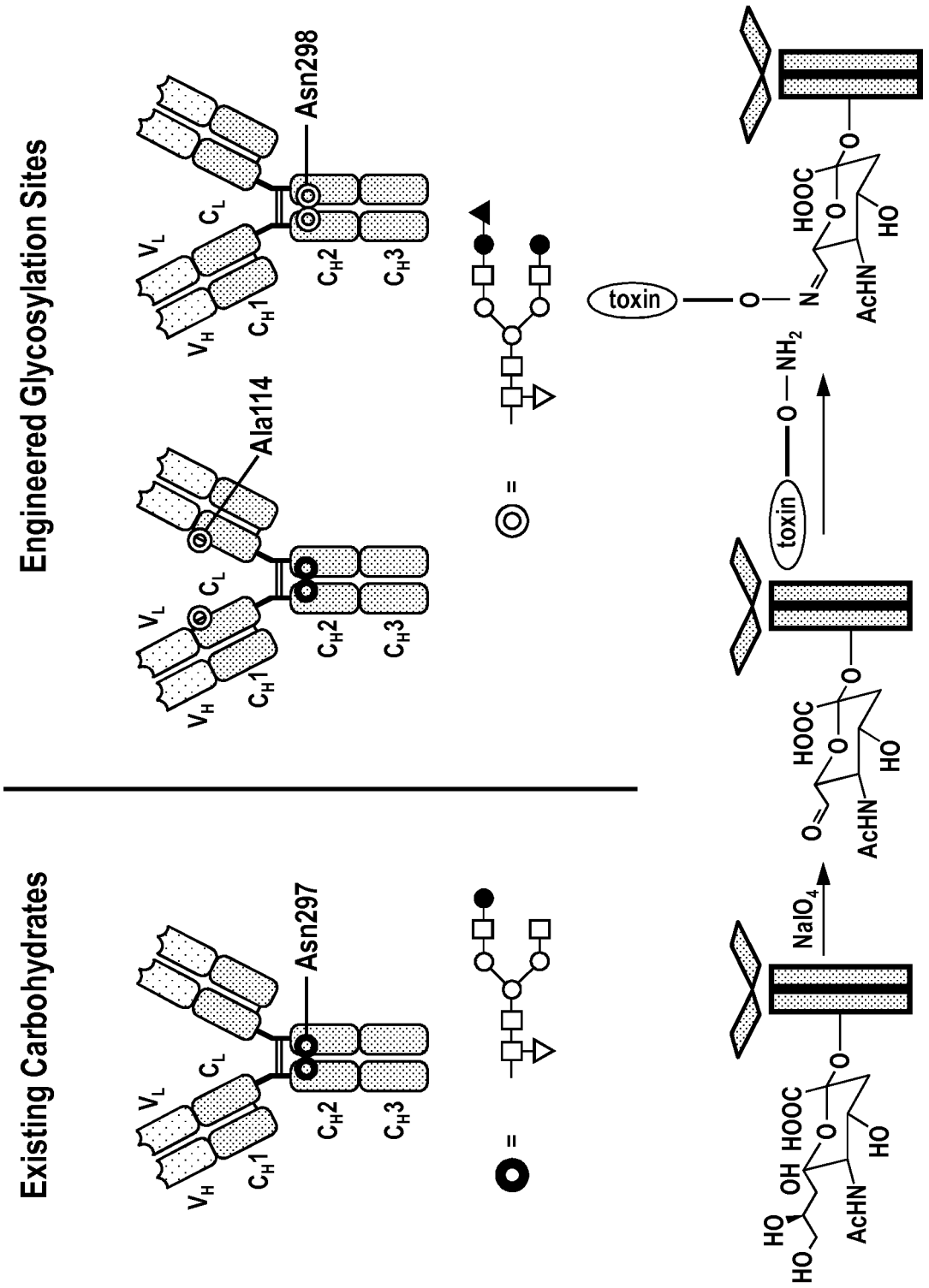

Engineered Glycosylation Sites
Existing Carbohydrates
Asn298
Ala114
Asn297
V_L
C_L
V_H
C_H1
C_H2
C_H3
toxin
$NaIO_4$
NH_2
HOOC
AcHN
HO
OH

Fig. 1

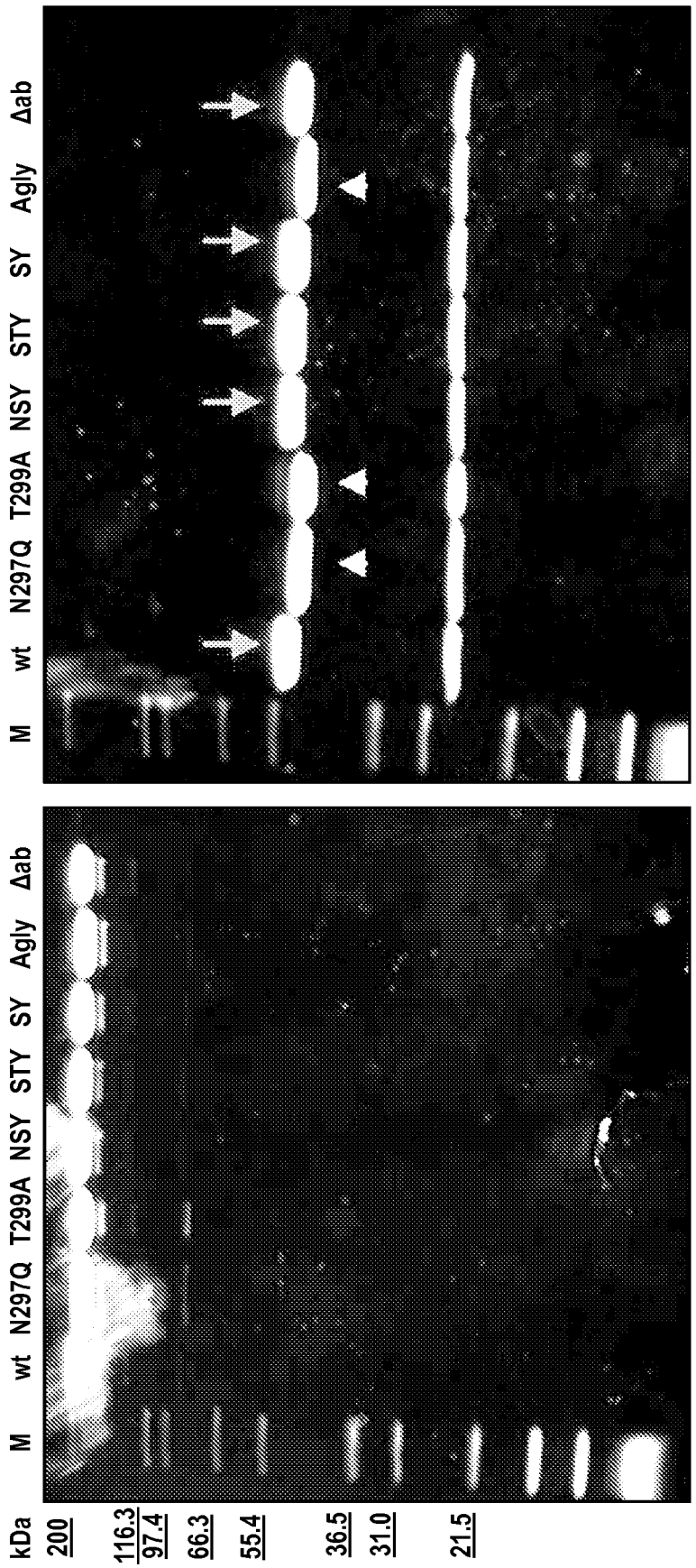

kDa
200
116.3
97.4
66.3
55.4
36.5
31.0
21.5
M
wt
N297Q
T299A
NSY
STY
SY
Agly
Δab
Non-reduced
Reduced

Fig. 2

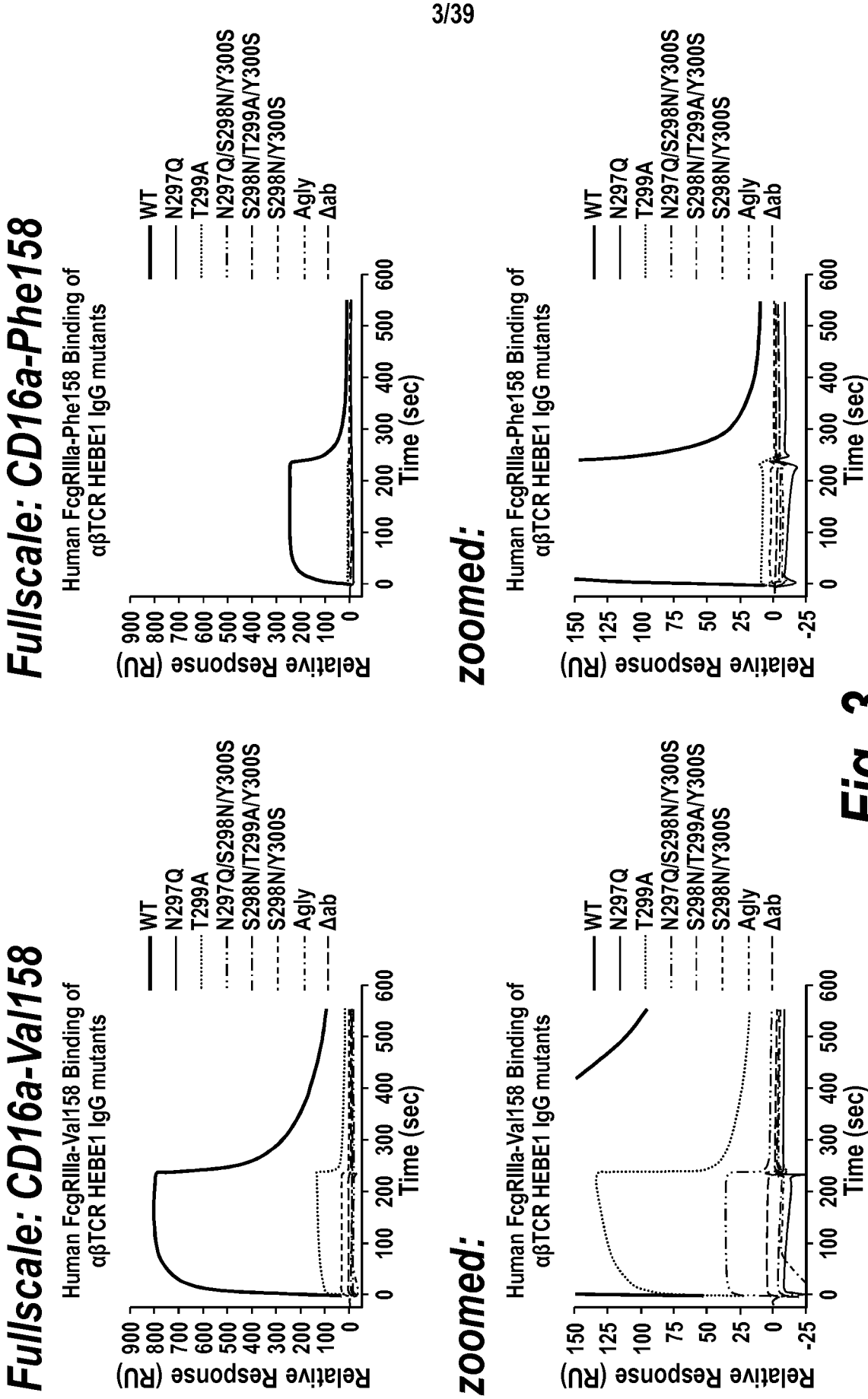

Fullscale: CD16a-Val158
Human FcgRIIIa-Val158 Binding of αβTCR HEBE1 IgG mutants
Relative Response (RU)
Time (sec)
WT
N297Q
T299A
N297Q/S298N/Y300S
S298N/T299A/Y300S
S298N/Y300S
Agly
Δab
zoomed:
Fullscale: CD16a-Phe158
Human FcgRIIIa-Phe158 Binding of αβTCR HEBE1 IgG mutants
zoomed:

Fig. 3

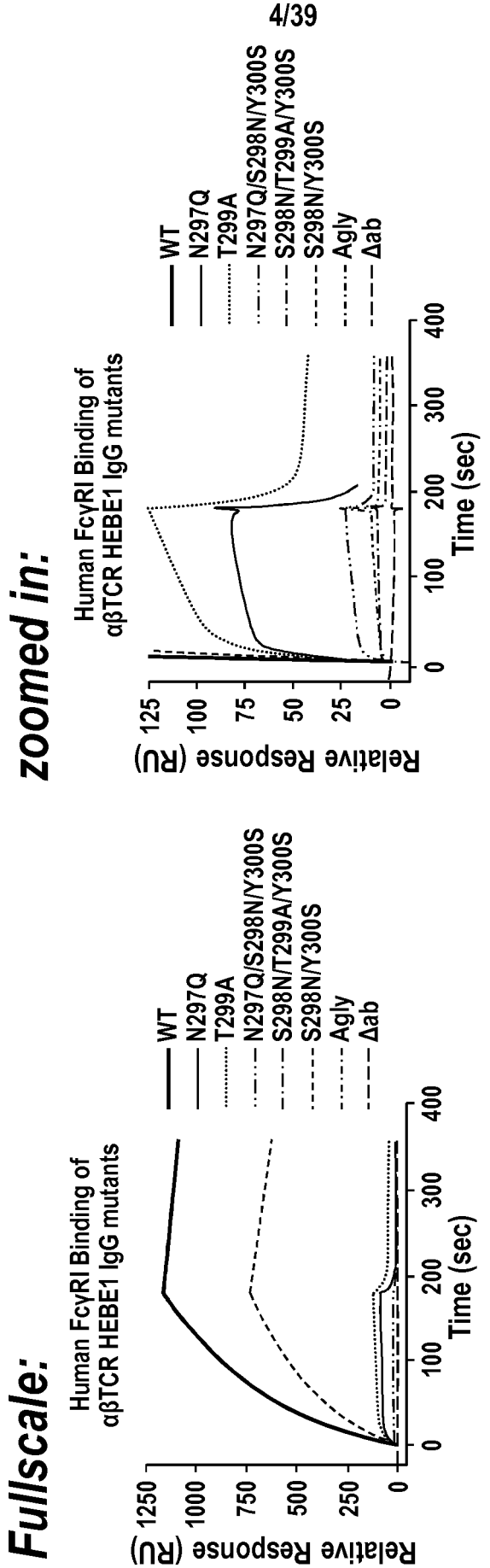

Fullscale:
Human FcγRI Binding of αβTCR HEBE1 IgG mutants
Relative Response (RU)
Time (sec)
WT
N297Q
T299A
N297Q/S298N/Y300S
S298N/T299A/Y300S
S298N/Y300S
Agly
Δab
zoomed in:
Human FcγRI Binding of αβTCR HEBE1 IgG mutants
Relative Response (RU)
Time (sec)
WT
N297Q
T299A
N297Q/S298N/Y300S
S298N/T299A/Y300S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

Fig. 4

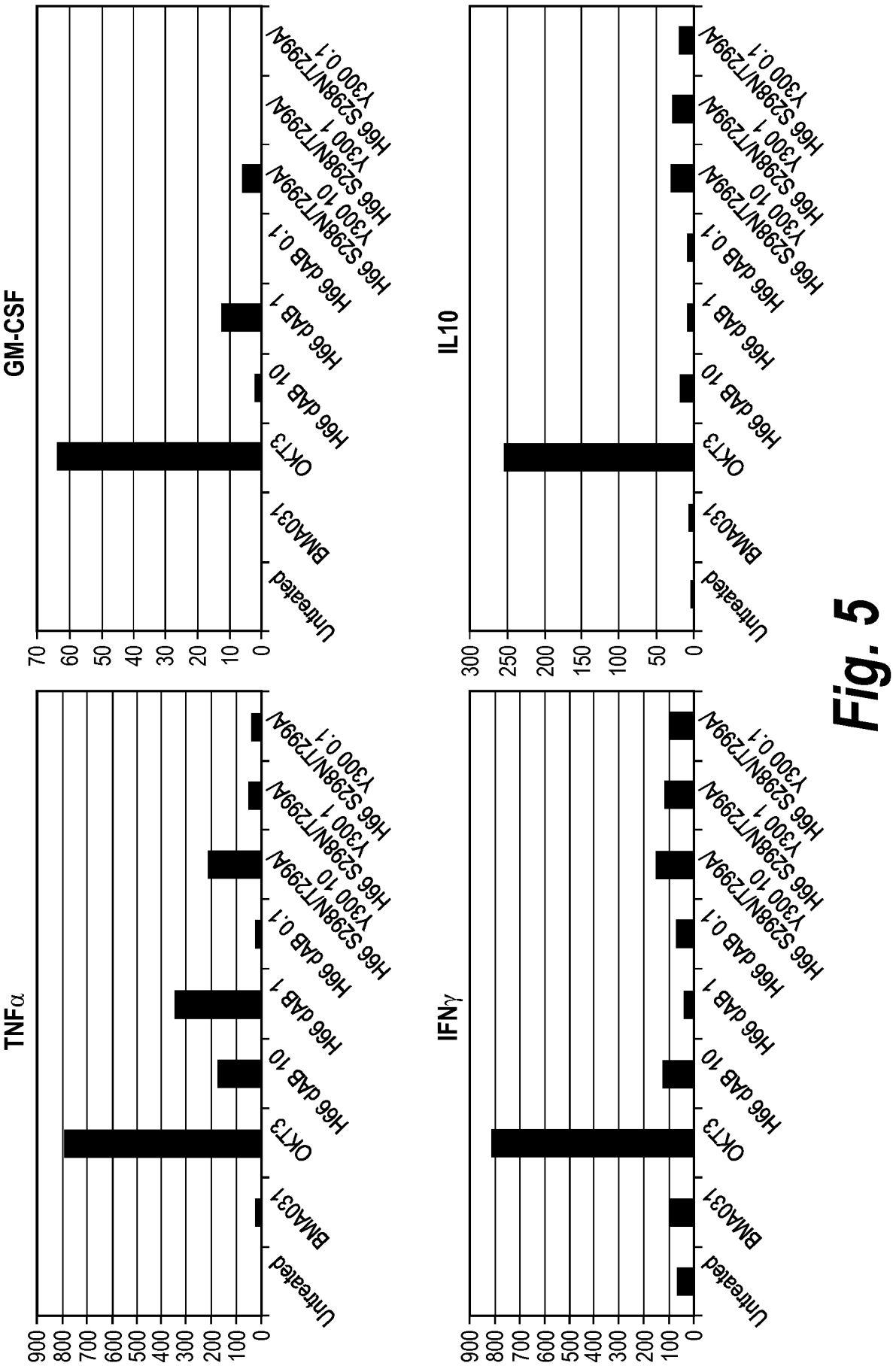

GM-CSF
IL10
TNFα
IFNγ
Untreated
BMA031
OKT3
H66 dAB 10
H66 dAB 1
H66 dAB 0.1
H66 S298N/T299A Y300 10
H66 S298N/T299A Y300 1
H66 S298N/T299A Y300 0.1

