Figure 1

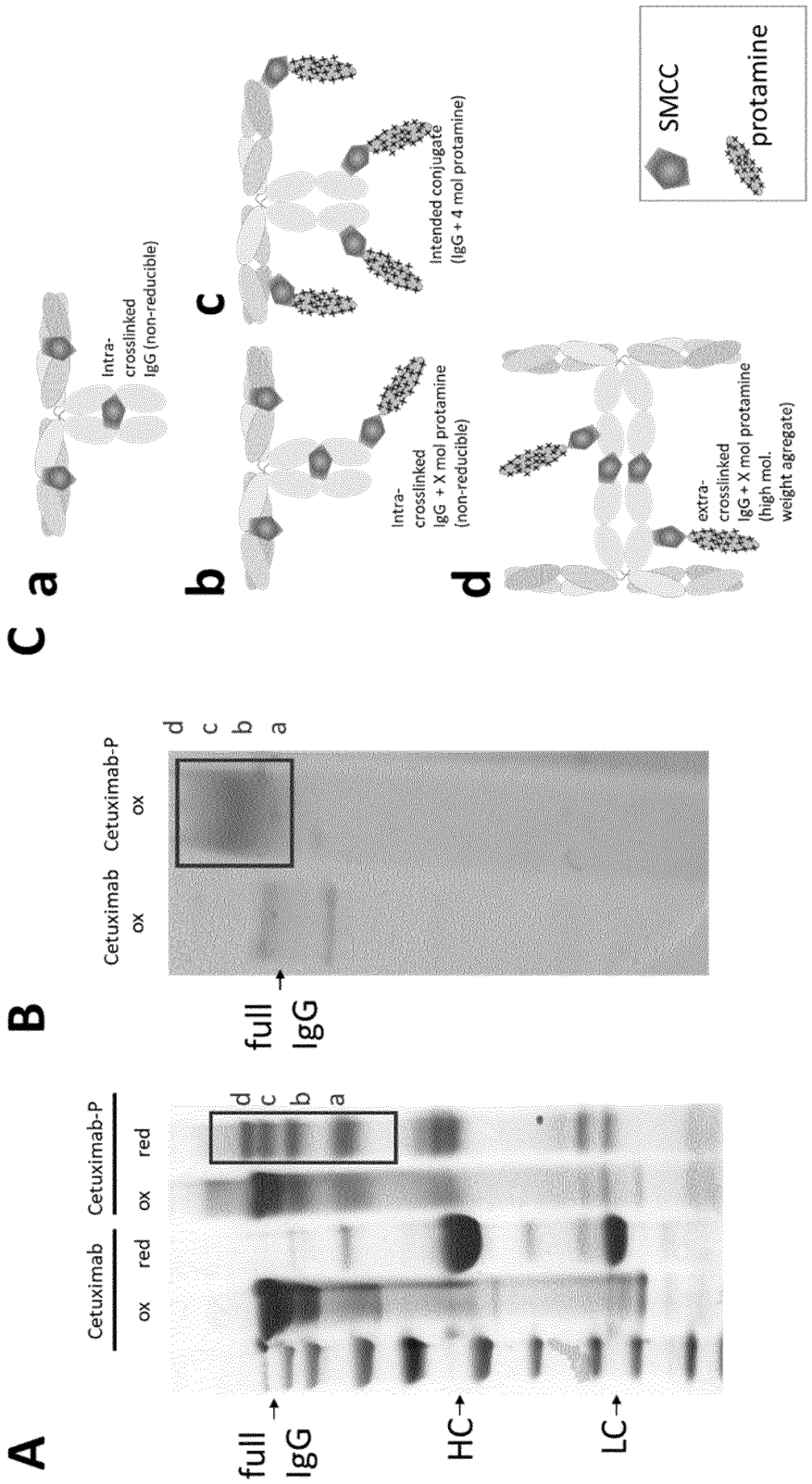

A
Cetuximab
Cetuximab-P
ox
red
ox
red
full IgG
HC
LC
d
c
b
a
B
Cetuximab
Cetuximab-P
ox
ox
full IgG
d
c
b
a
C
a
Intra-crosslinked IgG (non-reducible)
b
Intra-crosslinked IgG + X mol protamine (non-reducible)
c
Intended conjugate (IgG + 4 mol protamine)
d
extra-crosslinked IgG + X mol protamine (high mol. weight agregate)
SMCC
protamine

Figure 2

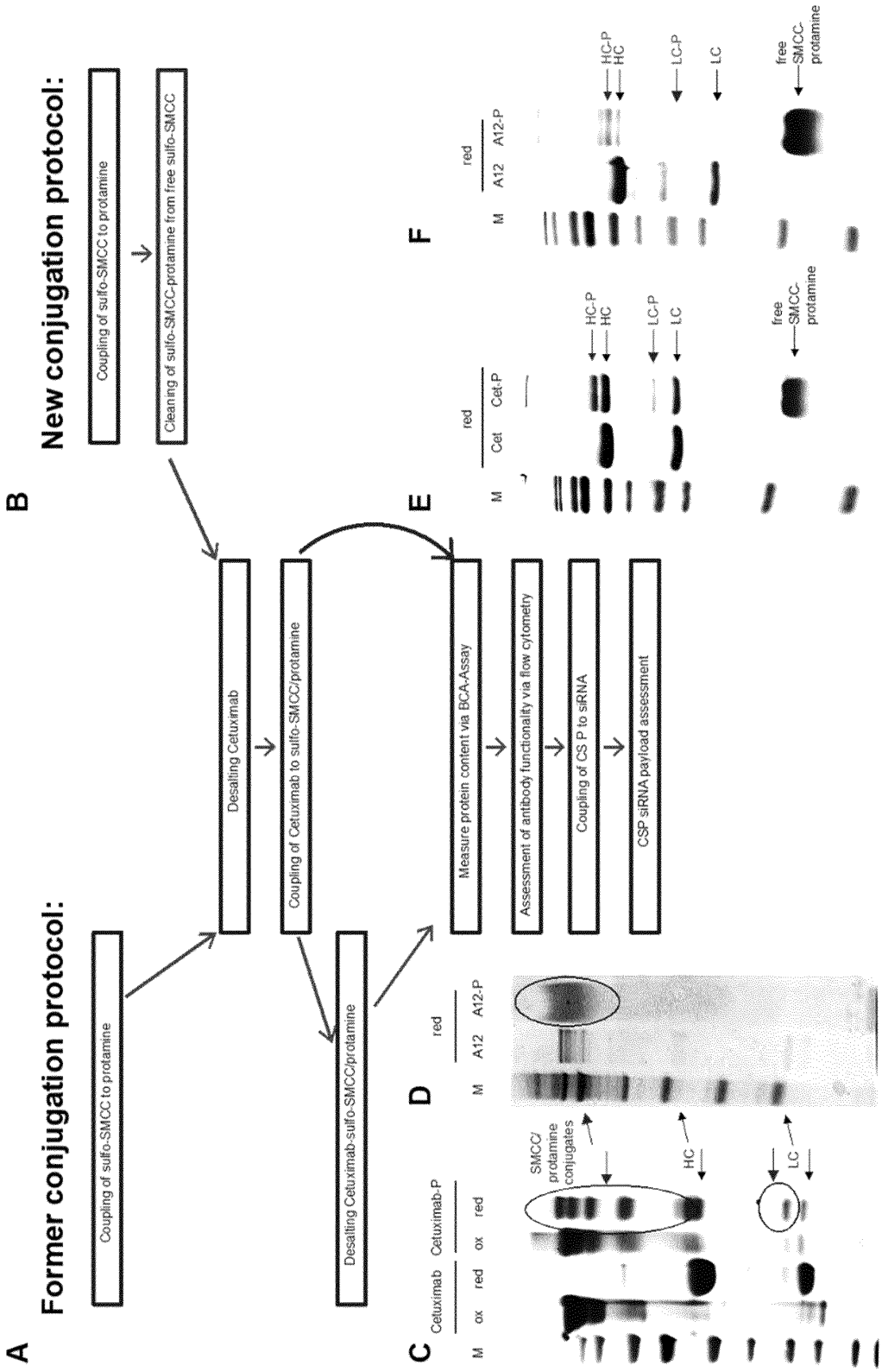
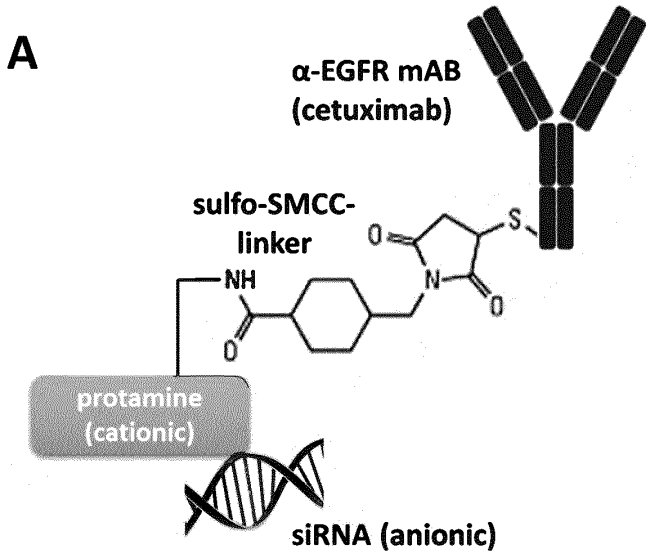

A
Former conjugation protocol:
Coupling of sulfo-SMCC to protamine
Desalting Cetuximab
Coupling of Cetuximab to sulfo-SMCC/protamine
Desalting Cetuximab-sulfo-SMCC/protamine
B
New conjugation protocol:
Coupling of sulfo-SMCC to protamine
Cleaning of sulfo-SMCC-protamine from free sulfo-SMCC
Measure protein content via BCA-Assay
Assessment of antibody functionality via flow cytometry
Coupling of CS P to siRNA
CSP siRNA payload assessment
C
Cetuximab
Cetuximab-P
M ox red ox red
SMCC/ protamine conjugates
HC
LC
D
red
M A12 A12-P
E
red
M Cet Cet-P
HC-P
HC
LC-P
LC
free SMCC-protamine
F
red
M A12 A12-P
HC-P
HC
LC-P
LC
free SMCC-protamine

Figure 3


A
α-EGFR mAB
(cetuximab)
sulfo-SMCC-
linker
NH
O
O
N
O
S
protamine
(cationic)
siRNA (anionic)

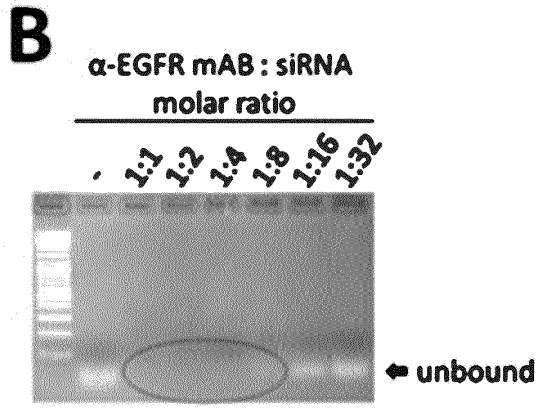

B
α-EGFR mAB : siRNA
molar ratio
-
1:1
1:2
1:4
1:8
1:16
1:32
unbound

Figure 3 (cont'd)

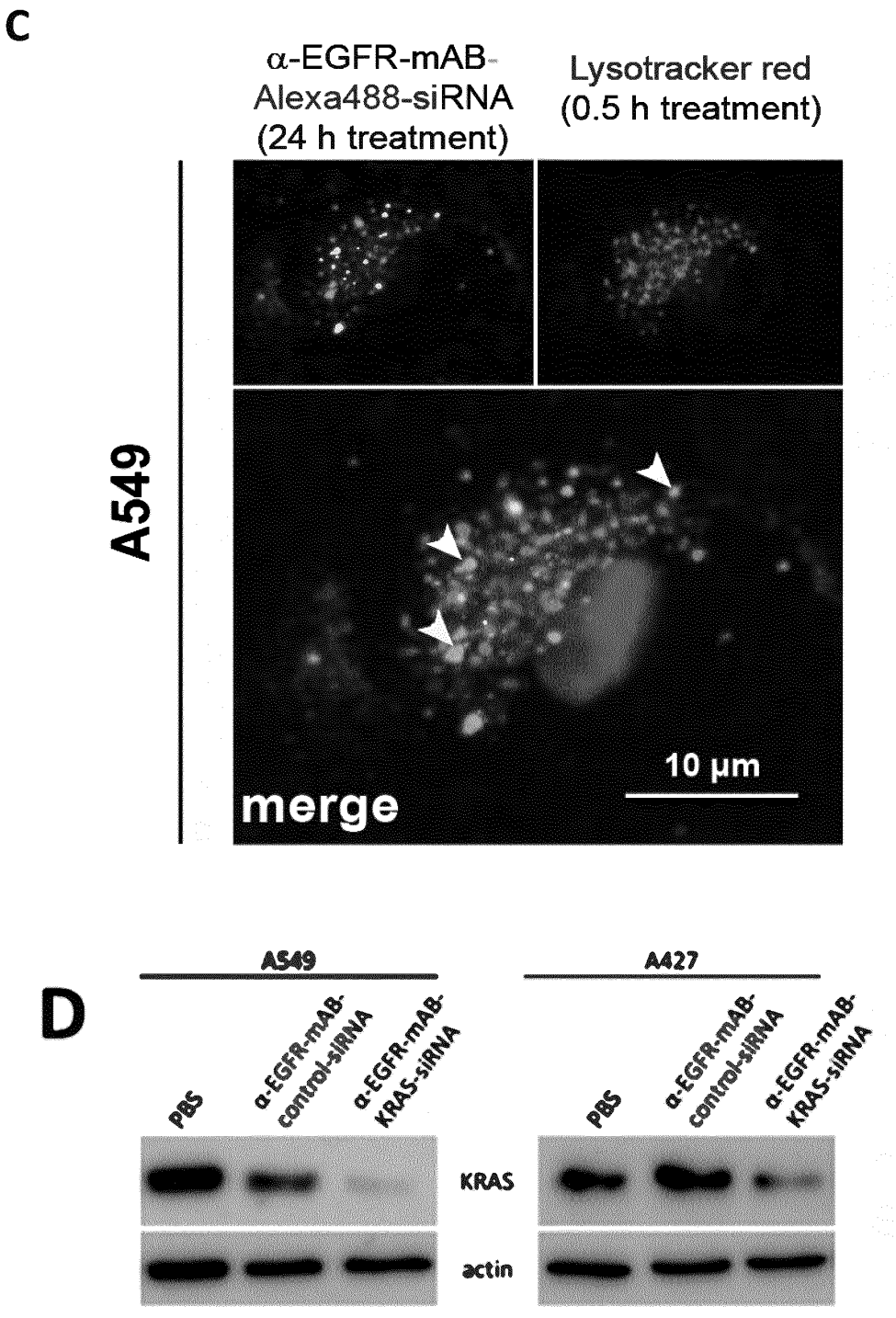


Figure 3 (cont'd)

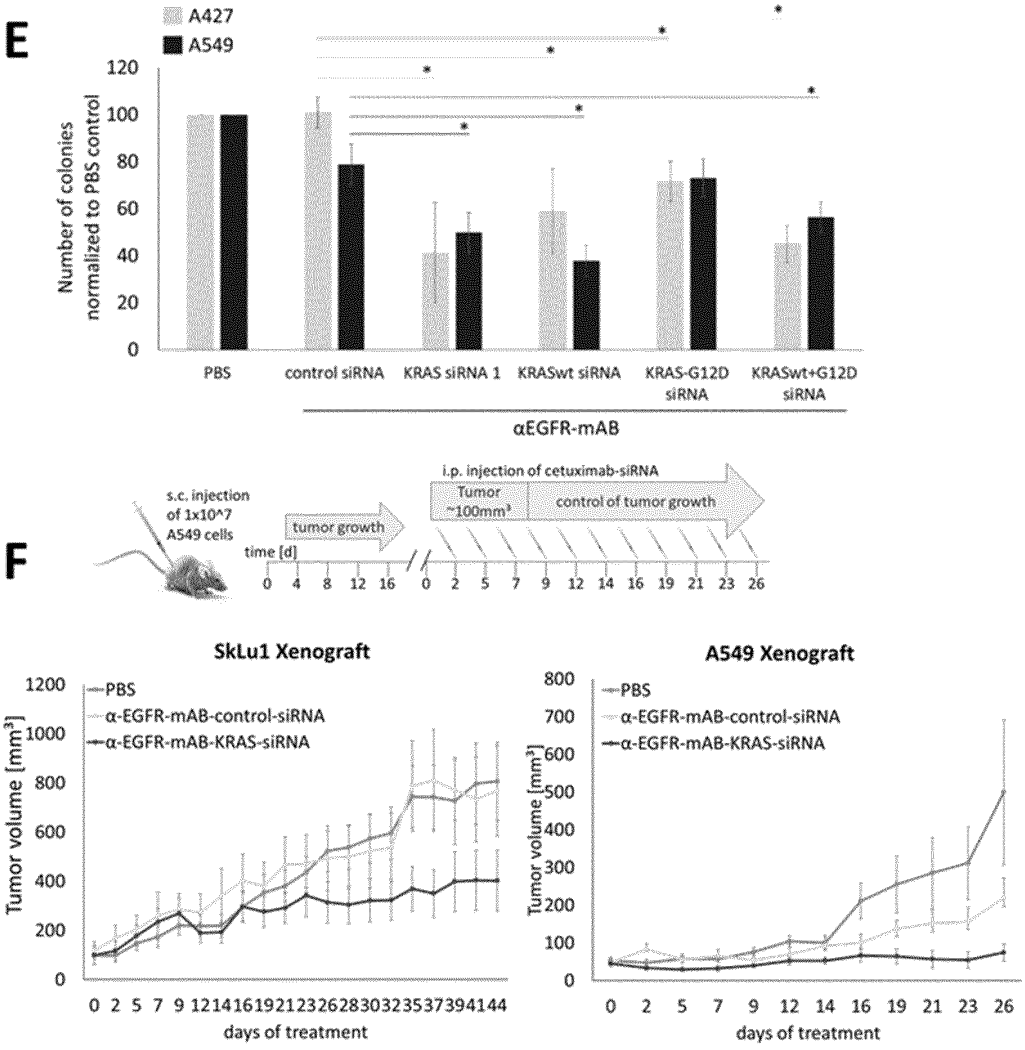


Figure 3 (cont'd)

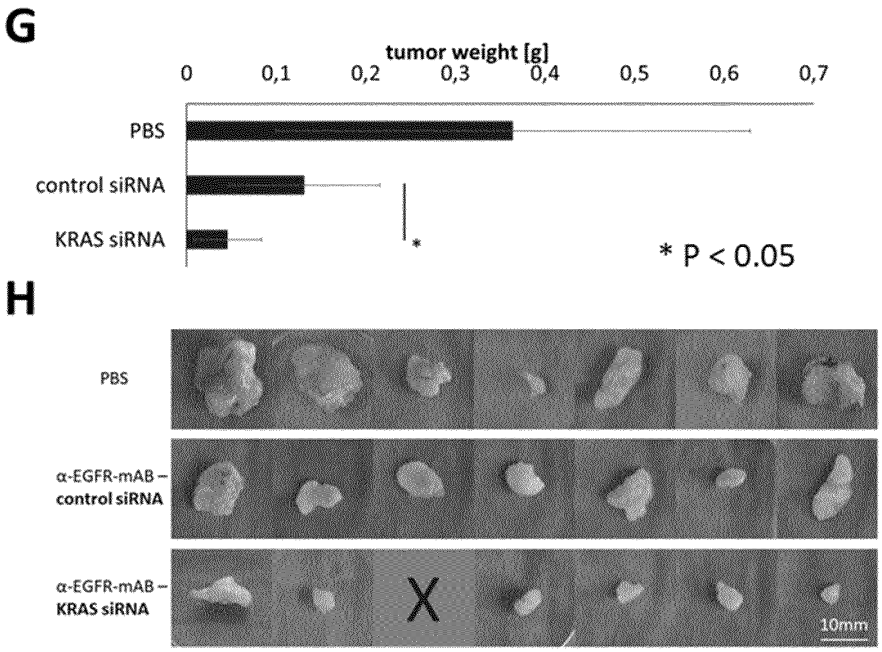


Figure 4

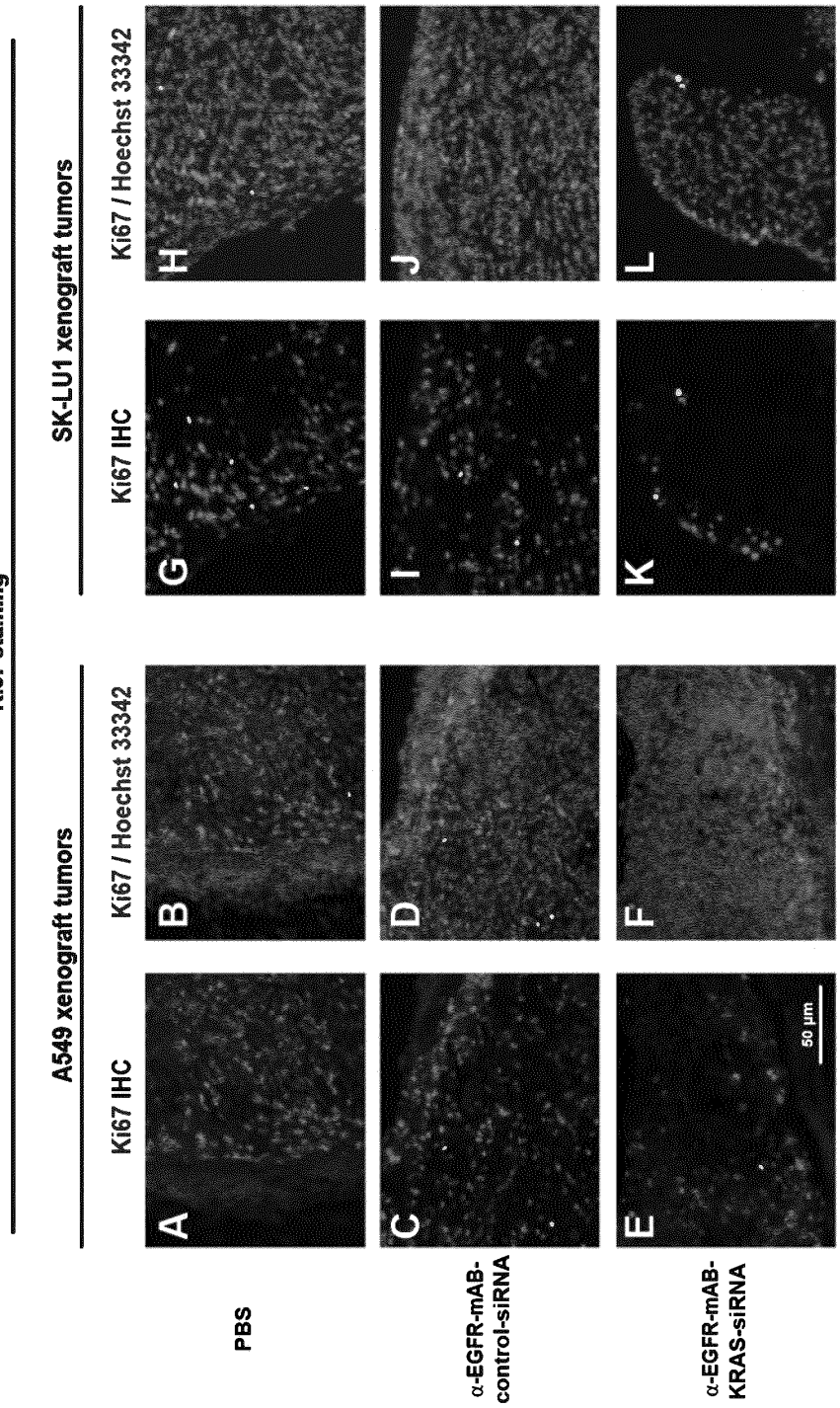

Ki67 staining
A549 xenograft tumors
SK-LU1 xenograft tumors
Ki67 IHC
Ki67 / Hoechst 33342
Ki67 IHC
Ki67 / Hoechst 33342
A
B
G
H
C
D
I
J
E
F
K
L
50 µm
PBS
α-EGFR-mAB-control-siRNA
α-EGFR-mAB-KRAS-siRNA

Figure 5

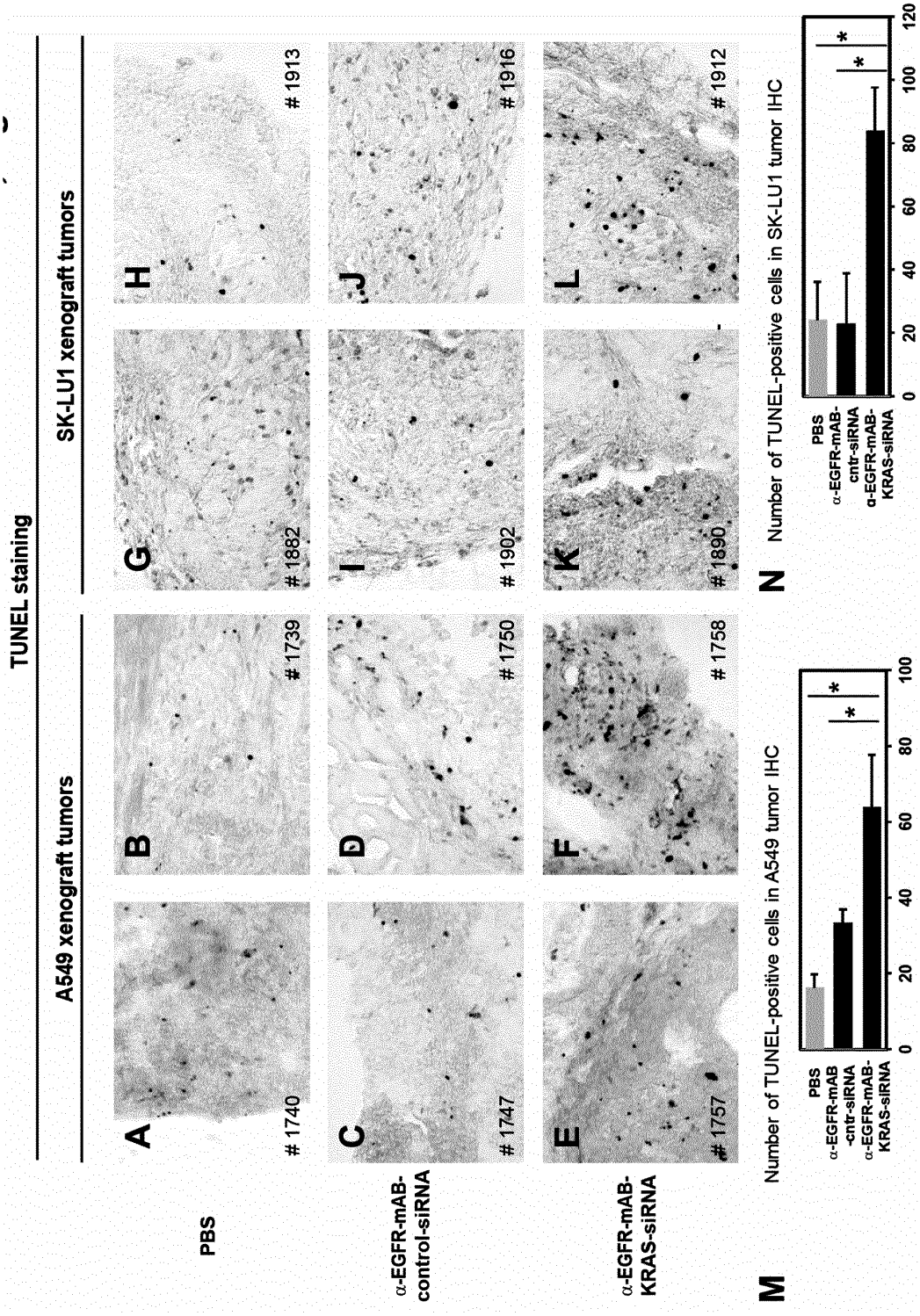

TUNEL staining
A549 xenograft tumors
SK-LU1 xenograft tumors
PBS
α-EGFR-mAB-control-siRNA
α-EGFR-mAB-KRAS-siRNA
A
1740
B
1739
G
1882
H
1913
C
1747
D
1750
I
1902
J
1916
E
1757
F
1758
K
1890
L
1912
M
Number of TUNEL-positive cells in A549 tumor IHC
PBS
α-EGFR-mAB-cntr-siRNA
α-EGFR-mAB-KRAS-siRNA
0
20
40
60
80
100
*
*
N
Number of TUNEL-positive cells in SK-LU1 tumor IHC
PBS
α-EGFR-mAB-cntr-siRNA
α-EGFR-mAB-KRAS-siRNA
0
20
40
60
80
100
120
*
*

Figure 6

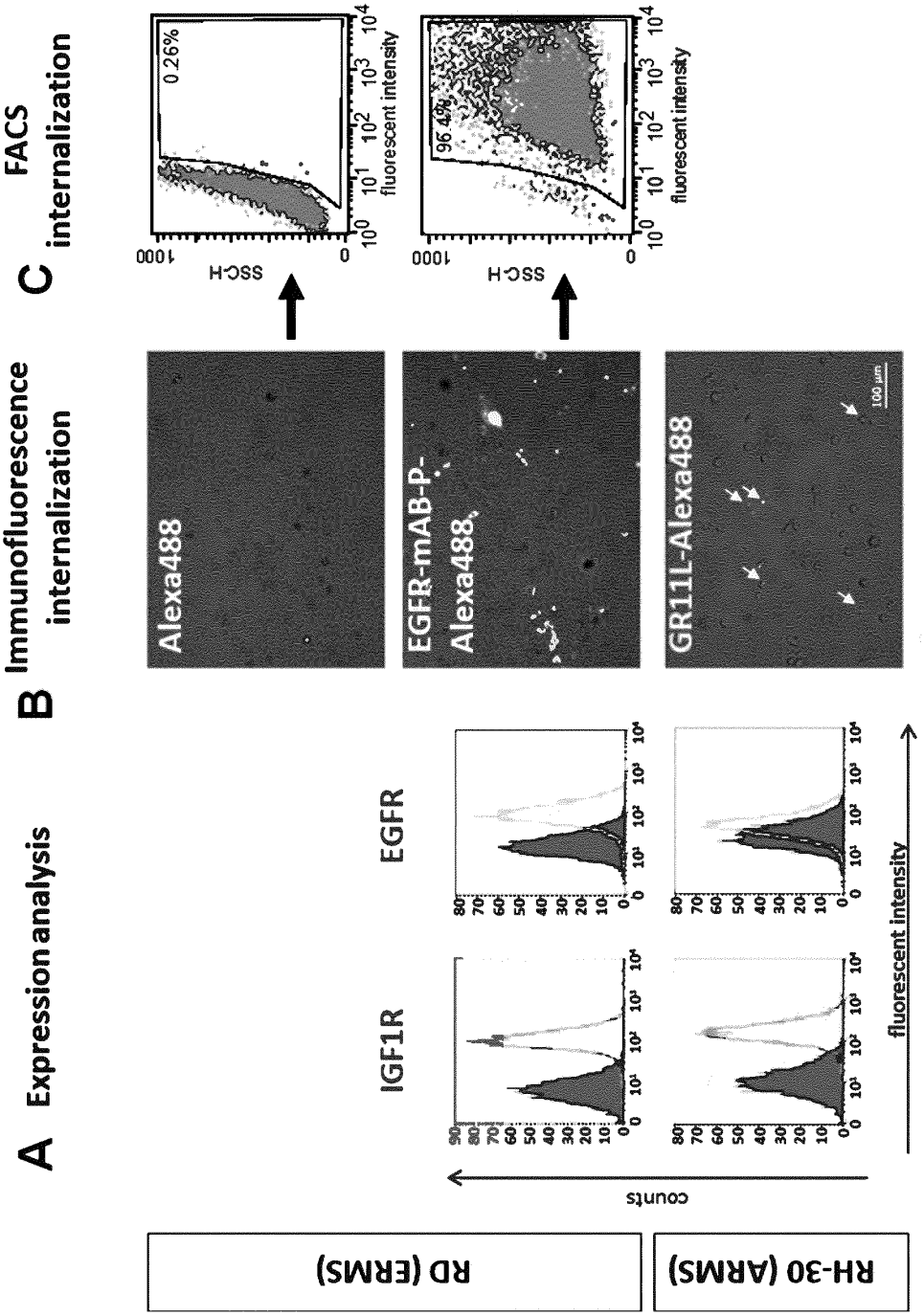

A
Expression analysis
B
Immunofluorescence internalization
C
FACS internalization
RH-30 (ARMS)
RD (ERMS)
IGF1R
EGFR
counts
fluorescent intensity
Alexa488
EGFR-mAB-P-Alexa488
GR11L-Alexa488
100 µm
SSC-H
fluorescent intensity
0.26%
96.4%

Figure 7

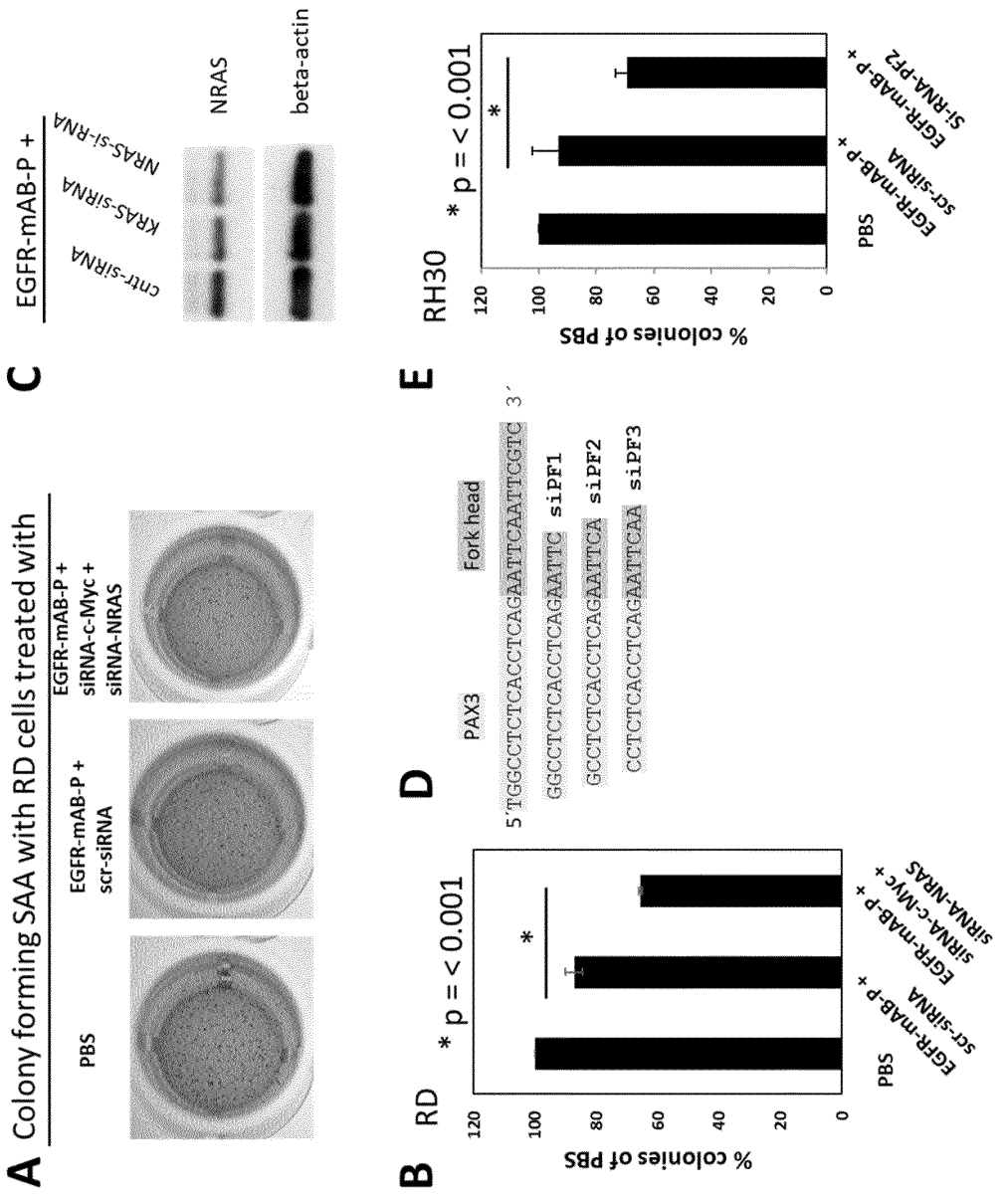

A
Colony forming SAA with RD cells treated with
PBS
EGFR-mAB-P + scr-siRNA
EGFR-mAB-P + siRNA-c-Myc + siRNA-NRAS
C
EGFR-mAB-P +
cntr-siRNA
KRAS-siRNA
NRAS-si-RNA
NRAS
beta-actin
B
RD
* p = < 0.001
% colonies of PBS
PBS
EGFR-mAB-P + scr-siRNA
EGFR-mAB-P + siRNA-c-Myc + siRNA-NRAS
D
PAX3
Fork head
5´TGGCCTCTCACCTCAGAATTCAATTCGTC 3´
GGCCTCTCACCTCAGAATTC siPF1
GCCTCTCACCTCAGAATTCA siPF2
CCTCTCACCTCAGAATTCAA siPF3
E
RH30
* p = < 0.001
% colonies of PBS
PBS
EGFR-mAB-P + scr-siRNA
EGFR-mAB-P + si-RNA-PF2

Figure 8

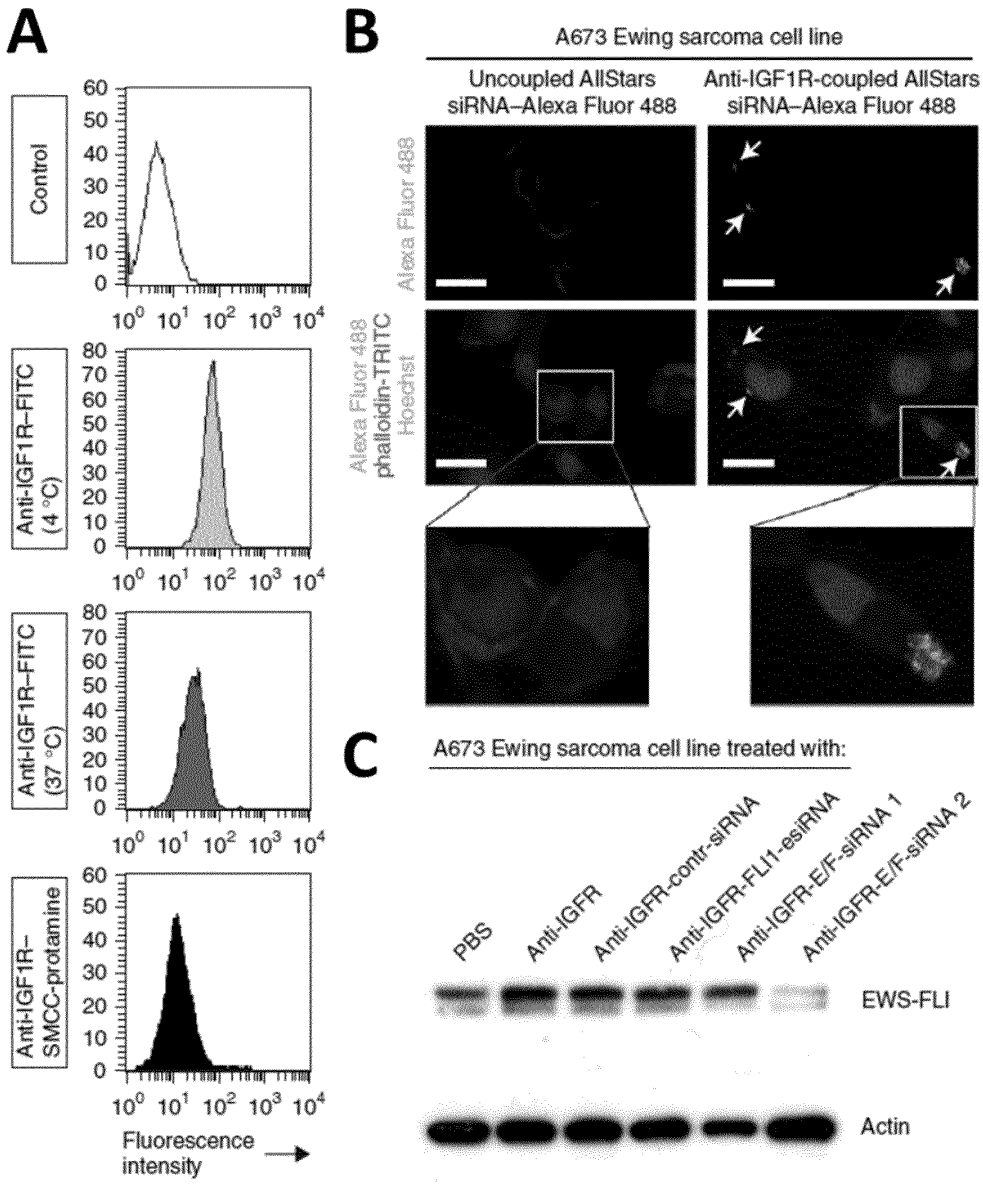

A
Control
Anti-IGF1R–FITC (4 °C)
Anti-IGF1R–FITC (37 °C)
Anti-IGF1R–SMCC-protamine
Fluorescence intensity
B
A673 Ewing sarcoma cell line
Uncoupled AllStars siRNA–Alexa Fluor 488
Anti-IGF1R-coupled AllStars siRNA–Alexa Fluor 488
Alexa Fluor 488
Alexa Fluor 488 phalloidin-TRITC Hoechst
C
A673 Ewing sarcoma cell line treated with:
PBS
Anti-IGFR
Anti-IGFR-contr-siRNA
Anti-IGFR-FLI1-esiRNA
Anti-IGFR-E/F-siRNA 1
Anti-IGFR-E/F-siRNA 2
EWS-FLI
Actin

