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(54) **COMPOSITIONS FOR INDUCING MODIFICATIONS OF TARGET ENDOGENOUS NUCLEIC ACID SEQUENCES IN NUCLEUSES OF EUKARYOTIC CELLS**

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(58) **Field of Classification Search**

None

See application file for complete search history.

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(57) **ABSTRACT**

The present disclosure relates to targeted genome editing in eukaryotic cells or organisms. More particularly, the present disclosure provides for compositions and methods that may induce modifications in target endogenous nucleic acid sequences in nucleuses of eukaryotic cells. For example, methods disclosed herein may comprise preparing a Cas9/RNA complex, wherein the Cas9/RNA complex comprises a Cas9 protein and a guide RNA. Methods may further comprise introducing the Cas9/RNA complex into a non-human embryo, wherein the Cas9/RNA complex induces a modification at a target endogenous nucleic acid of the non-human embryo to provide for a genome modified embryo. Methods may further comprise transferring the genome modified embryo into a foster mother and allowing the foster mother to produce a F0 animal having the modification at the target endogenous nucleic acid.

16 Claims, 61 Drawing Sheets

Specification includes a Sequence Listing.

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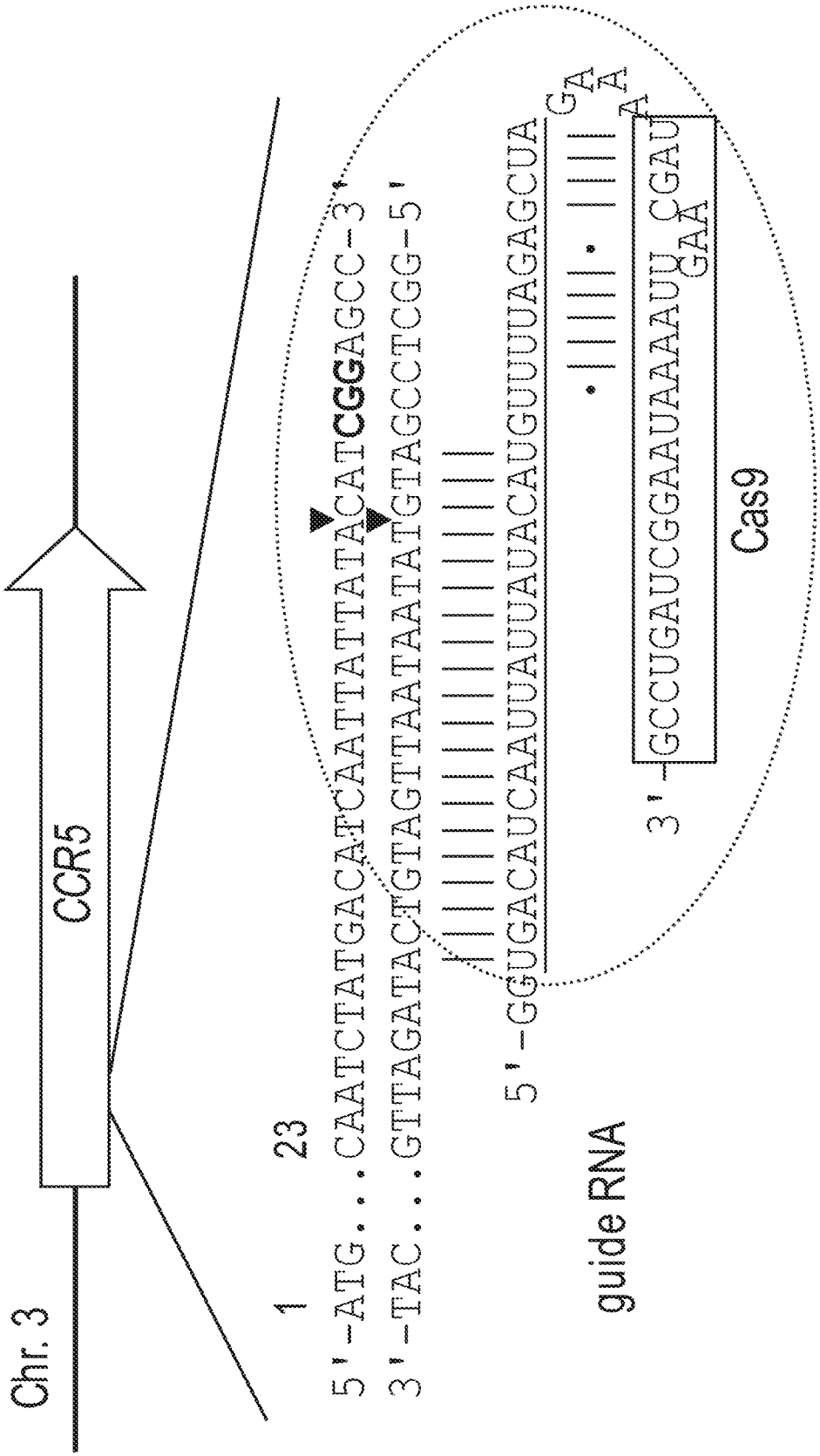
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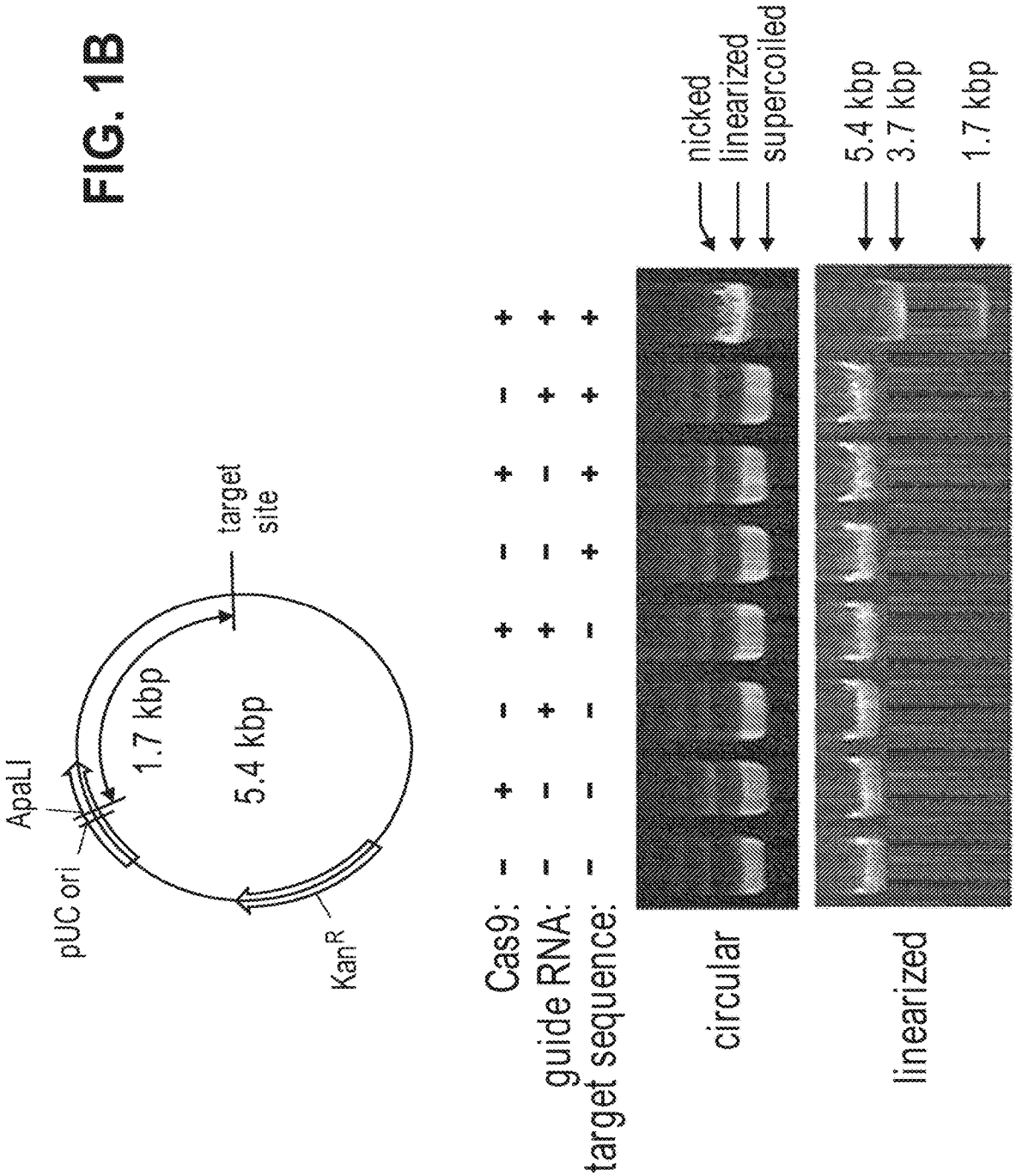
3'-TAC...GTTAGATACTGTAGTTAATAATATGTAGCCTCGG-5'
5'-GGUGACAUCAAUUAUUAUACAUGUUUUAGAGCUA
guide RNA
3'-GCCUGAUCGGAAUAAAAUU CGAU
GAA
GA
A
Cas9

FIG. 1A

FIG. 1B


ApaLI
pUC ori
1.7 kbp
5.4 kbp
target site
Kan^R
Cas9: - + - + + + - - + + + +
guide RNA: - - + + + - - + - + + +
target sequence: - - - - - + + + + + + +
nicked
linearized
supercoiled
circular
5.4 kbp
3.7 kbp
1.7 kbp
linearized

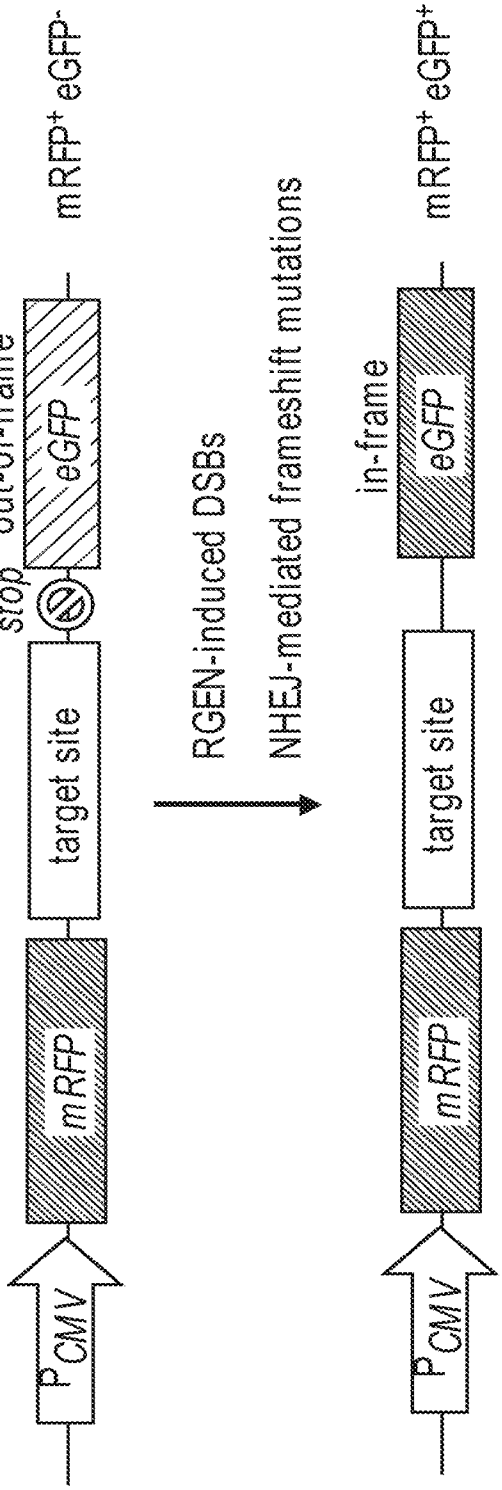

mRFP+ eGFP-
out-of-frame
stop
eGFP
target site
mRFP
P CMV
RGEN-induced DSBs
NHEJ-mediated frameshift mutations
mRFP+ eGFP+
in-frame
eGFP
target site
mRFP
P CMV

FIG. 2A

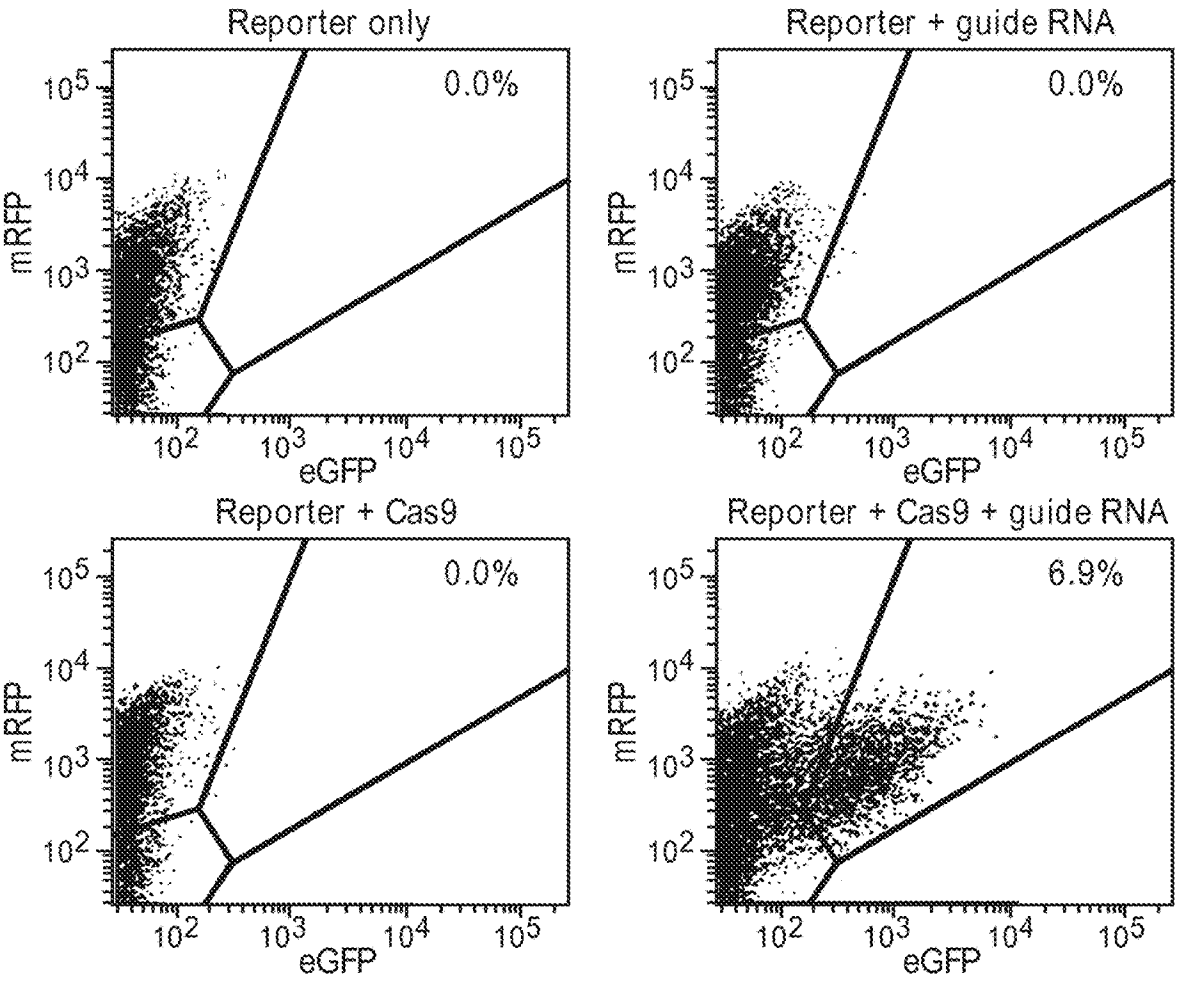

Reporter only
0.0%
Reporter + guide RNA
0.0%
Reporter + Cas9
0.0%
Reporter + Cas9 + guide RNA
6.9%
mRFP
eGFP
10^2
10^3
10^4
10^5

FIG. 2B

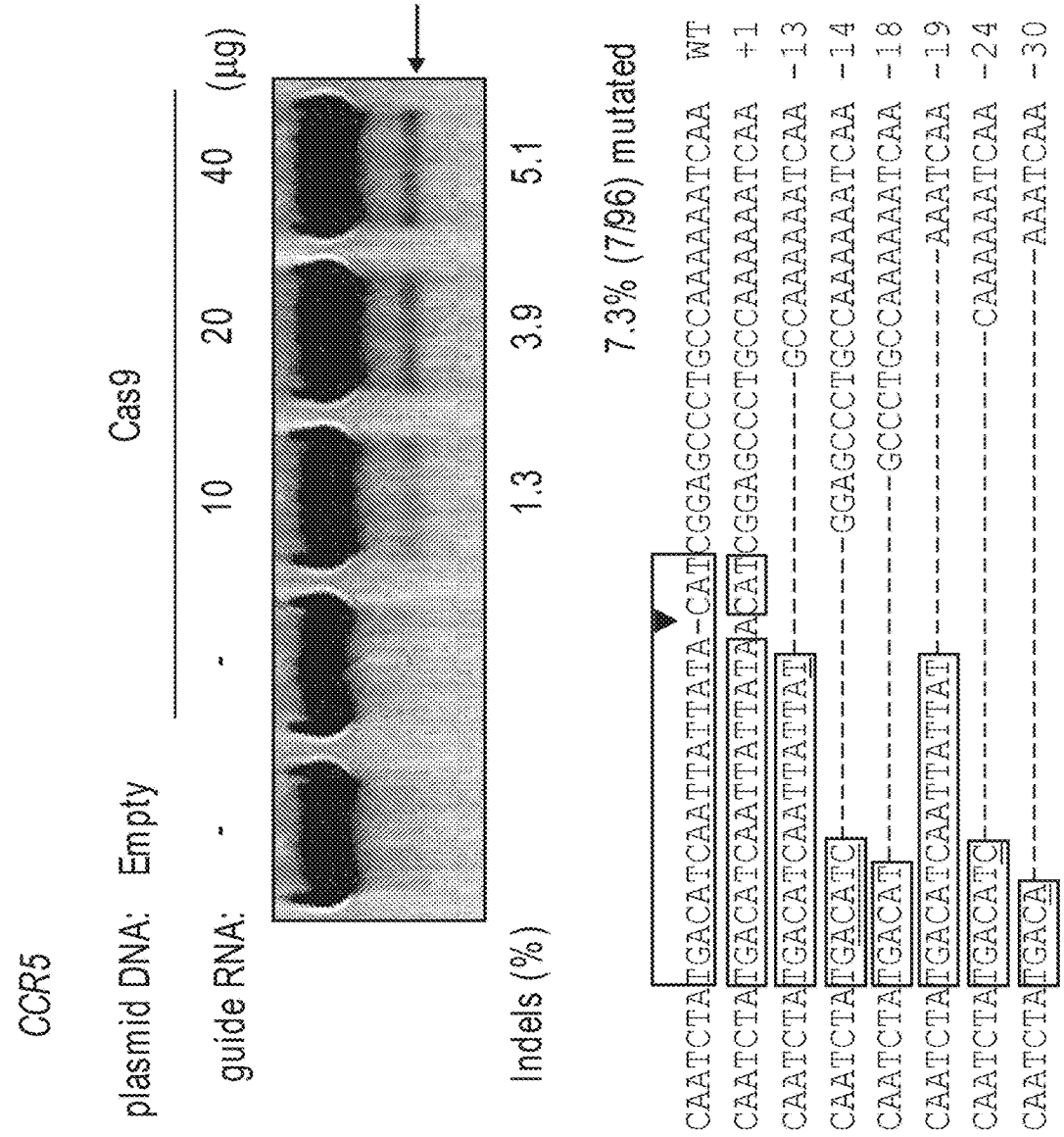

CCR5
Cas9
plasmid DNA: Empty
guide RNA: - - 10 20 40 (μg)
Indels (%) 1.3 3.9 5.1
7.3% (7/96) mutated
CAATCTATGACATCAATTATATA-CATCGGAGCCCTGCCAAAAATCAA WT
CAATCTATGACATCAATTATATAACATCGGAGCCCTGCCAAAAATCAA +1
CAATCTATGACATCAATTAT----------GCCAAAAAATCAA -13
CAATCTATGACATC----------GGAGCCCTGCCAAAAATCAA -14
CAATCTATGACAT----------GCCCTGCCAAAAATCAA -18
CAATCTATGACATCAATTATAT----------AAATCAA -19
CAATCTATGACATC----------CAAAAATCAA -24
CAATCTATGACA----------AAATCAA -30

FIG. 3A

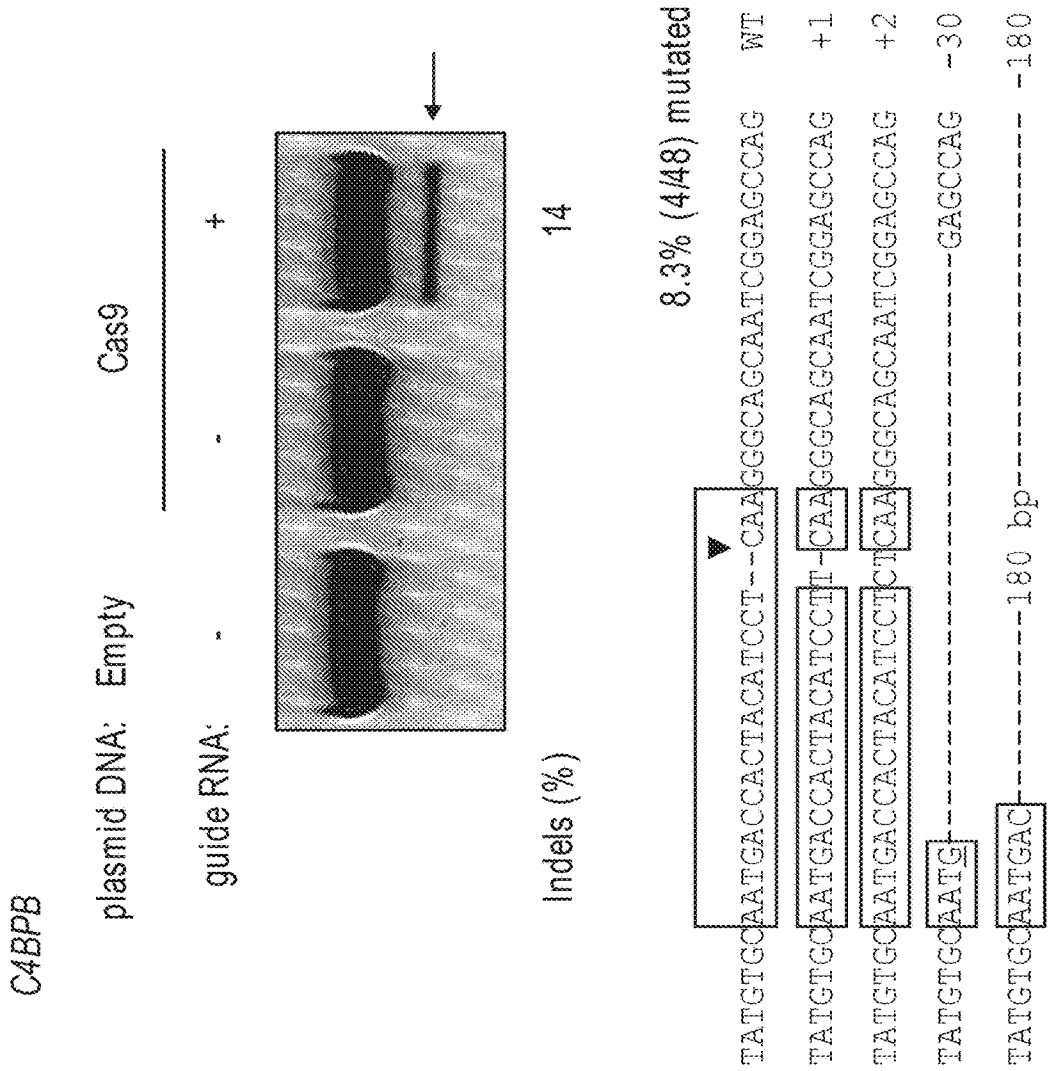

C4BPB
plasmid DNA: Empty
Cas9
guide RNA: - - +
Indels (%) 14
8.3% (4/48) mutated
TATGTGCAATGACCACTACATCCT--CAAGGGCAGCAATCGGAGCCAG WT
TATGTGCAATGACCACTACATCCTT-CAAGGGCAGCAATCGGAGCCAG +1
TATGTGCAATGACCACTACATCCTCTCAAGGGCAGCAATCGGAGCCAG +2
TATGTGCAATGT----------GAGCCAG -30
TATGTGCAATGAC----------180 bp----------180

FIG. 3B

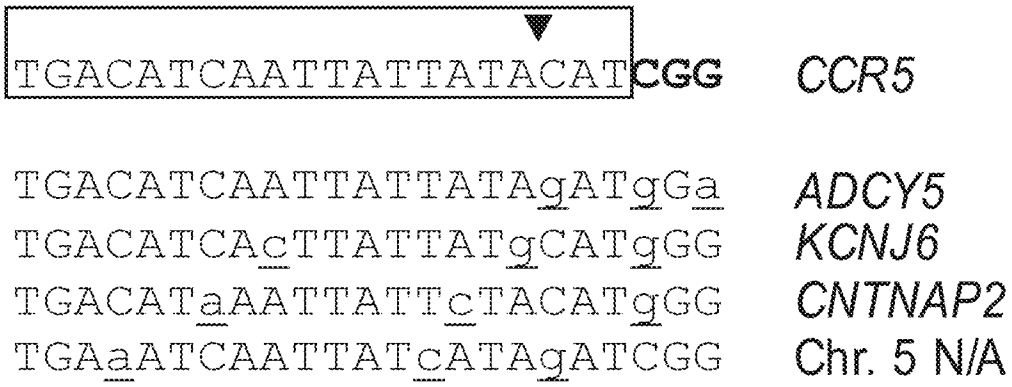

TGACATCAATTATTATACATCGG
CCR5
TGACATCAATTATTATAgATgGa
ADCY5
TGACATCAcTTATTATgCATgGG
KCNJ6
TGACATaAATTATTcTACATgGG
CNTNAP2
TGAaATCAATTATcATAgATCGG
Chr. 5 N/A

FIG. 4A

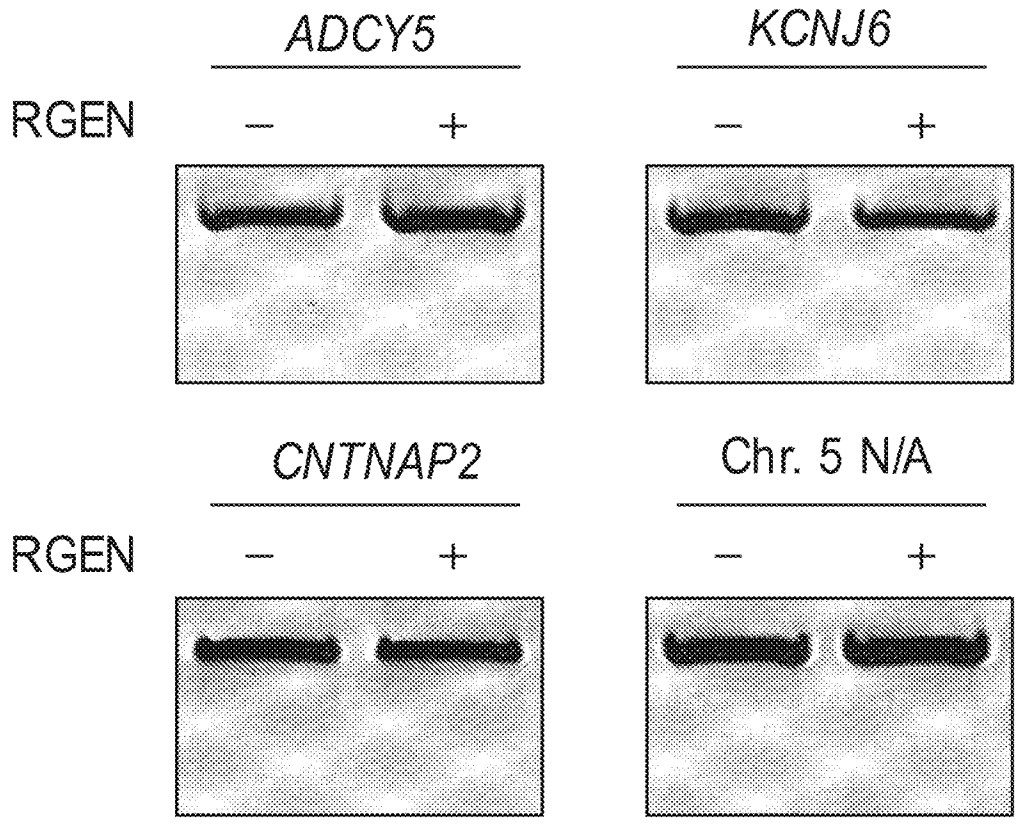
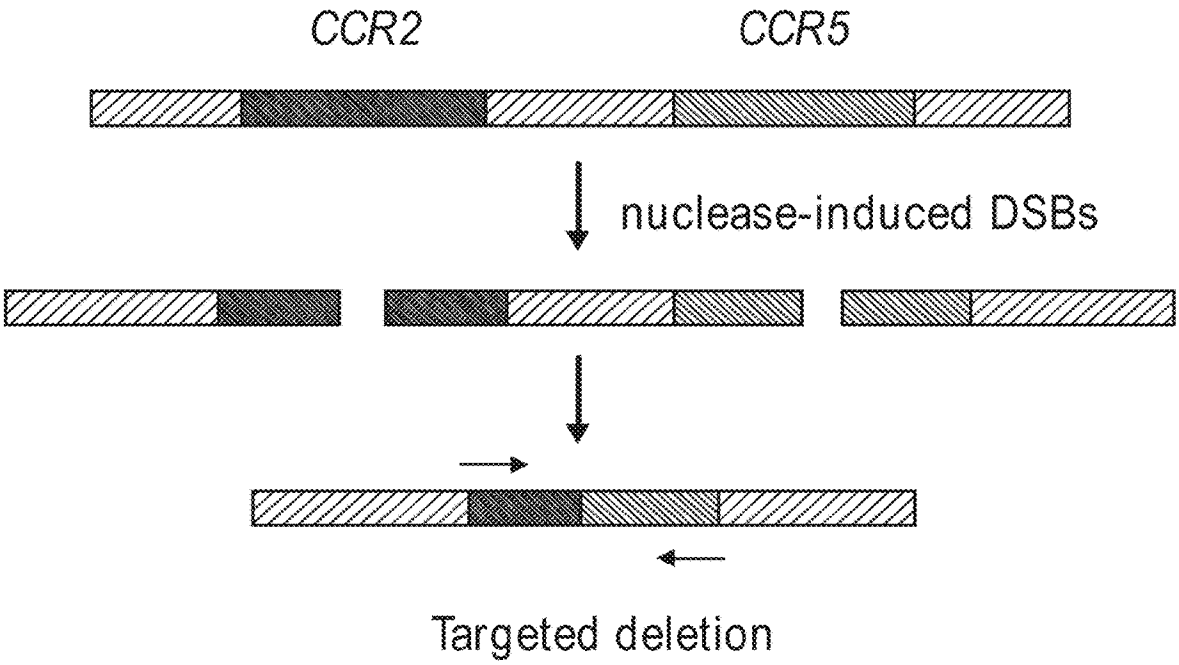

ADCY5
KCNJ6
RGEN
–
+
–
+
CNTNAP2
Chr. 5 N/A
RGEN
–
+
–
+

FIG. 4B


CCR2
CCR5
nuclease-induced DSBs
Targeted deletion

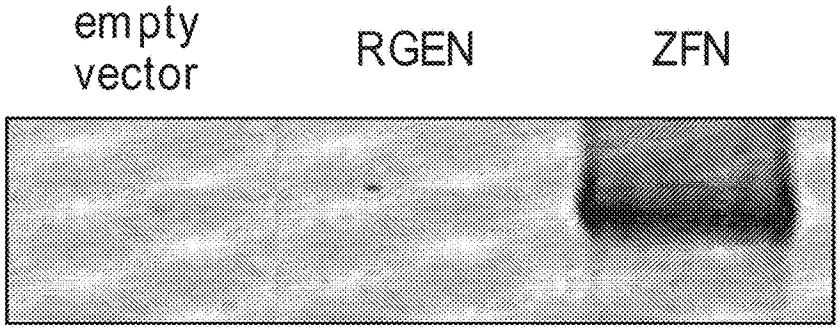

empty vector
RGEN
ZFN

FIG. 4C

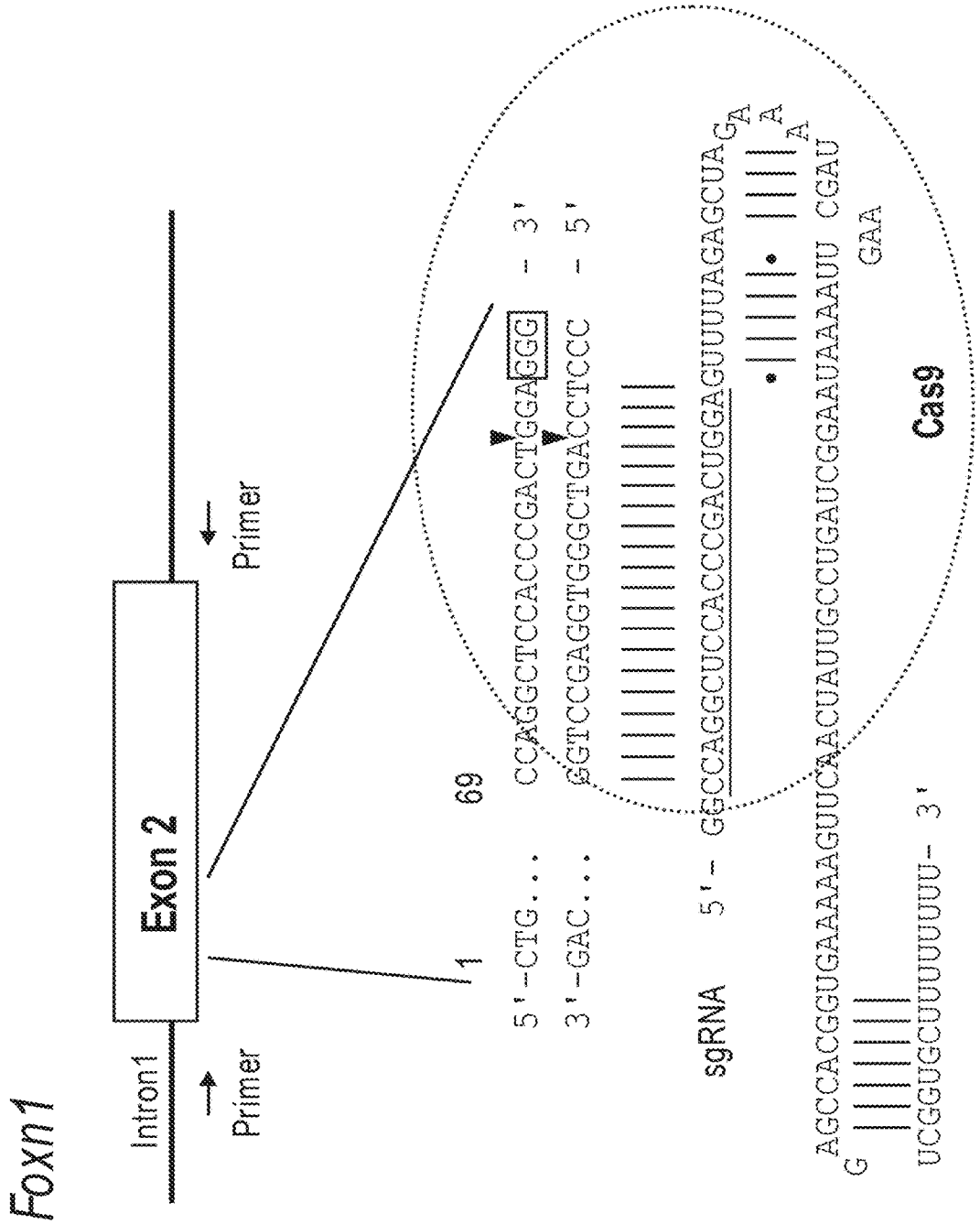

Foxn1
Intron1
Exon 2
Primer
Primer
1
69
5'-CTG...
3'-GAC...
CCAGGCTCCACCCGACTGGAGGG - 3'
GGTCCGAGGTGGGCTGACCTCCC - 5'
sgRNA 5'- GGCCAGGCUCCACCCGACUGGAGUUUAGAGCUA
AGCCACGGUGAAAAAGUUCAACUAUUGCCUGAUCGGAAUAAAAUU CGAU
G
UCGGUGCUUUUUUUU- 3'
GAA
Cas9

FIG. 5A

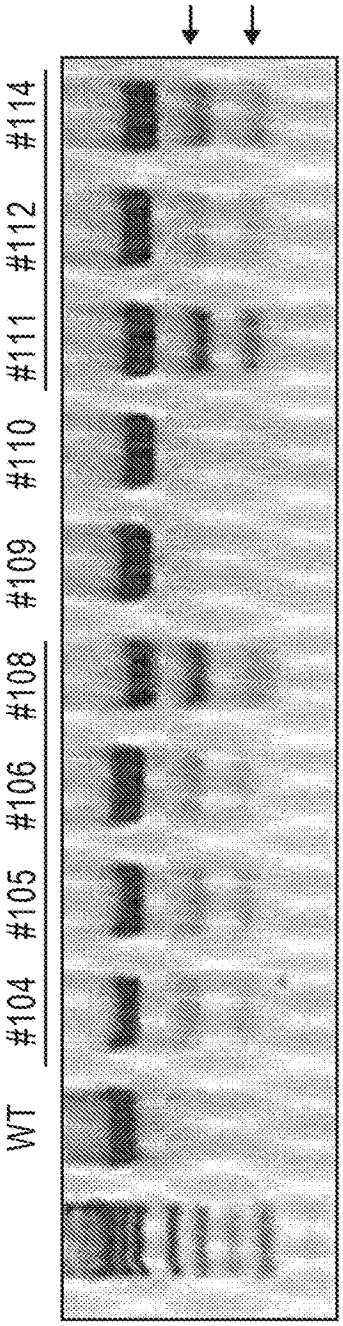

WT
#104
#105
#106
#108
#109
#110
#111
#112
#114

FIG. 5B

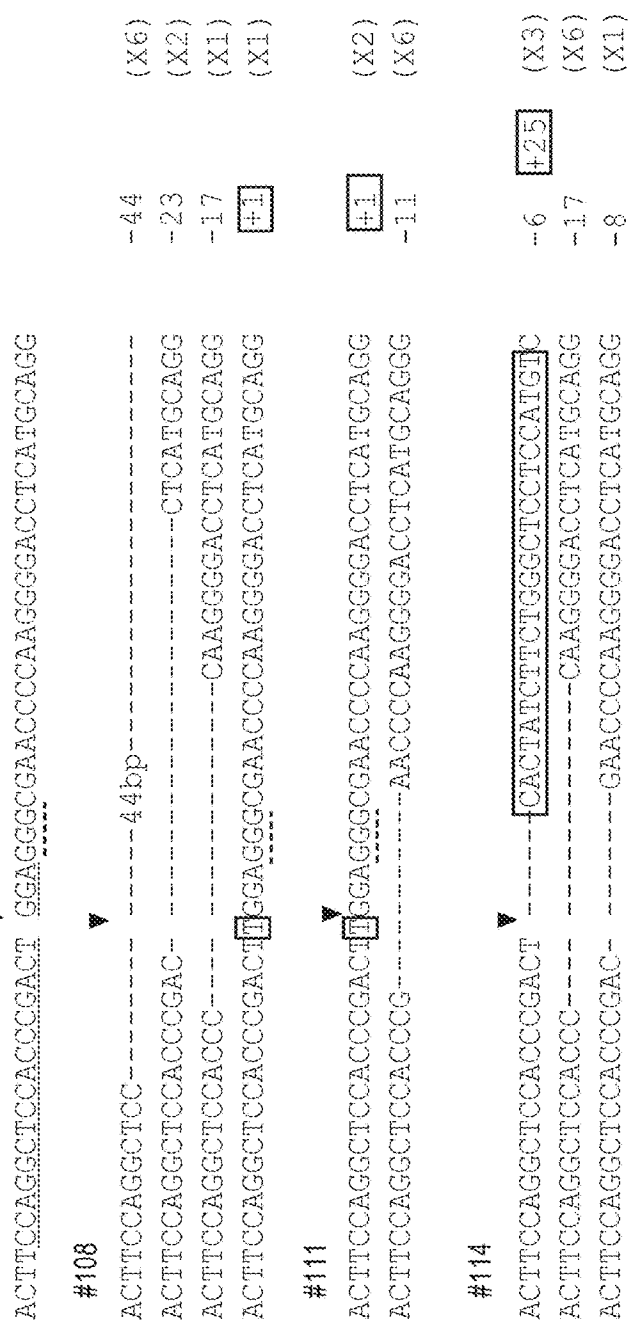

WT
ACTTCCAGGCTCCACCCGACT GGAGGGGCGAACCCCAAGGGGACCTCATGCAGG
#108
ACTTCCAGGCTCC---------44bp----------CTCATGCAGG -44 (X6)
ACTTCCAGGCTCCACCCGAC----------CTCATGCAGG -23 (X2)
ACTTCCAGGCTCCACCC----------CAAGGGGACCTCATGCAGG -17 (X1)
ACTTCCAGGCTCCACCCGACTGGAGGGGCGAACCCCAAGGGGACCTCATGCAGG +1 (X1)
#111
ACTTCCAGGCTCCACCCGACTGGAGGGGCGAACCCCAAGGGGACCTCATGCAGG +1 (X2)
ACTTCCAGGCTCCACCCG----------AACCCCAAGGGGACCTCATGCAGGG -11 (X6)
#114
ACTTCCAGGCTCCACCCGACT-----CACTATCTTCTGGGCTCCTCCATGTC -6 +25 (X3)
ACTTCCAGGCTCCACCC----------CAAGGGGACCTCATGCAGG -17 (X6)
ACTTCCAGGCTCCACCCGAC----------GAACCCCAAGGGGACCTCATGCAGG -8 (X1)

FIG. 5C

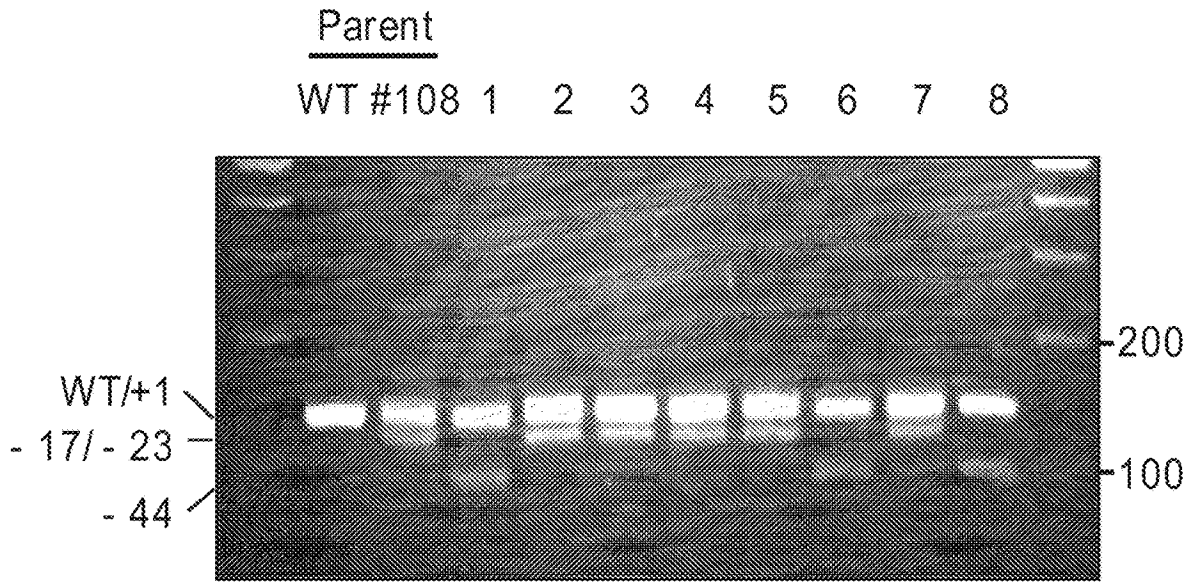


FIG. 5D

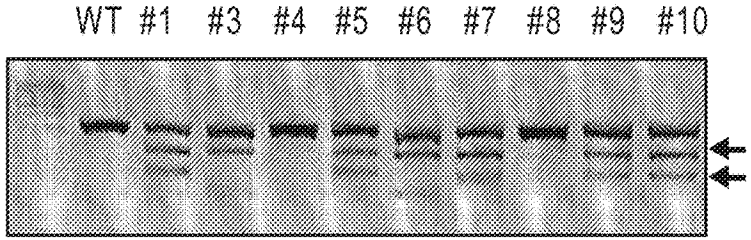


FIG. 6A

Cas9 mRNA (ng/μl)	sgRNA (ng/μl)	Tested embryos	Mutants (%)
10	1	27	9 (33)
10	10	49	28 (57)
10	100	45	41 (91)

FIG. 6B

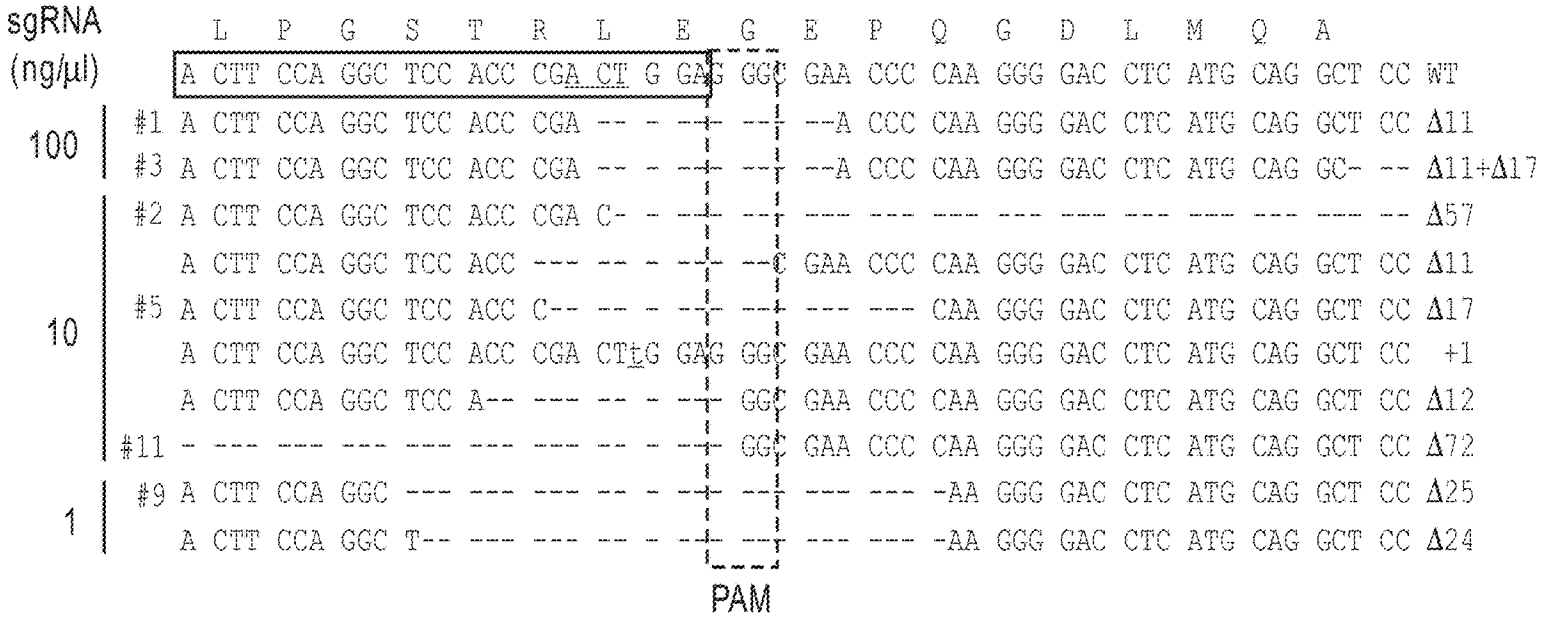
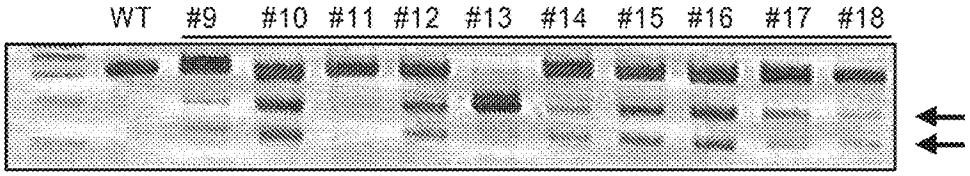


FIG. 6C

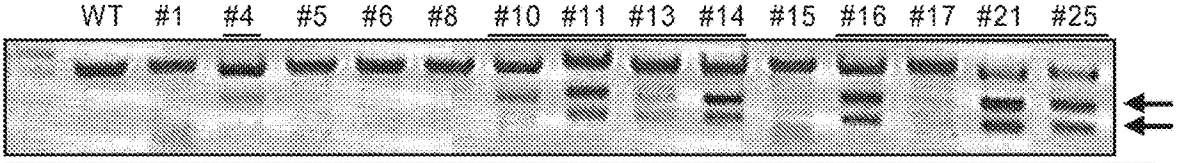
Pronucleous injection



Cas9 protein (nM)	sgRNA (nM)	Tested embryos	Mutants (%)
2	4	20	3 (15%)
20	40	15	5 (33%)
200	400	17	15 (88%)

FIG. 7A

Intra-cytoplasmic injection



Cas9 protein (nM)	sgRNA (nM)	Tested embryos	Mutants (%)
2	4	18	1 (6%)
20	40	19	6 (32%)
200	400	14	10 (71%)

FIG. 7B

Sequence	Indels	Embryo no.
ACTTCCAGGCTCCACCCGACTGGAGGGCGAACCCCAAGGGGACCTCATGCAG	WT	
ACTTCCAGGCGAACCCC----------AAGGGGACCTCATGCAG	Δ18	2
ACTTCCAGGCTCCAC----------AAGGGGACCTCATGCAG	Δ20	1
ACTTCCAGGCTCCACCC----------AAGGGGACCTCATGCCC	Δ19	1
ACTTCCAGGCTCCACCC----------CAAGGGGACCTCATGCAG	Δ17	1
ACTTCCAGGCTCCACCCGA----------ACCCCAAGGGGACCTCATGCAG	Δ11	3
ACTTCCAGGCTCCACCCGAA--GGAGGGCGAACCCCAAGGGGACCTCATGCA	Δ3+1	1
ACTTCCAGGCTCCACCCGACT--AGGGCGAACCCCAAGGGGACCTCATGCAG	Δ2	1
ACTTCCAGGCTCCACCCGACTGGGAGGGCGAACCCCAAGGGGACCTCATGCA	+1	1
ACTTCCAGGCTCCACCCGACTTGGAGGGCGAACCCCAAGGGGACCTCATGCA	+1	10
ACTTCCAGGCTCCACCCGA------GGCGAACCCCAAGGGGACCTCATGCAG	Δ6	1
ACTTCCAGGCTCCACCCGA-----GGGCGAACCCCAAGGGGACCTCATGCAG	Δ5	2
ACTTCCAGGCTCCACC----------TCATGCAG	Δ28	1
----------AGGGCGAACCCCAAGGGGACCTCATGCAG	Δ126	1
Total		26

