FIG. 1

Integrated Cassettes (integrated portions are between frt sites)

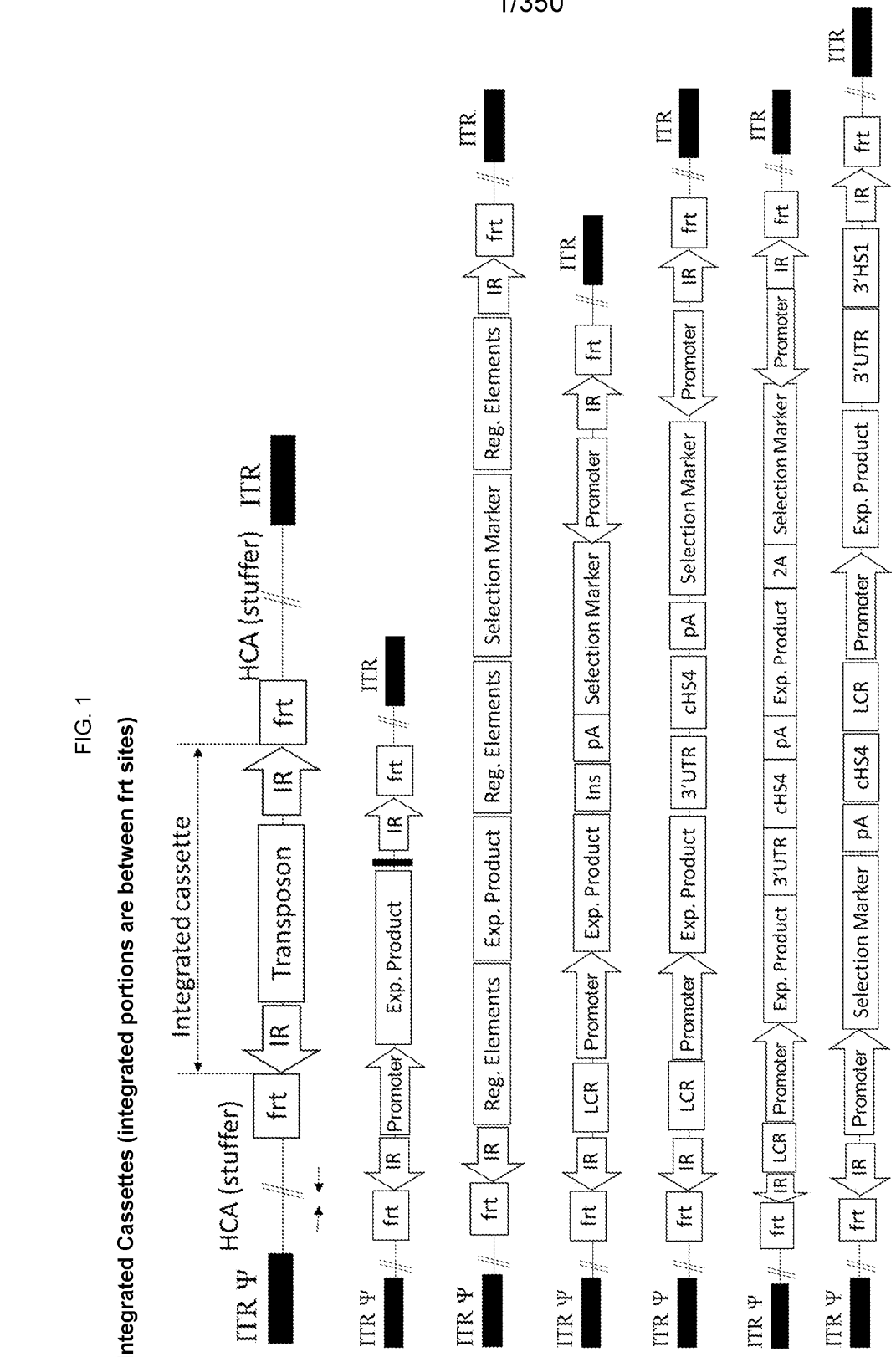


FIG. 1 cont'd

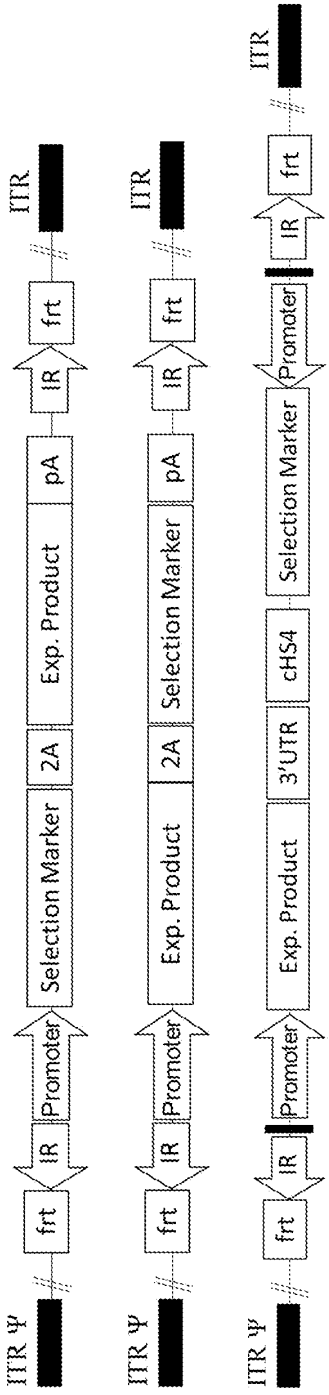

ITR Ψ
frt
IR
Promoter
Selection Marker
2A
Exp. Product
pA
IR
frt
ITR
ITR Ψ
frt
IR
Promoter
Exp. Product
2A
Selection Marker
pA
IR
frt
ITR
ITR Ψ
frt
IR
Promoter
Exp. Product
3'UTR
cHS4
Selection Marker
Promoter
IR
frt
ITR

FIG. 1 cont'd

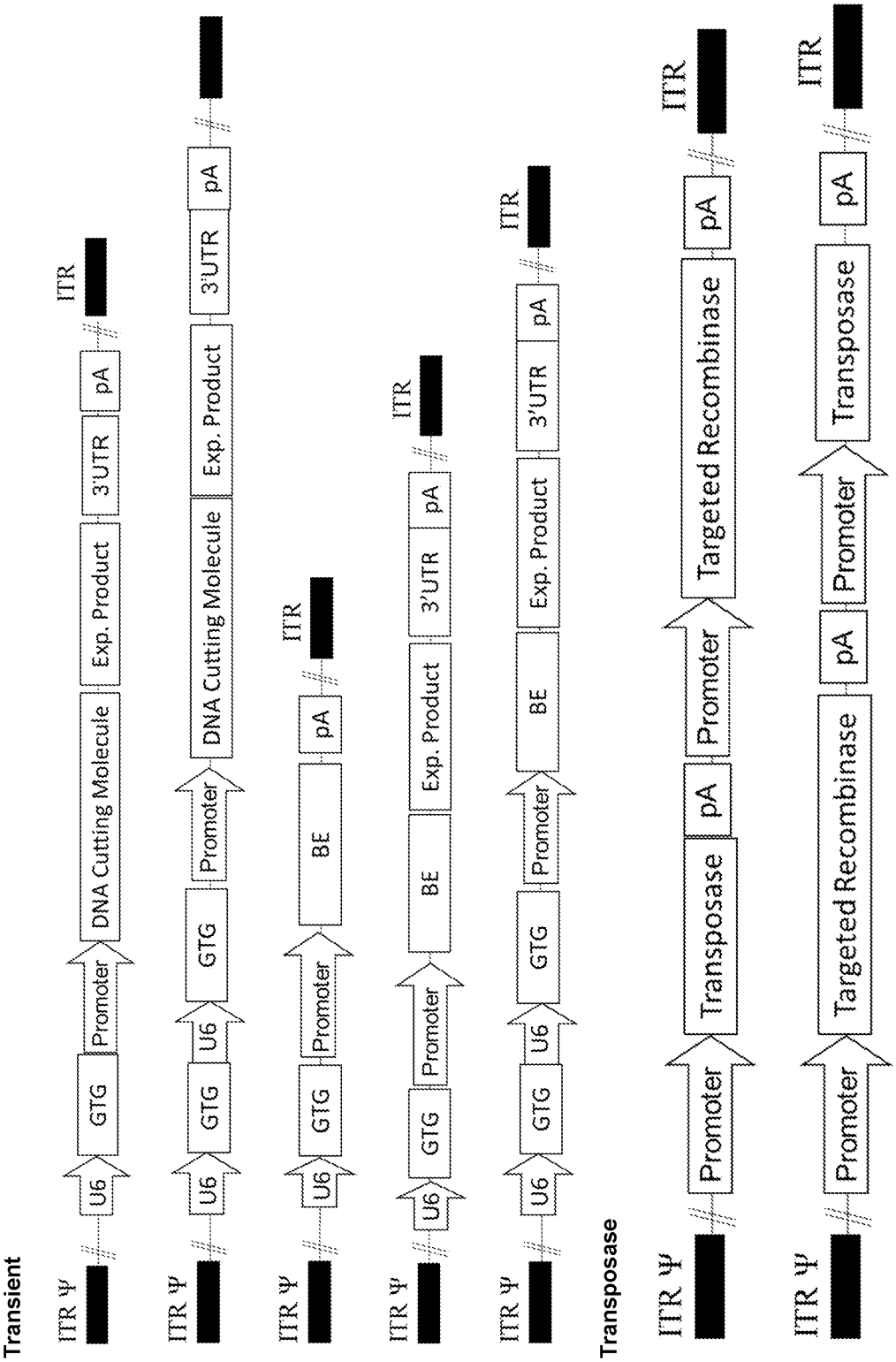

Transient
ITR Ψ
U6
GTG
Promoter
DNA Cutting Molecule
Exp. Product
3'UTR
pA
ITR
BE
Transposase
Targeted Recombinase

FIG. 2A

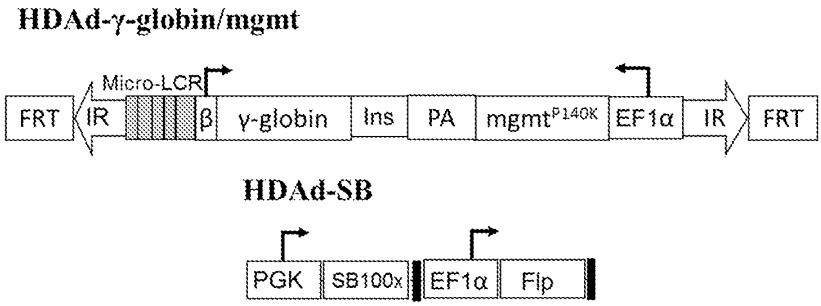


FIG. 2B

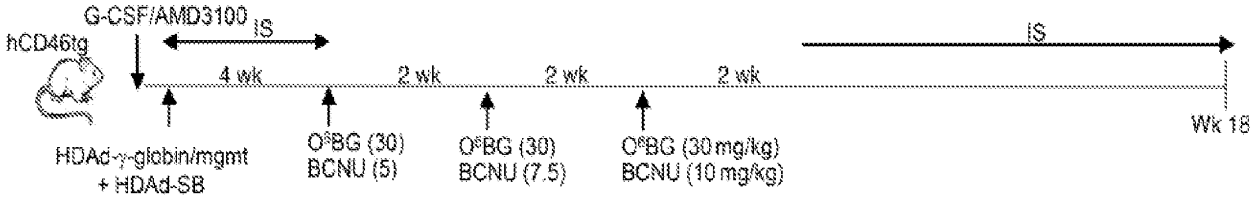


FIG. 2C

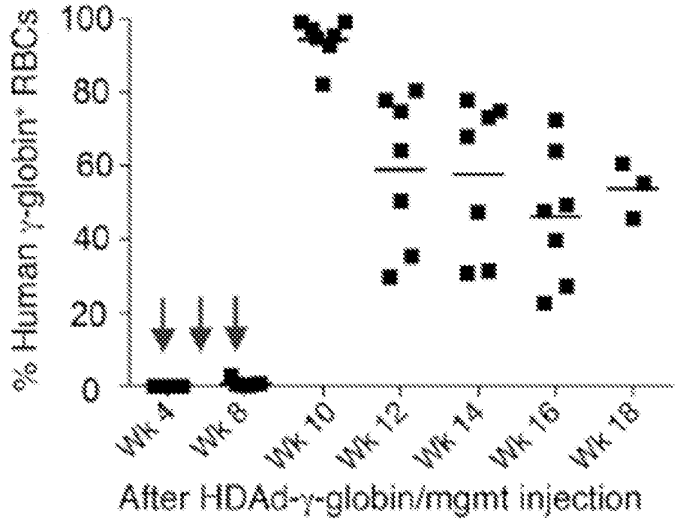


FIG. 2D

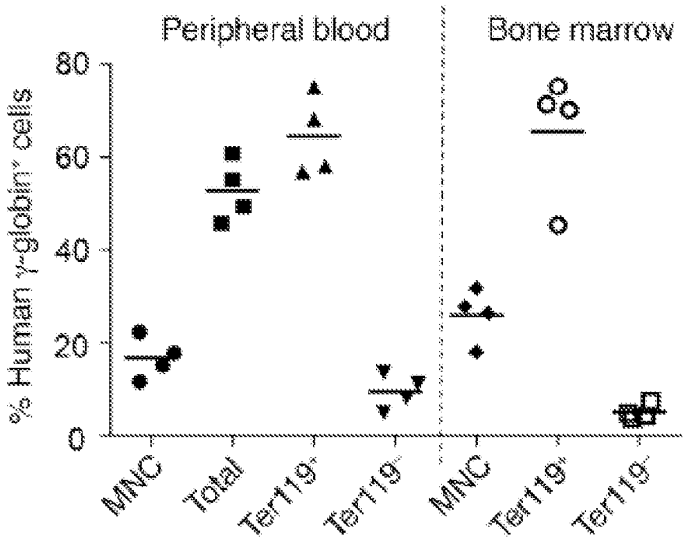

Peripheral blood
Bone marrow
% Human γ-globin+ cells
80
60
40
20
0
MNC
Total
Ter119+
Ter119-
MNC
Ter119+
Ter119-

FIG. 2E

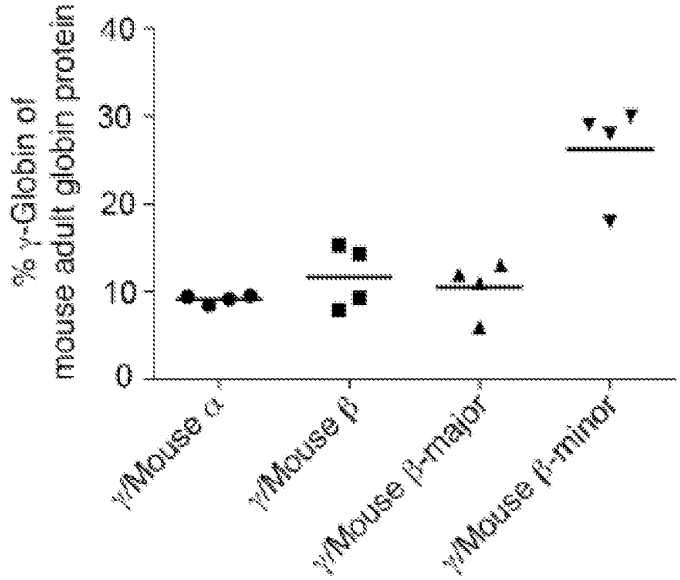

% γ-Globin of mouse adult globin protein
40
30
20
10
0
γ/Mouse α
γ/Mouse β
γ/Mouse β-major
γ/Mouse β-minor

FIG. 2F

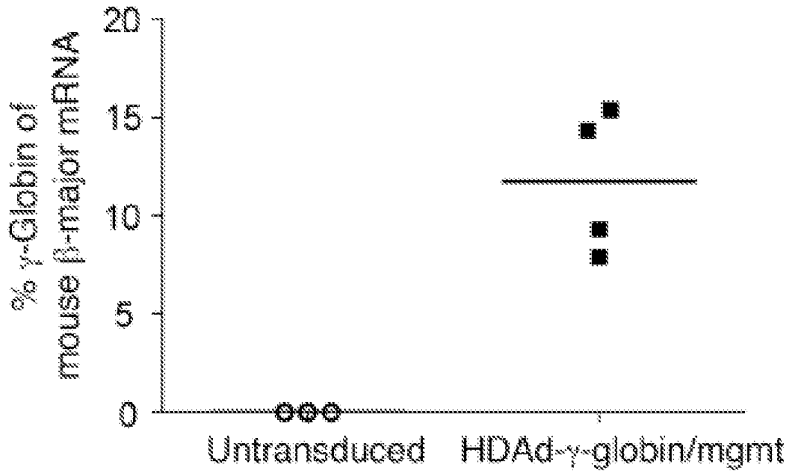

% γ-Globin of mouse β-major mRNA
20
15
10
5
0
Untransduced
HDAd-γ-globin/mgmt

FIG. 3

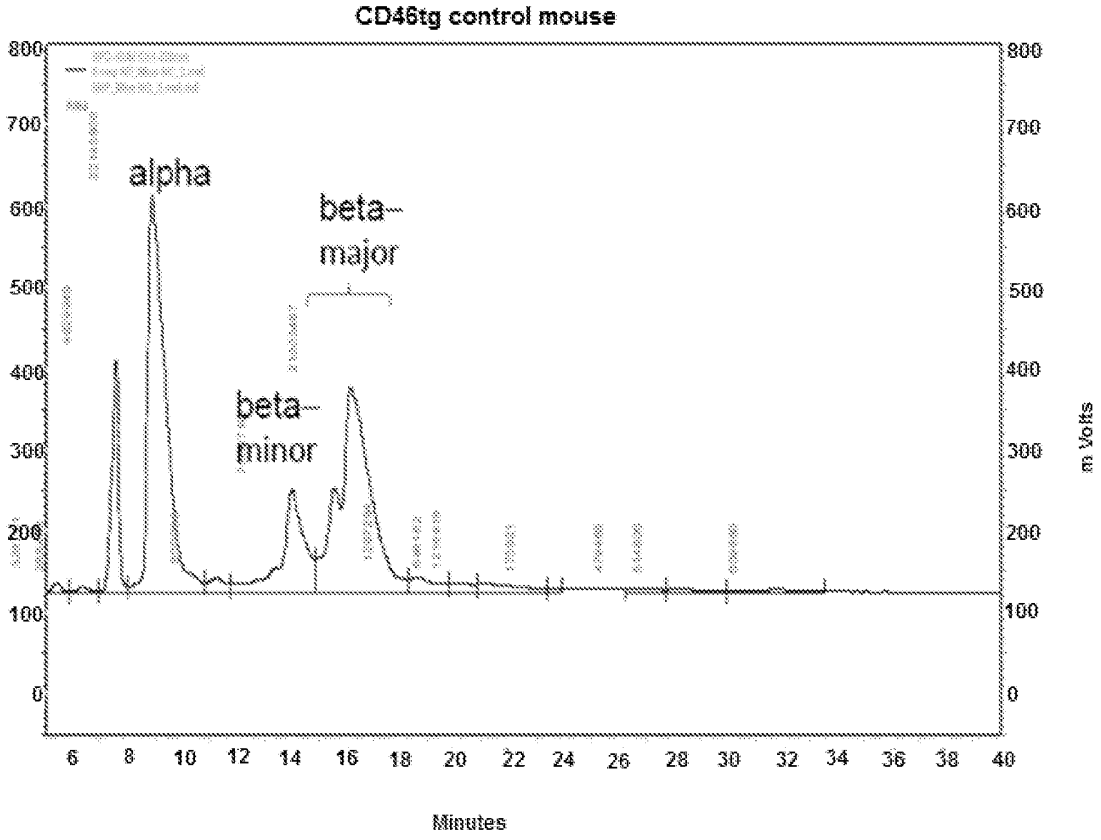

CD46tg control mouse
alpha
beta-major
beta-minor
800
700
600
500
400
300
200
100
0
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
Minutes
m Volts

FIG. 3 cont'd

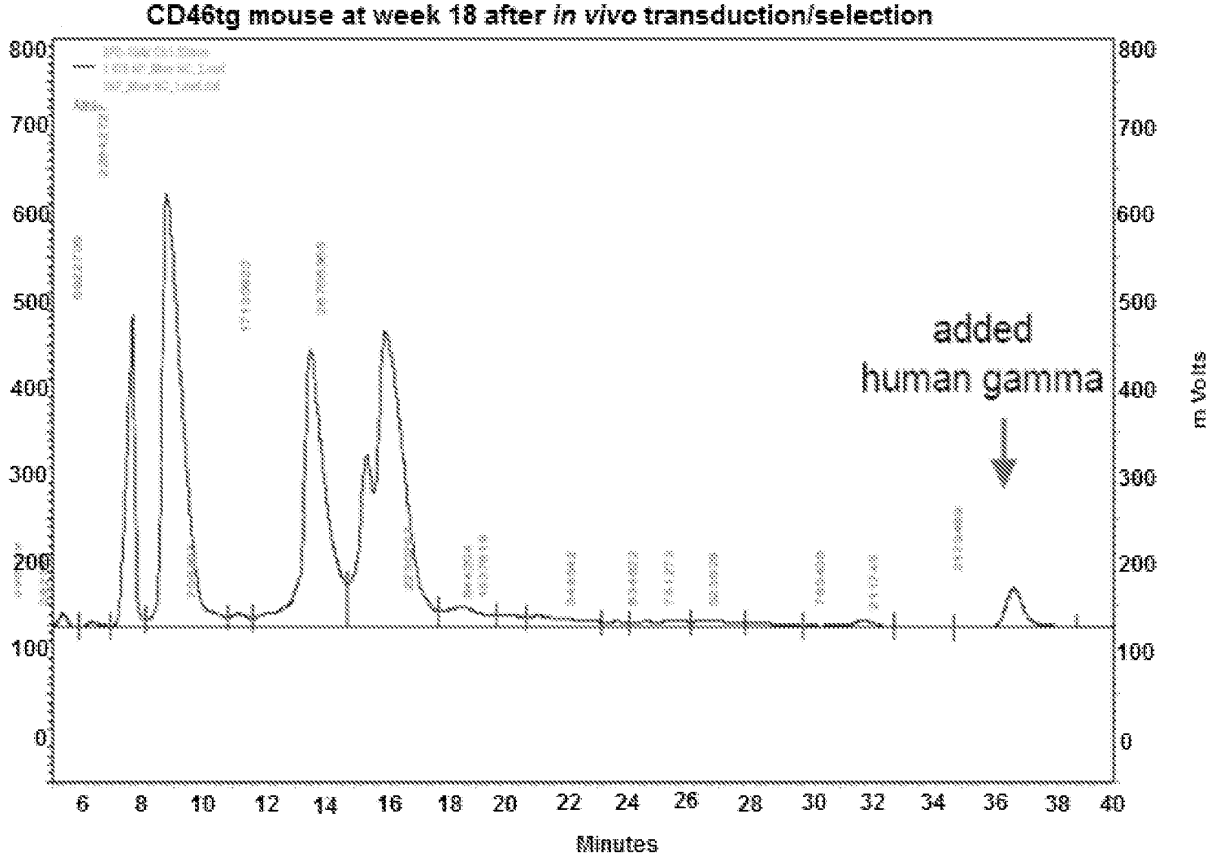

CD46tg mouse at week 18 after in vivo transduction/selection
added
human gamma
800
700
600
500
400
300
200
100
0
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
Minutes
m Volts

FIG. 4A

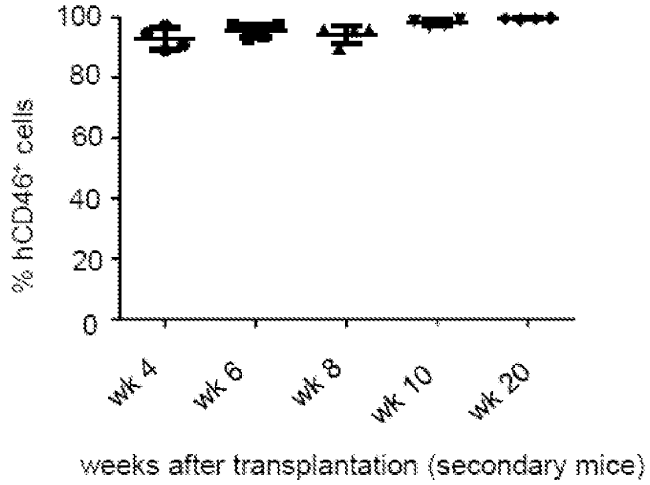


FIG. 4B

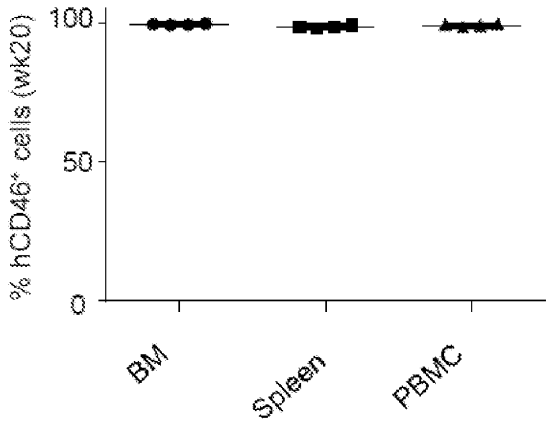


FIG. 4C

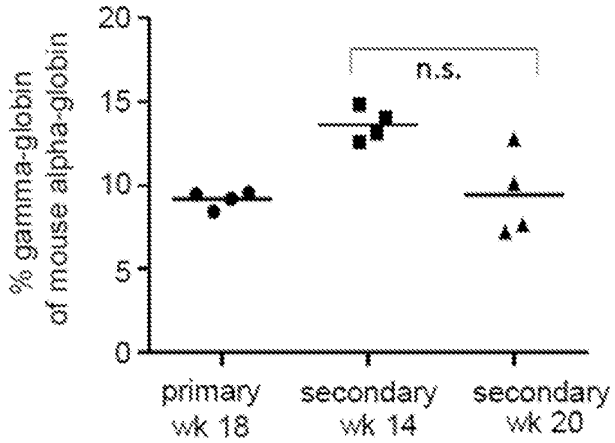


FIG. 5A



FIG. 5B

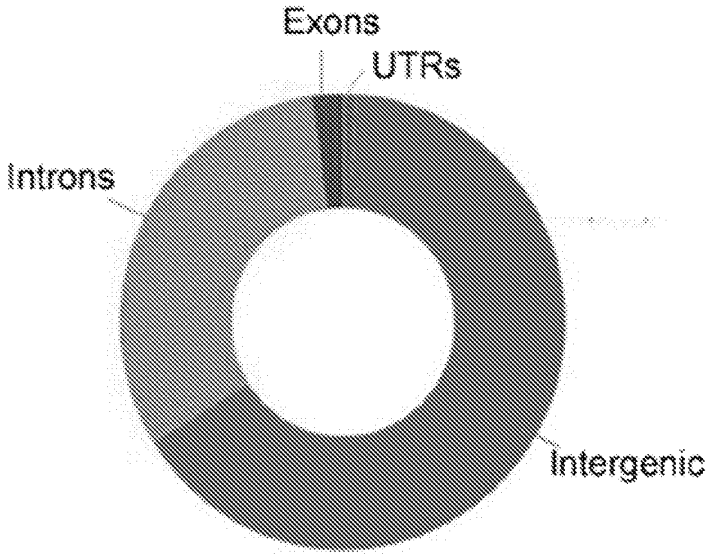

Exons
UTRs
Introns
Intergenic

FIG. 5C

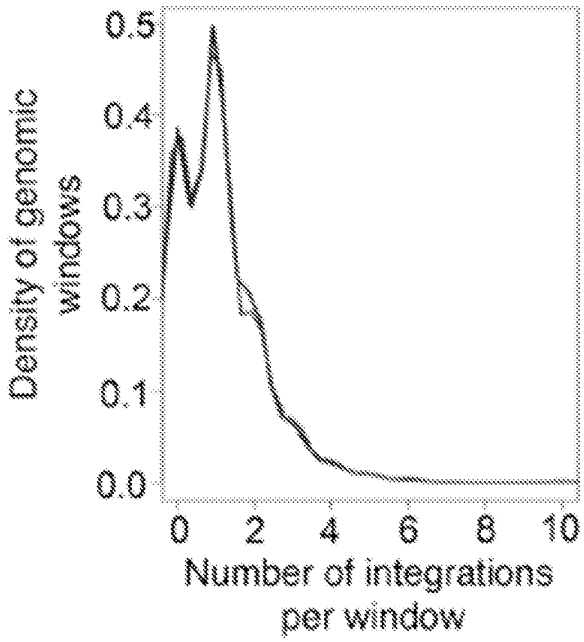

0.5
0.4
0.3
0.2
0.1
0.0
Density of genomic windows
0
2
4
6
8
10
Number of integrations per window

FIG. 5D

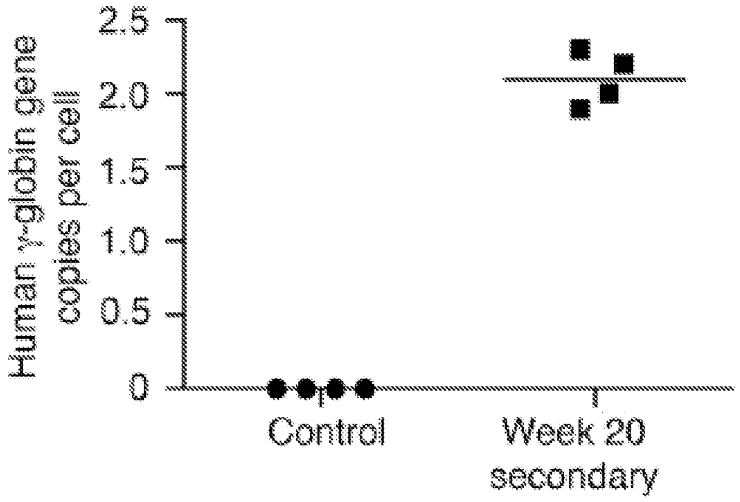

Human γ-globin gene copies per cell
2.5
2.0
1.5
1.0
0.5
0
Control
Week 20 secondary

