

Table S1. Primer sets for the amplification of CDRs from the oligonucleotide mixture.

Product name	Templates	Primers	
CDR-H1	Oligomix ^a	VH3-1-f	VH3-1-b
CDR-H2	Oligomix	VH3-2-f	VH3-2-b
Oligo-H3	Oligomix	lib-cdr-f	lib-cdr-b
CDR-H3(#1~#8)	H3(#1~#8) ^b	VH-3-f	VH-3-b
CDR-L1	Oligomix	VL-1-f	VL-1-b
CDR-L2	Oligomix	VL-2-f	VL-2-b
CDR-L3	Oligomix	VL-3-f	VL-3-b
CDR-K1	Oligomix	VK-1-f	VK-1-b
CDR-K2	Oligomix	VK-2-f	VK-2-b
CDR-K3	Oligomix	VK-3-f	VK-3-b

^a Oligonucleotide pool synthesized by parallel synthesis.

^b CDR-H3 oligonucleotides separated by length (9~16 aa).

Table S2. Primers for the amplification of the framework regions for single-CDR library construction.

Product name	Templates	Primers	
1	VHVL-pUC57 ^a	pUC57-b	VH3-1-f-rc
2	VHVL-pUC57	pUC57-b	VH3-2-f-rc
3	VHVL-pUC57	pUC57-b	VH-3-rc-F
4	VHVL-pUC57	pUC57-b	VL-1-f-rc
5	VHVL-pUC57	pUC57-b	VL-2-f-rc
6	VHVL-pUC57	pUC57-b	VL-3-f-rc
7	VHVK-pUC57 ^b	pUC57-b	VK-1-f-rc
8	VHVK-pUC57	pUC57-b	VK-2-f-rc
9	VHVK-pUC57	pUC57-b	VK-3-f-rc
10	VHVL-pFcF ^c	VH3-1-b-rc	hCH2-b
11	VHVL-pFcF	VH3-2-b-rc	hCH2-b
12	VHVL-pFcF	VH-3-rc-B	hCH2-b
13	VHVL-pFcF	VL-1-b-rc	hCH2-b
14	VHVL-pFcF	VL-2-b-rc	hCH2-b
15	VHVL-pFcF	VL-3-b-rc	hCH2-b
16	VHVK-pFcF ^d	VK-1-b-rc	hCH2-b
17	VHVK-pFcF	VK-2-b-rc	hCH2-b
18	VHVK-pFcF	VK-3-b-rc	hCH2-b

^a Codon-optimized VH3-23/JH4-(G₄S)₃-Vλ1-47/Jλ2 gene cloned in pUC57 vector.

^b Codon-optimized VH3-23/JH4-(G₄S)₃-Vκ3-20/Jκ1 gene cloned in pUC57 vector.

^c Codon-optimized VH3-23/JH4-(G₄S)₃-Vλ1-47/Jλ2 gene cloned in pFcF vector (a pcDNA3.1-derived vector with a pair of SfiI sites and a hIgG1 Fc tag).

^d Codon-optimized VH3-23/JH4-(G₄S)₃-Vκ3-20/Jκ1 gene cloned in pFcF vector (a pcDNA3.1-derived vector with a pair of SfiI sites and a hIgG1 Fc tag).

^{a-d} Codon-optimized scFv genes were synthesized with asymmetric SfiI restriction sites compatible with pComb3X phagemid vector at both ends.

Table S3. Assembly of single-CDR scFv libraries by overlap extension PCR.

Product name	Templates	Primers	
VHVL -H1	1 + CDR-H1 + 10		
VHVL -H2	2 + CDR-H2 + 11		
VHVL -H3	3 + CDR-H3 + 12		
VHVL -L1	4 + CDR-L1 + 13		
VHVL -L2	5 + CDR-L2 + 14	pUC57-in-b	hCH2-in-b
VHVL -L3	6 + CDR-L3 + 15		
VHVK -K1	7 + CDR-K1 + 16		
VHVK -K2	8 + CDR-K2 + 17		
VHVK -K3	9 + CDR-K3 + 18		

Table S4. Amplification of the proofread CDR and adjoining framework regions by PCR.

Product name	Templates	Primers	
VH3-1	VHVL -H1	pC3x-f	VH3-1-b
VH3-2	VHVL -H2	VH3-1-b-rc	VH3-2-b
VH3-H3	VHVL -H3	VH3-2-b-rc	VH-3-b
VL-1	VHVL -L1	VH-3-rc-B	VL-2-f-rc
VL-2	VHVL -L2	VL-2-f	VL-3-f-rc
VL-3	VHVL -L3	VL-3-f	pC3x-b
VK-1	VHVK -K1	VH-3-rc-B	VK-2-f-rc
VK-2	VHVK -K2	VK-2-f	VK-3-f-rc
VK-3	VHVK -K3	VK-3-f	pC3x-b

Table S5. Primers for the assembly of VH, VL/VK and scFv by OE-PCR.

