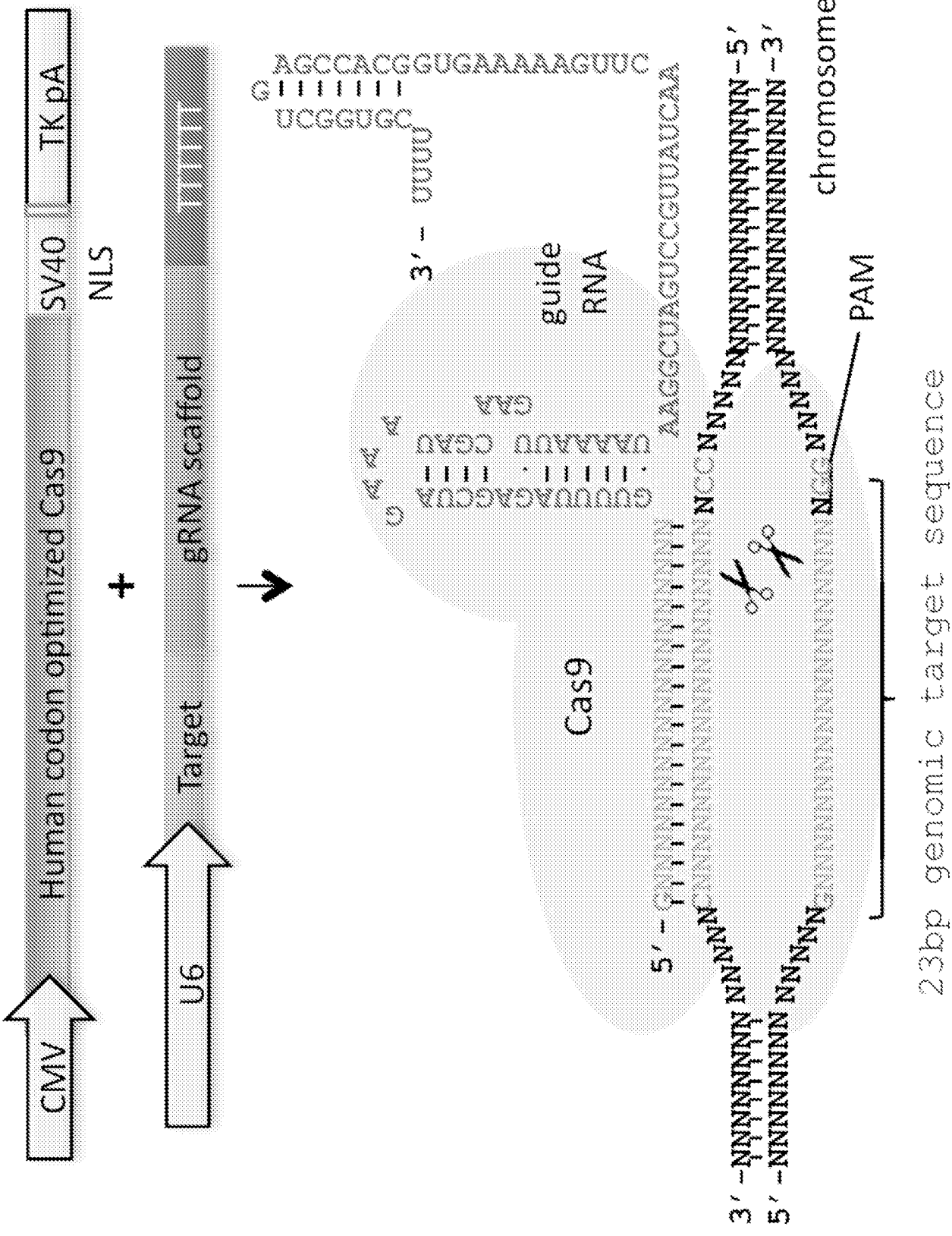
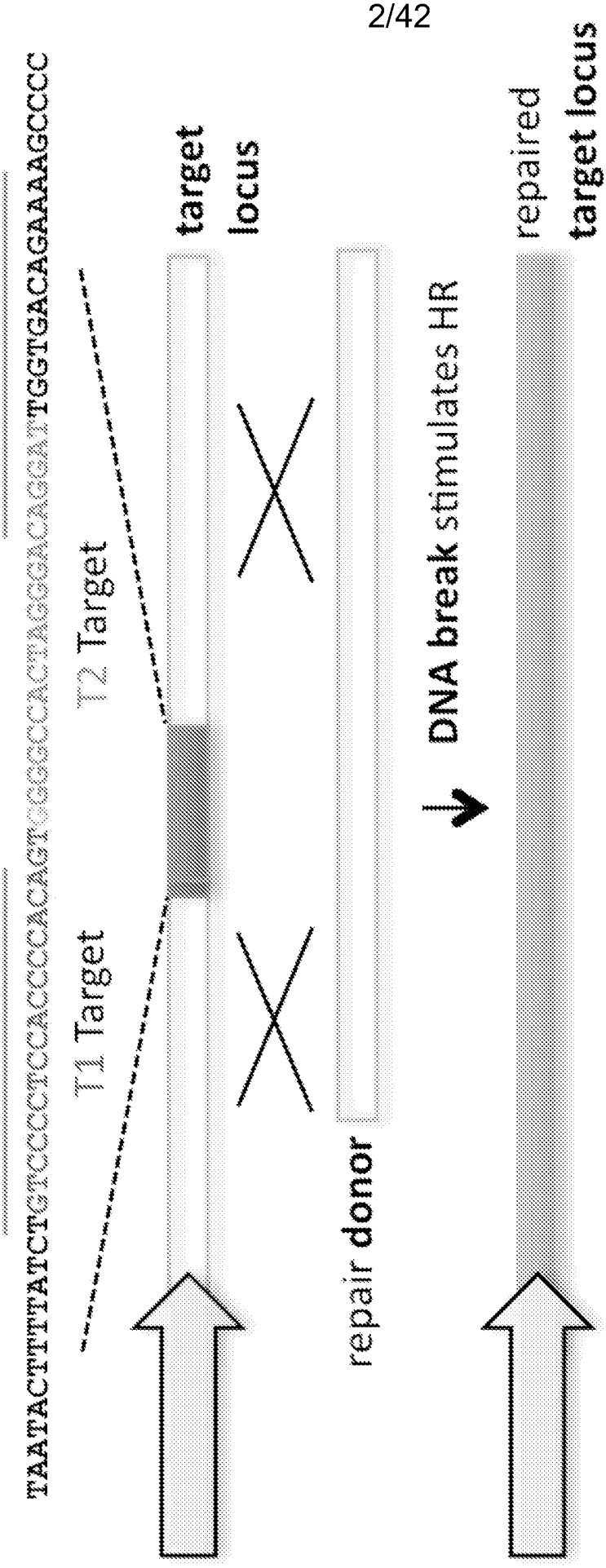

CMV
Human codon ophimized Cas9
SV40
NLS
TK pA
+
U6
Target
gRNA scaffold
AGCCACGGUGAAAAAGUUC
G
UCGGUGC
3' - uuuu
guide
RNA
Cas9
5' -
AAGGCUAGUCCGUAUCAA
chromosome
PAM
23bp genomic target sequence
3' -
5' -

FIG. 1A

FIG. 1B


AAVS1-TALENs target site
TAATACTTTTATCTGTCCCCTCCACCCCACAGTGGGCCACTAGGGACAGGATTGGTGACAGAAAAGCCCC
T1 Target
T2 Target
target locus
repair donor
DNA break stimulates HR
repaired target locus

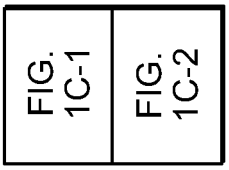

FIG. 1C-1
FIG. 1C-2

FIG. 1C

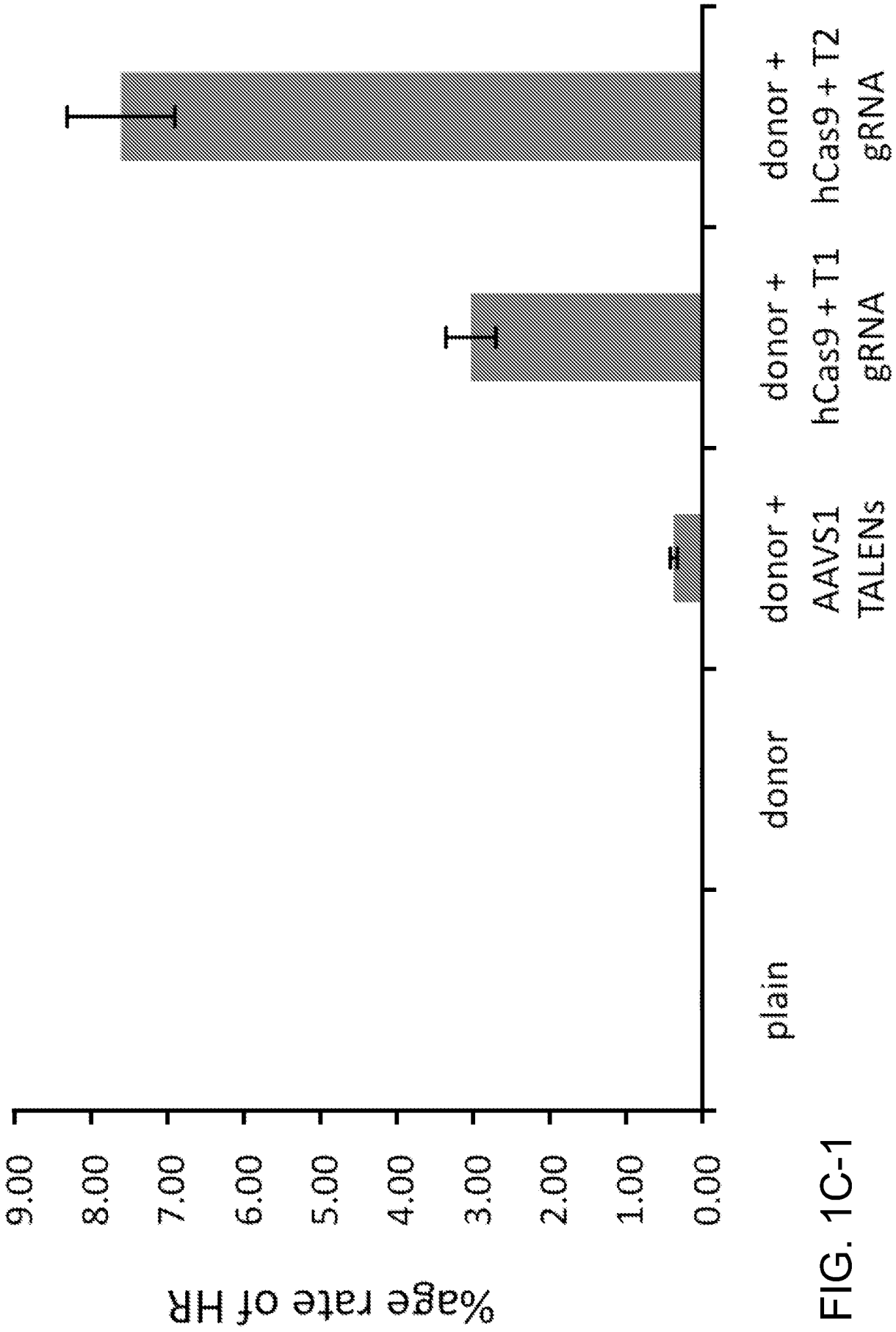

%age rate of HR
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
plain
donor
donor + AAVS1 TALENs
donor + hCas9 + T1 gRNA
donor + hCas9 + T2 gRNA

FIG. 1C-1

FIG. 1C-2

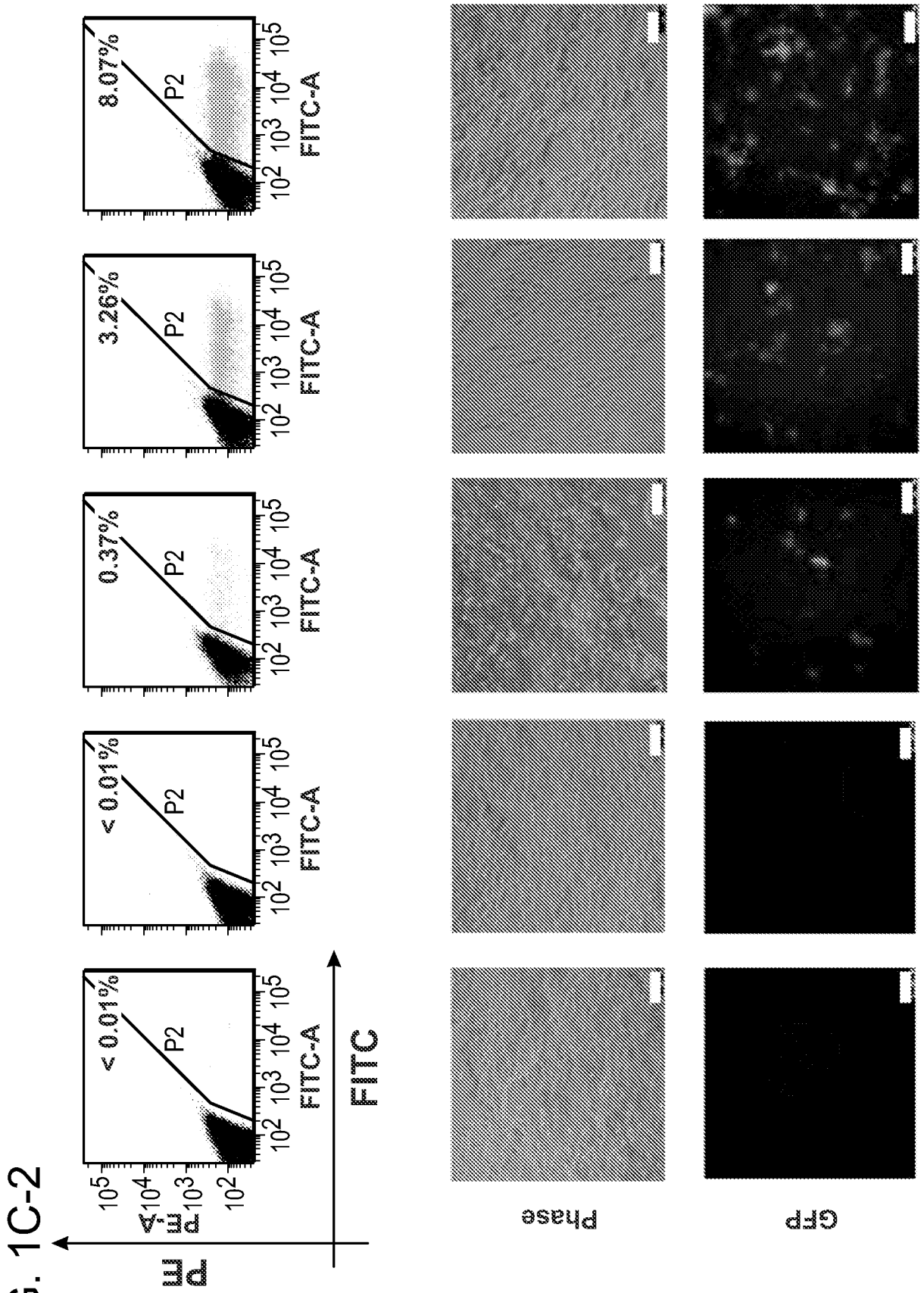


FIG. 2A

endogenous 'native' hAAVS1 locus sequence

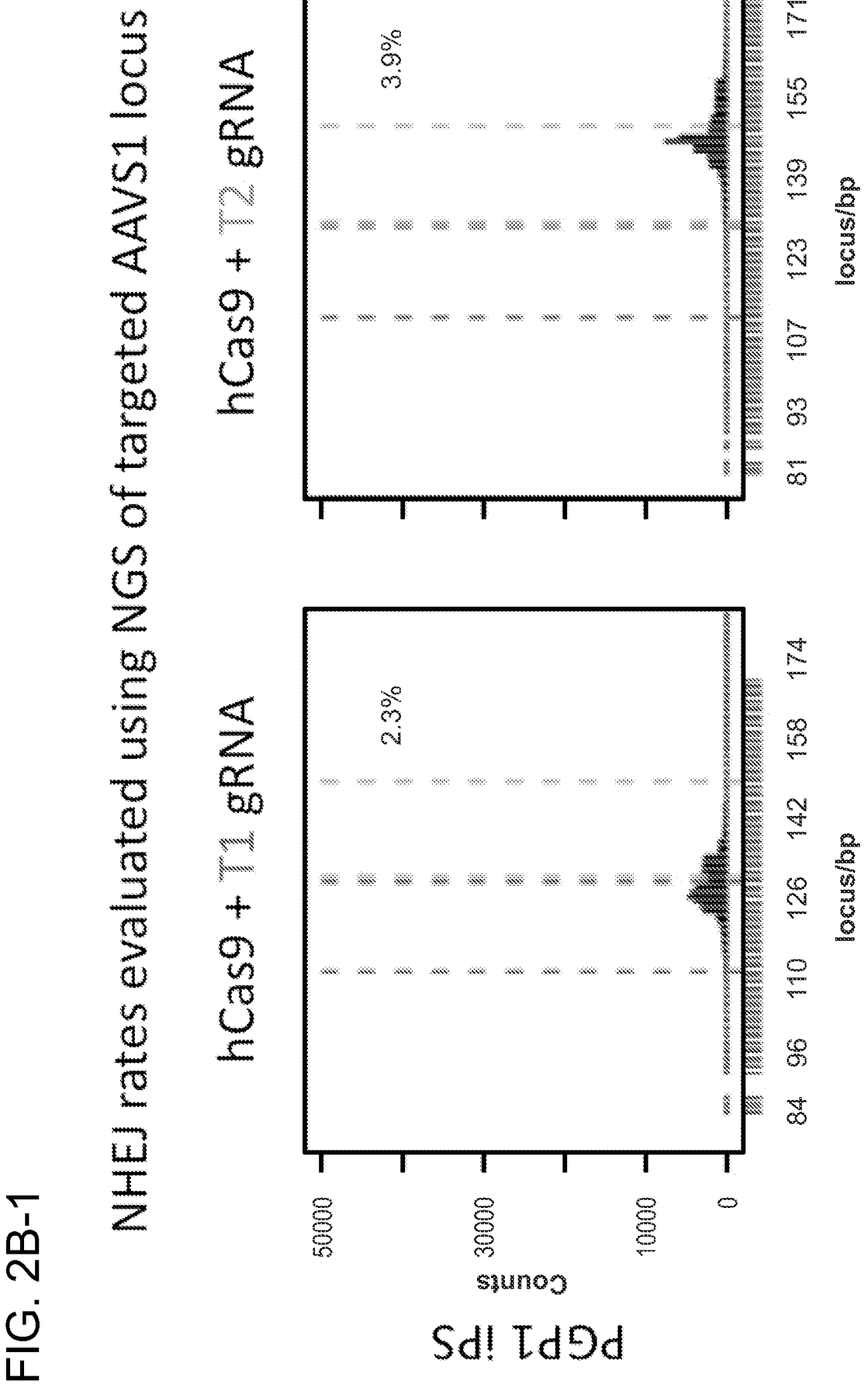
. . .TTATCTGTCCCCTCCACCCCACAGTGGGGCCACTAGGGACAGCATTGGTGA. . .

