Fig. S1.

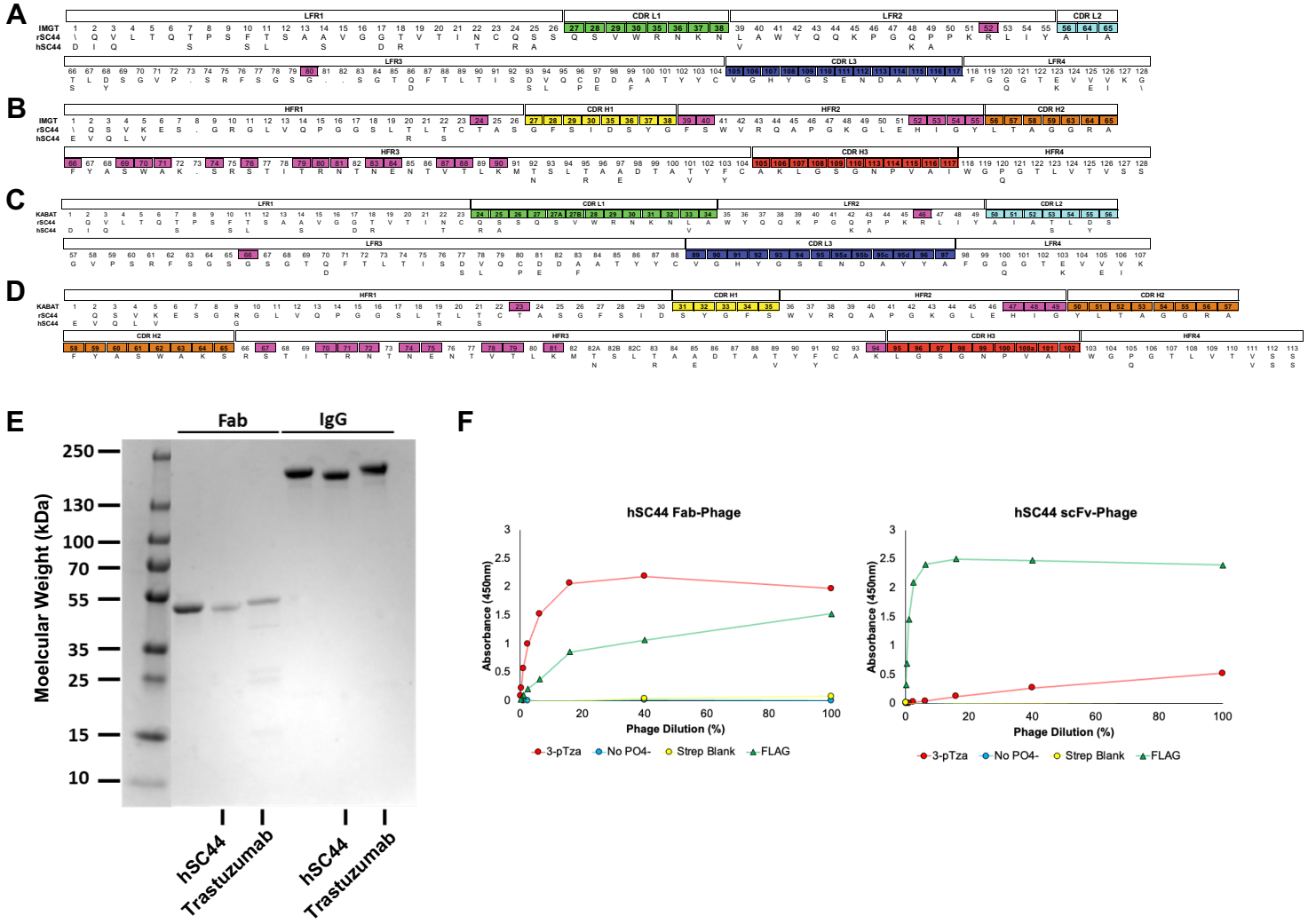
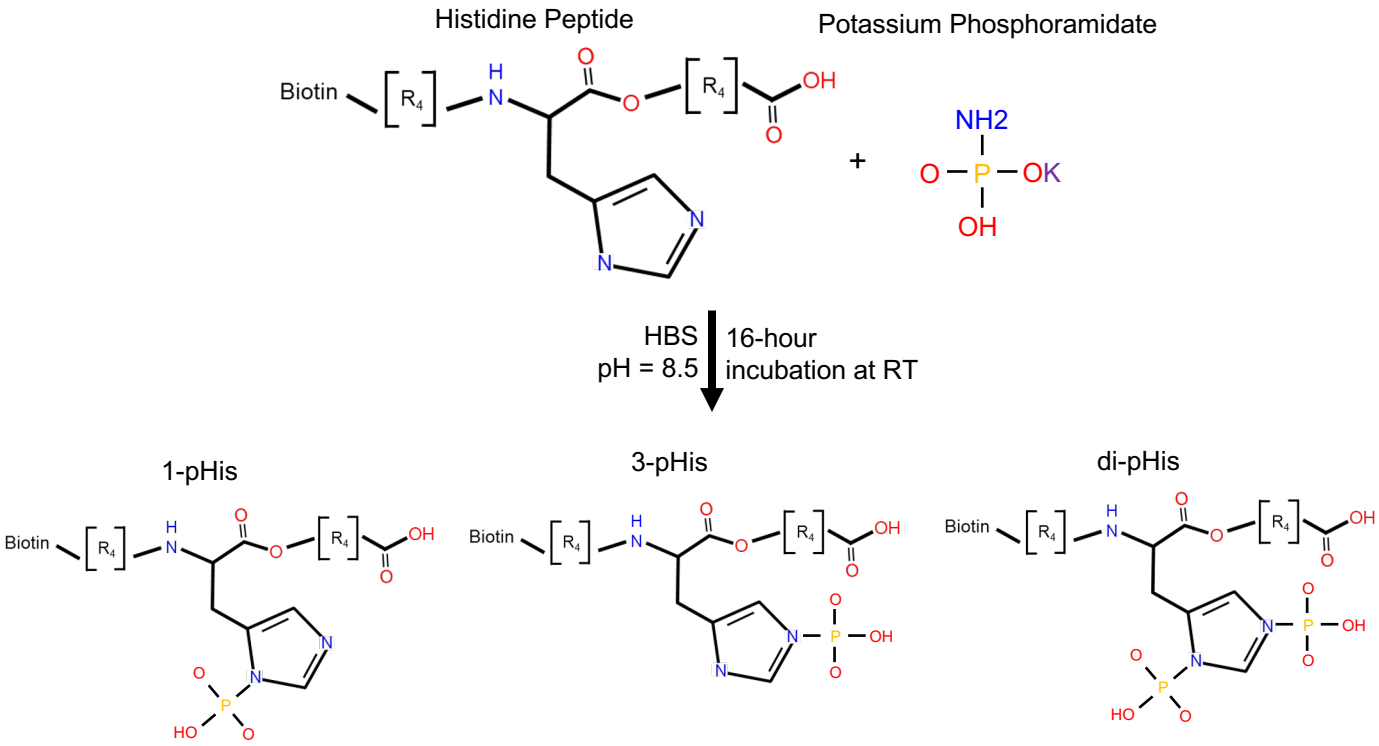

A
B
C
D
E
Fab
IgG
250
130
100
70
55
35
25
15
10
Moelcular Weight (kDa)
hSC44
Trastuzumab
hSC44
Trastuzumab
F
hSC44 Fab-Phage
hSC44 scFv-Phage
Absorbance (450nm)
Phage Dilution (%)
3-pTza
No PO4-
Strep Blank
FLAG

Fig. S2.

1. Phosphoramidate Reaction



2. Phosphohistidine Immunoprecipitation

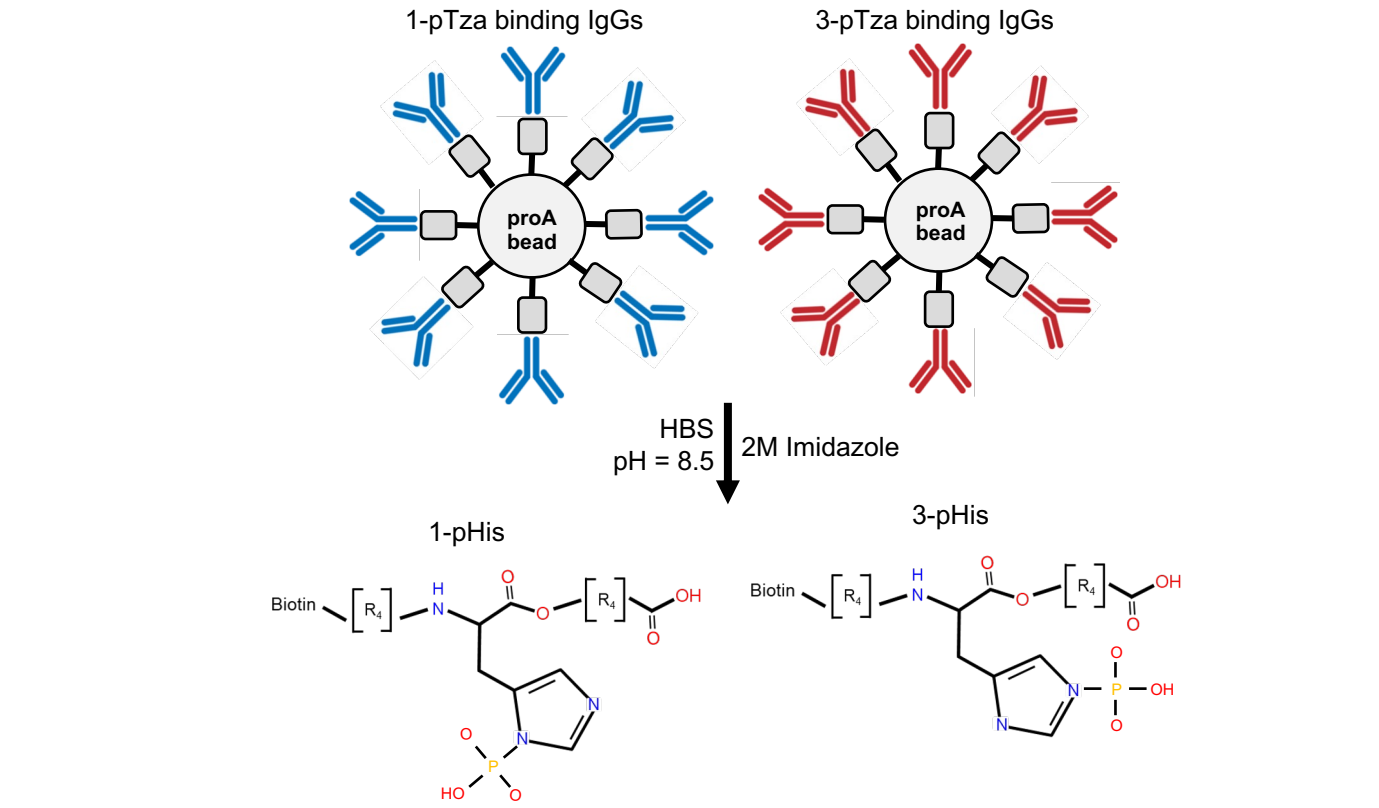


Fig. S3.

