Supplementary Material (ESI) for Molecular BioSystems

Supplementary data

Signal sequence as a determinant in expressing disulfide-stabilized single chain antibody variable fragments (sc-dsFv) against human VEGF†

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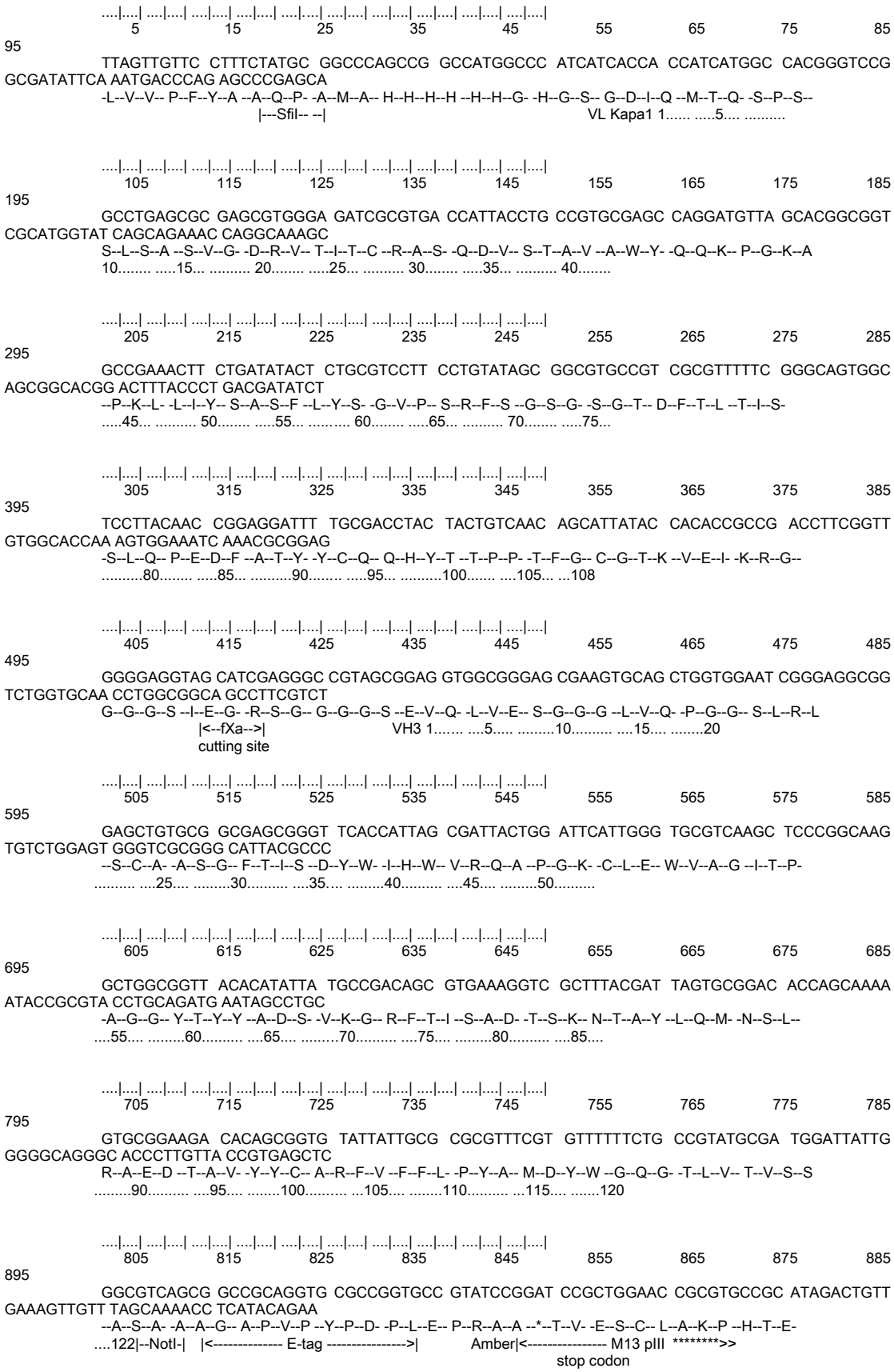

Supplementary Fig. 1 The DNA and amino acid sequence of anti-VEGF S5 sc-dsFv (fXa+).