T1 target T2 target

FIG. 2B-1	FIG. 2B-2	FIG. 2B-3

FIG. 2B

FIG. 2B-1


NHEJ rates evaluated using NGS of targeted AAVS1 locus
hCas9 + T1 gRNA
hCas9 + T2 gRNA
2.3%
3.9%
PGP1 iPS
Counts
50000
30000
10000
0
84 96 110 126 142 158 174
81 93 107 123 139 155 171
locus/bp
locus/bp

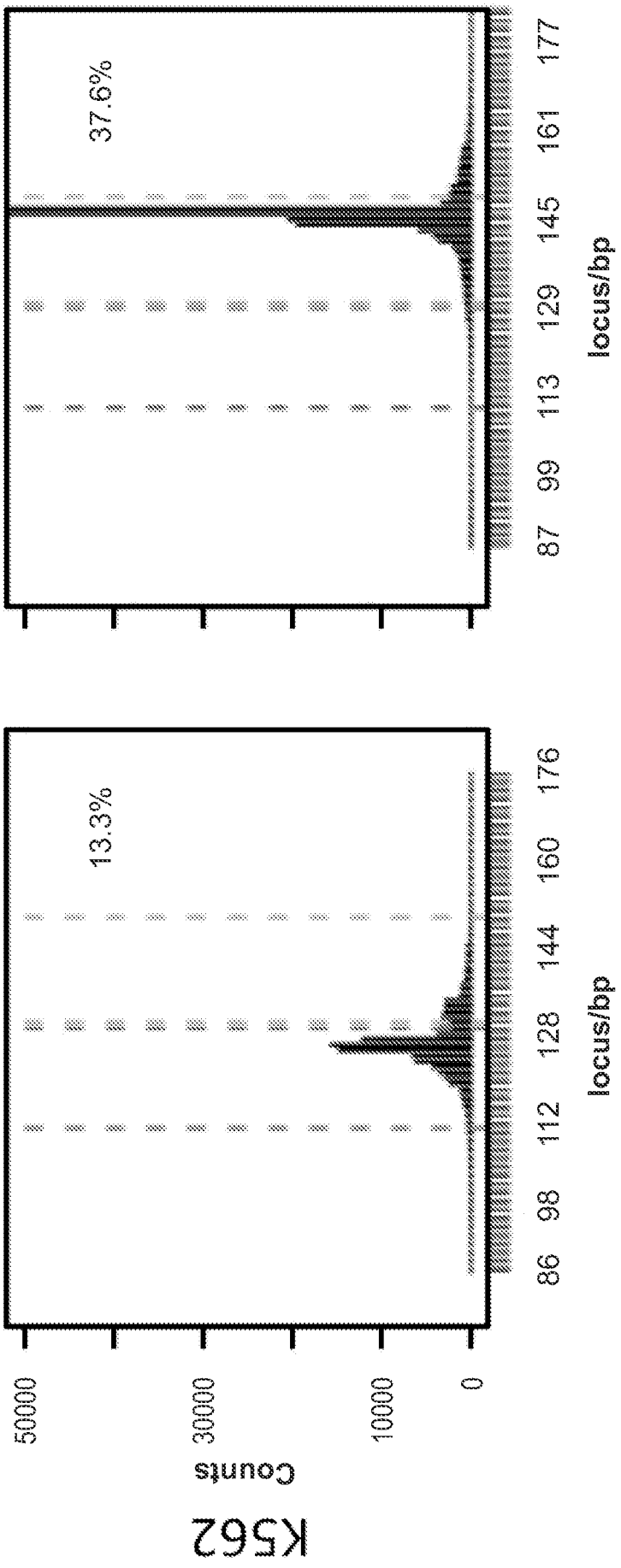

K562
Counts
50000
30000
10000
0
13.3%
37.6%
locus/bp
86 98 112 128 144 160 176
87 99 113 129 145 161 177

FIG. 2B-2

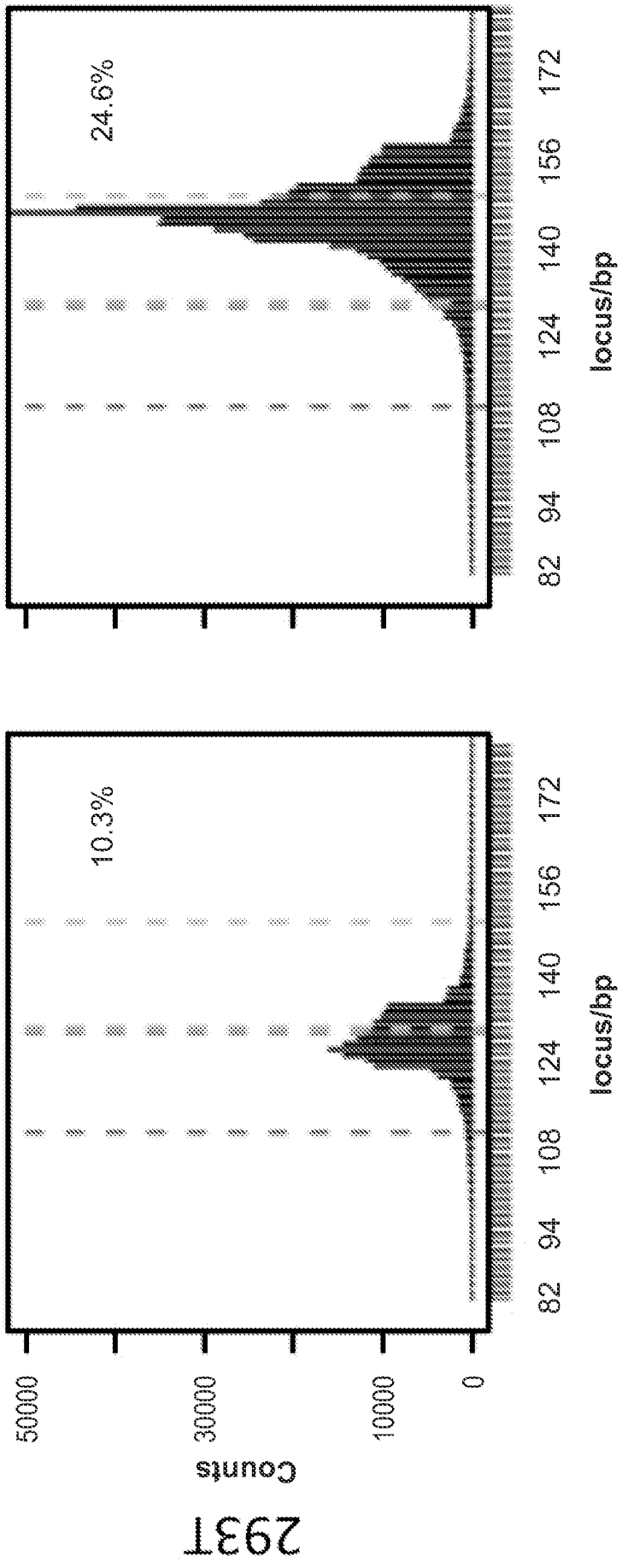

293T
Counts
50000
30000
10000
0
10.3%
24.6%
82
94
108
124
140
156
172
locus/bp

FIG. 2B-3

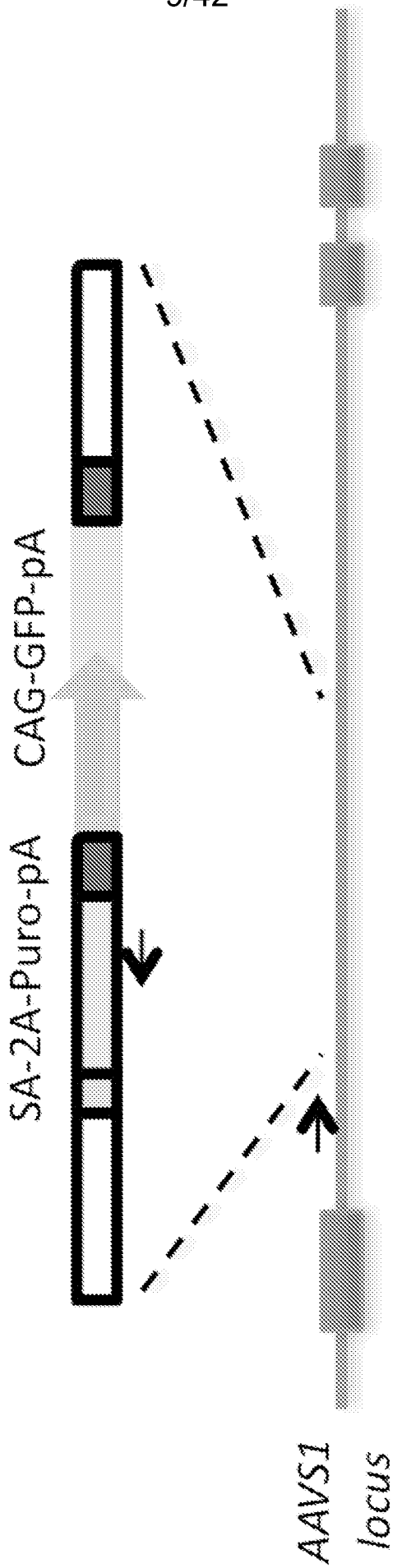


FIG. 2C

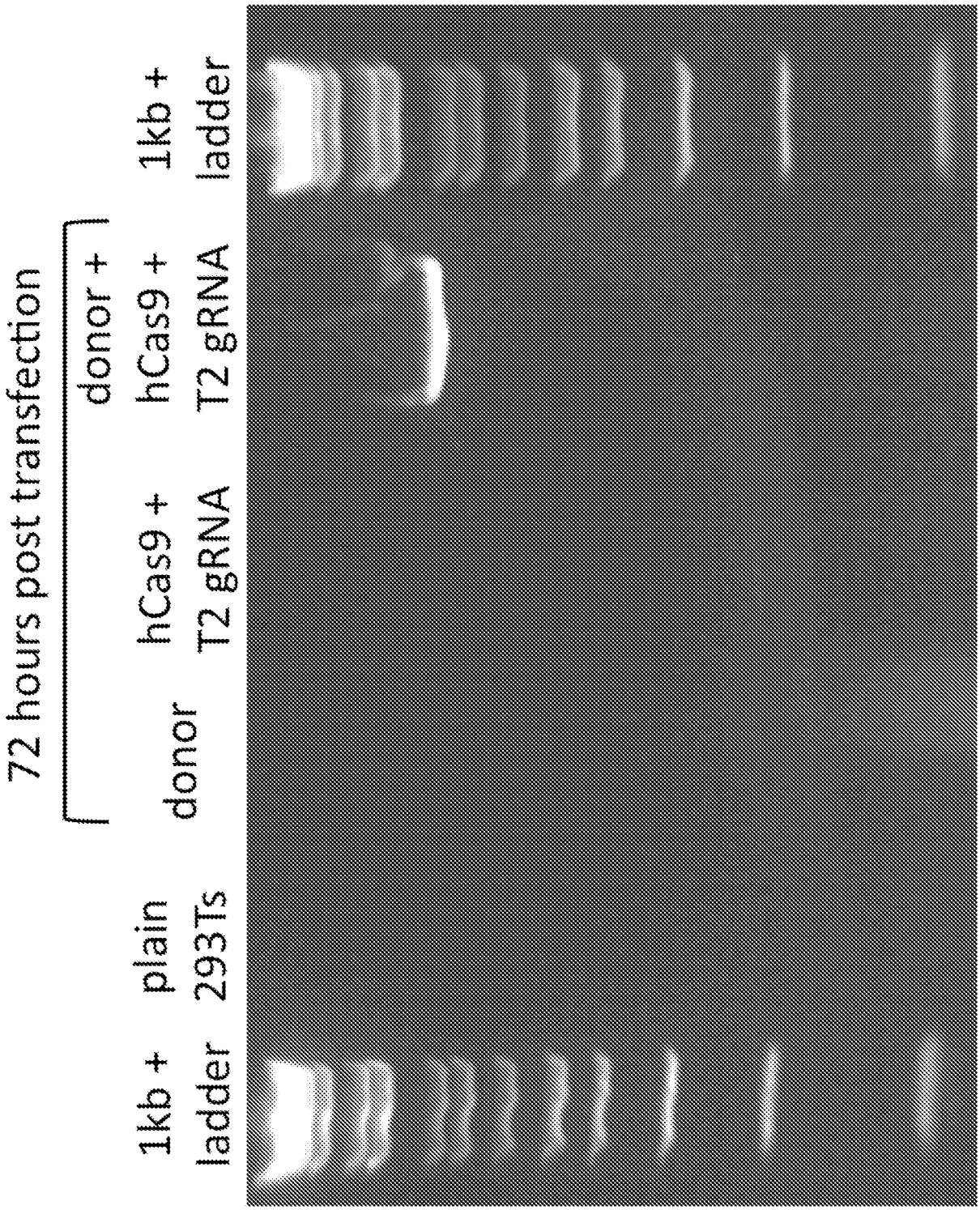

1kb + ladder
plain 293Ts
72 hours post transfection
donor
hCas9 + T2 gRNA
donor + hCas9 + T2 gRNA
1kb + ladder

FIG. 2D

FIG. 2E

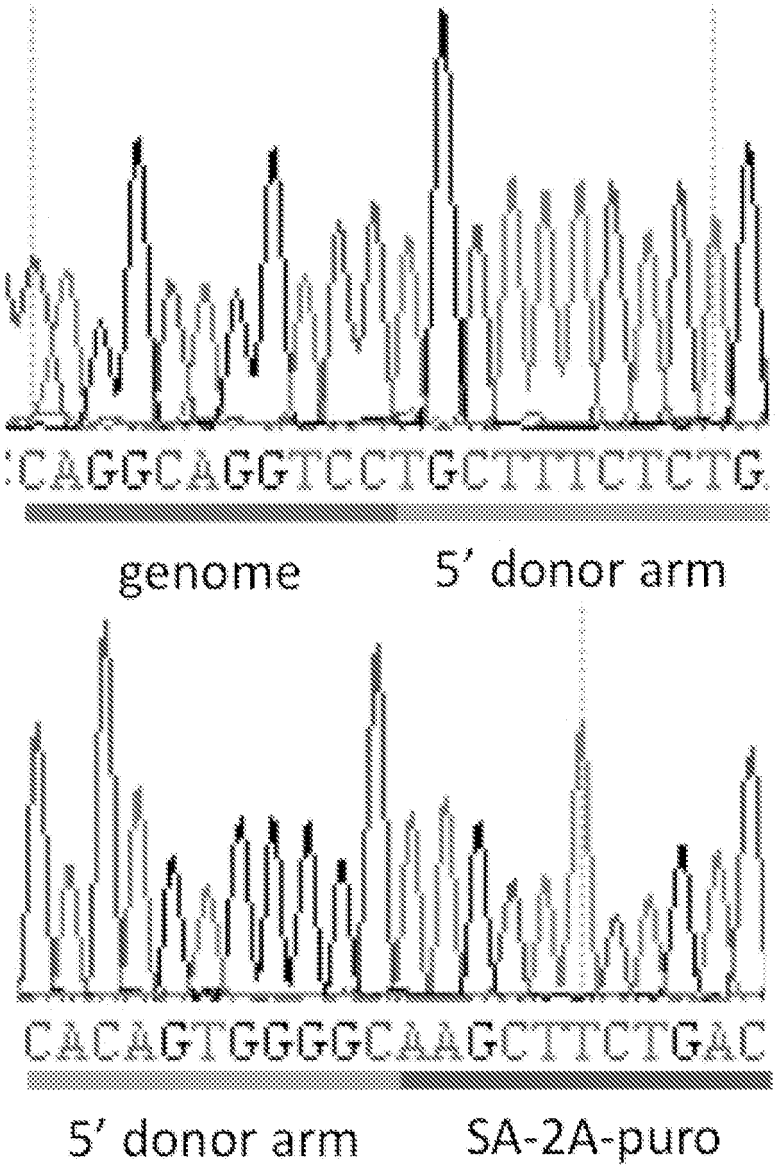

CAGGCAGGTCCTGCTTTCTCTG
genome
5′ donor arm
CACAGTGGGGCAAGCTTCTGAC
5′ donor arm
SA-2A-puro