FIG. 7C

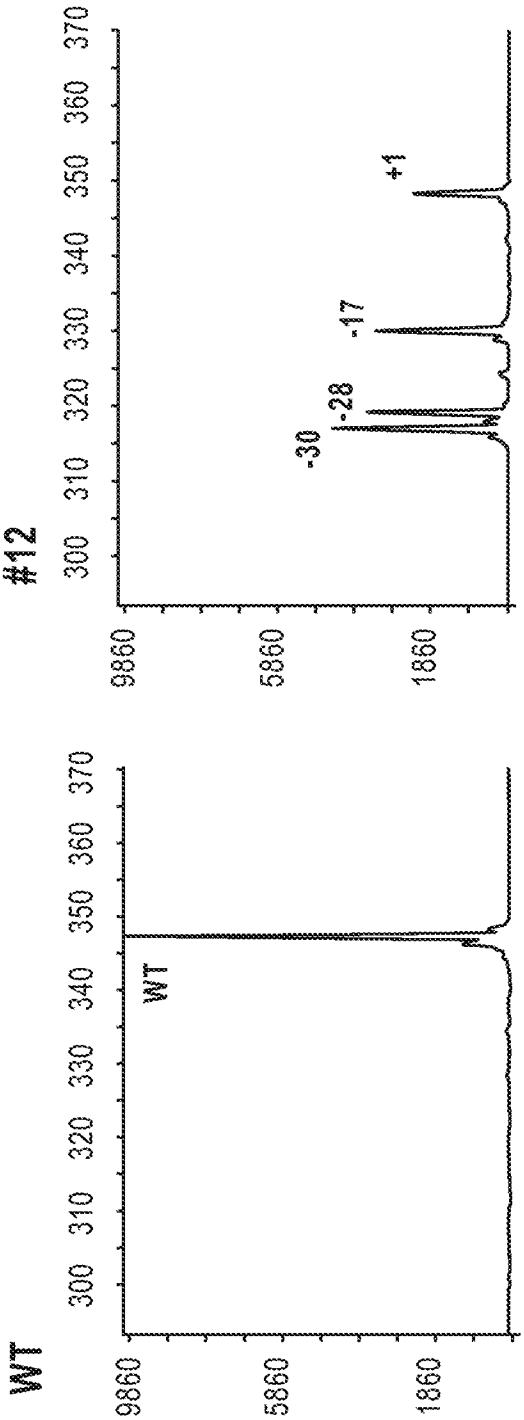


FIG. 8A

FIG. 8B

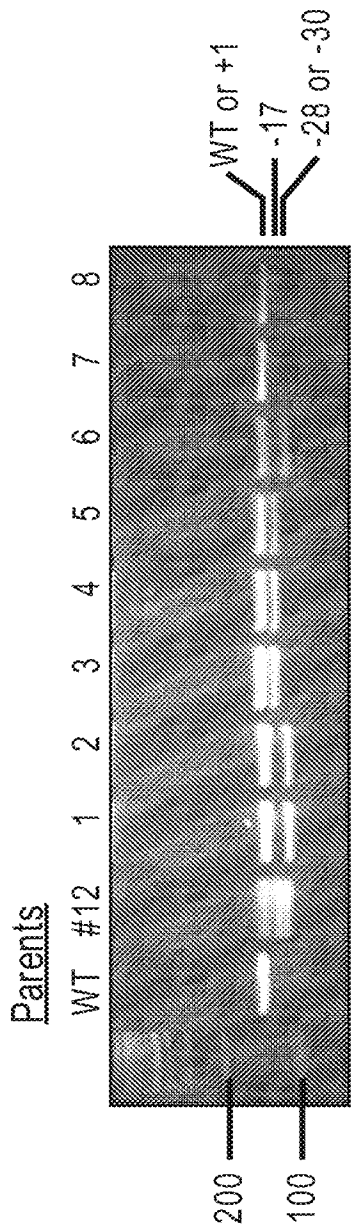


FIG. 8C

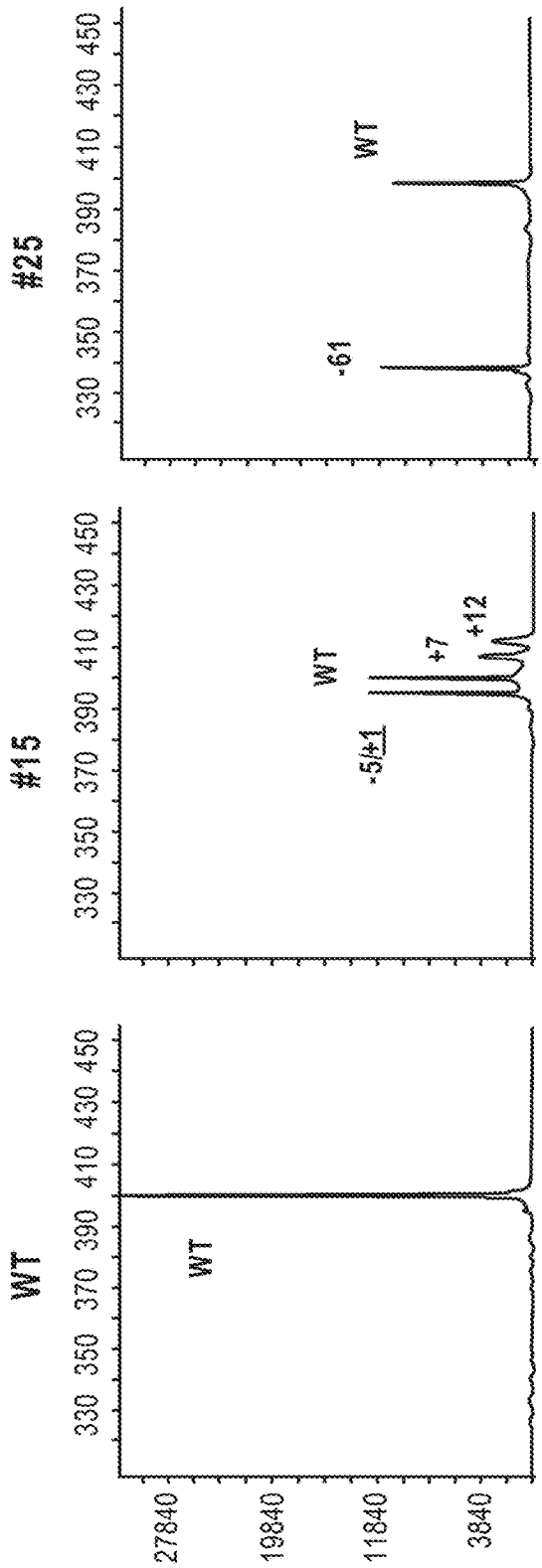

WT
#15
#25
330 350 370 390 410 430 450
WT
-5/+1
+7
+12
-61
27840
19840
11840
3840

FIG. 9A

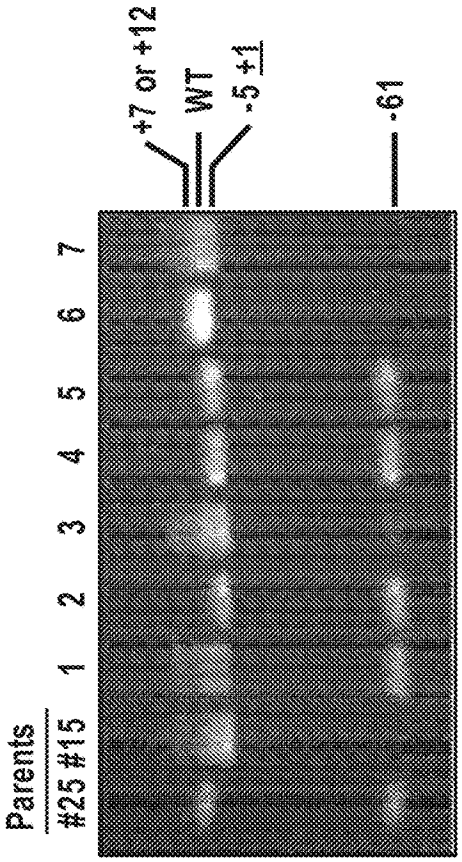

Parents
#25 #15 1 2 3 4 5 6 7
+7 or +12
WT
-5 +1
-61

FIG. 9B

CCR5 #4

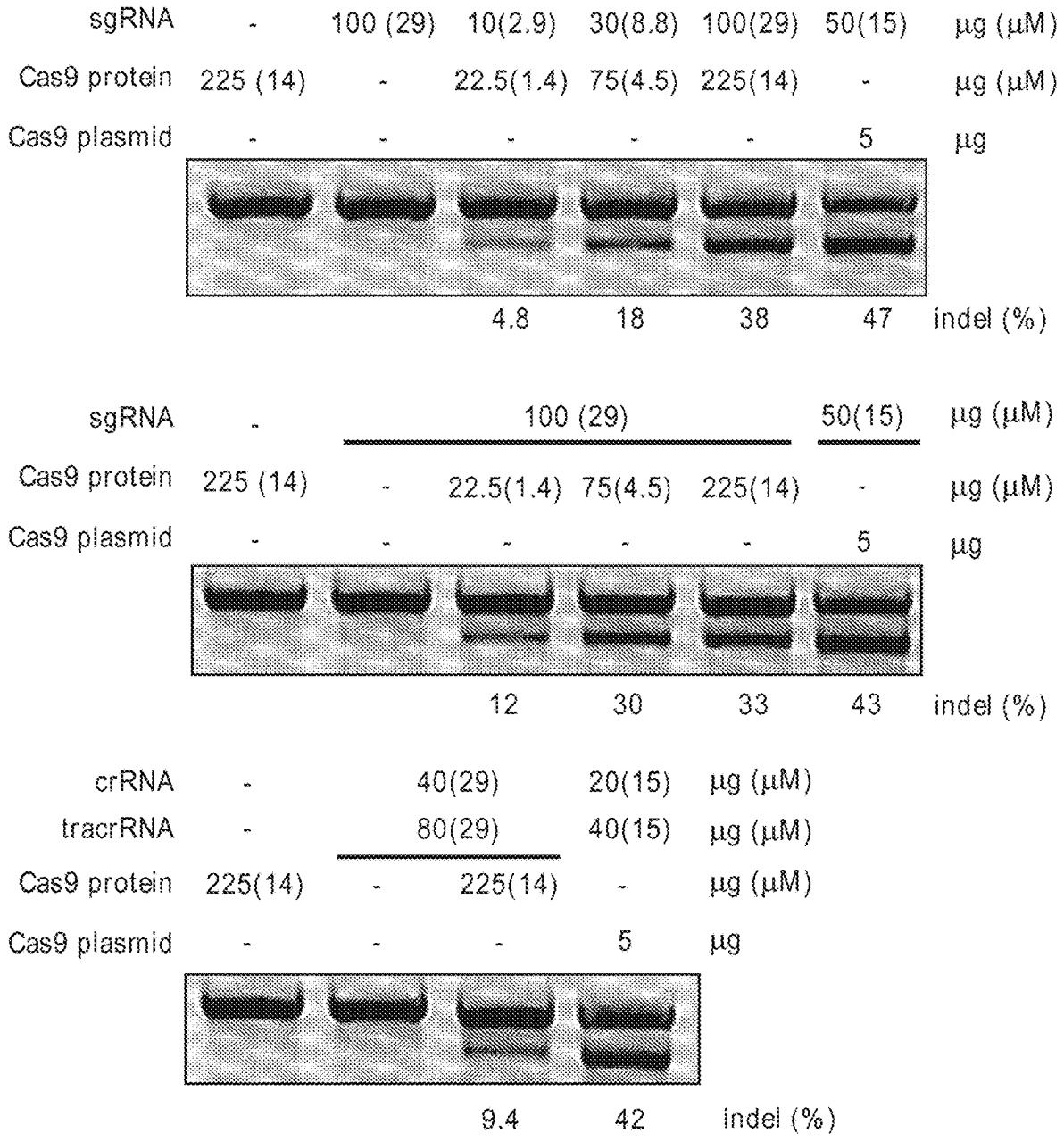


FIG. 10A

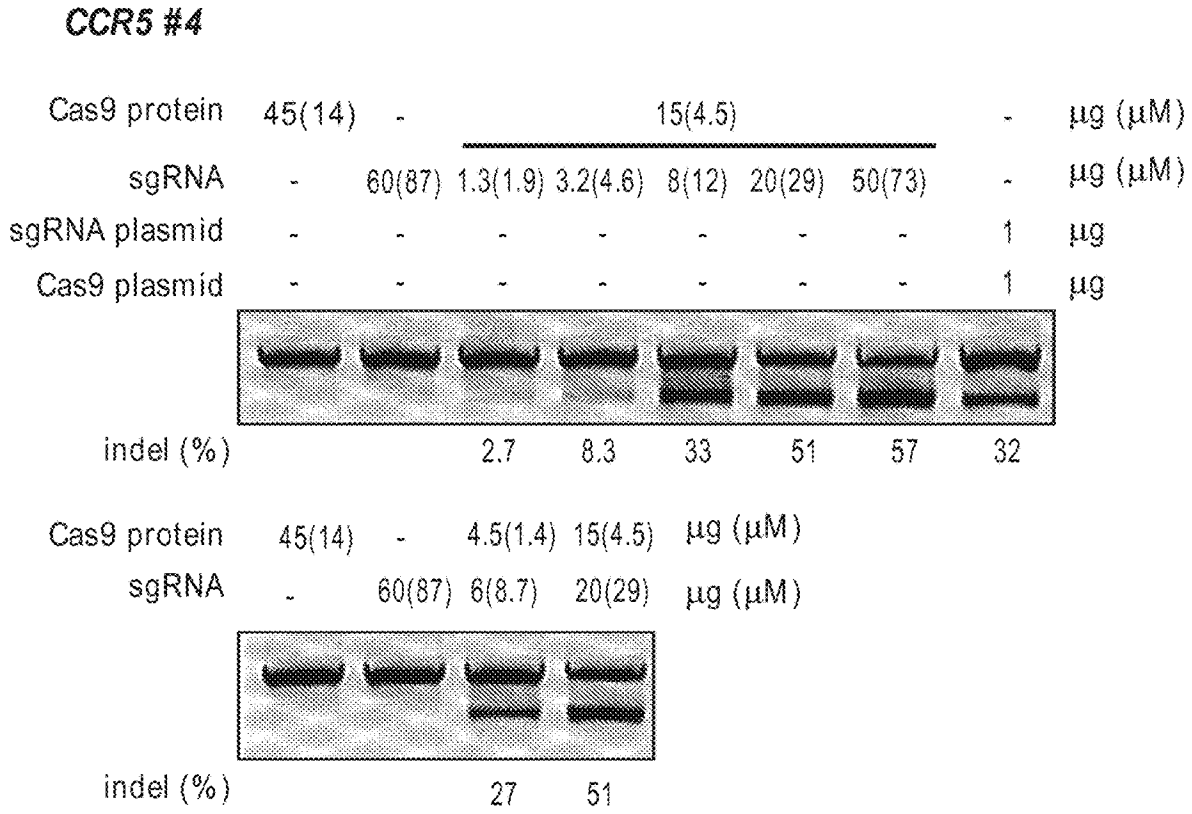

CCR5 #4
Cas9 protein 45(14) - 15(4.5) - μg (μM)
sgRNA - 60(87) 1.3(1.9) 3.2(4.6) 8(12) 20(29) 50(73) - μg (μM)
sgRNA plasmid - - - - - - - 1 μg
Cas9 plasmid - - - - - - - 1 μg
indel (%) 2.7 8.3 33 51 57 32
Cas9 protein 45(14) - 4.5(1.4) 15(4.5) μg (μM)
sgRNA - 60(87) 6(8.7) 20(29) μg (μM)
indel (%) 27 51

FIG. 10B

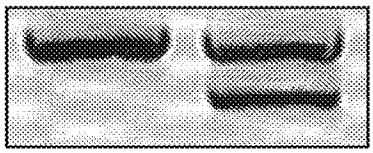
CCR5

```
CAATCTATGACATCAATTATTATA-CATCGGAGCCCTGCCAAAAAATCAA      WT
CAATCTATGACATCAATTATTAT-----CGGAGCCCTGCCAAAAAATCAA      -4
CAATCTATGACATCAATTAT-----CATCGGAGCCCTGCCAAAAAATCAA      -4
CAATCTATGACATCAATTAT--------CGGAGCCCTGCCAAAAAATCAA      -7
CAATCTATGACATCAATTATTAT--CATCGGAGCCCTGCCAAAAAATCAA      -1
CAATCTATGACATCAATTATTATAACATCGGAGCCCTGCCAAAAAATCAA      +1
CAATCTATGACAA----------------GAGCCCTGCCAAAAAATCAA  -17,+1
```

FIG. 10C

ABCC11

Cas9 protein	-	15(4.5)	μg (μM)
sgRNA	-	20(29)	μg (μM)



Indel (%)	35

FIG. 10D

ABCC11

```
TTCTCAAGGCAGCATCATACTTCCCCCACGGTGGGACAGCTGCCCTCCCTGG      WT
TTCTCAAGGCAGCATCATACTTCC------CTGGGACAGCTGCCCTCCCTGG      -6
TTCTCAAGGCAGCATCATACTTC---CACGGTGGGACAGCTGCCCTCCCTGG      -3
TTCTCAAGGCAGC-------------------------TGCCCTCCCTGG      -29
TTCTCAAGGCAGCATCATACTT-------------------CCCTCCCTGG      -20
TTCTCAAGGCAGCATCATACTT-------------------CCCTCCCTGG      -20
TTCTC-----------------------------------------------  -256
```

FIG. 10E

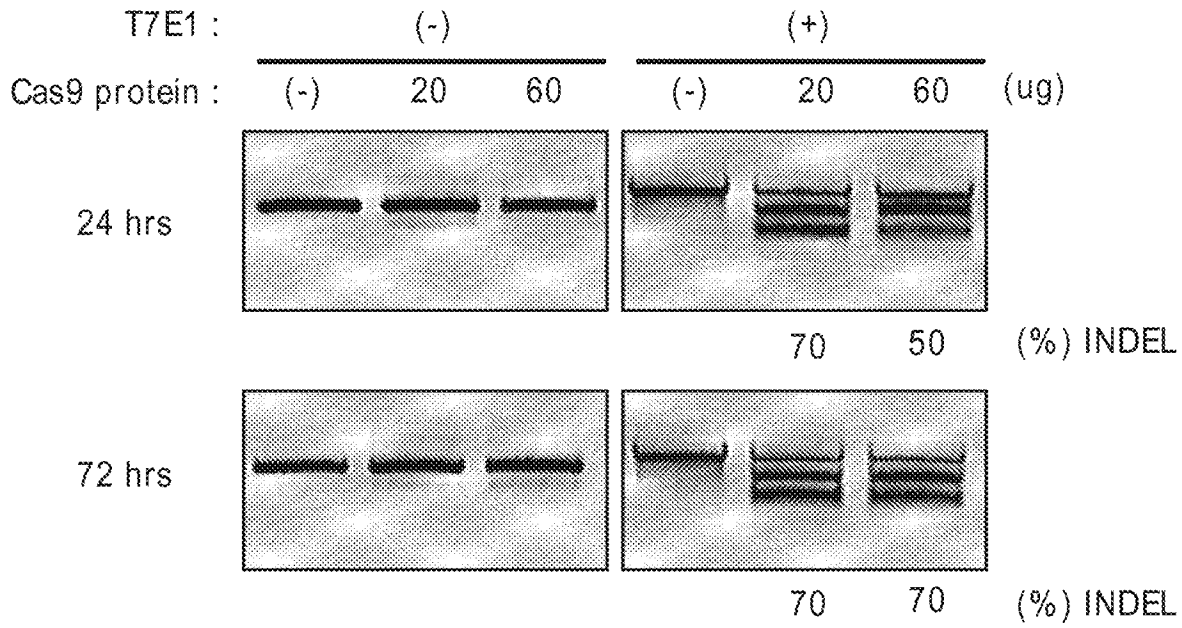

T7E1 :
(-)
(+)
Cas9 protein :
(-)
20
60
(-)
20
60
(ug)
24 hrs
70
50
(%) INDEL
72 hrs
70
70
(%) INDEL

FIG. 11

Target sequence 1
Target sequence 2
ACAAAGCGATTTTGAAAGATGGAAGCGCGGTGGTTAT//..... 190 bp .....//GGGGTGAAACTAAACTGGTCCACATGGTGGAAGATTG (wt)
ACAAAGCGATTTTGAAAGATGGAAGCGCGGTGGTTAT//..... 190 bp .....//GGGGTGAAACTAAA------ACATGGTGGAAGATTG (-7)
ACAAAGCGATTTTGAAAGATGGAAGCG--ACATGGTGGAAGATTG (-224)
ACAAAGCGATTTTGAAAGATGGAAGCG--CACATGGTGGAAGATTG (-223)
ACAAAGCGATTTTGAAAGATGGAAGCGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCACATGGTGGAAGATTG (-223+62)

FIG. 12

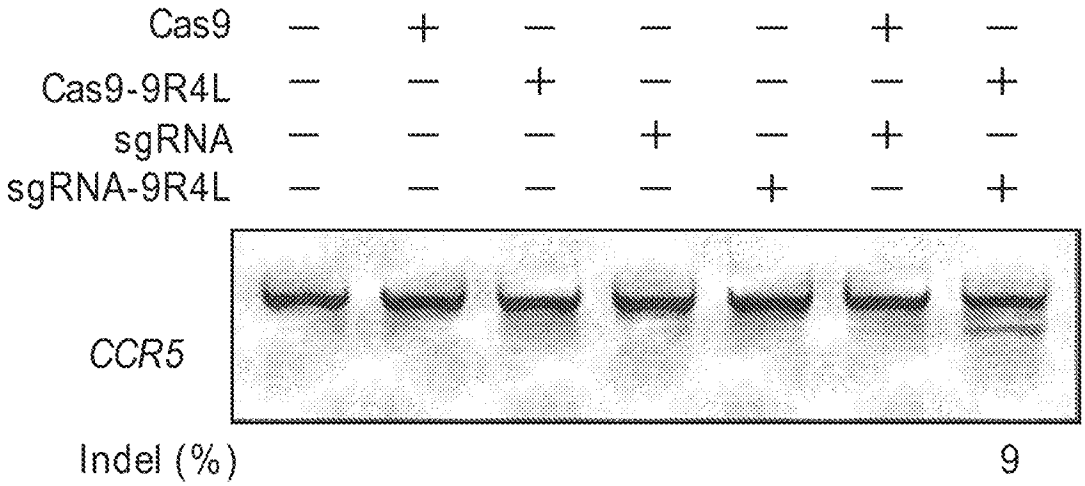


FIG. 13

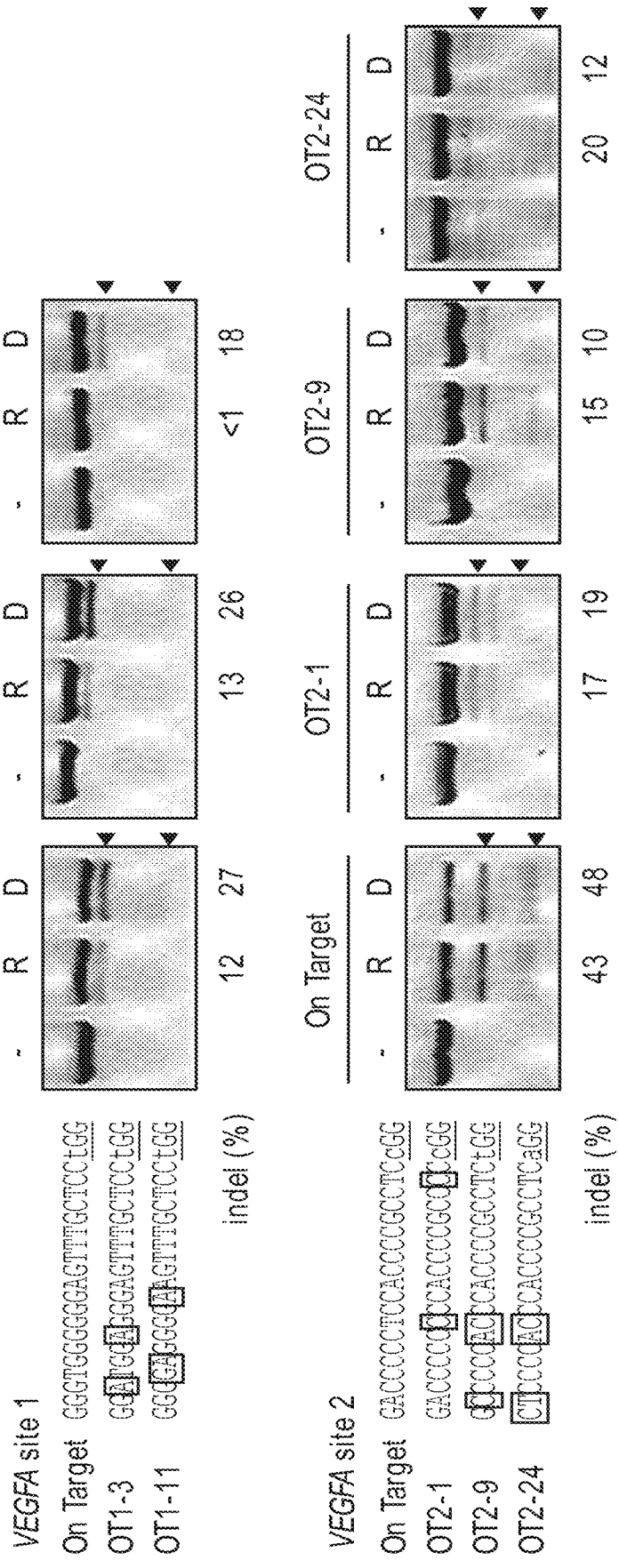

On Target
OT1-3
OT1-11
OT2-1
OT2-9
OT2-24
- R D
VEGFA site 1
VEGFA site 2
On Target GGGTGGGGGGAGTTTGCTCCTGG
OT1-3 GGGAGGGAGTTTGCTCCTGG
OT1-11 GGGGGGAGTTTGCTCCTGG
On Target GACCCCCTCCACCCCGCCTCCGG
OT2-1 GACCCCCTCCACCCCGCCTCCGG
OT2-9 GCCCCACCCCACCCCGCCTCTGG
OT2-24 CTCCCCACCCCACCCCGCCTCAGG
12 27
13 26
<1 18
43 48
17 19
15 10
20 12
indel (%)

FIG. 14A

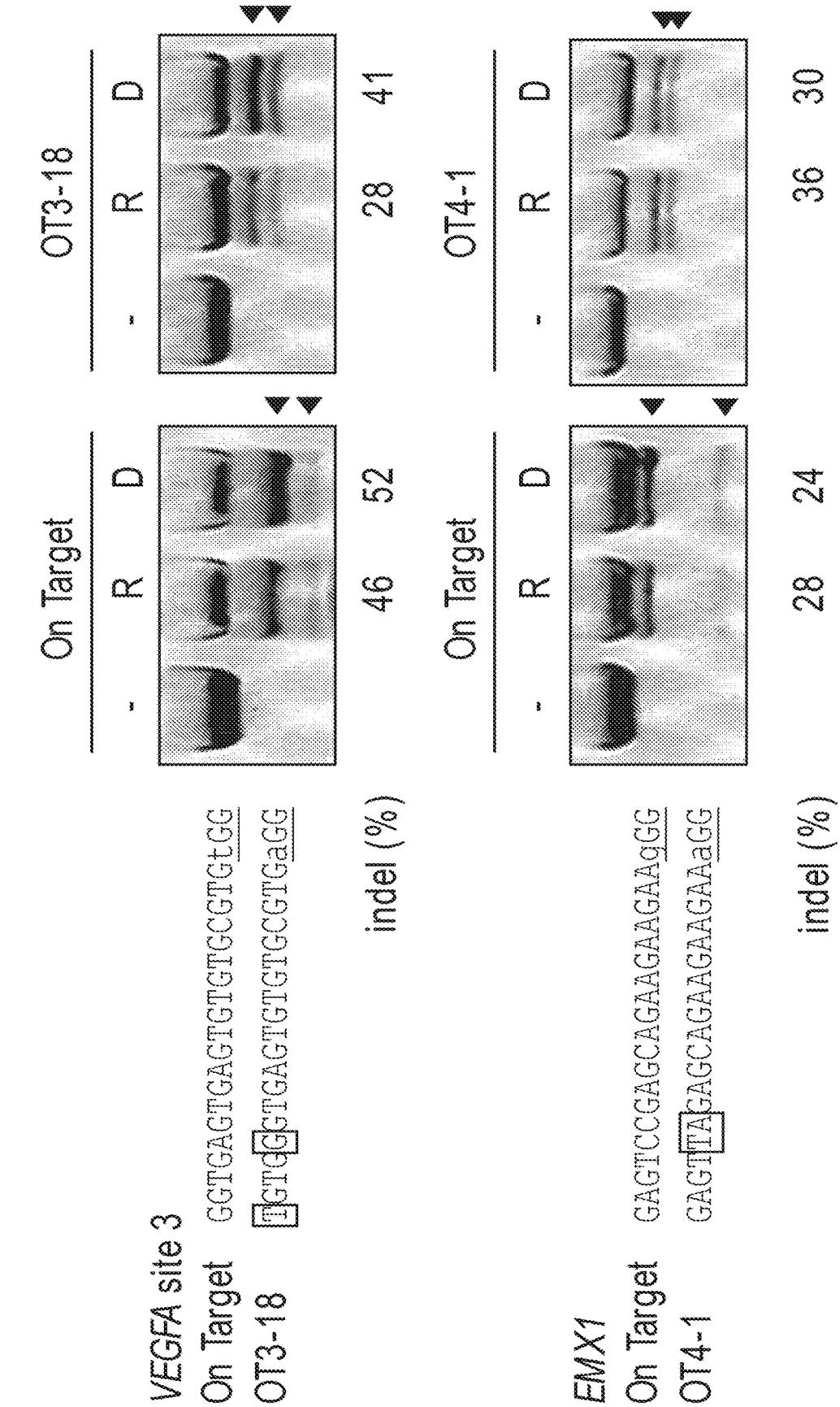

VEGFA site 3
On Target GGTGAGTGAGTGTGTGCGTGtGG
OT3-18 TGTGGGTGAGTGTGTGCGTGaGG
On Target
- R D
46 52
indel (%)
OT3-18
- R D
28 41
EMX1
On Target GAGTCCGAGCAGAAGAAGgGG
OT4-1 GAGTTAGAGCAGAAGAAGAaGG
On Target
- R D
28 24
indel (%)
OT4-1
- R D
36 30

FIG. 14B

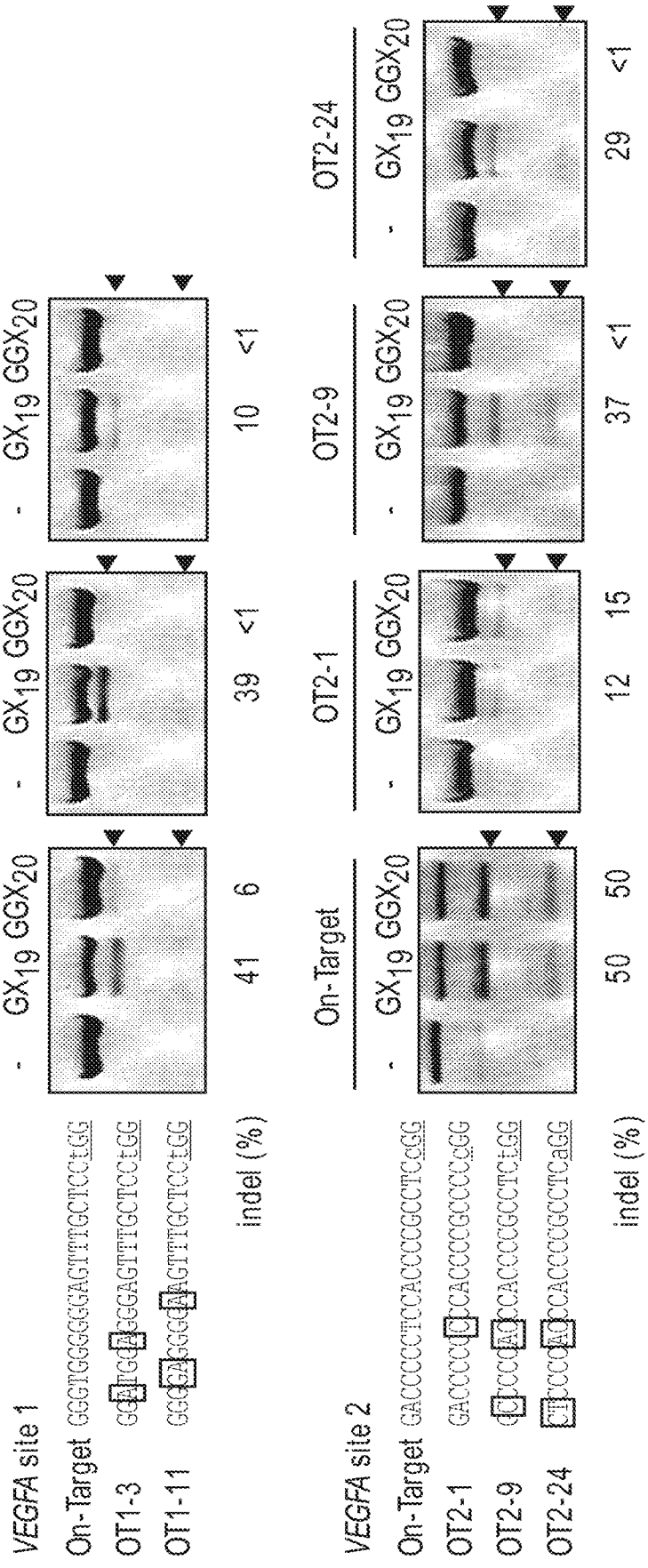

On-Target
OT1-3
OT1-11
- GX19 GGX20
- GX19 GGX20
- GX19 GGX20
VEGFA site 1
On-Target GGGTGGGGGGAGTTTGCTCCtGG
OT1-3 GGGTGGGGGAGTTTGCTCCtGG
OT1-11 GGGCAGGGGAAGTTTGCTCCtGG
indel (%)
41 6
39 <1
10 <1
On-Target
OT2-1
OT2-9
OT2-24
- GX19 GGX20
- GX19 GGX20
- GX19 GGX20
VEGFA site 2
On-Target GACCCCCTCCACCCCGCCTCcGG
OT2-1 GACCCCCTCACCCCGCCCCcGG
OT2-9 GCCCCAGCCACCCCGCCTCtGG
OT2-24 TTCCCCAGCCACCCCGCCTCaGG
indel (%)
50 50
12 15
37 <1
29 <1

FIG. 15A

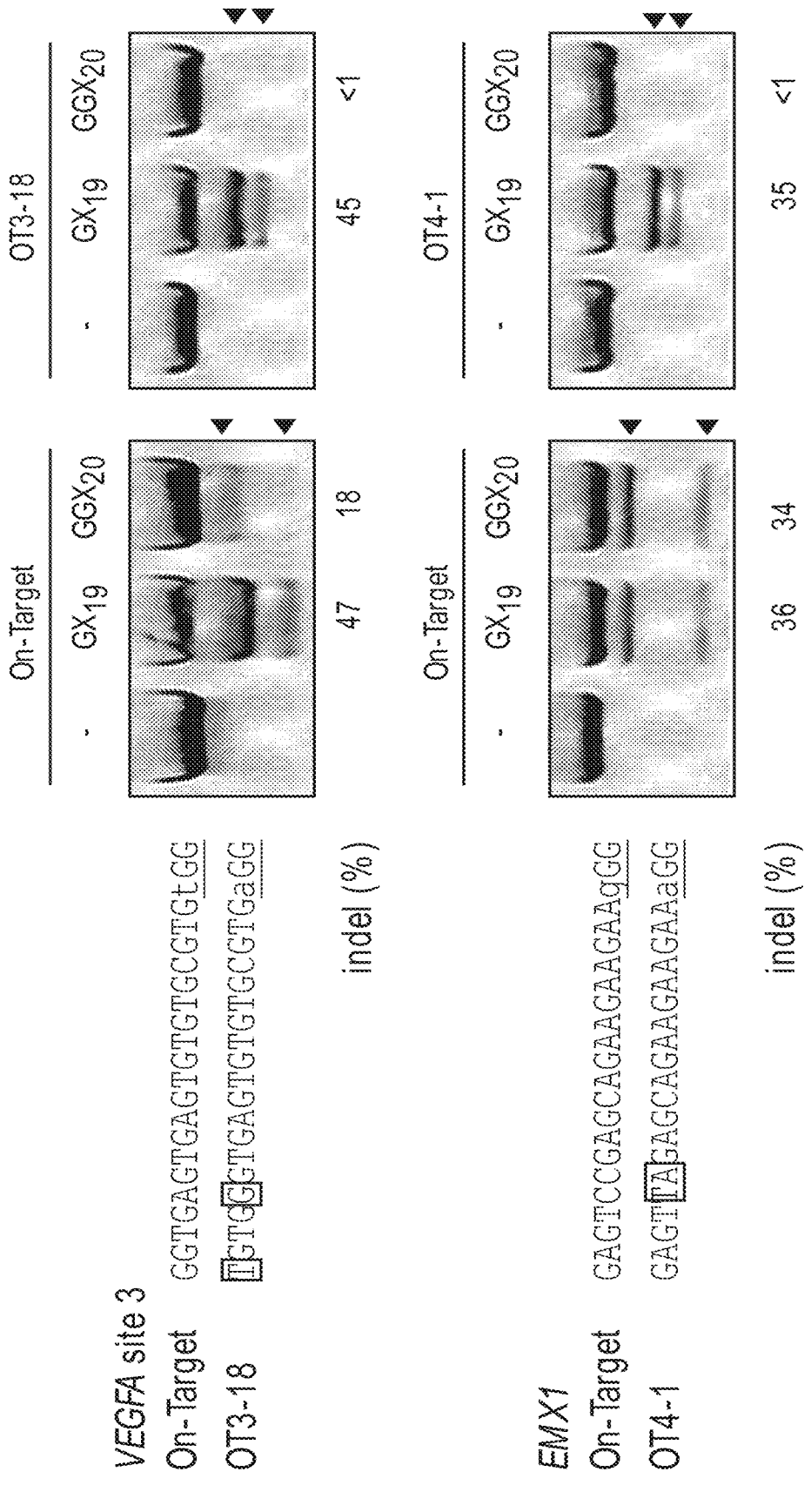

VEGFA site 3
On-Target GGTGAGTGAGTGTGTGCGTGtGG
OT3-18 TGTGGGTGAGTGTGTGCGTGaGG
EMX1
On-Target GAGTCCGAGCAGAAGAAGAAgGG
OT4-1 GAGTTAGCAGAAGAAGAAaGG
On-Target
OT3-18
OT4-1
- GX19 GGX20
indel (%)
47 18
45 <1
36 34
35 <1

FIG. 15B

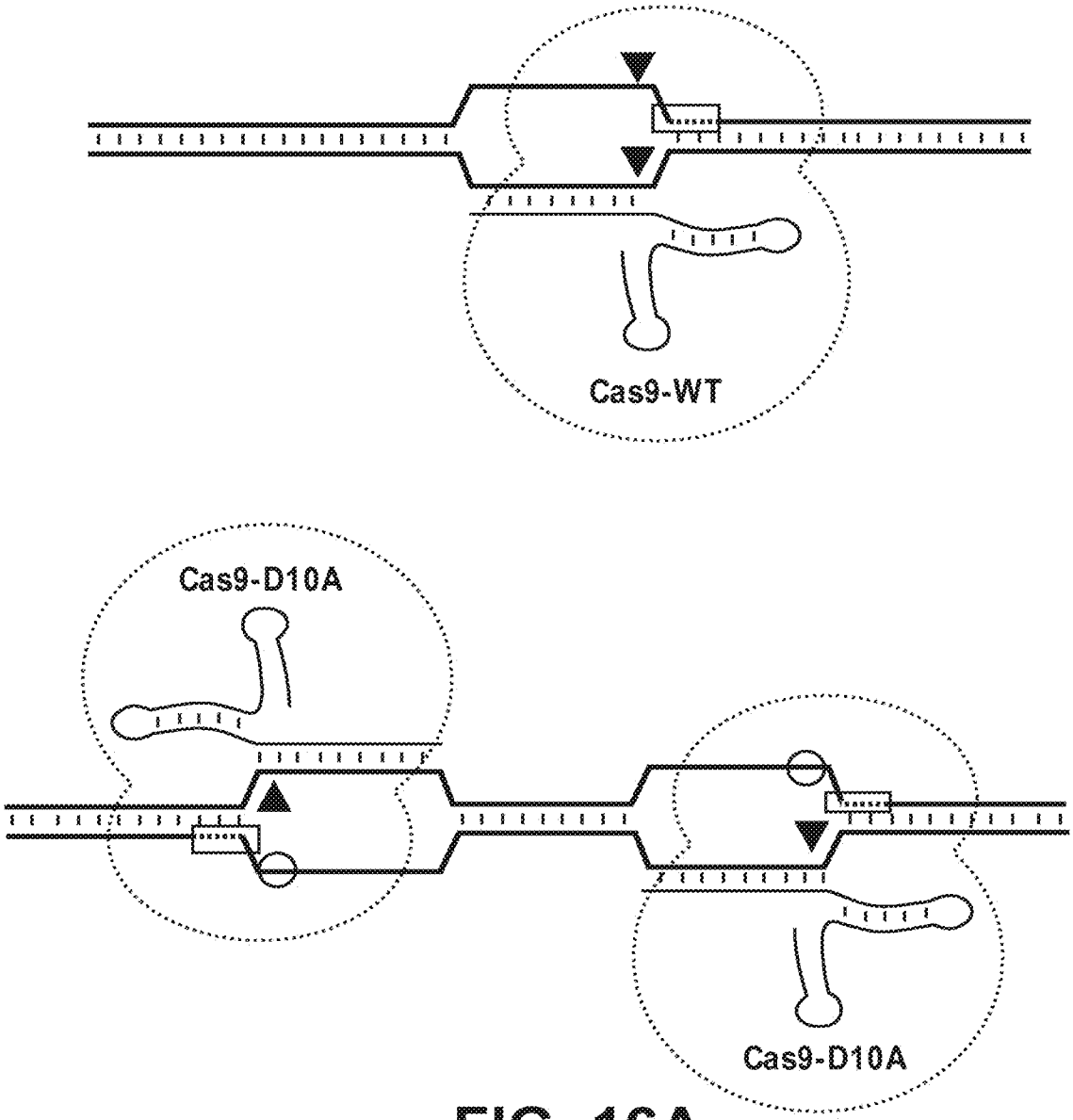


FIG. 16A

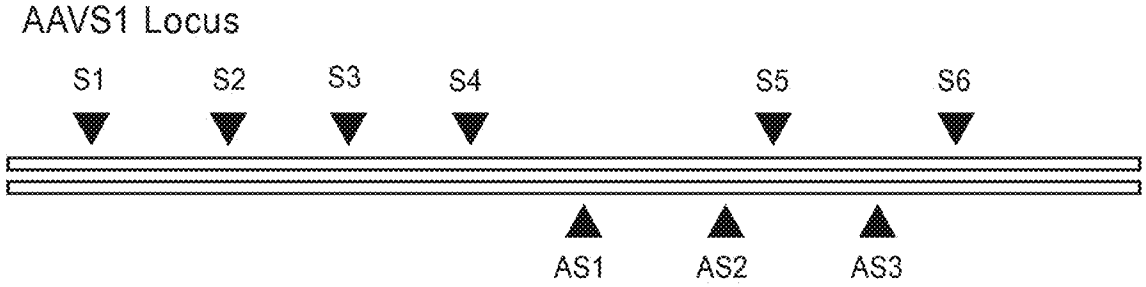


FIG. 16B

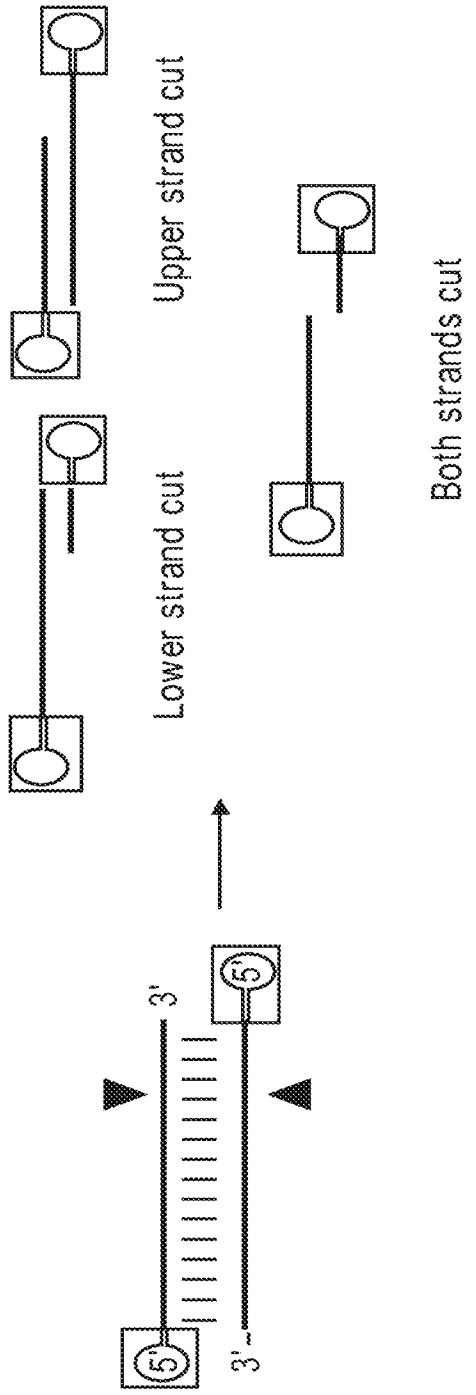


FIG. 16C

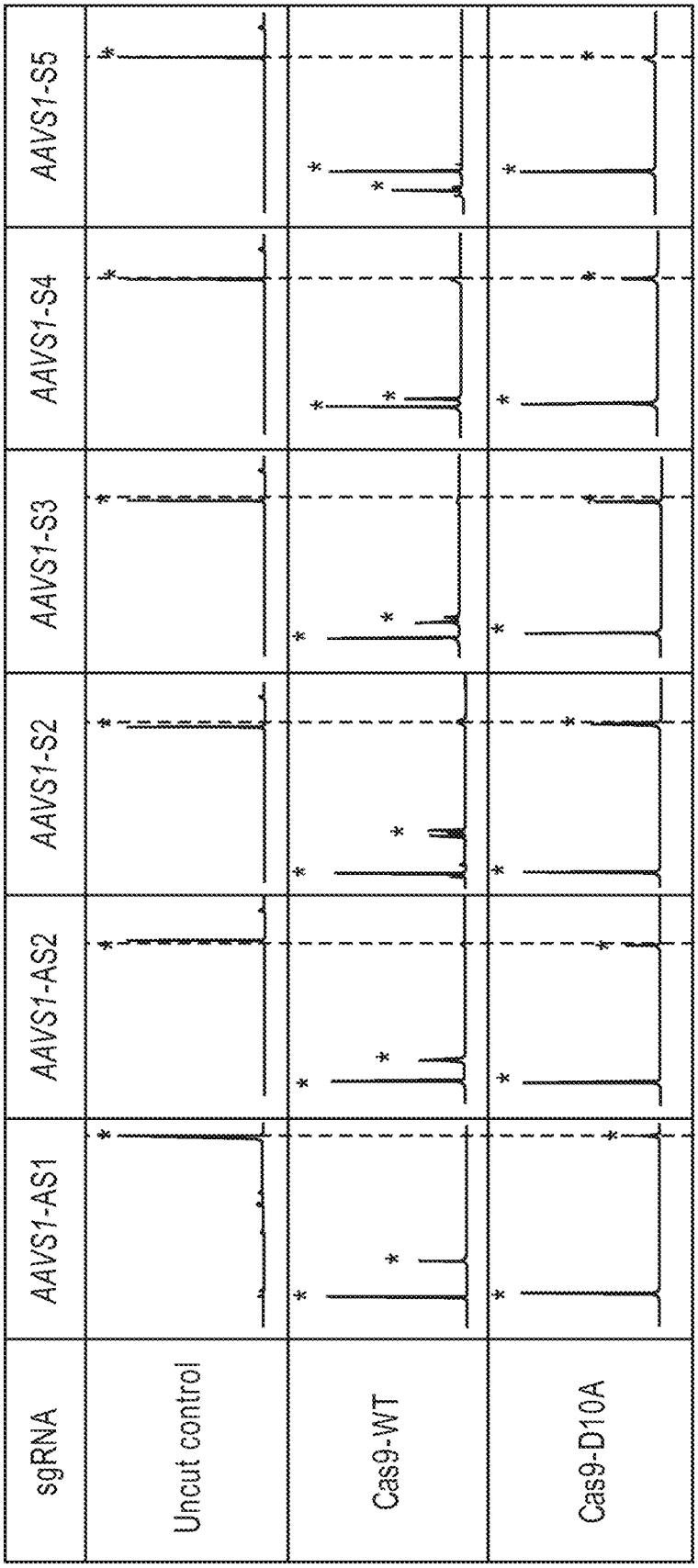

sgRNA
AAVS1-AS1
AAVS1-AS2
AAVS1-S2
AAVS1-S3
AAVS1-S4
AAVS1-S5
Uncut control
Cas9-WT
Cas9-D10A

FIG. 16D

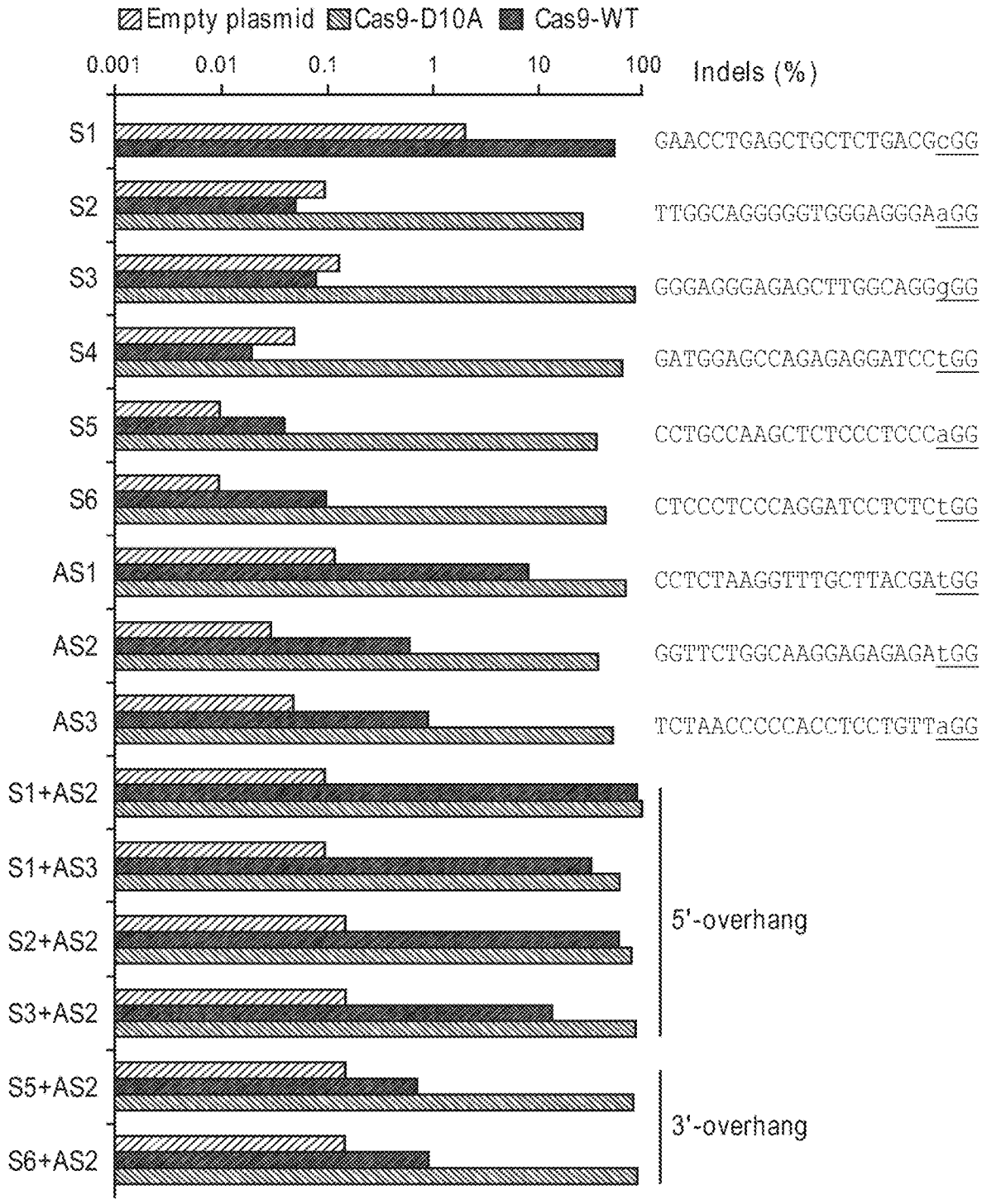

Empty plasmid
Cas9-D10A
Cas9-WT
0.001
0.01
0.1
1
10
100
Indels (%)
S1
GAACCTGAGCTGCTCTGACGcGG
S2
TTGGCAGGGGGTGGGAGGGAaGG
S3
GGGAGGGAGAGCTTGGCAGGgGG
S4
GATGGAGCCAGAGAGGATCCtGG
S5
CCTGCCAAGCTCTCCCTCCCaGG
S6
CTCCCTCCCAGGATCCTCTCtGG
AS1
CCTCTAAGGTTTGCTTACGAtGG
AS2
GGTTCTGGCAAGGAGAGAGAtGG
AS3
TCTAACCCCCACCTCCTGTTaGG
S1+AS2
S1+AS3
S2+AS2
S3+AS2
S5+AS2
S6+AS2
5'-overhang
3'-overhang

FIG. 17A

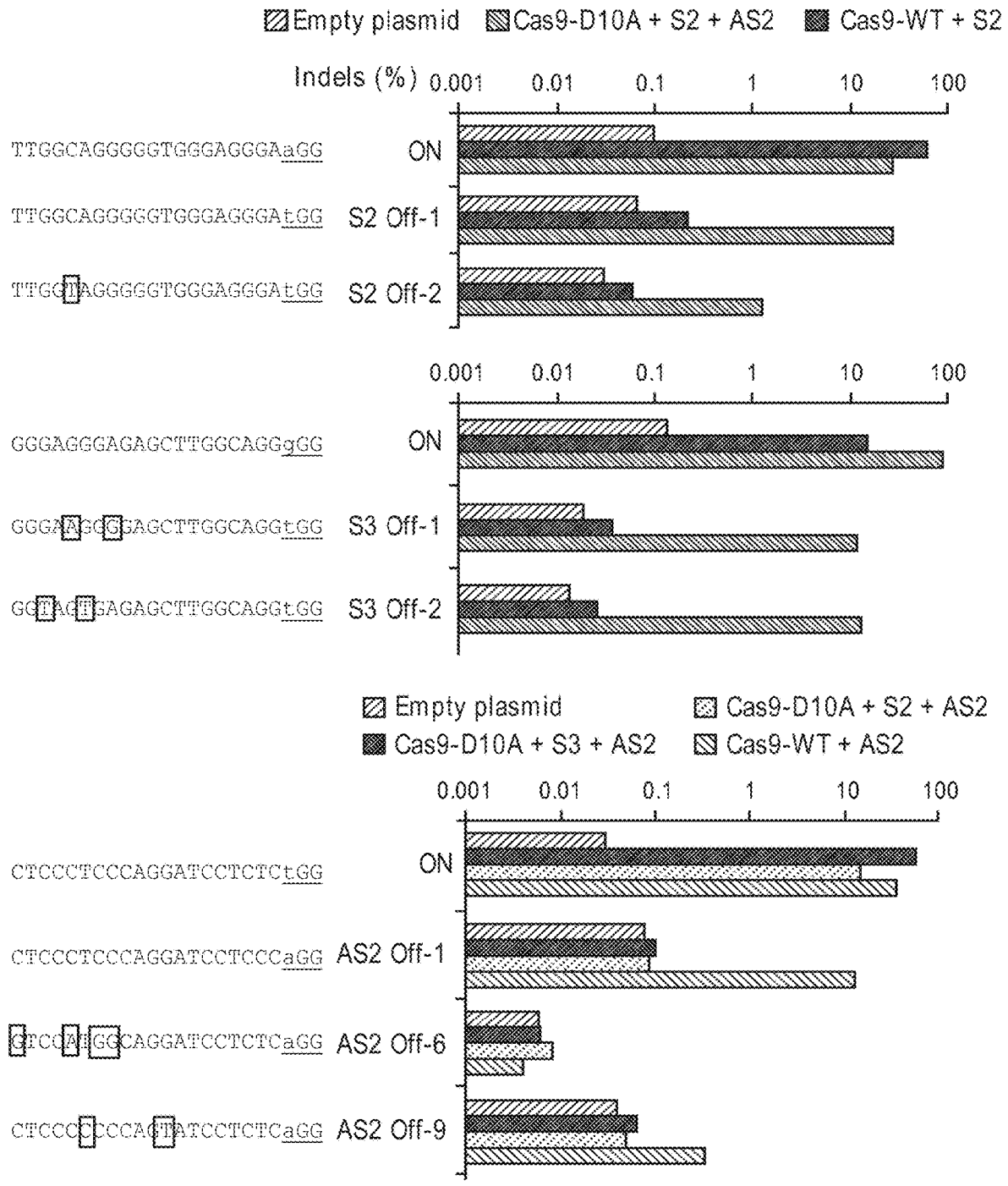

Empty plasmid
Cas9-D10A + S2 + AS2
Cas9-WT + S2
Indels (%)
0.001
0.01
0.1
1
10
100
TTGGCAGGGGGTGGGAGGGAaGG
ON
TTGGCAGGGGGTGGGAGGGAtGG
S2 Off-1
TTGGTAGGGGGTGGGAGGGAtGG
S2 Off-2
GGGAGGGAGAGCTTGGCAGGgGG
ON
GGGAAGGGGAGCTTGGCAGGtGG
S3 Off-1
GCTAGTGAGAGCTTGGCAGGtGG
S3 Off-2
Empty plasmid
Cas9-D10A + S2 + AS2
Cas9-D10A + S3 + AS2
Cas9-WT + AS2
CTCCCTCCCAGGATCCTCTCtGG
ON
CTCCCTCCCAGGATCCTCCCaGG
AS2 Off-1
GTCCAGGGCAGGATCCTCTCaGG
AS2 Off-6
CTCCCCCCCAGTATCCTCTCaGG
AS2 Off-9

