**Supplementary Material for:**

“Semi-automated Biopanning of Bacterial Display Libraries for Peptide Affinity Reagent Discovery and Analysis of Resulting Isolates”

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**EXPANDED PROTOCOL FOR NEW USERS:**

**1. Biopanning Bacterial Display Libraries Using autoMCS**

NOTE: This “Expanded Protocol for New Users” provides more detail than the more streamlined version of protocol in the manuscript itself; experienced users should see the truncated protocol. This autoMCS-based sorting protocol has been previously described 14. If an autoMCS device is unavailable for sorting, see the supplemental protocol from Sarkes *et al.* 2015 since a manual sorting protocol is also described in that work 14. The autoMCS protocol provided here has been further adapted to include additional negative sorting steps for improved specificity 1,15. Streptavidin-coated paramagnetic beads are used here (see table of materials) to capture biotinylated protein target, but other capture strategies, including direct coupling of protein to magnetic beads, should work similarly, changing or removing appropriate steps. A schematic of this biopanning protocol is illustrated in **Figure 1**. Aseptic technique should be exercised when possible.

* 1. ***Negative Sorting to Remove Binders Likely to Cross-React with Magnetic Beads***
		1. Inoculate 500 mL of Luria Broth Miller (LB) containing appropriate antibiotic with approximately 1 x 1011 cells of a diverse bacterial display sorting library (see table of materials).

NOTE: The bacterial display peptide library used here (see table of materials) contains approximately 109 – 1011 unique members 8,12 and is grown in LB containing 25 µg/mL chloramphenicol (LB Cm25). The remainder of this protocol will assume that this library is used. Growth and induction conditions can be adjusted accordingly if using a different species of bacteria, a plasmid with a different antibiotic resistance gene or promoter, etc. If beginning with a frozen cell stock, thaw the cells on ice before inoculation.

* + 1. Incubate at 37 °C with shaking at 225 RPM until the culture reaches an OD600 of 0.5 – 0.55 (approximately 30 – 90 min from frozen stock with 1 x 1011 cells). Induce peptide expression with 0.04% w/v L-arabinose by diluting a 4% stock 1:100, shaking at 225 RPM for 45 min at 37°C.

NOTE: Induction for longer than 45 min can improve expression level. Up to 90 min also works well. Addition of 2 mM ethylenediaminetetraacetic acid (EDTA, which facilitates peptide display 42) also improves expression level but may have unknown effects on the iron oxide-containing magnetic beads. Addition of EDTA has not yet been tested during sorting steps.

* + 1. Place induced culture on ice to inhibit further growth and expression. Centrifuge approximately 2 x 1011 cells at 6000 x g for 20 min at 4°C. Remove supernatant and resuspend cells in 1.5 mL PBS by gently swirling.

NOTE: An OD600 of 1.0 approximately equals 1 x 109 cells/mL for *E. coli*. **DO NOT VORTEX** as this may lyse the cells; resuspend by hand or by briefly shaking at ≤ 225 RPM, 4°C.

* + 1. Transfer cells to a 2 mL microcentrifuge tube or several tubes as needed and spin at 6000 x g for 5 min at RT (room temperature) or 4°C. Remove supernatant. Resuspend the cell pellet in 1 mL phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA; PBS-B).

NOTE: To prepare PBS-B, add the solid BSA to the surface of the PBS solution while stirring gently with a stir bar to mix. The BSA will float on the surface until fully dissolved.

* + 1. Wash 300 µL of streptavidin-coated beads (about 3 x 109 beads, see table of materials) in 1 mL PBS-B and centrifuge for 5 min at ≥ 5000 x g (at RT). Place tube in a bench top magnetic particle separator. Carefully remove supernatant, avoiding the pellet.

NOTE: Be sure to use the magnetic bead for the chosen capture strategy, if altered, for these negative sorting steps. If a magnetic particle separator is unavailable, be particularly cautious when removing the supernatant. It is better to leave behind a small amount of PBS-B than to lose magnetic beads.

* + 1. Resuspend the beads in the cell plus PBS-B mixture prepared above. Incubate at 4°C on a rotating platform for 45 min. Place samples on ice.
		2. Turn on autoMCS instrument to initialize the system. Prime lines using manufacturer’s run and wash buffers (see table of materials) by selecting **Wash Now** at the bottom of the screen in the **Separation** menu, then **Rinse** and **Run**.

NOTE: Perform routine maintenance following manufacturer’s instructions. Two magnetic columns (see table of materials) should be installed. Ensure there is sufficient buffer and space in the waste container for all priming and separation steps. The waste container should also contain sufficient decontaminant for the organism used (10% of the volume should be filled with bleach for *E. coli*).