FIG. 5E

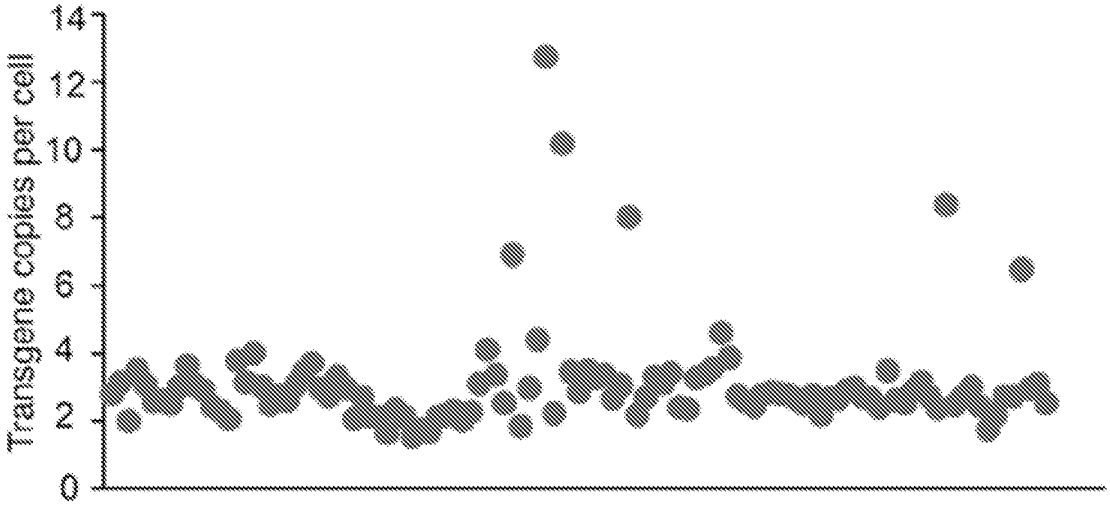

Transgene copies per cell
14
12
10
8
6
4
2
0

FIG. 6

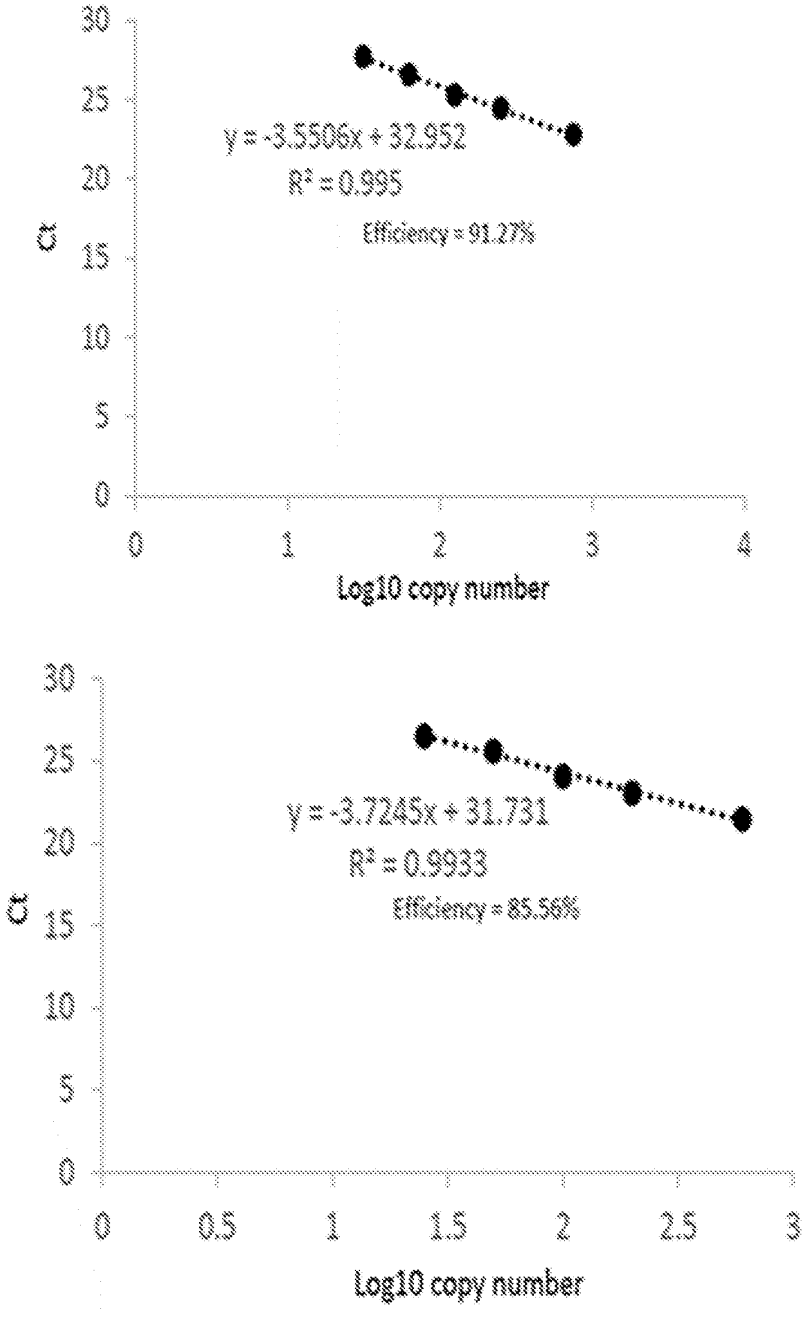

30
25
20
15
10
5
0
Ct
y = -3.5506x + 32.952
R² = 0.995
Efficiency = 91.27%
0
1
2
3
4
Log10 copy number
30
25
20
15
10
5
0
Ct
y = -3.7245x + 31.731
R² = 0.9933
Efficiency = 85.56%
0
0.5
1
1.5
2
2.5
3
Log10 copy number

FIG. 7A

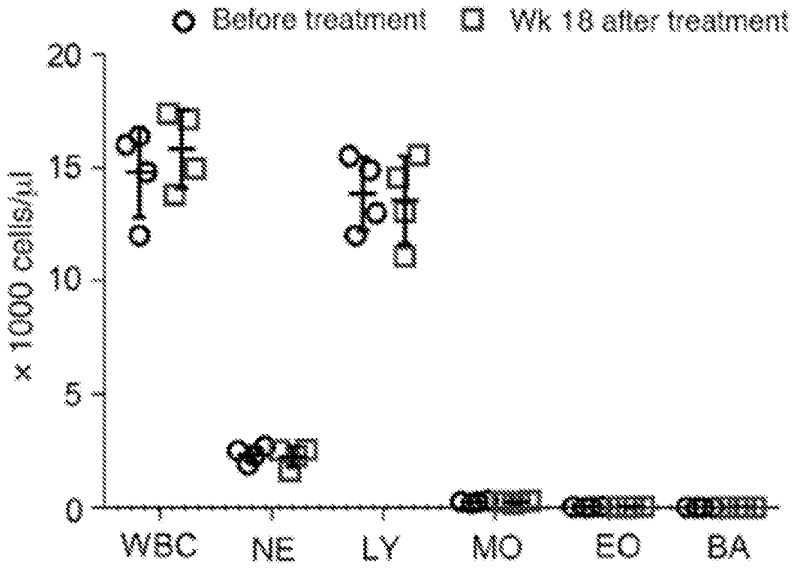

Before treatment
Wk 18 after treatment
20
15
10
5
0
× 1000 cells/μl
WBC
NE
LY
MO
EO
BA

FIG. 7B

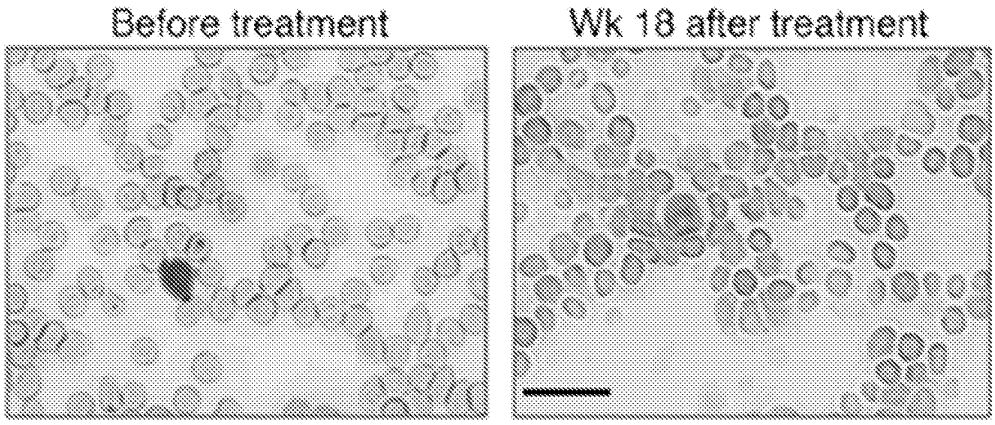

Before treatment
Wk 18 after treatment

FIG. 7C

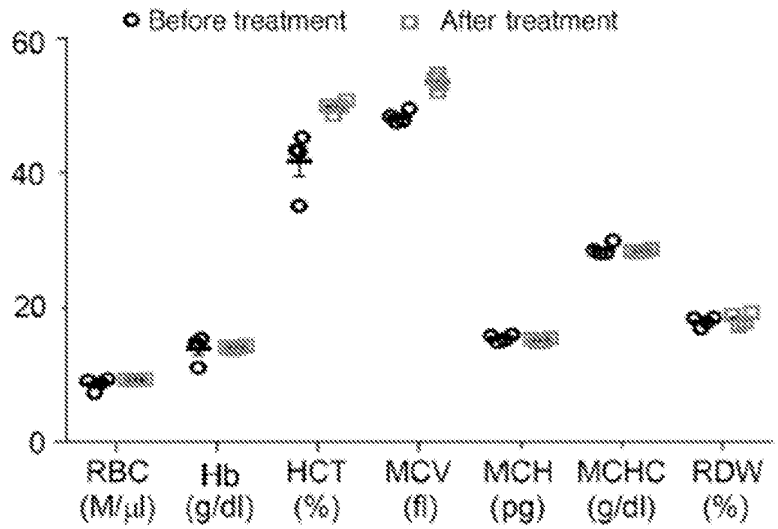

Before treatment
After treatment
60
40
20
0
RBC (M/μl)
Hb (g/dl)
HCT (%)
MCV (fl)
MCH (pg)
MCHC (g/dl)
RDW (%)

FIG. 7D

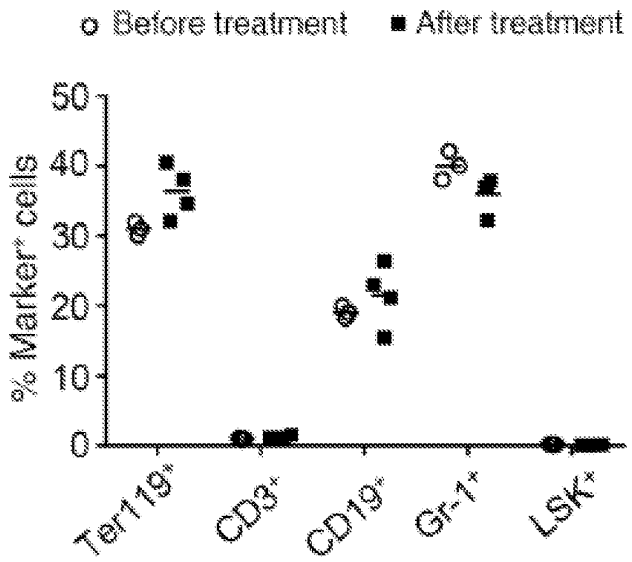

Before treatment
After treatment
% Marker+ cells
50
40
30
20
10
0
Ter119+
CD3+
CD19+
Gr-1+
LSK+

FIG. 7E

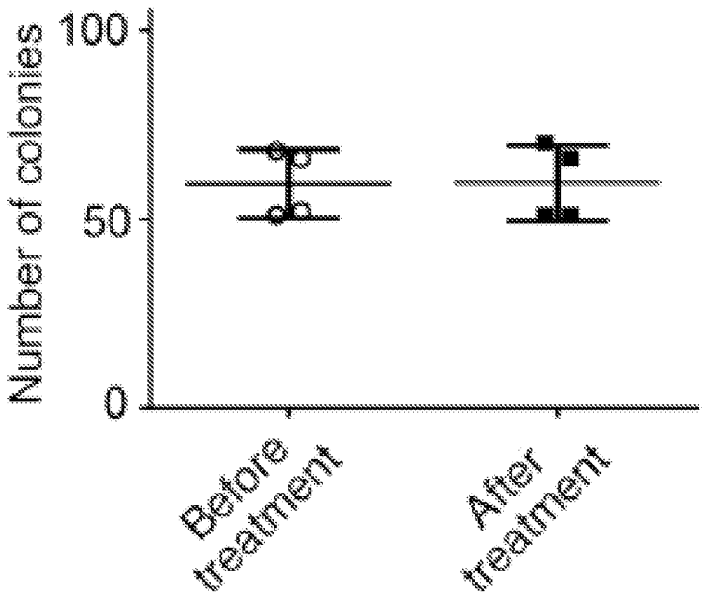

Number of colonies
100
50
0
Before treatment
After treatment

FIG. 8

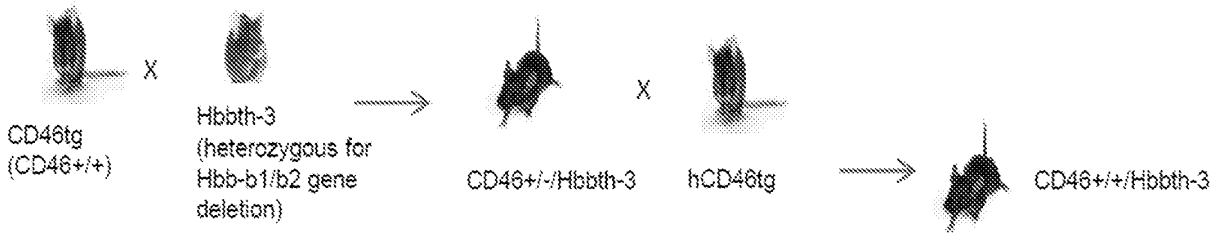

X
CD46tg
(CD46+/+)
Hbbth-3
(heterozygous for
Hbb-b1/b2 gene
deletion)
CD46+/-/Hbbth-3
X
hCD46tg
CD46+/+/Hbbth-3

FIG. 9A

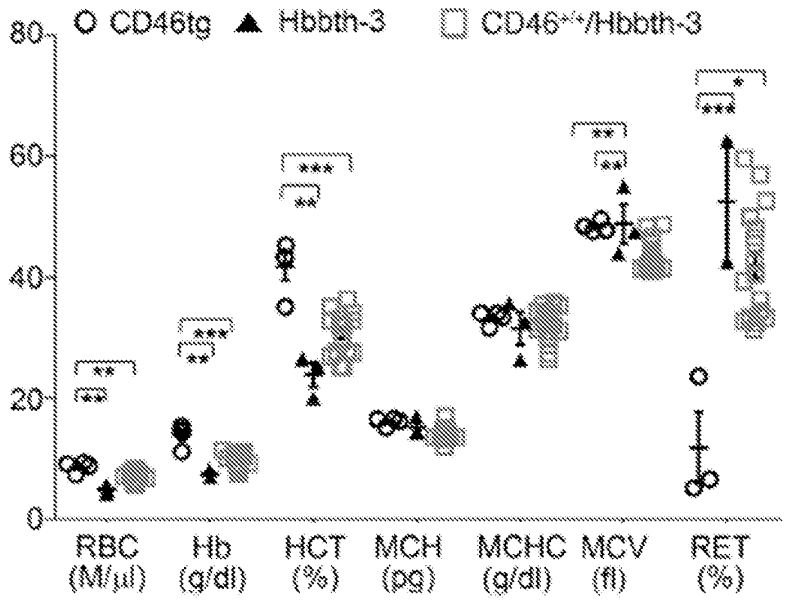

CD46tg
Hbbth-3
CD46-/-/Hbbth-3
80
60
40
20
0
RBC (M/μl)
Hb (g/dl)
HCT (%)
MCH (pg)
MCHC (g/dl)
MCV (fl)
RET (%)

FIG. 9B

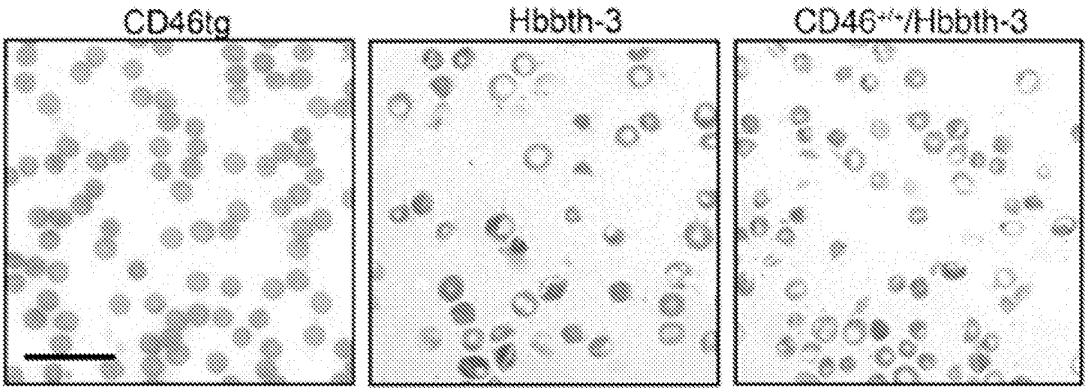

CD46tg
Hbbth-3
CD46-/-/Hbbth-3

FIG. 9C

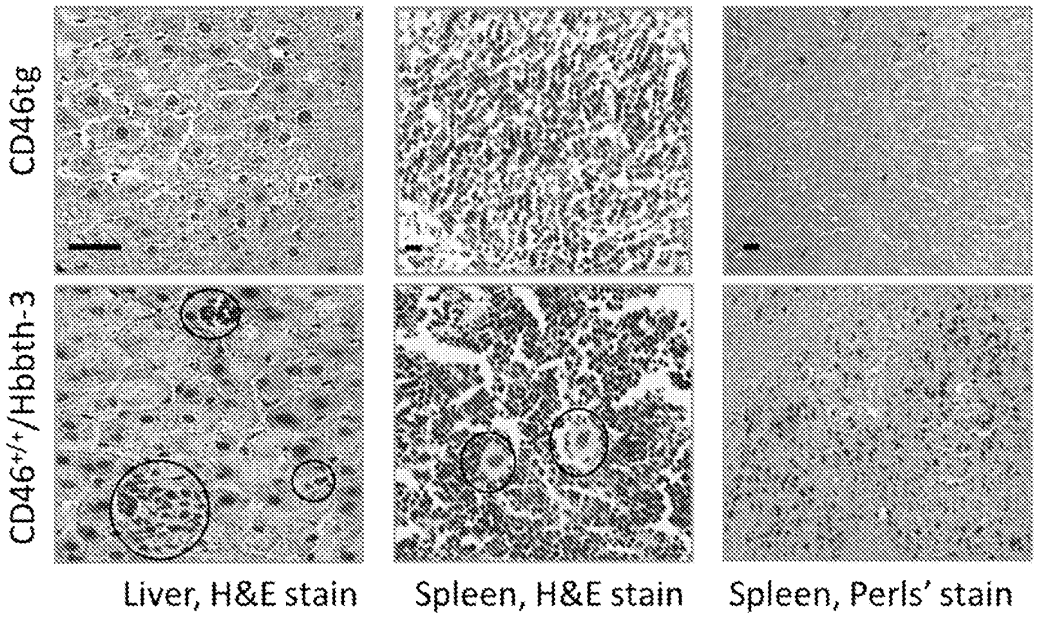

CD46tg
CD46+/+/Hbbth-3
Liver, H&E stain
Spleen, H&E stain
Spleen, Perls' stain

FIG. 10

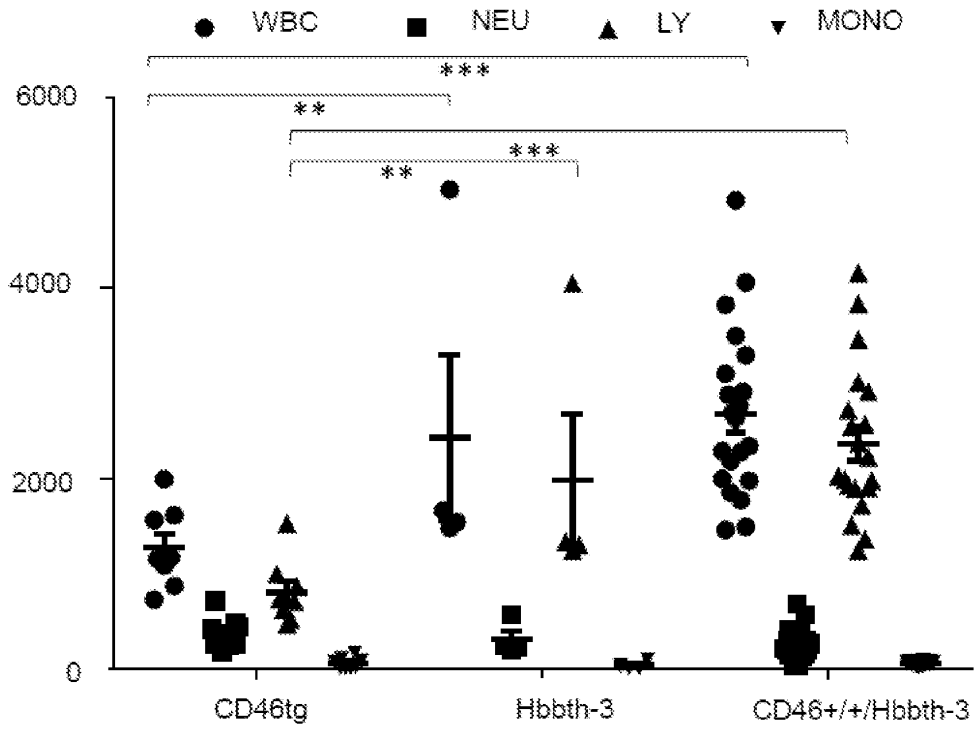

WBC
NEU
LY
MONO
***
**
***
**
6000
4000
2000
0
CD46tg
Hbbth-3
CD46+/+/Hbbth-3

FIG. 11

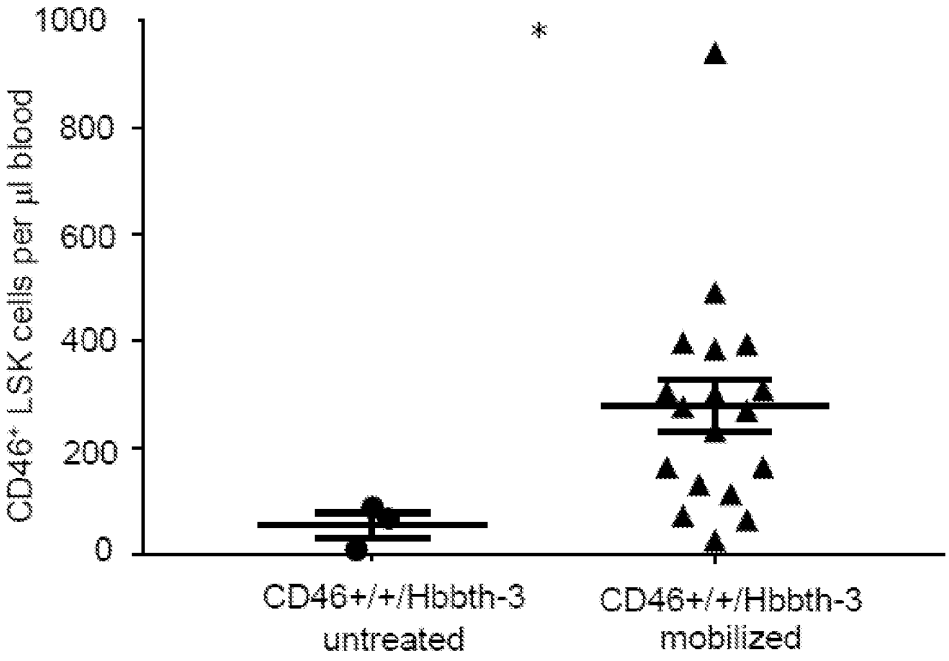

1000
800
600
400
200
0
CD46+ LSK cells per µl blood
*
CD46+/+/Hbbth-3
untreated
CD46+/+/Hbbth-3
mobilized

FIG. 12

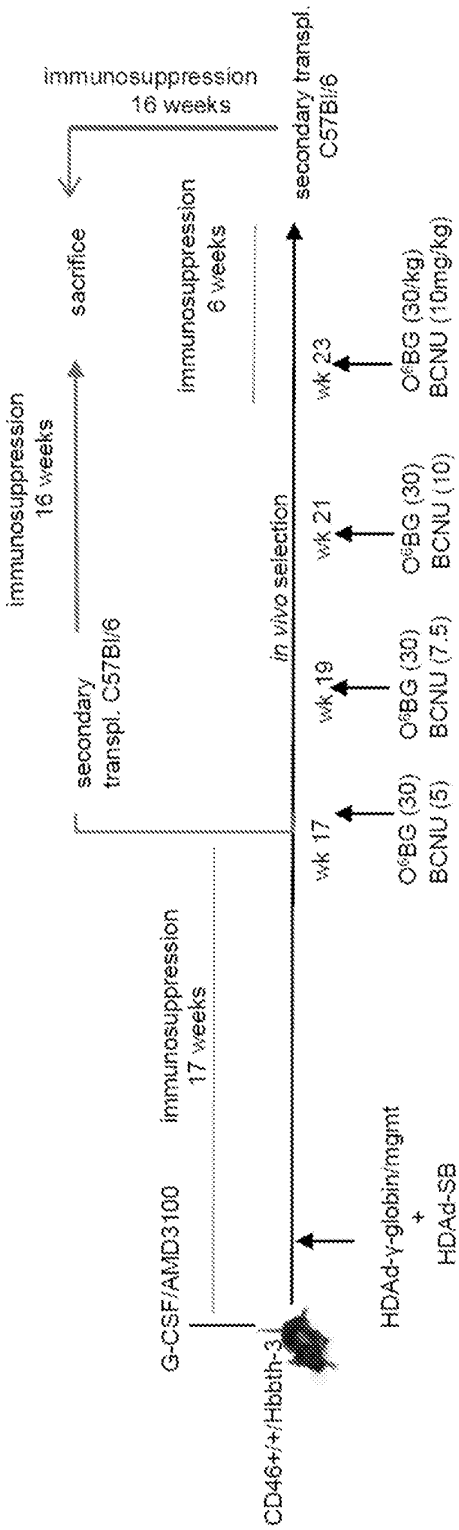

CD46+/+/Hbb^{th-3}
G-CSF/AMD3100
immunosuppression
17 weeks
HDAd-γ-globin/mgmt
+
HDAd-SB
(2x10^10 vp, twice
IV
wk 17
O6BG (30)
BCNU (5)
wk 19
O6BG (30)
BCNU (7.5)
in vivo selection
wk 21
O6BG (30)
BCNU (10)
wk 23
O6BG (30/kg)
BCNU (10mg/kg)
secondary transpl. C57Bl/6
immunosuppression
6 weeks
secondary
transpl. C57Bl/6
immunosuppression
16 weeks
sacrifice
immunosuppression
16 weeks
secondary transpl.
C57Bl/6

