

Supporting Information to:

Stringent Selection Drives Convergence Toward Omicron-like SARS-CoV-2 Receptor-Binding Motifs

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Supporting information part PS1 - platform for parallelized *in vitro* evolution

The pJYDC4 plasmid was created using a cut-and-paste replacement of the eUnaG2 reporter with SpyCatcher003. Similarly, the pJYDC6 plasmid was constructed using a designed monomeric avidin (DeMA). The design of this reporter was based on the PDB structure 1avd of rhizavidin. The design process was identical to the preparation of the eUnaG2 and DnbALFA reporters in Zahradník, J. *et al.* A Protein-Engineered, Enhanced Yeast Display Platform for Rapid Evolution of Challenging Targets. *ACS Synthetic Biology* 10, 3445-3460 (2021). [https://doi.org:10.1021/acssynbio.1c00395](https://doi.org/10.1021/acssynbio.1c00395)

>SpyCatcher003_S.cer_optimized

ATGGTTACCACTGAGCGGTCTGAGTGGTGAACAGGGTCCGAGCGGTGATATGACCACCGAAGAAGATAGCG
CAACCCATATCAAATTTAGCAAACGTGATGAAGATGGTCGTGAAGTGGCAGGCGCAACCATGGAAGTGCCTGA
TAGCAGCGGTAAACCATTAGCACCTGGATTAGTGTATGGTCACGTGAAAGATTTTATCTGTATCCGGGTAAA
TATACCTTCGTTGAAACCGCAGCACCGGATGGTTATGAAGTTGCAACCCCGATTGAATTCACCGTTAACGAAG
ATGGCCAGGTTACCGTTGATGGTGAAGCAACCGAAGGTGATGCACATACC

>DeMA_S.cer_optimized

TTGATGCCTCTAACTTCAAGGACTTCTCTTCTATTGCTGGTACTTCTACTACCTGGCAAAATCAACATGGTT
CCACTATGGTTATCACCGTTGATTCTCAAGGTAACGTTTCTGGTCAATACGTTAATAGAGCTGAAGGTACTGG
TTGTCAGAAATTCCTATATCCATTGACTGGTTGGGTTAACGGTACTTTCATTGATTTCTCTGTTACCTGGAAC
AACTCTACCGAAACTGTAACTCTGTTACTCAATGGACTGGTTACGCTCAAGTTAATGGTAACAACACTGAAA
TCGTTACCGATTGGAAGTTGGTTTATGAAGGTCCATCTGGTCCAGCTATTGGCAAGGTCAAGATACTTTTCA
ATACGTCCCACTACCGAG

pJYDC-barcF-RBDo-Wu plasmid sequence segment with expression of whole yeast display cassette used: eUnaG2 – Aga2p – barcode_F_fwd - RBDo-Wu_sequence - barcode_F_rev.

Left and right barcodes were integrated flanking the expression cassette within the expression construct. Their sequences are in the Table S1

>Expression_construct

ATGAGGTTCCCATCTATTTTACCGCTGTTGTTTTGCTGCTTCTCTGCTTTGGCTGCTCCAGCTAATGGTATGTTAGA
AAAATTTGTTGGCACCTGGAAGATCGAATCCTCTGAAAATTTTGGTGAATACTTGAAGGCTATCGGTGCCCCAAAAGAAT
TGGCTGATGCTGGTGATGCTACTACTCCAGTCTTGTACATTTCTCAAAAAGGATGGTGATAAGATGACCGTCAAGATTGAA
AACGGTCCACCAACTTTTTTGGATACCCAAGTTTCTTTCAAGTTGGGTGAAGAATTCGACGAATTTCCATCCGATAGAAG
AAAGGGTGTAAAGTCCGTTGTTAACTTGTCTGGTGAGAAGTTGGTTTACGTTCAAAAAGTGGGATGGTAAAGAAACCACTT
ACGTCAGAGAAATCAAGGACGGTAAATTGGTTGTTACTTTGACCATGGGTGATGTTGTTGCTGTTAGATCTTATAGAAGG
GCCTCTGAAGTTTCTGCACAGGAAGTACCAACTATATGCGAGCAAATCCCCTCACCAACTTTAGAAATCGACGCCGTACTC
TTTGTCAACGACTACTATTTTGGCCAACGGGAAGGCAATGCAAGGAGTTTTTGAATATTACAAATCAGTAACGTTTGTCA
GTAATTGCGGTTCTCACCCCTCAACAAGTAGCAAAGGCAGCCCCATAAACACACAGTATGTTTTTAAGGACAATAGCTCG
ACGATTgaaggtagatacccatagcagcttccagactacgctctgcaggctagtgggtggaggaggtctgggtggaggcgg
tagcggaggcggaCAAGGTACCGGAAGTACAAGTgctagccatattgggtTGCCCTTTTGGTGAAGTTTTTAACGCCACCA
GATTTGCATCTGTTTATGCTTGAACAGGAAGAGAATCAGCAACTGTGTTGCTGATTATTCTGTCTATATAAATCCGCA
TCATTTTCCACTTTTAAGTGTTATGGAGTGTCTCCTACTAAATTAATGATCTCTGCTTTACTAATGTCTATGCAGATTC
ATTTGTAATTAGAGGTGATGAAGTCAGACAAATCGCTCCAGGGCAAAGTGGAAAGATTGCTGATTATAAATTATAAATTAC
CAGATGATTTTACAGGCTGCGTTATAGCTTGAATCTAACAATCTTGATTCTAAGGTTGGTGGTAATTATAAATTACCTG
TATAGATTGTTTATGAAGTCTAATCTCAAACCTTTTGAGAGAGATATTTCAACTGAAATCTATCAGGCCGGTAGCACACC
TTGTAATGGTGTGAAGGTTTTAATTGTTACTTTCTTTTCAATCATATGGTTTCCAACCCACTAATGGTGTGGTTACC
AACCATACAGAGTAGTAGTACTTTCTTTTGAAGTTCTACATGCACCAGCAACTGTTTGTGGACCTAAAggatccAGATCC
GGAAATGGCAGTACCgaacaaagcttatttctgaagaggacttgtaa

- in red short AppS4 leader sequence; in green eUnaG2 reporter protein; in bold Aga2p sequence; yellow background highlighted sequences – fwd and rev barcodes; in blue RBD sequence; in magenta c-myc sequence

Table S1. Expression cassette barcodes for identification of plasmids in parallelized *in vitro* evolution

Barcode name	Barcode sequence	AA sequence
A_fwd	AGCAACGGTACTGGATCTAGT	SNGTGSS
A_rev	GGAGGCAACTCAAGTGGAAC	GGASSGT
B_fwd	AACACTTCAGGAGGTAGAGGA	NTSGGRG
B_rev	ACCGGATCTAATGGTTCAGGT	TGSNGSG
C_fwd	TCCTCTGGAACATAATGGCACA	SSGTNGT
C_rev	GGTTCAACTGGCGGTTCTCTCG	GSTGGSS
D_fwd	acaaacgggttccggcacaggc	TNGSGTG
D_rev	aataccgggttctggaaccgca	NTGSGTA
E_fwd	AGTGGCAATGCAACTGGTAGC	SGNATGS
E_rev	TCGGGCAATAGTGGATCATCT	SGNSGSS
F_fwd	CAAGGTACCGGAAGTACAAGT	QGTGSTS
F_rev	AGATCCGGAAATGGCAGTACC	RSGNGST
G_fwd	aacagcaccggcggcagcggc	NSTGGSG
G_rev	gctcgcgctgccgggtgcggcc	GRTGSAS
H_fwd	accaccaacagcagcggcagc	TTNSSGS
H_rev	gccgccgctgttcgcgggtgcg	RTANSGG

- lowercase letters show barcodes that were not used due to potential expression influence

Table S2. Initial RBD sequences, barcodes and plasmids

Original SARS-CoV2 sequence (Low Stringency selections, LSS)	Barcode	Plasmid (1 st round)
isolate Wuhan-Hu-1 (MN908947.3)	FF	pJYDC3
isolate SARS-CoV-2/human/ITA/VA-English-2021-01-22/2021 (MW521144.1)	CC	pJYDC3
isolate SARS-CoV-2/human/ITA/VA-South Africa 2021-02-03/2021 (MW560269.1)	AA	pJYDC3
MW560269.1 + Q498R (Abbreviated RY)	BB	pJYDC3
Omicron BA.1; isolate SARS-CoV-2/human/USA/TX-CDC-ASC210646153/2022 (OM652834.1)	EE	pJYDC3
Original SARS-CoV2 sequence (High Stringency selections, HSS)	Barcode	Plasmid (1 st round)
isolate Wuhan-Hu-1 (MN908947.3)	AA	pJYDC1
isolate Wuhan-Hu-1 (MN908947.3) + I358F mutation introduced by SDM	BB	pJYDC3
Omicron BA.1; isolate SARS-CoV-2/human/USA/TX-CDC-ASC210646153/2022 (OM652834.1)	C_fwd	pJYDC1
Omicron BA.2, isolate SARS-CoV-2/human/USA/CA-CDC-LC0582623/2022 (ON373904.1)	C_rev	pJYDC3

- combination of barcodes A_fwd + A_rev is simplified to AA; C_fwd means that only forward barcode was used in this case, C_rev means that only reverse barcode was used in this case

Table S3. Sample specific barcodes used in Illumina paired-end deep sequencing

Sample	Forward primer	Fwd-seq-barcode (5'-end)	Reverse primer	Rev-seq-barcode (5'-end)
HSS WT lib non-sorted	TTGCTGATTATAATTATAA ATTACCAGATGATTTT	Not used	CAAGTCCTCTTCAGAAAT AAGCTTTTGTTTCGGATCC	AGTTCCACTTGAGTT GCCTCC
HSS I358F non-sorted	TTGCTGATTATAATTATAA ATTACCAGATGATTTT	Not used	CAAGTCCTCTTCAGAAAT AAGCTTTTGTTTCGGATCC	TCCTGAACCATTAGA TCCGGT
HSS BA1 non-sorted	TTGCTGATTATAATTATAA ATTACCAGATGATTTT	Not used	CAAGTCCTCTTCAGAAAT AAGCTTTTGTTTCGGATCC	TGTGCCATTAGTTCC AGAGGA
HSS BA2 non-sorted	TTGCTGATTATAATTATAA ATTACCAGATGATTTT	Not used	CAAGTCCTCTTCAGAAAT AAGCTTTTGTTTCGGATCC	CGAGGAACCGCCAG TTGAACC
HSS WT lib 2	TTGCTGATTATAATTATAA ATTACCAGATGATTTT	Not used	CAAGTCCTCTTCAGAAAT AAGCTTTTGTTTCGGATCC	AGTTCCACTTGAGTT GCCTCC
HSS I358F lib 2	TTGCTGATTATAATTATAA ATTACCAGATGATTTT	Not used	CAAGTCCTCTTCAGAAAT AAGCTTTTGTTTCGGATCC	TCCTGAACCATTAGA TCCGGT
HSS BA1 lib 2	TTGCTGATTATAATTATAA ATTACCAGATGATTTT	Not used	CAAGTCCTCTTCAGAAAT AAGCTTTTGTTTCGGATCC	TGTGCCATTAGTTCC AGAGGA
HSS BA2 lib 2	TTGCTGATTATAATTATAA ATTACCAGATGATTTT	Not used	CAAGTCCTCTTCAGAAAT AAGCTTTTGTTTCGGATCC	CGAGGAACCGCCAG TTGAACC
HSS WT lib 4	TTGCTGATTATAATTATAA ATTACCAGATGATTTT	Not used	CAAGTCCTCTTCAGAAAT AAGCTTTTGTTTCGGATCC	AGTTCCACTTGAGTT GCCTCC
HSS I358F lib 4	TTGCTGATTATAATTATAA ATTACCAGATGATTTT	Not used	CAAGTCCTCTTCAGAAAT AAGCTTTTGTTTCGGATCC	TCCTGAACCATTAGA TCCGGT
HSS BA1 lib 4	TTGCTGATTATAATTATAA ATTACCAGATGATTTT	Not used	CAAGTCCTCTTCAGAAAT AAGCTTTTGTTTCGGATCC	TGTGCCATTAGTTCC AGAGGA
HSS BA2 lib 4	TTGCTGATTATAATTATAA ATTACCAGATGATTTT	Not used	CAAGTCCTCTTCAGAAAT AAGCTTTTGTTTCGGATCC	CGAGGAACCGCCAG TTGAACC
LSS WT	GTACAAGTGCTAGCCATA TGGGTTGCC	GCCCCT CT	GGCCTGATAGATTTCAGT TGAAATATCTCTC	TTCTCAGA
LSS Alpha	ATGGCACAGCTAGCCATA TGGGTTG	AACCTT AC	GGCCTGATAGATTTCAGT TGAAATATCTCTC	CCGTACAC
LSS Beta	GGATCTAGTGCTAGCCAT ATGGGTTG	GACAGC CC	ACCGGCCTGATAGATTTC AGTTG	TGCCGTGG
LSS RBD-v48	GTAGAGGAGCTAGCCATA TGGGTTG	ACGCAA TC	ACCGGCCTGATAGATTTC AGTTG	GCACCTAG
LSS BA.1	CTGGTAGCGCTAGCCATA TGGGTAC	AGATGA GT	GGCCTGATAGATTTCAGT TGAAATATCTCTC	CTCACAAT

Table S4. Codon frequencies upon HSS evolution of the RBM

Position	WT aa	BA.1 aa	BA.2 aa	Mutant aa	WT codon	Mutant codon	Freq Lib WT	Freq WT 2nd	Freq WT 4th	Freq Lib I358F	Freq I358F 2nd	Freq I358F 4th	Freq Lib BA.1	Freq BA.1 2nd	Freq BA.1 4th	Freq Lib BA.2	Freq BA.2 2nd	Freq BA.2 4th
440	N	K	K	K	AAT	AAG	5.4E-04	5.3E-01	6.8E-01	3.1E-04	5.5E-01	8.6E-01	9.8E-01	9.9E-01	9.7E-01	9.7E-01	9.9E-01	9.7E-01
444	K			T	AAG	ACG	3.8E-03	2.1E-01	1.9E-01	3.7E-03	5.5E-01	7.6E-01	4.1E-03	2.7E-01	1.1E-01	4.1E-03	9.7E-03	8.9E-03
444	K			R	AAG	AGG	1.8E-03	3.2E-01	4.6E-01	3.1E-03	2.2E-03	1.2E-03	8.4E-04	1.5E-01	6.7E-02	3.2E-03	6.7E-01	7.5E-01
445	V			V	GTT	GTC	2.2E-03	2.3E-03	7.4E-03	4.0E-03	3.0E-03	7.8E-03	1.0E-03	3.5E-01	5.7E-01	3.8E-03	1.7E-03	6.9E-03
446	G	S	G	S	GGT	AGT	2.3E-03	8.6E-03	1.2E-02	2.8E-03	2.0E-02	3.4E-02	9.9E-01	5.4E-01	7.8E-01	2.9E-03	2.2E-03	5.5E-03
450	N			D	AAT	GAT	1.5E-03	6.2E-01	6.9E-01	2.1E-03	9.3E-04	4.3E-03	9.0E-04	5.6E-01	7.3E-01	2.1E-03	7.1E-01	8.2E-01
452	L			K	CTG	AAG	8.0E-06	1.5E-02	1.6E-01	7.0E-06	1.5E-05	1.6E-05	4.0E-06	4.6E-03	3.6E-02	1.1E-05	5.5E-03	9.8E-02
452	L			M	CTG	ATG	2.6E-03	6.1E-01	5.3E-01	3.1E-03	1.8E-03	1.2E-03	2.3E-03	5.6E-01	6.9E-01	3.8E-03	7.1E-01	7.2E-01
452	L			R	CTG	CGG	8.9E-04	2.8E-01	2.7E-01	7.7E-04	5.8E-01	9.1E-01	7.5E-04	4.0E-01	2.6E-01	8.5E-04	1.3E-02	1.3E-02
453	Y			F	TAT	TTT	2.2E-03	1.6E-02	6.4E-03	3.4E-03	1.2E-01	2.1E-02	1.3E-03	5.9E-03	3.8E-03	3.2E-03	1.4E-01	1.7E-01
460	N			K	AAT	AAA	1.8E-03	3.0E-01	2.3E-01	2.2E-03	5.9E-01	9.2E-01	1.4E-03	3.4E-01	2.3E-01	2.1E-03	1.5E-01	1.7E-01
460	N			K	AAT	AAG	2.7E-04	6.3E-01	7.4E-01	2.4E-04	9.2E-03	2.9E-02	2.3E-04	5.8E-01	7.4E-01	2.7E-04	7.2E-01	8.1E-01
477	S	N	N	N	AGC	AAC	4.8E-03	8.4E-01	9.8E-01	5.1E-03	7.5E-01	9.8E-01	9.9E-01	9.9E-01	9.9E-01	9.8E-01	9.9E-01	9.9E-01
478	T	K	K	K	ACA	AAA	1.3E-03	8.1E-01	9.7E-01	1.1E-03	6.4E-01	9.5E-01	9.9E-01	9.9E-01	9.8E-01	9.7E-01	9.9E-01	9.7E-01
482	G			R	GGT	CGT	3.8E-04	1.9E-01	2.6E-01	2.9E-04	7.0E-06	7.2E-04	3.2E-04	1.0E-05	2.9E-03	3.5E-04	4.0E-06	2.0E-05
484	E			R	GAA	AGA	3.7E-05	4.6E-01	9.0E-01	1.6E-05	7.3E-03	1.0E-01	3.1E-05	6.3E-01	9.0E-01	2.4E-05	6.0E-01	7.1E-01
484	E	A	A	A	GAA	GCA	9.4E-04	4.0E-01	6.3E-02	1.1E-03	5.7E-01	6.4E-01	9.8E-01	3.6E-01	6.2E-02	9.8E-01	3.9E-01	2.7E-01
486	F			V	TTT	GTT	3.0E-03	2.7E-01	2.6E-02	2.6E-03	4.2E-01	5.2E-02	3.1E-03	1.2E-01	1.3E-02	2.6E-03	3.8E-03	2.5E-03
493	Q			K	CAA	AAA	6.5E-04	3.0E-04	5.0E-02	6.8E-04	8.6E-03	1.7E-01	2.0E-06	5.9E-04	8.0E-03	2.0E-06	1.9E-04	8.5E-05
493	Q	R	R	R	CAA	CGA	2.5E-03	1.0E-02	2.7E-01	4.0E-03	1.1E-01	6.5E-01	9.9E-01	5.7E-02	1.6E-02	9.8E-01	1.8E-01	1.5E-01
493	Q			G	CAA	GGA	3.0E-06	1.0E-06	2.5E-04	2.0E-06	3.0E-05	3.0E-04	7.1E-04	1.1E-01	7.1E-02	7.0E-04	1.5E-01	3.9E-01
496	G	S	G	S	GGT	AGT	1.6E-03	6.5E-03	7.7E-03	2.0E-03	3.5E-02	1.3E-02	9.9E-01	2.0E-01	6.7E-02	2.2E-03	4.0E-04	1.5E-03
498	Q			H	CAA	CAT	2.5E-03	9.1E-03	2.5E-05	4.4E-03	2.4E-01	2.9E-02	2.6E-05	1.2E-04	4.4E-05	3.5E-05	1.8E-05	3.0E-06
498	Q	R	R	R	CAA	CGA	3.4E-03	9.1E-01	9.0E-01	5.2E-03	7.0E-01	9.2E-01	9.9E-01	9.9E-01	9.5E-01	9.8E-01	9.8E-01	9.8E-01
501	N			T	AAT	ACT	5.2E-04	2.5E-03	3.0E-06	6.1E-04	2.3E-01	3.5E-02	2.0E-06	6.8E-05	2.6E-05	3.0E-06	2.3E-05	1.0E-06
501	N	Y	Y	Y	AAT	TAT	1.8E-03	9.6E-01	9.8E-01	2.4E-03	7.3E-01	9.6E-01	9.9E-01	9.9E-01	9.9E-01	9.8E-01	9.9E-01	9.8E-01
505	Y	H	H	H	TAC	CAC	2.1E-03	3.6E-01	6.2E-02	3.6E-03	1.2E-01	3.6E-02	9.9E-01	9.6E-01	6.6E-01	9.8E-01	9.9E-01	9.5E-01

Mutant codon frequencies (Freq), as determined from NGS of the non-selected first library (Lib) in comparison to the 2nd and 4th round of library propagation and selection (2nd and 4th). Codons with frequencies of >0.1 in at least one library are shown. Mutants are in relation to WT. Blue is for low and red for high frequencies.

Table S5. Frequencies of amino-acid mutations in RBD of the Spike protein of SARS-CoV-2 compared to frequencies of mutations in HSS selected clones

Residue	WT	BA1	BA2	var	1 Frequency SARS- CoV-2	2 Frequency SARS- CoV-2 (2023+)	3 Frequency HSS WT	4 Frequency HSS I358F	5 Frequency HSS BA.1	6 Frequency HSS BA.2
440	N	K	K	K	0.638	0.935	0.684	0.857	0.973	0.974
444	K			T	0.037	0.000	0.198	0.762	0.109	0.009
445	V			P	0.079	0.000	0.002	0.024	0.000	0.000
445	V			H	0.014	0.283				
446	G	S	G	S	0.444	0.819	0.012	0.034	0.778	0.005
446	G			G	0.543	0.181	0.961	0.940	0.453	0.975
450	N			D	0.018	0.298	0.694	0.004	0.733	0.816
452	L			R	0.269	0.167	0.265	0.913	0.256	0.013
452	L			Q	0.023	0.001	0.001	0.003	0.000	0.019
452	L			W	0.014	0.292				
455	L			S	0.013	0.282				
456	F			L	0.020	0.341	0.001	0.011	0.000	0.000
460	N			K	0.156	0.902	0.973	0.945	0.976	0.974
477	S	N	N	N	0.867	0.951	0.978	0.980	0.989	0.990
478	T	K	K	K	0.927	0.849	0.971	0.951	0.983	0.974
478	T			R	0.011	0.096				
481	N			K	0.010	0.298	0.011	0.001	0.006	0.001
484	E			R	0.001	0.002	0.900	0.105	0.903	0.709
484	E	A	A	A	0.860	0.651	0.063	0.641	0.062	0.271
484	E			K	0.018	0.290	0.003	0.078	0.003	0.002
486	F			V	0.108	0.114	0.026	0.052	0.013	0.003
486	F			P	0.068	0.777				
490	F			S	0.070	0.506	0.017	0.035	0.004	0.198
493	Q	R	R	R	0.675	0.000	0.273	0.670	0.016	0.146
496	G	S	G	S	0.541	0.000	0.008	0.013	0.067	0.002
496	G			G	0.453	0.999	0.972	0.951	0.794	0.967
498	Q	R	R	R	0.872	0.951	0.972	0.953	0.985	0.994
501	N	Y	Y	Y	0.894	0.953	0.983	0.958	0.994	0.993
505	Y	H	H	H	0.851	0.950	0.062	0.036	0.667	0.982

WT corresponds to the amino-acid in the isolate Wuhan-Hu-1 (MN908947.3) sequence. In 5th column named “var” is the aa which frequencies are shown in 1-6 for the corresponding variants or selections.

¹ Frequencies calculated from GISAID database in spikenuc1207 released by GISAID on Dec 7 2024.

² Frequencies calculated from GISAID database in spikenuc1207, deposited after 2023.

³ Frequencies as calculated from HSS of WT.

⁴ Frequencies as calculated from HSS of WT+I358F.