* + 1. Prime the system with PBS-B, using PBS-B as both Wash Buffer and Running Buffer in the next steps. Select the **Separation** menu from the upper navigation bar and select **Wash Now**. Select **Rinse** and **Run** to prime the system with fresh PBS-B.

NOTE: This is important for removing azide from the system, which inhibits bacterial growth. Fresh PBS-B (prepared same day) should be used to avoid contamination.

* + 1. Transfer cell and bead mixture from the incubation step (1.1.6) to a 15 mL conical tube. Rinse the tube with an additional 500 µl of fresh PBS-B and add this to the tube as well to recover as many cells as possible. Place this tube in the sample slot, and empty 15 mL conical tubes in the positive and negative selection slots, of a pre-chilled (4°C) rack (see table of materials). Place rack on instrument platform.
		2. Assign a separation program for each sample on the rack (up to 5 samples can be sorted in one run). Add a **Rinse** step in between each sample and after the last sample. Select **Run** to start the cell separation. Select **OK** to confirm that enough buffer is available.

NOTE: The **Posselds** separation program works well for negative sorting and positive sorting round 1 and **Posseld** is preferred for sorting rounds 2 – 4, but both of these programs have been successfully used for all negative and positive sorting rounds 14,15 and other programs may prove better for a particular application (described further in discussion).

* + 1. When the program is complete, remove the rack and retain the appropriate fraction. Here, for a negative sort, retain negative fractions (containing cells without beads). Place on ice.

NOTE: Before using sorted fractions to inoculate overnight cultures in step 1.1.12, an estimate of the number of cells isolated in each fraction can be obtained using a spot plating technique with serial dilutions, if desired 14.

* + 1. Use the negative sort fraction in its entirety to inoculate 1L of LB Cm25 with 0.2% w/v D-glucose by diluting 2% D-glucose 1:100. Grow overnight at 37 °C, shaking at 225 RPM.

NOTE: For better recovery of isolated cells, the tube should be rinsed several times with a small amount of the inoculation media.

* + 1. Before turning off the autoMCS instrument, change the run and wash buffers to the manufacturer’s run and wash buffers (see table of materials). Select **Wash Now**, then **Rinse** and **Run** (to remove PBS-B from the tubing). When finished, be sure there is 70% ethanol in the decontamination line, then press the **power icon** at the top right corner of the screen and select **Yes**.
		2. When the system shutdown is complete (the bottles will be purple), turn off the machine.
		3. The next day, use the overnight culture to make freezer stocks with about 1 x 1011 cells per vial in LB with 15% glycerol. Additionally, or alternatively, use this culture in the next step: additional negative sorting (section 1.2) or the first round of positive sorting (section 1.3).

NOTE: We typically freeze the streptavidin-depleted stocks so that they can be used for several experiments before proceeding to section 1.2 and/or section 1.3. The freezer stock with 1 x 1011 cells can be prepared by taking an OD600 measurement of the cell culture to estimate total cells, centrifuging the appropriate volume of the culture at 6000 x g to pellet cells, diluting the pellet to 2 x 1011 cells/mL in LB, and then diluting that culture in an equivalent volume of LB 30% glycerol. 1 mL stocks will then contain 1 x 1011 cells in 15% glycerol.

* 1. ***Negative Sorting to Remove Binders Likely to Cross-React with Other Targets of Interest***

NOTE: Section 1.2 can be skipped if no further negative sorting is needed or desired. In that case, proceed to section 1.3. Residual binding to any undesired targets can be assessed by FACS as in section 2: FACS Analysis of Sorting Rounds. It is recommended to follow section 1.2 if there is a specific similar protein that could be problematic to downstream applications, or if a different capture strategy is used that exposes the display library to additional proteins during sorting that could interfere with binding to the target protein.

1.2.1) For negative sorting against a specific protein target, such as a similar protein with potential to also bind to the discovered peptides 1,15, repeat growth and induction steps 1.1.1 – 1.1.3, beginning with a frozen stock of streptavidin-depleted library from step 1.1.15 or the overnight culture from step 1.1.12 (using OD600 to estimate and inoculate with 1 x 1011 cells).

1.2.2) Transfer cells to a 2 mL microcentrifuge tube or several tubes as needed and spin at 6000 x g for 5 min at RT or 4°C. Remove supernatant. Resuspend cell pellet in 1 mL PBS containing 600 nM biotinylated cross-reactive protein target. Incubate at 4 °C for 45 min on a rotating platform.

