Supporting Information

Designed phosphoprotein recognition in *Escherichia coli*

Nicholas Sawyer[1,2], Brandon M. Gassaway[4,5], Adrian D. Haimovich[5,6], Farren J. Isaacs[5,6], Jesse Rinehart[4,5], and Lynne Regan[1,2,3,*]

[1]Department of Molecular Biophysics and Biochemistry, [2]Integrated Graduate Program in Physical and Engineering Biology, [3]Department of Chemistry, [4]Department of Cellular and Molecular Physiology, [5]Systems Biology Institute, and [6]Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT 06520

* Corresponding author: lynne.regan@yale.edu

Table of Contents:

Supplementary Figure & Table Legends

Supplementary Figure 1. Tandem mass spectra for nonphosphopeptide (top) and phosphopeptide (bottom) from tryptic digests of GST fusion proteins. The peptide sequence, parent ion m/z, and MASCOT score are indicated for each spectrum. Ions with m/z consistent with one of the peptide fragments are labeled in both the sequence and spectra. "0" denotes the neutral loss of H_2O and "++" indicates an ion with z = 2.

Supplementary Figure 2. Construction of pNAS duet vectors. Plasmids are depicted as circles with genes overlaid indicating the direction of transcription for each. PCR primers are drawn in proximity to the plasmids based on their regions of sequence complementarity. PCR products are shown as linear fragments. Major assembly steps are indicated by numbers.

Supplementary Table 1. Dissociation constants (K_ds) for each TRAP with both the phosphopeptide target and non-phosphopeptide analogue. The TRAP which exhibits the largest difference between the dissociation constants for TRAP-ME(pS)VD and TRAP-MESVD interactions is highlighted in yellow.

Supplementary Table 2. Primers used for construction of TRAP variants.

Supplementary Table 3. Primers used for construction of pNAS duet vectors.

Supplementary Materials and Methods

Reagents

All restriction enzymes were obtained from New England Biolabs. PyBOP (benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate), 5(6)-carboxyfluorescein, Fmoc-Asp-OH Wang resin, and all Fmoc-amino acids were obtained from Novabiochem (EMD Millipore) and Santa Cruz Biotechnology . All PCR amplification was performed using Phusion^{TM} polymerase (New England Biolabs).

Peptide Synthesis

Fluorescein-tagged peptides (FLu-GGME-pS-VD-OH and FLu-GGMESVD-OH) were manually prepared starting with 1 millimole of Fmoc-Asp-OH Wang resin using standard Fmoc procedures with PyBOP activation (FLu stands for 5(6)-carboxyfluorescein, pS stands for phosphoserine, and OH indicates a free carboxyl terminus).[1] Coupling of 5(6)-carboxyfluorescein was allowed to proceed for longer than standard amino acids (8-12 hours overnight). After 5(6)-carboxyfluorescein coupling, the resin was washed 3 times each with DMF, DCM, and diethyl ether and dried under vacuum. Peptides were cleaved from dried resin using a solution of 95% TFA, 2.5% water, and 2.5% ethanedithiol. Cleaved peptides were precipitated using cold diethyl ether, extracted with water, and lyophilized. Each peptide was purified by HPLC using a Vydac 218TP510 (semi-preparative C18) column. Mass spectrometry (MALDI-TOF) was used to confirm peptide identity.

TRAP Cloning

Several TRAPs (R332/K334, R332/A334, T332/K334, and T332/A334) had been constructed in previous work[12] and were subcloned into the pPROEX-HTam vector by PCR amplification of the TRAP sequence with primers 2A_MGS_F and 2A_MGS_R (see Supplementary Table 2), digestion of PCR product and pPROEX-HTam vector with BamHI and HindIII, and ligation (wild type = T332/D334). The pPROEX-HTam expression vector is a modified pPROEX-HTa vector (Invitrogen) in which the BamHI restriction site is in-frame with the TEV-protease cleavable N-terminal hexahistidine tag. For other TRAPs (R332/R334, K332/K334, K332/R334, K332/A334, and T332/R334), mutations were introduced into the TPR2A gene by QuikChange (Stratagene) using various primers (see Supplementary Table 2) and pPROEX-HTam containing one of the previously constructed TRAPs as a template (the template was chosen for each TRAP such that the number of mutations introduced was minimized). TRAP sequences were confirmed by DNA sequencing (W. M. Keck Facility, Yale University) using the sequencing primer 5'-GAGCGGATAACAATTTCACACAGG-3' (similar to M13 reverse primer).