⁵ Frequencies as calculated from HSS of BA.1.

⁶ Frequencies as calculated from HSS of BA.2. Only frequencies >0.01 are shown. Empty field (ND) is for HSS frequencies which were <0.01.

Table S6 – Frequencies of combinations of mutations in the RBD between residues 498 and 501.

Library name	Sequence 498-QPTN-501 Occurrence (%)	Sequence 498-QPTY-501 Occurrence (%)	Sequence 498-HPTN-501 Occurrence (%)	Sequence 498-HPTY-501 Occurrence (%)	Sequence 498-HPTT/S-501 Occurrence (%)	Sequence 498-RPTY-501 Occurrence (%)	Other notable than previous > 0.01	Total sequences fully spanning the motif*
WT 7th lib LSS	0.280	14.030	4.420	0.470	56.210	15.060	('HPTS', 6.99), ('QPTT', 0.69), ('HPST', 0.31), ('RPPT', 0.24), ('HPTA', 0.17), ('QPTF', 0.12), ('HPTT', 0.1)	7865410
Alpha 7th lib LSS	1.430	38.630	11.920	0.460	6.910	36.190	('HPTS', 2.37), ('HPTA', 0.48), ('QPTT', 0.42), ('QPTF', 0.16), ('RPAV', 0.13)	12873967
Beta 7th lib LSS	0.220	2.940	0.230	0.010	0.080	93.840	('RPAV', 0.37), ('QPTF', 0.33), ('RPTD', 0.22), ('RPTF', 0.16), ('RPSV', 0.12), ('RSTY', 0.11)	20922
RBD v48 7th lib LSS	0.400	0.450	0.070	0.000	0.100	97.750	('RPAV', 0.24), ('RPSV', 0.2), ('RPTD', 0.1)	3211512
RBD BA.1 7th lib LSS	0.430	0.100	0.030	0.000	0.010	97.470	('RPAV', 0.54), ('RPSV', 0.51), ('LPTY', 0.14), ('RPTD', 0.13)	6918311
WT 4th lib HSS	0.910	0.130	0.000	0.000	0.000	96.360	('RRTY', 0.55), ('RHYY', 0.35), ('RSTY', 0.26), ('RPAV', 0.25), ('RPSV', 0.24), ('RPTN', 0.11)	749658
I358F 4th lib HSS	0.010	0.240	0.030	0.010	2.860	94.580	('YPTT', 0.39), ('RPSV', 0.18), ('RPAV', 0.18), ('RTTY', 0.17), ('LPTY', 0.15), ('RHYY', 0.13), ('RPTC', 0.12), ('RPTT', 0.11), ('RRTY', 0.11)	756757
BA.1 4th lib HSS	0.130	0.090	0.000	0.000	0.000	97.920	('RPAV', 0.37), ('RPSV', 0.36)	614899
BA.2 4th lib HSS	0.000	0.120	0.000	0.000	0.000	98.070	('RPSV', 0.34), ('RHYY', 0.32), ('RRTY', 0.16), ('RPAV', 0.14), ('RPTH', 0.14), ('RPTF', 0.13), ('RSTY', 0.12)	817816
GISAID (Spikenuc_1207)	37.720	11.580	0.000	0.000	0.000	47.990		646698

WT corresponds to the amino-acid in the isolate Wuhan-Hu-1 (MN908947.3) sequence and was used as a reference with a sequence 498-QPTN-501.

Table S7. Binding affinities and sequences of clones from Fig. 4.

Lineages	KD (nM)	339	345	346	356	358	368	371	373	375	376	378	392	405	408	417	428	440	444	445	446	450	452	459	460	462	470	477	478	483	484	486	493	496	498	501	505			
WT aa	4.36 (0.3)	G	T	R	K	I	L	S	S	S	T	K	F	D	R	K	D	N	K	V	G	N	L	S	N	K	T	S	T	V	E	F	Q	G	Q	N	Y			
WT I358F	4.24 (0.5)					F																																		
Alpha	4.4 (0.5)																																				Y			
Beta	3.15 (0.3)															N															K						Y			
BA.1	5.9 (0.3)	D					L	P	F							N		K			S							N	K		A		R	S	R	Y	H			
BA.2	7.0 (0.6)	D					F	P	F	A			N	S	N		K											N	K		A		R		R	Y	H			
XBB.1.5	1.9 (0.2)	H	T			I	F	P	F	A			N	S	N		K		P	S					K			N	K		A	P			R	Y	H			
LSS																																								
WT-c35	2.737 (0.33)					F																														G	H			
WT-cJ452	2.956 (0.23)					F																			T	K									G		Y			
WT-cJ428	2.613 (0.18)																							Q										L	G	H				
WT-c46	1.45 (0.14)																																			G	H	T		
WT-c71	2.316 (0.11)												E				K								K					A					G	H	S			
Alpha-c46	3.834 (0.34)																																			G	R	Y		
Alpha-c49	2.856 (0.4)																																			G		Y		
Alpha-c71	3.383 (0.4)																												N							G		Y		
Alpha-cJ71	3.804 (0.4)																												N							G		Y		
Beta-c56	2.9 (0.3)															N													N			K				G	R	Y		
Beta-c72	3.4 (0.3)															N		K							K							K				G	R	Y		
BA1-cJ43	4.235 (0.15)	D					L	P				I				N		K								E	N	K		A		R			R	Y	H			
BA1-cJ44	3.659 (0.12)	D					L	P				S				I		K			S							N	K		A		R	G	R	Y	H			
BA1-cJ715	4.589 (0.1)	D					L	P				L				I		K			S								N	K		A					R	Y		
BA1-c72	3.637 (0.08)	D	N				L	P								N	G	K										A	N	K		A					R	Y	H	
BA1-c73	1.973 (0.1)	A					L	P				R				I		K			S						R		N	K		A					R	Y	H	
HSS																																								
WT-c21	0.69 (0.04)																	K	R			D	M	K			N	K		R		Q	G	R	Y	H				
WT-c22	0.41(0.03)																	K	R			D	M	K			N	K		R		Q	G	R	Y					
WT-c41	0.6 (0.03)																					D	M	K			N	K		R						R	Y			
WT-c42	0.5 (0.04)																	K	R			D	M	K			N	K		R						R	Y			
WT-c43	0.49 (0.06)																	K	R			D	K	K			N	K		R		Q	G	R	Y					
WT-c44	0.44 (0.03)																	K	T			R		K			N	K		R		Q	G	R	Y					
I358F-c21	0.75 (0.06)					F												K	T			R		K			N	K						Q	G	R	Y			
I358F-c22	0.61 (0.04)					F												K	T			R		K			N	K		A	V			Q	G	R	Y			
I358F-c41	0.6 (0.07)					F												K	T			R		K			N	K		A		R			R	Y				
I358F-c42	0.44 (0.05)					F												K	T			R		K			N	K		R		R			R	Y				
I358F-c43	0.73 (0.07)					F												K	T			R		K			N	K		K						R	Y			
I358F-c44	0.5 (0.05)					F																R		K			N	K		A		R	G	R	Y					
I358F-c45	0.59 (0.06)					F												K	T			R		K			N	K		V		R	G	R	Y					
BA1-c21	1.2(0.12)	D					L	P	F							N		K	T			R		K			N	K		A		G			R	Y	H			
BA1-c41	1.7 (0.16)	D					L	P	F							N		K			S	D	M	K			N	K		R						R	Y	H		
BA1-c42	1.67 (0.17)	D					L	P	F							N		K			S	D	M	K			N	K		R							R	Y		
BA1-c43	1.68 (0.17)	D					L	P	F							N		K	A			R		K			N	K		R								R	Y	H
BA1-c44	0.96 (0.1)	D					L	P	F							N		K			S	D	M	K			N	K	I	R		Q	G	R	Y					
BA2-c21	1.1 (0.08)	D					F	P	F	A			N	S	N		K	R			D	M	K				N	K		R		Q	G	R	Y	H				
BA2-c41	0.86 (0.06)	D					F	P	F	A			N	S	N		K	R			D	K	K				N	K		R							R	Y	H	
BA2-c42	1.3 (0.16)	D					F	P	F	A			N	S	N		K	R			D	M	K				N	K		A		G	G	R	Y	H				
BA2-c43	1.1 (0.14)	D					F	P	F	A			N	S	N		K	R			D	K	K				N	K		R		Q	G	R	Y	H				

Binding affinities were calculated from at least 3 independent replicates using GraphPad v.10 and one site specific binding model (see Fig. 4 and the related source file). In parenthesis are $\pm$ 95% CI values. Mutant residues shown only for given position are relative to WT (first seq. with residues shown), as determined by Sanger sequencing of the selected clones. For HSS residues in gray are those in the stability determining region, which were not mutated.

Supporting information part PS2 – Analysis of error prone libraries for high stringency selections

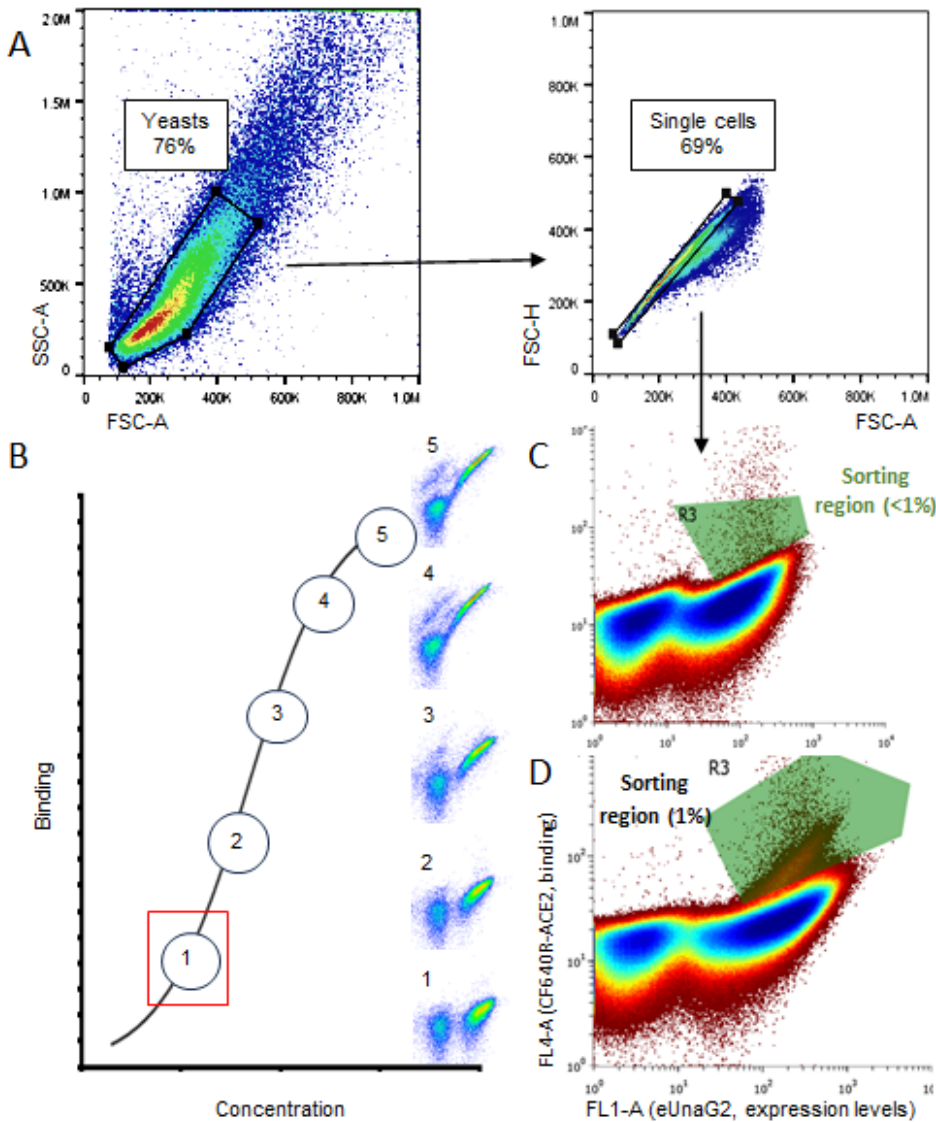


Fig. S1 – Gating and selection strategies for *in vitro* evolution of the SARS-CoV-2 RBD domain. (A) Gating strategy used during FACS sorting. Yeast cells were selected based on their forward and side scatter area parameters (FSC-A and SSC-A) and subsequently, single cells were gated using a diagonal plot of FCS-A versus height (FCS-H). (B) Sorting under different ACE-2 concentrations. The library was incubated with a range of ACE2 concentrations to identify the minimal concentration that promotes elevated signal (inset 1-3). Under these conditions, clones with stronger binding affinity had the highest competitive/signal advantage over the parental population. For LSS higher concentration conditions were used (insets 4–5). (C) Stepwise sorting strategy for HSS to enhance discrimination between improved clones and the parental population. Initially, below 1% of top signal cells were sorted in HSS. These selected cells were then cultured, expressed and subjected to a second round of sorting for higher enrichment of the enhanced binding population (D).

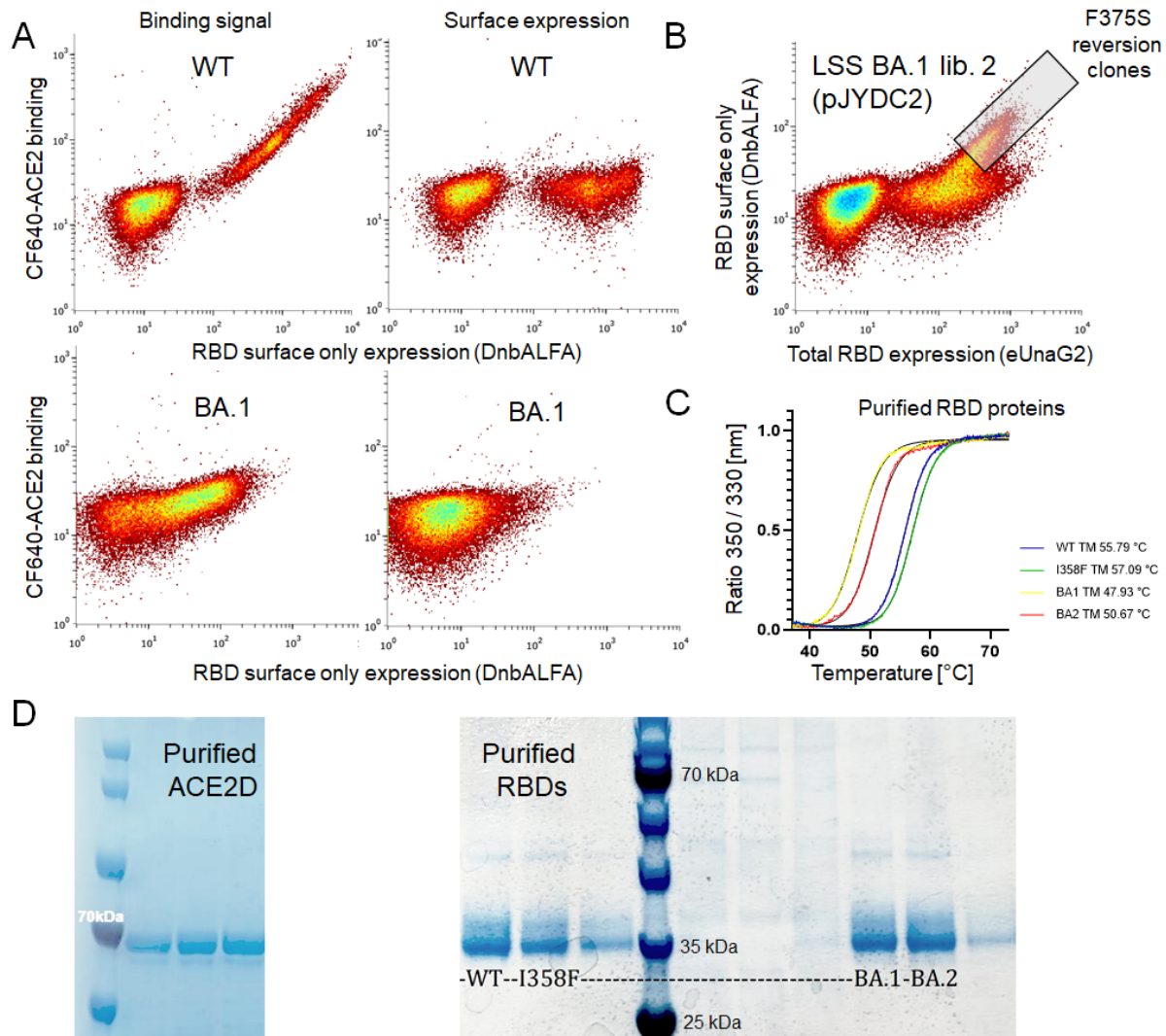


Fig. S2 – Yeast surface display and stability analysis of purified proteins. (A) Flow cytometry dot plot showing strong ACE2 binding and robust surface expression for the wild-type (WT) RBD, in contrast to markedly reduced binding and surface expression observed for the BA.1 RBD. (B) A dominant population displaying a reverted phenotype, relative to BA.1 in panel A, dominated in the population as early as the second round of selection. Surface versus total expression was assessed using the pJYDC2 plasmid. Sequencing identified the F375S reversion as responsible for the restored phenotype. (C) Tycho NT.6 (NanoTemper) thermal unfolding profiles of purified WT, WT+I358F, BA.1, and BA.2 RBDs, demonstrating substantial destabilization of both Omicron lineages relative to WT. (D) SDS-PAGE analysis of purified ACE2D and RBDs proteins with marker (BlueRay Prestained Protein Marker). Uncropped gels are provided in file: Source data for Fig. S2 after Supplementary Data Tables and Figures (this document).

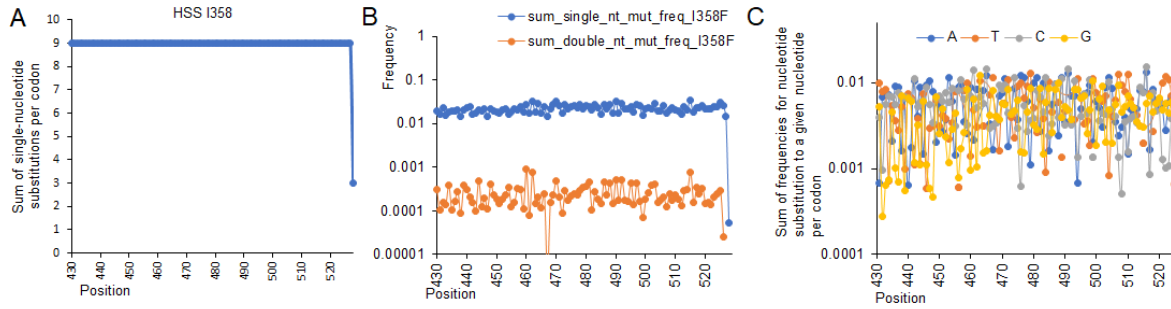


Fig. S3 – WT+I358F library quality assessment. (A) The number of observed single nucleotide substitutions per codon along the RBM, with 9 representing all mutations in all three positions. (B) Frequencies for all single- and double-nucleotide substitutions per codon, showing minimal positional deviations. (C) Sum of single-nucleotide mutation frequencies per mutant nucleotide and codon.

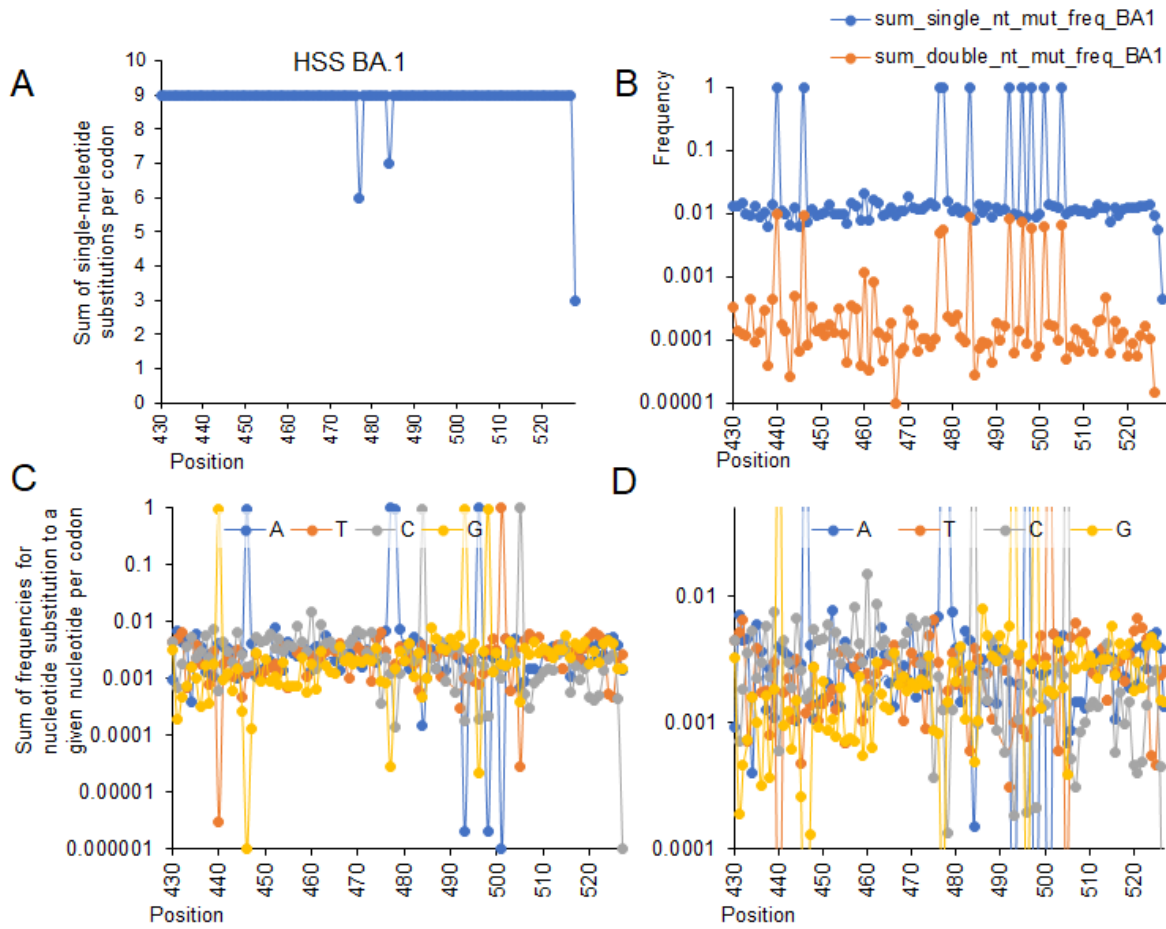


Fig. S4 – BA.1 library quality assessment. (A) The number of observed single nucleotide substitutions per codon along the RBM, with 9 representing all mutations in all three positions. (B) Frequencies for all single- and double-nucleotide substitutions per codon, showing minimal positional deviations. Note that the high frequencies in certain positions are of the BA.1 mutations. WT sequence was used as a reference. (C) Sum of single-nucleotide mutation frequencies per mutant nucleotide and codon with WT reference sequence. (D) Sum of single-nucleotide mutation frequencies per mutant nucleotide and codon – detail for frequencies from 0.001 to 0.01.

