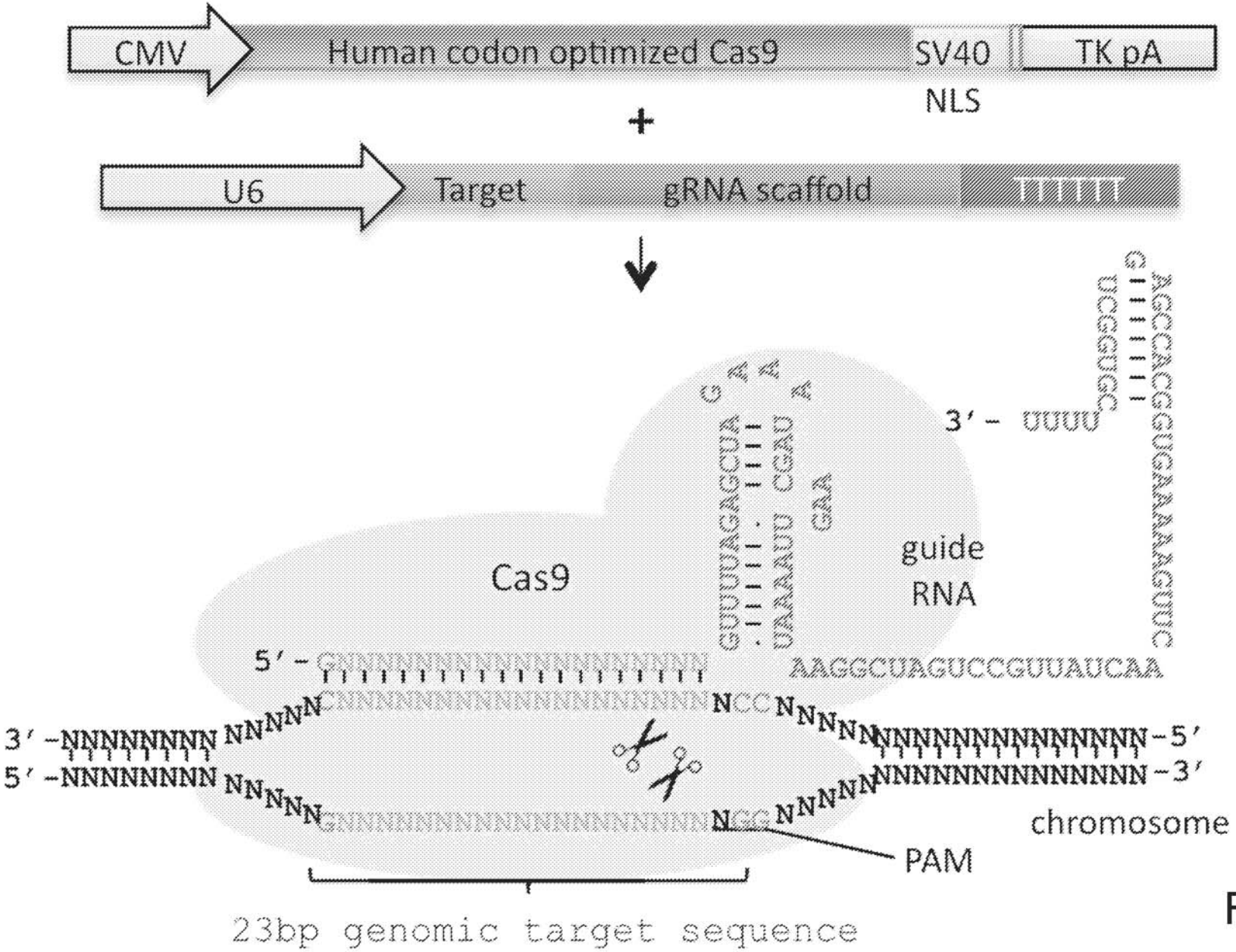

CMV
Human codon optimized Cas9
SV40 NLS
TK pA
+
U6
Target
gRNA scaffold
TTTTTT
Cas9
guide RNA
5′ – GNNNNNNNNNNNNNNNNNNN
AAGGCUAGUCCGUUAUCAA
3′ – UUUU
3′ –NNNNNNNN
5′ –NNNNNNNN
NCCNNNNNNNNNNNNNNN – 5′
NNNNNNNNNNNNNNNN – 3′
chromosome
PAM
23bp genomic target sequence

FIG. 1A

FIG. 1B

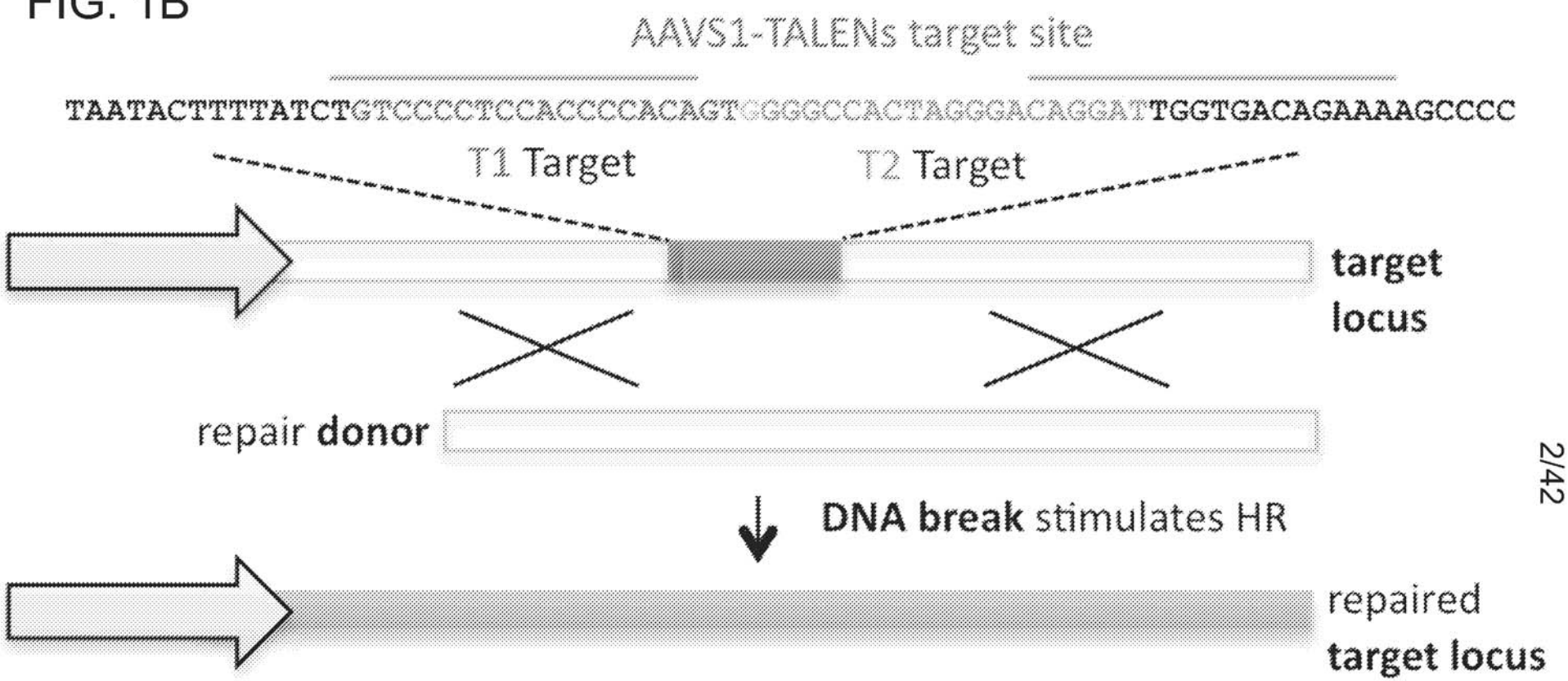


FIG. 1C-1
FIG. 1C-2

FIG. 1C

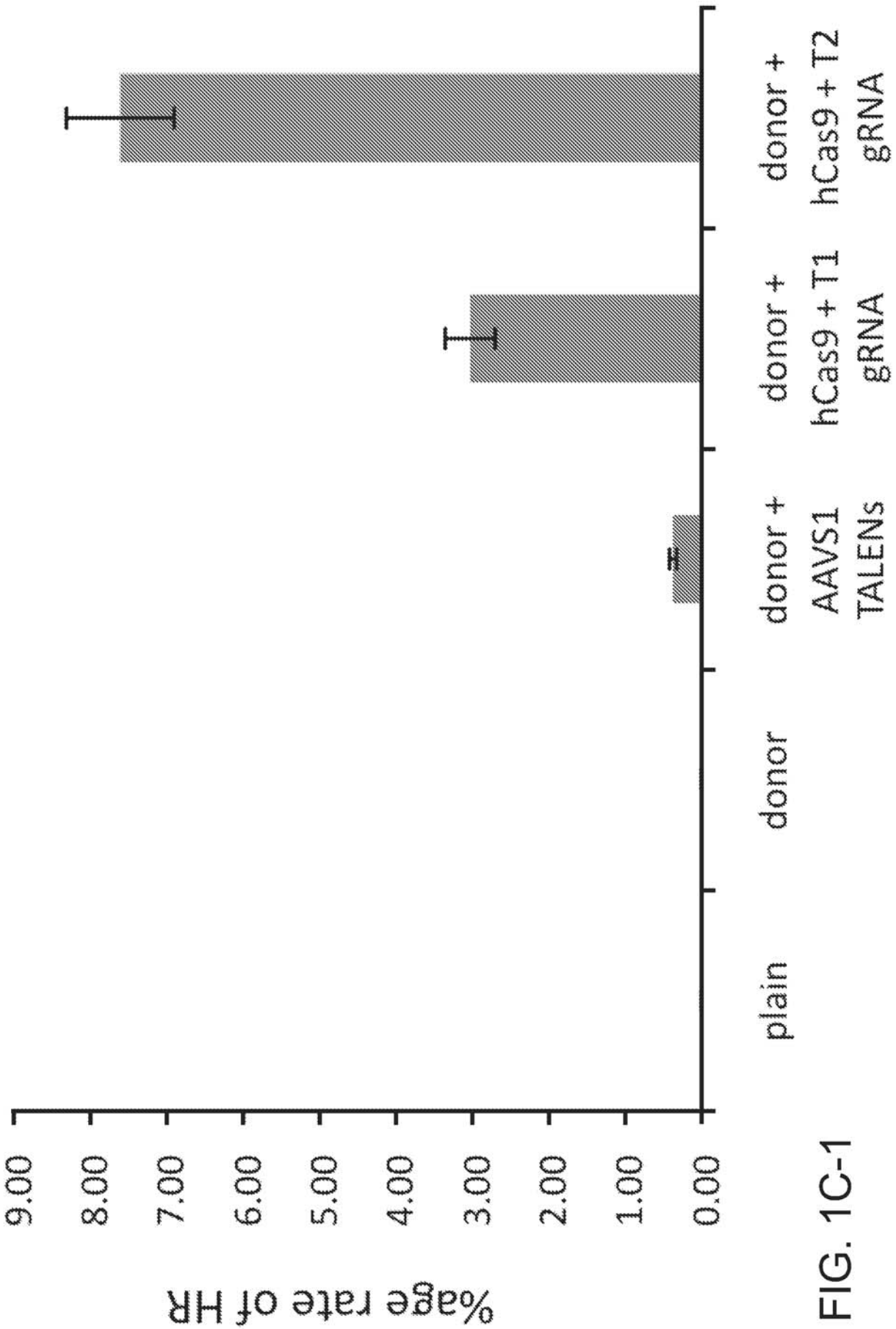

%age rate of HR
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
plain
donor
donor + AAVS1 TALENs
donor + hCas9 + T1 gRNA
donor + hCas9 + T2 gRNA

FIG. 1C-1

FIG. 1C-2

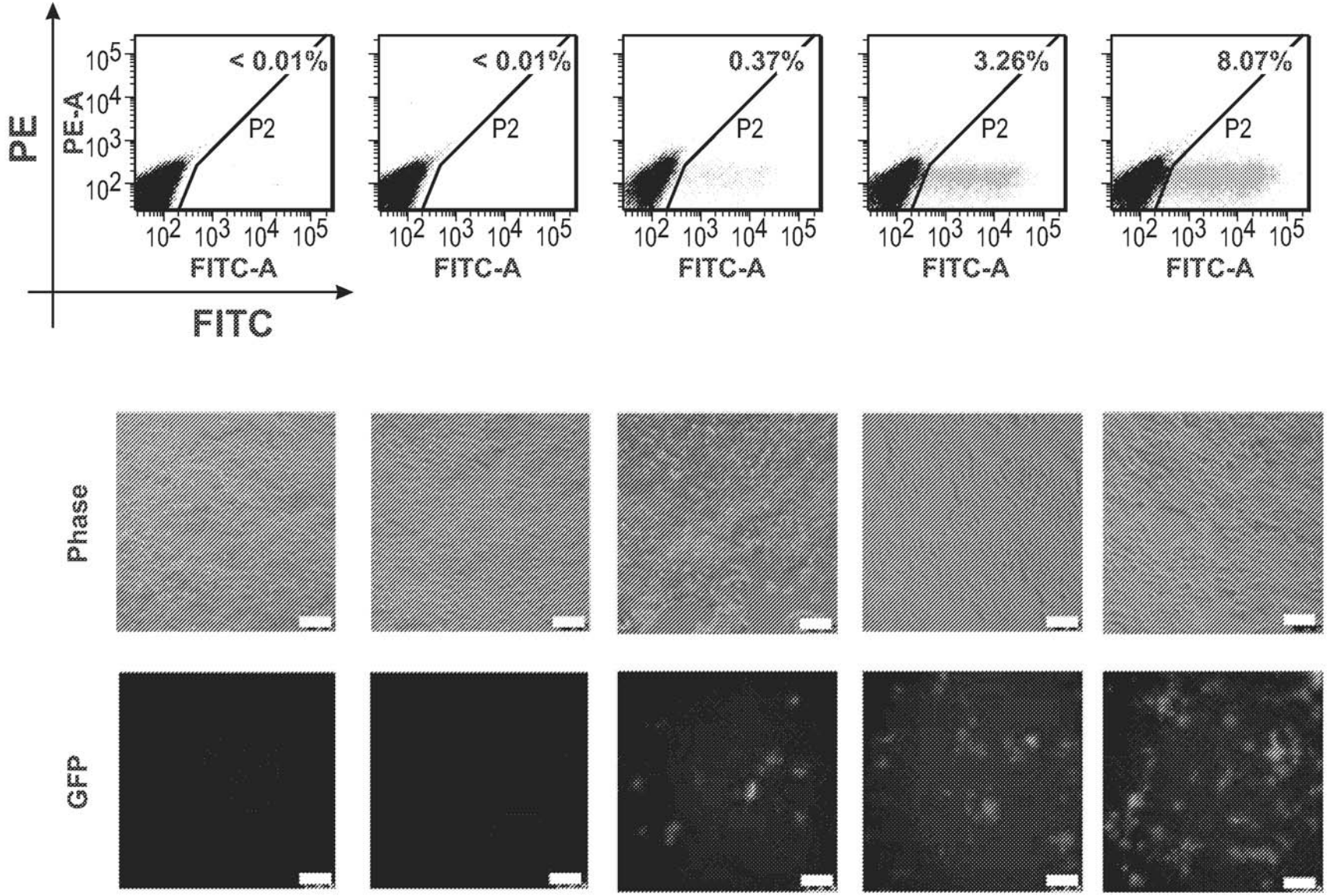


FIG. 2A

endogenous 'native' hAAVS1 locus sequence

..TTATCTGTCCCCTCCACCCCACAGTGGGGCCACTAGGGACAGGATTGGTGA..

T1 target T2 target

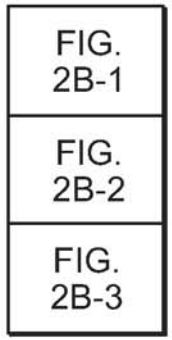


FIG. 2B

FIG. 2B-1

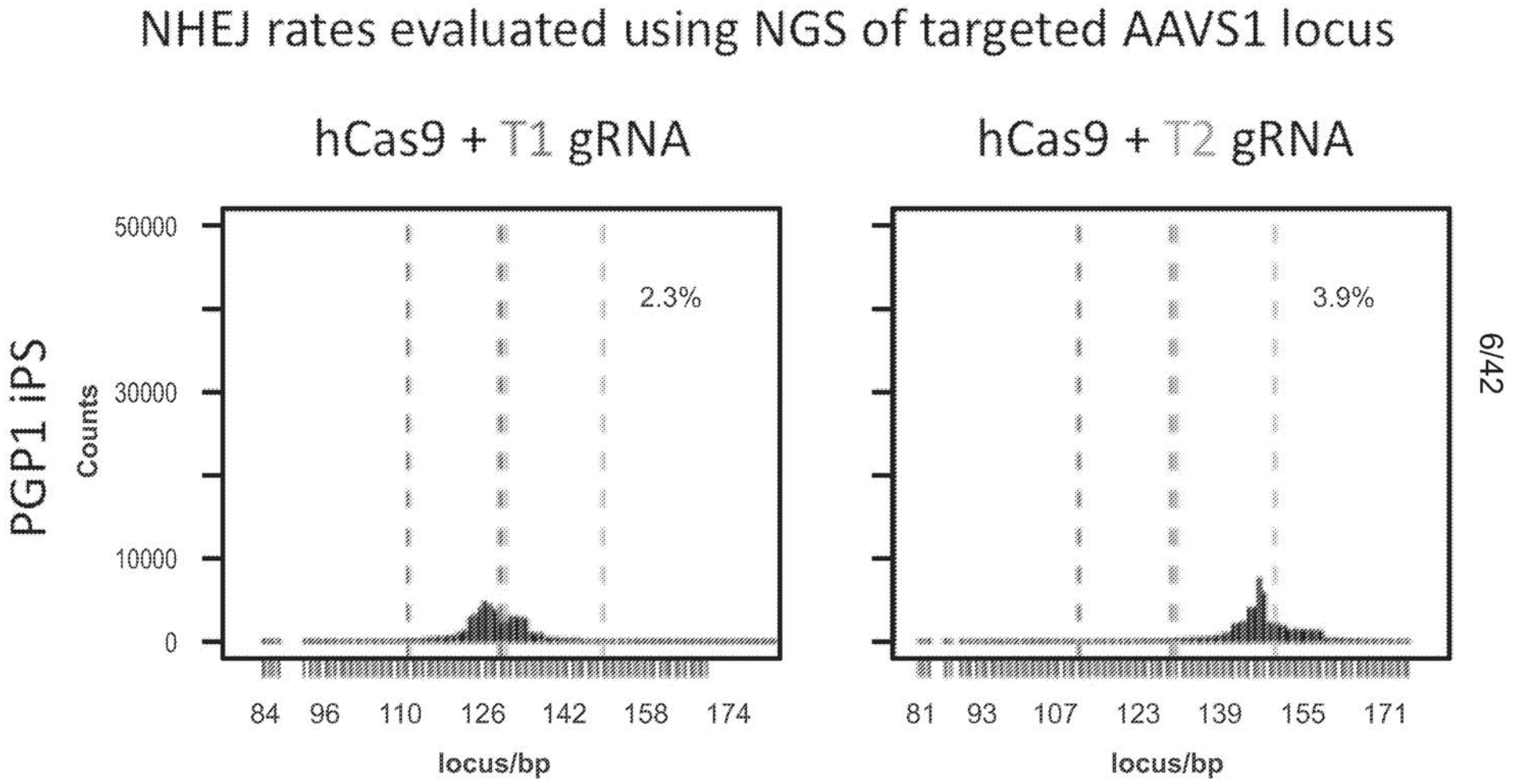

NHEJ rates evaluated using NGS of targeted AAVS1 locus
hCas9 + T1 gRNA
hCas9 + T2 gRNA
PGP1 iPS
Counts
50000
30000
10000
0
2.3%
3.9%
84 96 110 126 142 158 174
81 93 107 123 139 155 171
locus/bp
locus/bp

