Supplementary Methods

V_HH panning strategies. In the first round of pannings, 10 ug/mL of target antigen was coated onto a Nunc Immunotube, then blocked with a blocking solution (either 2% goat serum or 4% non-fat dried milk powder in PBS). The phage library was diluted 2-fold in blocking solution, then exposed to immobilized antigen for 1 hour. Unbound phages were washed away with 0.5% PBST, and the bound phages were eluted. Elution was carried out in two phases, first by adding an overnight growth of ER2738 *E. coli* (tetracycline resistant) for 15 minutes, and then secondarily by eluting remaining bound phages with a low pH glycine solution (pH 2.2) for 10 minutes. The glycine eluted phages were neutralized with Tris, combined with the ER2738 eluted phages and plated on SB-Agar-Ampicillin-Tetracycline-2% glucose plates to form a lawn. This lawn was grown overnight, then scraped and stored in 15% glycerol at -80C, to be used as input for the second round. To prepare the phage input for the second round, the same procedure as round 1 was repeated, where an aliquot of the Round 1 Elution was thawed and grown to log phase, then infected with helper phage. Phages were precipitated and prepared for screening as in round 1. In the second round, the antigen was coated on the immunotube at 1 ug/mL and blocked. The round 2 phage input was then diluted 200-fold in blocking solution, and binding to immobilized antigen was limited to 10 minutes before washing and elution. Eluted phages were serially 10-fold diluted and plated, and 95 individual *E. coli* colonies were chosen for further study, one in each well of a 96 well "master" plate (with one empty well as a negative growth control). Each well contained 200 uL SB media with ampicillin, tetracycline and 2% glucose. In some screens, two separate 96 well plates were chosen, resulting in 190 individual colonies. The remaining bulk of the second round elution was plated, and then scraped and stored at -80C.

To test chosen VHH colonies for binding to targets, a "soluble" plate was made. A few µL of each well of the master plate was transferred to a well of fresh media (160 uL SB ampicillin, tetracycline) in a new plate. The plate was grown to log phase, and then induced to express VHH-pIII fusion protein with 70 uL of 10 mM IPTG overnight (final concentration ~3 mM). Expressed VHH protein (with an epitopic E-tag situated between the VHH and pIII proteins) is targeted to the periplasm by a signal peptide, and some small amount leaks out of the bacterial outer membrane and into the supernatant. The next day the plate was spun down, and the supernatant was transferred to ELISA plates that had been coated with relevant antigens and then blocked. Binding was detected with anti-E tag antibody conjugated to HRP (Bethyl Labs),

developed with SureBlue TMB (KPL), and the absorbance was read at 450 nm on a VersaMax Microplate reader (Molecular Devices). The VHH coding regions of positive binding clones were then amplified by PCR, and DNA fingerprinted by digestion with two different restriction enzymes (BsaJI and BSTN1) to identify unique clones. All unique digestion patterns were then grown, miniprepped and sequenced, and the VHHs with unique protein sequences were chosen for expression and characterization.

The nine different screening strategies were as follows:
Screens 1 and 2: Immunotubes were directly coated with RTA (1) or RTB (2). Two plates of colonies against each target were chosen. This resulted in 11 anti-RTA (JIV- and JIY-) and 9 anti-RTB (JIW- and JIZ-) VHHs.

Screens 3 and 4: Immunotubes were directly coated with RTA (3) or RTB (4). One plate of colonies against each target was chosen. This resulted in 7 anti-RTA (JNM-) and 9 anti-RTB (JNN-) VHHs.

Screens 5 and 6: Immunotubes were coated with either ASF (5) or PB10 (6), and then ricin was captured. One plate of colonies against each target was chosen, and only VHHs double positive for binding to both targets were kept. This resulted in 10 anti-RTA, 2 anti-RTB and 1 anti-holotoxin VHHs (V1 and V2).

Screen 7: Immunotubes were coated with avidin, and then biotin-tagged peptides E12 and G3 were captured. One plate of colonies was chosen. None of the chosen colonies recognized the peptides, RTA or ricin.

Screen 8: Immunotubes were coated with ASF, and then RTB was captured. One plate of colonies was chosen. This resulted in 5 anti-RTB VHHs (V4).

Screen 9: Immunotubes were coated with JIV-F5, and then ricin was captured. JIV-F6 was added into the supernatant in saturating concentrations. One plate of colonies was chosen. This resulted in 3 anti-RTA VHHs, 8 anti-RTB VHHs, and 3 anti-holotoxin VHHs (V5).

VHH Nomenclature. VHH nomenclature is based on an incremental naming scheme unique to the institute that identified the V_HH. Screens 1-4 were performed at Tufts Cummings School of Veterinary Medicine, where each plate of colonies chosen after two rounds is given a three-letter code. Any VHH identified from that plate is named as the three-letter code, followed by the well that the VHH was grown in (i.e, JIV-F5 was from the plate named JIV, and well F5). Starting with screen 5, ricin panning was performed at the Wadsworth Center, where "VHH Phage Display Experiment 1" led to a plate of colonies named V1. Thus, V1B4 was from the "V1" plate, well B4, and so forth.

VHH Cloning and Expression. Once VHHs were sequenced and chosen for expression, the VHH coding insert was digested out of the phage display plasmid with AscI/NotI, run on and cut out of a gel, and Gene Cleaned (MP Biomedical, Santa Ana, CA). The insert was then added to a previously prepared linear expression plasmid (also cut with AscI/NotI, as well as an internal SpeI cut site) with Ampicillin resistance, a Thioredoxin expression partner for the VHH, and a C-terminal epitopic E-tag peptide, and ligated with T4 ligase. The plasmid was then transformed into Top 10 *E. coli* cells (Thermo Fisher Scientific, Waltham, MA) and plated on LB-Ampicillin plates. Two colonies were picked off the plate, grown in LB-Amp media, and their plasmids isolated. Plasmid DNA was sequenced to confirm the ligation and the expected VHH sequence. One correct clone was chosen for each VHH to be transformed for expression.

