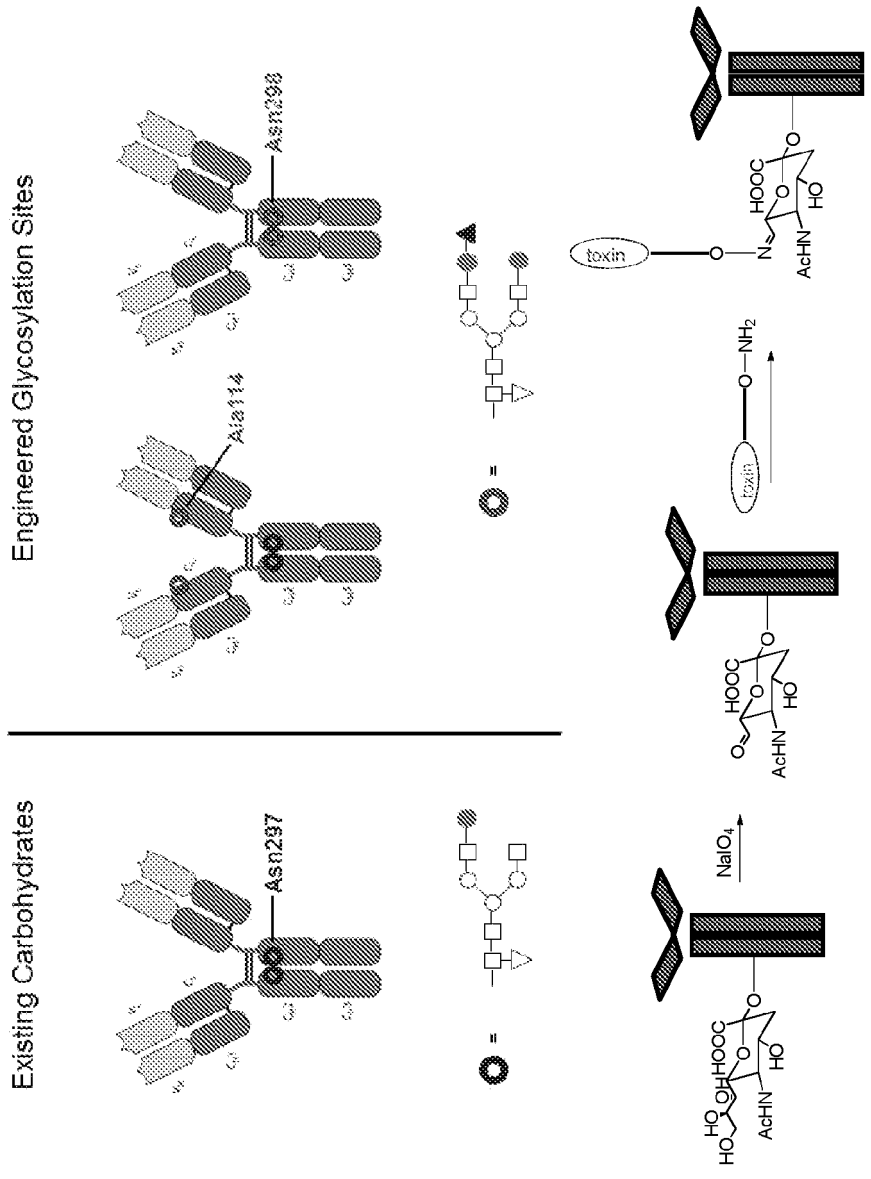

Engineered Glycosylation Sites
Existing Carbohydrates
Asn398
Ala114
Asn297
toxin
NaIO4
NH2
AcHN
HOOC
HO

Figure 1

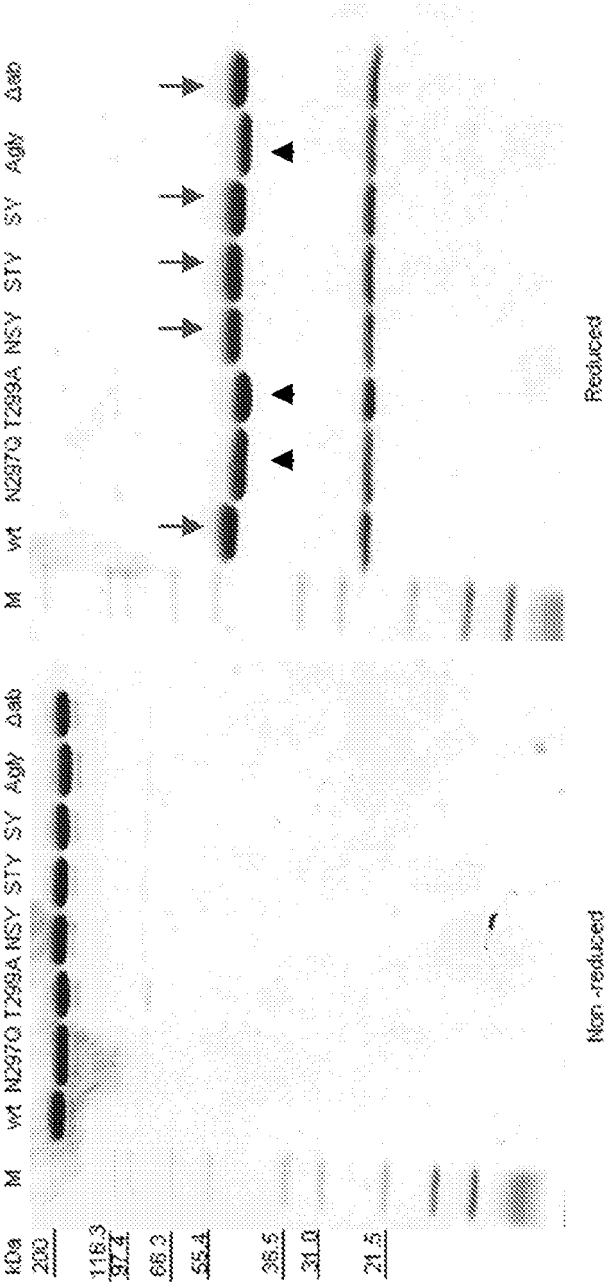

kDa
M
wt
N297Q
T299A
NSY
STY
SY
Agly
Δab
200
116.3
97.4
66.2
55.4
36.5
31.0
21.5
Non-reduced
Reduced

Figure 2

Figure 3

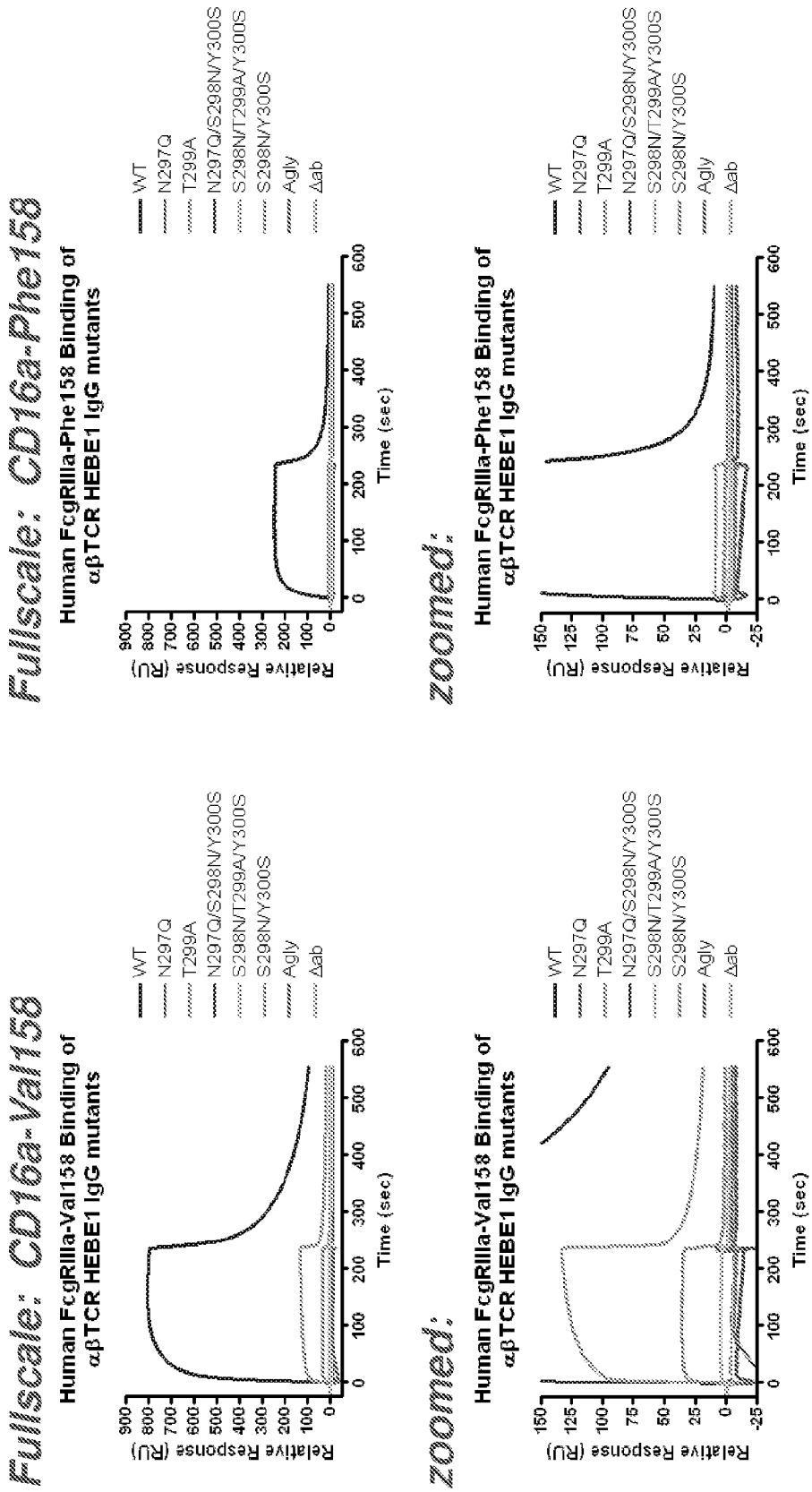

Fullscale: CD16a-Val158
Human FcgRIIIa-Val158 Binding of αβTCR HEBE1 IgG mutants
Relative Response (RU)
Time (sec)
WT
N297Q
T299A
N297Q/S298N/Y300S
S298N/T299A/Y300S
S298N/Y300S
Agly
Δab
Fullscale: CD16a-Phe158
Human FcgRIIIa-Phe158 Binding of αβTCR HEBE1 IgG mutants
Relative Response (RU)
Time (sec)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