FIG. 2B-2

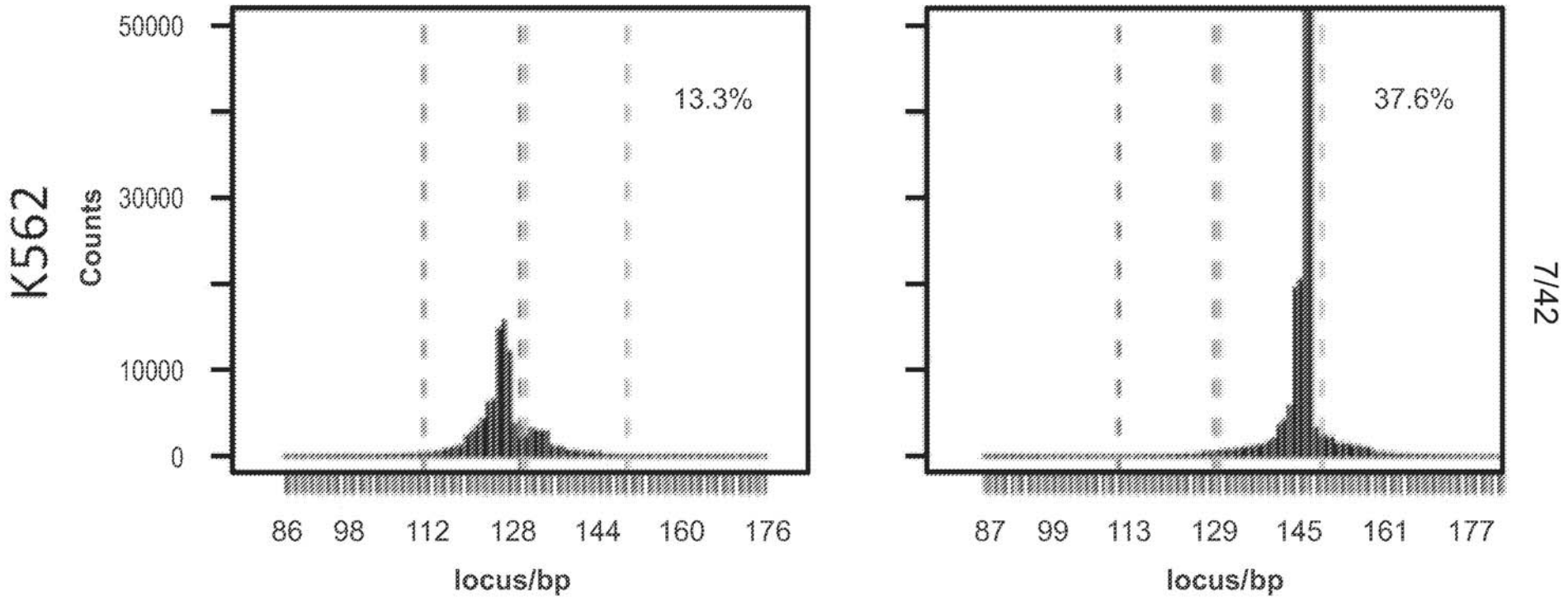

K562
Counts
50000
30000
10000
0
13.3%
86 98 112 128 144 160 176
locus/bp
37.6%
87 99 113 129 145 161 177
locus/bp

FIG. 2B-3

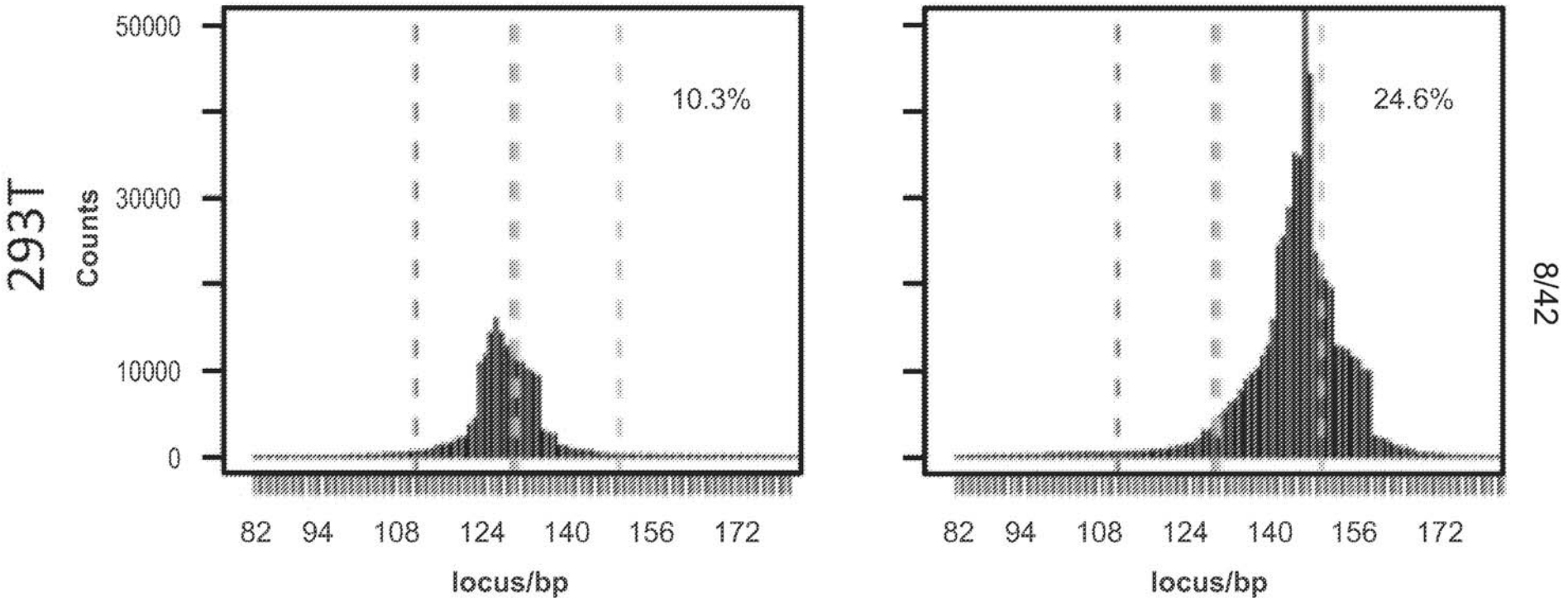

293T
Counts
50000
30000
10000
0
10.3%
24.6%
82
94
108
124
140
156
172
locus/bp

FIG. 2C

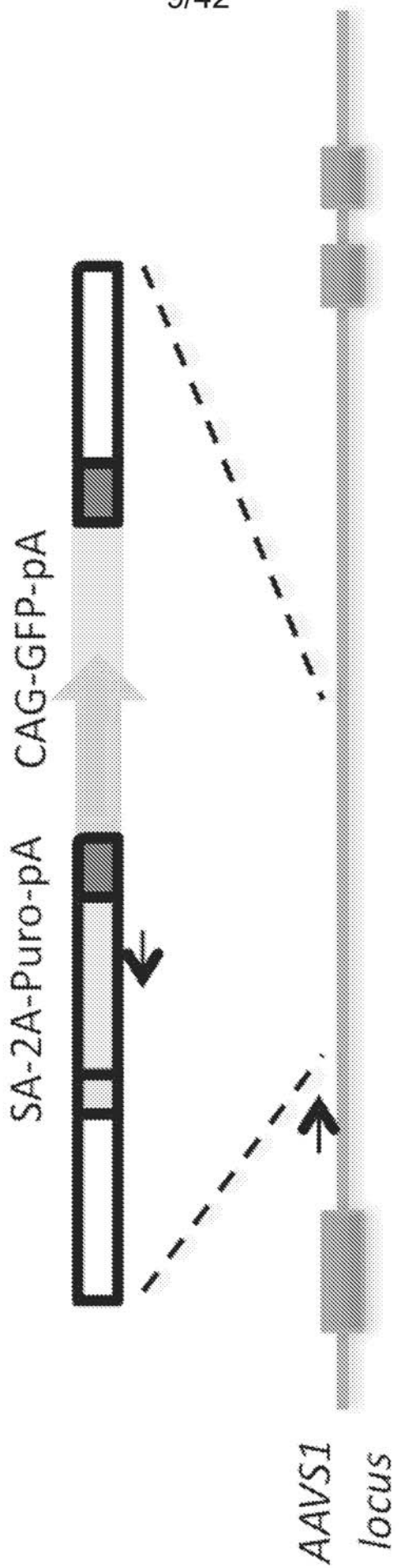


FIG. 2D

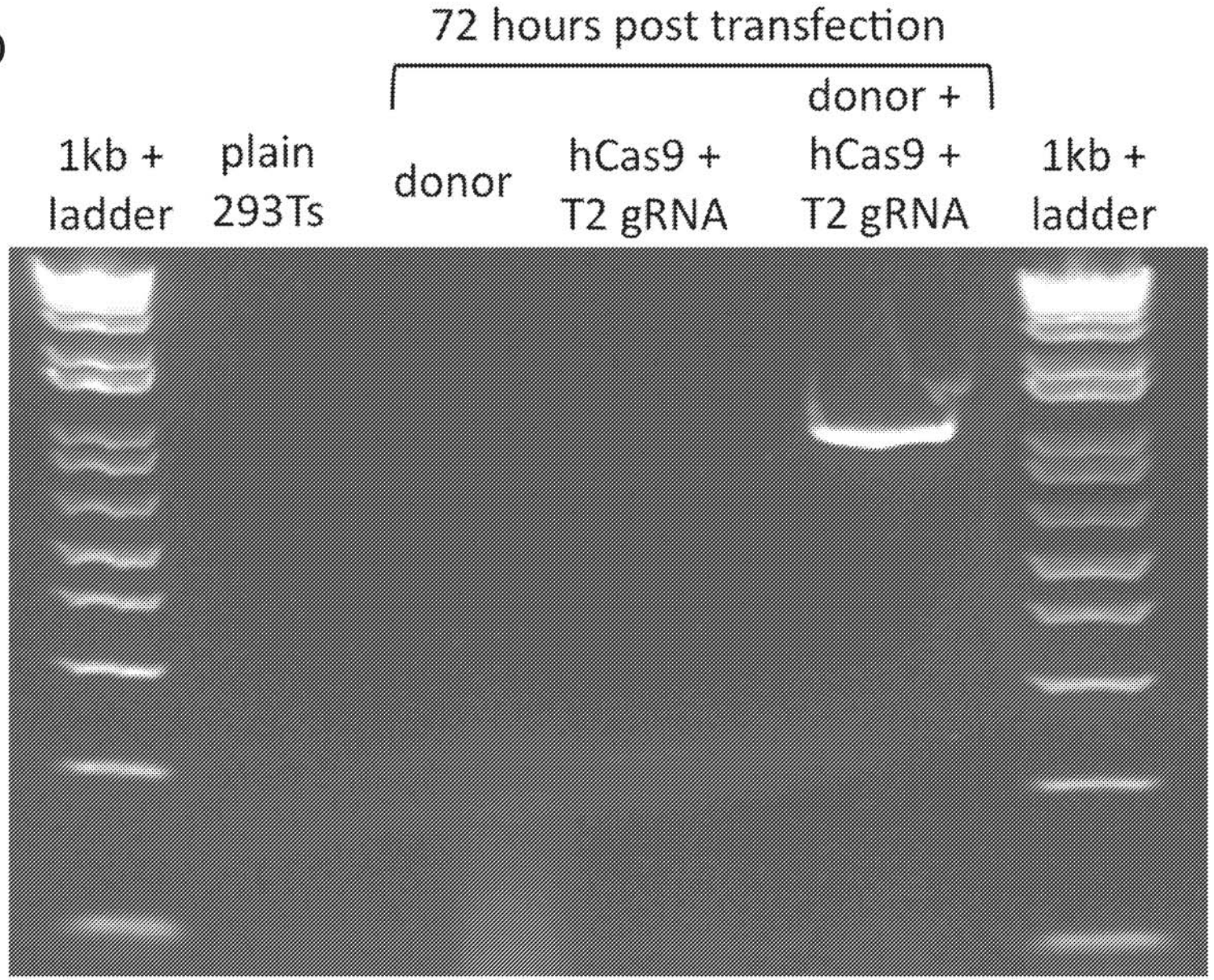

72 hours post transfection
1kb + ladder
plain 293Ts
donor
hCas9 + T2 gRNA
donor + hCas9 + T2 gRNA
1kb + ladder

FIG. 2E

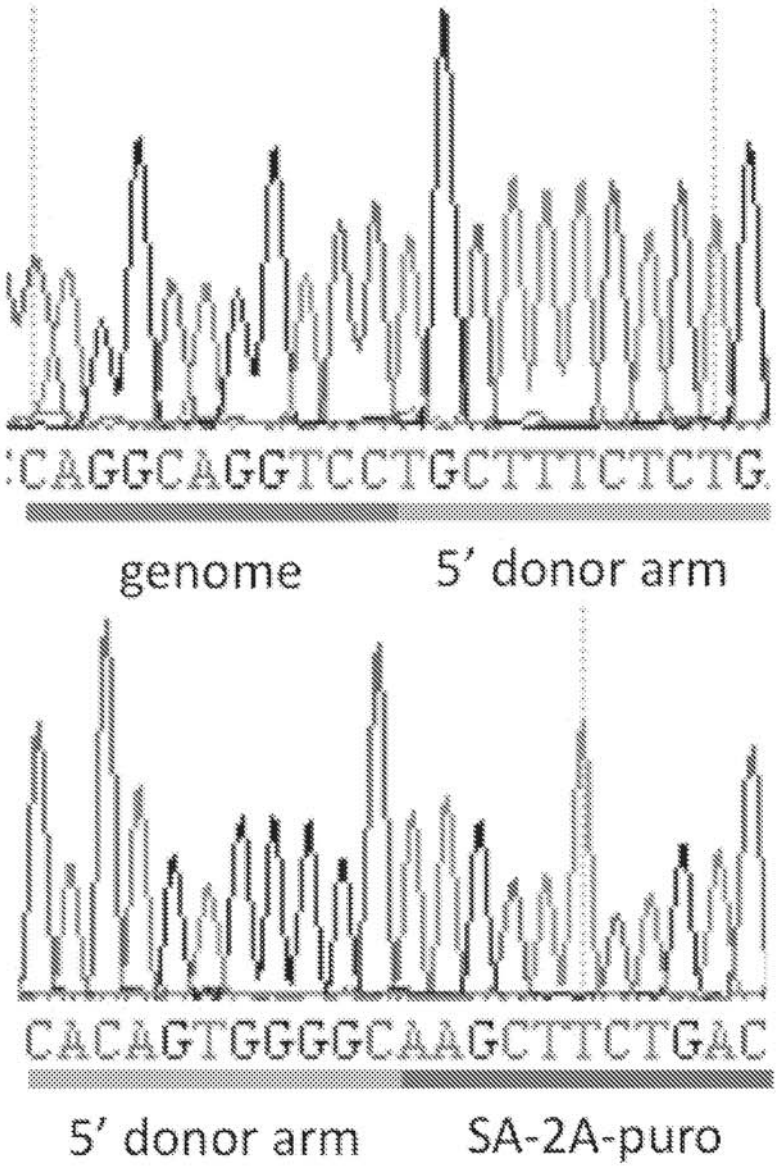

CAGGCAGGTCCTGCTTTCTCTG
genome
5' donor arm
CACAGTGGGGCAAGCTTCTGAC
5' donor arm
SA-2A-puro

FIG. 2F

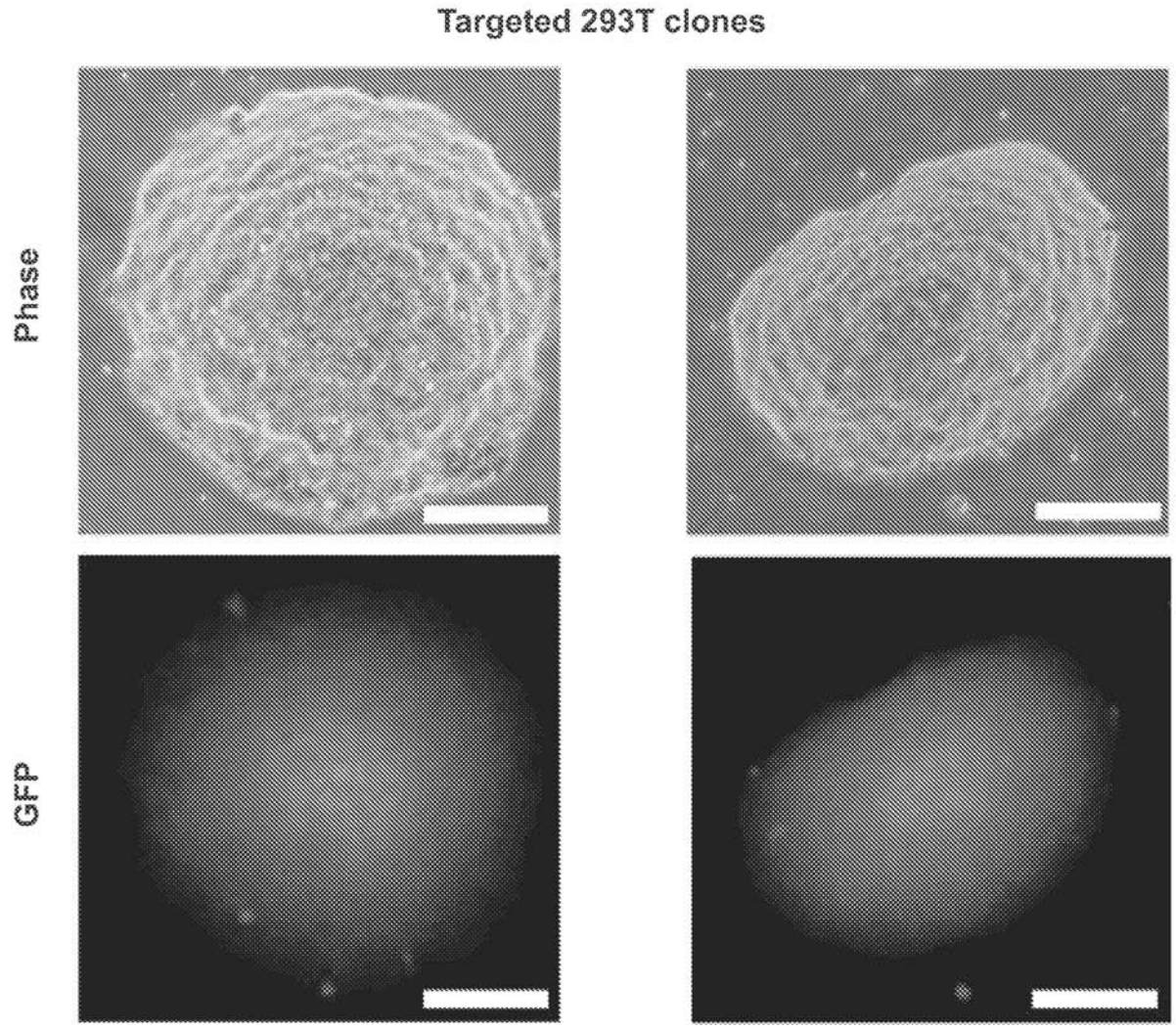
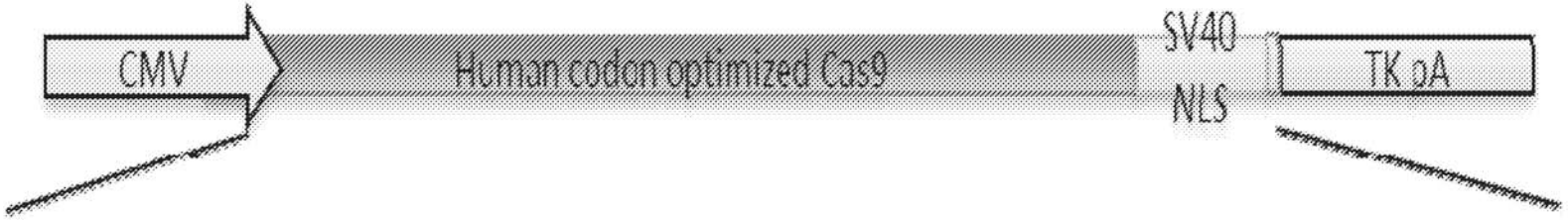