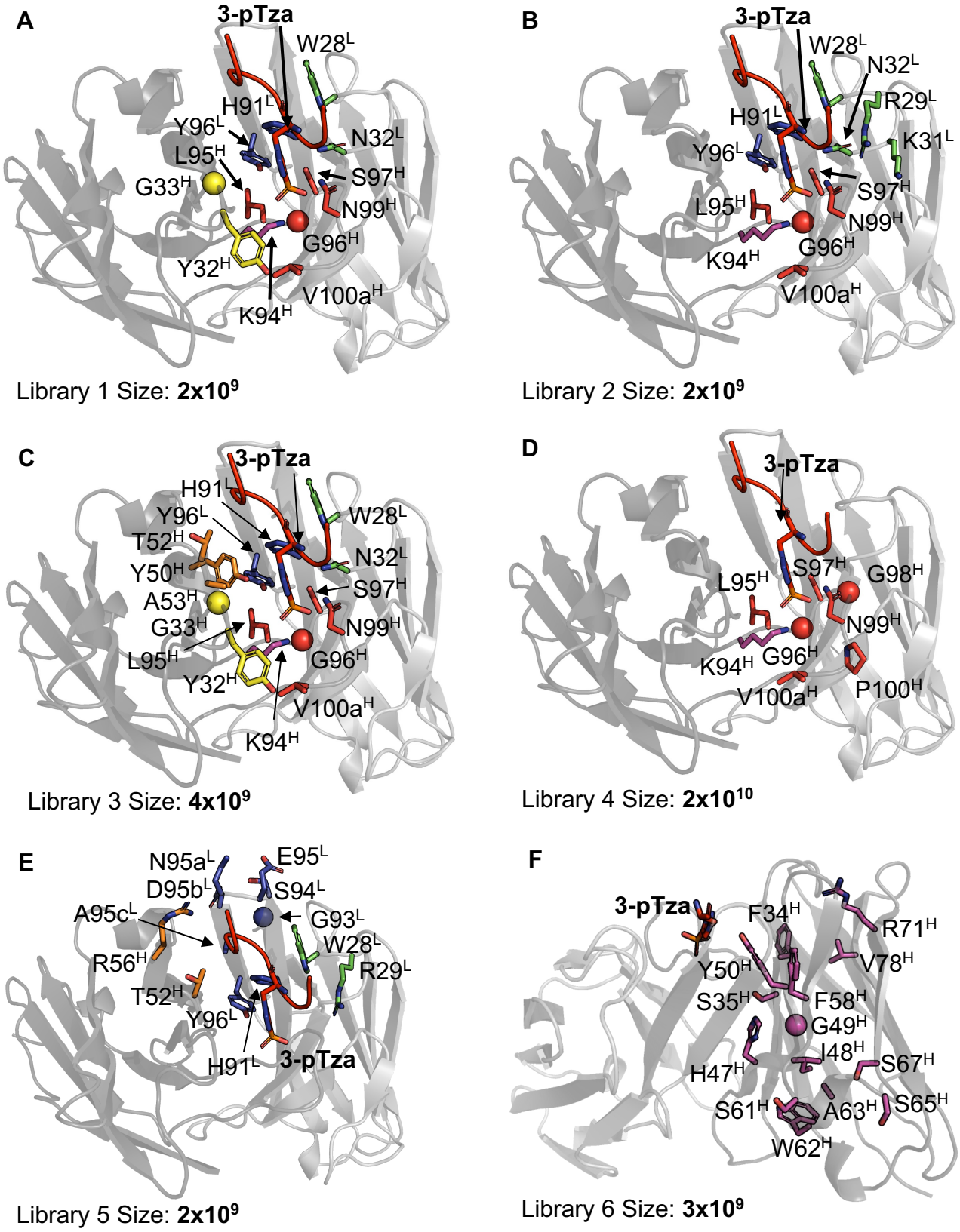

A
3-pTza
W28L
H91L
Y96L
N32L
L95H
S97H
G33H
N99H
G96H
Y32H
V100aH
K94H
Library 1 Size: 2x10^9
B
3-pTza
W28L
N32L
R29L
H91L
K31L
Y96L
S97H
L95H
N99H
G96H
K94H
V100aH
Library 2 Size: 2x10^9
C
3-pTza
H91L
Y96L
W28L
T52H
N32L
Y50H
S97H
A53H
G33H
N99H
L95H
G96H
Y32H
V100aH
K94H
Library 3 Size: 4x10^9
D
3-pTza
S97H
G98H
L95H
N99H
K94H
G96H
V100aH
P100H
Library 4 Size: 2x10^10
E
N95aL
E95L
D95bL
S94L
A95cL
G93L
W28L
R56H
R29L
T52H
Y96L
H91L
3-pTza
Library 5 Size: 2x10^9
F
3-pTza
F34H
R71H
Y50H
V78H
S35H
F58H
G49H
I48H
S67H
H47H
S61H
A63H
S65H
W62H
Library 6 Size: 3x10^9

Fig. S4.

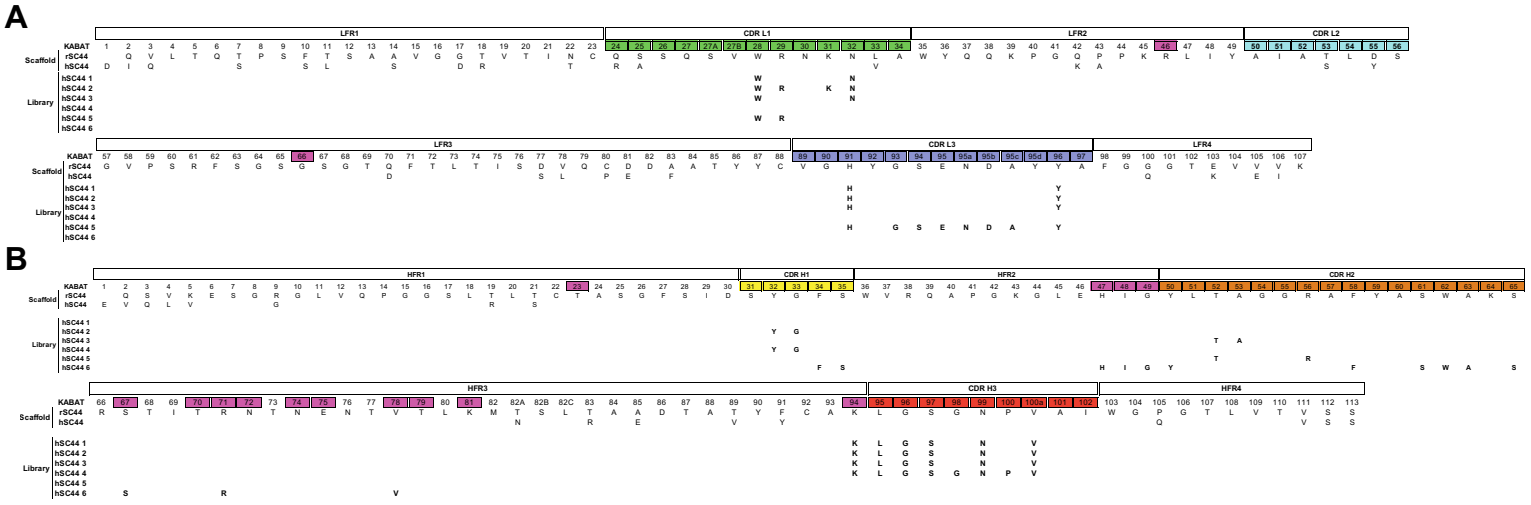

A
LFR1
CDR L1
LFR2
CDR L2
LFR3
CDR L3
LFR4
KABAT
rSC44
hSC44
Scaffold
Library
hSC44 1
hSC44 2
hSC44 3
hSC44 4
hSC44 5
hSC44 6
B
HFR1
CDR H1
HFR2
CDR H2
HFR3
CDR H3
HFR4

Fig. S5.

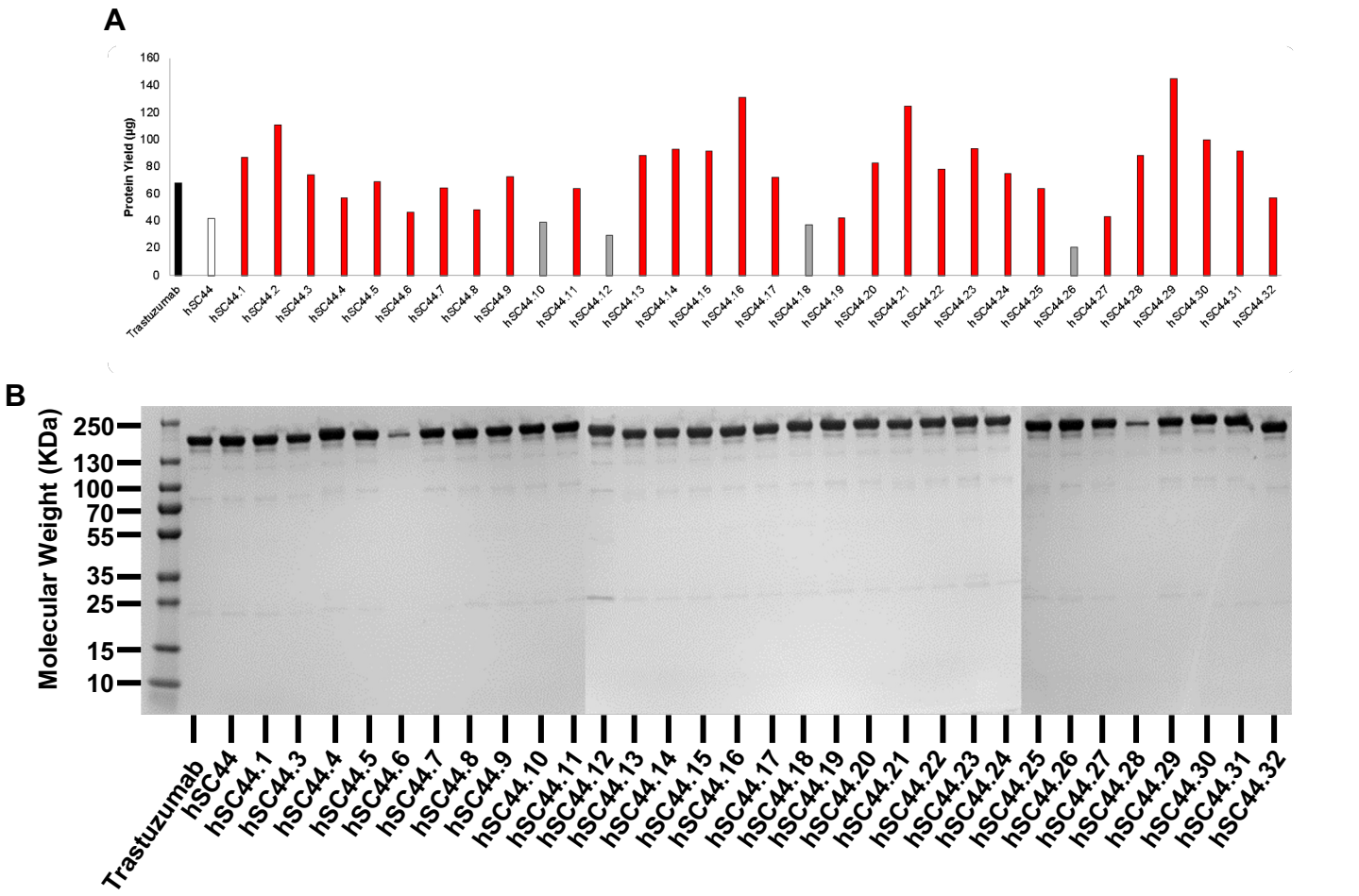

A
Protein Yield (µg)
0
20
40
60
80
100
120
140
160
Trastuzumab
hSC44
hSC44.1
hSC44.2
hSC44.3
hSC44.4
hSC44.5
hSC44.6
hSC44.7
hSC44.8
hSC44.9
hSC44.10
hSC44.11
hSC44.12
hSC44.13
hSC44.14
hSC44.15
hSC44.16
hSC44.17
hSC44.18
hSC44.19
hSC44.20
hSC44.21
hSC44.22
hSC44.23
hSC44.24
hSC44.25
hSC44.26
hSC44.27
hSC44.28
hSC44.29
hSC44.30
hSC44.31
hSC44.32
B
Molecular Weight (KDa)
250
130
100
70
55
35
25
15
10
Trastuzumab
hSC44
hSC44.1
hSC44.3
hSC44.4
hSC44.5
hSC44.6
hSC44.7
hSC44.8
hSC44.9
hSC44.10
hSC44.11
hSC44.12
hSC44.13
hSC44.14
hSC44.15
hSC44.16
hSC44.17
hSC44.18
hSC44.19
hSC44.20
hSC44.21
hSC44.22
hSC44.23
hSC44.24
hSC44.25
hSC44.26
hSC44.27
hSC44.28
hSC44.29
hSC44.30
hSC44.31
hSC44.32
C
Selected on 3-pTza (pools a/b)
Selected on 3-pHis (pools c/d)
Fold Change in EC50 (3-pHis) over hSC44
0
2
4
6
8
10
12
hSC44
hSC44.8
hSC44.20
hSC44.28
hSC44.29
hSC44.30
hSC44.31
hSC44.32
D
hSC44.20
hSC44.20.N32Y^L
hSC44.20.N32F^L
Absorbance (450 nm)
0
0.5
1
1.5
2
0.0
0.2
2.0
Peptide Concentration (Log10(ug/mL))
3-pTza
3-pHis
pTyr
pSer
pThr

Fig. S6.

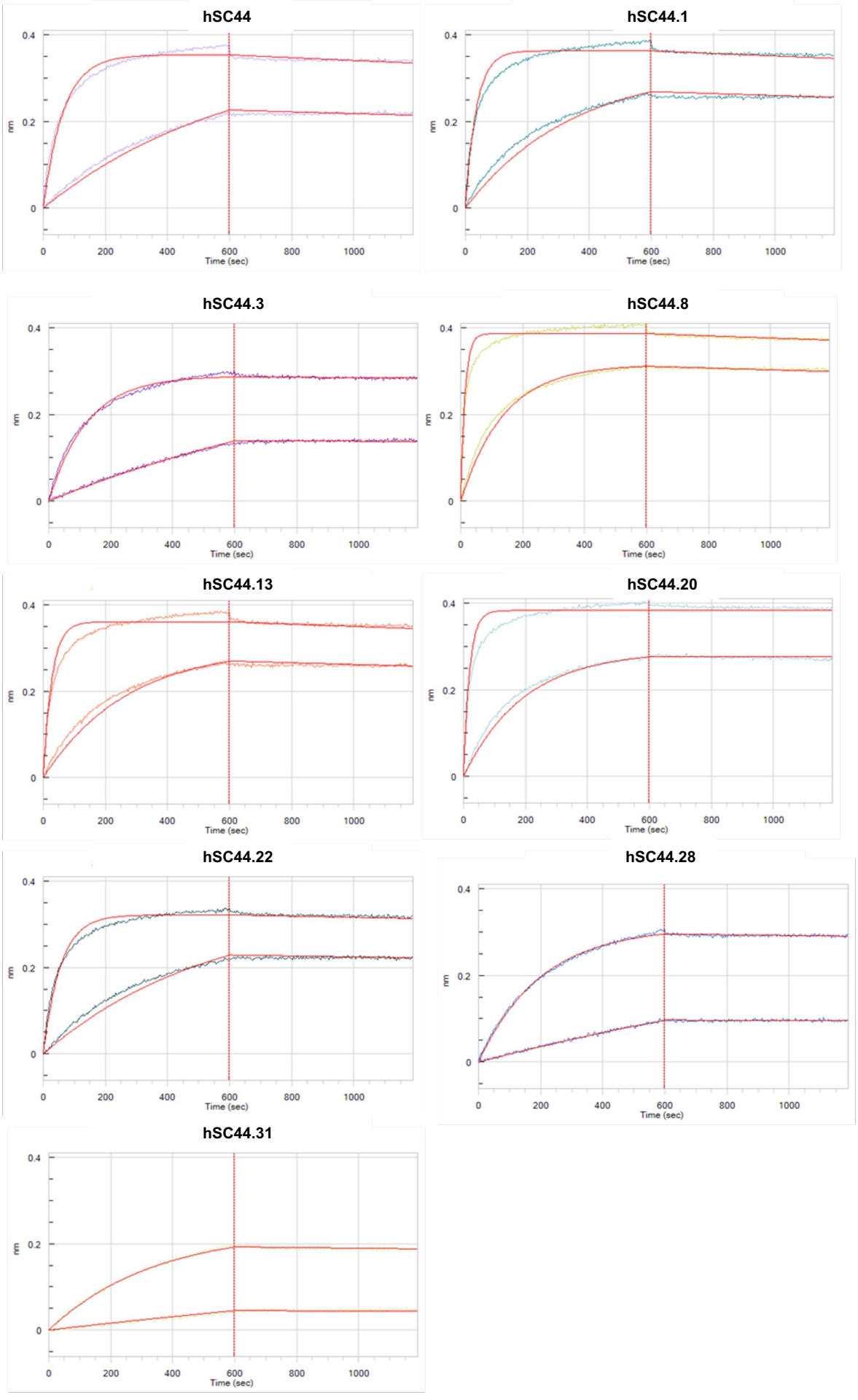

hSC44
hSC44.1
hSC44.3
hSC44.8
hSC44.13
hSC44.20
hSC44.22
hSC44.28
hSC44.31
nm
Time (sec)
0
0.2
0.4
0
200
400
600
800
1000

Fig. S7.

