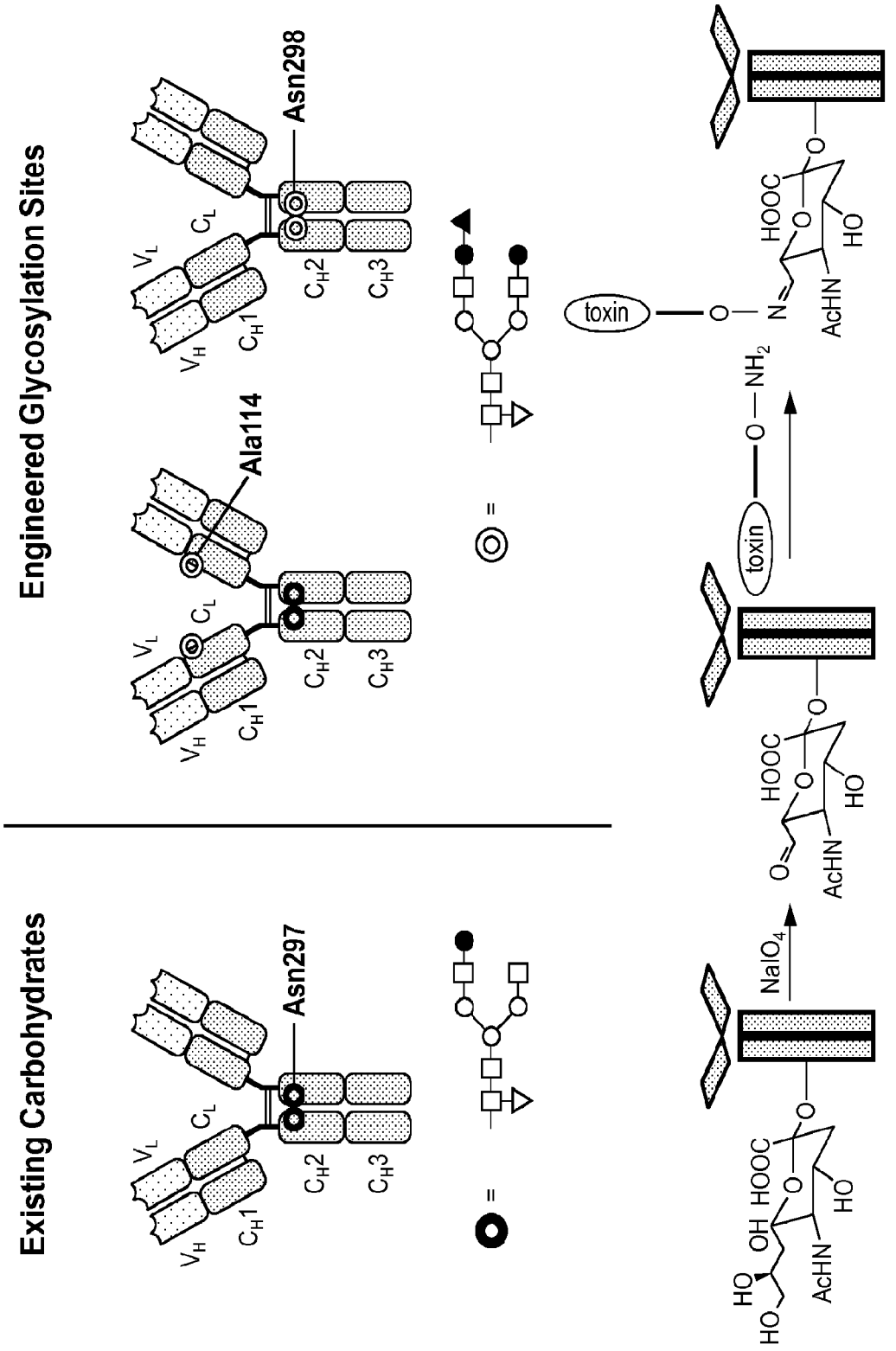

Engineered Glycosylation Sites
Existing Carbohydrates
Asn298
Ala114
Asn297
V_H
V_L
C_H1
C_L
C_H2
C_H3
toxin
$NaIO_4$
NH_2
HOOC
HO
AcHN
OH
HO

Fig. 1

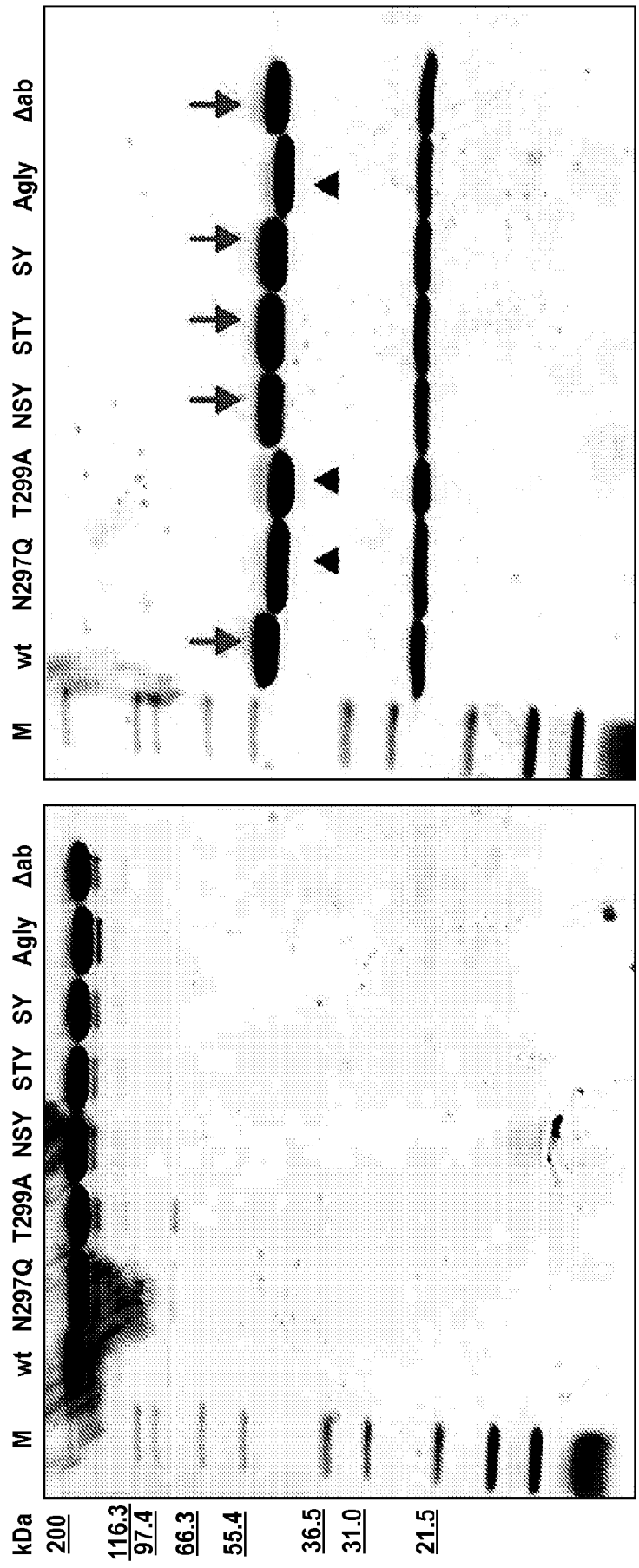

kDa
200
116.3
97.4
66.3
55.4
36.5
31.0
21.5
M
wt
N297Q
T299A
NSY
STY
SY
Agly
Δab
Non-reduced
Reduced

Fig. 2

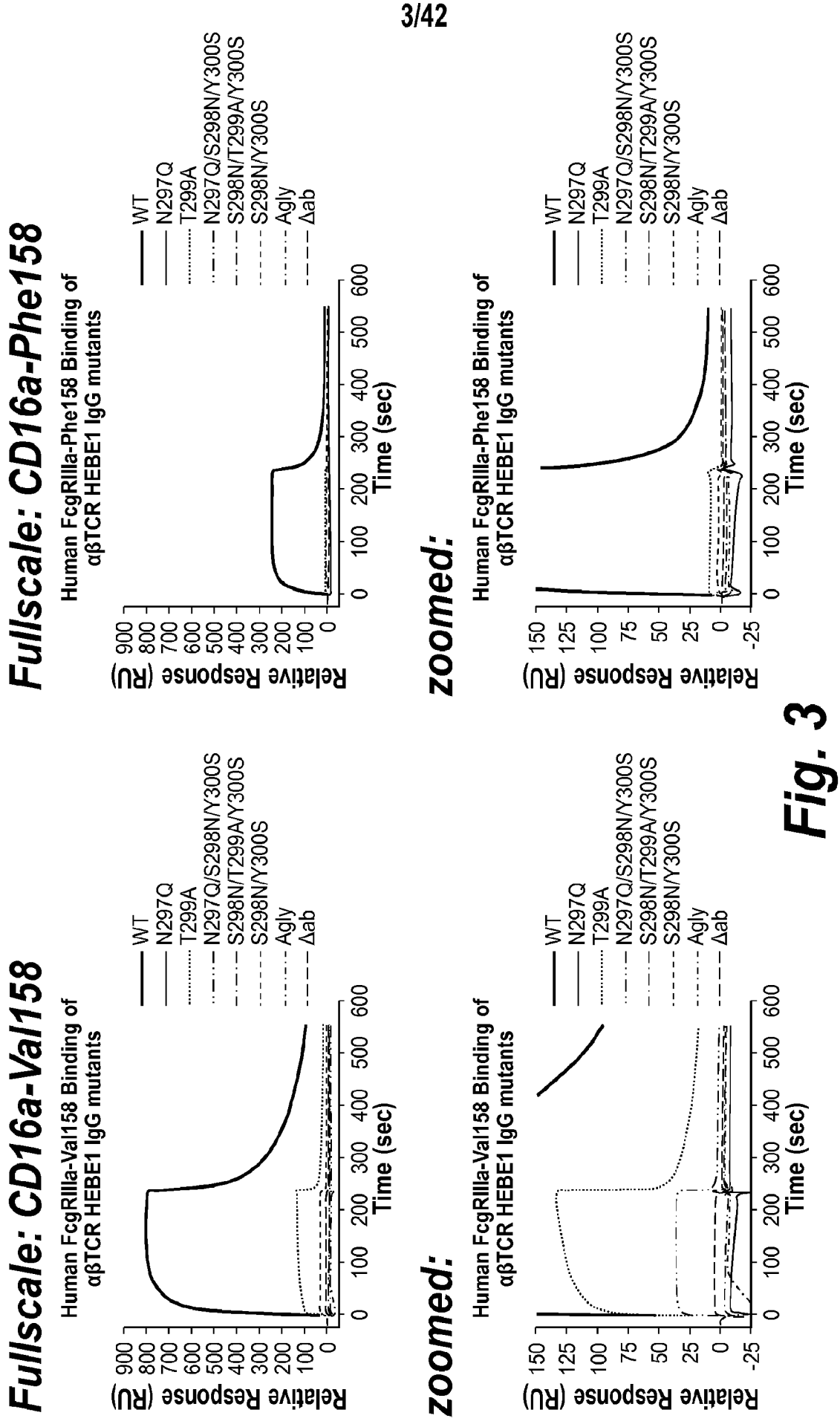

Fullscale: CD16a-Val158
Human FcgRIIIa-Val158 Binding of αβTCR HEBE1 IgG mutants
Relative Response (RU)
Time (sec)
WT
N297Q
T299A
N297Q/S298N/Y300S
S298N/T299A/Y300S
S298N/Y300S
Agly
Δab
zoomed:
Fullscale: CD16a-Phe158
zoomed:

Fig. 3

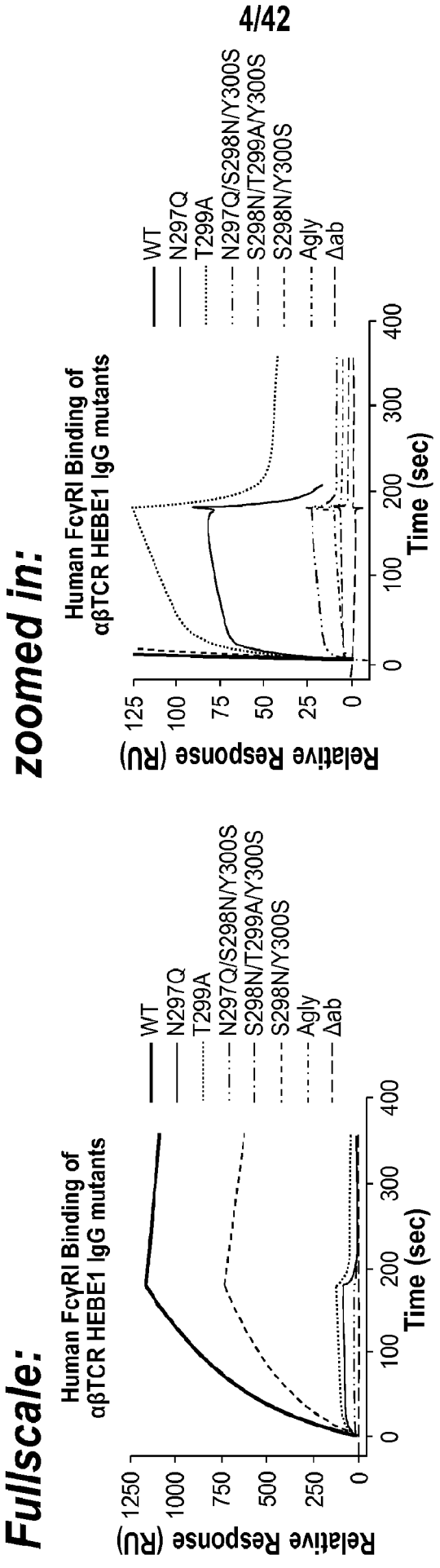

Fullscale:
Human FcγRI Binding of αβTCR HEBE1 IgG mutants
Relative Response (RU)
Time (sec)
WT
N297Q
T299A
N297Q/S298N/Y300S
S298N/T299A/Y300S
S298N/Y300S
Agly
Δab
zoomed in:
Human FcγRI Binding of αβTCR HEBE1 IgG mutants
Relative Response (RU)
Time (sec)
WT
N297Q
T299A
N297Q/S298N/Y300S
S298N/T299A/Y300S
S298N/Y300S
Agly
Δab

Fig. 4

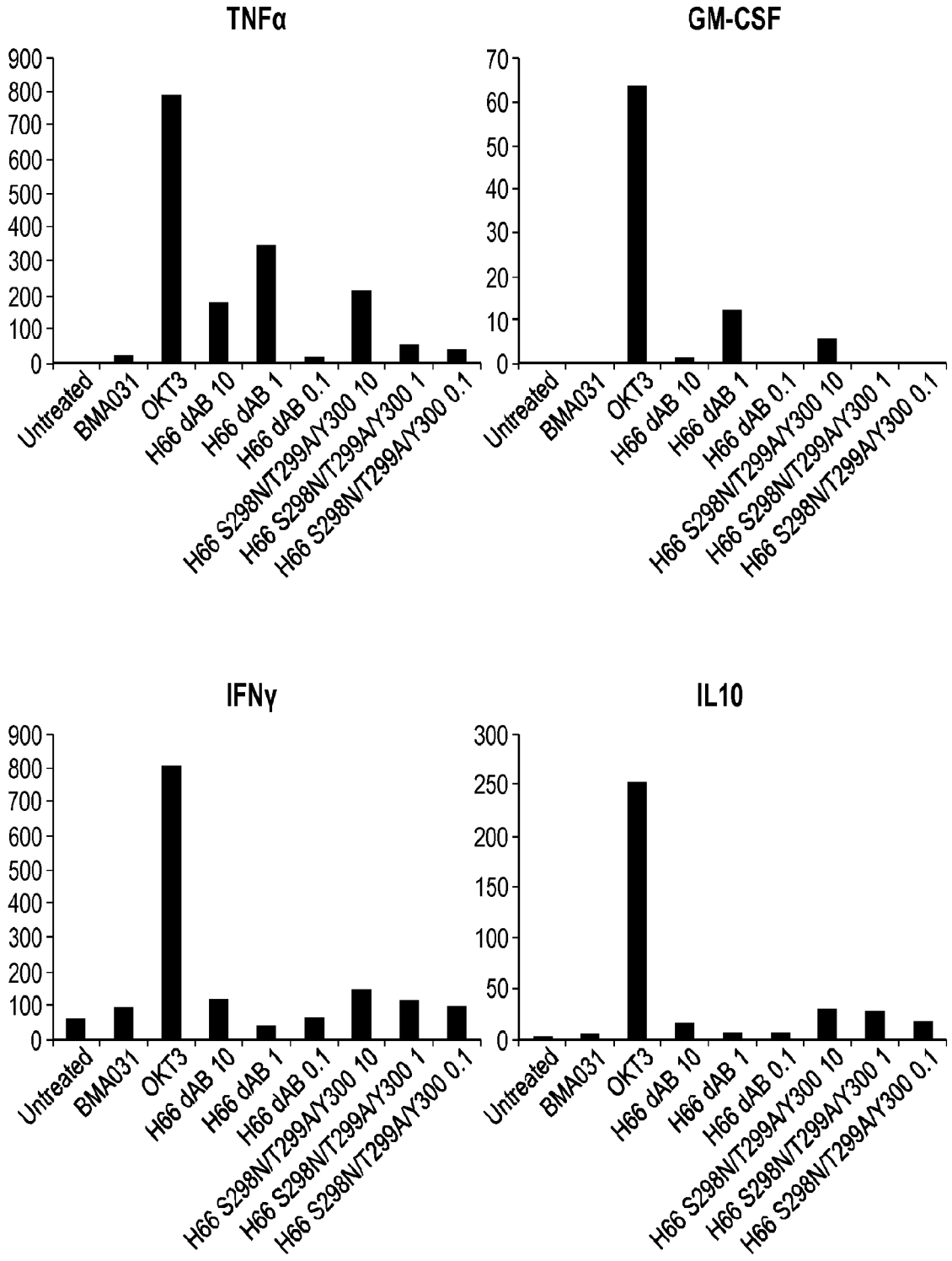

TNFα
900
800
700
600
500
400
300
200
100
0
Untreated
BMA031
OKT3
H66 dAB 10
H66 dAB 1
H66 dAB 0.1
H66 S298N/T299A/Y300 10
H66 S298N/T299A/Y300 1
H66 S298N/T299A/Y300 0.1
GM-CSF
70
60
50
40
30
20
10
0
IFNγ
IL10
300
250
200
150
100
50