FIG. 13A

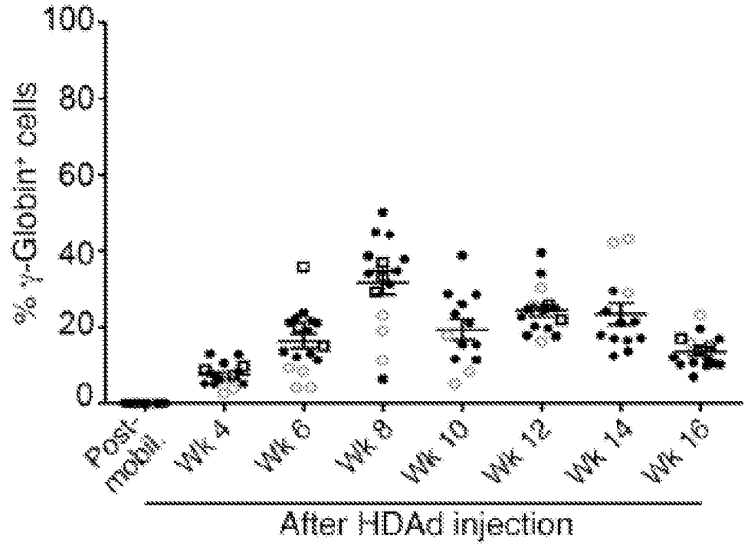


FIG. 13B

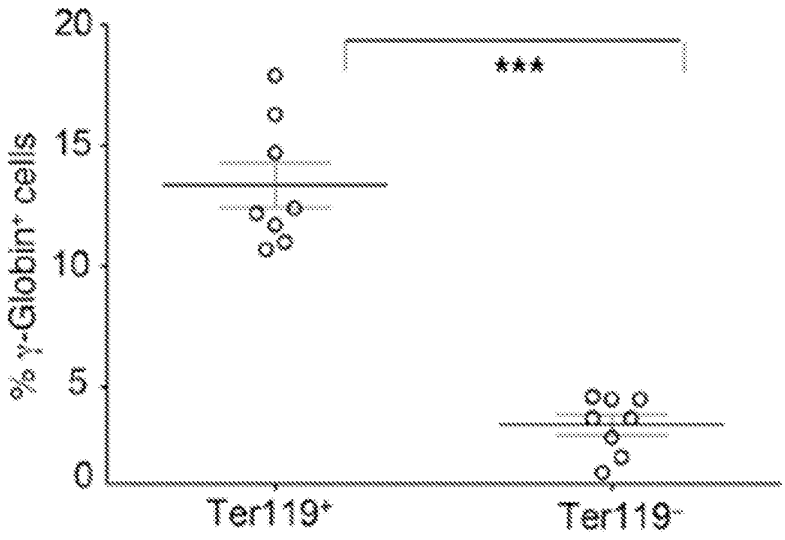


FIG. 13C

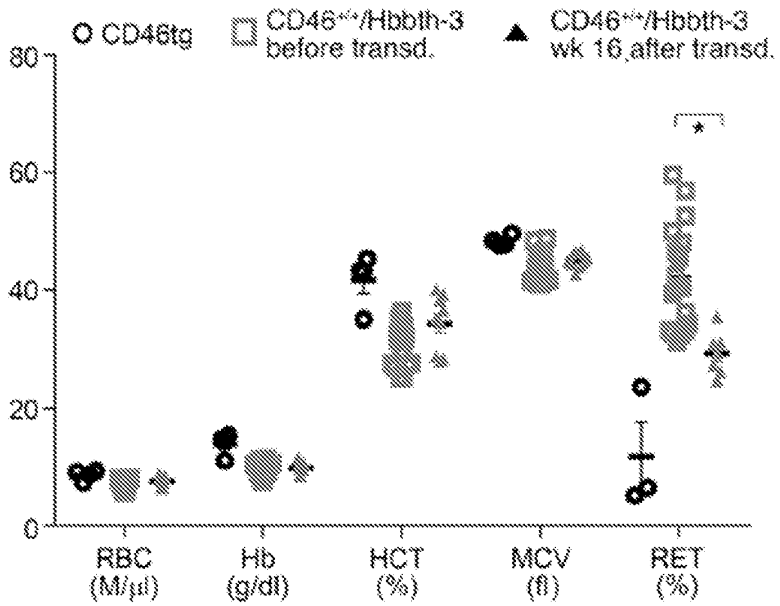


FIG. 13D

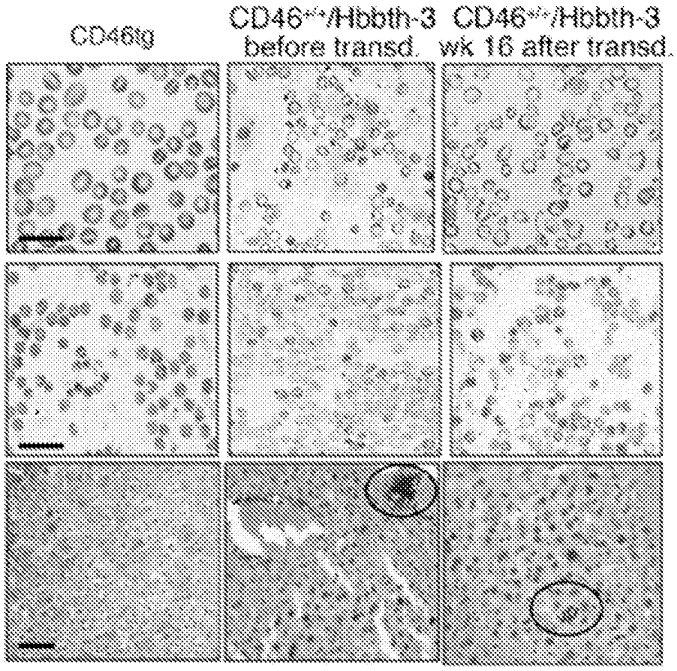


FIG. 13E

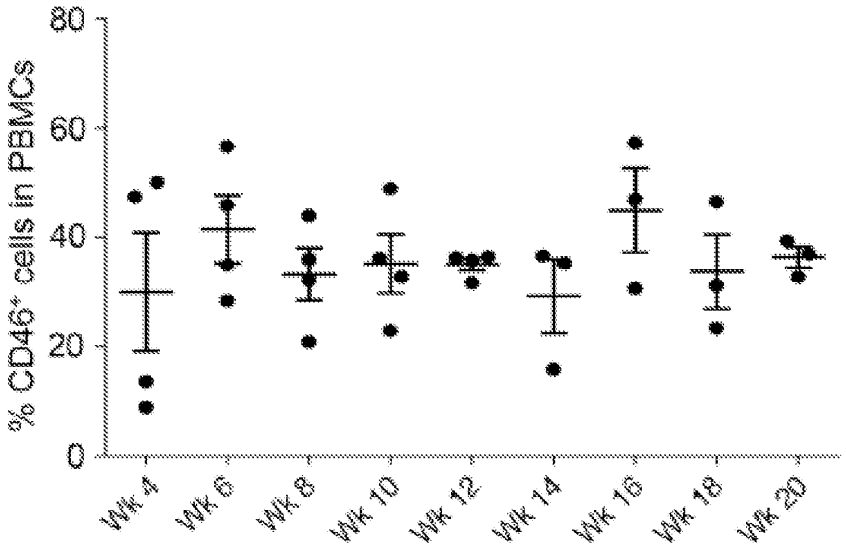


FIG. 13F

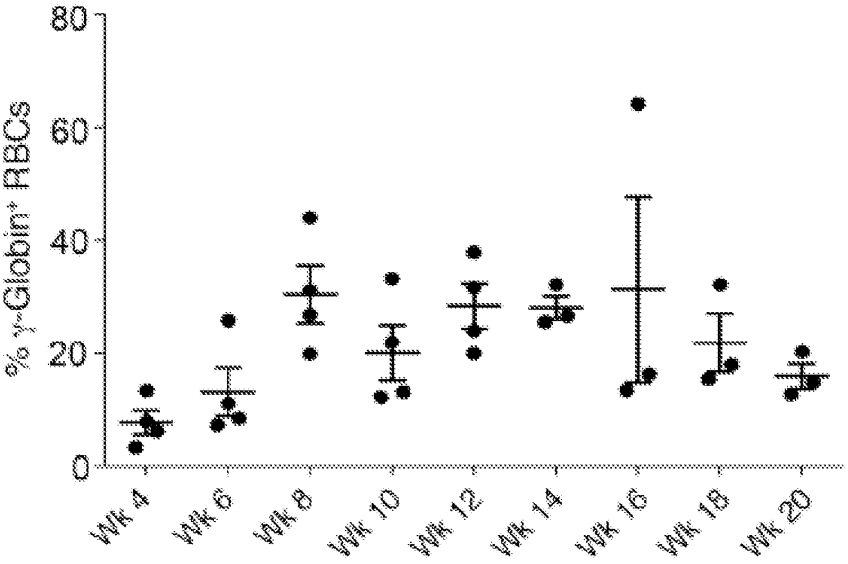


FIG. 14A

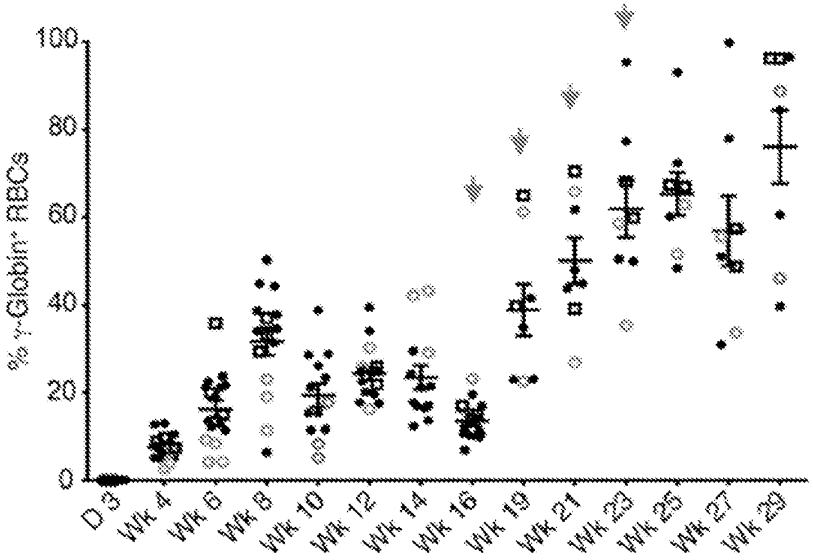


FIG. 14B

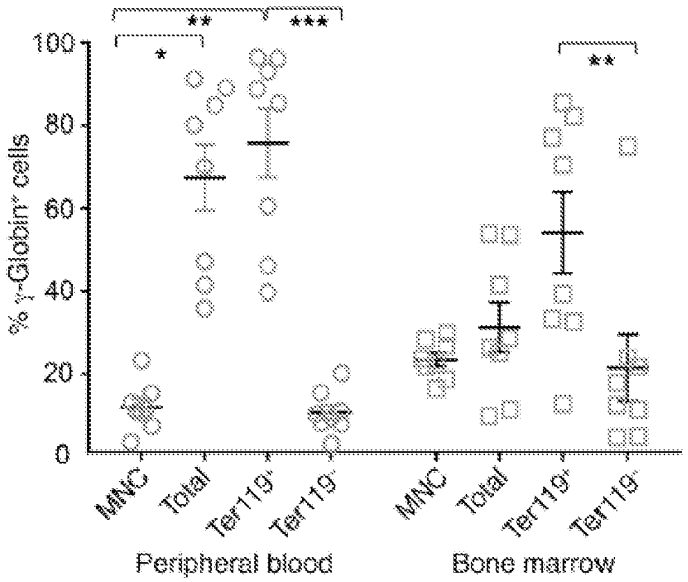


FIG. 14C

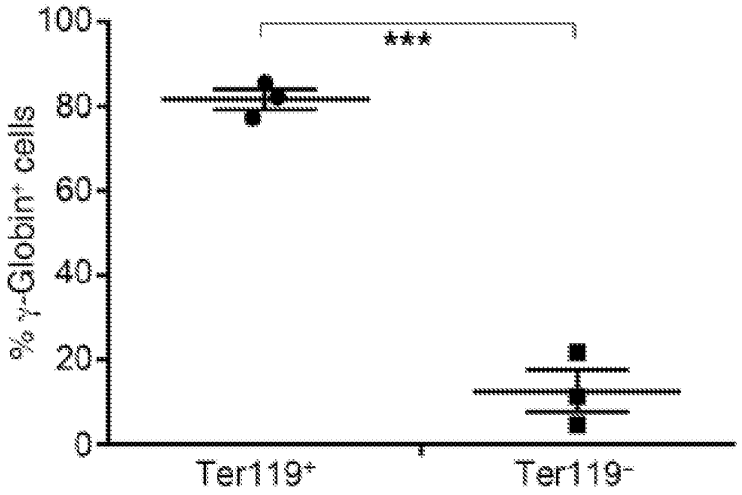


FIG. 14D

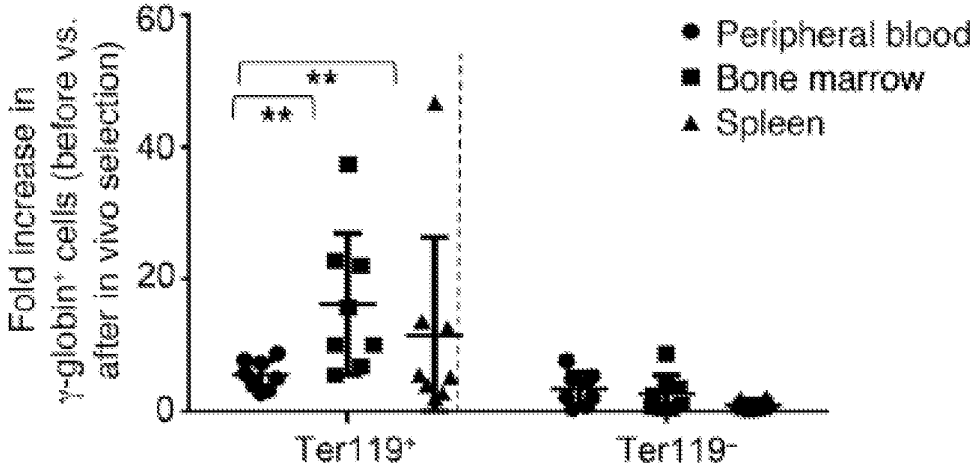


FIG. 14E

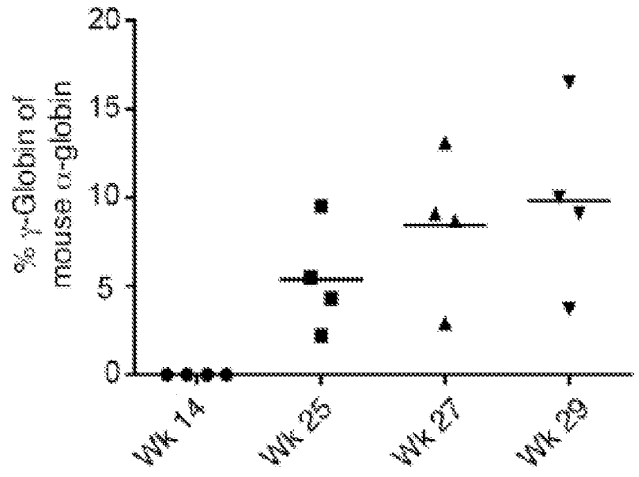


FIG. 14F

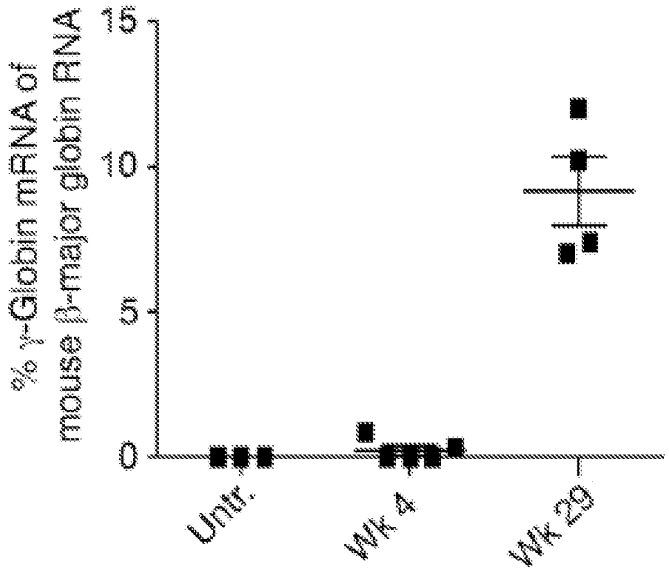


FIG. 15A

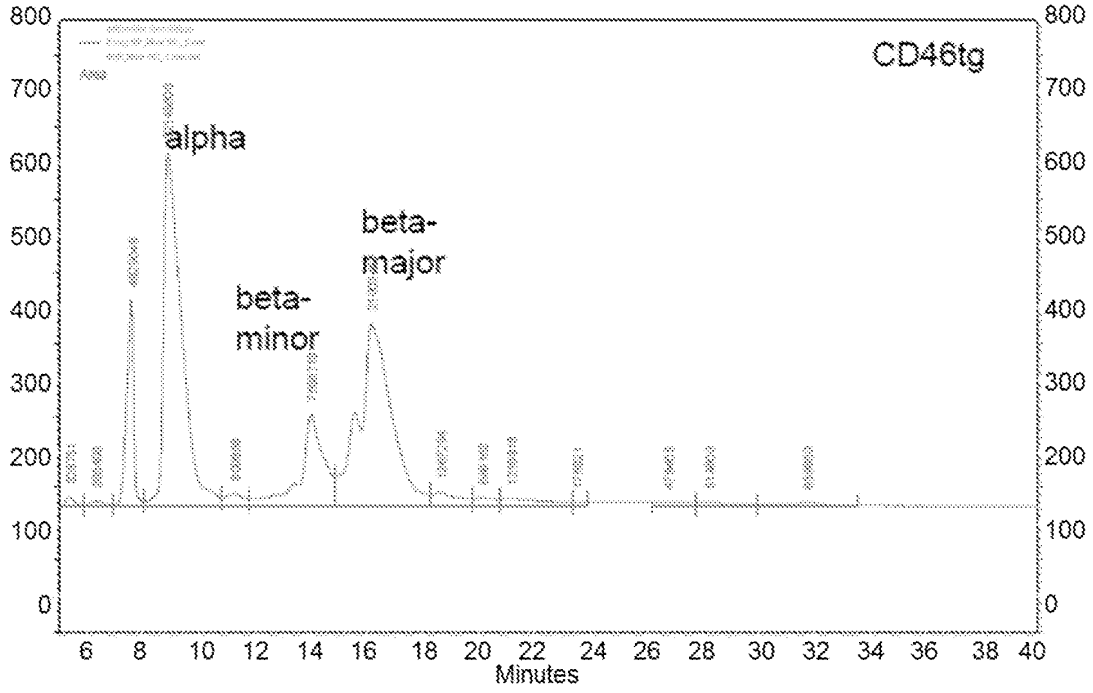


FIG. 15B

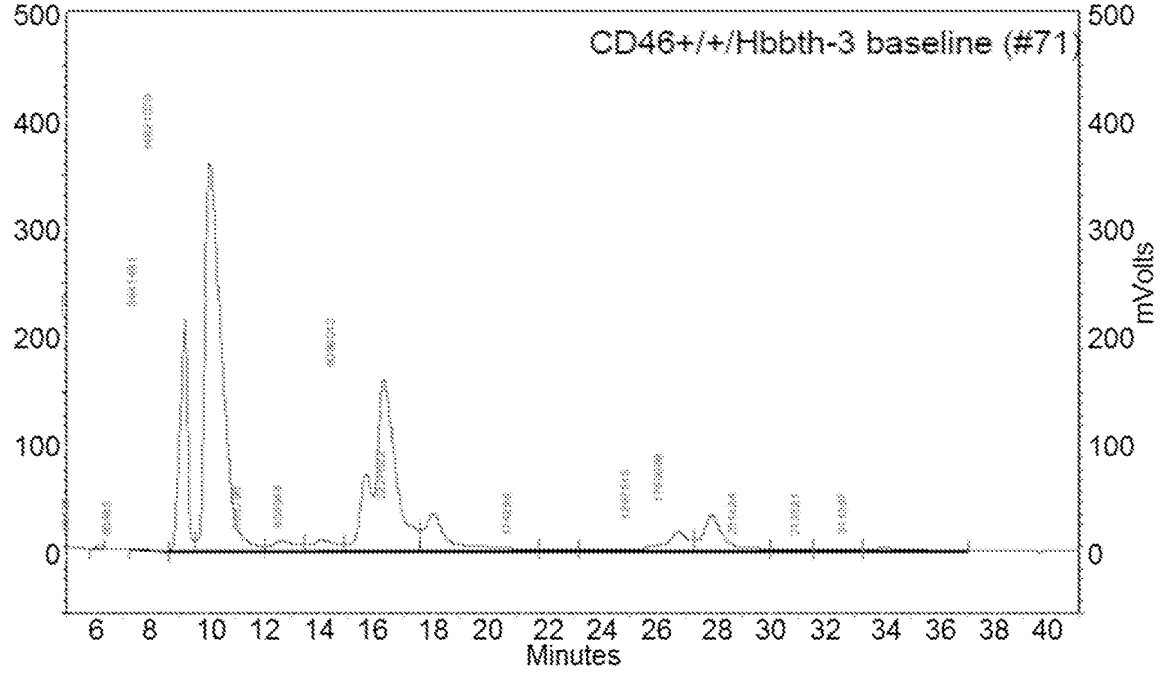


FIG. 15C

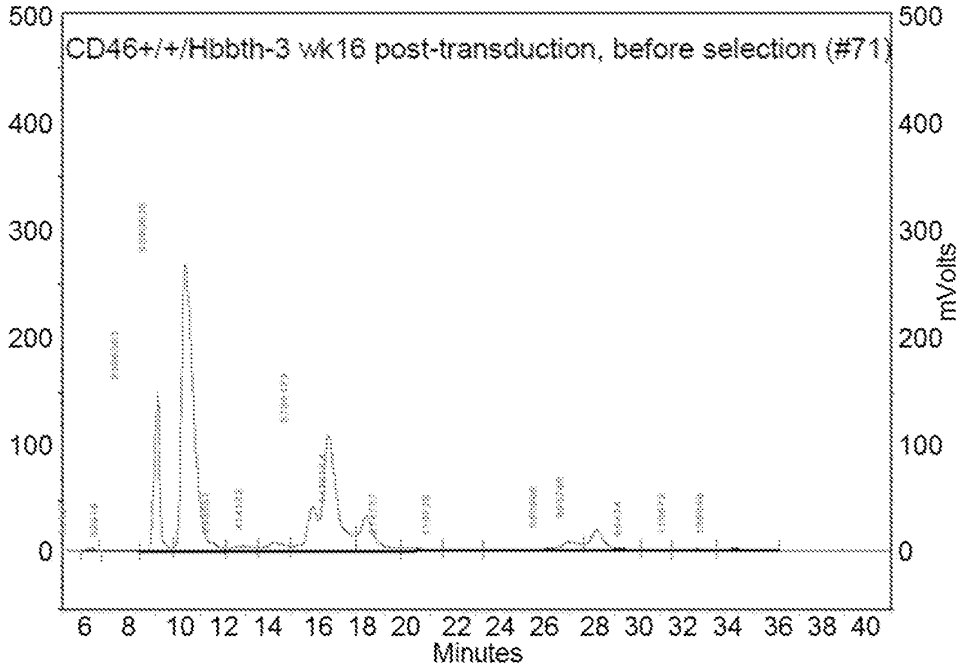


FIG. 15D

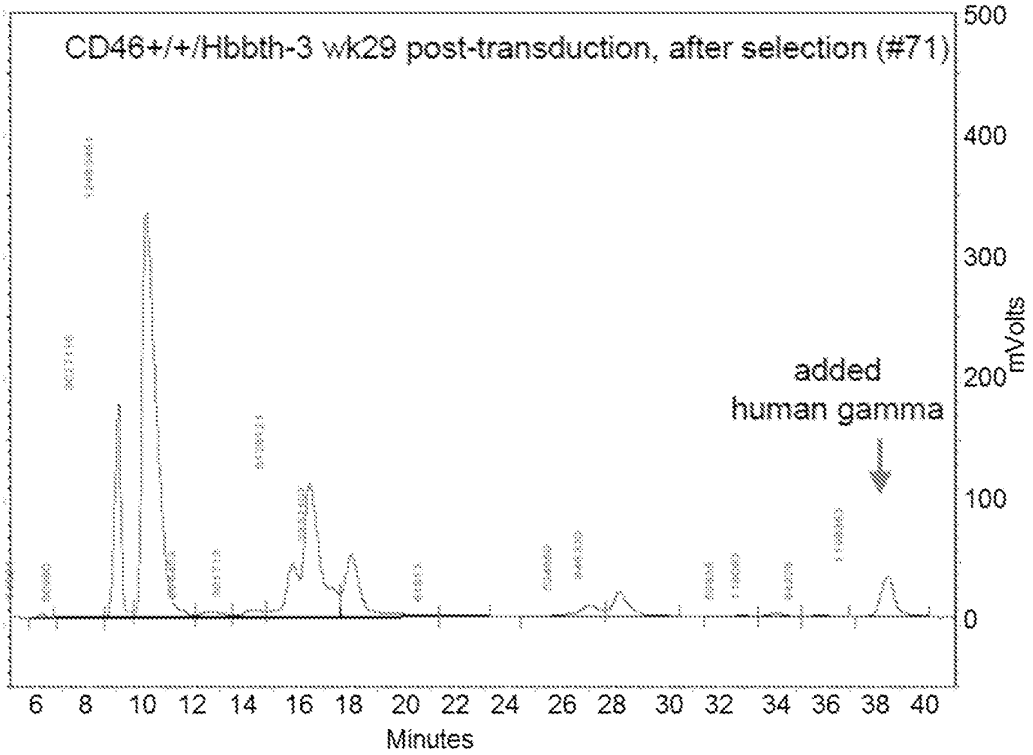


FIG. 16

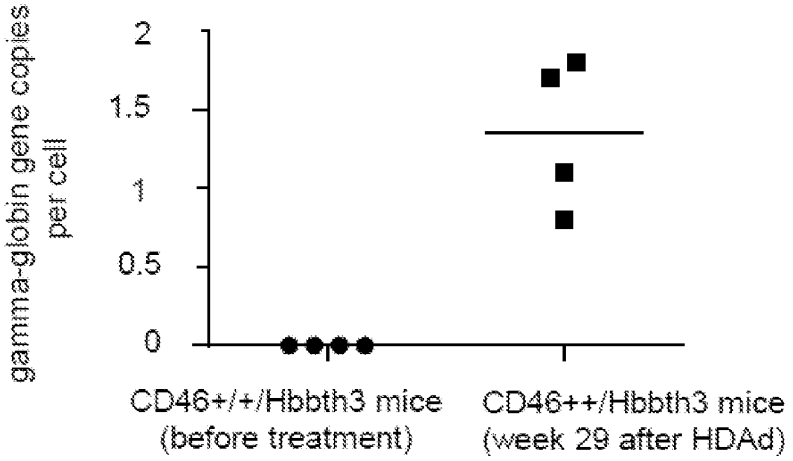


FIG. 17A

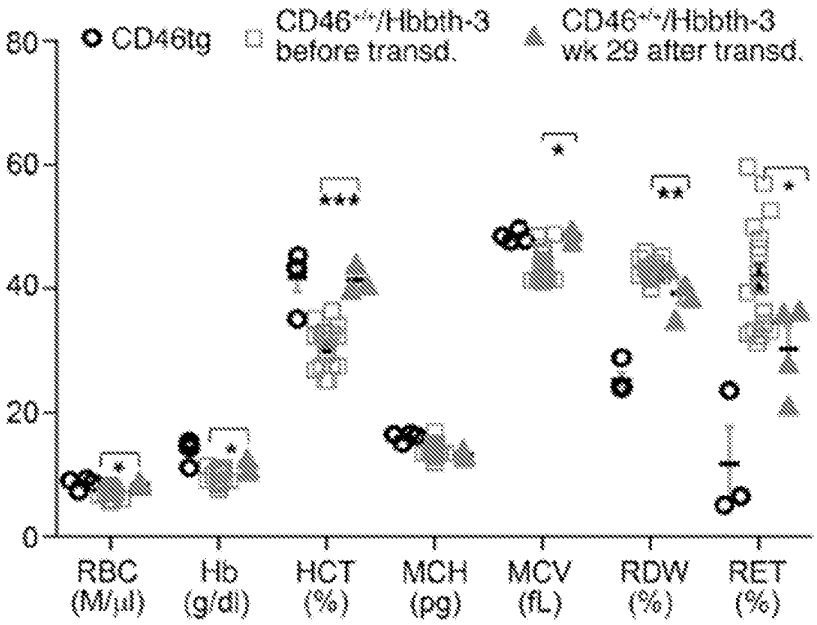


FIG. 17B

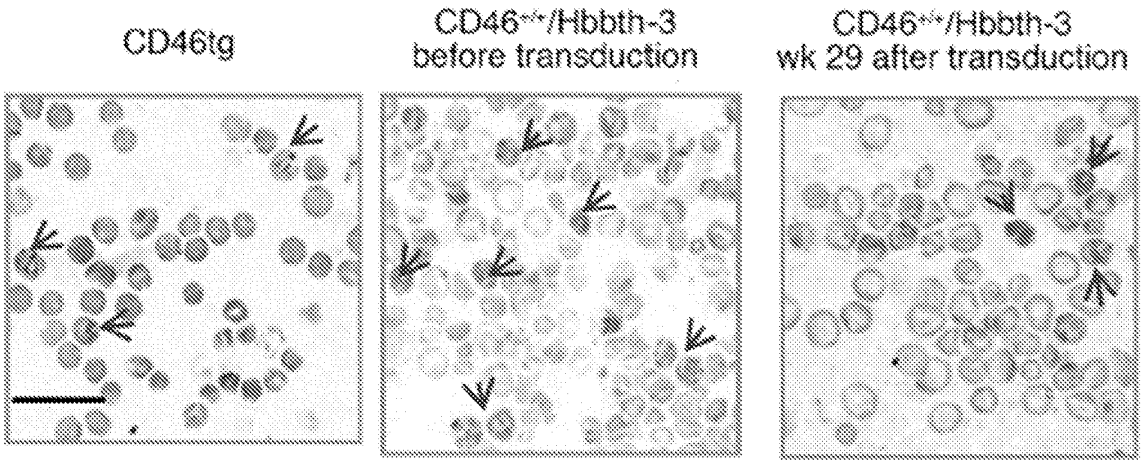


FIG. 17C

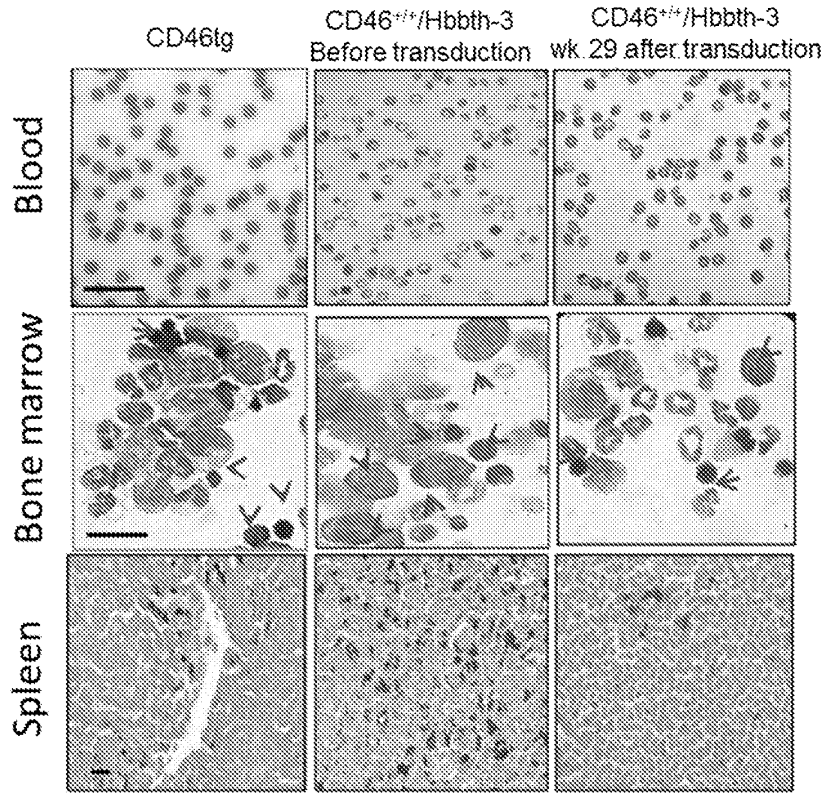


FIG. 17D

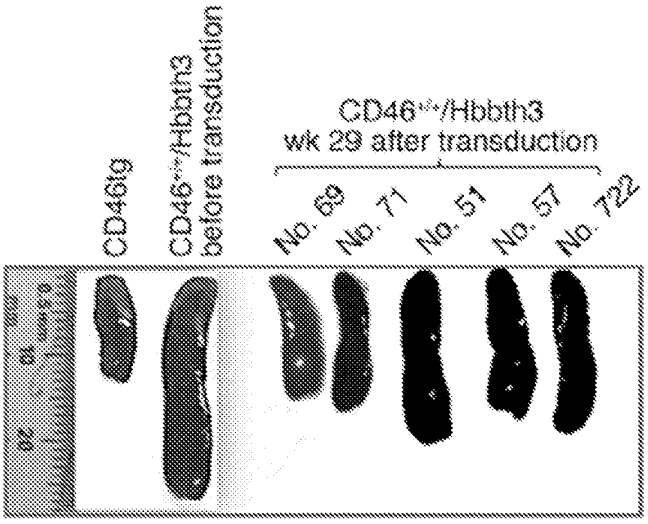


FIG. 17E

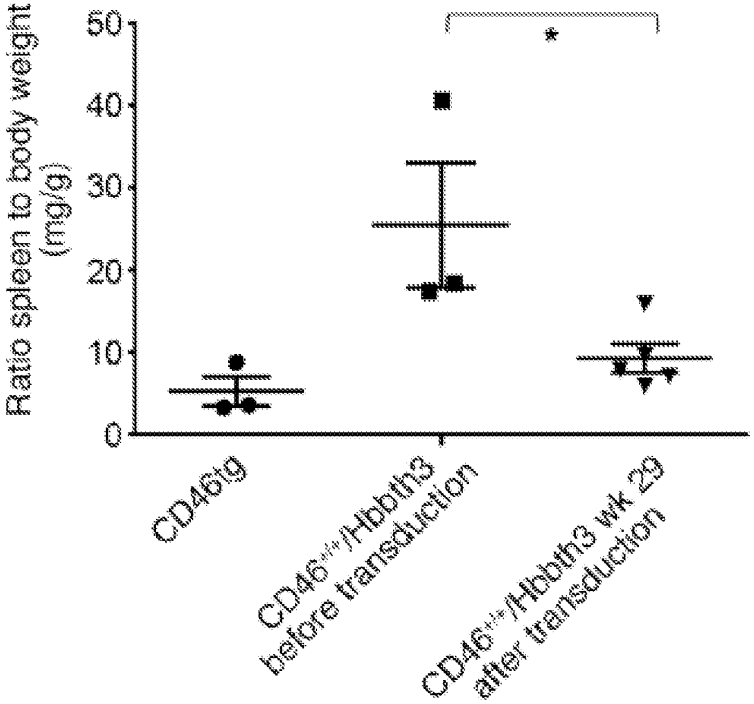


FIG. 18A

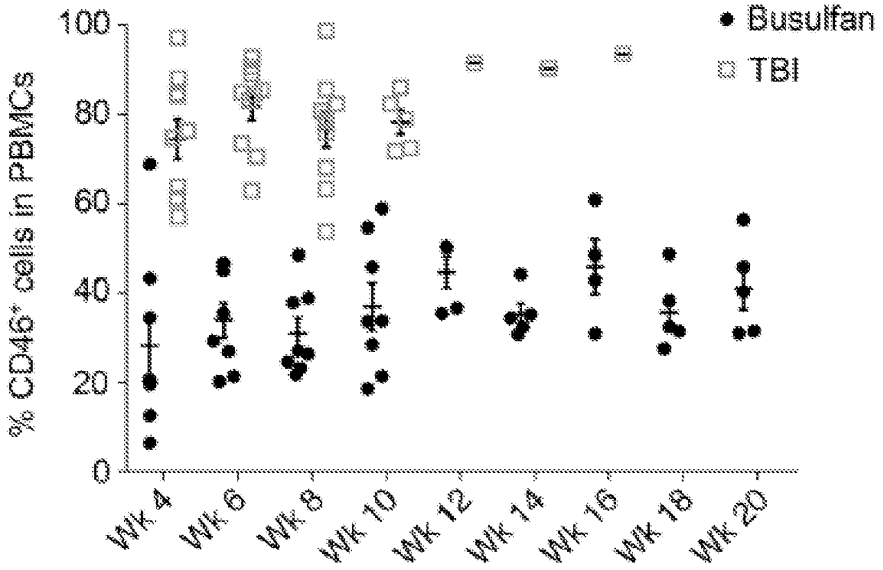


