µL of 1 M Tris-HCl (pH 11) to each coated well.

4.5.4. Pool the eluted phage from all 8 wells into a single 1.5 mL microcentrifuge tube. Pipette up and down during the transfer to make solutions homogenous and aspirate all liquid from the wells.

4.5.5. Add 10% BSA to the pooled eluted phage to a final concentration of 1%. For a typical round one elution volume of 950 µL, add 95 µL of 10% BSA. Store at 4 °C. This is the round one output.

5. Preparation for subsequent rounds of selection

5.1. Prepare a seed culture for culturing the phage input for the next round of selection as in step 3.1.

5.2. Coat the plate for the third round of selection as in step 3.2 with the changes noted below.

5.2.1. Only coat four wells per protein in a 96-well binding plate. Use half of the contents of the "round 2/3" or "round 4/5" tubes for the appropriate round. Dilute the contents of these tubes as needed, in order to avoid proteins settling out of the solution.

5.3. Prepare the phage input for the next round of selection.

5.3.1. Use half of the round one output from step 4.5.5 to inoculate 3 mL of mid- log phase cells from step 4.1. For a typical round one output, inoculate with 500 µL of the output.

5.3.2. Incubate at 37 °C for 30 min with 200 rpm orbital shaking.

5.3.3. Add M13K07 helper phage to a final concentration of 1×10^{10} PFU/mL.

5.3.4. Incubate at 37 °C for 1 h with 200 rpm orbital shaking.

5.3.5. Transfer the entire 3 mL of culture to 30 mL of 2YT/carb/kan. Grow overnight at 37 °C with 200 rpm orbital shaking.

5.3.6. The next day, transfer the culture into a 50 mL centrifuge tube and centrifuge at 11,000 x *g* for 10 min at 4 °C to precipitate cells.

5.3.7. Decant the supernatant into a new 50 mL centrifuge tube and mix with 8 mL of PEG/NaCl and incubate on ice for 10 min.

5.3.8. Centrifuge at 11,000 x *g* for 10 min at 4 °C, discard the supernatant, and centrifuge again for 2 min.

NOTE: Put the tube in the rotor in the same orientation the second time as it was the first to keep the pellet in the same place, therefore, making it easier to see.

5.3.9. Resuspend the phage pellet in 800 µL of PBT so that it is fully homogenous and no clumps are visible.

5.3.10. Transfer the phage solution to a 1.5 mL microcentrifuge tube and centrifuge at 16,200 x *g* for 4 min at 4 °C to pellet debris.

5.3.11. Transfer the supernatant to a new microcentrifuge tube. This is the input for the next

round of selection.

5.4. Optional: Titer the input and output solutions.

5.4.1. Use 500 µL of an *E. coli* seed culture to inoculate 5 mL of 2YT/tet and incubate at 37 °C with 200 rpm orbital shaking until bacteria is in mid-log phase (OD_{600} ≅ 0.6 – 0.8). This takes approximately 1 h.

5.4.2. Dilute phage input/output solutions to 1x 10^{-3}-1 x 10^{-5} in PBS and add 10 µL of each dilution to 90 µL of cultured cells.

NOTE: Use diluted phage solutions immediately because phage will adsorb to the tube over time, and transformations may produce false negatives.

5.4.3. Incubate at 37 °C for 20 min with 200 rpm orbital shaking.

5.4.4. Plate 5 µL on separate LB/carb plates.

NOTE: Amount plated is variable depends on previous results/personal preference.

6. Subsequent rounds of selection

6.1. Perform all subsequent rounds along with the preparation as done in steps 3 and 4, respectively, with differences noted below.

6.1.1. Use the input produced in the previous round as the phage source instead of a library. Coat 4 wells per target protein with 100 µL of the phage input acquired from the previous round. Store remaining phage inputs at 4 °C.

6.1.2. Make the washing step in 4.5.1 more stringent with each round of selection. Round one requires washing 4 times, round two requires 6 times, round three requires 8 times, and rounds four and five both require washing 10 times.

6.1.3. Reduce some volumes compared to those used in round one because subsequent rounds only coat four wells instead of eight. For example, in step 4.5.5 only 45 µL of 10% BSA is added to the phage output, and in step 5.3.1 only 250 µL of the phage output is used to inoculate cells.

7. Post selection processing and phage Isolation

7.1. Titer the input and output solutions for rounds four and five.

7.1.1. If not already done in step 5.4, follow the same steps to titer rounds four and five phage outputs, ideally producing a range of 30-300 colonies across a few plates.

7.2. Culture and isolate phage.

7.2.1. Aliquot 450 µL of 2YT/carb/M13K07 into every tube of a 96 mini tube culture box.

7.2.2. Pick well-isolated colonies from any of the plates from step 7.1 to inoculate each tube.

NOTE: Use the top half of the box for round four outputs and the bottom half for round five outputs.

7.2.3. Incubate overnight at 37 °C with 200 rpm orbital shaking.

7.2.4. Centrifuge at 1,200 x *g* for 10 min at 4 °C.

7.2.5. Transfer as much supernatant as possible to a new 96 mini tube culture box without disturbing the cell pellet and discard the old one. Store at 4 °C.

7.2.6. From the new box, transfer 100 µL from each tube to a 96 well non-binding plate containing 100 µL of 50% glycerol in all wells. Mix well and store at -80 °C as a backup stock.