FIG. 17B

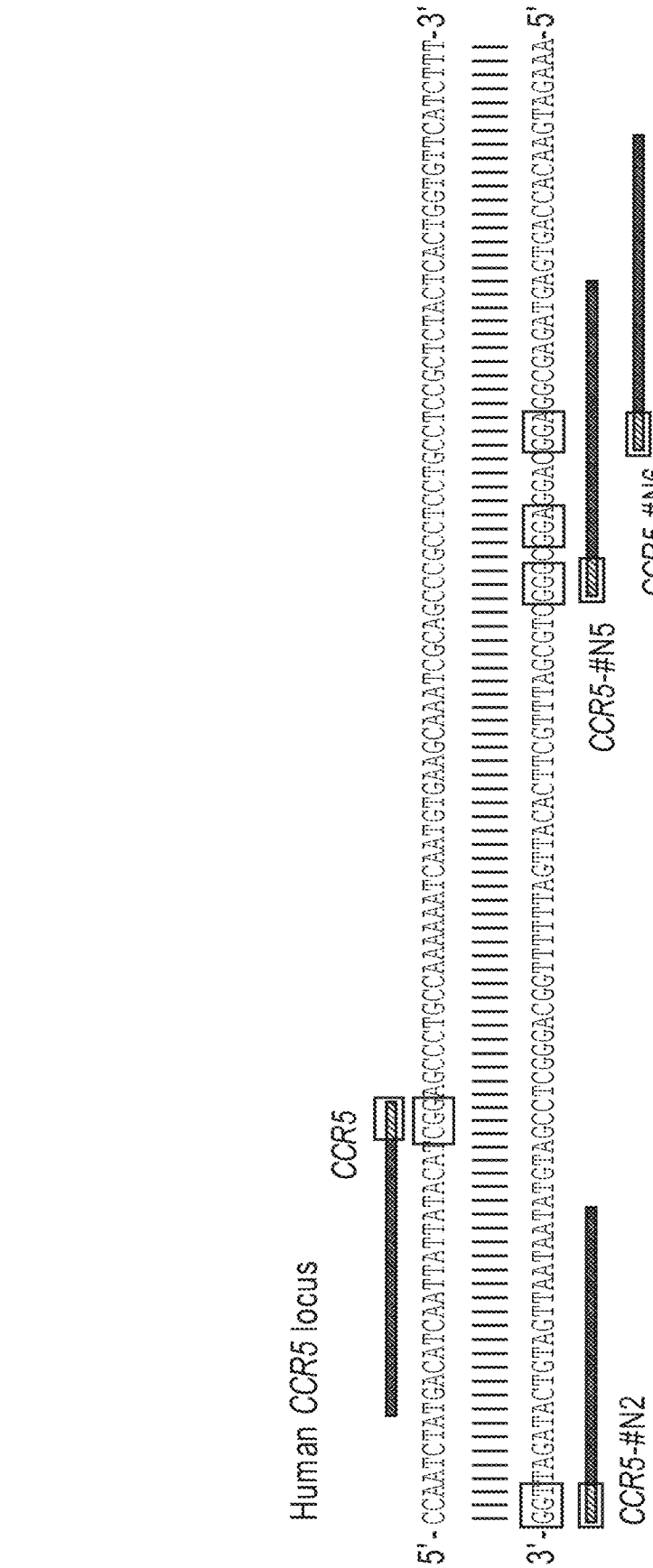

Human CCR5 locus
CCR5
5'- CCAATCTATGACATCAATTATTATACATCGGAGCCCTGCCAAAAAATCAATGTGAAGCAAATCGCAGCCCGCCTCCTGCCTCCGCTCTACTCACTGGTGTTCATCTTT-3'
3'-GGTTAGATACTGTAGTTAATAATATGTAGCCTCGGGACGGTTTTTTAGTTACACTTCGTTTAGCGTCGGCGACGGCAGATGAGTGACCACAAGTAGAAA-5'
CCR5-#N2
CCR5-#N5
CCR5-#N6

FIG. 18A

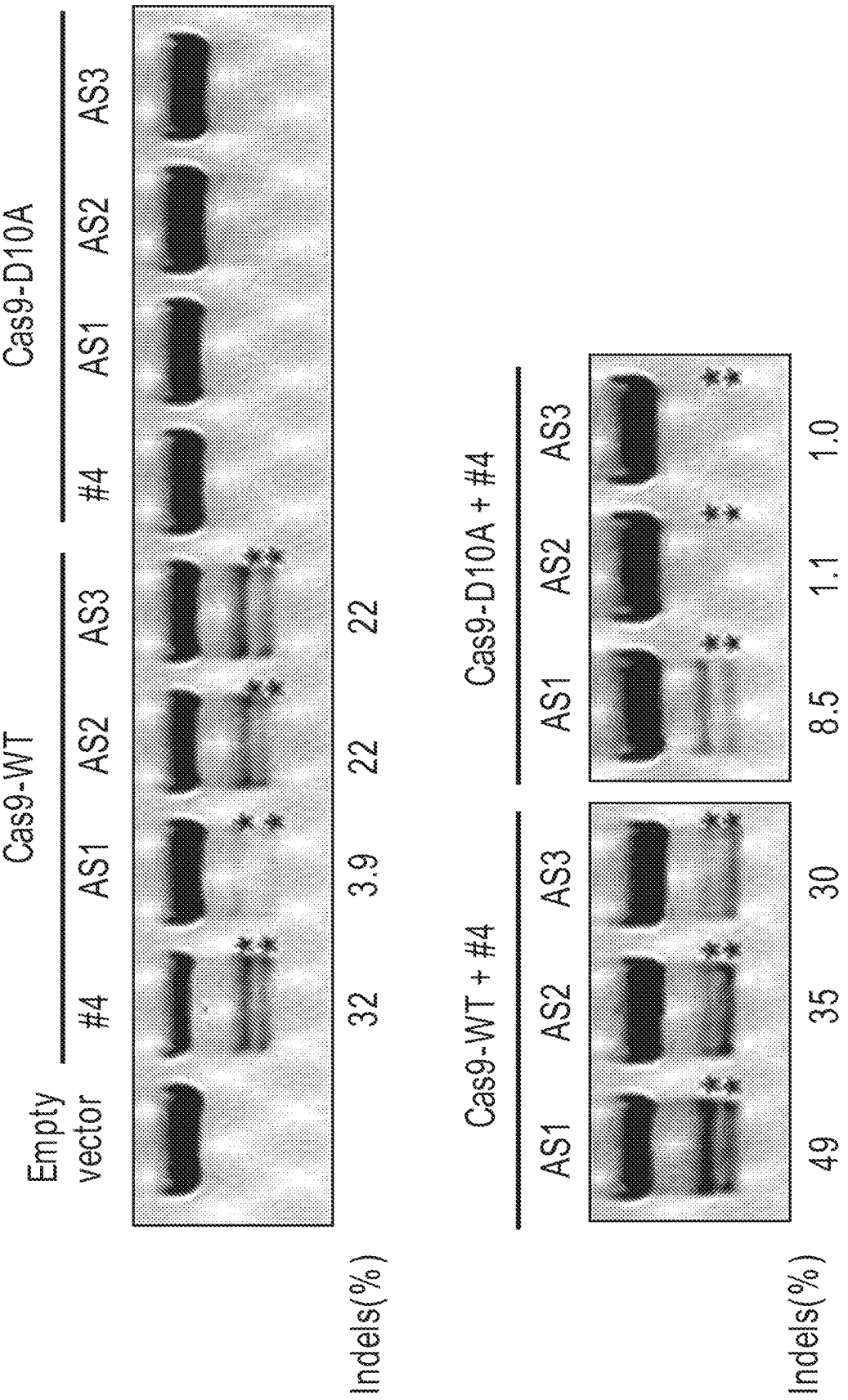

Empty vector
Cas9-WT
Cas9-D10A
#4
AS1
AS2
AS3
#4
AS1
AS2
AS3
Indels(%)
32
3.9
22
22
Cas9-WT + #4
Cas9-D10A + #4
AS1
AS2
AS3
AS1
AS2
AS3
Indels(%)
49
35
30
8.5
1.1
1.0

FIG. 18B

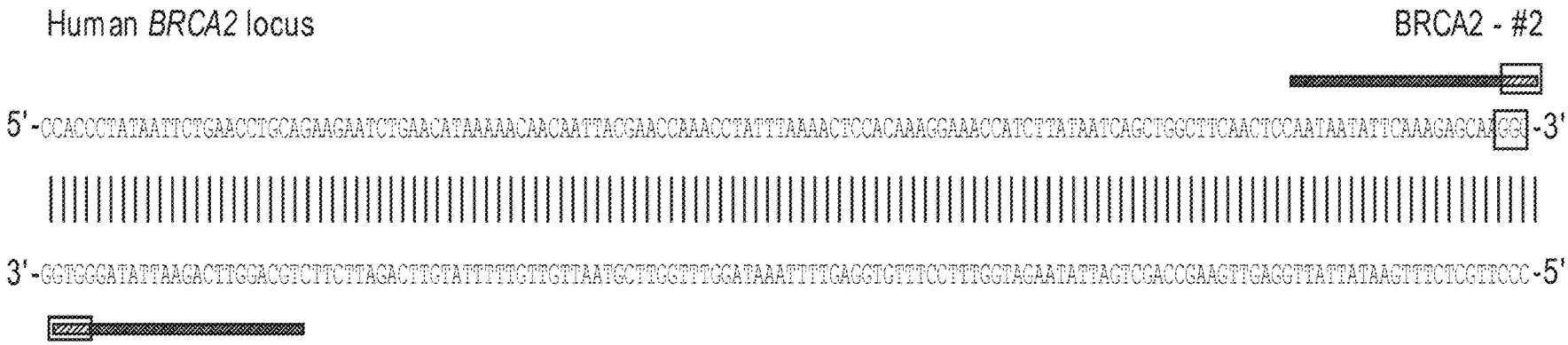


FIG. 18C

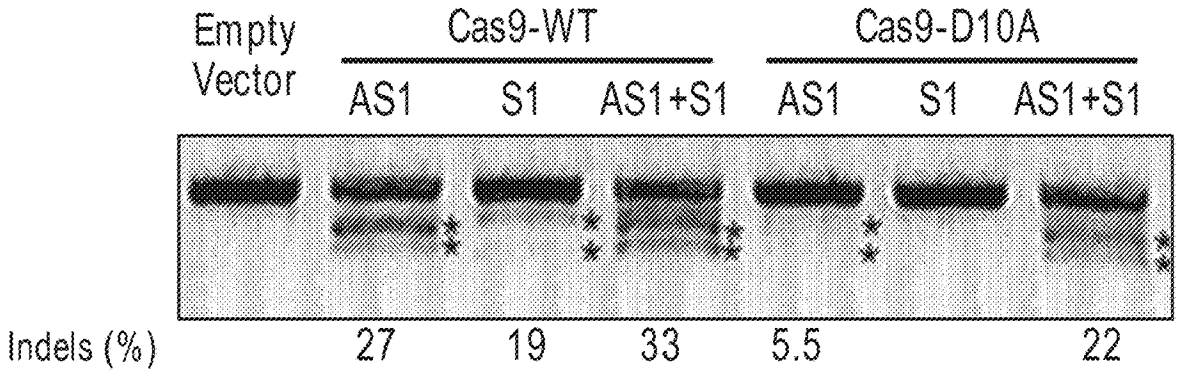


FIG. 18D

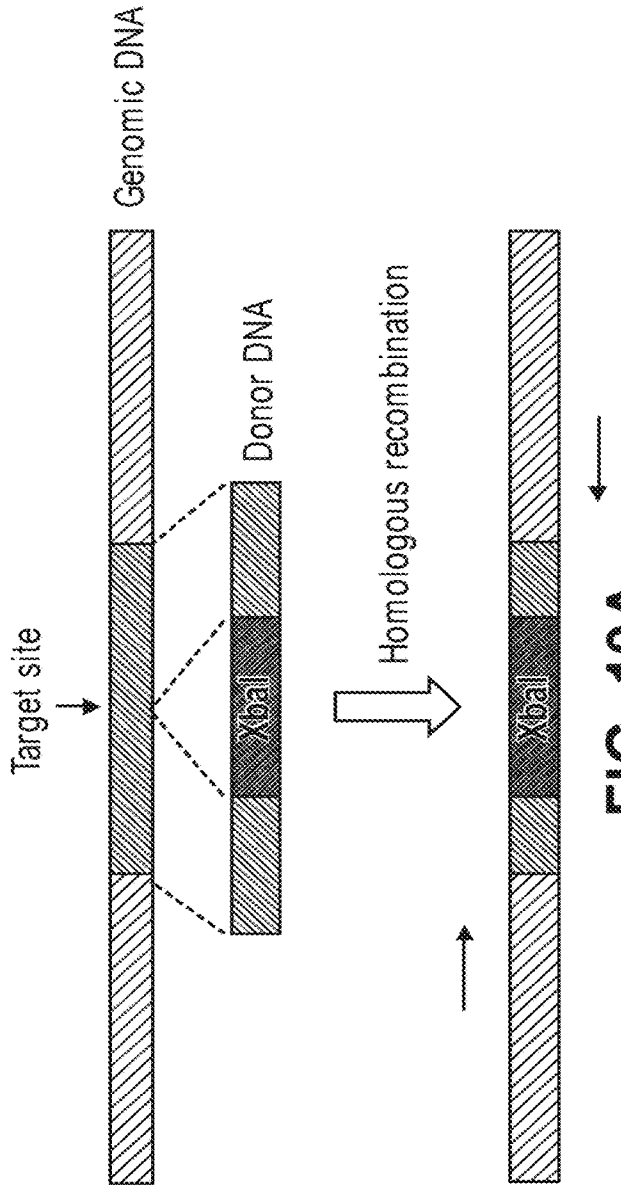


FIG. 19A

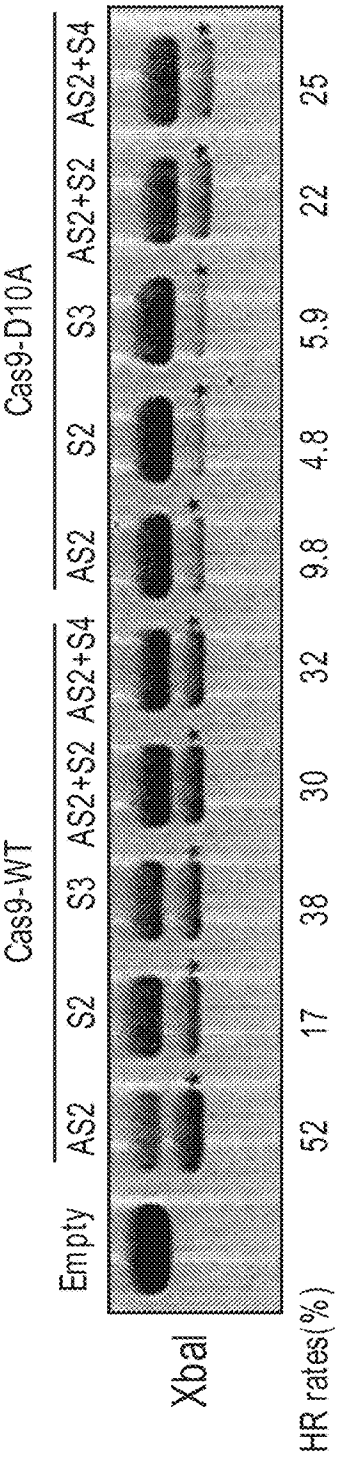


FIG. 19B

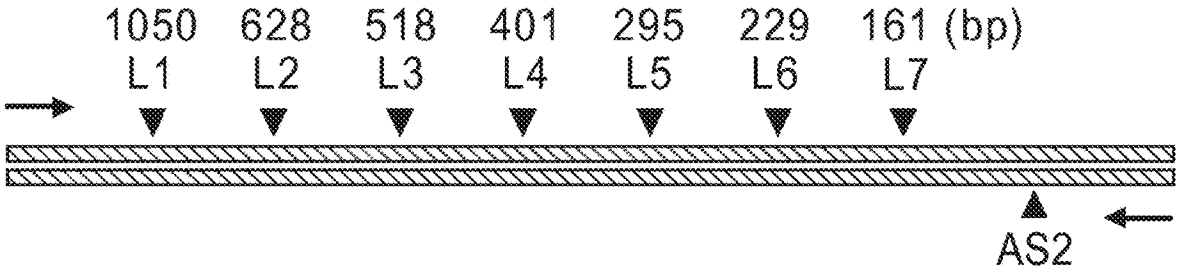


FIG. 20A

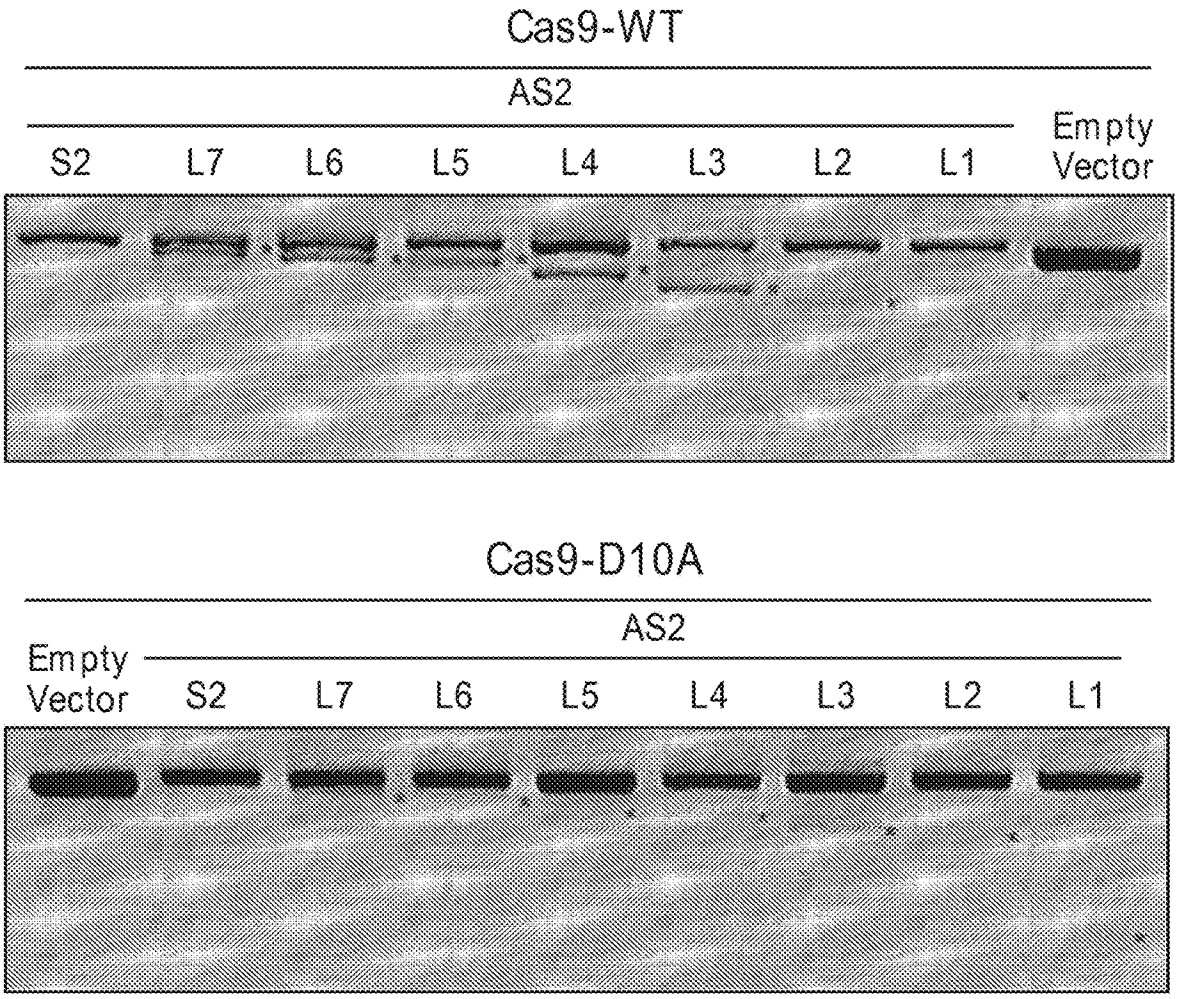


FIG. 20B

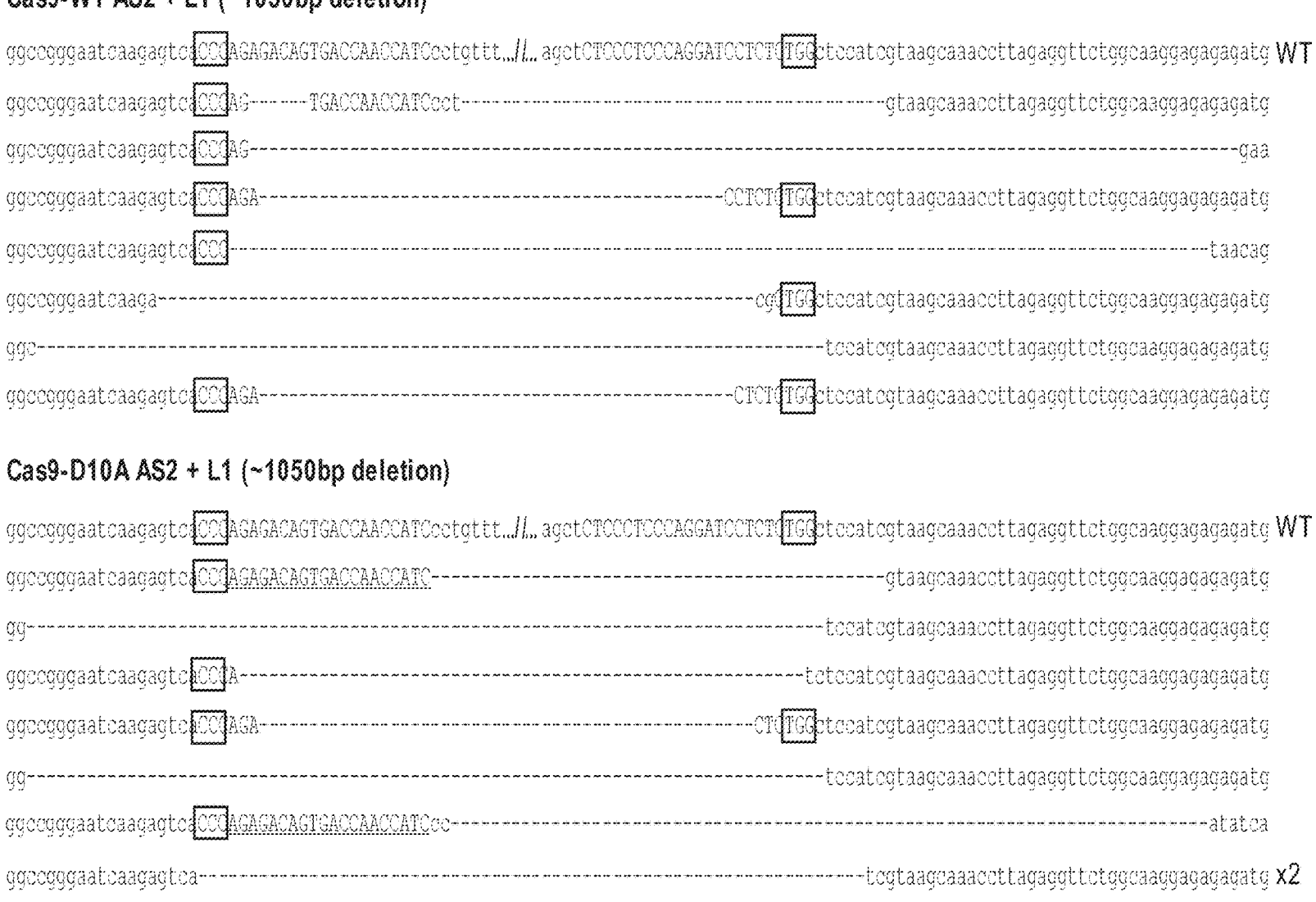

Cas9-WT AS2 + L1 (~1050bp deletion)
WT
Cas9-D10A AS2 + L1 (~1050bp deletion)
WT
x2

FIG. 20C

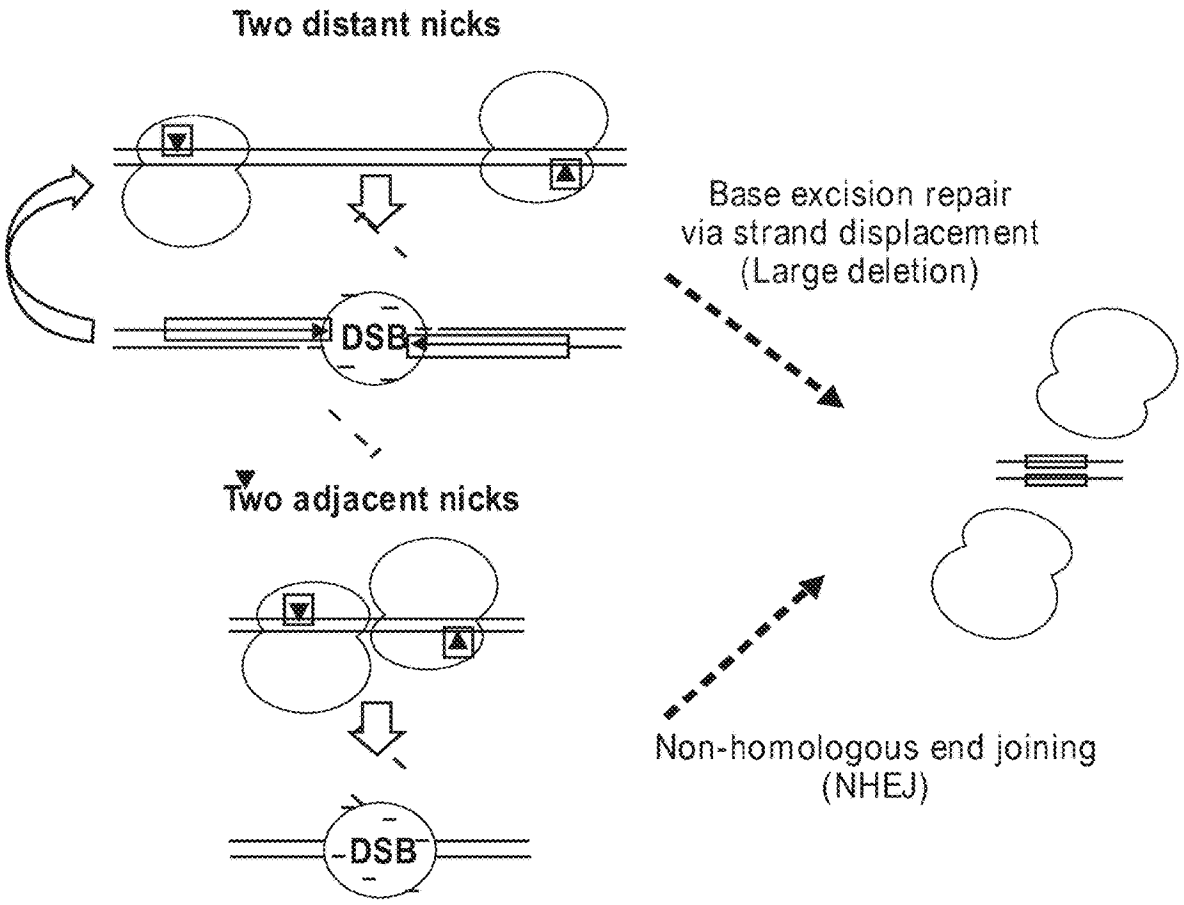

Two distant nicks
DSB
Base excision repair
via strand displacement
(Large deletion)
Two adjacent nicks
DSB
Non-homologous end joining
(NHEJ)

FIG. 20D

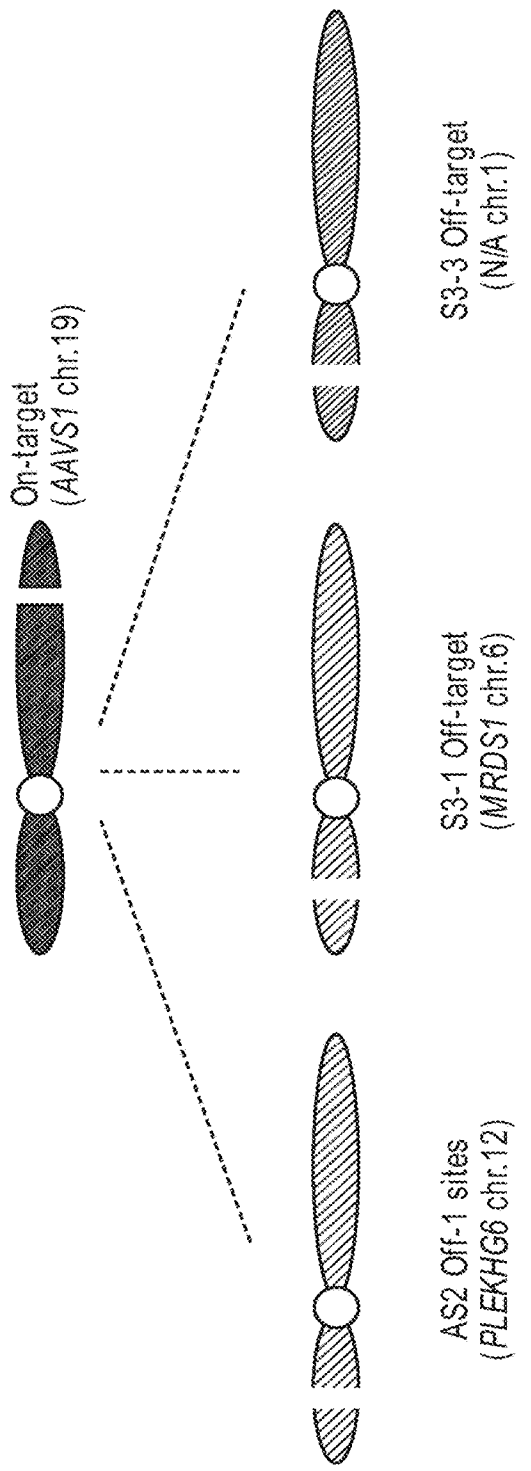
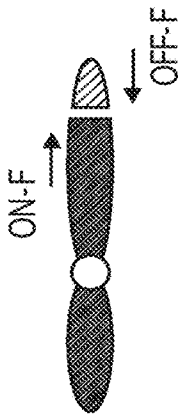


FIG. 21A



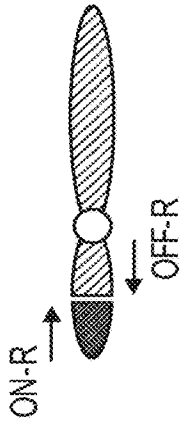


FIG. 21B

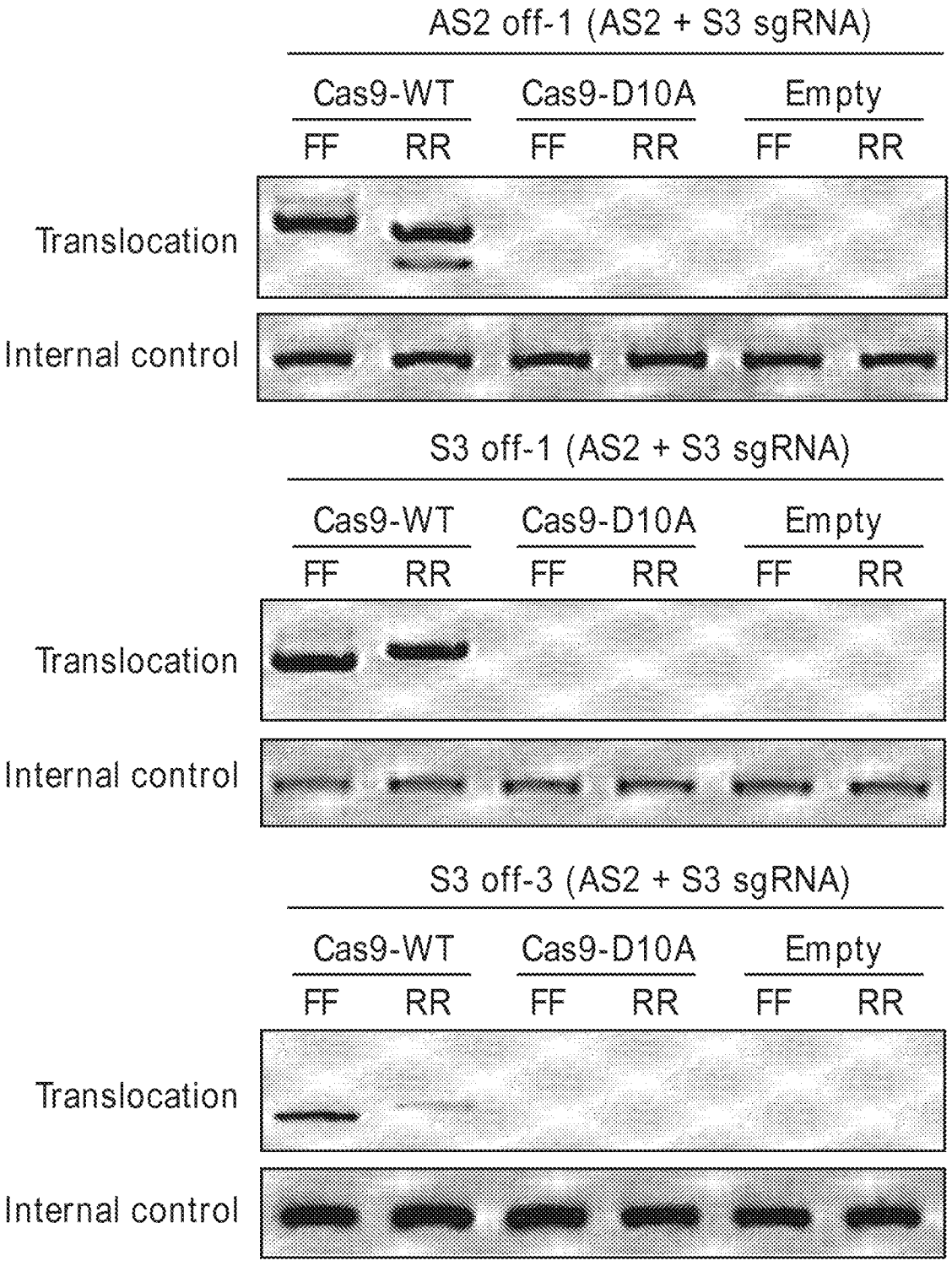

AS2 off-1 (AS2 + S3 sgRNA)
Cas9-WT
Cas9-D10A
Empty
FF
RR
FF
RR
FF
RR
Translocation
Internal control
S3 off-1 (AS2 + S3 sgRNA)
Cas9-WT
Cas9-D10A
Empty
FF
RR
FF
RR
FF
RR
Translocation
Internal control
S3 off-3 (AS2 + S3 sgRNA)
Cas9-WT
Cas9-D10A
Empty
FF
RR
FF
RR
FF
RR
Translocation
Internal control

FIG. 21C

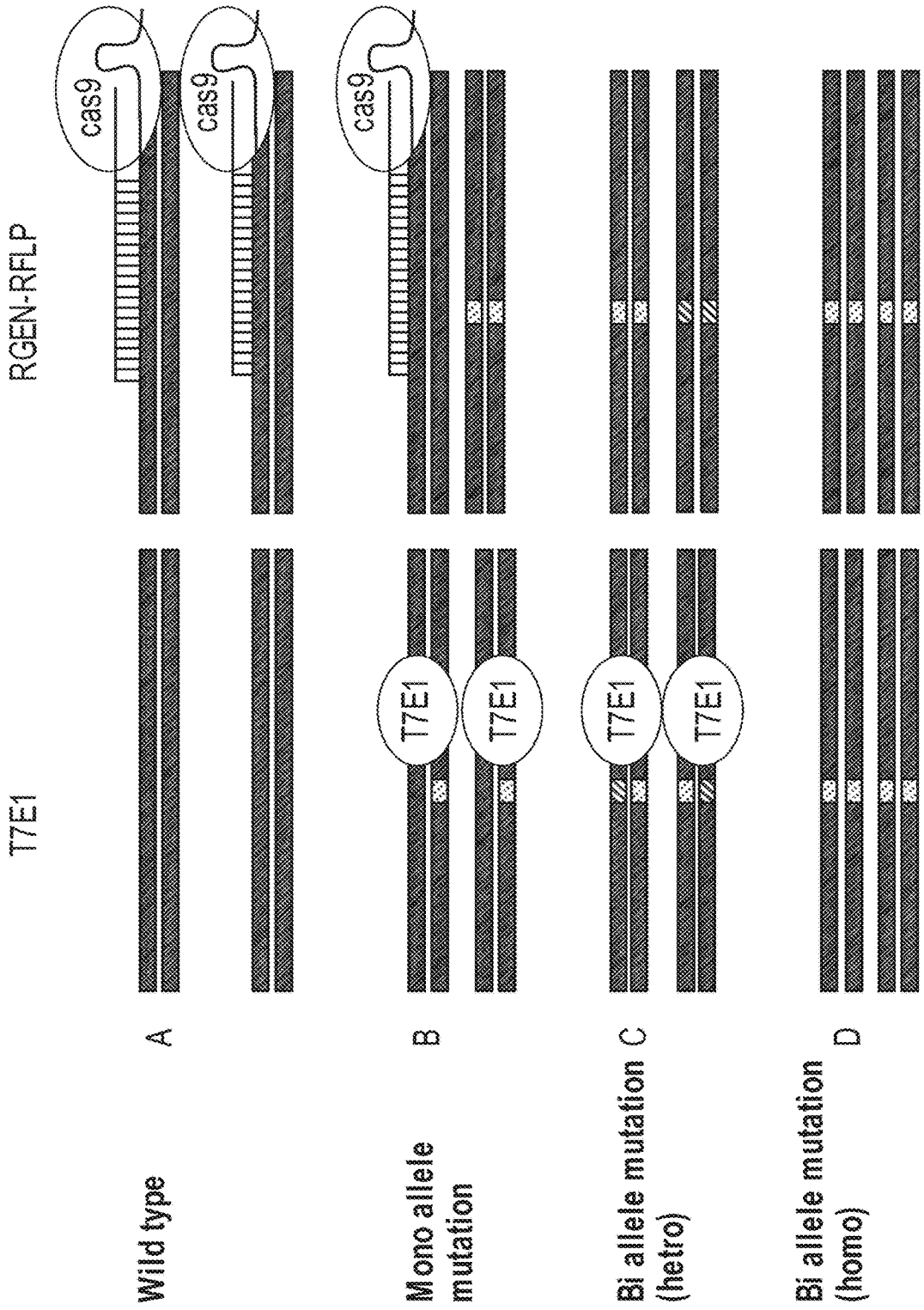

RGEN-RFLP
T7E1
cas9
cas9
cas9
T7E1
T7E1
T7E1
T7E1
Wild type
A
Mono allele mutation
B
Bi allele mutation (hetro)
C
Bi allele mutation (homo)
D

FIG. 22A

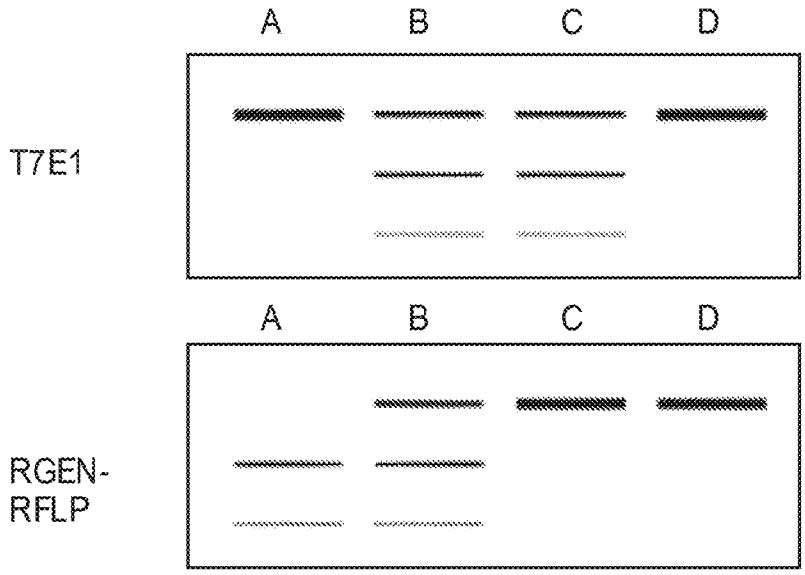


FIG. 22B

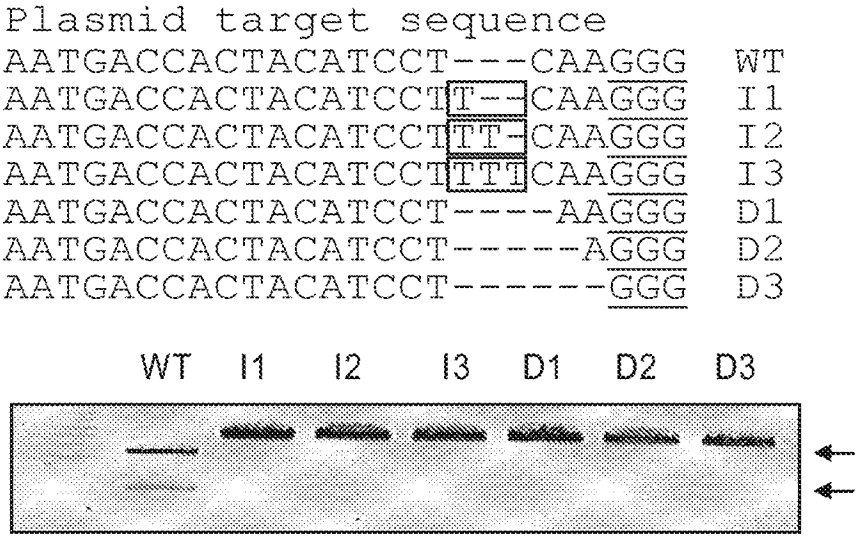


FIG. 23

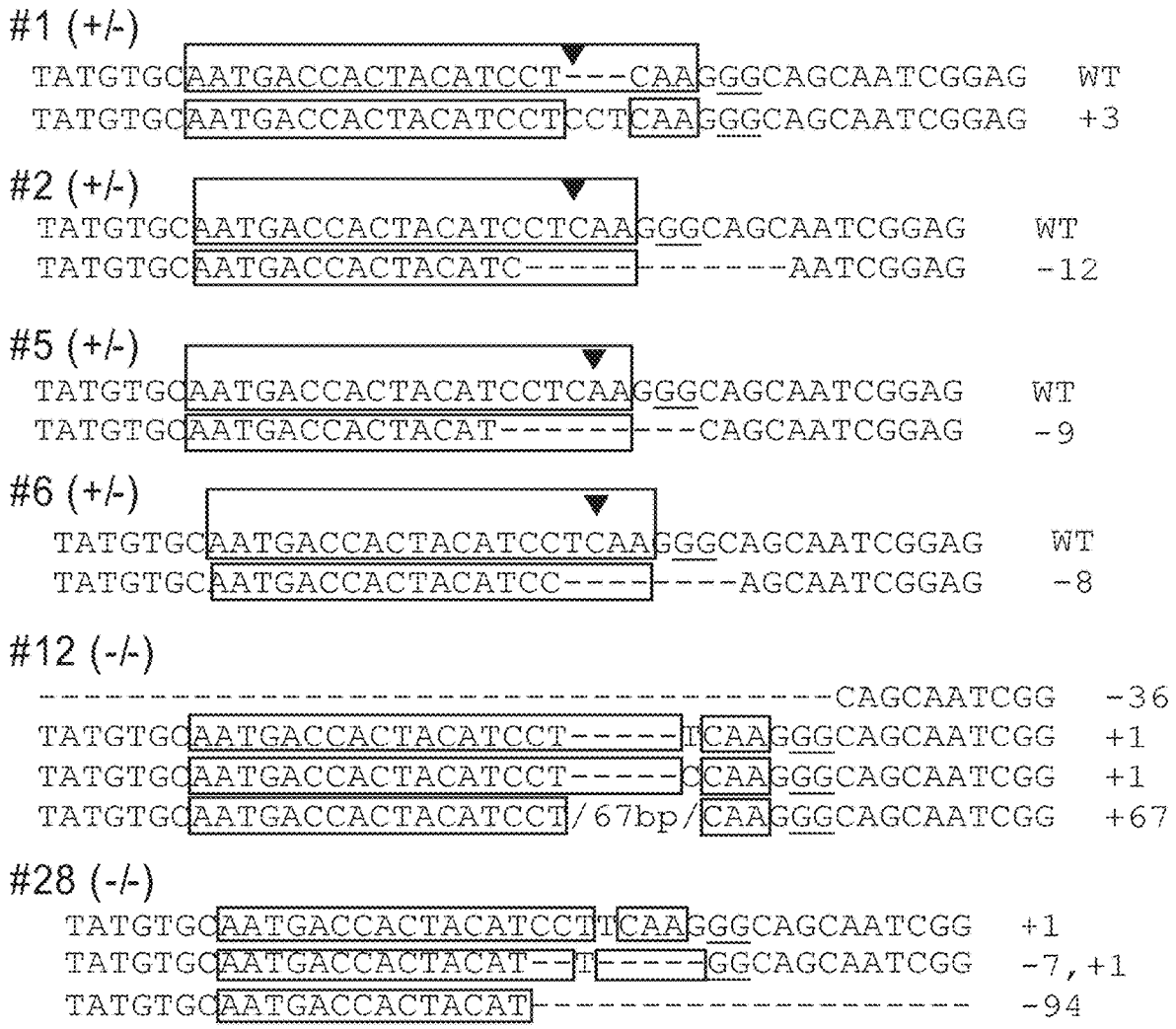

#1 (+/-)
TATGTGCAATGACCACTACATCCT---CAAGGGCAGCAATCGGAG WT
TATGTGCAATGACCACTACATCCTCCTCAAGGGCAGCAATCGGAG +3
#2 (+/-)
TATGTGCAATGACCACTACATCCTCAAGGGCAGCAATCGGAG WT
TATGTGCAATGACCACTACATC--------AATCGGAG -12
#5 (+/-)
TATGTGCAATGACCACTACATCCTCAAGGGCAGCAATCGGAG WT
TATGTGCAATGACCACTACAT------CAGCAATCGGAG -9
#6 (+/-)
TATGTGCAATGACCACTACATCCTCAAGGGCAGCAATCGGAG WT
TATGTGCAATGACCACTACATCC----AGCAATCGGAG -8
#12 (-/-)
CAGCAATCGG -36
TATGTGCAATGACCACTACATCCT-----TCAAGGGCAGCAATCGG +1
TATGTGCAATGACCACTACATCCT-----CCAAGGGCAGCAATCGG +1
TATGTGCAATGACCACTACATCCT/67bp/CAAGGGCAGCAATCGG +67
#28 (-/-)
TATGTGCAATGACCACTACATCCTTCAAGGGCAGCAATCGG +1
TATGTGCAATGACCACTACAT--T-----GGCAGCAATCGG -7,+1
TATGTGCAATGACCACTACAT---------- -94

FIG. 24A

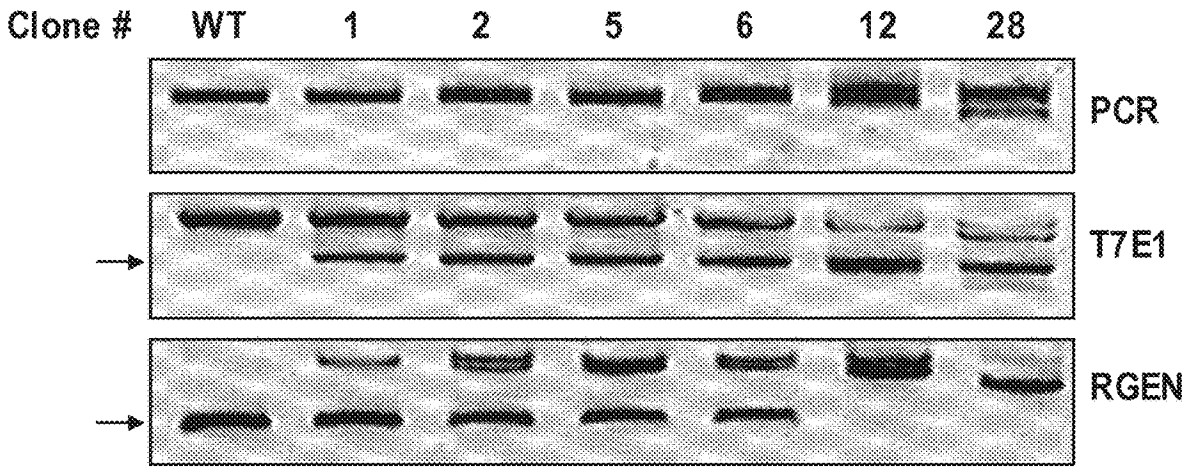


FIG. 24B

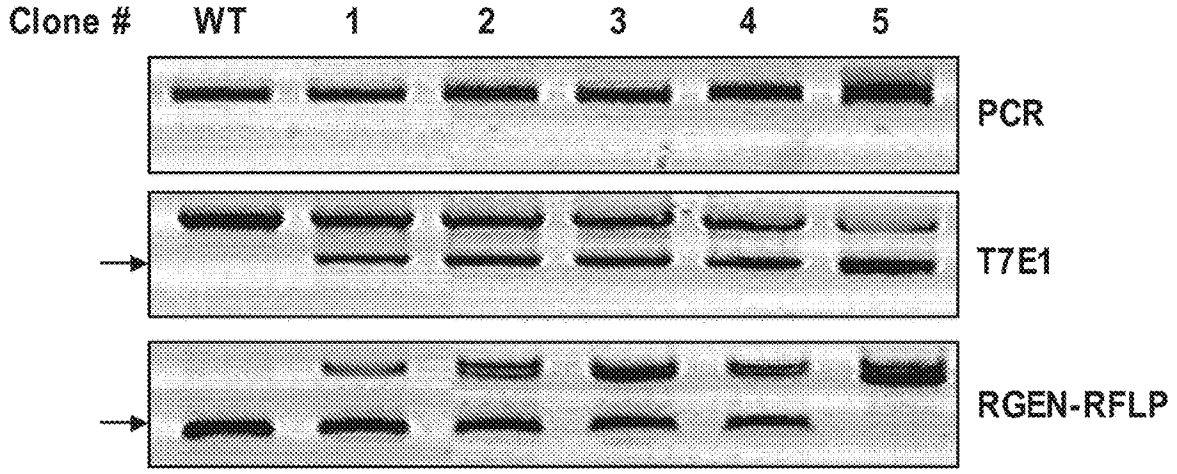


FIG. 25A

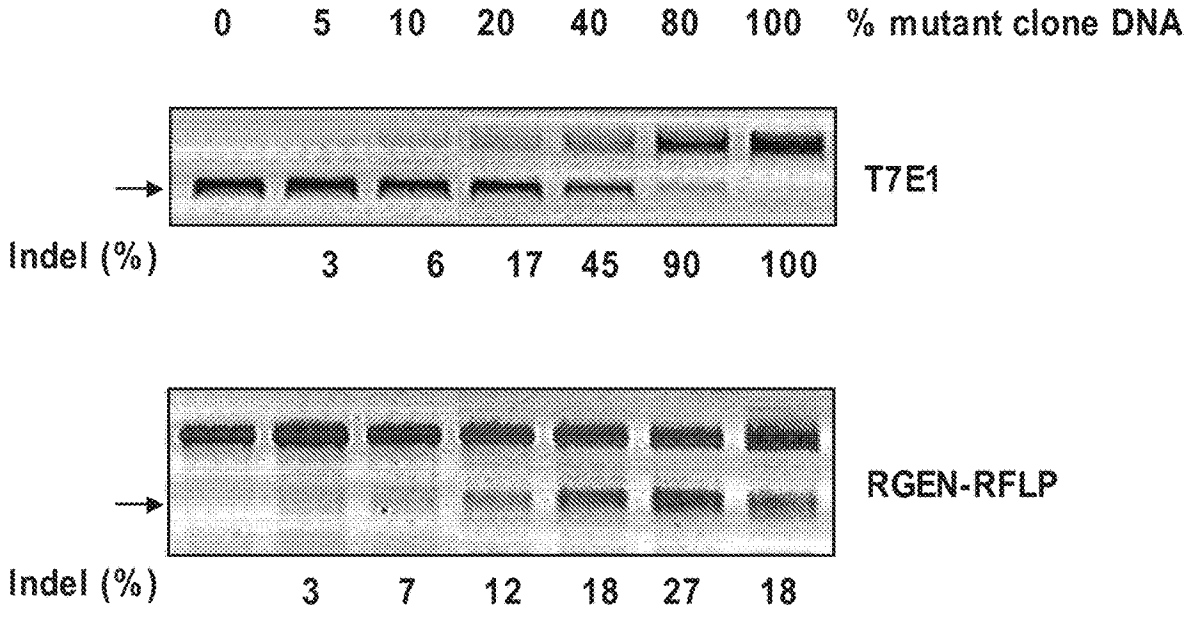
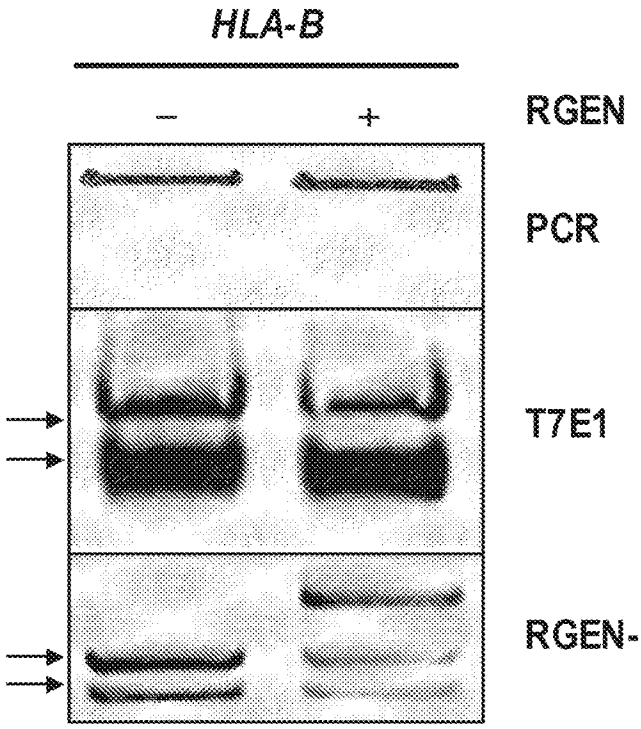


FIG. 25B


HLA-B
–
+
RGEN
PCR
T7E1
RGEN-RFLP

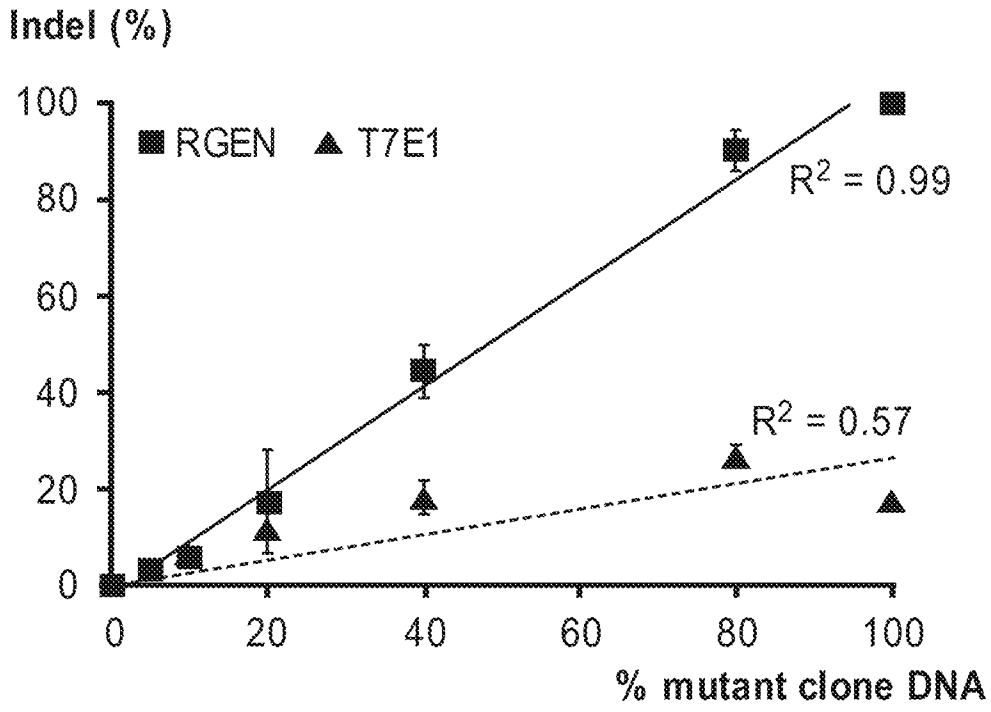

Indel (%)
100
80
60
40
20
0
RGEN
T7E1
R2 = 0.99
R2 = 0.57
0
20
40
60
80
100
% mutant clone DNA