Once the sequence was confirmed, the plasmid prep was then transformed into Rosetta-gami 2(DE3)pLacI *E. coli* cells (EMD Millipore, Billerica, MA) with chloramphenicol resistance, and grown on LB-Amp-Chlor-2% glucose plates overnight. A single colony was then chosen and grown in LB-Amp-Chlor-2% glucose media overnight. The next morning, this growth was used to seed a subculture of 130 mL of LB-Amp-Chlor media. Once grown to log phase, VHH expression was induced with 1 mM IPTG, and the suspension was shaken at 15° C overnight. The bacteria were then lysed using Bug Buster plus Lysonase Kit (EMD Millipore, Billerica MA), HexaHis-tagged thioredoxin-VHH protein in the supernatant was purified with a Ni-NTA agarose (Thermo Fisher Scientific, Waltham, MA) column, and eluted with 250 mM imidazole. Purified VHH was then dialyzed in a 7000 MWCO Slide-A-Lyzer dialysis cassette (Thermo Fisher Scientific, Waltham, MA) against 4L PBS overnight, with at least one buffer

change. Dialyzed protein was then sterilized using a Whatman Puradisc 0.2 micron filter (Thermo Fisher Scientific, Waltham, MA). Protein purity was verified by SDS PAGE (**Fig. 1A**), and concentration was determined by absorbance at 280 nm using the calculated extinction coefficient for each thioredoxin-VHH construct.

HX-MS analysis of RiVax with V_HHs. JNM-C12 protection of RiVax peptides is shown in **Figure S7A** and is shown mapped onto the structure of RTA in **Figure 6A**. As described in the methods section, regions that are strongly and intermediately protected are defined as the epitope region. Based on protection, the JNM-C12 binds to residues 92-109 spanning helix B and a 3/10-helix on the C-terminal end of helix B and residues 108-122 spanning strand h. There was also some deprotection of residues 249-255 spanning a 3/10- helix and a β-bridge, possibly due to allosteric effects induced by JNM-C12.

JNM-D1 protection of RiVax peptides is shown in **Figure S7B** and mapped onto the structure of RTA in **Figure 6B**. Based on protection, the JNM-D1 binds to residues 130-135, spanning a 3/10-helix on the C-terminal end of helix C, residues 123-129, spanning helix C, and in residues 205-210, spanning helix G. Interestingly, this V_HH induced strong deprotection (faster HX) in residues 249-255 that span a 3/10- helix and a β-bridge. This strong deprotection on the opposite side of RiVax might be due to allosteric effects induced by JNM-D1.

V1B11 protection of RiVax peptides is shown in **Figure S7C** and is shown mapped onto the structure of RTA in **Figure 6C**. Based on protection, V1B11 binds to residues 123-135, spanning helix C, residues 205-217, spanning helix G, and residues 247-255, spanning a 3/10-helix and a β-bridge,. The binding of V1B11 to residues 247-255 of RiVax is in contrast to the other two V_HHs, JNM-D1 and JNM-C12, where there is an increase in flexibility in residues 247-255. However, V1B11 and JNM-D1 share a similar epitope region on RiVax in residues 123-135, spanning helix C and residues 205-217, spanning helix G.

Supplemental References

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Supplementary Figure Legends

Figure S1. RTA secondary structure. RTA (PDB ID: 1RTC) is shown in green cartoon representation. Secondary structural elements are labeled.

Figure S2. Vero cell cytotoxicity assays. The 7 strong (legend, left, closed symbols) and 7 moderate (legend, right, open symbols) neutralizing V_HHs are shown. The horizontal dotted line at 50% cell viability intersects each curve at the IC_{50} value for that VHH. The vertical dotted lines are at the borderlines between neutralizing categories. VHHs with $IC_{50}s$ <10 nM are strong neutralizers, while those with $IC_{50}s$ between 10 and 100 nM are moderate neutralizers. The graph is zoomed in to concentrations between 100 pM and 1 μM to clearly show the delineation between strong and moderate neutralizers. Shown is a representative curve for each V_HH, with each concentration tested in triplicate, and error bars representing the standard deviation. In some cases, the error bars are too small to be visible.

Figure S3. Representative SPR binding curves. Shown are representative SPR binding curves for the same five VHHs as in Figure 1. (A) V5B6; (B) V5D5; (C) V5E4; (D) V5G1; (E) V5H6.

Figure S4. Alignment of ten published VHH:RTA co-crystal structures. RTA (PDB ID: 1RTC) is shown in gray surface representation. All ten published VHH:RTA co-crystal structures (see Table 2 for PDB IDs) are aligned showing epitope clustering. With respect to the left image, Cluster I VHHs are shown on the top right (JIV-F5 (light purple), JIV-G12 (blue), JIY-A7 (yellow), JIY-E5 (brown), JIY-G11 (pink) and JNM-F8 (orange)). The yellow VHH on the top left is JIY-D10, which straddles Clusters I and II. The three VHHs on the left side, JIY-E1 (gray), V1C7 (pink), and V5E1 (green) represent Cluster II.

Figure S5. Sandwich ELISA overestimates VHH epitopes. Left: The same image that is shown in Figure 2C, the co-crystal structure of V1C7 (red) and RTA (gray) (PDB ID: 5J56), with its sandwich ELISA predicted epitope colored orange and purple. Right: The same image, rotated up 90 degrees. In this orientation, it's notable that the predicted epitope overestimates the surface area contacted by V1C7 in the crystal structure. This likely represents steric clashes between the VHH and the mAbs used in the sandwich ELISA.

Figure S6. Heat map of anti-RTA VHHs bound to mAb captured ricin. Anti-RTA VHHs are listed down the left side, while mAbs representing the various RTA epitope clusters are shown along the top. The surrogate receptor ASF is also included. All VHHs are normalized to the mAb-ricin complex to which it bound strongest.

Figure S7. $\Delta\overline{HX}$ plots of RiVax in the presence of respective V_HHs as measured by HX−MS. **(A)** JNM-C12, **(B)** JNM-D1 and **(C)** V1B11. The x-axis is the peptide index. The residue ranges of the peptides are listed in **Table S5**. The $\Delta\overline{HX}$ on the y-axis was calculated as described in methods and in more detail elsewhere (R. Toth et al., *manuscript in preparation*). The bars (peptides) are colored based on protection category: dark blue indicates strong protection, medium blue is intermediate protection, and yellow is strong deprotection. The dashed lines

represent a confidence interval set at three times the standard error of $\Delta\overline{HX}$ for each peptide. Gaps in the confidence limit indicate that data for the corresponding peptides are not available.

