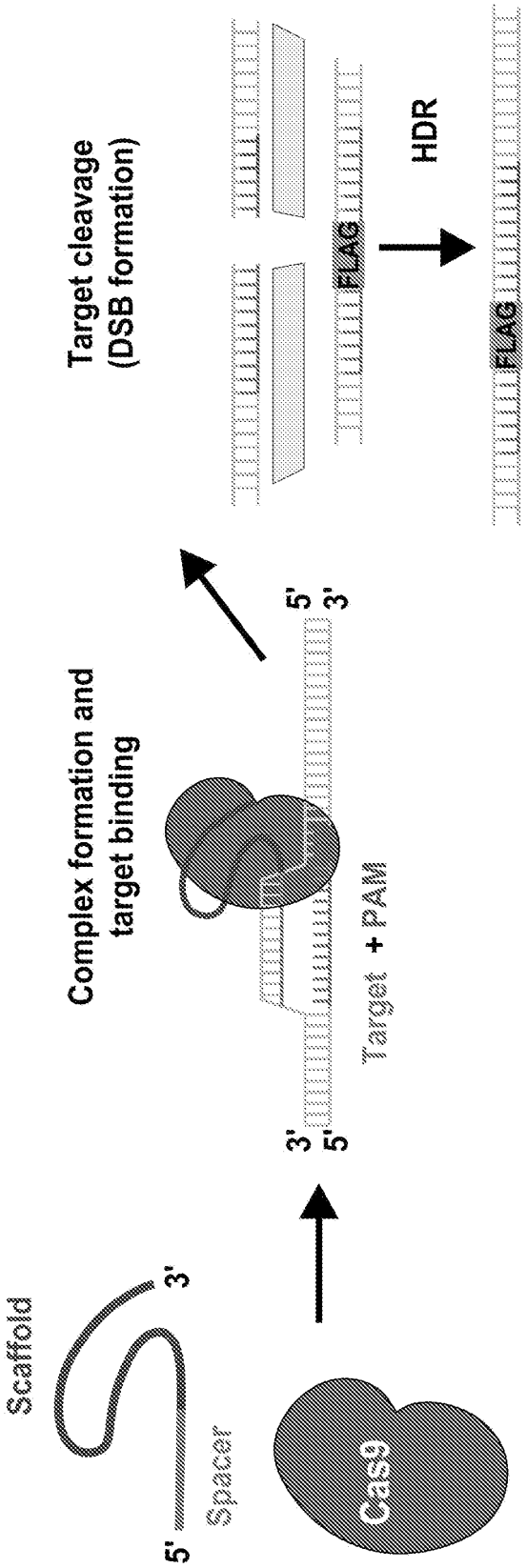
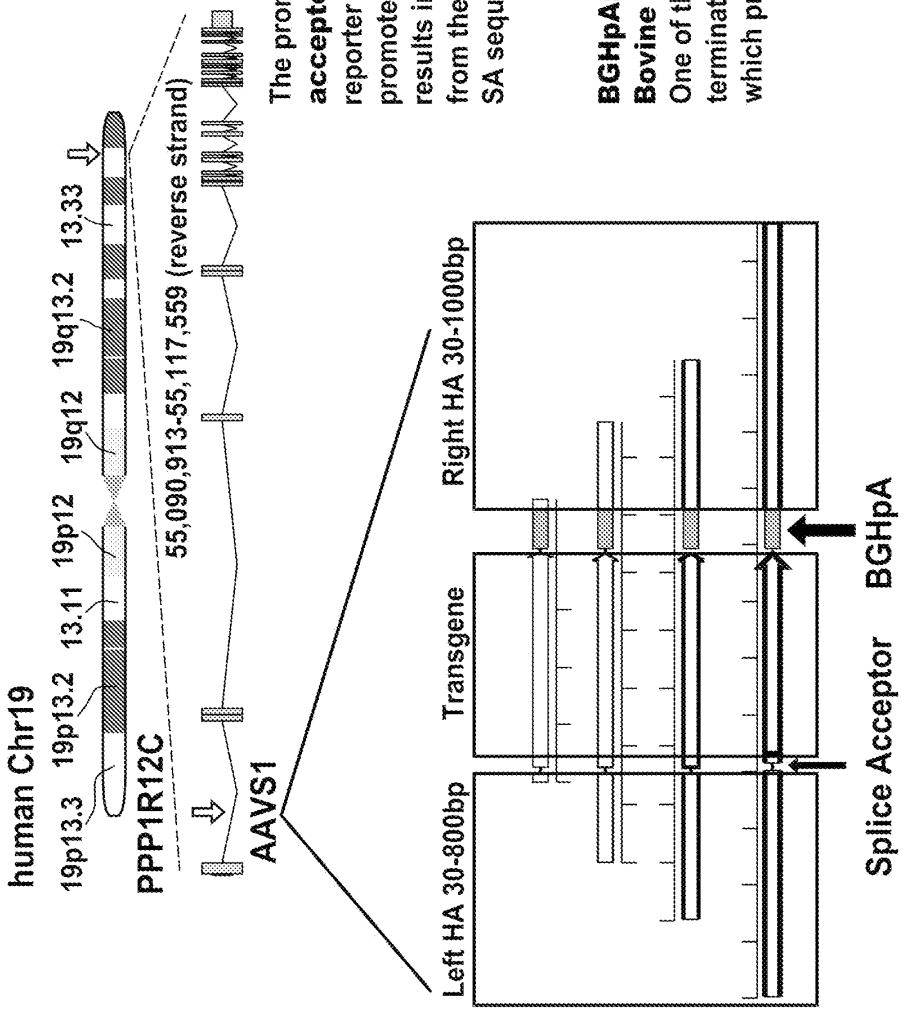

Scaffold
3'
5'
Spacer
Cas9
3'
5'
5'
3'
Target + PAM
Complex formation and target binding
Target cleavage (DSB formation)
FLAG
HDR
FLAG

FIG. 1



The promoterless reporter gene containes **splice acceptor (SA)** sequence. The promotorless reporter gene can be expressed when insertion of a promoter trap vector occurs in an intron and results in a transcriptional fusion due to splicing from the chromosomal splice donor (SD) site to the SA sequence.

BGHpA:
Bovine Growth Hormone Polyadenylation Signal
One of the commonly used mammalian expression terminators include the sequence motif AAUAAA which promotes both polyadenylation and termination.

FIG. 1 CONT.

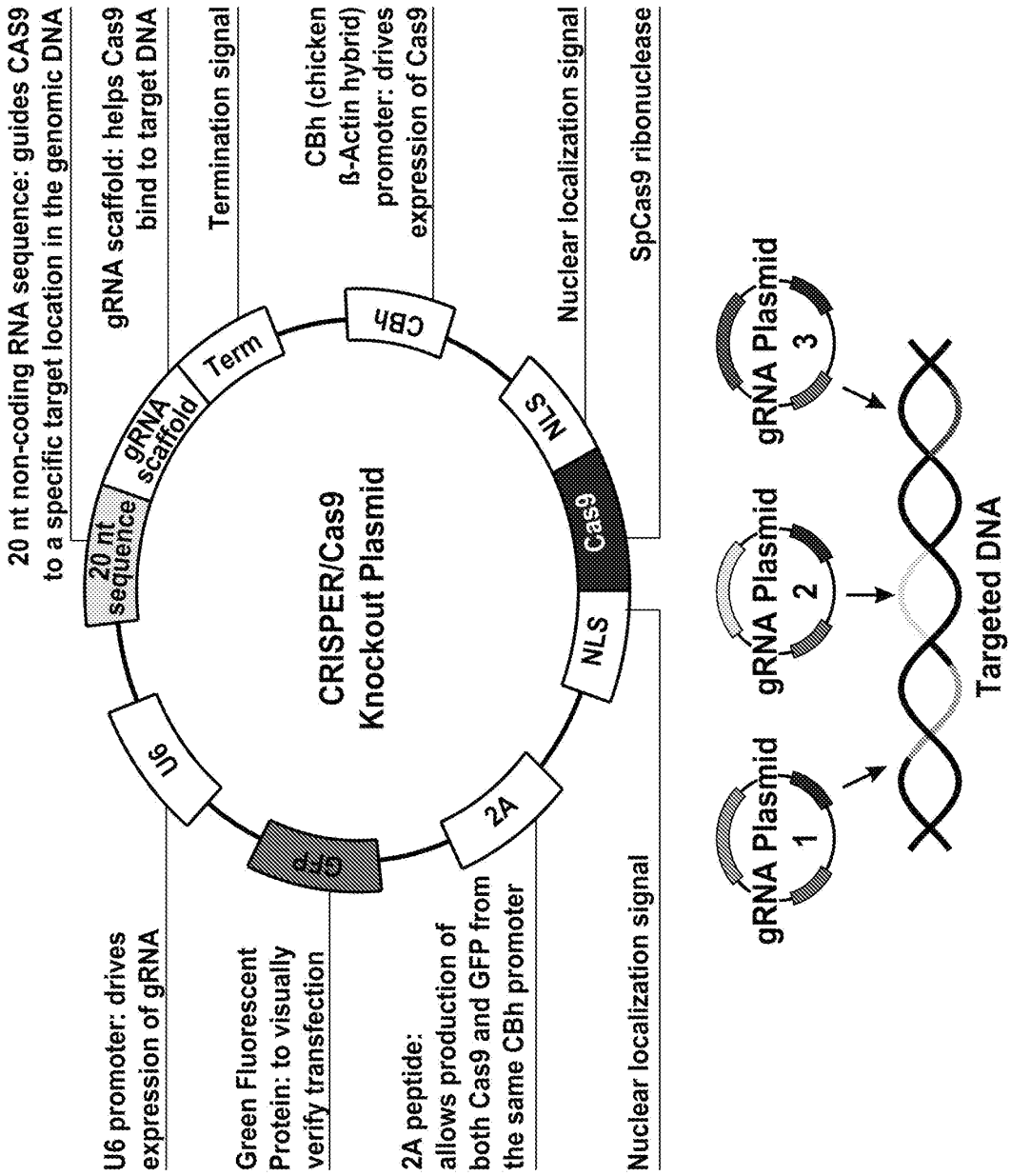

20 nt non-coding RNA sequence: guides CAS9 to a specific target location in the genomic DNA
gRNA scaffold: helps Cas9 bind to target DNA
Termination signal
CBh (chicken ß-Actin hybrid) promoter: drives expression of Cas9
Nuclear localization signal
SpCas9 ribonuclease
20 nt sequence
gRNA scaffold
Term
CBh
NLS
Cas9
NLS
2A
GFP
U6
CRISPER/Cas9 Knockout Plasmid
U6 promoter: drives expression of gRNA
Green Fluorescent Protein: to visually verify transfection
2A peptide: allows production of both Cas9 and GFP from the same CBh promoter
Nuclear localization signal
gRNA Plasmid 1
gRNA Plasmid 2
gRNA Plasmid 3
Targeted DNA

FIG. 2

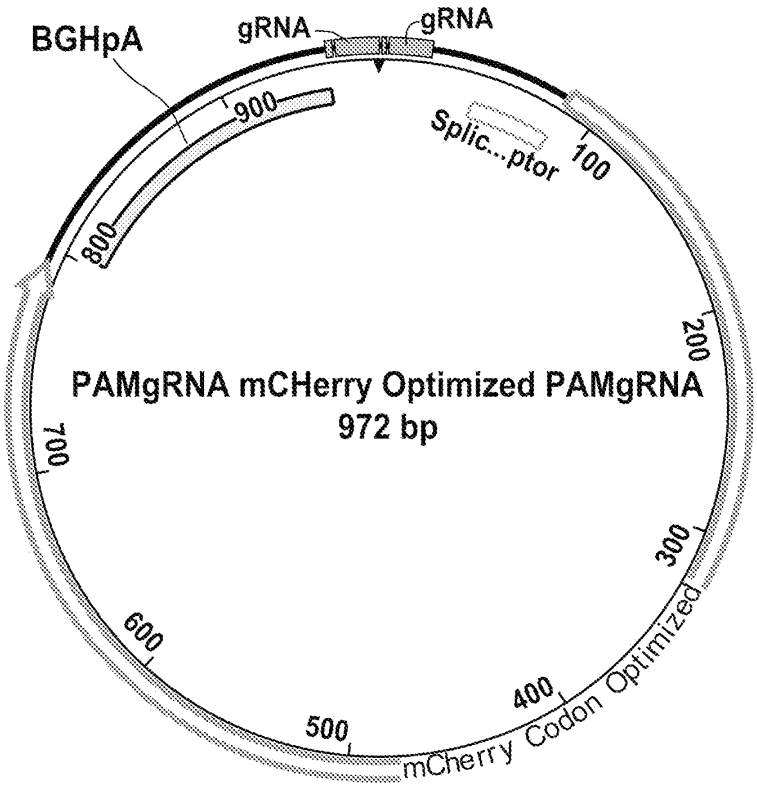

BGHpA
gRNA
gRNA
900
Splic...ptor
100
800
200
PAMgRNA mCHerry Optimized PAMgRNA
972 bp
700
300
600
400
500
mCherry Codon Optimized