NOTE: 600 nM is a suggested starting concentration, which can be altered if a noted benefit is observed. Biotinylation of protein target can be achieved and quantified using the reagents suggested in the table of materials.

1.2.3) Meanwhile, wash 100 µL of streptavidin-coated beads (about 1 x 109 beads) in 1 mL PBS-B and centrifuge for 5 min at ≥ 5000 x g (RT). Place tube in a bench top magnetic particle separator and carefully remove supernatant, avoiding the pellet.

NOTE: If a magnetic particle separator is unavailable, be particularly cautious when removing the supernatant. It is better to leave behind a small amount of PBS-B than to lose magnetic beads.

1.2.4) Centrifuge the target-bound cells from 1.2.2 at 6000 x g for 5 min (RT or 4°C) to remove any unbound target protein. Remove supernatant and resuspend the cell pellet in 1 mL PBS-B. Resuspend beads in entire volume of washed cells bound to cross-reactive protein target. Incubate at 4°C on a rotating platform for 30 min.

1.2.5) Place sample on ice and complete steps 1.1.7 – 1.1.15 for a negative sort, making appropriate freezer stocks. Continue to section 1.3 for a positive sort if all desired negative sorting has been achieved, or repeat section 1.2 to negative sort against another potential cross-reactive protein.

* 1. ***Round 1 Positive Sort***

NOTE: Round 1 positive sorting against a specific protein target is similar to a negative sort against a specific sorting target (see 1.2) except that the bead-containing, positive fraction is retained.

* + 1. Inoculate 500 mL of LB Cm25 with approximately 1 x 1011 cells of a diverse bacterial display sorting library that has been streptavidin-depleted and grown overnight (from step 1.1.12, using OD600 to estimate and inoculate with about 1 x 1011 cells), or frozen (from step 1.1.15) and thawed on ice, or that has been further depleted of binders to other cross-reactive protein targets (from step 1.2.5), as desired.

NOTE: An OD600 of 1.0 approximately equals 1 x 109 cells/mL for *E. coli*.

* + 1. Incubate at 37 °C with shaking at 225 RPM until the culture reaches an OD600 of 0.5 – 0.55. Induce peptide expression with 0.04% w/v L-arabinose by diluting a 4% stock 1:100, shaking at 225 RPM for 45 min at 37°C.
		2. Place induced culture on ice. Centrifuge approximately 2 x 1011 cells at 6000 x g for 20 min at 4°C. Remove supernatant and resuspend cells in 1.5 mL PBS by gently swirling (by hand or by briefly shaking at ≤ 225 RPM, 4°C).
		3. Transfer cells to a 2 mL microcentrifuge tube or several tubes as needed and spin at 6000 x g for 5 min at RT or 4°C. Remove supernatant.

1.3.5) Resuspend cell pellet in 1 mL PBS containing 600 nM biotinylated protein target of interest. Incubate at 4 °C for 45 min on a rotating platform.

NOTE: 600 nM is a suggested starting concentration, which can be altered if a noted benefit is observed. Biotinylation of protein target can be achieved and quantified using the reagents suggested in the table of materials. Biotinylation in PBS is typically successful if it is a suitable buffer for the protein of interest.

* + 1. Meanwhile, wash 100 µL of streptavidin-coated beads (about 1 x 109 beads) in 1 mL PBS-B and centrifuge for 5 min at ≥ 5000 x g (at RT). Place tube in a bench top magnetic particle separator, and carefully remove the supernatant, avoiding the pellet.
		2. When incubation with target is complete, centrifuge the target-bound cells from 1.3.5 at 6000 x g for 5 min (RT or 4°C) to remove any unbound target protein. Remove supernatant and resuspend the cell pellet in 1 mL PBS-B. Resuspend beads in entire volume of washed cells bound to protein target. Incubate at 4°C on a rotating platform for 30 min. Place on ice.

NOTE: If a magnetic particle separator is unavailable, be particularly cautious when removing the supernatant. It is better to leave behind a small amount of PBS-B than to lose magnetic beads.

1.3.8) Turn on autoMCS instrument to initialize the system. Prime lines using manufacturer’s run and wash buffers (see table of materials) by selecting **Wash Now** at the bottom of the screen in the **Separation** menu, then **Rinse** and **Run**.

NOTE: Perform routine maintenance following manufacturer’s instructions. Two magnetic columns (see table of materials) should be installed. Ensure there is sufficient buffer and space in the waste container for all priming and separation steps. The waste container should also contain sufficient decontaminant for the organism used (10% of the volume should be filled with bleach for *E. coli*).