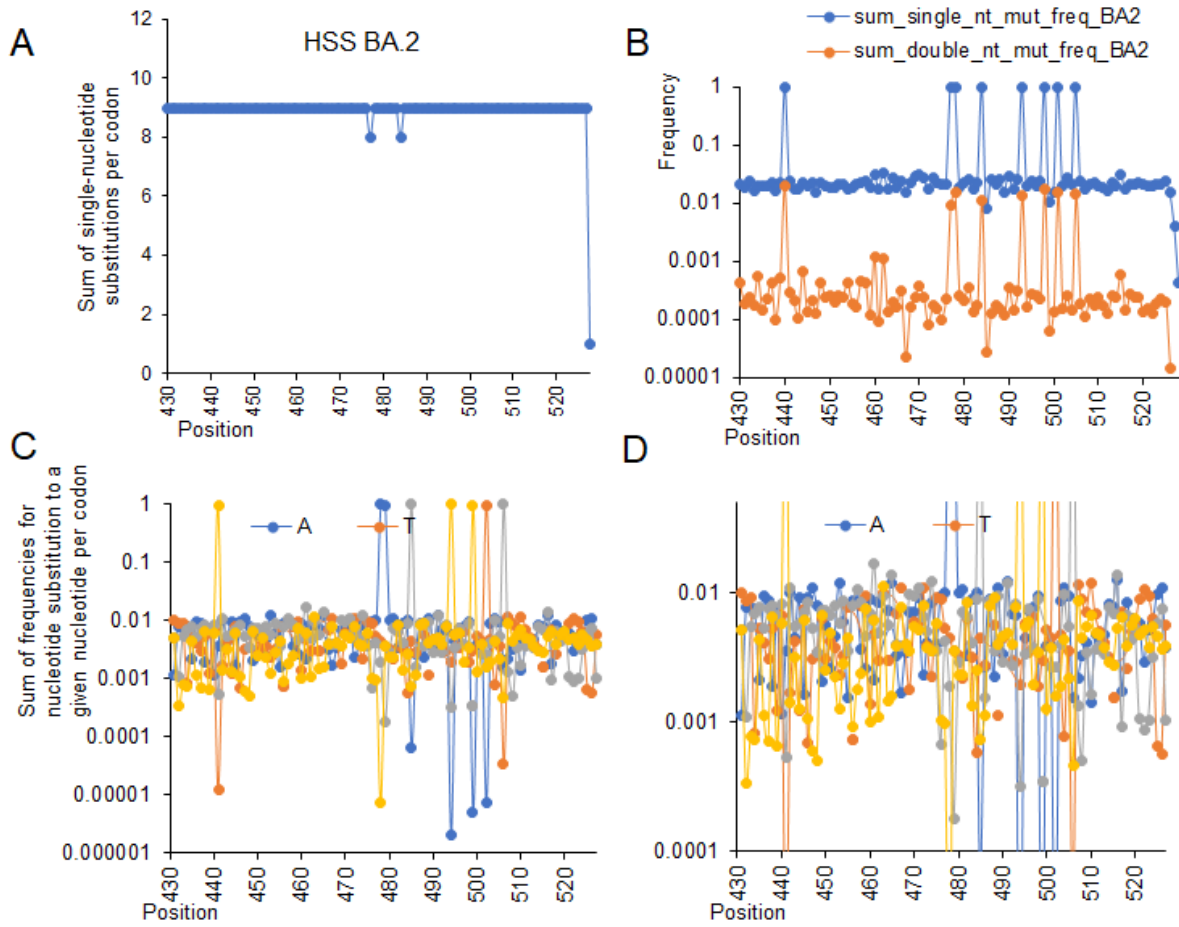


Fig. S5 – BA.2 library quality assessment. (A) The number of observed single nucleotide substitutions per codon along the RBM, with 9 representing all mutations in all three positions. (B) Frequencies for all single- and double-nucleotide substitutions per codon, showing minimal positional deviations. Note that the high frequencies in certain positions are of the BA.2 mutations. WT sequence was used as reference. (C) Sum of single-nucleotide mutation frequencies per mutant nucleotide and codon with WT reference sequence. (D) Sum of single-nucleotide mutation frequencies per mutant nucleotide and codon – detail for frequencies from 0.001 to 0.01.

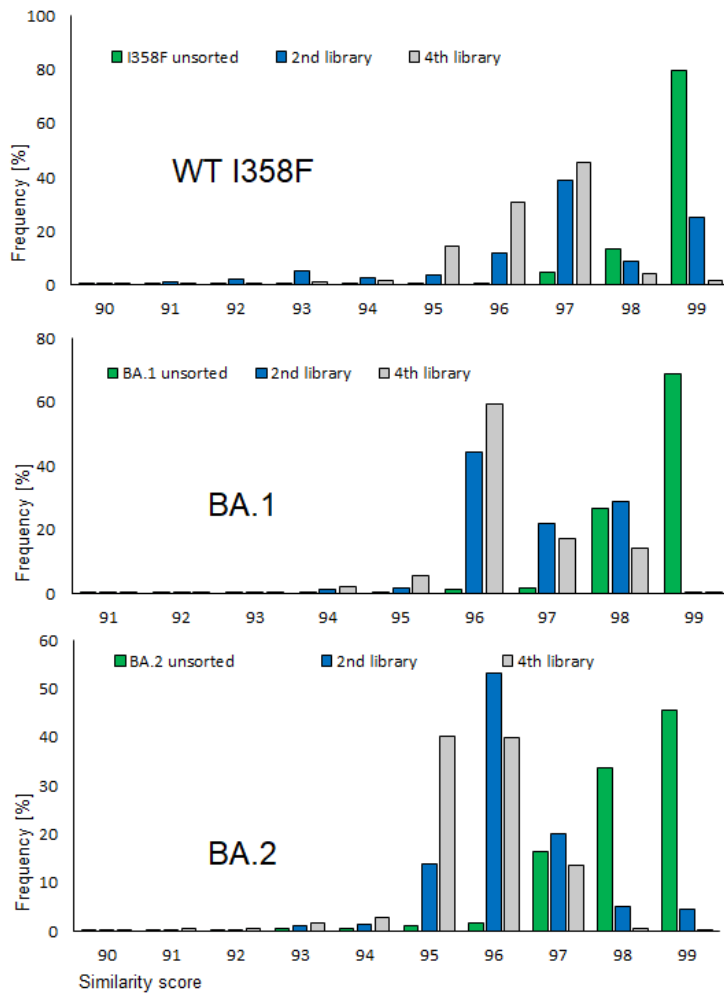


Fig. S6 – Comparison of similarity scores for individual sequencing reads in HSS libraries. Merged paired-end reads into a single sequence with WT, BA.1 and BA.2 sequences as the reference for the unsorted initial library. Populations after sorting in the 2nd and 4th rounds of the *in vitro* evolution are shown. The sequenced regions and corresponding merged paired-end read length for individual libraries differ, and therefore, similarity scores cannot be compared across libraries. More detailed distributions are shown in Fig. S7.

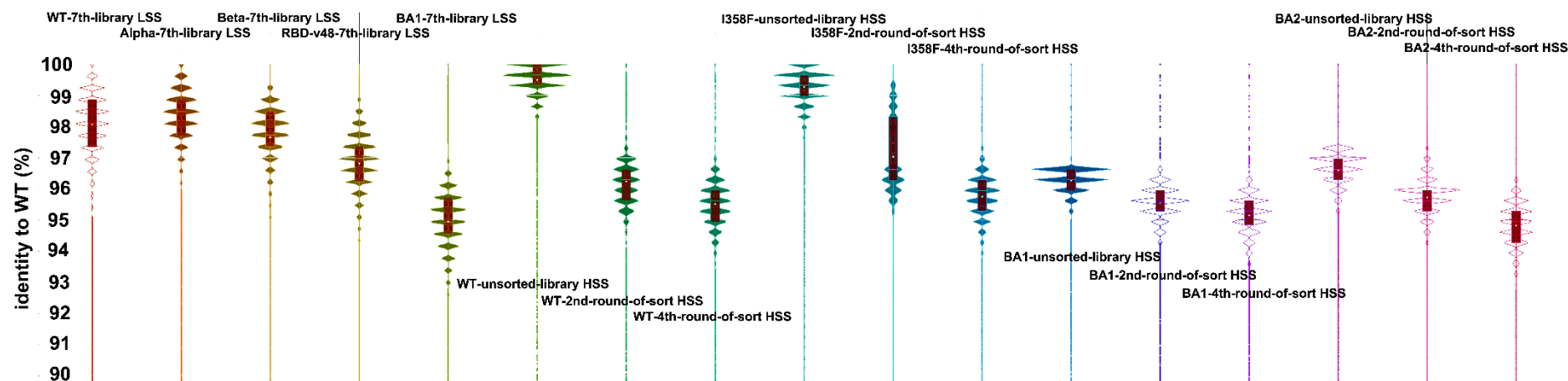


Fig. S7 – Distribution of nucleotide sequence identities among libraries. Sequences were aligned to Wuhan-Hu-1 sequence reference. The individual violin plots combined with Whisker's box plots show detailed distribution of sequence identities in each sample dataset. Notably, a white spot in the middle represents mean value. The bottom part of the violin with lower bounds has been cut away for clarity, considered uninformative. The figure shows decreasing sequence similarity with additional PCR-mutagenesis steps followed by functional selection for ACE2 receptor binding. The mutation load in 7th LSS BA.1 library closely matches with the one observed for 4th HSS BA.1. The sequence identities to the Wuhan S protein gene at the DNA-level of the RBM amplicons were obtained by BLASTN v2.17.0+ and the violin plots were rendered using ggViolin function from the ggiraphExtra v0.3.0 R package.

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WT	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	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330	331	332	333	334	335	336	337	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360	361	362	363	364	365	366	367	368	369	370	371	372	373	374	375	376	377	378	379	380	381	382	383	384	385	386	387	388	389	390	391	392	393	394	395	396	397	398	399	400	401	402	403	404	405	406	407	408	409	410	411	412	413	414	415	416	417	418	419	420	421	422	423	424	425	426	427	428	429	430	431	432	433	434	435	436	437	438	439	440	441	442	443	444	445	446	447	448	449	450	451	452	453	454	455	456	457	458	459	460	461	462	463	464	465	466	467	468	469	470	471	472	473	474	475	476	477	478	479	480	481	482	483	484	485	486	487	488	489	490	491	492	493	494	495	496	497	498	499	500	501	502	503	504	505	506	507	508	509	510	511	512	513	514	515	516	517	518	519	520	521	522	523	524	525	526	527	528	529	530	531	532	533	534	535	536	537	538	539	540	541	542	543	544	545	546	547	548	549	550	551	552	553	554	555	556	557	558	559	560	561	562	563	564	565	566	567	568	569	570	571	572	573	574	575	576	577	578	579	580	581	582	583	584	585	586	587	588	589	590	591	592	593	594	595	596	597	598	599	600	601	602	603	604	605	606	607	608	609	610	611	612	613	614	615	616	617	618	619	620	621	622	623	624	625	626	627	628	629	630	631	632	633	634	635	636	637	638	639	640	641	642	643	644	645	646	647	648	649	650	651	652	653	654	655	656	657	658	659	660	661	662	663	664	665	666	667	668	669	670	671	672	673	674	675	676	677	678	679	680	681	682	683	684	685	686	687	688	689	690	691	692	693	694	695	696	697	698	699	700	701	702	703	704	705	706	707	708	709	710	711	712	713	714	715	716	717	718	719	720	721	722	723	724	725	726	727	728	729	730	731	732	733	734	735	736	737	738	739	740	741	742	743	744	745	746	747	748	749	750	751	752	753	754	755	756	757	758	759	760	761	762	763	764	765	766	767	768	769	770	771	772	773	774	775	776	777	778	779	780	781	782	783	784	785	786	787	788	789	790	791	792	793	794	795	796	797	798	799	800	801	802	803	804	805	806	807	808	809	810	811	812	813	814	815	816	817	818	819	820	821	822	823	824	825	826	827	828	829	830	831	832	833	834	835	836	837	838	839	840	841	842	843	844	845	846	847	848	849	850	851	852	853	854	855	856	857	858	859	860	861	862	863	864	865	866	867	868	869	870	871	872	873	874	875	876	877	878	879	880	881	882	883	884	885	886	887	888	889	890	891	892	893	894	895	896	897	898	899	900	901	902	903	904	905	906	907	908	909	910	911	912	913	914	915	916	917	918	919	920	921	922	923	924	925	926	927	928	929	930	931	932	933	934	935	936	937	938	939	940	941	942	943	944	945	946	947	948	949	950	951	952	953	954	955	956	957	958	959	960	961	962	963	964	965	966	967	968	969	970	971	972	973	974	975	976	977	978	979	980	981	982	983	984	985	986	987	988	989	990	991	992	993	994	995	996	997	998	999	1000
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Fig. S8 – Translated Sanger sequencing results for selected clones for WT LSS libraries 1 to 7. Residues identical to the WT sequence are shown in blue, whereas mutated amino acids are indicated in white.

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Fig. S9 – Translated Sanger sequencing results for selected clones for Alpha LSS libraries 1 to 7. Residues identical to the WT sequence are shown in blue, whereas mutated amino acids are indicated in white. Characteristic residue for the Alpha variant relative to the WT RBD is highlighted in yellow (N501Y mutation).

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Fig. S10 – Translated Sanger sequencing results for selected clones for Beta LSS libraries 1 to 7. Residues identical to the WT sequence are shown in blue, whereas mutated amino acids are indicated in white. Characteristic Beta variant residues relative to the WT RBD are highlighted in yellow (K417N, E484K, N501Y mutations).

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Fig. S11 – Translated Sanger sequencing results for selected clones for RBD-v48 LSS libraries 1 to 7. Residues identical to the WT sequence are shown in blue, whereas mutated amino acids are indicated in white. Characteristic RBD-v48 variant residues relative to the WT RBD are highlighted in yellow (K417N, E484K, Q498R, N501Y mutations).

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CPDEVNATRFASVYVWNNKRISNCVDYSVLYLAPFTFKCYGVSPSTKLNLCFTNNVADS FVIRGDEVRIQAPGGTGADYNNKLPPDFTGCVIAWNSLDSKVGNNYNYLYLFRKSNLKPFFERDSTEIYQAGNKPNGVAGNCFYFPLSSPTTGVGQPPRRVSVLSFELLHAPATVCGPK

CPDEVNATRFASVYVWNNKRISNCVDYSVLYLAPFTFKCYGVSPSTKLNLCFTNNVADS FVIRGDEVRIQAPGGTGADYNNKLPPDFTGCVIAWNSLDSKVGNNYLYLFRKSNLKPFFERDSTEIYQAGNKPNGVAGNCFYFPLSSPTTGVGQPPRRVSVLSFELLHAPATVCGPK

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CPDEVNATRFASVYVWNNKRISNCVDYSVLYLAPFTFKCYGVSPSTKLNLCFTNNVADS FVIRGDEVRIQAPGGTGADYNNKLPPDFTGCVIAWNSLDSKVGNNYLYLFRKSNLKPFFERDSTEIYQAGNKPNGVAGNCFYFPLSSPTTGVGQPPRRVSVLSFELLHAPATVCGPK

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CPDEVNATRFASVYVWNNKRISNCVDYSVLYLAPFTFKCYGVSPSTKLNLCFTNNVADS FVIRGDEVRIQAPGGTGADYNNKLPPDFTGCVIAWNSLDSKVGNNYLYLFRKSNLKPFFERDSTEIYQAGNKPNGVAGNCFYFPLSSPTTGVGQPPRRVSVLSFELLHAPATVCGPK

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CPDEVNATRFASVYVWNNKRISNCVDYSVLYLAPFTFKCYGVSPSTKLNLCFTNNVADS FVIRGDEVRIQAPGGTGADYNNKLPPDFTGCVIAWNSLDSKVGNNYLYLFRKSNLKPFFERDSTEIYQAGNKPNGVAGNCFYFPLSSPTTGVGQPPRRVSVLSFELLHAPATVCGPK

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CPDEVNATRFASVYVWNNKRISNCVDYSVLYLAPFTFKCYGVSPSTKLNLCFTNNVADS FVIRGDEVRIQAPGGTGADYNNKLPPDFTGCVIAWNSLDSKVGNNYLYLFRKSNLKPFFERDSTEIYQAGNKPNGVAGNCFYFPLSSPTTGVGQPPRRVSVLSFELLHAPATVCGPK

CPDEVNATRFASVYVWNNKRISNCVDYSVLYLAPFTFKCYGVSPSTKLNLCFTNNVADS FVIRGDEVRIQAPGGTGADYNNKLPPDFTGCVIAWNSLDSKVGNNYLYLFRKSNLKPFFERDSTEIYQAGNKPNGVAGNCFYFPLSSPTTGVGQPPRRVSVLSFELLHAPATVCGPK

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CPDEVNATRFASVYVWNNKRISNCVDYSVLYLAPFTFKCYGVSPSTKLNLCFTNNVADS FVIRGDEVRIQAPGGTGADYNNKLPPDFTGCVIAWNSLDSKVGNNYLYLFRKSNLKPFFERDSTEIYQAGNKPNGVAGNCFYFPLSSPTTGVGQPPRRVSVLSFELLHAPATVCGPK

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BA.1 library	residue	WT
	382	CPTGEVFNATRFASVYAWNRKRIS
	383	ISNCVADYSVLYNKA
	384	AFSTFKCYGVSP
	385	PTKLN
	386	DLCT
	387	FTNVYADSV
	388	FVIRGDEV
	389	VRQIAPGQT
	390	GN
	391	IADYNYKLP
	392	DDFTGCV
	393	IAWNSN
	394	KLDSK
	395	VGGNYN
	396	LYRLFR
	397	KSNL
	398	KPFERD
	399	ISTE
	400	IYQAGN
	401	PCNGVAG
	402	FNCYF
	403	FLQSY
	404	SRPT
	405	YGVGHQ
	406	PPRVVLS
	407	FELL
	408	LHAPAT
	409	VCGRK
	410	CPTEVFNATRFASVYAWNRKRIS
	411	ISNCVADYSVLYNKA
	412	AFSTFKCYGVSP
	413	PTKLN
	414	DLCT
	415	FTNVYADSV
	416	FVIRGDEV
	417	VRQIAPGQT
	418	GN
	419	IADYNYKLP
	420	DDFTGCV
	421	IAWNSN
	422	KLDSK
	423	VGGNYN
	424	LYRLFR
	425	KSNL
	426	KPFERD
	427	ISTE
	428	IYQAGN
	429	PCNGVAG
	430	FNCYF
	431	FLQSY
	432	SRPT
	433	YGVGHQ
	434	PPRVVLS
	435	FELL
	436	LHAPAT
	437	VCGRK
	438	CPTEVFNATRFASVYAWNRKRIS
	439	ISNCVADYSVLYNKA
	440	AFSTFKCYGVSP
	441	PTKLN
	442	DLCT
	443	FTNVYADSV
	444	FVIRGDEV
	445	VRQIAPGQT
	446	GN
	447	IADYNYKLP
	448	DDFTGCV
	449	IAWNSN
	450	KLDSK
	451	VGGNYN
	452	LYRLFR
	453	KSNL
	454	KPFERD
	455	ISTE
	456	IYQAGN
	457	PCNGVAG
	458	FNCYF
	459	FLQSY
	460	SRPT
	461	YGVGHQ
	462	PPRVVLS
	463	FELL
	464	LHAPAT
	465	VCGRK
	466	CPTEVFNATRFASVYAWNRKRIS
	467	ISNCVADYSVLYNKA
	468	AFSTFKCYGVSP
	469	PTKLN
	470	DLCT
	471	FTNVYADSV
	472	FVIRGDEV
	473	VRQIAPGQT
	474	GN
	475	IADYNYKLP
	476	DDFTGCV
	477	IAWNSN
	478	KLDSK
	479	VGGNYN
	480	LYRLFR
	481	KSNL
	482	KPFERD
	483	ISTE
	484	IYQAGN
	485	PCNGVAG
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	487	FLQSY
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	817	KSNL
	818	KPFERD
	819	ISTE
	820	IYQAGN
	821	PCNGVAG
	822	FNCYF
	823	FLQSY
	824	SRPT
	825	YGVGHQ
	826	PPRVVLS
	827	FELL
	828	LHAPAT
	829	VCGRK
	830	CPTEVFNATRFASVYAWNRKRIS
	831	ISNCVADYSVLYNKA
	832	AFSTFKCYGVSP
	833	PTKLN
	834	DLCT
	835	FTNVYADSV
	836	FVIRGDEV
	837	