FIG. 25C

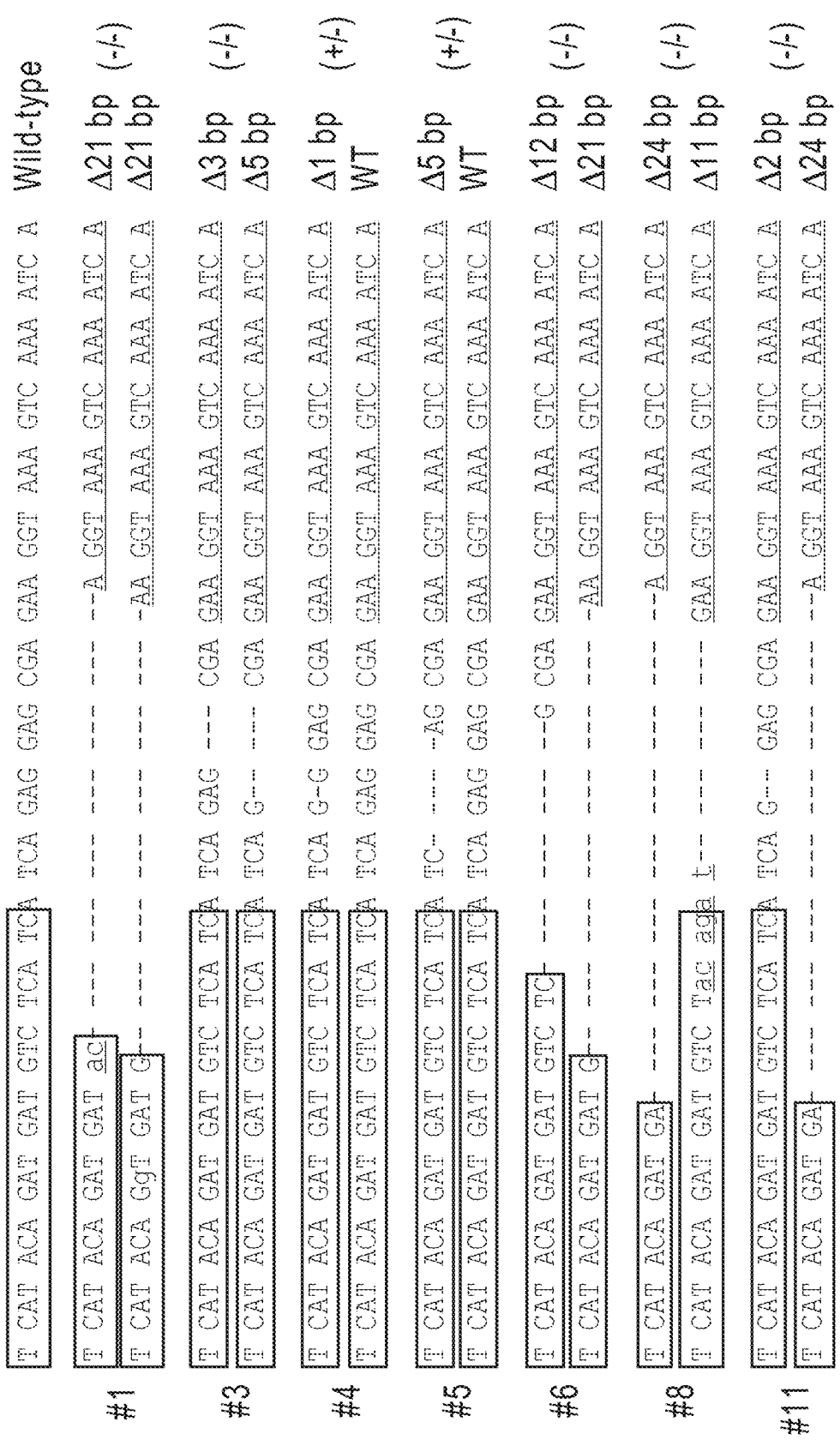

Wild-type
T CAT ACA GAT GAT GTC TCA TCA GAG GAG CGA GAA GGT AAA GTC AAA ATC A
#1
T CAT ACA GAT GAT ac --- --- --- --- --- --A GGT AAA GTC AAA ATC A Δ21 bp (-/-)
T CAT ACA GgT GAT G --- --- --- --- --- -AA GGT AAA GTC AAA ATC A Δ21 bp
#3
T CAT ACA GAT GAT GTC TCA TCA GAG --- --- CGA GAA GGT AAA GTC AAA ATC A Δ3 bp (-/-)
T CAT ACA GAT GAT GTC TCA TCA G-- --- --- CGA GAA GGT AAA GTC AAA ATC A Δ5 bp
#4
T CAT ACA GAT GAT GTC TCA TCA G-G GAG CGA GAA GGT AAA GTC AAA ATC A Δ1 bp (+/-)
T CAT ACA GAT GAT GTC TCA TCA GAG GAG CGA GAA GGT AAA GTC AAA ATC A WT
#5
T CAT ACA GAT GAT GTC TCA TC- --- --- --AG CGA GAA GGT AAA GTC AAA ATC A Δ5 bp (+/-)
T CAT ACA GAT GAT GTC TCA TCA GAG GAG CGA GAA GGT AAA GTC AAA ATC A WT
#6
T CAT ACA GAT GAT GTC TC --- --- --- --G CGA GAA GGT AAA GTC AAA ATC A Δ12 bp (-/-)
T CAT ACA GAT GAT G -- --- --- --- --- -AA GGT AAA GTC AAA ATC A Δ21 bp
#8
T CAT ACA GAT GA --- --- --- --- --- --A GGT AAA GTC AAA ATC A Δ24 bp (-/-)
T CAT ACA GAT GAT GTC Tac aga t-- --- --- --- --- GAA GGT AAA GTC AAA ATC A Δ11 bp
#11
T CAT ACA GAT GAT GTC TCA TCA TCA G-- GAG CGA GAA GGT AAA GTC AAA ATC A Δ2 bp (-/-)
T CAT ACA GAT GA --- --- --- --- --- --A GGT AAA GTC AAA ATC A Δ24 bp

FIG. 26A

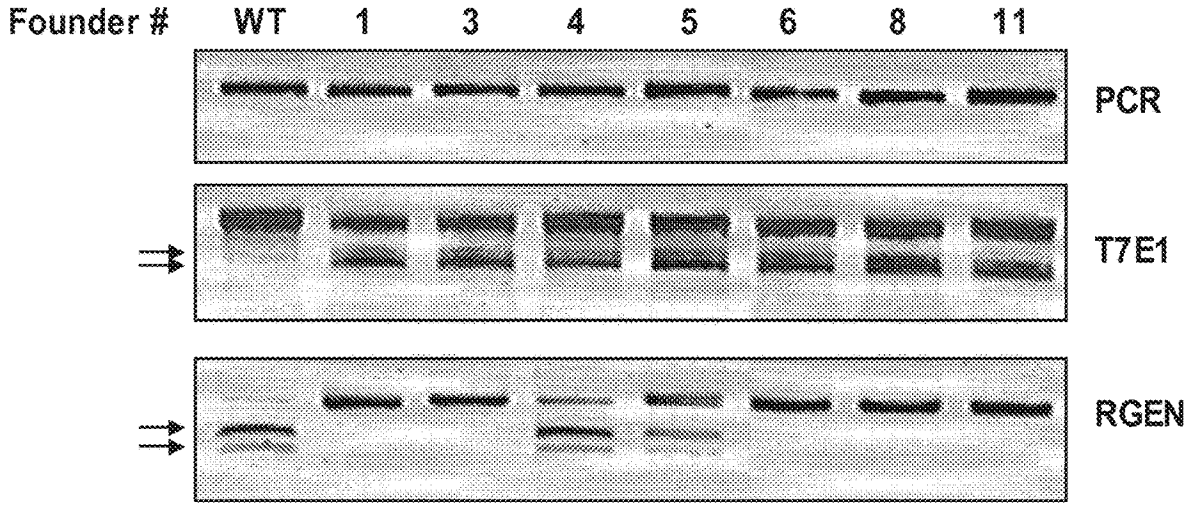


FIG. 26B

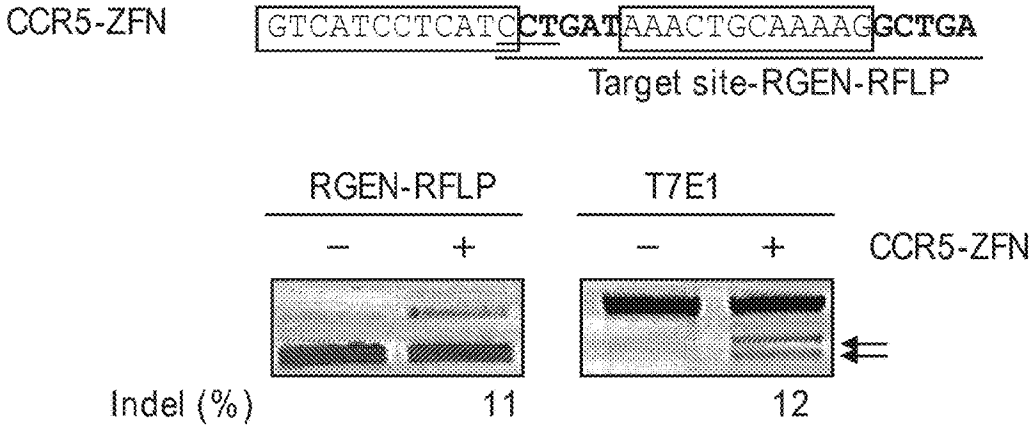


FIG. 27

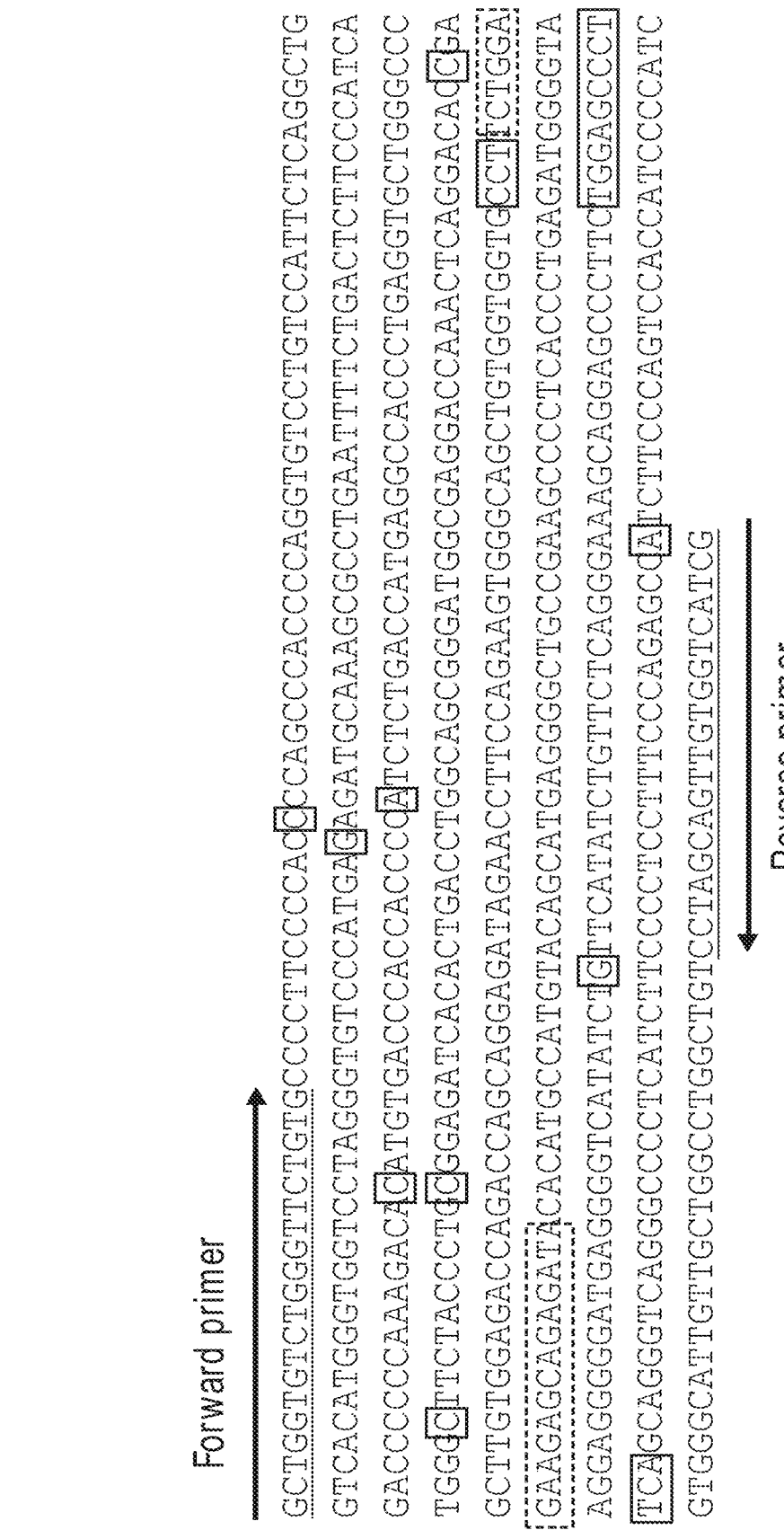
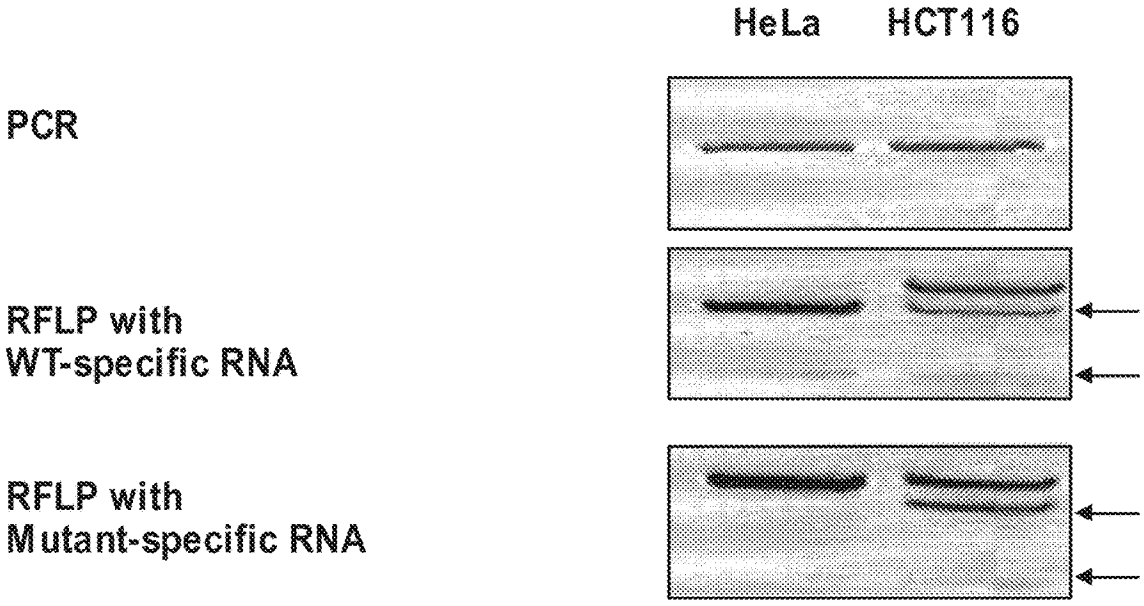

Forward primer
GCTGGTGTCTGGGTTCTGTGCCCCTTCCCCACCCAGCCCACCCCAGGTGTCCTGTCCATTCTCAGGCTG
GTCACATGGGTGGTCCTAGGGTGTCCCATGAGAGATGCAAAGCGCCTGAATTTTCTGACTCTTCCCATCA
GACCCCCCAAAGACACATGTGACCCACCACCCCCATCTCTGACCATGAGGCCACCCTGAGGTGCTGGGCCC
TGGGCTTCTACCCTGCGGAGATCACACTGACCTGGCAGCGGGATGGCGAGGACCAAACTCAGGACACGCGA
GCTTGTGGAGACCAGACCAGCAGGAGATAGAACCTTCCAGAAGTGGGCAGCTGTGGTGGTGCCTTCTGGA
GAAGAGCAGAGATACACATGCCATGTGCAGCATGAGGGGCTGCCGAAGCCCCTCACCCTGAGATGGGGTA
AGGAGGGGGATGAGGGGTCATATCTGTCATATCTGTTCTCAGGGAAGCAGGAGCCCTTCTGGAGCCCT
TCAGCAGGGTCAGGGCCCCTCATCTTCCCCTCCTTTCCCAGAGCCACTCTTCCCCAGTCCACCATCCCCATC
GTGGGCATTGTTGCTGGCCTGTCCTAGCAGTTGTGGTCATCG
Reverse primer

FIG. 28



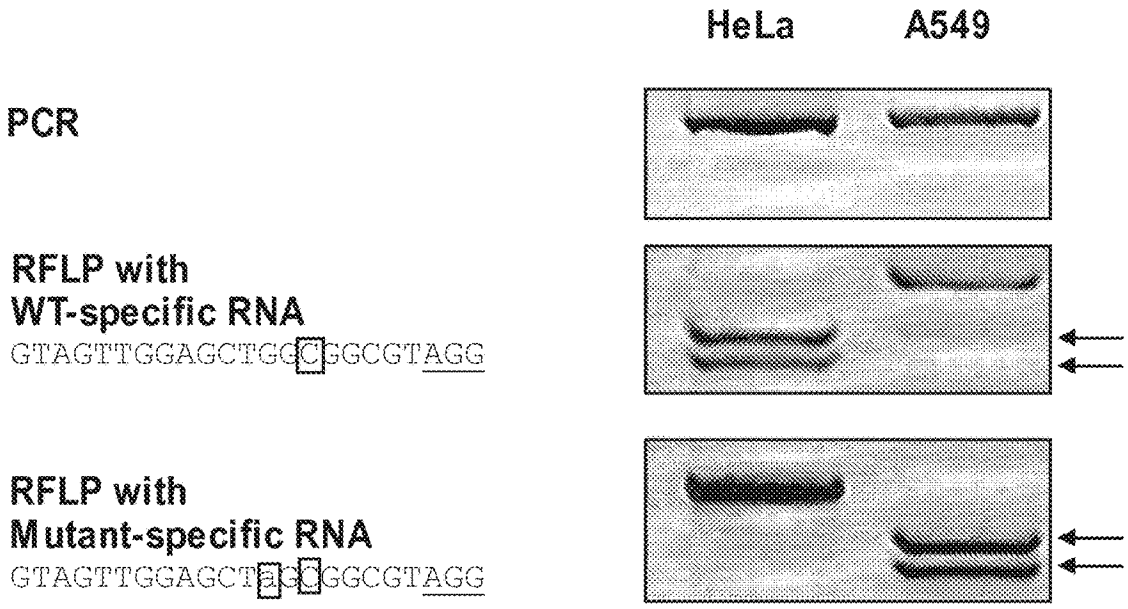
HeLa

ACTACCACAGCTCCTTCTCTGAGTGG wild-type

HCT116

ACTACCACAGCTCCTTCTCTGAGTGG wild-type
ACTACCACAGCTCCT---CTGAGTGG c.133-135 del TCT

FIG. 29A



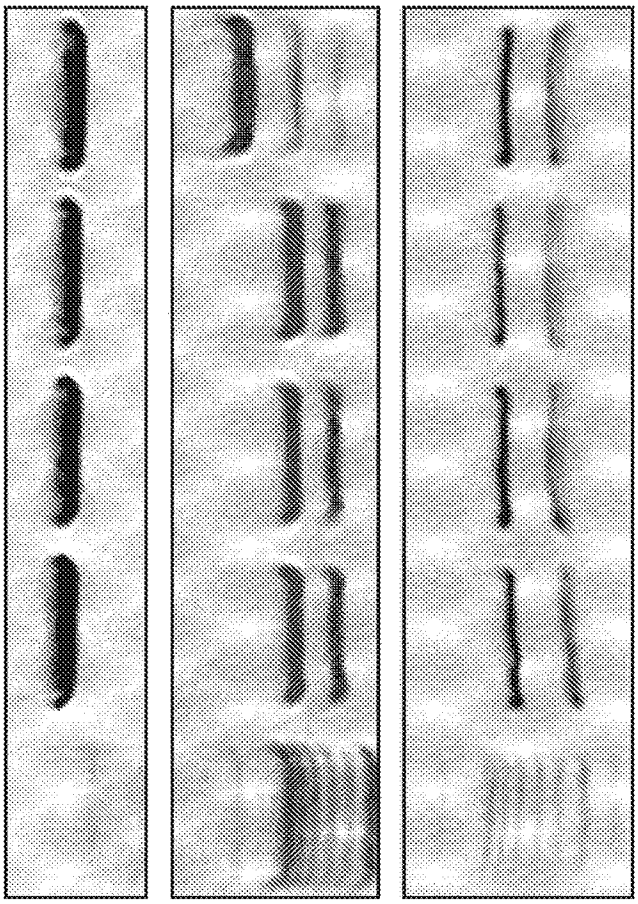
HeLa

GTAGTTGGAGCTGGTGGCGTAGG wild-type

A549

GTAGTTGGAGCTAGTGGCGTAGG c.34G>A

FIG. 29B



HEK293T

CCATACA----------TTAAAGATAGTCATCTTGGGG

HeLa

CCATACAGTCAGTATCAATTCTGGAAGAATTTCCAGACATTAAAGATAGTCATCTTGGGG

CCATACAGTCAGTATCAATTCTGGAAGAATTTCCAGACATTAAAGATAGTCATCTTGGGG

FIG. 30A

CCR5 WT

ccatacagtcagtatcaattctggaagaatttccagacattaaagatagtcatct

CCR5 WT target site

CCR5 Δ32 off-target site

CCR5 Δ32

ccataca----------ttaaagatagtcatct

CCR5 Δ32 on-target site

FIG. 30B

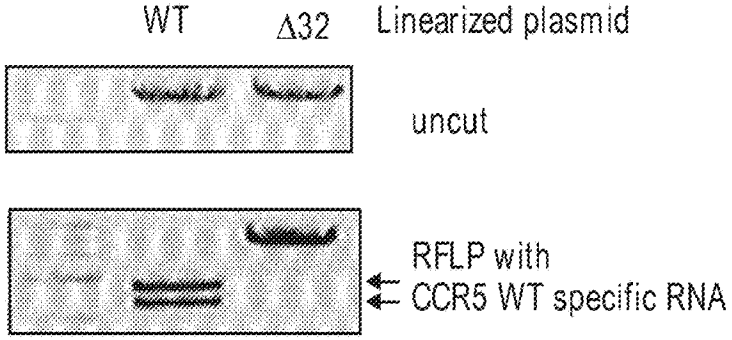
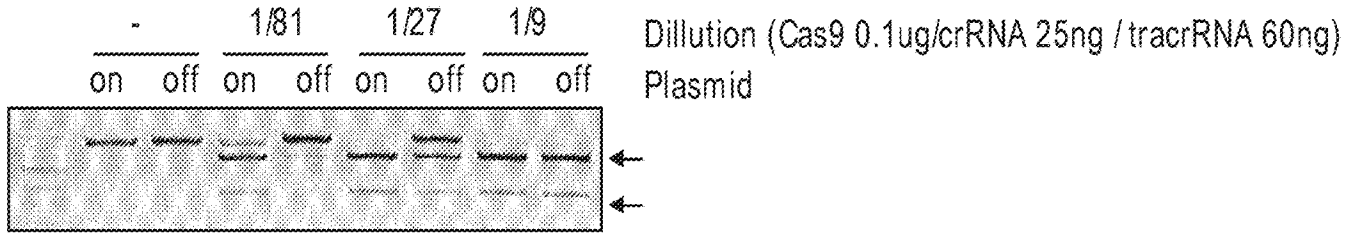


FIG. 30C



On agatgactatctttaatgtctgg

Off agatgactatctttaatgtagtgg

FIG. 30D

KRAS

HeLa

GTAGTTGGAGCTGGTGGCGTAGG Wild-type

A549

GTAGTTGGAGCTaGTGGCGTAGG c.34G>A

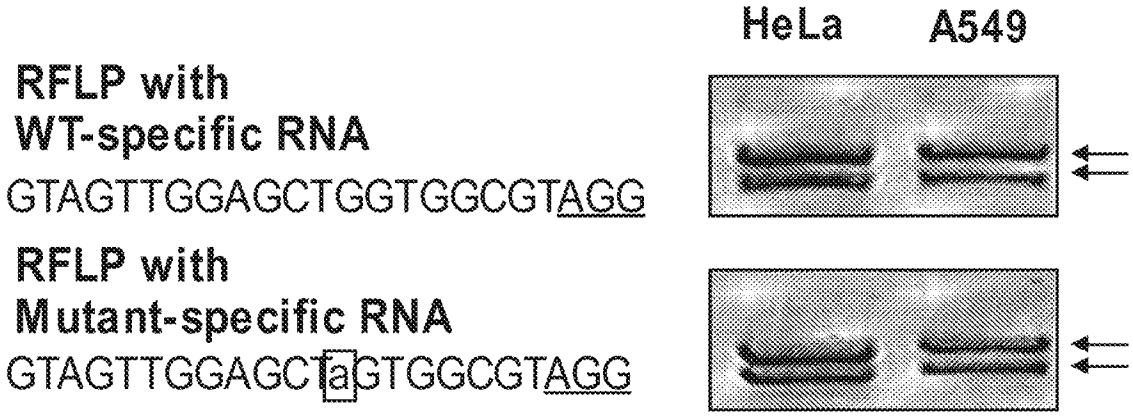
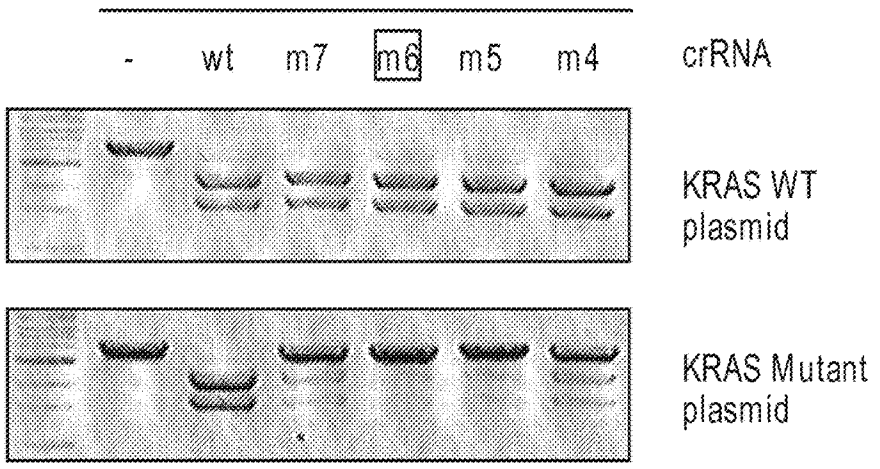


FIG. 31A

KRAS (WT) RNA target

GTAGTTGGAGCTGGTGGCGTAGG wt
GTAGTTGGAGCTGATGGCGTAGG m7
GTAGTTGGAGCTGGCGGCGTAGG m6
GTAGTTGGAGCTGGTAGCGTAGG m5
GTAGTTGGAGCTGGTGACGTAGG m4



KRAS (C.34G>A) RNA target

GTAGTTGGAGCTaGGTGGCGTAGG m 8
GTAGTTGGAGCTaATGGCGTAGG m 7,8
GTAGTTGGAGCTaGCGGCGTAGG m 6,8
GTAGTTGGAGCTaGTAGCGTAGG m 5,8
GTAGTTGGAGCTaGTGACGTAGG m 4,8

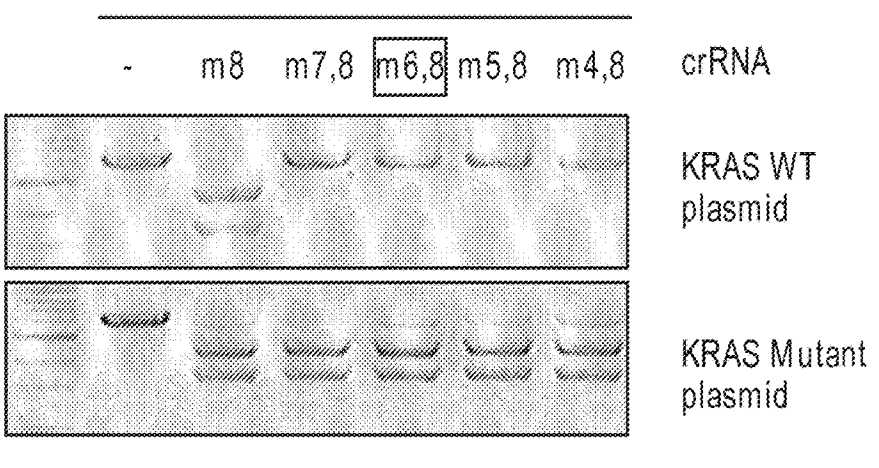


FIG. 31B

PIK3CA

HeLa

CAAATGAATGATGCACATCATGG Wild-type

HCT116

CAAATGAATGATGCACATCATGG Wild-type
CAAATGAATGATGCACgTCATGG C.3140A>G

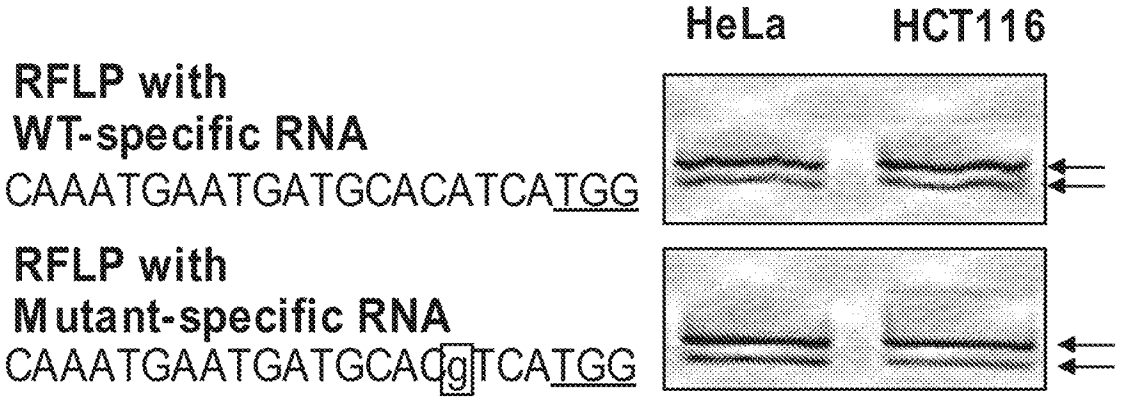


FIG. 32A

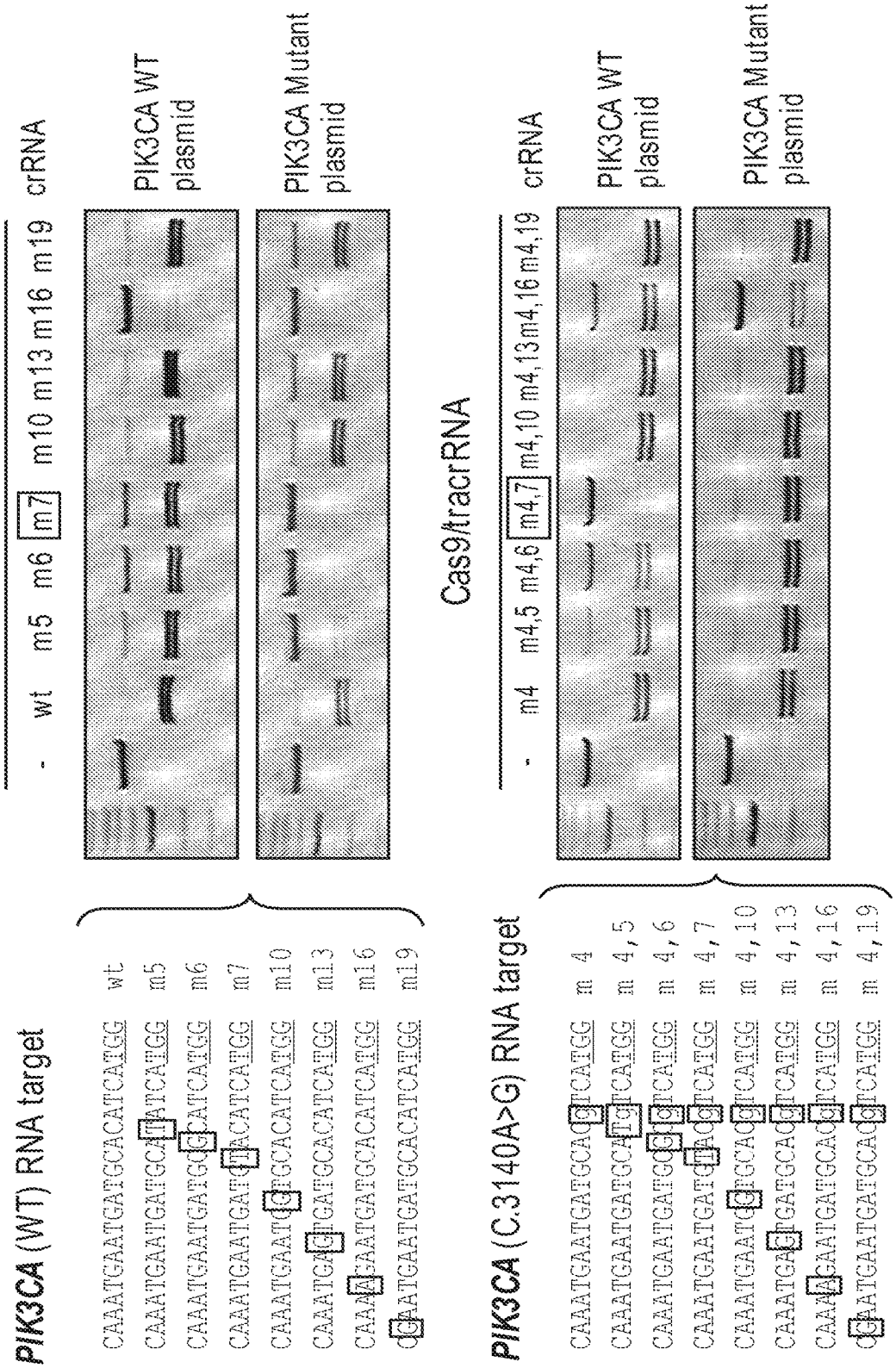

Cas9/tracrRNA
- wt m5 m6 m7 m10 m13 m16 m19 crRNA
PIK3CA WT plasmid
PIK3CA Mutant plasmid
PIK3CA (WT) RNA target
CAAATGAATGATGCACATCATGG wt
CAAATGAATGATGCATCATGG m5
CAAATGAATGATGGCATCATGG m6
CAAATGAATGATCACATCATGG m7
CAAATGAATGTGGCACATCATGG m10
CAAATGAGTGATGCACATCATGG m13
CAAAGAATGATGCACATCATGG m16
GAATGAATGATGCACATCATGG m19
Cas9/tracrRNA
- m4 m4,5 m4,6 m4,7 m4,10 m4,13 m4,16 m4,19 crRNA
PIK3CA WT plasmid
PIK3CA Mutant plasmid
PIK3CA (C.3140A>G) RNA target
CAAATGAATGATGCACGTCATGG m 4
CAAATGAATGATGCATGTCATGG m 4,5
CAAATGAATGATGGCGTCATGG m 4,6
CAAATGAATGATCACGTCATGG m 4,7
CAAATGAATGTGGCACGTCATGG m 4,10
CAAATGAGTGATGCACGTCATGG m 4,13
CAAAGAATGATGCACGTCATGG m 4,16
GAATGAATGATGCACGTCATGG m 4,19

FIG. 32B

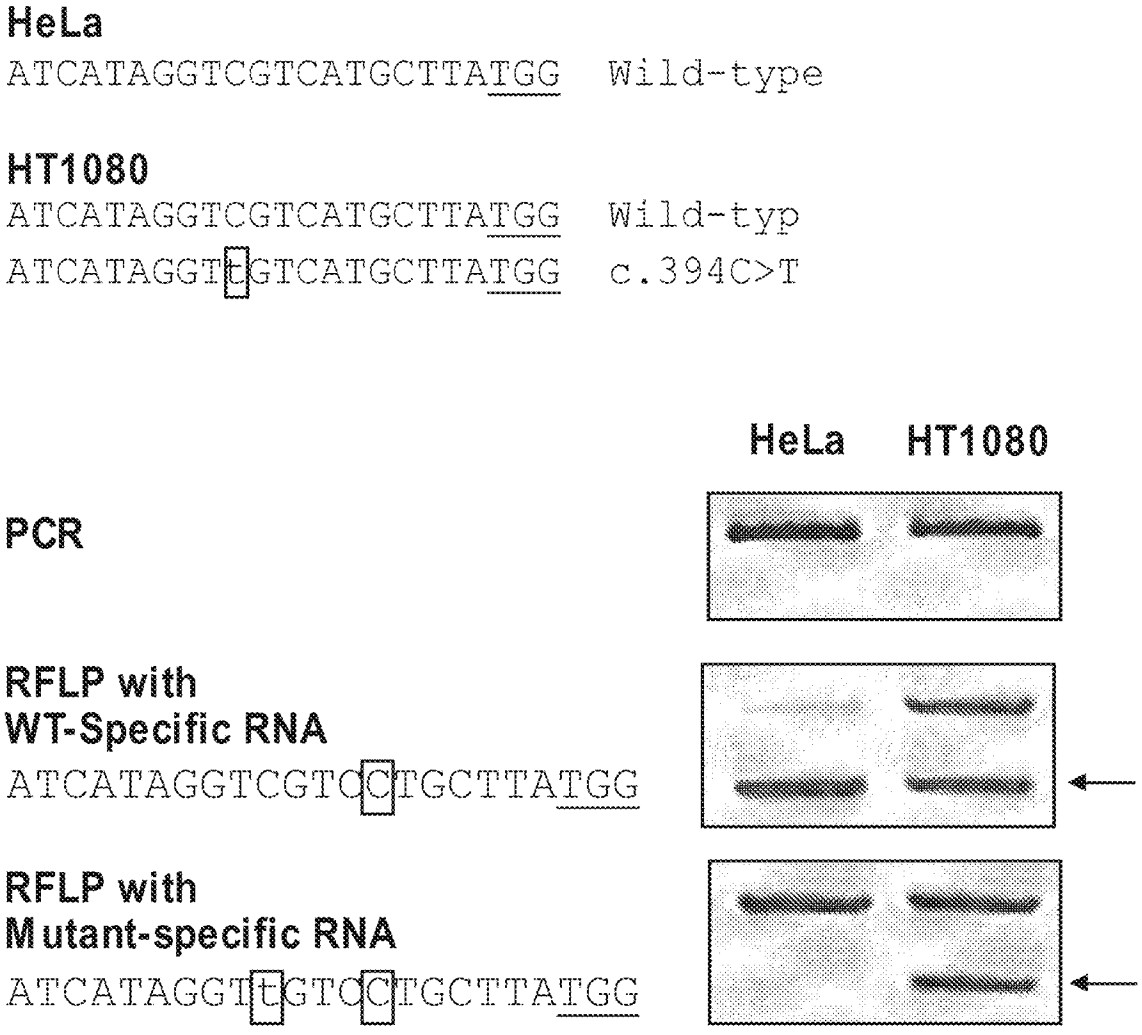

HeLa
ATCATAGGTCGTCATGCTTATGG Wild-type
HT1080
ATCATAGGTCGTCATGCTTATGG Wild-typ
ATCATAGGTTGTCATGCTTATGG c.394C>T
HeLa HT1080
PCR
RFLP with
WT-Specific RNA
ATCATAGGTCGTCCTGCTTATGG
RFLP with
Mutant-specific RNA
ATCATAGGTTGTCCTGCTTATGG

FIG. 33A

PIK3CA

HeLa

CAAATGAATGATGCACATCATGG Wild-type

HCT116

CAAATGAATGATGCACATCATGG Wild-type

CAAATGAATGATGCACgTCATGG C.3140A>G

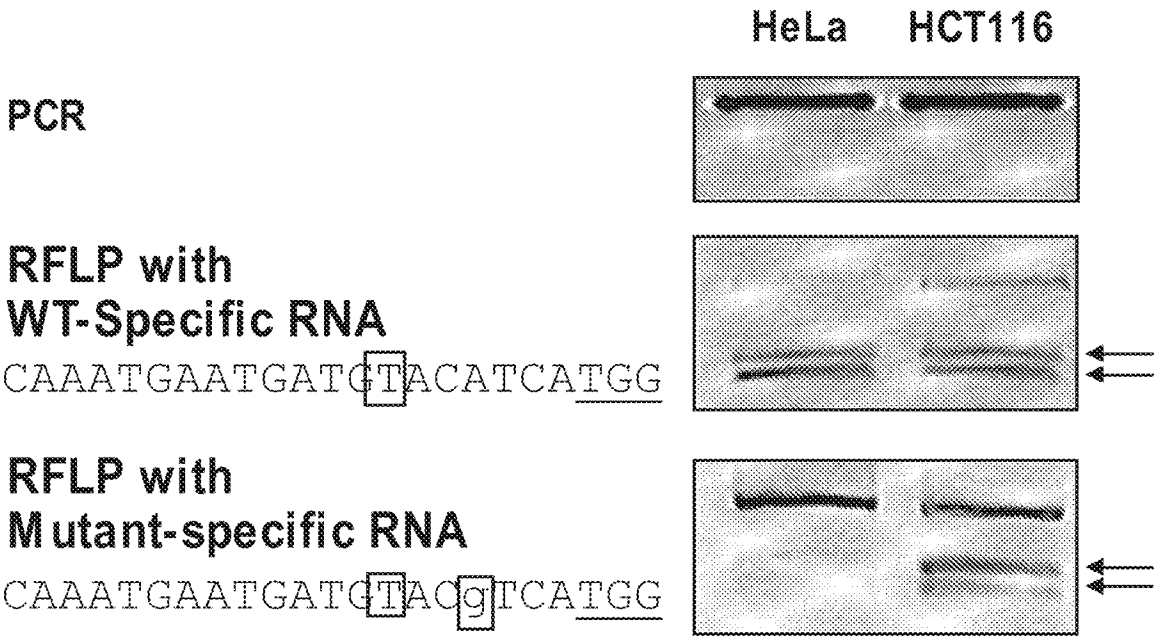


FIG. 33B

NRAS

HeLa

CTGGACAAGAAGAGTACAGTGCC Wild-type

HT1080

CTGGACAAGAAGAGTACAGTGCC Wild-type

CTGGA[a]AAGAAGAGTACAGTGCC c.181C>A

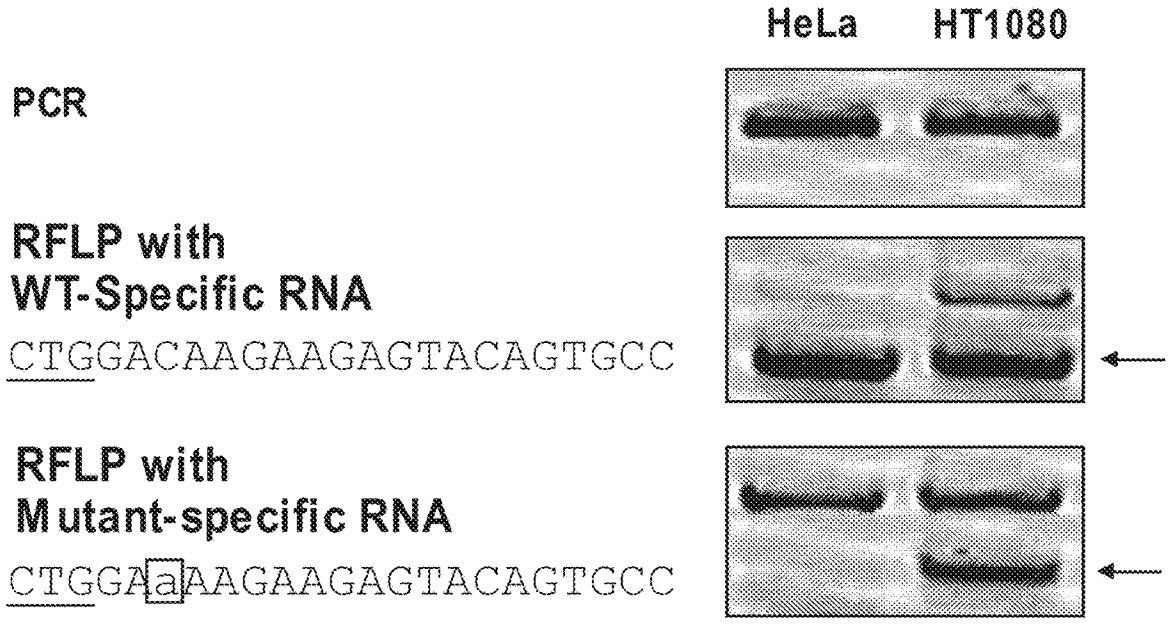


FIG. 33C

BRAF

HeLa

ACTCCATCGAGATTTCACTGTAG Wild-type

HT29

ACTCCATCGAGATTTCACTGTAG Wild-type

ACTCCATCGAGATTTCtCTGTAG (c.1799T>A)

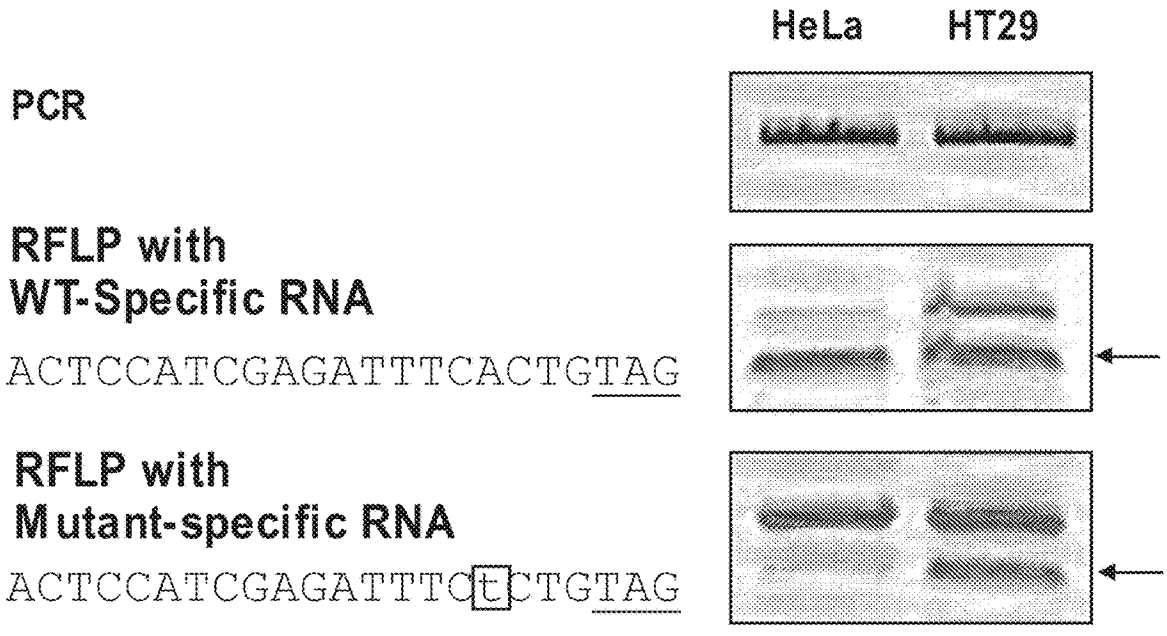


FIG. 33D

COMPOSITIONS FOR INDUCING MODIFICATIONS OF TARGET ENDOGENOUS NUCLEIC ACID SEQUENCES IN NUCLEUSES OF EUKARYOTIC CELLS

CROSS REFERENCE TO RELATED APPLICATIONS

The present application is a continuation application of U.S. application Ser. No. 18/932,745 filed Oct. 31, 2024, which is a continuation application of U.S. application Ser. No. 17/004,338 filed Aug. 27, 2020, which is a continuation application of U.S. application Ser. No. 14/685,568 filed Apr. 13, 2015, which is a continuation of PCT/KR2013/009488 filed Oct. 23, 2013, which claims priority to U.S. Provisional Application No. 61/837,481 filed on Jun. 20, 2013, U.S. Provisional Application No. 61/803,599 filed Mar. 20, 2013, and U.S. Provisional Application No. 61/717,324 filed Oct. 23, 2012, the entire contents of each aforementioned application are incorporated herein by reference.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted electronically in XML file format and is hereby incorporated by reference in its entirety. Said XML copy, created on Jan. 13, 2025, is named 00161_SL.xml and is 495,164 bytes in size.

TECHNICAL FIELD

The present invention relates to targeted genome editing in eukaryotic cells or organisms. More particularly, the present invention relates to a composition for cleaving a target DNA in eukaryotic cells or organisms comprising a guide RNA specific for the target DNA and Cas protein-encoding nucleic acid or Cas protein, and use thereof.

BACKGROUND ART

CRISPRs (Clustered Regularly Interspaced Short Palindromic Repeats) are loci containing multiple short direct repeats that are found in the genomes of approximately 40% of sequenced bacteria and 90% of sequenced archaea. CRISPR functions as a prokaryotic immune system, in that it confers resistance to exogenous genetic elements such as plasmids and phages. The CRISPR system provides a form of acquired immunity. Short segments of foreign DNA, called spacers, are incorporated into the genome between CRISPR repeats, and serve as a memory of past exposures. CRISPR spacers are then used to recognize and silence exogenous genetic elements in a manner analogous to RNAi in eukaryotic organisms.

Cas9, an essential protein component in the Type II CRISPR/Cas system, forms an active endonuclease when complexed with two RNAs termed CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA), thereby slicing foreign genetic elements in invading phages or plasmids to protect the host cells. crRNA is transcribed from the CRISPR element in the host genome, which was previously captured from such foreign invaders. Recently, Jinek et al. (1) demonstrated that a single-chain chimeric RNA produced by fusing an essential portion of crRNA and tracrRNA could replace the two RNAs in the Cas9/RNA complex to form a functional endonuclease.

CRISPR/Cas systems offer an advantage to zinc finger and transcription activator-like effector DNA-binding proteins, as the site specificity in nucleotide binding CRISPR-Cas proteins is governed by a RNA molecule instead of the DNA-binding protein, which can be more challenging to design and synthesize.

However, until now, a genome editing method using the RNA-guided endonuclease (RGEN) based on CRISPR/Cas system has not been developed.

Meanwhile, Restriction fragment length polymorphism (RFLP) is one of the oldest, most convenient, and least expensive methods of genotyping that is still used widely in molecular biology and genetics but is often limited by the lack of appropriate sites recognized by restriction endonucleases.

Engineered nuclease-induced mutations are detected by various methods, which include mismatch-sensitive T7 endonuclease I (T7E1) or Surveyor nuclease assays, RFLP, capillary electrophoresis of fluorescent PCR products, Dideoxy sequencing, and deep sequencing. The T7E1 and Surveyor assays are widely used but are cumbersome. Furthermore, these enzymes tend to underestimate mutation frequencies because mutant sequences can form homoduplexes with each other and cannot distinguish homozygous bi-allelic mutant clones from wildtype cells. RFLP is free of these limitations and therefore is a method of choice. Indeed, RFLP was one of the first methods to detect engineered nuclease-mediated mutations in cells and animals. Unfortunately, however, RFLP is limited by the availability of appropriate restriction sites. It is possible that no restriction sites are available at the target site of interest.

DISCLOSURE OF INVENTION

Technical Problem

Until now, a genome editing and genotyping method using the RNA-guided endonuclease (RGEN) based on CRISPR/Cas system has not been developed.

Under these circumstances, the present inventors have made many efforts to develop a genome editing method based on CRISPR/Cas system and finally established a programmable RNA-guided endonuclease that cleave DNA in a targeted manner in eukaryotic cells and organisms.

In addition, the present inventors have made many efforts to develop a novel method of using RNA-guided endonucleases (RGENs) in RFLP analysis. They have used RGENS to genotype recurrent mutations found in cancer and those induced in cells and organisms by engineered nucleases including RGENs themselves, thereby completing the present invention.

Solution to Problem

It is an object of the present invention to provide a composition for cleaving target DNA in eukaryotic cells or organisms comprising a guide RNA specific for target DNA or DNA that encodes the guide RNA, and Cas protein-encoding nucleic acid or Cas protein.

It is another object of the present invention to provide a composition for inducing targeted mutagenesis in eukaryotic cells or organisms, comprising a guide RNA specific for target DNA or DNA that encodes the guide RNA, and Cas protein-encoding nucleic acid or Cas protein.

It is still another object of the present invention to provide a kit for cleaving a target DNA in eukaryotic cells or organisms comprising a guide RNA specific for target DNA or DNA that encodes the guide RNA, and Cas protein-encoding nucleic acid or Cas protein.

It is still another object of the present invention to provide a kit for inducing targeted mutagenesis in eukaryotic cells or organisms comprising a guide RNA specific for target DNA or DNA that encodes the guide RNA, and Cas protein-encoding nucleic acid or Cas protein.

It is still another object of the present invention to provide a method for preparing a eukaryotic cell or organism comprising Cas protein and a guide RNA comprising a step of co-transfecting or serial-transfecting the eukaryotic cell or organism with a Cas protein-encoding nucleic acid or Cas protein, and a guide RNA or DNA that encodes the guide RNA.

It is still another object of the present invention to provide a eukaryotic cell or organism comprising a guide RNA specific for target DNA or DNA that encodes the guide RNA, and Cas protein-encoding nucleic acid or Cas protein.

It is still another object of the present invention to provide a method for cleaving a target DNA in eukaryotic cells or organisms comprising a step of transfecting the eukaryotic cells or organisms comprising a target DNA with a composition comprising a guide RNA specific for target DNA or DNA that encodes the guide RNA, and Cas protein-encoding nucleic acid or Cas protein.

It is still another object of the present invention to provide a method for inducing targeted mutagenesis in a eukaryotic cell or organism comprising a step of treating a eukaryotic cell or organism with a composition comprising a guide RNA specific for target DNA or DNA that encodes the guide RNA, and Cas protein-encoding nucleic acid or Cas protein.

It is still another object of the present invention to provide an embryo, a genome-modified animal, or genome-modified plant comprising a genome edited by a composition comprising a guide RNA specific for target DNA or DNA that encodes the guide RNA, and Cas protein-encoding nucleic acid or Cas protein.

It is still another object of the present invention to provide a method of preparing a genome-modified animal comprising a step of introducing the composition comprising a guide RNA specific for target DNA or DNA that encodes the guide RNA, and Cas protein-encoding nucleic acid or Cas protein into an embryo of an animal; and a step of transferring the embryo into a oviduct of pseudopregnant foster mother to produce a genome-modified animal.

It is still another object of the present invention to provide a composition for genotyping mutations or variations in an isolated biological sample, comprising a guide RNA specific for the target DNA sequence Cas protein.

It is still another object of the present invention to provide a method of using a RNA-guided endonuclease (RGEN) to genotype mutations induced by engineered nucleases in cells or naturally-occurring mutations or variations, wherein the RGEN comprises a guide RNA specific for target DNA and Cas protein.

It is still another object of the present invention to provide a kit for genotyping mutations induced by engineered nucleases in cells or naturally-occurring mutations or variations, comprising a RNA-guided endonuclease (RGEN), wherein the RGEN comprises a guide RNA specific for target DNA and Cas protein.

It is an object of the present invention to provide a composition for cleaving target DNA in eukaryotic cells or organisms comprising a guide RNA specific for target DNA or DNA that encodes the guide RNA, and Cas protein-encoding nucleic acid or Cas protein.

It is another object of the present invention to provide a composition for inducing targeted mutagenesis in eukaryotic cells or organisms, comprising a guide RNA specific for target DNA or DNA that encodes the guide RNA, and Cas protein-encoding nucleic acid or Cas protein.

It is still another object of the present invention to provide a kit for cleaving a target DNA in eukaryotic cells or organisms comprising a guide RNA specific for target DNA or DNA that encodes the guide RNA, and Cas protein-encoding nucleic acid or Cas protein.

It is still another object of the present invention to provide a kit for inducing targeted mutagenesis in eukaryotic cells or organisms comprising a guide RNA specific for target DNA or DNA that encodes the guide RNA, and Cas protein-encoding nucleic acid or Cas protein.

It is still another object of the present invention to provide a method for preparing a eukaryotic cell or organism comprising Cas protein and a guide RNA comprising a step of co-transfecting or serial-transfecting the eukaryotic cell or organism with a Cas protein-encoding nucleic acid or Cas protein, and a guide RNA or DNA that encodes the guide RNA.

It is still another object of the present invention to provide a eukaryotic cell or organism comprising a guide RNA specific for target DNA or DNA that encodes the guide RNA, and Cas protein-encoding nucleic acid or Cas protein.