FIG. 2F

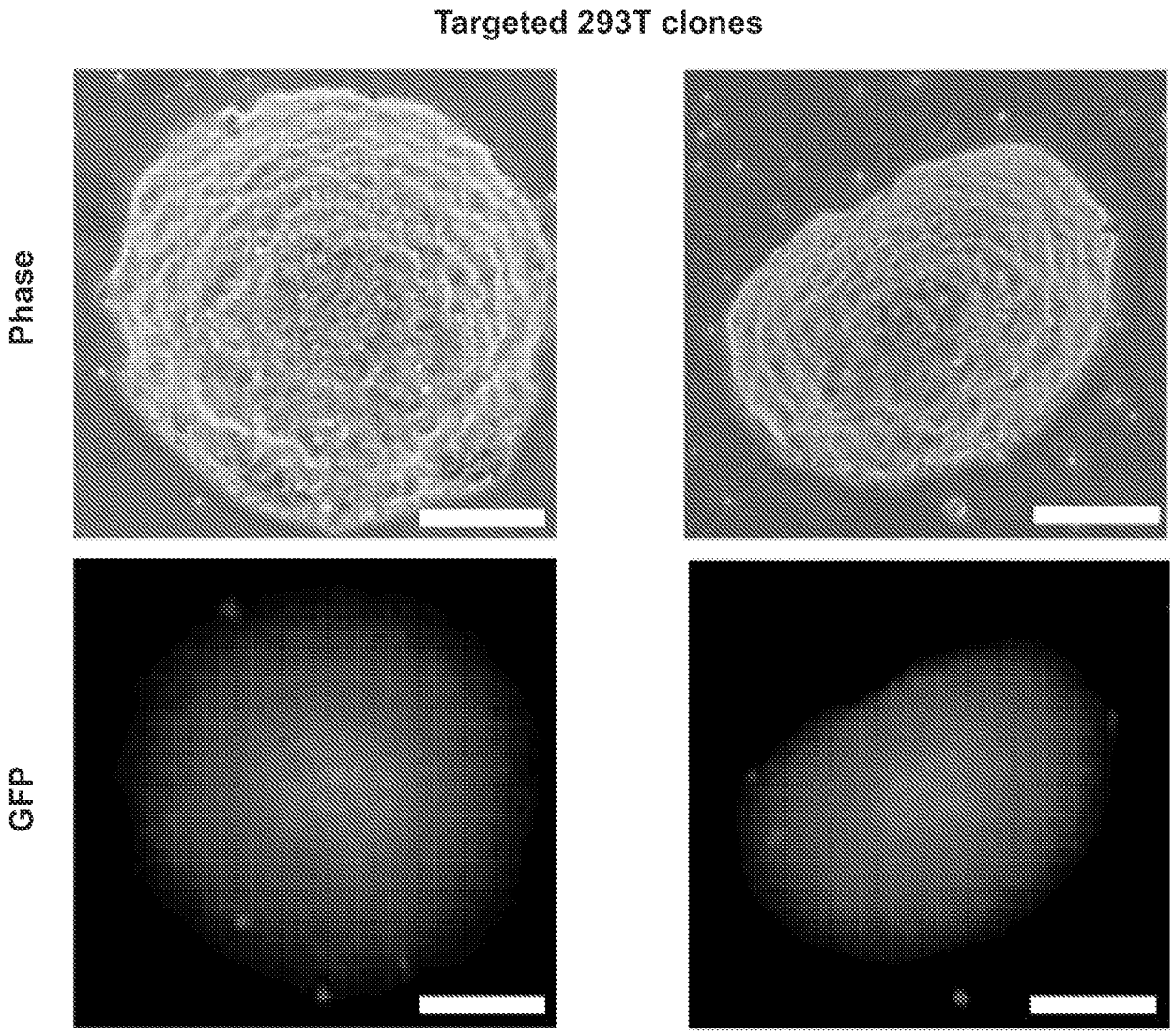
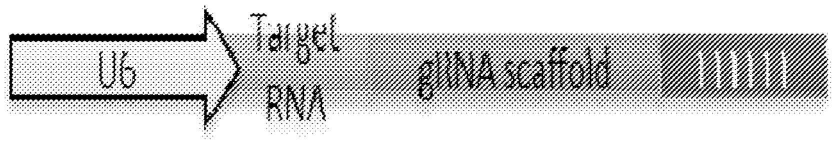


FIG. 3A-1

FIG. 3A

FIG. 3A-2

FIG. 3B



U6 promoter + target RNA + guide RNA scaffold:

TGTACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACTGGATCCGGTACCAAGGTCGGGCAGGAAGAGGGCCTATTTCCCATGATTCCTTCATATTTG
CATATACGATACAAGGCTGTTAGAGAGATAATTAGAATTAATTTGACTGTAAACACAAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTT
GGGTAGTTTGCAGTTTTAAAATTATGTTTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAA
GGACGAAACACCGNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGT
CGGTGCTTTTTTTCTAGACCCAGCTTTCTTGTACAAAGTTGGCATTA

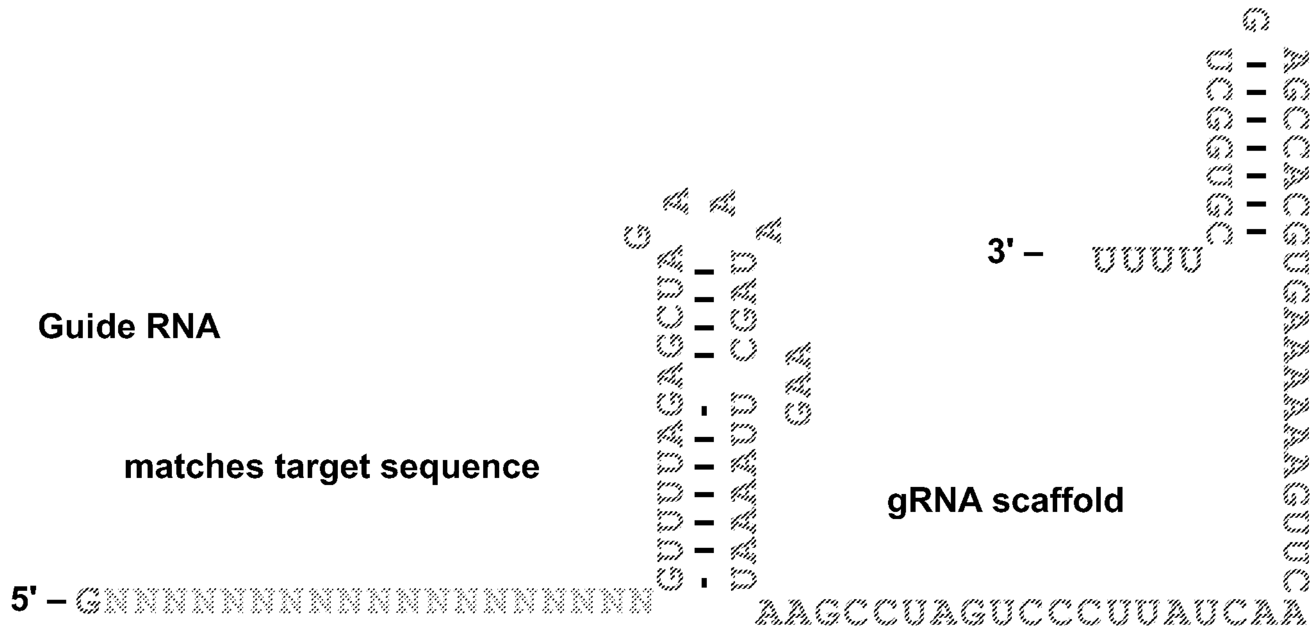


FIG. 3C

PAM

GNNNNNNNNNNNNNNNNNNNGG general form of target sequence

Name	Target Sequence
GFP gRNA Target 1	GTGAACCGCATCGAGCTGAAGGG
GFP gRNA Target 2	GGAGCGCACCATCTTCTTCAAGG
AAVS1 gRNA Target 1	GTCCCCTCCACCCCACAGTGGGG
AAVS1 gRNA Target 2	GGGGCCACTAGGGACAGGATTGG
DNMT3a gRNA Target 1	GCATGATGCGCGGCCCAAGGAGG
DNMT3a gRNA Target 2	GAGATGATCGCCCCTTCTTCTGG
DNMT3b gRNA Target	GAATTACTCACGCCCCAAGGAGG

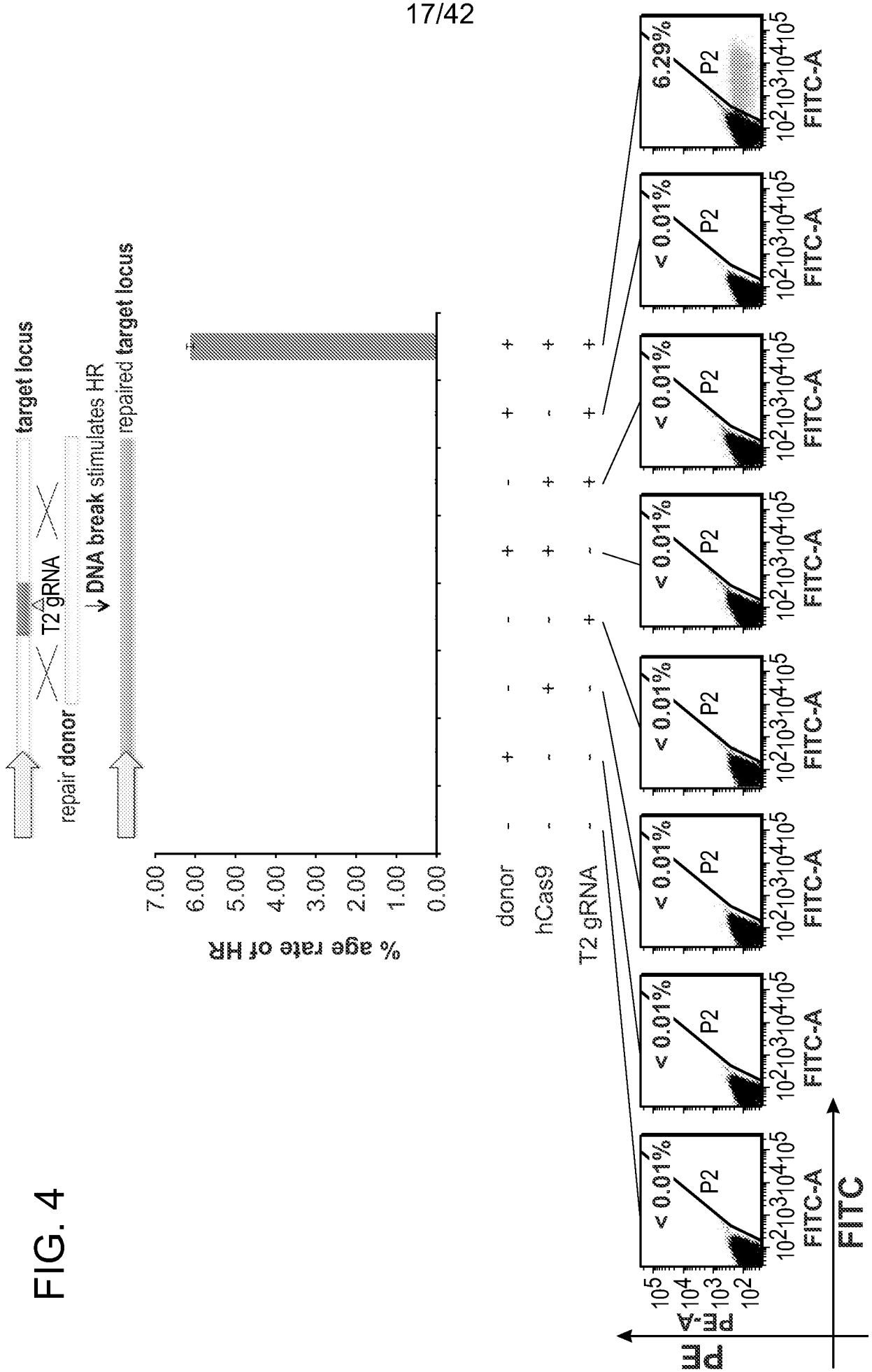

target locus
T2 gRNA
repair donor
DNA break stimulates HR
repaired target locus
% age rate of HR
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
donor
hCas9
T2 gRNA
< 0.01%
6.29%
P2
FITC-A
FITC

FIG. 4

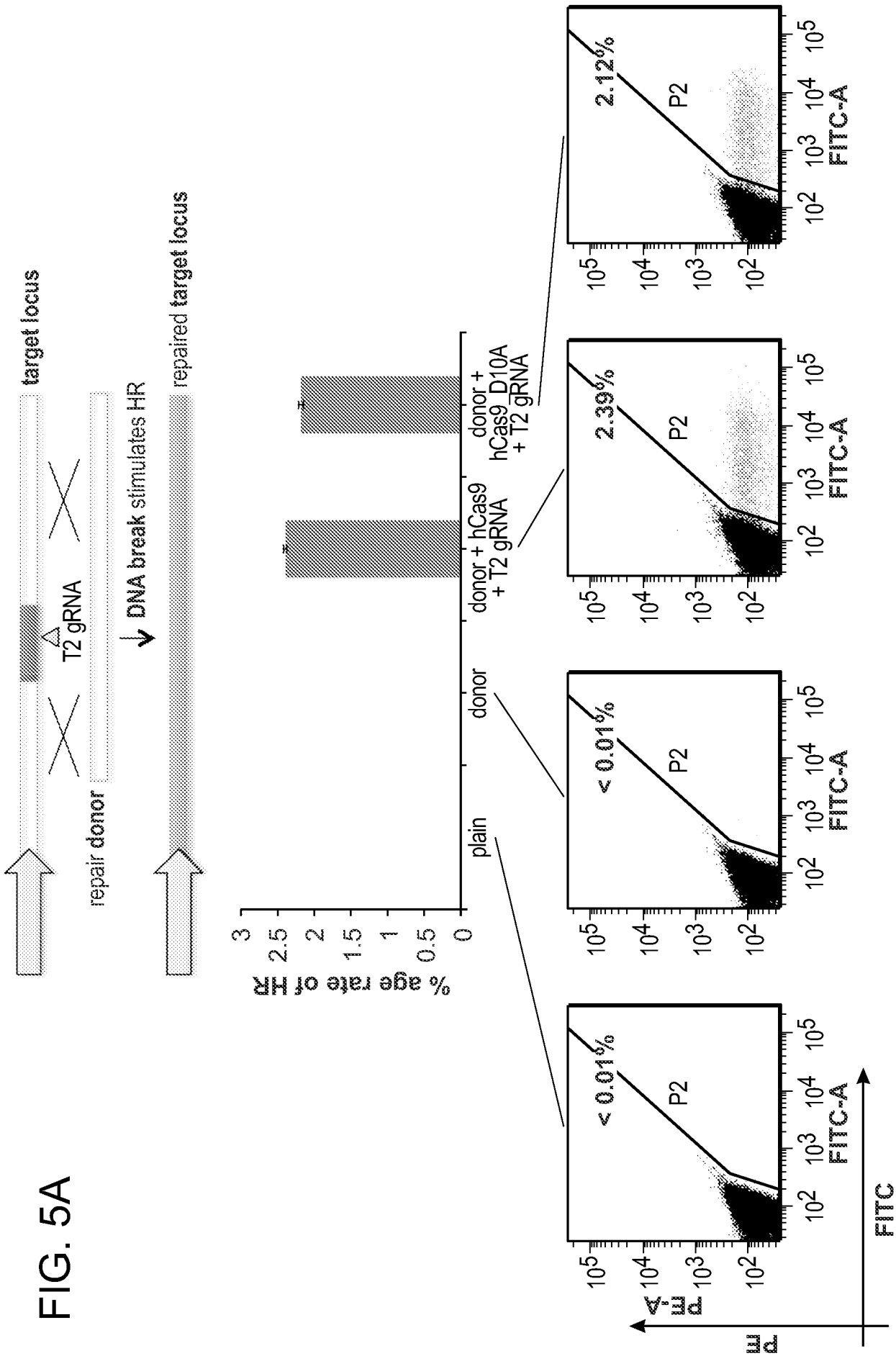

target locus
T2 gRNA
repair donor
DNA break stimulates HR
repaired target locus
% age rate of HR
3
2.5
2
1.5
1
0.5
0
plain
donor
donor + hCas9 + T2 gRNA
donor + hCas9_D10A + T2 gRNA
< 0.01%
< 0.01%
2.39%
2.12%
P2
PE-A
FITC-A
FITC
10^2
10^3
10^4
10^5

