Figure 1

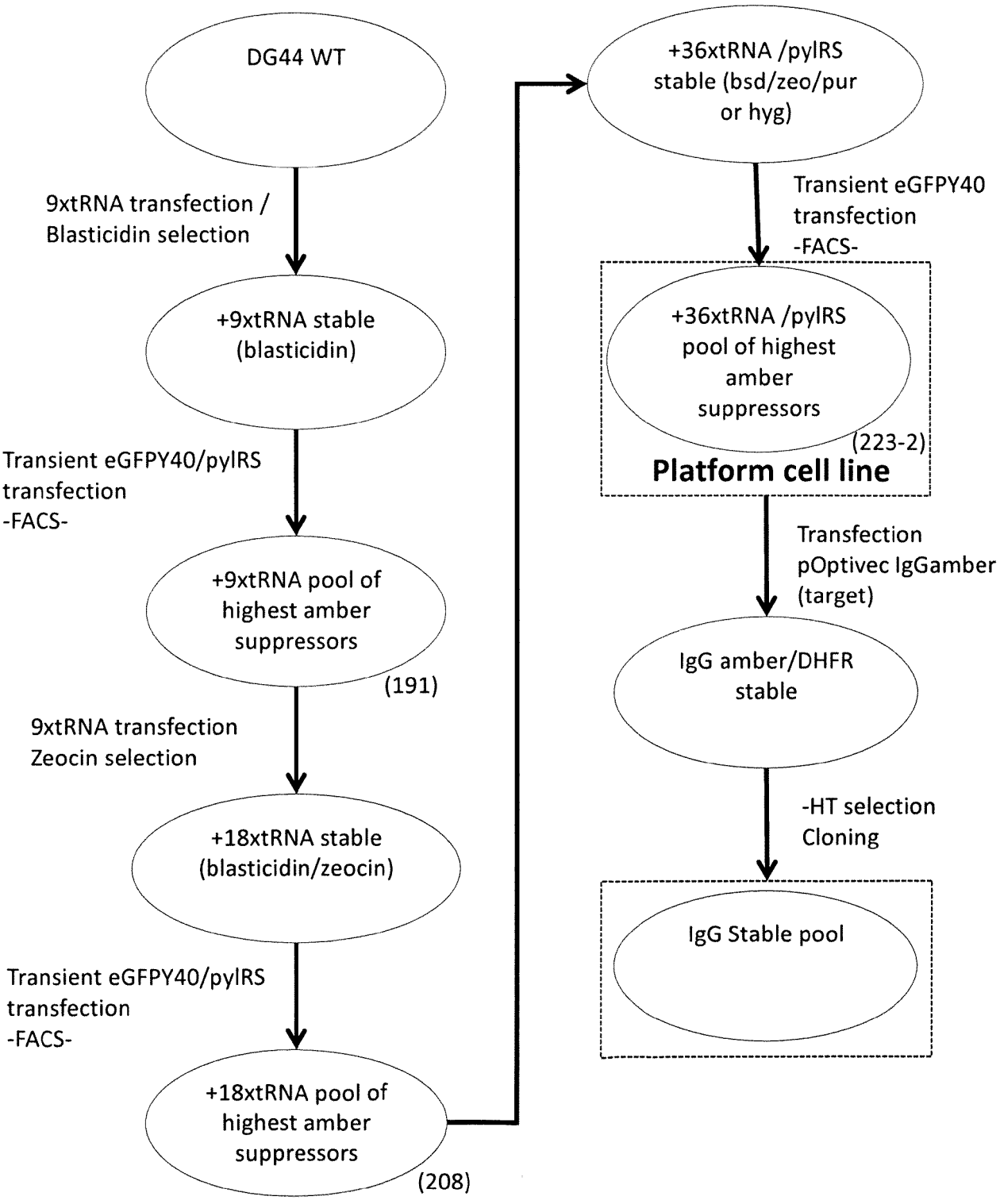

DG44 WT
9xtRNA transfection / Blasticidin selection
+9xtRNA stable (blasticidin)
Transient eGFPY40/pylRS transfection -FACS-
+9xtRNA pool of highest amber suppressors
(191)
9xtRNA transfection Zeocin selection
+18xtRNA stable (blasticidin/zeocin)
Transient eGFPY40/pylRS transfection -FACS-
+18xtRNA pool of highest amber suppressors
(208)
+36xtRNA /pylRS stable (bsd/zeo/pur or hyg)
Transient eGFPY40 transfection -FACS-
+36xtRNA /pylRS pool of highest amber suppressors
(223-2)
Platform cell line
Transfection pOptivec IgGamber (target)
IgG amber/DHFR stable
-HT selection Cloning
IgG Stable pool

Figure 2

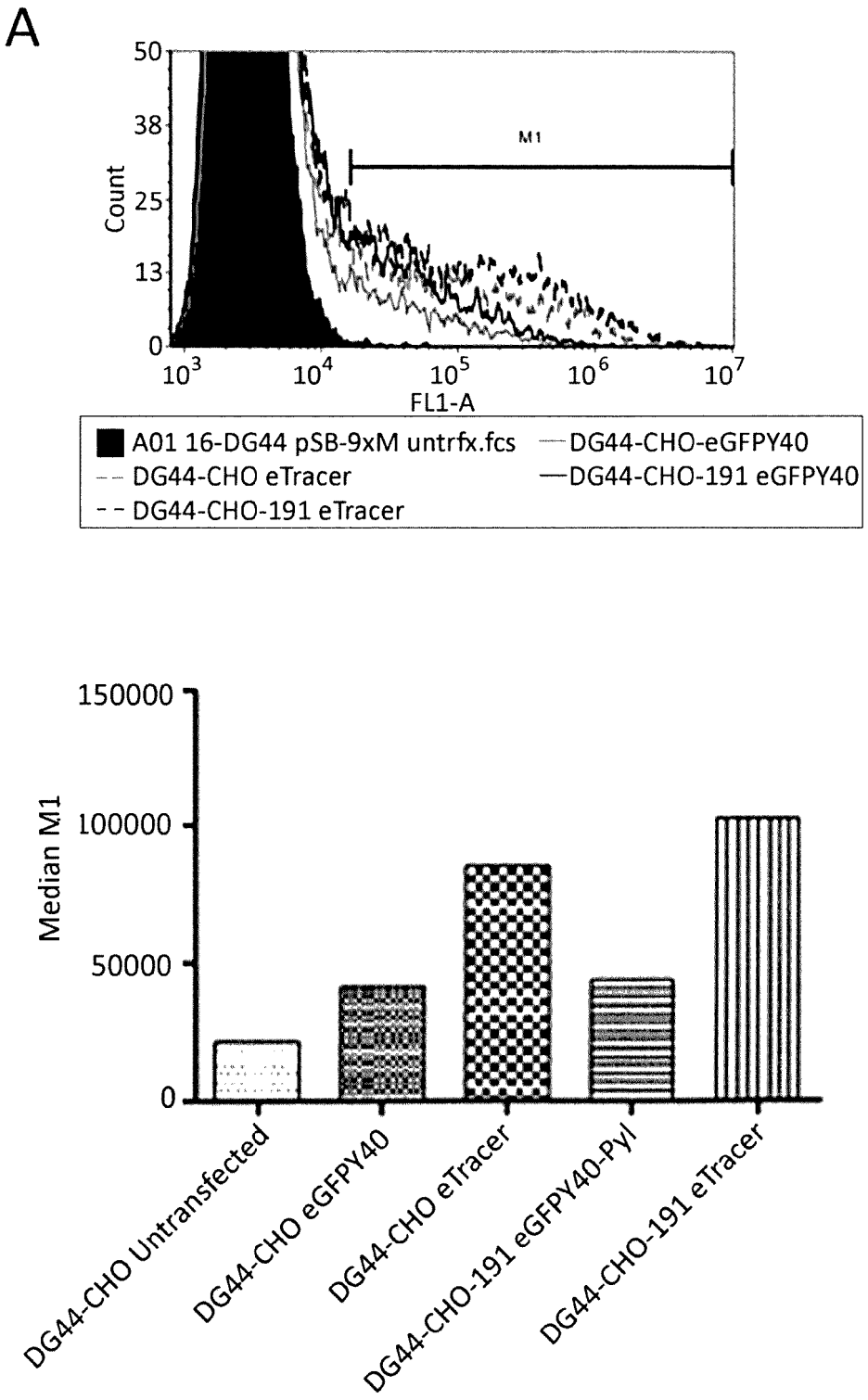
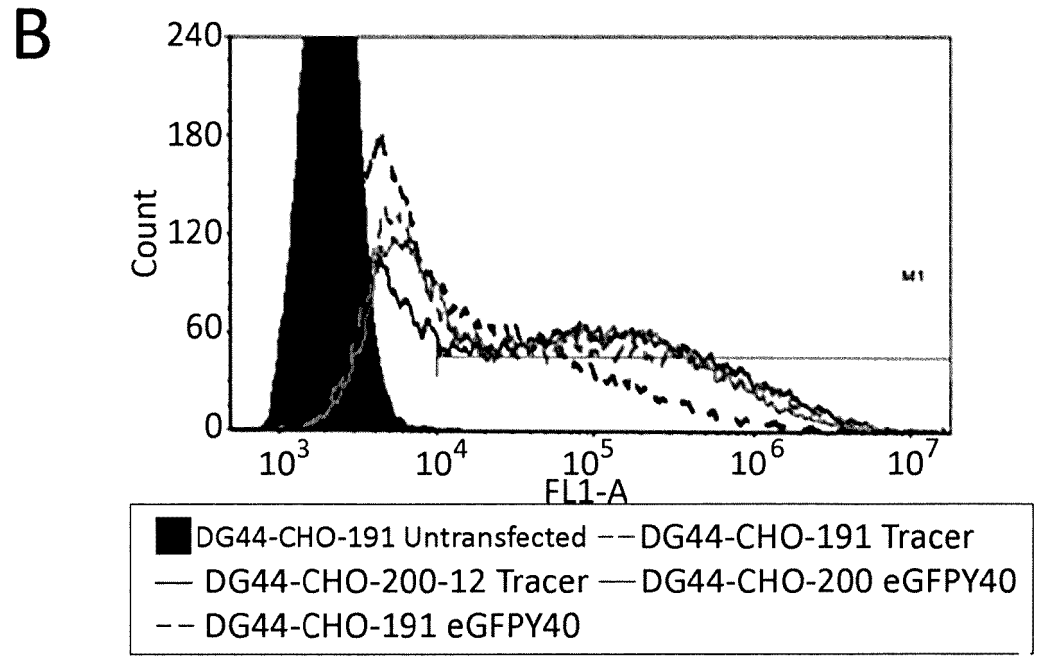

A
50
38
25
13
0
Count
M1
$10^3$
$10^4$
$10^5$
$10^6$
$10^7$
FL1-A
A01 16-DG44 pSB-9xM untrfx.fcs
DG44-CHO eTracer
DG44-CHO-191 eTracer
DG44-CHO-eGFPY40
DG44-CHO-191 eGFPY40
150000
100000
50000
0
Median M1
DG44-CHO Untransfected
DG44-CHO eGFPY40
DG44-CHO eTracer
DG44-CHO-191 eGFPY40-Pyl
DG44-CHO-191 eTracer

Figure 2 cont



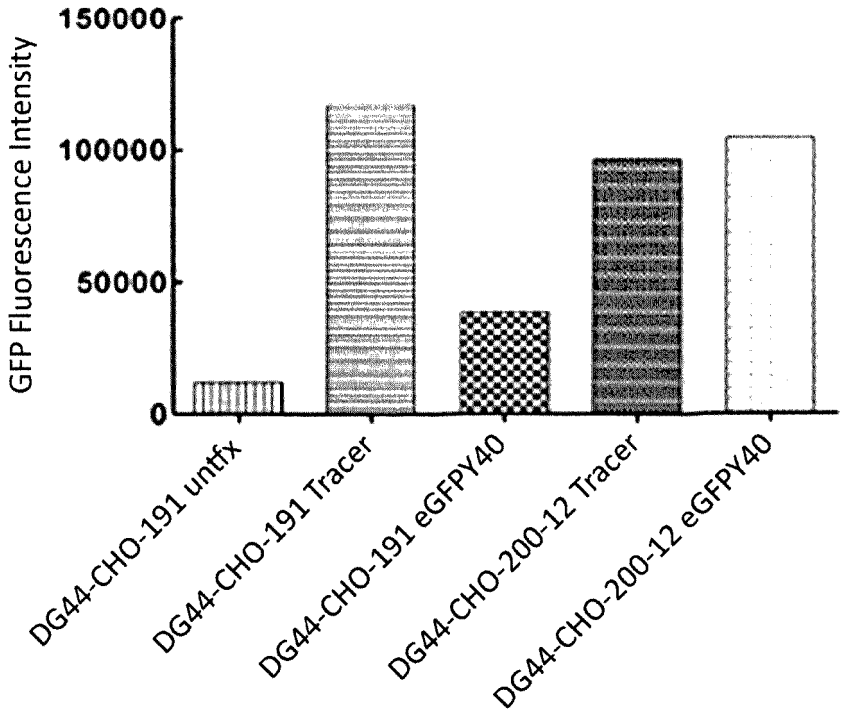
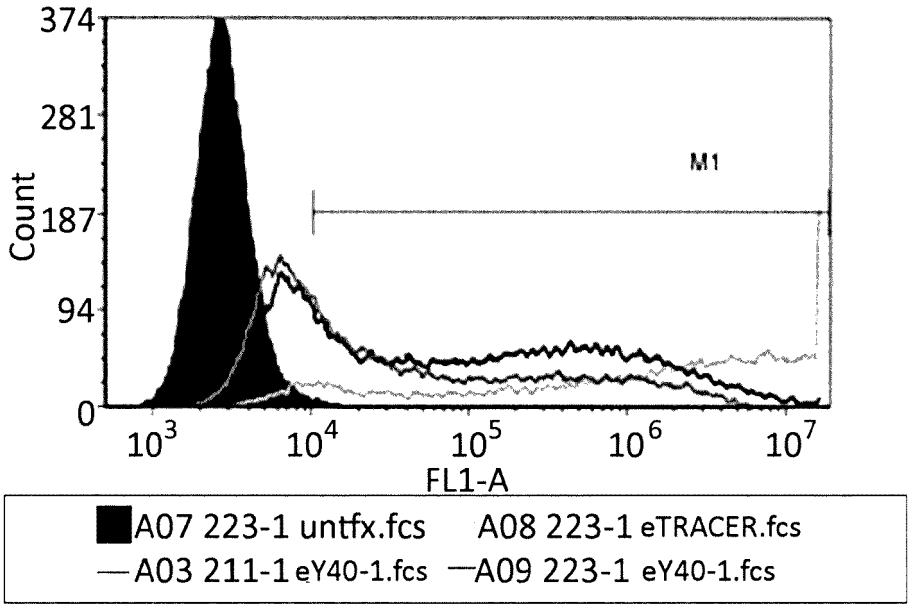



Figure 2 cont

C



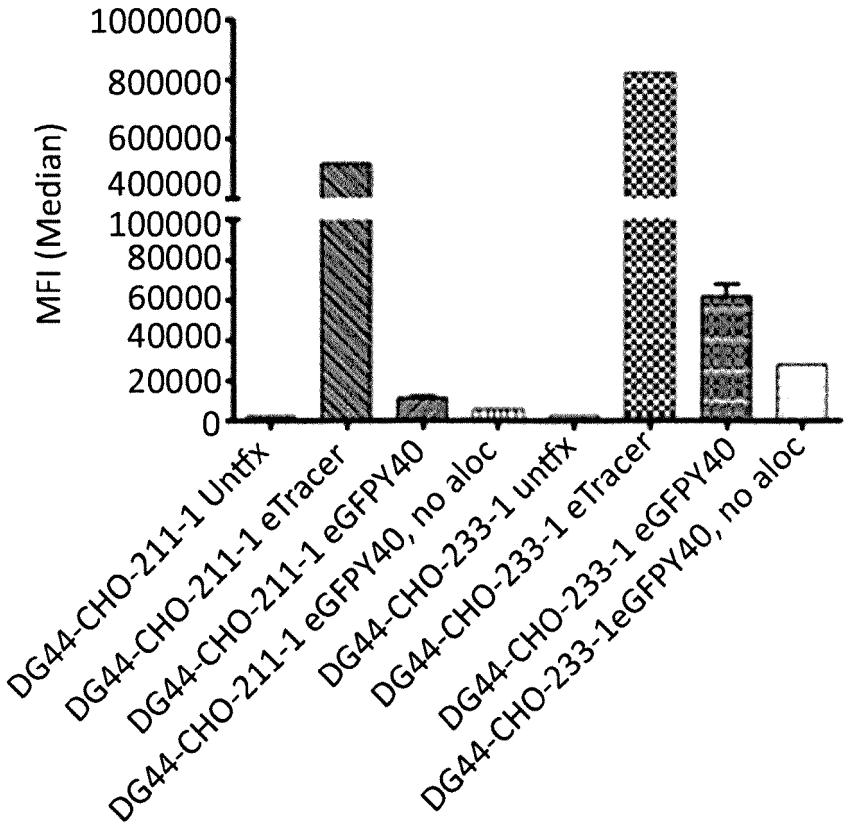



Figure 3

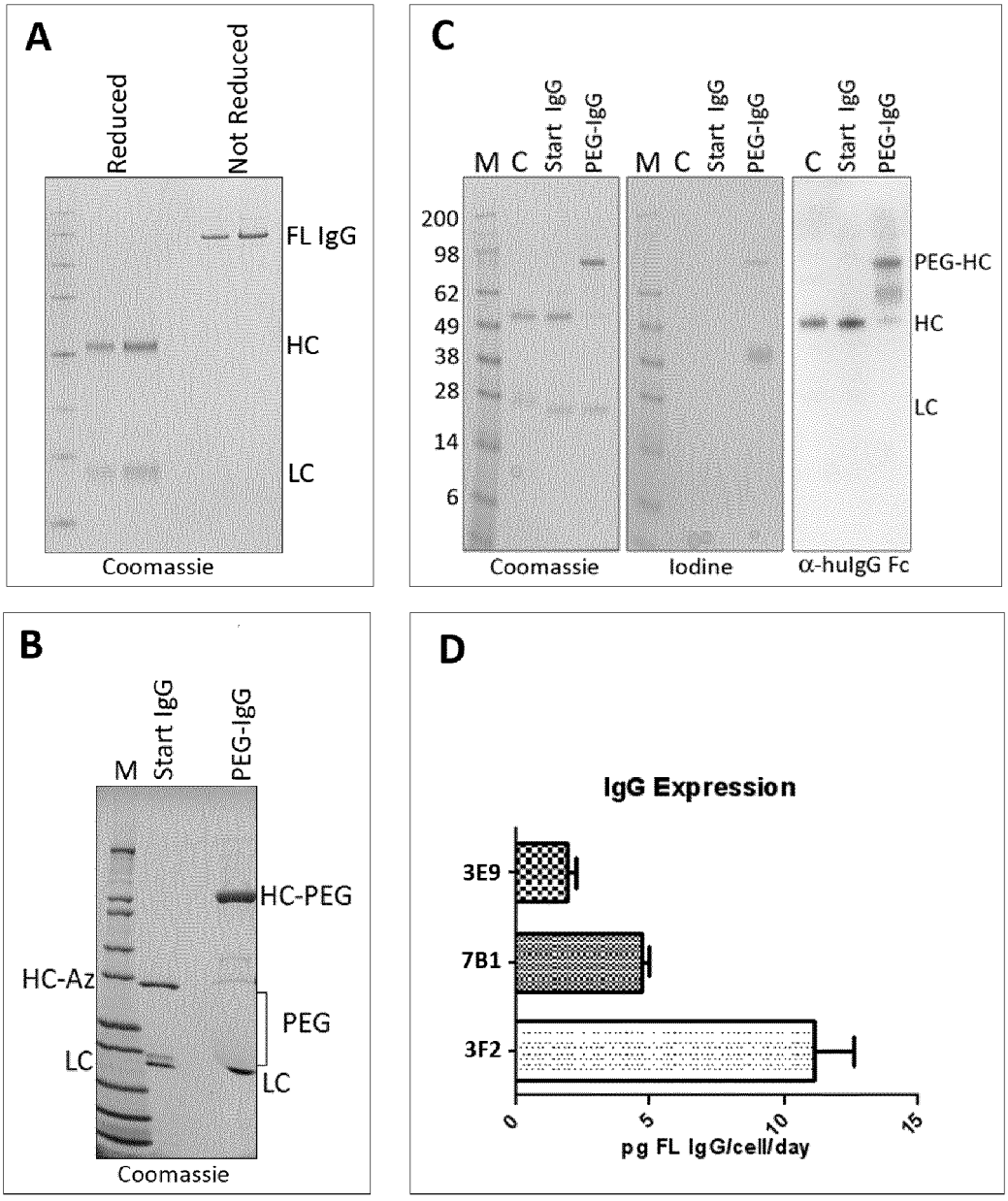
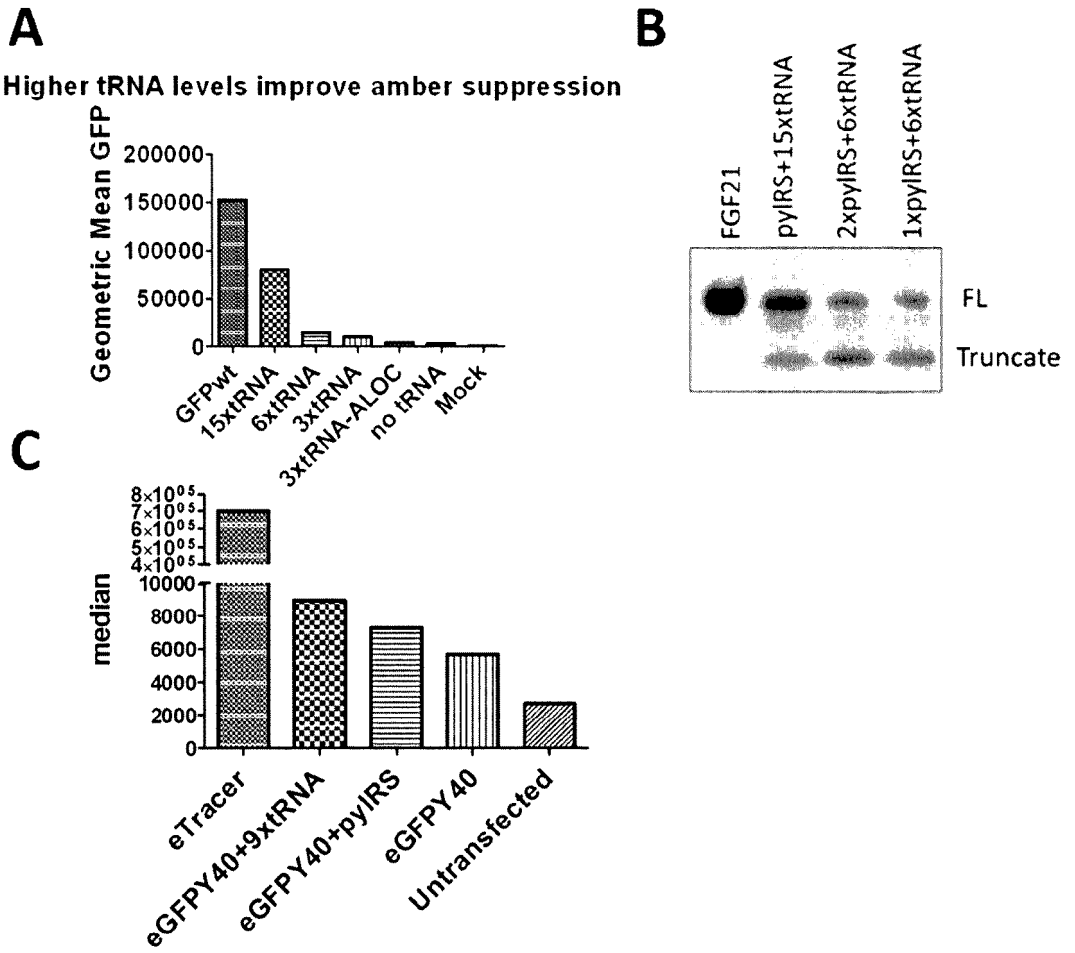