REPRESENTATIVE RESULTS:

Binders produced from phage display can be verified and analyzed in many ways. It is recommended to first proceed with sequencing the phage with primers that flank the diversified insert in the phagemid library. An ideal phage display experiment will show a clear bias towards several sequences (**Figure 1**). Other sequences will also be present but with a lower count, appearing more as background noise. In the example provided, where phage display was performed between ubiquitin variants (UbVs) and wildtype UBE4B, there is a particular bias towards sequences #1-4. An example Python script for organizing and analyzing sequencing files has been provided ("phageDisplaySeqAnalysis.py"). To confirm the significance of the binders, enzyme-linked immunosorbent assays (ELISA) can be used as a quick measure of relative binding affinity to the wildtype target protein, mutated target protein, as well as off-target proteins (**Figure 1**). Differences in binding affinities between the new binders and the wildtype binding protein, upon which the new binders were based, can be determined by normalizing the ELISA absorbance of the binders to that of the wildtype protein (not shown) or other proteins that demonstrate no appreciable binding with the target protein (**Figure 1**). This example demonstrates the tendency of the enriched sequences to have higher binding affinity for the target protein. An example Python script for organizing and analyzing sequencing files has been provided ("phageDisplayElisaAnalysis.py"). Additionally, an example Python script specifically for analyzing UbV results has been provided ("phageDisplayUbvAnalysis.py"). Ideally, binder sequences that are more numerous will possess higher relative binding affinity than less numerous sequences, which are presumably background noise. It is possible that unstable binders will be low in number but demonstrate appreciable binding through ELISAs. These binders should be further investigated to determine if the ELISA scores are artifacts or if they are indeed binders worthy of further characterization.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative results for a ubiquitin variant (UbV) selection against UBE4B. (**A**) UbVs were ordered from highest to lowest frequency (counts). Sequences represent the diversified ubiquitin region in the UbV with all the randomized residues (specifically, residues 2, 4, 6, 8-12, 14, 42, 44, 46-49, 62-64, 66, 68, and 70-78). All ELISA absorbances were normalized against 96 averaged BSA ELISA scores and 96 averaged GST ELISA scores. Darker green represents stronger relative binding. GST was included as a control for non-specific binding due to the fact that the target protein is GST-tagged. (**B**) Graphical summary of the ELISA results from panel A.

Figure 2. Suggested order of steps for performing phage display. Dashed lines indicate processes that are carried over to the next day or from the previous day. All subsequent rounds after round three proceed similarly to round three, excluding round five where no phage input preparation is necessary unless more rounds are being performed.

Figure 3. Suggested labware and labels for setting up a phage display experiment. Purpose and relevant steps in the protocol are indicated. R: round; I: input; O: output.

Figure 4. Appearances of typical pellets encountered during the phage display procedure. The phage library pellet presents as a streak along the side of the tube and can be recentrifuged to concentrate the pellet in the bottom of the tube. Phage input pellets and debris pellets appear more typical.

DISCUSSION:

As mentioned in step 2.1 (protein preparation), a variety of methods can be used to assess the protein concentration, and each will have unique benefits and drawbacks based on the specific target protein used for phage display. A source of detailed descriptions and protocols for popular methods has been provided previously[11].

Using the phage retained by a previous round of phage display as the input for a subsequent round enriches the good binders by gradually removing binders that bound weakly, transiently, or by chance. By rounds four and five there will ideally be a clear bias towards a small and specific set of peptide sequences, demonstrating binding preferences of the target protein.

This protocol has been optimized for use with a UbV library. While these steps may generally apply for other kinds of libraries (e.g., peptide, antibody, etc.), they will likely need to be adapted to accommodate such non-UbV libraries. As mentioned in step 4.3.1, library diversity and concentration should be known prior to proceeding with phage display. For more information on how these library attributes are determined, please see the protocol used to create the UbV libraries used in this procedure[12].

The phage display results can be very clear cut and present obvious candidate binders to pursue further characterization. For example, binders that are both highly frequent and have significantly higher binding, as measured by ELISA, are clear candidates for further study.

However, when the frequency of a particular binder does not positively correlate with the ELISA data, this may present some confusion as to how to single out interesting binders. In the example provided (**Figure 1**), it would be recommended to pick a combination of the most common binders, even if they have a low binding affinity, and those with higher binding affinity, even if they appear infrequent. Infrequent binders with high ELISA scores may not be as common due to instability during phage display, which would negatively influence their prevalence in this final data. As such, these are worth investigating as much as the highly frequent binders are.

Isolated phage solutions can be contaminated easily. It is recommended to proceed with DNA sequencing as soon as possible using primers that flank the region of the diversified peptide. Another typical post-display analysis is to perform ELISAs of the binders with their target protein. Additionally, ELISAs can be performed with the binders and mutated/truncated versions of their target proteins, which can give a rough idea of their probable binding mode. It is very important to note that phage solutions ought to be mixed well prior to use in any experiment if they have been sitting for a while. The phage can adsorb to the walls of the tube or settle out of the solution and may produce false negatives.

The most time-efficient way of carrying out this procedure is illustrated in **Figure 2**. Begin every round with preparing the bacterial culture necessary for growing the phage input for the subsequent round the next day. While this culture is growing, coated plates can be blocked. While the plates are being blocked, the library can be prepared (for round one) or the phage input can be prepared (for subsequent rounds). On the day of round four there is no need to prepare a seed culture and thus on the day of round five there is no need to do any preparations for the next round's phage input unless one intends to do more than five rounds of selection. To further economize time, a suggested labware setup has also been provided (**Figure 3**). Additionally, phage pellets are not always distinct, so pictures of typical pellet appearances encountered during this procedure have been provided (**Figure 4**).

If there are any issues with pelleting the phage during the preparation of the input for a subsequent round, the experiment may need to be halted for a day while new phage are grown. These can be recovered by going back to the phage saved in the tubes for the input for that round. For example, if the phage necessary for the third round of display cannot be pelleted for some reason, you can return to the "R3I" tube that contains 400 µL of phage input for round three. This can only be repeated once if coating four wells with 100 µL.

