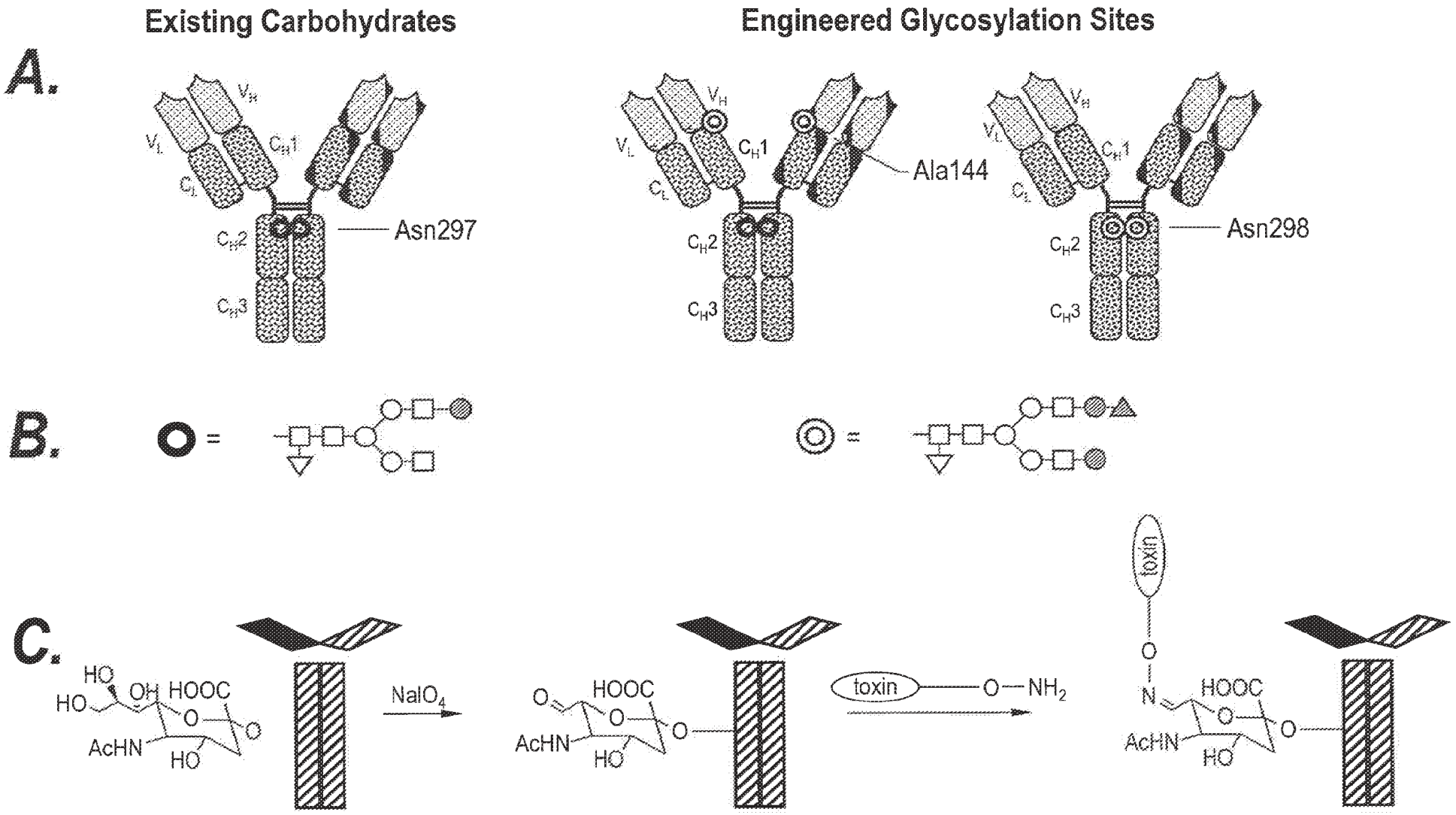

Existing Carbohydrates
Engineered Glycosylation Sites
A.
Asn297
Ala144
Asn298
B.
C.
NaIO4
toxin
O—NH2

Fig. 1

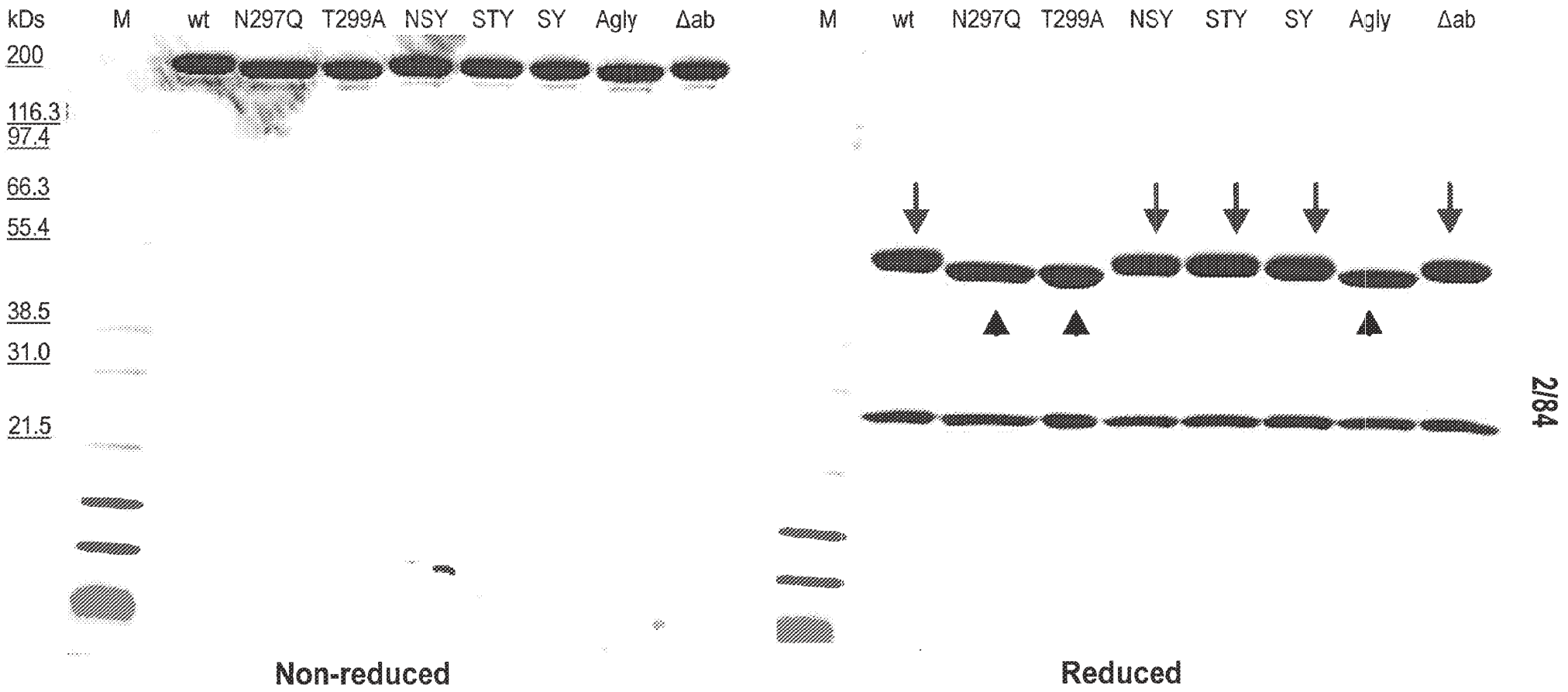
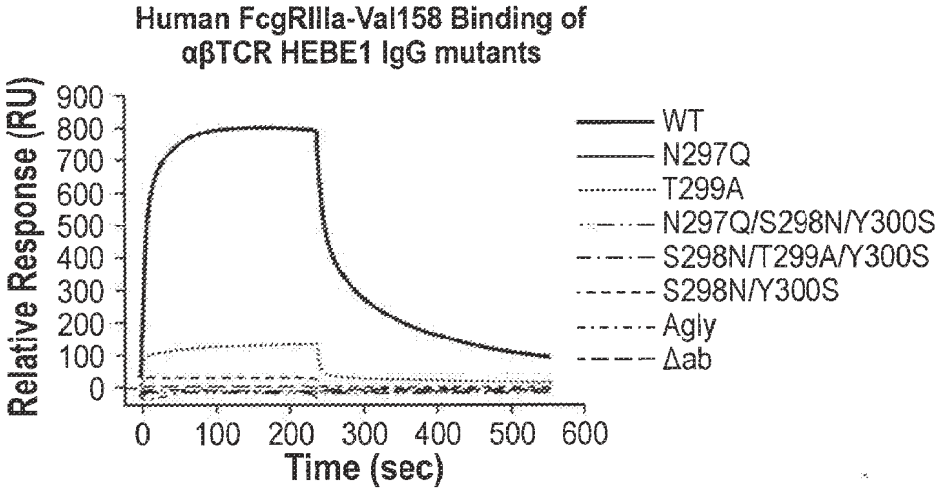
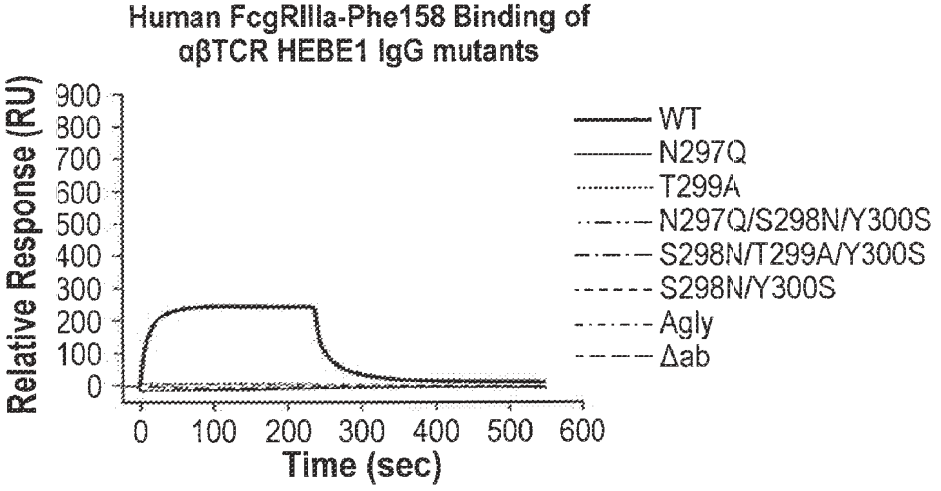

kDs
M
wt
N297Q
T299A
NSY
STY
SY
Agly
Δab
200
116.3
97.4
66.3
55.4
38.5
31.0
21.5
Non-reduced
Reduced

Fig. 2

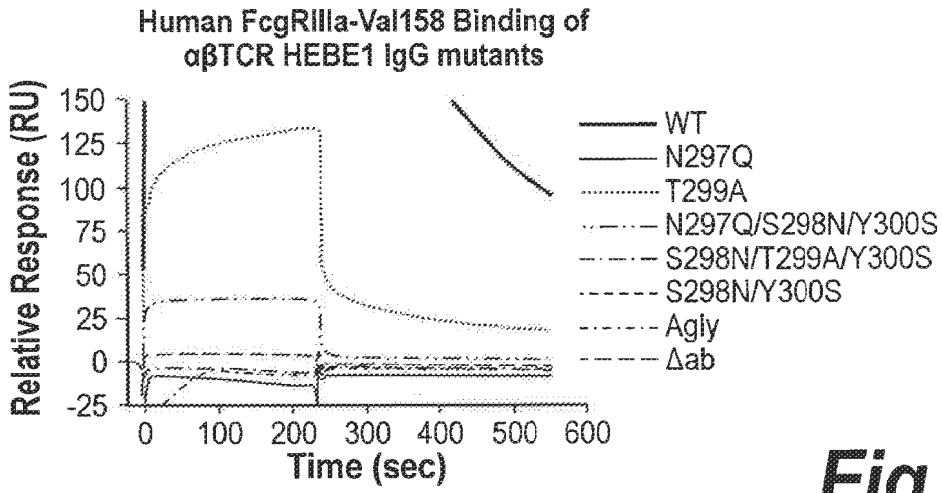
Fullscale: CD16a-Val158



Fullscale: CD16a-Phe158



zoomed:



zoomed:

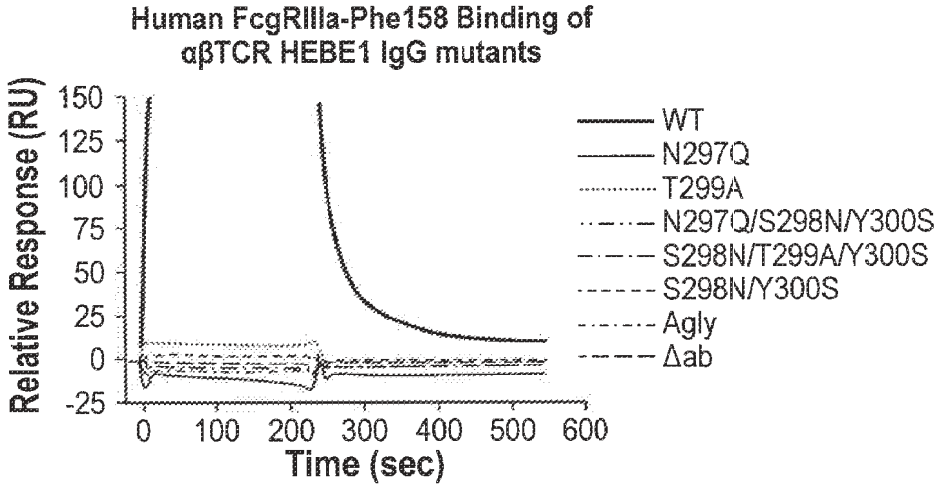


Fig. 3

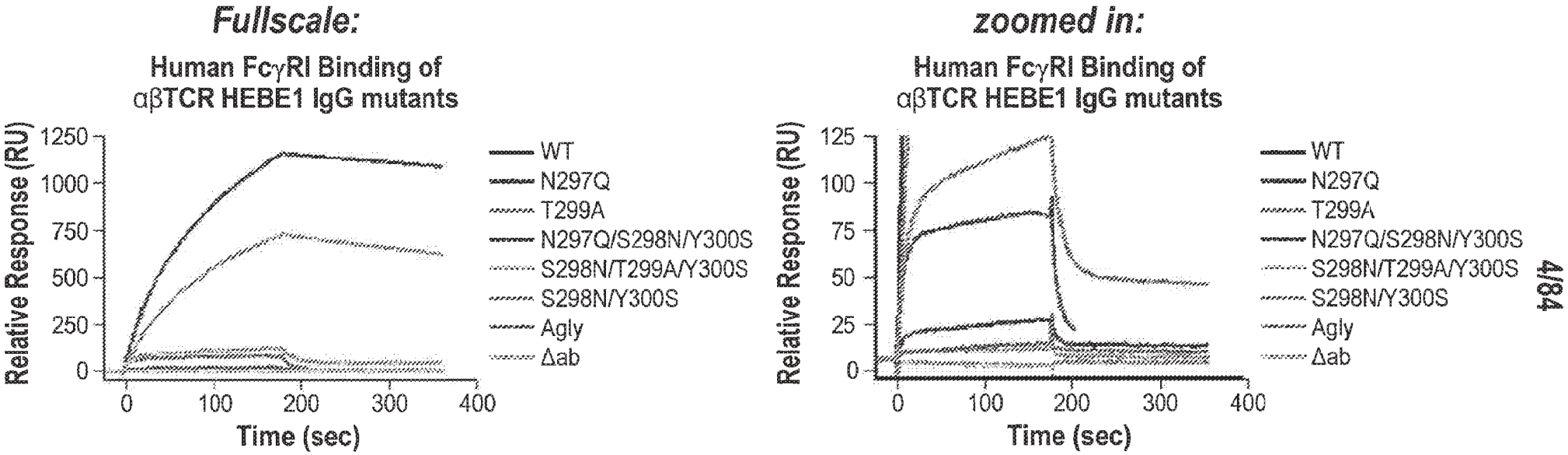

Fullscale:
Human FcγRI Binding of
αβTCR HEBE1 IgG mutants
Relative Response (RU)
1250
1000
750
500
250
0
0
100
200
300
400
Time (sec)
WT
N297Q
T299A
N297Q/S298N/Y300S
S298N/T299A/Y300S
S298N/Y300S
Agly
Δab
zoomed in:
Human FcγRI Binding of
αβTCR HEBE1 IgG mutants
Relative Response (RU)
125
100
75
50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

Fig. 4

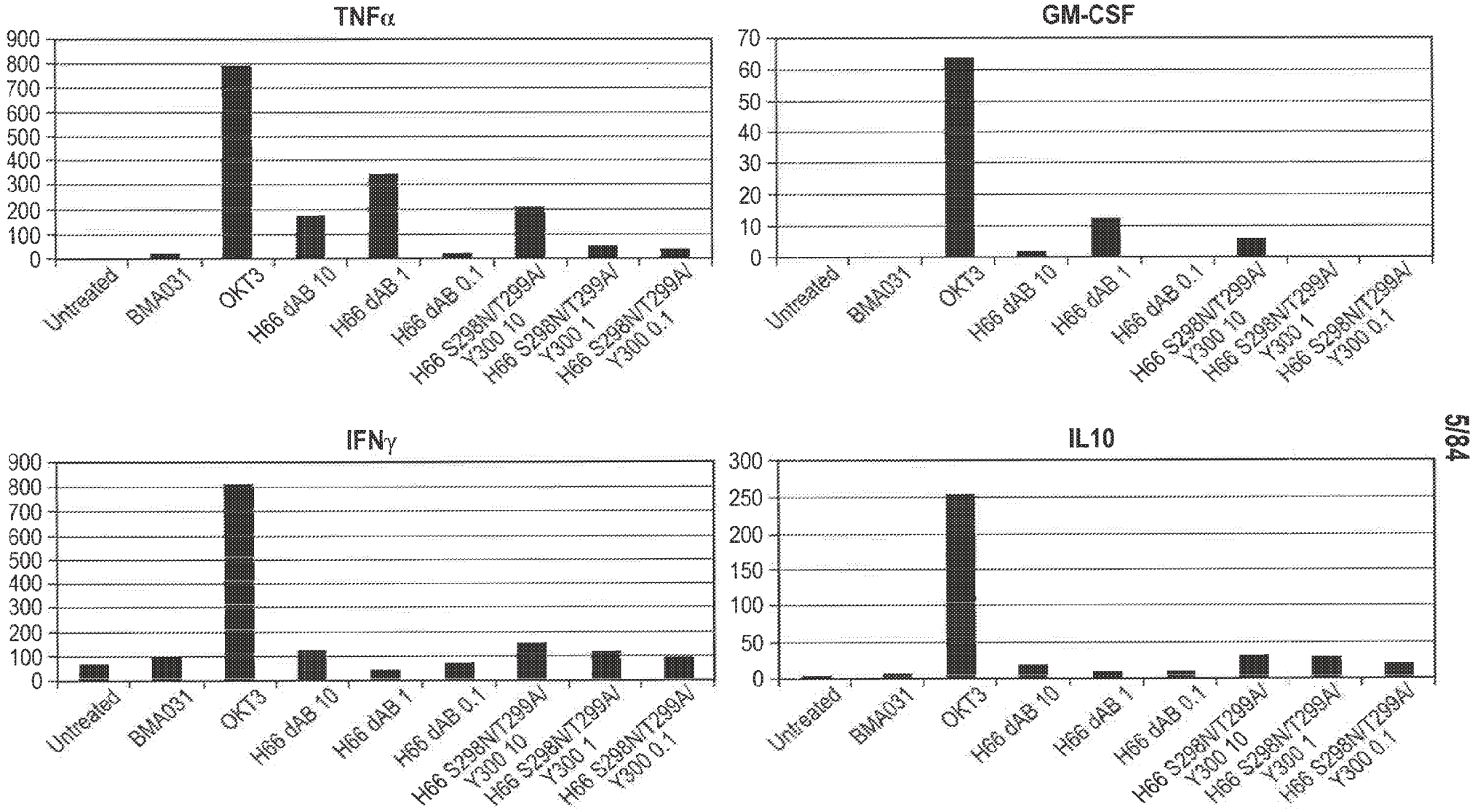

TNFα
900
800
700
600
500
400
300
200
100
0
Untreated
BMA031
OKT3
H66 dAB 10
H66 dAB 1
H66 dAB 0.1
H66 S298N/T299A/ Y300 10
H66 S298N/T299A/ Y300 1
H66 S298N/T299A/ Y300 0.1
GM-CSF
70
60
50
40
30
20
10
0
IFNγ
IL10
300
250
200
150
100
50

Fig. 5

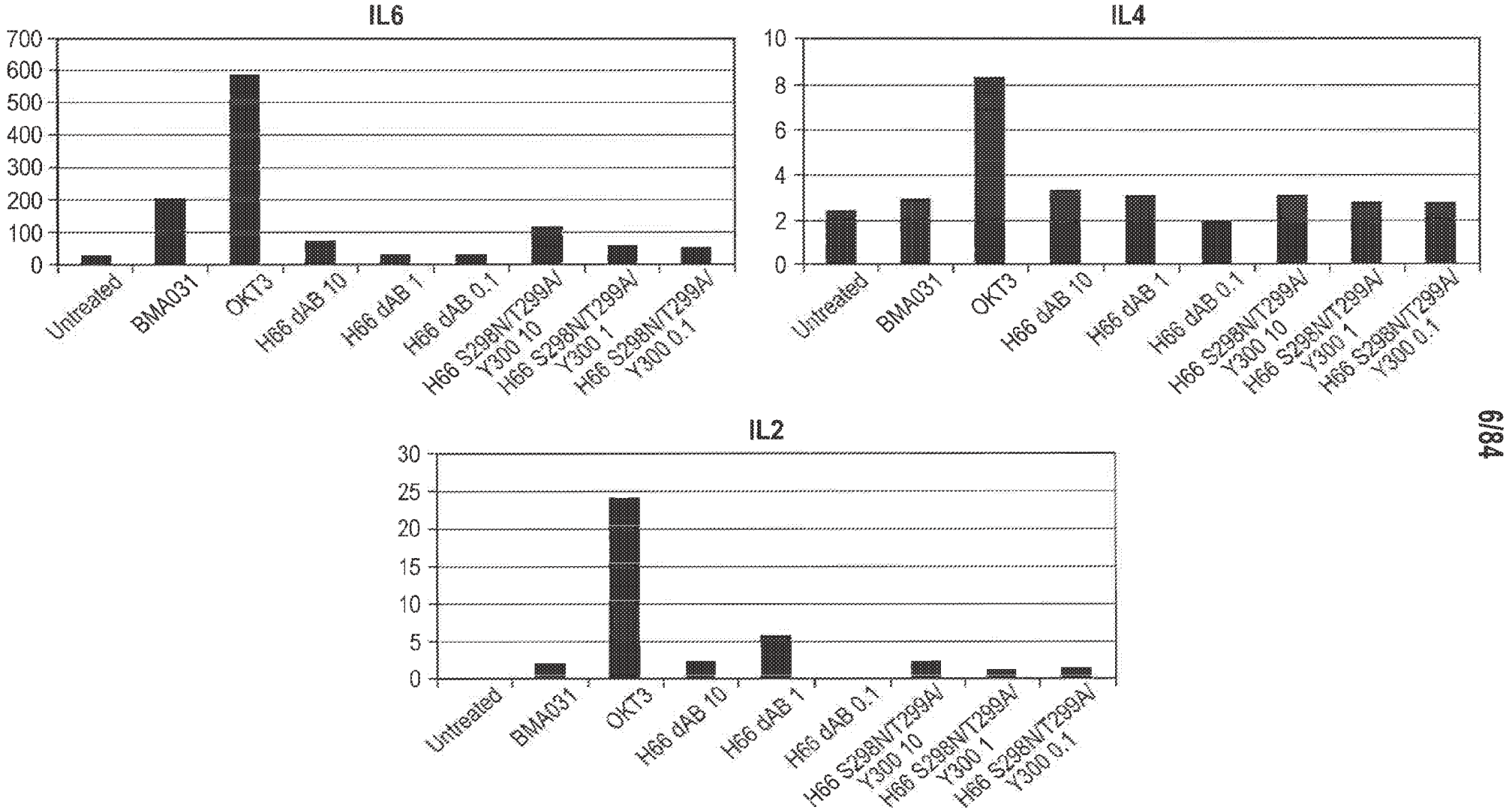

IL6
700
600
500
400
300
200
100
0
Untreated
BMA031
OKT3
H66 dAB 10
H66 dAB 1
H66 dAB 0.1
H66 S298N/T299A/ Y300 10
H66 S298N/T299A/ Y300 1
H66 S298N/T299A/ Y300 0.1
IL4
10
8
6
4
2
0
Untreated
BMA031
OKT3
H66 dAB 10
H66 dAB 1
H66 dAB 0.1
H66 S298N/T299A/ Y300 10
H66 S298N/T299A/ Y300 1
H66 S298N/T299A/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

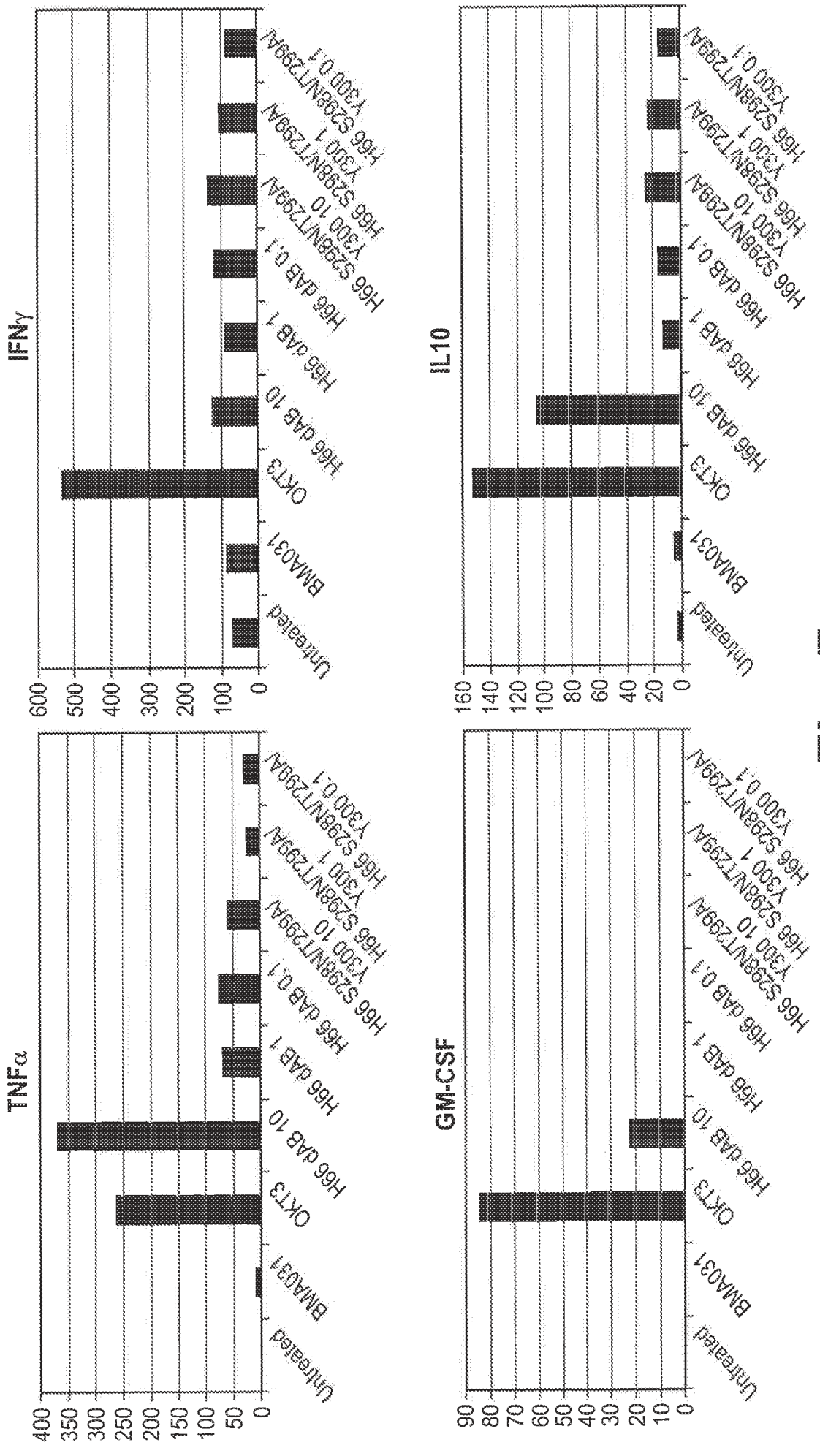

IFNγ
600
500
400
300
200
100
0
Untreated
BMA031
OKT3
H66 dAb 10
H66 dAb 1
H66 dAb 0,1
H66 S298N/T299A Y300 10
H66 S298N/T299A Y300 1
H66 S298N/T299A Y300 0,1
IL10
160
140
120
100
80
60
40
20
0
GM-CSF
90
80
70
60
50
40
30
20
10
0
TNFα
400
350
300
250
200
150
100
50
0

Fig. 7

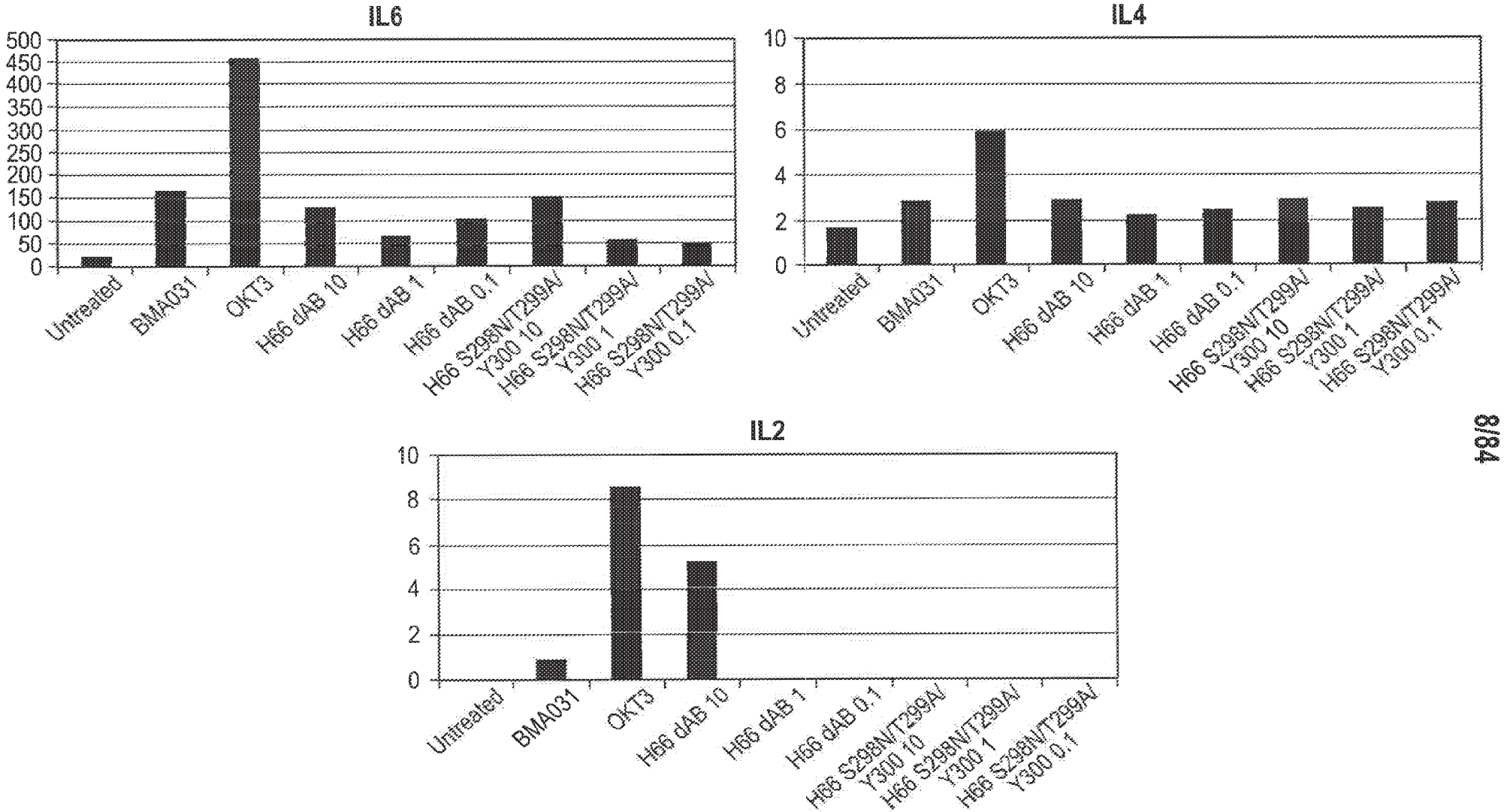
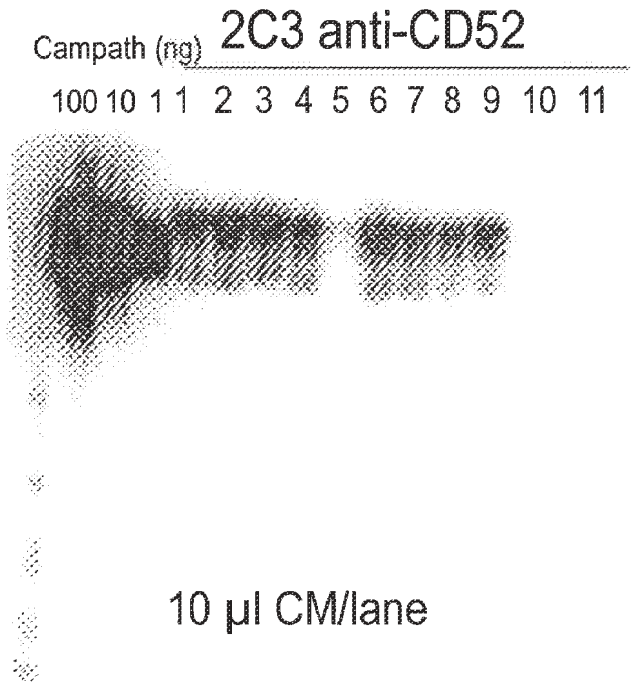

IL6
500
450
400
350
300
250
200
150
100
50
0
Untreated
BMA031
OKT3
H66 dAB 10
H66 dAB 1
H66 dAB 0.1
H66 S298N/T299A/ Y300 10
H66 S298N/T299A/ Y300 1
H66 S298N/T299A/ Y300 0.1
IL4
10
8
6
4
2
0
Untreated
BMA031
OKT3
H66 dAB 10
H66 dAB 1
H66 dAB 0.1
H66 S298N/T299A/ Y300 10
H66 S298N/T299A/ Y300 1
H66 S298N/T299A/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

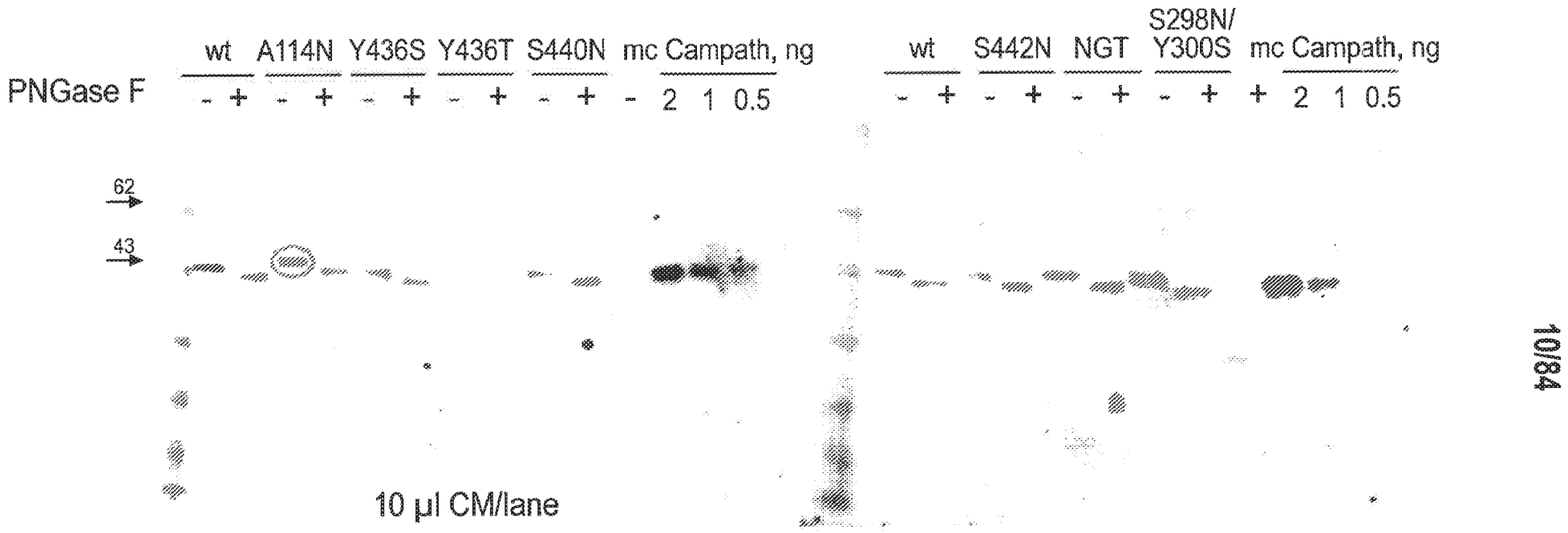

wt
A114N
Y436S
Y436T
S440N
mc Campath, ng
PNGase F
- + - + - + - + - + - 2 1 0.5
wt
S442N
NGT
S298N/
Y300S
mc Campath, ng
- + - + - + - + + 2 1 0.5
62
43
10 µl CM/lane

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

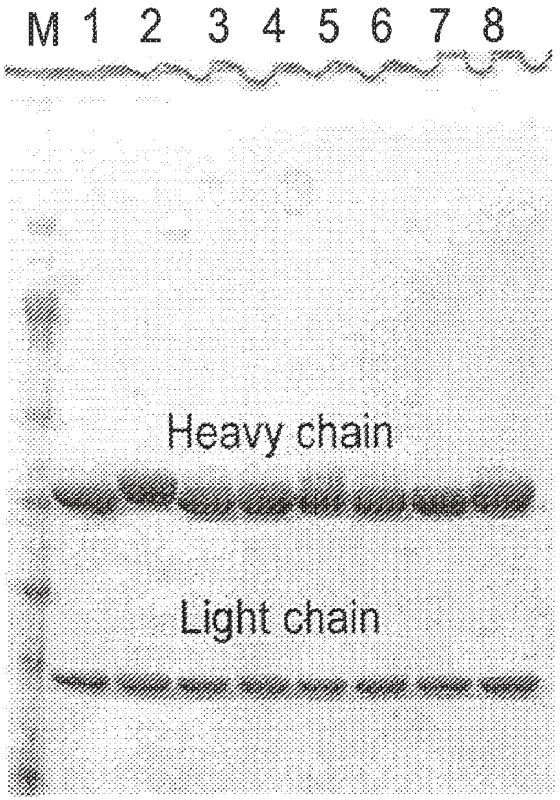
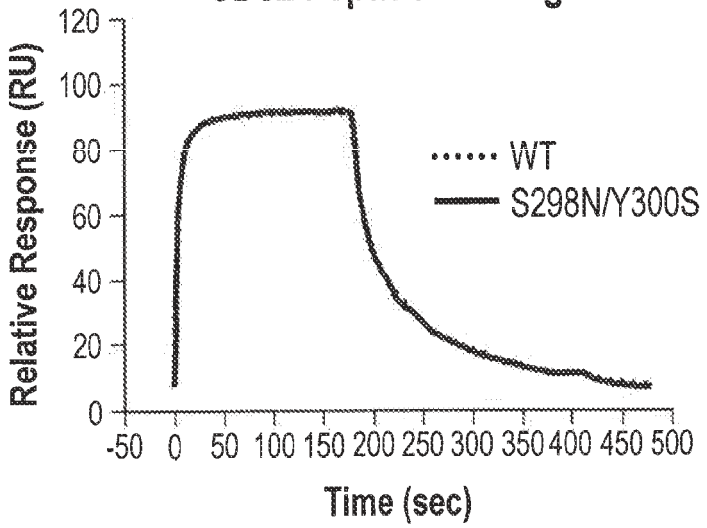
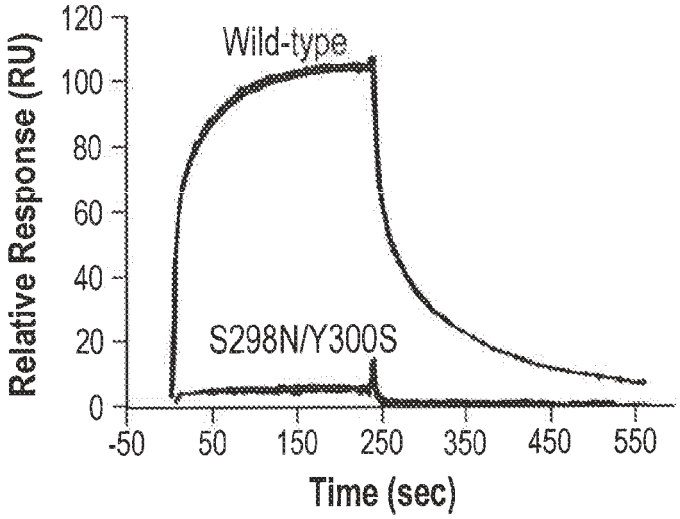


Fig. 11

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)
120
100
80
60
40
20
0
-50 50 150 250 350 450 550
Time (sec)
Wild-type
S298N/Y300S

C.