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Lib	98A	WT	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	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330	331	332	333	334	335	336	337	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360	361	362	363	364	365	366	367	368	369	370	371	372	373	374	375	376	377	378	379	380	381	382	383	384	385	386	387	388	389	390	391	392	393	394	395	396	397	398	399	400	401	402	403	404	405	406	407	408	409	410	411	412	413	414	415	416	417	418	419	420	421	422	423	424	425	426	427	428	429	430	431	432	433	434	435	436	437	438	439	440	441	442	443	444	445	446	447	448	449	450	451	452	453	454	455	456	457	458	459	460	461	462	463	464	465	466	467	468	469	470	471	472	473	474	475	476	477	478	479	480	481	482	483	484	485	486	487	488	489	490	491	492	493	494	495	496	497	498	499	500	501	502	503	504	505	506	507	508	509	510	511	512	513	514	515	516	517	518	519	520	521	522	523	524	525	526	527	528	529	530	531	532	533	534	535	536	537	538	539	540	541	542	543	544	545	546	547	548	549	550	551	552	553	554	555	556	557	558	559	560	561	562	563	564	565	566	567	568	569	570	571	572	573	574	575	576	577	578	579	580	581	582	583	584	585	586	587	588	589	590	591	592	593	594	595	596	597	598	599	600	601	602	603	604	605	606	607	608	609	610	611	612	613	614	615	616	617	618	619	620	621	622	623	624	625	626	627	628	629	630	631	632	633	634	635	636	637	638	639	640	641	642	643	644	645	646	647	648	649	650	651	652	653	654	655	656	657	658	659	660	661	662	663	664	665	666	667	668	669	670	671	672	673	674	675	676	677	678	679	680	681	682	683	684	685	686	687	688	689	690	691	692	693	694	695	696	697	698	699	700	701	702	703	704	705	706	707	708	709	710	711	712	713	714	715	716	717	718	719	720	721	722	723	724	725	726	727	728	729	730	731	732	733	734	735	736	737	738	739	740	741	742	743	744	745	746	747	748	749	750	751	752	753	754	755	756	757	758	759	760	761	762	763	764	765	766	767	768	769	770	771	772	773	774	775	776	777	778	779	780	781	782	783	784	785	786	787	788	789	790	791	792	793	794	795	796	797	798	799	800	801	802	803	804	805	806	807	808	809	810	811	812	813	814	815	816	817	818	819	820	821	822	823	824	825	826	827	828	829	830	831	832	833	834	835	836	837	838	839	840	841	842	843	844	845	846	847	848	849	850	851	852	853	854	855	856	857	858	859	860	861	862	863	864	865	866	867	868	869	870	871	872	873	874	875	876	877	878	879	880	881	882	883	884	885	886	887	888	889	890	891	892	893	894	895	896	897	898	899	900	901	902	903	904	905	906	907	908	909	910	911	912	913	914	915	916	917	918	919	920	921	922	923	924	925	926	927	928	929	930	931	932	933	934	935	936	937	938	939	940	941	942	943	944	945	946	947	948	949	950	951	952	953	954	955	956	957	958	959	960	961	962	963	964	965	966	967	968	969	970	971	972	973	974	975	976	977	978	979	980	981	982	983	984	985	986	987	988	989	990	991	992	993	994	995	996	997	998	999	1000	1001	1002	1003	1004	1005	1006	1007	1008	1009	1010	1011	1012	1013	1014	1015	1016	1017	1018	1019	1020	1021	1022	1023	1024	1025	1026	1027	1028	1029	1030	1031	1032	1033	1034	1035	1036	1037	1038	1039	1040	1041	1042	1043	1044	1045	1046	1047	1048	1049	1050	1051	1052	1053	1054	1055	1056	1057	1058	1059	1060	1061	1062	1063	1064	1065	1066	1067	1068	1069	1070	1071	1072	1073	1074	1075	1076	1077	1078	1079	1080	1081	1082	1083	1084	1085	1086	1087	1088	1089	1090	1091	1092	1093	1094	1095	1096	1097	1098	1099	1100	1101	1102	1103	1104	1105	1106	1107	1108	1109	1110	1111	1112	1113	1114	1115	1116	1117	1118	1119	1120	1121	1122	1123	1124	1125	1126	1127	1128	1129	1130	1131	1132	1133	1134	1135	1136	1137	1138	1139	1140	1141	1142	1143	1144	1145	1146	1147	1148	1149	1150	1151	1152	1153	1154	1155	1156	1157	1158	1159	1160	1161	1162	1163	1164	1165	1166	1167	1168	1169	1170	1171	1172	1173	1174	1175	1176	1177	1178	1179	1180	1181	1182	1183	1184	1185	1186	1187	1188	1189	1190	1191	1192	1193	1194	1195	1196	1197	1198	1199	1200	1201	1202	1203	1204	1205	1206	1207	1208	1209	1210	1211	1212	1213	1214	1215	1216	1217	1218	1219	1220	1221	1222	1223	1224	1225	1226	1227	1228	1229	1230	1231	1232	1233	1234	1235	1236	1237	1238	1239	1240	1241	1242	1243	1244	1245	1246	1247	1248	1249	1250	1251	1252	1253	1254	1255	1256	1257	1258	1259	1260	1261	1262	1263	1264	1265	1266	1267	1268	1269	1270	1271	1272	1273	1274	1275	1276	1277	1278	1279	1280	1281	1282	1283	1284	1285	1286	1287	1288	1289	1290	1291	1292	1293	1294	1295	1296	1297	1298	1299	1300	1301	1302	1303	1304	1305	1306	1307	1308	1309	1310	1311	1312	1313	1314	1315	1316	1317	1318	1319	1320	1321	1322	1323	1324	1325	1326	1327	1328	1329	1330	1331	1332	1333	1334	1335	1336	1337	1338	1339	1340	1341	1342	1343	1344	1345	1346	1347	1348	1349	1350	1351	1352	1353	1354	1355	1356	1357	1358	1359	1360	1361	1362	1363	1364	1365	1366	1367	1368	1369	1370	1371	1372	1373	1374	1375	1376	1377	1378	1379	1380	1381	1382	1383	1384	1385	1386	1387	1388	1389	1390	1391	1392	1393	1394	1395	1396	1397	1398	1399	1400	1401	1402	1403	1404	1405	1406	1407	1408	1409	1410	1411	1412	1413	1414	1415	1416	1417	1418	1419	1420	1421	1422	1423	1424	1425	1426	1427	1428	1429	1430	1431	1432	1433	1434	1435	1436	1437	1438	1439	1440	1441	1442	1443	1444	1445	1446	1447	1448	1449	1450	1451	1452	1453	1454	1455	1456	1457	1458	1459	1460	1461	1462	1463	1464	1465	1466	1467	1468	1469	1470	1471	1472	1473	1474	1475	1476	1477	1478	1479	1480	1481	1482	1483	1484	148
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Shoshany *et al.* (2026) Stringent Selection Drives Convergence Toward Omicron-like SARS-CoV-2 Receptor-Binding Motifs

res. Number.	430	431	432	433	434	435	436	437	438	439	440	441	442	443	444	445	446	447	448	449	450	451	452	453	454	455	456	457	458	459	460	461	462	463	464	465	466	467	468	469	470	471	472	473	474	475	476	477	478	479	480	481	482	483	484	485	486	487	488	489	490	491	492	493	494	495	496	497	498	499	500	501	502	503	504	505	506	507	508	509	510	511	512	513	514	515	516	517	518	519	520	521	522	523	524	525	526	527	528
WT	T	G	C	V	I	A	W	N	S	N	L	D	S	K	V	G	G	N	Y	L	R	L	R	F	K	S	N	L	K	P	F	E	R	D	I	S	T	E	I	Y	Q	A	G	S	T	P	C	N	G	V	G	F	N	C	Y	F	F	L	Q	S	Y	G	F	A	P	T	N	G	V	G	O	P	Y	R	V	V	L	S	F	E	L	L	H	A	P	A	T	V	C	G	P	K							
WT	T	G	C	V	I	A	W	N	S	N	L	D	S	K	V	G	G	N	Y	L	R	L	R	F	K	S	N	L	K	P	F	E	R	D	I	S	T	E	I	Y	Q	A	G	S	T	P	C	N	G	V	G	F	N	C	Y	F	F	L	Q	S	Y	G	F	A	P	T	N	G	V	G	O	P	Y	R	V	V	L	S	F	E	L	L	H	A	P	A	T	V	C	G	P	K							
Alpha	T	G	C	V	I	A	W	N	S	N	L	D	S	K	V	G	G	N	Y	L	R	L	R	F	K	S	N	L	K	P	F	E	R	D	I	S	T	E	I	Y	Q	A	G	S	T	P	C	N	G	V	G	F	N	C	Y	F	F	L	Q	S	Y	G	F	A	P	T	N	G	V	G	O	P	Y	R	V	V	L	S	F	E	L	L	H	A	P	A	T	V	C	G	P	K							
WT	T	G	C	V	I	A	W	N	S	N	L	D	S	K	V	G	G	N	Y	L	R	L	R	F	K	S	N	L	K	P	F	E	R	D	I	S	T	E	I	Y	Q	A	G	S	T	P	C	N	G	V	G	F	N	C	Y	F	F	L	Q	S	Y	G	F	A	P	T	N	G	V	G	O	P	Y	R	V	V	L	S	F	E	L	L	H	A	P	A	T	V	C	G	P	K							
WT	T	G	C	V	I	A	W	N	S	N	L	D	S	K	V	G	G	N	Y	L	R	L	R	F	K	S	N	L	K	P	F	E	R	D	I	S	T	E	I	Y	Q	A	G	S	T	P	C	N	G	V	G	F	N	C	Y	F	F	L	Q	S	Y	G	F	A	P	T	N	G	V	G	O	P	Y	R	V	V	L	S	F	E	L	L	H	A	P	A	T	V	C	G	P	K							
WT	T	G	C	V	I	A	W	N	S	N	L	D	S	K	V	G	G	N	Y	L	R	L	R	F	K	S	N	L	K	P	F	E	R	D	I	S	T	E	I	Y	Q	A	G	S	T	P	C	N	G	V	G	F	N	C	Y	F	F	L	Q	S	Y	G	F	A	P	T	N	G	V	G	O	P	Y	R	V	V	L	S	F	E	L	L	H	A	P	A	T	V	C	G	P	K							
WT	T	G	C	V	I	A	W	N	S	N	L	D	S	K	V	G	G	N	Y	L	R	L	R	F	K	S	N	L	K	P	F	E	R	D	I	S	T	E	I	Y	Q	A	G	S	T	P	C	N	G	V	G	F	N	C	Y	F	F	L	Q	S	Y	G	F	A	P	T	N	G	V	G	O	P	Y	R	V	V	L	S	F	E	L	L	H	A	P	A	T	V	C	G	P	K							
WT	T	G	C	V	I	A	W	N	S	N	L	D	S	K	V	G	G	N	Y	L	R	L	R	F	K	S	N	L	K	P	F	E	R	D	I	S	T	E	I	Y	Q	A	G	S	T	P	C	N	G	V	G	F	N	C	Y	F	F	L	Q	S	Y	G	F	A	P	T	N	G	V	G	O	P	Y	R	V	V	L	S	F	E	L	L	H	A	P	A	T	V	C	G	P	K							
WT	T	G	C	V	I	A	W	N	S	N	L	D	S	K	V	G	G	N	Y	L	R	L	R	F	K	S	N	L	K	P	F	E	R	D	I	S	T	E	I	Y	Q	A	G	S	T	P	C	N	G	V	G	F	N	C	Y	F	F	L	Q	S	Y	G	F	A	P	T	N	G	V	G	O	P	Y	R	V	V	L	S	F	E	L	L	H	A	P	A	T	V	C	G	P	K							
WT	T	G	C	V	I	A	W	N	S	N	L	D	S	K	V	G	G	N	Y	L	R	L	R	F	K	S	N	L	K	P	F	E	R	D	I	S	T	E	I	Y	Q	A	G	S	T	P	C	N	G	V	G	F	N	C	Y	F	F	L	Q	S	Y	G	F	A	P	T	N	G	V	G	O	P	Y	R	V	V	L	S	F	E	L	L	H	A	P	A	T	V	C	G	P	K							
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WT	T	G	C	V	I	A	W	N	S	N	L	D	S	K	V	G	G	N	Y	L	R	L	R	F	K	S	N	L	K	P	F	E	R	D	I	S	T	E	I	Y	Q	A	G	S	T	P	C	N	G	V	G	F	N	C	Y	F	F	L	Q	S	Y	G	F	A	P	T	N	G	V	G	O	P	Y	R	V	V	L	S	F	E	L	L	H	A	P	A	T	V	C	G	P	K							
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WT	T	G	C	V	I	A	W	N	S	N	L	D	S	K	V	G	G	N	Y	L	R	L	R	F	K	S	N	L	K	P	F	E	R	D	I	S	T	E	I	Y	Q	A	G	S	T	P	C	N	G	V	G	F	N	C	Y	F	F	L	Q	S	Y	G	F	A	P	T	N	G	V	G	O	P	Y	R	V	V	L	S	F	E	L	L	H	A	P	A	T	V	C	G	P	K							
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WT	T	G	C	V	I	A	W	N	S	N	L	D	S	K	V	G	G	N	Y	L	R	L	R	F	K	S	N	L	K	P	F	E	R	D	I	S	T	E	I	Y	Q	A	G	S	T	P	C	N	G	V	G	F	N	C	Y	F	F	L	Q	S	Y	G	F	A	P	T	N	G	V	G	O	P	Y	R	V	V	L	S	F	E	L	L	H	A	P	A	T	V	C	G	P	K							
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[illegible]

Fig. S14 – Multiple sequence alignment of selected 30 highest-frequency clones identified by Illumina sequencing from the LSS libraries (WT, Alpha, Beta, RBD v48, BA.1). Positions identical to wild-type (WT) are indicated by a dot, while amino acid substitutions are shown explicitly. The WT, specific library starting sequence (Alpha, Beta, RBD v48), and BA.1 reference sequences are shown in the header. Residues corresponding to WT are highlighted in blue, while residues carrying mutations characteristic for a given variant are highlighted in yellow. The number displayed to the left of each sequence indicates the absolute number of occurrences of that identical clone observed in the dataset.

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	L	DS	KV	GG	NY	NY	L	Y	L	R	L	R	K	S	N	L	K	P	F	E	R	D	S	T	E	I	Y	Q	A	G	N

Fig. S15 – Multiple sequence alignment of selected 30 highest-frequency clones identified by Illumina sequencing from the WT HSS libraries (unsorted, round 2 and round 4). Positions identical to wild-type (WT) are indicated by a dot, while amino acid substitutions are shown explicitly. The WT, BA.1, and BA.2 reference sequences are shown in the header. Residues corresponding to WT are highlighted in blue, while residues carrying mutations characteristic for a given variant are highlighted in yellow. The number displayed to the left of each sequence indicates the absolute number of occurrences of that identical clone observed in the dataset.

Shoshany *et al.* (2026) Stringent Selection Drives Convergence Toward Omicron-like SARS-CoV-2 Receptor-Binding Motifs

[illegible]

Fig. S16 – Multiple sequence alignment of selected 30 highest-frequency clones identified by Illumina sequencing from the I358F HSS libraries (unsorted, round 2 and round 4). Positions identical to wild-type (WT) are indicated by a dot, while amino acid substitutions are shown explicitly. The WT, BA.1, and BA.2 reference sequences are shown in the header. Residues corresponding to WT are highlighted in blue, while residues carrying mutations characteristic for a given variant are highlighted in yellow. The number displayed to the left of each sequence indicates the absolute number of occurrences of that identical clone observed in the dataset.

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[illegible]

Fig. S17 – Multiple sequence alignment of selected 30 highest-frequency clones identified by Illumina sequencing from the BA.1 HSS libraries (unsorted, round 2 and round 4). Positions identical to wild-type (WT) are indicated by a dot, while amino acid substitutions are shown explicitly. The WT, BA.1, and BA.2 reference sequences are shown in the header. Residues corresponding to WT are highlighted in blue, while residues carrying mutations characteristic for a given variant are highlighted in yellow. The number displayed to the left of each sequence indicates the absolute number of occurrences of that identical clone observed in the dataset.

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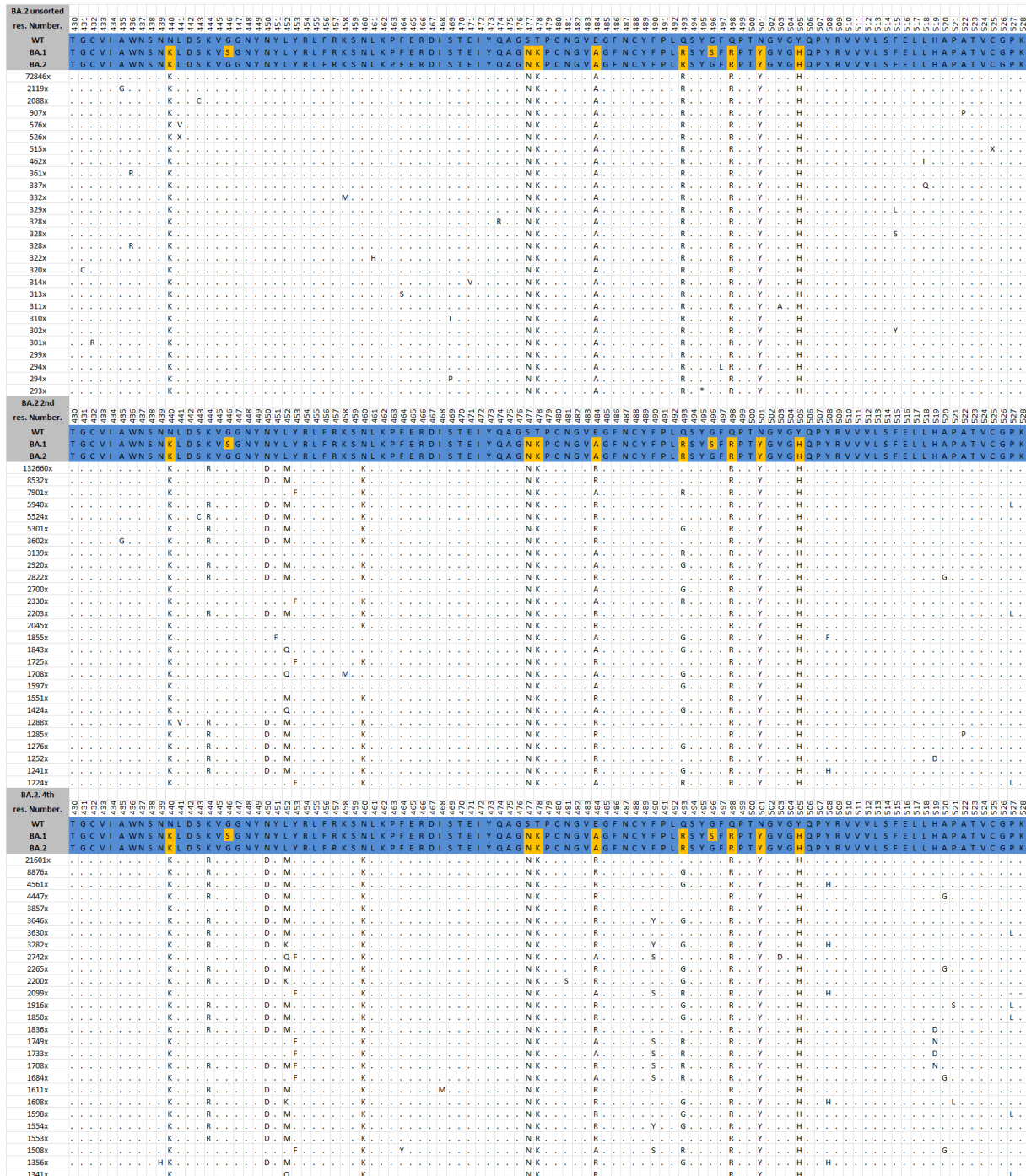
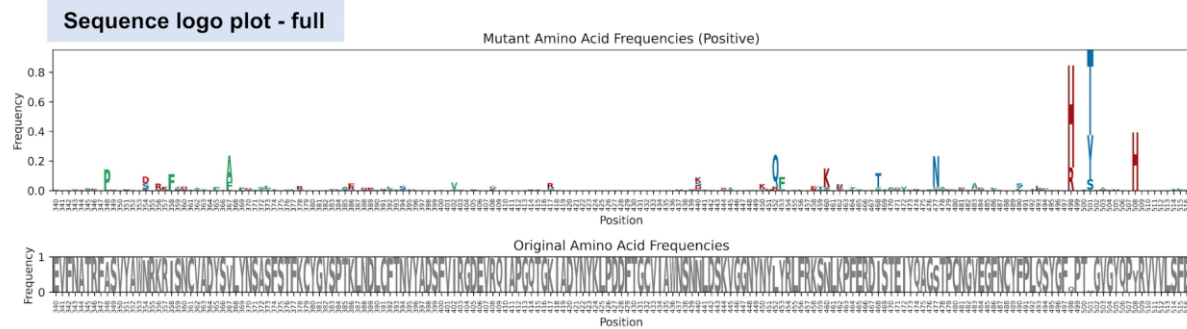


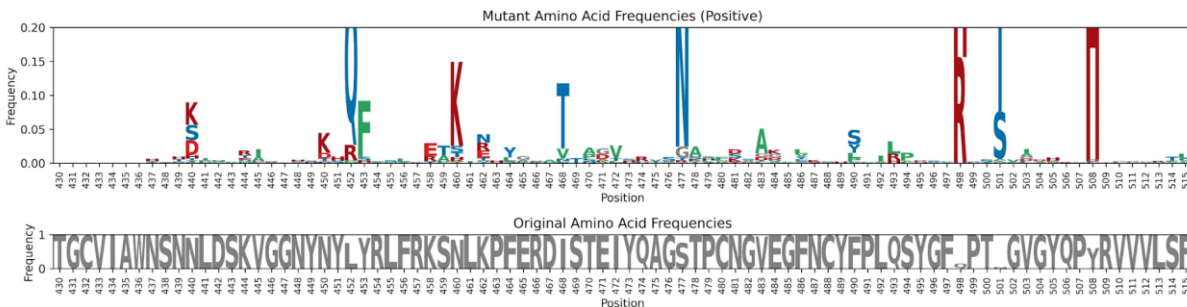
Fig. S18 – Multiple sequence alignment of selected 30 highest-frequency clones identified by Illumina sequencing from the BA.2 HSS libraries (unsorted, round 2 and round 4). Positions identical to wild-type (WT) are indicated by a dot, while amino acid substitutions are shown explicitly. The WT, BA.1, and BA.2 reference sequences are shown in the header. Residues corresponding to WT are highlighted in blue, while residues carrying mutations characteristic for a given variant are highlighted in yellow. The number displayed to the left of each sequence indicates the absolute number of occurrences of that identical clone observed in the dataset.

Supporting information part PS3 – Analysis of mutations in low stringency selection libraries

A WT 7th library low stringency selection (LSS)



B Sequence logo plot – low freq detail



C Mutation scatter plot

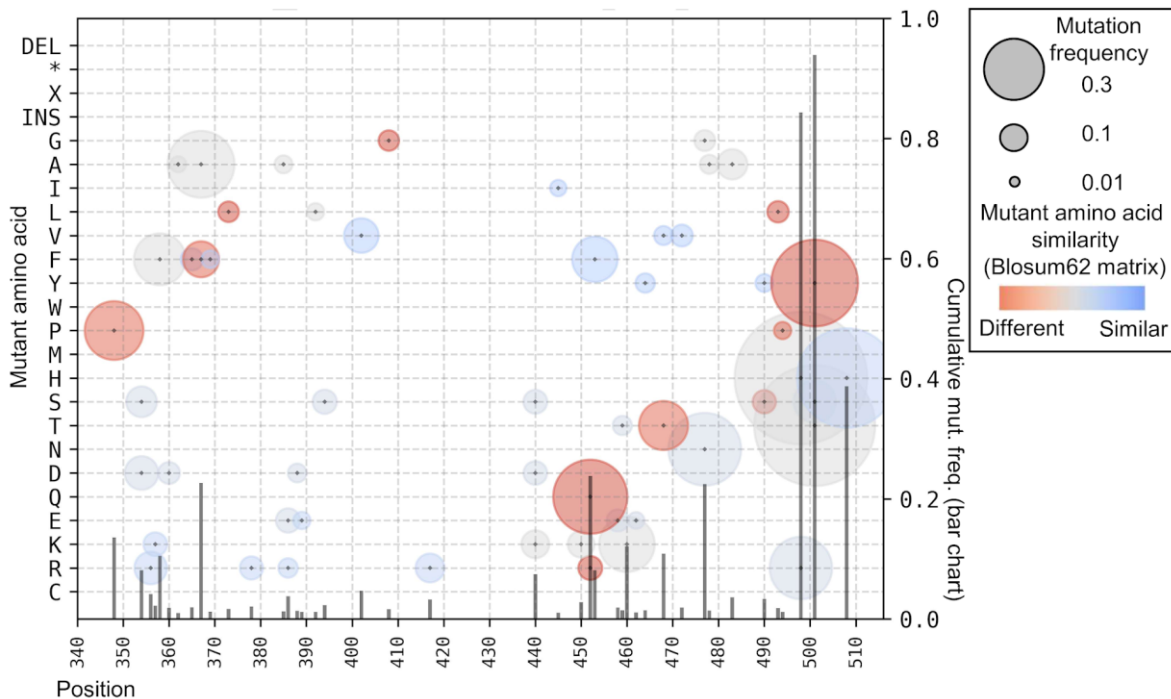


Fig. S19 – Logo plots and mutation scatter plot for WT 7th library under LSS. (A) Sequence logo plot (top panel) showing mutations in the library relative to the WT sequence. The bottom panel (in gray) displays the complementary frequency of the original amino acid at each position. (B) Sequence logo plot focusing on less frequent mutations in the library, with the y-axis frequency range set to 0–0.2. (C) Mutation scatter plot illustrating mutations in the population with a frequency ≥ 0.01 and their evolutionary distance from the original residues.

