Video Article

A Protocol for Phage Display and Affinity Selection Using Recombinant Protein Baits

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Abstract

Using recombinant phage as a scaffold to present various protein portions encoded by a directionally cloned cDNA library to immobilized bait molecules is an efficient means to discover interactions. The technique has largely been used to discover protein-protein interactions but the bait molecule to be challenged need not be restricted to proteins. The protocol presented here has been optimized to allow a modest number of baits to be screened in replicates to maximize the identification of independent clones presenting the same protein. This permits greater confidence that interacting proteins identified are legitimate interactors of the bait molecule. Monitoring the phage titer after each affinity selection round provides information on how the affinity selection is progressing as well as on the efficacy of negative controls. One means of titering the phage, and how and what to prepare in advance to allow this process to progress as efficiently as possible, is presented. Attributes of amplicons retrieved following isolation of independent plaque are highlighted that can be used to ascertain how well the affinity selection has progressed. Trouble shooting techniques to minimize false positives or to bypass persistently recovered phage are explained. Means of reducing viral contamination flare up are discussed.

Video Link

The video component of this article can be found at http://www.jove.com/video/50685/

Introduction

Why use phage display and affinity selection instead of the myriad other techniques available for discovering and investigating protein interactions with other molecules? Phage display can claim some unique advantages over other methods of detecting protein-ligand interactions[1-3] including the following:

Very wide repertoire of bait molecules

The foremost reason is in the diversity of molecules capable of acting as bait in affinity selection[4]. Phage display is a very powerful means of isolating protein fragments that interact with other proteins, nucleotides, carbohydrates, *etc.*[5]. Essentially, if a polymer/molecule can be attached to a recoverable support, it can be screened for affinity with phage displayed proteins. Additionally, the bait molecule can be accessed to determine if it retains biological activity when immobilized[6], if conditions used to reduce/eliminate its activity are effective[6] or, to introduce post-translational modifications to the bait prior to affinity selection.

Phage resistance to extraneous factors

A second reason for using phage display, is that it is possible that some baits may require environmental stress (heat, high osmolyte concentrations, specific cofactors, etc.) to capture their interacting proteins, or the phage displayed proteins may need to be somehow altered prior to affinity selection. The primary factor being studied is the interaction between bait and protein, not the condition that allows the interaction to occur or if it is lethal to the test organism. The T7 phage is particularly well suited to such studies because it can withstand harsh experimental conditions both intact and viable (*e.g.* the published thermal maximum for T7 viability is ~60 °C[7]). As an example of altering phage displayed proteins, when examining the *Arabidopsis thaliana* seed proteome for protein substrates of the repair enzyme, PROTEIN ISOASPARTYL METHYLTRANSFERASE (PIMT), the virus used in these studies had been "aged" for one week, prior to each affinity selection round, to encourage introduction of isoaspartate (isoAsp) residues in susceptible proteins[6] which is not possible in organisms that can recognize and repair/metabolize such abnormalities.

Metabolically inert

Furthermore, the phage are usually resistant to metabolic poisons and interfering small molecules that would, at the least, result in pleiotropic effects on metabolically active test organisms. Following the stringency washes, the poison is removed before a large volume of bacteria are introduced for infection so the poison is diluted to a range innocuous to the bacteria or subsequent phage replication. While investigating protein

targets of the PIMT repair enzyme, S-Adenosyl Methionine (AdoMet) was used to activate the micro-titer-plate-well immobilized enzyme to permit target protein capture while relying on S-Adenosyl Homocysteine (AdoHcy) to inactivate the enzyme and provide a useful negative control secure in the knowledge that the virus would not be adversely influenced by either AdoMet or AdoHcy [6]. Additionally, members of certain LATE EMBRYOGENESIS ABUNDANT (LEA) protein families, investigated in this lab, are known to alter their shape in the presence of additives such as sucrose [8], which can attain concentrations as high as 200 mM in soybean seeds at the point of physiological maturity [9]. The virus is not anticipated to be influenced by addition of 200 mM sucrose in each affinity selection round, possibly necessary for certain LEA-client protein interactions, which is not the case for autonomously viable test organisms [10].

This lab has focused on discovering protein-protein interactions in mature, dehydrated or germinating seeds that underlie the mechanism of stored proteome protection during dehydration [11] or repair of components of the stored proteome that are susceptible to isoAsp formation once the seed has imbibed [6]. Thus, the production and purification of the recombinant proteins required as bait in an active state, and ensuring that they remain so, before and after they are immobilized, although frequently difficult, is a cornerstone to our work. However, as each recombinant protein production scenario is different, optimizing conditions for recombinant protein production will not be addressed here. Users are urged to attempt, wherever possible, to determine if the immobilized protein is still functional (*e.g.* if it is an enzyme, do an enzyme assay in the microtiter plate wells). This will provide some confidence that the bait is biologically active and therefore, that any discovered interactions are somewhat more likely to have biological relevance.

Protocol

A graphic depiction of the procedure described below (**Figure 1**) highlights the two primary components for affinity selection using a phage display library: A) a phage display cDNA library likely to encode proteins with affinity for the bait and; B) a purified recombinant bait protein. Production of bait (recombinant protein) has been extensively examined and literature outlining best practices for securing soluble, active recombinant protein from *E. coli* [12-13], eukaryotic yeast [14], insect [15-16], plant [17-18], or mammalian [19-20] cells abound.

In the following protocol, a hexahistidyl tagged recombinant protein has been used as bait. This allows verification that the bait proteins remain in the wells after overnight incubation and washing steps.