A
Reduced
Not Reduced
FL IgG
HC
LC
Coomassie
C
M C Start IgG PEG-IgG
M C Start IgG PEG-IgG
C Start IgG PEG-IgG
200
98
62
49
38
28
14
6
PEG-HC
HC
LC
Coomassie
Iodine
α-huIgG Fc
B
M
Start IgG
PEG-IgG
HC-PEG
HC-Az
PEG
LC
LC
Coomassie
D
IgG Expression
3E9
7B1
3F2
0
5
10
15
pg FL IgG/cell/day

Figure 4


A
Higher tRNA levels improve amber suppression
Geometric Mean GFP
200000
150000
100000
50000
0
GFPwt
15xtRNA
6xtRNA
3xtRNA
3xtRNA-ALOC
no tRNA
Mock
B
FGF21
pylRS+15xtRNA
2xpylRS+6xtRNA
1xpylRS+6xtRNA
FL
Truncate
C
median
8×10^05
7×10^05
6×10^05
5×10^05
4×10^05
10000
8000
6000
4000
2000
0
eTracer
eGFPY40+9xtRNA
eGFPY40+pylRS
eGFPY40
Untransfected

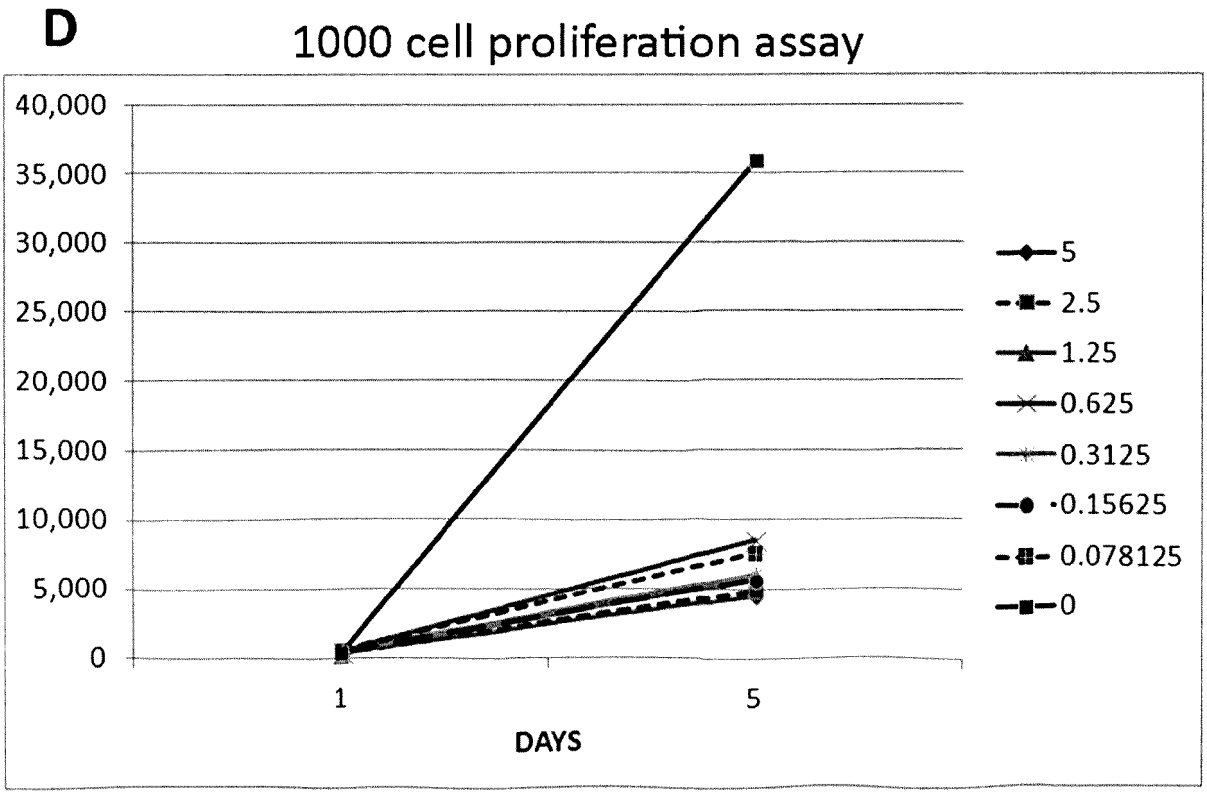

D
1000 cell proliferation assay
40,000
35,000
30,000
25,000
20,000
15,000
10,000
5,000
0
1
5
DAYS
5
2.5
1.25
0.625
0.3125
0.15625
0.078125
0

Figure 4 cont

E

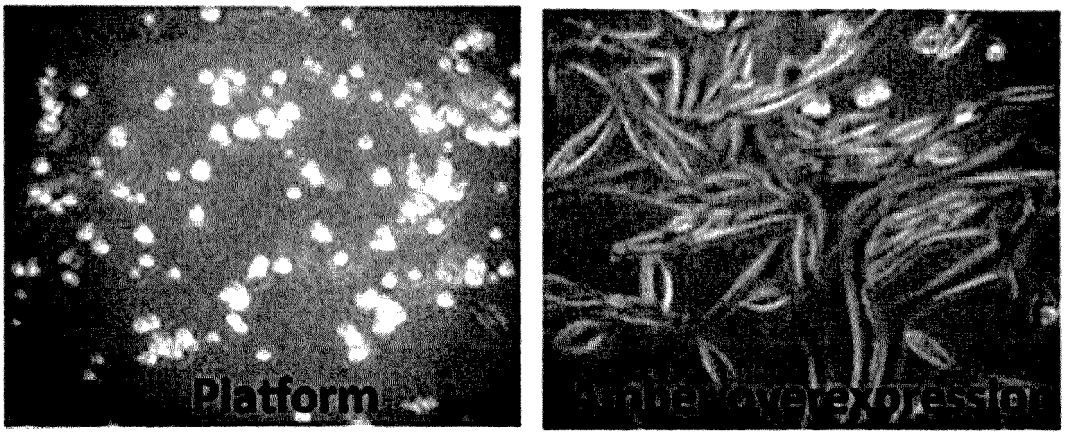


Figure 5

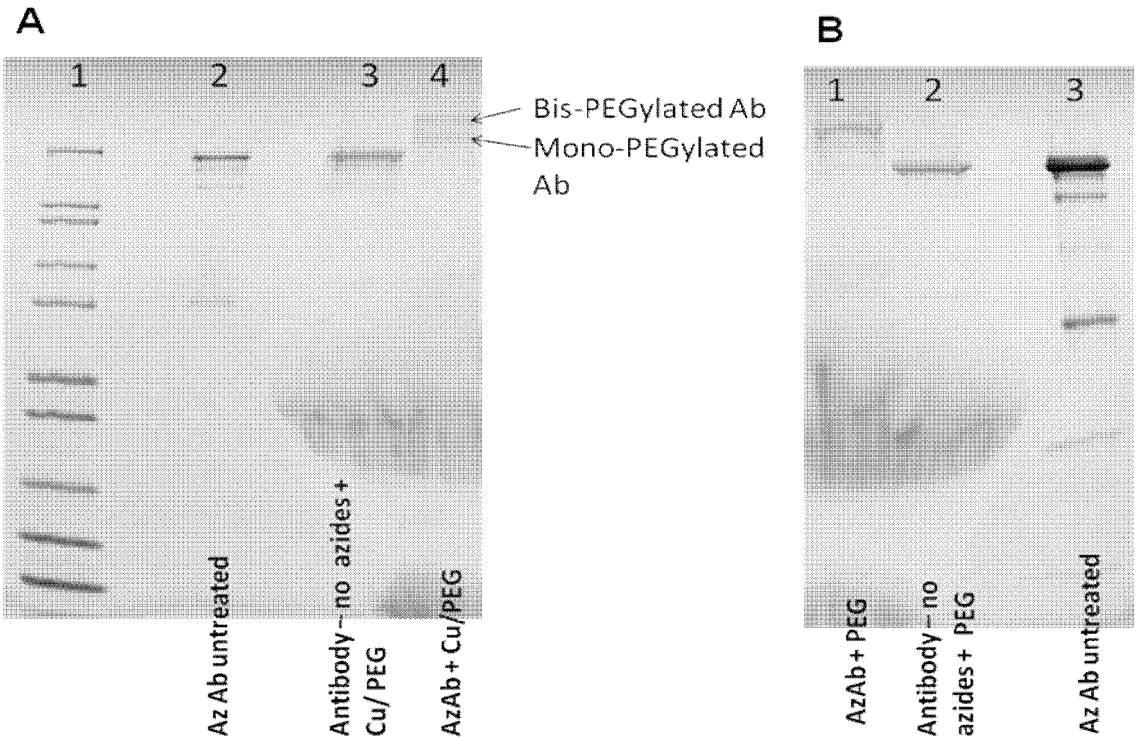



Figure 6

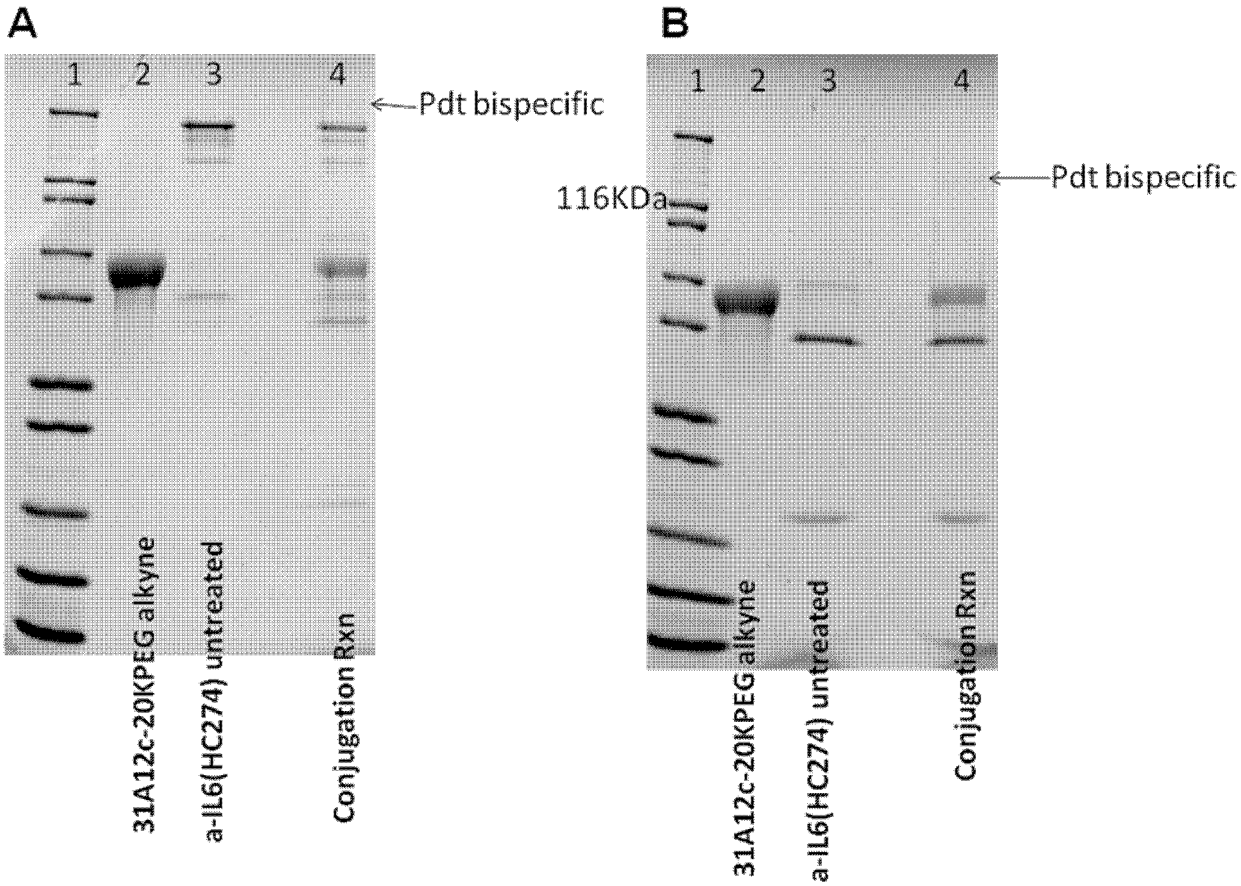


Figure 7

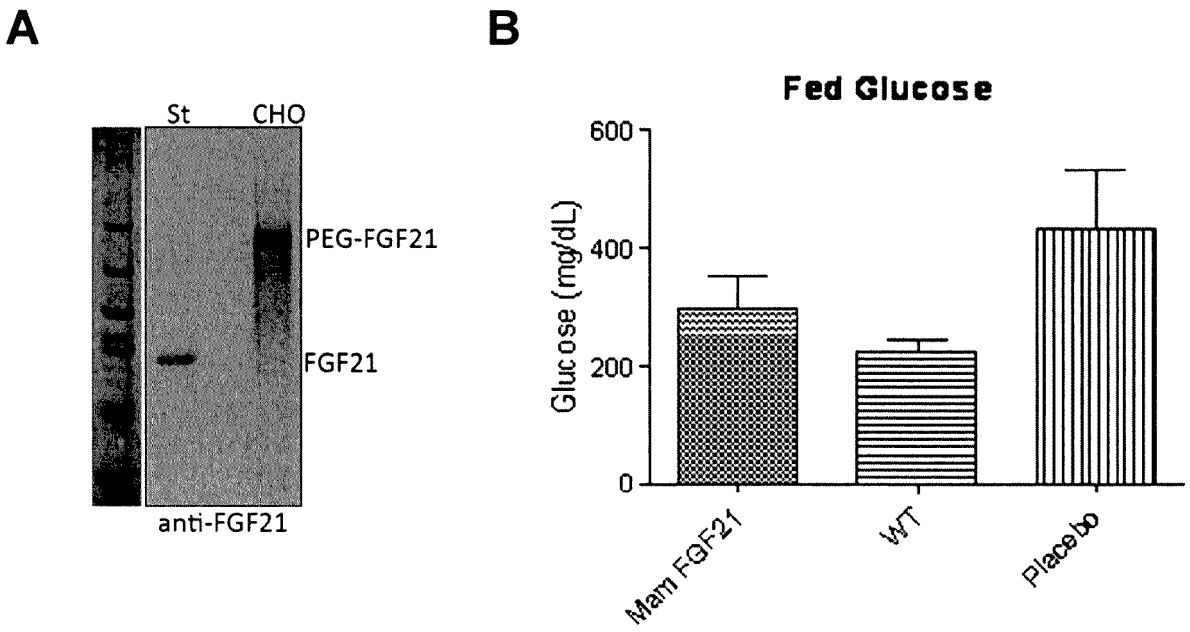



Figure 8

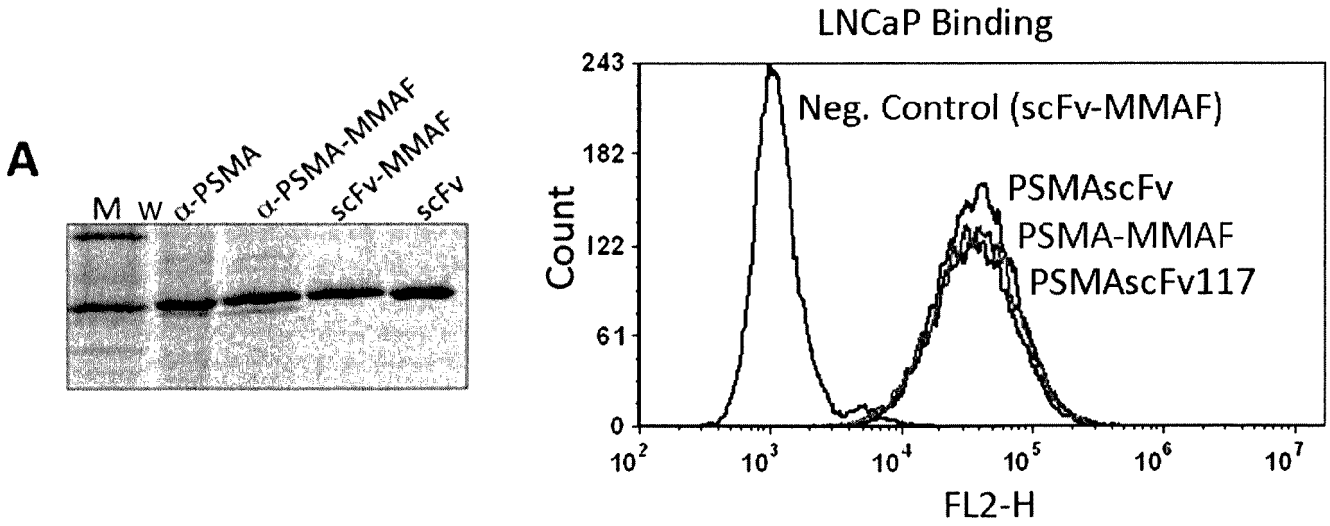
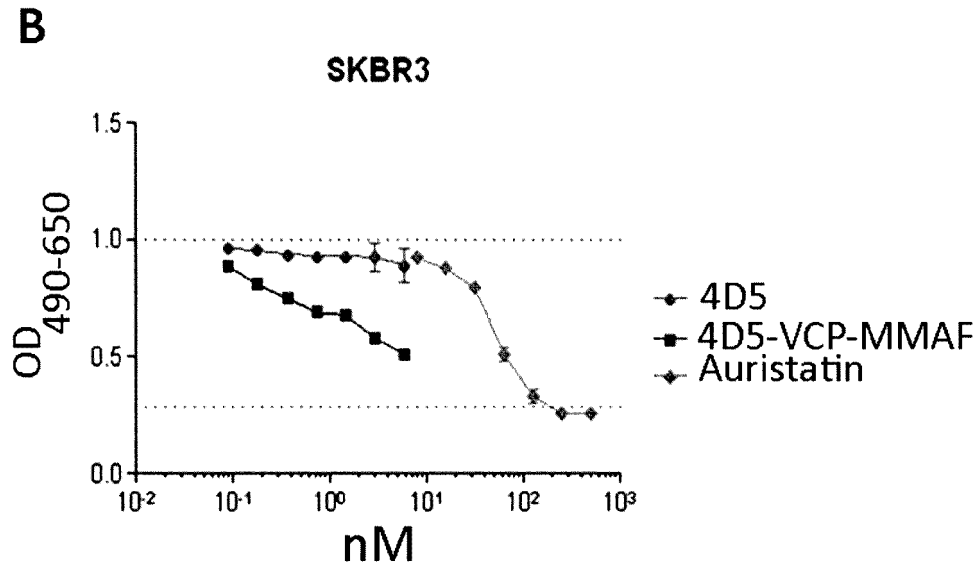