FIG. 18B

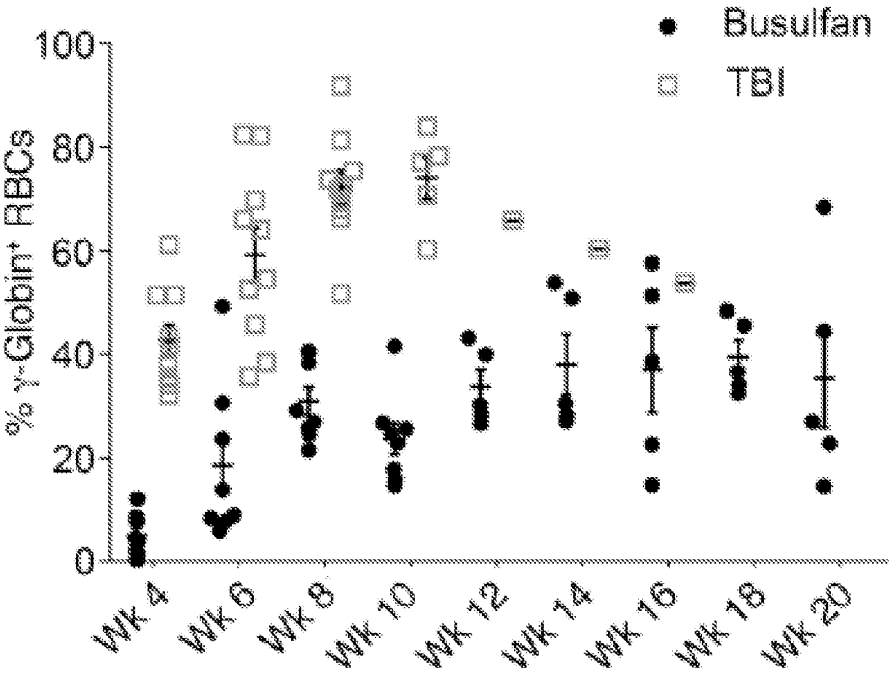


FIG. 18C

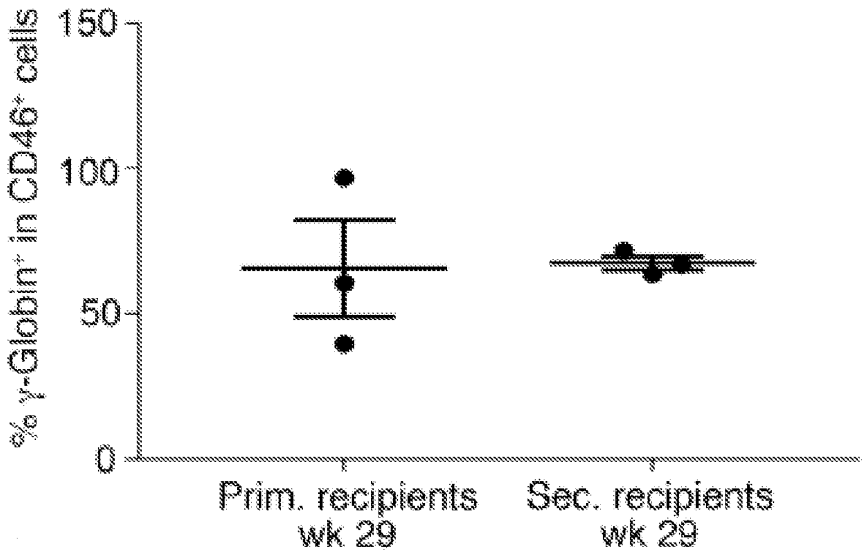


FIG. 18D

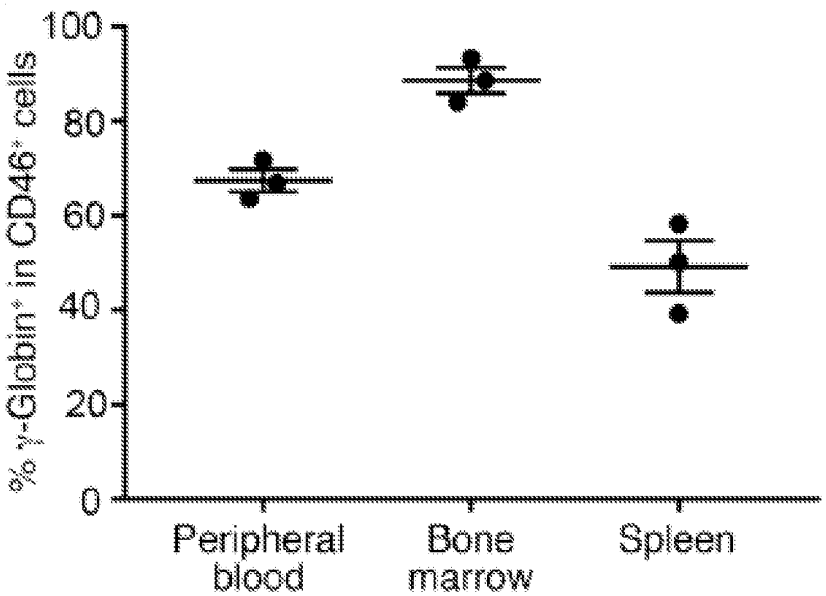


FIG. 18E

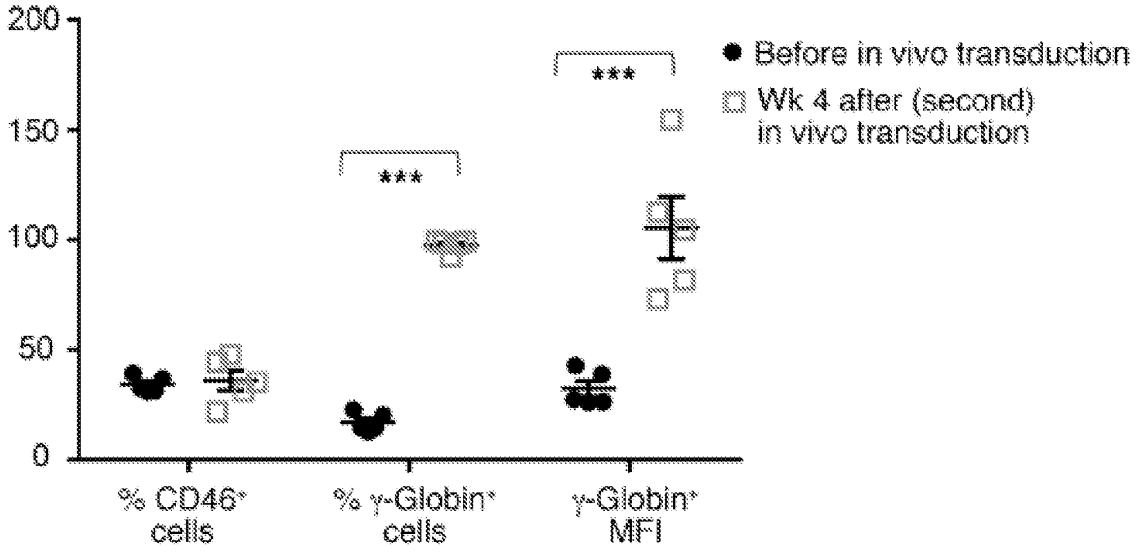


FIG. 19A

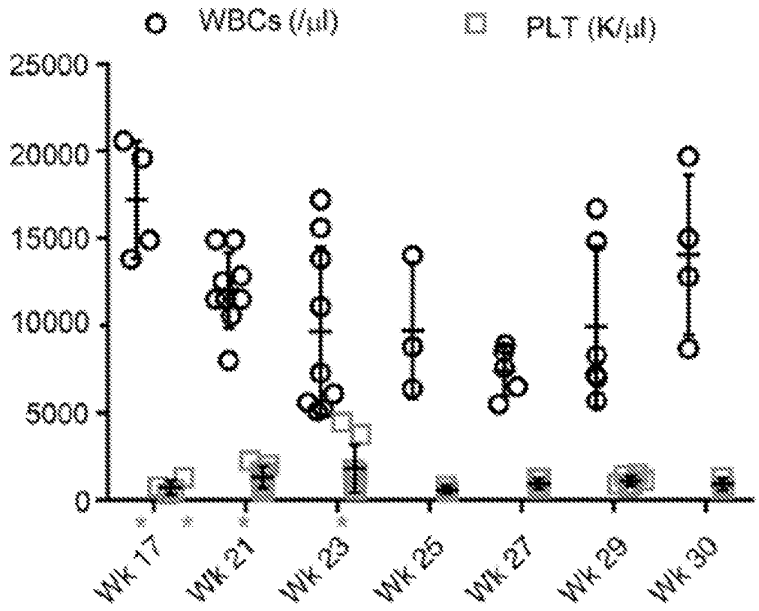


FIG. 19B

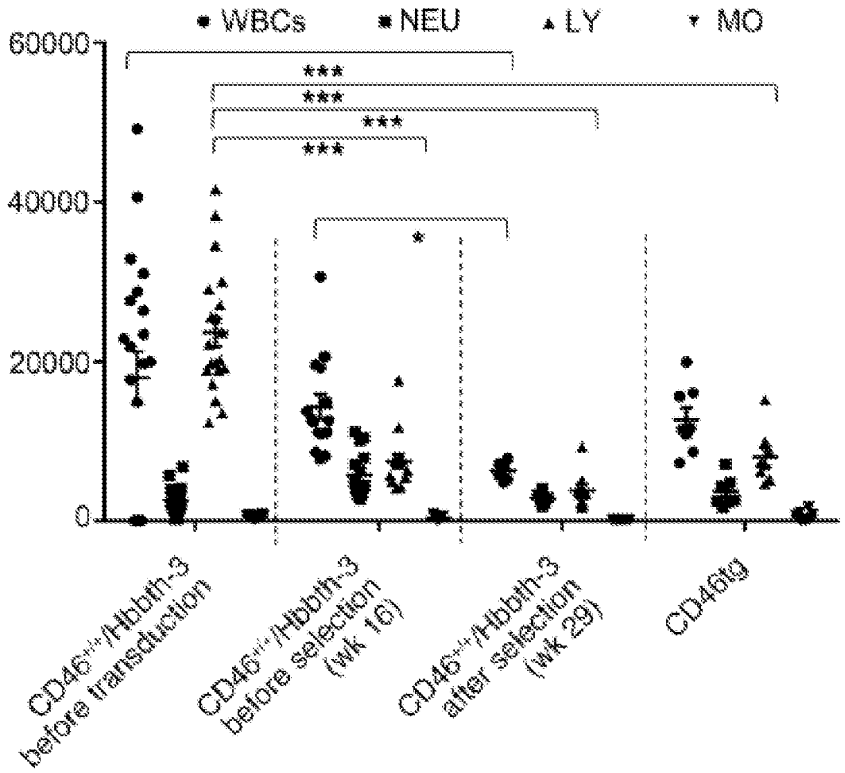


FIG. 19C

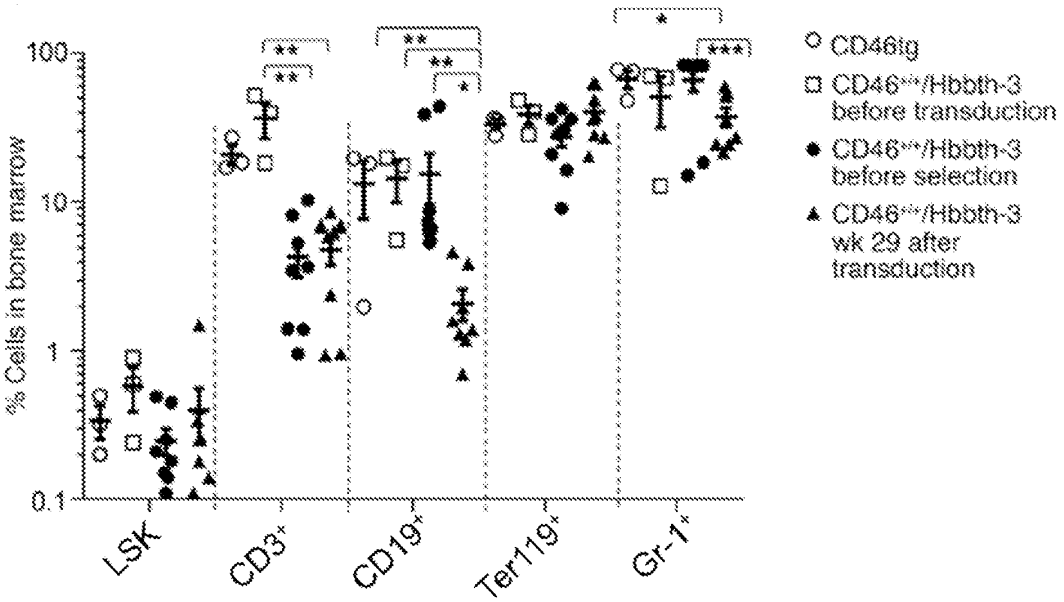


FIG. 19D

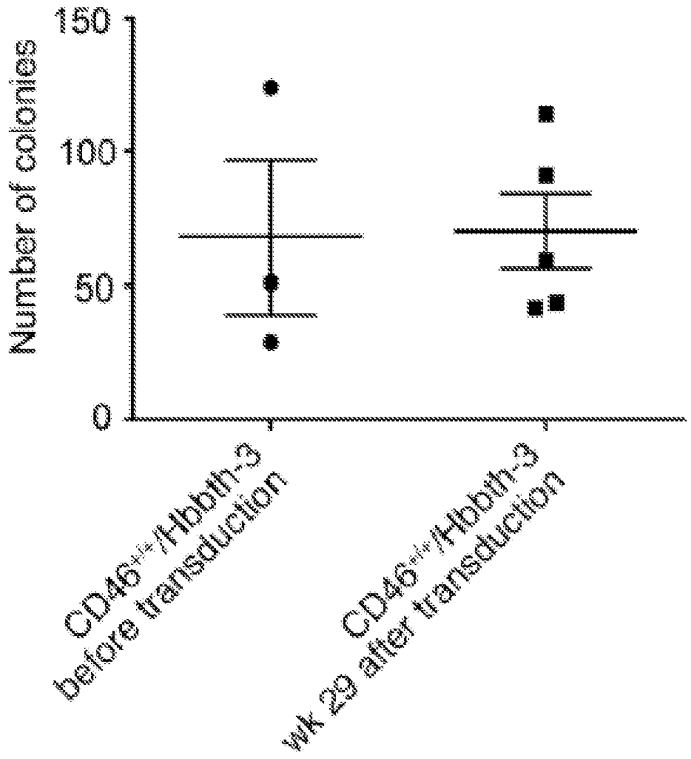

Number of colonies
150
100
50
0
CD46$^{ki/ki}$/Hbbth-3 before transduction
CD46$^{ki/ki}$/Hbbth-3 wk 29 after transduction

FIG. 20A

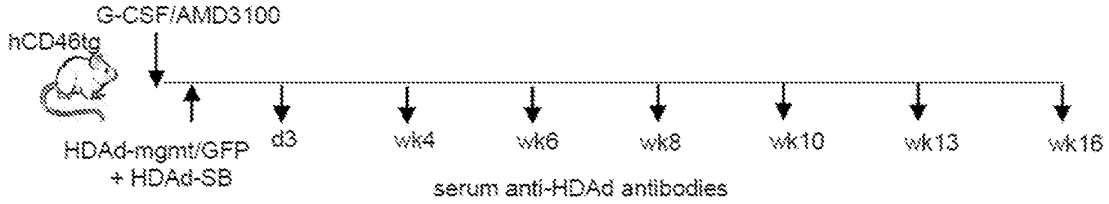


FIG. 20B

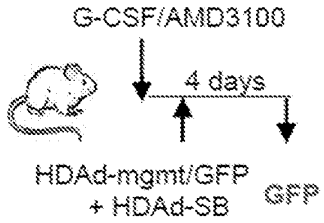


FIG. 20C

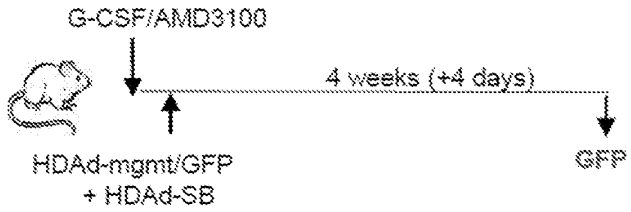


FIG. 20D

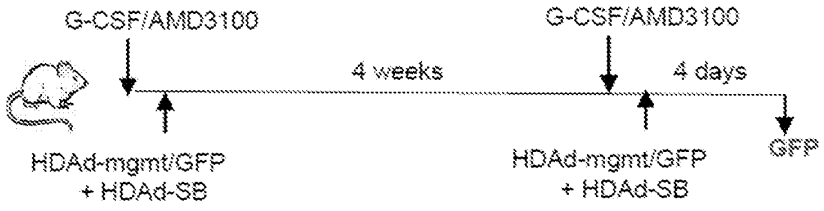


FIG. 20E

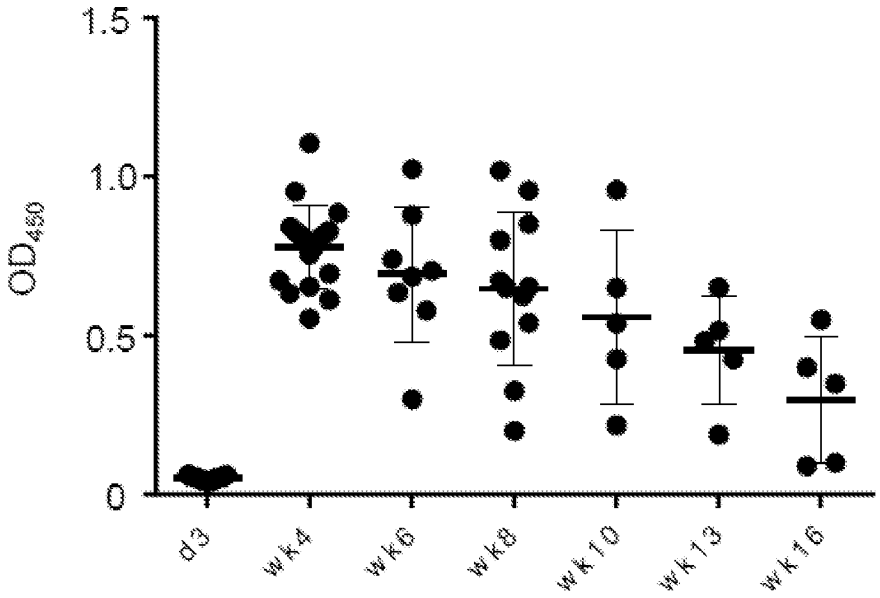


FIG. 20F

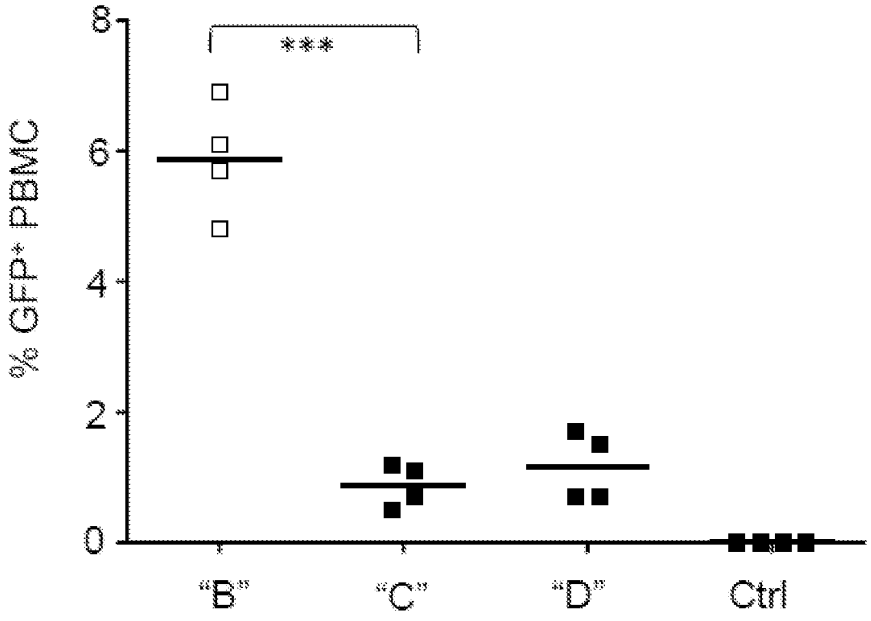


FIG. 21A

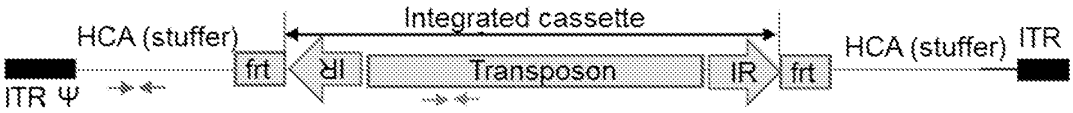


FIG. 21B

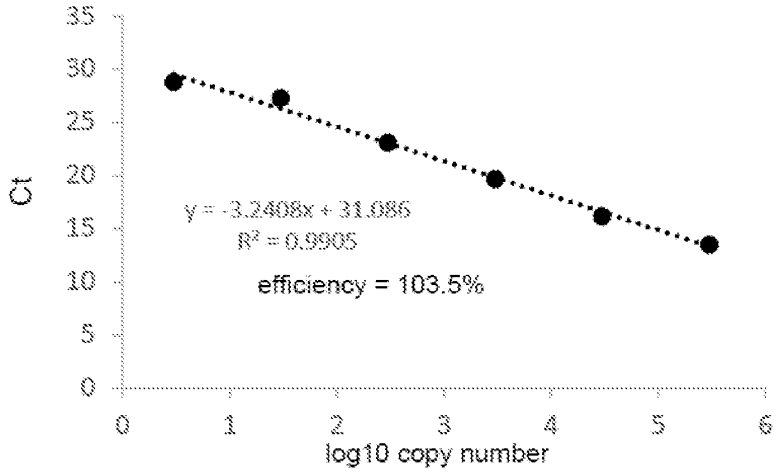


FIG. 21C

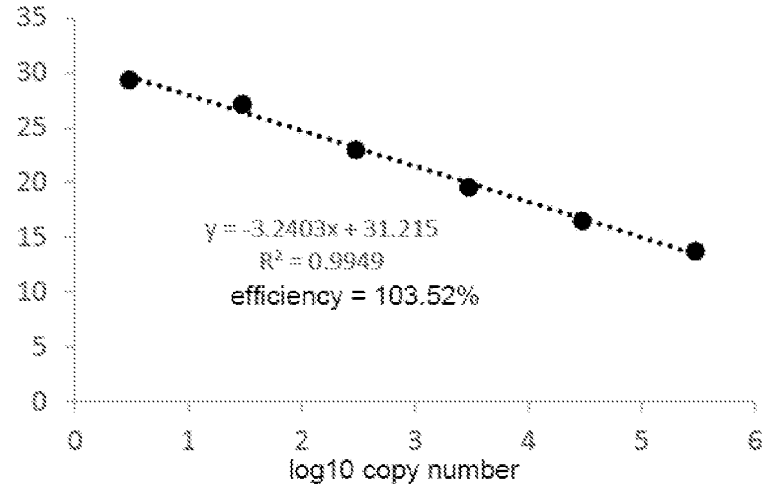


FIG. 21D

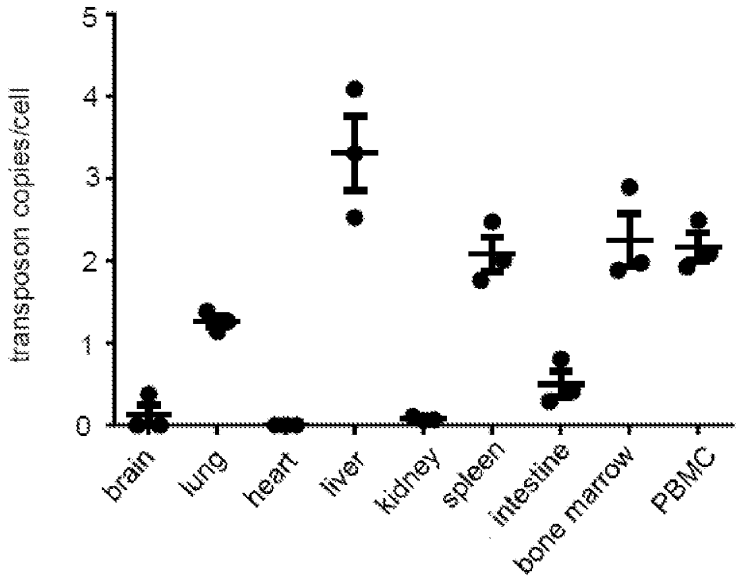

transposon copies/cell
5
4
3
2
1
0
brain
lung
heart
liver
kidney
spleen
intestine
bone marrow
PBMC

