FIG. 1

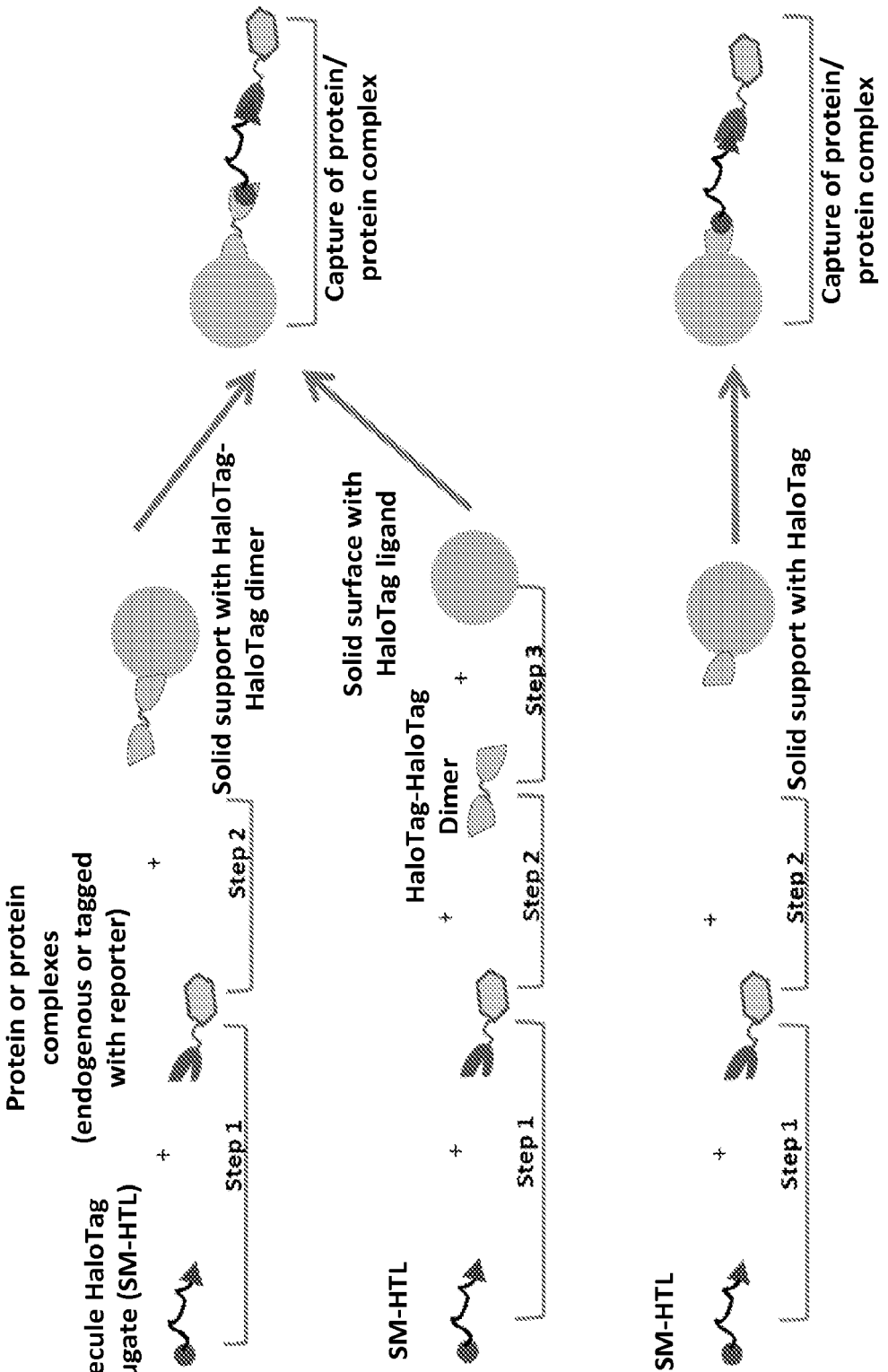

Small Molecule HaloTag ligand conjugate (SM-HTL)
Protein or protein complexes (endogenous or tagged with reporter)
Step 1
Step 2
Solid support with HaloTag-HaloTag dimer
Capture of protein/ protein complex
SM-HTL
HaloTag-HaloTag Dimer
Step 1
Step 2
Step 3
Solid surface with HaloTag ligand
Capture of protein/ protein complex
SM-HTL
Step 1
Step 2
Solid support with HaloTag
Capture of protein/ protein complex

FIG. 2

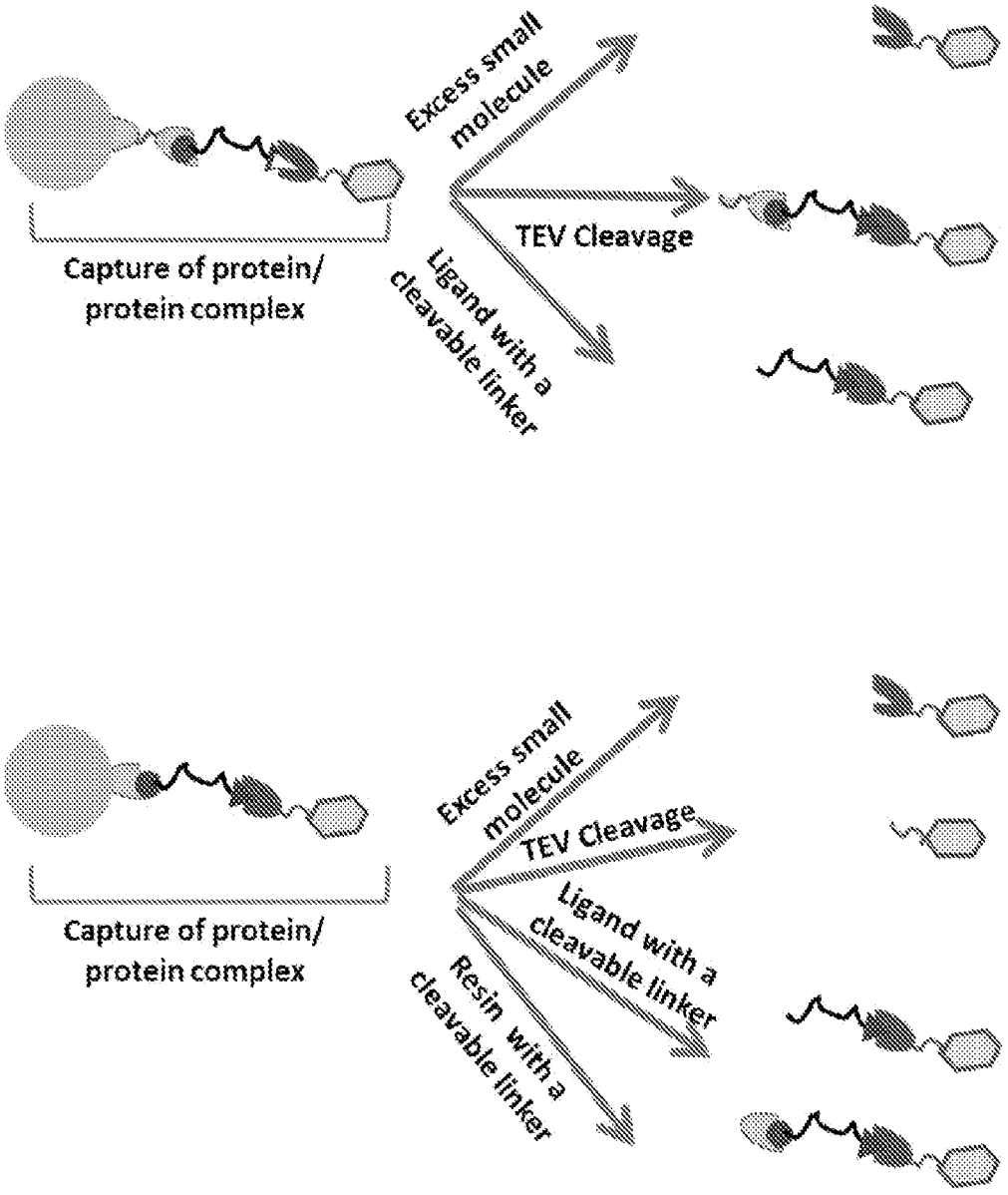

Excess small molecule
TEV Cleavage
Ligand with a cleavable linker
Capture of protein/ protein complex
Excess small molecule
TEV Cleavage
Ligand with a cleavable linker
Resin with a cleavable linker
Capture of protein/ protein complex