1.3.9) Prime the system with PBS-B, using PBS-B as both Wash Buffer and Running Buffer in the next steps. Select the **Separation** menu from the upper navigation bar and select **Wash Now**. Select **Rinse** and **Run** to prime the system with fresh PBS-B.

NOTE: Bottles from manufacturer’s buffers can be saved and reused for storage of PBS-B

1.3.10) Transfer cell and bead mixture from incubation step above to a 15 mL conical tube, rinsing the tube with an additional 500 µl of PBS-B and pooling the wash with the sample. Place this tube in the sample slot, and empty 15 mL conical tubes in the positive and negative selection slots, of a pre-chilled (4°C) rack (see table of materials). Place rack on instrument platform with all caps removed from the tubes.

1.3.11) Assign a separation program for each sample on the rack (up to 5 samples can be sorted in one run). Add a **Rinse** step in between each sample and after the last sample. Select **Run** to start the cell separation. Select **OK** or **CONTINUE** to confirm that enough buffer is available.

NOTE: The **Posselds** separation program works well for negative sorting and positive sorting round 1 and **Posseld** is preferred for sorting rounds 2 – 4, but both of these programs have been successfully used for all negative and positive sorting rounds 14,15 and other programs may prove better for a particular application (described further in discussion).

1.3.12) When the program is complete, remove the rack and retain the appropriate fraction. Here, for a positive sort, retain positive fractions (containing cells and beads). Place on ice.

1.3.13) Before turning off the autoMCS instrument, change the run and wash buffers to the manufacturer’s run and wash buffers (see table of materials). Select **Wash Now**, then **Rinse** and **Run**. When finished, be sure there is 70% ethanol in the decontamination line, then press the **power icon** at the top right corner of the screen and select **Yes**.

* + 1. When the system shutdown is complete (the bottles will be purple), turn off the machine.

1.3.15) Use the positive sort fraction in its entirety to inoculate 1L of LB Cm25 with 0.2% w/v D-glucose. Grow overnight at 37 °C, shaking at 225 RPM.

NOTE: Before using sorted fractions to inoculate overnight cultures, an estimate of the number of cells isolated in each fraction can be obtained using a spot plating technique with serial dilutions, if desired 14.

1.3.16) The next day, use the overnight culture to make freezer stocks in LB containing 15% glycerol, and/or continue to positive sorting round 2 in section 1.4.

NOTE: Freezer stock can be prepared by diluting culture in an equivalent volume of LB 30% glycerol).

***1.4 Subsequent Positive Sorting Rounds***

NOTE: Typically, four sorting rounds are recommended, although three sorting rounds are generally sufficient 14,15. When to stop sorting is assisted by the FACS Analysis of Sorting Rounds described in section 2; it is possible to continue sorting beyond four rounds if a continued benefit is noted. Concentrations of target and magnetic bead volume decrease with each subsequent positive sorting round.

1.4.1) Using the overnight culture from the previous sorting round (step 1.3.16 or 1.4.7), inoculate 5 mL LB Cm25 with 100 µl cells (1:50 dilution). Incubate at 37°C with shaking at 225 RPM until the culture reaches an OD600 of 0.5 – 0.55 (approximately 90-120 min).

NOTE: A frozen cell stock from the previous sorting round can also be used for inoculation, but if the concentration of the cell stock is 1x1011 cells/mL, add the 100 µL of cells to a larger volume of LB (such as 1 L).

1.4.2) Induce peptide expression with 0.04% w/v L-arabinose, shaking at 225 RPM for 45 min at 37°C. Place induced cells on ice.

NOTE: This induced culture can also be used to assess binding affinity and specificity for the sorting round it originated from, as described in section 2 below. Induction for longer than 45 min can improve expression level; up to 90 min also works well.

1.4.3) Centrifuge 1 x 108 cells at 6000 x g for 5 min at RT or 4°C. Remove supernatant and resuspend cell pellet in 50 µL PBS containing half the concentration of biotinylated protein target of interest used for the previous round of sorting (therefore 300 nM for round 2, 150 nM for round 3, and 75 nM for round 4 from our suggested starting point). Incubate for 45 min on ice (or at 4°C a rotating platform).

1.4.4) Meanwhile, wash streptavidin-coated beads (15 µL for round 2, 8 µL for round 3, and 4 µL for round 4) in 1 mL PBS-B and centrifuge for 5 min at ≥ 5000 x g (at RT). Place tube in a bench top magnetic particle separator and carefully remove supernatant, avoiding the pellet. Resuspend beads in 50 µL PBS-B.

NOTE: If a magnetic particle separator is unavailable, be particularly cautious when removing the supernatant. It is better to leave behind a small amount of PBS-B than to lose magnetic beads.