Figure 8 cont



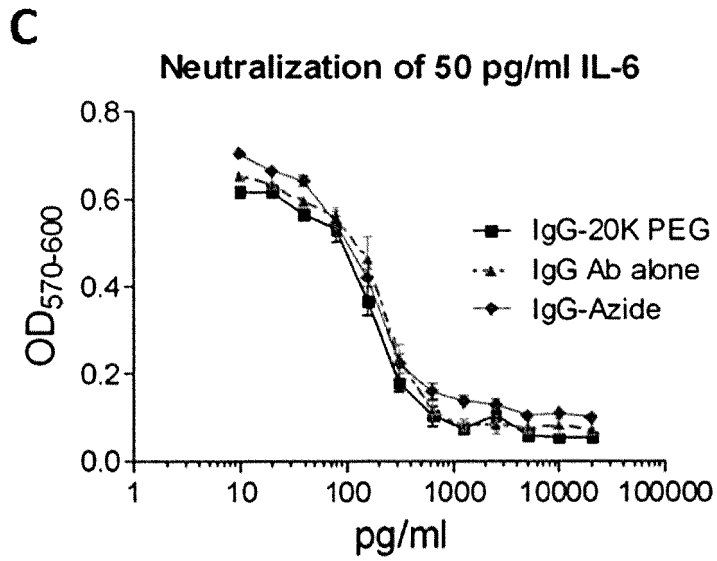



Figure 9

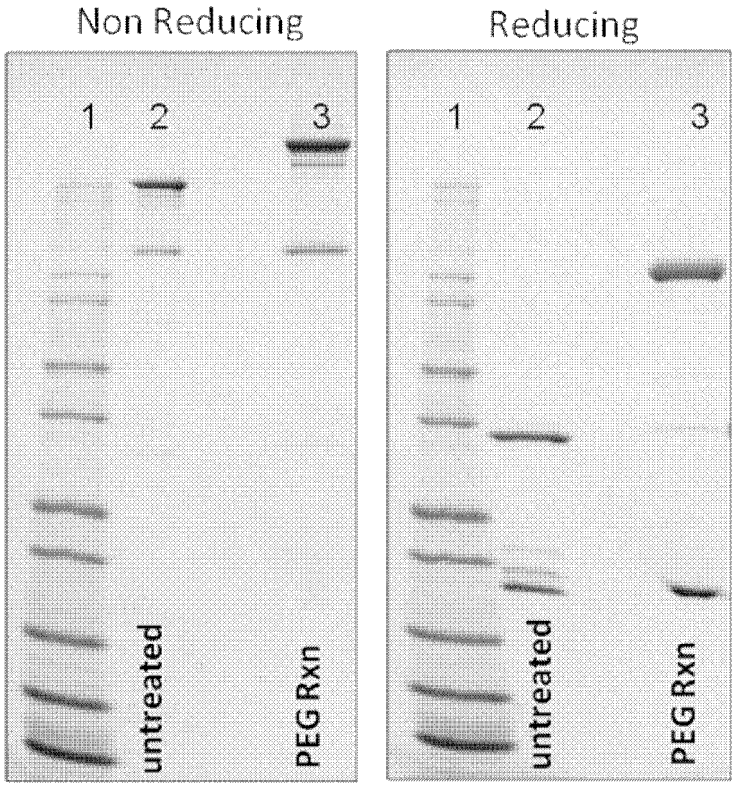



Figure 10

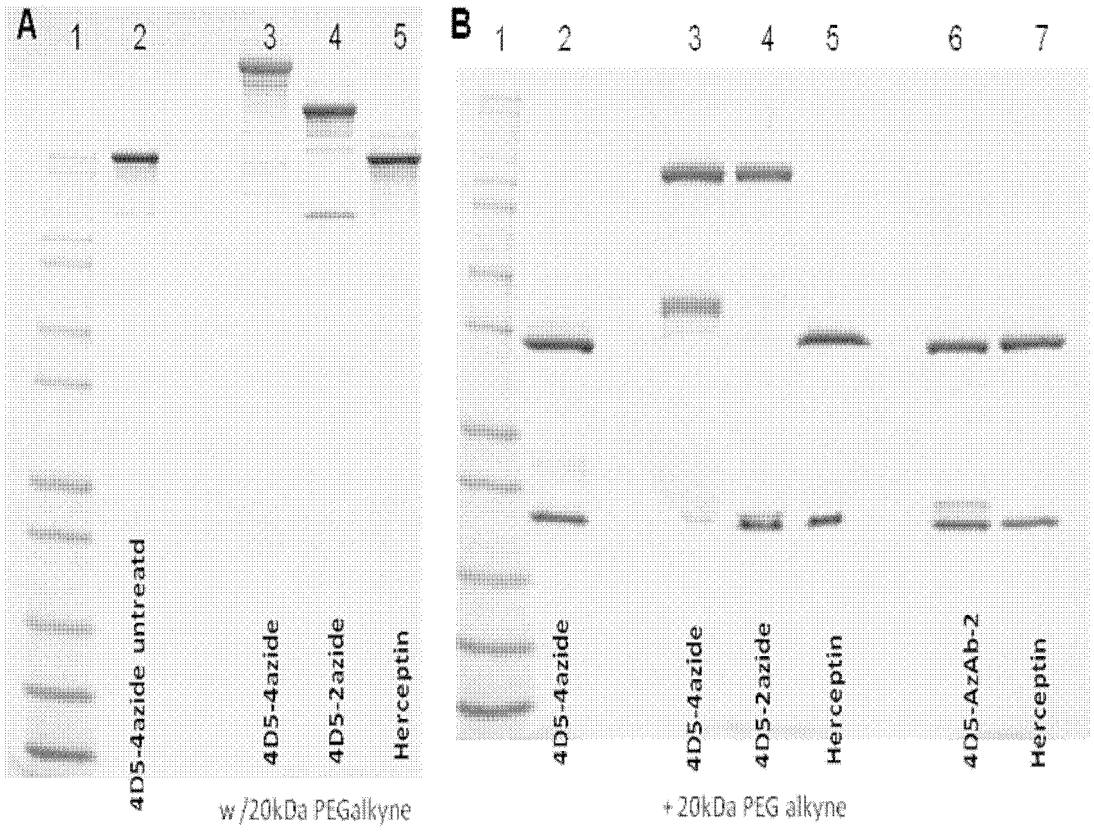


Figure 11

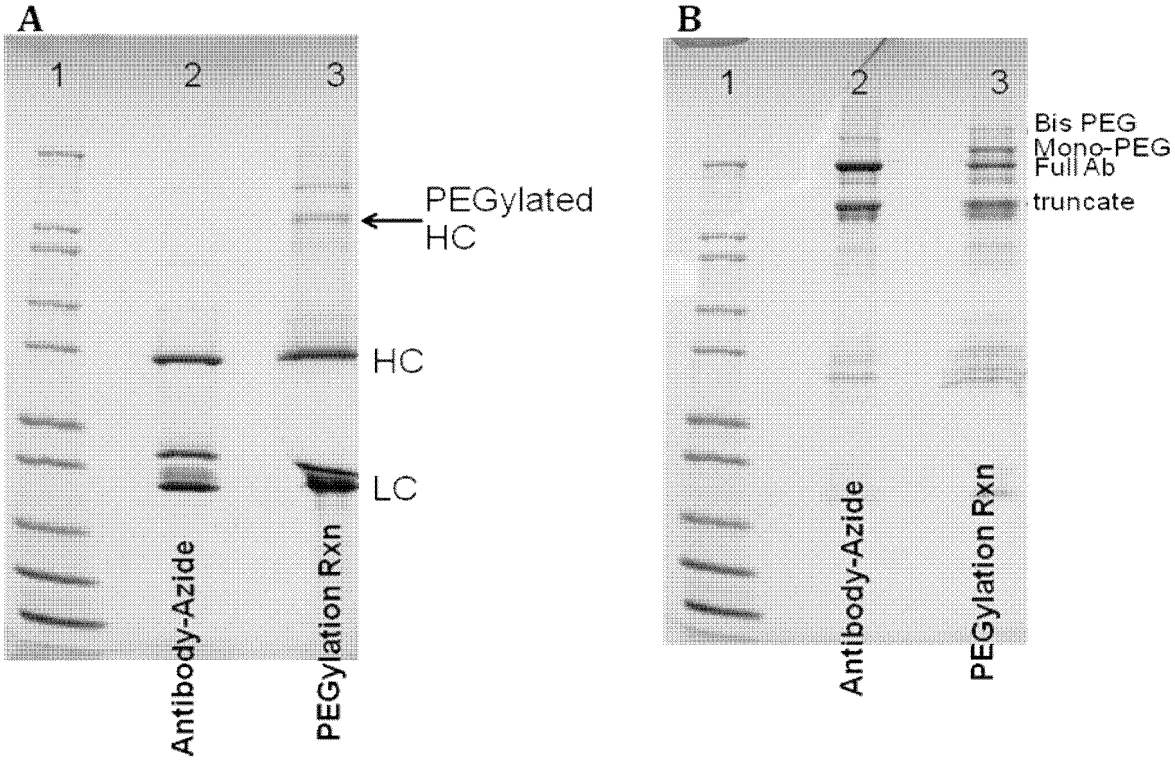



Figure 12

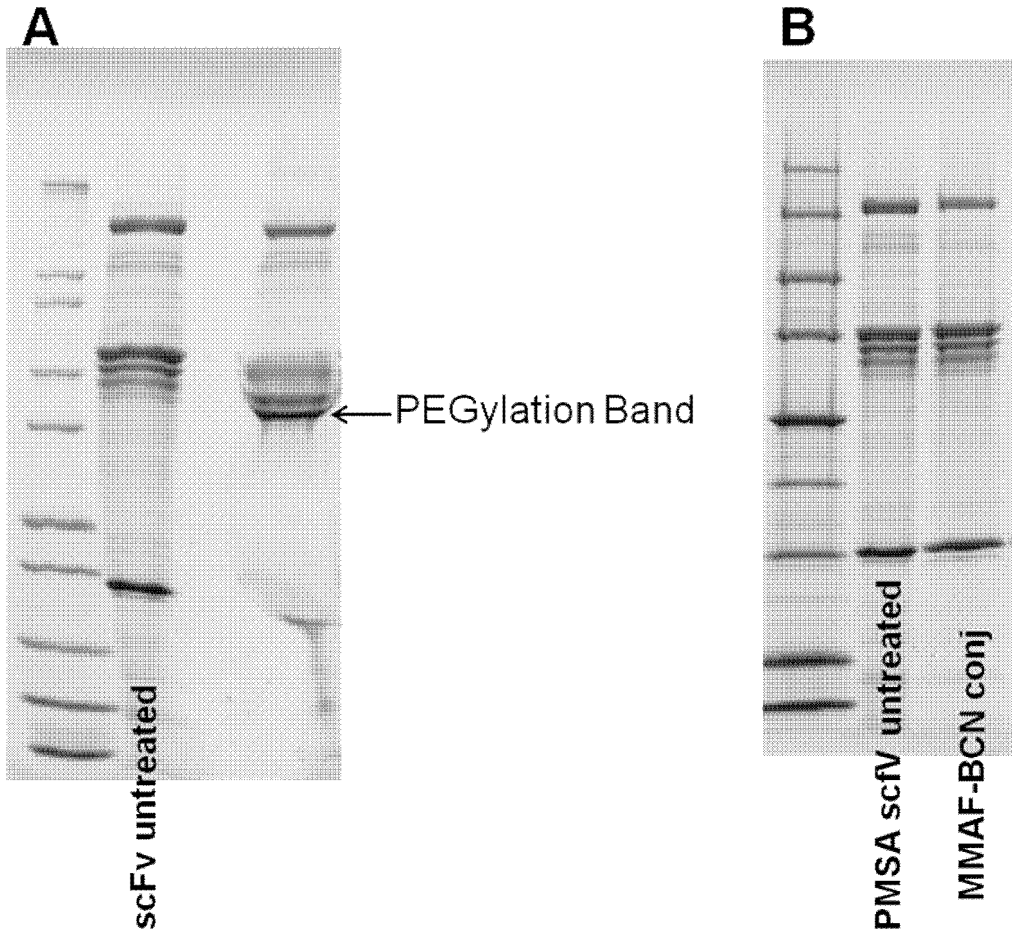
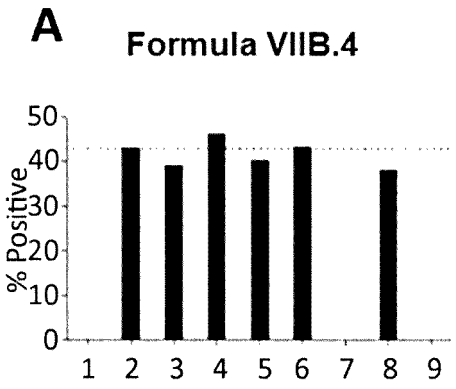


Figure 13




1. untransfected
2. GFP
3. GFP + Lys Azide (0.5 nM)
4. GFP + Decoy (2 nM)
5. GFP + Decoy (1 nM)
6. GFP + Decoy (0.5 nM)
7. GFP + Lys azide + Decoy (2 nM)
8. GFP + Lys azide + decoy (1 nM)
9. GFP + Lys azide + Decoy (0.5 n

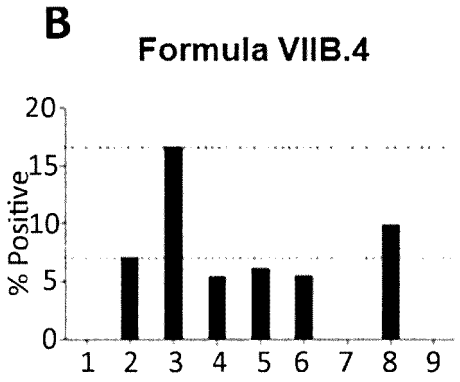
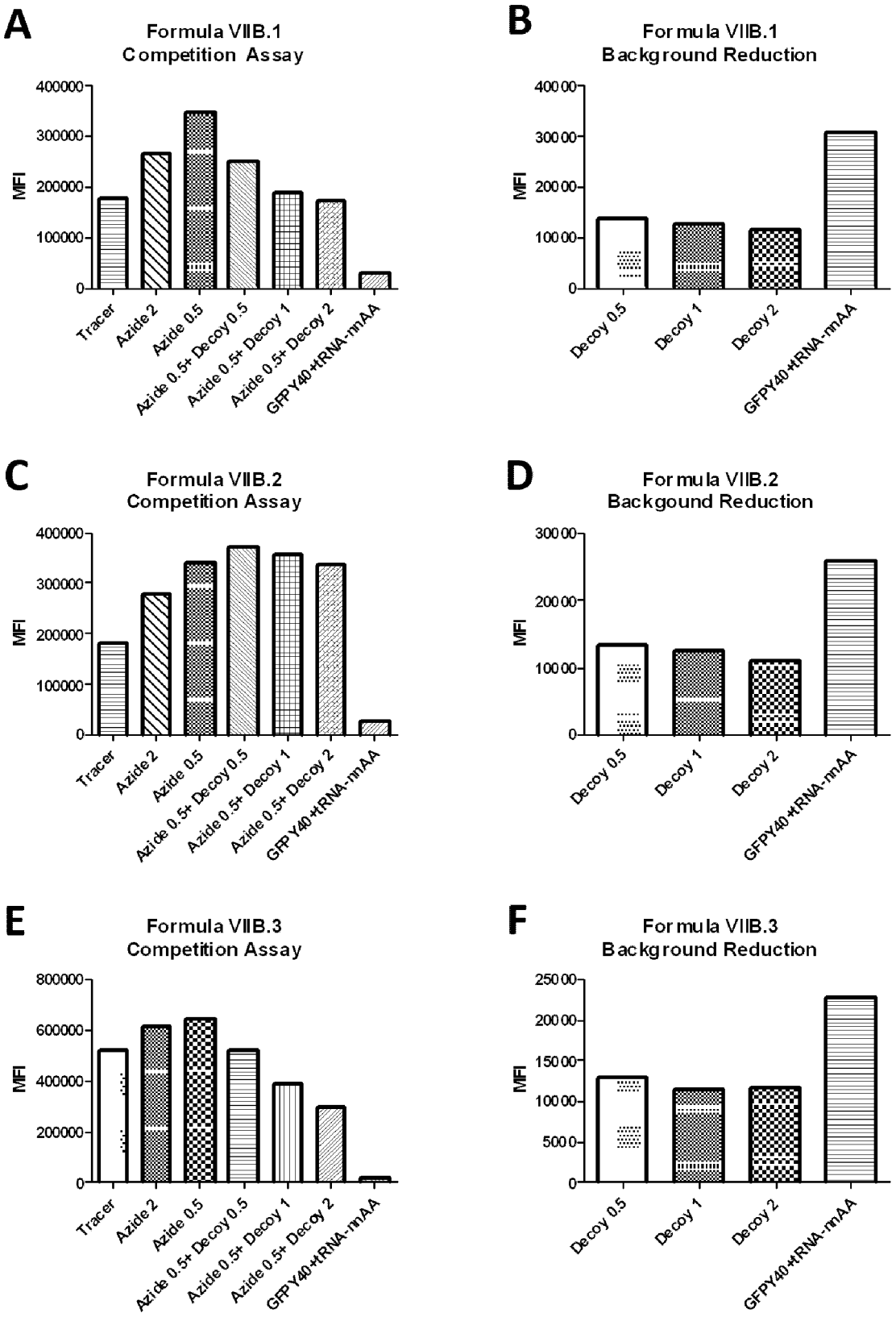
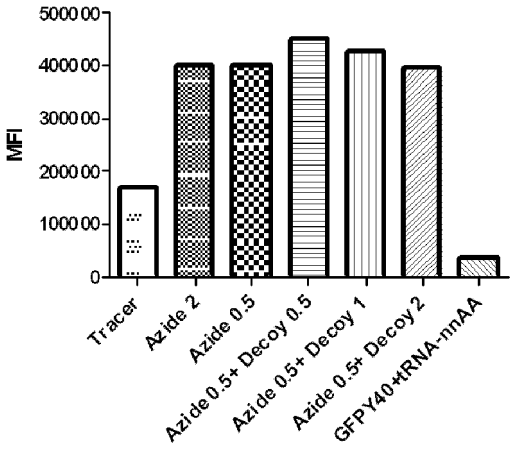


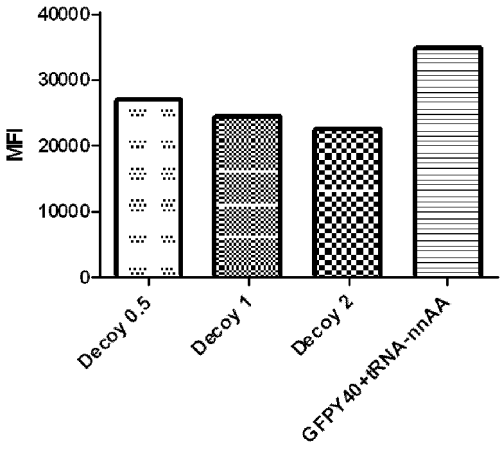
Figure 14


A
Formula VIIB.1
Competition Assay
MFI
400000
300000
200000
100000
0
Tracer
Azide 2
Azide 0.5
Azide 0.5+ Decoy 0.5
Azide 0.5+ Decoy 1
Azide 0.5+ Decoy 2
GFPY40+tRNA-mAA
B
Formula VIIB.1
Background Reduction
MFI
40000
30000
20000
10000
0
Decoy 0.5
Decoy 1
Decoy 2
GFPY40+tRNA-mAA
C
Formula VIIB.2
Competition Assay
MFI
400000
300000
200000
100000
0
Tracer
Azide 2
Azide 0.5
Azide 0.5+ Decoy 0.5
Azide 0.5+ Decoy 1
Azide 0.5+ Decoy 2
GFPY40+tRNA-mAA
D
Formula VIIB.2
Backgound Reduction
MFI
30000
20000
10000
0
Decoy 0.5
Decoy 1
Decoy 2
GFPY40+tRNA-mAA
E
Formula VIIB.3
Competition Assay
MFI
800000
600000
400000
200000
0
Tracer
Azide 2
Azide 0.5
Azide 0.5+ Decoy 0.5
Azide 0.5+ Decoy 1
Azide 0.5+ Decoy 2
GFPY40+tRNA-mAA
F
Formula VIIB.3
Background Reduction
MFI
25000
20000
15000
10000
5000
0
Decoy 0.5
Decoy 1
Decoy 2
GFPY40+tRNA-mAA

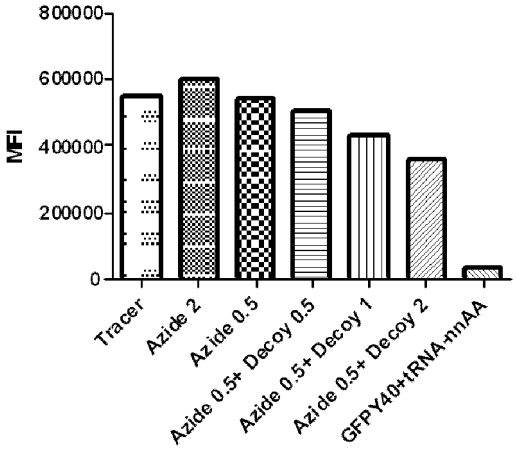
G


Formula VIIB.5
Competition Assay
MFI
5000000
4000000
3000000
2000000
1000000
0
Tracer
Azide 2
Azide 0.5
Azide 0.5+ Decoy 0.5
Azide 0.5+ Decoy 1
Azide 0.5+ Decoy 2
GFPY40+tRNA-nnAA

H


Formula VIIB.5
Background Reduction
MFI
40000
30000
20000
10000
0
Decoy 0.5
Decoy 1
Decoy 2
GFPY40+tRNA-nnAA

I


Formula VIIB.6
Competition Assay
MFI
800000
600000
400000
200000
0
Tracer
Azide 2
Azide 0.5
Azide 0.5+ Decoy 0.5
Azide 0.5+ Decoy 1
Azide 0.5+ Decoy 2
GFPY40+tRNA-nnAA