Figure 9

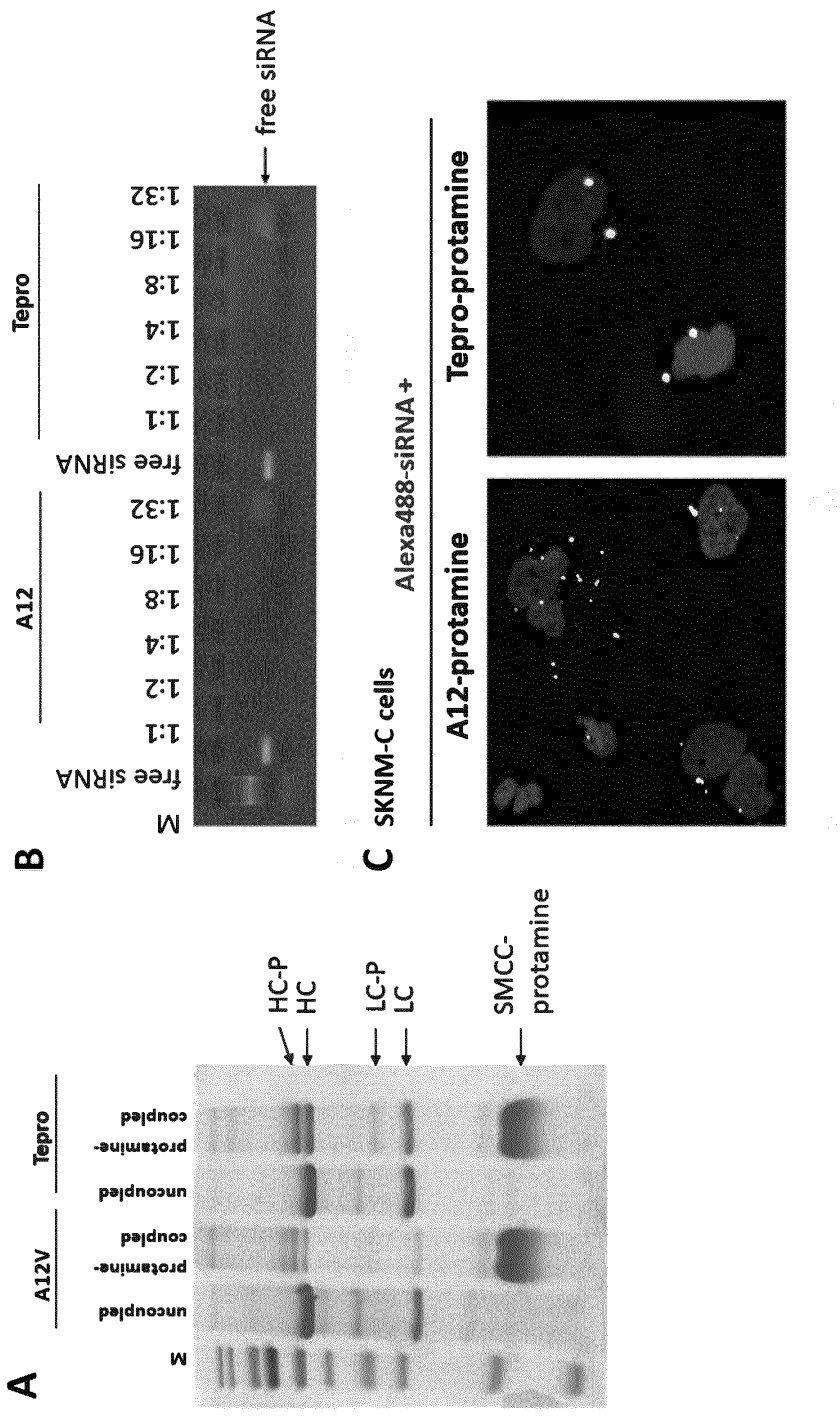

A
A12V
Tepro
M
unconjugated
protamine-coupled
unconjugated
protamine-coupled
HC-P
HC
LC-P
LC
SMCC-protamine
B
M
free siRNA
A12
1:1
1:2
1:4
1:8
1:16
1:32
free siRNA
Tepro
1:1
1:2
1:4
1:8
1:16
1:32
free siRNA
C
SKNM-C cells
Alexa488-siRNA+
A12-protamine
Tepro-protamine

Figure 10

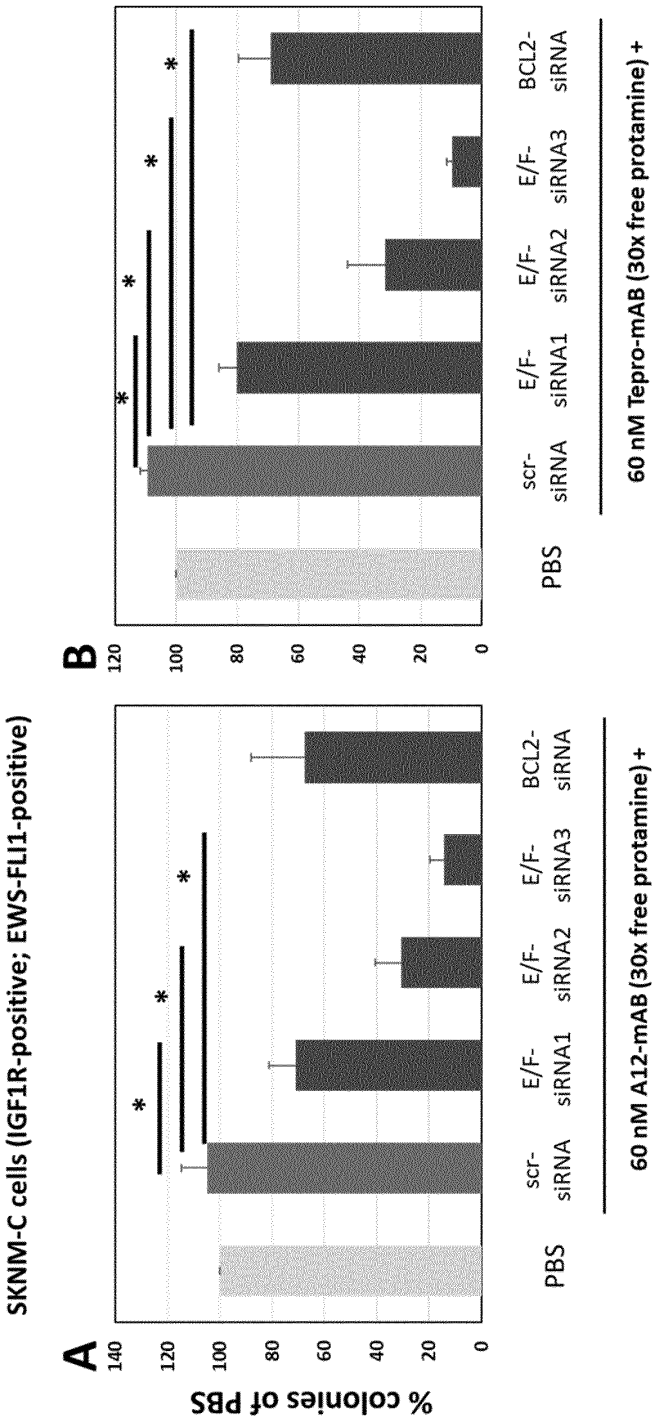

SKNM-C cells (IGF1R-positive; EWS-FLI1-positive)
A
% colonies of PBS
140
120
100
80
60
40
20
0
*
*
*
*
PBS
scr-siRNA
E/F-siRNA1
E/F-siRNA2
E/F-siRNA3
BCL2-siRNA
60 nM A12-mAB (30x free protamine) +
B
120
100
80
60
40
20
0
*
*
*
*
PBS
scr-siRNA
E/F-siRNA1
E/F-siRNA2
E/F-siRNA3
BCL2-siRNA
60 nM Tepro-mAB (30x free protamine) +

Figure 11

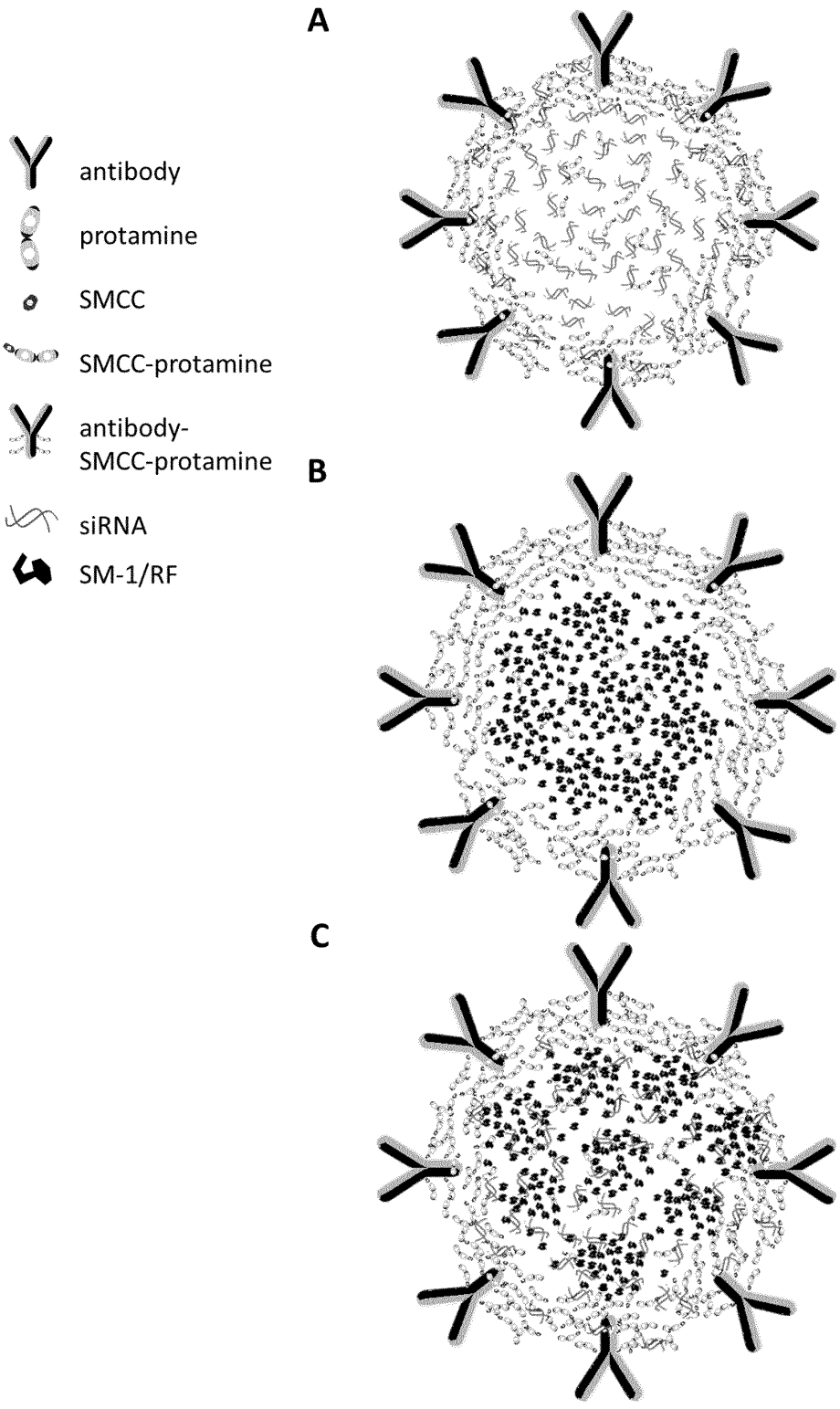

A
B
C
antibody
protamine
SMCC
SMCC-protamine
antibody-
SMCC-protamine
siRNA
SM-1/RF

Figure 12

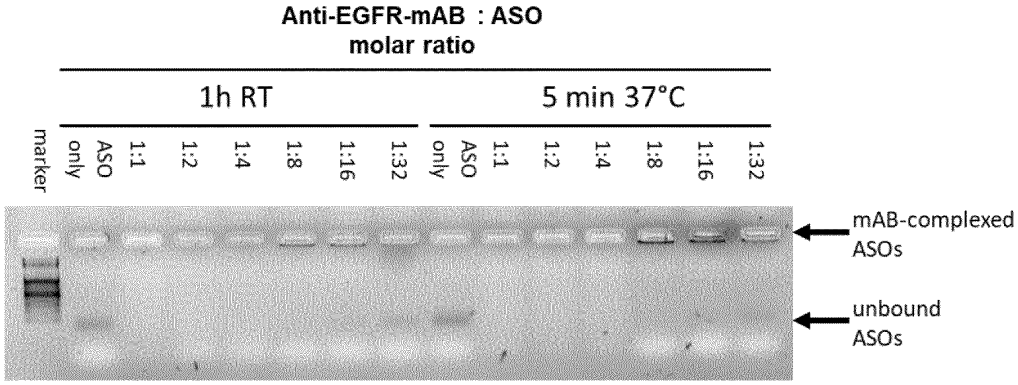

Anti-EGFR-mAB : ASO
molar ratio
1h RT
5 min 37°C
marker
ASO only
1:1
1:2
1:4
1:8
1:16
1:32
ASO only
1:1
1:2
1:4
1:8
1:16
1:32
mAB-complexed ASOs
unbound ASOs

Figure 13

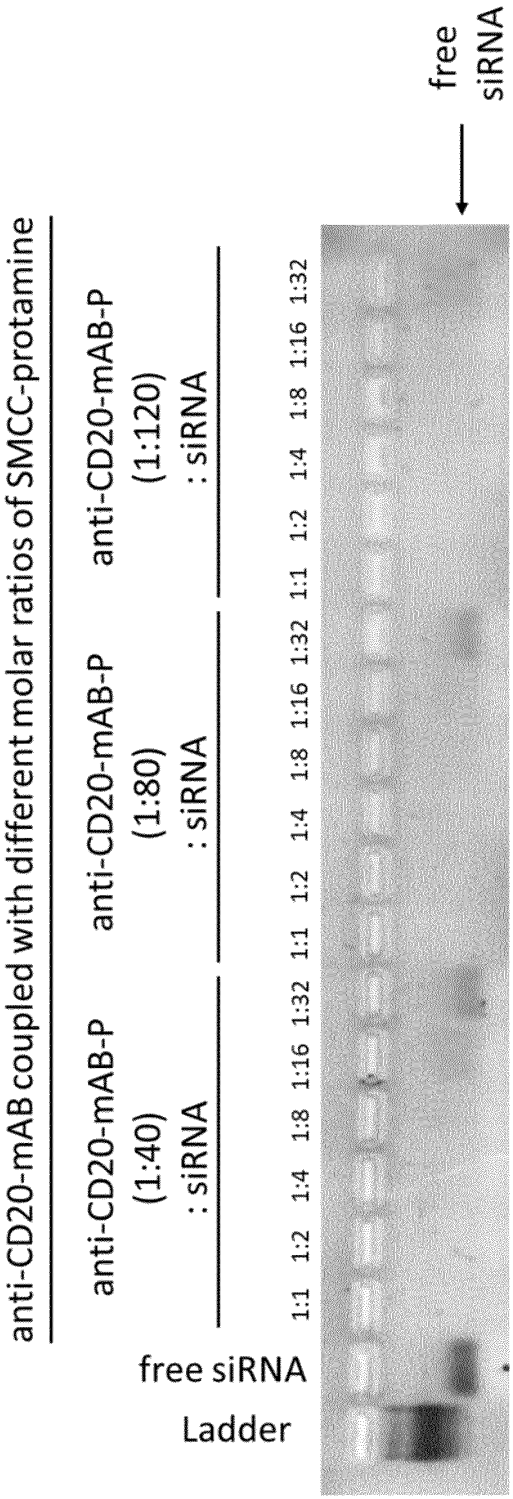

free siRNA
anti-CD20-mAB coupled with different molar ratios of SMCC-protamine
anti-CD20-mAB-P (1:40) : siRNA
anti-CD20-mAB-P (1:80) : siRNA
anti-CD20-mAB-P (1:120) : siRNA
1:1 1:2 1:4 1:8 1:16 1:32 1:1 1:2 1:4 1:8 1:16 1:32 1:1 1:2 1:4 1:8 1:16 1:32
free siRNA
Ladder

Figure 14

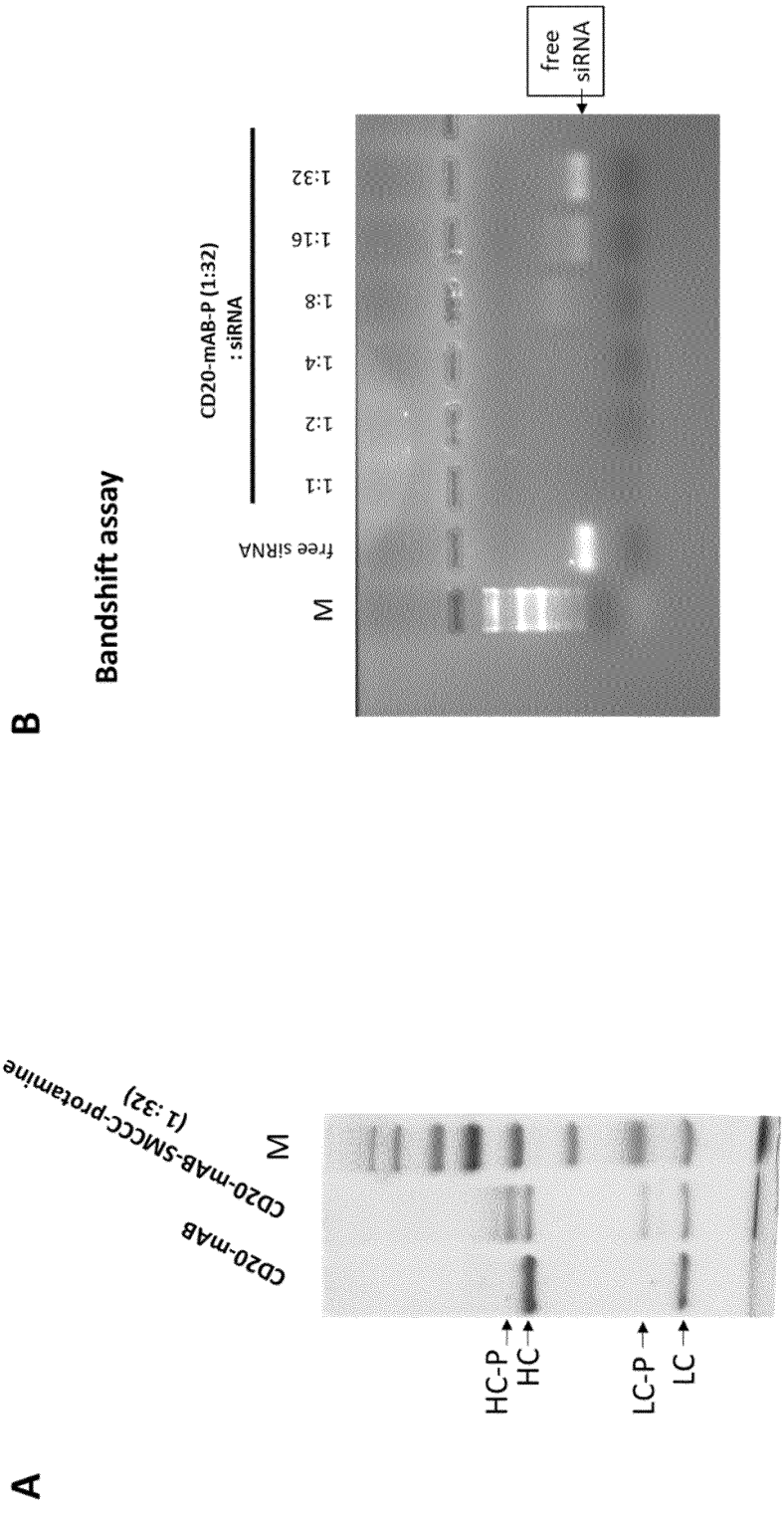

A
M
CD20-mAB-SMCCC-proteamine (1 :32)
CD20-mAB
HC-P
HC
LC-P
LC
B
Bandshift assay
M
free siRNA
CD20-mAB-P (1:32) : siRNA
1:1
1:2
1:4
1:8
1:16
1:32
free siRNA

Figure 15

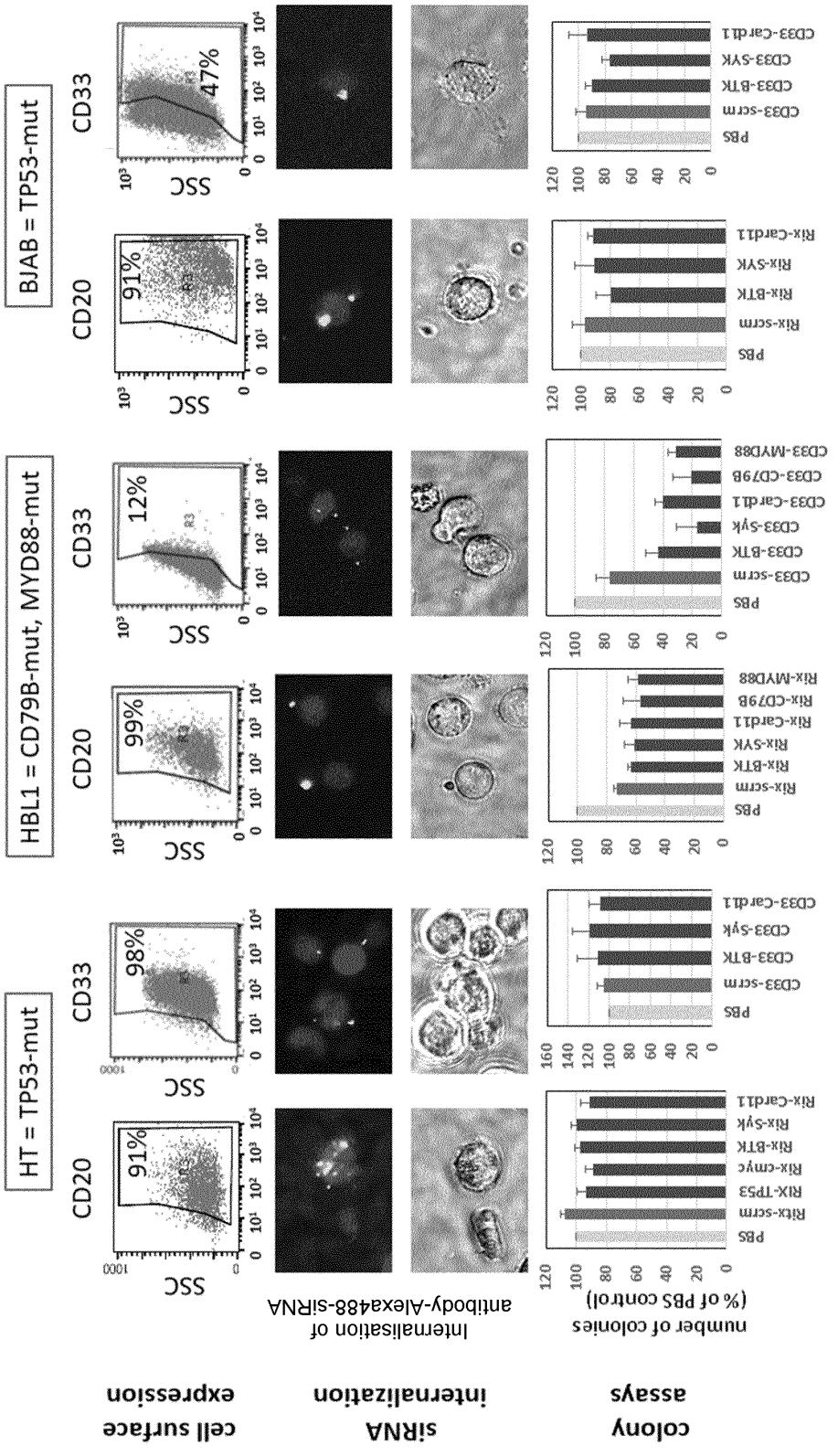

cell surface expression
siRNA internalization
colony assays
HT = TP53-mut
HBL1 = CD79B-mut, MYD88-mut
BJAB = TP53-mut
CD20
CD33
SSC
91%
98%
99%
12%
47%
Internalisation of antibody-Alexa488-siRNA
number of colonies (% of PBS control)
PBS
Rtx-scrm
RIX-TP53
Rix-cmyc
Rix-BTK
Rix-Syk
Rix-Card11
Rix-scrm
Rix-BTK
Rix-SYK
Rix-Card11
Rix-CD79B
Rix-MYD88
CD33-scrm
CD33-BTK
CD33-SYK
CD33-Card11
CD33-CD79B
CD33-MYD88

Figure 16

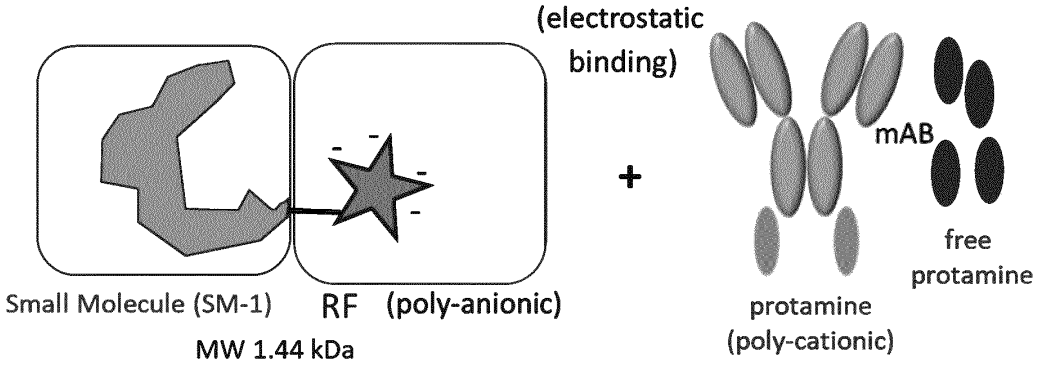


Figure 17

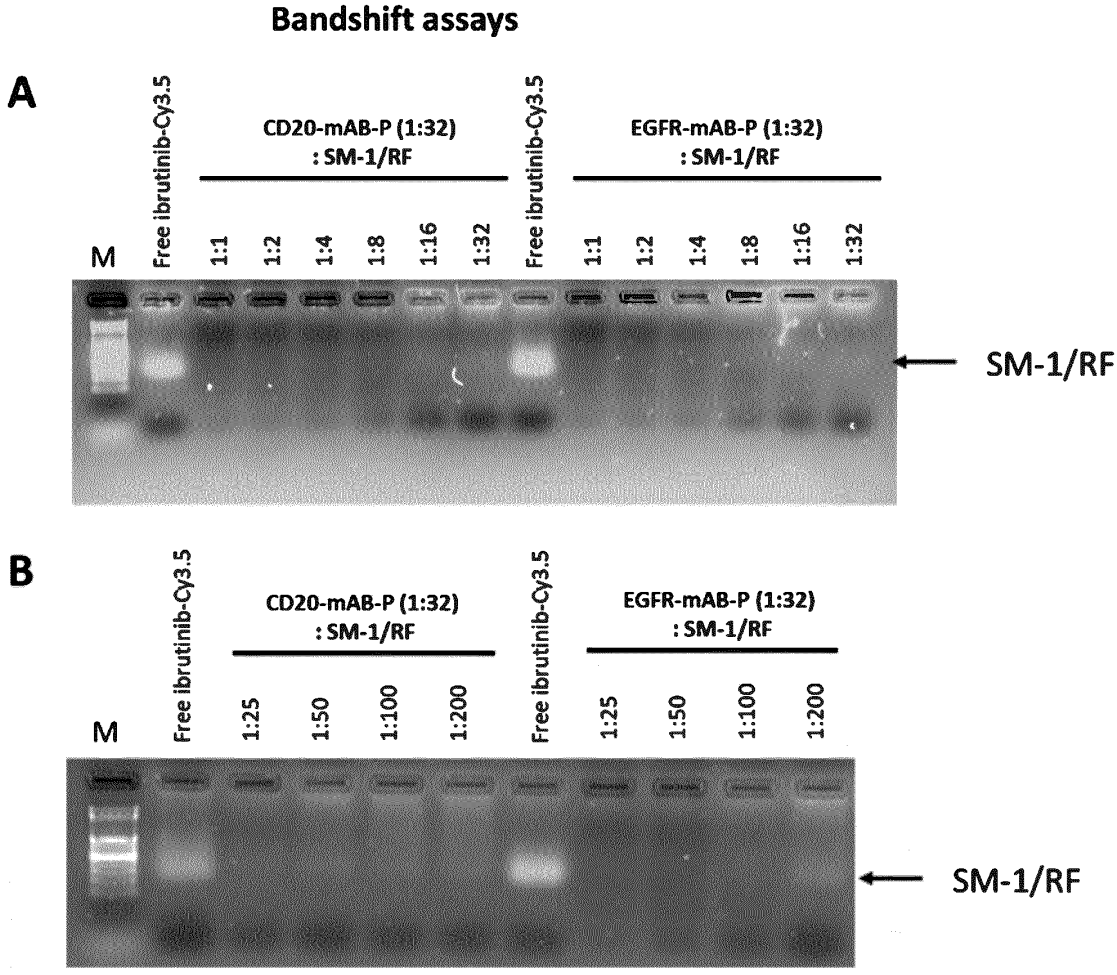


Figure 18

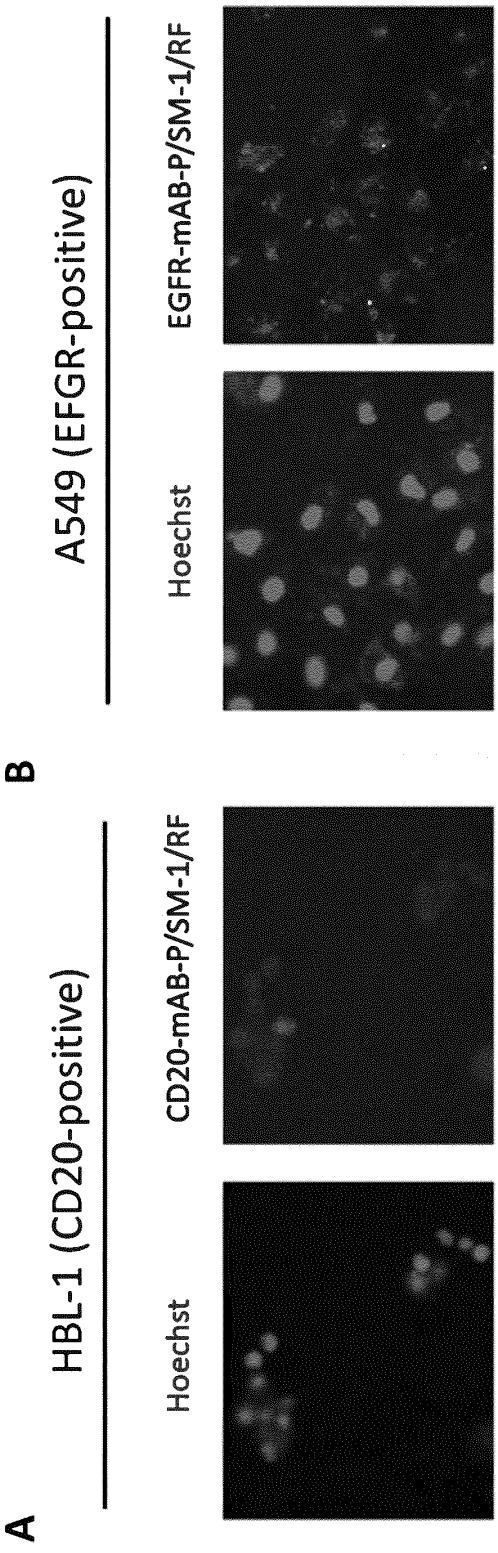

A
HBL-1 (CD20-positive)
Hoechst
CD20-mAB-P/SM-1/RF
B
A549 (EFGR-positive)
Hoechst
EGFR-mAB-P/SM-1/RF

Figure 19

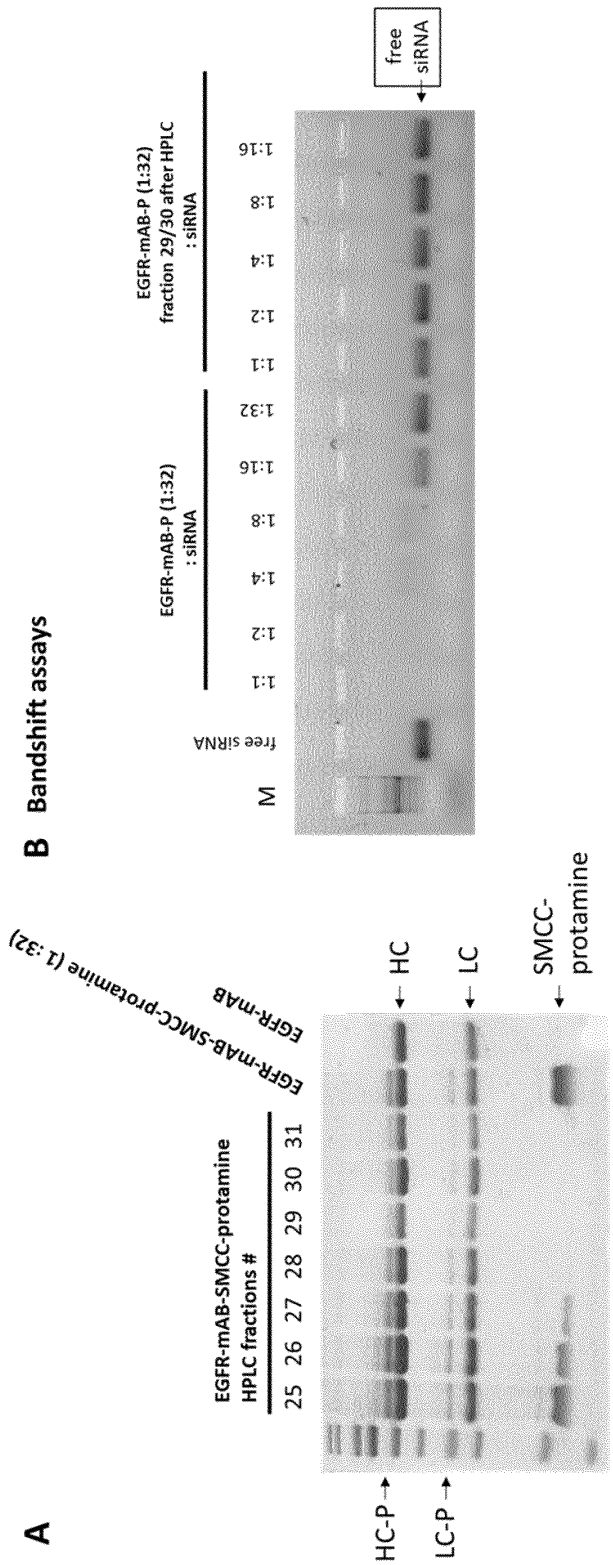

A
HC-P
LC-P
HC
LC
SMCC-protamine
EGFR-mAB-SMCC-protamine (1 :32)
EGFR-mAB
EGFR-mAB-SMCC-protamine HPLC fractions #
25 26 27 28 29 30 31
B Bandshift assays
M
free siRNA
1:1 1:2 1:4 1:8 1:16 1:32
EGFR-mAB-P (1:32) : siRNA
1:1 1:2 1:4 1:8 1:16
EGFR-mAB-P (1:32) fraction 29/30 after HPLC : siRNA
free siRNA

Figure 20

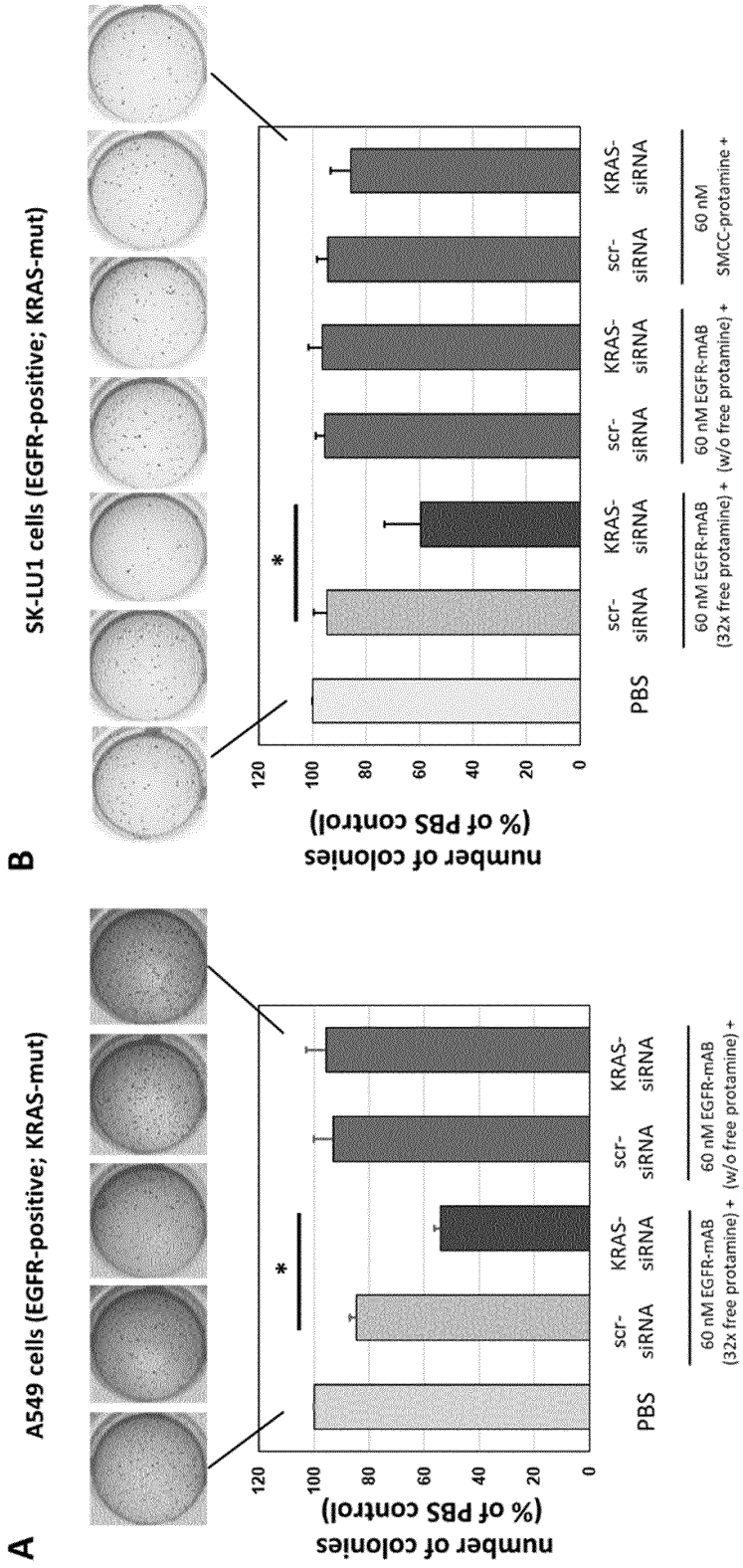

A
A549 cells (EGFR-positive; KRAS-mut)
number of colonies
(% of PBS control)
120
100
80
60
40
20
0
*
PBS
scr-siRNA
KRAS-siRNA
scr-siRNA
KRAS-siRNA
60 nM EGFR-mAB (32x free protamine) +
60 nM EGFR-mAB (w/o free protamine) +
B
SK-LU1 cells (EGFR-positive; KRAS-mut)
number of colonies
(% of PBS control)
120
100
80
60
40
20
0
*
PBS
scr-siRNA
KRAS-siRNA
scr-siRNA
KRAS-siRNA
scr-siRNA
KRAS-siRNA
60 nM EGFR-mAB (32x free protamine) +
60 nM EGFR-mAB (w/o free protamine) +
60 nM SMCC-protamine +