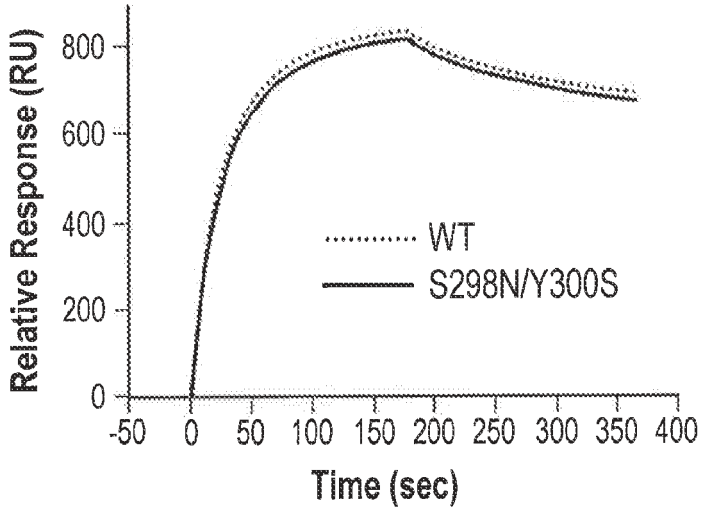

Mouse FcRn Binding
Relative Response (RU)
800
600
400
200
0
-50 0 50 100 150 200 250 300 350 400
Time (sec)
WT
S298N/Y300S

Fig. 12

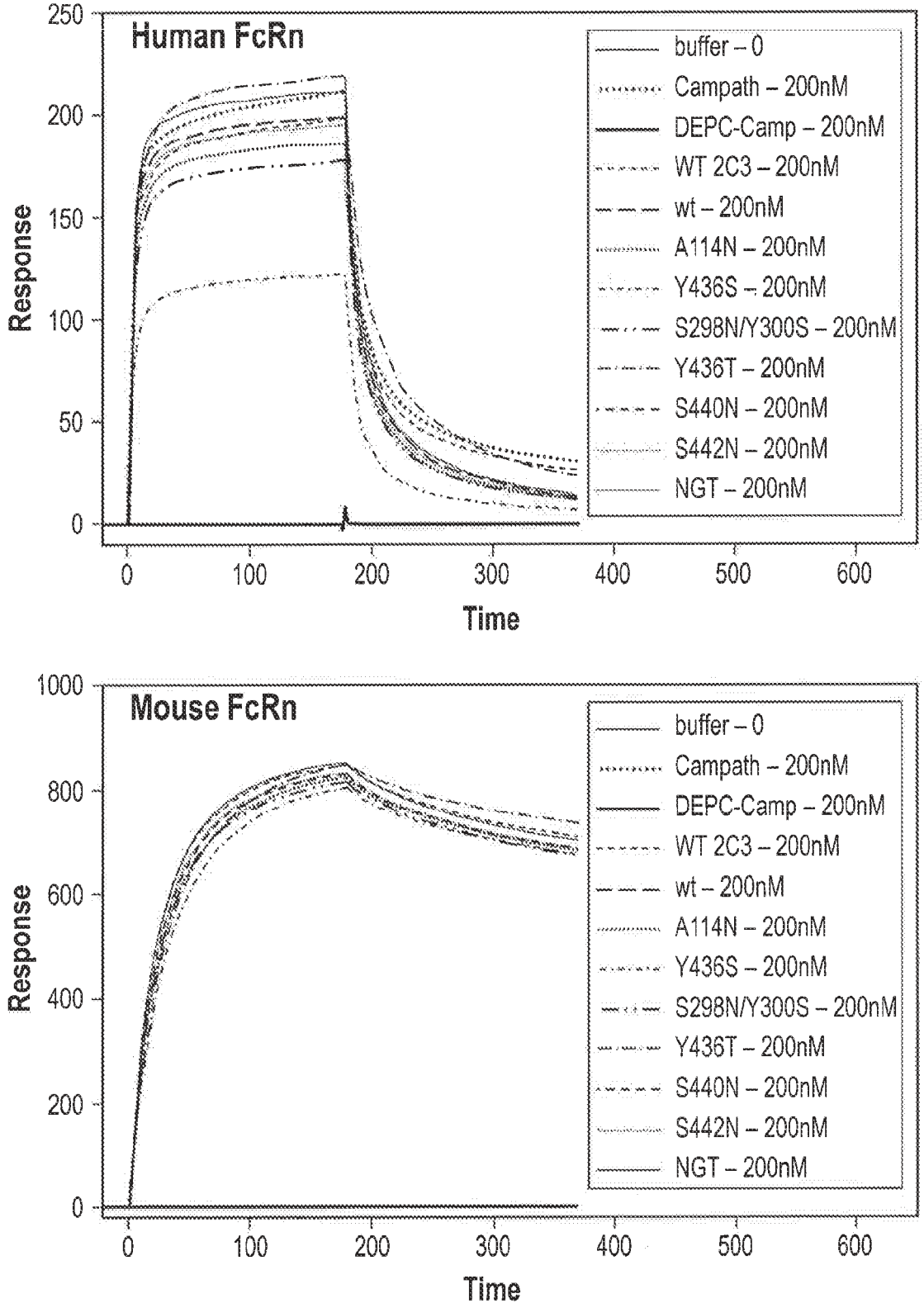
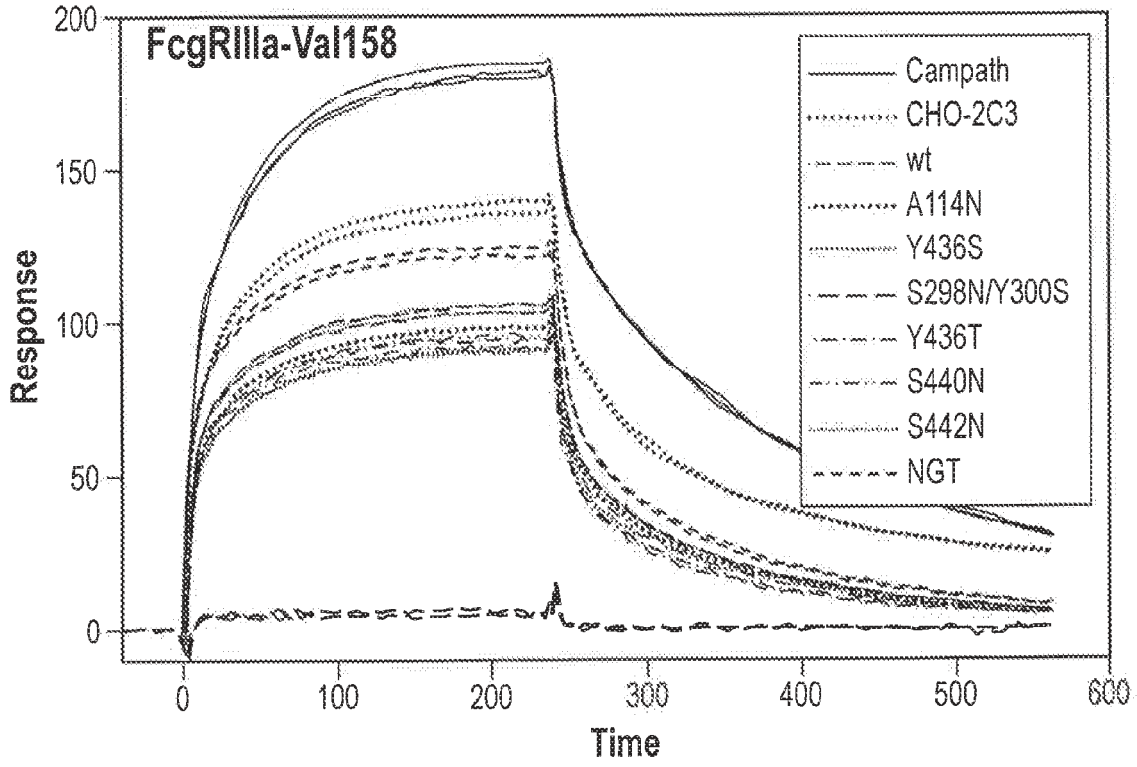

Human FcRn
Response
Time
buffer – 0
Campath – 200nM
DEPC-Camp – 200nM
WT 2C3 – 200nM
wt – 200nM
A114N – 200nM
Y436S – 200nM
S298N/Y300S – 200nM
Y436T – 200nM
S440N – 200nM
S442N – 200nM
NGT – 200nM
Mouse FcRn
Response
Time
buffer – 0
Campath – 200nM
DEPC-Camp – 200nM
WT 2C3 – 200nM
wt – 200nM
A114N –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

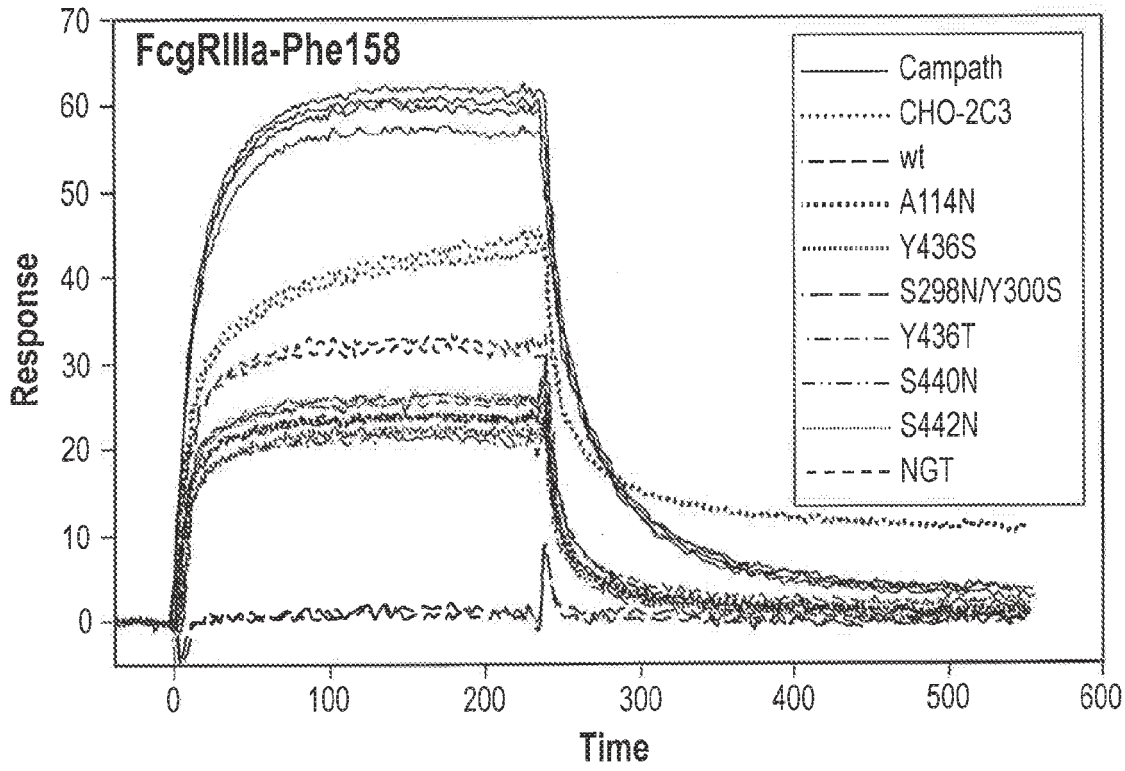
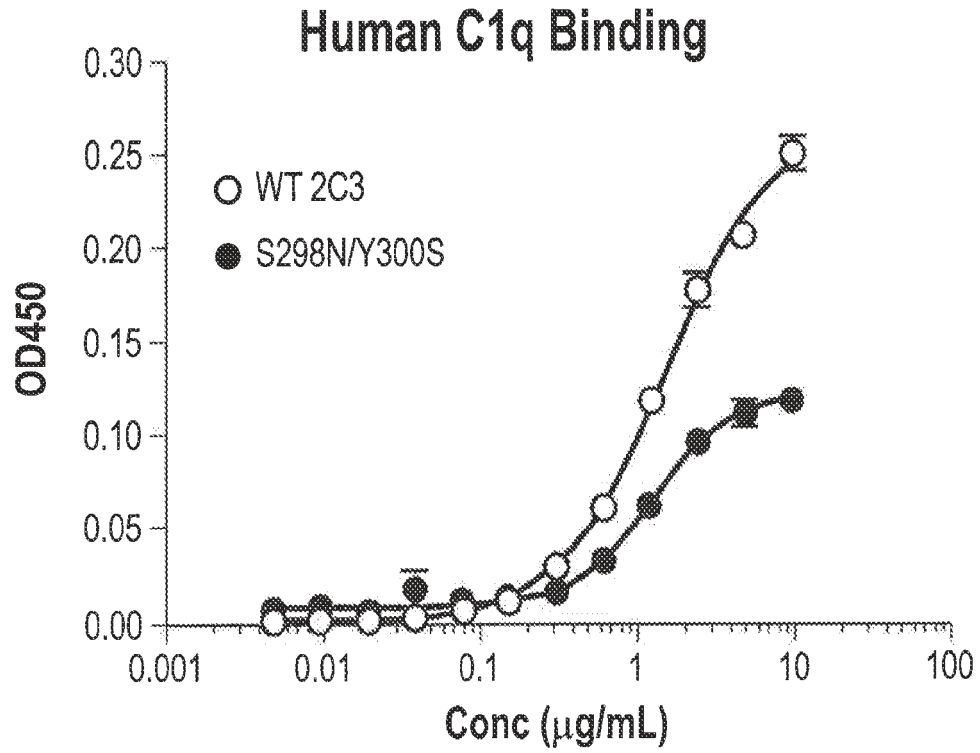

FcgRIIIa-Phe158
Campath
CHO-2C3
wt
A114N
Y436S
S298N/Y300S
Y436T
S440N
S442N
NGT
Response
Time
0
10
20
30
40
50
60
70
0
100
200
300
400
500
600

Fig. 14

A.


Human C1q Binding
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.

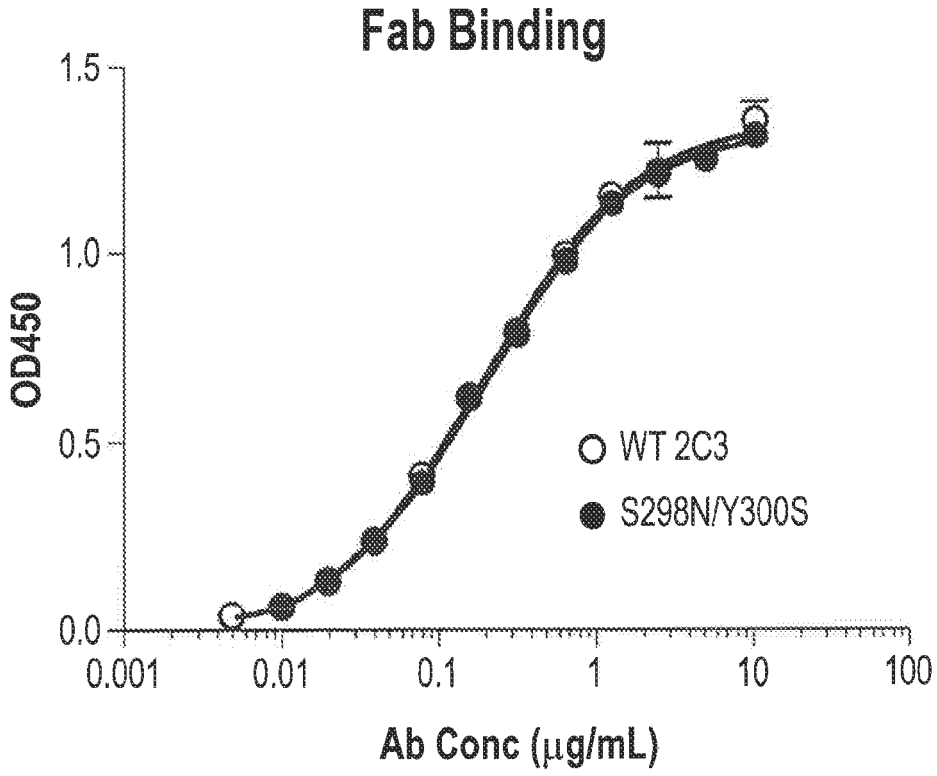

Fab Binding
1.5
1.0
0.5
0.0
OD450
WT 2C3
S298N/Y300S
0.001
0.01
0.1
1
10
100
Ab Conc (μg/mL)

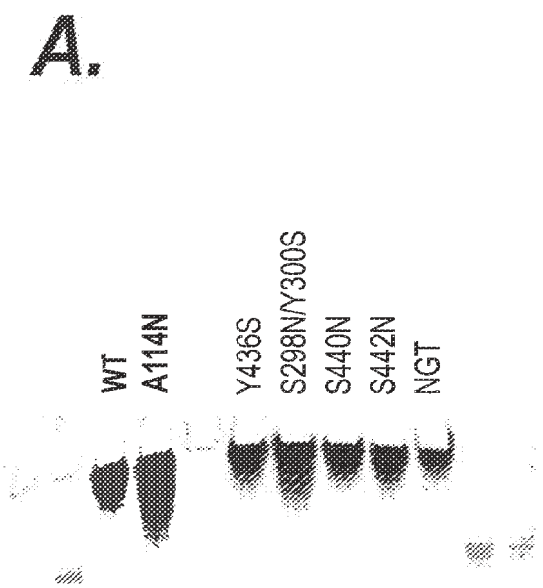
Fig. 15

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

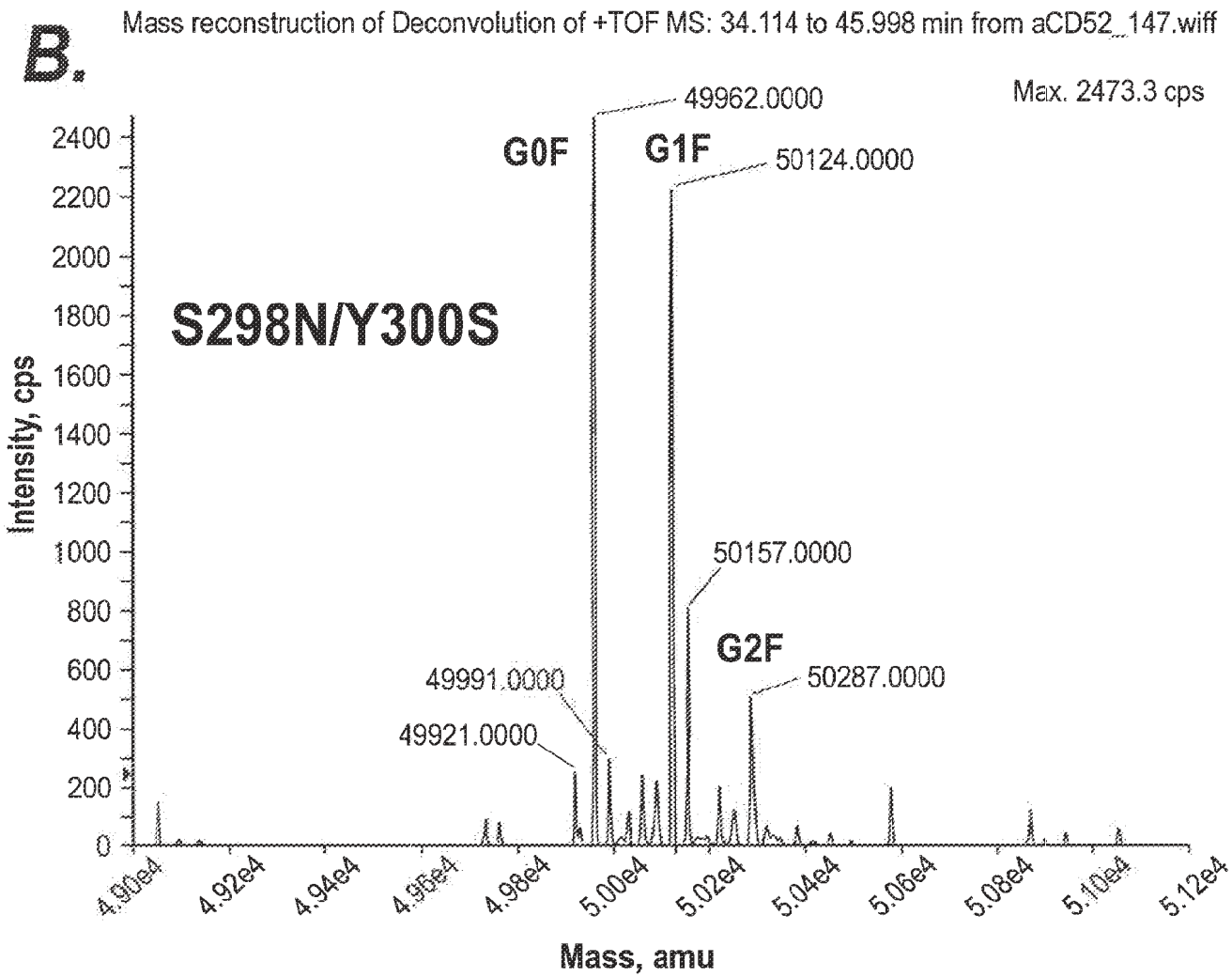

B.
Mass reconstruction of Deconvolution of +TOF MS: 34.114 to 45.998 min from aCD52_147.wiff
Max. 2473.3 cps
S298N/Y300S
G0F
G1F
G2F
49962.0000
50124.0000
50157.0000
50287.0000
49991.0000
49921.0000
Intensity, cps
Mass, amu

Fig. 18

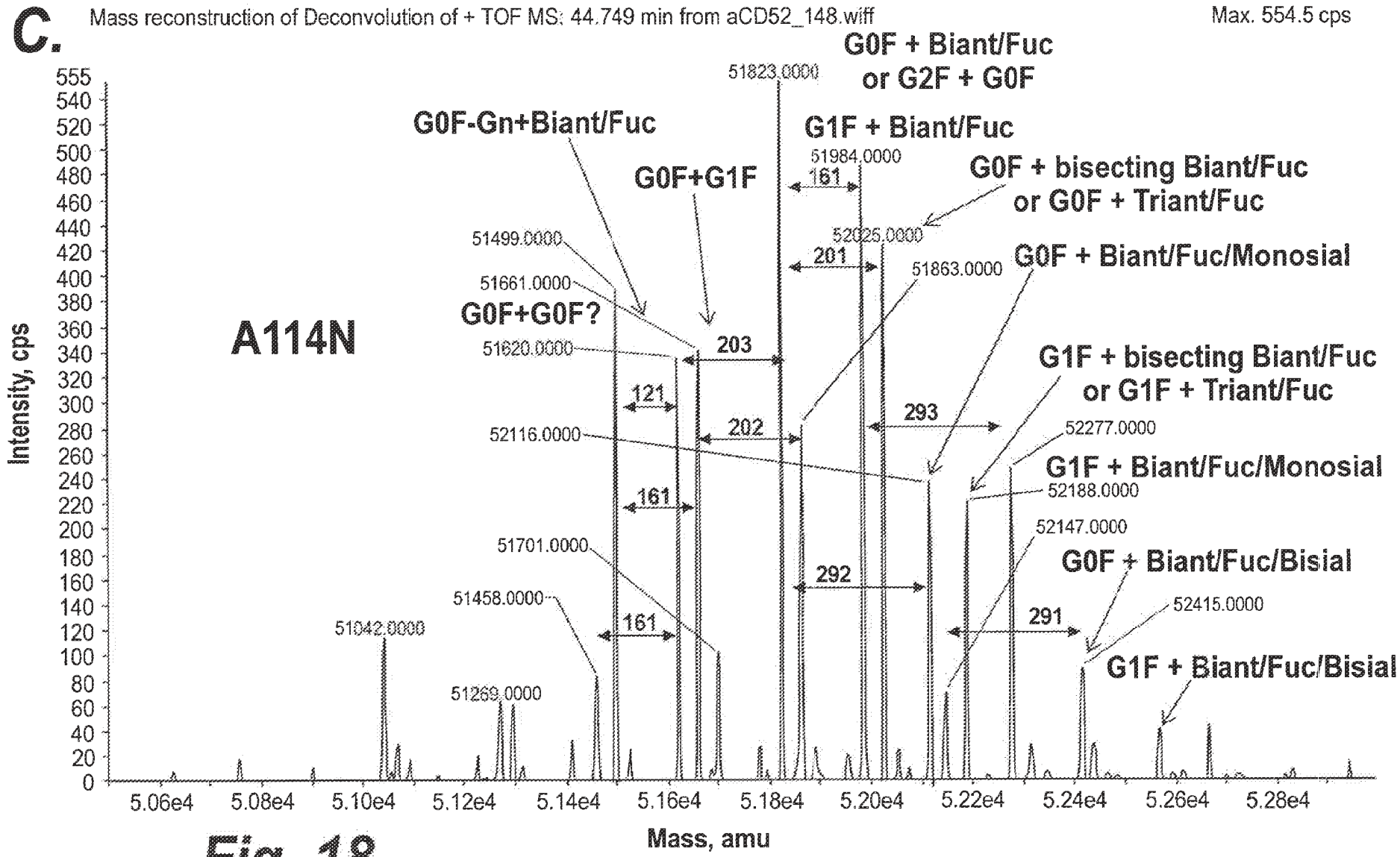

C.
Mass reconstruction of Deconvolution of + TOF MS: 44.749 min from aCD52_148.wiff
Max. 554.5 cps
A114N
Intensity, cps
Mass, amu
555
540
520
500
480
460
440
420
400
380
360
340
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
5.06e4
5.08e4
5.10e4
5.12e4
5.14e4
5.16e4
5.18e4
5.20e4
5.22e4
5.24e4
5.26e4
5.28e4
51823.0000
G0F + Biant/Fuc
or G2F + G0F
G0F-Gn+Biant/Fuc
G1F + Biant/Fuc
51984.0000
161
G0F+G1F
G0F + bisecting Biant/Fuc
or G0F + Triant/Fuc
52025.0000
51499.0000
201
51661.0000
51863.0000
G0F + Biant/Fuc/Monosial
G0F+G0F?
51620.0000
203
G1F + bisecting Biant/Fuc
or G1F + Triant/Fuc
121
293
202
52277.0000
52116.0000
G1F + Biant/Fuc/Monosial
52188.0000
161
52147.0000
51701.0000
G0F + Biant/Fuc/Bisial
292
51458.0000
52415.0000
291
51042.0000
161
G1F + Biant/Fuc/Bisial
51269.0000

Fig. 18

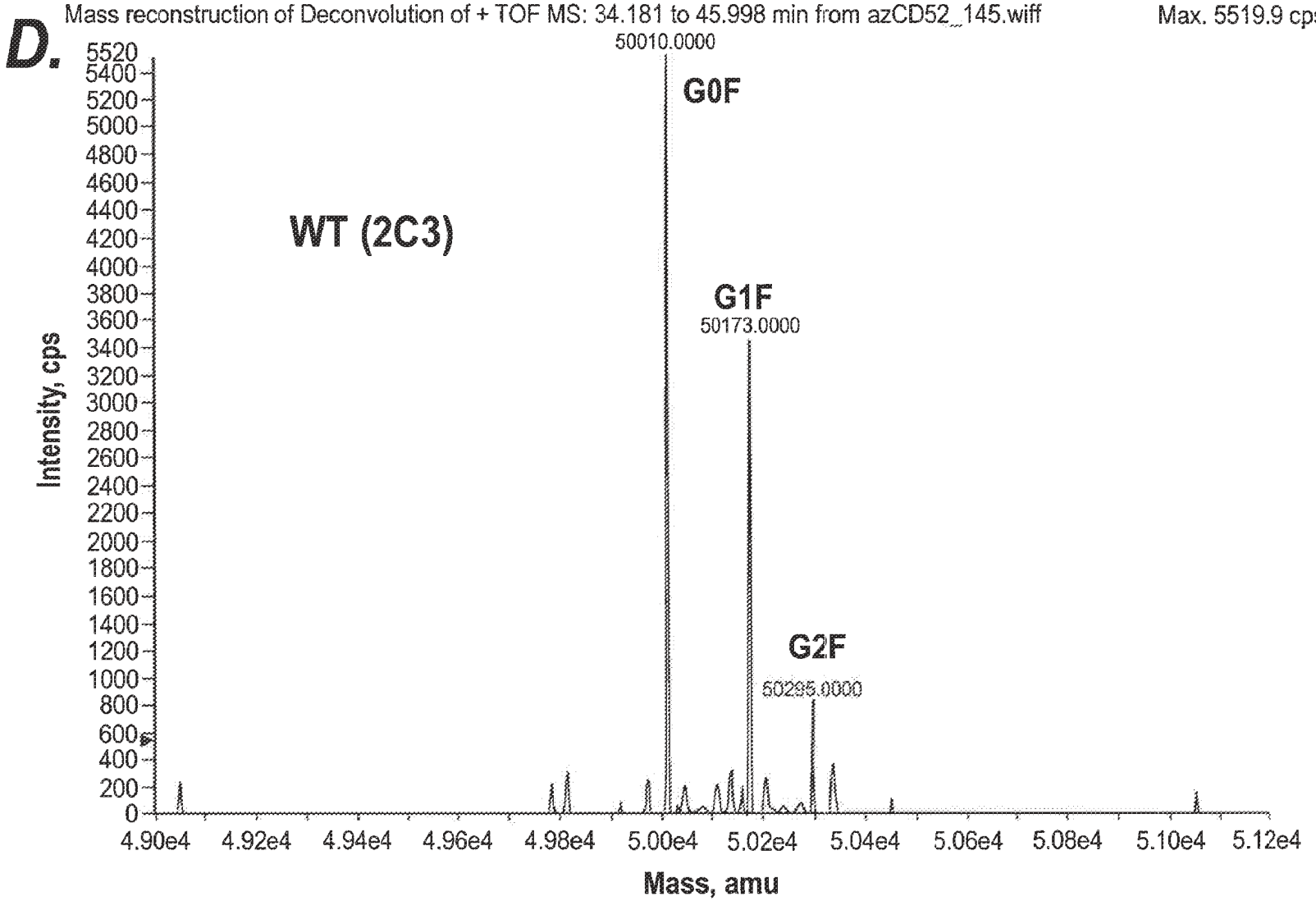

D.
Mass reconstruction of Deconvolution of + TOF MS: 34.181 to 45.998 min from azCD52_145.wiff
Max. 5519.9 cps
50010.0000
G0F
WT (2C3)
G1F
50173.0000
G2F
50295.0000
Intensity, cps
5520
5400
5200
5000
4800
4600
4400
4200
4000
3800
3600
3400
3200
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
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
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.

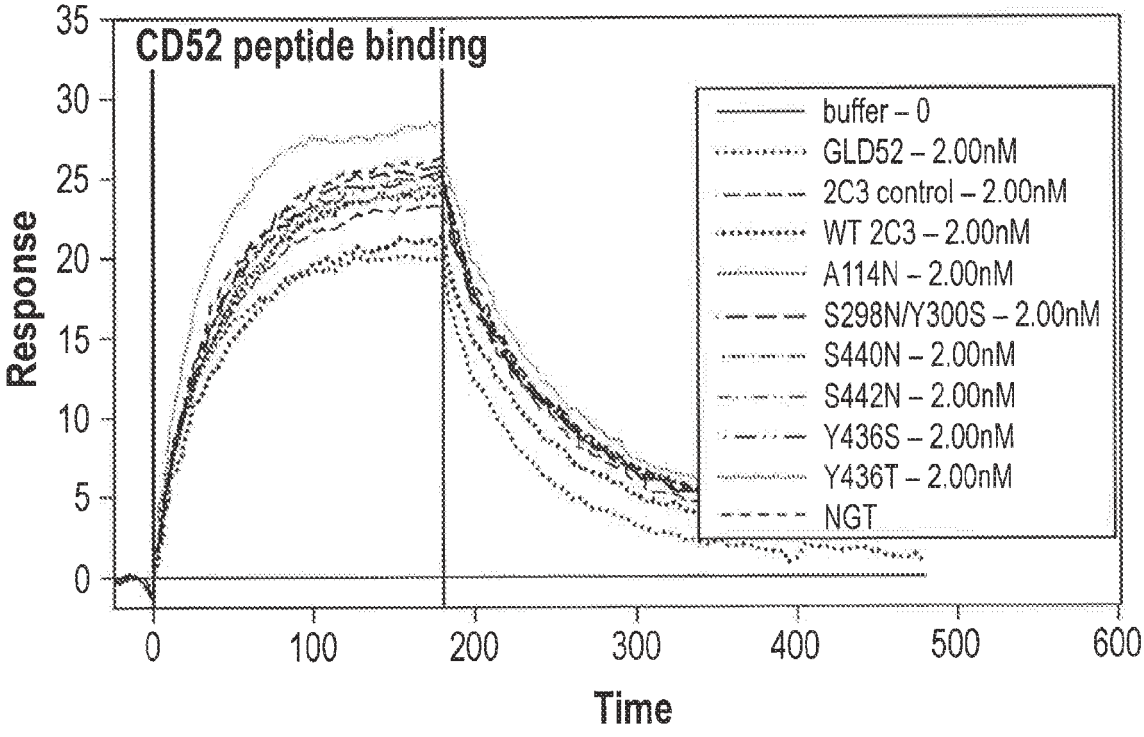


Fig. 19

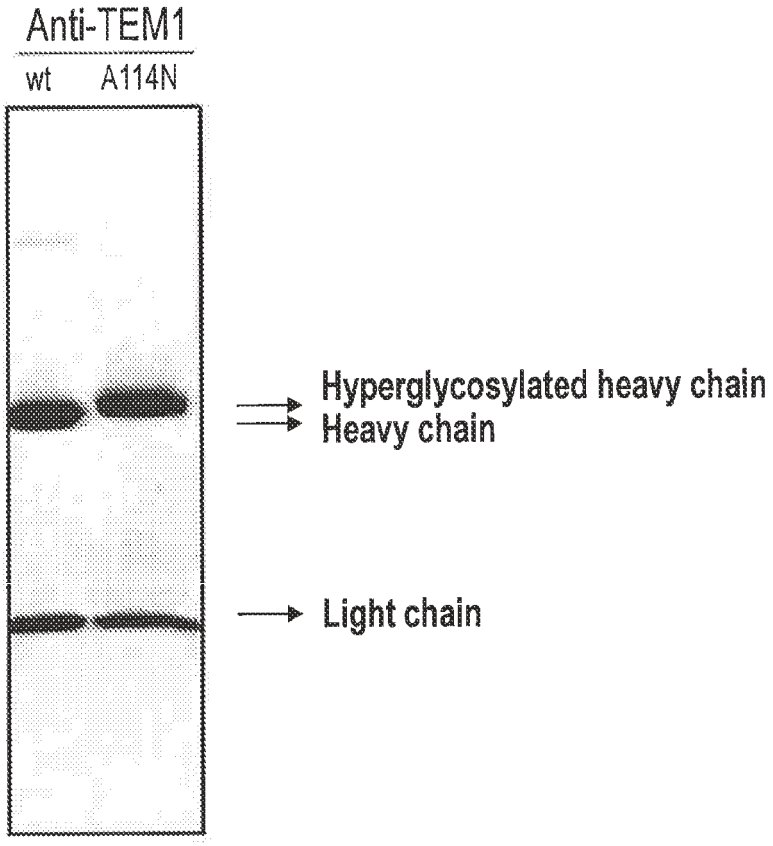

Anti-TEM1
wt
A114N
Hyperglycosylated heavy chain
Heavy chain
Light chain

Fig. 20

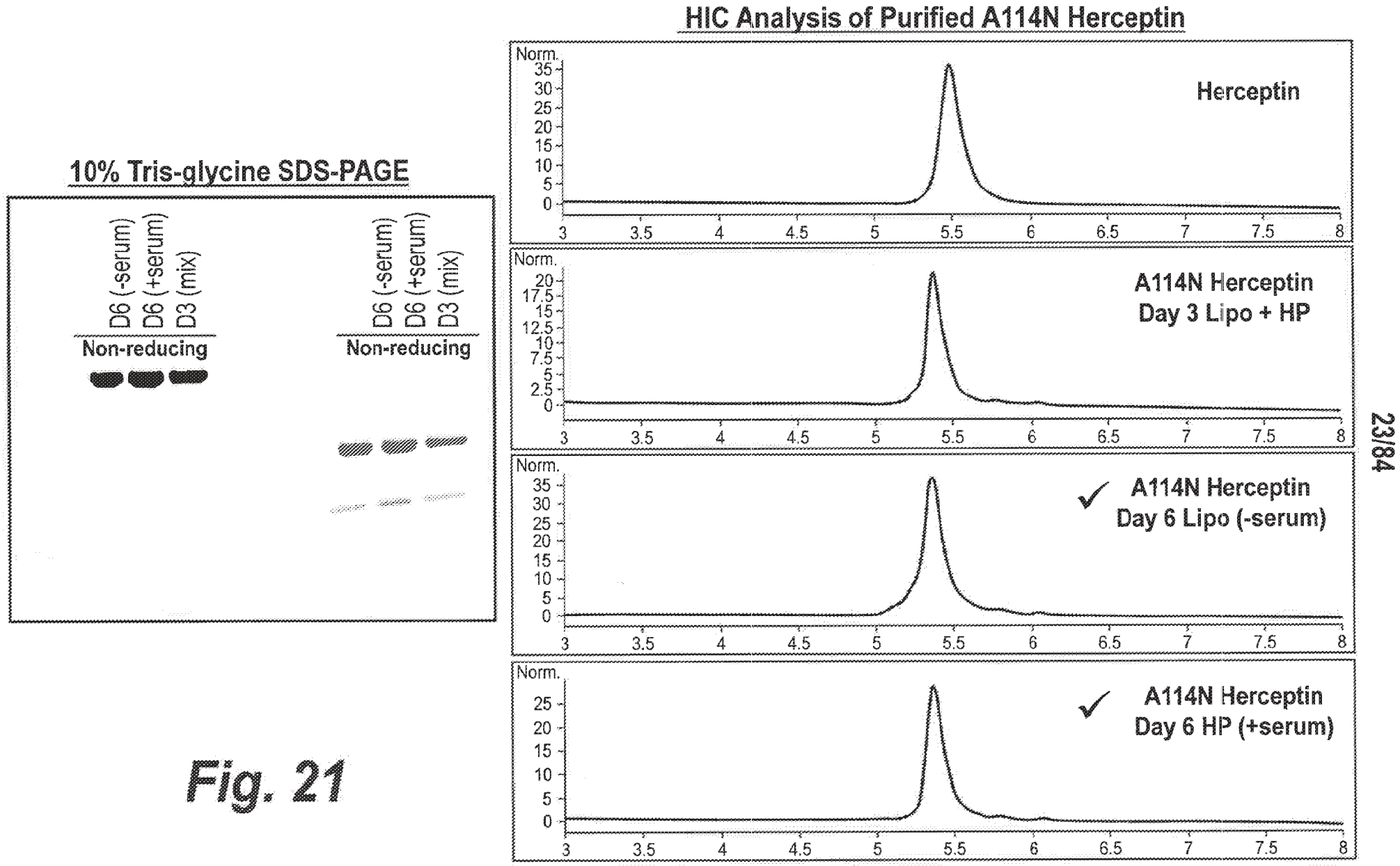

10% Tris-glycine SDS-PAGE
D6 (-serum)
D6 (+serum)
D3 (mix)
Non-reducing
D6 (-serum)
D6 (+serum)
D3 (mix)
Non-reducing
HIC Analysis of Purified A114N Herceptin
Norm.
Herceptin
A114N Herceptin
Day 3 Lipo + HP
A114N Herceptin
Day 6 Lipo (-serum)
A114N Herceptin
Day 6 HP (+serum)