J

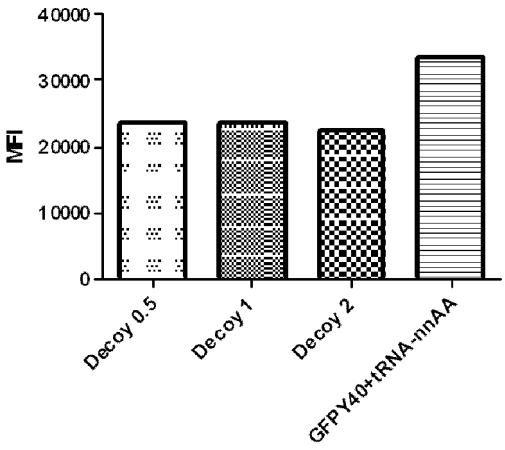
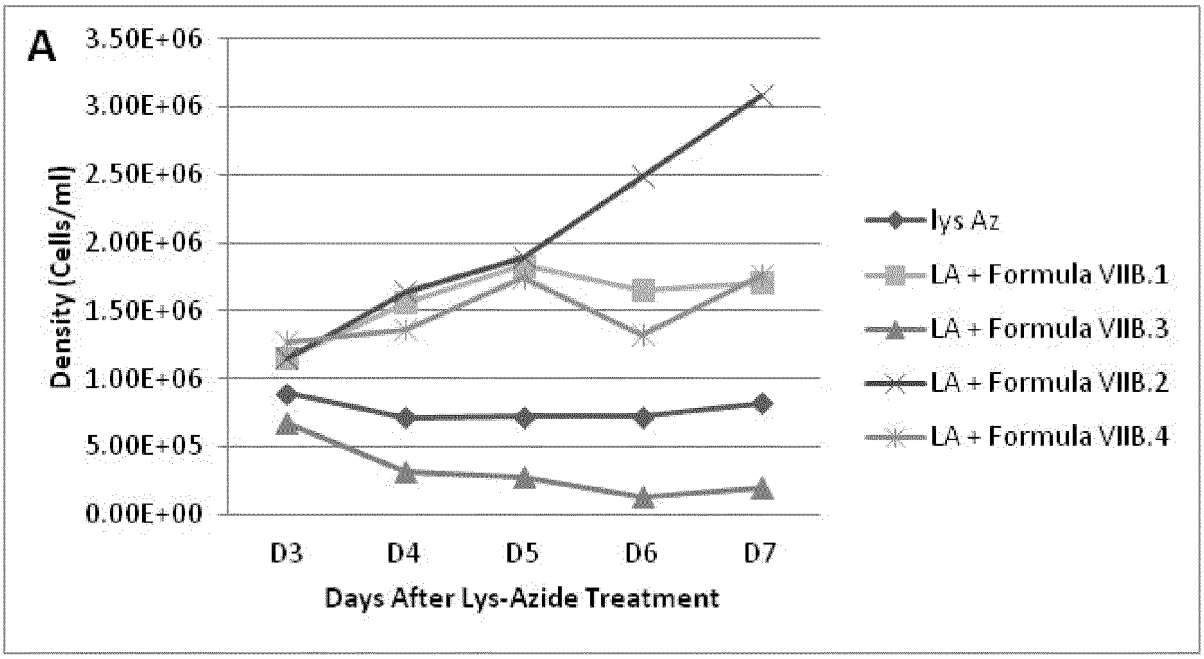

Formula VIIB.6
Background Reduction
MFI
40000
30000
20000
10000
0
Decoy 0.5
Decoy 1
Decoy 2
GFPY40+tRNA-nnAA

Figure 15


A
3.50E+06
3.00E+06
2.50E+06
2.00E+06
1.50E+06
1.00E+06
5.00E+05
0.00E+00
Density (Cells/ml)
D3
D4
D5
D6
D7
Days After Lys-Azide Treatment
lys Az
LA + Formula VIIB.1
LA + Formula VIIB.3
LA + Formula VIIB.2
LA + Formula VIIB.4

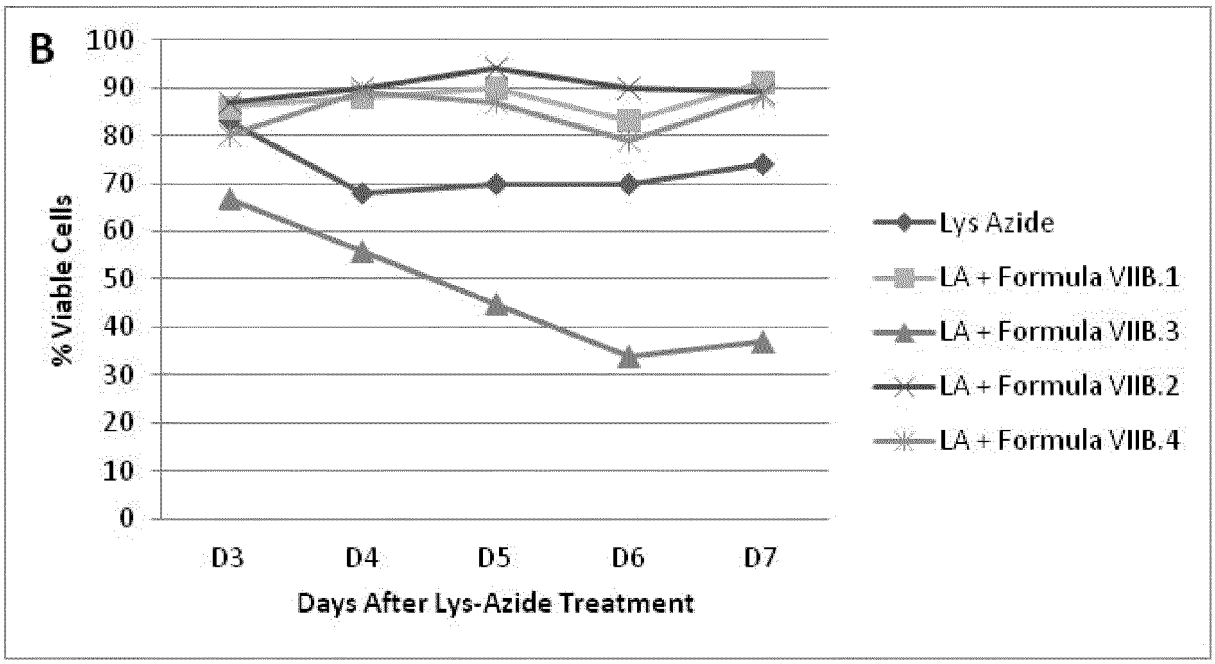

B
100
90
80
70
60
50
40
30
20
10
0
% Viable Cells
D3
D4
D5
D6
D7
Days After Lys-Azide Treatment
Lys Azide
LA + Formula VIIB.1
LA + Formula VIIB.3
LA + Formula VIIB.2
LA + Formula VIIB.4

Figure 16

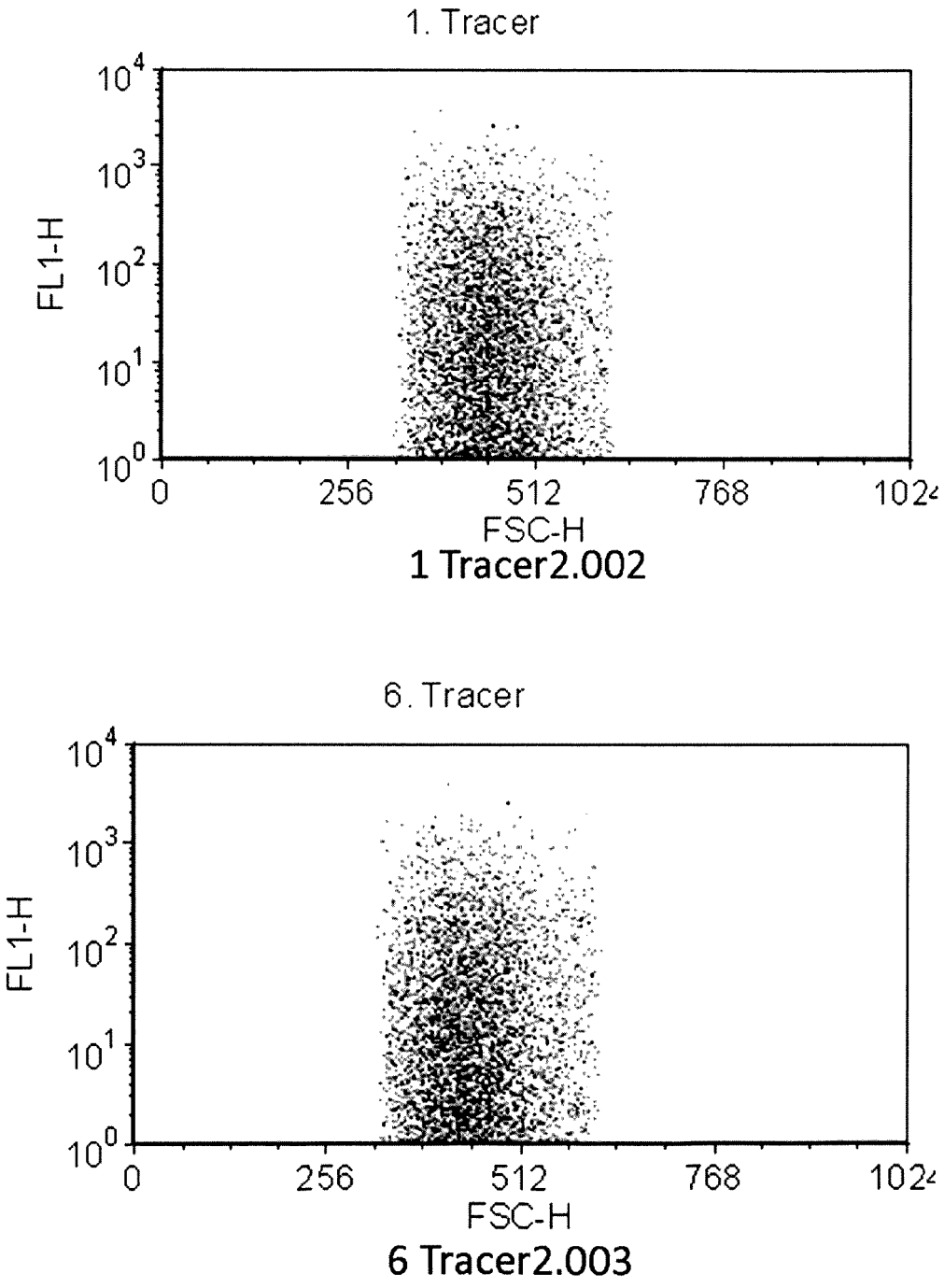
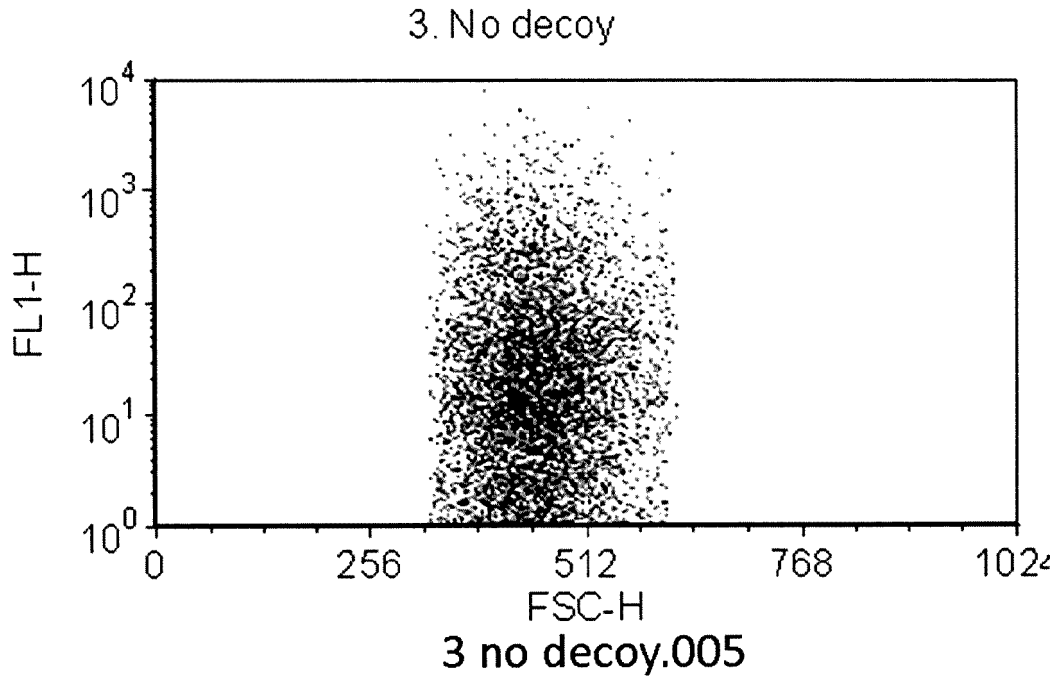

1. Tracer
FL1-H
FSC-H
1 Tracer2.002
6. Tracer
FL1-H
FSC-H
6 Tracer2.003

Figure 16 cont



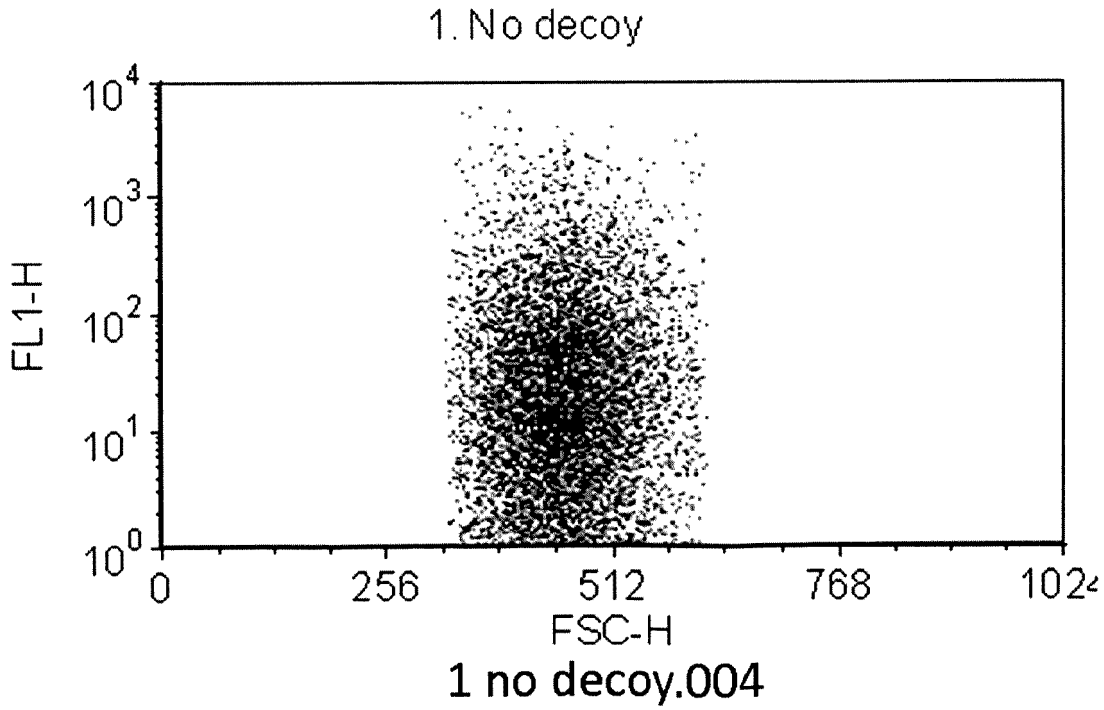
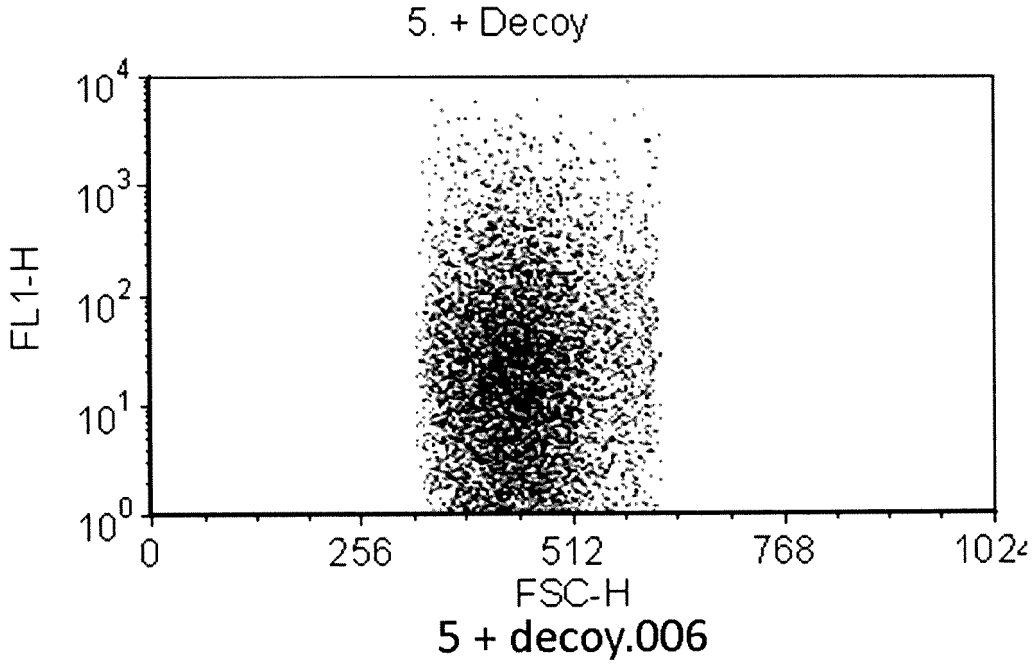



Figure 16 cont



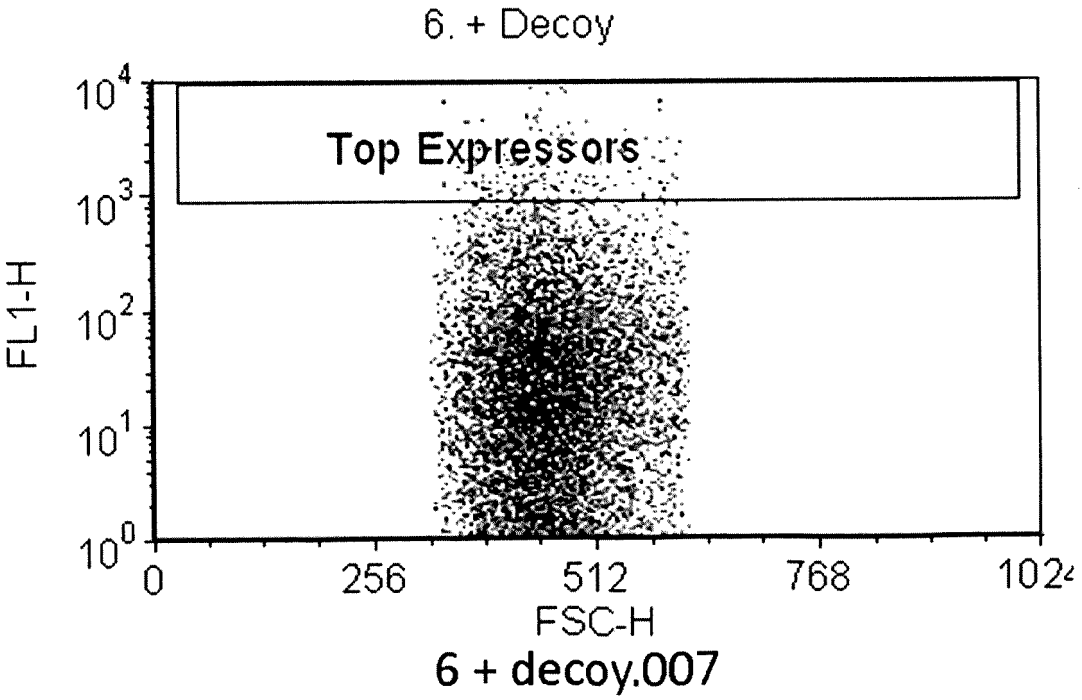



Figure 17

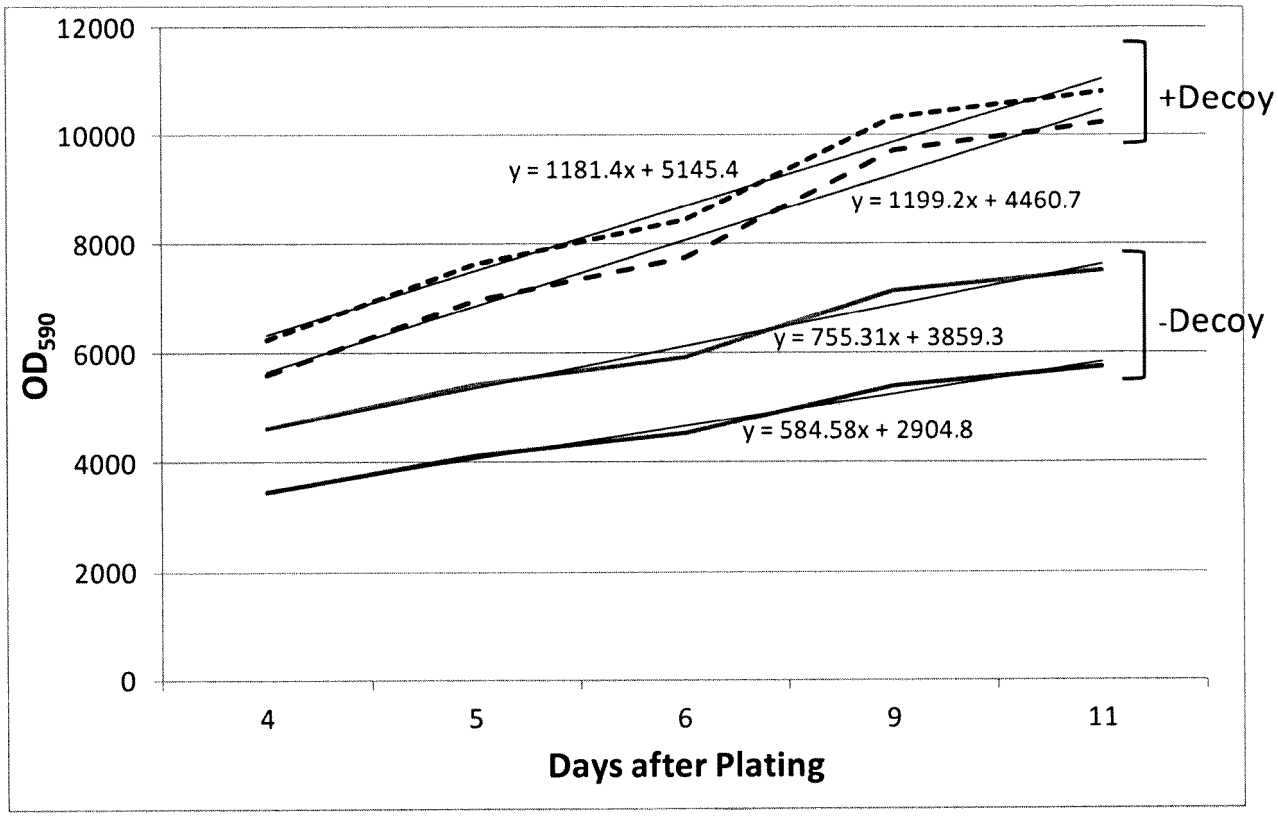

12000
10000
8000
6000
4000
2000
0
OD590
4
5
6
9
11
Days after Plating
y = 1181.4x + 5145.4
y = 1199.2x + 4460.7
y = 755.31x + 3859.3
y = 584.58x + 2904.8
+Decoy
-Decoy