It is still another object of the present invention to provide a method for cleaving a target DNA in eukaryotic cells or organisms comprising a step of transfecting the eukaryotic cells or organisms comprising a target DNA with a composition comprising a guide RNA specific for target DNA or DNA that encodes the guide RNA, and Cas protein-encoding nucleic acid or Cas protein.

It is still another object of the present invention to provide a method for inducing targeted mutagenesis in a eukaryotic cell or organism comprising a step of treating a eukaryotic cell or organism with a composition comprising a guide RNA specific for target DNA or DNA that encodes the guide RNA, and Cas protein-encoding nucleic acid or Cas protein.

It is still another object of the present invention to provide an embryo, a genome-modified animal, or genome-modified plant comprising a genome edited by a composition comprising a guide RNA specific for target DNA or DNA that encodes the guide RNA, and Cas protein-encoding nucleic acid or Cas protein.

It is still another object of the present invention to provide a method of preparing a genome-modified animal comprising a step of introducing the composition comprising a guide RNA specific for target DNA or DNA that encodes the guide RNA, and Cas protein-encoding nucleic acid or Cas protein into an embryo of an animal; and a step of transferring the embryo into a oviduct of pseudopregnant foster mother to produce a genome-modified animal.

It is still another object of the present invention to provide a composition for genotyping mutations or variations in an isolated biological sample, comprising a guide RNA specific for the target DNA sequence Cas protein.

It is still another object of the present invention to provide a composition for genotyping nucleic acid sequences in pathogenic microorganisms in an isolated biological sample, comprising a guide RNA specific for the target DNA sequence and Cas protein.

It is still another object of the present invention to provide a kit for genotyping mutations or variations in an isolated biological sample, comprising the composition, specifically comprising a RNA-guided endonuclease (RGEN), wherein the RGEN comprises a guide RNA specific for target DNA and Cas protein.

It is still another object of the present invention to provide a method of genotyping mutations or variations in an

isolated biological sample, using the composition, specifically comprising a RNA-guided endonuclease (RGEN), wherein the RGEN comprises a guide RNA specific for target DNA and Cas protein.

Advantageous Effects of Invention

The present composition for cleaving a target DNA or inducing a targeted mutagenesis in eukaryotic cells or organisms, comprising a guide RNA specific for the target DNA and Cas protein-encoding nucleic acid or Cas protein, the kit comprising the composition, and the method for inducing targeted mutagenesis provide a new convenient genome editing tools. In addition, because custom RGENs can be designed to target any DNA sequence, almost any single nucleotide polymorphism or small insertion/deletion (indel) can be analyzed via RGEN-mediated RFLP, therefore, the composition and method of the present invention may be used in detection and cleaving naturally-occurring variations and mutations.

BRIEF DESCRIPTION OF DRAWINGS

FIGS. **1**A and **1**B show Cas9-catalyzed cleavage of plasmid DNA in vitro. FIG. **1**A: Schematic representation of target DNA (SEQ ID NO: 112) and chimeric RNA sequences (SEQ ID NO: 113). Triangles indicate cleavage sites. The PAM sequence recognized by Cas9 is shown in bold. The sequences in the guide RNA (SEQ ID NO: 113) derived from crRNA and tracrRNA are shown in box and underlined, respectively. FIG. **1**B: In vitro cleavage of plasmid DNA by Cas9. An intact circular plasmid or ApaLI-digested plasmid was incubated with Cas9 and guide RNA.

FIGS. **2**A and **2**B show Cas9-induced mutagenesis at an episomal target site. FIG. **2**A: Schematic overview of cell-based assays using a RFP-GFP reporter. GFP is not expressed from this reporter because the GFP sequence is fused to the RFP sequence out-of-frame. The RFP-GFP fusion protein is expressed only when the target site between the two sequences is cleaved by a site-specific nuclease. FIG. **2**B: Flow cytometry of cells transfected with Cas9. The percentage of cells that express the RFP-GFP fusion protein is indicated.

FIGS. **3**A and **3**B show RGEN-driven mutations at endogenous chromosomal sites. FIG. **3**A: CCR5 locus. FIG. **3**B: C4BPB locus. (Top) The T7E1 assay was used to detect RGEN-driven mutations. Arrows indicate the expected position of DNA bands cleaved by T7E1. Mutation frequencies (Indels (%)) were calculated by measuring the band intensities. (Bottom) DNA sequences of the wild-type (WT) CCR5 (SEQ ID NO: 114) and C4BPB (SEQ ID NO: 122) and mutant clones. DNA sequences of RGEN-induced mutations at the CCR5 locus: +1 (SEQ ID NO: 115), −13 (SEQ ID NO: 116), −14 (SEQ ID NO: 117), −18 (SEQ ID NO: 118), −19 (SEQ ID NO: 119), −24 (SEQ ID NO: 120), and −30 (SEQ ID NO: 121). DNA sequences of RGEN-induced mutations at the C4BPB locus: +1 (SEQ ID NO: 122), +2 (SEQ ID NO: 123), −30 (SEQ ID NO: 125), and −180 (SEQ ID NO: 126). The region of the target sequence complementary to the guide RNA is shown in box. The PAM sequence is shown in bold. Triangles indicate the cleavage site. Bases corresponding to microhomologies are underlined. The column on the right indicates the number of inserted or deleted bases.

FIGS. **4**A, **4**B, and **4**C show that RGEN-driven off-target mutations are undetectable. FIG. **4**A: On-target and potential off-target sequences. The human genome was searched in silico for potential off-target sites. Four sites were identified, ADCY5 (SEQ ID NO: 128), KCNJ6 (SEQ ID NO: 129), CNTNAP2 (SEQ ID NO: 130), and Chr. 5 N/A (SEQ ID NO: 131), each of which carries 3-base mismatches with the CCR5 on-target (SEQ ID NO: 127). Mismatched bases are underlined. FIG. **4**B: The T7E1 assay was used to investigate whether these sites were mutated in cells transfected with the Cas9/RNA complex. No mutations were detected at these sites. N/A (not applicable), an intergenic site. FIG. **4**C: Cas9 did not induce off-target-associated chromosomal deletions. The CCR5-specific RGEN and ZFN were expressed in human cells. PCR was used to detect the induction of the 15-kb chromosomal deletions in these cells.

FIGS. **5**A, **5**B, **5**C, and **5**D show RGEN-induced Foxn1 gene targeting in mice. FIG. **5**A: A schematic diagram depicting target DNA (SEQ ID NO: 132) and a sgRNA specific to exon 2 of the mouse Foxn1 gene (SEQ ID NO: 133). PAM in exon 2 is shown in a box and the sequence in the sgRNA that is complementary to exon 2 is underlined. Triangles indicate cleavage sites. FIG. **5**B: Representative T7E1 assays demonstrating gene-targeting efficiencies of Cas9 mRNA plus Foxn1-specific sgRNA that were delivered via intra-cytoplasmic injection into one-cell stage mouse embryos. Numbers indicate independent founder mice generated from the highest dose. Arrows indicate bands cleaved by T7E1. FIG. **5**C: DNA sequences of wild-type (WT) Foxn1 (SEQ ID NO: 134) and mutant alleles (SEQ ID NOs. 135-141) observed in three Foxn1 mutant founders identified in FIG. **5**B. DNA sequences of mutant alleles in founder #108: −44(SEQ ID NO: 135), −23 (SEQ ID NO: 136), −17 (SEQ ID NO: 137), and +1 (SEQ ID NO: 138). DNA sequences of mutant alleles in founder #111: +1 (SEQ ID NO: 138) and −11 (SEQ ID NO: 139). DNA sequences of mutant alleles in founder #114: −6 (SEQ ID NO: 140), −17 (SEQ ID NO: 137), and −8 (SEQ ID NO: 141). The number of occurrences is shown in parentheses. FIG. **5**D: PCR genotyping of F1 progenies derived from crossing Foxn1 founder #108 and wild-type FVB/NTac. Note the segregation of the mutant alleles found in Foxn1 founder #108 in the progenies.

FIGS. **6**A, **6**B, and **6**C show Foxn1 gene targeting in mouse embryos by intra-cytoplasmic injection of Cas9 mRNA and Foxn1-sgRNA. FIG. **6**A: A representative result of a T7E1 assay monitoring the mutation rate after injecting the highest dose. Arrows indicate bands cleaved by T7E1. FIG. **6**B: A summary of T7E1 assay results. Mutant fractions among in vitro cultivated embryos obtained after intra-cytoplasmic injection of the indicated RGEN doses are indicated. FIG. **6**C: DNA sequences of wild-type (WT) Foxn1 (SEQ ID NO: 143) and Foxn1 mutant alleles (SEQ ID Nos. 144-152) identified from a subset of T7E1-positive mutant embryos. The DNA sequences of the mutant alleles are: Δ11 (SEQ ID NO: 144), Δ11+Δ17 (SEQ ID NO: 145) Δ57 (SEQ ID NO: 146), Δ17 (SEQ ID NO: 147), +1 (SEQ ID NO: 148), Δ12 (SEQ ID NO: 149, Δ72 (SEQ ID NO: 150), Δ25 (SEQ ID NO: 151), Δ24 (SEQ ID NO: 152). The target sequence of the wild-type allele is denoted in box.

FIGS. **7**A, **7**B, and **7**C show Foxn1 gene targeting in mouse embryos using the recombinant Cas9 protein: Foxn1-sgRNA complex. FIG. **7**A and FIG. **7**B are representative T7E1 assays results and their summaries. Embryos were cultivated in vitro after they underwent pronuclear (FIG. **7**A) or intra-cytoplasmic injection (FIG. **7**B). Underlined numbers indicate T7E1-positive mutant founder mice. FIG. **7**C: DNA sequences of wild-type (WT) Foxn1 (SEQ ID NO: 153) and Foxn1 mutant alleles (SEQ ID NOS. 154-166) identified from the in vitro cultivated embryos that were

obtained by the pronucleus injection of recombinant Cas9 protein: Foxn1-sgRNA complex at the highest dose. The target sequence of the wild-type allele is denoted in box. The DNA sequences of the mutant alleles are: Δ18 (SEQ ID NO: 154), Δ20 (SEQ ID NO: 155), Δ19 (SEQ ID NO: 156), Δ17 (SEQ ID NO: 157), Δ11 (SEQ ID NO: 158), Δ3+1 (SEQ ID NO: 159), Δ2 (SEQ ID NO: 160), +1, Embryo 1 (SEQ ID NO: 161), +1, Embryo 10 (SEQ ID NO: 162), Δ6 (SEQ ID NO: 163), Δ5 (SEQ ID NO: 164), Δ28 (SEQ ID NO: 165), and Δ126 (SEQ ID NO: 166).

FIGS. **8**A, **8**B, and **8**C show Germ-line transmission of the mutant alleles found in Foxn1 mutant founder #12. FIG. **8**A: wild type fPCR analysis. FIG. **8**B: Foxn1 mutant founder #12 fPCR analysis. FIG. **8**C: PCR genotyping of wild-type FVB/NTac, the founder mouse, and their F1 progenies.

FIGS. **9**A and **9**B show Genotypes of embryos generated by crossing Prkdc mutant founders. Prkdc mutant founders ♂25 and ♀15 were crossed and E13.5 embryos were isolated. FIG. **9**A: fPCR analysis of wild-type, founder ♂25, and founder ♀15. Note that, due to the technical limitations of fPCR analysis, these results showed small differences from the precise sequences of the mutant alleles; e.g., from the sequence analysis, Δ269/Δ61/WT and Δ5+1/+7/+12/WT were identified in founders ♂25 and ♀15, respectively. FIG. **9**B: Genotypes of the generated embryos.

FIGS. **10**A, **10**B, **10**C, **10**D, and **10**E show Cas9 protein/sgRNA complex induced targeted mutation at CCR5 gene (FIGS. **10**A-**10**C) and ABCC11 gene (FIGS. **10**D-**10**E). FIG. **10**A: Results of a T7E1 assay monitoring the mutation rate at CCR5 locus after introducing Cas9 protein and sgRNA or Cas9 protein and crRNA+tracrRNA into K562 cells. FIG. **10**B: Results of a T7E1 assay using 1/5 scaled down doses of Cas9 protein and sgRNA. FIG. **10**C: Wild-type (WT) CCR5 sequence (SEQ ID NO: 114) and Cas protein induced mutant sequences (SEQ ID NOs. 167-171 and 115) identified in CCR5 locus. The DNA sequences of the mutant sequences are: −4 (SEQ ID NO: 167), −4 (SEQ ID NO: 168), −7 (SEQ ID NO: 169), −1 (SEQ ID NO: 170), +1 (SEQ ID NO: 115), and −17, +1 (SEQ ID NO: 171). FIG. **10**D: Results of a T7E1 assay monitoring the mutation rate at ABCC11 locus after introducing Cas9 protein and sgRNA into K562 cells. FIG. **10**E: Wild-type (WT) ABCC11 sequence (SEQ ID NO: 172) and Cas9 protein induced mutant sequences (SEQ ID NOs. 173-176) identified in ABCC11 locus. The DNA sequences of the mutant sequences are: −6 (SEQ ID NO: 173), −3 (SEQ ID NO: 174), −29 (SEQ ID NO: 175), −20 (SEQ ID NO: 176), and −256 (TTCTC).

FIG. **11** shows recombinant Cas9 protein-induced mutations in Arabidopsis protoplasts.

FIG. **12** shows wild type BRI1 sequence (SEQ ID NO: 177) and recombinant Cas9 protein-induced mutant sequences (SEQ ID NOs. 178-181) in the *Arabidopsis* BRI1 gene. The DNA sequences of the mutant sequences are: −7 (SEQ ID NO: 178), −224 (SEQ ID NO: 179), −223 (SEQ ID NO: 180), and −223, +62 (SEQ ID NO: 181).

FIG. **13** shows T7E1 assay showing endogenous CCR5 gene disruption in 293 cells by treatment of Cas9-mal-9R4L and sgRNA/C9R4LC complex.

FIGS. **14**A and **14**B show mutation frequencies at on-target and off-target sites of RGENs reported in Fu et al. (2013). T7E1 assays analyzing genomic DNA from K562 cells (R) transfected serially with 20 μg of Cas9-encoding plasmid and with 60 μg and 120 μg of in vitro transcribed GX19 crRNA and tracrRNA, respectively (1×10^6 cells), or (D) co-transfected with 1 μg of Cas9-encoding plasmid and 1 μg of GX19 SgRNA expression plasmid (2×10^5 cells). FIG. **14**A: VEGFA site 1 on target sequence (SEQ ID NO: 182) and off target sequences, OT1-3 (SEQ ID NO: 183) and OT1-11 (SEQ ID NO: 184). VEGFA site 2 on target sequence (SEQ ID NO: 185) and off target sequences OT2-1 (SEQ ID NO: 186), OT2-9 (SEQ ID NO: 187) and OT2-24 (SEQ ID NO: 188). FIG. **14**B: VEGFA site 3 on target sequence (SEQ ID NO: 189) and off target sequence OT3-18 (SEQ ID NO: 190) and EMX1 on target sequence (SEQ ID NO: 191) and off target sequence OT4-1 (SEQ ID NO: 192).

FIGS. **15**A and **15**B show comparison of guide RNA structure. Mutation frequencies of the RGENs reported in Fu et al. (2013) were measured at on-target and off-target sites using the T7E1 assay. K562 cells were co-transfected with the Cas9-encoding plasmid and the plasmid encoding GX19 sgRNA or GGX20 sgRNA. Off-target sites (OT1-3 etc.) are labeled as in Fu et al. (2013). FIG. **15**A: VEGFA site 1 on target sequence (SEQ ID NO: 182) and off target sequences OT1-3 (SEQ ID NO: 183 and OT1-11 (SEQ ID NO: 184). VEGFA site 2 on target sequence (SEQ ID NO: 185) and off target sequences OT2-1 (SEQ ID NO: 186), OT2-9 (SEQ ID NO: 187), and OT2-24 (SEQ ID NO: 188). FIG. **15**B: VEGFA site 3 on target sequence (SEQ ID NO: 189) and off target sequence OT3-18 (SEQ ID NO: 190) and EMX1 on target sequence (SEQ ID NO: 191) and off target sequence OT4-1 (SEQ ID NO: 192).

FIGS. **16**A, **16**B, **16**C, and **16**D show that in vitro DNA cleavage by Cas9 nickases. FIG. **16**A: Schematic overview of the Cas9 nuclease and the paired Cas9 nickase. The PAM sequences and cleavage sites are shown in box. FIG. **16**B: Target sites in the human AAVS1 locus. The position of each target site is shown in triangle. FIG. **16**C: Schematic overview of DNA cleavage reactions. FAM dyes (shown in box) were linked to both 5' ends of the DNA substrate. FIG. **16**D: DSBs and SSBs analyzed using fluorescent capillary electrophoresis. Fluorescently-labeled DNA substrates were incubated with Cas9 nucleases or nickases before electrophoresis.

FIGS. **17**A and **17**B show comparison of Cas9 nuclease and nickase behavior. FIG. **17**A: On-target mutation frequencies associated with Cas9 nucleases (WT), nickases (D10A), and paired nickases at the following target sequences of the AAVS1 locus: S1 (SEQ ID NO: 193, S2 (SEQ ID NO: 194), S3 (SEQ ID NO: 195), S4 (SEQ ID NO: 196), S5 (SEQ ID NO: 197), S6 (SEQ ID NO: 198), AS1 (SEQ ID NO: 199), AS2 (SEQ ID NO: 200), and AS3 (SEQ ID NO: 201). Paired nickases that would produce 5' overhangs or 3' overhangs are indicated. FIG. **17**B: Analysis of off-target effects of Cas9 nucleases and paired nickases. A total of seven potential off-target sites (SEQ ID NOs. 202-208) for three sgRNAs were analyzed. The mutation frequency for the S2 on-target sequence (SEQ ID NO: 194) was compared to the off-target sequences, S2 Off-1 (SEQ ID NO: 202) and S2 Off-2 (SEQ ID NO: 203). The mutation frequency for the S3 on-target sequence (SEQ ID NO: 195) was compared to the off-target sequences, S3 Off-1 (SEQ ID NO: 204) and S3 Off-2 (SEQ ID NO: 205). The mutation frequency for the AS2 on-target sequence (SEQ ID NO: 198) was compared to the off-target sequences, AS2Off-1 (SEQ ID NO: 206), AS2 Off-6 (SEQ ID NO: 207), and AS2 Off-9 (SEQ ID NO: 208).

FIGS. **18**A, **18**B, **18**C, and **18**D show paired Cas9 nickases tested at other endogenous human loci. The sgRNA target sites at the human CCR5 locus (FIG. **18**A; SEQ ID NO: 209) and the BRCA2 locus (FIG. **18**C; SEQ ID NO: 210). PAM sequences are indicated in a box. Genome editing activities at CCR5 (FIG. **18**B) and BRCA2 (FIG.

18D) target sites were detected by the T7E1 assay. The repair of two nicks that would produce 5' overhangs led to the formation of indels much more frequently than did those producing 3' overhangs.

FIGS. **19**A and **19**B show that paired Cas9 nickases mediate homologous recombination. FIG. **19**A: Strategy to detect homologous recombination. Donor DNA included an XbaI restriction enzyme site between two homology arms, whereas the endogenous target site lacked this site. A PCR assay was used to detect sequences that had undergone homologous recombination. To prevent amplification of contaminating donor DNA, primers specific to genomic DNA were used. FIG. **19**B: Efficiency of homologous recombination. Only amplicons of a region in which homologous recombination had occurred could be digested with XbaI; the intensities of the cleavage bands were used to measure the efficiency of this method.

FIGS. **20**A, **20**B, **20**C, and **20**D show DNA splicing induced by paired Cas9 nickases. FIG. **20**A: The target sites of paired nickases in the human AAVS1 locus. The distances between the AS2 site and each of the other sites are shown. Arrows indicate PCR primers. FIG. **20**B: Genomic deletions detected using PCR. Asterisks indicate deletion-specific PCR products. FIG. **20**C: DNA sequences of wild-type (WT) (SEQ ID NO: 211 and 332) and the following deletion-specific PCR products (SEQ ID Nos. 212-218) obtained using AS2 sgRNAs or deletion-specific PCR products (SEQ ID NOs. 219-224) using L1 sgRNAs. Target site PAM sequences are shown in box and sgRNA-matching sequences are shown in capital letters. Intact sgRNA-matching sequences are underlined. FIG. **20**D: A schematic model of paired Cas9 nickase-mediated chromosomal deletions. Newly-synthesized DNA strands are shown in box.

FIGS. **21**A, **21**B, and **21**C show that paired Cas9 nickases do not induce translocations. FIG. **21**A: Schematic overview of chromosomal translocations between the on-target and off-target sites. FIG. **21**B: PCR amplification to detect chromosomal translocations. FIG. **21**C: Translocations induced by Cas9 nucleases but not by the nickase pair.

FIGS. **22**A and **22**B show a conceptual diagram of the T7E1 and RFLP assays. FIG. **22**A: Comparison of assay cleavage reactions in four possible scenarios after engineered nuclease treatment in a diploid cell: (A) wild type, (B) a monoallelic mutation, (C) different biallelic mutations (hetero), and (D) identical biallelic mutations (homo). Black lines represent PCR products derived from each allele; dashed and dotted boxes indicate insertion/deletion mutations generated by NHEJ. FIG. **22**B: Expected results of T7E1 and RGEN digestion resolved by electrophoresis.

FIG. **23** shows in vitro cleavage assay of a linearized plasmid containing the C4BPB target site bearing indels. DNA sequences of individual plasmid substrates (upper panel): WT (SEQ ID NO: 104), I1 (SEQ ID NO: 225), I2 (SEQ ID NO: 226), I3 (SEQ ID NO: 227), D1 (SEQ ID NO: 228), D2 (SEQ ID NO: 229), and D3 (SEQ ID NO: 230). The PAM sequence is underlined. Inserted bases are shown in box. Arrows (bottom panel) indicate expected positions of DNA bands cleaved by the wild-type-specific RGEN after electrophoresis.

FIGS. **24**A and **24**B show genotyping of mutations induced by engineered nucleases in cells via RGEN-mediated RFLP. FIG. **24**A: Genotype of C4BPB wild type (SEQ ID NO: 231) and the following mutant K562 cell clones: +3 (SEQ ID NO: 232, −12 (SEQ ID NO: 233), −9 (SEQ ID NO: 234), −8 (SEQ ID NO: 235), −36 (SEQ ID NO: 236), +1 (SEQ ID NO: 237), +1 (SEQ ID NO: 238), +67 (SEQ ID NO: 239), −7, +1 (SEQ ID NO: 240), −94 (SEQ ID NO: 241). FIG. **24**B: Comparison of the mismatch-sensitive T7E1 assay with RGEN-mediated RFLP analysis. Black arrows indicate the cleavage product by treatment of T7E1 enzyme or RGENS.

FIGS. **25**A, **25**B, and **25**C show genotyping of RGEN-induced mutations via the RGEN-RFLP technique. FIG. **25**A: Analysis of C4BPB-disrupted clones using RGEN-RFLP and T7E1 assays. Arrows indicate expected positions of DNA bands cleaved by RGEN or T7E1. FIG. **25**B: Quantitative comparison of RGEN-RFLP analysis with T7E1 assays. Genomic DNA samples from wild-type and C4BPB-disrupted K562 cells were mixed in various ratios and subjected to PCR amplification. FIG. **25**C: Genotyping of RGEN-induced mutations in the HLA-B gene in HeLa cells with RFLP and T7E1 analyses.

FIGS. **26**A and **26**B show genotyping of mutations induced by engineered nucleases in organisms via RGEN-mediated RFLP. FIG. **26**A: Genotype of Pibf1 wild-type (WT) (SEQ ID NO: 242) and the following mutant founder mice: #1 (SEQ ID NO: 243 and SEQ ID NO: 244), #3 (SEQ ID NO: 245 and SEQ ID NO: 246), #4 (SEQ ID NO: 247 and SEQ ID NO: 242), #5 (SEQ ID NO: 246 and SEQ ID NO: 242), #6 (SEQ ID NO: 248 and SEQ ID NO: 249), #8 (SEQ ID NO: 250 and SEQ ID NO: 251), and #11 (SEQ ID NO: 252 and SEQ ID NO: 250). FIG. **26**B: Comparison of the mismatch-sensitive T7E1 assay with RGEN-mediated RFLP analysis. Black arrows indicate the cleavage product by treatment of T7E1 enzyme or RGENS.

FIG. **27** shows RGEN-mediated genotyping of ZFN-induced mutations at a wild-type CCR5 sequence (SEQ ID NO: 253). The ZFN target site is shown in box. Black arrows indicate DNA bands cleaved by T7E1.

FIG. **28** shows polymorphic sites in a region of the human HLA-B gene (SEQ ID NO: 254). The sequence, which surrounds the RGEN target site, is that of a PCR amplicon from HeLa cells. Polymorphic positions are shown in box. The RGEN target site and the PAM sequence are shown in dashed and bolded box, respectively. Primer sequences are underlined.

FIGS. **29**A and **29**B show genotyping of oncogenic mutations via RGEN-RFLP analysis. FIG. **29**A: A recurrent mutation (c.133-135 deletion of TCT; SEQ ID NO: 256) in the human CTNNB1 gene in HCT116 cells was detected by RGENS. The wild-type CTNNB1 sequence is represented by SEQ ID NO: 255. HeLa cells were used as a negative control. FIG. **29**B: Genotyping of the KRAS substitution mutation (c.34 G>A) in the A549 cancer cell line with RGENs that contain mismatched guide RNA that are WT-specific (SEQ ID NO: 257) or mutant-specific (SEQ ID NO: 258). Mismatched nucleotides are shown in box. HeLa cells were used as a negative control. Arrows indicate DNA bands cleaved by RGENs. DNA sequences confirmed by Sanger sequencing are shown: wild-type (SEQ ID NO: 259) and c. 34G>A (SEQ ID NO: 260).

FIGS. **30**A, **30**B, **30**C, and **30**D show genotyping of the CCR5 delta32 allele in HEK293T cells via RGEN-RFLP analysis. FIG. **30**A: RGEN-RFLP assays of cell lines. DNA sequences of the wild-type CCR5 locus (SEQ ID NO: 262) and delta 32 mutation (SEQ ID NO: 261) are shown. K562, SKBR3, and HeLa cells were used as wild-type controls. Arrows indicate DNA bands cleaved by RGENs. FIG. **30**B: DNA sequence of wild-type (SEQ ID NO: 263) and delta32 CCR5 alleles (SEQ ID NO: 264). Both on-target and off-target sites of RGENs used in RFLP analysis are underlined. A single-nucleotide mismatch between the two sites is shown in box. The PAM sequence is underlined. FIG. **30**C: In vitro cleavage of plasmids harboring WT or del32 CCR5

alleles using the wild-type-specific RGEN. FIG. **30**D Confirming the presence of an off-target site of the CCR5-delta32-specific RGEN at the CCR5 locus. In vitro cleavage assays of plasmids harboring either on-target (SEQ ID NO: 265) or off-target sequences (SEQ ID NO: 266) using various amounts of the del32-specific RGEN.

FIGS. **31**A and **31**B show genotyping of a KRAS point mutation (c.34 G>A). FIG. **31**A: RGEN-RFLP analysis of the KRAS mutation (c.34 G>A) in cancer cell lines. PCR products from HeLa cells (used as a wild-type control) or A549 cells, which are homozygous for the point mutation, were digested with RGENs with perfectly matched crRNA specific to the wild-type sequence (SEQ ID NO: 259) or the mutant sequence (SEQ ID NO: 260). KRAS genotypes in these cells were confirmed by Sanger sequencing. FIG. **31**B: Plasmids harboring either the wild-type (SEQ ID NO: 259) or mutant KRAS sequences (SEQ ID NO: 260) were digested using RGENs with perfectly matched crRNAs or attenuated, one-base mismatched crRNAs: m7 (SEQ ID NO: 267), m6 (SEQ ID NO: 257), m5 (SEQ ID NO: 268), m4 (SEQ ID NO: 269), m8 (SEQ ID NO: 260), m7, 8 (SEQ ID NO: 270), m6, 8 (SEQ ID NO: 258), m5, 8 (SEQ ID NO: 271), and m4, 8 (SEQ ID NO: 272). Attenuated crRNAs that were chosen for genotyping are labeled in box above the gels.

FIGS. **32**A and **32**B show genotyping of a PIK3CA point mutation (c.3140 A>G). FIG. **32**A: RGEN-RFLP analysis of the PIK3CA mutation (c.3140 A>G) in cancer cell lines. PCR products from HeLa cells (used as a wild-type control) or HCT116 cells that are heterozygous for the point mutation were digested with RGENs with perfectly matched crRNA specific to the wild-type sequence (SEQ ID NO: 273) or the mutant sequence (SEQ ID NO: 274). PIK3CA genotypes in these cells were confirmed by Sanger sequencing. FIG. **32**B: Plasmids harboring either the wild-type PIK3CA sequence (SEQ ID NO: 273) or mutant PIK3CA sequence (SEQ ID NO: 274) were digested using RGENs with perfectly matched crRNAs or attenuated, one-base mismatched crRNAs: m5 (SEQ ID NO: 275), m6 (SEQ ID NO: 276), m7 (SEQ ID NO: 277), m10 (SEQ ID NO: 278), m13 (SEQ ID NO: 279), m16 (SEQ ID NO: 280), m19 (SEQ ID NO: 281), m4 (SEQ ID NO: 274), m4, 5 (SEQ ID NO: 282), m4, 6 (SEQ ID NO: 283), m4, 7 (SEQ ID NO: 284), m4, 10 (SEQ ID NO: 285), m4, 13 (SEQ ID NO: 286), m4, 16 (SEQ ID NO: 287), and m4, 19 (SEQ ID NO: 288). Attenuated crRNAs that were chosen for genotyping are labeled in box above the gels.

FIGS. **33**A, **33**B, **33**C, and **33**D show genotyping of recurrent point mutations in cancer cell lines. FIG. **33**A: RGEN-RFLP assays to distinguish between a wild-type IDH gene sequence (SEQ ID NO: 289) and a recurrent oncogenic point mutation sequence in the IDH gene (c.394c>T; SEQ ID NO: 290). RGENs with attenuated, one-base mismatched crRNAs, SEQ ID NO: 291 (WT-Specific RNA) and SEQ ID NO: 292 (Mutant-Specific RNA), distinguished the wild type and mutant IDH sequences. FIG. **33**B: RGEN-RFLP assays to distinguish between a wild-type PIK3CA gene sequence (SEQ ID NO: 271) and a recurrent oncogenic point mutation sequence in the PIK3CA gene (c.3140A>G; SEQ ID NO: 273). RGENs with attenuated, one-base mismatched crRNAs, SEQ ID NO: 275 (WT-Specific RNA) and SEQ ID NO: 284 (Mutant-Specific RNA), distinguished the wild type and mutant PIK3CA sequences. FIG. **33**C: RGEN-RFLP assays to distinguish between a wild-type NRAS gene sequence (SEQ ID NO: 293) and a recurrent oncogenic point mutation sequence in the NRAS gene (c.181C>A; SEQ ID NO: 294). RGENs with perfectly matched crRNAs, SEQ ID NO: 293 (WT-Specific RNA) and SEQ ID NO: 294 (Mutant-Specific RNA), distinguished the wild type and mutant NRAS sequences. FIG. **33**D: RGEN-RFLP assays to distinguish between a wild-type BRAF gene sequence (SEQ ID NO: 295) and a recurrent oncogenic point mutation sequence in the BRAF gene (c.1799T>A; SEQ ID NO: 296). RGENs with perfectly matched crRNAs, SEQ ID NO: 295 (WT-Specific RNA) and SEQ ID NO: 296 (Mutant-Specific RNA), distinguished the wild type and mutant BRAF sequences. Genotypes of each cell line confirmed by Sanger sequencing are shown. Mismatched nucleotides are shown in box. Black arrows indicate DNA bands cleaved by RGENS.

BEST MODE FOR CARRYING OUT THE INVENTION

In accordance with one aspect of the invention, the present invention provides a composition for cleaving target DNA in eukaryotic cells or organisms comprising a guide RNA specific for target DNA or DNA that encodes the guide RNA, and Cas protein-encoding nucleic acid or Cas protein. In addition, the present invention provides a use of the composition for cleaving target DNA in eukaryotic cells or organisms comprising a guide RNA specific for target DNA or DNA that encodes the guide RNA, and Cas protein-encoding nucleic acid or Cas protein.

In the present invention, the composition is also referred to as a RNA-guided endonuclease (RGEN) composition.

ZFNs and TALENs enable targeted mutagenesis in mammalian cells, model organisms, plants, and livestock, but the mutation frequencies obtained with individual nucleases are widely different from each other. Furthermore, some ZFNs and TALENs fail to show any genome editing activities. DNA methylation may limit the binding of these engineered nucleases to target sites. In addition, it is technically challenging and time-consuming to make customized nucleases.

The present inventors have developed a new RNA-guided endonuclease composition based on Cas protein to overcome the disadvantages of ZENs and TALENS.

Prior to the present invention, an endonuclease activity of Cas proteins has been known. However, it has not been known whether the endonuclease activity of Cas protein would function in an eukaryotic cell because of the complexity of the eukaryotic genome. Further, until now, a composition comprising Cas protein or Cas protein-encoding nucleic acid and a guide RNA specific for the target DNA to cleave a target DNA in eukaryotic cells or organisms has not been developed.

Compared to ZENs and TALENs, the present RGEN composition based on Cas protein can be more readily customized because only the synthetic guide RNA component is replaced to make a new genome-editing nuclease. No sub-cloning steps are involved to make customized RNA guided endonucleases. Furthermore, the relatively small size of the Cas gene (for example, 4.2 kbp for Cas9) as compared to a pair of TALEN genes (~6 kbp) provides an advantage for this RNA-guided endonuclease composition in some applications such as virus-mediated gene delivery. Further, this RNA-guided endonuclease does not have off-target effects and thus does not induce unwanted mutations, deletion, inversions, and duplications. These features make the present RNA-guided endonuclease composition a scalable, versatile, and convenient tool for genome engineering in eukaryotic cells and organisms. In addition, RGEN can be designed to target any DNA sequence, almost any single nucleotide polymorphism or small insertion/deletion (indel)

can be analyzed via RGEN-mediated RFLP. The specificity of RGENs is determined by the RNA component that hybridizes with a target DNA sequence of up to 20 base pairs (bp) in length and by the Cas9 protein that recognizes the protospacer-adjacent motif (PAM). RGENS are readily reprogrammed by replacing the RNA component. Therefore, RGENs provide a platform to use simple and robust R00FLP analysis for various sequence variations.

The target DNA may be an endogenous DNA, or artificial DNA, preferably, endogenous DNA.

As used herein, the term "Cas protein" refers to an essential protein component in the CRISPR/Cas system, forms an active endonuclease or nickase when complexed with two RNAs termed CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA).

The information on the gene and protein of Cas are available from GenBank of National Center for Biotechnology Information (NCBI), without limitation.

The CRISPR-associated (cas) genes encoding Cas proteins are often associated with CRISPR repeat-spacer arrays. More than forty different Cas protein families have been described. Of these protein families, Cas1 appears to be ubiquitous among different CRISPR/Cas systems. There are three types of CRISPR-Cas system. Among them, Type II CRISPR/Cas system involving Cas9 protein and crRNA and tracrRNA is representative and is well known. Particular combinations of cas genes and repeat structures have been used to define 8 CRISPR subtypes (Ecoli, Ypest, Nmeni, Dvulg, Tneap, Hmari, Apern, and Mtube).

The Cas protein may be linked to a protein transduction domain. The protein transduction domain may be poly-arginine or a TAT protein derived from HIV, but it is not limited thereto.

The present composition may comprise Cas component in the form of a protein or in the form of a nucleic acid encoding Cas protein.

In the present invention, Cas protein may be any Cas protein provided that it has an endonuclease or nickase activity when complexed with a guide RNA.

Preferably, Cas protein is Cas9 protein or variants thereof.

The variant of the Cas9 protein may be a mutant form of Cas9 in which the catalytic aspartate residue is changed to any other amino acid. Preferably, the other amino acid may be an alanine, but it is not limited thereto.

Further, Cas protein may be the one isolated from an organism such as *Streptococcus* sp., preferably *Streptococcus pyogenes* or a recombinant protein, but it is not limited thereto.

The Cas protein derived from *Streptococcus pyogenes* may recognize NGG trinucleotide. The Cas protein may comprise an amino acid sequence of SEQ ID NO: 109, but it is not limited thereto.

The term "recombinant" when used with reference, e.g., to a cell, nucleic acid, protein, or vector, indicates that the cell, nucleic acid, protein or vector, has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. Thus, for example, a recombinant Cas protein may be generated by reconstituting Cas protein-encoding sequence using the human codon table.

As for the present invention, Cas protein-encoding nucleic acid may be a form of vector, such as plasmid comprising Cas-encoding sequence under a promoter such as CMV or CAG. When Cas protein is Cas9, Cas9 encoding sequence may be derived from *Streptococcus* sp., and preferably derived from *Streptococcus pyogenes*. For example, Cas9 encoding nucleic acid may comprise the nucleotide sequence of SEQ ID. NO: 1. Moreover, Cas9 encoding nucleic acid may comprise the nucleotide sequence having homology of at least 50% to the sequence of SEQ ID NO: 1, preferably at least 60, 70, 80, 90, 95, 97, 98, or 99% to the SEQ ID NO:1, but it is not limited thereto. Cas9 encoding nucleic acid may comprise the nucleotide sequence of SEQ ID NOs. 108, 110, 106, or 107.

As used herein, the term "guide RNA" refers to a RNA which is specific for the target DNA and can form a complex with Cas protein and bring Cas protein to the target DNA.

In the present invention, the guide RNA may consist of two RNA, i.e., CRISPR RNA (crRNA) and transactivating crRNA (tracrRNA) or be a single-chain RNA (sgRNA) produced by fusion of an essential portion of crRNA and tracrRNA.

The guide RNA may be a dualRNA comprising a crRNA and a tracrRNA.

If the guide RNA comprises the essential portion of crRNA and tracrRNA and a portion complementary to a target, any guide RNA may be used in the present invention.

The crRNA may hybridize with a target DNA.

The RGEN may consist of Cas protein, and dualRNA (invariable tracrRNA and target-specific crRNA), or Cas protein and sgRNA (fusion of an essential portion of invariable tracrRNA and target-specific crRNA), and may be readily reprogrammed by replacing crRNA.

The guide RNA further comprises one or more additional nucleotides at the 5' end of the single-chain guide RNA or the crRNA of the dualRNA.

Preferably, the guide RNA further comprises 2-additional guanine nucleotides at the 5' end of the single-chain guide RNA or the crRNA of the dualRNA.

The guide RNA may be transferred into a cell or an organism in the form of RNA or DNA that encodes the guide RNA. The guide RNA may be in the form of an isolated RNA, RNA incorporated into a viral vector, or is encoded in a vector. Preferably, the vector may be a viral vector, plasmid vector, or agrobacterium vector, but it is not limited thereto.

A DNA that encodes the guide RNA may be a vector comprising a sequence coding for the guide RNA. For example, the guide RNA may be transferred into a cell or organism by transfecting the cell or organism with the isolated guide RNA or plasmid DNA comprising a sequence coding for the guide RNA and a promoter.

Alternatively, the guide RNA may be transferred into a cell or organism using virus-mediated gene delivery.

When the guide RNA is transfected in the form of an isolated RNA into a cell or organism, the guide RNA may be prepared by in vitro transcription using any in vitro transcription system known in the art. The guide RNA is preferably transferred to a cell in the form of isolated RNA rather than in the form of plasmid comprising encoding sequence for a guide RNA. As used herein, the term "isolated RNA" may be interchangeable to "naked RNA". This is cost- and time-saving because it does not require a step of cloning. However, the use of plasmid DNA or virus-mediated gene delivery for transfection of the guide RNA is not excluded.

The present RGEN composition comprising Cas protein or Cas protein-encoding nucleic acid and a guide RNA can specifically cleave a target DNA due to a specificity of the guide RNA for a target and an endonuclease or nickase activity of Cas protein.

As used herein, the term "cleavage" refers to the breakage of the covalent backbone of a nucleotide molecule.

In the present invention, a guide RNA may be prepared to be specific for any target which is to be cleaved. Therefore, the present RGEN composition can cleave any target DNA by manipulating or genotyping the target-specific portion of the guide RNA.

The guide RNA and the Cas protein may function as a pair. As used herein, the term "paired Cas nickase" may refer to the guide RNA and the Cas protein functioning as a pair. The pair comprises two guide RNAs. The guide RNA and Cas protein may function as a pair, and induce two nicks on different DNA strand. The two nicks may be separated by at least 100 bps, but are not limited thereto.

In the Example, the present inventors confirmed that paired Cas nickase allow targeted mutagenesis and large deletions of up to 1-kbp chromosomal segments in human cells. Importantly, paired nickases did not induce indels at off-target sites at which their corresponding nucleases induce mutations. Furthermore, unlike nucleases, paired nickases did not promote unwanted translocations associated with off-target DNA cleavages. In principle, paired nickases double the specificity of Cas9-mediated mutagenesis and will broaden the utility of RNA-guided enzymes in applications that require precise genome editing such as gene and cell therapy.

In the present invention, the composition may be used in the genotyping of a genome in the eukaryotic cells or organisms in vitro.

In one specific embodiment, the guide RNA may comprise the nucleotide sequence of Seq ID. No. 1, wherein the portion of nucleotide position 3~22 is a target-specific portion and thus, the sequence of this portion may be changed depending on a target.

As used herein, a eukaryotic cell or organism may be yeast, fungus, protozoa, plant, higher plant, and insect, or amphibian cells, or mammalian cells such as CHO, HeLa, HEK293, and COS-1, for example, cultured cells (in vitro), graft cells and primary cell culture (in vitro and ex vivo), and in vivo cells, and also mammalian cells including human, which are commonly used in the art, without limitation.

In one specific embodiment, it was found that Cas9 protein/single-chain guide RNA could generate site-specific DNA double-strand breaks in vitro and in mammalian cells, whose spontaneous repair induced targeted genome mutations at high frequencies.

Moreover, it was found that gene-knockout mice could be induced by the injection of Cas9 protein/guide RNA complexes or Cas9 mRNA/guide RNA into one-cell stage embryo and germ-line transmittable mutations could be generated by Cas9/guide RNA system.

Using Cas protein rather than a nucleic acid encoding Cas protein to induce a targeted mutagenesis is advantageous because exogeneous DNA is not introduced into an organism. Thus, the composition comprising Cas protein and a guide RNA may be used to develop therapeutics or value-added crops, livestock, poultry, fish, pets, etc.

In accordance with another aspect of the invention, the present invention provides a composition for inducing targeted mutagenesis in eukaryotic cells or organisms, comprising a guide RNA specific for target DNA or DNA that encodes the guide RNA, and Cas protein-encoding nucleic acid or Cas protein. In addition, the present invention provides a use of the composition for inducing targeted mutagenesis in eukaryotic cells or organisms, comprising a guide RNA specific for target DNA or DNA that encodes the guide RNA, and Cas protein-encoding nucleic acid or Cas protein.

A guide RNA, Cas protein-encoding nucleic acid or Cas protein are as described in the above.

In accordance with another aspect of the invention, the present invention provides a kit for cleaving a target DNA or inducing targeted mutagenesis in eukaryotic cells or organisms comprising a guide RNA specific for target DNA or DNA that encodes the guide RNA, and Cas protein-encoding nucleic acid or Cas protein.

A guide RNA, Cas protein-encoding nucleic acid or Cas protein are as described in the above.

The kit may comprise a guide RNA and Cas protein-encoding nucleic acid or Cas protein as separate components or as one composition.

The present kit may comprise some additional components necessary for transferring the guide RNA and Cas component to a cell or an organism. For example, the kit may comprise an injection buffer such as DEPC-treated injection buffer, and materials necessary for analysis of mutation of a target DNA, but are not limited thereto.

In accordance with another aspect, the present invention provides a method for preparing a eukaryotic cell or organism comprising Cas protein and a guide RNA comprising a step of co-transfecting or serial-transfecting the eukaryotic cell or organism with a Cas protein-encoding nucleic acid or Cas protein, and a guide RNA or DNA that encodes the guide RNA.

A guide RNA, Cas protein-encoding nucleic acid or Cas protein are as described in the above.

In the present invention, a Cas protein-encoding nucleic acid or Cas protein and a guide RNA or DNA that encodes the guide RNA may be transferred into a cell by various methods known in the art, such as microinjection, electroporation, DEAE-dextran treatment, lipofection, nanoparticle-mediated transfection, protein transduction domain mediated transduction, virus-mediated gene delivery, and PEG-mediated transfection in protoplast, and so on, but are not limited thereto. Also, a Cas protein encoding nucleic acid or Cas protein and a guide RNA may be transferred into an organism by various method known in the art to administer a gene or a protein such as injection. A Cas protein-encoding nucleic acid or Cas protein may be transferred into a cell in the form of complex with a guide RNA, or separately. Cas protein fused to a protein transduction domain such as Tat can also be delivered efficiently into cells.

Preferably, the eukaryotic cell or organism is co-transfected or serial-transfected with a Cas9 protein and a guide RNA.

The serial-transfection may be performed by transfection with Cas protein-encoding nucleic acid first, followed by second transfection with naked guide RNA. Preferably, the second transfection is after 3, 6, 12, 18, 24 hours, but it is not limited thereto.

In accordance with another aspect, the present invention provides a eukaryotic cell or organism comprising a guide RNA specific for target DNA or DNA that encodes the guide RNA, and Cas protein-encoding nucleic acid or Cas protein.

The eukaryotic cells or organisms may be prepared by transferring the composition comprising a guide RNA specific for target DNA or DNA that encodes the guide RNA, and Cas protein-encoding nucleic acid or Cas protein into the cell or organism.

The eukaryotic cell may be yeast, fungus, protozoa, higher plant, and insect, or amphibian cells, or mammalian cells such as CHO, HeLa, HEK293, and COS-1, for example, cultured cells (in vitro), graft cells and primary cell culture (in vitro and ex vivo), and in vivo cells, and also mammalian cells including human, which are commonly

used in the art, without limitation. Further the organism may be yeast, fungus, protozoa, plant, higher plant, insect, amphibian, or mammal.

In accordance with another aspect of the invention, the present invention provides a method for cleaving a target DNA or inducing targeted mutagenesis in eukaryotic cells or organisms, comprising a step of treating a cell or organism comprising a target DNA with a composition comprising a guide RNA specific for target DNA or DNA that encodes the guide RNA, and Cas protein-encoding nucleic acid or Cas protein.

The step of treating a cell or organism with the composition may be performed by transferring the present composition comprising a guide RNA specific for target DNA or DNA that encodes the guide RNA, and Cas protein-encoding nucleic acid or Cas protein into the cell or organism.

As described in the above, such transfer may be performed by microinjection, transfection, electroporation, and so on.

In accordance with another aspect of the invention, the present invention provides an embryo comprising a genome edited by the present RGEN composition comprising a guide RNA specific for target DNA or DNA that encodes the guide RNA, and Cas protein-encoding nucleic acid or Cas protein.

Any embryo can be used in the present invention, and for the present invention, the embryo may be an embryo of a mouse. The embryo may be produced by injecting PMSG (Pregnant Mare Serum Gonadotropin) and hCG (human Chorionic Gonadotropin) into a female mouse of 4 to 7 weeks and the super-ovulated female mouse may be mated to males, and the fertilized embryos may be collected from oviducts.

The present RGEN composition introduced into an embryo can cleave a target DNA complementary to the guide RNA by the action of Cas protein and cause a mutation in the target DNA. Thus, the embryo into which the present RGEN composition has been introduced has an edited genome.

In one specific embodiment, it was found that the present RGEN composition could cause a mutation in a mouse embryo and the mutation could be transmitted to offspring.

A method for introducing the RGEN composition into the embryo may be any method known in the art, such as microinjection, stem cell insertion, retrovirus insertion, and so on. Preferably, a microinjection technique can be used.

In accordance with another aspect, the present invention provides a genome-modified animal obtained by transferring the embryo comprising a genome edited by the present RGEN composition into the oviducts of an animal.

In the present invention, the term "genome-modified animal" refers to an animal of which genome has been modified in the stage of embryo by the present RGEN composition and the type of the animal is not limited.

The genome-modified animal has mutations caused by a targeted mutagenesis based on the present RGEN composition. The mutations may be any one of deletion, insertion, translocation, inversion. The site of mutation depends on the sequence of guide RNA of the RGEN composition.

The genome-modified animal having a mutation of a gene may be used to determine the function of the gene.

In accordance with another aspect of the invention, the present invention provides a method of preparing a genome-modified animal comprising a step of introducing the present RGEN composition comprising a guide RNA specific for the target DNA or DNA that encodes the guide RNA and Cas protein-encoding nucleic acid or Cas protein into an embryo of an animal; and a step of transferring the embryo into a oviduct of pseudopregnant foster mother to produce a genome-modified animal.

The step of introducing the present RGEN composition may be accomplished by any method known in the art such as microinjection, stem cell insertion, retroviral insertion, and so on.

In accordance with another aspect of the invention, the present invention provides a plant regenerated form the genome-modified protoplasts prepared by the method for eukaryotic cells comprising the RGEN composition.

In accordance with another aspect of the invention, the present invention provides a composition for genotyping mutations or variations in an isolated biological sample, comprising a guide RNA specific for the target DNA sequence Cas protein. In addition, the present invention provides a composition for genotyping nucleic acid sequences in pathogenic microorganisms in an isolated biological sample, comprising a guide RNA specific for the target DNA sequence and Cas protein.

A guide RNA, Cas protein-encoding nucleic acid or Cas protein are as described in the above.

As used herein the term "genotyping" refers to the "Restriction fragment length polymorphism (RFLP) assay".

RFLP may be used in 1) the detection of indel in cells or organisms induced by the engineered nucleases, 2) the genotyping naturally-occurring mutations or variations in cells or organisms, or 3) the genotyping the DNA of infected pathogenic microorganisms including virus or bacteria, etc.

The mutations or variation may be induced by engineered nucleases in cells.

The engineered nuclease may be a Zinc Finger Nuclease (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), or RGENs, but it is not limited thereto.

As used herein the term "biological sample" includes samples for analysis, such as tissues, cells, whole blood, serum, plasma, saliva, sputum, cerebrospinal fluid or urine, but is not limited thereto.

The mutations or variation may be a naturally-occurring mutations or variations.

The mutations or variations are induced by the pathogenic microorganisms. Namely, the mutations or variations occur due to the infection of pathogenic microorganisms, when the pathogenic microorganisms are detected, the biological sample is identified as infected.

The pathogenic microorganisms may be virus or bacteria, but are not limited thereto.

Engineered nuclease-induced mutations are detected by various methods, which include mismatch-sensitive Surveyor or T7 endonuclease I (T7E1) assays, RFLP analysis, fluorescent PCR, DNA melting analysis, and Sanger and deep sequencing. The T7E1 and Surveyor assays are widely used but often underestimate mutation frequencies because the assays detect heteroduplexes (formed by the hybridization of mutant and wild-type sequences or two different mutant sequences); they fail to detect homoduplexes formed by the hybridization of two identical mutant sequences. Thus, these assays cannot distinguish homozygous biallelic mutant clones from wild-type cells nor heterozygous biallelic mutants from heterozygous monoallelic mutants (FIG. **22**). In addition, sequence polymorphisms near the nuclease target site can produce confounding results because the enzymes can cleave heteroduplexes formed by hybridization of these different wild-type alleles. RFLP analysis is free of these limitations and therefore is a method of choice. Indeed, RFLP analysis was one of the first methods used to detect

engineered nuclease-mediated mutations. Unfortunately, however, it is limited by the availability of appropriate restriction sites.

In accordance with another aspect of the invention, the present invention provides a kit for genotyping mutations or variations in an isolated biological sample, comprising the composition for genotyping mutations or variations in an isolated biological sample. In addition, the present invention provides a kit for genotyping nucleic acid sequences in pathogenic microorganisms in an isolated biological sample, comprising a guide RNA specific for the target DNA sequence and Cas protein.

A guide RNA, Cas protein-encoding nucleic acid or Cas protein are as described in the above.

In accordance with another aspect of the invention, the present invention provides a method of genotyping mutations or variations in an isolated biological sample, using the composition for genotyping mutations or variations in an isolated biological sample. In addition, the present invention provides a method of genotyping nucleic acid sequences in pathogenic microorganisms in an isolated biological sample, comprising a guide RNA specific for the target DNA sequence and Cas protein.

A guide RNA, Cas protein-encoding nucleic acid or Cas protein are as described in the above.

Mode for the Invention

Hereinafter, the present invention will be described in more detail with reference to Examples. However, these Examples are for illustrative purposes only, and the invention is not intended to be limited by these Examples.

Example 1: Genome Editing Assay

1-1. DNA Cleavage Activity of Cas9 Protein

Firstly, the DNA cleavage activity of Cas9 derived from *Streptococcus pyogenes* in the presence or absence of a chimeric guide RNA in vitro was tested.

To this end, recombinant Cas9 protein that was expressed in and purified from *E. coli* was used to cleave a predigested or circular plasmid DNA that contained the 23-base pair (bp) human CCR5 target sequence. A Cas9 target sequence consists of a 20-bp DNA sequence complementary to crRNA or a chimeric guide RNA and the trinucleotide (5'-NGG-3') protospacer adjacent motif (PAM) recognized by Cas9 itself (FIG. **1**A).

Specifically, the Cas9-coding sequence (4,104 bp), derived from *Streptococcus pyogenes* strain MI GAS (NC_002737.1), was reconstituted using the human codon usage table and synthesized using oligonucleotides. First, 1-kb DNA segments were assembled using overlapping ~35-mer oligonucleotides and Phusion™ polymerase (New England Biolabs) and cloned into T-vector (SolGent). A full-length Cas9 sequence was assembled using four 1-kbp DNA segments by overlap PCR. The Cas9-encoding DNA segment was subcloned into p3s, which was derived from pcDNA3.1 (Invitrogen). In this vector, a peptide tag (NH2-GGSGPPKKKRKVYPYDVPDYA-COOH, SEQ ID NO: 2) containing the HA epitope and a nuclear localization signal (NLS) was added to the C-terminus of Cas9. Expression and nuclear localization of the Cas9 protein in HEK 293T cells were confirmed by western blotting using anti-HA antibody (Santa Cruz).

Then, the Cas9 cassette was subcloned into pET28-b(+) and transformed into BL21(DE3). The expression of Cas9 was induced using 0.5 mM IPTG for 4 h at 25° C. The Cas9 protein containing the His6-tag at the C terminus was purified using Ni-NTA agarose resin (Qiagen) and dialyzed against 20 mM HEPES (pH 7.5), 150 mM KCl, 1 mM DTT, and 10% glycerol (1). Purified Cas9 (50 nM) was incubated with super-coiled or pre-digested plasmid DNA (300 ng) and chimeric RNA (50 nM) in a reaction volume of 20 μl in NEB buffer 3 for 1 h at 37° C. Digested DNA was analyzed by electrophoresis using 0.8% agarose gels.

Cas9 cleaved the plasmid DNA efficiently at the expected position only in the presence of the synthetic RNA and did not cleave a control plasmid that lacked the target sequence (FIG. **1**B).

1-2. DNA Cleavage by Cas9/Guide RNA Complex in Human Cells

A RFP-GFP reporter was used to investigate whether the Cas9/guide RNA complex can cleave the target sequence incorporated between the RFP and GFP sequences in mammalian cells.

In this reporter, the GFP sequence is fused to the RFP sequence out-of-frame (2). The active GFP is expressed only when the target sequence is cleaved by site-specific nucleases, which causes frameshifting small insertions or deletions (indels) around the target sequence via error-prone non-homologous end-joining (NHEJ) repair of the double-strand break (DSB) (FIG. **2**).

The RFP-GFP reporter plasmids used in this study were constructed as described previously (2). Oligonucleotides corresponding to target sites (Table 1) were synthesized (Macrogen) and annealed. The annealed oligonucleotides were ligated into a reporter vector digested with EcoRI and BamHI.