FIG. 5A

FIG. 5B

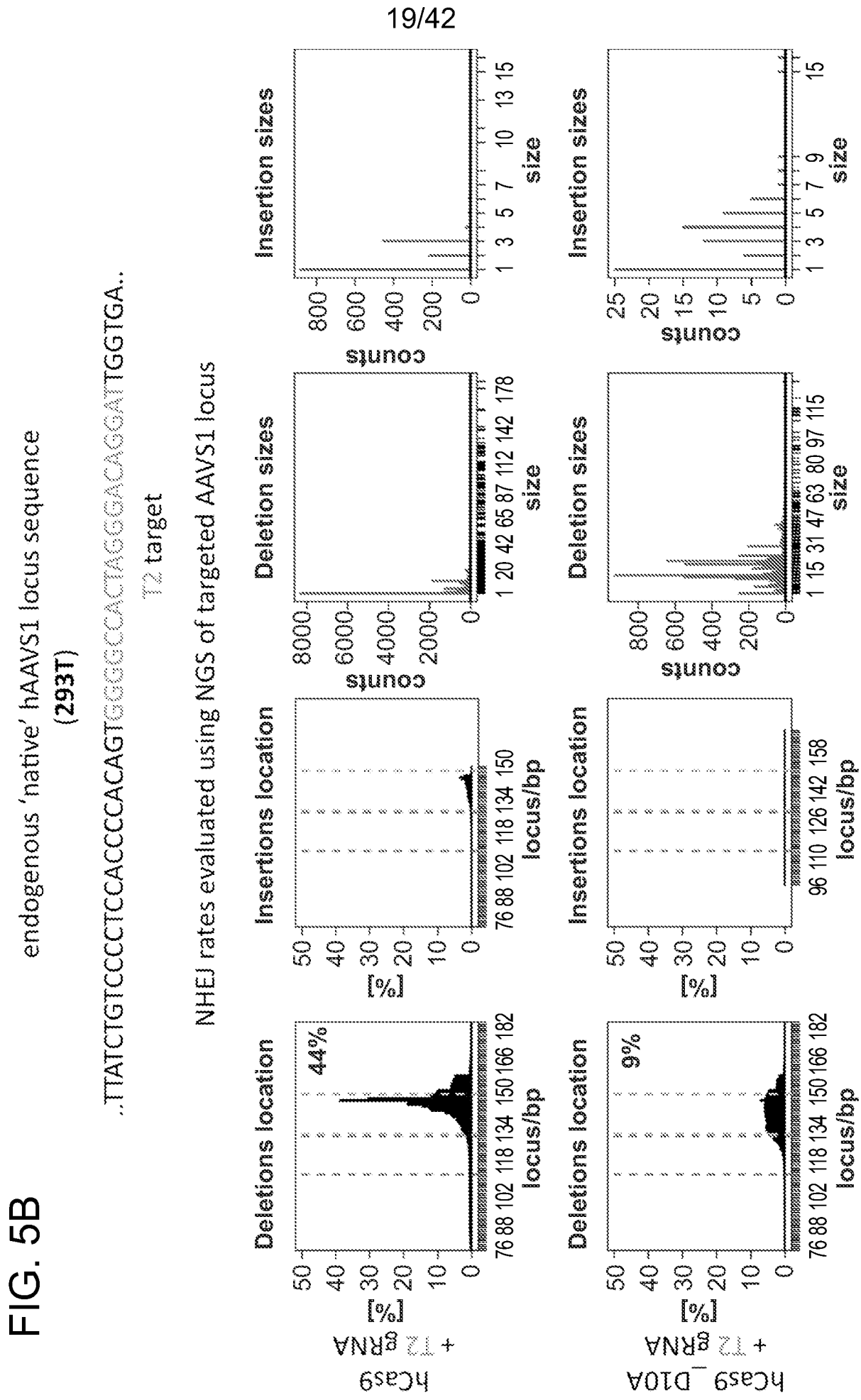

endogenous 'native' hAAVS1 locus sequence
(293T)
..TTATCTGTCCCCTCCACCCCACAGTGGGGCCACTAGGGACAGGATTGGTGA..
T2 target
NHEJ rates evaluated using NGS of targeted AAVS1 locus
hCas9 + T2 gRNA
hCas9_D10A + T2 gRNA
Deletions location
Insertions location
Deletion sizes
Insertion sizes
44%
9%
[%]
counts
locus/bp
size
76 88 102 118 134 150 166 182
96 110 126 142 158
1 20 42 65 87 112 142 178
1 15 31 47 63 80 97 115
1 3 5 7 10 13 15
1 3 5 7 9 15

FIG. 6A

68bp DNA fragment from the AAVS1 locus

wt cell line locus sequence: TAATACTTTTATCTGTCCCCTCCACCCCACAGTGGGGCCACTAGGGACAGGATTGGTGACAGAAAAGCCCC

mut(A) cell line locus sequence: TAATACTTTTATCTGTCAAAAAAACCCCACAGTGGGGCCACTAGGGACAGGATTGGTGACAGAAAAGCCCC

mut(G) cell line locus sequence: TAATACTTTTATCTCTGGGGGACCCCACAGTGGGGCCACTAGGGACAGGATTGGTGACAGAAAAGCCCC

FIG. 6

FIG. 6B

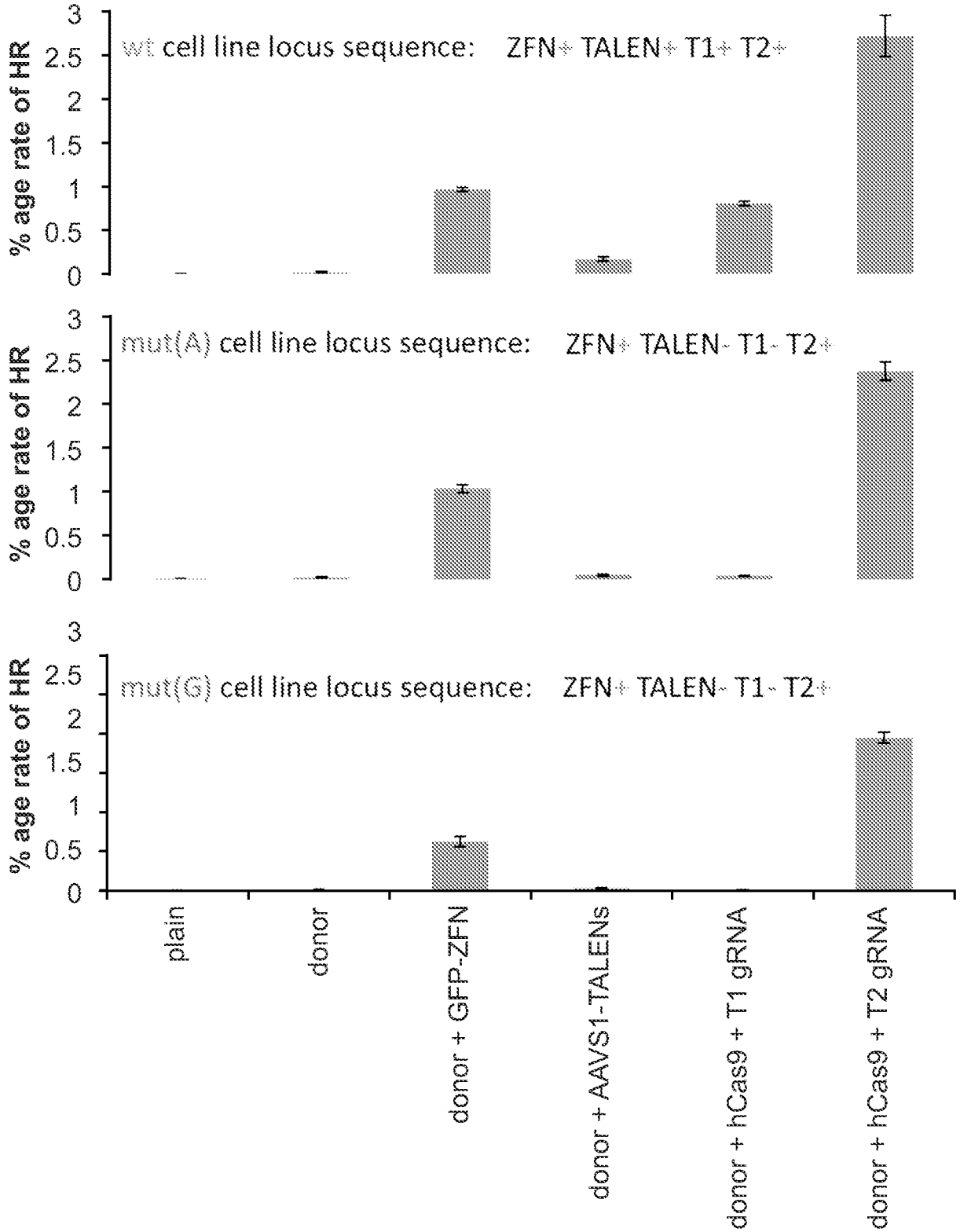

wt cell line locus sequence: ZFN+ TALEN+ T1+ T2+
mut(A) cell line locus sequence: ZFN+ TALEN- T1- T2+
mut(G) cell line locus sequence: ZFN+ TALEN- T1- T2+
% age rate of HR
0
0.5
1
1.5
2
2.5
3
plain
donor
donor + GFP-ZFN
donor + AAVS1-TALENs
donor + hCas9 + T1 gRNA
donor + hCas9 + T2 gRNA

FIG. 7

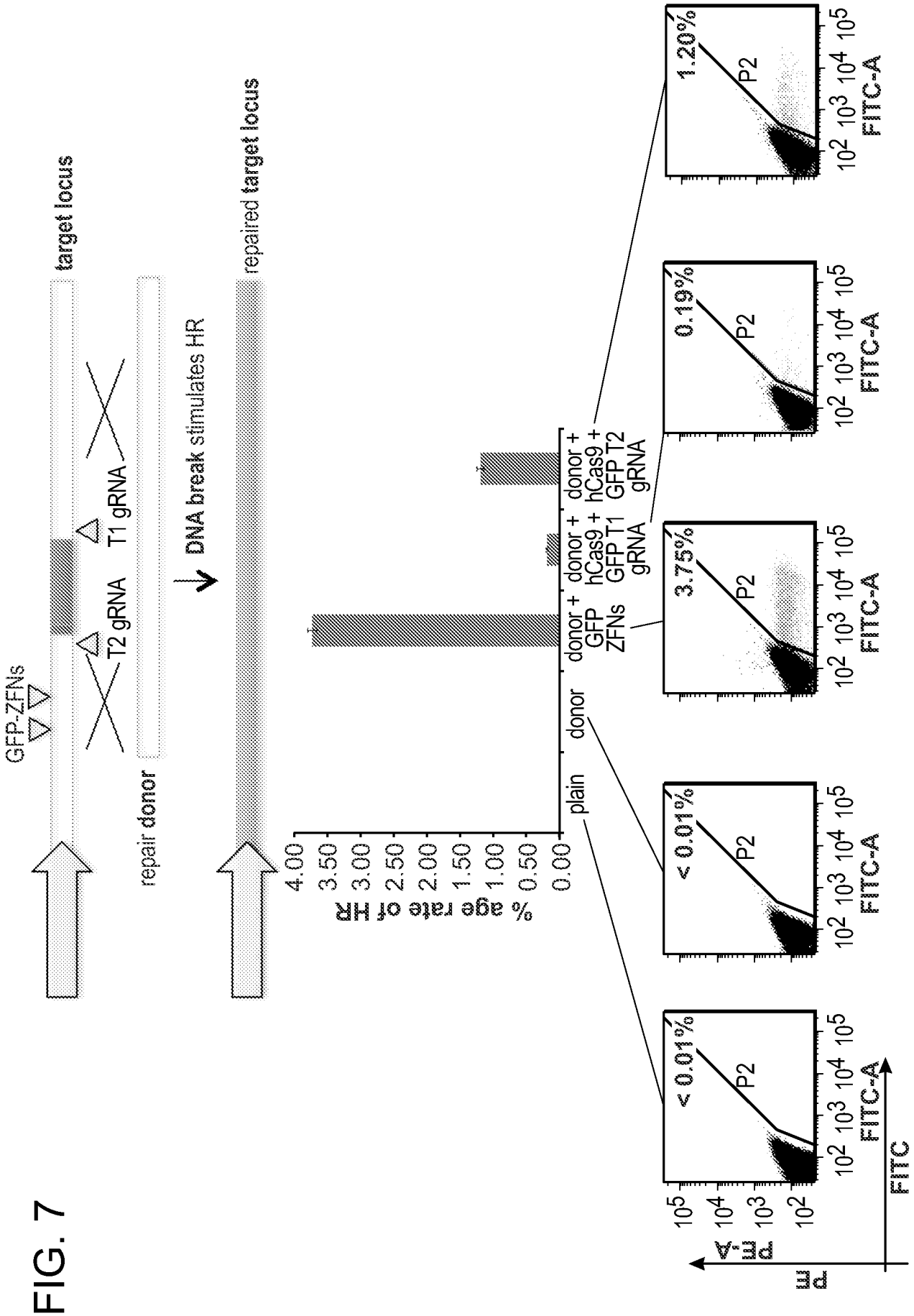

GFP-ZFNs
target locus
T2 gRNA
T1 gRNA
repair donor
DNA break stimulates HR
repaired target locus
% age rate of HR
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
plain
donor
donor + GFP ZFNs
donor + hCas9 + GFP T1 gRNA
donor + hCas9 + GFP T2 gRNA
< 0.01%
< 0.01%
3.75%
0.19%
1.20%
P2
SSC-A
FITC-A
FITC

FIG. 8A

58bp DNA fragments from the DNMT3a or DNMT3b loci

DNMT3a cell line locus sequence: TAATGCATGATGCCGGCCCCAAGGAGGGAGATGATCGGCCCTTCTTCTGGCTCTTTGAGAA

DNMT3b cell line locus sequence: TAATGAATTACTCACGCCCCAAGGAGGGTGATGACCGGCCGTTCTTCTGGATGTTTGAGAA