FIG. 3A

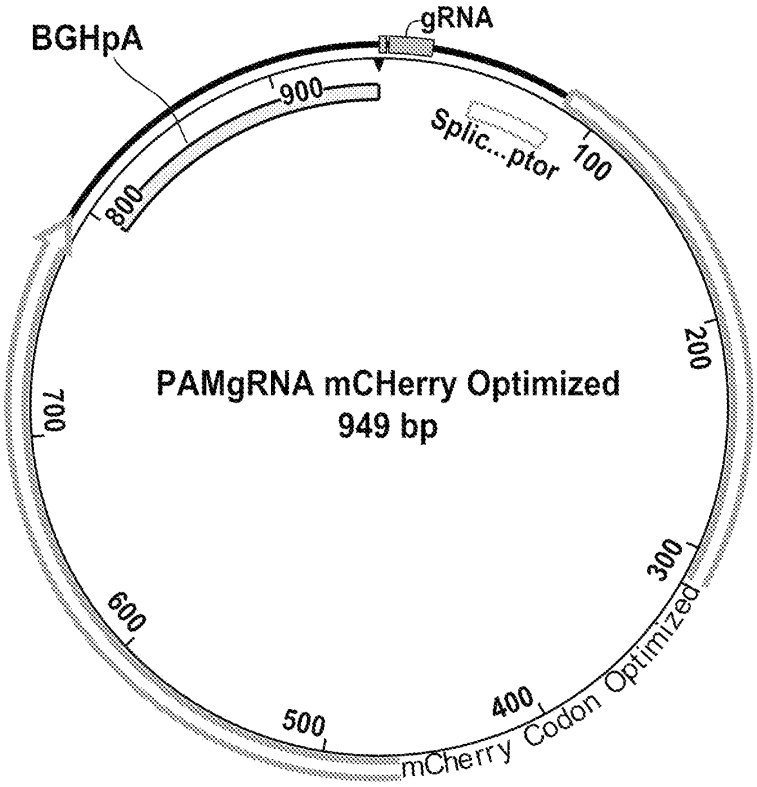

BGHpA
gRNA
900
Splic...ptor
100
800
200
PAMgRNA mCHerry Optimized
949 bp
700
300
600
400
500
mCherry Codon Optimized

FIG. 3B

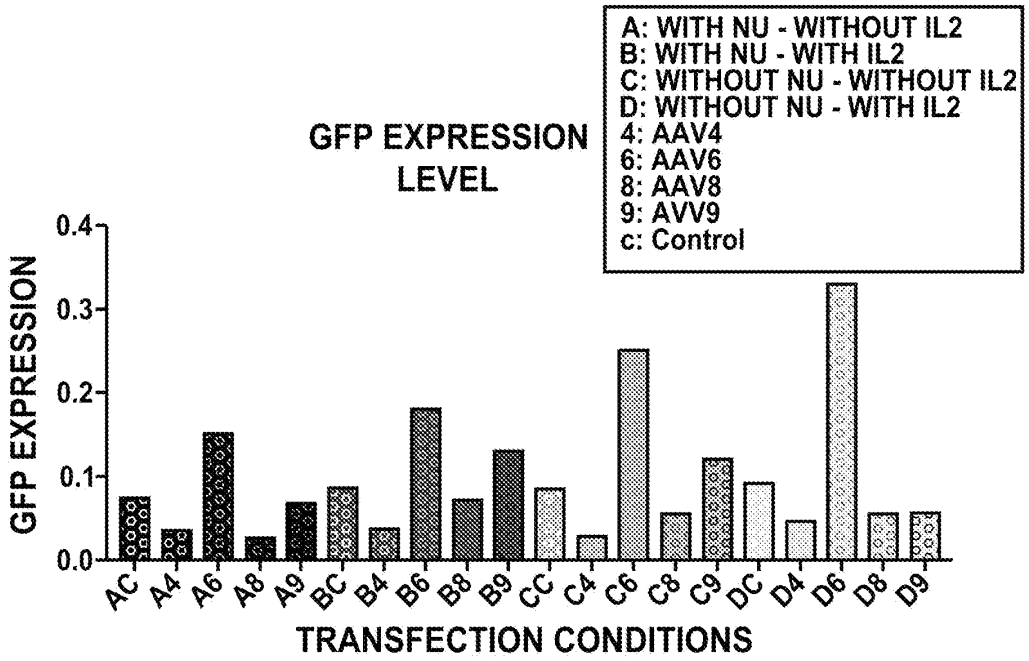

A: WITH NU - WITHOUT IL2
B: WITH NU - WITH IL2
C: WITHOUT NU - WITHOUT IL2
D: WITHOUT NU - WITH IL2
4: AAV4
6: AAV6
8: AAV8
9: AVV9
c: Control
GFP EXPRESSION LEVEL
GFP EXPRESSION
0.4
0.3
0.2
0.1
0.0
AC A4 A6 A8 A9 BC B4 B6 B8 B9 CC C4 C6 C8 C9 DC D4 D6 D8 D9
TRANSFECTION CONDITIONS

FIG. 4A

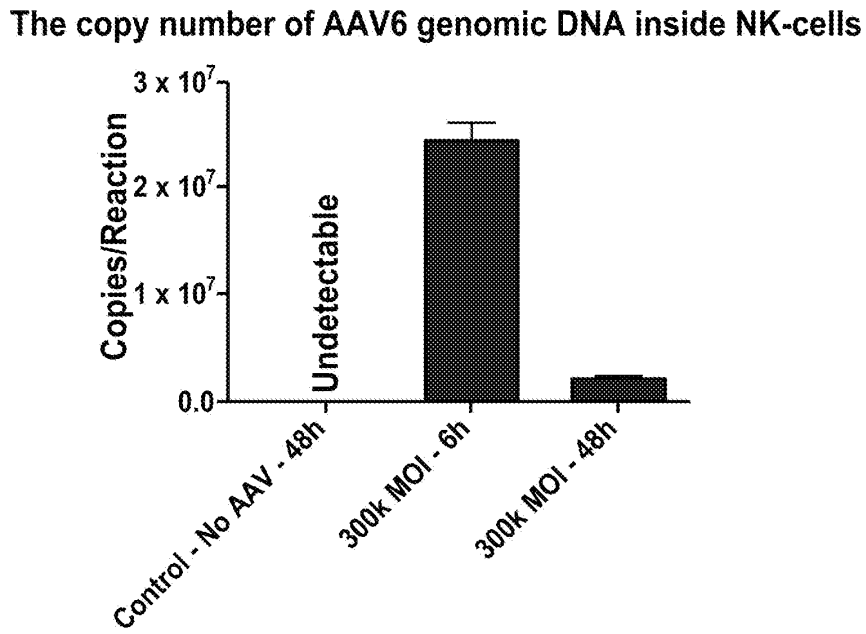

The copy number of AAV6 genomic DNA inside NK-cells
Copies/Reaction
3 x 10^7
2 x 10^7
1 x 10^7
0.0
Undetectable
Control - No AAV - 48h
300k MOI - 6h
300k MOI - 48h

FIG. 4B

Testing new primers to confirm the integration of mCherry reporter gene into AAVS in HEK293 cells
1200bp-mCherry1_forward: AACTCTGCCCTCTAACGCTG: within mCherry gene
1200bp-mCherry1_reverse: CCGTCCTCGAAGTTCATCAC: 1200bp out of the mCherry gene

1200bp-mCherry2_forward: CGCCTACAACGTCAACATC: 1200bp out of the mCherry gene
1200bp-mCherry2_reverse: GAATCCCTCCTCTCTGAACC: within mCherry gene

These new primers amplify the mCherry just in case of integration (Not the residual plasmids)

1. **NC**-AAVS1-No Elecrtoporation **Old primers** to amplify the AAVS1 gene flanking the Cas9 target site.
2. **scPAMPAM+AAVBB** DNA electroporation - **OLD Primers** (This could amplify also the residual plasmids)
3. **ss800+AAVBB** DNA electroporation - **OLD Primers** (This could amplify also the residual plasmids)

4. **NC**-No electroporation -**1200bp-mCherry1 Primers**. This should amplify just the mCherry if is integrated
5. **scPAMPAM+AAVBB** DNA electroporation - 1200bp-mCherry1 Primers. This should amplify just the mCherry if is integrated
6. **Ss800+AAVBB** DNA electroporation - 1200bp-mCherry1 Primers. This should amplify just the mCherry if is integrated

7. **NC**-No electroporation -**1200bp-mCherry2 Primers**. This should amplify just the mCherry if is integrated
8. **scPAMPAM+AAVBB** DNA electroporation-1200bp mCherry2 Primers. This should amplify just the mCherry if is integrated
9. **Ss800+AAVBB** DNA electroporation - 1200bp-mCherry2 Primers. This should amplify just the mCherry if is integrated

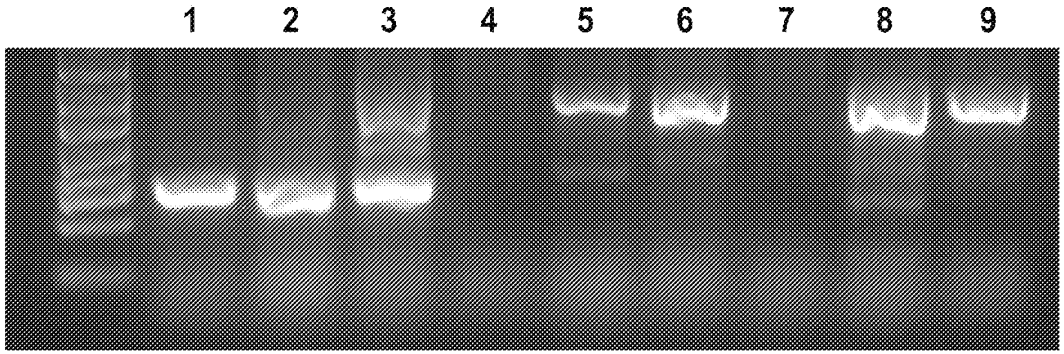
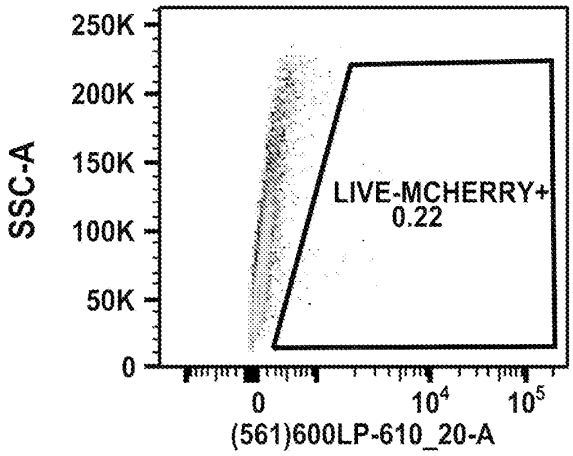
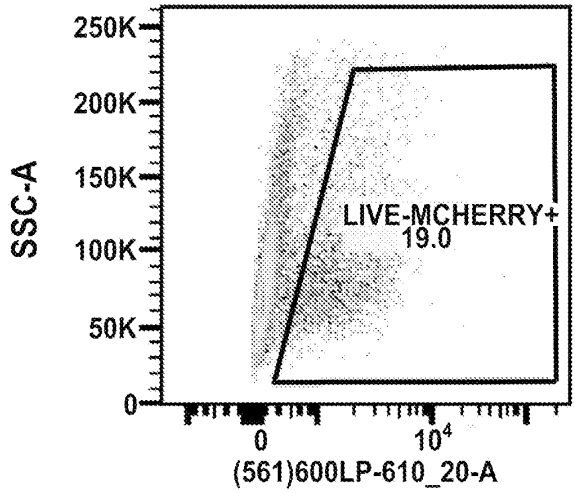


