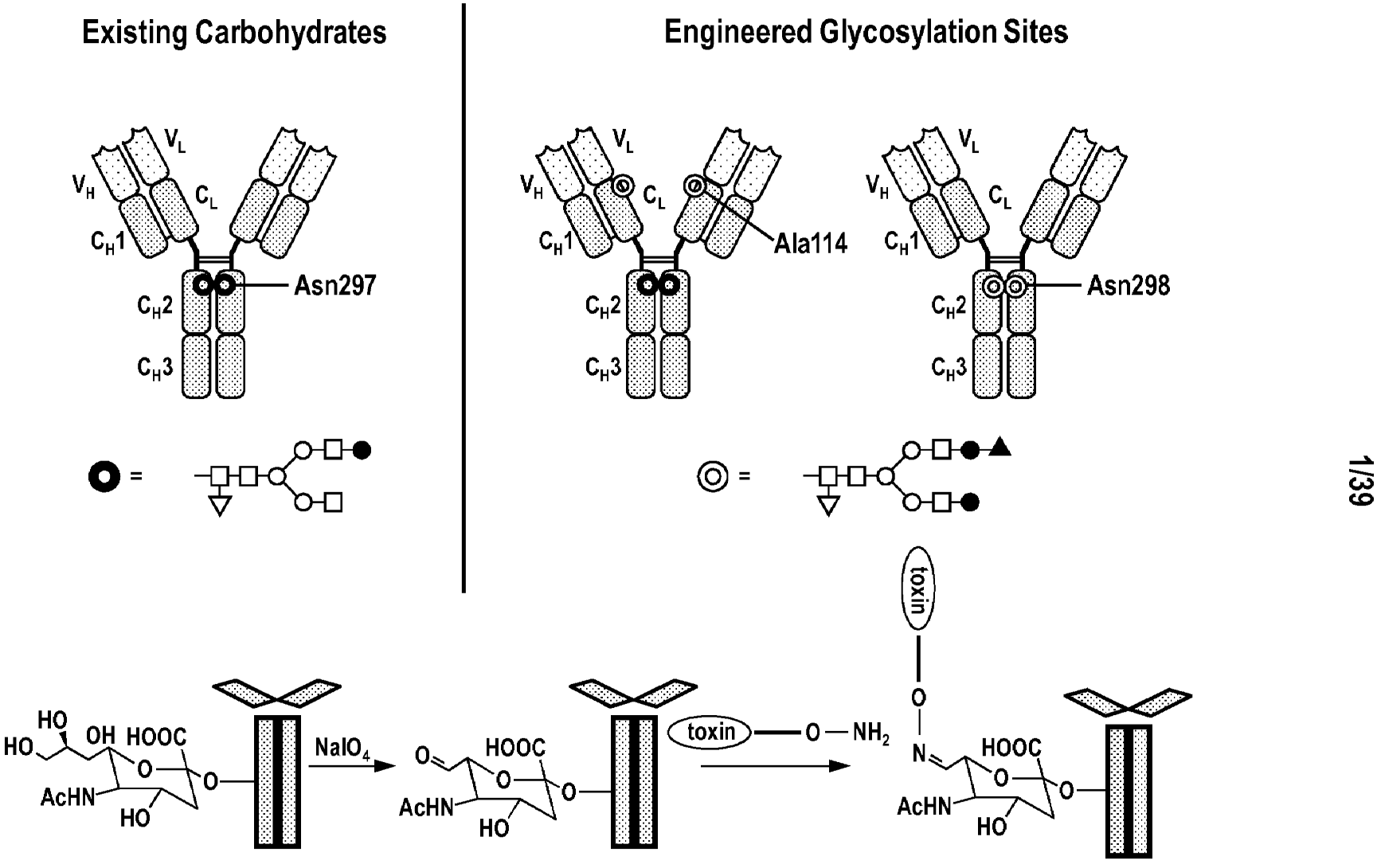

Existing Carbohydrates
Engineered Glycosylation Sites
V_L
V_H
C_L
C_H1
C_H2
C_H3
Asn297
Ala114
Asn298
=
=
toxin
$NaIO_4$
NH_2
HO
OH
HOOC
AcHN
HO
O
N

Fig. 1

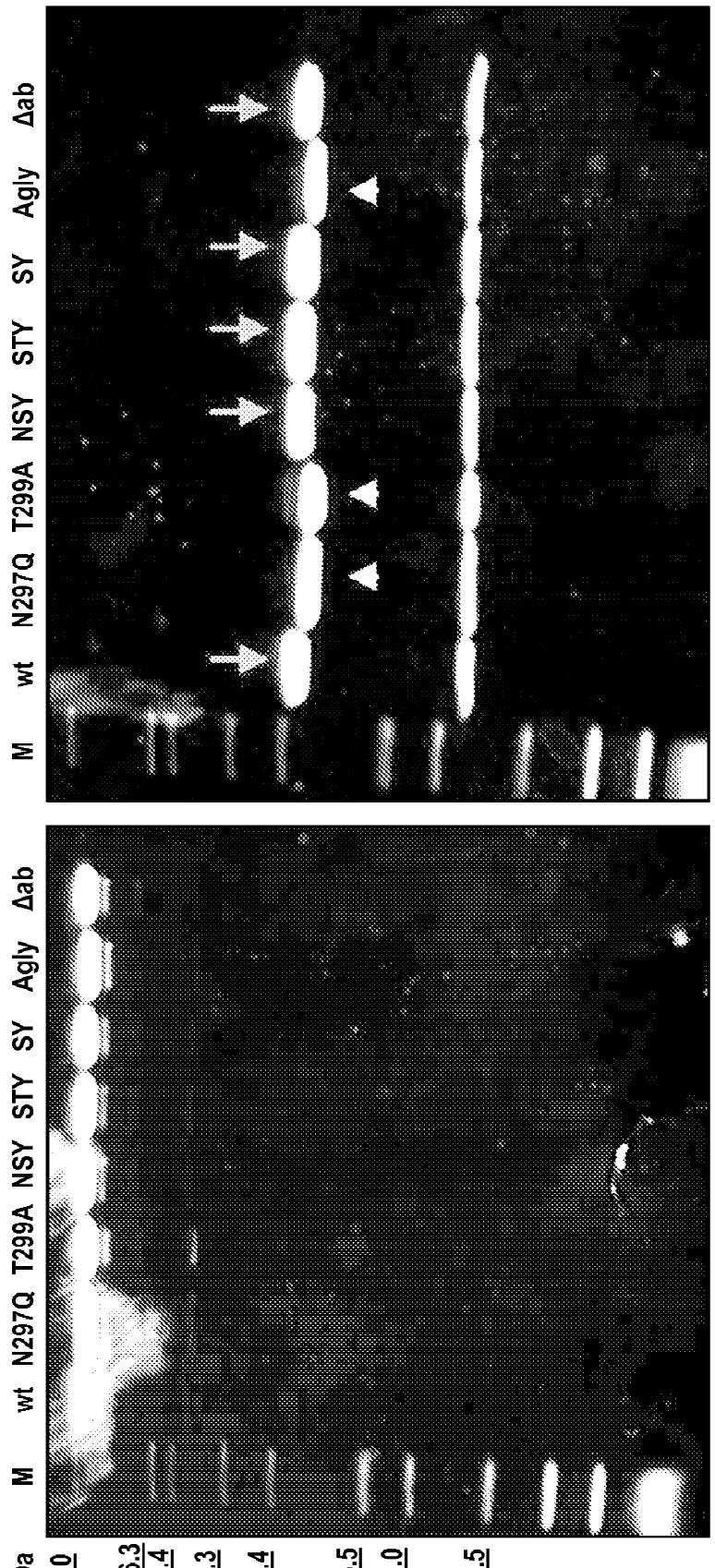
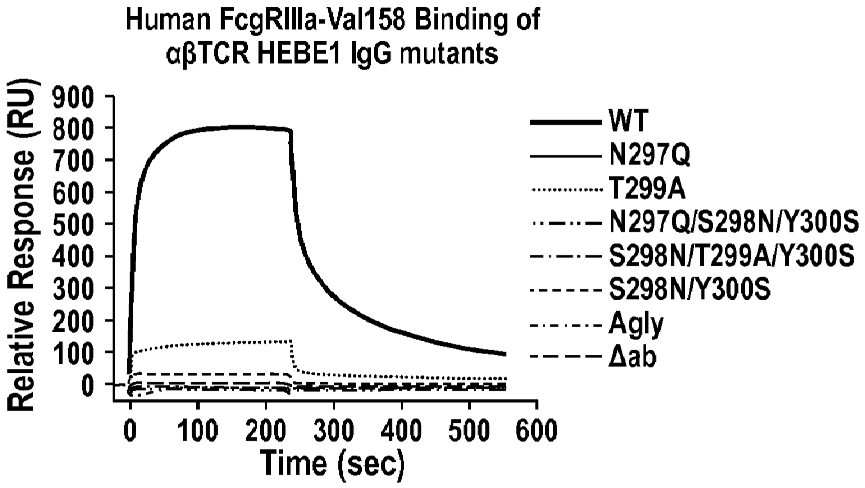
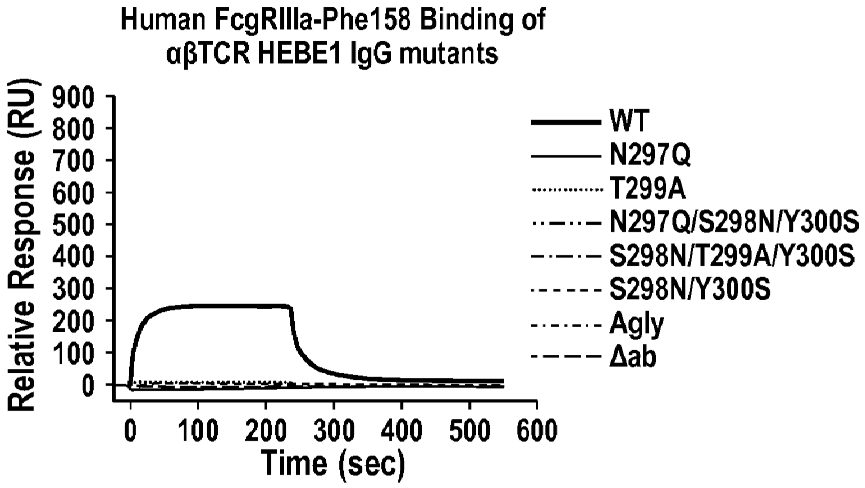

kDa
M
wt
N297Q
T299A
NSY
STY
SY
Agly
Δab
200
116.3
97.4
66.3
55.4
36.5
31.0
21.5
Non-reduced
Reduced

Fig. 2

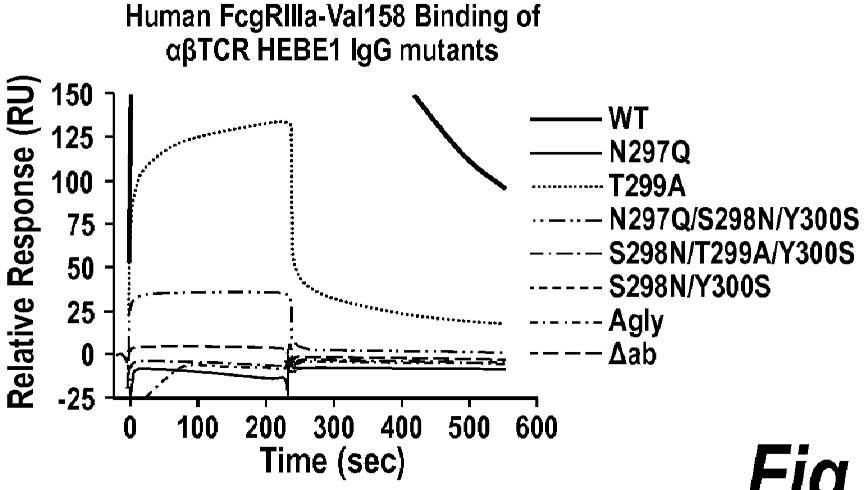
Fullscale: CD16a-Val158



Fullscale: CD16a-Phe158



zoomed:



zoomed:

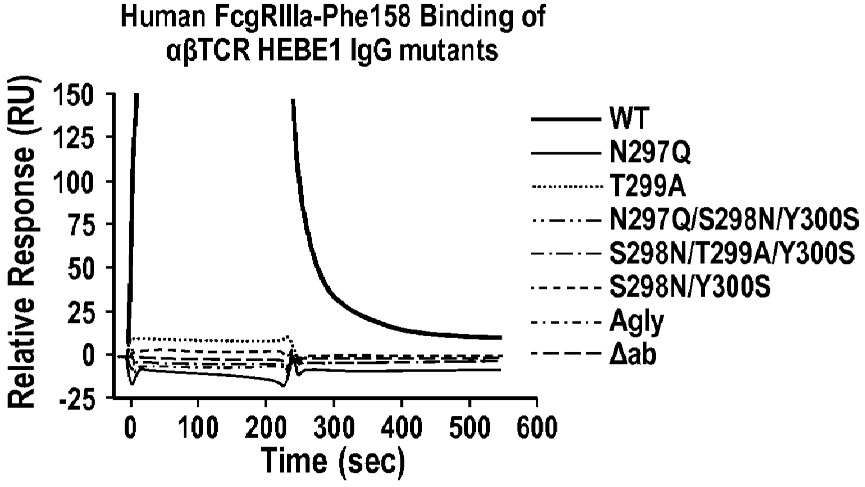


Fig. 3

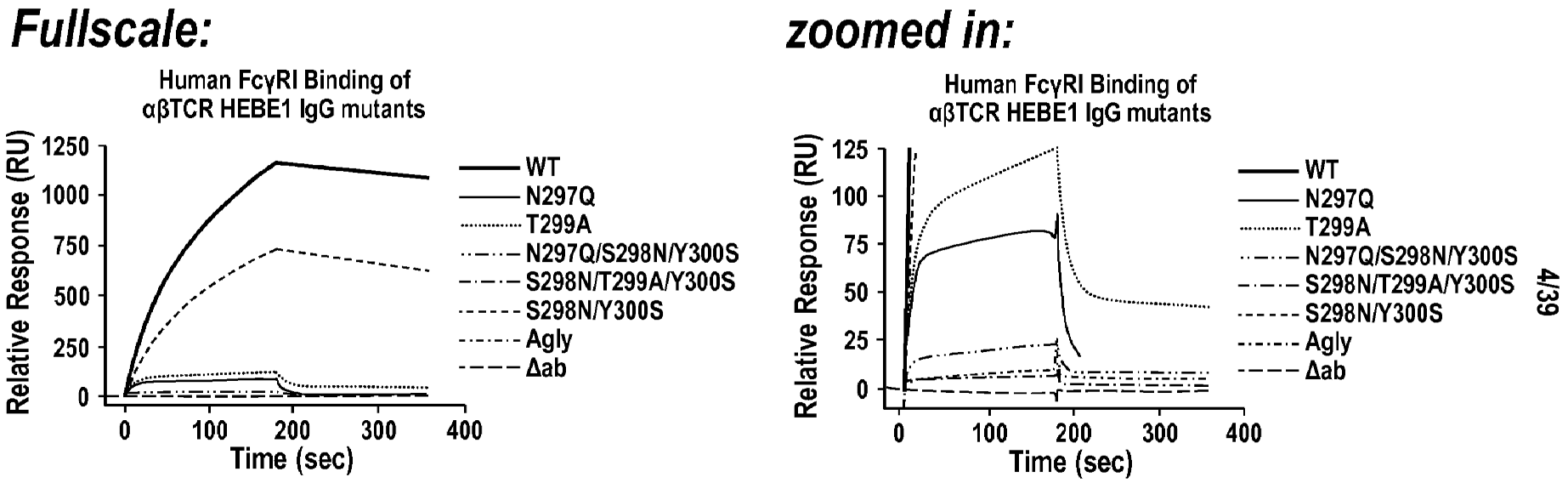

Fullscale:
Human FcγRI Binding of
αβTCR HEBE1 IgG mutants
Relative Response (RU)
1250
1000
750
500
250
0
0
100
200
300
400
Time (sec)
WT
N297Q
T299A
N297Q/S298N/Y300S
S298N/T299A/Y300S
S298N/Y300S
Agly
Δab
zoomed in:
Human FcγRI Binding of
αβTCR HEBE1 IgG mutants
Relative Response (RU)
125
100
75
50
25
0
0
100
200
300
400
Time (sec)
WT
N297Q
T299A
N297Q/S298N/Y300S
S298N/T299A/Y300S
S298N/Y300S
Agly
Δab