HEK 293T cells were co-transfected with Cas9-encoding plasmid (0.8 μg) and the RFP-GFP reporter plasmid (0.2 μg) in a 24-well plate using Lipofectamine™ 2000 (Invitrogen).

Meanwhile, the in vitro transcribed chimeric RNA had been prepared as follows. RNA was in vitro transcribed through run-off reactions using the MEGAshortscript™ T7 kit (Ambion) according to the manufacturer's manual. Templates for RNA in vitro transcription were generated by annealing two complementary single strand DNAs or by PCR amplification (Table 1). Transcribed RNA was resolved on a 8% denaturing urea-PAGE gel. The gel slice containing RNA was cut out and transferred to probe elution buffer. RNA was recovered in nuclease-free water followed by phenol: chloroform extraction, chloroform extraction, and ethanol precipitation. Purified RNAs were quantified by spectrometry.

At 12 h post transfection, chimeric RNA (1 μg) prepared by in vitro transcription was transfected using Lipofectamine 2000.

At 3d post-transfection, transfected cells were subjected to flow cytometry and cells expressing both RFP and GFP were counted.

It was found that GFP-expressing cells were obtained only when the cells were transfected first with the Cas9 plasmid and then with the guide RNA 12 h later (FIG. **2**), demonstrating that RGENs could recognize and cleave the target DNA sequence in cultured human cells. Thus GFP-expressing cells were obtained by serial-transfection of the Cas9 plasmid and the guide RNA rather than co-transfection.

TABLE 1

Gene		sequence (5' to 3')	SEQ ID NO.
Oligonucleotides used for the construction of the reporter plasmid			
CCR5	F	AATTCATGACATCAATTATTATACATCGGAGGAG	3
	R	GATCCTCCTCCGATGTATAATAATTGATGTCATG	4
Primers used in the T7E1 assay			
CCR5	F1	CTCCATGGTGCTATAGAGCA	5
	F2	GAGCCAAGCTCTCCATCTAGT	6
	R	GCCCTGTCAAGAGTTGACAC	7
C4BPB	F1	TATTTGGCTGGTTGAAAGGG	8
	R1	AAAGTCATGAAATAAACACACCCA	9
	F2	CTGCATTGATATGGTAGTACCATG	10
	R2	GCTGTTCATTGCAATGGAATG	11
Primers used for the amplification of off-target sites			
ADCY5	F1	GCTCCCACCTTAGTGCTCTG	12
	R1	GGTGGCAGGAACCTGTATGT	13
	F2	GTCATTGGCCAGAGATGTGGA	14
	R2	GTCCCATGACAGGCGTGTAT	15
KCNJ6	F	GCCTGGCCAAGTTTCAGTTA	16
	R1	TGGAGCCATTGGTTTGCATC	17
	R2	CCAGAACTAAGCCGTTTCTGAC	18
CNTNAP2	F1	ATCACCGACAACCAGTTTCC	19
	F2	TGCAGTGCAGACTCTTTCCA	20
	R	AAGGACACAGGGCAACTGAA	21
N/A Chr.5	F1	TGTGGAACGAGTGGTGACAG	22
	R1	GCTGGATTAGGAGGCAGGATTC	23
	F2	GTGCTGAGAACGCTTCATAGAG	24
	R2	GGACCAAACCACATTCTTCTCAC	25
Primers used for the detection of chromosomal deletions			
Deletion	F	CCACATCTCGTTCTCGGTTT	26
	R	TCACAAGCCCACAGATATTT	27

1-3. Targeted Disruption of Endogenous Genes in Mammalian Cells by RGEN

To test whether RGENs could be used for targeted disruption of endogenous genes in mammalian cells, genomic DNA isolated from transfected cells using T7 endonuclease I (T7E1), a mismatch-sensitive endonuclease that specifically recognizes and cleaves heteroduplexes formed by the hybridization of wild-type and mutant DNA sequences was analyzed (3).

To introduce DSBs in mammalian cells using RGENs, 2×10^6 K562 cells were transfected with 20 μg of Cas9-encoding plasmid using the 4D-Nucleofector™, SF Cell Line 4D-Nucleofector® X Kit, Program FF-120 (Lonza) according to the manufacturer's protocol. For this experiment, K562 (ATCC, CCL-243) cells were grown in RPMI-1640 with 10% FBS and the penicillin/streptomycin mix (100 U/ml and 100 μg/ml, respectively).

After 24 h, 10-40 μg of in vitro transcribed chimeric RNA was nucleofected into 1×10^6 K562 cells. The in vitro transcribed chimeric RNA had been prepared as described in the Example 1-2.

Cells were collected two days after RNA transfection and genomic DNA was isolated. The region including the target site was PCR-amplified using the primers described in Table 1. The amplicons were subjected to the T7E1 assay as described previously (3). For sequencing analysis, PCR products corresponding to genomic modifications were purified and cloned into the T-Blunt vector using the T-Blunt PCR Cloning Kit (SolGent). Cloned products were sequenced using the M13 primer.

It was found that mutations were induced only when the cells were transfected serially with Cas9-encoding plasmid and then with guide RNA (FIG. **3**). Mutation frequencies (Indels (%) in FIG. **3**A) estimated from the relative DNA band intensities were RNA-dosage dependent, ranging from 1.3% to 5.1%. DNA sequencing analysis of the PCR amplicons corroborated the induction of RGEN-mediated mutations at the endogenous sites. Indels and microhomologies, characteristic of error-prone NHEJ, were observed at the target site. The mutation frequency measured by direct sequencing was 7.3% (=7 mutant clones/96 clones), on par with those obtained with zinc finger nucleases (ZFNs) or transcription-activator-like effector nucleases (TALENS).

Serial-transfection of Cas9 plasmid and guide RNA was required to induce mutations in cells. But when plasmids that encode guide RNA, serial transfection was unnecessary and cells were co-transfected with Cas9 plasmid and guide RNA-encoding plasmid.

In the meantime, both ZFNs and TALENs have been successfully developed to disrupt the human CCR5 gene (3-6), which encodes a G-protein-coupled chemokine receptor, an essential co-receptor of HIV infection. A CCR5-specific ZEN is now under clinical investigation in the US for the treatment of AIDS (7). These ZFNs and TALENs, however, have off-target effects, inducing both local mutations at sites whose sequences are homologous to the on-target sequence (6, 8-10) and genome rearrangements that arise from the repair of two concurrent DSBs induced at on-target and off-target sites (11-12). The most striking off-target sites associated with these CCR5-specific engineered nucleases reside in the CCR2 locus, a close homolog of CCR5, located 15-kbp upstream of CCR5. To avoid off-target mutations in the CCR2 gene and unwanted deletions, inversions, and duplications of the 15-kbp chromo-

somal segment between the CCR5 on-target and CCR2 off-target sites, the present inventors intentionally chose the target site of our CCR5-specific RGEN to recognize a region within the CCR5 sequence that has no apparent homology with the CCR2 sequence.

The present inventors investigated whether the CCR5-specific RGEN had off-target effects. To this end, we searched for potential off-target sites in the human genome by identifying sites that are most homologous to the intended 23-bp target sequence. As expected, no such sites were found in the CCR2 gene. Instead, four sites, each of which carries 3-base mismatches with the on-target site, were found (FIG. **4**A). The T7E1 assays showed that mutations were not detected at these sites (assay sensitivity, ~0.5%), demonstrating exquisite specificities of RGENS (FIG. **4**B). Furthermore, PCR was used to detect the induction of chromosomal deletions in cells separately transfected with plasmids encoding the ZEN and RGEN specific to CCR5. Whereas the ZEN induced deletions, the RGEN did not (FIG. **4**C).

Next, RGENs was reprogrammed by replacing the CCR5-specific guide RNA with a newly-synthesized RNA designed to target the human C4BPB gene, which encodes the beta chain of C4b-binding protein, a transcription factor. This RGEN induced mutations at the chromosomal target site in K562 cells at high frequencies (FIG. **3**B). Mutation frequencies measured by the T7E1 assay and by direct sequencing were 14% and 8.3% (=4 mutant clones/48 clones), respectively. Out of four mutant sequences, two clones contained a single-base or two-base insertion precisely at the cleavage site, a pattern that was also observed at the CCR5 target site. These results indicate that RGENs cleave chromosomal target DNA at expected positions in cells.

Example 2: Proteinaceous RGEN-Mediated Genome Editing

RGENs can be delivered into cells in many different forms. RGENs consist of Cas9 protein, crRNA, and tracrRNA. The two RNAs can be fused to form a single-chain guide RNA (sgRNA). A plasmid that encodes Cas9 under a promoter such as CMV or CAG can be transfected into cells. crRNA, tracrRNA, or sgRNA can also be expressed in cells using plasmids that encode these RNAs. Use of plasmids, however, often results in integration of the whole or part of the plasmids in the host genome. The bacterial sequences incorporated in plasmid DNA can cause unwanted immune response in vivo. Cells transfected with plasmid for cell therapy or animals and plants derived from DNA-transfected cells must go through a costly and lengthy regulation procedure before market approval in most developed countries. Furthermore, plasmid DNA can persist in cells for several days post-transfection, aggravating off-target effects of RGENS.

Here, we used recombinant Cas9 protein complexed with in vitro transcribed guide RNA to induce targeted disruption of endogenous genes in human cells. Recombinant Cas9 protein fused with the hexa-histidine tag was expressed in and purified from *E. coli* using standard Ni ion affinity chromatography and gel filtration. Purified recombinant Cas9 protein was concentrated in storage buffer (20 mM HEPES pH 7.5, 150 mM KCl, 1 mM DTT, and 10% glycerol). Cas9 protein/sgRNA complex was introduced directly into K562 cells by nucleofection: 1×10^6 K562 cells were transfected with 22.5-225 (1.4-14 μM) of Cas9 protein mixed with 100 ug (29 μM) of in vitro transcribed sgRNA (or crRNA 40 ug and tracrRNA 80 ug) in 100 μl solution using the 4D-Nucleofector, SF Cell Line 4D-Nucleofector X Kit, Program FF-120 (Lonza) according to the manufacturer's protocol. After nucleofection, cells were placed in growth media in 6-well plates and incubated for 48 hr. When 2×10^5 K562 cells were transfected with ⅕ scale-downed protocol, 4.5-45 μg of Cas9 protein mixed with 6-60 ug of in vitro transcribed sgRNA (or crRNA 8 μg and tracrRNA 16 μg) were used and nucleofected in 20 μl solution. Nucleofected cells were then placed in growth media in 48-well plates. After 48 hr, cells were collected and genomic DNA was isolated. The genomic DNA region spanning the target site was PCR-amplified and subjected to the T7E1 assay.

As shown in FIG. **10**, Cas9 protein/sgRNA complex induced targeted mutation at the CCR5 locus at frequencies that ranged from 4.8 to 38% in a sgRNA or Cas9 protein dose-dependent manner, on par with the frequency obtained with Cas9 plasmid transfection (45%). Cas9 protein/crRNA/tracrRNA complex was able to induce mutations at a frequency of 9.4%. Cas9 protein alone failed to induce mutations. When 2×10^5 cells were transfected with ⅕ scale-downed doses of Cas9 protein and sgRNA, mutation frequencies at the CCR5 locus ranged from 2.7 to 57% in a dose-dependent manner, greater than that obtained with co-transfection of Cas9 plasmid and sgRNA plasmid (32%).

We also tested Cas9 protein/sgRNA complex that targets the ABCC11 gene and found that this complex induced indels at a frequency of 35%, demonstrating general utility of this method.

TABLE 2

Sequences of guide RNA

Target	RNA type	RNA sequence (5' to 3')	Length	SEQ ID NO
CCR5	sgRNA	GGUGACAUCAAUUAUUAUACAUGUUUUAGAGCUAG AAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCA ACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU	104 bp	28
	crRNA	GGUGACAUCAAUUAUUAUACAUGUUUUAGAGCUAU GCUGUUUUG	44 bp	29
	tracrRNA	GGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAA GGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCG AGUCGGUGCUUUUUUU	86 bp	30

Example 3: RNA-Guided Genome Editing in Mice

To examine the gene-targeting potential of RGENs in pronuclear (PN)-stage mouse embryos, the forkhead box N1 (Foxn1) gene, which is important for thymus development and keratinocyte differentiation (Nehls et al., 1996), and the protein kinase, DNA activated, catalytic polypeptide (Prkdc)

gene, which encodes an enzyme critical for DNA DSB repair and recombination (Taccioli et al., 1998) were used.

To evaluate the genome-editing activity of the Foxn1-RGEN, we injected Cas9 mRNA (10-ng/μl solution) with various doses of the sgRNA (FIG. **5***a*) into the cytoplasm of PN-stage mouse embryos, and conducted T7 endonuclease I (T7E1) assays (Kim et al. 2009) using genomic DNAs obtained from in vitro cultivated embryos (FIG. **6***a*).

Alternatively, we directly injected the RGEN in the form of recombinant Cas9 protein (0.3 to 30 ng/μl) complexed with the two-fold molar excess of Foxn1-specific sgRNA (0.14 to 14 ng/μl) into the cytoplasm or pronucleus of one-cell mouse embryos, and analyzed mutations in the Foxn1 gene using in vitro cultivated embryos (FIG. **7**).

Specifically, Cas9 mRNA and sgRNAs were synthesized in vitro from linear DNA templates using the mMESSAGE mMACHINE T7 Ultra kit (Ambion) and MEGAshortscript T7 kit (Ambion), respectively, according to the manufacturers' instructions, and were diluted with appropriate amounts of diethyl pyrocarbonate (DEPC, Sigma)-treated injection buffer (0.25 mM EDTA, 10 mM Tris, pH 7.4). Templates for sgRNA synthesis were generated using oligonucleotides listed in Table 3. Recombinant Cas9 protein was obtained from ToolGen, Inc.

TABLE 3

RNA Name	Direction	Sequence (5' to 3')	SEQ ID NO
Foxn1 #1 sgRNA	F	GAAATTAATACGACTCACTATAGG*CAGTCTGACG* *TCACACTTCCG*GTTTTAGAGCTAGAAATAGCAAGT TAAAATAAGGCTAGTCCG	31
Foxn1 #2 sgRNA	F	GAAATTAATACGACTCACTATAGG*ACTTCCAGGC* *TCCACCCGAC*GTTTTAGAGCTAGAAATAGCAAGT TAAAATAAGGCTAGTCCG	32
Foxn1 #3 sgRNA	F	GAAATTAATACGACTCACTATAGG*CCAGGCTCCA* *CCCGACTGG*AGTTTTAGAGCTAGAAATAGCAAGT TAAAATAAGGCTAGTCCG	33
Foxn1 #4 sgRNA	F	GAAATTAATACGACTCACTATAGG*ACTGGAGGGC* *GAACCCCAAGG*TTTTAGAGCTAGAAATAGCAAGT TAAAATAAGGCTAGTCCG	34
Foxn1 #5 sgRNA	F	GAAATTAATACGACTCACTATAGG*ACCCCAAGGG* *GACCTCATGC*GTTTTAGAGCTAGAAATAGCAAGT TAAAATAAGGCTAGTCCG	35
Prkdc #1 sgRNA	F	GAAATTAATACGACTCACTATAGG*TTAGTTTTTT* *CCAGAGACTT*GTTTTAGAGCTAGAAATAGCAAGT TAAAATAAGGCTAGTCCG	36
Prkdc #2 sgRNA	F	GAAATTAATACGACTCACTATAGG*TTGGTTTGCT* *TGTGTTTATC*GTTTTAGAGCTAGAAATAGCAAGT TAAAATAAGGCTAGTCCG	37
Prkdc #3 sgRNA	F	GAAATTAATACGACTCACTATAGGCACAAGCAAA *CCAAAGTCTC*GTTTTAGAGCTAGAAATAGCAAGT TAAAATAAGGCTAGTCCG	38
Prkdc #4 sgRNA	F	GAAATTAATACGACTCACTATAGG*CCTCAATGCT* *AAGCGACTTC*GTTTTAGAGCTAGAAATAGCAAGT TAAAATAAGGCTAGTCCG	39

All animal experiments were performed in accordance with the Korean Food and Drug Administration (KFDA) guidelines. Protocols were reviewed and approved by the Institutional Animal Care and Use Committees (IACUC) of the Laboratory Animal Research Center at Yonsei University (Permit Number: 2013-0099). All mice were maintained in the specific pathogen-free facility of the Yonsei Laboratory Animal Research Center. FVB/NTac (Taconic) and ICR mouse strains were used as embryo donors and foster mothers, respectively. Female FVB/NTac mice (7-8 weeks old) were super-ovulated by intra-peritoneal injections of 5 IU pregnant mare serum gonadotropin (PMSG, Sigma) and 5 IU human chorionic gonadotropin (hCG, Sigma) at 48-hour intervals. The super-ovulated female mice were mated to FVB/NTac stud males, and fertilized embryos were collected from oviducts.

Cas9 mRNA and sgRNAs in M2 medium (Sigma) were injected into the cytoplasm of fertilized eggs with well-recognized pronuclei using a Piezo-driven micromanipulator (Prime Tech).

In the case of injection of recombinant Cas9 protein, the recombinant Cas9 protein: Foxn1-sgRNA complex was diluted with DEPC-treated injection buffer (0.25 mM EDTA, 10 mM Tris, pH 7.4) and injected into male pronuclei using a TransferMan NK2 micromanipulator and a Femto-Jet® microinjector (Eppendorf).

The manipulated embryos were transferred into the oviducts of pseudopregnant foster mothers to produce live animals, or were cultivated in vitro for further analyses.

To screen F0 mice and in vitro cultivated mouse embryos with RGEN-induced mutations, T7E1 assays were performed as previously described using genomic DNA samples from tail biopsies and lysates of whole embryos (Cho et al., 2013).

Briefly, the genomic region encompassing the RGEN target site was PCR-amplified, melted, and re-annealed to form heteroduplex DNA, which was treated with T7 endonuclease 1 (New England Biolabs), and then analyzed by agarose gel electrophoresis. Potential off-target sites were identified by searching with bowtie 0.12.9 and were also similarly monitored by T7E1 assays. The primer pairs used in these assays were listed in Tables 4 and 5.

TABLE 4

Primers used in the T7E1 assay

Gene	Direction	Sequence(5' to 3')	SEQ ID NO
Foxn1	F1	GTCTGTCTATCATCTCTTCCCTTCTCTCC	40
	F2	TCCCTAATCCGATGGCTAGCTCCAG	41
	R1	ACGAGCAGCTGAAGTTAGCATGC	42
	R2	CTACTCAATGCTCTTAGAGCTACCAGG CTTGC	43
Prkdc	F	GACTGTTGTGGGGAGGGCCG	44
	F2	GGGAGGGCCGAAAGTCTTATTTTG	45
	R1	CCTGAAGACTGAAGTTGGCAGAAGTGAG	46
	R2	CTTTAGGGCTTCTTCTCTACAATCACG	47

TABLE 5

Primers used for amplification of off-target sites

Gene	Notation	Direction	Sequence(5' to 3')	SEQ ID NO
Foxn1	off 1	F	CTCGGTGTGTAGCCCTGAC	48
		R	AGACTGGCCTGGAACTCACAG	49
	off 2	F	CACTAAAGCCTGTCAGGAAGCCG	50
		R	CTGTGGAGAGCACACAGCAGC	51
	off 3	F	GCTGCGACCTGAGACCATG	52
		R	CTTCAATGGCTTCCTGCTTAGGCTAC	53
	off 4	F	GGTTCAGATGAGGCCATCCTTTC	54
		R	CCTGATCTGCAGGCTTAACCCTTG	55
Prkdc	off 1	F	CTCACCTGCACATCACATGTGG	56
		R	GGCATCCACCCTATGGGGTC	57
	off 2	F	GCCTTGACCTAGAGCTTAAAGAGCC	58
		R	GGTCTTGTTAGCAGGAAGGACACTG	59
	off 3	F	AAAACTCTGCTTGATGGGATATGTGGG	60
		R	CTCTCACTGGTTATCTGTGCTCCTTC	61
	off 4	F	GGATCAATAGGTGGTGGGGGATG	62
		R	GTGAATGACACAATGTGACAGCTTCAG	63
	off 5	F	CACAAGACAGACCTCTCAACATTCAGTC	64
		R	GTGCATGCATATAATCCATTCTGATTGCTCTC	65
	off 6	F1	GGGAGGCAGAGGCAGGT	66
		F2	GGATCTCTGTGAGTTTGAGGCCA	67
		R1	GCTCCAGAACTCACTCTTAGGCTC	68

Mutant founders identified by the T7E1 assay were further analyzed by fPCR. Appropriate regions of genomic DNA were sequenced as described previously (Sung et al., 2013). For routine PCR genotyping of F1 progenies, the following primer pairs were used for both wild-type and mutant alleles:

(SEQ ID NO: 69)
5'-CTACTCCCTCCGCAGTCTGA-3'
and

(SEQ ID NO: 70)
5'-CCAGGCCTAGGTTCCAGGTA-3'

for the Foxn1 gene,

(SEQ ID NO: 71)
5'-CCCCAGCATTGCAGATTTCC-3'
and

(SEQ ID NO: 72)
5'-AGGGCTTCTTCTCTACAATCACG-3'

for Prkdc gene.

In the case of injection of Cas9 mRNA, mutant fractions (the number of mutant embryos/the number of total embryos) were dose-dependent, ranging from 33% (1 ng/μl sgRNA) to 91% (100 ng/μl) (FIG. **6***b*). Sequence analysis confirmed mutations in the Foxn1 gene; most mutations were small deletions (FIG. **6***c*), reminiscent of those induced by ZFNs and TALENS (Kim et al., 2013).

In the case of injection of Cas9 protein, these injection doses and methods minimally affected the survival and development of mouse embryos in vitro: over 70% of RGEN-injected embryos hatched out normally in both experiments. Again, mutant fractions obtained with Cas9 protein injection were dose-dependent, and reached up to 88% at the highest dose via pronucleus injection and to 71% via intra-cytoplasmic injection (FIGS. **7***a* and **7***b*). Similar to the mutation patterns induced by Cas9 mRNA plus sgRNA (FIG. **6***c*), those induced by the Cas9 protein-sgRNA complex were mostly small deletions (FIG. **7***c*). These results clearly demonstrate that RGENs have high gene-targeting activity in mouse embryos.

Encouraged by the high mutant frequencies and low cytotoxicity induced by RGENs, we produced live animals by transferring the mouse embryos into the oviducts of pseudo-pregnant foster mothers.

Notably, the birth rates were very high, ranging from 58% to 738, and were not affected by the increasing doses of Foxn1-sgRNA (Table 6).

TABLE 6

RGEN-mediated gene-targeting in FVB/NTac mice

Target Gene	Cas9 mRNA + sgRNA (ng/μl)	Injected embryos	Transferred embryos (%)	Total newborns (%)	Live newborns * (%)	Founders † (%)
Foxn1	10 + 1	76	62 (82)	45 (73)	31 (50)	12 (39)
	10 + 10	104	90 (87)	52 (58)	58 (64)	33 (57)
	10 + 100	100	90 (90)	62 (69)	58 (64)	54 (93)
	Total	280	242 (86)	159 (66)	147 (61)	99 (67)
Prkdc	50 + 50	73	58 (79)	35 (60)	33 (57)	11 (33)
	50 + 100	79	59 (75)	22 (37)	21 (36)	7 (33)
	50 + 250	94	73 (78)	37 (51)	37 (51)	21 (57)
	Total	246	190 (77)	94 (49)	91 (48)	39 (43)

Out of 147 newborns, we obtained 99 mutant founder mice. Consistent with the results observed in cultivated embryos (FIG. **6***b*), mutant fractions were proportional to the doses of Foxn1-sgRNA, and reached up to 93% (100 ng/μl Foxn1-sgRNA) (Tables 6 and 7, FIG. **5***b*).

TABLE 7

DNA sequences of Foxn1 mutant alleles identified from a subset of T7E1-positive mutant founders

ACTTCCAGGCTCCACCCGACTGGAGGGCGAACCCCAAGGGGACCTCATGCAGG (SEQ ID NO: 134)	del + ins	#	Founder mice
ACTTCCAGGC----------AACCCCAAGGGGACCTCATGCAGG (SEQ ID NO: 297)	Δ19	1	20
ACTTCCAGGC----------GAACCCCAAGGGGACCTCATGCAGG (SEQ ID NO: 298)	Δ18	1	115
ACTTCCAGGCTCC---------- (SEQ ID NO: 299)	Δ60	1	19
ACTTCCAGGCTCC---------- (SEQ ID NO: 300)	Δ44	1	108
ACTTCCAGGCTCC----------CAAGGGGACCTCATGCAGG (SEQ ID NO: 301)	Δ21	1	64
ACTTCCAGGCTCC----------TTAGGAGGCGAACCCCAAGGGGACCTCA (SEQ ID NO: 302)	Δ12 + 6	1	126
ACTTCCAGGCTCCACC----------TCATGCAGG (SEQ ID NO: 303)	Δ28	1	5
ACTTCCAGGCTCCACCC----------CCAAGGGACCTCATG (SEQ ID NO: 304)	Δ21 + 4	1	61
ACTTCCAGGCTCCACCC----------AAGGGGACCTCATGCAGG (SEQ ID NO: 305)	Δ18	2	95, 29
ACTTCCAGGCTCCACCC----------CAAGGGGACCTCATGCAGG (SEQ ID NO: 306)	Δ17	7	12, 14, 27, 66, 108, 114, 126
ACTTCCAGGCTCCACCC----------ACCCAAGGGGACCTCATGCAG (SEQ ID NO: 307)	Δ15 + 1	1	32
ACTTCCAGGCTCCACCC----------CACCCAAGGGGACCTCATGCA (SEQ ID NO: 308)	Δ15 + 2	1	124
ACTTCCAGGCTCCACCC----------ACCCCAAGGGGACCTCATGCAGG (SEQ ID NO: 309)	Δ13	1	32
ACTTCCAGGCTCCACCC--------GGCGAACCCCAAGGGGACCTCATGCAGG (SEQ ID NO: 310)	Δ8	1	110
ACTTCCAGGCTCCACCCT----------GGGGACCTCATGCAGG (SEQ ID NO: 311)	Δ20 + 1	1	29

TABLE 7-continued

DNA sequences of Foxn1 mutant alleles identified from a subset of T7E1-positive mutant founders

ACTTCCAGGCTCCACCCG----------AACCCCAAGGGGACCTCATGCAGG (SEQ ID NO: 312)	Δ11	1	111
ACTTCCAGGCTCCACCCGA----------ACCTCATGCAGG (SEQ ID NO: 313)	Δ22	1	79
ACTTCCAGGCTCCACCCGA----------GGGGACCTCATGCAGG (SEQ ID NO: 314)	Δ18	2	13, 127
ACTTCCAGGCTCCACCCCA----------AGGGGACCTCATGCAGG (SEQ ID NO: 315)	Δ17	1	24
ACTTCCAGGCTCCACCCGA----------ACCCCAAGGGGACCTCATGCAGG (SEQ ID NO: 316)	Δ11	5	14, 53, 58, 69, 124
ACTTCCAGGCTCCACCCGA----------GACCCCAAGGGGACCTCATGCAGG (SEQ ID NO: 317)	Δ10	1	14
ACTTCCAGGCTCCACCCGA-----GGGCGAACCCCAAGGGGACCTCATGCAGG (SEQ ID NO: 318)	Δ5	3	53, 79, 115
ACTTCCAGGCTCCACCCGAC----------CTCATGCAGG (SEQ ID NO: 319)	Δ23	1	108
ACTTCCAGGCTCCACCCGAC----------CCCCAAGGGGACCTCATGCAGG (SEQ ID NO: 320)	Δ11	1	3
ACTTCCAGGCTCCACCCGAC----------GAAGGGCCCCAAGGGGACCTCA (SEQ ID NO: 321)	Δ11 + 6	1	66
ACTTCCAGGCTCCACCCGAC--------GAACCCCAAGGGGACCTCATGCAGG (SEQ ID NO: 322)	Δ8	2	3, 66
ACTTCCAGGCTCCACCCGAC-----GGCGAACCCCAAGGGGACCTCATGCAGG (SEQ ID NO: 323)	Δ5	1	27
ACTTCCAGGCTCCACCCGAC--GTGCTTGAGGGCGAACCCCAAGGGGACCTCA (SEQ ID NO: 324)	Δ2 + 6	2	5
ACTTCCAGGCTCCACCCGACT------CACTATCTTCTGGGCTCCTCCATGTC (SEQ ID NO: 325)	Δ6 + 25	2	21, 114
ACTTCCAGGCTCCACCCGACT----TGGCGAACCCCAAGGGGACCTCATGCAG (SEQ ID NO: 326)	Δ4 + 1	1	53
ACTTCCAGGCTCCACCCGACT--TGCAGGGCGAACCCCAAGGGGACCTCATGC (SEQ ID NO: 327)	Δ2 + 3	1	126
ACTTCCAGGCTCCACCCGACTTGGAGGGCGAACCCCAAGGGGACCTCATGCAG (SEQ ID NO: 328)	+1	15	3, 5, 12, 19, 29, 55, 56, 61, 66, 68, 81, 108, 111, 124, 127
ACTTCCAGGCTCCACCCGACTTTGGAGGGCGAACCCCAAGGGGACCTCATGCA (SEQ ID NO: 329)	+2	2	79, 120
ACTTCCAGGCTCCACCCGACTGTTGGAGGGCGAACCCCAAGGGGACCTCATGC (SEQ ID NO: 330)	+3	1	55
ACTTCCAGGCTCCACCCGACTGGAG(+455)GGCGAACCCCAAGGGGACCTCC (SEQ ID NO: 331)	+455	1	13

To generate Prkdc-targeted mice, we applied a 5-fold higher concentration of Cas9 mRNA (50 ng/μl) with increasing doses of Prkdc-sgRNA (50, 100, and 250 ng/μl). Again, the birth rates were very high, ranging from 51% to 60%, enough to produce a sufficient number of newborns for the analysis (Table 6). The mutant fraction was 57% (21 mutant founders among 37 newborns) at the maximum dose of Prkdc-sgRNA. These birth rates obtained with RGENs were approximately 2- to 10-fold higher than those with TALENs reported in our previous study (Sung et al., 2013). These results demonstrate that RGENs are potent gene-targeting reagents with minimal toxicity.

To test the germ-line transmission of the mutant alleles, we crossed the Foxn1 mutant founder #108, a mosaic with

four different alleles (FIG. **5***c*, and Table 8) with wild-type mice, and monitored the genotypes of F1 offspring.

TABLE 8

Genotypes of Foxn1 mutant mice

Founder NO.	sgRNA (ng/ml)	Genotyping Summary	Detected alleles
58*	1	not determined	Δ11
19	100	bi-allelic	Δ60/+1
20	100	bi-allelic	Δ67/Δ19
13	100	bi-allelic	Δ18/+455
32	10	bi-allelic (heterozygote)	Δ13/Δ15+1
115	10	bi-allelic (heterozygote)	Δ18/Δ5
111	10	bi-allelic (heterozygote)	Δ11/+1
110	10	bi-allelic (homozygote)	Δ8/Δ8
120	10	bi-allelic (homozygote)	+2/+2
81	100	heterozygote	+1/WT
69	100	homozygote	Δ11/Δ11
55	1	mosaic	Δ18/Δ1/+1/+3
56	1	mosaic	Δ127/Δ41/Δ2/+1
127	1	mosaic	Δ18/+1/WT
53	1	mosaic	Δ11/Δ5/Δ4+1/WT
27	10	mosaic	Δ17/Δ5/WT
29	10	mosaic	Δ18/Δ20+1/+1
95	10	mosaic	Δ18/Δ14/Δ8/Δ4
108	10	mosaic	+1/Δ17/Δ23/Δ44
114	10	mosaic	Δ17/Δ8/Δ6+25
124	10	mosaic	Δ11/Δ15+2/+1
126	10	mosaic	Δ17/Δ2+3/Δ12+6
12	100	mosaic	Δ30/Δ28/Δ17/+1
5	100	mosaic	Δ28/Δ11/Δ2+6/+1
14	100	mosaic	Δ17/Δ11/Δ10
21	100	mosaic	Δ127/Δ41/Δ2/Δ6+25
24	100	mosaic	Δ17/+1/WT
64	100	mosaic	Δ31/Δ21/+1/WT
68	100	mosaic	Δ17/Δ11/+1/WT
79	100	mosaic	Δ22/Δ5/+2/WT
61	100	mosaic	Δ21+4/Δ6/+1/+9
66**	100	mosaic	Δ17/Δ8/Δ11+6/+1/WT
3	100	mosaic	Δ11/Δ8/+1

Underlined alleles were sequenced.
Alleles in red, detected by sequencing, but not by fPCR.
*only one clone sequenced.
**Not determined by fPCR.

As expected, all the progenies were heterozygous mutants possessing the wild-type allele and one of the mutant alleles (FIG. **5***d*). We also confirmed the germ-line transmission in independent founder mice of Foxn1 (FIG. **8**) and Prkdc (FIG. **9**). To the best of our knowledge, these results provide the first evidence that RGEN-induced mutant alleles are stably transmitted to F1 progenies in animals.

Example 4: RNA-Guided Genome Editing in Plants

4-1. Production of Cas9 Protein

The Cas9 coding sequence (4104 bps), derived from *Streptococcus pyogenes* strain M1 GAS (NC_002737.1), was cloned to pET28-b(+) plasmid. A nuclear targeting sequence (NLS) was included at the protein N terminus to ensure the localization of the protein to the nucleus. pET28-b(+) plasmid containing Cas9 ORF was transformed into BL21(DE3). Cas9 was then induced using 0.2 mM IPTG for 16 hrs at 18° C. and purified using Ni-NTA agarose beads (Qiagen) following the manufacturer's instructions. Purified Cas9 protein was concentrated using Ultracel—100K (Millipore).

4-2. Production of Guide RNA

The genomic sequence of the Arabidopsis gene encoding the BRI1 was screened for the presence of a NGG motif, the so called protospacer adjacent motif (PAM), in an exon which is required for Cas9 targeting To disrupt the BRI1 gene in Arabidopsis, we identified two RGEN target sites in an exon that contain the NGG motif. sgRNAs were produced in vitro using template DNA. Each template DNA was generated by extension with two partially overlapped oligonucleotides (Macrogen, Table X1) and Phusion polymerase (Thermo Scientific) using the following conditions—98° C. 30 sec {98° C. 10 sec, 54° C. 20 sec, 72° C. 2 min}×20, 72° C. 5 min.

TABLE 9

Oligonucleotides for the production of the template DNA for in vitro transcription

Oligonucleotides	Sequence (5'-3')	SEQ ID NO
BRI1 target 1 (Forward)	GAAATTAATACGACTCACTATAGGTTTGAAAGATGG AAGCGCGGGTTTTAGAGCTAGAAATAGCAAGTTAAA ATAAGGCTAGTCCG	73
BRI1 target 2 (Forward)	GAAATTAATACGACTCACTATAGGTGAAACTAAACT GGTCCACAGTTTTAGAGCTAGAAATAGCAAGTTAAA ATAAGGCTAGTCCG	74
Universal (Reverse)	AAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTG ATAACGGACTAGCCTTATTTTAACTTGC	75

The extended DNA was purified and used as a template for the in vitro production of the guide RNA's using the MEGAshortscript T7 kit (Life Technologies). Guide RNA were then purified by Phenol/Chloroform extraction and ethanol precipitation. To prepare Cas9/sgRNA complexes, 10 μl of purified Cas9 protein (12 μg/μl) and 4 μl each of two sgRNAs (11 μg/μl) were mixed in 20 μl NEB3 buffer (New England Biolabs) and incubated for 10 min at 37° C.

4-3. Transfection of Cas9/sgRNA Complex to Protoplast

The leaves of 4-week-old Arabidopsis seedlings grown aseptically in petri dishes were digested in enzyme solution (1% cellulose R10, 0.5% macerozyme R10, 450 mM mannitol, 20 mM MES pH 5.7 and CPW salt) for 8~16 hrs at 25° C. with 40 rpm shaking in the dark. Enzyme/protoplast solutions were filtered and centrifuged at 100×g for 3~5 min. Protoplasts were re-suspended in CPW solution after counting cells under the microscope (×100) using a hemacytometer. Finally, protoplasts were re-suspended at 1×10^6/ml in MMG solution (4 mM HEPES pH 5.7, 400 mM mannitol and 15 mM MgCl2). To transfect the protoplasts with Cas9/sgRNA complex, 200 μL (200,000 protoplasts) of the protoplast suspension were gently mixed with 3.3 or 10 μL of Cas9/sgRNA complex [Cas9 protein (6 μg/μL) and two sgRNAs (2.2 μg/μL each)] and 200 μL of 40% polyethylene glycol transfection buffer (40% PEG4000, 200 mM mannitol and 100 mM CaCl2) in 2 ml tubes. After 5~20 min incubation at room temperature, transfection was stopped by adding wash buffer with W5 solution (2 mM MES pH 5.7, 154 mM NaCl, 125 mM CaCl2 and 5 mM KCl). Protoplasts were then collected by centrifugation for 5 min at 100×g, washed with 1 ml of W5 solution, centrifuged for another 5 min at 100×g. The density of protoplasts was adjusted to 1×10^5/ml and they were cultured in modified KM 8p liquid medium with 400 mM glucose.

4-4. Detection of Mutations in *Arabidopsis* Protoplasts and Plants

After 24 hr or 72 hr post-transfection, protoplasts were collected and genomic DNA was isolated. The genomic DNA region spanning the two target sites was PCR-amplified and subjected to the T7E1 assay. As shown in FIG. **11**, indels were induced by RGENs at high frequencies that ranged from 50% to 70%. Surprisingly, mutations were induced at 24 hr post-transfection. Apparently, Cas9 protein functions immediately after transfection. PCR products were purified and cloned into T-Blunt PCR Cloning Kit (Solgent). Plasmids were purified and subjected to Sanger sequencing with M13F primer. One mutant sequence had a 7-bp deletion at one site (FIG. **12**). The other three mutant sequences had deletions of ~220-bp DNA segments between the two RGEN site.

Example 5: Cas9 Protein Transduction Using a Cell-Penetrating Peptide or Protein Transduction Domain

5-1. Construction of his-Cas9-Encoding Plasmid

Cas9 with a cysteine at the C-terminal was prepared by PCR amplification using the previously described Cas9 plasmid {Cho, 2013 #166} as the template and cloned into pET28-(a) vector (Novagen, Merk Millipore, Germany) containing His-tag at the N-terminus.

5-2. Cell Culture

293T (Human embryonic kidney cell line), and HeLa (human ovarian cancer cell line) were grown in DMEM (GIBCO-BRL Rockville) supplemented with 10% FBS and 1% penicillin and streptomycin.

5-3. Expression and Purification of Cas9 Protein

To express the Cas9 protein, *E. coli* BL21 cells were transformed with thepET28-(a) vector encoding Cas9 and plated onto Luria-Bertani (LB) agar medium containing 50 μg/mL kanamycin (Amresco, Solon, OH). Next day, a single colony was picked and cultured in LB broth containing 50 μg/mL kanamycin at 37° C. overnight. Following day, this starter culture at 0.1 OD600 was inoculated into Luria broth containing 50 μg/mL kanamycin and incubated for 2 hrs at 37° C. until OD600 reached to 0.6-0.8. To induce Cas9 protein expression, the cells were cultured at 30° C. overnight after addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (Promega, Madison, WI) to the final concentration of 0.5 mM.

The cells were collected by centrifugation at 4000 rpm for 15-20 mins, resuspended in a lysis buffer (20 mM Tris-Cl pH8.0, 300 mM NaCl, 20 mM imidazole, 1× protease inhibitor cocktail, 1 mg/ml lysozyme), and lysed by sonication (40% duty, 10 sec pulse, 30 sec rest, for 10 mins on ice). The soluble fraction was separated as the supernatant after centrifugation at 15,000 rpm for 20 mins at 4° C. Cas9 protein was purified at 4° C. using a column containing Ni-NTA agarose resin (QIAGEN) and AKTA prime instrument (AKTA prime, GE Healthcare, UK). During this chromatography step, soluble protein fractions were loaded onto Ni-NTA agarose resin column (GE Healthcare, UK) at the flow rate of 1 mL/min. The column was washed with a washing buffer (20 mM Tris-Cl pH8.0, 300 mM NaCl, 20 mM imidazole, 1× protease inhibitor cocktail) and the bound protein was eluted at the flow rate of 0.5 ml/min with an elution buffer (20 mM Tris-Cl pH8.0, 300 mM NaCl, 250 mM imidazole, 1× protease inhibitor cocktail). The pooled eluted fraction was concentrated and dialyzed against storage buffer (50 mM Tris-HCl, pH8.0, 200 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 20% Glycerol). Protein concentration was quantitated by Bradford assay (Biorad, Hercules, CA) and purity was analyzed by SDS-PAGE using bovine serum albumin as the control.

5-4. Conjugation of Cas9 to 9R4L

1 mg Cas9 protein diluted in PBS at the concentration of 1 mg/mL and 50 μg of maleimide-9R4L peptide in 25 μL DW (Peptron, Korea) were gently mixed using a rotor at room temperature for 2 hrs and at 4° C. overnight. To remove unconjugated maleimide-9R4L, the samples were dialyzed using 50kDamolecular weight cutoff membrane against of DPBS (pH 7.4) at 4° C. for 24 hrs. Cas9-9R4L protein was collected from the dialysis membrane and the protein amount was determined using Bradford assay.

5-5. Preparation of sgRNA-9R4L

sgRNA (1 μg) was gently added to various amounts of C9R4LC peptide (ranging from 1 to 40 weight ratio) in 100 μl of DPBS (pH 7.4). This mixture was incubated at room temperature for 30 mins and diluted to 10 folds using RNAse-free deionized water. The hydrodynamic diameter and z-potential of the formed nanoparticles were measured using dynamic light scattering (Zetasizer-nano analyzer ZS; Malvern instruments, Worcestershire, UK).

5-6. Cas9 Protein and sgRNA Treatments

Cas9-9R4L and sgRNA-C9R4LC were treated to the cells as follows: lug of sgRNA and 15 μg of C9R4LC peptide were added to 250 mL of OPTIMEM medium and incubated at room temperature for 30 mins. At 24 hrs after seeding, cells were washed with OPTIMEM medium and treated with sgRNA-C9R4LC complex for 4 hrs at 37° C. Cells were washed again with OPTIMEM medium and treated with Cas9-9R4L for 2 hrs at 37° C. After treatment, culture media was replaced with serum-containing complete medium and incubated at 37° C. for 24 hrs before the next treatment.

Same procedure was followed for multiple treatments of Cas9 and sgRNA for three consecutive days.

5-7. Cas9-9R4L and sgRNA-9R4L Can Edit Endogenous Genes in Cultured Mammalian Cells Without the Use of Additional Delivery Tools

To determine whether Cas9-9R4L and sgRNA-9R4L can edit endogenous genes in cultured mammalian cells without the use of additional delivery tools, we treated 293 cells with Cas9-9R4L and sgRNA-9R4L targeting the CCR5 gene and analyzed the genomic DNA. T7E1 assay showed that 9% of CCR5 gene was disrupted in cells treated with both Cas9-9R4L and sgRNA-9R4L and that the CCR5 gene disruption was not observed in control cells including those untreated, treated with either Cas9-9R or sgRNA-9R4L, or treated with both unmodified Cas-9 and sgRNA (FIG. **13**), suggesting that the treatment with Cas9-9R4L protein and sgRNA conjugated with 9R4L, but not unmodified Cas9 and sgRNA, can lead to efficient genome editing in mammalian cells.

Example 6: Control of Off-Target Mutation According to Guide RNA Structure

Recently, three groups reported that RGENs had off-target effects in human cells. To our surprise, RGENs induced mutations efficiently at off-target sites that differ by 3 to 5 nucleotides from on-target sites. We noticed, however, that there were several differences between our RGENs and those used by others. First, we used dualRNA, which is crRNA plus tracrRNA, rather than single-guide RNA (sgRNA) that is composed of essential portions of crRNA and tracrRNA. Second, we transfected K562 cells (but not HeLa cells) with synthetic crRNA rather than plasmids encoding crRNA. HeLa cells were transfected with crRNA-encoding plasmids. Other groups used sgRNA-encoding plasmids. Third, our guide RNA had two additional guanine nucleotides at the 5' end, which are required for efficient transcription by T7 polymerase in vitro. No such additional nucleotides were included in the sgRNA used by others. Thus, the RNA sequence of our guide RNA can be shown as 5'-GGX_{20}, whereas 5'-GX_{19}, in which X_{20} or GX_{19} corresponds to the 20-bp target sequence, represents the sequence used by others. The first guanine nucleotide is required for transcription by RNA polymerase in cells. To test whether off-target RGEN effects can be attributed to these differences, we chose four RGENs that induced off-target mutations in human cells at high frequencies (13). First, we compared our method of using in vitro transcribed dualRNA with the method of transfecting sgRNA-encoding plasmids in K562 cells and measured mutation frequencies at the on-target and off-target sites via the T7E1 assay. Three RGENs showed comparable mutation frequencies at on-target and off-target sites regardless of the composition of guide RNA. Interestingly, one RGEN (VEFGA site 1) did not induce indels at one validated off-target site, which differs by three nucleotides from the on-target site (termed OT1-11, FIG. **14**), when synthetic dualRNA was used. But the synthetic dualRNA did not discriminate the other validated off-target site (OT1-3), which differs by two nucleotides from the on-target site.

Next, we tested whether the addition of two guanine nucleotides at the 5' end of sgRNA could make RGENs more specific by comparing 5'-GGX_{20} (or 5'-$GGGX_{19}$) sgRNA with 5'-GX_{19} sgRNA. Four GX_{19} sgRNAs complexed with Cas9 induced indels equally efficiently at on-target and off-target sites, tolerating up to four nucleotide mismatches. In sharp contrast, GGX_{20} sgRNAs discriminated off-target sites effectively. In fact, the T7E1 assay barely detected RGEN-induced indels at six out of the seven validated off-target sites when we used the four GGX_{20} sgRNAs (FIG. **15**). We noticed, however, that two GGX_{20} sgRNAs (VEGFA sites 1 and 3) were less active at on-target sites than were the corresponding GX_{19} sgRNAs. These results show that the extra nucleotides at the 5' end can affect mutation frequencies at on-target and off-target sites, perhaps by altering guide RNA stability, concentration, or secondary structure.

These results suggest that three factors—the use of synthetic guide RNA rather than guide RNA-encoding plasmids, dualRNA rather than sgRNA, and GGX_{20} sgRNA rather than GX_{19} sgRNA-have cumulative effects on the discrimination of off-target sites.

Example 7: Paired Cas9 Nickases

In principle, single-strand breaks (SSBs) cannot be repaired by error-prone NHEJ but still trigger high fidelity homology-directed repair (HDR) or base excision repair. But nickase-induced targeted mutagenesis via HDR is much less efficient than is nuclease-induced mutagenesis. We reasoned that paired Cas9 nickases would produce composite DSBs, which trigger DNA repair via NHEJ or HDR, leading to efficient mutagenesis (FIG. **16**A). Furthermore, paired nickases would double the specificity of Cas9-based genome editing.

We first tested several Cas9 nucleases and nickases designed to target sites in the AAVS1 locus (FIG. **16**B) in vitro via fluorescent capillary electrophoresis. Unlike Cas9 nucleases that cleaved both strands of DNA substrates, Cas9 nickases composed of guide RNA and a mutant form of Cas9 in which a catalytic aspartate residue is changed to an alanine (D10A Cas9) cleaved only one strand, producing site-specific nicks (FIG. **16**C, D). Interestingly, however, some nickases (AS1, AS2, AS3, and S6 in FIG. **17**A) induced indels at target sites in human cells, suggesting that nicks can be converted to DSBs, albeit inefficiently, in vivo. Paired Cas9 nickases producing two adjacent nicks on opposite DNA strands yielded indels at frequencies that ranged from 14% to 91%, comparable to the effects of paired nucleases (FIG. **17**A). The repair of two nicks that would produce 5' overhangs led to the formation of indels much more frequently than those producing 3' overhangs at three genomic loci (FIG. **17**A and FIG. **18**). In addition, paired nickases enabled targeted genome editing via homology-directed repair more efficiently than did single nickases (FIG. **19**).

We next measured mutation frequencies of paired nickases and nucleases at off-target sites using deep sequencing. Cas9 nucleases complexed with three sgRNAs induced off-target mutations at six sites that differ by one or two nucleotides from their corresponding on-target sites with frequencies that ranged from 0.5% to 10% (FIG. **17**B). In contrast, paired Cas9 nickases did not produce indels above the detection limit of 0.1% at any of the six off-target sites. The S2 Off-1 site that differs by a single nucleotide at the first position in the PAM (i.e., N in NGG) from its on-target site can be considered as another on-target site. As expected, the Cas9 nuclease complexed with the S2 sgRNA was equally efficient at this site and the on-target site. In sharp contrast, D10A Cas9 complexed with the S2 and AS2 sgRNAs discriminated this site from the on-target site by a factor of 270 fold. This paired nickase also discriminated the

AS2 off-target sites (Off-1 and Off-9 in FIG. **17**B) from the on-target site by factors of 160 fold and 990 fold, respectively.

Example 8: Chromosomal DNA Splicing Induced by Paired Cas9 Nickases

Two concurrent DSBs produced by engineered nucleases such as ZENs and TALENs can promote large deletions of the intervening chromosomal segments has been reported. We tested whether two SSBs induced by paired Cas9 nickases can also produce deletions in human cells. We used PCR to detect deletion events and found that seven paired nickases induced deletions of up to 1.1-kbp chromosomal segments as efficiently as paired Cas9 nucleases did (FIG. **20**A, B). DNA sequences of the PCR products confirmed the deletion events (FIG. **20**C). Interestingly, the sgRNA-matching sequence remained intact in two out of seven deletion-specific PCR amplicons (underlined in FIG. **20**C). In contrast, Cas9 nuclease pairs did not produce sequences that contained intact target sites. This finding suggests that two distant nicks were not converted to two separate DSBs to promote deletions of the intervening chromosomal segment. In addition, it is unlikely that two nicks separated by more than a 100 bp can produce a composite DSB with large overhangs under physiological conditions because the melting temperature is very high.

We propose that two distant nicks are repaired by strand displacement in a head-to-head direction, resulting in the formation of a DSB in the middle, whose repair via NHEJ causes small deletions (FIG. **20**D). Because the two target sites remain intact during this process, nickases can induce SSBs again, triggering the cycle repeatedly until the target sites are deleted. This mechanism explains why two offset nicks producing 5' overhangs but not those producing 3' overhangs induced indels efficiently at three loci.

We then investigated whether Cas9 nucleases and nickases can induce unwanted chromosomal translocations that result from NHEJ repair of on-target and off-target DNA cleavages (FIG. **21**A). We were able to detect translocations induced by Cas9 nucleases using PCR (FIG. **21**B, C). No such PCR products were amplified using genomic DNA isolated from cells transfected with the plasmids encoding the AS2+S3 Cas9 nickase pair. This result is in line with the fact that both AS2 and S3 nickases, unlike their corresponding nucleases, did not produce indels at off-target sites (FIG. **17**B).

These results suggest that paired Cas9 nickases allow targeted mutagenesis and large deletions of up to 1-kbp chromosomal segments in human cells. Importantly, paired nickases did not induce indels at off-target sites at which their corresponding nucleases induce mutations. Furthermore, unlike nucleases, paired nickases did not promote unwanted translocations associated with off-target DNA cleavages. In principle, paired nickases double the specificity of Cas9-mediated mutagenesis and will broaden the utility of RNA-guided enzymes in applications that require precise genome editing such as gene and cell therapy. One caveat to this approach is that two highly active sgRNAs are needed to make an efficient nickase pair, limiting targetable sites. As shown in this and other studies, not all sgRNAs are equally active. When single clones rather than populations of cells are used for further studies or applications, the choice of guide RNAs that represent unique sequences in the genome and the use of optimized guide RNAs would suffice to avoid off-target mutations associated with Cas9 nucleases. We propose that both Cas9 nucleases and paired nickases are powerful options that will facilitate precision genome editing in cells and organisms.

Example 9: Genotyping with CRISPR/Cas-Derived RNA-Guided Endonucleases

Next, we reasoned that RGENs can be used in Restriction fragment length polymorphism (RFLP) analysis, replacing conventional restriction enzymes. Engineered nucleases including RGENs induce indels at target sites, when the DSBs caused by the nucleases are repaired by the error-prone non-homologous end-joining (NHEJ) system. RGENs that are designed to recognize the target sequences cannot cleave mutant sequences with indels but will cleave wild-type target sequences efficiently.

9-1. RGEN Components

crRNA and tracrRNA were prepared by in vitro transcription using MEGAshortcript T7 kit (Ambion) according to the manufacturer's instruction. Transcribed RNAs were resolved on a 8% denaturing urea-PAGE gel. The gel slice containing RNA was cut out and transferred to elution buffer. RNA was recovered in nuclease-free water followed by phenol: chloroform extraction, chloroform extraction, and ethanol precipitation. Purified RNA was quantified by spectrometry. Templates for crRNA were prepared by annealing an oligonucleotide whose sequence is shown as 5'-GAAATTAATACGACTCACTATAGGX_{20}GTTTTAGAGCTATGCTGTTTTG-3' (SEQ ID NO: 76), in which X_{20} is the target sequence, and its complementary oligonucleotide. The template for tracrRNA was synthesized by extension of forward and reverse oligonucleotides (5'-GAAATTAATACGACTCACTATAGGAACCATTCAAAACAGCATAGCAAGTTAAAATAAGGCTA GTCCG-3' (SEQ ID NO: 77) and 5'-AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACT TGCTATG-3' (SEQ ID NO: 78)) using Phusion polymerase (New England Biolabs).

9-2. Recombinant Cas9 Protein Purification

The Cas9 DNA construct used in our previous Example, which encodes Cas9 fused to the His6-tag at the C terminus, was inserted in the pET-28a expression vector. The recombinant Cas9 protein was expressed in *E. coli* strain BL21 (DE3) cultured in LB medium at 25° C. for 4 hours after induction with 1 mM IPTG. Cells were harvested and resuspended in buffer containing 20 mM Tris PH 8.0, 500 mM NaCl, 5 mM imidazole, and 1 mM PMSF. Cells were frozen in liquid nitrogen, thawed at 4° C., and sonicated. After centrifugation, the Cas9 protein in the lysate was bound to Ni-NTA agarose resin (Qiagen), washed with buffer containing 20 mM Tris pH 8.0, 500 mM NaCl, and 20 mM imidazole, and eluted with buffer containing 20 mM Tris pH 8.0, 500 mM NaCl, and 250 mM imidazole. Purified Cas9 protein was dialyzed against 20 mM HEPES (pH 7.5), 150 mM KCl, 1 mM DTT, and 10% glycerol and analyzed by SDS-PAGE.

9-3. T7 Endonuclease I Assay

The T7E1 assay was performed as following. In brief, PCR products amplified using genomic DNA were denatured at 95° C., reannealed at 16° C., and incubated with 5 units of T7 Endonuclease I (New England BioLabs) for 20 min at 37° C. The reaction products were resolved using 2 to 2.5% agarose gel electrophoresis.

9-4. RGEN-RFLP Assay

PCR products (100-150 ng) were incubated for 60 min at 37° C. with optimized concentrations (Table 10) of Cas9 protein, tracrRNA, crRNA in 10 μl NEB buffer 3 (1×). After

the cleavage reaction, RNase A (4 μg) was added, and the reaction mixture was incubated for 30 min at 37° C. to remove RNA. Reactions were stopped with 6× stop solution buffer containing 30% glycerol, 1.2% SDS, and 100 mM EDTA. Products were resolved with 1-2.5% agarose gel electrophoresis and visualized with EtBr staining.