The titer of output phage for rounds four to five is typically in the range of 10^6 to 10^8 PFU/mL. If titering is not of interest, simply having enough colonies present to fill up a 96 tube mini culture box should be sufficient to provide an accurate representation of the phage diversity and titer does not necessarily matter. However, if the titer of the output phage is low and more colonies are desired, phage can be reamplified by repeating step 5.3. Essentially, the phage can be reamplified by taking the output for the desired selection round, inoculating mid-log phase cells, adding helper phage and carbenicillin, growing the culture overnight, and harvesting the phage via PEG precipitation as previously described.

Other display methods do exist, and each possesses their own advantages and drawbacks relative to phage display. In vivo display methods, such as cell-surface display, may increase the likelihood of proper protein folding and also permit post-translational modifications to occur; however, these methods are constrained by having to use considerably smaller library sizes[13] and expressing proteins polyvalently. Polyvalent expression introduces avidity effects that interfere with and mask the intrinsic affinity of the peptide, which is of greater interest when generating novel binders. Phage display bypasses this issue because it has been adapted for monovalent display, thereby facilitating the selection of binders with genuinely improved affinities[14–16]. Other in vitro display methods are similarly not impeded by the limitations of in vivo display methods but present their own unique challenges. For example, ribosome display may be used to probe larger libraries ($10^{13\text{-}14}$)[17], however, the output of the selections is in the form of mRNA molecules which are inherently less stable than the phage-encapsulated DNA output of phage display[18]. Other in vitro display methods, such as mRNA/cDNA display, cis activity-based display (CIS) and covalent antibody display (CAD) have demonstrated problems with efficiency, stability, and inconsistency[19–23].

Phage display itself is limited by library sizes being restricted by the efficiency of bacterial transformation and by not permitting libraries with sequences that interfere with phage/bacterial growth[16], but generally, these limitations are negligible, and phage display has been successful at producing highly specific and potent binders of target proteins[2, 5, 10, 24, 25]. This can not only be utilized in medical research to develop new therapeutics but also to elucidate protein and enzyme characteristics and learn more about protein interactions involved in important biological pathways.

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DISCLOSURES:

The authors declare no conflict of interests.

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Figure 1

A

UbV	Randomised Residues	Count	Max Values: Normalized Absorbance (BSA)	Max Values: Normalized Absorbance (GST)
1	KFKLTSNRTRIAGMLIKKTHILKSRLARR	9	34.2	15.7
2	QFKYPGNRPRVAGKLQPVTHVLTLRVKAA	8	33.5	14.8
3	QFKRIGQRPSIAGKLLKRTHIVRLLHGDR	7	38.0	18.0
4	QFKLTGHSTRIAGKMRFKHFLLRPRSLFR	7	36.7	17.0
5	SFRFTRKRSSIAGKQLTKTYVLRFQGVKK	5	38.8	19.1
6	RFKAQGERSRIAGMQRKTTHLSRVRVYDS	5	36.0	17.1
7	KFKRNGNRPRIAGNQQKVTHVSRPPGIPK	5	36.2	16.2
8	QRKLTGTRTSIAGNQLELTHLLRLRGAGS	4	28.6	11.4
9	LFKISGTRANITGKQKKVTHLRRIRGETV	3	38.2	18.2
10	NFKLSKRRTRIAGKQQKKTHLLRLSAVER	3	35.4	16.2
11	PFKLTGKRTSIAGNMLKVTHLLRHSKVTA	3	35.6	13.0
12	QFKLRGKTTRIAGLKQKKHYLLRLRVRSY	3	28.9	12.2
13	QKKYPGTTTRIGGKQLKLTHVLIVRAPNI	2	34.7	17.2
14	QFKISGNRARITGKSQKKTHLFRRVGTLA	2	30.7	15.8
15	ERKFTGKKTSIAGKHLKRTHVFRLRDKLE	2	30.9	15.7
16	TFKLPGTRIRIAGYQLKKTHLGSLRDRIA	1	32.1	16.8
17	HFKPTWNRTRIAGMKQKKTFVLTTRRRYE	1	31.9	16.8
18	QFRLAGRRTRITGMHITKTHLWRIEAQPN	1	30.4	16.0
19	QFKFSRTRTRIAGGEQKKTHLLIPRRAVA	1	26.6	14.0
20	MFKLPGKRRRIAGKQAKVTHVLRLRSVPL	1	26.3	13.8
21	QYKLRQPRTRIAGQLLIKTHVLTLRGSHA	1	25.6	13.4
22	PFKRAKKRTTIAGKHVKKTYLLRHRRTSG	1	25.5	13.4
23	PHKLTRPITNIAGKKQAKTHLFRLRRAHS	1	24.2	12.7
24	QFKPTRQRPRITGIQHKKTHLLRLLAAVR	1	23.4	12.3
25	KFKLTGSRSRVGGKKLMKTHLMRLRGHAS	1	22.5	11.8
26	QKKLMGERPSIAGKQRKHTHILRLRGPER	1	19.4	10.2
27	AFKFTRETSRIAGYQKTKHHLLRLRSTLS	1	15.5	8.2

