

## Supplementary Material

### **Stability-diversity tradeoffs impose fundamental constraints on selection of synthetic human V<sub>H</sub>/V<sub>L</sub> single-domain antibodies from *in vitro* display libraries**

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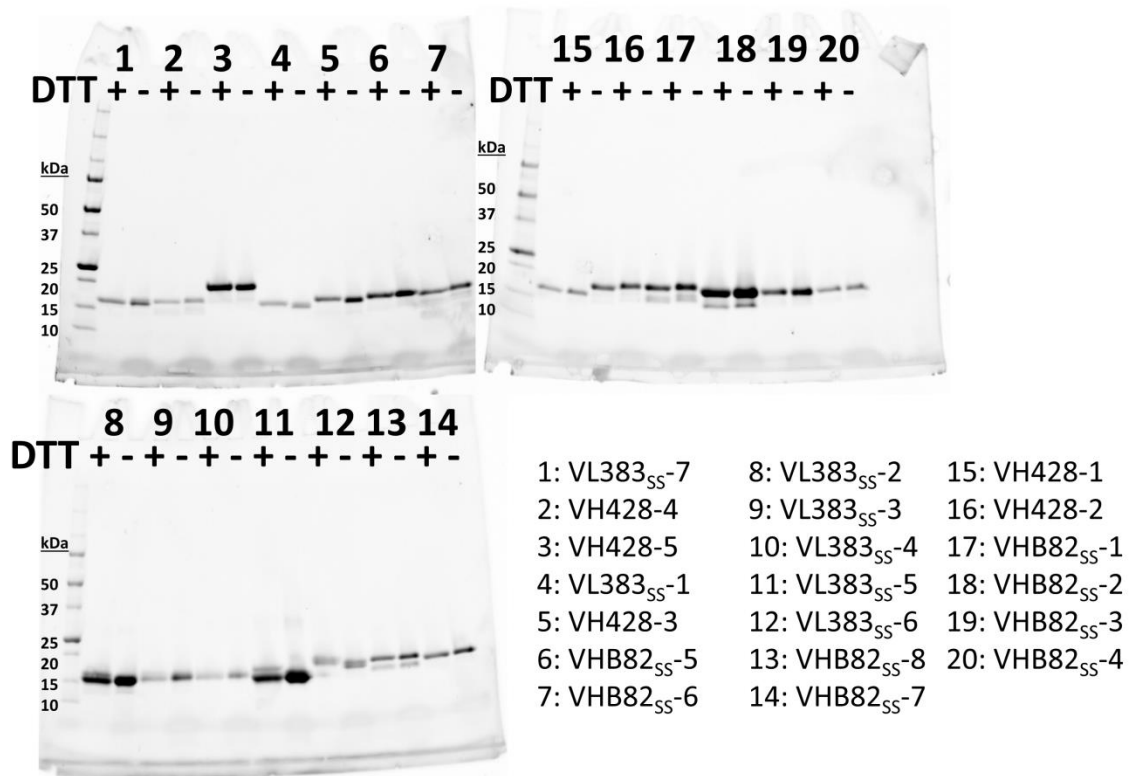
[jamshid.tanha@nrc-cnrc.gc.ca](mailto:jamshid.tanha@nrc-cnrc.gc.ca)