Fig. 5

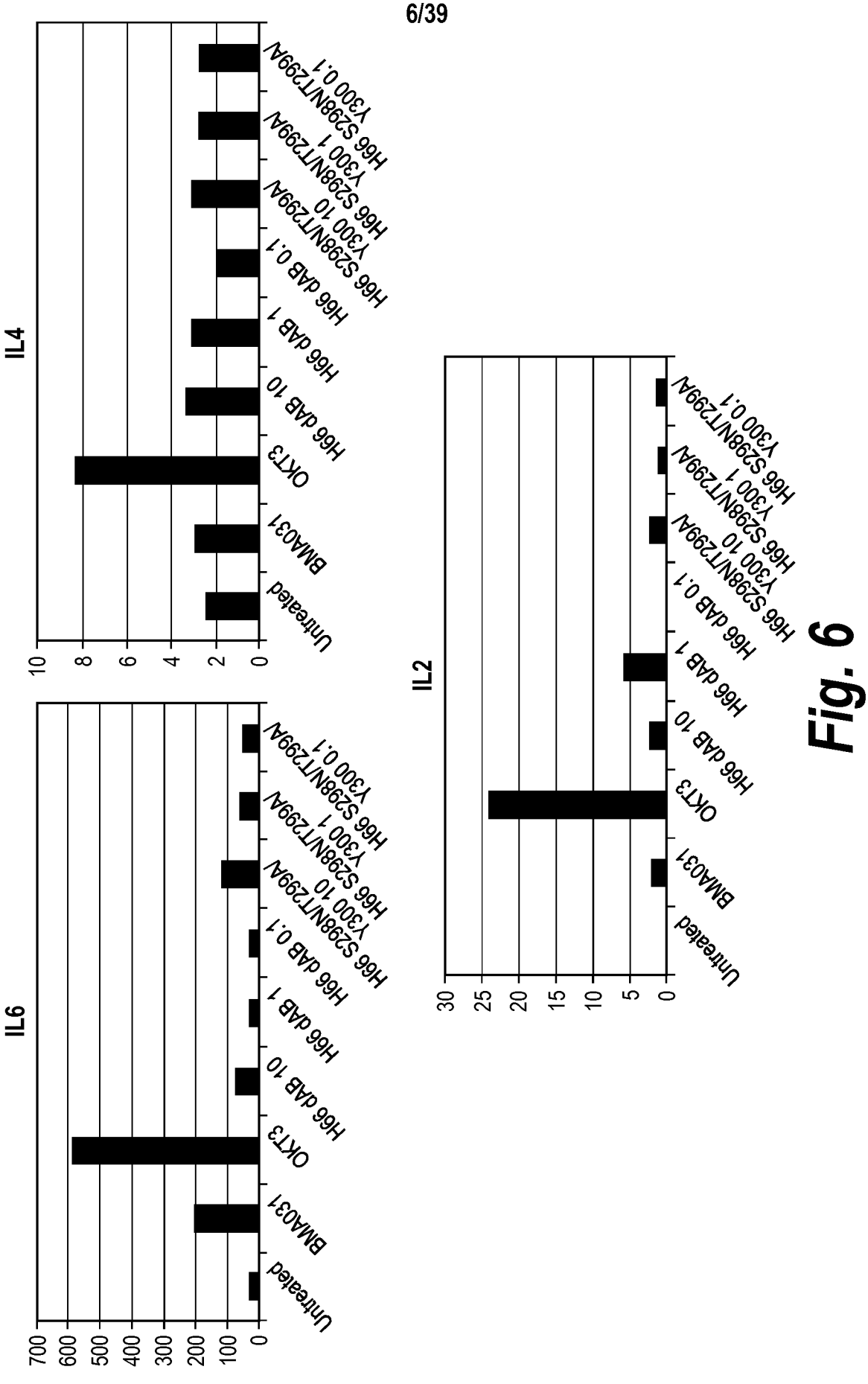

IL6
700
600
500
400
300
200
100
0
Untreated
BMA031
OKT3
H66 dAB 10
H66 dAB 1
H66 dAB 0.1
H66 S298N/T299A Y300 10
H66 S298N/T299A Y300 1
H66 S298N/T299A Y300 0.1
IL4
10
8
6
4
2
0
Untreated
BMA031
OKT3
H66 dAB 10
H66 dAB 1
H66 dAB 0.1
H66 S298N/T299A Y300 10
H66 S298N/T299A Y300 1
H66 S298N/T299A Y300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

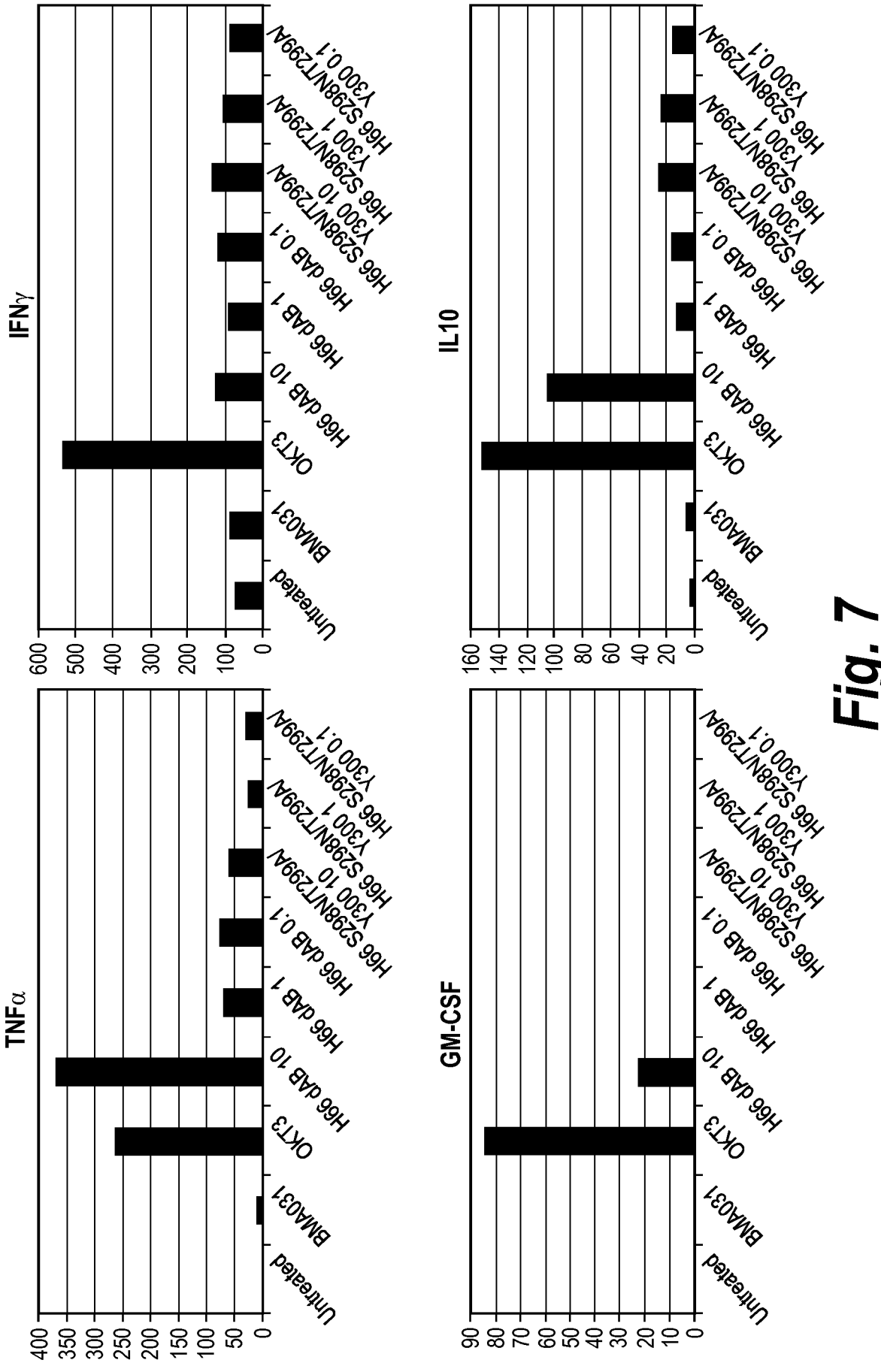

IFNγ
IL10
TNFα
GM-CSF
Untreated
BMA031
OKT3
H66 dAB 10
H66 dAB 1
H66 dAB 0.1
H66 S298N/T299A Y300 10
H66 S298N/T299A Y300 1
H66 S298N/T299A Y300 0.1

Fig. 7

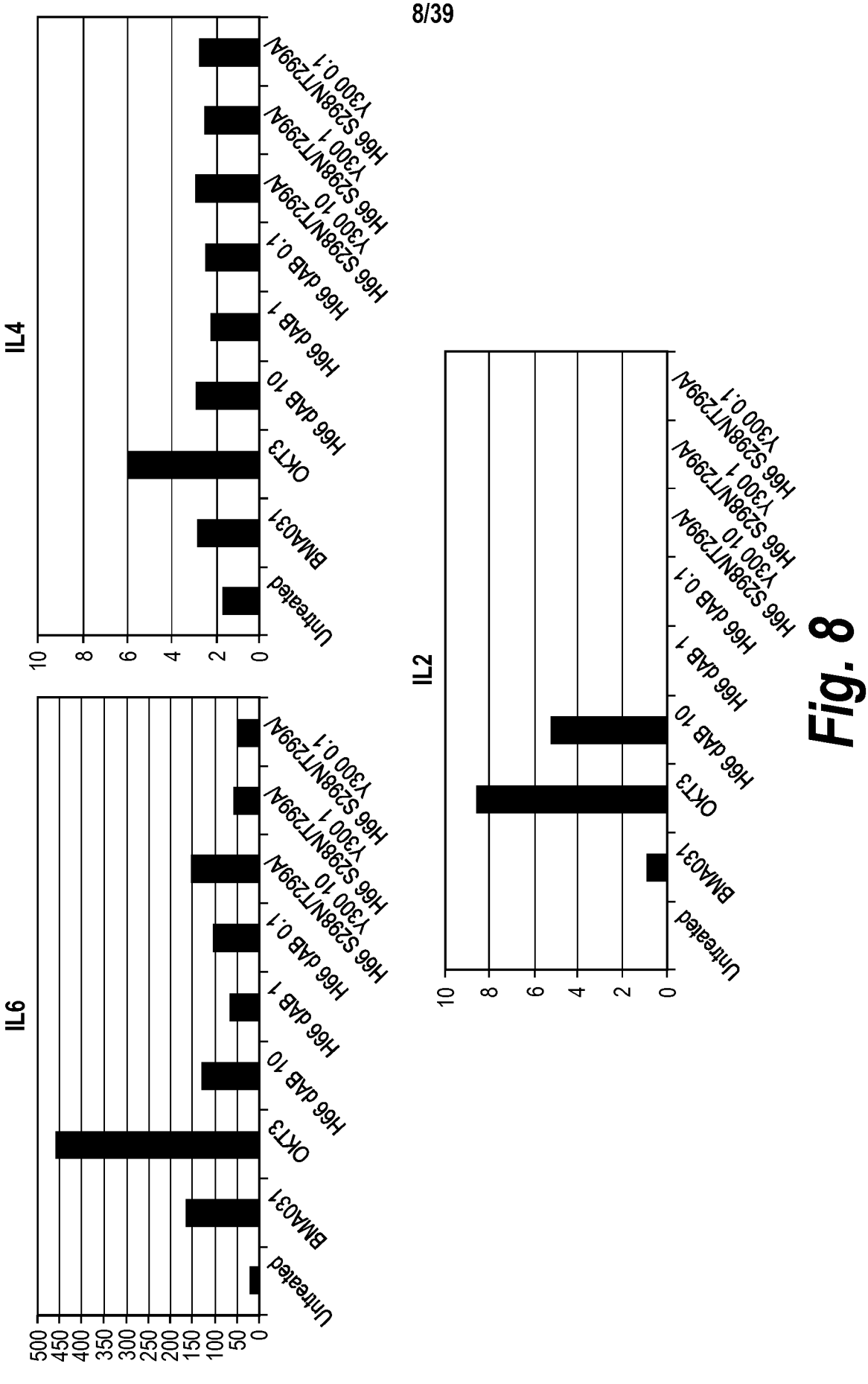
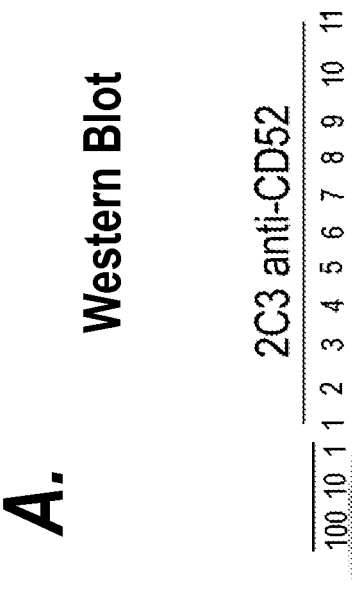

IL4
IL6
IL2
Untreated
BMA031
OKT3
H66 dAB 10
H66 dAB 1
H66 dAB 0.1
H66 S298N/T299A Y300 10
H66 S298N/T299A Y300 1
H66 S298N/T299A Y300 0.1

Fig. 8

A. **Western Blot**



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	1.60E-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

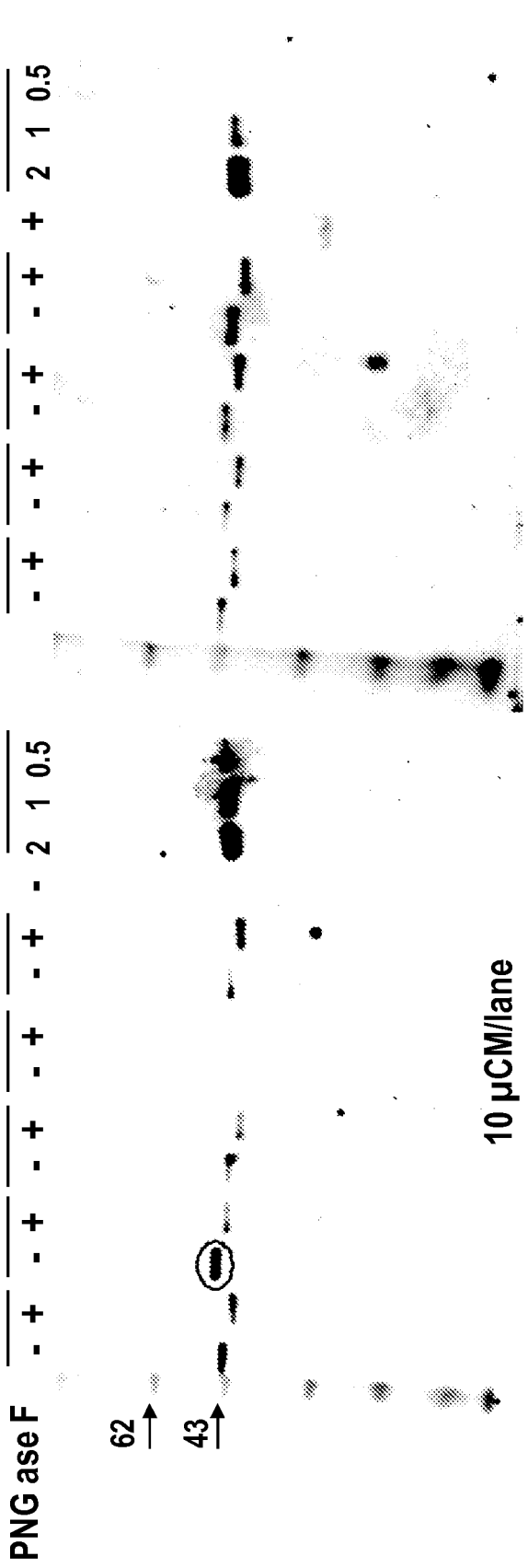
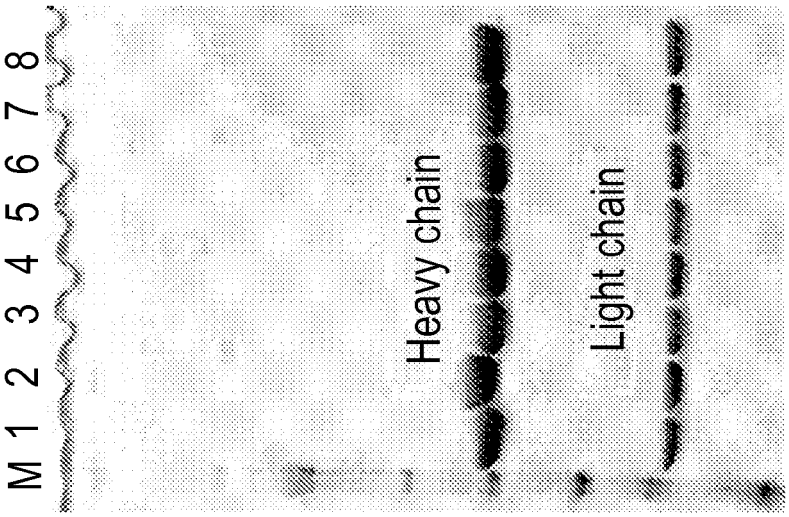