Fig. 4

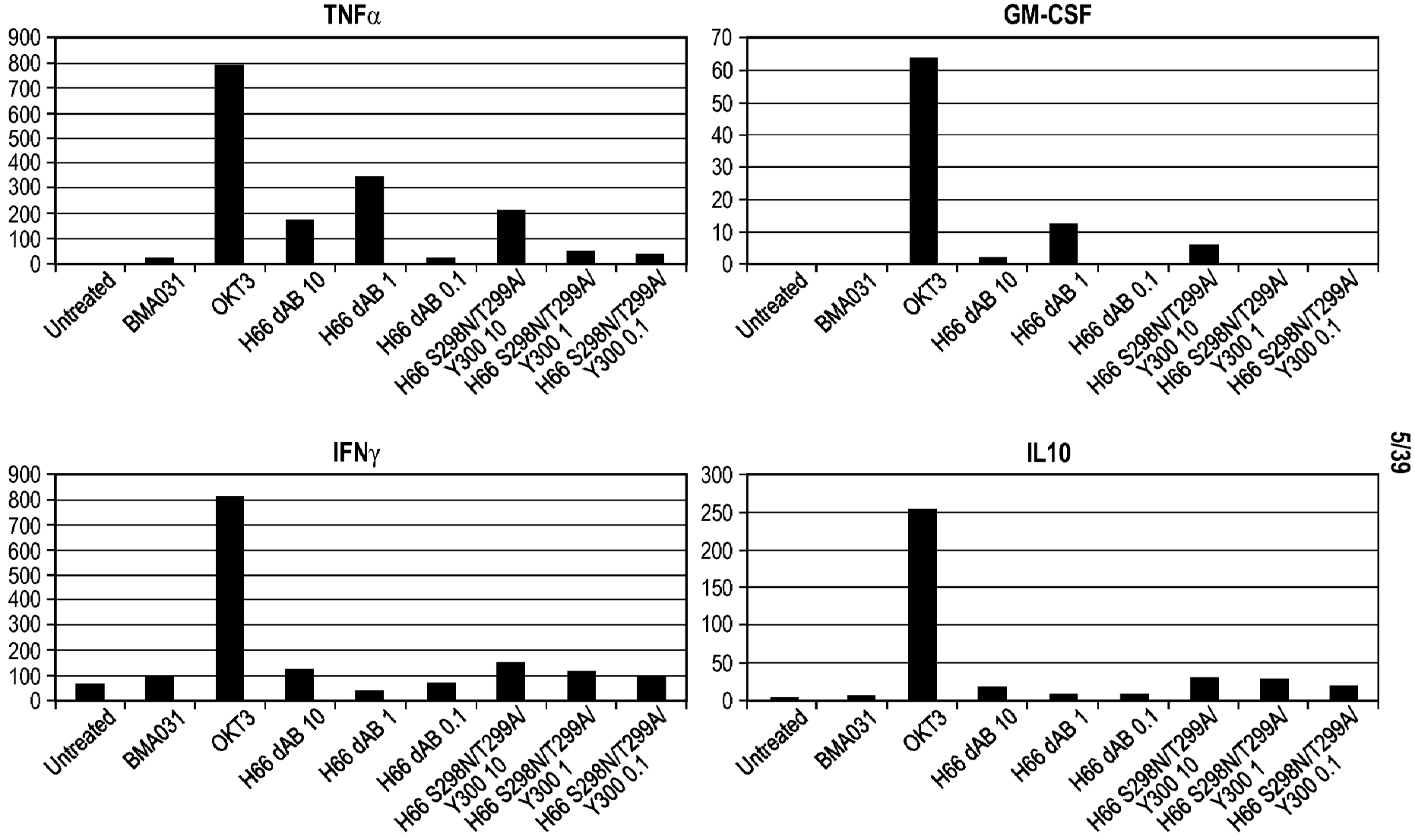

TNFα
900
800
700
600
500
400
300
200
100
0
Untreated
BMA031
OKT3
H66 dAB 10
H66 dAB 1
H66 dAB 0.1
H66 S298N/T299A/ Y300 10
H66 S298N/T299A/ Y300 1
H66 S298N/T299A/ Y300 0.1
GM-CSF
70
60
50
40
30
20
10
0
IFNγ
IL10
300
250
200
150
100
50

Fig. 5

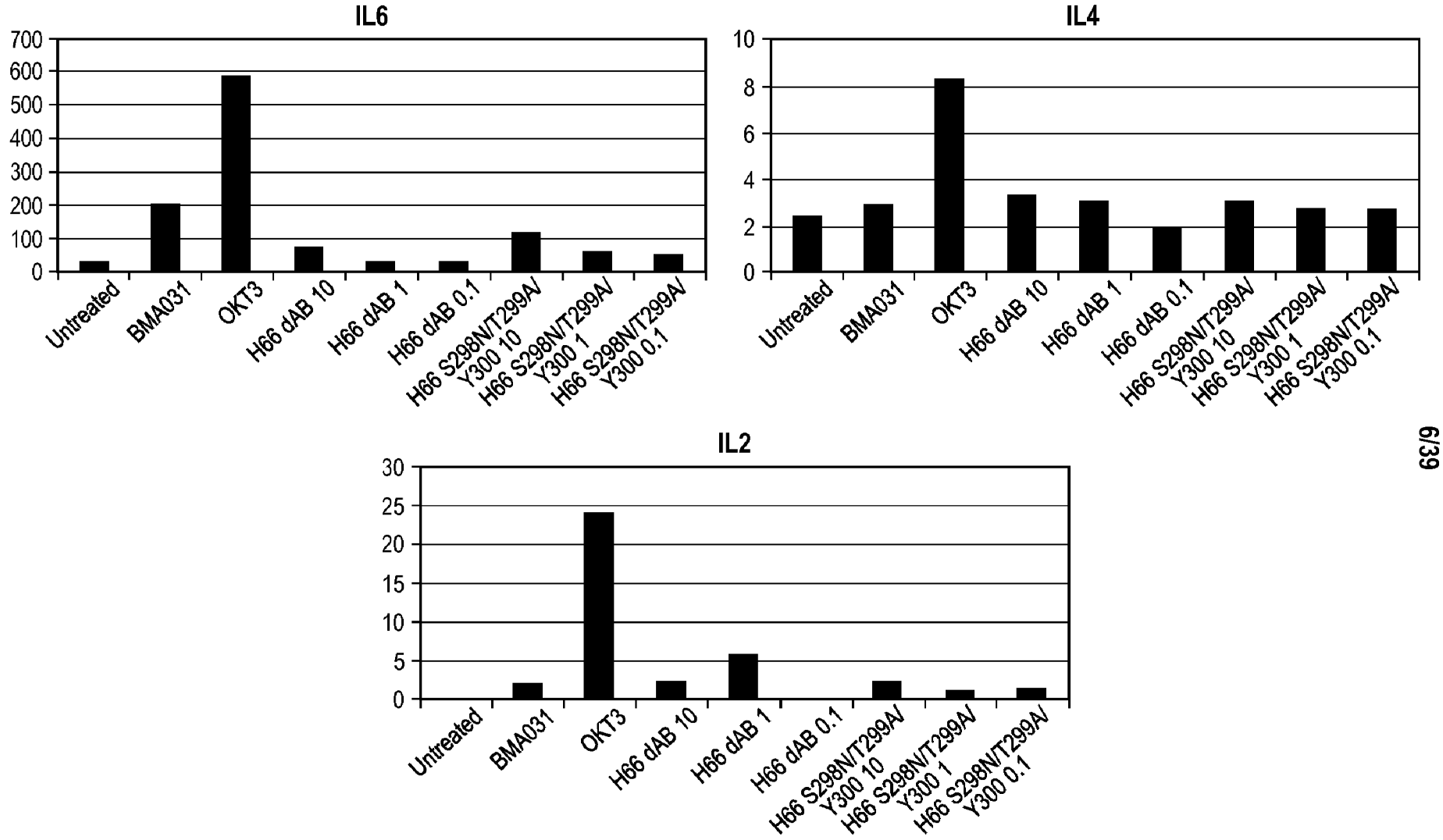

IL6
700
600
500
400
300
200
100
0
Untreated
BMA031
OKT3
H66 dAB 10
H66 dAB 1
H66 dAB 0.1
H66 S298N/T299A/ Y300 10
H66 S298N/T299A/ Y300 1
H66 S298N/T299A/ Y300 0.1
IL4
10
8
6
4
2
0
Untreated
BMA031
OKT3
H66 dAB 10
H66 dAB 1
H66 dAB 0.1
H66 S298N/T299A/ Y300 10
H66 S298N/T299A/ Y300 1
H66 S298N/T299A/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

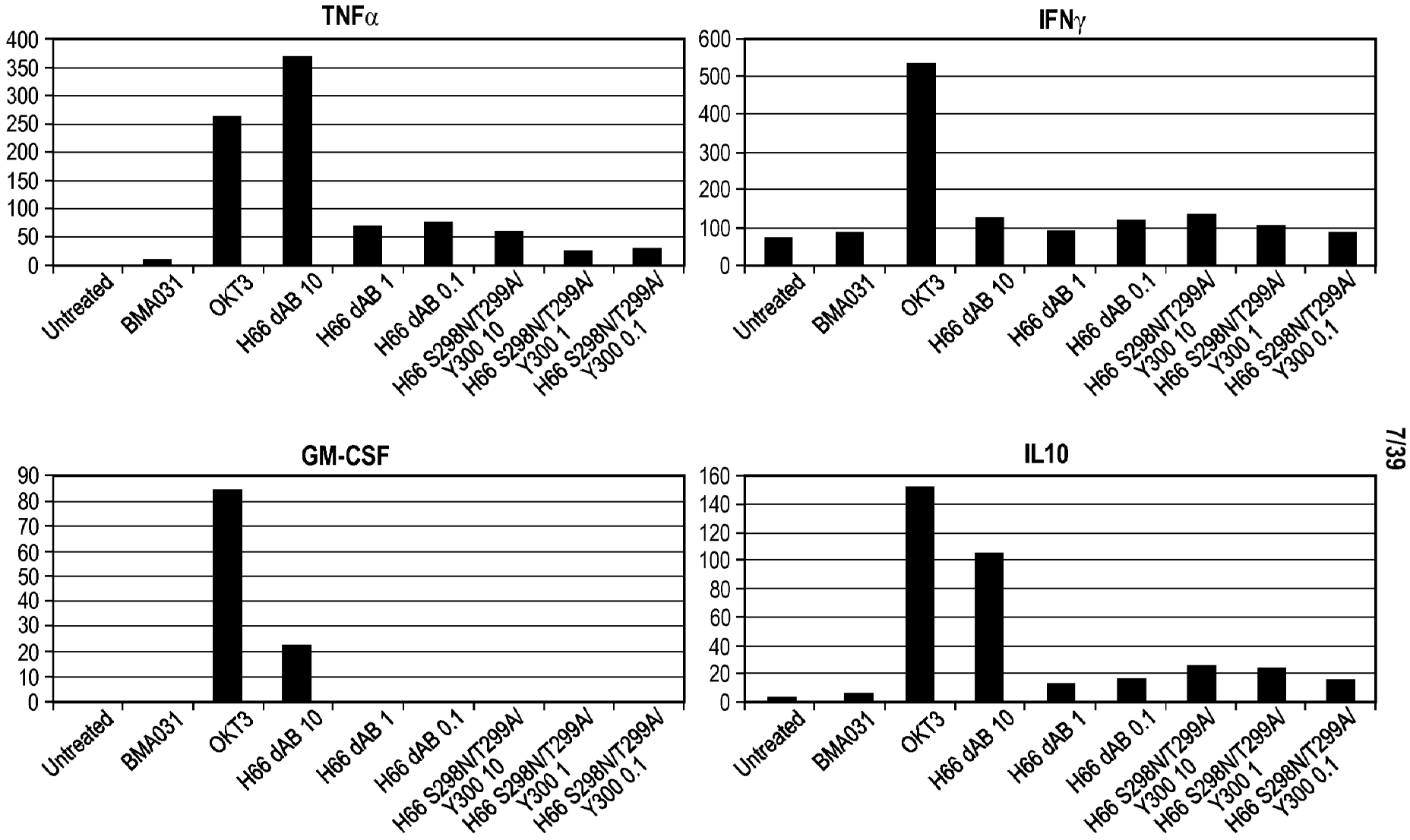

TNFα
400
350
300
250
200
150
100
50
0
IFNγ
600
500
400
300
200
100
0
GM-CSF
90
80
70
60
50
40
30
20
10
0
IL10
160
140
120
100
80
60
40
20
0
Untreated
BMA031
OKT3
H66 dAB 10
H66 dAB 1
H66 dAB 0.1
H66 S298N/T299A/ Y300 10
H66 S298N/T299A/ Y300 1
H66 S298N/T299A/ Y300 0.1

Fig. 7

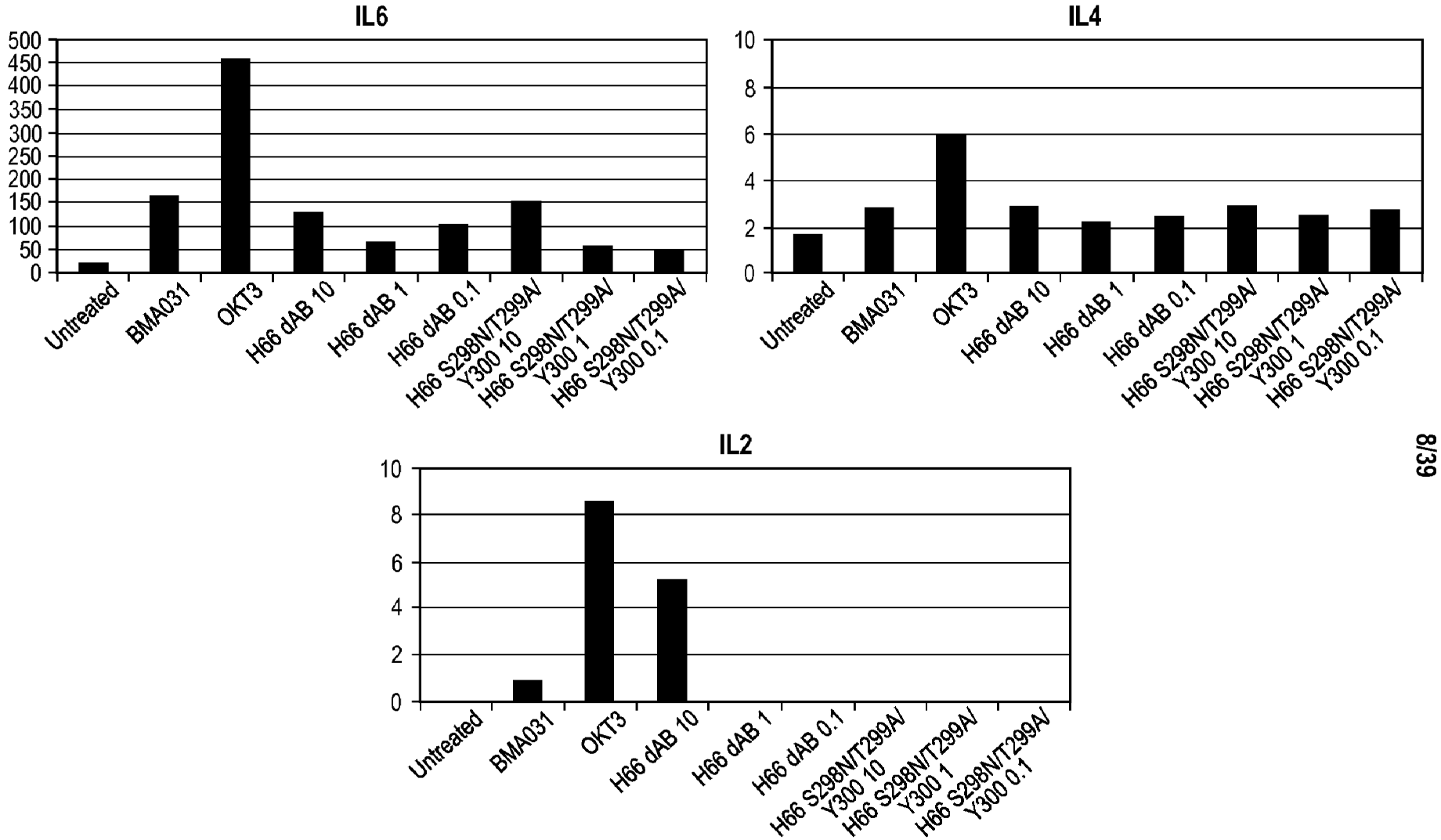
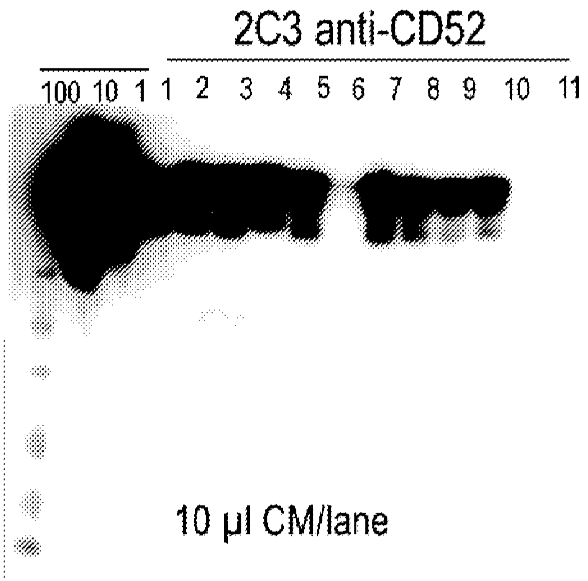

IL6
500
450
400
350
300
250
200
150
100
50
0
Untreated
BMA031
OKT3
H66 dAB 10
H66 dAB 1
H66 dAB 0.1
H66 S298N/T299A/ Y300 10
H66 S298N/T299A/ Y300 1
H66 S298N/T299A/ Y300 0.1
IL4
10
8
6
4
2
0
Untreated
BMA031
OKT3
H66 dAB 10
H66 dAB 1
H66 dAB 0.1
H66 S298N/T299A/ Y300 10
H66 S298N/T299A/ Y300 1
H66 S298N/T299A/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

Western Blot



1 - WT
2 - WT
3 - A114N
4 - Y436S
5 - Y436T
6 - S440N
7 - S442N
8 - NGT
9 - 298N/Y300S
10 - Mock
11 - Media