1.4.5) After the 45 min incubation with target in step 1.4.3, centrifuge the cells at 6000 x g for 5 min at RT or 4°C, remove the supernatant, and resuspend the cells in the 50 µL washed beads from 1.4.4. Keep sample on ice and complete steps 1.3.7 – 1.3.13 for a positive sort.

1.4.6) Inoculate a 5 mL culture of LB Cm25 supplemented with 0.2% w/v D-glucose with the entire positive, bead-containing fraction from the sort.

NOTE: Before using sorted fractions to inoculate overnight cultures, an estimate of the number of cells isolated in each fraction can be obtained using a spot plating technique with serial dilutions, if desired 14.

1.4.7) The next day, use the overnight culture to make freezer stocks in LB containing 15% glycerol and/or continue to the next positive sorting round, returning to step 1.4.1.

NOTE: Freezer stock can be prepared by diluting culture in an equivalent volume of LB 30% glycerol. Using the induced culture from 1.4.2 to assess binding affinity and specificity as described in section 2 below can help determine when to stop sorting. If two sorting rounds in a row show similar binding affinity, or a later round shows decreased binding affinity for the target of interest, this a desirable place to stop sorting. After the final round, return to step 1.4.1 but stop at 1.4.2.

**2. FACS Analysis of Sorting Rounds**

2.1) Using the induced cells from step 1.4.2 for each sorting round, or identical cultures, add 5 µL induced cells to 25 µL of each of the following prepared solutions for binding assessment (see also table of materials and table below): PBS alone; PBS with 150 nM YPet Mona (YPet 12,13; positive control for expression), if available; 900 nM protein target conjugated to a fluorescent dye, such as amine-reactive dye with emission/excitation at 493nm⁄518nm (Target-488); 900 nM cross-reactive protein target(s) used for negative sorting labeled with the same fluorescent dye (Cross-Reactive Protein-488); and 900 nM streptavidin-R-phycoerythrin (SAPE, pink in appearance). Incubate on ice for 45 min.

Note: If continuing directly from step 1.4.2, this step will always be done the day after the biopanning for that round was completed since the down-selected library was grown overnight then subcultured and induced the following day. Cultures grown and induced similarly from frozen sorting round stocks can also be used; in that case, all rounds can be tested and compared in a single experiment. Also note that SAPE is chosen as a negative control here due to the streptavidin-conjugated magnetic beads used for capture. If the capture method is changed, be sure to choose a similarly appropriate negative control for comparison. Additionally, if creating or using an alternative bacterial display library, fixed tags other than the P2X peptide which binds to YPet Mona could be incorporated into the display scaffold for use as a positive control.

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| **Reagent** | **Purpose** | **Notes** |
| PBS Alone | Negative control, creation of gates | Buffer can be changed to accommodate your system |
| PBS with 150 nM YPet Mona | Positive control for expression | Different positive control strategies can be used if sorting library is changed |
| PBS with 900 nM Target-488 | Assessment of peptide affinity for your target | Protein target conjugated to a fluorescent dye (i.e. amine reactive dye with emission/excitation at 493nm/518nM) |
| PBS with 900 nM Cross-Reactive Protein-488 | Assessment of peptide specificity | Cross-reactive protein target(s) used for negative sorting labeled with the same fluorescent dye as above |
| PBS with 900 nM SAPE | Negative control for assessment of binding to magnetic beads | Streptavidin-R-phycoerythrin for use with streptavidin-coated beads; can be changed if capture strategy is changed |

2.2) Centrifuge cells at 6000 x g for 5 min at RT or 4°C. Remove supernatant and store samples on ice.

NOTE: Keep the hinge of the microcentrifuge tube facing outward during centrifugation to ensure the pellet is in a known location (at the bottom of the tube on the same side as the hinge) as it will be difficult to see. Carefully remove supernatant by drawing liquid from the opposite side of the pellet. Cell pellets can be stored on ice until all samples are ready for analysis.

2.3) Turn on the FACS instrument, open the software, start-up the system, and calibrate instrument following manufacturer’s instructions 43,44.

2.4) Within the FACS software, click on “Administrator” and click on desired folder or create a “New Folder.” Click on the “New Experiment” icon at the top left of the screen. Right click icon to “rename” experiment. Within that experiment, click on “Global Worksheets”.

2.5) Click on the “Dot Plot” icon, then click on the global worksheet spreadsheet to create this scatterplot. Click on the dot plot graph itself, then select the “Inspector” icon at the top left of the screen and adjust the axes to biexponential display by selecting the boxes next to “Y Axis” and “X Axis” in the open window. Close the biexponential display window by clicking the X.