Fig. 21

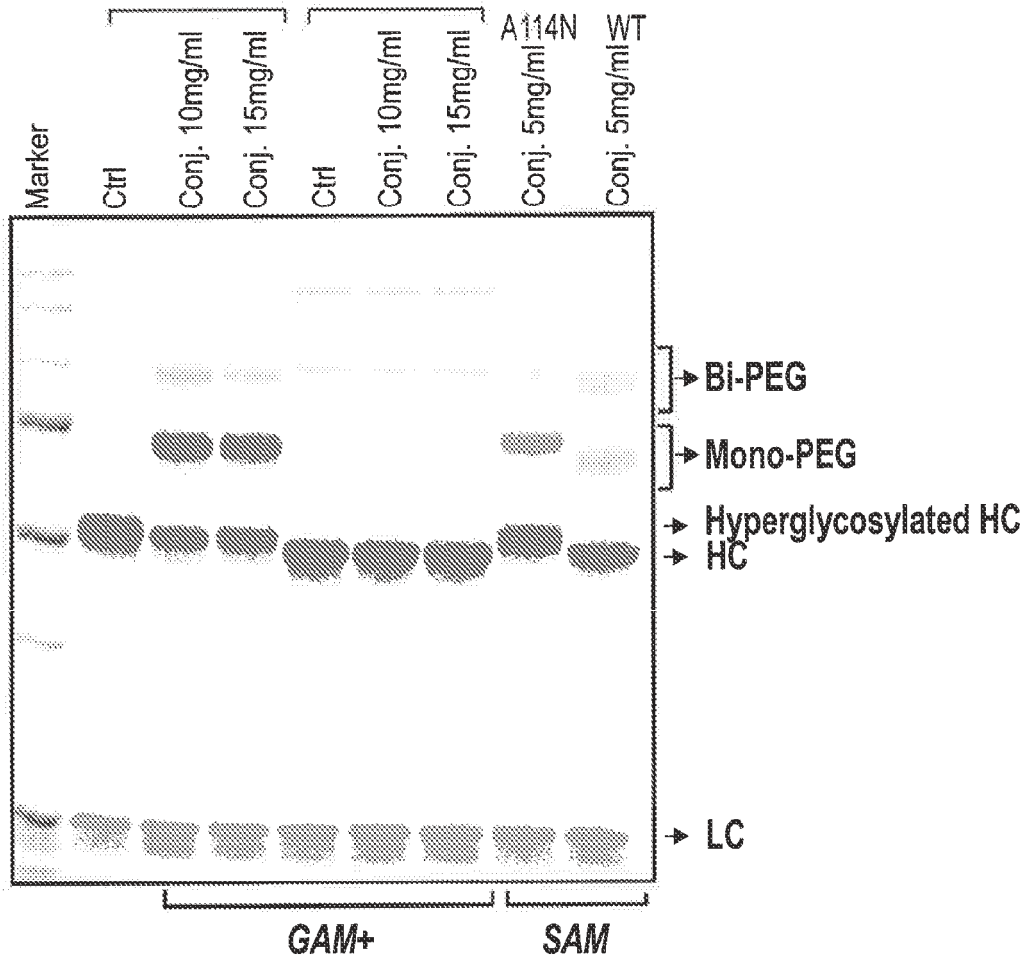

Marker
Ctrl
Conj. 10mg/ml
Conj. 15mg/ml
Ctrl
Conj. 10mg/ml
Conj. 15mg/ml
A114N
Conj. 5mg/ml
WT
Conj. 5mg/ml
Bi-PEG
Mono-PEG
Hyperglycosylated HC
HC
LC
GAM+
SAM

Fig. 22

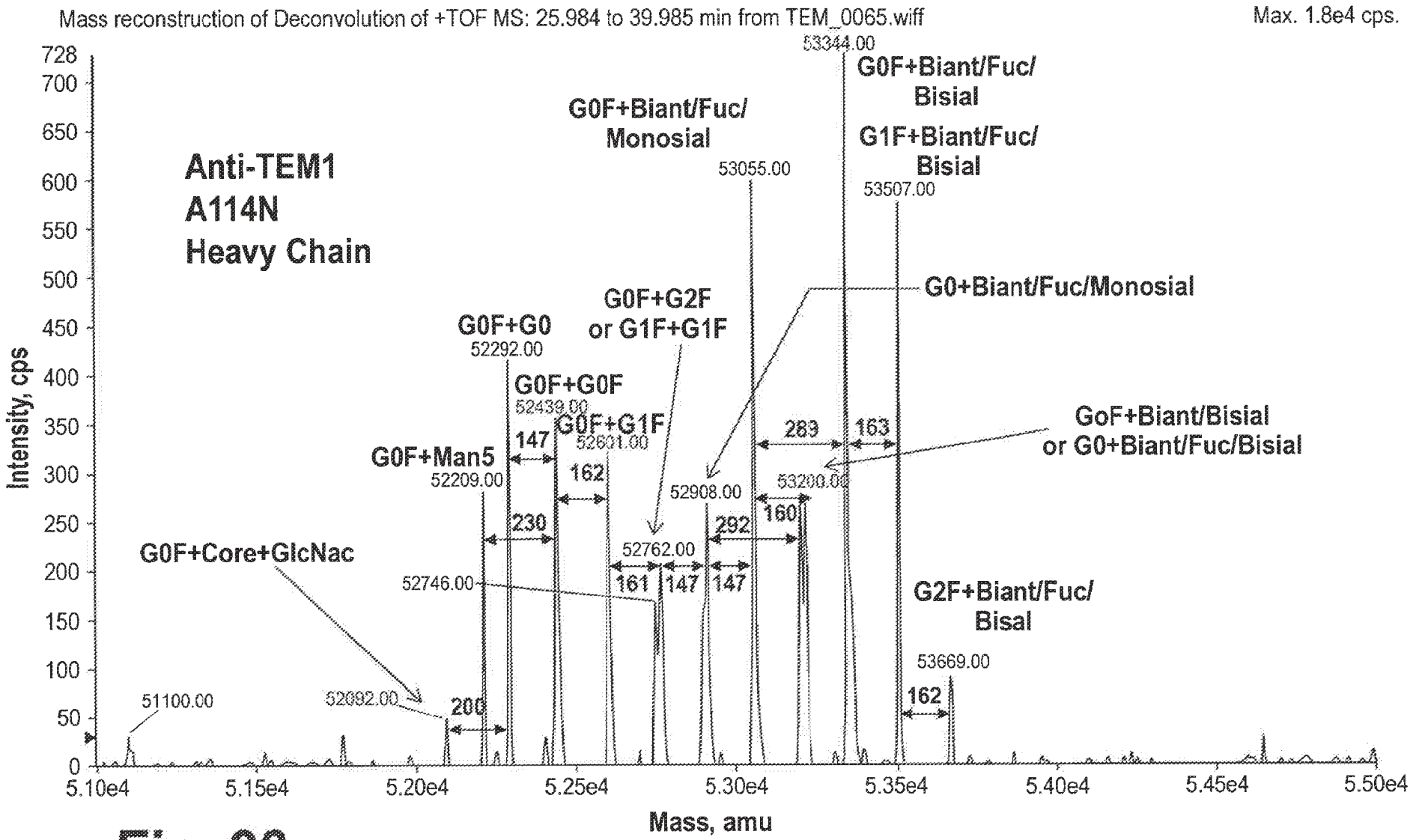

Mass reconstruction of Deconvolution of +TOF MS: 25.984 to 39.985 min from TEM_0065.wiff
Max. 1.8e4 cps.
Anti-TEM1
A114N
Heavy Chain
G0F+Biant/Fuc/
Bisial
G1F+Biant/Fuc/
Bisial
G0F+Biant/Fuc/
Monosial
G0+Biant/Fuc/Monosial
G0F+G2F
or G1F+G1F
G0F+G0
G0F+G0F
G0F+G1F
G0F+Man5
GoF+Biant/Bisial
or G0+Biant/Fuc/Bisial
G0F+Core+GlcNac
G2F+Biant/Fuc/
Bisal
53344.00
53055.00
53507.00
52292.00
52439.00
52601.00
52209.00
52908.00
53200.00
52762.00
52746.00
52092.00
51100.00
53669.00
147
162
230
200
283
163
160
292
161
147
147
Intensity, cps
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
Mass, amu

Fig. 23

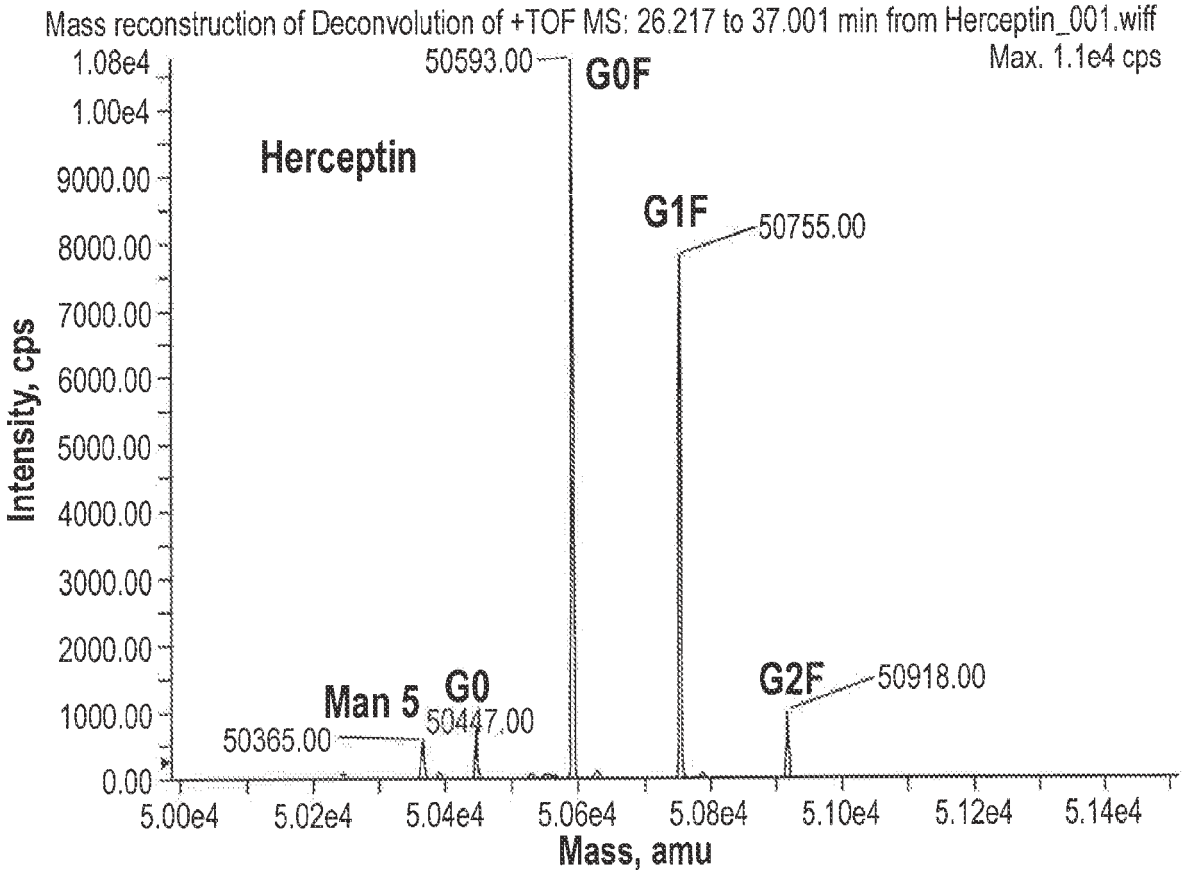

Mass reconstruction of Deconvolution of +TOF MS: 26.217 to 37.001 min from Herceptin_001.wiff
Max. 1.1e4 cps
Herceptin
50593.00
G0F
G1F
50755.00
Man 5
G0
50365.00
50447.00
G2F
50918.00
Intensity, cps
Mass, amu
Mass reconstruction of Deconvolution of +TOF MS: 26.401 to 37.051 min from Herceptin_002.wiff
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
52418.00
52334.00
52460.00
52738.00
52666.00
52623.00
+K, G0F+Hybrid
51326.00
52049.00
+K, G0F/G0F
52698.00
+K,
G0F+biant/fuc/
bisial
+K,Man5+core/
fuc+GlcNac
52212.00
52786.00
Non-Mutant
G1F
52212.00
51822.00
51980.00
+K,
G1F+biant/fuc/
bisial
Non-Mutant
G0F
51594.00
53120.00
52988.00
51851.00
50757.00
50594.00
53282.00
Intensity, cps
Mass, amu

Fig. 24

A.

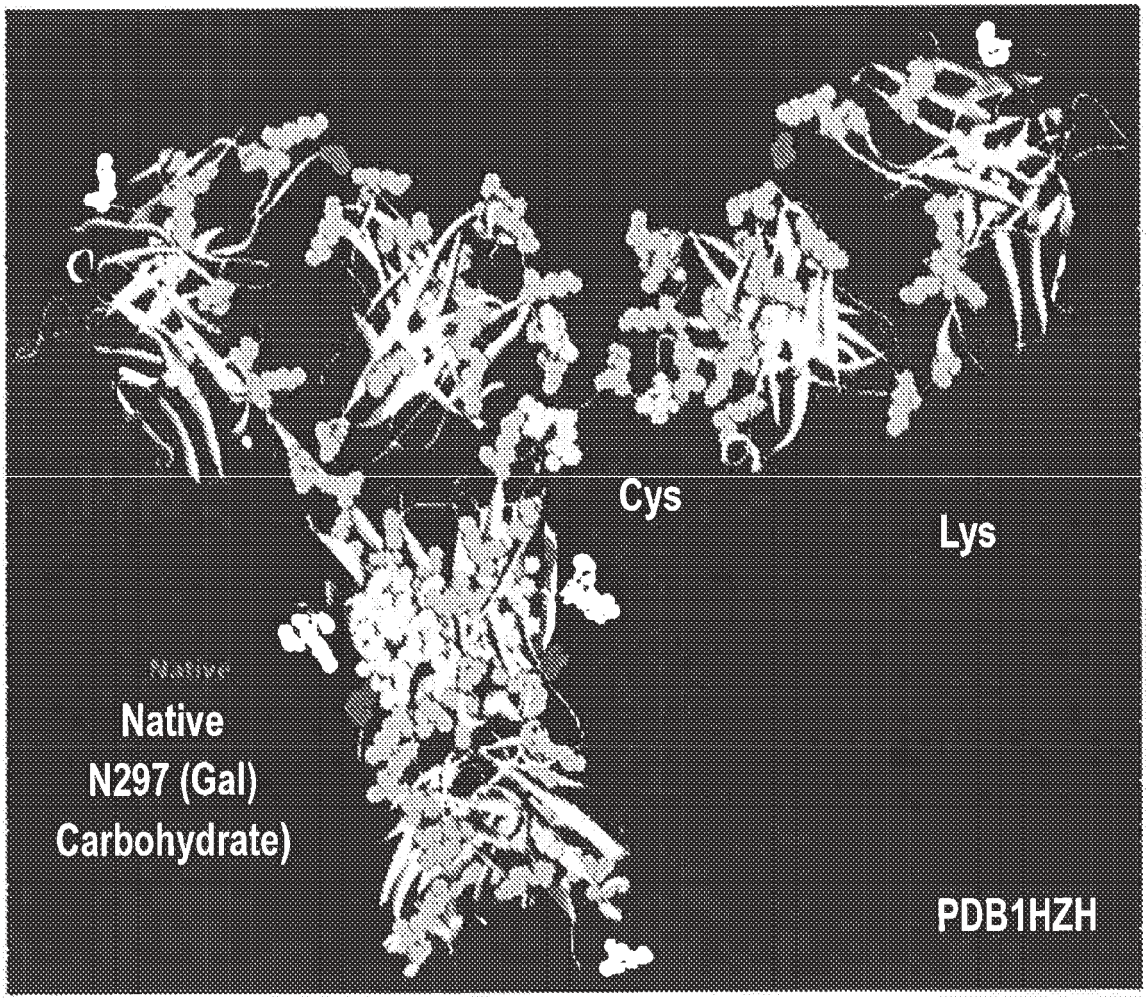
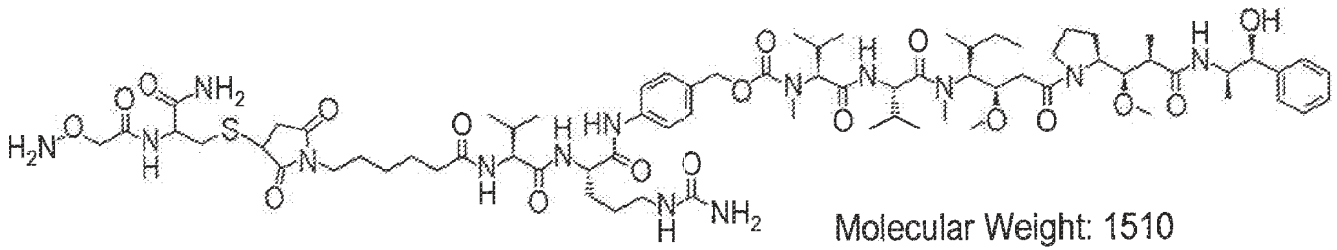


Fig. 25

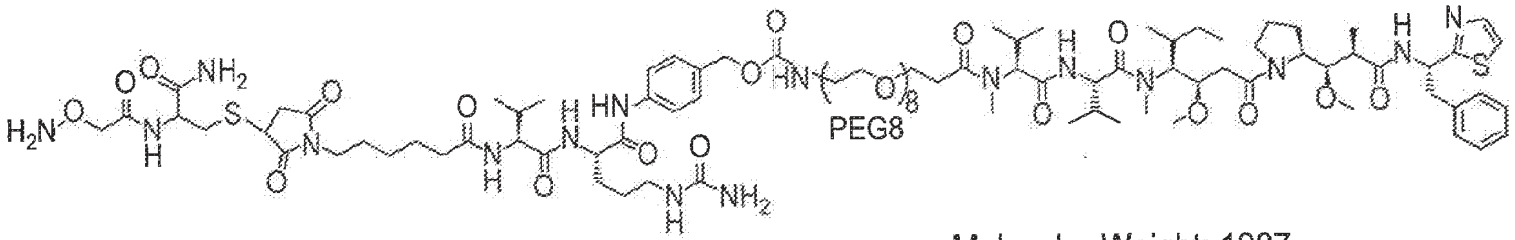
B.

Aminooxy-Cys-MC-VC-PABC-MMAE



Molecular Weight: 1510

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



Molecular Weight: 1987

Fig. 25

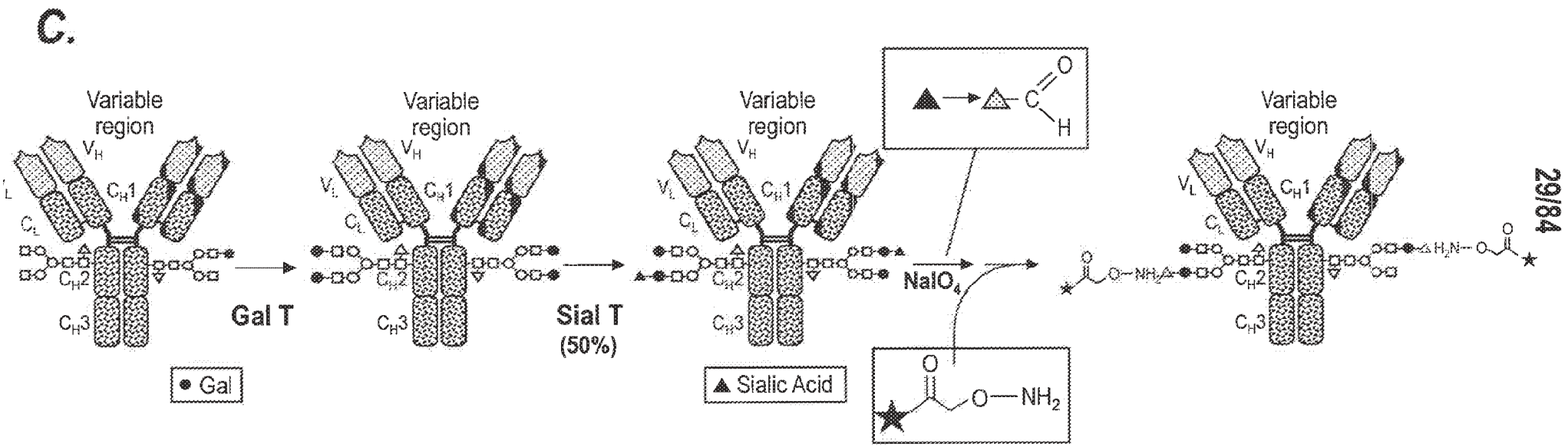

C.
Variable region
V_H
V_L
C_H1
C_L
C_H2
C_H3
Gal T
Sial T
(50%)
$NaIO_4$
● Gal
▲ Sialic Acid
O
C
H
O—NH_2
H_2N—O

Fig. 25

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

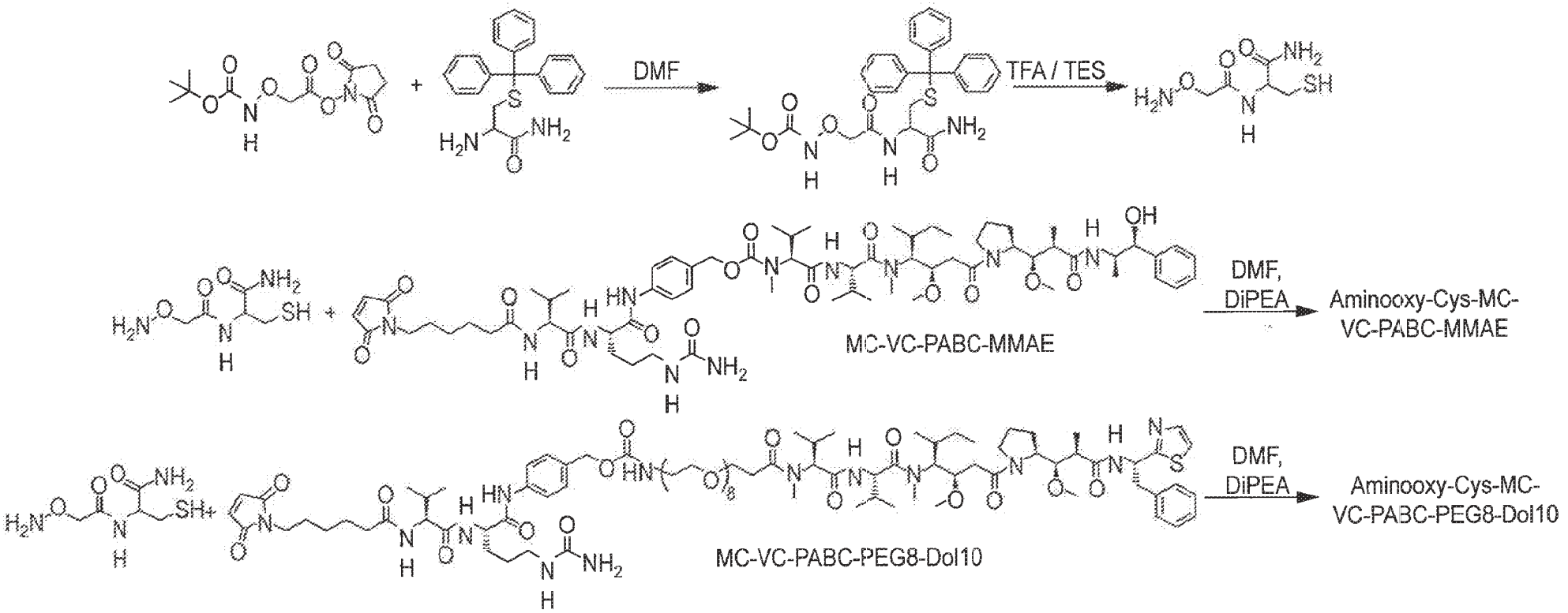
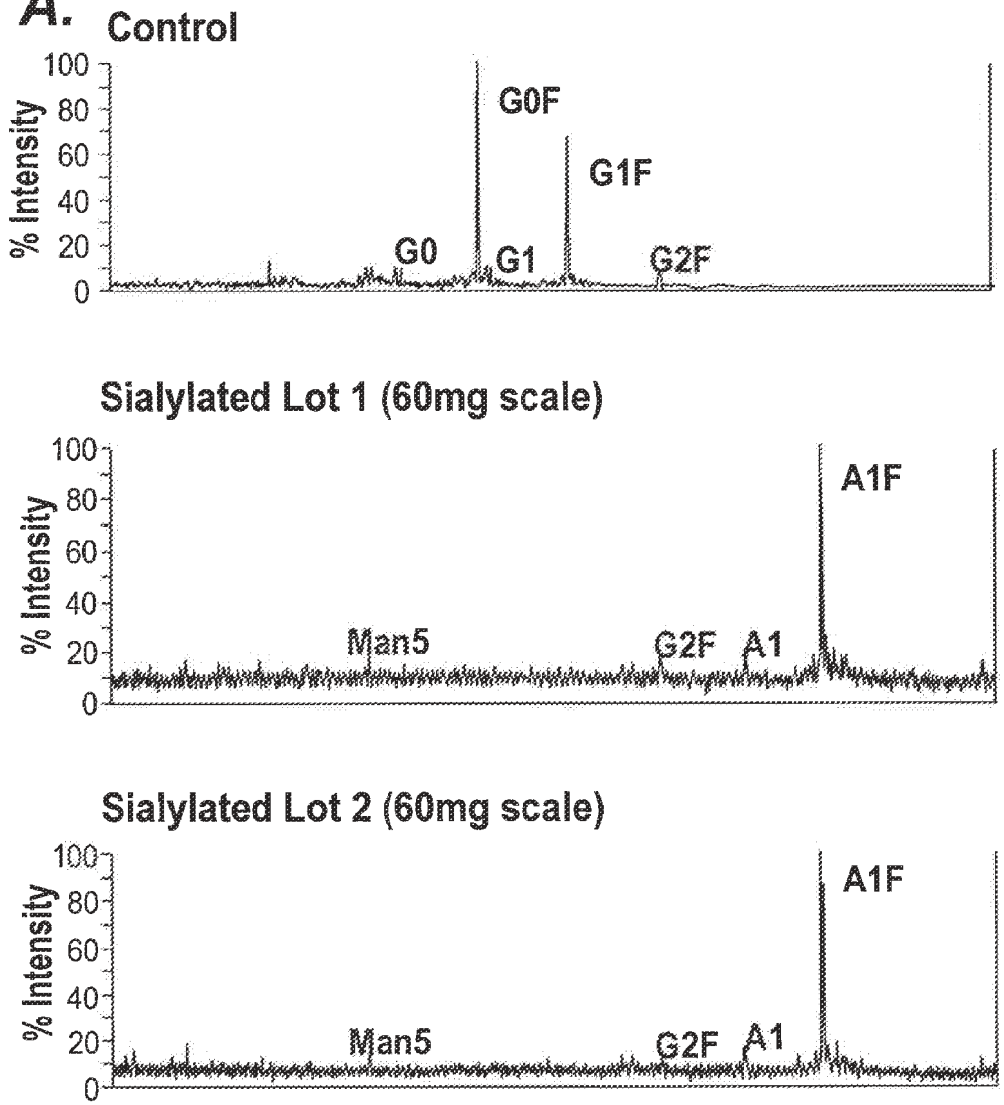


Fig. 26

A.



B.

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

C.

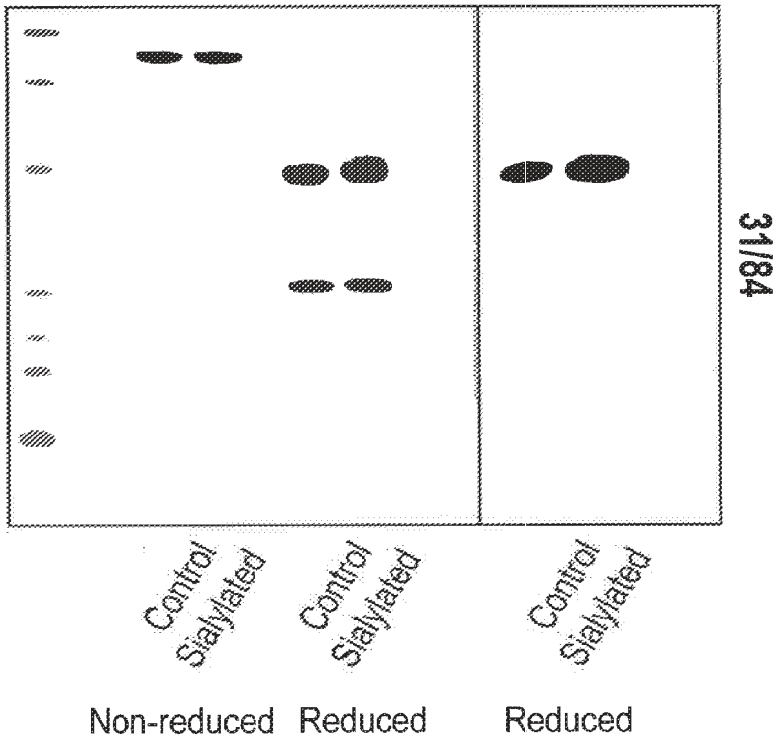
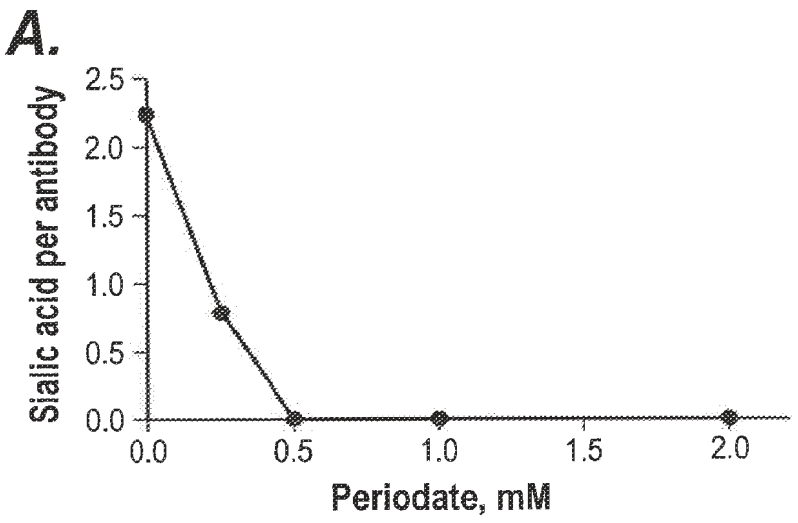
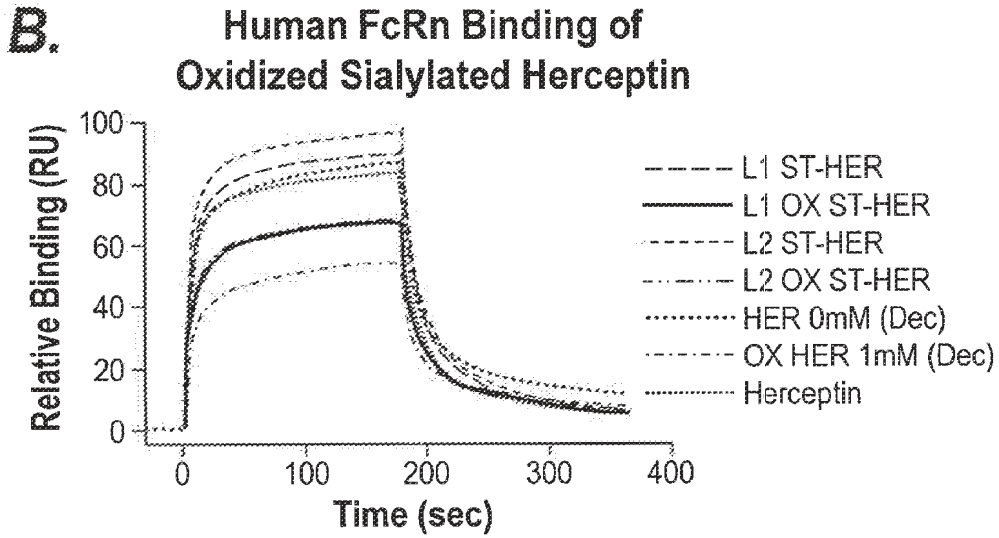
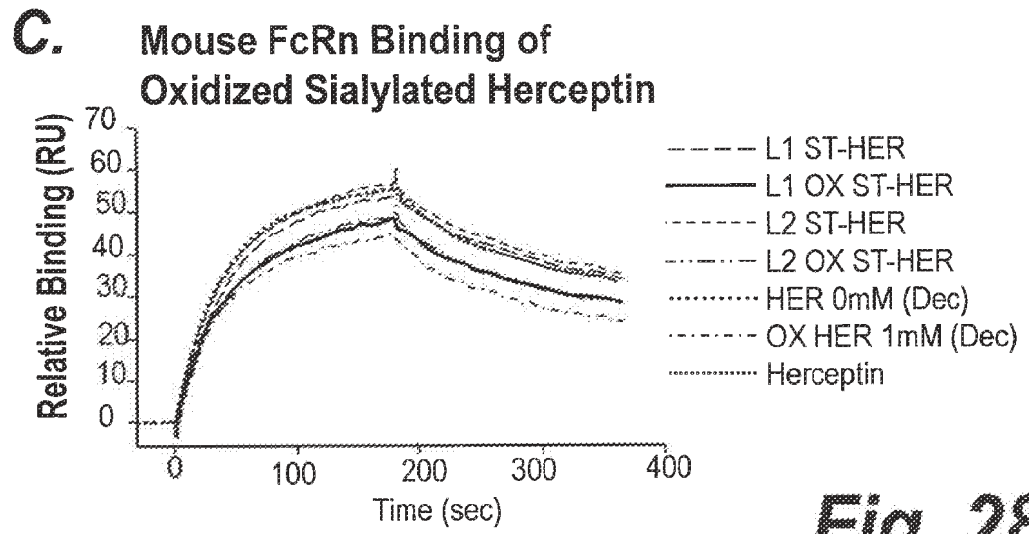


Fig. 27


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

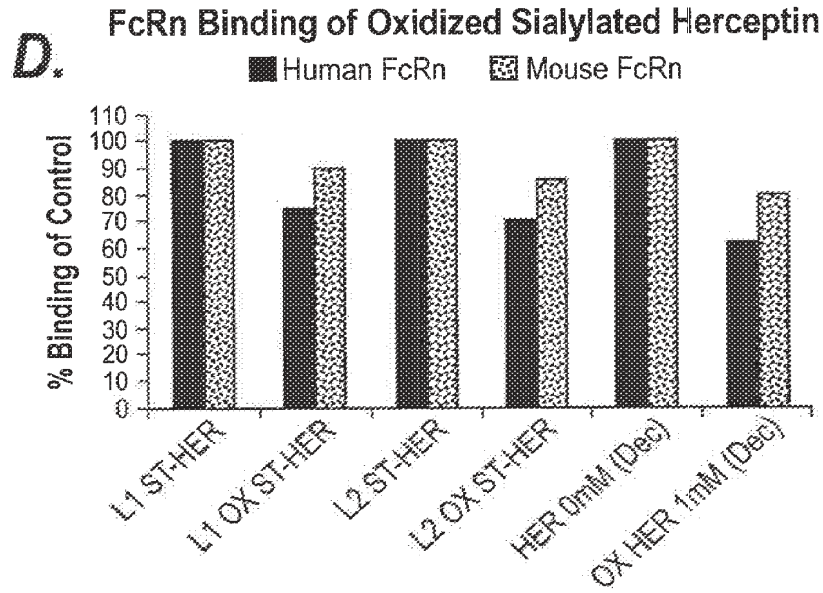

D.
FcRn Binding of Oxidized Sialylated Herceptin
Human FcRn
Mouse FcRn
% Binding of Control
110
100
90
80
70
60
50
40
30
20
10
0
L1 ST-HER
L1 OX ST-HER
L2 ST-HER
L2 OX ST-HER
HER 0mM (Dec)
OX HER 1mM (Dec)

Fig. 28

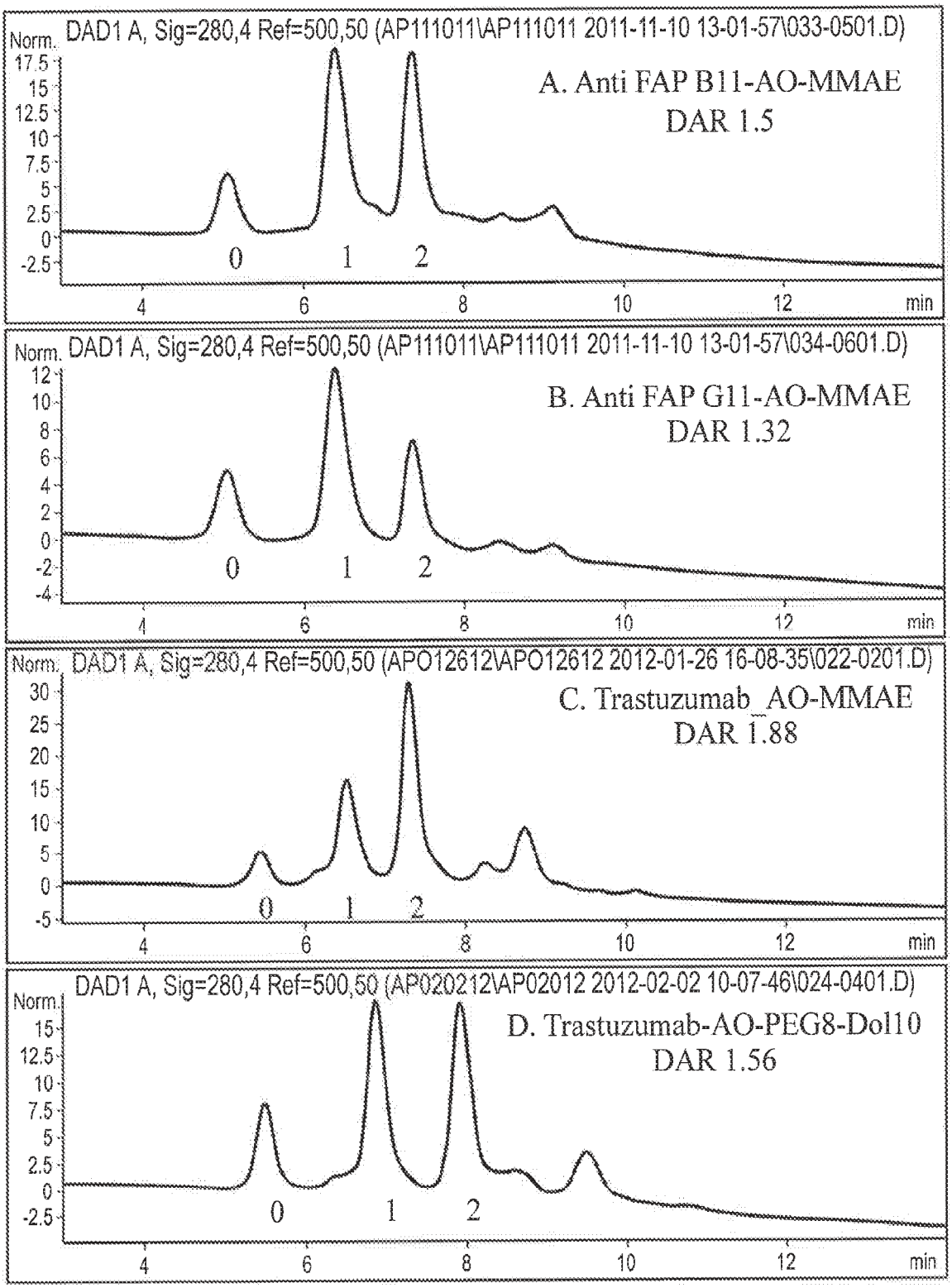

Norm.
DAD1 A, Sig=280,4 Ref=500,50 (AP111011\AP111011 2011-11-10 13-01-57\033-0501.D)
A. Anti FAP B11-AO-MMAE
DAR 1.5
0
1
2
min
DAD1 A, Sig=280,4 Ref=500,50 (AP111011\AP111011 2011-11-10 13-01-57\034-0601.D)
B. Anti FAP G11-AO-MMAE
DAR 1.32
DAD1 A, Sig=280,4 Ref=500,50 (APO12612\APO12612 2012-01-26 16-08-35\022-0201.D)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