B

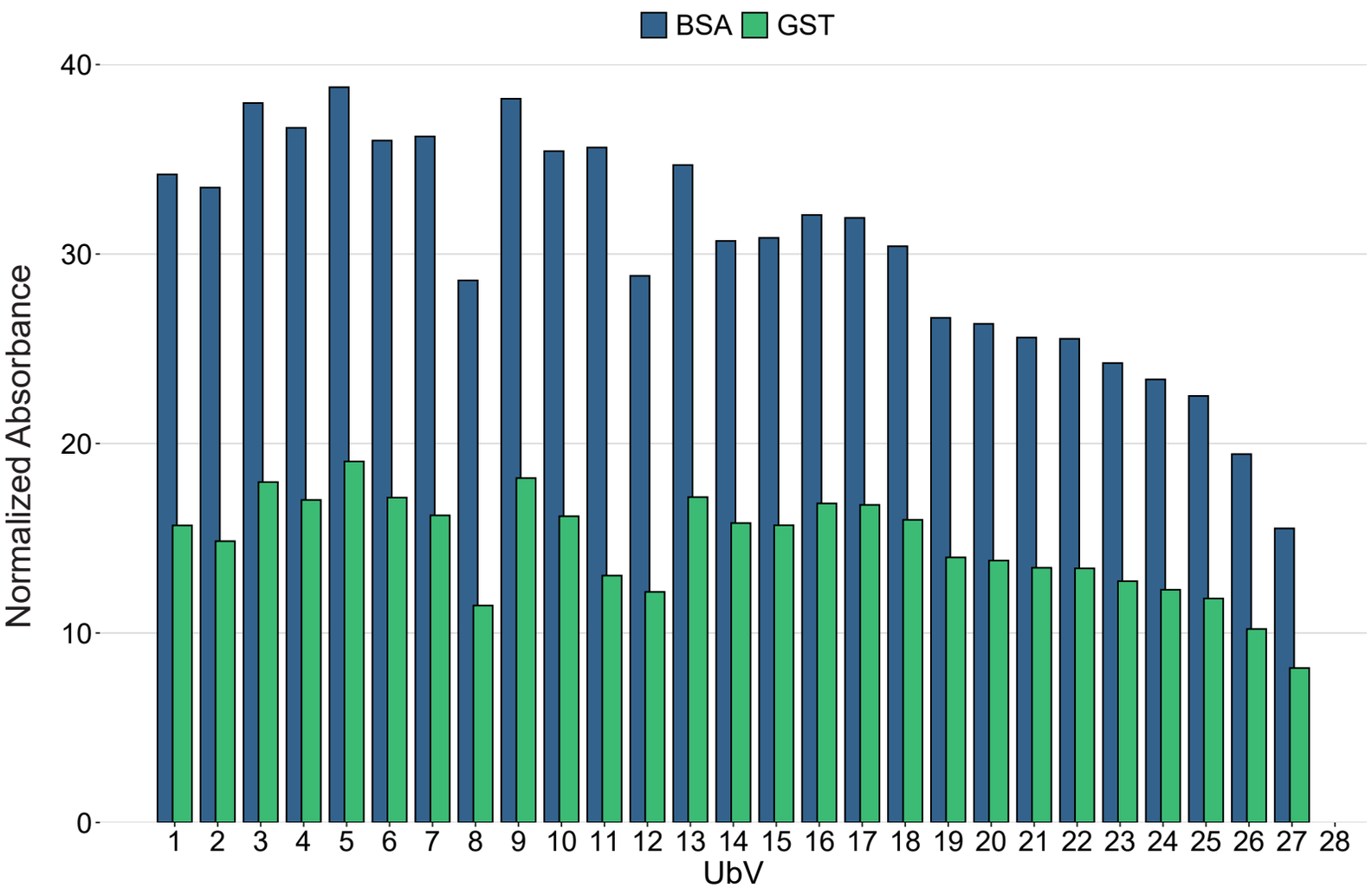


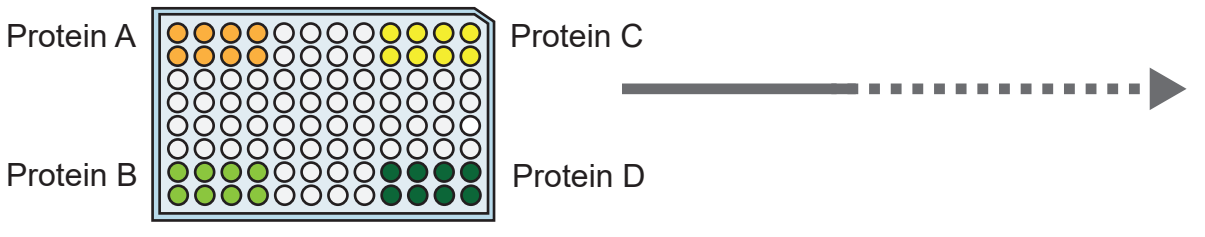
Figure 2

Click here to access/download;Figure;figure2 2.pdf

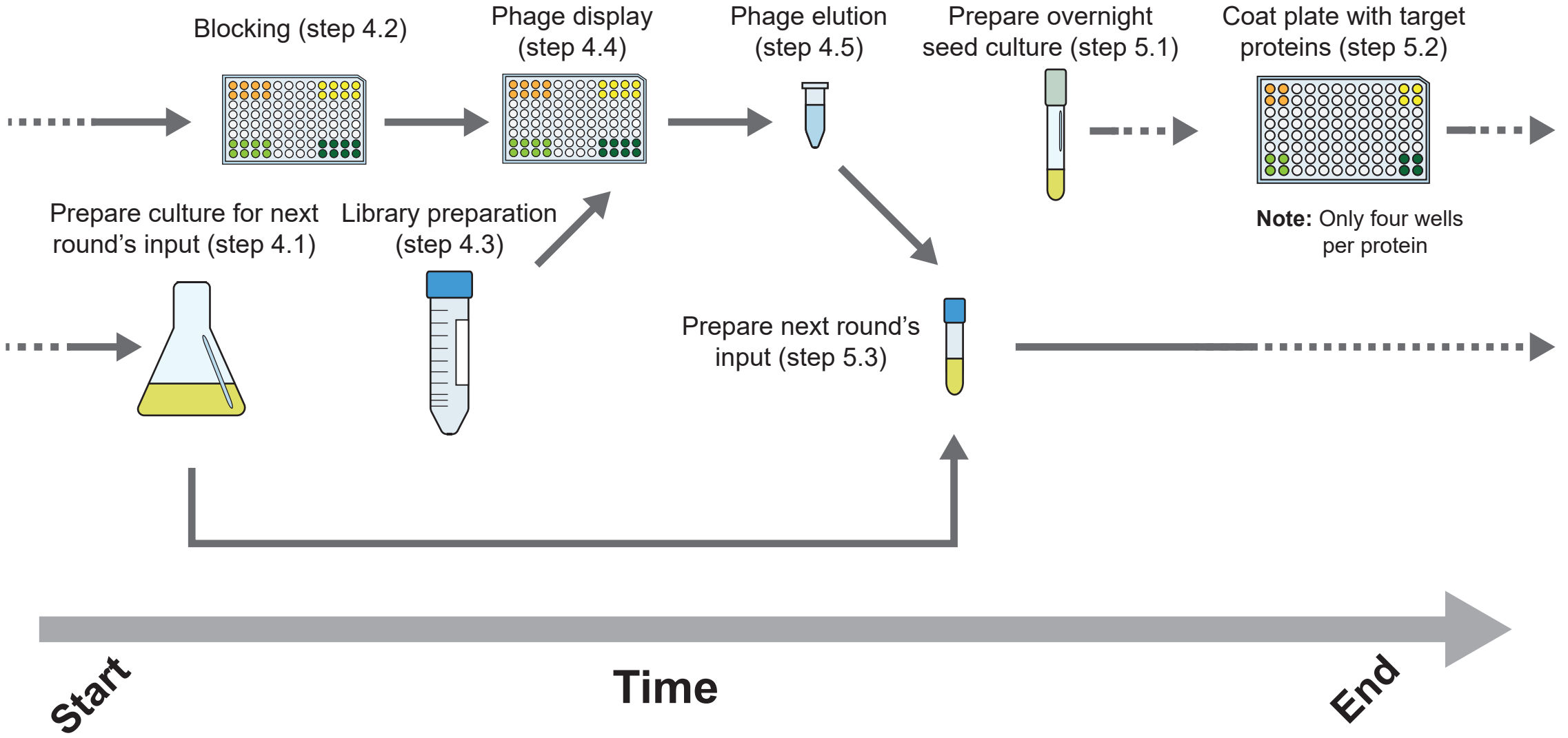
Day 1 Prior to Round One (Step 3)

Prepare overnight seed culture (step 3.1)