FIG. 3A-1
FIG. 3A-2

FIG. 3A

FIG. 3A-1



gccaccATGGACAAGAAGTACTCCATTGGGCTCGATATCGGCACAAACAGCGTCGGCTGGGCCGTCATTACGGACGAGTACAAGGTGCCGAGCAAAAAATTCAAAGTTCTGGGCAATACCGA
TCGCCACAGCATAAAGAAGAACCTCATTGGCGCCCTCCTGTTCGACTCCGGGGAGACGGCCGAAGCCACGCGGCTCAAAAGAACAGCACGGCGCAGATATACCCGCAGAAAGAATCGGATCT
GCTACCTGCAGGAGATCTTTAGTAATGAGATGGCTAAGGTGGATGACTCTTTCTTCCATAGGCTGGAGGAGTCCTTTTTGGTGGAGGAGGATAAAAAGCACGAGCGCCACCCAATCTTTGGC
AATATCGTGGACGAGGTGGCGTACCATGAAAAGTACCCAACCATATATCATCTGAGGAAGAAGCTTGTAGACAGTACTGATAAGGCTGACTTGCGGTTGATCTATCTCGCGCTGGCGCATAT
GATCAAATTTCGGGGACACTTCCTCATCGAGGGGGACCTGAACCCAGACAACAGCGATGTCGACAAACTCTTTATCCAACTGGTTCAGACTTACAATCAGCTTTTCGAAGAGAACCCGATCA
ACGCATCCGGAGTTGACGCCAAAGCAATCCTGAGCGCTAGGCTGTCCAAATCCCGGCGGCTCGAAAACCTCATCGCACAGCTCCCTGGGGAGAAGAAGAACGGCCTGTTTGGTAATCTTATC
GCCCTGTCACTCGGGCTGACCCCCAACTTTAAATCTAACTTCGACCTGGCCGAAGATGCCAAGCTTCAACTGAGCAAAGACACCTACGATGATGATCTCGACAATCTGCTGGCCCAGATCGG
CGACCAGTACGCAGACCTTTTTTTGGCGGCAAAGAACCTGTCAGACGCCATTCTGCTGAGTGATATTCTGCGAGTGAACACGGAGATCACCAAAGCTCCGCTGAGCGCTAGTATGATCAAGC
GCTATGATGAGCACCACCAAGACTTGACTTTGCTGAAGGCCCTTGTCAGACAGCAACTGCCTGAGAAGTACAAGGAAATTTTCTTCGATCAGTCTAAAAATGGCTACGCCGGATACATTGAC
GGCGGAGCAAGCCAGGAGGAATTTTACAAATTTATTAAGCCCATCTTGGAAAAAATGGACGGCACCGAGGAGCTGCTGGTAAAGCTTAACAGAGAAGATCTGTTGCGCAAACAGCGCACTTT
CGACAATGGAAGCATCCCCCACCAGATTCACCTGGGCGAACTGCACGCTATCCTCAGGCGGCAAGAGGATTTCTACCCCTTTTTGAAAGATAACAGGGAAAAGATTGAGAAAATCCTCACAT
TTCGGATACCCTACTATGTAGGCCCCCTCGCCCGGGGAAATTCCAGATTCGCGTGGATGACTCGCAAATCAGAAGAGACCATCACTCCCTGGAACTTCGAGGAAGTCGTGGATAAGGGGGCC
TCTGCCCAGTCCTTCATCGAAAGGATGACTAACTTTGATAAAAATCTGCCTAACGAAAAGGTGCTTCCTAAACACTCTCTGCTGTACGAGTACTTCACAGTTTATAACGAGCTCACCAAGGT
CAAATACGTCACAGAAGGGATGAGAAAGCCAGCATTCCTGTCTGGAGAGCAGAAGAAAGCTATCGTGGACCTCCTCTTCAAGACGAACCGGAAAGTTACCGTGAAACAGCTCAAAGAAGACT

FIG. 3A-2

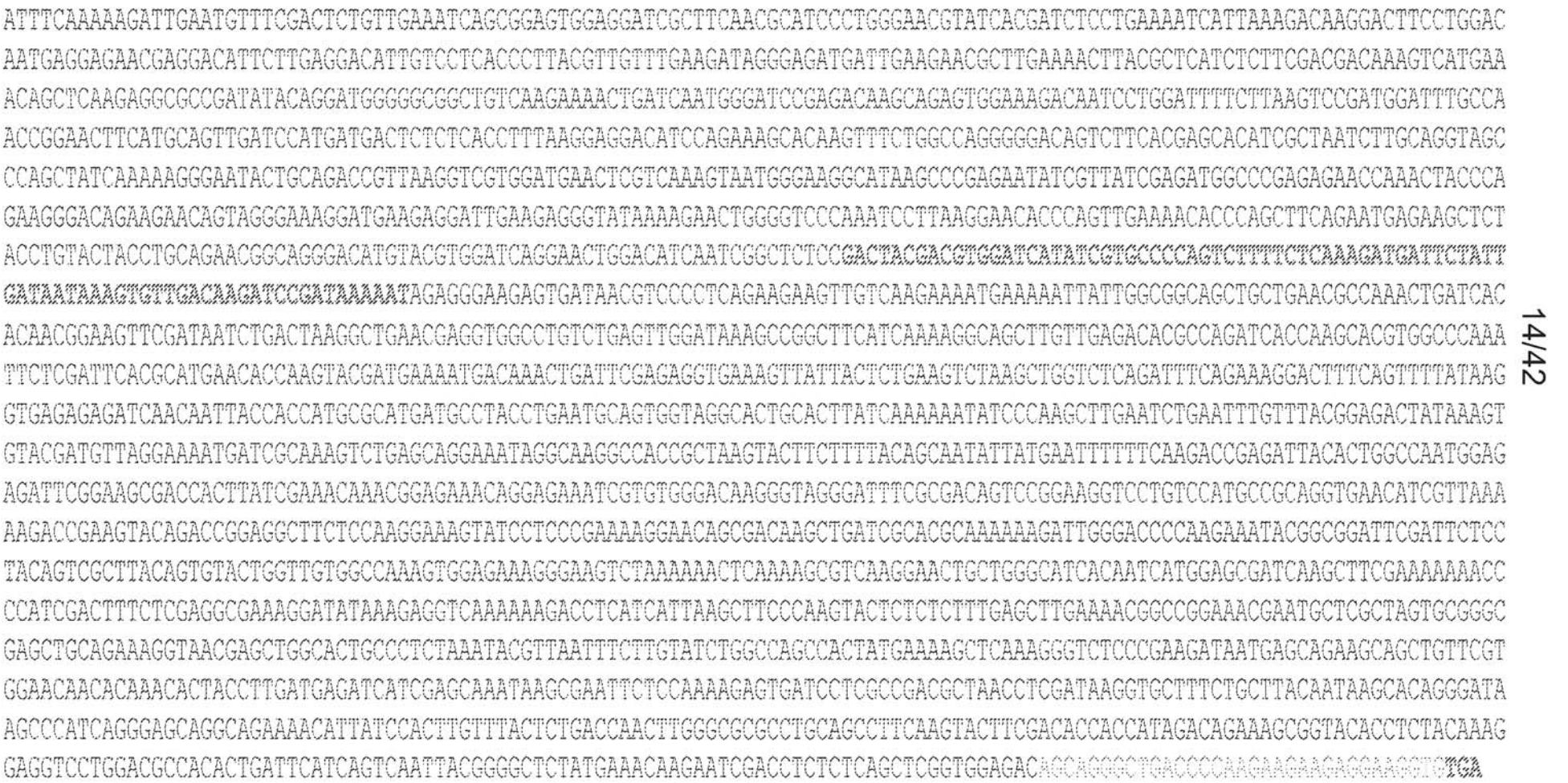

ATTTCAAAAAGATTGAATGTTTCGACTCTGTTGAAATCAGCGGAGTGGAGGATCGCTTCAACGCATCCCTGGGAACGTATCACGATCTCCTGAAAATCATTAAAGACAAGGACTTCCTGGAC
AATGAGGAGAACGAGGACATTCTTGAGGACATTGTCCTCACCCTTACGTTGTTTGAAGATAGGGAGATGATTGAAGAACGCTTGAAAACTTACGCTCATCTCTTCGACGACAAAGTCATGAA
ACAGCTCAAGAGGCGCCGATATACAGGATGGGGGCGGCTGTCAAGAAAACTGATCAATGGGATCCGAGACAAGCAGAGTGGAAAGACAATCCTGGATTTTCTTAAGTCCGATGGATTTGCCA
ACCGGAACTTCATGCAGTTGATCCATGATGACTCTCTCACCTTTAAGGAGGACATCCAGAAAGCACAAGTTTCTGGCCAGGGGGACAGTCTTCACGAGCACATCGCTAATCTTGCAGGTAGC
CCAGCTATCAAAAAGGGAATACTGCAGACCGTTAAGGTCGTGGATGAACTCGTCAAAGTAATGGGAAGGCATAAGCCCGAGAATATCGTTATCGAGATGGCCCGAGAGAACCAAACTACCCA
GAAGGGACAGAAGAACAGTAGGGAAAGGATGAAGAGGATTGAAGAGGGTATAAAAGAACTGGGGTCCCAAATCCTTAAGGAACACCCAGTTGAAAACACCCAGCTTCAGAATGAGAAGCTCT
ACCTGTACTACCTGCAGAACGGCAGGGACATGTACGTGGATCAGGAACTGGACATCAATCGGCTCTCCGACTACGACGTGGATCATATCGTGCCCCAGTCTTTTCTCAAAGATGATTCTATT
GATAATAAAGTGTTGACAAGATCCCGATAAAAATAGAGGGAAGAGTGATAACGTCCCCTCAGAAGAAGTTGTCAAGAAAATGAAAAATTATTGGCGGCAGCTGCTGAACGCCAAACTGATCAC
ACAACGGAAGTTCGATAATCTGACTAAGGCTGAACGAGGTGGCCTGTCTGAGTTGGATAAAGCCGGCTTCATCAAAAGGCAGCTTGTTGAGACACGCCAGATCACCAAGCACGTGGCCCAAA
TTCTCGATTCACGCATGAACACCAAGTACGATGAAAATGACAAACTGATTCGAGAGGTGAAAGTTATTACTCTGAAGTCTAAGCTGGTCTCAGATTTCAGAAAGGACTTTCAGTTTTATAAG
GTGAGAGAGATCAACAATTACCACCATGCGCATGATGCCTACCTGAATGCAGTGGTAGGCACTGCACTTATCAAAAAATATCCCAAGCTTGAATCTGAATTTGTTTACGGAGACTATAAAGT
GTACGATGTTAGGAAAATGATCGCAAAGTCTGAGCAGGAAATAGGCAAGGCCACCGCTAAGTACTTCTTTTACAGCAATATTATGAATTTTTTCAAGACCGAGATTACACTGGCCAATGGAG
AGATTCGGAAGCGACCACTTATCGAAACAAACGGAGAAACAGGAGAAATCGTGTGGGACAAGGGTAGGGATTTCGCGACAGTCCGGAAGGTCCTGTCCATGCCGCAGGTGAACATCGTTAAA
AAGACCGAAGTACAGACCGGAGGCTTCTCCAAGGAAAGTATCCTCCCGAAAAGGAACAGCGACAAGCTGATCGCACGCAAAAAAGATTGGGACCCCAAGAAATACGGCGGATTCGATTCTCC
TACAGTCGCTTACAGTGTACTGGTTGTGGCCAAAGTGGAGAAAGGGAAGTCTAAAAAACTCAAAAGCGTCAAGGAACTGCTGGGCATCACAATCATGGAGCGATCAAGCTTCGAAAAAAACC
CCATCGACTTTCTCGAGGCGAAAGGATATAAAGAGGTCAAAAAAGACCTCATCATTAAGCTTCCCAAGTACTCTCTCTTTGAGCTTGAAAACGGCCGGAAACGAATGCTCGCTAGTGCGGGC
GAGCTGCAGAAAGGTAACGAGCTGGCACTGCCCTCTAAATACGTTAATTTCTTGTATCTGGCCAGCCACTATGAAAAGCTCAAAGGGTCTCCCGAAGATAATGAGCAGAAGCAGCTGTTCGT
GGAACAACACAAACACTACCTTGATGAGATCATCGAGCAAATAAGCGAATTCTCCAAAAGAGTGATCCTCGCCGACGCTAACCTCGATAAGGTGCTTTCTGCTTACAATAAGCACAGGGATA
AGCCCATCAGGGAGCAGGCAGAAAACATTATCCACTTGTTTACTCTGACCAACTTGGGCGCGCCTGCAGCCTTCAAGTACTTCGACACCACCATAGACAGAAAGCGGTACACCTCTACAAAG
GAGGTCCTGGACGCCACACTGATTCATCAGTCAATTACGGGGCTCTATGAAACAAGAATCGACCTCTCTCAGCTCGGTGGAGAC

FIG. 3B



U6 promoter + target RNA + guide RNA scaffold:

TGTACAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACTGGATCCGGTACCAAGGTCGGGCAGGAAGAGGGCCTATTTCCCATGATTCCTTCATATTTG
CATATACGATACAAGGCTGTTAGAGAGATAATTAGAATTAATTTGACTGTAAACACAAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTT
GGGTAGTTTGCAGTTTTAAAATTATGTTTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAA
GGACGAAACACCGNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGT
CGGTGCTTTTTTTCTAGACCCAGCTTTCTTGTACAAAGTTGGCATTA