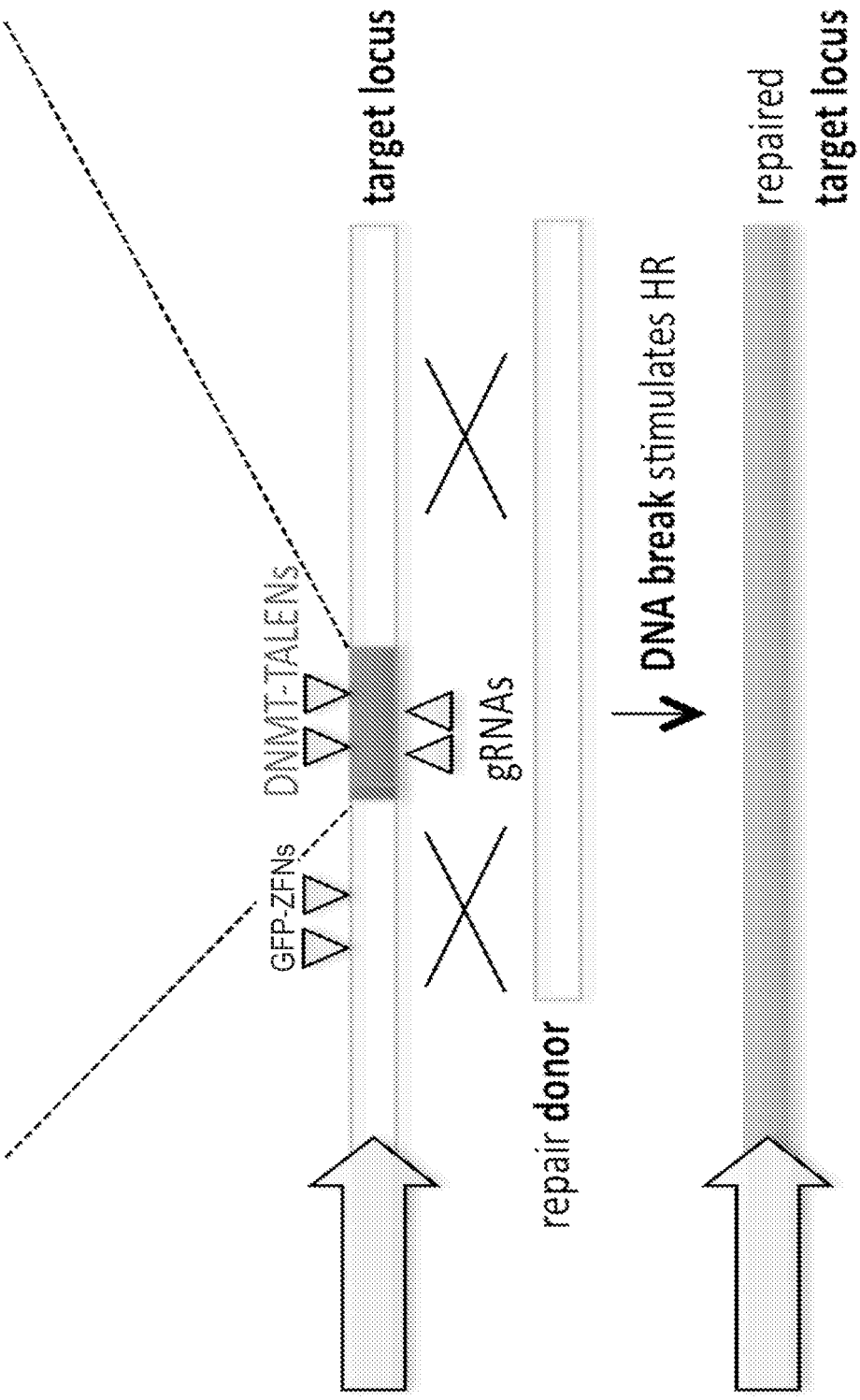


FIG. 8

FIG. 8A
FIG. 8B

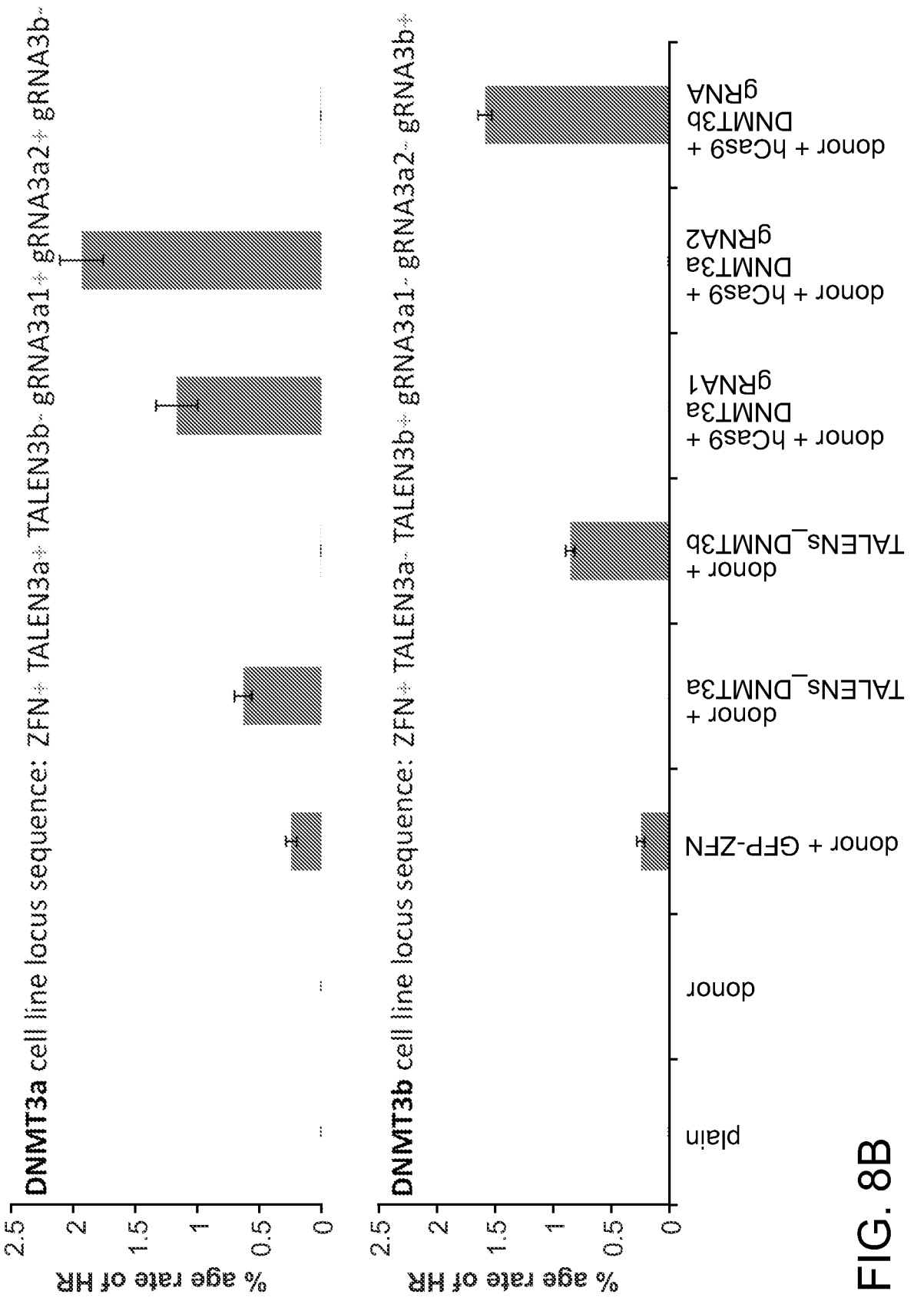


FIG. 8B

FIG. 9A	FIG. 9B	FIG. 9C

FIG. 9

FIG. 9A

endogenous 'native' hAAVS1 locus sequence
(**PGP1 iPS**)

..TTATCTGTCCCTCCACCCACAGTGGGGCCACTAGGGACAGGATTGGTGA..

T1 target T2 target

NHEJ rates evaluated using NGS of targeted AAVS1 locus

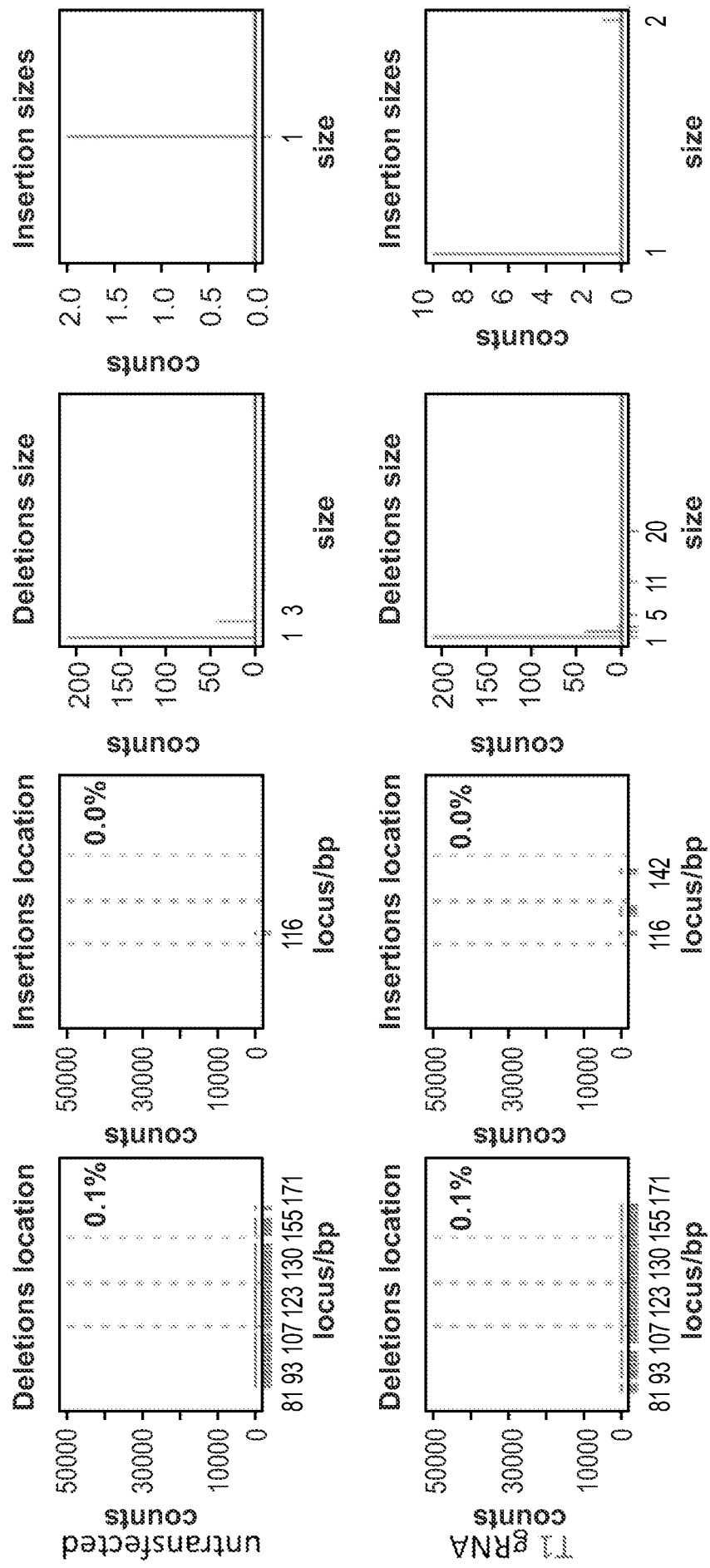


FIG. 9B

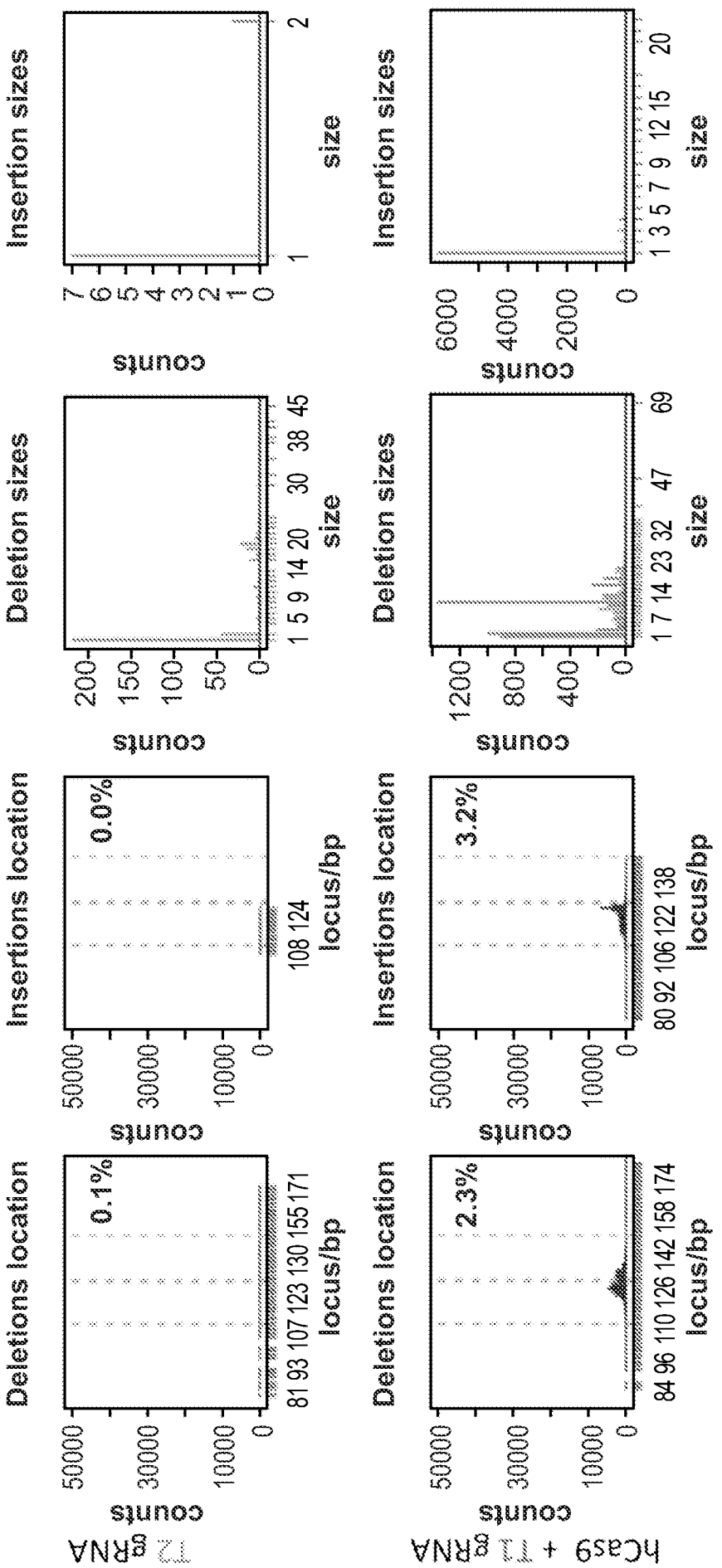

hCas9 + T1 gRNA
T2 gRNA
Deletions location
Insertions location
Deletion sizes
Insertion sizes
counts
locus/bp
size
0.1%
0.0%
2.3%
3.2%
81 93 107 123 130 155 171
108 124
84 96 110 126 142 158 174
80 92 106 122 138
1 5 9 14 20 30 38 45
1 7 14 23 32 47 69
1 3 5 7 9 12 15 20
0
10000
30000
50000
100
150
200
400
800
1200
2000
4000
6000
1
2
3
4
5
6
7

FIG. 9C

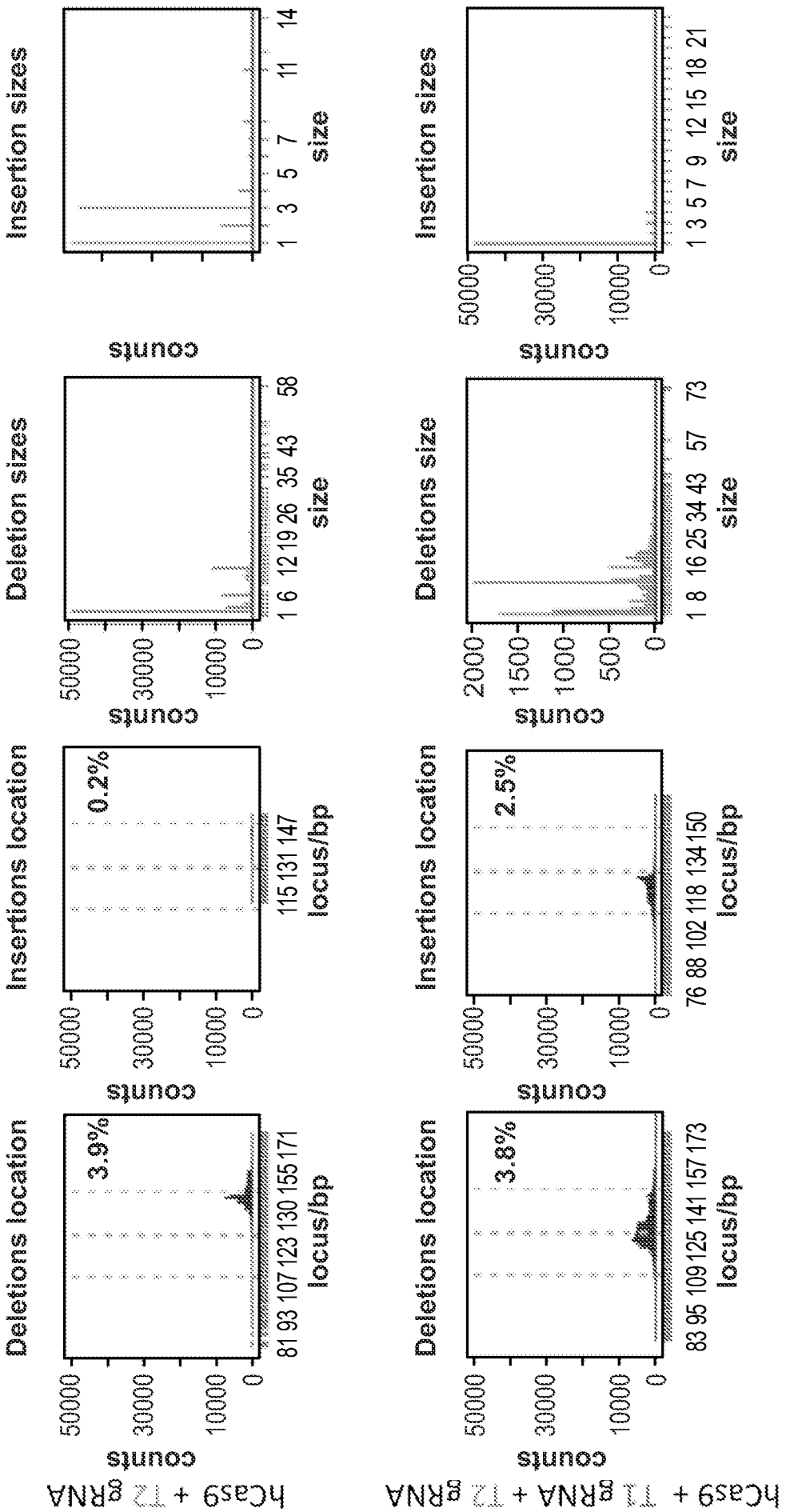

hCas9 + T2 gRNA
hCas9 + T1 gRNA + T2 gRNA
Deletions location
Insertions location
Deletion sizes
Insertion sizes
Deletions size
counts
locus/bp
size
3.9%
0.2%
2.5%
3.8%
50000
30000
10000
0
2000
1500
1000
500
81 93 107 123 130 155 171
115 131 147
1 6 12 19 26 35 43 58
1 3 5 7 11 14
76 88 102 118 134 150
83 95 109 125 141 157 173
1 8 16 25 34 43 57 73
1 3 5 7 9 12 15 18 21

FIG. 10

FIG. 10A	FIG. 10B

FIG. 10A

endogenous 'native' hAAVS1 locus sequence
(K562)

..TTATCTGTCCCCTCCACCCCACAGTGGGGCCACTAGGGACAGGATTGGTGA..