	CDR-H1 (H31-H35B)											CDR-H2 (H50-65)											CDR-H3 (H95-102)														
	FR1			FR2			FR3			FR4			FR1			FR2			FR3			FR4			FR1			FR2			FR3			FR4			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33				
VH428	123456789012345678901234567890	12345	67890123456789	012abc3456789012345	67890123456789012345	6789012345678901234	567890abcd12	34567890123	1234567890123	12345678901234567890	12345678901234567890	12345678901234567890	12345678901234567890	12345678901234567890	12345678901234567890	12345678901234567890	12345678901234567890	12345678901234567890	12345678901234567890	12345678901234567890	12345678901234567890	12345678901234567890	12345678901234567890	12345678901234567890	12345678901234567890	12345678901234567890	12345678901234567890	12345678901234567890	12345678901234567890	12345678901234567890	12345678901234567890	12345678901234567890					
VH428 <sub>SS</sub>	QLQLQESGGGLVQPGGSLRLS	CAASGFTFS	SYAMS	WFRQAPGKGLEWVG	FIRSKAYGGTTEYAASVKG	RFTISRDDSKSIAYLQMN	SLRAEDTAMYICAR	RAKDGYNSPEDY	WGQGT	TLTVSS																											
VH420	QVQLVESGGGLVQPGGSLRLS	CAASGFTFS	SYAMS	WFRQAPGKGLEWVC	FIRSKAYGGTTEYAASVKG	RFTISRDDSKSIAYLQMN	SLRAEDTAMYICAR	RAKDGYNSPEDY	WGQGT	TLTVSS																											
VH420 <sub>SS</sub>	QVQLVESGGGLVQPGGSLRLS	CAASGFTFS	NAWMT	WVRQAPGKGLEWVG	RIKTKTDGGTTYAAPVKG	RFTISRDDSKNTLYLQMN	SLKTEDTAVYYCTT	DRDHSS----	GS	WGQGT	TLTVSS																										
VH429	EVQLVESGGTLVQPGGSLRLS	CAASGFTFI	NYAMS	WVRQAPDKGLDWVS	TISN--NGGATYYADSVKG	RFTISRDNNSNTLYLQMN	SLRPDDTAVYYCAK	GPINTGRY--	GD	WGQGT	TLTVSS																										
VH429 <sub>SS</sub>	EVQLVESGGTLVQPGGSLRLS	CAASGFTFI	NYAMS	WVRQAPDKGLDWVC	TISN--NGGATYYADSVKG	RFTISRDNNSNTLYLQMN	SLRPDDTAVYYCAK	GPINTGRY--	GD	WGQGT	TLTVSS																										
VHB82	QVQLQESGGGLVQPGGSLRLS	CAASGFTFS	SYAMS	WVRQAPGKGLEWVS	AISG--SGGSTYYADSVKG	RFTISRDNNSKNTLYLQMN	SLRAEDTAVYYCGT	DMEV-----		WGKGT	TLTVSS																										
VHB82 <sub>SS</sub>	QVQLQESGGGLVQPGGSLRLS	CAASGFTFS	SYAMS	WVRQAPGKGLEWVC	AISG--SGGSTYYADSVKG	RFTISRDNNSKNTLYLQMN	SLRAEDTAVYYCGT	DMEV-----		WGKGT	TLTVSS																										
VHM81	EVQLVQSGGGLVQPGSLRLS	CAASGFTFD	DYAMH	WVRQAPGKGLEWVS	GISG--SGASTYYADSVKG	RFTISRDNNSKNTLYLQMN	SLRAGDTALYYCAR	QSITGPTGAFDV		WGQGT	MTVTVSS																										
VHM81 <sub>SS</sub>	EVQLVQSGGGLVQPGSLRLS	CAASGFTFD	DYAMH	WVRQAPGKGLEWVC	GISG--SGASTYYADSVKG	RFTISRDNNSKNTLYLQMN	SLRAGDTALYYCAR	QSITGPTGAFDV		WGQGT	MTVTVSS																										