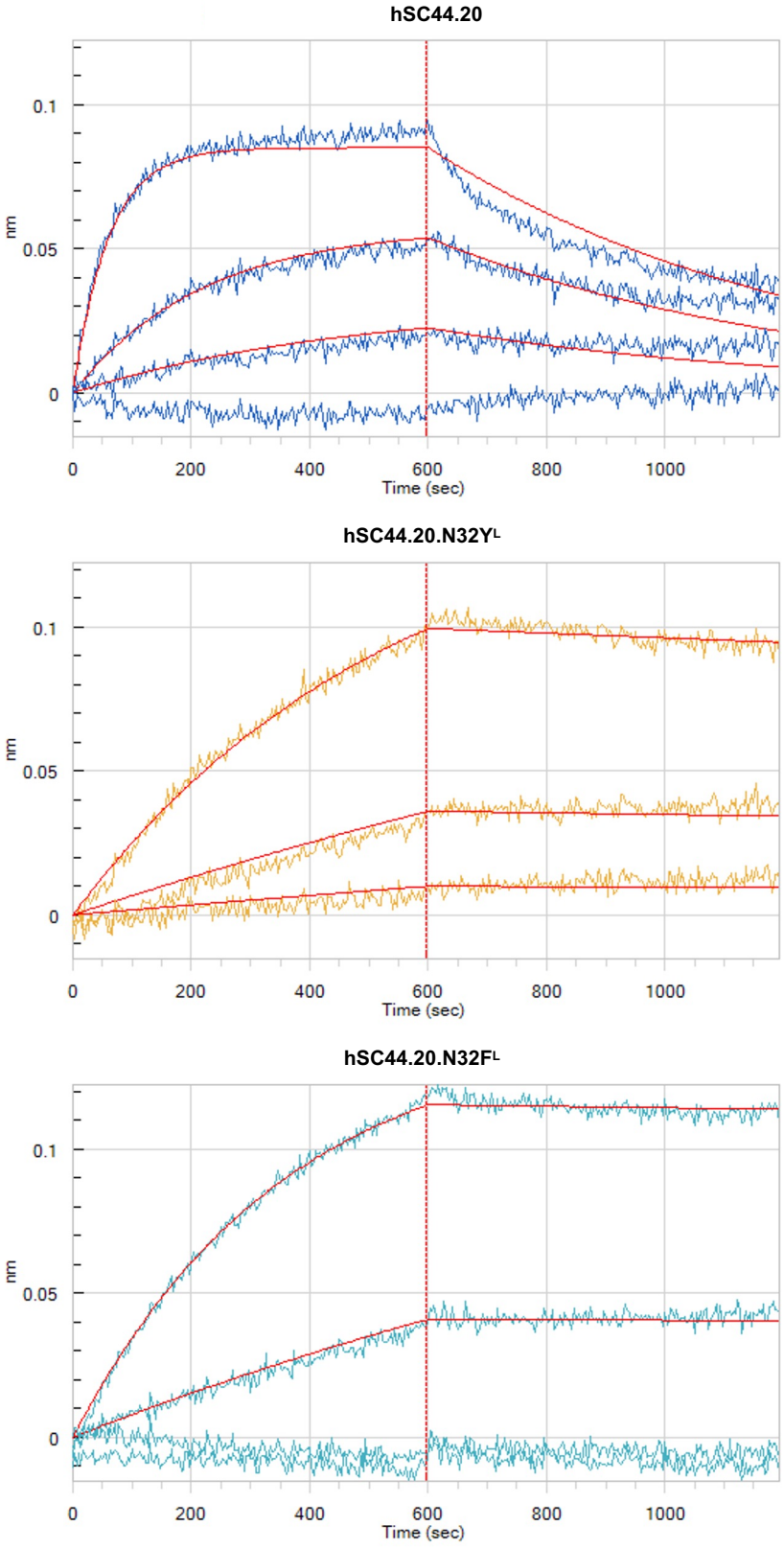

hSC44.20
0.1
0.05
0
nm
0
200
400
600
800
1000
Time (sec)
hSC44.20.N32Y^L
0.1
0.05
0
nm
0
200
400
600
800
1000
Time (sec)
hSC44.20.N32F^L
0.1
0.05
0
nm
0
200
400
600
800
1000
Time (sec)

Fig. S8.

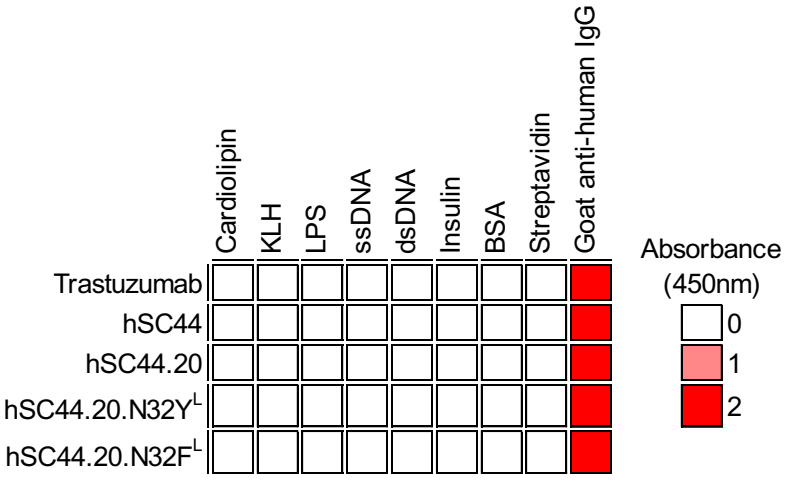

Cardiolipin
KLH
LPS
ssDNA
dsDNA
Insulin
BSA
Streptavidin
Goat anti-human IgG
Trastuzumab
hSC44
hSC44.20
hSC44.20.N32Y^L
hSC44.20.N32F^L
Absorbance
(450nm)
0
1
2

Fig. S9.

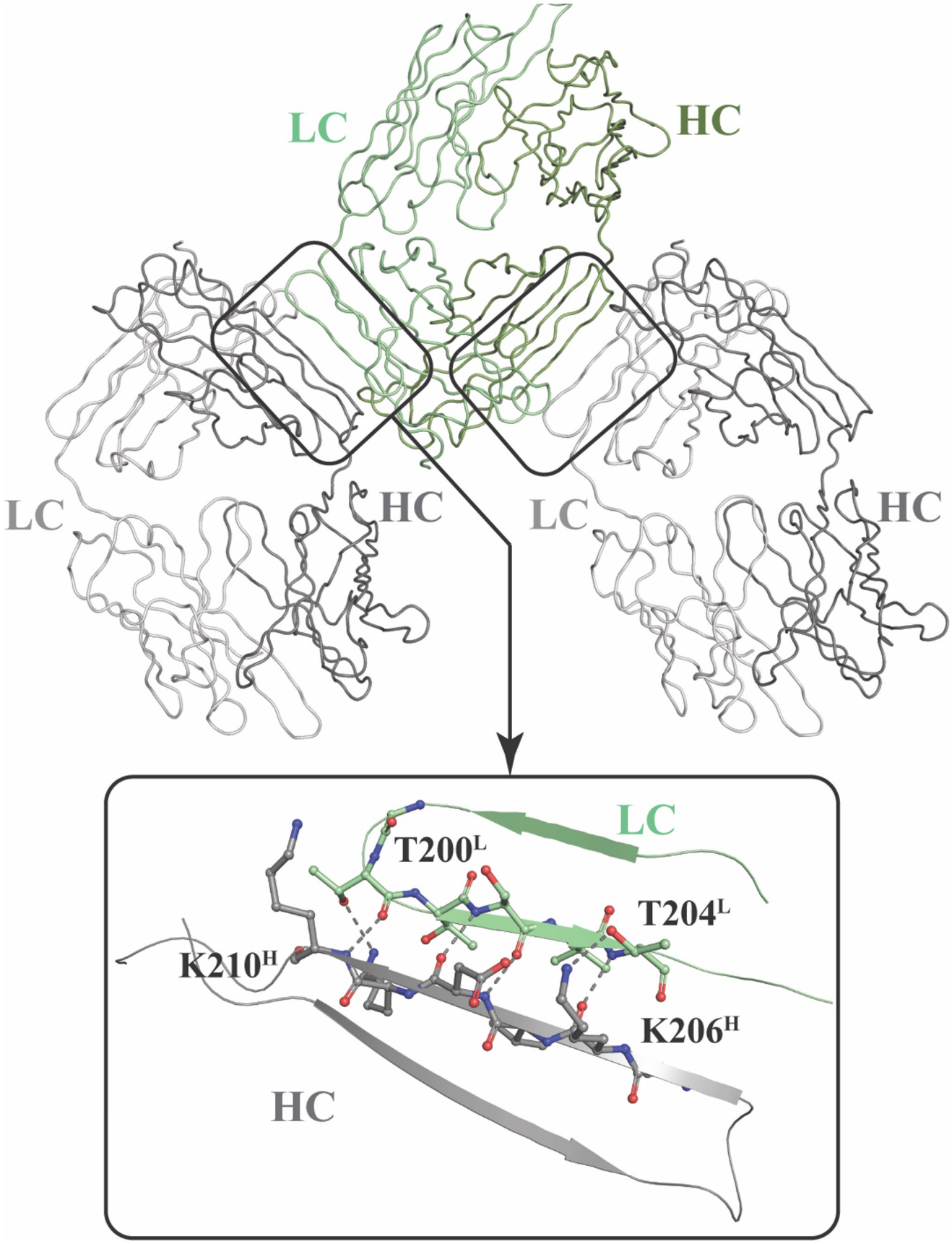


Fig. S10.

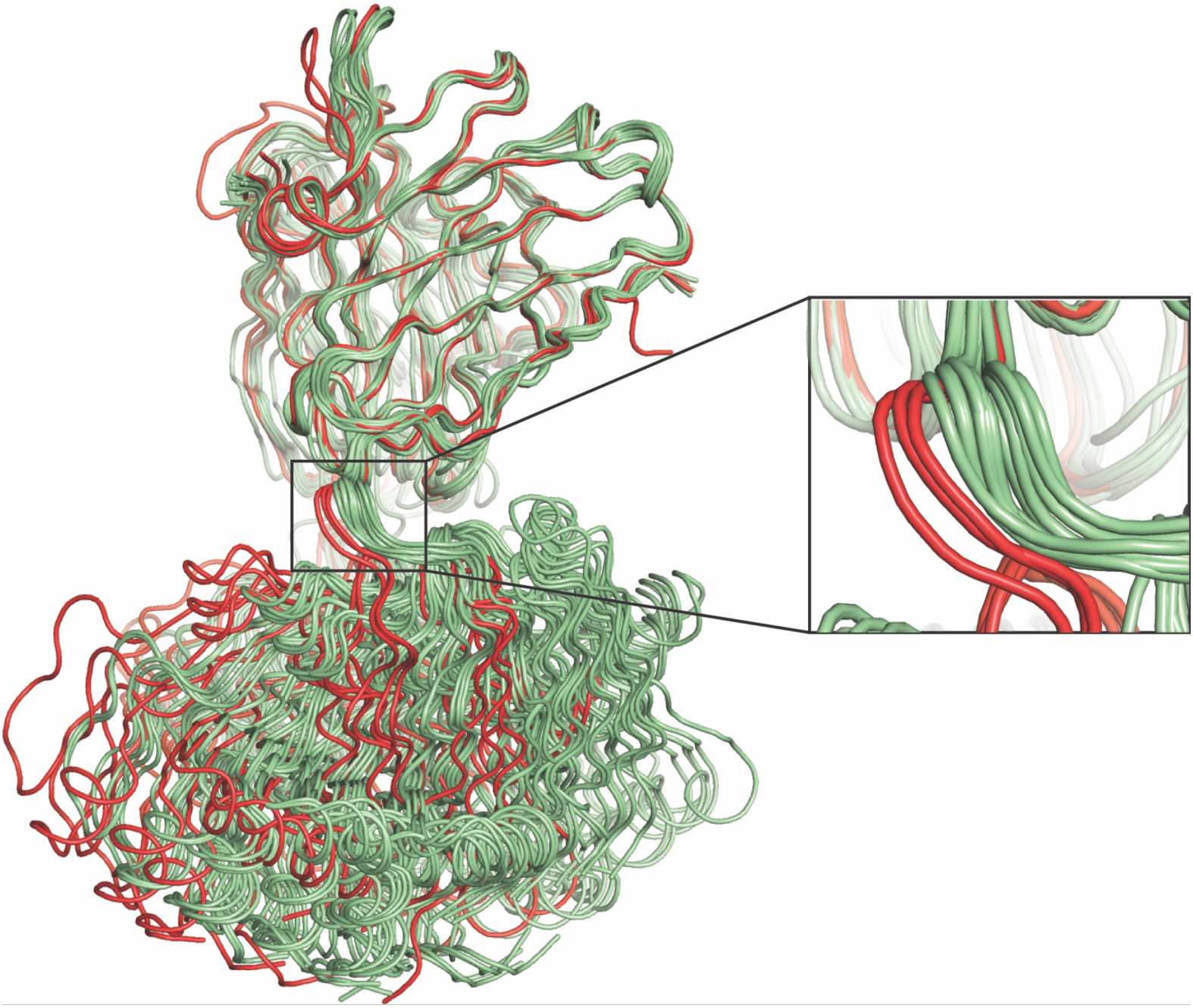


Fig. S11.