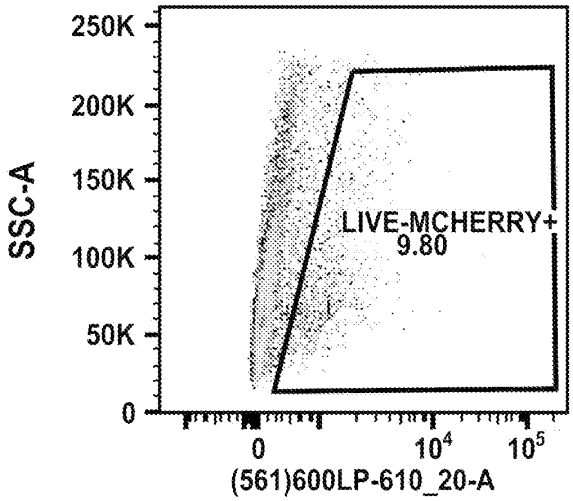
FIG. 5A



Specimen_001_Unstained.fcs
LIVE
13612

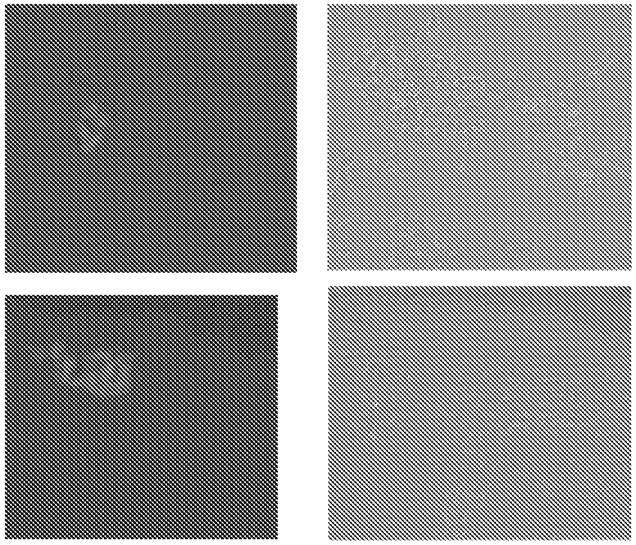


Specimen_001_800 BP.fcs
LIVE
15592



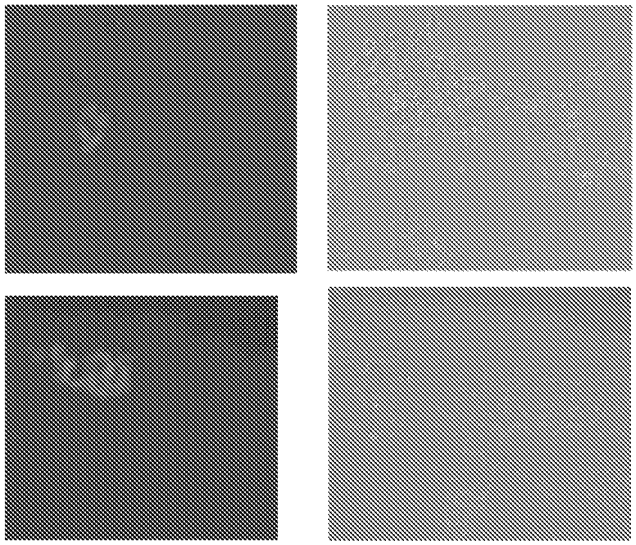
Specimen_001_PAMg.fcs
LIVE
11559

FIG. 5B



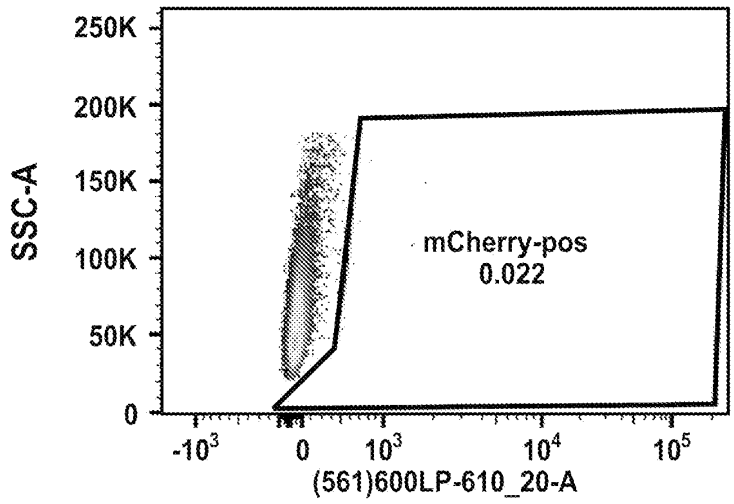
mCherry positive HEK-293 cells
Electroporated with pDNA of
PAMg condition.
Cas9/RNP+pDNA encoding
mCHerry
5 Days post electroporation

FIG. 5C



mCherry positive HEK-293 cells
Electroporated with pDNA of
PAMg condition.
Cas9/RNP+pDNA encoding
mCHerry
5 Days post electroporation

FIG. 6A


250K
200K
150K
100K
50K
0
SSC-A
mCherry-pos
0.022
-10^3
0
10^3
10^4
10^5
(561)600LP-610_20-A

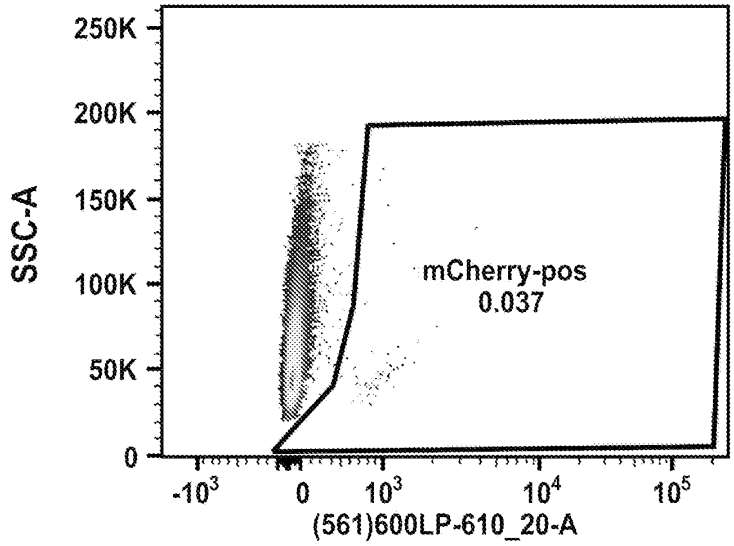

250K
200K
150K
100K
50K
0
SSC-A
mCherry-pos
0.037
-10^3
0
10^3
10^4
10^5
(561)600LP-610_20-A

FIG. 6B

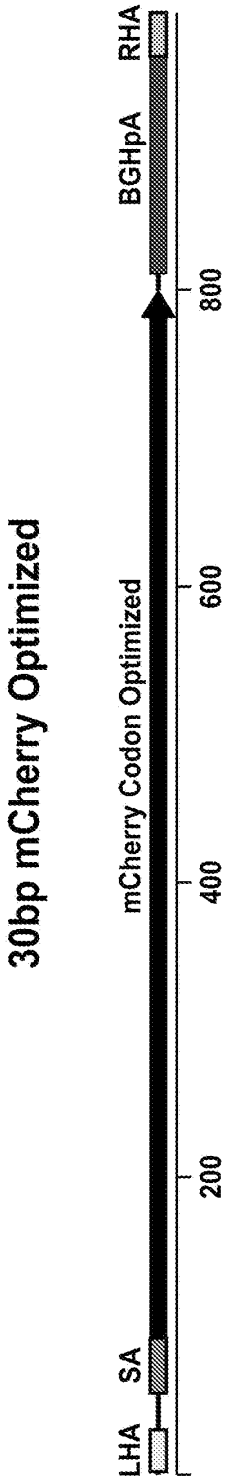

30bp mCherry Optimized
mCherry Codon Optimized
LHA
SA
BGHpA
RHA
200
400
600
800

FIG. 7A

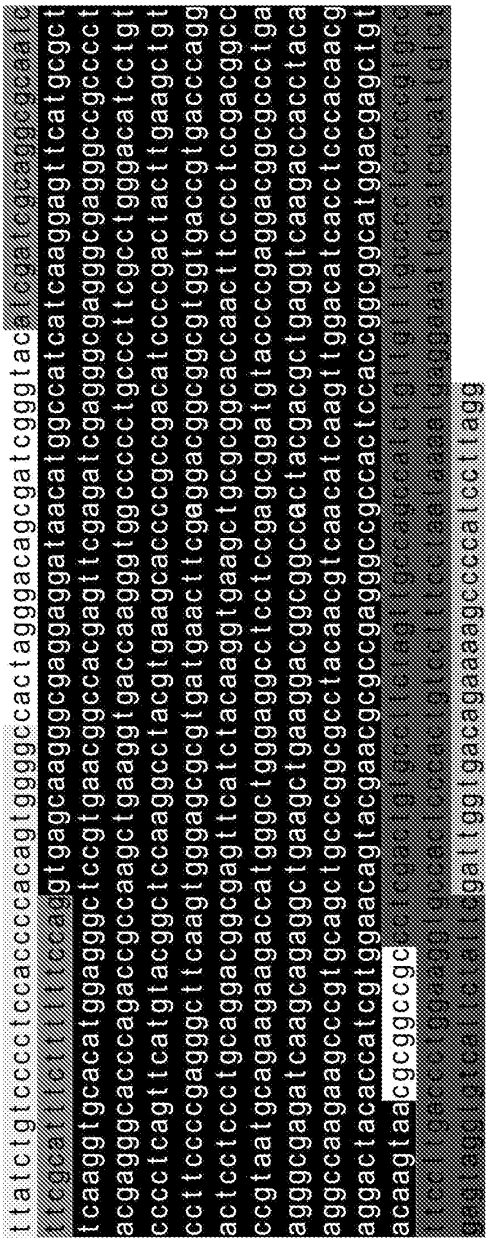


FIG. 7B

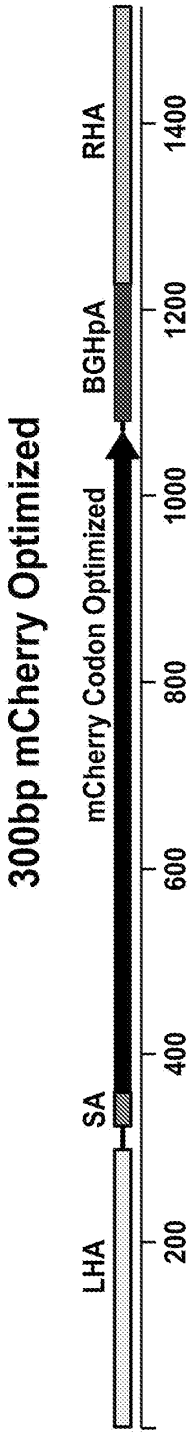

300bp mCherry Optimized
LHA
SA
mCherry Codon Optimized
BGHpA
RHA
200
400
600
800
1000
1200
1400

FIG. 7C

FIG. 7D

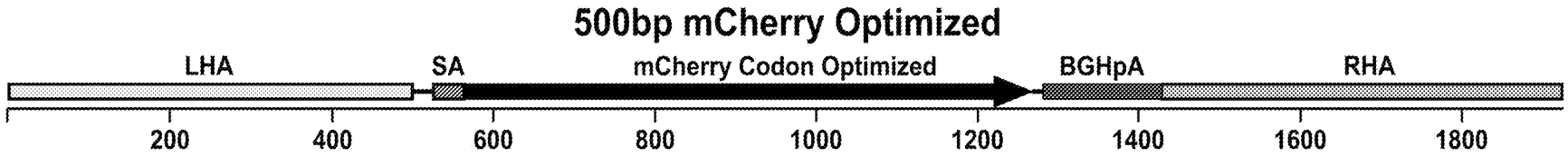


FIG. 7E

tccctttccttctccttctggggcctgtgccatctctcgtttcttaggatggccttctccgacggatgtctcccttgcgtcc
cccctcccccttcttgtaggcctgcatcatcaccgtttttctggacaaccccaaagtaccccgtctccctggctttagccacct
ctccatcctcttgctttctttgcctggacaccccgttctcctgtggattcgggtcacctctcactcctttcatttgggcagct
cccctacccccccttacctctctagtctgtgctagctcttccagcccccctgtcatggcatcttccaggggtccgagagctcagc
tagtcttcttcctccaacccgggcccctatgtccacttcaggacagcatgtttgctgcctccagggatcctgtgtccccgagc
tgggaccaccttatattcccagggccggttaatgtggctctggttctgggtacttttatctgtcccctccaccccacagtggg
gccactagggacagcgatcgggtacatcgatcgcagcgcgcaatcttcgcatttctttttccaggtgagcaagggcgaggagg
ataacatggccatcatcaaggagttcatgcgcttcaaggtgcacatggagggctccgtgaacggccacgagttcgagatcgag
ggcgagggcgagggccgcccctacgagggcacccagaccgccaagctgaaggtgaccaagggtggccccctgcccttcgcctg
ggacatcctgtcccctcagttcatgtacggctccaaggcctacgtgaagcaccccgccgacatccccgactacttgaagctgt
ccttccccgagggcttcaagtgggagcgcgtgatgaacttcgaggacggcggcgtggtgaccgtgacccaggactcctccctg
caggacggcgagttcatctacaaggtgaagctgcgcggcaccaacttcccctccgacggccccgtaatgcagaagaagaccat
gggctgggaggcctcctccgagcggatgtaccccgaggacggcgccctgaagggcgagatcaagcagaggctgaagctgaagg
acggcggccactacgacgctgaggtcaagaccacctacaaggccaagaagcccgtgcagctgcccggcgcctacaacgtcaac
atcaagttggacatcacctcccacaacgaggactacaccatcgtggaacagtacgaacgcgccgagggccgccactccaccgg
cggcatggacgagctgtacaagtaacgcggccgc[illegible]
[illegible]
[illegible]gattggtgacagaaaagccccatccttaggcctcctccttcctagtctcctgatattgggtctaaccc
ccacctcctgttaggcagattccttatctggtgacacacccccatttcctggagccatctctccttgccagaacctctaag
gtttgcttacgatggagccagagacgatc tgggagggagagcttggcagggggtgggagggaagggggggatgcgtgacctg
cccggttctcagtggccaccctgc ctaccctctcccagaacctgagctgctctgacgcggctgtctggtgcgtttc ctgat
cctggtgctgcagcttccttacacttcccaagaggagaagcagtttggaaaaacaaaatcagaataagttggtcctgagttct
aactttggctcttcacctttctagtccccaatttatattgttcctccgtgcgtcagttttacctgtgagataaggccagtagc
cagccccgtcctggcag