1. ELISA for Recombinant Protein Retention in the Microtiter Plate Wells

1. Mark a microtiter plate with permanent ink, and designate which wells will contain which protein concentrations. Perform this in 3 replications of wells. Include 3 replications of a negative control concentration series (non-hexahistidyl-tagged protein; BSA works well as this background check).
2. Wash the plate extensively with water and remove water by smacking the plate upside down on 4 layers paper towel between washes.
3. Immobilize, in the first 3 wells, in 100 µl of Tris, pH 7.5, (or buffer of choice) the highest concentration of the recombinant protein (10 $\mu g \cdot ml^{-1}$). Add to the next three wells the recombinant protein at (1.0 $\mu g \cdot ml^{-1}$), etc, down to 1.0 $ng \cdot ml^{-1}$. Do the same with the BSA concentration series.
4. Cover dishes with plastic wrap and leave overnight at 4 °C.
5. Next morning, remove protein by smacking plate upside down on 4-5 layers paper towel.
6. Wash wells 5 times with 200 µl 1x TBS each time, leaving the buffer in the wells for ~1 min each time and removing the buffer wash by smacking the plate upside down on the paper towels after each wash.
7. Block the ELISA plate on a rotary shaker using 200 µl 5% (w/v) BSA or 200 µl 5% (w/v) blocking reagent in TBS at room temperature for 2 hr (or sitting stationary overnight at 4 °C if convenient) wrapped in plastic wrap.
8. Remove excess blocking solution by smacking the plate upside down on the paper towels. Wash 4 x 1 min each time with 200 µl of 1x TBS, removing the wash solution by smacking the plate upside down.
9. Dilute penta-HIS primary antibody 1/2000 in blocking buffer and deliver 100 µl to each well. Incubate 1-2 hr at room temperature with the plate stationary.
10. Remove the primary antibody by smacking the plate upside down on the paper towels. Wash 4 x 1 min each time with 200 µl of 1x TBST. Remove each wash by smacking the plate upside down.
11. Dilute the secondary antibody (Goat anti mouse alkaline phosphatase conjugate), in blocking buffer and incubate 100 µl in each well for 1 hr at room temperature with the plate stationary.
12. Remove the secondary antibody by smacking the plate upside down on the paper towels. Wash 4 x 1 min each time with 200 µl of 1x TBST. Remove each wash by smacking the plate upside down.
13. Place 200 µl of *para*-nitrophenylphosphate (pNPP) substrate solution in each well. The substrate is a solid at -20 °C so make aliquots and retrieve the requisite number of aliquots necessary for detection out of the freezer well ahead of time so it is completely thawed by this stage.
14. At 30 min stop the reaction by adding 50 µl of 3M NaOH to each well.
15. Read the absorbance at 405 nm immediately on the ELISA plate reader.

Note: If the recombinant protein of interest does not attach itself to the wells, it is possible to alter the composition/pH of the buffer considerably (Carbonate buffer pH 10.0) or add chaotropes (urea) to attempt to assist protein attachment to the microtiter plate wells. However, re-establishing that the recombinant protein: 1) remains bound to the microtiter plate wells under the conditions for the affinity selection and; 2) retains its biological activity following removal of the high pH/chaotrope is recommended.

2. Growing Bacterial Host (BLT5403) for Titering

1. Autoclave (**Figure 2A**) ten 250 ml Erlenmeyer flasks and 3 culture tubes. Make LB liquid and LB agar for pouring solid media plates. Cool LB agar to 50 °C and add ampicillin to 100 µg/ml before asceptically pouring into Petri dishes in a flow hood (**Figure 2B**).

2. Also in a flow hood (**Figure 2B**) streak an LB, 100 µg/ml ampicillin (LB^{AMP100}) agar plate for single colonies of BLT5403 from stock kept in 15% (v/v) glycerol at -80 °C (**Figure 2C**). Place plate upside down at 37 °C overnight in an incubator for growth (**Figure 2D**).
3. In the morning, inoculate 50 ml LB^{AMP100} in a 250 ml Erlenmeyer flask with a single colony picked from the plate. Incubate at 37 °C, 200 rpm in a horizontal shaker (**Figure 2E, F**), and use a spectrophotometer to monitor cell density at OD_{600} = 0.5 to 0.6 (**Figure 2H**).
4. Pour the cells into a 50 ml Falcon tube or similar, and keep at 4 °C until use (**Figure 2G**). Cells will usually remain viable for ~1 week.
5. Make 500 ml LB, place it in a baffled Fernbach flask and autoclave it. Once it is sterile, place the flask at 4 °C (**Figure 2G**) or room temperature until the night of "DAY ONE" below.

Note: The host bacterial cells BLT5403, expressing a plasmid-borne source of the T7 native capsid protein without which the T7 mid-, and low-copy vectors cannot replicate successfully, are extremely susceptible to the virus. It is imperative to avoid contamination. Contamination will eventually occur at which point all surfaces in contact with the virus/infected bacteria must be wiped with 70% (v/v) ethanol or scrubbed using detergent (**Figure 2I**). If this is ineffective, a thorough decontamination of all surfaces that can withstand Envirocide (**Figure 2J**) is required. Start BLT5403 cells from freezer stock each new round of affinity selection to help mitigate contamination of the stock in 4 °C, and ensure vigorous cells are being used in the protocol. Filter barrier pipet tips are essential.

3. Affinity Selection

DAY ONE

1. Mark a microtiter plate with permanent ink, and designate which wells will contain which proteins/amendments. (Due to contamination issues, plates are never reused). Perform this in 3 replications of wells for each protein and treatment.
2. Wash the plate extensively with water and remove water by smacking the plate upside down on 4 layers paper towel between washes. Immobilize, in 100 µl of Tris, pH 7.5, (or buffer of choice) the recombinant protein (10 µg/ml) in six wells, or BSA (10 µg/ml) in three wells, for a total of 9 wells for first round of affinity selection. Cover dish with plastic wrap and leave overnight at 4 °C (**Figure 2G**).
3. Ensure that there are 9 x 250 ml Erlenmeyer flasks (see 2.1 above) cleaned, autoclaved, and labeled appropriately (coding with colored tape works well, **Figure 2F**).
4. Calculate the volume of phage lysate to use for each well based on the titer of the library being used and the number of phage to be screened.
5. Ensure that fresh BLT5403 cells are ready to use for titering this affinity selection round tomorrow (**Figure 3A**).
6. Ensure sufficient 1x TBS (150 mM NaCl, 50 mM Tris Base, pH 7.6) and 1x TTBS (1x TBS + 0.05% (v/v) Tween 20) are available to perform 10 x 200 µl washes for the number of wells being used. Additionally, pre-incubate the TTBS at the affinity selection temperature. Ensure that a backup, sealed, 50 ml Falcon tube of 1 x TTBS exists at the affinity selection temperature with sufficient 1x TTBS.
7. Prepare 3 treatments x 3 replications x 3 triplicates of 1.5 ml Eppendorf tubes (27 total) with 990 µl of $LB^{100\ AMP}$ each and label appropriately (*e.g.* 10^{-2}). These are for the infected bacteria retrieved from the microtiter plate wells tomorrow. Mark the dilution on one side of the tube (10^{-2}) and an ascending number on the other side ("1"). For each Eppendorf tube, also label one borosilicate tube and one $LB^{100\ AMP}$ plate. In this way, the plates and borosilicate test tubes are numbered 1, 2, 3, 4, 5, etc. to make titering go faster. Afterward the simple number can be matched to the dilution and the treatment on the Eppendorf tube (*e.g.* the #12 tube was the third triplicate of the first replication of the BSA control for dilution 10^{-5}). Store Eppendorf tubes and $LB^{100\ AMP}$ plates overnight at 4 °C (**Figure. 2G, 3B**).

For example: Start labeling 1.5 ml Eppendorf tubes for three 10^{-2} dilutions of phage from the BSA replication one well as 1, 2, and 3 (three triplicate readings of phage titer for replication one), and then mark borosilicate test tubes 1, 2, and 3 in which the infected bacteria will be mixed with uninfected bacteria and top agarose before pouring on $LB^{100\ AMP}$ agar plates (**Figure 3C**).