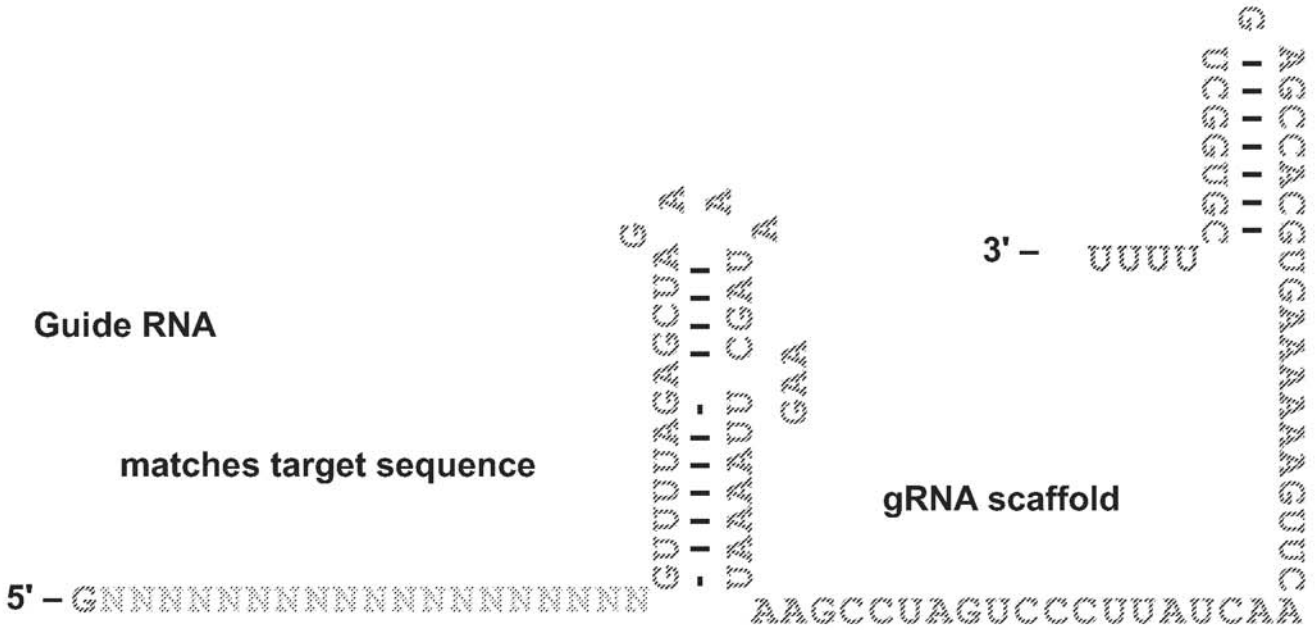


FIG. 3C

PAM

GNNNNNNNNNNNNNNNNNNNNGG 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

FIG. 4

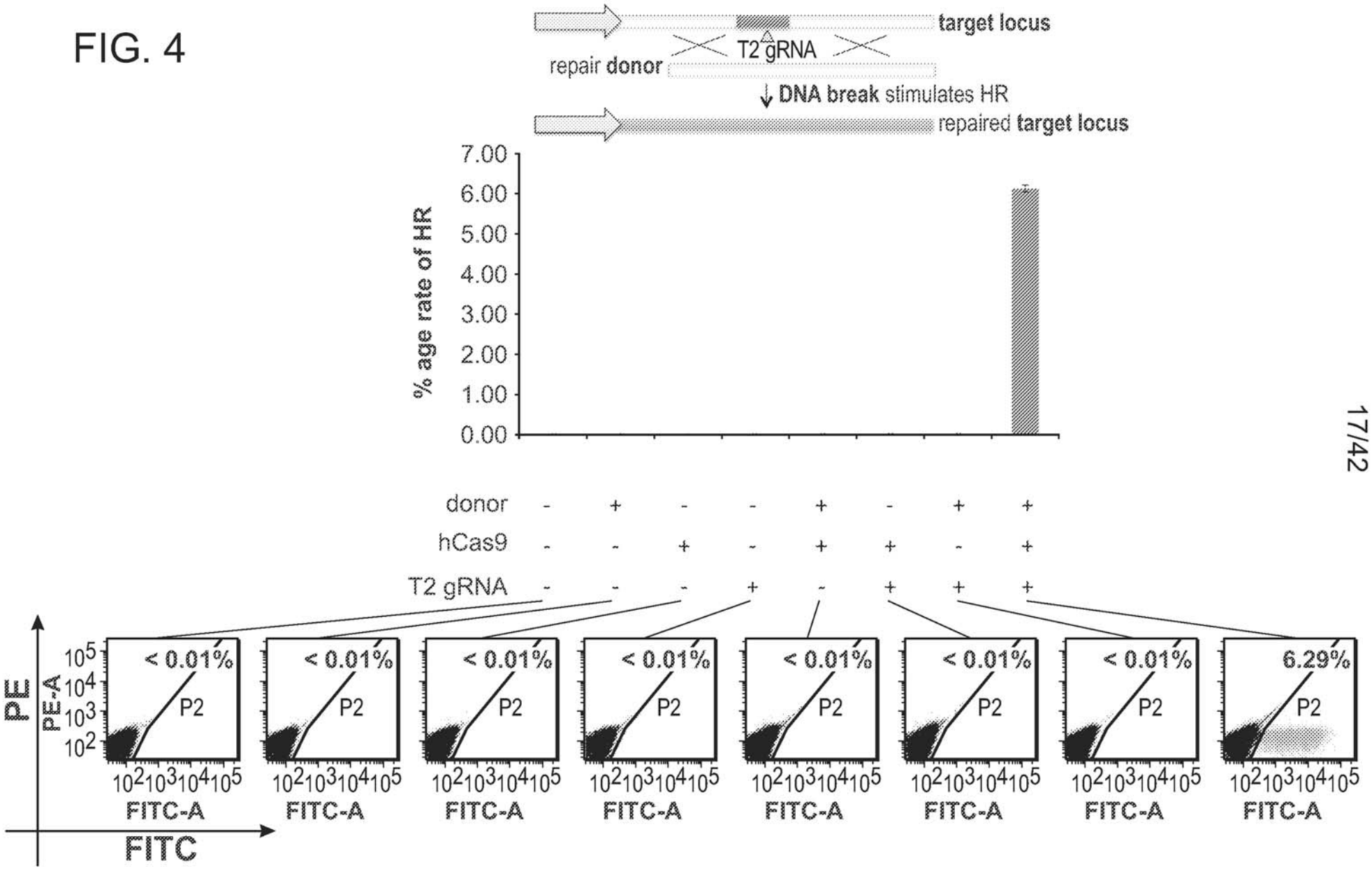

target locus
T2 gRNA
repair donor
DNA break stimulates HR
repaired target locus
% age rate of HR
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
donor - + - - + - + +
hCas9 - - + - + + - +
T2 gRNA - - - + - + + +
PE
PE-A
FITC
FITC-A
< 0.01%
6.29%
P2

FIG. 5A

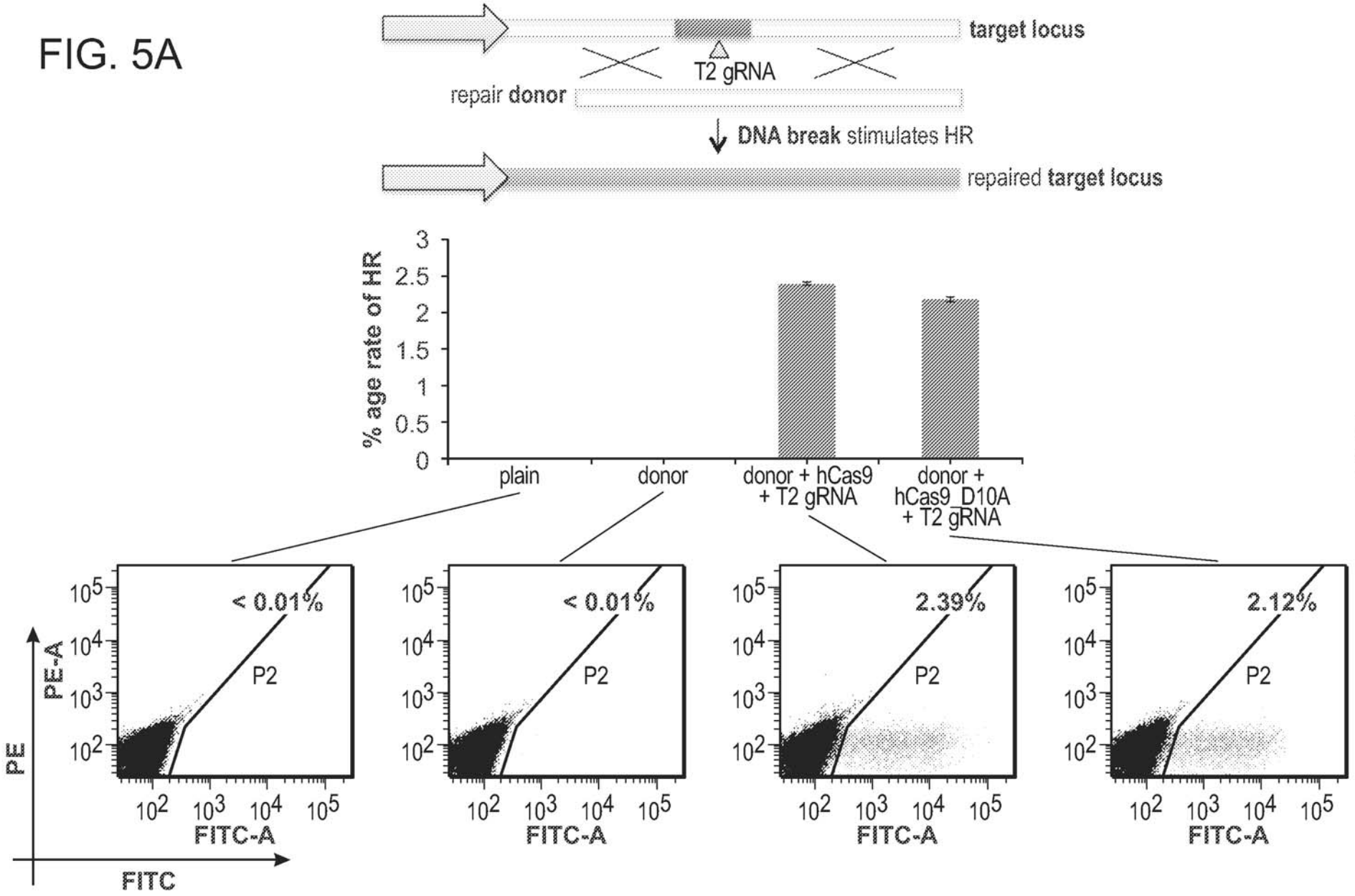

target locus
T2 gRNA
repair donor
DNA break stimulates HR
repaired target locus
% age rate of HR
3
2.5
2
1.5
1
0.5
0
plain
donor
donor + hCas9 + T2 gRNA
donor + hCas9_D10A + T2 gRNA
PE
PE-A
FITC
FITC-A
< 0.01%
< 0.01%
2.39%
2.12%
P2
P2
P2
P2

FIG. 5B

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

..TTATCTGTCCCCTCCACCCCACAGTGGGGCCACTAGGGACAGGATTGGTGA..
T2 target

NHEJ rates evaluated using NGS of targeted AAVS1 locus

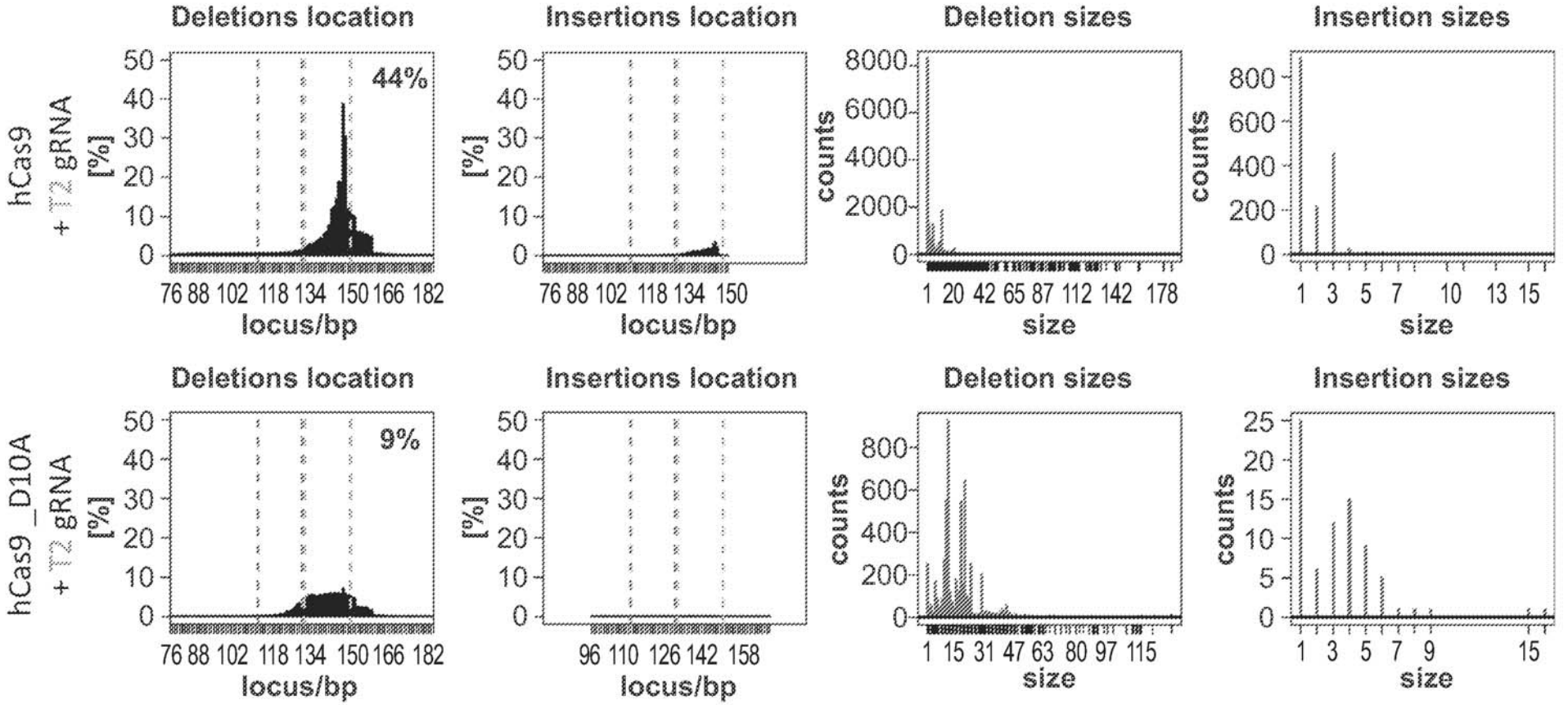


FIG. 6A

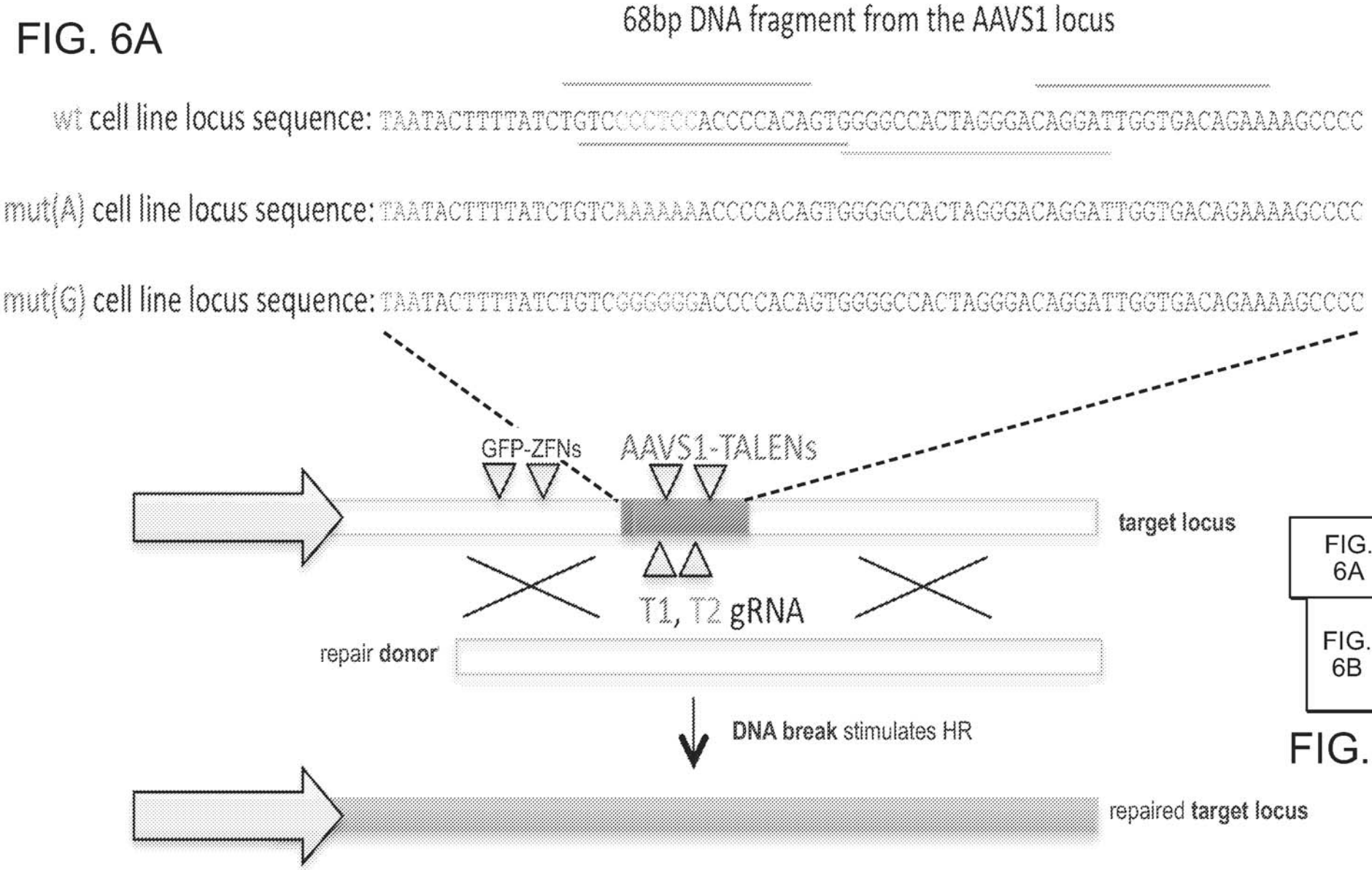


FIG. 6A
FIG. 6B

FIG. 6

FIG. 6B

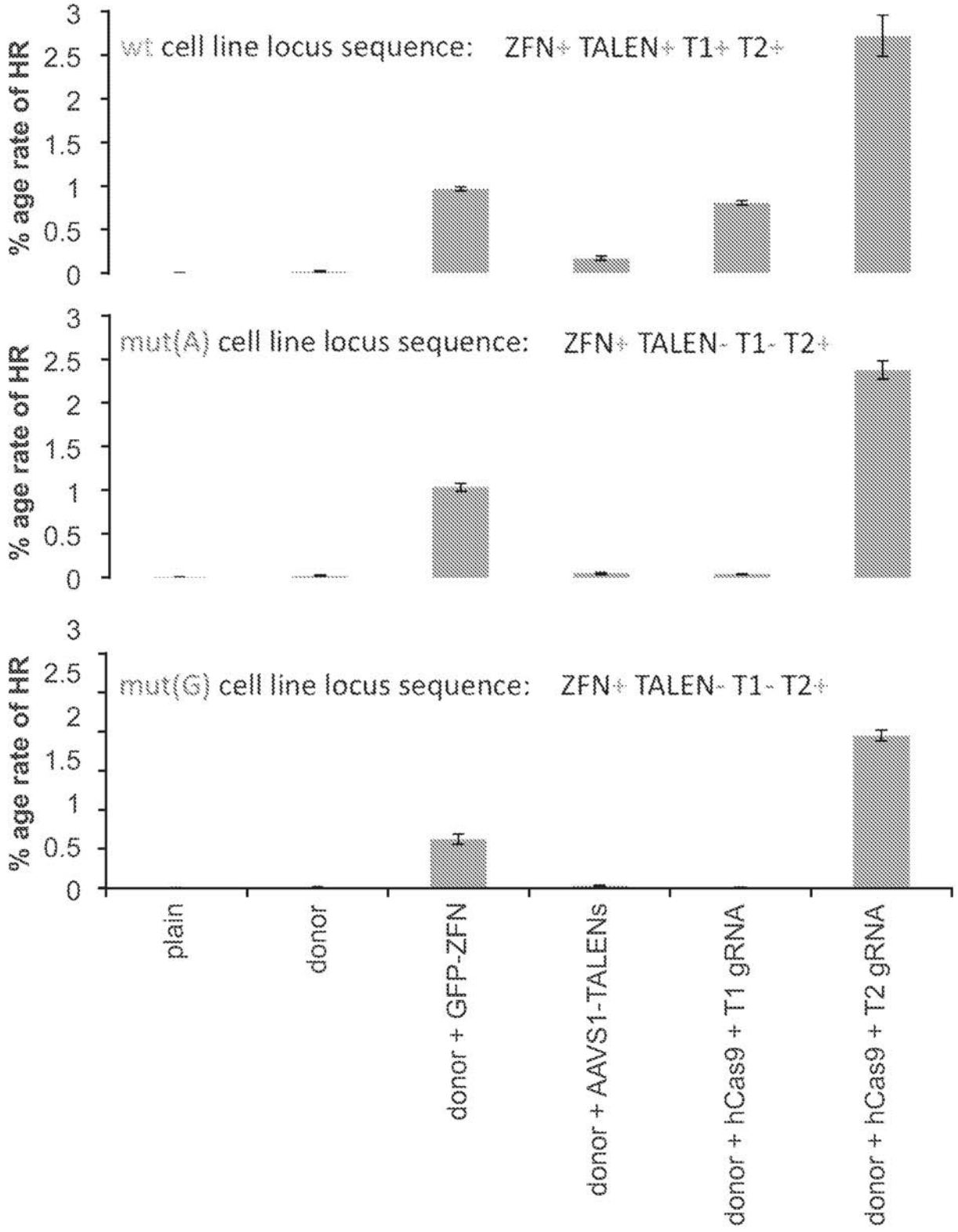

wt cell line locus sequence: ZFN+ TALEN+ T1+ T2+
mut(A) cell line locus sequence: ZFN+ TALEN- T1- T2+
mut(G) cell line locus sequence: ZFN+ TALEN- T1- T2+
% age rate of HR
0
0.5
1
1.5
2
2.5
3
plain
donor
donor + GFP-ZFN
donor + AAVS1-TALENs
donor + hCas9 + T1 gRNA
donor + hCas9 + T2 gRNA