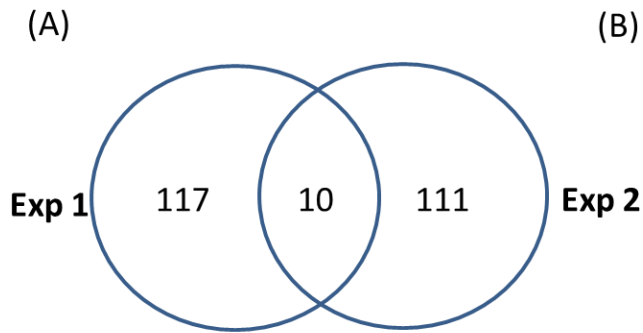
	CDR-L1 (L24-34)											CDR-L2 (L50-56)											CDR-L3 (L89-97)															
	FR1			FR2			FR3			FR4			FR1			FR2			FR3			FR4			FR1			FR2			FR3			FR4				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33					
VL382	12345678901234567890123	4567890a1234	567890123456789	0123456	78901234567890123456789	0123456	789012345678901234567890123456789012345678	901234567	8901234567	8901234567	8901234567	8901234567	8901234567	8901234567	8901234567	8901234567	8901234567	8901234567	8901234567	8901234567	8901234567	8901234567	8901234567	8901234567	8901234567	8901234567	8901234567	8901234567	8901234567	8901234567	8901234567	8901234567	8901234567	8901234567	8901234567			
VL382 <sub>SS</sub>	EIVMTQSPGTL	SLSPGDRATLSC	RASQDIS-TYLA	WYQKPGQAPRLLIY	SASRRAT	GIPDRFSGSGGTDFTLT	ISRLEPEDFAVYYC	QQYSSSPKT	FGQGT	KVTIVL																												
VL383	ETTLTQSPGTL	SLSPGERATLSC	RASQSVS-KNLA	WYQKPGQSPRLLIH	SISTRAT	GVPARFSGSGGTDFTLT	ISRLEPEDFAVYYC	QQYGNPQT	FGQGT	KVEIK																												
VL383 <sub>SS</sub>	ETTLTQSPGTL	SLSPGERATLSC	RASQSVS-KNLA	WYQKPGQSPRLLCH	SISTRAT	GVPARFSCGSGGTDFTLT	ISRLEPEDFAVYYC	QQYGNPQT	FGQGT	KVEIK																												
VL325	EIVLTQSPPTL	SLSPGERATLSC	RASQSVG-RYLA	WYQKPGQAPRLLVF	DTSNRAP	GVPARFSGRSGTLFTLT	ISSLEPEDSAVYFC	QQYSSSPRT	FGHGT	KVTIVL																												
VL325 <sub>SS</sub>	EIVLTQSPPTL	SLSPGERATLSC	RASQSVG-RYLA	WYQKPGQAPRLLCF	DTSNRAP	GVPARFSCRSGTLFTLT	ISSLEPEDSAVYFC	QQYSSSPRT	FGQGT	KVTIVL																												
VL330	DIQMTQSPFSL	SAFVGDRTITC	RASESVG-NLSL	WYQLKPGKNPRLVLS	GGSFQVS	GVSARFSGSGAGTLFTLT	ITGLRLDSDATYYC	QQSDAVPRT	FGHGT	KVSVL																												
VL330 <sub>SS</sub>	DIQMTQSPFSL	SAFVGDRTITC	RASESVG-NLSL	WYQLKPGKNPRLICS	GGSFQVS	GVSARFSCSGAGTLFTLT	ITGLRLDSDATYYC	QQSDAVPRT	FGHGT	KVSVL																												
VL335	EIVMTQSPATL	SLSPGERATLSC	RASQSVSSSLA	WYQKPGQAPRLLIY	GTSNRAT	GIPDRFSGSGGTHFTLT	INRLEPGDFAVYYC	QQYSSSPRT	FGQGT	KVTIVL																												
VL335 <sub>SS</sub>	EIVMTQSPATL	SLSPGERATLSC	RASQSVSSSLA	WYQKPGQAPRLLICY	GTSNRAT	GIPDRFSCSGGTHFTLT	INRLEPGDFAVYYC	QQYSSSPRT	FGQGT	KVTIVL																												

**Supplementary Figure S1.** Full-length amino acid sequences of human autonomous V<sub>H</sub>/V<sub>L</sub> sdAb scaffolds used in this study. Numbering is according to Kabat.

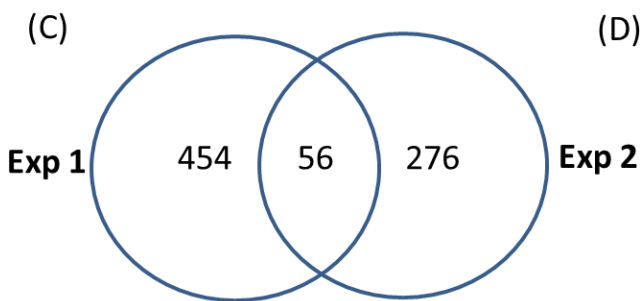
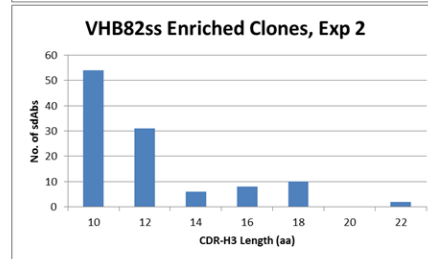
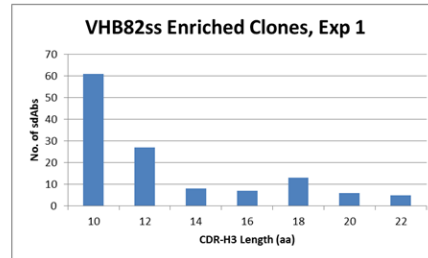