8. For the serial dilutions, label Eppendorf tubes and, to each, add 900 µl of $LB^{100\ AMP}$. Once the initial dilutions (10^{-2}) of infected bacteria are made for each protein/treatment, replication, and triplicate, tomorrow, they will be mixed well and 100 µl taken from them and added to the appropriate Eppendorf tube containing 900 µl $LB^{100\ AMP}$ for the 10^{-3} dilution (tubes 4, 5, and 6 for replication one BSA control). These will be mixed well in their turn and the 10^{-3} dilutions will be used to make the 10^{-4} dilutions (7, 8, 9). Use the 10^{-4} dilutions for the 10^{-5} dilutions (10, 11, 12), etc. to get the titer for the first of the three BSA replications (wells).
9. The same will be done for BSA replication two (well 2), BSA replication three (well 3), the three replications of the poisoned bait, and finally the three replications of the bait wells. At the end, there should be tubes labeled from 1 to 135 (135 is the last triplicate of the last replication of the bait at the maximum dilution of 10^{-6}). Store these Eppendorf tubes overnight at 4 °C (**Figure 3C** shows the Eppendorf- and borosilicate-tubes for all dilutions of the triplicates of the three replications (the three wells) of BSA.
10. Start 2 or 3 culture tubes of BLT5403 cells (picked from single colonies from a freshly streaked overnight LB^{AMP100} plate; from step 2.2 above) growing in the incubating shaker (**Figure 2E, F**) as an overnight culture. To the 500 ml LB (point 2.5 above), add ampicillin to 100 µg/ml and place the Fernbach flask in the clamp in the shaking incubator so it will be at 37 °C and aerated the following morning (**Figure 2F**).

Note: Round three and four may require approximate dilutions of as much as 10^{-10} volume of phage to volume of to LB^{AMP100}.

DAY TWO

11. Next morning, place the autoclaved, empty 9 x 250 ml Erlenmeyer flasks (one for each microtiter plate well) in the clamps in the incubating shaker. Innoculate the 500 ml $LB^{100\ AMP}$ with 500 µl from one of the overnight cultures of BLT5403 cells started last night (see 3.8 above), reseal and start it growing (37 °C, 220 rpm). Turn on the spectrophotometer (**Figure 2H**) and set it to read absorbance at 600 nm.
12. Remove the microtiter plate from 4 °C, remove the plastic film, and remove any unbound protein from the plate by washing 10 times with 200 µl each time of 1x TBS pH 7.5 and smacking the plate upside down on the paper towel between washes. Block with 200 µl 5% (w/v) BSA or 200 µl 5% (w/v) blocking reagent in TBS for 1 hr (or overnight if convenient) with the plate wrapped in plastic wrap.

13. Wash off the blocking solution 10 times with 200 µl each time of 1x TBST, smacking the plate upside down on 4 layers of paper towel between washes. It is possible to refill the wells with 200 µl water at this stage, cover them with plastic wrap and put them at 4 °C to store for maximum 1 week.

Note: Start the culture soon enough that, by the time the phage infected-BLT5403 from the completion of the affinity selection are ready to add, the cells in the 500 ml are between 0.6 and 1.0 OD_{600}. If they grow much beyond 1.0, lysis may not occur due to resource restrictions as cells approach stationary phase. If the cells do not attain an OD_{600} of 0.6 at least prior to the introduction of phage, the multiplicity of infection can be too high, rapidly killing the cells, which can influence the representation of the lysate. If the cells are not yet approaching an OD_{600} of 0.6 by the completion of the affinity selection, inoculate the flask with more BLT5403 from the second (or even from both second and third) test tubes shaking at 37 °C (**Figure 2F**). If an OD_{600} of 1.0 is approaching too fast, turn off the heater and the shaker and open the lid to cool the culture and starve the bacteria for oxygen. Recommence heating/shaking once the phage have been added.

FROM HERE USE FILTER BARRIER TIPS TO AVOID PHAGE CROSS-CONTAMINATION

14. Once the flask is inoculated, put the phage library (100 µl) in with the proteins, re-seal the plate with plastic wrap and incubate at room temperature for 1 hr.
15. At 55 min, record the OD_{600} of the cells. The doubling time is roughly every 20 min. Act accordingly (see "Note" above).
16. At the end of one hour remove the plastic wrap from the plate and immediately wash by shaking out the phage into the transformed waste and then rapidly adding 200 µl of 1x TBST. (Use a multipipettor with a 1 = 100 µl head and the pipettor set on 2 [*i.e.* delivers 200 µl per plunger depression] for this and it goes quickly). Set a timer for 1 min. After 1 min, shake out buffer into transformed waste, smack plate upside down on paper towel and place back on bench (25 °C plate). Repeat washing steps ten times.
17. After the 10^{th} wash, take 200 µl of BLT5403 cells from the 50 ml Falcon tube at 4 °C and put them in the bottom of the well from which the last wash of TTBS has been removed. Wrap the plates in plastic wrap and put at 37 °C for 20 min to get any phage still stuck to the bait to infect the bacteria (see note in discussion concerning persistently retrieved phage and/or high background from indiscriminately binding phage).
18. At the end of 20 min, unwrap the plates and take 10 µl bacteria from the BSA at 25 °C replication one well and put it in the 990 µl of $LB^{100\ AMP}$ marked "1". Repeat twice more for that well (tubes 2 and 3) resulting in 3 triplicates of 1/100 dilutions of the phage from that well. This is BSA replication one sampled three times. These dilutions will be used to estimate the average titer ± SEM for that replication of BSA.
19. Do the same for replication 2 and 3 of BSA (three triplicate samples of 10 µl each), for the three replicated wells of the poisoned bait protein, and for the bait protein. Set these 27 Eppendorf tubes aside and get the remaining 170 µl of the infected bacteria in the wells growing toward lysis.
20. If the bacteria in the flask is at OD_{600} = 0.6 to 1.0, decant 50 ml cells from the Fernbach flask into each of the nine 250 ml Erlenmeyer flasks. Take the remaining 170 µl of phage-infected BLT5403 bacteria in the BSA replication 1 well and add it to the 50 ml cells marked "BSA Rep 1" (yellow tape). Do this for all nine wells and put them shaking in the incubator (**Figure 2F**).
21. Monitor the cells in the 37 °C incubating shaker from time-to-time for lysis (about 3 hr from the time of inoculation). Make the dilutions from the 27 initial dilutions (10^{-2}) in the appropriate, labeled Eppendorf tubes during this time.
22. To perform these dilutions from the initial 27 dilutions from 3.12 above, take 100 µl of the LB with bacteria from Eppendorf tube 1 (BSA replication one, first of the triplicate samples from this 1/100 dilution from this well) and add it to the 900 µl of LB in Eppendorf tube 4 (1 x 10^{-4} dilution of BSA replication one, first of the triplicate samples from this well).
23. Discard the filter barrier tip for a new one before getting 100 µl of the LB with bacteria from Eppendorf tube 4 to add to the 900 µl of LB in Eppendorf tube 7 (1 x 10^{-5} dilution of BSA replication one, first of the triplicate samples from this well). Continue the dilutions (down to 1 x 10^{-6}) for all triplicates of all replications of all proteins and treatments (135 tubes in total).
24. Once the cells have lysed, bring the culture to 0.5 M NaCl by adding 5 ml from a 5 M NaCl stock and transfer to a labeled centrifuge bottle.
25. Centrifuge at 8,000 x g for 10 min at 4 °C. Decant supernatant (virus) into a clean, sterile 50 ml Falcon tube.
26. Add a few drops of chloroform to the decanted supernatant in the Falcon tube.
27. Store at 4 °C for the next round of affinity selection.