Figure 4

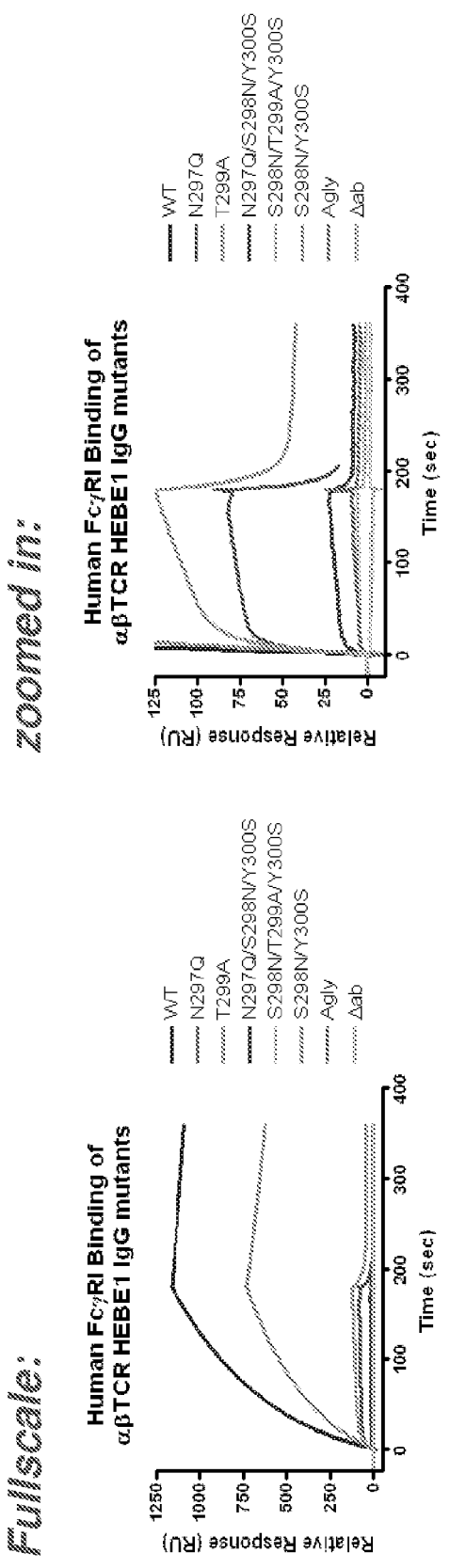
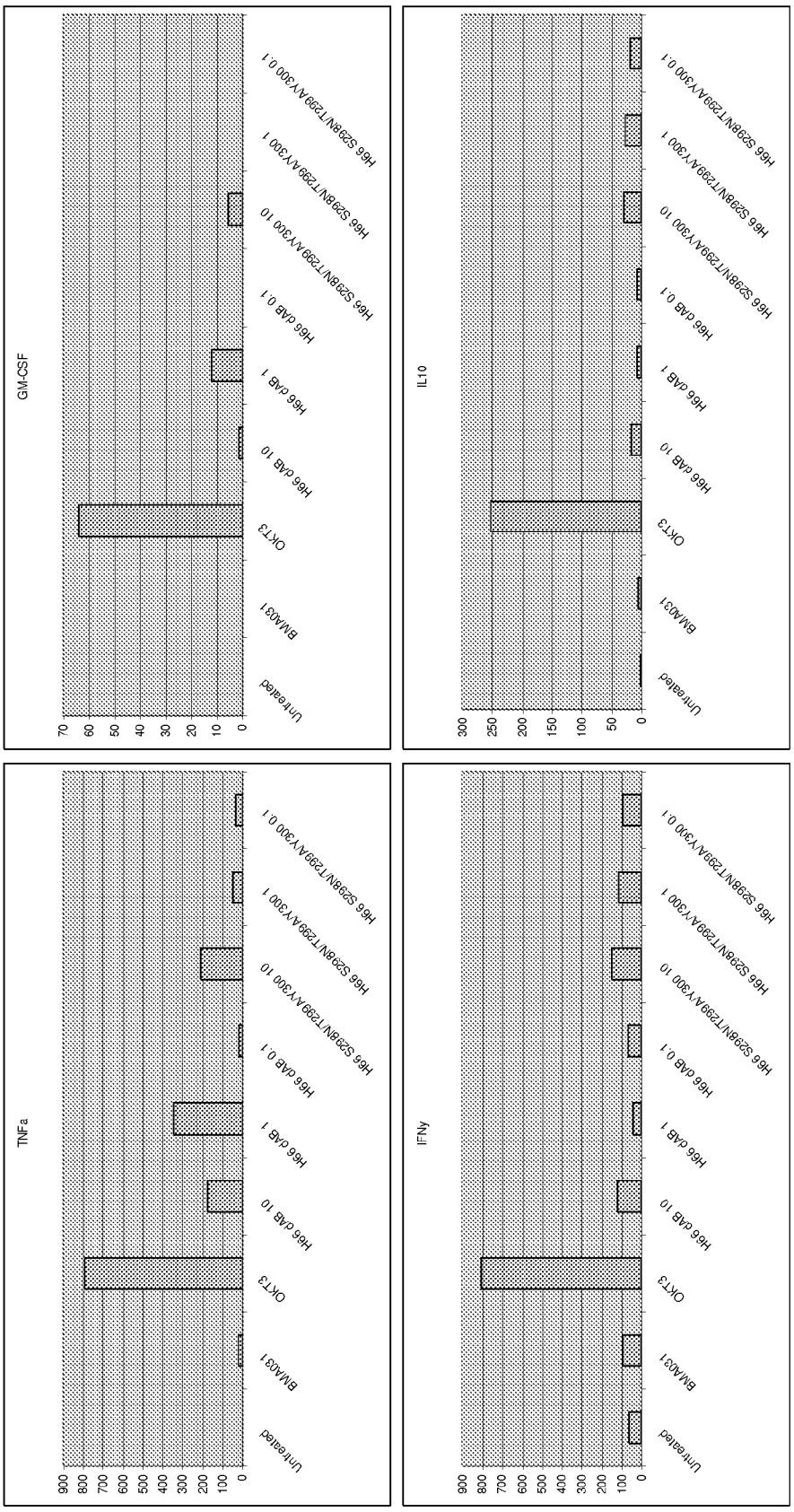

Fullscale:
Human FcγRI Binding of αβTCR HEBE1 IgG mutants
Relative Response (RU)
Time (sec)
WT
N297Q
T299A
N297Q/S298N/Y300S
S298N/T299A/Y300S
S298N/Y300S
Agly
Δab
zoomed in:
Human FcγRI Binding of αβTCR HEBE1 IgG mutants
Relative Response (RU)
Time (sec)
WT
N297Q
T299A
N297Q/S298N/Y300S
S298N/T299A/Y300S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

Figure 5


GM-CSF
IL10
TNFa
IFNγ
Untreated
BM-A03.1
OKT3
H66 cAB 10
H66 cAB 1
H66 cAB 0.1
H66 S298N/T299A/V300 10
H66 S298N/T299A/V300 1
H66 S298N/T299A/V300 0.1

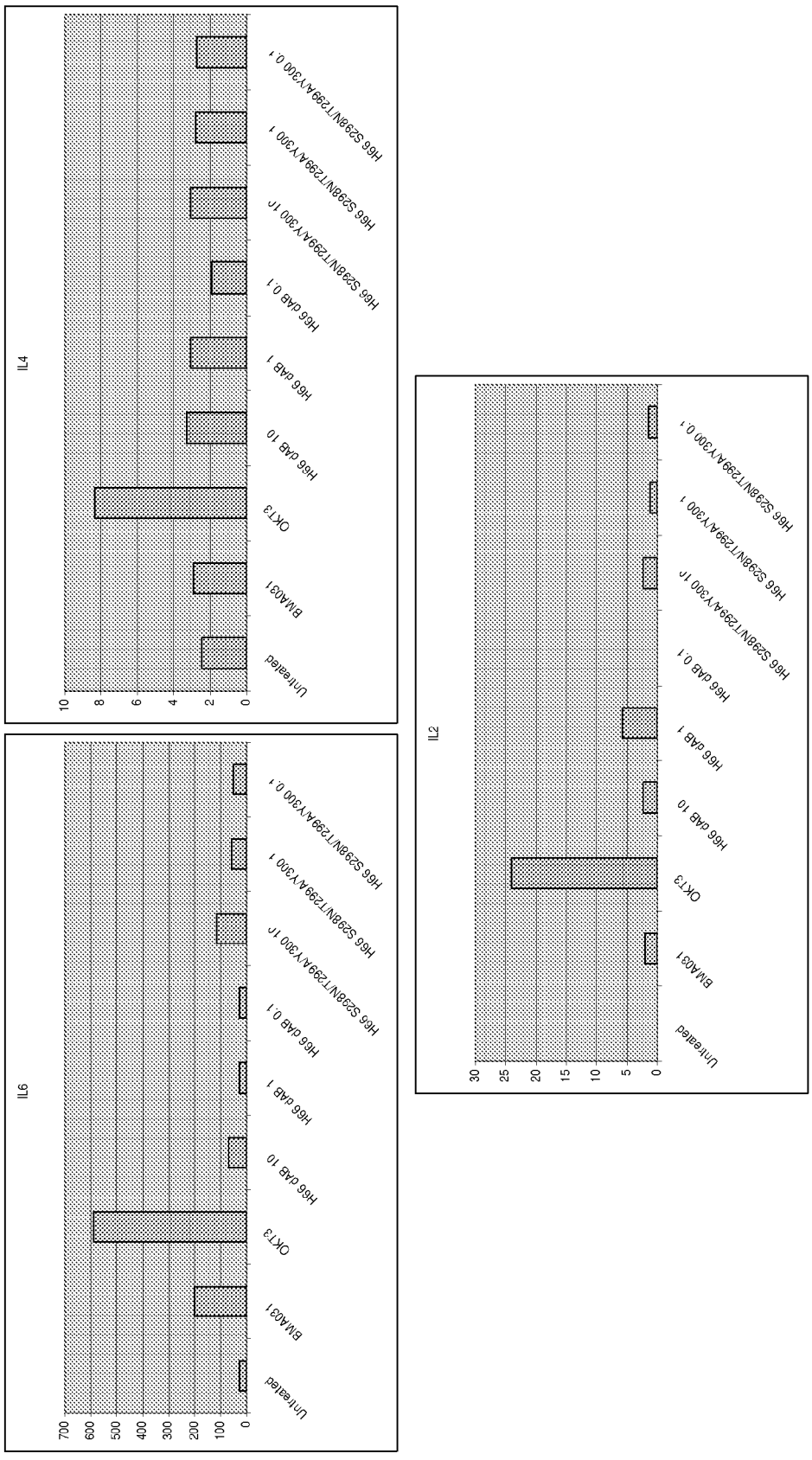
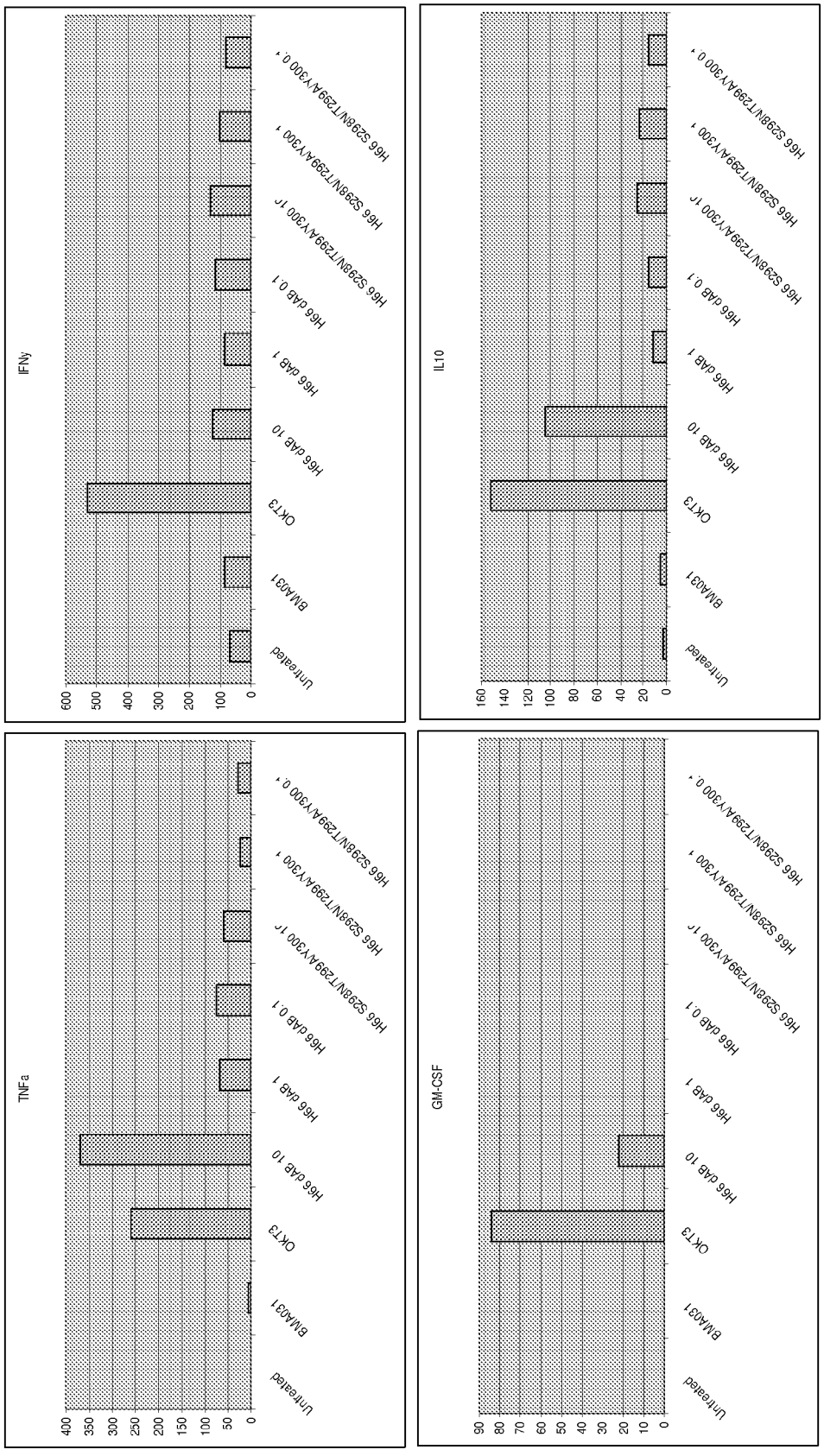

IL4
10
8
6
4
2
0
Untreated
BMA031
OKT3
H66 dAB 10
H66 dAB 1
H66 dAB 0.1
H66 S298N/T299A/Y300 1r
H66 S298N/T299A/Y300 1
H66 S298N/T299A/Y300 0.1
IL6
700
600
500
400
300
200
100
0
Untreated
BMA031
OKT3
H66 dAB 10
H66 dAB 1
H66 dAB 0.1
H66 S298N/T299A/Y300 1r
H66 S298N/T299A/Y300 1
H66 S298N/T299A/Y300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

Figure 6

Figure 7


IFNγ
TNFα
IL10
GM-CSF
Untreated
BMA031
OKT3
H66 dAB 10
H66 dAB 1
H66 dAB 0.1
H66 S298N/T299A/V300 1F
H66 S298N/T299A/V300 1
H66 S298N/T299A/V300 0.1