Fig. 5

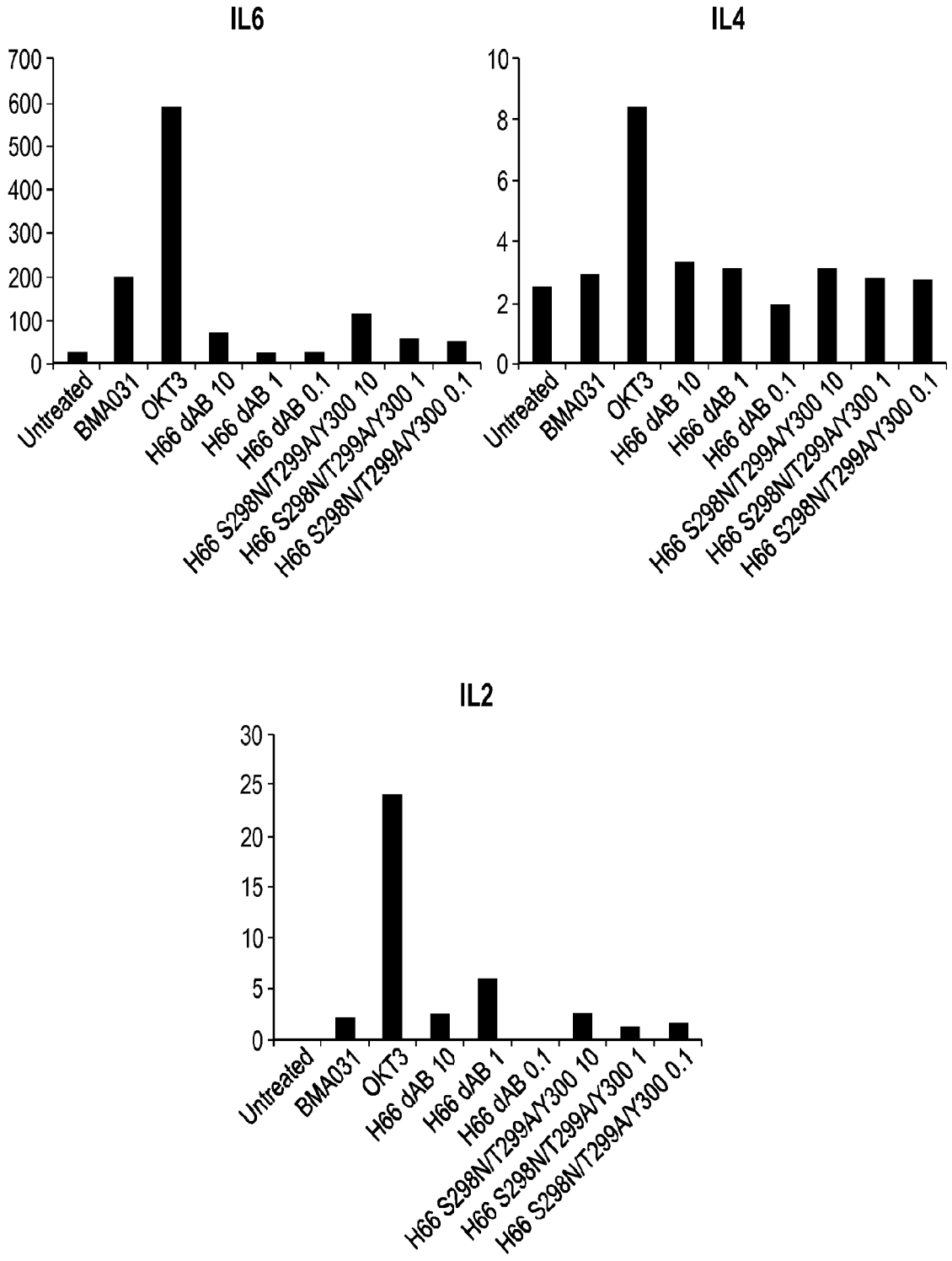

IL6
700
600
500
400
300
200
100
0
Untreated
BMA031
OKT3
H66 dAB 10
H66 dAB 1
H66 dAB 0.1
H66 S298N/T299A/Y300 10
H66 S298N/T299A/Y300 1
H66 S298N/T299A/Y300 0.1
IL4
10
8
6
4
2
0
Untreated
BMA031
OKT3
H66 dAB 10
H66 dAB 1
H66 dAB 0.1
H66 S298N/T299A/Y300 10
H66 S298N/T299A/Y300 1
H66 S298N/T299A/Y300 0.1
IL2
30
25
20
15
10
5
0
Untreated
BMA031
OKT3
H66 dAB 10
H66 dAB 1
H66 dAB 0.1
H66 S298N/T299A/Y300 10
H66 S298N/T299A/Y300 1
H66 S298N/T299A/Y300 0.1

Fig. 6

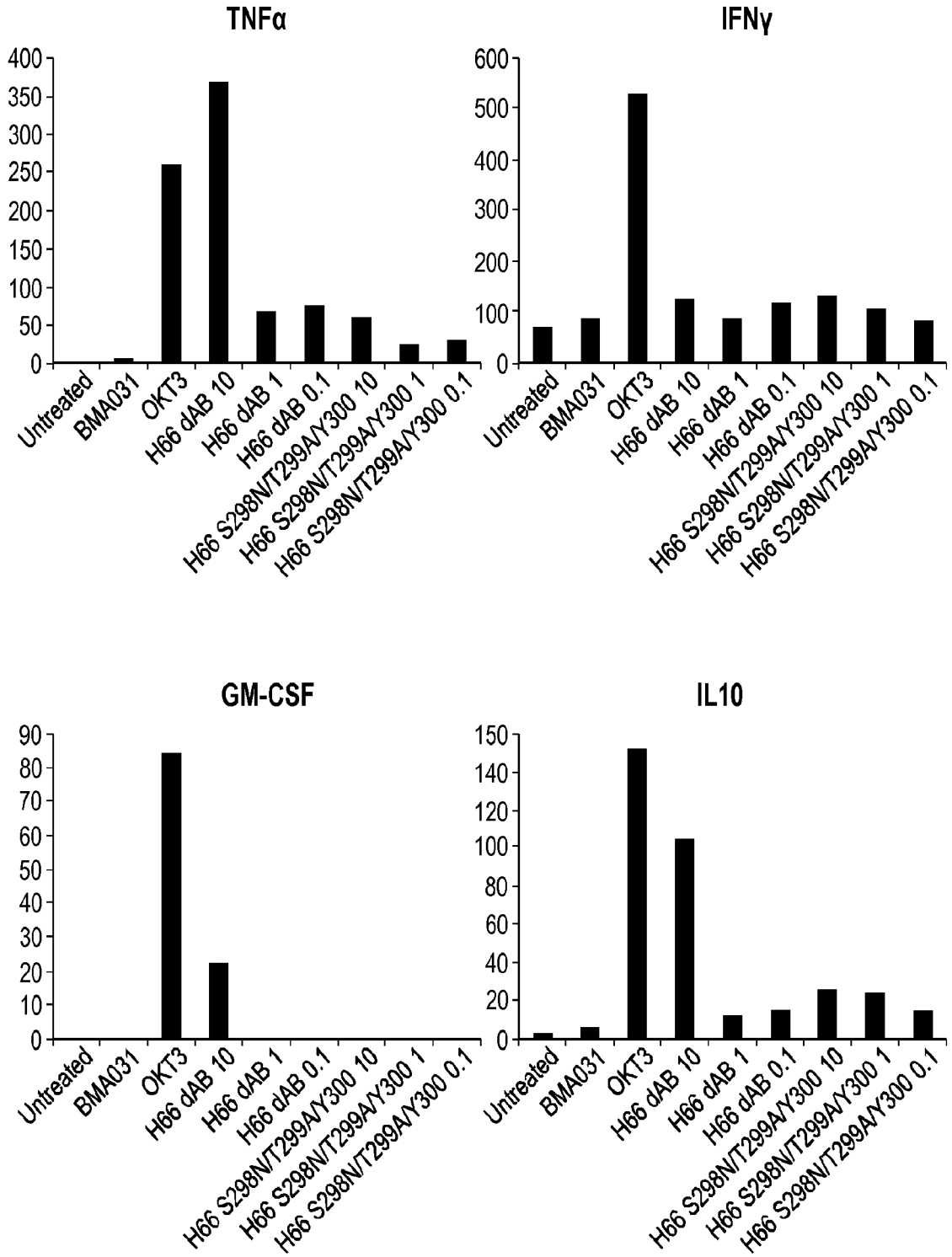

TNFα
IFNγ
GM-CSF
IL10
Untreated
BMA031
OKT3
H66 dAB 10
H66 dAB 1
H66 dAB 0.1
H66 S298N/T299A/Y300 10
H66 S298N/T299A/Y300 1
H66 S298N/T299A/Y300 0.1

Fig. 7

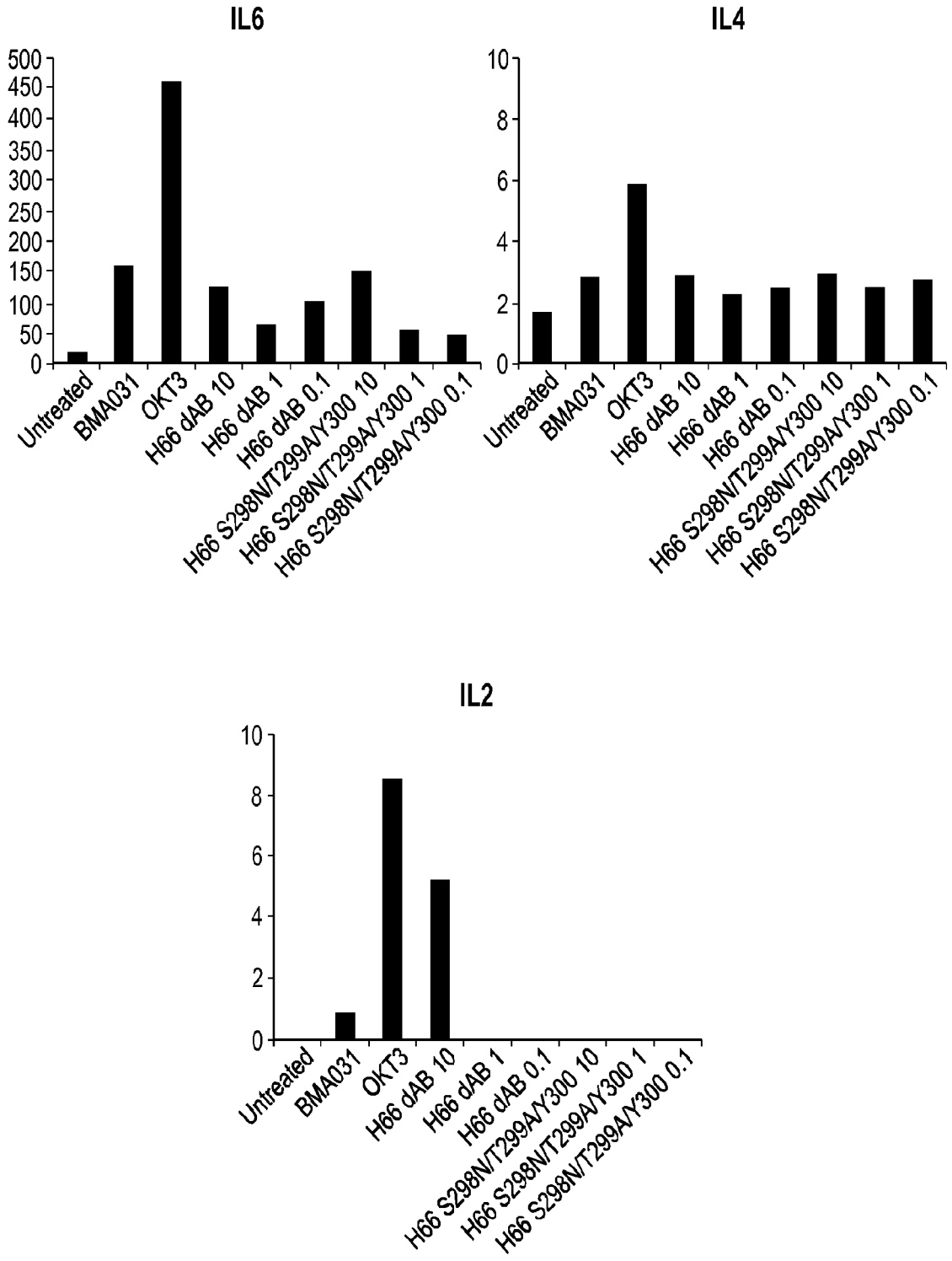

IL6
500
450
400
350
300
250
200
150
100
50
0
Untreated
BMA031
OKT3
H66 dAB 10
H66 dAB 1
H66 dAB 0.1
H66 S298N/T299A/Y300 10
H66 S298N/T299A/Y300 1
H66 S298N/T299A/Y300 0.1
IL4
10
8
6
4
2
0
Untreated
BMA031
OKT3
H66 dAB 10
H66 dAB 1
H66 dAB 0.1
H66 S298N/T299A/Y300 10
H66 S298N/T299A/Y300 1
H66 S298N/T299A/Y300 0.1
IL2
10
8
6
4
2
0
Untreated
BMA031
OKT3
H66 dAB 10
H66 dAB 1
H66 dAB 0.1
H66 S298N/T299A/Y300 10
H66 S298N/T299A/Y300 1
H66 S298N/T299A/Y300 0.1

Fig. 8

A

B

Biacore

Unknown	Conc (µg/mL)	Relative Response (RU)	Calc Conc (M)	Calc Conc (µg/mL)
2C3 in media	3.00	855.75	2.45E-08	3.669
2C3 in media	3.00	860.96	2.47E-08	3.710
2C3 in media	3.00	866.89	2.51E-08	3.758
A114N		146.5	1.60E-09	0.240
Mock		87.84	8.62E-10	0.129
NGT		124.52	1.30E-09	0.196
S298N/Y300S		112.14	1.15E-09	0.172
S440N		146.36	160E-09	0.240
S442N		121.21	1.26E-09	0.189
WT (Katya)		148.41	1.63E-09	0.244
WT (Tim)		148.08	1.62E-09	0.244
Y436S		158.72	1.78E-09	0.267
Y436T		84.27	8.23E-10	0.123
incubated media		87.38	8.57E-10	0.129

Fig. 9

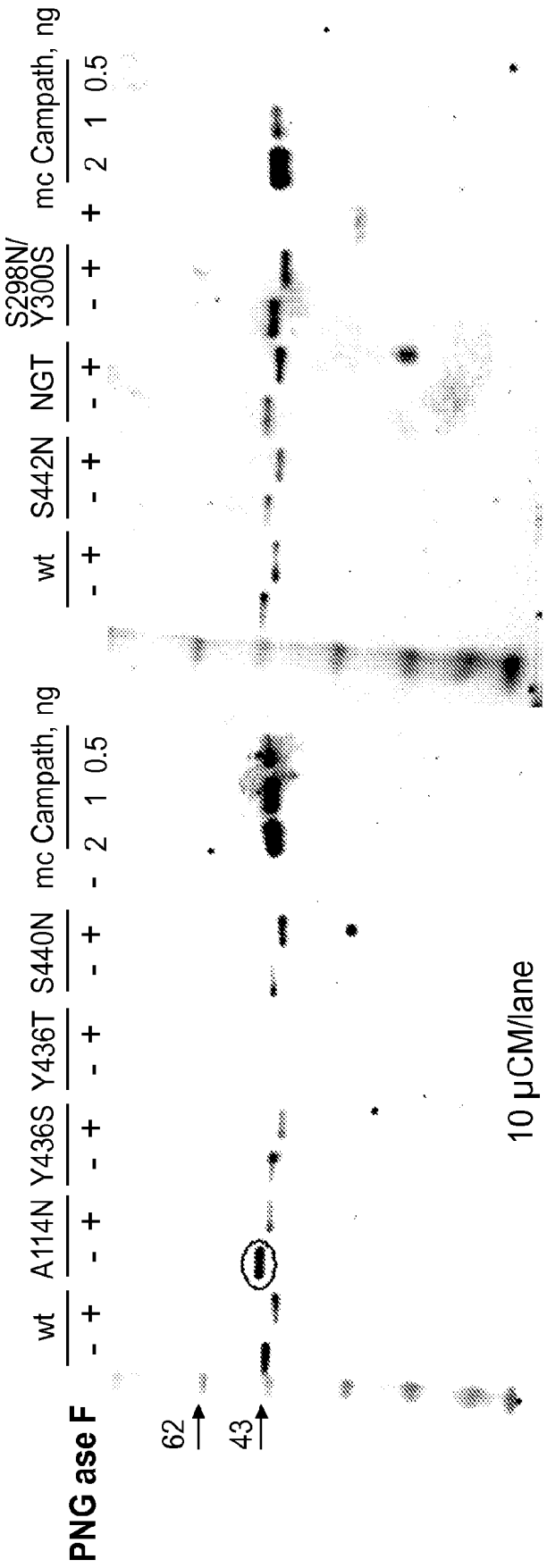
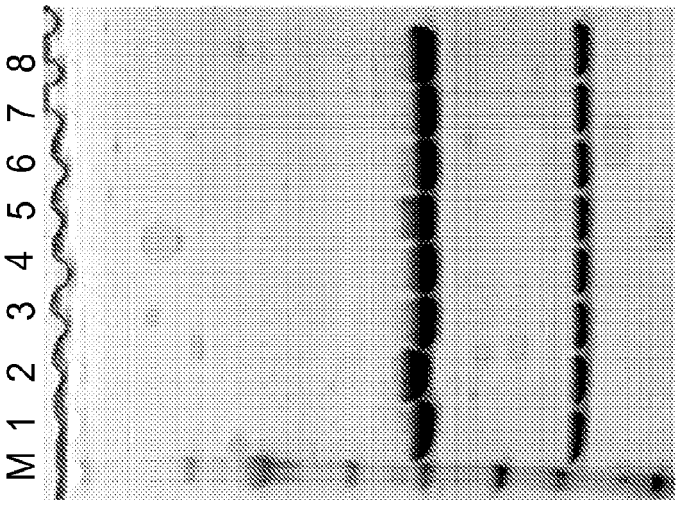

PNG ase F
wt
A114N
Y436S
Y436T
S440N
mc Campath, ng
wt
S442N
NGT
S298N/ Y300S
mc Campath, ng
62
43
10 µCM/lane

Fig. 10



Lane	Mutant	mg/ml
1	wt	2.66
2	A114N	2.99
3	Y436S	1.61
4	S298N/Y300S	0.99
5	Y436T	0.41
6	S440N	1.21
7	S442N	1.62
8	NGT	2.21