FIG. 7

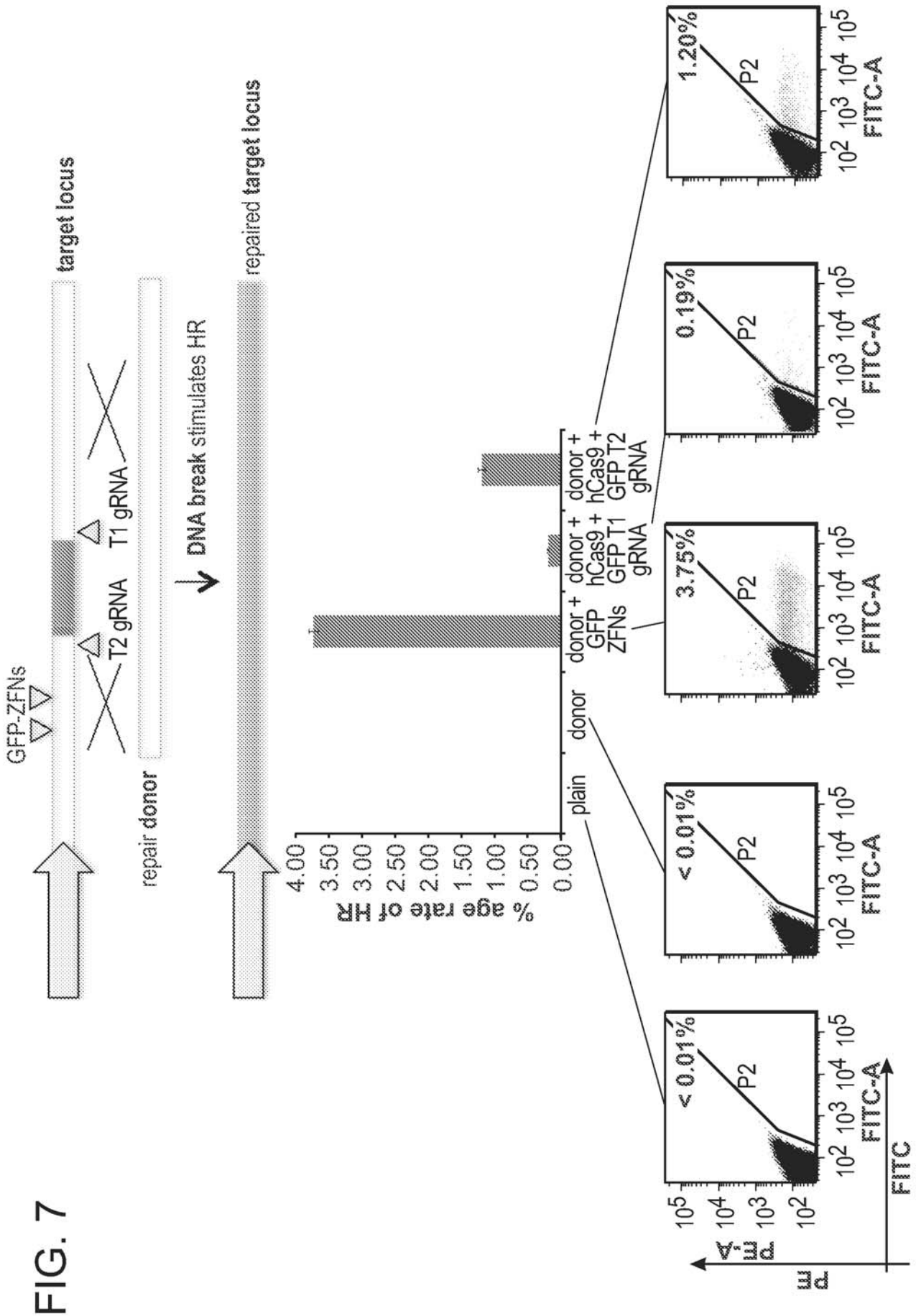

GFP-ZFNs
target locus
T2 gRNA
T1 gRNA
repair donor
DNA break stimulates HR
repaired target locus
% age rate of HR
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
plain
donor
donor + GFP ZFNs
donor + hCas9 + GFP T1 gRNA
donor + hCas9 + GFP T2 gRNA
< 0.01%
< 0.01%
3.75%
0.19%
1.20%
P2
FITC-A
PE-A
FITC

FIG. 8A

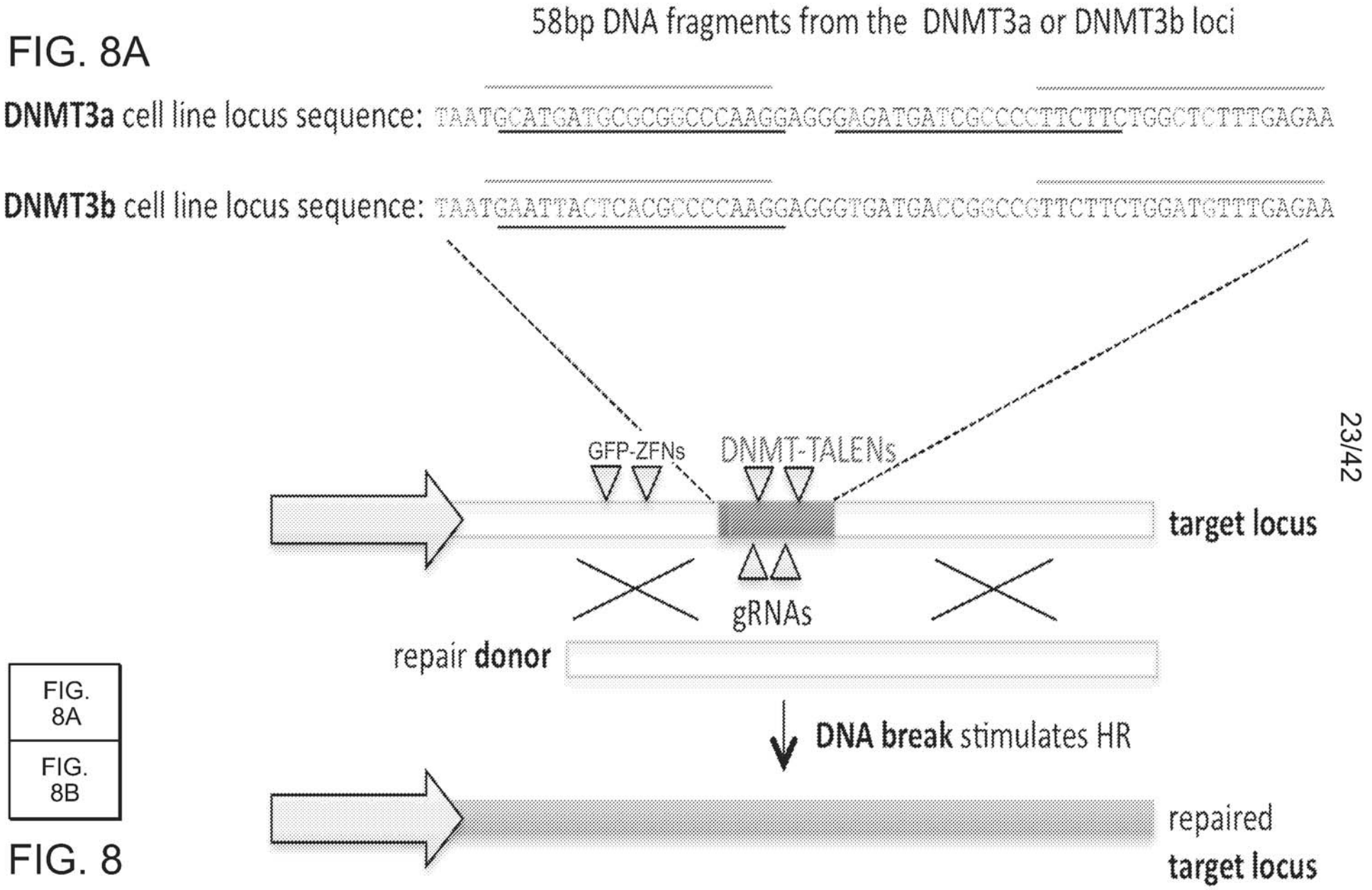


FIG. 8A
FIG. 8B

FIG. 8

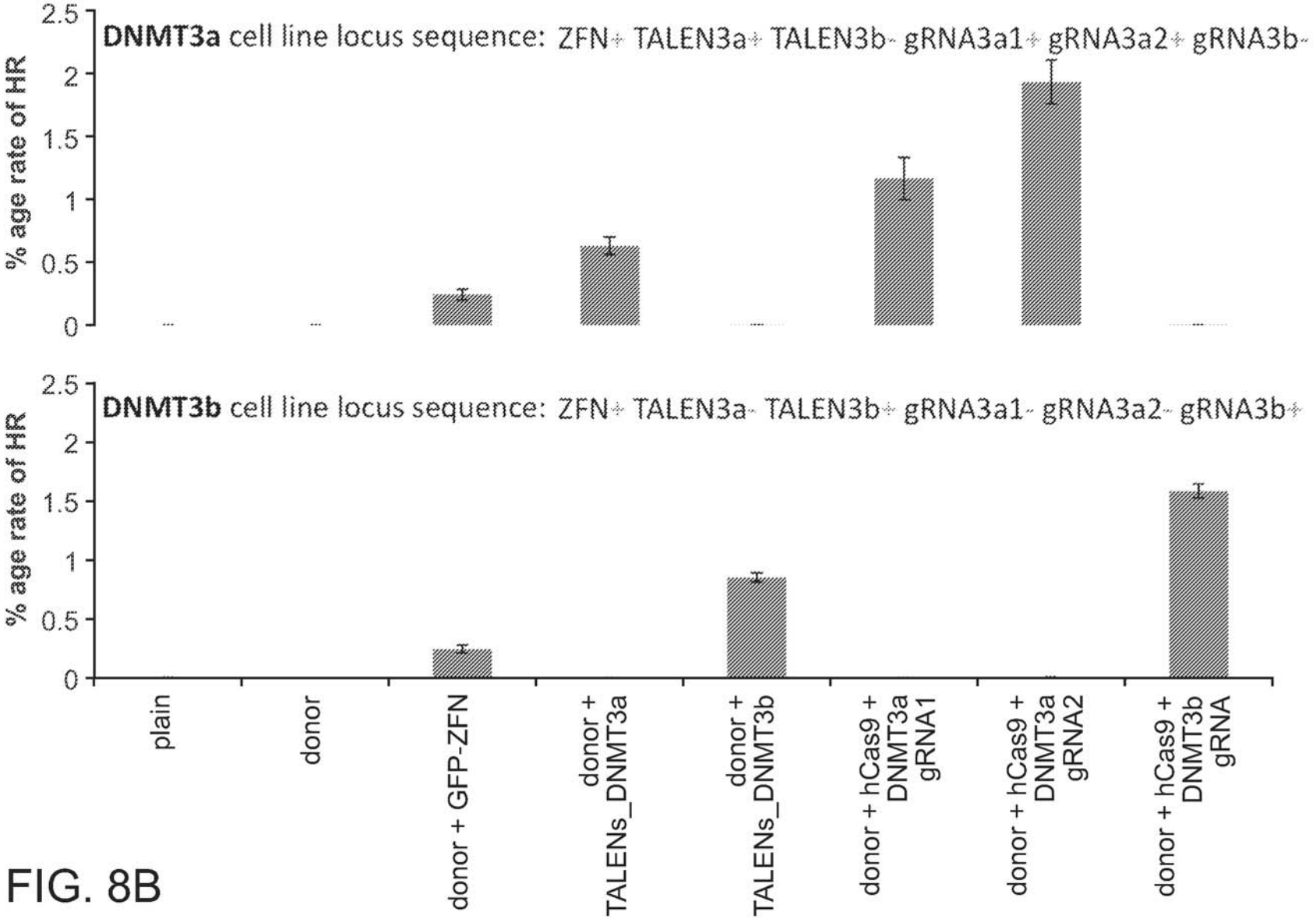


FIG. 8B

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

FIG. 9

FIG. 9A

endogenous 'native' hAAVS1 locus sequence
(PGP1 iPS)

..TTATCTGTCCCCTCCACCCCACAGTGGGGCCACTAGGGACAGGATTGGTGA..

T1 target T2 target

NHEJ rates evaluated using NGS of targeted AAVS1 locus

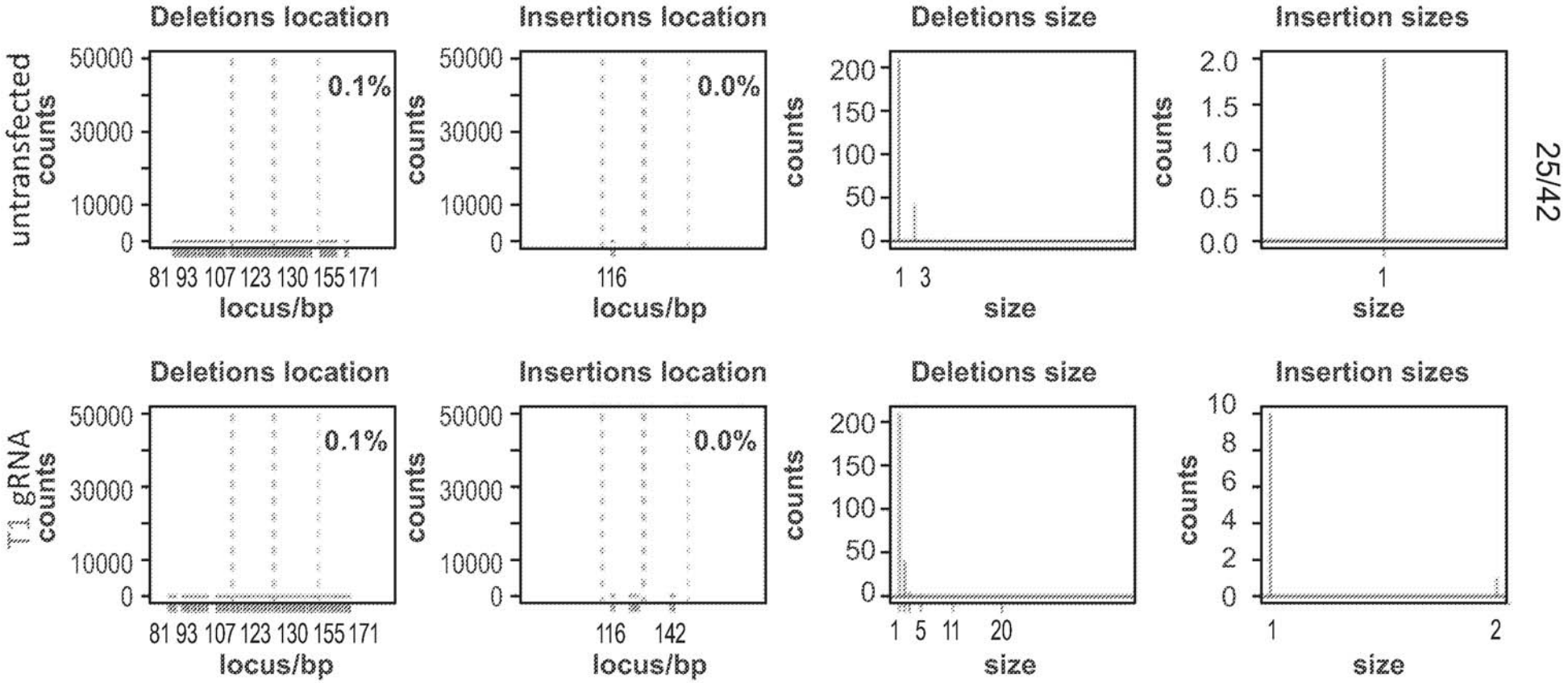


FIG. 9B

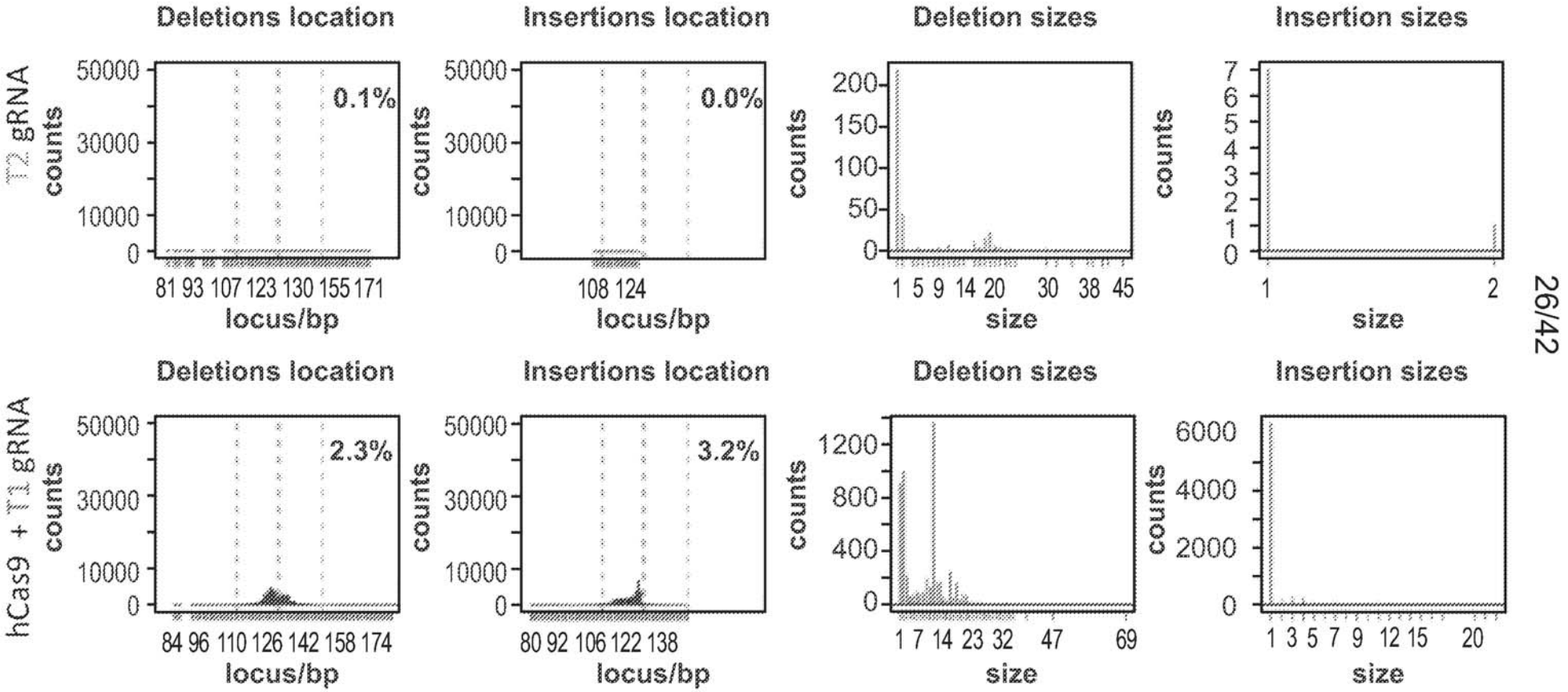

Deletions location
Insertions location
Deletion sizes
Insertion sizes
T2 gRNA
counts
0.1%
0.0%
locus/bp
size
hCas9 + T1 gRNA
2.3%
3.2%