TRAP Expression and Purification

TRAPs were expressed as fusion proteins with TEV protease-cleavable N-terminal hexahistidine tags in BL21(DE3) Gold cells using ZYM-5052 autoinduction media. For each TRAP, 500 mL of cell culture was grown overnight at 25°C with shaking at 250 rpm as recommend in ref. 2.

Cells were harvested by centrifugation (approximately 5 mL of cells per culture). Cells were lysed by resuspending each cell pellet in 20 mL of lysis buffer (50 mM Tris pH 7.4, 300 mM NaCl, 1 mg/mL lysozyme, 5 mM β-mercaptoethanol, 1 Complete EDTA-free protease inhibitor tablet (Roche Applied Science) per 20 mL), incubating on ice for at least 1 hour, and sonication. Insoluble cellular debris was removed by centrifugation. The clarified lysate was incubated with 5 mL of Ni-NTA agarose resin at 4°C for 10 minutes. The flow-through sample was passed over the column a second time to maximize fusion protein retention. The column was then washed three times with buffers containing 50 mM Tris pH 7.4, 20 mM imidazole, and varying NaCl concentration (5 column volumes per wash, washes 1 & 3 contained 300 mM NaCl while wash 2 contained 1 M NaCl). Protein was eluted from the column using elution buffer (50 mM Tris pH 7.4, 300 mM NaCl, 250 mM imidazole) and collected as a 20 mL fraction.

Eluate was dialyzed 3 times at 4°C in buffers containing 50 mM Tris pH 7.4 and varying salt concentrations (dialysis 1 = 1 L containing 2 M NaCl for 4 hours; dialysis 2 = 2 L containing 300 mM NaCl overnight; dialysis 3 = 1 L containing 300 mM NaCl for 4 hours). The 2 M NaCl dialysis step helped removed fluorescent contamination from the eluate. During the overnight dialysis step, concentrated TEV protease was added to the sample to cleave off the hexahistidine tag. Dialysis in ≥300 mM NaCl is important for both TRAP and TEV protease stability.

After dialysis, 50 mM Tris pH 7.4, 2 M NaCl was added to the sample to increase the final NaCl concentration to 1M to reduce non-specific binding to the Ni-NTA agarose resin. The sample was then incubated with 5 mL of Ni-NTA agarose resin and incubated at 4°C for 10 minutes. The flow-through sample was passed over the column a second time to maximize retention of uncleaved protein. The flow-through sample containing cleaved protein was collected and dialyzed twice in 2L of 50 mM Tris pH 7.4, 300 mM NaCl (first dialysis for 4 hours, second dialysis overnight).

Protein samples were concentrated by centrifugation to 1-2 mL total volume using Amicon Ultra centrifugal filters (3000 MWCO, EMD Millipore) and stored at -80°C until use. Final concentrations were 200 μM-1 mM.

pNAS Duet Vector Assembly

The overall process for pNAS vector assembly is illustrated in Supplementary Figure 2. All primers used for PCR amplification are listed in Supplementary Table 3.

1. Unique XhoI, BglII, and PstI sites were added to the pMB2G vector by PCR amplification of two halves of pMB2G with the primer pairs A+B and C+D, digestion of both fragments with XhoI and PstI, and ligation to create the vector MB2r.

2. The ColE1 origin of replication in MB2r was replaced by a fragment containing the p15A origin of replication from PMRBAD-linkCGFP (and a unique SalI restriction site) by PCR amplification of the GFP-bla fragment of MB2r with primers A+E, PCR amplification of the p15A origin of replication from PMRBAD-linkCGFP using primers F+G, digestion of both fragments with PstI and SalI, and ligation to create the vector p15A.

3. To make the 15Ad duet with GFP and mCherry under the control of two orthogonal promoters, the mCherry gene was first incorporated downstream of the P_{LtetO} promoter in the pZE21G vector. This was accomplished by PCR amplification of the mCherry open reading frame (ORF) from pPROEX-HTam/mCherry-MEEVF using primers H+I, digestion of the PCR fragment and the pZE21G vector with KpnI and HindIII, and ligation to create the vector pZE21-mCherry.