Fig. 11

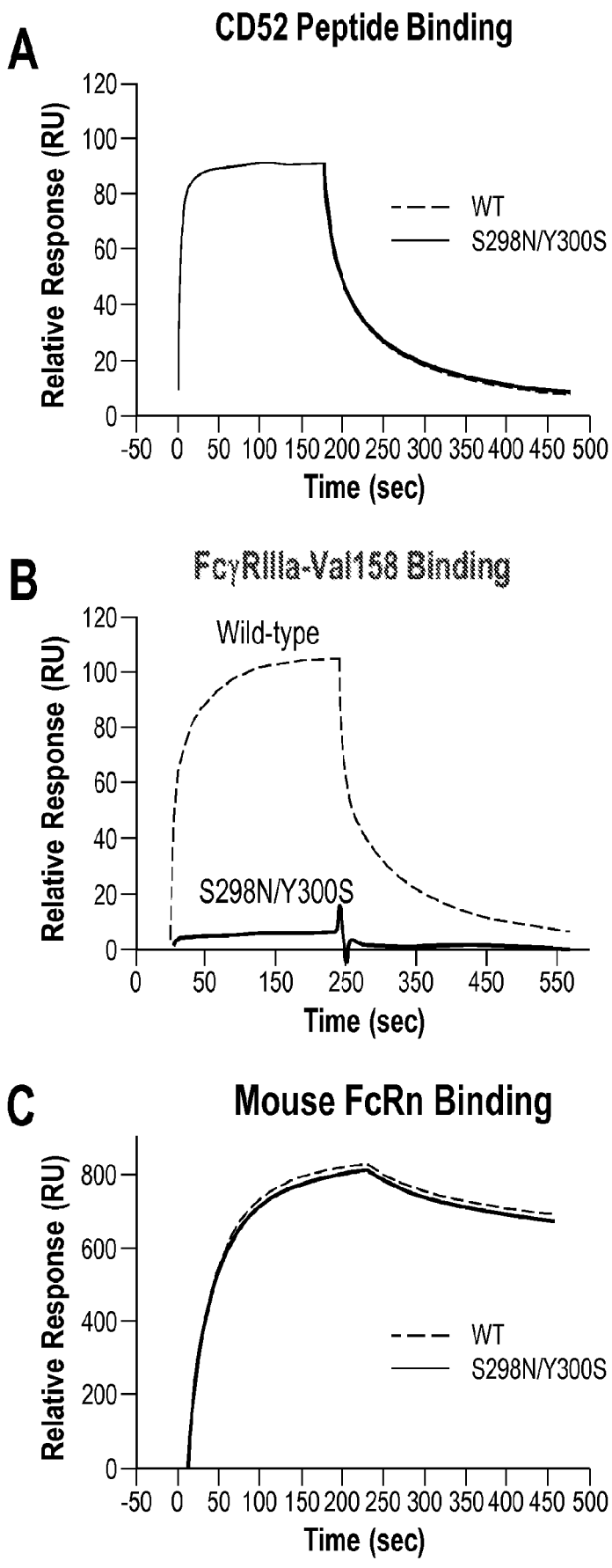

A
CD52 Peptide Binding
Relative Response (RU)
120
100
80
60
40
20
0
-50 0 50 100 150 200 250 300 350 400 450 500
Time (sec)
WT
S298N/Y300S
B
FcγRIIIa-Val158 Binding
Relative Response (RU)
Wild-type
S298N/Y300S
0 50 150 250 350 450 550
Time (sec)
C
Mouse FcRn Binding
Relative Response (RU)
800
600
400
200
0
-50 0 50 100 150 200 250 300 350 400 450 500
Time (sec)
WT
S298N/Y300S

Fig. 12

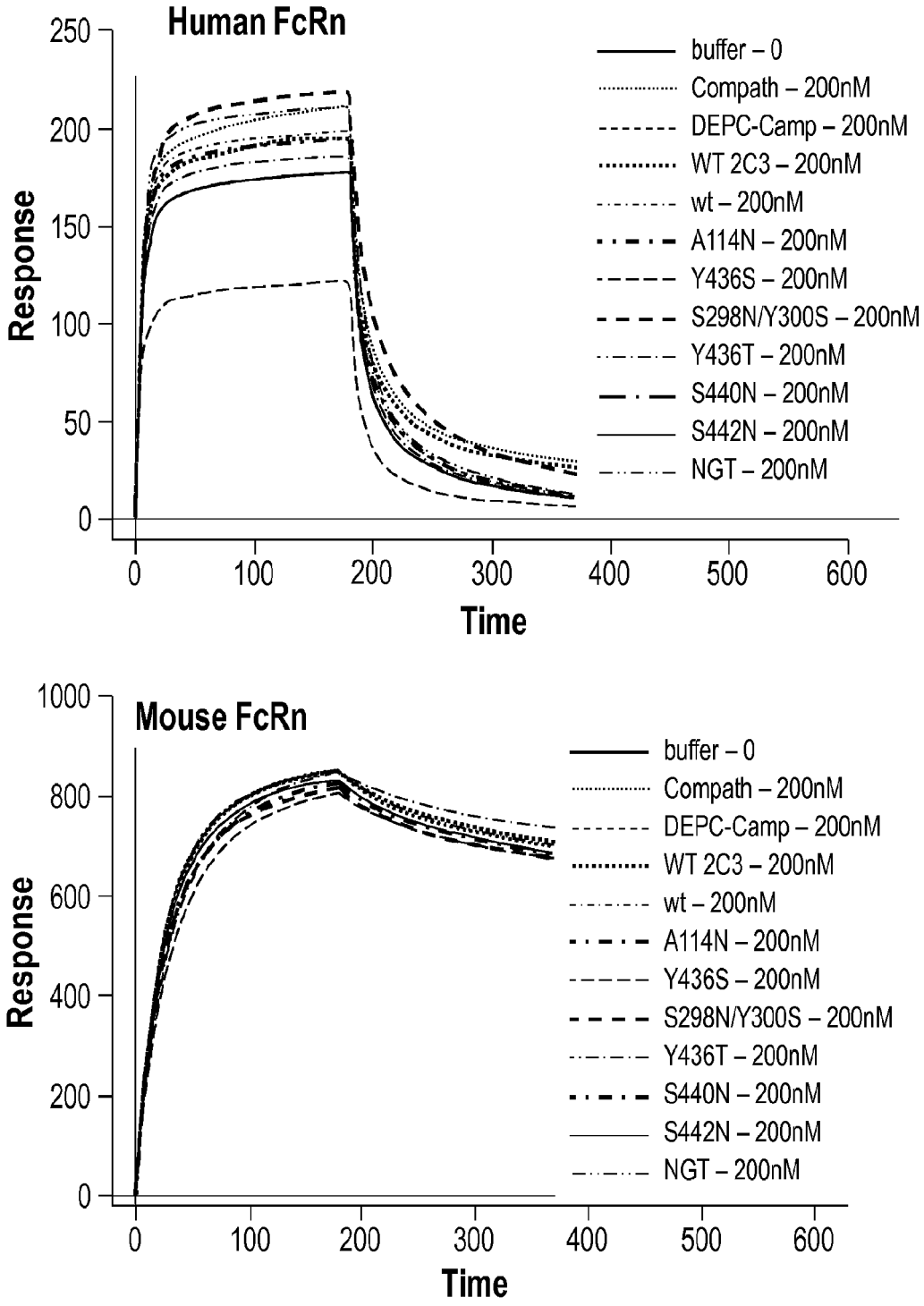

Human FcRn
Response
Time
buffer – 0
Compath – 200nM
DEPC-Camp – 200nM
WT 2C3 – 200nM
wt – 200nM
A114N – 200nM
Y436S – 200nM
S298N/Y300S – 200nM
Y436T – 200nM
S440N – 200nM
S442N – 200nM
NGT – 200nM
Mouse FcRn
Response
Time
buffer – 0
Compath – 200nM
DEPC-Camp – 200nM
WT 2C3 – 200nM
wt – 200nM
A114N – 200nM
Y436S – 200nM
S298N/Y300S – 200nM
Y436T – 200nM
S440N – 200nM
S442N – 200nM
NGT – 200nM

Fig. 13

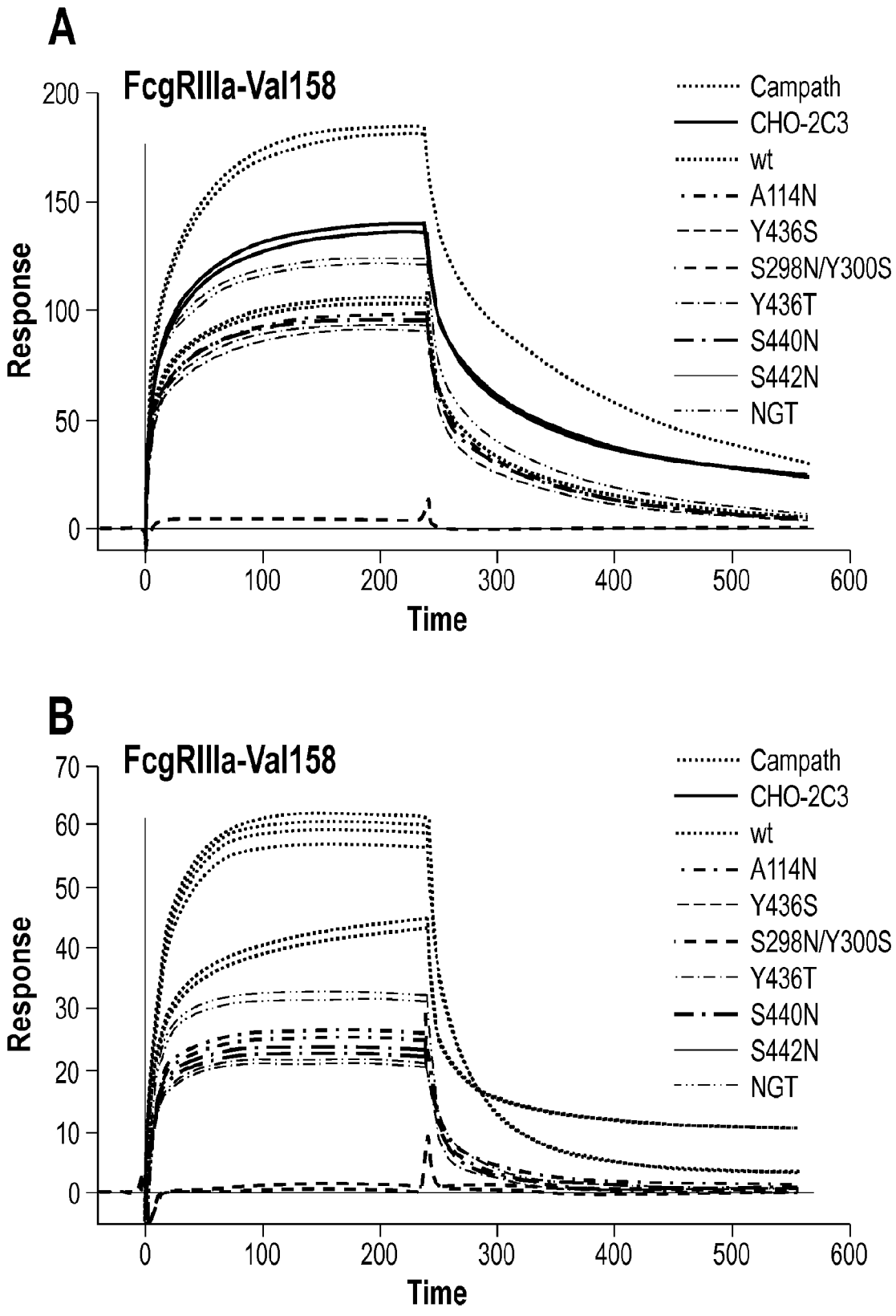

A
FcgRIIIa-Val158
Response
Time
Campath
CHO-2C3
wt
A114N
Y436S
S298N/Y300S
Y436T
S440N
S442N
NGT
B
FcgRIIIa-Val158
Response
Time
Campath
CHO-2C3
wt
A114N
Y436S
S298N/Y300S
Y436T
S440N
S442N
NGT

Fig. 14

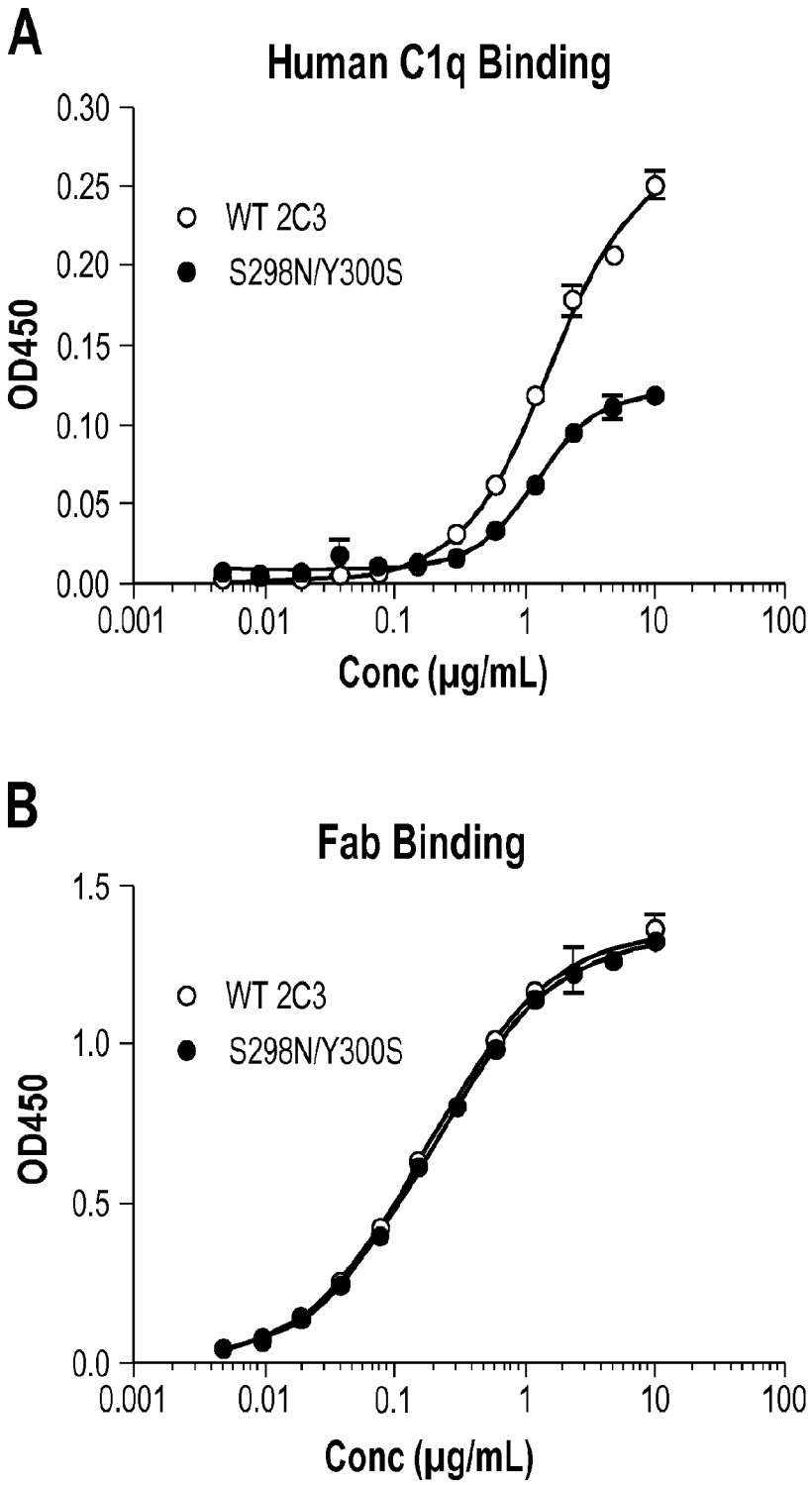

A
Human C1q Binding
0.30
0.25
0.20
0.15
0.10
0.05
0.00
OD450
WT 2C3
S298N/Y300S
0.001
0.01
0.1
1
10
100
Conc (µg/mL)
B
Fab Binding
1.5
1.0
0.5
0.0
OD450
WT 2C3
S298N/Y300S
0.001
0.01
0.1
1
10
100
Conc (µg/mL)

Fig. 15

Sample	k_a(x10^6/Ms)	k_d(x10^{-2}/s)	R_{max}(RU)	K_D(nM)
GLD52	7.0	1.7	67.0	2.44
WT2C3	6.0	1.1	64.2	1.75
A114N	4.7	1.1	59.5	2.45
Y436S	5.9	1.0	66.9	1.73
S298N?Y300S	5.7	1.0	63.3	1.80
Y436T	4.8	0.9	65.7	1.95
S440N	5.8	1.1	66.8	1.84
S442N	5.7	1.1	66.2	1.85
NGT	7.9	1.1	70.2	1.35

Fig. 16

Sample	K on ($x10^6 M^{-1}s^{-1}$)	K off ($x10^{-2}s^{-1}$)	KD (nM)
WT 2C3	5.2	1.1	2.1
A114N	5.3	1.3	2.4

