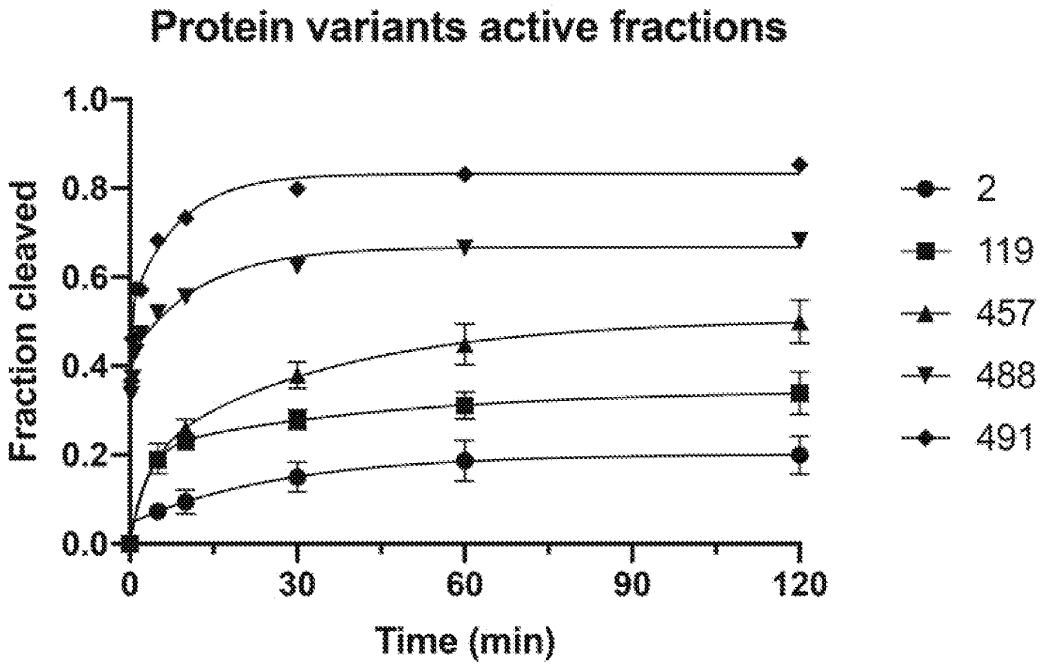

Protein variants active fractions
Fraction cleaved
1.0
0.8
0.6
0.4
0.2
0.0
0
30
60
90
120
Time (min)
2
119
457
488
491

FIG. 1

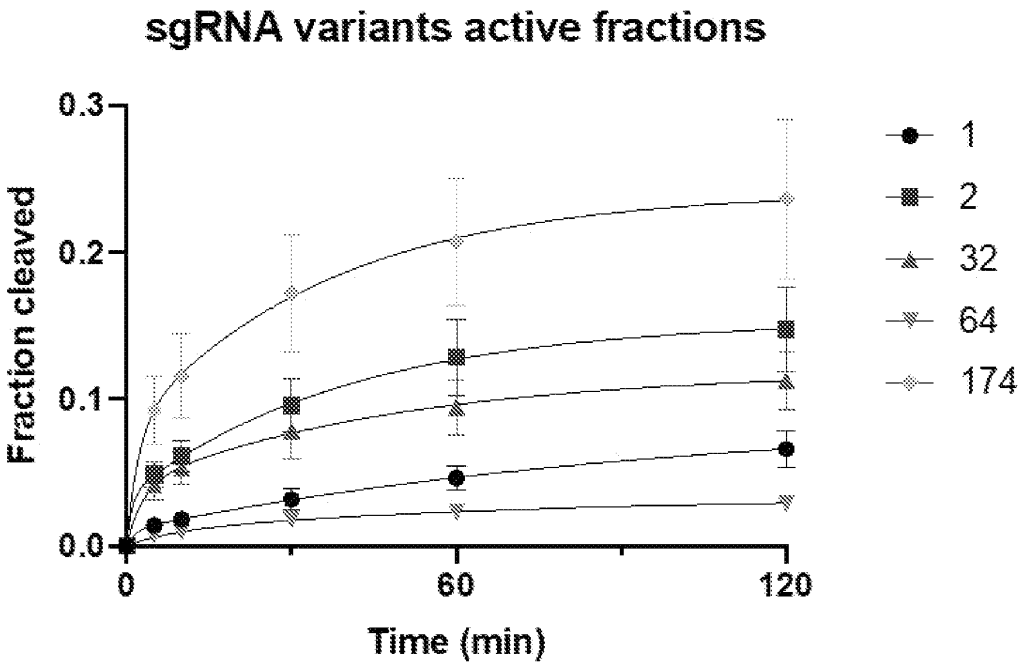

sgRNA variants active fractions
Fraction cleaved
0.3
0.2
0.1
0.0
0
60
120
Time (min)
1
2
32
64
174

FIG. 2

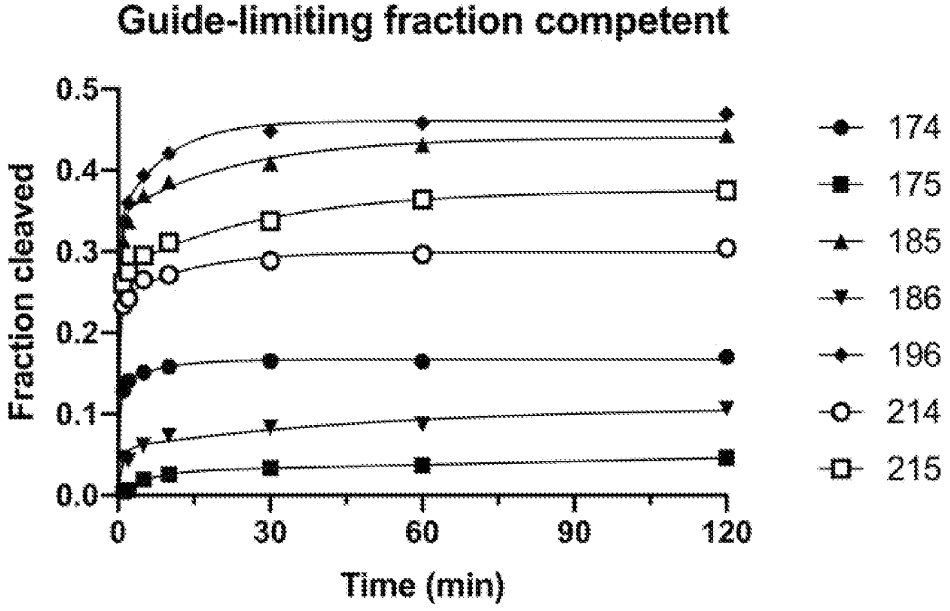

Guide-limiting fraction competent
Fraction cleaved
0.5
0.4
0.3
0.2
0.1
0.0
0
30
60
90
120
Time (min)
174
175
185
186
196
214
215

FIG. 3

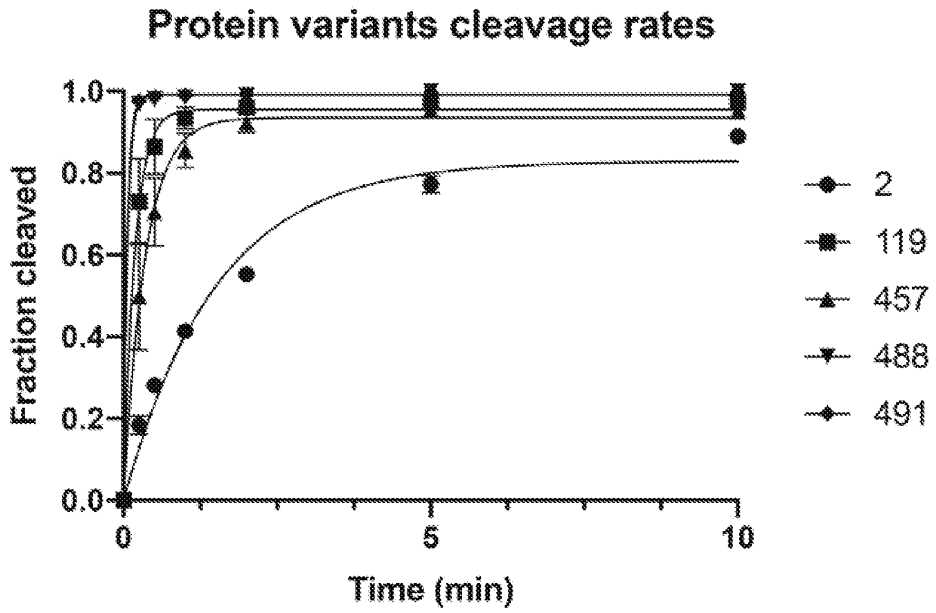

Protein variants cleavage rates
Fraction cleaved
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
Time (min)
2
119
457
488
491

FIG. 4

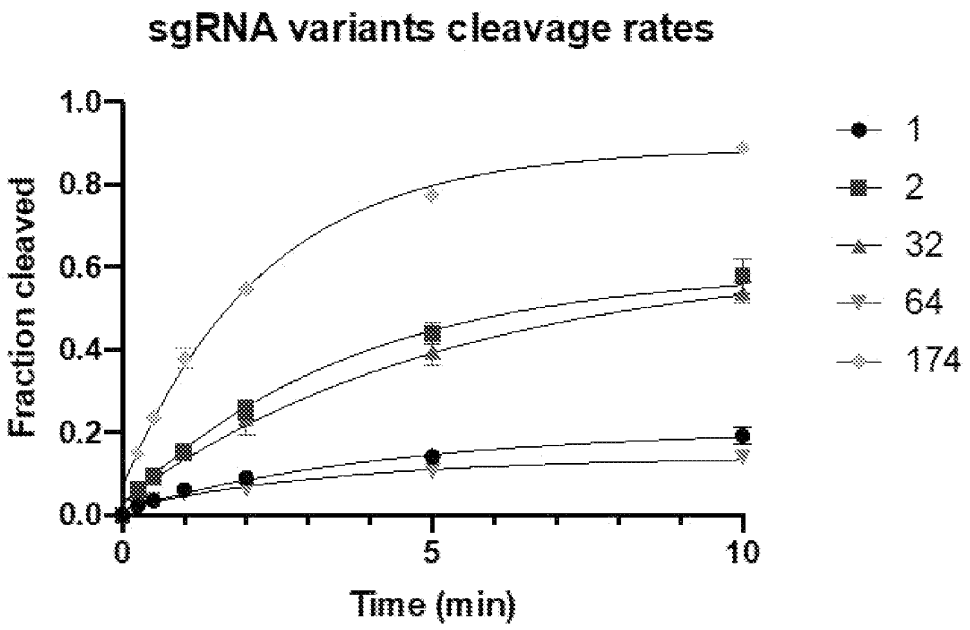

sgRNA variants cleavage rates
Fraction cleaved
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
Time (min)
1
2
32
64
174

FIG. 5

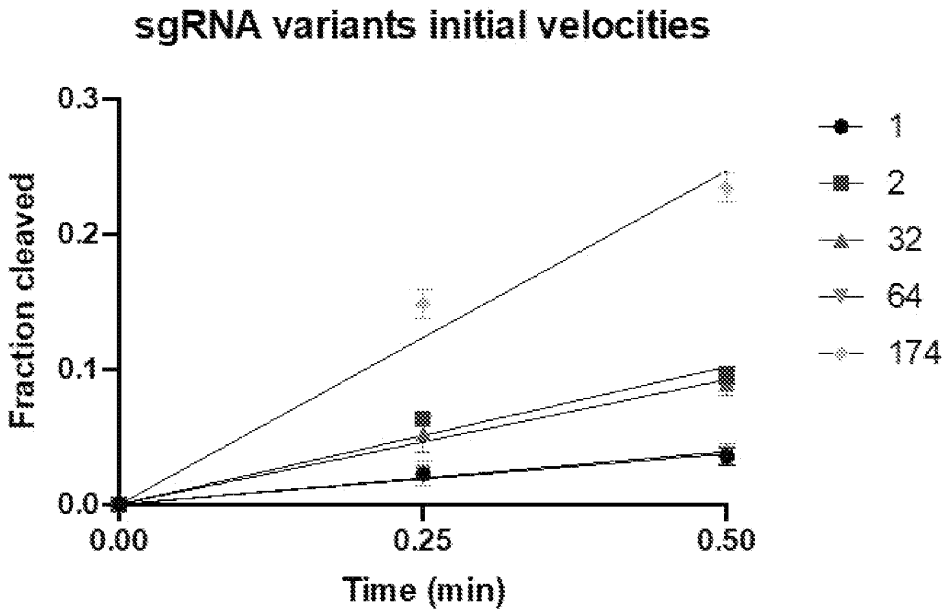

sgRNA variants initial velocities
Fraction cleaved
0.3
0.2
0.1
0.0
0.00
0.25
0.50
Time (min)
1
2
32
64
174

FIG. 6

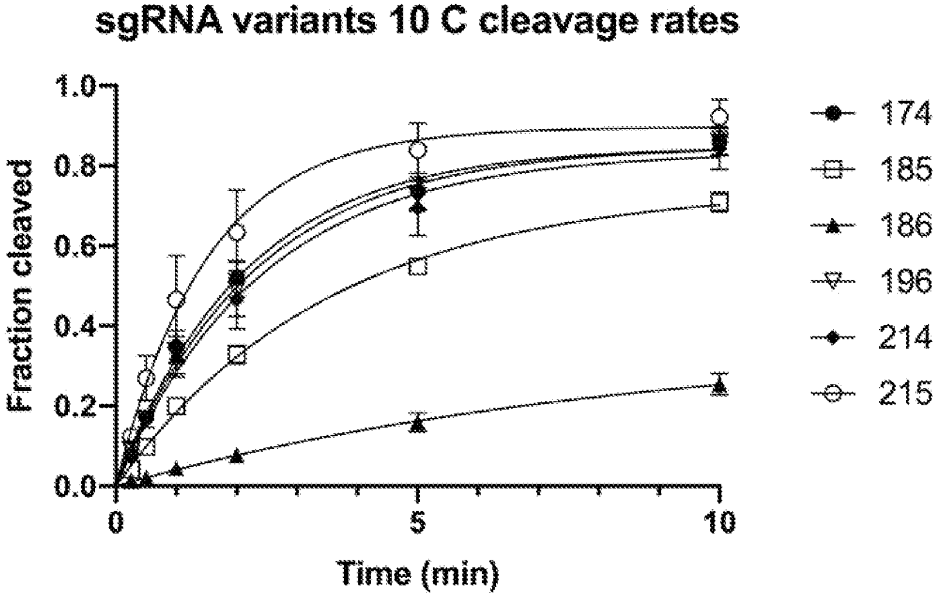

sgRNA variants 10 C cleavage rates
Fraction cleaved
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
Time (min)
174
185
186
196
214
215

FIG. 7

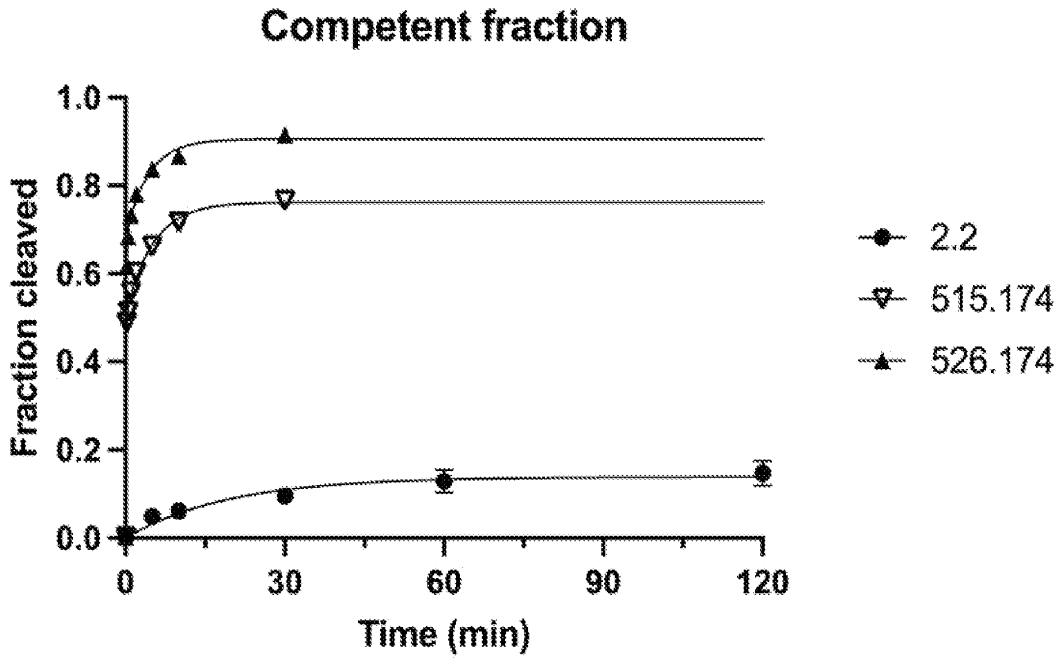

Competent fraction
Fraction cleaved
1.0
0.8
0.6
0.4
0.2
0.0
0
30
60
90
120
Time (min)
2.2
515.174
526.174

FIG. 8

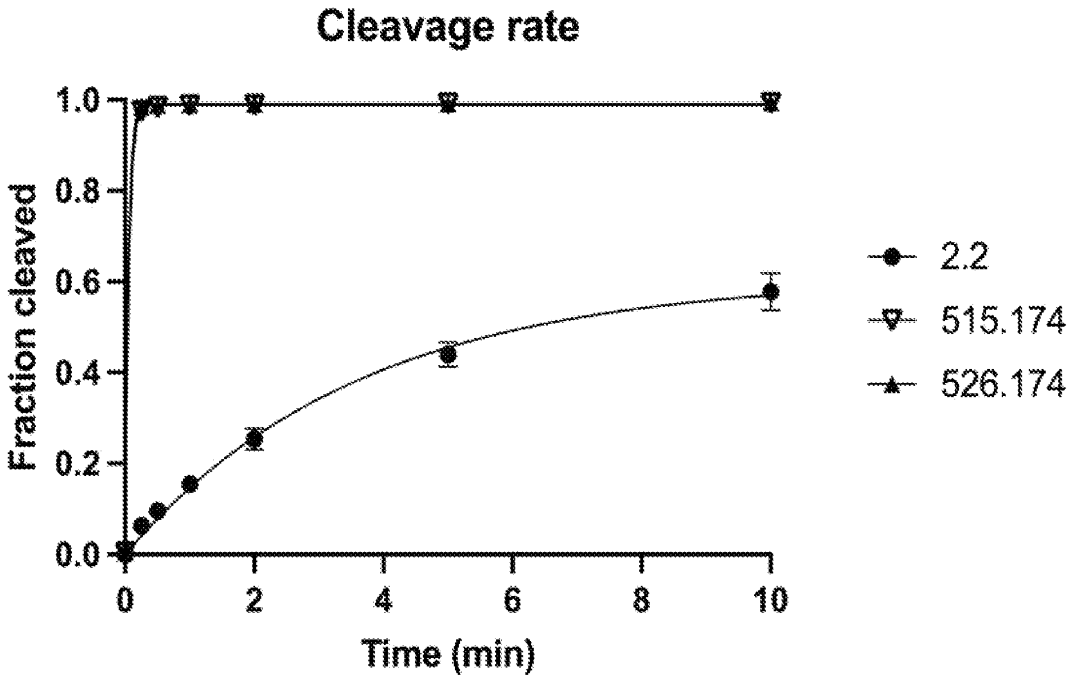

Cleavage rate
Fraction cleaved
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
Time (min)
2.2
515.174
526.174

FIG. 9

Cleavage rate on NTC PAMs

FIG. 10A

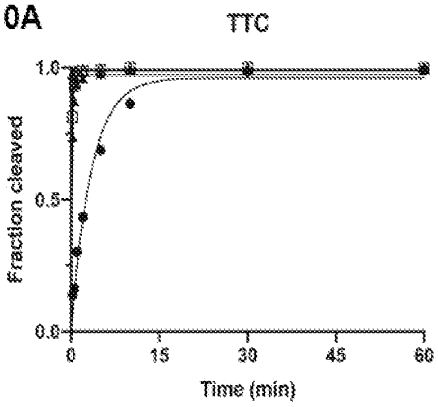


FIG. 10B

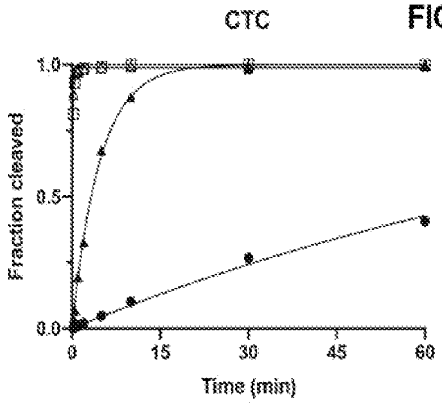


FIG. 10C

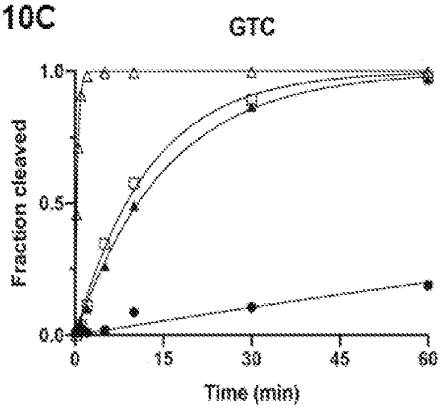
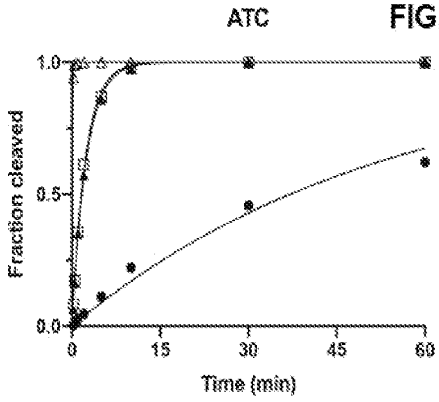