2.6) Create a “New Tube” by clicking the icon at the top left of the screen and rename the specimen with appropriate information by right clicking the specimen icon under “global worksheet” and selecting “rename”. Expand the specimen by clicking the (+) sign, then double click on the tube icon, right click on it, and again select “rename” to describe the sample.

2.7) Use this dot plot graph with default SSC-A vs FSC-A to run a negative control sample in PBS alone by double clicking on that tube or selecting the arrow next to it. Just before running the sample, resuspend the cell pellet in 500 µL ice cold FACS running buffer and transfer sample to a FACS tube (see table of materials), mixing well by pipetting and flicking the tube. Place the resuspended cells on the sample injection tube (SIT) of the instrument (gently move the aspirator arm to the side to do this). Press “Acquire Data” with “Events to Display” at 30,000 - 50,000 and “Events to Record” at 10,000, flow rate at low (to start; increase if needed), and “SIT flush” selected.

NOTE: Appropriate speed (low, medium, or high) is the speed at which the number of events/s is approximately between 200 and 2000. Use this range for all steps.

2.8) While acquiring, adjust photomultiplier tube (PMT) voltage threshold if needed by selecting the “Threshold” tab. Click on and change the “Value”. Adjust voltage for forward and side scatter (FSC and SSC) such that negative control cells in PBS alone fall slightly below the center.

NOTE: Additional parameters (such as SSC) can be added in the “Threshold” tab using the “add” icon. Typically, PMT voltages of about 700 V to 1000V for both SSC and FSC work well for *E. coli*.

2.9) If the sample is reading properly, adjust the flow rate to display 200-2000 events/s and press “Record Data”. When finished recording, remove tube from SIT and place on ice. Choose the “Polygon Gate” icon and use the mouse to draw a gate around the majority of the cell population in the scatterplot. Right click on dot plot and select “Show Population Hierarchy”.

2.10) Use this “P1” gate as a parent by clicking on “P1” in the population hierarchy. Create a new dot plot following step 2.5. Right click each axis and change the Y-axis to FITC-A and the X-axis to FSC-A. Gate the negative control (PBS alone) using the “polygon gate” icon, with the gate as tight as possible around the top and left side of the population.

NOTE: Double check the population hierarchy to ensure that the P1 gate is a parent of the P2 gate. If it is not, it will appear in line with the P1 gate and “All Events” will be the parent; delete the gate and re-create it following the step 2.10.

2.11) Select “P2” in the population hierarchy, right click, and select “invert gate”. Right click on the dot plot with P2 gate and select “Show Populations”. Select the populations “P2” and “Not P2” for display. Less than 1% of the population should fall outside the gate, as determined by “Not P2”; adjust gate if necessary.

2.12) Right click the dot plot with P2 gate and select “Create Statistics View”. Right click the statistics view window and select “Edit Statistics View”. Click the “Populations” tab and add or remove populations, as desired, including the parent population, P1 (P2, and Not P2 should already be shown). Click on the “Statistics” tab and add “FITC-A Median” and any other statistics of interest. Close the window by pressing OK.

2.13) Create a similar dot plot graph for PE-A vs FSC-A and gate using PBS alone sample (previous plots can be duplicated and altered, don’t forget to adjust Y-axis, invert the gate, and display the correct populations). Use this spreadsheet to run all samples (including negative control cells incubated with each fluorophore-labeled target) in this manner, recording about 10,000 events for each.

NOTE: The cells that fall outside the gate after incubation with the Target-488 protein, the YPet positive control, etc. are binders to that protein, and the value from “Not PX” (where X is the number of the gated population for that graph) should be recorded as % bound. When using SAPE as a negative control, use the PE-A vs FSC-A plot. The median fluorescence intensity (MFI) of P1 should also be recorded as this gives information beyond % binding, to show the extent of binding in relative terms, and is more robust in the presence of outliers 45. Median fluorescence intensity can be normalized (nMFI) by dividing MFI of each peptide by the MFI of a scaffold only negative control (with no N-terminal peptide), or a clone containing a peptide sequence that does not bind the target of interest, after incubation with the same target conjugated to the same fluorophore 14. If these negative control cells are unavailable, uninduced cells incubated with the same target and labeled with the same fluorophore can also be used for normalization.

**3. Sequence Analysis of Potential Candidates and Assessment of Binding Affinity**

***3.1) Peptide Sequence Determination***

3.1.1) Select and sequence tens or hundreds of bacterial colonies from the final round(s) of biopanning (typically rounds 3 and/or 4 are sufficient 14).