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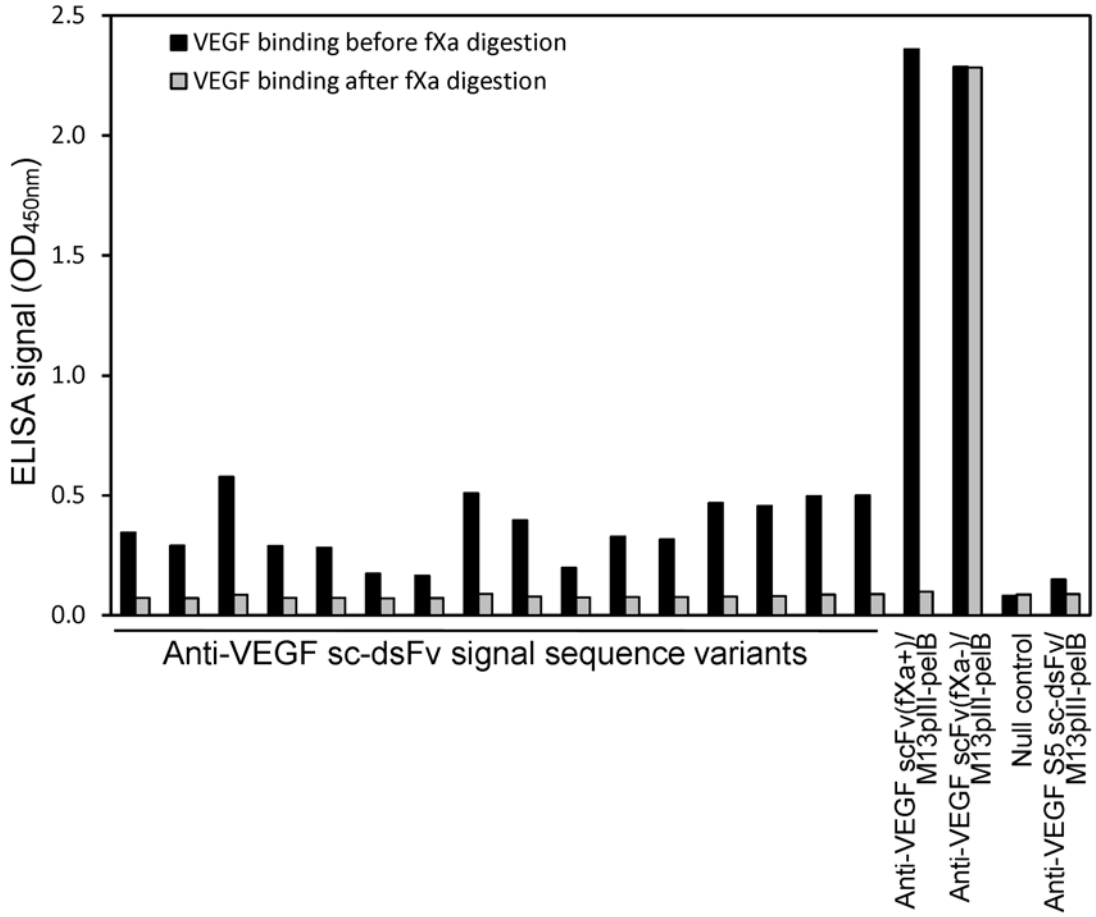
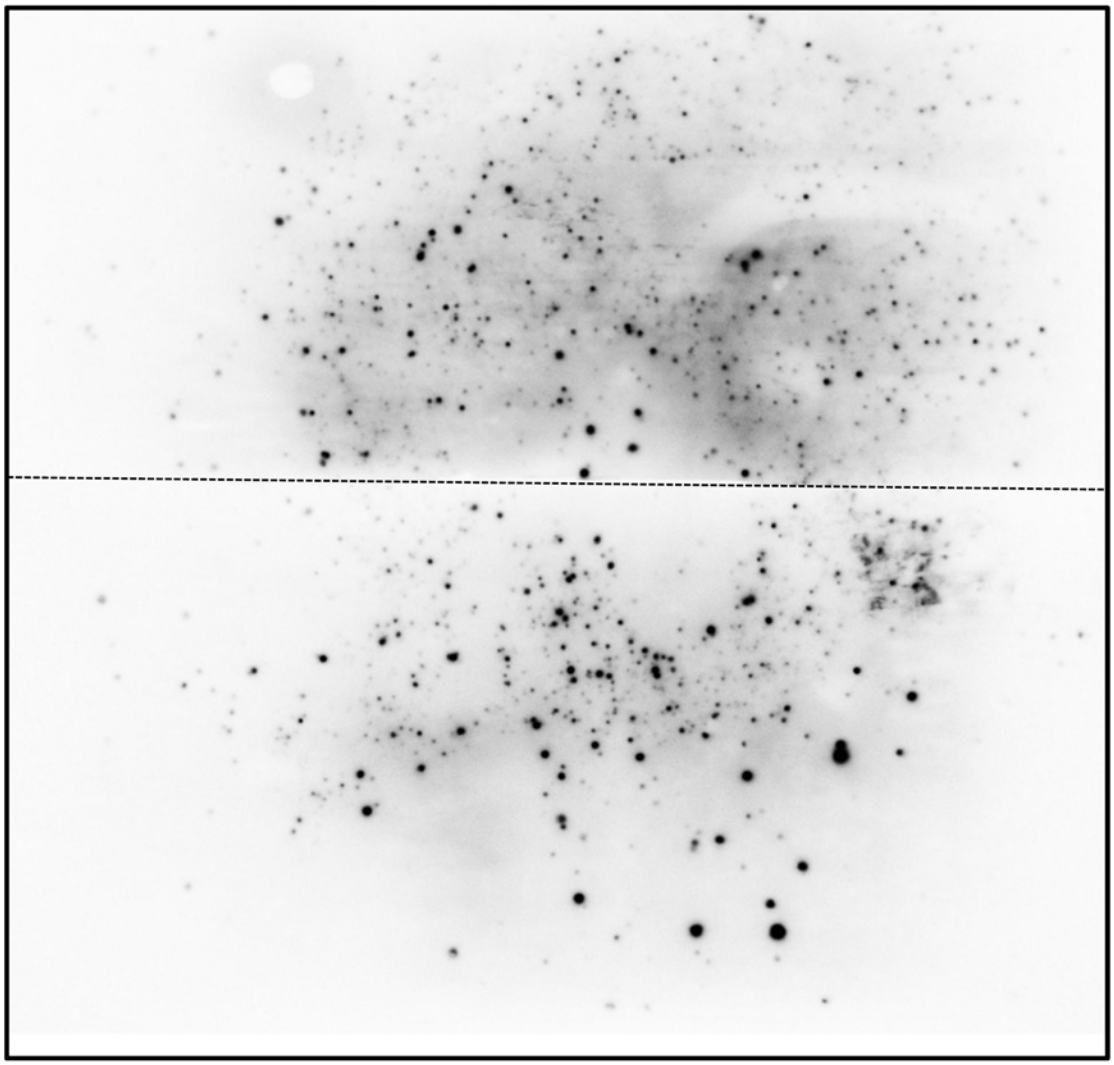
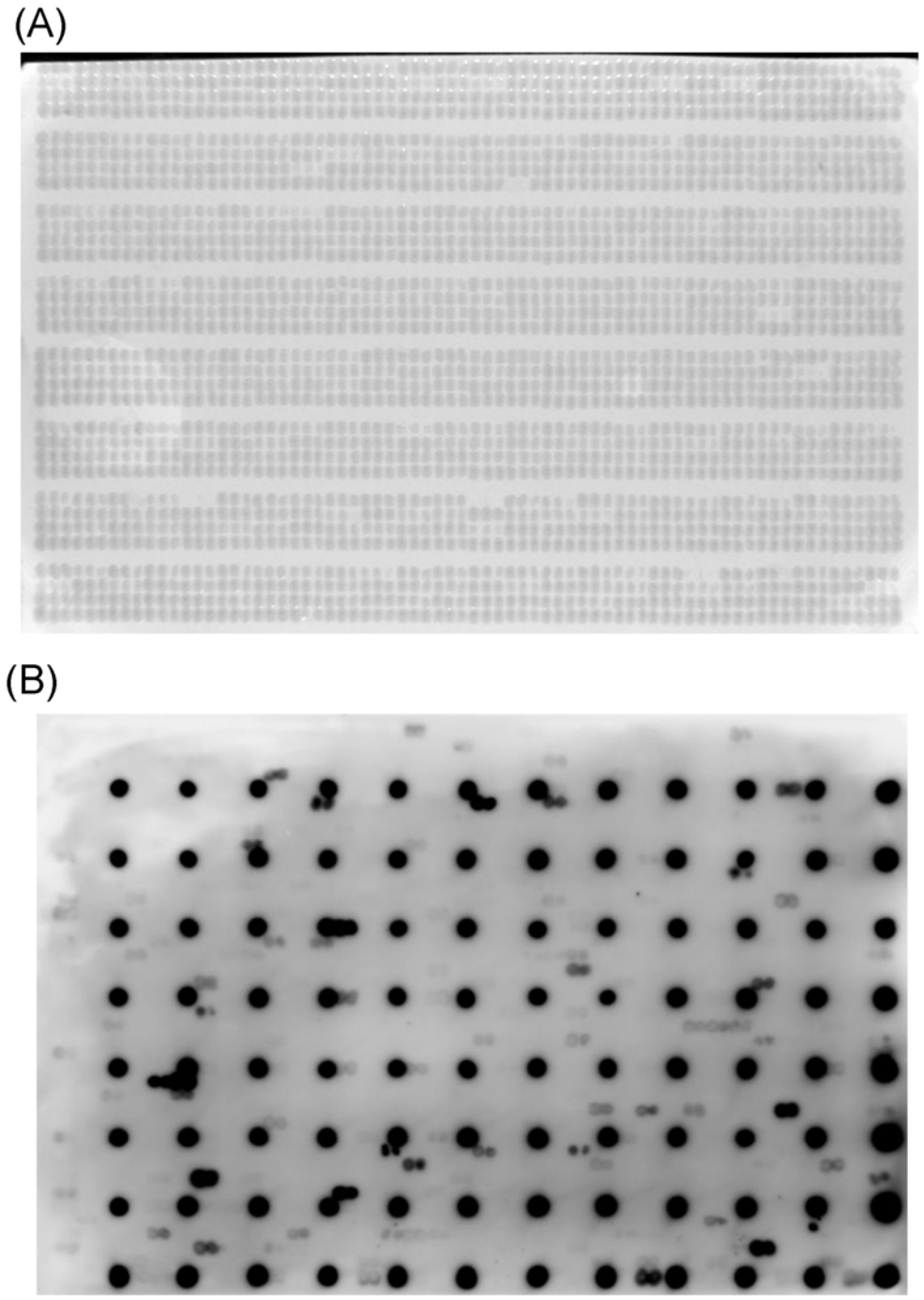