Fig. 17

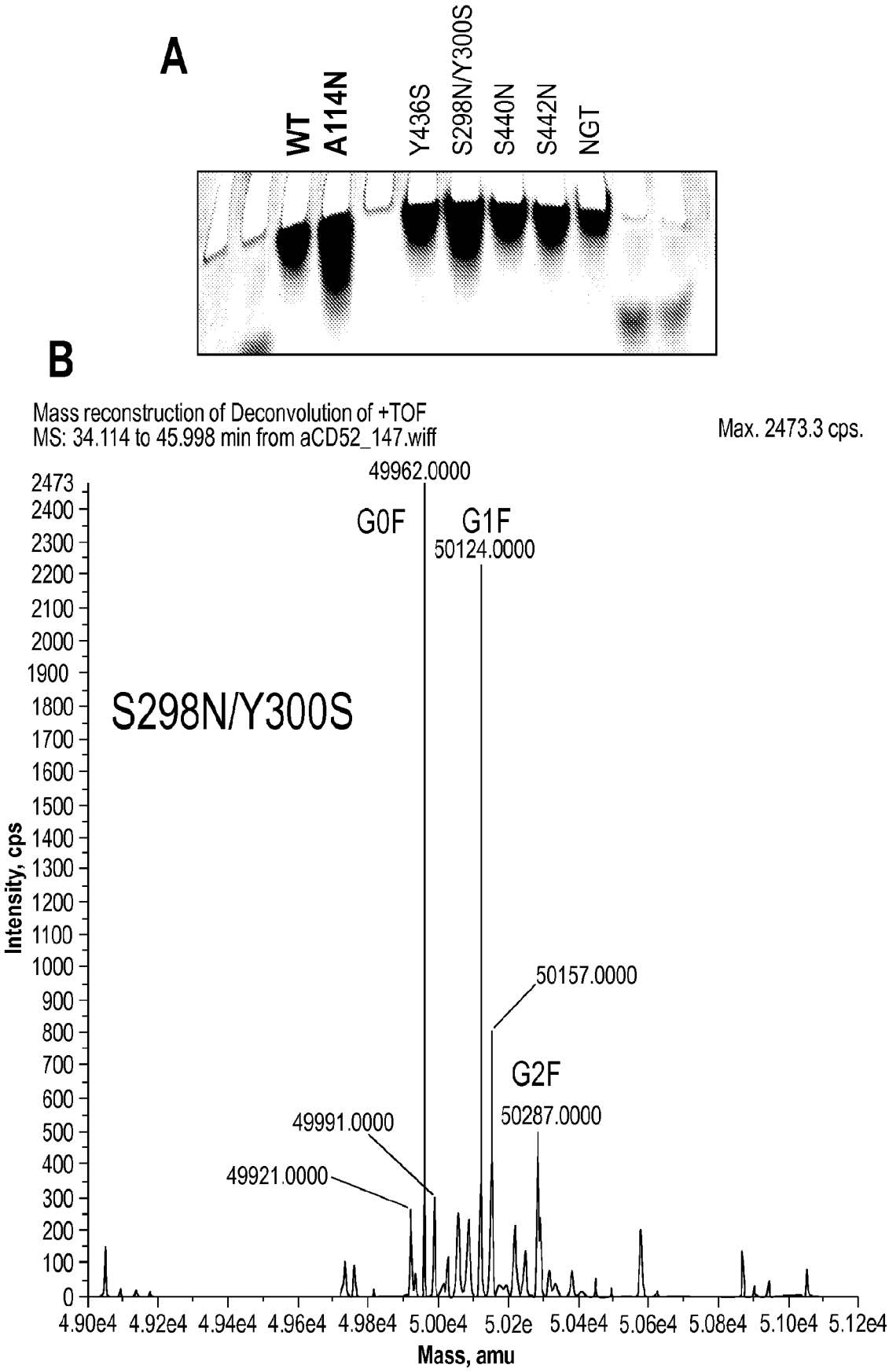

A
WT
A114N
Y436S
S298N/Y300S
S440N
S442N
NGT
B
Mass reconstruction of Deconvolution of +TOF
MS: 34.114 to 45.998 min from aCD52_147.wiff
Max. 2473.3 cps.
49962.0000
G0F
G1F
50124.0000
S298N/Y300S
50157.0000
G2F
50287.0000
49991.0000
49921.0000
Intensity, cps
Mass, amu

Fig. 18

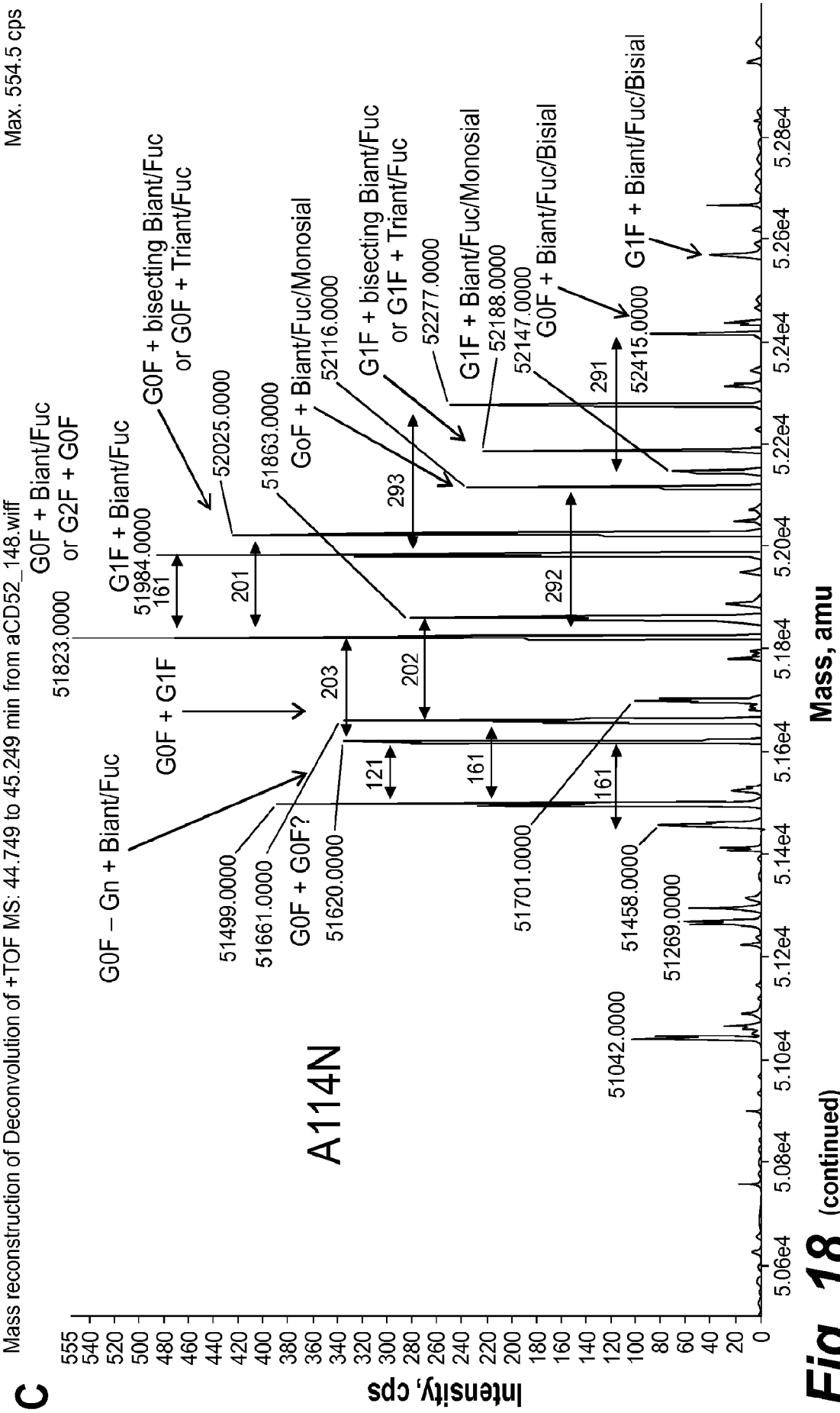


Fig. 18 (continued)

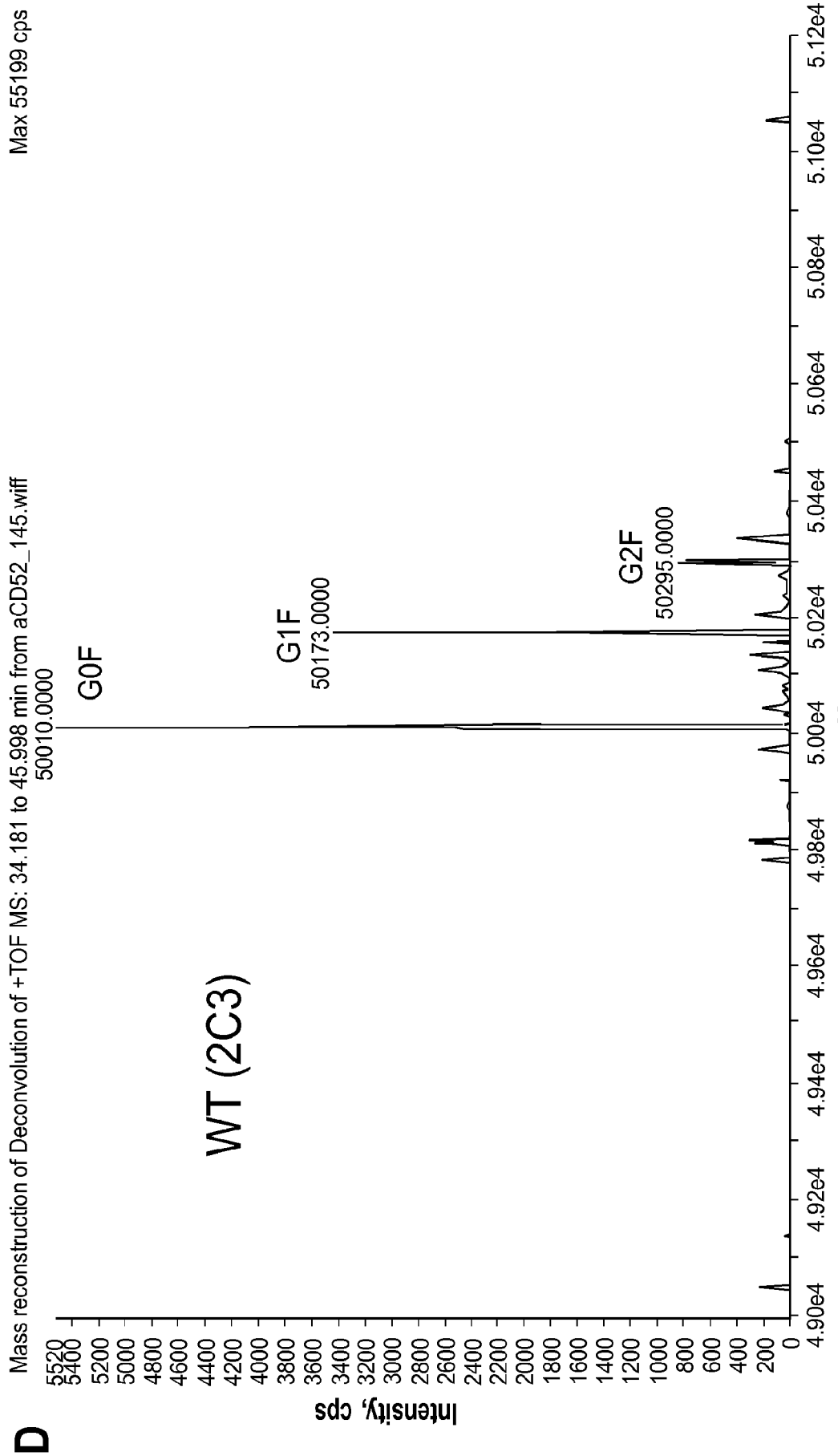


Fig. 18 (continued)

A

Sample	lot #	Octet Conc (µg/mL)
Mock media	11/23/2009	too low
wt 2C3	11/23/2009	2.54
A114N	11/23/2009	2.83
S298N/Y300S	11/23/2009	1.36
S440N	11/23/2009	1.32
S442N	11/23/2009	1.21
Y436S	11/23/2009	1.92
Y436T	11/23/2009	0.34
NGT	11/23/2009	1.90

B

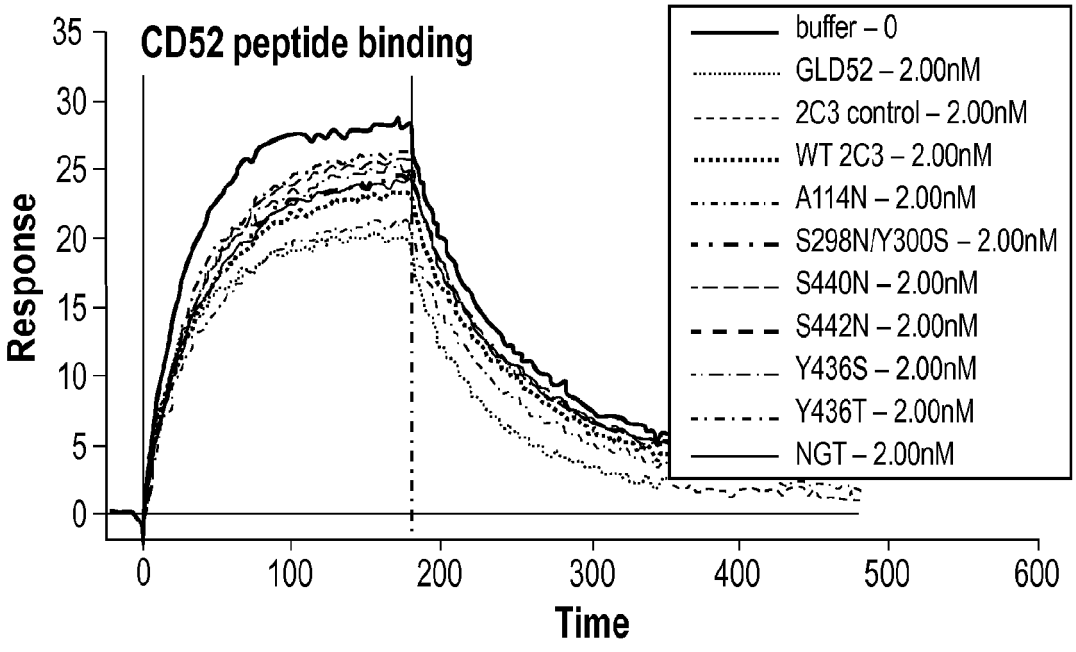


Fig. 19

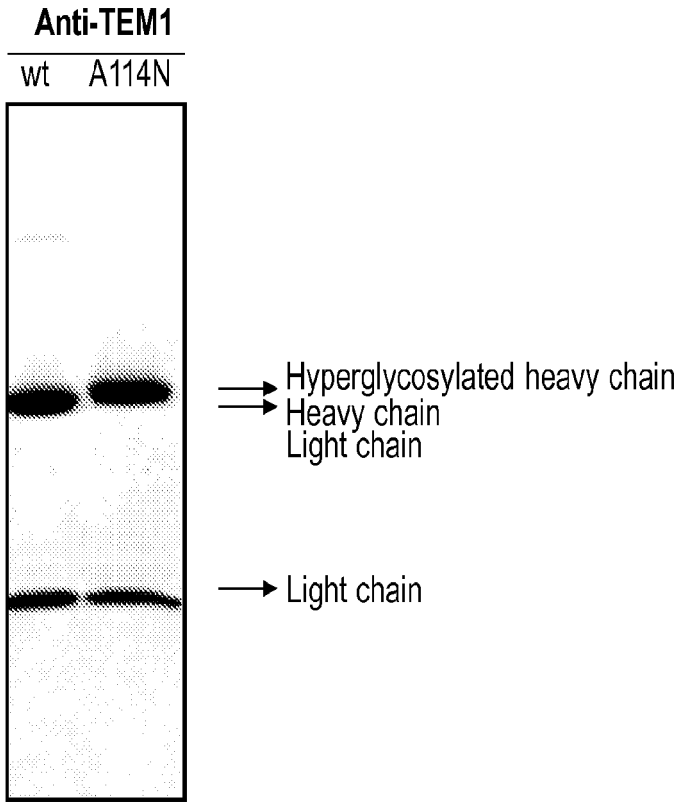
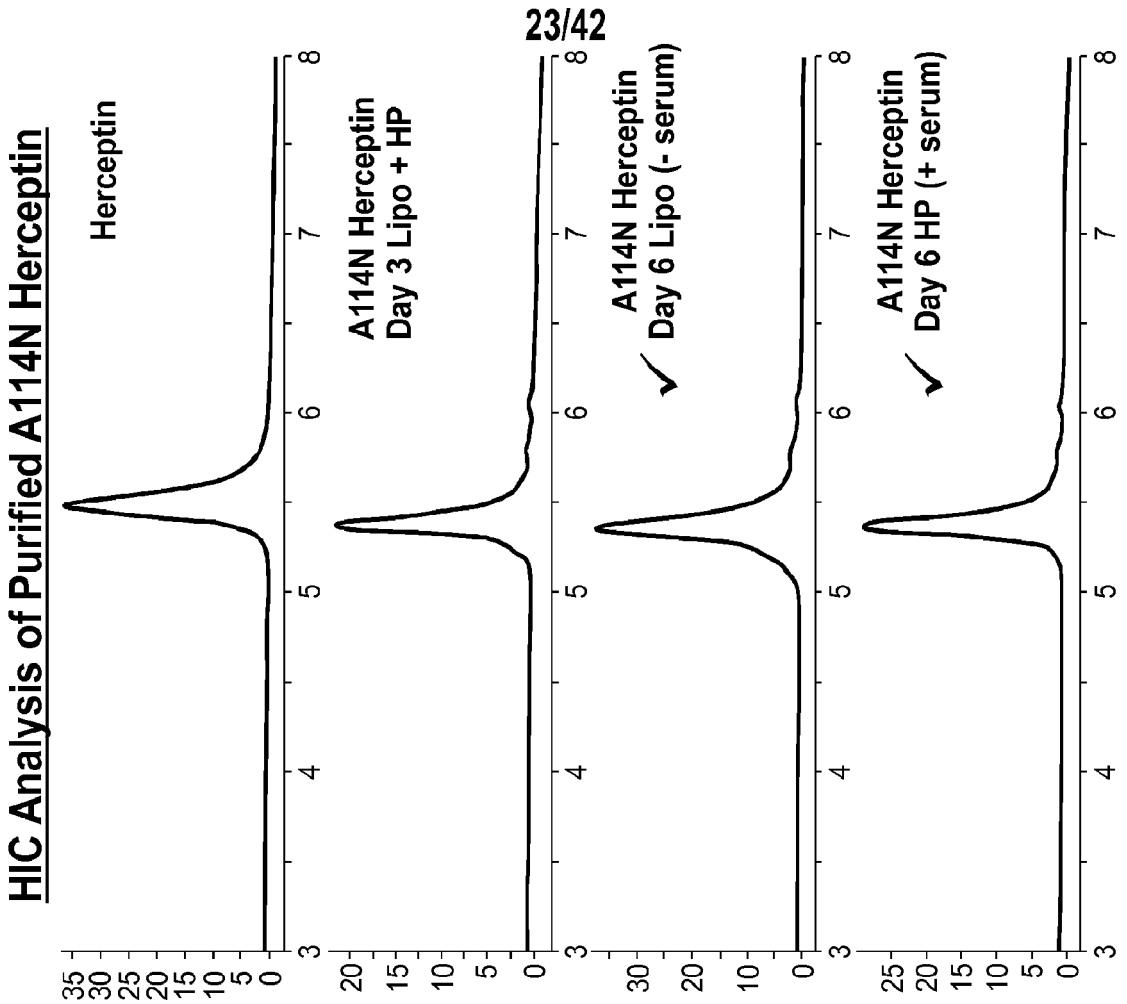

Anti-TEM1
wt
A114N
Hyperglycosylated heavy chain
Heavy chain
Light chain
Light chain

Fig. 20


HIC Analysis of Purified A114N Herceptin
Herceptin
A114N Herceptin
Day 3 Lipo + HP
A114N Herceptin
Day 6 Lipo (- serum)
A114N Herceptin
Day 6 HP (+ serum)