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

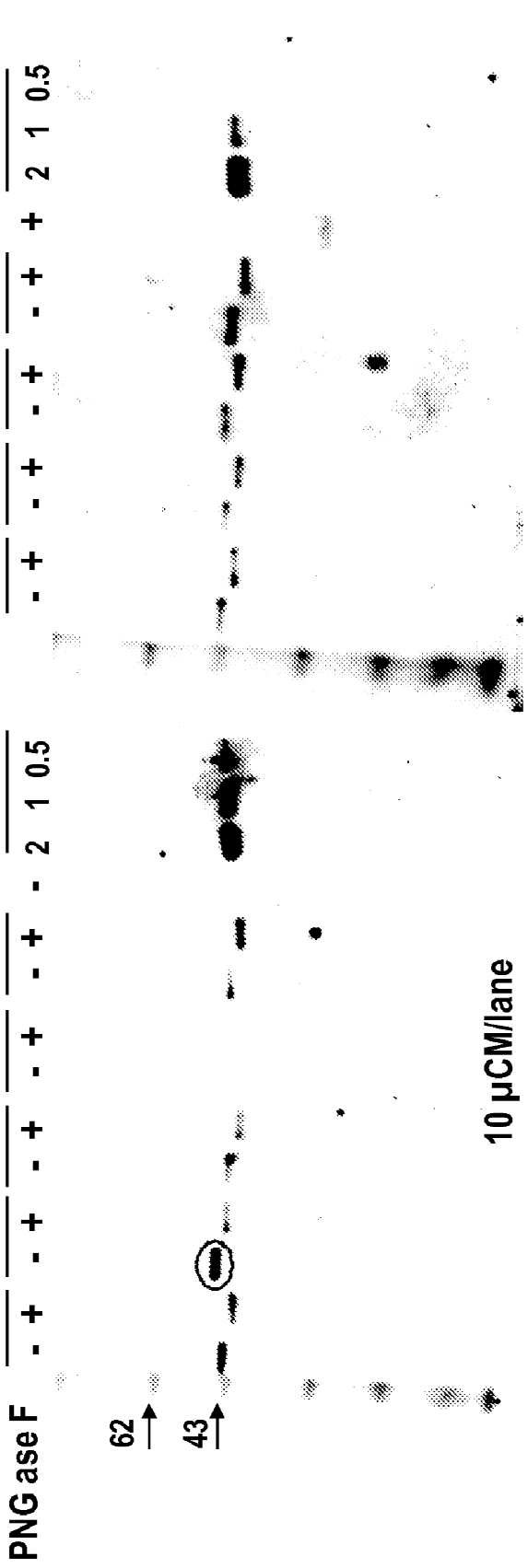

PNG ase F
wt
A114N
Y436S
Y436T
S440N
mc Campath, ng
wt
S442N
NGT
S298N/ Y300S
mc Campath, ng
62
43
10 µCM/lane

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

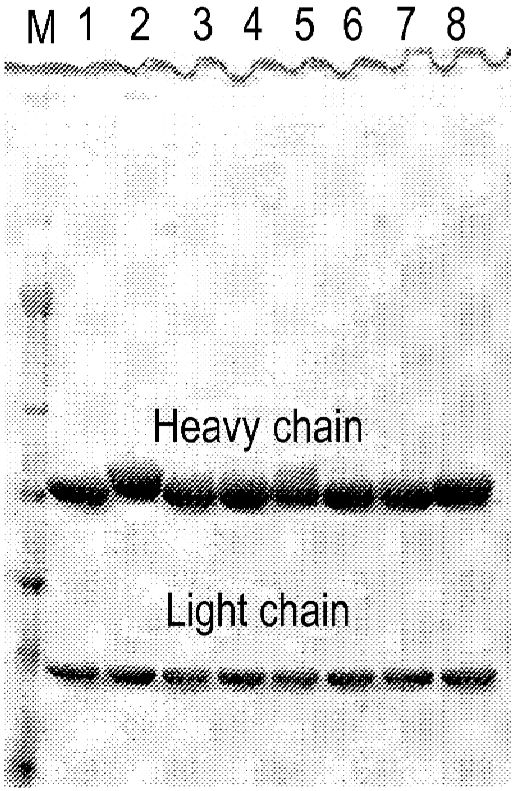


Fig. 11

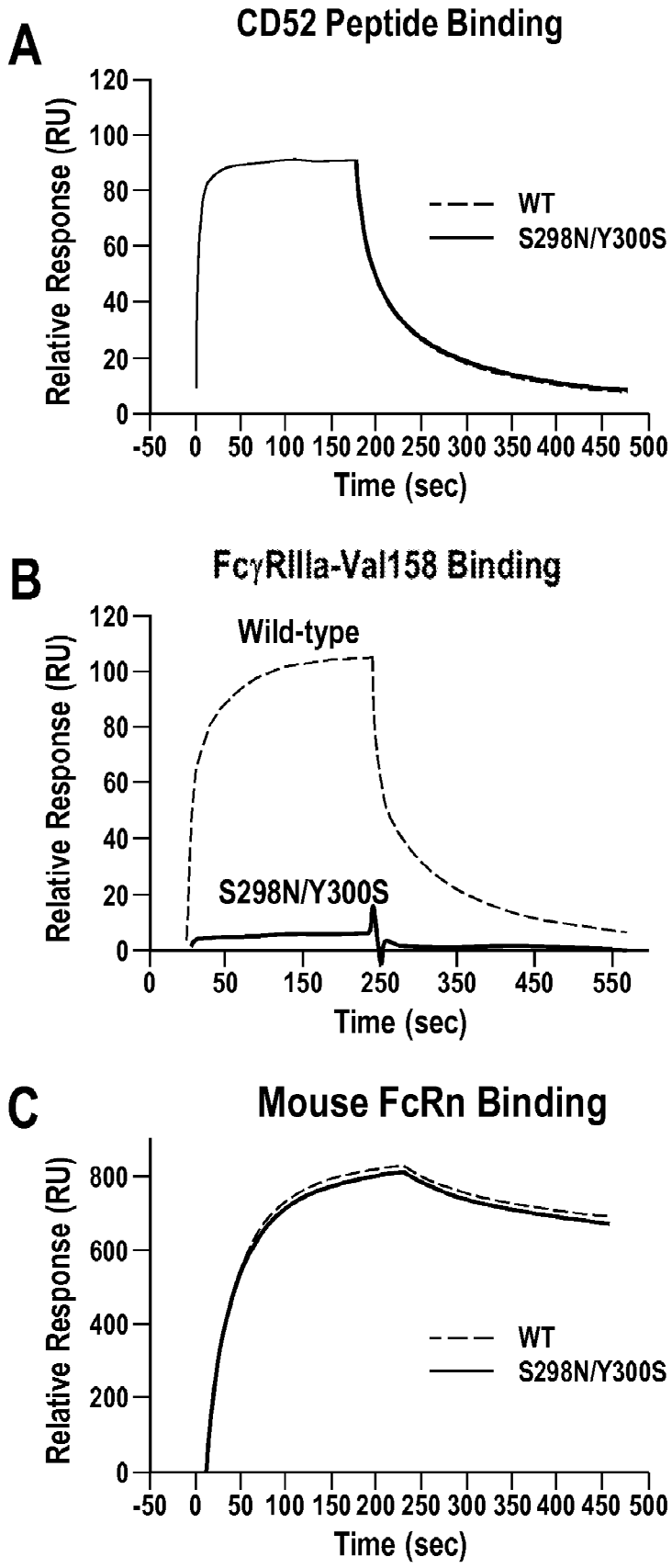

A
CD52 Peptide Binding
Relative Response (RU)
120
100
80
60
40
20
0
-50 0 50 100 150 200 250 300 350 400 450 500
Time (sec)
WT
S298N/Y300S
B
FcγRIIIa-Val158 Binding
Wild-type
S298N/Y300S
Relative Response (RU)
0 50 150 250 350 450 550
Time (sec)
C
Mouse FcRn Binding
Relative Response (RU)
800
600
400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

Fig. 12

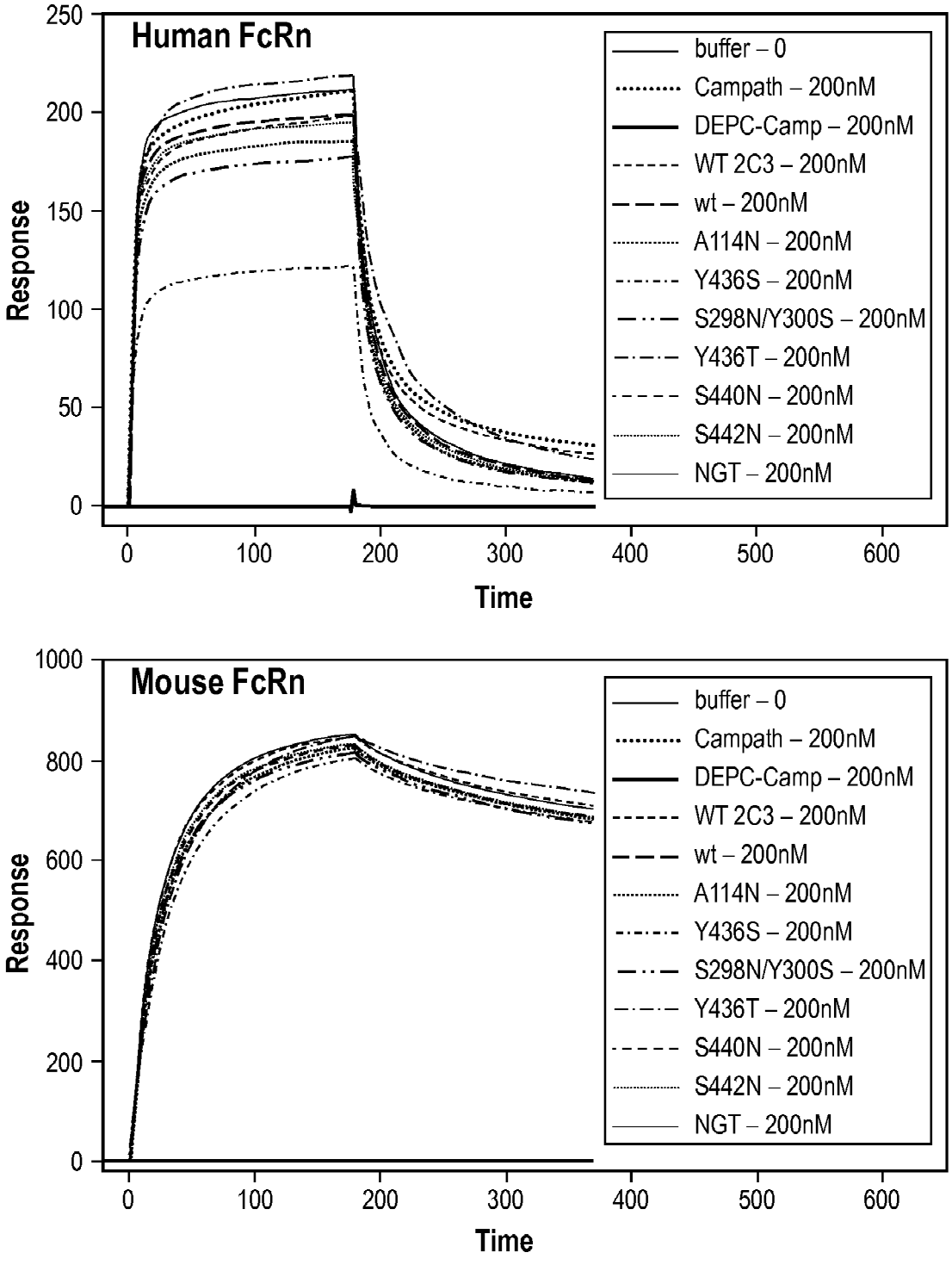
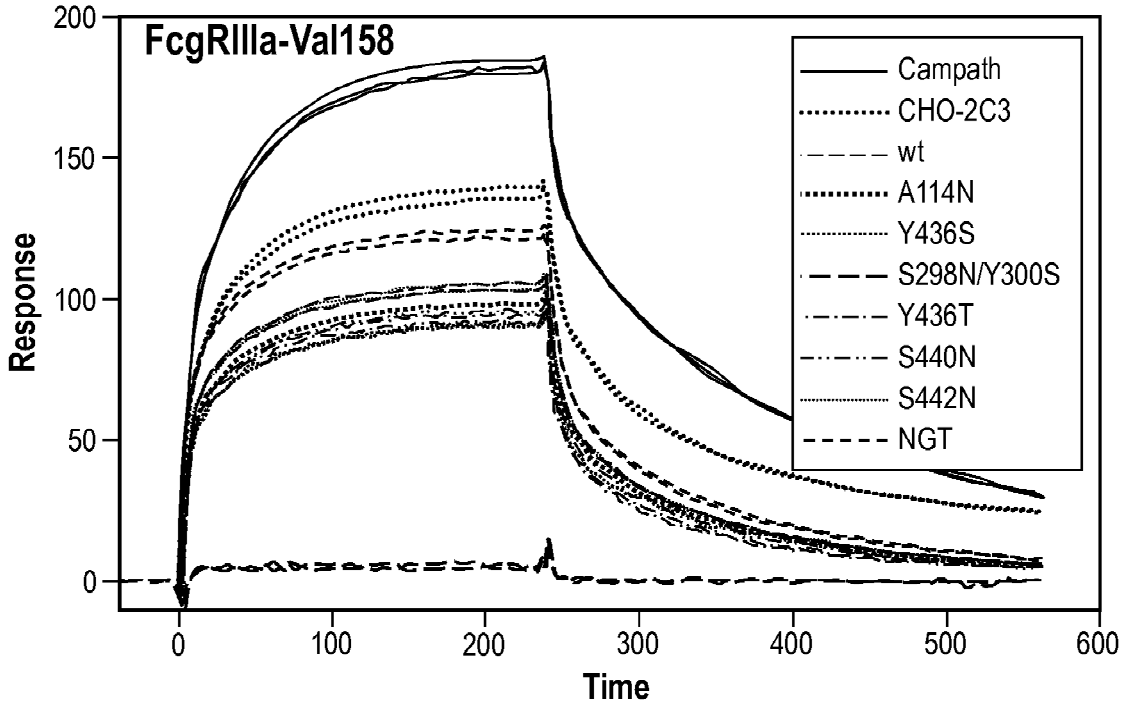

Human FcRn
Response
Time
250
200
150
100
50
0
0
100
200
300
400
500
600
buffer – 0
Campath – 200nM
DEPC-Camp – 200nM
WT 2C3 – 200nM
wt – 200nM
A114N – 200nM
Y436S – 200nM
S298N/Y300S – 200nM
Y436T – 200nM
S440N – 200nM
S442N – 200nM
NGT – 200nM
Mouse FcRn
Response
Time
1000
800
600
400
200
0
0
100
200
300
400
500
600
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.

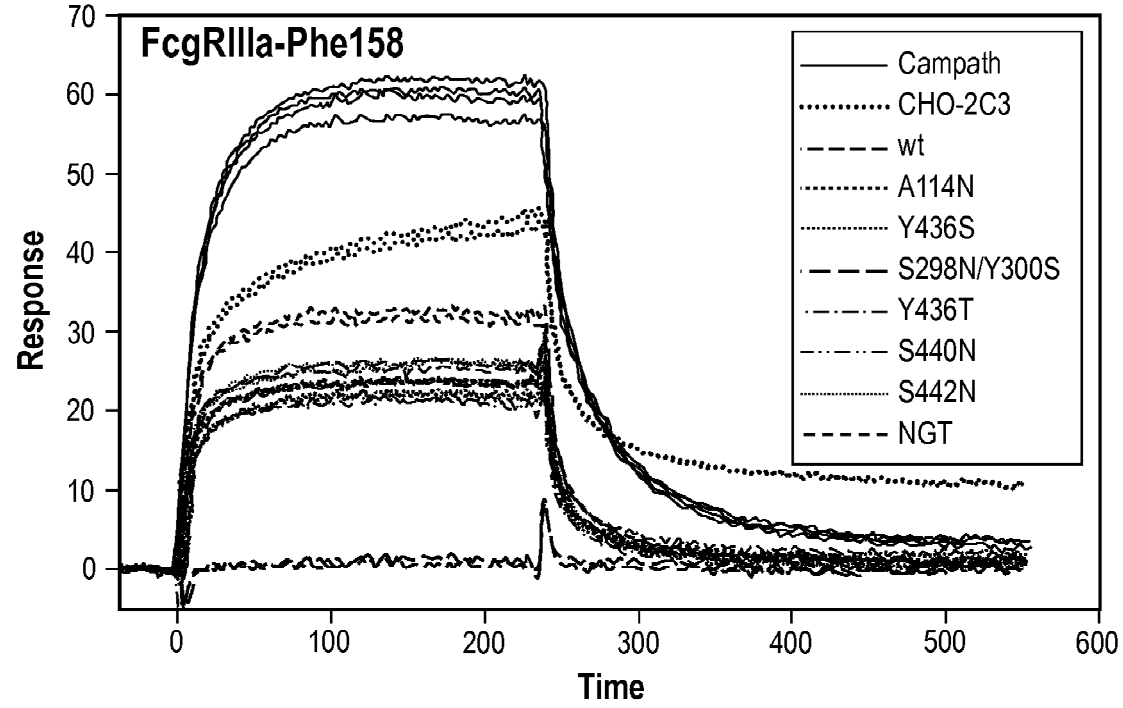
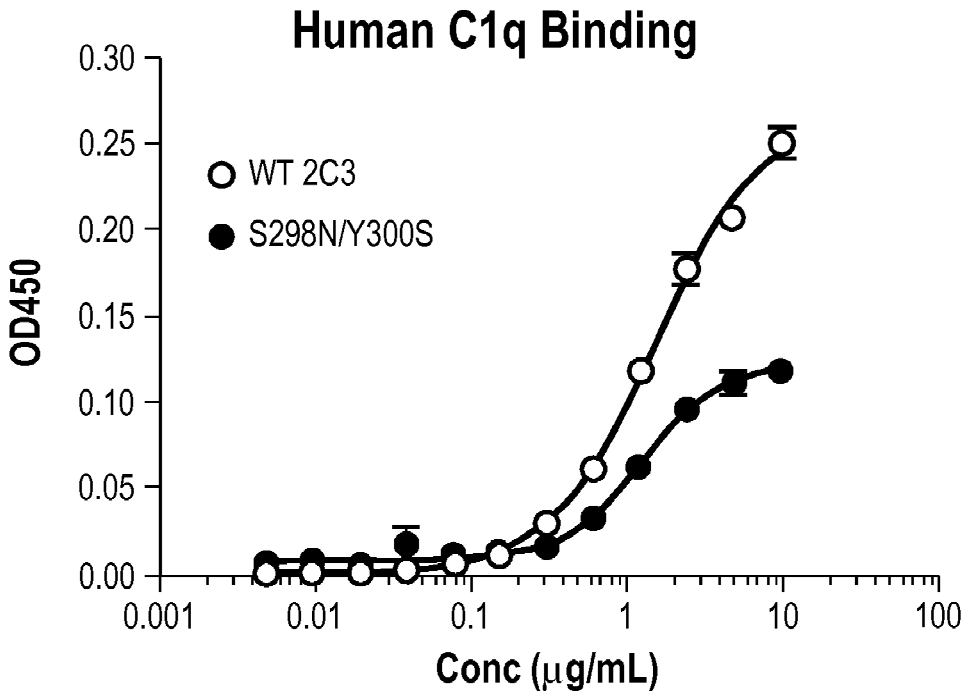

FcgRIIIa-Phe158
Campath
CHO-2C3
wt
A114N
Y436S
S298N/Y300S
Y436T
S440N
S442N
NGT
Response
Time
0
10
20
30
40
50
60
70
0
100
200
300
400
500
600

Fig. 14

A.