Figure 21

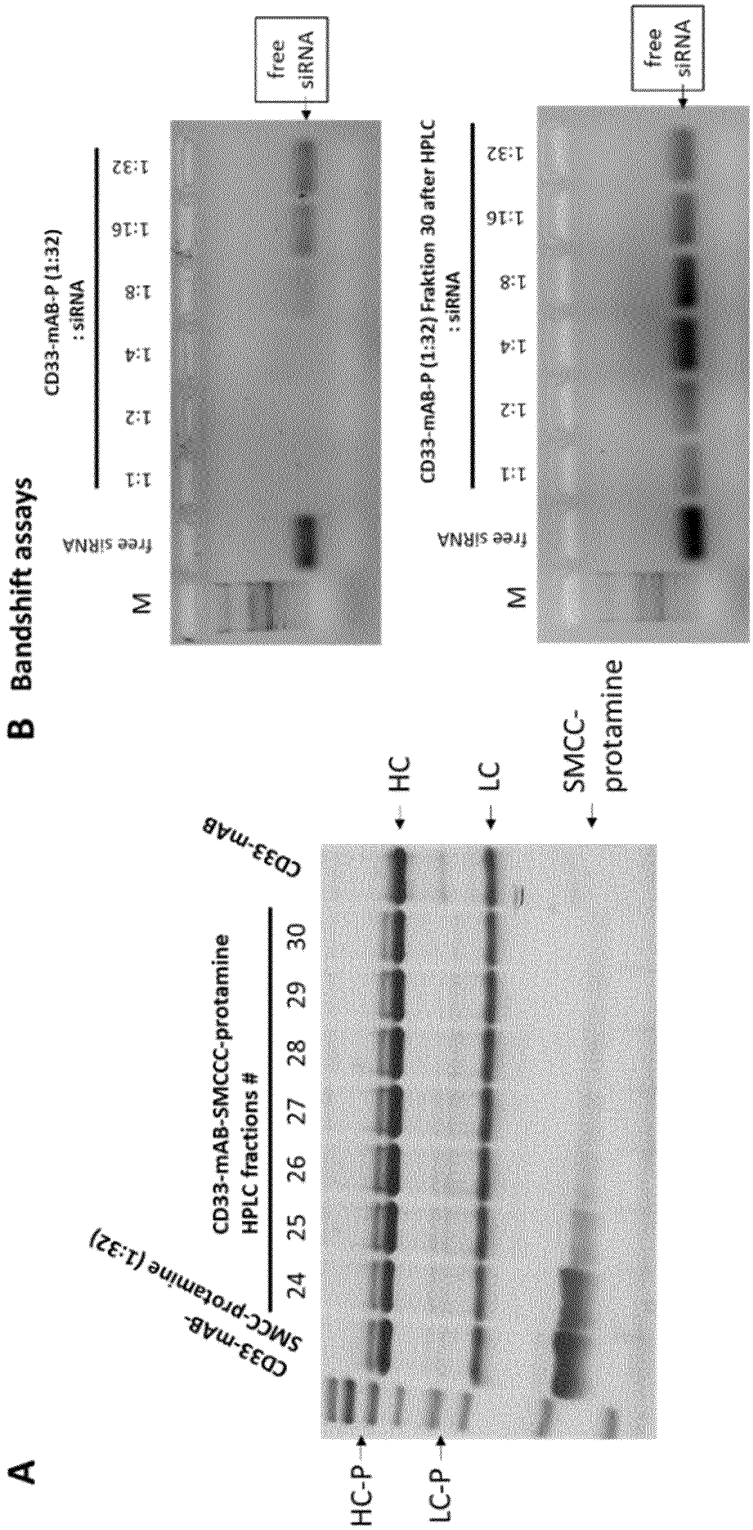

A
HC-P
LC-P
CD33-mAB-SMCC-protamine (1:32)
CD33-mAB-SMCCC-protamine HPLC fractions #
24 25 26 27 28 29 30
CD33-mAB
HC
LC
SMCC-protamine
B Bandshift assays
M
free siRNA
CD33-mAB-P (1:32) : siRNA
1:1 1:2 1:4 1:8 1:16 1:32
free siRNA
CD33-mAB-P (1:32) Fraktion 30 after HPLC : siRNA
1:1 1:2 1:4 1:8 1:16 1:32
free siRNA

Figure 21 (cont'd)

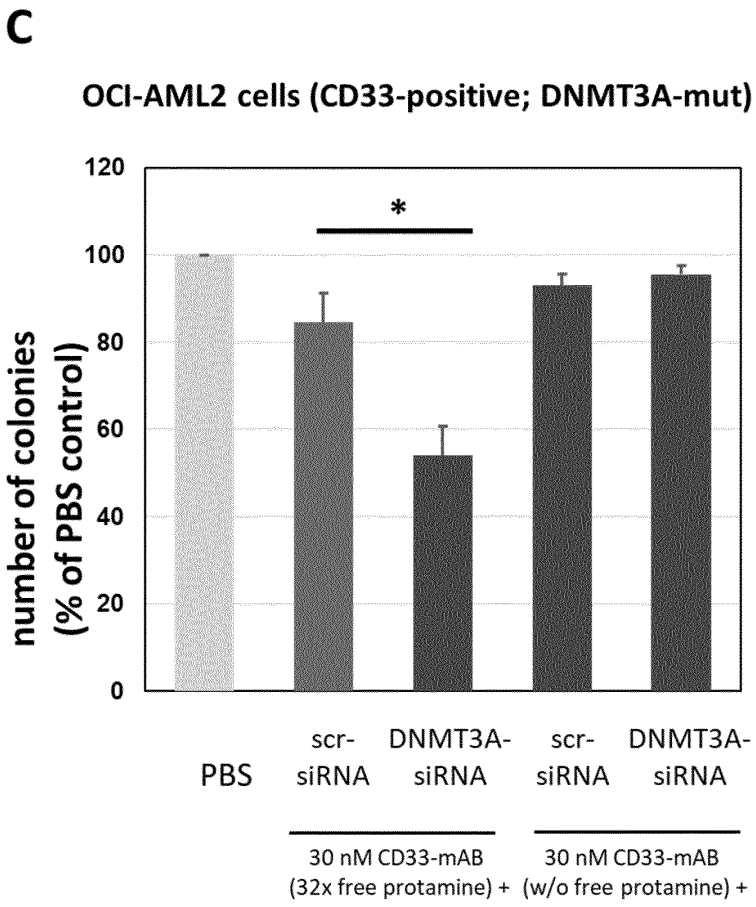


Figure 22

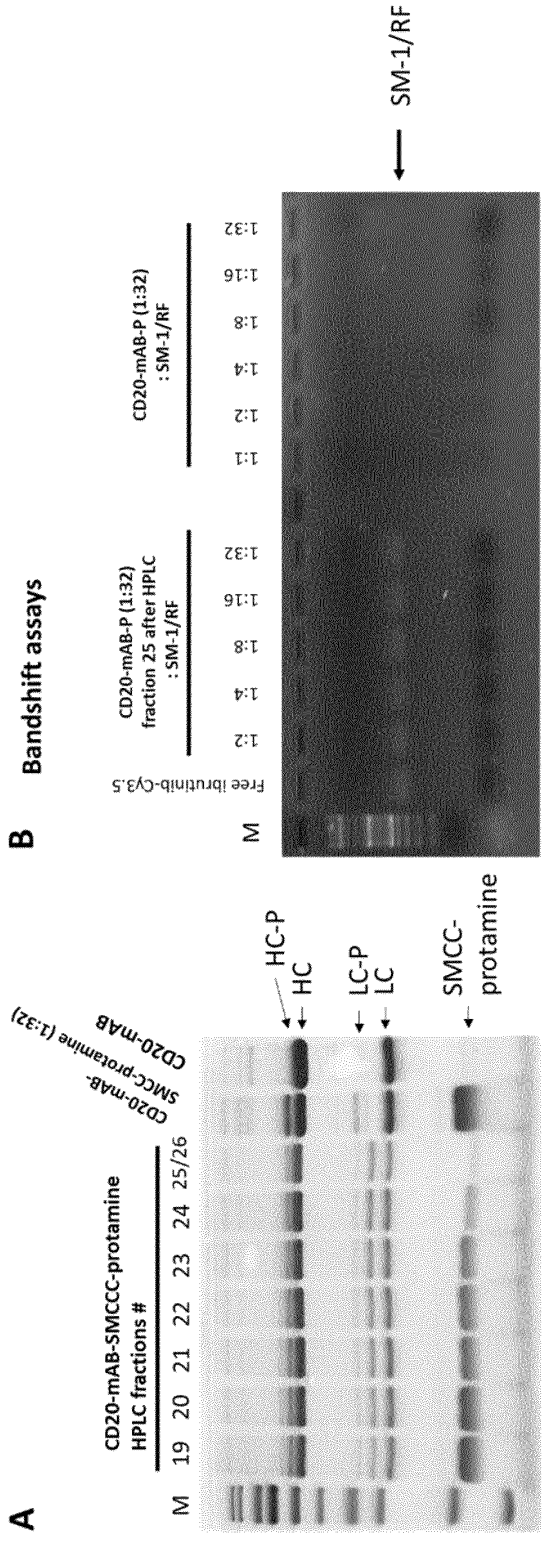

A
M
19
20
21
22
23
24
25/26
CD20-mAB-5MCCC-protamine
HPLC fractions #
CD20-mAB-SMCC-protamine (1:32)
CD20-mAB
HC-P
HC
LC-P
LC
SMCC-protamine
B
Bandshift assays
M
Free Ibrutinib-Cy3.5
1:2
1:4
1:8
1:16
1:32
CD20-mAB-P (1:32) fraction 25 after HPLC : SM-1/RF
1:1
1:2
1:4
1:8
1:16
1:32
CD20-mAB-P (1:32) : SM-1/RF
SM-1/RF

Figure 23

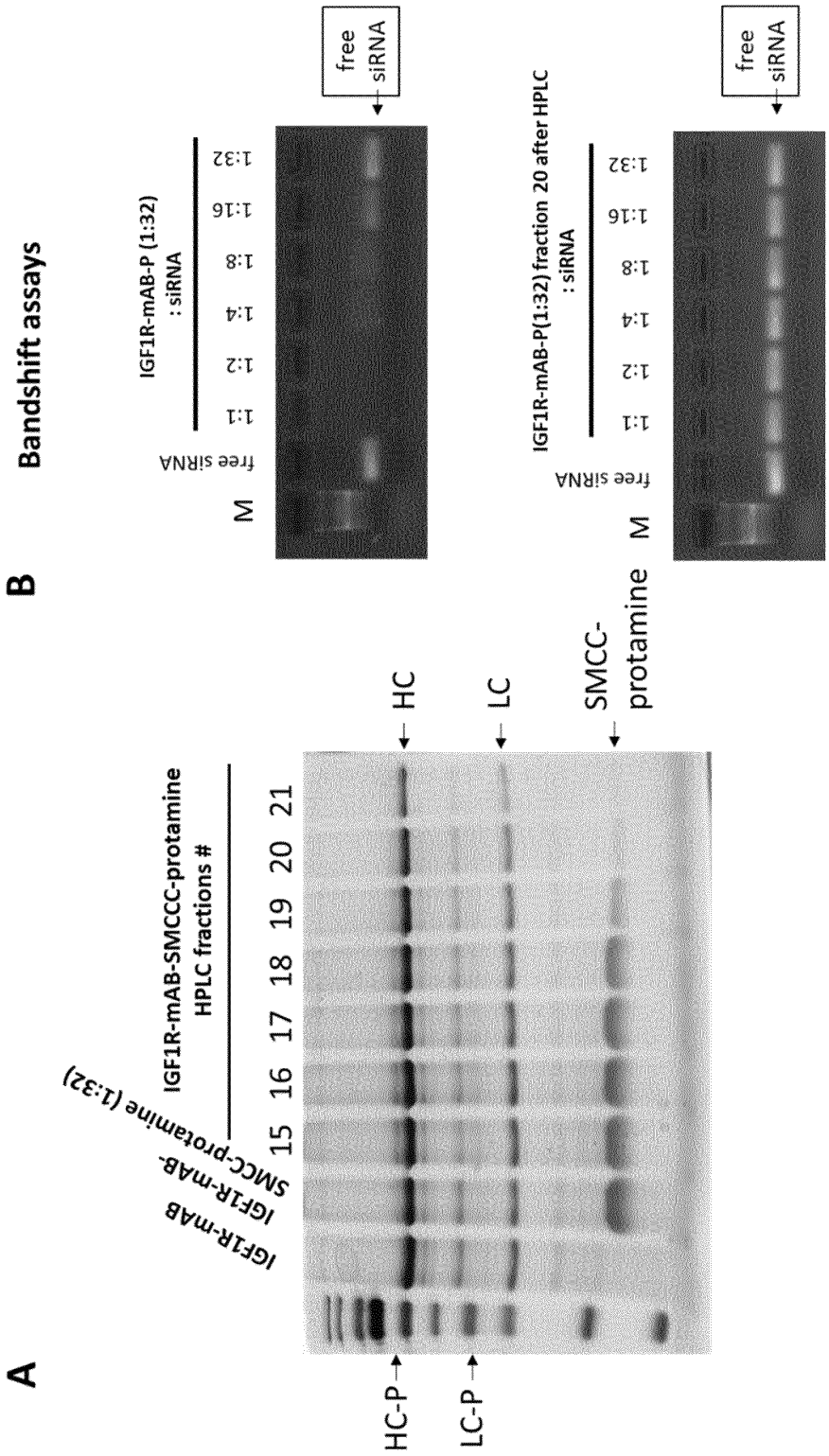

A
IGF1R-mAB
IGF1R-mAB-SMCC-protamine (1:32)
IGF1R-mAB-SMCC-protamine HPLC fractions #
15 16 17 18 19 20 21
HC
LC
SMCC-protamine
HC-P
LC-P
B
Bandshift assays
IGF1R-mAB-P (1:32) : siRNA
M
free siRNA
1:1
1:2
1:4
1:8
1:16
1:32
free siRNA
IGF1R-mAB-P(1:32) fraction 20 after HPLC : siRNA
M
free siRNA
1:1
1:2
1:4
1:8
1:16
1:32
free siRNA

Figure 24

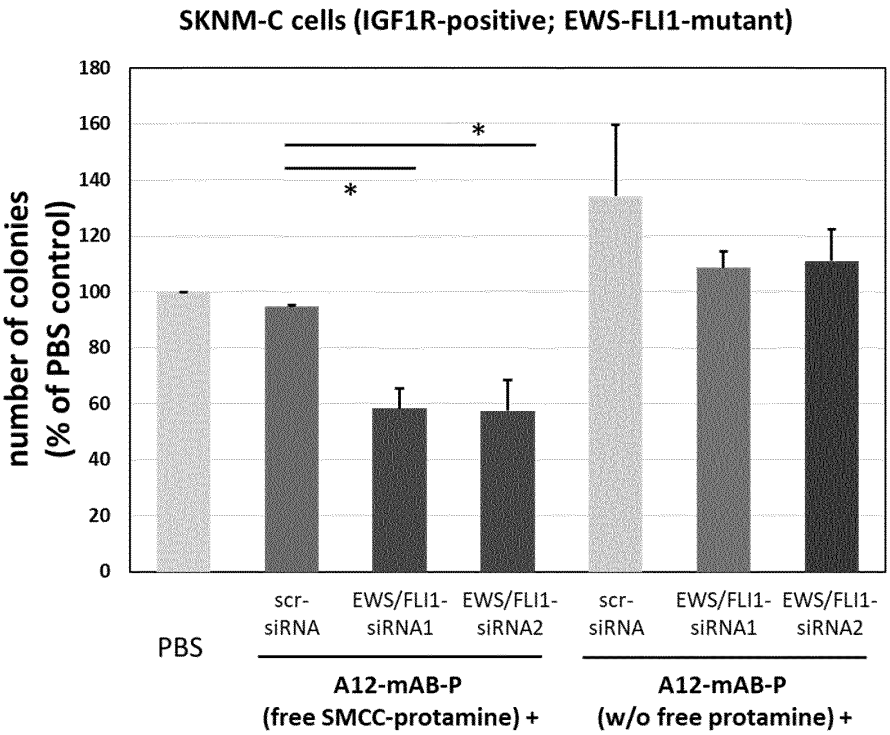


Figure 25

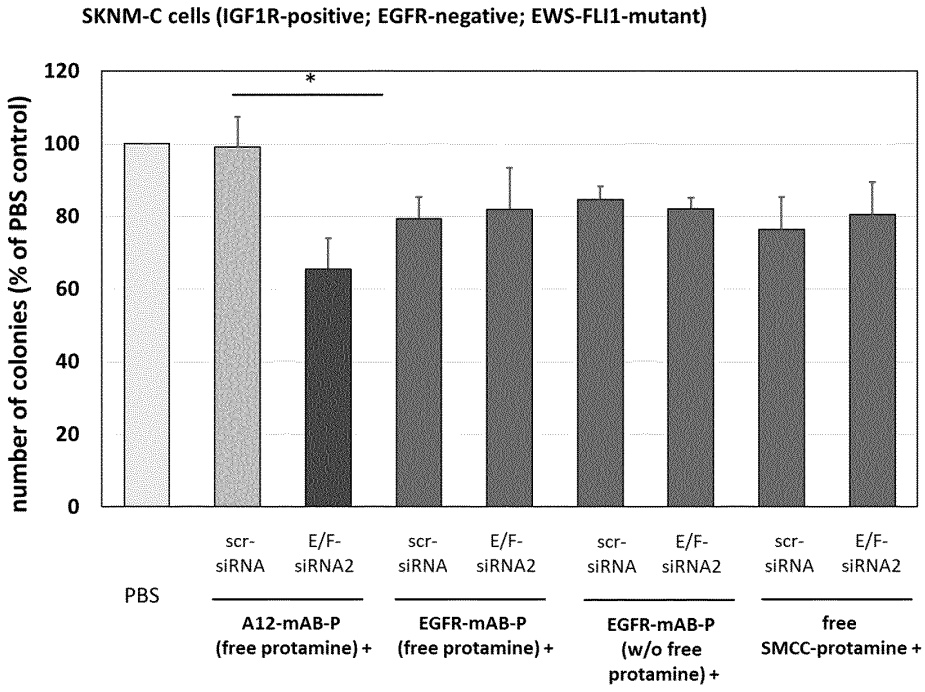


Figure 26

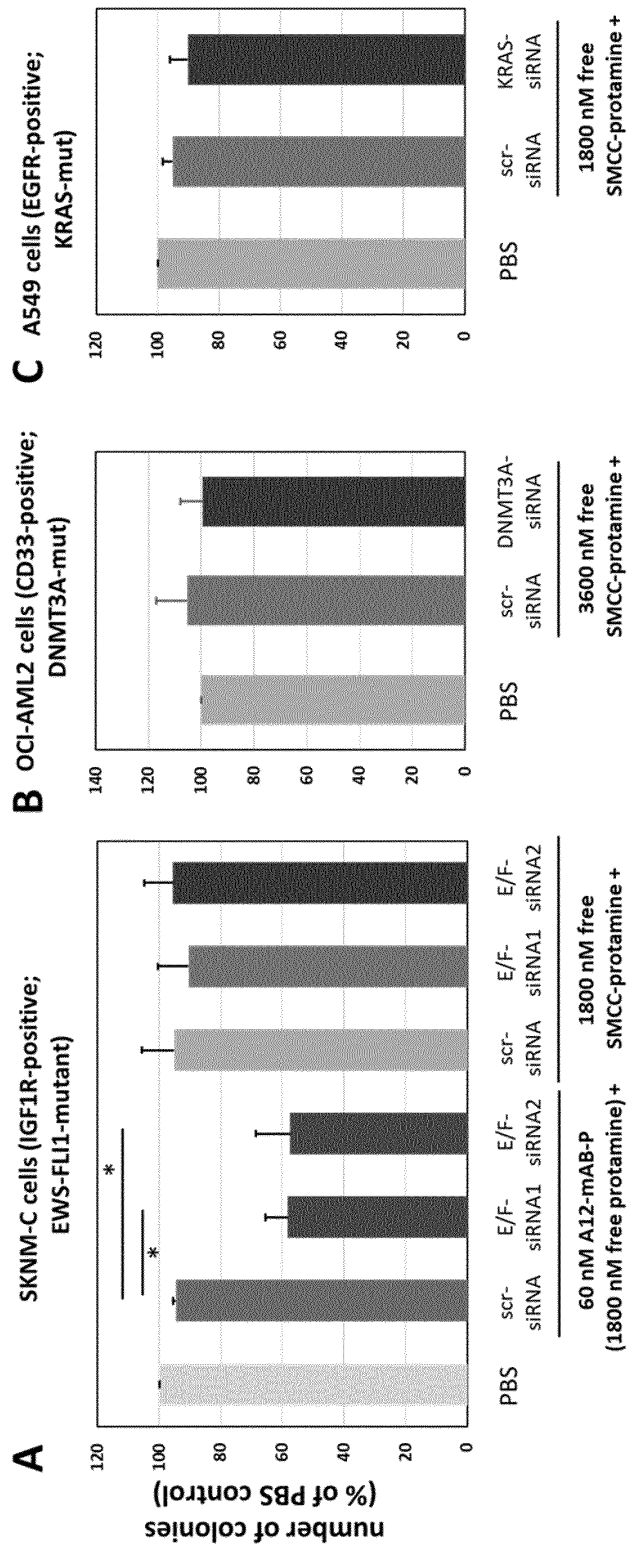

A
SKNM-C cells (IGF1R-positive; EWS-FLI1-mutant)
number of colonies (% of PBS control)
120
100
80
60
40
20
0
*
*
PBS
scr-siRNA
E/F-siRNA1
E/F-siRNA2
scr-siRNA
E/F-siRNA1
E/F-siRNA2
60 nM A12-mAB-P (1800 nM free protamine) +
1800 nM free SMCC-protamine +
B
OCI-AML2 cells (CD33-positive; DNMT3A-mut)
140
120
100
80
60
40
20
0
PBS
scr-siRNA
DNMT3A-siRNA
3600 nM free SMCC-protamine +
C
A549 cells (EGFR-positive; KRAS-mut)
120
100
80
60
40
20
0
PBS
scr-siRNA
KRAS-siRNA
1800 nM free SMCC-protamine +

Figure 27

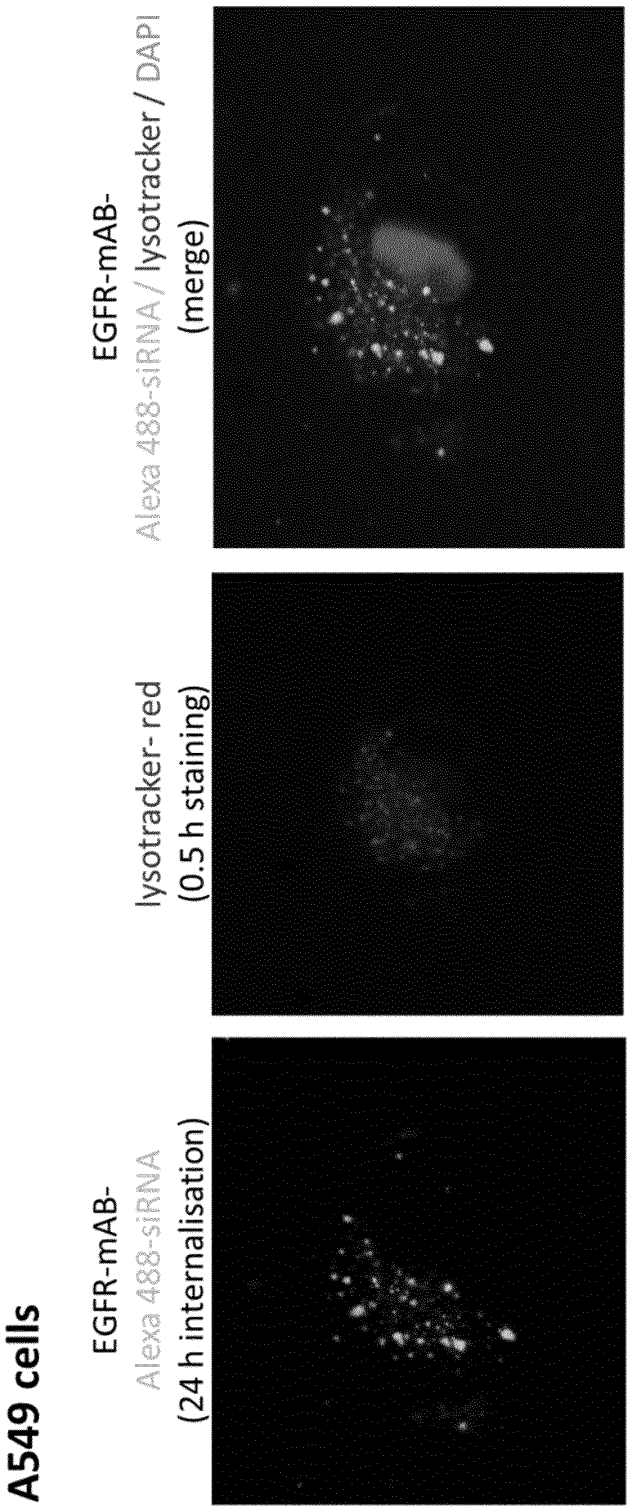

A549 cells
EGFR-mAB-
Alexa 488-siRNA
(24 h internalisation)
lysotracker- red
(0.5 h staining)
EGFR-mAB-
Alexa 488-siRNA / lysotracker / DAPI
(merge)

Figure 28

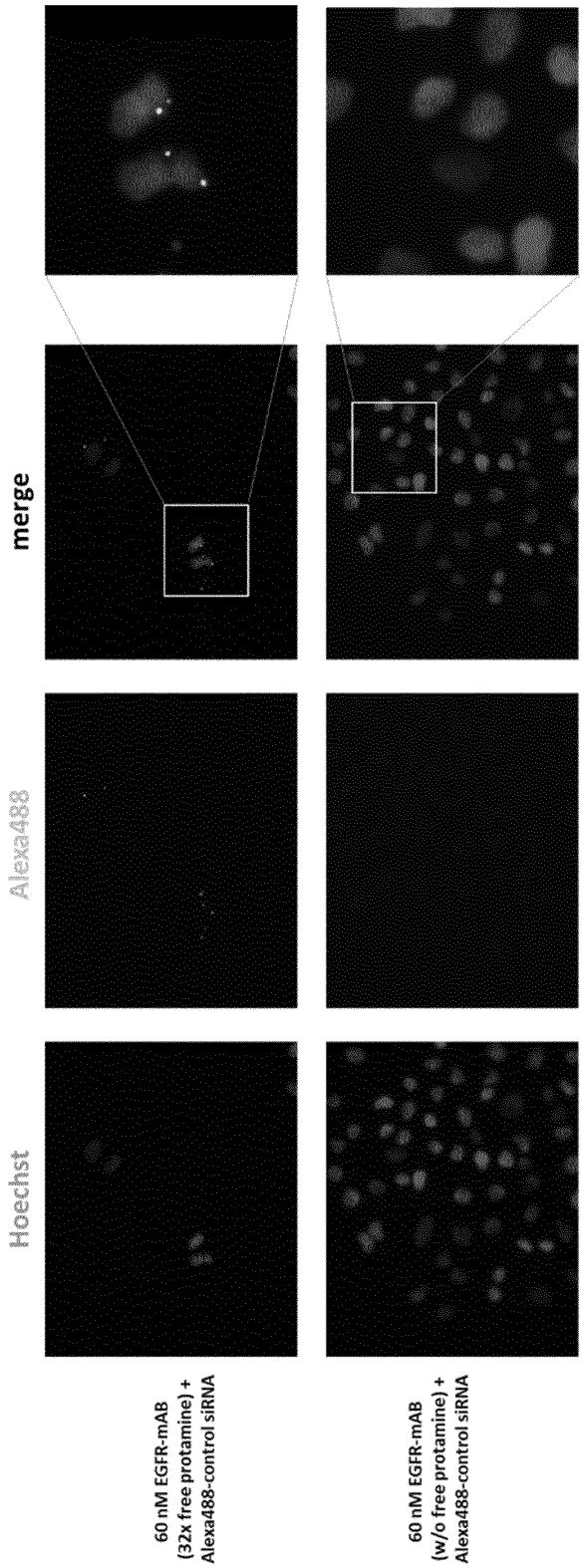

merge
Alexa488
Hoechst
60 nM EGFR-mAB (32x free protamine) + Alexa488-control siRNA
60 nM EGFR-mAB (w/o free protamine) + Alexa488-control siRNA

Figure 29

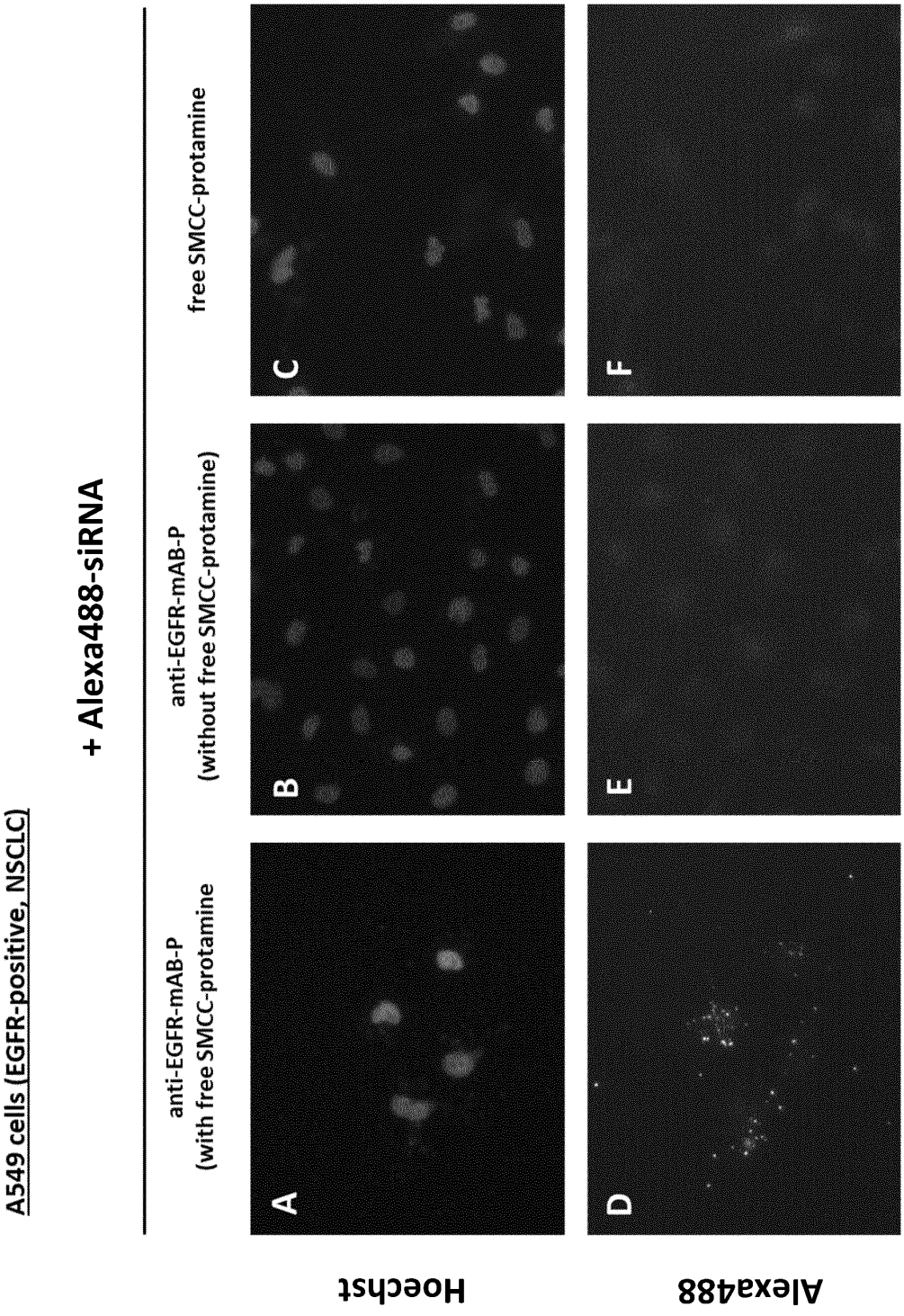

A549 cells (EGFR-positive, NSCLC)
+ Alexa488-siRNA
anti-EGFR-mAB-P (with free SMCC-protamine
anti-EGFR-mAB-P (without free SMCC-protamine)
free SMCC-protamine
A
B
C
D
E
F
Hoechst
Alexa488

Figure 30

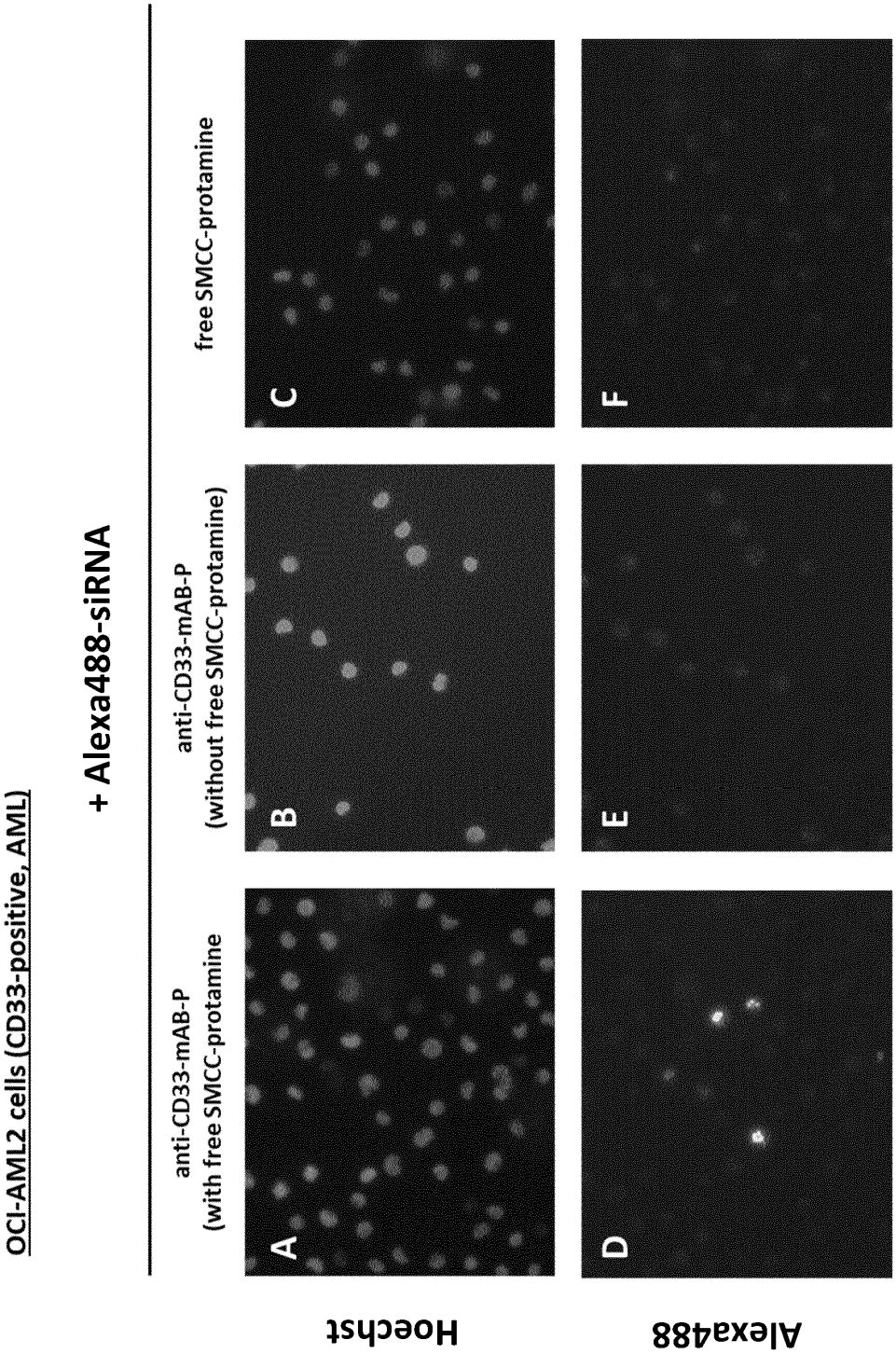

OCI-AML2 cells (CD33-positive, AML)
+ Alexa488-siRNA
anti-CD33-mAB-P (with free SMCC-protamine
anti-CD33-mAB-P (without free SMCC-protamine)
free SMCC-protamine
A
B
C
D
E
F
Hoechst
Alexa488