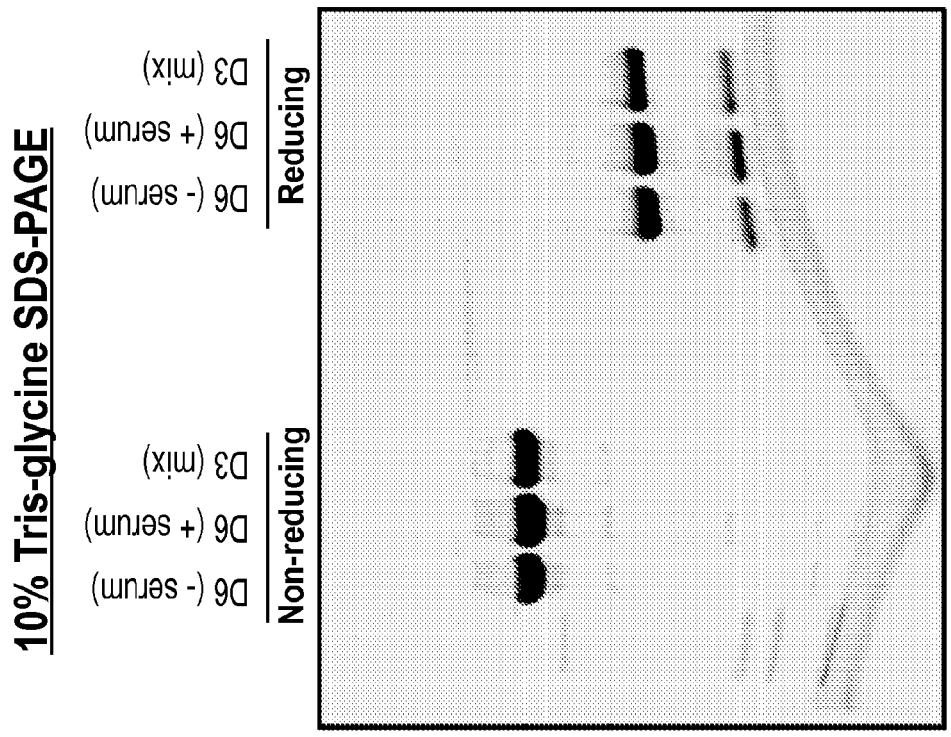

10% Tris-glycine SDS-PAGE
Non-reducing
D6 (- serum)
D6 (+ serum)
D3 (mix)
Reducing
D6 (- serum)
D6 (+ serum)
D3 (mix)

Fig. 21

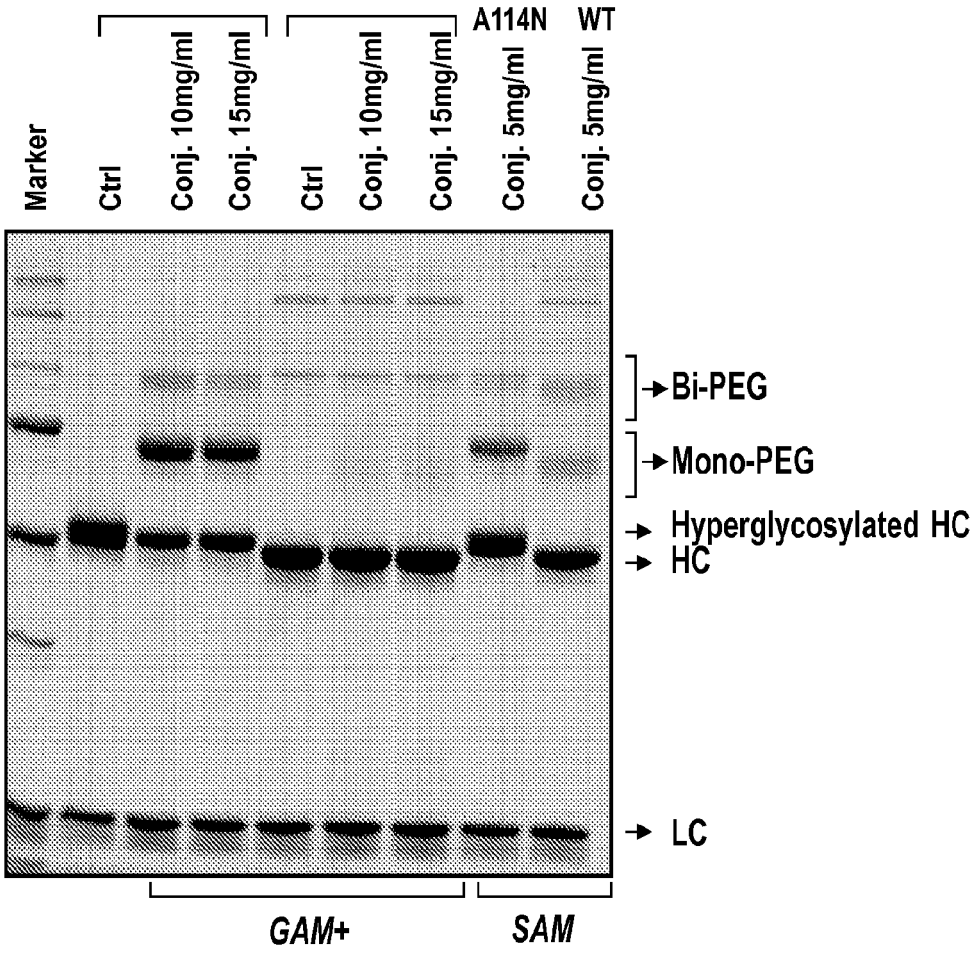

Marker
Ctrl
Conj. 10mg/ml
Conj. 15mg/ml
Ctrl
Conj. 10mg/ml
Conj. 15mg/ml
A114N
Conj. 5mg/ml
WT
Conj. 5mg/ml
Bi-PEG
Mono-PEG
Hyperglycosylated HC
HC
LC
GAM+
SAM

Fig. 22

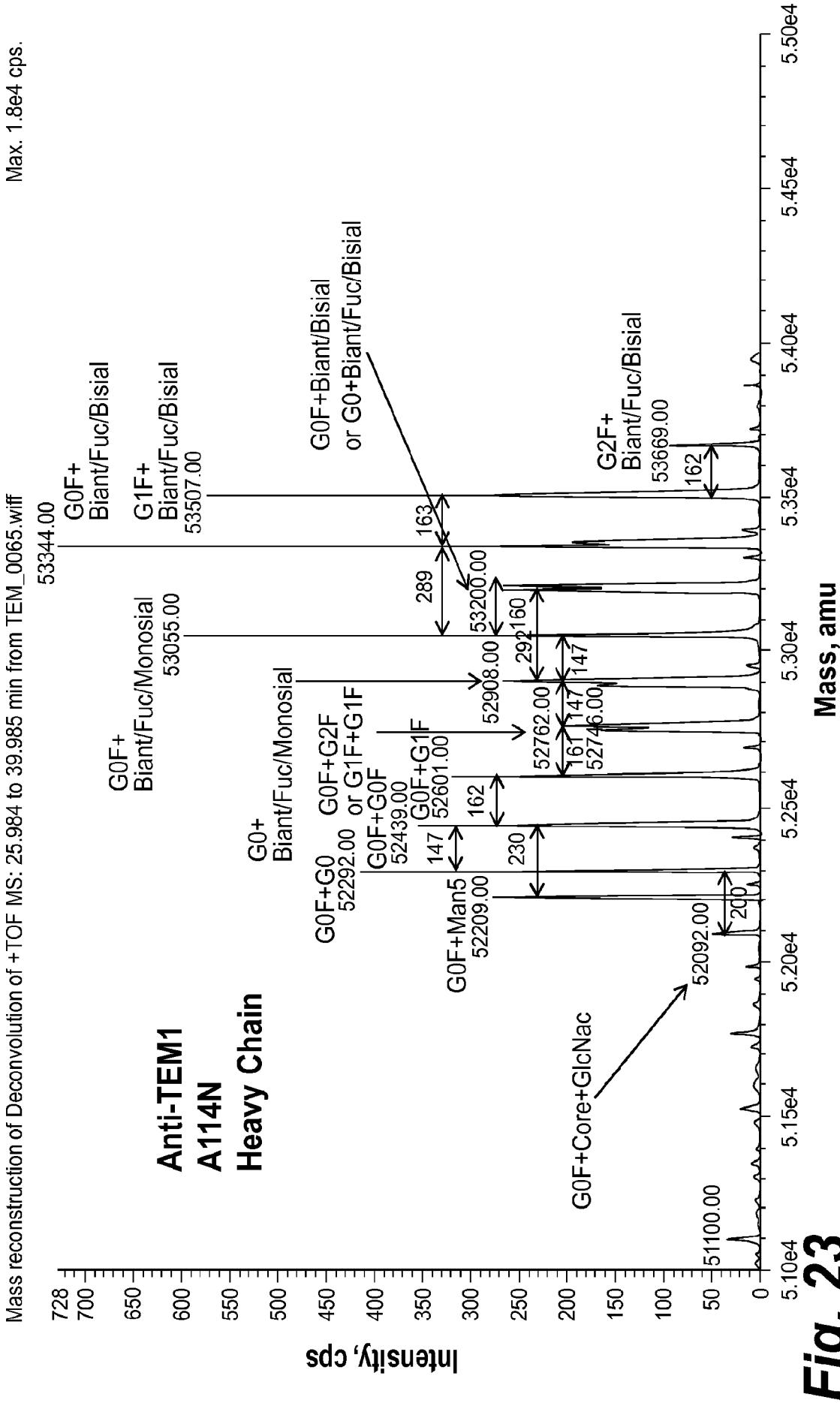

Mass reconstruction of Deconvolution of +TOF MS: 25.984 to 39.985 min from TEM_0065.wiff
Max. 1.8e4 cps.
Anti-TEM1
A114N
Heavy Chain
Intensity, cps
Mass, amu
G0F+
Biant/Fuc/Bisial
53344.00
G1F+
Biant/Fuc/Bisial
53507.00
G0F+Biant/Bisial
or G0+Biant/Fuc/Bisial
G2F+
Biant/Fuc/Bisial
53669.00
G0F+
Biant/Fuc/Monosial
53055.00
G0+
Biant/Fuc/Monosial
G0F+G0
52292.00
G0F+G2F
or G1F+G1F
G0F+G0F
52439.00
G0F+G1F
52601.00
G0F+Man5
52209.00
G0F+Core+GlcNac
52092.00
51100.00
53200.00
52908.00
52762.00
52746.00
163
289
160
292
147
147
161
162
147
230
200
728
700
650
600
550
500
450
400
350
300
250
200
150
100
50
0
5.10e4
5.15e4
5.20e4
5.25e4
5.30e4
5.35e4
5.40e4
5.45e4
5.50e4

Fig. 23

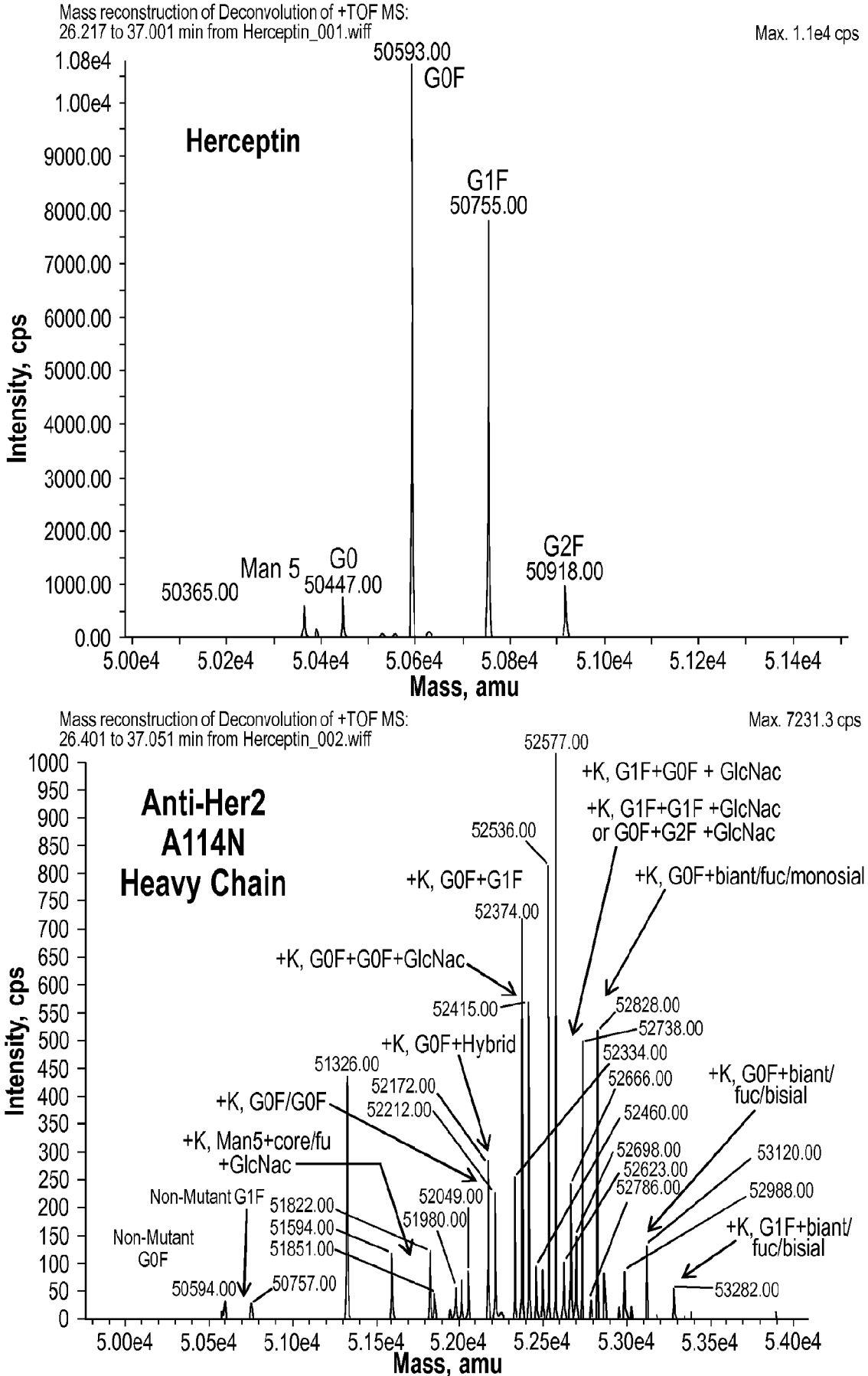

Mass reconstruction of Deconvolution of +TOF MS:
26.217 to 37.001 min from Herceptin_001.wiff
Max. 1.1e4 cps
Herceptin
50593.00
G0F
G1F
50755.00
G2F
50918.00
Man 5
50365.00
G0
50447.00
Intensity, cps
Mass, amu
Mass reconstruction of Deconvolution of +TOF MS:
26.401 to 37.051 min from Herceptin_002.wiff
Max. 7231.3 cps
Anti-Her2
A114N
Heavy Chain
52577.00
+K, G1F+G0F + GlcNac
+K, G1F+G1F +GlcNac
or G0F+G2F +GlcNac
52536.00
+K, G0F+G1F
+K, G0F+biant/fuc/monosial
52374.00
+K, G0F+G0F+GlcNac
52415.00
52828.00
52738.00
+K, G0F+Hybrid
52334.00
51326.00
52666.00
+K, G0F+biant/
fuc/bisial
52172.00
52212.00
+K, G0F/G0F
52460.00
+K, Man5+core/fu
+GlcNac
52698.00
53120.00
52623.00
52786.00
Non-Mutant G1F
51822.00
52049.00
52988.00
51594.00
51980.00
Non-Mutant
G0F
51851.00
+K, G1F+biant/
fuc/bisial
50594.00
50757.00
53282.00
Intensity, cps
Mass, amu

Fig. 24

A

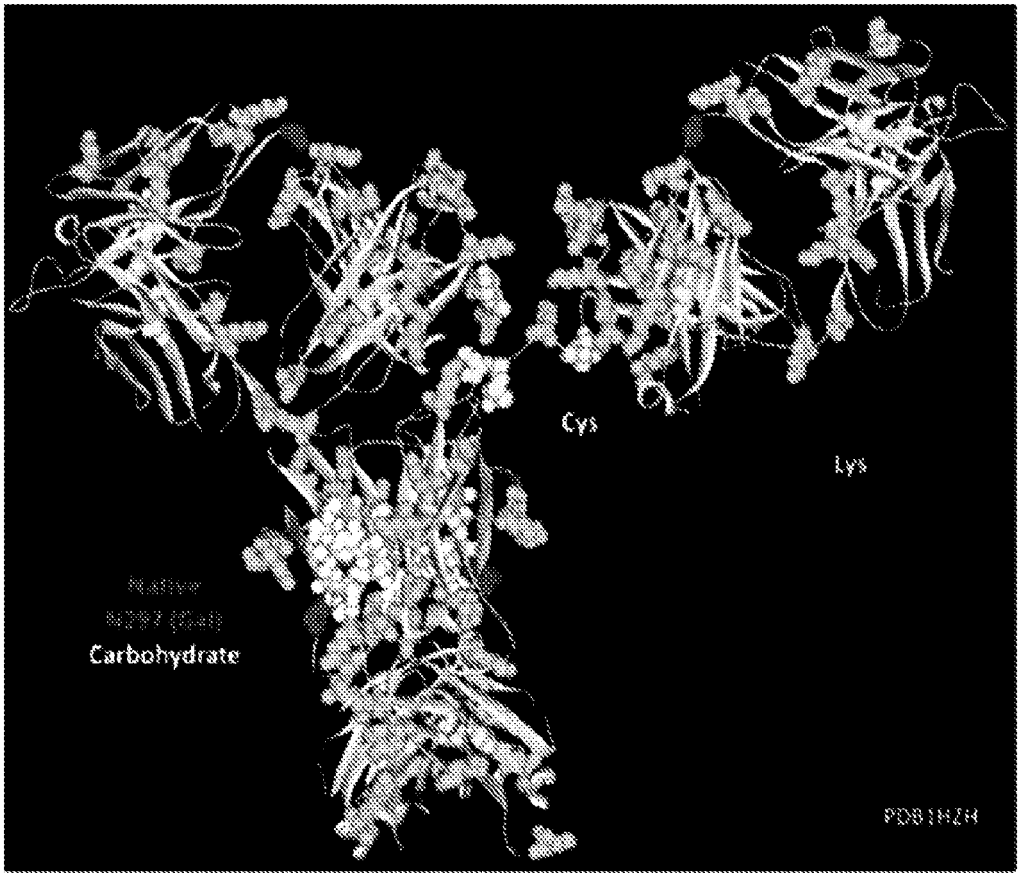
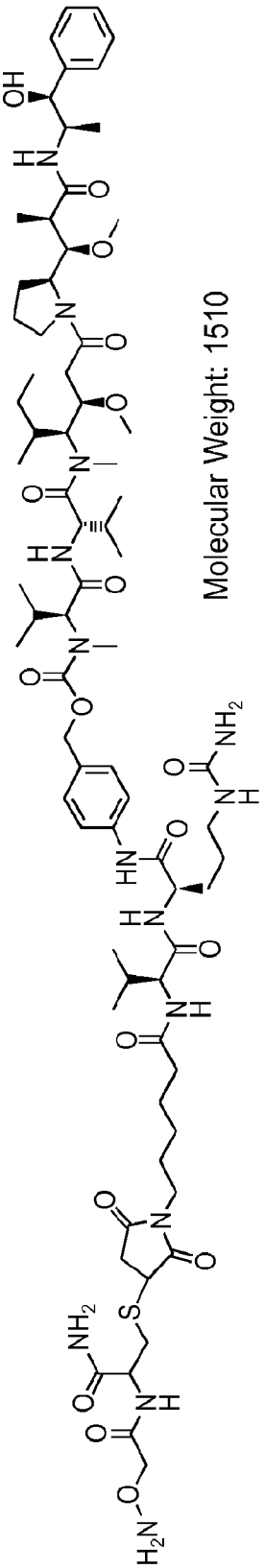