FIG. 10D



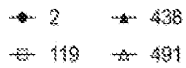


FIG. 11A

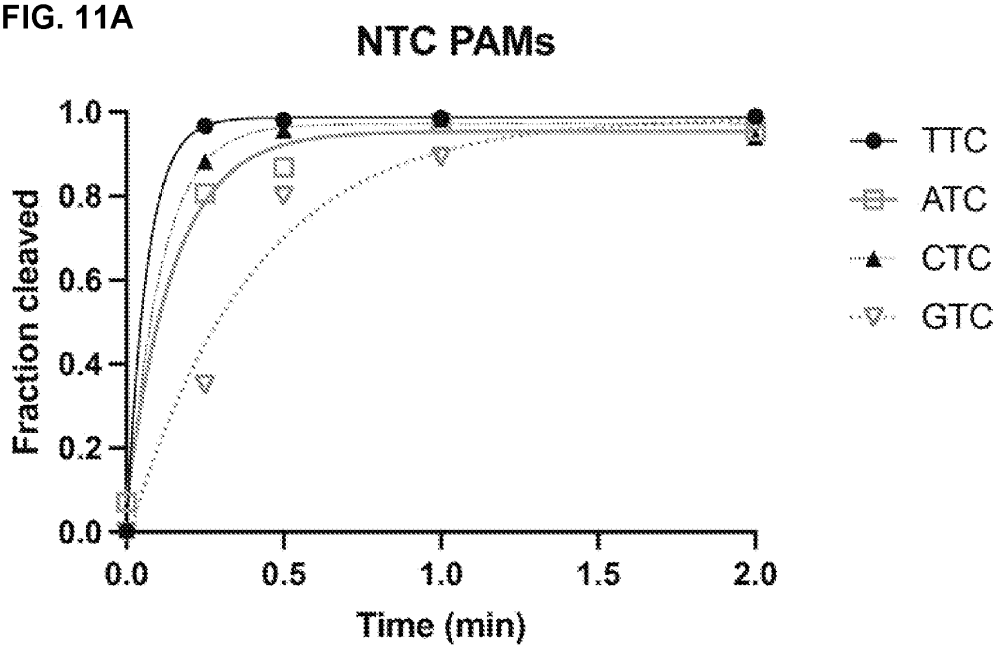


FIG. 11B

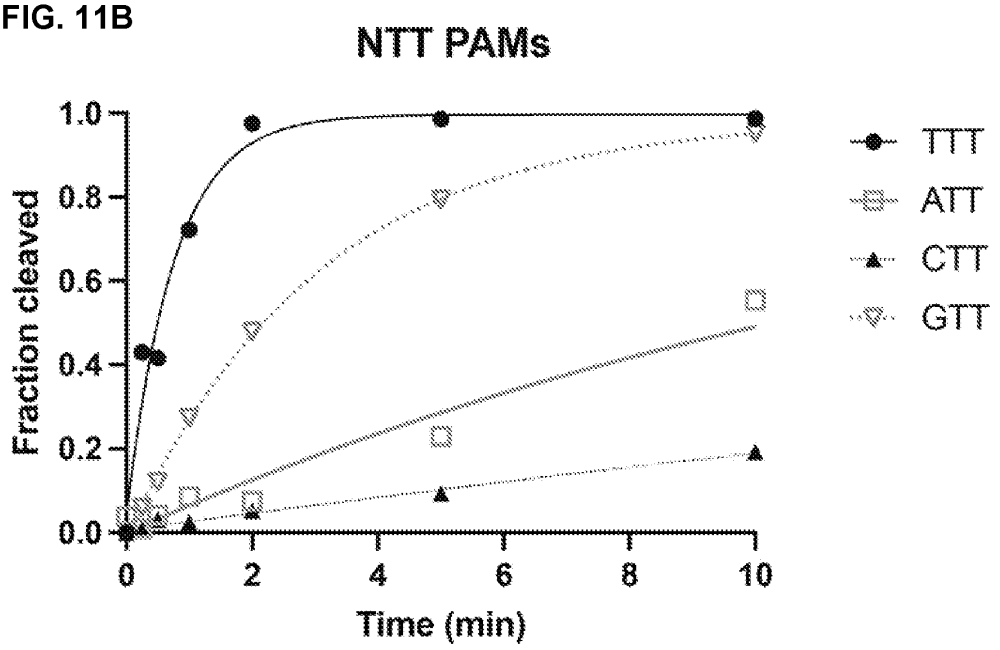


FIG. 12A

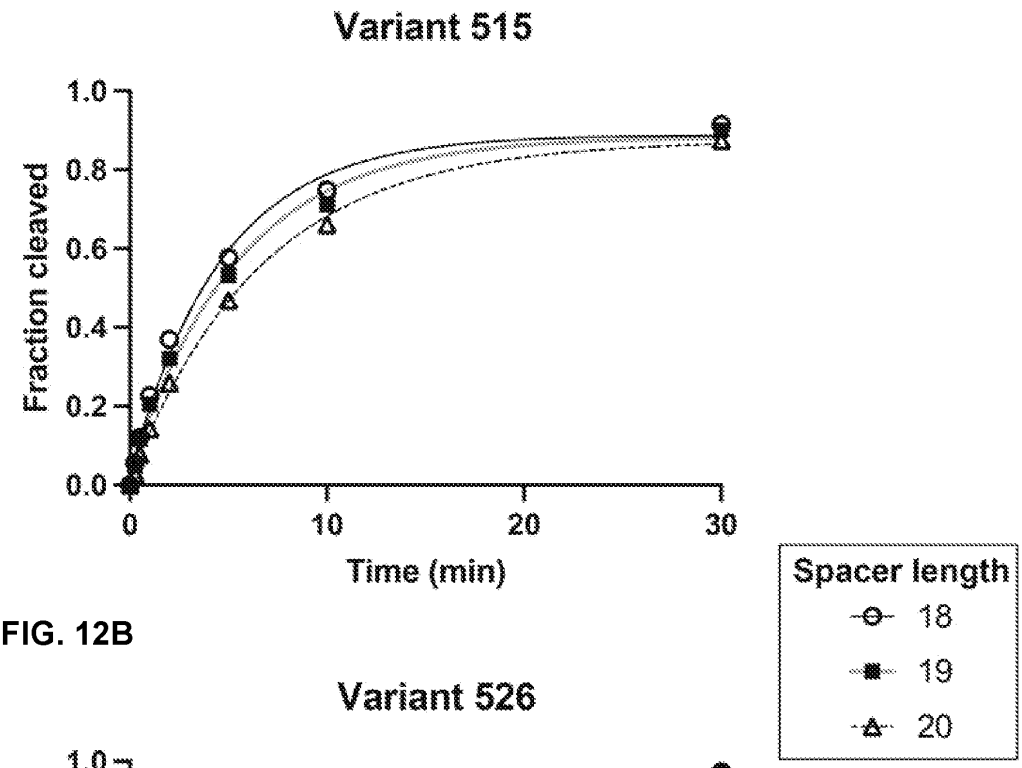
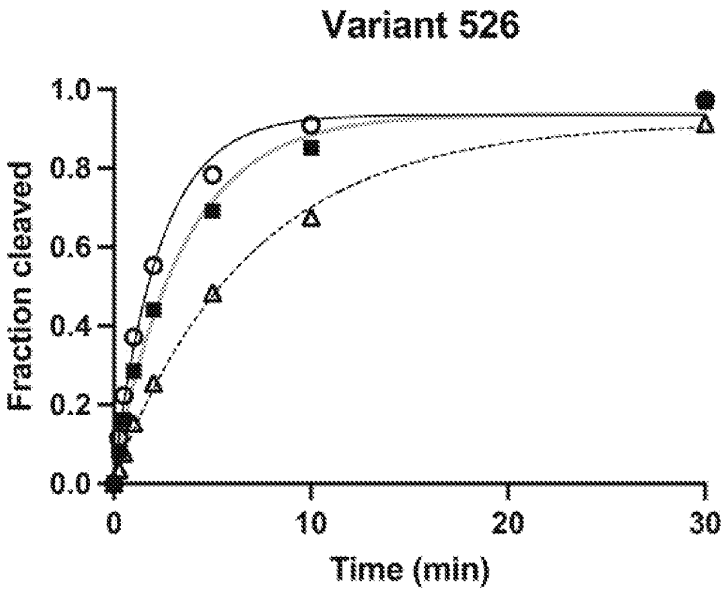


FIG. 12B



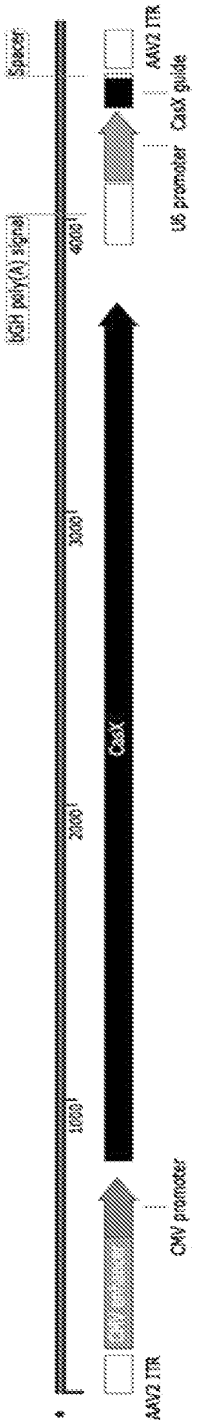

Spacer
AAV2 ITR
CasX guide
U6 promoter
bGH poly(A) signal
4000
3000
2000
CasX
1000
CMV promoter
AAV2 ITR

FIG. 13

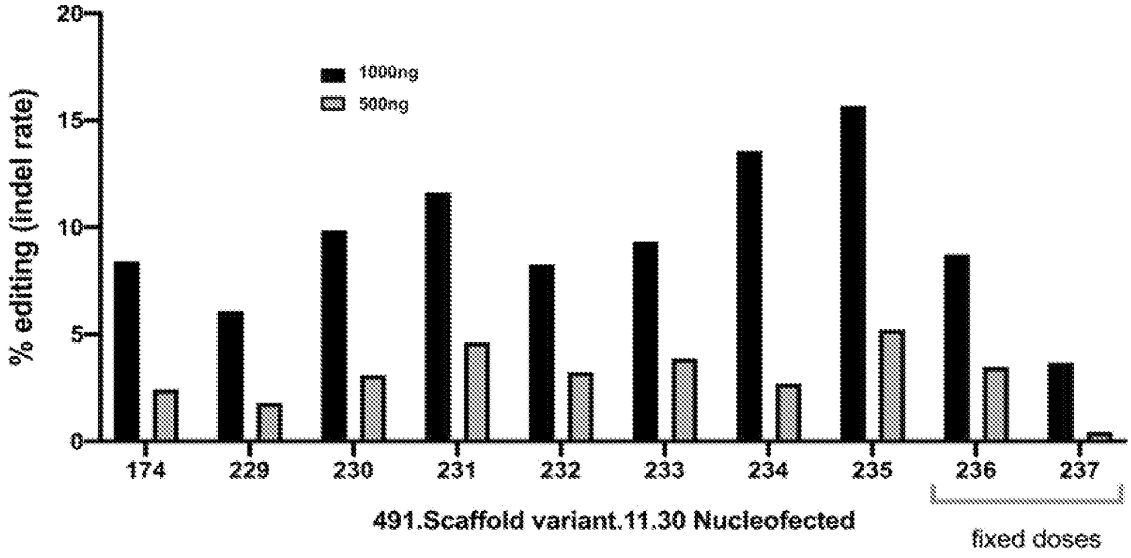

20
15
10
5
0
% editing (indel rate)
1000ng
500ng
174
229
230
231
232
233
234
235
236
237
491.Scaffold variant.11.30 Nucleofected
fixed doses

FIG. 14

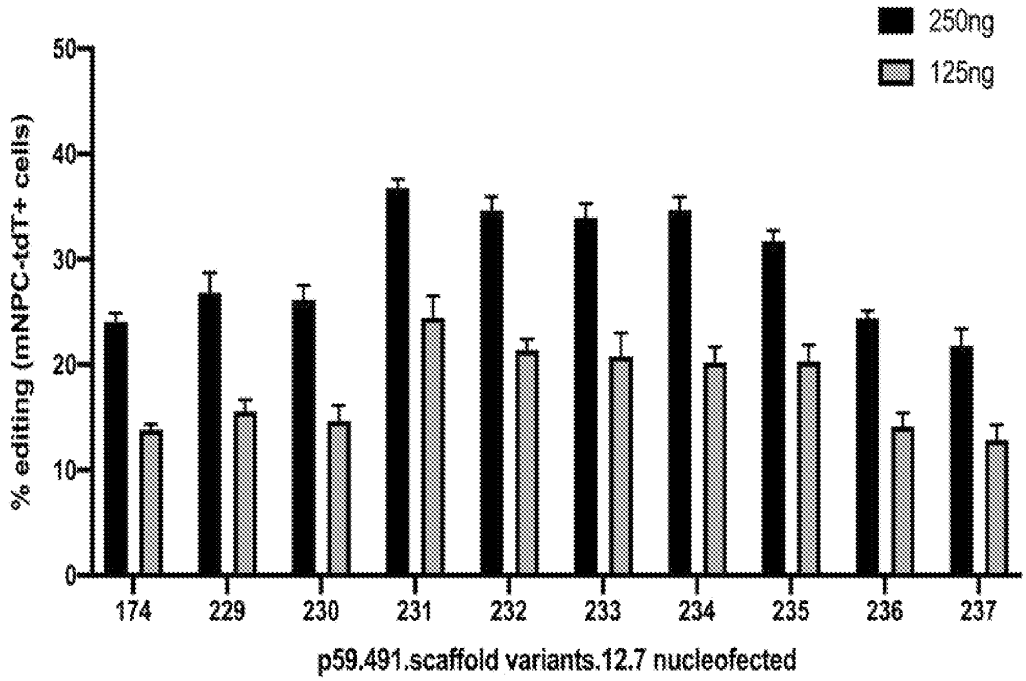
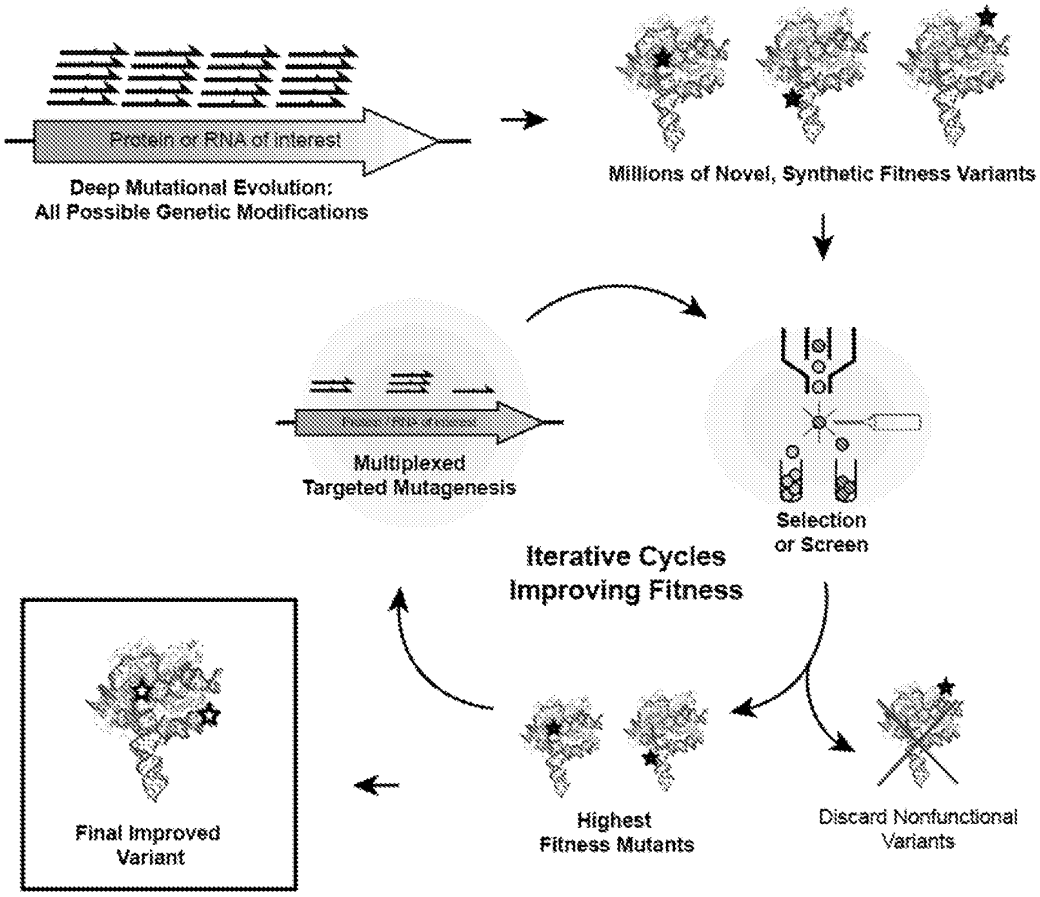

250ng
125ng
% editing (mNPC-tdT+ cells)
0
10
20
30
40
50
174
229
230
231
232
233
234
235
236
237
p59.491.scaffold variants.12.7 nucleofected

FIG. 15



Methods of Generating CasX Protein and guide RNA Variants

FIG. 16

FIG. 17A

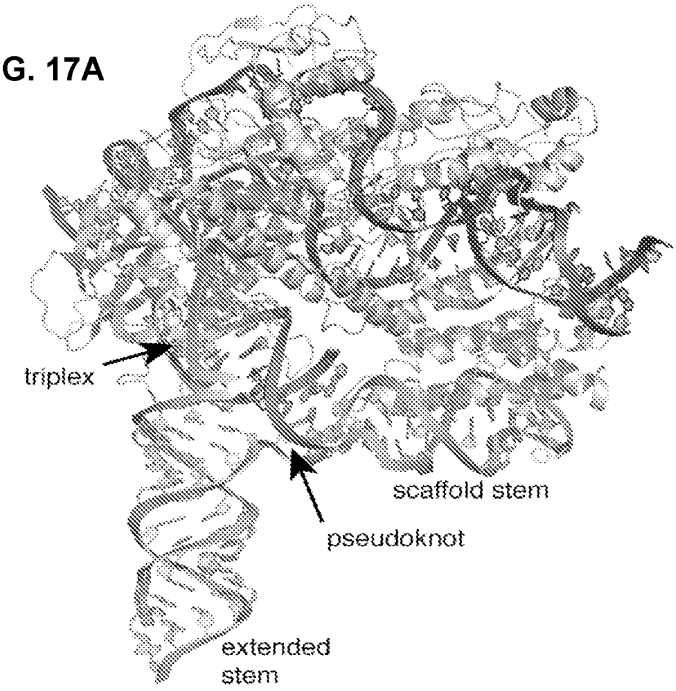
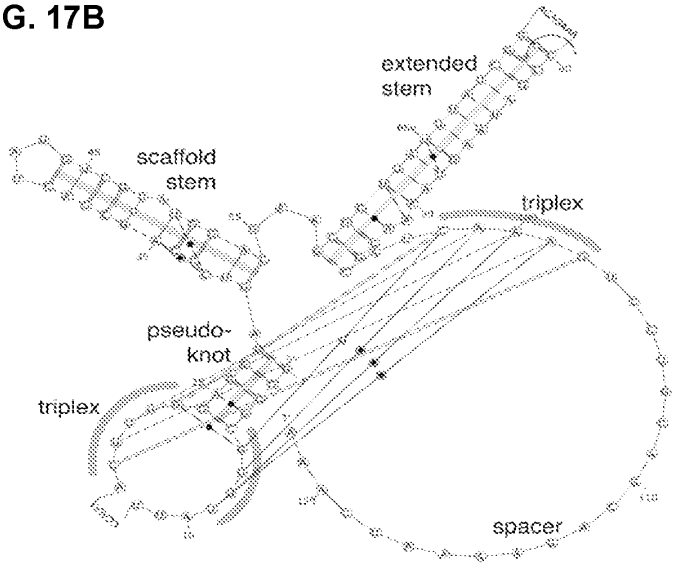


FIG. 17B



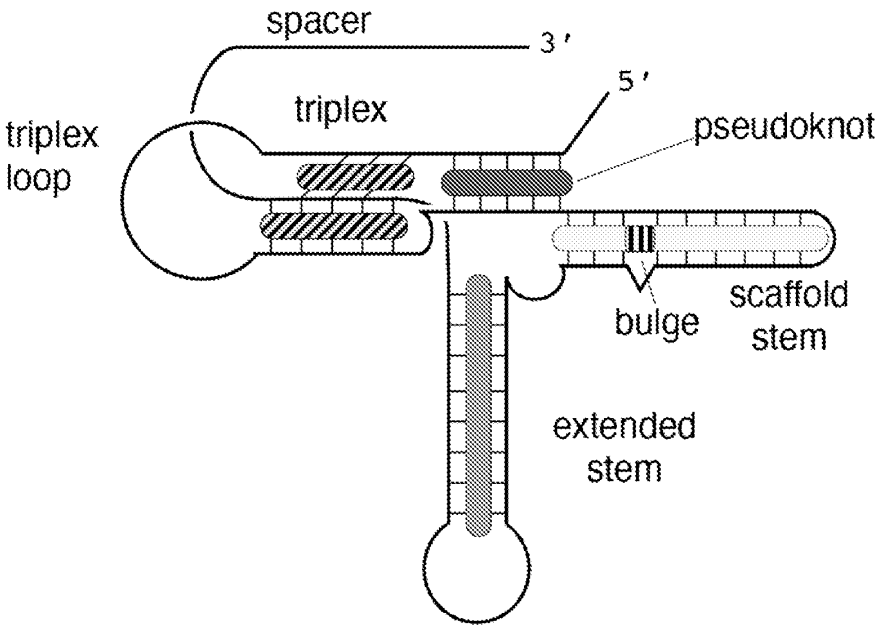

spacer
3'
5'
triplex
triplex loop
pseudoknot
bulge
scaffold stem
extended stem

FIG. 18

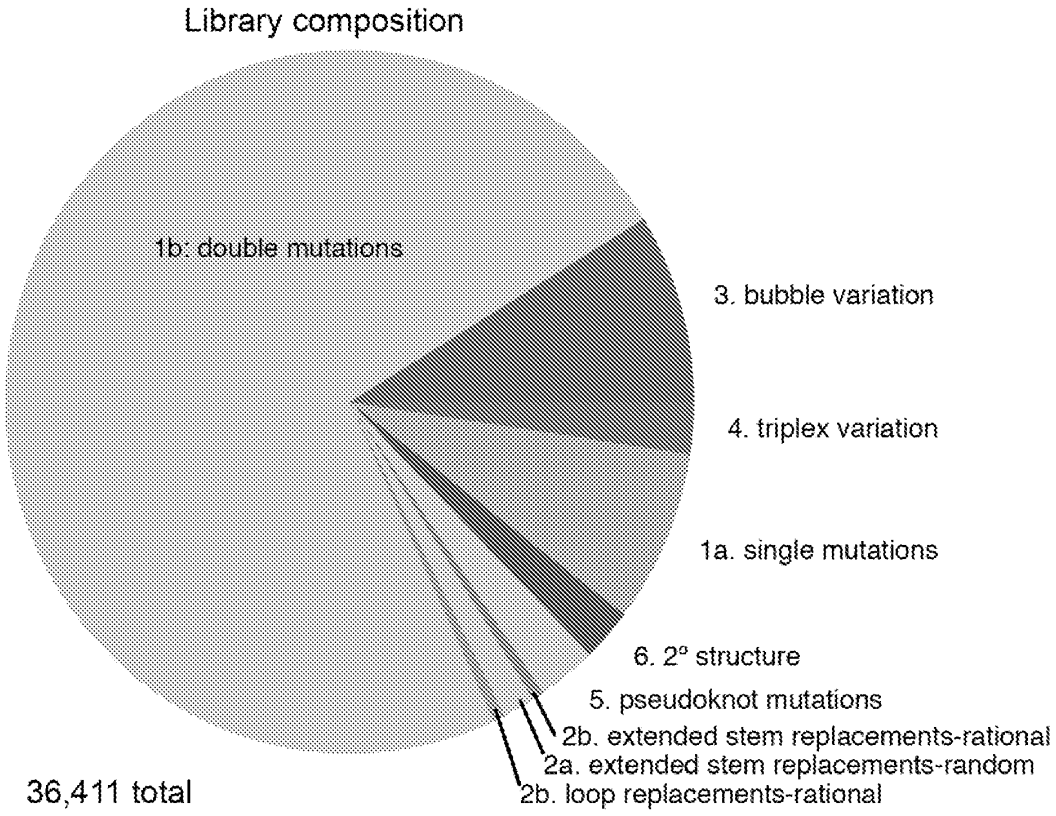

Library composition
1b: double mutations
3. bubble variation
4. triplex variation
1a. single mutations
6. 2° structure
5. pseudoknot mutations
2b. extended stem replacements-rational
2a. extended stem replacements-random
2b. loop replacements-rational
36,411 total