Figure S8. Heat map of anti-RTB VHHs bound to mAb captured ricin. Anti-RTB VHHs are listed down the left side, while anti-RTB mAbs and mAbs representing RTA Cluster 2 are shown along the top. The surrogate receptor ASF is also included. All VHHs are normalized to the mAb-ricin complex to which it bound strongest.

Supplementary Tables

Table S1. Non-Expressed V_HHs

VHH	Screen	Genbank	Specificity[a]	Family
JNM-F2	3	KY824624	A	JIY-D10
JNM-F3	3	KY824625	A	JNM-A11
JNM-G5	3	KY824626	A	JIV-F6
JNN-A8	4	KY824627	B	JIZ-B7
JNN-B10	4	KY824628	B	JIZ-B7
V1A8	5	KY824629	A	JIY-D9
V1A12	5	KY824630	A	JNM-B5
V2F11	6	KY824631	A	JIV-F6
V4C1	8	KY824632	B	V4C6
V4C7	8	KY824633	B	V2C11
V4E3	8	KY824634	B	V2C11
V4H5	8	KY824635	B	JIZ-B7
V5F4	9	KY824636	A	-[b]
V5G9	9	KY824637	B	V4A1

[a], A=RTA, B=RTB; [b], V5F4 is a unique sequence and does not belong to a sequence family; V_HH expression in *E. coli* failed.

Table S2. V_HH Binding Kinetics to Ricin[a]			
V_HH	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_D (M)
JIV-F5	5.44E+05	1.05E-05	1.93E-11
JIV-F6	1.94E+05	3.61E-04	1.86E-09
JIV-G12	9.30E+05	1.20E-04	1.29E-10
JIY-A7	1.50E+05	6.50E-04	4.33E-09
JIY-D9	3.06E+05	5.31E-05	1.74E-10
JIY-D10	4.40E+05	5.00E-05	1.14E-10
JIY-E1	3.50E+05	2.40E-04	6.86E-10
JIY-E3	9.53E+05	2.82E-04	2.96E-10
JIY-E5	1.10E+05	2.10E-05	1.91E-10
JIY-G11	1.50E+05	8.30E-05	5.53E-10
JIZ-B7	3.06E+05	9.35E-06	3.06E-11
JNM-A11	4.45E+05	8.92E-05	2.00E-10
JNM-B5	4.70E+05	2.82E-05	6.00E-11
JNM-C12	5.07E+05	5.01E-05	9.88E-11
JNM-D1	1.80E+05	2.15E-04	1.19E-09
JNM-E4	2.88E+05	2.40E-05	8.33E-11
JNM-F8	9.37E+05	1.90E-04	2.03E-10
JNM-G4	1.23E+06	1.57E-04	1.28E-10
V1B4	6.55E+04	2.68E-04	4.09E-09
V1B10	8.29E+04	7.60E-05	9.17E-10
V1B11	2.76E+04	2.44E-04	8.84E-09
V1C7	9.10E+04	2.90E-04	3.19E-09
V1D3	3.15E+05	1.45E-04	4.60E-10
V1G6	3.05E+04	1.63E-04	5.34E-09
V2A11	2.97E+04	5.41E-05	1.82E-09
V2B8	1.96E+04	6.36E-04	3.24E-08
V2B9	1.47E+05	1.51E-04	1.03E-09
V2C11	3.45E+05	5.32E-06	1.54E-11
V2D4	2.75E+04	8.79E-05	3.20E-09
V2E8	5.09E+04	2.92E-04	5.74E-09
V2G10	8.48E+04	9.84E-05	1.16E-09
V4A1	2.49E+05	1.66E-04	6.67E-10
V5A2	2.15E+05	3.14E-04	1.46E-09
V5B1	1.38E+05	2.99E-04	2.17E-09
V5B6	1.76E+05	1.17E-04	6.68E-10
V5C1	6.37E+05	1.92E-04	3.01E-10
V5C4	3.85E+04	1.93E-04	5.01E-09
V5D1	4.25E+05	9.20E-05	2.16E-10
V5D5	5.91E+04	3.27E-04	5.54E-09

V5E1	2.17E+05	4.14E-06	1.91E-11
V5E4	4.82E+05	2.90E-06	6.01E-12
V5G1	1.89E+05	2.00E-05	1.06E-10
V5G6	1.98E+05	2.45E-04	1.24E-09
V5G12	1.64E+05	1.63E-04	9.94E-10
V5H2	1.82E+05	3.96E-04	2.18E-09
V5H6	3.00E+04	6.96E-04	2.32E-08

[a], Binding kinetics were determined by SPR, as described in the Materials and Methods.

Table S3. RTA-specific mouse mAbs		
mAb	**Cluster**	**Ref.**
PB10	I	[1]
WECB2		[2]
SWB1	I-II	unpublished
SyH7	II	[1]
PA1		[2]
TB12		[2]
PH12		[2]
IB2	III	[2]
GD12	IV	[3]

Table S4. RTB-specific mouse mAbs	
mAb	**Ref.**
24B11	[4]
SylH3	[5]
JB11	[5]
JB4	[6]
BJF9	[6]