Supplementary Fig. 2 VEGF binding activity and fXa resistance for anti-VEGF sc-dsFv expressed in *E. coli* strain HB2151. 16 clones for which the overnight supernatant had the highest VEGF-binding activity were selected from $5x10^6$ variants with colony filter screening (Supplementary Figure 3). The binding activity of soluble sc-dsFv proteins from overnight supernatant were further analyzed with ELISA. Two micrograms of VEGF were coated in each well, and the binding capacities of scFv/sc-dsFv were detected with HRP-conjugated protein L as shown in the black histogram. Diminishing of VEGF-binding affinity due to the fXa treatment was shown in the light-gray histogram. The fXa positive control, anti-VEGF scFv (fXa+)/M13pIII-pelB, has an IEGR fXa cutting site in the linker peptide between the two variable domains (linker peptide sequence: -G_4SIEGRSG_4S-), but does not have interface disulfide bond linkage. The fXa negative control, anti-VEGF scFv (fXa-)/M13pIII-pelB, has neither fXa cutting site (linker peptide sequence: -G_4SG_4SG_4S-) nor interface disulfide bond linkage. The null control phagemid contains double TAA stop codon in the signal peptide sequence, and thus the bacteria harboring the null phagemid did not express the fusion protein. Anti-VEGF S5 sc-dsFv/M13pIII-pelB (sequence shown in Supplementary Figure 1) is different from anti-VEGF scFv (fXa+)/M13pIII-pelB only in one pair of cysteins (L:Gln100Cys & H:Gly44Cys) introduced in the interface between the two variable domains.