FIG. 9C

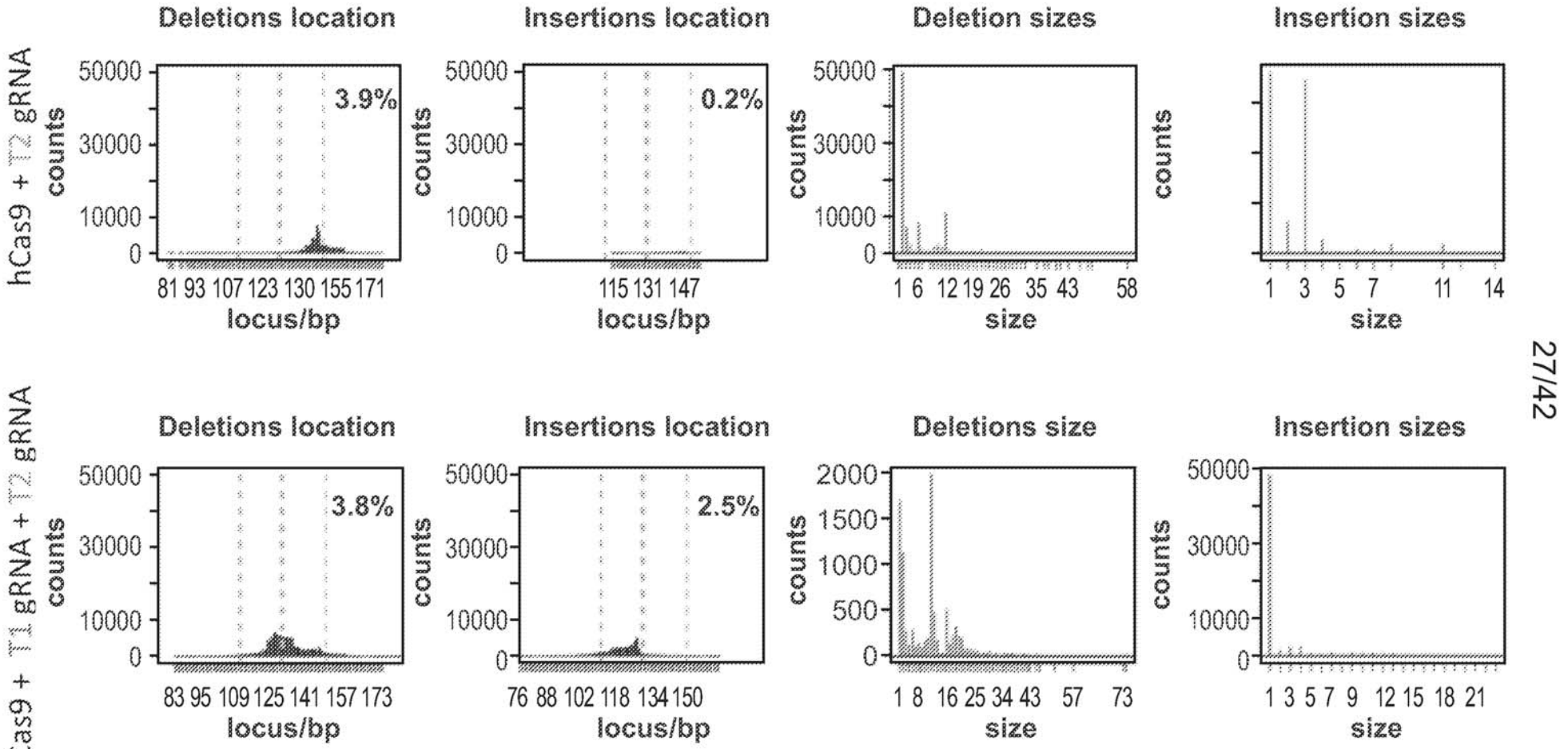

hCas9 + T2 gRNA
Deletions location
Insertions location
Deletion sizes
Insertion sizes
counts
3.9%
0.2%
81 93 107 123 130 155 171
115 131 147
1 6 12 19 26 35 43 58
1 3 5 7 11 14
locus/bp
size
hCas9 + T1 gRNA + T2 gRNA
Deletions location
Insertions location
Deletions size
Insertion sizes
3.8%
2.5%
83 95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

FIG. 10A

endogenous 'native' hAAVS1 locus sequence
(K562)

..TTATCTGTCCCCTCCACCCCACAGTGGGGCCACTAGGGACAGGATTGGTGA..
T1 target T2 target

NHEJ rates evaluated using NGS of targeted AAVS1 locus

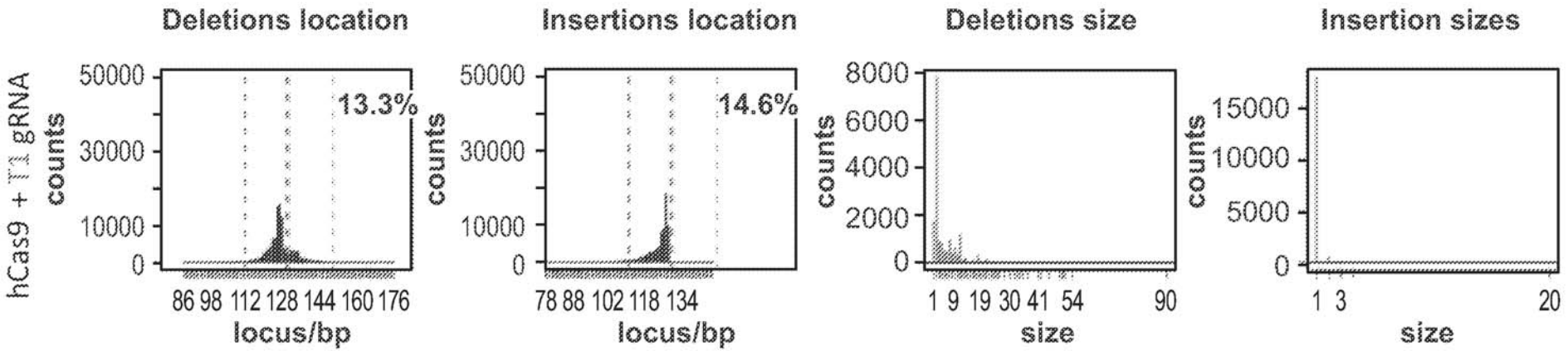


FIG. 10

FIG. 10A
FIG. 10B

FIG. 10B

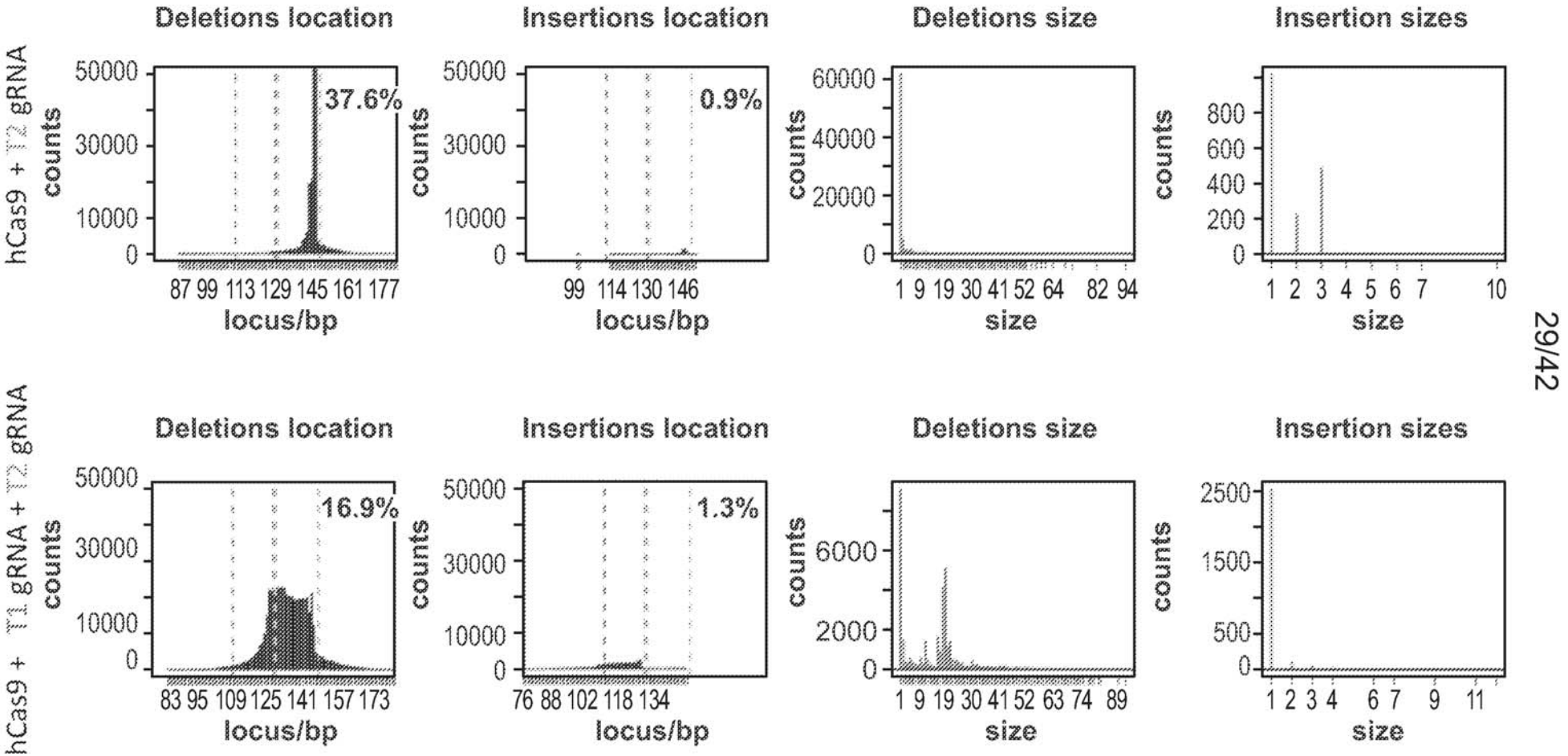

hCas9 + T2 gRNA
Deletions location
Insertions location
Deletions size
Insertion sizes
counts
locus/bp
size
37.6%
0.9%
hCas9 + T1 gRNA + T2 gRNA
16.9%
1.3%

FIG. 11A

endogenous 'native' hAAVS1 locus sequence
(293T)

..TTATCTGTCCCCTCCACCCCACAGTGGGGCCACTAGGGACAGGATTGGTGA..
T1 target T2 target

NHEJ rates evaluated using NGS of targeted AAVS1 locus

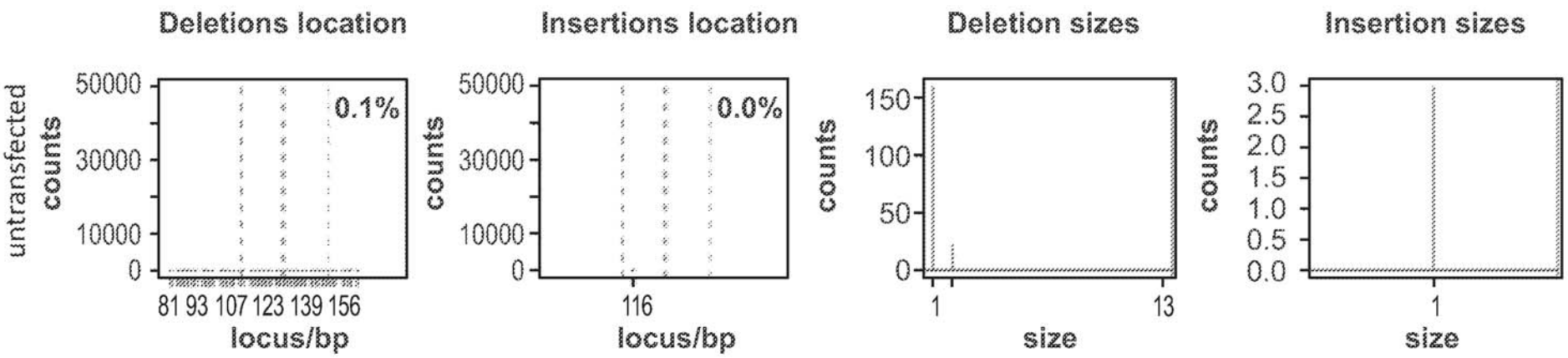


FIG. 11A
FIG. 11B
FIG. 11

FIG. 11B

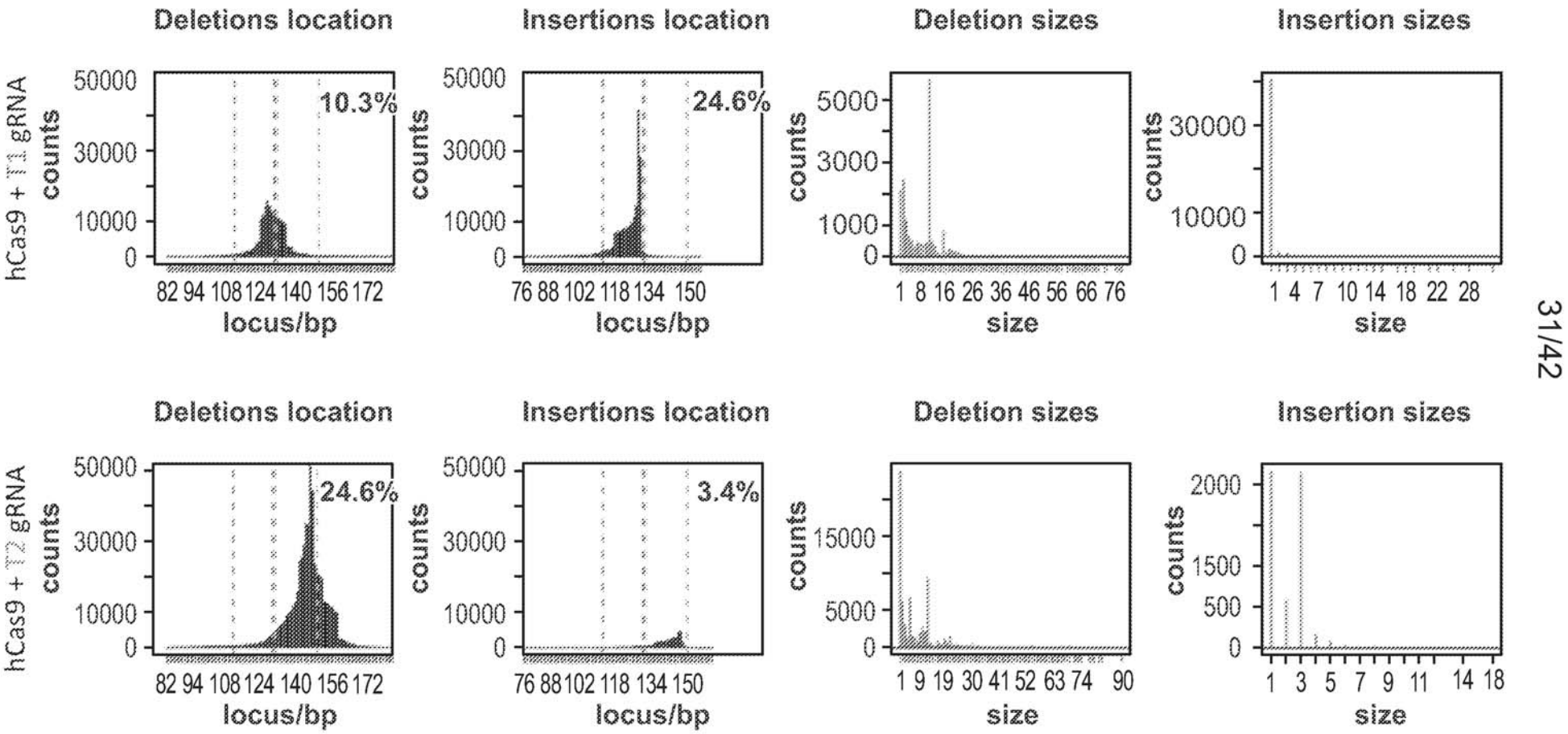

Deletions location
Insertions location
Deletion sizes
Insertion sizes
hCas9 + T1 gRNA
counts
10.3%
24.6%
locus/bp
size
hCas9 + T2 gRNA
24.6%
3.4%

FIG. 12A

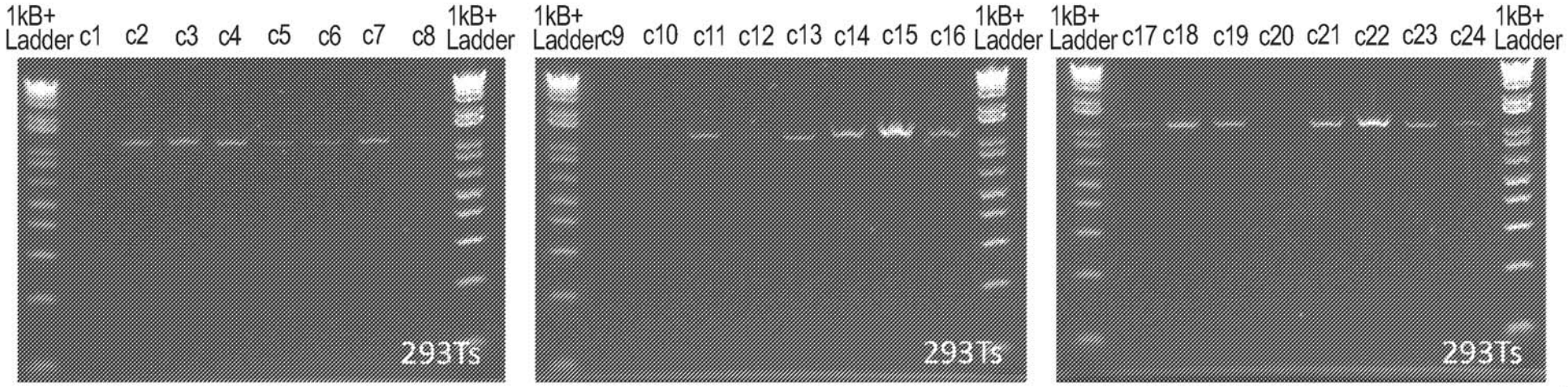


FIG. 12B

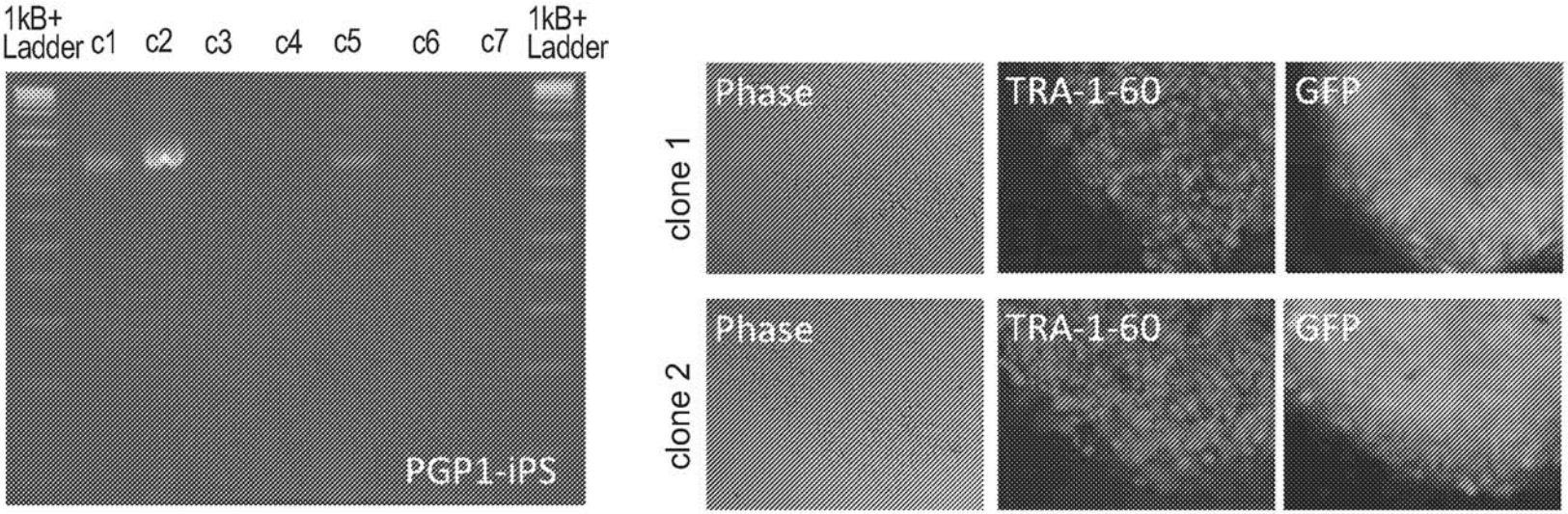


FIG. 12C

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

...GGTACTTTTATCTGTCCCCTCCACCCCACAGTGGGGCCACTAGGGACAGGATTGGTGACAGAAAAGCCCCATCCTTAGGCCTCCTCCTTCCTAGTCTCCTGATATTGGGTC...