4. DAY THREE

1. Autoclave or bleach all labware that has been used in generating this affinity selection round to prepare for the next round.

5. Titering

1. Number as many solid $LB^{100\ AMP}$ plates as there are dilutions in ascending order (there should now be 1 plate for every borosilicate tube and Eppendorf tube, each with the SAME number). Put the plates in the 37 °C incubator (**Figure 3D**).
2. Melt top agarose (1.0 g Bacto Tryptone, 0.5 g Yeast Extract, 0.5 g NaCl, 0.6 g agarose, make to 100 ml, autoclave) by **loosening** the top agarose bottle cap and alternately microwaving the bottle and swirling it to mix it until the top agarose has completely melted. Put the bottle in the water bath to cool to 50 °C (**Figure 3E**).
3. Take a borosilicate 10 ml pipet with a pipet pump attached. Light a Bunsen burner. Turn a Styrofoam Falcon tube holder or cooler lid upside down on the bench and place the LB100 AMP plates 1 through 6 (fresh out of the 37 °C incubator) on it, Plate 1 uppermost (**Figure 4A**). The Styrofoam keeps the plates warm.
4. Put 250 µl BLT5403 cells from 4 °C into each of the numbered borosilicate test tubes prepared on day one above (section 3.7 and **Figure 4B, C**). Arrange the numbered borosilicate tubes in the same ascending series as the dilutions in the 1.5 ml Eppendorf tubes (**Figure 4A**).
5. Put 100 µl from the dilution in Eppendorf tube 1 in the 250 µl of cells in borosilicate test tube 1 (**Figure 4D, E**). Heat the first 5 inches of the pipet over the flame a bit (not too much! ~3 swipes, **Figure 4F**) and plunge into the molten top agarose. Pull up 3 ml and deliver QUICKLY into the test tube on top of the cells/phage (**Figure 4G**).

6. Mix by flicking with a finger while holding the tube with the other hand (**Figure 4H**) and pour the contents on the plate (**Figure 4I**). Tilt the plate and use the test tube to chase bubbles and dry spots until the whole plate is covered by the top agarose. Set it aside, upright on the bench to cool.
7. Discard the borosilicate tube, eject the filter barrier tip, get a fresh one and continue until all the dilutions have been plated. Retrieve $LB^{100\ AMP}$ plates from the incubator as they are depleted but no more than 6 at a time or they cool and the top agarose solidifies too quickly.
8. Once the top agarose is solid, turn the plates upside down (IMPORTANT TO DO THIS. Otherwise, the agar/agarose "weeps", condenses on the lid, drops down on the top agarose and runs over it, taking the phage with it making it impossible to titer accurately) and EITHER: 1) put the plates in the 37 °C incubator [be back 4 hr later to count the titer as T7 is aggressive] OR: 2) Leave them upside down on the bench and they are ready the next morning to count titer.
9. Taking all necessary precautions to protect against the glass shattering, or injury if it does, put the top agarose bottle cap back on **loosely** and microwave the top agarose until it boils. Do this twice. Let it cool, tighten the cap and put it away. Turn the water bath down to its usual temperature or shut it off. Put the dilutions in the 4 °C fridge. They will be needed to interpret the titers and/or redo some of the titering.

6. Determining and Calculating Titer

1. Examine the plaque formed on the plates and discard those with confluent lysis (**Figure 5A** *e.g.* 10^{-2} dilution). Determine which of the remaining serial dilutions have produced sufficiently few plaque to enable an accurate tally (**Figure 5A, B**). Record the number of plaque from these plates (**Table 1**; **Figure 5B**).
2. Multiply the number of plaque by the dilution and then multiply this number by 100 to get the number of plaque forming units per ml of infected bacteria taken from the wells (*i.e.* only 10 µl of infected bacteria were taken for each of the triplicates and so this must be multiplied by 100 to get counts per ml). Average the number of Plaque Forming Units (PFU) per milliliter, (PFU/ml of undiluted infected bacteria taken out of the microtiter plate well), across the three triplicates taken from this well.
3. Form a Grand Average of the tallies for the three replicate wells and calculate the standard error of this mean (**Table 1**). This is what will be recorded in the figure of the increase in phage titer for each affinity selection round and treatment (**Figure 5C**).

7. Plaque Isolation, PCR and Sequencing

1. At the end of affinity selection round 4, select 18 individual, well defined, plaque colonies and, using a sterile Pasteur Pipet, toothpick, yellow pipet tip or similar device, core these plaque from the titering plates. If using tubes or tips, first wet the inside with the Tris-HCl, pH 8.5 in the Eppendorf tube (**Figure 5D**).
2. If using tubes, make sure the borosilicate tube holds no excess drops of Tris, depress the bulb and, with it still compressed, push the borosilicate pipet tip through a well isolated plaque right to the plastic Petri dish below (**Figure 5E**). Release the bulb with the pipet tip still in the agar/top agarose and suck up the core (**Figure 5F**, see arrow).
3. Expel the core into 100 µl Tris-HCl, pH 8.5 in the appropriate tube (**Figure 5G**) and vortex briefly.
4. Heat 20 µl from this volume at 65 °C for 10 min.
5. Centrifuge the heated volume at 13,000 x g in a benchtop centrifuge at room temperature for 10 min. Take 3 µl from the supernatant of this aliquot to be used in PCR reactions with T7-UP and T7-DOWN primers.
6. For each of the 18 isolated plaque, heated solutions, make a 50 µl PCR reaction using an annealing temperature of 58 °C and 35 cycles.
7. Run 20 µl of each reaction on a 1% (w/v) agarose gel containing 0.015% (v/v) ethidium bromide. Visualize with a transilluminator using appropriate eye protection and nitrile lab gloves. (CAUTION: ethidium bromide is a potent mutagen.) Photograph the gel and dispose of contaminated materials properly (**Figure 6A, B**).
8. If the amplicons are single products, and not from empty vector (product runs near 100 bp on a 1% (w/v) TAE agarose gel; **Figure 6A**, B arrows), clean the remaining 30 µl for use as a sequencing template. If not a single product on the gel (**Figure 6B**, plaque 15), cut out the most prominent band(s) from the gel and extract the DNA from the gel using a kit.
9. Sequence the amplicons that are not empty vectors using the T7-UP primer.
10. Examine each sequence for the linker arms and, from this information, determine where the commencement of the insert is located. Use the Basic Local Alignment Search Tool (BLAST; National Library of Medicine) to determine the identity of the encoded cDNA, whether the insert is in frame and, if so, what portion of the protein coding sequence (CDS) has been displayed and captured by the bait.