Human C1q Binding
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.

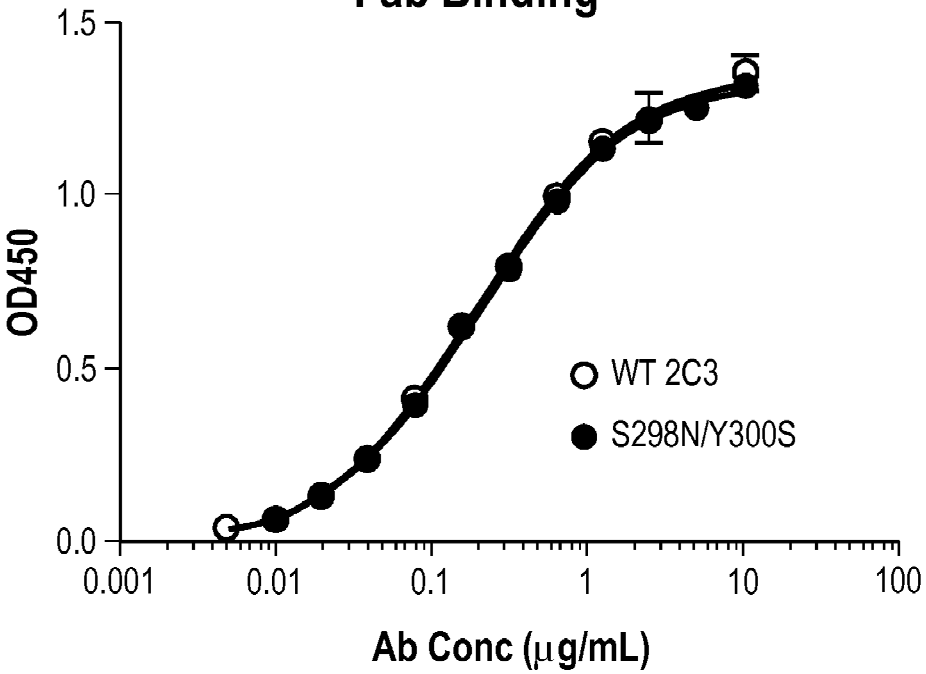

Fab Binding
1.5
1.0
0.5
0.0
OD450
WT 2C3
S298N/Y300S
0.001
0.01
0.1
1
10
100
Ab Conc (μg/mL)

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

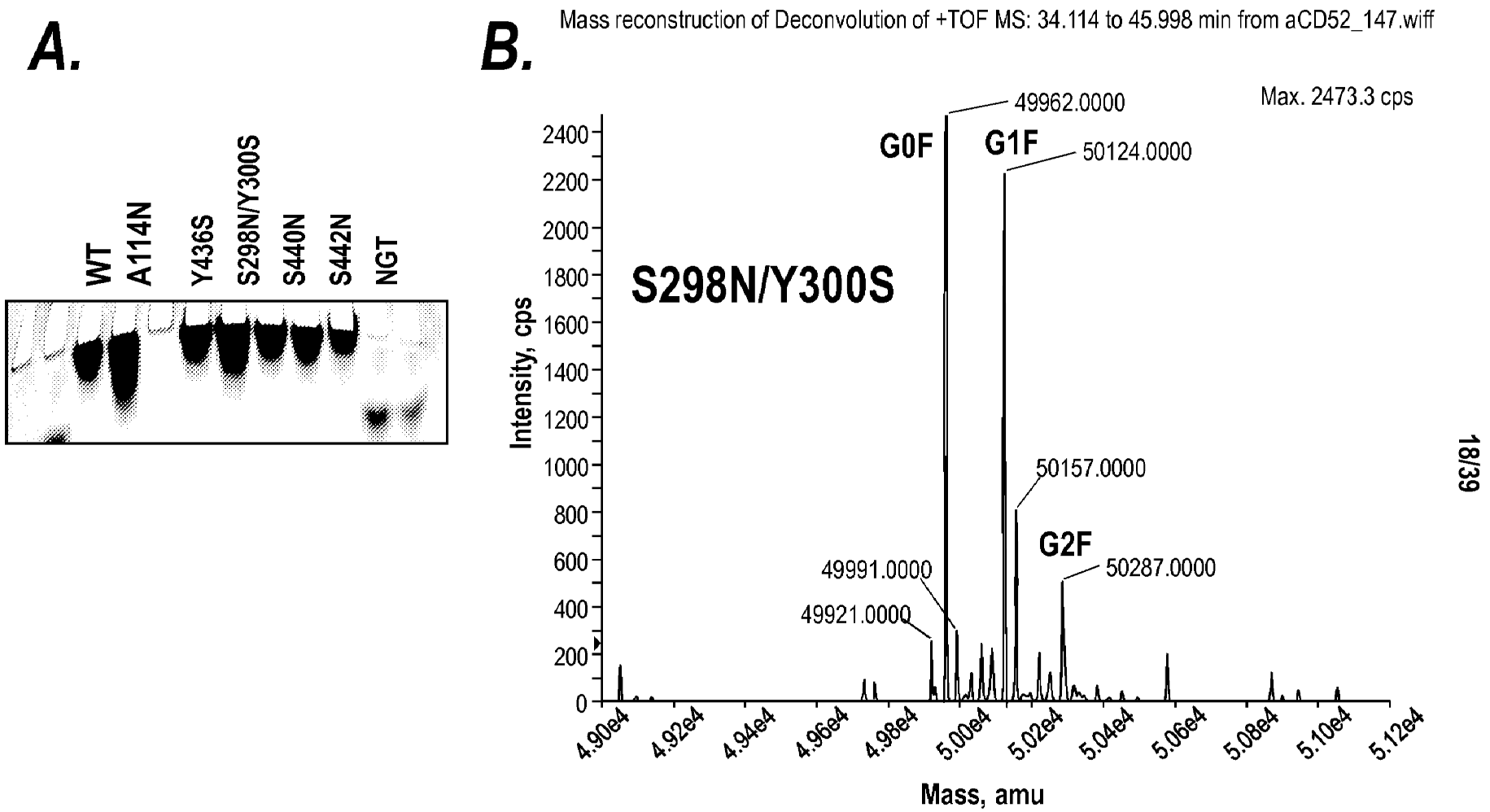

A.
WT
A114N
Y436S
S298N/Y300S
S440N
S442N
NGT
B.
Mass reconstruction of Deconvolution of +TOF MS: 34.114 to 45.998 min from aCD52_147.wiff
Max. 2473.3 cps
S298N/Y300S
G0F
G1F
G2F
49962.0000
50124.0000
50157.0000
50287.0000
49991.0000
49921.0000
Intensity, cps
Mass, amu

Fig. 18

C.

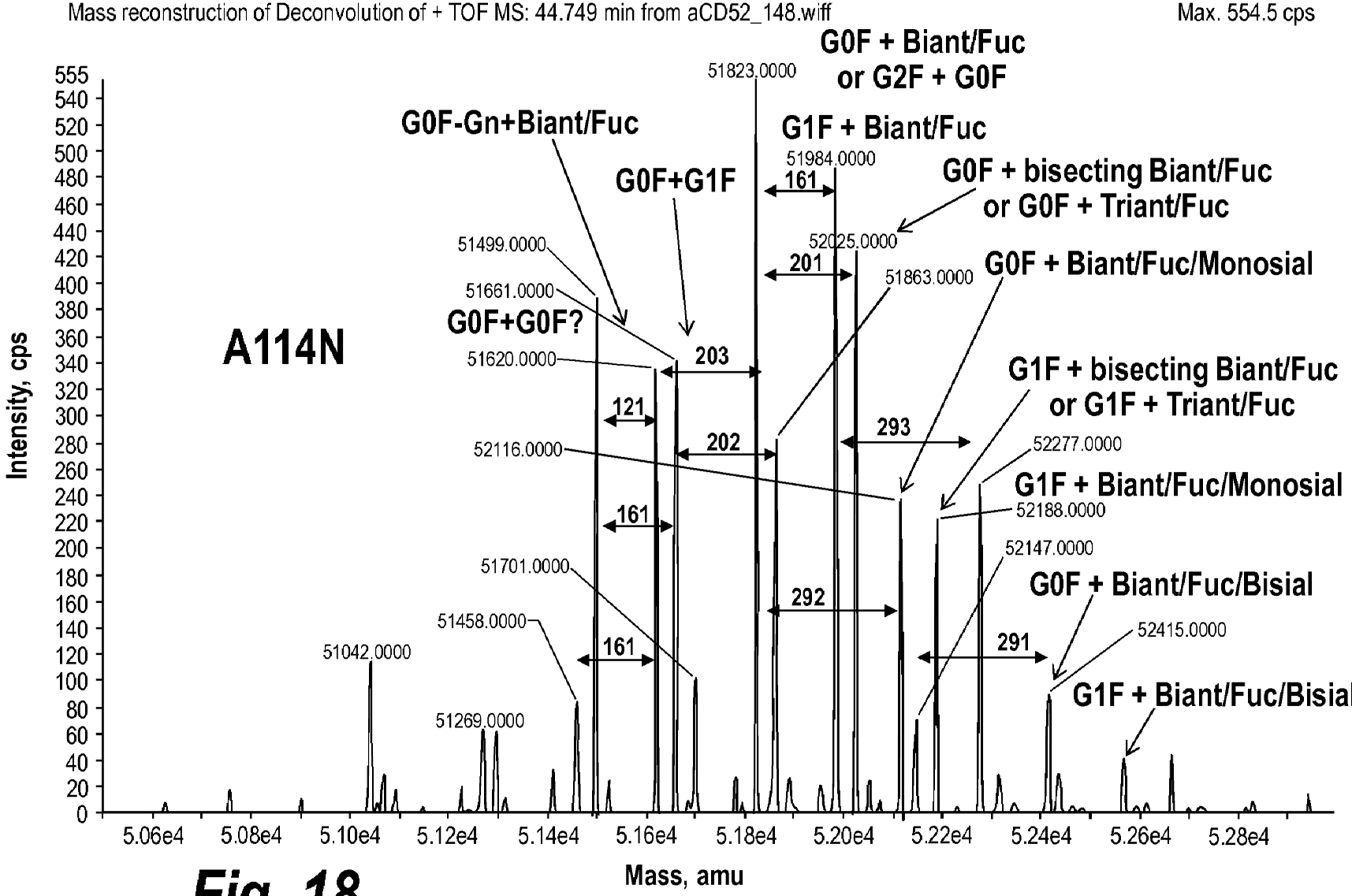

Mass reconstruction of Deconvolution of + TOF MS: 44.749 min from aCD52_148.wiff
Max. 554.5 cps
A114N
Intensity, cps
Mass, amu
G0F + Biant/Fuc
or G2F + G0F
51823.0000
G0F-Gn+Biant/Fuc
G1F + Biant/Fuc
51984.0000
G0F+G1F
161
G0F + bisecting Biant/Fuc
or G0F + Triant/Fuc
52025.0000
51499.0000
201
51863.0000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

Fig. 18

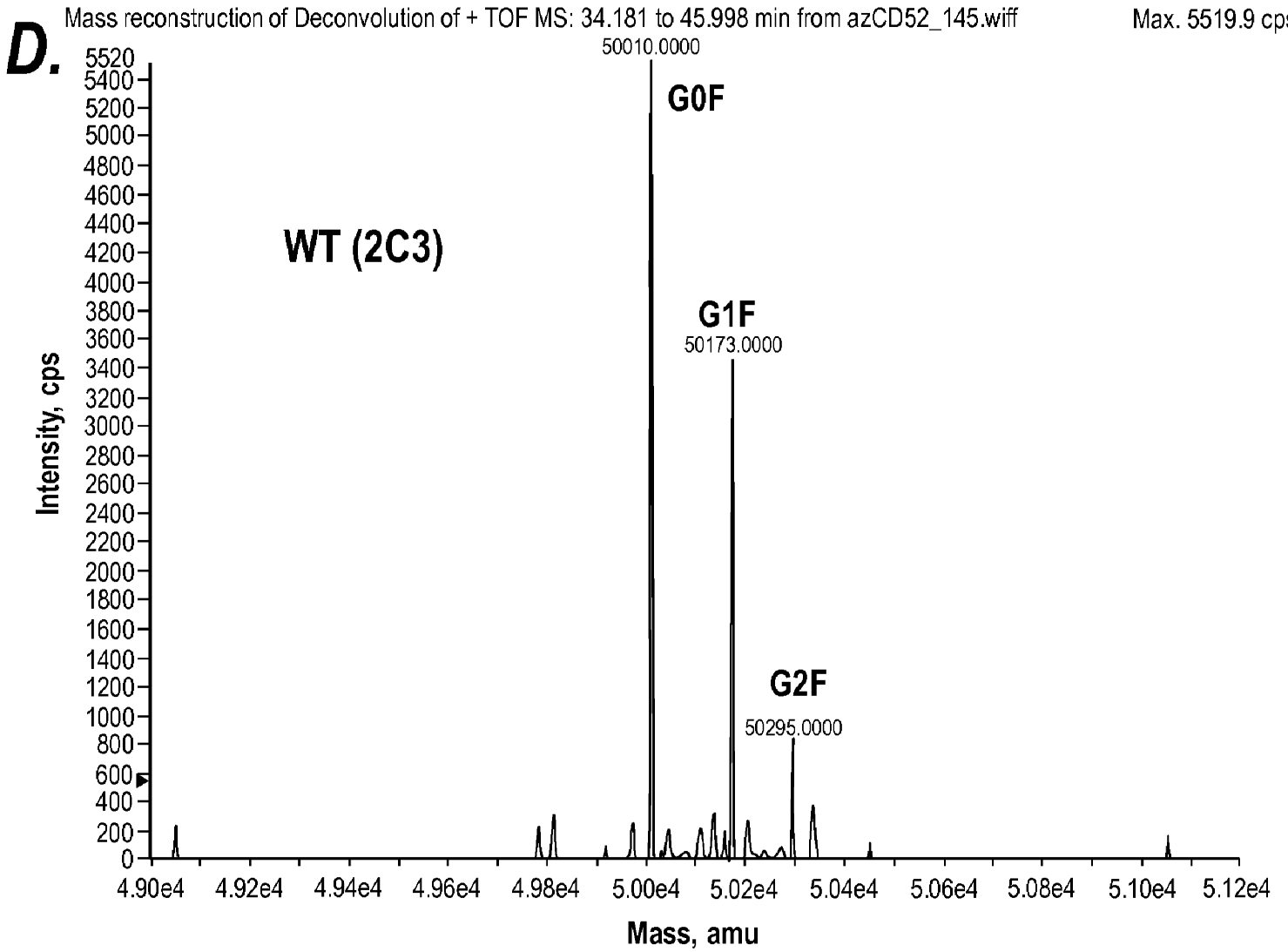

D.
Mass reconstruction of Deconvolution of + TOF MS: 34.181 to 45.998 min from azCD52_145.wiff
Max. 5519.9 cps
50010.0000
G0F
WT (2C3)
G1F
50173.0000
G2F
50295.0000
Intensity, cps
Mass, amu
5520
5400
5200
5000
4800
4600
4400
4200
4000
3800
3600
3400
3200
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
4.90e4
4.92e4
4.94e4
4.96e4
4.98e4
5.00e4
5.02e4
5.04e4
5.06e4
5.08e4
5.10e4
5.12e4

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.