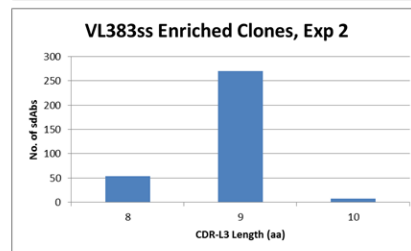
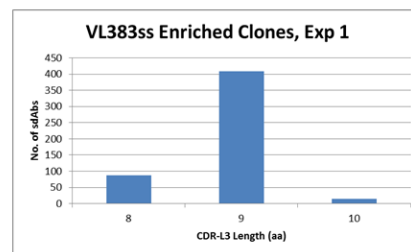
**Supplementary Figure S3.** SDS-PAGE in reducing and non-reducing conditions of all antigen-specific sdAbs isolated from the VL383<sub>SS</sub>, VH428 and VHB82<sub>SS</sub> synthetic human V<sub>H</sub>/V<sub>L</sub> sdAb libraries. 1-5 µg of each IMAC-purified sdAb was electrophoresed in a Mini-PROTEAN<sup>®</sup> TGX stain-free gel (Bio-Rad); the loading buffer for the samples contained 40 mM Tris, pH 6.8, 6% (v/v) glycerol, 2% (w/v) SDS and 0.05% (w/v) bromophenol blue. DTT was added to some samples at 15 mM (+ DTT). Molecular weight marker is Bio-Rad Precision Plus Protein<sup>™</sup> Unstained Protein Standards, Cat. #1610363.



VHB82<sub>SS</sub>, Helper Phage Rescue,  
3 rounds protA selection +  
amplification in *E. coli*, unique  
sdAbs found at  $\geq 6$  copies



VL383<sub>SS</sub>, Helper Phage Rescue,  
3 rounds protL selection +  
amplification in *E. coli*, unique  
sdAbs found at  $\geq 10$  copies



**Supplementary Figure S4.** Properties of human  $V_H$  and  $V_L$  sdAbs enriched by stability selection. (A) Overlap of unique  $V_H$  sdAbs enriched from the VHB82<sub>SS</sub> library by replicate stability selection. (B) CDR-H3 length distribution of unique  $V_H$  sdAbs enriched from the VHB82<sub>SS</sub> library by replicate stability selection. (C) Overlap of unique  $V_L$  sdAbs enriched from the VL383<sub>SS</sub> library by replicate stability selection. (D) CDR-L3 length distribution of unique  $V_L$  sdAbs enriched from the VL383<sub>SS</sub> library by replicate stability selection.