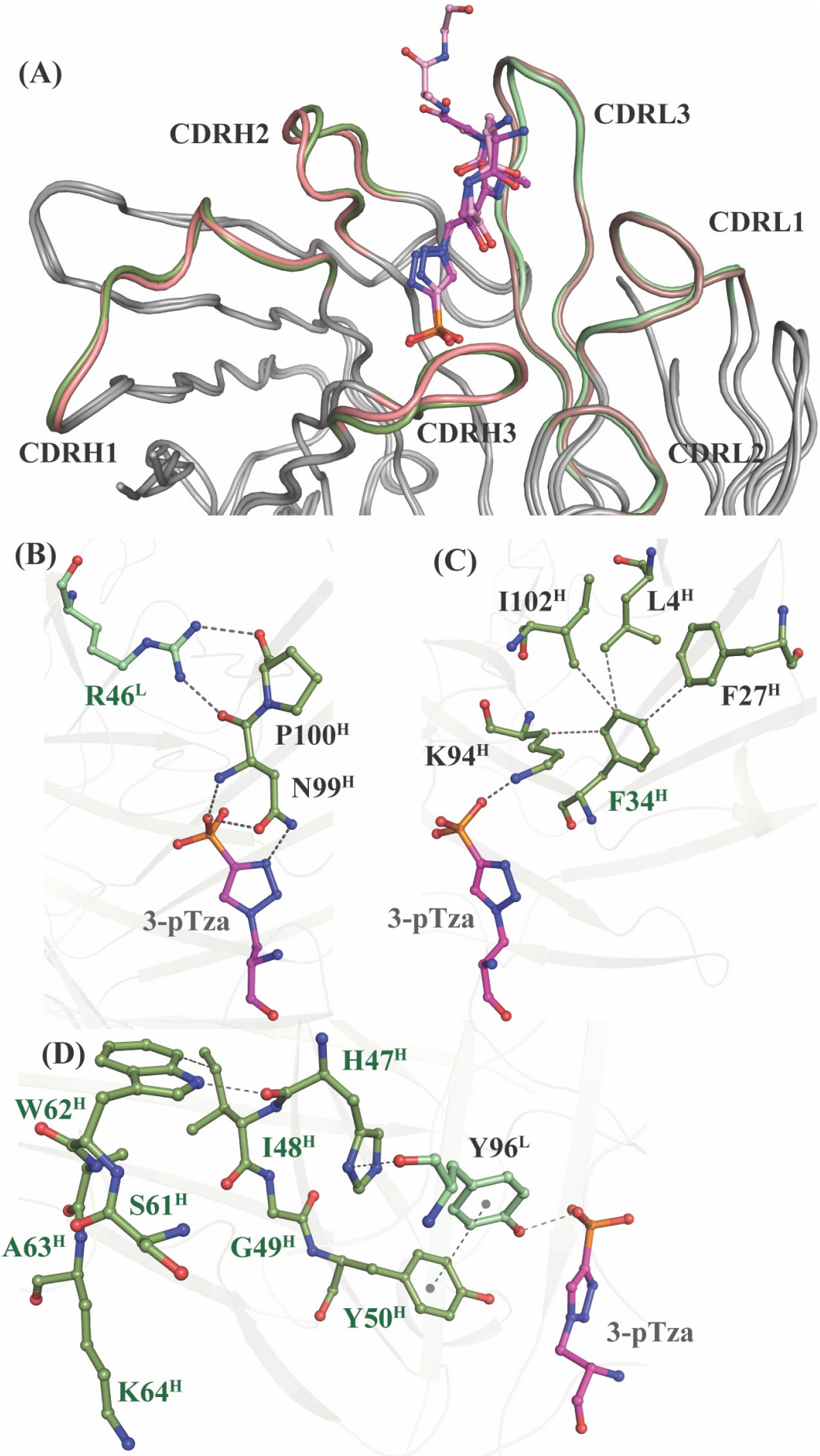

(A)
CDRH2
CDRL3
CDRL1
CDRH1
CDRH3
CDRL2
(B)
R46L
P100H
N99H
3-pTza
(C)
I102H
L4H
F27H
K94H
F34H
3-pTza
(D)
H47H
W62H
I48H
Y96L
S61H
A63H
G49H
Y50H
3-pTza
K64H

Fig. S12.

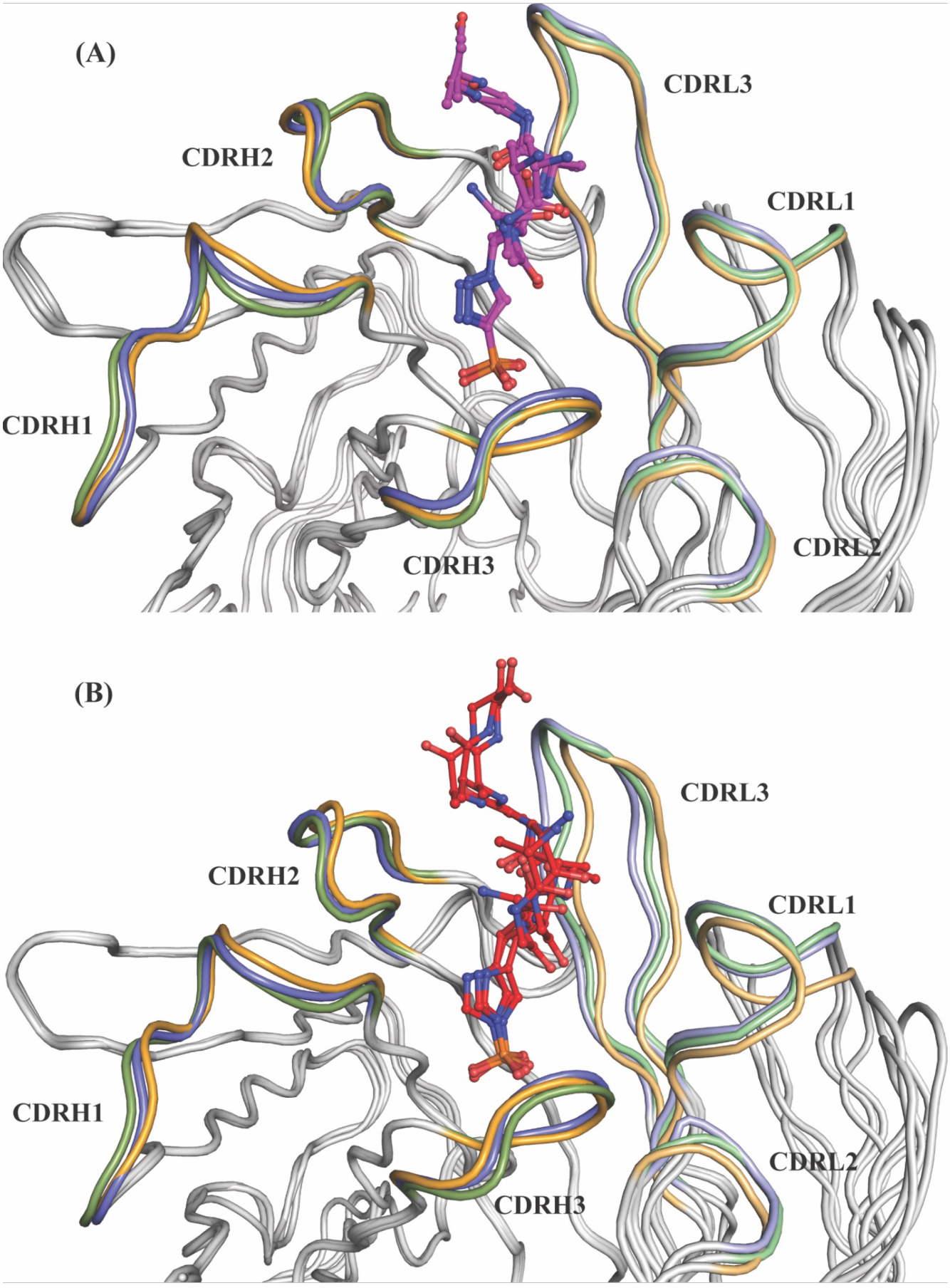

(A)
CDRL3
CDRH2
CDRL1
CDRH1
CDRH3
CDRL2
(B)
CDRL3
CDRH2
CDRL1
CDRH1
CDRL2
CDRH3

Fig. S13.

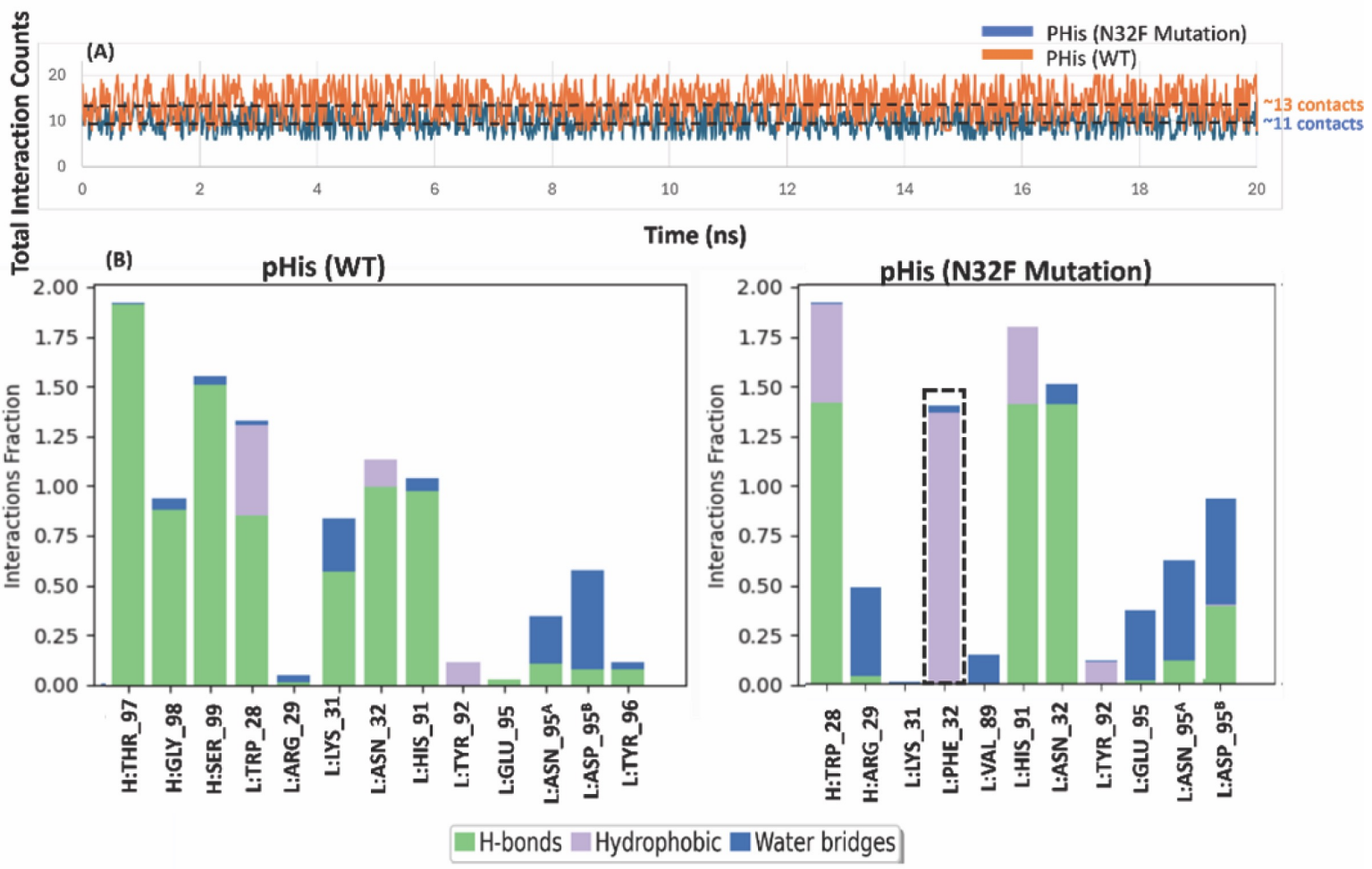

(A)
Total Interaction Counts
PHis (N32F Mutation)
PHis (WT)
~13 contacts
~11 contacts
Time (ns)
(B)
pHis (WT)
pHis (N32F Mutation)
Interactions Fraction
H:THR_97
H:GLY_98
H:SER_99
L:TRP_28
L:ARG_29
L:LYS_31
L:ASN_32
L:HIS_91
L:TYR_92
L:GLU_95
L:ASN_95A
L:ASP_95B
L:TYR_96
H:TRP_28
H:ARG_29
L:LYS_31
L:PHE_32
L:VAL_89
L:HIS_91
L:ASN_32
L:TYR_92
L:GLU_95
L:ASN_95A
L:ASP_95B
H-bonds
Hydrophobic
Water bridges

Fig. S14.