Figure 31

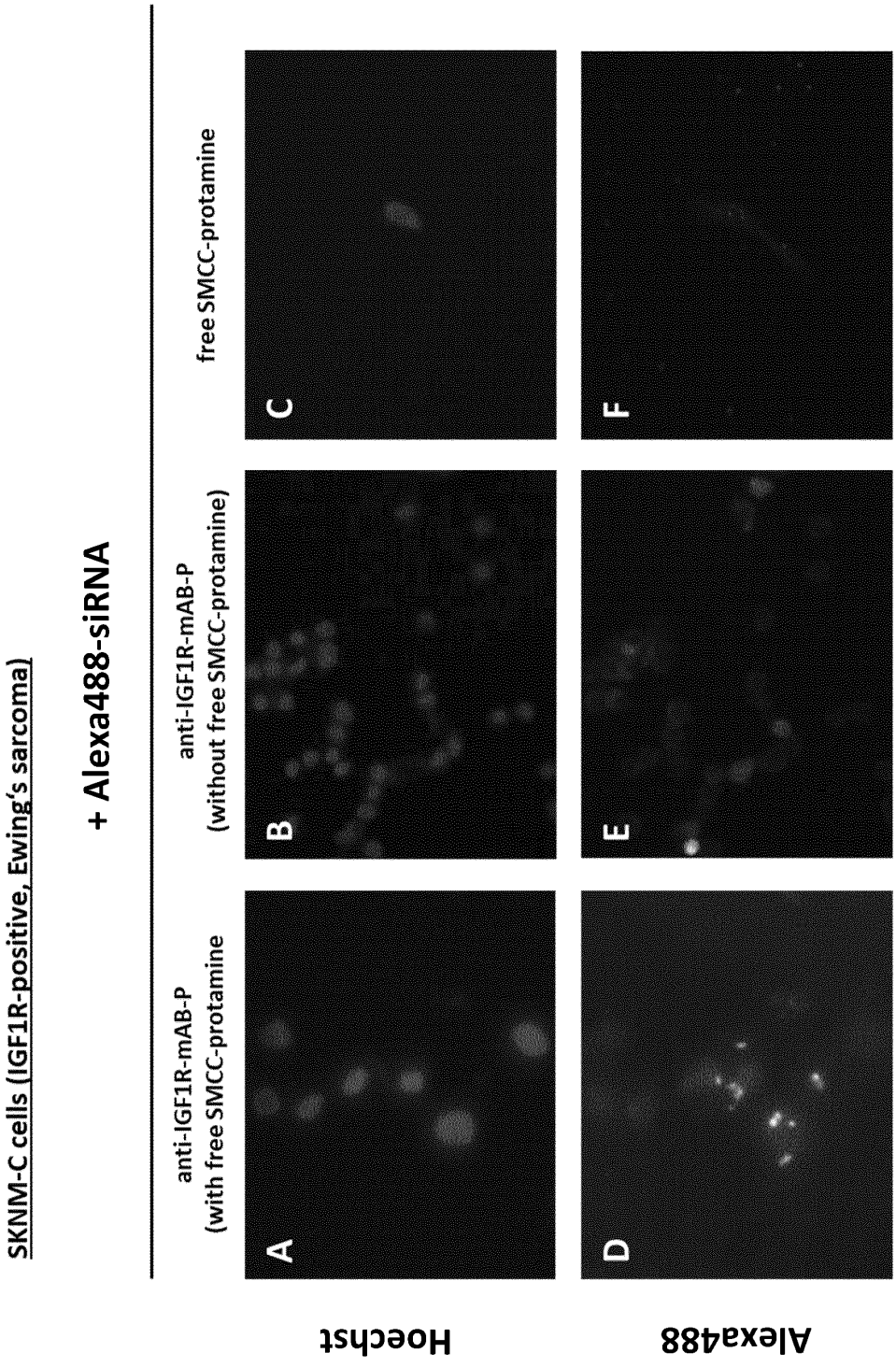

SKNM-C cells (IGF1R-positive, Ewing's sarcoma)
+ Alexa488-siRNA
anti-IGF1R-mAB-P (with free SMCC-protamine
anti-IGF1R-mAB-P (without free SMCC-protamine)
free SMCC-protamine
A
B
C
D
E
F
Hoechst
Alexa488

Figure 32

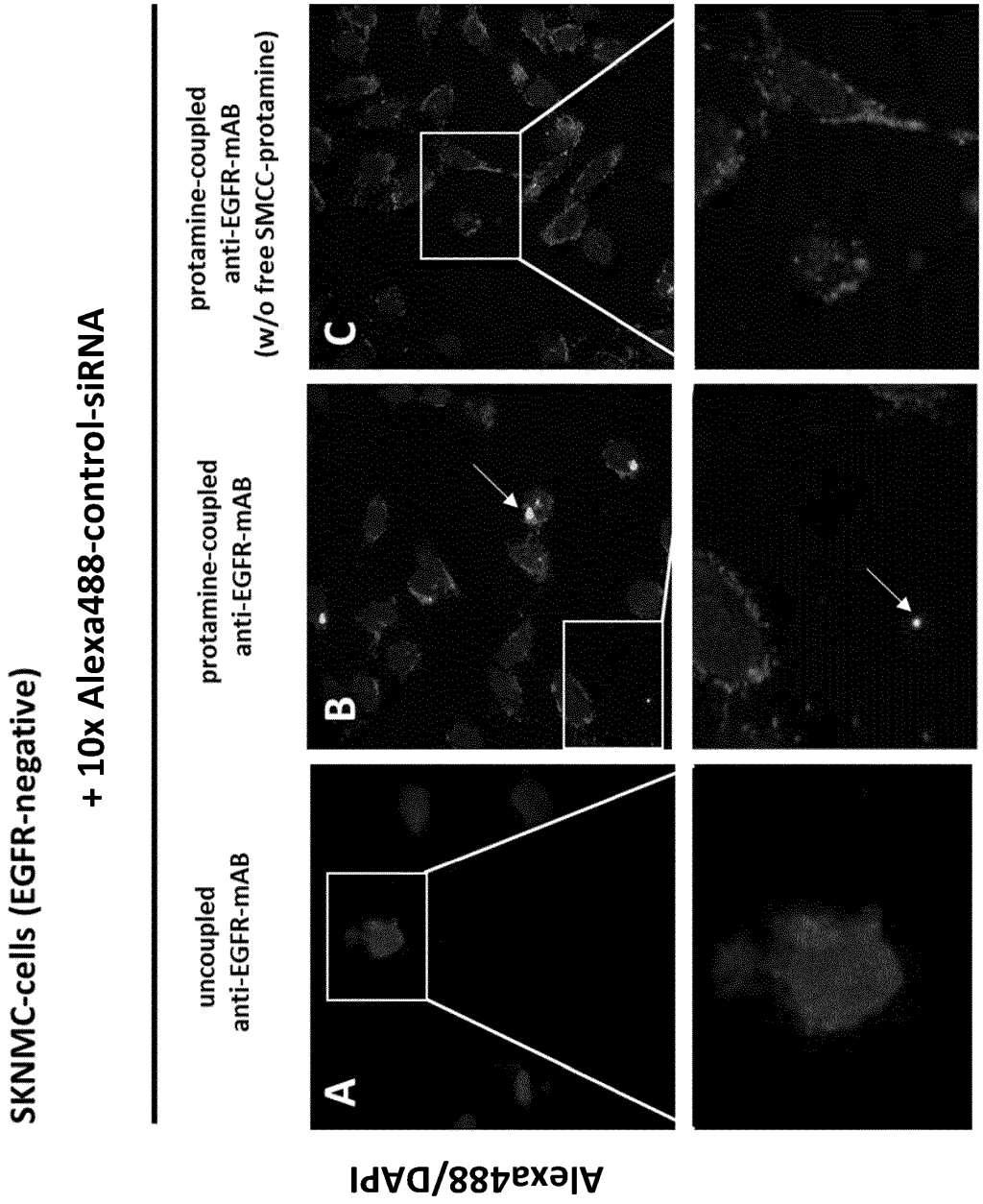

SKNMC-cells (EGFR-negative)
+ 10x Alexa488-control-siRNA
uncoupled anti-EGFR-mAB
protamine-coupled anti-EGFR-mAB
protamine-coupled anti-EGFR-mAB (w/o free SMCC-protamine)
A
B
C
Alexa488/DAPI

Figure 33

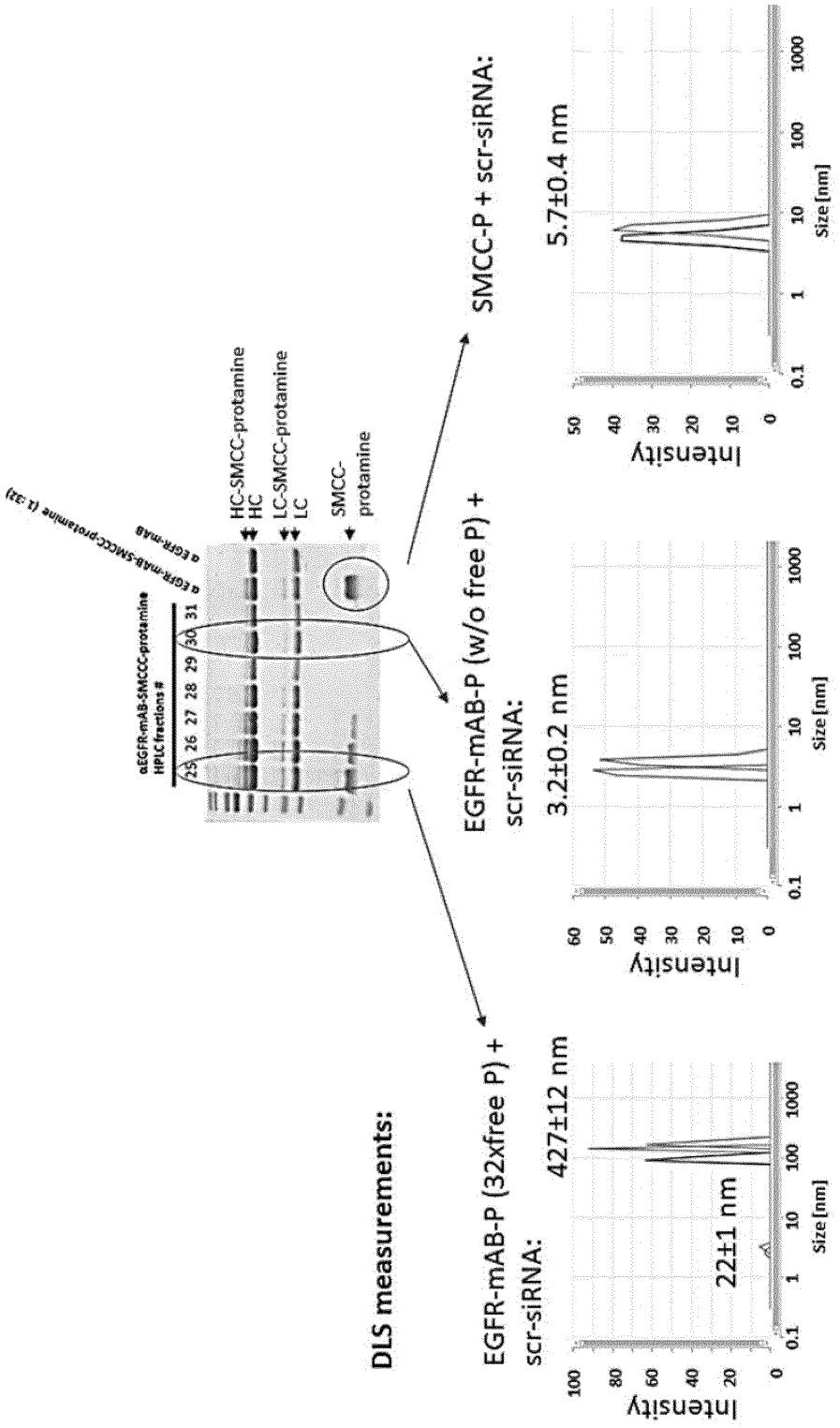

DLS measurements:
EGFR-mAB-P (32xfree P) + scr-siRNA:
427±12 nm
22±1 nm
EGFR-mAB-P (w/o free P) + scr-siRNA:
3.2±0.2 nm
SMCC-P + scr-siRNA:
5.7±0.4 nm
Intensity
Size [nm]
HC-SMCC-protamine HC
LC-SMCC-protamine LC
SMCC-protamine
αEGFR-mAB-SMCCC-protamine HPLC fractions #
25 26 27 28 29 30 31
α EGFR-mAB-SMCCC-protamine (1:32)
α EGFR-mAB

Figure 34

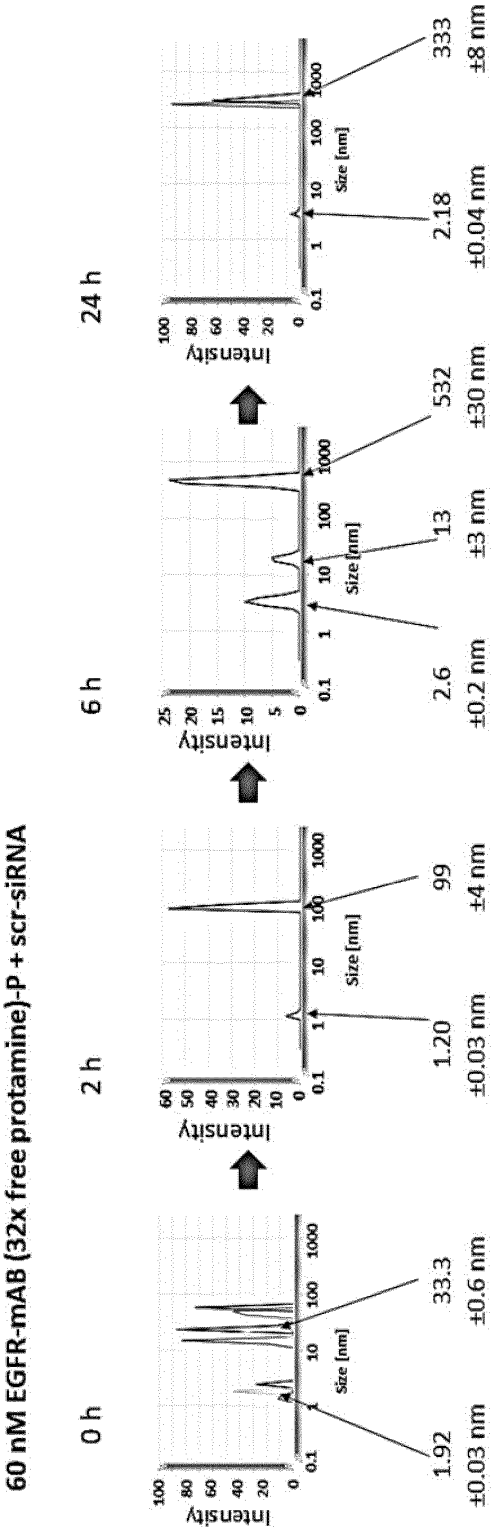

60 nM EGFR-mAB (32x free protamine)-P + scr-siRNA
0 h
2 h
6 h
24 h
Intensity
Size [nm]
1.92
33.3
±0.03 nm
±0.6 nm
1.20
99
±0.03 nm
±4 nm
2.6
13
532
±0.2 nm
±3 nm
±30 nm
2.18
333
±0.04 nm
±8 nm

Figure 35

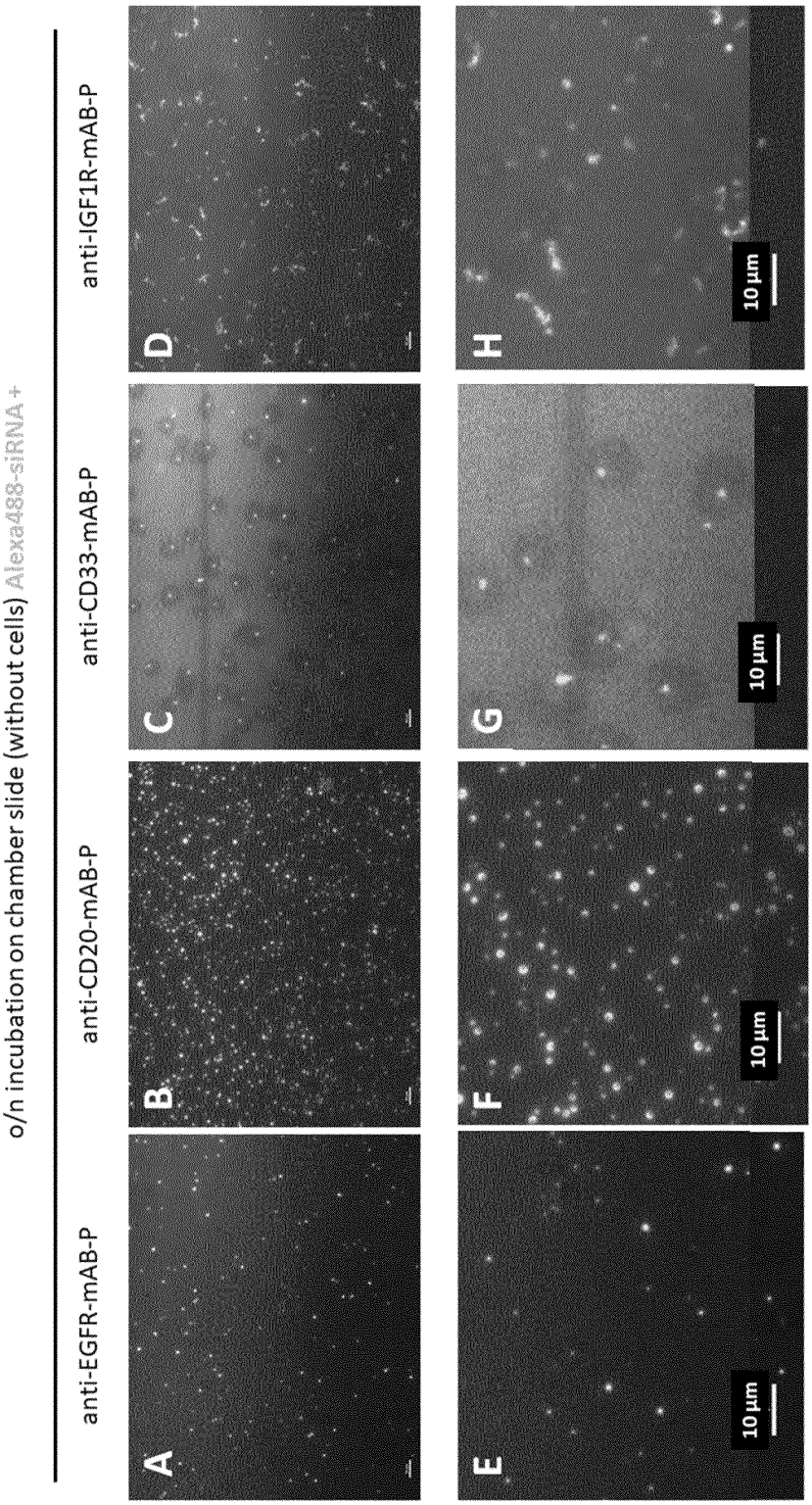

o/n incubation on chamber slide (without cells) Alexa488-siRNA +
anti-EGFR-mAB-P
anti-CD20-mAB-P
anti-CD33-mAB-P
anti-IGF1R-mAB-P
A
B
C
D
E
F
G
H
10 µm

Figure 36

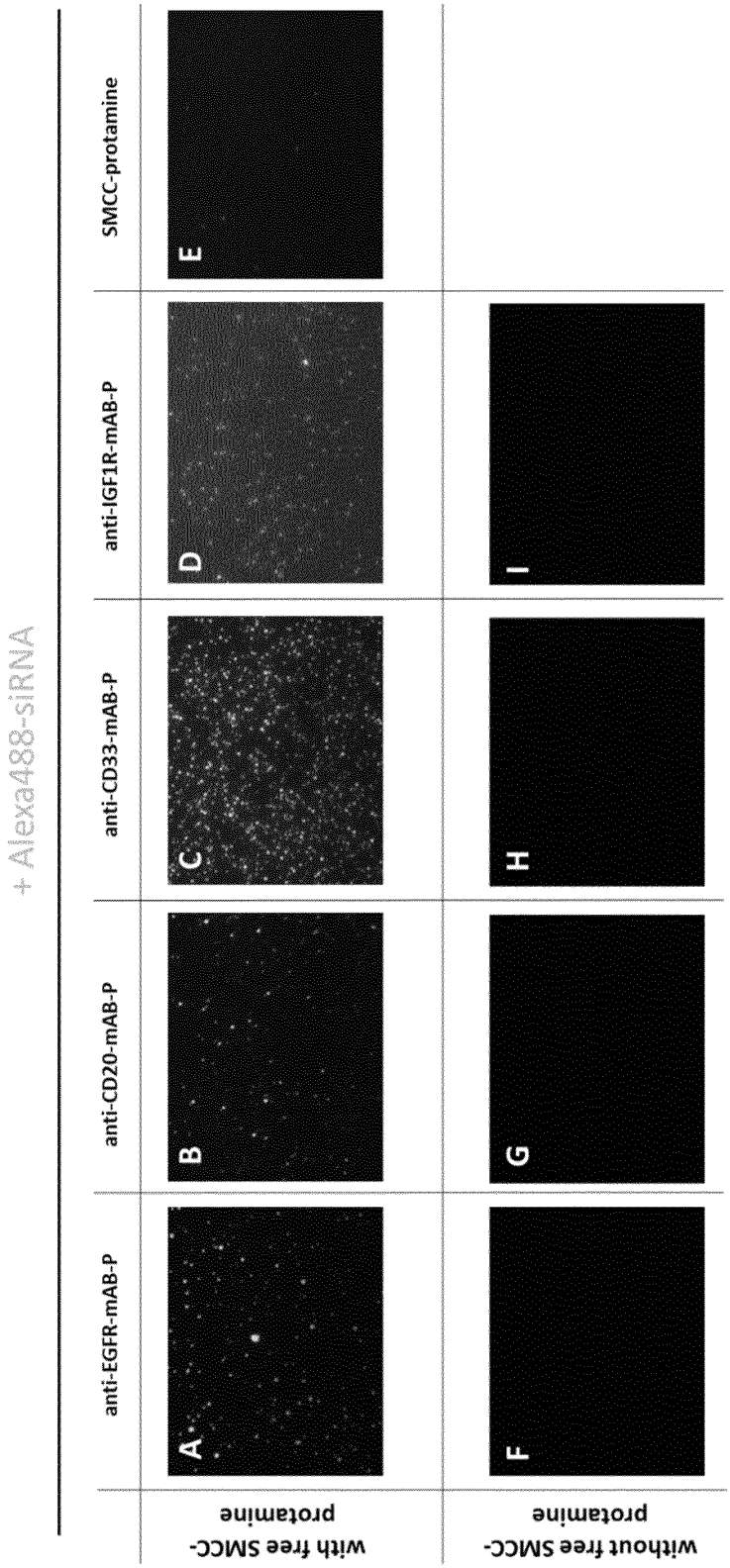

+ Alexa488-siRNA
anti-EGFR-mAB-P
anti-CD20-mAB-P
anti-CD33-mAB-P
anti-IGF1R-mAB-P
SMCC-protamine
with free SMCC-protamine
without free SMCC-protamine
A
B
C
D
E
F
G
H
I

Figure 37

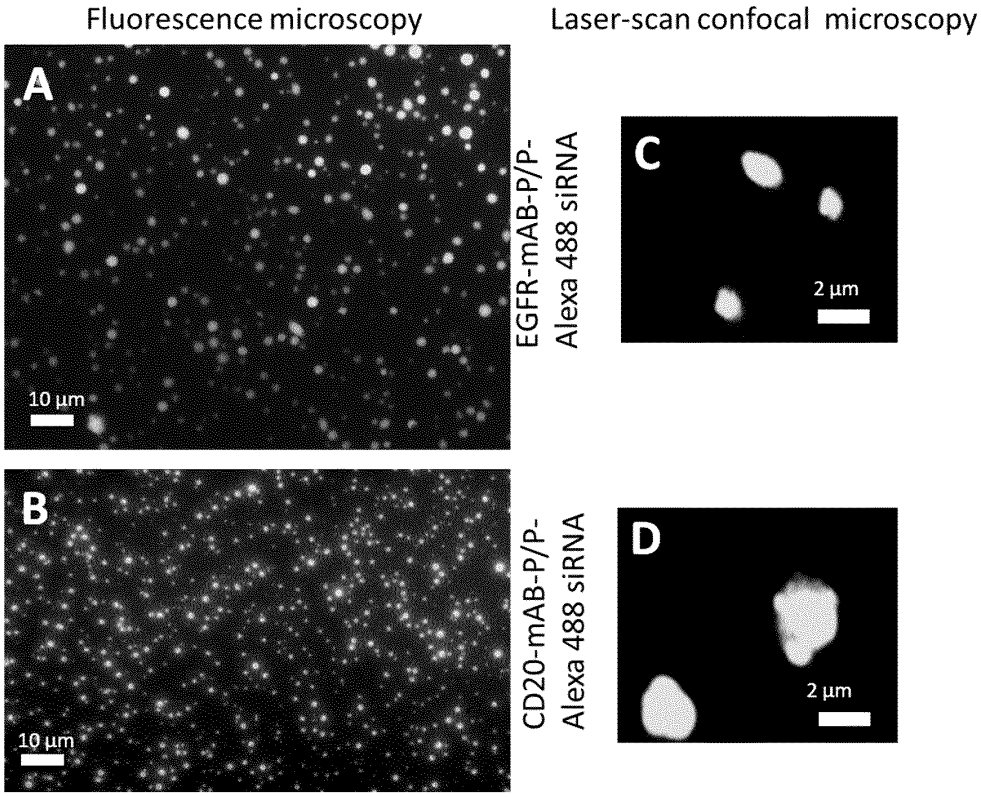


Figure 38

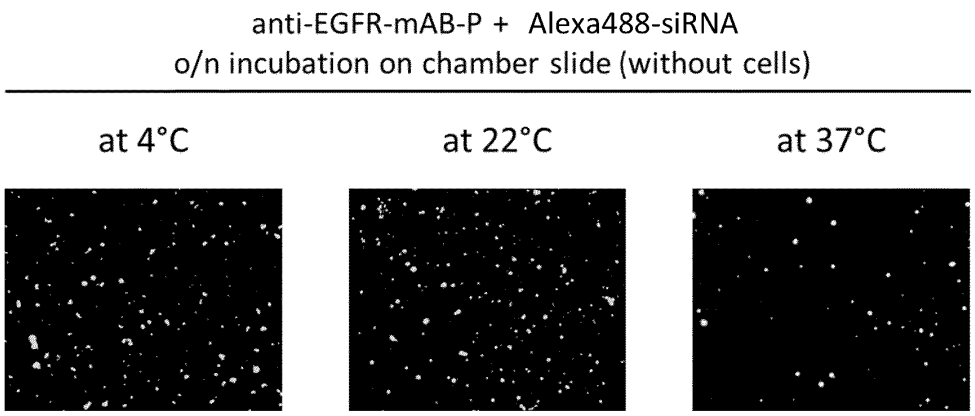


Figure 39

A

cetuximab concentration (nM)	SMCC-protamine concentration (nM)	EGFR-mAB : SMCC-protamine conjugation ratio
60	60	1:1
60	192	1:3.2
60	600	1:10
60	1920	1:32
60	3000	1:50
60	6000	1:100

B

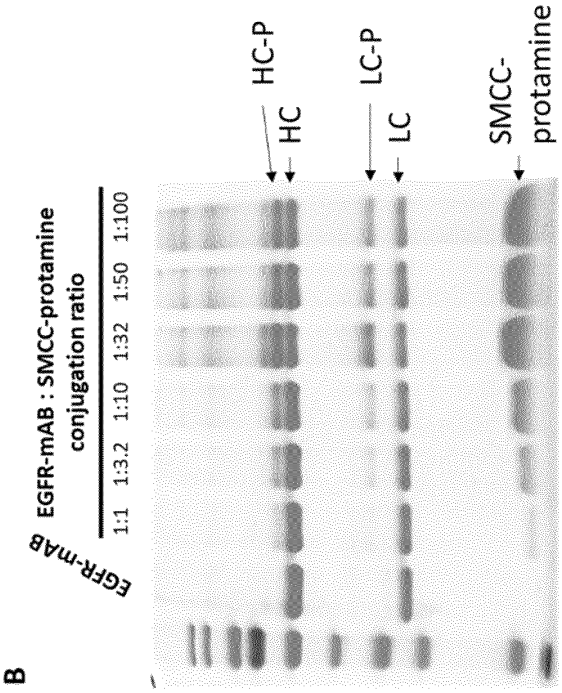


Figure 40

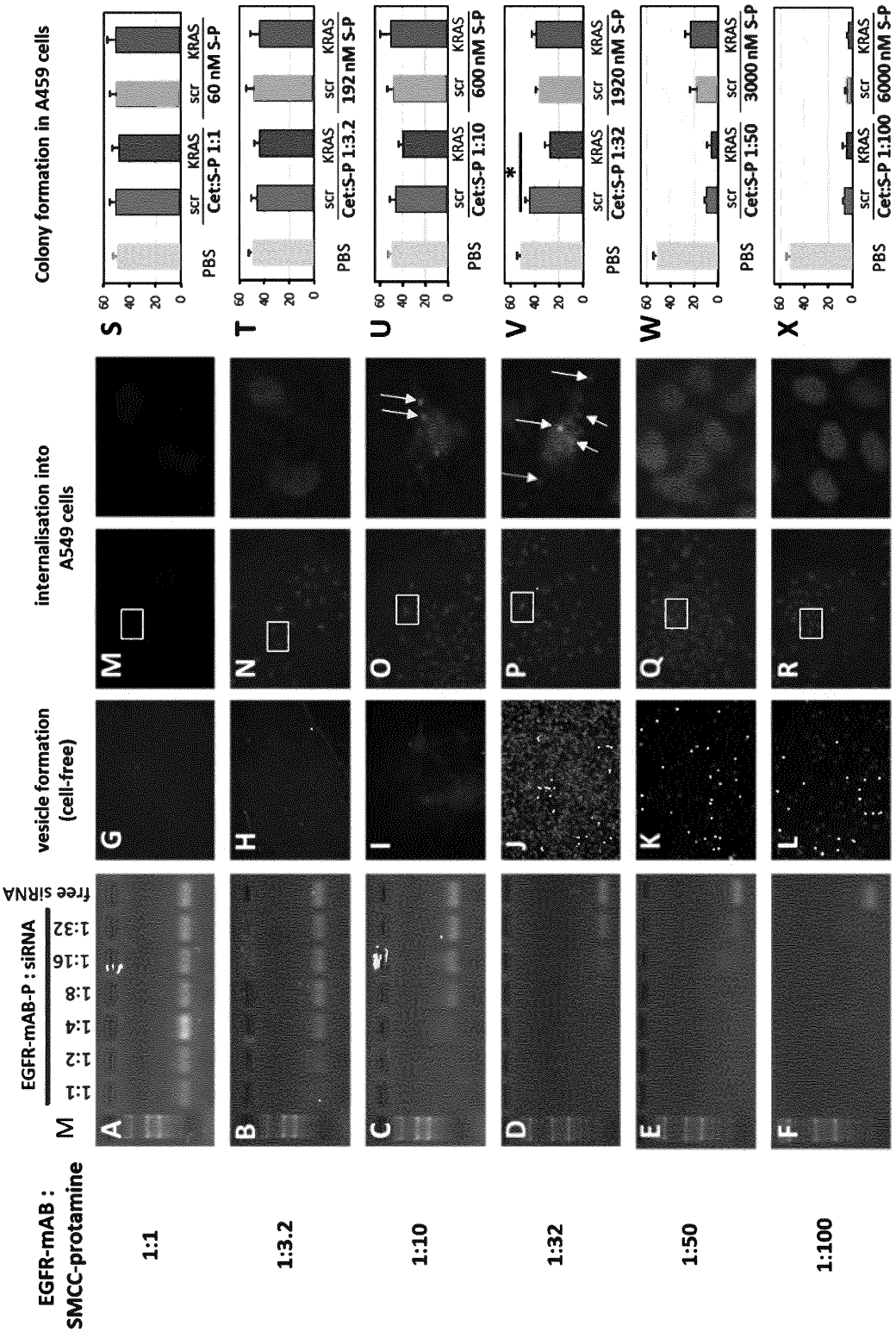

EGFR-mAB : SMCC-protamine
1:1
1:3.2
1:10
1:32
1:50
1:100
M
EGFR-mAB-P : siRNA
1:1
1:2
1:4
1:8
1:16
1:32
free siRNA
vesicle formation (cell-free)
internalisation into A549 cells
Colony formation in A459 cells
PBS
scr
KRAS
Cet:S-P 1:1
60 nM S-P
Cet:S-P 1:3.2
192 nM S-P
Cet:S-P 1:10
600 nM S-P
Cet:S-P 1:32
1920 nM S-P
Cet:S-P 1:50
3000 nM S-P
Cet:S-P 1:100
6000 nM S-P

Figure 41

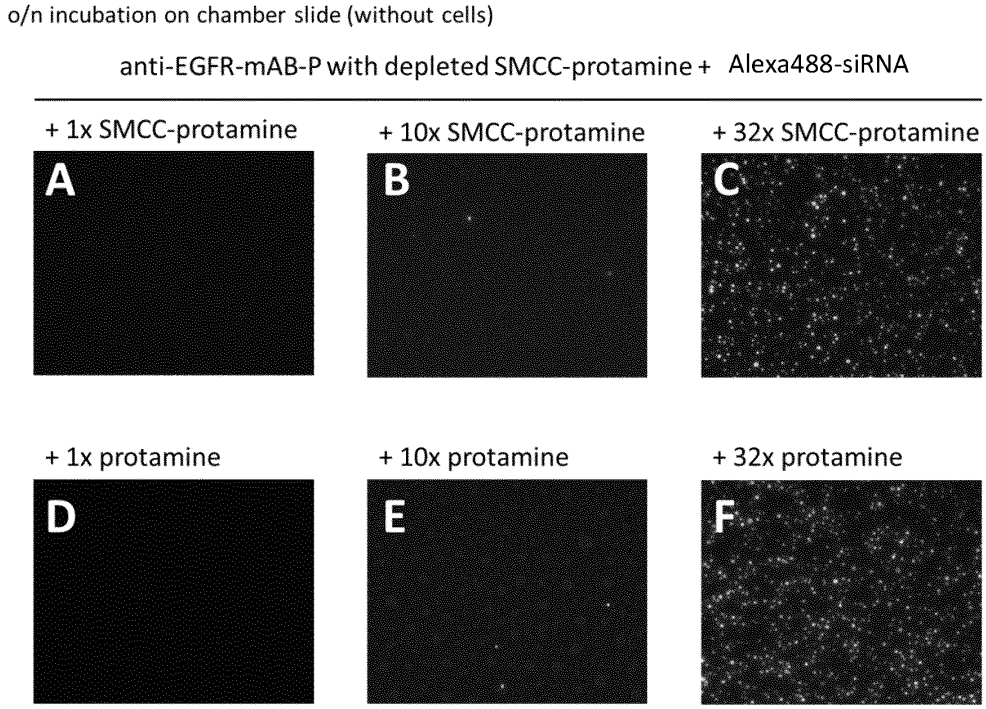


Figure 42

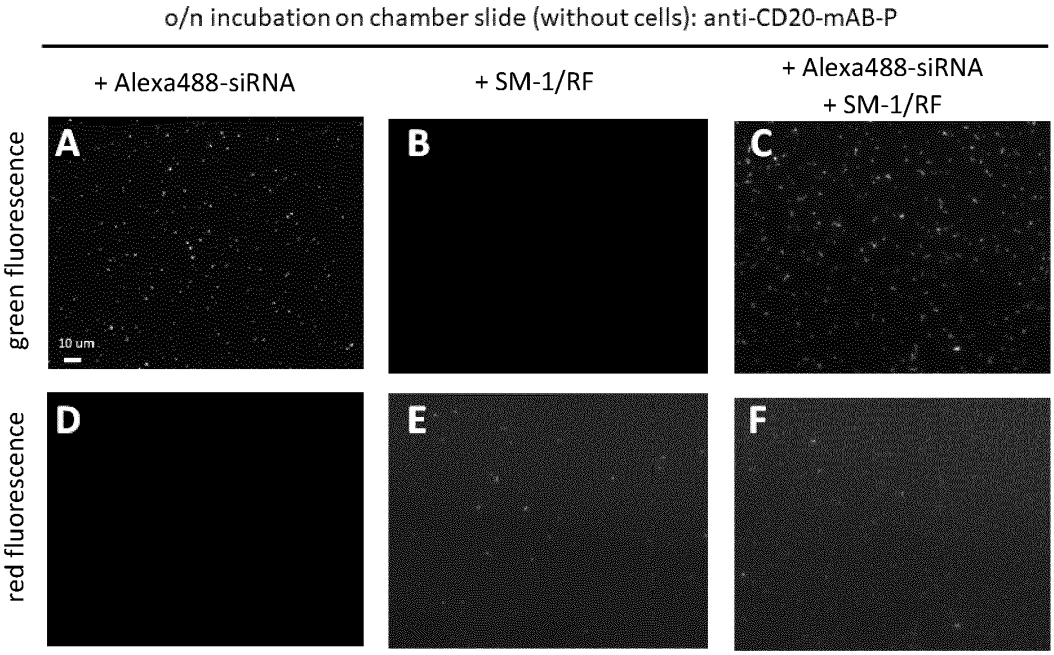


Figure 43

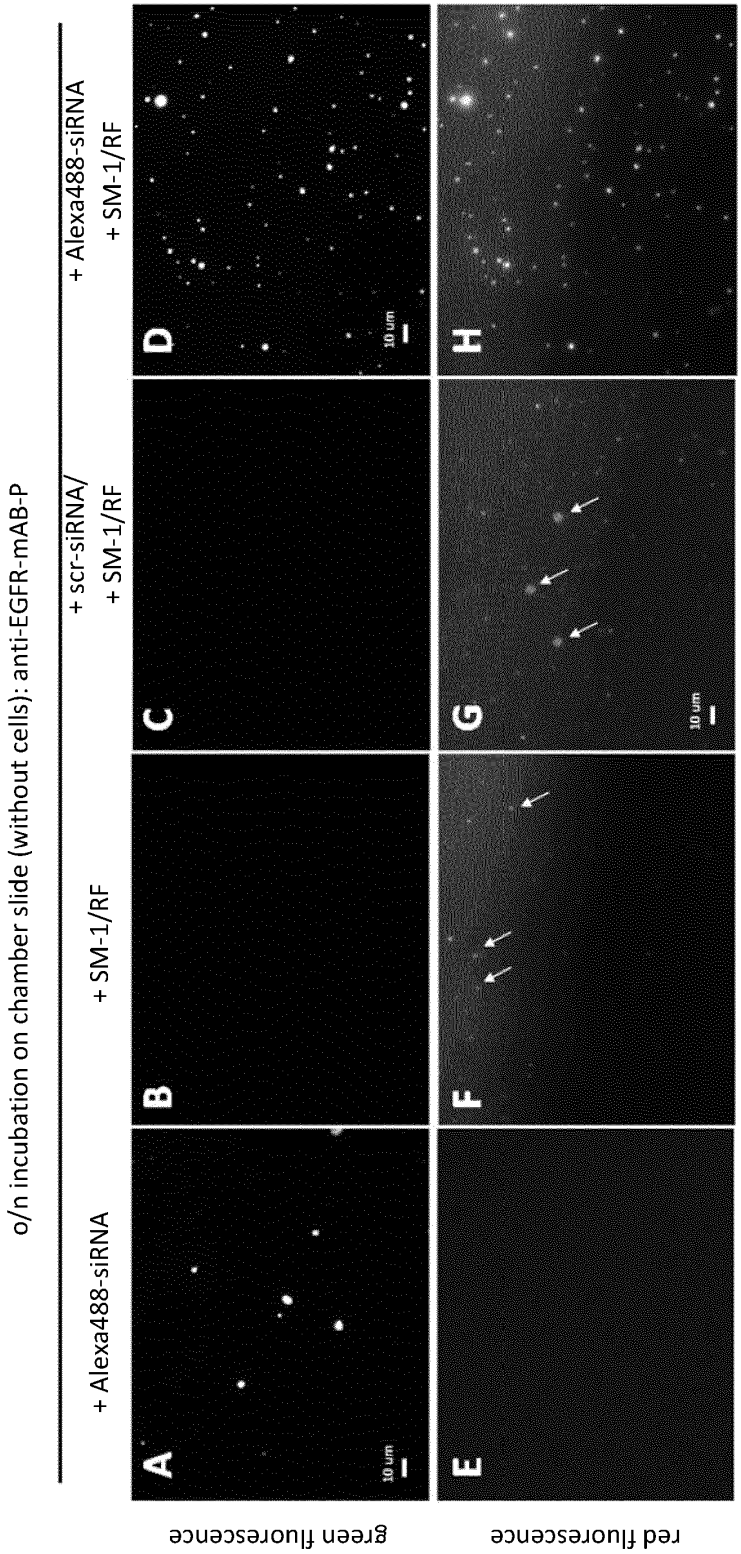

o/n incubation on chamber slide (without cells): anti-EGFR-mAB-P
+ Alexa488-siRNA
+ SM-1/RF
+ scr-siRNA/
+ SM-1/RF
+ Alexa488-siRNA
+ SM-1/RF
A
B
C
D
E
F
G
H
10 um
green fluorescence
red fluorescence

