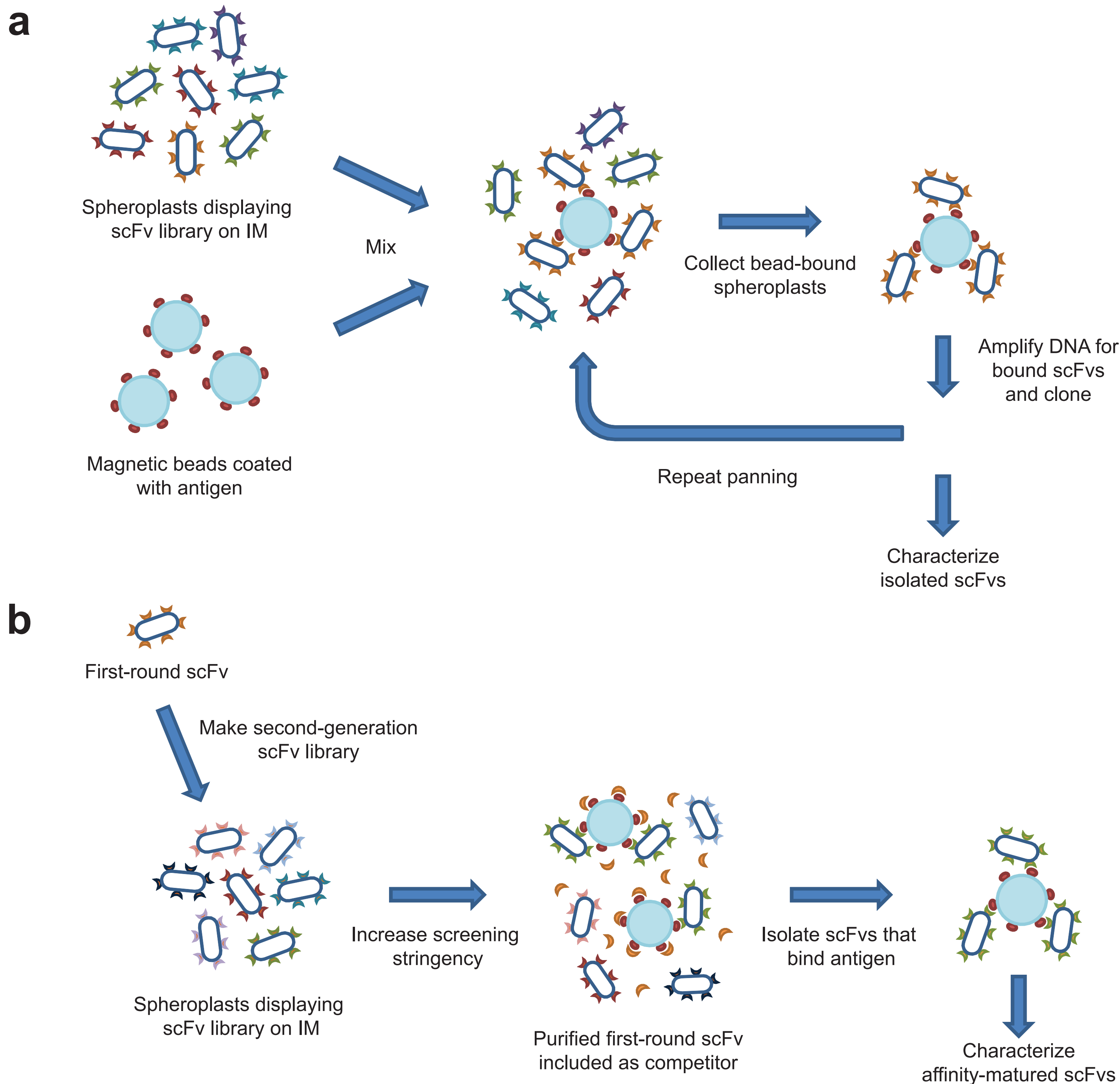
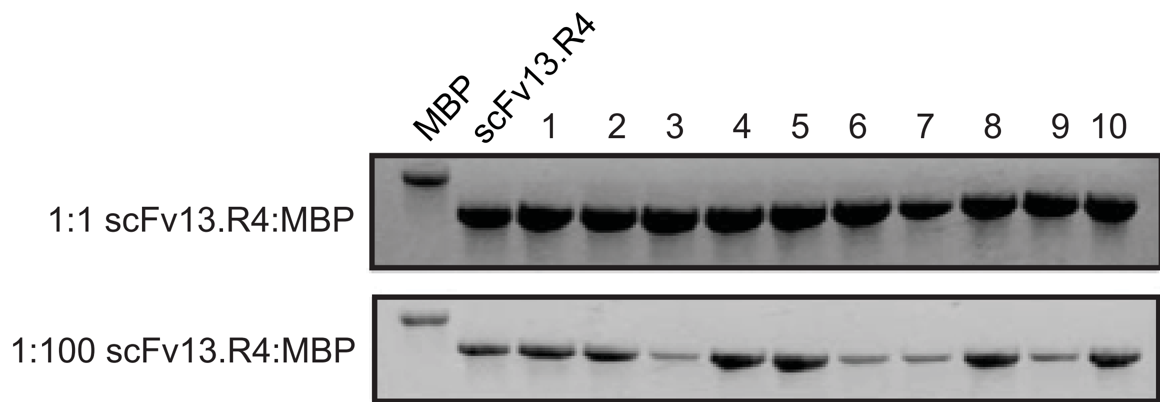
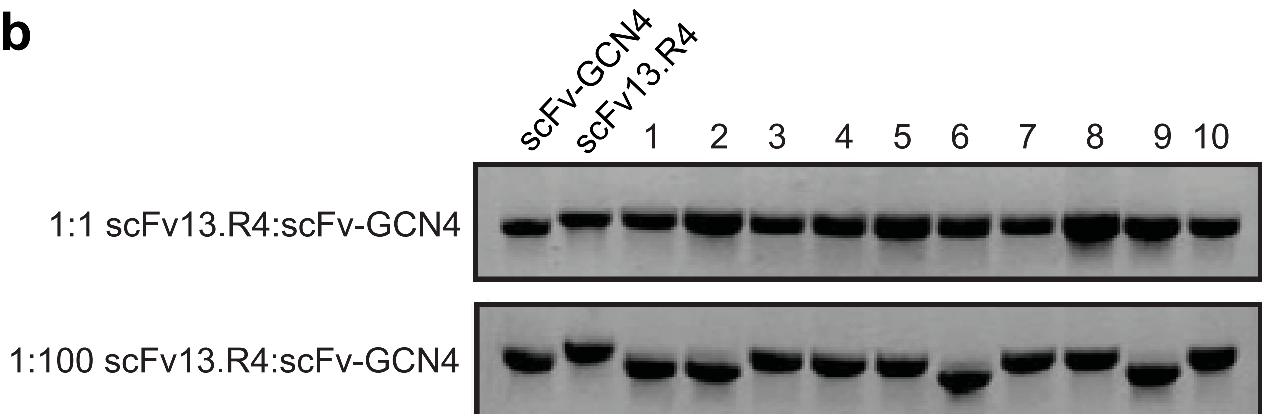
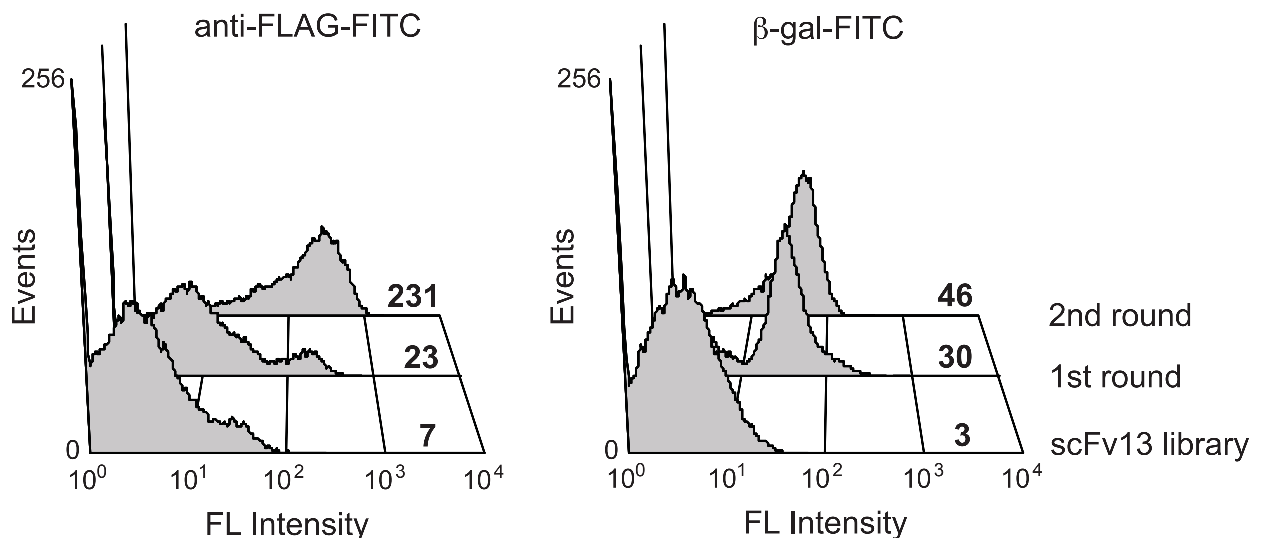


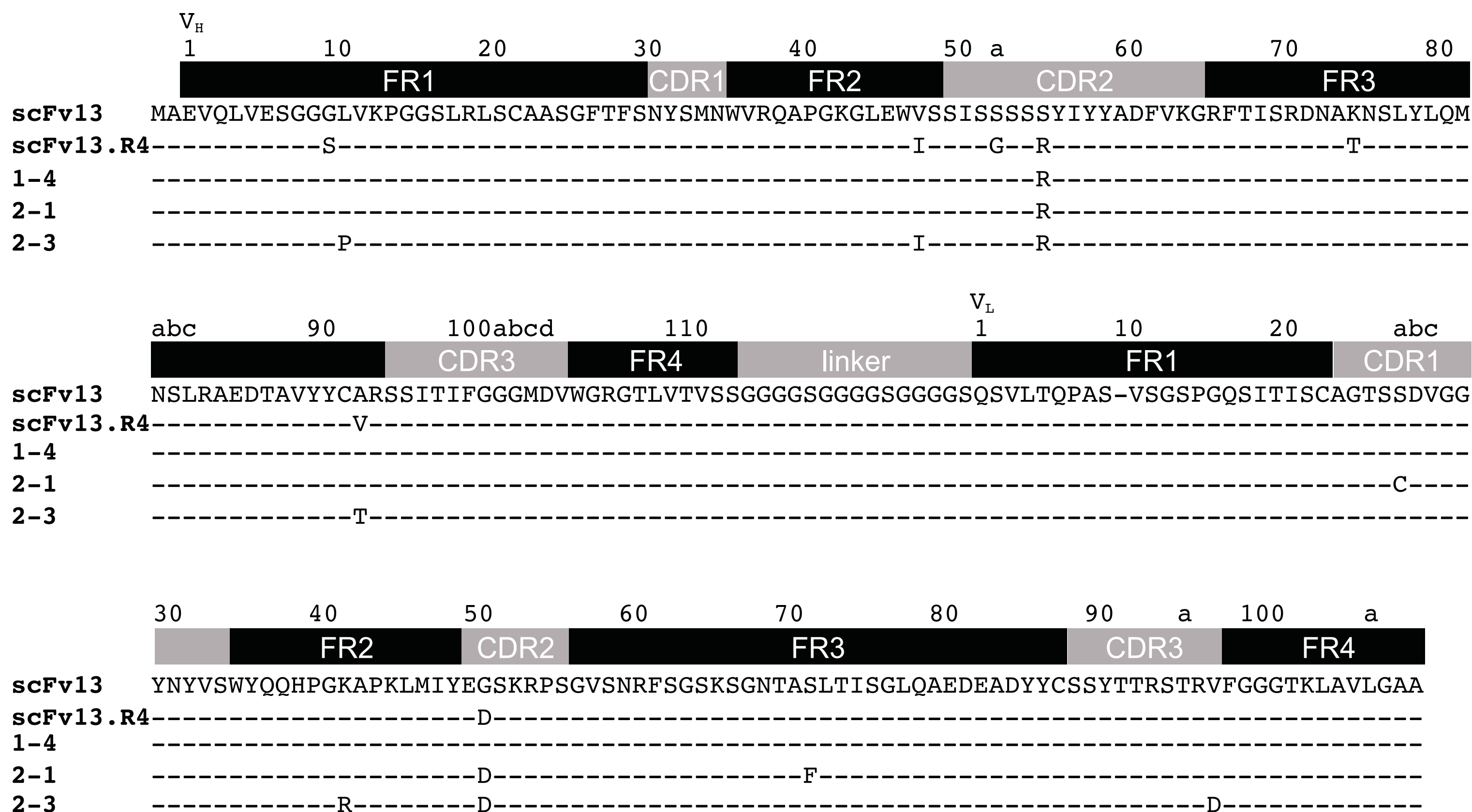
Supplemental Figure S1. Screening strategies for isolating scFvs using MAD-TRAP. (a) First round of screening. An error-prone PCR library was created from scFv13 wt. Spheroplasts were generated from cells displaying this library on the inner membrane (IM) and mixed with magnetic beads coated with