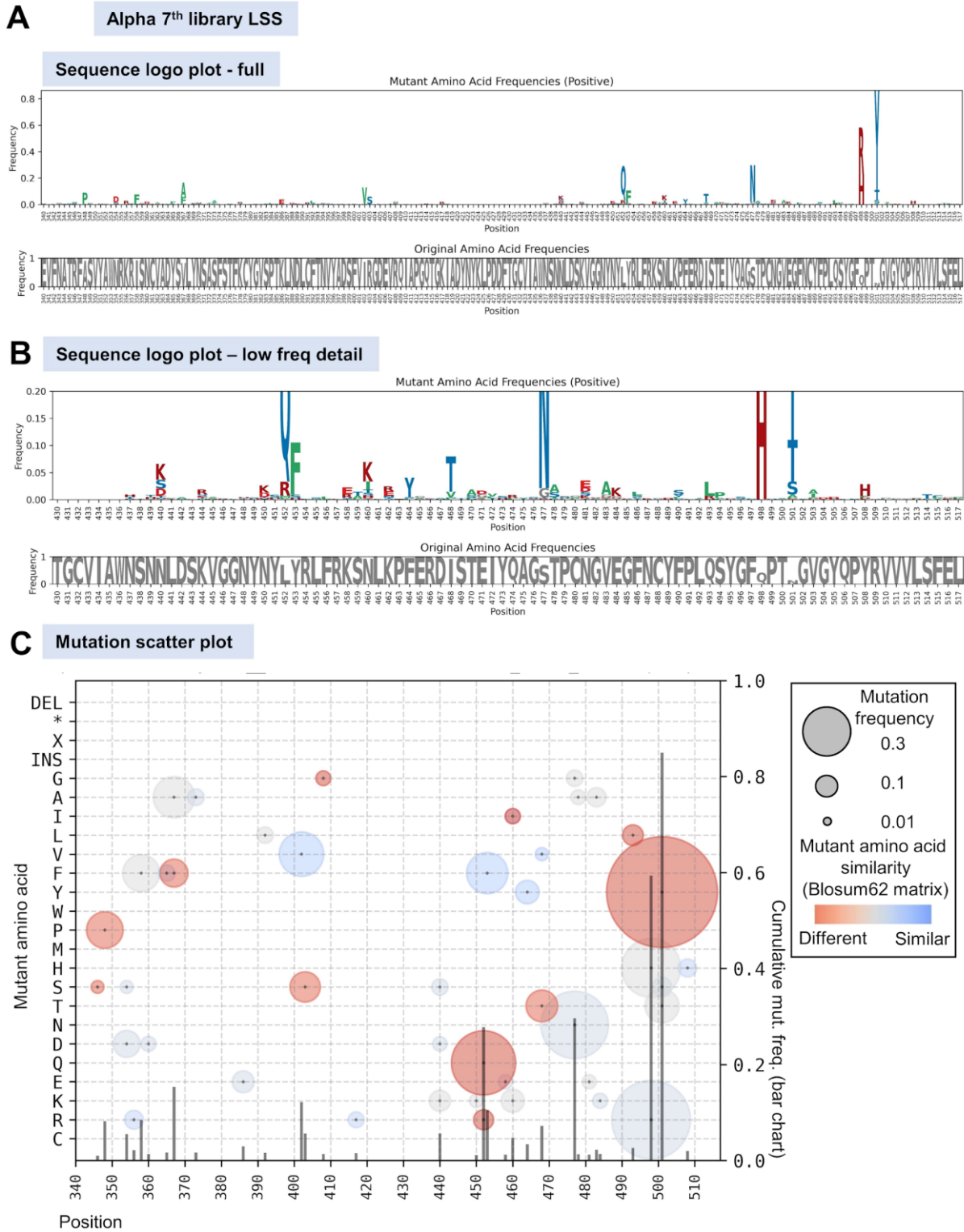


Fig. S20 – Logo plots and mutation scatter plot for Alpha 7th library under LSS. (A) Sequence logo plot (top panel) showing mutations in the library relative to the WT sequence. The bottom panel (in gray) displays the complementary frequency of the original amino acid at each position. (B) Sequence logo plot focusing on less frequent mutations in the library, with the y-axis frequency range set to 0–0.2. (C) Mutation scatter plot illustrating mutations in the population with a frequency ≥ 0.01 and their evolutionary distance from the original residues.

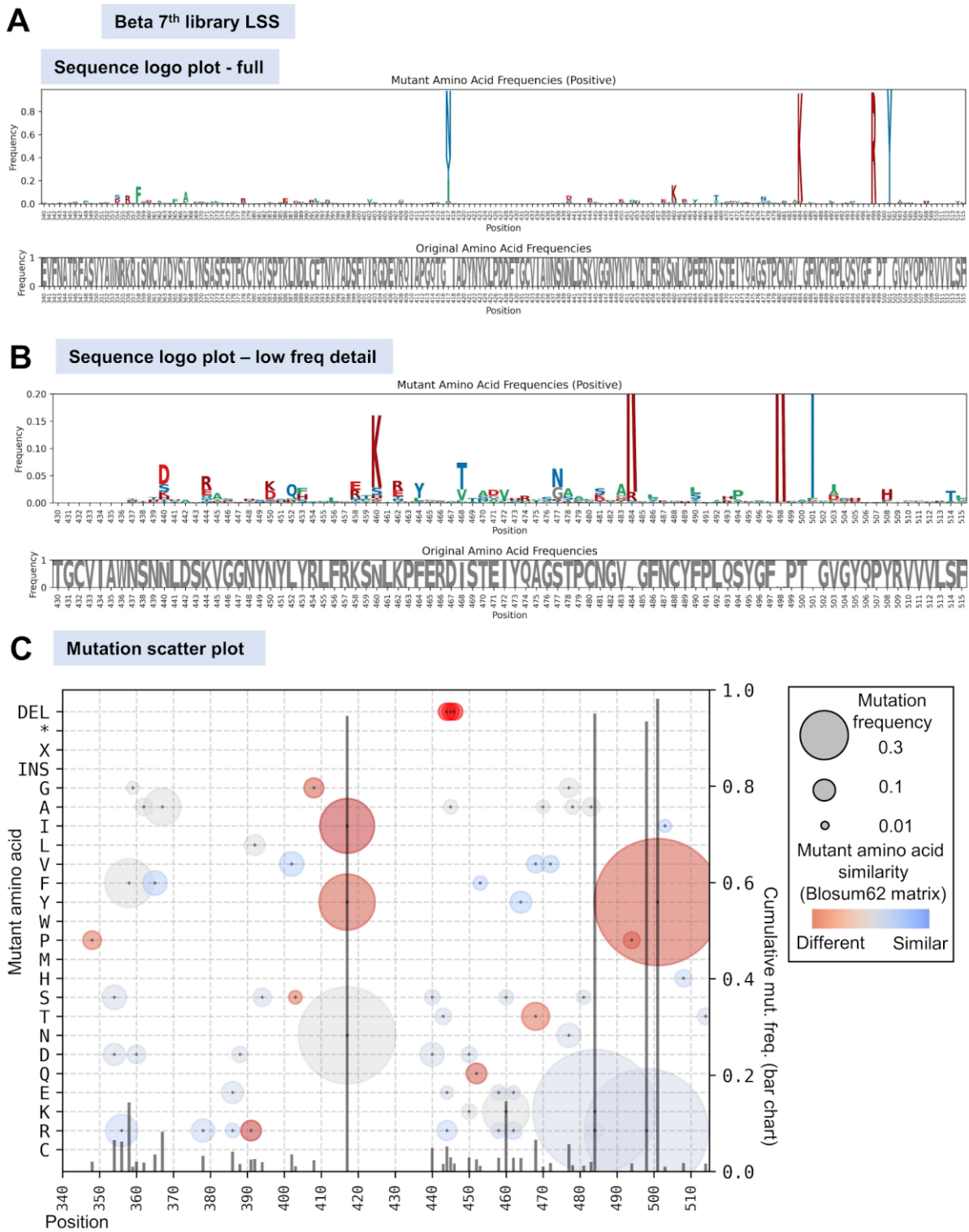


Fig. S21 – Logo plots and mutation scatter plot for Beta 7th library under LSS. (A) Sequence logo plot (top panel) showing mutations in the library relative to the WT sequence. The bottom panel (in gray) displays the complementary frequency of the original amino acid at each position. (B) Sequence logo plot focusing on less frequent mutations in the library, with the y-axis frequency range set to 0–0.2. (C) Mutation scatter plot illustrating mutations in the population with a frequency ≥ 0.01 and their evolutionary distance from the original residues.



Fig. S22 – Logo plots and mutation scatter plot for v48 7th library under LSS. (A) Sequence logo plot (top panel) showing mutations in the library relative to the WT sequence. The bottom panel (in gray) displays the complementary frequency of the original amino acid at each position. (B) Sequence logo plot focusing on less frequent mutations in the library, with the y-axis frequency range set to 0–0.2. (C) Mutation scatter plot illustrating mutations in the population with a frequency ≥ 0.01 and their evolutionary distance from the original residues.

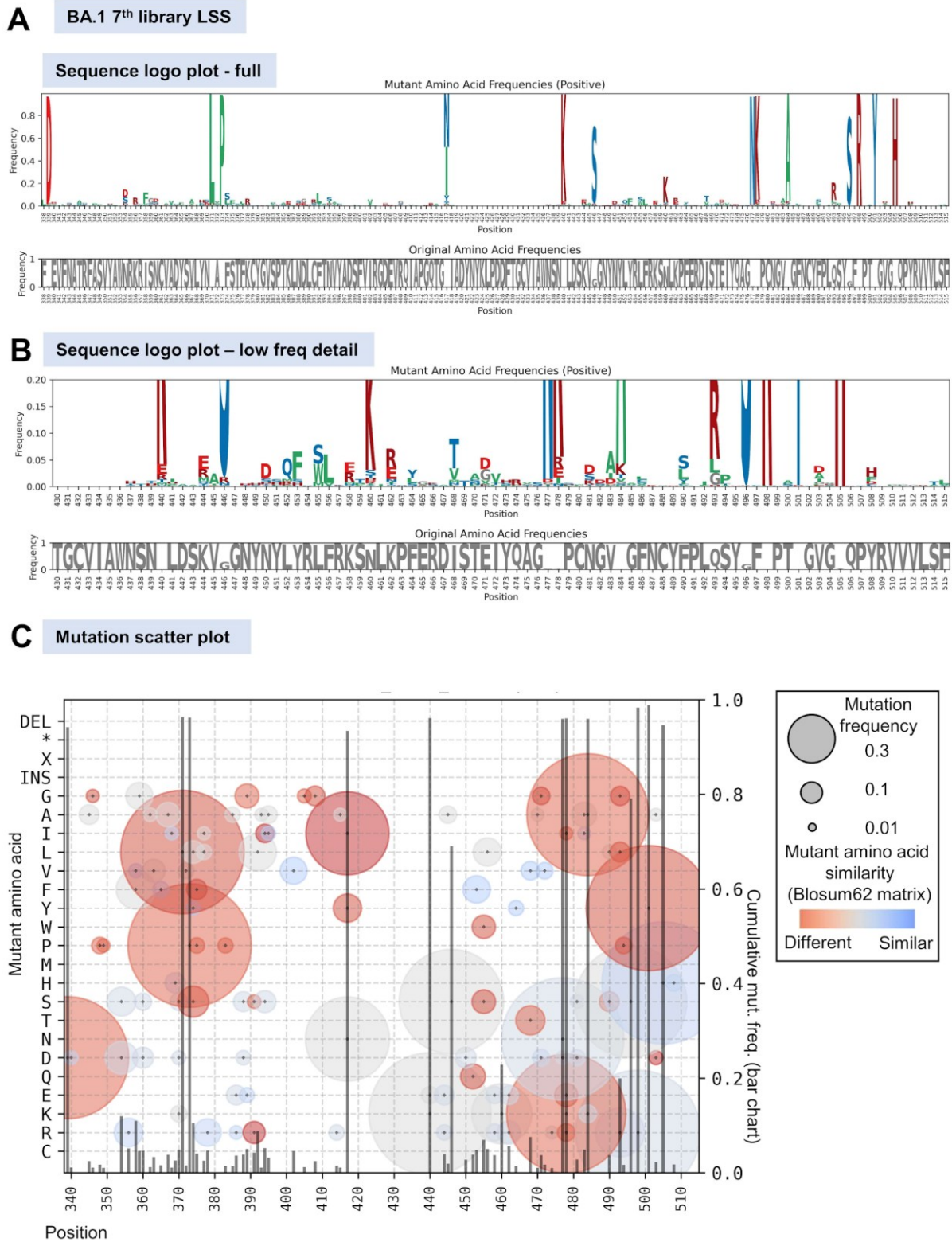
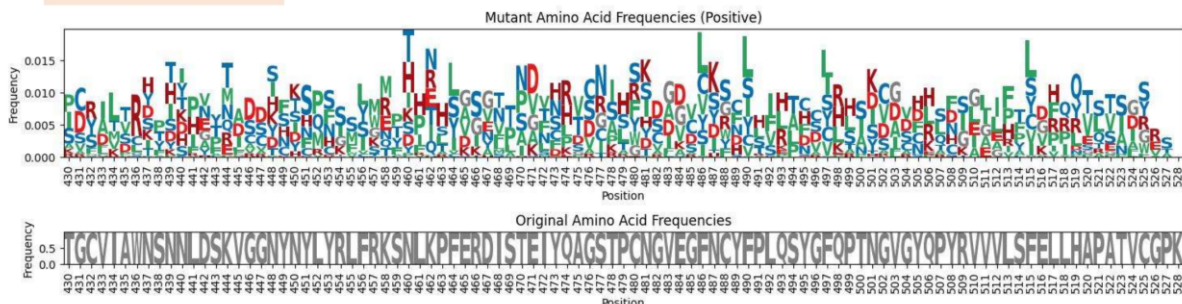


Fig. S23 – Logo plots and mutation scatter plot for BA.1 7th library under LSS. (A) Sequence logo plot (top panel) showing mutations in the library relative to the WT sequence. The bottom panel (in gray) displays the complementary frequency of the original amino acid at each position. (B) Sequence logo plot focusing on less frequent mutations in the library, with the y-axis frequency range set to 0–0.2. (C) Mutation scatter plot illustrating mutations in the population with a frequency ≥ 0.01 and their evolutionary distance from the original residues.

Supporting information part PS4 – Analysis of mutations at different stages of high stringency selection libraries

A WT non-sorted lib. HSS

Sequence logo plot



B Mutation scatter plot

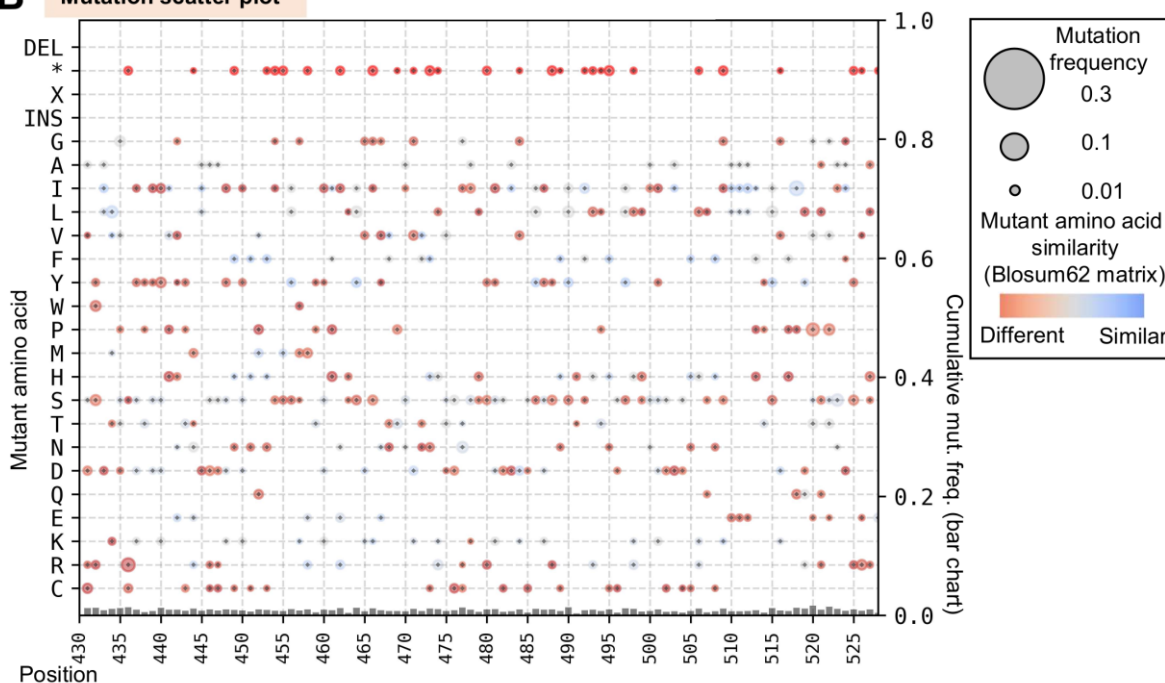


Fig. S24 – Logo plot and mutation scatter plot for WT non-selected library for HSS. (A) Sequence logo plot (top panel) showing mutations in the library relative to the WT sequence. The bottom panel (in gray) displays the complementary frequency of the original amino acid at each position. (B) Mutation scatter plot illustrating mutations in the population and their evolutionary distance from the original residues.

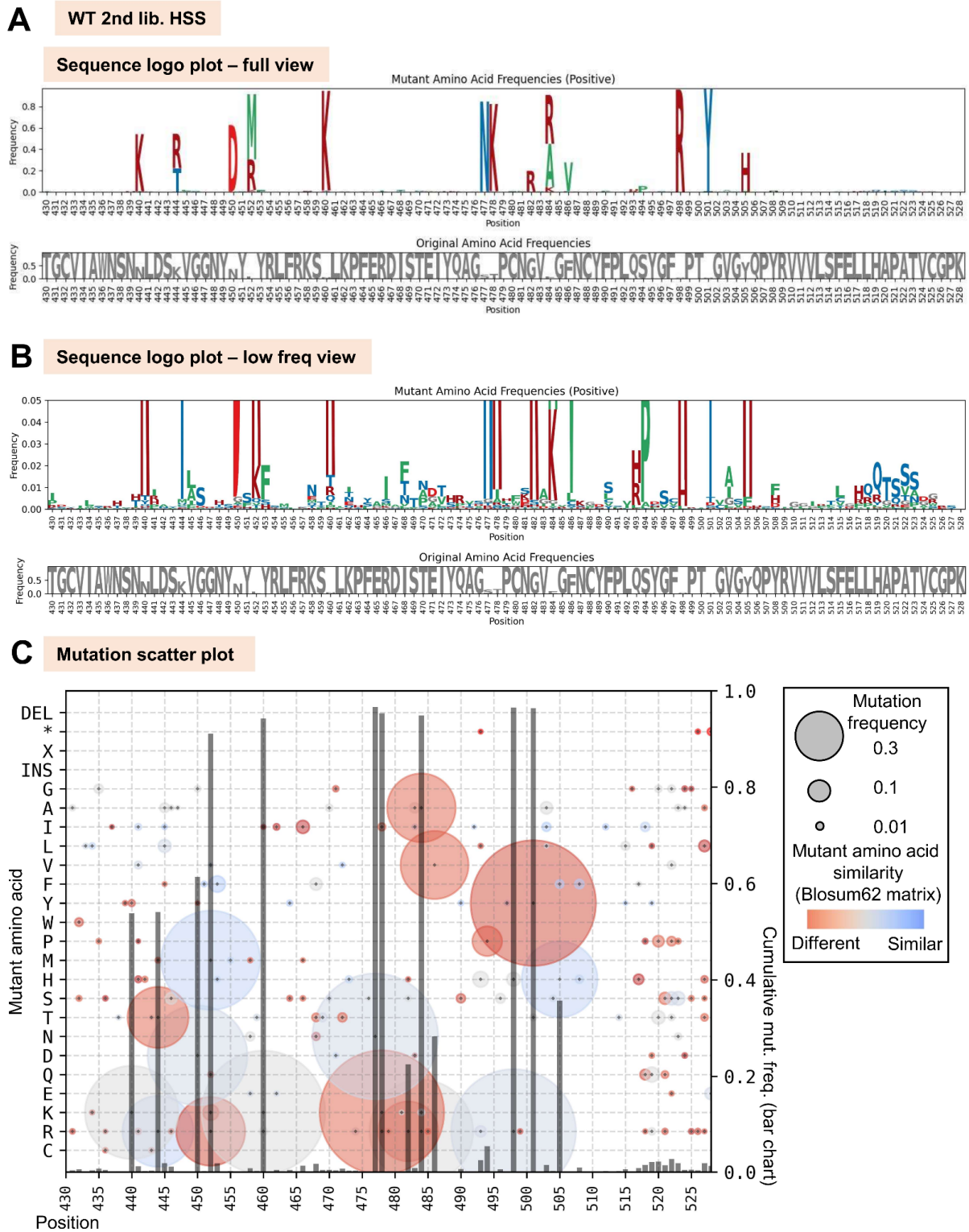


Fig. S25 – Logo plots and mutation scatter plot for WT 2nd library selected under HSS. (A) Sequence logo plot (top panel) showing mutations in the library relative to the WT sequence. The bottom panel (in gray) displays the complementary frequency of the original amino acid at each position. (B) Sequence logo plot focusing on less frequent mutations in the library, with the y-axis frequency range set to 0–0.2. (C) Mutation scatter plot illustrating mutations in the population and their evolutionary distance from the original residues.

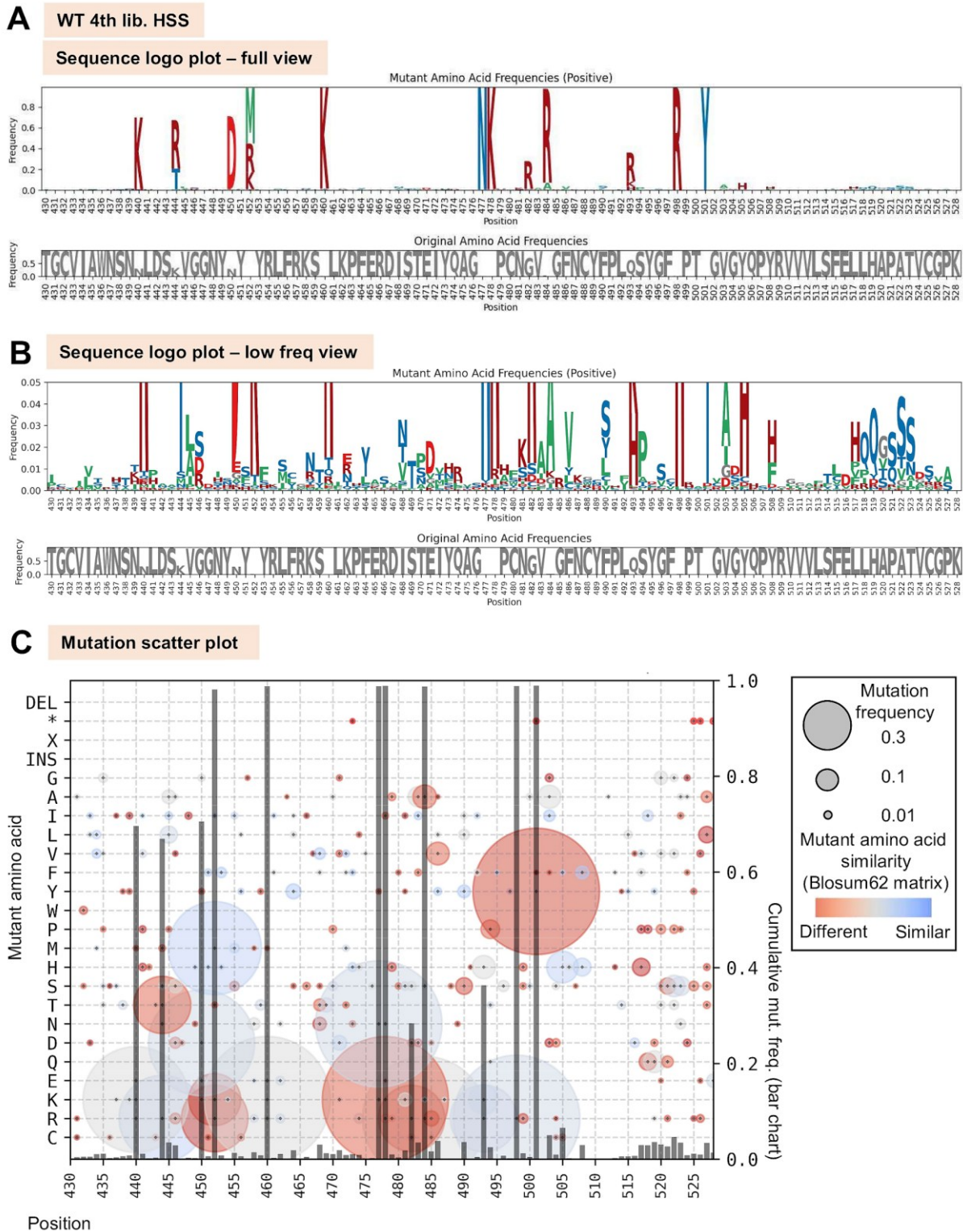


Fig. S26 – Logo plots and mutation scatter plot for WT 4th library selected under HSS. (A) Sequence logo plot (top panel) showing mutations in the library relative to the WT sequence. The bottom panel (in gray) displays the complementary frequency of the original amino acid at each position. (B) Sequence logo plot focusing on less frequent mutations in the library, with the y-axis frequency range set to 0–0.2. (C) Mutation scatter plot illustrating mutations in the population and their evolutionary distance from the original residues.