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

5'-...ATAICTGTCCCCTCCACCCCACAGTGGGGCCACTAGGGACAGGAATTGGTGACAGAAAAGCCCCATCCTTAGGCCTCCTCCTTCCTAGTCT-3'

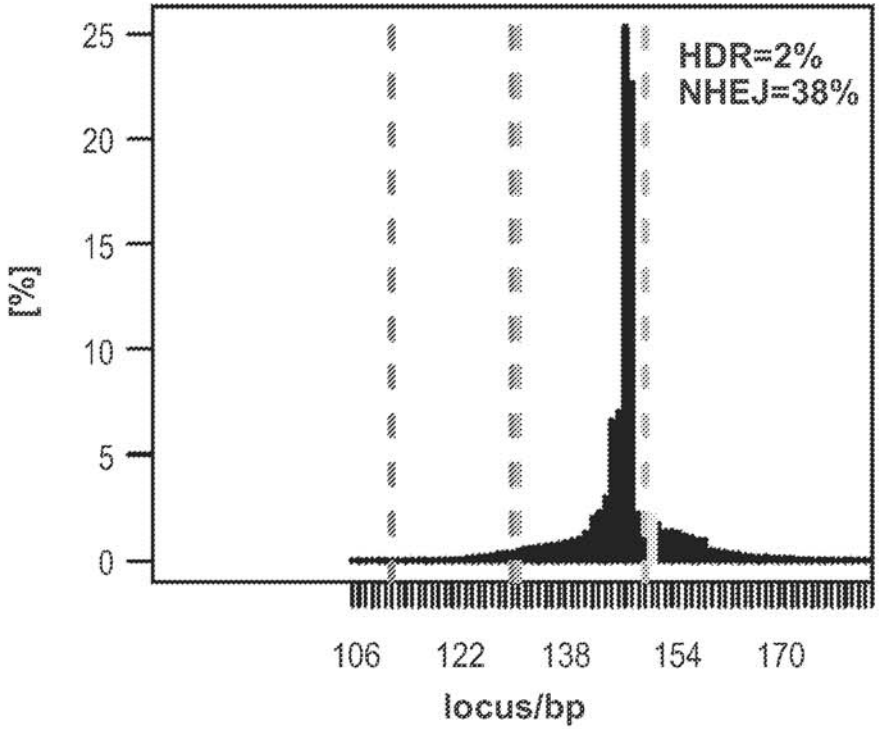


FIG. 13A-1

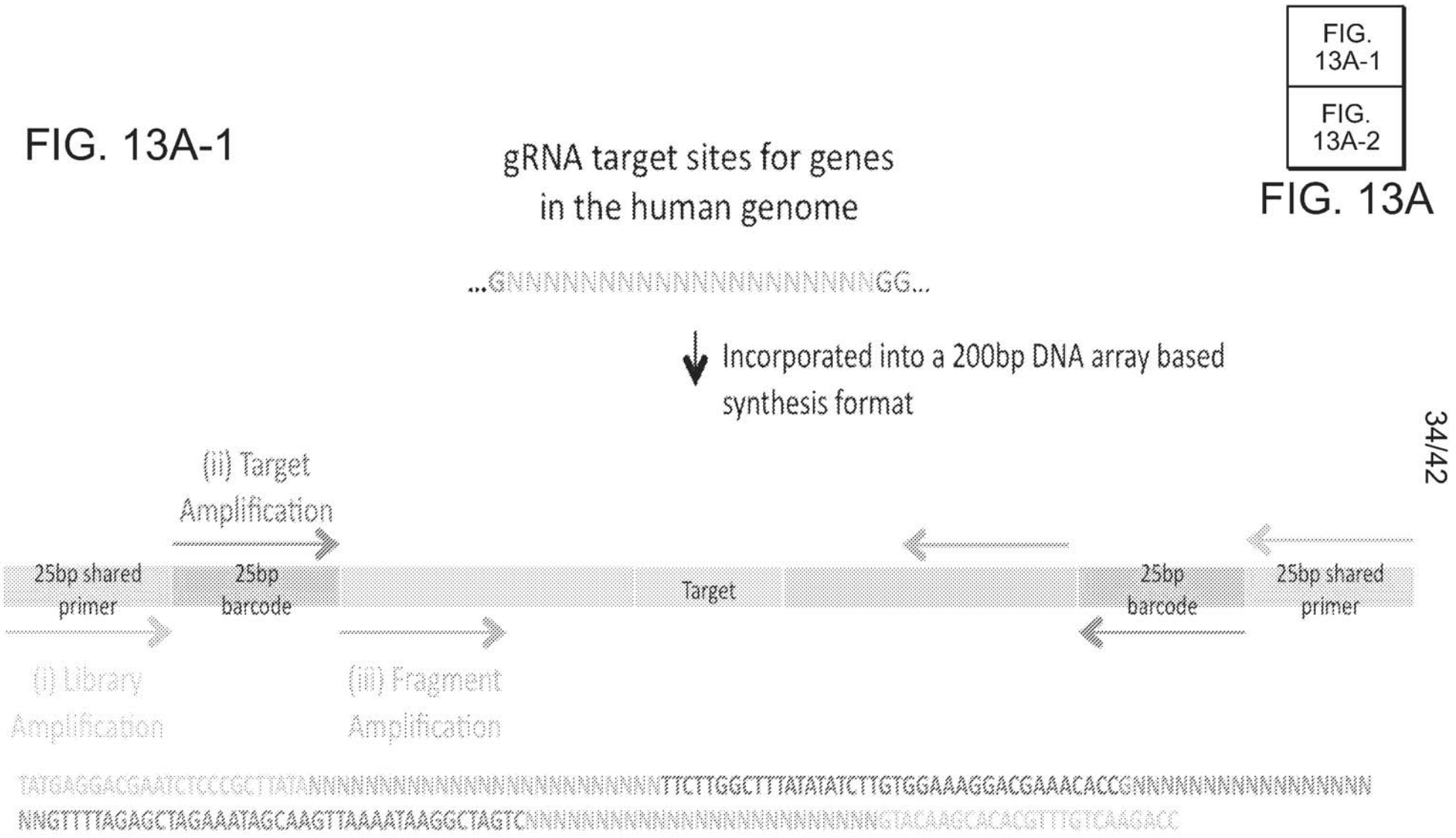


FIG. 13A-2

FIG. 13A

FIG. 13A-2

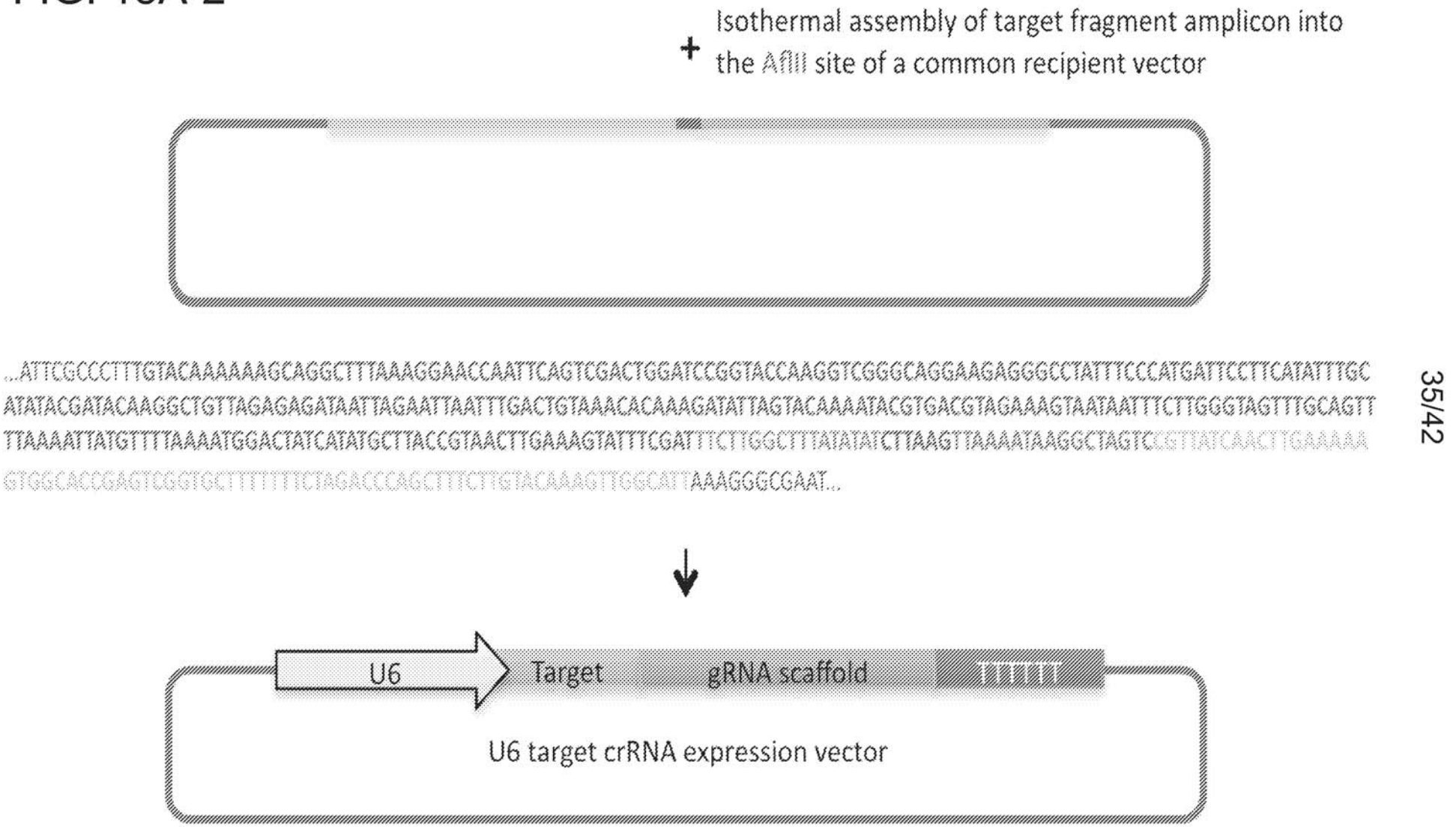

+ Isothermal assembly of target fragment amplicon into the AflII site of a common recipient vector
...ATTCGCCCTTTGTACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACTGGATCCGGTACCAAGGTCGGGCAGGAAGAGGGCCTATTTCCCATGATTCCTTCATATTTGC
ATATACGATACAAGGCTGTTAGAGAGATAATTAGAATTAATTTGACTGTAAACACAAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTT
TTAAAATTATGTTTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAA
GTGGCACCGAGTCGGTGCTTTTTTTCTAGACCCAGCTTTCTTGTACAAAGTTGGCATTAAAGGGCGAAT...
U6
Target
gRNA scaffold
TTTTTT
U6 target crRNA expression vector

FIG. 13B

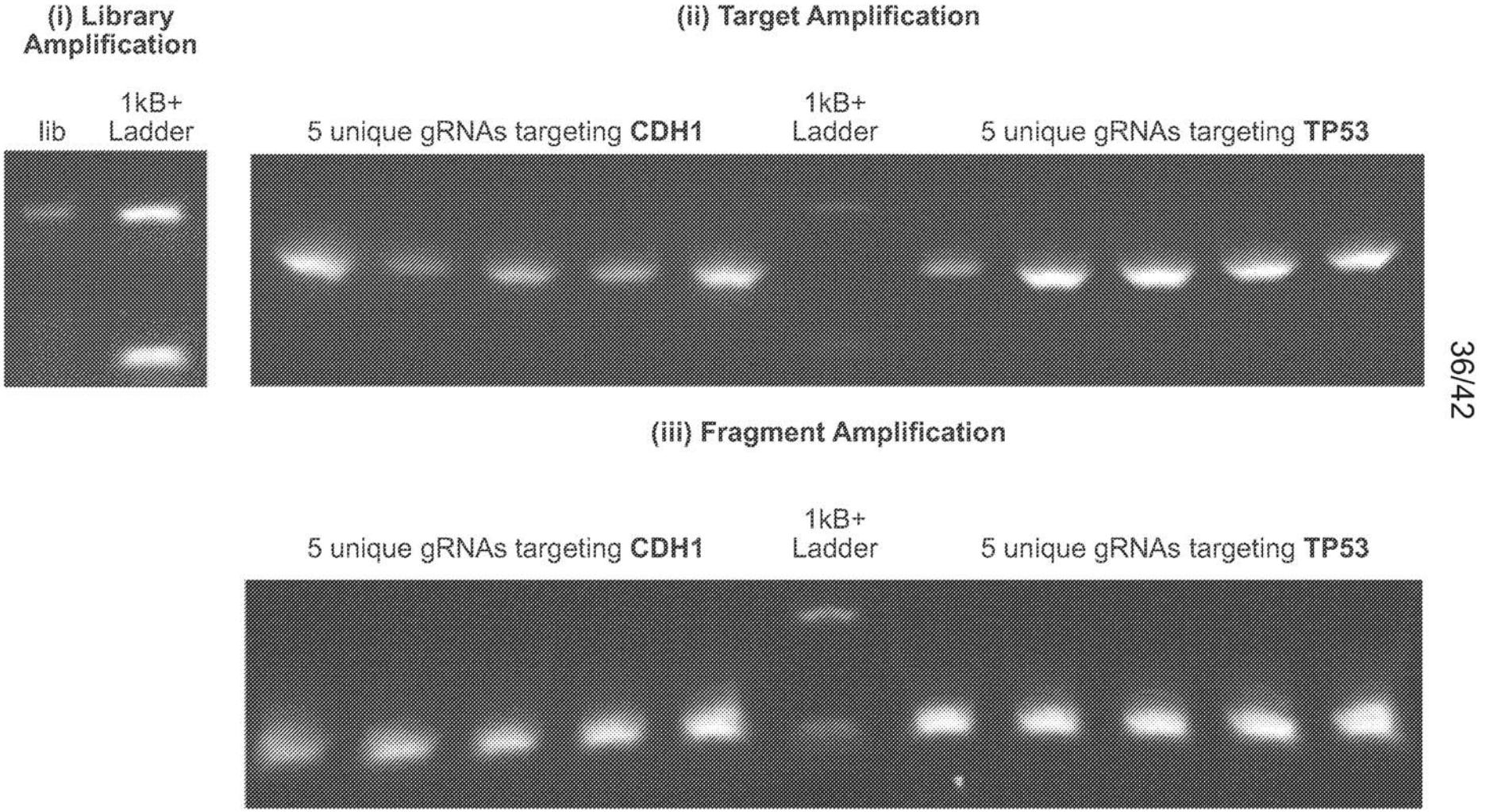

(i) Library Amplification
lib
1kB+ Ladder
(ii) Target Amplification
5 unique gRNAs targeting CDH1
1kB+ Ladder
5 unique gRNAs targeting TP53
(iii) Fragment Amplification
5 unique gRNAs targeting CDH1
1kB+ Ladder
5 unique gRNAs targeting TP53