4. The P_{LtetO}-mCherry-terminator fragment was then added to the p15A vector by PCR amplification from pZE21-mCherry using primers J+K, digestion of the fragment and the p15A vector with XhoI and BglII, and ligation to create the 15Ad vector.

5. An unexpected multiple cloning site artifact from the pZE21-mCherry vector was removed by PCR amplification of a shortened mCherry terminator region using primers L+K, digestion of the fragment and 15Ad vector with HindIII and BglII, and ligation to create the 15Ads vector.

6. The araC gene was added to the 15Ads vector by PCR amplification of araC from pMRBAD-linkCGFP using primers M+N, digestion of the fragment and 15Ads vector with BamHI and SphI, and ligation to create the pNAS1 vector.

7. The pNAS1F vector was created by PCR amplification of the P_{LtetO}…araC and P_{BAD}-GFP-rrnB terminator fragments from the pNAS1 vector using primer pairs O+P and Q+R, respectively, digestion of both fragments with AatII and SacI, and ligation. This step added a unique SacI restriction site to the 3' end of the GFP ORF, such that both ORFs are now flanked by unique restriction sites (KpnI & HindIII for P_{LtetO}, AatII & SacI for P_{BAD}).

8. pNAS1B is a permuted form of the pNAS1F vector in which the positions of the mCherry and bla genes are swapped. This was accomplished by PCR amplification of pNAS1F with three primer pairs: S+B, T+U, V+W. The S+B fragment was digested with AvrII and XhoI. The T+U fragment was digested with XhoI and BglII. The V+W fragment was digested with BglII and AvrII. All three fragments were simultaneously ligated to create the pNAS1B vector.

All restriction digests were carried out overnight at 37°C. All ligations were carried out for a minimum of 12 hours at 16°C. Ligation reactions were transformed into DH10B cells by electroporation. Each assembly step was verified by digest and/or sequencing.

Integration of GST Fusion and Split mCherry Genes into pNAS Duet Vectors
Each pNAS duet vector contains the araC gene and p15A origin of replication from pMRBAD-link-CGFP,[16] the ampicillin resistance and GFP genes from pMB2G, and mCherry under the control of the pZE21G-derived P_{LtetO} promoter.[15] For mass spectrometry, genes encoding the GST-peptide fusion proteins were cloned individually

into pNAS1F for expression under the control of the P_{LtetO} promoter. For split fluorescent protein assembly experiments, both halves of mCherry were sequentially cloned into the same pNAS1B duet vector such that N-mCherry and its fusion proteins are expressed under the control of the P_{LtetO} promoter and C-mCherry and its fusion proteins are expressed under the control of the P_{BAD} promoter. The sequence of the pNAS1B plasmid containing N-mCherry and C-mCherry is provided below.

GST Fusion Protein Expression and Purification

Starter cultures were prepared by inoculating a single EcAR7 colony (transformed with both the Sep-OTS and a pNAS1F duet vector containing a GST fusion protein under the control of the P_{LtetO} promoter) into 25 mL of 2xYT supplemented with 100 µg/mL ampicillin and 25 µg/mL kanamycin. Starter cultures were grown for approximately 24 hours at 30°C with shaking at 250 rpm. Expression cultures were prepared by inoculating 10 mL of starter culture into 1 L of 2xYT supplemented with 100 µg/mL ampicillin and 25 µg/mL kanamycin. Expression cultures were grown at 30°C with shaking at 250 rpm until OD_{600} reached 0.8 (4.5-5 hours), at which point IPTG, phosphoserine, and anhydrotetracycline were added to final concentrations of 1 mM, 2 mM, and 100 ng/mL, respectively, to simultaneously induce expression of the Sep-OTS components and GST-peptide fusion protein as well as provide an enriched source of phosphoserine. Cultures were shifted to 20°C for protein expression and allowed to grow for an additional 20 hours with shaking at 250 rpm. Cells were harvested by centrifugation and frozen at -80°C until protein purification.