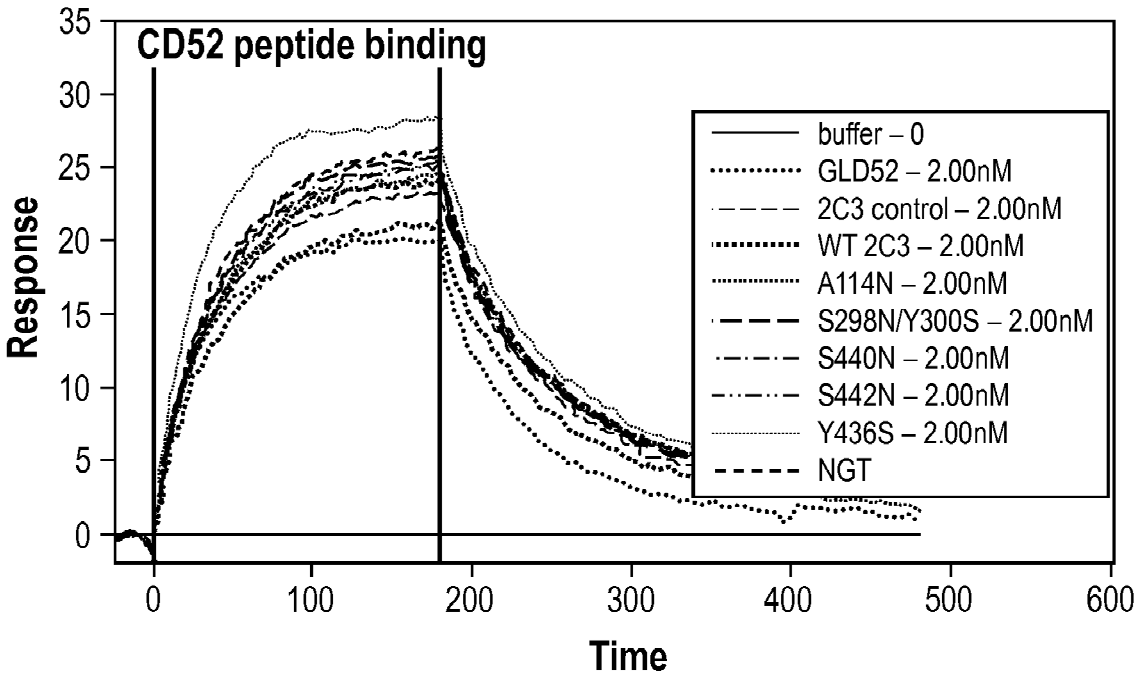


Fig. 19

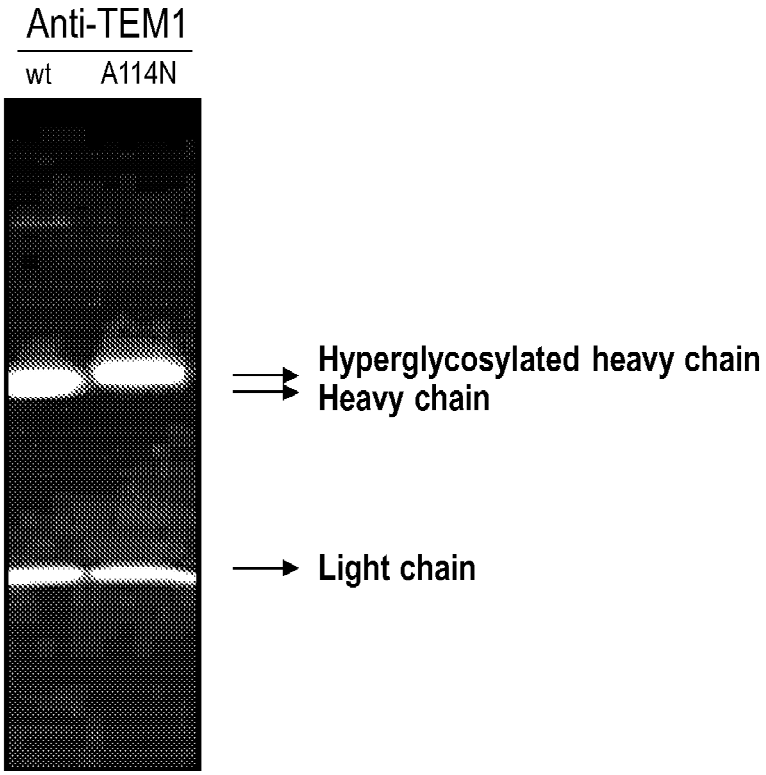

Anti-TEM1
wt
A114N
Hyperglycosylated heavy chain
Heavy chain
Light chain

Fig. 20

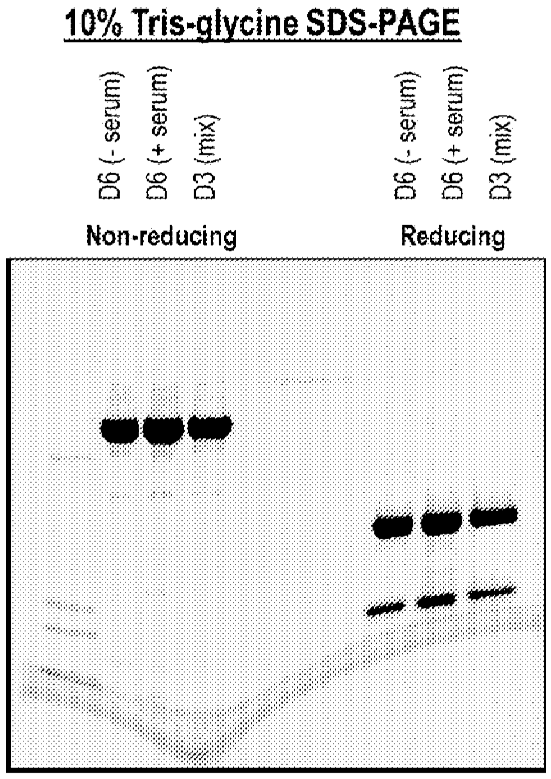
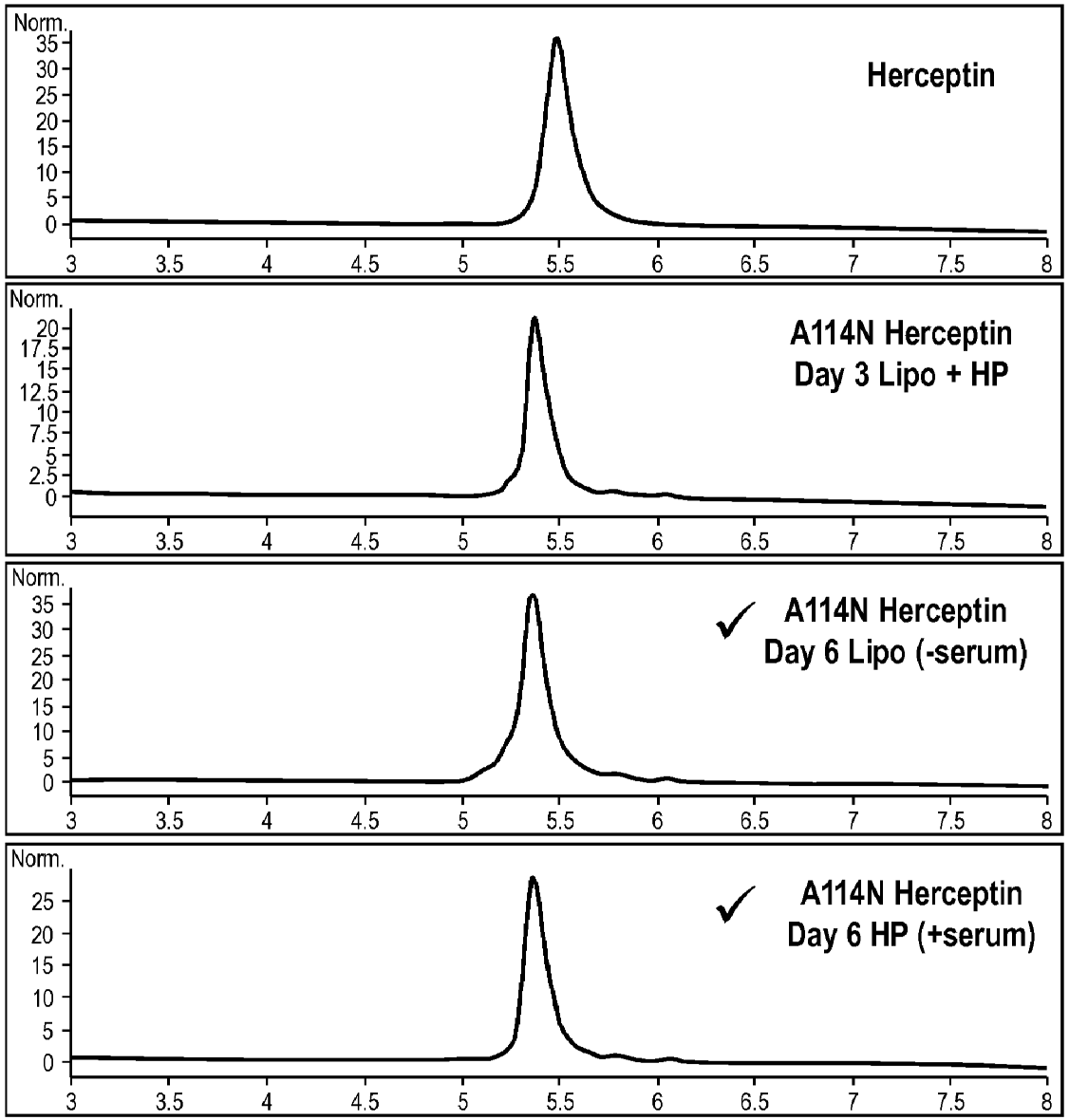

10% Tris-glycine SDS-PAGE
D6 (- serum)
D6 (+ serum)
D3 (mix)
D6 (- serum)
D6 (+ serum)
D3 (mix)
Non-reducing
Reducing

Fig. 21


HIC Analysis of Purified A114N Herceptin
Norm.
Herceptin
A114N Herceptin
Day 3 Lipo + HP
A114N Herceptin
Day 6 Lipo (-serum)
A114N Herceptin
Day 6 HP (+serum)
3
3.5
4
4.5
5
5.5
6
6.5
7
7.5
8

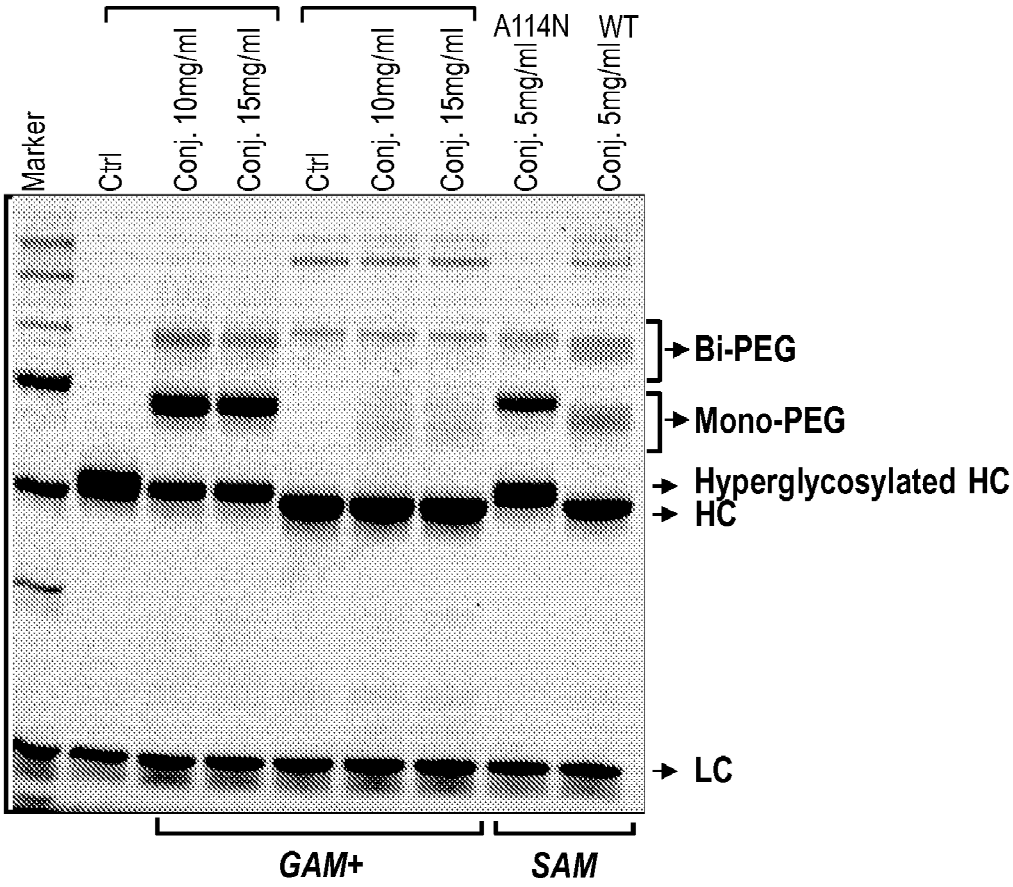

Marker
Ctrl
Conj. 10mg/ml
Conj. 15mg/ml
Ctrl
Conj. 10mg/ml
Conj. 15mg/ml
A114N
Conj. 5mg/ml
WT
Conj. 5mg/ml
Bi-PEG
Mono-PEG
Hyperglycosylated HC
HC
LC
GAM+
SAM

Fig. 22

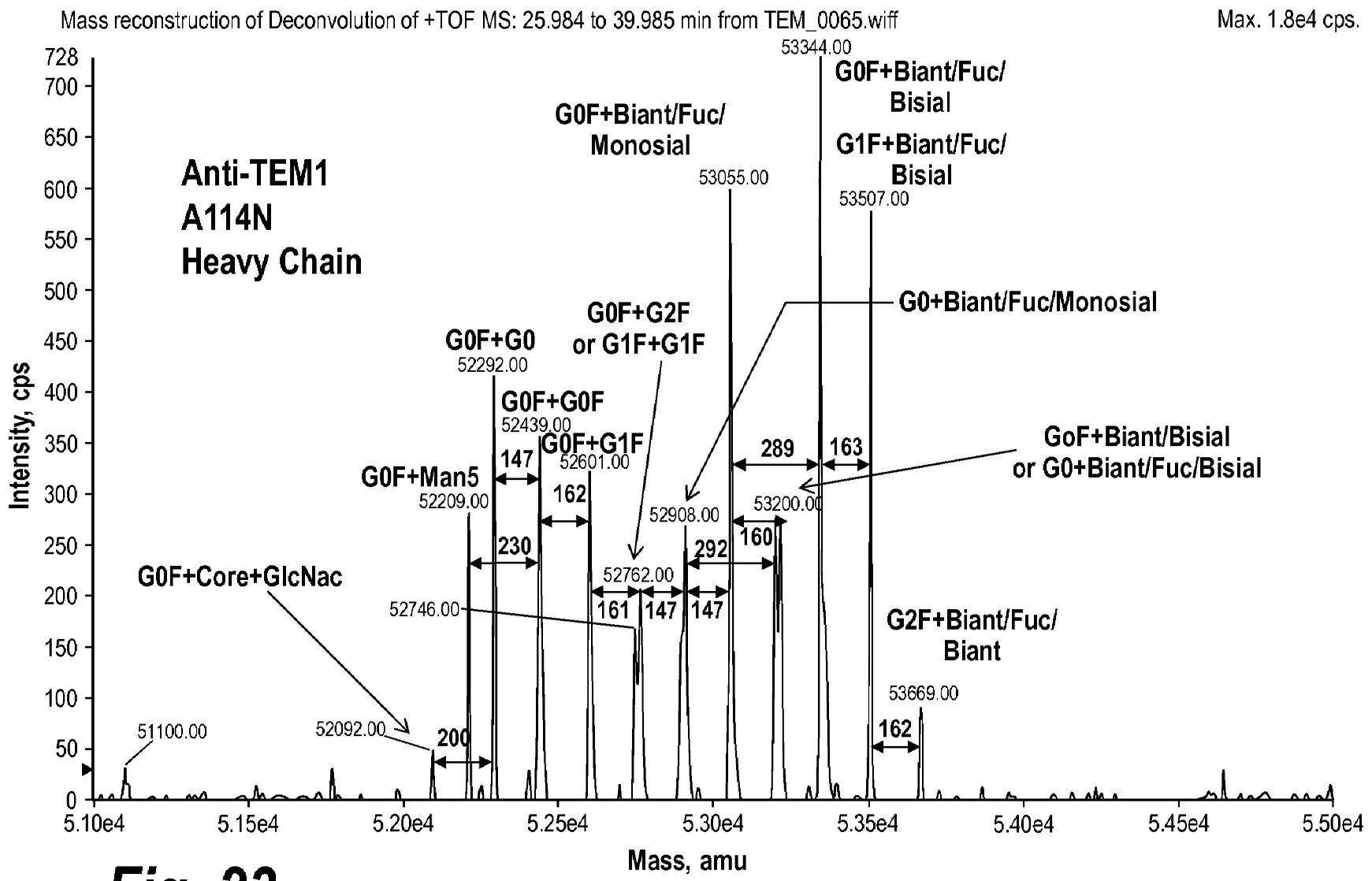

Mass reconstruction of Deconvolution of +TOF MS: 25.984 to 39.985 min from TEM_0065.wiff
Max. 1.8e4 cps.
Anti-TEM1
A114N
Heavy Chain
G0F+Biant/Fuc/
Bisial
53344.00
G0F+Biant/Fuc/
Monosial
G1F+Biant/Fuc/
Bisial
53055.00
53507.00
G0+Biant/Fuc/Monosial
G0F+G2F
or G1F+G1F
G0F+G0
52292.00
G0F+G0F
52439.00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