PNG ase F
wt
A114N
Y436S
Y436T
S440N
mc Campath, ng
2 1 0.5
wt
S442N
NGT
S298N/
Y300S
mc Campath, ng
2 1 0.5
62
43
10 µCM/lane

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

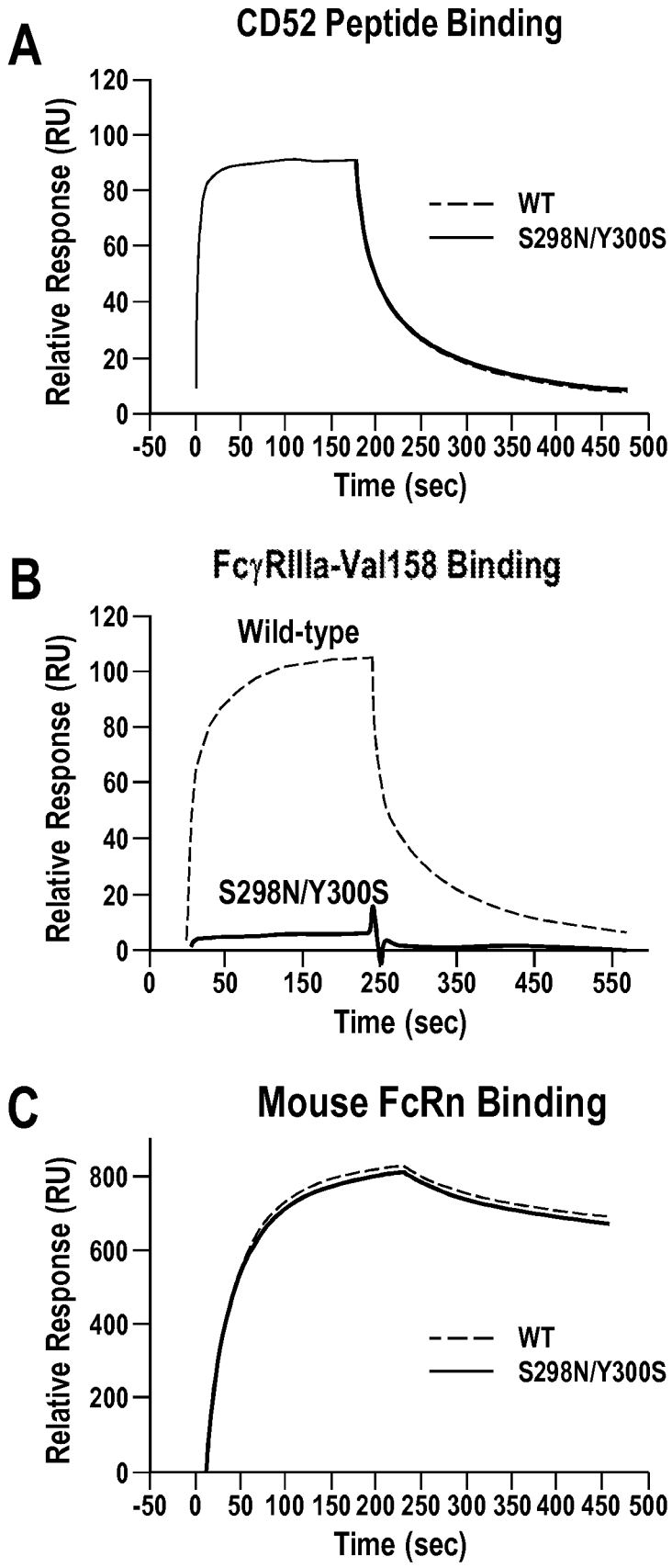

A
CD52 Peptide Binding
Relative Response (RU)
120
100
80
60
40
20
0
-50 0 50 100 150 200 250 300 350 400 450 500
Time (sec)
WT
S298N/Y300S
B
FcγRIIIa-Val158 Binding
Wild-type
S298N/Y300S
Relative Response (RU)
120
100
80
60
40
20
0
0 50 150 250 350 450 550
Time (sec)
C
Mouse FcRn Binding
Relative Response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

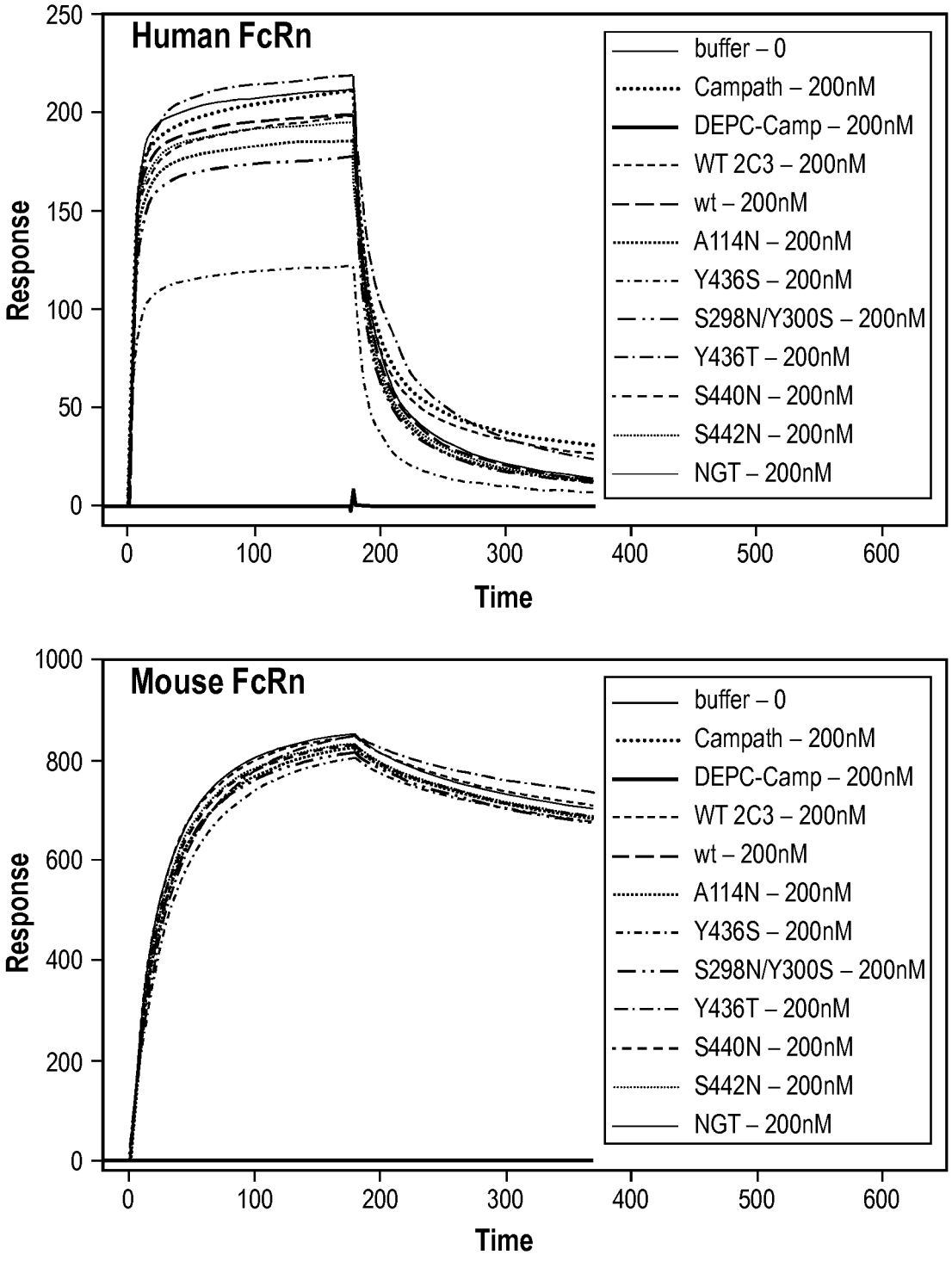
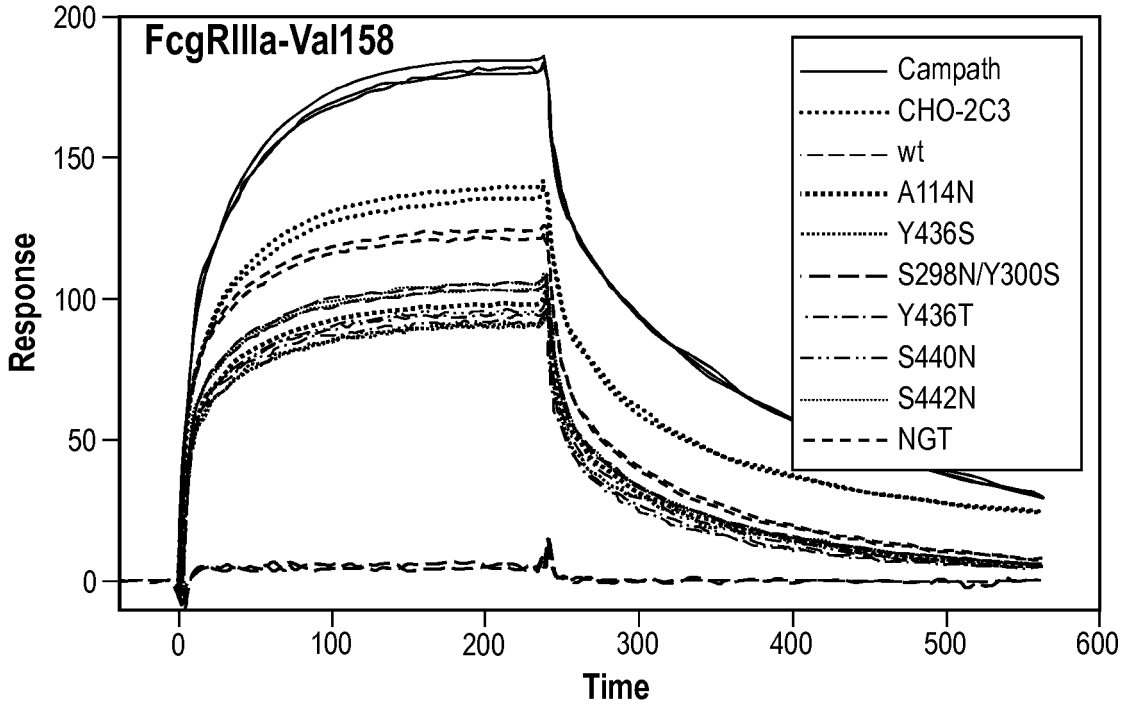

Human FcRn
Response
Time
buffer – 0
Campath – 200nM
DEPC-Camp – 200nM
WT 2C3 – 200nM
wt – 200nM
A114N – 200nM
Y436S – 200nM
S298N/Y300S – 200nM
Y436T – 200nM
S440N – 200nM
S442N – 200nM
NGT – 200nM
Mouse FcRn
Response
Time
buffer – 0
Campath – 200nM
DEPC-Camp – 200nM
WT 2C3 – 200nM
wt – 200nM
A114N –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

Fig. 13

A.


FcgRIIIa-Val158
Campath
CHO-2C3
wt
A114N
Y436S
S298N/Y300S
Y436T
S440N
S442N
NGT
Response
Time
0
50
100
150
200
0
100
200
300
400
500
600

B.

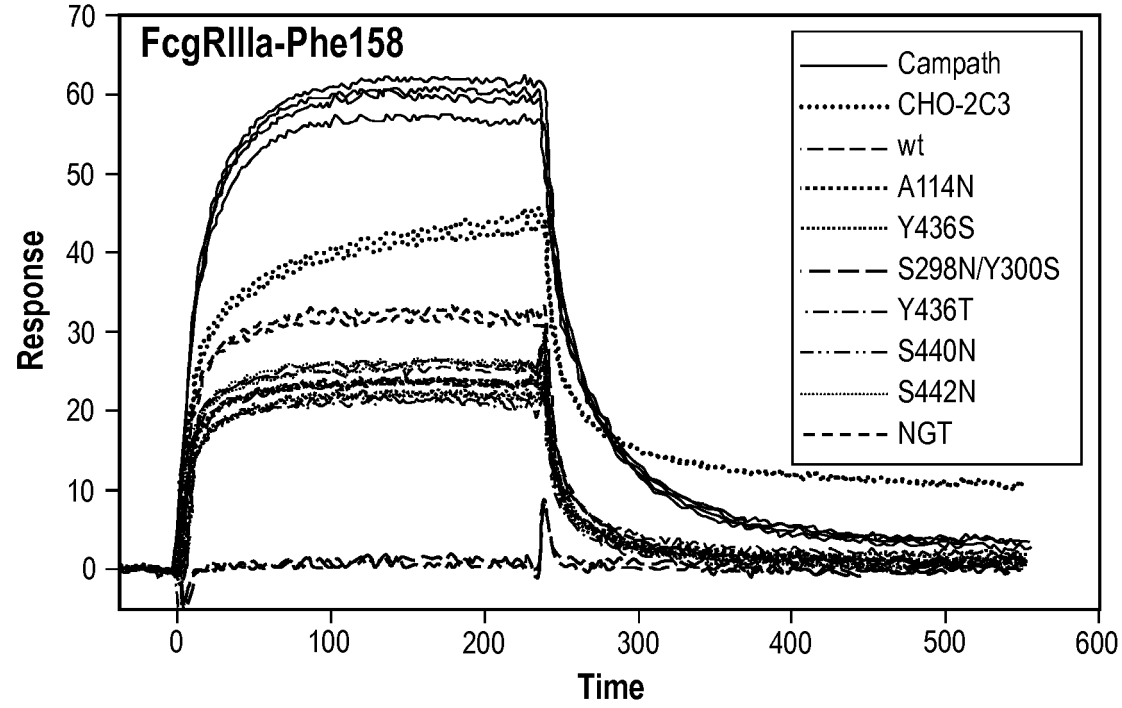

FcgRIIIa-Phe158
Campath
CHO-2C3
wt
A114N
Y436S
S298N/Y300S
Y436T
S440N
S442N
NGT
Response
Time
0
10
20
30
40
50
60
70
0
100
200
300
400
500
600

Fig. 14

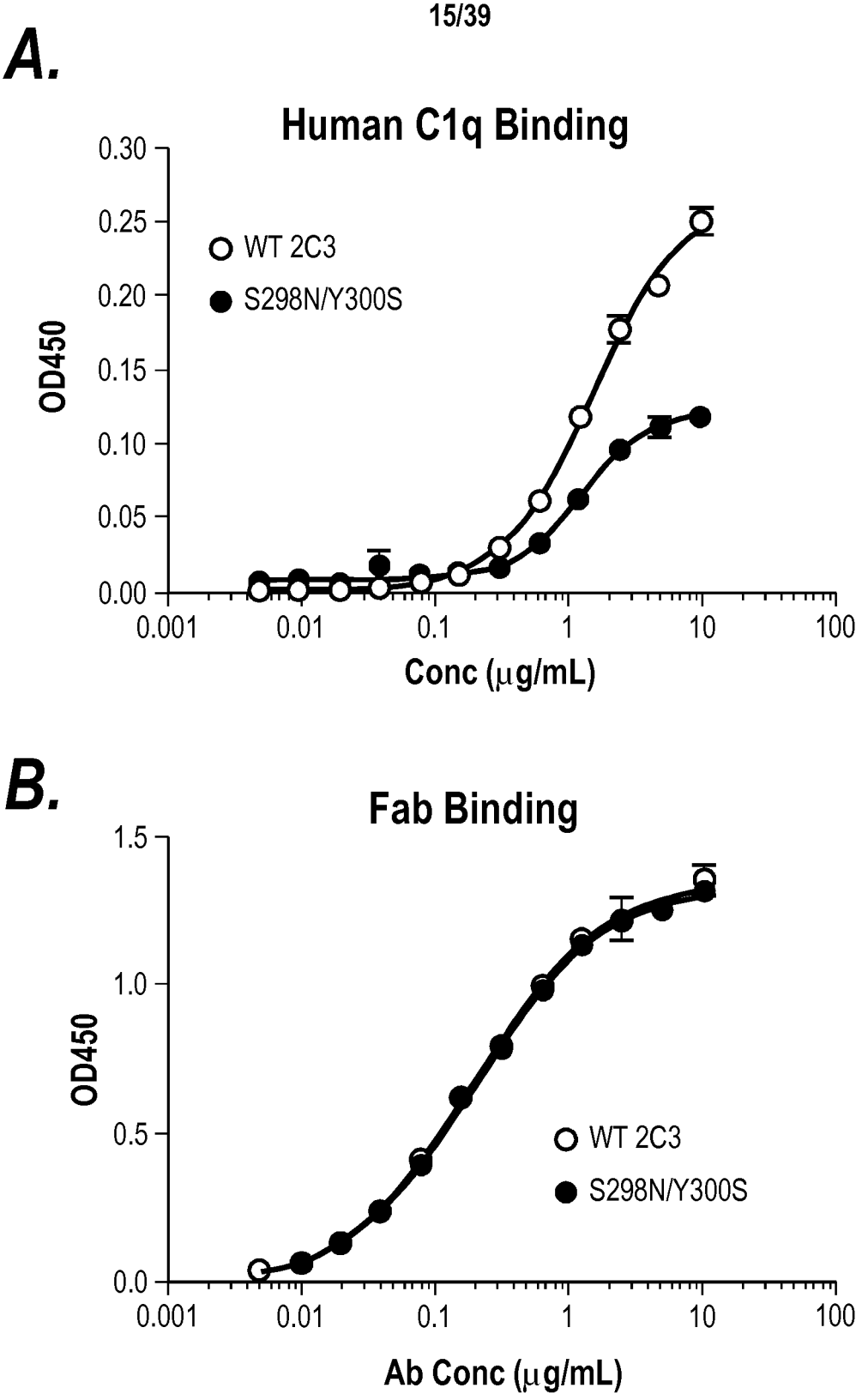

A.
Human C1q Binding
OD450
0.30
0.25
0.20
0.15
0.10
0.05
0.00
WT 2C3
S298N/Y300S
0.001
0.01
0.1
1
10
100
Conc (μg/mL)
B.
Fab Binding
OD450
1.5
1.0
0.5
0.0
WT 2C3
S298N/Y300S
0.001
0.01
0.1
1
10
100
Ab Conc (μg/mL)