For purification, each cell pellet was resuspended in 5 mL of phosphoprotein lysis buffer (50 mM Tris pH 7.5, 500 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 10% glycerol, 5 mM DTT, 1 mg/mL lysozyme, 50 mM NaF, 1 mM activated Na_3VO_4, 1 Complete EDTA-free protease inhibitor tablet per 40 mL) and incubated on ice for 30 minutes. Cells were further lysed by sonication followed by centrifugation to remove insoluble material. Clarified lysate was added to 200 µL of glutathione resin and incubated at 4°C for 1 hour. Resin was centrifuged at 500 x g for 5 minutes at 4°C, and supernatant was removed by pipetting. Resin was washed 3 times by resuspending resin in 6 mL of wash buffer (phosphoprotein lysis buffer without lysozyme), centrifuging under the same conditions, and removing supernatant by pipetting. Glutathione buffer (phosphoprotein wash buffer with 50 mM fresh reduced glutathione, pH adjusted to 8.0) was incubated with the resin for 1 hour at 4°C to elute GST fusion proteins from the resin. Resin was then centrifuged under the same conditions, and the supernatant containing purified proteins was collected. Sample buffer was replaced with storage buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM DTT, 20% glycerol) by several rounds of concentration and dilution using Amicon Ultra centrifugal filters (10,000 MWCO). Buffer-exchanged samples were stored at -80°C.

Mass Spectrometry Sample Preparation

In-gel digestion is performed similarly to previously described methods.[3] Briefly, purified GST-peptide fusion proteins were visualized with Coomassie Blue staining (R-250), excised from the gel, and cut into 1 mm^3 pieces. Gel pieces were sequentially washed with fresh solutions containing 50% (v/v) acetonitrile (ACN), 25 mM NH_4HCO_3 and 50%

(v/v) ACN, 5 mM NH_4HCO_3. Proteins were then digested overnight at 37°C in a solution containing 45 mM NH_4CO_3, 5% ACN, and 13.33 ng/µl trypsin (Promega).
Peptides were extracted from gel pieces with 1.67% FA in 66.6%ACN.
Phosphopeptides were enriched by reconstitution in 20 µl of binding buffer (50% ACN, 0.5% TFA) and loading onto stage tips assembled in-house (1x0.6 mm punch of Empore C18 extraction disks (3M) in a 200 µl pipette tip, loaded with 400 µg of Titanshere TiO_2 microshperes (GL Sciences)).[4] Tips were washed twice with binding buffer and once with 80% ACN, 0.1% FA. Peptides were eluted with 1% NH_4OH followed by 80% ACN, 0.1% FA. Peptides were dried and reconstituted in a solution of 19% formic acid, 27 mM sodium phosphate, pH 8.2, and 0.02% TFA for LC/MS/MS analysis.
Chromatographic separation was performed on an LTQ Orbitrap Velos (Thermo Fisher Scientific) with a Waters nanoAcquity system with a vented split configuration on a 32 mm 150 µm ID trap column packed in-house with 3 µm Magic C18AQ 200A resin (Bruker) and a 200 mm 75 µm ID PicoFrit column (New Objective) packed in-house with 1.9 µm ReproSil-Pur C18-AQ 120A resin (Dr. Maisch) using a nonlinear, 90-minute gradient from 5% to 95% ACN (with 0.1% FA). Peptides were detected in data dependent mode using a Top 10 HCD method. Data was searched using MASCOT v 2.4.0.

The pNAS1B sequence containing N-mCherry and C-mCherry is provided below and annotated as follows:

Red = N-mCherry ORF (linker sequence in bold and underlined)
Aqua = unique restriction sites for creating N-mCherry gene fusions (5’ SalI, 3’ HindIII)
Gray = C-mCherry ORF (linker sequence in bold and underlined)
Pink = unique restriction sites for creating C-mCherry gene fusions (5’ AatII, 3’ NotI)

pNAS1B plasmid sequence:

AGATCTTCCCTATCAGTGATAGAGATTGACATCCCTATCAGTGATAGAGATACTGA
GCACATCAGCAGGACGCACTGACCGAATTCATTAAAGAGGAGAAAGGTACCATGG
CATCCGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTTCATGCG
CTTCAAGGTGCACATGGAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGG
CGAGGGCGAGGGCCGCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGA
CCAAGGGTGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCTCAGTTCATGTA
CGGCTCCAAGGCCTACGTGAAGCACCCCGCCGACATCCCCGACTACTTGAAGCTG
TCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGC
GTGGTGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCGAGTTCATCTACAAGG
TGAAGCTGCGCGGCACCAACTTCCCCTCCGACGGCCCCGTAATGCAGAAGAAGAC
CATGGGCTGGGAGGCCTCCTCCGAGCGGATGTACCCCGAGGAC**GGTGGCTCTGG**
CTCTGGGTCGACTGGTTAATGAAAGCTTCATGGTACGCGTGCTGCCAGGCATCAA
ATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTC
GGTGAACGCTCTCCTGAGTAGGACAAATCCGCCGCCCTCCTAGGATCAGCGCTAG
CGGAGTGTATACTGGCTTACTATGTTGGCACTGATGAGGGTGTCAGTGAAGTGCTT
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GAGCGGAAATGGCTTACGAACGGGGCGGAGATTTCCTGGAAGATGCCAGGAAGA
TACTTAACAGGGAAGTGAGAGGGCCGCGGCAAAGCCGTTTTTCCATAGGCTCCGC
CCCCCTGACAAGCATCACGAAATCTGACGCTCAAATCAGTGGTGGCGAAACCCGA
CAGGACTATAAAGATACCAGGCGTTTCCCCCTGGCGGCTCCCTCGTGCGCTCTCC
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TCGTTACCAATTATGACAACTTGACGGCTACATCATTCACTTTTTCTTCACAACCGG
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GATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTT
ACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGT
GAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCA
CTGAGCGA

N-mCherry protein sequence (linker underlined):

MASVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKVTK
GGPLPFAWDILSPQFMYGSKAYVKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTV
TQDSSLQDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEASSERMYPEDGGSGSGSTG
**

C-mCherry protein sequence (linker underlined):