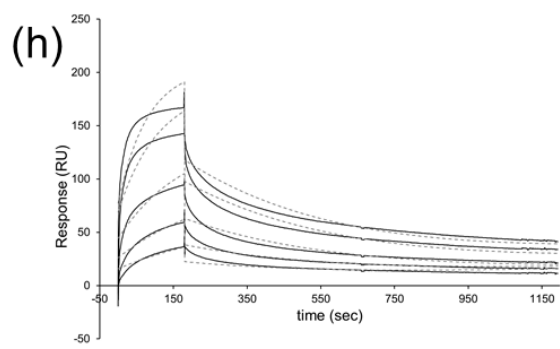
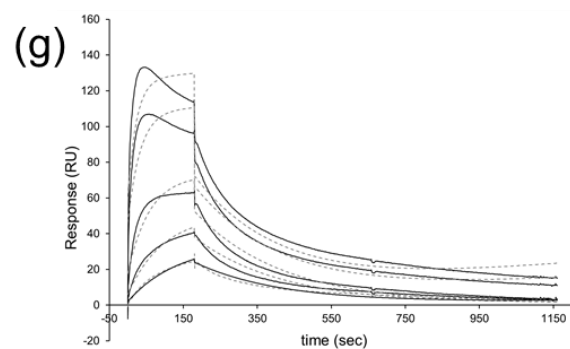
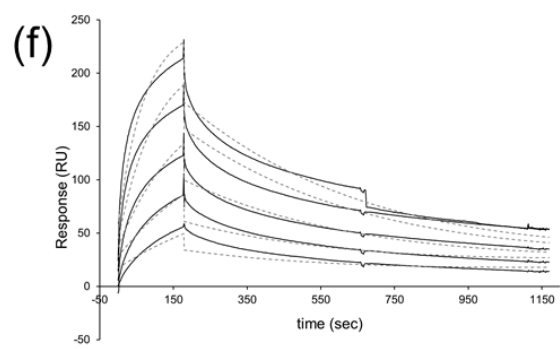
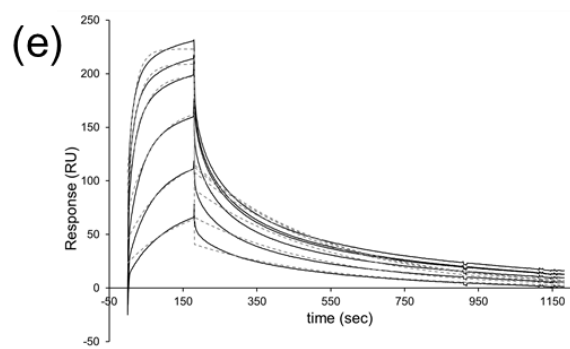
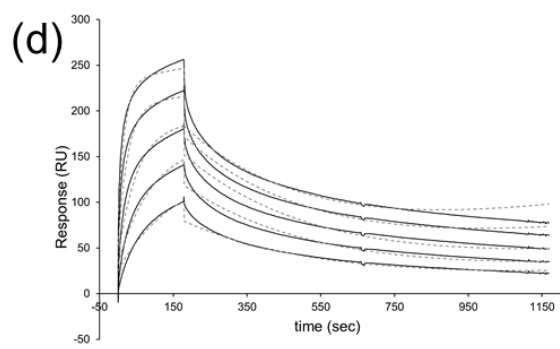
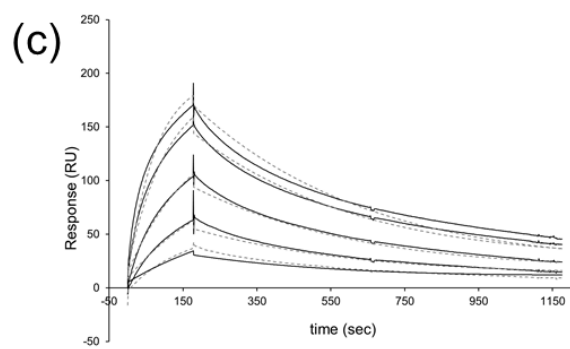
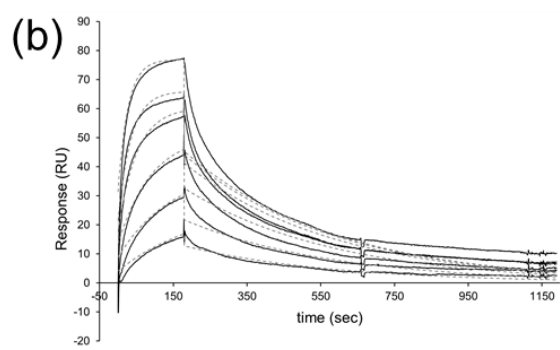
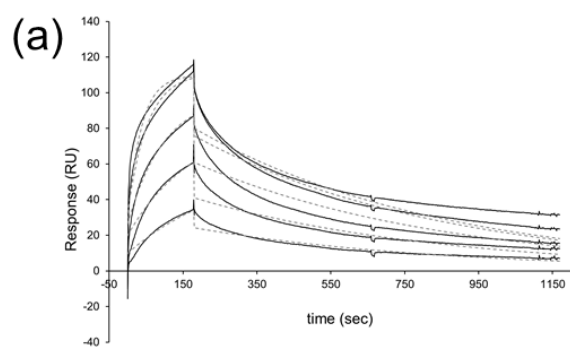
Product name	Templates	Primers	
<i>Primers for the assembly of VH, VL, or VK domains</i>			
VH3	VH3-1 + VH3-2 + VH3-H3	pC3x-f	VH-3-b
VL1	VL-1 + VL-2 + VL-3	VH-3-rc-B	pC3x-b
VK3	VK-1 + VK-2 + VK-3	VH-3-rc-B	pC3x-b
<i>Amplification of the final scFv gene</i>			
VH3VL1	VH3 + VL1	pC3x-f	pC3x-b
VH3VK3	VH3 + VK3	pC3x-f	pC3x-b