TABLE 10

Concentration of RGEN components in RFLP assays

Target Name	Cas9 (ng/μl)	crRNA (ng/μl)	tracrRNA (ng/μl)
C4BPB	100	25	60
PIBF-NGG-RGEN	100	25	60
HLA-B	1.2	0.3	0.7
CCR5-ZFN	100	25	60
CTNNB1 Wild type specific	30	10	20
CTNNB1 mutant specific	30	10	20
CCR5 WT-specific	100	25	60
CCR5 Δ32-specific	10	2.5	6
KRAS WT specific(wt)	30	10	20
KRAS mutant specific(m8)	30	10	20

TABLE 10-continued

Concentration of RGEN components in RFLP assays

Target Name	Cas9 (ng/μl)	crRNA (ng/μl)	tracrRNA (ng/μl)
KRAS WT specific (m6)	30	10	20
KRAS mutant specific (m6, 8)	30	10	20
PIK3CA WT specific (wt)	100	25	60
PIK3CA mutant specific(m4)	30	10	20
PIK3CA WT specific (m7)	100	25	60
PIK3CA mutant specific(m4, 7)	30	10	20
BRAF WT-specific	30	10	20
BRAF mutant-specific	100	25	60
NRAS WT-specific	100	25	60
NRAS mutant-specific	30	10	20
IDH WT-specific	30	10	20
IDH mutant-specific	30	10	20
PIBF-NAG-RGEN	30	10	60

TABLE 11

Primers

Gene(site)	Direction	Sequence(5' to 3')	SEQ ID NO
CCR5(RGEN)	F1	CTCCATGGTGCTATAGAGCA	79
	F2	GAGCCAAGCTCTCCATCTAGT	80
	R	GCCCTGTCAAGAGTTGACAC	81
CCR5(ZFN)	F	GCACAGGGTGGAACAAGATGGA	82
	R	GCCAGGTACCTATCGATTGTCAGG	83
CCR5(del32)	F	GAGCCAAGCTCTCCATCTAGT	84
	R	ACTCTGACTG GGTCACCAGC	85
C4BPB	F1	*TATTTGGCTGGTTGAAAGGG*	86
	R1	*AAAGTCATGAAATAAACACACCCA*	87
	F2	*CTGCATTGATATGGTAGTACCATG*	88
	R2	*GCTGTTCATTGCAATGGAATG*	89
CTNNB1	F	ATGGAGTTGGACATGGCCATGG	90
	R	ACTCACTATCCACAGTTCAGCATTTACC	91
KRAS	F	*TGGAGATAGCTGTCAGCAACTTT*	92
	R	*CAACAA AGCAAAGGTAAAGTTGGTAATAG*	93
PIK3CA	F	*GGTTTCAGGAGATGTGTTACAAGGC*	94
	R	*GATTGTGCAATTCCTATGCAATCGGTC*	95
NRAS	F	*CACTGGGTACTTAATCTGTAGCCTC*	96
	R	*GGTTCCAAGTCATTCCCAGTAGC*	97
IDH1	F	*CATCACTGCAGTTGTAGGTTATAACTATCC*	98
	R	*TTGAAAACCACAGATCTGGTTGAACC*	99
BRAF	F	*GGAGTGCCAAGAGAATATCTGG*	100
	R	*CTGAAACTGGTTTCAAAATATTCGTTTTAAGG*	101
PIBF	F	*GCTCTGTATGCCCTGTAGTAGG*	102
	R	*TTTGCATCTGACCTTACCTTTG*	103

9-5. Plasmid Cleavage Assay

Restriction enzyme-treated linearized plasmid (100 ng) was incubated for 60 min at 37° C. with Cas9 protein (0.1 μg), tracrRNA (60 ng), and crRNA (25 ng) in 10 μl NEB 3 buffer (1×). Reactions were stopped with 6× stop solution containing 30% glycerol, 1.2% SDS, and 100 mM EDTA. Products were resolved with 1% agarose gel electrophoresis and visualized with EtBr staining.

9-6. Strategy of RFLP

New RGENs with desired DNA specificities can be readily created by replacing crRNA; no de novo purification of custom proteins is required once recombinant Cas9 protein is available. Engineered nucleases, including RGENs, induce small insertions or deletions (indels) at target sites when the DSBs caused by the nucleases are repaired by error-prone non-homologous end-joining (NHEJ). RGENs that are designed to recognize the target sequences cleave wild-type sequences efficiently but cannot cleave mutant sequences with indels (FIG. **22**).

We first tested whether RGENs can differentially cleave plasmids that contain wild-type or modified C4BPB target sequences that harbor 1- to 3-base indels at the cleavage site. None of the six plasmids with these indels were cleaved by a C4BPB-specific RGEN5 composed of target-specific crRNA, tracrRNA, and recombinant Cas9 protein (FIG. **23**). In contrast, the plasmid with the intact target sequence was cleaved efficiently by this RGEN.

9-7. Detection of Mutations Induced by the Same RGENs Using RGEN-Mediated RFLP

Next, to test the feasibility of RGEN-mediated RFLP for detection of mutations induced by the same RGENs, we utilized gene-modified K562 human cancer cell clones established using an RGEN targeting C4BPB gene (Table 12).

TABLE 12

Target sequence of RGENs used in this study

Gene	Target sequence	SEQ ID NO
human C4BPB	AATGACCACTACATCCTCAAGGG	104
mouse Pibf1	AGATGATGTCTCATCATCAGAGG	105

C4BPB mutant clones used in this study have various mutations ranging from 94 bp deletion to 67 bp insertion (FIG. **24**A). Importantly, all mutations occurred in mutant clones resulted in the loss of RGEN target site. Among 6 C4BPB clones analyzed, 4 clones have both wildtype and mutant alleles (+/−) and 2 clones have only mutant alleles (−/−).

The PCR products spanning the RGEN target site amplified from wildtype K562 genomic DNA were digested completely by the RGEN composed of target-specific crRNA, tracrRNA, and recombinant Cas9 protein expressed in and purified from *E. coli* (FIG. **24**B/Lane 1). When the C4BPB mutant clones were subjected to RFLP analysis using the RGEN, PCR amplicons of +/− clones that contained both wildtype and mutant alleles were partially digested, and those of −/− cloned that did not contain the wildtype allele were not digested at all, yielding no cleavage products corresponding to the wildtype sequence (FIG. **24**B). Even a single-base insertion at the target site blocked the digestion (#12 and #28 clones) of amplified mutant alleles by the C4BPB RGEN, showing the high specificity of RGEN-mediated RFLP. We subjected the PCR amplicons to the mismatch-sensitive T7E1 assay in parallel (FIG. **24**B). Notably, the T7E1 assay was not able to distinguish −/− clones from +/− clones. To make it matters worse, the T7E1 assay cannot distinguish homozygous mutant clones that contain the same mutant sequence from wildtype clones, because annealing of the same mutant sequence will form a homoduplex. Thus, RGEN-mediated RFLP has a critical advantage over the conventional mismatch-sensitive nuclease assay in the analysis of mutant clones induced by engineered nucleases including ZENS, TALENS and RGENS.

9-8. Quantitative Assay for RGEN-RFLP Analysis

We also investigated whether RGEN-RFLP analysis is a quantitative method. Genomic DNA samples isolated from the C4BPB null clone and the wild-type cells were mixed at various ratios and used for PCR amplifications. The PCR products were subjected to RGEN genotyping and the T7E1 assay in parallel (FIG. **25***b*). As expected, DNA cleavage by the RGEN was proportional to the wild type to mutant ratio. In contrast, results of the T7E1 assay correlated poorly with mutation frequencies inferred from the ratios and were inaccurate, especially at high mutant %, a situation in which complementary mutant sequences can hybridize with each other to form homoduplexes.

9-9. Analysis of Mutant Mouse Founders Using a RGEN-Mediated RFLP Genotyping

We also applied RGEN-mediated RFLP genotyping (RGEN genotyping in short) to the analysis of mutant mouse founders that had been established by injection of TALENs into mouse one-cell embryos (FIG. **26**A). We designed and used an RGEN that recognized the TALEN target site in the Pibf1 gene (Table 10). Genomic DNA was isolated from a wildtype mouse and mutant mice and subjected to RGEN genotyping after PCR amplification. RGEN genotyping successfully detected various mutations, which ranged from one to 27-bp deletions (FIG. **26**B). Unlike the T7E1 assay, RGEN genotyping enabled differential detection of +/− and −/− founder.

9-10. Detection of Mutations Induced in Human Cells by a CCR5-Specific ZFN Using RGENS

In addition, we used RGENs to detect mutations induced in human cells by a CCR5-specific ZFN, representing yet another class of engineered nucleases (FIG. **27**). These results show that RGENs can detect mutations induced by nucleases other than RGENs themselves. In fact, we expect that RGENs can be designed to detect mutations induced by most, if not all, engineered nucleases. The only limitation in the design of an RGEN genotyping assay is the requirement for the GG or AG (CC or CT on the complementary strand) dinucleotide in the PAM sequence recognized by the Cas9 protein, which occurs once per 4 bp on average. Indels induced anywhere within the seed region of several bases in crRNA and the PAM nucleotides are expected to disrupt RGEN-catalyzed DNA cleavage. Indeed, we identified at least one RGEN site in most (98%) of the ZEN and TALEN sites.

9-11. Detection of Polymorphisms or Variations Using RGEN

Next, we designed and tested a new RGEN that targets a highly polymorphic locus, HLA-B, that encodes Human Leukocyte Antigen B (a.k.a. MHC class I protein) (FIG. **28**). HeLa cells were transfected with RGEN plasmids, and the genomic DNA was subjected to T7E1 and RGEN-RFLP analyses in parallel. T7E1 produced false positive bands that resulted from sequence polymorphisms near the target site (FIG. **25***c*). As expected, however, the same RGEN used for gene disruption cleaved PCR products from wild-type cells completely but those from RGEN-transfected cells partially,

indicating the presence of RGEN-induced indels at the target site. This result shows that RGEN-RFLP analysis has a clear advantage over the T7E1 assay, especially when it is not known whether target genes have polymorphisms or variations in cells of interest.

9-12. Detection of Recurrent Mutations Found in Cancer and Naturally-Occurring Polymorphisms Through RGEN-RFLP Analysis

RGEN-RFLP analysis has applications beyond genotyping of engineered nuclease-induced mutations. We sought to use RGEN genotyping to detect recurrent mutations found in cancer and naturally-occurring polymorphisms. We chose the human colorectal cancer cell line, HCT116, which carries a gain-of-function 3-bp deletion in the oncogenic CTNNB1 gene encoding beta-catenin. PCR products amplified from HCT116 genomic DNA were cleaved partially by both wild-type-specific and mutant-specific RGENs, in line with the heterozygous genotype in HCT116 cells (FIG. **29***a*). In sharp contrast, PCR products amplified from DNA from HeLa cells harboring only wild-type alleles were digested completely by the wild-type-specific RGEN and were not cleaved at all by the mutation-specific RGEN.

We also noted that HEK293 cells harbor the 32-bp deletion (de132) in the CCR5 gene, which encodes an essential co-receptor of HIV infection: Homozygous del32 CCR5 carriers are immune to HIV infection. We designed one RGEN specific to the del32 allele and the other to the wild-type allele. As expected, the wild-type-specific RGEN cleaved the PCR products obtained from K562, SKBR3, or HeLa cells (used as wild-type controls) completely but those from HEK293 cells partially (FIG. **30***a*), confirming the presence of the uncleavable del32 allele in HEK293 cells. Unexpectedly, however, the del32-specific RGEN cleaved the PCR products from wild-type cells as efficiently as those from HEK293 cells. Interestingly, this RGEN had an off-target site with a single-base mismatch immediately downstream of the on-target site (FIG. **30**). These results suggest that RGENs can be used to detect naturally-occurring indels but cannot distinguish sequences with single nucleotide polymorphisms or point mutations due to their off-target effects.

To genotype oncogenic single-nucleotide variations using RGENs, we attenuated RGEN activity by employing a single-base mismatched guide RNA instead of a perfectly-matched RNA. RGENs that contained the perfectly-matched guide RNA specific to the wild-type sequence or mutant sequence cleaved both sequences (FIGS. **31***a* and **32***a*). In contrast, RGENS that contained a single-base mismatched guide RNA distinguished the two sequences, enabling genotyping of three recurrent oncogenic point mutations in the KRAS, PIK3CA, and IDH1 genes in human cancer cell lines (FIG. **29***b* and FIGS. **33***a, b*). In addition, we were able to detect point mutations in the BRAF and NRAS genes using RGENs that recognize the NAG PAM sequence (FIGS. **33***c, d*). We believe that we can use RGEN-RFLP to genotype almost any, if not all, mutations or polymorphisms in the human and other genomes.

The above data proposes RGENS as providing a platform to use simple and robust RFLP analysis for various sequence variations. With high flexibility in reprogramming target sequence, RGENs can be used to detect various genetic variations (single nucleotide variations, small insertion/deletions, structural variations) such as disease-related recurring mutations, genotypes related to drug-response by a patient and also mutations induced by engineered nucleases in cells. Here, we used RGEN genotyping to detect mutations induced by engineered nucleases in cells and animals. In principle, one could also use RGENs that will specifically detect and cleave naturally-occurring variations and mutations.

Based on the above description, it should be understood by those skilled in the art that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention without departing from the technical idea or essential features of the invention as defined in the following claims. In this regard, the above-described examples are for illustrative purposes only, and the invention is not intended to be limited by these examples. The scope of the present invention should be understood to include all of the modifications or modified form derived from the meaning and scope of the following claims or its equivalent concepts.

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FEATURE                 Location/Qualifiers
misc_feature            1..20
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
misc_feature            1..20
                        note = R primer for CCR5
source                  1..20
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 7
gccctgtcaa gagttgacac                                            20

SEQ ID NO: 8            moltype = DNA  length = 20
FEATURE                 Location/Qualifiers
misc_feature            1..20
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
misc_feature            1..20
                        note = F1 primer for C4BPB
source                  1..20
```

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                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 8
tatttggctg gttgaaaggg                                              20

SEQ ID NO: 9         moltype = DNA  length = 24
FEATURE              Location/Qualifiers
misc_feature         1..24
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
misc_feature         1..24
                     note = R1 primer for C4BPB
source               1..24
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 9
aaagtcatga aataaacaca ccca                                         24

SEQ ID NO: 10        moltype = DNA  length = 24
FEATURE              Location/Qualifiers
misc_feature         1..24
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
misc_feature         1..24
                     note = F2 primer for C4BPB
source               1..24
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 10
ctgcattgat atggtagtac catg                                         24

SEQ ID NO: 11        moltype = DNA  length = 21
FEATURE              Location/Qualifiers
misc_feature         1..21
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
misc_feature         1..21
                     note = R2 primer for C4BPB
source               1..21
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 11
gctgttcatt gcaatggaat g                                            21

SEQ ID NO: 12        moltype = DNA  length = 20
FEATURE              Location/Qualifiers
misc_feature         1..20
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
misc_feature         1..20
                     note = F1 primer for ADCY5
source               1..20
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 12
gctcccacct tagtgctctg                                              20

SEQ ID NO: 13        moltype = DNA  length = 20
FEATURE              Location/Qualifiers
misc_feature         1..20
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
misc_feature         1..20
                     note = R1 primer for ADCY5
source               1..20
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 13
ggtggcagga acctgtatgt                                              20

SEQ ID NO: 14        moltype = DNA  length = 21
FEATURE              Location/Qualifiers
misc_feature         1..21
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
misc_feature         1..21
                     note = F2 primer for ADCY5
source               1..21
                     mol_type = other DNA
```

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```
                    organism = synthetic construct
SEQUENCE: 14
gtcattggcc agagatgtgg a                                              21

SEQ ID NO: 15       moltype = DNA  length = 20
FEATURE             Location/Qualifiers
misc_feature        1..20
                    note = Description of Artificial Sequence: Synthetic
                     oligonucleotide
misc_feature        1..20
                    note = R2 primer for ADCY5
source              1..20
                    mol_type = other DNA
                    organism = synthetic construct
SEQUENCE: 15
gtcccatgac aggcgtgtat                                                20

SEQ ID NO: 16       moltype = DNA  length = 20
FEATURE             Location/Qualifiers
misc_feature        1..20
                    note = Description of Artificial Sequence: Synthetic
                     oligonucleotide
misc_feature        1..20
                    note = F primer for KCNJ6
source              1..20
                    mol_type = other DNA
                    organism = synthetic construct
SEQUENCE: 16
gcctggccaa gtttcagtta                                                20

SEQ ID NO: 17       moltype = DNA  length = 20
FEATURE             Location/Qualifiers
misc_feature        1..20
                    note = Description of Artificial Sequence: Synthetic
                     oligonucleotide
misc_feature        1..20
                    note = R1 primer for KCNJ6
source              1..20
                    mol_type = other DNA
                    organism = synthetic construct
SEQUENCE: 17
tggagccatt ggtttgcatc                                                20

SEQ ID NO: 18       moltype = DNA  length = 22
FEATURE             Location/Qualifiers
misc_feature        1..22
                    note = Description of Artificial Sequence: Synthetic
                     oligonucleotide
misc_feature        1..22
                    note = R2 primer for KCNJ6
source              1..22
                    mol_type = other DNA
                    organism = synthetic construct
SEQUENCE: 18
ccagaactaa gccgtttctg ac                                             22

SEQ ID NO: 19       moltype = DNA  length = 20
FEATURE             Location/Qualifiers
misc_feature        1..20
                    note = Description of Artificial Sequence: Synthetic
                     oligonucleotide
misc_feature        1..20
                    note = F1 primer for CNTNAP2
source              1..20
                    mol_type = other DNA
                    organism = synthetic construct
SEQUENCE: 19
atcaccgaca accagtttcc                                                20

SEQ ID NO: 20       moltype = DNA  length = 20
FEATURE             Location/Qualifiers
misc_feature        1..20
                    note = Description of Artificial Sequence: Synthetic
                     oligonucleotide
misc_feature        1..20
                    note = F2 primer for CNTNAP2
source              1..20
                    mol_type = other DNA
                    organism = synthetic construct
```

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```
SEQUENCE: 20
tgcagtgcag actctttcca                                              20

SEQ ID NO: 21          moltype = DNA  length = 20
FEATURE                Location/Qualifiers
misc_feature           1..20
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
misc_feature           1..20
                       note = R primer for CNTNAP2
source                 1..20
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 21
aaggacacag ggcaactgaa                                              20

SEQ ID NO: 22          moltype = DNA  length = 20
FEATURE                Location/Qualifiers
misc_feature           1..20
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
misc_feature           1..20
                       note = F1 primer for N/A Chr. 5
source                 1..20
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 22
tgtggaacga gtggtgacag                                              20

SEQ ID NO: 23          moltype = DNA  length = 22
FEATURE                Location/Qualifiers
misc_feature           1..22
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
misc_feature           1..22
                       note = R1 primer for N/A Chr. 5
source                 1..22
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 23
gctggattag gaggcaggat tc                                           22

SEQ ID NO: 24          moltype = DNA  length = 22
FEATURE                Location/Qualifiers
misc_feature           1..22
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
misc_feature           1..22
                       note = F2 primer for N/A Chr. 5
source                 1..22
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 24
gtgctgagaa cgcttcatag ag                                           22

SEQ ID NO: 25          moltype = DNA  length = 23
FEATURE                Location/Qualifiers
misc_feature           1..23
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
misc_feature           1..23
                       note = R2 primer for N/A Chr. 5
source                 1..23
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 25
ggaccaaacc acattcttct cac                                          23

SEQ ID NO: 26          moltype = DNA  length = 20
FEATURE                Location/Qualifiers
misc_feature           1..20
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
misc_feature           1..20
                       note = F primer for deletion
source                 1..20
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 26
```

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```
ccacatctcg ttctcggttt                                              20

SEQ ID NO: 27          moltype = DNA  length = 20
FEATURE                Location/Qualifiers
misc_feature           1..20
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
misc_feature           1..20
                       note = R primer for deletion
source                 1..20
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 27
tcacaagccc acagatattt                                              20

SEQ ID NO: 28          moltype = RNA  length = 105
FEATURE                Location/Qualifiers
misc_feature           1..105
                       note = Description of Artificial Sequence: Synthetic
                        polynucleotide
misc_feature           1..105
                       note = sgRNA for CCR5
source                 1..105
                       mol_type = other RNA
                       organism = synthetic construct
SEQUENCE: 28
ggtgacatca attattatac atgttttaga gctagaaata gcaagttaaa ataaggctag  60
tccgttatca acttgaaaaa gtggcaccga gtcggtgctt ttttt                  105

SEQ ID NO: 29          moltype = RNA  length = 44
FEATURE                Location/Qualifiers
misc_feature           1..44
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
misc_feature           1..44
                       note = crRNA for CCR5
source                 1..44
                       mol_type = other RNA
                       organism = synthetic construct
SEQUENCE: 29
ggtgacatca attattatac atgttttaga gctatgctgt tttg                   44

SEQ ID NO: 30          moltype = RNA  length = 86
FEATURE                Location/Qualifiers
misc_feature           1..86
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
misc_feature           1..86
                       note = tracrRNA for CCR5
source                 1..86
                       mol_type = other RNA
                       organism = synthetic construct
SEQUENCE: 30
ggaaccattc aaaacagcat agcaagttaa aataaggcta gtccgttatc aacttgaaaa  60
agtggcaccg agtcggtgct tttttt                                       86

SEQ ID NO: 31          moltype = DNA  length = 86
FEATURE                Location/Qualifiers
misc_feature           1..86
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
misc_feature           1..86
                       note = Foxn1 #1 sgRNA
source                 1..86
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 31
gaaattaata cgactcacta taggcagtct gacgtcacac ttccgtttta gagctagaaa  60
tagcaagtta aaataaggct agtccg                                       86

SEQ ID NO: 32          moltype = DNA  length = 86
FEATURE                Location/Qualifiers
misc_feature           1..86
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
misc_feature           1..86
                       note = Foxn1 #2 sgRNA
source                 1..86
                       mol_type = other DNA
```

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```
                     organism = synthetic construct
SEQUENCE: 32
gaaattaata cgactcacta taggacttcc aggctccacc cgacgtttta gagctagaaa  60
tagcaagtta aaataaggct agtccg                                       86

SEQ ID NO: 33          moltype = DNA  length = 86
FEATURE                Location/Qualifiers
misc_feature           1..86
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
misc_feature           1..86
                       note = Foxn1 #3 sgRNA
source                 1..86
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 33
gaaattaata cgactcacta taggccaggc tccacccgac tggagtttta gagctagaaa  60
tagcaagtta aaataaggct agtccg                                       86

SEQ ID NO: 34          moltype = DNA  length = 86
FEATURE                Location/Qualifiers
misc_feature           1..86
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
misc_feature           1..86
                       note = Foxn1 #4 sgRNA
source                 1..86
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 34
gaaattaata cgactcacta taggactgga gggcgaaccc caaggtttta gagctagaaa  60
tagcaagtta aaataaggct agtccg                                       86

SEQ ID NO: 35          moltype = DNA  length = 86
FEATURE                Location/Qualifiers
misc_feature           1..86
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
misc_feature           1..86
                       note = Foxn1 #5 sgRNA
source                 1..86
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 35
gaaattaata cgactcacta taggacccca aggggacctc atgcgtttta gagctagaaa  60
tagcaagtta aaataaggct agtccg                                       86

SEQ ID NO: 36          moltype = DNA  length = 86
FEATURE                Location/Qualifiers
misc_feature           1..86
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
misc_feature           1..86
                       note = Prkdc #1 sgRNA
source                 1..86
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 36
gaaattaata cgactcacta taggttagtt ttttccagag acttgtttta gagctagaaa  60
tagcaagtta aaataaggct agtccg                                       86

SEQ ID NO: 37          moltype = DNA  length = 86
FEATURE                Location/Qualifiers
misc_feature           1..86
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
misc_feature           1..86
                       note = Prkdc #2 sgRNA
source                 1..86
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 37
gaaattaata cgactcacta taggttggtt tgcttgtgtt tatcgtttta gagctagaaa  60
tagcaagtta aaataaggct agtccg                                       86

SEQ ID NO: 38          moltype = DNA  length = 86
FEATURE                Location/Qualifiers
misc_feature           1..86
                       note = Description of Artificial Sequence: Synthetic
```

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```
                      oligonucleotide
misc_feature          1..86
                      note = Prkdc #3 sgRNA
source                1..86
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 38
gaaattaata cgactcacta taggcacaag caaaccaaag tctcgtttta gagctagaaa  60
tagcaagtta aaataaggct agtccg                                       86

SEQ ID NO: 39         moltype = DNA  length = 86
FEATURE               Location/Qualifiers
misc_feature          1..86
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
misc_feature          1..86
                      note = Prkdc #4 sgRNA
source                1..86
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 39
gaaattaata cgactcacta taggcctcaa tgctaagcga cttcgtttta gagctagaaa  60
tagcaagtta aaataaggct agtccg                                       86

SEQ ID NO: 40         moltype = DNA  length = 29
FEATURE               Location/Qualifiers
misc_feature          1..29
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
misc_feature          1..29
                      note = F1 primer for Foxn1
source                1..29
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 40
gtctgtctat catctcttcc cttctctcc                                    29

SEQ ID NO: 41         moltype = DNA  length = 25
FEATURE               Location/Qualifiers
misc_feature          1..25
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
misc_feature          1..25
                      note = F2 primer for Foxn1
source                1..25
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 41
tccctaatcc gatggctagc tccag                                        25

SEQ ID NO: 42         moltype = DNA  length = 23
FEATURE               Location/Qualifiers
misc_feature          1..23
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
misc_feature          1..23
                      note = R1 primer for Foxn1
source                1..23
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 42
acgagcagct gaagttagca tgc                                          23

SEQ ID NO: 43         moltype = DNA  length = 32
FEATURE               Location/Qualifiers
misc_feature          1..32
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
misc_feature          1..32
                      note = R2 primer for Foxn1
source                1..32
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 43
ctactcaatg ctcttagagc taccaggctt gc                                32

SEQ ID NO: 44         moltype = DNA  length = 20
FEATURE               Location/Qualifiers
misc_feature          1..20
```

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```
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
misc_feature         1..20
                     note = F primer for Prkdc
source               1..20
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 44
gactgttgtg gggagggccg                                              20

SEQ ID NO: 45        moltype = DNA  length = 24
FEATURE              Location/Qualifiers
misc_feature         1..24
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
misc_feature         1..24
                     note = F2 primer for Prkdc
source               1..24
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 45
gggagggccg aaagtcttat tttg                                         24

SEQ ID NO: 46        moltype = DNA  length = 28
FEATURE              Location/Qualifiers
misc_feature         1..28
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
misc_feature         1..28
                     note = R1 primer for Prkdc
source               1..28
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 46
cctgaagact gaagttggca gaagtgag                                     28

SEQ ID NO: 47        moltype = DNA  length = 27
FEATURE              Location/Qualifiers
misc_feature         1..27
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
misc_feature         1..27
                     note = R2 primer for Prkdc
source               1..27
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 47
ctttagggct tcttctctac aatcacg                                      27

SEQ ID NO: 48        moltype = DNA  length = 38
FEATURE              Location/Qualifiers
misc_feature         1..38
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
misc_feature         1..38
                     note = F primer for Foxn1
source               1..38
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 48
ctcggtgtgt agccctgacc tcggtgtgta gccctgac                          38

SEQ ID NO: 49        moltype = DNA  length = 21
FEATURE              Location/Qualifiers
misc_feature         1..21
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
misc_feature         1..21
                     note = R primer for Foxn1
source               1..21
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 49
agactggcct ggaactcaca g                                            21

SEQ ID NO: 50        moltype = DNA  length = 23
FEATURE              Location/Qualifiers
misc_feature         1..23
                     note = Description of Artificial Sequence: Synthetic
```

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```
                      oligonucleotide
misc_feature          1..23
                      note = F primer for Foxn1
source                1..23
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 50
cactaaagcc tgtcaggaag ccg                                          23

SEQ ID NO: 51         moltype = DNA  length = 21
FEATURE               Location/Qualifiers
misc_feature          1..21
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
misc_feature          1..21
                      note = R primer for Foxn1
source                1..21
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 51
ctgtggagag cacacagcag c                                            21

SEQ ID NO: 52         moltype = DNA  length = 19
FEATURE               Location/Qualifiers
misc_feature          1..19
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
misc_feature          1..19
                      note = F primer for Foxn1
source                1..19
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 52
gctgcgacct gagaccatg                                               19

SEQ ID NO: 53         moltype = DNA  length = 26
FEATURE               Location/Qualifiers
misc_feature          1..26
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
misc_feature          1..26
                      note = R primer for Foxn1
source                1..26
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 53
cttcaatggc ttcctgctta ggctac                                       26

SEQ ID NO: 54         moltype = DNA  length = 23
FEATURE               Location/Qualifiers
misc_feature          1..23
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
misc_feature          1..23
                      note = F primer for Foxn1
source                1..23
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 54
ggttcagatg aggccatcct ttc                                          23

SEQ ID NO: 55         moltype = DNA  length = 24
FEATURE               Location/Qualifiers
misc_feature          1..24
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
misc_feature          1..24
                      note = R primer for Foxn1
source                1..24
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 55
cctgatctgc aggcttaacc cttg                                         24

SEQ ID NO: 56         moltype = DNA  length = 22
FEATURE               Location/Qualifiers
misc_feature          1..22
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
```

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```
misc_feature          1..22
                      note = F primer for Prkdc
source                1..22
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 56
ctcacctgca catcacatgt gg                                          22

SEQ ID NO: 57         moltype = DNA  length = 20
FEATURE               Location/Qualifiers
misc_feature          1..20
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
misc_feature          1..20
                      note = R primer for Prkdc
source                1..20
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 57
ggcatccacc ctatggggtc                                             20

SEQ ID NO: 58         moltype = DNA  length = 25
FEATURE               Location/Qualifiers
misc_feature          1..25
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
misc_feature          1..25
                      note = F primer for Prkdc
source                1..25
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 58
gccttgacct agagcttaaa gagcc                                       25

SEQ ID NO: 59         moltype = DNA  length = 25
FEATURE               Location/Qualifiers
misc_feature          1..25
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
misc_feature          1..25
                      note = R primer for Prkdc
source                1..25
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 59
ggtcttgtta gcaggaagga cactg                                       25

SEQ ID NO: 60         moltype = DNA  length = 27
FEATURE               Location/Qualifiers
misc_feature          1..27
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
misc_feature          1..27
                      note = F primer for Prkdc
source                1..27
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 60
aaaactctgc ttgatgggat atgtggg                                     27

SEQ ID NO: 61         moltype = DNA  length = 26
FEATURE               Location/Qualifiers
misc_feature          1..26
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
misc_feature          1..26
                      note = R primer for Prkdc
source                1..26
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 61
ctctcactgg ttatctgtgc tccttc                                      26

SEQ ID NO: 62         moltype = DNA  length = 23
FEATURE               Location/Qualifiers
misc_feature          1..23
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
misc_feature          1..23
```

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```
                     note = F primer for Prkdc
source               1..23
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 62
ggatcaatag gtggtggggg atg                                          23

SEQ ID NO: 63        moltype = DNA  length = 27
FEATURE              Location/Qualifiers
misc_feature         1..27
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
misc_feature         1..27
                     note = R primer for Prkdc
source               1..27
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 63
gtgaatgaca caatgtgaca gcttcag                                      27

SEQ ID NO: 64        moltype = DNA  length = 28
FEATURE              Location/Qualifiers
misc_feature         1..28
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
misc_feature         1..28
                     note = F primer for Prkdc
source               1..28
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 64
cacaagacag acctctcaac attcagtc                                     28

SEQ ID NO: 65        moltype = DNA  length = 32
FEATURE              Location/Qualifiers
misc_feature         1..32
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
misc_feature         1..32
                     note = R primer for Prkdc
source               1..32
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 65
gtgcatgcat ataatccatt ctgattgctc tc                                32

SEQ ID NO: 66        moltype = DNA  length = 17
FEATURE              Location/Qualifiers
misc_feature         1..17
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
misc_feature         1..17
                     note = F1 primer for Prkdc
source               1..17
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 66
gggaggcaga ggcaggt                                                 17

SEQ ID NO: 67        moltype = DNA  length = 23
FEATURE              Location/Qualifiers
misc_feature         1..23
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
misc_feature         1..23
                     note = F2 primer for Prkdc
source               1..23
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 67
ggatctctgt gagtttgagg cca                                          23

SEQ ID NO: 68        moltype = DNA  length = 24
FEATURE              Location/Qualifiers
misc_feature         1..24
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
misc_feature         1..24
                     note = R1 primer for Prkdc
```

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```
source                  1..24
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 68
gctccagaac tcactcttag gctc                                          24

SEQ ID NO: 69           moltype = DNA  length = 20
FEATURE                 Location/Qualifiers
misc_feature            1..20
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
misc_feature            1..20
                        note = Primer for Foxn1
source                  1..20
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 69
ctactccctc cgcagtctga                                               20

SEQ ID NO: 70           moltype = DNA  length = 20
FEATURE                 Location/Qualifiers
misc_feature            1..20
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
misc_feature            1..20
                        note = Primer for Foxn1
source                  1..20
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 70
ccaggcctag gttccaggta                                               20

SEQ ID NO: 71           moltype = DNA  length = 20
FEATURE                 Location/Qualifiers
misc_feature            1..20
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
misc_feature            1..20
                        note = Primer for Prkdc
source                  1..20
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 71
ccccagcatt gcagatttcc                                               20

SEQ ID NO: 72           moltype = DNA  length = 23
FEATURE                 Location/Qualifiers
misc_feature            1..23
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
misc_feature            1..23
                        note = Primer for Prkdc
source                  1..23
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 72
agggcttctt ctctacaatc acg                                           23

SEQ ID NO: 73           moltype = DNA  length = 86
FEATURE                 Location/Qualifiers
misc_feature            1..86
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
misc_feature            1..86
                        note = BRI1 target 1
source                  1..86
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 73
gaaattaata cgactcacta taggtttgaa agatggaagc gcgggtttta gagctagaaa  60
tagcaagtta aaataaggct agtccg                                        86

SEQ ID NO: 74           moltype = DNA  length = 86
FEATURE                 Location/Qualifiers
misc_feature            1..86
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
misc_feature            1..86
                        note = BRI1 target 2
```

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```
source                  1..86
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 74
gaaattaata cgactcacta taggtgaaac taaactggtc cacagtttta gagctagaaa  60
tagcaagtta aaataaggct agtccg                                       86

SEQ ID NO: 75           moltype = DNA  length = 64
FEATURE                 Location/Qualifiers
misc_feature            1..64
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
misc_feature            1..64
                        note = Universal
source                  1..64
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 75
aaaaaagcac cgactcggtg ccacttttc aagttgataa cggactagcc ttattttaac  60
ttgc                                                               64

SEQ ID NO: 76           moltype = DNA  length = 65
FEATURE                 Location/Qualifiers
misc_feature            1..65
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
misc_feature            1..65
                        note = Templates for crRNA
misc_difference         25..44
                        note = modified_base - a, c, t, g, unknown or other
source                  1..65
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 76
gaaattaata cgactcacta taggnnnnnn nnnnnnnnnn nnnngtttta gagctatgct  60
gtttt                                                              65

SEQ ID NO: 77           moltype = DNA  length = 67
FEATURE                 Location/Qualifiers
misc_feature            1..67
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
misc_feature            1..67
                        note = tracrRNA
source                  1..67
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 77
gaaattaata cgactcacta taggaaccat tcaaaacagc atagcaagtt aaaataaggc  60
tagtccg                                                            67

SEQ ID NO: 78           moltype = DNA  length = 69
FEATURE                 Location/Qualifiers
misc_feature            1..69
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
misc_feature            1..69
                        note = tracrRNA
source                  1..69
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 78
aaaaaaagca ccgactcggt gccacttttt caagttgata acggactagc cttattttaa  60
cttgctatg                                                          69

SEQ ID NO: 79           moltype = DNA  length = 20
FEATURE                 Location/Qualifiers
misc_feature            1..20
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
misc_feature            1..20
                        note = Primer
source                  1..20
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 79
ctccatggtg ctatagagca                                              20

SEQ ID NO: 80           moltype = DNA  length = 21
```

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```
FEATURE                 Location/Qualifiers
misc_feature            1..21
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
misc_feature            1..21
                        note = Primer
source                  1..21
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 80
gagccaagct ctccatctag t                                              21

SEQ ID NO: 81           moltype = DNA  length = 20
FEATURE                 Location/Qualifiers
misc_feature            1..20
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
misc_feature            1..20
                        note = Primer
source                  1..20
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 81
gccctgtcaa gagttgacac                                                20

SEQ ID NO: 82           moltype = DNA  length = 22
FEATURE                 Location/Qualifiers
misc_feature            1..22
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
misc_feature            1..22
                        note = Primer
source                  1..22
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 82
gcacagggtg gaacaagatg ga                                             22

SEQ ID NO: 83           moltype = DNA  length = 24
FEATURE                 Location/Qualifiers
misc_feature            1..24
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
misc_feature            1..24
                        note = Primer
source                  1..24
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 83
gccaggtacc tatcgattgt cagg                                           24

SEQ ID NO: 84           moltype = DNA  length = 21
FEATURE                 Location/Qualifiers
misc_feature            1..21
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
misc_feature            1..21
                        note = Primer
source                  1..21
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 84
gagccaagct ctccatctag t                                              21

SEQ ID NO: 85           moltype = DNA  length = 20
FEATURE                 Location/Qualifiers
misc_feature            1..20
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
misc_feature            1..20
                        note = Primer
source                  1..20
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 85
actctgactg ggtcaccagc                                                20

SEQ ID NO: 86           moltype = DNA  length = 20
FEATURE                 Location/Qualifiers
```

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misc_feature 1..20
note = Description of Artificial Sequence: Synthetic oligonucleotide
misc_feature 1..20
note = Primer
source 1..20
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 86
tatttggctg gttgaaaggg 20

SEQ ID NO: 87 moltype = DNA length = 24
FEATURE Location/Qualifiers
misc_feature 1..24
note = Description of Artificial Sequence: Synthetic oligonucleotide
misc_feature 1..24
note = Primer
source 1..24
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 87
aaagtcatga aataaacaca ccca 24

SEQ ID NO: 88 moltype = DNA length = 24
FEATURE Location/Qualifiers
misc_feature 1..24
note = Description of Artificial Sequence: Synthetic oligonucleotide
misc_feature 1..24
note = Primer
source 1..24
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 88
ctgcattgat atggtagtac catg 24

SEQ ID NO: 89 moltype = DNA length = 21
FEATURE Location/Qualifiers
misc_feature 1..21
note = Description of Artificial Sequence: Synthetic oligonucleotide
misc_feature 1..21
note = Primer
source 1..21
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 89
gctgttcatt gcaatggaat g 21

SEQ ID NO: 90 moltype = DNA length = 22
FEATURE Location/Qualifiers
misc_feature 1..22
note = Description of Artificial Sequence: Synthetic oligonucleotide
misc_feature 1..22
note = Primer
source 1..22
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 90
atggagttgg acatggccat gg 22

SEQ ID NO: 91 moltype = DNA length = 28
FEATURE Location/Qualifiers
misc_feature 1..28
note = Description of Artificial Sequence: Synthetic oligonucleotide
misc_feature 1..28
note = Primer
source 1..28
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 91
actcactatc cacagttcag catttacc 28

SEQ ID NO: 92 moltype = DNA length = 23
FEATURE Location/Qualifiers
misc_feature 1..23

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```
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
misc_feature         1..23
                     note = Primer
source               1..23
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 92
tggagatagc tgtcagcaac ttt                                          23

SEQ ID NO: 93        moltype = DNA  length = 29
FEATURE              Location/Qualifiers
misc_feature         1..29
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
misc_feature         1..29
                     note = Primer
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 93
caacaaagca aaggtaaagt tggtaatag                                    29

SEQ ID NO: 94        moltype = DNA  length = 25
FEATURE              Location/Qualifiers
misc_feature         1..25
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
misc_feature         1..25
                     note = Primer
source               1..25
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 94
ggtttcagga gatgtgttac aaggc                                        25

SEQ ID NO: 95        moltype = DNA  length = 27
FEATURE              Location/Qualifiers
misc_feature         1..27
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
misc_feature         1..27
                     note = Primer
source               1..27
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 95
gattgtgcaa ttcctatgca atcggtc                                      27

SEQ ID NO: 96        moltype = DNA  length = 25
FEATURE              Location/Qualifiers
misc_feature         1..25
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
misc_feature         1..25
                     note = Primer
source               1..25
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 96
cactgggtac ttaatctgta gcctc                                        25

SEQ ID NO: 97        moltype = DNA  length = 23
FEATURE              Location/Qualifiers
misc_feature         1..23
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
misc_feature         1..23
                     note = Primer
source               1..23
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 97
ggttccaagt cattcccagt agc                                          23

SEQ ID NO: 98        moltype = DNA  length = 30
FEATURE              Location/Qualifiers
misc_feature         1..30
                     note = Description of Artificial Sequence: Synthetic
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```
                      oligonucleotide
misc_feature          1..30
                      note = Primer
source                1..30
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 98
catcactgca gttgtaggtt ataactatcc                                    30

SEQ ID NO: 99         moltype = DNA  length = 26
FEATURE               Location/Qualifiers
misc_feature          1..26
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
misc_feature          1..26
                      note = Primer
source                1..26
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 99
ttgaaaacca cagatctggt tgaacc                                        26

SEQ ID NO: 100        moltype = DNA  length = 22
FEATURE               Location/Qualifiers
misc_feature          1..22
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
misc_feature          1..22
                      note = Primer
source                1..22
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 100
ggagtgccaa gagaatatct gg                                            22

SEQ ID NO: 101        moltype = DNA  length = 32
FEATURE               Location/Qualifiers
misc_feature          1..32
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
misc_feature          1..32
                      note = Primer
source                1..32
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 101
ctgaaactgg tttcaaaata ttcgttttaa gg                                 32

SEQ ID NO: 102        moltype = DNA  length = 22
FEATURE               Location/Qualifiers
misc_feature          1..22
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
misc_feature          1..22
                      note = Primer
source                1..22
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 102
gctctgtatg ccctgtagta gg                                            22

SEQ ID NO: 103        moltype = DNA  length = 22
FEATURE               Location/Qualifiers
misc_feature          1..22
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
misc_feature          1..22
                      note = Primer
source                1..22
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 103
tttgcatctg accttacctt tg                                            22

SEQ ID NO: 104        moltype = DNA  length = 23
FEATURE               Location/Qualifiers
misc_feature          1..23
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
```

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misc_feature 1..23
note = Target sequence of RGEN
source 1..23
mol_type = other DNA
organism = synthetic construct

SEQUENCE: 104
aatgaccact acatcctcaa ggg 23

SEQ ID NO: 105 moltype = DNA length = 23
FEATURE Location/Qualifiers
misc_feature 1..23
note = Description of Artificial Sequence: Synthetic oligonucleotide
misc_feature 1..23
note = Target sequence of RGEN
source 1..23
mol_type = other DNA
organism = synthetic construct

SEQUENCE: 105
agatgatgtc tcatcatcag agg 23

SEQ ID NO: 106 moltype = DNA length = 4170
FEATURE Location/Qualifiers
misc_feature 1..4170
note = Description of Artificial Sequence: Synthetic polynucleotide
misc_feature 1..4170
note = Cas9-coding sequence in p3s-Cas9HC (humanized, C-term tagging, human cell experiments)
source 1..4170
mol_type = other DNA
organism = synthetic construct

SEQUENCE: 106
atggacaaga agtacagcat cggcctggac atcggtacca acagcgtggg ctgggccgtg 60
atcaccgacg agtacaaggt gcccagcaag aagttcaagg tgctgggcaa caccgaccgc 120
cacagcatca agaagaacct gatcggcgcc ctgctgttcg acagcggcga gaccgccgag 180
gccacccgcc tgaagcgcac cgcccgccgc cgctacaccc gccgcaagaa ccgcatctgc 240
tacctgcagg agatcttcag caacgagatg gccaaggtgg acgacagctt cttccaccgc 300
ctggaggaga gcttcctggt ggaggaggac aagaagcacg agcgccaccc catcttcggc 360
aacatcgtgg acgaggtggc ctaccacgag aagtacccca ccatctacca cctgcgcaag 420
aagctggtgg acagcaccga caaggccgac ctgcgcctga tctacctggc cctggcccac 480
atgatcaagt tccgcggcca cttcctgatc gagggcgacc tgaaccccga caacagcgac 540
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aacctgccca acgagaaggt gctgcccaag cacagcctgc tgtacgagta cttcaccgtg 1560
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agcggcgagc agaagaaggc catcgtggac ctgctgttca agaccaaccg caaggtgacc 1680
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gtggagaaca cccagctgca gaacgagaag ctgtacctgt actacctgca gaacggccgc 2460
gacatgtacg tggaccagga gctggacatc aaccgcctga gcgactacga cgtggaccac 2520
atcgtgcccc agagcttcct gaaggacgac agcatcgaca acaaggtgct gacccgcagc 2580
gacaagaacc gcggcaagag cgacaacgtg cccagcgagg aggtggtgaa gaagatgaag 2640
aactactggc gccagctgct gaacgccaag ctgatcaccc agcgcaagtt cgacaacctg 2700
accaaggccg agcgcggcgg cctgagcgag ctggacaagg ccggcttcat caagcgccag 2760

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ctggtggaga cccgccagat caccaagcac gtggcccaga tcctggacag ccgcatgaac  2820
accaagtacg acgagaacga caagctgatc cgcgaggtga aggtgatcac cctgaagagc  2880
aagctggtga gcgacttccg caaggacttc cagttctaca aggtgcgcga gatcaacaac  2940
taccaccacg cccacgacgc ctacctgaac gccgtggtgg gcaccgccct gatcaagaag  3000
taccccaagc tggagagcga gttcgtgtac ggcgactaca aggtgtacga cgtgcgcaag  3060
atgatcgcca agagcgagca ggagatcggc aaggccaccg ccaagtactt cttctacagc  3120
aacatcatga acttcttcaa gaccgagatc accctggcca acggcgagat ccgcaagcgc  3180
cccctgatcg agaccaacgg cgagaccggc gagatcgtgt gggacaaggg ccgcgacttc  3240
gccaccgtgc gcaaggtgct gagcatgccc caggtgaaca tcgtgaagaa gaccgaggtg  3300
cagaccggcg gcttcagcaa ggagagcatc ctgcccaagc gcaacagcga caagctgatc  3360
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tacagcgtgc tggtggtggc caaggtggag aagggcaaga gcaagaagct gaagagcgtg  3480
aaggagctgc tgggcatcac catcatggag cgcagcagct tcgagaagaa ccccatcgac  3540
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tacagcctgt tcgagctgga gaacggccgc aagcgcatgc tggccagcgc cggcgagctg  3660
cagaagggca acgagctggc cctgcccagc aagtacgtga acttcctgta cctggccagc  3720
cactacgaga agctgaaggg cagccccgag gacaacgagc agaagcagct gttcgtggag  3780
cagcacaagc actacctgga cgagatcatc gagcagatca gcgagttcag caagcgcgtg  3840
atcctggccg acgccaacct ggacaaggtg ctgagcgcct acaacaagca ccgcgacaag  3900
cccatccgcg agcaggccga gaacatcatc cacctgttca ccctgaccaa cctgggcgcc  3960
cccgccgcct tcaagtactt cgacaccacc atcgaccgca agcgctacac cagcaccaag  4020
gaggtgctgg acgccaccct gatccaccag agcatcaccg gtctgtacga gacccgcatc  4080
gacctgagcc agctgggcgg cgacggcggc tccggacctc caaagaaaaa gagaaaagta  4140
tacccctacg acgtgcccga ctacgcctaa                                   4170

SEQ ID NO: 107          moltype = DNA  length = 4194
FEATURE                 Location/Qualifiers
misc_feature            1..4194
                        note = Description of Artificial Sequence: Synthetic
                         polynucleotide
misc_feature            1..4194
                        note = Cas9 coding sequence in p3s-Cas9HN (humanized codon,
                         N-term tagging (underlined), human cell experiments)
source                  1..4194
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 107
atggtgtacc cctacgacgt gcccgactac gccgaattgc ctccaaaaaa gaagagaaag  60
gtagggatcc gaattcccgg ggaaaaaccg gacaagaagt acagcatcgg cctggacatc  120
ggtaccaaca gcgtgggctg ggccgtgatc accgacgagt acaaggtgcc cagcaagaag  180
ttcaaggtgc tgggcaacac cgaccgccac agcatcaaga agaacctgat cggcgccctg  240
ctgttcgaca gcggcgagac cgccgaggcc acccgcctga agcgcaccgc ccgccgccgc  300
tacacccgcc gcaagaaccg catctgctac ctgcaggaga tcttcagcaa cgagatggcc  360
aaggtggacg acagcttctt ccaccgcctg gaggagagct tcctggtgga ggaggacaag  420
aagcacgagc gccaccccat cttcggcaac atcgtggacg aggtggccta ccacgagaag  480
taccccacca tctaccacct gcgcaagaag ctggtggaca gcaccgacaa ggccgacctg  540
cgcctgatct acctggccct ggcccacatg atcaagttcc gcggccactt cctgatcgag  600
ggcgacctga accccgacaa cagcgacgtg gacaagctgt tcatccagct ggtgcagacc  660
tacaaccagc tgttcgagga gaaccccatc aacgccagcg gcgtggacgc caaggccatc  720
ctgagcgccc gcctgagcaa gagccgccgc ctggagaacc tgatcgccca gctgccccgc  780
gagaagaaga acggcctgtt cggcaacctg atcgccctga gcctgggcct gaccccaac  840
ttcaagagca acttcgacct ggccgaggac gccaagctgc agctgagcaa ggacacctac  900
gacgacgacc tggacaacct gctggcccag atcggcgacc agtacgccga cctgttcctg  960
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atcaccaagg ccccctgag cgccagcatg atcaagcgct acgacgagca ccaccaggac  1080
ctgaccctgc tgaaggccct ggtgcgccag cagctgcccg agaagtacaa ggagatcttc  1140
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ttctacaagt tcatcaagcc catcctggag aagatggacg gcaccgagga gctgctggtg  1260
aagctgaacc gcgaggacct gctgcgcaag cagcgcacct tcgacaacgg cagcatcccc  1320
caccagatcc acctgggcga gctgcacgcc atcctgcgcc gccaggagga cttctacccc  1380
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accatcaccc cctggaactt cgaggaggtg gtggacaagg gcgccagcgc ccagagcttc  1560
atcgagcgca tgaccaactt cgacaagaac ctgcccaacg agaaggtgct gcccaagcac  1620
agcctgctgt acgagtactt caccgtgtac aacgagctga ccaaggtgaa gtacgtgacc  1680
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misc_feature            1..4107
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source                  1..4107
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                        organism = synthetic construct
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SEQ ID NO: 109          moltype = AA  length = 1368
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REGION                  1..1368
                        note = Description of Artificial Sequence: Synthetic
                         polypeptide
REGION                  1..1368
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source                  1..1368
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SEQ ID NO: 110          moltype = DNA  length = 4221
FEATURE                 Location/Qualifiers
misc_feature            1..4221
                        note = Description of Artificial Sequence: Synthetic
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misc_feature            1..4221
                        note = Cas9-coding sequence in pET-Cas9N3T for the
                         production of recombinant Cas9 protein in E. coli
                         (humanized codon; hexa-His-tag and a nuclear localization
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source                  1..4221
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SEQ ID NO: 111 moltype = AA length = 1406
FEATURE Location/Qualifiers
REGION 1..1406
note = Description of Artificial Sequence: Synthetic polypeptide
REGION 1..1406

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ASGVDAKAIL SARLSKSRRL ENLIAQLPGE KKNGLFGNLI ALSLGLTPNF KSNFDLAEDA  300
KLQLSKDTYD DDLDNLLAQI GDQYADLFLA AKNLSDAILL SDILRVNTEI TKAPLSASMI  360
KRYDEHHQDL TLLKALVRQQ LPEKYKEIFF DQSKNGYAGY IDGGASQEEF YKFIKPILEK  420
MDGTEELLVK LNREDLLRKQ RTFDNGSIPH QIHLGELHAI LRRQEDFYPF LKDNREKIEK  480
ILTFRIPYYV GPLARGNSRF AWMTRKSEET ITPWNFEEVV DKGASAQSFI ERMTNFDKNL  540
PNEKVLPKHS LLYEYFTVYN ELTKVKYVTE GMRKPAFLSG EQKKAIVDLL FKTNRKVTVK  600
QLKEDYFKKI ECFDSVEISG VEDRFNASLG TYHDLLKIIK DKDFLDNEEN EDILEDIVLT  660
LTLFEDREMI EERLKTYAHL FDDKVMKQLK RRRYTGWGRL SRKLINGIRD KQSGKTILDF  720
LKSDGFANRN FMQLIHDDSL TFKEDIQKAQ VSGQGDSLHE HIANLAGSPA IKKGILQTVK  780
VVDELVKVMG RHKPENIVIE MARENQTTQK GQKNSRERMK RIEEGIKELG SQILKEHPVE  840
NTQLQNEKLY LYYLQNGRDM YVDQELDINR LSDYDVDHIV PQSFLKDDSI DNKVLTRSDK  900
NRGKSDNVPS EEVVKKMKNY WRQLLNAKLI TQRKFDNLTK AERGGLSELD KAGFIKRQLV  960
ETRQITKHVA QILDSRMNTK YDENDKLIRE VKVITLKSKL VSDFRKDFQF YKVREINNYH  1020
HAHDAYLNAV VGTALIKKYP KLESEFVYGD YKVYDVRKMI AKSEQEIGKA TAKYFFYSNI  1080
MNFFKTEITL ANGEIRKRPL IETNGETGEI VWDKGRDFAT VRKVLSMPQV NIVKKTEVQT  1140
GGFSKESILP KRNSDKLIAR KKDWDPKKYG GFDSPTVAYS VLVVAKVEKG KSKKLKSVKE  1200
LLGITIMERS SFEKNPIDFL EAKGYKEVKK DLIIKLPKYS LFELENGRKR MLASAGELQK  1260
GNELALPSKY VNFLYLASHY EKLKGSPEDN EQKQLFVEQH KHYLDEIIEQ ISEFSKRVIL  1320
ADANLDKVLS AYNKHRDKPI REQAENIIHL FTLTNLGAPA AFKYFDTTID RKRYTSTKEV  1380
LDATLIHQSI TGLYETRIDL SQLGGD                                       1406

SEQ ID NO: 112       moltype = DNA  length = 34
FEATURE              Location/Qualifiers
source               1..34
                     mol_type = unassigned DNA
                     organism = Homo sapiens
SEQUENCE: 112
caatctatga catcaattat tatacatcgg agcc                               34

SEQ ID NO: 113       moltype = RNA  length = 64
FEATURE              Location/Qualifiers
misc_feature         1..64
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..64
                     mol_type = other RNA
                     organism = synthetic construct
SEQUENCE: 113
ggtgacatca attattatac atgttttaga gctagaaata gcaagttaaa ataaggctag  60
tccg                                                                64

SEQ ID NO: 114       moltype = DNA  length = 49
FEATURE              Location/Qualifiers
misc_feature         1..49
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..49
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 114
caatctatga catcaattat tatacatcgg agccctgcca aaaaatcaa               49

SEQ ID NO: 115       moltype = DNA  length = 50
FEATURE              Location/Qualifiers
misc_feature         1..50
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..50
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 115
caatctatga catcaattat tataacatcg gagccctgcc aaaaaatcaa              50

SEQ ID NO: 116       moltype = DNA  length = 36
FEATURE              Location/Qualifiers
misc_feature         1..36
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..36
                     mol_type = other DNA
```

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                     organism = synthetic construct
SEQUENCE: 116
caatctatga catcaattat tatgccaaaa aatcaa                              36

SEQ ID NO: 117       moltype = DNA  length = 35
FEATURE              Location/Qualifiers
misc_feature         1..35
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..35
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 117
caatctatga catcggagcc ctgccaaaaa atcaa                               35

SEQ ID NO: 118       moltype = DNA  length = 31
FEATURE              Location/Qualifiers
misc_feature         1..31
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..31
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 118
caatctatga catgccctgc caaaaaatca a                                   31

SEQ ID NO: 119       moltype = DNA  length = 30
FEATURE              Location/Qualifiers
misc_feature         1..30
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..30
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 119
caatctatga catcaattat tataaatcaa                                     30

SEQ ID NO: 120       moltype = DNA  length = 25
FEATURE              Location/Qualifiers
misc_feature         1..25
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..25
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 120
caatctatga catccaaaaa atcaa                                          25

SEQ ID NO: 121       moltype = DNA  length = 19
FEATURE              Location/Qualifiers
misc_feature         1..19
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..19
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 121
caatctatga caaaatcaa                                                 19