Figure 18

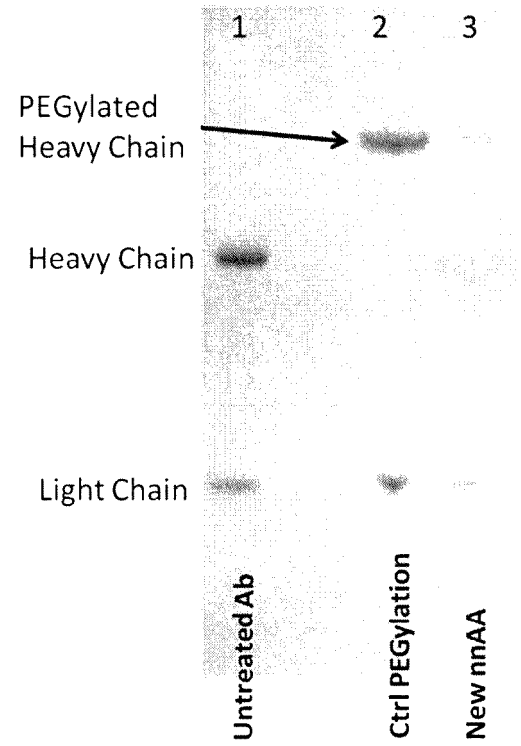

1
2
3
PEGylated
Heavy Chain
Heavy Chain
Light Chain
Untreated Ab
Ctrl PEGylation
New nnAA

Figure 19

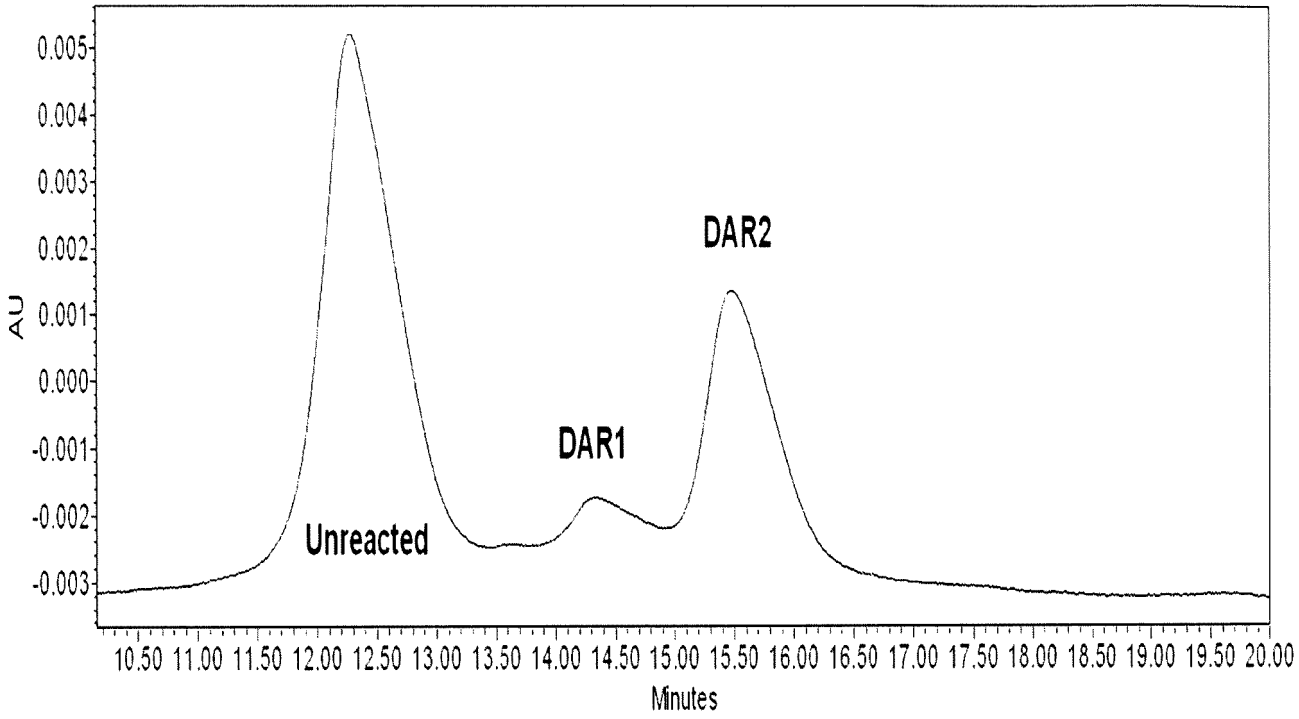

0.005
0.004
0.003
0.002
0.001
0.000
-0.001
-0.002
-0.003
AU
DAR2
DAR1
Unreacted
10.50 11.00 11.50 12.00 12.50 13.00 13.50 14.00 14.50 15.00 15.50 16.00 16.50 17.00 17.50 18.00 18.50 19.00 19.50 20.00
Minutes

Figure 20

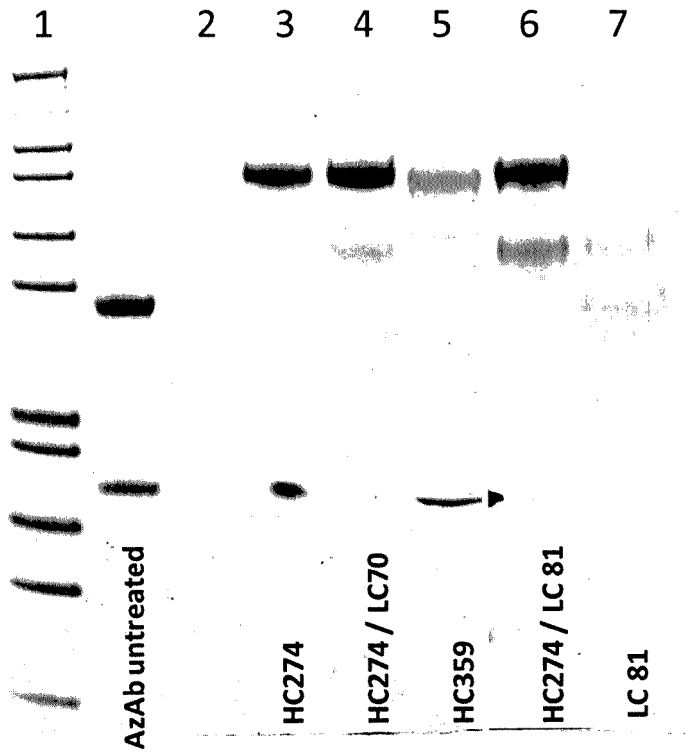



Figure 21

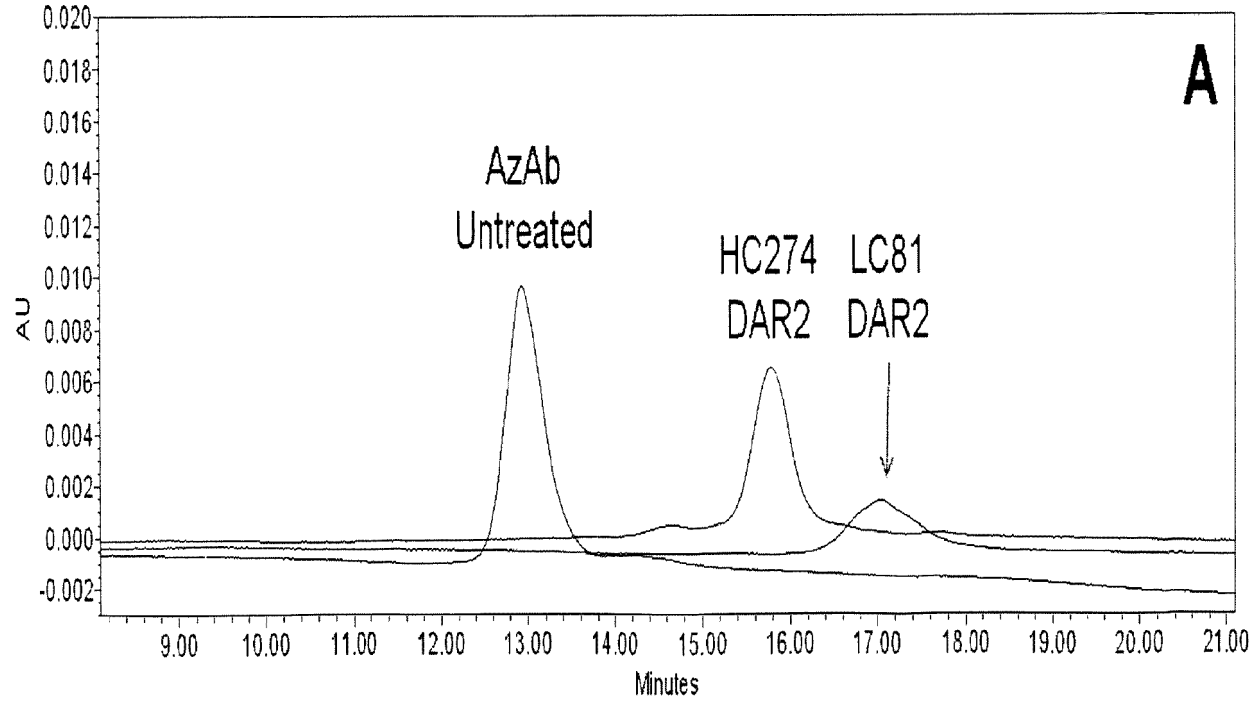
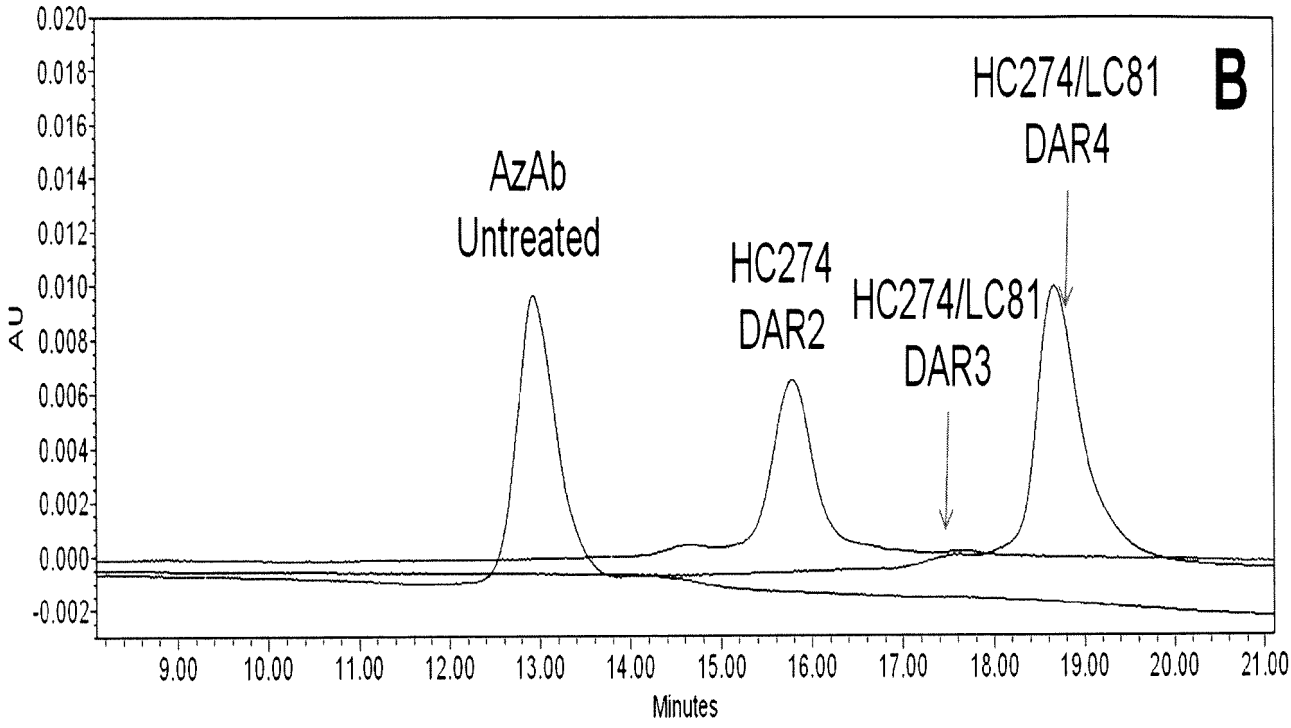


Figure 21 cont



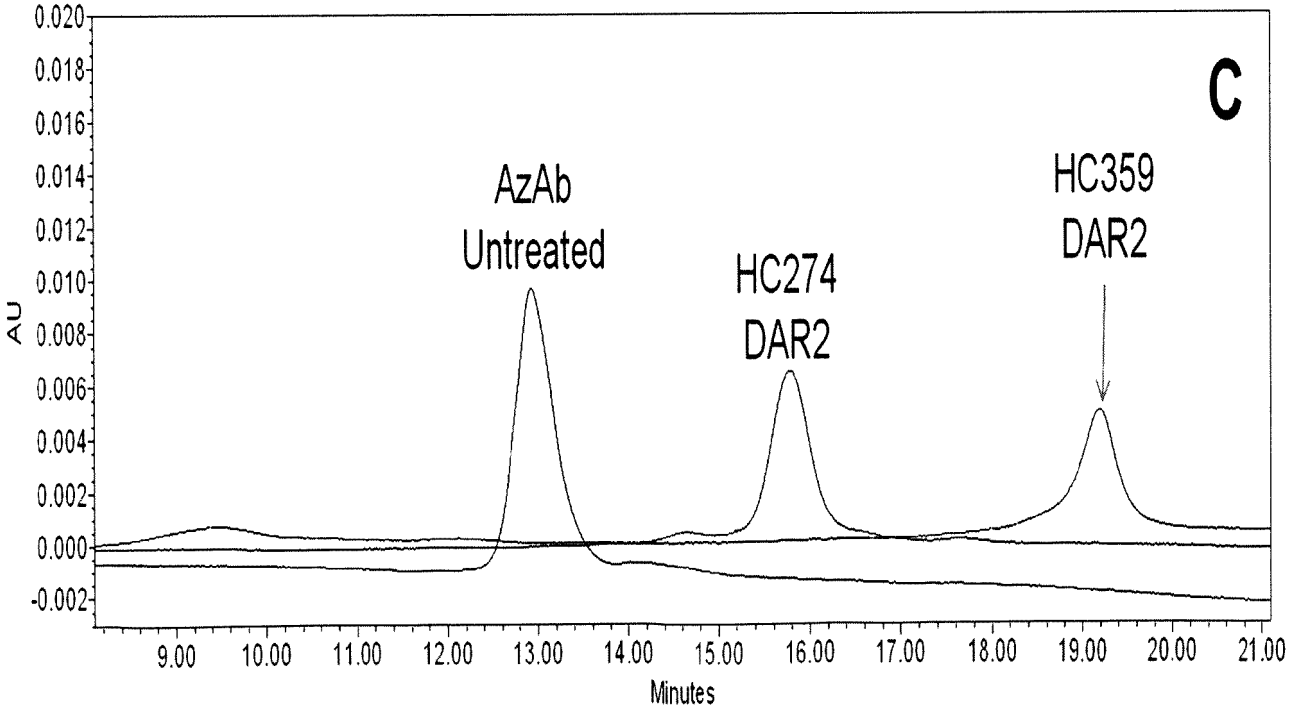



Figure 22.

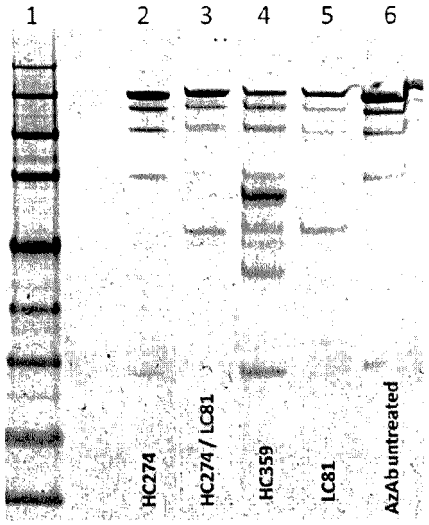



Figure 23

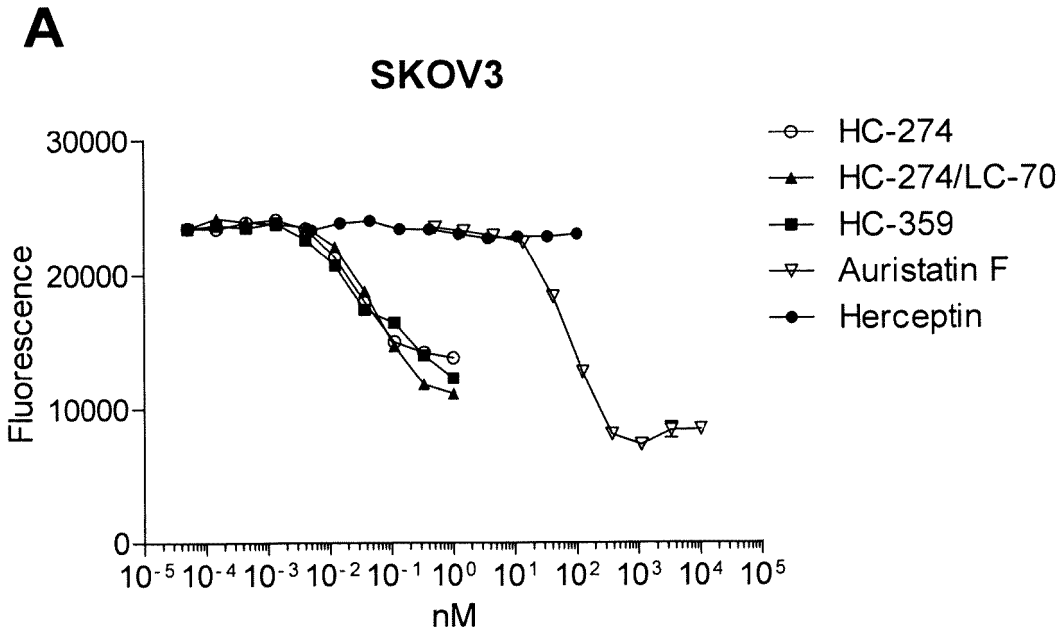
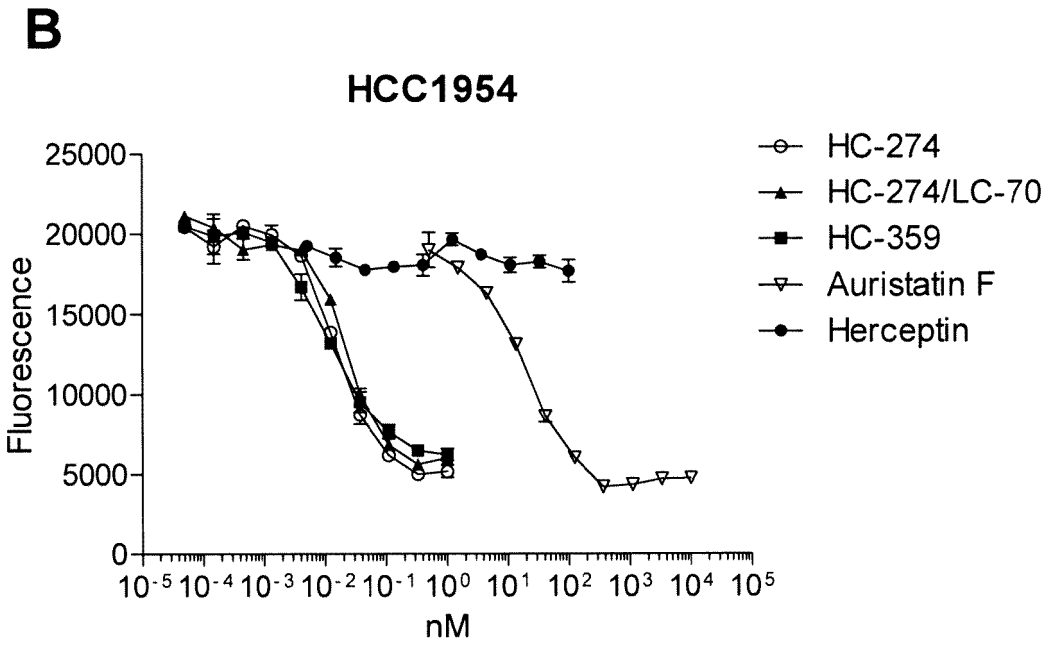