FIG. 7F

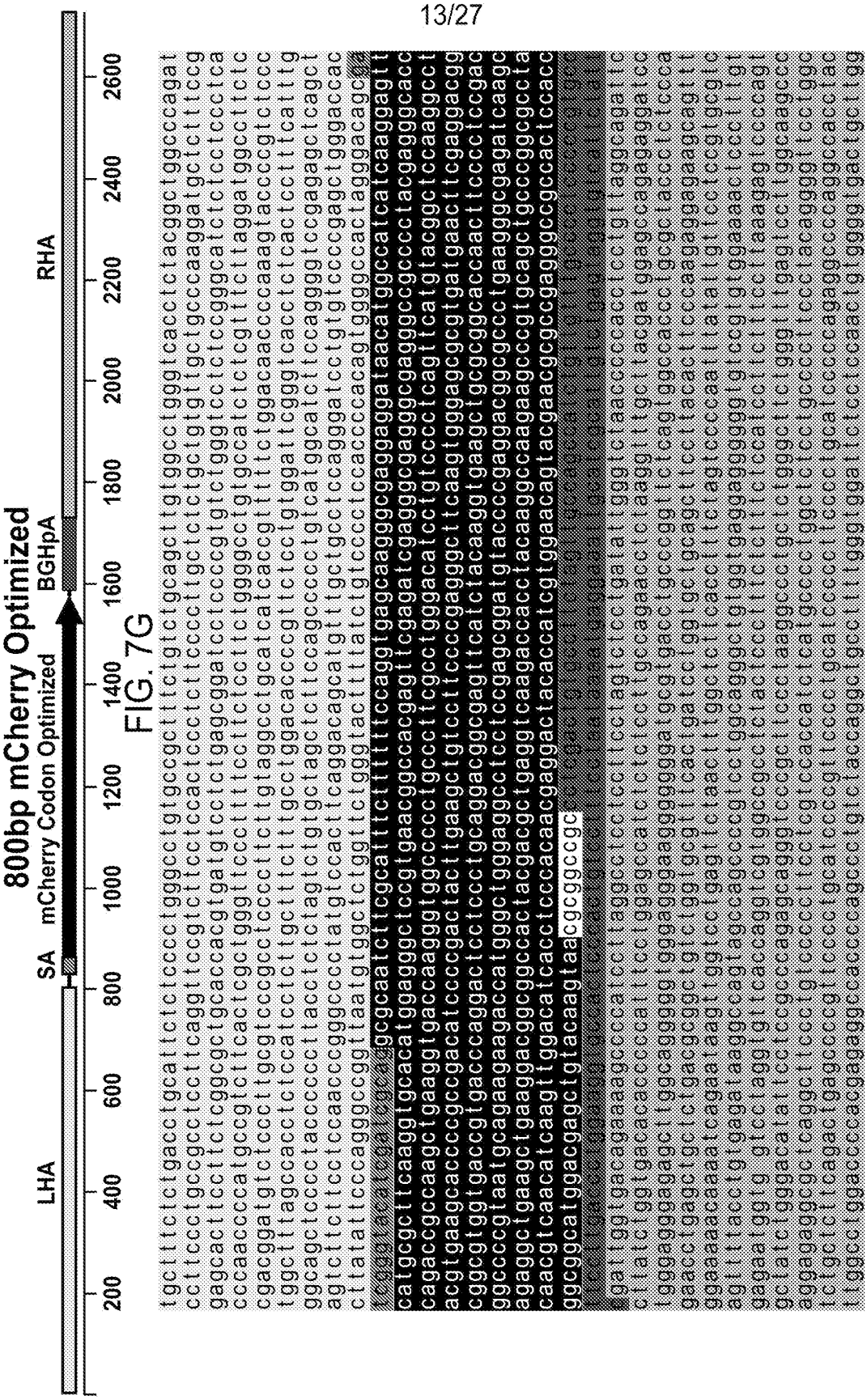

800bp mCherry Optimized
LHA
SA
mCherry Codon Optimized
BGHpA
RHA
200
400
600
800
1000
1200
1400
1600
1800
2000
2200
2400
2600

FIG. 7G

FIG. 7H

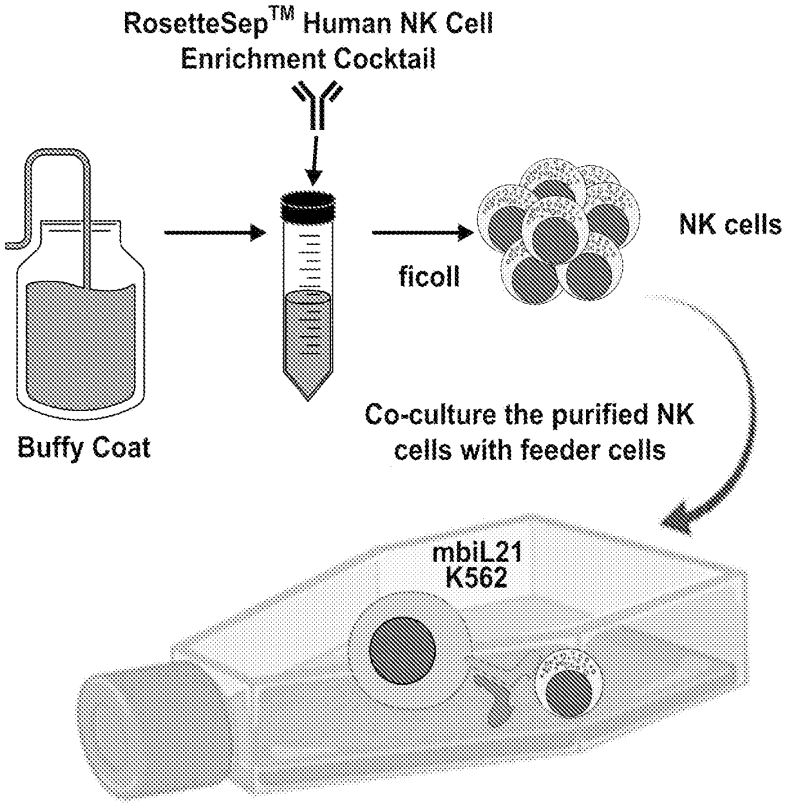

RosetteSep™ Human NK Cell
Enrichment Cocktail
Buffy Coat
ficoll
NK cells
Co-culture the purified NK
cells with feeder cells
mbiL21
K562

FIG. 8

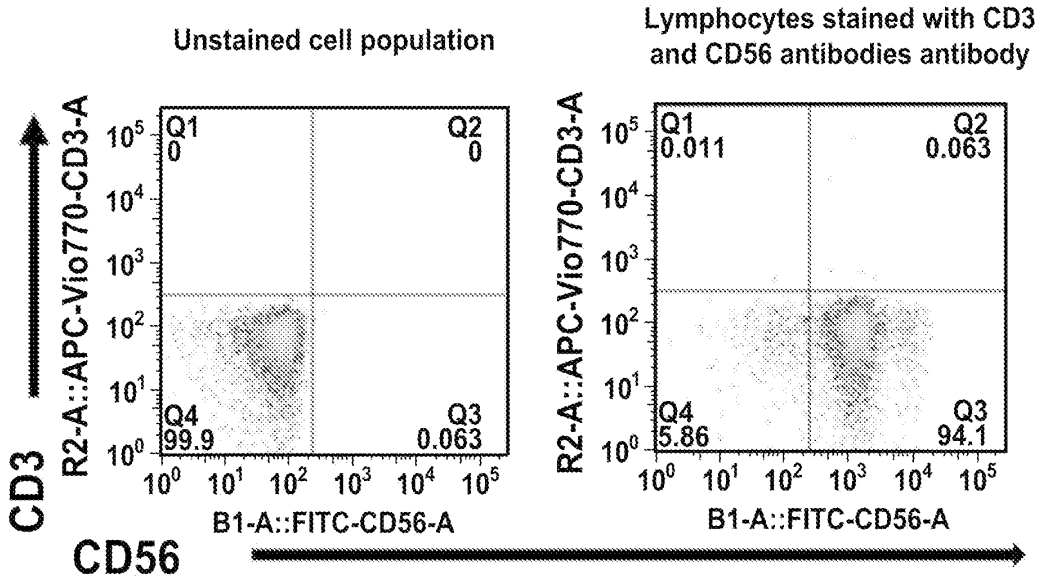

Unstained cell population
Lymphocytes stained with CD3
and CD56 antibodies antibody
R2-A::APC-Vio770-CD3-A
B1-A::FITC-CD56-A
Q1
0
Q2
0
Q4
99.9
Q3
0.063
Q1
0.011
Q2
0.063
Q4
5.86
Q3
94.1
CD3
CD56

FIG. 9

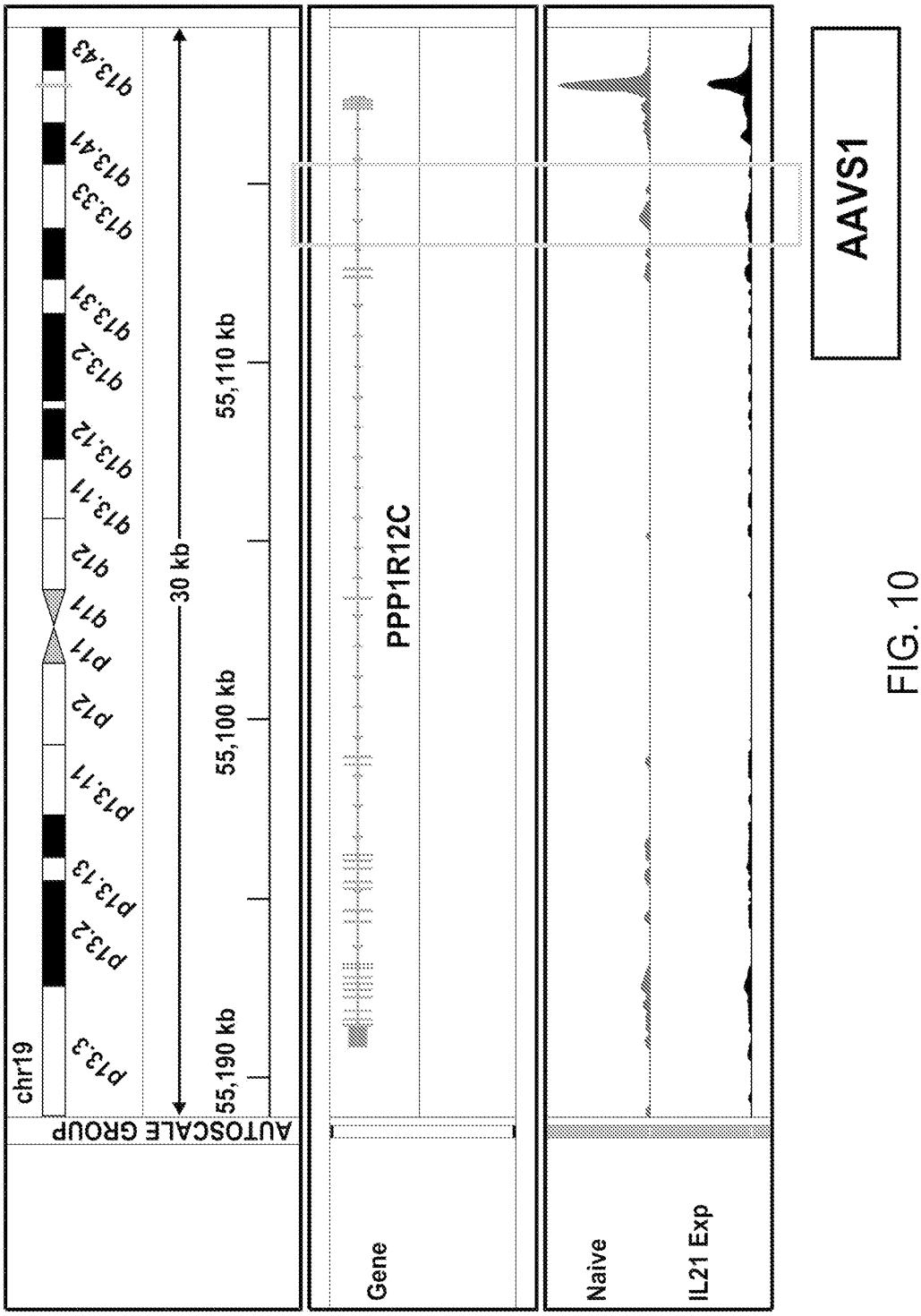

chr19
p13.3
p13.2
p13.13
p13.11
p12
p11.1
q11
q12
q13.11
q13.12
q13.2
q13.31
q13.33
q13.41
q13.43
30 kb
55,190 kb
55,100 kb
55,110 kb
AUTOSCALE GROUP
PPP1R12C
Gene
Naive
IL21 Exp
AAVS1