Representative Results

Being able to impair the capacity of the bait to interact with phage-displayed proteins (metabolically poisoning the bait) provides a potent negative control for this technique. It is also advisable to determine if the bait, when bound to the microtiter plate well, retains its function. Both of these checks will increase the confidence that interacting, phage-displayed proteins recovered by the non-poisoned bait are legitimate.

Sampling three triplicates from each well adds considerably to the time and work involved in the affinity selection but provides a more accurate estimate of the titer within each well than sampling only once. While most of the titers for the triplicates are in good agreement, note that the triplicates for replication 2 of the BSA in round four vary widely (**Table 1**). By sampling several times, the final estimated tallies are not as susceptible to pipetting errors and a more accurate estimate of the actual titer and the variation around this estimate, well-to-well, are obtained (**Table 1**, **Figure 5C**).

Increasing phage titer, preferentially for the non-poisoned bait, as the number of affinity selection rounds increase, also provides confidence that the technique is working (**Figure 5C**). The retention of an increasing percentage of phage containing insert in non-poisoned bait wells as the number of affinity selections increases is also auspicious (**Figure 6A, B**).

After advanced rounds of affinity selection, an increase in the number of independently recovered phage that have insert (discernible as PCR amplicons greater in size than ~100 bp; **Figure 6B**), relative to the first round (**Figure 6A**), and the settling of these amplicons on a few identically sized bands (note lanes 5-9, 11-18 in **Figure 6B**), is a good indication that particular clones are being selected in the affinity selection

(**Figure 6A, B**). After sequencing a number of plaque obtained in the final affinity selection round, retrieval of a similar region (different clones) of the same protein is independent verification that the portion of the displayed protein has a *bona fide* affinity for the bait. These independently recovered phage also provide information on what part of the entire protein is capable of interacting with the bait. If the phage displayed cDNA library has been constructed using random priming, a great diversity of partial- and full-length-protein coverage can be anticipated (within the limits of the phage to tolerate the cDNA insertion size), leading to multiple, independent clones covering the same portion of the protein. Even the contiguous out-of-frame hits should be scrutinized to determine if they encode a similar motif to which the bait binds (This assumes that the libraries were not constructed using ORF-technology [3]).

Round	Treatment	Replication	Dilution	1.00E-02	1.00E-03	1.00E-04	1.00E-05	1.00E-06	Actual titer	Average	per mL	Grand average	std dev	std err
4	BSA	1	Triplicate 1	4.41E+02	**	**	**	**	4.41E+04	4.07E+04	4.07E+06	4.60E+06	6.11E+05	3.53E+05
			Triplicate 2	3.23E+02	**	**	**	**	3.23E+04					
			Triplicate 3	4.56E+02	**	**	**	**	4.56E+04					
		2	Triplicate 1	5.10E+02	**	**	**	**	5.10E+04	5.27E+04	5.27E+06			
			Triplicate 2	4.68E+02	**	**	**	**	4.68E+04					
			Triplicate 3	6.02E+02	**	**	**	**	6.02E+04					
		3	Triplicate 1	4.75E+02	**	**	**	**	4.75E+04	4.47E+04	4.47E+06			
			Triplicate 2	4.21E+02	**	**	**	**	4.21E+04					
			Triplicate 3	4.45E+02	**	**	**	**	4.45E+04					
	poisoned bait	1	Triplicate 1	2.25E+02	**	**	**	**	2.25E+04	2.81E+04	2.81E+06	2.87E+06	1.62E+05	9.35E+04
			Triplicate 2	3.05E+02	**	**	**	**	3.05E+04					
			Triplicate 3	3.12E+02	**	**	**	**	3.12E+04					
		2	Triplicate 1	3.16E+02	**	**	**	**	3.16E+04	3.05E+04	3.05E+06			
			Triplicate 2	3.01E+02	**	**	**	**	3.01E+04					
			Triplicate 3	2.98E+02	**	**	**	**	2.98E+04					
		3	Triplicate 1	2.86E+02	**	**	**	**	2.86E+04	2.74E+04	2.74E+06			
			Triplicate 2	2.59E+02	**	**	**	**	2.59E+04					
			Triplicate 3	2.78E+02	**	**	**	**	2.78E+04					
	bait	1	Triplicate 1	--	6.77E+02	**	**	**	6.77E+05	6.74E+05	6.74E+07	6.57E+07	3.61E+06	2.08E+06
			Triplicate 2	--	7.00E+02	**	**	**	7.00E+05					
			Triplicate 3	--	6.46E+02	**	**	**	6.46E+05					
		2	Triplicate 1	--	5.41E+02	**	**	**	5.41E+05	6.16E+05	6.16E+07			
			Triplicate 2	--	6.83E+02	**	**	**	6.83E+05					
			Triplicate 3	--	6.24E+02	**	**	**	6.24E+05					
		3	Triplicate 1	--	7.12E+02	**	**	**	7.12E+05	6.82E+05	6.82E+07			
			Triplicate 2	--	6.45E+02	**	**	**	6.45E+05					
			Triplicate 3	--	6.89E+02	**	**	**	6.89E+05					

Table 1. Calculations for the average titer/ml infected bacteria from the wells of the fourth round of affinity selection for the three treatments. The numbers for each triplicate of the 10^{-3} dilution of the plaque taken from the first replication of the bait well are taken from **Figure 5B.** Click here to view larger table.