Figure 23 cont



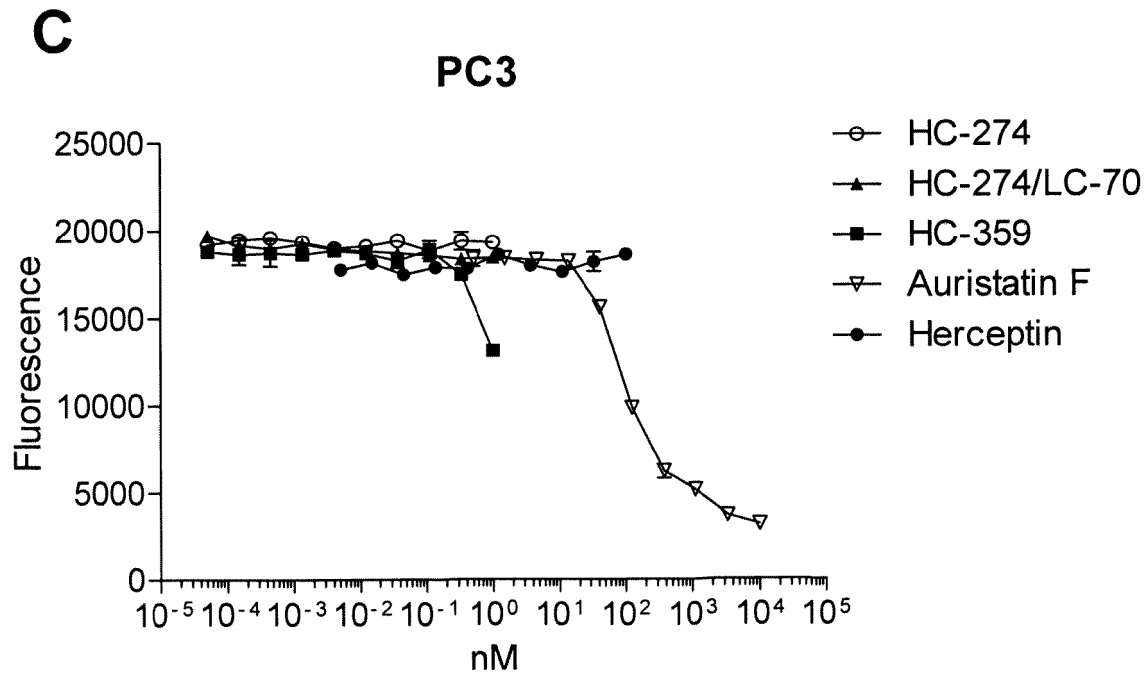
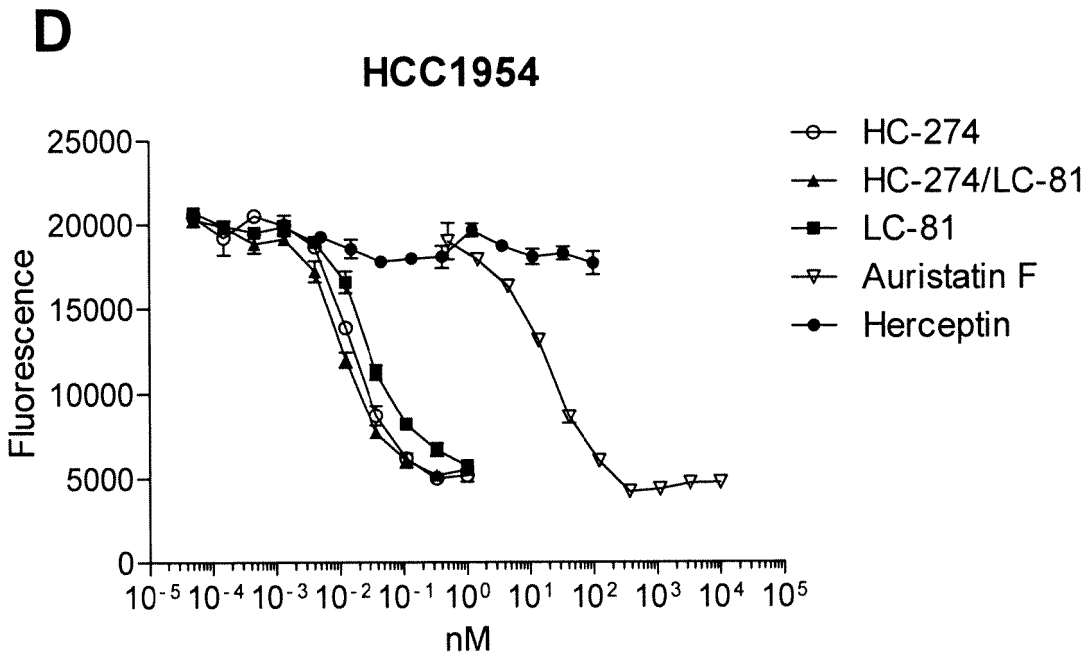



Figure 23 cont



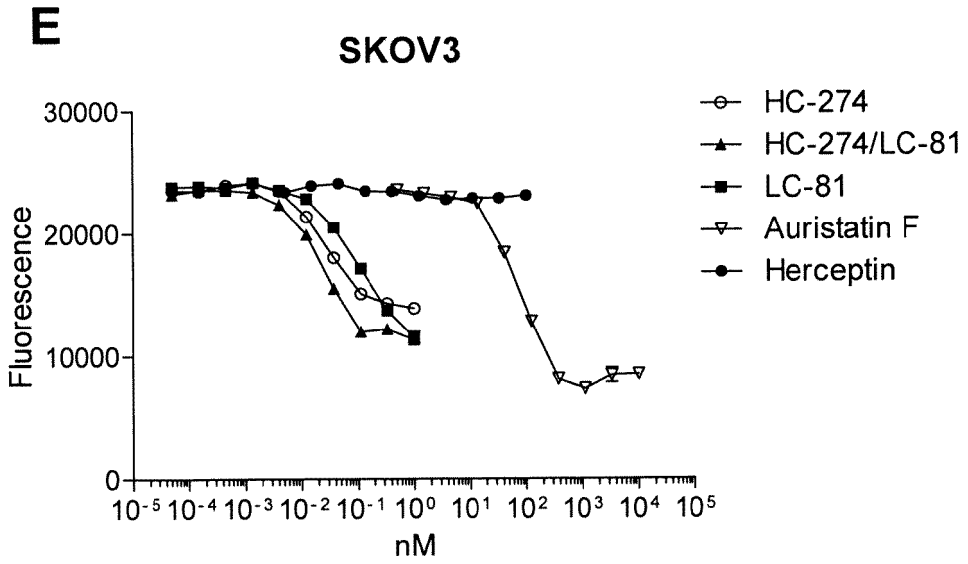



Figure 23 cont

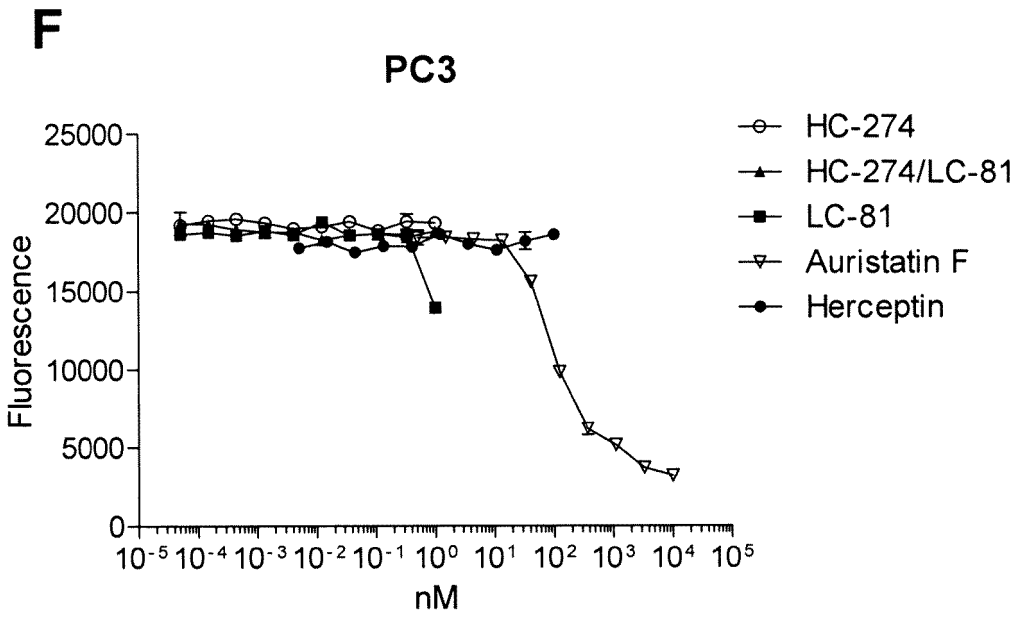


Figure 24

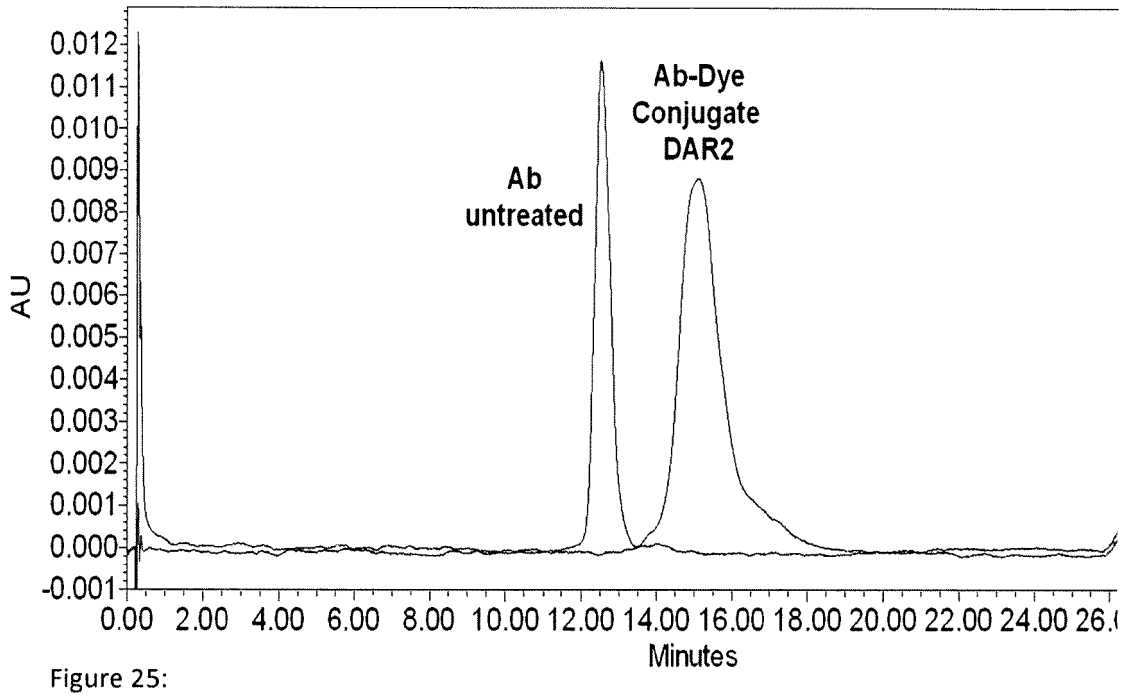



Figure 25:

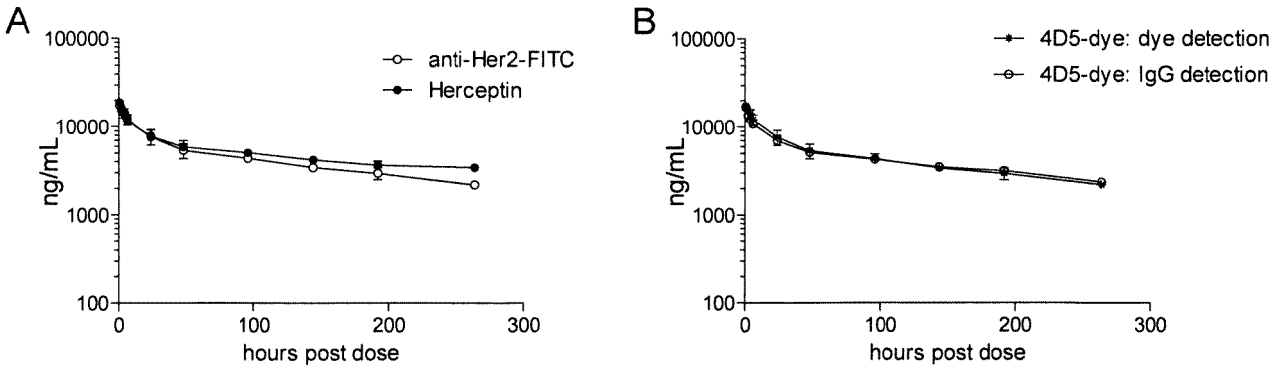
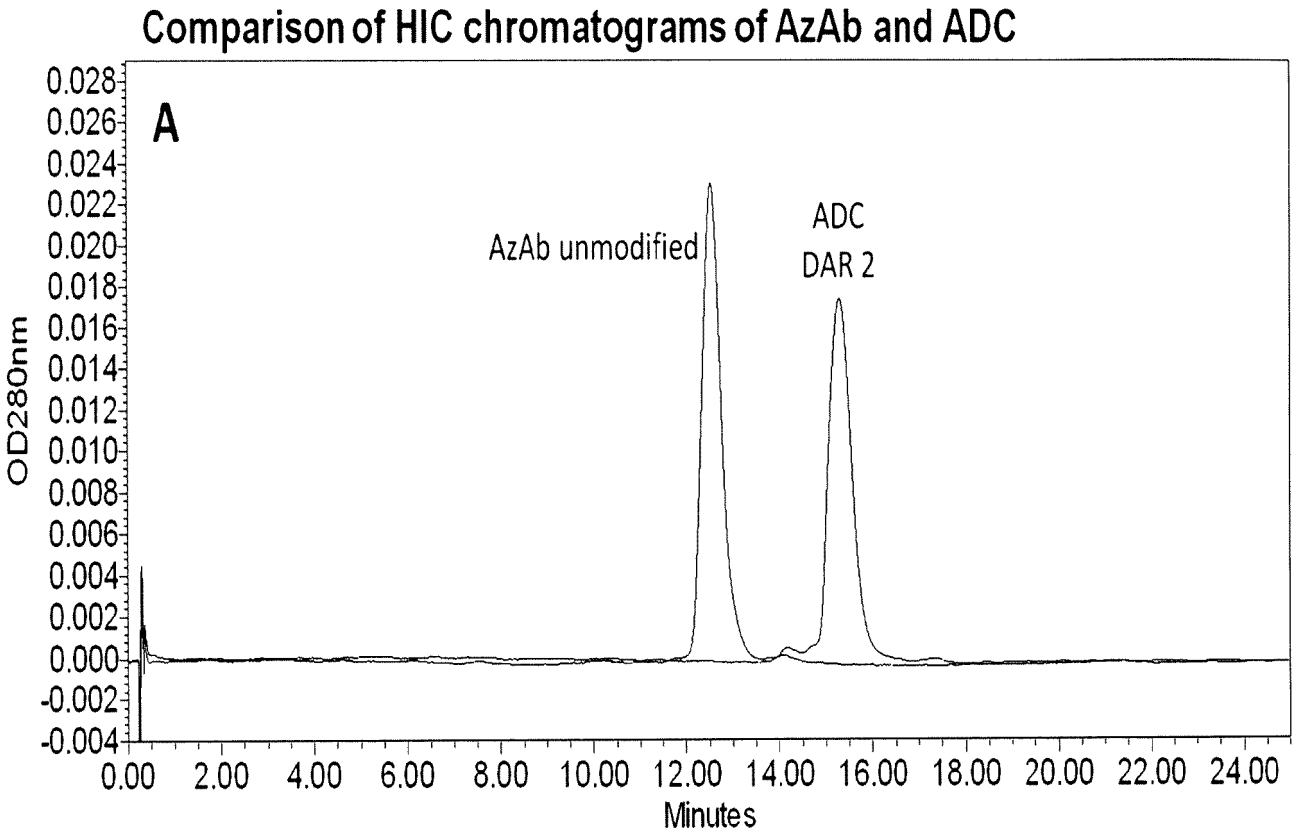


Figure 26


Comparison of HIC chromatograms of AzAb and ADC
A
AzAb unmodified
ADC
DAR 2
OD280nm
0.028
0.026
0.024
0.022
0.020
0.018
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
-0.002
-0.004
0.00 2.00 4.00 6.00 8.00 10.00 12.00 14.00 16.00 18.00 20.00 22.00 24.00
Minutes