MGAAAGGSGGALKGEIKQRLKLKDGGHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITS
HNEDYTIVEQYERAEGRHSTGGMDELYK**

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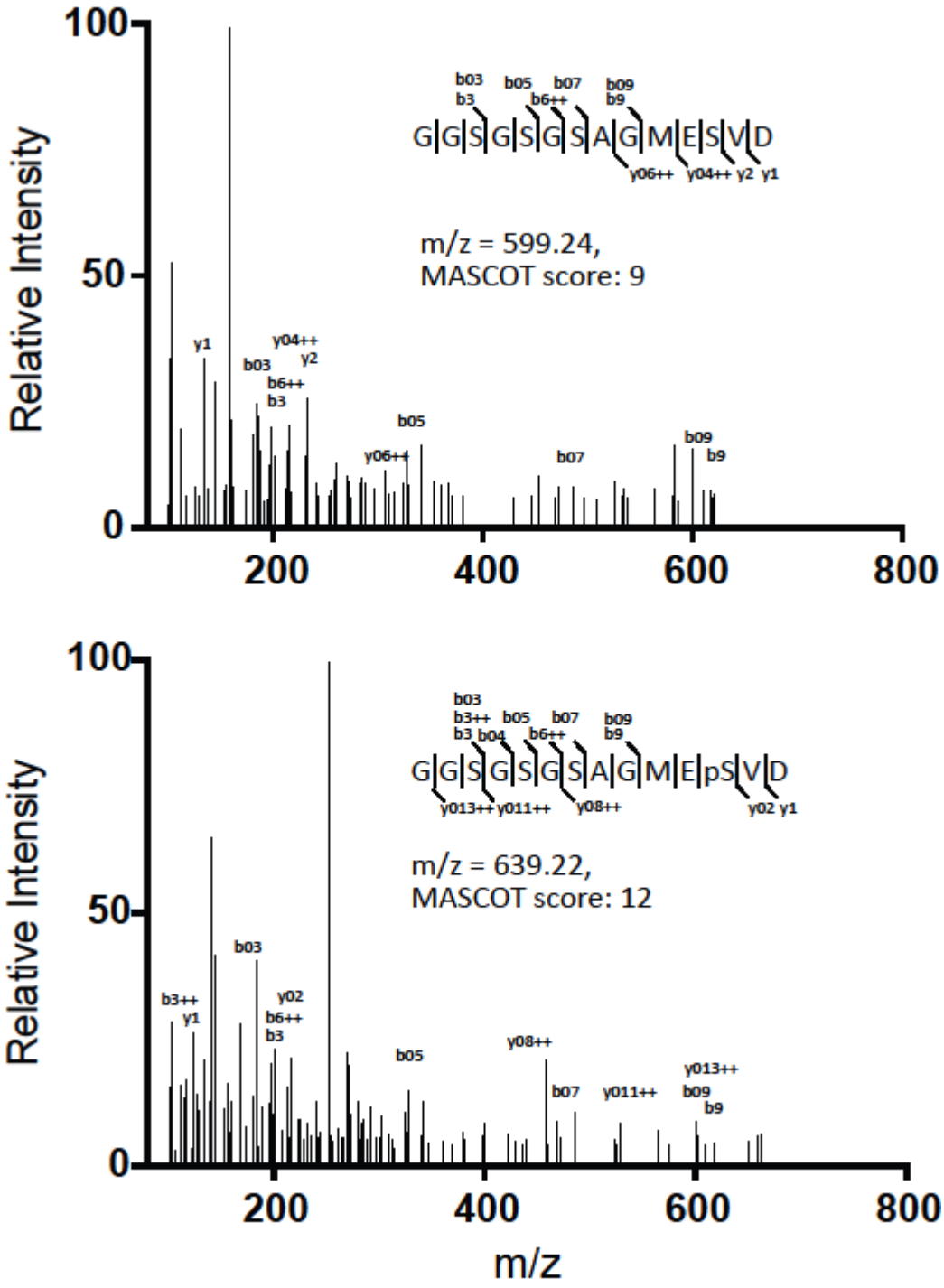
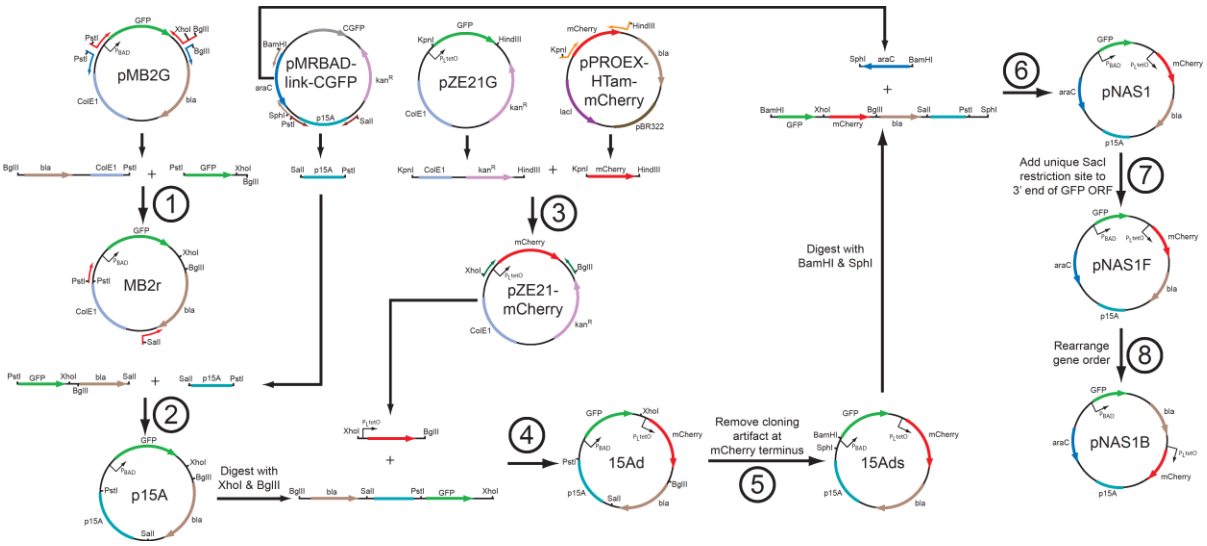