the target antigen (β-galactosidase). A magnet was used to collect the bead-bound spheroplasts, and the DNA encoding the scFvs displayed on the bound spheroplasts was recovered by PCR amplification. The resulting scFv sub-library was then cloned back into the IM display plasmid, and the panning process was repeated before characterizing the isolated clones. (b) Second round of screening (affinity maturation). A second-generation error-prone PCR library was created from the most promising clone isolated during the first round of screening (clone 1-4). The screening process was repeated as described above, but, to increase the stringency of the screen, purified clone 1-4 was included as a competitor in the binding reaction.



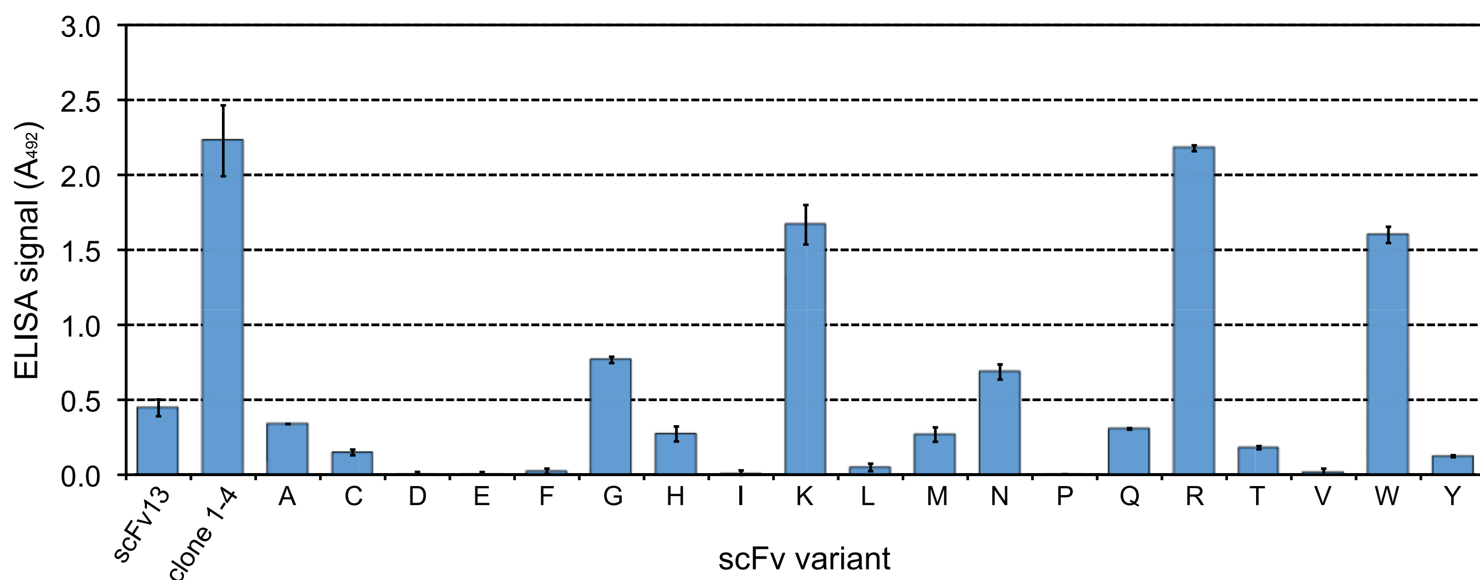




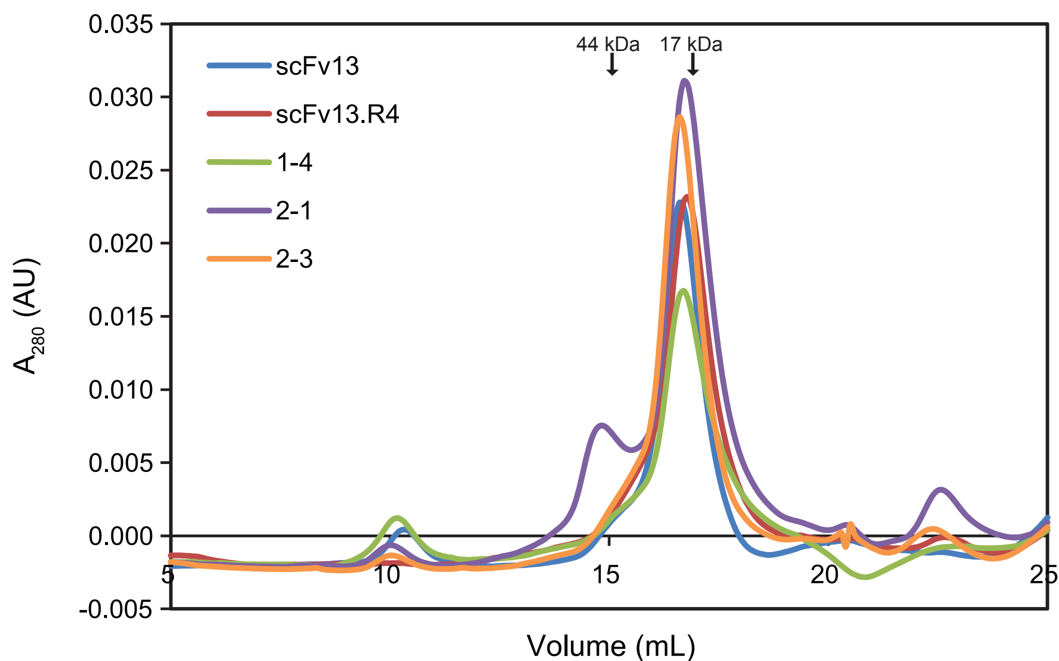
Supplemental Figure S2. Clonal enrichment using MAD-TRAP. (a) PCR analysis of colonies isolated from mixtures of scFv13.R4 and MBP. Spheroplasts of cells expressing scFv13.R4 and MBP were mixed at ratios of 1:1 and 1:100 and panned against β-gal beads. After amplification from bead-bound spheroplasts, PCR products were analyzed by gel electrophoresis to determine the identity of the isolated proteins. (b) PCR analysis of colonies isolated from libraries of scFv13.R4 and scFv-GCN4. Spheroplasts of cells expressing scFv13.R4 and scFv-GCN4 were mixed at ratios of 1:1 and 1:100 and panned against β-gal beads. After amplification from bead-bound spheroplasts, PCR products were analyzed by gel electrophoresis to determine the identity of the isolated proteins. (c) FC analysis of scFv13 random mutagenesis library panning. Spheroplasts expressing the initial scFv13 random mutagenesis library and spheroplasts expressing the libraries resulting from the first and second round of β-gal bead panning were interrogated for the presence of Ti-2 using an anti-FLAG FITC antibody and for antigen binding activity using β-gal-FITC. Median fluorescence values are shown directly in the histogram.