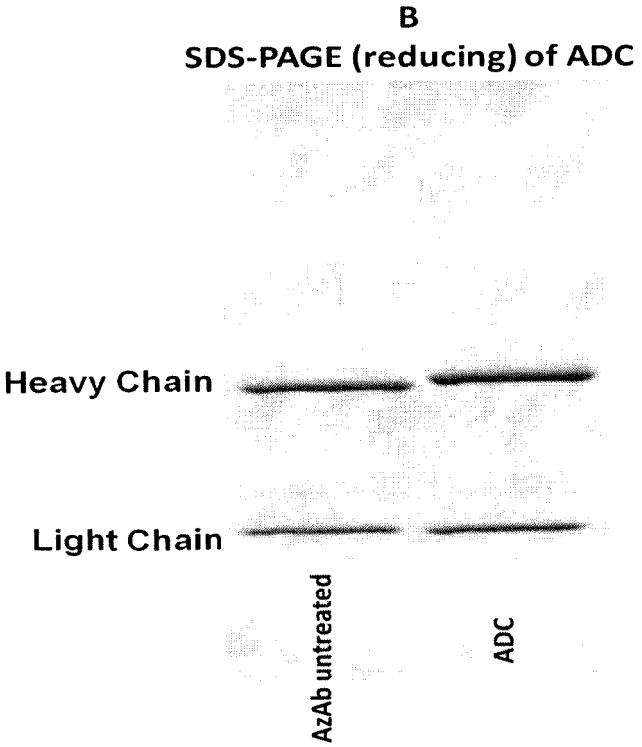

B
SDS-PAGE (reducing) of ADC
Heavy Chain
Light Chain
AzAb untreated
ADC

Figure 27

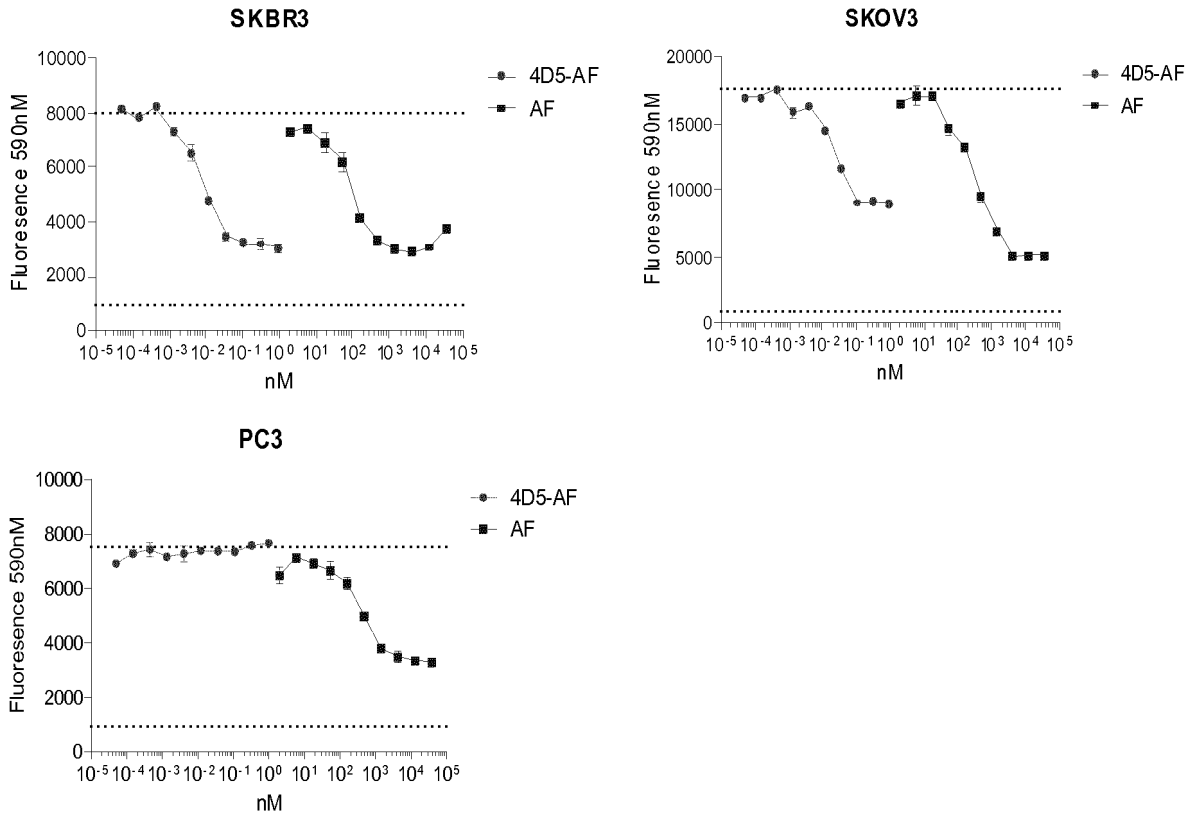



Figure 28

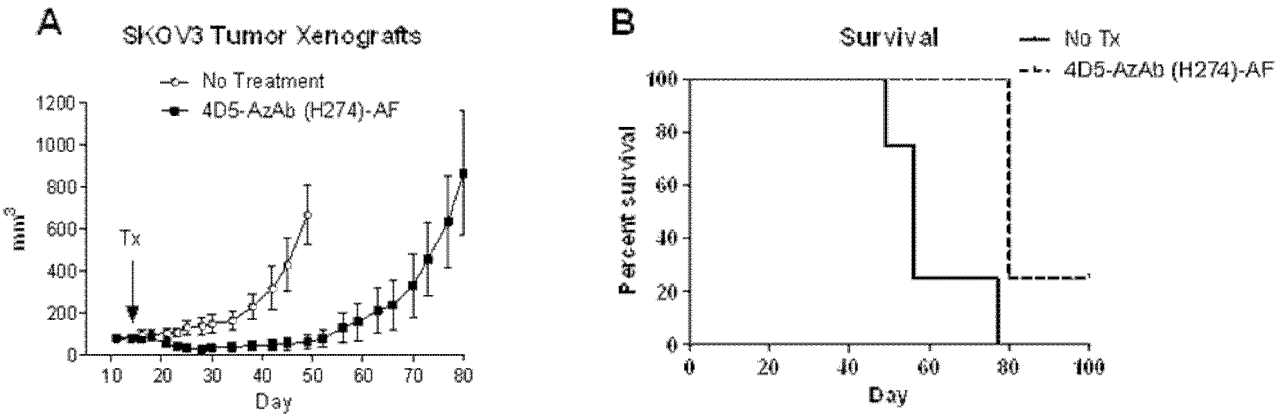


Figure 29

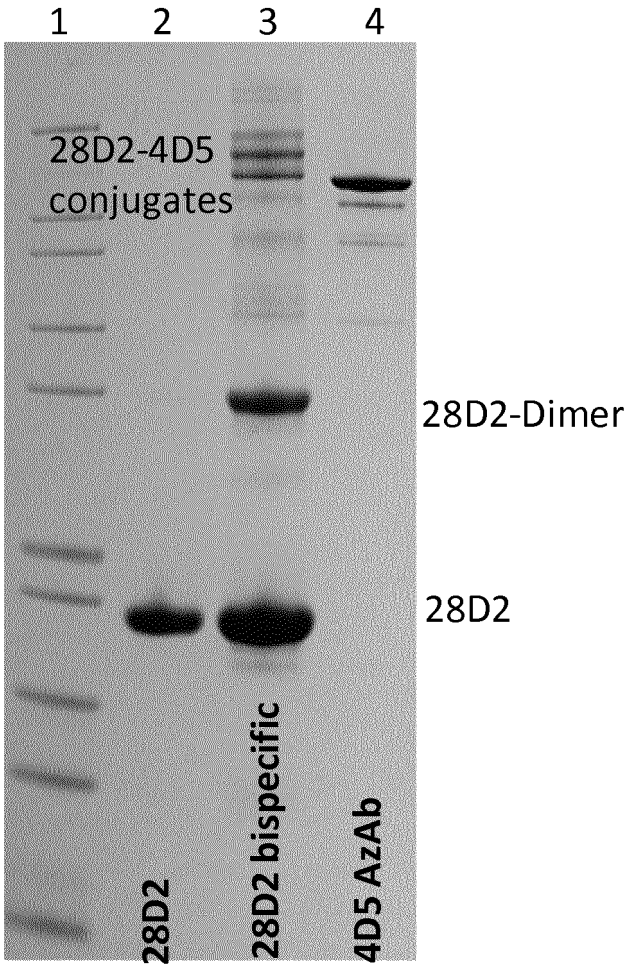

1
2
3
4
28D2-4D5
conjugates
28D2-Dimer
28D2
28D2
28D2 bispecific
4D5 AzAb

Figure 30

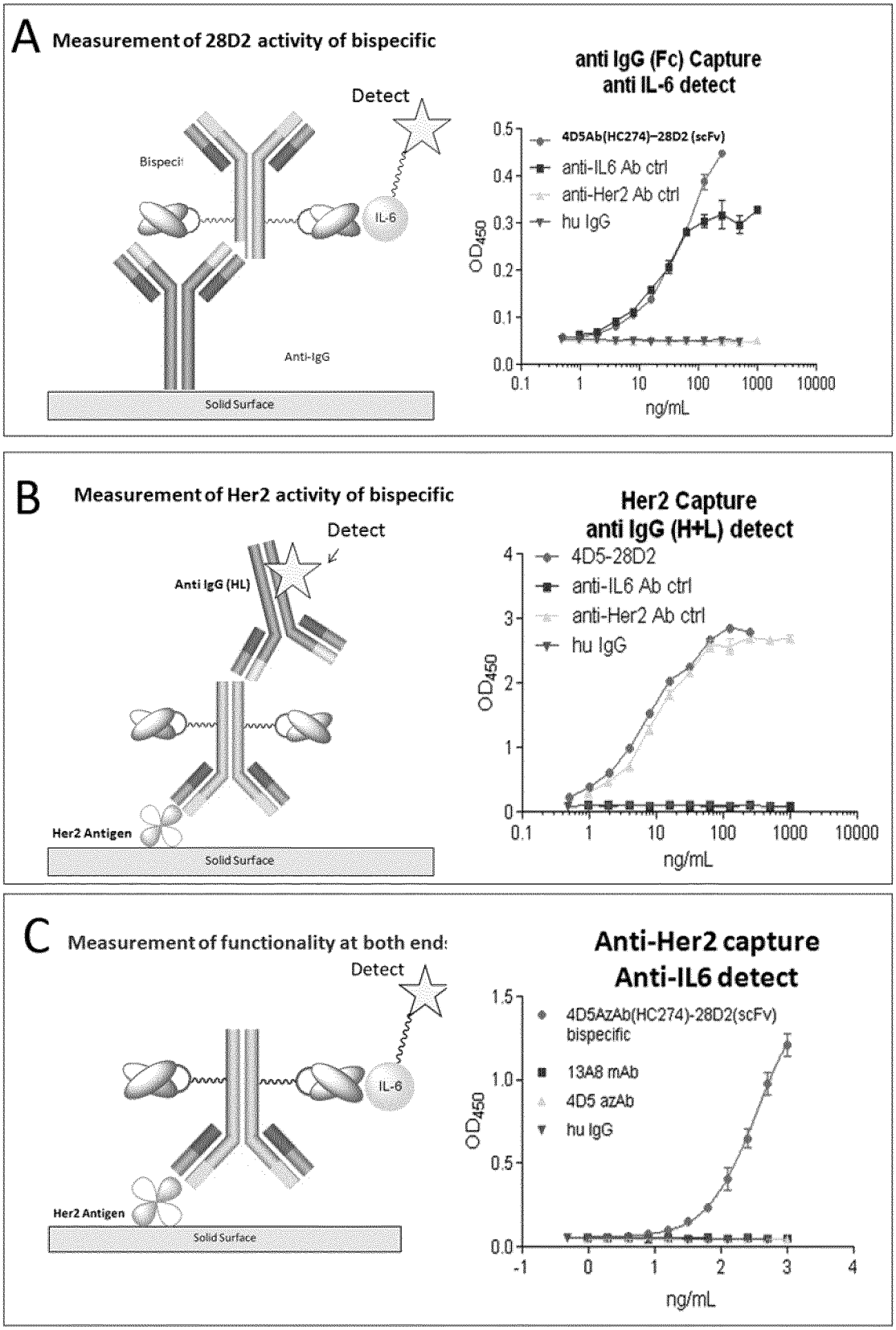

A
Measurement of 28D2 activity of bispecific
Detect
Bispecif
IL-6
Anti-IgG
Solid Surface
anti IgG (Fc) Capture
anti IL-6 detect
4D5Ab(HC274)–28D2 (scFv)
anti-IL6 Ab ctrl
anti-Her2 Ab ctrl
hu IgG
OD450
ng/mL
B
Measurement of Her2 activity of bispecific
Detect
Anti IgG (HL)
Her2 Antigen
Solid Surface
Her2 Capture
anti IgG (H+L) detect
4D5-28D2
anti-IL6 Ab ctrl
anti-Her2 Ab ctrl
hu IgG
OD450
ng/mL
C
Measurement of functionality at both end
Detect
IL-6
Her2 Antigen
Solid Surface
Anti-Her2 capture
Anti-IL6 detect
4D5AzAb(HC274)-28D2(scFv)
bispecific
13A8 mAb
4D5 azAb
hu IgG
OD450
ng/mL

Figure 31

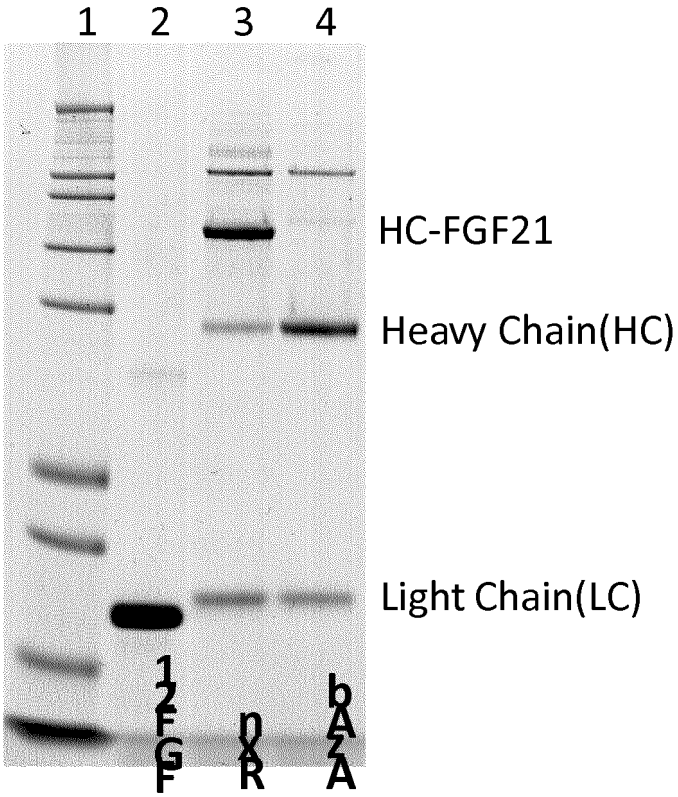



Figure 32

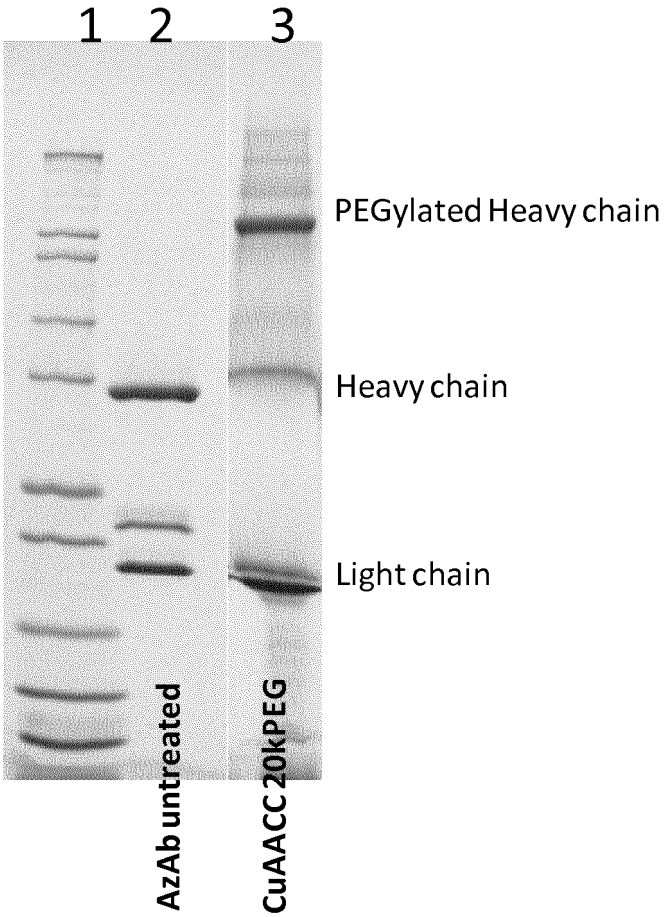


Figure 33

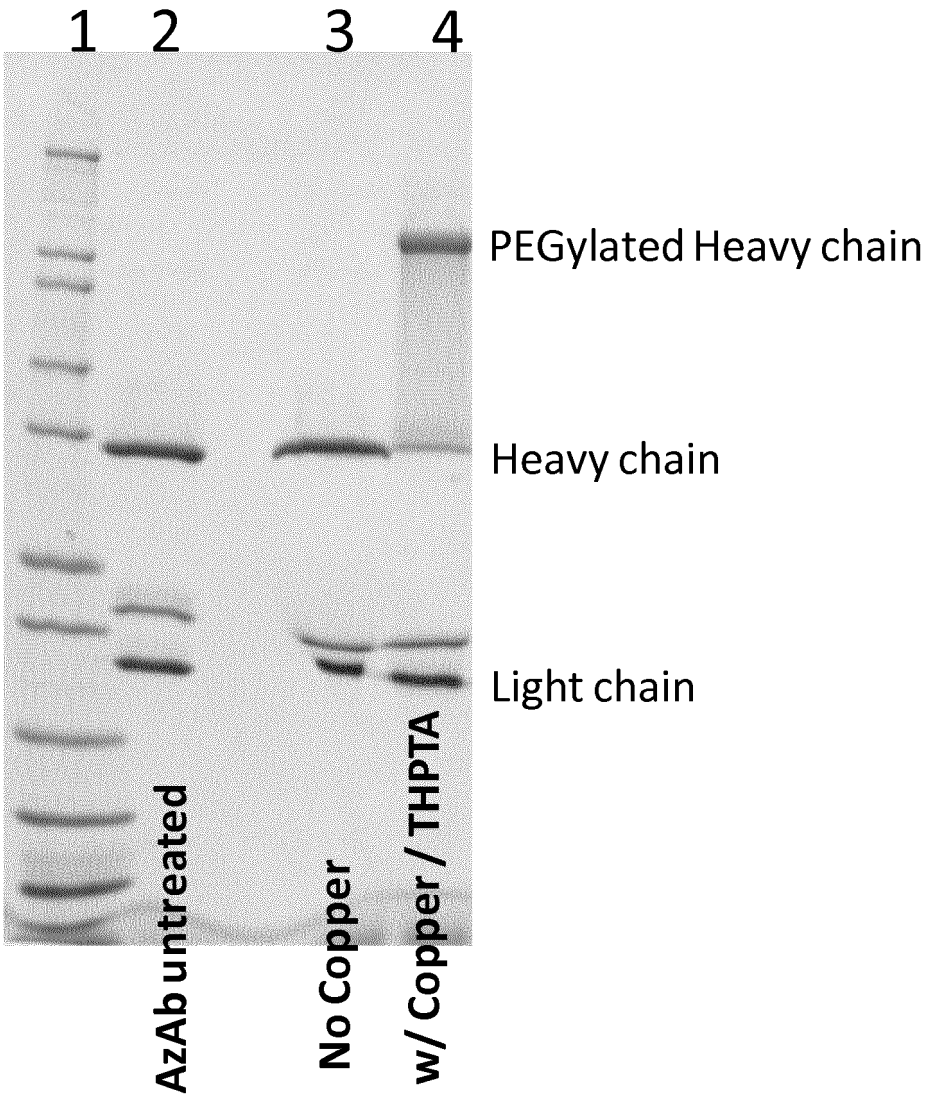

1
2
3
4
PEGylated Heavy chain
Heavy chain
Light chain
AzAb untreated
No Copper
w/ Copper / THPTA

Figure 34

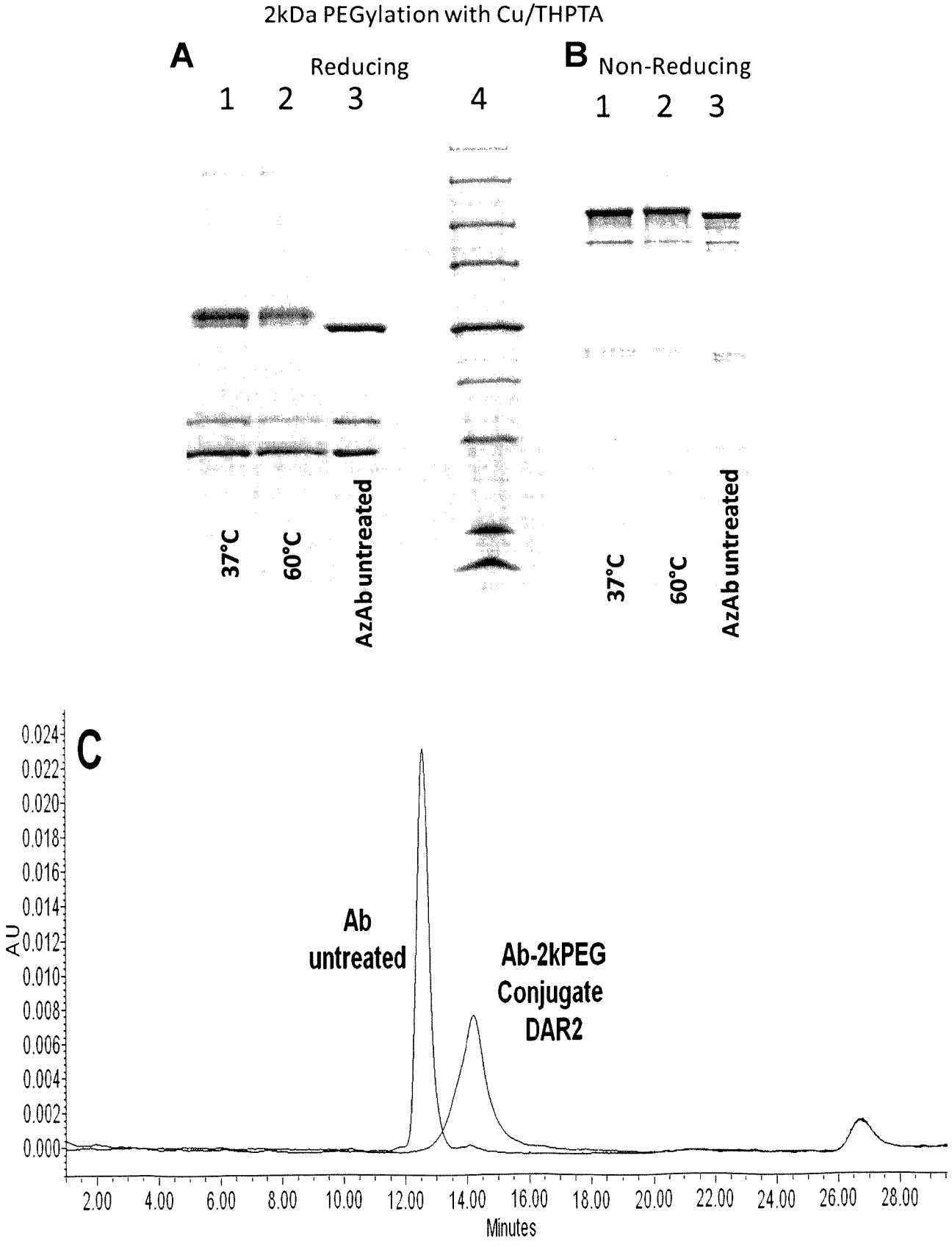

2kDa PEGylation with Cu/THPTA
A
Reducing
1
2
3
4
37°C
60°C
AzAb untreated
B
Non-Reducing
1
2
3
37°C
60°C
AzAb untreated
C
AU
0.024
0.022
0.020
0.018
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
Ab
untreated
Ab-2kPEG
Conjugate
DAR2
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
26.00
28.00
Minutes

Figure 35

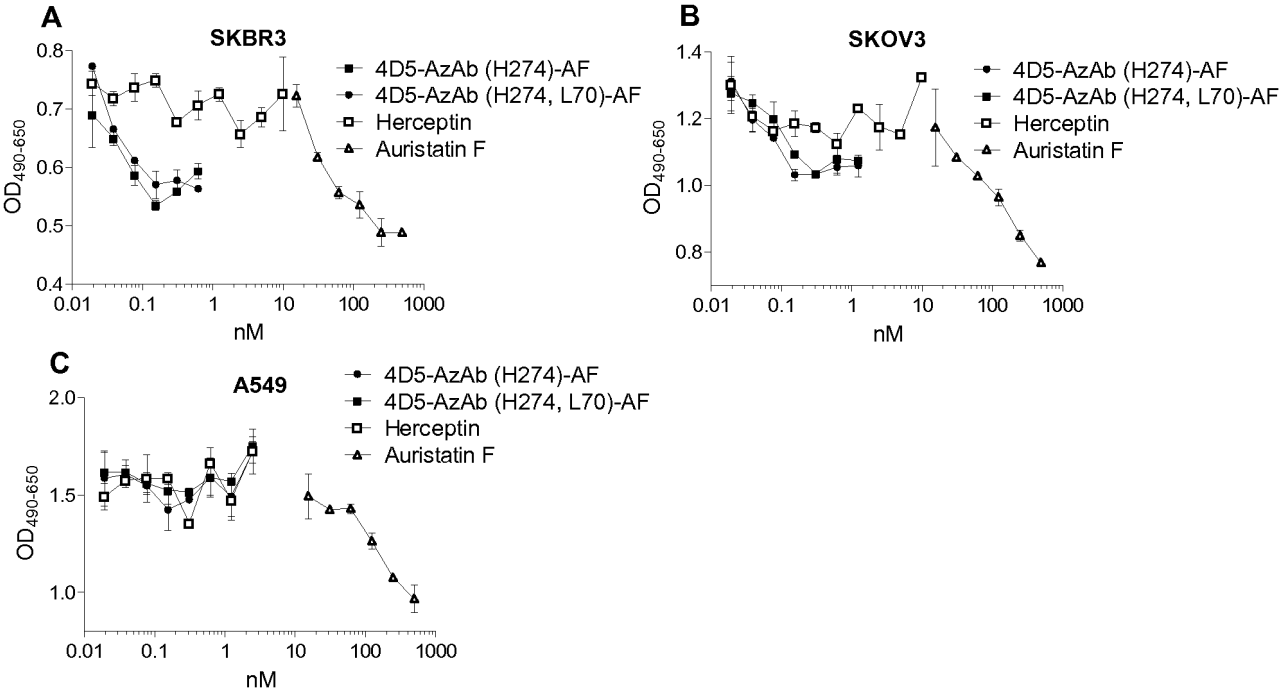



Figure 36 :