SEQ ID NO: 122       moltype = DNA  length = 46
FEATURE              Location/Qualifiers
source               1..46
                     mol_type = unassigned DNA
                     organism = Homo sapiens
SEQUENCE: 122
tatgtgcaat gaccactaca tcctcaaggg cagcaatcgg agccag                    46

SEQ ID NO: 123       moltype = DNA  length = 47
FEATURE              Location/Qualifiers
misc_feature         1..47
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..47
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 123
tatgtgcaat gaccactaca tccttcaagg gcagcaatcg gagccag                   47

SEQ ID NO: 124       moltype = DNA  length = 48
```

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```
FEATURE                 Location/Qualifiers
misc_feature            1..48
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..48
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 124
tatgtgcaat gaccactaca tcctctcaag ggcagcaatc ggagccag                48

SEQ ID NO: 125          moltype = DNA  length = 18
FEATURE                 Location/Qualifiers
misc_feature            1..18
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..18
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 125
tatgtgcaat ggagccag                                                 18

SEQ ID NO: 126          moltype = DNA  length = 13
FEATURE                 Location/Qualifiers
misc_feature            1..13
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..13
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 126
tatgtgcaat gac                                                      13

SEQ ID NO: 127          moltype = DNA  length = 23
FEATURE                 Location/Qualifiers
source                  1..23
                        mol_type = unassigned DNA
                        organism = Homo sapiens
SEQUENCE: 127
tgacatcaat tattatacat cgg                                           23

SEQ ID NO: 128          moltype = DNA  length = 23
FEATURE                 Location/Qualifiers
source                  1..23
                        mol_type = unassigned DNA
                        organism = Homo sapiens
SEQUENCE: 128
tgacatcaat tattatagat gga                                           23

SEQ ID NO: 129          moltype = DNA  length = 23
FEATURE                 Location/Qualifiers
source                  1..23
                        mol_type = unassigned DNA
                        organism = Homo sapiens
SEQUENCE: 129
tgacatcact tattatgcat ggg                                           23

SEQ ID NO: 130          moltype = DNA  length = 23
FEATURE                 Location/Qualifiers
source                  1..23
                        mol_type = unassigned DNA
                        organism = Homo sapiens
SEQUENCE: 130
tgacataaat tattctacat ggg                                           23

SEQ ID NO: 131          moltype = DNA  length = 23
FEATURE                 Location/Qualifiers
source                  1..23
                        mol_type = unassigned DNA
                        organism = Homo sapiens
SEQUENCE: 131
tgaaatcaat tatcatagat cgg                                           23

SEQ ID NO: 132          moltype = DNA  length = 23
FEATURE                 Location/Qualifiers
source                  1..23
                        mol_type = unassigned DNA
                        organism = Homo sapiens
SEQUENCE: 132
ccaggctcca cccgactgga ggg                                           23
```

```
SEQ ID NO: 133          moltype = RNA  length = 106
FEATURE                 Location/Qualifiers
misc_feature            1..106
                        note = Description of Artificial Sequence: Synthetic
                         polynucleotide
source                  1..106
                        mol_type = other RNA
                        organism = synthetic construct
SEQUENCE: 133
ggccaggctc cacccgactg gagttttaga gctagaaata gcaagttaaa ataaggctag  60
tccgttatca acttgaaaaa gtggcaccga gtcggtgctt tttttt                 106

SEQ ID NO: 134          moltype = DNA  length = 53
FEATURE                 Location/Qualifiers
source                  1..53
                        mol_type = unassigned DNA
                        organism = Homo sapiens
SEQUENCE: 134
acttccaggc tccacccgac tggagggcga accccaaggg gacctcatgc agg          53

SEQ ID NO: 135          moltype = DNA  length = 13
FEATURE                 Location/Qualifiers
misc_feature            1..13
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..13
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 135
acttccaggc tcc                                                      13

SEQ ID NO: 136          moltype = DNA  length = 30
FEATURE                 Location/Qualifiers
misc_feature            1..30
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..30
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 136
acttccaggc tccacccgac ctcatgcagg                                    30

SEQ ID NO: 137          moltype = DNA  length = 36
FEATURE                 Location/Qualifiers
misc_feature            1..36
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..36
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 137
acttccaggc tccaccccaa ggggacctca tgcagg                             36

SEQ ID NO: 138          moltype = DNA  length = 54
FEATURE                 Location/Qualifiers
misc_feature            1..54
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..54
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 138
acttccaggc tccacccgac ttggagggcg aaccccaagg ggacctcatg cagg         54

SEQ ID NO: 139          moltype = DNA  length = 43
FEATURE                 Location/Qualifiers
misc_feature            1..43
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..43
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 139
acttccaggc tccacccgaa ccccaagggg acctcatgca ggg                     43

SEQ ID NO: 140          moltype = DNA  length = 47
FEATURE                 Location/Qualifiers
misc_feature            1..47
```

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```
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..47
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 140
acttccaggc tccacccgac tcactatctt ctgggctcct ccatgtc                47

SEQ ID NO: 141       moltype = DNA  length = 45
FEATURE              Location/Qualifiers
misc_feature         1..45
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..45
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 141
acttccaggc tccacccgac gaaccccaag gggacctcat gcagg                  45

SEQ ID NO: 142       moltype = AA  length = 18
FEATURE              Location/Qualifiers
source               1..18
                     mol_type = protein
                     organism = Homo sapiens
SEQUENCE: 142
LPGSTRLEGE PQGDLMQA                                                18

SEQ ID NO: 143       moltype = DNA  length = 57
FEATURE              Location/Qualifiers
source               1..57
                     mol_type = unassigned DNA
                     organism = Homo sapiens
CDS                  2..55
SEQUENCE: 143
acttccaggc tccacccgac tggagggcga accccaaggg gacctcatgc aggctcc     57

SEQ ID NO: 144       moltype = DNA  length = 46
FEATURE              Location/Qualifiers
misc_feature         1..46
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..46
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 144
acttccaggc tccacccgaa ccccaagggg acctcatgca ggctcc                 46

SEQ ID NO: 145       moltype = DNA  length = 43
FEATURE              Location/Qualifiers
misc_feature         1..43
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..43
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 145
acttccaggc tccacccgaa ccccaagggg acctcatgca ggc                    43

SEQ ID NO: 146       moltype = DNA  length = 20
FEATURE              Location/Qualifiers
misc_feature         1..20
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..20
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 146
acttccaggc tccacccgac                                              20

SEQ ID NO: 147       moltype = DNA  length = 40
FEATURE              Location/Qualifiers
misc_feature         1..40
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..40
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 147
acttccaggc tccaccccaa ggggacctca tgcaggctcc                        40
```

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```
SEQ ID NO: 148          moltype = DNA  length = 58
FEATURE                 Location/Qualifiers
misc_feature            1..58
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..58
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 148
acttccaggc tccacccgac ttggagggcg aaccccaagg ggacctcatg caggctcc    58

SEQ ID NO: 149          moltype = DNA  length = 46
FEATURE                 Location/Qualifiers
misc_feature            1..46
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..46
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 149
acttccaggc tccaggcgaa ccccaagggg acctcatgca ggctcc                  46

SEQ ID NO: 150          moltype = DNA  length = 32
FEATURE                 Location/Qualifiers
misc_feature            1..32
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..32
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 150
ggcgaacccc aaggggacct catgcaggct cc                                  32

SEQ ID NO: 151          moltype = DNA  length = 32
FEATURE                 Location/Qualifiers
misc_feature            1..32
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..32
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 151
acttccaggc aaggggacct catgcaggct cc                                  32

SEQ ID NO: 152          moltype = DNA  length = 33
FEATURE                 Location/Qualifiers
misc_feature            1..33
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..33
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 152
acttccaggc taaggggacc tcatgcaggc tcc                                 33

SEQ ID NO: 153          moltype = DNA  length = 52
FEATURE                 Location/Qualifiers
source                  1..52
                        mol_type = unassigned DNA
                        organism = Homo sapiens
SEQUENCE: 153
acttccaggc tccacccgac tggagggcga accccaaggg gacctcatgc ag          52

SEQ ID NO: 154          moltype = DNA  length = 34
FEATURE                 Location/Qualifiers
misc_feature            1..34
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..34
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 154
acttccaggc gaaccccaag gggacctcat gcag                                34

SEQ ID NO: 155          moltype = DNA  length = 32
FEATURE                 Location/Qualifiers
misc_feature            1..32
                        note = Description of Artificial Sequence: Synthetic
```

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```
                      oligonucleotide
source                1..32
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 155
acttccaggc tccacaaggg gacctcatgc ag                                  32

SEQ ID NO: 156        moltype = DNA  length = 34
FEATURE               Location/Qualifiers
misc_feature          1..34
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
source                1..34
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 156
acttccaggc tccacccaag gggacctcat gccc                                34

SEQ ID NO: 157        moltype = DNA  length = 35
FEATURE               Location/Qualifiers
misc_feature          1..35
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
source                1..35
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 157
acttccaggc tccaccccaa ggggacctca tgcag                               35

SEQ ID NO: 158        moltype = DNA  length = 41
FEATURE               Location/Qualifiers
misc_feature          1..41
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
source                1..41
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 158
acttccaggc tccacccgaa ccccaagggg acctcatgca g                        41

SEQ ID NO: 159        moltype = DNA  length = 50
FEATURE               Location/Qualifiers
misc_feature          1..50
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
source                1..50
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 159
acttccaggc tccacccgaa ggagggcgaa ccccaagggg acctcatgca               50

SEQ ID NO: 160        moltype = DNA  length = 50
FEATURE               Location/Qualifiers
misc_feature          1..50
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
source                1..50
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 160
acttccaggc tccacccgac tagggcgaac cccaagggga cctcatgcag               50

SEQ ID NO: 161        moltype = DNA  length = 52
FEATURE               Location/Qualifiers
misc_feature          1..52
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
source                1..52
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 161
acttccaggc tccacccgac tgggagggcg aaccccaagg ggacctcatg ca            52

SEQ ID NO: 162        moltype = DNA  length = 52
FEATURE               Location/Qualifiers
misc_feature          1..52
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
source                1..52
```

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                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 162
acttccaggc tccacccgac ttggagggcg aaccccaagg ggacctcatg ca          52

SEQ ID NO: 163       moltype = DNA  length = 46
FEATURE              Location/Qualifiers
misc_feature         1..46
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..46
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 163
acttccaggc tccacccgag gcgaacccca aggggacctc atgcag                 46

SEQ ID NO: 164       moltype = DNA  length = 47
FEATURE              Location/Qualifiers
misc_feature         1..47
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..47
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 164
acttccaggc tccacccgag ggcgaacccc aaggggacct catgcag                47

SEQ ID NO: 165       moltype = DNA  length = 24
FEATURE              Location/Qualifiers
misc_feature         1..24
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..24
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 165
acttccaggc tccacctcat gcag                                         24

SEQ ID NO: 166       moltype = DNA  length = 29
FEATURE              Location/Qualifiers
misc_feature         1..29
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 166
agggcgaacc ccaaggggac ctcatgcag                                    29

SEQ ID NO: 167       moltype = DNA  length = 45
FEATURE              Location/Qualifiers
misc_feature         1..45
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..45
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 167
caatctatga catcaattat tatcggagcc ctgccaaaaa atcaa                  45

SEQ ID NO: 168       moltype = DNA  length = 45
FEATURE              Location/Qualifiers
misc_feature         1..45
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..45
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 168
caatctatga catcaattat catcggagcc ctgccaaaaa atcaa                  45

SEQ ID NO: 169       moltype = DNA  length = 42
FEATURE              Location/Qualifiers
misc_feature         1..42
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..42
                     mol_type = other DNA
                     organism = synthetic construct
```

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SEQUENCE: 169
caatctatga catcaattat cggagccctg ccaaaaaatc aa 42

SEQ ID NO: 170 moltype = DNA length = 48
FEATURE Location/Qualifiers
misc_feature 1..48
note = Description of Artificial Sequence: Synthetic oligonucleotide
source 1..48
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 170
caatctatga catcaattat tatcatcgga gccctgccaa aaaatcaa 48

SEQ ID NO: 171 moltype = DNA length = 33
FEATURE Location/Qualifiers
misc_feature 1..33
note = Description of Artificial Sequence: Synthetic oligonucleotide
source 1..33
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 171
caatctatga caagagccct gccaaaaaat caa 33

SEQ ID NO: 172 moltype = DNA length = 52
FEATURE Location/Qualifiers
source 1..52
mol_type = unassigned DNA
organism = Homo sapiens
SEQUENCE: 172
ttctcaaggc agcatcatac ttcccccacg gtgggacagc tgccctccct gg 52

SEQ ID NO: 173 moltype = DNA length = 46
FEATURE Location/Qualifiers
misc_feature 1..46
note = Description of Artificial Sequence: Synthetic oligonucleotide
source 1..46
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 173
ttctcaaggc agcatcatac ttccctggga cagctgccct ccctgg 46

SEQ ID NO: 174 moltype = DNA length = 49
FEATURE Location/Qualifiers
misc_feature 1..49
note = Description of Artificial Sequence: Synthetic oligonucleotide
source 1..49
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 174
ttctcaaggc agcatcatac ttccacggtg ggacagctgc cctccctgg 49

SEQ ID NO: 175 moltype = DNA length = 25
FEATURE Location/Qualifiers
misc_feature 1..25
note = Description of Artificial Sequence: Synthetic oligonucleotide
source 1..25
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 175
ttctcaaggc agctgccctc cctgg 25

SEQ ID NO: 176 moltype = DNA length = 32
FEATURE Location/Qualifiers
misc_feature 1..32
note = Description of Artificial Sequence: Synthetic oligonucleotide
source 1..32
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 176
ttctcaaggc agcatcatac ttccctccct gg 32

SEQ ID NO: 177 moltype = DNA length = 264
FEATURE Location/Qualifiers

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```
misc_difference        38..227
                       note = modified_base - a, c, t, g, unknown or other
source                 1..264
                       mol_type = unassigned DNA
                       organism = Homo sapiens
SEQUENCE: 177
acaaagcgat tttgaaagat ggaagcgcgg tggctatnnn nnnnnnnnnn nnnnnnnnnn  60
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn  120
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn  180
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnggg gtgaaactaa  240
actggtccac acggcggaag attg                                         264

SEQ ID NO: 178         moltype = DNA  length = 257
FEATURE                Location/Qualifiers
misc_feature           1..257
                       note = Description of Artificial Sequence: Synthetic
                        polynucleotide
misc_difference        38..227
                       note = modified_base - a, c, t, g, unknown or other
source                 1..257
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 178
acaaagcgat tttgaaagat ggaagcgcgg tggctatnnn nnnnnnnnnn nnnnnnnnnn  60
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn  120
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn  180
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnggg gtgaaactaa  240
aacacggcgg aagattg                                                  257

SEQ ID NO: 179         moltype = DNA  length = 43
FEATURE                Location/Qualifiers
misc_feature           1..43
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
source                 1..43
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 179
acaaagcgat tttgaaagat ggaagcgaca cggcggaaga ttg                     43

SEQ ID NO: 180         moltype = DNA  length = 44
FEATURE                Location/Qualifiers
misc_feature           1..44
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
source                 1..44
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 180
acaaagcgat tttgaaagat ggaagcgcac acggcggaag attg                    44

SEQ ID NO: 181         moltype = DNA  length = 106
FEATURE                Location/Qualifiers
misc_feature           1..106
                       note = Description of Artificial Sequence: Synthetic
                        polynucleotide
source                 1..106
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 181
acaaagcgat tttgaaagat ggaagcgaaa tagcaagtta aaataaggct agtccgttat  60
caacttgaaa aagtggcacc gagtcggtgc acacggcgga agattg                  106

SEQ ID NO: 182         moltype = DNA  length = 23
FEATURE                Location/Qualifiers
misc_feature           1..23
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
source                 1..23
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 182
gggtgggggg agtttgctcc tgg                                           23

SEQ ID NO: 183         moltype = DNA  length = 23
FEATURE                Location/Qualifiers
misc_feature           1..23
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
```

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source                  1..23
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 183
ggatggaggg agtttgctcc tgg                                          23

SEQ ID NO: 184          moltype = DNA  length = 23
FEATURE                 Location/Qualifiers
misc_feature            1..23
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..23
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 184
ggggagggga agtttgctcc tgg                                          23

SEQ ID NO: 185          moltype = DNA  length = 23
FEATURE                 Location/Qualifiers
misc_feature            1..23
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..23
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 185
gacccctcc accccgcctc cgg                                           23

SEQ ID NO: 186          moltype = DNA  length = 23
FEATURE                 Location/Qualifiers
misc_feature            1..23
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..23
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 186
gaccccccc accccgcccc cgg                                           23

SEQ ID NO: 187          moltype = DNA  length = 23
FEATURE                 Location/Qualifiers
misc_feature            1..23
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..23
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 187
gcccccaccc accccgcctc tgg                                          23

SEQ ID NO: 188          moltype = DNA  length = 23
FEATURE                 Location/Qualifiers
misc_feature            1..23
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..23
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 188
ctccccaccc accccgcctc agg                                          23

SEQ ID NO: 189          moltype = DNA  length = 23
FEATURE                 Location/Qualifiers
misc_feature            1..23
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..23
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 189
ggtgagtgag tgtgtgcgtg tgg                                          23

SEQ ID NO: 190          moltype = DNA  length = 23
FEATURE                 Location/Qualifiers
misc_feature            1..23
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..23
                        mol_type = other DNA
```

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                     organism = synthetic construct
SEQUENCE: 190
tgtgggtgag tgtgtgcgtg agg                                          23

SEQ ID NO: 191       moltype = DNA  length = 23
FEATURE              Location/Qualifiers
misc_feature         1..23
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..23
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 191
gagtccgagc agaagaagaa ggg                                          23

SEQ ID NO: 192       moltype = DNA  length = 23
FEATURE              Location/Qualifiers
misc_feature         1..23
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..23
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 192
gagttagagc agaagaagaa agg                                          23

SEQ ID NO: 193       moltype = DNA  length = 23
FEATURE              Location/Qualifiers
misc_feature         1..23
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..23
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 193
gaacctgagc tgctctgacg cgg                                          23

SEQ ID NO: 194       moltype = DNA  length = 23
FEATURE              Location/Qualifiers
misc_feature         1..23
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..23
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 194
ttggcagggg gtgggaggga agg                                          23

SEQ ID NO: 195       moltype = DNA  length = 23
FEATURE              Location/Qualifiers
misc_feature         1..23
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..23
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 195
gggagggaga gcttggcagg ggg                                          23

SEQ ID NO: 196       moltype = DNA  length = 23
FEATURE              Location/Qualifiers
misc_feature         1..23
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..23
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 196
gatggagcca gagaggatcc tgg                                          23

SEQ ID NO: 197       moltype = DNA  length = 23
FEATURE              Location/Qualifiers
misc_feature         1..23
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..23
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 197
```

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cctgccaagc tctccctccc agg                                          23

SEQ ID NO: 198         moltype = DNA  length = 23
FEATURE                Location/Qualifiers
misc_feature           1..23
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
source                 1..23
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 198
ctccctccca ggatcctctc tgg                                          23

SEQ ID NO: 199         moltype = DNA  length = 23
FEATURE                Location/Qualifiers
misc_feature           1..23
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
source                 1..23
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 199
cctctaaggt ttgcttacga tgg                                          23

SEQ ID NO: 200         moltype = DNA  length = 23
FEATURE                Location/Qualifiers
misc_feature           1..23
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
source                 1..23
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 200
ggttctggca aggagagaga tgg                                          23

SEQ ID NO: 201         moltype = DNA  length = 23
FEATURE                Location/Qualifiers
misc_feature           1..23
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
source                 1..23
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 201
tctaaccccc acctcctgtt agg                                          23

SEQ ID NO: 202         moltype = DNA  length = 23
FEATURE                Location/Qualifiers
misc_feature           1..23
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
source                 1..23
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 202
ttggcagggg gtgggaggga tgg                                          23

SEQ ID NO: 203         moltype = DNA  length = 23
FEATURE                Location/Qualifiers
misc_feature           1..23
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
source                 1..23
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 203
ttggtagggg gtgggaggga tgg                                          23

SEQ ID NO: 204         moltype = DNA  length = 23
FEATURE                Location/Qualifiers
misc_feature           1..23
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
source                 1..23
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 204
gggaagggga gcttggcagg tgg                                          23
```

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SEQ ID NO: 205          moltype = DNA  length = 23
FEATURE                 Location/Qualifiers
misc_feature            1..23
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..23
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 205
ggtagtgaga gcttggcagg tgg                                          23

SEQ ID NO: 206          moltype = DNA  length = 23
FEATURE                 Location/Qualifiers
misc_feature            1..23
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..23
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 206
ctccctccca ggatcctccc agg                                          23

SEQ ID NO: 207          moltype = DNA  length = 23
FEATURE                 Location/Qualifiers
misc_feature            1..23
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..23
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 207
gtccatggca ggatcctctc agg                                          23

SEQ ID NO: 208          moltype = DNA  length = 23
FEATURE                 Location/Qualifiers
misc_feature            1..23
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..23
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 208
ctccccccca gtatcctctc agg                                          23

SEQ ID NO: 209          moltype = DNA  length = 108
FEATURE                 Location/Qualifiers
source                  1..108
                        mol_type = unassigned DNA
                        organism = Homo sapiens
SEQUENCE: 209
ccaatctatg acatcaatta ttatacatcg gagccctgcc aaaaaatcaa tgtgaagcaa  60
atcgcagccc gcctcctgcc tccgctctac tcactggtgt tcatcttt               108

SEQ ID NO: 210          moltype = DNA  length = 138
FEATURE                 Location/Qualifiers
source                  1..138
                        mol_type = unassigned DNA
                        organism = Homo sapiens
SEQUENCE: 210
ccaccctata attctgaacc tgcagaagaa tctgaacata aaaacaacaa ttacgaacca  60
aacctattta aaactccaca aaggaaacca tcttataatc agctggcttc aactccaata  120
atattcaaag agcaaggg                                                138

SEQ ID NO: 211          moltype = DNA  length = 49
FEATURE                 Location/Qualifiers
source                  1..49
                        mol_type = unassigned DNA
                        organism = Homo sapiens
SEQUENCE: 211
ggccgggaat caagagtcac ccagagacag tgaccaacca tccctgttt               49

SEQ ID NO: 212          moltype = DNA  length = 77
FEATURE                 Location/Qualifiers
misc_feature            1..77
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..77
                        mol_type = other DNA
                        organism = synthetic construct
```

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SEQUENCE: 212
ggccgggaat caagagtcac ccagtgacca accatccctg taagcaaacc ttagaggttc  60
tggcaaggag agagatg                                                 77

SEQ ID NO: 213         moltype = DNA  length = 27
FEATURE                Location/Qualifiers
misc_feature           1..27
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
source                 1..27
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 213
ggccgggaat caagagtcac ccaggaa                                      27

SEQ ID NO: 214         moltype = DNA  length = 79
FEATURE                Location/Qualifiers
misc_feature           1..79
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
source                 1..79
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 214
ggccgggaat caagagtcac ccagacctct ctggctccat cgtaagcaaa ccttagaggt  60
tctggcaagg agagagatg                                               79

SEQ ID NO: 215         moltype = DNA  length = 28
FEATURE                Location/Qualifiers
misc_feature           1..28
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
source                 1..28
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 215
ggccgggaat caagagtcac cctaacag                                     28

SEQ ID NO: 216         moltype = DNA  length = 66
FEATURE                Location/Qualifiers
misc_feature           1..66
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
source                 1..66
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 216
ggccgggaat caagacgctg gctccatcgt aagcaaacct tagaggttct ggcaaggaga  60
gagatg                                                             66

SEQ ID NO: 217         moltype = DNA  length = 47
FEATURE                Location/Qualifiers
misc_feature           1..47
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
source                 1..47
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 217
ggctccatcg taagcaaacc ttagaggttc tggcaaggag agagatg                47

SEQ ID NO: 218         moltype = DNA  length = 78
FEATURE                Location/Qualifiers
misc_feature           1..78
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
source                 1..78
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 218
ggccgggaat caagagtcac ccagactctc tggctccatc gtaagcaaac cttagaggtt  60
ctggcaagga gagagatg                                                78

SEQ ID NO: 219         moltype = DNA  length = 80
FEATURE                Location/Qualifiers
misc_feature           1..80
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
source                 1..80
```

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                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 219
ggccgggaat caagagtcac ccagagacag tgaccaacca tcgtaagcaa accttagagg  60
ttctggcaag gagagagatg                                              80

SEQ ID NO: 220       moltype = DNA  length = 46
FEATURE              Location/Qualifiers
misc_feature         1..46
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..46
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 220
ggtccatcgt aagcaaacct tagaggttct ggcaaggaga gagatg                  46

SEQ ID NO: 221       moltype = DNA  length = 69
FEATURE              Location/Qualifiers
misc_feature         1..69
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..69
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 221
ggccgggaat caagagtcac ccatctccat cgtaagcaaa ccttagaggt tctggcaagg  60
agagagatg                                                          69

SEQ ID NO: 222       moltype = DNA  length = 76
FEATURE              Location/Qualifiers
misc_feature         1..76
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..76
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 222
ggccgggaat caagagtcac ccagactctg gctccatcgt aagcaaacct tagaggttct  60
ggcaaggaga gagatg                                                  76

SEQ ID NO: 223       moltype = DNA  length = 50
FEATURE              Location/Qualifiers
misc_feature         1..50
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..50
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 223
ggccgggaat caagagtcac ccagagacag tgaccaacca tcccatatca              50

SEQ ID NO: 224       moltype = DNA  length = 59
FEATURE              Location/Qualifiers
misc_feature         1..59
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..59
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 224
ggccgggaat caagagtcat cgtaagcaaa ccttagaggt tctggcaagg agagagatg   59

SEQ ID NO: 225       moltype = DNA  length = 24
FEATURE              Location/Qualifiers
misc_feature         1..24
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..24
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 225
aatgaccact acatccttca aggg                                         24

SEQ ID NO: 226       moltype = DNA  length = 25
FEATURE              Location/Qualifiers
misc_feature         1..25
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
```

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source                  1..25
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 226
aatgaccact acatcctttc aaggg                                          25

SEQ ID NO: 227          moltype = DNA  length = 26
FEATURE                 Location/Qualifiers
misc_feature            1..26
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..26
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 227
aatgaccact acatcctttt caaggg                                         26

SEQ ID NO: 228          moltype = DNA  length = 22
FEATURE                 Location/Qualifiers
misc_feature            1..22
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..22
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 228
aatgaccact acatcctaag gg                                             22

SEQ ID NO: 229          moltype = DNA  length = 21
FEATURE                 Location/Qualifiers
misc_feature            1..21
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..21
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 229
aatgaccact acatcctagg g                                              21

SEQ ID NO: 230          moltype = DNA  length = 20
FEATURE                 Location/Qualifiers
misc_feature            1..20
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..20
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 230
aatgaccact acatcctggg                                                20

SEQ ID NO: 231          moltype = DNA  length = 42
FEATURE                 Location/Qualifiers
source                  1..42
                        mol_type = unassigned DNA
                        organism = Homo sapiens
SEQUENCE: 231
tatgtgcaat gaccactaca tcctcaaggg cagcaatcgg ag                       42

SEQ ID NO: 232          moltype = DNA  length = 45
FEATURE                 Location/Qualifiers
misc_feature            1..45
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..45
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 232
tatgtgcaat gaccactaca tcctcctcaa gggcagcaat cggag                    45

SEQ ID NO: 233          moltype = DNA  length = 30
FEATURE                 Location/Qualifiers
misc_feature            1..30
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..30
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 233
tatgtgcaat gaccactaca tcaatcggag                                     30
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SEQ ID NO: 234 moltype = DNA length = 33
FEATURE Location/Qualifiers
misc_feature 1..33
note = Description of Artificial Sequence: Synthetic oligonucleotide
source 1..33
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 234
tatgtgcaat gaccactaca tcagcaatcg gag 33

SEQ ID NO: 235 moltype = DNA length = 34
FEATURE Location/Qualifiers
misc_feature 1..34
note = Description of Artificial Sequence: Synthetic oligonucleotide
source 1..34
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 235
tatgtgcaat gaccactaca tccagcaatc ggag 34

SEQ ID NO: 236 moltype = DNA length = 10
FEATURE Location/Qualifiers
misc_feature 1..10
note = Description of Artificial Sequence: Synthetic oligonucleotide
source 1..10
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 236
cagcaatcgg 10

SEQ ID NO: 237 moltype = DNA length = 41
FEATURE Location/Qualifiers
misc_feature 1..41
note = Description of Artificial Sequence: Synthetic oligonucleotide
source 1..41
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 237
tatgtgcaat gaccactaca tccttcaagg gcagcaatcg g 41

SEQ ID NO: 238 moltype = DNA length = 41
FEATURE Location/Qualifiers
misc_feature 1..41
note = Description of Artificial Sequence: Synthetic oligonucleotide
source 1..41
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 238
tatgtgcaat gaccactaca tcctccaagg gcagcaatcg g 41

SEQ ID NO: 239 moltype = DNA length = 41
FEATURE Location/Qualifiers
misc_feature 1..41
note = Description of Artificial Sequence: Synthetic oligonucleotide
source 1..41
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 239
tatgtgcaat gaccactaca tcctbcaagg gcagcaatcg g 41

SEQ ID NO: 240 moltype = DNA length = 34
FEATURE Location/Qualifiers
misc_feature 1..34
note = Description of Artificial Sequence: Synthetic oligonucleotide
source 1..34
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 240
tatgtgcaat gaccactaca ttggcagcaa tcgg 34

SEQ ID NO: 241 moltype = DNA length = 21

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FEATURE                 Location/Qualifiers
misc_feature            1..21
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..21
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 241
tatgtgcaat gaccactaca t                                              21

SEQ ID NO: 242          moltype = DNA  length = 53
FEATURE                 Location/Qualifiers
source                  1..53
                        mol_type = unassigned DNA
                        organism = Homo sapiens
SEQUENCE: 242
tcatacagat gatgtctcat catcagagga gcgagaaggt aaagtcaaaa tca           53

SEQ ID NO: 243          moltype = DNA  length = 32
FEATURE                 Location/Qualifiers
misc_feature            1..32
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..32
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 243
tcatacagat gatacaggta aagtcaaaat ca                                  32

SEQ ID NO: 244          moltype = DNA  length = 32
FEATURE                 Location/Qualifiers
misc_feature            1..32
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..32
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 244
tcatacaggt gatgaaggta aagtcaaaat ca                                  32

SEQ ID NO: 245          moltype = DNA  length = 50
FEATURE                 Location/Qualifiers
misc_feature            1..50
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..50
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 245
tcatacagat gatgtctcat catcagagcg agaaggtaaa gtcaaaatca               50

SEQ ID NO: 246          moltype = DNA  length = 48
FEATURE                 Location/Qualifiers
misc_feature            1..48
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..48
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 246
tcatacagat gatgtctcat catcagcgag aaggtaaagt caaaatca                 48

SEQ ID NO: 247          moltype = DNA  length = 52
FEATURE                 Location/Qualifiers
misc_feature            1..52
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..52
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 247
tcatacagat gatgtctcat catcagggag cgagaaggta aagtcaaaat ca            52

SEQ ID NO: 248          moltype = DNA  length = 41
FEATURE                 Location/Qualifiers
misc_feature            1..41
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..41
```

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                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 248
tcatacagat gatgtctcgc gagaaggtaa agtcaaaatc a                          41

SEQ ID NO: 249       moltype = DNA  length = 32
FEATURE              Location/Qualifiers
misc_feature         1..32
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..32
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 249
tcatacagat gatgaaggta aagtcaaaat ca                                    32

SEQ ID NO: 250       moltype = DNA  length = 29
FEATURE              Location/Qualifiers
misc_feature         1..29
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 250
tcatacagat gaaggtaaag tcaaaatca                                        29

SEQ ID NO: 251       moltype = DNA  length = 42
FEATURE              Location/Qualifiers
misc_feature         1..42
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..42
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 251
tcatacagat gatgtctaca gatgaaggta aagtcaaaat ca                         42

SEQ ID NO: 252       moltype = DNA  length = 51
FEATURE              Location/Qualifiers
misc_feature         1..51
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..51
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 252
tcatacagat gatgtctcat catcaggagc gagaaggtaa agtcaaaatc a               51

SEQ ID NO: 253       moltype = DNA  length = 34
FEATURE              Location/Qualifiers
misc_feature         1..34
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..34
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 253
gtcatcctca tcctgataaa ctgcaaaagg ctga                                  34

SEQ ID NO: 254       moltype = DNA  length = 606
FEATURE              Location/Qualifiers
source               1..606
                     mol_type = unassigned DNA
                     organism = Homo sapiens
SEQUENCE: 254
gctggtgtct gggttctgtg ccccttcccc accccagccc accccaggtg tcctgtccat  60
tctcaggctg gtcacatggg tggtcctagg gtgtcccatg agagatgcaa agcgcctgaa  120
ttttctgact cttcccatca gaccccccaa agacacatgt gacccaccac cccatctctg  180
accatgaggc caccctgagg tgctgggccc tgggcttcta ccctgcggag atcacactga  240
cctggcagcg ggatggcgag gaccaaactc aggacaccga gcttgtggag accagaccag  300
caggagatag aaccttccag aagtgggcag ctgtggtggt gccttctgga gaagagcaga  360
gatacacatg ccatgtacag catgaggggc tgccgaagcc cctcaccctg agatggggta  420
aggaggggga tgaggggtca tatctgttca tatctgttct cagggaaagc aggagccctt  480
ctggagccct tcagcagggt cagggcccct catcttcccc tcctttccca gagccatctt  540
cccagtccac catccccatc gtgggcattg ttgctggcct ggctgtccta gcagttgtgg  600
tcatcg                                                             606

SEQ ID NO: 255       moltype = DNA  length = 26
```

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```
FEATURE               Location/Qualifiers
source                1..26
                      mol_type = unassigned DNA
                      organism = Homo sapiens
SEQUENCE: 255
actaccacag ctccttctct gagtgg                                        26

SEQ ID NO: 256        moltype = DNA  length = 23
FEATURE               Location/Qualifiers
misc_feature          1..23
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
source                1..23
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 256
actaccacag ctcctctgag tgg                                           23

SEQ ID NO: 257        moltype = DNA  length = 23
FEATURE               Location/Qualifiers
source                1..23
                      mol_type = unassigned DNA
                      organism = Homo sapiens
SEQUENCE: 257
gtagttggag ctggcggcgt agg                                           23

SEQ ID NO: 258        moltype = DNA  length = 23
FEATURE               Location/Qualifiers
misc_feature          1..23
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
source                1..23
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 258
gtagttggag ctagcggcgt agg                                           23

SEQ ID NO: 259        moltype = DNA  length = 23
FEATURE               Location/Qualifiers
source                1..23
                      mol_type = unassigned DNA
                      organism = Homo sapiens
SEQUENCE: 259
gtagttggag ctggtggcgt agg                                           23

SEQ ID NO: 260        moltype = DNA  length = 23
FEATURE               Location/Qualifiers
misc_feature          1..23
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
source                1..23
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 260
gtagttggag ctagtggcgt agg                                           23

SEQ ID NO: 261        moltype = DNA  length = 28
FEATURE               Location/Qualifiers
misc_feature          1..28
                      note = Description of Artificial Sequence: Synthetic
                       oligonucleotide
source                1..28
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 261
ccatacatta aagatagtca tcttgggg                                      28

SEQ ID NO: 262        moltype = DNA  length = 60
FEATURE               Location/Qualifiers
source                1..60
                      mol_type = unassigned DNA
                      organism = Homo sapiens
SEQUENCE: 262
ccatacagtc agtatcaatt ctggaagaat ttccagacat taaagatagt catcttgggg  60

SEQ ID NO: 263        moltype = DNA  length = 55
FEATURE               Location/Qualifiers
source                1..55
                      mol_type = unassigned DNA
```

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                     organism = Homo sapiens
SEQUENCE: 263
ccatacagtc agtatcaatt ctggaagaat ttccagacat taaagatagt catct       55

SEQ ID NO: 264         moltype = DNA  length = 23
FEATURE                Location/Qualifiers
misc_feature           1..23
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
source                 1..23
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 264
ccatacatta aagatagtca tct                                          23

SEQ ID NO: 265         moltype = DNA  length = 23
FEATURE                Location/Qualifiers
source                 1..23
                       mol_type = unassigned DNA
                       organism = Homo sapiens
SEQUENCE: 265
agatgactat ctttaatgtc tgg                                          23

SEQ ID NO: 266         moltype = DNA  length = 23
FEATURE                Location/Qualifiers
source                 1..23
                       mol_type = unassigned DNA
                       organism = Homo sapiens
SEQUENCE: 266
agatgactat ctttaatgta tgg                                          23

SEQ ID NO: 267         moltype = DNA  length = 23
FEATURE                Location/Qualifiers
misc_feature           1..23
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
source                 1..23
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 267
gtagttggag ctgatggcgt agg                                          23

SEQ ID NO: 268         moltype = DNA  length = 23
FEATURE                Location/Qualifiers
misc_feature           1..23
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
source                 1..23
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 268
gtagttggag ctggtagcgt agg                                          23

SEQ ID NO: 269         moltype = DNA  length = 23
FEATURE                Location/Qualifiers
misc_feature           1..23
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
source                 1..23
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 269
gtagttggag ctggtgacgt agg                                          23

SEQ ID NO: 270         moltype = DNA  length = 23
FEATURE                Location/Qualifiers
misc_feature           1..23
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
source                 1..23
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 270
gtagttggag ctaatggcgt agg                                          23

SEQ ID NO: 271         moltype = DNA  length = 23
FEATURE                Location/Qualifiers
misc_feature           1..23
                       note = Description of Artificial Sequence: Synthetic
```

-continued

```
                     oligonucleotide
source               1..23
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 271
gtagttggag ctagtagcgt agg                                          23

SEQ ID NO: 272       moltype = DNA  length = 23
FEATURE              Location/Qualifiers
misc_feature         1..23
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..23
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 272
gtagttggag ctagtgacgt agg                                          23

SEQ ID NO: 273       moltype = DNA  length = 23
FEATURE              Location/Qualifiers
source               1..23
                     mol_type = unassigned DNA
                     organism = Homo sapiens
SEQUENCE: 273
caaatgaatg atgcacatca tgg                                          23

SEQ ID NO: 274       moltype = DNA  length = 23
FEATURE              Location/Qualifiers
misc_feature         1..23
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..23
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 274
caaatgaatg atgcacgtca tgg                                          23

SEQ ID NO: 275       moltype = DNA  length = 23
FEATURE              Location/Qualifiers
misc_feature         1..23
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..23
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 275
caaatgaatg atgcatatca tgg                                          23

SEQ ID NO: 276       moltype = DNA  length = 23
FEATURE              Location/Qualifiers
misc_feature         1..23
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..23
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 276
caaatgaatg atgcgcatca tgg                                          23

SEQ ID NO: 277       moltype = DNA  length = 23
FEATURE              Location/Qualifiers
misc_feature         1..23
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..23
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 277
caaatgaatg atgtacatca tgg                                          23

SEQ ID NO: 278       moltype = DNA  length = 23
FEATURE              Location/Qualifiers
misc_feature         1..23
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..23
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 278
```

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caaatgaatg gtgcacatca tgg 23

SEQ ID NO: 279 moltype = DNA length = 23
FEATURE Location/Qualifiers
misc_feature 1..23
note = Description of Artificial Sequence: Synthetic oligonucleotide
source 1..23
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 279
caaatgagtg atgcacatca tgg 23

SEQ ID NO: 280 moltype = DNA length = 23
FEATURE Location/Qualifiers
misc_feature 1..23
note = Description of Artificial Sequence: Synthetic oligonucleotide
source 1..23
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 280
caaaagaatg atgcacatca tgg 23

SEQ ID NO: 281 moltype = DNA length = 23
FEATURE Location/Qualifiers
misc_feature 1..23
note = Description of Artificial Sequence: Synthetic oligonucleotide
source 1..23
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 281
cgaatgaatg atgcacatca tgg 23

SEQ ID NO: 282 moltype = DNA length = 23
FEATURE Location/Qualifiers
misc_feature 1..23
note = Description of Artificial Sequence: Synthetic oligonucleotide
source 1..23
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 282
caaatgaatg atgcatgtca tgg 23

SEQ ID NO: 283 moltype = DNA length = 23
FEATURE Location/Qualifiers
misc_feature 1..23
note = Description of Artificial Sequence: Synthetic oligonucleotide
source 1..23
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 283
caaatgaatg atgcgcgtca tgg 23

SEQ ID NO: 284 moltype = DNA length = 23
FEATURE Location/Qualifiers
misc_feature 1..23
note = Description of Artificial Sequence: Synthetic oligonucleotide
source 1..23
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 284
caaatgaatg atgtacgtca tgg 23

SEQ ID NO: 285 moltype = DNA length = 23
FEATURE Location/Qualifiers
misc_feature 1..23
note = Description of Artificial Sequence: Synthetic oligonucleotide
source 1..23
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 285
caaatgaatg gtgcacgtca tgg 23

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```
SEQ ID NO: 286          moltype = DNA  length = 23
FEATURE                 Location/Qualifiers
misc_feature            1..23
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..23
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 286
caaatgagtg atgcacgtca tgg                                          23

SEQ ID NO: 287          moltype = DNA  length = 23
FEATURE                 Location/Qualifiers
misc_feature            1..23
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..23
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 287
caaaagaatg atgcacgtca tgg                                          23

SEQ ID NO: 288          moltype = DNA  length = 23
FEATURE                 Location/Qualifiers
misc_feature            1..23
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..23
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 288
cgaatgaatg atgcacgtca tgg                                          23

SEQ ID NO: 289          moltype = DNA  length = 23
FEATURE                 Location/Qualifiers
source                  1..23
                        mol_type = unassigned DNA
                        organism = Homo sapiens
SEQUENCE: 289
atcataggtc gtcatgctta tgg                                          23

SEQ ID NO: 290          moltype = DNA  length = 23
FEATURE                 Location/Qualifiers
misc_feature            1..23
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..23
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 290
atcataggtt gtcatgctta tgg                                          23

SEQ ID NO: 291          moltype = DNA  length = 23
FEATURE                 Location/Qualifiers
source                  1..23
                        mol_type = unassigned DNA
                        organism = Homo sapiens
SEQUENCE: 291
atcataggtc gtcctgctta tgg                                          23

SEQ ID NO: 292          moltype = DNA  length = 23
FEATURE                 Location/Qualifiers
misc_feature            1..23
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..23
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 292
atcataggtt gtcctgctta tgg                                          23

SEQ ID NO: 293          moltype = DNA  length = 23
FEATURE                 Location/Qualifiers
source                  1..23
                        mol_type = unassigned DNA
                        organism = Homo sapiens
SEQUENCE: 293
ctggacaaga agagtacagt gcc                                          23
```

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```
SEQ ID NO: 294          moltype = DNA  length = 23
FEATURE                 Location/Qualifiers
misc_feature            1..23
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..23
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 294
ctggaaaaga agagtacagt gcc                                          23

SEQ ID NO: 295          moltype = DNA  length = 23
FEATURE                 Location/Qualifiers
source                  1..23
                        mol_type = unassigned DNA
                        organism = Homo sapiens
SEQUENCE: 295
actccatcga gatttcactg tag                                          23

SEQ ID NO: 296          moltype = DNA  length = 23
FEATURE                 Location/Qualifiers
misc_feature            1..23
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..23
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 296
actccatcga gatttctctg tag                                          23

SEQ ID NO: 297          moltype = DNA  length = 34
FEATURE                 Location/Qualifiers
misc_feature            1..34
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..34
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 297
acttccaggc aaccccaagg ggacctcatg cagg                              34

SEQ ID NO: 298          moltype = DNA  length = 35
FEATURE                 Location/Qualifiers
misc_feature            1..35
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..35
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 298
acttccaggc gaaccccaag gggacctcat gcagg                             35

SEQ ID NO: 299          moltype = DNA  length = 13
FEATURE                 Location/Qualifiers
misc_feature            1..13
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..13
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 299
acttccaggc tcc                                                     13

SEQ ID NO: 300          moltype = DNA  length = 13
FEATURE                 Location/Qualifiers
misc_feature            1..13
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..13
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 300
acttccaggc tcc                                                     13

SEQ ID NO: 301          moltype = DNA  length = 32
FEATURE                 Location/Qualifiers
misc_feature            1..32
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
```

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source 1..32
mol_type = other DNA
organism = synthetic construct

SEQUENCE: 301
acttccaggc tcccaagggg acctcatgca gg 32

SEQ ID NO: 302 moltype = DNA length = 41
FEATURE Location/Qualifiers
misc_feature 1..41
note = Description of Artificial Sequence: Synthetic oligonucleotide
source 1..41
mol_type = other DNA
organism = synthetic construct

SEQUENCE: 302
acttccaggc tccttaggag gcgaacccca aggggacctc a 41

SEQ ID NO: 303 moltype = DNA length = 25
FEATURE Location/Qualifiers
misc_feature 1..25
note = Description of Artificial Sequence: Synthetic oligonucleotide
source 1..25
mol_type = other DNA
organism = synthetic construct

SEQUENCE: 303
acttccaggc tccacctcat gcagg 25

SEQ ID NO: 304 moltype = DNA length = 32
FEATURE Location/Qualifiers
misc_feature 1..32
note = Description of Artificial Sequence: Synthetic oligonucleotide
source 1..32
mol_type = other DNA
organism = synthetic construct

SEQUENCE: 304
acttccaggc tccaccccca agggacctca tg 32

SEQ ID NO: 305 moltype = DNA length = 35
FEATURE Location/Qualifiers
misc_feature 1..35
note = Description of Artificial Sequence: Synthetic oligonucleotide
source 1..35
mol_type = other DNA
organism = synthetic construct

SEQUENCE: 305
acttccaggc tccacccaag gggacctcat gcagg 35

SEQ ID NO: 306 moltype = DNA length = 36
FEATURE Location/Qualifiers
misc_feature 1..36
note = Description of Artificial Sequence: Synthetic oligonucleotide
source 1..36
mol_type = other DNA
organism = synthetic construct

SEQUENCE: 306
acttccaggc tccaccccaa ggggacctca tgcagg 36

SEQ ID NO: 307 moltype = DNA length = 38
FEATURE Location/Qualifiers
misc_feature 1..38
note = Description of Artificial Sequence: Synthetic oligonucleotide
source 1..38
mol_type = other DNA
organism = synthetic construct

SEQUENCE: 307
acttccaggc tccacccacc caaggggacc tcatgcag 38

SEQ ID NO: 308 moltype = DNA length = 38
FEATURE Location/Qualifiers
misc_feature 1..38
note = Description of Artificial Sequence: Synthetic oligonucleotide
source 1..38
mol_type = other DNA

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                     organism = synthetic construct
SEQUENCE: 308
acttccaggc tccaccccac ccaaggggac ctcatgca                          38

SEQ ID NO: 309       moltype = DNA  length = 40
FEATURE              Location/Qualifiers
misc_feature         1..40
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..40
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 309
acttccaggc tccacccacc ccaaggggac ctcatgcagg                        40

SEQ ID NO: 310       moltype = DNA  length = 45
FEATURE              Location/Qualifiers
misc_feature         1..45
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..45
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 310
acttccaggc tccacccggc gaaccccaag gggacctcat gcagg                  45

SEQ ID NO: 311       moltype = DNA  length = 34
FEATURE              Location/Qualifiers
misc_feature         1..34
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..34
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 311
acttccaggc tccaccctgg ggacctcatg cagg                              34

SEQ ID NO: 312       moltype = DNA  length = 42
FEATURE              Location/Qualifiers
misc_feature         1..42
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..42
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 312
acttccaggc tccacccgaa ccccaagggg acctcatgca gg                     42

SEQ ID NO: 313       moltype = DNA  length = 31
FEATURE              Location/Qualifiers
misc_feature         1..31
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..31
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 313
acttccaggc tccacccgaa cctcatgcag g                                 31

SEQ ID NO: 314       moltype = DNA  length = 35
FEATURE              Location/Qualifiers
misc_feature         1..35
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..35
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 314
acttccaggc tccacccgag gggacctcat gcagg                             35

SEQ ID NO: 315       moltype = DNA  length = 36
FEATURE              Location/Qualifiers
misc_feature         1..36
                     note = Description of Artificial Sequence: Synthetic
                      oligonucleotide
source               1..36
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 315
```

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```
acttccaggc tccaccccaa ggggacctca tgcagg                              36

SEQ ID NO: 316         moltype = DNA  length = 42
FEATURE                Location/Qualifiers
misc_feature           1..42
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
source                 1..42
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 316
acttccaggc tccacccgaa ccccaagggg acctcatgca gg                       42

SEQ ID NO: 317         moltype = DNA  length = 43
FEATURE                Location/Qualifiers
misc_feature           1..43
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
source                 1..43
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 317
acttccaggc tccacccgag accccaaggg gacctcatgc agg                      43

SEQ ID NO: 318         moltype = DNA  length = 48
FEATURE                Location/Qualifiers
misc_feature           1..48
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
source                 1..48
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 318
acttccaggc tccacccgag ggcgaacccc aaggggacct catgcagg                 48

SEQ ID NO: 319         moltype = DNA  length = 30
FEATURE                Location/Qualifiers
misc_feature           1..30
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
source                 1..30
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 319
acttccaggc tccacccgac ctcatgcagg                                     30

SEQ ID NO: 320         moltype = DNA  length = 42
FEATURE                Location/Qualifiers
misc_feature           1..42
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
source                 1..42
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 320
acttccaggc tccacccgac ccccaagggg acctcatgca gg                       42

SEQ ID NO: 321         moltype = DNA  length = 42
FEATURE                Location/Qualifiers
misc_feature           1..42
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
source                 1..42
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 321
acttccaggc tccacccgac gaagggcccc aaggggacct ca                       42

SEQ ID NO: 322         moltype = DNA  length = 45
FEATURE                Location/Qualifiers
misc_feature           1..45
                       note = Description of Artificial Sequence: Synthetic
                        oligonucleotide
source                 1..45
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 322
acttccaggc tccacccgac gaaccccaag gggacctcat gcagg                    45
```

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```
SEQ ID NO: 323          moltype = DNA  length = 48
FEATURE                 Location/Qualifiers
misc_feature            1..48
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..48
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 323
acttccaggc tccacccgac ggcgaacccc aaggggacct catgcagg               48

SEQ ID NO: 324          moltype = DNA  length = 51
FEATURE                 Location/Qualifiers
misc_feature            1..51
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..51
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 324
acttccaggc tccacccgac gtgcttgagg gcgaacccca aggggacctc a           51

SEQ ID NO: 325          moltype = DNA  length = 47
FEATURE                 Location/Qualifiers
misc_feature            1..47
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..47
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 325
acttccaggc tccacccgac tcactatctt ctgggctcct ccatgtc                47

SEQ ID NO: 326          moltype = DNA  length = 49
FEATURE                 Location/Qualifiers
misc_feature            1..49
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..49
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 326
acttccaggc tccacccgac ttggcgaacc ccaaggggac ctcatgcag              49

SEQ ID NO: 327          moltype = DNA  length = 51
FEATURE                 Location/Qualifiers
misc_feature            1..51
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..51
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 327
acttccaggc tccacccgac ttgcagggcg aaccccaagg ggacctcatg c           51

SEQ ID NO: 328          moltype = DNA  length = 53
FEATURE                 Location/Qualifiers
misc_feature            1..53
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..53
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 328
acttccaggc tccacccgac ttggagggcg aaccccaagg ggacctcatg cag         53

SEQ ID NO: 329          moltype = DNA  length = 53
FEATURE                 Location/Qualifiers
misc_feature            1..53
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..53
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 329
acttccaggc tccacccgac tttggagggc gaaccccaag gggacctcat gca         53

SEQ ID NO: 330          moltype = DNA  length = 53
FEATURE                 Location/Qualifiers
```

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```
misc_feature            1..53
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
source                  1..53
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 330
acttccaggc tccacccgac tgttggaggg cgaaccccaa ggggacctca tgc          53

SEQ ID NO: 331          moltype = DNA  length = 502
FEATURE                 Location/Qualifiers
misc_feature            1..502
                        note = Description of Artificial Sequence: Synthetic
                         oligonucleotide
misc_difference         26..480
                        note = misc_feature - a, c, t, g, unknown or other
source                  1..502
                        mol_type = other DNA
                        organism = synthetic construct
SEQUENCE: 331
acttccaggc tccacccgac tggagnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn  60
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn  120
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn  180
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn  240
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn  300
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn  360
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn  420
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn  480
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aggagagaga tg                                                      72
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The invention claimed is:

1. A method for producing a genome-modified non-human animal, the method comprising:

providing a Cas9/RNA complex comprising a Cas9 protein and a guide RNA by providing the Cas9 protein and the guide RNA to a cell-free buffer wherein the guide RNA comprises a CRISPR RNA (crRNA) and a transactivating crRNA (tracrRNA), and wherein the guide RNA is provided in a molar excess over the Cas9 protein in the cell-free buffer;

introducing the Cas9/RNA complex into a non-human embryo, wherein the Cas9/RNA complex induces a modification at a target endogenous nucleic acid of the non-human embryo to provide for a genome modified embryo;

transferring the genome modified embryo into a foster mother; and

allowing the foster mother to produce a F0 animal having the modification at the target endogenous nucleic acid.

2. The method of claim **1**, further comprising:

mating the F0 animal with a wild-type non-human animal or another genome-modified non-human animal of the same species as the F0 animal having the modification at the target endogenous nucleic acid to provide a F1 offspring having the modification at the target endogenous nucleic acid.

3. The method of claim **1**, wherein introducing the Cas9/RNA complex into the non-human embryo comprises introducing the Cas9/RNA complex into a pronucleus of the non-human embryo.

4. The method of claim **1**, wherein introducing the Cas9/RNA complex into the non-human embryo comprises introducing the Cas9/RNA complex into a cytoplasm of the non-human embryo.

5. The method of claim **1**, wherein the Cas9/RNA complex is introduced into the non-human embryo by electroporation.

6. The method of claim **1**, wherein the Cas9/RNA complex is introduced into the non-human embryo by microinjection.

7. The method of claim **1**, wherein the crRNA is fused to the tracrRNA.

8. The method of claim **1**, wherein the crRNA comprises i) a first portion to be hybridized with a portion of the tracrRNA, and ii) a second portion complementary to the target endogenous nucleic acid of the non-human embryo; and

wherein the target endogenous nucleic acid comprises i) a first strand having a 20-base pair region complementary to the second portion of the crRNA and ii) a second strand having a trinucleotide protospacer adjacent motif (PAM).

9. The method of claim **1**, wherein providing the Cas9/RNA complex comprises

preparing the Cas9 protein;

preparing the guide RNA; and

preparing the cell-free buffer.

10. The method of claim **1**, wherein the molar excess is an at least two-fold molar excess.

11. A method for producing a genome-modified non-human animal, the method comprising:

preparing a Cas9 protein;

preparing a single-guide RNA (sgRNA):

preparing a cell-free buffer;

preparing a Cas9/sgRNA complex by providing the Cas9 protein and the sgRNA to the cell-free buffer, wherein the sgRNA is provided in a molar excess over the Cas9 protein; and

introducing the Cas9/sgRNA complex into a non-human embryo, wherein the non-human embryo comprises a target endogenous nucleic acid in the nucleus, and wherein the Cas9/sgRNA complex induces modification of the target endogenous nucleic acid of the non-human embryo to provide for a genome modified embryo.

12. The method of claim **11**, wherein the molar excess is an at least two-fold molar excess.

13. The method of claim **11**, wherein introducing the Cas9/sgRNA complex into the non-human embryo comprises introducing the Cas9/sgRNA complex into a pronucleus of the non-human embryo.

14. The method of claim **11**, wherein introducing the Cas9/sgRNA complex into the non-human embryo comprises introducing the Cas9/sgRNA complex into a cytoplasm of the non-human embryo.

15. The method of claim **11**, wherein the Cas9/sgRNA complex is introduced into the non-human embryo by electroporation.

16. The method of claim **11**, wherein the Cas9/sgRNA complex is introduced into the non-human embryo by microinjection.

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