FIG. 22A

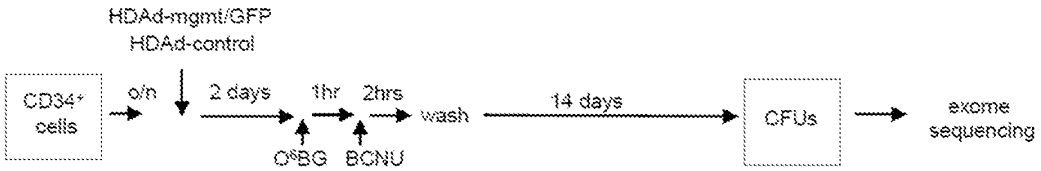


FIG. 22B

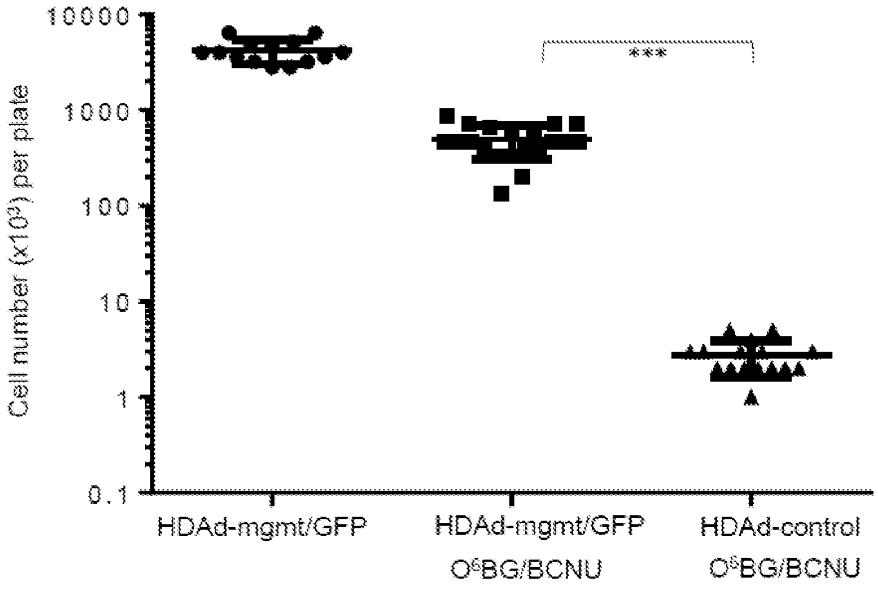


FIG. 22C

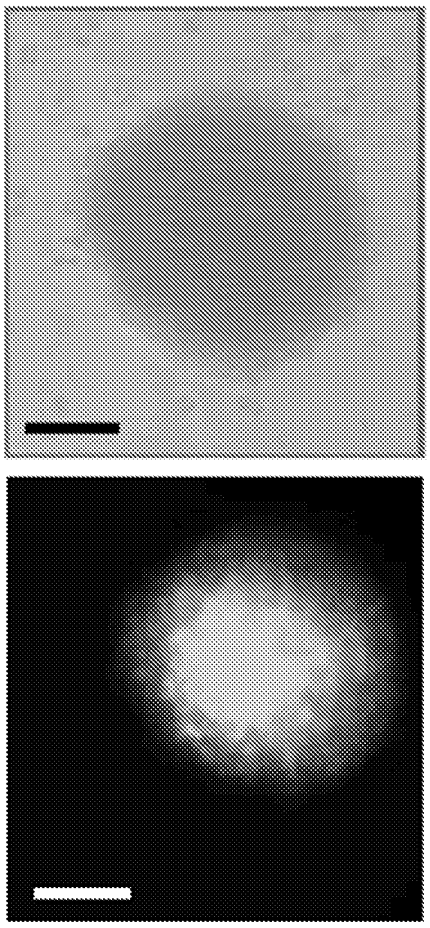


FIG. 23

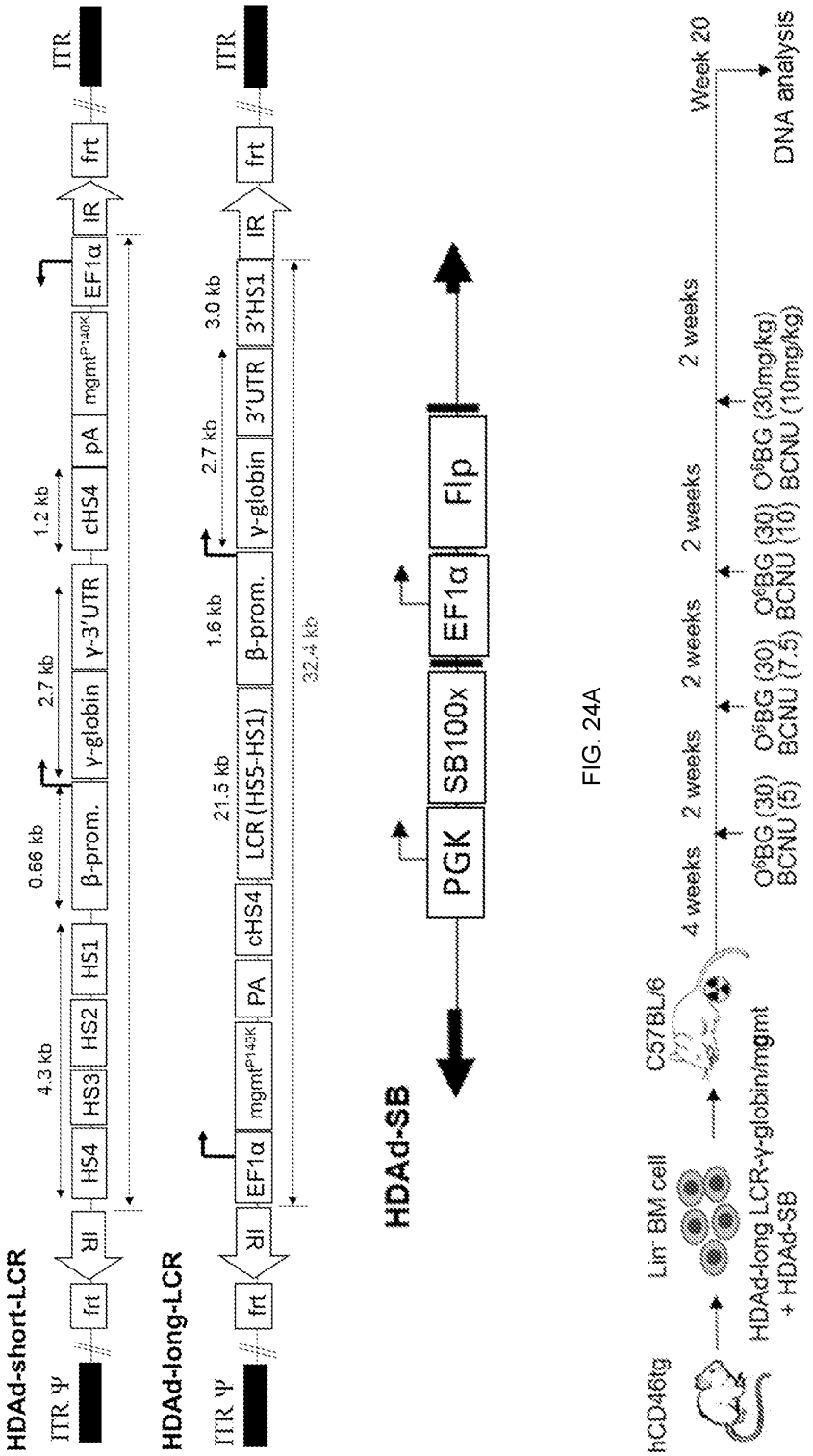


FIG. 24A

FIG. 24C

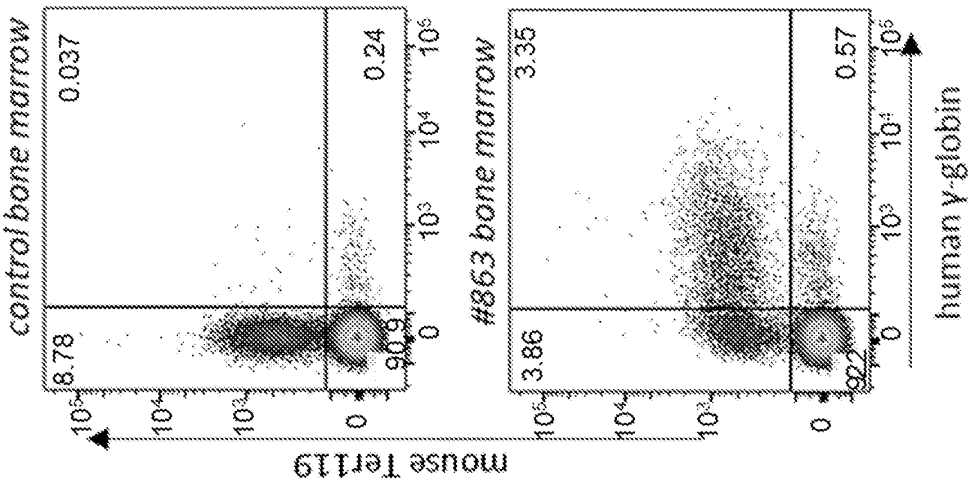


FIG. 24B

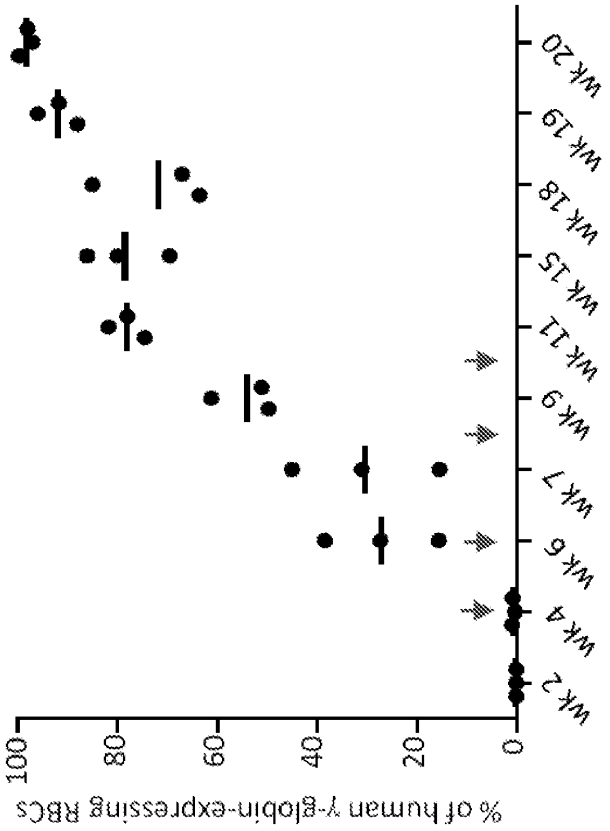


FIG. 24D

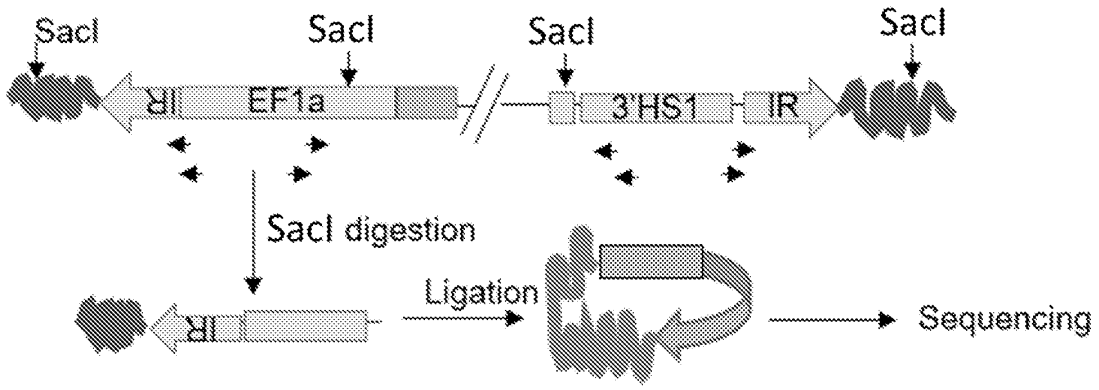


FIG. 24E

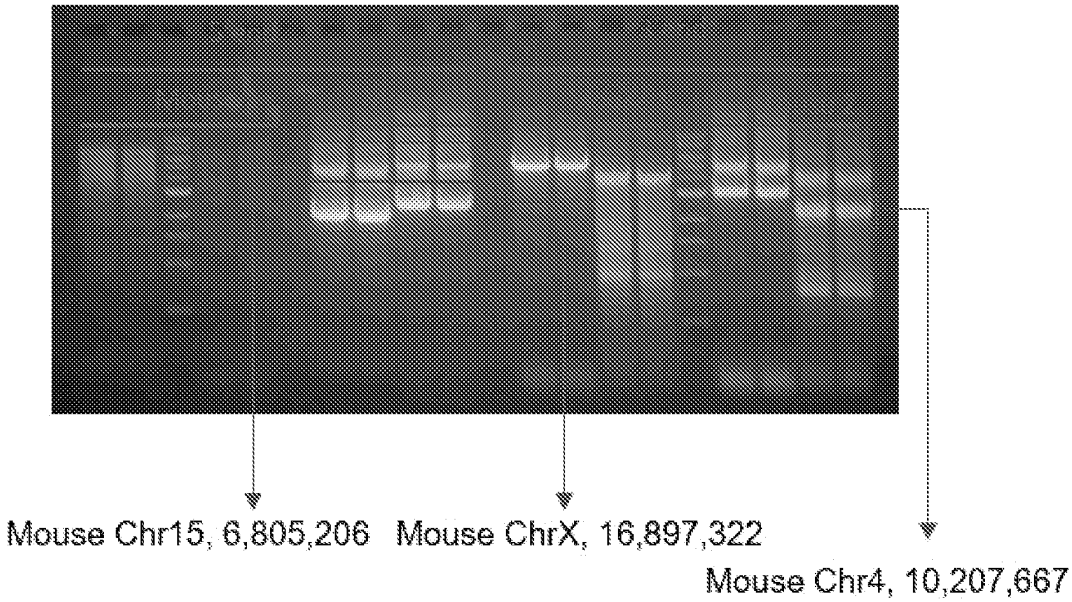


FIG. 24F

CCCTGGGATTCCCCAAGGCAGGGGCGAGTCCTTTTGTATGAATTACTCAAATCGATAACTAGAAACTTAATTAACAACGA
GATCTTATAATTTGCATACTTCTGCCTGCTGGGGACTTTCCACACCCTAGCTGACACAAGAATTTGAAATACATCCACAG
GTACACCTCCAATTGACTCAAATGATGTCAATTAGTCTATCATAATCTTCTAAAGCCATGACATCATTTTAACTGGAATT
TTCCAAGCTGTTTAAAGGCACAGTCAACTTAGTGTATGTAAACTTCTGACCCACTGGAATTGTGATACAGTGAATTATAA
GTGAAATAATCTGTCTGTAAACAATTGTTGGAAAAATGACTTGTGTCATGCACAAAGTAGATGTCCTAACTGACTTGCCA
AAACTATTGTTTGTTAACAAGAAATTTGTGGAGTAGTTGAAAAACGAGTTTTAATGACTCCAACTTAAGTGTATGTAAAC
TTCCGACTTCAACTG[TA]**AGAATGGCCCATTCATCTATAGTAGCACACAATATTTGCATTTGTGCGACAGTATAAGGGACA**
ATTATGCTATCAGGCATTTTTCCAAAGTGAGTAATCGAAGTTTTTATACCTTTGTGTGCCATGTTTGCTACCATGGTGGG
ATAATCTTACACGCGTTCTCGCGACCGGCCAGGAAAGACGCAACAAACCGGAATCTTCTGCGGCAAAAGCTTTATTGCTT

SEQ ID NO: 1

TAGAAACTTAATTAACAACGAGATCTTATAATTTGCATACTTCTGCCTGCTGGGGACTTTCCACACCCTAGCTGACACAA
GAATTTGAAATACATCCACAGGTACACCTCCAATTGACTCAAATGATGTCAATTAGTCTATCATAATCTTCTAAAGCCAT
GACATCATTTTAACTGGAATTTTCCAAGCTGTTTAAAGGCACAGTCAACTTAGTGTATGTAAACTTCTGACCCACTGGAA
TTGTGATACAGTGAATTATAAGTGAAATAATCTGTCTGTAAACAATTGTTGGAAAAATGACTTGTGTCATGCACAAAGTA
GATGTCCTAACTGACTTGCCAAAACTATTGTTTGTTAACAAGAAATTTGTGGAGTAGTTGAAAAACGAGTTTTAATGACT
CCAACTTAAGTGTATGTAAACTTCCGACTTCAACTG[TA]**CAAGTAGACCAAATATCCATATACATAAAAGAAAAAAATAGA**
AAAAATTTCTAGTGACAGAAAAATGACAAAGAACATACTGCTTTATTACTACTATTAAGATGTTTGCTTCCATTACACTC
ATATGAGTCATGATATTTTTTCTTCATTTTTTTCTANTNNCACTNGAAAT

SEQ ID NO: 2

GTTGCTAGGAATGAGCCAAATTCATCTGTATTAAACAGTGGGAGCTTGTGGAAGGCTACTCGAAATGTTTGACCCAAGTT
AAACAATTTAAAGGCAATGCTACCAAATACTAATTGAGTGTATGTTAACTTCTGACCCACTGGGAATGTGATGAAAGAAA
TAAAAGCTGAAATGAATCATTCTCTCTACTATTATTCTGATATTTCACATTCTTAAAATAAAGTGGTGATCCTAACTGAC
CTTAAGACAGGGAATCTTTACTCGGATTAAATGTCAGGAATTGTGAAAAAGTGAGTTTAAATGTATTTGGCTAAGGTGTA
TGTAAACTTCCGACTTCAACTG[TA]**TATCCTCCCCGTTGCACCCTCTTGATGATGCTGAGATGAACACAGATGCTCACTCC**
TTGAGGGCTCTAAGCTTATGCTGACACAGACACAGGTGCTCACTTCTATGAATGGCCTAAGATTTGAGGACATCATGAGG
ACAAGTGTGATAAAATCTTGGAACAACCTCCCAGAGGTCT

SEQ ID NO: 3

5'end vector sequence
Sleeping beauty IR/DR sequence
Integration junction (chr15, 6805206)

5'end vector sequence
Sleeping beauty IR/DR sequence
Integration junction (chrX, 16897322)

3'end vector sequence
Sleeping beauty IR/DR sequence
Integration junction (chr4, 10207667)

FIG. 25A

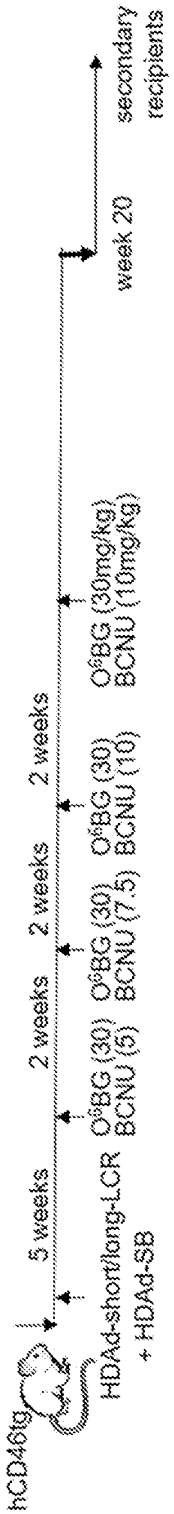

hCD46tg
G-CSF/AMD3100
5 weeks
HDAd-short/long-LCR
+ HDAd-SB
2 weeks
O6BG (30)
BCNU (5)
2 weeks
O6BG (30)
BCNU (7.5)
2 weeks
O6BG (30)
BCNU (10)
O6BG (30mg/kg)
BCNU (10mg/kg)
week 20
secondary
recipients