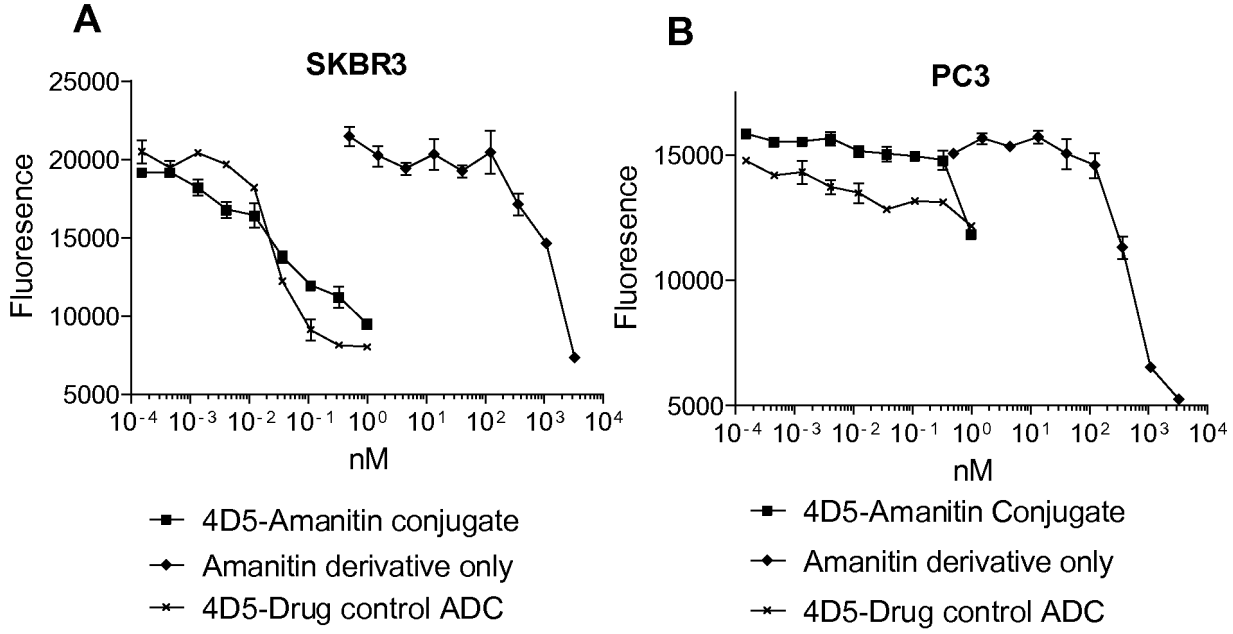


Figure 37

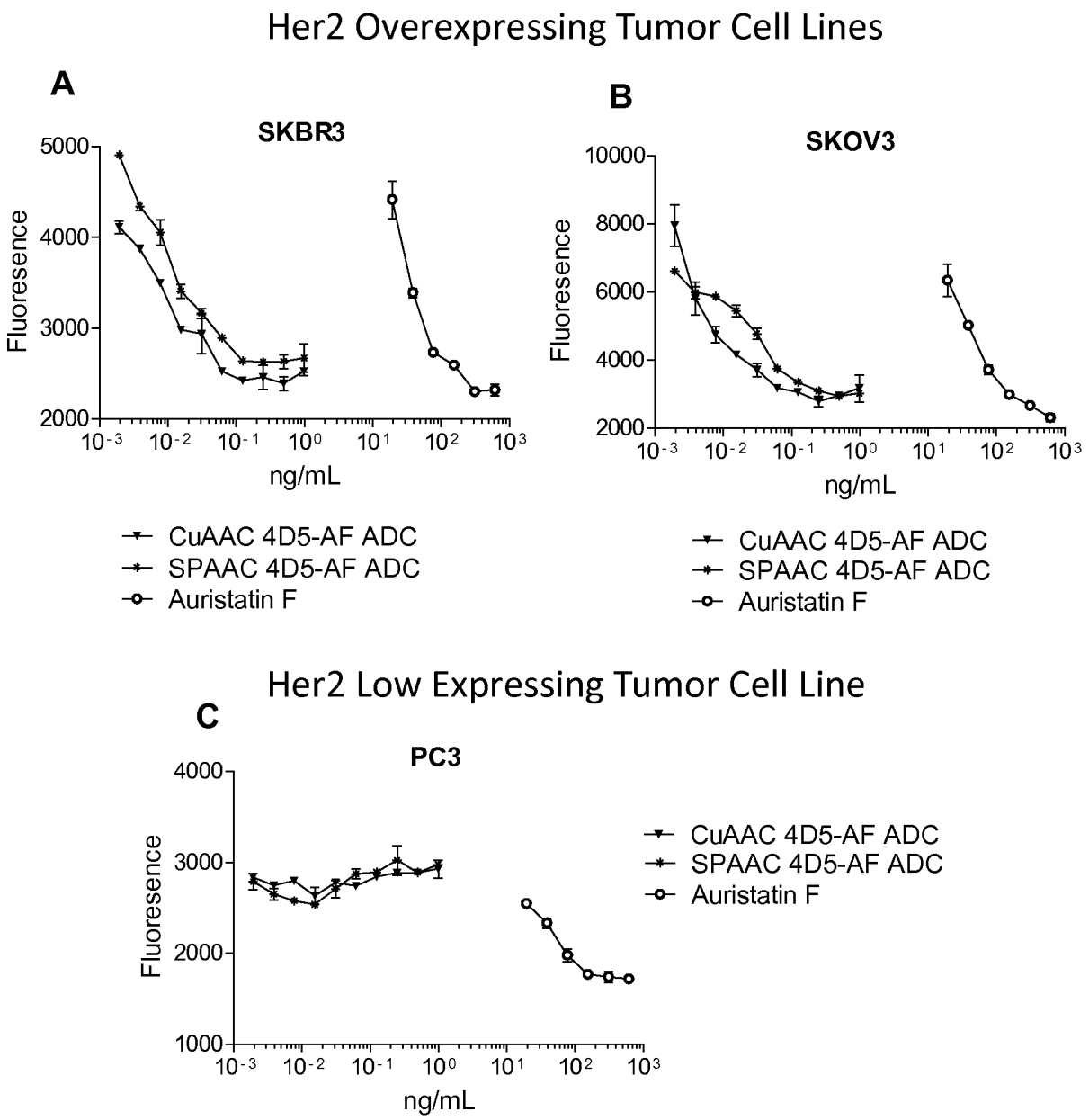

Her2 Overexpressing Tumor Cell Lines
A
SKBR3
Fluoresence
5000
4000
3000
2000
10-3 10-2 10-1 100 101 102 103
ng/mL
B
SKOV3
Fluoresence
10000
8000
6000
4000
2000
10-3 10-2 10-1 100 101 102 103
ng/mL
CuAAC 4D5-AF ADC
SPAAC 4D5-AF ADC
Auristatin F
CuAAC 4D5-AF ADC
SPAAC 4D5-AF ADC
Auristatin F
Her2 Low Expressing Tumor Cell Line
C
PC3
Fluoresence
4000
3000
2000
1000
10-3 10-2 10-1 100 101 102 103
ng/mL
CuAAC 4D5-AF ADC
SPAAC 4D5-AF ADC
Auristatin F

Figure 38

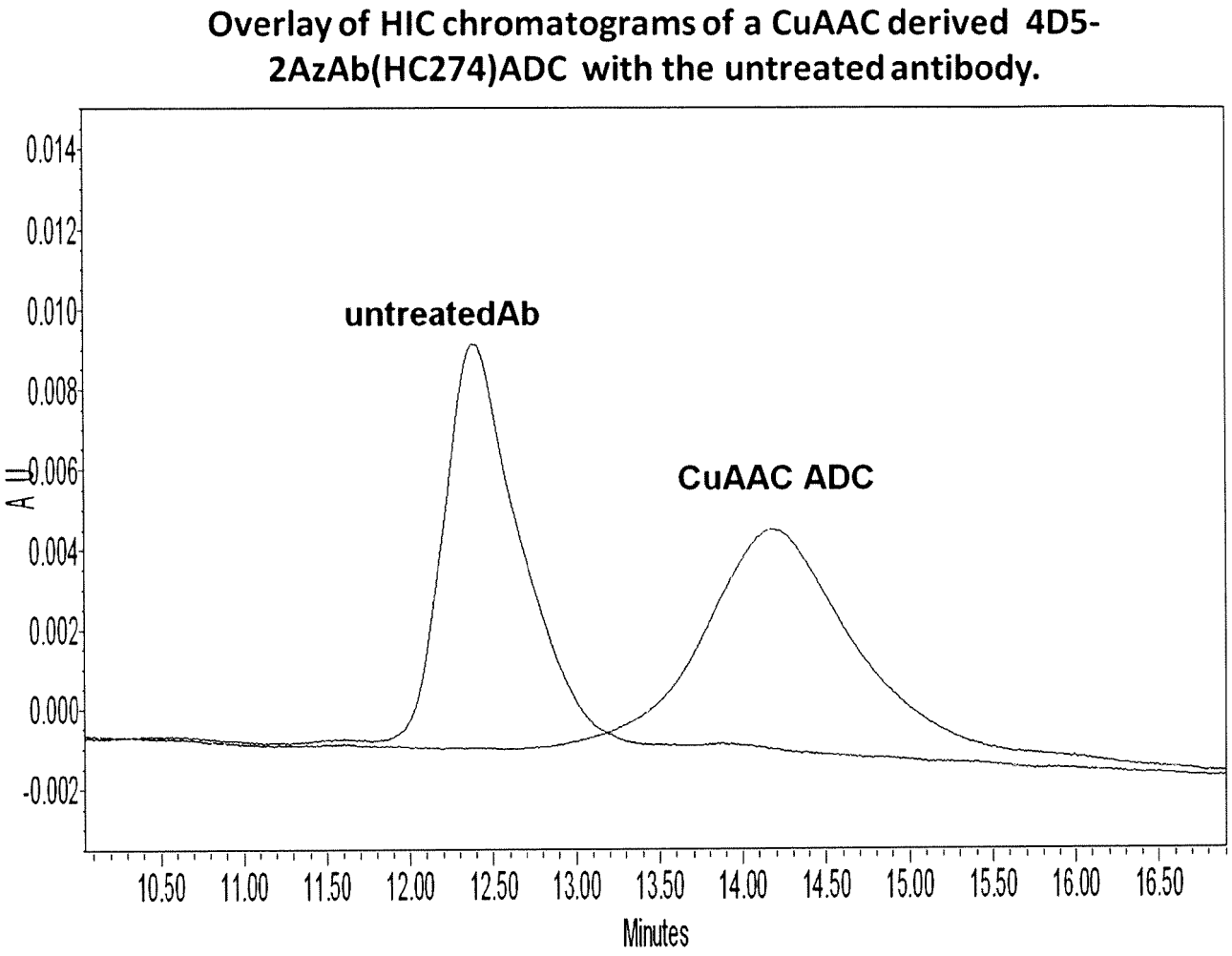



Figure 39

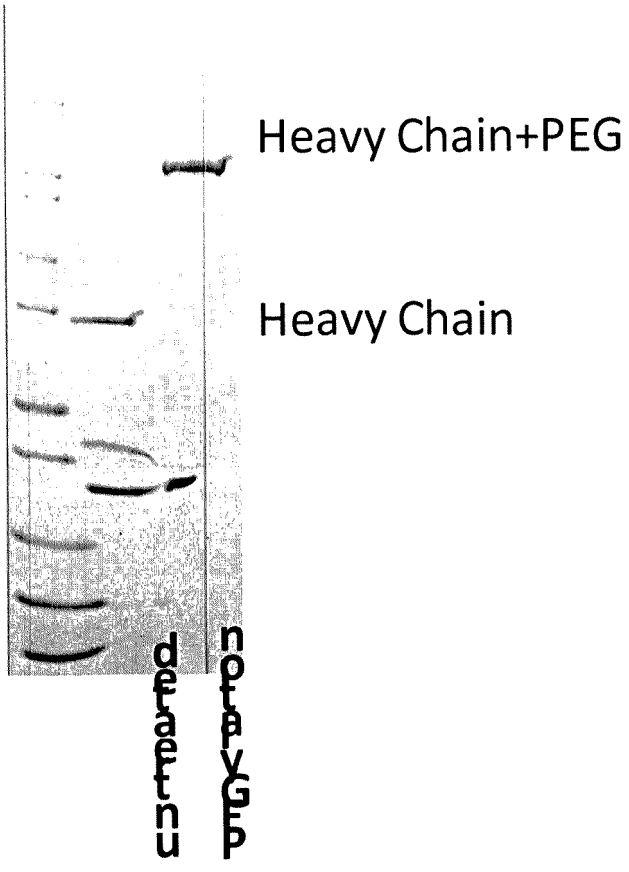


Figure 40

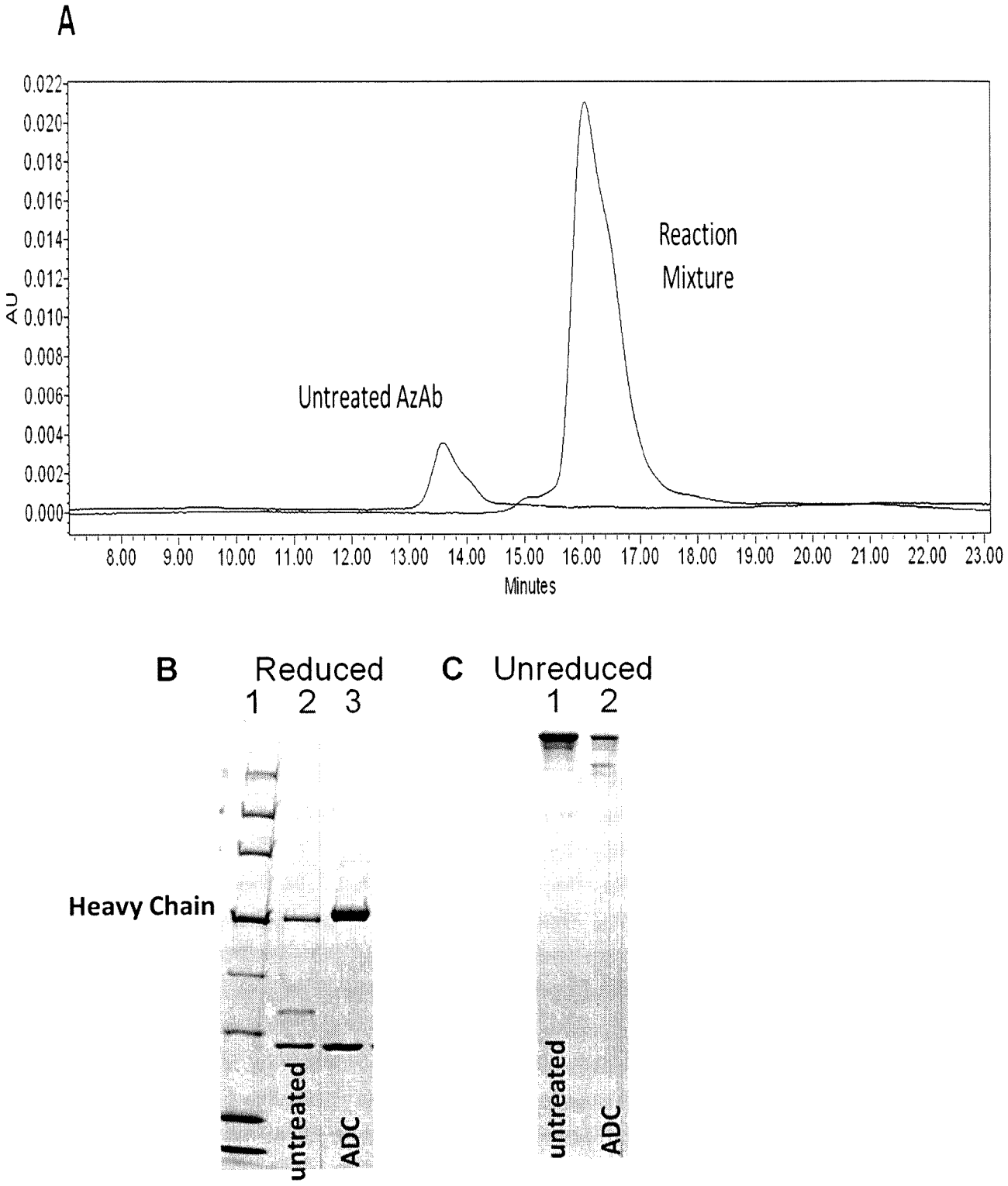

A
AU
0.022
0.020
0.018
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
Reaction Mixture
Untreated AzAb
8.00 9.00 10.00 11.00 12.00 13.00 14.00 15.00 16.00 17.00 18.00 19.00 20.00 21.00 22.00 23.00
Minutes
B Reduced
1 2 3
Heavy Chain
untreated
ADC
C Unreduced
1 2
untreated
ADC

Figure 41

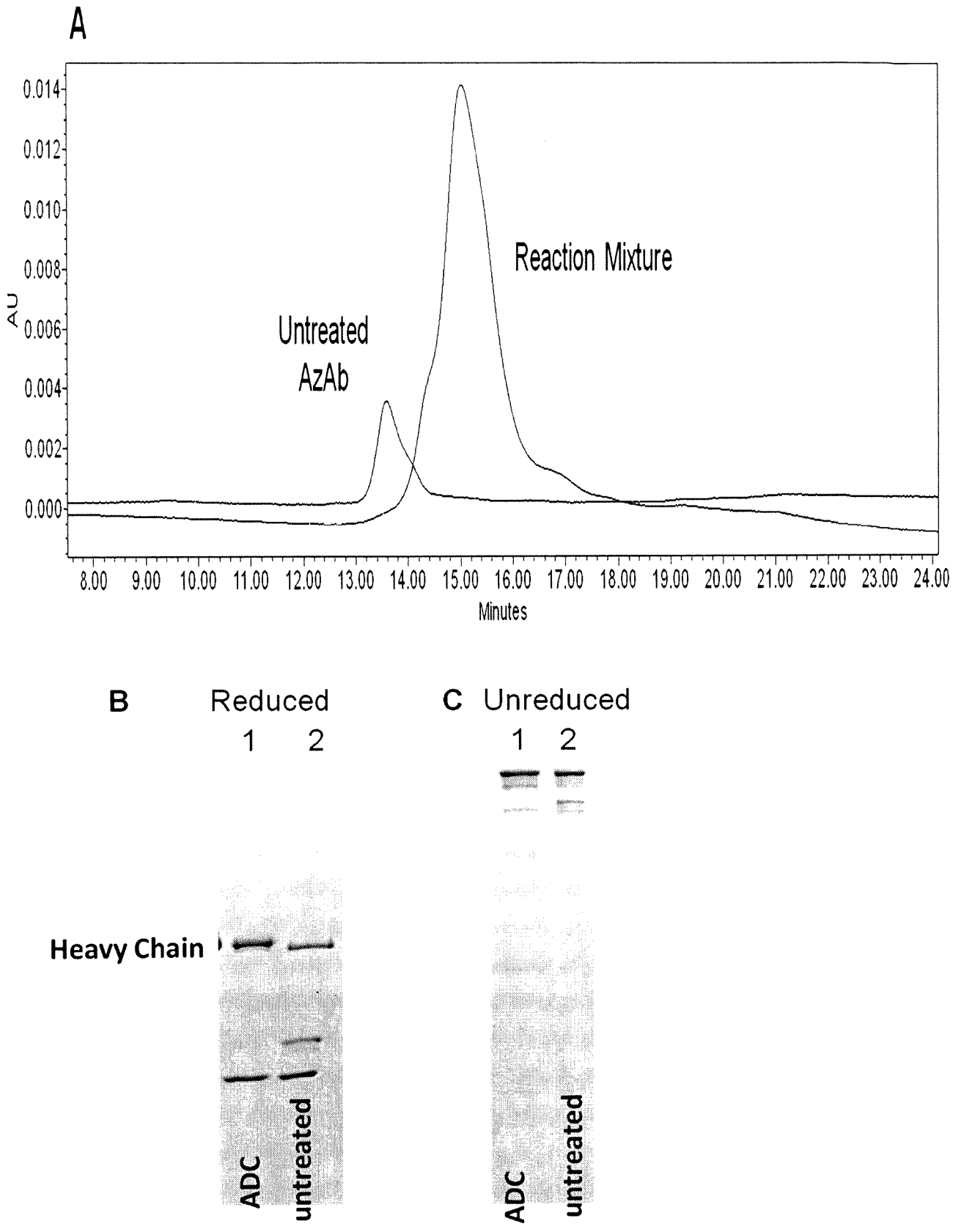
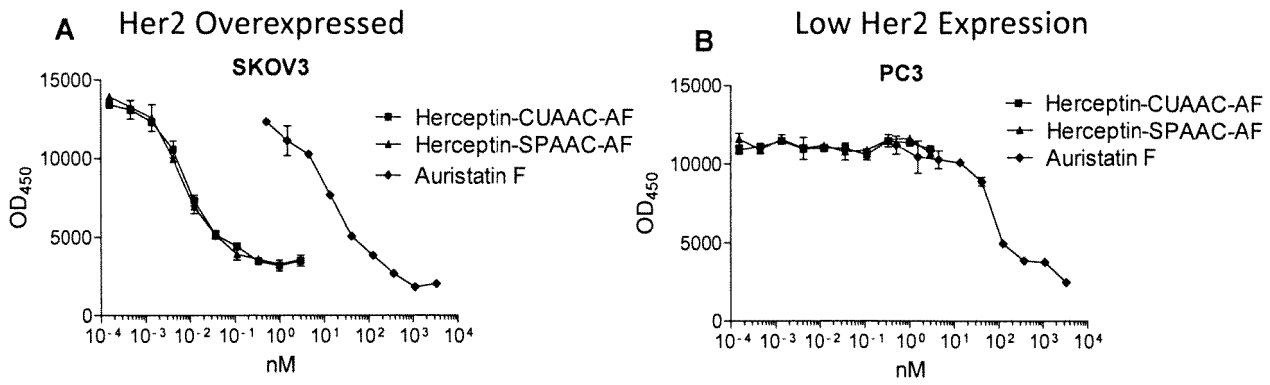

A
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
AU
Reaction Mixture
Untreated
AzAb
8.00 9.00 10.00 11.00 12.00 13.00 14.00 15.00 16.00 17.00 18.00 19.00 20.00 21.00 22.00 23.00 24.00
Minutes
B Reduced
1 2
Heavy Chain
ADC
untreated
C Unreduced
1 2
ADC
untreated

Figure 42


A
Her2 Overexpressed
SKOV3
15000
10000
5000
0
OD450
10-4 10-3 10-2 10-1 100 101 102 103 104
nM
Herceptin-CUAAC-AF
Herceptin-SPAAC-AF
Auristatin F
B
Low Her2 Expression
PC3
15000
10000
5000
0
OD450
10-4 10-3 10-2 10-1 100 101 102 103 104
nM
Herceptin-CUAAC-AF
Herceptin-SPAAC-AF
Auristatin F