FIG. 19

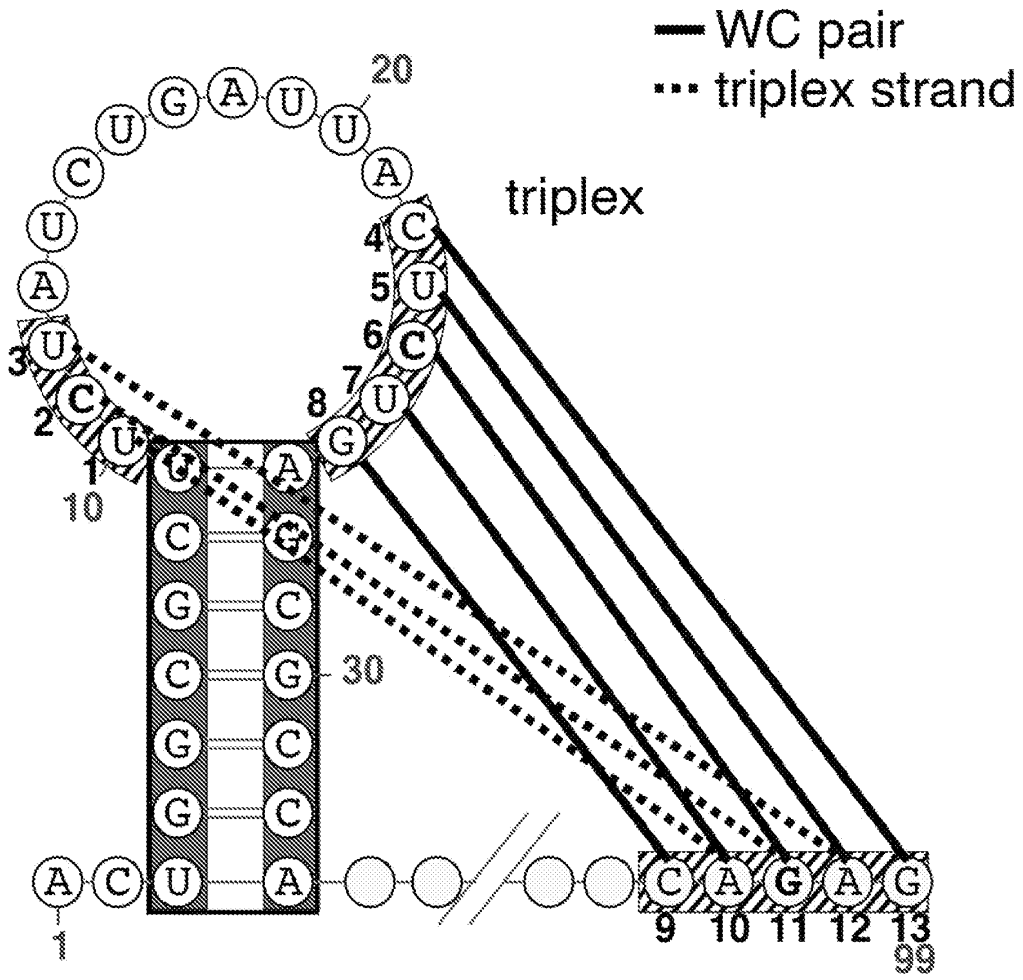


FIG. 20

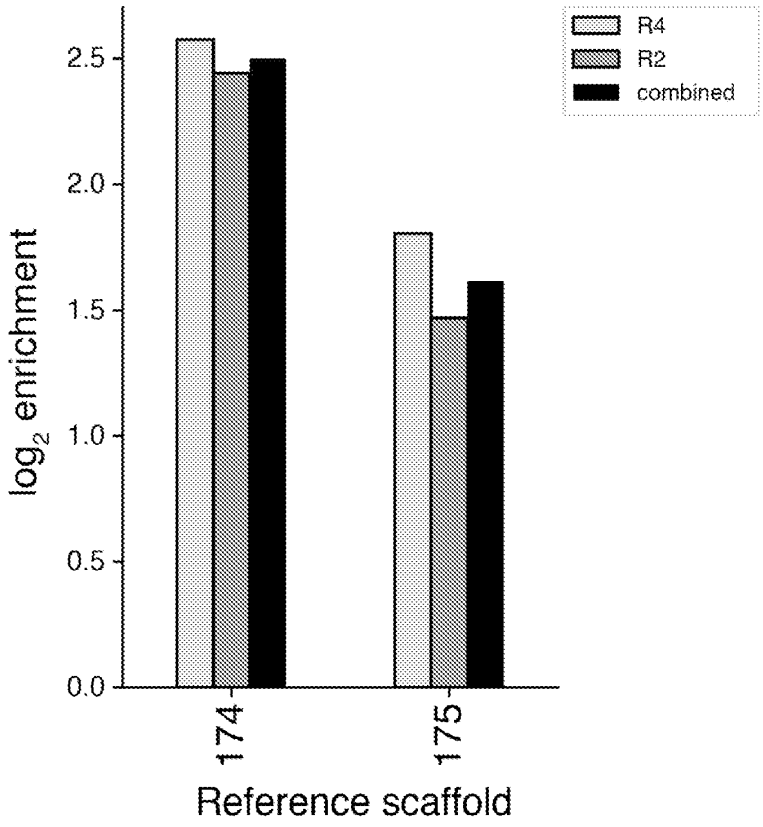

R4
R2
combined
2.5
2.0
1.5
1.0
0.5
0.0
log2 enrichment
174
175
Reference scaffold

FIG. 21

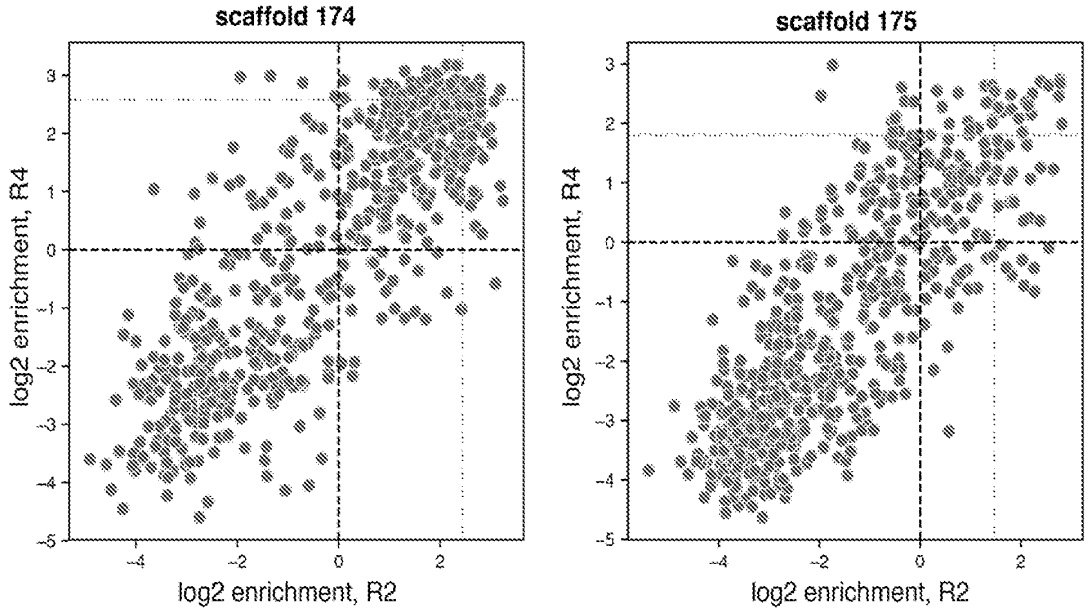

scaffold 174
log2 enrichment, R4
log2 enrichment, R2
scaffold 175
log2 enrichment, R4
log2 enrichment, R2

FIG. 22

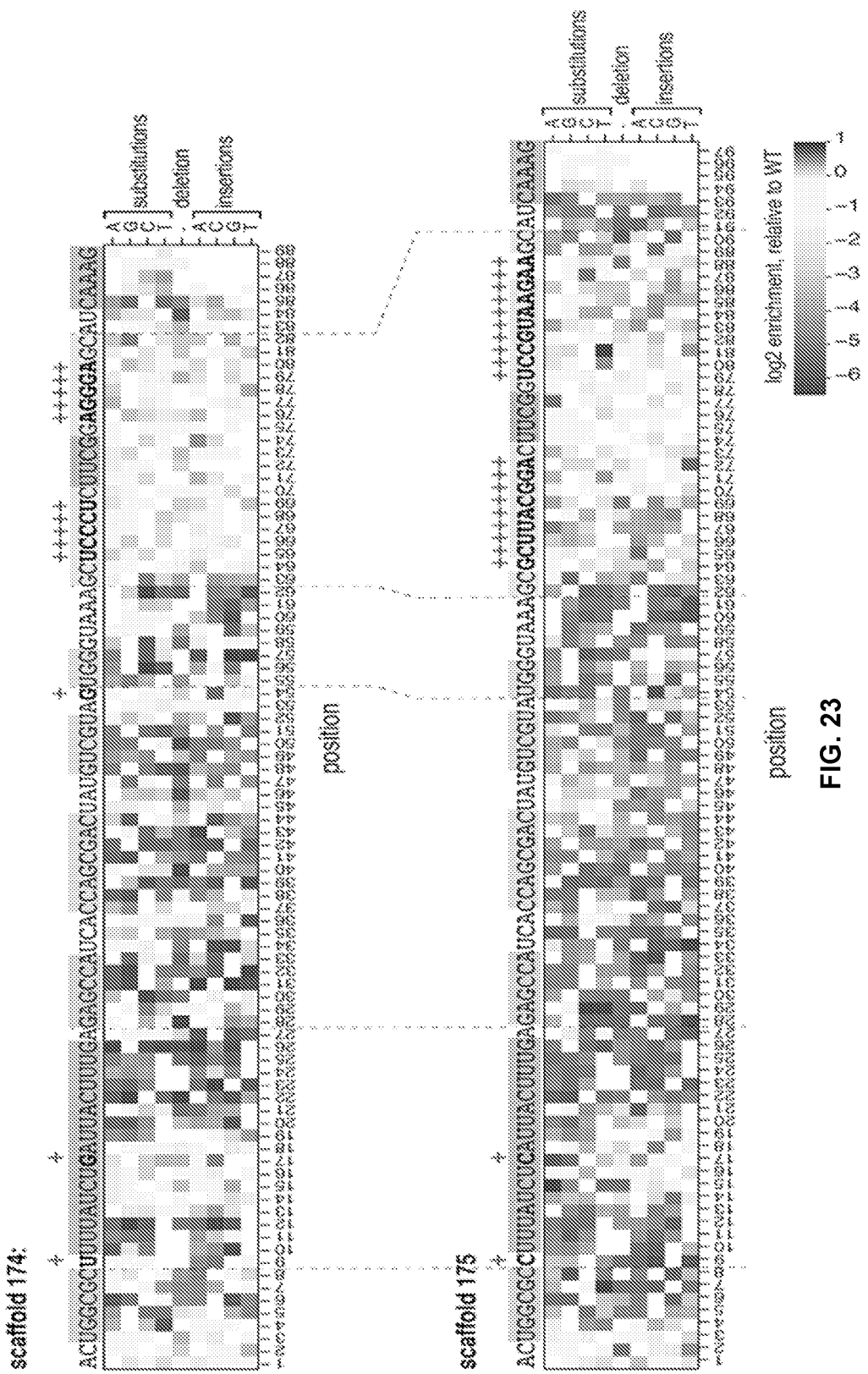

scaffold 174:
scaffold 175
position
position
substitutions
deletion
insertions
log2 enrichment, relative to WT

FIG. 23

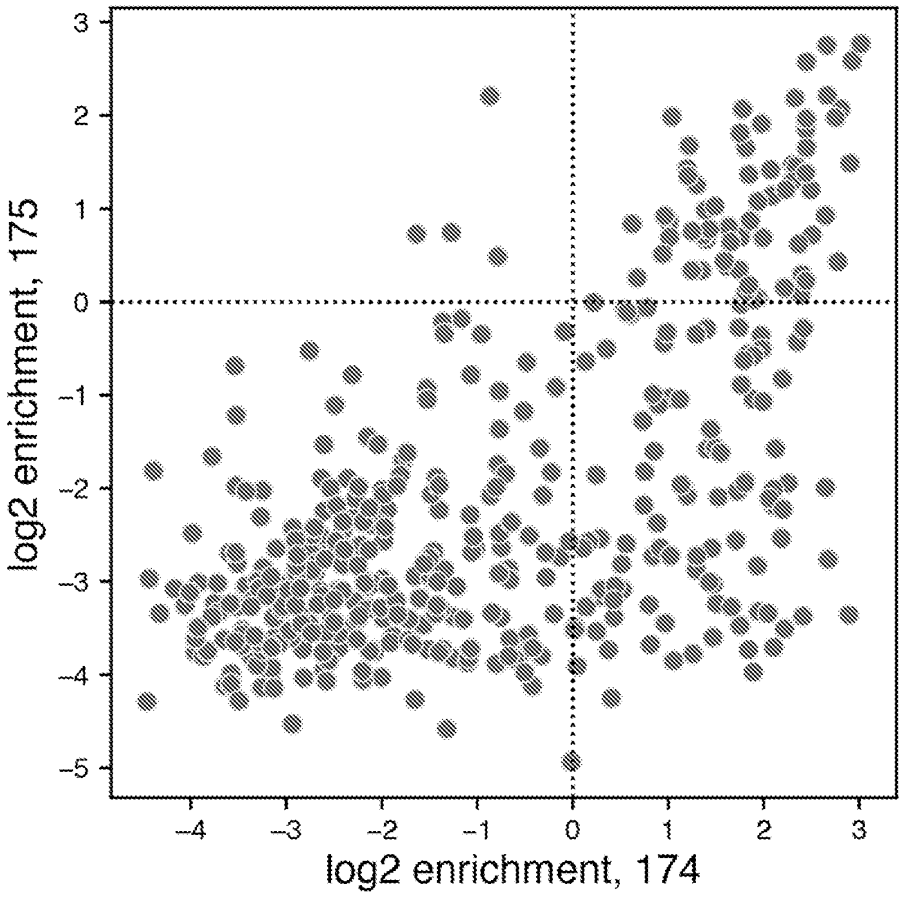

log2 enrichment, 175
log2 enrichment, 174
3
2
1
0
−1
−2
−3
−4
−5
−4
−3
−2
−1
0
1
2
3

FIG. 24

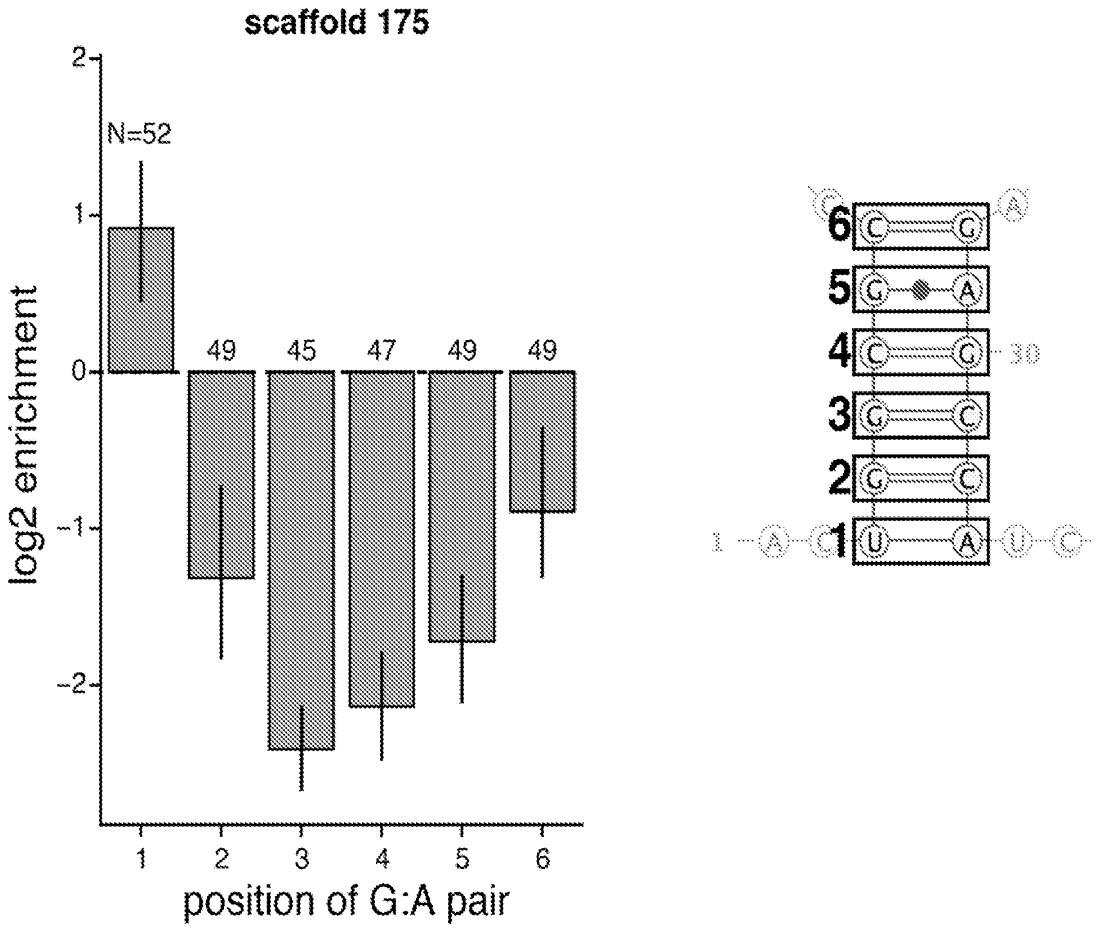

scaffold 175
log2 enrichment
2
1
0
−1
−2
N=52
49
45
47
49
49
1
2
3
4
5
6
position of G:A pair
6
5
4
3
2
1
30

FIG. 25

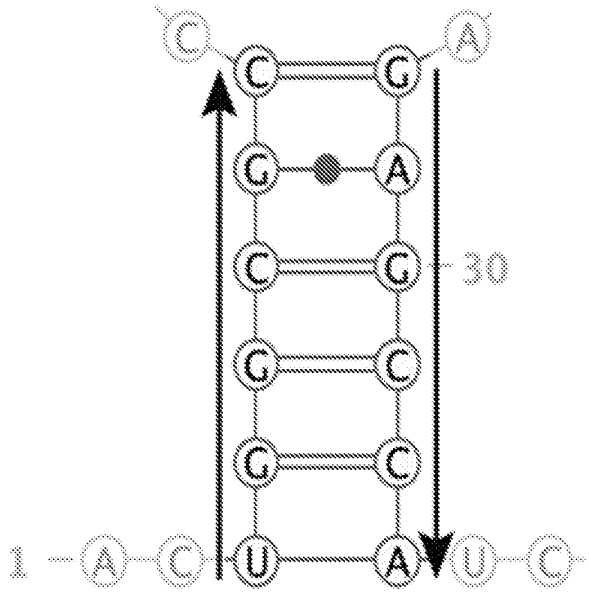


FIG. 26

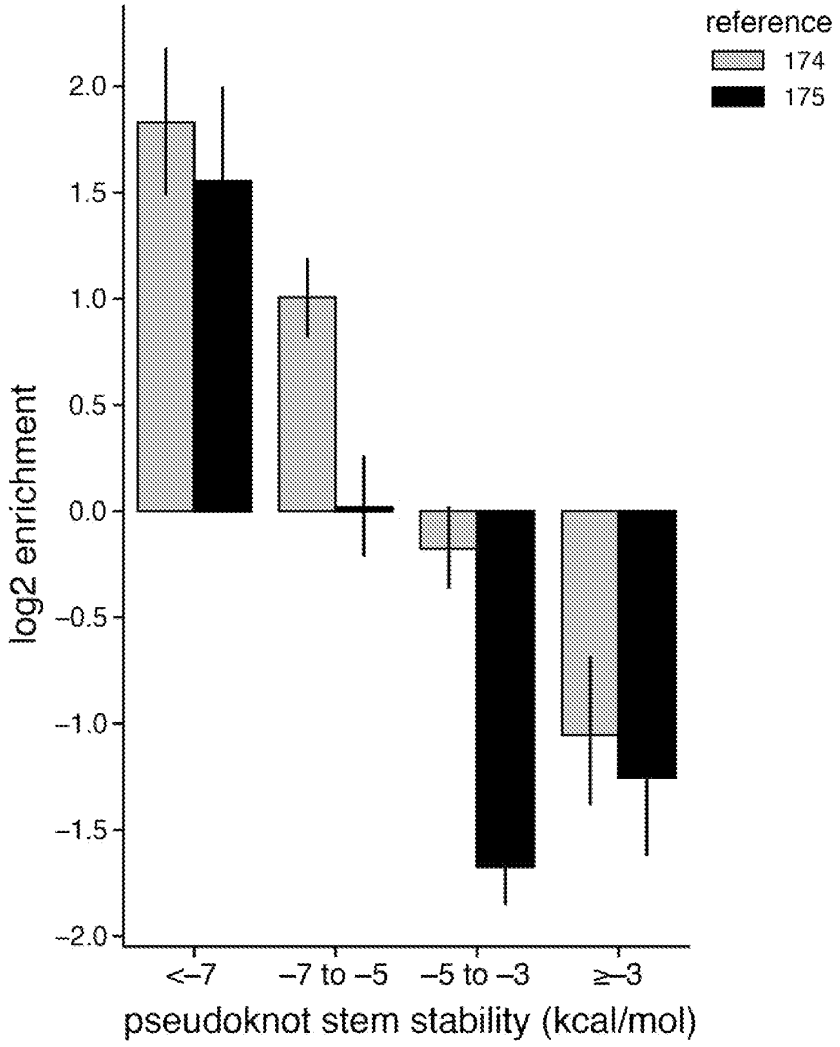

reference
174
175
2.0
1.5
1.0
0.5
0.0
−0.5
−1.0
−1.5
−2.0
log2 enrichment
<−7
−7 to −5
−5 to −3
≥−3
pseudoknot stem stability (kcal/mol)