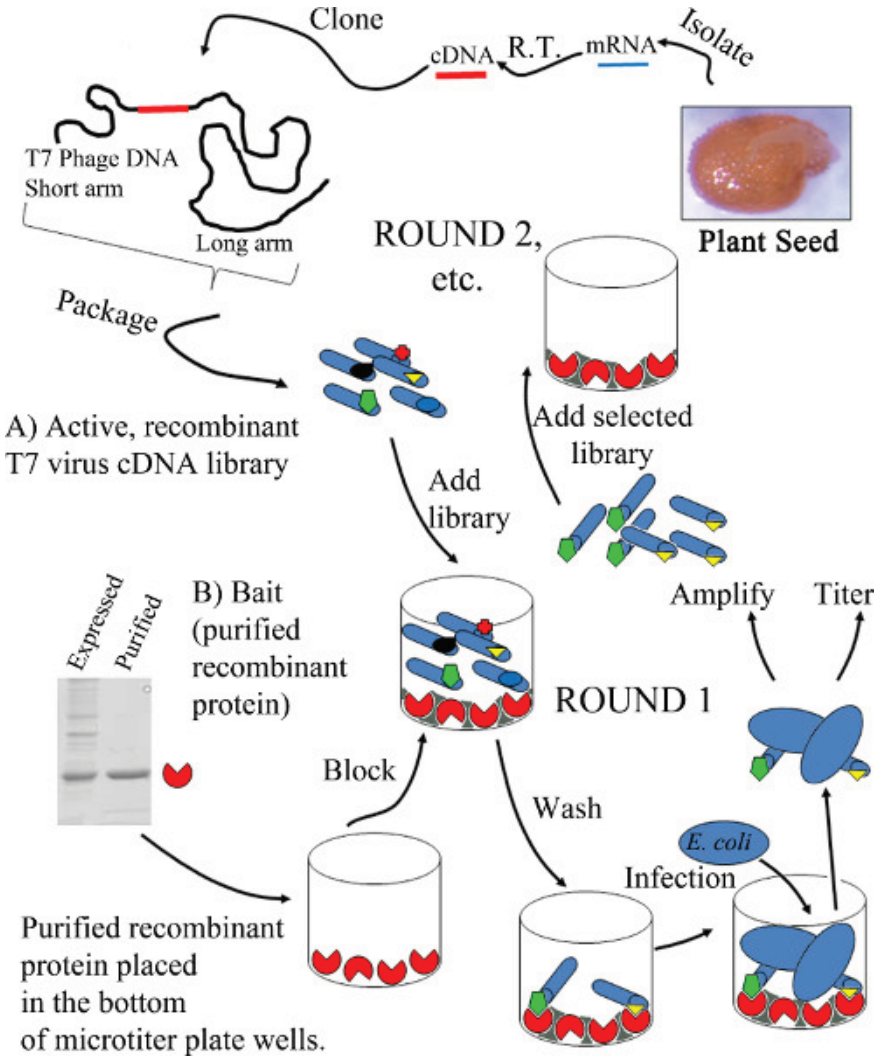

Figure 1. Overall graphic depiction of the phage display process. Two primary requirements include; **A)** access to a phage displayed library, preferably constructed using randomly primed, poly-A selected RNA, for generating the cDNAs; and **B)** a bait that is, in so far as possible, verifiable as biologically active, even when immobilized on a solid support and, if possible, rendered inactive by addition of an inhibitor (*e.g.* competitively inhibited from binding bait protein). RT: reverse transcription.

Figure 2. Some of the equipment and material necessary for performing phage display. Sterile technique requires access to both: **A)** an autoclave and; **B)** a laminar flow hood. Phage libraries and BLT5403 bacterial stock require -80 °C temperatures for prolonged storage **C)** Growing phage and bacteria requires, **D-F)** 37 °C incubators, one of which, **E-F)** is capable of growing liquid cultures (orbital). Optimizing the experiment through color coding, **F)** minimizes mistakes. BLT5403 cells for titering can be stored for up to one week, **G)** at 4 °C. Optimizing the placement of the, **H)** spectrophotometer for following cell densities next to the orbiting shaker can save time. Frequently treating surfaces and pipets with, **I)** powerful detergent and **J)** Envirocide, can help to minimize the risk of viral contamination.

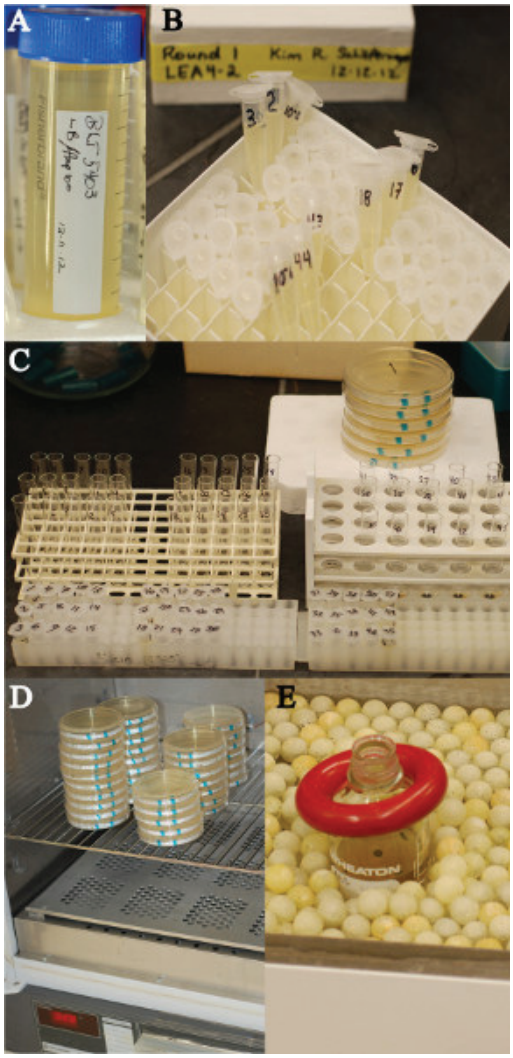

Figure 3. One possible set-up useful for permitting smooth affinity selection. **A)** The BLT5403 are grown a day or two prior to titering and are plated, without viral introduction, to determine that they are not contaminated with virus. Titering on the day of the affinity selection can be facilitated by preparing beforehand by: **B)** pre-labeling the Eppendorf and; **C)** borosilicate tubes to be used. **B)** Aliquoting the dilution solutions of LB^{100AMP} and storing them at 4 °C overnight. **D)** Place the pre-numbered, LB^{100AMP} agar-containing, Petri dishes at 37 °C to warm approximately 1 hr before titering. **E)** Melting the sterile top agarose (loosen the cap) and placing it in a pre-heated 50 °C water bath to cool to this temperature an hour before titering commences. Use a lead donut to avoid the bottle capsizing as top agarose is removed.