FIG. 10

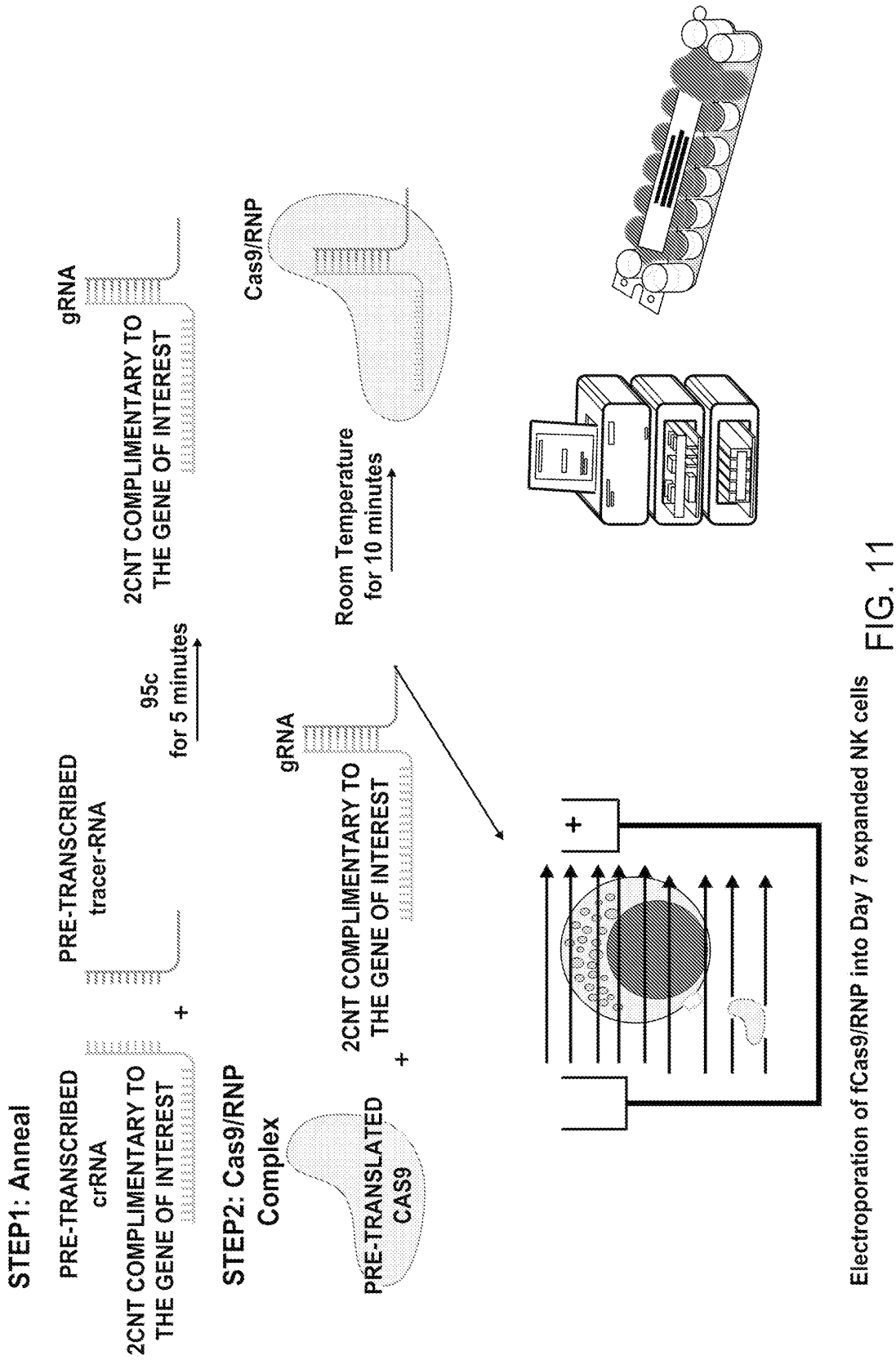

STEP1: Anneal
PRE-TRANSCRIBED crRNA
2CNT COMPLIMENTARY TO THE GENE OF INTEREST
+
PRE-TRANSCRIBED tracer-RNA
95c for 5 minutes
gRNA
2CNT COMPLIMENTARY TO THE GENE OF INTEREST
STEP2: Cas9/RNP Complex
PRE-TRANSLATED CAS9
+
gRNA
2CNT COMPLIMENTARY TO THE GENE OF INTEREST
Cas9/RNP
Room Temperature for 10 minutes
+
Electroporation of fCas9/RNP into Day 7 expanded NK cells

FIG. 11

Contributions | Indel Distribution | Traces

Status
SUCCEEDED
0

PAM Sequence
TGG

r^2
0.99

Guide Target
GGGGCCACTAGGGACAGGAT

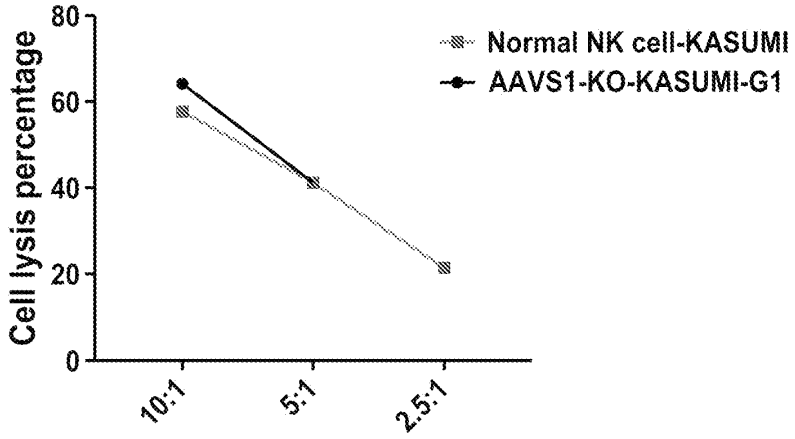
ICE
86

KO-Score
85

RELATIVE CONTRIBUTION OF EACH SEQUENCE (NORMALIZED)

INDEL	CONTRIBUTION	SEQUENCE	
-1	82.2%	CCCACAGTGGGGCCACTAGGGACAG	-ATTGGTGACAGAAAAGCCCCATCCTTAGGCCTCCTCCTTCCTAGTCTCC
+ 0	13%	CCCACAGTGGGGCCACTAGGGACAG	GATTGGTGACAGAAAAGCCCCATCCTTAGGCCTCCTCCTTCCTAGTCTCC
-4	1.1%	CCCACAGTGGGGCCACTAGGGA---	-ATTGGTGACAGAAAAGCCCCATCCTTAGGCCTCCTCCTTCCTAGTCTCC
-21	0.7%	CCCACAGTGGGGCCAC---------	----------AAAAGCCCCATCCTTAGGCCTCCTCCTTCCTAGTCTCC
-9	0.6%	CCCACAGTGGGGCCAC---------	GATTGGTGACAGAAAAGCCCCATCCTTAGGCCTCCTCCTTCCTAGTCTCC
-7	0.5%	CCCACAGTGGGGCCACTAGGGA---	----GGTGACAGAAAAGCCCCATCCTTAGGCCTCCTCCTTCCTAGTCTCC
-28	0.2%	CCCACAGTGGGG----------	----------AGCCCCATCCTTAGGCCTCCTCCTTCCTAGTCTCC
-6	0.1%	CCCACAGTGGGGCCACTAGGGA---	---TGGTGACAGAAAAGCCCCATCCTTAGGCCTCCTCCTTCCTAGTCTCC
-20	0.1%	CCCACAGTGGGG----------	-------GACAGAAAAGCCCCATCCTTAGGCCTCCTCCTTCCTAGTCTCC
-11	0.1%	CCCACAGTGGGGCC----------	GATTGGTGACAGAAAAGCCCCATCCTTAGGCCTCCTCCTTCCTAGTCTCC
-6	0.1%	CCCACAGTGGGGCCACTAGGG----	--TTGGTGACAGAAAAGCCCCATCCTTAGGCCTCCTCCTTCCTAGTCTCC
-8	0.1%	CCCACAGTGGGGCCACT--------	GATTGGTGACAGAAAAGCCCCATCCTTAGGCCTCCTCCTTCCTAGTCTCC
-25	0.1%	CCCACAGTGGGG----------	----------AAAAGCCCCATCCTTAGGCCTCCTCCTTCCTAGTCTCC
-25	0%	CCCACAGTGGGGC----------	----------AAAGCCCCATCCTTAGGCCTCCTCCTTCCTAGTCTCC
-21	0%	CCCACAGTGGGGC----------	---------CAGAAAAGCCCCATCCTTAGGCCTCCTCCTTCCTAGTCTCC
-30	0%	CCCACAGTGG----------	----------AGCCCCATCCTTAGGCCTCCTCCTTCCTAGTCTCC

FIG. 12



Cytotoxcity of normal NK and AAVS-KO NK cells
againts AML (Kasumi-Cell line)