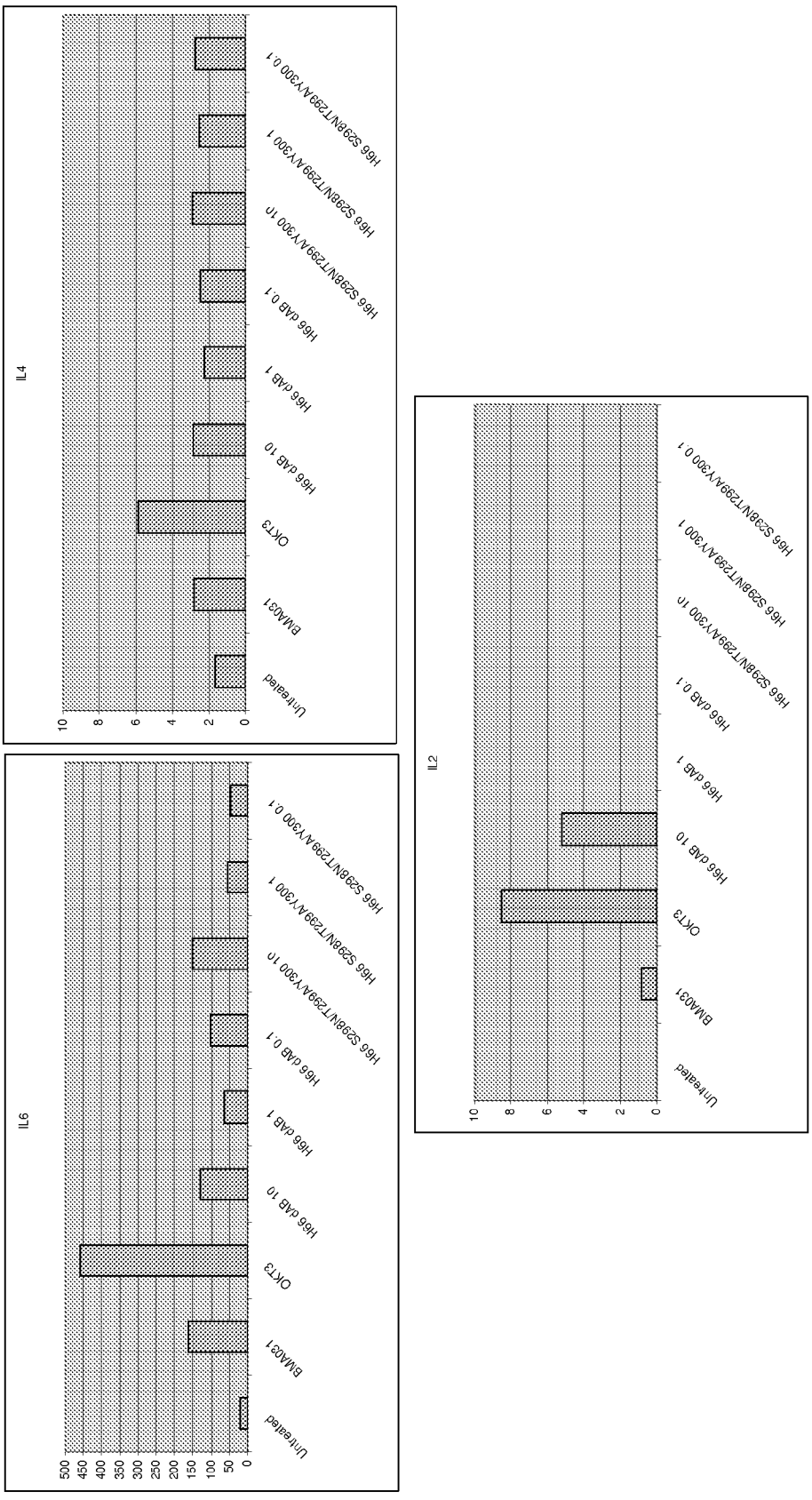
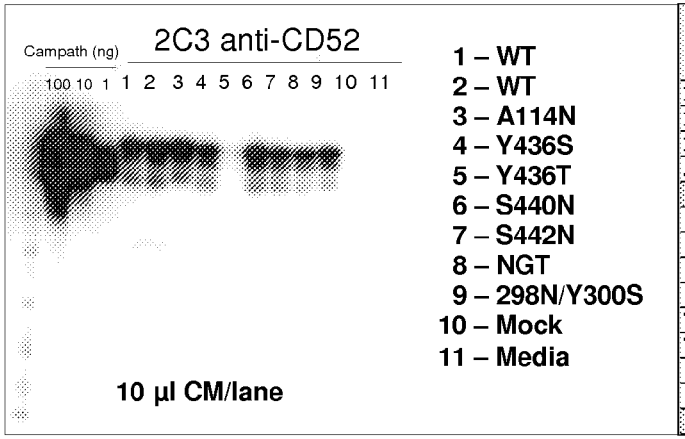

IL4
IL2
IL6
Untreated
BMA031
OKT3
H66 dAB 10
H66 dAB 1
H66 dAB 0.1
H66 S298N/T299A/Y300 1r
H66 S298N/T299A/Y300 1
H66 S298N/T299A/Y300 0.1

Figure 8

Figure 9

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

Figure 10

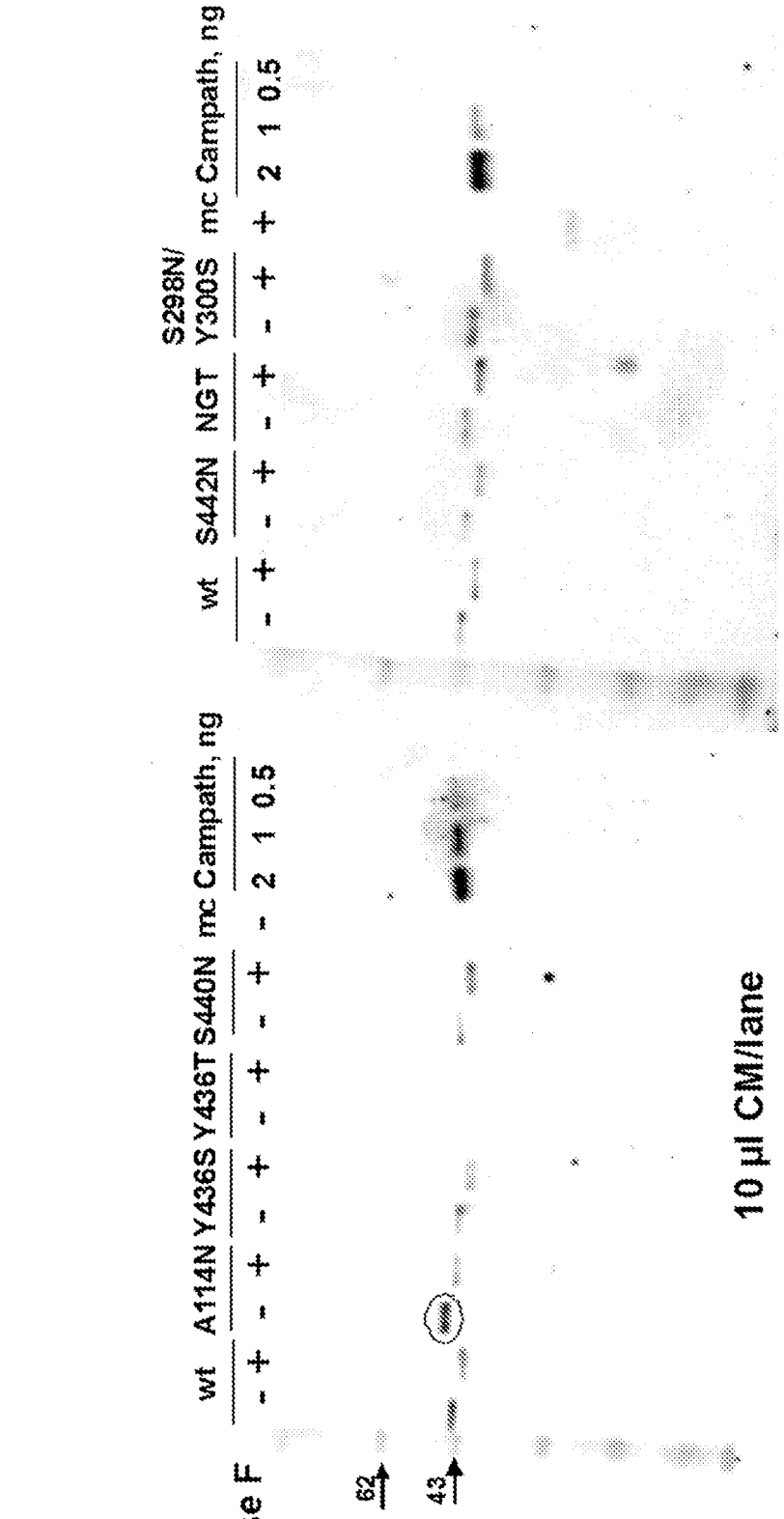

PNGase F
wt
A114N
Y436S
Y436T
S440N
mc Campath, ng
- + - + - + - + - 2 1 0.5
62
43
10 µl CM/lane
S298N/
Y300S
wt
S442N
NGT
mc Campath, ng
- + - + - + - + + 2 1 0.5

Figure 11

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

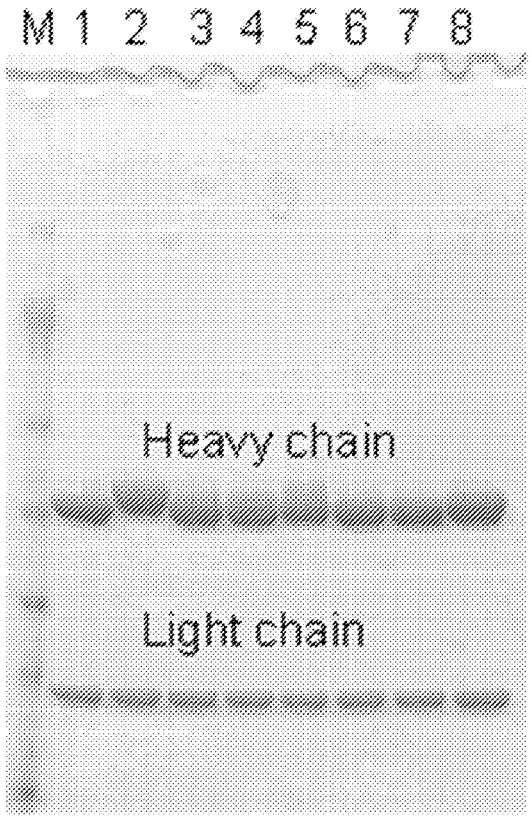
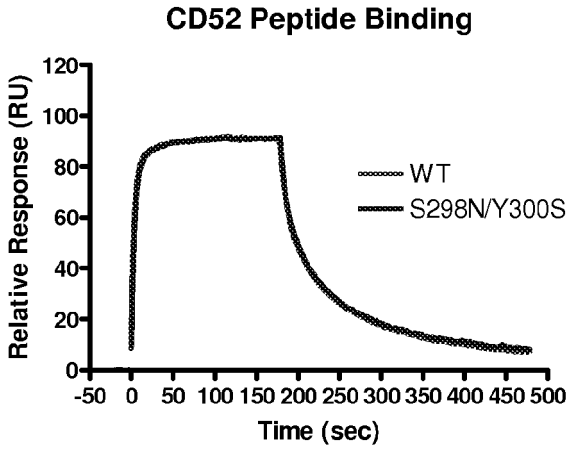
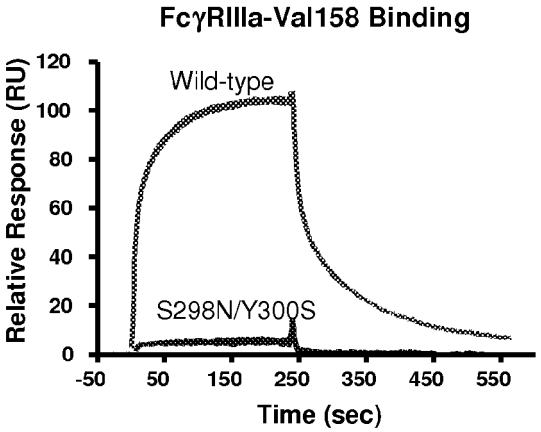


Figure 12

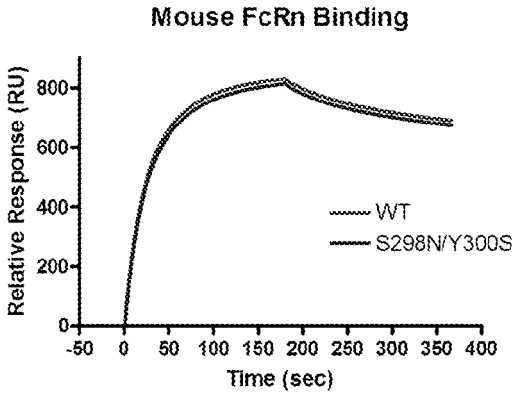
A.



B.



C.