Table S6. Sequences of the primer used in this work.*

Primer name	Sequence (5'→3')
hCH2-b	CTTGACCTCAGGGTCTTC
hCH2-in-b	CCA GGA GTT CAG GTG CTG
lib-cdr-b	CTGAGTCGATGACCTACG
lib-cdr-f	GTCAGTCACGCTCTAAGG
pC3x-b	AAC CAT CGA TAG CAG CAC CG
pC3x-f	GCA CGA CAG GTT TCC CGA C
pUC57-b	TTCGCCATTTCAGGCTGCG
pUC57-in-b	GGA TGT GCT GCA AGG CGA
VH3-1-b	TTACCTGGTGCCTGTCTG
VH3-1-b-rc	CAGACAGGCACCAGGTAA
VH3-1-f	CTCCGGATTCACTTTCAGC
VH3-1-f-rc	GCTGAAAGTGAATCCGGAG
VH3-2-b	GCGTGAGATGGTGAAGCG
VH3-2-b-rc	CGCTTCACCATCTCACGC
VH3-2-f	GGACTGGAGTGGGTCTCT
VH3-2-f-rc	AGAGACCCACTCCAGTCC
VH-3-b	CAGAGTACCTTGTCCTCCA
VH-3-f	CACTGCCGTGTATTACTGC
VH-3-rc-B	TGGGGACAAGGTACTCTG
VH-3-rc-F	GCAGTAATACACGGCAGTG
VK-1-b	CCAGGTTTCTGTTGGTACCA
VK-1-b-rc	TGGTACCAACAGAAACCTGG
VK-1-f	CGCGCAACTCTGTCTTGT
VK-1-f-rc	ACAAGACAGAGTTGCGCG
VK-2-b	GAACCTGTCTGGGATGCC
VK-2-b-rc	GGCATCCCAGACAGGTTC
VK-2-f	CCACGCCTGCTCATCTAT
VK-2-f-rc	ATAGATGAGCAGGCGTGG
VK-3-b	ACCTTCGTTCCCTGACCA
VK-3-b-rc	TGGTCAGGGAACGAAGGT
VK-3-f	GACTTCGCAGTTTACTATTGT
VK-3-f-rc	ACAATAGTAAACTGCGAAGTC
VL-1-b	CTGGGAGTTGCTGATACCA
VL-1-b-rc	TGGTATCAGCAACTCCCAG
VL-1-f	CGCGTCACCATCAGCTGC
VL-1-f-rc	GCAGCTGATGGTGACGCG
VL-2-b	AAAGCGATCAGGCACACC
VL-2-b-rc	GGTGTGCCTGATCGCTTT

VL-2-f	CTCCTAAGCTCCTGATTTAC
VL-2-f-rc	GTAAATCAGGAGCTTAGGAG
VL-3-b	AGTTTGGTCCCACCGCCG
VL-3-b-rc	CGGCGGTGGGACCAAAC
VL-3-f	CGAGGCTGACTATTACTGC
VL-3-f-rc	GCAGTAATAGTCAGCCTCG

* Oligonucleotides were $\geq 85\%$ pure after purification using Oligonucleotide Purification Cartridge