Fig. 29

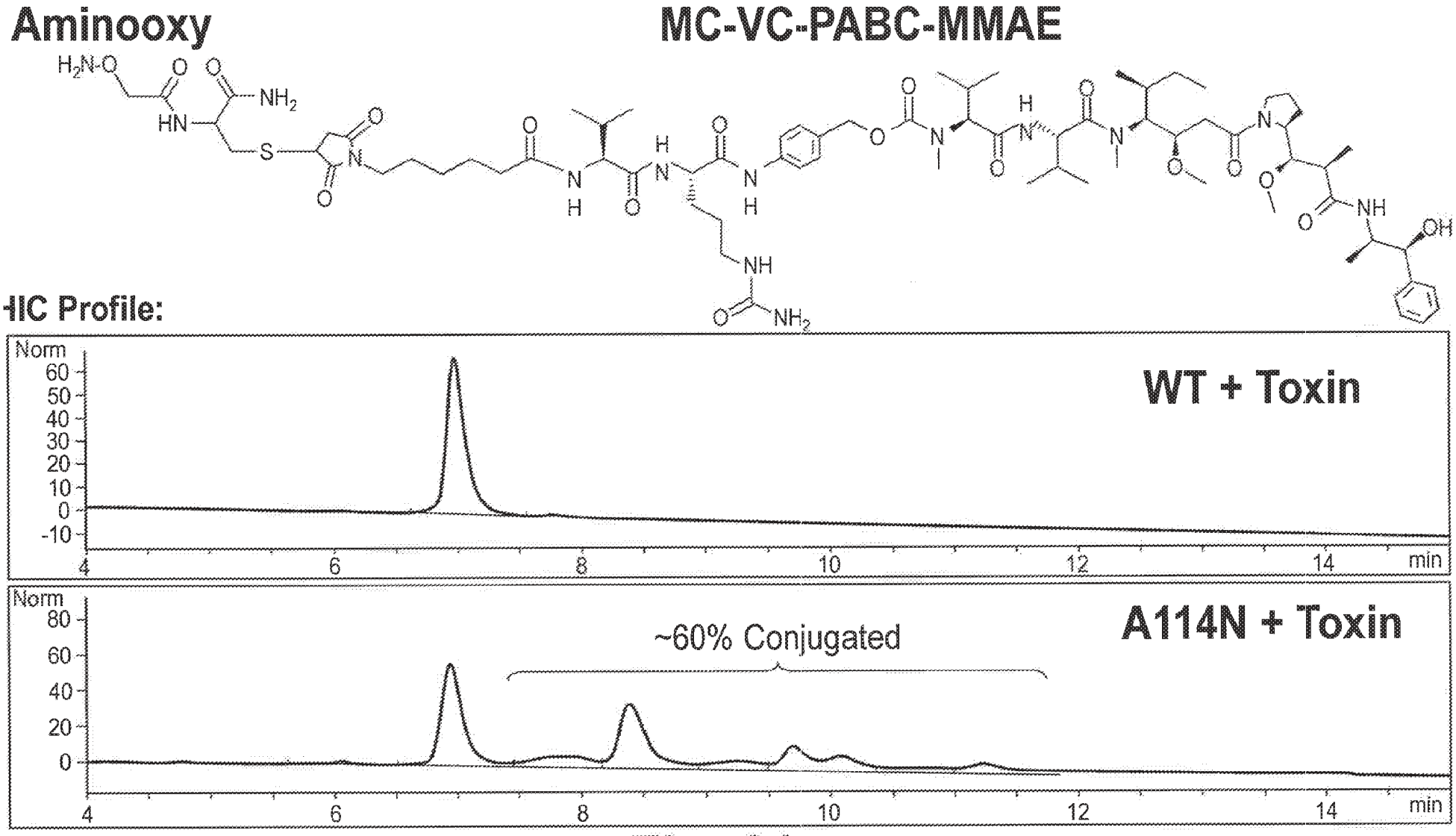

Aminooxy
MC-VC-PABC-MMAE
HIC Profile:
Norm
60
50
40
30
20
10
0
-10
4
6
8
10
12
14
min
WT + Toxin
Norm
80
60
40
20
0
~60% Conjugated
A114N + Toxin

Fig. 30

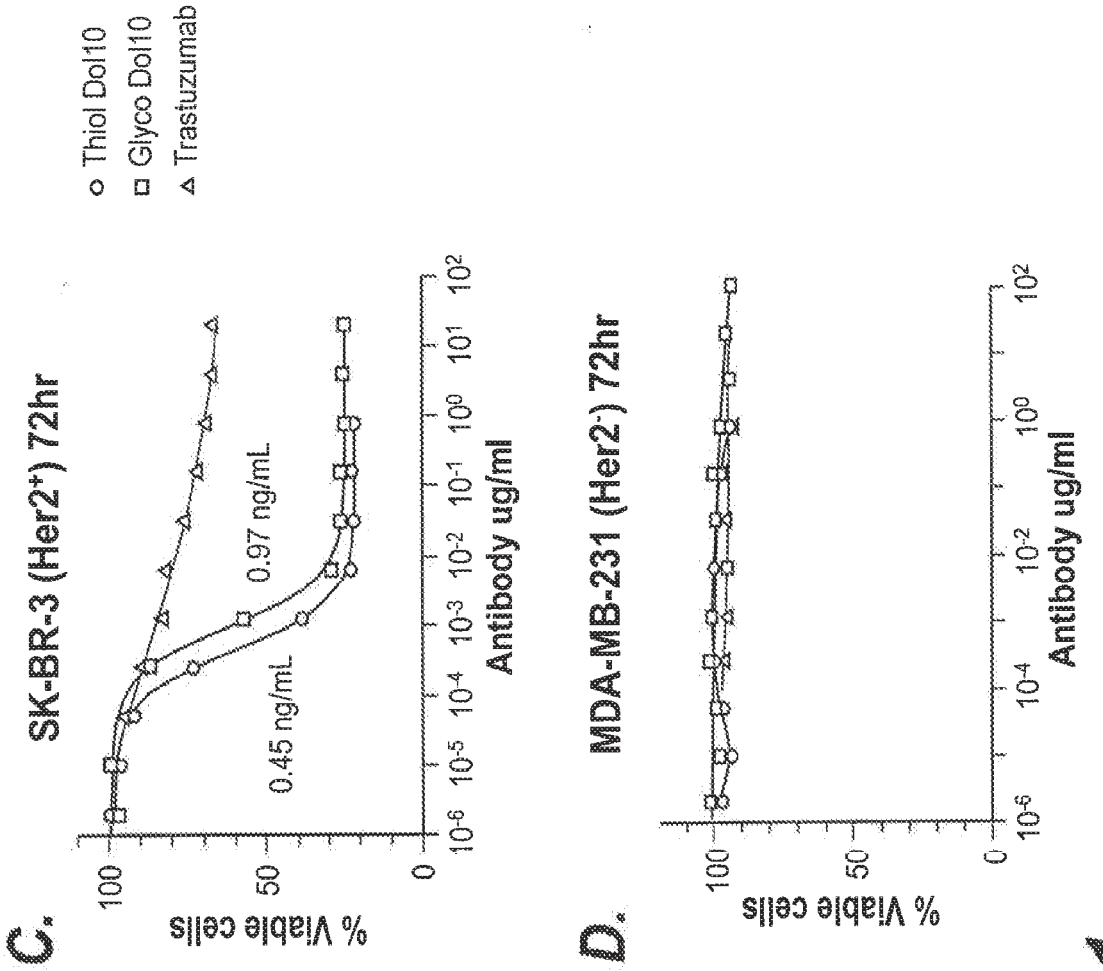


Figure 31: please align the legend so that symbols and words line up

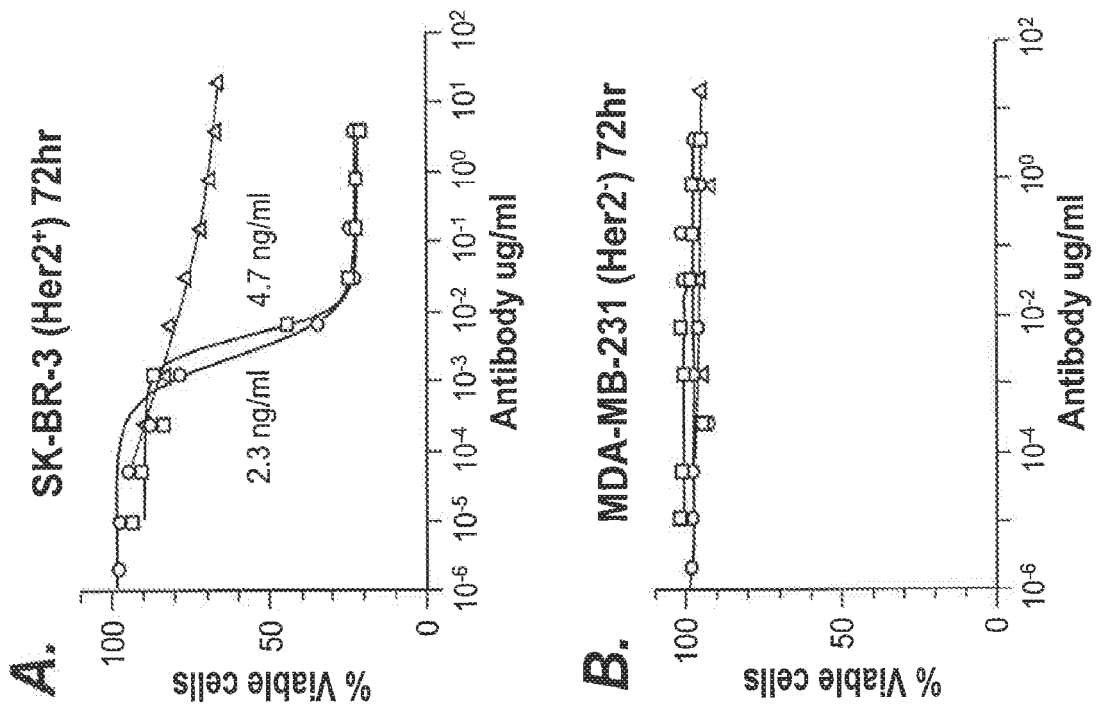


Fig. 31

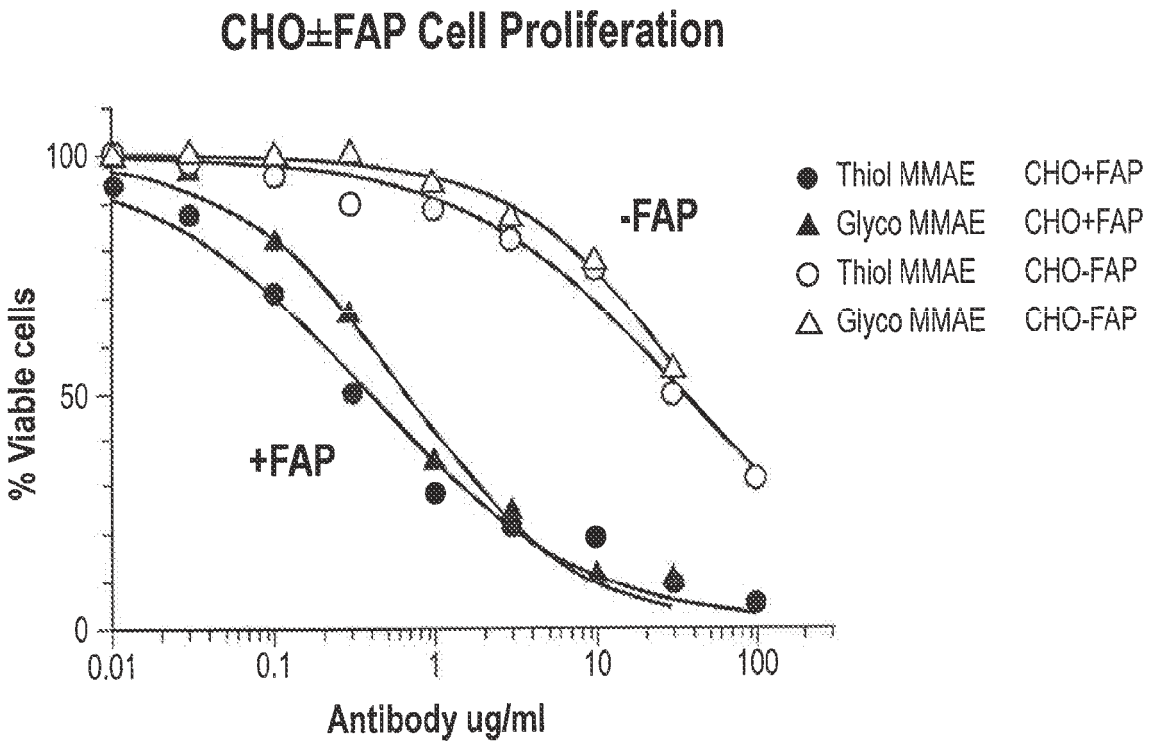

CHO±FAP Cell Proliferation
% Viable cells
100
50
0
0.01
0.1
1
10
100
Antibody ug/ml
-FAP
+FAP
Thiol MMAE CHO+FAP
Glyco MMAE CHO+FAP
Thiol MMAE CHO-FAP
Glyco MMAE CHO-FAP

Fig. 32

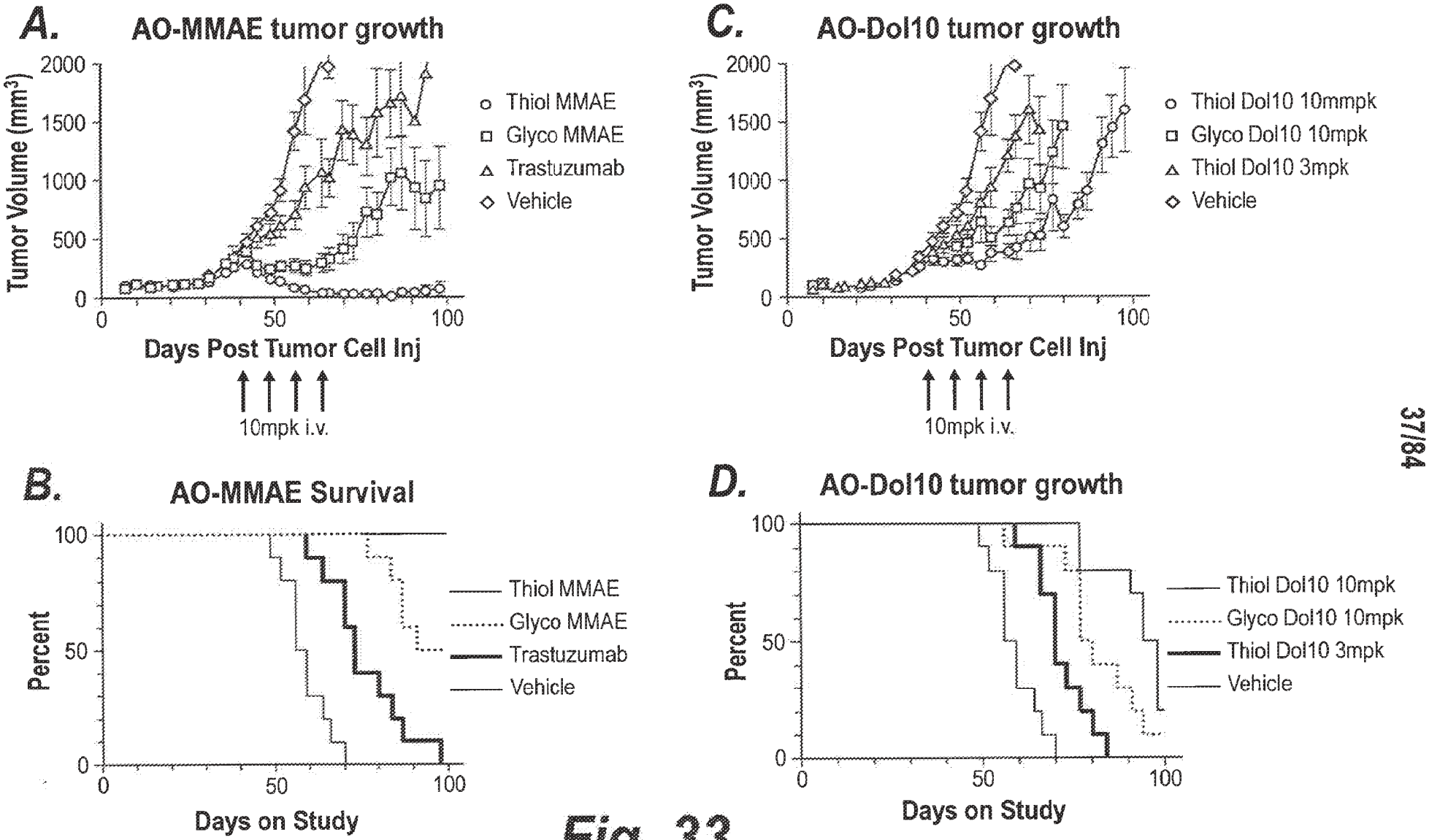

A.
AO-MMAE tumor growth
Tumor Volume (mm³)
2000
1500
1000
500
0
0
50
100
Days Post Tumor Cell Inj
10mpk i.v.
Thiol MMAE
Glyco MMAE
Trastuzumab
Vehicle
C.
AO-Dol10 tumor growth
Tumor Volume (mm³)
2000
1500
1000
500
0
0
50
100
Days Post Tumor Cell Inj
10mpk i.v.
Thiol Dol10 10mmpk
Glyco Dol10 10mpk
Thiol Dol10 3mpk
Vehicle
B.
AO-MMAE Survival
Percent
100
50
0
0
50
100
Days on Study
Thiol MMAE
Glyco MMAE
Trastuzumab
Vehicle
D.
AO-Dol10 tumor growth
Percent
100
50
0
0
50
100
Days on Study
Thiol Dol10 10mpk
Glyco Dol10 10mpk
Thiol Dol10 3mpk
Vehicle

Fig. 33

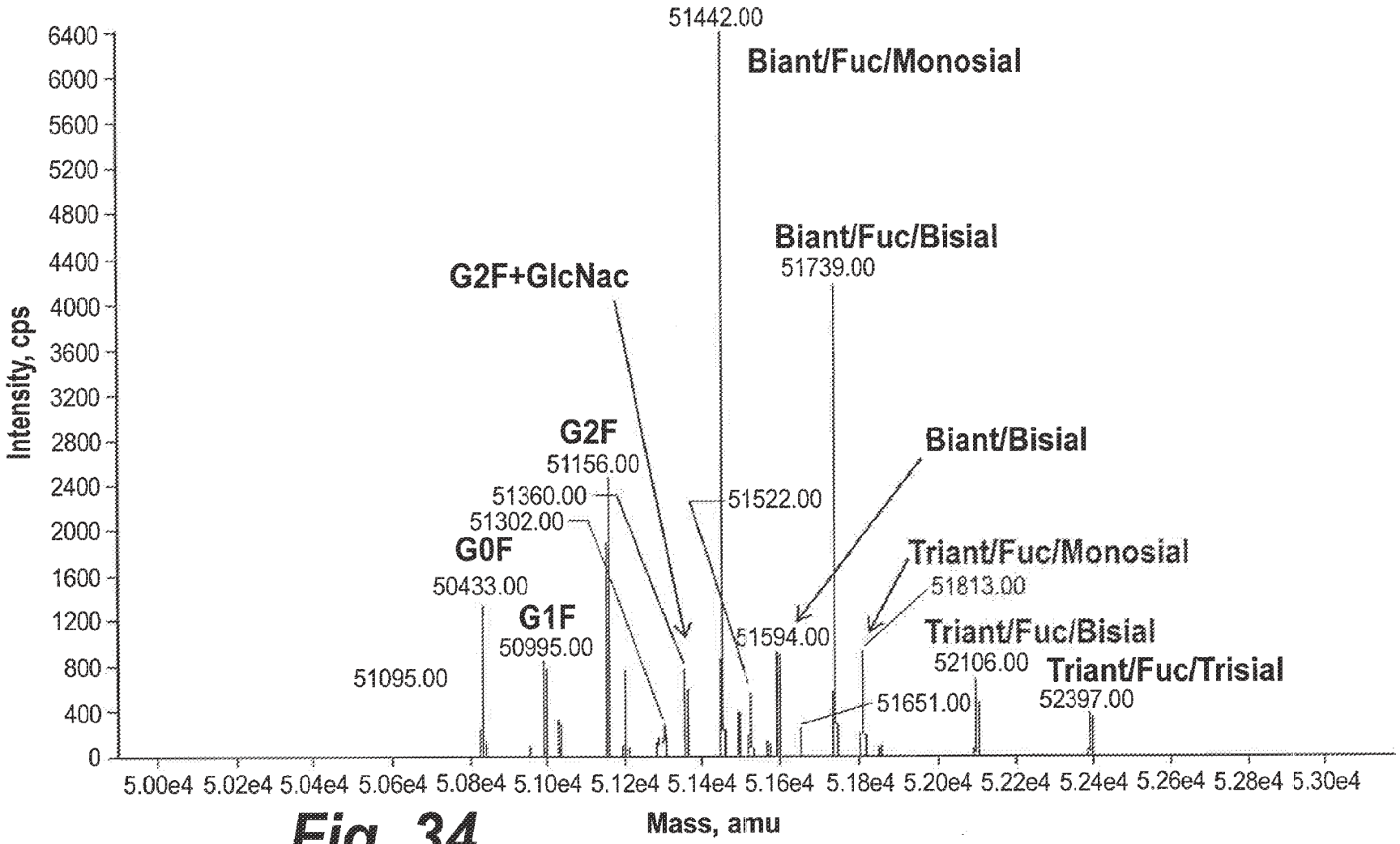

LC/MS of S298N/T299A/Y300S (NNAS) heavy chain
Intensity, cps
6400
6000
5600
5200
4800
4400
4000
3600
3200
2800
2400
2000
1600
1200
800
400
0
5.00e4 5.02e4 5.04e4 5.06e4 5.08e4 5.10e4 5.12e4 5.14e4 5.16e4 5.18e4 5.20e4 5.22e4 5.24e4 5.26e4 5.28e4 5.30e4
Mass, amu
51442.00
Biant/Fuc/Monosial
Biant/Fuc/Bisial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

Fig. 34

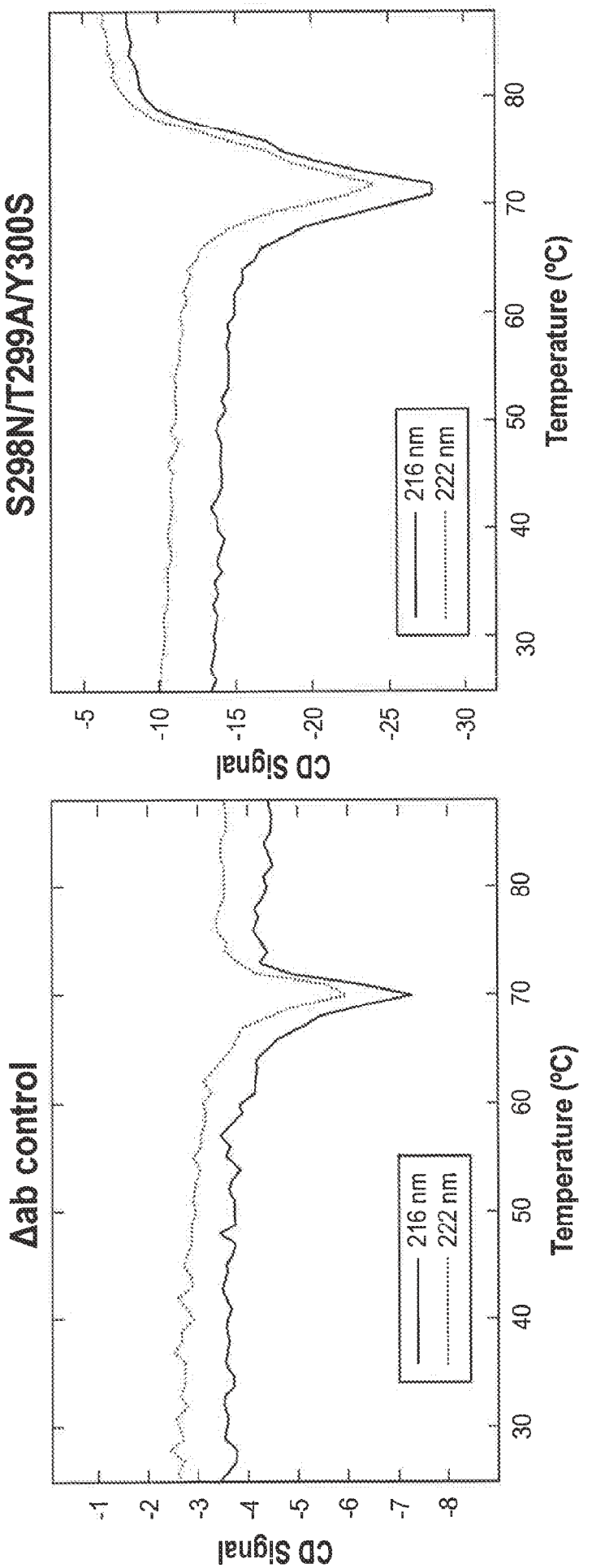

Δab control
CD Signal
-1
-2
-3
-4
-5
-6
-7
-8
30
40
50
60
70
80
Temperature (°C)
216 nm
222 nm
S298N/T299A/Y300S
CD Signal
-5
-10
-15
-20
-25
-30
30
40
50
60
70
80
Temperature (°C)
216 nm
222 nm

Fig. 35

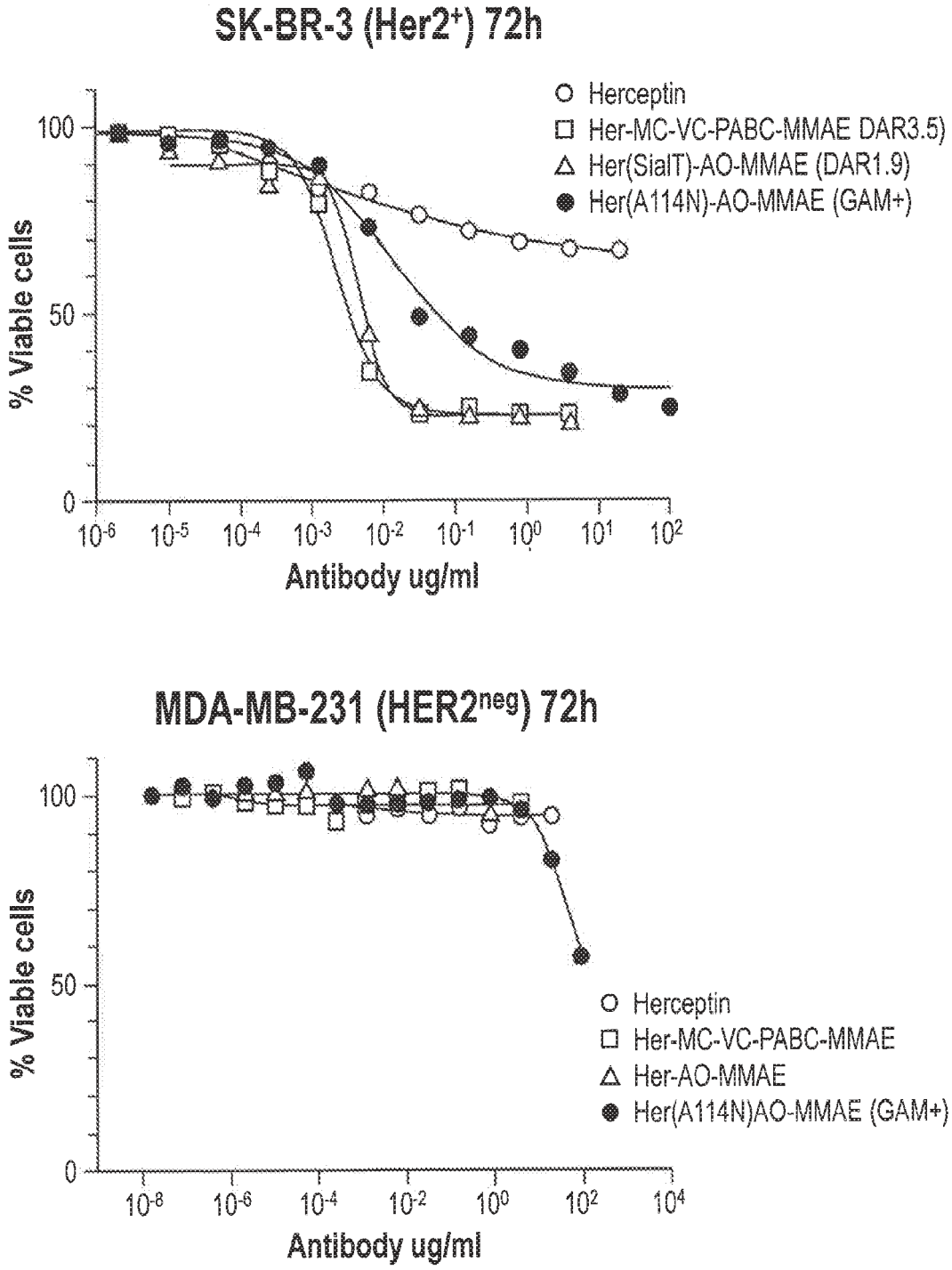

SK-BR-3 (Her2+) 72h
Herceptin
Her-MC-VC-PABC-MMAE DAR3.5)
Her(SialT)-AO-MMAE (DAR1.9)
Her(A114N)-AO-MMAE (GAM+)
% Viable cells
100
50
0
10-6 10-5 10-4 10-3 10-2 10-1 100 101 102
Antibody ug/ml
MDA-MB-231 (HER2neg) 72h
Herceptin
Her-MC-VC-PABC-MMAE
Her-AO-MMAE
Her(A114N)AO-MMAE (GAM+)
% Viable cells
100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

Fig. 36

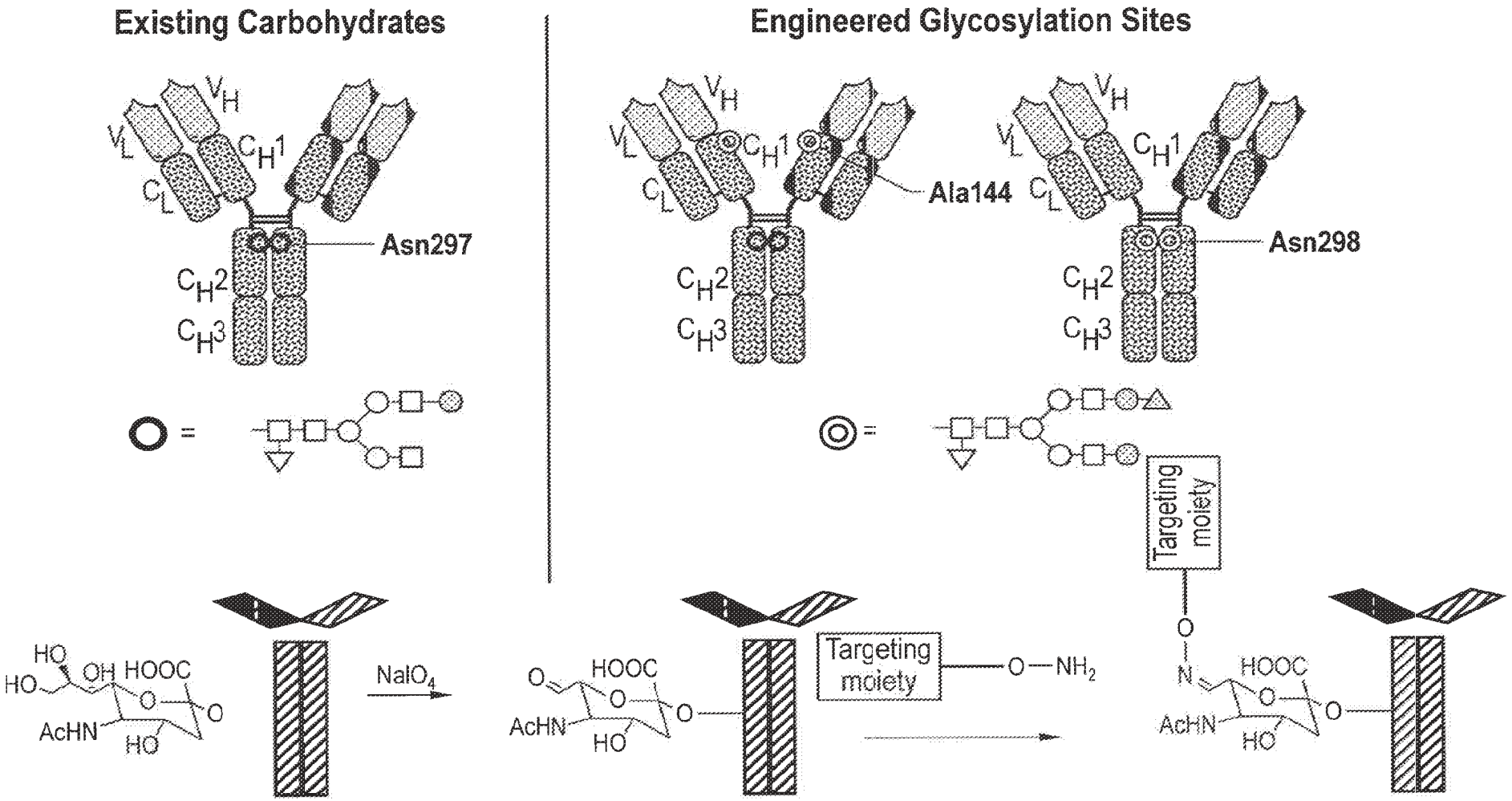

Existing Carbohydrates
Engineered Glycosylation Sites
Asn297
Ala144
Asn298
Targeting moiety
NaIO4
Targeting moiety
O—NH2

Fig. 37

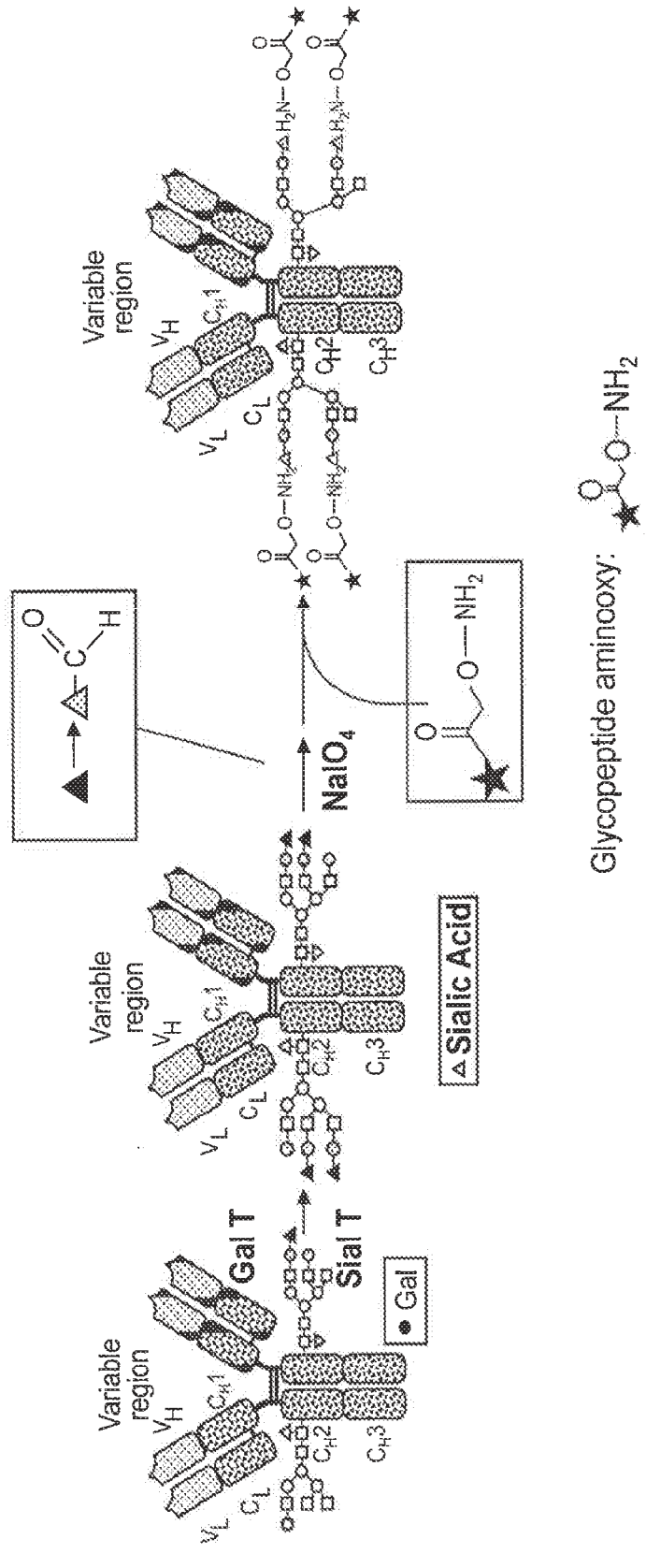

Variable region
V_H
C_H1
V_L
C_L
C_H2
C_H3
Gal T
Sial T
• Gal
Variable region
V_H
C_H1
V_L
C_L
C_H2
C_H3
△Sialic Acid
$NaIO_4$
Variable region
V_H
C_H1
V_L
C_L
C_H2
C_H3
Glycopeptide aminooxy:
NH_2