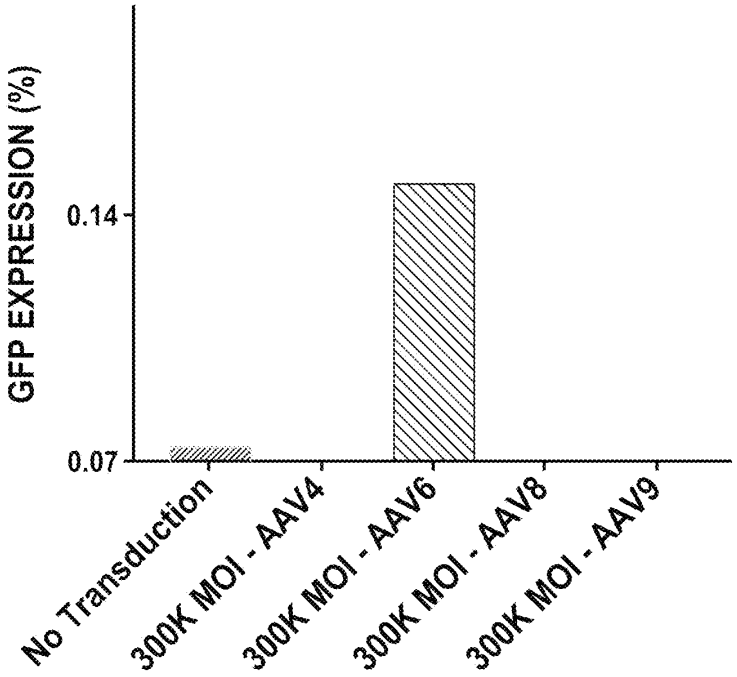


FIG. 13

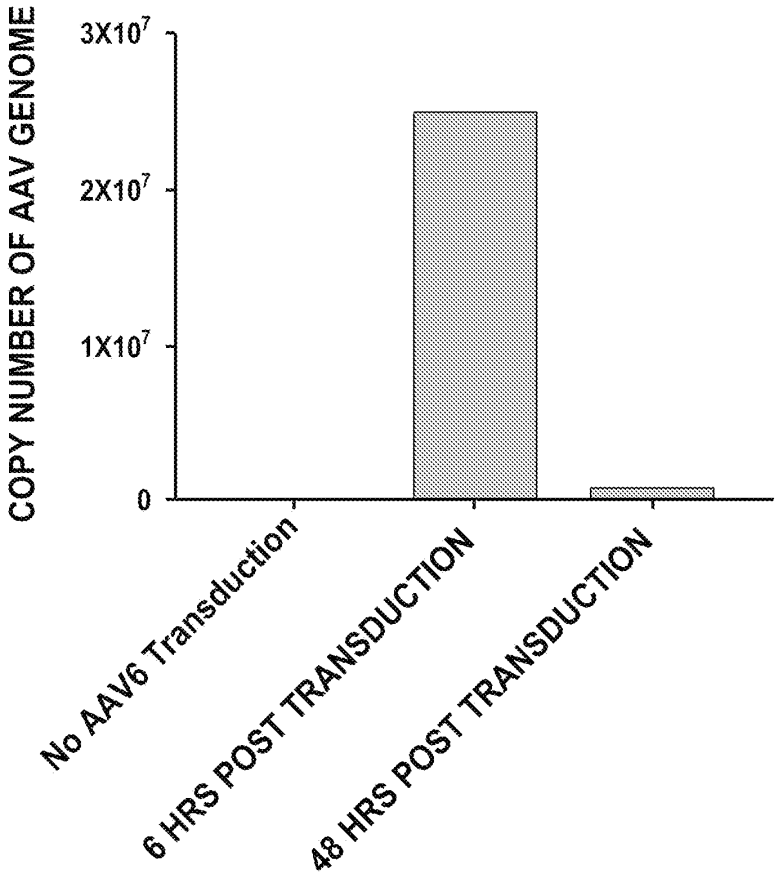

COPY NUMBER OF AAV GENOME
3X10^7
2X10^7
1X10^7
0
No AAV6 Transduction
6 HRS POST TRANSDUCTION
48 HRS POST TRANSDUCTION

FIG. 14

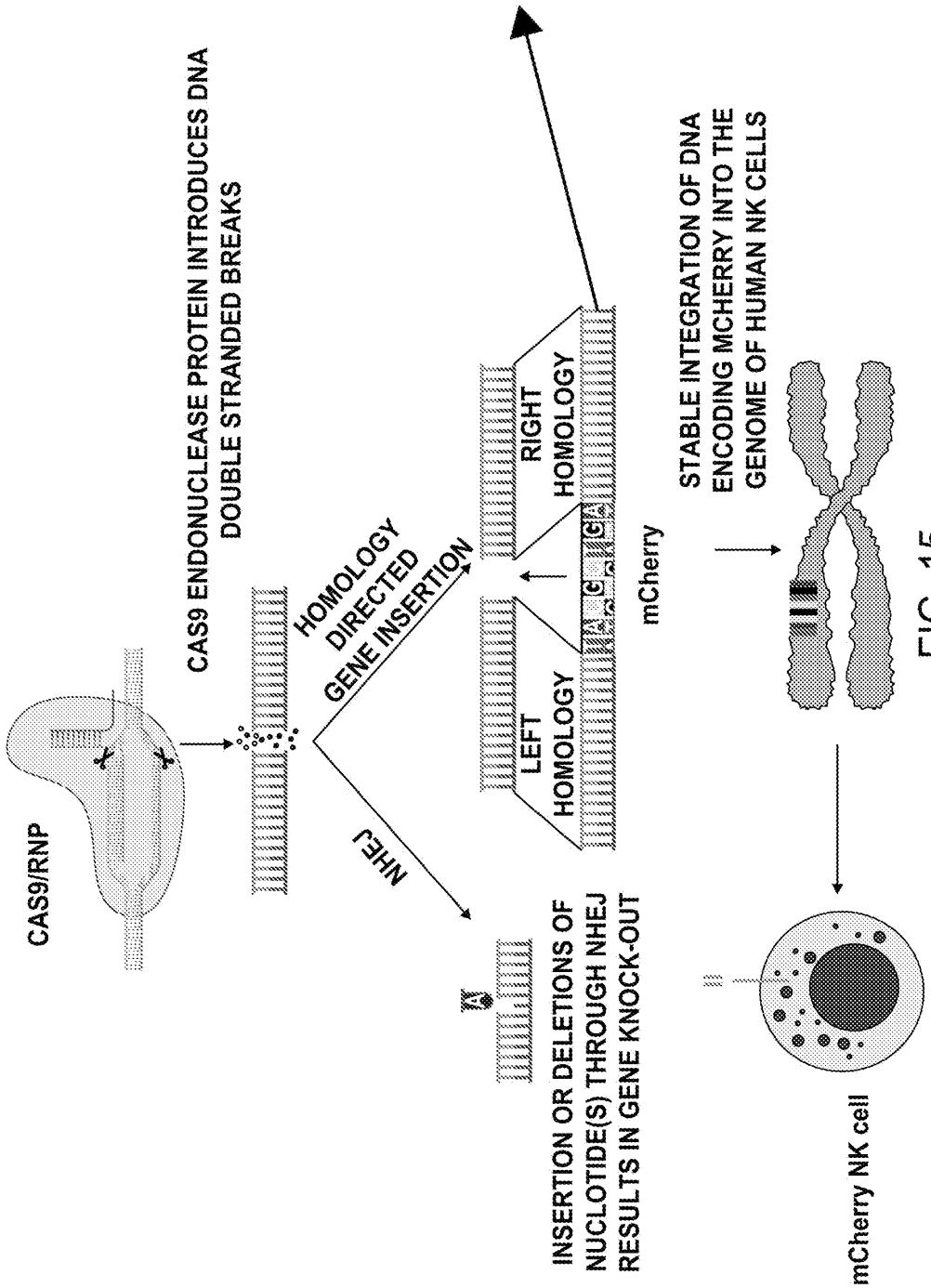

CAS9/RNP
CAS9 ENDONUCLEASE PROTEIN INTRODUCES DNA DOUBLE STRANDED BREAKS
HOMOLOGY DIRECTED GENE INSERTION
NHEJ
LEFT HOMOLOGY
RIGHT HOMOLOGY
mCherry
INSERTION OR DELETIONS OF NUCLOTIDE(S) THROUGH NHEJ RESULTS IN GENE KNOCK-OUT
STABLE INTEGRATION OF DNA ENCODING MCHERRY INTO THE GENOME OF HUMAN NK CELLS
mCherry NK cell

FIG. 15

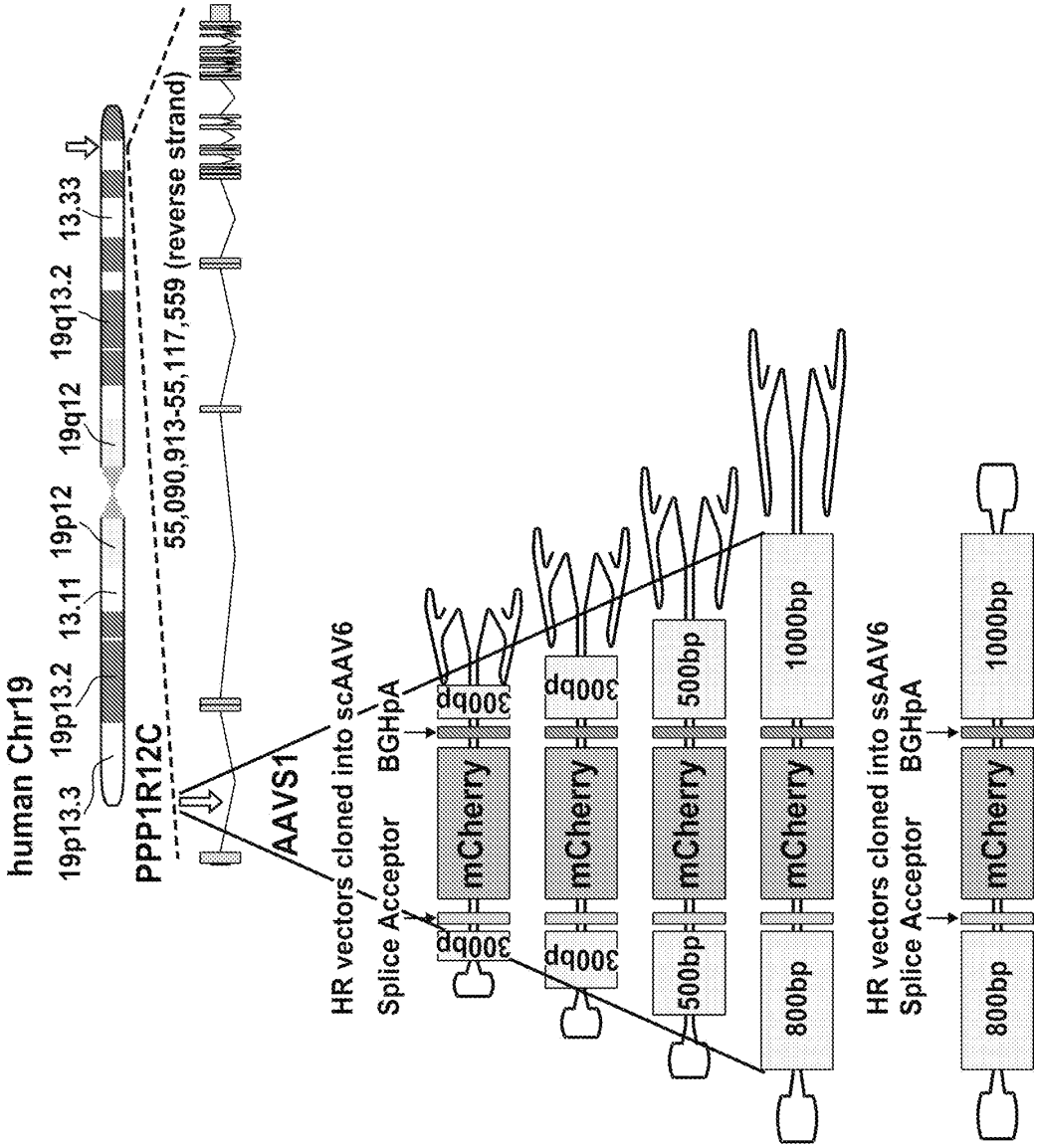

human Chr19
19p13.3
19p13.2
13.11
19p12
19q12
19q13.2
13.33
PPP1R12C
55,090,913-55,117,559 (reverse strand)
AAVS1
HR vectors cloned into scAAV6
Splice Acceptor
BGHpA
300bp
mCherry
300bp
300bp
mCherry
300bp
500bp
mCherry
500bp
1000bp
mCherry
800bp
HR vectors cloned into ssAAV6
Splice Acceptor
BGHpA
1000bp
mCherry
800bp

FIG. 15 CONT.

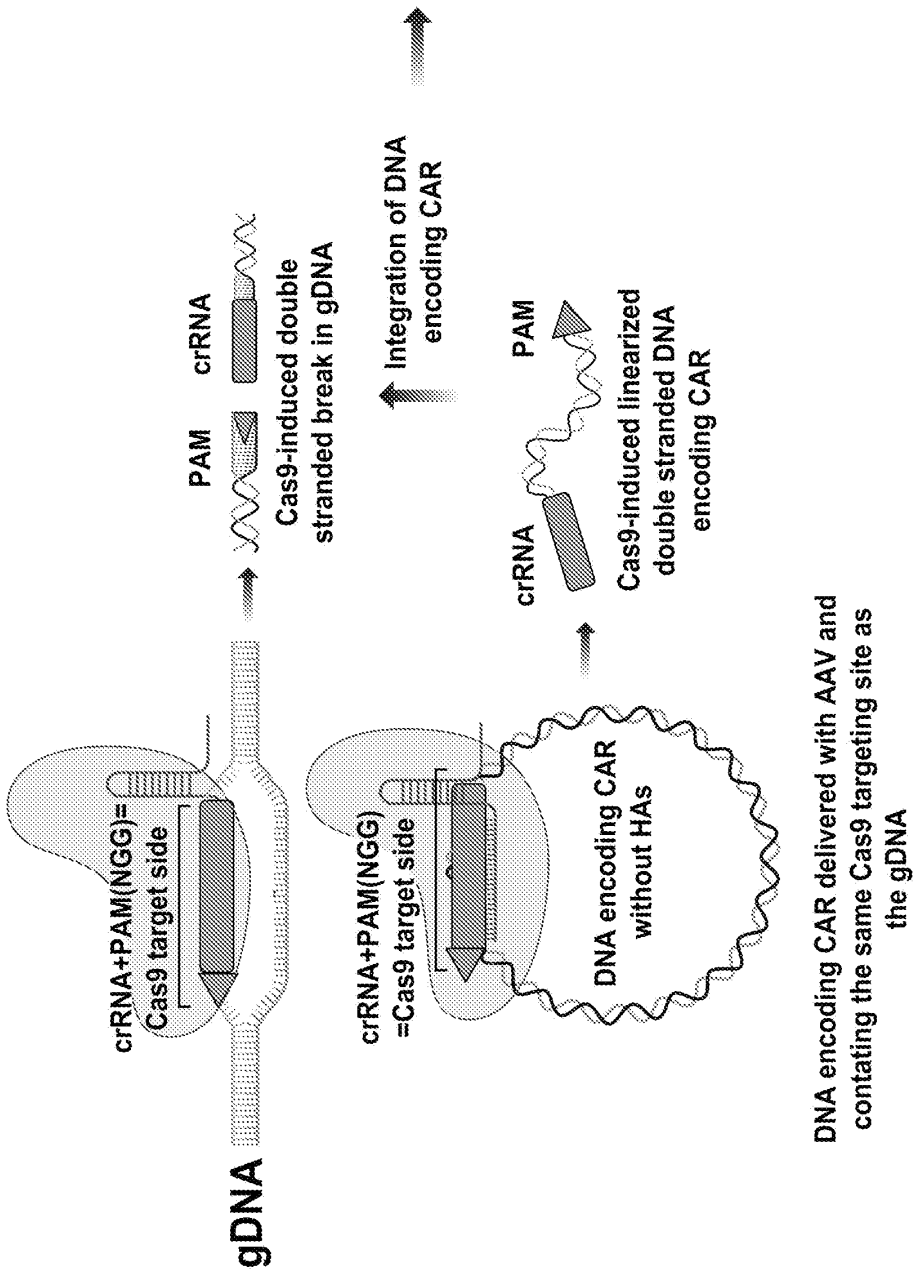

gDNA
crRNA+PAM(NGG)=
Cas9 target side
PAM
crRNA
Cas9-induced double
stranded break in gDNA
crRNA+PAM(NGG)
=Cas9 target side
DNA encoding CAR
without HAs
Integration of DNA
encoding CAR
crRNA
PAM
Cas9-induced linearized
double stranded DNA
encoding CAR
DNA encoding CAR delivered with AAV and
contating the same Cas9 targeting site as
the gDNA