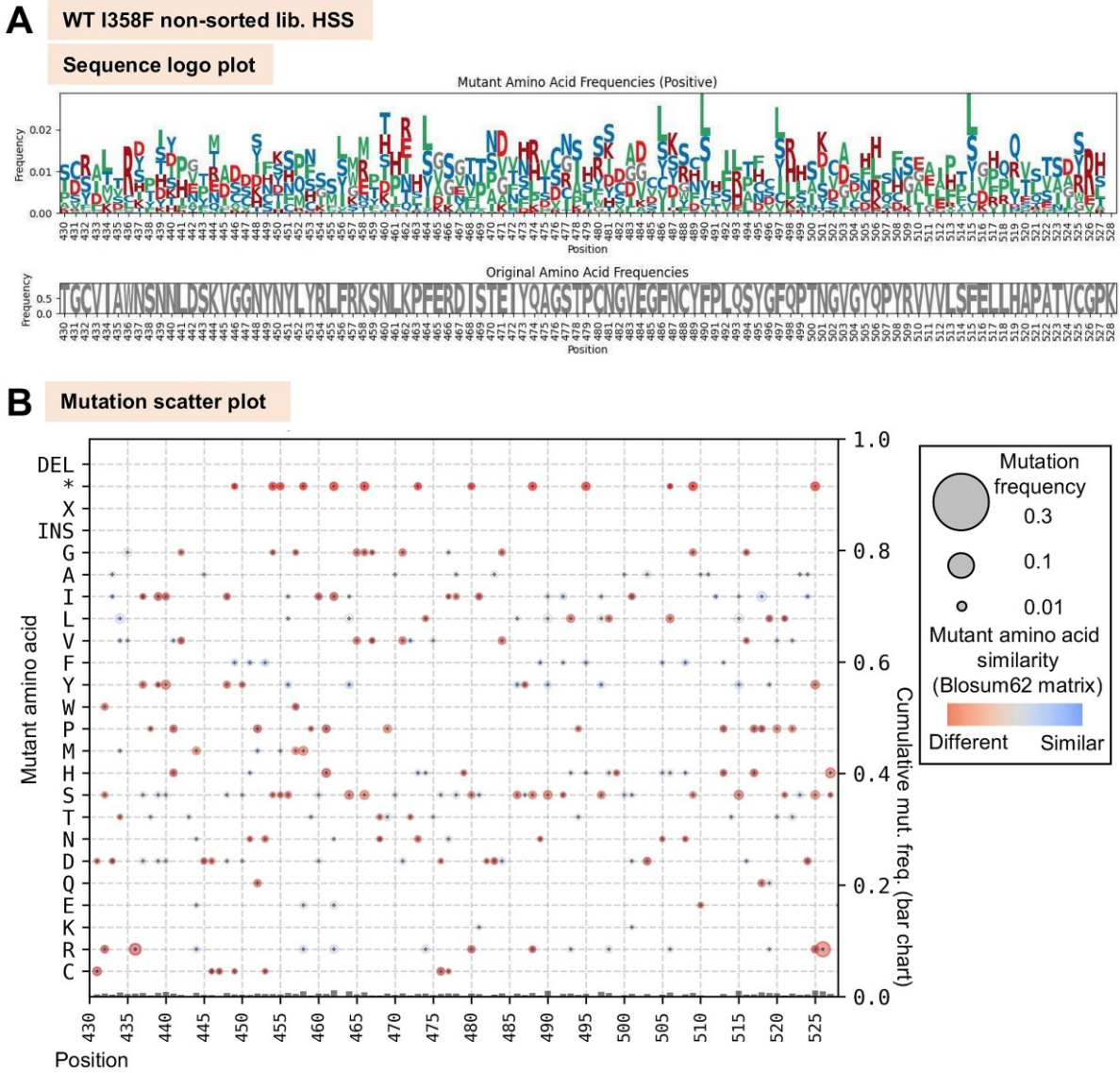


Fig. S27 – Logo plot and mutation scatter plot for WT I358F non-selected library for HSS. (A) Sequence logo plot (top panel) showing mutations in the library relative to the WT sequence. The bottom panel (in gray) displays the complementary frequency of the original amino acid at each position. (B) Mutation scatter plot illustrating mutations in the population and their evolutionary distance from the original residues.

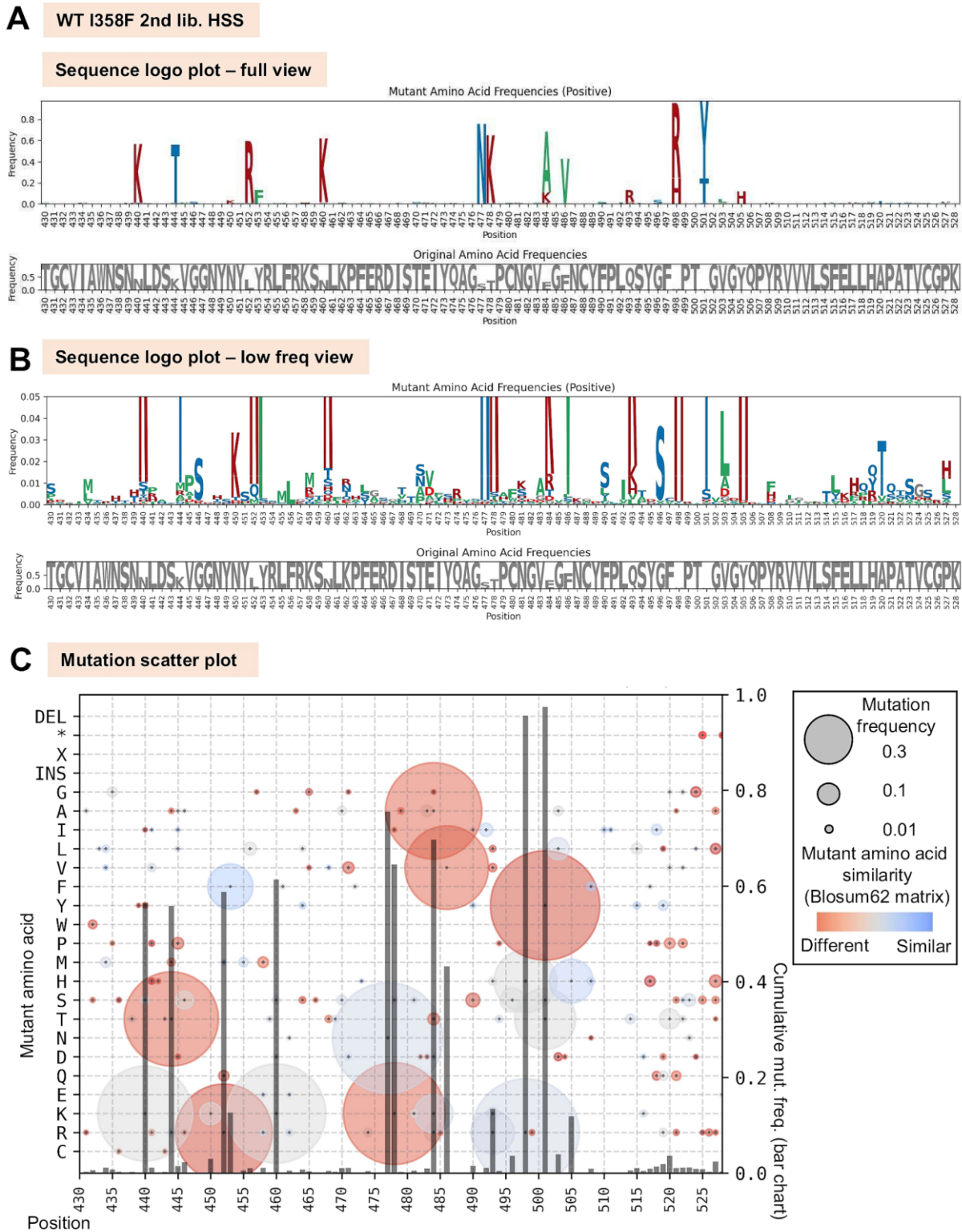


Fig. S28 – Logo plots and mutation scatter plot for WT I358F 2nd library selected under HSS. (A) Sequence logo plot (top panel) showing mutations in the library relative to the WT sequence. The bottom panel (in gray) displays the complementary frequency of the original amino acid at each position. (B) Sequence logo plot focusing on less frequent mutations in the library, with the y-axis frequency range set to 0–0.2. (C) Mutation scatter plot illustrating mutations in the population and their evolutionary distance from the original residues.

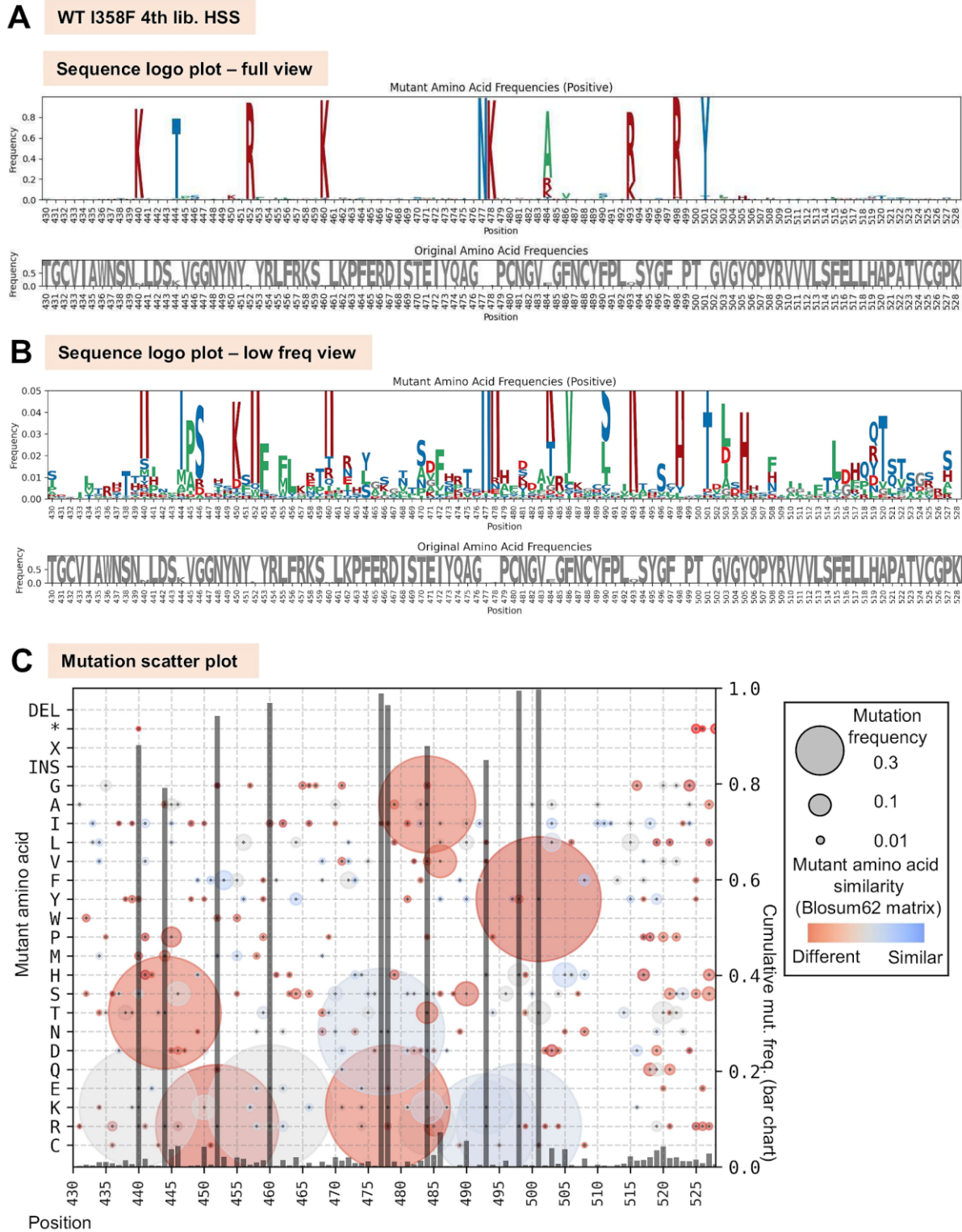


Fig. S29 – Logo plots and mutation scatter plot for WT I358F 4th library selected under HSS. (A) Sequence logo plot (top panel) showing mutations in the library relative to the WT sequence. The bottom panel (in gray) displays the complementary frequency of the original amino acid at each position. (B) Sequence logo plot focusing on less frequent mutations in the library, with the y-axis frequency range set to 0–0.2. (C) Mutation scatter plot illustrating mutations in the population and their evolutionary distance from the original residues.

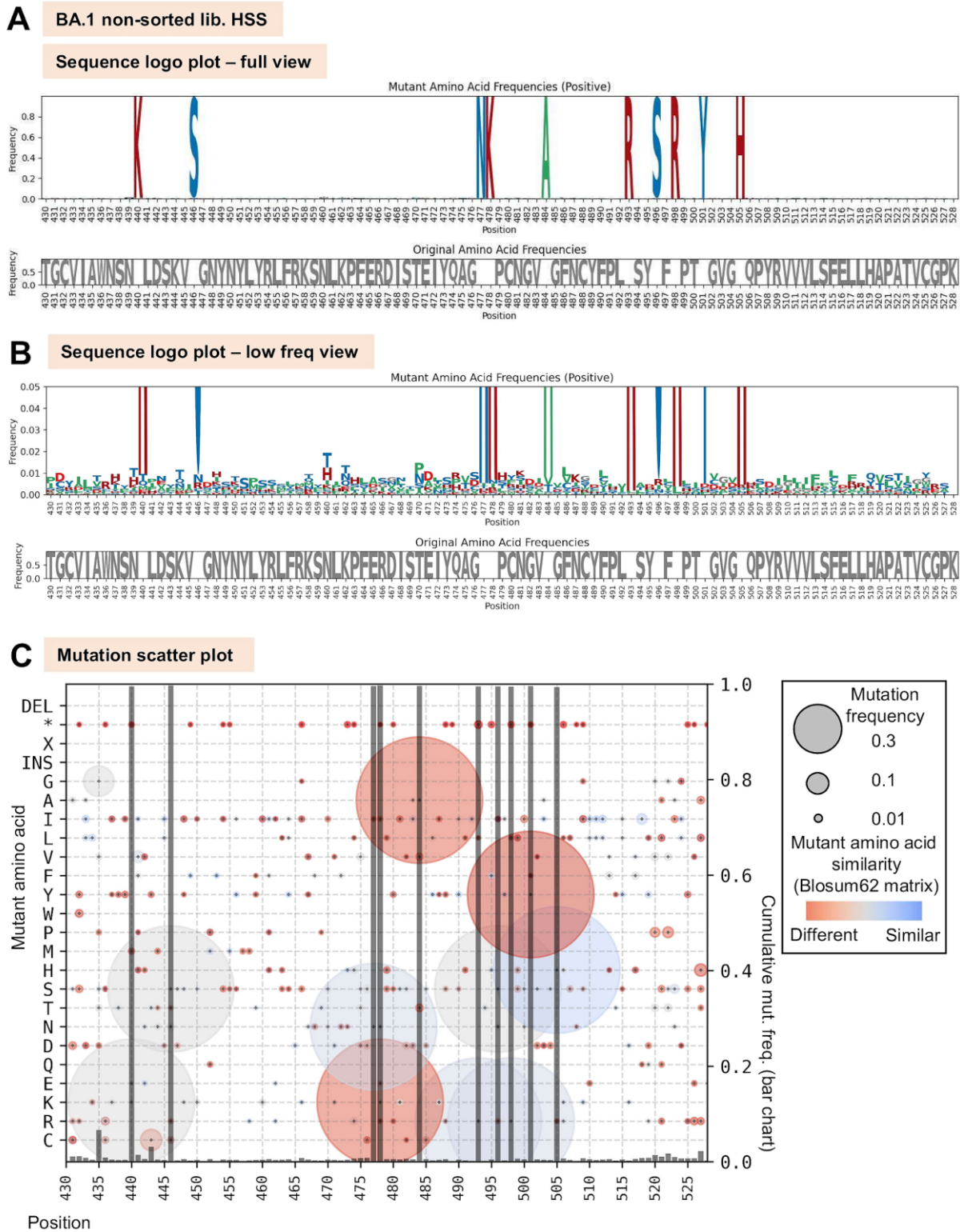


Fig. S30 – Logo plots and mutation scatter plot for BA.1 non-selected library selected under HSS. (A) Sequence logo plot (top panel) showing mutations in the library relative to the WT sequence. The bottom panel (in gray) displays the complementary frequency of the original amino acid at each position. (B) Sequence logo plot focusing on less frequent mutations in the library, with the y-axis frequency range set to 0–0.2. (C) Mutation scatter plot illustrating mutations in the population and their evolutionary distance from the original residues.

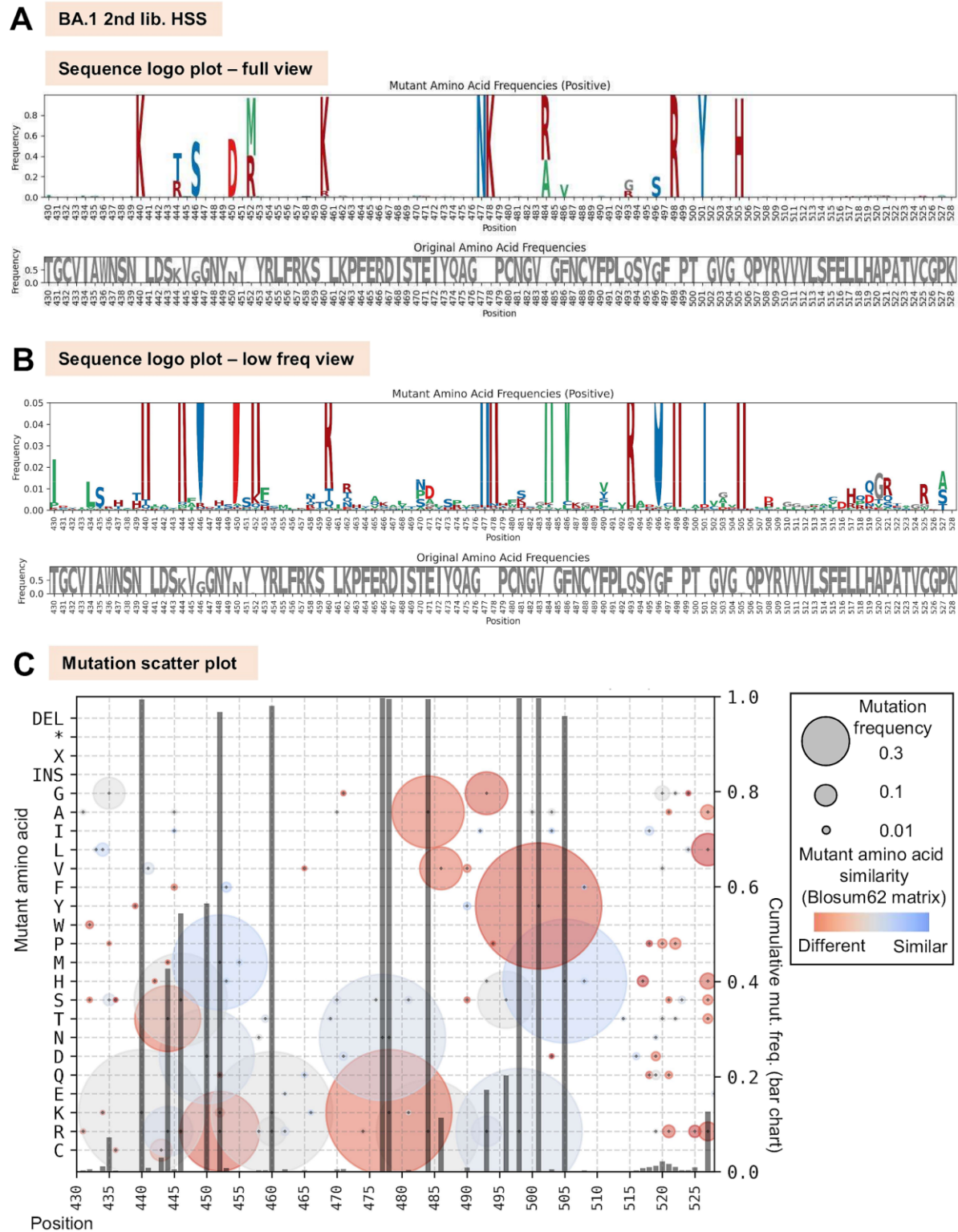


Fig. S31 – Logo plots and mutation scatter plot for BA.1 2nd library selected under HSS. (A) Sequence logo plot (top panel) showing mutations in the library relative to the WT sequence. The bottom panel (in gray) displays the complementary frequency of the original amino acid at each position. (B) Sequence logo plot focusing on less frequent mutations in the library, with the y-axis frequency range set to 0–0.2. (C) Mutation scatter plot illustrating mutations in the population and their evolutionary distance from the original residues.