Fig. 38

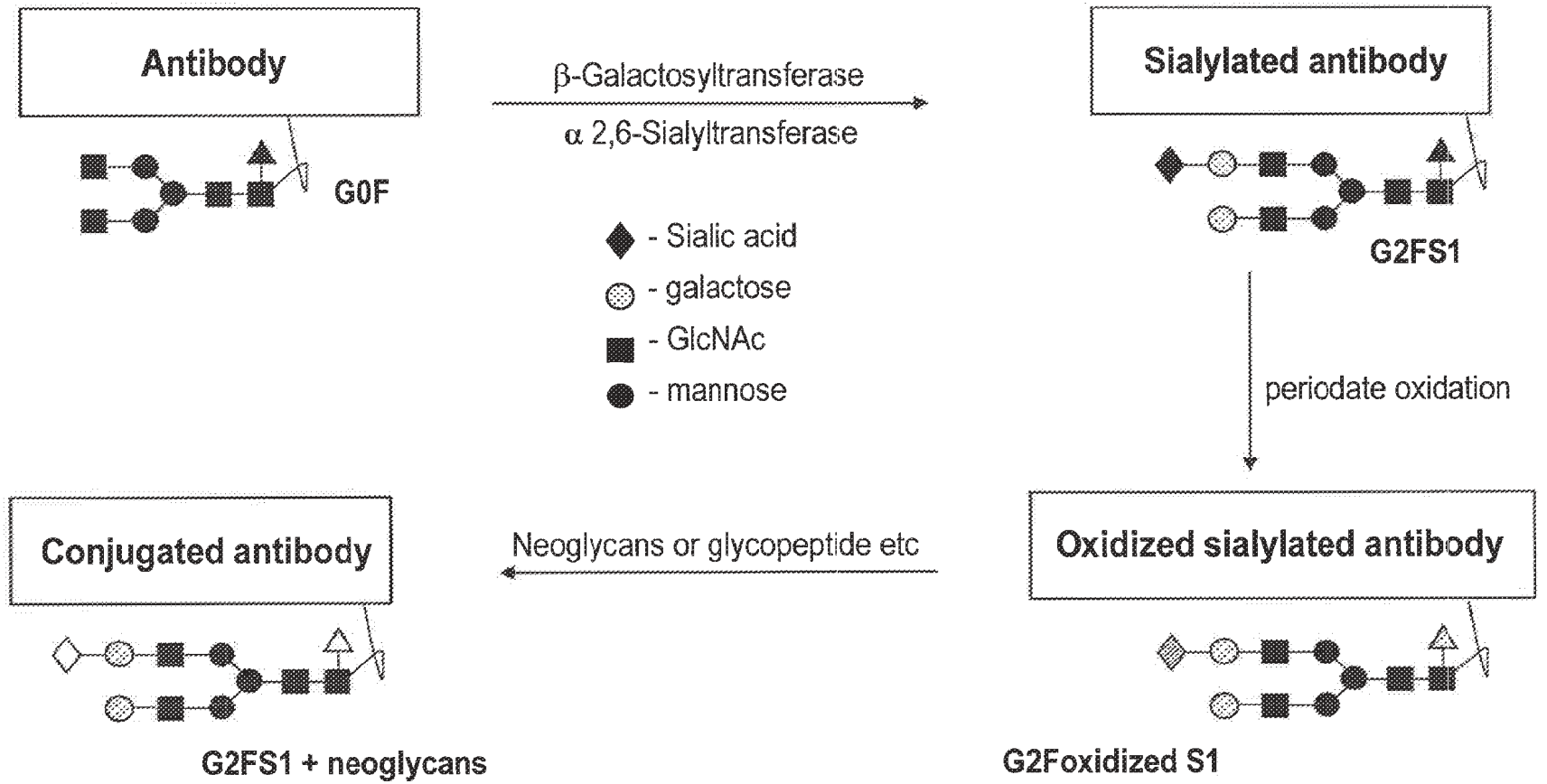
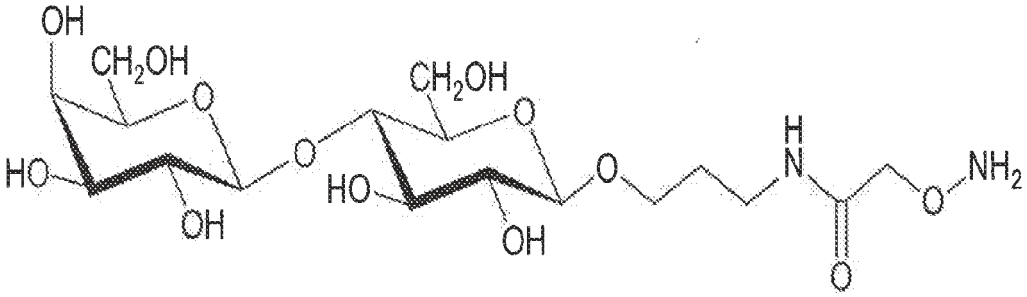

Antibody
G0F
β-Galactosyltransferase
α 2,6-Sialyltransferase
- Sialic acid
- galactose
- GlcNAc
- mannose
Sialylated antibody
G2FS1
periodate oxidation
Oxidized sialylated antibody
G2Foxidized S1
Neoglycans or glycopeptide etc
Conjugated antibody
G2FS1 + neoglycans

Fig. 39

Lactose aminooxy for native glycans (SAM or GAM):



Exact Mass: 472.19
Molecular Weight: 472.44

bisM6P hexamannose aminooxy (SAM or GAM):

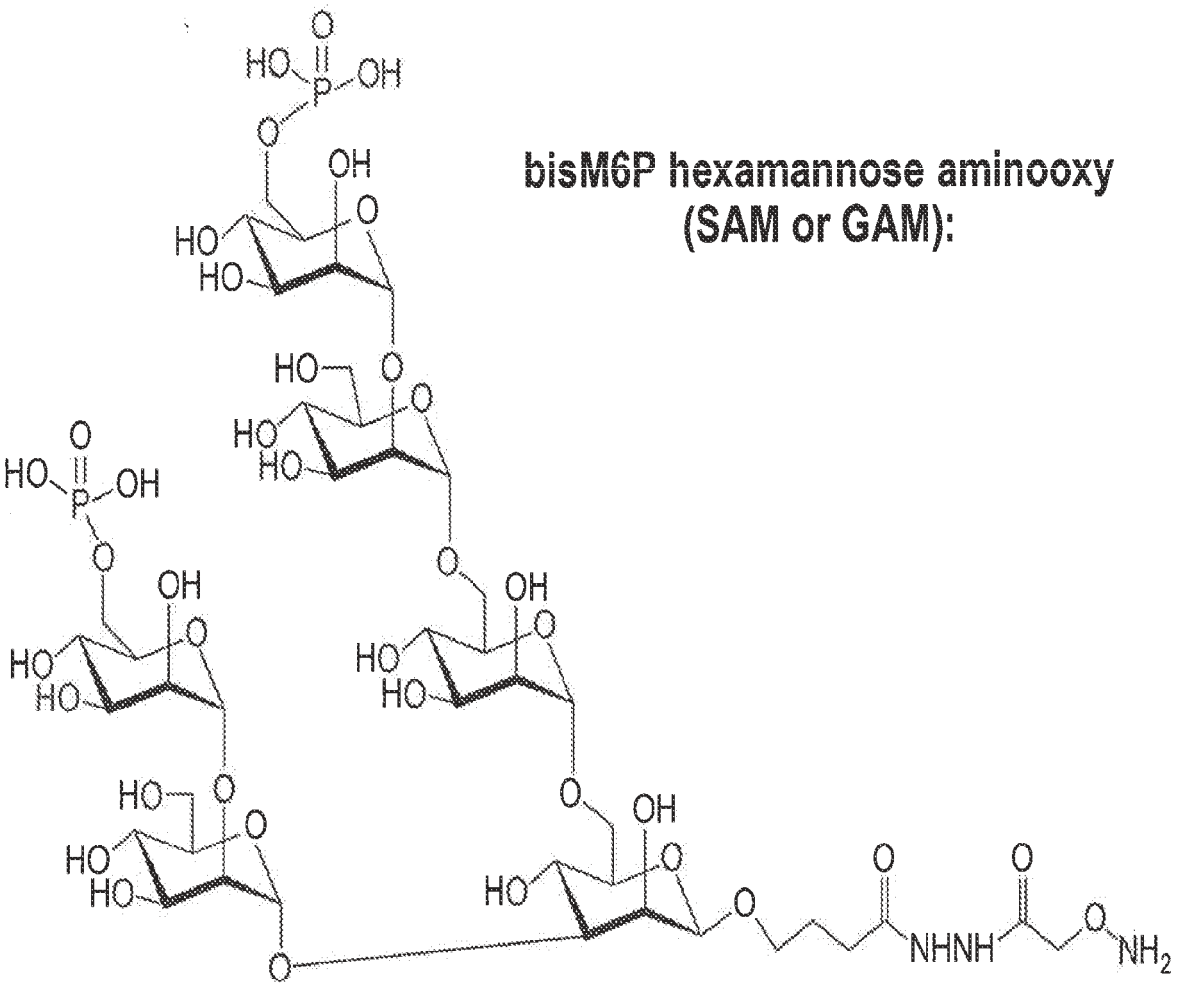
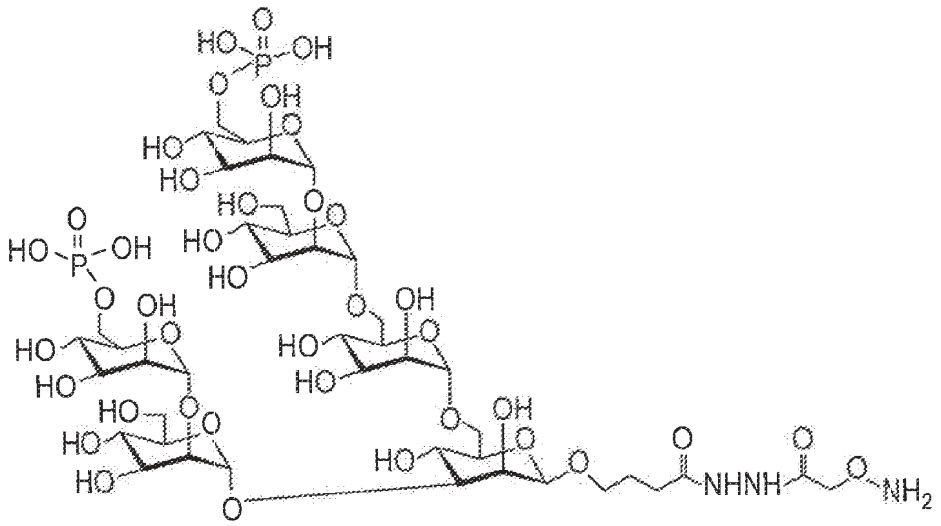
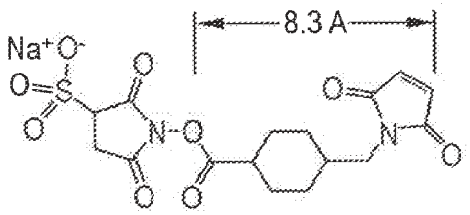


Fig. 40

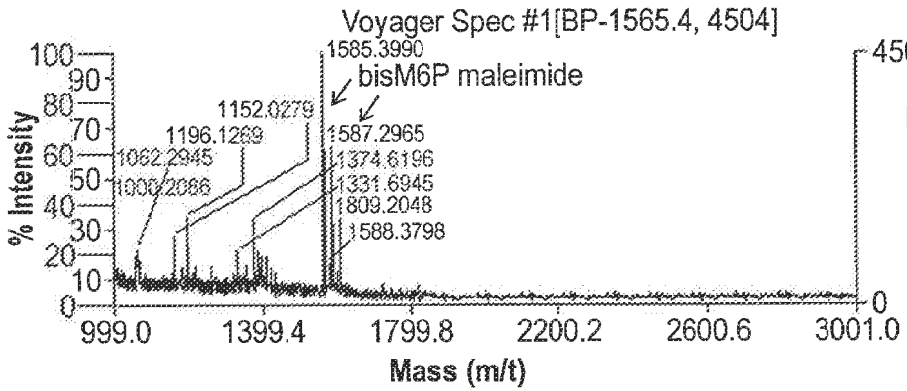
Fig. 41

bisM6P hexamannose aminooxy (SAM on Fc glycans):

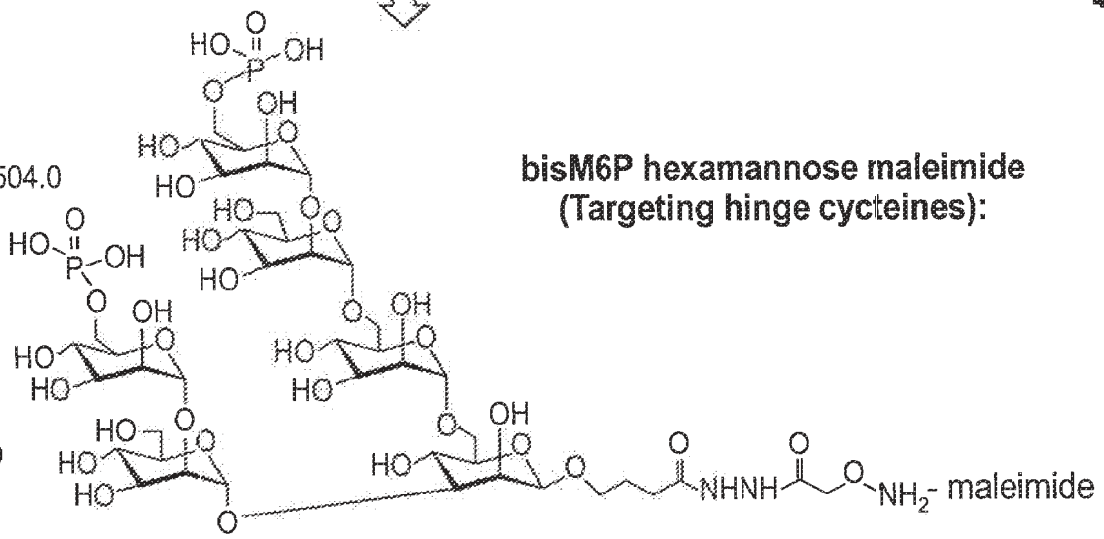




Sulfo SMCC



bisM6P hexamannose maleimide (Targeting hinge cycteines):



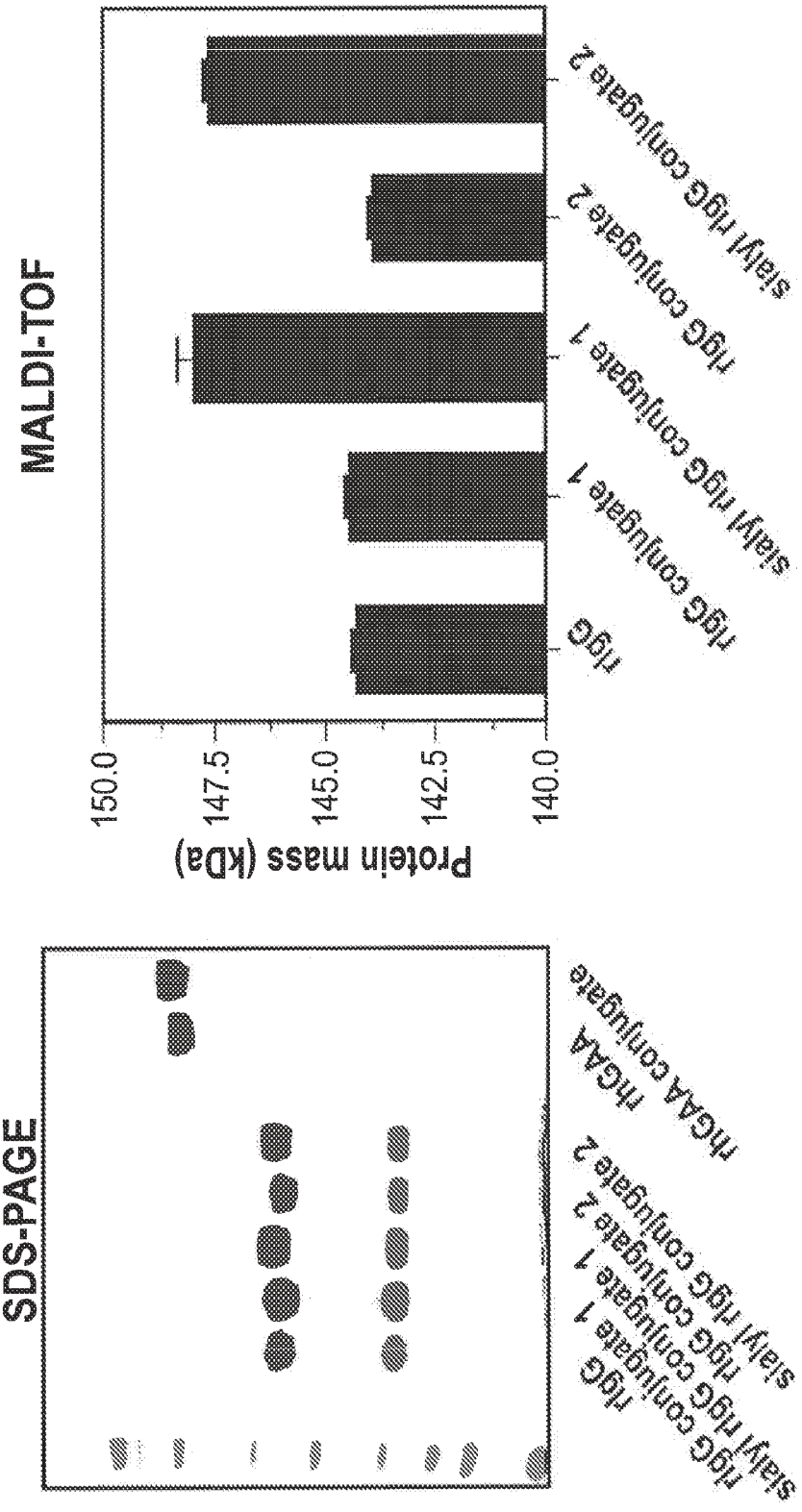

MALDI-TOF
Protein mass (kDa)
150.0
147.5
145.0
142.5
140.0
rIgG
rIgG conjugate 1
sialyl rIgG conjugate 1
rIgG conjugate 2
sialyl rIgG conjugate 2
SDS-PAGE
rIgG
rIgG conjugate 1
sialyl rIgG conjugate 1
rIgG conjugate 2
sialyl rIgG conjugate 2
rhGAA
rhGAA conjugate

Fig. 42

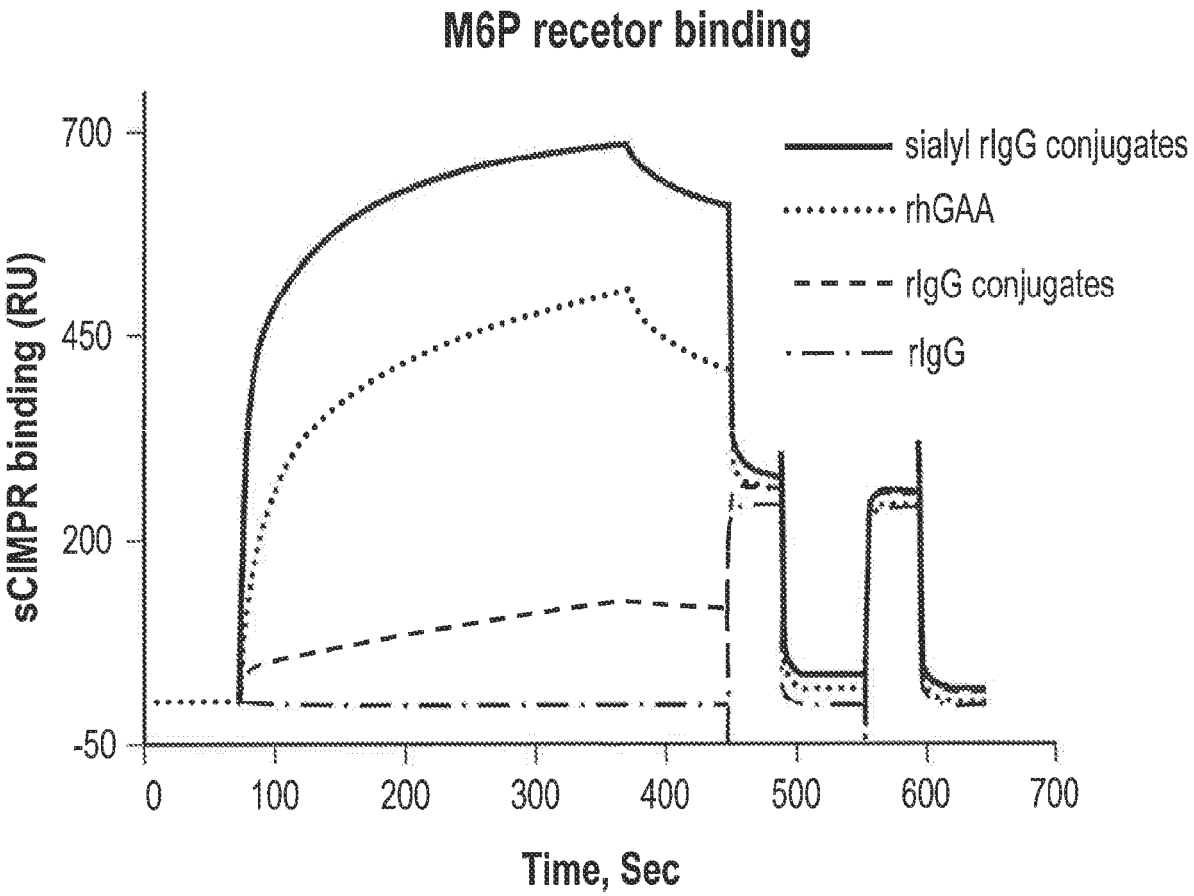

M6P recetor binding
sialyl rIgG conjugates
rhGAA
rIgG conjugates
rIgG
sCIMPR binding (RU)
700
450
200
-50
0
100
200
300
400
500
600
700
Time, Sec

Fig. 43

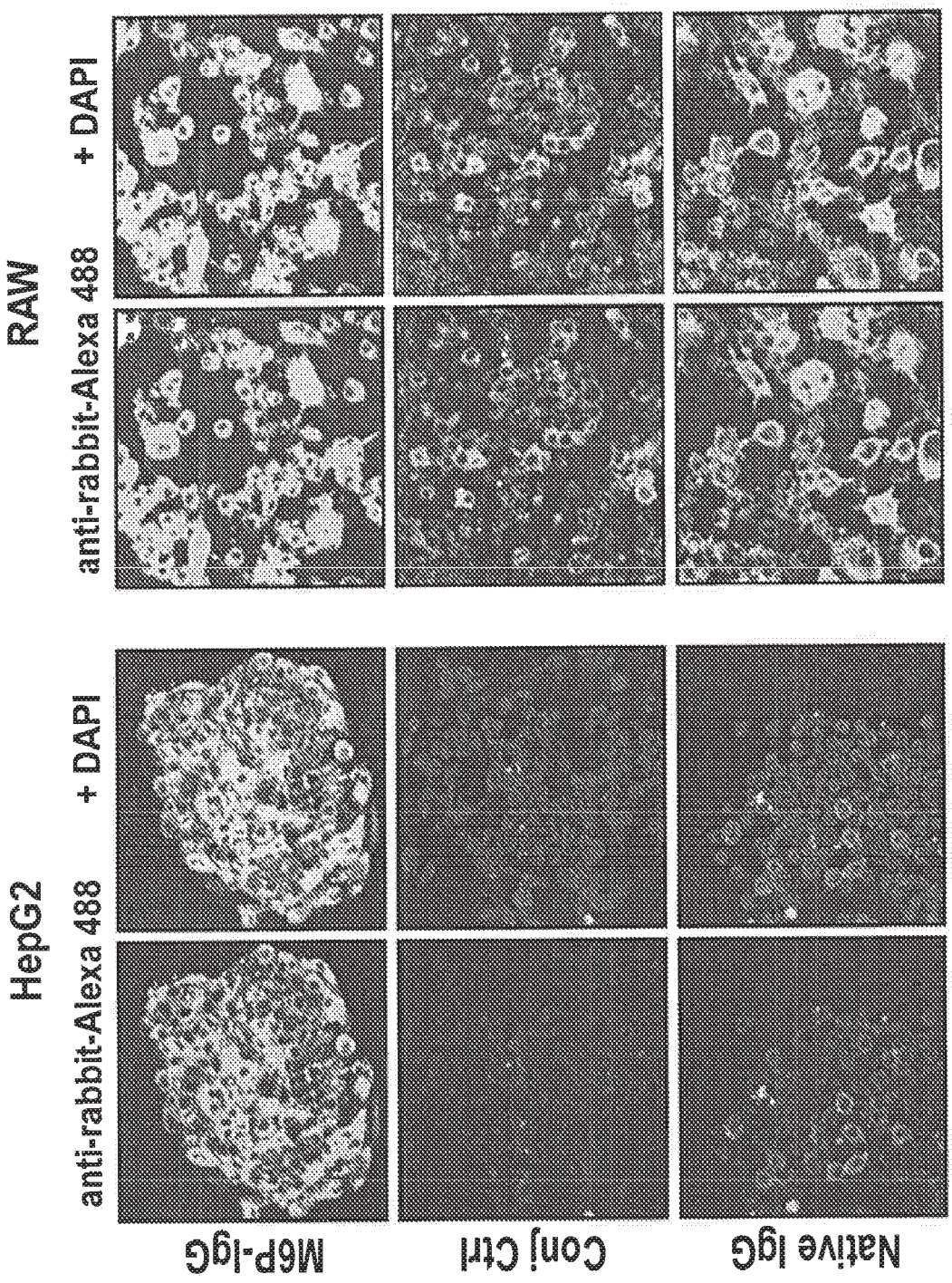

HepG2
RAW
anti-rabbit-Alexa 488
+ DAPI
anti-rabbit-Alexa 488
+ DAPI
M6P-IgG
Conj ctrl
Native IgG

Fig. 44

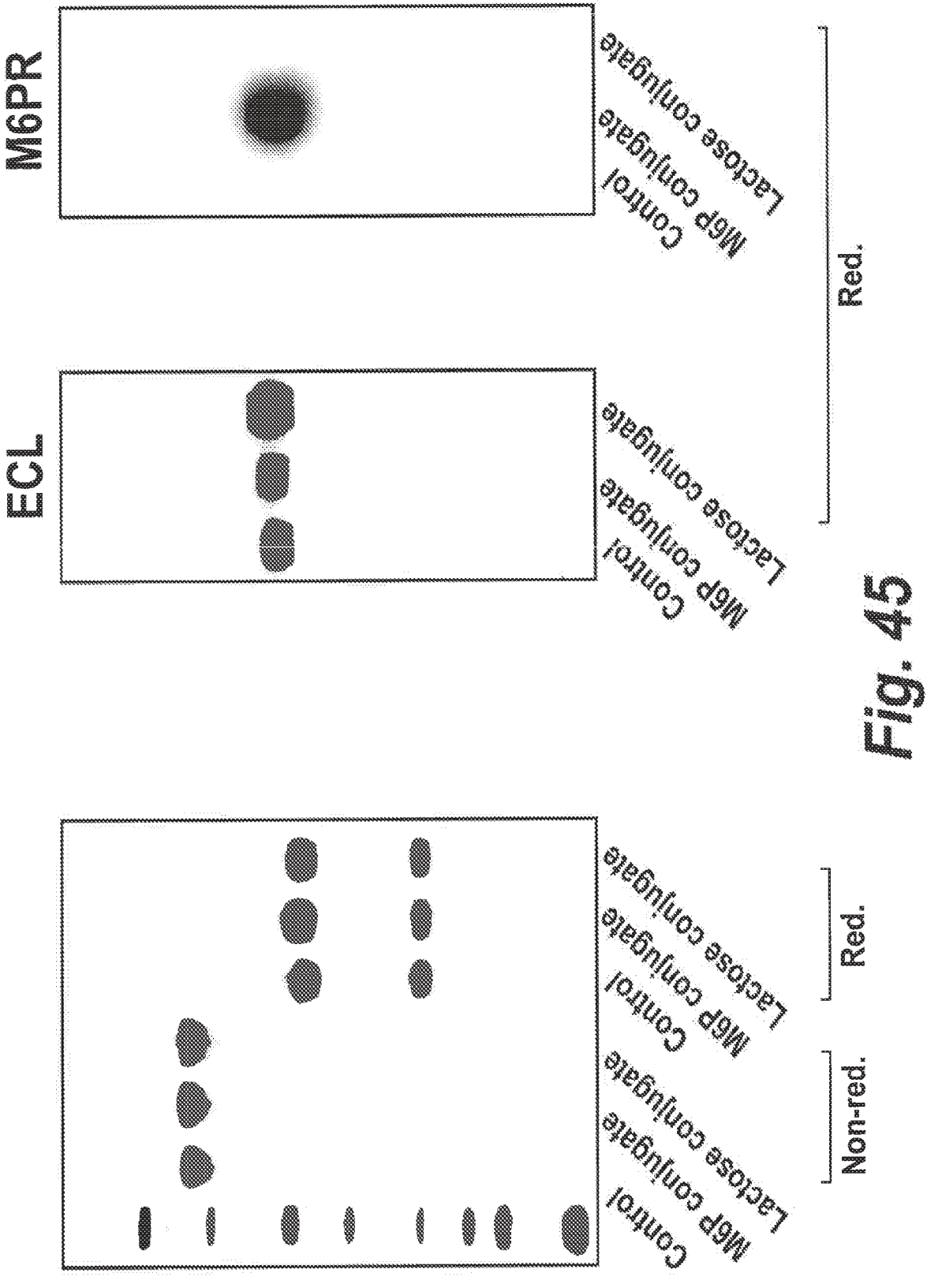

M6PR
ECL
Control
M6p conjugate
Lactose conjugate
Red.
Non-red.
Red.

Fig. 45

Samples	MW (kDa)	# Glycan
Control	145.42 ± 0.24	0.00
M6P conjugate	147.95 ± 0.09	1.92
Lactose conjugate	146.35 ± 0.36	1.96

Fig. 46

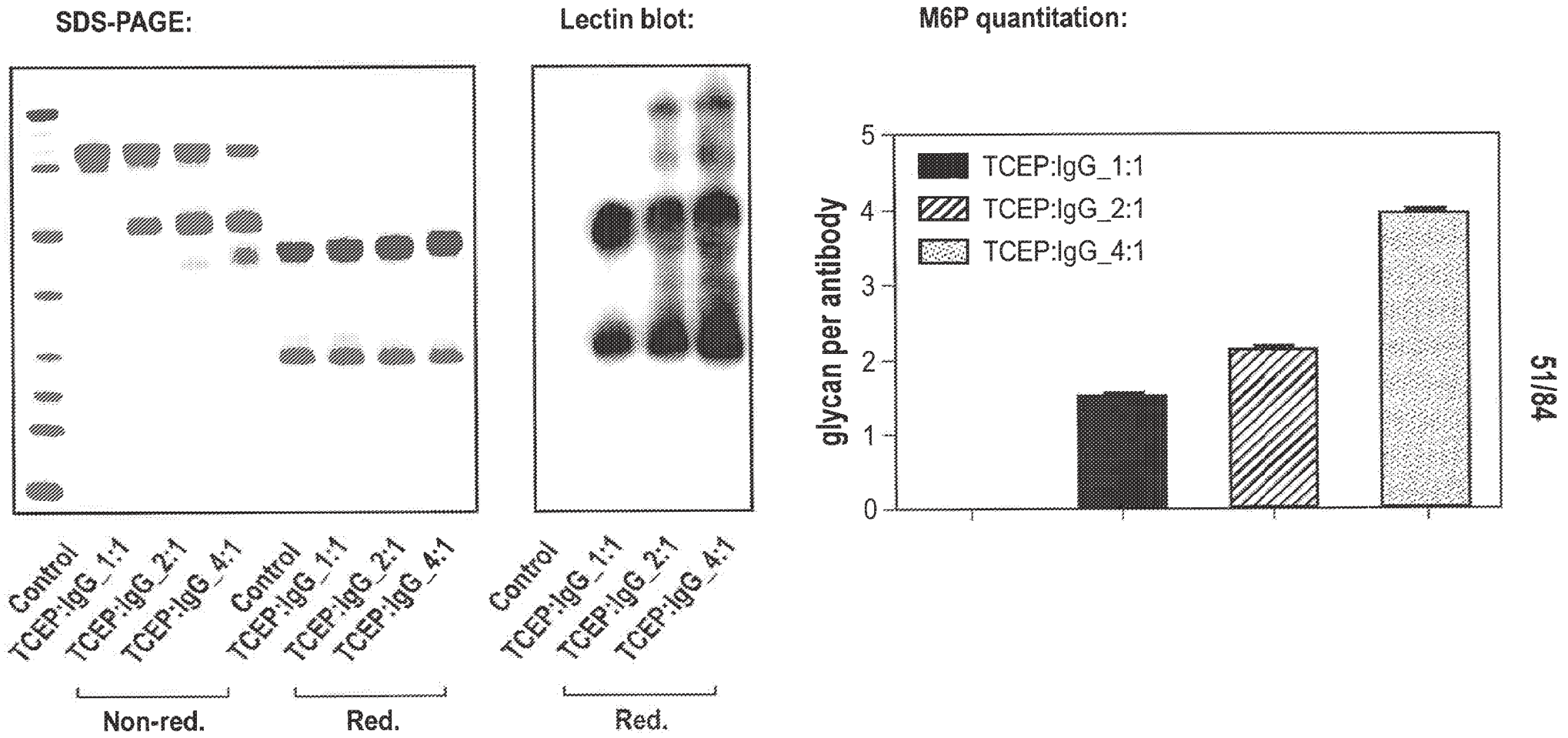

SDS-PAGE:
Lectin blot:
M6P quantitation:
Control
TCEP:IgG_1:1
TCEP:IgG_2:1
TCEP:IgG_4:1
Control
TCEP:IgG_1:1
TCEP:IgG_2:1
TCEP:IgG_4:1
Control
TCEP:IgG_1:1
TCEP:IgG_2:1
TCEP:IgG_4:1
Non-red.
Red.
Red.
glycan per antibody
0
1
2
3
4
5
TCEP:IgG_1:1
TCEP:IgG_2:1
TCEP:IgG_4:1

Fig. 47

Galactose quantitation:

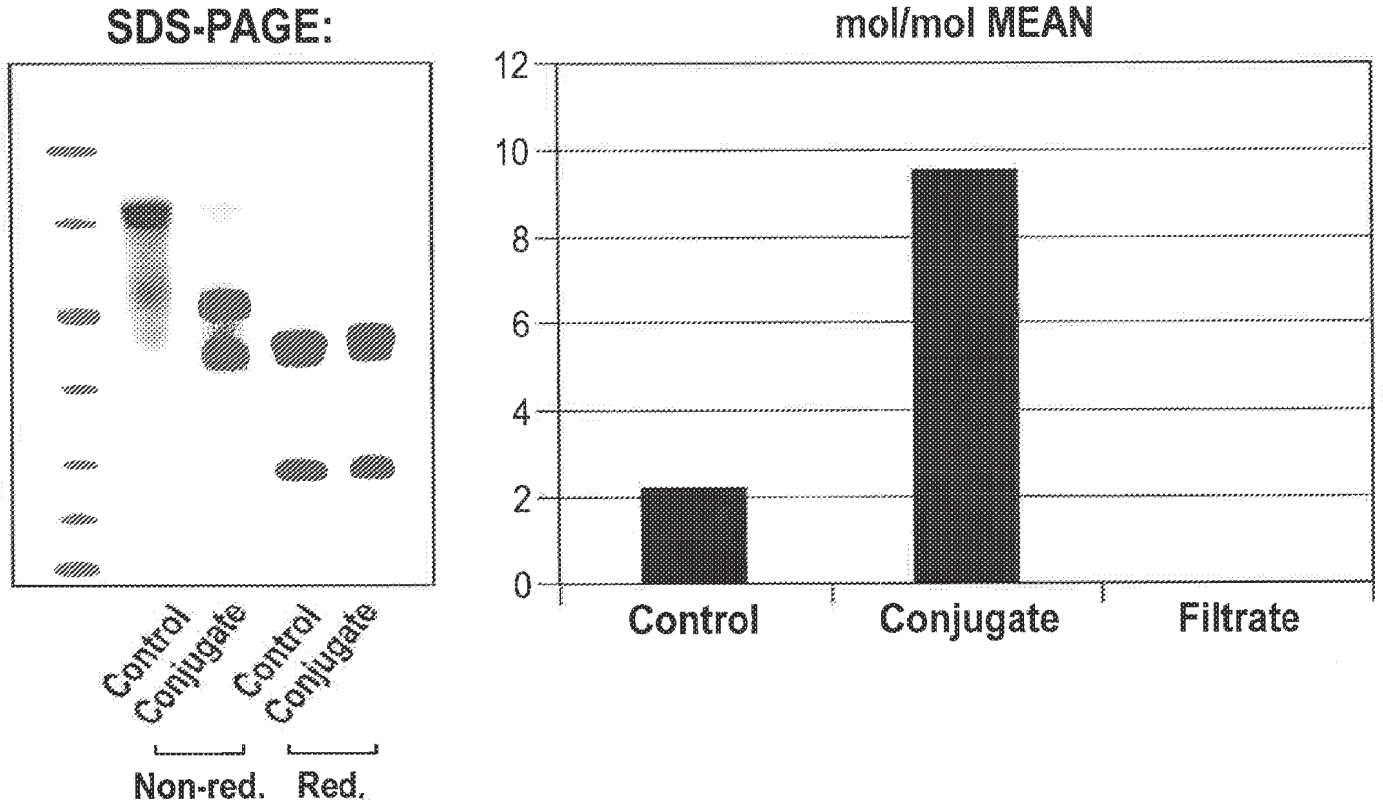


Fig. 48

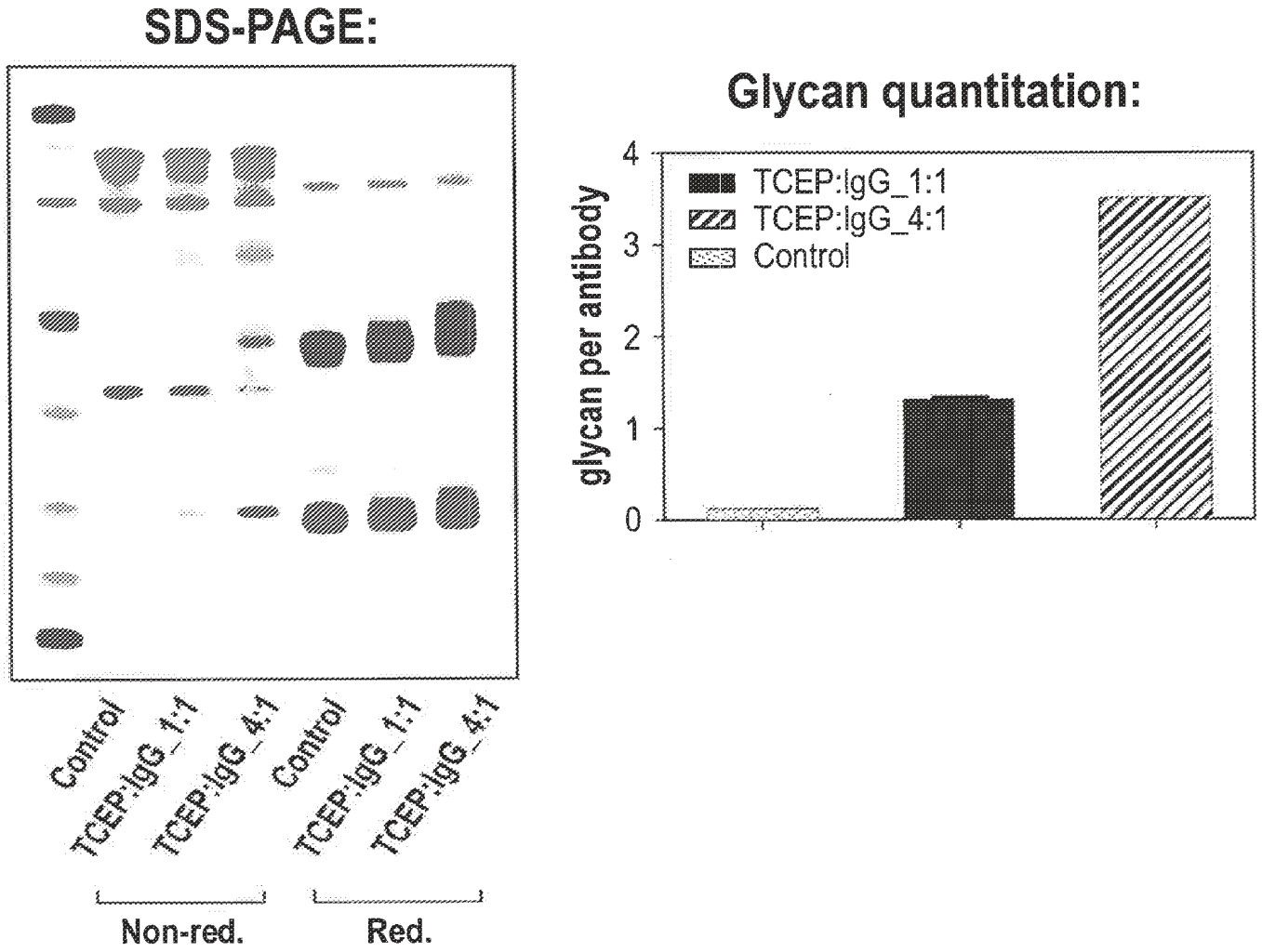
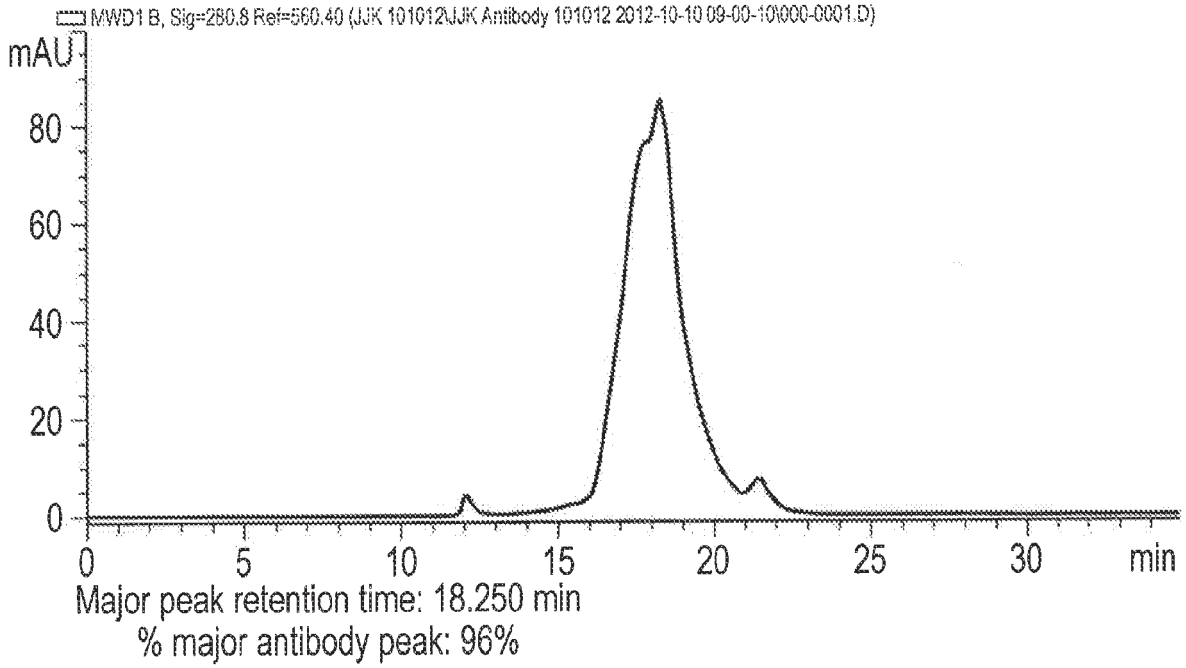