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

Fig. 17

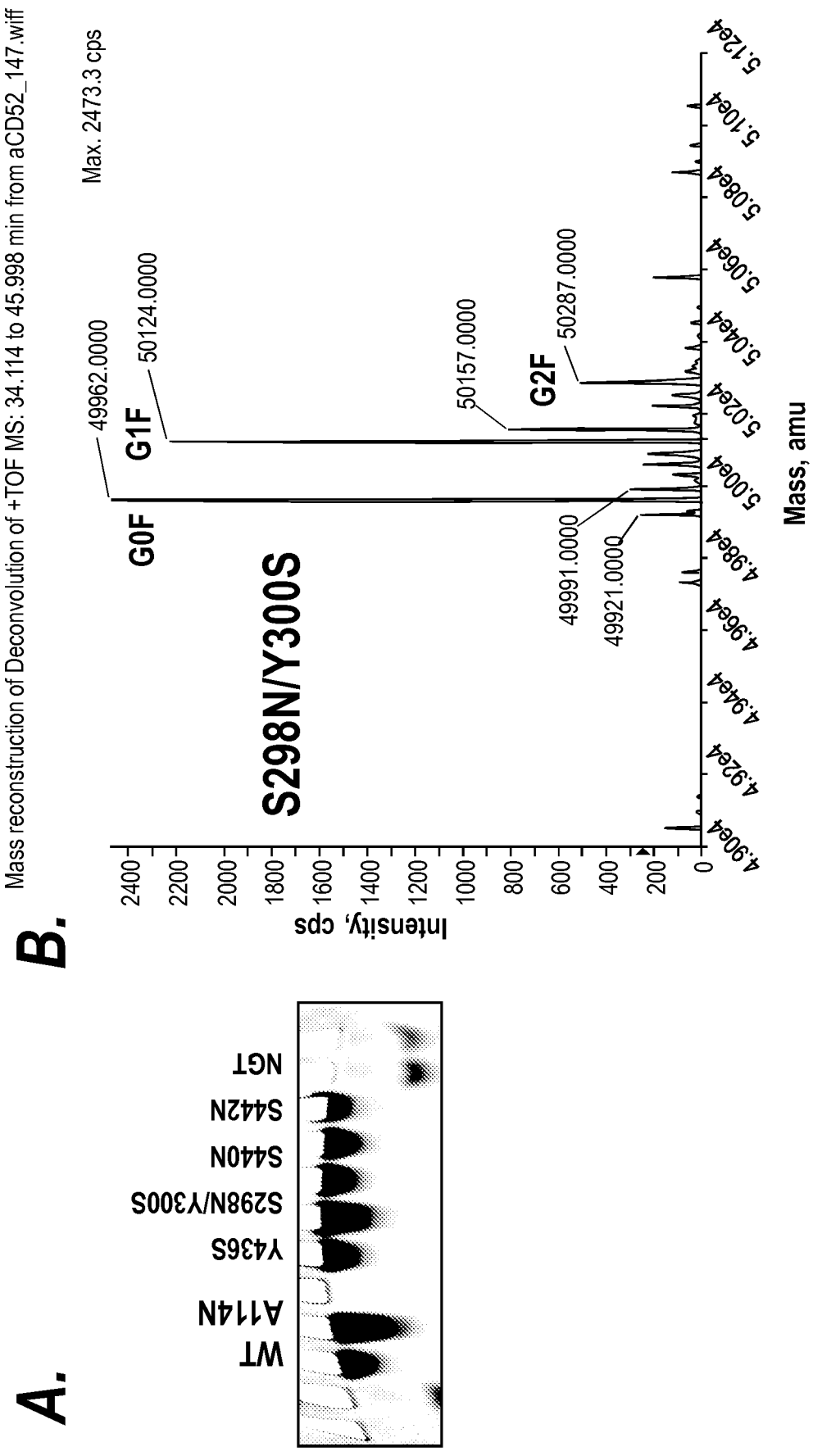

A.
WT
A114N
Y436S
S298N/Y300S
S440N
S442N
NGT
B.
Mass reconstruction of Deconvolution of +TOF MS: 34.114 to 45.998 min from aCD52_147.wiff
Max. 2473.3 cps
S298N/Y300S
G0F
G1F
G2F
49962.0000
50124.0000
50157.0000
50287.0000
49991.0000
49921.0000
Intensity, cps
Mass, amu

Fig. 18

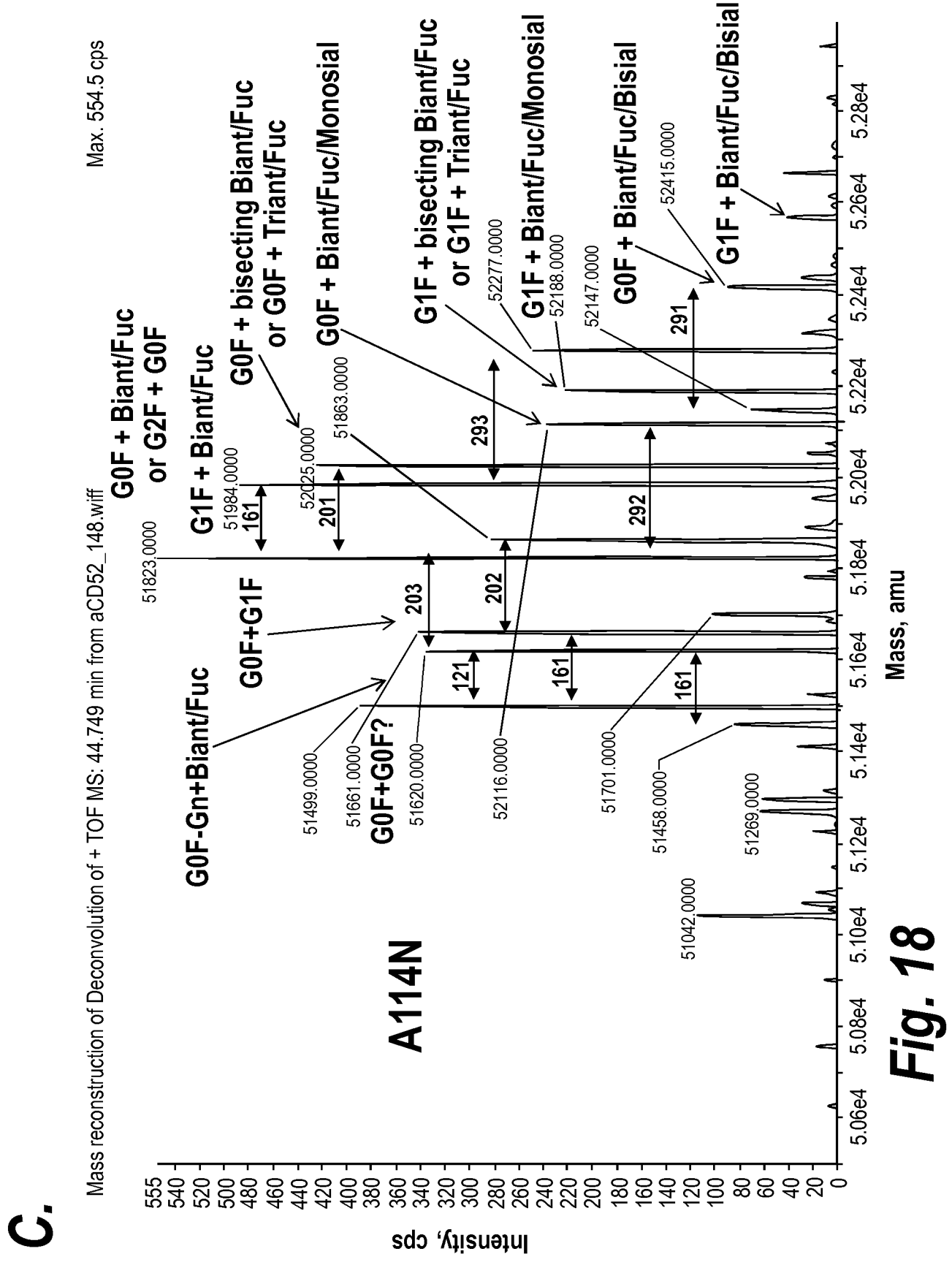

C.
Mass reconstruction of Deconvolution of + TOF MS: 44.749 min from aCD52_148.wiff
Max. 554.5 cps
A114N
G0F-Gn+Biant/Fuc
G0F+G1F
G0F+G0F?
G1F + Biant/Fuc
G0F + Biant/Fuc
or G2F + G0F
G0F + bisecting Biant/Fuc
or G0F + Triant/Fuc
G0F + Biant/Fuc/Monosial
G1F + bisecting Biant/Fuc
or G1F + Triant/Fuc
G1F + Biant/Fuc/Monosial
G0F + Biant/Fuc/Bisial
G1F + Biant/Fuc/Bisial
51823.0000
51984.0000
52025.0000
51863.0000
52277.0000
52188.0000
52147.0000
52415.0000
51499.0000
51661.0000
51620.0000
52116.0000
51701.0000
51458.0000
51269.0000
51042.0000
161
201
293
203
202
292
291
121
161
161
Intensity, cps
Mass, amu

Fig. 18

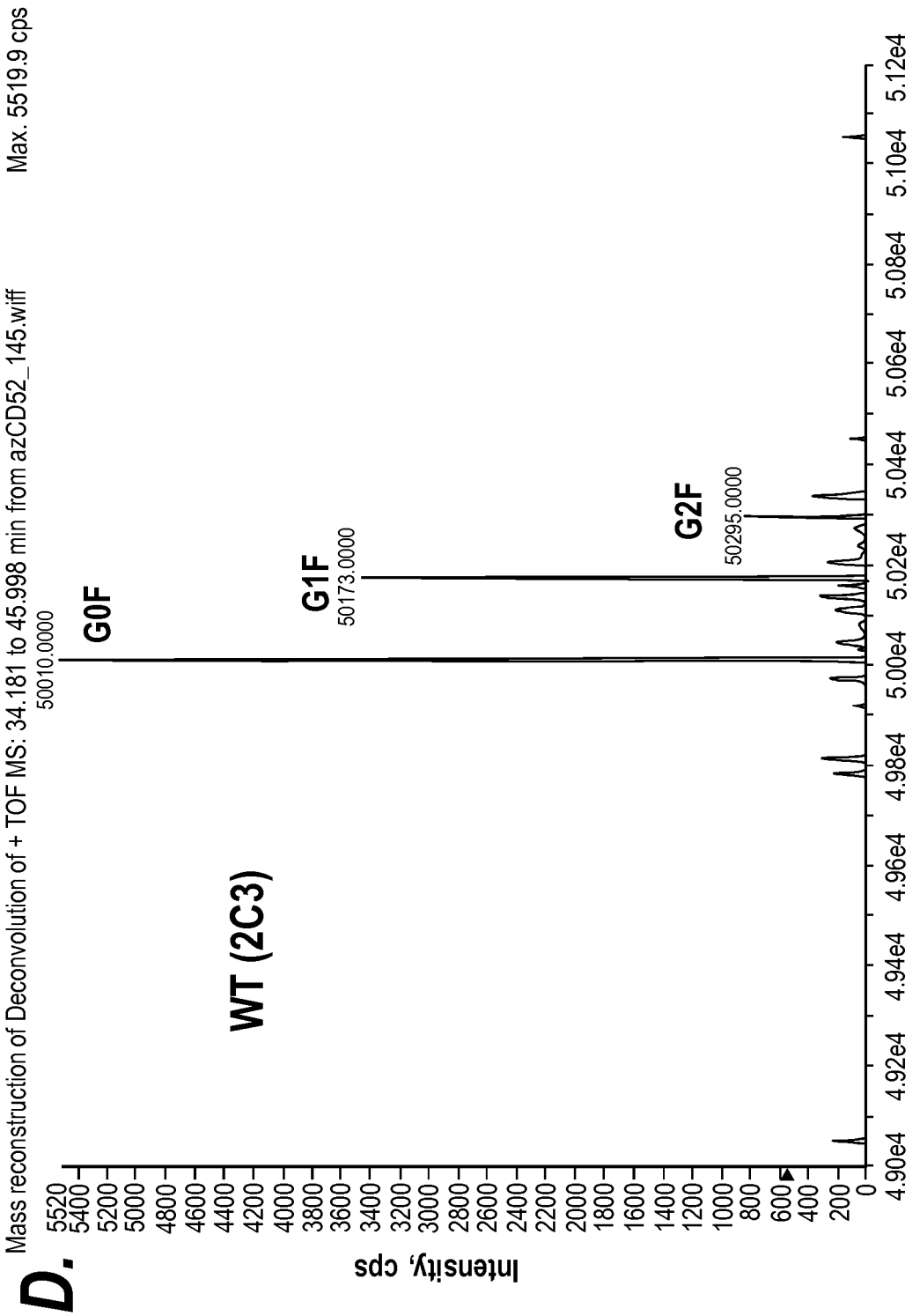

D.
Mass reconstruction of Deconvolution of + TOF MS: 34.181 to 45.998 min from azCD52_145.wiff
Max. 5519.9 cps
WT (2C3)
G0F
50010.0000
G1F
50173.0000
G2F
50295.0000
Intensity, cps
Mass, amu

Fig. 18

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.