T1 target T2 target

NHEJ rates evaluated using NGS of targeted AAVS1 locus

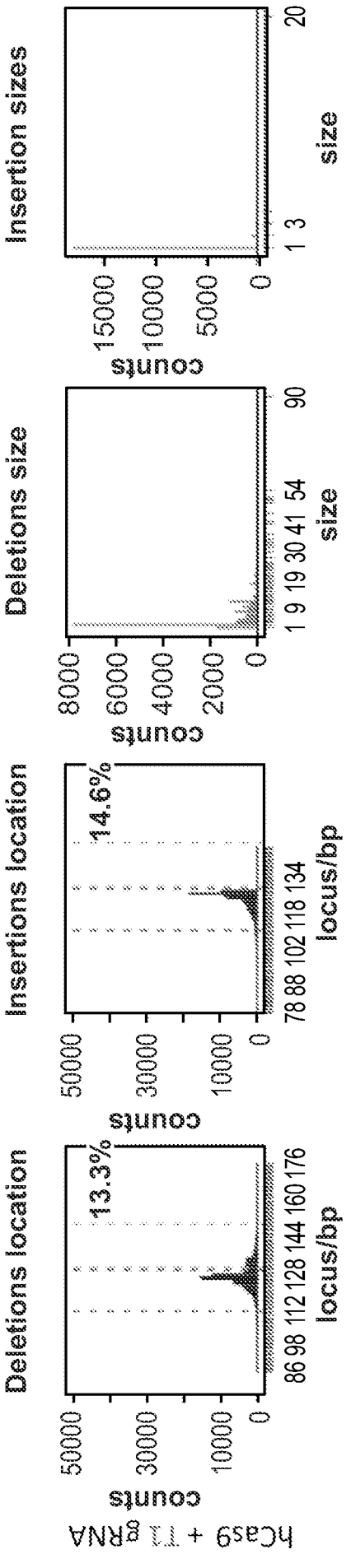


FIG. 10B

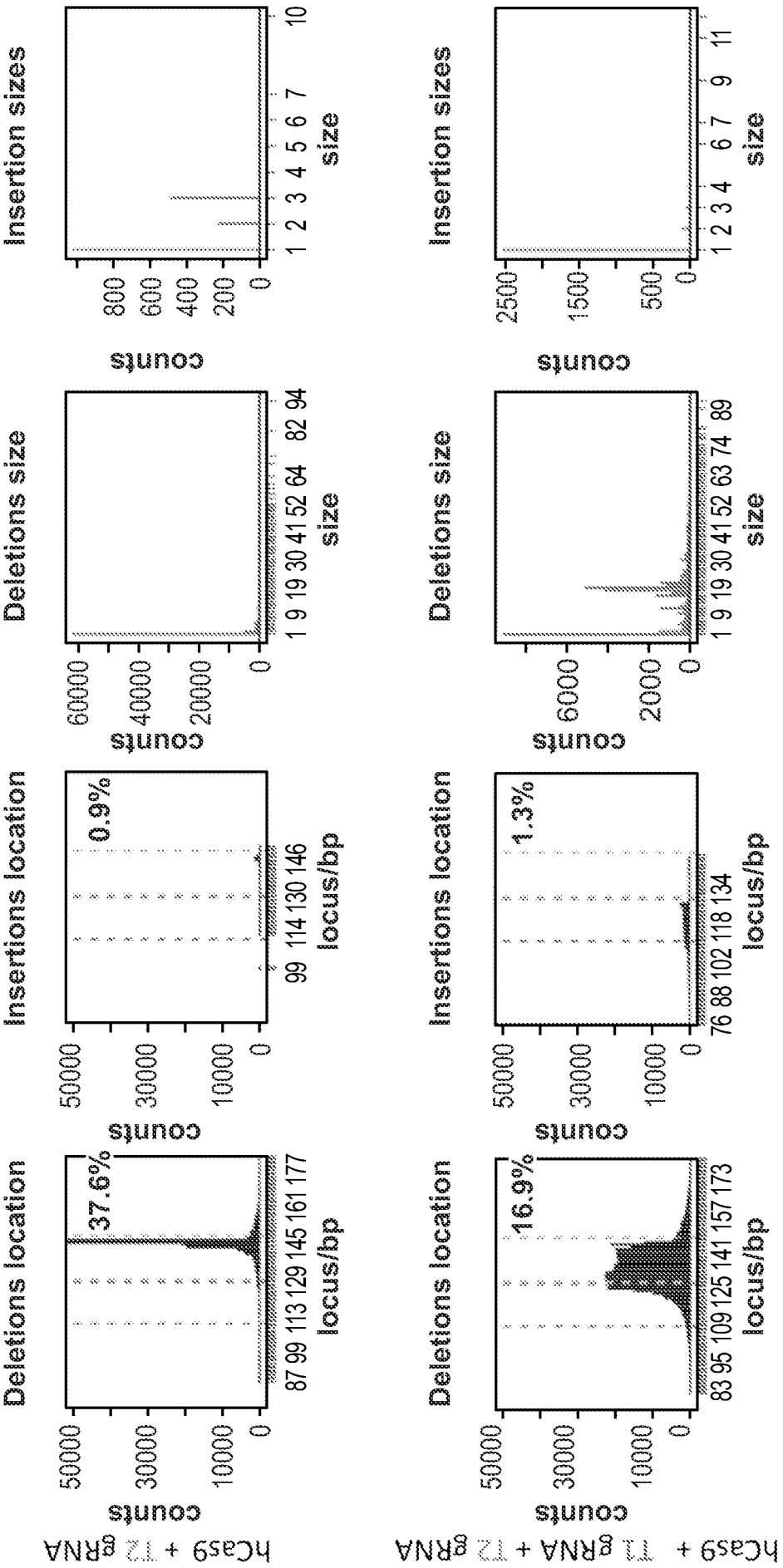

hCas9 + T2 gRNA
hCas9 + T1 gRNA + T2 gRNA
Deletions location
Insertions location
Deletions size
Insertion sizes
counts
locus/bp
size
37.6%
0.9%
16.9%
1.3%
87 99 113 129 145 161 177
99 114 130 146
83 95 109 125 141 157 173
76 88 102 118 134
1 9 19 30 41 52 64 82 94
1 9 19 30 41 52 63 74 89
1 2 3 4 5 6 7 10
1 2 3 4 6 7 9 11

FIG. 11

FIG. 11A
FIG. 11B

FIG. 11A

endogenous 'native' hAAVS1 locus sequence
(**293T**)

..TTATCTGTCCCCTCCACCCACAGTGGGGCCACTAGGGACAGGATTGGTGA..

T1 target T2 target

NHEJ rates evaluated using NGS of targeted AAVS1 locus

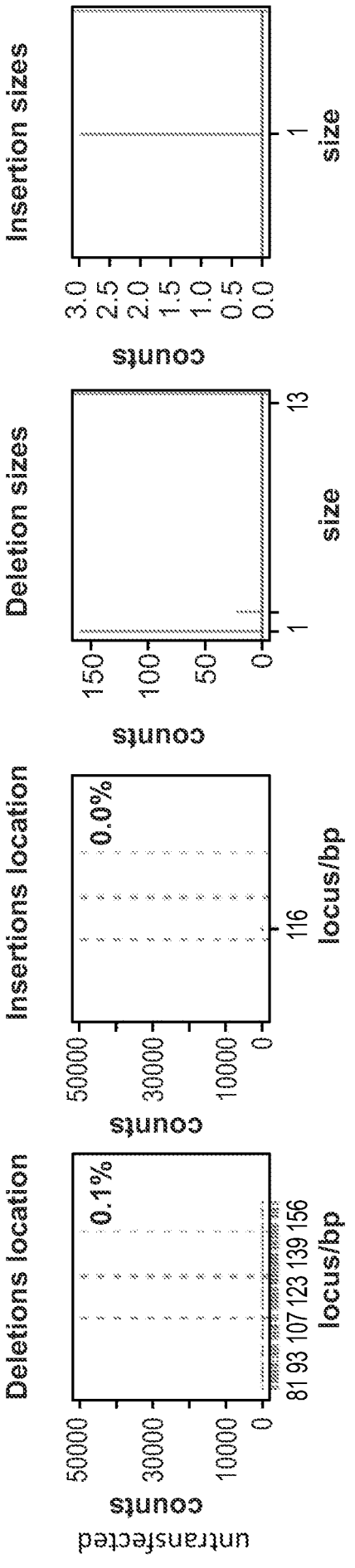


FIG. 11B

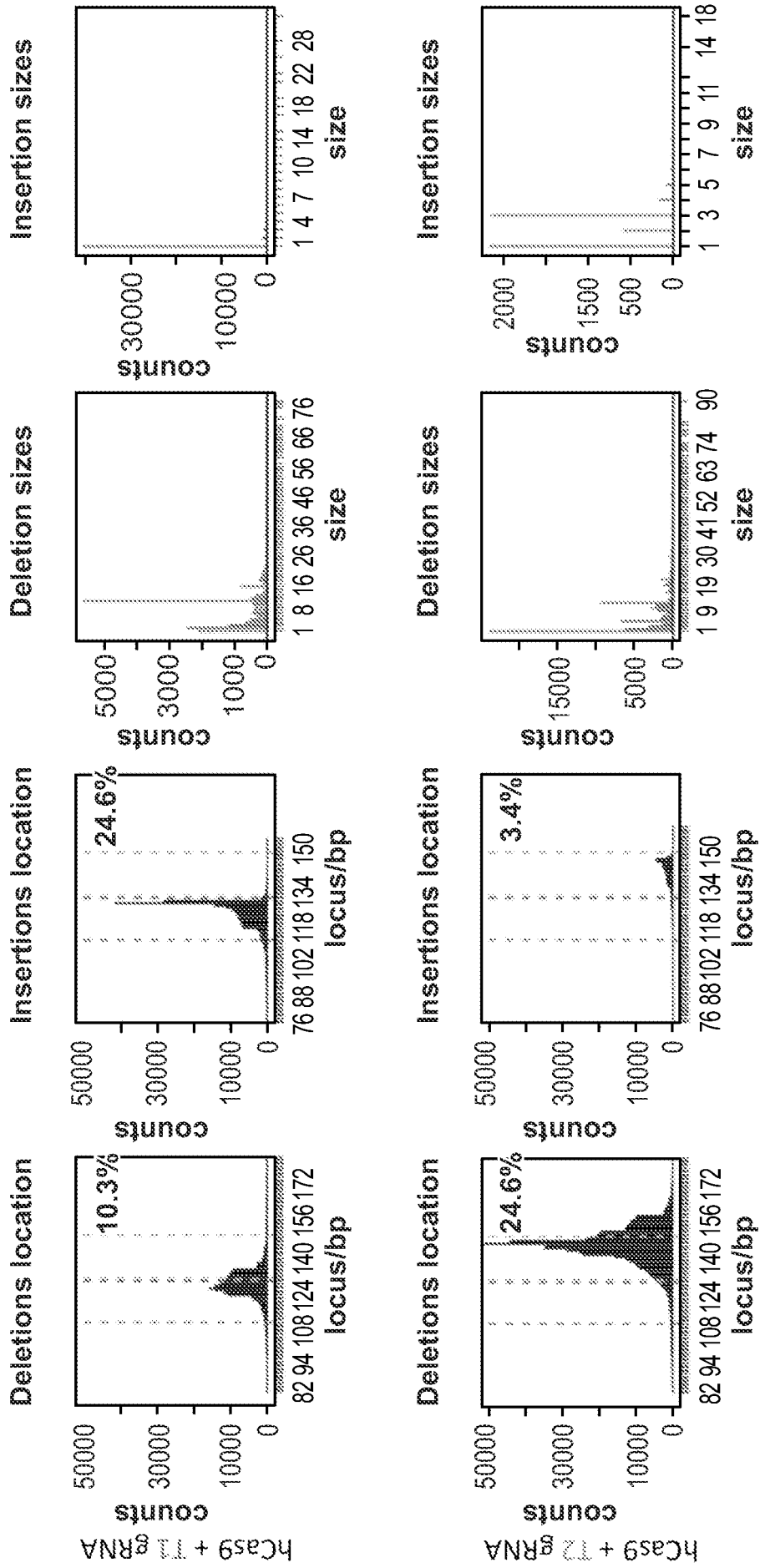

hCas9 + T1 gRNA
hCas9 + T2 gRNA
Deletions location
Insertions location
Deletion sizes
Insertion sizes
counts
locus/bp
size
10.3%
24.6%
3.4%
24.6%
82 94 108 124 140 156 172
76 88 102 118 134 150
1 8 16 26 36 46 56 66 76
1 9 19 30 41 52 63 74 90
1 4 7 10 14 18 22 28
1 3 5 7 9 11 14 18

FIG. 12A

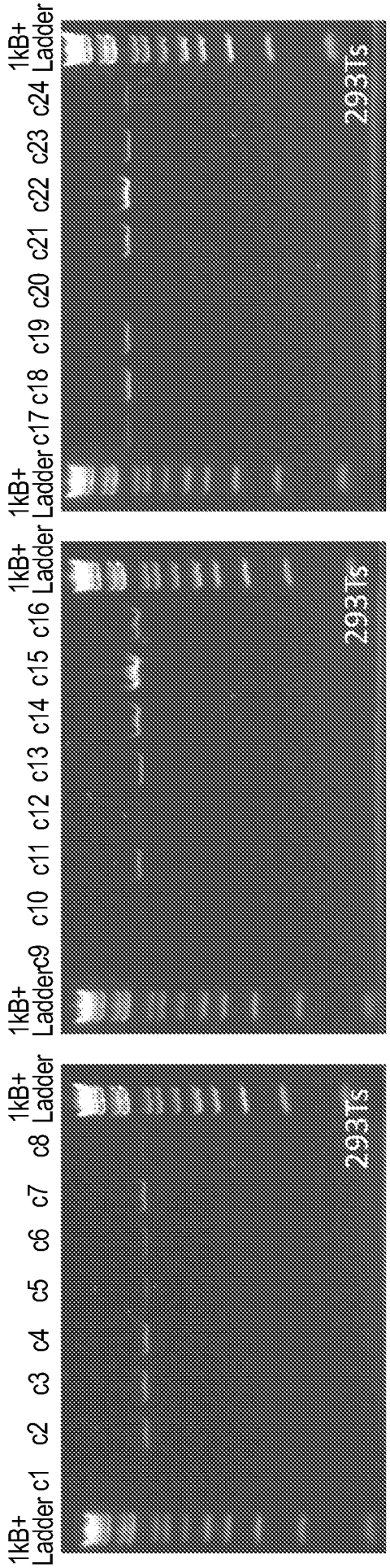


FIG. 12B

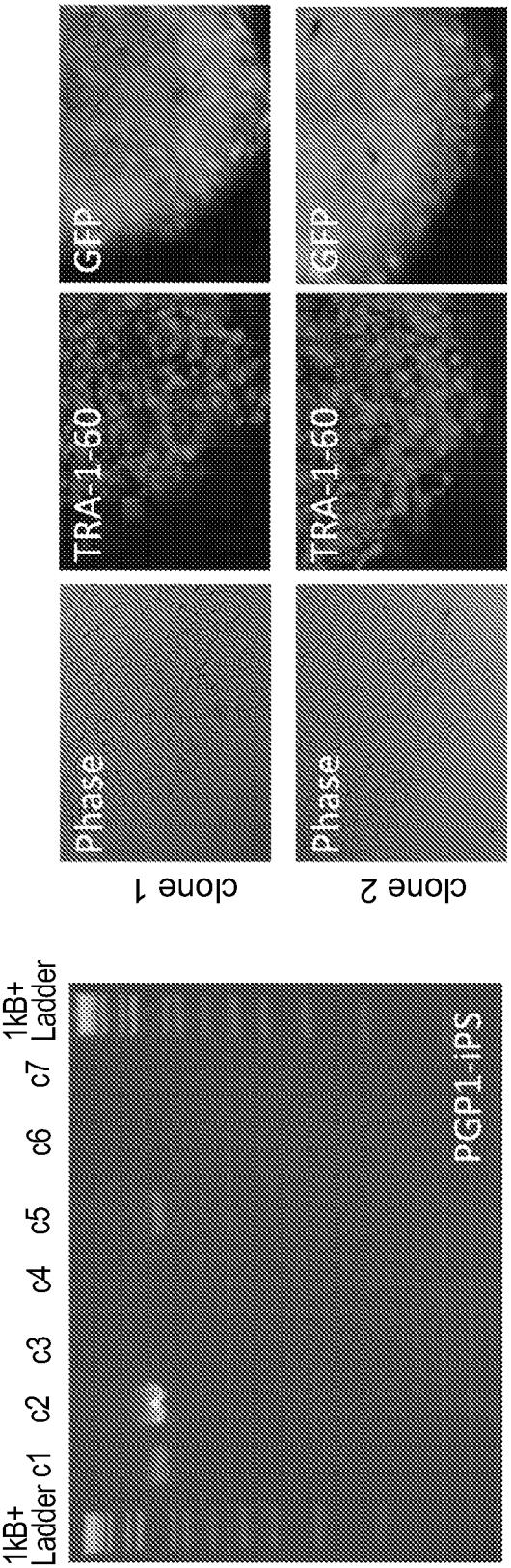


FIG. 12C

endogenous 'native' hAAVS1 locus sequence, with the AAVS1 T2 gRNA target site highlighted

...GGTACTTTTATTCTGTCCCTCCACCCCACAGTGGGGCCACTAGAACAGATTGTGACAGAAAAGGCCATCCTTAGGCCTCCTCCTTCCTAGTCTCCTGATATTGGGTC...