SDS-PAGE:
Control
TCEP:IgG_1:1
TCEP:IgG_4:1
Control
TCEP:IgG_1:1
TCEP:IgG_4:1
Non-red.
Red.
Glycan quantitation:
glycan per antibody
0
1
2
3
4
TCEP:IgG_1:1
TCEP:IgG_4:1
Control

Fig. 49

Control:



Lactose maleimide conjugate:

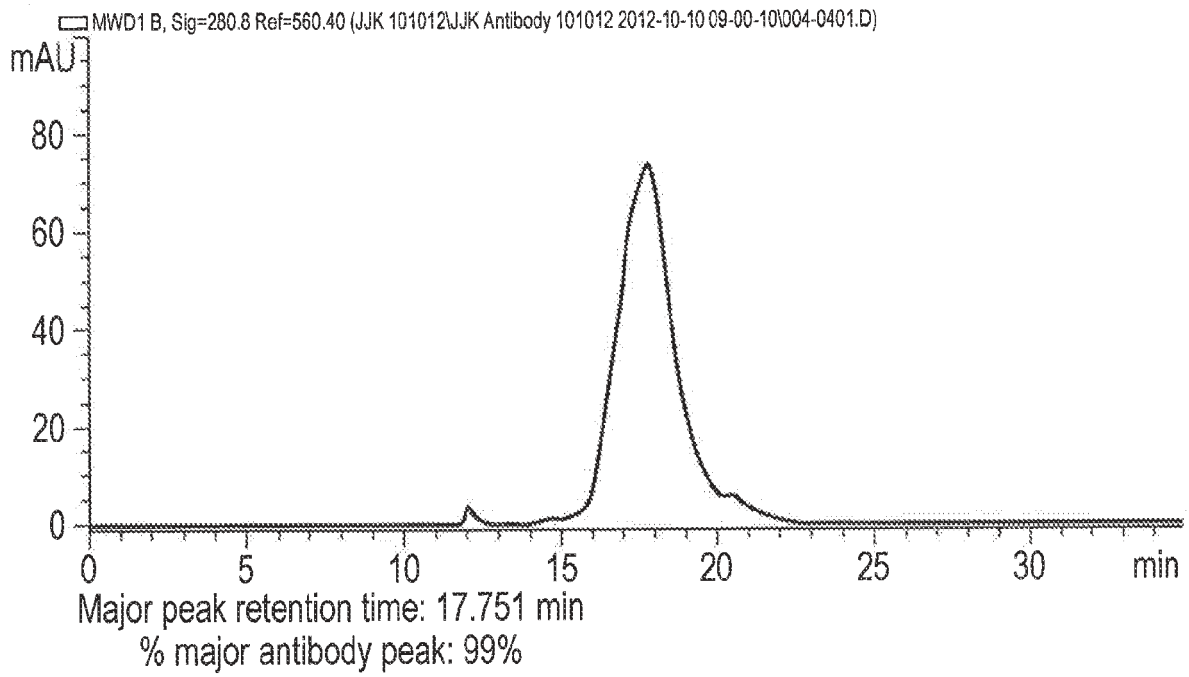
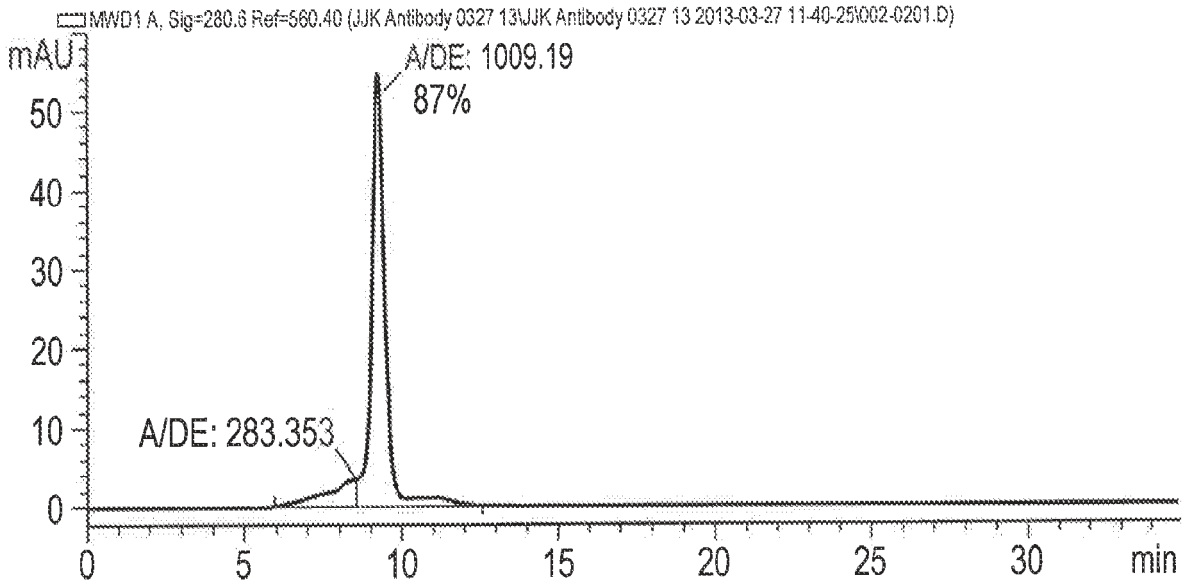


Fig. 50

Control:



M6P conjugate:

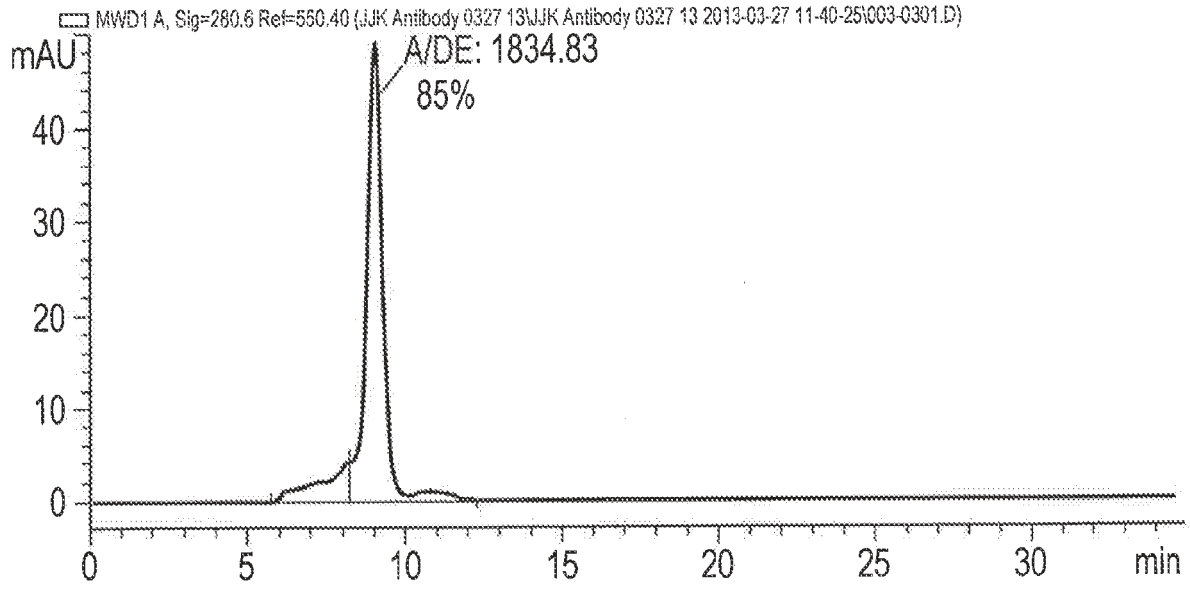
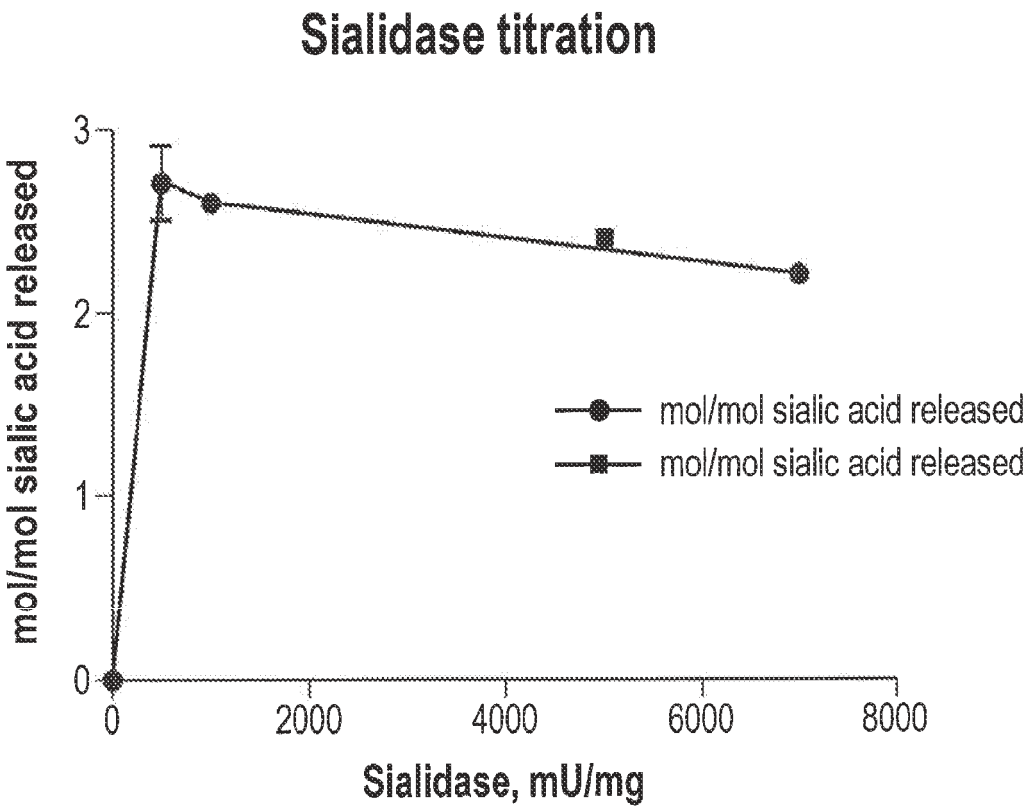


Fig. 51



	mol per mol sialic acid
NNAS	2.4 ± 0
Sialylated NNAS	3.7 ± 0.2
Desialylated and galactosylated NNAS	0 ± 0

Fig. 52

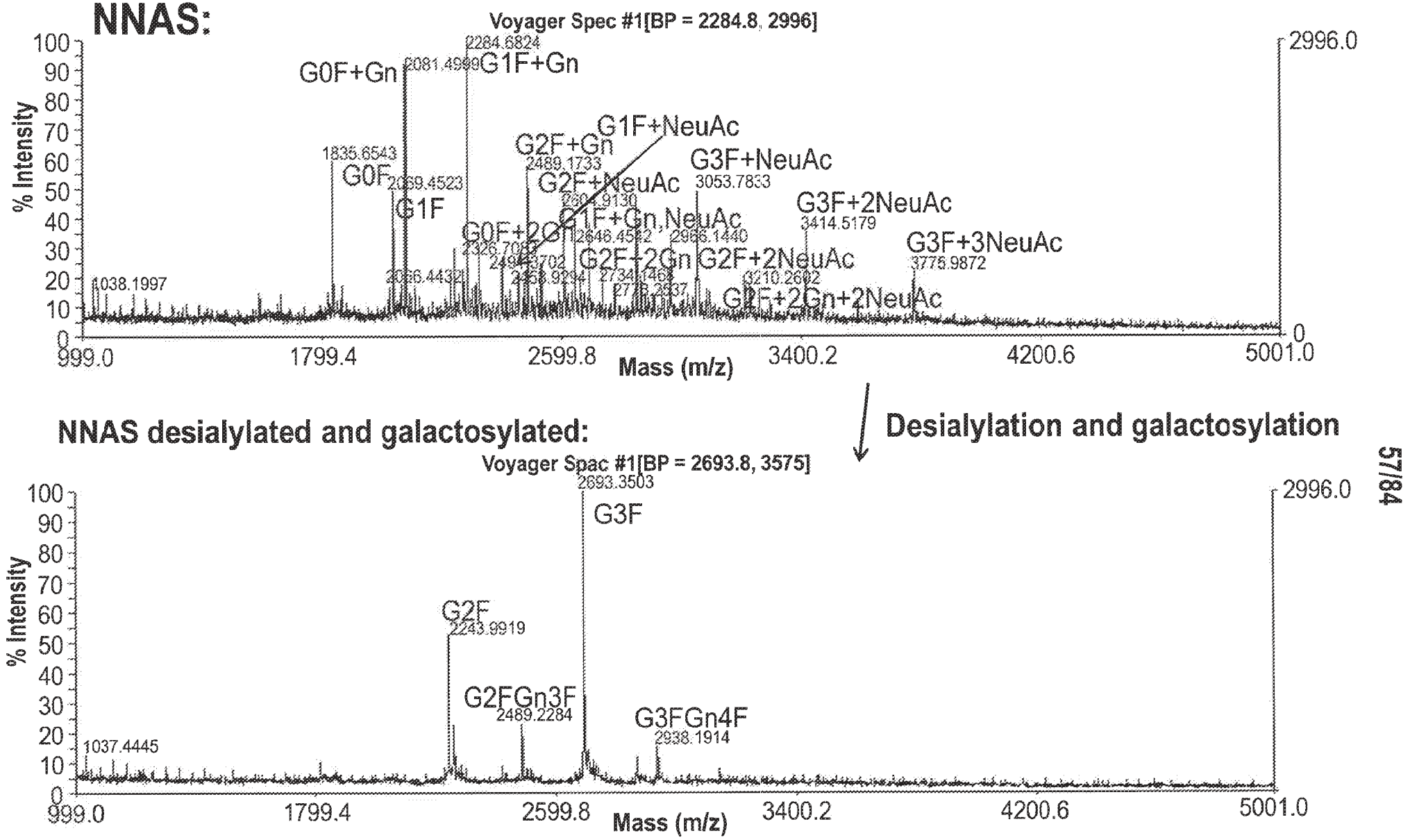

NNAS:
Voyager Spec #1[BP = 2284.8, 2996]
% Intensity
Mass (m/z)
2284.6824
2081.4999
G0F+Gn
G1F+Gn
1836.6543
G0F
2069.4523
G1F
G2F+Gn
2489.1733
G1F+NeuAc
G2F+NeuAc
2891.9130
G1F+Gn,NeuAc
G0F+2Gn
2646.4512
2966.1440
G2F+2Gn
G2F+2NeuAc
G3F+NeuAc
3053.7833
G3F+2NeuAc
3414.5179
G3F+3NeuAc
3775.9872
G2F+2Gn+2NeuAc
1038.1997
2996.0
999.0
1799.4
2599.8
3400.2
4200.6
5001.0
NNAS desialylated and galactosylated:
Desialylation and galactosylation
Voyager Spac #1[BP = 2693.8, 3575]
2693.3503
G3F
G2F
2243.9919
G2FGn3F
2489.2284
G3FGn4F
2938.1914
1037.4445

Fig. 53

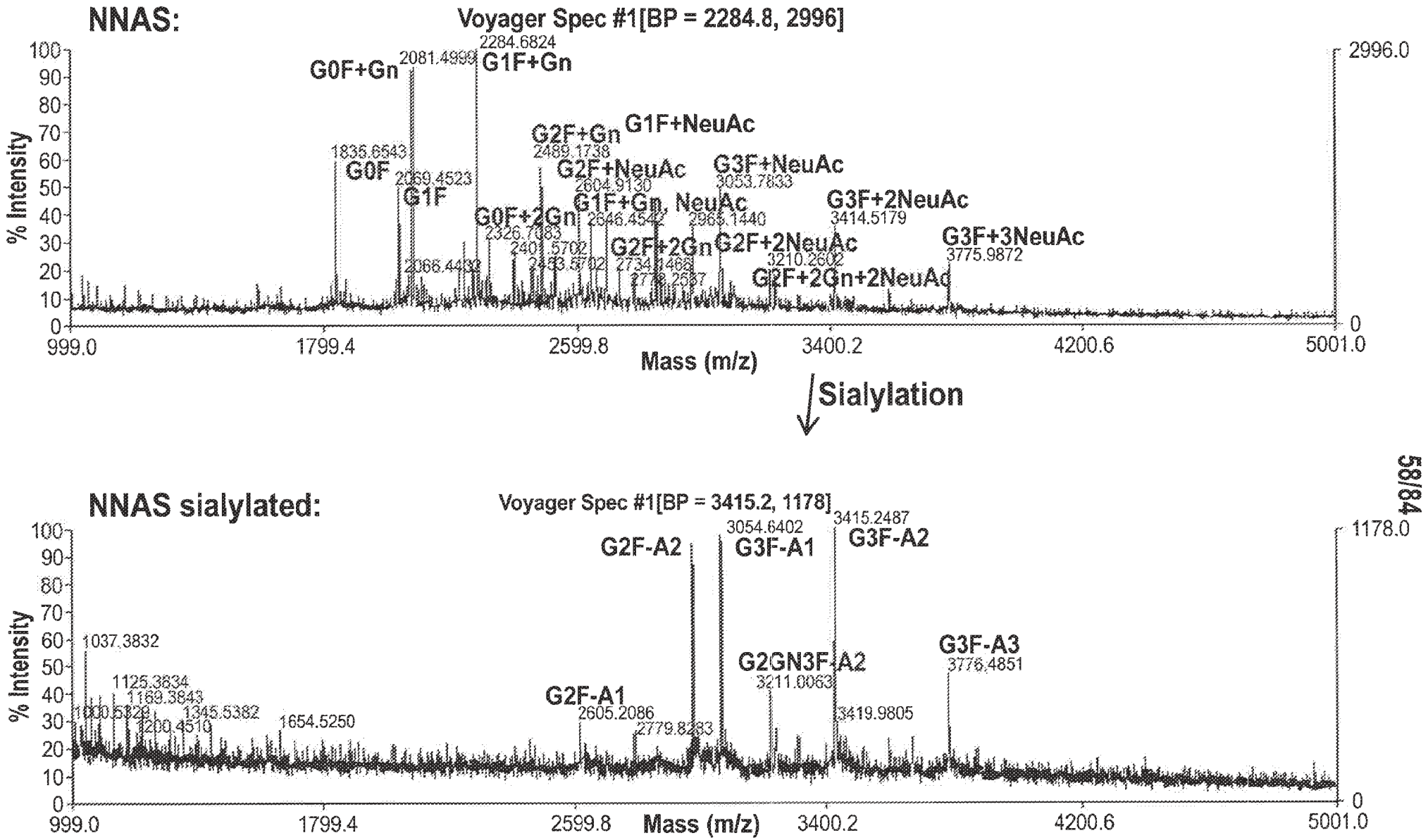
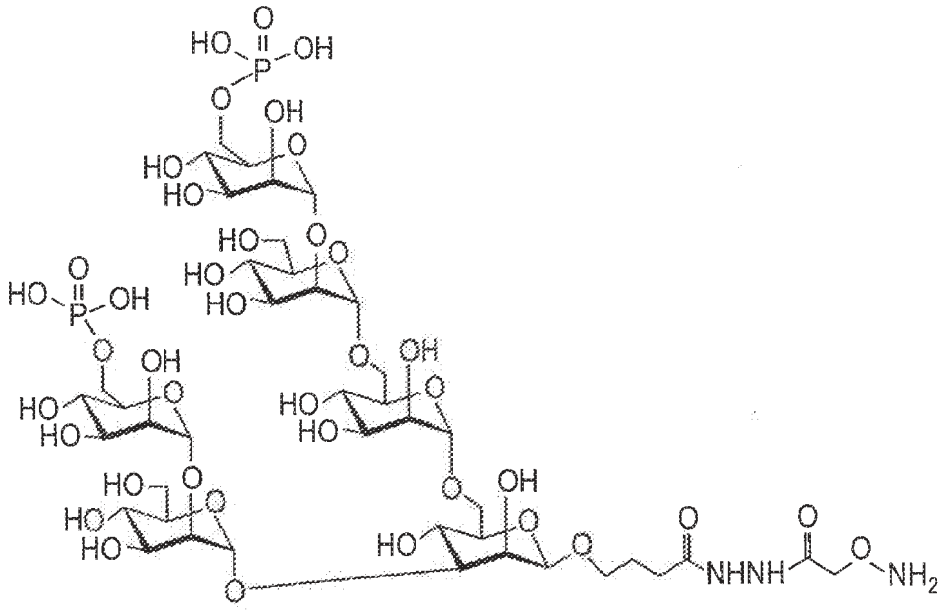
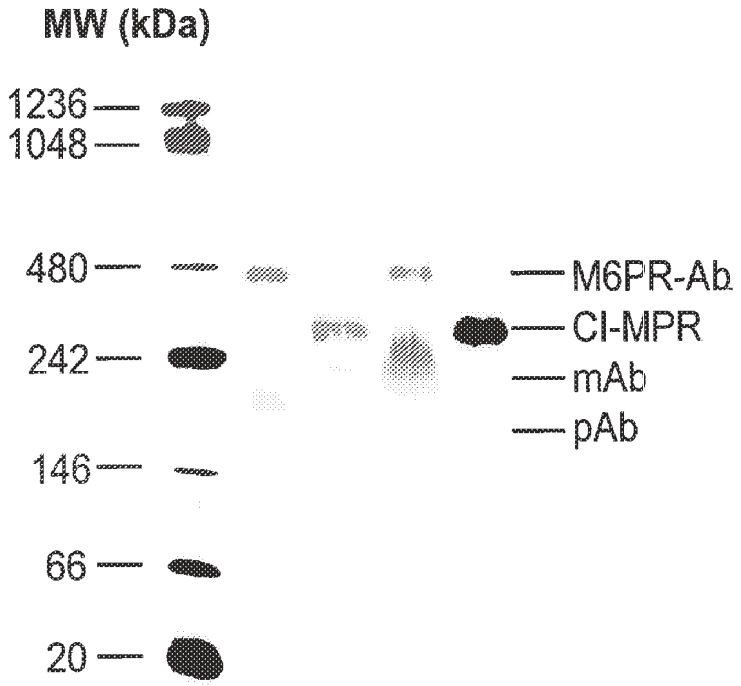

NNAS:
Voyager Spec #1[BP = 2284.8, 2996]
% Intensity
Mass (m/z)
G0F+Gn
2081.4999
2284.6824
G1F+Gn
G2F+Gn
G1F+NeuAc
2489.1738
1836.6843
G0F
2069.4523
G1F
G2F+NeuAc
2604.9130
G3F+NeuAc
3053.7833
G1F+Gn, NeuAc
2646.4542
2965.1440
G0F+2Gn
2326.7083
2401.5702
G2F+2Gn
G2F+2NeuAc
3210.2602
G3F+2NeuAc
3414.5179
G3F+3NeuAc
3775.9872
G2F+2Gn+2NeuAc
2996.0
999.0
1799.4
2599.8
3400.2
4200.6
5001.0
Sialylation
NNAS sialylated:
Voyager Spec #1[BP = 3415.2, 1178]
% Intensity
Mass (m/z)
G2F-A2
3054.6402
G3F-A1
3415.2487
G3F-A2
1037.3832
1125.3834
1169.3843
1345.5382
1654.5250
G2F-A1
2605.2086
2779.8283
G2GN3F-A2
3211.0063
3419.9805
G3F-A3
3776.4851
1178.0
999.0
1799.4
2599.8
3400.2
4200.6
5001.0

Fig. 54

bisM6P hexasaccharide aminooxy (SAM):





M6P conjugate at Fc (pAb, 3 glycans)	*			
mAb 3F11		*		
M6P conjugate at Fc (mAb, 1.5 glycans)			*	
pAb				*

Fig. 55

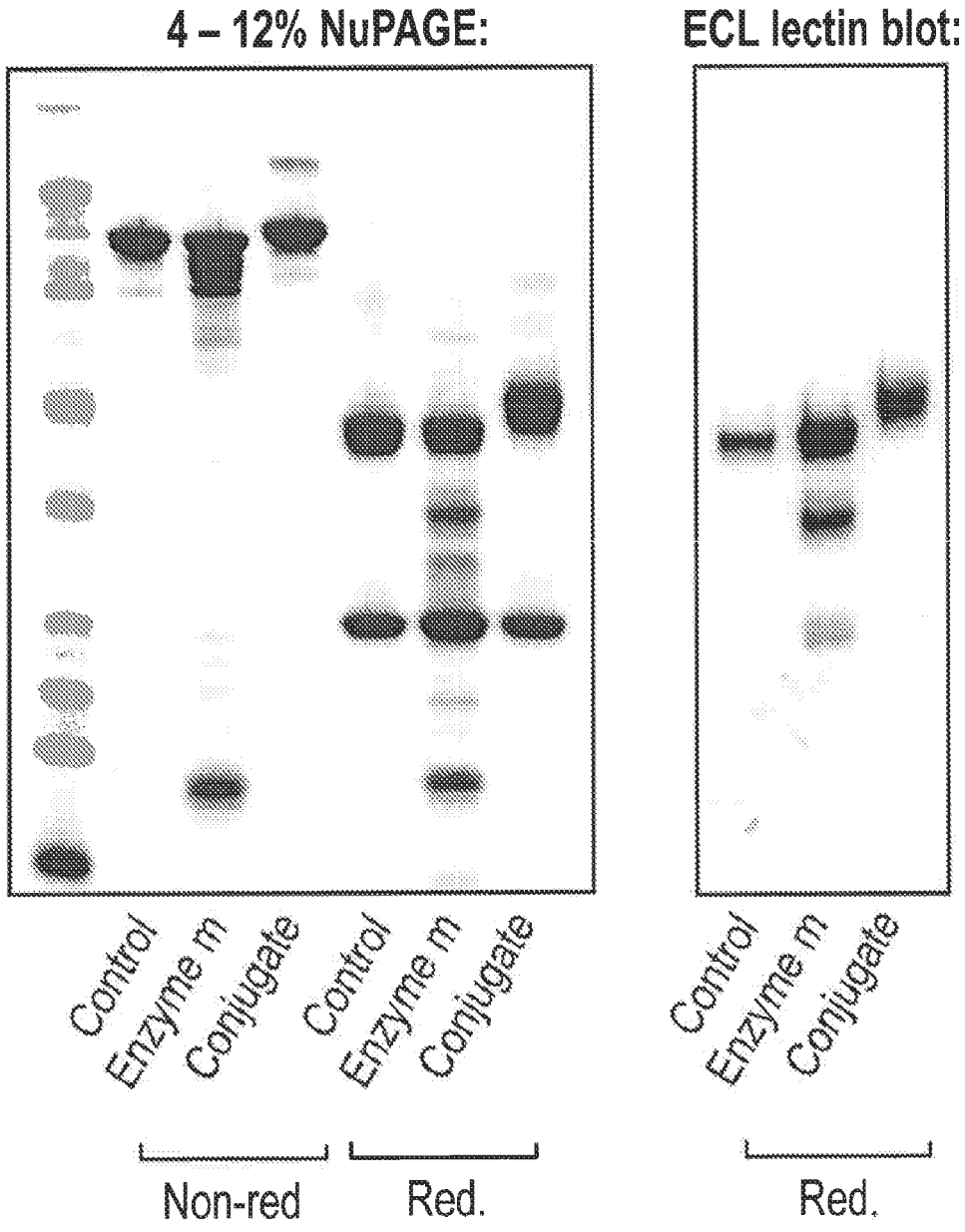

4 – 12% NuPAGE:
ECL lectin blot:
Control
Enzyme m
Conjugate
Control
Enzyme m
Conjugate
Control
Enzyme m
Conjugate
Non-red
Red.
Red.

Fig. 56

	mol galactose per mol AB	mol glycopeptide per mol Ab
NNAS	1.4 + 0	0
NNAS desialylated/galactosylated	7.4 + 0.3	0
NNAS conjugate	9.4 + 0	3.1

Fig. 57

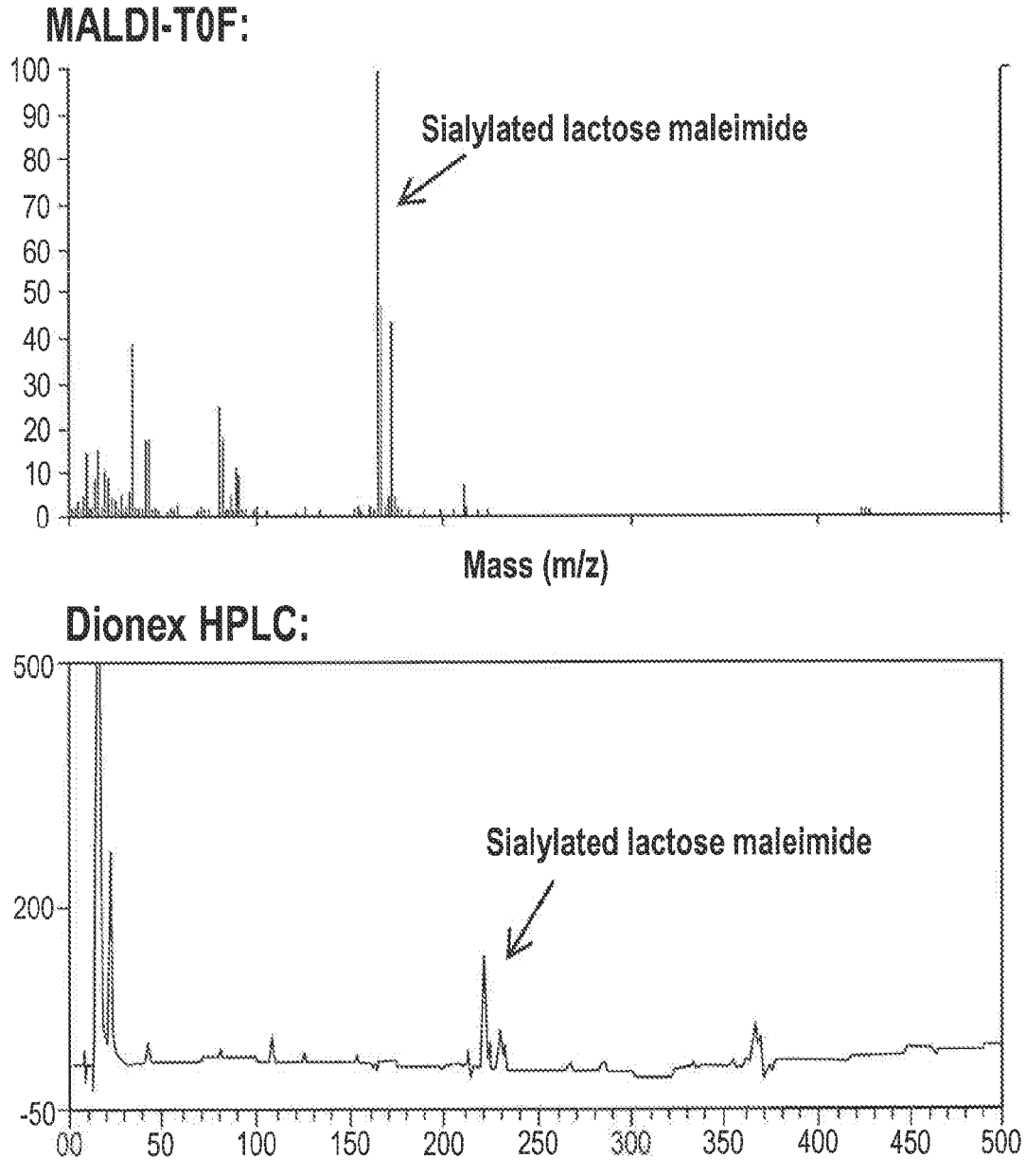


Fig. 58

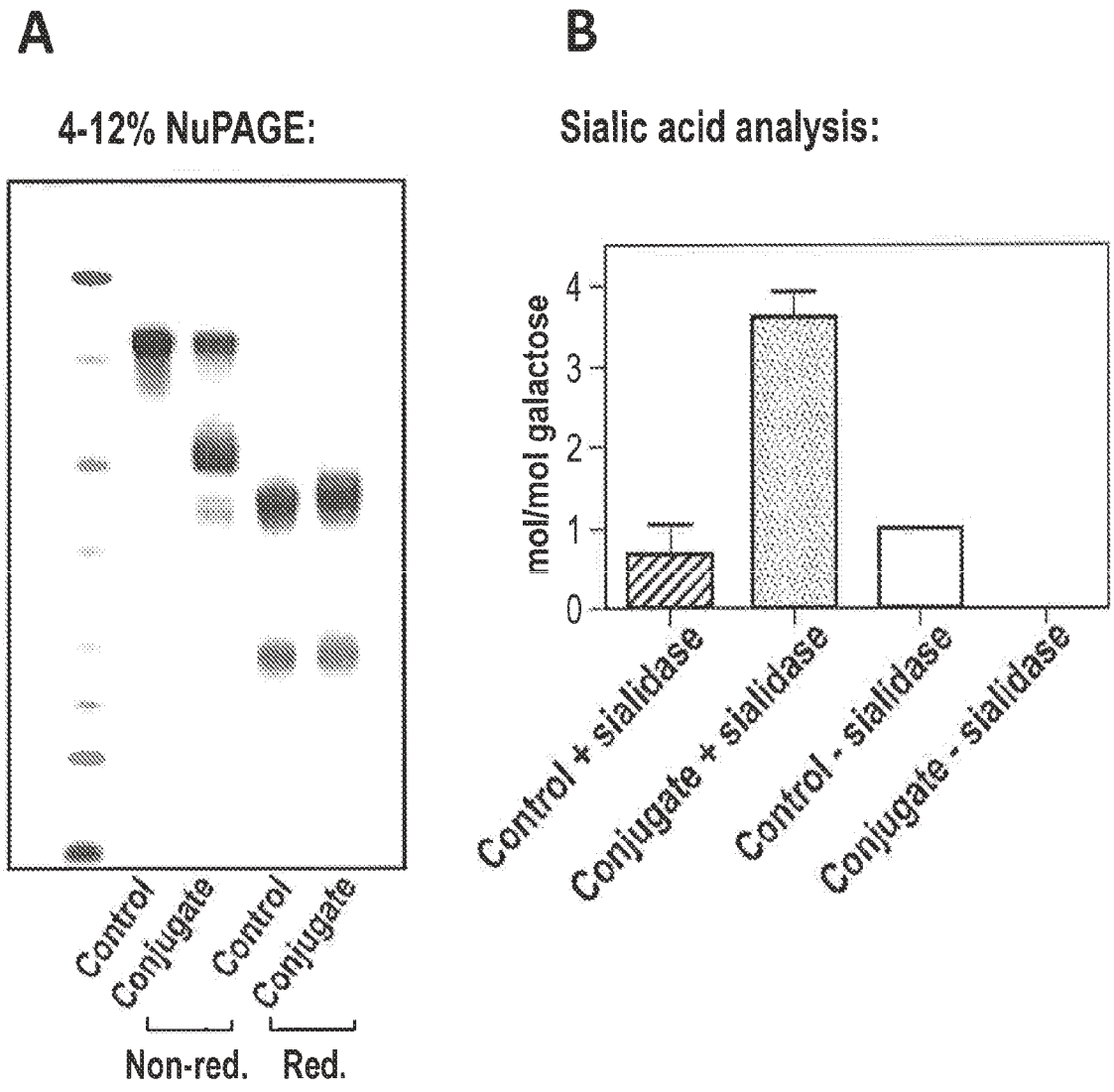

A
4-12% NuPAGE:
Control
Conjugate
Control
Conjugate
Non-red.
Red.
B
Sialic acid analysis:
mol/mol galactose
0
1
2
3
4
Control + sialidase
Conjugate + sialidase
Control - sialidase
Conjugate - sialidase

Fig. 59

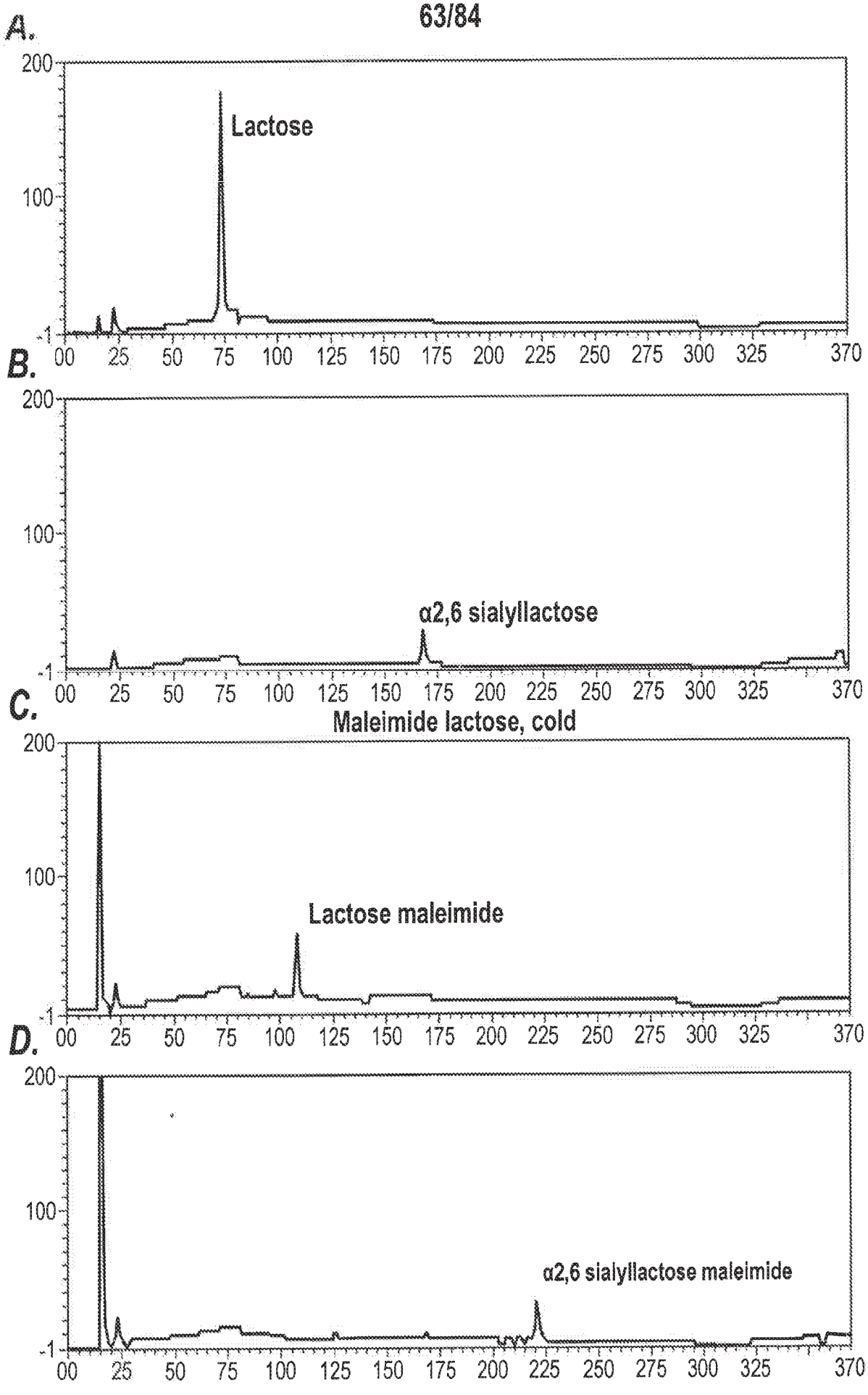

A.
Lactose
B.
α2,6 sialyllactose
C.
Maleimide lactose, cold
Lactose maleimide
D.
α2,6 sialyllactose maleimide
200
100
-1
00 25 50 75 100 125 150 175 200 225 250 275 300 325 370