FIG. 3A

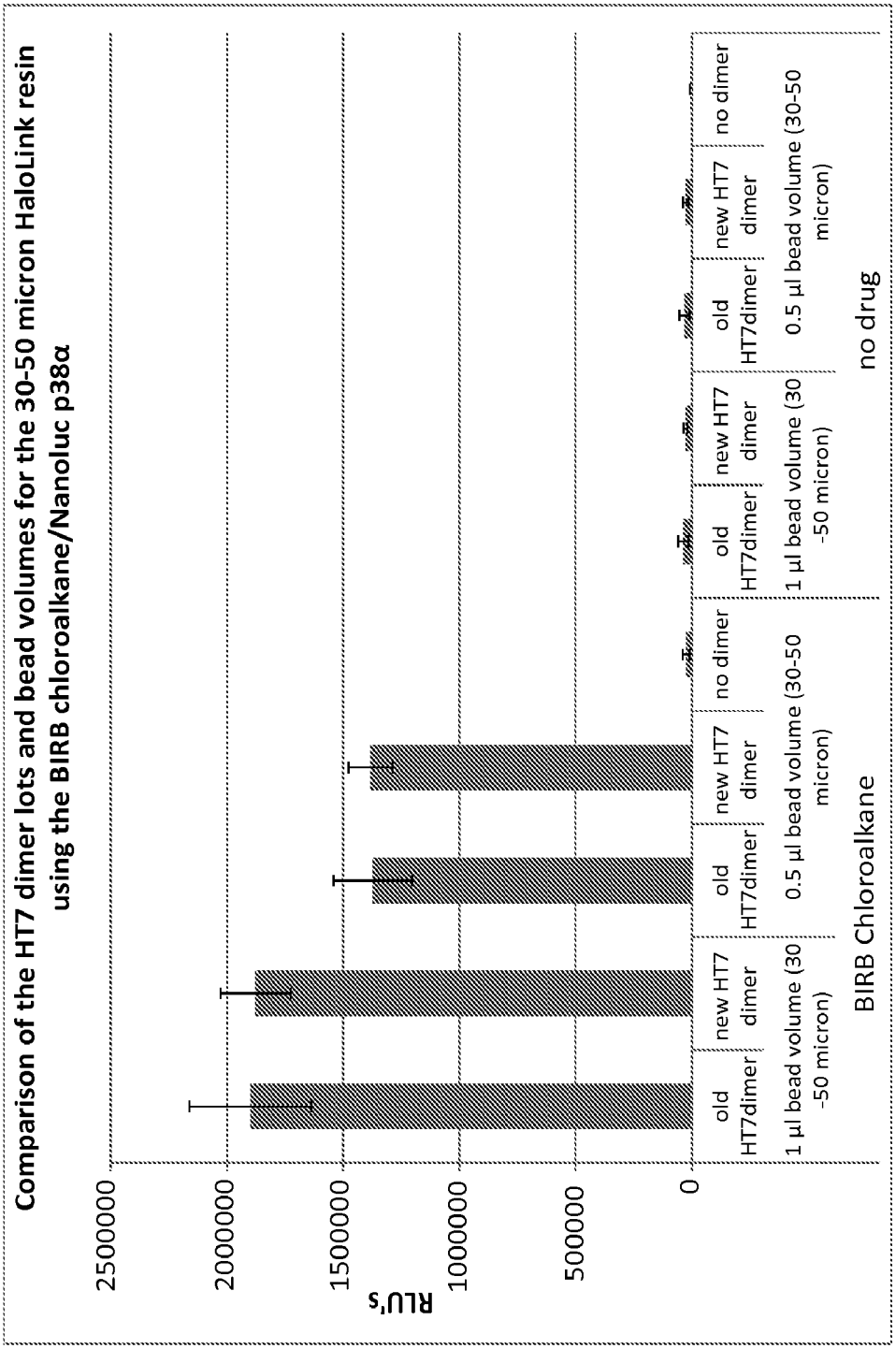

Comparison of the HT7 dimer lots and bead volumes for the 30-50 micron HaloLink resin using the BIRB chloroalkane/Nanoluc p38α
RLU's
2500000
2000000
1500000
1000000
500000
0
old HT7dimer
new HT7 dimer
no dimer
old HT7dimer
new HT7 dimer
no dimer
old HT7dimer
new HT7 dimer
no dimer
1 µl bead volume (30 -50 micron)
0.5 µl bead volume (30-50 micron)
1 µl bead volume (30 -50 micron)
0.5 µl bead volume (30-50 micron)
BIRB Chloroalkane
no drug

FIG. 3B

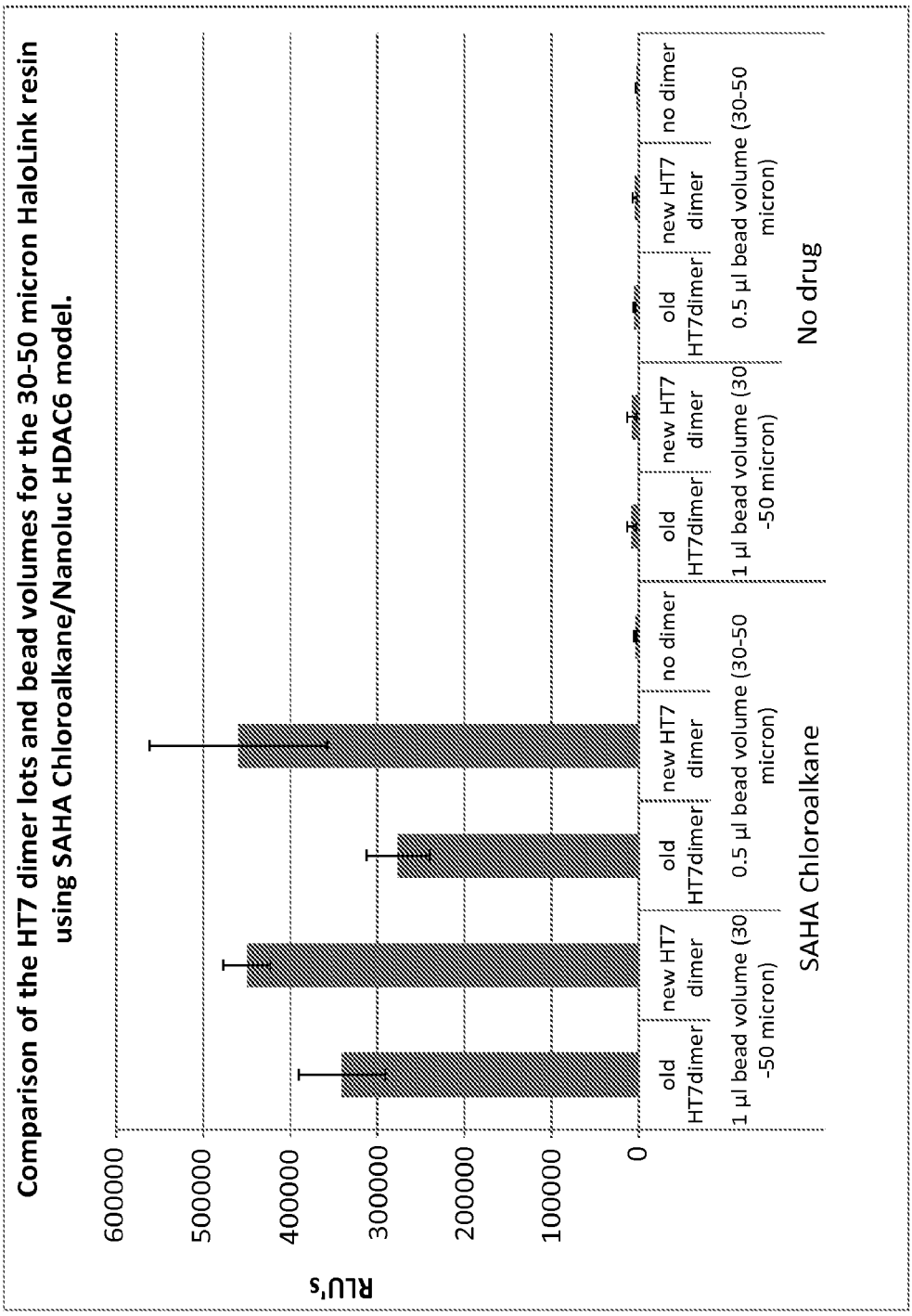

Comparison of the HT7 dimer lots and bead volumes for the 30-50 micron HaloLink resin using SAHA Chloroalkane/Nanoluc HDAC6 model.
RLU's
600000
500000
400000
300000
200000
100000
0
old HT7dimer
new HT7 dimer
no dimer
old HT7dimer
new HT7 dimer
no dimer
old HT7dimer
new HT7 dimer
no dimer
1 µl bead volume (30 -50 micron)
0.5 µl bead volume (30-50 micron)
1 µl bead volume (30 -50 micron)
0.5 µl bead volume (30-50 micron)
SAHA Chloroalkane
No drug