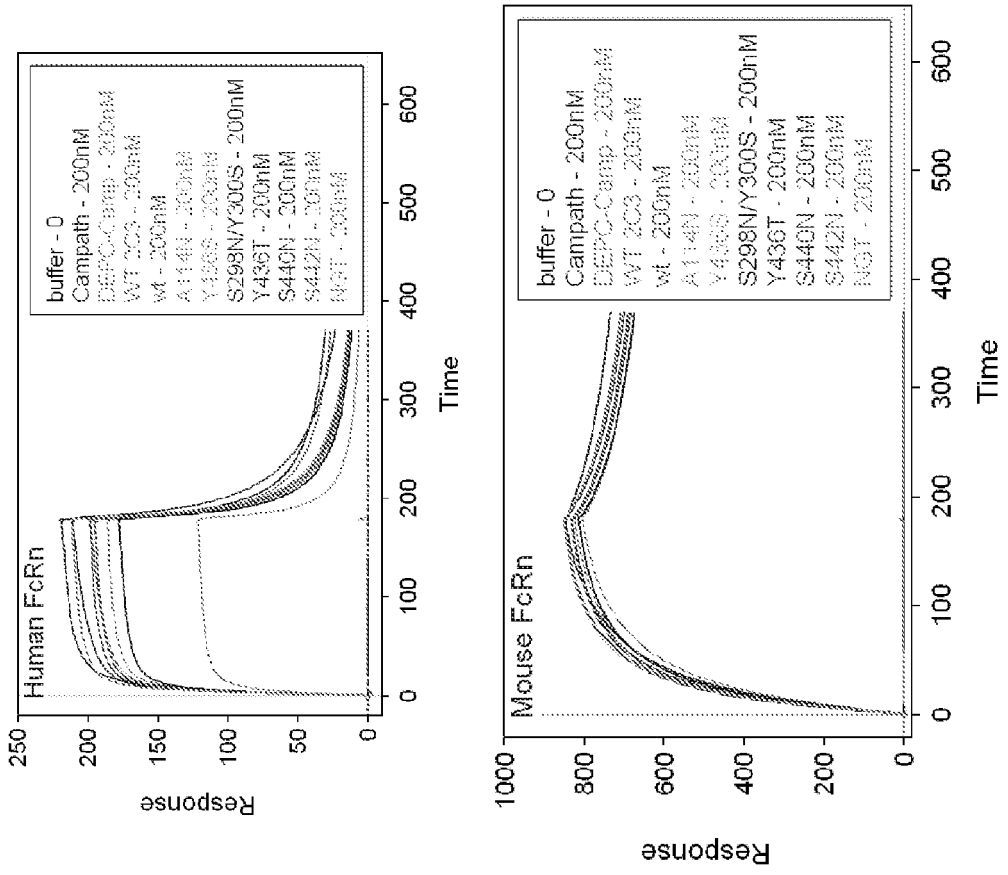
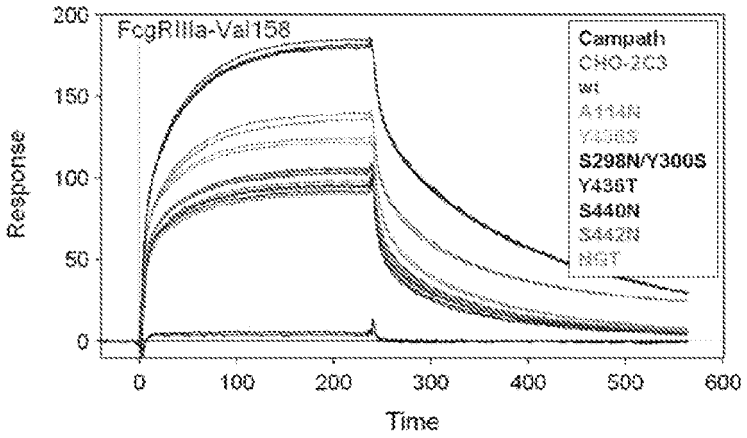

Human FcRn
buffer - 0
Campath - 200nM
BEP/C-Camp - 200nM
WT 2C3 - 200nM
wt - 200nM
A114N - 200nM
Y436S - 200nM
S298N/Y300S - 200nM
Y436T - 200nM
S440N - 200nM
S442N - 200nM
NGT - 200nM
Response
Time
0
50
100
150
200
250
0
100
200
300
400
500
600
Mouse FcRn
buffer - 0
Campath - 200nM
BEP/C-Camp - 200nM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

Figure 13

Figure 14

A.


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

B.

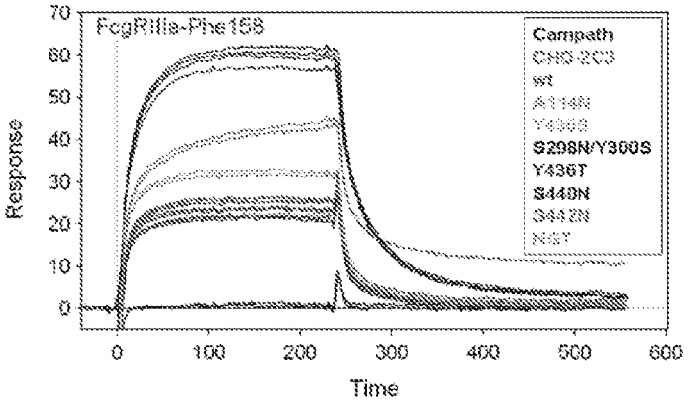
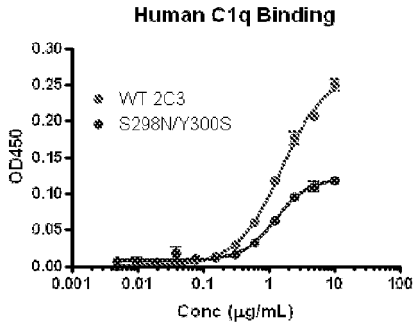

FcgRIIIa-Phe158
Response
Time
Campath
CHO-2C3
wt
A114N
Y436S
S298N/Y300S
Y436T
S440N
S442N
NGT
0
10
20
30
40
50
60
70
0
100
200
300
400
500
600

Figure 15

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.

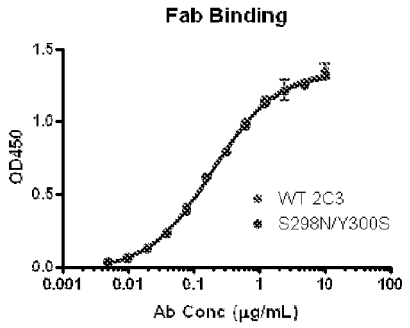

Fab Binding
OD450
1.5
1.0
0.5
0.0
WT 2C3
S298N/Y300S
0.001
0.01
0.1
1
10
100
Ab Conc (µg/mL)

Figure 16

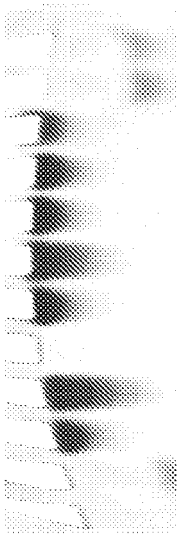
Sample	k_a ($x10^6$/Ms)	k_d ($x10^{-2}$/s)	R_{max} (RU)	K_D (nM)
GLD52	7.0	1.7	67.0	2.44
WT 2C3	6.0	1.1	64.2	1.75
A114N	4.7	1.1	59.5	2.45
Y436S	5.9	1.0	66.9	1.73
S298N/Y300S	5.7	1.0	63.3	1.80
Y436T	4.8	0.9	65.7	1.95
S440N	5.8	1.1	66.8	1.84
S442N	5.7	1.1	66.2	1.85
NGT	7.9	1.1	70.2	1.35

Figure 17

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

Figure 18

A.


WT
A114N
Y430S
S298N/Y300S
S442N
S442N
NGT

B.

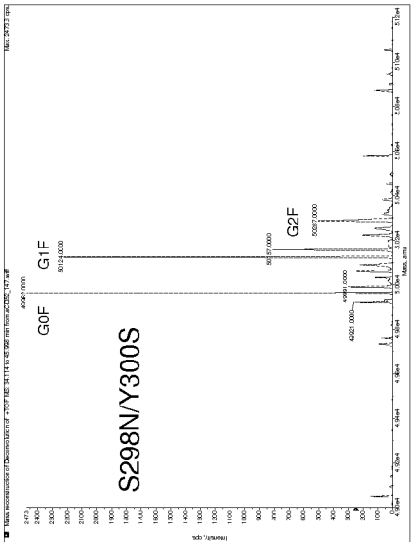
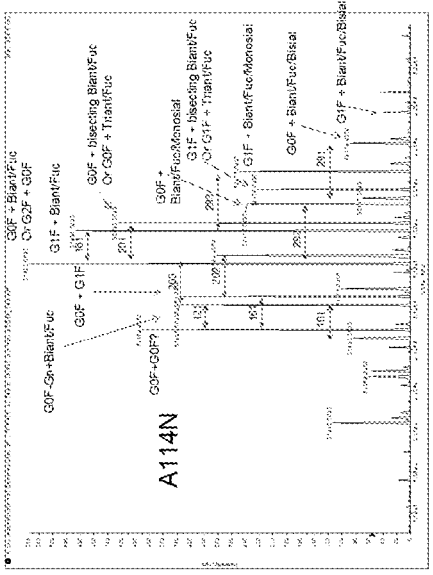

G0F
G1F
G2F
S298N/Y300S

Figure 18

C.


A114N
G0F-Gn+BiantFuc
G0F+G0F?
G0F + BiantFuc Or G2F + G0F
G1F + BiantFuc
G0F + G1F
G0F + bisecting BiantFuc Or G0F + TriantFuc
G0F + BiantFuc:Monosial
G1F + bisecting BiantFuc Or G1F + TriantFuc
G1F + BiantFuc:Monosial
G0F + BiantFuc:Bisial
G1F + BiantFuc:Bisial

D.

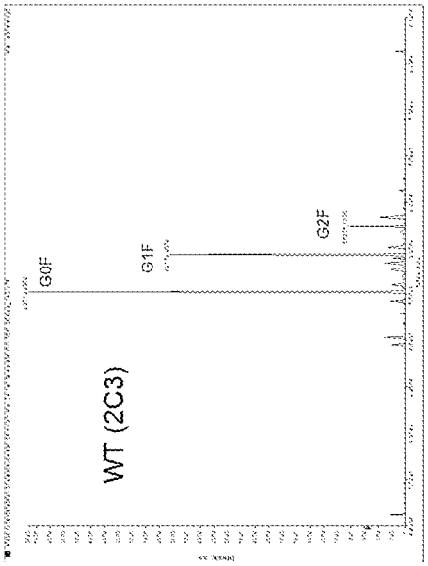
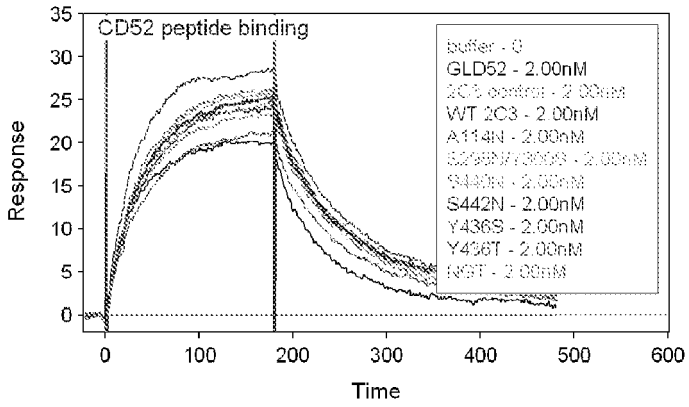

WT (2C3)
G0F
G1F
G2F

Figure 19

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.



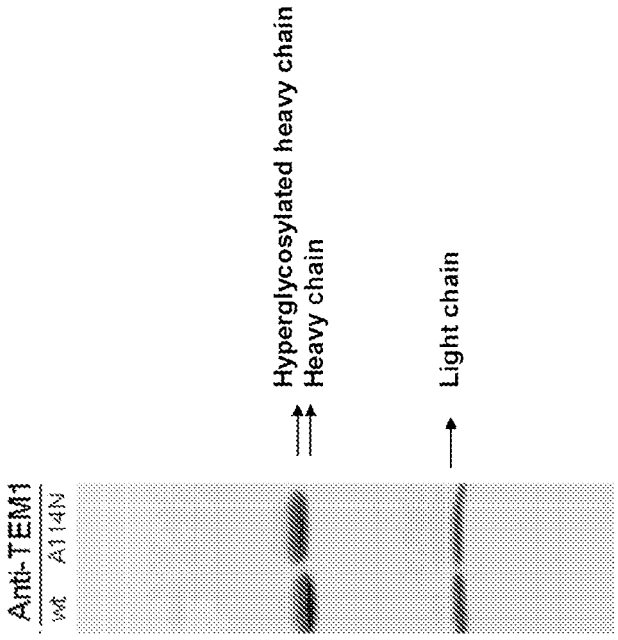
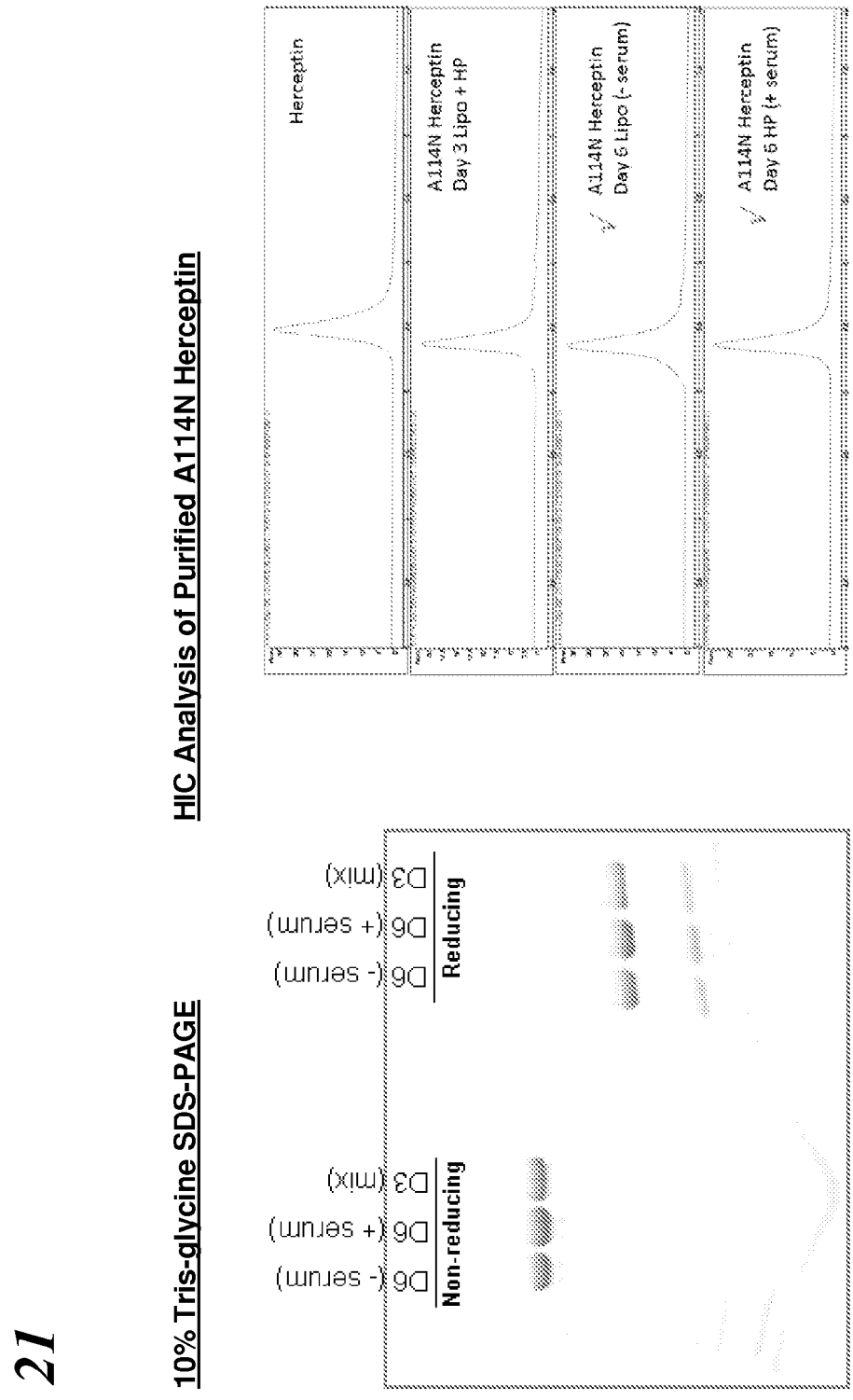