Coat plate with target proteins (step 3.2)

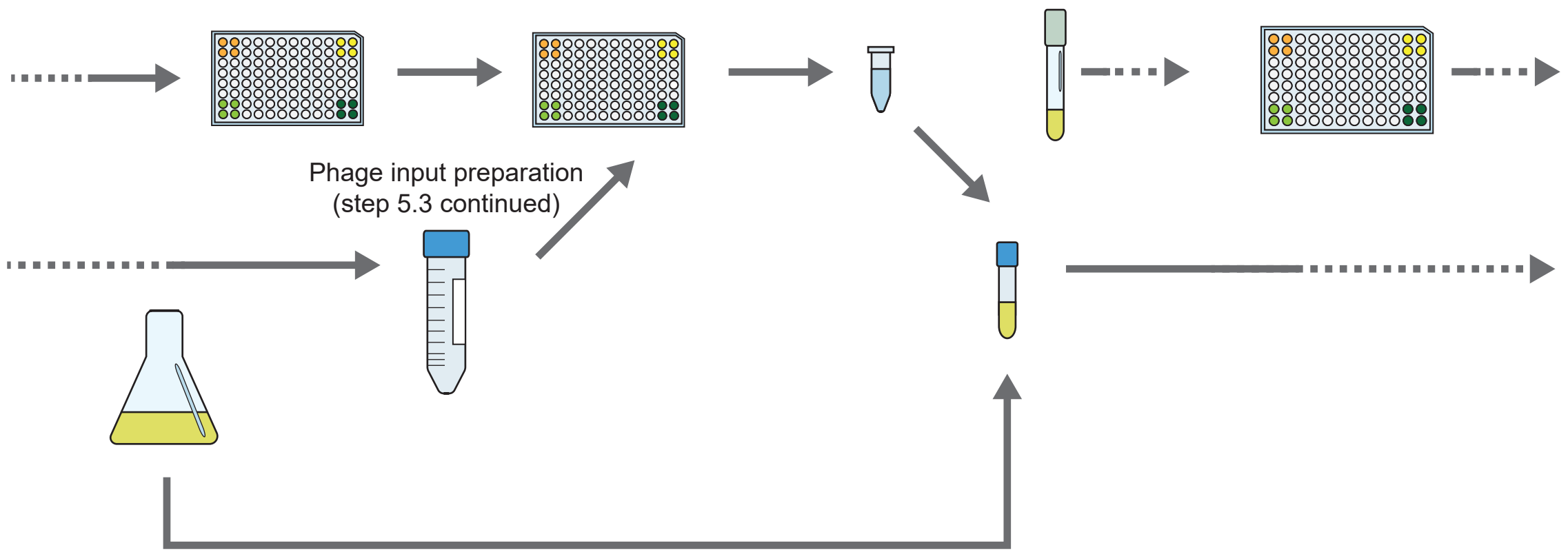


Day 2 Round One (Steps 4 & 5)



Day 3 Round Two (Steps 4 & 5 with changes noted in step 6)

Note: General steps that remain the same are not labeled.