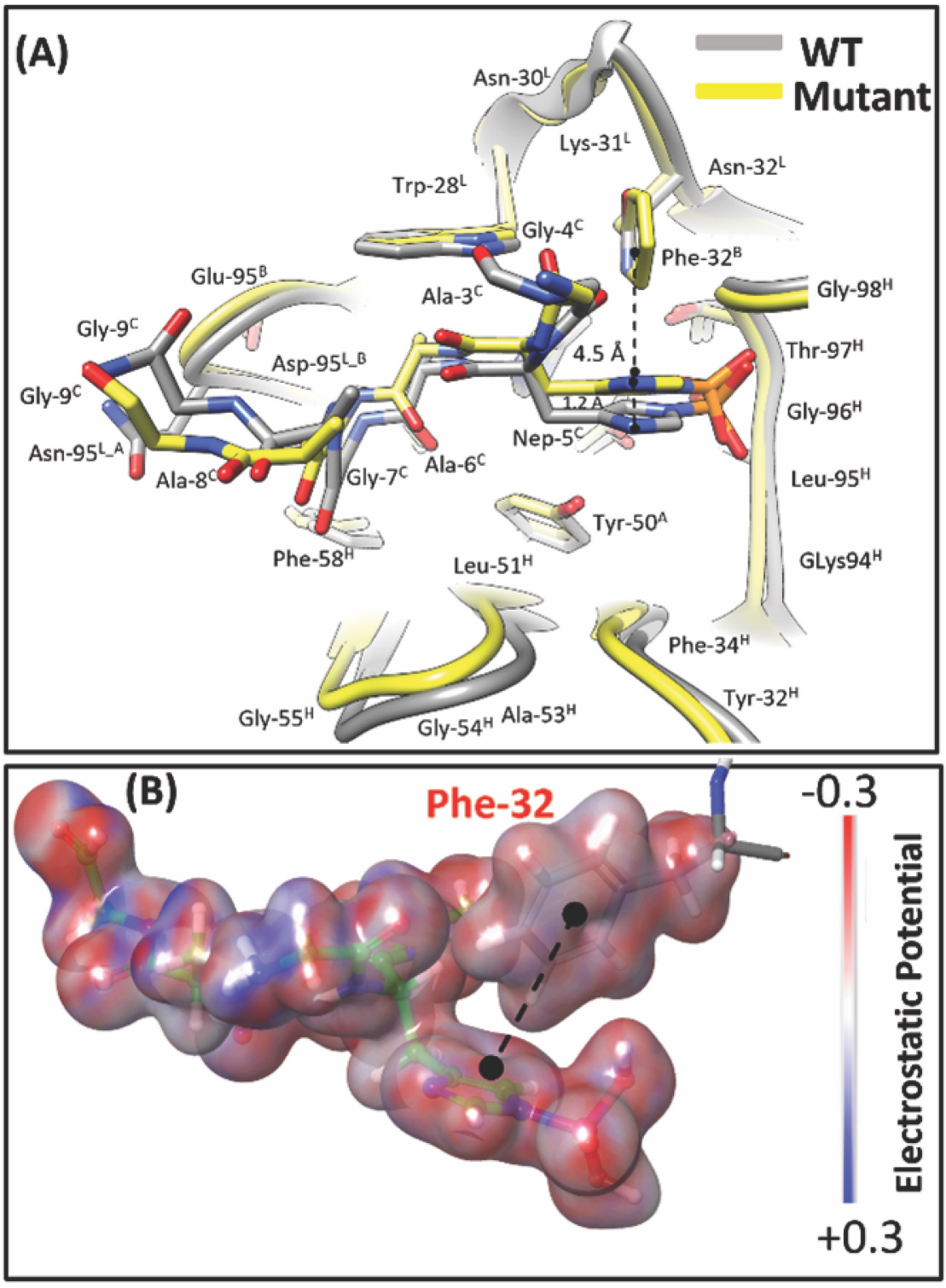

(A)
WT
Mutant
Asn-30L
Lys-31L
Asn-32L
Trp-28L
Gly-4C
Phe-32B
Glu-95B
Ala-3C
Gly-98H
Gly-9C
Asp-95L_B
4.5 Å
Thr-97H
Gly-9C
1.2 Å
Gly-96H
Nep-5C
Asn-95L_A
Ala-8C
Gly-7C
Ala-6C
Leu-95H
Tyr-50A
Phe-58H
Leu-51H
GLys94H
Phe-34H
Tyr-32H
Gly-55H
Gly-54H
Ala-53H
(B)
Phe-32
-0.3
Electrostatic Potential
+0.3

Fig. S15.

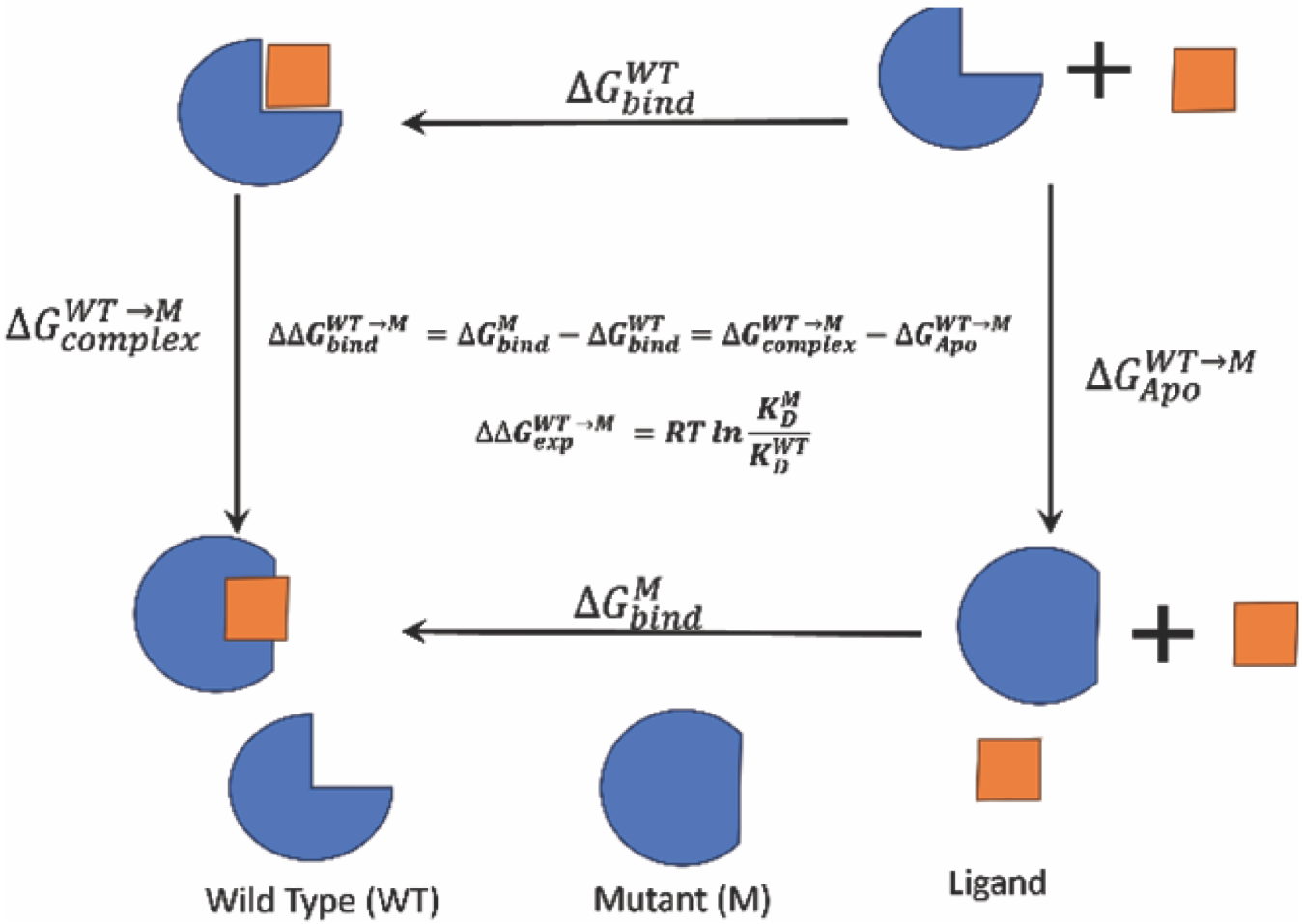

ΔG_{bind}^{WT}
$\Delta G_{complex}^{WT \rightarrow M}$
$\Delta\Delta G_{bind}^{WT \rightarrow M} = \Delta G_{bind}^{M} - \Delta G_{bind}^{WT} = \Delta G_{complex}^{WT \rightarrow M} - \Delta G_{Apo}^{WT \rightarrow M}$
$\Delta\Delta G_{exp}^{WT \rightarrow M} = RT\ ln \frac{K_D^M}{K_D^{WT}}$
$\Delta G_{Apo}^{WT \rightarrow M}$
ΔG_{bind}^{M}
Wild Type (WT)
Mutant (M)
Ligand

Fig. S16.

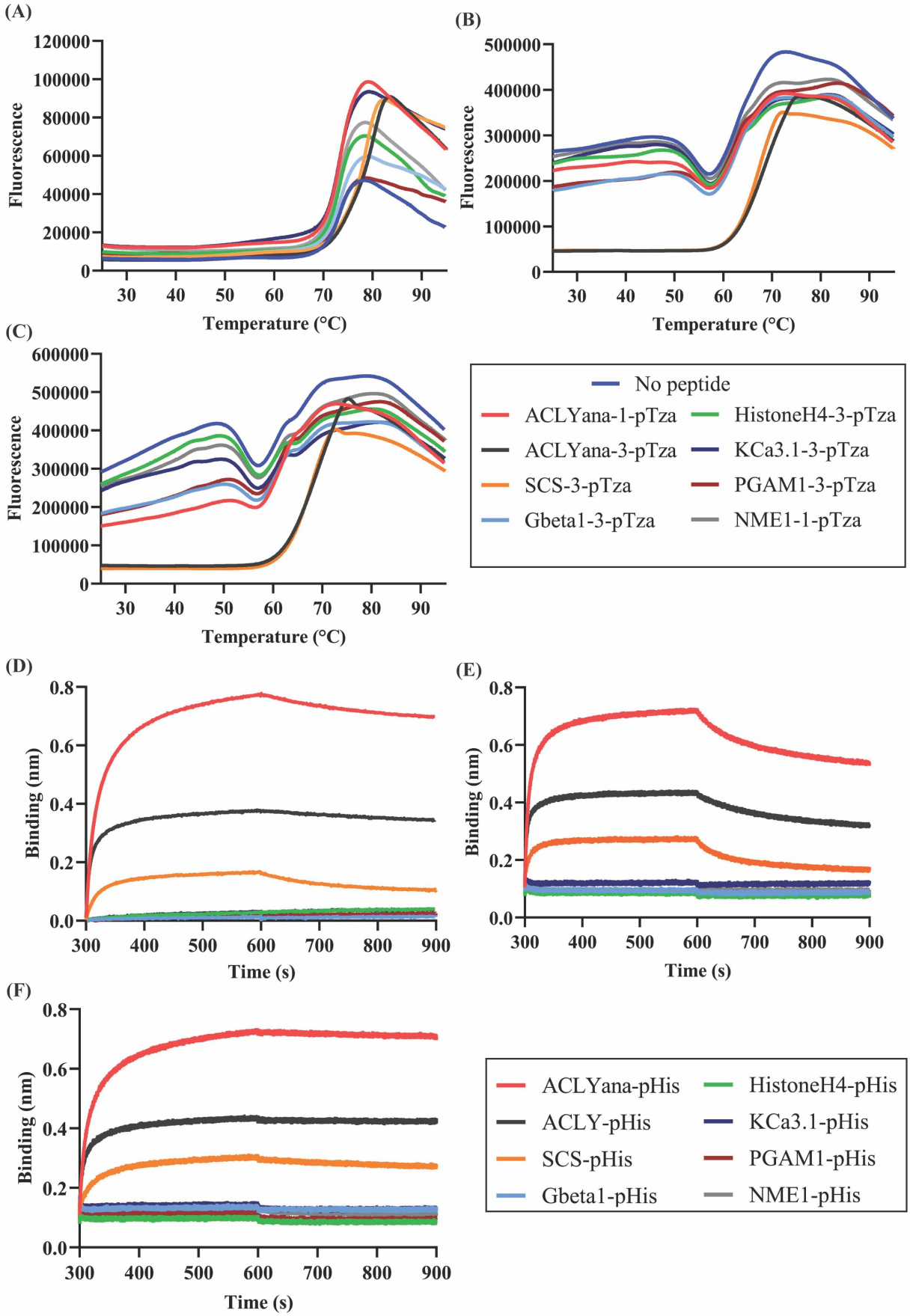

(A)
Fluorescence
Temperature (°C)
(B)
Fluorescence
Temperature (°C)
(C)
Fluorescence
Temperature (°C)
No peptide
ACLYana-1-pTza
ACLYana-3-pTza
SCS-3-pTza
Gbeta1-3-pTza
HistoneH4-3-pTza
KCa3.1-3-pTza
PGAM1-3-pTza
NME1-1-pTza
(D)
Binding (nm)
Time (s)
(E)
Binding (nm)
Time (s)
(F)
Binding (nm)
Time (s)
ACLYana-pHis
ACLY-pHis
SCS-pHis
Gbeta1-pHis
HistoneH4-pHis
KCa3.1-pHis
PGAM1-pHis
NME1-pHis

Fig. S17.