Anti-TEM1
wt A114N
Hyperglycosylated heavy chain
Heavy chain
Light chain

Figure 20

Figure 21


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

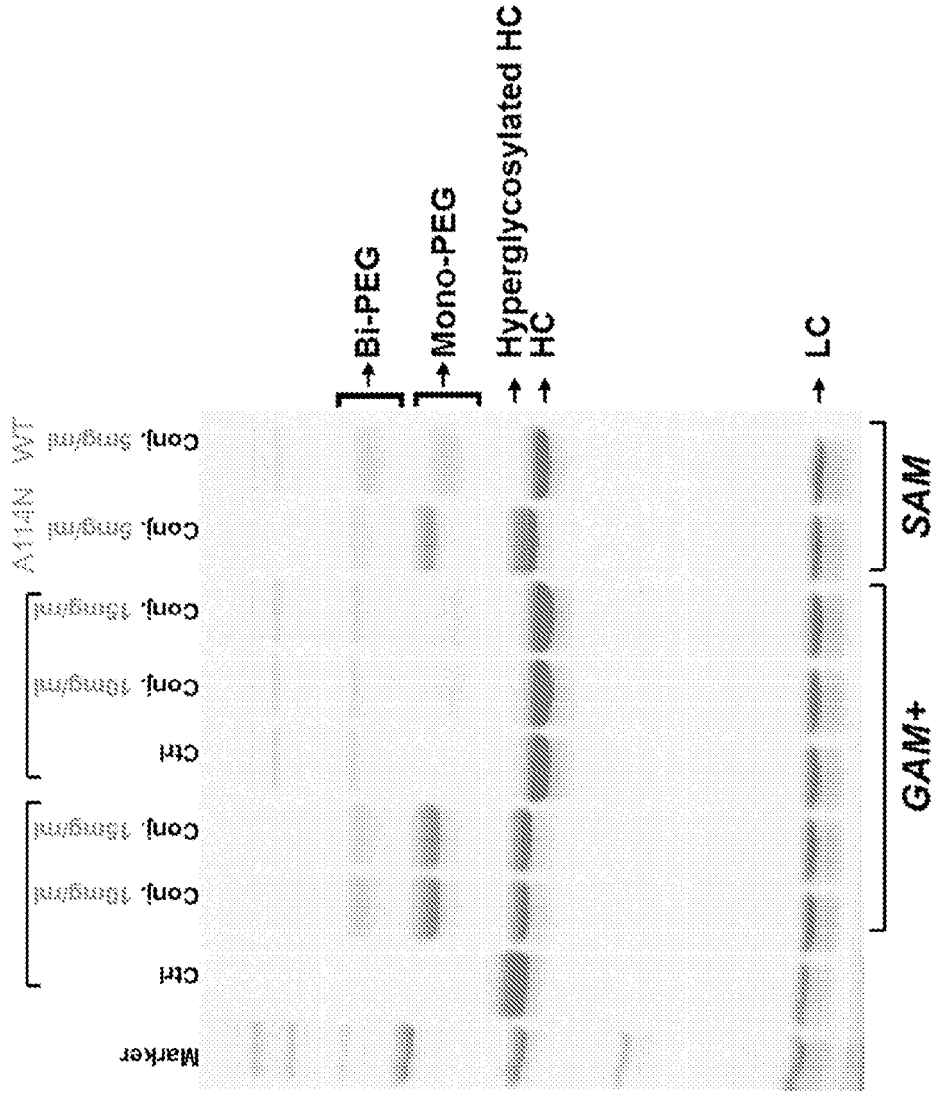
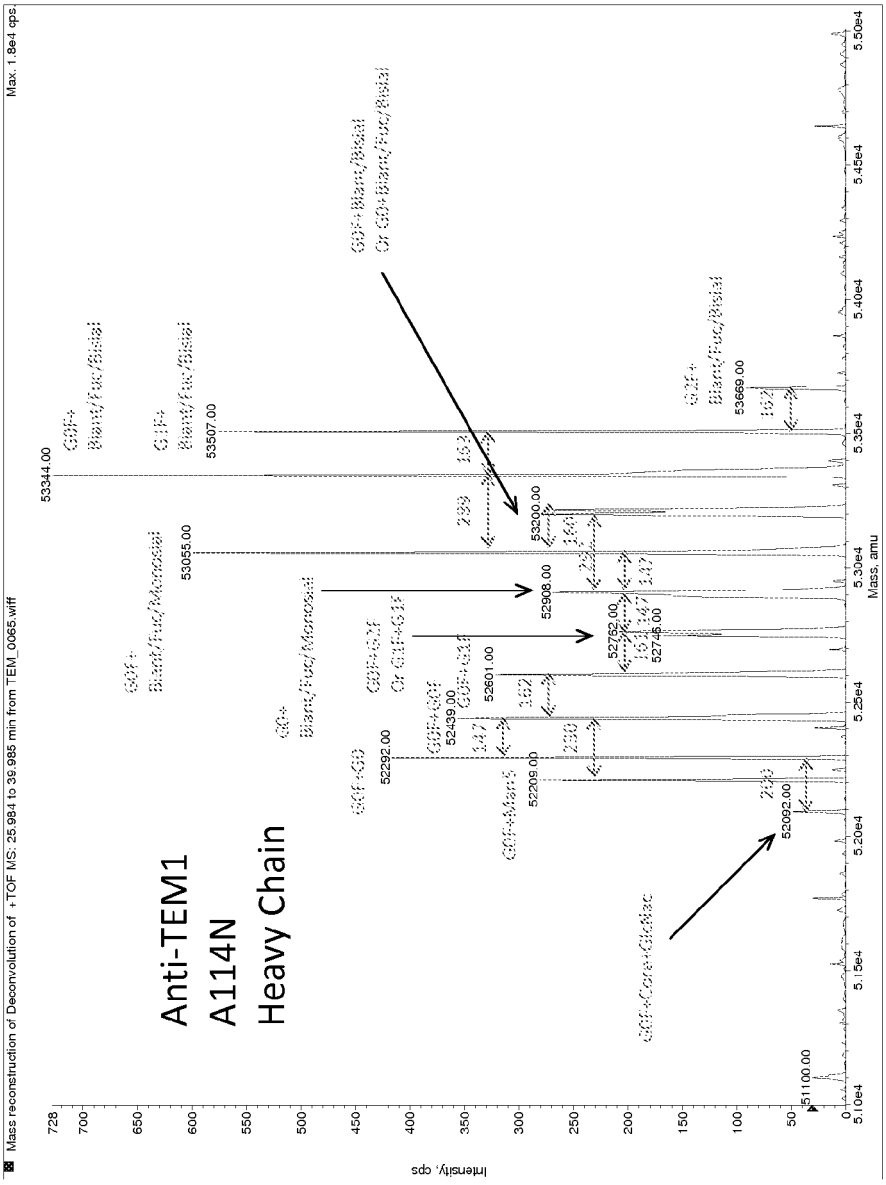

Bi-PEG
Mono-PEG
Hyperglycosylated HC
HC
LC
A114N WT
Conj. 5mg/ml
Conj. 5mg/ml
Conj. 15mg/ml
Conj. 15mg/ml
ctrl
Conj. 15mg/ml
Conj. 15mg/ml
ctrl
Marker
SAM
GAM+

Figure 22

Figure 23


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
53344.00
53507.00
53055.00
52908.00
53200.00
52762.00
52746.00
52601.00
52439.00
52292.00
52209.00
52092.00
53669.00
51100.00

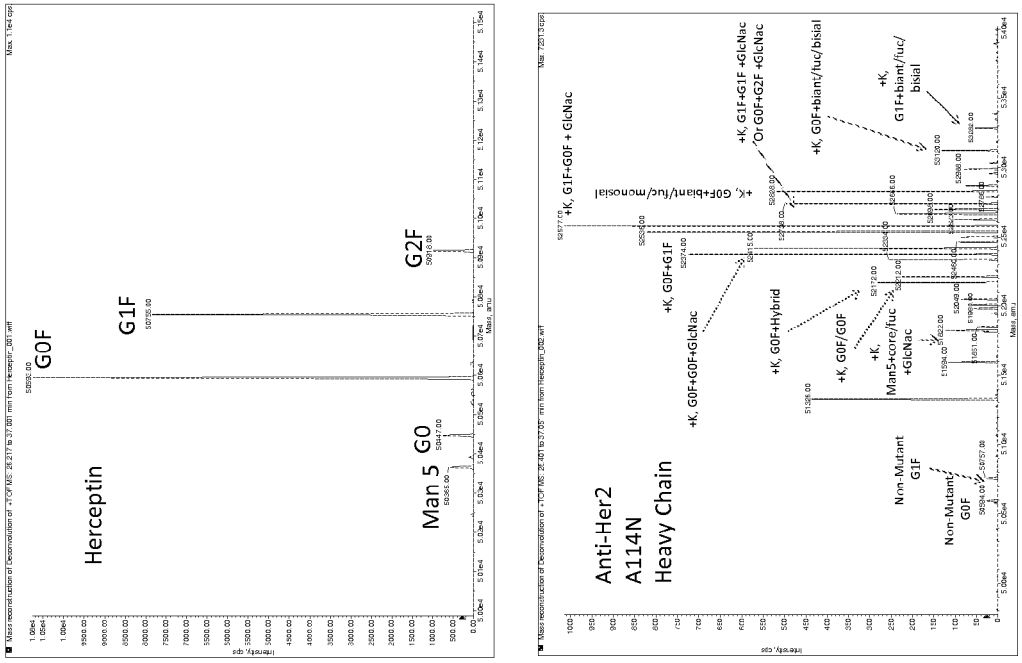

Herceptin
G0F
G1F
G2F
Man 5 G0
Anti-Her2
A114N
Heavy Chain
+K, G1F+G0F + GlcNac
+K, G0F+biant/fuc/monosial
+K, G1F+G1F +GlcNac
Or G0F+G2F +GlcNac
+K, G0F+biant/fuc/bisial
+K, G1F+biant/fuc/ bisial
+K, G0F+G0F+GlcNac
+K, G0F+G1F
+K, G0F+Hybrid
+K, G0F/G0F
+K, Man5+core/fuc +GlcNac
Non-Mutant G1F
Non-Mutant G0F