Fig. 25

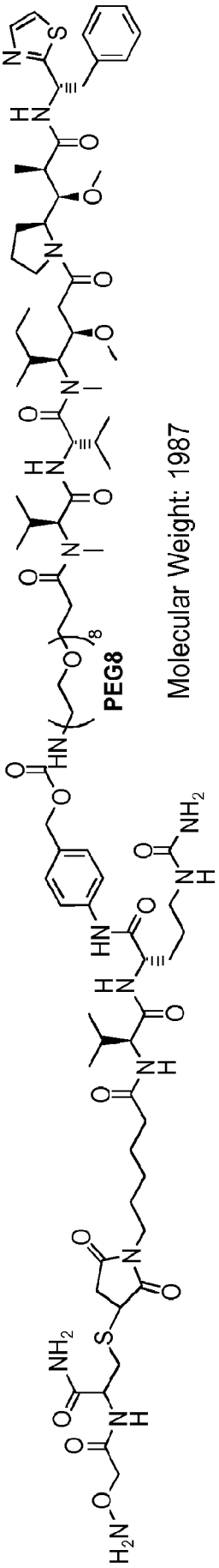
B

Aminooxy-Cys_MC-VC-PABC-MMAE



Molecular Weight: 1510

Aminooxy-Cys-MC-VC-PABC-PEG8-Dol10



Molecular Weight: 1987

Fig. 25
(continued)

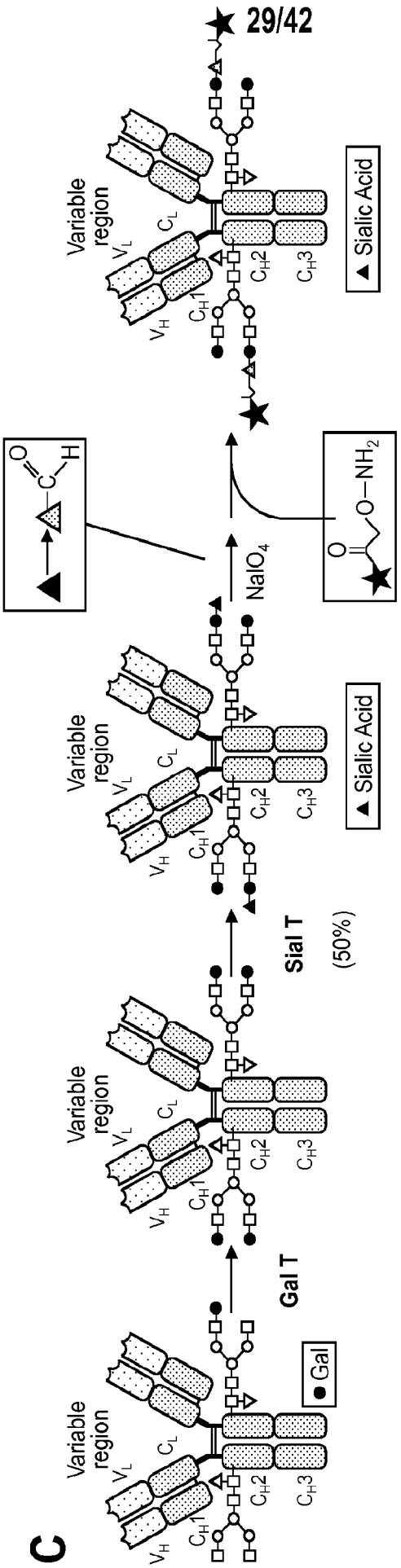


Fig. 25
(continued)

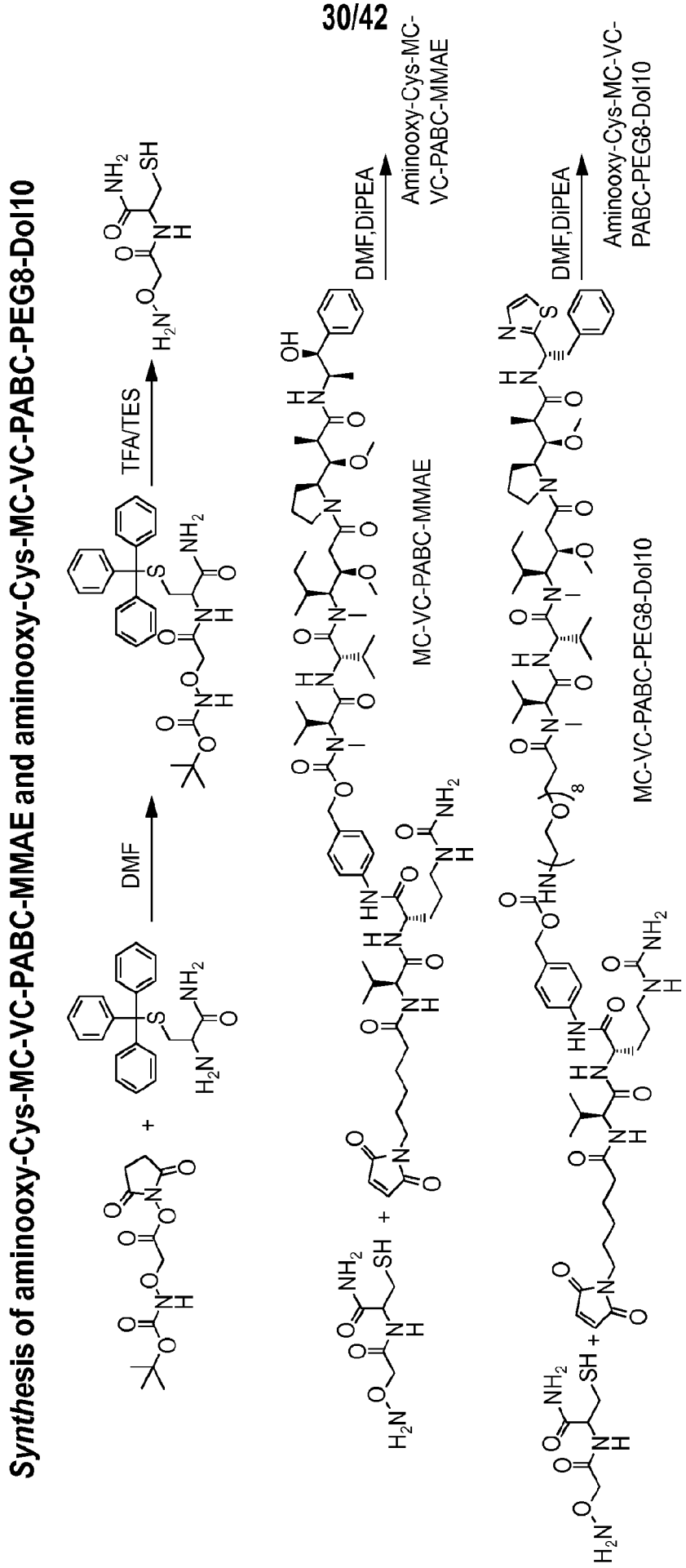

Synthesis of aminooxy-Cys-MC-VC-PABC-MMAE and aminooxy-Cys-MC-VC-PABC-PEG8-Dol10
DMF
TFA/TES
DMF,DiPEA
Aminooxy-Cys-MC-VC-PABC-MMAE
MC-VC-PABC-MMAE
DMF,DiPEA
Aminooxy-Cys-MC-VC-PABC-PEG8-Dol10
MC-VC-PABC-PEG8-Dol10

Fig. 26

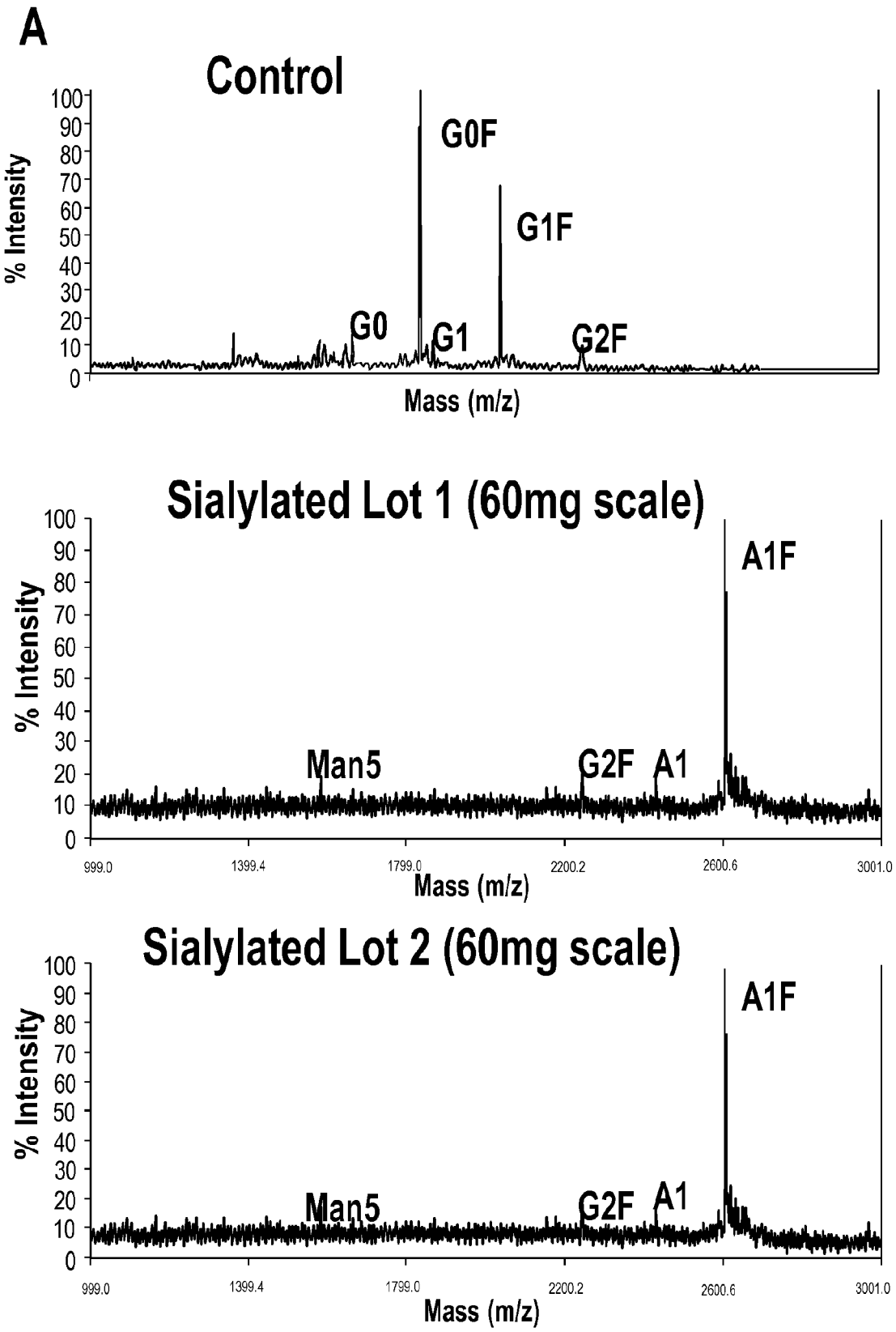

A
Control
% Intensity
100
90
80
70
60
50
40
30
20
10
0
G0F
G1F
G0
G1
G2F
Mass (m/z)
Sialylated Lot 1 (60mg scale)
% Intensity
A1F
Man5
G2F
A1
999.0
1399.4
1799.0
2200.2
2600.6
3001.0
Mass (m/z)
Sialylated Lot 2 (60mg scale)
% Intensity
A1F
Man5
G2F
A1
999.0
1399.4
1799.0
2200.2
2600.6
3001.0
Mass (m/z)

Fig. 27

B

	Sialic acid, mol/mol
Control	0
Sialylated Lot 1	2.2 ± 0
Sialylated Lot2	2.3 ± 0.1

C

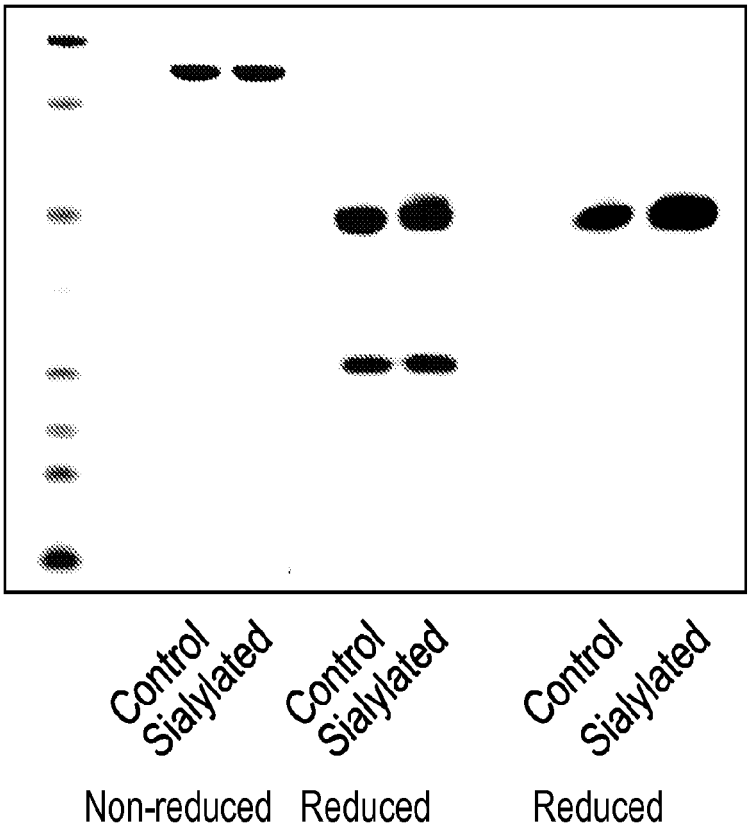
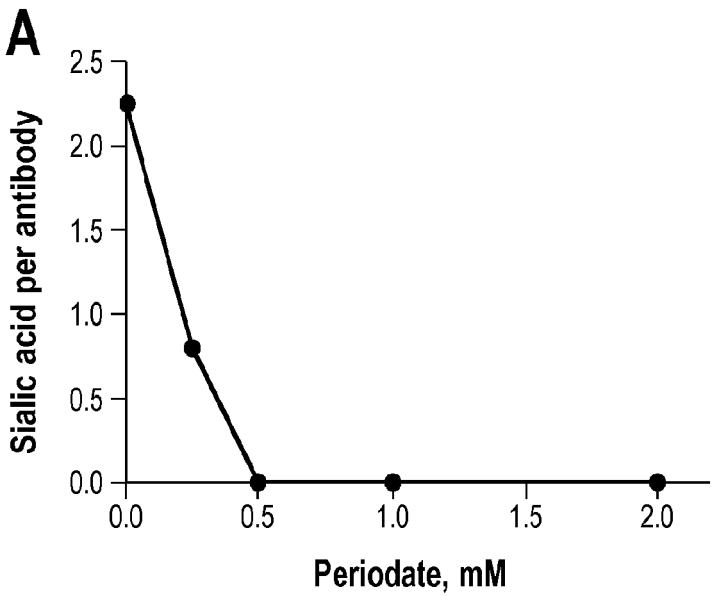


Fig. 27

(continued)


A
Sialic acid per antibody
2.5
2.0
1.5
1.0
0.5
0.0
0.0
0.5
1.0
1.5
2.0
Periodate, mM