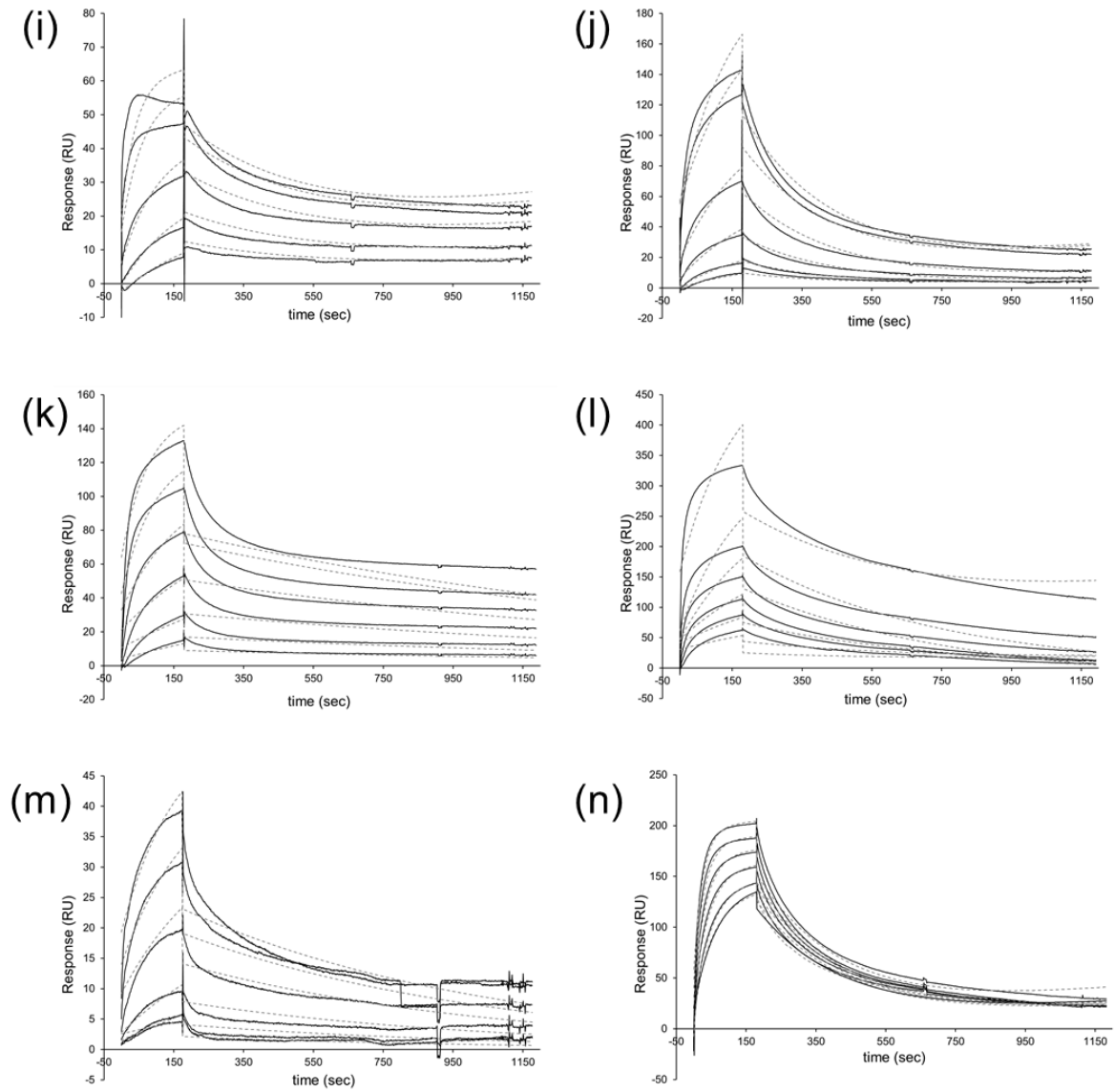


Figure S1. SPR sensorgrams (solid lines) and 1:1 Langmuir binding fitting (dashed lines) of OPAL-T clones. (a) AIMP1-C6 ([scFv] = 40–480 nM), (b) AIMP1-D4 ([scFv] = 67–1,070 nM), (c) AIMP1-E7 ([scFv] = 54–860 nM), (d) BCMA-A3 ([scFv] = 50–600 nM), (e) BCMA-B5 ([scFv] = 40–640 nM), (f) BCMA-D11 ([scFv] = 43–690 nM), (g) CARS-B6 ([scFv] = 31–375 nM), (h) CARS-D7 ([scFv] = 55–870 nM), (i) CDRS-D11 ([scFv] = 66–800 nM), (j) CARS-F4 ([scFv] = 5.7–91 nM), (k) CD22-D1 ([scFv] = 45–900 nM), (l) HEWL-H5 ([scFv] = 62–990 nM), (m) SARS-D6 ([scFv] = 5.7–91 nM), and (n) SARS-F4 ([scFv] = 80–230 nM).