FIG. 3C

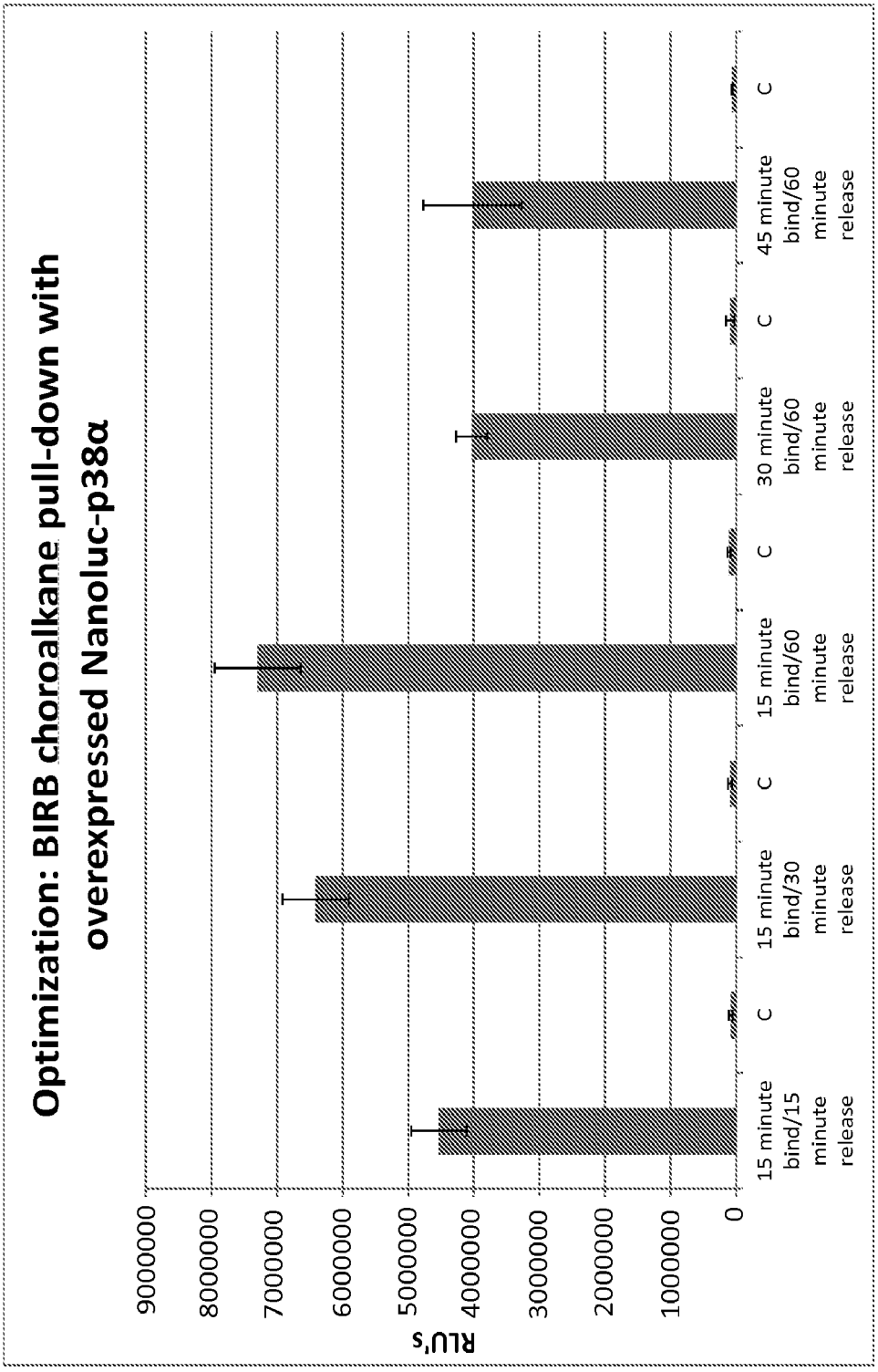

Optimization: BIRB choroalkane pull-down with overexpressed Nanoluc-p38α
RLU's
9000000
8000000
7000000
6000000
5000000
4000000
3000000
2000000
1000000
0
15 minute bind/15 minute release
C
15 minute bind/30 minute release
C
15 minute bind/60 minute release
C
30 minute bind/60 minute release
C
45 minute bind/60 minute release
C

FIG. 3D

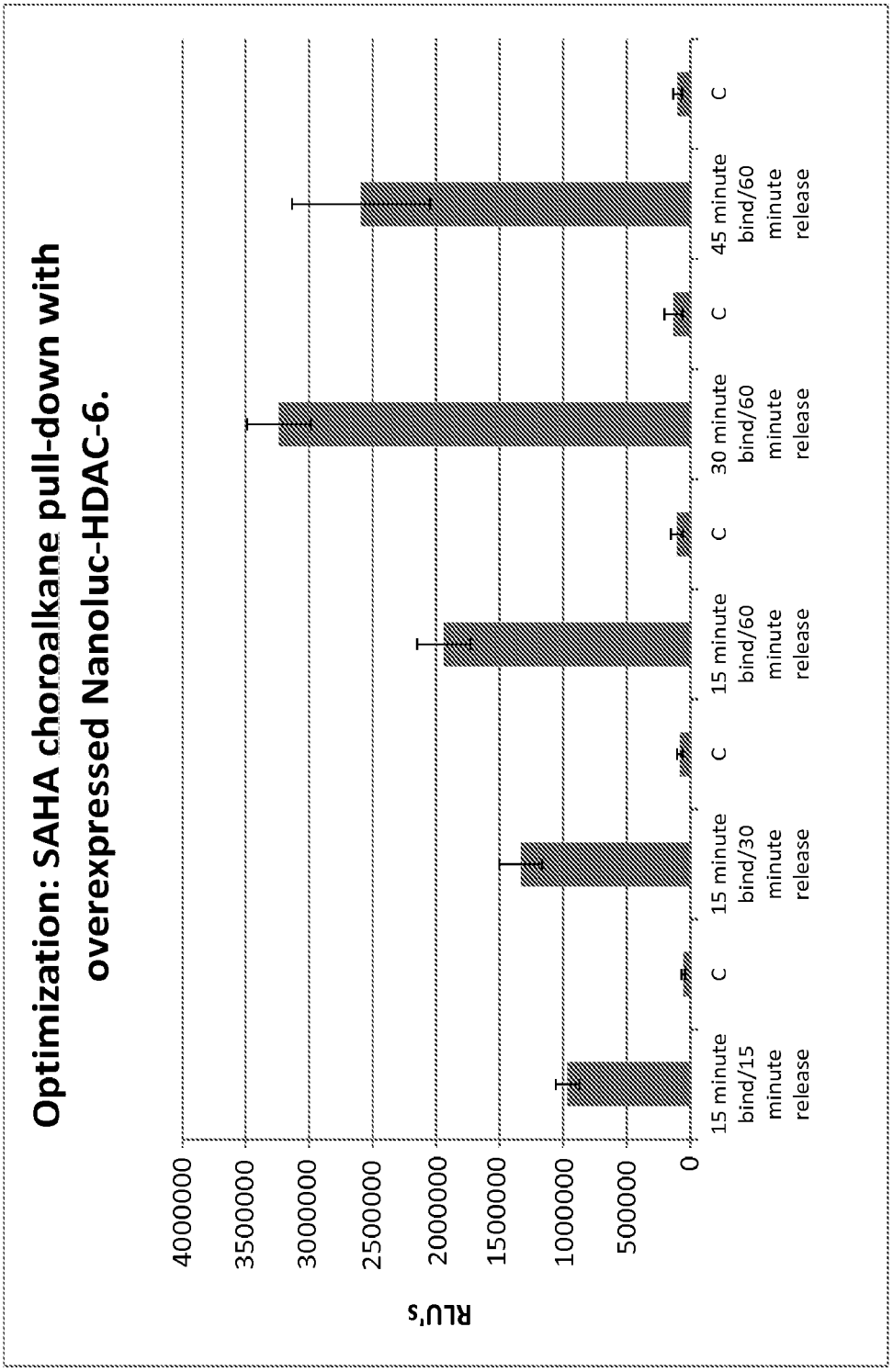

Optimization: SAHA choroalkane pull-down with overexpressed Nanoluc-HDAC-6.
RLU's
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
15 minute bind/15 minute release
C
15 minute bind/30 minute release
C
15 minute bind/60 minute release
C
30 minute bind/60 minute release
C
45 minute bind/60 minute release
C