Figure 24

Figure 25

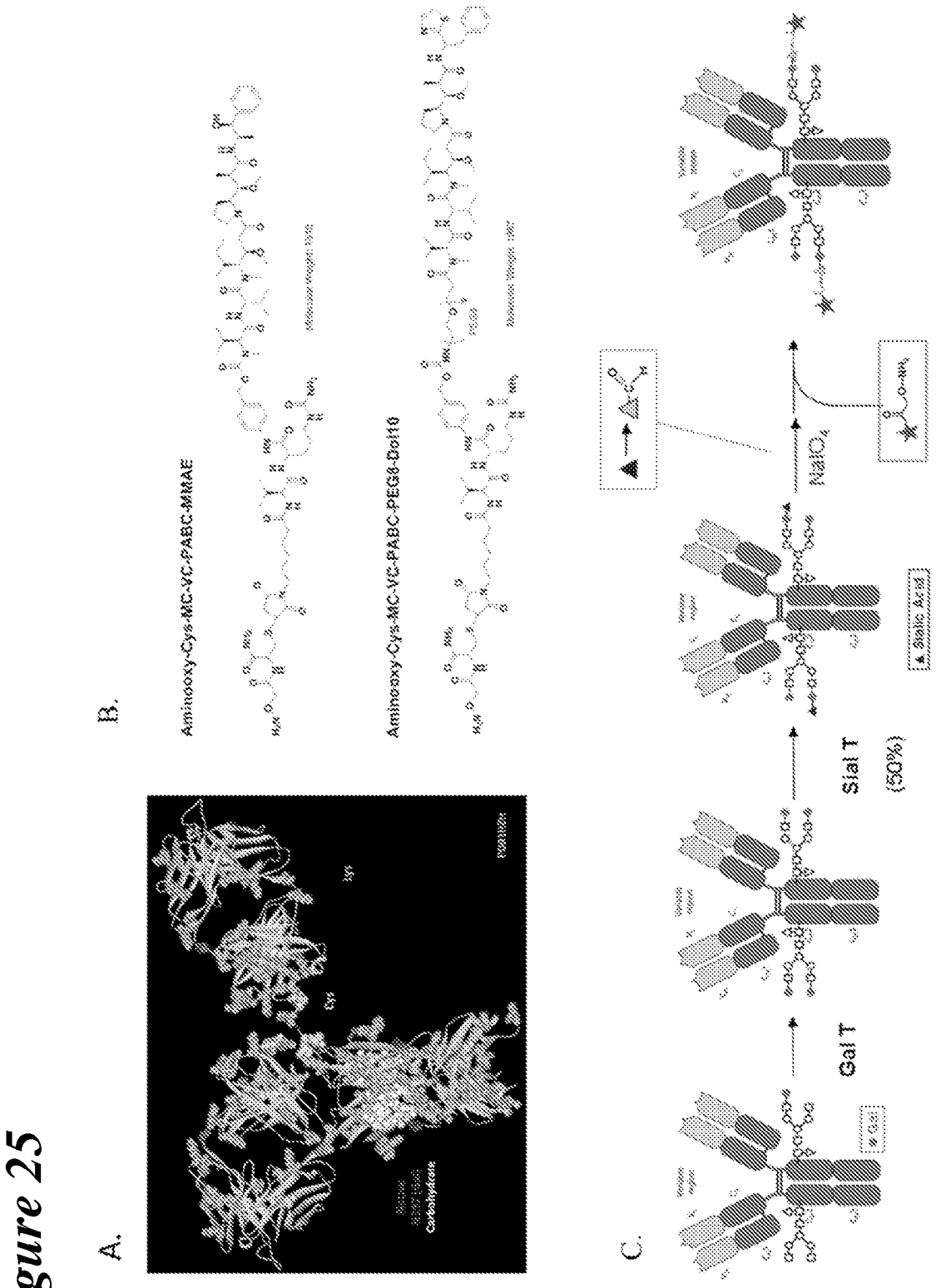

A.
B.
Aminooxy-Cys-MC-VC-PABC-MMAE
Aminooxy-Cys-MC-VC-PABC-PEG8-Dol10
C.
Gal T
Sial T
(50%)
NaIO4
Sialic Acid

Figure 26

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

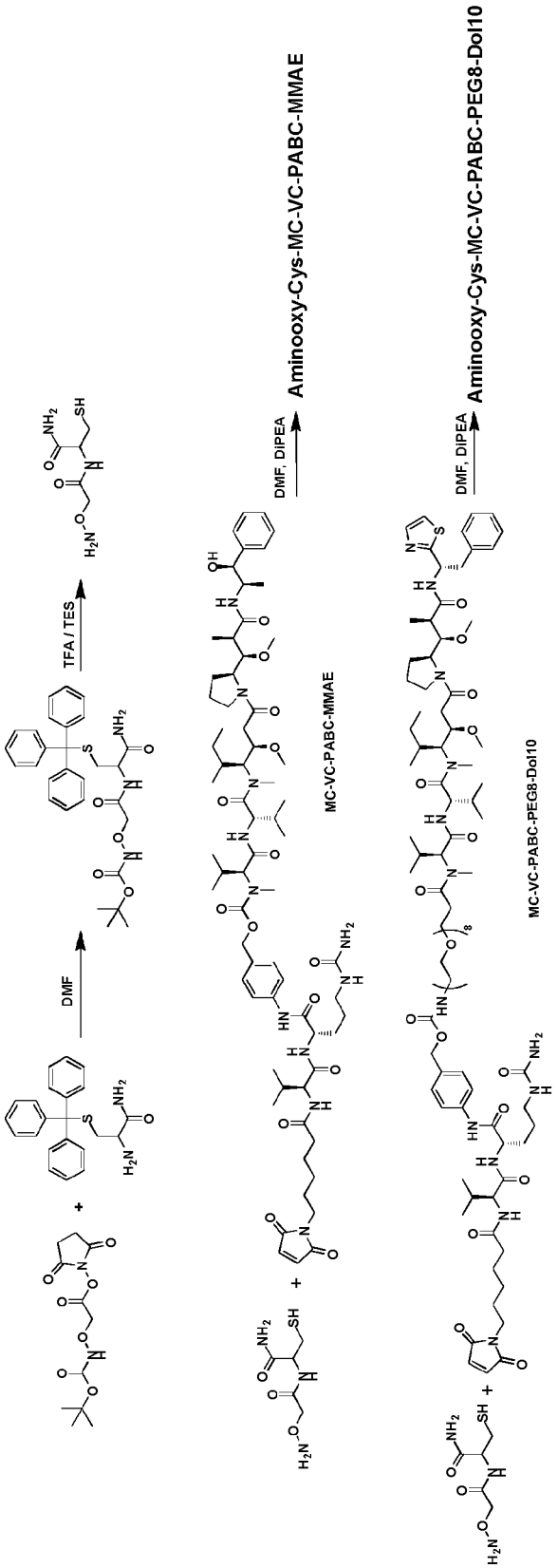
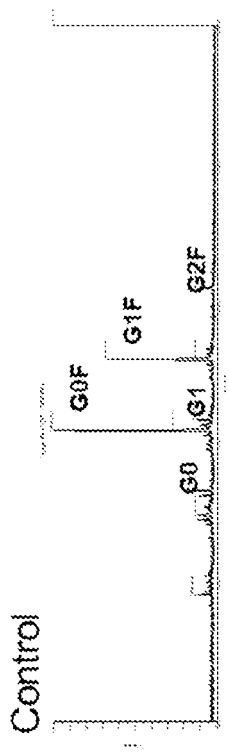


Figure 27

A.



B.

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

C.

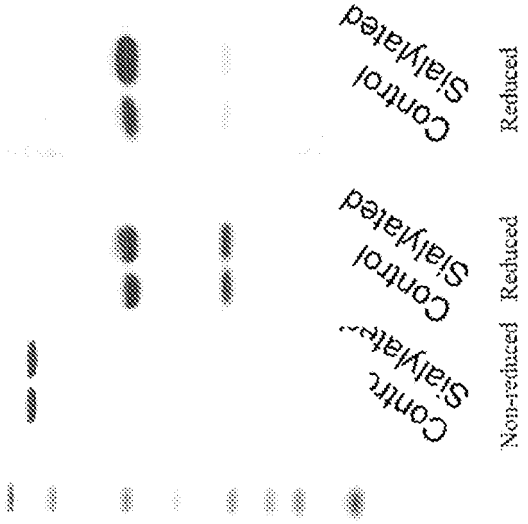


Figure 28

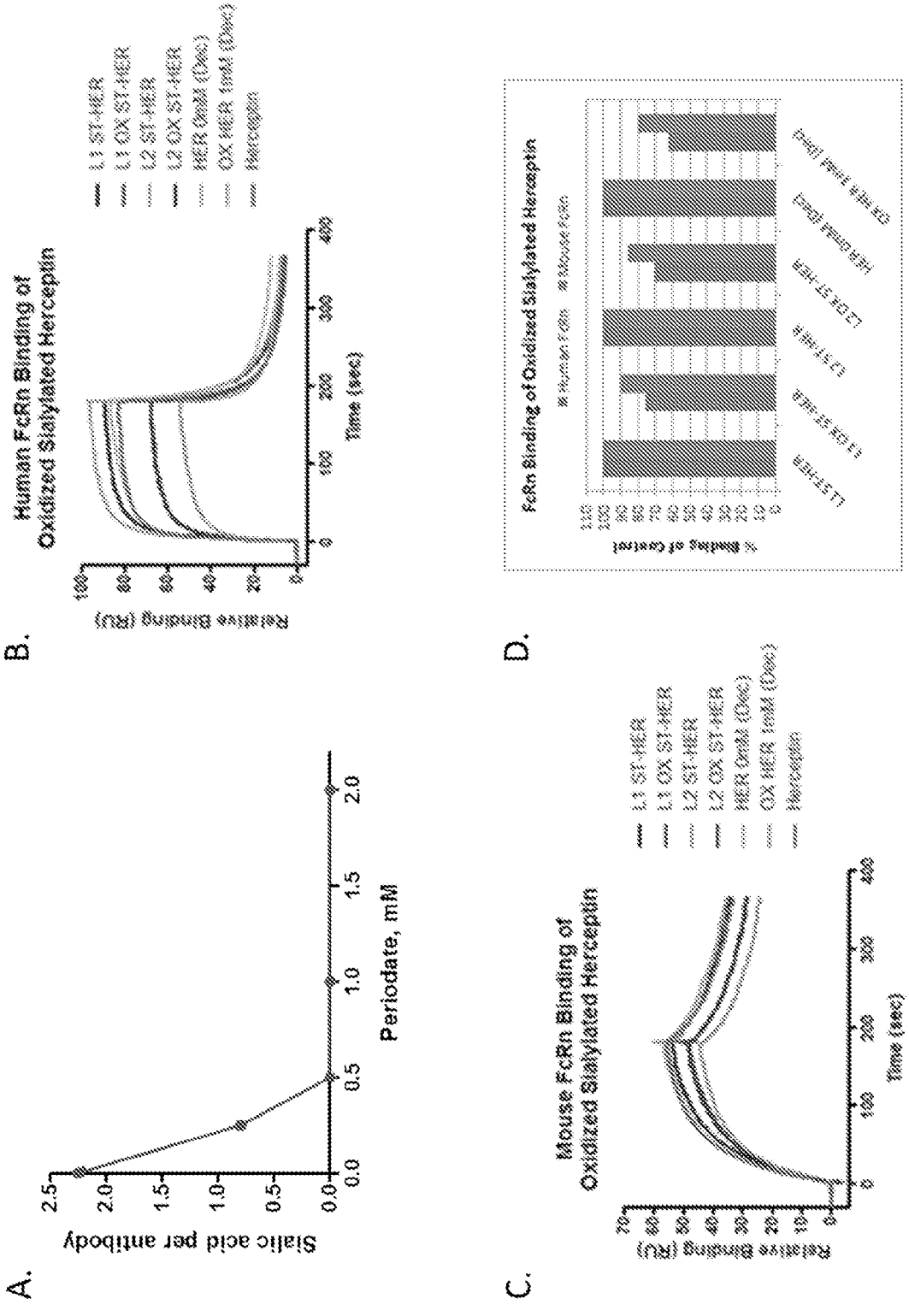

A.
Sialic acid per antibody
Periodate, mM
B.
Human FcRn Binding of Oxidized Sialylated Herceptin
Relative Binding (RU)
Time (sec)
L1 ST-HER
L1 OX ST-HER
L2 ST-HER
L2 OX ST-HER
HER OxM (Dec)
OX HER 1mM (Dec)
Herceptin
C.
Mouse FcRn Binding of Oxidized Sialylated Herceptin
Relative Binding (RU)
Time (sec)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

Figure 29

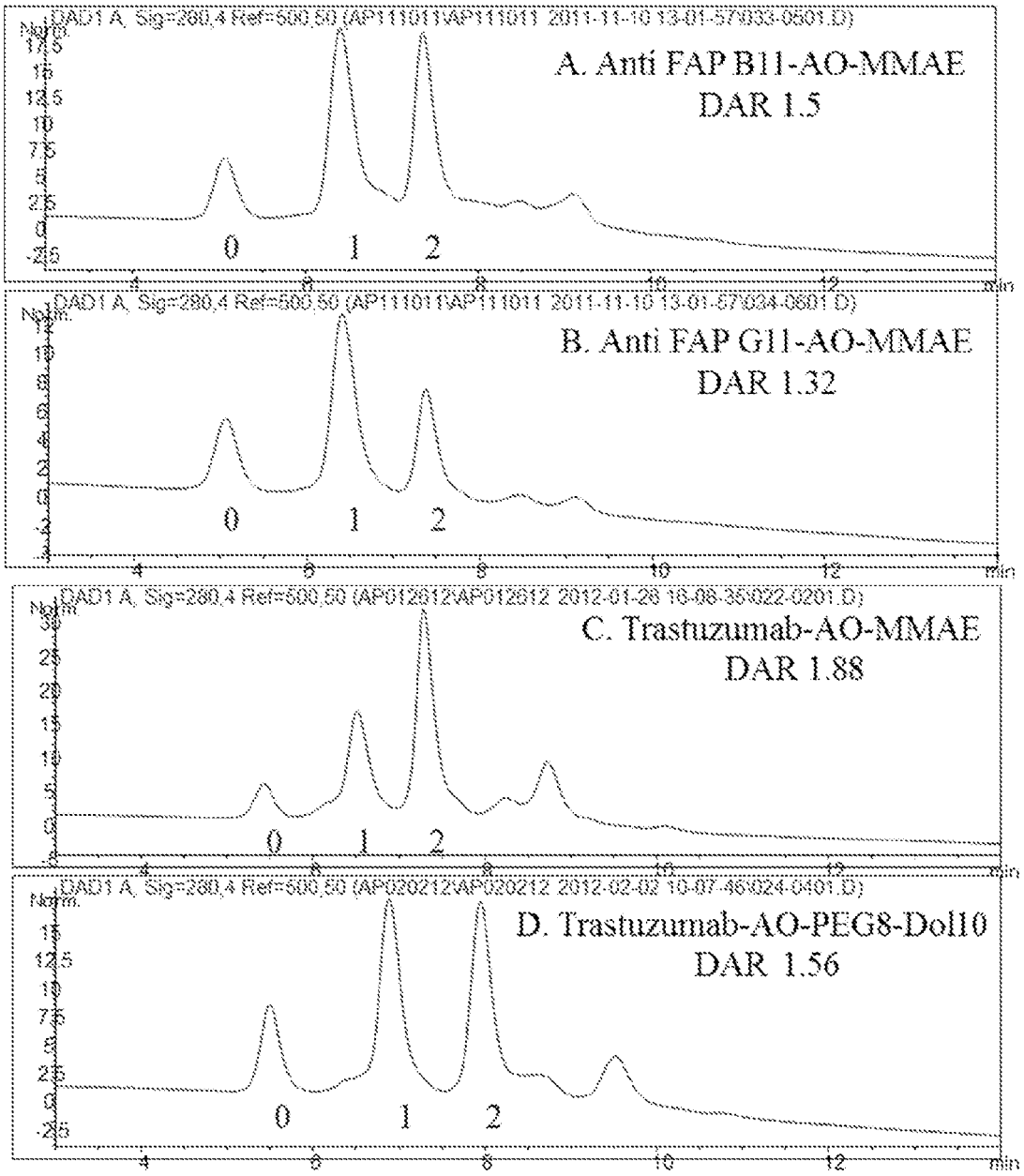

A. Anti FAP B11-AO-MMAE
DAR 1.5
B. Anti FAP G11-AO-MMAE
DAR 1.32
C. Trastuzumab-AO-MMAE
DAR 1.88
D. Trastuzumab-AO-PEG8-Dol10
DAR 1.56
0
1
2
min