FIG. 14A

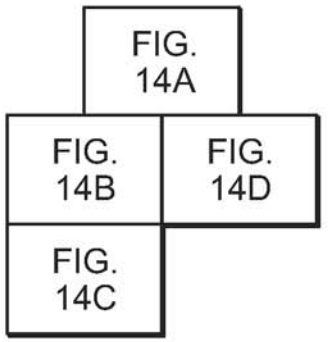


FIG. 14

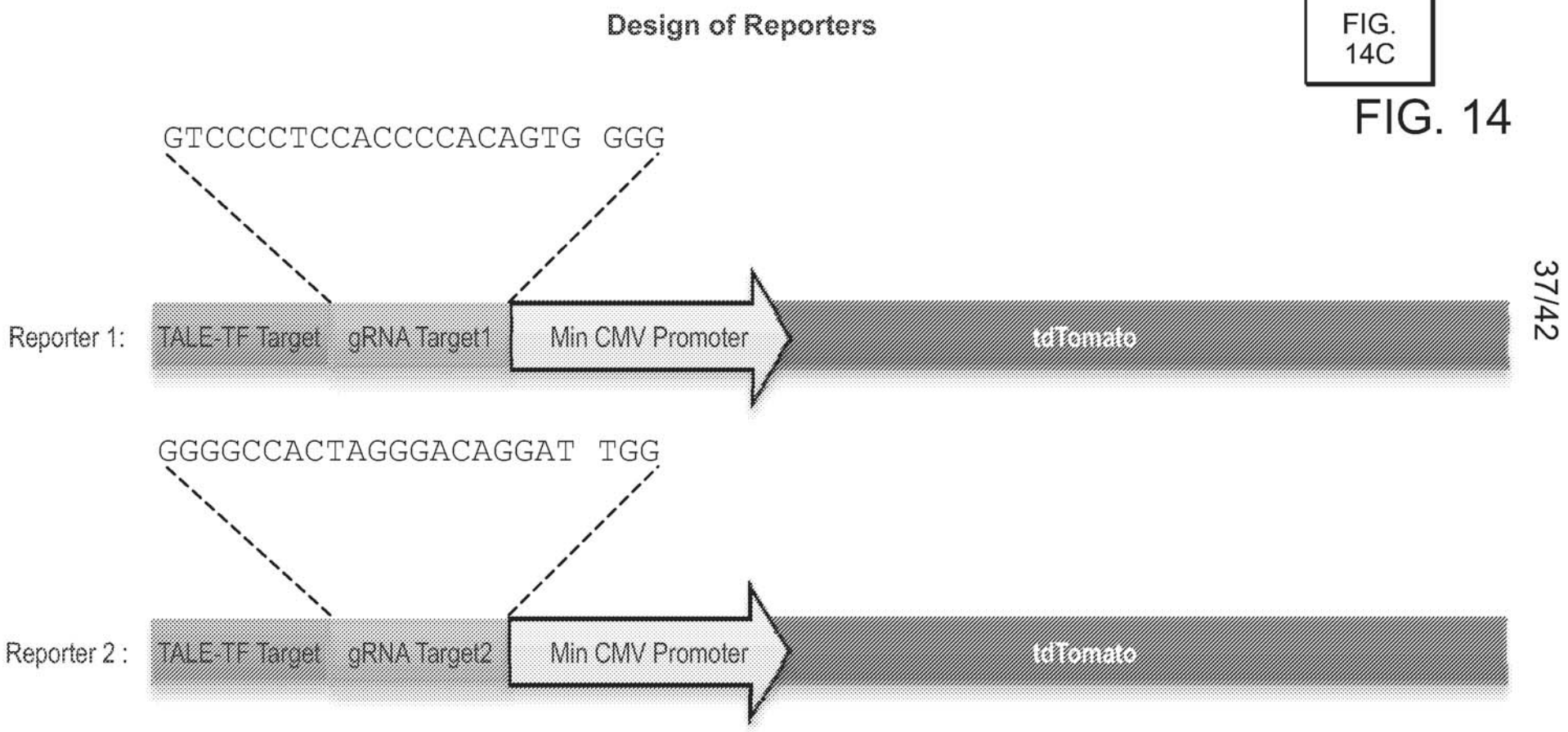


FIG. 14B

(a) Fluorescence Activated Cell Sorting (FACS) Analysis

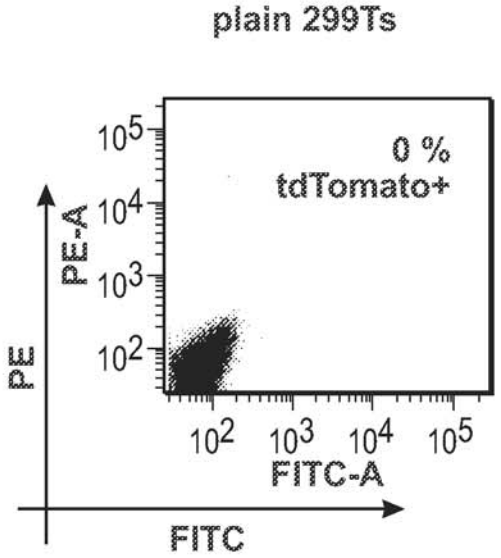


FIG. 14C

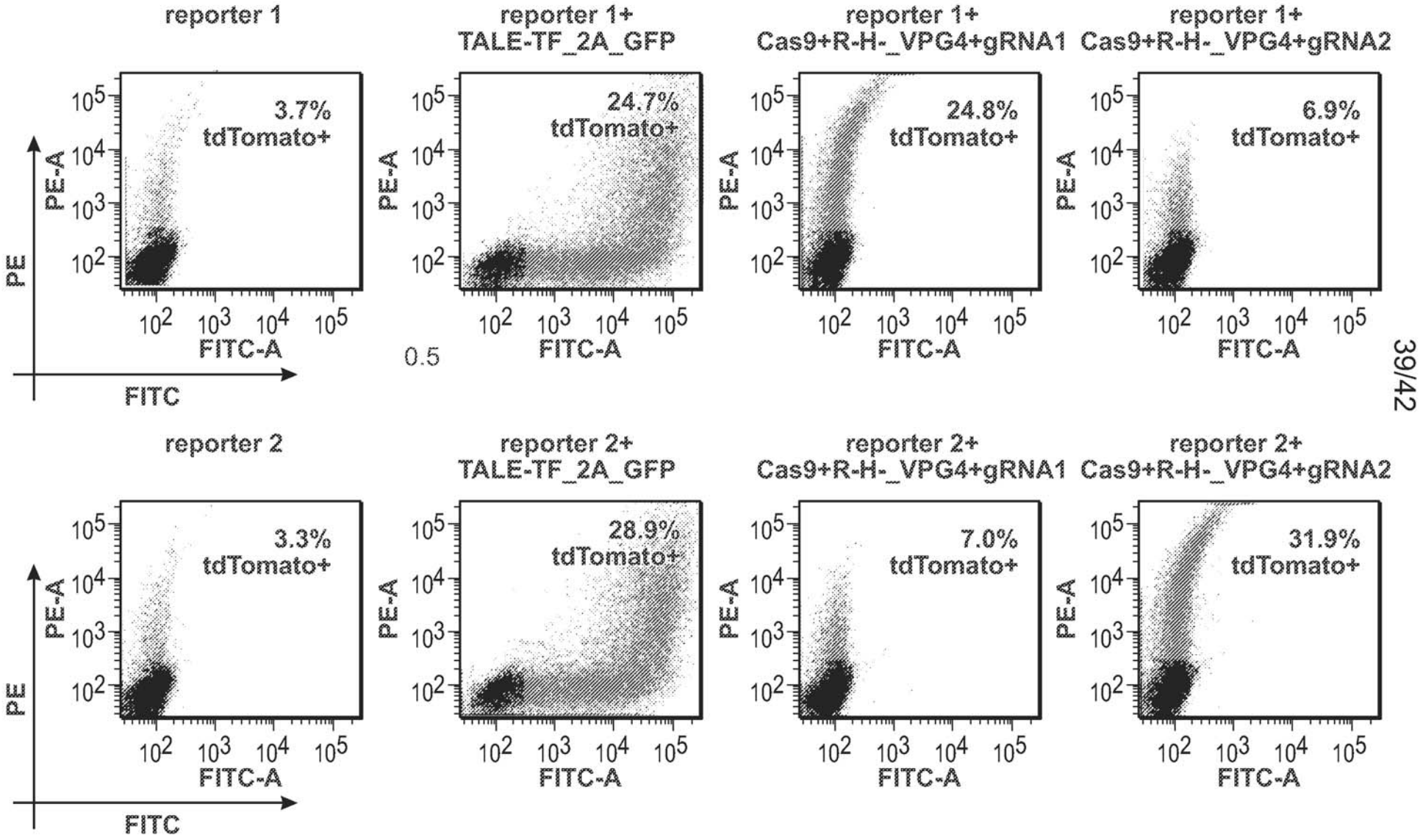

reporter 1
reporter 1+ TALE-TF_2A_GFP
reporter 1+ Cas9+R-H-_VPG4+gRNA1
reporter 1+ Cas9+R-H-_VPG4+gRNA2
3.7% tdTomato+
24.7% tdTomato+
24.8% tdTomato+
6.9% tdTomato+
reporter 2
reporter 2+ TALE-TF_2A_GFP
reporter 2+ Cas9+R-H-_VPG4+gRNA1
reporter 2+ Cas9+R-H-_VPG4+gRNA2
3.3% tdTomato+
28.9% tdTomato+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

FIG. 14D

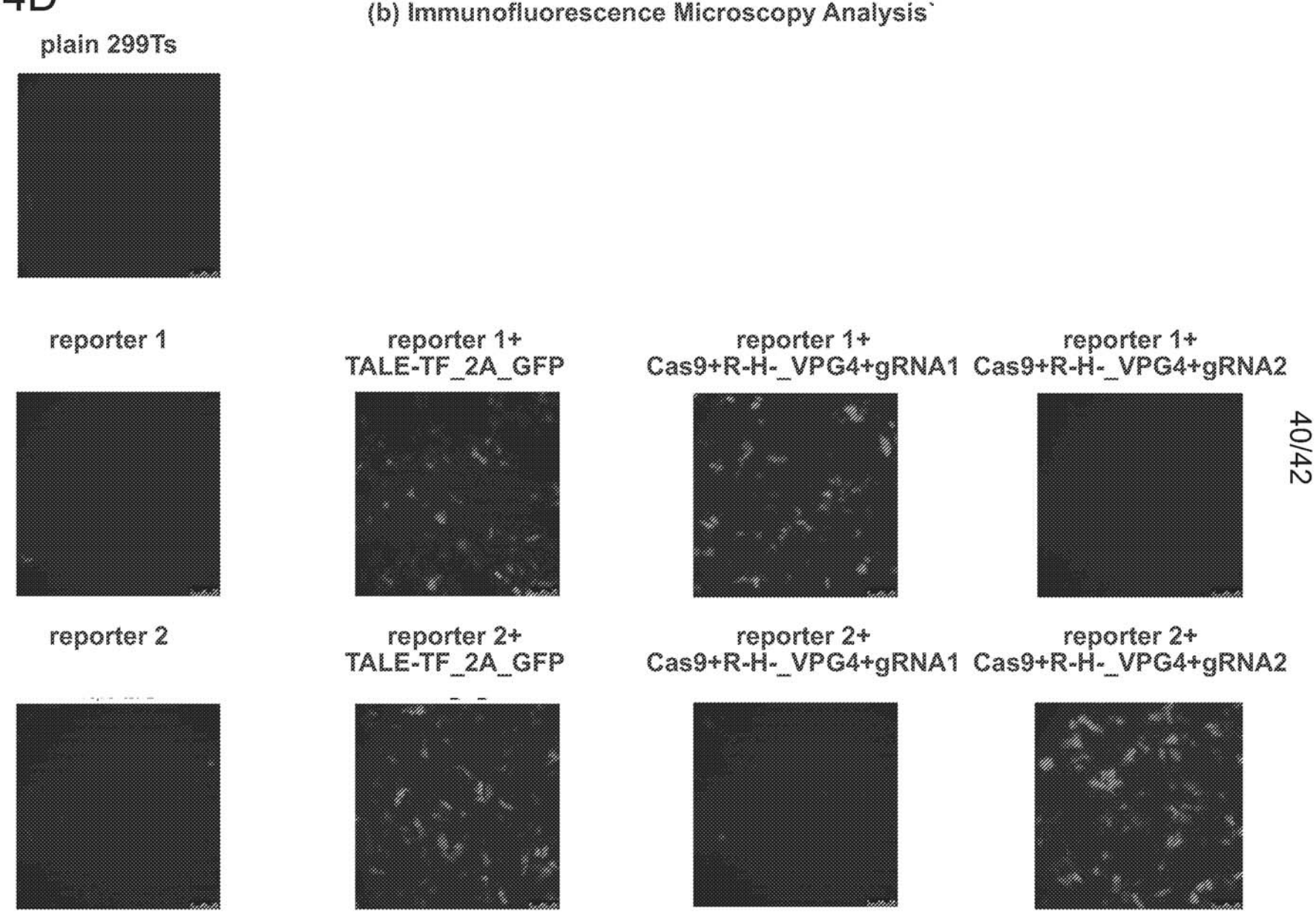

(b) Immunofluorescence Microscopy Analysis`
plain 299Ts
reporter 1
reporter 1+ TALE-TF_2A_GFP
reporter 1+ Cas9+R-H-_VPG4+gRNA1
reporter 1+ Cas9+R-H-_VPG4+gRNA2
reporter 2
reporter 2+ TALE-TF_2A_GFP
reporter 2+ Cas9+R-H-_VPG4+gRNA1
reporter 2+ Cas9+R-H-_VPG4+gRNA2

FIG. 15

FIG. 15A
FIG. 15B

FIG. 15A

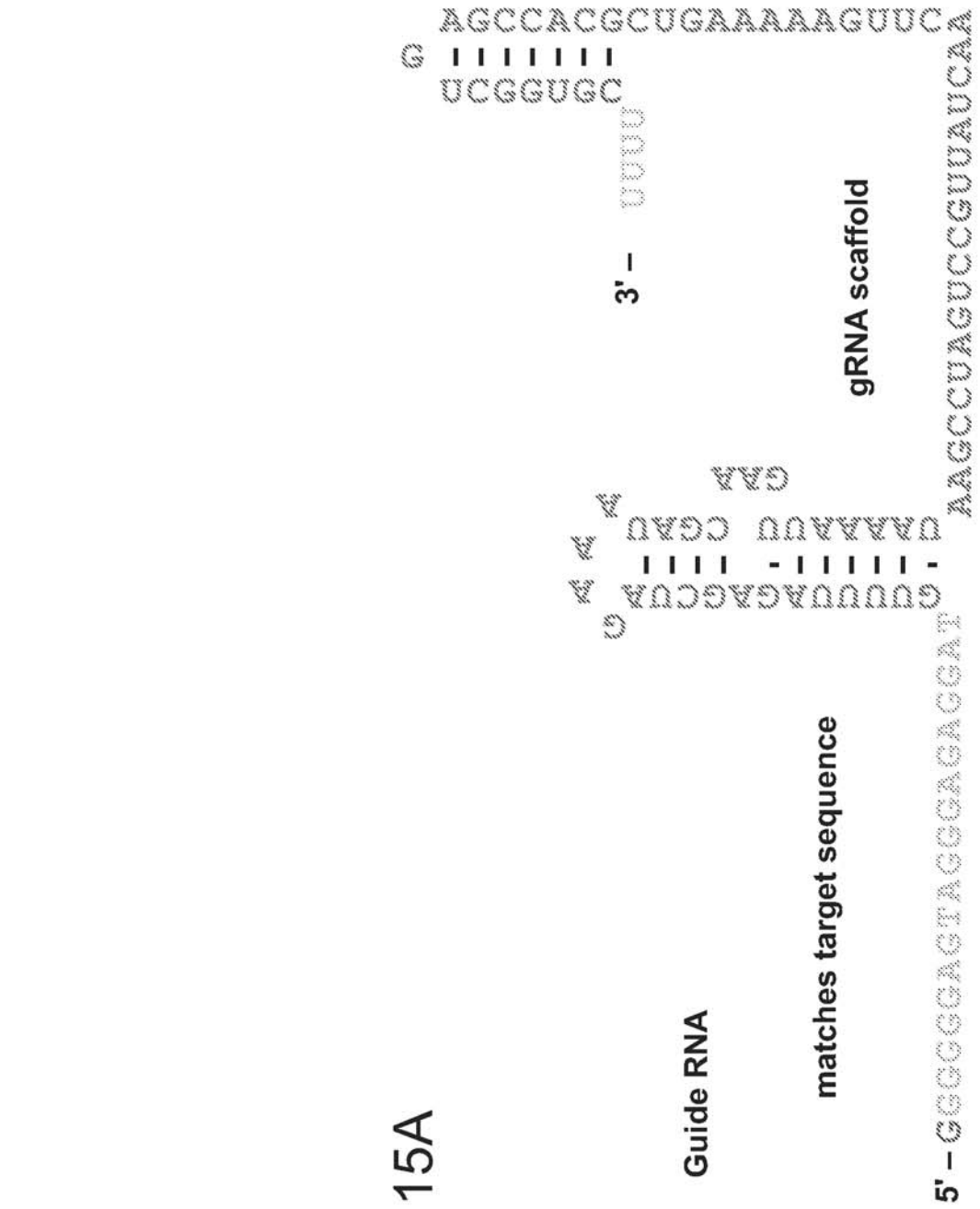
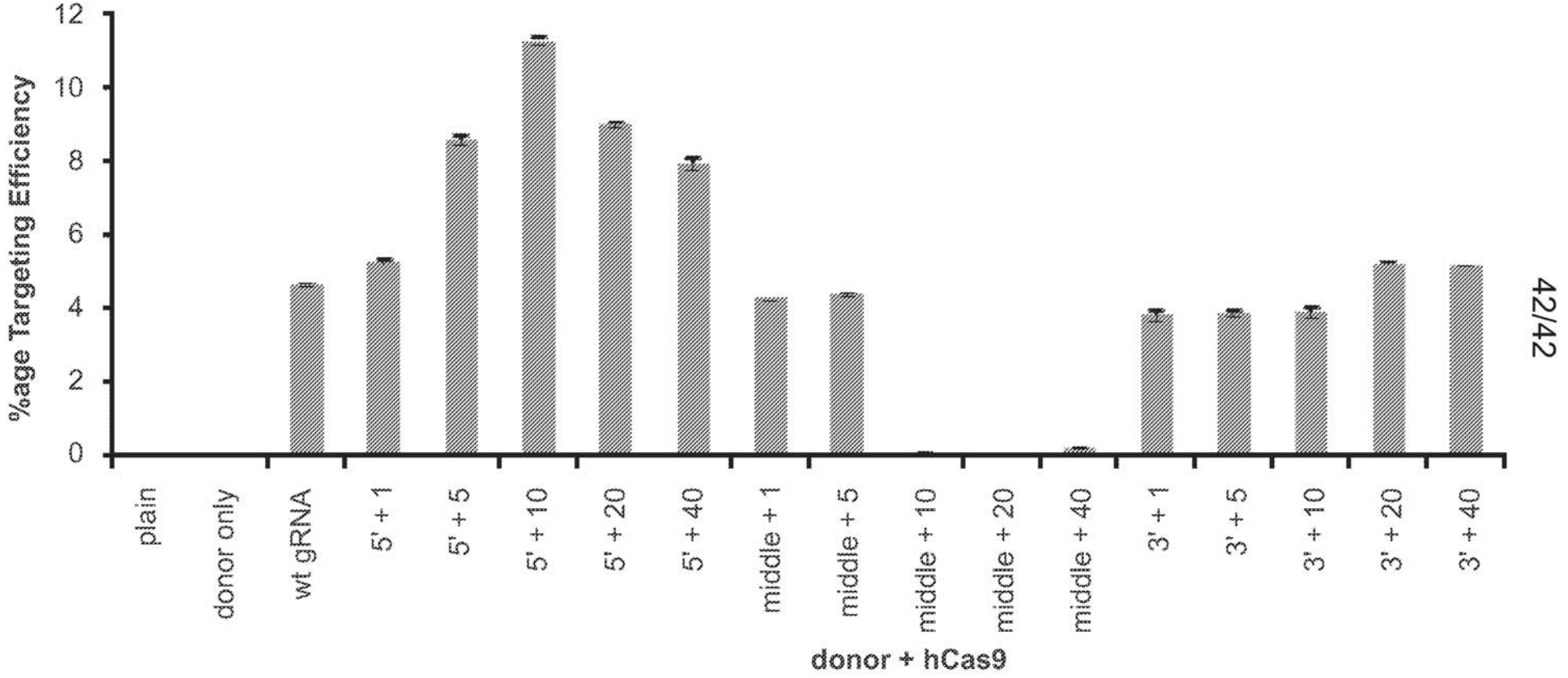


FIG. 15B


%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