VL383 <sub>ss</sub> Randomization Position, Helper Phase Rescue, 3 Rounds of Stability Selection, REPLICATE 1													
	CDR-L1				CDR-L2				CDR-L3				
	36 (X1)	37 (X1)	38 (X1)	57 (X1)	66 (X1)	68 (X1)	107 (X1)	108 (X2)	109(X2)	113(X2)	114(X2)	116(X2)	
*	0.6	1.6	1.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	11.4	0.0
A	16.3	5.9	9.6	35.3	10.8	13.1	3.9	14.5	13.2	7.1	0.0	0.0	9.0
C	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
D	44.1	28.8	25.5	7.3	15.7	24.3	12.2	7.5	10.6	14.3	5.5	2.5	2.5
E	0.0	0.0	0.0	0.0	0.0	0.0	0.2	10.6	12.8	7.1	17.6	8.8	8.8
F	1.2	0.2	0.6	0.4	1.0	0.6	2.7	2.5	4.5	14.3	2.2	2.4	2.4
G	0.0	0.0	0.0	0.0	0.2	3.5	0.4	4.5	2.8	14.3	5.5	3.9	3.9
H	10.2	21.0	26.1	1.0	19.8	17.5	11.6	12.2	7.1	0.0	8.0	10.4	10.4
I	0.2	1.0	0.0	6.7	12.7	1.6	1.2	1.0	1.9	0.0	1.8	1.4	1.4
K	0.0	0.0	0.0	0.0	0.0	0.0	0.2	5.9	2.4	0.0	5.3	2.9	2.9
L	1.0	1.0	0.0	5.9	0.6	6.7	1.8	0.8	1.0	0.9	7.1	2.0	0.0
M	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
N	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
P	4.3	6.5	1.4	16.9	0.4	22.2	3.7	0.8	1.2	14.3	6.5	11.4	11.4
Q	0.0	0.0	0.0	0.0	0.0	0.0	0.8	8.8	6.6	7.1	5.7	2.4	2.4
R	0.0	0.2	0.0	0.0	3.5	0.0	0.0	3.9	1.9	0.0	2.9	0.4	0.4
S	11.4	12.4	19.2	18.6	7.1	9.4	16.7	6.3	10.4	0.0	3.9	9.8	9.8
T	5.3	18.8	2.9	6.3	11.8	2.0	8.8	2.5	9.0	7.1	4.7	3.3	3.3
V	0.8	1.0	0.0	6.5	6.1	1.2	1.6	0.6	1.9	0.0	2.7	5.3	5.3
W	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.4	1.9	0.0	4.7	5.5	5.5
Y	4.7	2.7	7.5	0.6	4.3	2.9	35.3	15.1	10.9	7.1	9.6	20.6	20.6

VL383 <sub>ss</sub> Randomization Position, Helper Phase Rescue, 3 Rounds of Stability Selection, REPLICATE 2													
	CDR-L1				CDR-L2				CDR-L3				
	36 (X1)	37 (X1)	38 (X1)	57 (X1)	66 (X1)	68 (X1)	107 (X1)	108 (X2)	109(X2)	113(X2)	114(X2)	116(X2)	
*	0.3	1.8	0.9	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A	15.4	8.1	9.9	38.9	11.4	14.5	4.5	15.7	10.8	12.5	10.2	9.3	9.3
C	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
D	46.4	27.4	26.5	6.9	19.0	27.1	12.3	6.0	7.9	25.0	6.0	3.6	3.6
E	0.0	0.0	0.0	0.0	0.0	0.0	0.0	11.7	18.0	12.5	17.5	7.2	7.2
F	1.8	0.3	0.9	0.3	0.6	0.6	2.7	3.0	5.0	12.5	2.4	2.1	2.1
G	0.0	0.0	0.0	0.0	0.3	3.3	0.3	3.3	3.6	0.0	3.9	4.5	4.5
H	9.6	19.0	27.4	0.3	17.8	16.6	11.1	13.9	8.3	0.0	6.3	9.0	9.0
I	0.3	1.2	0.0	6.6	10.8	1.5	1.2	0.9	2.2	0.0	2.1	1.2	1.2
K	0.0	0.0	0.0	0.0	0.0	0.0	0.3	5.7	1.1	0.0	6.9	3.3	3.3
L	0.9	0.3	4.8	0.9	8.1	1.5	0.9	1.8	0.7	12.5	2.1	0.0	0.0
M	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
N	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
P	6.3	6.6	1.5	16.0	0.9	20.2	3.3	0.3	0.7	0.0	7.5	10.8	10.8
Q	0.0	0.0	0.0	0.0	0.0	0.0	0.3	8.4	6.8	12.5	8.4	2.7	2.7
R	0.0	0.0	0.0	0.0	3.3	0.0	0.0	3.9	2.5	0.0	3.3	0.6	0.6
S	8.7	13.0	19.0	15.4	5.4	8.7	15.4	6.0	10.1	0.0	4.5	10.8	10.8
T	6.3	17.5	2.7	8.1	12.7	1.2	9.9	2.4	7.9	0.0	3.6	4.5	4.5
V	1.2	1.2	0.0	6.3	6.3	1.8	1.8	0.6	1.1	0.0	2.7	4.2	4.2
W	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.1	2.2	0.0	4.2	5.7	5.7
Y	2.7	3.6	6.0	0.3	3.0	3.0	35.8	14.2	11.2	12.5	8.1	20.2	20.2

**Supplementary Figure S5.** Amino acid representation at each randomization position in VL sdAb clones ( $n_1=510$  and  $n_2=332$ ) enriched from the VL383<sub>ss</sub> library by replicate stability selection. Asterisks denote stop codons.