FIG. 27

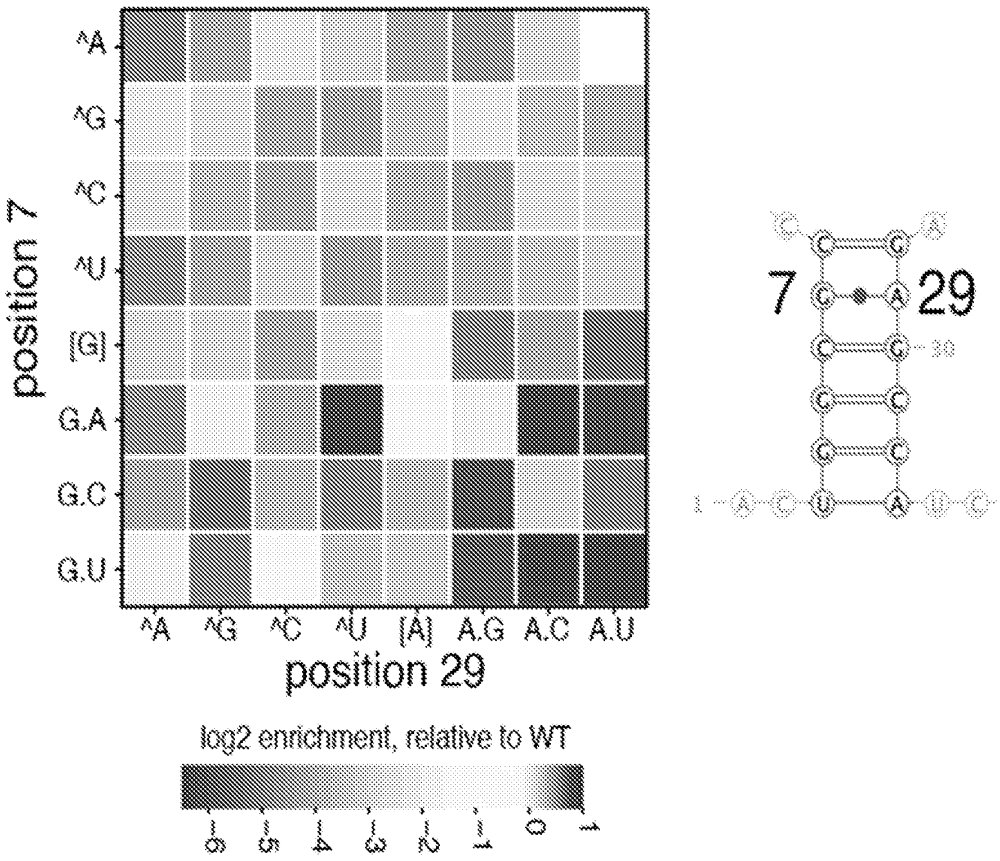

position 7
^A
^G
^C
^U
[G]
G.A
G.C
G.U
^A ^G ^C ^U [A] A.G A.C A.U
position 29
log2 enrichment, relative to WT
7
29

FIG. 28

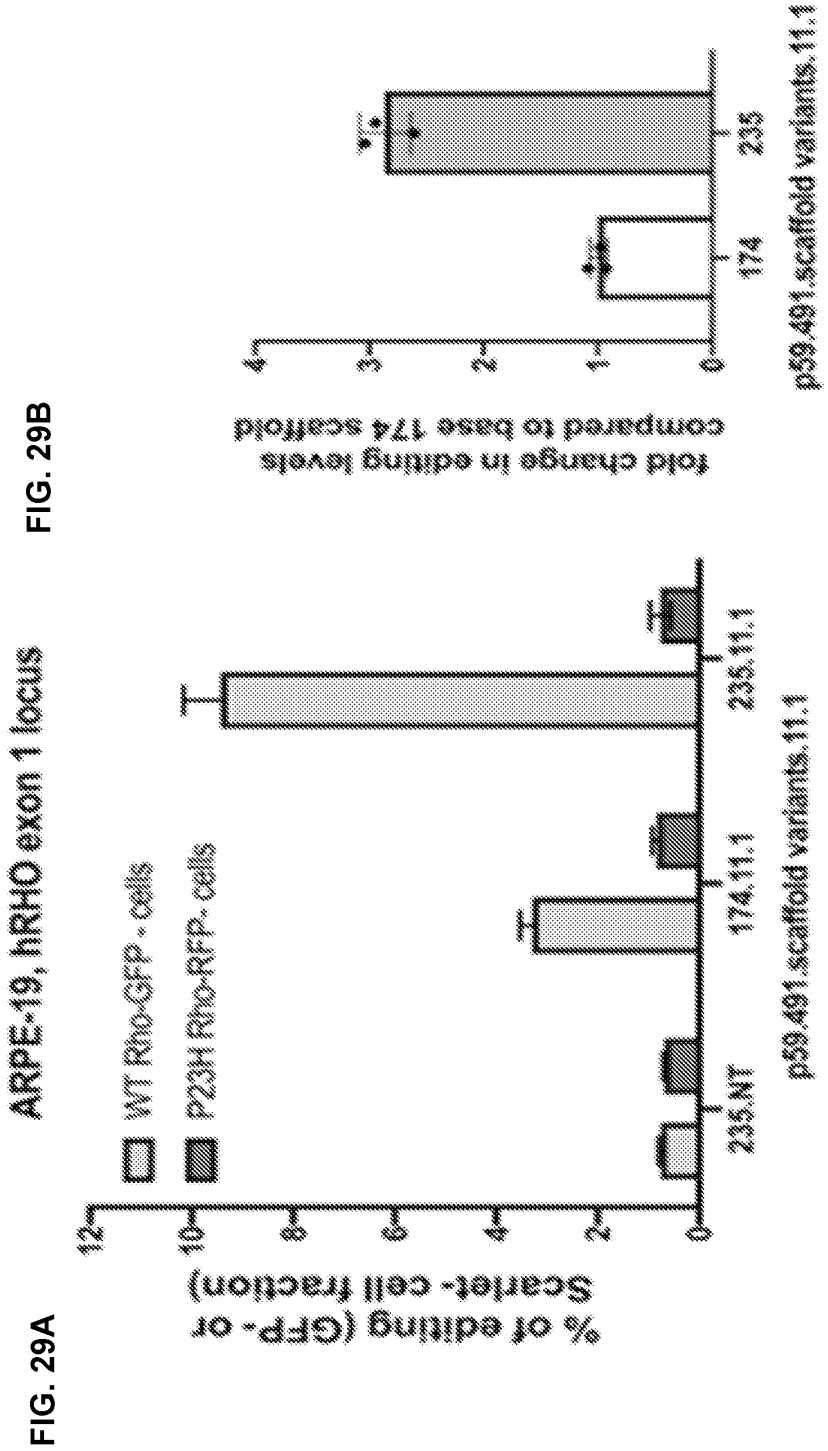


FIG. 29A

FIG. 29B

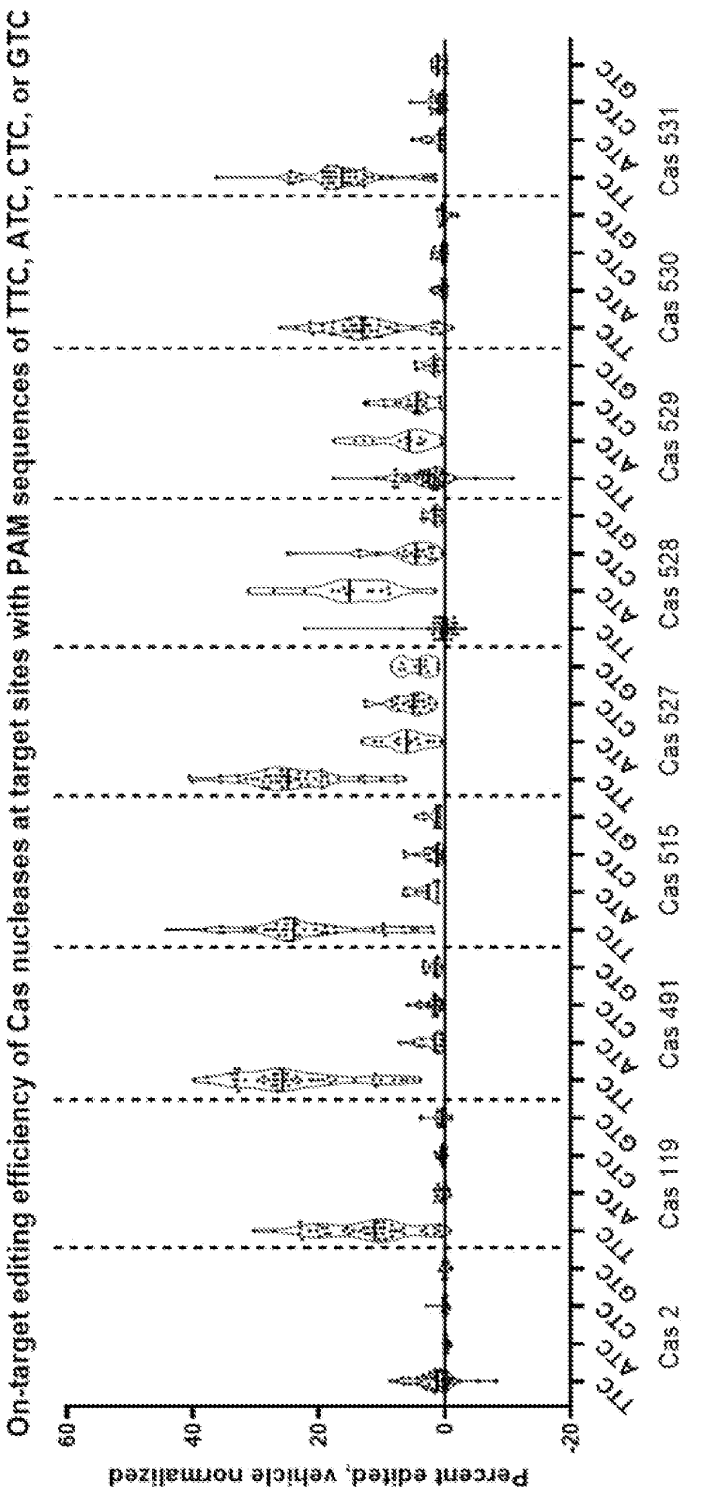

On-target editing efficiency of Cas nucleases at target sites with PAM sequences of TTC, ATC, CTC, or GTC
Percent edited, vehicle normalized
60
40
20
0
-20
TTC ATC CTC GTC TTC ATC CTC GTC TTC ATC CTC GTC TTC ATC CTC GTC TTC ATC CTC GTC TTC ATC CTC GTC TTC ATC CTC GTC
Cas 2
Cas 119
Cas 491
Cas 515
Cas 527
Cas 528
Cas 529
Cas 530
Cas 531
Cas Protein

FIG. 30

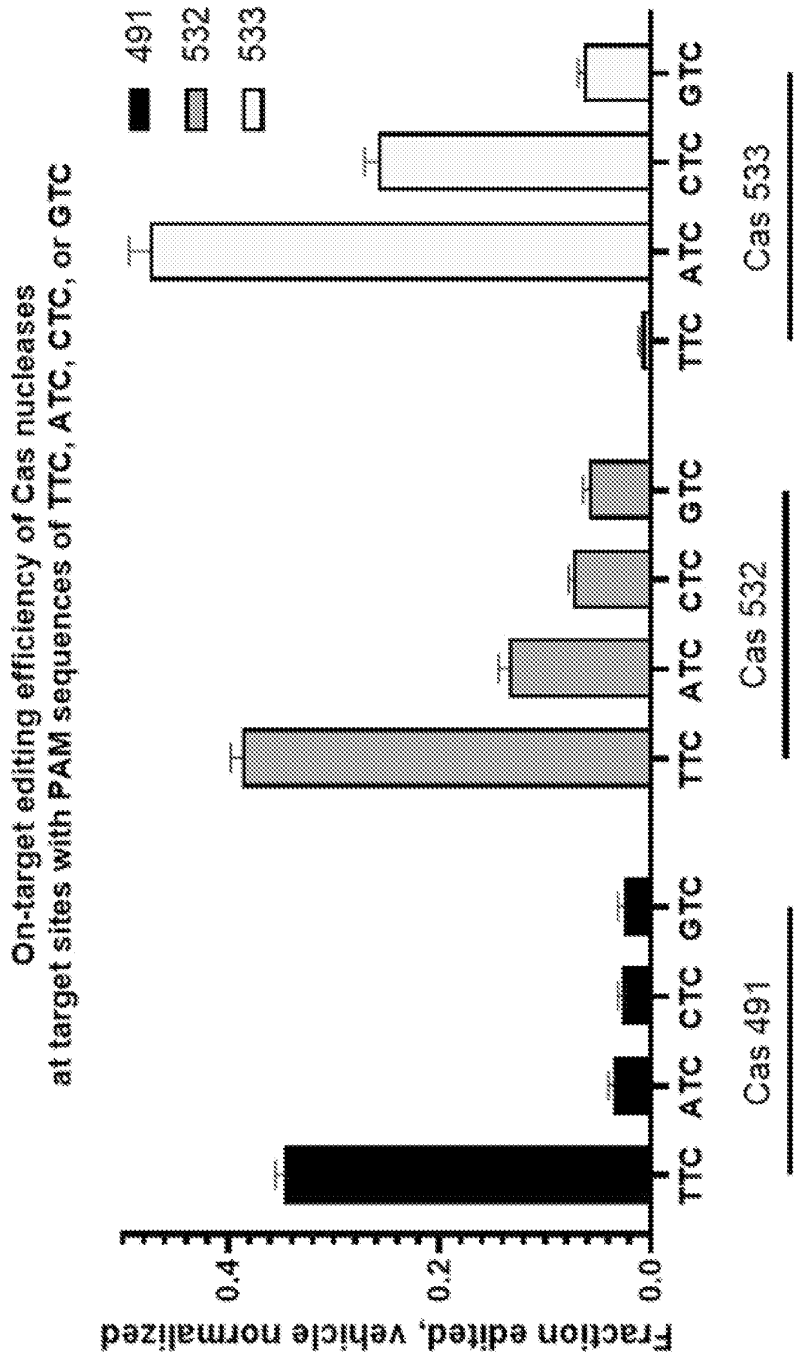

On-target editing efficiency of Cas nucleases
at target sites with PAM sequences of TTC, ATC, CTC, or GTC
491
532
533
Fraction edited, vehicle normalized
0.0
0.2
0.4
TTC
ATC
CTC
GTC
TTC
ATC
CTC
GTC
TTC
ATC
CTC
GTC
Cas 491
Cas 532
Cas 533
Cas Protein

FIG. 31

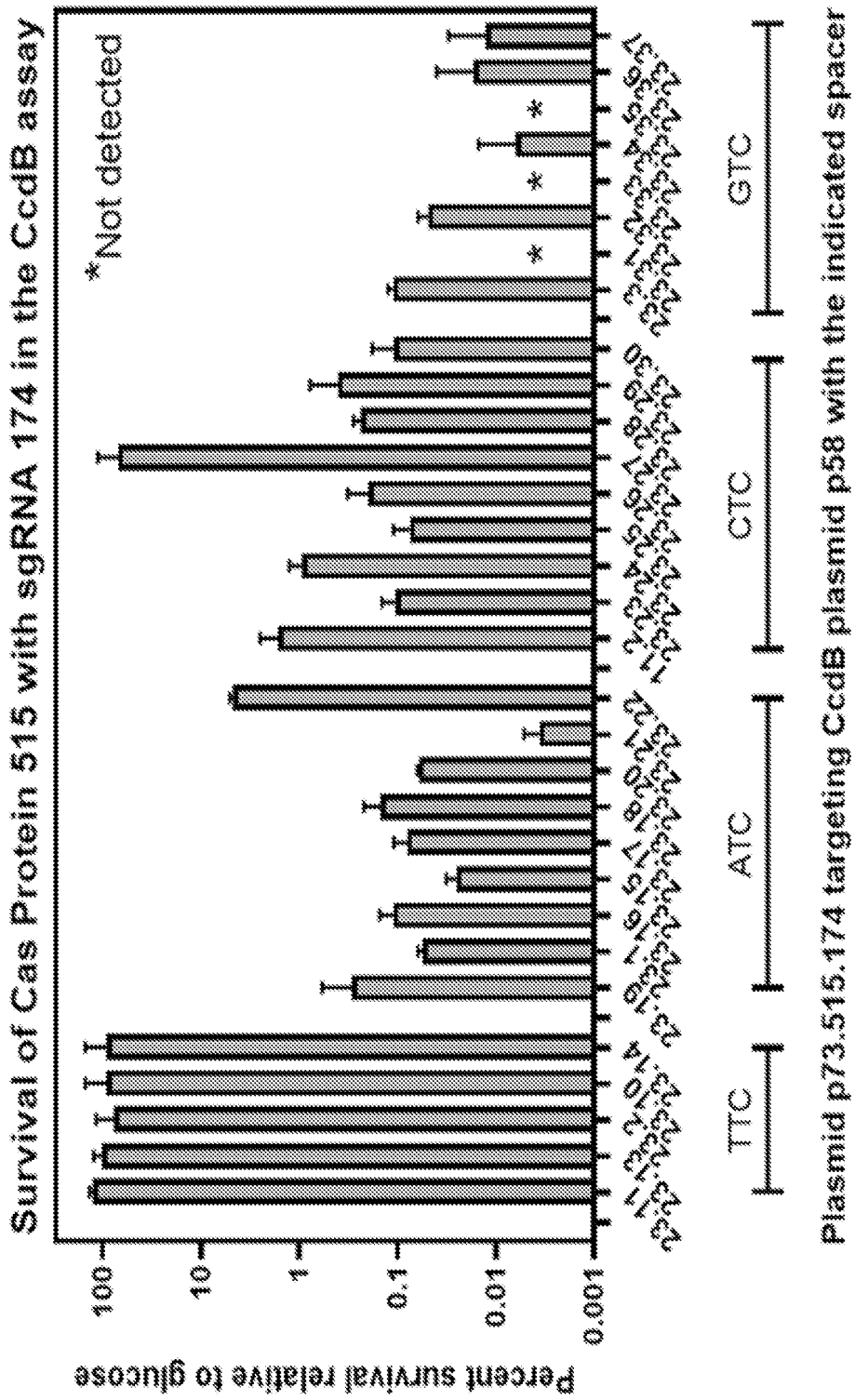

Survival of Cas Protein 515 with sgRNA 174 in the CcdB assay
*Not detected
Percent survival relative to glucose
100
10
1
0.1
0.01
0.001
TTC
ATC
CTC
GTC
Plasmid p73.515.174 targeting CcdB plasmid p58 with the indicated spacer