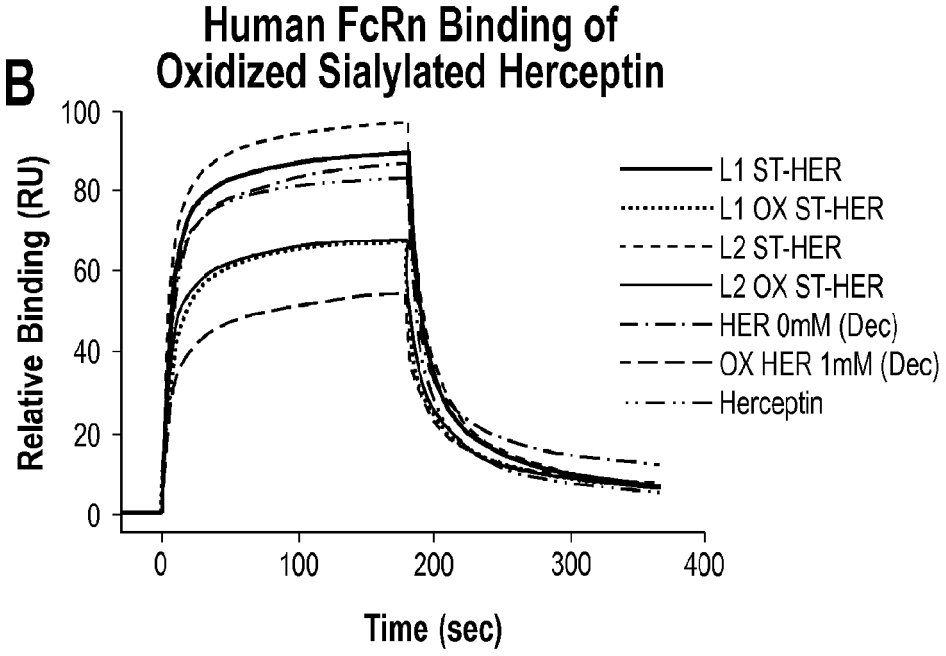
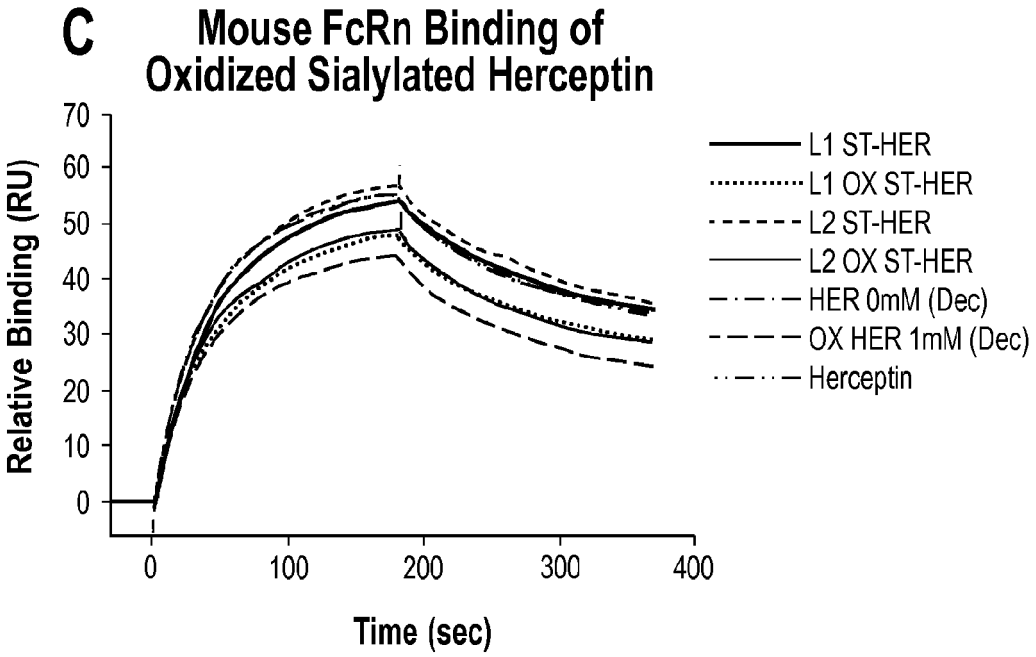

B
Human FcRn Binding of
Oxidized Sialylated Herceptin
Relative Binding (RU)
100
80
60
40
20
0
0
100
200
300
400
Time (sec)
L1 ST-HER
L1 OX ST-HER
L2 ST-HER
L2 OX ST-HER
HER 0mM (Dec)
OX HER 1mM (Dec)
Herceptin

Fig. 28



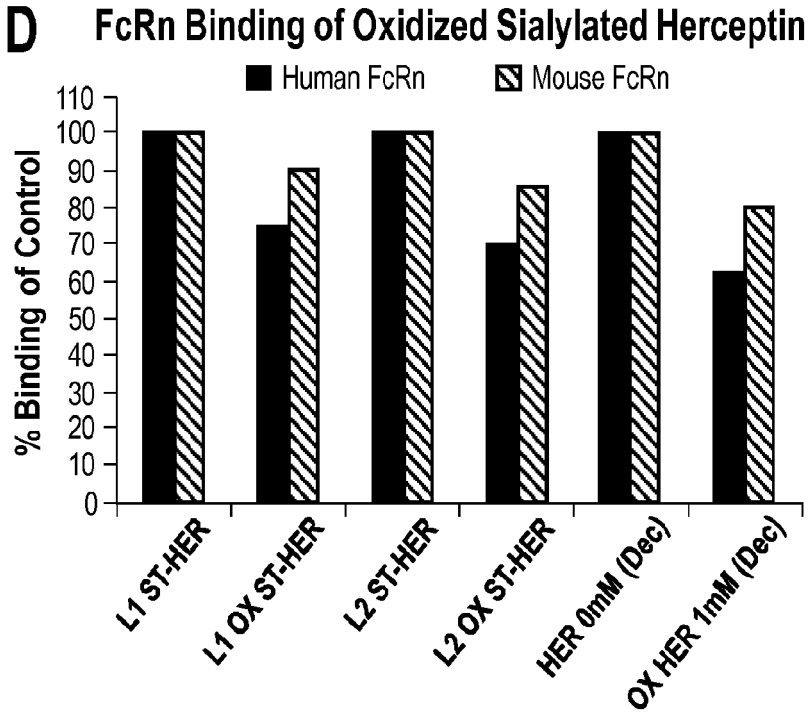


Fig. 28

(continued)

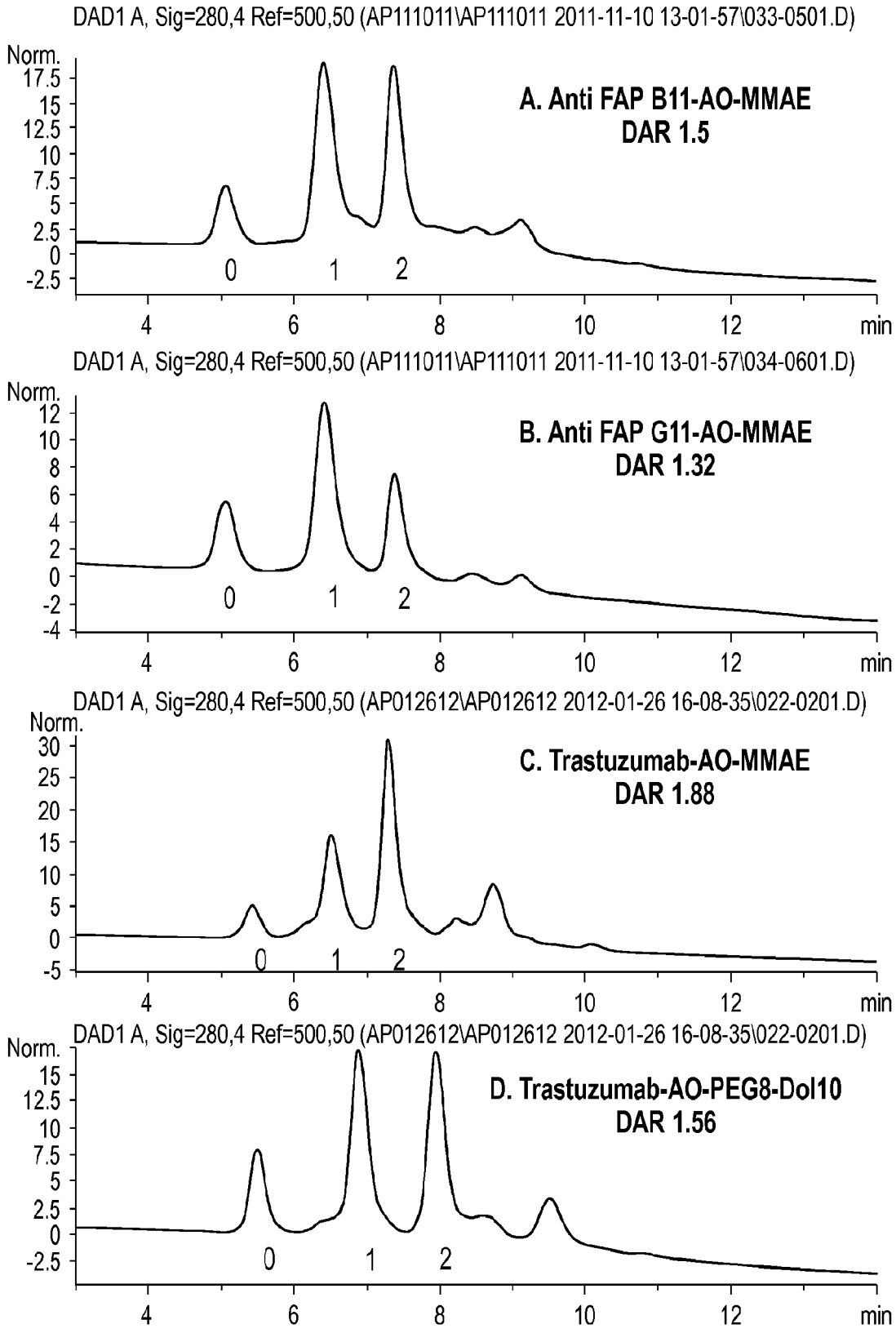

DAD1 A, Sig=280,4 Ref=500,50 (AP111011\AP111011 2011-11-10 13-01-57\033-0501.D)
Norm.
A. Anti FAP B11-AO-MMAE
DAR 1.5
0
1
2
min
DAD1 A, Sig=280,4 Ref=500,50 (AP111011\AP111011 2011-11-10 13-01-57\034-0601.D)
Norm.
B. Anti FAP G11-AO-MMAE
DAR 1.32
0
1
2
min
DAD1 A, Sig=280,4 Ref=500,50 (AP012612\AP012612 2012-01-26 16-08-35\022-0201.D)
Norm.
C. Trastuzumab-AO-MMAE
DAR 1.88
0
1
2
min
DAD1 A, Sig=280,4 Ref=500,50 (AP012612\AP012612 2012-01-26 16-08-35\022-0201.D)
Norm.
D. Trastuzumab-AO-PEG8-Dol10
DAR 1.56
0
1
2
min

Fig. 29

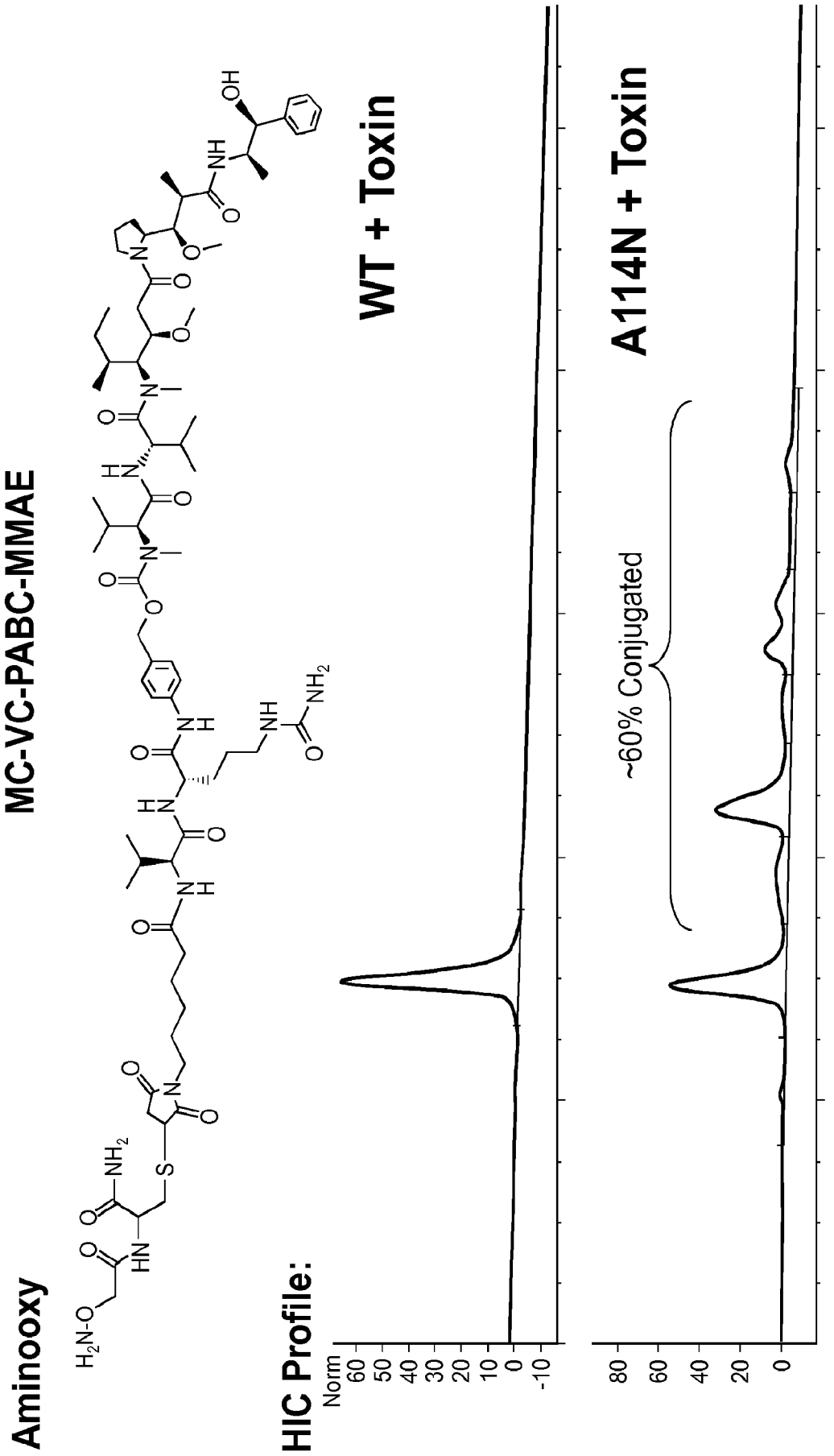

Aminooxy
MC-VC-PABC-MMAE
HIC Profile:
Norm
60
50
40
30
20
10
0
-10
WT + Toxin
A114N + Toxin
~60% Conjugated
80
60
40
20
0

Fig. 30

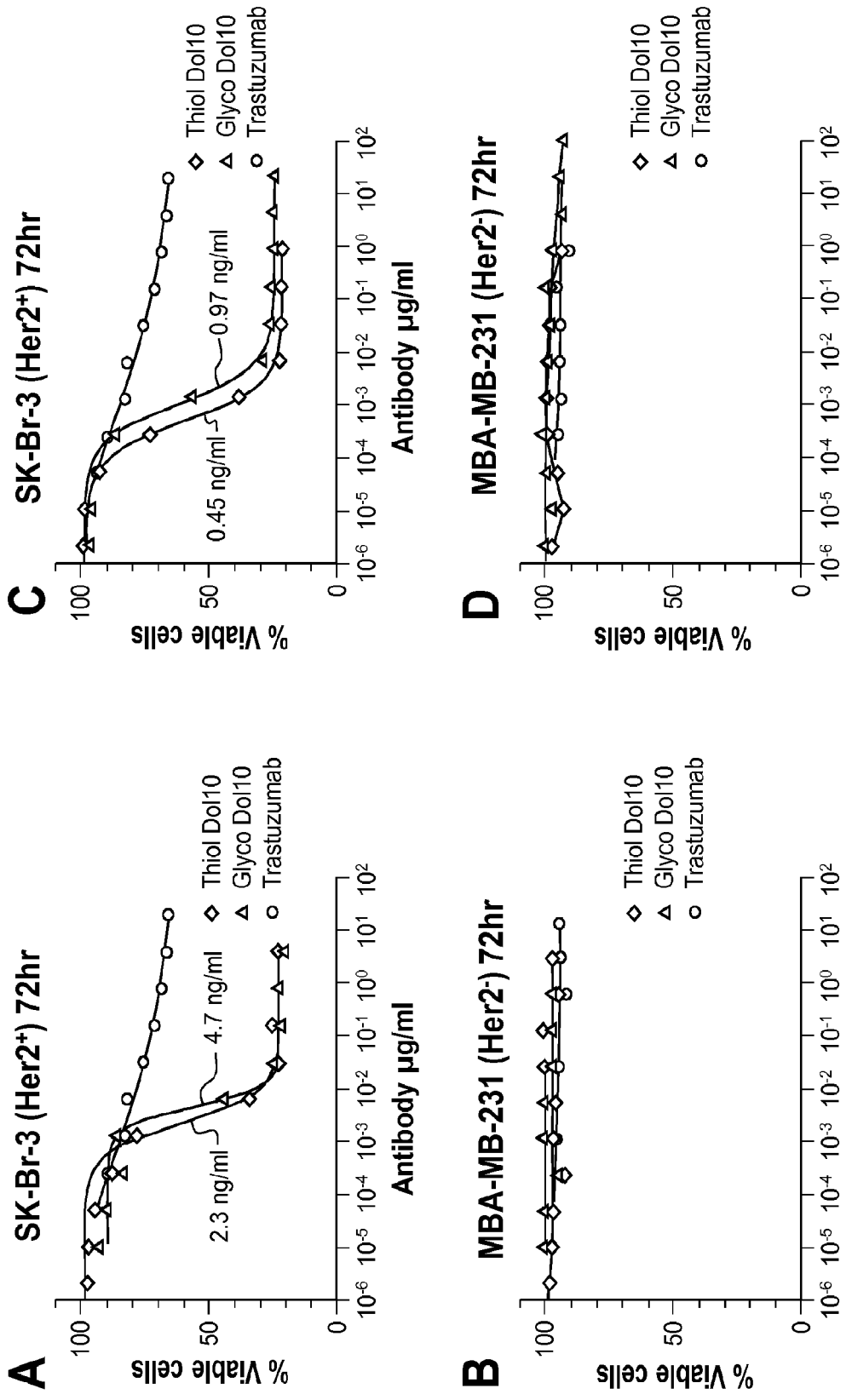

A
SK-Br-3 (Her2+) 72hr
% Viable cells
Antibody µg/ml
Thiol Dol10
Glyco Dol10
Trastuzumab
4.7 ng/ml
2.3 ng/ml
B
MBA-MB-231 (Her2-) 72hr
C
SK-Br-3 (Her2+) 72hr
0.97 ng/ml
0.45 ng/ml
D
MBA-MB-231 (Her2-) 72hr