Figure 30

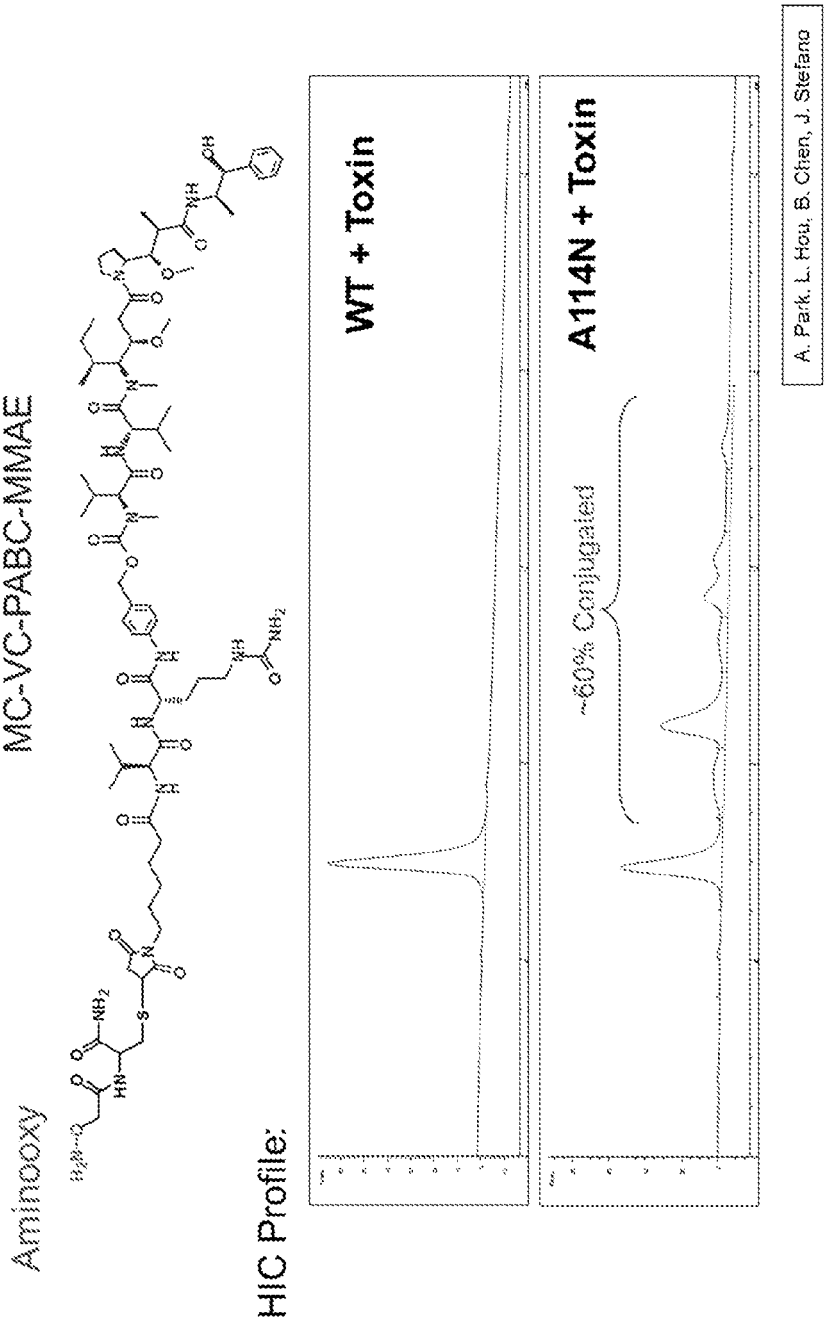

Aminooxy
MC-VC-PABC-MMAE
HIC Profile:
WT + Toxin
A114N + Toxin
~60% Conjugated
A. Park, L. Hou, B. Chen, J. Stefano

Figure 31

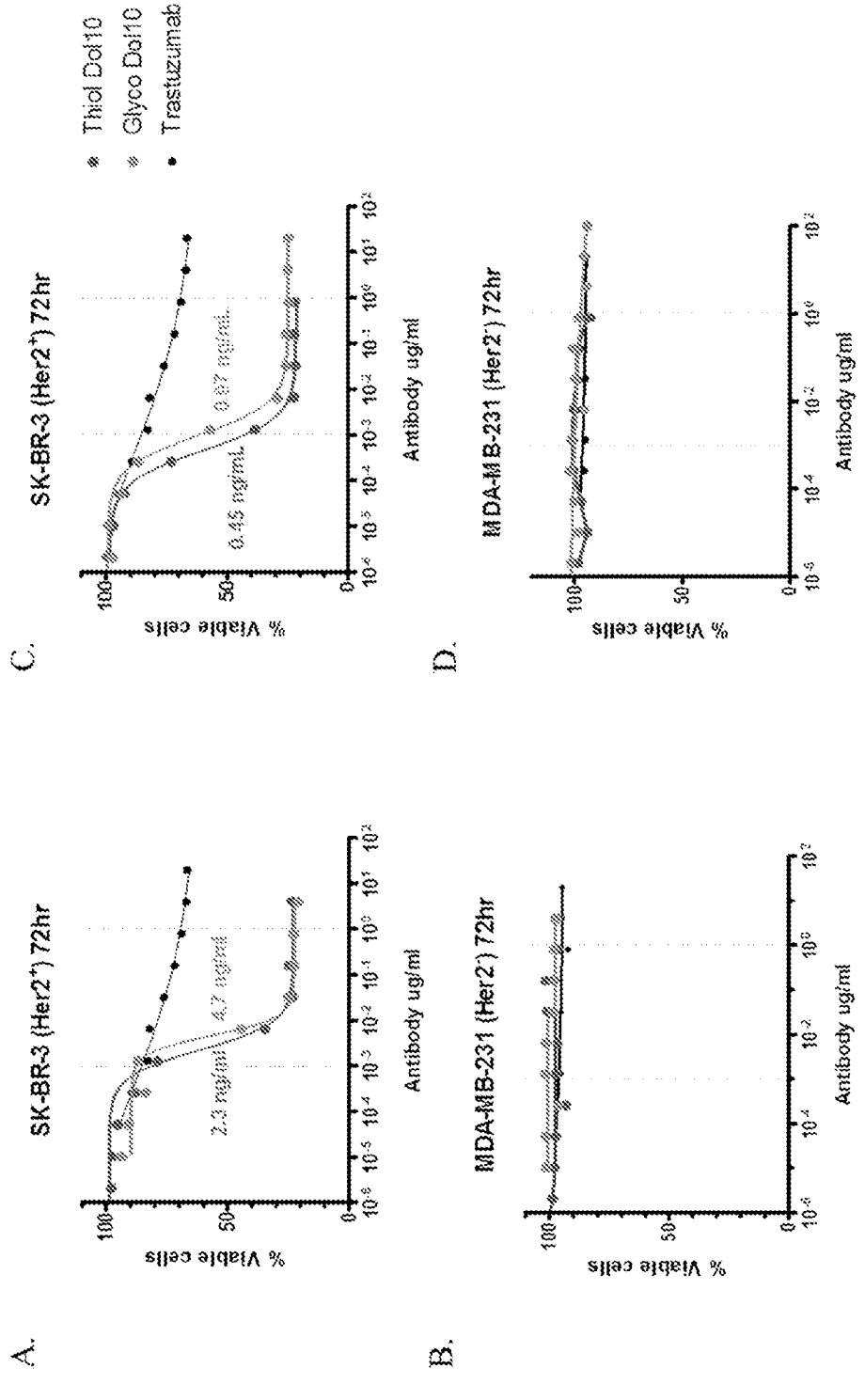

A.
SK-BR-3 (Her2+) 72hr
% Viable cells
Antibody ug/ml
2.3 ng/ml
4.7 ng/ml
B.
MDA-MB-231 (Her2-) 72hr
% Viable cells
Antibody ug/ml
C.
SK-BR-3 (Her2+) 72hr
% Viable cells
Antibody ug/ml
0.45 ng/ml
0.97 ng/ml
Thiol Dol10
Glyco Dol10
Trastuzumab
D.
MDA-MB-231 (Her2-) 72hr
% Viable cells
Antibody ug/ml

Figure 32

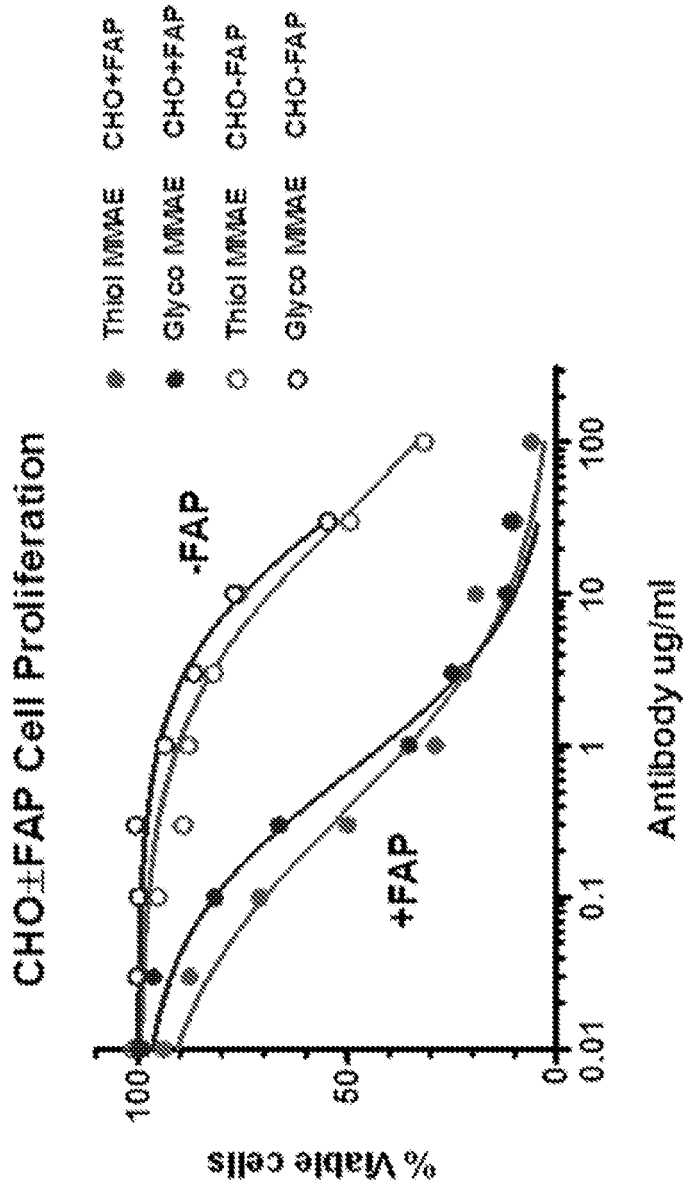
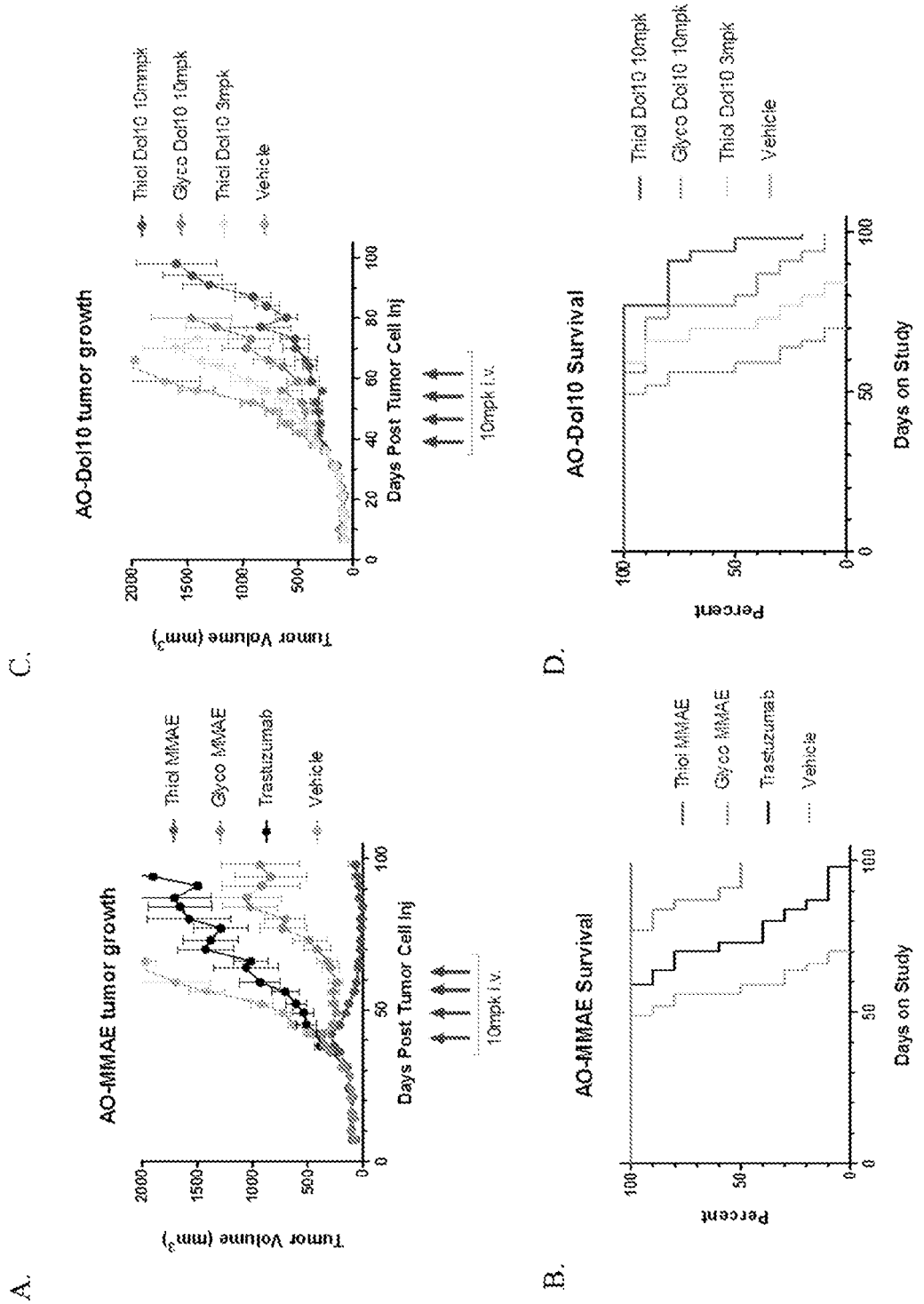