FIG. 32

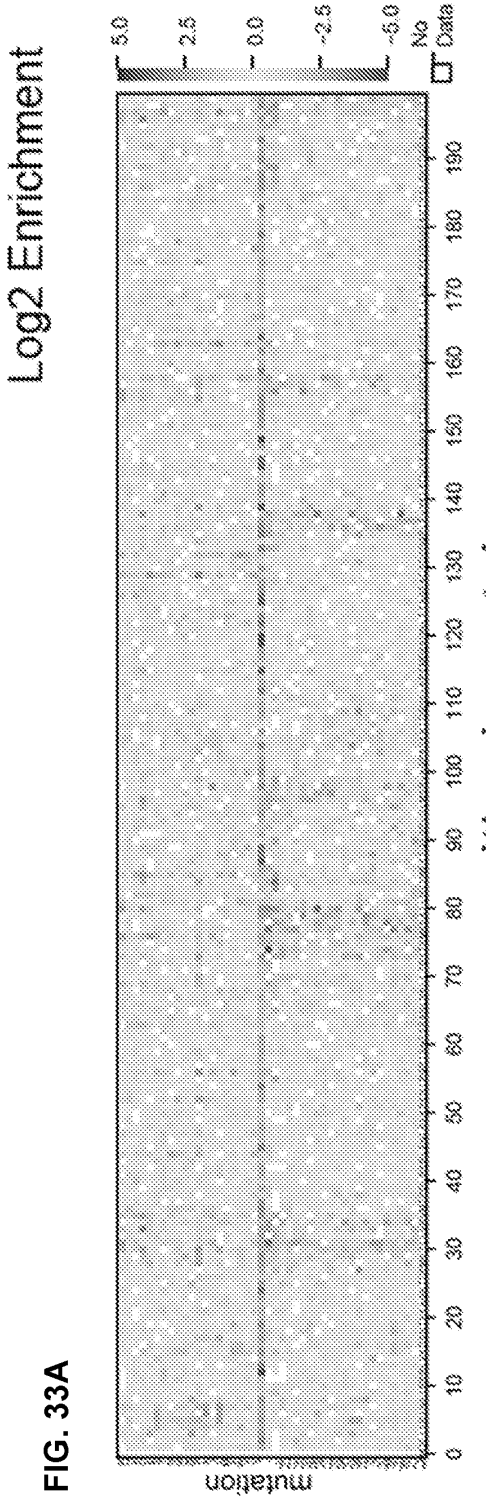


FIG. 33A

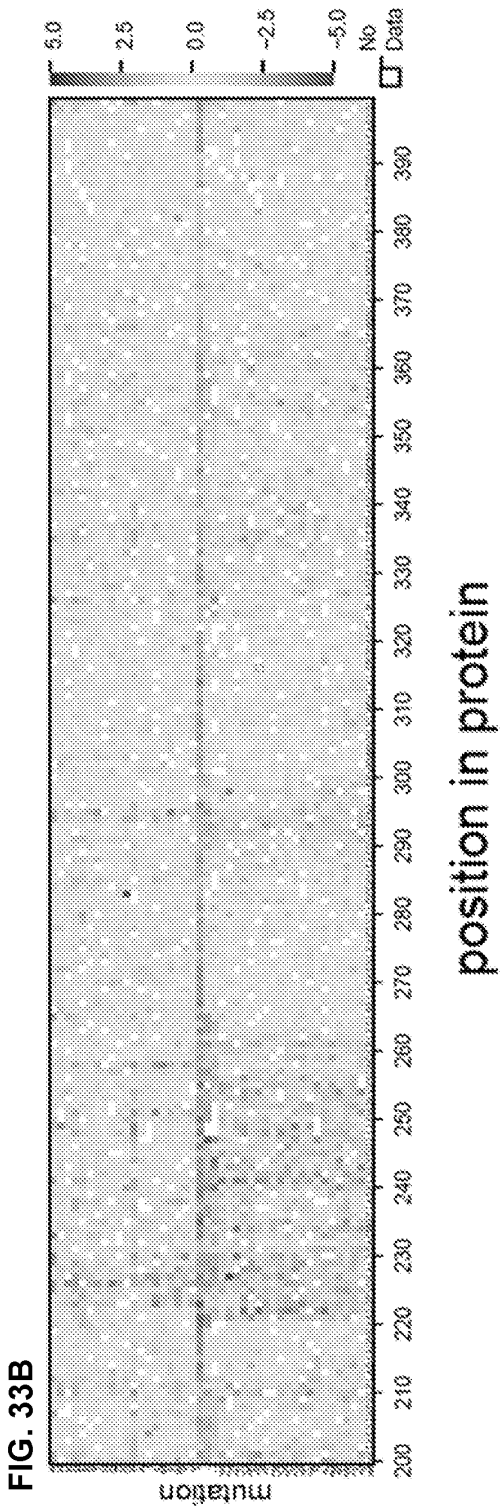


FIG. 33B

FIG. 33C

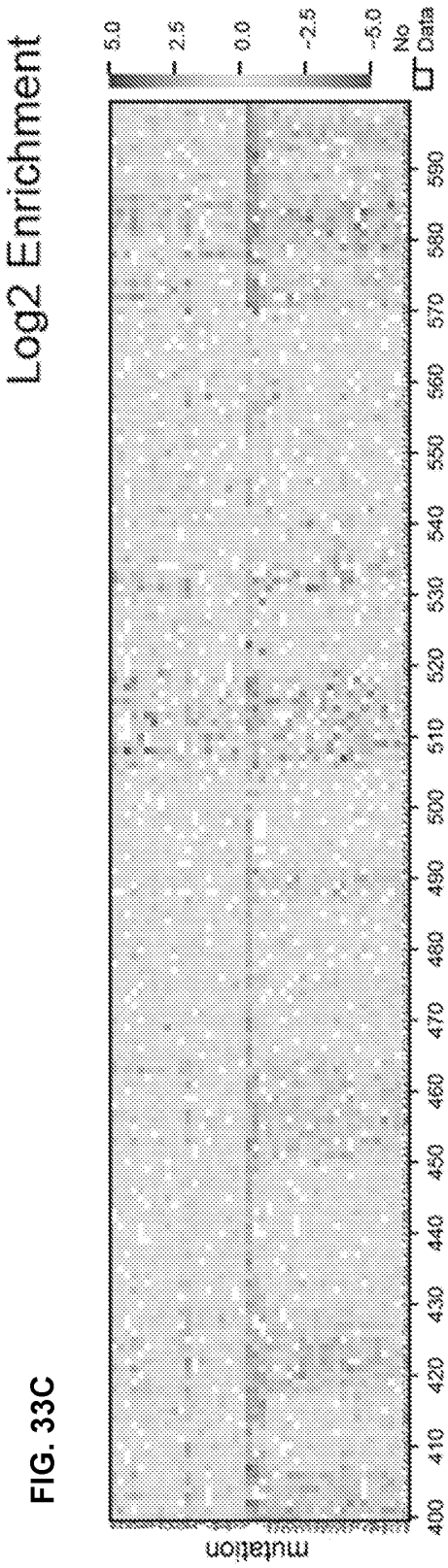
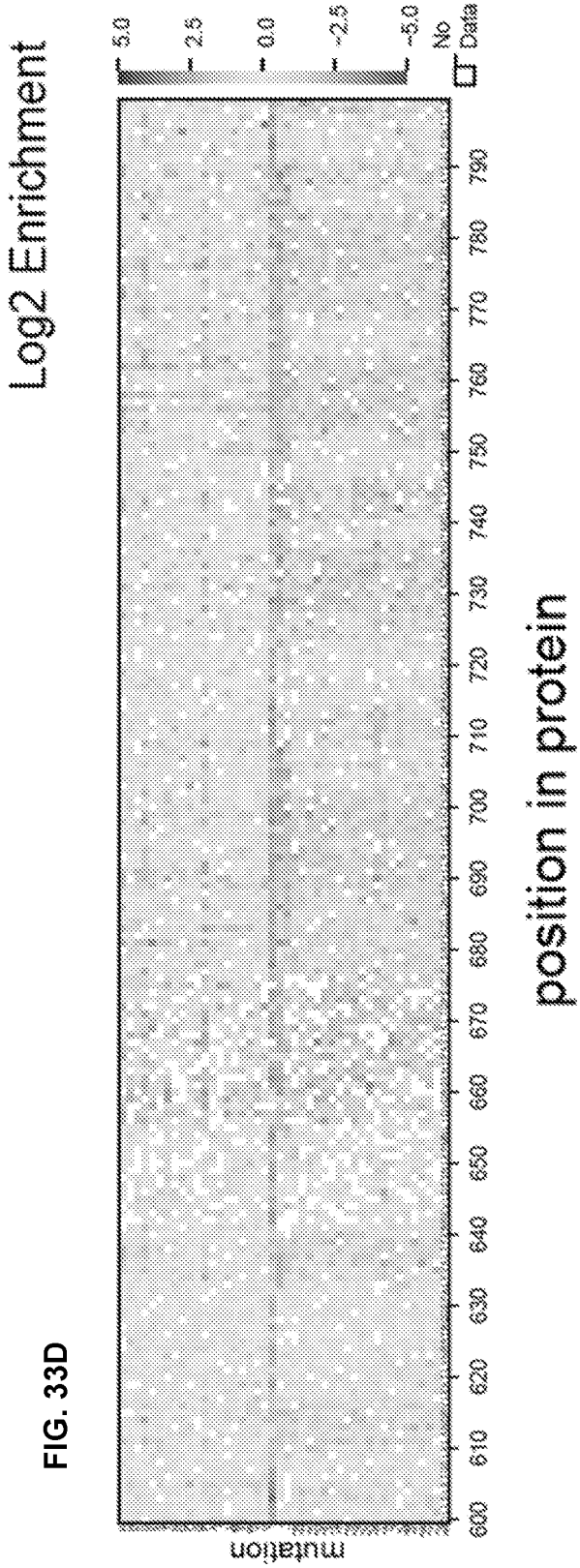


FIG. 33D



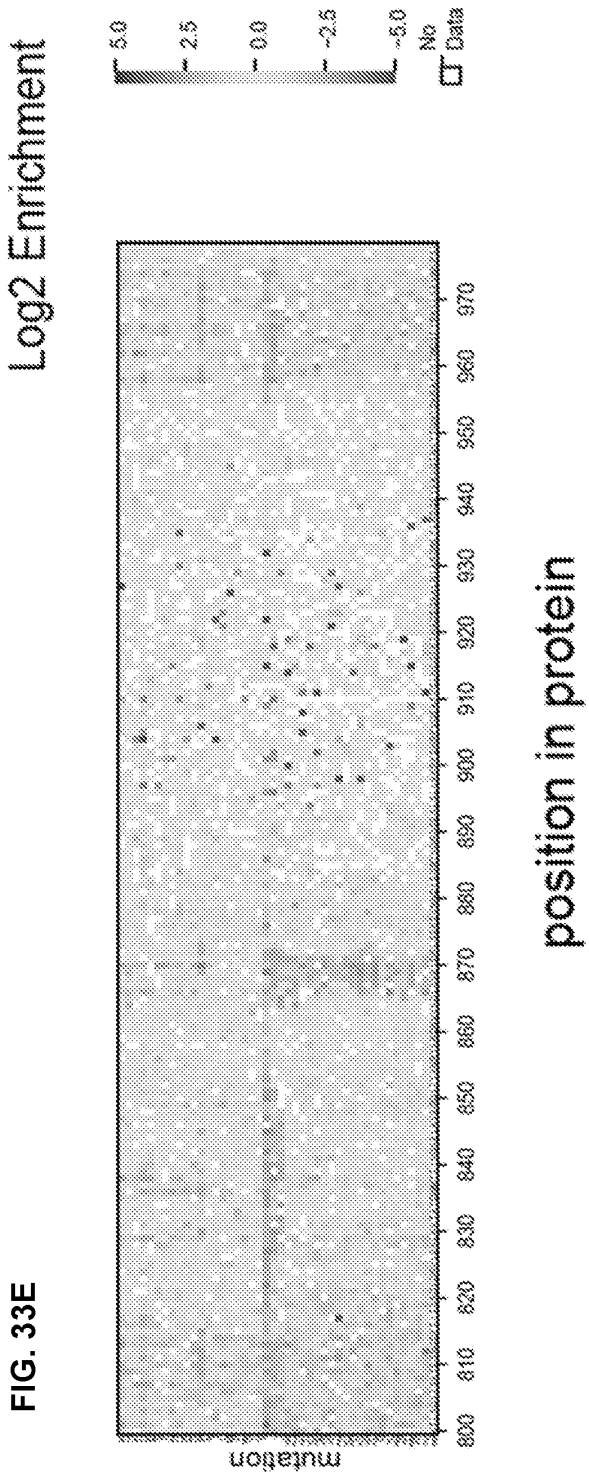

Log2 Enrichment
5.0
2.5
0.0
-2.5
-5.0
No Data
mutation
position in protein
810
820
830
840
850
860
870
880
890
900
910
920
930
940
950
960
970

FIG. 33E

FIG. 34A

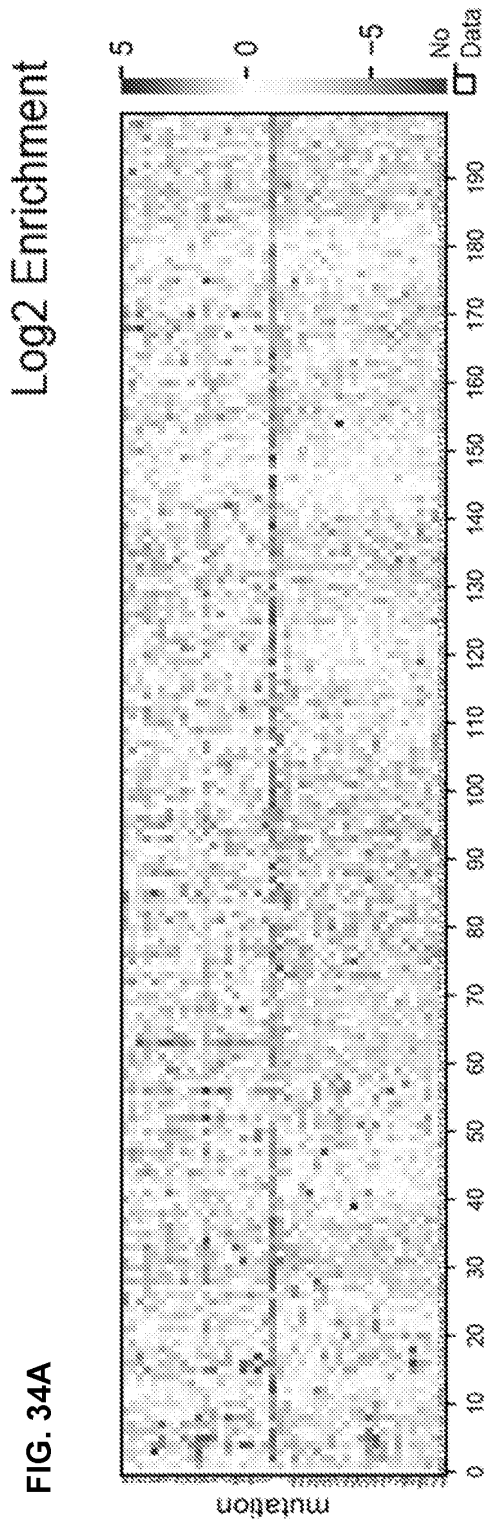
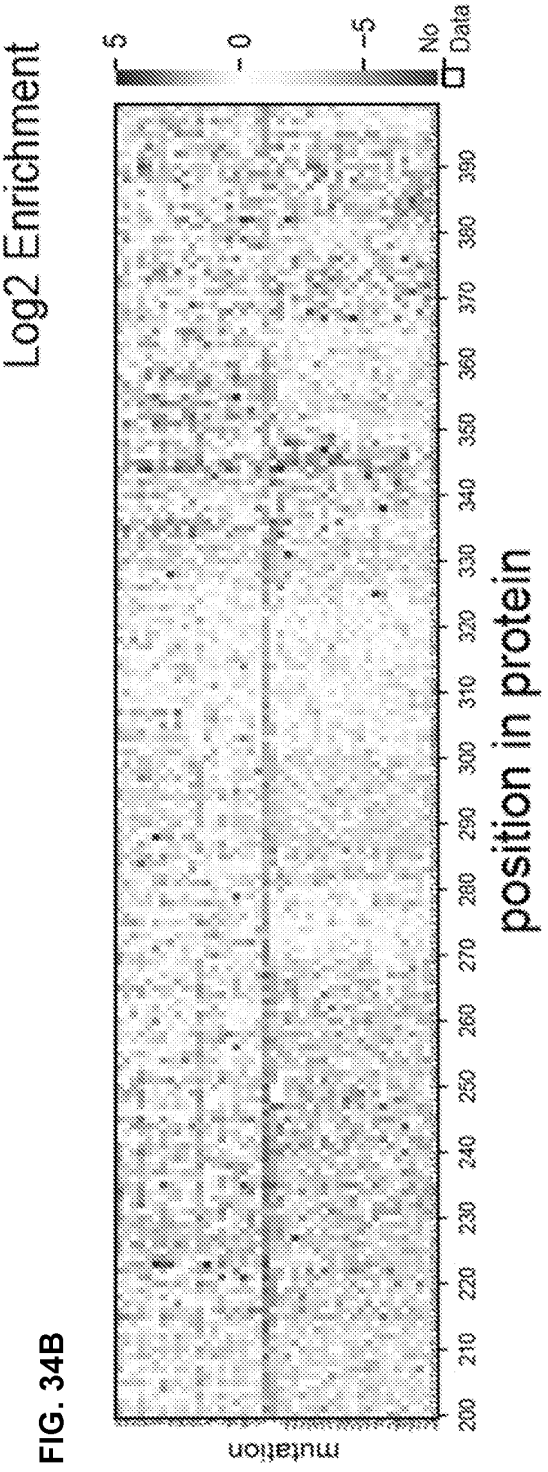


FIG. 34B



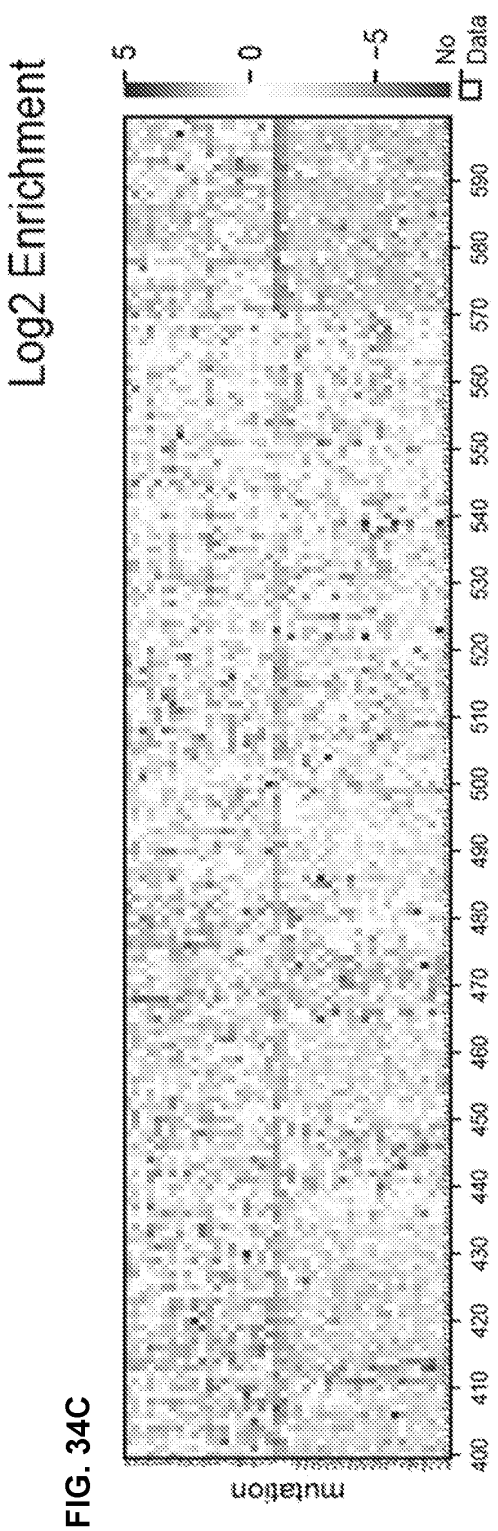


FIG. 34C

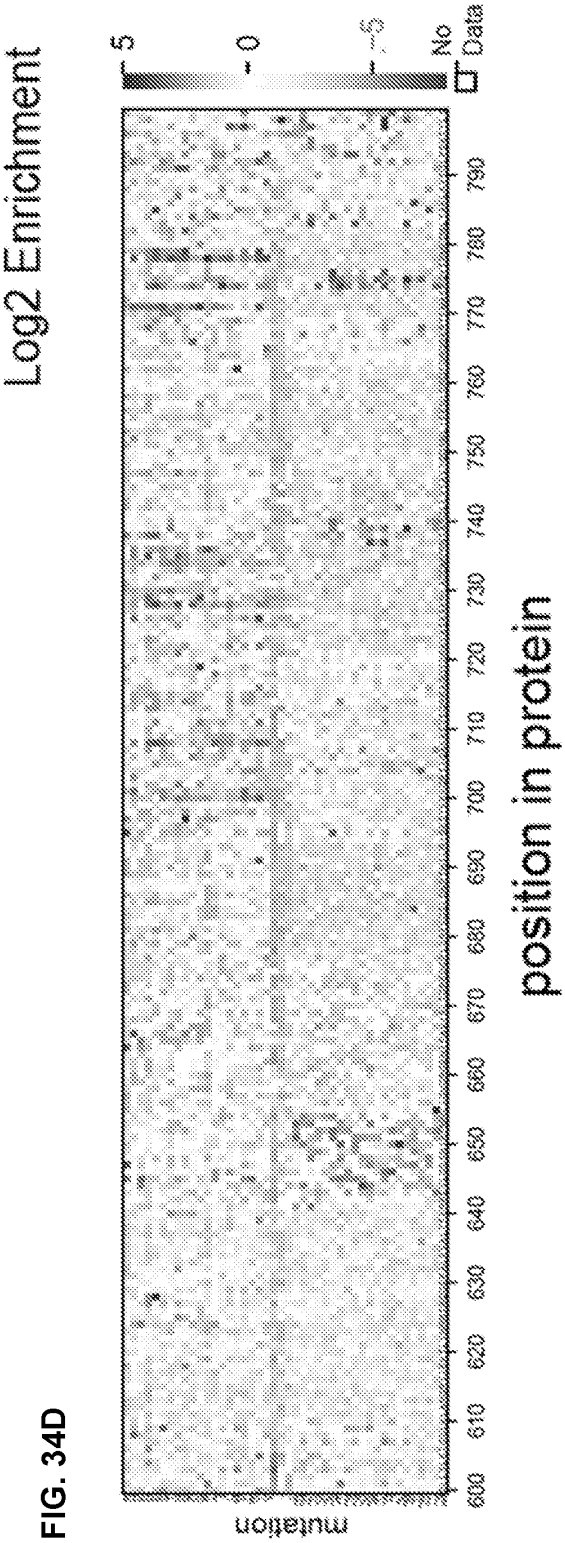


FIG. 34D

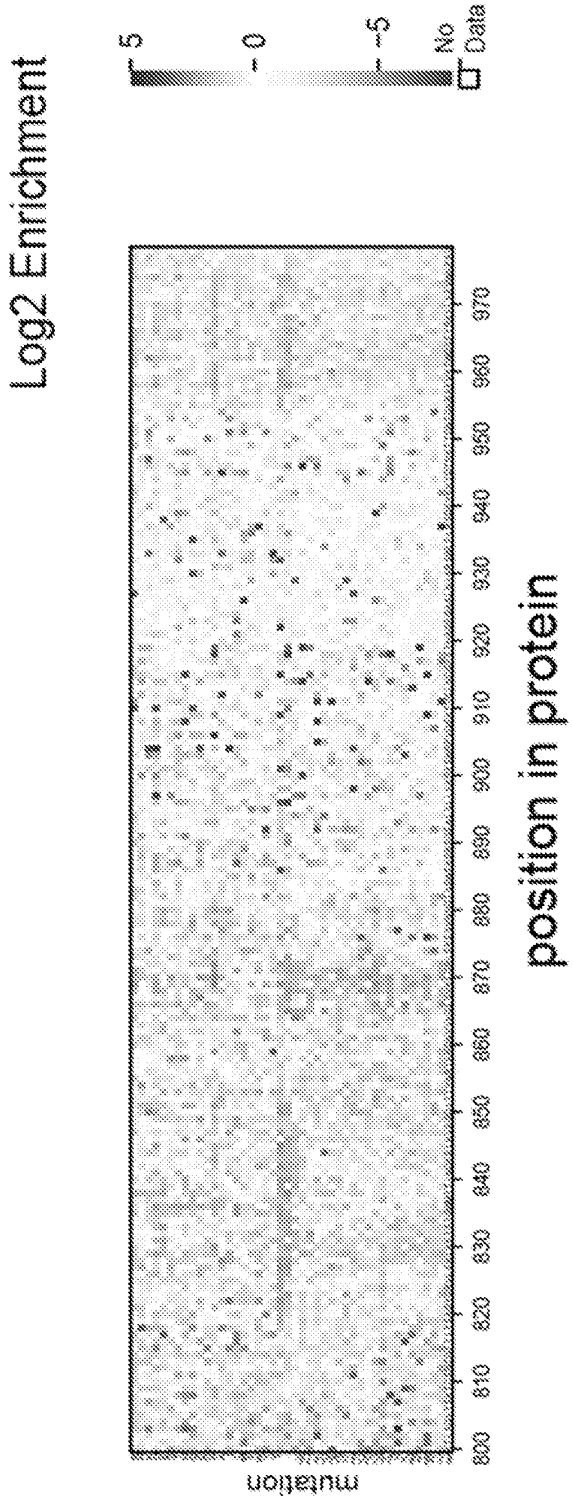

Log2 Enrichment
5
0
-5
No Data
position in protein
mutation
800
810
820
830
840
850
860
870
880
890
900
910
920
930
940
950
960
970