Fig. 60

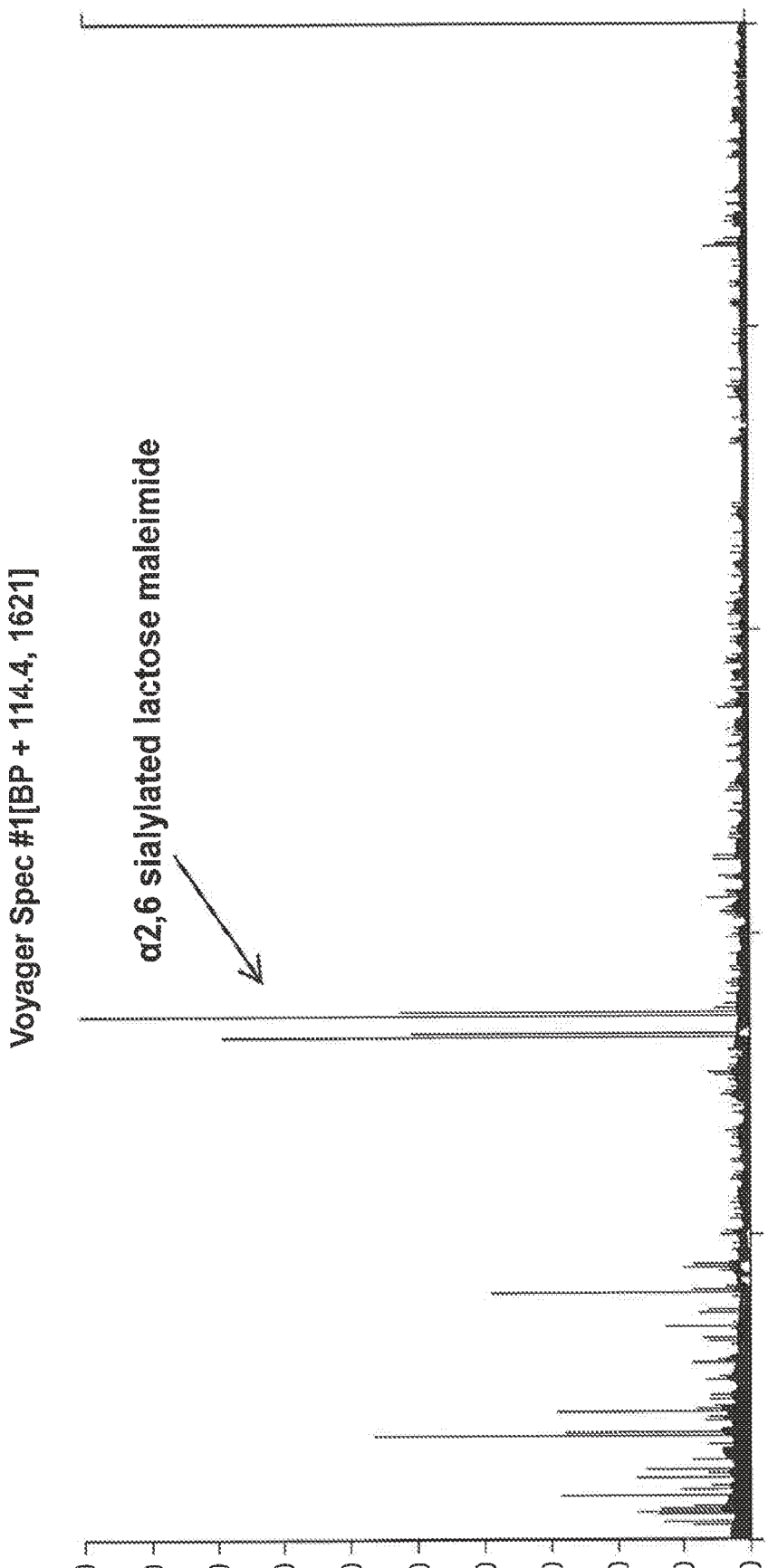
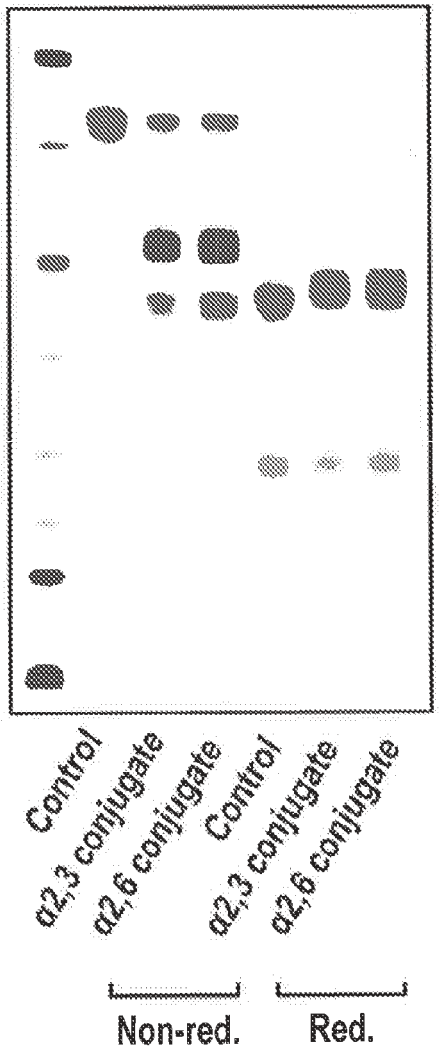

Voyager Spec #1[BP + 114.4, 1621]
α2,6 sialylated lactose maleimide
% Intensity
100
90
80
70
60
50
40
30
20
10
0
Mass (m/z)

Fig. 61

A.

4-12% NuPAGE:



B.

Sialic acid analysis:

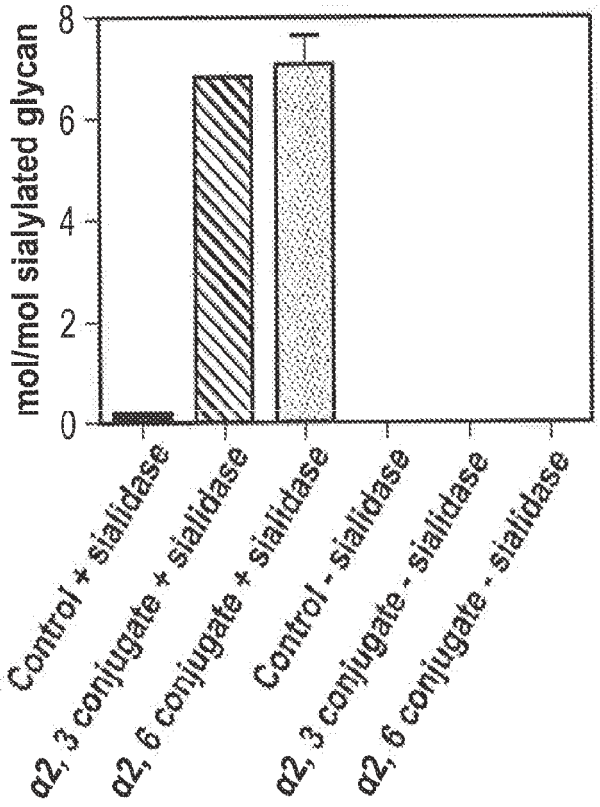


Fig. 62

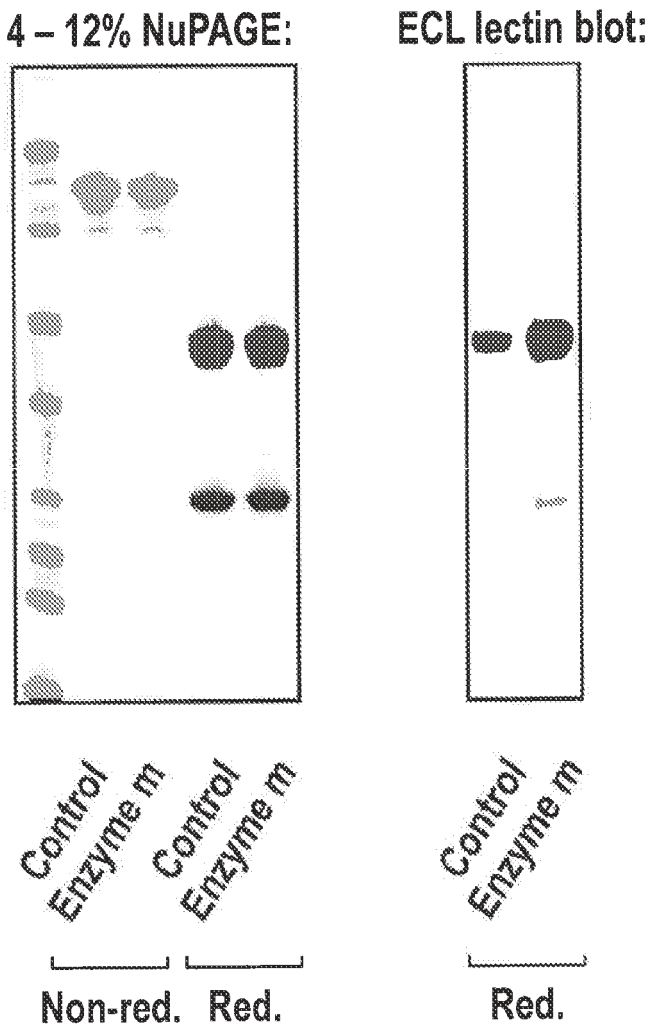

4 – 12% NuPAGE:
ECL lectin blot:
Control
Enzyme m
Control
Enzyme m
Control
Enzyme m
Non-red.
Red.
Red.

Fig. 63

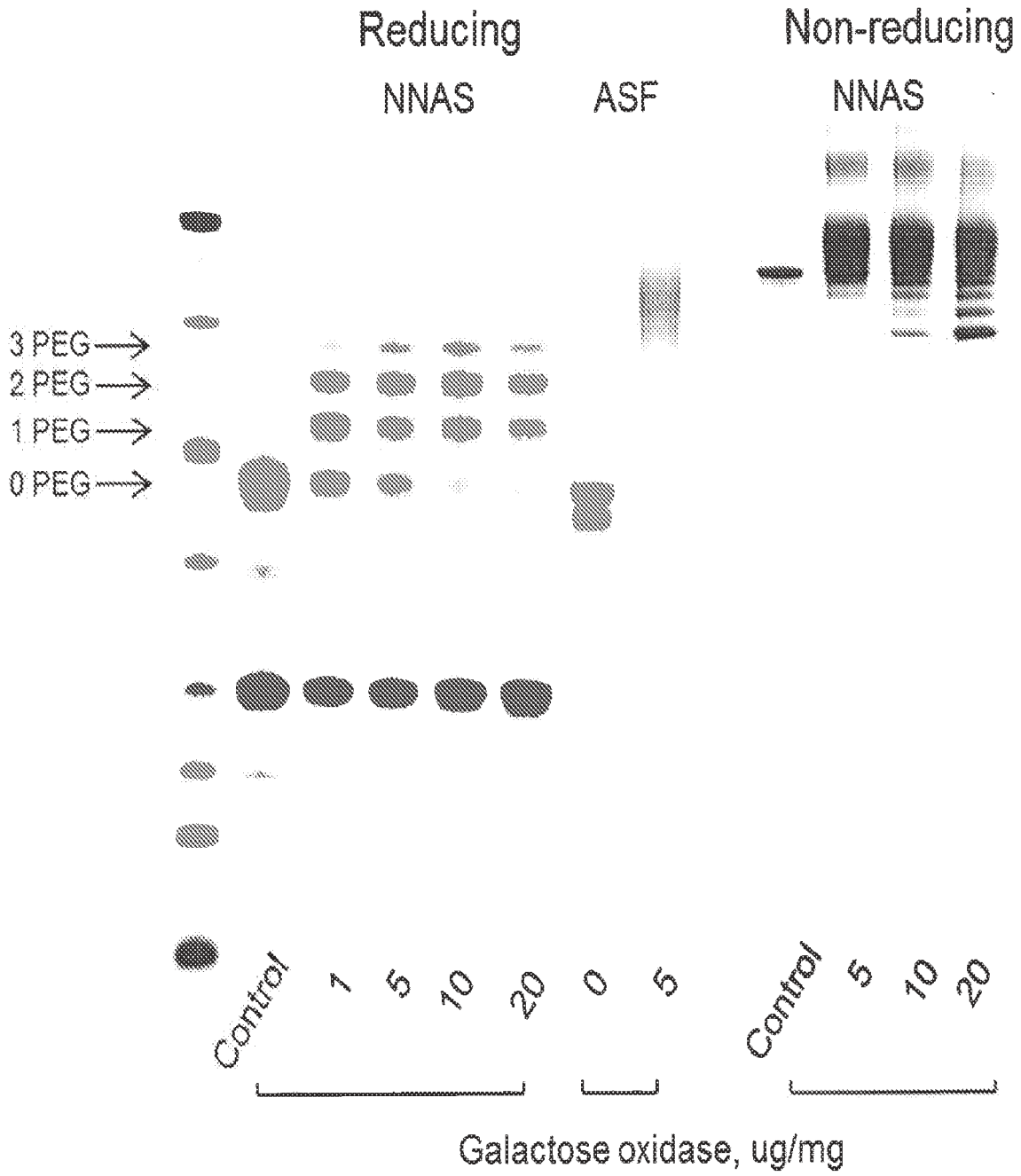

Reducing
Non-reducing
NNAS
ASF
NNAS
3 PEG
2 PEG
1 PEG
0 PEG
Control
1
5
10
20
0
5
Control
5
10
20
Galactose oxidase, ug/mg

Fig. 64

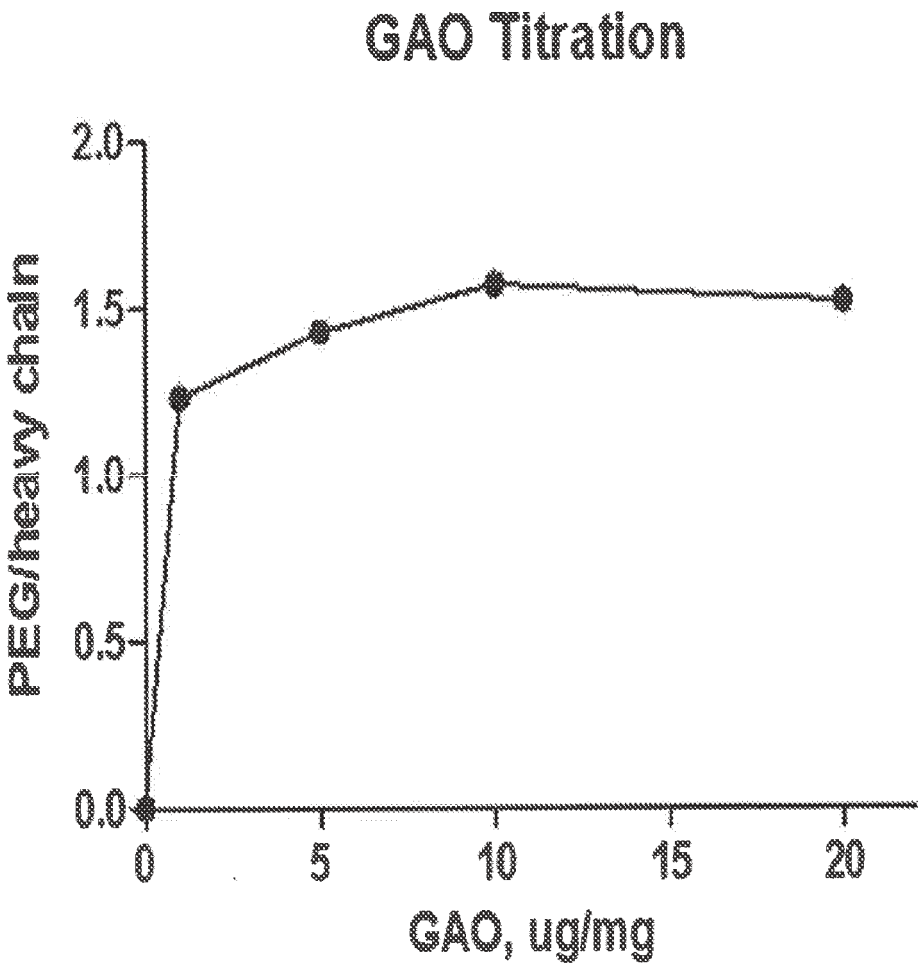

GAO Titration
PEG/heavy chain
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
GAO, ug/mg

Fig. 65

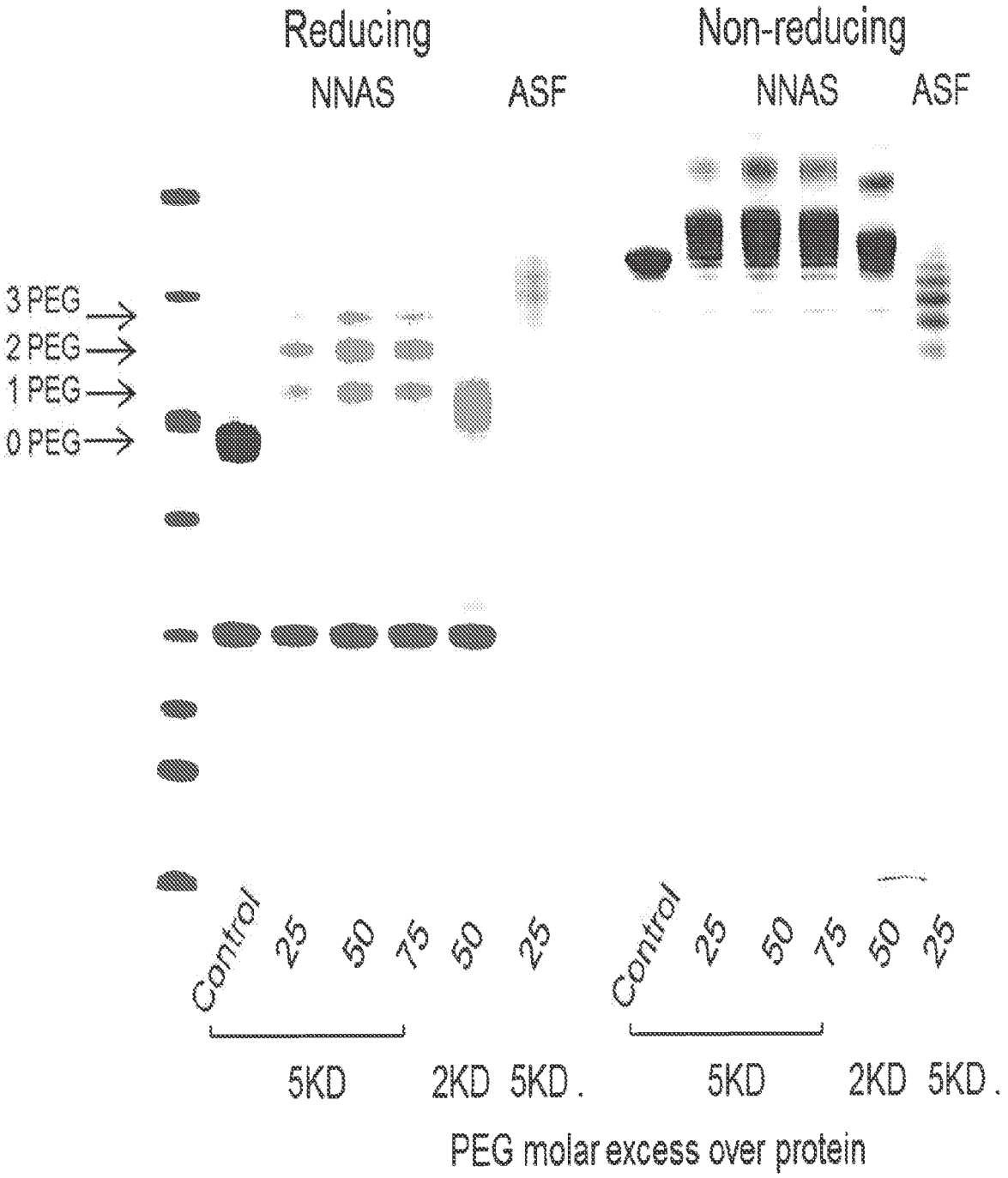

Reducing
Non-reducing
NNAS
ASF
NNAS
ASF
3 PEG
2 PEG
1 PEG
0 PEG
Control
25
50
75
50
25
Control
25
50
75
50
25
5KD
2KD
5KD.
5KD
2KD
5KD.
PEG molar excess over protein

Fig. 66

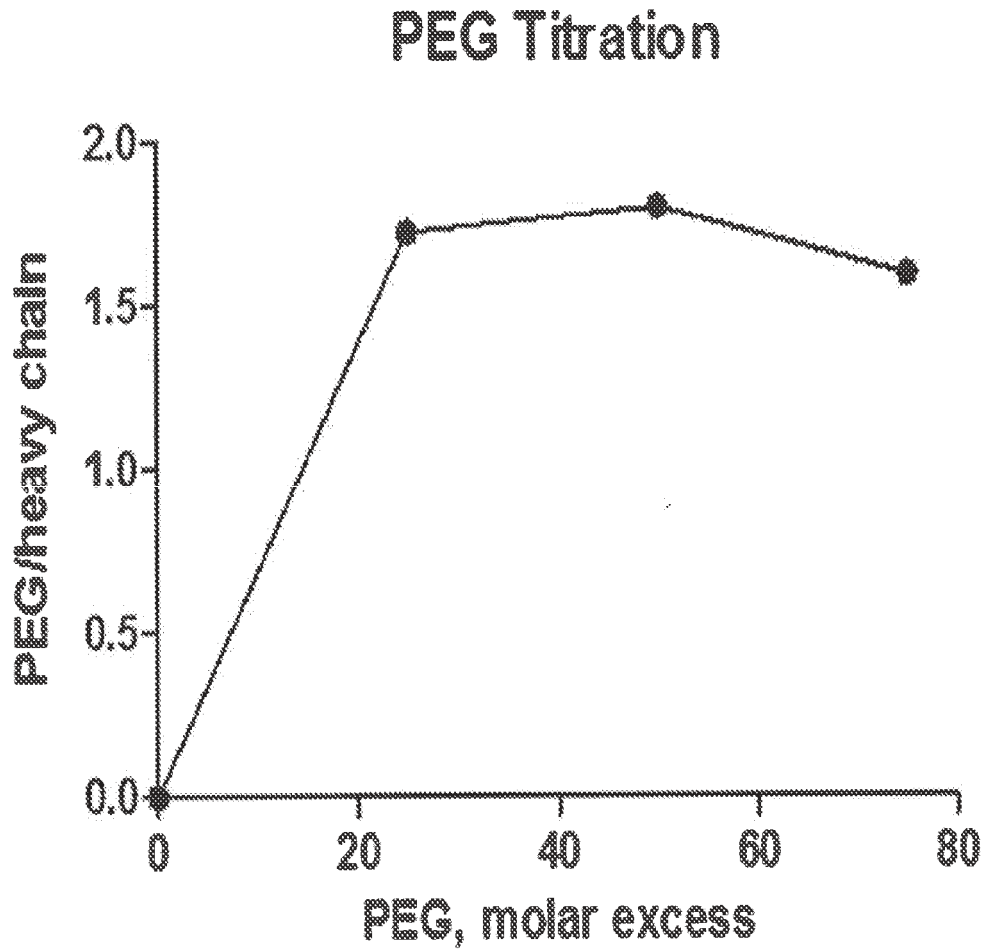
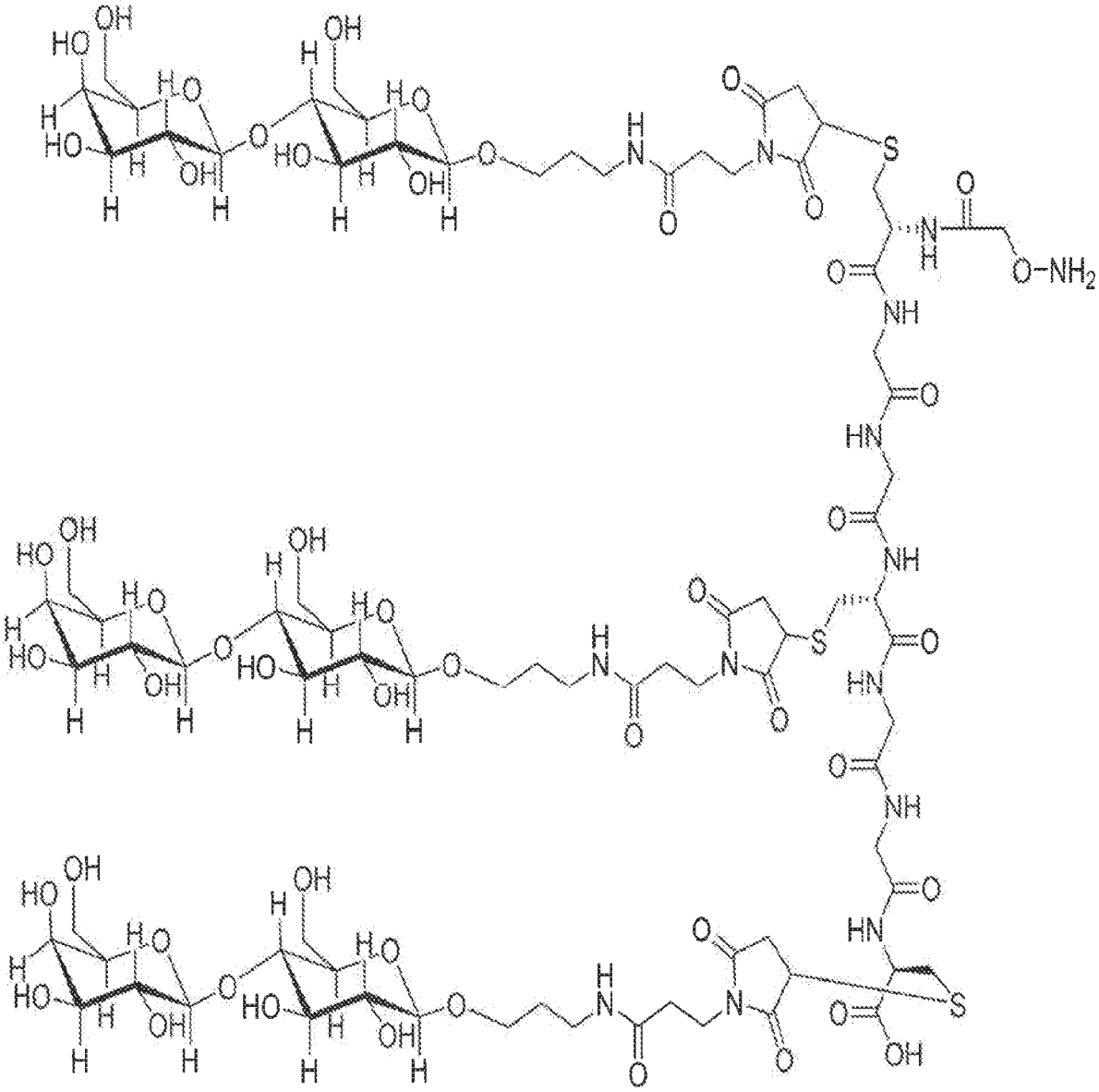

PEG Titration
PEG/heavy chain
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
PEG, molar excess

Fig. 67



Glycopeptide aminooxy: lactose$_3$-Cys$_3$-Gly$_4$

Fig. 68

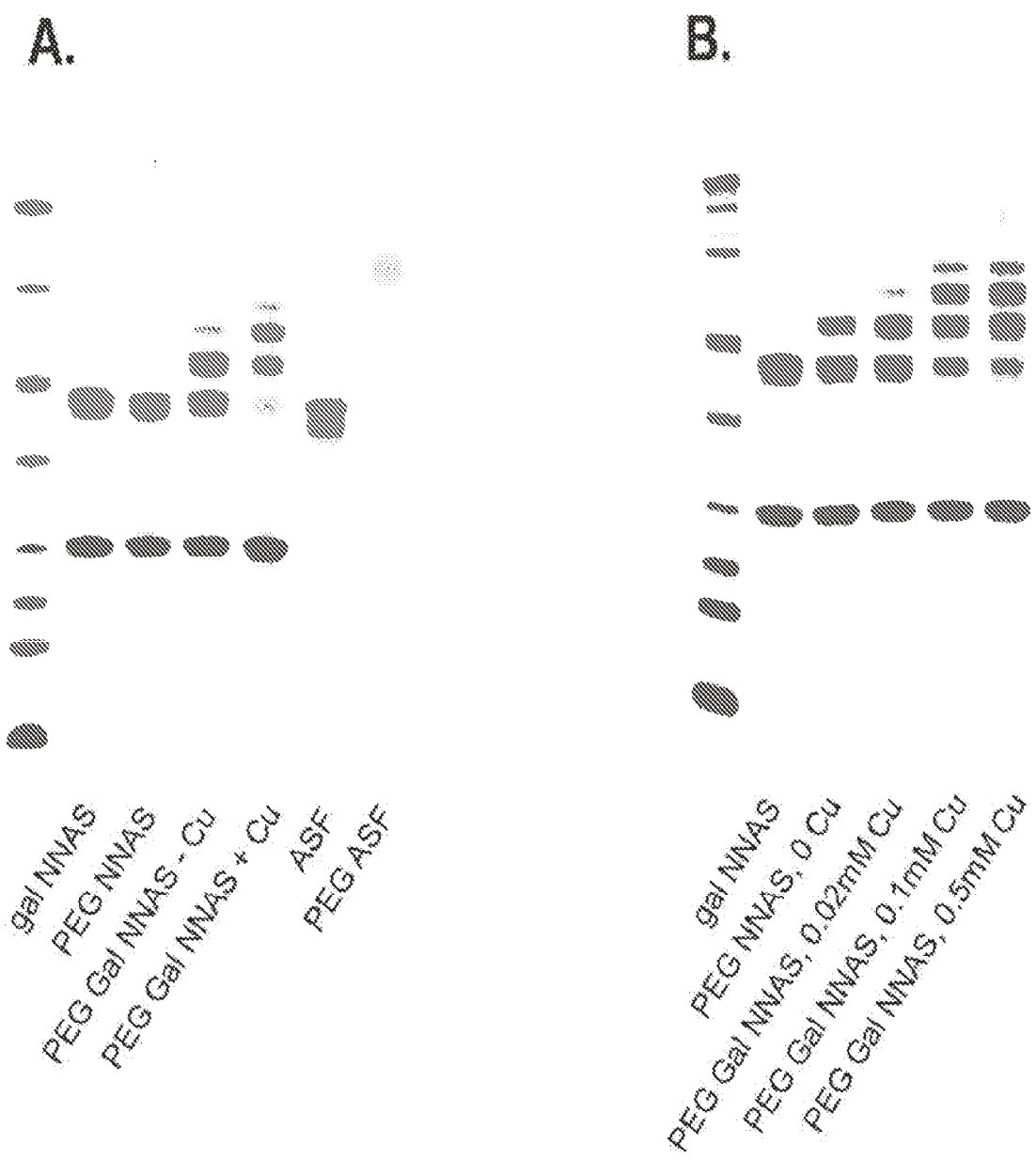

A.
gal NNAS
PEG NNAS
PEG Gal NNAS - Cu
PEG Gal NNAS + Cu
ASF
PEG ASF
B.
gal NNAS
PEG NNAS, 0 Cu
PEG Gal NNAS, 0.02mM Cu
PEG Gal NNAS, 0.1mM Cu
PEG Gal NNAS, 0.5mM Cu

Fig. 69

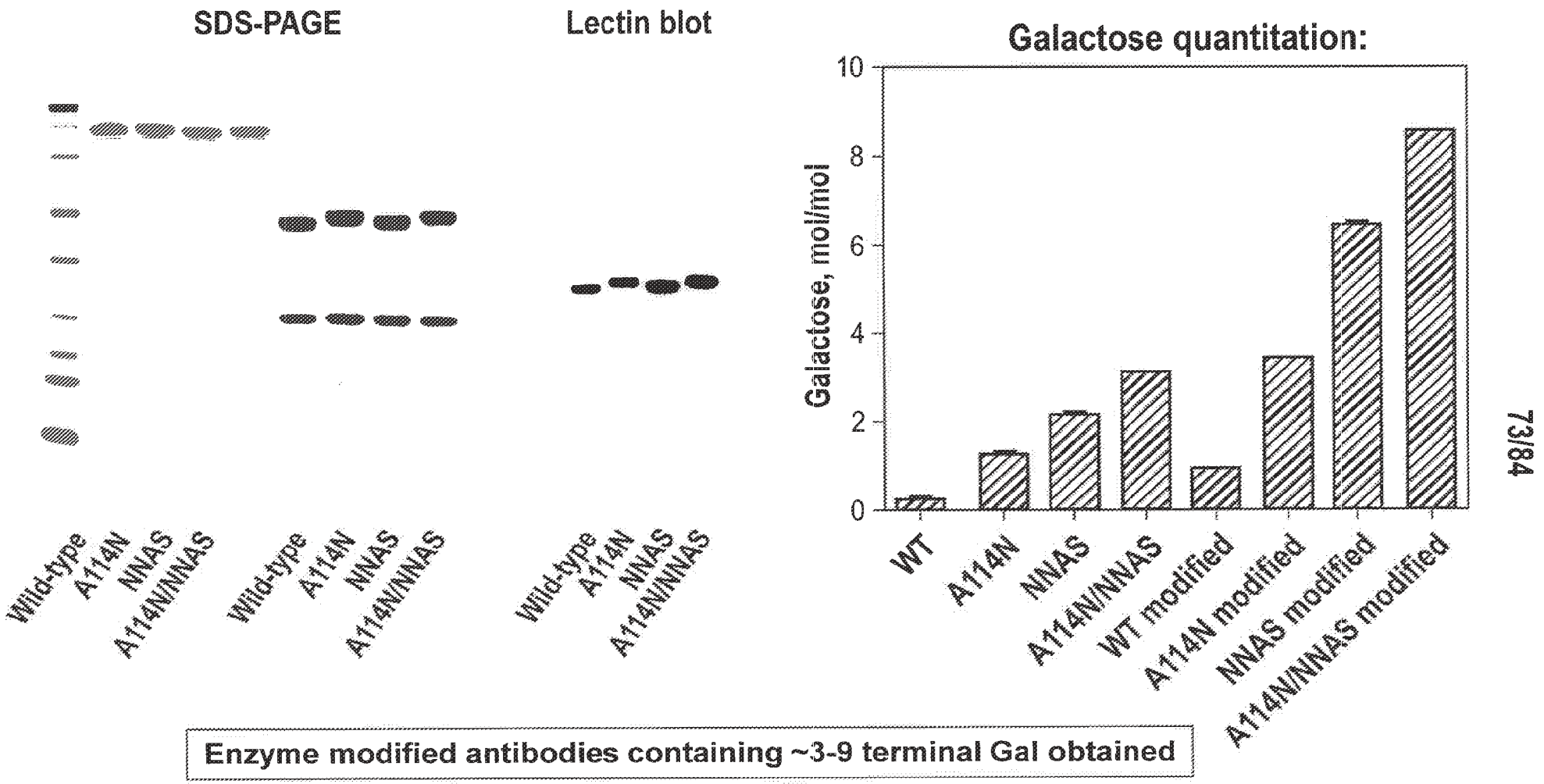
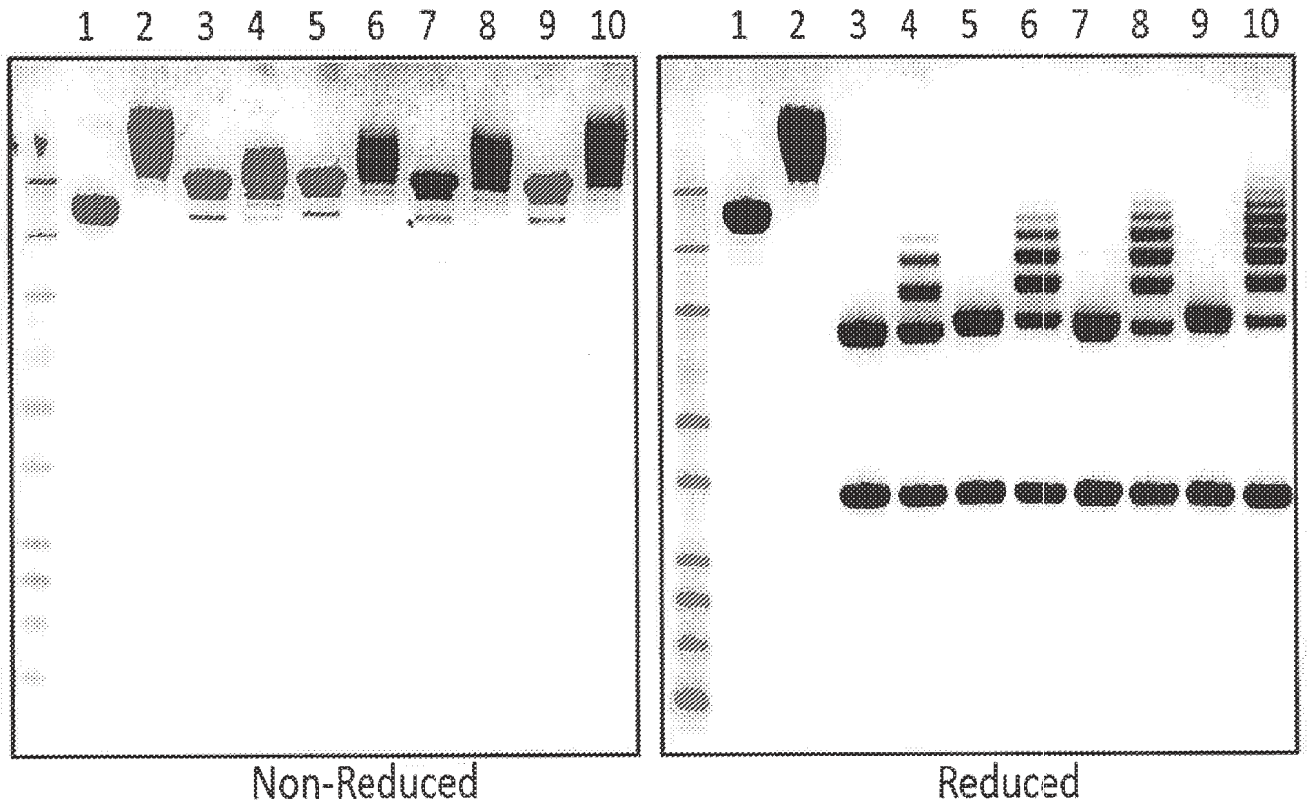