FIG. 4

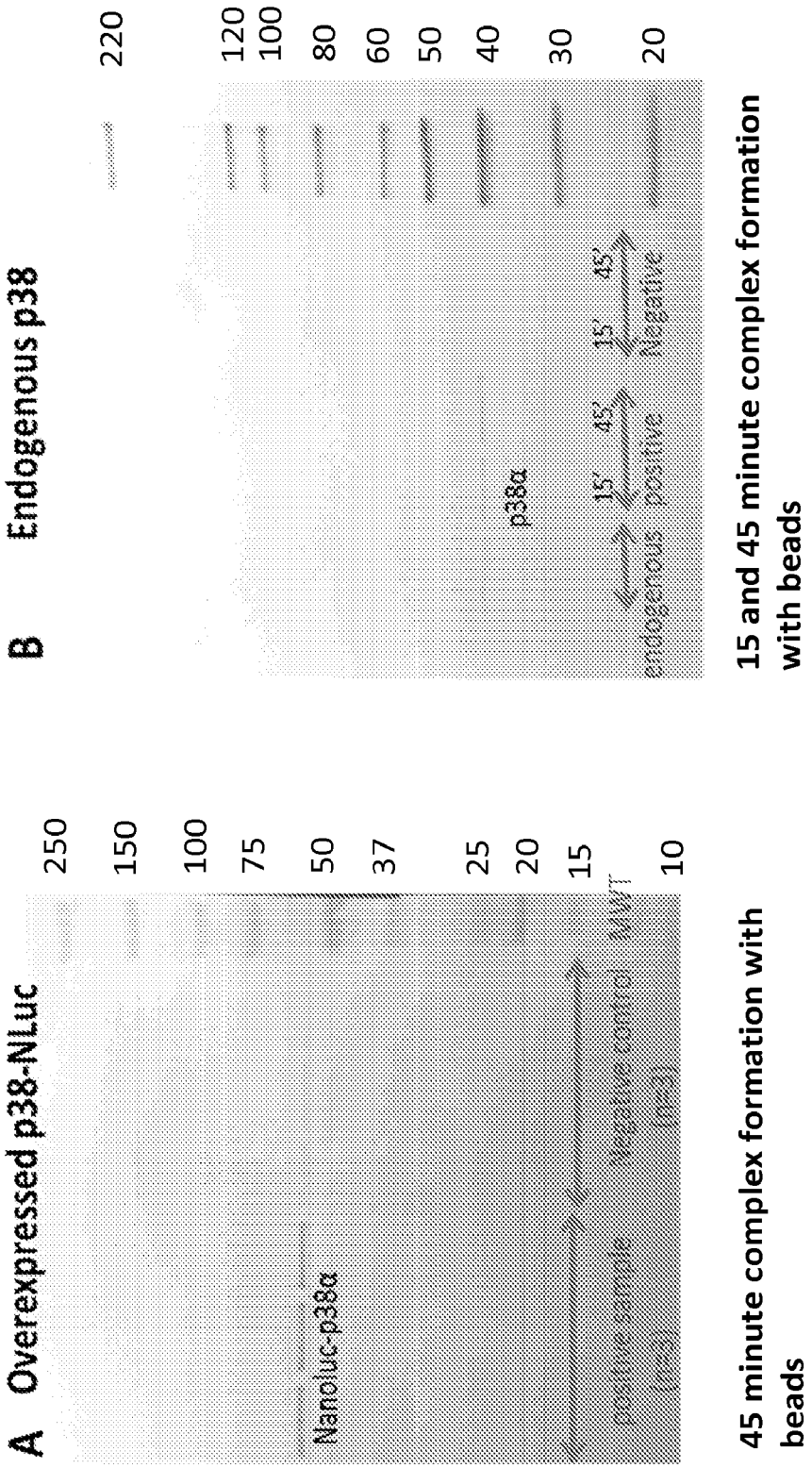

A Overexpressed p38-NLuc
250
150
100
75
50
37
25
20
15
10
Nanoluc-p38α
positive control MW
(no)
45 minute complex formation with beads
B Endogenous p38
220
120
100
80
60
50
40
30
20
p38α
15'
45'
endogenous positive
15'
45'
Negative
15 and 45 minute complex formation with beads

FIG. 5

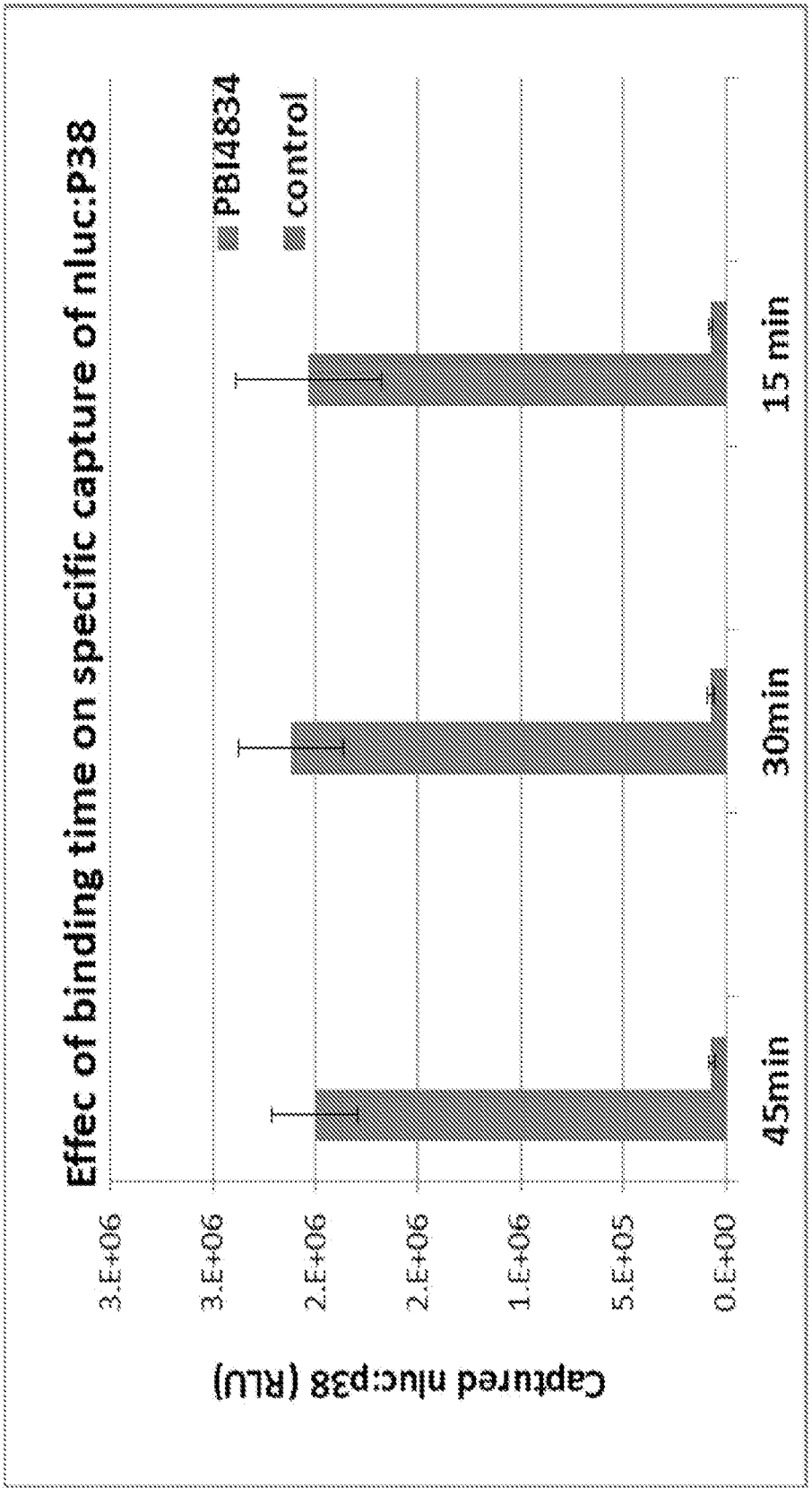

Effec of binding time on specific capture of nluc:P38
PBI4834
control
Captured nluc:p38 (RLU)
3.E+06
3.E+06
2.E+06
2.E+06
1.E+06
5.E+05
0.E+00
45min
30min
15 min