FIG. 16A

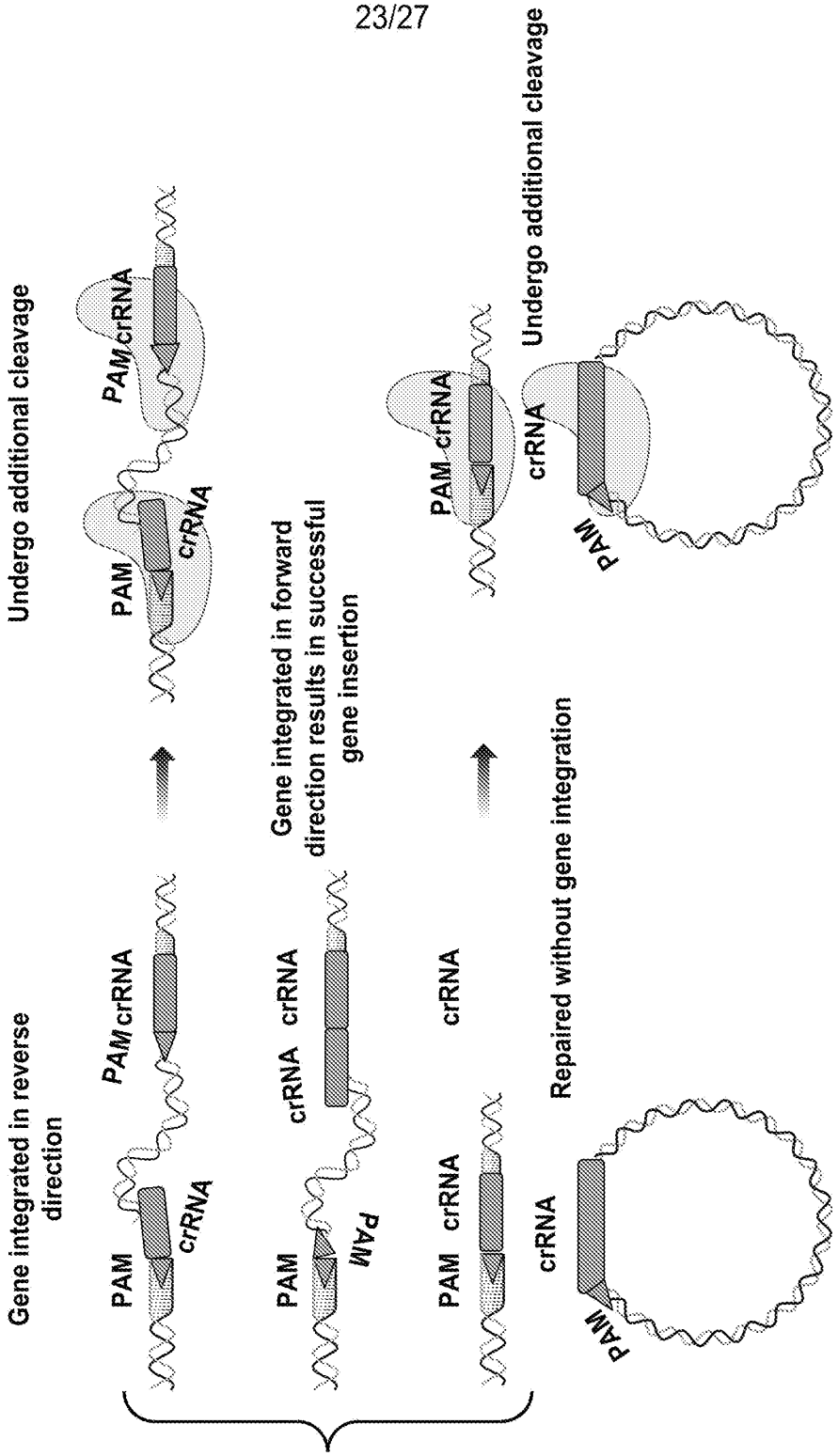

Gene integrated in reverse direction
PAM
crRNA
PAM crRNA
Undergo additional cleavage
PAM
crRNA
PAM crRNA
Gene integrated in forward direction results in successful gene insertion
crRNA
crRNA
PAM
PAM
crRNA
PAM crRNA
Undergo additional cleavage
PAM crRNA
crRNA
PAM
Repaired without gene integration
crRNA
PAM

FIG. 16A CONT.

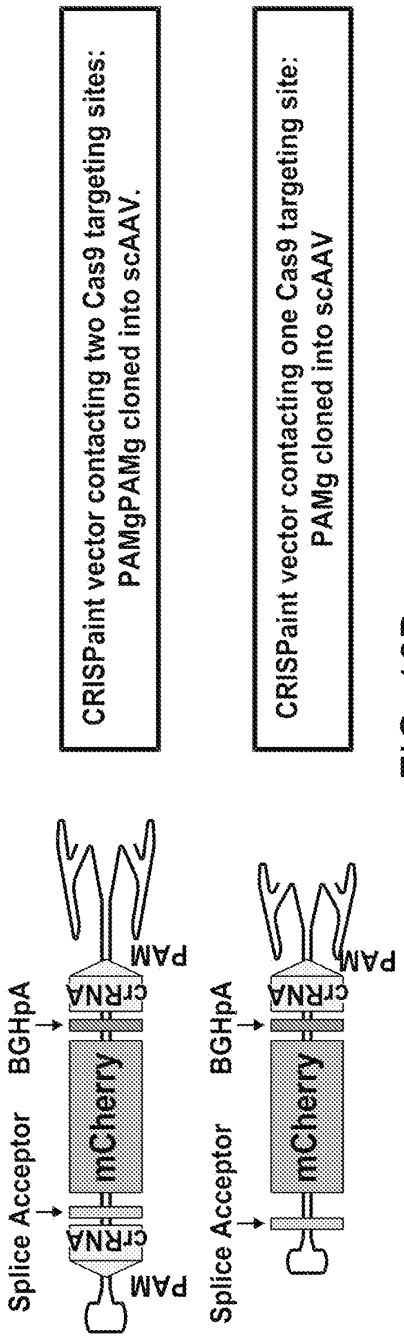


FIG. 16B

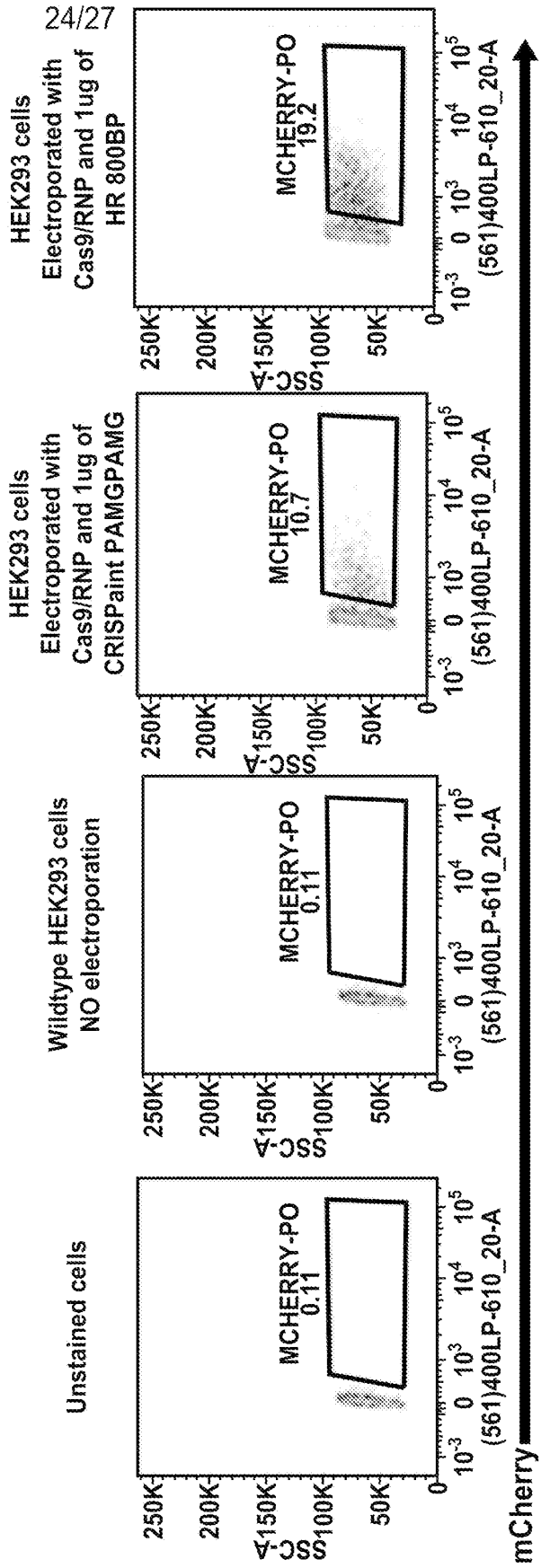


FIG. 17

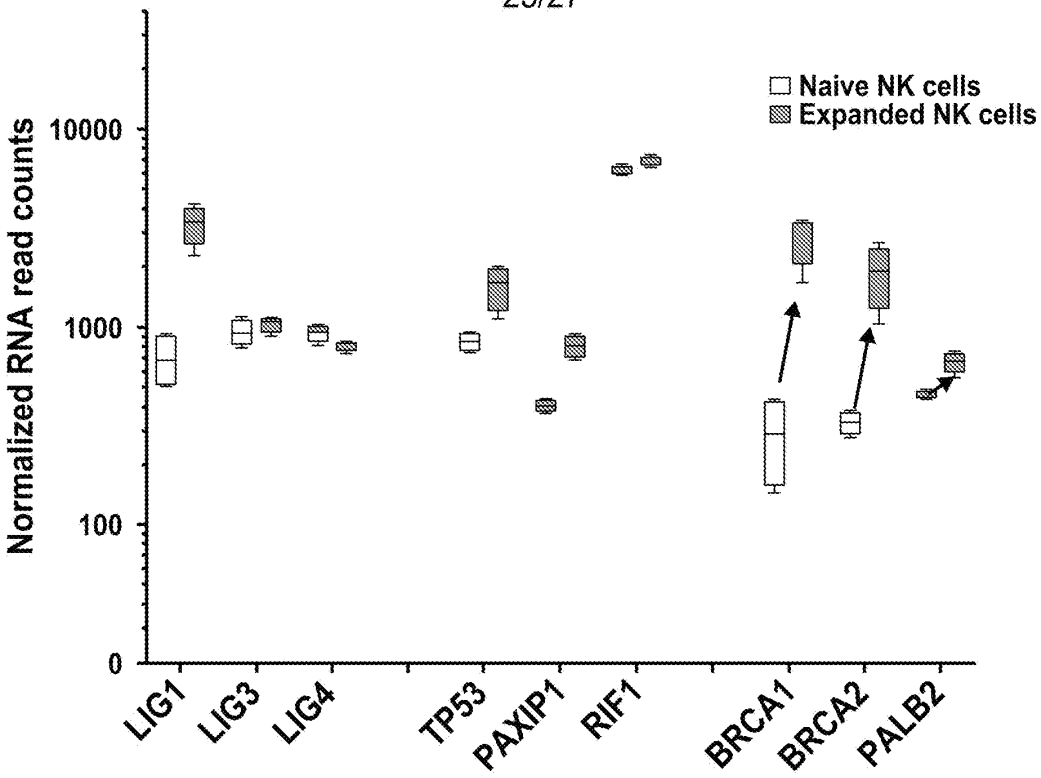

Naive NK cells
Expanded NK cells
Normalized RNA read counts
10000
1000
100
0
LIG1
LIG3
LIG4
TP53
PAXIP1
RIF1
BRCA1
BRCA2
PALB2