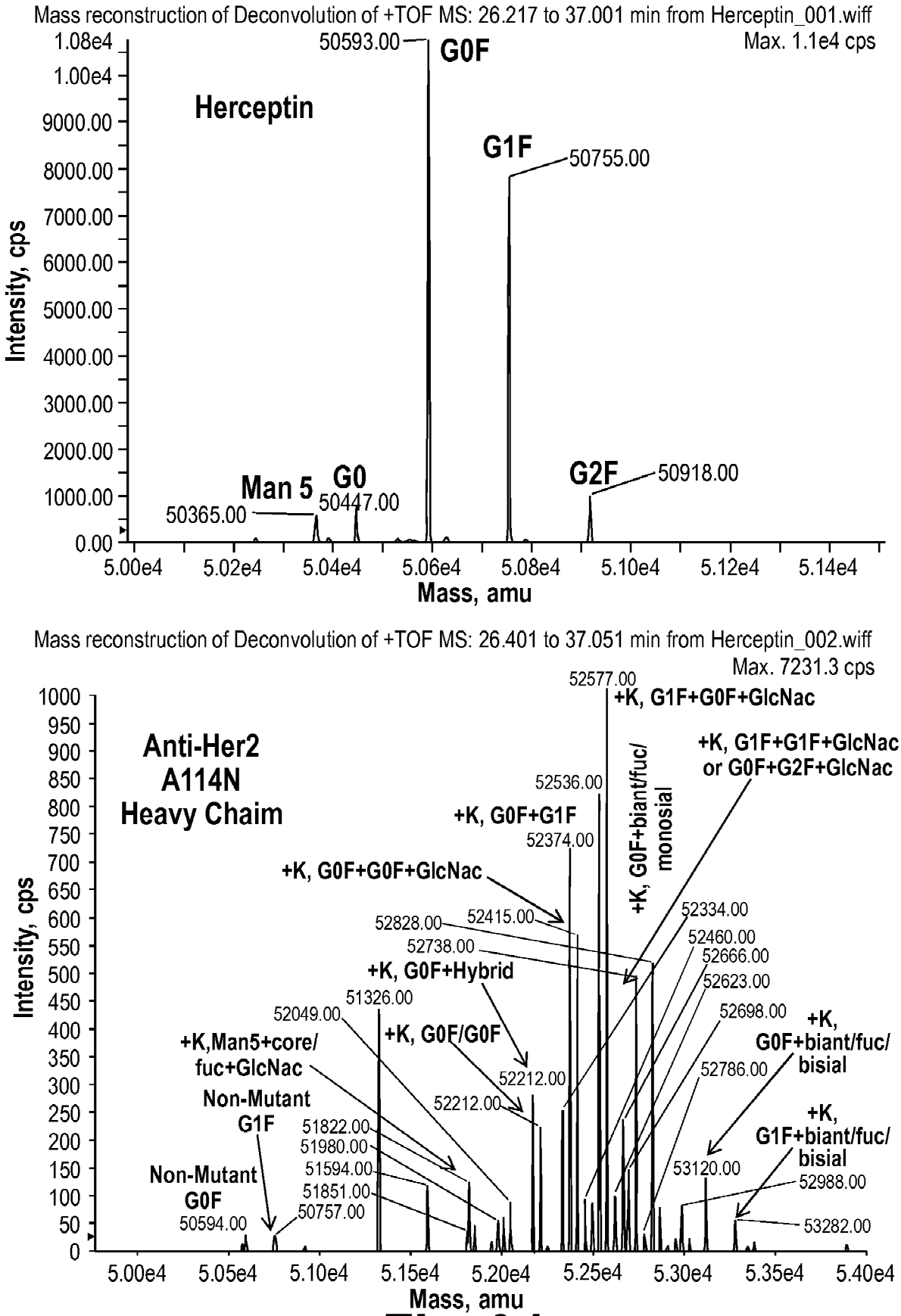

Mass reconstruction of Deconvolution of +TOF MS: 26.217 to 37.001 min from Herceptin_001.wiff
Max. 1.1e4 cps
Herceptin
50593.00
G0F
G1F
50755.00
Man 5
G0
50365.00
50447.00
G2F
50918.00
Intensity, cps
Mass, amu
Mass reconstruction of Deconvolution of +TOF MS: 26.401 to 37.051 min from Herceptin_002.wiff
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.

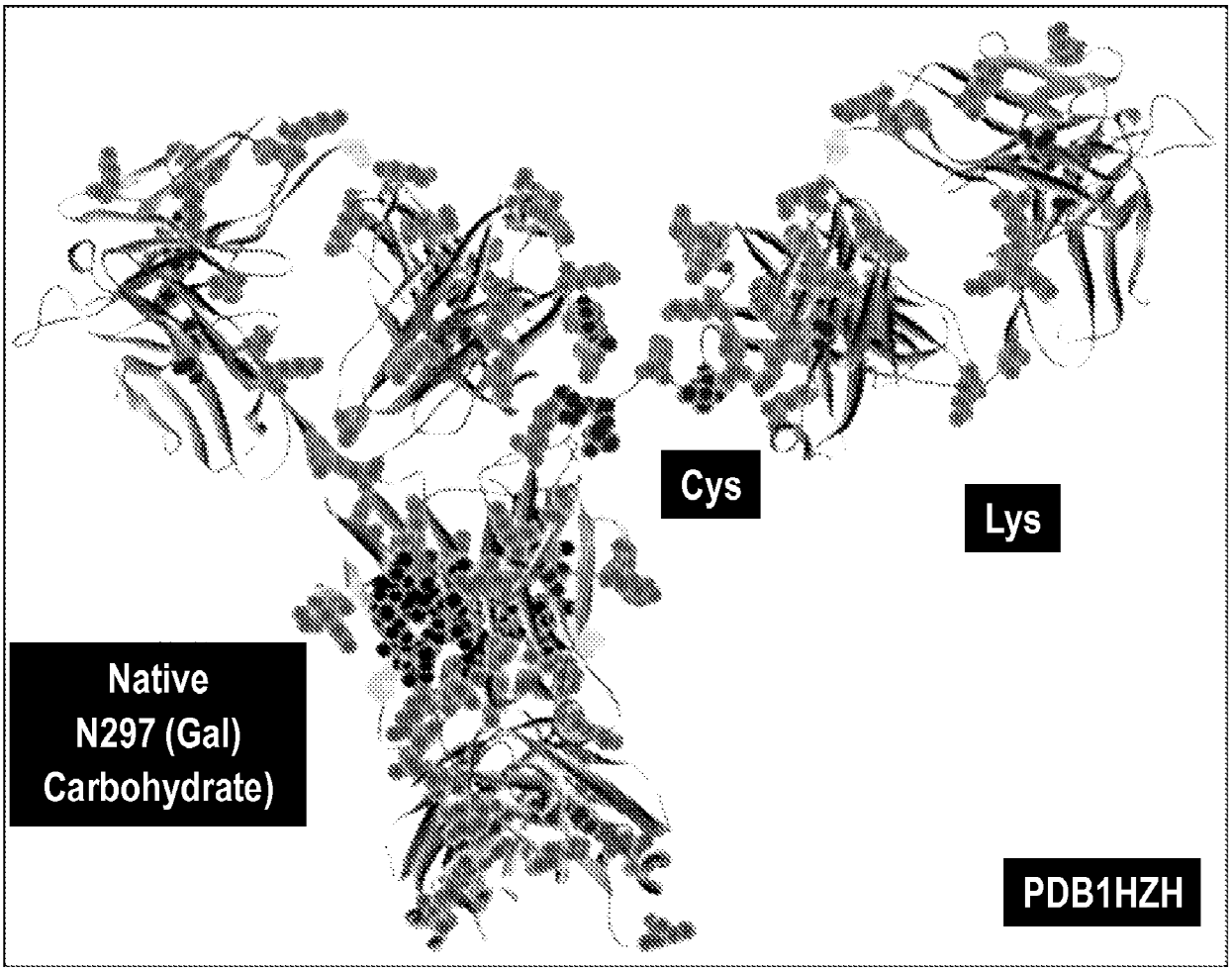
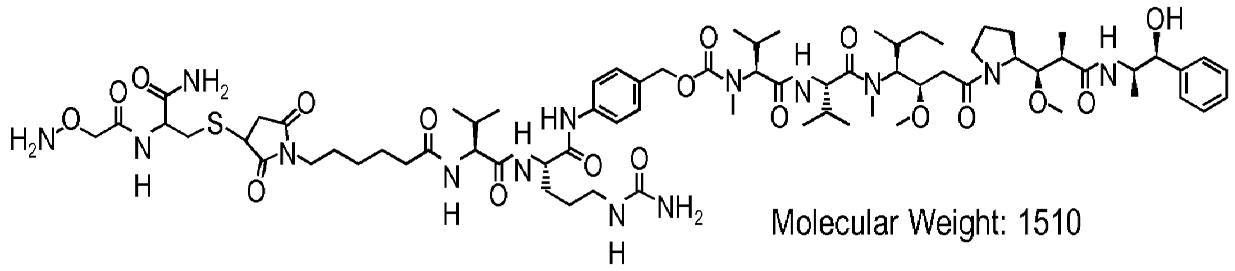


Fig. 25

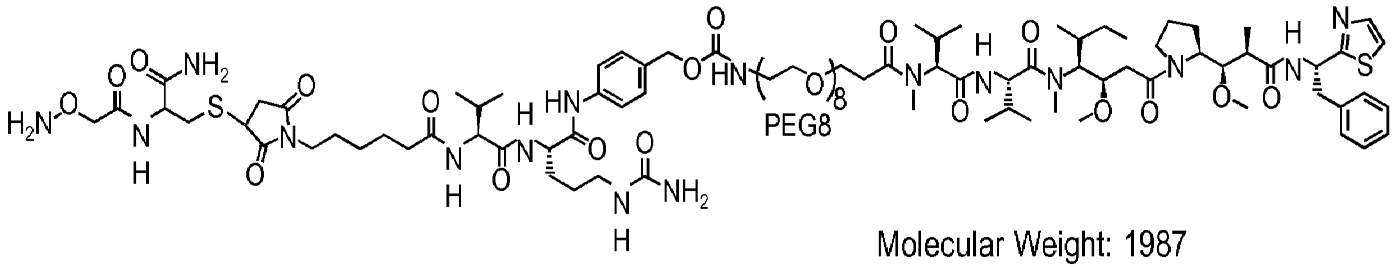
B.

Aminooxy-Cys-MC-VC-PABC-MMAE



Molecular Weight: 1510

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



Molecular Weight: 1987

Fig. 25

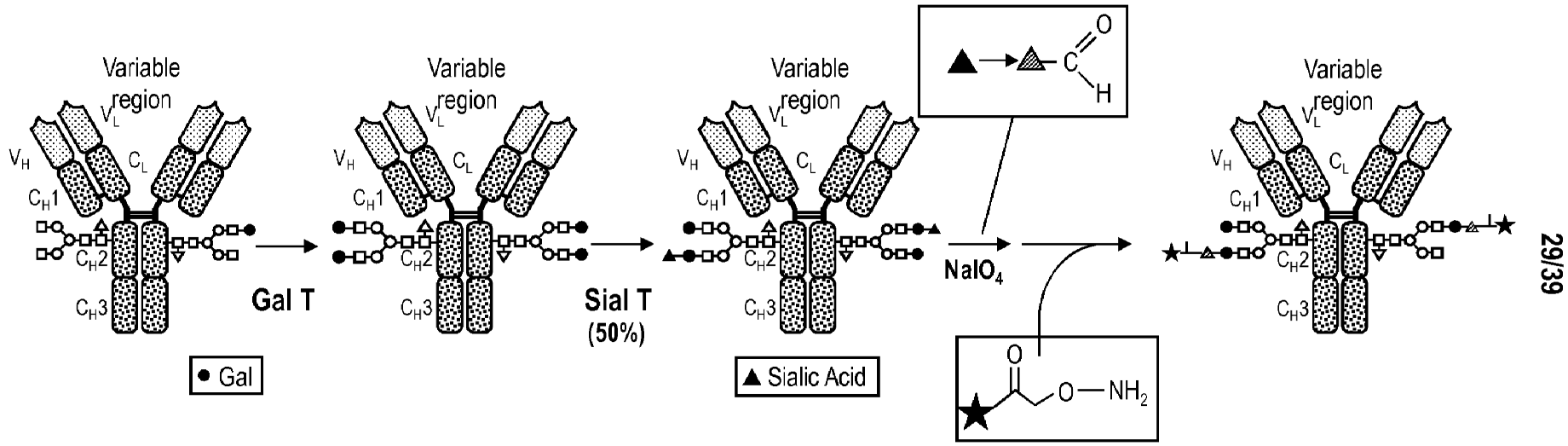

C.
Variable region
V_L
V_H
C_L
C_H1
C_H2
C_H3
Gal T
Sial T
(50%)
$NaIO_4$
O
C
H
O
O—NH_2
● Gal
▲ Sialic Acid

Fig. 25

Synthesis of aminooxy-Cys-MC-VC-PABC-MMAE and aminooxy-Cys-MC-VC-PABC-PEG8-Dol10

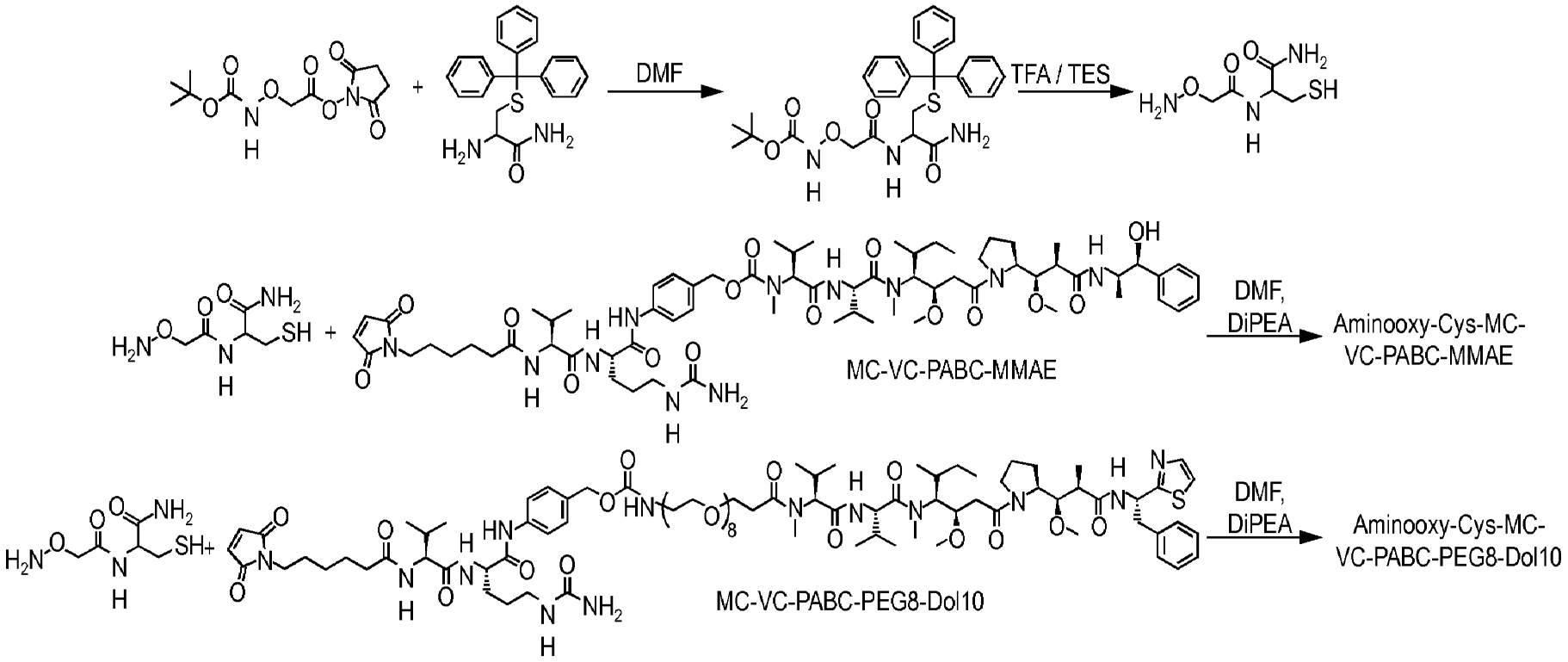
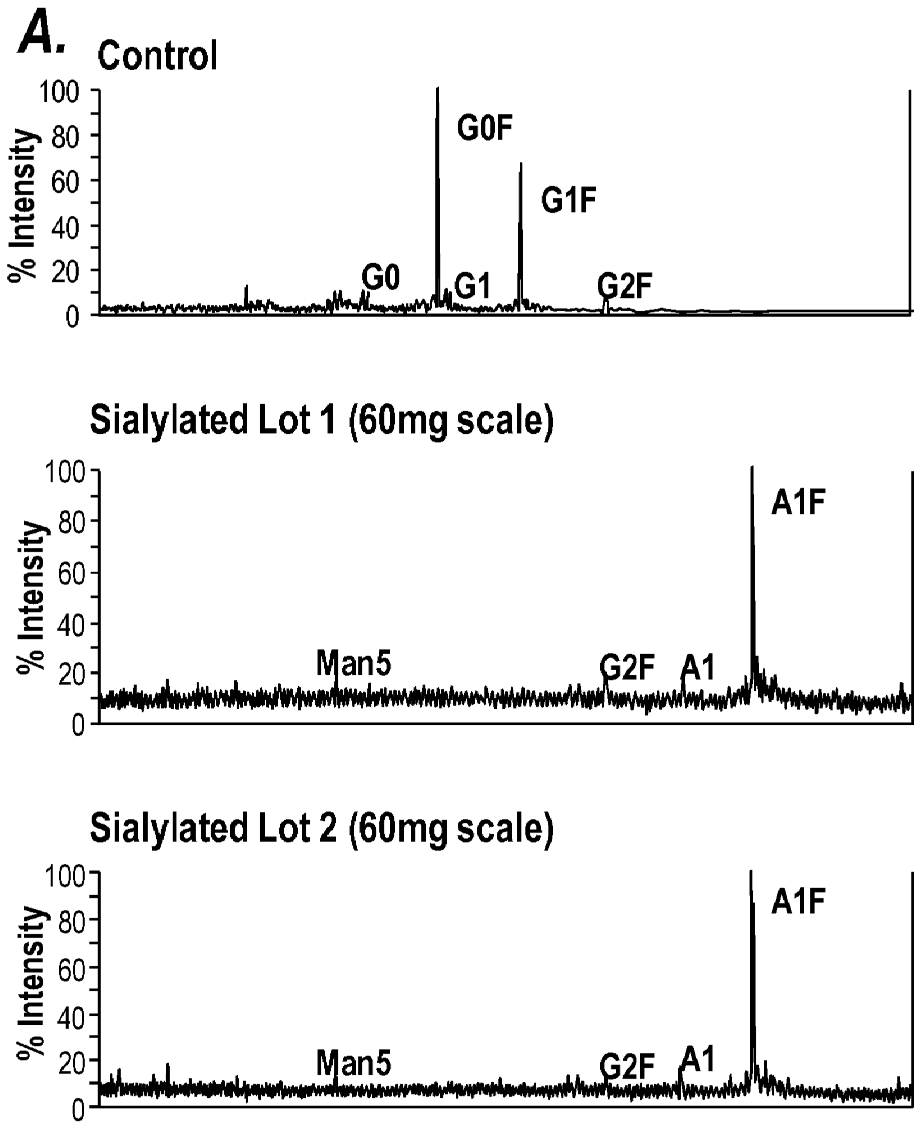


Fig. 26



B.