Day 4 Round Three (Steps 4 & 5 with changes noted in step 6)

Day 5 Round Four (Steps 4 & 5 with changes noted in step 6)

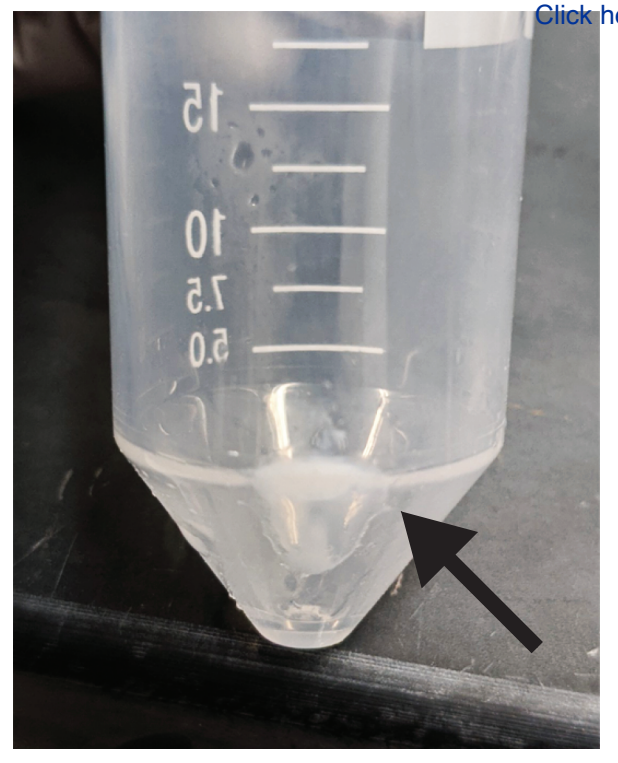
Day 5 Round Five (Step 4 with changes noted in step 6)

Figure 3

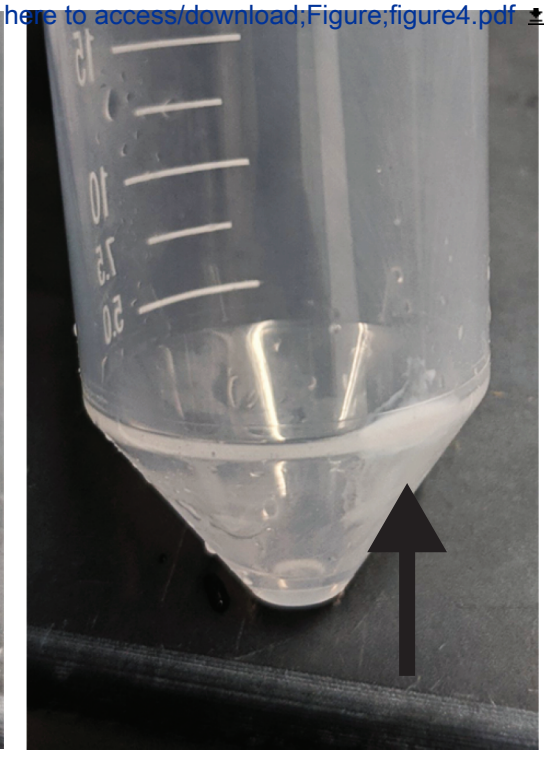
Tube	Label				Purpose
1.5 mL microcentrifuge tubes	R1 - A R2/3 - A R4/5 - A	R1 - B R2/3 - B R4/5 - B	R1 - C R2/3 - C R4/5 - C	R1 - D R2/3 - D R4/5 - D	Protein preparation Step 2.2.1
	R2I - A R3I - A R4I - A R5I - A	R2I - B R3I - B R4I - B R5I - B	R2I - C R3I - C R4I - C R5I - C	R2I - D R3I - D R4I - D R5I - D	Final phage inputs Step 5.3.11
	R1O - A R2O - A R3O - A R4O - A R5O - A	R1O - B R2O - B R3O - B R4O - B R5O - B	R1O - C R2O - C R3O - C R4O - C R5O - C	R1O - D R2O - D R3O - D R4O - D R5O - D	Final phage outputs Step 4.5.5 (and subsequent rounds)
	R2I - A (p) R3I - A (p) R4I - A (p) R5I - A (p)	R2I - A (p) R3I - A (p) R4I - A (p) R5I - A (p)	R2I - A (p) R3I - A (p) R4I - A (p) R5I - A (p)	R2I - A (p) R3I - A (p) R4I - A (p) R5I - A (p)	Phage input debris pellets Step 5.3.10
50 mL centrifuge tubes	Library				Phage library preparation Step 4.3.1
	R2I - A (p) R3I - A (p) R4I - A (p) R5I - A (p)	R2I - A (p) R3I - A (p) R4I - A (p) R5I - A (p)	R2I - A (p) R3I - A (p) R4I - A (p) R5I - A (p)	R2I - A (p) R3I - A (p) R4I - A (p) R5I - A (p)	Phage input cell pellets Step 5.3.6
	R2I - A (peg) R3I - A (peg) R4I - A (peg) R5I - A (peg)	R2I - A (peg) R3I - A (peg) R4I - A (peg) R5I - A (peg)	R2I - A (peg) R3I - A (peg) R4I - A (peg) R5I - A (peg)	R2I - A (peg) R3I - A (peg) R4I - A (peg) R5I - A (peg)	Phage input precipitation Step 5.3.8
Culture tubes	R2I - A R3I - A R4I - A R5I - A	R2I - B R3I - B R4I - B R5I - B	R2I - C R3I - C R4I - C R5I - C	R2I - D R3I - D R4I - D R5I - D	Phage input culturing Step 5.3.1
Test tubes	R2I R3I R4I R5I				Phage input seed culture Step 3.1.1 (and subsequent rounds)
250 mL Erlenmeyer flasks	R2I - A R3I - A R4I - A R5I - A	R2I - B R3I - B R4I - B R5I - B	R2I - C R3I - C R4I - C R5I - C	R2I - D R3I - D R4I - D R5I - D	Phage input mid-log phase culture Step 4.1.1
	R2I R3I R4I R5I				Phage input mid-log overnight culture Step 5.3.5