Figure 44

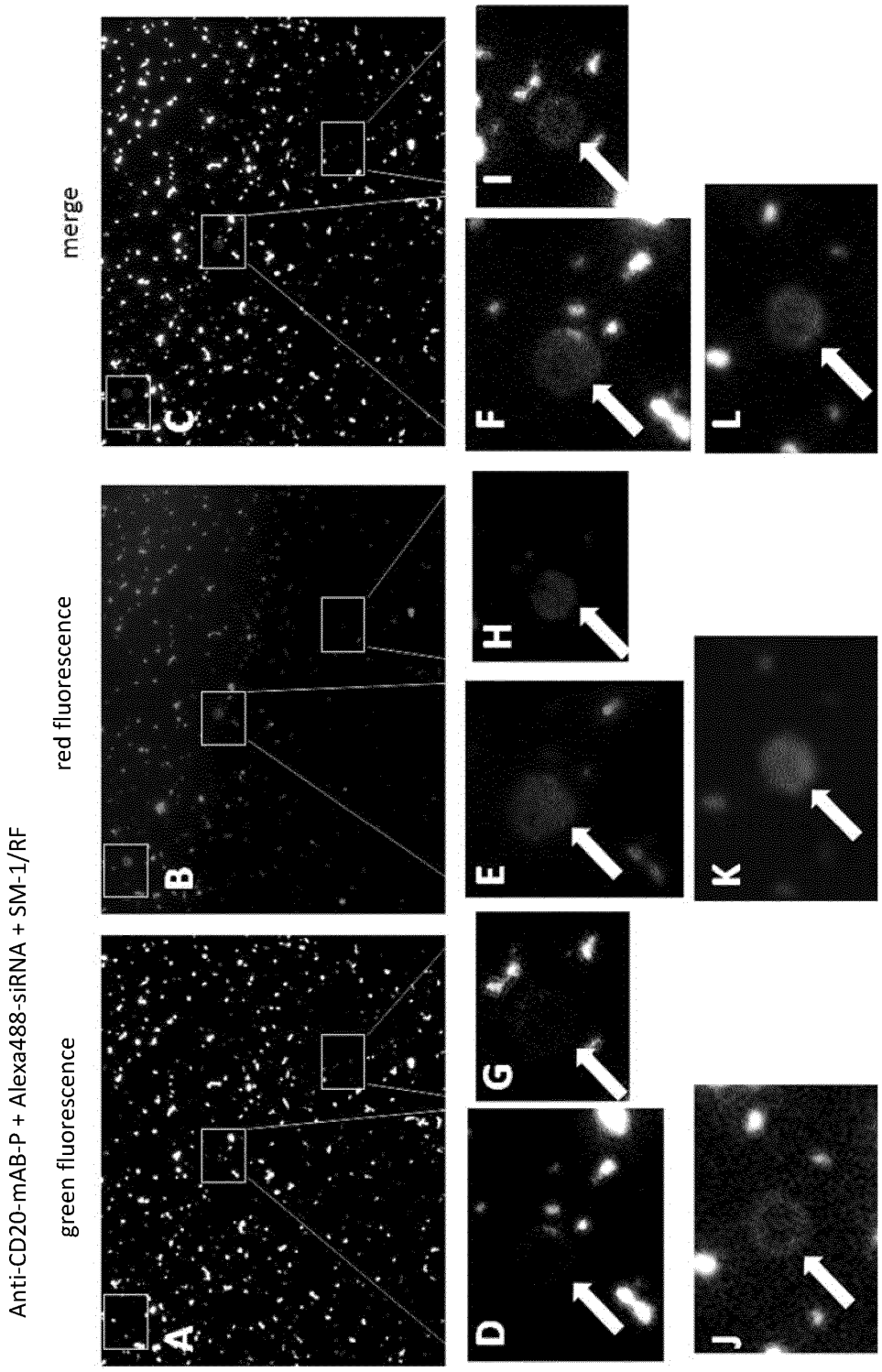

Anti-CD20-mAB-P + Alexa488-siRNA + SM-1/RF
green fluorescence
red fluorescence
merge
A
B
C
D
E
F
G
H
I
J
K
L

Figure 45

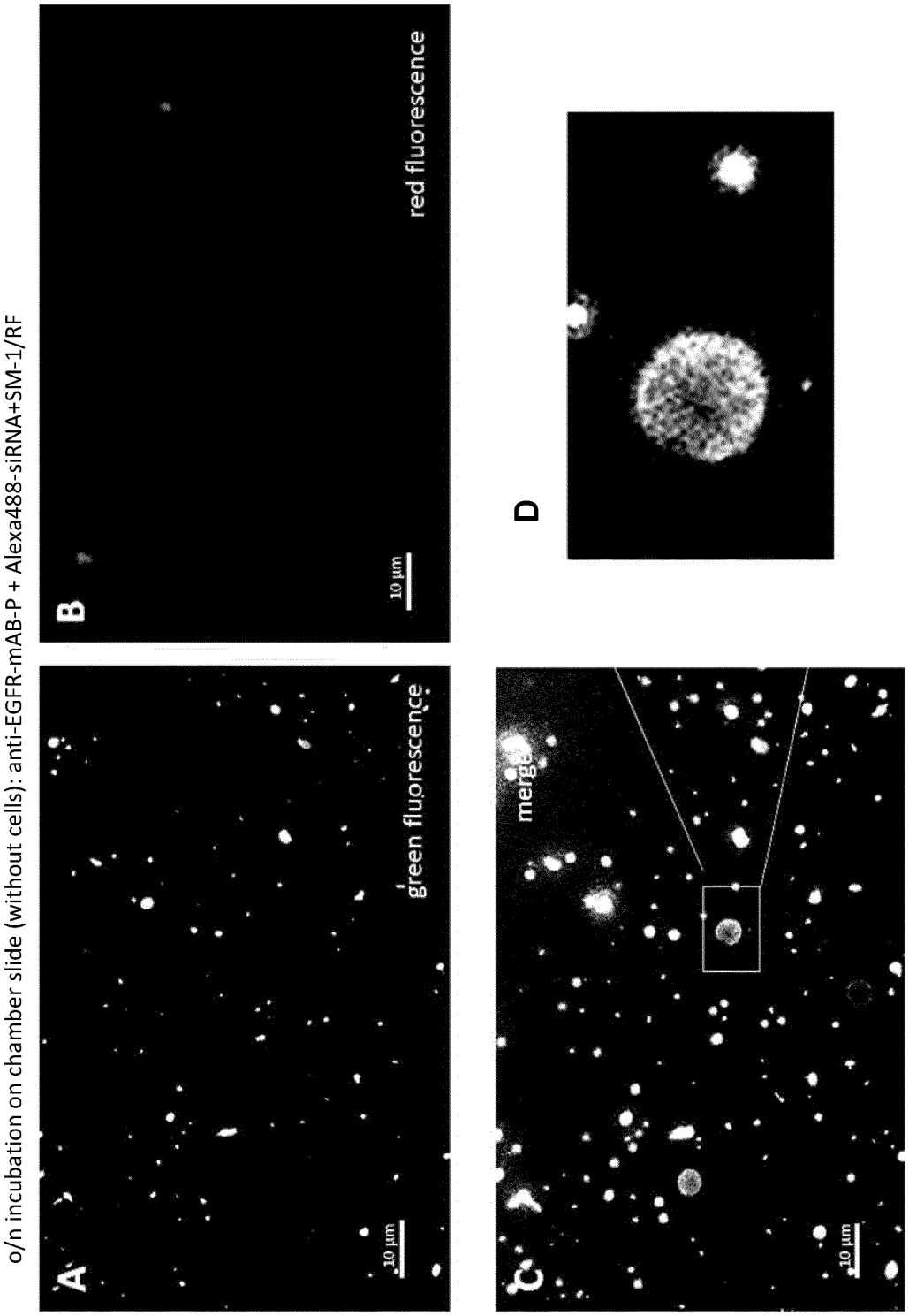

o/n incubation on chamber slide (without cells): anti-EGFR-mAB-P + Alexa488-siRNA+SM-1/RF
A
green fluorescence
10 µm
B
red fluorescence
10 µm
C
merge
10 µm
D

Figure 46

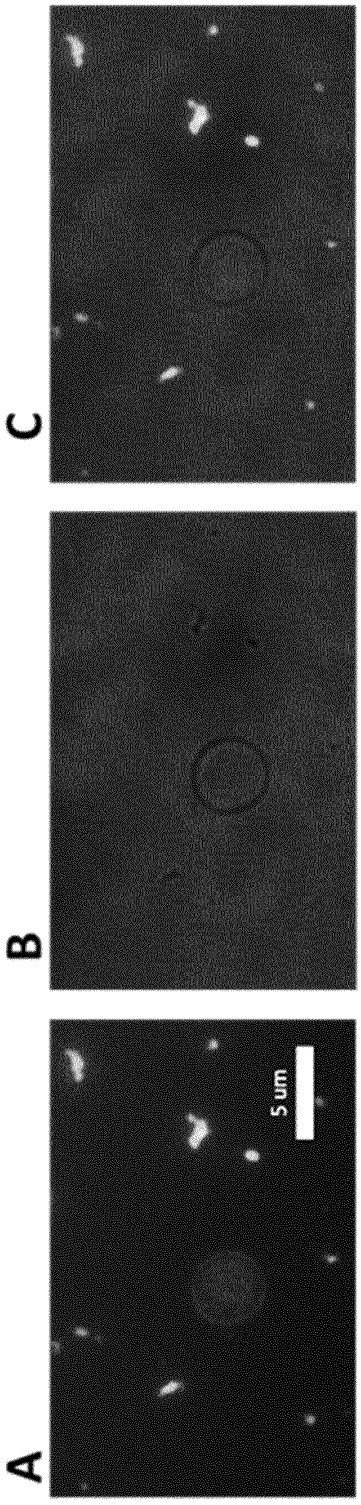


Figure 47

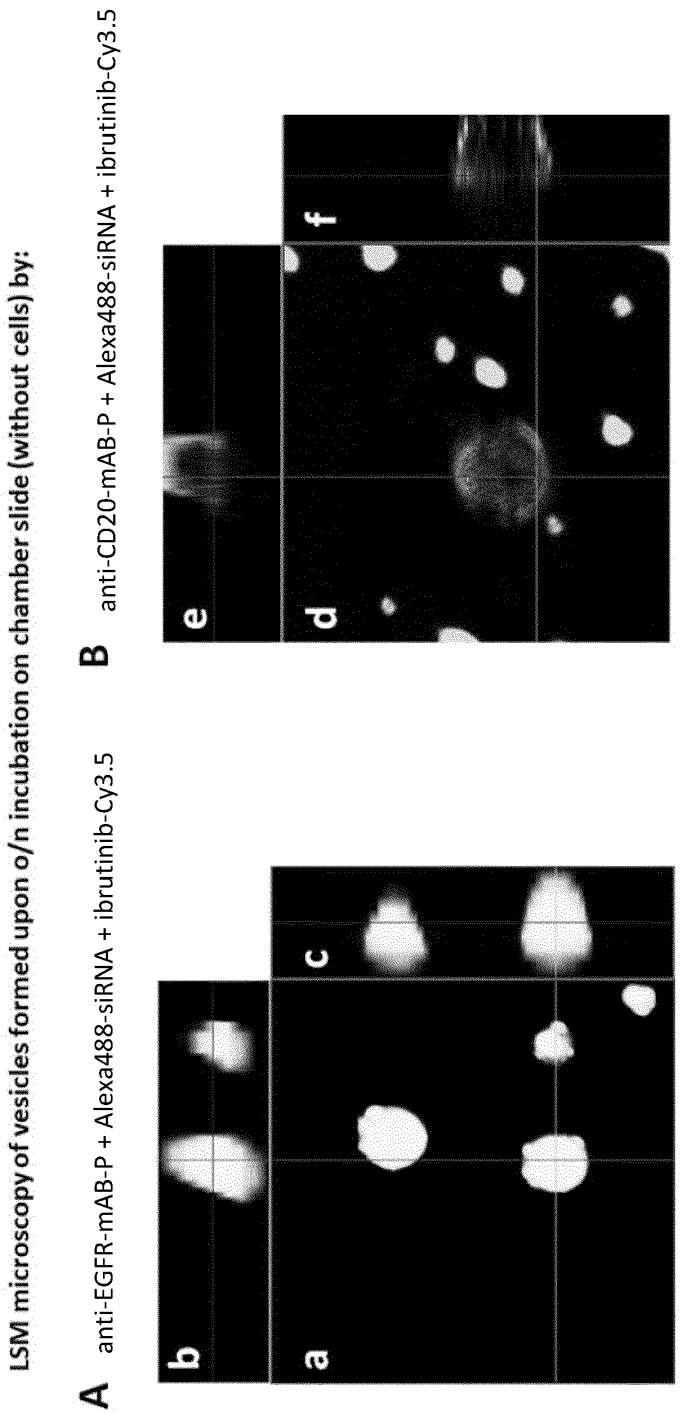

LSM microscopy of vesicles formed upon o/n incubation on chamber slide (without cells) by:
A anti-EGFR-mAB-P + Alexa488-siRNA + ibrutinib-Cy3.5
B anti-CD20-mAB-P + Alexa488-siRNA + ibrutinib-Cy3.5
a
b
c
d
e
f

Figure 48

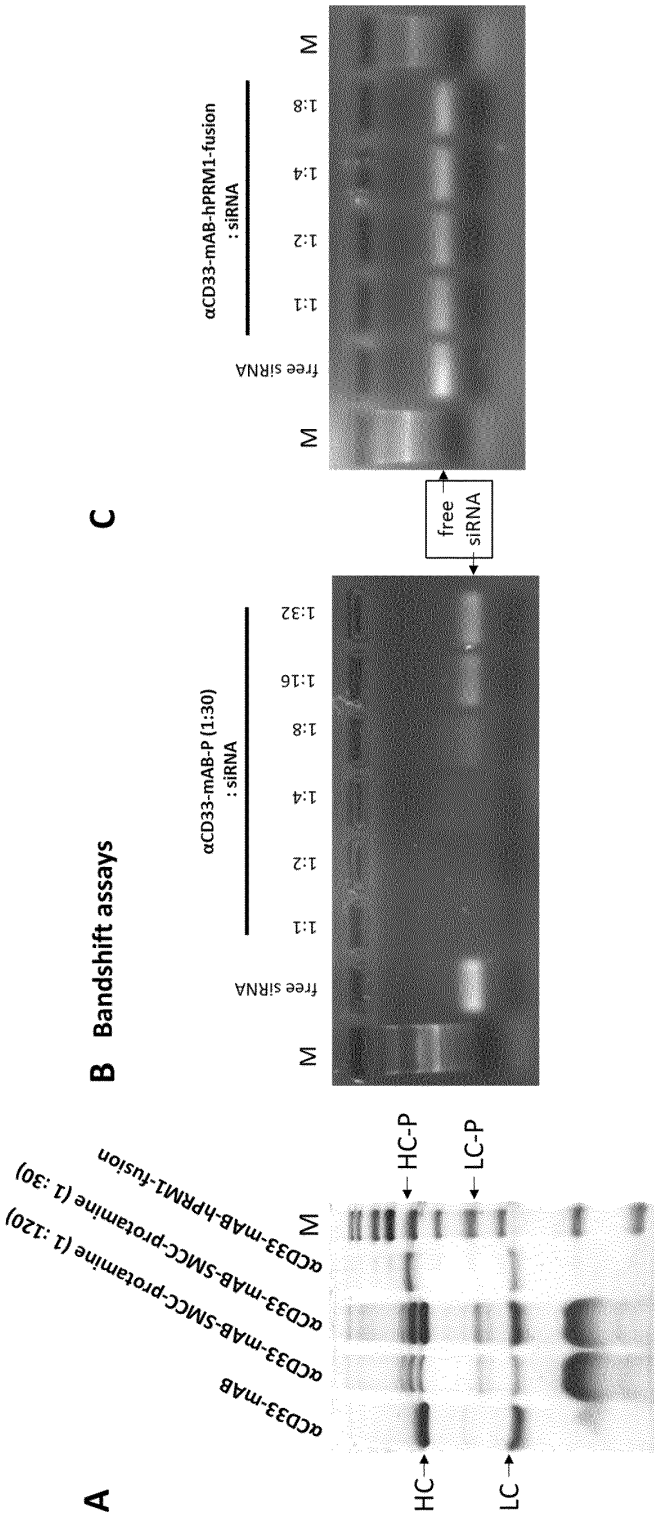

A
αCD33-mAB
αCD33-mAB-SMCC-protamine (1 :120)
αCD33-mAB-SMCC-protamine (1 :30)
αCD33-mAB-hPRM1-fusion
M
HC-P
LC-P
HC
LC
B Bandshift assays
αCD33-mAB-P (1:30) : siRNA
free siRNA
1:1
1:2
1:4
1:8
1:16
1:32
M
C
free siRNA
αCD33-mAB-hPRM1-fusion : siRNA
free siRNA
1:1
1:2
1:4
1:8
M

Figure 49

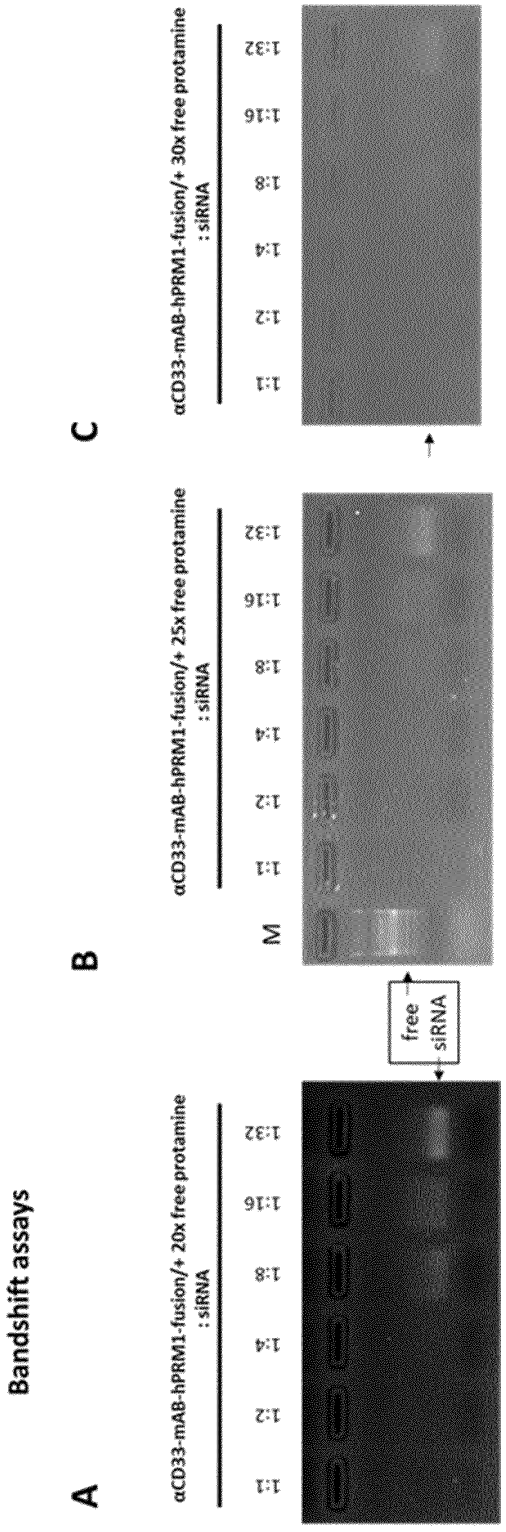

Bandshift assays
A
αCD33-mAB-hPRM1-fusion/+ 20x free protamine : siRNA
1:1
1:2
1:4
1:8
1:16
1:32
free siRNA
B
M
αCD33-mAB-hPRM1-fusion/+ 25x free protamine : siRNA
1:1
1:2
1:4
1:8
1:16
1:32
C
αCD33-mAB-hPRM1-fusion/+ 30x free protamine : siRNA
1:1
1:2
1:4
1:8
1:16
1:32

Figure 50

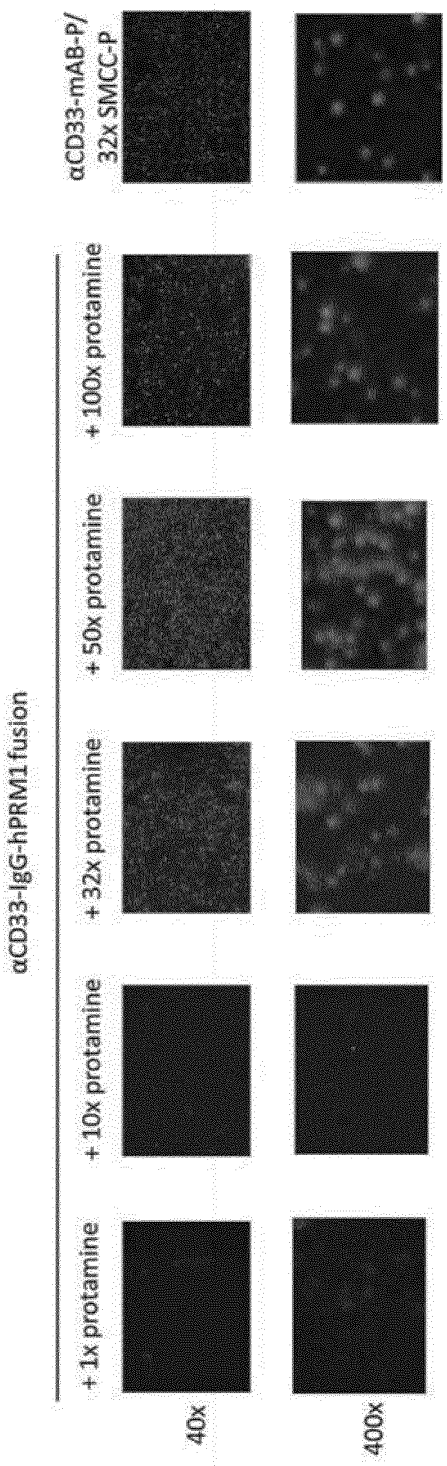

αCD33-IgG-hPRM1 fusion
+ 1x protamine
+ 10x protamine
+ 32x protamine
+ 50x protamine
+ 100x protamine
αCD33-mAB-P/
32x SMCC-P
40x
400x

Figure 51

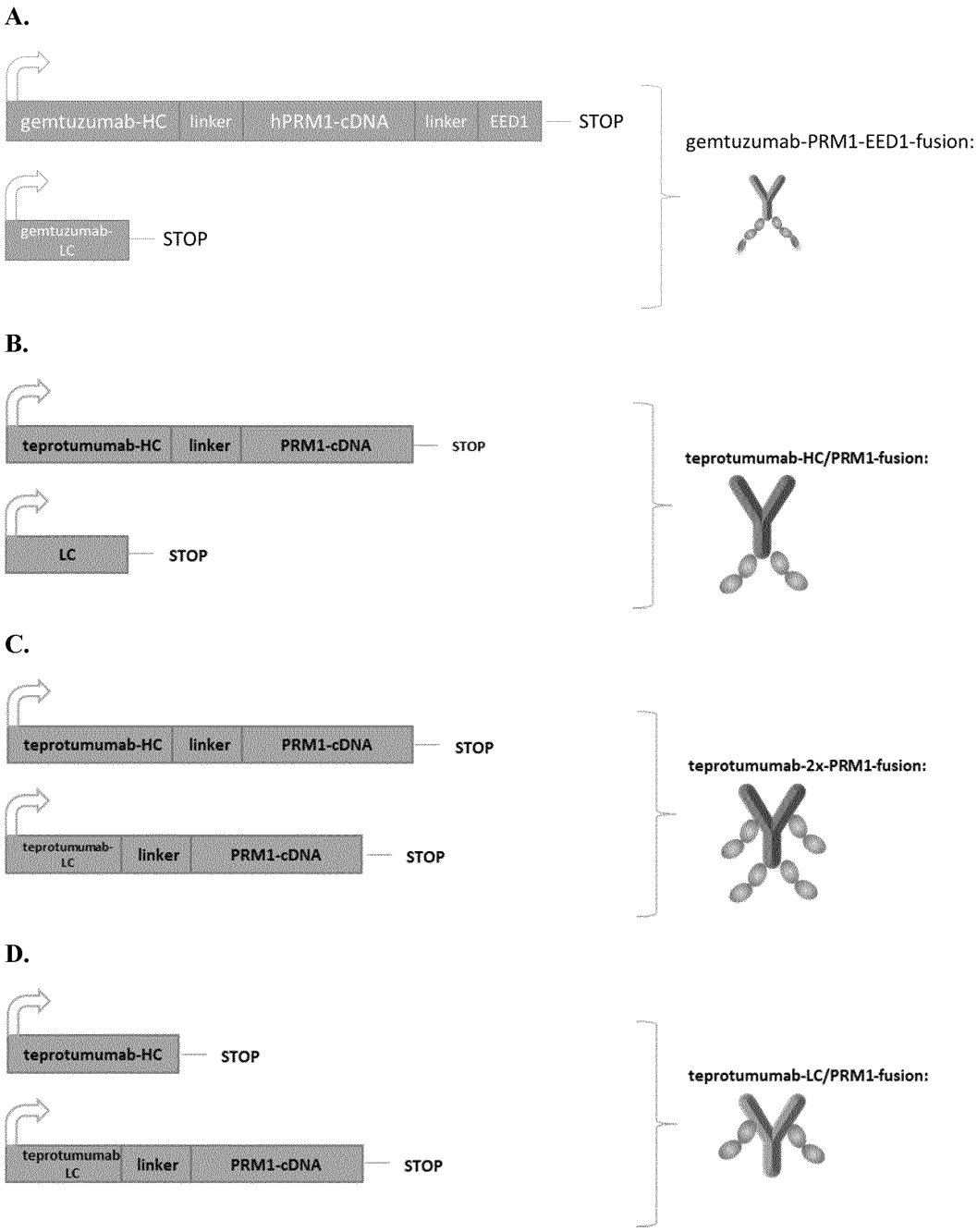

A.
gemtuzumab-HC
linker
hPRM1-cDNA
linker
EED1
STOP
gemtuzumab-LC
STOP
gemtuzumab-PRM1-EED1-fusion:
B.
teprotumumab-HC
linker
PRM1-cDNA
STOP
LC
STOP
teprotumumab-HC/PRM1-fusion:
C.
teprotumumab-HC
linker
PRM1-cDNA
STOP
teprotumumab-LC
linker
PRM1-cDNA
STOP
teprotumumab-2x-PRM1-fusion:
D.
teprotumumab-HC
STOP
teprotumumab LC
linker
PRM1-cDNA
STOP
teprotumumab-LC/PRM1-fusion:

Figure 52

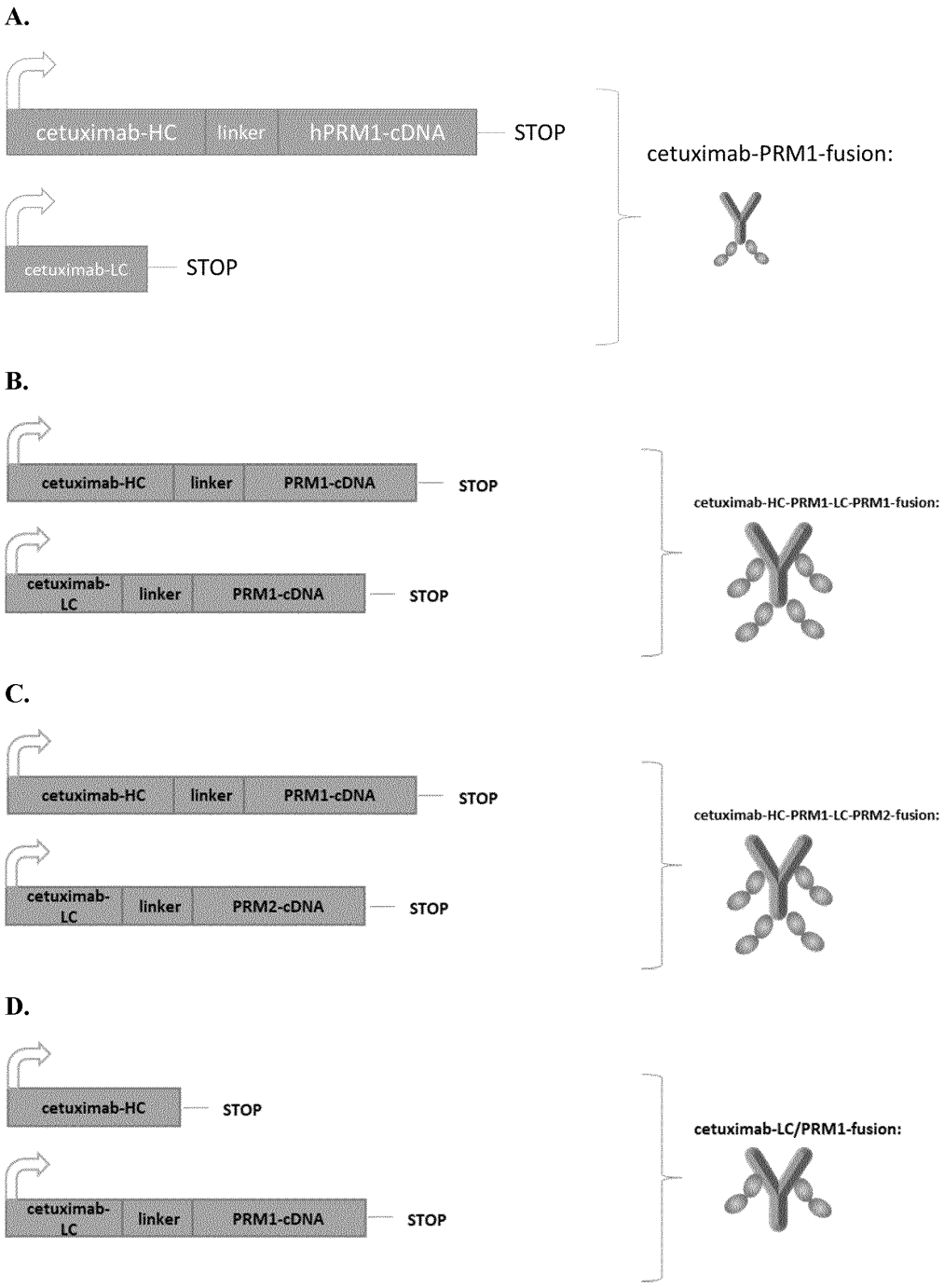

A.
cetuximab-HC
linker
hPRM1-cDNA
STOP
cetuximab-LC
STOP
cetuximab-PRM1-fusion:
B.
cetuximab-HC
linker
PRM1-cDNA
STOP
cetuximab-LC
linker
PRM1-cDNA
STOP
cetuximab-HC-PRM1-LC-PRM1-fusion:
C.
cetuximab-HC
linker
PRM1-cDNA
STOP
cetuximab-LC
linker
PRM2-cDNA
STOP
cetuximab-HC-PRM1-LC-PRM2-fusion:
D.
cetuximab-HC
STOP
cetuximab-LC
linker
PRM1-cDNA
STOP
cetuximab-LC/PRM1-fusion:

Figure 52 (cont'd)

E.

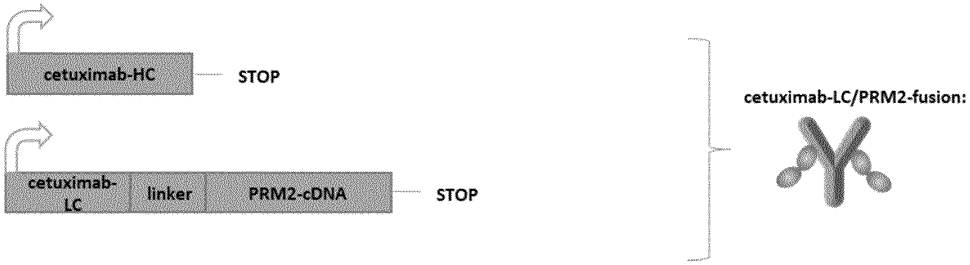


Figure 53

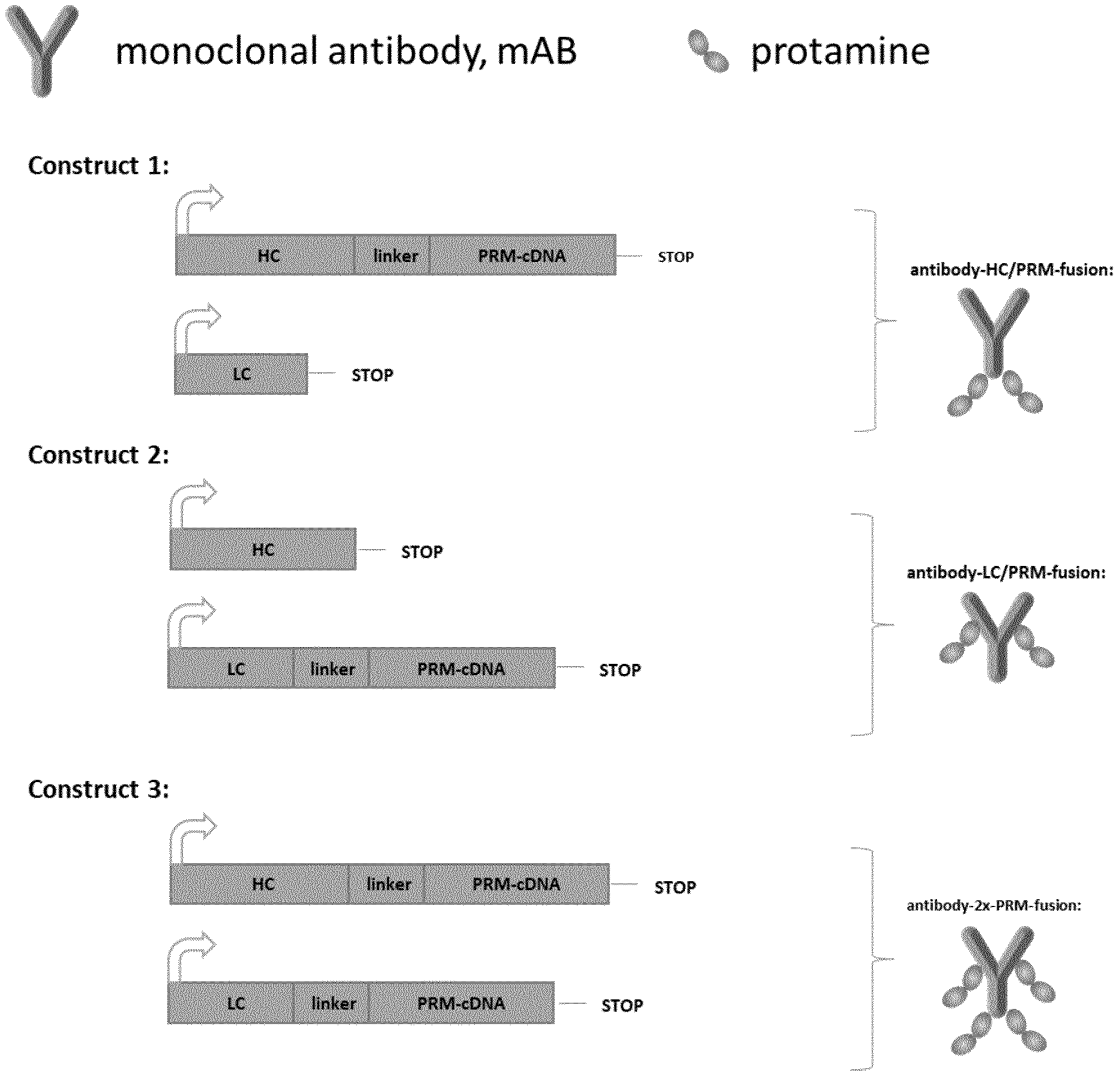


Figure 54

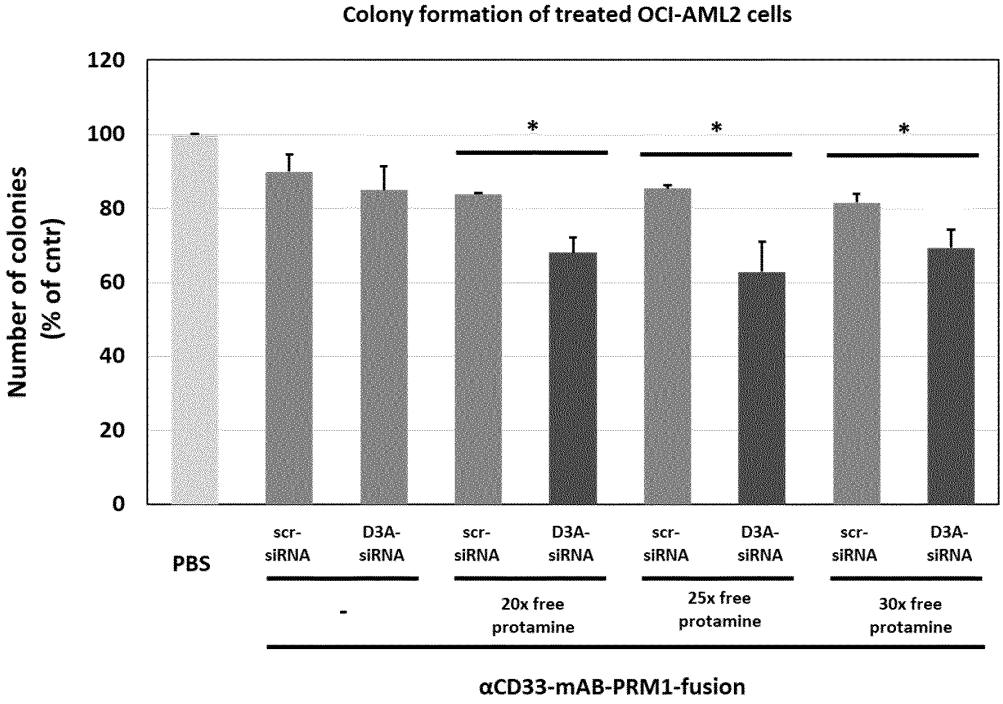


Figure 55

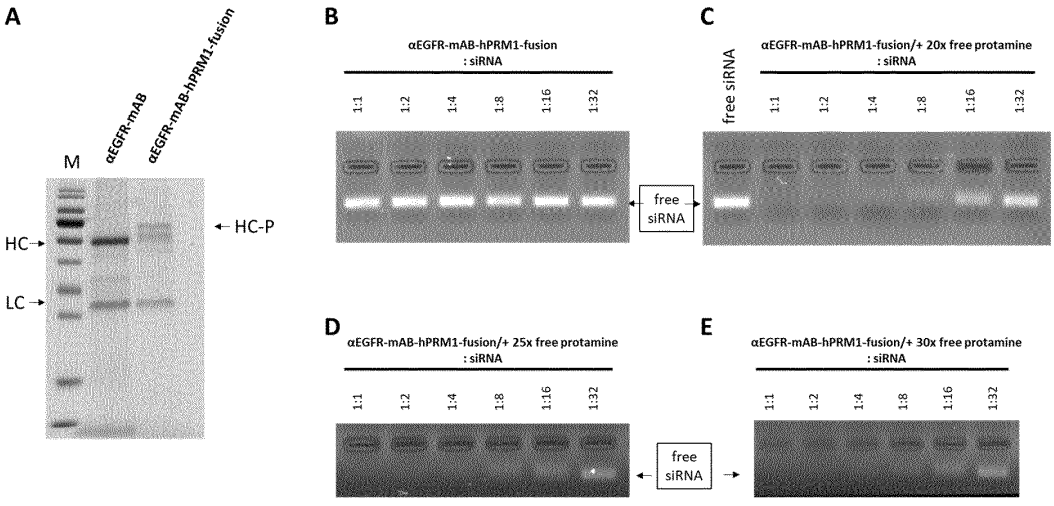


Figure 56

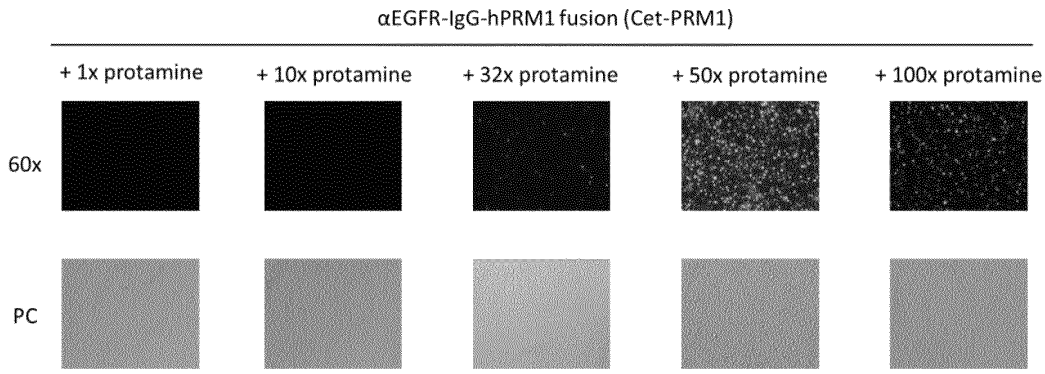
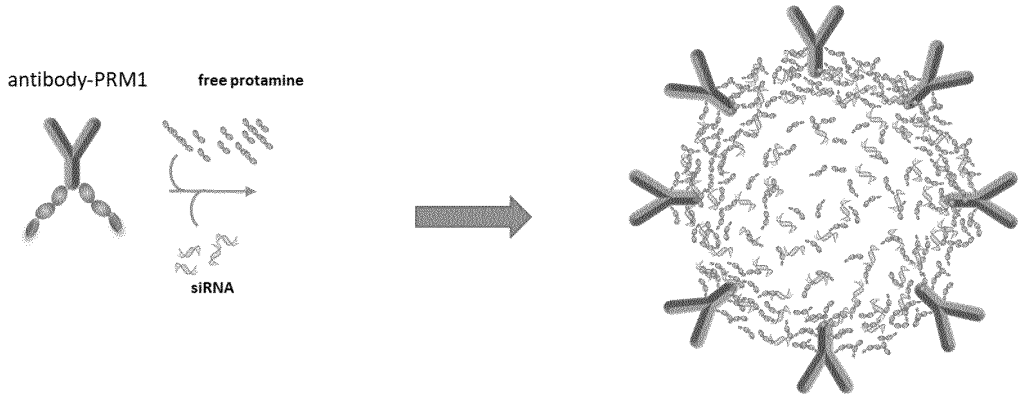