FIG. 18

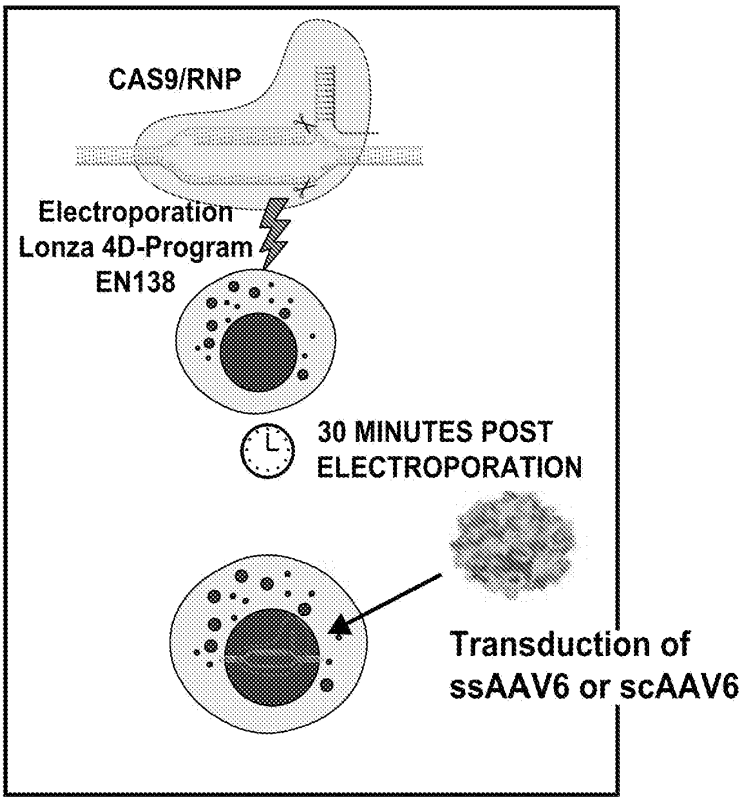

CAS9/RNP
Electroporation
Lonza 4D-Program
EN138
30 MINUTES POST
ELECTROPORATION
Transduction of
ssAAV6 or scAAV6

FIG. 19

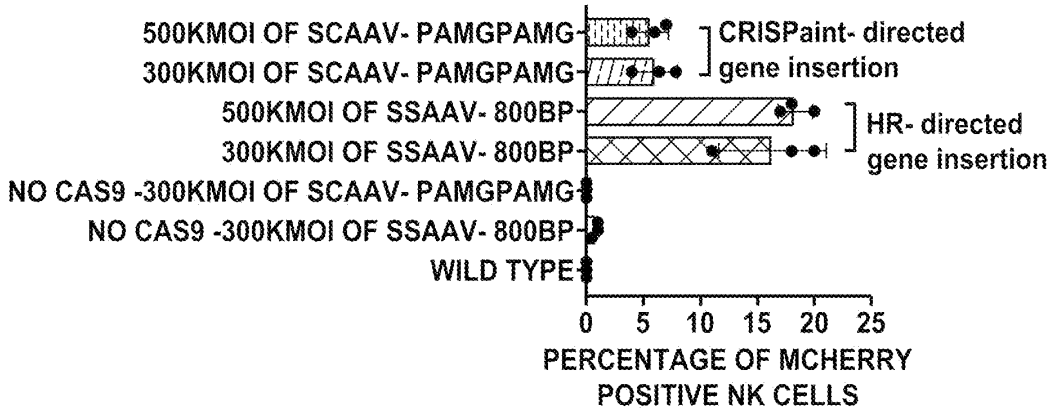

500KMOI OF SCAAV- PAMGPAMG
300KMOI OF SCAAV- PAMGPAMG
500KMOI OF SSAAV- 800BP
300KMOI OF SSAAV- 800BP
NO CAS9 -300KMOI OF SCAAV- PAMGPAMG
NO CAS9 -300KMOI OF SSAAV- 800BP
WILD TYPE
CRISPaint- directed gene insertion
HR- directed gene insertion
0 5 10 15 20 25
PERCENTAGE OF MCHERRY POSITIVE NK CELLS

FIG. 20

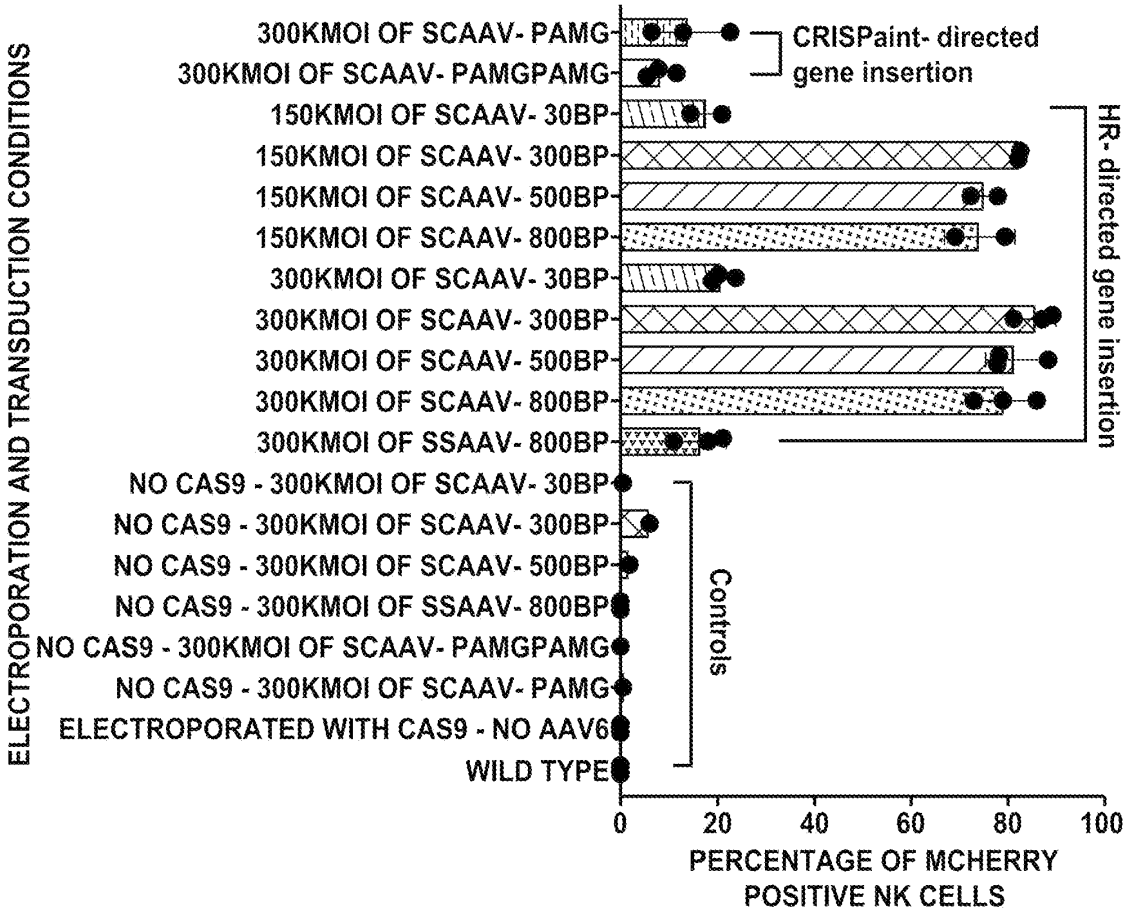

ELECTROPORATION AND TRANSDUCTION CONDITIONS
300KMOI OF SCAAV- PAMG
300KMOI OF SCAAV- PAMGPAMG
150KMOI OF SCAAV- 30BP
150KMOI OF SCAAV- 300BP
150KMOI OF SCAAV- 500BP
150KMOI OF SCAAV- 800BP
300KMOI OF SCAAV- 30BP
300KMOI OF SCAAV- 300BP
300KMOI OF SCAAV- 500BP
300KMOI OF SCAAV- 800BP
300KMOI OF SSAAV- 800BP
NO CAS9 - 300KMOI OF SCAAV- 30BP
NO CAS9 - 300KMOI OF SCAAV- 300BP
NO CAS9 - 300KMOI OF SCAAV- 500BP
NO CAS9 - 300KMOI OF SSAAV- 800BP
NO CAS9 - 300KMOI OF SCAAV- PAMGPAMG
NO CAS9 - 300KMOI OF SCAAV- PAMG
ELECTROPORATED WITH CAS9 - NO AAV6
WILD TYPE
CRISPaint- directed gene insertion
HR- directed gene insertion
Controls
0 20 40 60 80 100
PERCENTAGE OF MCHERRY POSITIVE NK CELLS

FIG. 21

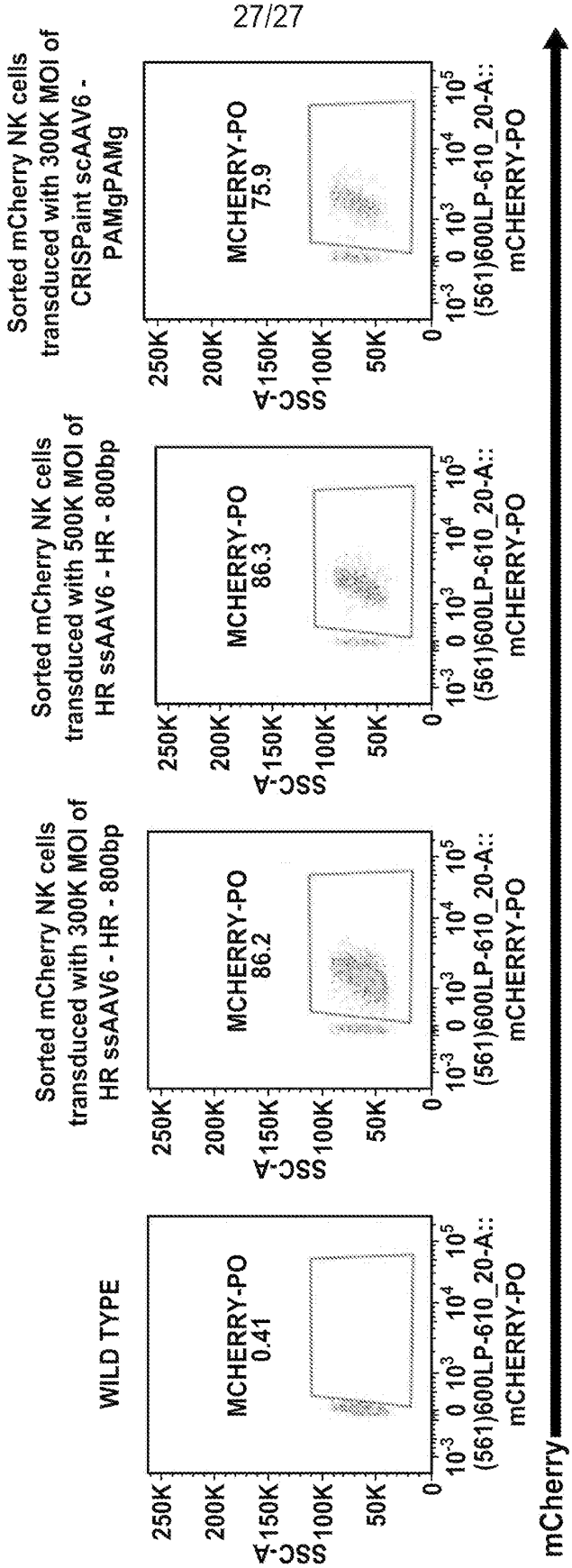

WILD TYPE
MCHERRY-PO
0.41
Sorted mCherry NK cells transduced with 300K MOI of HR ssAAV6 - HR - 800bp
MCHERRY-PO
86.2
Sorted mCherry NK cells transduced with 500K MOI of HR ssAAV6 - HR - 800bp
MCHERRY-PO
86.3
Sorted mCherry NK cells transduced with 300K MOI of CRISPaint scAAV6 - PAMgPAMg
MCHERRY-PO
75.9
SSC-A
250K
200K
150K
100K
50K
0
10^{-3} 0 10^3 10^4 10^5
(561)600LP-610_20-A::
mCHERRY-PO
mCherry

FIG. 22