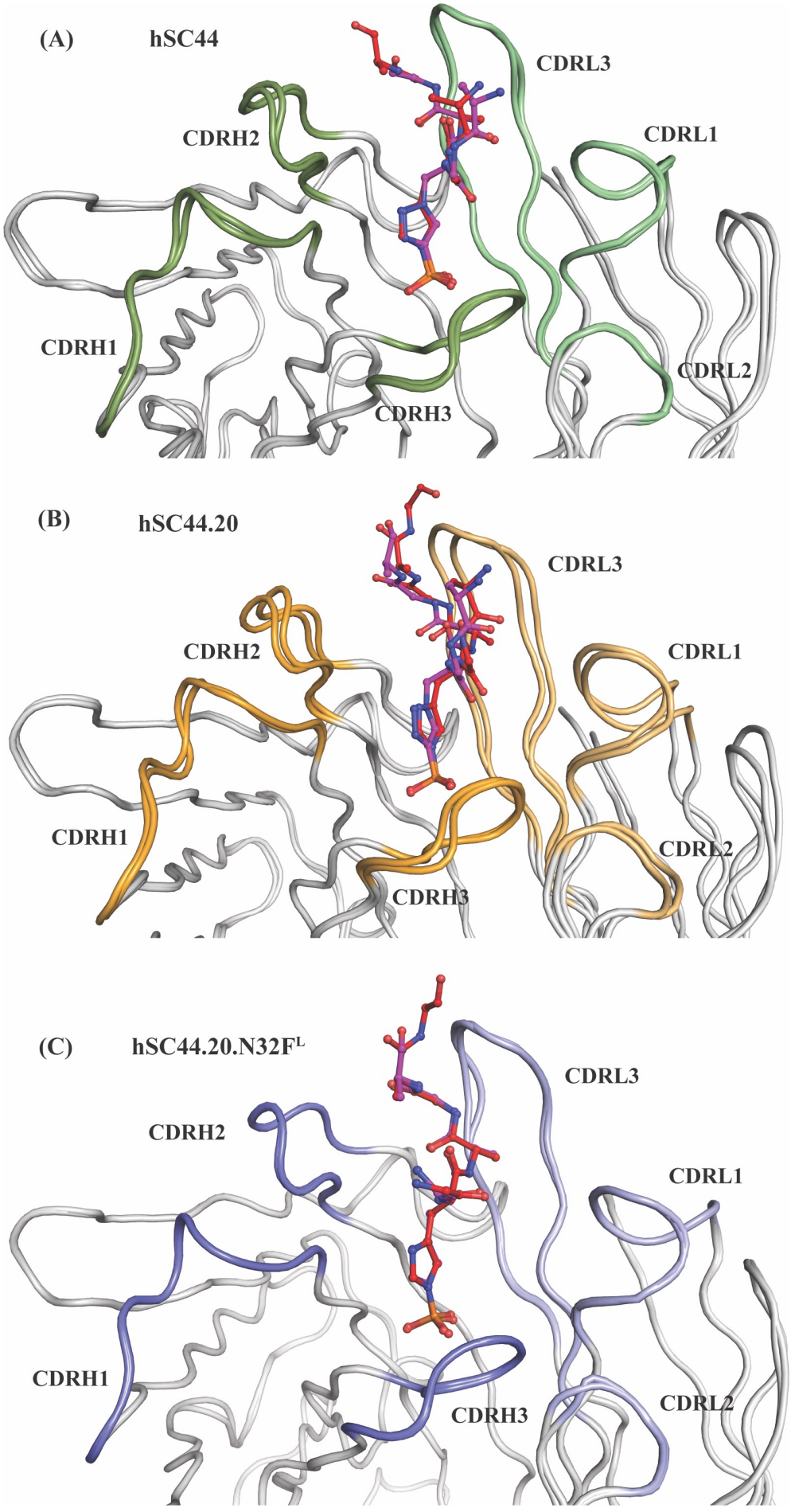

(A)
hSC44
CDRL3
CDRH2
CDRL1
CDRH1
CDRL2
CDRH3
(B)
hSC44.20
CDRL3
CDRH2
CDRL1
CDRH1
CDRL2
CDRH3
(C)
hSC44.20.N32F^L
CDRL3
CDRH2
CDRL1
CDRH1
CDRH3
CDRL2

Fig. S18.

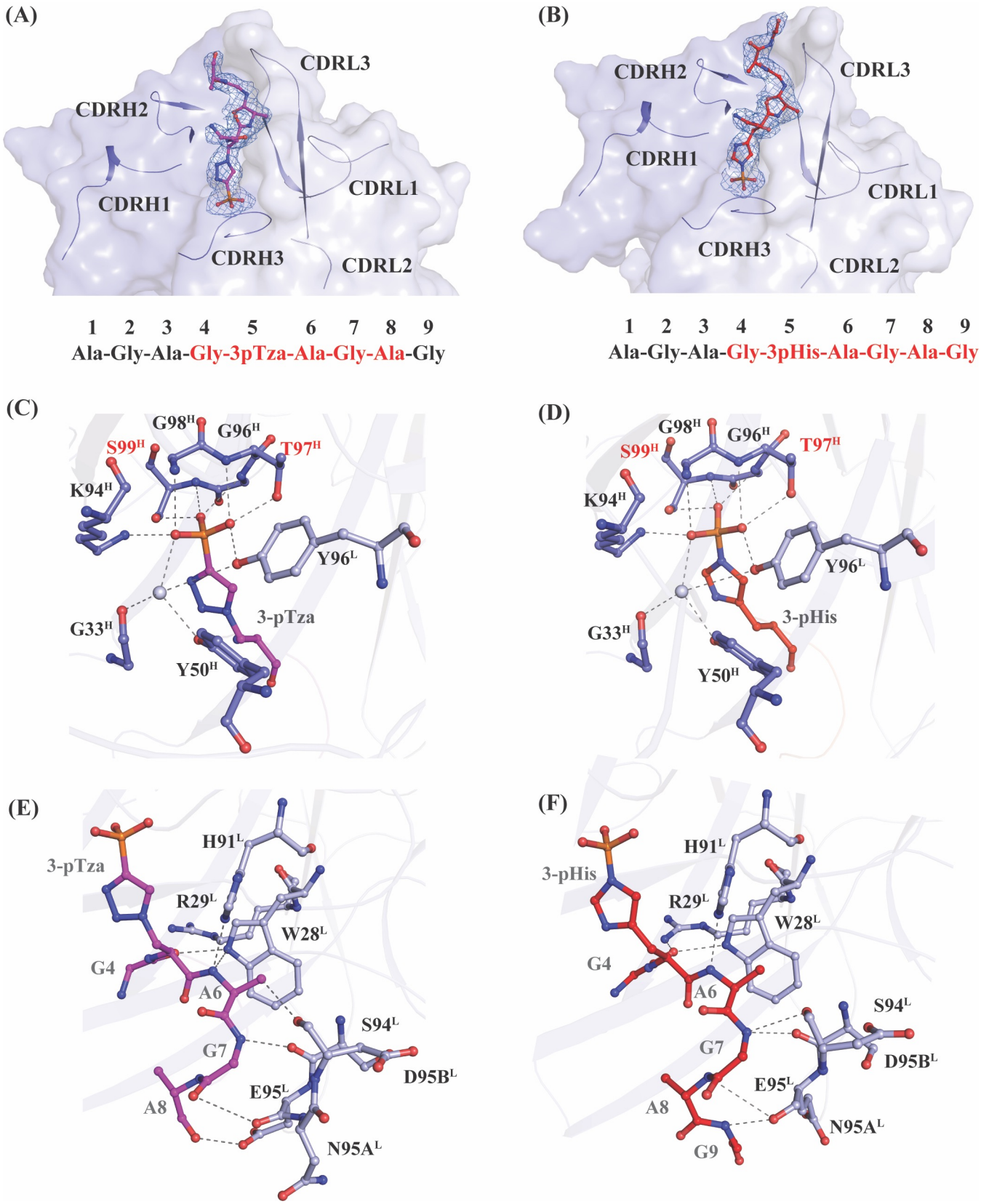

(A)
CDRL3
CDRH2
CDRL1
CDRH1
CDRH3
CDRL2
1 2 3 4 5 6 7 8 9
Ala-Gly-Ala-Gly-3pTza-Ala-Gly-Ala-Gly
(B)
CDRH2
CDRL3
CDRH1
CDRL1
CDRH3
CDRL2
1 2 3 4 5 6 7 8 9
Ala-Gly-Ala-Gly-3pHis-Ala-Gly-Ala-Gly
(C)
G98H
G96H
S99H
T97H
K94H
Y96L
3-pTza
G33H
Y50H
(D)
G98H
G96H
S99H
T97H
K94H
Y96L
3-pHis
G33H
Y50H
(E)
3-pTza
H91L
R29L
W28L
G4
A6
S94L
G7
D95BL
E95L
A8
N95AL
(F)
3-pHis
H91L
R29L
W28L
G4
A6
S94L
G7
D95BL
E95L
A8
N95AL
G9

Fig. S19.

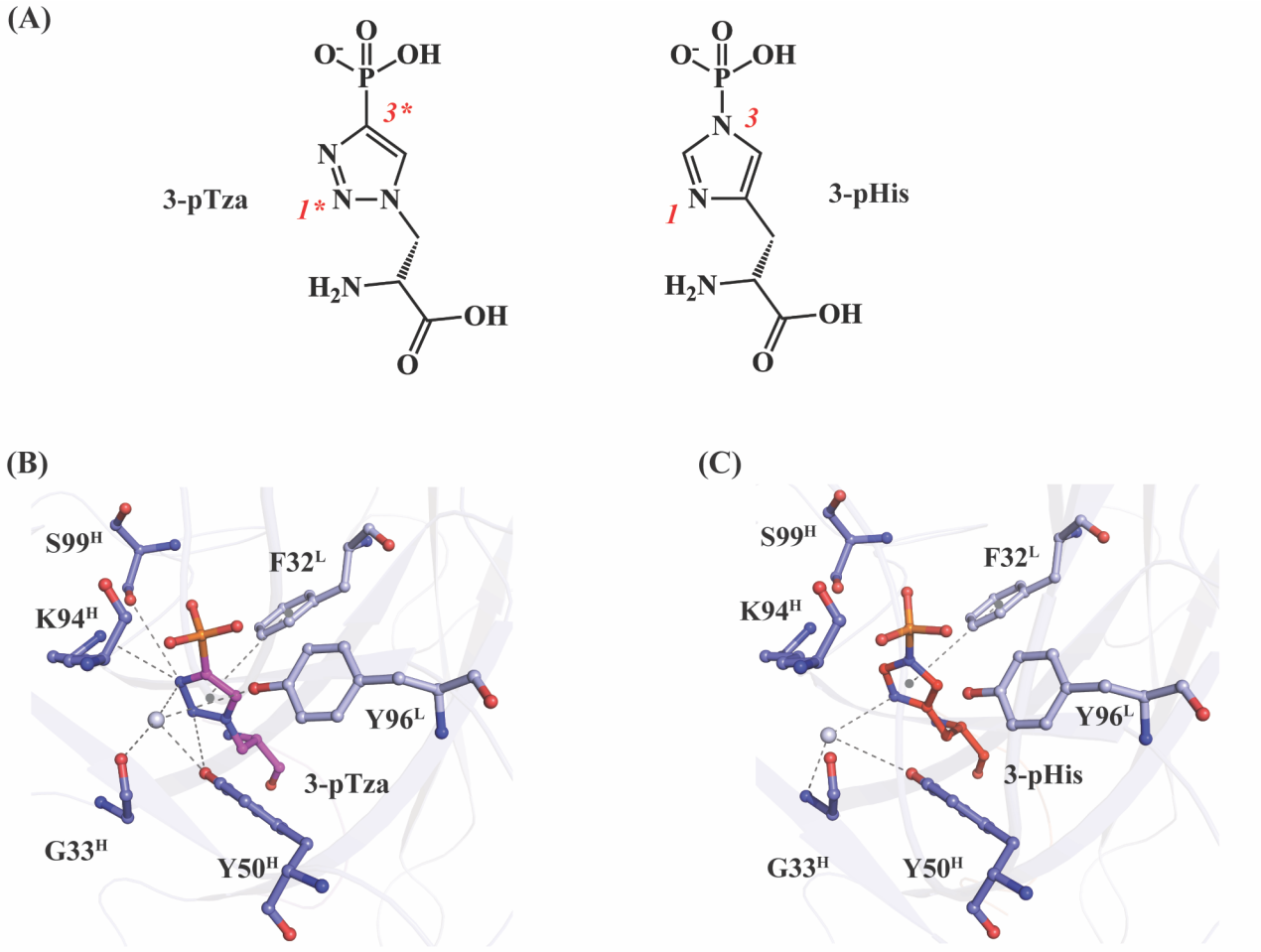

(A)
3-pTza
3-pHis
(B)
S99H
F32L
K94H
Y96L
3-pTza
G33H
Y50H
(C)
S99H
F32L
K94H
Y96L
3-pHis
G33H
Y50H

Fig. S20.