Figure 57



mAB-protamine/free protamine/siRNA nanoparticle

Figure 58

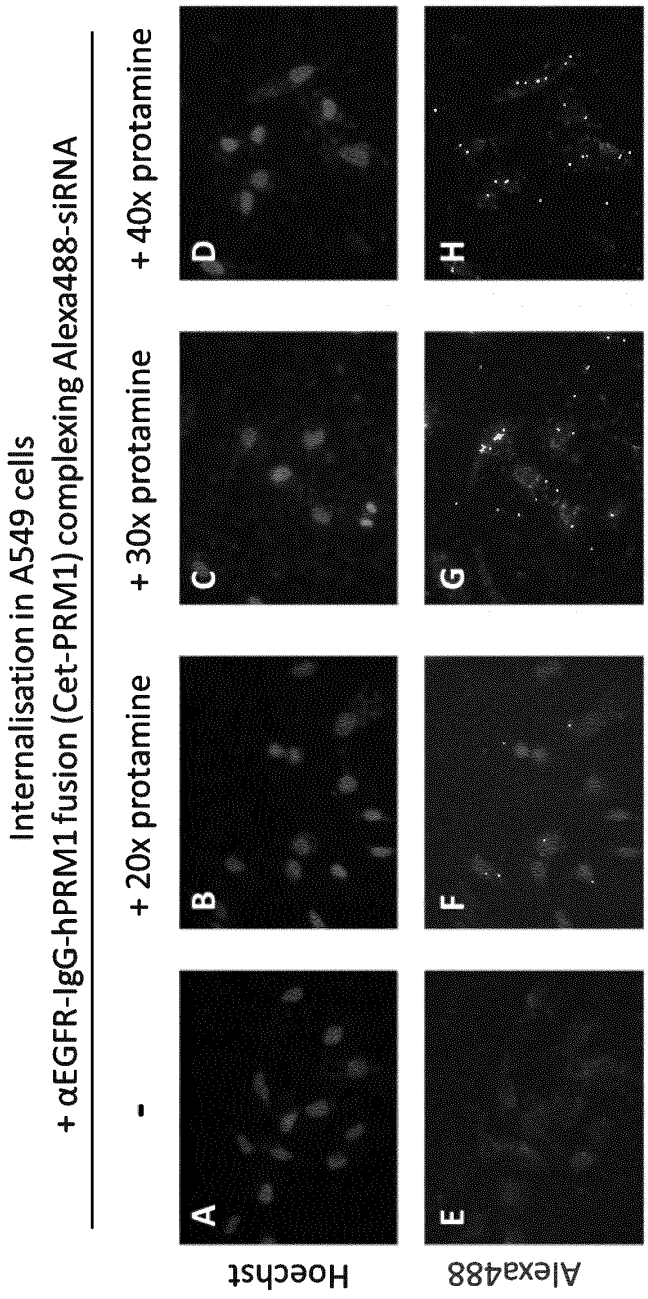

Internalisation in A549 cells
+ αEGFR-IgG-hPRM1 fusion (Cet-PRM1) complexing Alexa488-siRNA
-
+ 20x protamine
+ 30x protamine
+ 40x protamine
Hoechst
Alexa488
A
B
C
D
E
F
G
H

Figure 59

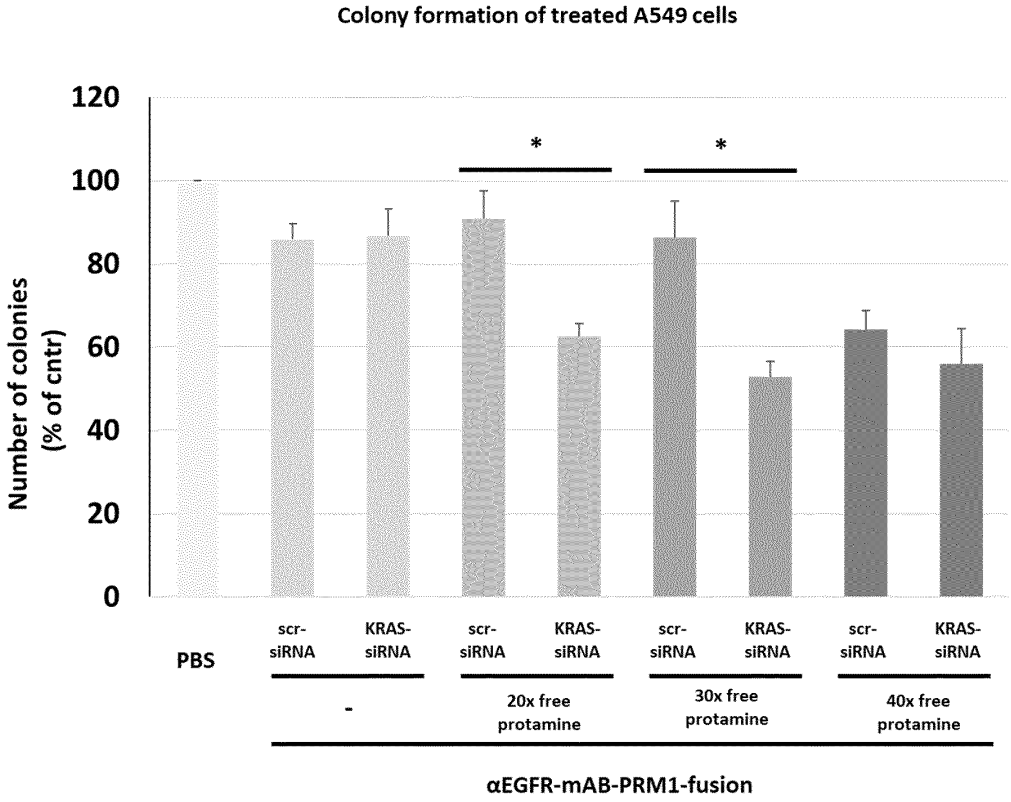

Colony formation of treated A549 cells
Number of colonies (% of cntr)
120
100
80
60
40
20
0
*
*
PBS
scr-siRNA
KRAS-siRNA
scr-siRNA
KRAS-siRNA
scr-siRNA
KRAS-siRNA
scr-siRNA
KRAS-siRNA
-
20x free protamine
30x free protamine
40x free protamine
αEGFR-mAB-PRM1-fusion

Figure 60

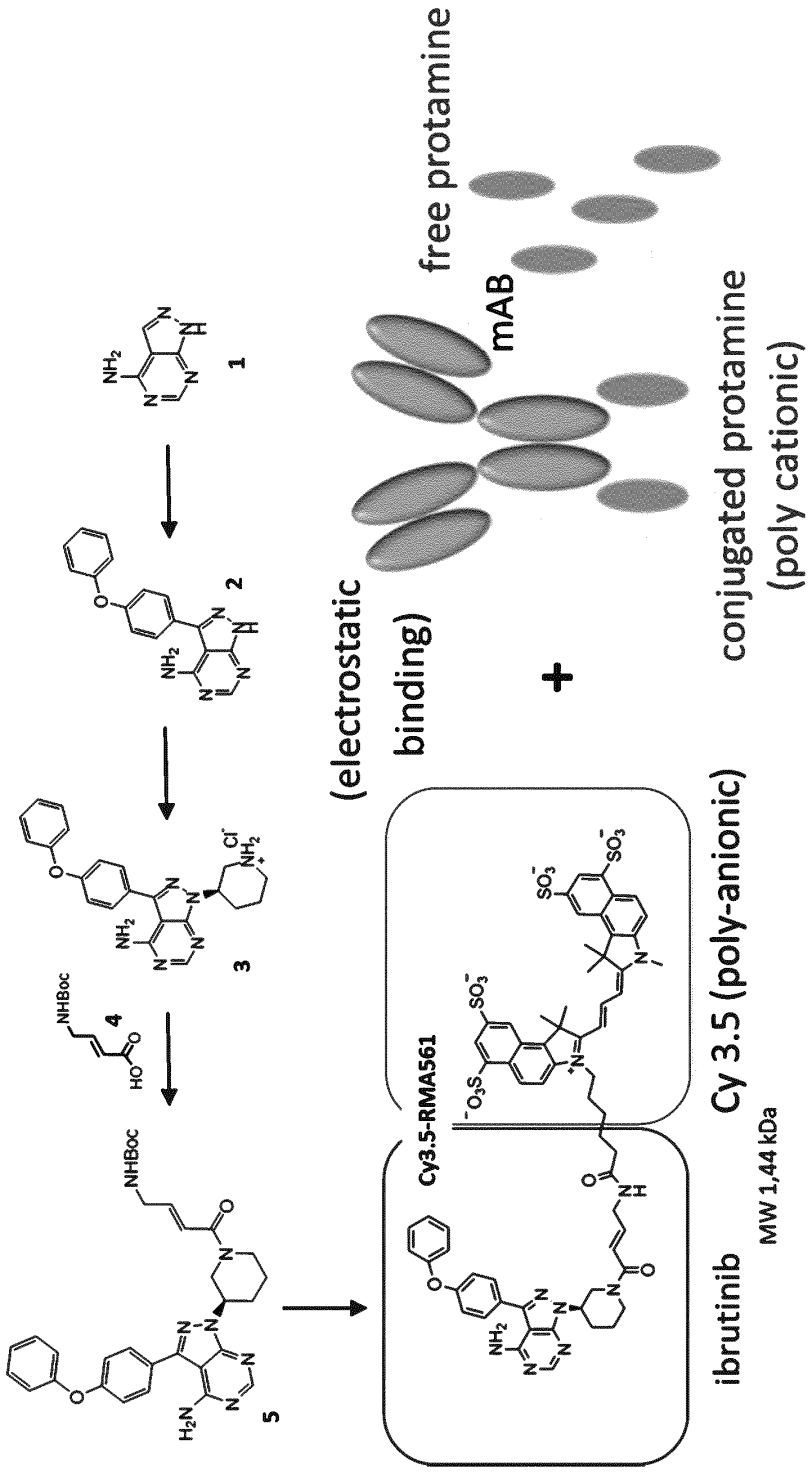
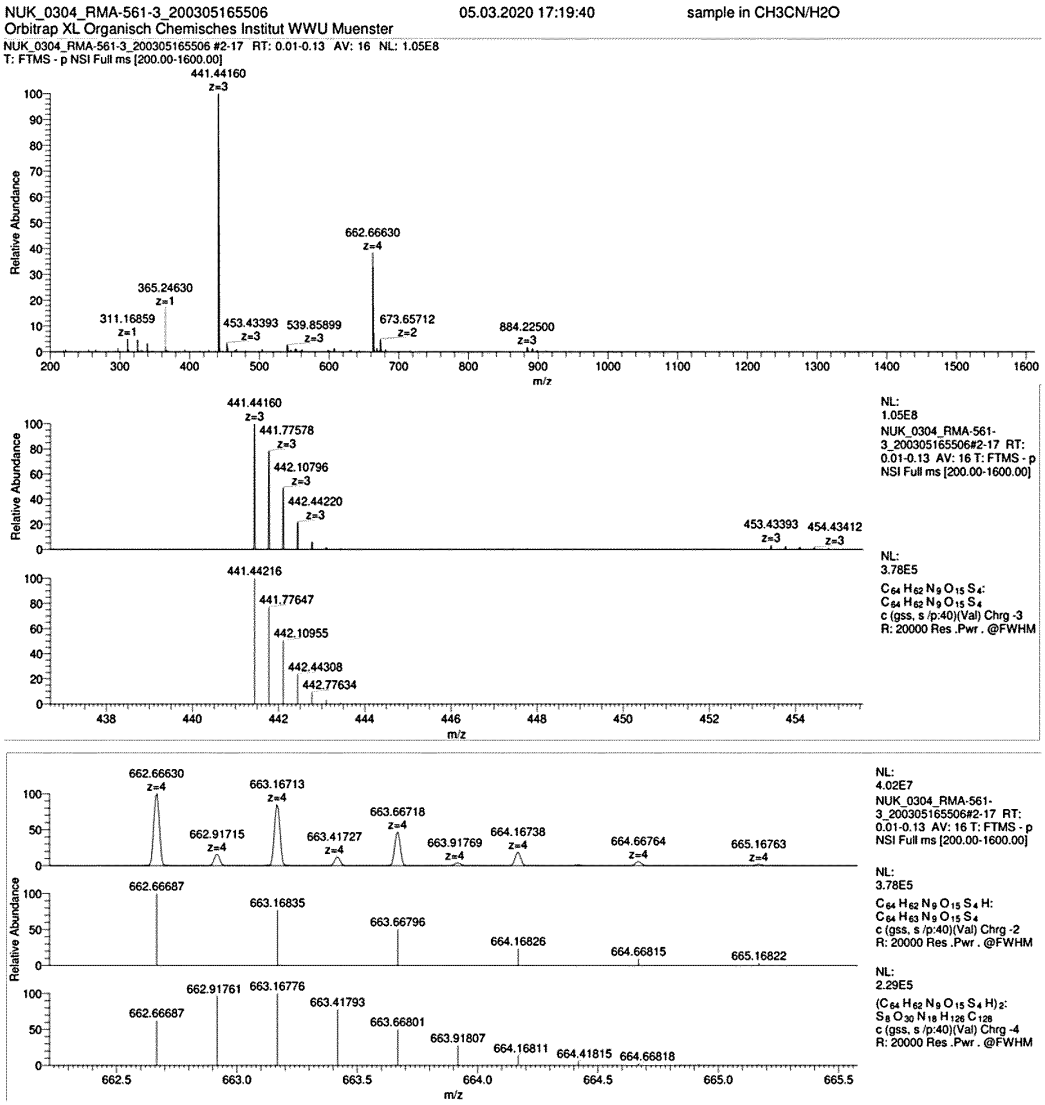

free protamine
mAB
conjugated protamine
(poly cationic)
(electrostatic
binding)
+
Cy3.5-RMA561
Cy 3.5 (poly-anionic)
MW 1,44 kDa
ibrutinib
1
2
3
4
5

Figure 61


NUK_0304_RMA-561-3_200305165506
05.03.2020 17:19:40
sample in CH3CN/H2O
Orbitrap XL Organisch Chemisches Institut WWU Muenster
NUK_0304_RMA-561-3_200305165506 #2-17 RT: 0.01-0.13 AV: 16 NL: 1.05E8
T: FTMS - p NSI Full ms [200.00-1600.00]
441.44160 z=3
662.66630 z=4
365.24630 z=1
311.16859 z=1
453.43393 z=3
539.85899 z=3
673.65712 z=2
884.22500 z=3
Relative Abundance
m/z
441.44160 z=3
441.77578 z=3
442.10796 z=3
442.44220 z=3
453.43393 z=3
454.43412 z=3
NL: 1.05E8
NUK_0304_RMA-561-3_200305165506#2-17 RT: 0.01-0.13 AV: 16 T: FTMS - p NSI Full ms [200.00-1600.00]
441.44216
441.77647
442.10955
442.44308
442.77634
NL: 3.78E5
C64 H62 N9 O15 S4: C64 H62 N9 O15 S4 c (gss, s /p:40)(Val) Chrg -3 R: 20000 Res .Pwr . @FWHM

662.66630 z=4
663.16713 z=4
662.91715 z=4
663.41727 z=4
663.66718 z=4
663.91769 z=4
664.16738 z=4
664.66764 z=4
665.16763 z=4
NL: 4.02E7
NUK_0304_RMA-561-3_200305165506#2-17 RT: 0.01-0.13 AV: 16 T: FTMS - p NSI Full ms [200.00-1600.00]
662.66687
663.16835
663.66796
664.16826
664.66815
665.16822
NL: 3.78E5
C64 H62 N9 O15 S4 H: C64 H63 N9 O15 S4 c (gss, s /p:40)(Val) Chrg -2 R: 20000 Res .Pwr . @FWHM
662.91761
663.16776
663.41793
662.66687
663.66801
663.91807
664.16811
664.41815
664.66818
NL: 2.29E5
(C64 H62 N9 O15 S4 H)2: S8 O30 N18 H126 C128 c (gss, s /p:40)(Val) Chrg -4 R: 20000 Res .Pwr . @FWHM
Relative Abundance
m/z

Figure 62

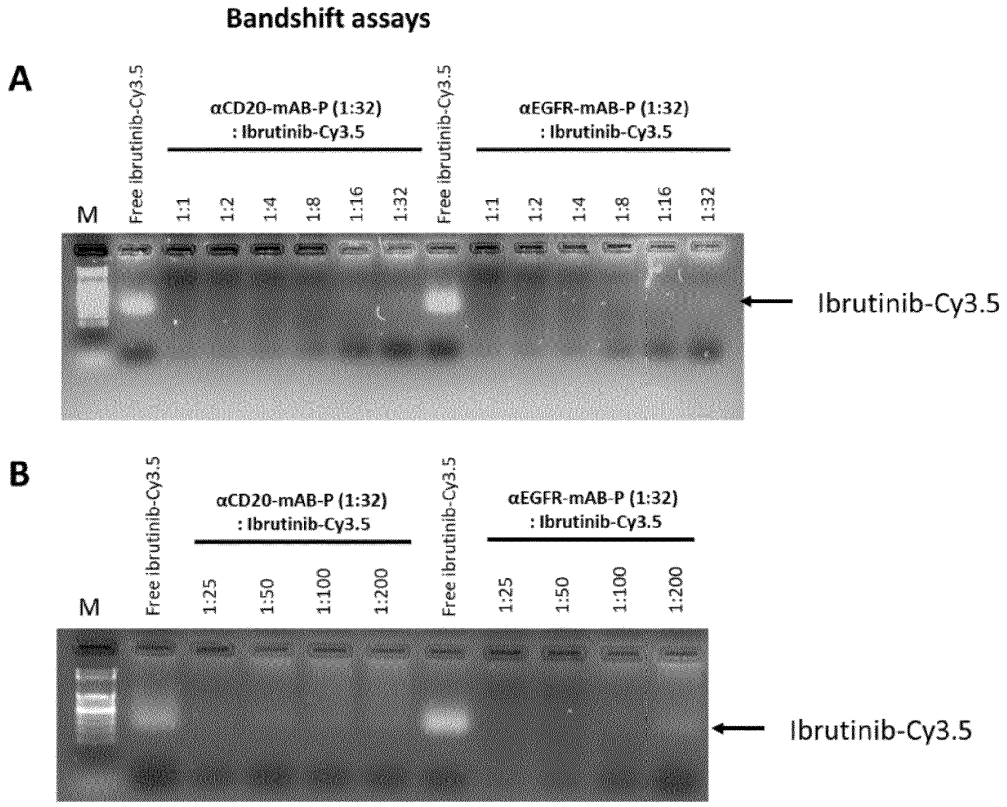


Figure 63

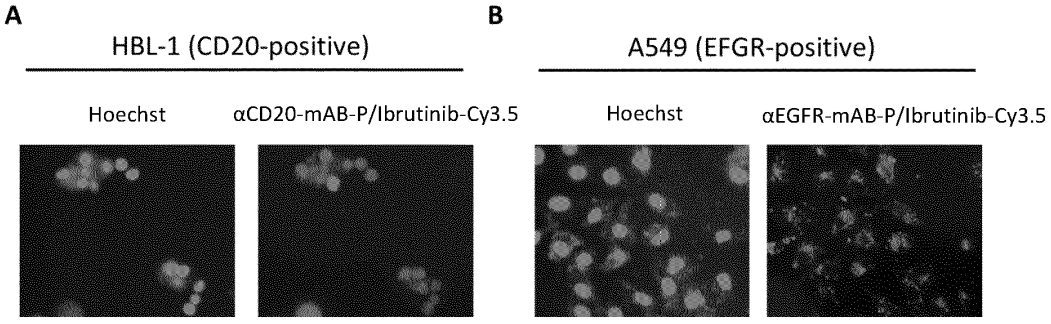


Figure 64

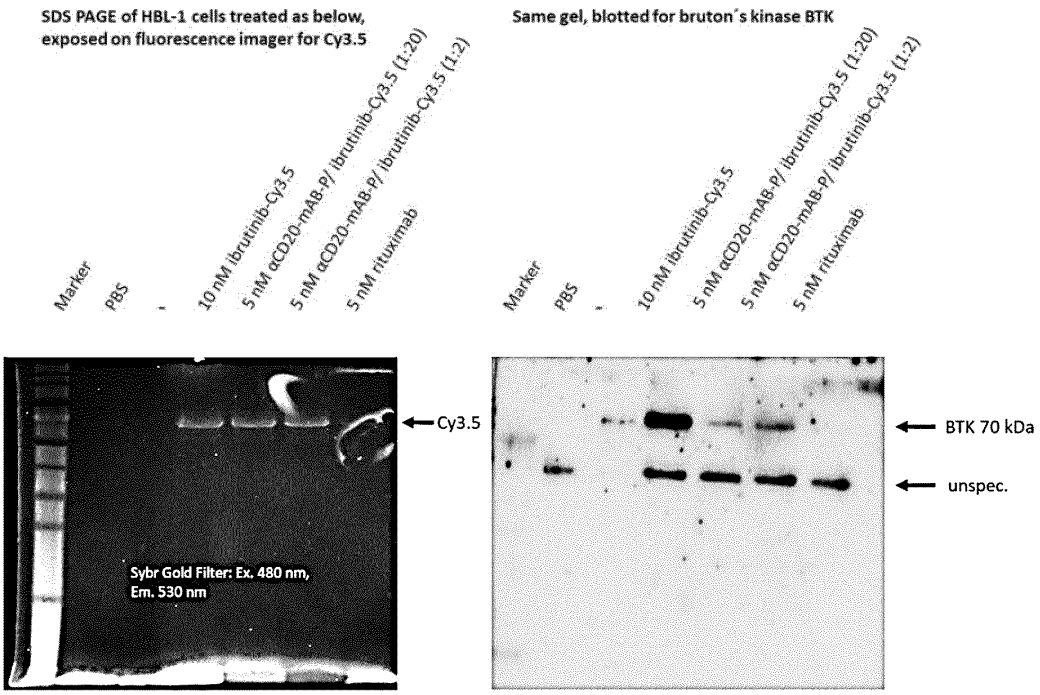


Figure 65

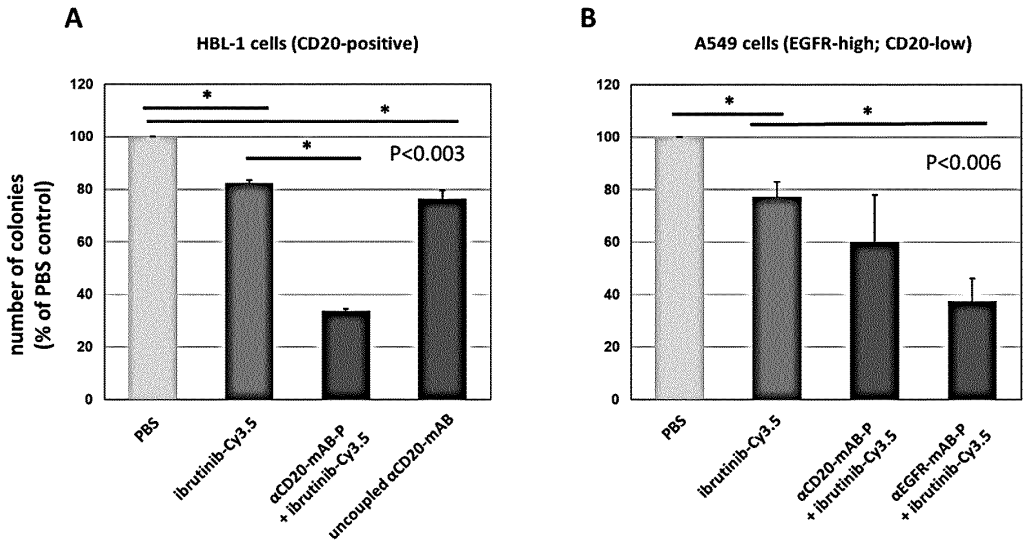


Figure 66

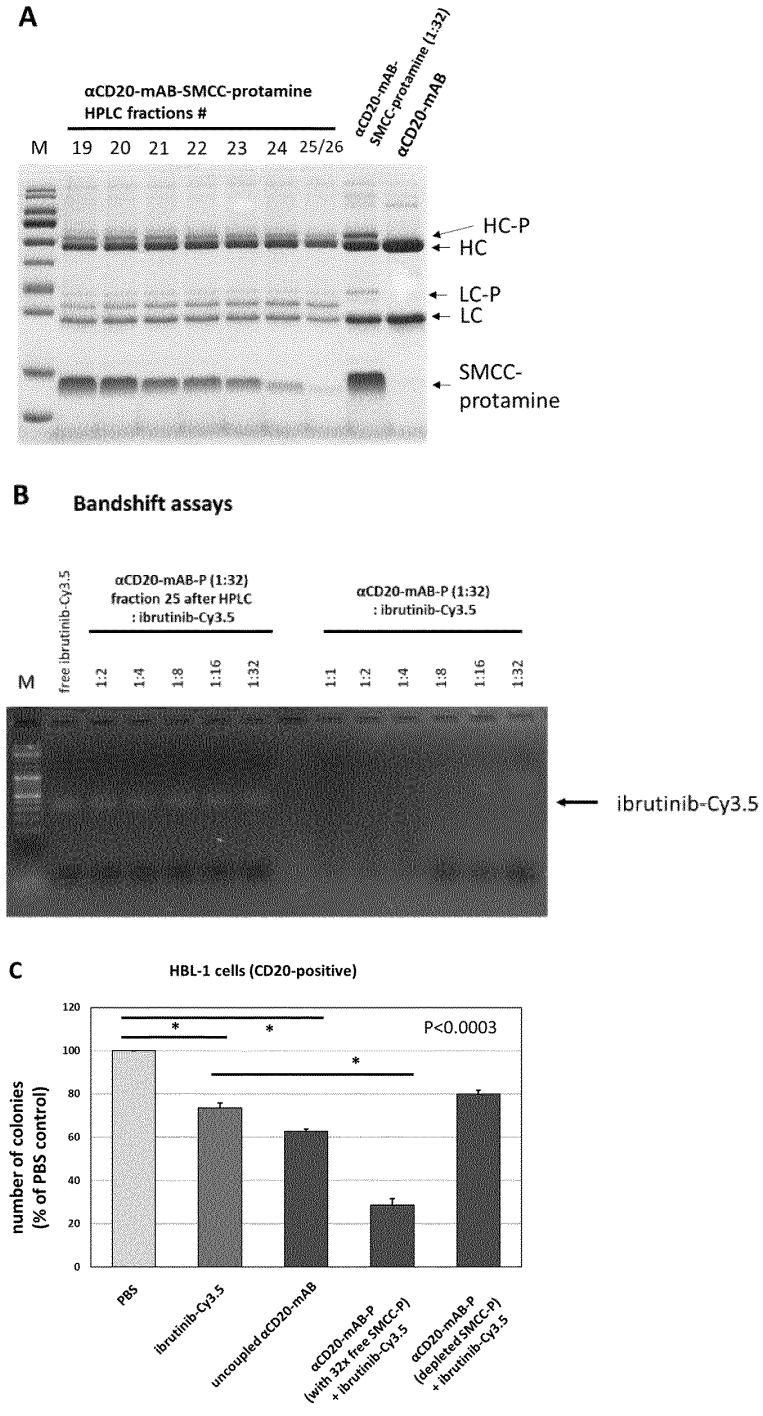

A
αCD20-mAB-SMCC-protamine
HPLC fractions #
M 19 20 21 22 23 24 25/26
αCD20-mAB-SMCC-protamine (1:32)
αCD20-mAB
HC-P
HC
LC-P
LC
SMCC-protamine
B
Bandshift assays
free ibrutinib-Cy3.5
αCD20-mAB-P (1:32) fraction 25 after HPLC : ibrutinib-Cy3.5
αCD20-mAB-P (1:32) : ibrutinib-Cy3.5
M 1:2 1:4 1:8 1:16 1:32 1:1 1:2 1:4 1:8 1:16 1:32
ibrutinib-Cy3.5
C
HBL-1 cells (CD20-positive)
number of colonies (% of PBS control)
P<0.0003
PBS
ibrutinib-Cy3.5
uncoupled αCD20-mAB
αCD20-mAB-P (with 32x free SMCC-P) + ibrutinib-Cy3.5
αCD20-mAB-P (depleted SMCC-P) + ibrutinib-Cy3.5

Figure 67

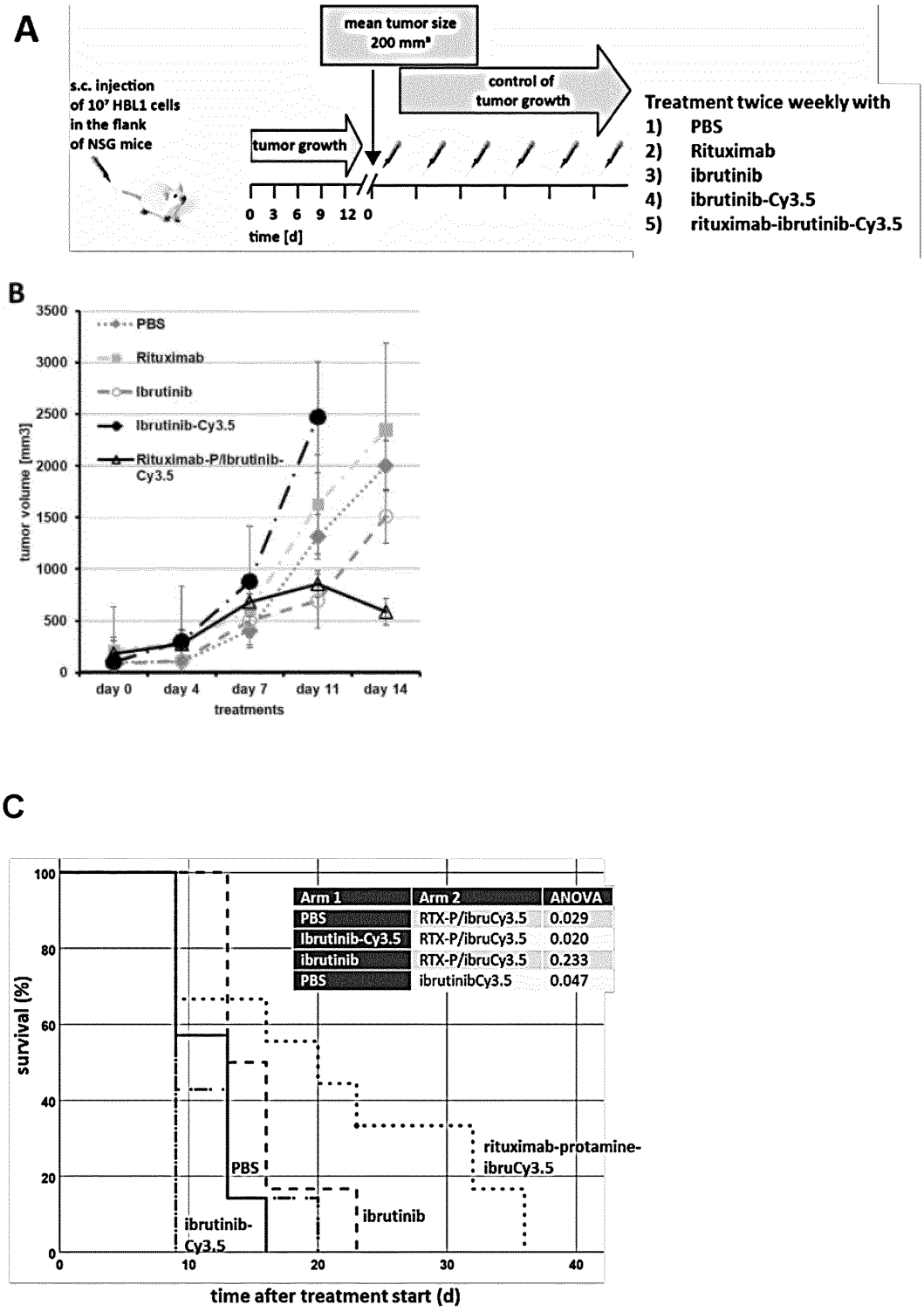

A
mean tumor size
200 mm³
control of
tumor growth
s.c. injection
of 10⁷ HBL1 cells
in the flank
of NSG mice
tumor growth
0 3 6 9 12 0
time [d]
Treatment twice weekly with
1) PBS
2) Rituximab
3) ibrutinib
4) ibrutinib-Cy3.5
5) rituximab-ibrutinib-Cy3.5
B
PBS
Rituximab
Ibrutinib
Ibrutinib-Cy3.5
Rituximab-P/Ibrutinib-Cy3.5
tumor volume [mm3]
day 0
day 4
day 7
day 11
day 14
treatments
C
Arm 1 Arm 2 ANOVA
PBS RTX-P/ibruCy3.5 0.029
Ibrutinib-Cy3.5 RTX-P/ibruCy3.5 0.020
ibrutinib RTX-P/ibruCy3.5 0.233
PBS ibrutinibCy3.5 0.047
survival (%)
rituximab-protamine-ibruCy3.5
PBS
ibrutinib
ibrutinib-Cy3.5
time after treatment start (d)