FIG. 6A

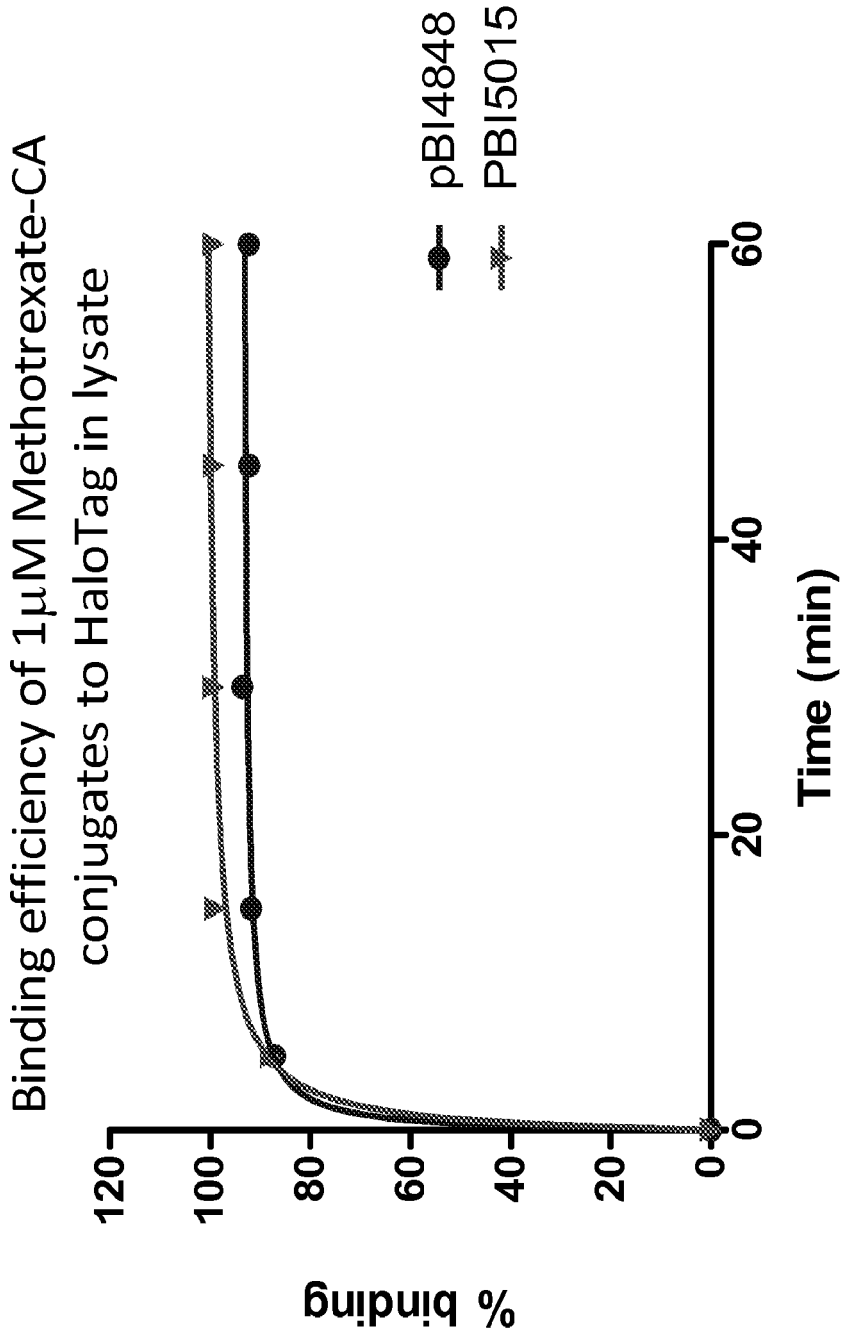
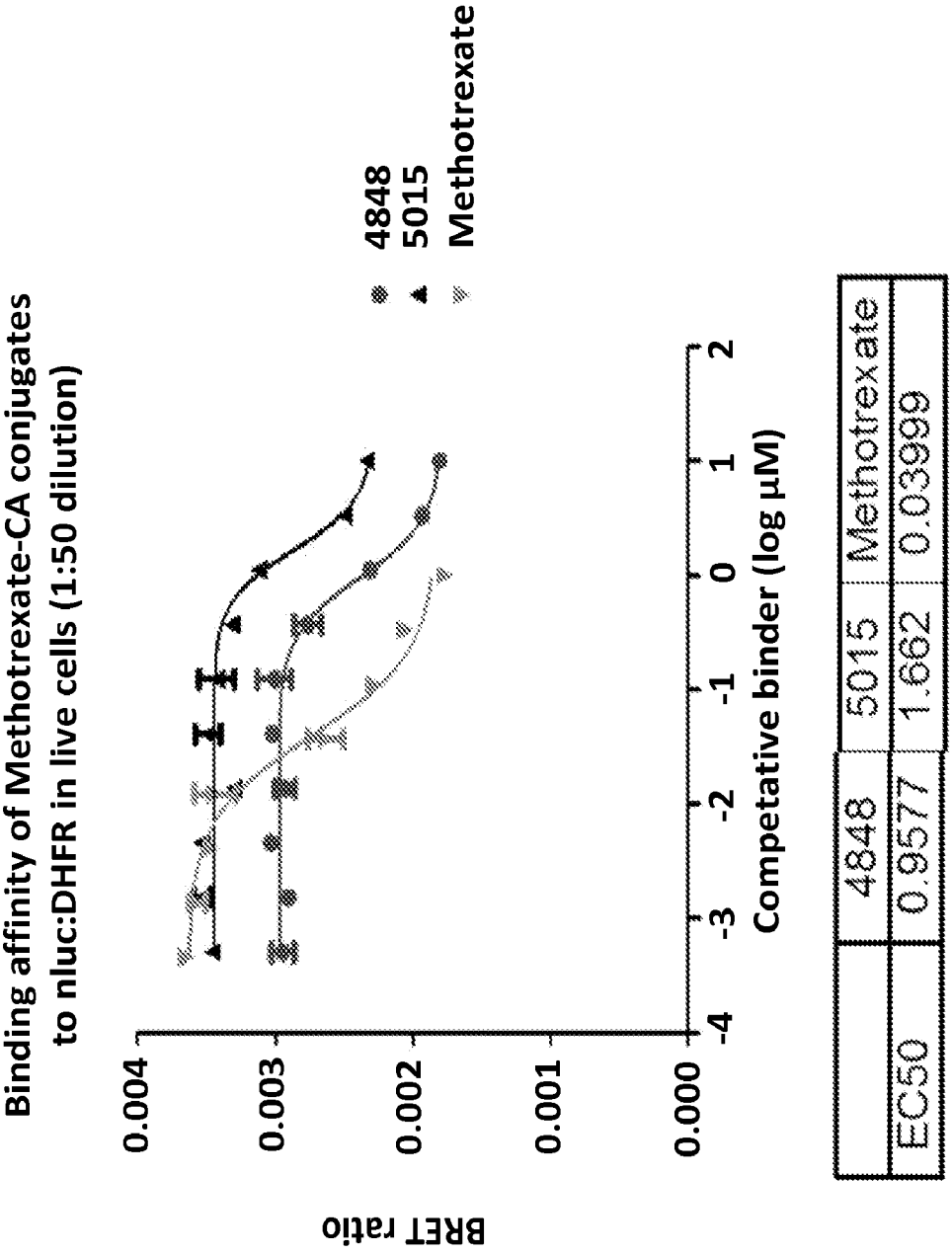

Binding efficiency of 1µM Methotrexate-CA conjugates to HaloTag in lysate
pBI4848
PBI5015
% binding
Time (min)
0
20
40
60
80
100
120
0
20
40
60

FIG. 6B



	4848	5015	Methotrexate
EC50	0.9577	1.662	0.03999

FIG. 6C

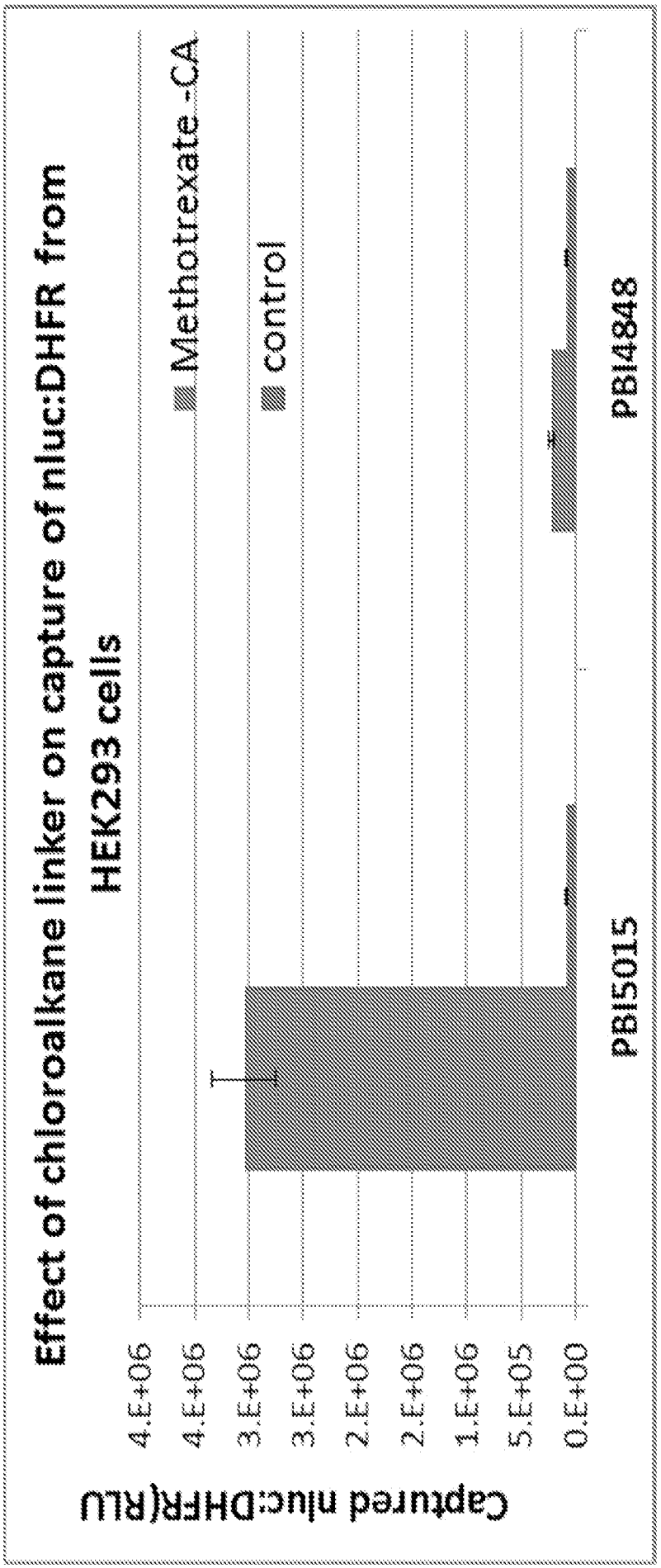

Effect of chloroalkane linker on capture of nluc:DHFR from HEK293 cells
Methotrexate -CA
control
Captured nluc:DHFR(RLU
4.E+06
4.E+06
3.E+06
3.E+06
2.E+06
2.E+06
1.E+06
5.E+05
0.E+00
PBI5015
PBI4848