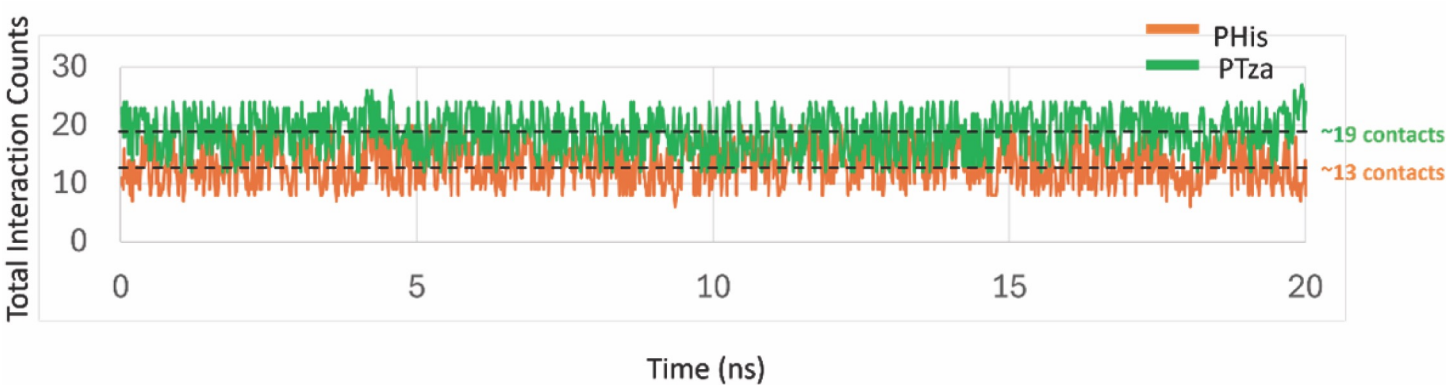

Total Interaction Counts
30
20
10
0
0
5
10
15
20
Time (ns)
PHis
PTza
~19 contacts
~13 contacts

Fig. S21

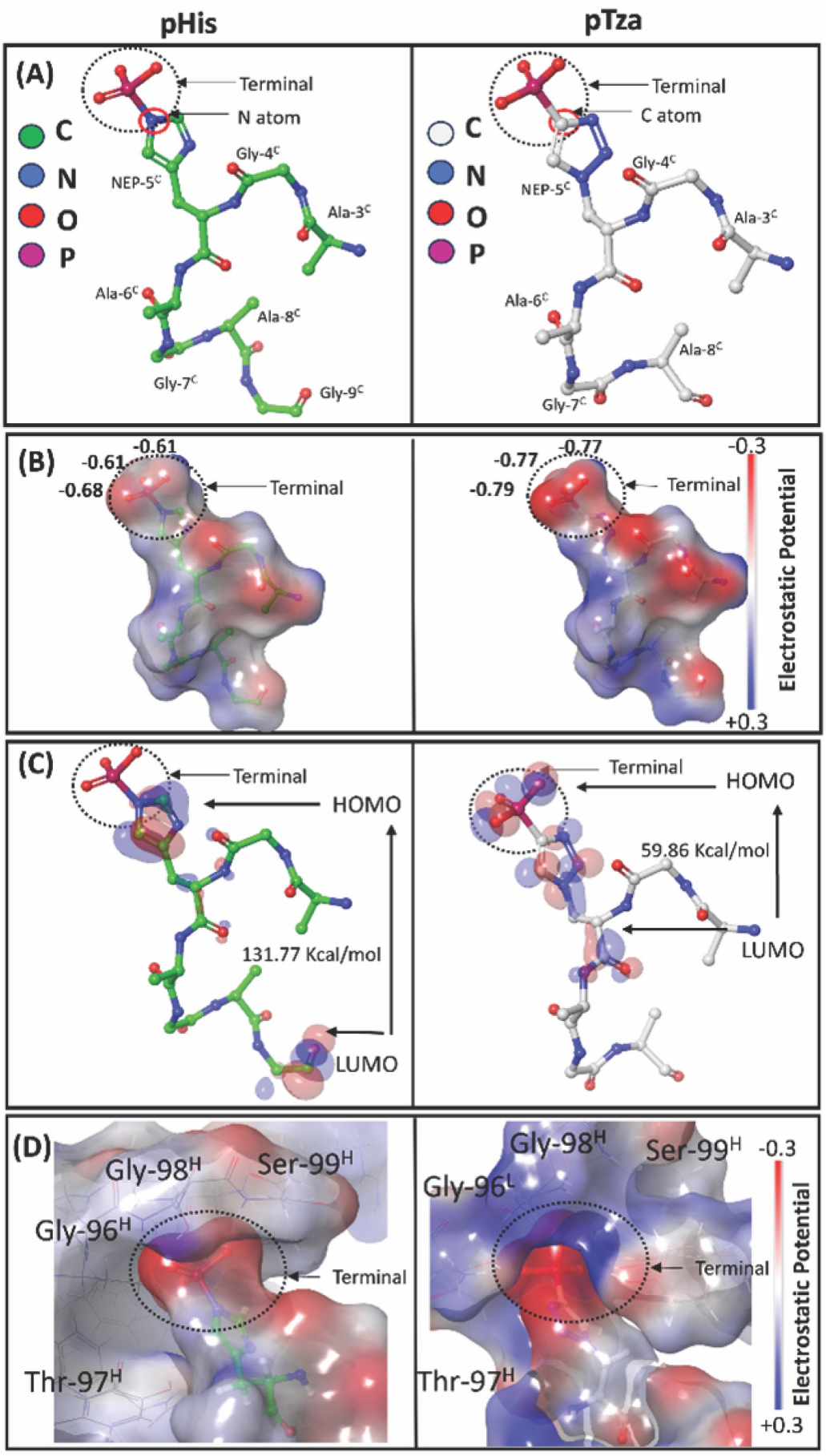

pHis
pTza
(A)
C
N
O
P
Terminal
N atom
C atom
Gly-4C
NEP-5C
Ala-3C
Ala-6C
Ala-8C
Gly-7C
Gly-9C
(B)
-0.61
-0.61
-0.68
-0.77
-0.77
-0.79
-0.3
+0.3
Electrostatic Potential
(C)
HOMO
LUMO
131.77 Kcal/mol
59.86 Kcal/mol
(D)
Gly-98H
Ser-99H
Gly-96H
Gly-96L
Thr-97H

Fig. S22.

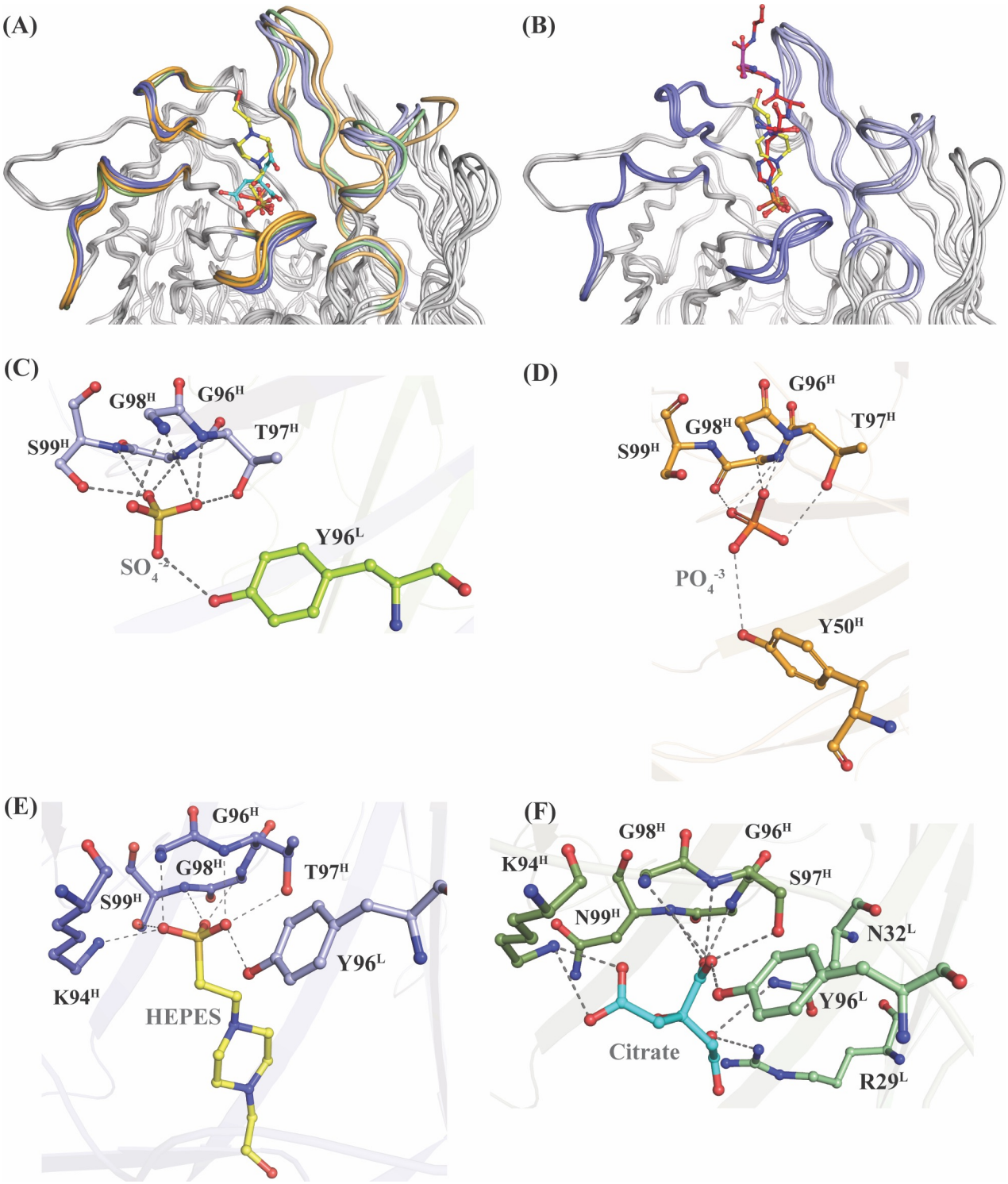

(A)
(B)
(C)
G98H
G96H
S99H
T97H
Y96L
SO4-2
(D)
G96H
G98H
T97H
S99H
PO4-3
Y50H
(E)
G96H
G98H
T97H
S99H
Y96L
K94H
HEPES
(F)
G98H
G96H
K94H
S97H
N99H
N32L
Y96L
Citrate
R29L

Fig. S23.

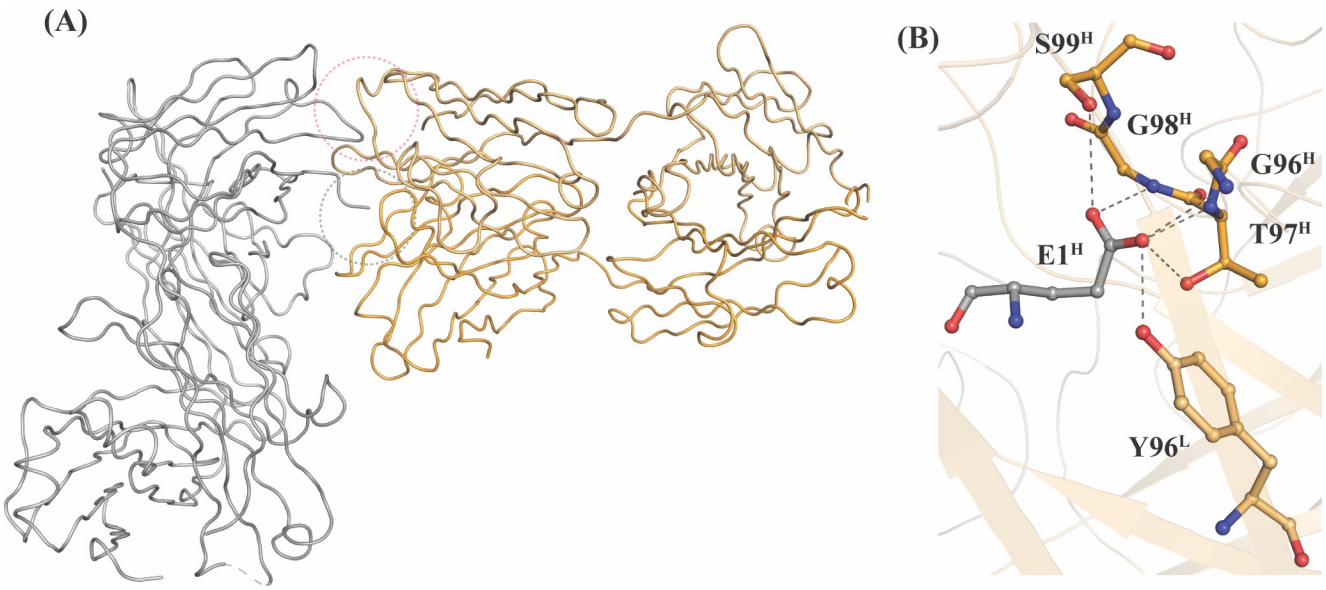


Table S1.

Table S1. Mutagenic oligonucleotides used to generate libraries hSC44 1-6.

Name	Mutagenic Oligonucleotide Sequence
hSC44.L1.1	CGTGCCAGTCAGTCCGTG(N4:10101070)(N3:10107010)(N3)(N2:10701010)(N3)(N4)AAC(N1:70101010)(N1)(N1)(N1)(N1)(N2)GTAGCCTGGTATCAACAG
hSC44.L1.2	CGTGCCAGTCAGTCCGTG(N4:10101070)(N3:10107010)(N3)CGTAACAAG(N1:70101010)(N1)(N2:10701010)GTAGCCTGGTATCAACAG
hSC44.L3.1	TACTGTGTGGGC(N2:10701010)(N1:70101010)(N4:10101070)TATGGCAGCGAAAACGATGCGTAT(N4)(N1)(N2)GCGTTCGGACAGGGTACC
hSC44.L3.2	TACTGTGTGGGC(N2:10701010)(N1:70101010)(N4:10101070)TAT(N3:10107010)(N3)(N2)(N1)(N3)(N2)(N3)(N1)(N1)(N1)(N1)(N2)(N3)(N1)(N4)(N3)(N2)(N3)TAT(N4)(N1)(N2)GCGTTCGGACAGGGTACC
hSC44.H1.1	GGCTTCAGCATTGATAGC(N4:10101070)(N1:70101010)(N4)(N3:10107010)(N3)(N2:10701010)TTTAGCTGGGTGCGTCAGGCC
hSC44.H2.1	CTGGAACATATTGGC(N4:10101070)(N1:70101010)(N4)CTG(N1)(N2:10701010)(N2)(N3:10107010)(N2)(N2)GGCGGCCGTGCGTTTTATGCC
hSC44.H2.2	CTGGAACATATTGGCTATCTG(N1:70101010)(N2:10701010)(N2)GCGGGCGGC(N2)(N3:10107010)(N4:10101070)GCGTTTTATGCCAGCTGG
hSC44.H3.1	GTCTATTATTGTGCT(N1:70101010)(N1)(N1)(N2:10701010)(N4:10101070)(N4)(N3:10107010)(N3)(N2)(N1)(N3)(N2)(N3)(N3)(N2)(N1)(N1)(N2)(N2)(N2)(N3)(N3)(N4)(N4)GCGATTTGGGGTCAAGGAACC
hSC44.H3.2	GTCTATTATTGTGCT(N1:70101010)(N1)(N3:10107010)(N2:10701010)(N4:10101070)(N4)(N3)(N3)(N2)(N1)(N3)(N2)GGC(N1)(N1)(N2)CCG(N3)(N4)(N4)GCGATTTGGGGTCAAGGAACC
hSC44.V.1	AGCATTGATAGCTATGGC(N4:10101070)(N4)(N4)(N1:70101010)(N3:10107010)(N2:10701010)TGGGTGCGTCAGGCCCCG
hSC44.V.2	GGTAAGGGCCTGGAA(N2:10701010)(N1:70101010)(N4:10101070)(N1)(N4)(N4)GGC(N4)(N1)(N4)CTGACCGCGGGC
hSC44.V.3	GGCCGTGCG(N4:10101070)(N4)(N4)TATGCC(N1:70101010)(N3:10107010)(N2:10701010)(N4)(N3)(N3)(N3)(N2)(N2)AAG(N1)(N3)(N2)CGT(N1)(N3)(N2)ACTATAACC(N2)(N3)(N4)AACACAAAC
hSC44.V.4	GAAAACACA(N3:10107010)(N4:10101070)(N4)ACCCTAAAAATGAACAGC

Table S2.