The abolishment of the strong scFv(fXa+)-VEGF binding after the fXa treatment shown in this figure was attributed to the cleavage of the linker peptide, leading to the dissociation of the variable domains, which in turn resulted in the diminishing affinity against VEGF (Lee et al., to be published). On the other hand, the fXa negative control, scFv(fXa-), does not have the -IEGR- substrate sequence in the linker peptide and thus was insensitive to the fXa treatment. As shown in the Figure, all the S5 anti-VEGF sc-dsFv's from the selected signal sequence variants bound to VEGF before the fXa treatment, but the affinity was completely abolished after the treatment of fXa. The result shown here suggests that the anti-VEGF sc-dsFv's produced from the selected signal sequence variants were similar in responding to fXa treatment to the fXa positive control anti-VEGF scFv(fXa+), which does not have the interface disulfide bond. That is, the interface disulfide bond was not formed in any of the selected anti-VEGF sc-dsFv from the non-suppressor *E. coli* strain HB2151



Supplementary Fig. 3 Selection of variants with sc-dsFv secretion by colony filter screening. About $5x10^6$ cfu phages were infected to *E. coli* strain HB2151, which were spread onto nitrocellulose membrane (21x21 cm^2). The secreted sc-dsFv's were captured on a VEGF-coated membrane. The signals were detected with HRP conjugated protein L and developed with ECL, as shown in this Figure. The dashed line indicates that the two images were taken separately.