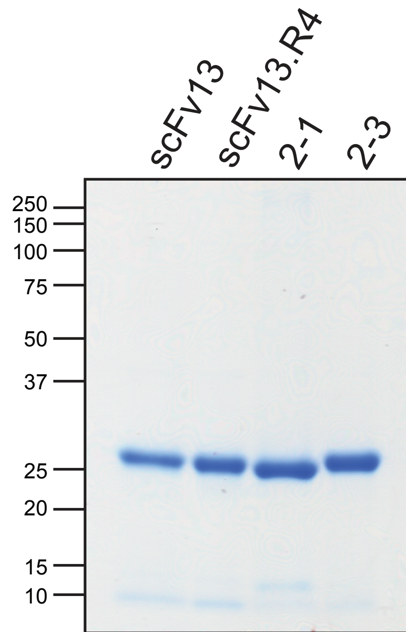
Supplemental Figure S3. Amino acid sequences of anti-β-gal scFv clones isolated using MAD-TRAP. Sequences for scFv13.R4, clone 1-4 from round 1, and clones 2-1 and 2-3 from round 2 are aligned with the sequence for scFv13. scFv13 was the parent scFv for the random mutagenesis library, and scFv13.R4 was isolated in a previous study after four rounds of directed evolution (Martineau et al., 1998). Sequences are numbered using the Kabat numbering system (Abhinandan and Martin, 2008). The scFv sequences begin with the heavy chain (V_H),which is linked to the light chain (V_L) by a flexible amino acid linker.



Supplemental Figure S4. ELISA data for binding activity of saturation mutagenesis clones. Residue S55 in the V_H domain of scFv13 was mutated to all 20 amino acids using a random NNK mutagenesis strategy. Cells expressing each of the scFv S55X (where X is the amino acid indicated) variants in the cytoplasm were lysed to obtain the soluble fraction. Samples were normalized by total protein concentration in the soluble fraction. scFvs bound to β-gal were detected with an anti-6x-His antibody. Data are the average of two replicates, and error bars represent standard error of the mean.



Supplemental Figure S5. Gel filtration analysis of scFvs. scFv samples were purified from *E. coli* lysates, and gel filtration was run to evaluate the multimeric state of the scFvs. Samples were run on a Superdex 200 10/300 GL column at 0.25 mL/min at 4°C in buffer containing 50 mM sodium phosphate (pH 7.5) and 150 mM sodium chloride. For each scFv the major peak is present at a size consistent with scFv monomer.



Supplemental Figure S6. Representative Coomassie-stained gel for purified scFvs. scFvs were expressed in the *E. coli* cytoplasm with a 6x-His tag and purified using Ni-NTA spin columns. A high molecular weight impurity was removed using a 100 kDa molecular weight cut-off column. Purified scFvs were normalized by total protein concentration, run on a 12% polyacrylamide gel, and visualized using Coomassie stain.