FIG. 7

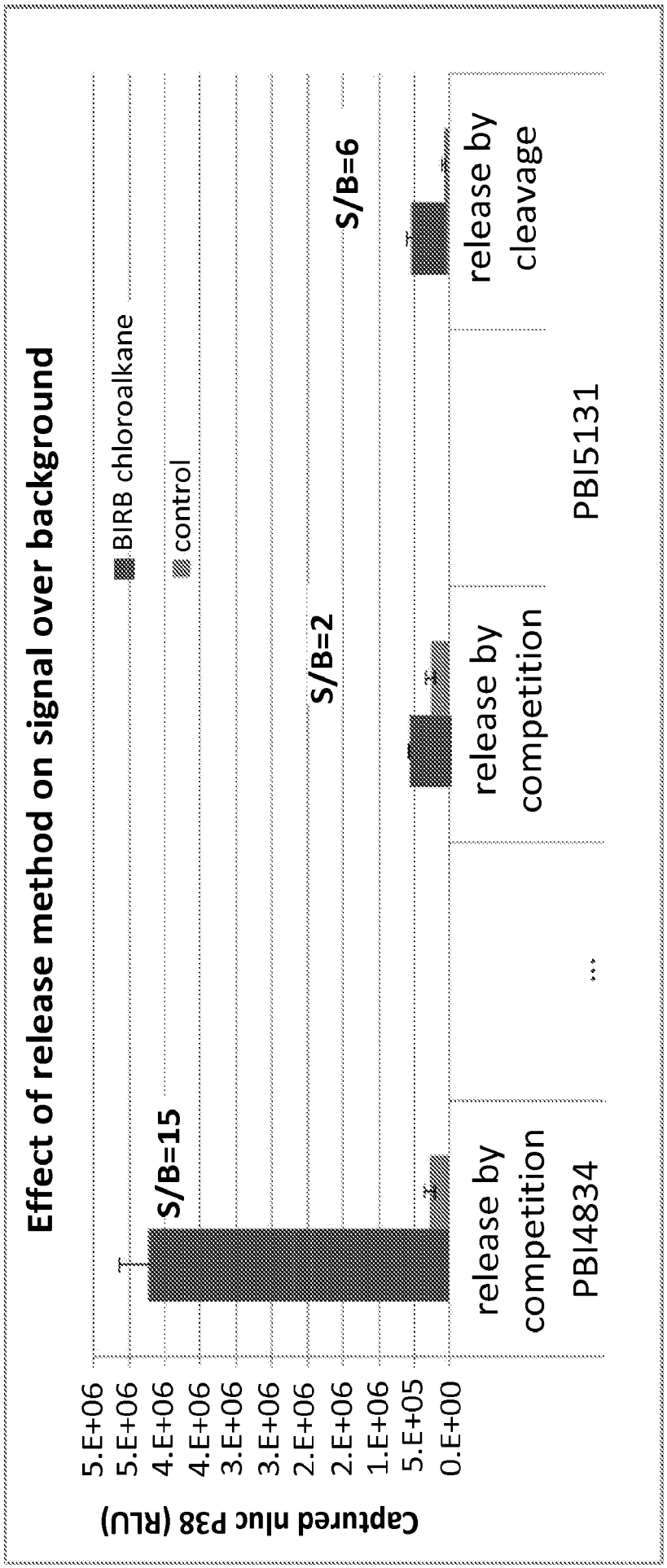

Effect of release method on signal over background
BIRB chloroalkane
control
S/B=15
S/B=2
S/B=6
Captured nluc P38 (RLU)
5.E+06
5.E+06
4.E+06
4.E+06
3.E+06
3.E+06
2.E+06
2.E+06
1.E+06
5.E+05
0.E+00
release by competition
PBI4834
...
release by competition
release by cleavage
PBI5131

FIG. 8A

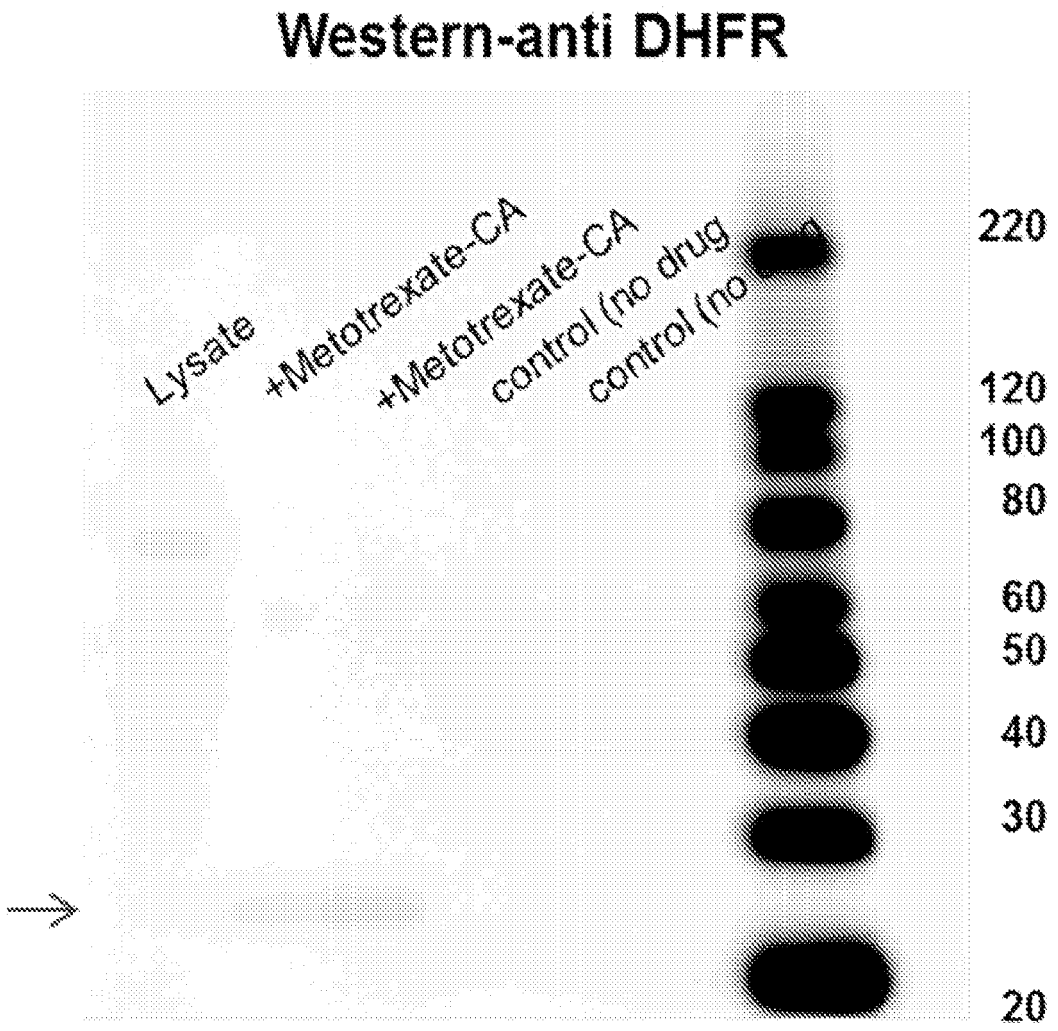

Western-anti DHFR
Lysate
+Metotrexate-CA
+Metotrexate-CA
control (no drug
control (no
220
120
100
80
60
50
40
30
20

FIG. 8B

Identified Proteins (6)	MW (kDA)	MTX-CA (SPC)	Control (SpC)	MTX-CA NSAF	Control NSAF
Dihydrofolate reductase OS=Homo sapiens GN=DHFR PE=1 SV=5	*21*	*77*	*0*	*0.189940*	*0*
Peroxiredoxin-2 OS=Homo sapiens GN=PRDX2 PE=1 SV=5	22	11	0	0.025901	0
Serpin B12 OS=Homo sapiens GN=SERPINB12 PE=1 SV-1	46	7	2	0.007883	0.001849
Collagen alpha-1(I) chain OS=Homo sapiens GN=COL1A1 PE=1 SV=5	*139*	*9*	*0*	*0.003354*	*0*
Collagen alpha-2(I) chain OS=Homo sapiens GN=COL1A2 PE=1 SV=7	*139*	*8*	*0*	*0.003213*	*0*
Myosin-9 OS=Homo sapiens GN=MYH9 PE=1 SV=4	227	13	0	0.002967	0