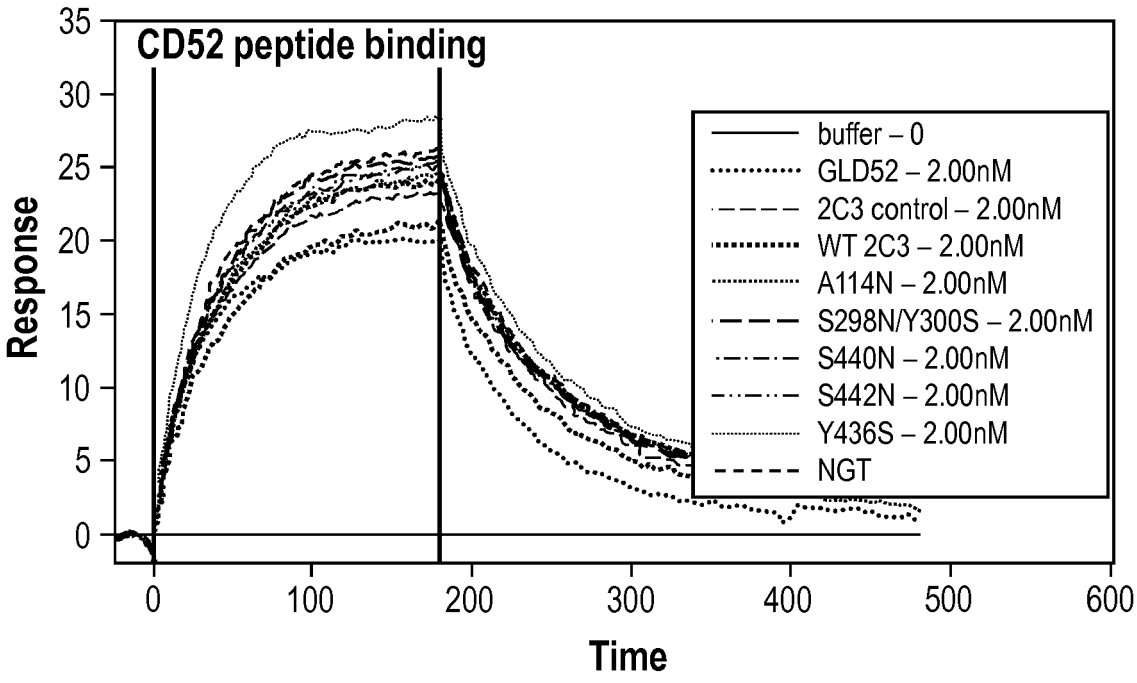


Fig. 19

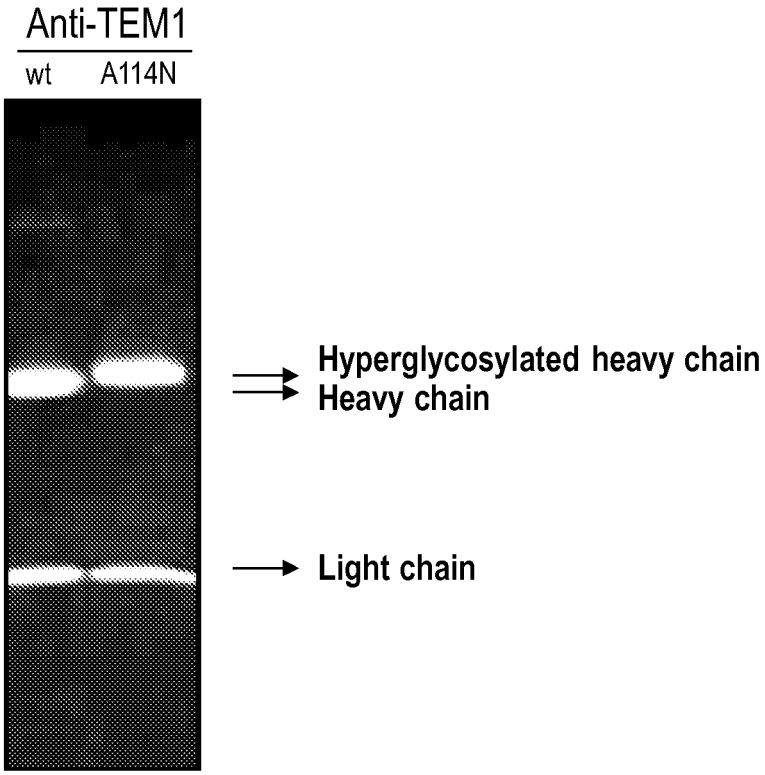
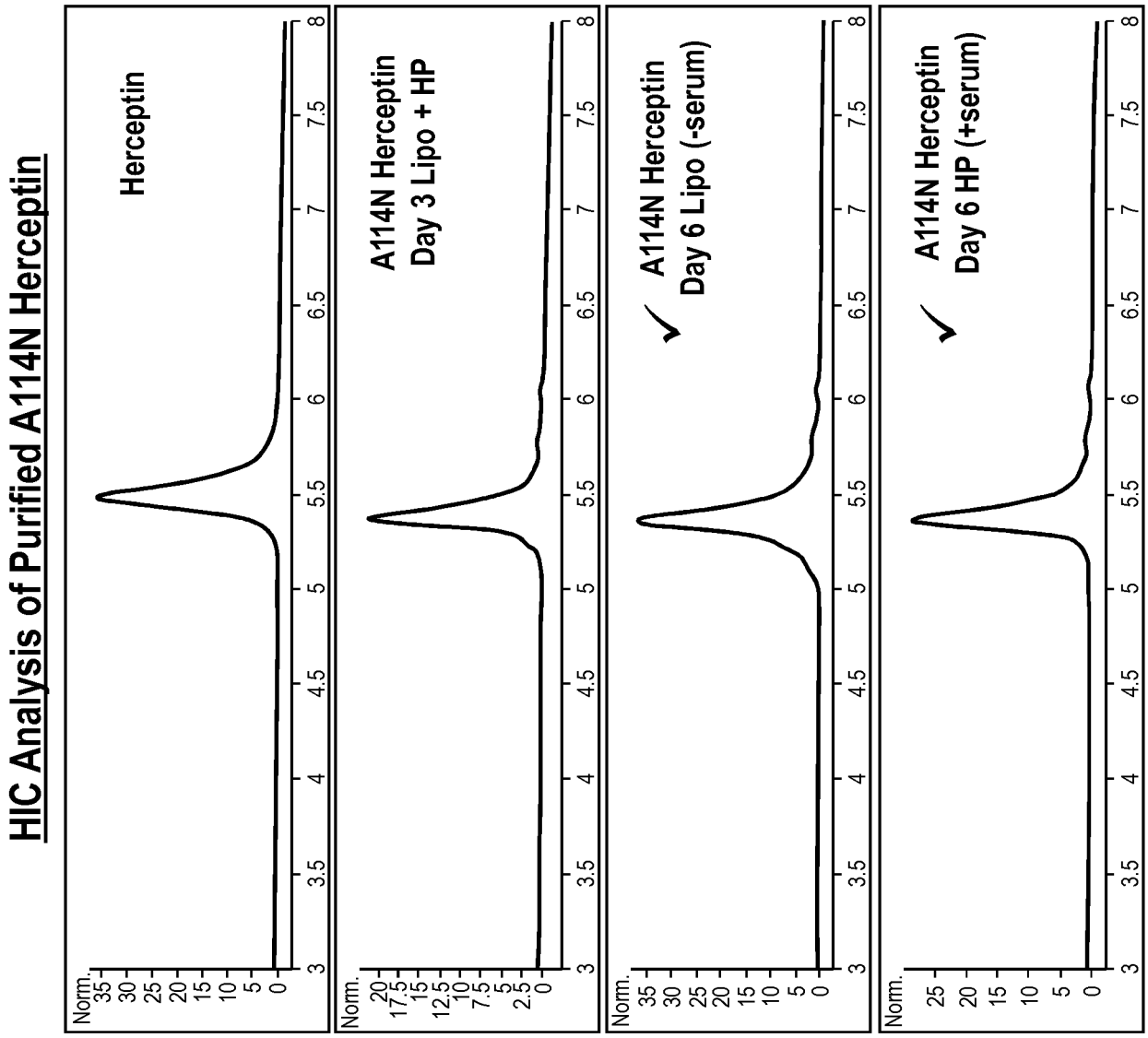

Anti-TEM1
wt
A114N
Hyperglycosylated heavy chain
Heavy chain
Light chain

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)
Norm.
3
3.5
4
4.5
5
5.5
6
6.5
7
7.5
8

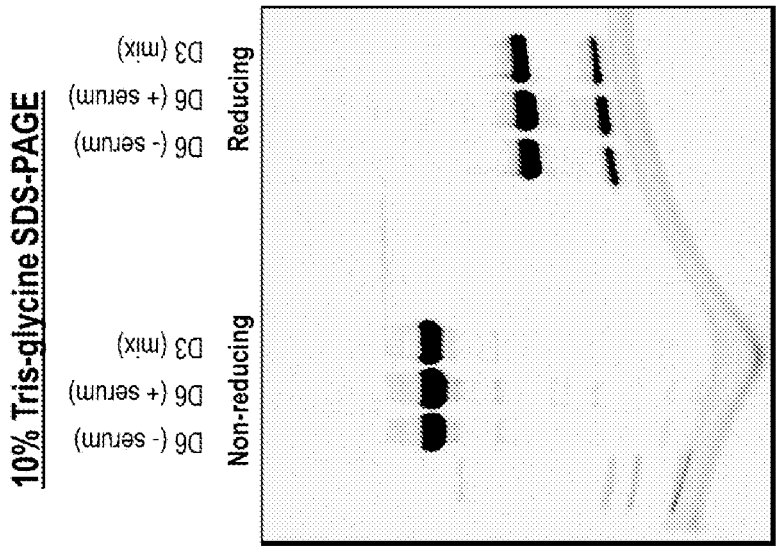

10% Tris-glycine SDS-PAGE
Non-reducing
Reducing
D6 (- serum)
D6 (+ serum)
D3 (mix)

Fig. 21

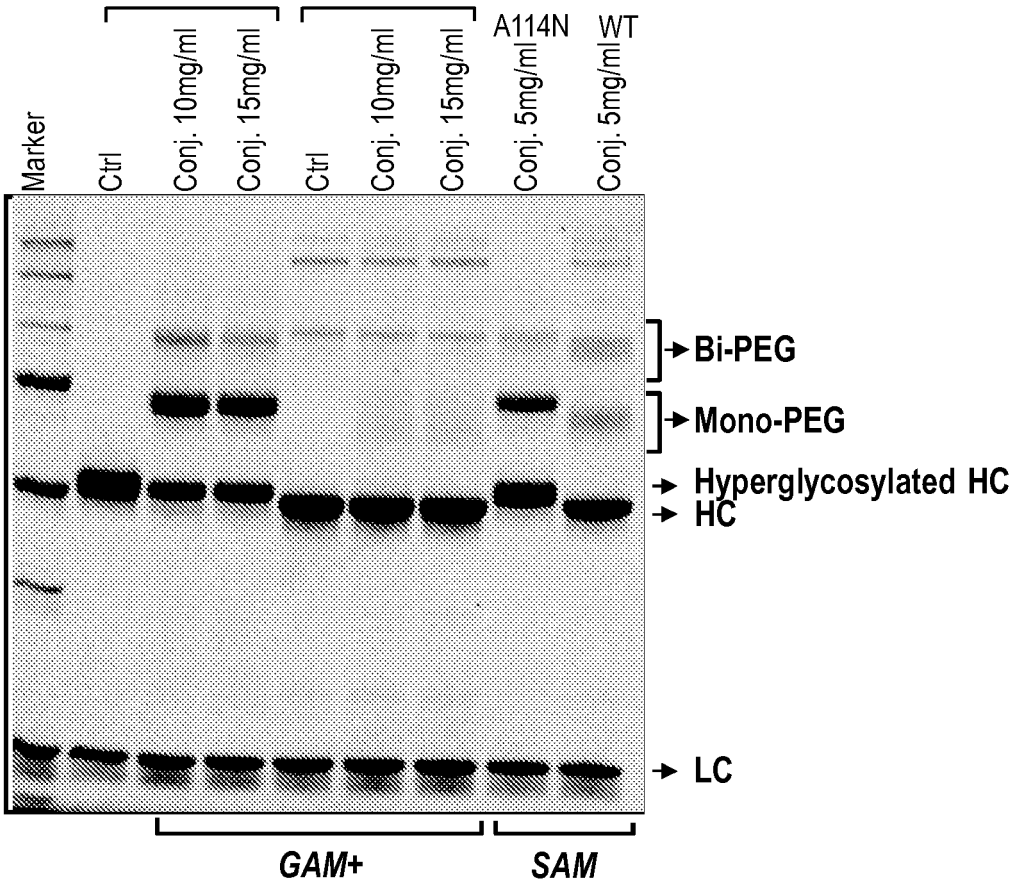

Marker
Ctrl
Conj. 10mg/ml
Conj. 15mg/ml
Ctrl
Conj. 10mg/ml
Conj. 15mg/ml
A114N
Conj. 5mg/ml
WT
Conj. 5mg/ml
Bi-PEG
Mono-PEG
Hyperglycosylated HC
HC
LC
GAM+
SAM

Fig. 22

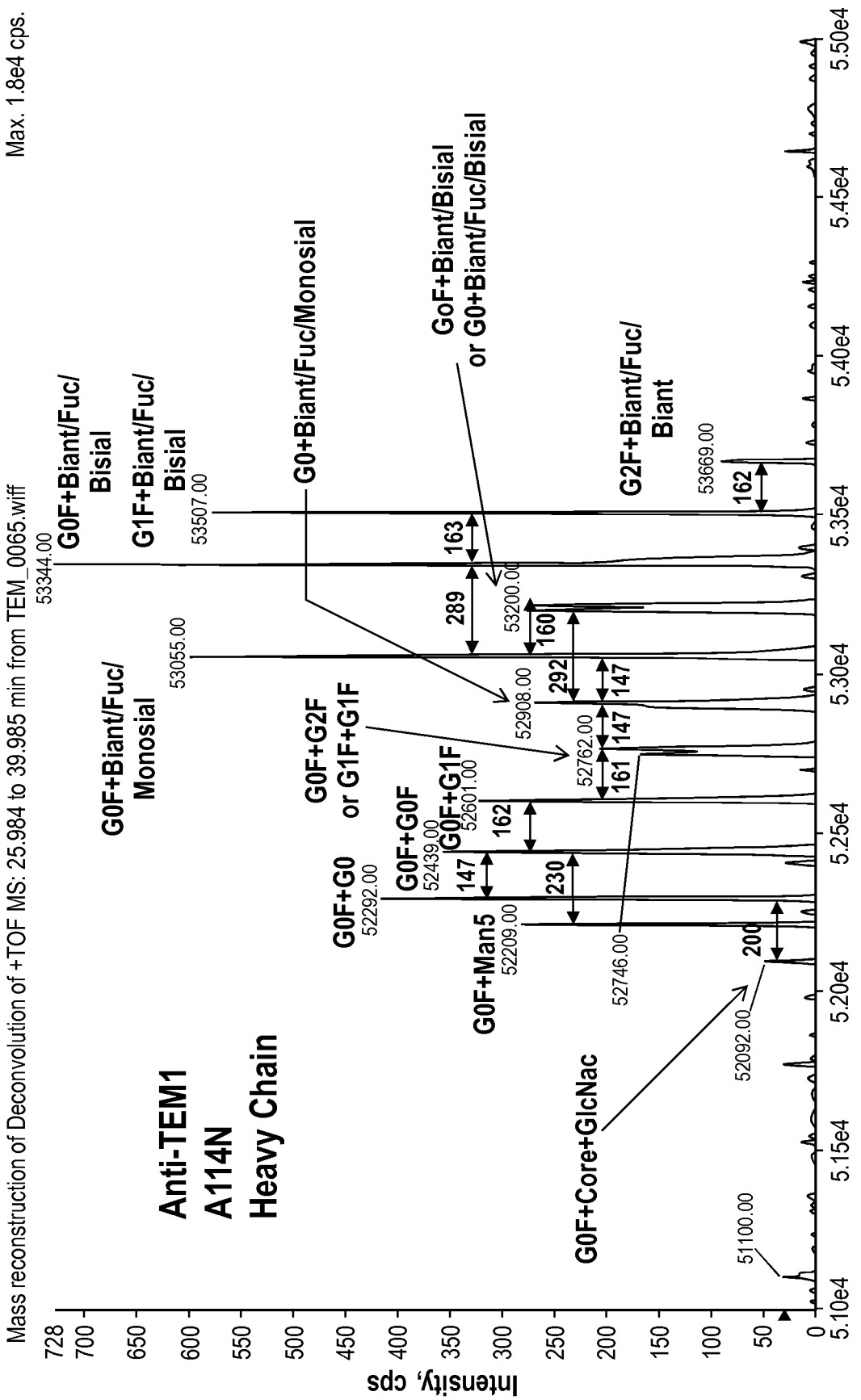
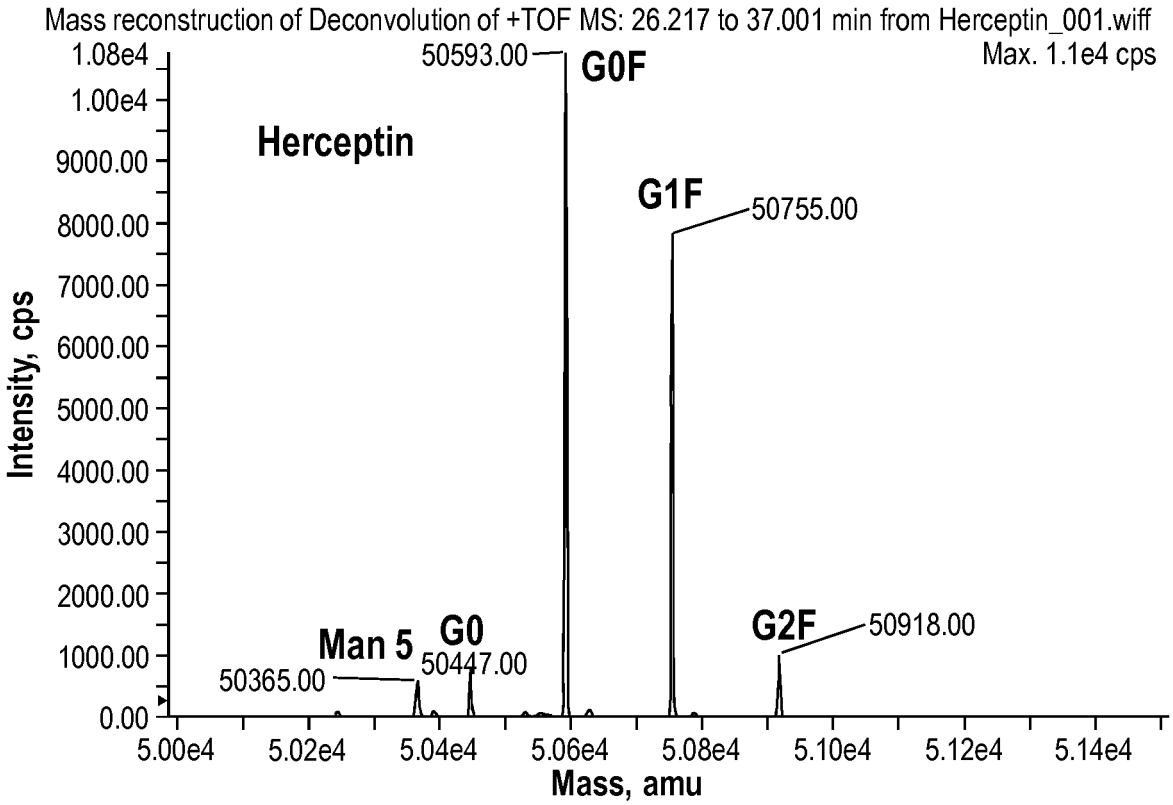

Mass reconstruction of Deconvolution of +TOF MS: 25.984 to 39.985 min from TEM_0065.wiff
Max. 1.8e4 cps.
Anti-TEM1
A114N
Heavy Chain
G0F+Biant/Fuc/
Bisial
G1F+Biant/Fuc/
Bisial
G0F+Biant/Fuc/
Monosial
G0+Biant/Fuc/Monosial
G0F+Biant/Bisial
or G0+Biant/Fuc/Bisial
G2F+Biant/Fuc/
Biant
G0F+G2F
or G1F+G1F
G0F+G1F
G0F+G0F
G0F+G0
G0F+Man5
G0F+Core+GlcNac
53344.00
53507.00
53055.00
53200.00
52908.00
52762.00
52601.00
52439.00
52292.00
52209.00
52746.00
52092.00
51100.00
53669.00
163
289
160
292
147
147
161
162
147
230
200
162
Intensity, cps
Mass, amu
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
Man 5
G0
50447.00
50365.00
G2F
50918.00
Intensity, cps
Mass, amu