Supplementary Fig. 4 Selection of variants with sc-dsFv secretion by colony array screening. A: *E. coli* strain ER2738 harboring phagemids were gridded onto nitrocellulose membrane (13x10 cm^2). The membrane was put onto agar medium at 37^oC until the colony formed, as shown in the Figure. B: The secreted sc-dsFv's were captured on a VEGF-coated membrane. The signals were detected with HRP conjugated protein L and developed with ECL. The result is shown in this panel. The prominent dot in each unit area is the positive control anti-VEGF scFv(fXa+)/M13pIII-pelB. The samples were spotted in double repeats.

Supplementary Table 1 Signal sequence lists. (A). Library L2, n=50; (B). Library L3, n=67; (C). Library L4, n=78.

(A)

Wild-type: VKKLLFAIPLVVPFYAAQPAMAHHH

Library: VKKLLXXXXXXXXXXAAQPAMAHHH

Selected sequences[a]:

SLVPIFPFST	ASPFFASYLW	qPALLFFSLR
WLWSTPLFPH	PLVWSLSPSq	FYPTLLHFSq
SLVYFFPFYP	LSTTVqTSLV	LSLASPYLFP
SLWLSSLSVL	LqPSFMLWLY	TLVqLFWPSS
SYWLDFIqVL	qSLLLLRALL	SLLLPLAPPN
qRTVAAAYFW	LVSLISSRLS	LqLSFYTWLS
RPALVYLWSV	VPVLFLRFSL	qSSIIFFYLR
qWGIRSVLLL	qCCLLMSRLL	PqSLLPAIMV
qSTVFFSWLS	LLSqLVLHLR	PqLLAASAVT
LYLSSAFSYD	NSLLIISSLR	qLCSLALWFR
qPVLFSFFIR	LqILMLRTFY	PILTSLTSDA
LPYFLSRFNT	qRLLFAGSFY	SLAFFLRVYF
FLSWLGLGSR	PFLFPMLPqP	qFSFFLYFLR
SVSLSSYSFY	FGSYLLSRAL	GPLLMFFqNY

qLLIIIYWIR	PAWLSLFFDT	ASCILVRAIL
PWLFPFHAYP	LGFTLWHSDV	LqYLFLLKLS
STLAFLRLFT	FALSLYAYSR	

(B)

Wild-type: VKKLLFAIPLVVPFYAAQPAMAHHH

Library: VKKLLFAIPLXXXXXXXXXXMAHHH

Selected sequences:

CVSVRSPAFA	LSQFQQAqPP	LASLSARCHG
TLFLQRSSLA	YFPLAPqPLA	YLVCSRPLHA
YLSMTRSGAA	SLLLCNqVFG	VCTLSSRAFS
YLIKPPEGFS	CVPCLSTRGA	LLSTIKTSFS
SCFLSRSAFS	YYFVSRPVPG	LTTLSRPSFS
YLAAPRSTVA	TLTPLSRGFP	FFSLSRYSLA
YFVLVRESSS	VGRFFYSEPS	SLFFSARAIA
GLFTIRDSFA	AMLEPTRSSA	FCALSRFTHA
WLGITKPVWS	FFASMRHTqA	VLSLSRTFSG
YLVSSKNSYP	FLLSPRSSVA	LWSLSSRGMT
VSMARPSSAL	TLILSHRSSA	LLSLHRFSFA
LAVFERPTYG	FSPTSQEIRH	VLSLNRGVFA
IPLLTRHPPA	MTTLASRTHA	SLLPLSRCFA
CVALSRSQAT	SWSLCRPVCA	MLQCYRSPLA
LLFRSVALAE	LTFRSPMSLA	LSVFSVRSST
SVqLMWPPRV	WCALSRQSMP	WALTSRSILA

YLMVHRPLSG	AASILqKSSA	LSALSSQTRA
SLYFSLEARA	WLSISLPSRG	VVSLRSFAFS
WTTLTGKALS	LTCYQSqSIA	MLSLRPTAAA
YILSPRLPPP	ISTLHFRAFG	SAFKIAPLWA
FHLCARPVFS	VLSLSRTSSG	TAAVqPPVWS
FLFRSSAPGq	WCqLSPLVYP	
LFEIPARSFA	FSMLPLNASq	

(C)

Wild-type: VKKLLFAIPLVVPFYAAQPAMAHHH

Library: VKKLLFAIPLVVPFYXXXXXXXXXX

Selected sequences:

TRSALAFFLP	ASSMSqYRQN	CRSSSSIFPL
TRSCFAFMLP	SNSHPqSMHL	TSGMSRLRSW
TTRVNAFMLV	AFSFASSqLV	SCGFSRLSKA
STFARSFMLT	AFSLSRTSSK	ARSYSRPPSI
SRSMSLHPTA	SqSATRVPLL	IFPIEASARR
ARSMQSFPTS	TRSMLLPGTS	GRVSPSRSEF
AqSMAVPIST	SRVSVAFMLM	QPVRSPRTPP
FPLSSRAFML	GASSWWLFPS	RSSPqSMSLI
PTNAIAFFLq	ARSMASTPLA	ARTSTMRTDW
PTRLFAFMLT	SRSYMLLSRP	SPLARPSARP
SSSMqPPPDN	VHALARKSQF	CRSGTFGNIG
GYSMSqSGLT	AqSRLRVYPP	ARSLSSYNAV
AVALqPPVSV	PVRqLHTNLR	TRAFSSPLSN
TPRAFCFMLP	PqPSRGFMLI	ARCFSSPTWP
SRNAPCFMLP	SQLHqSPGNP	SAALARSPPT
TRYSHAFMLI	SRGFSMAFFP	ARSFSSPPGP

SRALTSISGM	AQALTTRGLA
SqSMQPSSSL	SFSMTRSSPL
ARSLTQFSSV	SRALTMTPSF
ARALQSPSSM	SRSMSTSPIL
SRALSQMSTI	SRAFSSTPAM
SFSGTRWSYL	VYPARFPAKT
SYAMFSPRqL	ANALqPFQqL
CHAFSPSSKR	TAAMqYPPTT
SFSFTRqPLP	FTPLPRSRIP
TRSMVFPAKV	TCAFSGqRVE
SRSFSSPSIT	ARSFSAFPHG
SRSMTLKGPE	ACSFTRSYPS
SRAFSSPSGS	
TRSMSSLPSP	
ARSLSSPLTL	
GYAMqSPNYY	
SSALSSqPLV	
ARCFSSPVAL	
CLGRSMAPGP	

[a] For clearance, only 10 amino acids in the randomized sequence region (represented by X) are listed. “q” stands for TAG ambor stop which was translated into glutamine.

Supplementary Table 2 Quantitative measurements of VEGF binding, fXa resistance, and secretion quantity for sc-dsFv from optimum signal sequence variants. A. Library L2, B. Library L3, C. Library L4.

(A)

	Phage		sc-dsFv			SEQ[d]
	VEGF binding[a]	fXa resistance[b] (%)	VEGF binding	fXa resistance (%)	Quantity[c]	
L2-Eu2	0.851 ± 0.045	83.91 ± 5.55	0.077 ± 0.006	11.12 ± 0.04	0.0510	WLWSTPLFPH
L2-Eu3	0.258 ± 0.014	87.66 ± 5.89	0.070 ± 0.007	31.62 ± 2.62	0.0191	SLVYFFPFYP
L2-Eu6	0.726 ± 0.074	95.44 ± 7.90	0.041 ± 0.000	4.2 ± 0.17	0.0230	qRTVAAAYFW
L2-Eu9	0.609 ± 0.053	100.00 ± 0.00	0.052 ± 0.001	4.25 ± 0.58	0.0104	qSTVFFSWLS
L2-Eu11	0.716 ± 0.048	96.60 ± 5.89	0.053 ± 0.001	3.18 ± 0.28	0.0224	qPVLFSFFIR
L2-Eu12	0.388 ± 0.038	53.42 ± 3.31	0.065 ± 0.002	21.24 ± 2.59	0.1223	LPYFLSRFNT
L2-Eu15	1.288 ± 0.057	63.77 ± 5.01	0.072 ± 0.003	8.06 ± 0.29	0.1753	ASPFFASYLW
L2-Eu16	1.029 ± 0.080	84.48 ± 3.56	0.053 ± 0.004	5.62 ± 0.41	0.0956	PLVWSLSPSq
L2-Eu18	0.736 ± 0.040	68.36 ± 3.78	0.049 ± 0.002	3.64 ± 0.38	0.0475	LqPSFMLWLY
L2-Eu20	1.171 ± 0.095	63.61 ± 5.38	0.047 ± 0.001	4.03 ± 0.26	0.1029	LVSLISSRLS
L2-Eu21	1.226 ± 0.101	71.44 ± 3.02	0.049 ± 0.001	4.82 ± 0.30	0.1214	VPVLFLRFSL
L2-Eu25	0.599 ± 0.015	72.65 ± 0.49	0.047 ± 0.001	3.37 ± 0.18	0.0144	LqlLMLRTFY
L2-Eu35	0.773 ± 0.067	59.07 ± 4.54	0.054 ± 0.004	5.47 ± 0.78	0.0203	LqLSFYTWLS
L2-Eu38	0.118 ± 0.011	36.69 ± 1.00	0.039 ± 0.001	5.76 ± 0.54	0.0781	PqLLAASAVT
L2-Eu44	0.918 ± 0.043	60.47 ± 5.62	0.066 ± 0.004	3.94 ± 0.08	0.0138	qLLIIIYWIR
anti-VEGF scFv (fXa+)/M13pIII-pelB	1.000 ± 0.071	8.80 ± 0.56	1.000 ± 0.060	1.65 ± 0.27	1.0000	FAIPLVVPFY
anti-VEGF scFv (fXa-)/M13pIII-pelB	0.598 ± 0.027	99.62 ± 1.64	0.994 ± 0.016	99.24 ± 3.90	nd	FAIPLVVPFY
anti-VEGF S5 sc-dsFv (fXa+)/M13pIII-pelB	0.014 ± 0.003	nd[e]	0.022 ± 0.001	nd	nd	FAIPLVVPFY