In bold Known targets of Methotrexate including DHFR

FIG. 9A

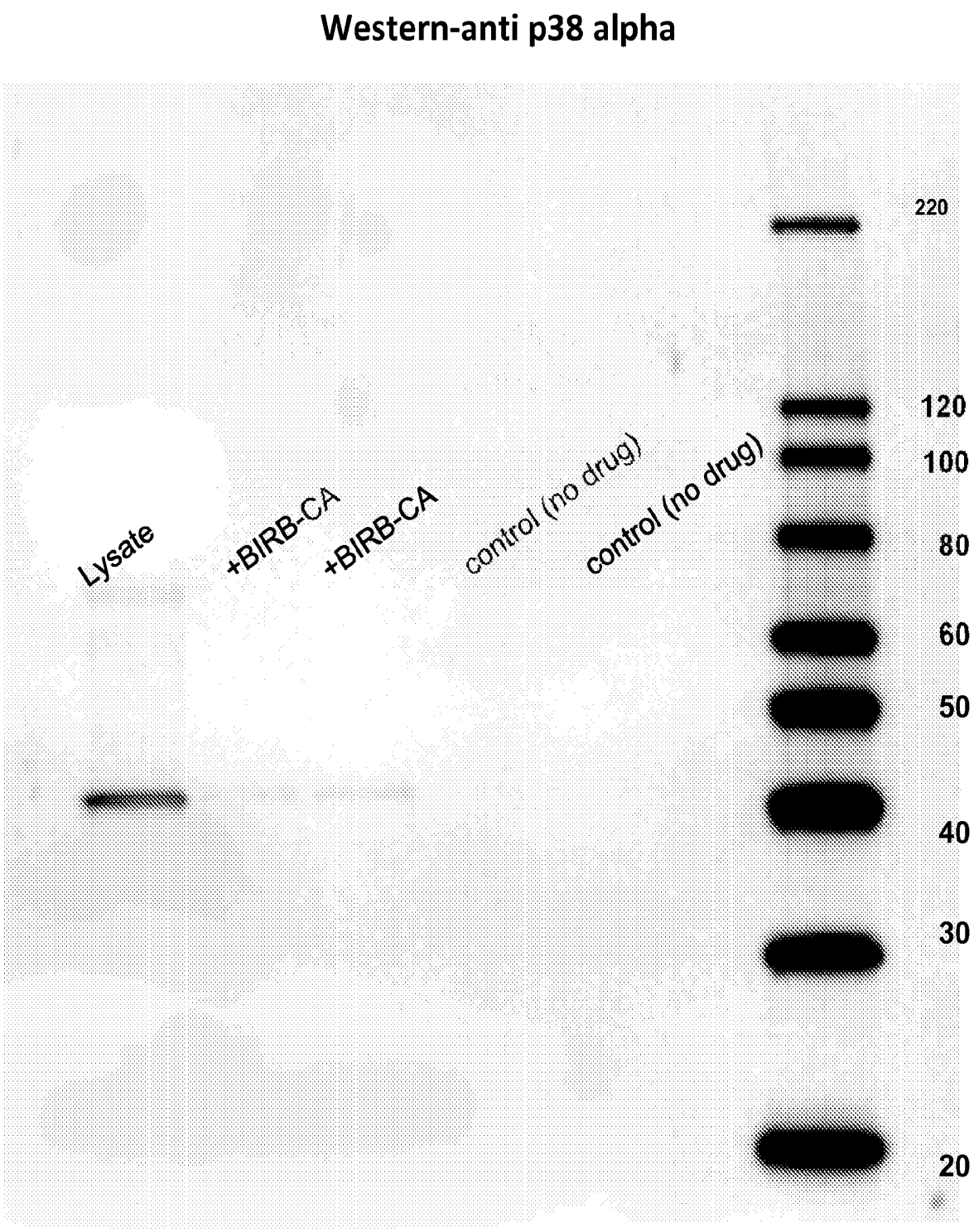

Western-anti p38 alpha
Lysate
+BIRB-CA
+BIRB-CA
control (no drug)
control (no drug)
220
120
100
80
60
50
40
30
20

FIG. 9B

Identified Proteins (23)	MW (kDa)	BIRB-CA (SPC)	control (SpC)	BIRB-CA NSAF	control NSAF
10 kDa heat shock protein, mitochondrial OS=Homo sapiens GN=HSPE1 PE=1 SV=2	11	7	0	0.022362	0
14-3-3 protein epsilon OS=Homo sapiens GN=YWHAE PE=1 SV=1	29	8	0	0.009694	0
L-lactate dehydrogenase B chain OS=Homo sapiens GN=LDHB PE=1 SV=2	37	10	0	0.009497	0
Alpha-enolase OS=Homo sapiens GN=ENO1 PE=1 SV=2	47	12	0	0.008972	0
→ Mitogen-activated protein kinase 14 OS=Homo sapiens GN=MAPK14 PE=1 SV=3	41	10	0	0.008571	0
Probable ATP-dependent RNA helicase DDX17 OS=Homo sapiens GN=DDX17 PE=1 SV=1	72	17	0	0.008297	0
Prohibitin OS=Homo sapiens GN=PHB PE=1 SV=1	30	6	0	0.007028	0
60 kDa heat shock protein, mitochondrial OS=Homo sapiens GN=HSPD1 PE=1 SV=2	61	12	2	0.006913	0.001685
L-lactate dehydrogenase A chain OS=Homo sapiens GN=LDHA PE=1 SV=2	37	7	0	0.006648	0
Fructose-bisphosphate aldolase A OS=Homo sapiens GN=ALDOA PE=1 SV=2	39	7	0	0.006307	0
14-3-3 protein gamma OS=Homo sapiens GN=YWHAG PE=1 SV=2	28	5	0	0.006275	0
Heat shock protein HSP 90-beta OS=Homo sapiens GN=HSP90AB1 PE=1 SV=4	83	13	0	0.005504	0
D-3-phosphoglycerate dehydrogenase OS=Homo sapiens GN=PHGDH PE=1 SV=4	57	8	0	0.004932	0
Tubulin beta chain OS=Homo sapiens GN=TUBB PE=1 SV=2	50	7	0	0.004920	0
Heat shock protein HSP 90-alpha OS=Homo sapiens GN=HSP90AA1 PE=1 SV=5	85	11	0	0.004548	0
→ Cyclin-dependent kinase 8 OS=Homo sapiens GN=CDK8 PE=1 SV=1	53	5	0	0.003315	0
Protein-glutamine gamma-glutamyltransferase E OS=Homo sapiens GN=TGM3 PE=1 SV	77	7	0	0.003195	0
Protein enabled homolog OS=Homo sapiens GN=ENAH PE=1 SV=2	67	6	0	0.003147	0
T-complex protein 1 subunit eta OS=Homo sapiens GN=CCT7 PE=1 SV=2	59	5	0	0.002978	0
ATP-dependent RNA helicase A OS=Homo sapiens GN=DHX9 PE=1 SV=4	141	9	0	0.002243	0
Protein Shroom3 OS=Homo sapiens GN=SHROOM3 PE=1 SV=2	217	11	0	0.001781	0
Myosin-9 OS=Homo sapiens GN=MYH9 PE=1 SV=4	227	11	0	0.001703	0
FACT complex subunit SPT16 OS=Homo sapiens GN=SUPT16H PE=1 SV=1	120	5	0	0.001464	0
→ STE20-like serine/threonine-protein kinase OS=Homo sapiens GN=SLK PE=1 SV=1	143	3	0	0.00073721	0