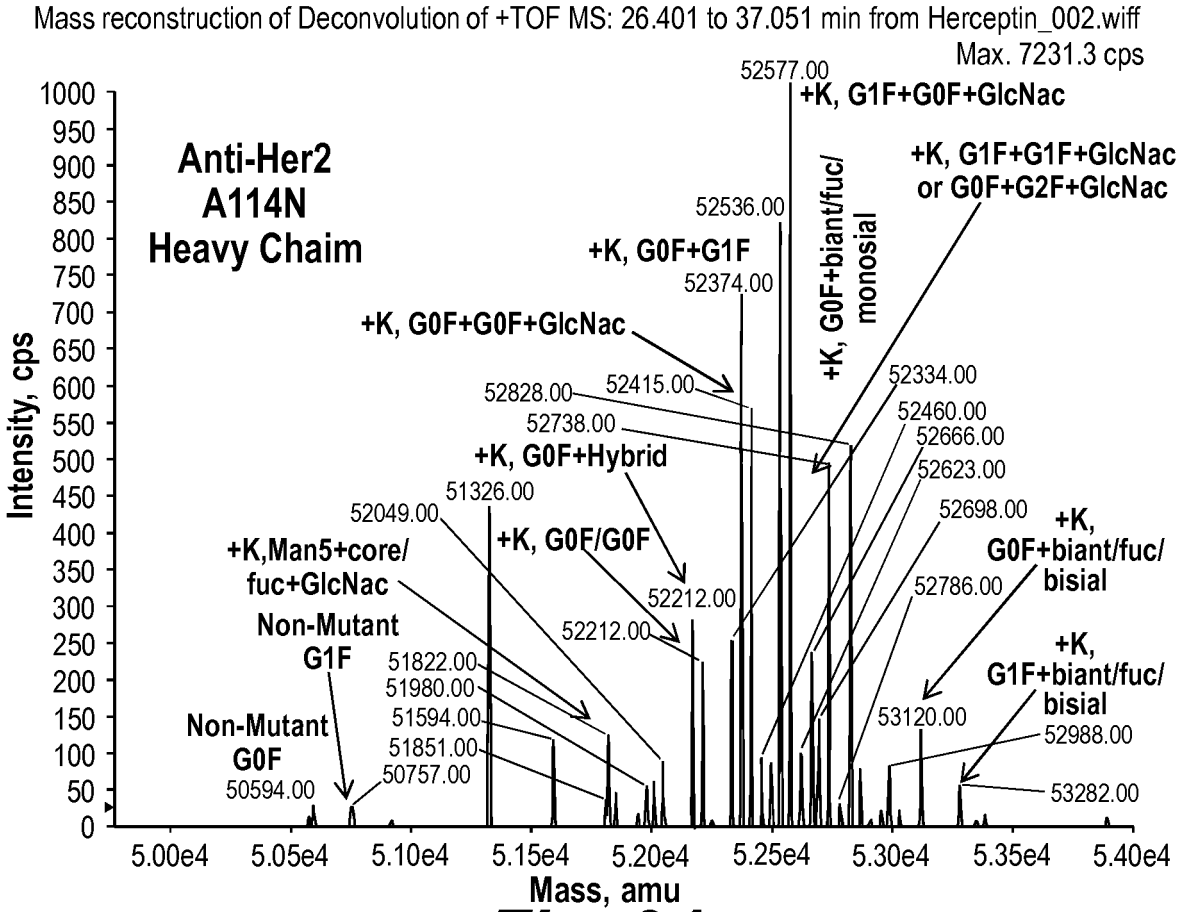

Mass reconstruction of Deconvolution of +TOF MS: 26.401 to 37.051 min from Herceptin_002.wiff
Max. 7231.3 cps
Anti-Her2
A114N
Heavy Chaim
52577.00
+K, G1F+G0F+GlcNac
+K, G1F+G1F+GlcNac
or G0F+G2F+GlcNac
52536.00
+K, G0F+G1F
52374.00
+K, G0F+biant/fuc/
monosial
+K, G0F+G0F+GlcNac
52828.00
52415.00
52334.00
52738.00
52460.00
52666.00
+K, G0F+Hybrid
52623.00
51326.00
52049.00
52698.00
+K, G0F/G0F
+K,
G0F+biant/fuc/
bisial
+K,Man5+core/
fuc+GlcNac
52212.00
52786.00
Non-Mutant
G1F
52212.00
+K,
G1F+biant/fuc/
bisial
51822.00
51980.00
51594.00
53120.00
Non-Mutant
G0F
51851.00
52988.00
50594.00
50757.00
53282.00
Intensity, cps
Mass, amu

Fig. 24

A.

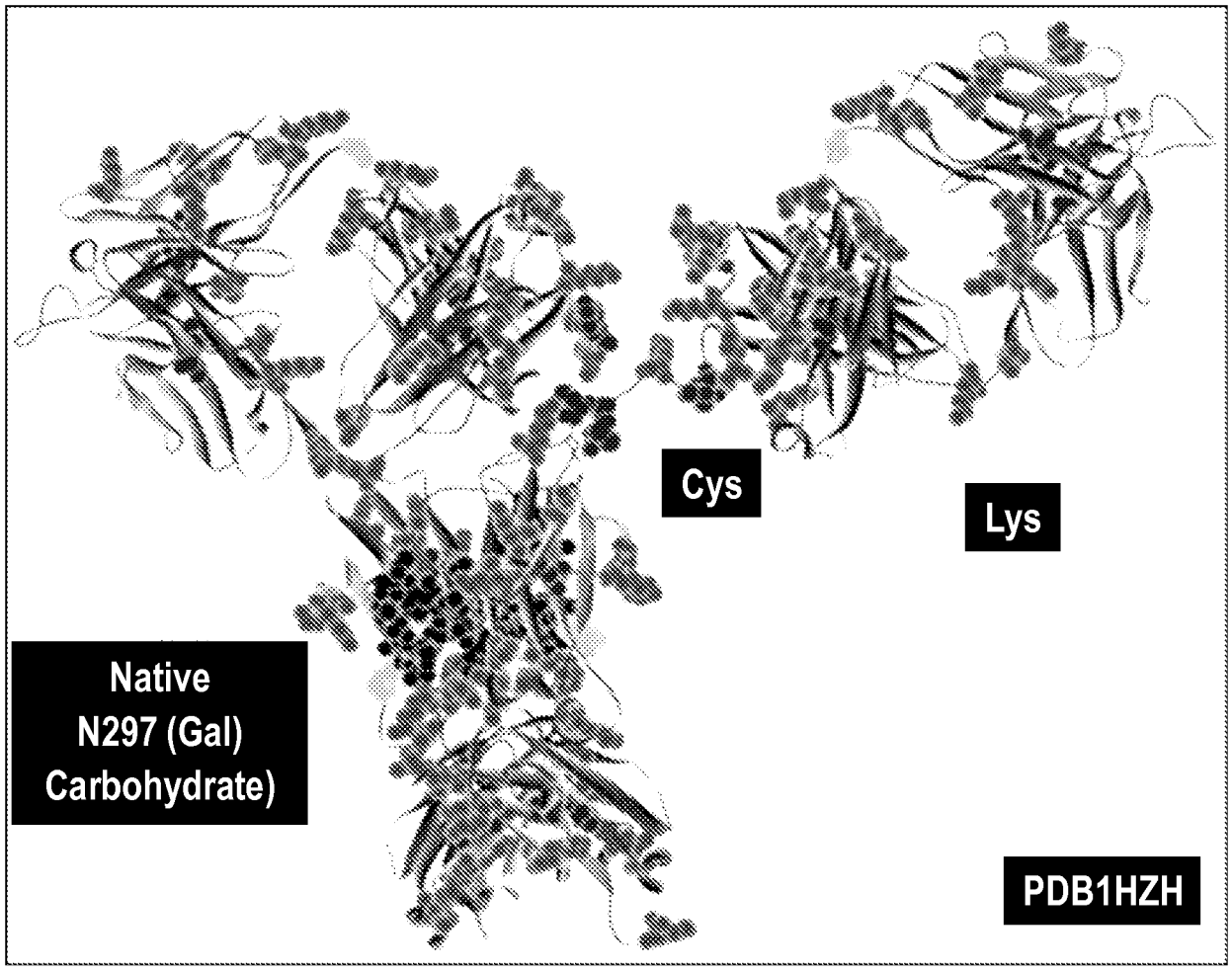
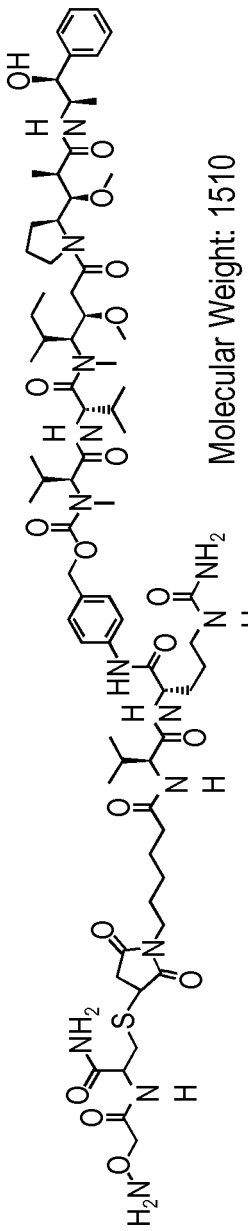


Fig. 25

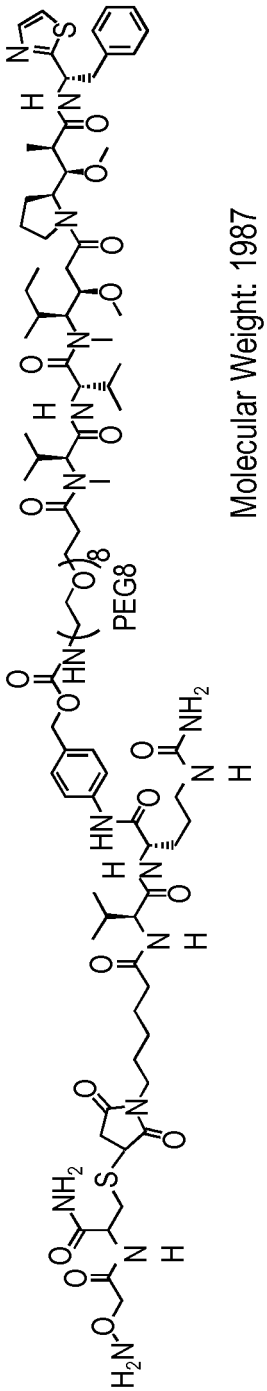
B.

Aminooxy-Cys-MC-VC-PABC-MMAE



Molecular Weight: 1510

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



Molecular Weight: 1987

Fig. 25

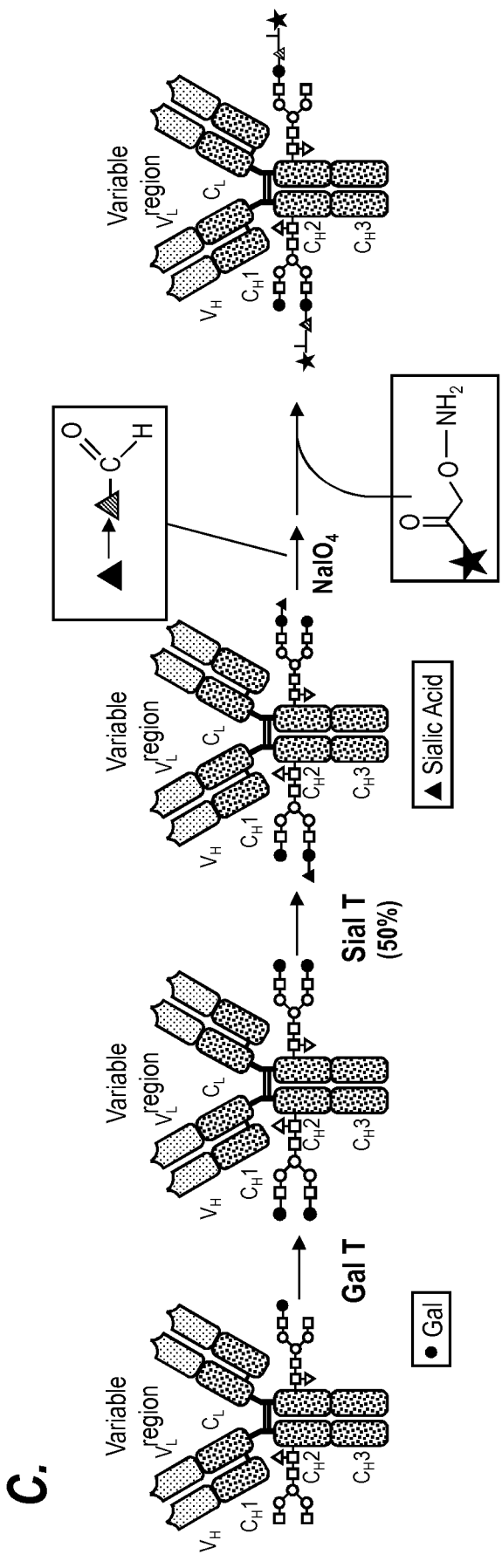

C.
Variable region
V_L
C_L
V_H
C_H1
C_H2
C_H3
Gal T
• Gal
Sial T
(50%)
▲ Sialic Acid
$NaIO_4$
O
C
H
O
O
NH_2

Fig. 25

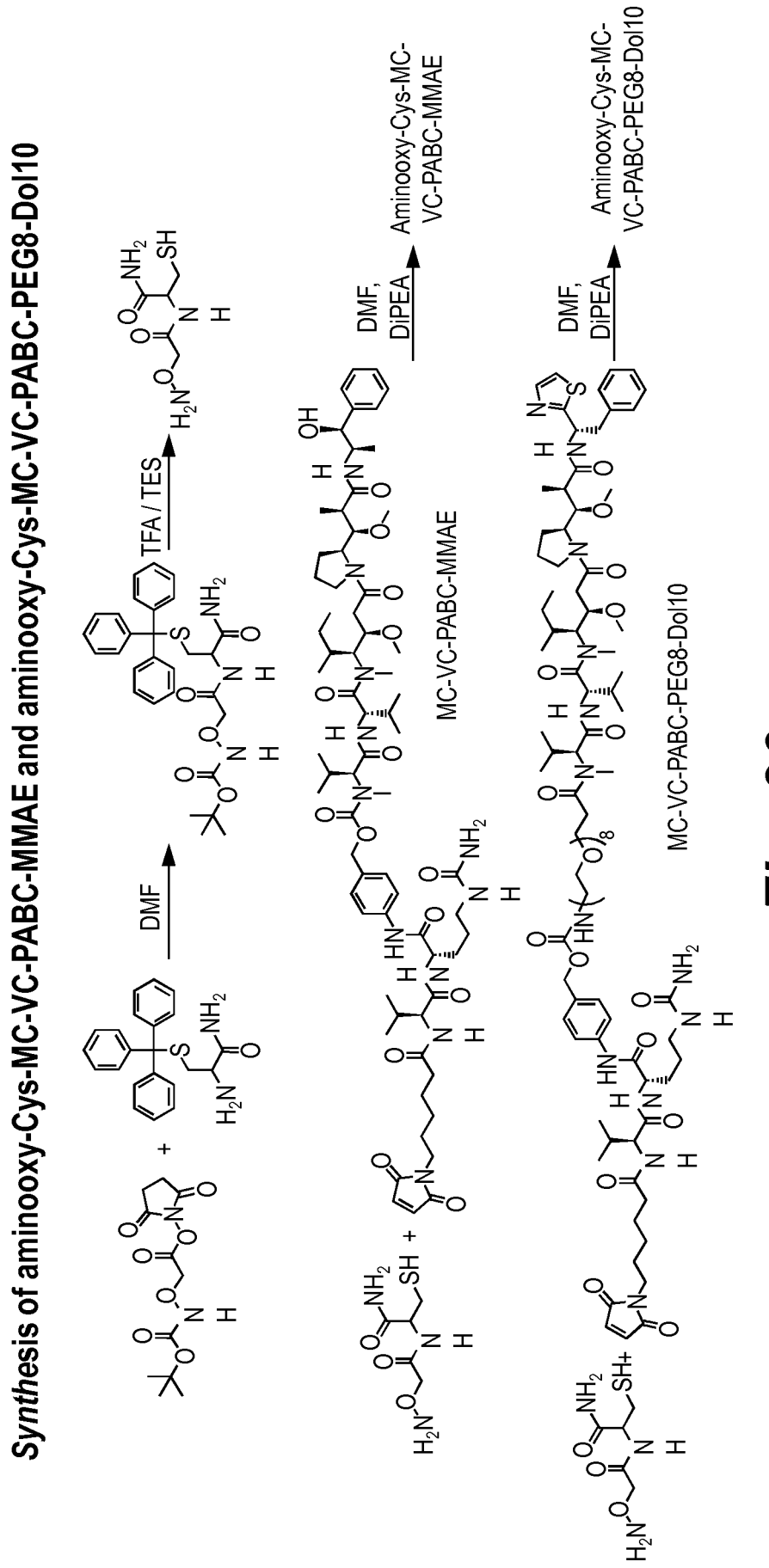
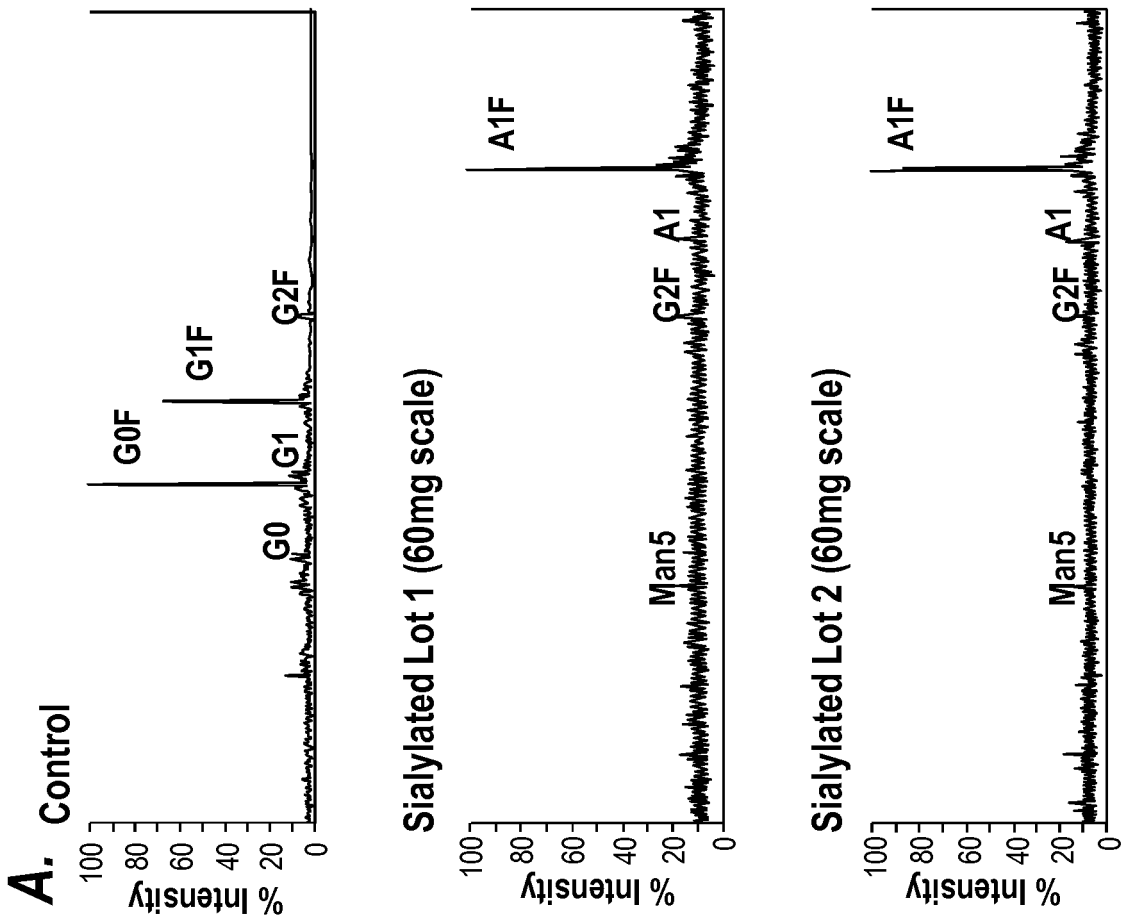

Synthesis of aminooxy-Cys-MC-VC-PABC-MMAE and aminooxy-Cys-MC-VC-PABC-PEG8-Dol10
DMF
TFA / TES
DMF, DiPEA
Aminooxy-Cys-MC-VC-PABC-MMAE
MC-VC-PABC-MMAE
DMF, DiPEA
Aminooxy-Cys-MC-VC-PABC-PEG8-Dol10
MC-VC-PABC-PEG8-Dol10

Fig. 26



B.