Targeting of AAVS1 locus in K562s : hCas9 + AAVS1 T2 gRNA + 90mer *oligo* donor

5'- TTATTCTGTCCCTCCACCCCACAGTGGGGCCACTAGAACAGATTGTGACAGAAAAGGCCATCCTTAGGCCTCCTCCTTCCTAGTCTCCTGATATTGGGTC-3'

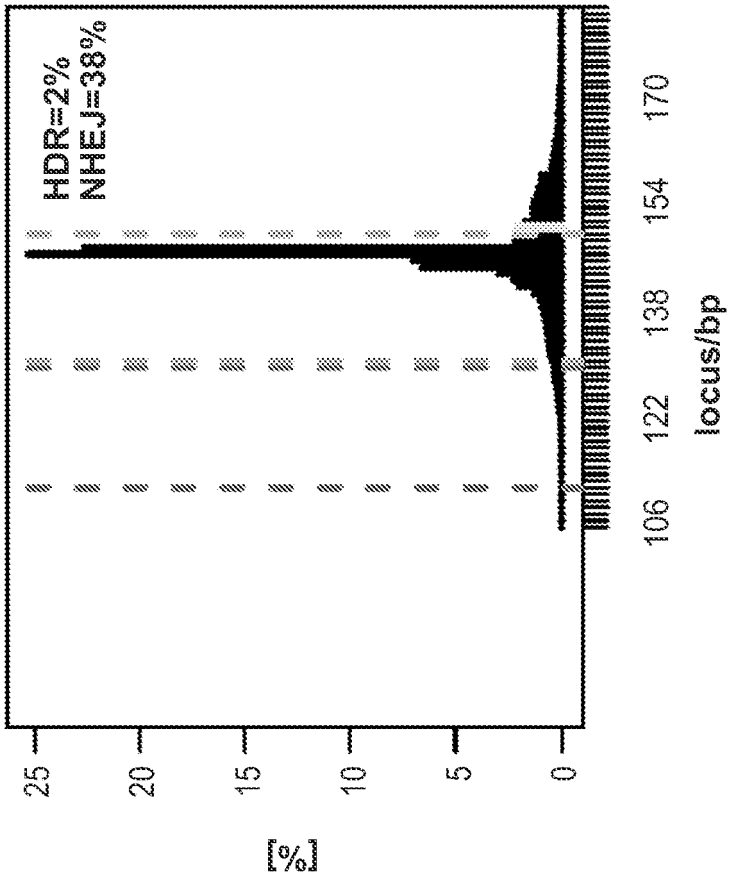


FIG. 13A-1 FIG. 13A-2

FIG. 13A

FIG. 13A-2

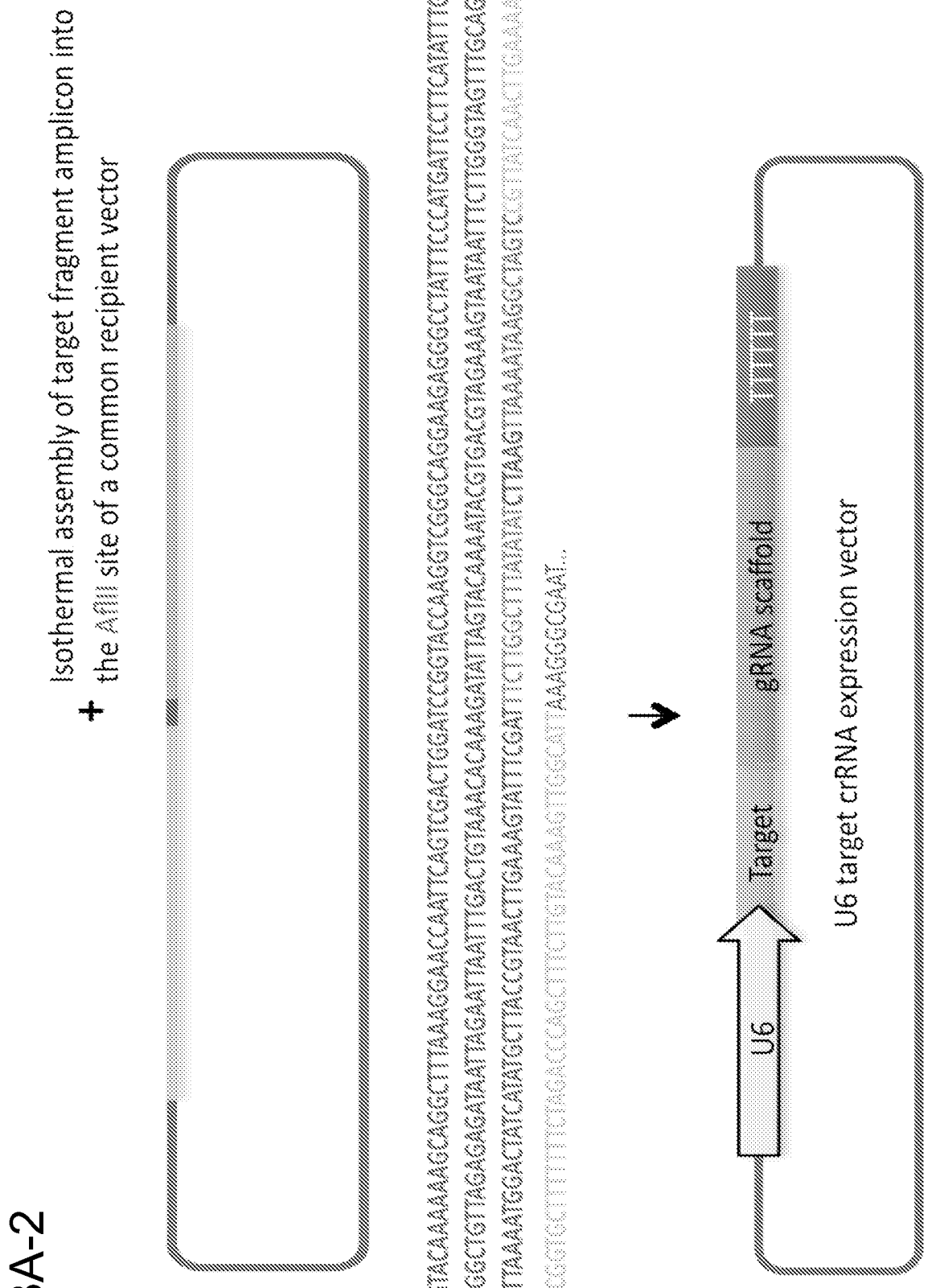
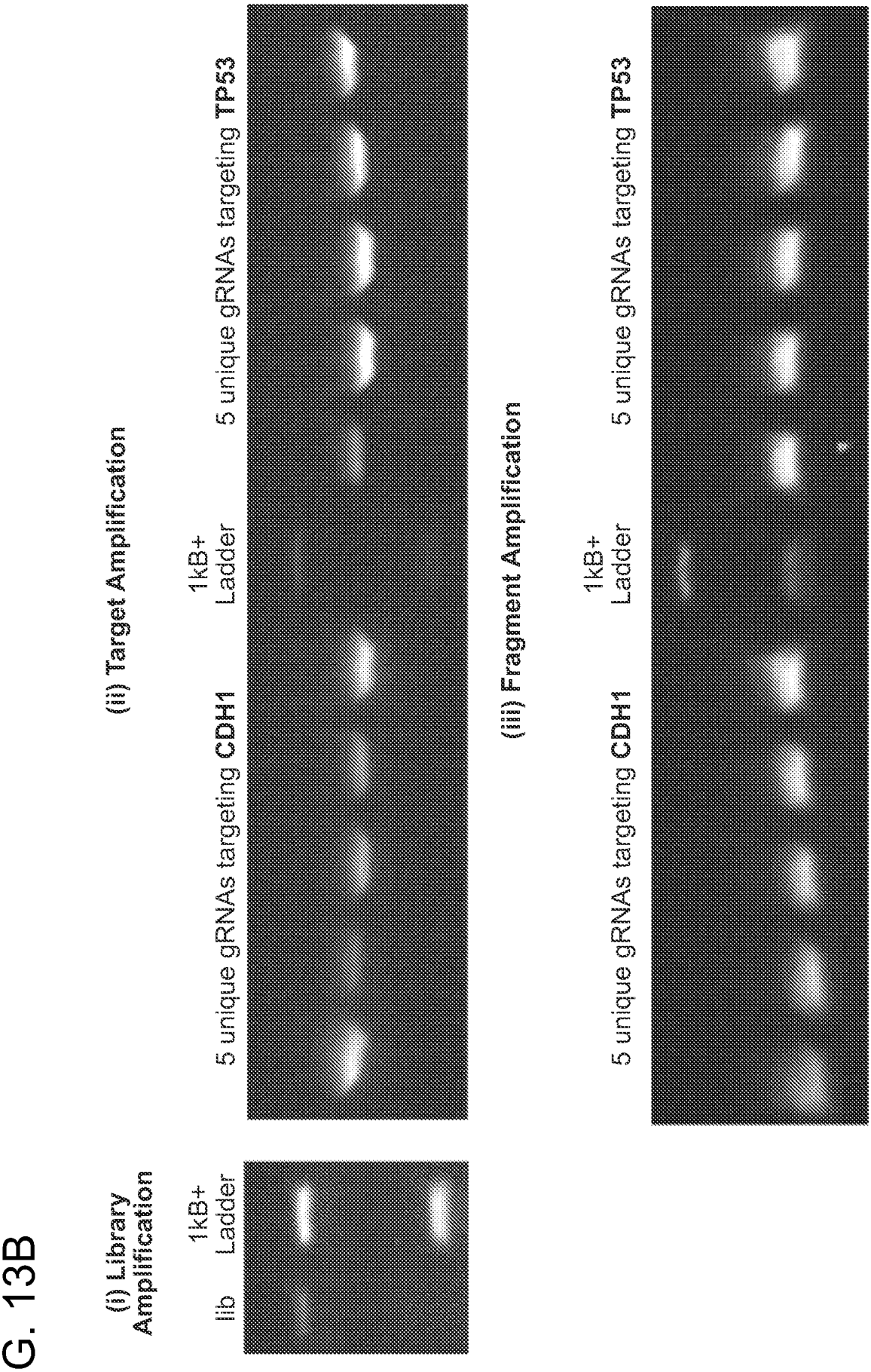

+ Isothermal assembly of target fragment amplicon into the AflIII site of a common recipient vector
...ATTGCCCTTTGTACAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACTGGATCCGGTACCAAGGTCGGGCAGGAAGAGGGCCTATTTCCCATGATTCCTTCATATTTGC
ATATACGATACAAGGCTGTTAGAGAGATAATTAGAATTAATTTGACTGTAAACACAAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTT
TTAAAATTATGTTTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATATCTTAAGTTAAAAAAAGGCTAGTCCGTTATCAACTTGAAAAA
GTGGCACCGAGTCGGTGCTTTTTTTGTAGACCCAGCTTTCTTGTACAAAGTTGGCATTAAAGGGCGAAT...
U6
Target
gRNA scaffold
TTTTTT
U6 target crRNA expression vector

FIG. 13B


(i) Library Amplification
lib
1kB+ Ladder
(ii) Target Amplification
5 unique gRNAs targeting CDH1
1kB+ Ladder
5 unique gRNAs targeting TP53
(iii) Fragment Amplification
5 unique gRNAs targeting CDH1
1kB+ Ladder
5 unique gRNAs targeting TP53