FIG. 34E

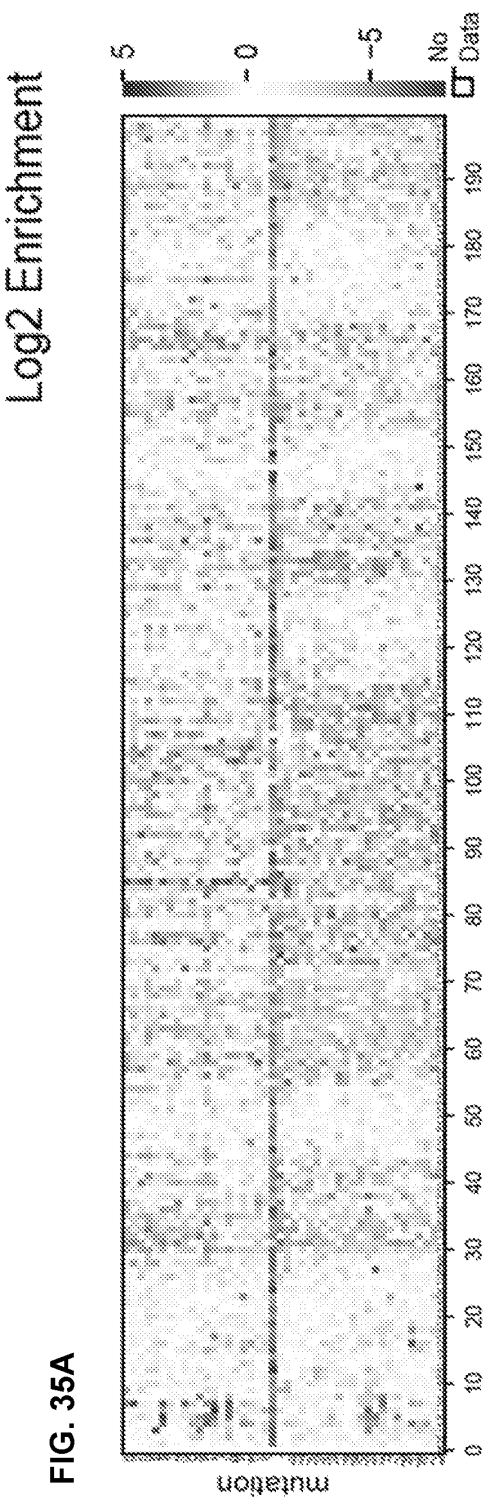


FIG. 35A

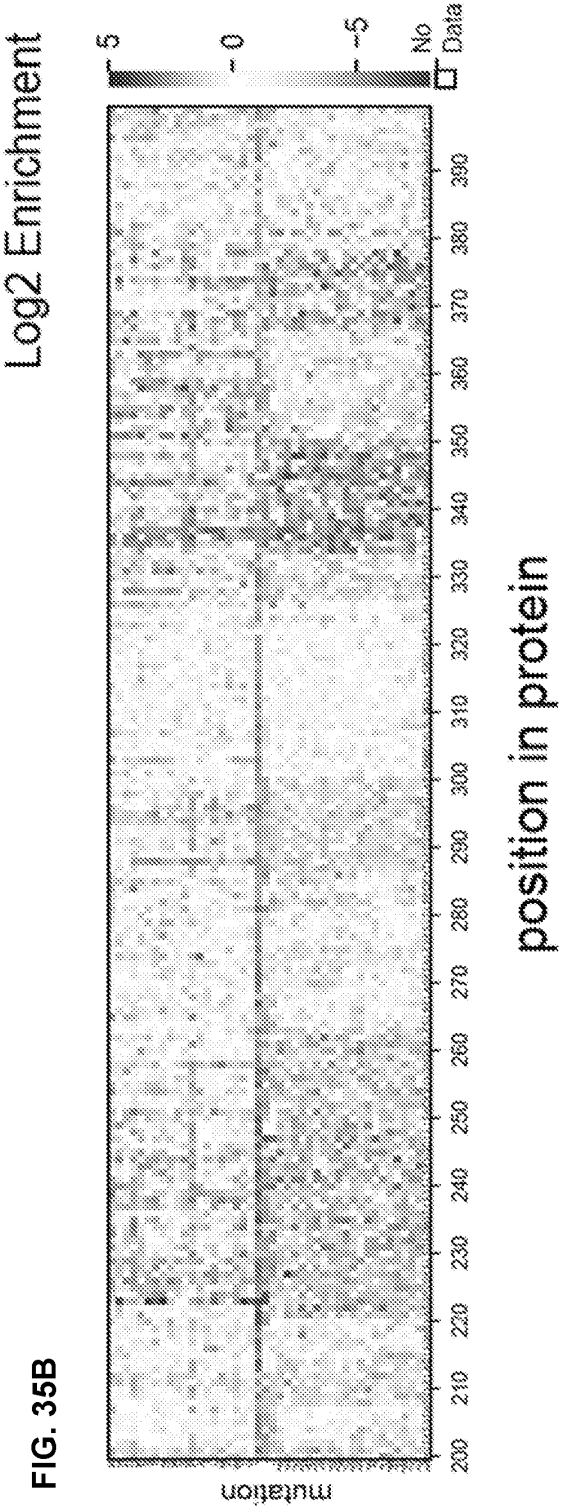


FIG. 35B

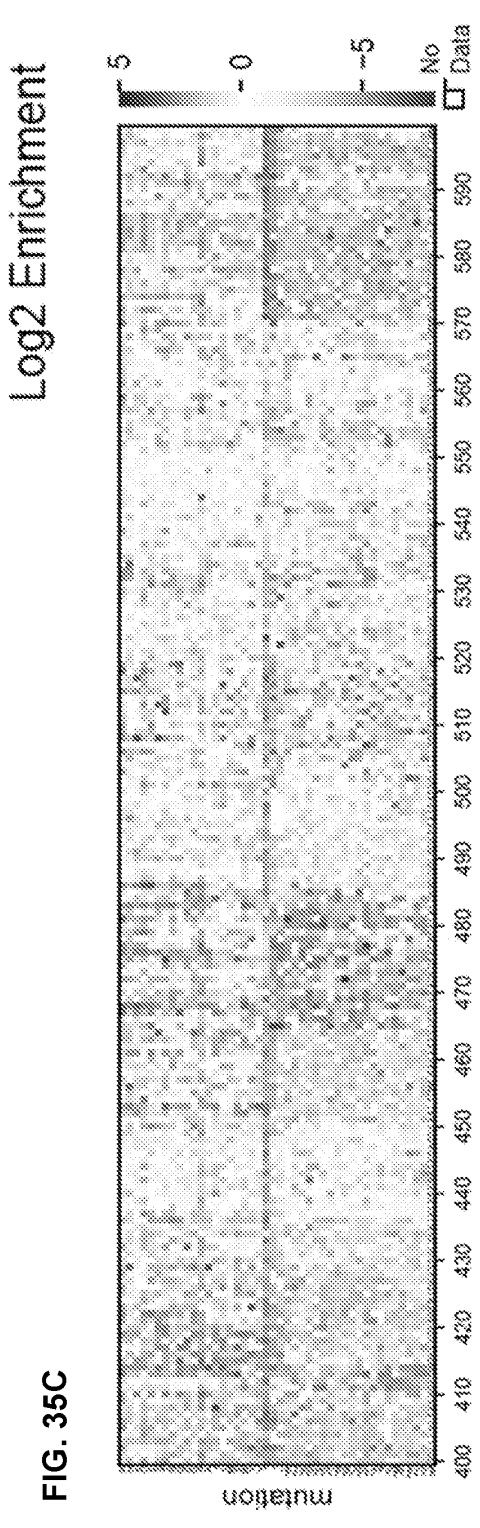


FIG. 35C

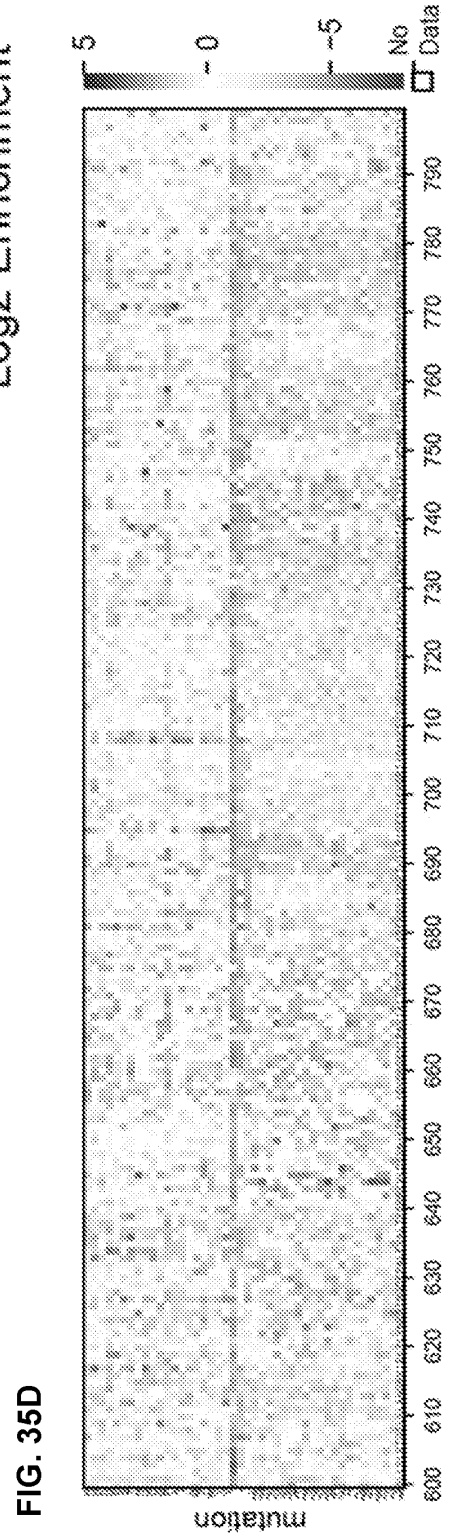


FIG. 35D

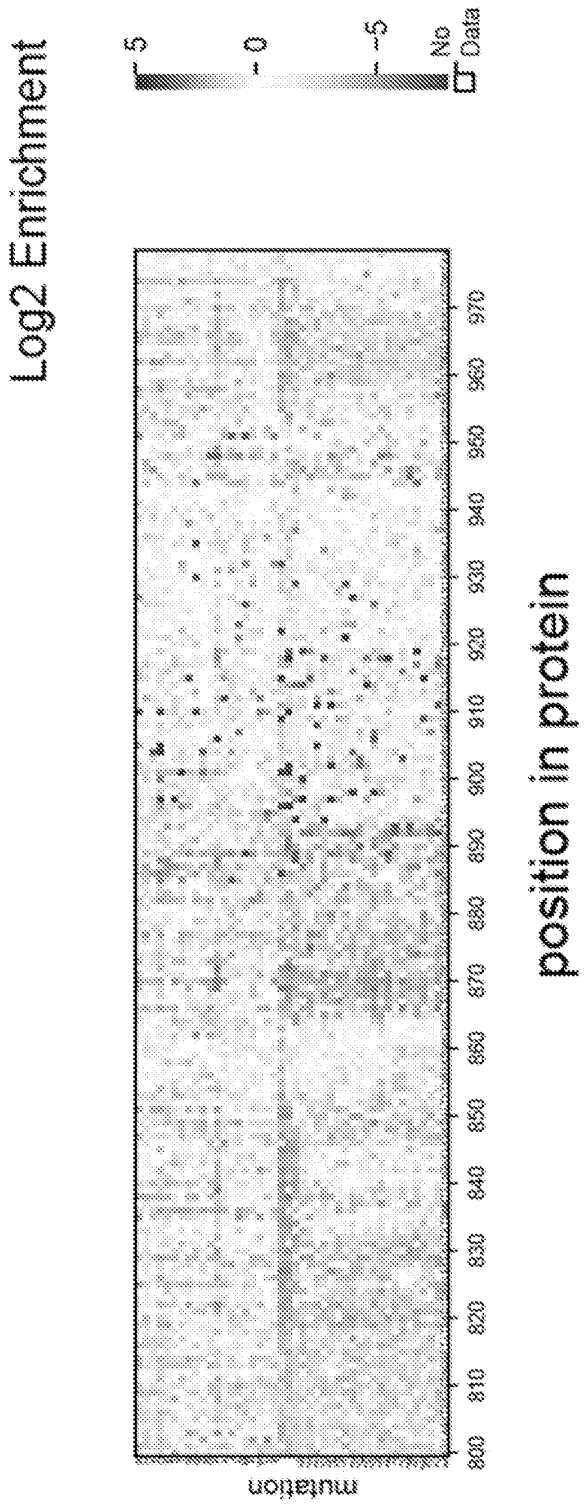

Log2 Enrichment
5
0
-5
No Data
position in protein
mutation
800
810
820
830
840
850
860
870
880
890
900
910
920
930
940
950
960
970

FIG. 35E

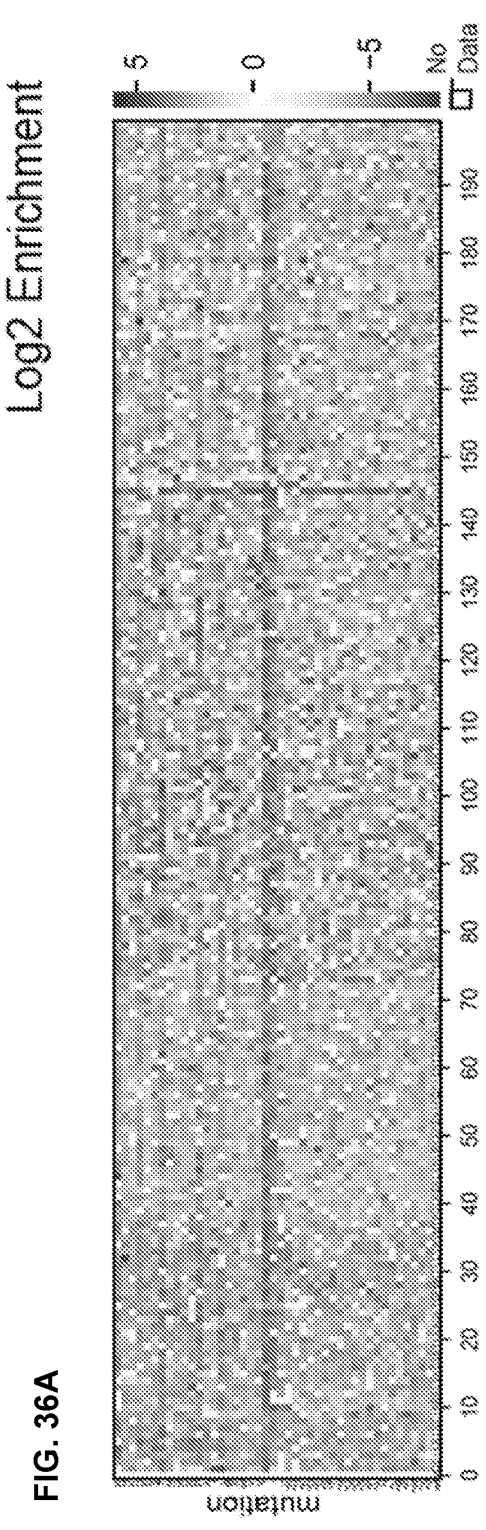


FIG. 36A

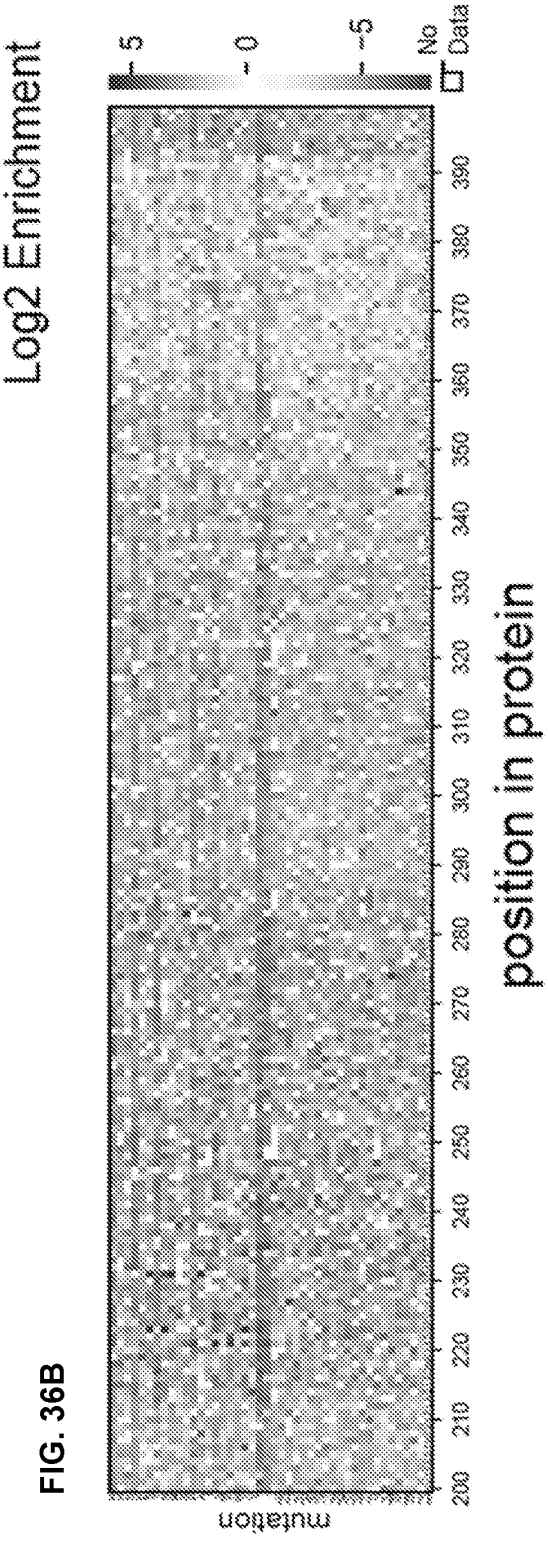


FIG. 36B

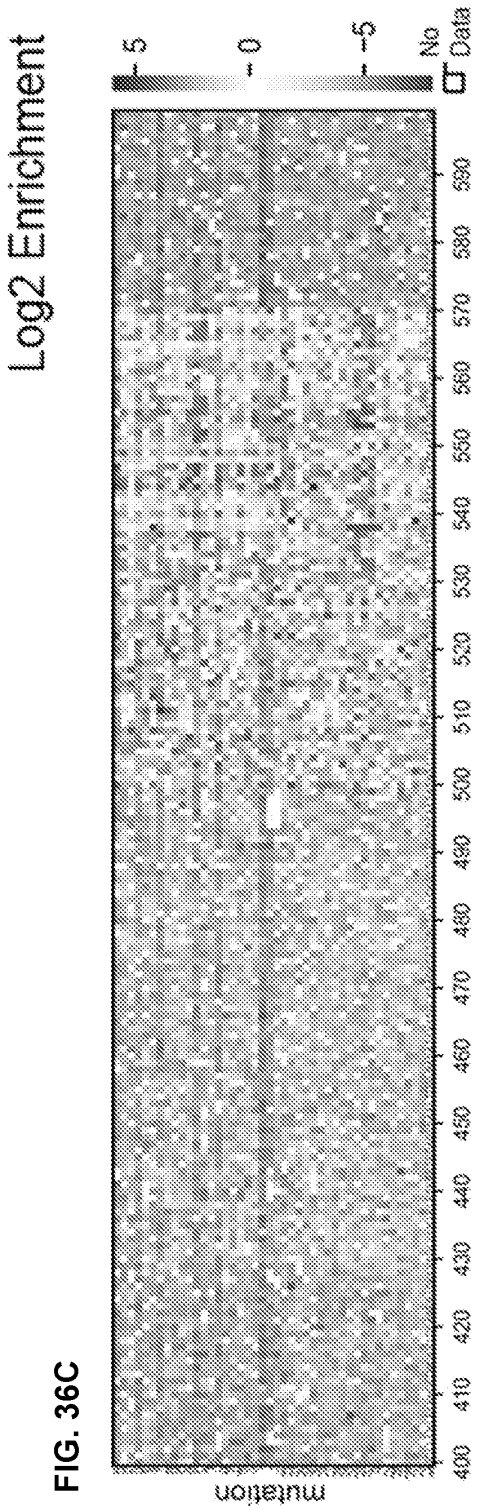


FIG. 36C

FIG. 36D

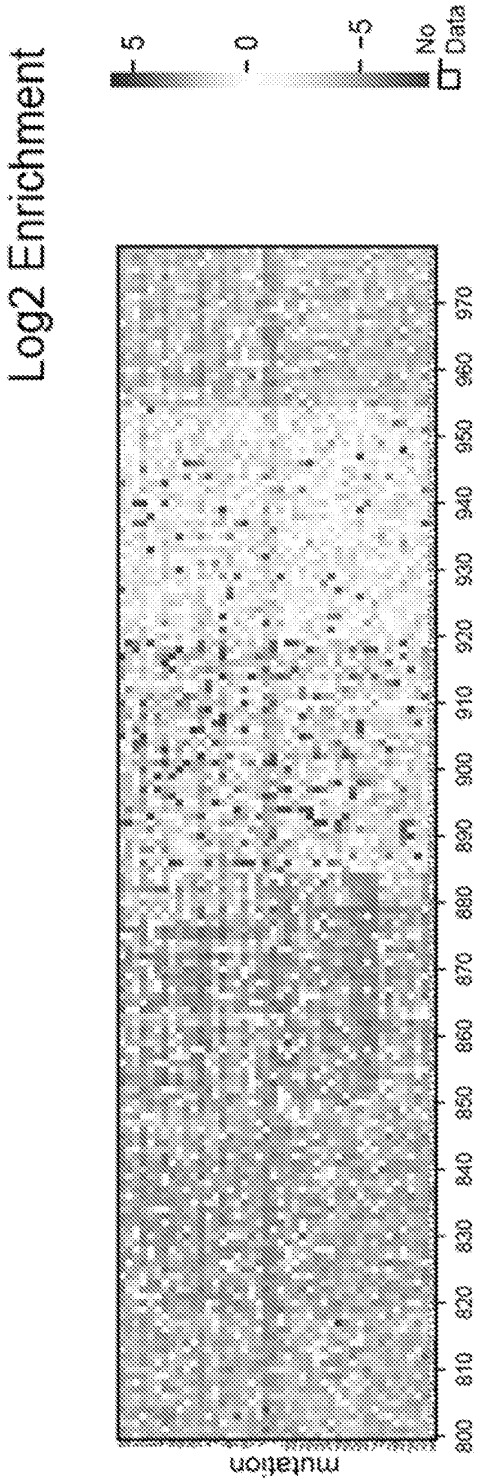

Log2 Enrichment
5
0
-5
No Data
position in protein
mutation
800
810
820
830
840
850
860
870
880
890
900
910
920
930
940
950
960
970

FIG. 36E

FIG. 37A

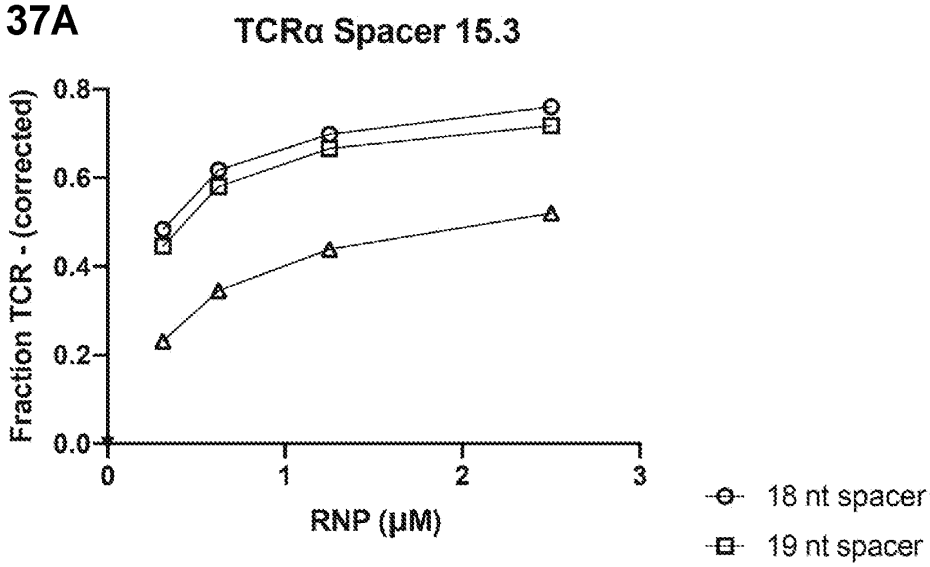
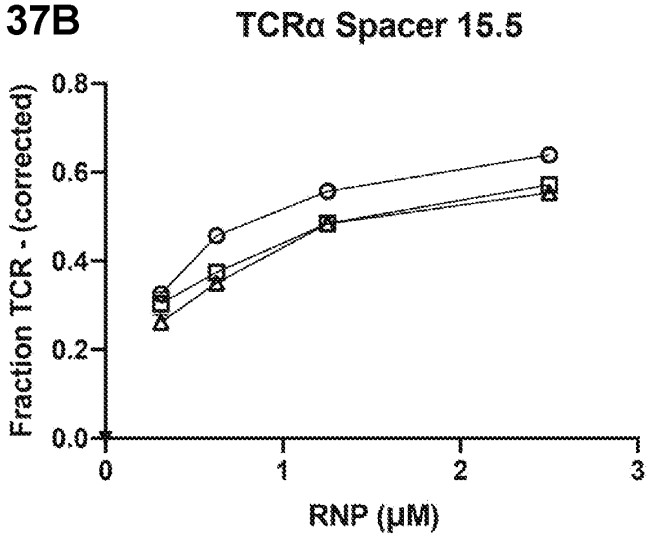