	Sialic acid, mol/mol
Control	0
Sialylated Lot 1	2.2 ± 0
Sialylated Lot 2	2.3 ± 0.1

C.

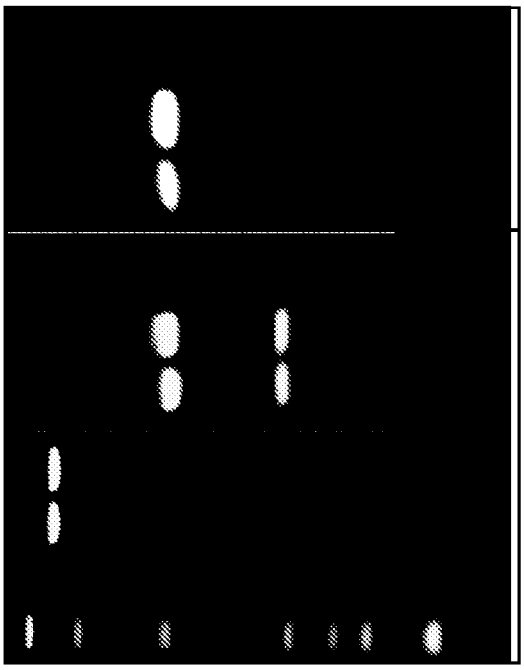


Fig. 27

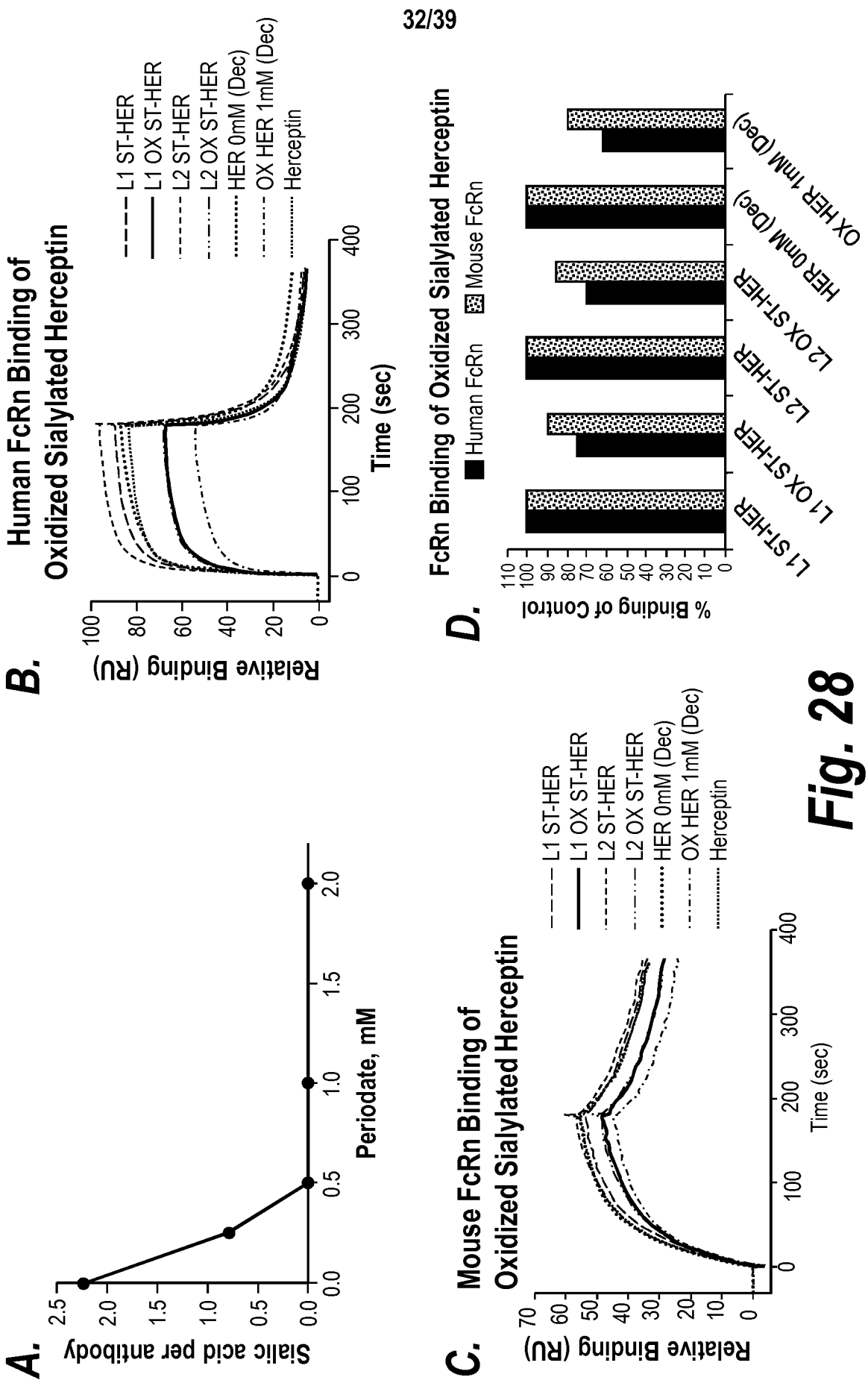

A.
Sialic acid per antibody
Periodate, mM
B.
Human FcRn Binding of Oxidized Sialylated Herceptin
Relative Binding (RU)
Time (sec)
L1 ST-HER
L1 OX ST-HER
L2 ST-HER
L2 OX ST-HER
HER 0mM (Dec)
OX HER 1mM (Dec)
Herceptin
C.
Mouse FcRn Binding of Oxidized Sialylated Herceptin
Relative Binding (RU)
Time (sec)
D.
FcRn Binding of Oxidized Sialylated Herceptin
Human FcRn
Mouse FcRn
% Binding of Control
L1 ST-HER
L1 OX ST-HER
L2 ST-HER
L2 OX ST-HER
HER 0mM (Dec)
OX HER 1mM (Dec)

Fig. 28

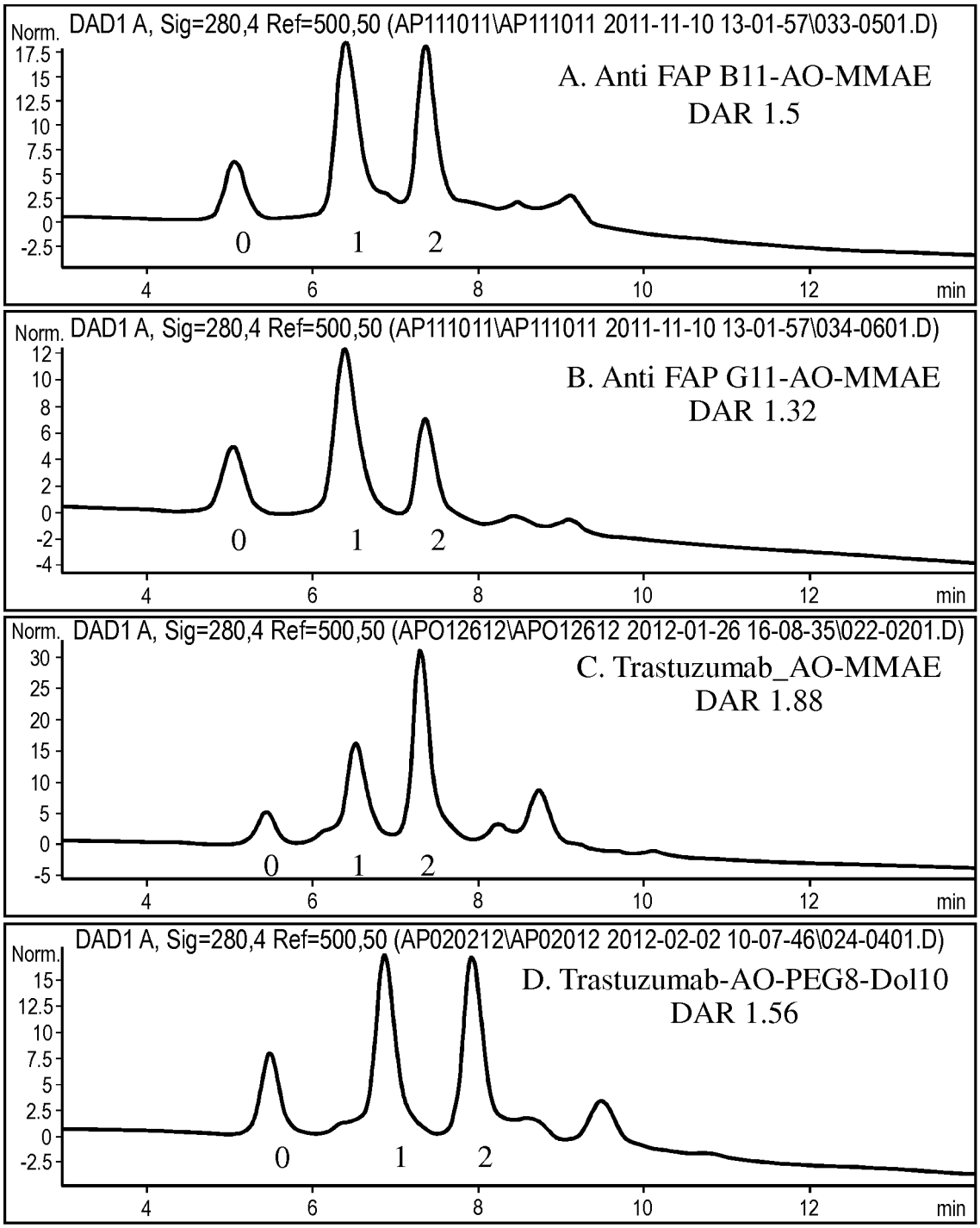

Norm.
DAD1 A, Sig=280,4 Ref=500,50 (AP111011\AP111011 2011-11-10 13-01-57\033-0501.D)
A. Anti FAP B11-AO-MMAE
DAR 1.5
0
1
2
min
Norm.
DAD1 A, Sig=280,4 Ref=500,50 (AP111011\AP111011 2011-11-10 13-01-57\034-0601.D)
B. Anti FAP G11-AO-MMAE
DAR 1.32
0
1
2
min
Norm.
DAD1 A, Sig=280,4 Ref=500,50 (APO12612\APO12612 2012-01-26 16-08-35\022-0201.D)
C. Trastuzumab_AO-MMAE
DAR 1.88
0
1
2
min
Norm.
DAD1 A, Sig=280,4 Ref=500,50 (AP020212\AP02012 2012-02-02 10-07-46\024-0401.D)
D. Trastuzumab-AO-PEG8-Dol10
DAR 1.56
0
1
2
min

Fig. 29

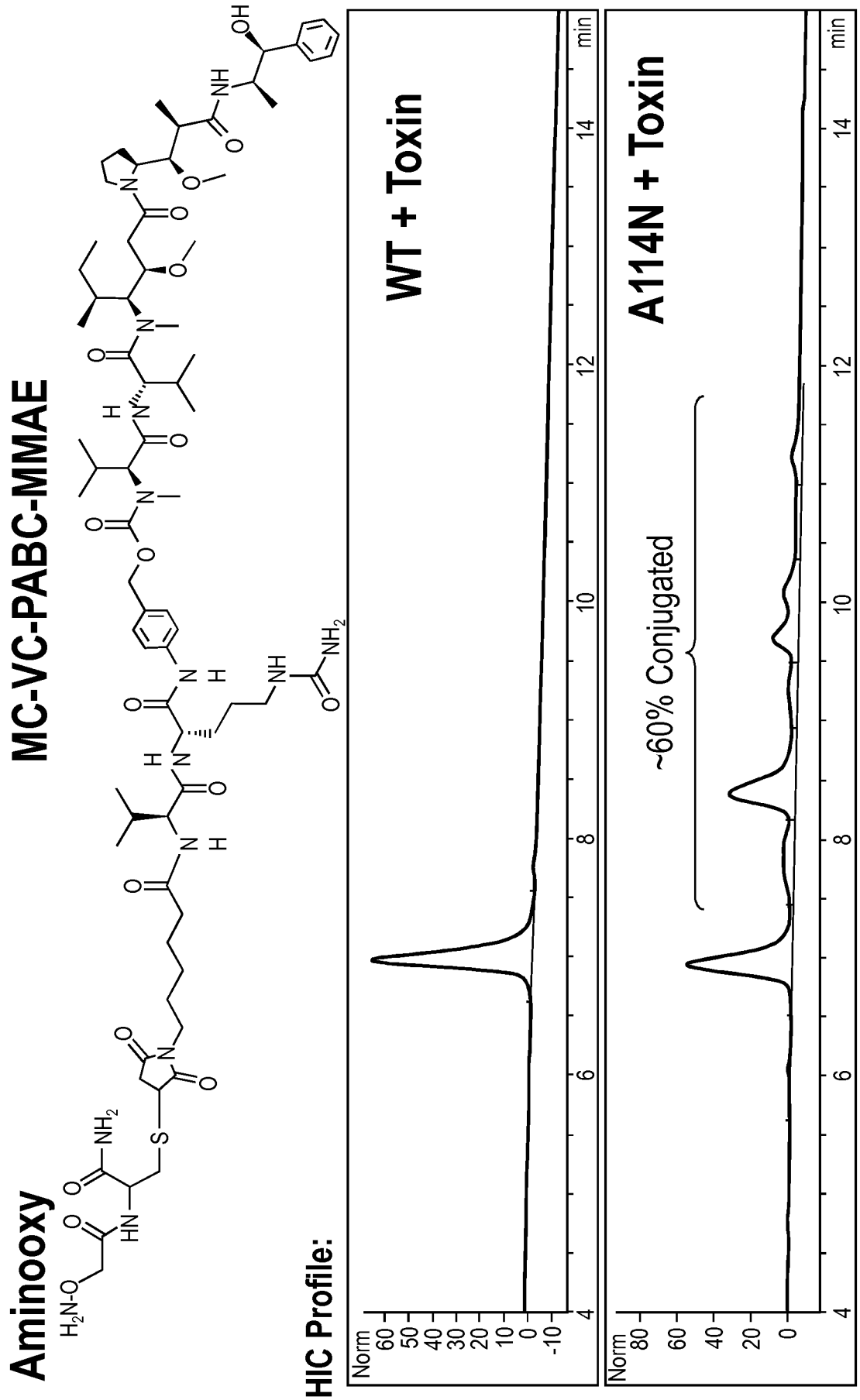

Aminooxy
MC-VC-PABC-MMAE
HIC Profile:
WT + Toxin
A114N + Toxin
~60% Conjugated
Norm
min