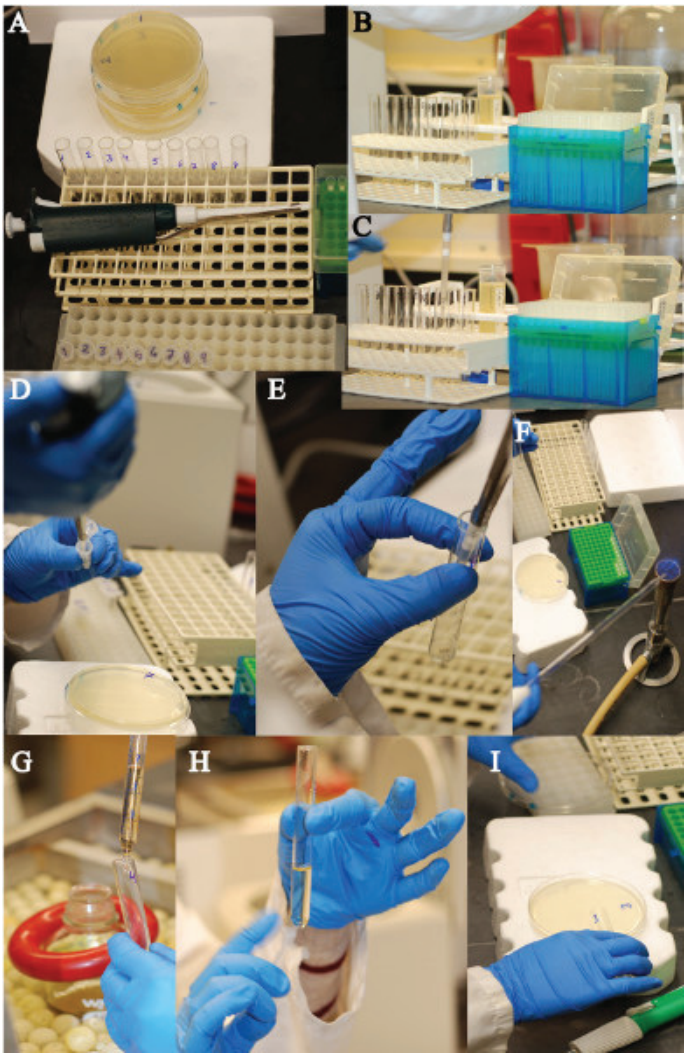

Figure 4. Titering the phage recovered from a round of affinity selection. Plating commences as soon as possible after infection to prevent artificial inflation of the titer. **A)** The first set of virus-infected BLT5403 dilutions and the borosilicate tubes assembled in racks. Using filter barrier tips: **B-C)** 250 µl BLT5403 cells from the stock from 4 °C are transferred to the borosilicate tubes. Using filter barrier tips: **D-E)** The BLT5403 in the first borosilicate tube is infected with the first virus dilution. **F)** The borosilicate pipet is heated over an open flame to prevent clogging and to keep the top agarose warm. Do not heat the pipet excessively or the bacteria will be scalded and die. **G)** 3 ml molten top agarose are quickly retrieved and poured over the bacteria in the borosilicate tube. **H)** The tube is flicked briefly to mix the contents and; **I)** The contents poured onto the pre-warmed LB^{100AMP} agar plates, using the tube to move bubbles to the sides of the plate and to ensure the top agarose covers the whole plate.

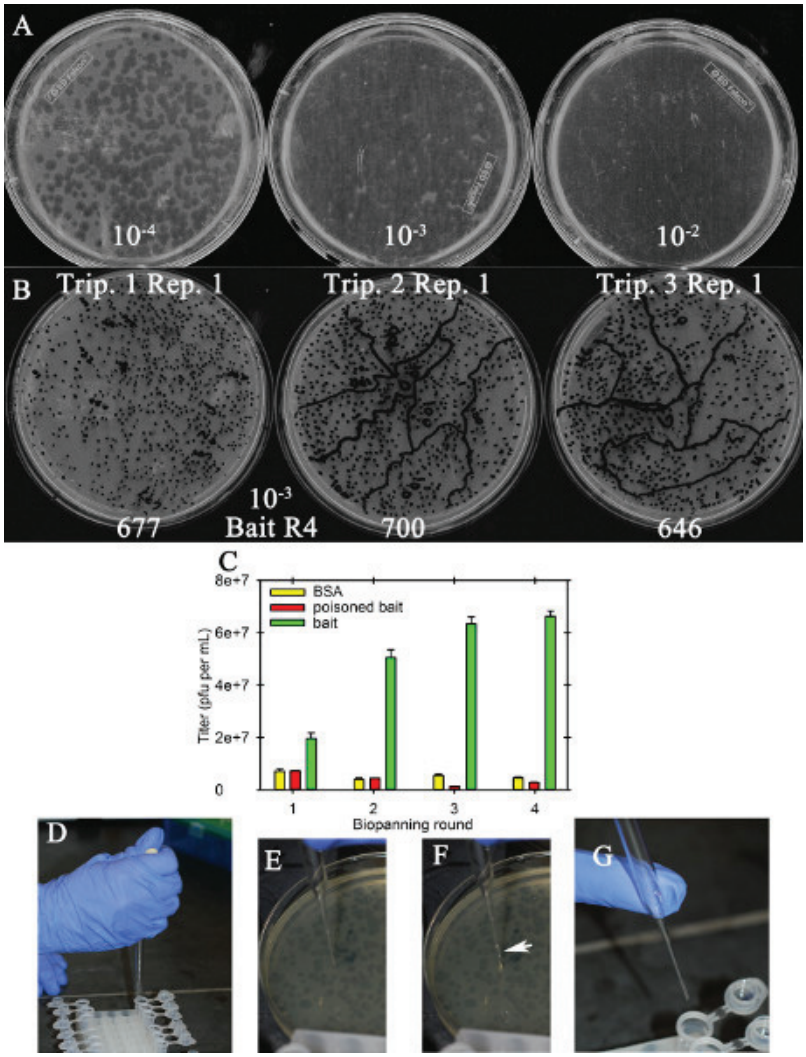

Figure 5. Typical titer results. **A)** The lower dilutions (10^{-2}) almost invariably result in confluent lysis for all treatments in the first affinity selection round (the virus in the figure have been grown overly long resulting in excessive plaque size to facilitate photography). **B)** The appropriate dilutions of plaque are counted using a sharpie to keep track of tallied plaque. **C)** The titer increases drastically for bait wells while remaining more-or-less stable for BSA or poisoned bait. In later rounds, titer from bait wells plateaus and further affinity selection is unproductive. **D)** A well isolated plaque has been chosen for sequencing and collected by wetting the Pasture pipet with solution from the Eppendorf tube, being sure to eliminate the excess liquid least it run over multiple plaque and contaminate the core. **E)** The pipet has been pushed through the plaque with the pipet bulb already compressed. **F)** The bulb has been released while the pipet is still in the top agarose, sucking the plaque up into the pipet (arrow). **G)** The cored plaque is about to be delivered into the liquid in the appropriate Eppendorf tube. Phage dilutions (volume of infected bacteria per volume of LB^{100AMP}) are depicted on the Petri dishes (*e.g.* 10^{-3} = 1/1000 dilution). The three triplicate samples from replication well 1 are abbreviated as Trip. 1, Trip. 2, and Trip. 3, all for replication 1 (Rep. 1= well 1). The numbers at the bottom of the plates in **B)** are the actual plaque numbers tallied on the plates. These are for the 10^{-3} dilution of the bacteria from the fourth round of affinity selection (R4 in figure) over the bait. Click here to view larger figure.