Figure 4

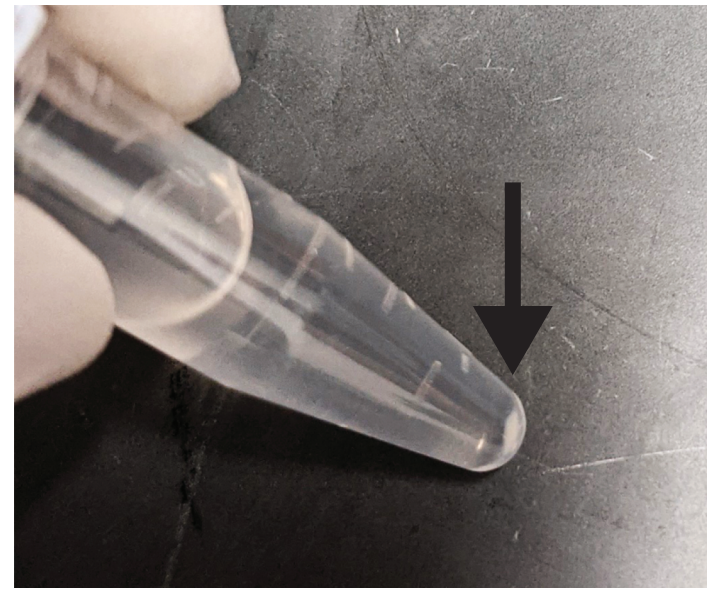
Phage library
pellet (step 4.3.4)



Phage input pellet:
First pelleting (step 5.3.8)



Phage input pellet:
Second pelleting (step 5.3.8)



Phage input debris
pellet (step 5.3.10)

JoVE62950
Using Phage Display to Develop Ubiquitin Variant Modulators for E3 ligases

Response to Editor and Reviewers

Editorial comments:

Point 1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response 1: We have checked the whole manuscript and all spelling, grammatical, and formatting errors have been corrected.

Point 2. Please revise the following lines to avoid previously published work: 53-54.

Response 2: Thank you for this suggestion. We have reworded this part to avoid previously published work and added more details to make the point clearer.

Point 3. 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. For example: OmniMax, Axygen Mini Tube System, etc.

Response 3: We have removed all commercial language from the manuscript.

Point 4. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. 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. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) 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.

Response 4: We have checked and confirmed that it contains all the details for readers to replicate the protocol.

Point 5. Line 127-128: Is there any specific technique used to find the concentration of the target protein.

Response 5: Measuring protein absorbance at 280 nm has been recommended in **step 2.1** as the most convenient way to do so.

Point 6. Line 168-169: During the re-centrifugation is there any medium added or is the tube placed back in the centrifuge after discarding the supernatant post first centrifugation.

Response 6: No medium is added after the first centrifugation and the second centrifugation is only intended to pull down any remaining supernatant. This has been clarified in **step 4.3.4**.

Point 7. Lines 237-248: 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.

Response 7: The tense has been corrected in the aforementioned places.

Point 8. Please include a one-line space between each protocol step and then highlight up to 3 pages 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.

Response 8: The spacing has been corrected and the highlighted steps (in yellow) have been reviewed to ensure they include the most critical points.

Point 9. Please include a title and a description of each figure and/or table in the Figure and Table Legends section. All figures and/or tables showing data must include measurement definitions, scale bars, and error bars (if applicable).

Response 9: Figure titles have been updated and made clearer where necessary.

Point 10. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials and sort the table in alphabetical order.

Response 10: We have removed the symbols. The table has also been sorted in alphabetical order.

Reviewer #1:

Manuscript Summary:
The manuscript describes a protocol for phage display of ubiquitin binding variants. It is a detailed protocol with clear instructions for each step of the process, as well as some introduction about potential applications.

We sincerely thank the reviewer for the positive comments on our manuscript.

Major Concerns:

No major concerns

Minor Concerns:

Point 1: No minor concerns besides ensuring that temperature is listed everywhere that it is a variable - for example, is there a range for room temperature?

Response 1: Thanks for this suggestion. Room temperature has been defined as ~20-25 °C in all relevant places in the text.

Reviewer #2:

Manuscript Summary:
In this review, Roscow and Zhang describe the procedure for performing phage display with an already-established ubiquitin variant library. The description of the protocol for selection is comprehensive and would provide a solid basis for performing selection with phage.

However, there are clarity issues in the way it is written. I am aware that the major part of JOVE is the video that accompanies the text, and I suspect many of these issues would be resolved by the video. Regardless, some points are included below. Note that these are not comprehensive, but are some errors or unclear sections that I noted.

We agree with the reviewer that the clarity can be improved. In addition to address all the points raised by this reviewer (see below), we went through the text thoroughly to make sure all procedures were described clearly and readers can follow with no issues. As the reviewer indicated, the accompanied video will provide further information in case any points are not clear.

Minor Concerns:

Point 1: Line 50 - I would say that the majority of Ub-protein interactions happen via this surface, but needs to be reworded to account for other potential interactions. Perhaps better to say '...the vast majority recognize a common surface...' ?

Response 1: This is a very good point. Thank you for the suggestion. Indeed other types of interactions can happen between ubiquitin and proteins of interest. The text has been revised as suggested in the 2nd paragraph of Introduction, and now more accurately reflect the nature of ubiquitin interactions.