Figure 68

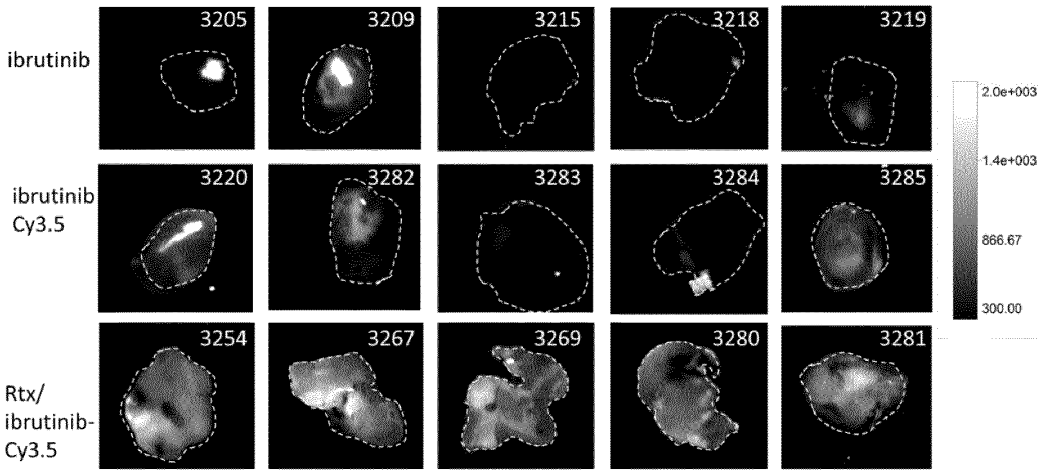


Figure 69

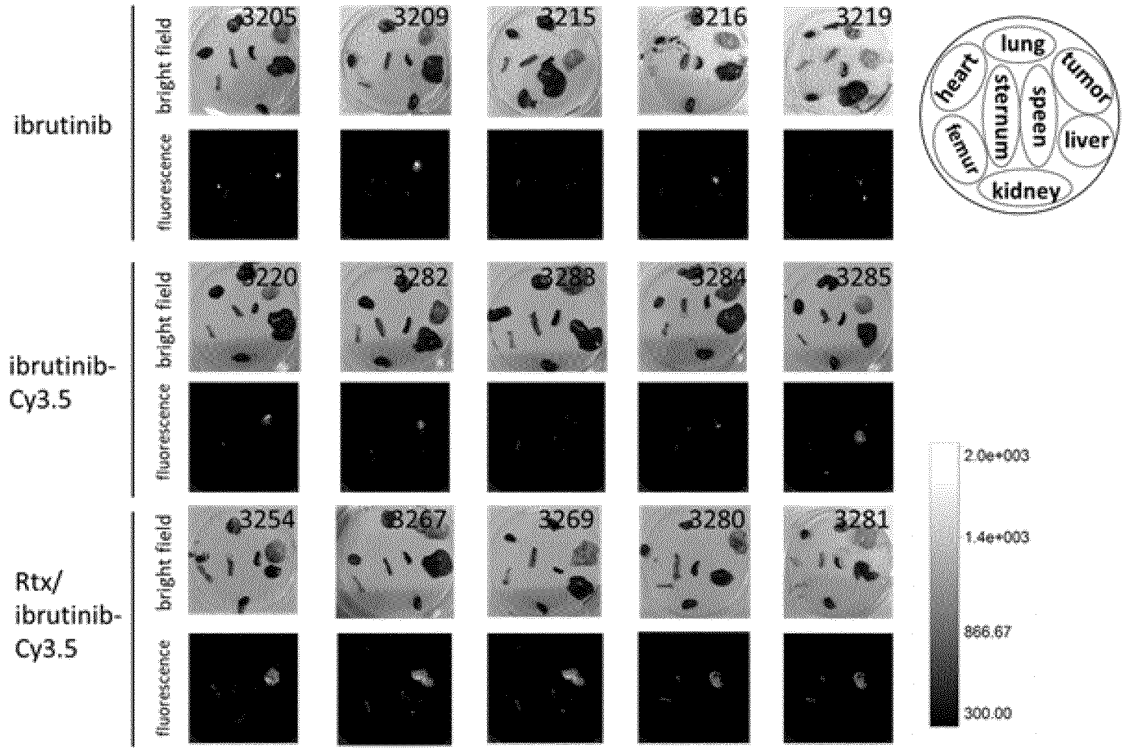


Figure 70

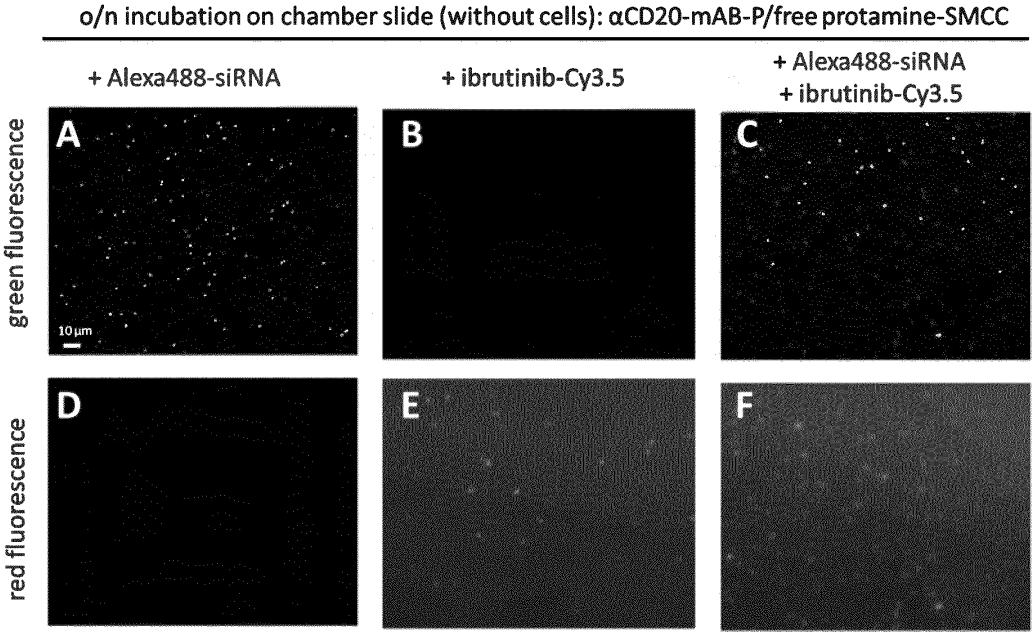


Figure 71

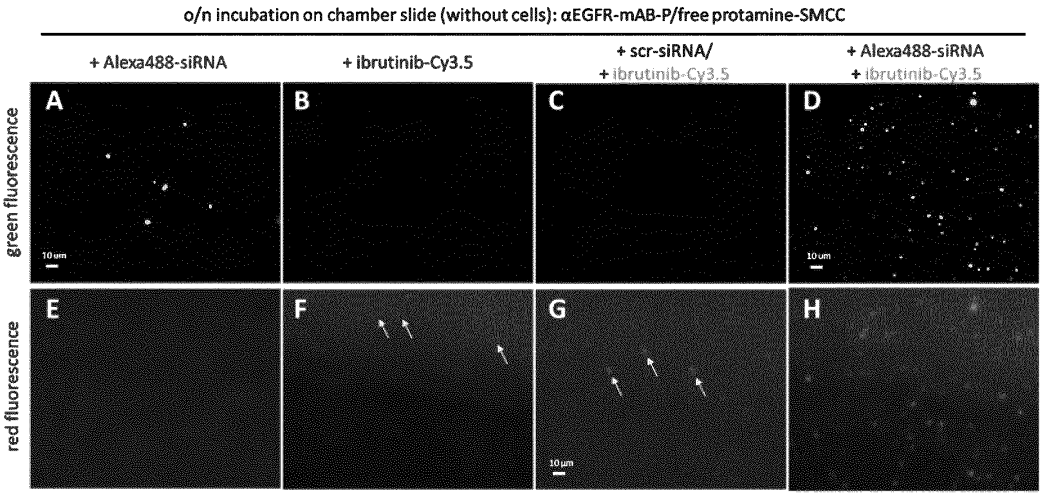


Figure 72

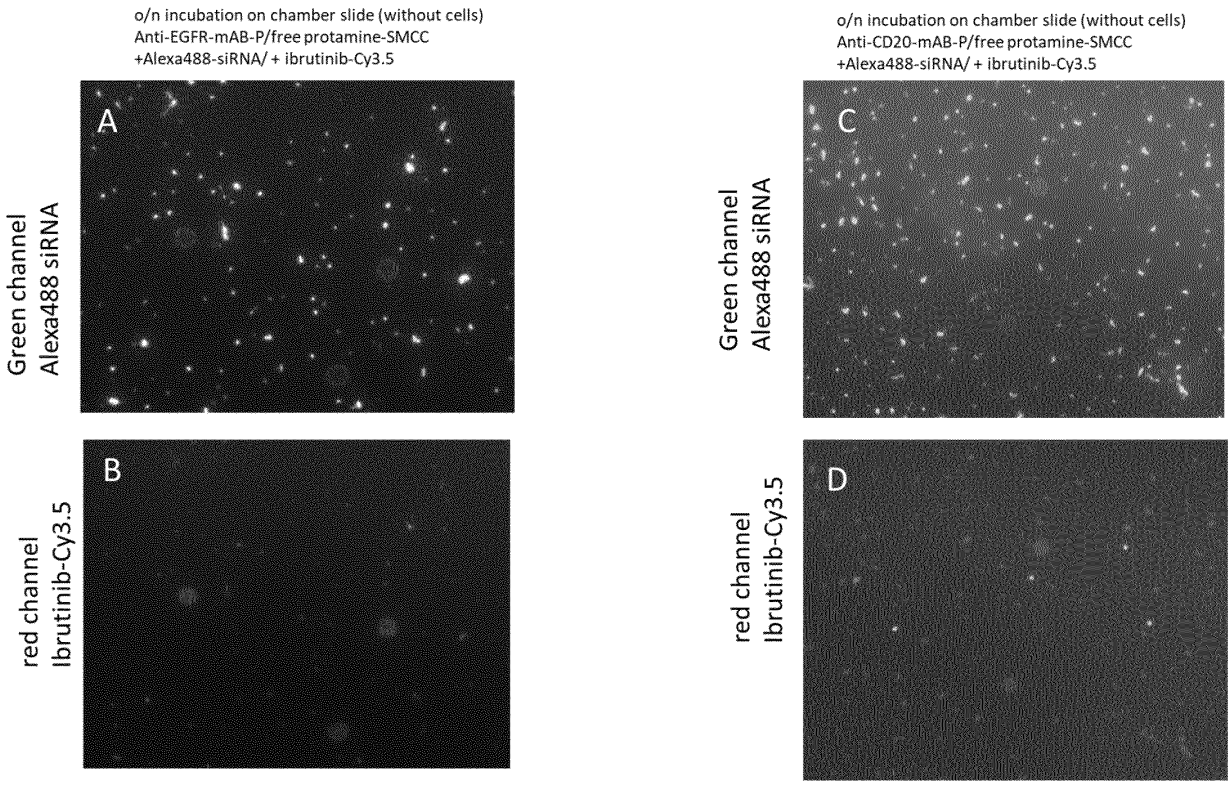
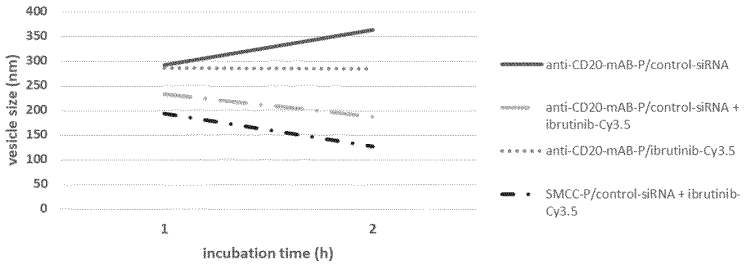
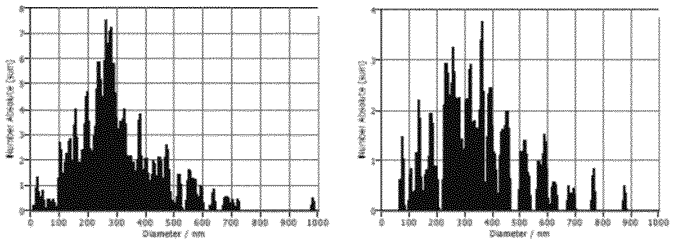

o/n incubation on chamber slide (without cells)
Anti-EGFR-mAB-P/free protamine-SMCC
+Alexa488-siRNA/ + ibrutinib-Cy3.5
A
Green channel
Alexa488 siRNA
B
red channel
Ibrutinib-Cy3.5
o/n incubation on chamber slide (without cells)
Anti-CD20-mAB-P/free protamine-SMCC
+Alexa488-siRNA/ + ibrutinib-Cy3.5
C
Green channel
Alexa488 siRNA
D
red channel
Ibrutinib-Cy3.5

Figure 73

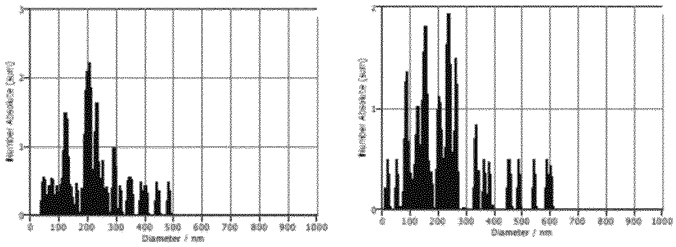
A



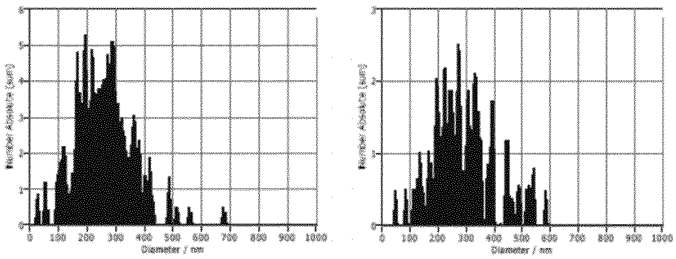
B αCD20-mAB-P + control-siRNA



C αCD20-mAB-P + ibrutinib-Cy3.5



D αCD20-mAB-P + control-siRNA + ibrutinib-Cy3.5



E free SMCC-P + control-siRNA + ibrutinib-Cy3.5

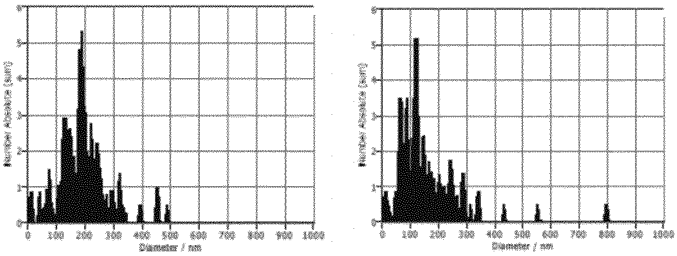
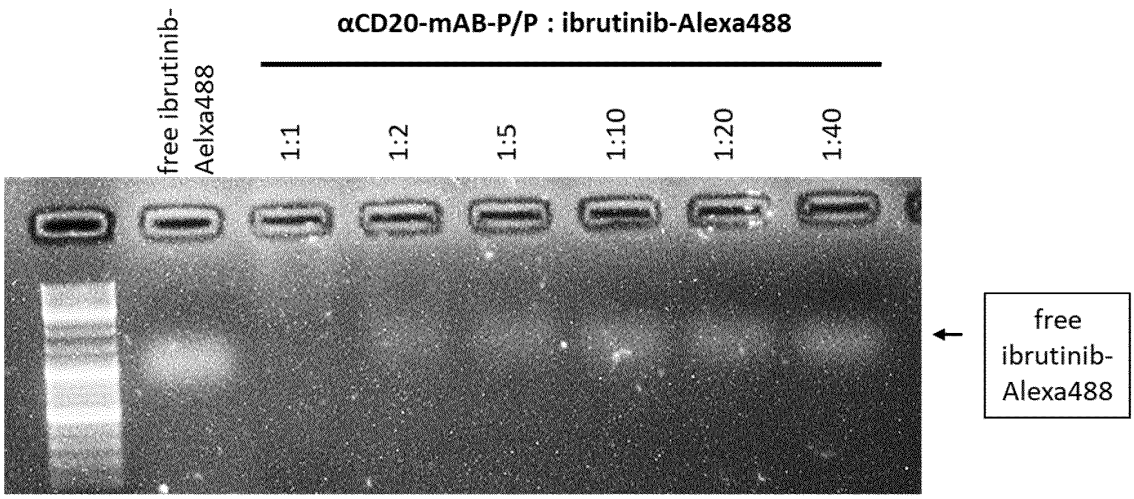


Figure 74

A


αCD20-mAB-P/P : ibrutinib-Alexa488
free ibrutinib-Aelxa488
1:1
1:2
1:5
1:10
1:20
1:40
free ibrutinib-Alexa488

B

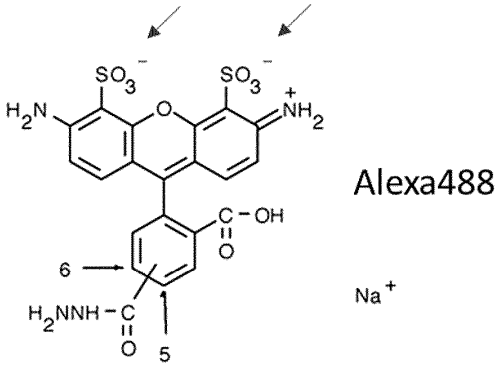

SO3 -
SO3 -
H2N
O
+NH2
C-OH
O
6
H2NNH-C
O
5
Alexa488
Na+

Figure 74 (cont'd)

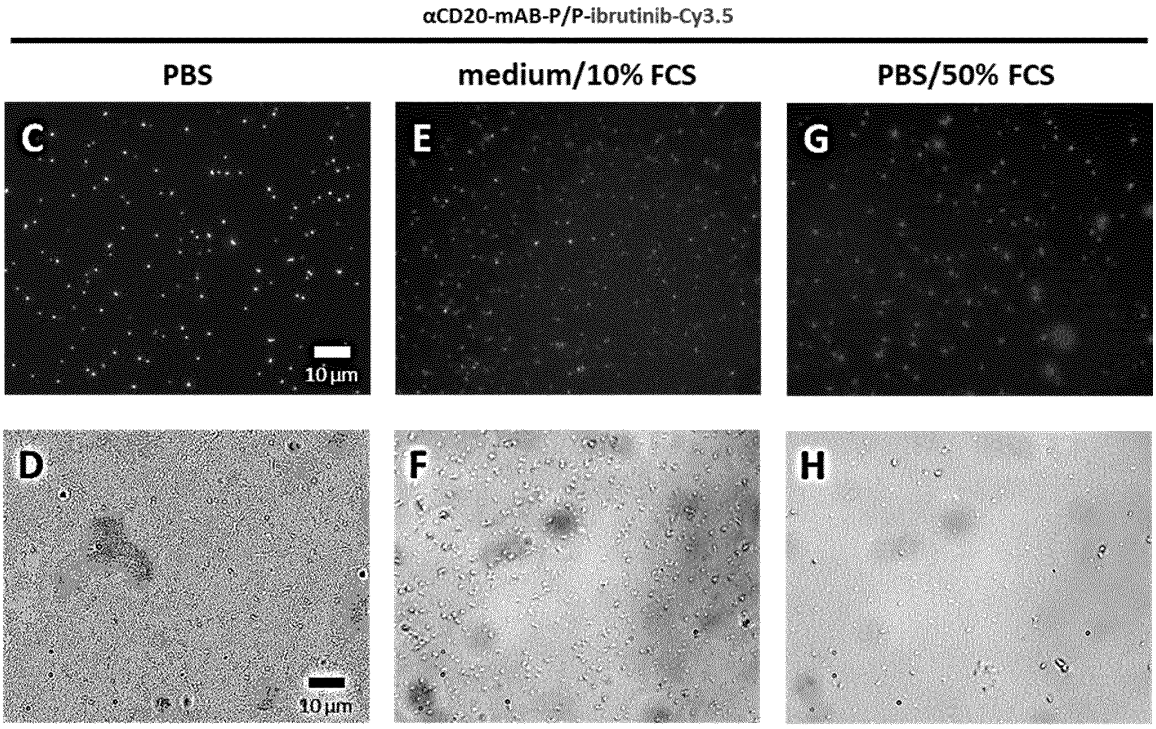


Figure 75

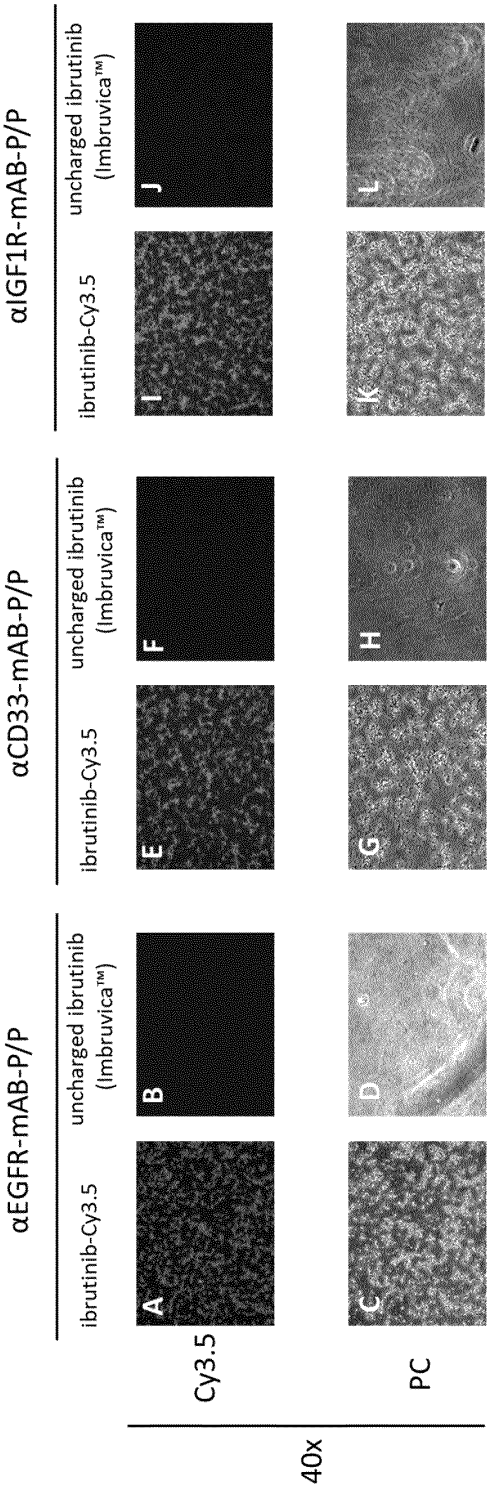

αEGFR-mAB-P/P
αCD33-mAB-P/P
αIGF1R-mAB-P/P
ibrutinib-Cy3.5
uncharged ibrutinib (Imbruvica™)
ibrutinib-Cy3.5
uncharged ibrutinib (Imbruvica™)
ibrutinib-Cy3.5
uncharged ibrutinib (Imbruvica™)
A
B
E
F
I
J
C
D
G
H
K
L
Cy3.5
PC
40x

Figure 76

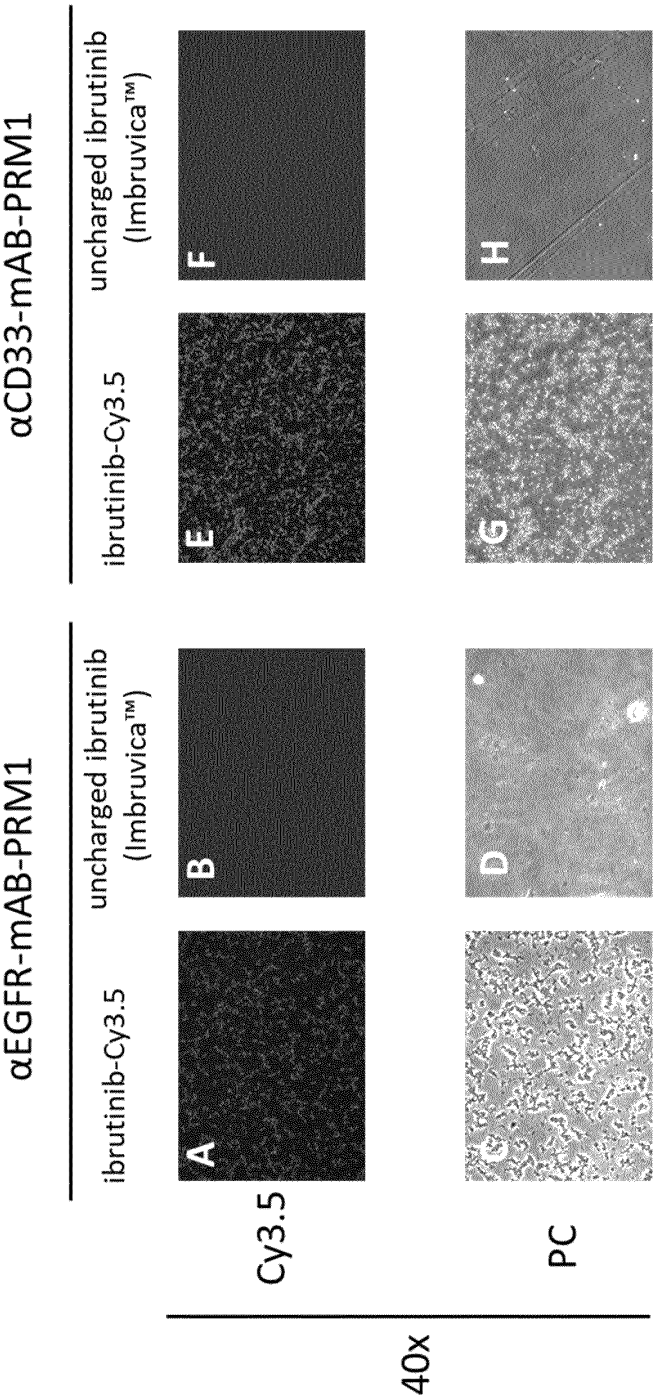

αEGFR-mAB-PRM1
αCD33-mAB-PRM1
ibrutinib-Cy3.5
uncharged ibrutinib (Imbruvica™)
ibrutinib-Cy3.5
uncharged ibrutinib (Imbruvica™)
A
B
E
F
D
G
H
Cy3.5
PC
40x

Figure 77

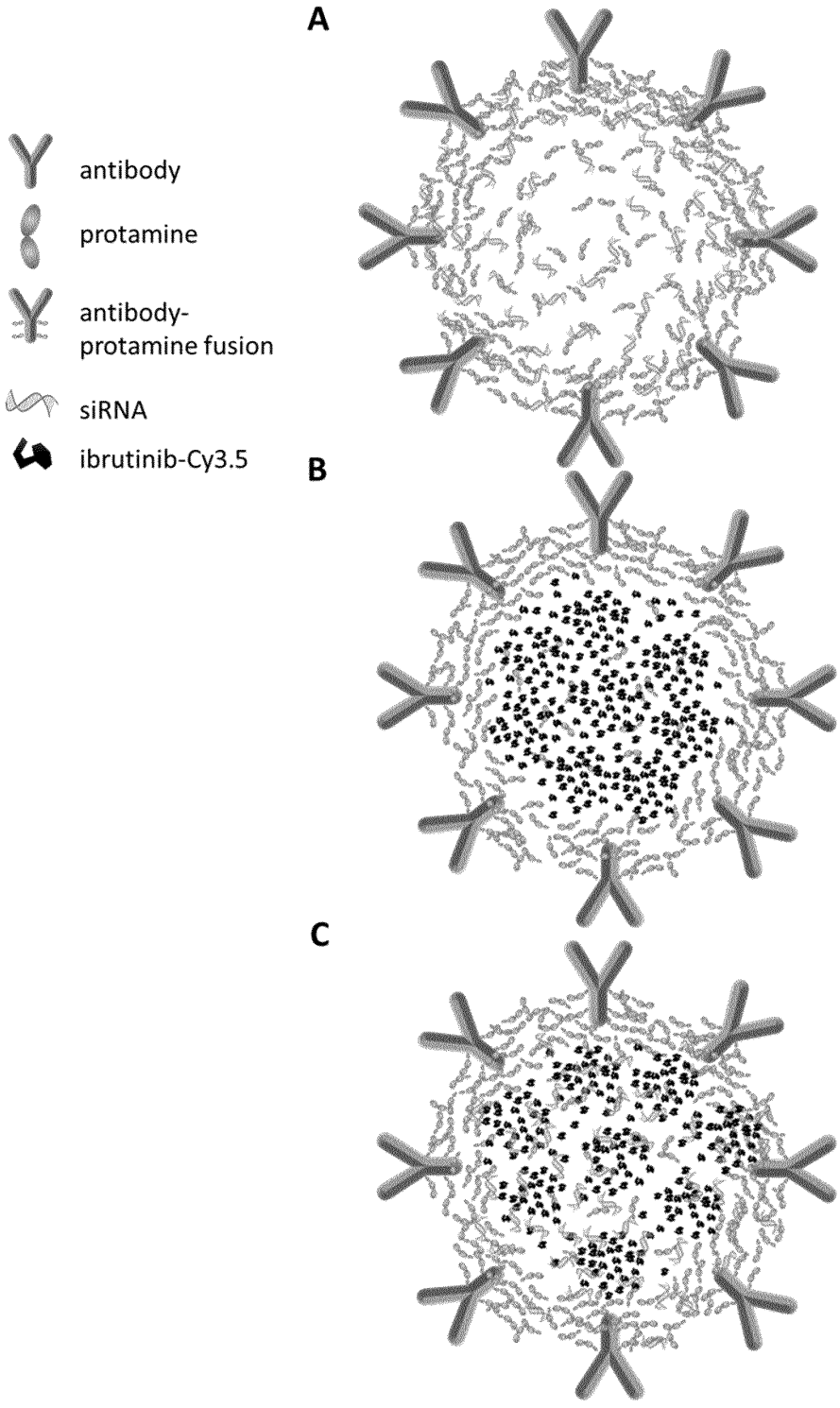

antibody
protamine
antibody-
protamine fusion
siRNA
ibrutinib-Cy3.5
A
B
C

Figure 78

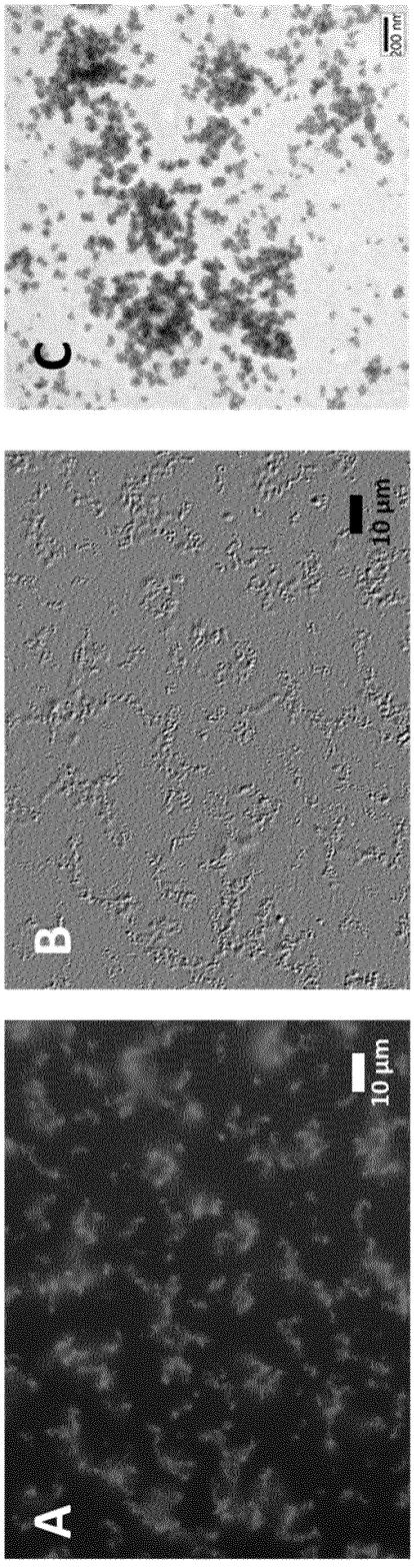


Figure 79

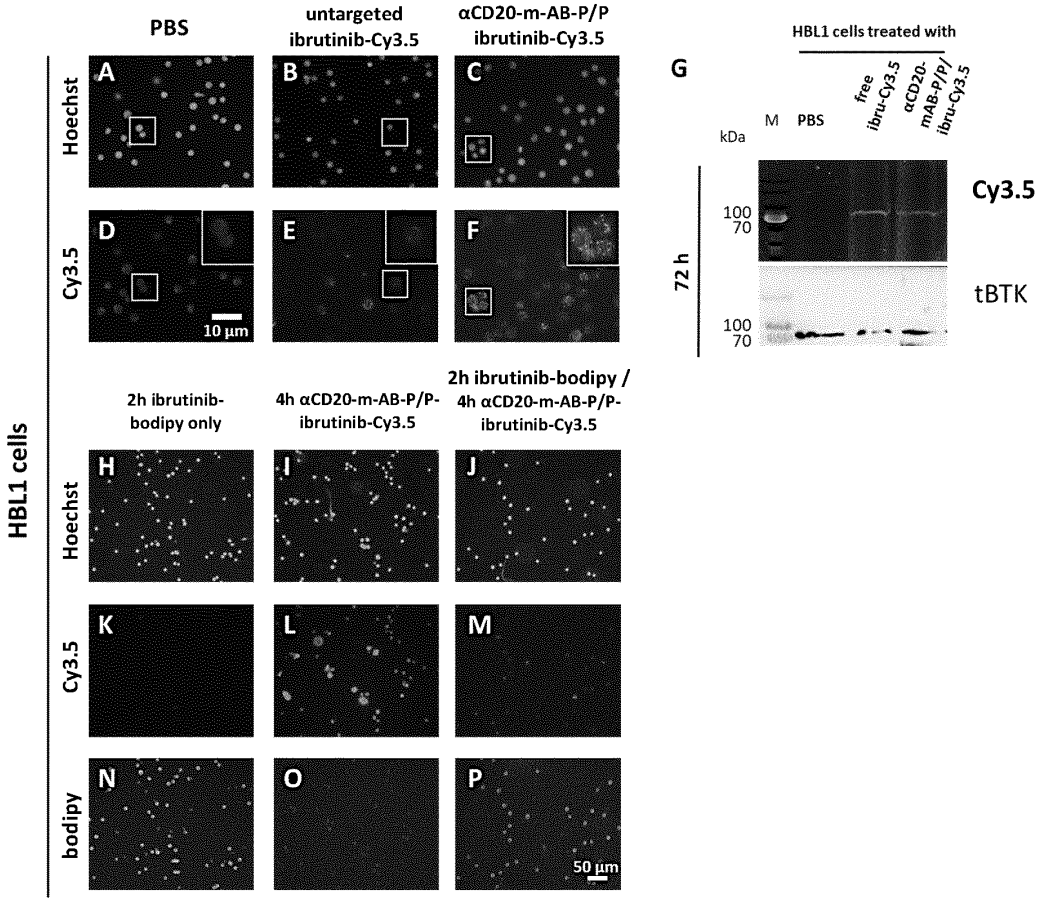

PBS
untargeted ibrutinib-Cy3.5
αCD20-m-AB-P/P ibrutinib-Cy3.5
HBL1 cells
Hoechst
Cy3.5
A
B
C
D
E
F
10 µm
2h ibrutinib-bodipy only
4h αCD20-m-AB-P/P-ibrutinib-Cy3.5
2h ibrutinib-bodipy / 4h αCD20-m-AB-P/P-ibrutinib-Cy3.5
H
I
J
K
L
M
bodipy
N
O
P
50 µm
G
HBL1 cells treated with
free ibru-Cy3.5
αCD20-mAB-P/P/ ibru-Cy3.5
M
PBS
kDa
72 h
100
70
Cy3.5
tBTK

Figure 80

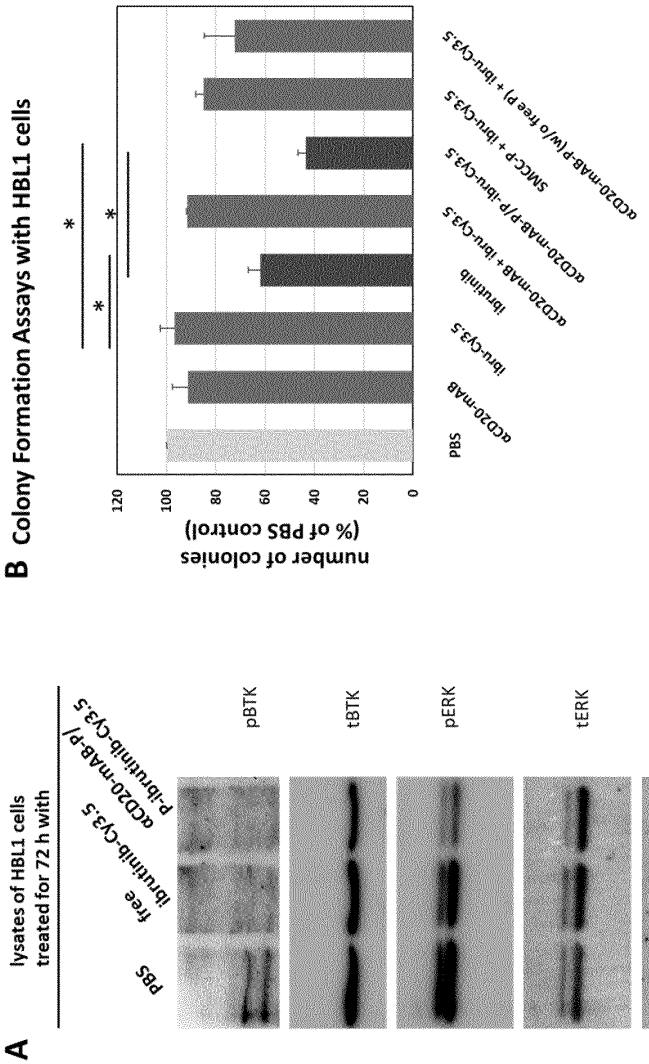

B Colony Formation Assays with HBL1 cells
number of colonies (% of PBS control)
0
20
40
60
80
100
120
PBS
αCD20-mAB
Ibru-Cy3.5
Ibrutinib
αCD20-mAB + Ibru-Cy3.5
αCD20-mAB-P/P-Ibru-Cy3.5
SMCC-P + Ibru-Cy3.5
αCD20-mAB-P(w/o free P) + Ibru-Cy3.5
A
lysates of HBL1 cells treated for 72 h with
PBS
free Ibrutinib-Cy3.5
αCD20-mAB-P/P-Ibrutinib-Cy3.5
pBTK
tBTK
pERK
tERK
β-Aktin

Figure 81

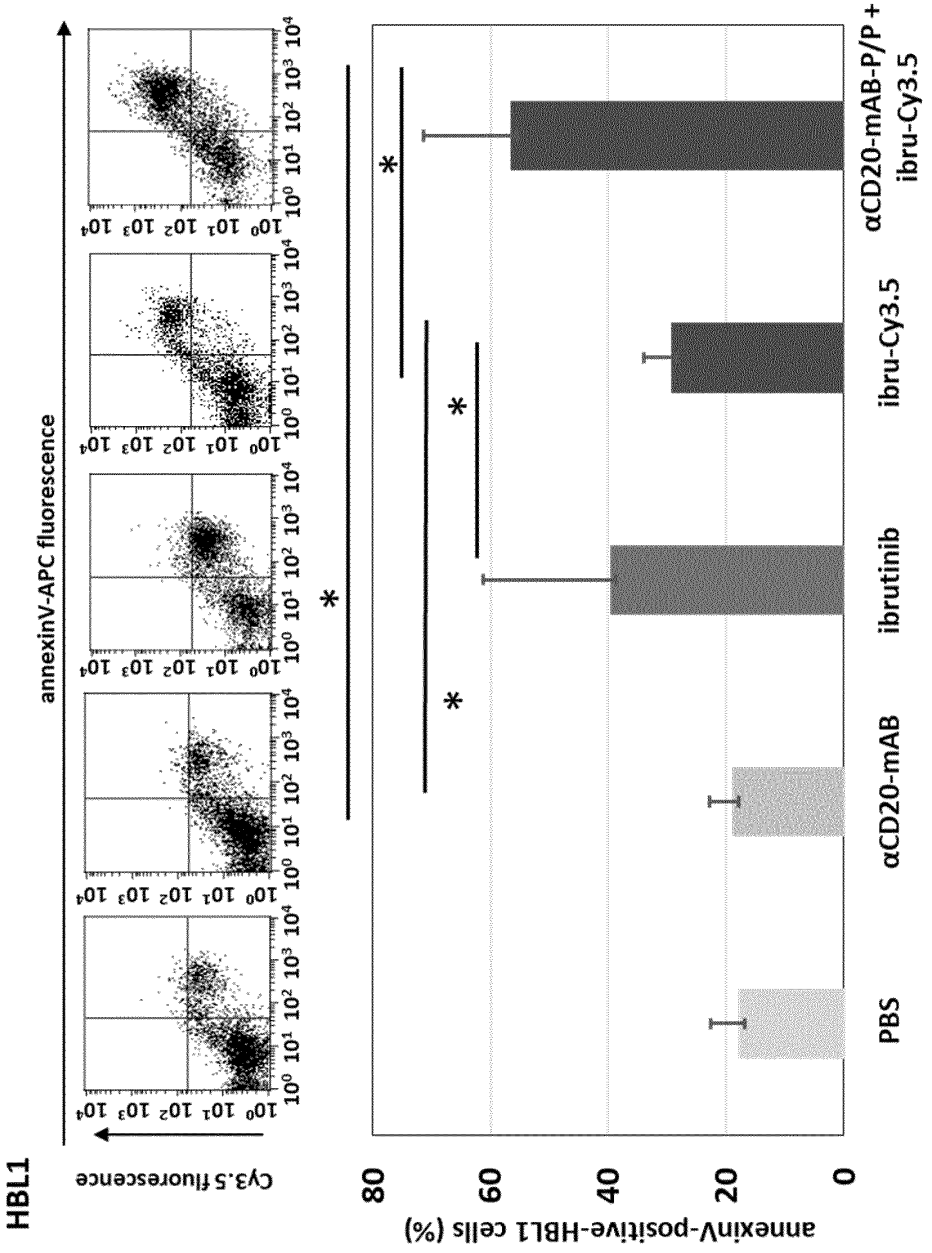

HBL1
annexinV-APC fluorescence
Cy3.5 fluorescence
annexinV-positive-HBL1 cells (%)
0
20
40
60
80
PBS
αCD20-mAB
ibrutinib
ibru-Cy3.5
αCD20-mAB-P/P + ibru-Cy3.5
*
*
*
*

Figure 82

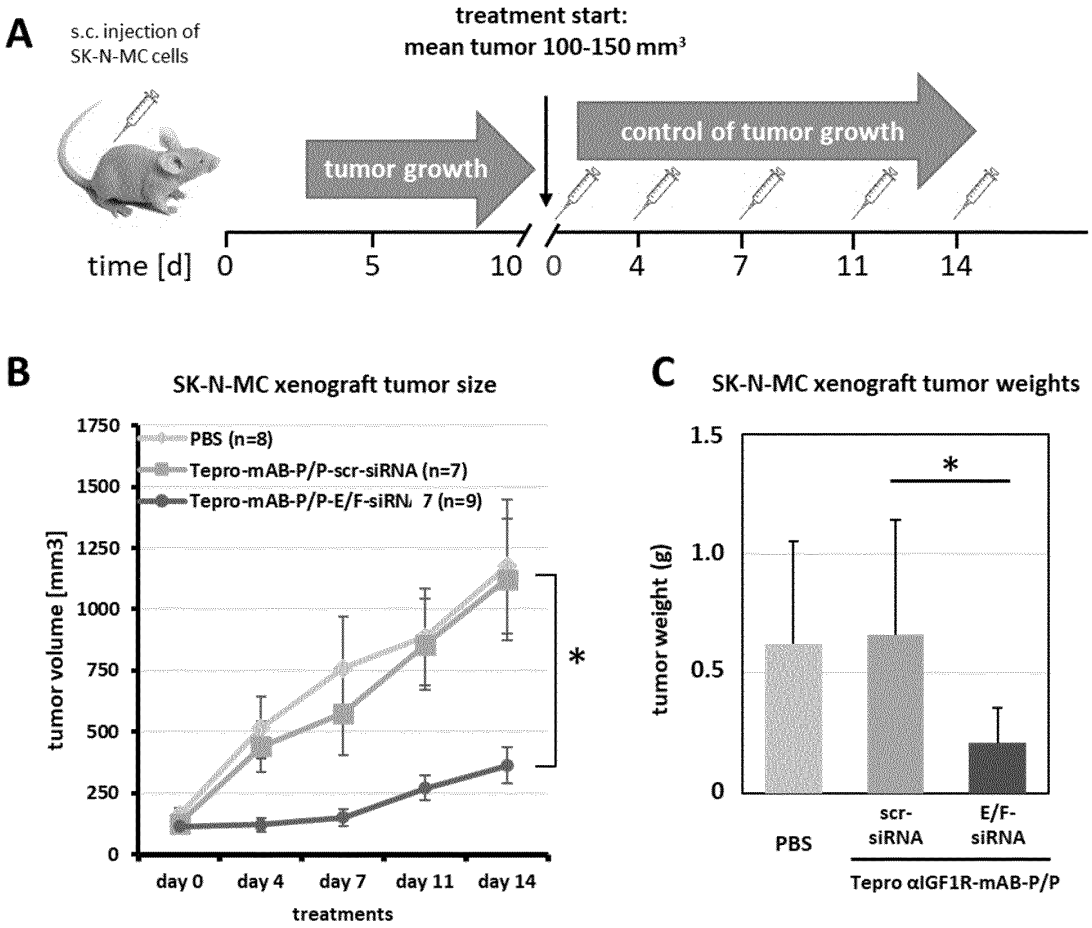

A
s.c. injection of SK-N-MC cells
treatment start: mean tumor 100-150 mm³
tumor growth
control of tumor growth
time [d] 0 5 10 0 4 7 11 14
B
SK-N-MC xenograft tumor size
PBS (n=8)
Tepro-mAB-P/P-scr-siRNA (n=7)
Tepro-mAB-P/P-E/F-siRNA (n=9)
tumor volume [mm3]
1750
1500
1250
1000
750
500
250
0
day 0 day 4 day 7 day 11 day 14
treatments
*
C
SK-N-MC xenograft tumor weights
tumor weight (g)
1.5
1.0
0.5
0
*
PBS
scr-siRNA
E/F-siRNA
Tepro αIGF1R-mAB-P/P