VL3835S Randomization Position, Helper Phase Rescue, 3 Rounds of Stability Selection, REPLICATE 1													
	CDR-L1			CDR-L2			CDR-L3			CDR-L3			
	36 (X1)	37 (X1)	38 (X1)	57 (X1)	66 (X1)	68 (X1)	107 (X1)	108 (X2)	109 (X2)	113 (X2)	114 (X2)	116 (X2)	
*	0.4	1.0	0.5	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A	18.6	8.0	10.9	34.2	10.4	12.8	7.3	12.8	9.7	10.3	9.4	9.4	9.5
C	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
D	37.3	25.8	22.2	9.5	19.9	27.0	16.0	8.4	10.1	9.0	6.5	4.2	4.2
E	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	11.5	14.3	12.7	16.7	10.0
F	1.4	0.6	1.0	0.8	1.5	1.6	3.3	2.8	3.8	3.7	2.9	3.7	3.7
G	0.3	0.1	0.1	0.0	0.1	1.7	0.2	5.8	4.1	8.4	4.9	5.2	5.2
H	11.0	18.4	25.2	2.4	16.1	13.5	9.8	11.5	7.6	6.6	7.1	7.8	7.8
I	1.1	0.9	0.3	6.8	8.9	3.1	1.8	2.2	2.7	3.1	3.0	3.3	3.3
K	0.0	0.0	0.0	0.0	0.0	0.0	0.1	4.8	2.6	3.6	5.7	3.0	3.0
L	1.2	0.7	5.1	1.9	9.4	2.4	2.2	2.2	1.4	2.9	1.9	0.2	0.2
M	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
N	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
P	6.0	6.7	3.3	17.2	3.2	18.9	6.1	2.7	3.3	8.4	7.6	9.9	9.9
Q	0.0	0.0	0.0	0.0	0.0	0.0	0.6	6.9	6.4	6.5	6.3	3.3	3.3
R	0.0	0.1	0.1	0.0	1.7	0.1	0.1	3.0	2.4	2.8	3.1	1.5	1.5
S	10.4	12.3	19.0	12.1	6.5	9.7	13.7	5.7	8.5	5.2	4.7	8.2	8.2
T	4.7	20.0	4.9	6.6	11.6	1.9	8.5	3.7	9.7	4.6	4.7	5.3	5.3
V	2.2	1.3	0.4	6.4	5.8	2.2	1.8	1.4	1.7	2.1	2.1	3.9	3.9
W	0.0	0.0	0.0	0.0	0.0	0.0	0.1	3.6	3.0	4.3	4.5	6.0	6.0
Y	5.2	3.9	6.9	1.8	4.7	4.9	28.1	10.9	8.4	5.8	8.9	14.9	14.9