FIG. 37B



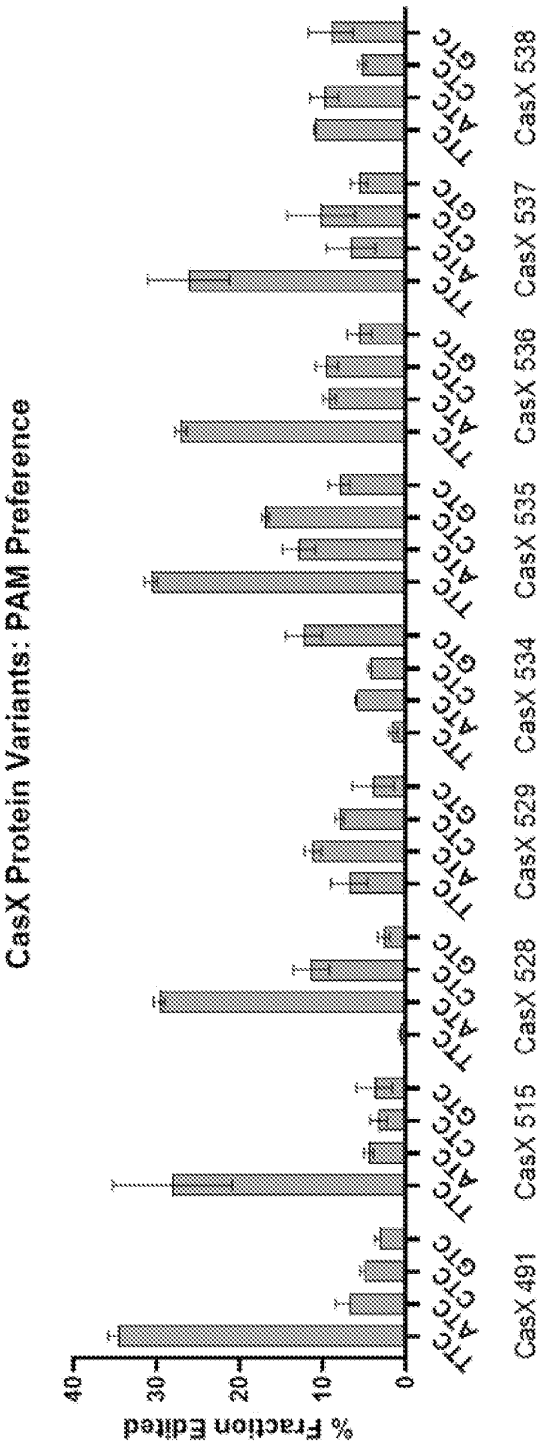

CasX Protein Variants: PAM Preference
% Fraction Edited
0
10
20
30
40
TTCATCGTC
TTCATCGTG
TTCATCGTT
TTCATCGTA
TTCATCGTC
TTCATCGTG
TTCATCGTT
TTCATCGTA
TTCATCGTC
TTCATCGTG
TTCATCGTT
TTCATCGTA
TTCATCGTC
TTCATCGTG
TTCATCGTT
TTCATCGTA
TTCATCGTC
TTCATCGTG
TTCATCGTT
TTCATCGTA
CasX 491
CasX 515
CasX 528
CasX 529
CasX 534
CasX 535
CasX 536
CasX 537
CasX 538
CasX Protein Variant

FIG. 38

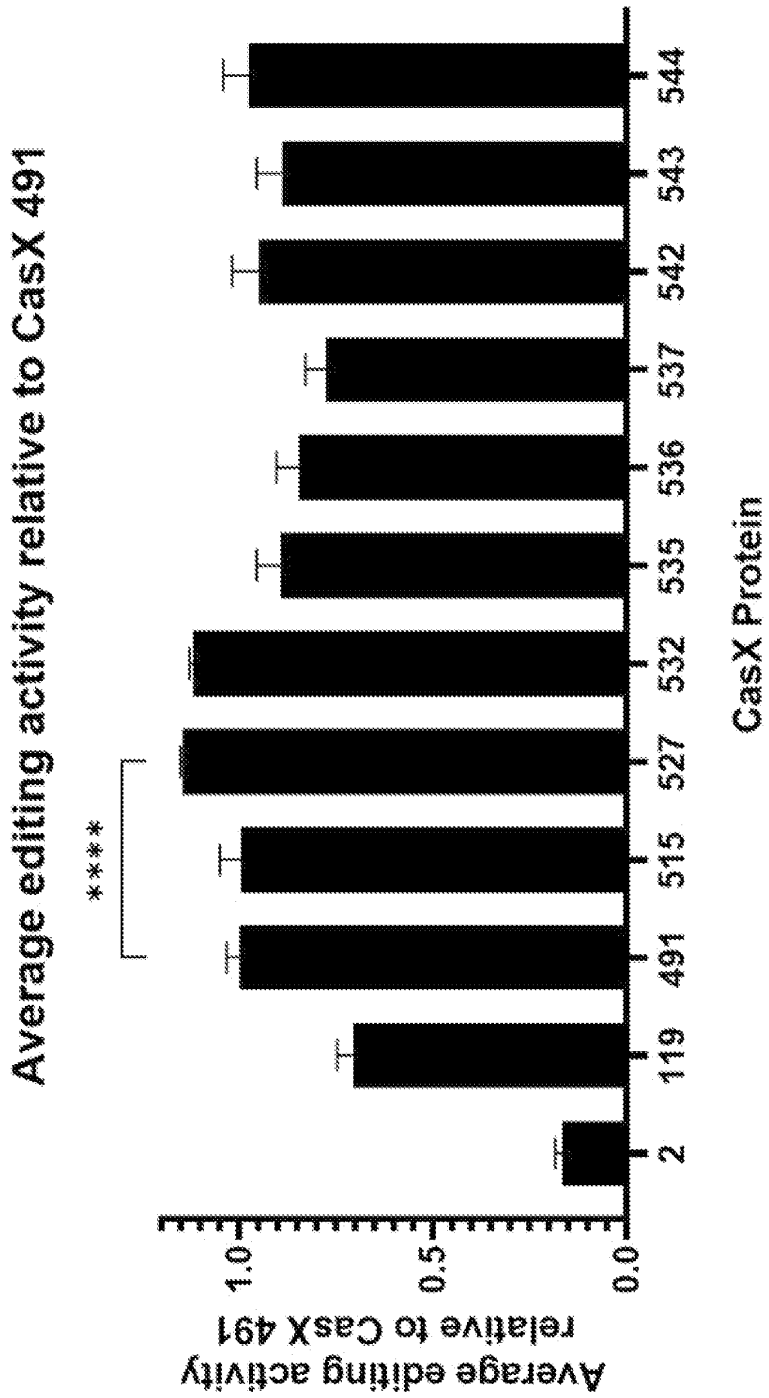

Average editing activity relative to CasX 491
****
Average editing activity relative to CasX 491
0.0
0.5
1.0
2
119
491
515
527
532
535
536
537
542
543
544
CasX Protein

FIG. 39

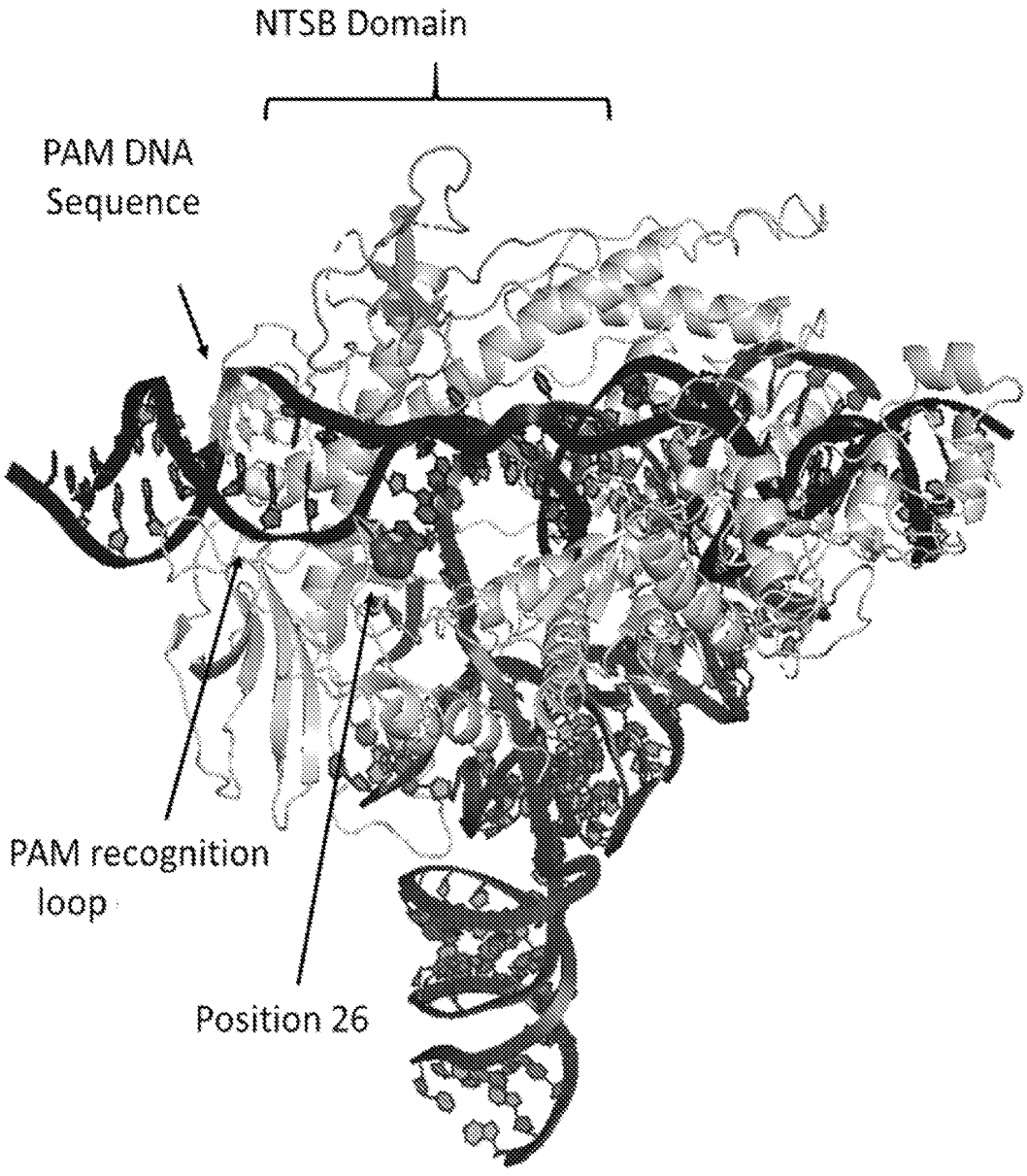

NTSB Domain
PAM DNA
Sequence
PAM recognition
loop
Position 26

FIG. 40

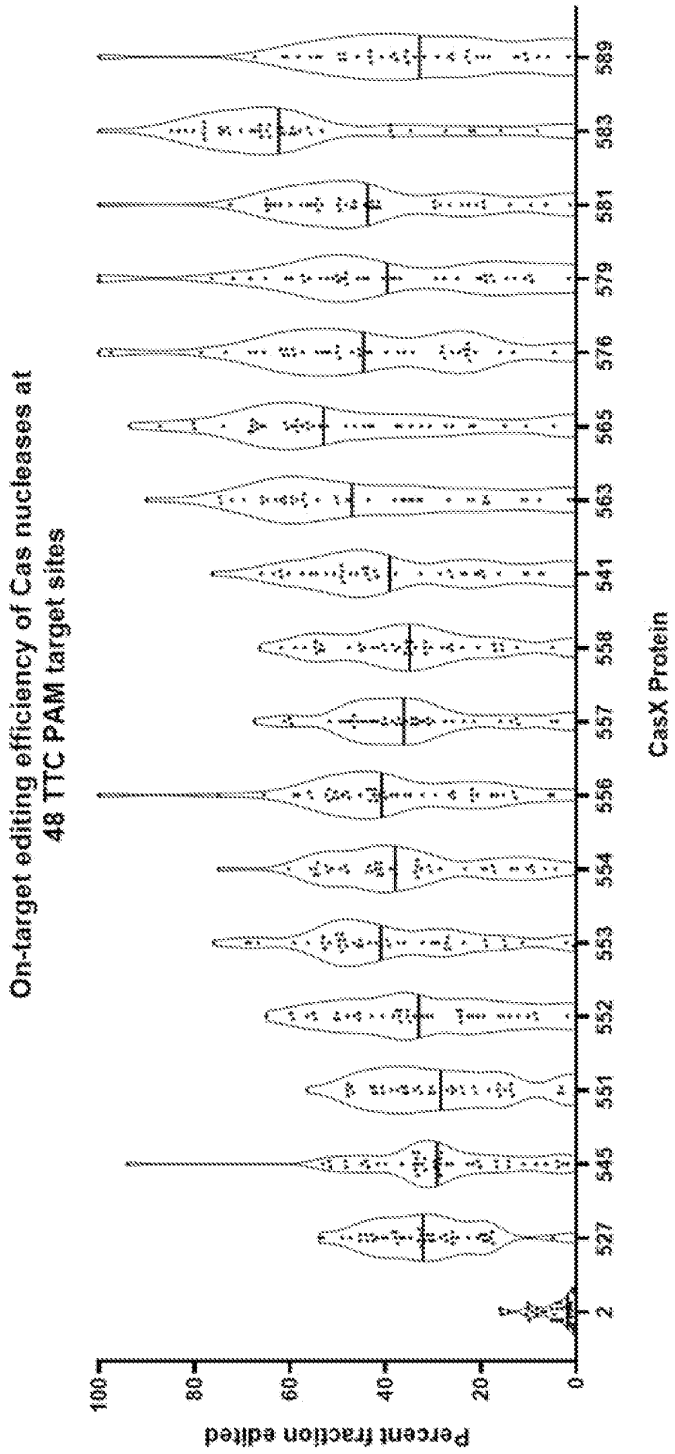

On-target editing efficiency of Cas nucleases at
48 TTC PAM target sites
Percent fraction edited
0
20
40
60
80
100
2
527
545
551
552
553
554
556
557
558
563
565
576
579
581
583
589
CasX Protein

FIG. 41

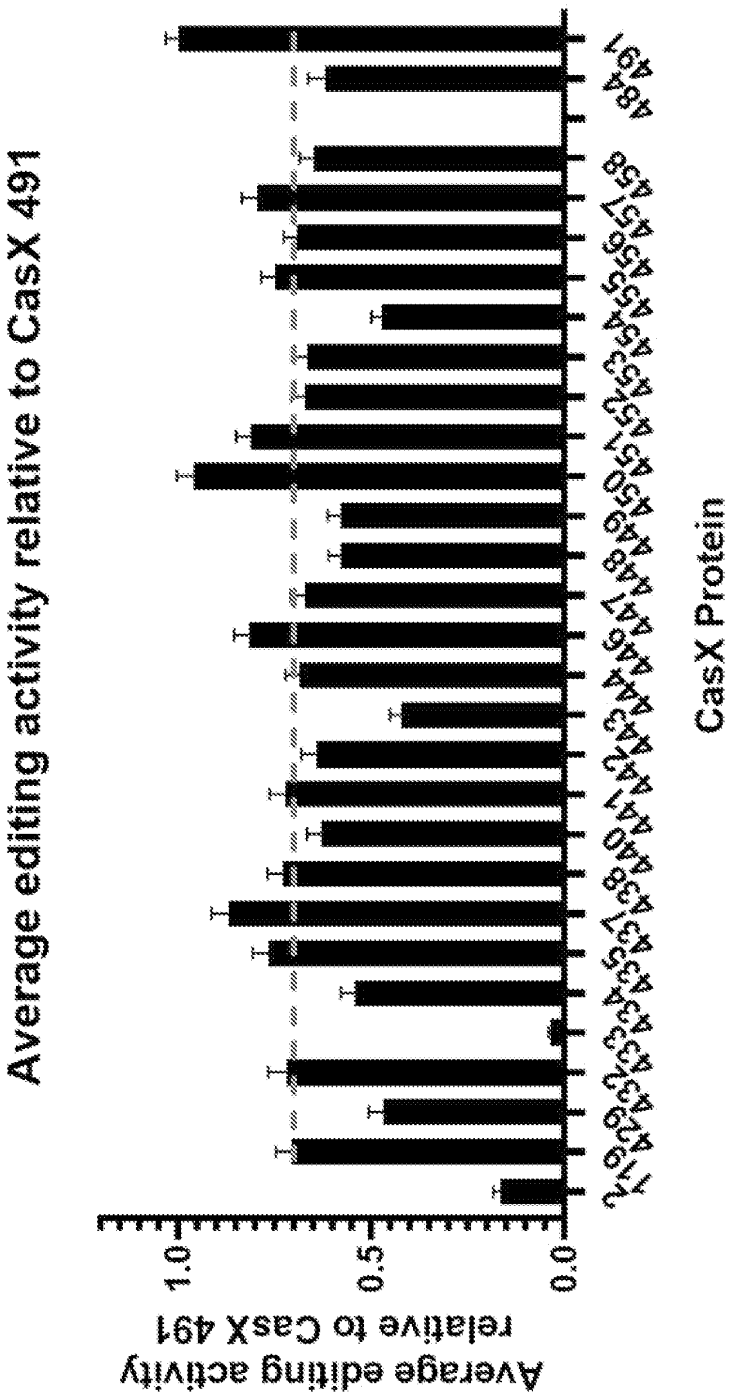

Average editing activity relative to CasX 491
Average editing activity relative to CasX 491
1.0
0.5
0.0
CasX Protein

FIG. 42

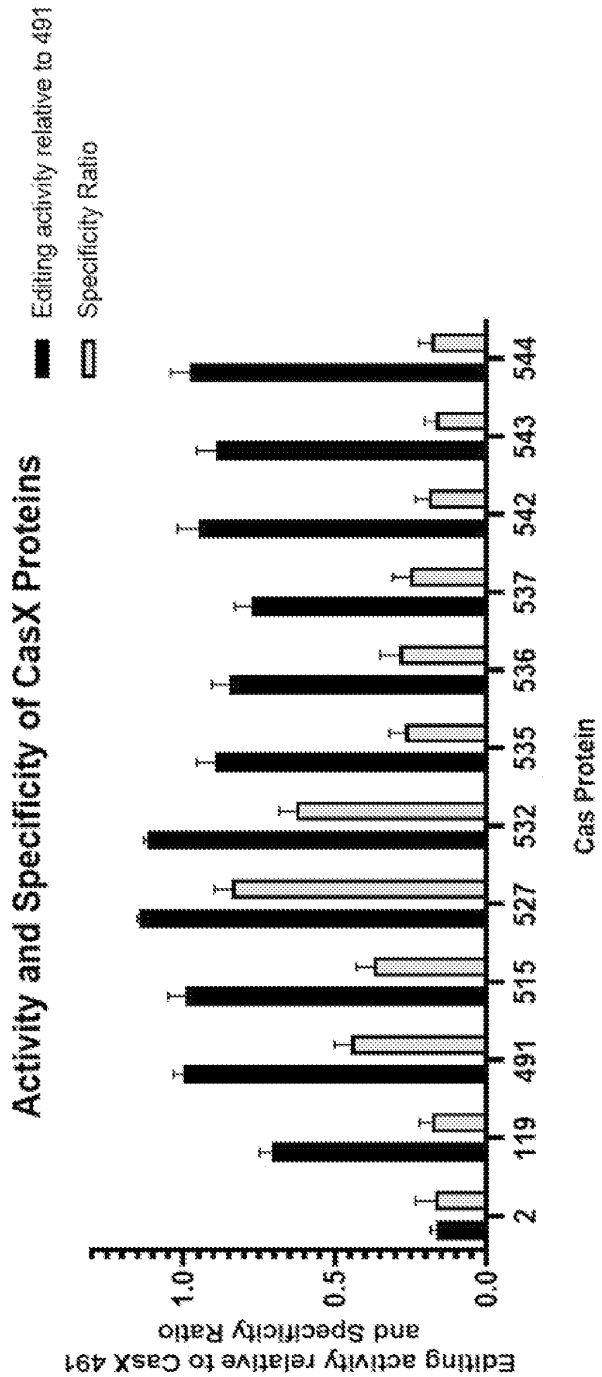

Activity and Specificity of CasX Proteins
Editing activity relative to 491
Specificity Ratio
Editing activity relative to CasX 491
and Specificity Ratio
0.0
0.5
1.0
2
119
491
515
527
532
535
536
537
542
543
544
Cas Protein

FIG. 43

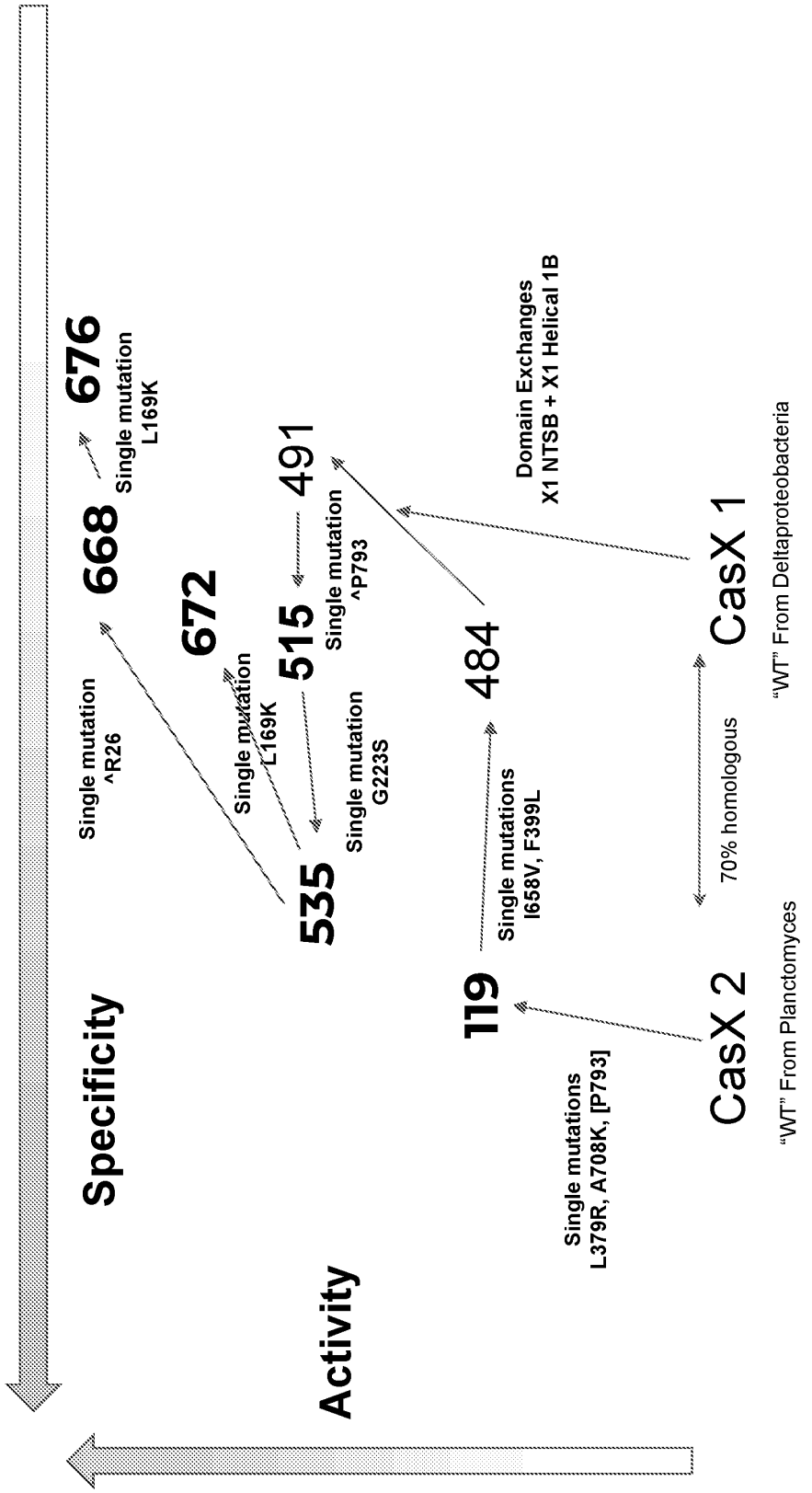

Specificity
Activity
676
Single mutation
L169K
668
Single mutation
^R26
672
Single mutation
L169K
515
Single mutation
G223S
491
Single mutation
^P793
535
484
Domain Exchanges
X1 NTSB + X1 Helical 1B
Single mutations
I658V, F399L
119
Single mutations
L379R, A708K, [P793]
CasX 2
"WT" From Planctomyces
70% homologous
CasX 1
"WT" From Deltaproteobacteria