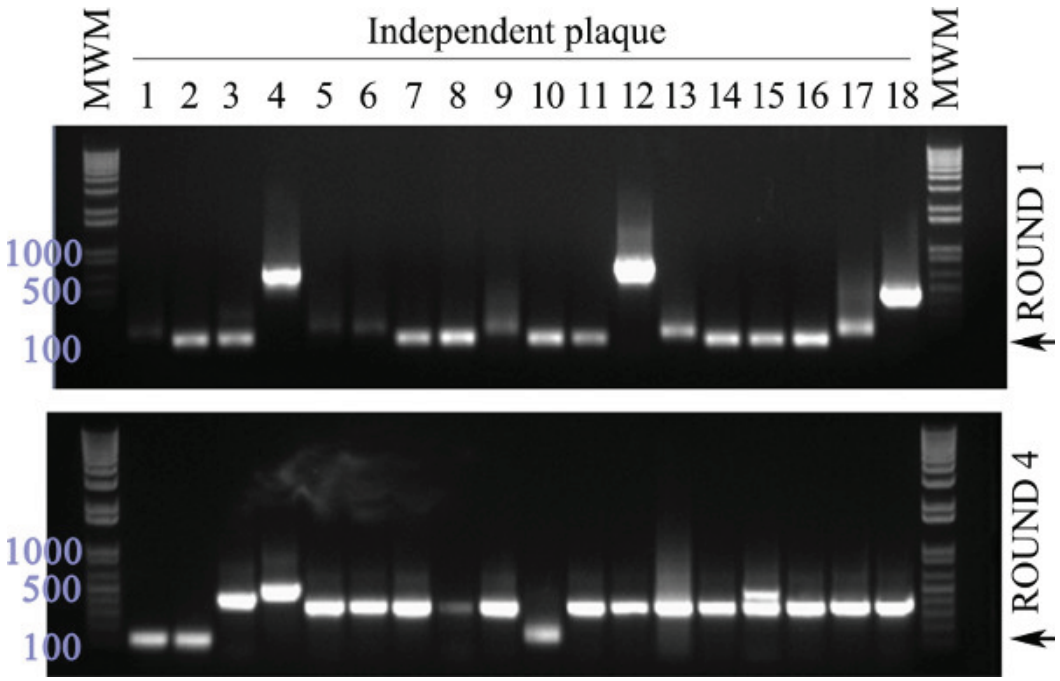

Figure 6. Typical PCR results for affinity selection over an appropriate bait. **A)** The percentage of empty vector plaque (arrows) is usually quite high initially but, in bait wells; **B)** decreases in subsequent rounds. The plaque containing insert has tended to settle on those containing identically sized insert in this later affinity selection round. MWM: Molecular Weight Marker.

Discussion

By running the experiment in three replicated wells, independently acquired phage of the same protein binding to the bait can be distinguished even if they are the same clone (*i.e.* no difference in the nucleotide sequence of the CDS region that has been acquired but these have been retrieved from independent wells). Otherwise, the only way to distinguish among phage encoding the same protein binding to the bait is if they are independently reverse transcribed regions that differ in some part of the nucleotide sequence.

The use of one Fernbach flask in which to grow all of the bacteria for use in amplifying the affinity selected phage permits a much less hectic monitoring of bacterial growth leading up to infection following affinity selection than trying to monitor 9 Erlenmeyer flasks. Decanting these bacteria, just prior to infection, into pre-warmed Erlenmeyer flasks also eliminates sub-library titer discrepancies due to alterations in the multiplicity of infection due to poor bacterial growth in specific flasks. Thus, alterations in phage titer from round-to-round should depend on the number of phage retained in the wells and the variation in titer among replicated wells should be minimal.

One exceptional advantage using phage display is the great power the technique has in identifying phage-displayed-protein that have an affinity for a particular bait. Due to the efficiency with which the phage replicate in the BLT5403 host, even a single phage representing a rare CDS that is retained in a well in the first affinity selection round, should the coat-fused protein have a high affinity for the bait, will be represented in the second round by much greater numbers. By the fourth round, this phage should constitute the majority of the phage present in the lysed culture.

This capacity of the phage to replicate to high titer is also a limitation of the technique. If a particular bait has an interacting protein partner for which it has high affinity in the library, it is very difficult to capture proteins that may legitimately bind the bait but at a lower affinity as these will tend to be outcompeted by the high affinity binding protein. The use of next generation sequencing techniques to identify such rare phage is one possible means of circumventing this limitation [1]. Furthermore, the susceptibility of the BLT5403 to the T7 phage results in bouts of "contamination" throughout the lab that require perseverance, rather harsh decontaminants (*e.g.* Envirocide) and excellent sterile technique to overcome. Contamination can result in a considerable loss of time in the progress of affinity selection as one tries to identify and eliminate the source.

One means that has limited success in partitioning phage into tight and loose "binders" is to try and recover the loose binders first by introducing into the wells a solution (1% SDS or similar chaotrope) designed to remove phage that are less tightly bound to the bait. This solution can then be removed first, prior to the introduction of the bacteria into the wells which are anticipated to be infected by those phage remaining bound to the bait. This technique can also reduce background, spuriously binding phage, considerably. However, if this technique is pursued, the number of sub-libraries, replicates and duplicates affinity selected in subsequent rounds is doubled, which adds considerably to the time and expense.

Following the course of phage titer increase over several rounds of affinity selection can run through a large volume of LB and a large number of $LB^{100\ AMP}$ plates, filter barrier tips, Eppendorf tubes, *etc*. This is expensive as is the sequencing required to examine even a modest number of phage. Titering a large number of dilutions of several replicates of treatments in triplicate requires considerable time so the importance of setting up as much of the material as possible the day prior to the affinity selection is paramount.

One exciting possibility that is currently being explored is to use the location of the independent phage proteins, binding to a bait, to model the physical attributes and the three dimensional structure of the region of the protein interacting with the bait. For example, examining the attributes of protein targets that bound to a set of homologous LATE EMBRYOGENESIS ABUNDANT (LEA) proteins from Arabidopsis (*Arabidopsis thaliana*) and Soybean (*Glycine max*), one important conclusion was that the LEA proteins bound to the region of the proteins that were the most (or among the most) hydrophilic of the binding proteins [11]. Retrospectively, given that the LEA proteins are hypothesized to protect their client molecules from denaturation during dehydration, it can be surmised that the region of the client molecules most susceptible to dysfunction without LEA protection might be those most capable of binding water (hydrophilic).

It is advisable to minimize the time between the introduction of the bacteria into the wells and their retrieval so the titer is not inflated. Ensure that the dilutions are sufficient by plating some BLT5403 without viral infection to verify that the bacterial stock is not contaminated and that the greatest dilutions are sufficient to avoid confluent lysis.

Disclosures

The authors have nothing to disclose.

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