(B)

	Phage		Sc-dsFv			SEQ
	VEGF binding	fXa resistance (%)	VEGF binding	fXa resistance (%)	Quantity	
L3-Eu2	0.756 ± 0.099	81.48 ± 3.44	0.088 ± 0.001	8.70 ± 0.21	0.0331	TLFLQRSSLA
L3-Eu3	1.426 ± 0.104	94.66 ± 5.26	0.090 ± 0.002	10.03 ± 0.56	0.0333	YLSMTRSGAA
L3-Eu4	0.847 ± 0.093	95.00 ± 4.66	0.078 ± 0.001	18.46 ± 0.55	0.0513	YLIKPPEGFS
L3-Eu5	0.522 ± 0.065	77.79 ± 6.15	0.074 ± 0.001	13.73 ± 0.95	0.0162	SCFLSRSAFS
L3-Eu7	1.762 ± 0.125	92.36 ± 7.24	0.087 ± 0.001	16.37 ± 0.70	0.0245	YFVLVRESSS
L3-Eu8	0.302 ± 0.050	62.35 ± 6.53	0.071 ± 0.001	17.08 ± 0.41	0.0369	GLFTIRDSFA
L3-Eu15	0.164 ± 0.048	33.16 ± 1.61	0.090 ± 0.002	10.23 ± 0.30	0.0076	LLFRSVALAE
L3-Eu19	1.204 ± 0.079	62.55 ± 1.34	0.072 ± 0.001	17.67 ± 0.75	0.1271	SLLLCNqVFG
L3-YJ1	1.110 ± 0.102	62.93 ± 0.85	0.058 ± 0.001	18.94 ± 0.27	0.0236	WCALSRQSMP
L3-YJ3	2.245 ± 0.072	71.71 ± 3.01	0.066 ± 0.001	20.63 ± 1.20	0.0163	YLVCSRPLHA
L3-YJ4	2.304 ± 0.051	92.50 ± 2.96	0.095 ± 0.002	14.46 ± 1.73	0.0225	VCTLSSRAFS
L3-YJ6	2.313 ± 0.011	80.01 ± 3.40	0.081 ± 0.002	17.18 ± 0.49	0.0182	LTTLSRPSFS
L3-YJ8	0.865 ± 0.045	55.74 ± 2.94	0.054 ± 0.001	18.80 ± 0.80	0.0129	SLFFSARAIA
L3-YJ19	2.111 ± 0.157	76.68 ± 4.51	0.090 ± 0.001	20.65 ± 1.14	0.0303	SLYFSLEARA
L3-YJ20	2.066 ± 0.159	66.69 ± 6.78	0.099 ± 0.001	12.90 ± 0.36	0.0217	WTTLTGKALS
L3-YJ24	1.823 ± 0.046	78.48 ± 4.24	0.099 ± 0.001	12.84 ± 1.16	0.0611	LFEIPARSFA
L3-YJ25	1.300 ± 0.104	63.75 ± 0.11	0.096 ± 0.001	5.54 ± 0.05	0.0190	AASILqKSSA
anti-VEGF scFv (fXa+)/M13pIII-pelB	1.000 ± 0.071	8.80 ± 0.56	1.000 ± 0.060	1.65 ± 0.27	1.0000	VVPFYAAQPA
anti-VEGF scFv (fXa-)/M13pIII-pelB	0.598 ± 0.027	99.62 ± 1.64	0.994 ± 0.016	99.24 ± 3.90	nd	VVPFYAAQPA
anti-VEGF S5 sc-dsFv (fXa+)/M13pIII-pelB	0.014 ± 0.003	nd	0.022 ± 0.001	nd	nd	VVPFYAAQPA

(C)