Fig. 31

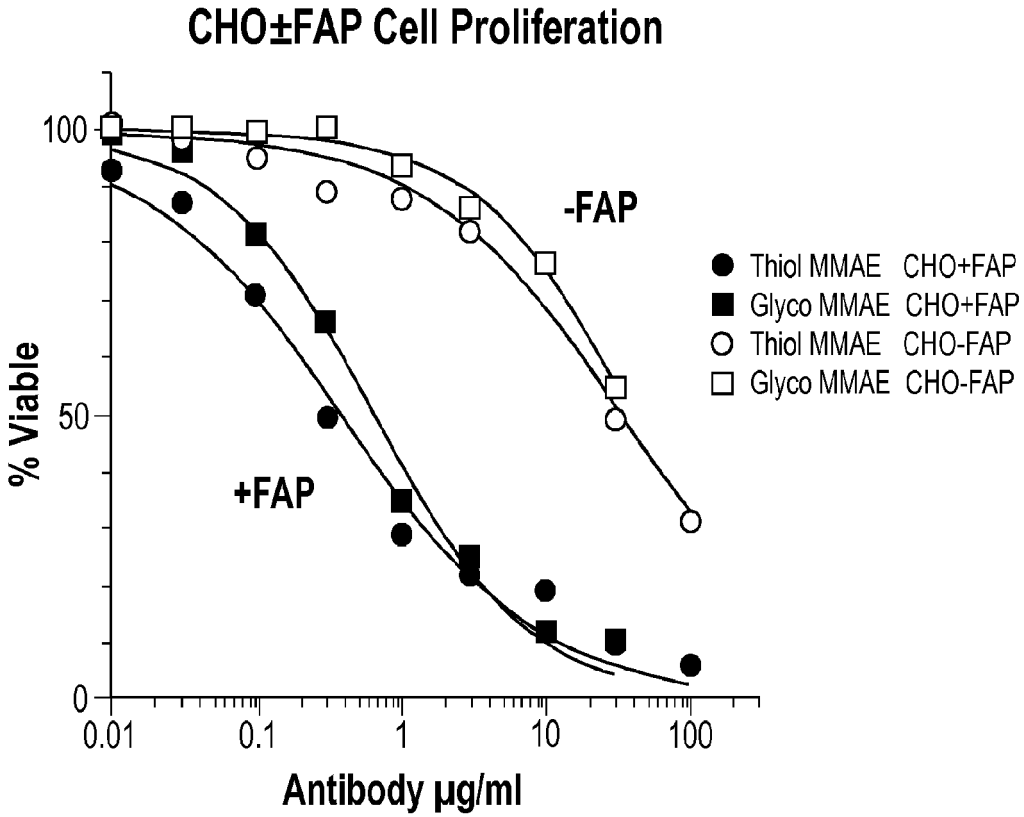

CHO±FAP Cell Proliferation
100
50
0
% Viable
-FAP
+FAP
0.01
0.1
1
10
100
Antibody µg/ml
Thiol MMAE CHO+FAP
Glyco MMAE CHO+FAP
Thiol MMAE CHO-FAP
Glyco MMAE CHO-FAP

Fig. 32

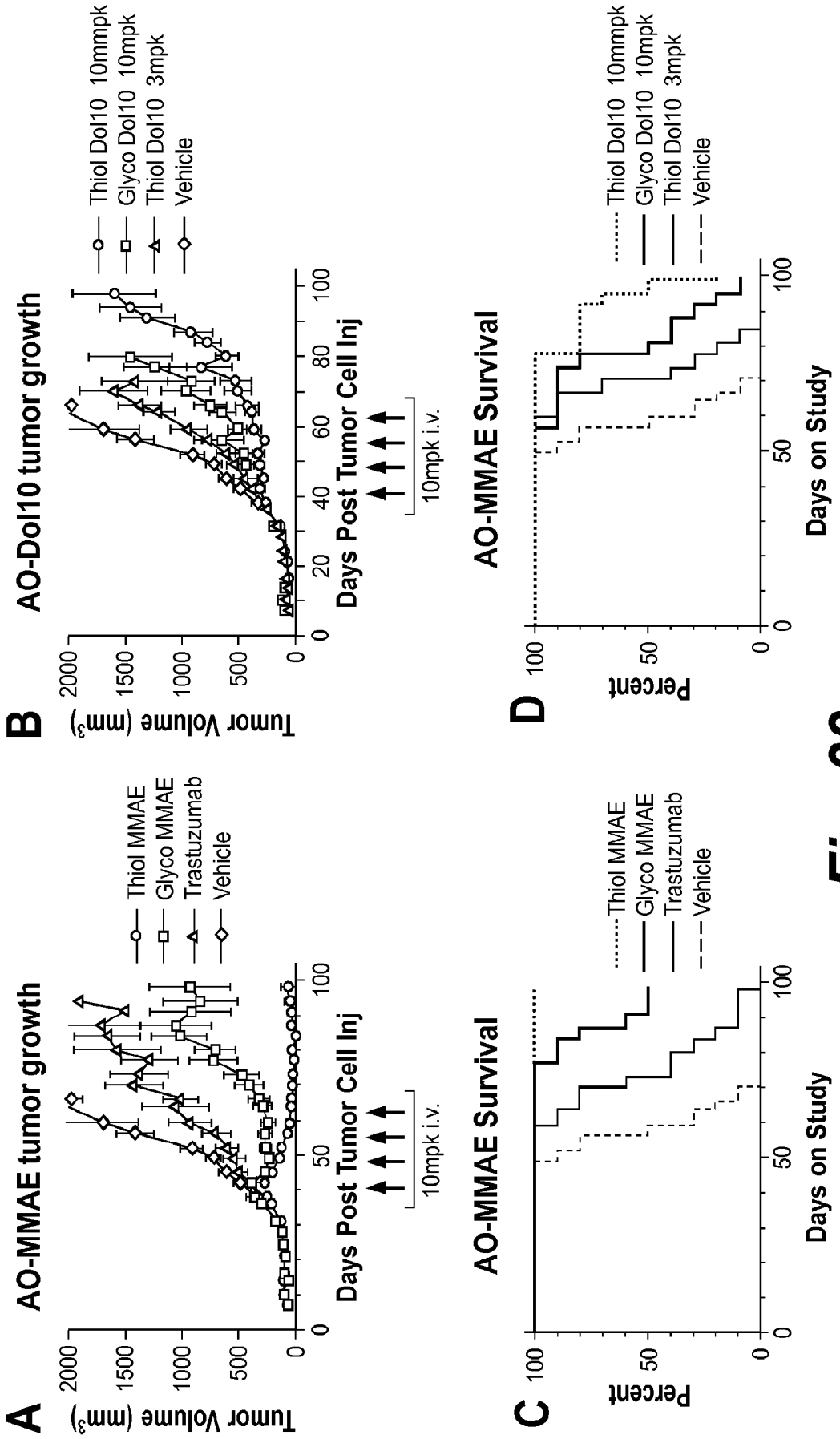

A
AO-MMAE tumor growth
Tumor Volume (mm3)
Days Post Tumor Cell Inj
10mpk i.v.
Thiol MMAE
Glyco MMAE
Trastuzumab
Vehicle
B
AO-Dol10 tumor growth
Tumor Volume (mm3)
Days Post Tumor Cell Inj
10mpk i.v.
Thiol Dol10 10mmpk
Glyco Dol10 10mpk
Thiol Dol10 3mpk
Vehicle
C
AO-MMAE Survival
Percent
Days on Study
Thiol MMAE
Glyco MMAE
Trastuzumab
Vehicle
D
AO-MMAE Survival
Percent
Days on Study
Thiol Dol10 10mmpk
Glyco Dol10 10mpk
Thiol Dol10 3mpk
Vehicle

Fig. 33

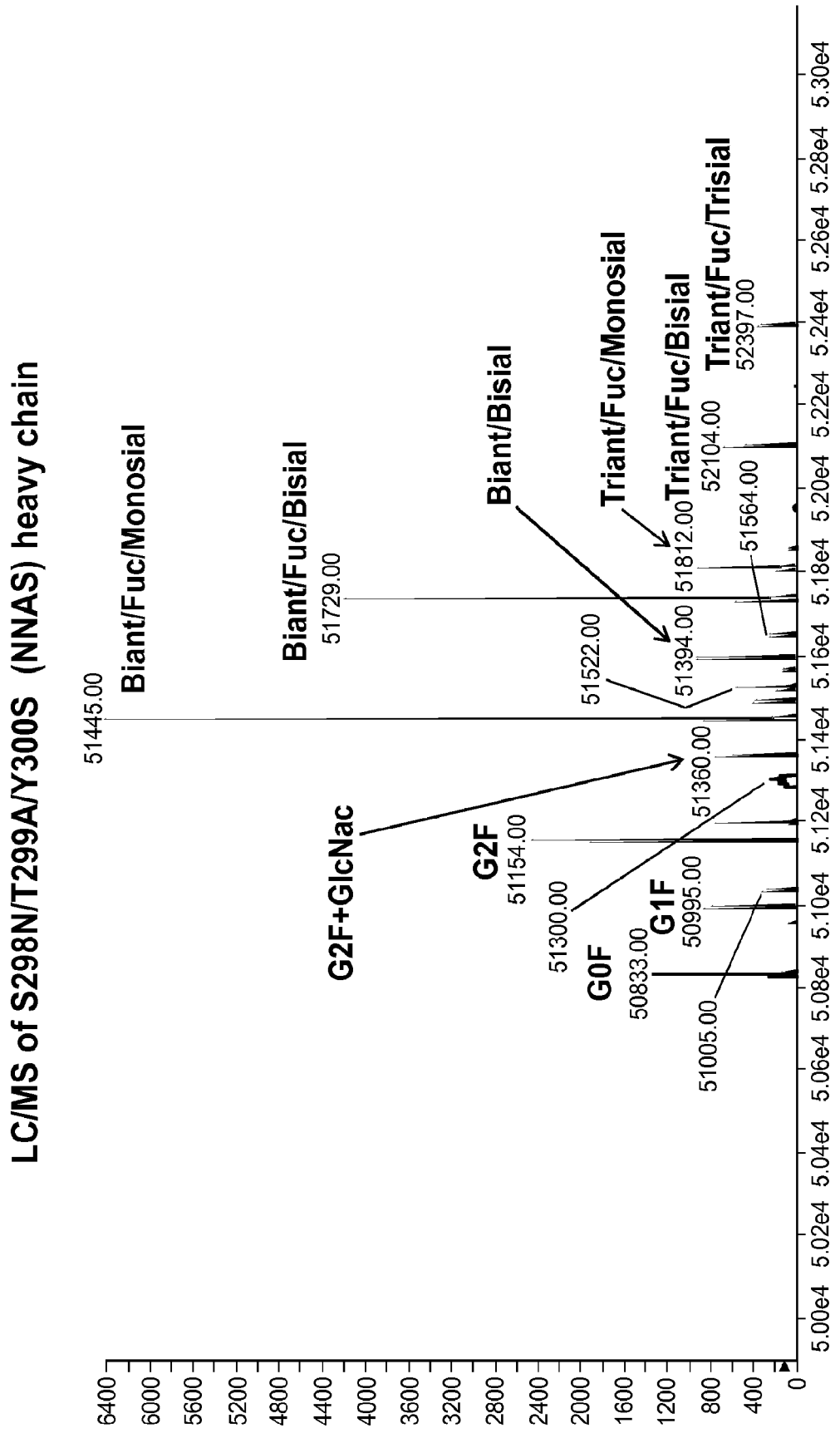

LC/MS of S298N/T299A/Y300S (NNAS) heavy chain
Biant/Fuc/Monosial
51445.00
Biant/Fuc/Bisial
51729.00
Biant/Bisial
51522.00
Triant/Fuc/Monosial
51394.00
Triant/Fuc/Bisial
51812.00
Triant/Fuc/Trisial
52397.00
52104.00
51564.00
G2F+GlcNac
51360.00
G2F
51154.00
51300.00
G1F
50995.00
G0F
50833.00
51005.00
Mass, amu
5.00e4 5.02e4 5.04e4 5.06e4 5.08e4 5.10e4 5.12e4 5.14e4 5.16e4 5.18e4 5.20e4 5.22e4 5.24e4 5.26e4 5.28e4 5.30e4
0 400 800 1200 1600 2000 2400 2800 3200 3600 4000 4400 4800 5200 5600 6000 6400

Fig. 34

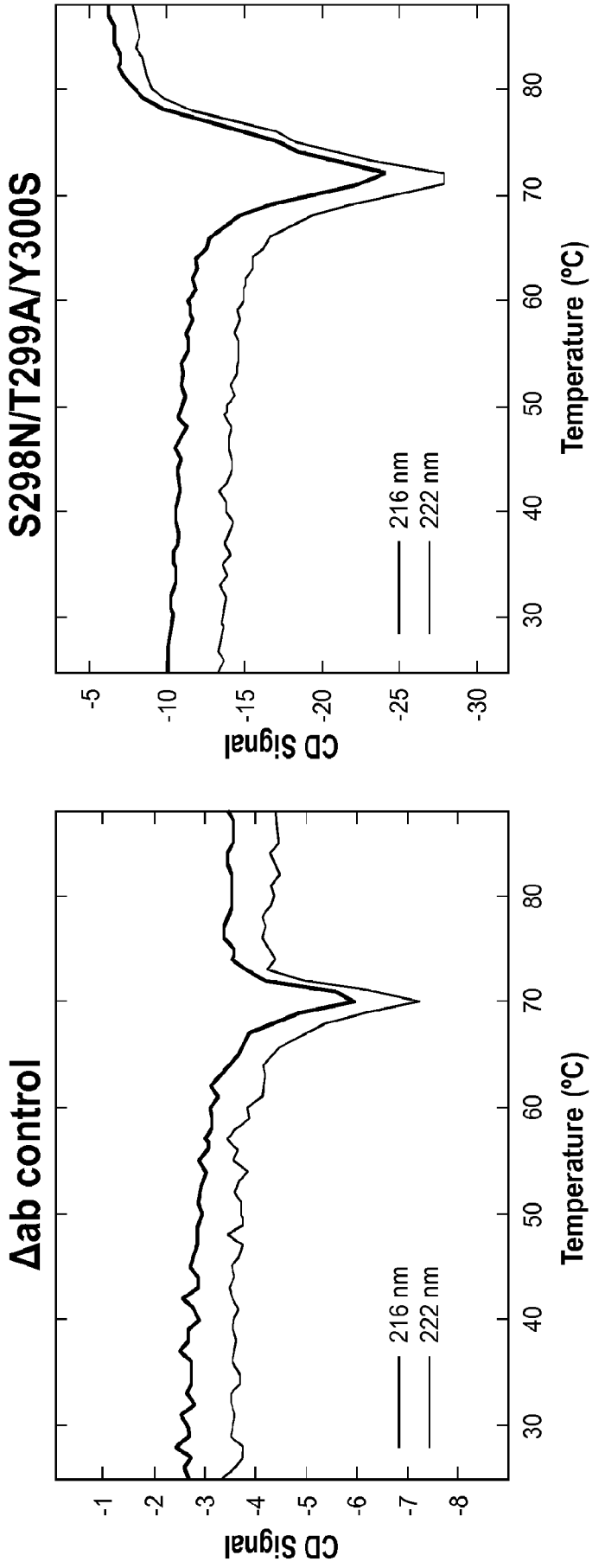

S298N/T299A/Y300S
CD Signal
Temperature (°C)
216 nm
222 nm
Δab control
CD Signal
Temperature (°C)
216 nm
222 nm

Fig. 35

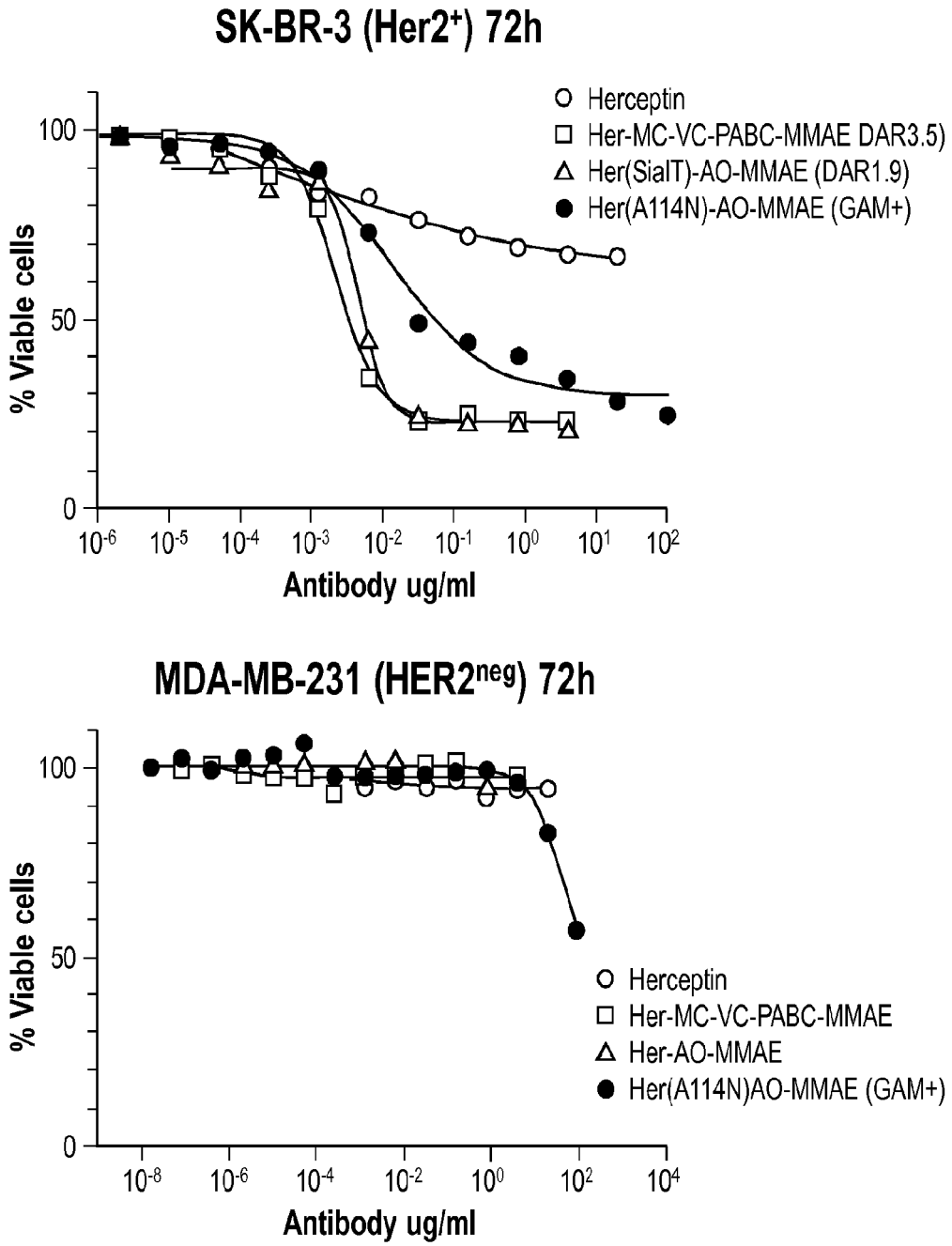

SK-BR-3 (Her2+) 72h
Herceptin
Her-MC-VC-PABC-MMAE DAR3.5)
Her(SialT)-AO-MMAE (DAR1.9)
Her(A114N)-AO-MMAE (GAM+)
% Viable cells
100
50
0
10-6 10-5 10-4 10-3 10-2 10-1 100 101 102
Antibody ug/ml
MDA-MB-231 (HER2neg) 72h
Herceptin
Her-MC-VC-PABC-MMAE
Her-AO-MMAE
Her(A114N)AO-MMAE (GAM+)
% Viable cells
100
50
0
10-8 10-6 10-4 10-2 100 102 104
Antibody ug/ml

Fig. 36