Table S2. Final concentrations of reagents used on various days of affinity selections.

	Day 1	Day 2	Day 3	Day 4	Day 5
BSA†	1%	1%	1%	1%	1%
Streptavidin†	10µg/mL	-	10µg/mL	-	10µg/mL
Neutravidin†	-	10µg/mL	-	10µg/mL	-
No PO_4^- Peptide*,†	-	10nM	25nM	50nM	100nM
1-pTza Peptide*,†	-	1nM	5nM	10nM	20nM
3-pTza/pHis Peptide*,‡	100nM	100nM	50nM	25nM	10nM
No. Wells	24	12	8	6	4
No. Washes	4	6	8	10	12

†Negative selections, ‡Positive Selections

*Captured using 2µg/mL streptavidin/neutravidin on alternating days

Table S3.

Table S3. Elbow angles of variants of hSC44 Fabs

Fab	Ligand	Protomers	Elbow angle (°)
hSC44.S1C	ACLYana-3-pTza peptide	HL	138.9
		AB	157.3
hSC44.S1C	ACLYana-3-pHis peptide	HL	137.8
		AB	157.1
hSC44.S1C	-	HL	138.9
		AB	159.7
hSC44.S1C.20	ACLYana-3-pTza peptide	HL	135.9
hSC44.S1C.20	ACLYana-3-pHis peptide	HL	135.8
hSC44.S1CE.20	-	HL	165.3
hSC44.S1CE.20	-	HL	172.6
hSC44.S1C.20.N32F^{L}	ACLYana-3-pTza peptide	HL	165.1
		AB	147.9
hSC44.S1C.20.N32F^{L}	ACLYana-3-pHis peptide	HL	165.4
		AB	149.0
hSC44.S1C.20.N32F^{L}	-	HL	156.2
hSC44.S1CE.20.N32F^{L}	-	HL	156.1

Table S4.

Table S4. Data collection and refinement statistics for newly solved Fab-Antigen crystal structures.

Table S4. X-ray data collection and refinement statistics for newly solved Fab-Antigen structures.

Structure Name	**hSC44.S1C**	**hSC44.S1C**	**hSC44.S1C**	**hSC44.S1C.20**	**hSC44.S1C.20**	**hSC44.S1CE.20**	**hSC44.S1CE.20**	**hSC44.S1C.20.N32F^{L}**	**hSC44.S1C.20. N32F^{L}**	**hSC44.S1C.20.N32F^{L}**	**hSC44.S1CE.20.N32F^{L}**
Combining site ligand	**AGAG-3pTza-AGAG**	**AGAG-3pHis-AGAG**	**No peptide (citrate)**	**AGAG-3pTza-AGAG**	**AGAG-3pHis-AGAG**	**No peptide (GluH1')**	**No peptide (PO4)**	**AGAG-3pTza-AGAG**	**AGAG-3pHis-AGAG**	**No peptide (SO4)**	**No peptide (HEPES)**
Data collection											
Beamline	SSRL 12-1	SSRL 12-1	APS 23-ID-B	APS 23-ID-D	SSRL 12-1	ALS 5.0.1	ALS 5.0.1	ALS 5.0.1	ALS 5.0.1	ALS 5.0.1	ALS 5.0.1
Wavelength (Å)	0.97946	0.97946	1.03317	1.0332	0.97946	0.97741	0.97741	0.97741	0.97741	0.97741	0.97741
Resolution (Å)[a]	39.34-1.95	38.72-2.20	46.91-1.75	41.04-1.85	34.82-1.90	47.17-2.45	49.79-2.40	47.25-1.98	47.19-2.09	48.05-1.94	46.27-1.84
Space group	$P2_12_12$	$P2_12_12$	$P2_12_12$	C2	$P2_1$	$P2_12_12_1$	$P4_32_12$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Unit cell (Å)	92.50, 139.62, 73.62	93.25, 139.06, 73.71	93.82, 138.85, 73.21	79.96, 73.12, 88.07	44.11, 72.61, 70.33	49.7, 73.3, 148.5	72.3, 72.3, 205.9	71.55, 73.95, 239.65	71.28, 74.03, 239.28	69.47, 73.25, 96.11	48.57, 72.07, 152.2
(°)	90, 90, 90	90, 90, 90	90, 90, 90	90, 111.24, 90	90, 98.21, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90
Total reflections	733,426	637,037	688,286	155,586	148,666	127,101	219,439	538,821	418,880	284,579	293,288
Unique reflections	68,051(3151)	46,924(2432)	95,210(4089)	40,163(1984)	33,299(1333)	19,822(839)	22,383(1088)	89,758(4417)	71,747(3417)	36,687(1806)	46,607(2191)
Multiplicity	10.8(7.8)	12.9(9.4)	7.2(5.5)	3.9(3.3)	4.5(3.6)	6.4(5.7)	9.8(6.2)	6.0(5.6)	5.8(5.2)	7.8(7.6)	6.3(4.5)
Completeness (%)	97.5(91.3)	99.9(99.9)	98.1(85.3)	99.8(99.4)	96.1(77.1)	96.3(84.2)	100(99.7)	100(100)	95.2(92.7)	100(100)	99.2(94.4)
Mean (I)/ σ_I	13.1(1.7)	11.8(1.8)	10.0(1.6)	6.9(1.1)	9.9(2.8)	19.1(2.1)	28.0(2.7)	8.9(1.5)	13.9(1.5)	11.4(1.0)	16.0(1.2)
R_{merge}[b](%)	14.3(193)	22.1(483)	12.4(117)	13.1(128)	16.2(120)	6.3(85.4)	7.1(76.1)	12.5(135)	10.1(130)	18.0(238)	10.0(178)
R_{meas}[c](%)	14.9(205)	23.0(512)	13.3(129)	15.1(151)	18.3(141)	6.9(94.1)	7.5(83.0)	13.7(150)	11.0(144)	19.3(254)	10.8(200)
R_{pim}[d](%)	4.4(67.3)	6.4(166)	4.9(52.9)	7.4(79.2)	8.2(71.9)	2.7(38.7)	2.3(32.2)	5.6(63.6)	4.4(61.1)	6.7(88.4)	4.2(90.4)
$CC_{1/2}$[e](%)	99.8(47.2)	99.8(56.5)	99.7(41.1)	99.5(44.1)	99.2(55.0)	99.9(70.6)	99.9(80.2)	99.5(39.6)	99.7(43.2)	99.3(37.4)	99.6(37.1)
Refinement											
Refinement resolution (Å)[a]	39.34-1.95	38.72-2.20	46.91-1.75	41.04-1.85	34.82-1.90	47.17-2.45	49.79-2.40	47.25-1.98	47.2-2.09	48.05-1.94	46.27-1.84
# reflections in refinement (work/free)	64,623/3393	46,924/2489	90,339/4802	38,147/1978	31,562/1721	18,793/985	21,221/1079	85,368/4291	68,152/3509	34,775/1846	44,224/2299
R_{work}/R_{free} (%)	21.2/26.0	23.0/27.5	21.0/23.9	20.5/24.3	17.2/21.3	24.4/29.0	25.8/29.3	22.4/25.7	21.8/24.5	21.8/24.7	20.4/23.6
# atoms (Fab/Peptide/Solvent)	6556/64/223	6556/69/64	6532/na/476	6514/57/283	6526/69/188	3286/na/10	3287/na/34	6589/64/206	6552/72/334	3278/na/149	3336/na/480
RMS (bonds)	0.006	0.002	0.006	0.003	0.005	0.005	0.003	0.008	0.002	0.003	0.005
RMS (angles)	0.91	0.6	0.83	0.75	0.77	0.71	0.61	0.94	0.52	0.66	0.86
Ramachandran favoured/allowed/ outliers (%)	96.9/2.9/0.2	97.6/2.4/0	96.7/2.9/0.4	97.7/2.3/0	97.7/2.1/0.2	95.1/4.7/0.2	96.0/4.0/0	96.4/3.6/0	97.3/2.6/0.1	97.7/2.3/0	98.4/1.6/0
Ramachandran plot Z score	0.02	-0.1	0.2	-0.1	0.8	-1.4	-1.6	-0.29	-0.81	0.06	0.39
Clashscore[f]	3.1	1.8	3.7	0.6	1.5	3.2	4.6	2.1	1.4	2.6	3.5
Wilson B ($Å^2$)	30	41	22	22	21	53	51	32	36	27	25
Average B ($Å^2$) for all atoms/Fab/ Peptide/Solvent	40/38/54/34	56/56/85/43	34/34/na/30	29/29/38/32	25/25/22/27	63/63/na/46	72/72/na/51	42/42/56/36	43/43/56/41	30/30/46/29	31/30/20/36
PDB ID	**8UJI**	**8UIT**	**8UIO**	**8UIH**	**8UIG**	**8UHT**	**8UHS**	**8UHP**	**8UHN**	**8UHJ**	**8UHH**

[a]Numbers in parentheses are for highest resolution shell

[b]$R_{merge} = \Sigma_{hkl} \Sigma_{i=1,n} |I_i(hkl) - \langle I(hkl)\rangle| / \Sigma_{hkl} \Sigma_{i=1,n} I_i(hkl)$

[c]$R_{meas} = \Sigma_{hkl} \sqrt{(n/n-1)} \Sigma_{i=1,n} |I_i(hkl) - \langle I(hkl)\rangle| / \Sigma_{hkl} \Sigma_{i=1,n} I_i(hkl)$

[d]$R_{pim} = \Sigma_{hkl} \sqrt{(1/n-1)} \Sigma_{i=1,n} |I_i(hkl) - \langle I(hkl)\rangle| / \Sigma_{hkl} \Sigma_{i=1,n} I_i(hkl)$

[e]$CC_{1/2}$ = Pearson Correlation Coefficient between two random half datasets

[f] Number of unfavorable all-atom steric overlaps ≥ 0.4Å per 1000 atoms

Table S5.

Table S5. Constructs with ligand, crystallization conditions and cryoprotectant used for crystallization experiments

pHis Fab	Ligand	Crystallization condition	Cryoprotectant
hSC44.S1C (C1)	AGAG-3-pTza-AGAG	0.2 M Lithium citrate, 20% PEG3350	30 % Ethylene glycol
hSC44.S1C (A7)	AGAG-3-pHis-AGAG	0.2 M Calcium chloride, 20% PEG3350	30 % Ethylene glycol
hSC44.S1C (A2)	No ligand	0.2 M Lithium citrate, 20% PEG3350	30 % Ethylene glycol
hSC44.S1C.20 (J13)	AGAG-3-pTza-AGAG	0.2 M tri-potassium citrate, 20% PEG3350	25% PEG400
hSC44.S1C.20 (B5)	AGAG-3-pHis-AGAG	0.2 M tri-potassium citrate, 20% PEG3350	25% PEG400
hSC44.S1CE.20 (C16)	No ligand	0.1 M Tris pH 8.5, 8% PEG8000	30 % Ethylene glycol
hSC44.S1CE.20 (A10)	No ligand	0.1 M HEPES pH 7.5, 20% PEG4000, 10% 2-propanol	30 % Ethylene glycol
hSC44.S1C.20.N32F^{L} (E12)	AGAG-3-pTza-AGAG	0.08 M Sodium Cacodylate pH 6.5, 0.16 M Calcium acetate, 20% glycerol, 14.4% PEG8000	10% glycerol
hSC44.S1C.20.N32F^{L} (E14)	AGAG-3-pHis-AGAG	0.08 M Sodium Cacodylate pH 6.5, 0.16 M Calcium acetate, 20% glycerol, 14.4% PEG8000	10% glycerol
hSC44.S1C.20.N32F^{L} (C8)	No ligand	0.1 M Tris pH 8.5, 0.2 M Lithium sulfate, 40% PEG400	Well solution
hSC44.S1CE.20.N32F^{L} (C15)	No ligand	0.1 M HEPES pH 7.5, 20% PEG4000, 10% 2-propanol	30 % Ethylene glycol

Table S6.

Table S6: Changes in the ligand binding free energies caused by the N32F^{L} mutation. The first column shows the binding free energy differences from FEP calculations ($\Delta\Delta \boldsymbol{G}_{\boldsymbol{bind}}^{\boldsymbol{WT}\rightarrow\boldsymbol{M}}$) of 3-pHis, whereas the last column shows the experimentally obtained binding free energy differences.

System	Relative Binding Free energy $\Delta\Delta G_{bind}$ (Kcal/mol)	K_D(M)	Experimental binding free energy $\Delta\Delta G_{bind}$ (Kcal/mol)
pHis_SC44H.20.F (N32F^{L})	-1.1±0.4	1.68E-09	$RT \ln \frac{K_D^M}{K_D^{WT}}$ =-1.4
pHis_SC44H.20.F (WT)		2.10E-08	