	Phage		Sc-dsFv			SEQ
	VEGF binding	fXa resistance (%)	VEGF binding	fXa resistance (%)	Quantity	
L4-Eu1	0.182 ± 0.016	48.40 ± 1.44	0.111 ± 0.003	5.70 ± 0.26	0.0196	TRSALAFFLP
L4-Eu2	1.729 ± 0.102	62.21 ± 3.74	0.444 ± 0.025	12.25 ± 0.33	0.2401	TRSCFAFMLP
L4-Eu3	0.610 ± 0.046	47.60 ± 2.05	0.109 ± 0.001	8.21 ± 0.36	0.1218	TTRVNAFMLV
L4-Eu9	0.226 ± 0.020	39.39 ± 1.99	0.304 ± 0.011	6.03 ± 0.27	0.0287	FPLSSRAFML
L4-Eu15	0.171 ± 0.008	39.78 ± 2.87	0.133 ± 0.004	6.45 ± 0.29	0.1200	TPRAFCFMLP
L4-Eu28	0.217 ± 0.001	44.01 ± 1.26	0.067 ± 0.006	5.46 ± 0.04	0.0709	VHALARKSQF
L4-Eu30	0.196 ± 0.013	43.94 ± 3.00	0.072 ± 0.005	6.87 ± 0.50	0.0326	PVRqLHTNLR
L4-Eu31	0.145 ± 0.001	40.12 ± 3.23	0.120 ± 0.010	3.42 ± 0.19	0.0002	PqPSRGFMLI
L4-Eu32	0.240 ± 0.008	57.89 ± 2.43	0.047 ± 0.002	7.71 ± 0.76	0.0196	SQLHqSPGNP
L4-Eu39	0.155 ± 0.005	45.07 ± 1.13	0.137 ± 0.009	3.75 ± 0.21	0.0247	GRVSPSRSEF
L4-CM2	2.075 ± 0.050	87.73 ± 2.38	0.123 ± 0.014	15.60 ± 1.22	0.0139	ARSLSSYNAV
L4-CM8	0.906 ± 0.093	69.01 ± 1.56	0.131 ± 0.015	32.13 ± 2.45	0.0199	SqSMQPSSSL
L4-CM13	1.486 ± 0.129	80.55 ± 6.87	0.069 ± 0.007	30.45 ± 2.04	0.0160	SYAMFSPRqL
L4-CM15	2.002 ± 0.136	63.77 ± 2.48	0.060 ± 0.026	21.21 ± 1.41	0.0178	SFSFTRqPLP
L4-YJ2	1.421 ± 0.092	62.03 ± 2.11	0.054 ± 0.007	3.85 ± 0.22	0.0223	CLGRSMAPGP
L4-YJ6	1.926 ± 0.173	65.55 ± 1.10	0.205 ± 0.017	21.66 ± 1.21	0.0169	SRSMSTSPIL
L4-YJ10	0.748 ± 0.068	68.47 ± 3.98	0.164 ± 0.014	37.79 ± 1.37	0.0173	TAAMqYPPTT
L4-Eu14	1.142 ± 0.422	63.42 ± 4.80	0.121 ± 0.034	21.40 ± 4.11	0.0523	AVALqPPVSV
L4-CM17	2.042 ± 0.100	64.91 ± 10.6	0.063 ± 0.027	48.51 ± 9.92	nd	SRSFSSPSIT
L4-CM20	2.633 ± 0.832	71.43 ± 12.1	0.064 ± 0.024	7.54 ± 3.93	nd	TRSMSSLPSP
anti-VEGF scFv (fXa+)/M13pIII-pelB	1.000 ± 0.071	8.80 ± 0.56	1.000 ± 0.060	1.65 ± 0.27	1.0000	AAQPAMAHHH
anti-VEGF scFv (fXa-)/M13pIII-pelB	0.598 ± 0.027	99.62 ± 1.64	0.994 ± 0.016	99.24 ± 3.90	nd	AAQPAMAHHH
anti-VEGF S5 sc-dsFv (fXa+)/M13pIII-pelB	0.014 ± 0.003	nd	0.022 ± 0.001	nd	nd	AAQPAMAHHH

[a] VEGF binding is the ELISA signal against VEGF and normalized with anti-VEGF scFv (fXa+). The standard deviations are derived by three repeats of the measurements, except for the western blot measurement, which was done once.

[b] FXa resistance is ELISA signal in the presence of fXa over that in the absence of fXa. The standard deviations are derived by three repeats of the measurements.

[c] The quantity was measured with western blot and normalized with the quantity of the

positive control anti-VEGF scFv(fXa+).

[d] For clearance, only 10 amino acids in the randomized sequence region are listed. q stands for TAG ambor stop condon which can be translated into glutamine.

[e] nd: not measured.