NOTE: Typically, we send a grid plate with at least 100 colonies from the final sorting round directly to the sequencing facility, with no plasmid isolation required 46. Sequencing can be performed for other sorting rounds as well if interested, but typically sequencing the final round is sufficient 14. If available, a larger population could be analyzed by next generation sequencing and a similar analysis to that described here could be applied.

3.1.2) Analyze the sequence data using the macro file that we have developed (see supporting information for code, “Sub eCPX\_Sequencing”) to specifically analyze the sequences generated from biopanning the 15mer bacterial display library listed in the table of materials. Use the spreadsheet software listed in the table of materials, or other preferred compatible software.

NOTE: A number of tools are available online that can also aid in the DNA sequence analysis 47. If preferred, use a different established method for sequence analysis and skip to step 3.1.3.

3.1.2.1) Download the set of sequence files (.seq files, text documents also work) and extract them into a new folder. If necessary, move or copy the folder to the computer’s hard drive (as opposed to a network folder) for improved speed.

3.1.2.2) Open a new spreadsheet window. Make sure to enable macros and enable all features, if those messages pop up.

NOTE: Steps 3.1.2.3-3.1.2.6 should only need to be completed the first time the macro is used. For subsequent analysis, simply "**Run the Macro**” starting at step 3.1.2.7.

3.1.2.3) Copy the entire contents of the macro found in the “Sub eCPX\_Sequencing” File.

NOTE: The current version is set up with flanking sequences for the 15-mer library listed in the table of materials and will translate the amino acids between them. Additional sequences can be inputted by copying 5’ flanking sequences in column A and 3’ flanking sequences in column B of the spreadsheet, up to 10 sequences for each, before running the macro. Sequences added to columns A and B take priority over the sequences within the code. Alternatively, the code can be modified to incorporate additional sequences that will be searched for each time. Specifically, line 25 ("GGCCAGTCTGGCCAG”, 5’ Flanking sequence) and line 34 ("GGCTCGAGC", 3’ flanking sequence) and associated lines can be modified or duplicated to incorporate additional sequences to search for (lines 27 and 29 and associated script are already duplicated from line 25 to assist with this process). The additional presence of the search sequences already in the macro will typically not affect the result if the desired search sequence is added in columns A and B or in lines 25 and 34 of the code, unless it is coincidentally part of the peptide insert sequence or is found elsewhere in the sequence query.

3.1.2.4) In the blank spreadsheet file, select the “View” tab, then double click on "Macros." Select the personal.xlb folder. If this is unavailable, record a macro first to make this appear.

3.1.2.5) Click "create" or "step into", depending on what can be highlighted. Paste the entire macro into the module.

3.1.2.6) Click the save icon or go to file, save. Exit the module. If a window pops up, press OK.

3.1.2.7) **Run the Macro:** Go to the folder where the desired .seq files are located. Click on the bar next to the icon of the folder at the top to view the folder location. Copy it.

3.1.2.8) Go to the new spreadsheet window. Select the view tab, then double click on macros. Select “eCPX\_Sequencing” from the list and click "run."

3.1.2.9) In the box that pops up, paste the file location copied in 3.1.2.7 and hit enter. The macro should start going through the sequences and organizing them into tables on various sheets.

3.1.2.10) Use the “Summary Table” sheet to determine if it will be necessary to check trace files for errors and make corrections manually (if sequences contain “X” or could not be translated).

NOTE: The “Summary Table” displays the translated peptide sequences, sorted by amino acid sequence (from A to Z), unless a sequencing error prevented translation. An “X” denotes an individual amino acid that could not be determined. The “AA sequences” sheet also has a “Read OK?” column to describe the total number of amino acids read or if there was a “5’ ERROR”, a “3’ ERROR”, or both (which gives the message “CHECK SEQ”). If there is a 5’ or 3’ error only, the macro will assume the peptide is a 15-mer and will attempt to read the peptide insert sequence starting from the flanking sequence that did match the input search sequence. In those cases, check trace files for errors and make corrections manually. Other tabs display additional information on the details of the sort or display the data formatted for additional processing (e.g., the “FASTA” sheet has the data in the FASTA format). Individual amino acid analysis is displayed in the “Freq Table” sheet.

3.1.3) Once the sequences are translated, organized, and any sequencing errors corrected, check the sorted peptide sequence list on the “Summary Table” sheet for any repeating sequences.

NOTE: It is helpful to look for repeating sequences first since these are likely to bind well and may show obvious trends, such as a consensus sequence.