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

C.

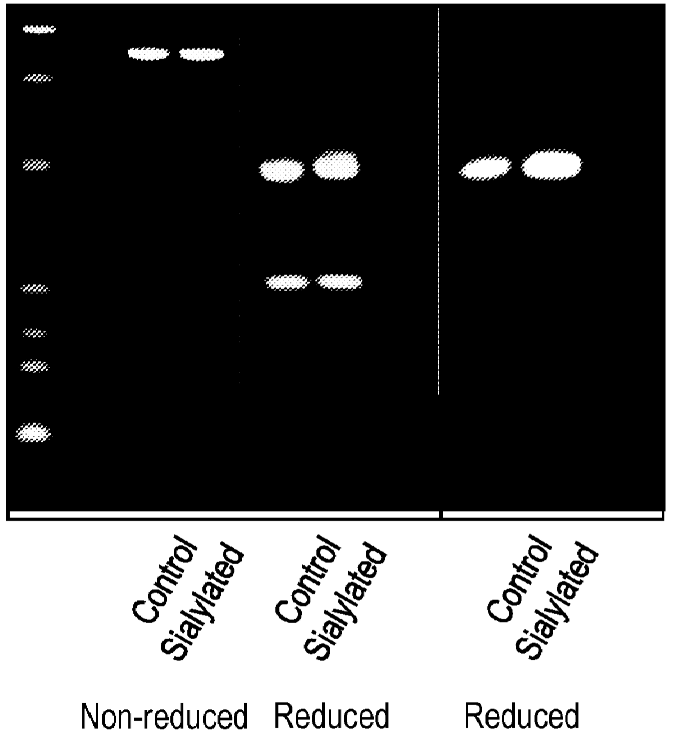


Fig. 27

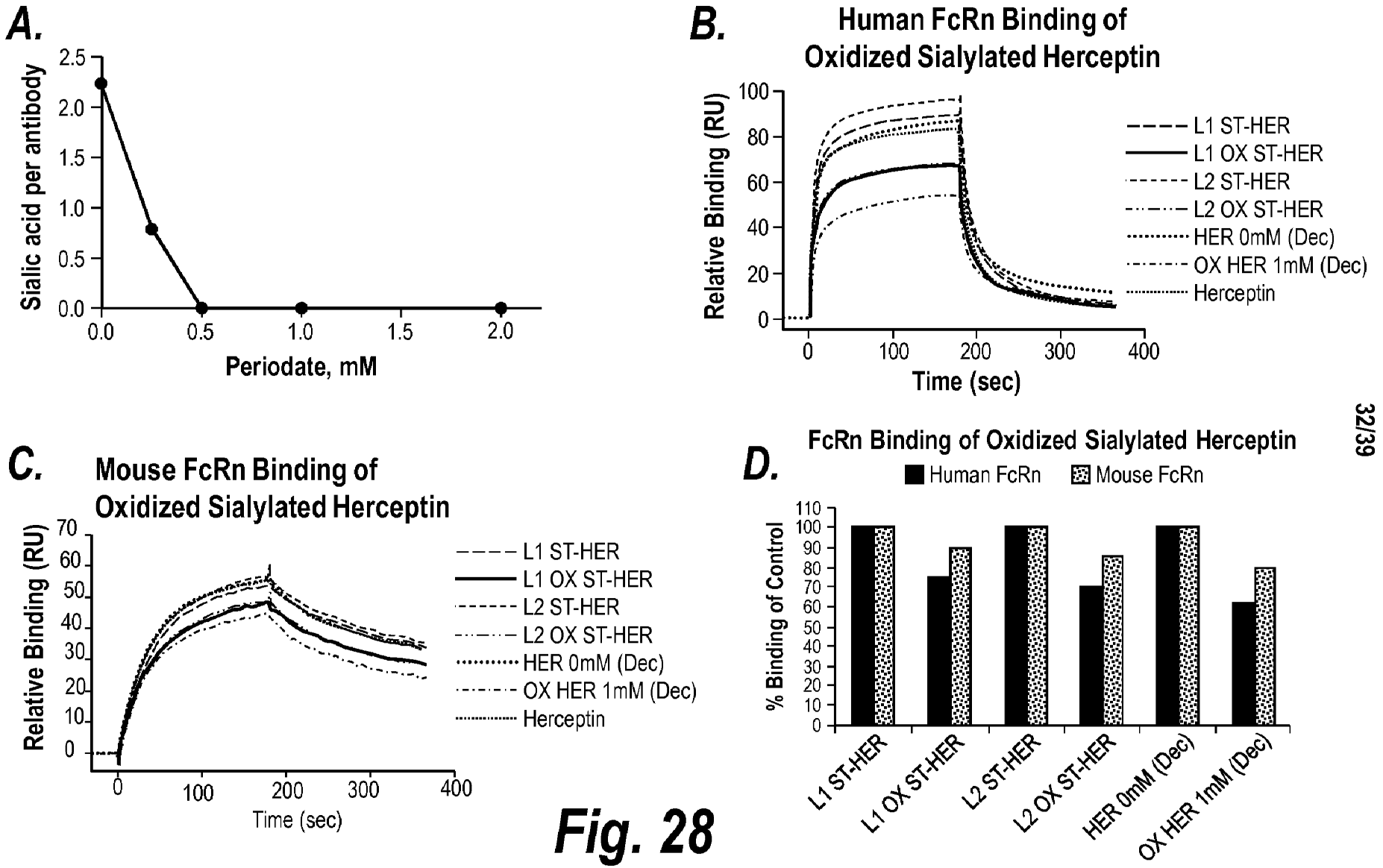

A.
Sialic acid per antibody
2.5
2.0
1.5
1.0
0.5
0.0
0.0
0.5
1.0
1.5
2.0
Periodate, mM
B.
Human FcRn Binding of
Oxidized Sialylated Herceptin
Relative Binding (RU)
100
80
60
40
20
0
0
100
200
300
400
Time (sec)
L1 ST-HER
L1 OX ST-HER
L2 ST-HER
L2 OX ST-HER
HER 0mM (Dec)
OX HER 1mM (Dec)
Herceptin
C.
Mouse FcRn Binding of
Oxidized Sialylated Herceptin
Relative Binding (RU)
70
60
50
40
30
20
10
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
D.
FcRn Binding of Oxidized Sialylated Herceptin
Human FcRn
Mouse FcRn
% Binding of Control
110
100
90
80
70
60
50
40
30
20
10
0
L1 ST-HER
L1 OX ST-HER
L2 ST-HER
L2 OX ST-HER
HER 0mM (Dec)
OX HER 1mM (Dec)

Fig. 28

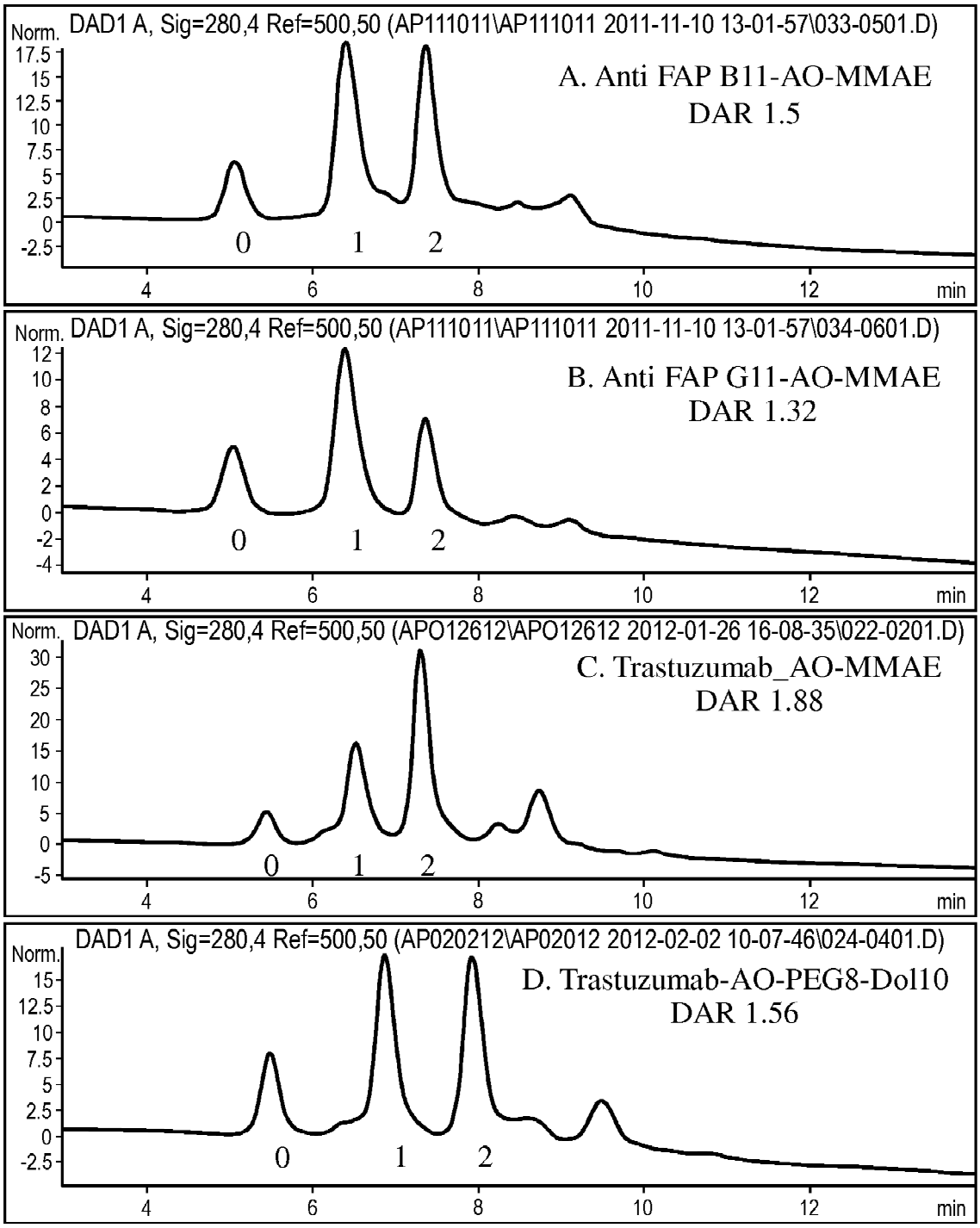
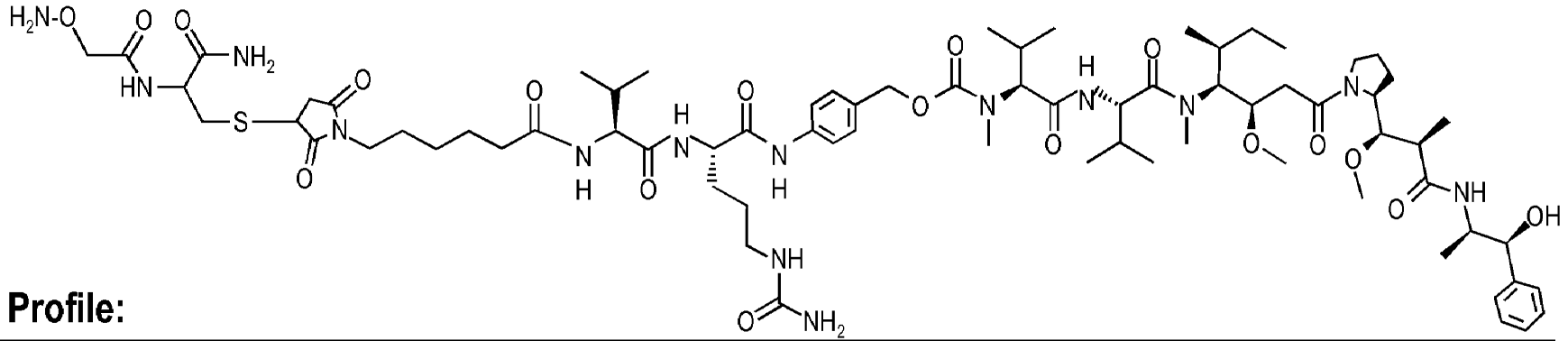

Norm.
DAD1 A, Sig=280,4 Ref=500,50 (AP111011\AP111011 2011-11-10 13-01-57\033-0501.D)
A. Anti FAP B11-AO-MMAE
DAR 1.5
0
1
2
min
Norm.
DAD1 A, Sig=280,4 Ref=500,50 (AP111011\AP111011 2011-11-10 13-01-57\034-0601.D)
B. Anti FAP G11-AO-MMAE
DAR 1.32
0
1
2
min
Norm.
DAD1 A, Sig=280,4 Ref=500,50 (APO12612\APO12612 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:

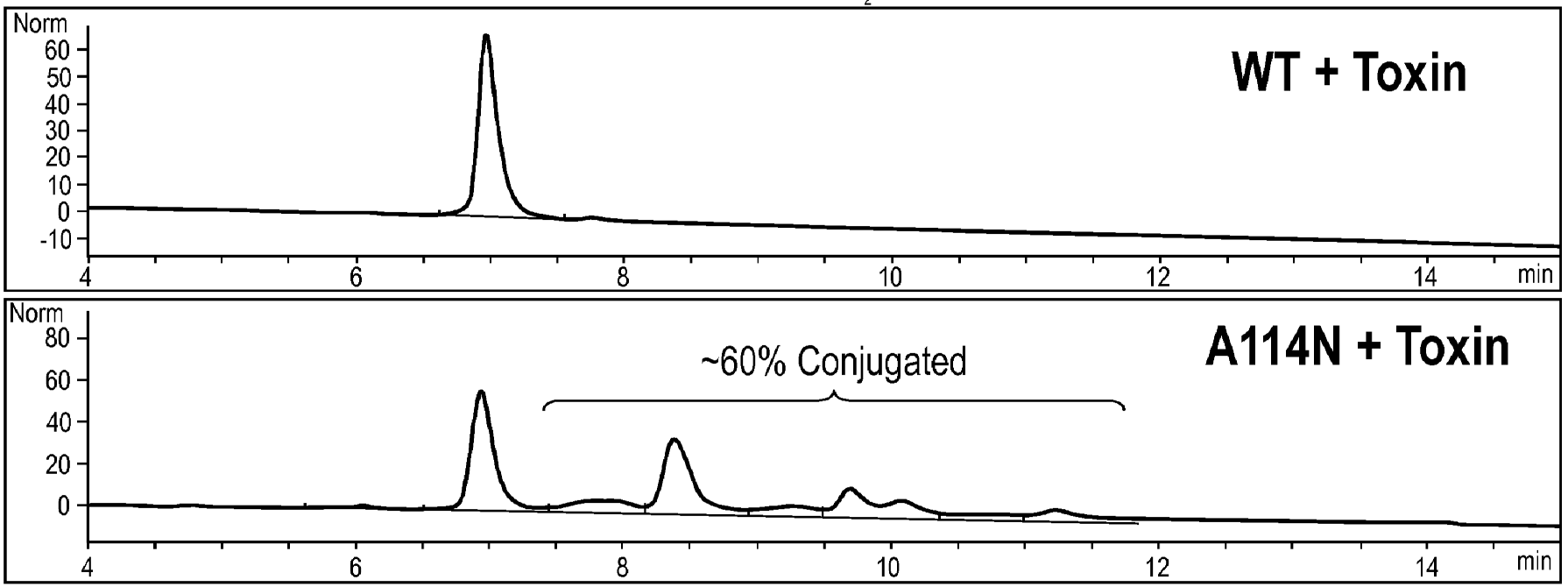


Fig. 30

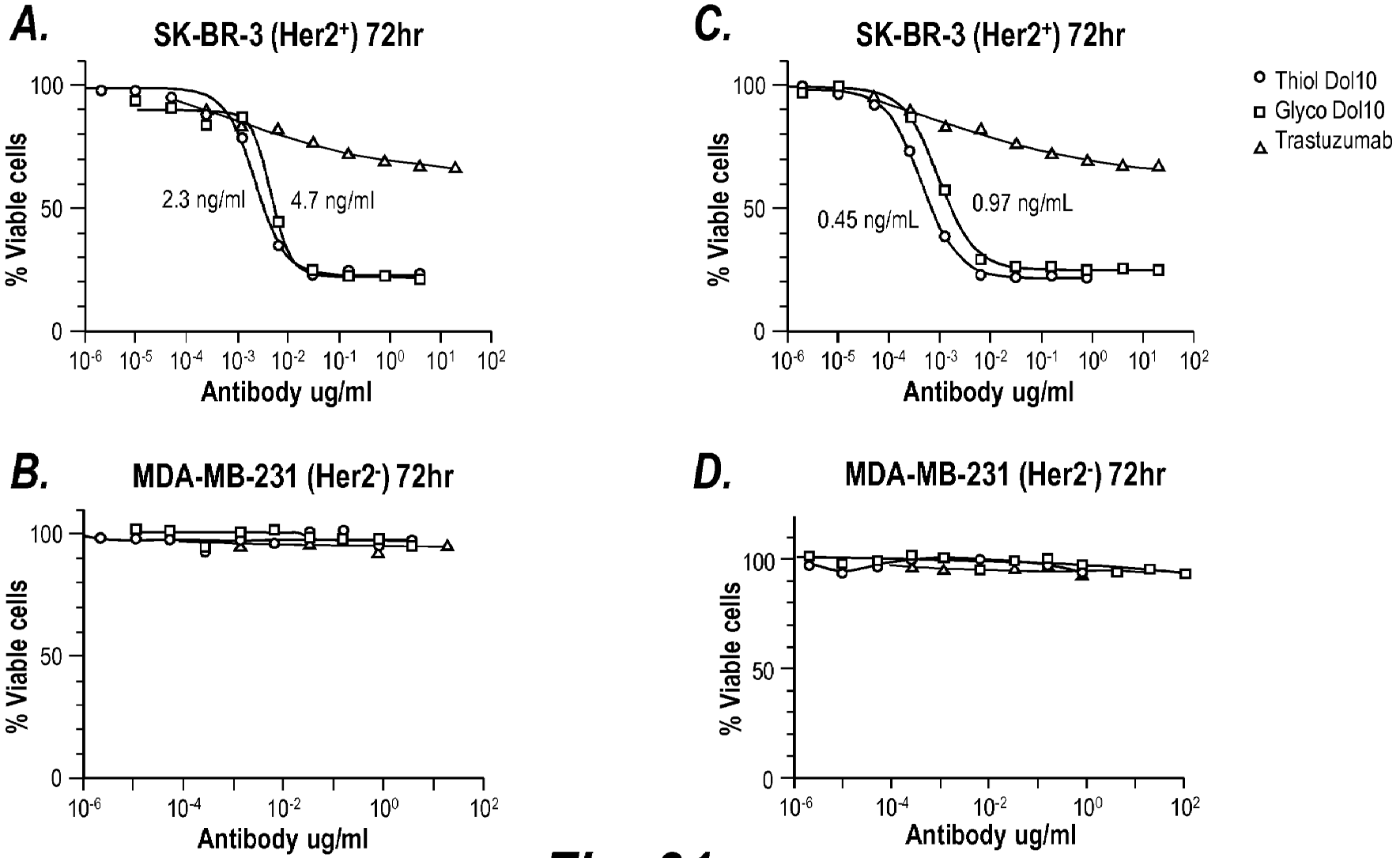

A.
SK-BR-3 (Her2+) 72hr
% Viable cells
Antibody ug/ml
2.3 ng/ml
4.7 ng/ml
B.
MDA-MB-231 (Her2-) 72hr
% Viable cells
Antibody ug/ml
C.
SK-BR-3 (Her2+) 72hr
% Viable cells
Antibody ug/ml
0.45 ng/mL
0.97 ng/mL
Thiol Dol10
Glyco Dol10
Trastuzumab
D.
MDA-MB-231 (Her2-) 72hr
% Viable cells
Antibody ug/ml