Fig. 30

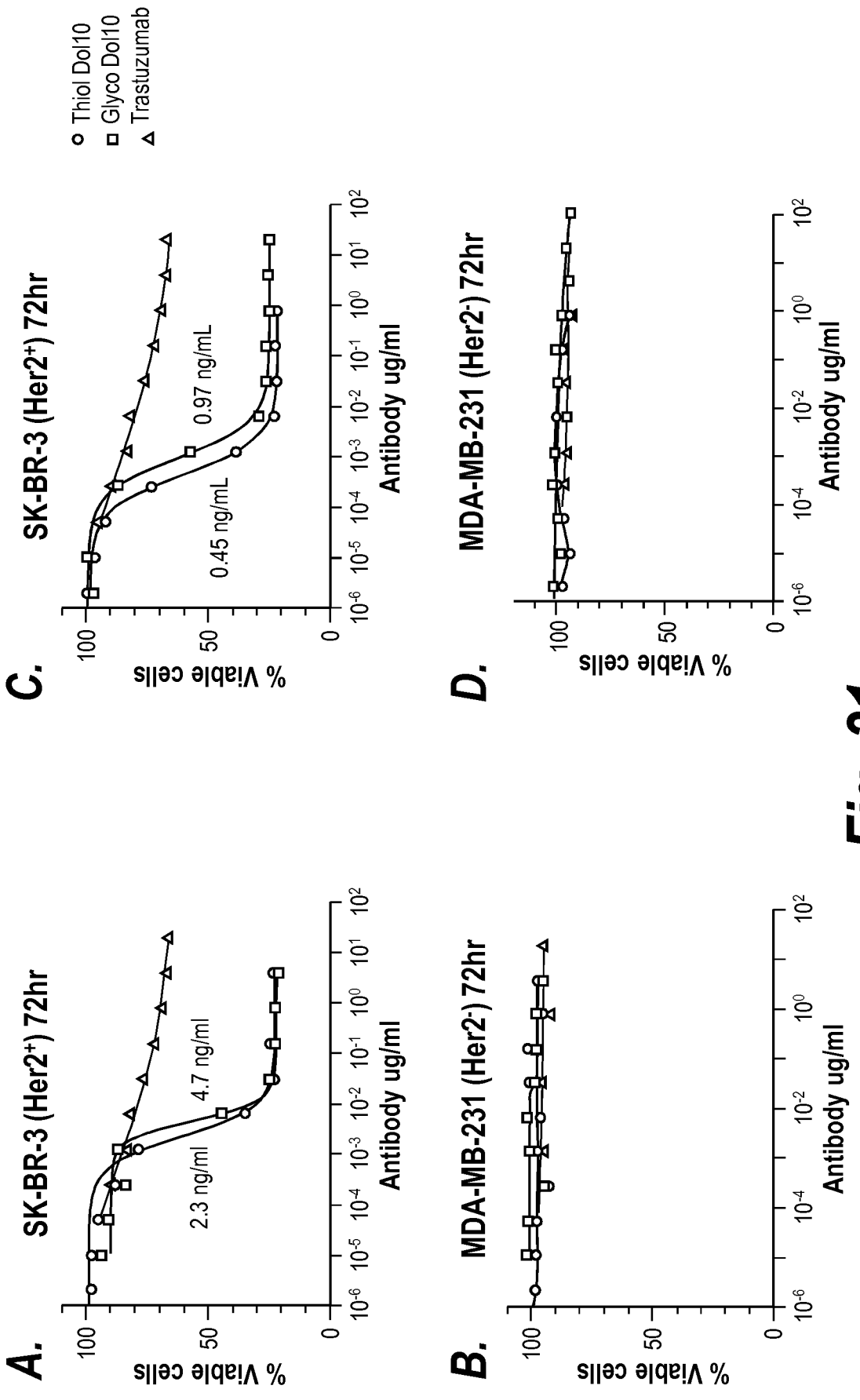

A.
SK-BR-3 (Her2+) 72hr
% Viable cells
Antibody ug/ml
2.3 ng/ml
4.7 ng/ml
B.
MDA-MB-231 (Her2-) 72hr
% Viable cells
Antibody ug/ml
C.
SK-BR-3 (Her2+) 72hr
Thiol Dol10
Glyco Dol10
Trastuzumab
% Viable cells
Antibody ug/ml
0.45 ng/mL
0.97 ng/mL
D.
MDA-MB-231 (Her2-) 72hr
% Viable cells
Antibody ug/ml

Fig. 31

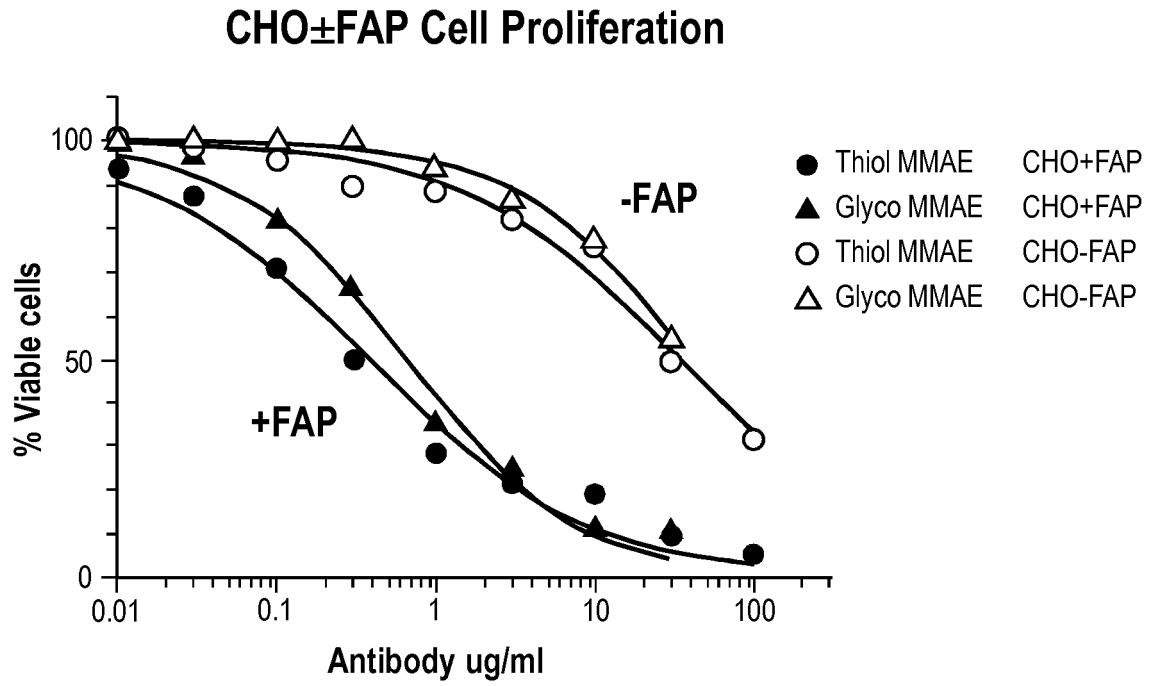

CHO±FAP Cell Proliferation
% Viable cells
100
50
0
0.01
0.1
1
10
100
Antibody ug/ml
-FAP
+FAP
Thiol MMAE CHO+FAP
Glyco MMAE CHO+FAP
Thiol MMAE CHO-FAP
Glyco MMAE CHO-FAP

Fig. 32

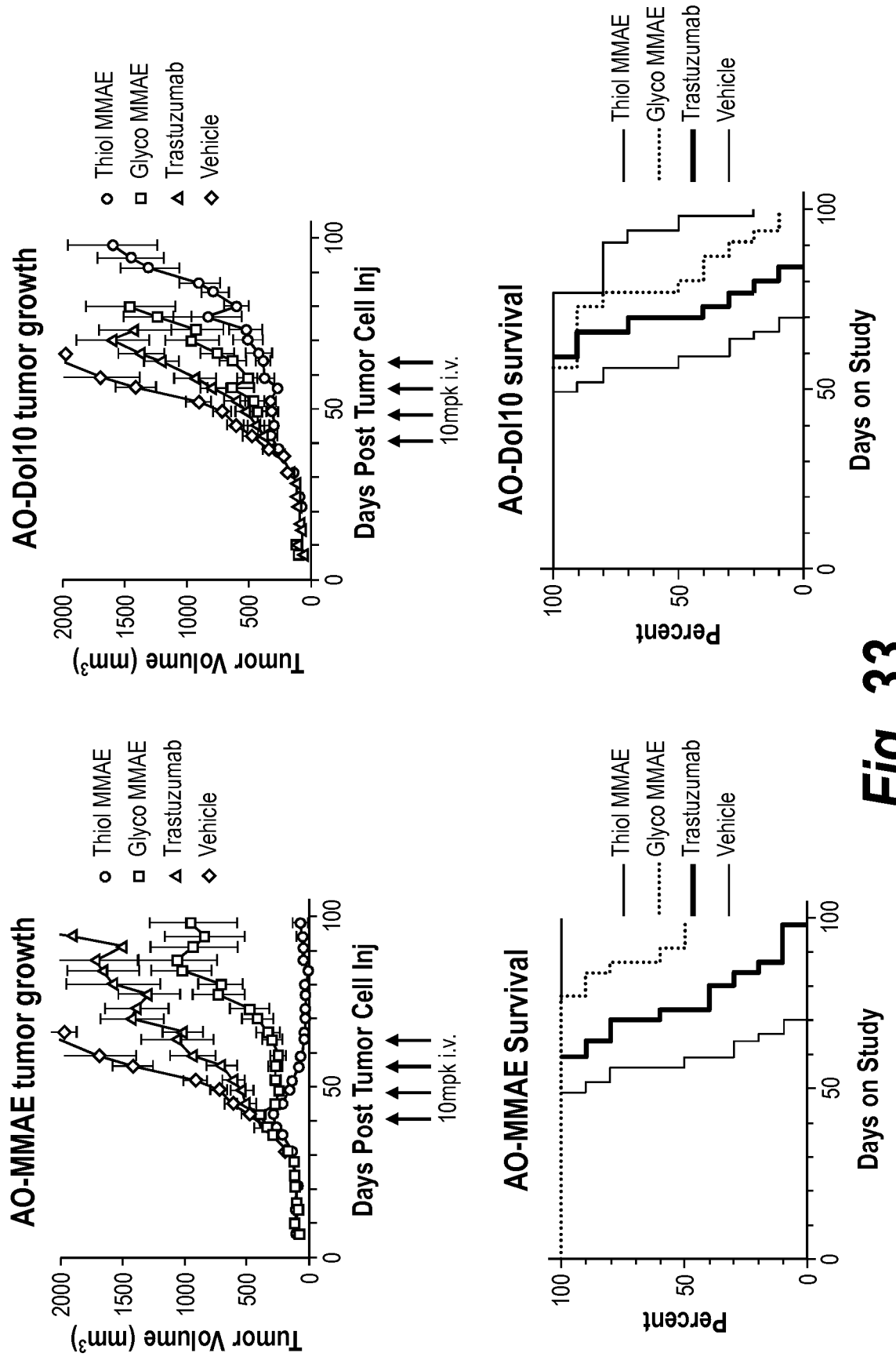

AO-Dol10 tumor growth
Tumor Volume (mm3)
Days Post Tumor Cell Inj
10mpk i.v.
Thiol MMAE
Glyco MMAE
Trastuzumab
Vehicle
AO-Dol10 survival
Percent
Days on Study
AO-MMAE tumor growth
AO-MMAE Survival

Fig. 33

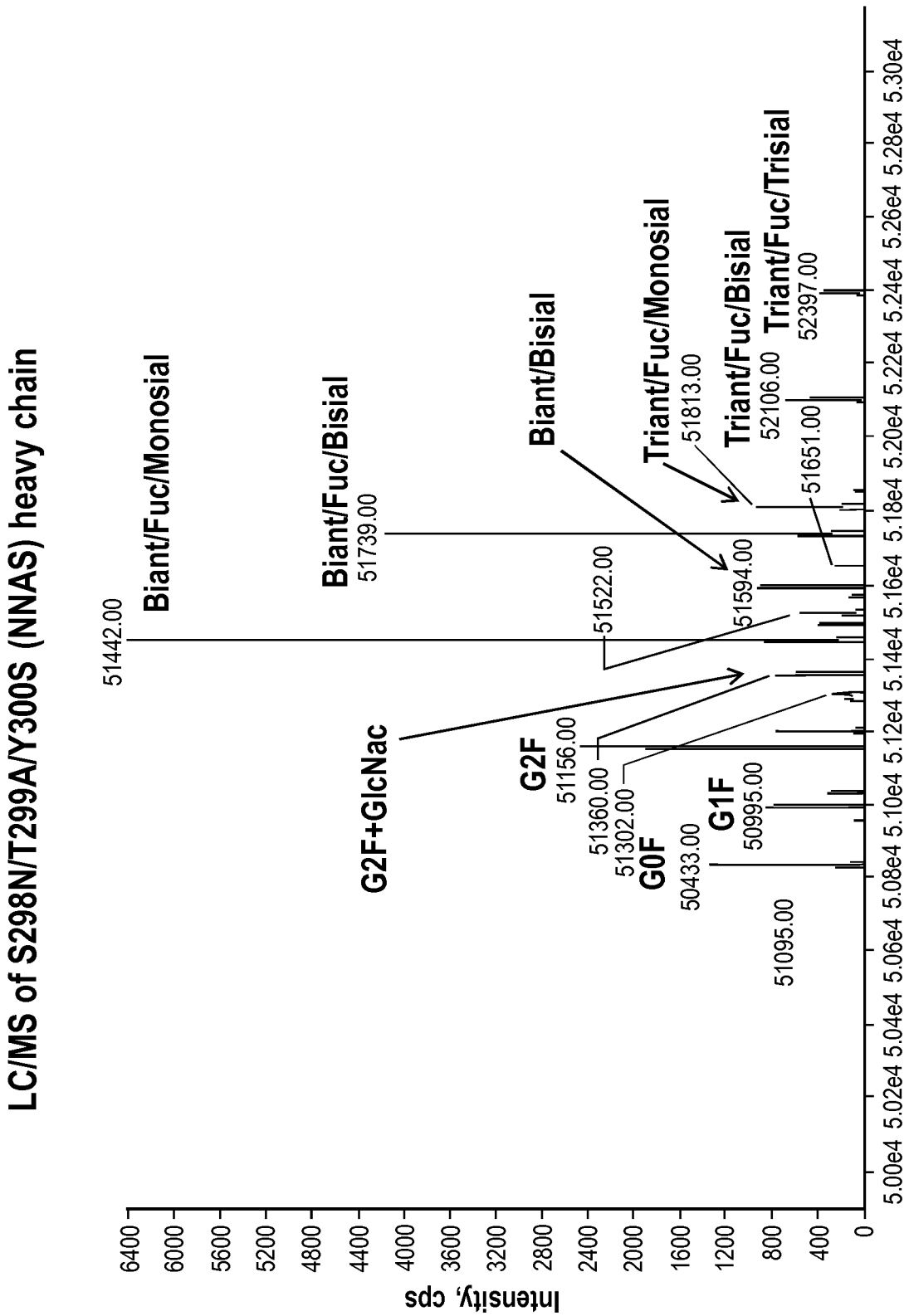

LC/MS of S298N/T299A/Y300S (NNAS) heavy chain
Intensity, cps
Mass, amu
Biant/Fuc/Monosial
51442.00
Biant/Fuc/Bisial
51739.00
Biant/Bisial
51594.00
Triant/Fuc/Monosial
51813.00
Triant/Fuc/Bisial
52106.00
Triant/Fuc/Trisial
52397.00
G2F+GlcNac
51522.00
G2F
51156.00
51360.00
G0F
51302.00
50433.00
G1F
50995.00
51095.00
51651.00
6400
6000
5600
5200
4800
4400
4000
3600
3200
2800
2400
2000
1600
1200
800
400
0
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

Fig. 34

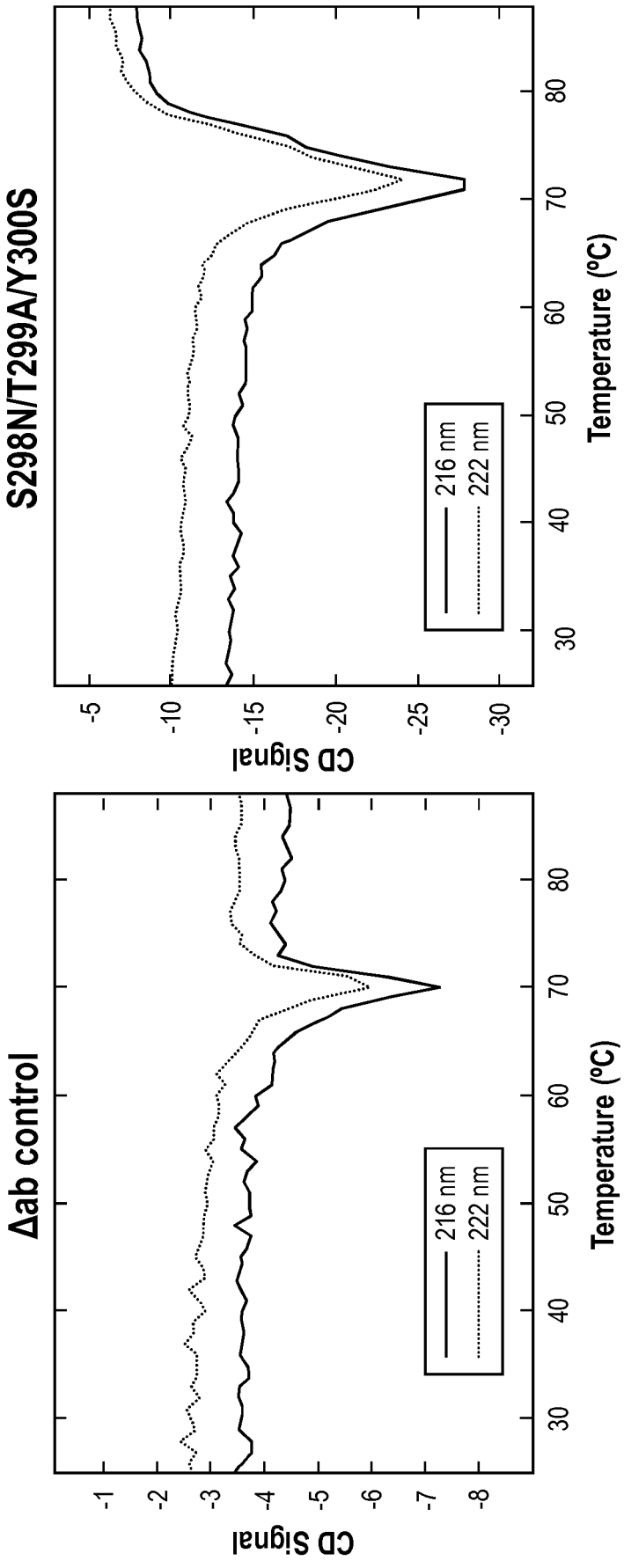

Δab control
S298N/T299A/Y300S
CD Signal
Temperature (°C)
216 nm
222 nm
-1
-2
-3
-4
-5
-6
-7
-8
-5
-10
-15
-20
-25
-30
30
40
50
60
70
80

Fig. 35