Table S5. RiVax peptic peptides

	Amino acid residue #			Amino acid residue #			Amino acid residue #			Amino acid residue #	
Pep #	start	end	Pep #	start	end	Pep #	start	end	Pep #	start	end
1	1	11	**36**	92	103	**71**	162	168	**106**	217	232
2	12	20	**37**	92	107	**72**	165	168	**107**	217	240
3	12	24	**38**	93	99	**73**	165	171	**108**	218	225
4	21	24	**39**	93	107	**74**	168	171	**109**	218	232
5	25	32	**40**	102	107	**75**	169	173	**110**	220	232
6	25	37	**41**	103	107	**76**	172	181	**111**	221	232
7	28	37	**42**	104	107	**77**	175	181	**112**	226	232
8	33	59	**43**	104	109	**78**	178	181	**113**	226	240
9	37	59	**44**	108	117	**79**	182	186	**114**	227	240
10	38	45	**45**	108	118	**80**	182	187	**115**	232	240
11	38	55	**46**	108	122	**81**	182	188	**116**	232	243
12	38	57	**47**	118	122	**82**	182	190	**117**	232	248
13	38	59	**48**	119	126	**83**	182	204	**118**	233	243
14	56	59	**49**	123	126	**84**	187	204	**119**	233	244
15	58	61	**50**	123	129	**85**	188	204	**120**	233	246
16	58	68	**51**	123	133	**86**	189	204	**121**	233	248
17	60	68	**52**	123	135	**87**	189	206	**122**	240	243
18	60	69	**53**	127	133	**88**	191	204	**123**	241	244
19	62	68	**54**	127	135	**89**	191	207	**124**	241	246
20	69	72	**55**	130	135	**90**	195	204	**125**	241	248
21	69	73	**56**	130	151	**91**	205	210	**126**	243	248
22	69	74	**57**	133	144	**92**	205	214	**127**	244	248
23	70	74	**58**	134	146	**93**	205	216	**128**	245	248
24	72	79	**59**	134	151	**94**	205	217	**129**	247	253
25	72	91	**60**	136	146	**95**	207	214	**130**	247	254
26	73	79	**61**	136	147	**96**	207	216	**131**	247	255
27	73	91	**62**	136	151	**97**	207	217	**132**	249	253
28	75	79	**63**	146	150	**98**	208	214	**133**	249	254
29	75	91	**64**	147	150	**99**	208	216	**134**	249	255
30	80	91	**65**	147	151	**100**	208	217	**135**	255	267
31	80	92	**66**	148	151	**101**	211	216	**136**	256	267
32	84	91	**67**	152	161	**102**	211	217	**137**	257	267
33	92	99	**68**	152	164	**103**	212	216	**138**	258	267
34	92	101	**69**	153	164	**104**	217	220			
35	92	102	**70**	162	167	**105**	217	225			

Figure S1

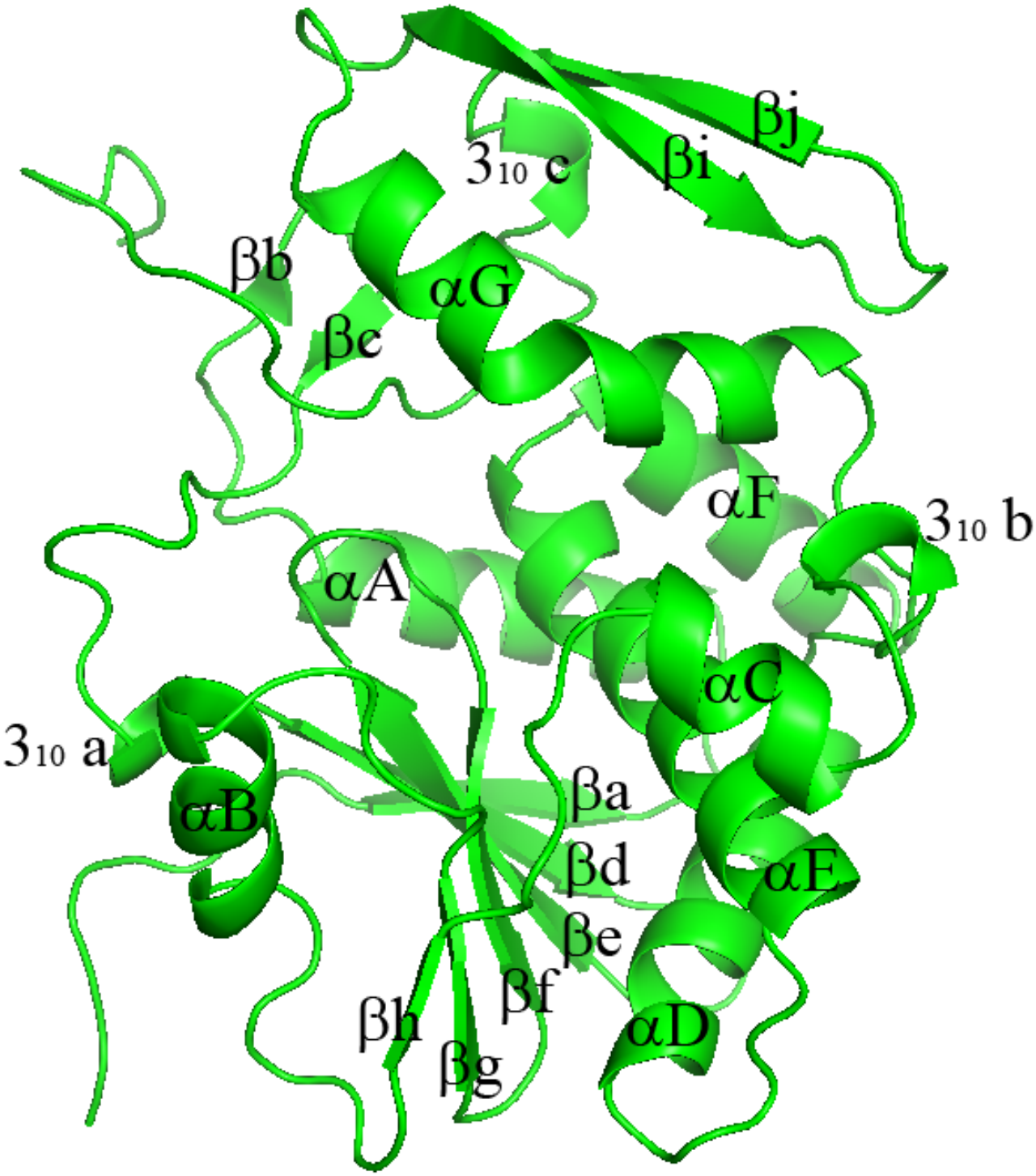
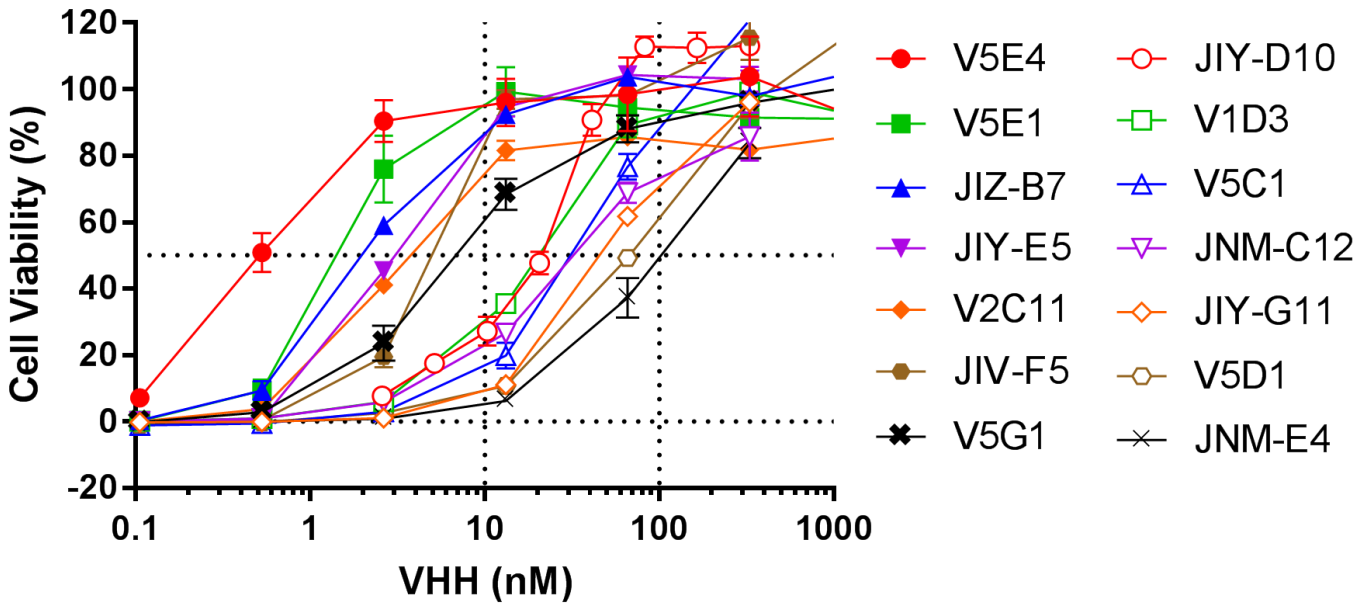