Fig. 31

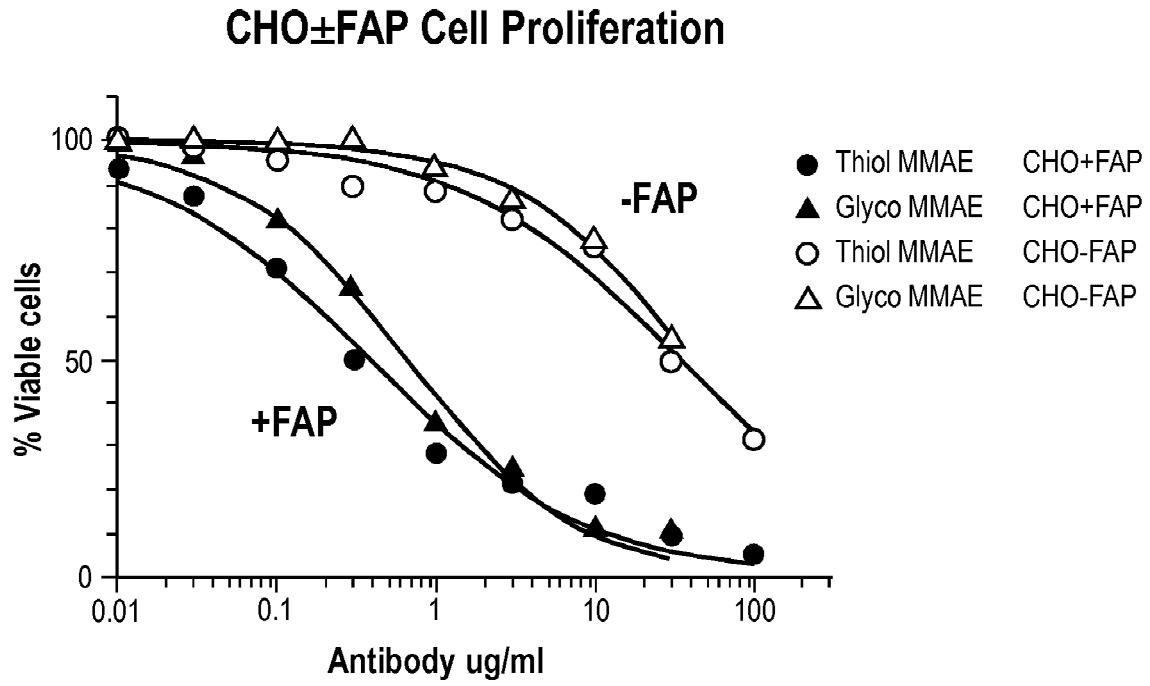

CHO±FAP Cell Proliferation
% Viable cells
100
50
0
0.01
0.1
1
10
100
Antibody ug/ml
-FAP
+FAP
Thiol MMAE CHO+FAP
Glyco MMAE CHO+FAP
Thiol MMAE CHO-FAP
Glyco MMAE CHO-FAP

Fig. 32

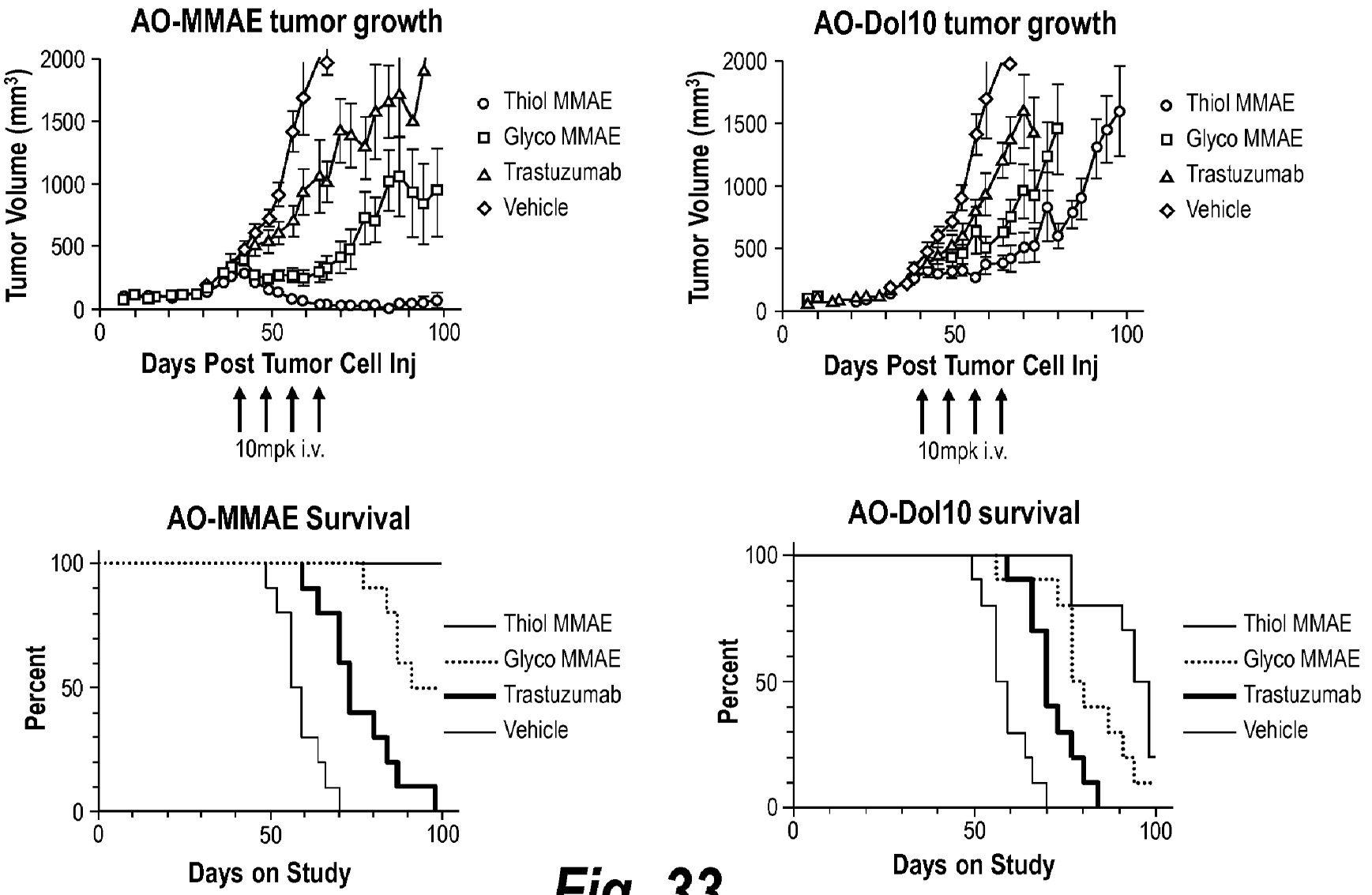

AO-MMAE tumor growth
Tumor Volume (mm3)
2000
1500
1000
500
0
0
50
100
Days Post Tumor Cell Inj
10mpk i.v.
Thiol MMAE
Glyco MMAE
Trastuzumab
Vehicle
AO-Dol10 tumor growth
Tumor Volume (mm3)
2000
1500
1000
500
0
0
50
100
Days Post Tumor Cell Inj
10mpk i.v.
Thiol MMAE
Glyco MMAE
Trastuzumab
Vehicle
AO-MMAE Survival
Percent
100
50
0
0
50
100
Days on Study
Thiol MMAE
Glyco MMAE
Trastuzumab
Vehicle
AO-Dol10 survival
Percent
100
50
0
0
50
100
Days on Study
Thiol MMAE
Glyco MMAE
Trastuzumab
Vehicle

Fig. 33

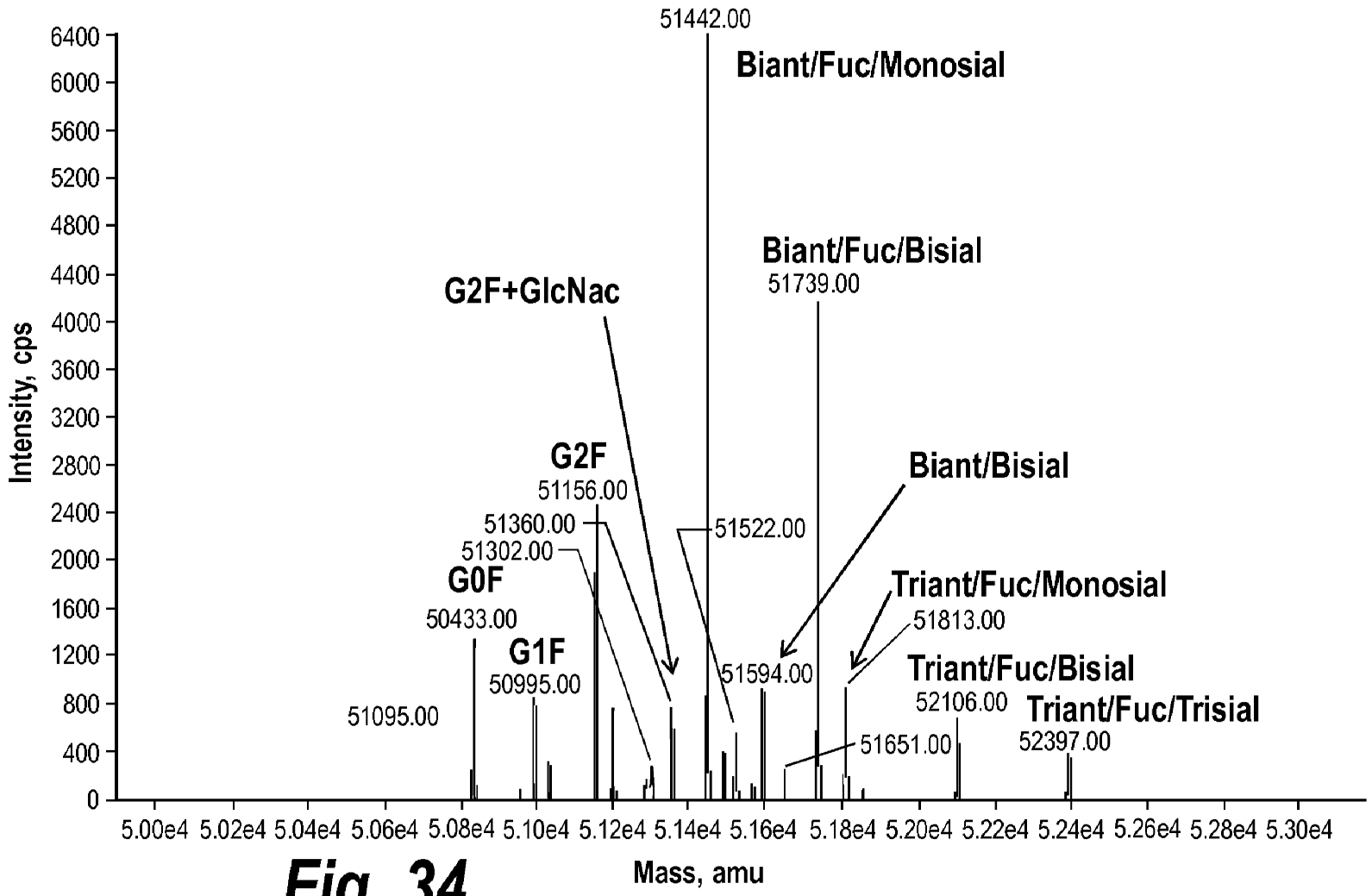

LC/MS of S298N/T299A/Y300S (NNAS) heavy chain
Intensity, cps
6400
6000
5600
5200
4800
4400
4000
3600
3200
2800
2400
2000
1600
1200
800
400
0
5.00e4 5.02e4 5.04e4 5.06e4 5.08e4 5.10e4 5.12e4 5.14e4 5.16e4 5.18e4 5.20e4 5.22e4 5.24e4 5.26e4 5.28e4 5.30e4
Mass, amu
51442.00
Biant/Fuc/Monosial
Biant/Fuc/Bisial
51739.00
G2F+GlcNac
G2F
51156.00
51360.00
51302.00
51522.00
Biant/Bisial
G0F
50433.00
G1F
50995.00
51095.00
Triant/Fuc/Monosial
51813.00
51594.00
Triant/Fuc/Bisial
52106.00
Triant/Fuc/Trisial
52397.00
51651.00

Fig. 34

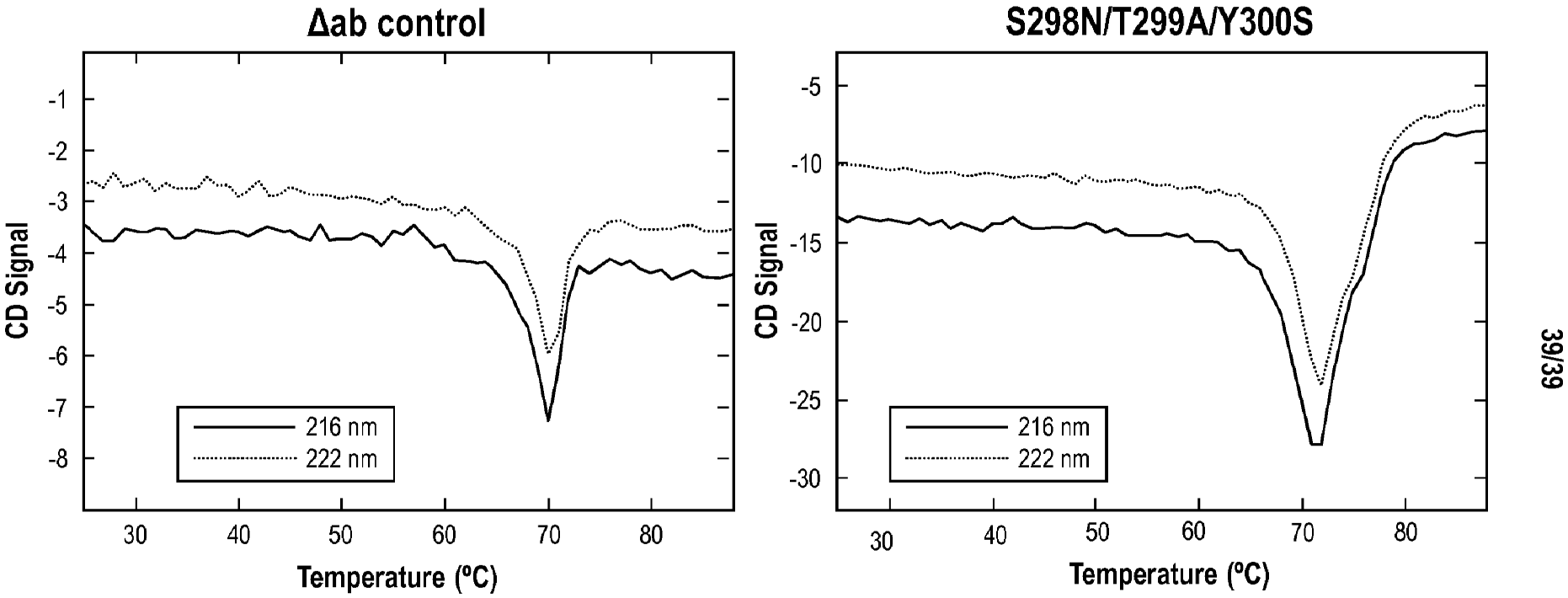

Δab control
S298N/T299A/Y300S
CD Signal
Temperature (ºC)
216 nm
222 nm
-1
-2
-3
-4
-5
-6
-7
-8
-10
-15
-20
-25
-30
30
40
50
60
70
80

Fig. 35