VL3835S Randomization Position, Helper Phase Rescue, 3 Rounds of Stability Selection, REPLICATE 2													
	CDR-L1			CDR-L2			CDR-L3			CDR-L3			
	36 (X1)	37 (X1)	38 (X1)	57 (X1)	66 (X1)	68 (X1)	107 (X1)	108 (X2)	109 (X2)	113 (X2)	114 (X2)	116 (X2)	
*	0.4	0.9	0.4	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A	18.0	8.2	11.5	34.1	10.6	12.8	7.4	12.5	8.8	9.7	9.0	9.4	9.4
C	0.0	0.0	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
D	37.9	27.0	23.3	9.5	20.4	27.5	16.4	8.3	10.5	9.0	6.7	4.4	4.4
E	0.0	0.0	0.0	0.0	0.0	0.0	0.3	11.8	14.7	12.6	16.9	10.0	10.0
F	1.4	0.6	1.1	0.9	1.6	1.7	3.5	2.8	3.6	3.1	3.0	3.8	3.8
G	0.3	0.1	0.1	0.0	0.1	1.7	0.2	6.0	4.2	8.4	4.6	5.4	5.4
H	11.2	18.4	25.0	2.6	15.4	13.0	9.5	11.5	7.7	6.7	7.1	7.5	7.5
I	1.2	0.9	0.4	6.9	8.3	3.1	1.9	2.3	2.8	3.0	3.1	3.4	3.4
K	0.0	0.0	0.0	0.0	0.0	0.0	0.1	4.8	2.6	4.0	5.5	3.1	3.1
L	1.3	0.8	4.4	2.0	9.6	2.4	2.2	2.2	1.6	3.0	2.0	0.2	0.2
M	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
N	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
P	5.7	6.8	3.3	17.4	3.5	18.4	6.1	2.9	3.4	9.0	7.8	10.0	10.0
Q	0.0	0.0	0.0	0.0	0.0	0.0	0.5	6.8	6.5	6.4	6.3	3.4	3.4
R	0.0	0.1	0.1	0.0	1.6	0.1	0.1	3.0	2.4	2.9	3.1	1.7	1.7
S	10.2	12.1	18.4	11.3	6.9	9.7	13.2	5.6	8.1	5.3	4.9	8.3	8.3
T	4.6	18.5	4.6	6.5	11.6	2.0	8.8	3.8	9.5	4.6	4.7	5.5	5.5
V	2.3	1.3	0.3	6.5	5.8	2.3	1.8	1.4	1.8	1.9	2.1	3.3	3.3
W	0.0	0.0	0.0	0.0	0.0	0.0	0.1	3.8	3.2	4.4	4.5	6.2	6.2
Y	5.3	4.1	6.9	2.0	4.6	5.1	27.9	10.5	8.5	5.8	8.6	14.3	14.3

**Supplementary Figure S7.** Amino acid representation at each randomization position in all VL sdAbs present after three rounds of replicate stability selection of the VL383ss library. Asterisks denote stop codons.





**Supplementary Table SI.** Sequences of oligonucleotide primers used in this study.

<b>Name</b>	<b>Sequence (5'→3')</b>	<b>Purpose</b>
M13F	TTG TAA AAC GAC GGC CAG	PCR Amplification of assembled library
M13R	GGA AAC AGC TAT GAC CAT G	PCR Amplification of assembled library
-96GIII	CCC TCA TAG TTA GCG TAA CGA TCT	Colony-PCR Sequencing
fdTGIII	GTG AAA AAA TTA TTA TTC GCA ATT CCT	Colony-PCR Sequencing
seqF-428	CGC TCT TCC GAT CTC TG NNNNN CAG CTG CAG CTG CAG GAG TCG GGG	NGS
seqF-B82S	CGC TCT TCC GAT CTC TG NNNNN CAG GTG CAG CTG CAG GAG TCG GGG	NGS
seqF-383S	CGC TCT TCC GAT CTC TG NNNNN GAA ACG ACA CTC ACG CAG TCT CCA	NGS
seqR-428	TGC TCT TCC GAT CTG AC NNNNN TGA GGA GAC GGT GAC TAG TGT TCC	NGS
seqR-B82s	TGC TCT TCC GAT CTG AC NNNNN TGA AGA GAC GGT GAC CGT GGT ACC	NGS
seqR-383S	TGC TCT TCC GAT CTG AC NNNNN TTT GAT CTC CAC CTT GGT CCC CTG	NGS

**Supplementary Table SII.** Expression yields from overnight *E. coli* TG1 cultures,  $T_m$ s and solubility profiles of potential human  $V_H$  sdAb scaffolds considered for use in this study.