FIG. 25B

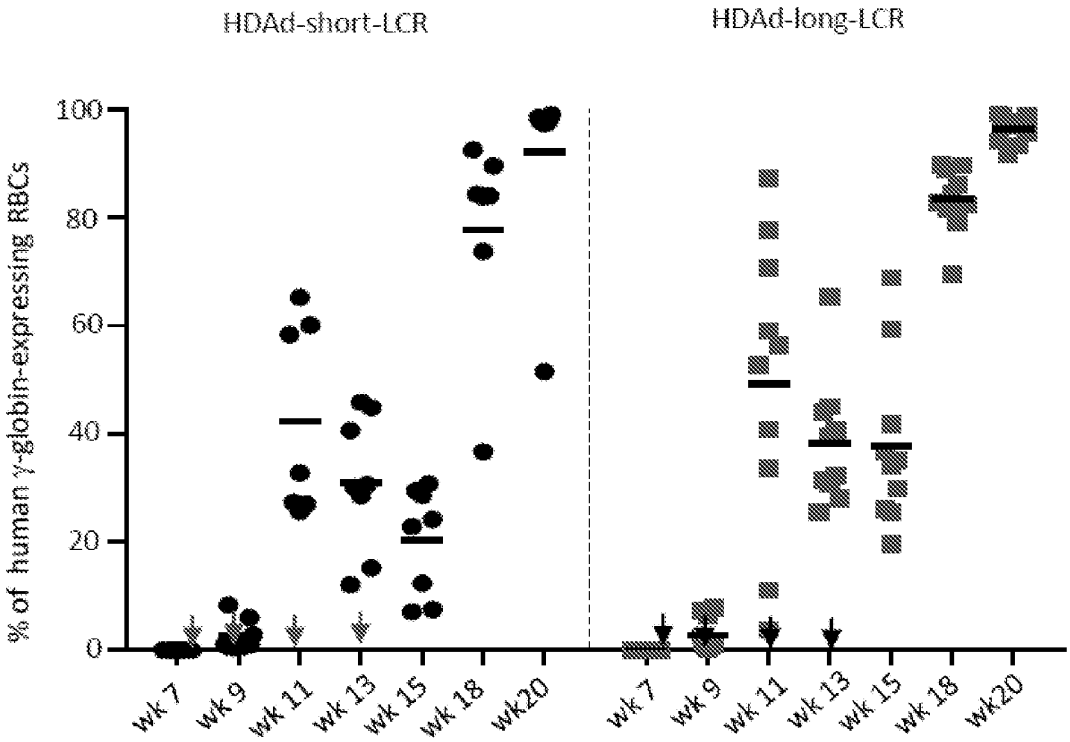


FIG. 25C

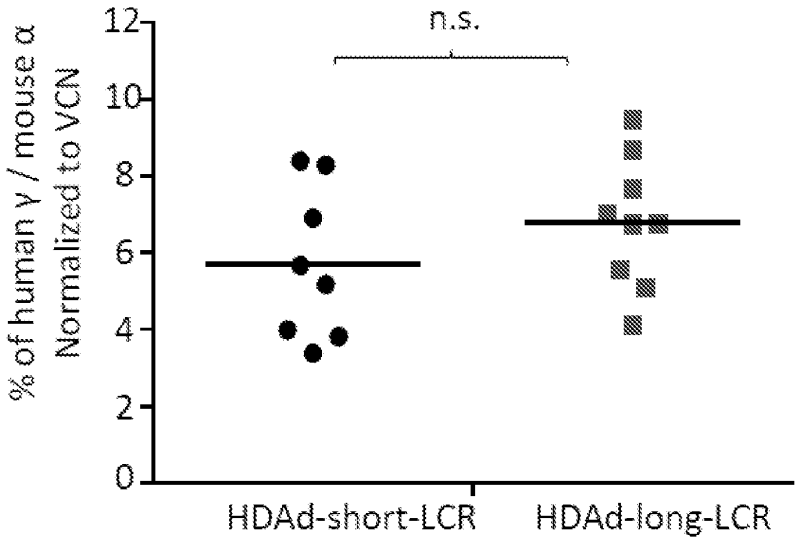


FIG. 25D

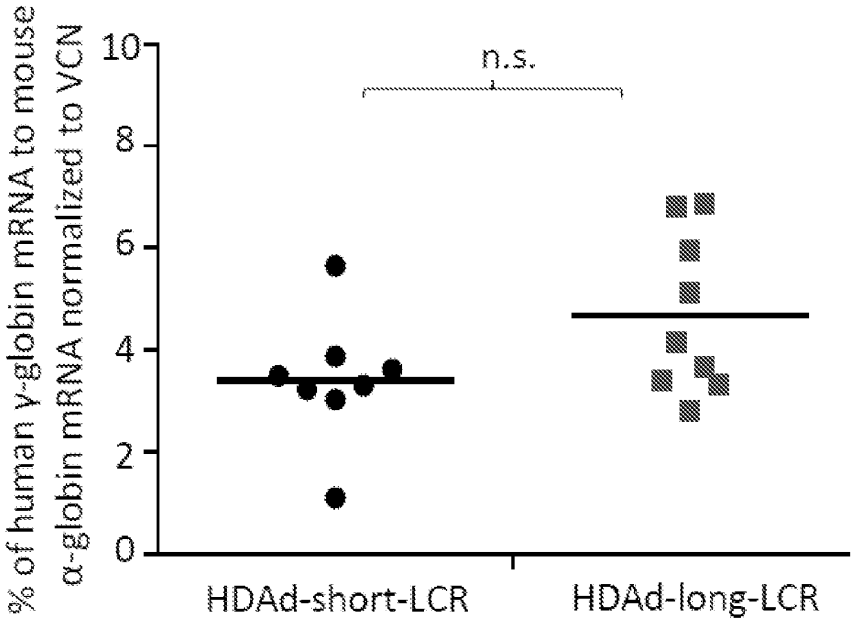


FIG. 25E

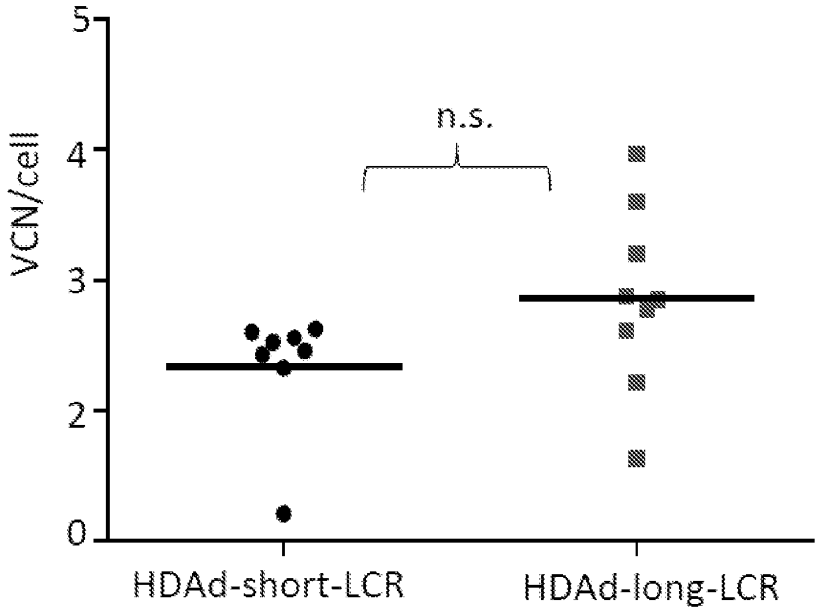


FIG. 26A

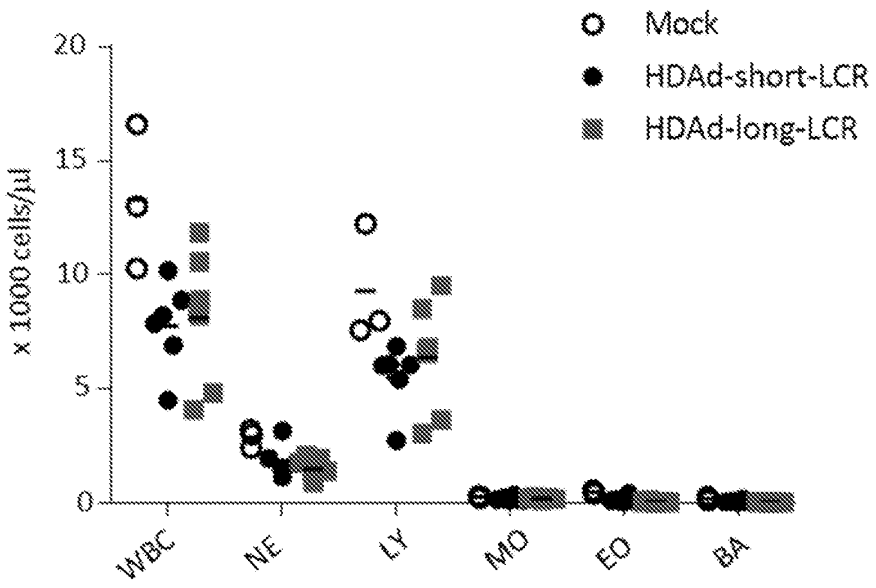


FIG. 26B

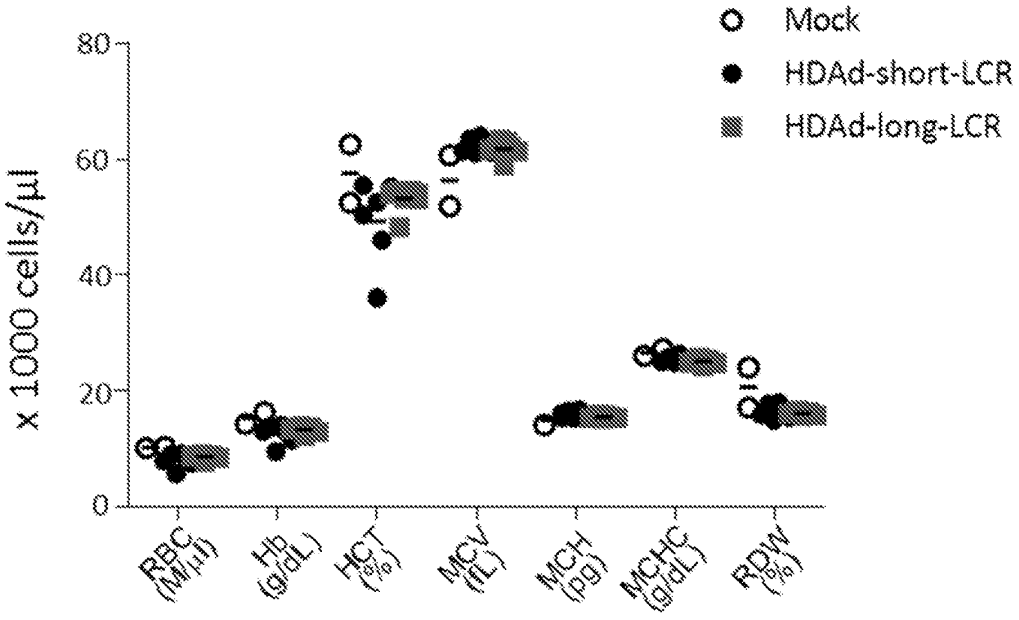


FIG. 26C

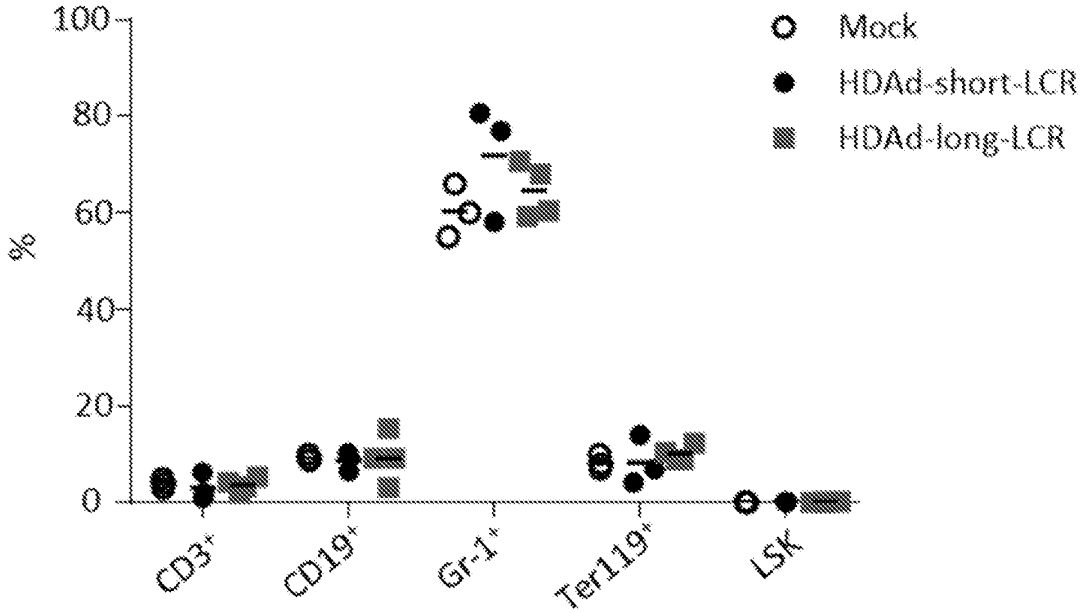


FIG. 26D

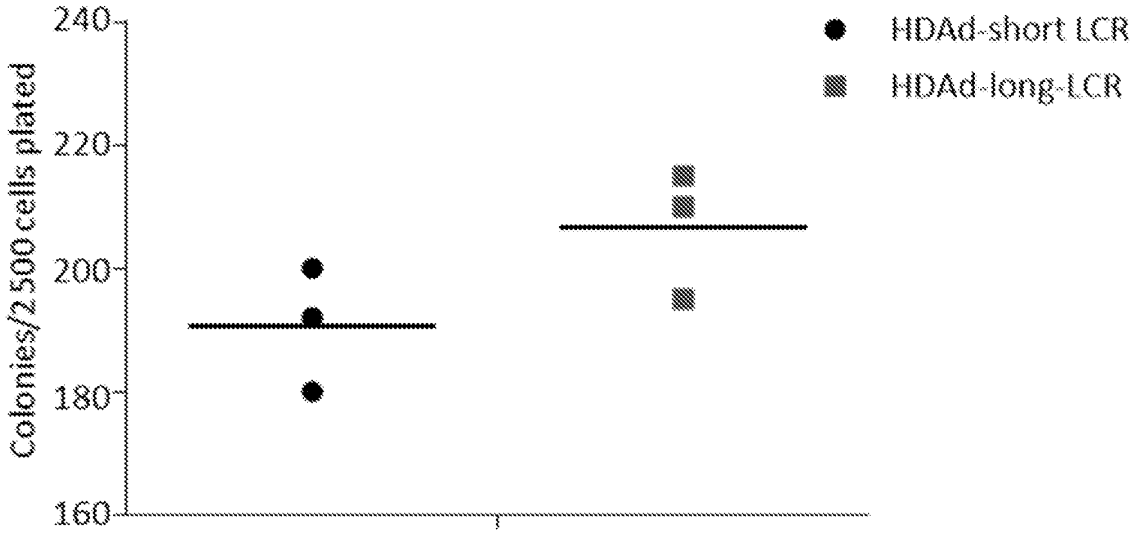


FIG. 27

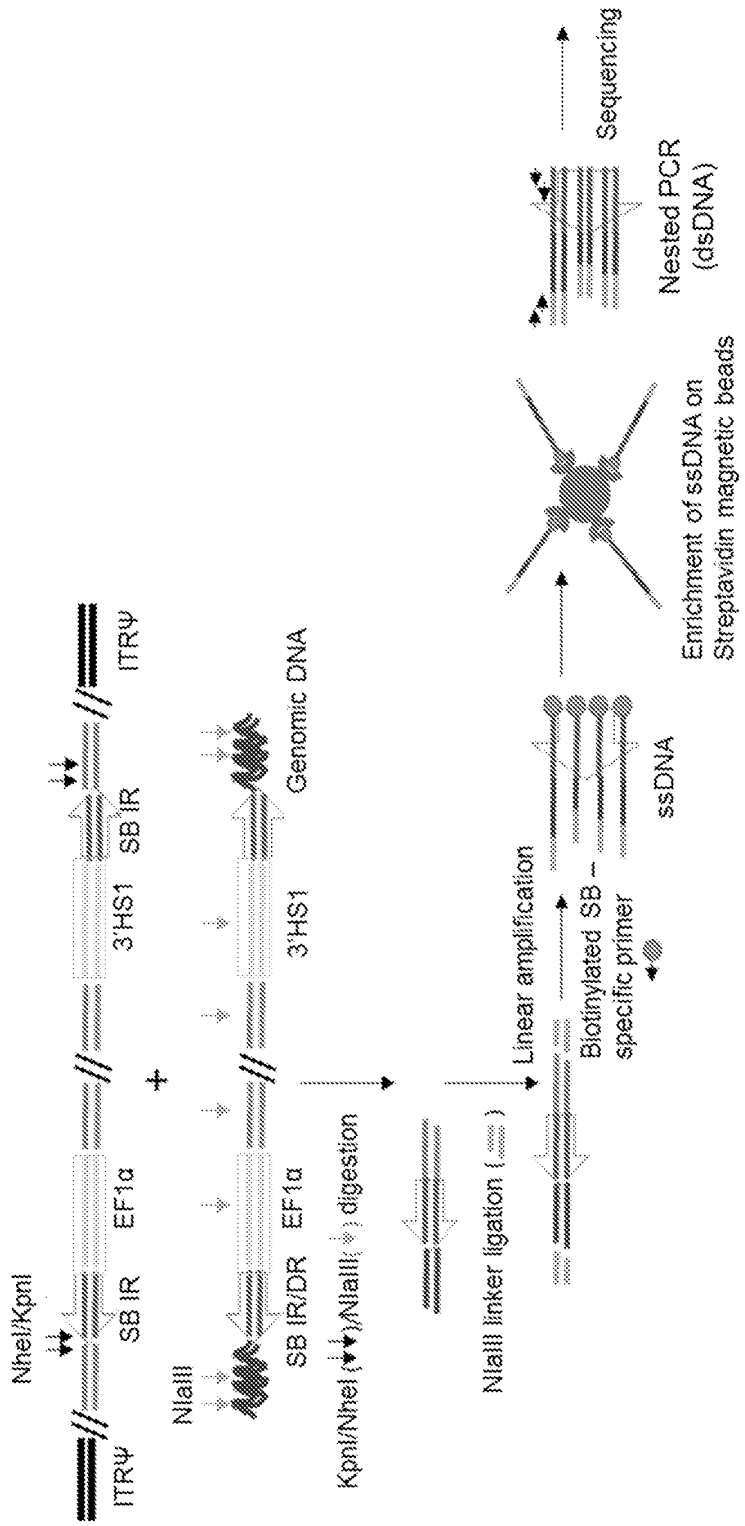

NheI/KpnI
ITRΨ
SB IR
EF1α
3'HS1
SB IR
ITRΨ
NlaIII
SB IR/DR
EF1α
3'HS1
Genomic DNA
KpnI/NheI (▼▼)/NlaIII (▼) digestion
NlaIII linker ligation (═)
Linear amplification
Biotinylated SB – specific primer
ssDNA
Enrichment of ssDNA on Streptavidin magnetic beads
Nested PCR (dsDNA)
Sequencing

FIG. 28A

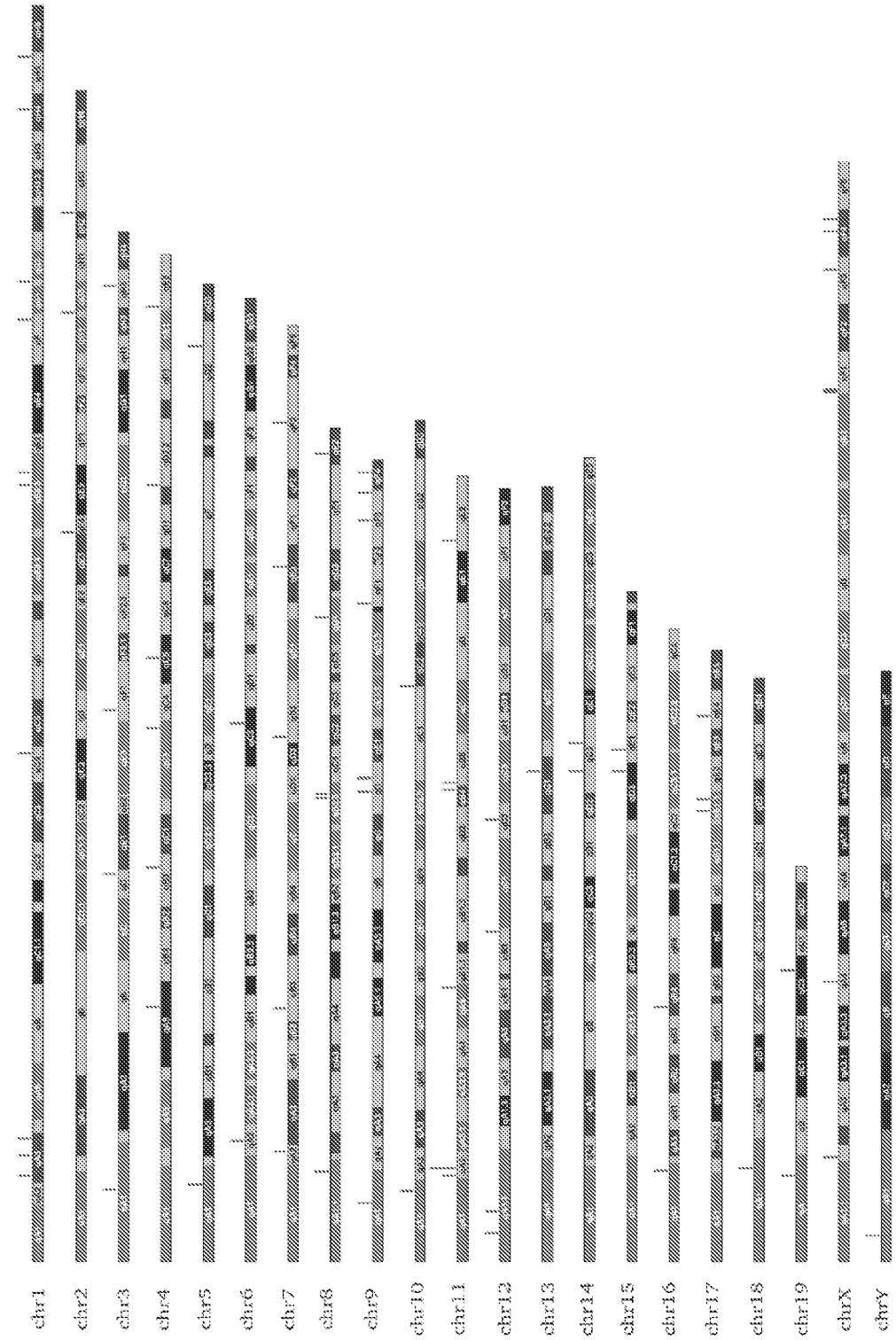

chr1
chr2
chr3
chr4
chr5
chr6
chr7
chr8
chr9
chr10
chr11
chr12
chr13
chr14
chr15
chr16
chr17
chr18
chr19
chrX
chrY

FIG 28B

Sleeping beauty IR/DR sequence
Integration junction (chr7, 79796094)
ACTTAAGTGT ATGTAAACTT CCGACTTCAA CTGTAGGCTA CCTGATTCTC TGGCCATCTC TGCCCACTAC CATG

SEQ ID NO: 4

Sleeping beauty IR/DR sequence
Integration junction (repeat region)
ACTTAAGTGT ATGTAAACTT CCGACTTCAA CTGTAAATTT TCCACCTTTT TCAGTTTTCC TGGCCATATT TCATG

SEQ ID NO: 5

FIG. 28C

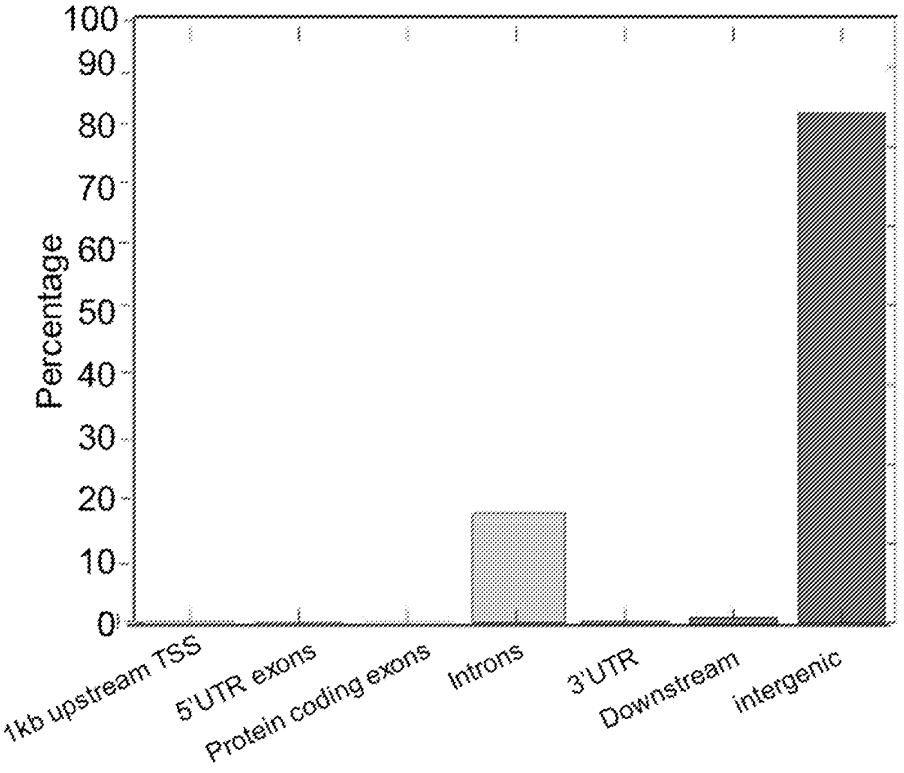


FIG. 28D

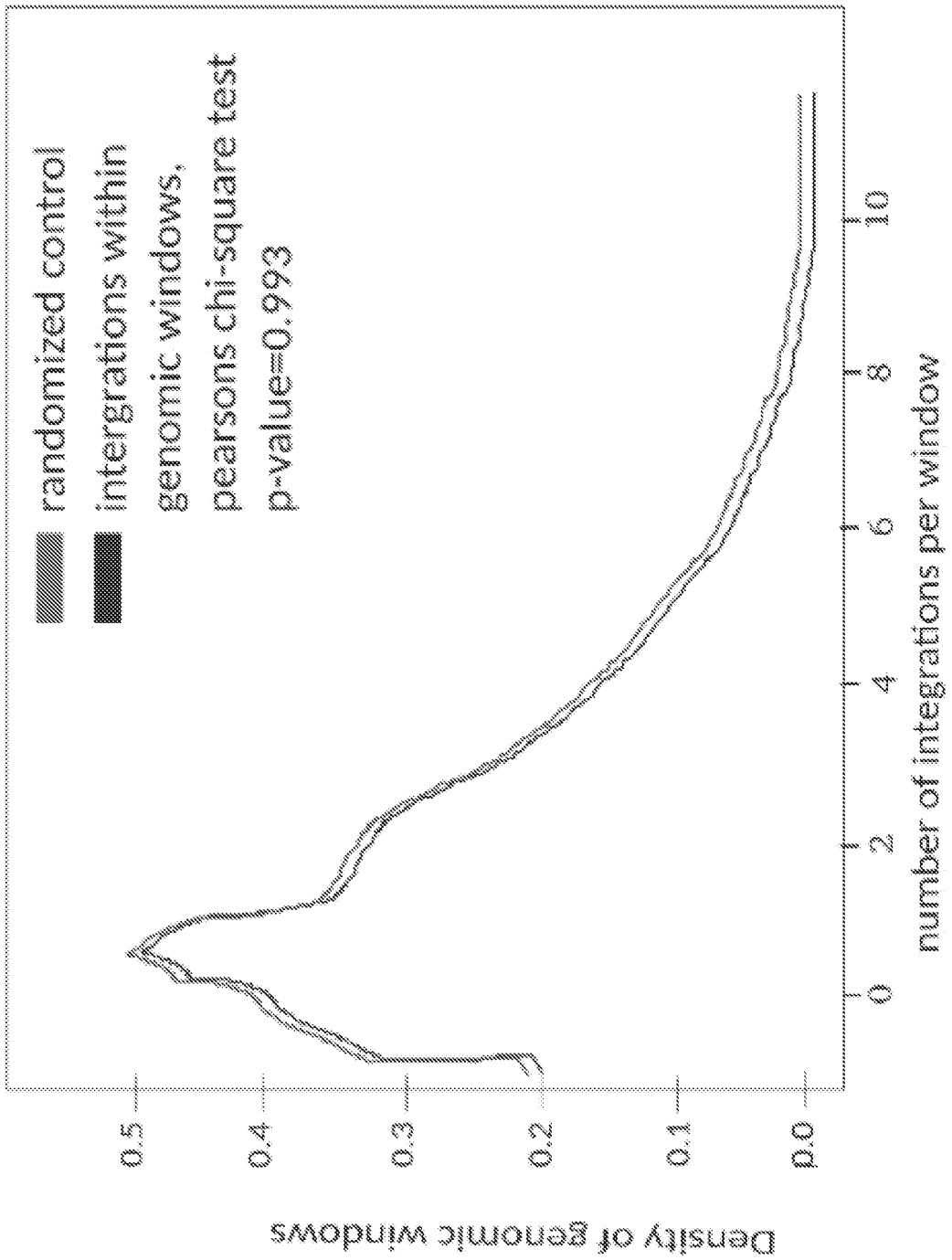

randomized control
intergrations within genomic windows,
pearsons chi-square test
p-value=0.993
Density of genomic windows
0.0
0.1
0.2
0.3
0.4
0.5
0
2
4
6
8
10
number of integrations per window

FIG. 29A

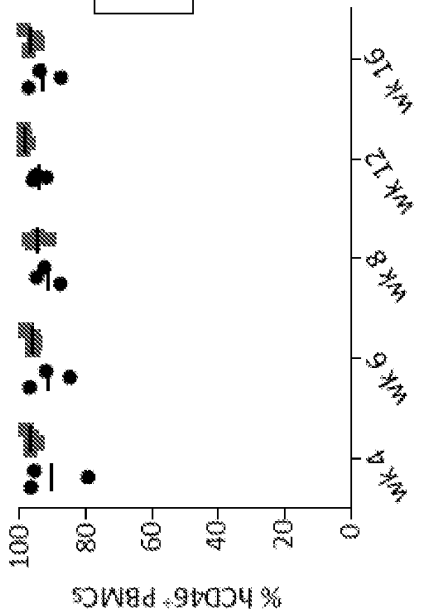


FIG. 29B

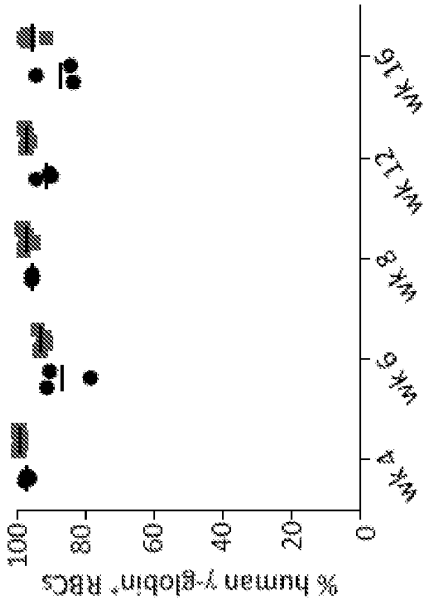


FIG. 29C

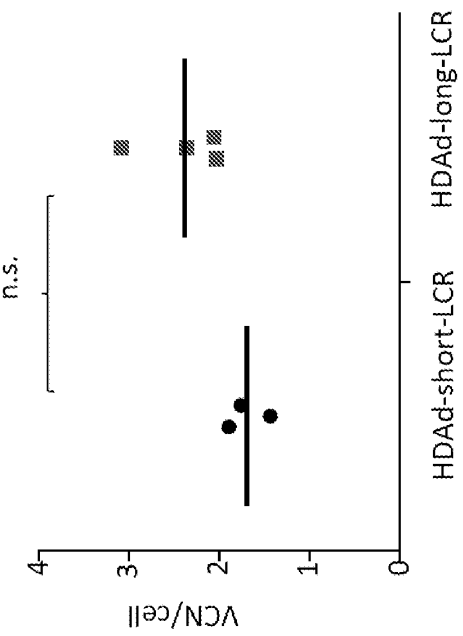


FIG.29D

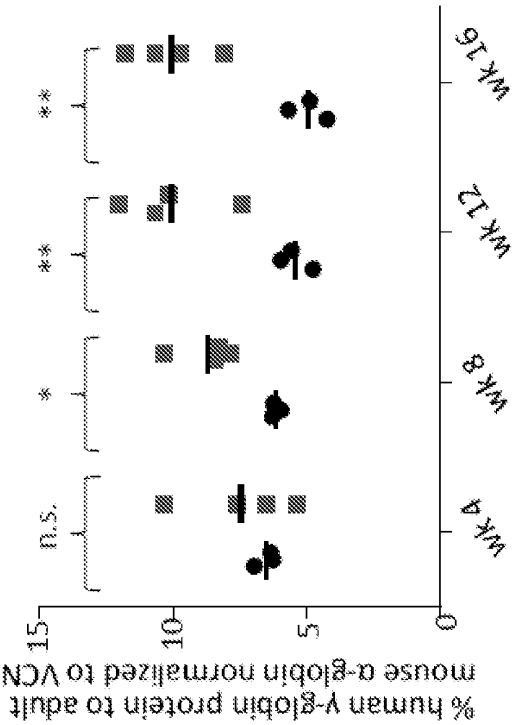


FIG. 29E

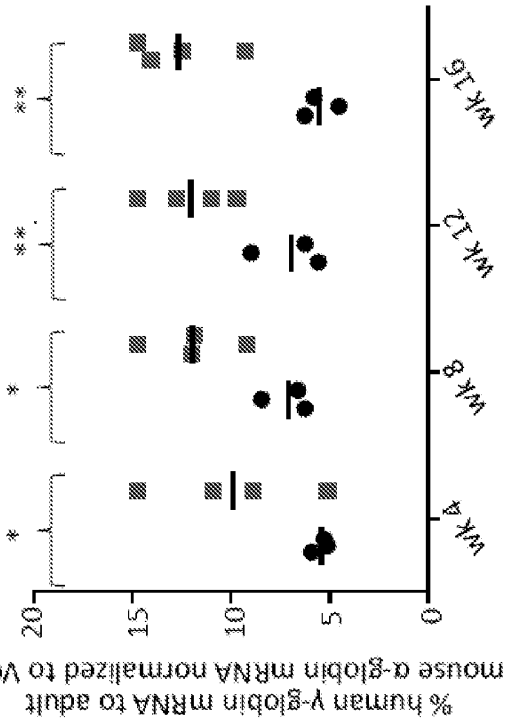


FIG. 29F

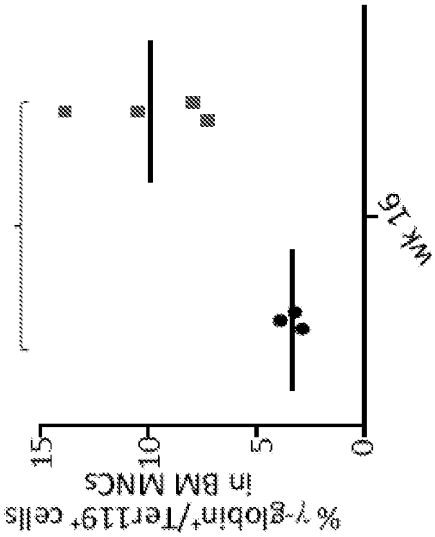


FIG. 29G

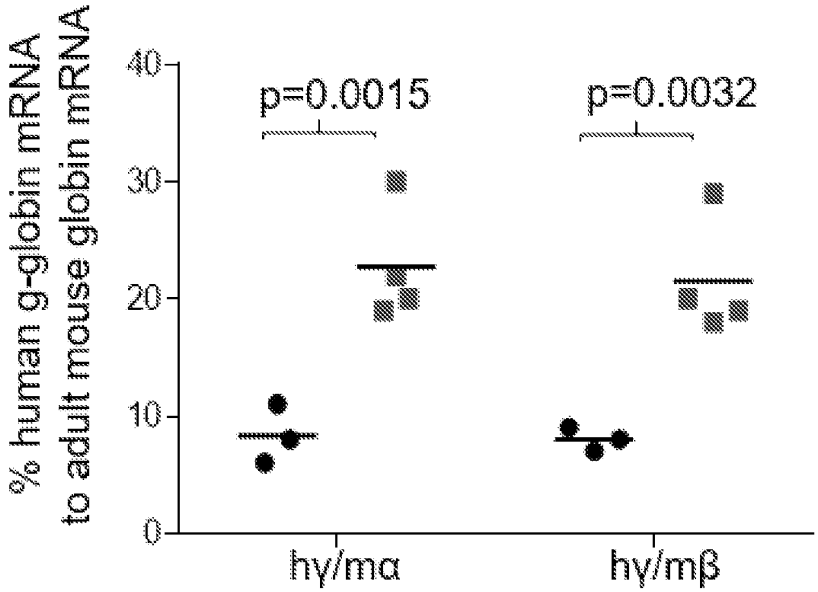

% human g-globin mRNA
to adult mouse globin mRNA
40
30
20
10
0
p=0.0015
p=0.0032
hγ/mα
hγ/mβ