SDS-PAGE
Lectin blot
Galactose quantitation:
Galactose, mol/mol
0
2
4
6
8
10
Wild-type
A114N
NNAS
A114N/NNAS
Wild-type
A114N
NNAS
A114N/NNAS
Wild-type
A114N
NNAS
A114N/NNAS
WT
A114N
NNAS
A114N/NNAS
WT modified
A114N modified
NNAS modified
A114N/NNAS modified
Enzyme modified antibodies containing ~3-9 terminal Gal obtained

Fig. 70

	Mol per mol, Mean ± SD
Wild-type	0.1 ± 0
A114N	0.5 ± 0
NNAS	2.3 ± 0
A114N/NNAS	2.7 ± 0

Fig. 71



1 GAA
2 GAA, 25x PEG
3 Her2 WT
4 Her2 WT, 25x PEG
5 Her2 A114N
6 Her2 A114N, 25x PEG
7 Her2 NNAS
8 Her2 NNAS, 25x PEG
9 Her2 A114N/NNAS
10 Her2 A114N/NNAS, 25x PEG

Fig. 72

	Mol per mol, Mean + SD
Wild-type	1.4
A114N	2.7
NNAS	3.4
A114N/NNAS	4.6

Fig. 73

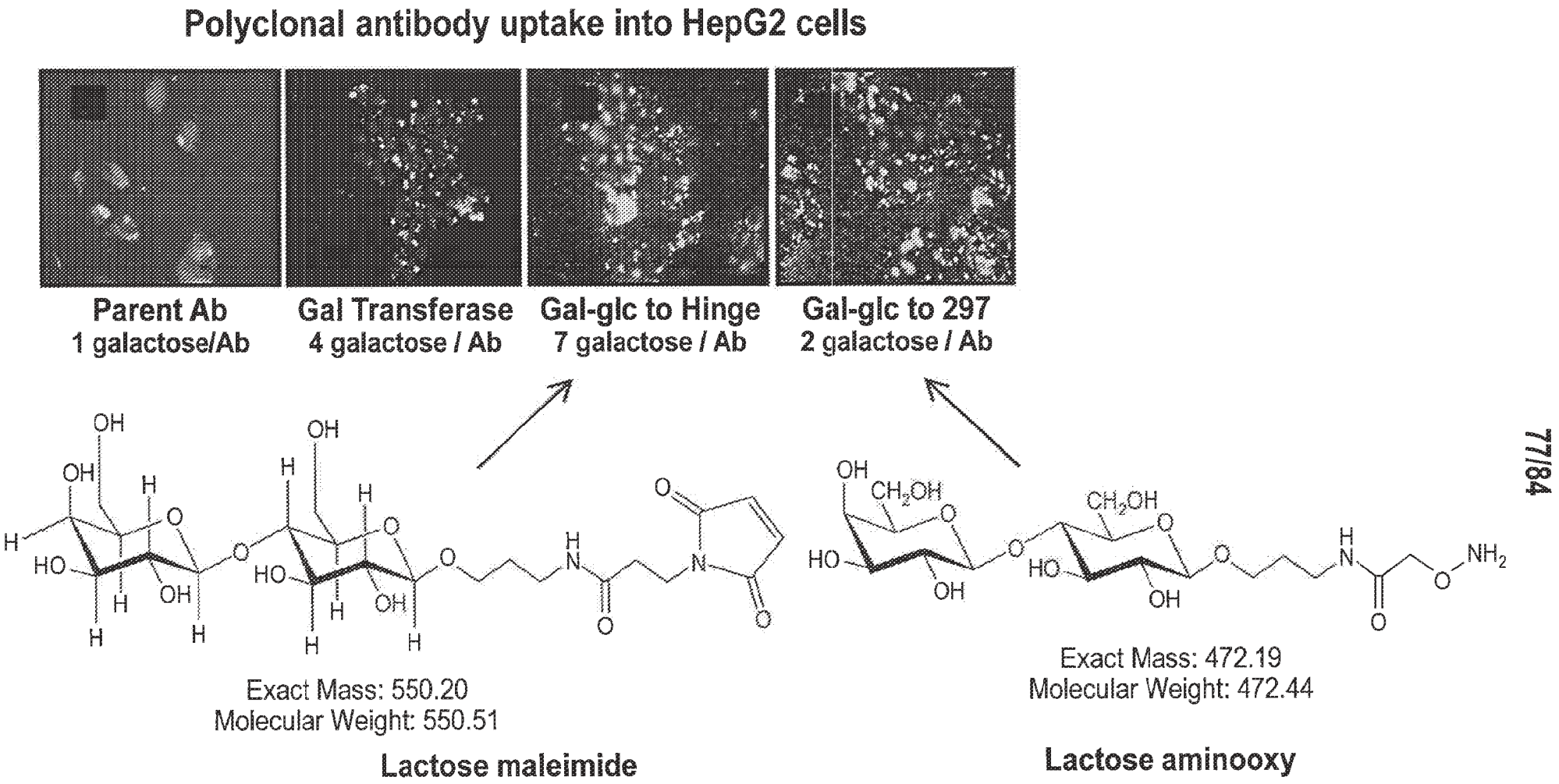

Polyclonal antibody uptake into HepG2 cells
Parent Ab
1 galactose/Ab
Gal Transferase
4 galactose / Ab
Gal-glc to Hinge
7 galactose / Ab
Gal-glc to 297
2 galactose / Ab
Exact Mass: 550.20
Molecular Weight: 550.51
Lactose maleimide
Exact Mass: 472.19
Molecular Weight: 472.44
Lactose aminooxy

Fig. 74

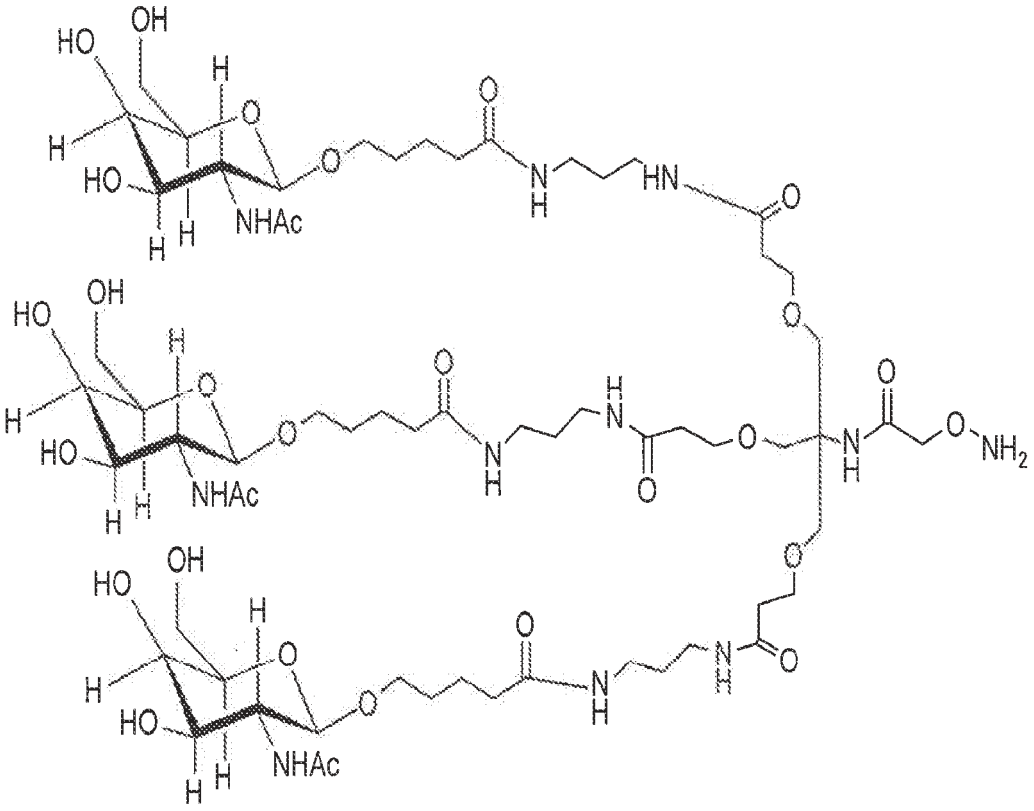
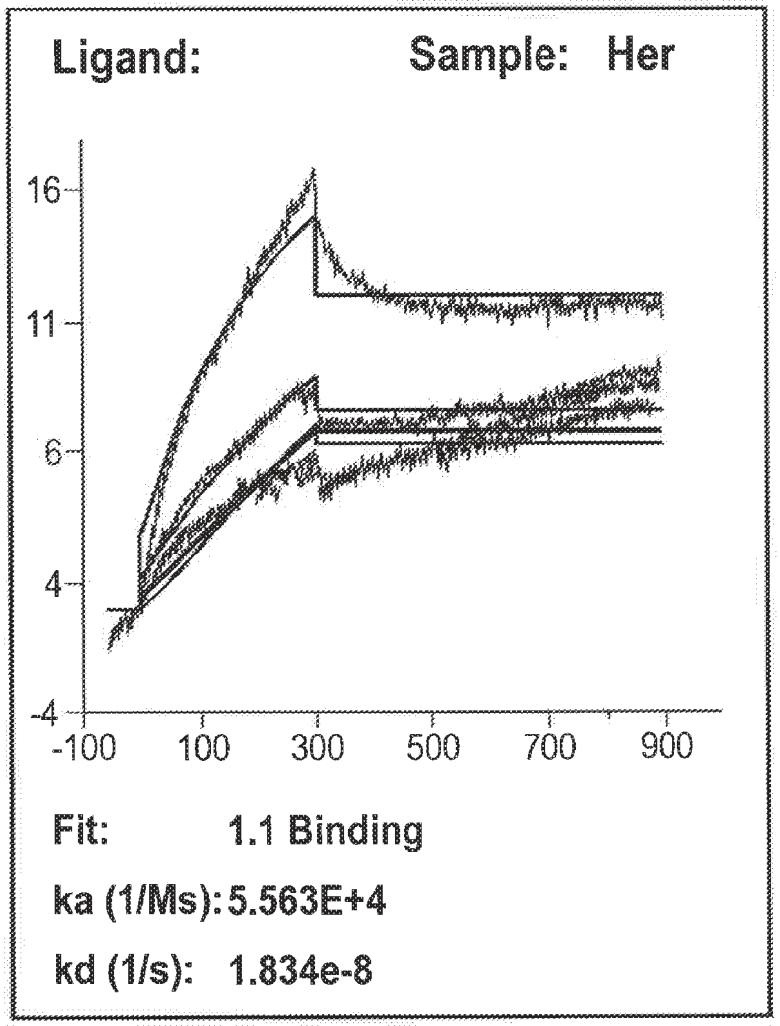
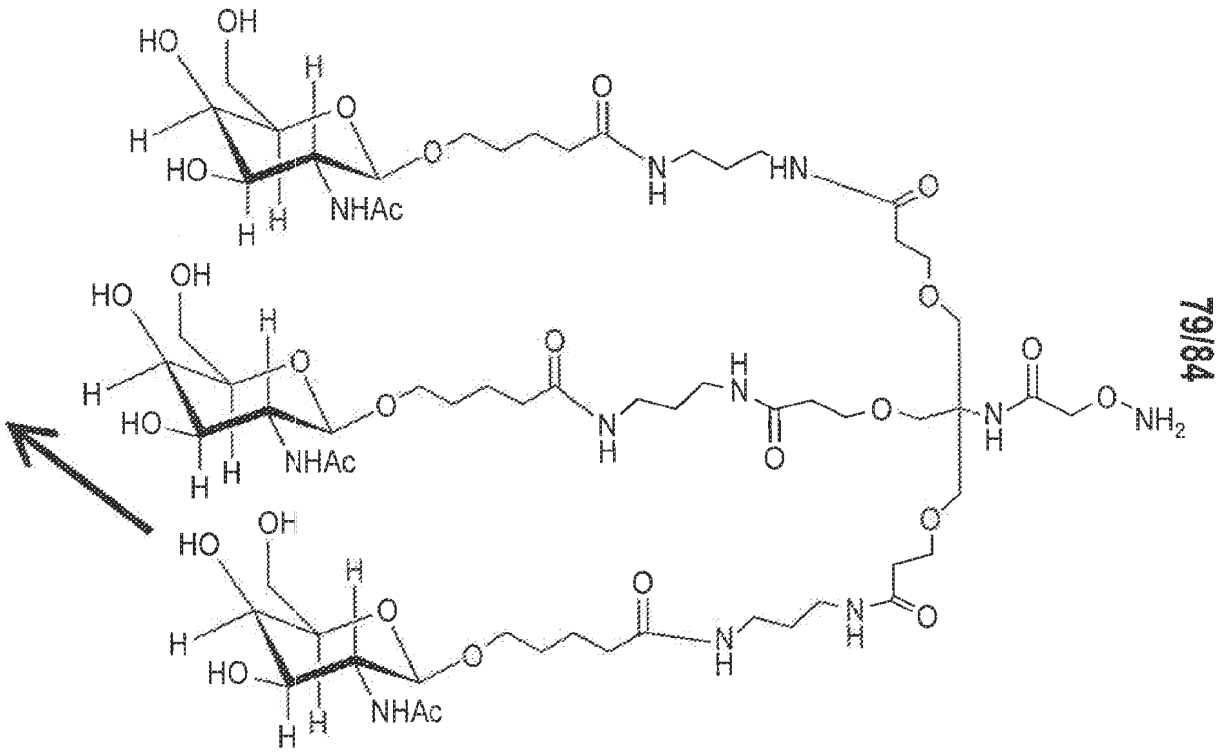

HO
OH
H
O
H
HO
H
H
NHAc
O
O
N
H
HN
O
O
O
HO
OH
H
O
H
HO
H
H
NHAc
O
O
N
H
H
N
O
O
O
N
H
O
O
NH2
O
HO
OH
H
O
H
HO
H
H
NHAc
O
O
N
H
N
H
O

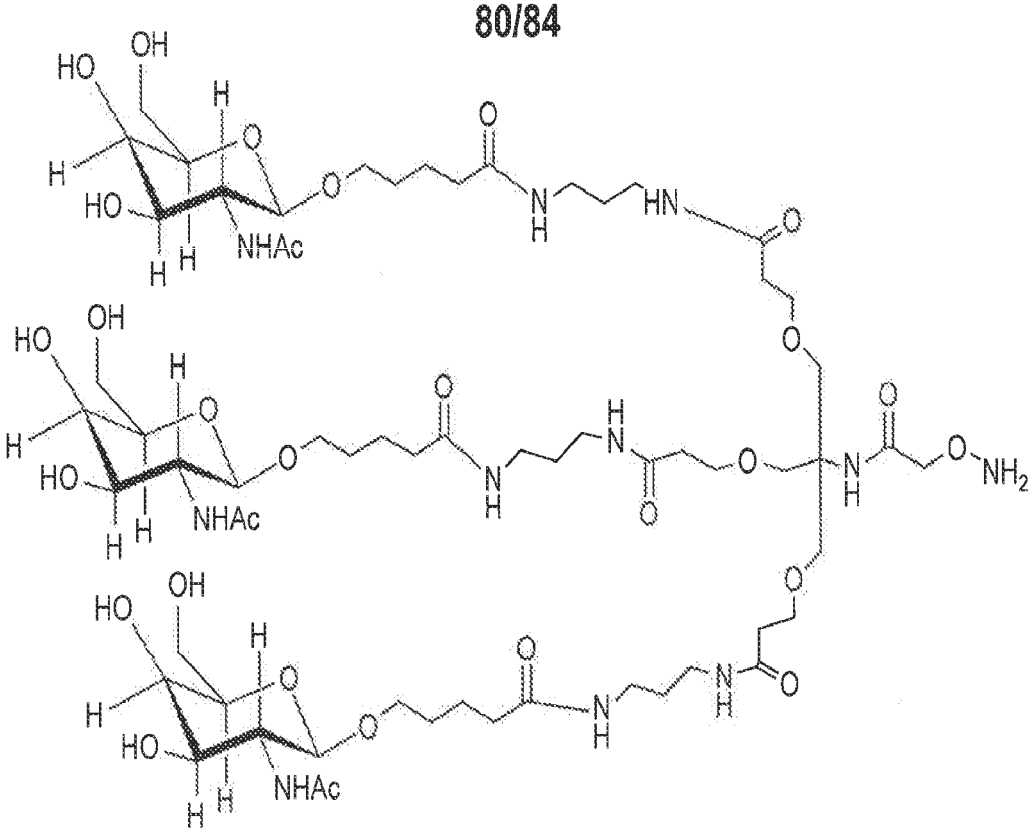
Fig. 75



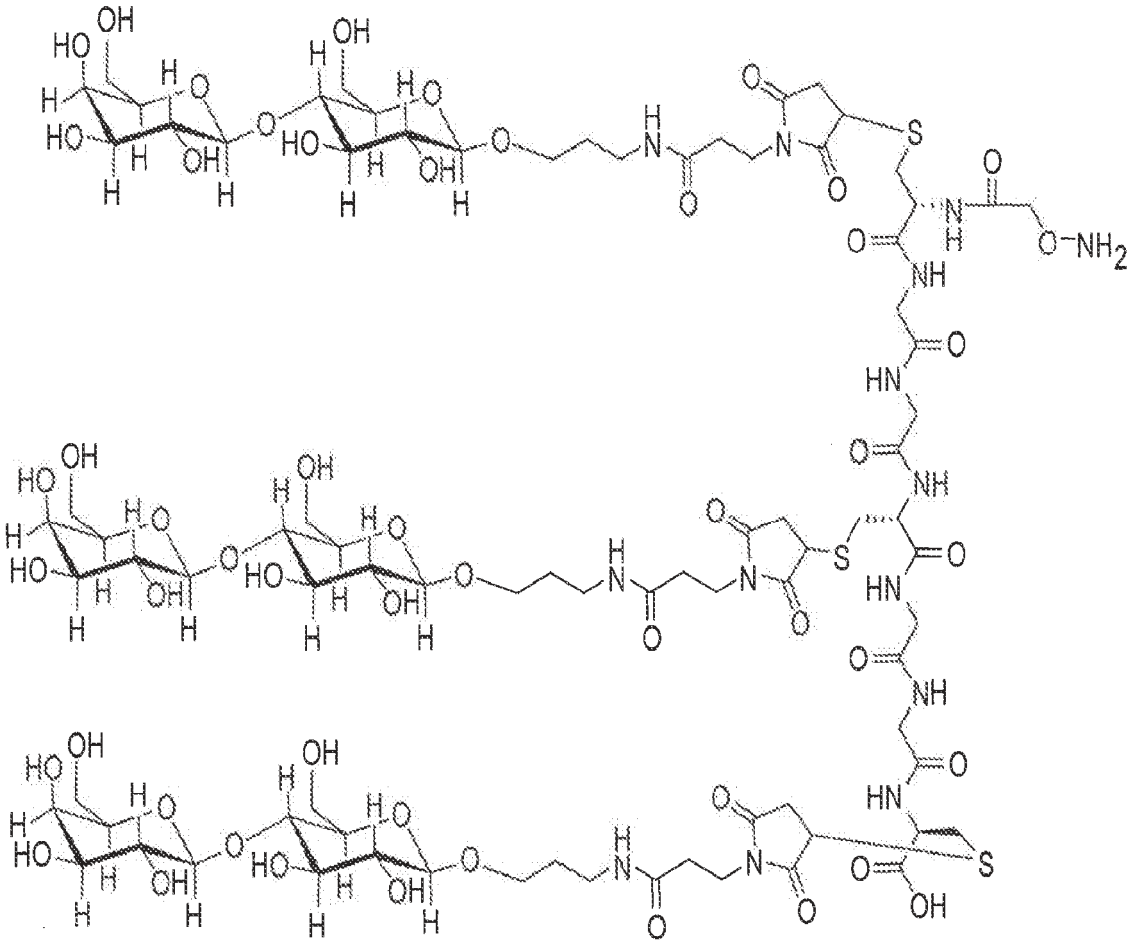


Trivalent GalNAc containing glycan

Fig. 76

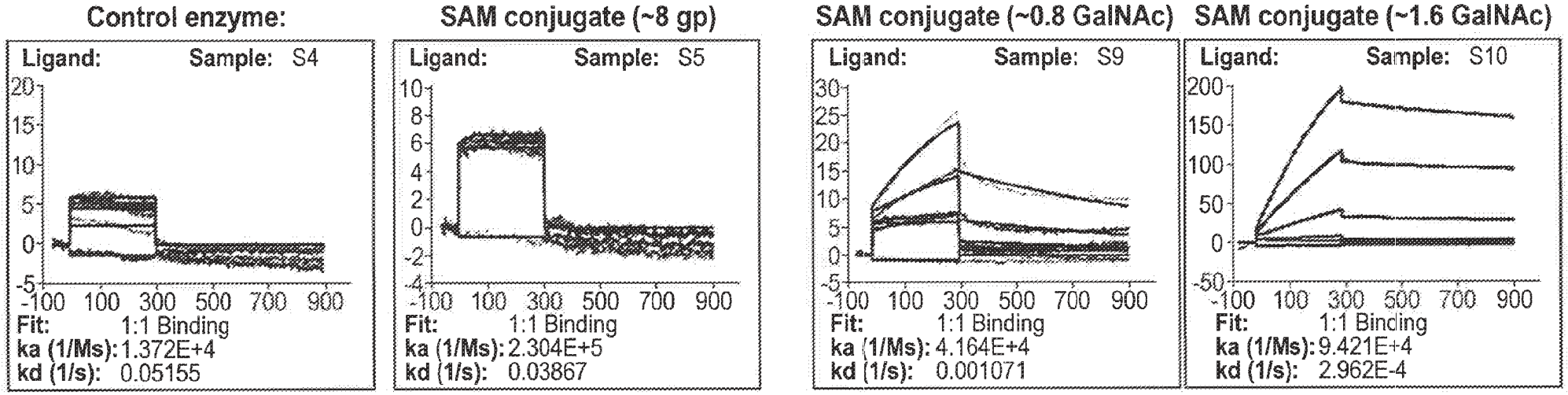


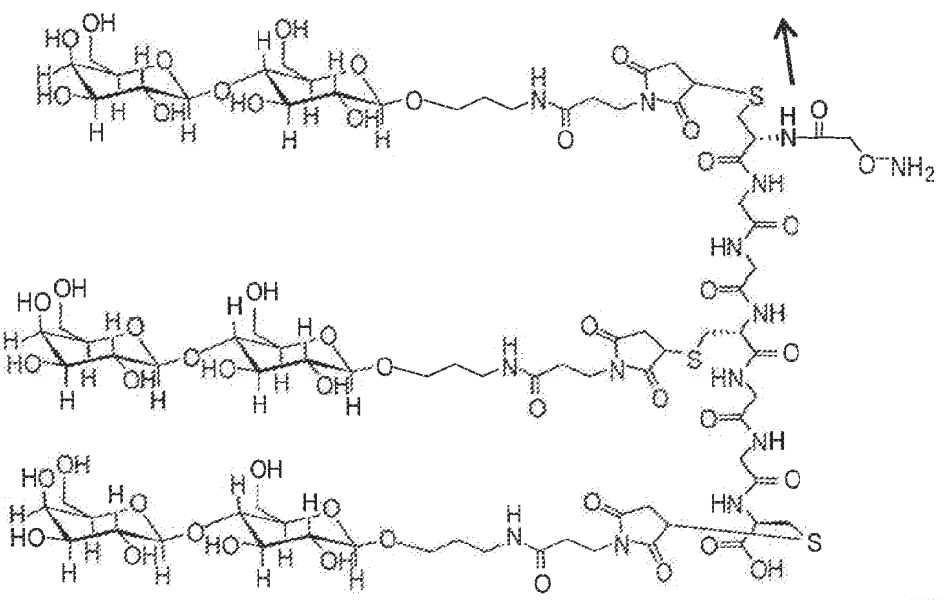
Trivalent GalNAc containing glycan



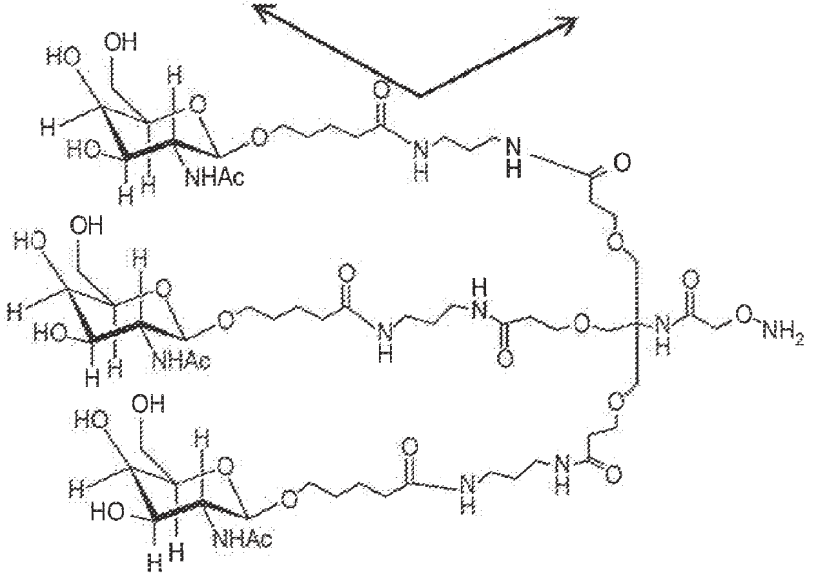
Trivalent Galactose containing glycopeptide

Fig. 77





Trivalent Galactose containing glycopeptide



Trivalent GalNAc containing glycan

Fig. 78

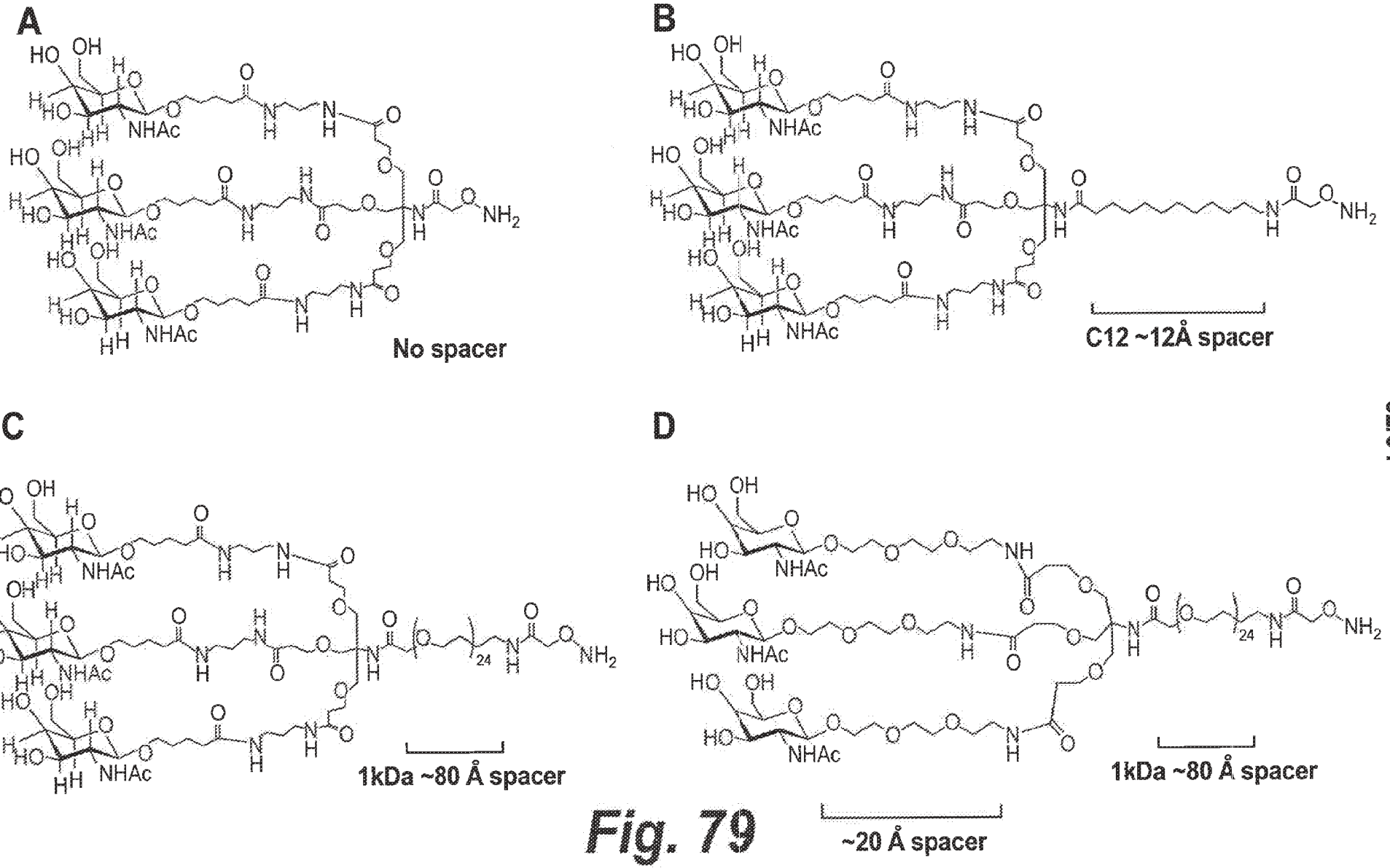
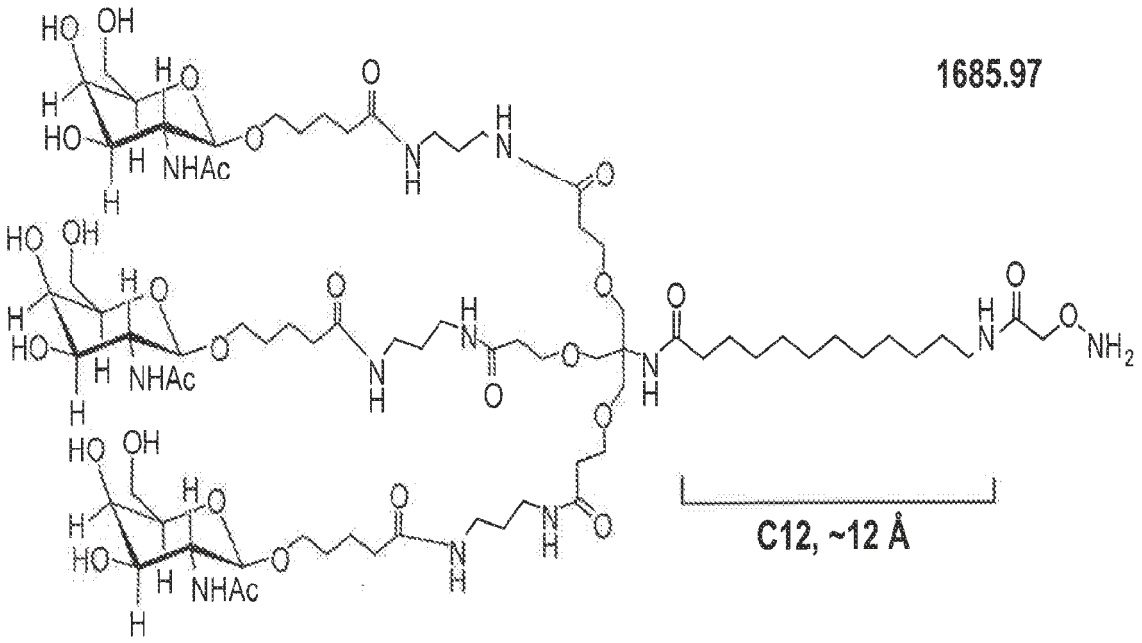

A
No spacer
B
C12 ~12Å spacer
C
1kDa ~80 Å spacer
D
1kDa ~80 Å spacer
~20 Å spacer

Fig. 79



Trivalent GalNAc glycan C12

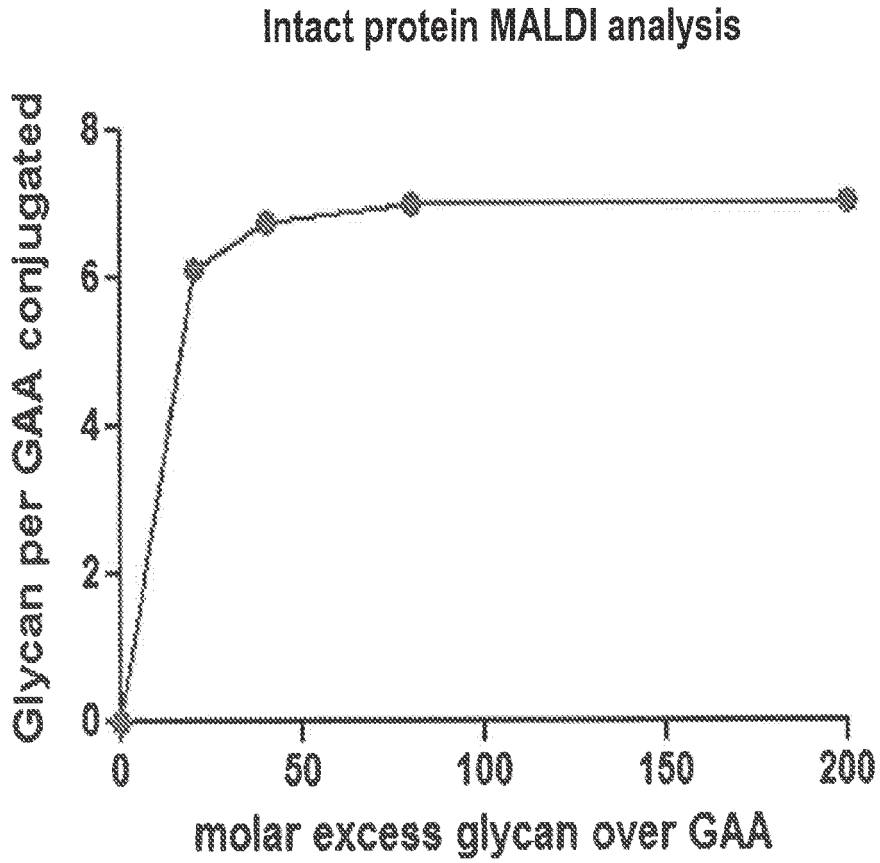
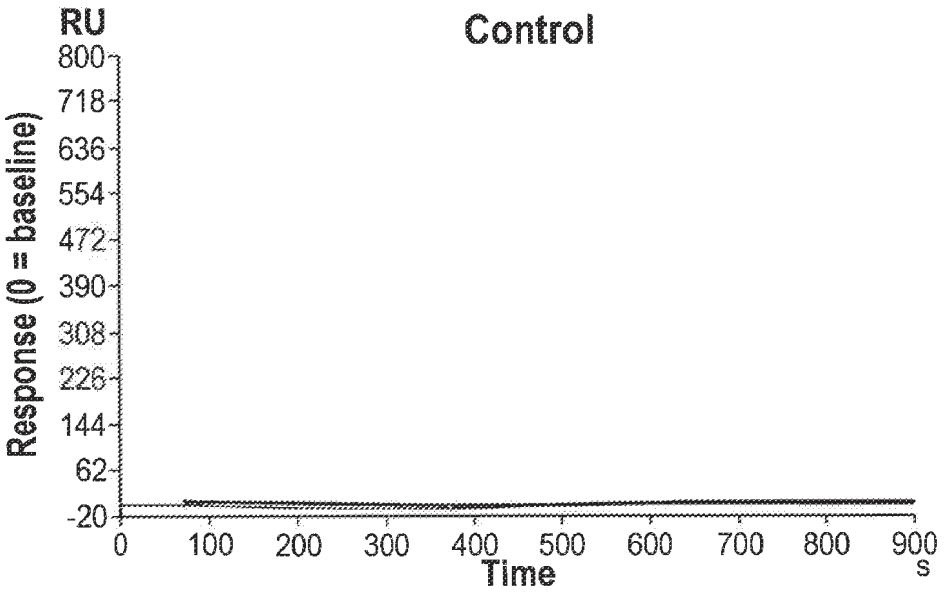
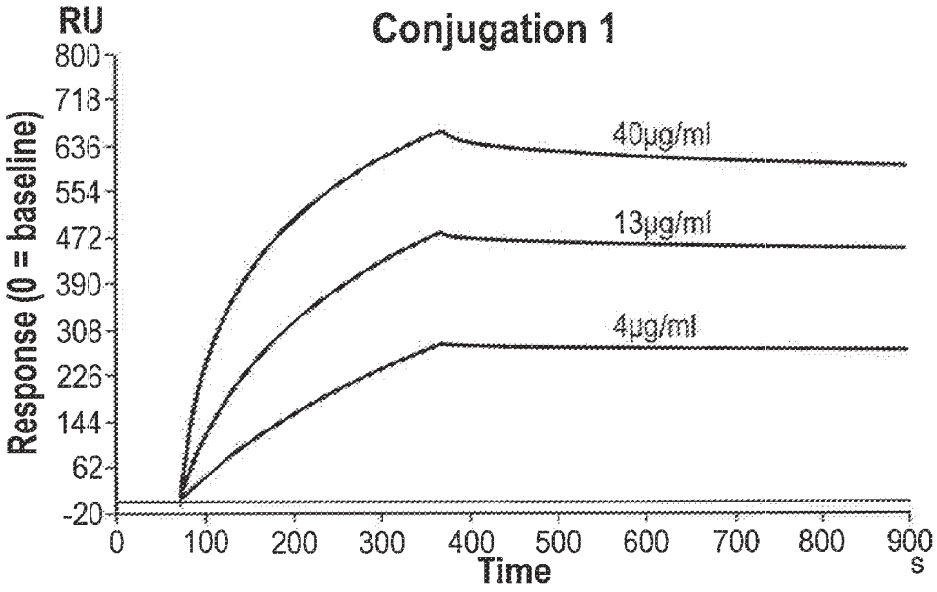


Fig. 80


RU
Control
Response (0 = baseline)
800
718
636
554
472
390
308
226
144
62
-20
0
100
200
300
400
500
600
700
800
900
Time
s


RU
Conjugation 1
Response (0 = baseline)
800
718
636
554
472
390
308
226
144
62
-20
40µg/ml
13µg/ml
4µg/ml
0
100
200
300
400
500
600
700
800
900
Time
s

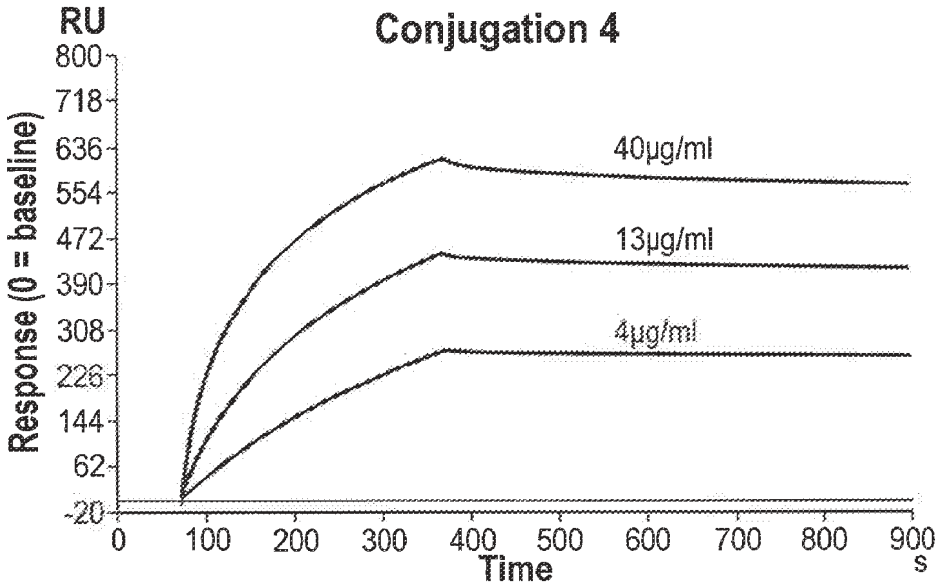

RU
Conjugation 4
Response (0 = baseline)
800
718
636
554
472
390
308
226
144
62
-20
40µg/ml
13µg/ml
4µg/ml
0
100
200
300
400
500
600
700
800
900
Time
s

Fig. 81