Supplementary Figure 1



Supplementary Figure 2

TRAP Name	Binding affinity for MEpSVD (K_d, μM)	Binding affinity for MESVD (K_d, μM)
RR	2.08 ± 0.20	29.10 ± 1.83
RK	2.15 ± 0.18	66.96 ± 9.30
KK	2.27 ± 0.20	45.32 ± 4.53
KR	2.67 ± 0.23	26.12 ± 2.11
TK	4.63 ± 0.31	48.21 ± 3.43
RA	5.77 ± 0.45	71.40 ± 9.61
TR	6.02 ± 0.45	53.02 ± 6.61
KA	6.16 ± 0.40	46.68 ± 9.05
TA	17.66 ± 1.12	99.41 ± 26.78

Supplementary Table 1

Primer Name	Sequence
2A_F_MGS	ATATTAATGGGATCCAAGCAGGCACTGAAAGAAAAAGAGCTGGGG
2A_R_MGS	CATCGAAAGCTTTAATTATCATTGCTCCTTCAGGATTTTCTCTGCCTGCTG
K2_K4_F	GCAGAACACCGAAAGCCAAAAGTGCTCAAGAAATGC
K2_K4_R	GCATTTCTTGAGCACTTTTGGCTTTCGGTGTTCTGC
R2_R4_F	GCAGAACACCGACGCCCACGTGTGCTCAAGAAATGC
R2_R4_R	GCATTTCTTGAGCACACGTGGGCGTCGGTGTTCTGC
K2_R4_F	GCAGAACACCGAAAGCCACGTGTGCTCAAGAAATGC
K2_R4_R	GCATTTCTTGAGCACACGTGGCTTTCGGTGTTCTGC
K2_A4_F	GCAGAACACCGAAAGCCAGCAGTGCTCAAGAAATGC
K2_A4_R	GCATTTCTTGAGCACTGCTGGCTTTCGGTGTTCTGC
2A_T2R4_F	GCAGAACACCGAACCCCACGTGTGCTCAAGAAATGC
2A_T2R4_R	GCATTTCTTGAGCACACGTGGGGTTCGGTGTTCTGC

Supplementary Table 2

Primer Name	Sequence
A	ATTATACTGCAGAGATCAATTCGCGCGCGAAGG
B	TTAAATCTCGAGGAGTTTGTAGAAACGCAAAAAGGCCATCC
C	ATATATCTCGAGATTAATAATTAAAGATCTTTCAAATATGTATCCGCTCATGAGACAATAACCC
D	TAGTTACTGCAGGCTGCCTCGCGCGTTTCG
E	ATTAAAGTCGACTCGCTCAGTGGAACGAAAACTCACG
F	TATTTAGTCGACATCAGCGCTAGCGGAGTGTATACTGG
G	TTAATACTGCAGTTAATAAGATGATCTTCTTGAGATCGTTTTGGTCTGCG
H	TATTAAGGTACCATGGCATCCGTGAGCAAGGGCGAGGAGG
I	TATATTAAGCTTTTATCACTTGTACAGCTCGTCCATGCCG
J	TAAATTCTCGAGTCCCTATCAGTGATAGAGATTGACATCC
K	TTAATTAGATCTAGGGCGGCGGATTTGTCC
L	TAATATAAGCTTCATGGTACGCGTGCTGCCAGGCATCAAATAAAACGAAAGGCTCAGTCG
M	TATATAGCATGCTGCATAATGTGCCTGTCAAATGGACGAAG
N	CGTCAGGTAGGATCCGCTAATCTTATGG
O	TTATTTGAGCTCTTGGCTGTTTTGGCGGATGAGAGAAG
P	ACTCATGACGTCATTCCTCCTGTTAGC
Q	GCTAACAGGAGGAATGACGTCATGAGT
R	ATAATTGAGCTCTTATTTGTAGAGTTCATCCATGCCATGTGTAATC
S	TATTTACCTAGGATCAGCGCTAGCGGAGTGTATACTGG
T	ATATATCTCGAGTTCAAATATGTATCCGCTCATGAGACAATAACCC
U	ATTAAAAGATCTTCGCTCAGTGGAACGAAAACTCACG
V	TAAATTAGATCTTCCCTATCAGTGATAGAGATTGACATCC
W	TTAATTCCTAGGAGGGCGGCGGATTTGTCC

Supplementary Table 3