3.1.4) Grow overnight cultures for sequenced colonies of interest (including the repeats and/or peptides with noticeable trends at a minimum) in 5 mL LB Cm25 at 37°C, shaking at 225 RPM, and save freezer stocks the next day in LB containing 15% glycerol, as in step 1.4.7. Test the peptides with repeating sequences for binding affinity and specificity using the FACS methods described in section 3.3 and use the FASTA format of the sequence list (full list and repeats only) to align the sequences using preferred alignment and analysis programs, as in section 3.2.

***3.2) Sequence Alignment Using Clustal Omega, Kalign, and Similar Programs***

3.2.1) Copy sequences in FASTA format from the FASTA spreadsheet of the macro, or otherwise create a list of FASTA files from the sequences to be aligned (such as those from step 3.1.3).

NOTE: Column A contains the FASTA files in numerical order by “Seq #” while column B displays them sorted by “AA sequence” from the “Summary Table” sheet. The list of peptide sequences sorted by amino acid sequence will display unusable sequences at the top, such as empty vector (as “# Empty”) and those sequences in which neither the 5’ or 3’ search criteria were found (as “# Value”). This feature allows for easy update or removal of those sequences from the analysis.

3.2.2) Open Clustal Omega48 or Kalign49 software by going to the website 50,51, or use other preferred software for sequence alignment. Copy and paste the FASTA sequence list and input it into the box below “sequences in any supported format”. Change “gap open penalty” to 30 under “more options” in Kalign (this is not possible in Clustal Omega), but otherwise keep default settings before clicking “submit”.

NOTE: ClustalW Output works well and gap penalties can be increased (to a maximum of 30) in Kalign for improved alignment of the actual displayed peptides, since gaps are not present. Decreasing gap penalty could be of use to see if a matured version with a spacer amino acid in that gap could yield a better binder, based on alignment to other similar sequences.

3.2.3) Analyze sequence alignment using Jalview 52,53 software directly within Clustal Omega and Kalign online software by selecting the “result summary” tab above the alignment and then clicking the “Jalview” icon.

NOTE: If preferred, or for analysis of sequence alignments generated by alternative programs, download 54 the software separately and copy and paste the alignment into the analysis window, which is opened by clicking “File”, “Input Alignment”, and “from Textbox”. This provides a means to easily determine if a consensus sequence is present in the input sequence alignment.

***3.3) Comparing Binding Affinity and Specificity Using FACS***

3.3.1) Inoculate 5 mL LB Cm25 supplemented with 0.2% w/v D-glucose with each individual isolate of interest (from step 3.1.4 and/or colonies of interest from further sequence analysis in section 3.2, etc.) and an appropriate negative control (display scaffold only or a peptide that does not bind target, as described in the note for step 2.13). Grow overnight at 37 °C, shaking at 225 RPM.

3.3.2) Use the overnight cultures to inoculate 3 mL LB Cm25 (NO GLUCOSE) with 60 µl cells (1:50 dilution). Incubate at 37°C with shaking at 225 RPM until the culture reaches an OD600 of 0.5 – 0.55 (approximately 90-120 min). Induce peptide expression with 0.04% w/v L-arabinose plus 2 mM EDTA (for facilitation of peptide display 42), shaking at 225 RPM for 45 min at 37°C.

3.3.3) Place induced cultures on ice. For each isolate, add 5 µL cells to 25 µL PBS alone or PBS containing: 150 nM YPet 12,13 (positive control for expression), if available; 250 nM Target-488; 250 nM Cross-Reactive Protein(s)-488; and 250 nM SAPE (see 2.1 for more detail on purpose of each reagent). Incubate on ice for 45 min.

NOTE: See step 2.1 for further explanation of labeled targets and negative controls.

3.3.4) Centrifuge cells at 6000 x g for 5 min at RT or 4°C. Carefully remove supernatant by drawing liquid from the opposite side of the pellet. Store cell pellets on ice until all samples and are ready for analysis.

NOTE: Keep the hinge of the microcentrifuge tube facing outward to ensure that the pellet is in a known location, at the bottom on the side of the hinge, as it will be hard to see. Cell pellets can be stored on ice at this point until all samples and are ready for analysis.

3.3.5) Resuspend each cell pellet in 500 µL ice cold FACS running buffer, mixing well by pipetting and flicking the tube, just before reading using a flow cytometer (see table of materials).

NOTE: See step 2.13 notes for further explanation of gating and calculation of nMFI to compare binding affinity and specificity. Non-binders or non-specific binders can now be removed from further analysis, and the highest affinity binders should be re-assessed by sequence alignment following section 3.2 to look for further trends that may have been missed in the initial sequenced population. Sections 3.2 and 3.3 may be repeated as needed as new trends and consensus sequences are noted. This analysis can include more random sequences if trends are not seen.