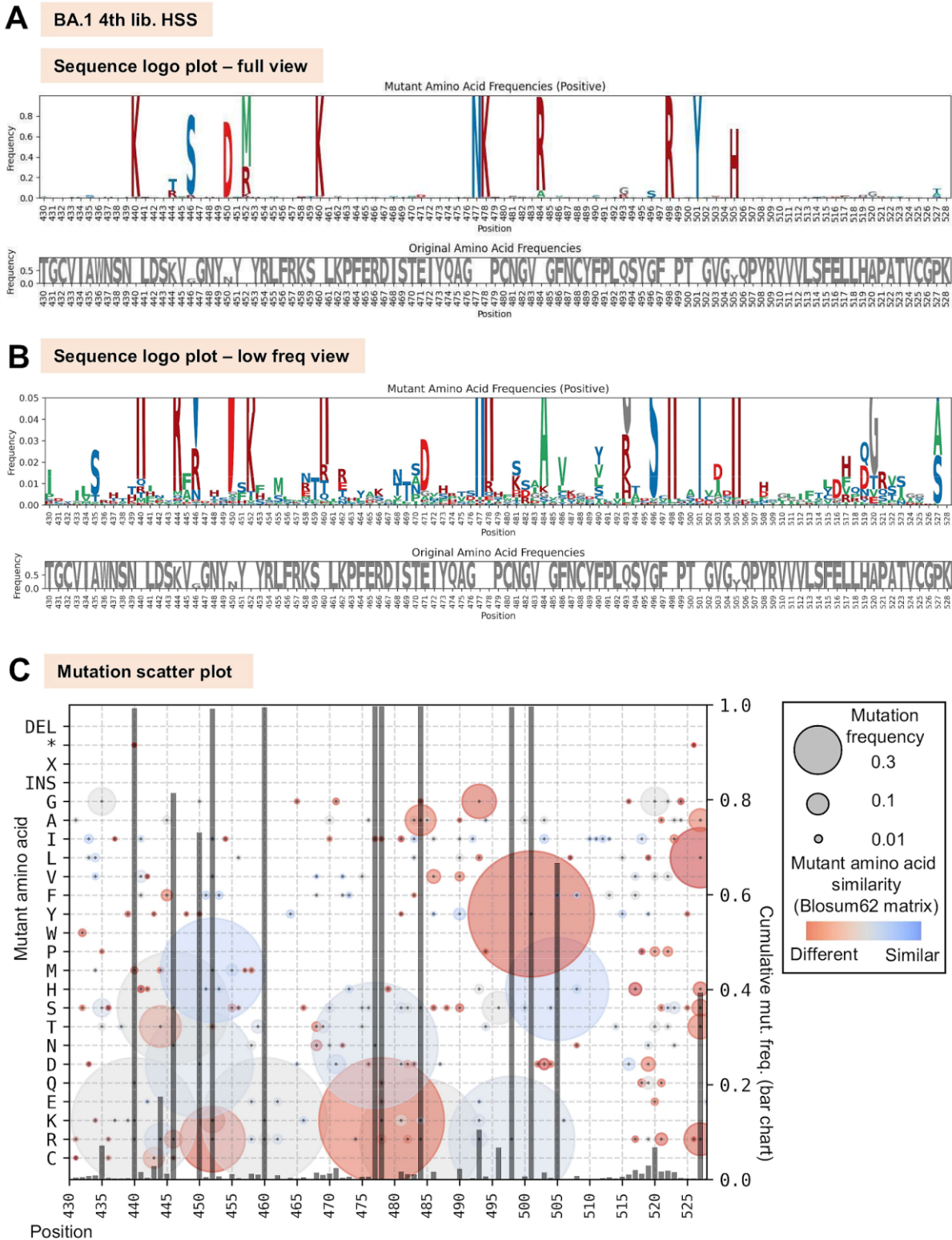


Fig. S32 – Logo plots and mutation scatter plot for BA.1 4th library selected under HSS. (A) Sequence logo plot (top panel) showing mutations in the library relative to the WT sequence. The bottom panel (in gray) displays the complementary frequency of the original amino acid at each position. (B) Sequence logo plot focusing on less frequent mutations in the library, with the y-axis frequency range set to 0–0.2. (C) Mutation scatter plot illustrating mutations in the population and their evolutionary distance from the original residues.

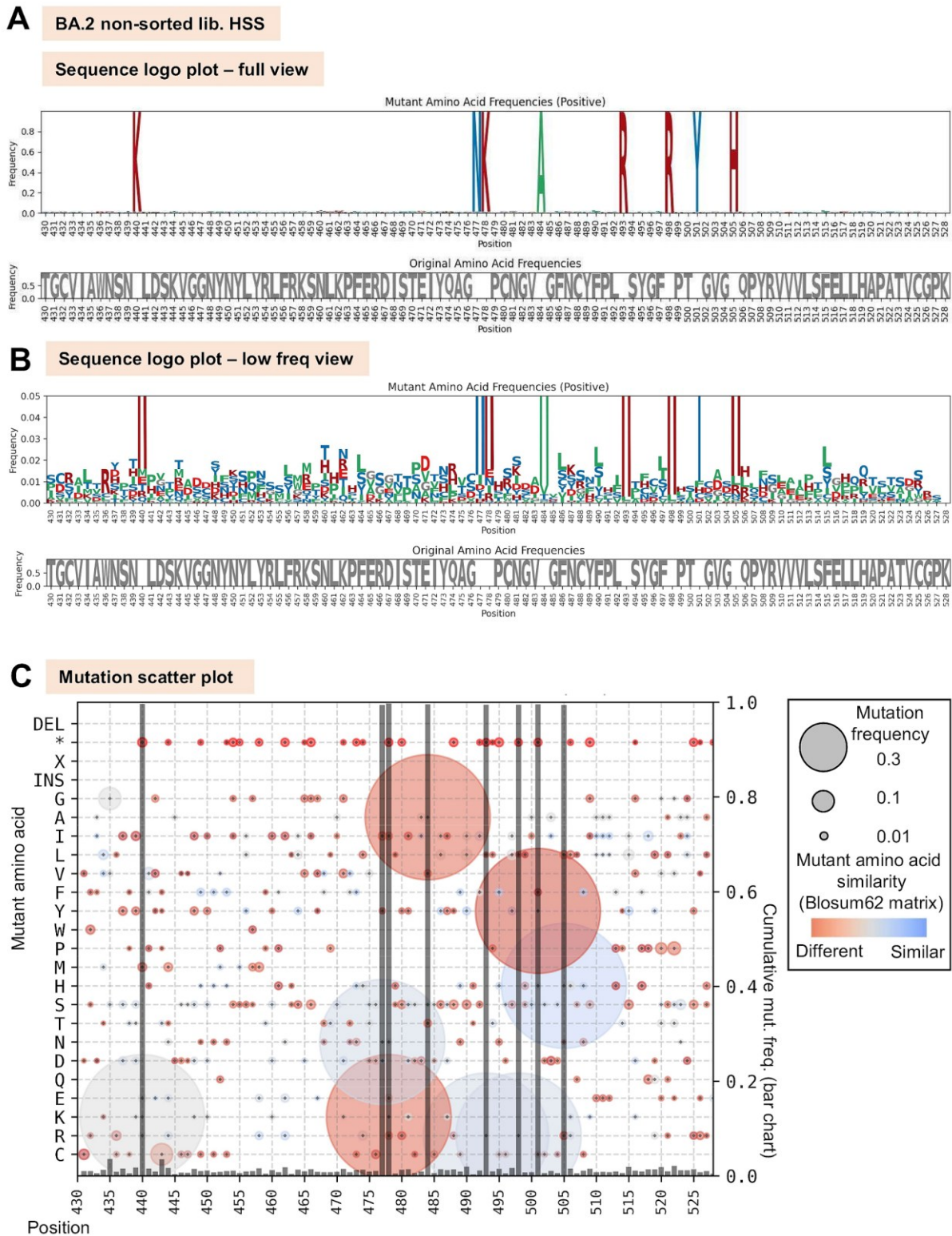


Fig. S33 – Logo plots and mutation scatter plot for BA.2 non-selected library for HSS. (A) Sequence logo plot (top panel) showing mutations in the library relative to the WT sequence. The bottom panel (in gray) displays the complementary frequency of the original amino acid at each position. (B) Sequence logo plot focusing on less frequent mutations in the library, with the y-axis frequency range set to 0–0.2. (C) Mutation scatter plot illustrating mutations in the population and their evolutionary distance from the original residues.

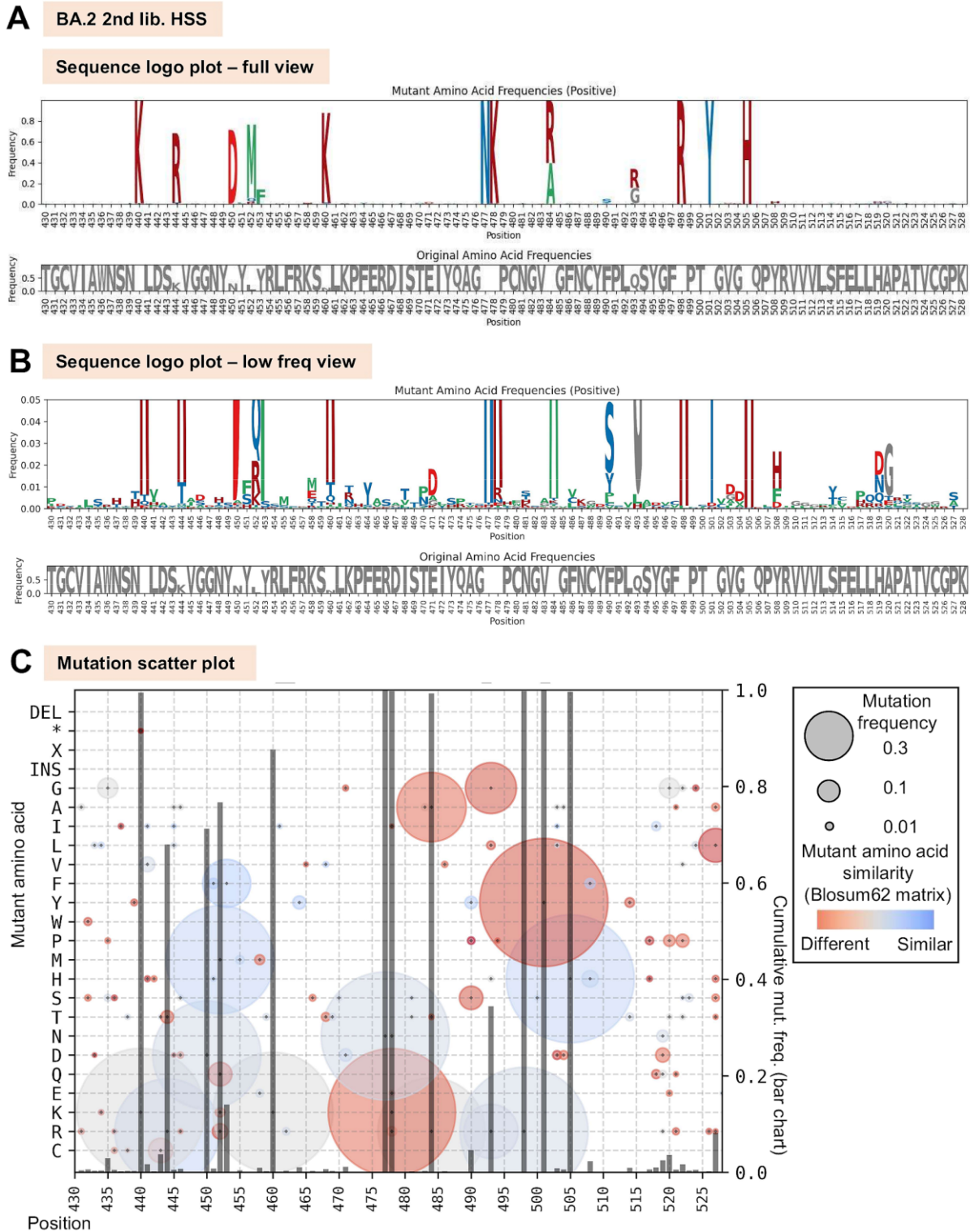


Fig. S34 – Logo plots and mutation scatter plot for BA.2 2nd library selected under HSS. (A) Sequence logo plot (top panel) showing mutations in the library relative to the WT sequence. The bottom panel (in gray) displays the complementary frequency of the original amino acid at each position. (B) Sequence logo plot focusing on less frequent mutations in the library, with the y-axis frequency range set to 0–0.2. (C) Mutation scatter plot illustrating mutations in the population and their evolutionary distance from the original residues.

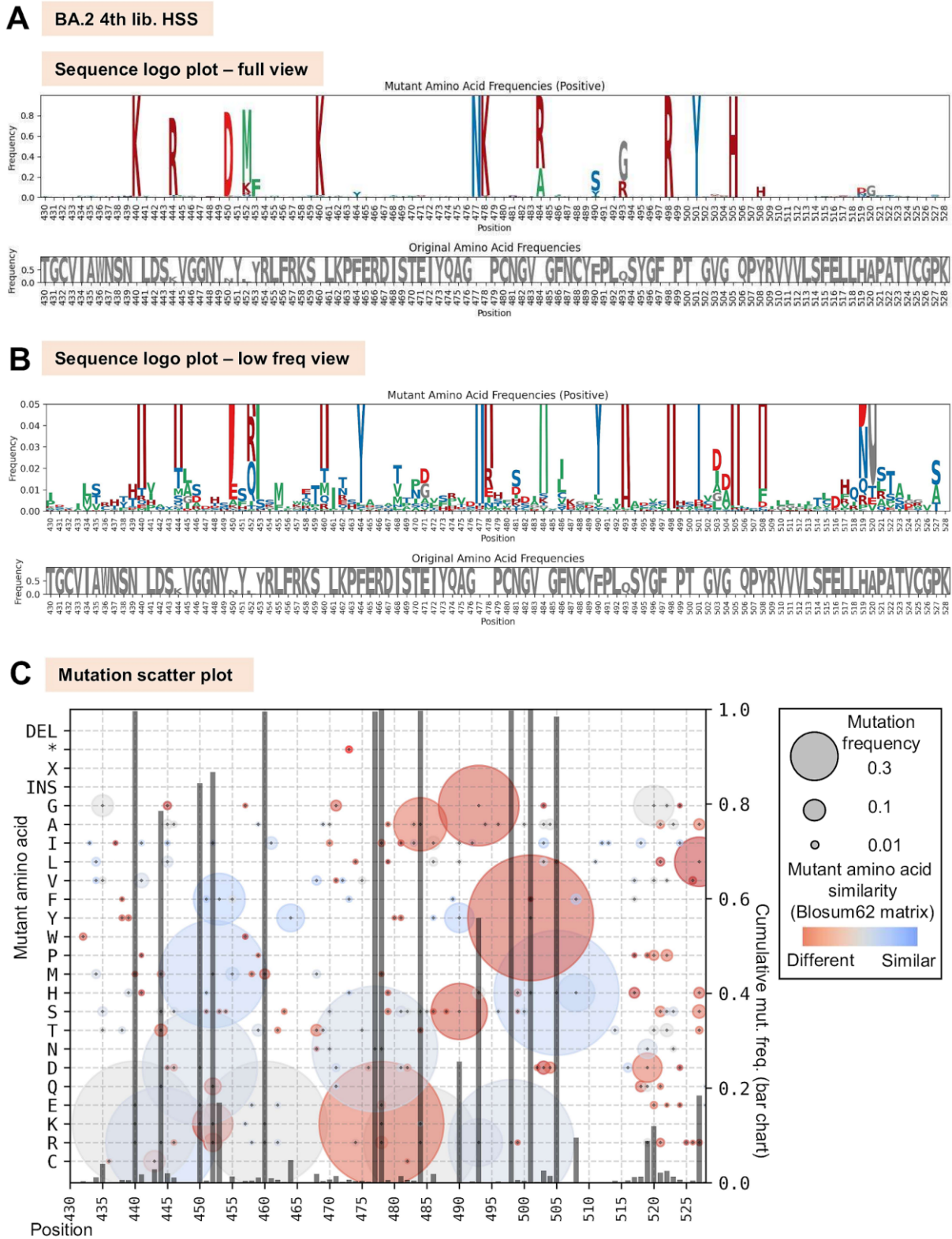
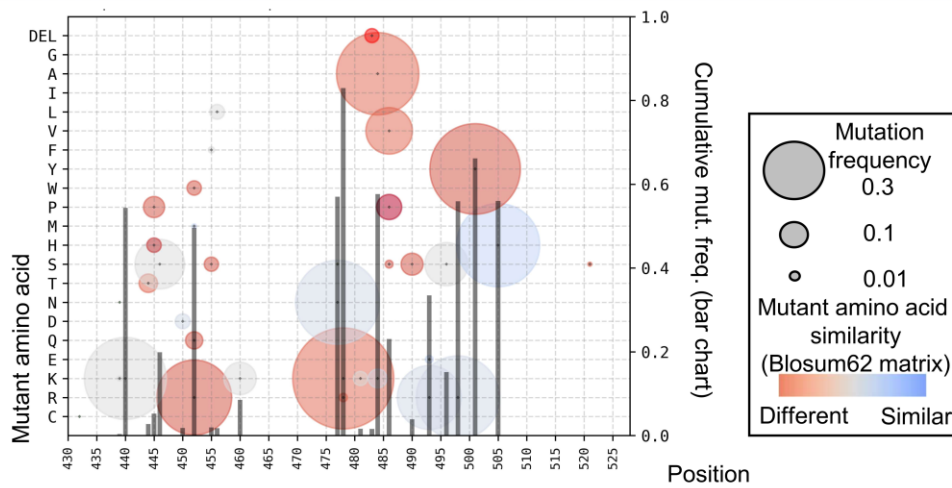
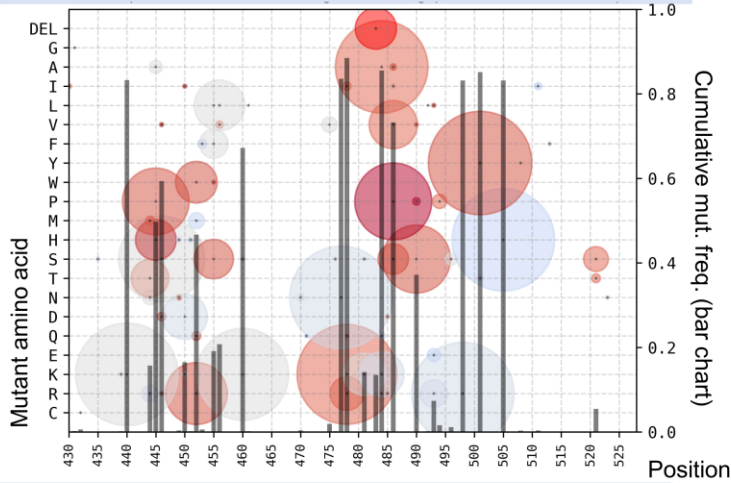


Fig. S35 – Logo plots and mutation scatter plot for BA.2 4th library selected under HSS. (A) Sequence logo plot (top panel) showing mutations in the library relative to the WT sequence. The bottom panel (in gray) displays the complementary frequency of the original amino acid at each position. (B) Sequence logo plot focusing on less frequent mutations in the library, with the y-axis frequency range set to 0–0.2. (C) Mutation scatter plot illustrating mutations in the population and their evolutionary distance from the original residues.

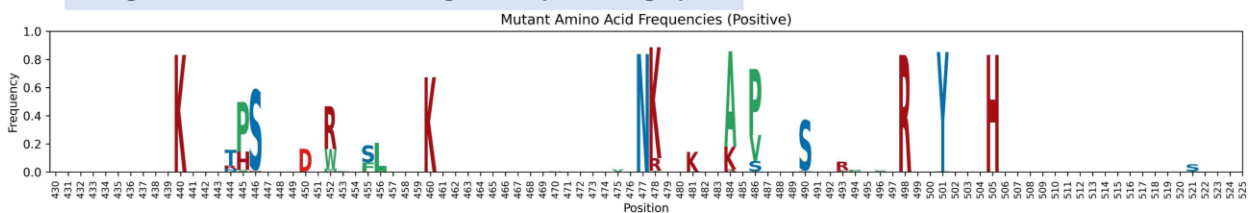
A All GISAID SARS-CoV-2 sequences (7th December 2024, GISAID_spikenuc1207 dataset)



B Pango named SARS-CoV-2 lineages (12th September 2024)



C Pango named SARS-CoV-2 lineages – sequence logo plot



D Pango named SARS-CoV-2 lineages without BA.2.86 and descendants – sequence logo plot

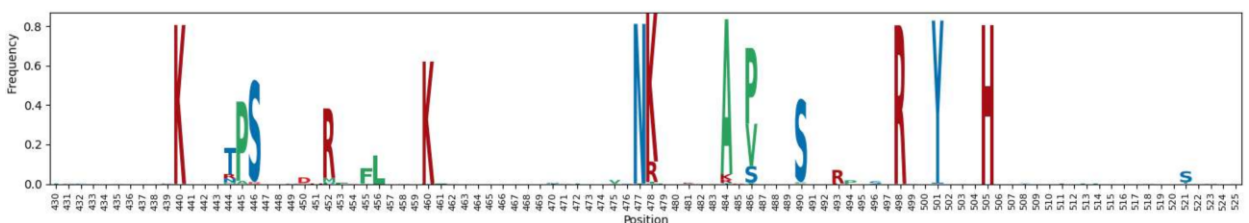


Fig. S36 – SARS-CoV-2 sequences and lineages analysis. (A) Full RBM mutation scatter plot for the complete set of all GISAID SARS-CoV-2 sequences above 0.1 % threshold obtained from a spikenuc1207 file released on December 7, 2024 by GISAID. (B) RBM (amino acids 430 – 528) mutation scatter plot for the complete set of all SARS-CoV-2 Pango named lineages (released on September 12, 2024) with lowered threshold 0.1 % due to disbalances introduced when lineages were merged into a single ancestral sequence, (C) Sequence logo plot for the complete set of all SARS-CoV-2 Pango named lineages. Deletion is not shown. (D) Sequence logo plot for the set of SARS-CoV-2 Pango named lineages without BA.2.86 and its descendants (“late” Omicron lineages).

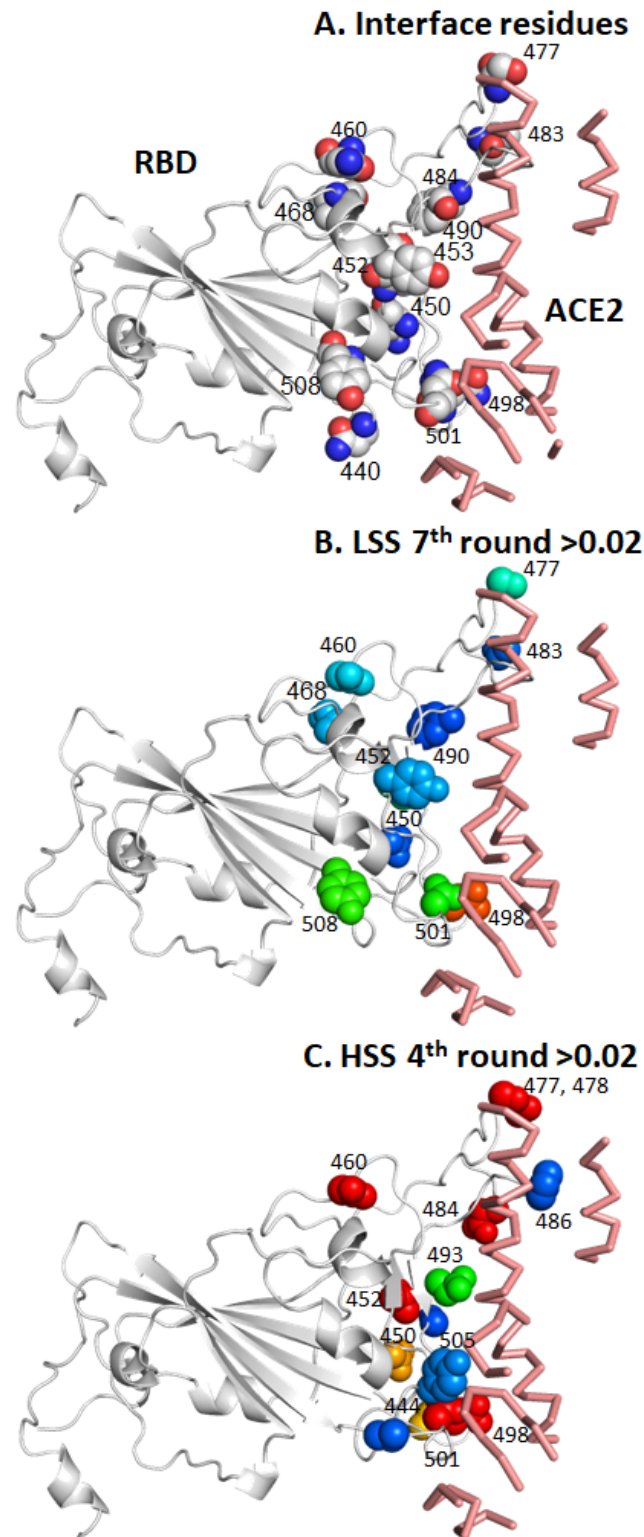


Fig. S37 – Structure representation of the mutations accumulated at higher frequencies following LSS and HSS. (A) Interface residues numbering based on Wuhan-Hu-1 (MN908947.3) Spike protein sequence similar to Jun Lan *et al.* (2020) Nature **581**: 215-220 (<https://www.nature.com/articles/s41586-020-2180-5>). (B, C) Residues with mutations frequencies >0.02, color coded according to frequency: blue (0.02) to red (1). Images were created by using PyMOL v3.1.6.1 and SARS-CoV RBD–ACE2 complex (PDB: 2AJF).