Point 2: line 56-58 should be clarified as it mixes up protein and DNA in an unclear way

Response 2: We agree that the original description is not clear. We have modified this section (3rd paragraph of Introduction) and the Abstract to avoid any confusion.

Point 3: 77 - 'against' a target protein of interest?

Response 3: Thanks for noting this. We have revised this sentence to fix the error.

Point 4: 84: PBS - recipe?

Response 4: PBS is prepared from a 10xPBS solution that is commercially available (Table of Materials). We have made this clear in the text.

Point 5: 84: H2O - deioinised? Tap water?

Response 5: All references to water have been changed to reflect that it is specifically ultrapure water being used.

Point 6: 101: LB mixture? Miller, etc?

Response 6: This is a mixture that is pre-made by a manufacturer (Table of Materials). This has been clarified in the relevant lines in the text.

Point 7: 115: Tris

Response 7: We have revised this accordingly.

Point 8: 127: Are there any ways you would recommend to quantify protein (ie; Abs280, Bradford, etc.) What do you mean by account for full length/truncated etc?

Response 8: Absorbance at 280 nm is sufficient to determine protein concentrations for this purpose and has been noted in **step 2.1** and the 1st paragraph of Discussion. Regarding the note of accounting for changes of protein length or tags, we were referring to changes in molecular weight while calculating for molarity from mass concentration.

Point 9: The authors should discuss options for immobilising proteins. On line 144 they say to use GST as a negative control, but can one also use MBP or lysozyme, etc.? What if you want to immobilise via a biotin tag?

Response 9: Yes, other epitope tags can be used. We have modified the text to make this point clear (**step 3.2.2**). The negative control should be the same as whatever tag was used to purify the protein of interest. This way, any interference due to interactions with the peptide tag can be accounted for. If the protein is biotin-tagged, then the immobilization is done by first coating the plates with neutravidin/streptavidin (room temperature overnight), followed by 1-hour incubation with biotinylated proteins in the next day.

Point 10: 148-222 why is this section highlighted?

Response 10: This section was highlighted as an indication of the steps to be filmed into the video. As filming can take place over only a single day, only steps from a single round can be represented in the video. The steps corresponding to the first round of display contain the most critical and most numerous steps. After this round, subsequent rounds proceed very similarly. Since the first round forms the foundation for all subsequent rounds, it is imperative to perform this successfully for later rounds to be of any value.

Point 11: 163-172: are we aiming for a particular concentration of phage? How do we determine phage concentration?

Response 11: Yes, we are aiming for 100x library diversity and this has been explained in **step 4.3.1**. Further details are given along with a reference to previous methods in the 3rd paragraph of Discussion.

Point 12: 177: the authors should explain why you would do a control plate first. For the titration section

Response 12: This is to account for any undesirable interactions between the binder and that tag on the target protein of interest. This has been noted in **step 3.2.2**.

Point 13: 214: 11 000 rpm or 11 000 g?

Response 13: This is RCF and has been corrected accordingly in **step 5.3.8**.

Point 14: 224-233: What is the intended outcome of the titrations? Would it make sense to compare titrations of selection against a control (like GST) with selection against your target protein?

Response 14: Yes, this is precisely the reason for the control plate mentioned in **step 3.2.2** and **step 4.4.1**.

Point 15: 232-233: inconsistent - is it 10-100 uL or 5 uL for titrations?

Response 15: This has been simplified in **step 5.4.4** to just say 5 µL.

Point 16: 262: without disturbing the pellet

Response 16: This has been added to **step 7.2.5** for clarity.

Point 17: 350-364: the authors should discuss yeast display as well

Response 17: Thank you for this suggestion. Yeast display was not elaborated upon on its own because it is one of the cell-surface display methods, which we spoke about in general terms in the Discussion (2nd from last paragraph).

Point 18: 369: Can similar techniques as described in the review be used for other kinds of libraries (eg: peptide, antibodies)? If so, the authors should mention this in the discussion.

Response 18: The general steps should be very similar, but the protocols described here are specific for UbV selections. We have noted this limitation in the 3rd paragraph of Discussion to indicate that various aspects of the protocol were adapted and optimised for UbV selections.

Point 19: Figure 1 - needs a better caption. Why is the ELISA against GST included in this figure? Why is only one section of diversified ubiquitin shown in this Figure? What trend are we meant to observe here?

Response 19: GST was included as a control for non-specific binding due to the fact that the target protein is GST-tagged. There is only one section of diversified ubiquitin but that includes all the randomized residues. The trend is that binding affinity (as shown in

normalized ELISA value) increase as the sequences increase in frequency. We have modified the legend to reflect all these points.

Point 20: Figure 2 - how were the values normalised? What is the 'ELISA score'?

Response 20: "ELISA score" has been corrected to "Normalized Absorbance" (**new Figure 1B**). These values were the actual absorbance value normalised again the controls (BSA or GST).

Point 21: The layout of Figure 3 makes it difficult to understand (again, this may be clarified by the video)

Response 21: We apologize for the confusion. We have now explained the process in more detail in the legend (**new Figure 2**). Moreover, in the video we will show the actual steps so that it can be understood more easily.

Point 22: A general question, and perhaps out of the scope of the review, but could you add some tips for how to decide which UbVs to pursue more comprehensively? In Figure 1 and 2, there is no obvious 'winner' phage, but if one wanted to clone a subset of these and express them recombinantly, a way to pare them down would be helpful.

Response 22: This is a very good question. We have now provided suggestions in the 4th paragraph of Discussion as to which UbVs should be pursued more comprehensively in downstream analysis.

ELISA analysis code

Sequence analysis code

UbV analysis code