Figure 83

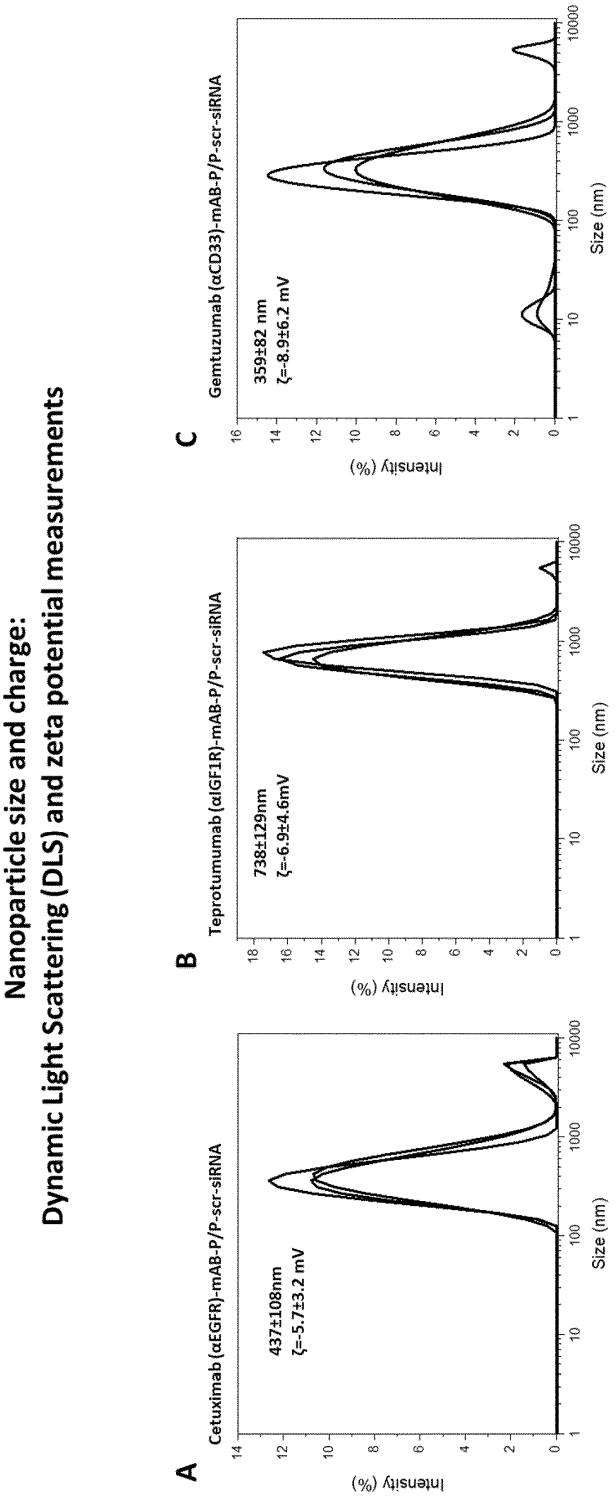

Nanoparticle size and charge:
Dynamic Light Scattering (DLS) and zeta potential measurements
A
Cetuximab (αEGFR)-mAB-P/P-scr-siRNA
437±108nm
ζ=-5.7±3.2 mV
B
Teprotumumab (αIGF1R)-mAB-P/P-scr-siRNA
738±129nm
ζ=-6.9±4.6mV
C
Gemtuzumab (αCD33)-mAB-P/P-scr-siRNA
359±82 nm
ζ=-8.9±6.2 mV
Intensity (%)
Size (nm)

Figure 84

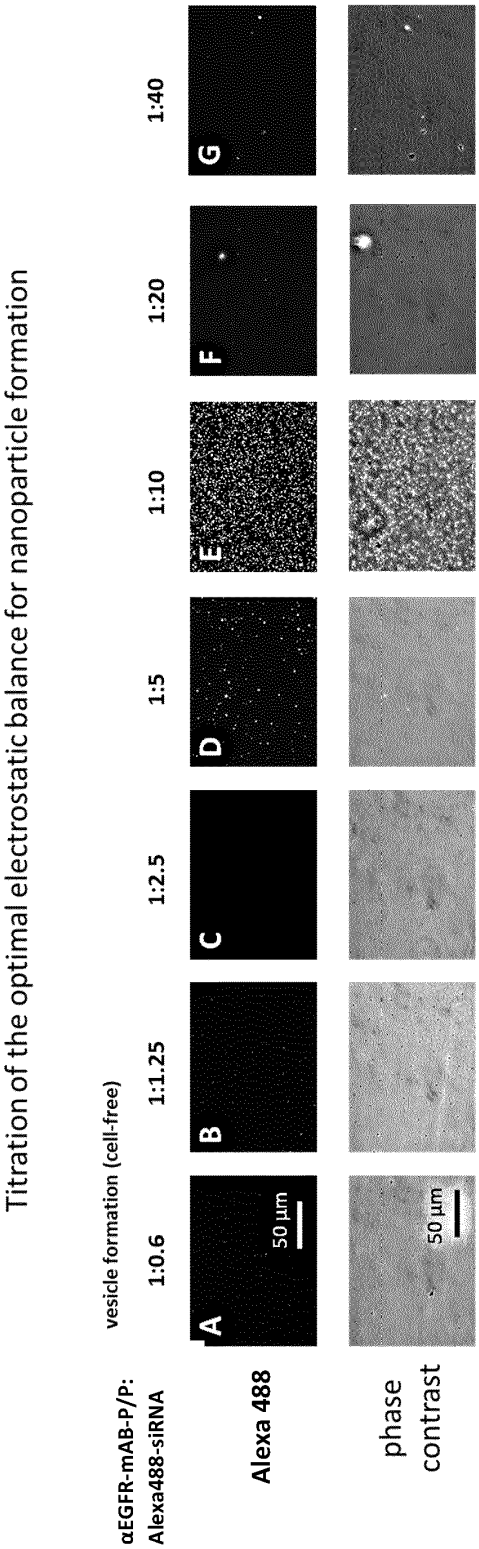

Titration of the optimal electrostatic balance for nanoparticle formation
vesicle formation (cell-free)
αEGFR-mAB-P/P:
Alexa488-siRNA
1:0.6
1:1.25
1:2.5
1:5
1:10
1:20
1:40
A
B
C
D
E
F
G
50 µm
50 µm
Alexa 488
phase contrast

Figure 85

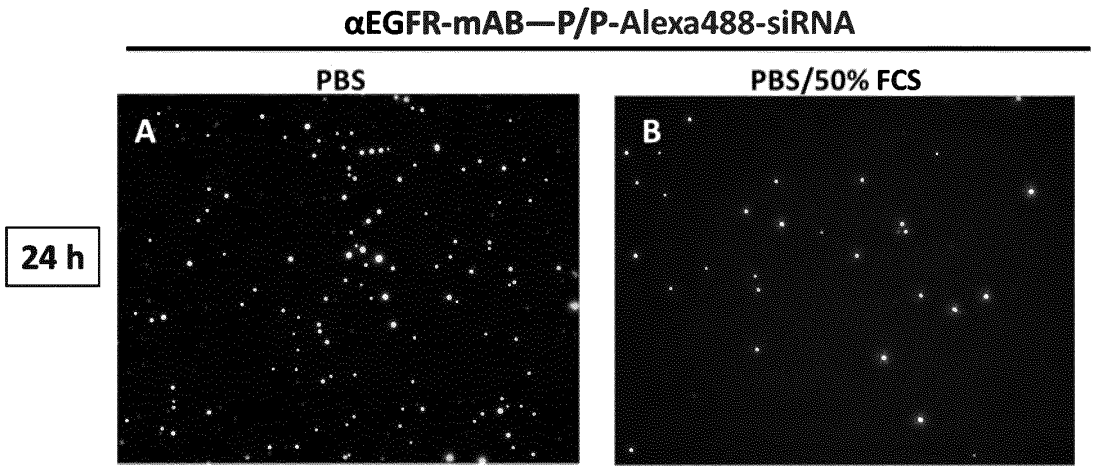


Figure 86

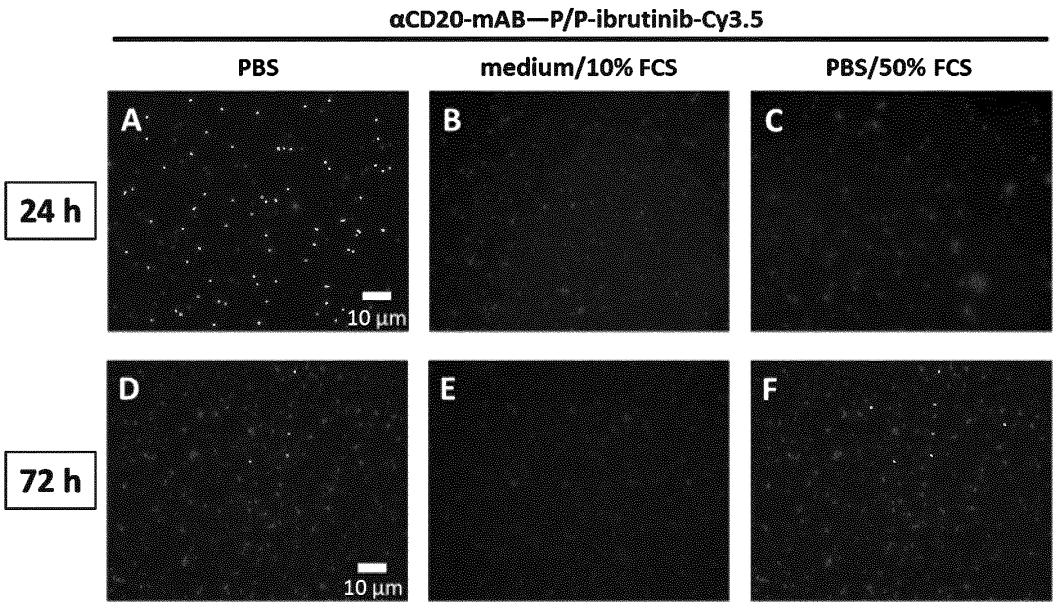


Figure 87

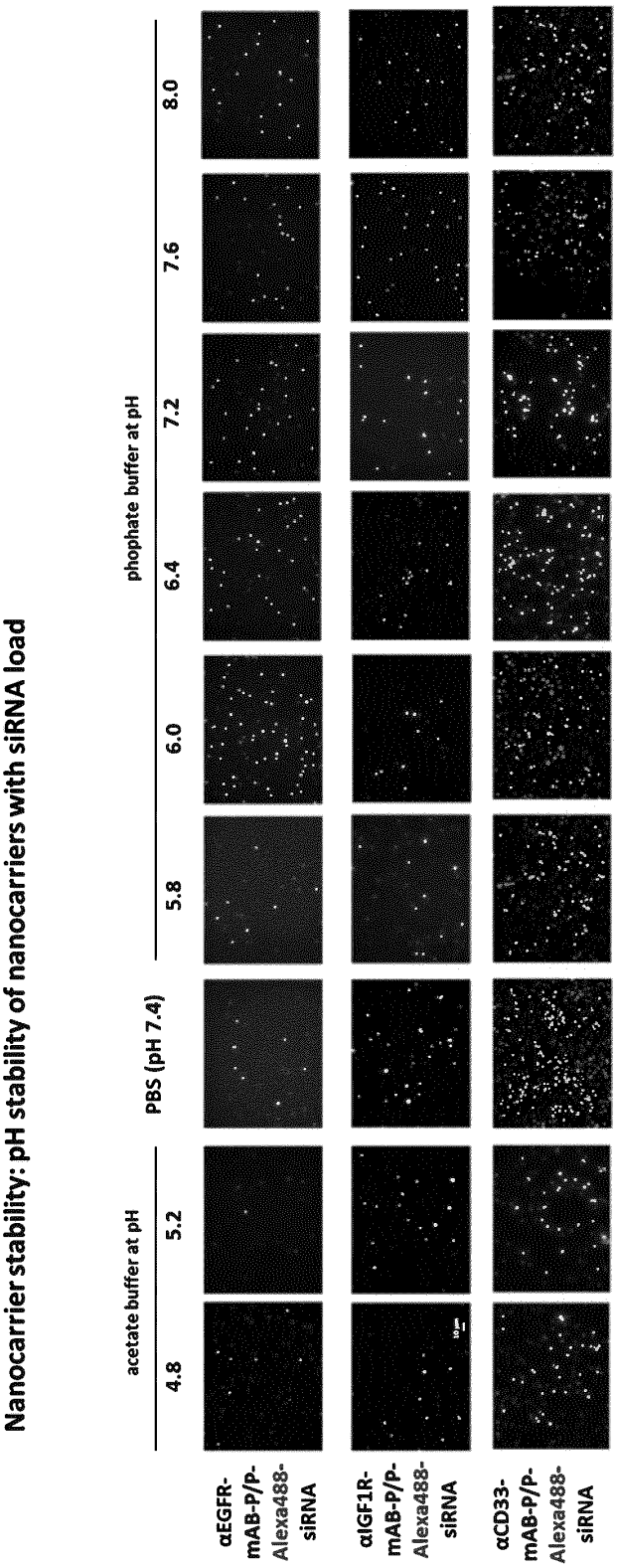

Nanocarrier stability: pH stability of nanocarriers with siRNA load
acetate buffer at pH
4.8
5.2
PBS (pH 7.4)
phophate buffer at pH
5.8
6.0
6.4
7.2
7.6
8.0
αEGFR-mAB-P/P-Alexa488-siRNA
αIGF1R-mAB-P/P-Alexa488-siRNA
αCD33-mAB-P/P-Alexa488-siRNA

Figure 88

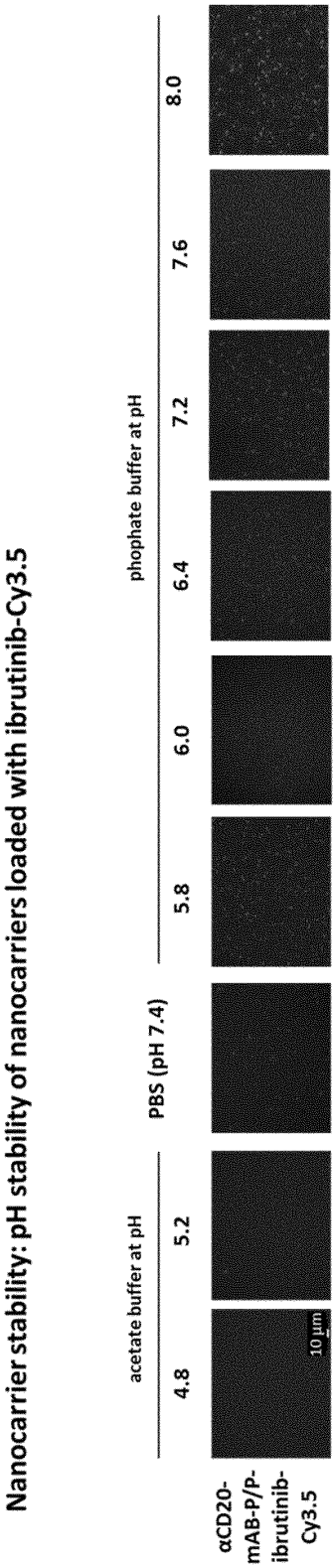

Nanocarrier stability: pH stability of nanocarriers loaded with ibrutinib-Cy3.5
acetate buffer at pH
4.8
5.2
PBS (pH 7.4)
phophate buffer at pH
5.8
6.0
6.4
7.2
7.6
8.0
αCD20-mAB-P/P-ibrutinib-Cy3.5
10 µm

Figure 89

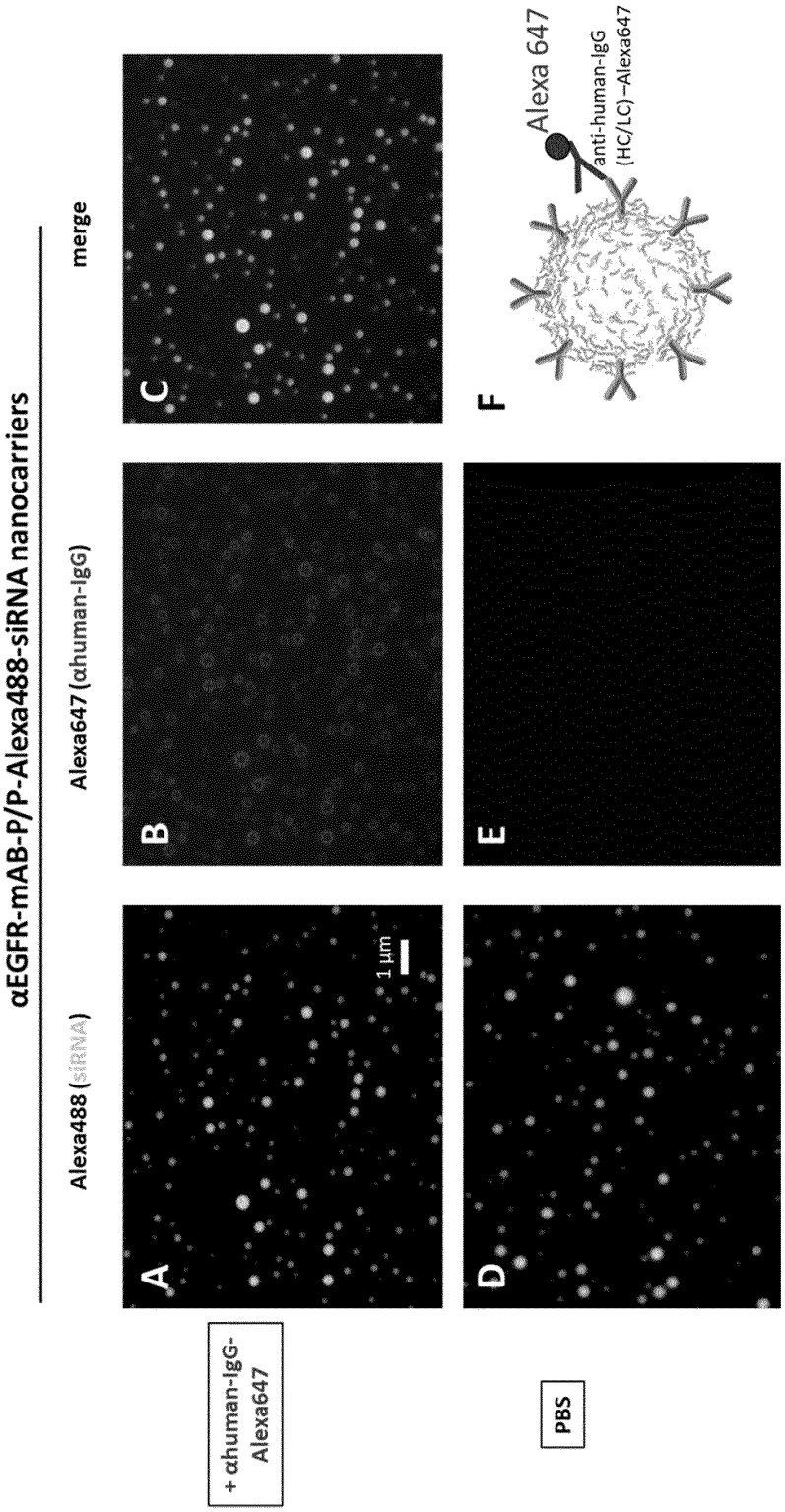

αEGFR-mAB-P/P-Alexa488-siRNA nanocarriers
Alexa488 (siRNA)
Alexa647 (αhuman-IgG)
merge
A
B
C
D
E
F
1 µm
+ αhuman-IgG-Alexa647
PBS
Alexa 647
anti-human-IgG (HC/LC)–Alexa647

Figure 90

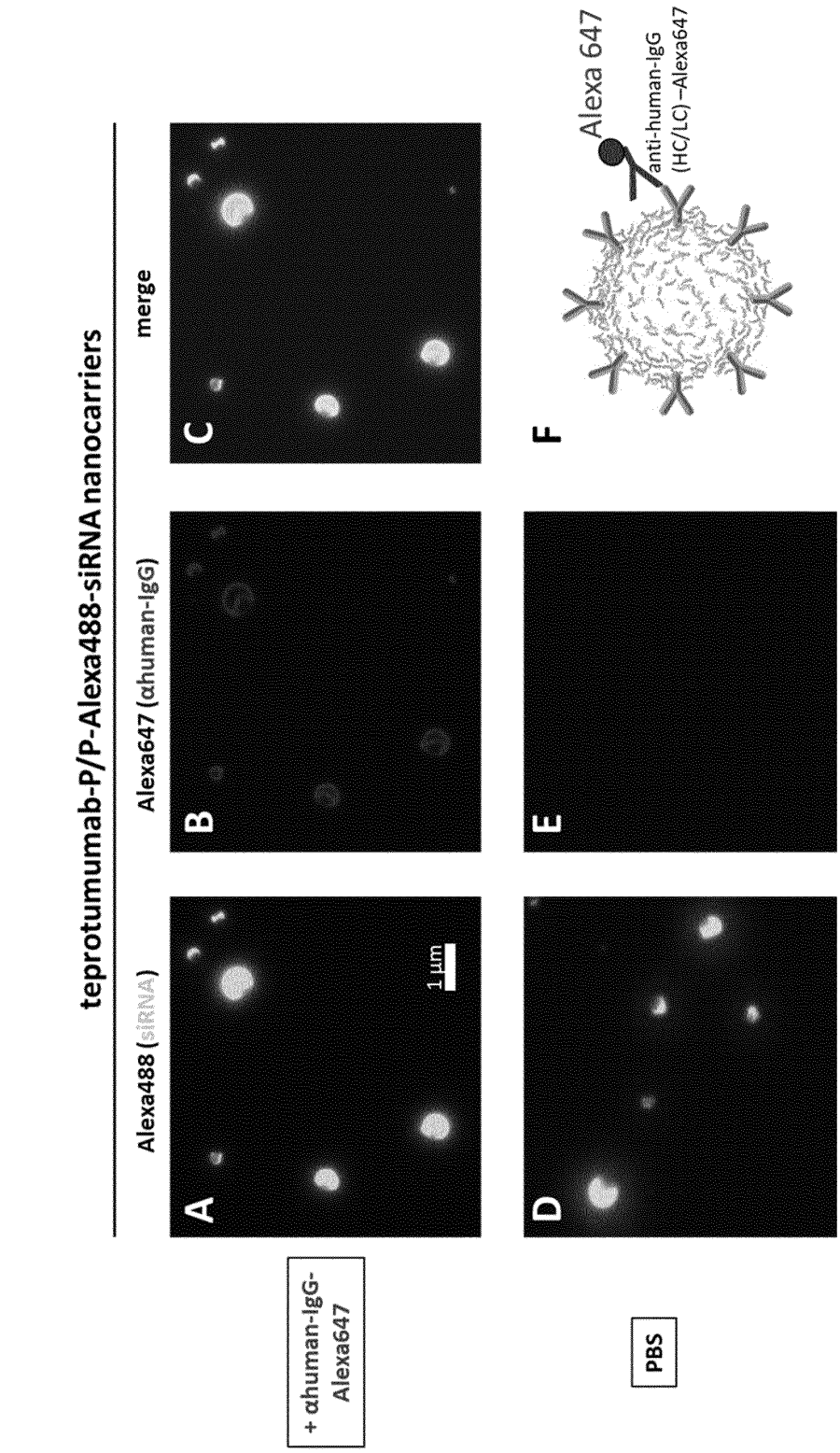
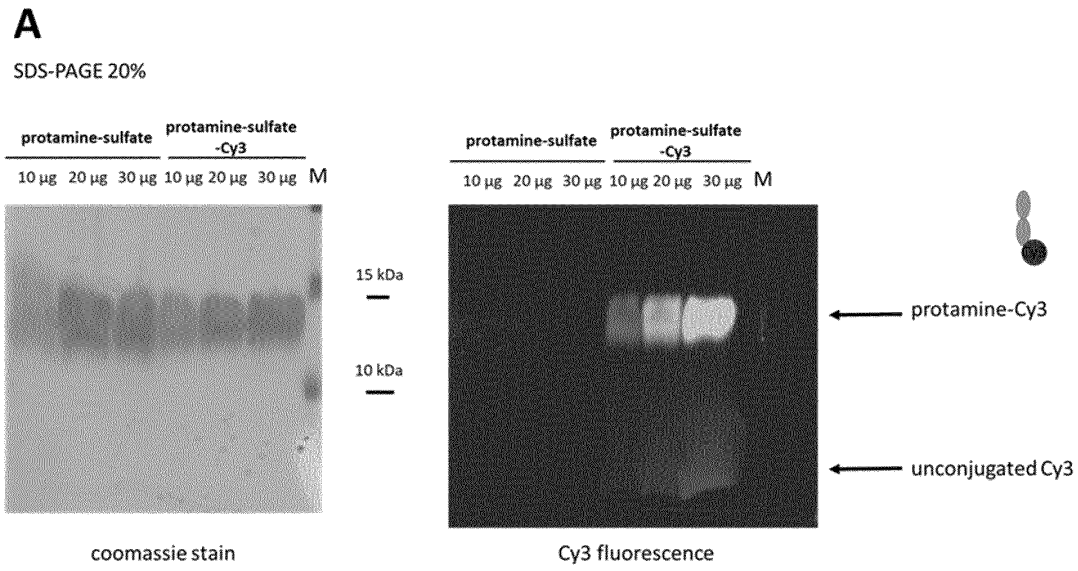

teprotumumab-P/P-Alexa488-siRNA nanocarriers
Alexa488 (siRNA)
Alexa647 (αhuman-IgG)
merge
A
B
C
D
E
F
1 µm
+ αhuman-IgG-Alexa647
PBS
Alexa 647
anti-human-IgG (HC/LC)–Alexa647

Figure 91

Visualisation of the free protamine in the nanocarrier complex:



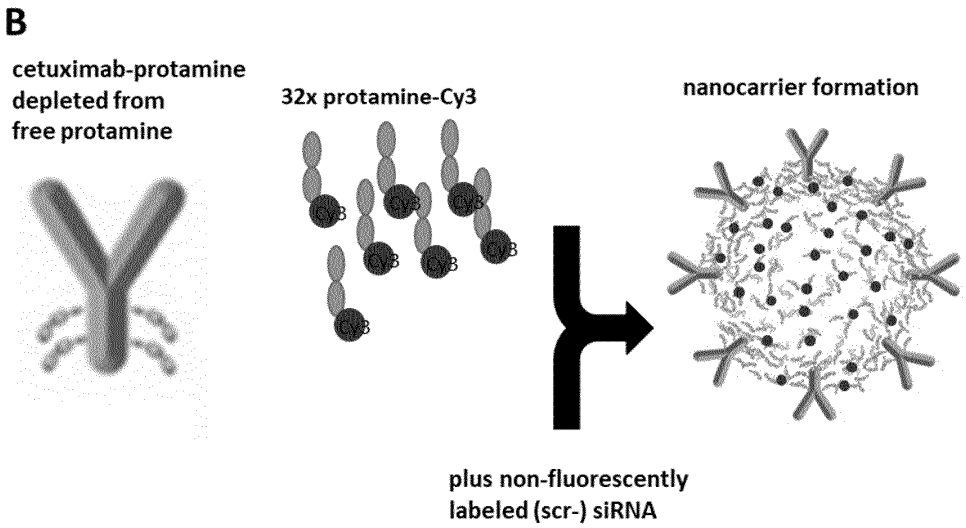


Figure 91 (cont'd)

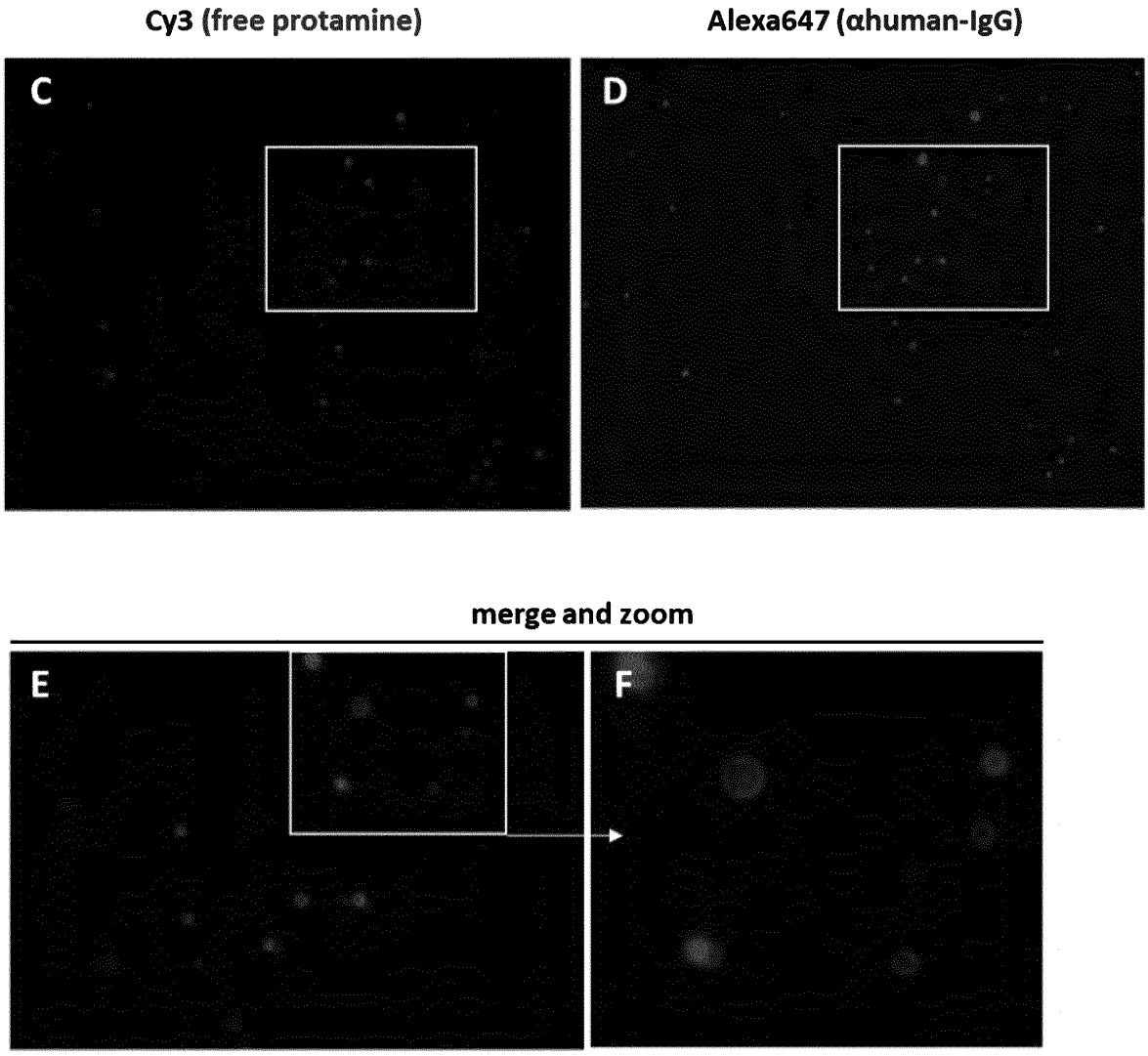


Figure 92

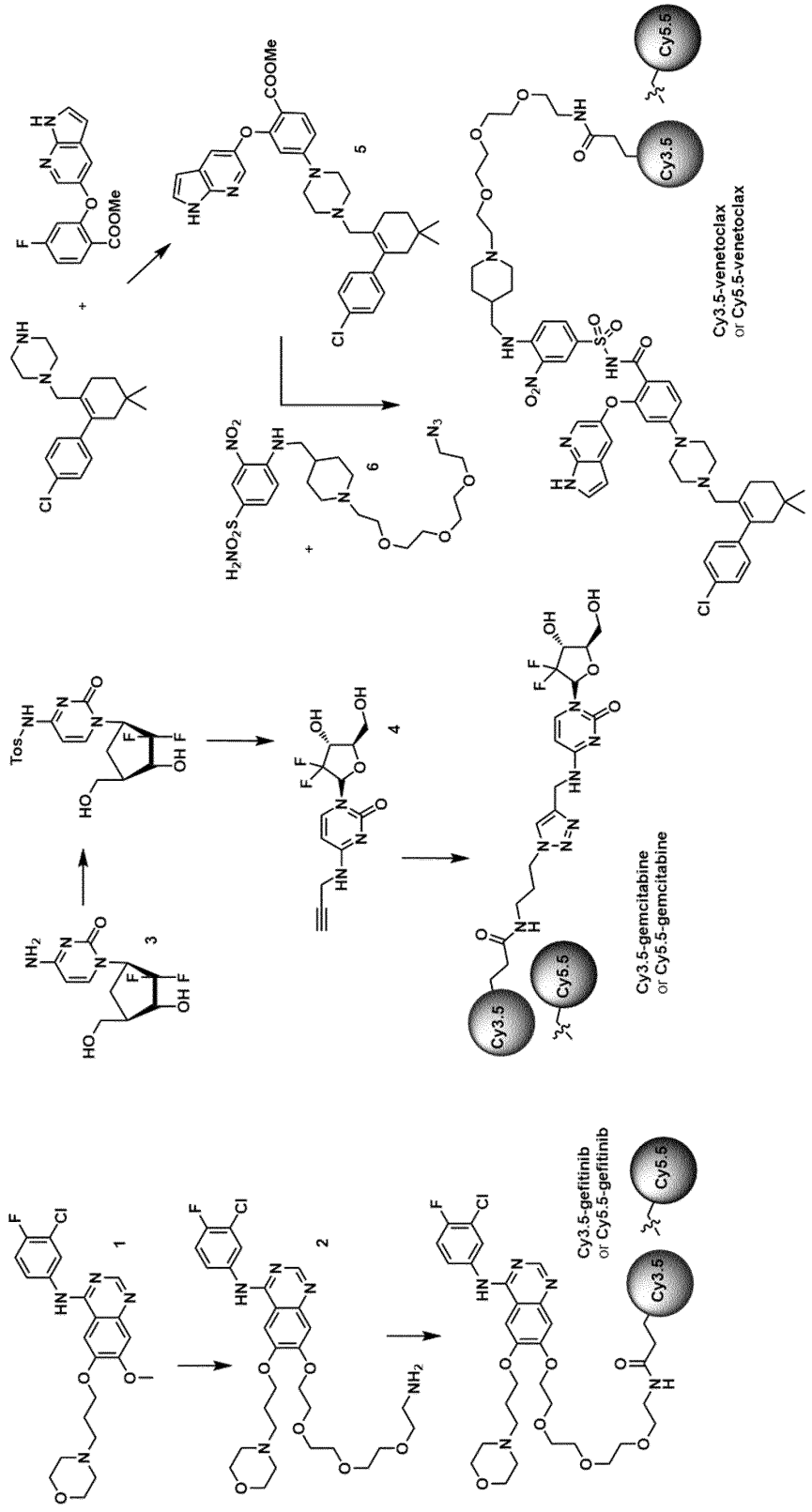
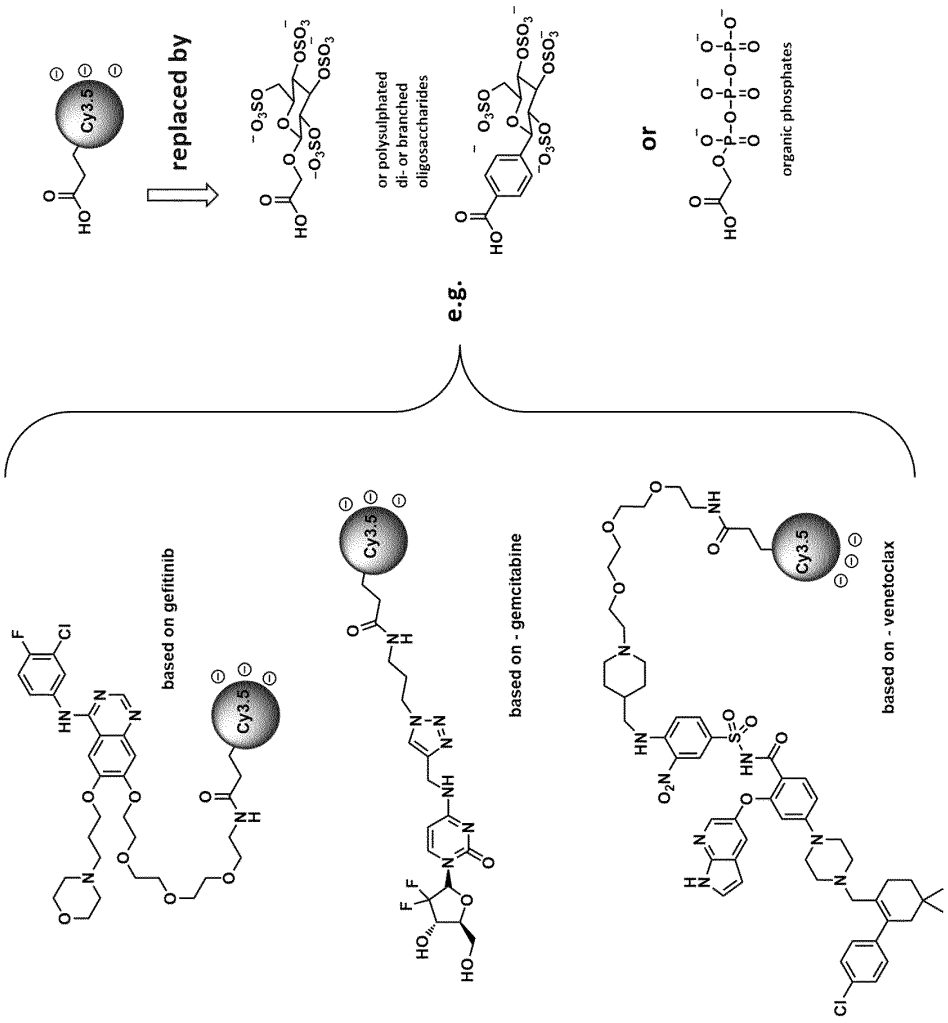

Cy3.5-venetoclax
or Cy5.5-venetoclax
Cy3.5-gemcitabine
or Cy5.5-gemcitabine
Cy3.5-gefitinib
or Cy5.5-gefitinib
Cy3.5
Cy5.5

Figure 93


Cy3.5
replaced by
or polysulphated
di- or branched
oligosaccharides
or
organic phosphates
e.g.
based on gefitinib
based on - gemcitabine
based on - venetoclax