FIG. 29H

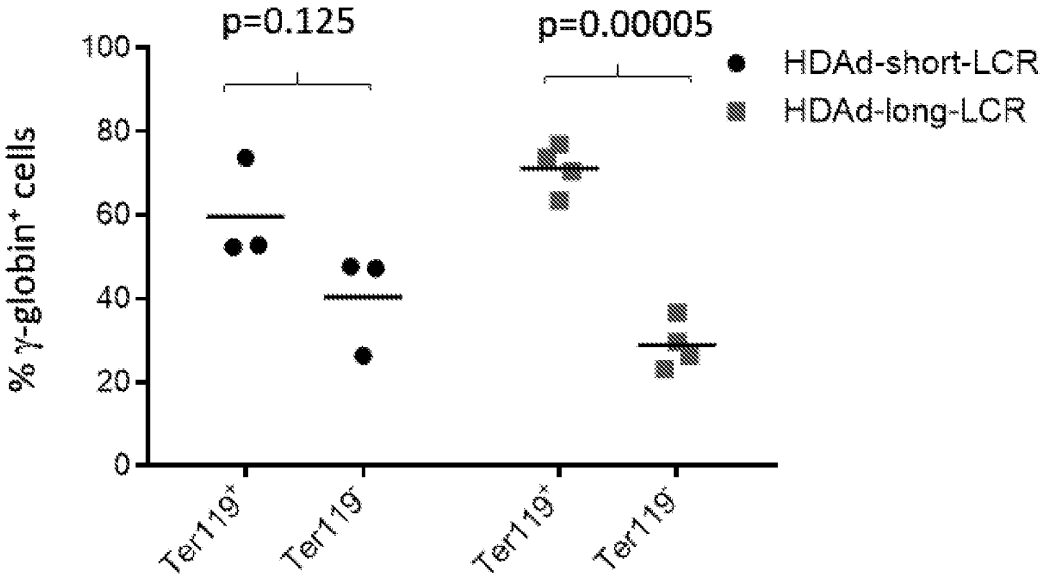


FIG. 29I

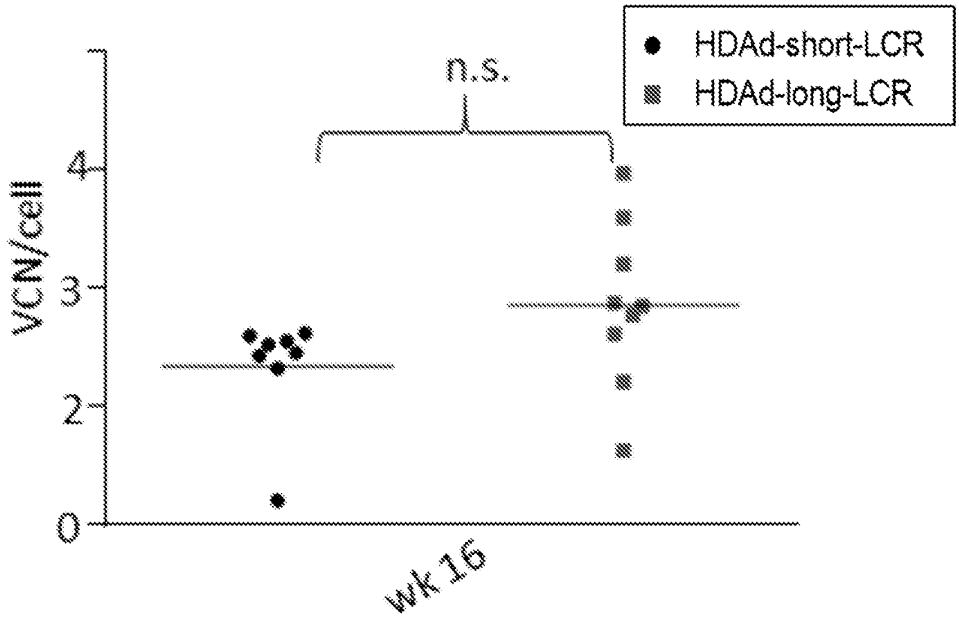


FIG. 30A

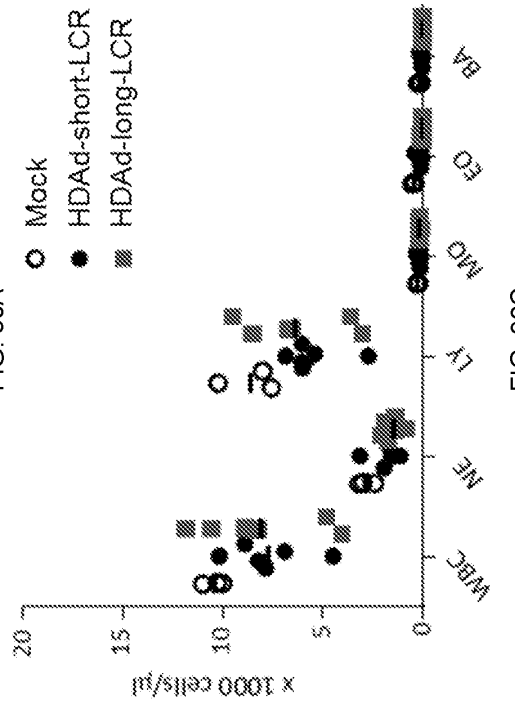


FIG. 30B

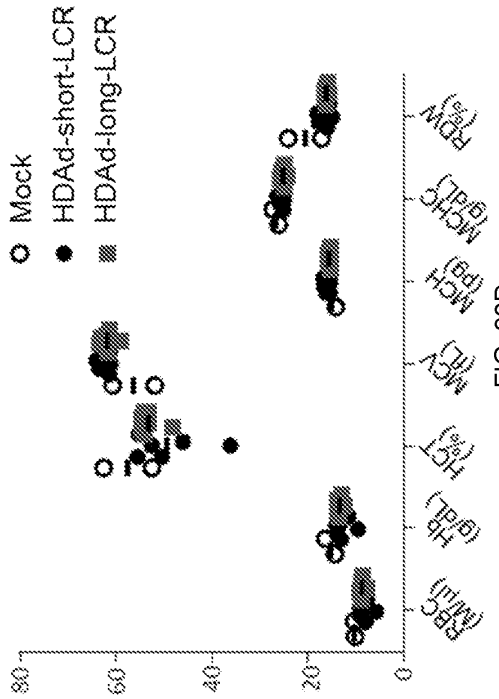


FIG. 30C

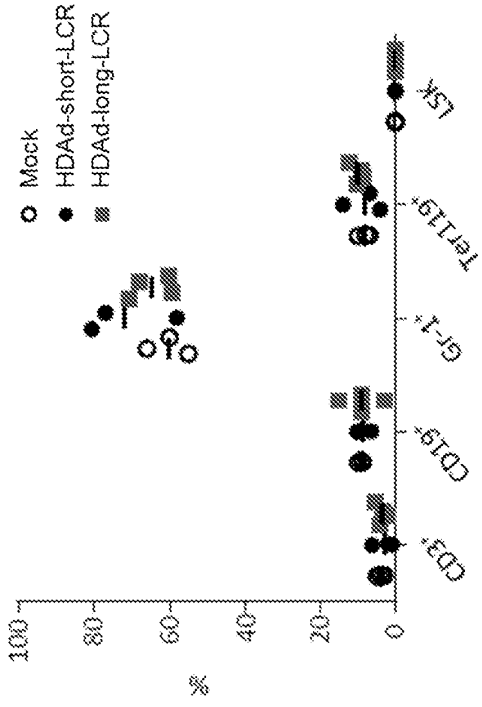


FIG. 30D

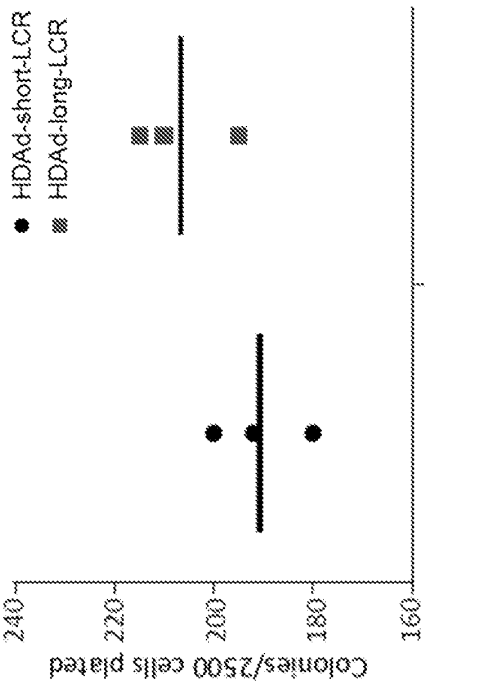


FIG. 31A

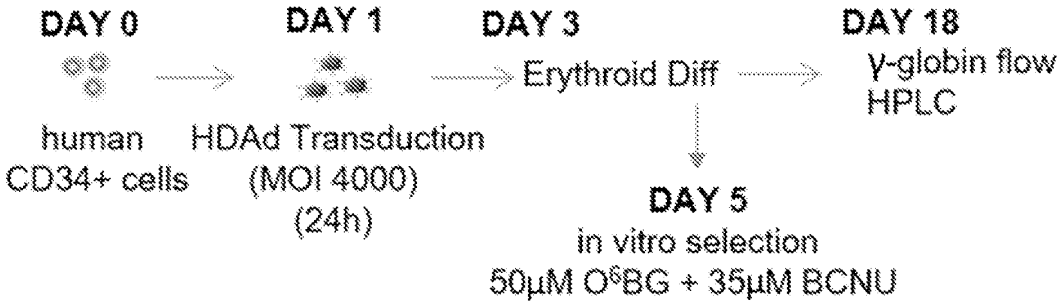


FIG. 31B

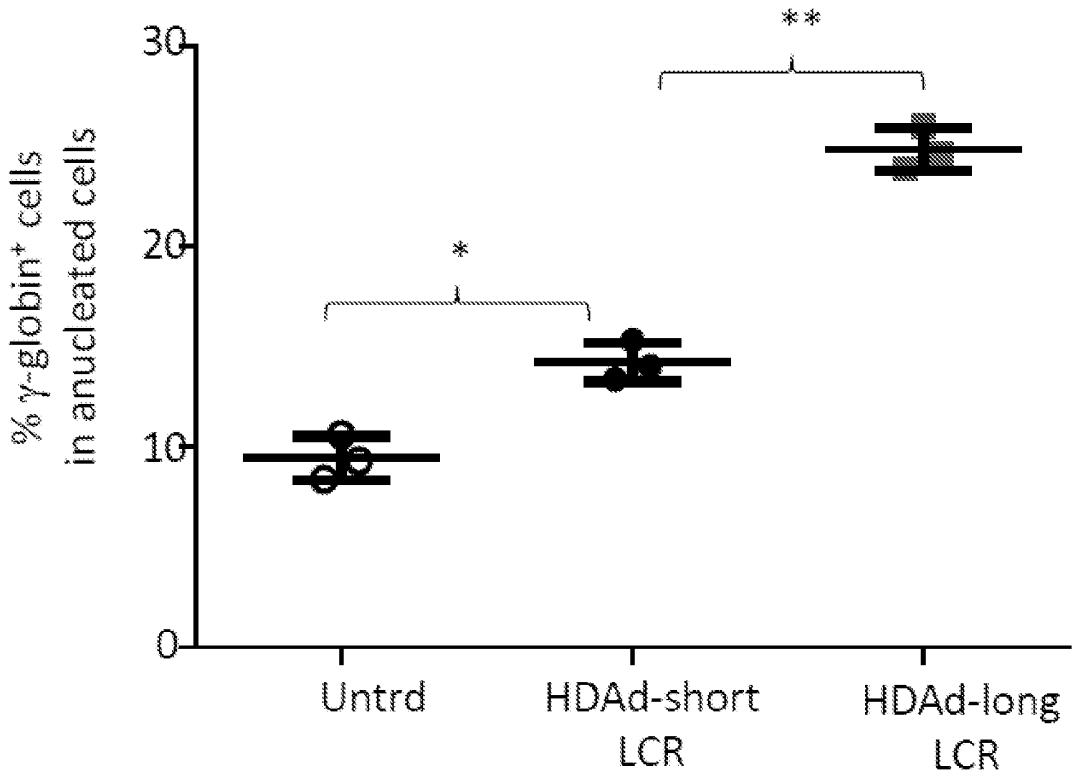


FIG. 31C

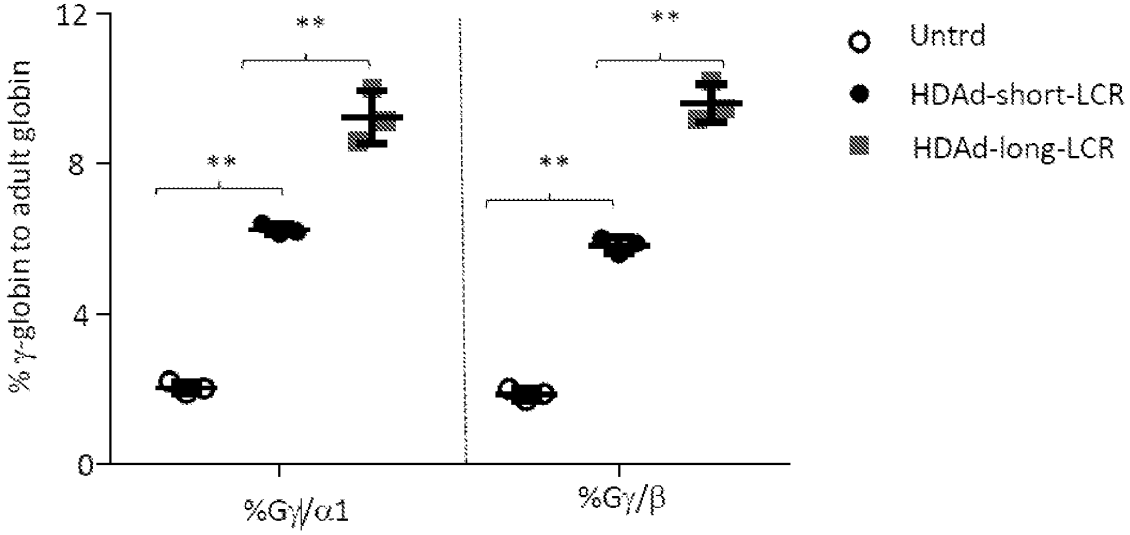


FIG. 31D

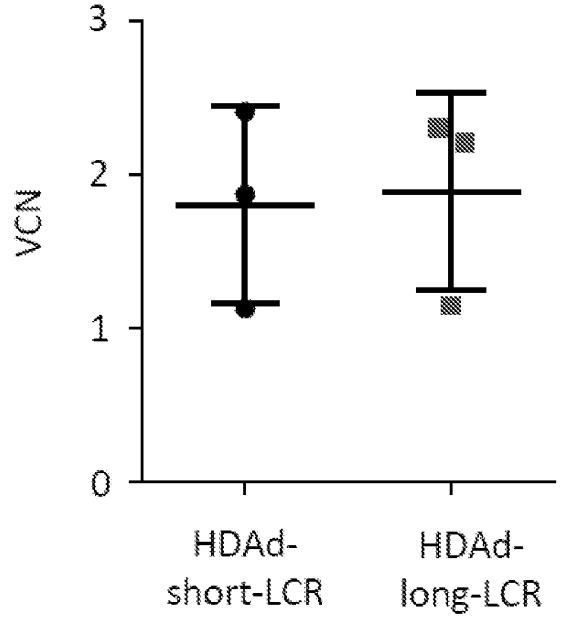


FIG. 32A

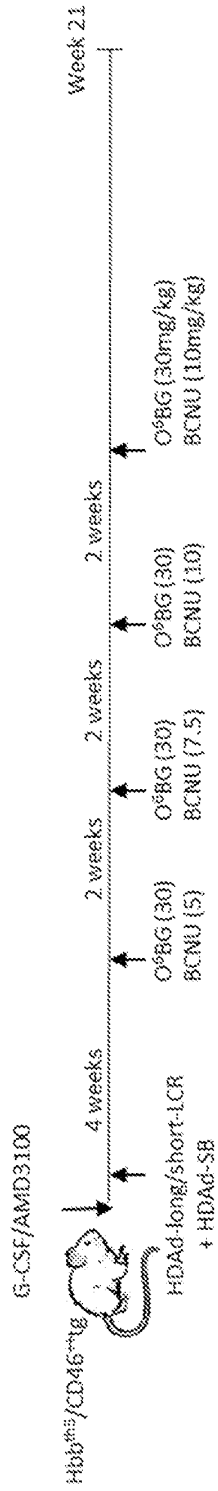


FIG. 32B

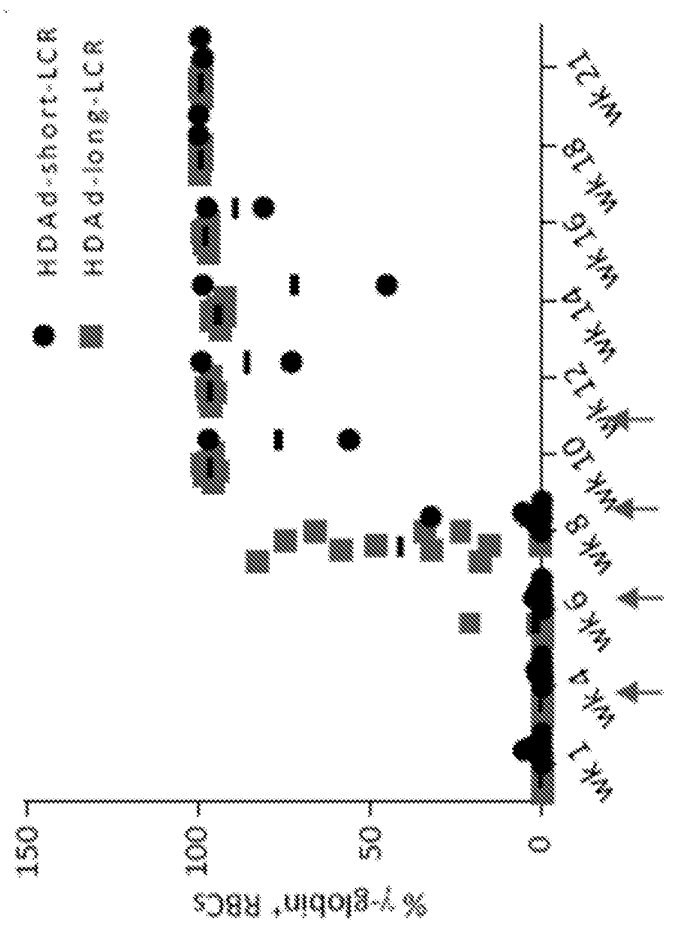


FIG. 32C

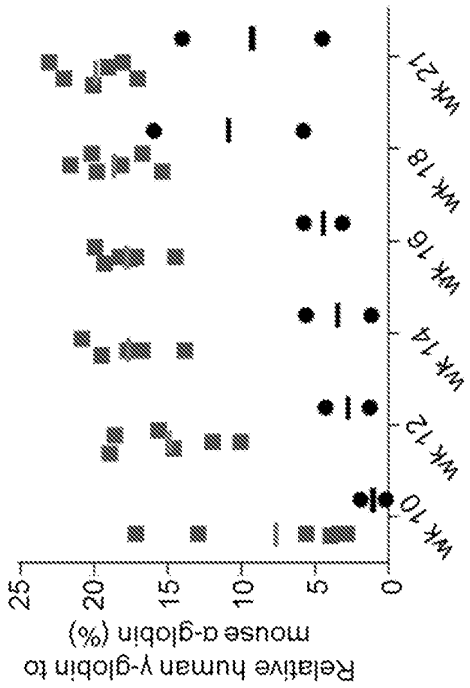


FIG. 32D

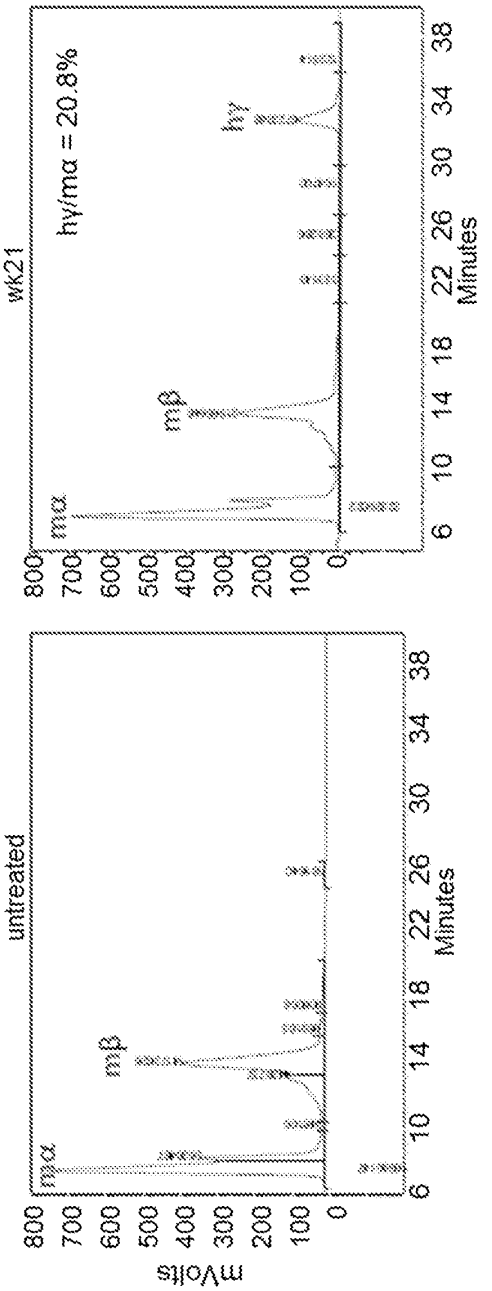


FIG. 32E

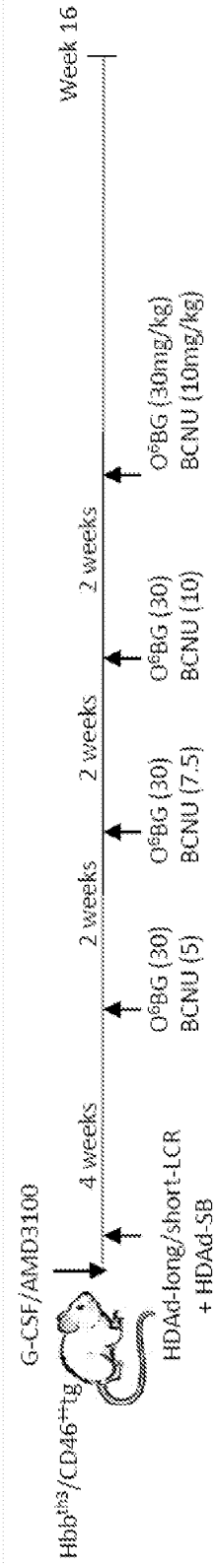
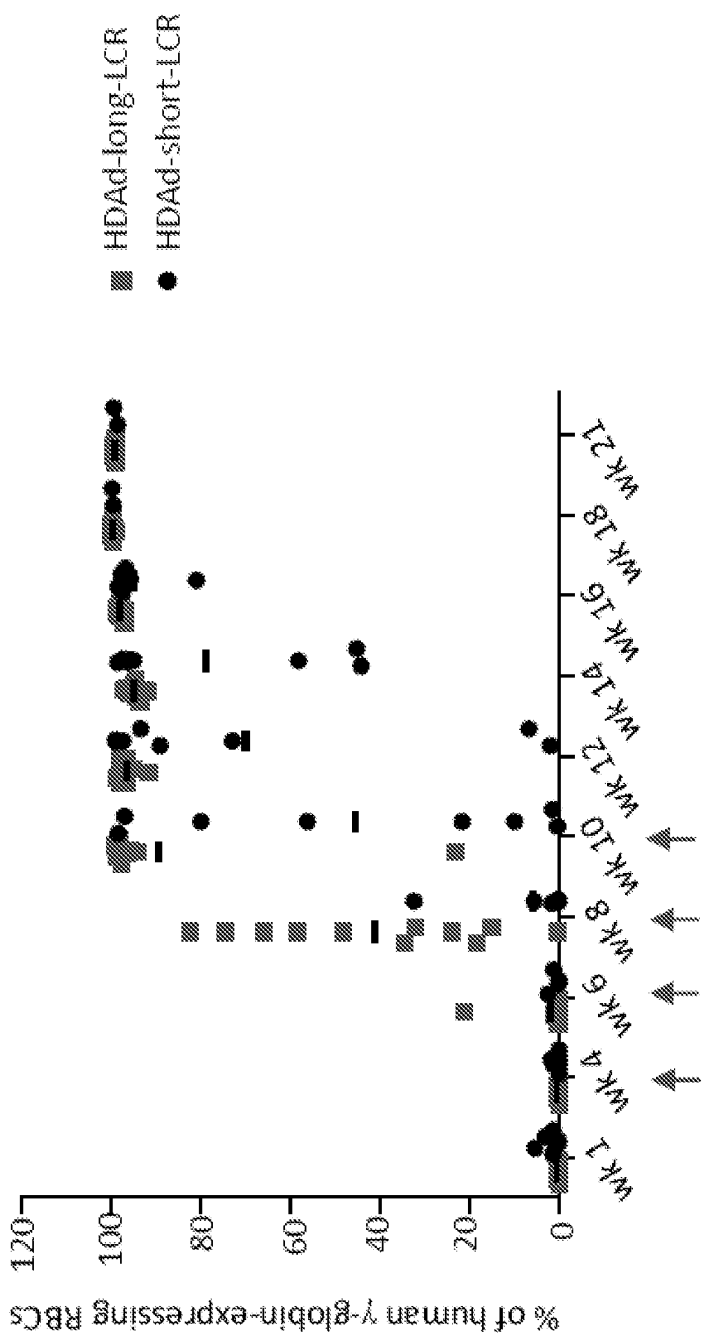


FIG. 32F



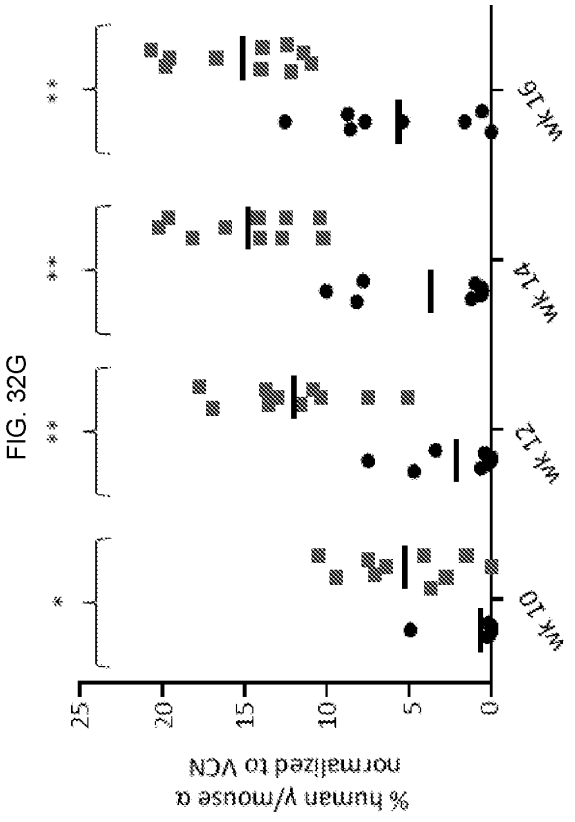


FIG. 32G

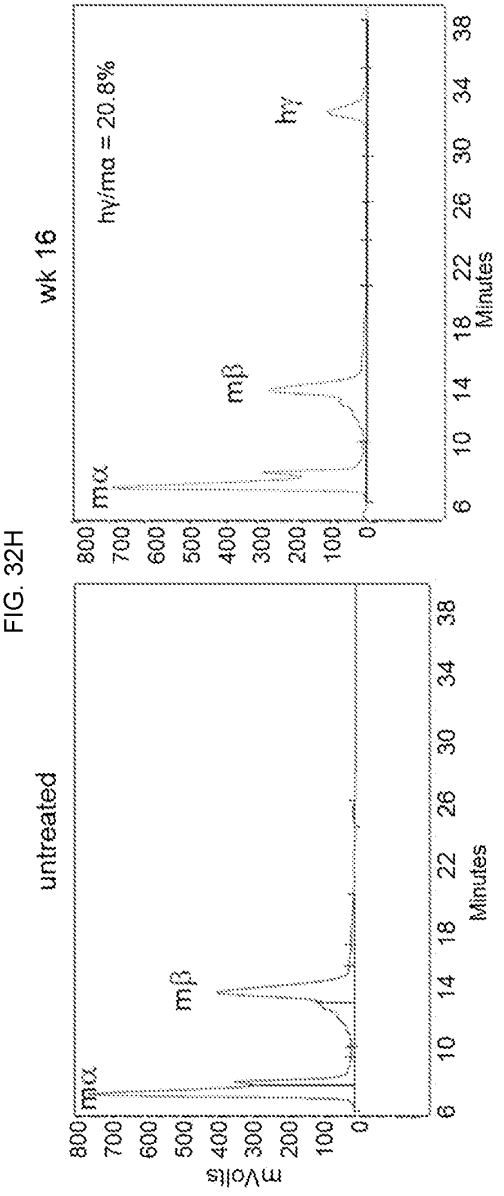


FIG. 32H

FIG. 33A

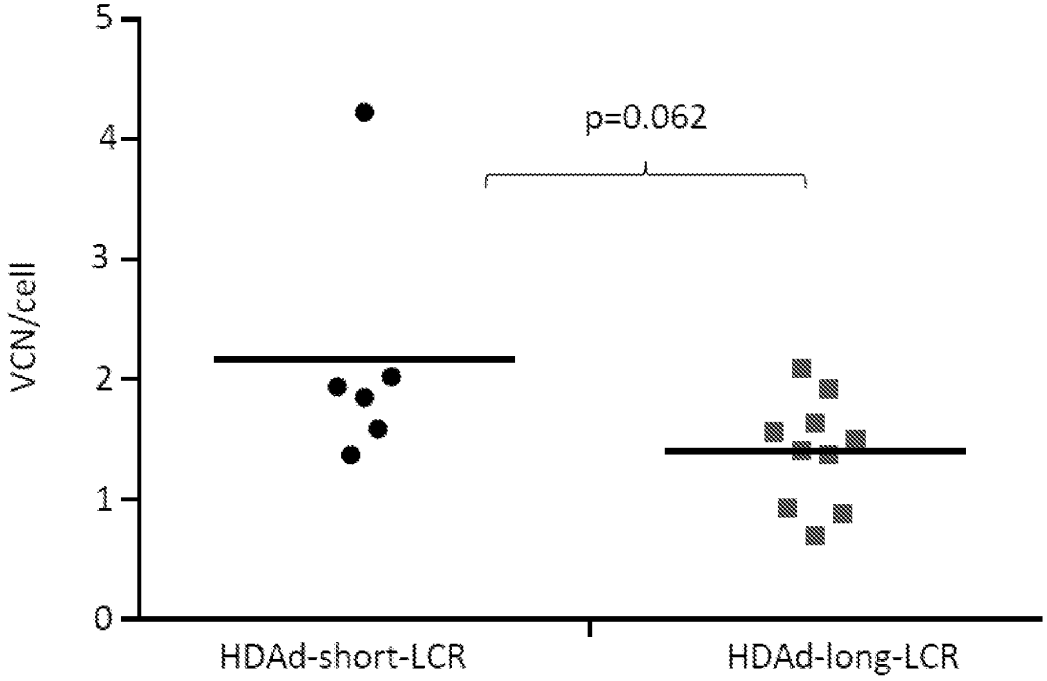


FIG. 33B

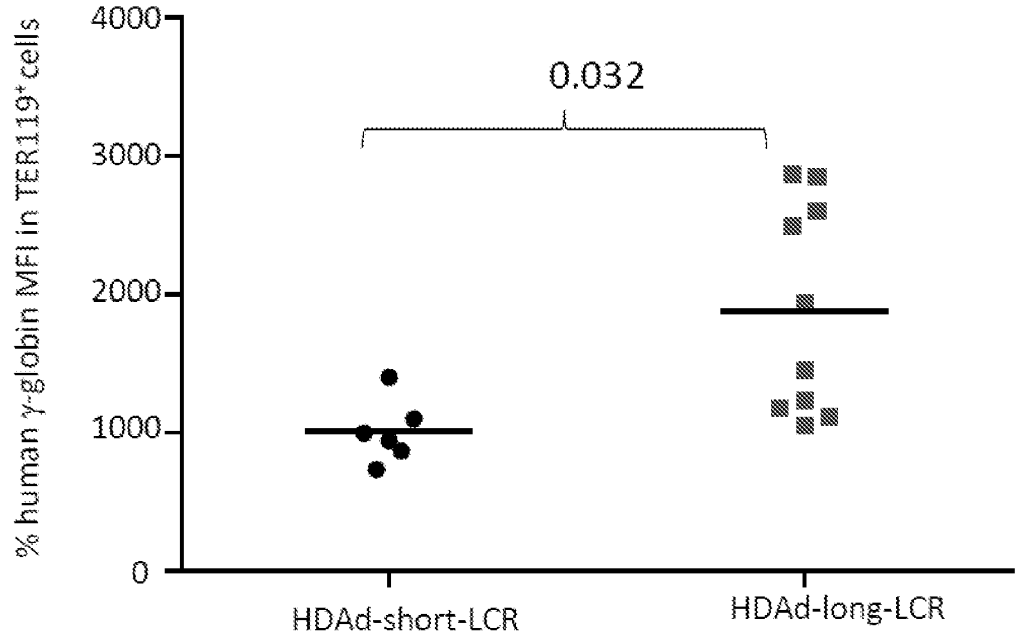


FIG. 34

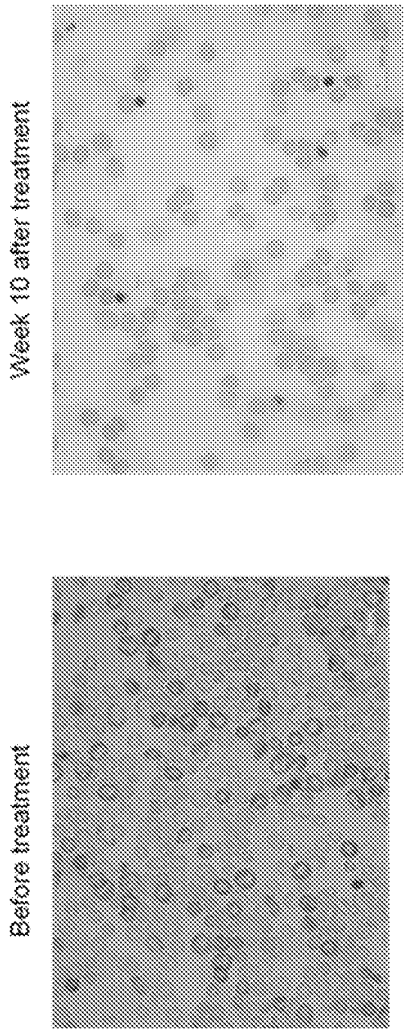

Week 10 after treatment
Before treatment

FIG. 35

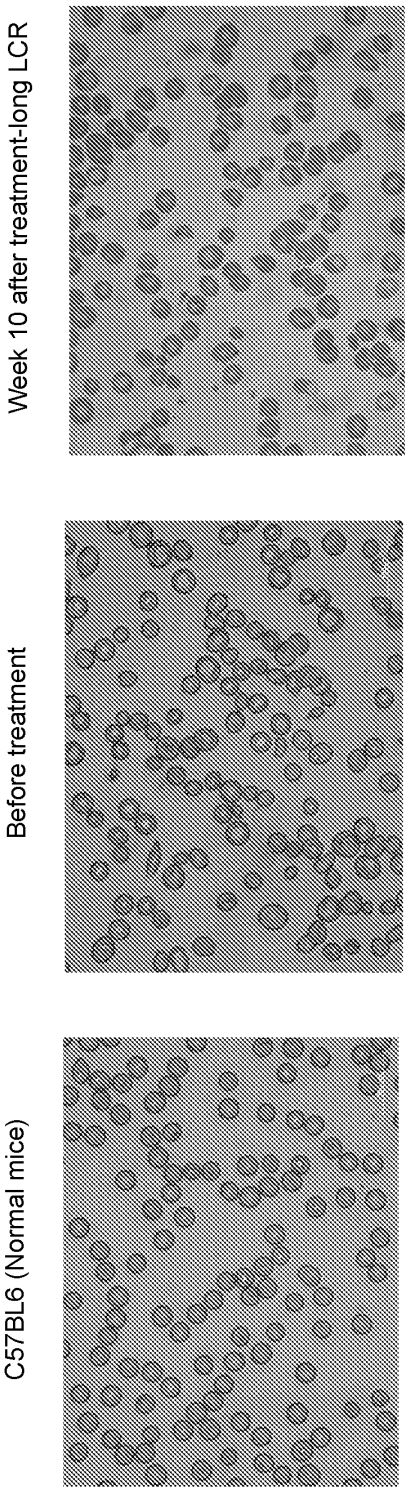

Week 10 after treatment-long LCR
Before treatment
C57BL6 (Normal mice)