CHO±FAP Cell Proliferation
Thiol MMAE CHO+FAP
Glyco MMAE CHO+FAP
Thiol MMAE CHO-FAP
Glyco MMAE CHO-FAP
-FAP
+FAP
% Viable cells
100
50
0
0.01
0.1
1
10
100
Antibody ug/ml

Figure 33


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

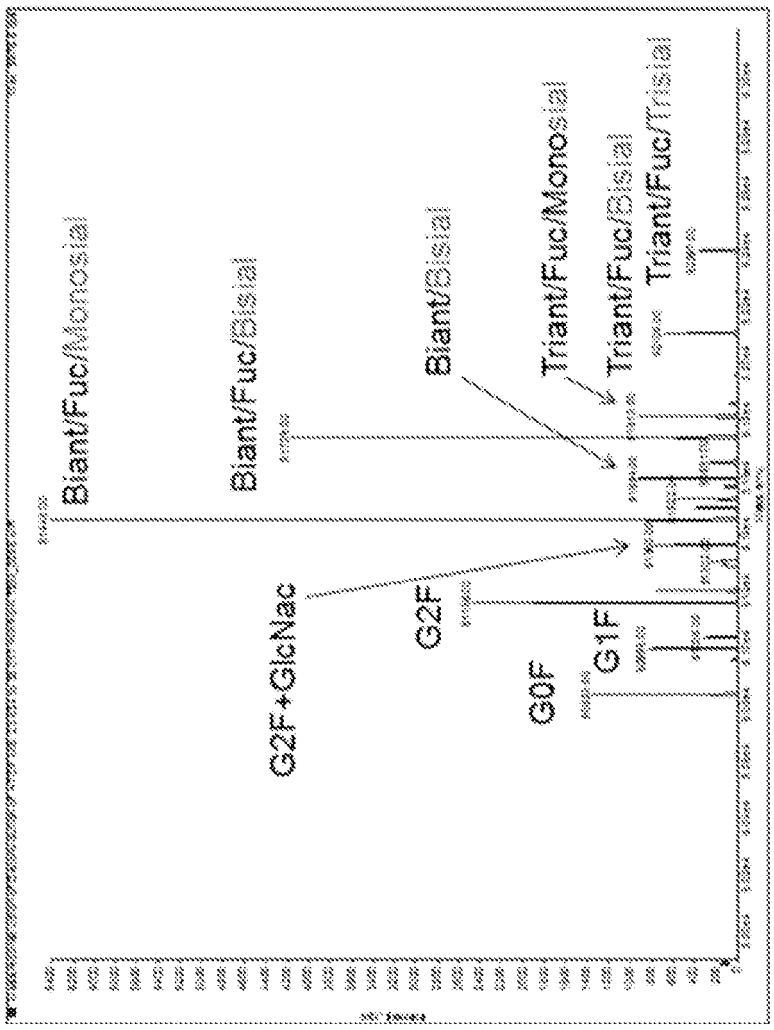

LC/MS of S298N/T299A/Y300S (NNAS) heavy chain
Biant/Fuc/Monosial
Biant/Fuc/Bisial
Biant/Bisial
Triant/Fuc/Monosial
Triant/Fuc/Bisial
Triant/Fuc/Trisial
G2F+GlcNac
G2F
G1F
G0F

Figure 34

Figure 35

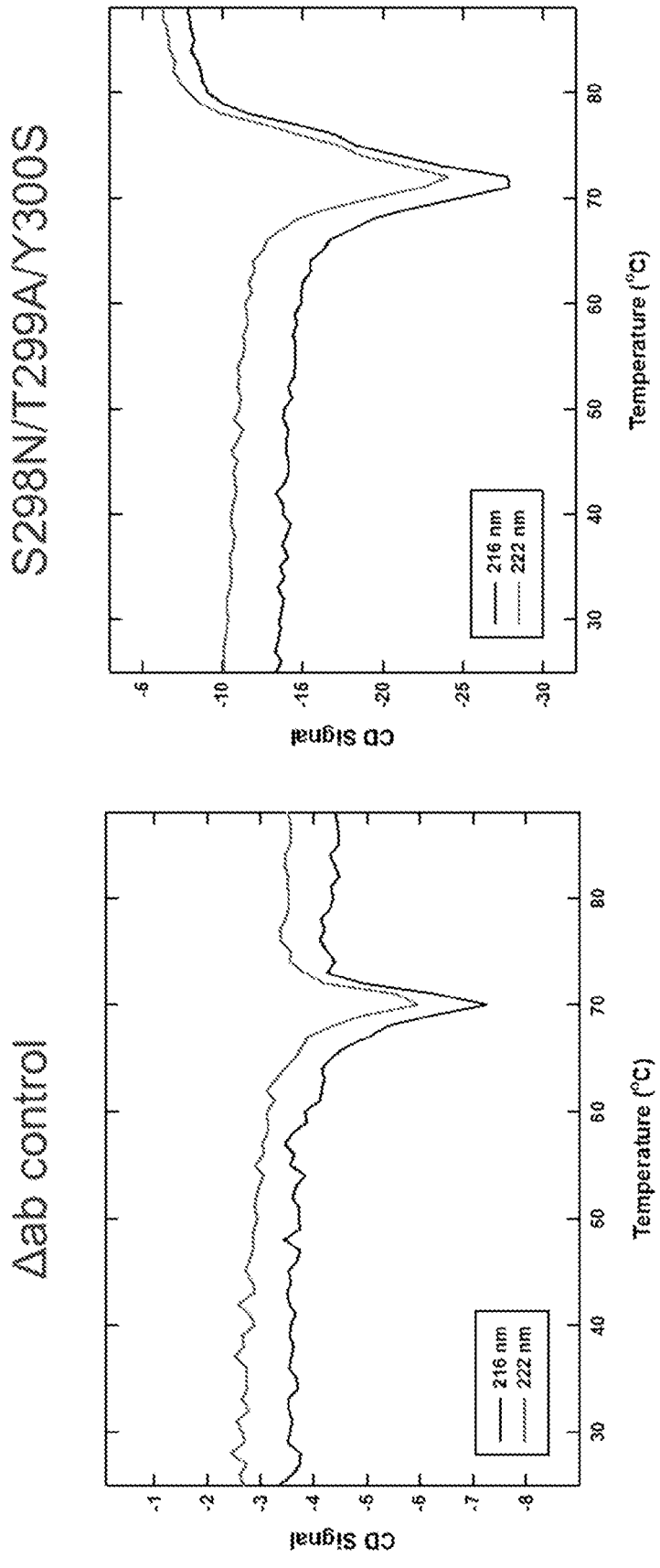
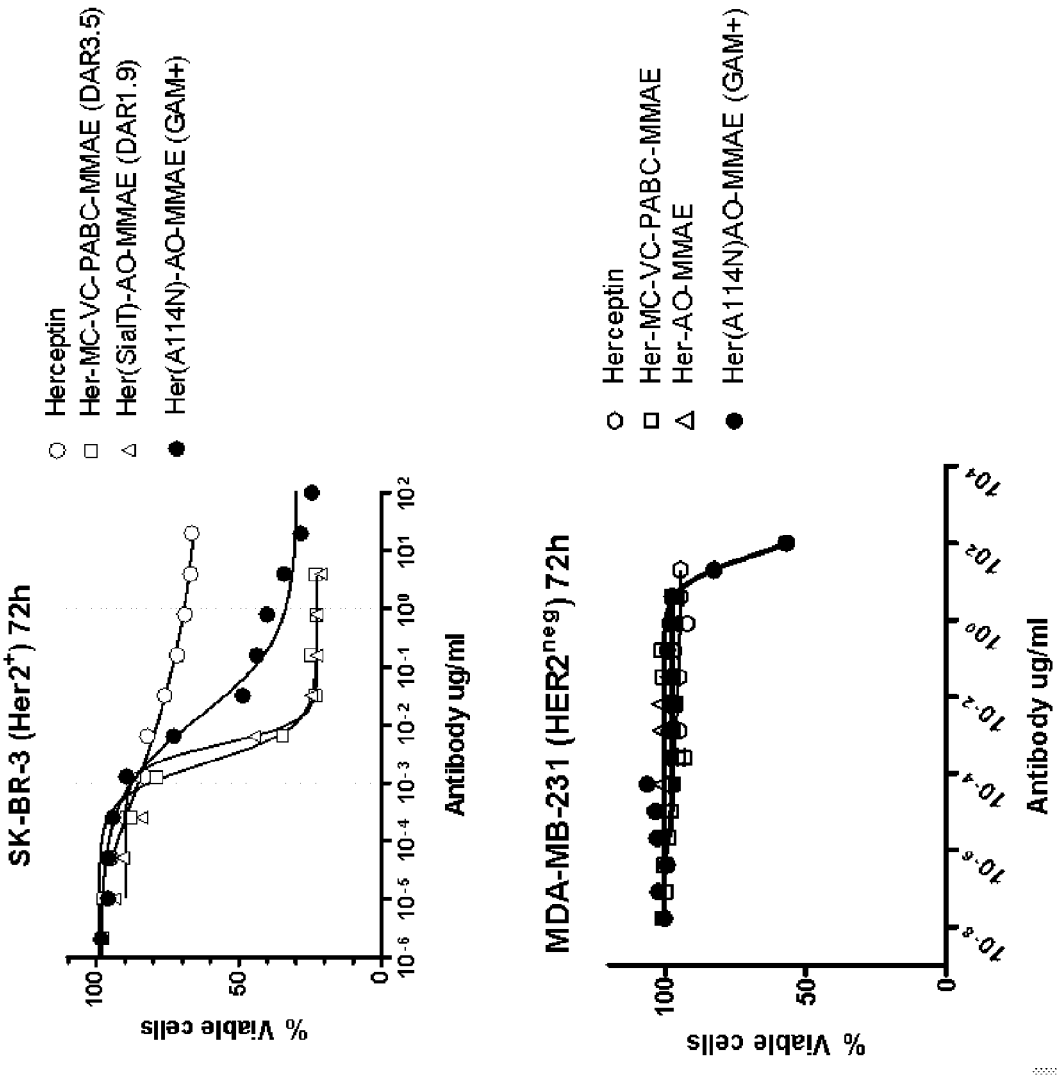

Δab control
S298N/T299A/Y300S
CD signal
Temperature (°C)
216 nm
222 nm
-1
-2
-3
-4
-5
-6
-7
-8
-10
-15
-20
-25
-30
30
40
50
60
70
80

Figure 36


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
Antibody ug/ml
MDA-MB-231 (HER2neg) 72h
Herceptin
Her-MC-VC-PABC-MMAE
Her-AO-MMAE
Her(A114N)AO-MMAE (GAM+)
% Viable cells
Antibody ug/ml