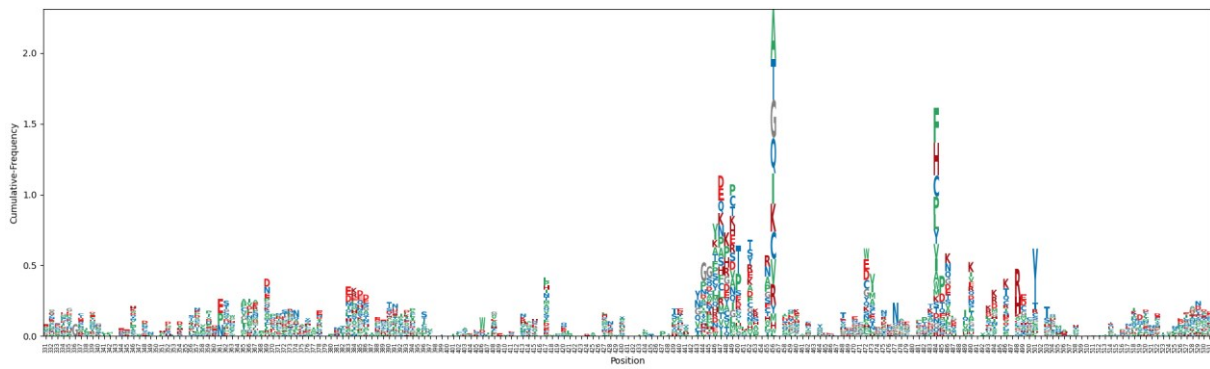


Fig. S38 – Sequence logo plot of RBD mutations that reduce human polyclonal plasma binding averaged across all individuals and time points. Data generated by Allison J. Greaney and colleagues (*Prof. Jesse D. Bloom laboratory*, ²⁴).

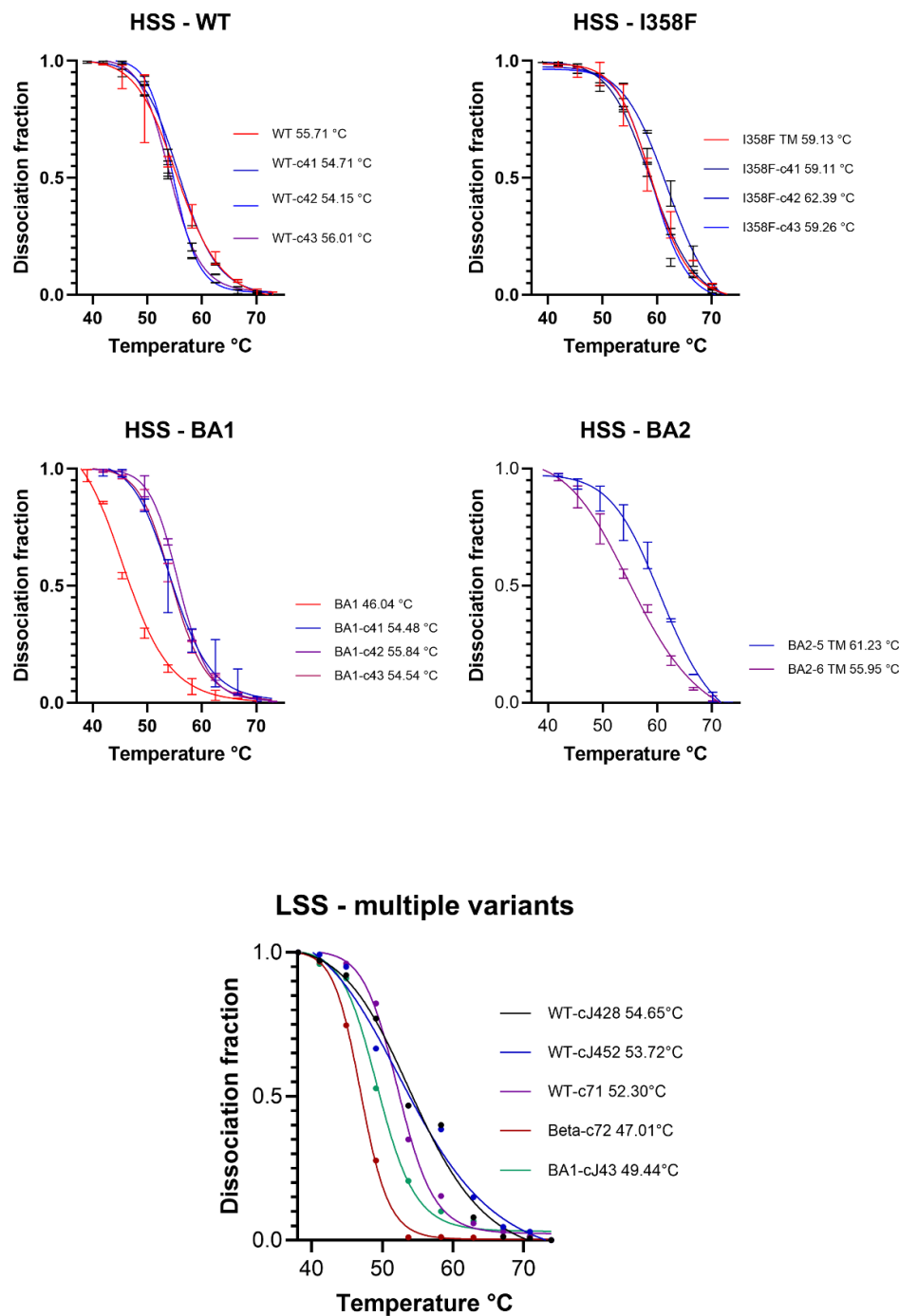


Figure S39 – Determination of the melting temperature (T_m) of RBD variants displayed on the yeast surface. Yeast cells expressing individual RBD clones on their surface were incubated at varying temperatures (38–72 °C) for 15 min to induce thermal denaturation. Following heat treatment, cells were cooled and incubated with fluorescently labeled ACE2 (10–20 nM) to assess the fraction of non-denaturated, binding-competent RBDs. After washing with PBSF, median fluorescence intensity values in the red channel were quantified by flow cytometry. Normalized values were fitted to a four-parameter logistic (4PL) sigmoidal curve using GraphPad Prism, from which T_m values were estimated.

Shoshany *et al.* (2026) Stringent Selection Drives Convergence Toward Omicron-like SARS-CoV-2 Receptor-Binding Motifs

SARS-CoV-1 library	clone	WT (CoV)
1	1	C P F S E V F N A T I F P S V Y A W E R K K I S N C V A D Y S V L N S T F S T F A C Y G V S A T L N D L C F S N Y A D S F V K G D D V Q I A P G Q T G V I A D Y N Y L P D D F M G C V L A W N T N I D A T S T G N Y K Y R L R G K L R P F E D I S N V F S P D G K C T P A L N C W P L N D Y G F T T T G I G Y Q P R V V V L S F
1	2	C P F S E V F N A T I F P S V Y A W E R K K I S N C V A D Y S V L N S T F S T F A C Y G V S A T L N D L C F S N Y A D S F V K G D D V Q I A P G Q T G V I A D Y N Y L P D D F M G C V L A W N T N I D A T S T G N Y K Y R L R G K L R P F E D I S N V F S P D G K C T P A L N C W P L N D Y G F T T T G I G Y Q P R V V V L S F
1	3	C P F S E V F N A T I F P S V Y A W E R K K I S N C V A D Y S V L N S T F S T F A C Y G V S A T L N D L C F S N Y A D S F V K G D D V Q I A P G Q T G V I A D Y N Y L P D D F M G C V L A W N T N I D A T S T G N Y K Y R L R G K L R P F E D I S N V F S P D G K C T P A L N C W P L N D Y G F T T T G I G Y Q P R V V V L S F
2	1	C P F S E V F N A T I F P S V Y A W E R K K I S N C V A D Y S V L N S T F S T F A C Y G V S A T L N D L C F S N Y A D S F V K G D D V Q I A P G Q T G V I A D Y N Y L P D D F M G C V L A W N T N I D A T S T G N Y K Y R L R G K L R P F E D I S N V F S P D G K C T P A L N C W P L N D Y G F T T T G I G Y Q P R V V V L S F
2	2	C P F S E V F N A T I F P S V Y A W E R K K I S N C V A D Y S V L N S T F S T F A C Y G V S A T L N D L C F S N Y A D S F V K G D D V Q I A P G Q T G V I A D Y N Y L P D D F M G C V L A W N T N I D A T S T G N Y K Y R L R G K L R P F E D I S N V F S P D G K C T P A L N C W P L N D Y G F T T T G I G Y Q P R V V V L S F
2	3	C P F S E V F N A T I F P S V Y A W E R K K I S N C V A D Y S V L N S T F S T F A C Y G V S A T L N D L C F S N Y A D S F V K G D D V Q I A P G Q T G V I A D Y N Y L P D D F M G C V L A W N T N I D A T S T G N Y K Y R L R G K L R P F E D I S N V F S P D G K C T P A L N C W P L N D Y G F T T T G I G Y Q P R V V V L S F
3	1	C P F S E V F N A T I F P S V Y A W E R K K I S N C V A D Y S V L N S T F S T F A C Y G V S A T L N D L C F S N Y A D S F V K G D D V Q I A P G Q T G V I A D Y N Y L P D D F M G C V L A W N T N I D A T S T G N Y K Y R L R G K L R P F E D I S N V F S P D G K C T P A L N C W P L N D Y G F T T T G I G Y Q P R V V V L S F
3	2	C P F S E V F N A T I F P S V Y A W E R K K I S N C V A D Y S V L N S T F S T F A C Y G V S A T L N D L C F S N Y A D S F V K G D D V Q I A P G Q T G V I A D Y N Y L P D D F M G C V L A W N T N I D A T S T G N Y K Y R L R G K L R P F E D I S N V F S P D G K C T P A L N C W P L N D Y G F T T T G I G Y Q P R V V V L S F
3	3	C P F S E V F N A T I F P S V Y A W E R K K I S N C V A D Y S V L N S T F S T F A C Y G V S A T L N D L C F S N Y A D S F V K G D D V Q I A P G Q T G V I A D Y N Y L P D D F M G C V L A W N T N I D A T S T G N Y K Y R L R G K L R P F E D I S N V F S P D G K C T P A L N C W P L N D Y G F T T T G I G Y Q P R V V V L S F
4	1	C P F S E V F N A T I F P S V Y A W E R K K I S N C V A D Y S V L N S T F S T F A C Y G V S A T L N D L C F S N Y A D S F V K G D D V Q I A P G Q T G V I A D Y N Y L P D D F M G C V L A W N T N I D A T S T G N Y K Y R L R G K L R P F E D I S N V F S P D G K C T P A L N C W P L N D Y G F T T T G I G Y Q P R V V V L S F
4	2	C P F S E V F N A T I F P S V Y A W E R K K I S N C V A D Y S V L N S T F S T F A C Y G V S A T L N D L C F S N Y A D S F V K G D D V Q I A P G Q T G V I A D Y N Y L P D D F M G C V L A W N T N I D A T S T G N Y K Y R L R G K L R P F E D I S N V F S P D G K C T P A L N C W P L N D Y G F T T T G I G Y Q P R V V V L S F
4	3	C P F S E V F N A T I F P S V Y A W E R K K I S N C V A D Y S V L N S T F S T F A C Y G V S A T L N D L C F S N Y A D S F V K G D D V Q I A P G Q T G V I A D Y N Y L P D D F M G C V L A W N T N I D A T S T G N Y K Y R L R G K L R P F E D I S N V F S P D G K C T P A L N C W P L N D Y G F T T T G I G Y Q P R V V V L S F
5	1	C P F S E V F N A T I F P S V Y A W E R K K I S N C V A D Y S V L N S T F S T F A C Y G V S A T L N D L C F S N Y A D S F V K G D D V Q I A P G Q T G V I A D Y N Y L P D D F M G C V L A W N T N I D A T S T G N Y K Y R L R G K L R P F E D I S N V F S P D G K C T P A L N C W P L N D Y G F T T T G I G Y Q P R V V V L S F
5	2	C P F S E V F N A T I F P S V Y A W E R K K I S N C V A D Y S V L N S T F S T F A C Y G V S A T L N D L C F S N Y A D S F V K G D D V Q I A P G Q T G V I A D Y N Y L P D D F M G C V L A W N T N I D A T S T G N Y K Y R L R G K L R P F E D I S N V F S P D G K C T P A L N C W P L N D Y G F T T T G I G Y Q P R V V V L S F
5	3	C P F S E V F N A T I F P S V Y A W E R K K I S N C V A D Y S V L N S T F S T F A C Y G V S A T L N D L C F S N Y A D S F V K G D D V Q I A P G Q T G V I A D Y N Y L P D D F M G C V L A W N T N I D A T S T G N Y K Y R L R G K L R P F E D I S N V F S P D G K C T P A L N C W P L N D Y G F T T T G I G Y Q P R V V V L S F

Fig S40 – Translated Sanger sequencing results for randomly selected SARS-CoV-1 affinity maturation clones. Residues identical to the SARS-CoV-1 WT sequence are shown in gray, whereas mutated amino acids are indicated in white.

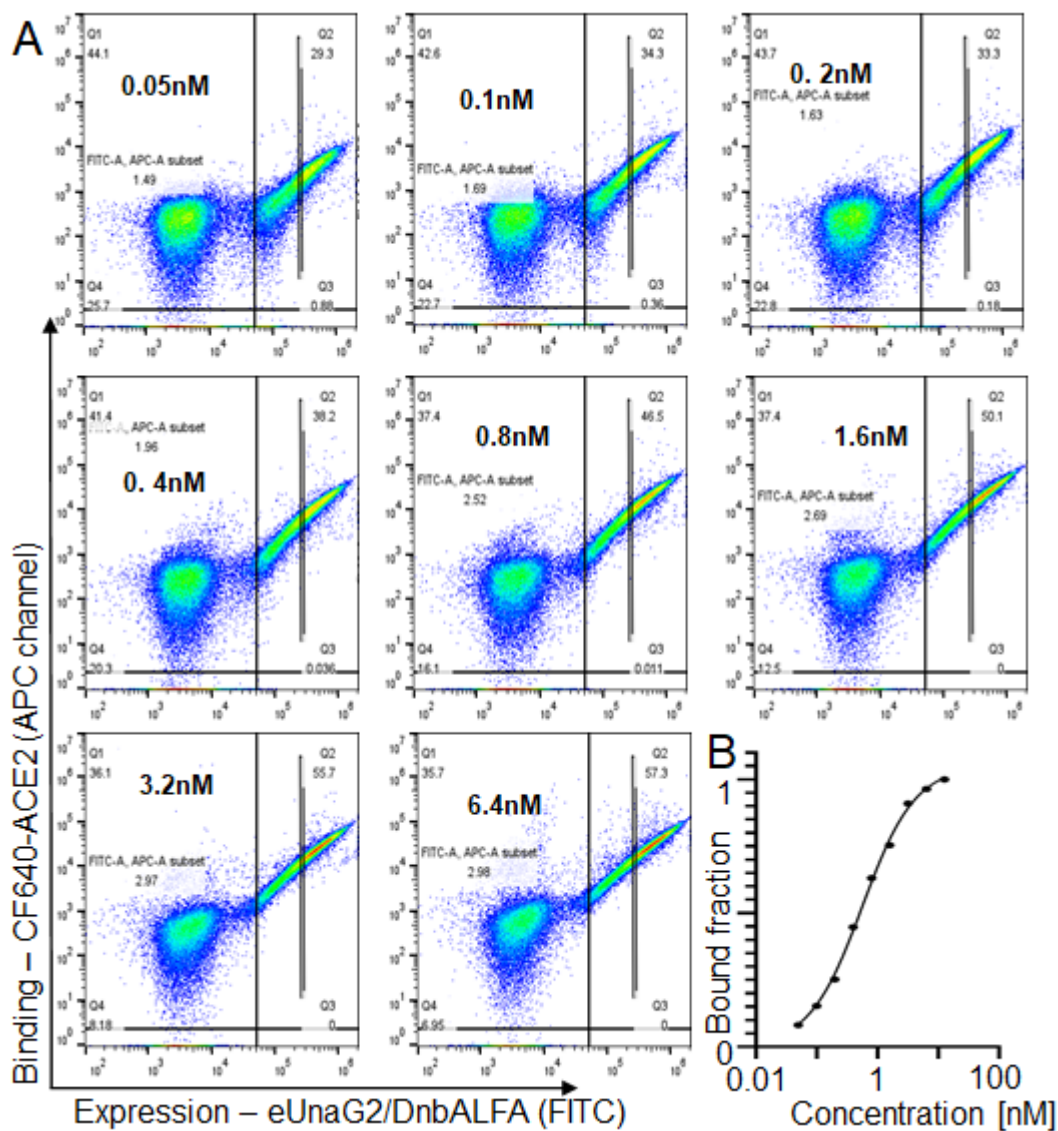
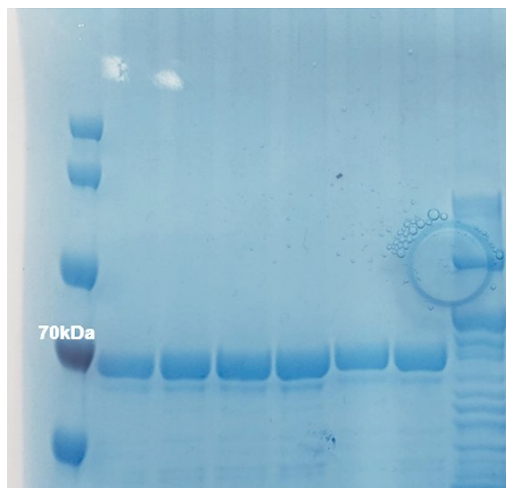
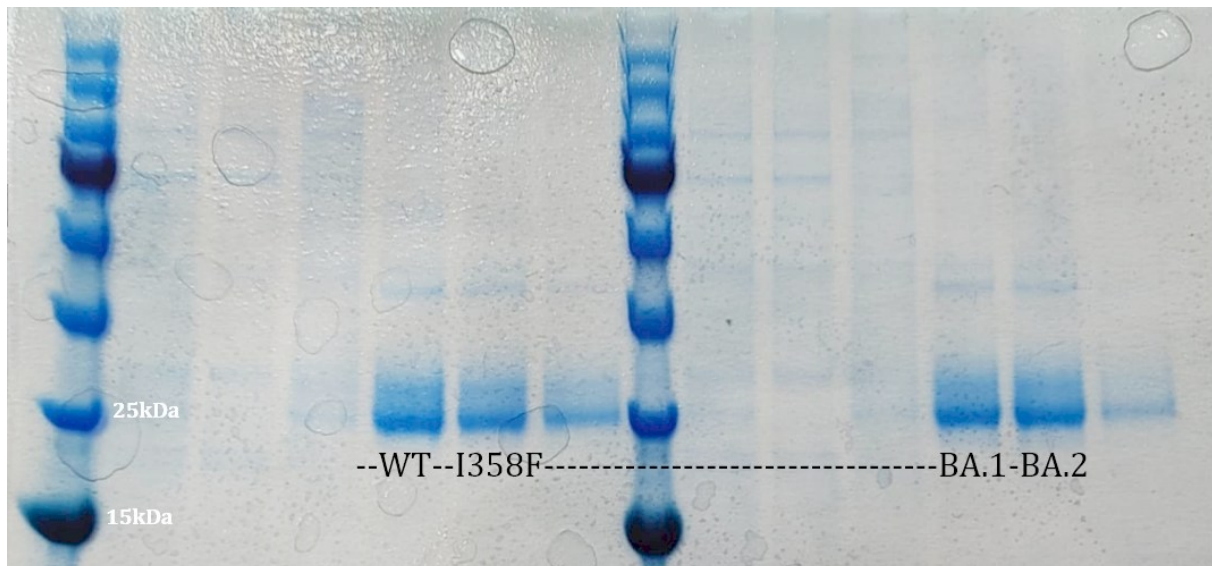
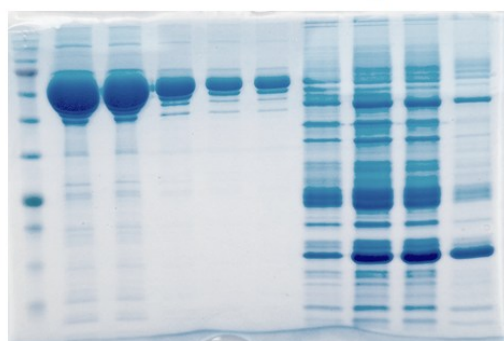


Fig. S41 – Gating strategy for yeast display-based binding affinity measurements. (A) A narrow vertical subset/subpopulation was defined to normalize the expression rates (FITC-A signal intensity, x-axis 10^0 - 10^6) across samples. Specific binding (APC-A signal intensity, y-axis 10^0 - 10^7) for each measurement was determined by subtracting the negative binding (Q1 median APC-A intensity) from the positive binding (Q2>subset median APC-A intensity). (B) Specific binding values at each ACE-2 concentration were fitted using GraphPad (Methods).



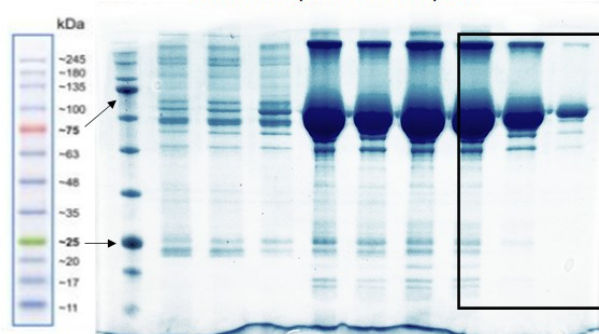
ACE2D-NiNTA-bdSUMO-cleavage



Sumo protease cleavage
fractions
(concentrated and
subjected to Superdex 75
16/600)

Imidazole elution fractions
after protease cleavage
(NiNTA resin bound fraction
control)

ACE2D-Superdex 75 16/600



Used in subsequent
Experiments

Source data for Fig. S2 – Uncropped gels and full purification process for ACE2D.