Figure S2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100
80
60
40
20
0
-20
0.1
1
10
100
1000
VHH (nM)
V5E4
V5E1
JIZ-B7
JIY-E5
V2C11
JIV-F5
V5G1
JIY-D10
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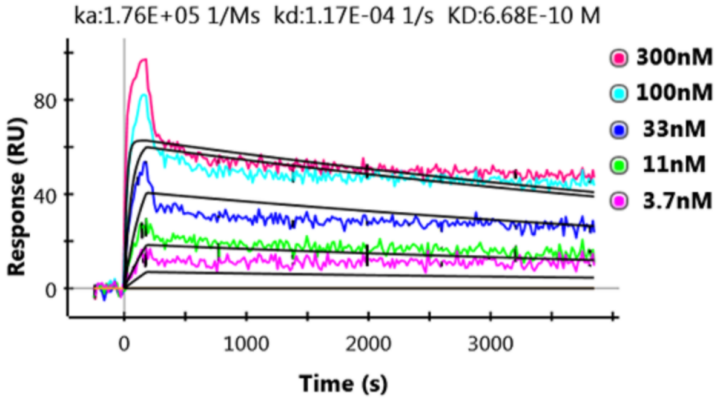
V5C1
JNM-C12
JIY-G11
V5D1
JNM-E4

Figure S3

A. V5B6



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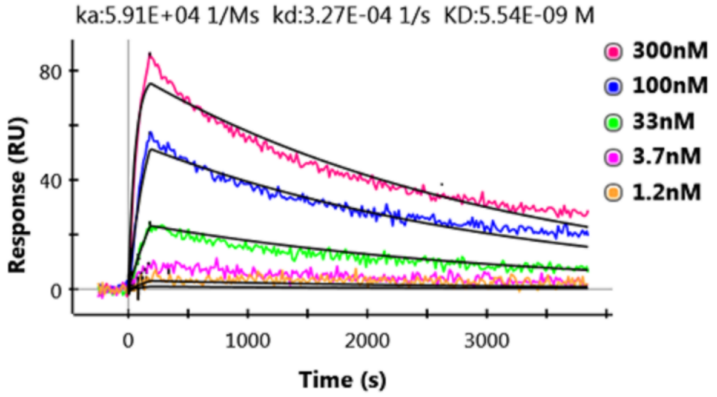

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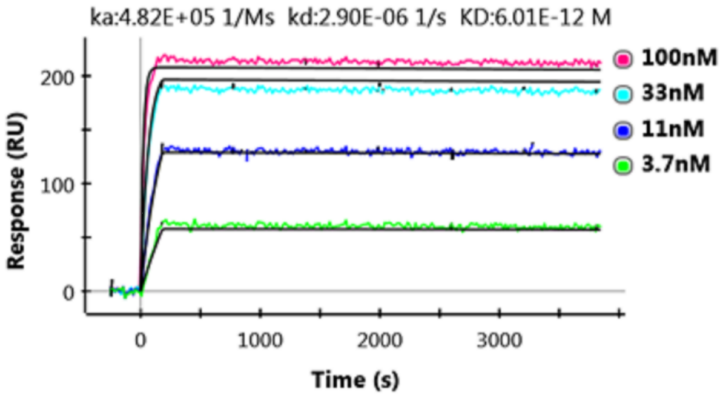

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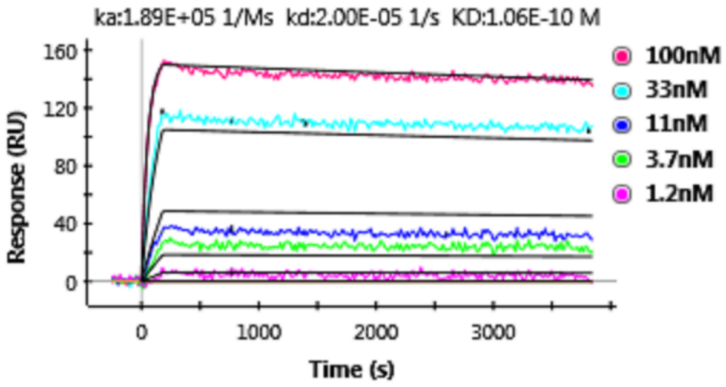