→ In red Known targets of BRIB796 including p38alpha =MAPK14

FIG. 10

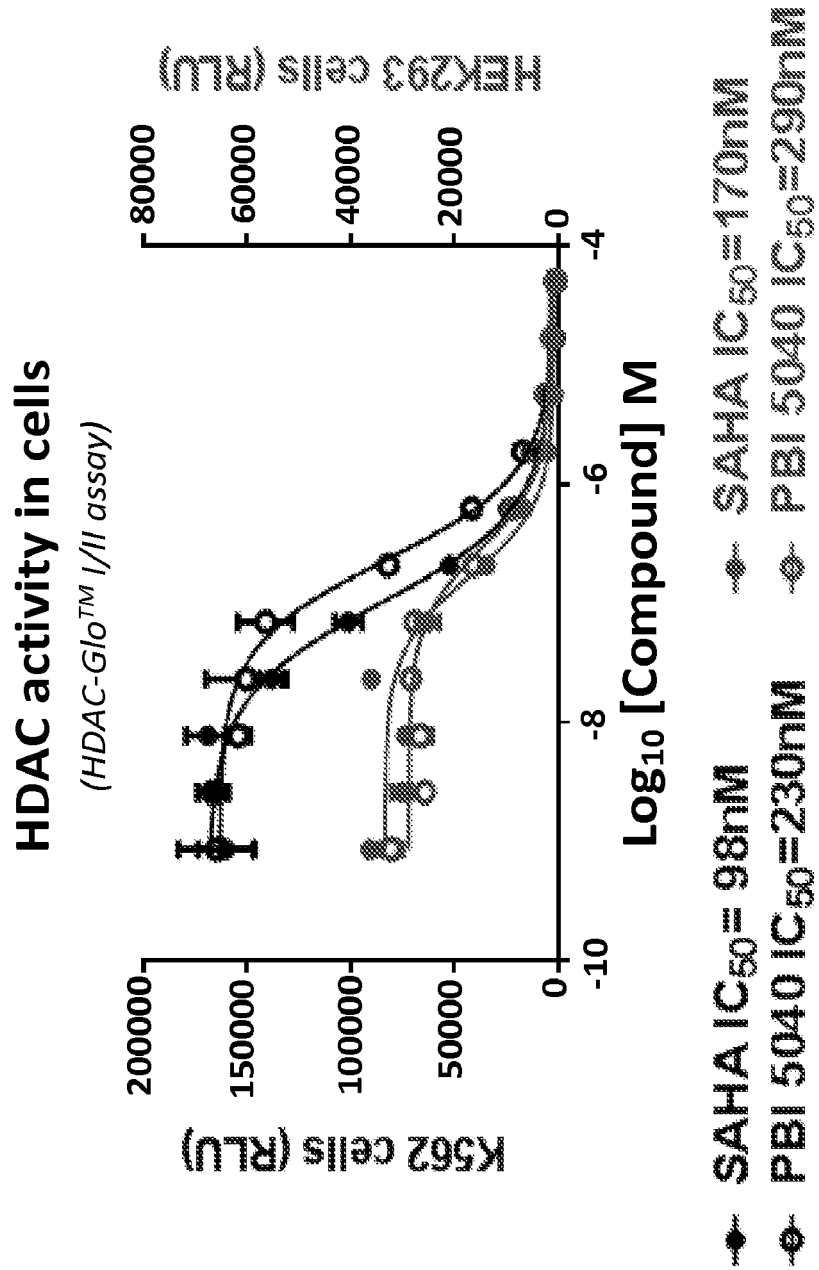
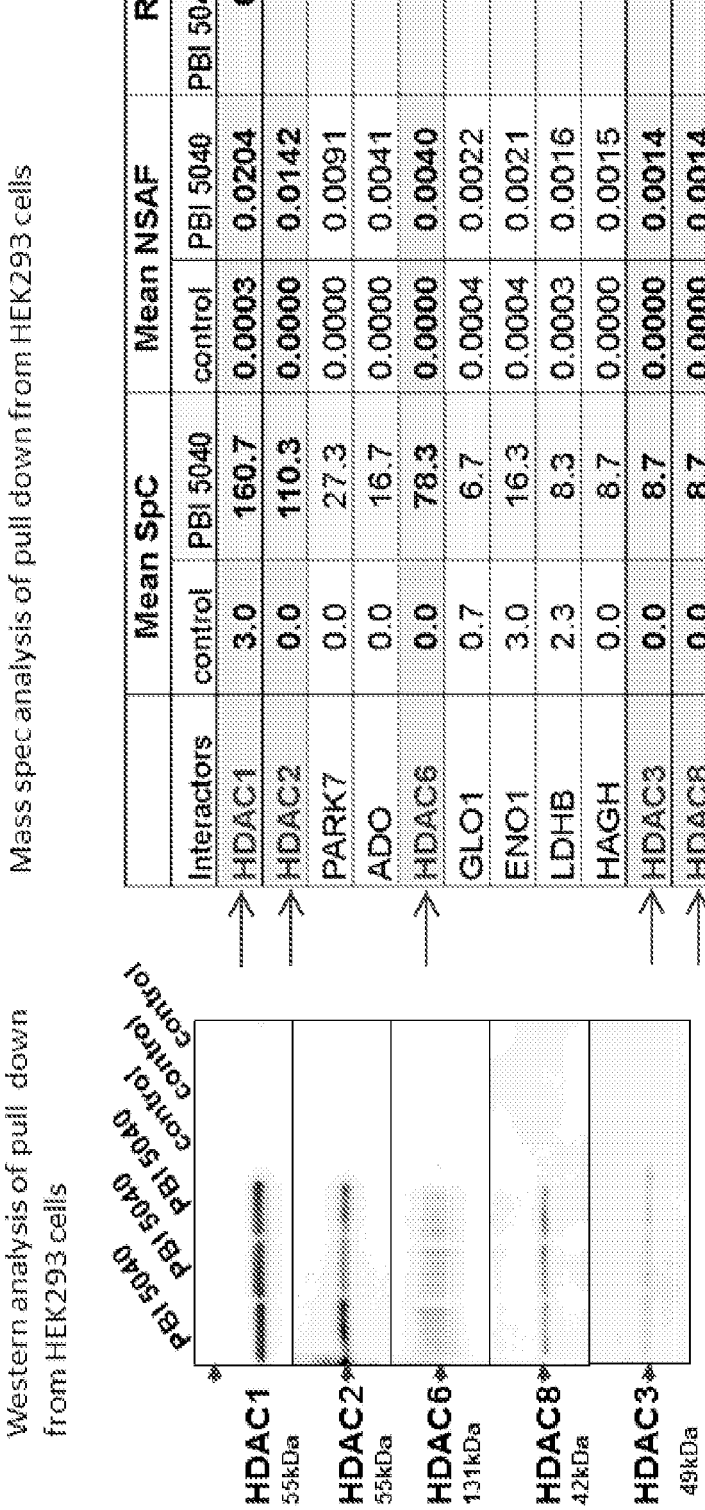

HDAC activity in cells
(HDAC-Glo™ I/II assay)
K562 cells (RLU)
HEK293 cells (RLU)
Log10 [Compound] M
SAHA IC50= 98nM
PBI 5040 IC50=230nM
SAHA IC50=170nM
PBI 5040 IC50=290nM

FIG. 11



Mass spec analysis of pull down from HEK293 cells

	Mean SpC		Mean NSAF		Ratio
Interactors	control	PBI 5040	control	PBI 5040	PBI 5040/control
HDAC1	3.0	160.7	0.0003	0.0204	62.5
HDAC2	0.0	110.3	0.0000	0.0142	
PARK7	0.0	27.3	0.0000	0.0091	
ADO	0.0	16.7	0.0000	0.0041	
HDAC6	0.0	78.3	0.0000	0.0040	
GLO1	0.7	6.7	0.0004	0.0022	5.0
ENO1	3.0	16.3	0.0004	0.0021	5.5
LDHB	2.3	8.3	0.0003	0.0016	4.6
HAGH	0.0	8.7	0.0000	0.0015	
HDAC3	0.0	8.7	0.0000	0.0014	
HDAC8	0.0	8.7	0.0000	0.0014	
CPPED1	0.0	6.7	0.0000	0.0014	
CRKL	1.3	5.3	0.0002	0.0011	5.2
FARSB	2.3	9.0	0.0002	0.0010	4.4

- In red all known direct targets of SAHA
- Results rare represented as the mean of 3 replicate pull down experiments

FIG. 12

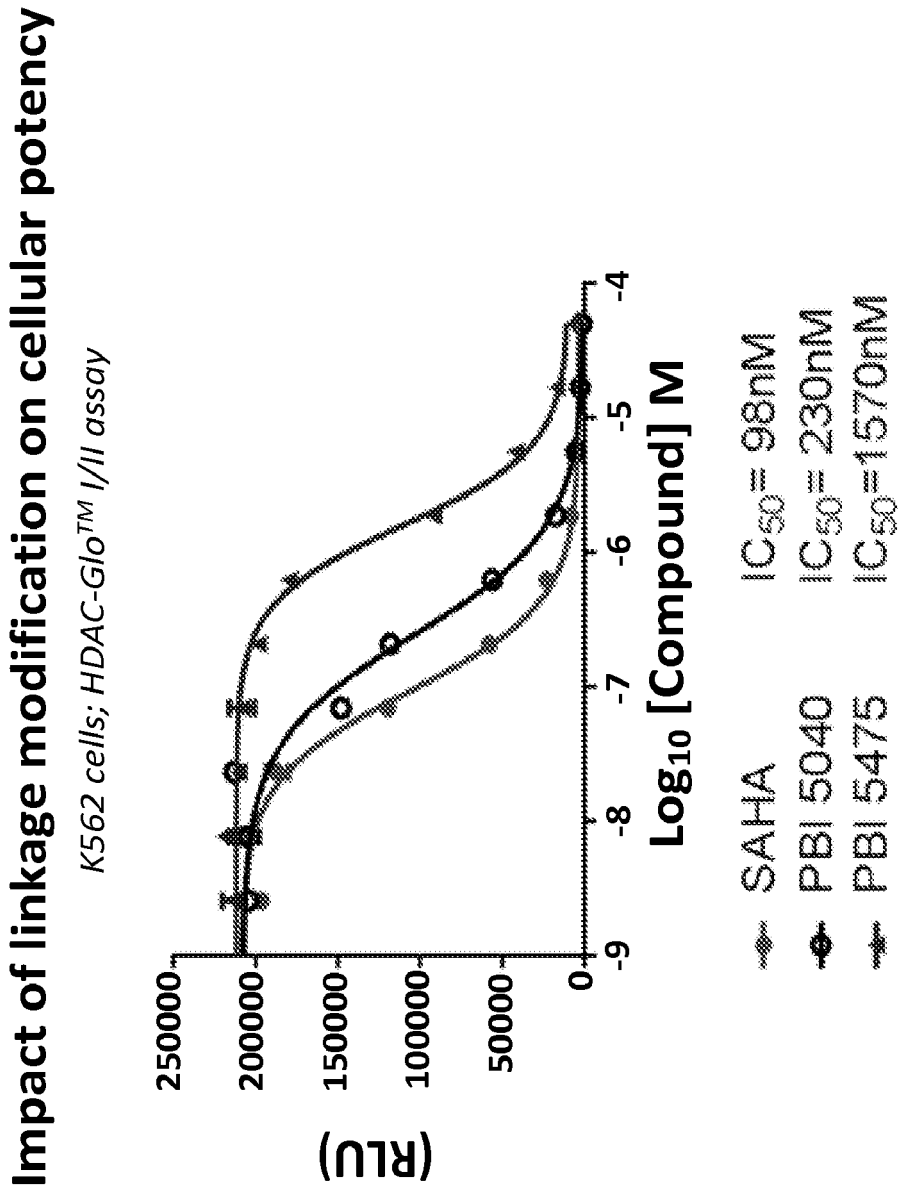

Impact of linkage modification on cellular potency
K562 cells; HDAC-Glo™ I/II assay
(RLU)
250000
200000
150000
100000
50000
0
-9
-8
-7
-6
-5
-4
Log10 [Compound] M
SAHA IC50= 98nM
PBI 5040 IC50= 230nM
PBI 5475 IC50=1570nM

FIG. 13

Western analysis of pull down from K562 cells