$V_H$ sdAb scaffold	Disulfide Linkages (IMGT numbering)	Expression Yield (mg/L)	$T_m$ (° C)	Monomer (%)
VH44	23-104	8.2	64.2	>95
VH44 <sub>SS</sub>	23-104, 54-78	2.0	74.5	72.3
<b>VHB82</b>	<b>23-104</b>	<b>12.8</b>	<b>57.9</b>	<b>&gt;95</b>
<b>VHB82<sub>SS</sub></b>	<b>23-104, 54-78</b>	<b>15.6</b>	<b>72.9</b>	<b>80.8</b>
VH419	23-104	3.4	56.9	>95
VH419 <sub>SS</sub>	23-104, 54-78	0.6	67.4	>95
<b>VH429</b>	<b>23-104</b>	<b>3.4</b>	<b>58.5</b>	<b>&gt;95</b>
<b>VH429<sub>SS</sub></b>	<b>23-104, 54-78</b>	<b>16.0</b>	<b>71.8</b>	<b>&gt;95</b>
VHM41	23-104	1.8	60.8	>95
VHM41 <sub>SS</sub>	23-104, 54-78	2.1	72.2	63.7
<b>VHM81</b>	<b>23-104</b>	<b>32.8</b>	<b>66.9</b>	<b>&gt;95</b>
<b>VHM81<sub>SS</sub></b>	<b>23-104, 54-78</b>	<b>1.9</b>	<b>76.8</b>	<b>85.0</b>
<b>VH428</b>	<b>23-104</b>	<b>40.0</b>	<b>62.3</b>	<b>&gt;95</b>
<b>VH428<sub>SS</sub></b>	<b>23-104, 54-78</b>	<b>9.6</b>	<b>73.1</b>	<b>&gt;95</b>
<b>VH420</b>	<b>23-104</b>	<b>8.1</b>	<b>57.8</b>	<b>&gt;95</b>
<b>VH420<sub>SS</sub></b>	<b>23-104, 54-78</b>	<b>33.6</b>	<b>67.3</b>	<b>&gt;95</b>
VH414	23-104	11.8	55.3	>95
VH414 <sub>SS</sub>	23-104, 54-78	nd	64.7	>95
VH423	23-104	2.4	55.0	>95
VH423 <sub>SS</sub>	23-104, 54-78	nd	70.7	>95
VH413	23-104	5.8	54.2	>95
VH413 <sub>SS</sub>	23-104, 54-78	<0.1	nd	nd

nd, not determined

$V_H$  sdAb scaffolds in bold red font were selected for CDR-shuffling experiments.

**Supplementary Table SIII.** Expression yields from overnight *E. coli* TG1 cultures,  $T_m$ s and solubility profiles of potential human V<sub>L</sub> sdAb scaffolds considered for use in this study.

V <sub>L</sub> sdAb scaffold	Disulfide Linkages (IMGT numbering)	Expression Yield (mg/L)	$T_m$ (° C)	Monomer (%)
VL324	23-104	2.5 – 7.0	66.1	>95
VL324 <sub>SS</sub>	23-104, 54-78	0.5 – 1.1	73.4	>95
<b>VL325</b>	<b>23-104</b>	<b>34.8</b>	<b>68.5</b>	<b>&gt;95</b>
<b>VL325<sub>SS</sub></b>	<b>23-104, 54-78</b>	<b>39.0</b>	<b>82.5</b>	<b>&gt;95</b>
<b>VL335</b>	<b>23-104</b>	<b>36.4</b>	<b>61.7</b>	<b>&gt;95</b>
<b>VL335<sub>SS</sub></b>	<b>23-104, 54-78</b>	<b>33.8</b>	<b>79.0</b>	<b>&gt;95</b>
VL342	23-104	1.0 – 7.7	58.4	>95
VL342 <sub>SS</sub>	23-104, 54-78	1.7 - 10.8	63.8	>95
VL364	23-104	0.3 – 77.0	57.0	>95
VL364 <sub>SS</sub>	23-104, 54-78	4.7	72.3	>95
VL389	23-104	3.0 – 16.7	51.9	>95
VL389 <sub>SS</sub>	23-104, 54-78	6.5	66.3	>95
<b>VL383</b>	<b>23-104</b>	<b>6.8</b>	<b>57.3</b>	<b>&gt;95</b>
<b>VL383<sub>SS</sub></b>	<b>23-104, 54-78</b>	<b>47.0</b>	<b>73.8</b>	<b>85.3</b>
<b>VL382</b>	<b>23-104</b>	<b>2.9</b>	<b>70.1</b>	<b>&gt;95</b>
<b>VL382<sub>SS</sub></b>	<b>23-104, 54-78</b>	<b>22.8</b>	<b>83.3</b>	<b>&gt;95</b>
<b>VL330</b>	<b>23-104</b>	<b>1.8</b>	<b>62.8</b>	<b>&gt;95</b>
<b>VL330<sub>SS</sub></b>	<b>23-104, 54-78</b>	<b>16.3</b>	<b>83.7</b>	<b>&gt;95</b>

nd, not determined

V<sub>L</sub> sdAb scaffolds in bold red font were selected for CDR-shuffling experiments.