FIG. 44

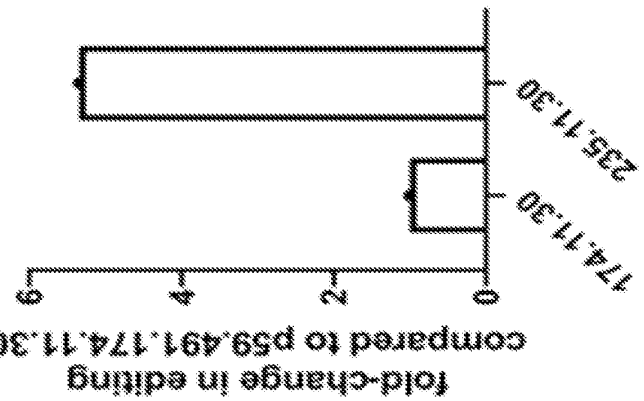


FIG. 45B

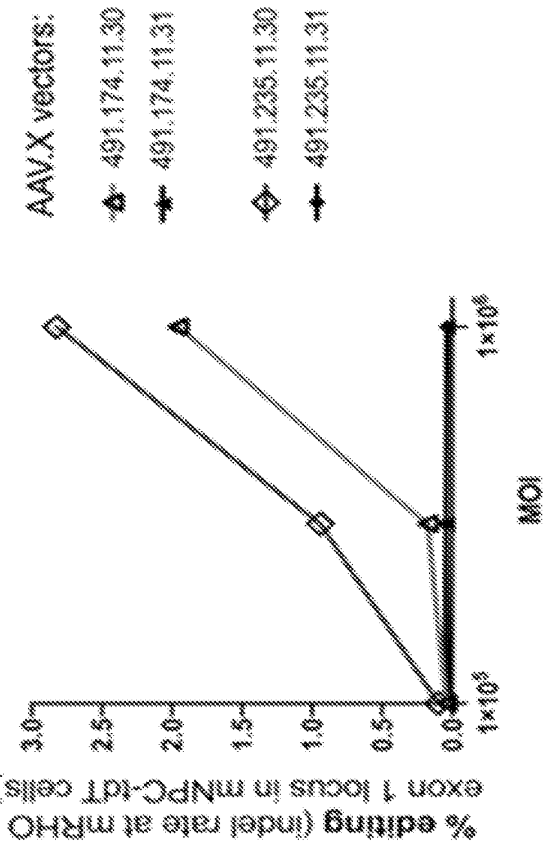


FIG. 45A

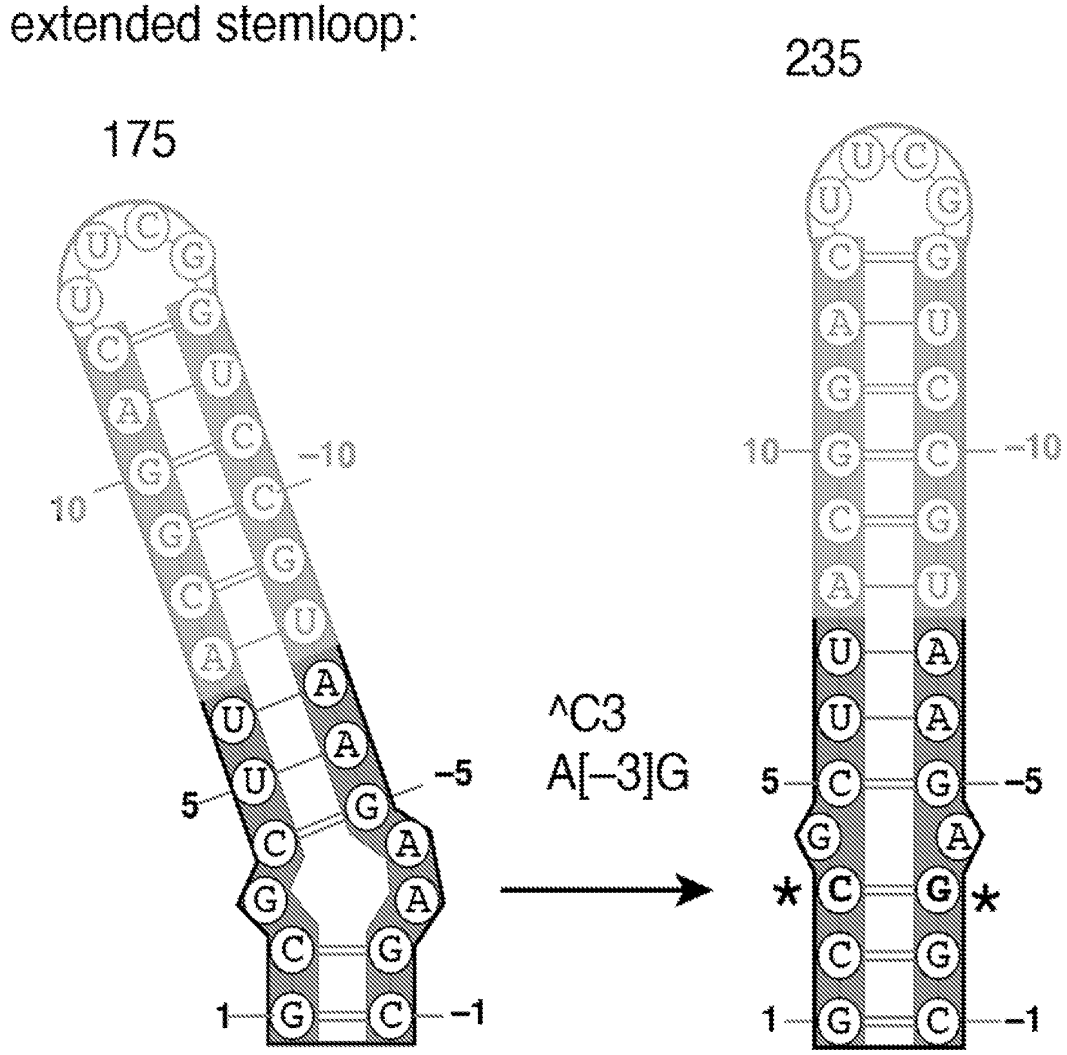


FIG. 46

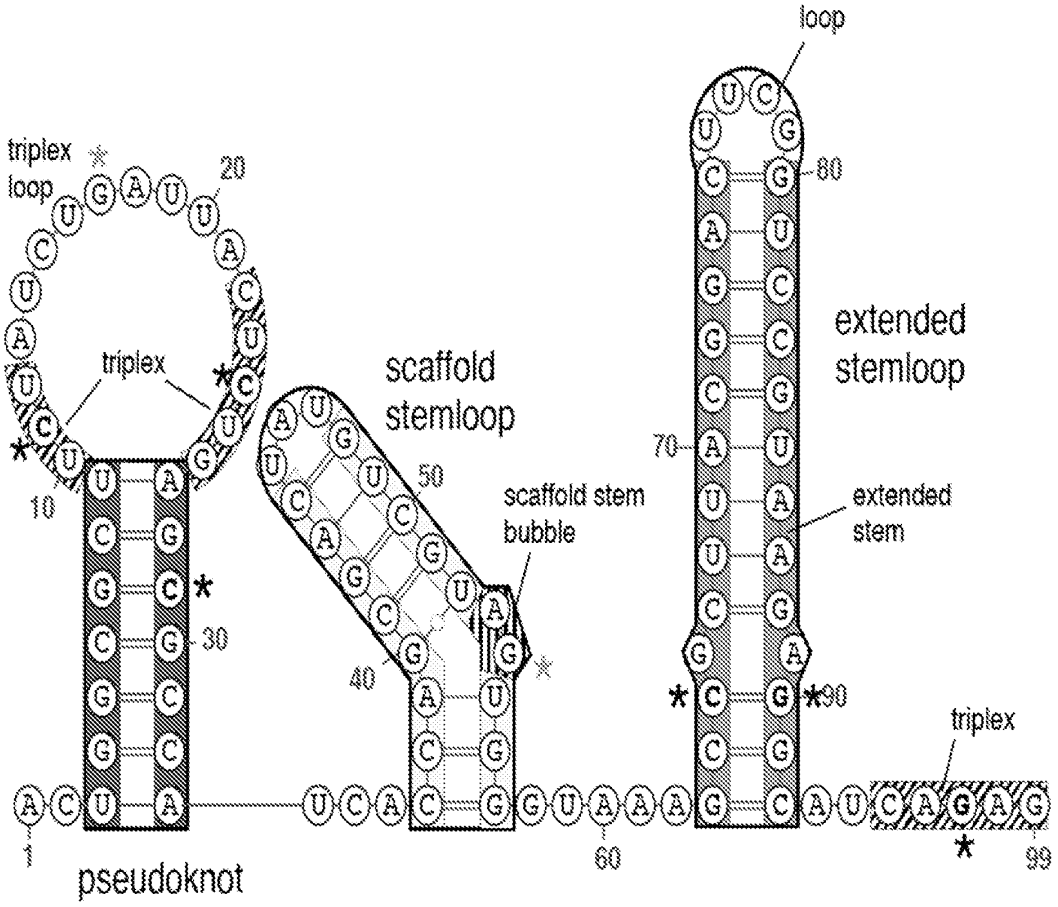

triplex
loop
triplex
scaffold
stemloop
scaffold stem
bubble
loop
extended
stemloop
extended
stem
triplex
pseudoknot
1
10
20
30
40
50
60
70
80
90
99

FIG. 47

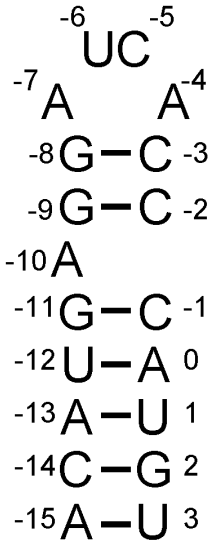


FIG. 48

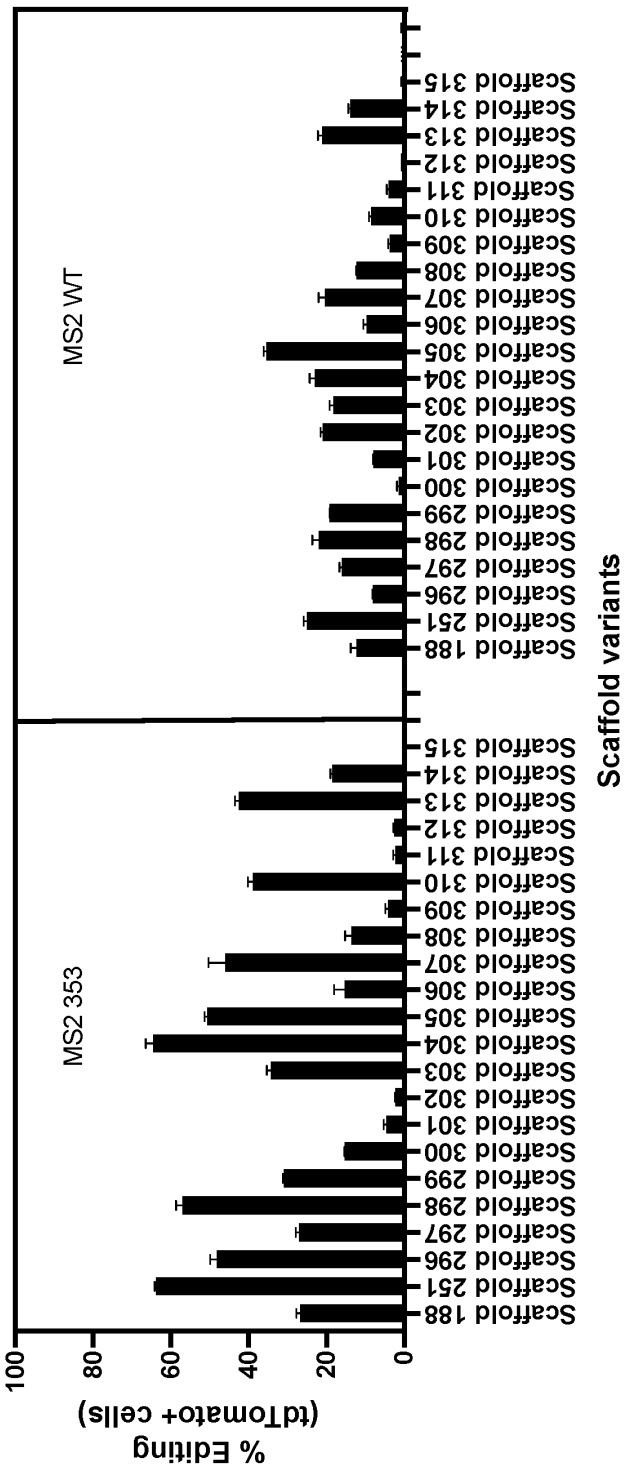

% Editing
(tdTomato+ cells)
100
80
60
40
20
0
MS2 353
MS2 WT
Scaffold 188
Scaffold 251
Scaffold 296
Scaffold 297
Scaffold 298
Scaffold 299
Scaffold 300
Scaffold 301
Scaffold 302
Scaffold 303
Scaffold 304
Scaffold 305
Scaffold 306
Scaffold 307
Scaffold 308
Scaffold 309
Scaffold 310
Scaffold 311
Scaffold 312
Scaffold 313
Scaffold 314
Scaffold 315
Scaffold variants

FIG. 49

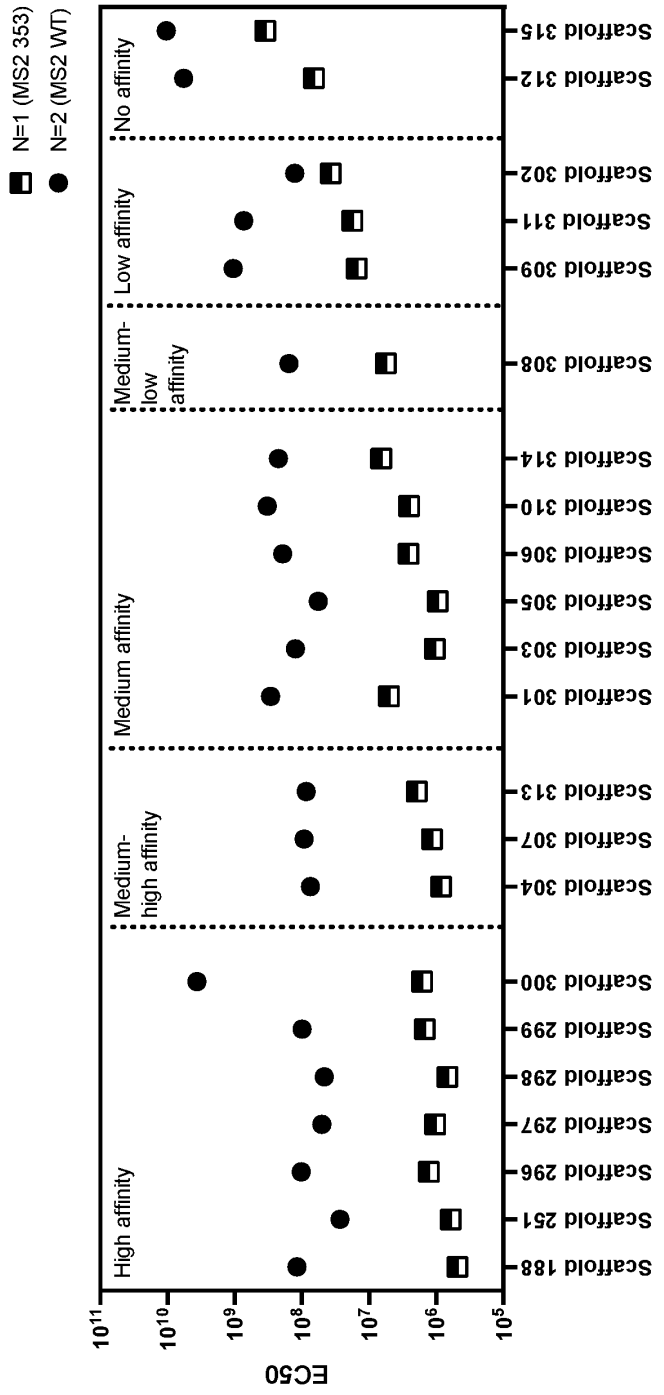

N=1 (MS2 353)
N=2 (MS2 WT)
EC50
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
High affinity
Medium-high affinity
Medium affinity
Medium-low affinity
Low affinity
No affinity
Scaffold 188
Scaffold 251
Scaffold 296
Scaffold 297
Scaffold 298
Scaffold 299
Scaffold 300
Scaffold 304
Scaffold 307
Scaffold 313
Scaffold 301
Scaffold 303
Scaffold 305
Scaffold 306
Scaffold 310
Scaffold 314
Scaffold 308
Scaffold 309
Scaffold 311
Scaffold 302
Scaffold 312
Scaffold 315
Scaffold variants

FIG. 50

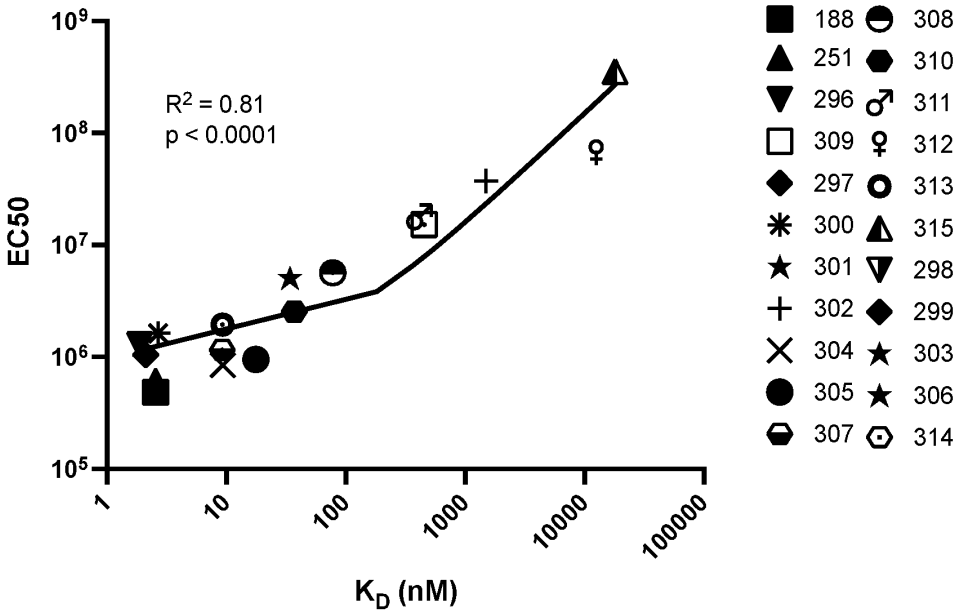

10^9
10^8
10^7
10^6
10^5
EC50
$R^2 = 0.81$
$p < 0.0001$
1
10
100
1000
10000
100000
K_D (nM)
188
251
296
309
297
300
301
302
304
305
307
308
310
311
312
313
315
298
299
303
306
314

FIG. 51

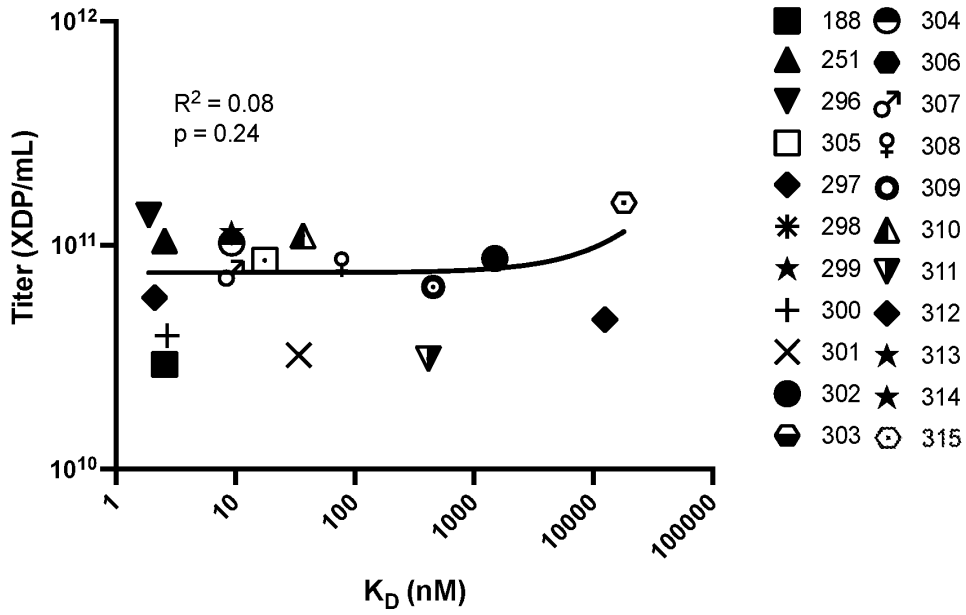

10^{12}
10^{11}
10^{10}
Titer (XDP/mL)
R^2 = 0.08
p = 0.24
1
10
100
1000
10000
100000
K_D (nM)
188
251
296
305
297
298
299
300
301
302
303
304
306
307
308
309
310
311
312
313
314
315

FIG. 52