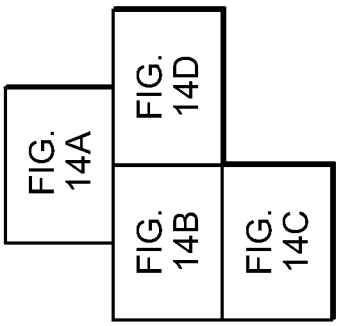


FIG. 14

FIG. 14A

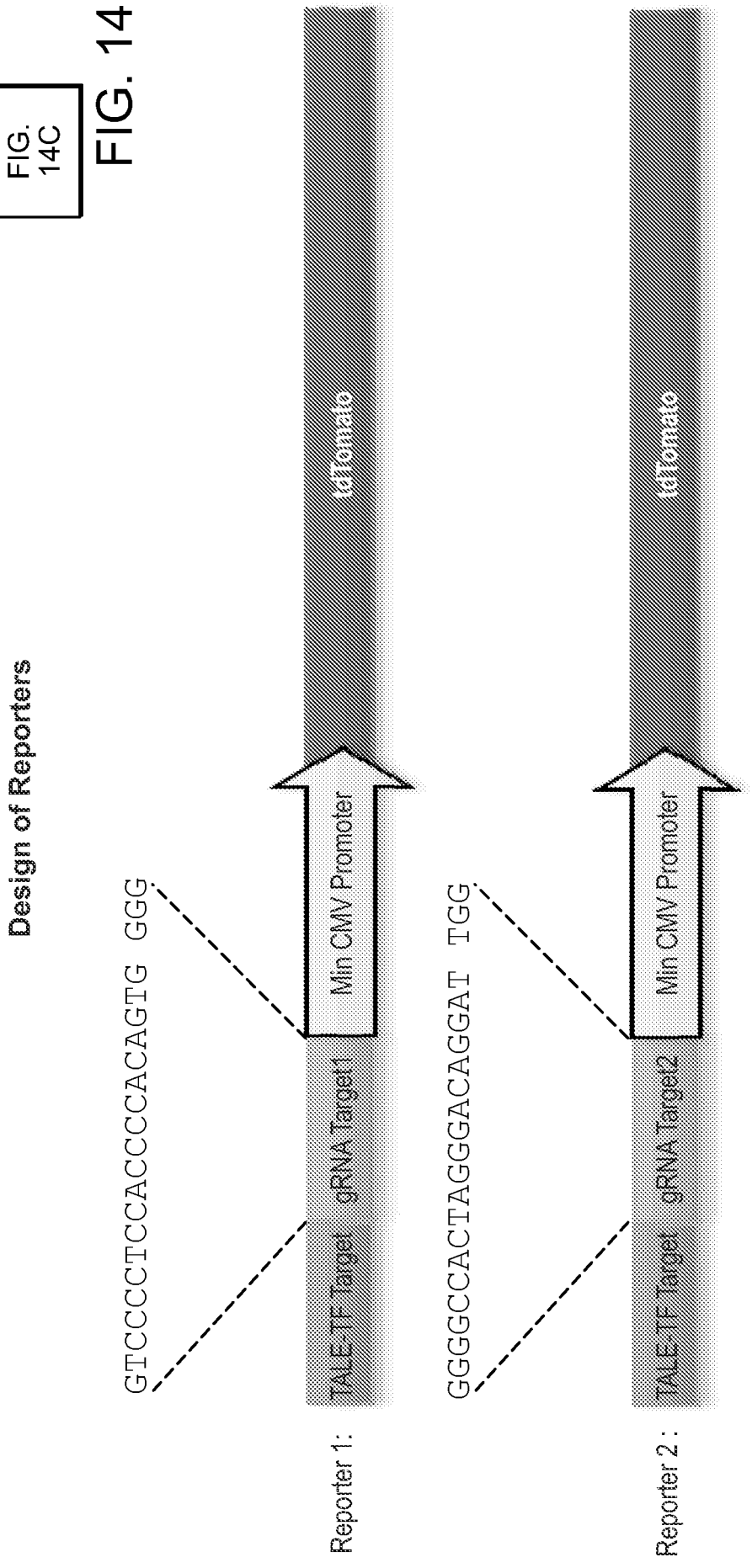


FIG. 14B

(a) Fluorescence Activated Cell Sorting (FACS) Analysis

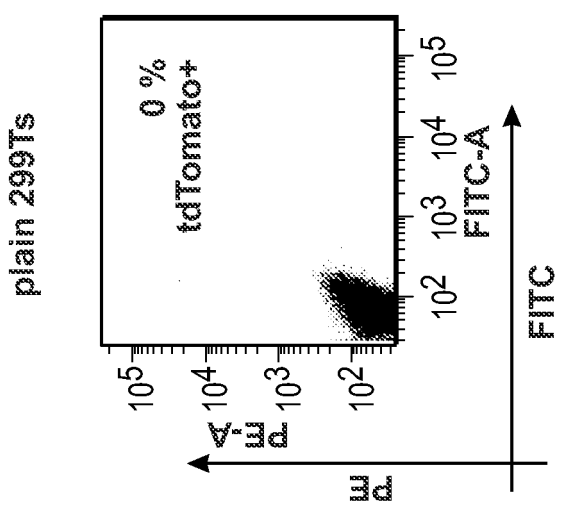


FIG. 14C

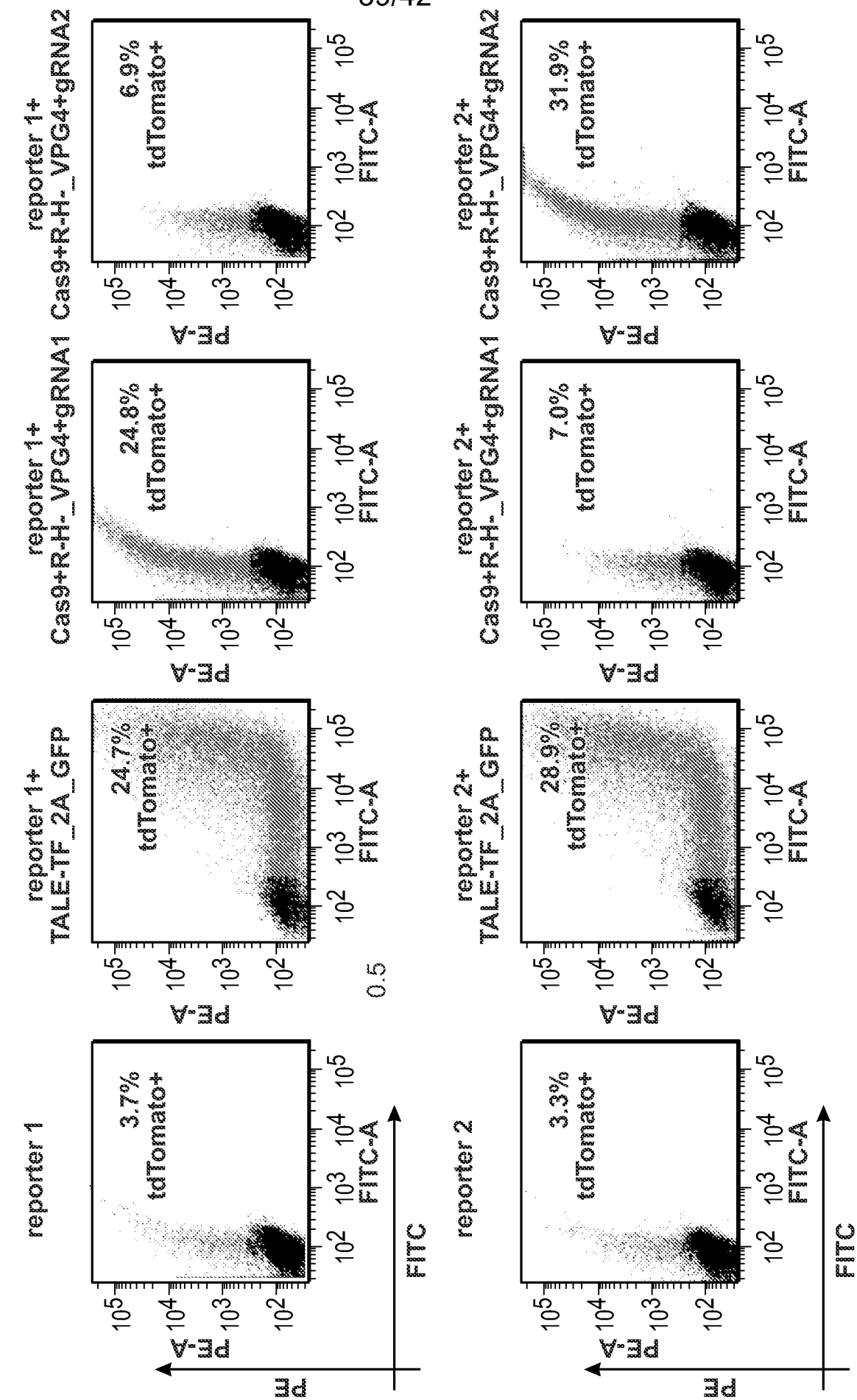
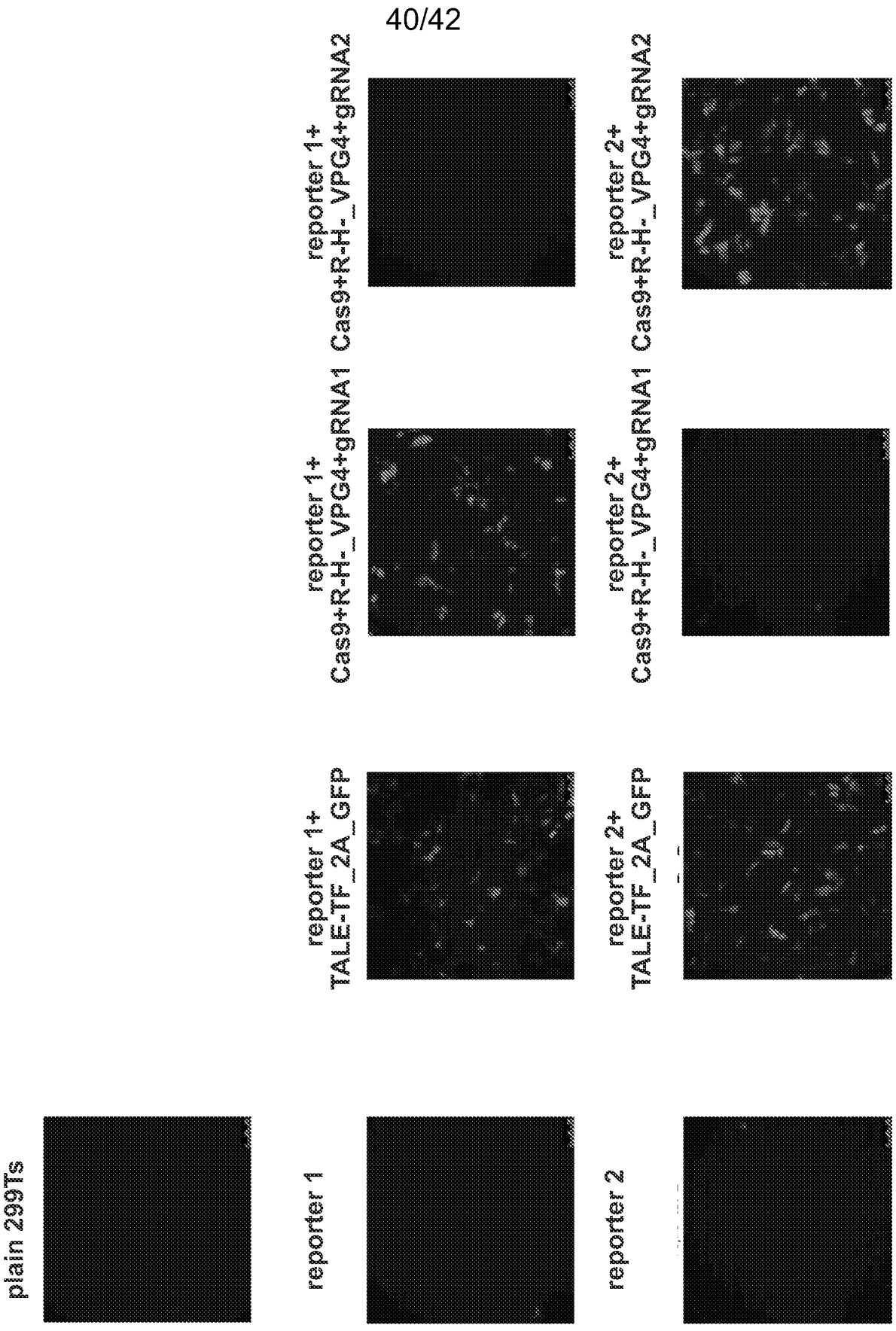

reporter 1
3.7%
tdTomato+
reporter 1+
TALE-TF_2A_GFP
24.7%
tdTomato+
reporter 1+
Cas9+R-H-_VPG4+gRNA1
24.8%
tdTomato+
reporter 1+
Cas9+R-H-_VPG4+gRNA2
6.9%
tdTomato+
reporter 2
3.3%
tdTomato+
reporter 2+
TALE-TF_2A_GFP
28.9%
tdTomato+
reporter 2+
Cas9+R-H-_VPG4+gRNA1
7.0%
tdTomato+
reporter 2+
Cas9+R-H-_VPG4+gRNA2
31.9%
tdTomato+
PE-Tx-Red-YG-A
FITC-A
FITC
0.5

FIG. 14D


(b) Immunofluorescence Microscopy Analysis
plain 293Ts
reporter 1
reporter 2
reporter 1+ TALE-TF_2A_GFP
reporter 1+ Cas9+R-H-_VPG4+gRNA1
reporter 1+ Cas9+R-H-_VPG4+gRNA2
reporter 2+ TALE-TF_2A_GFP
reporter 2+ Cas9+R-H-_VPG4+gRNA1
reporter 2+ Cas9+R-H-_VPG4+gRNA2

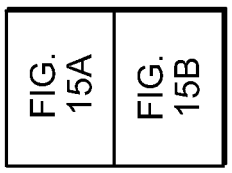


FIG. 15

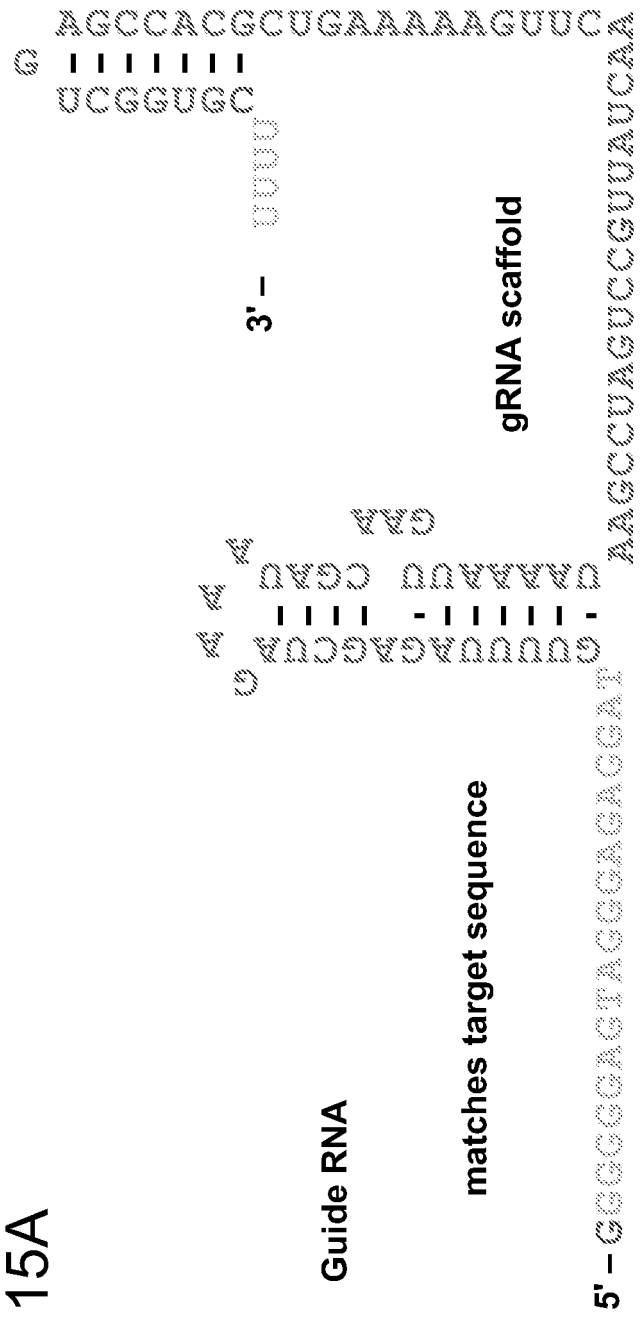


FIG. 15A

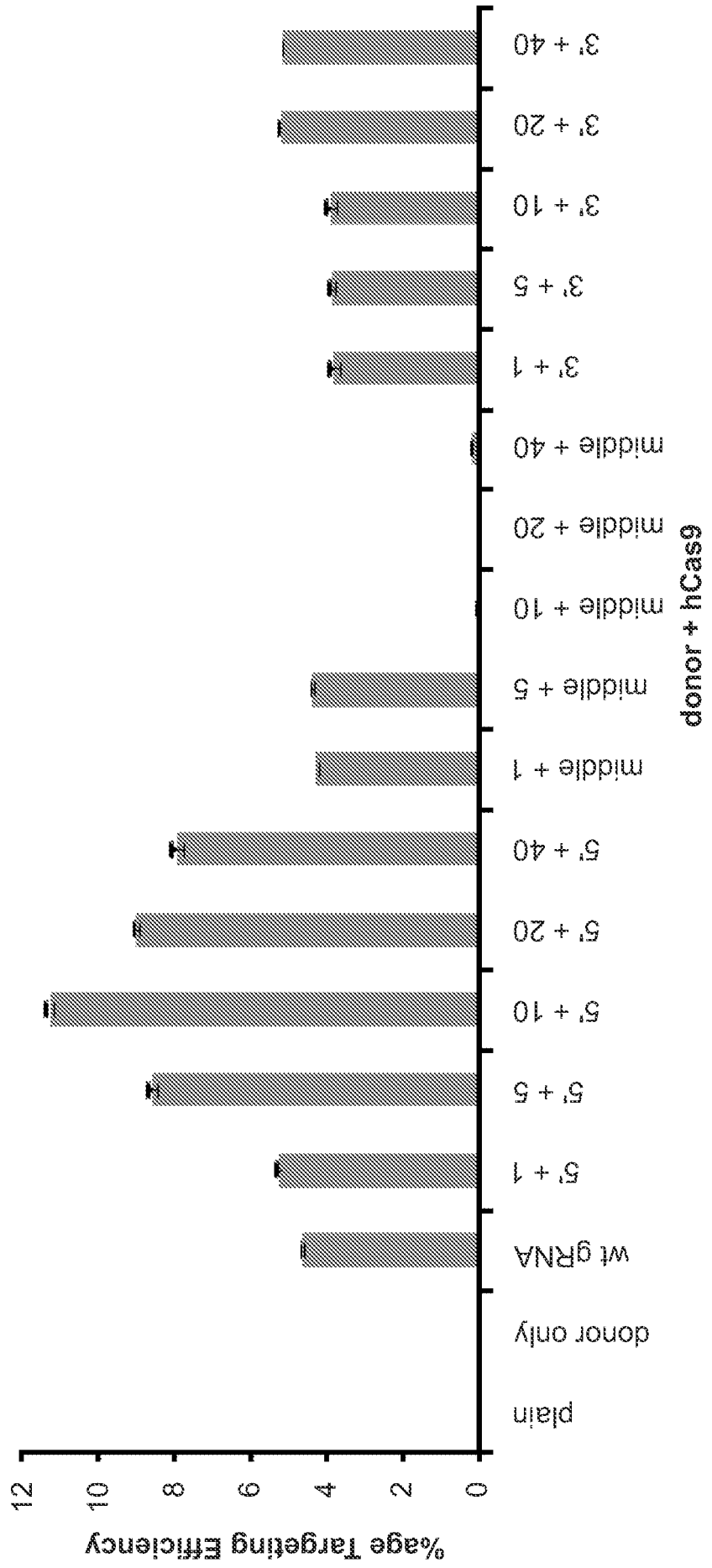

%age Targeting Efficiency
0
2
4
6
8
10
12
plain
donor only
wt gRNA
5' + 1
5' + 5
5' + 10
5' + 20
5' + 40
middle + 1
middle + 5
middle + 10
middle + 20
middle + 40
3' + 1
3' + 5
3' + 10
3' + 20
3' + 40
donor + hCas9

FIG. 15B