E. V5H6

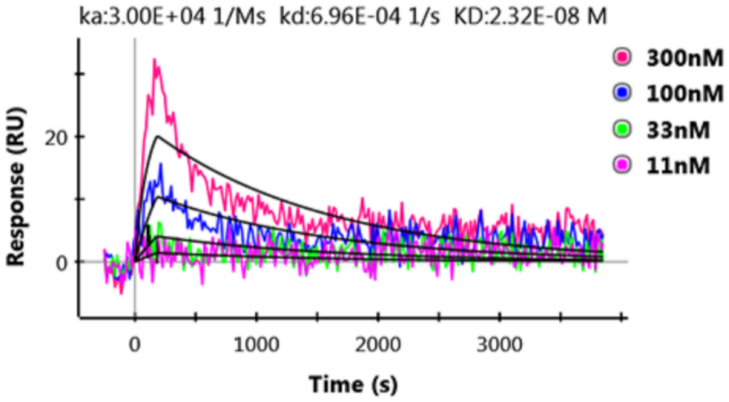


Figure S4

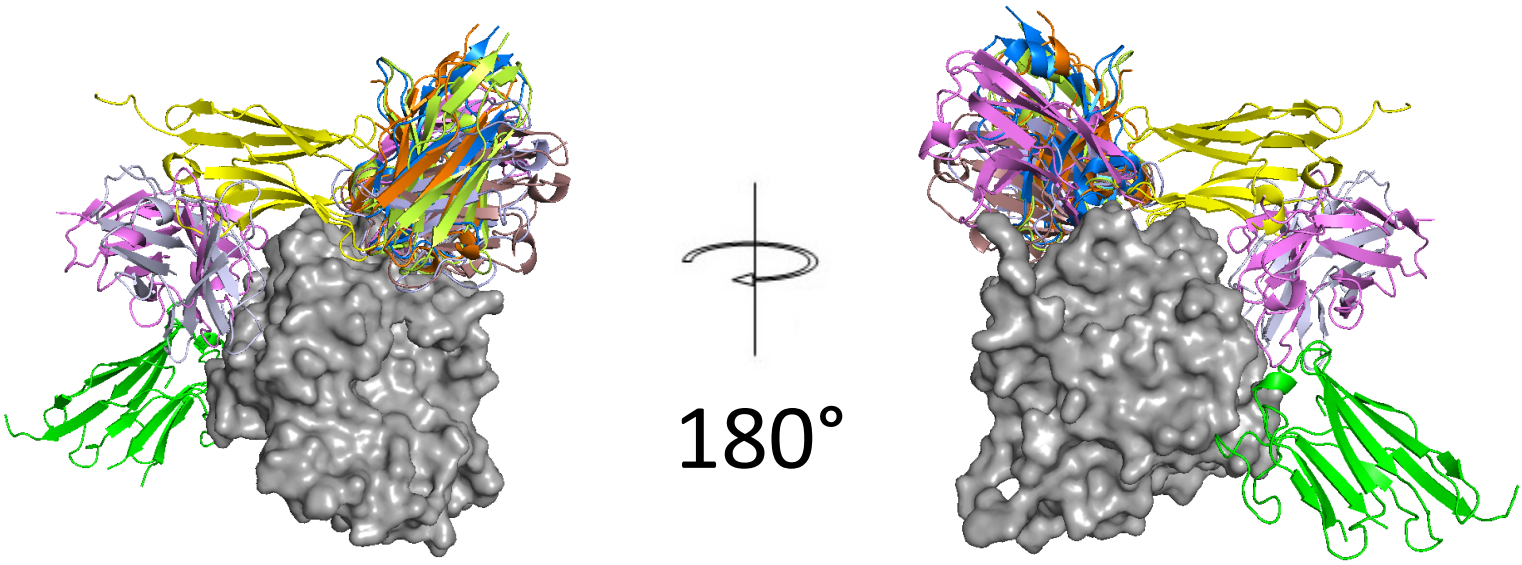


Figure S5

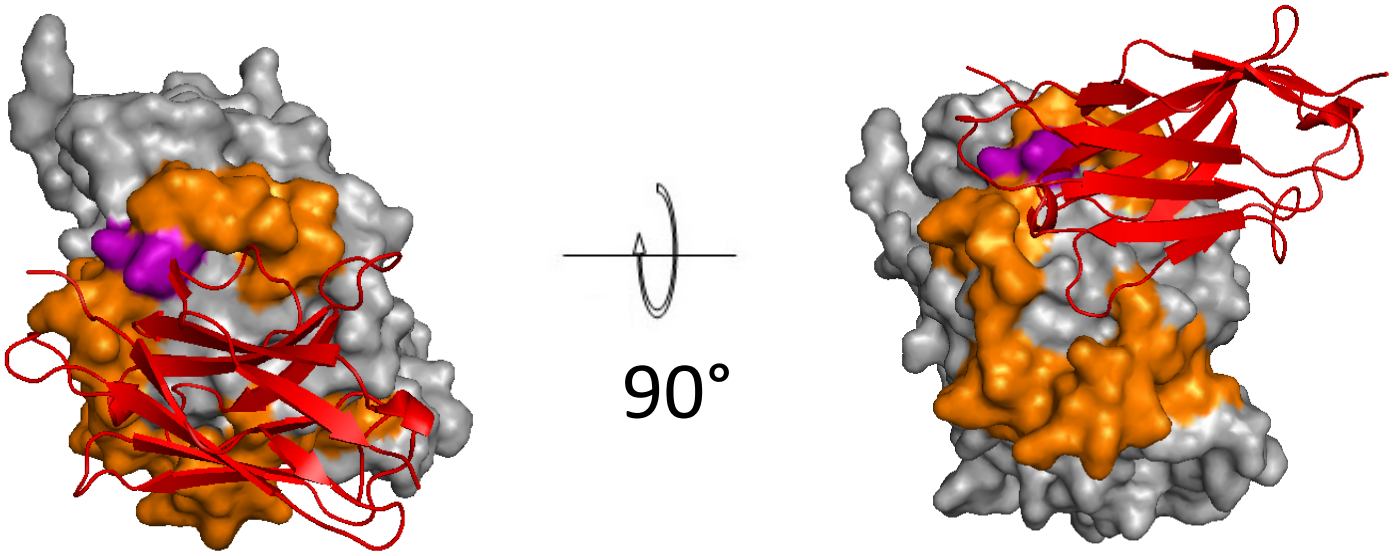


Figure S6

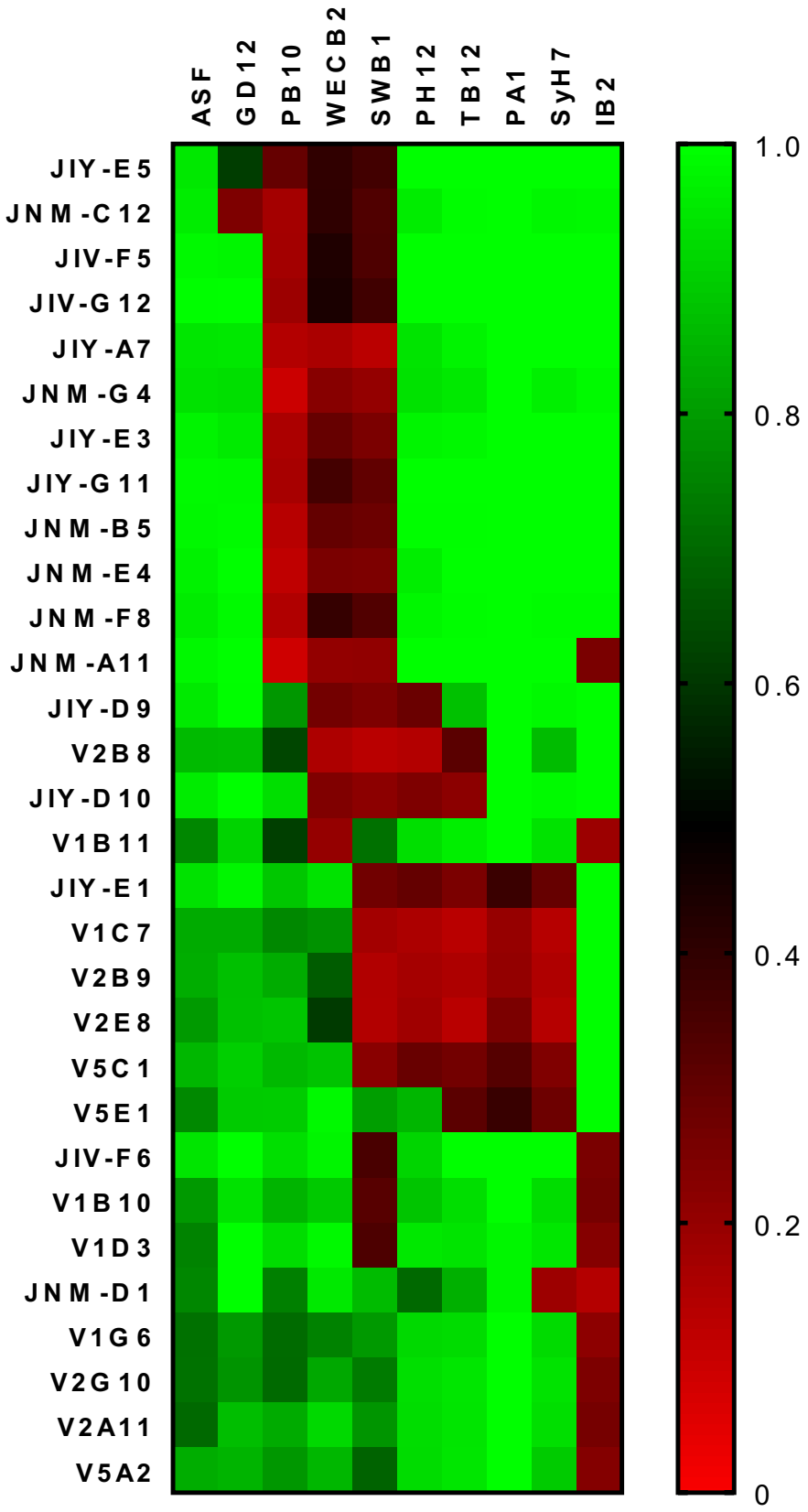

ASF
GD12
PB10
WECB2
SWB1
PH12
TB12
PA1
SyH7
IB2
JIY-E5
JNM-C12
JIV-F5
JIV-G12
JIY-A7
JNM-G4
JIY-E3
JIY-G11
JNM-B5
JNM-E4
JNM-F8
JNM-A11
JIY-D9
V2B8
JIY-D10
V1B11
JIY-E1
V1C7
V2B9
V2E8
V5C1
V5E1
JIV-F6
V1B10
V1D3
JNM-D1
V1G6
V2G10
V2A11
V5A2
1.0
0.8
0.6
0.4
0.2
0

Figure S7

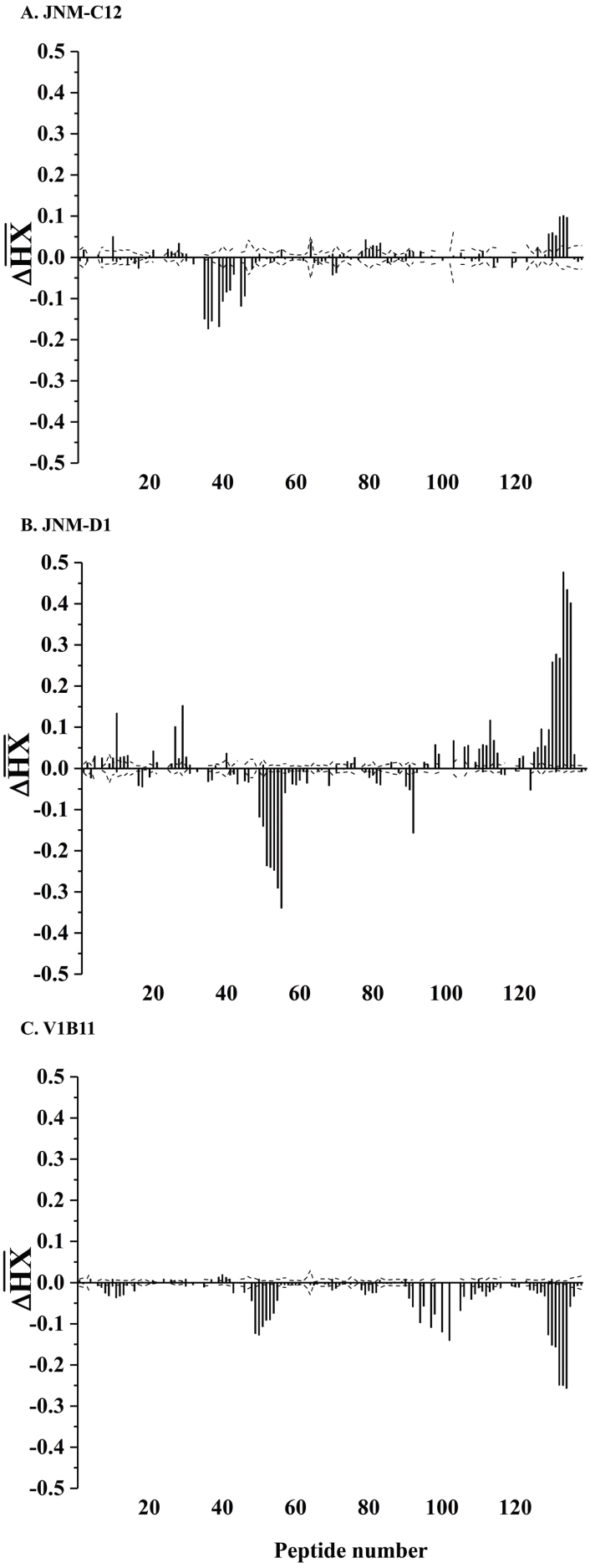
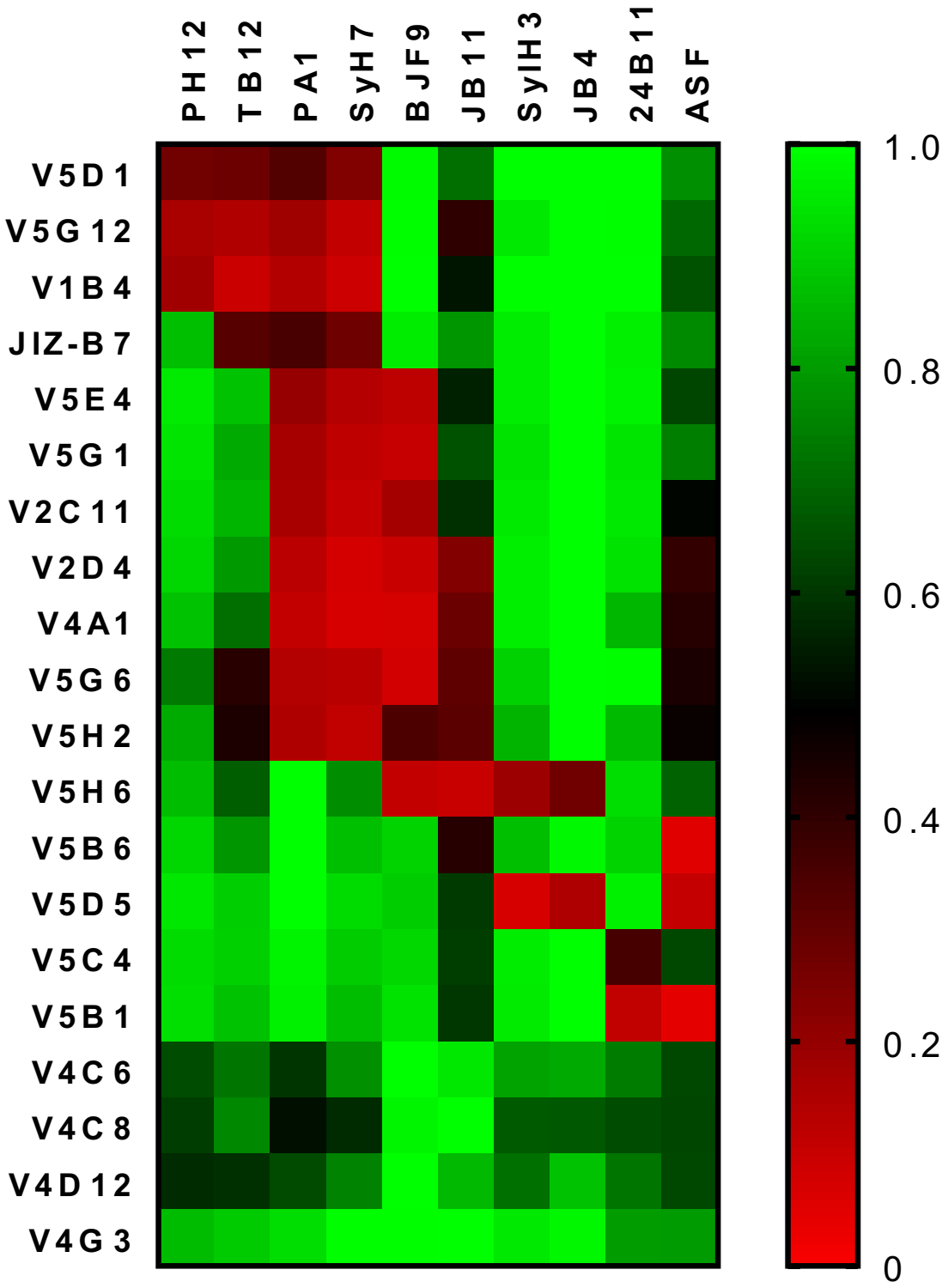

A. JNM-C12
B. JNM-D1
C. V1B11
ΔHX
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.4
-0.5
20
40
60
80
100
120
Peptide number

Figure S8


PH12
TB12
PA1
SyH7
BJF9
JB11
SylH3
JB4
24B11
ASF
V5D1
V5G12
V1B4
JIZ-B7
V5E4
V5G1
V2C11
V2D4
V4A1
V5G6
V5H2
V5H6
V5B6
V5D5
V5C4
V5B1
V4C6
V4C8
V4D12
V4G3
1.0
0.8
0.6
0.4
0.2
0