FIG. 36A

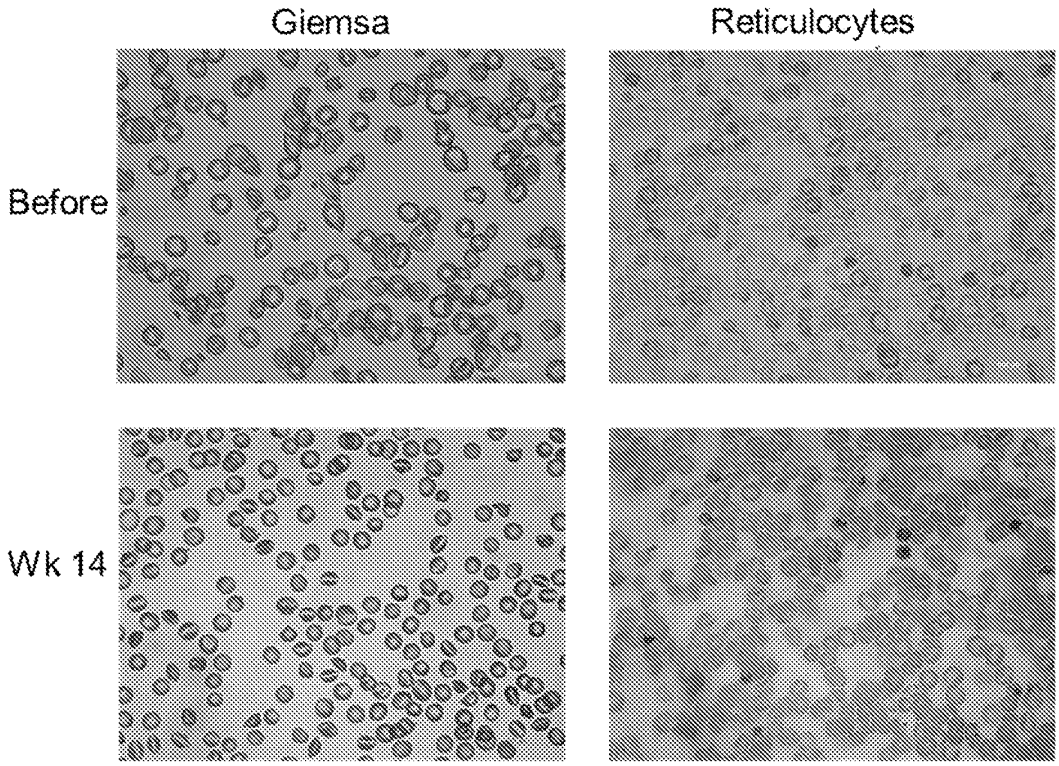


FIG. 36B

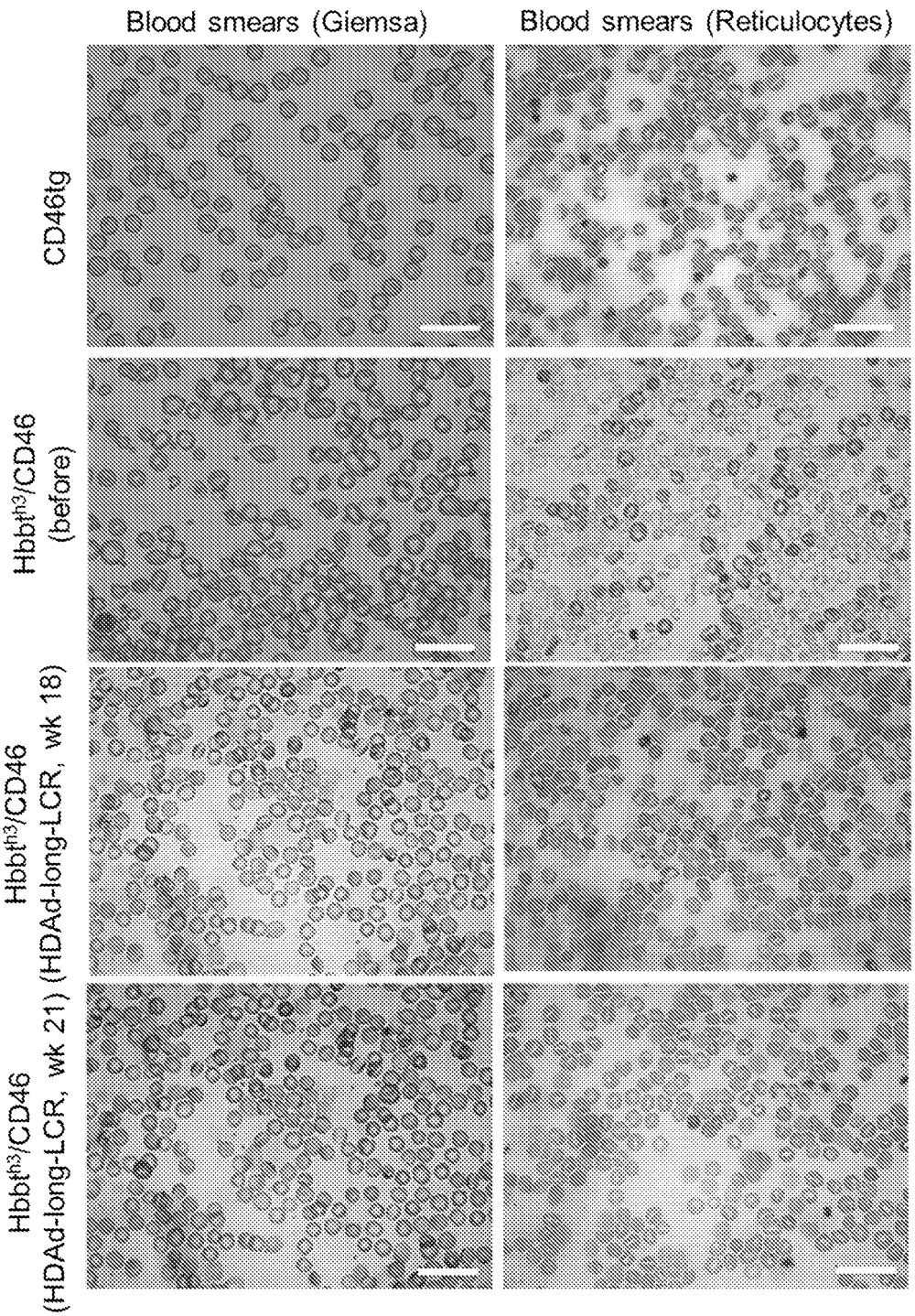

Blood smears (Giemsa)
Blood smears (Reticulocytes)
CD46tg
Hbbth3/CD46 (before)
Hbbth3/CD46 (HDAd-long-LCR, wk 18)
Hbbth3/CD46 (HDAd-long-LCR, wk 21)

FIG. 36C

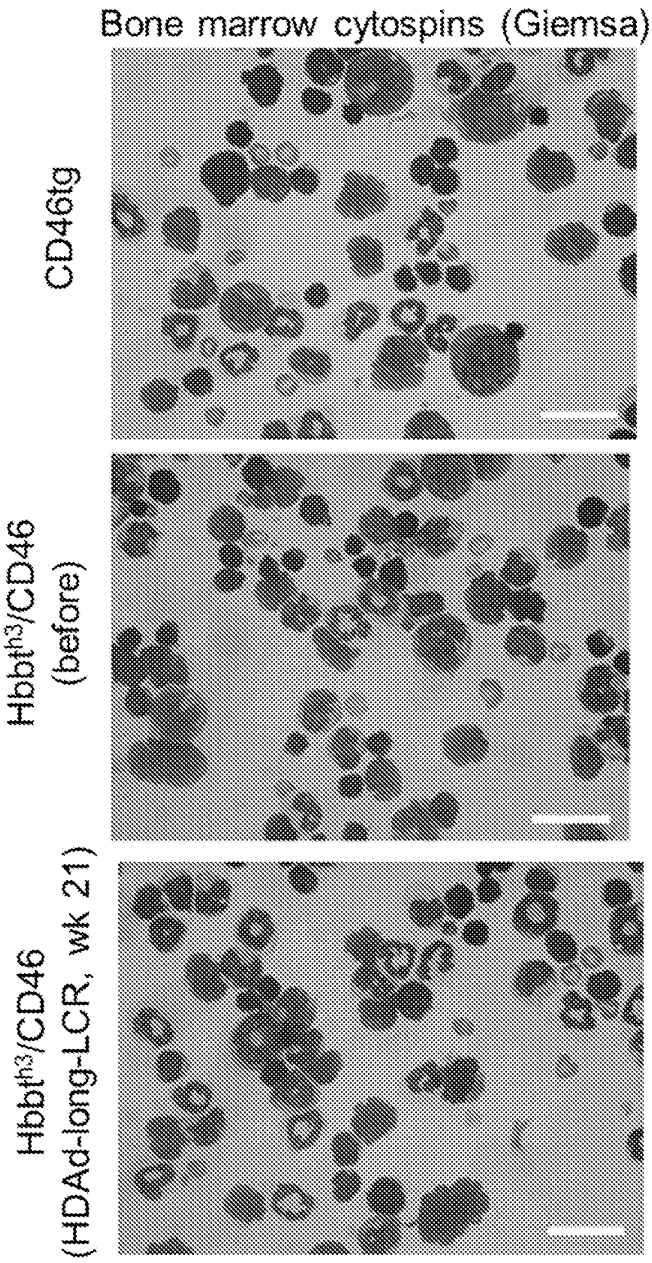

Bone marrow cytospins (Giemsa)
CD46tg
Hbbth3/CD46 (before)
Hbbth3/CD46 (HDAd-long-LCR, wk 21)

FIG. 37A

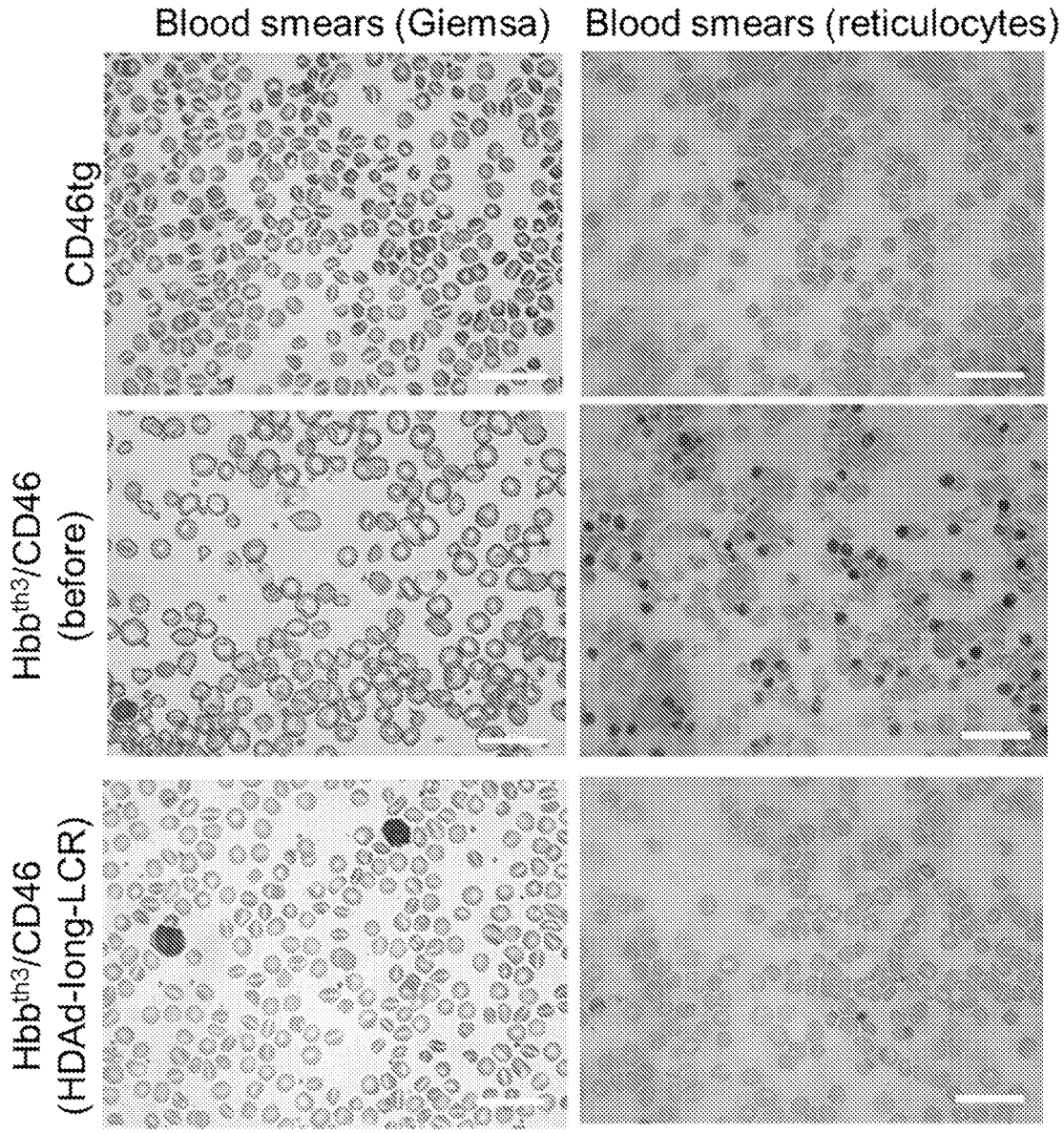

Blood smears (Giemsa)
Blood smears (reticulocytes)
CD46tg
Hbb^th3/CD46 (before)
Hbb^th3/CD46 (HDAd-long-LCR)

FIG. 37B

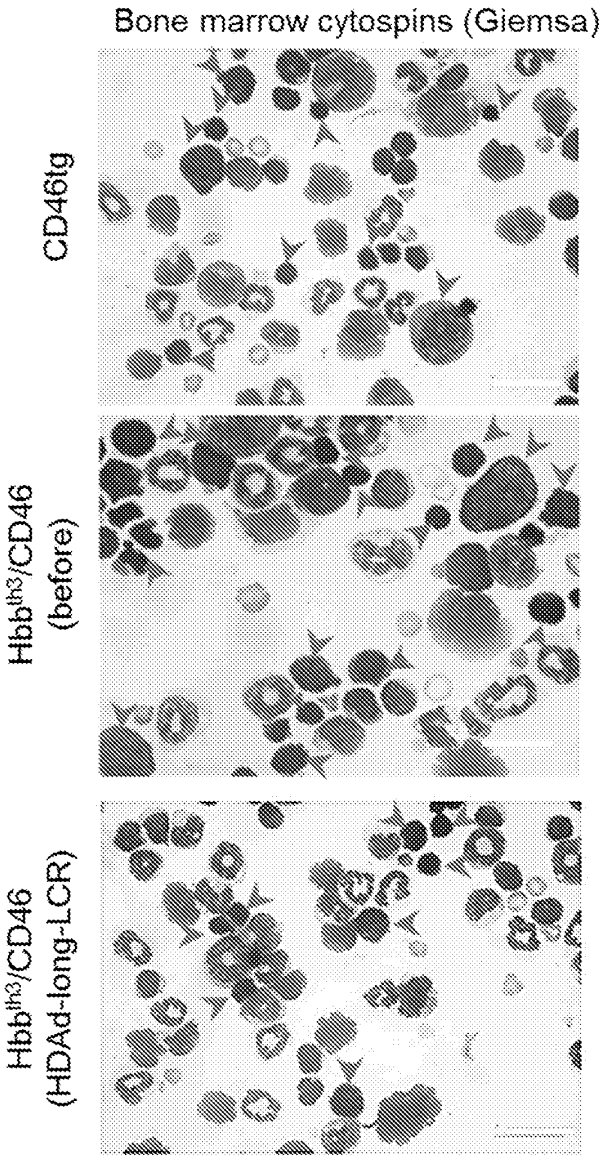

Bone marrow cytospins (Giemsa)
CD46tg
Hbb^th3/CD46 (before)
Hbb^th3/CD46 (HDAd-long-LCR)

FIG. 38

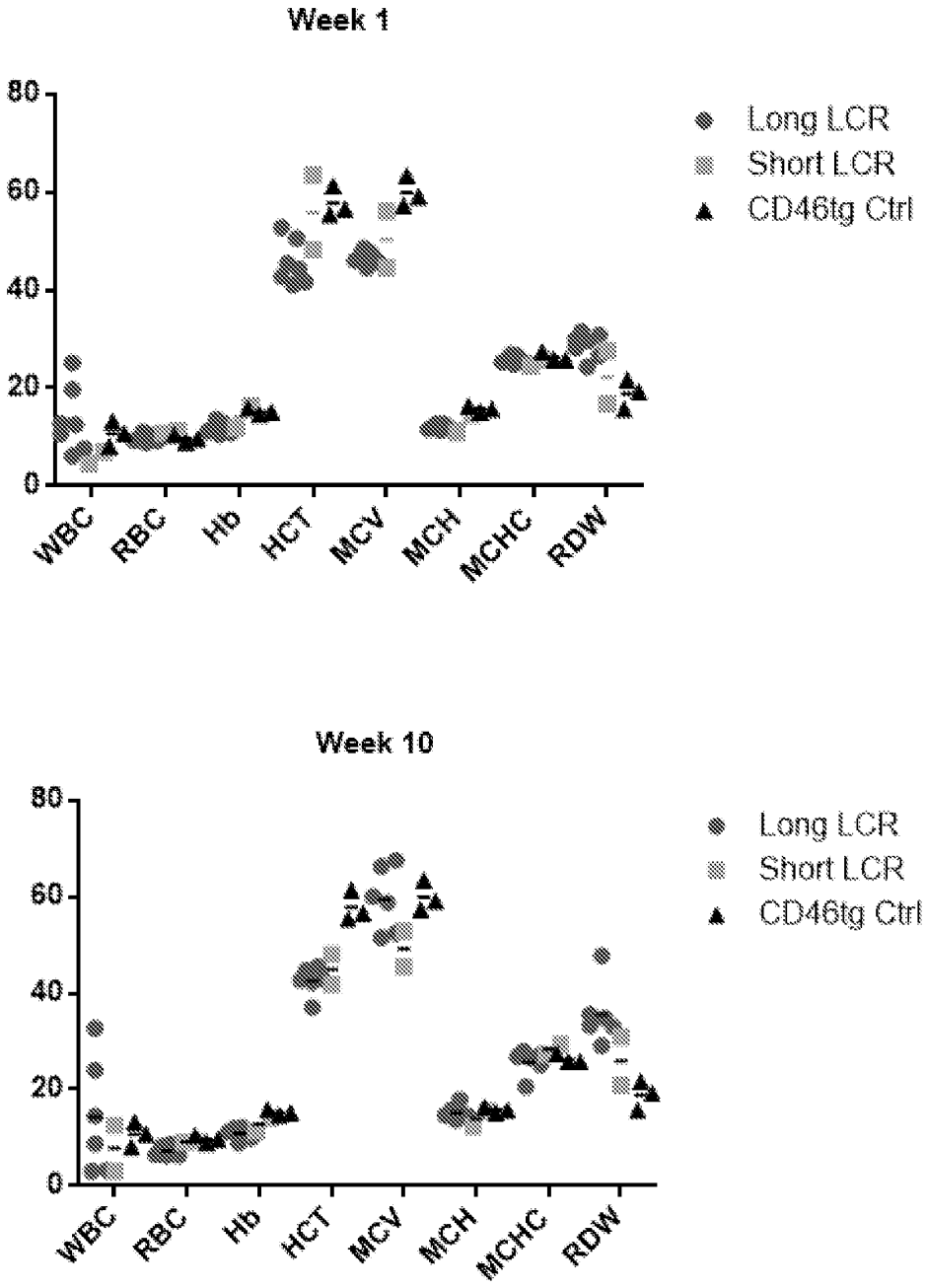

Week 1
80
60
40
20
0
WBC
RBC
Hb
HCT
MCV
MCH
MCHC
RDW
Long LCR
Short LCR
CD46tg Ctrl
Week 10
80
60
40
20
0
WBC
RBC
Hb
HCT
MCV
MCH
MCHC
RDW
Long LCR
Short LCR
CD46tg Ctrl

FIG. 39A

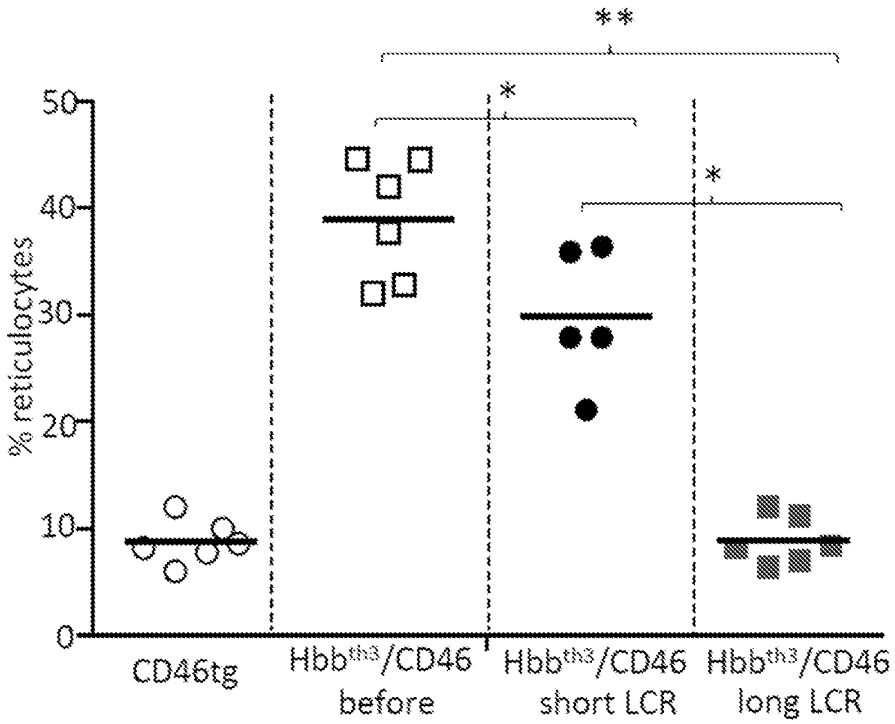

**
*
*
50
40
30
20
10
0
% reticulocytes
CD46tg
Hbb^th3/CD46 before
Hbb^th3/CD46 short LCR
Hbb^th3/CD46 long LCR

FIG. 39B

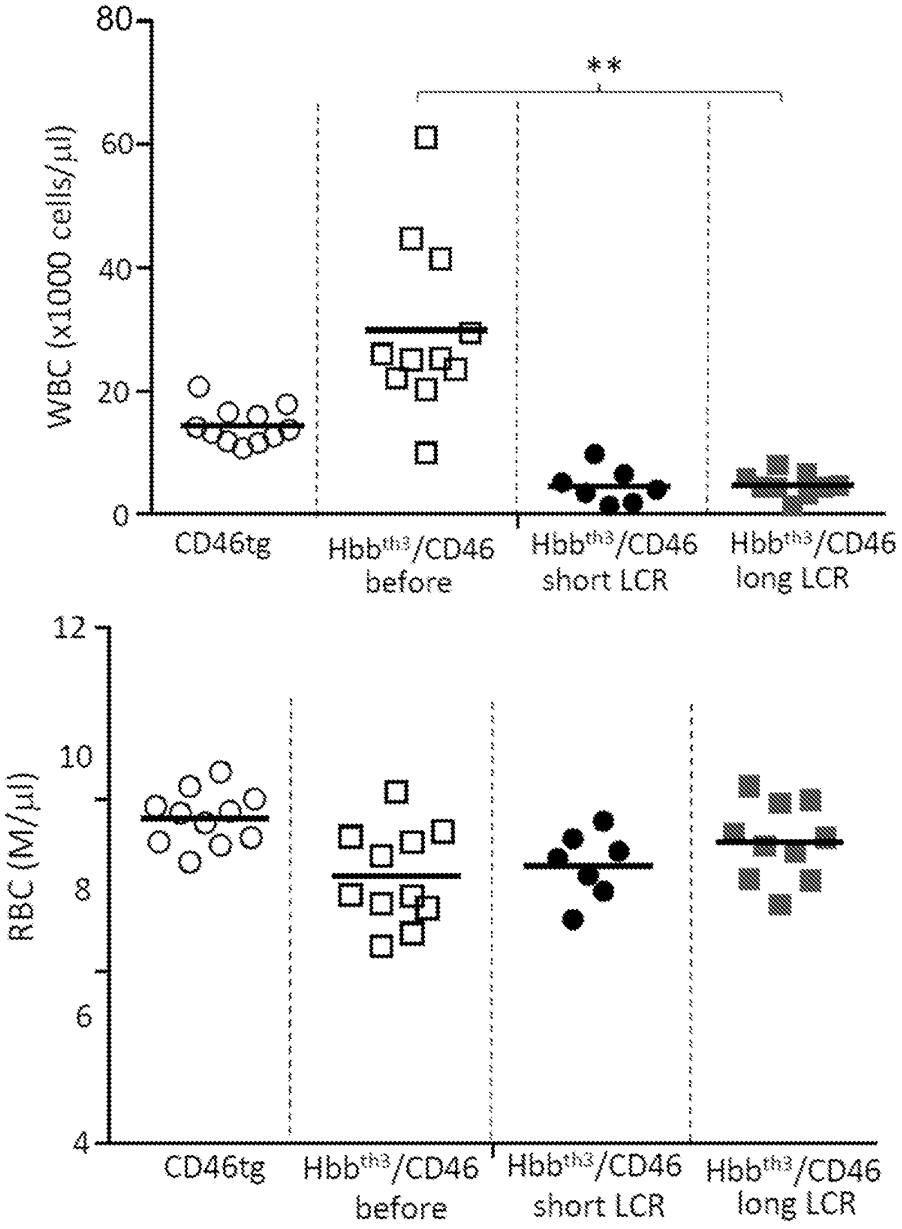

80
60
40
20
0
WBC (x1000 cells/μl)
**
CD46tg
Hbb^th3/CD46 before
Hbb^th3/CD46 short LCR
Hbb^th3/CD46 long LCR
12
10
8
6
4
RBC (M/μl)
CD46tg
Hbb^th3/CD46 before
Hbb^th3/CD46 short LCR
Hbb^th3/CD46 long LCR

FIG. 39B cont'd

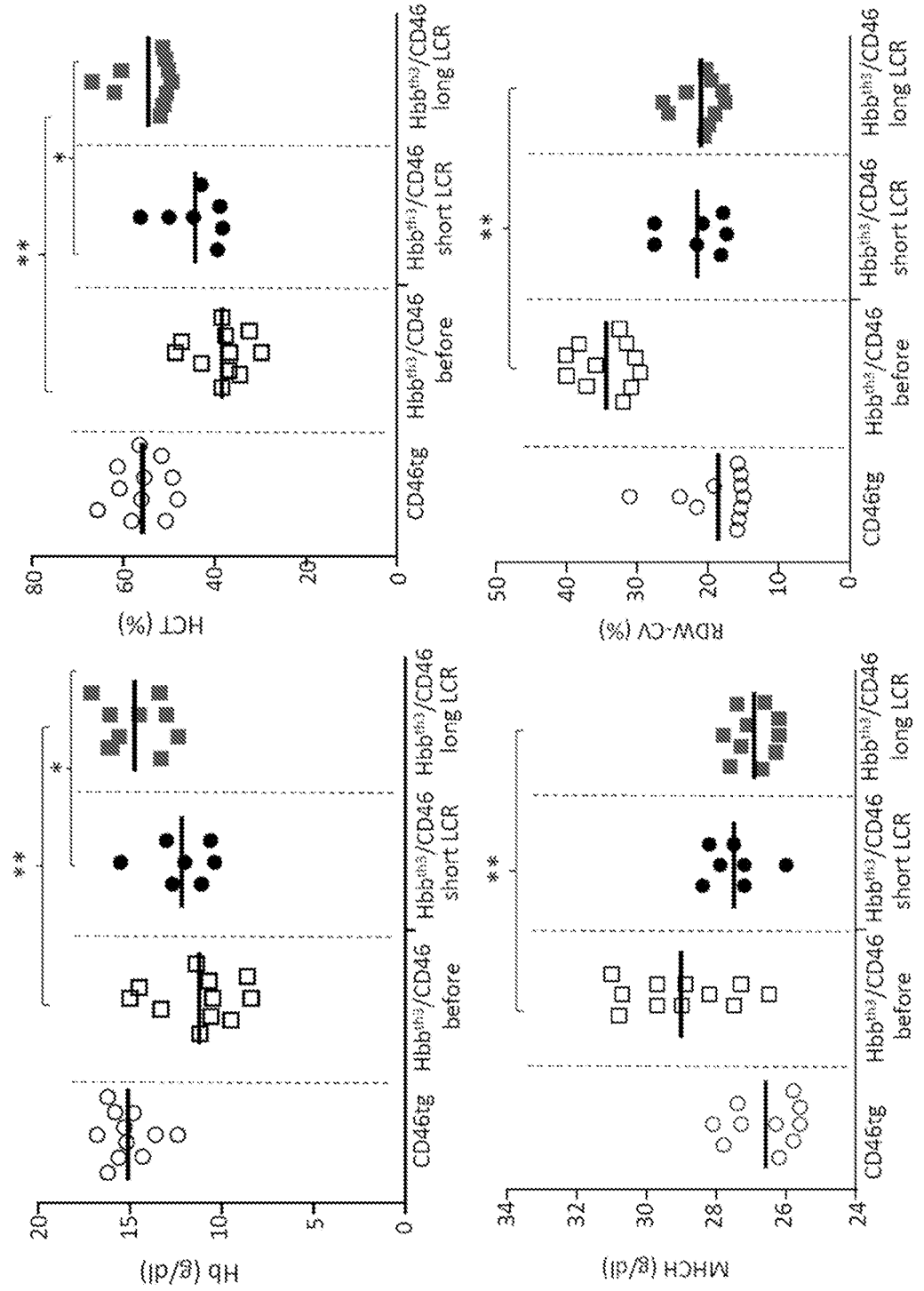
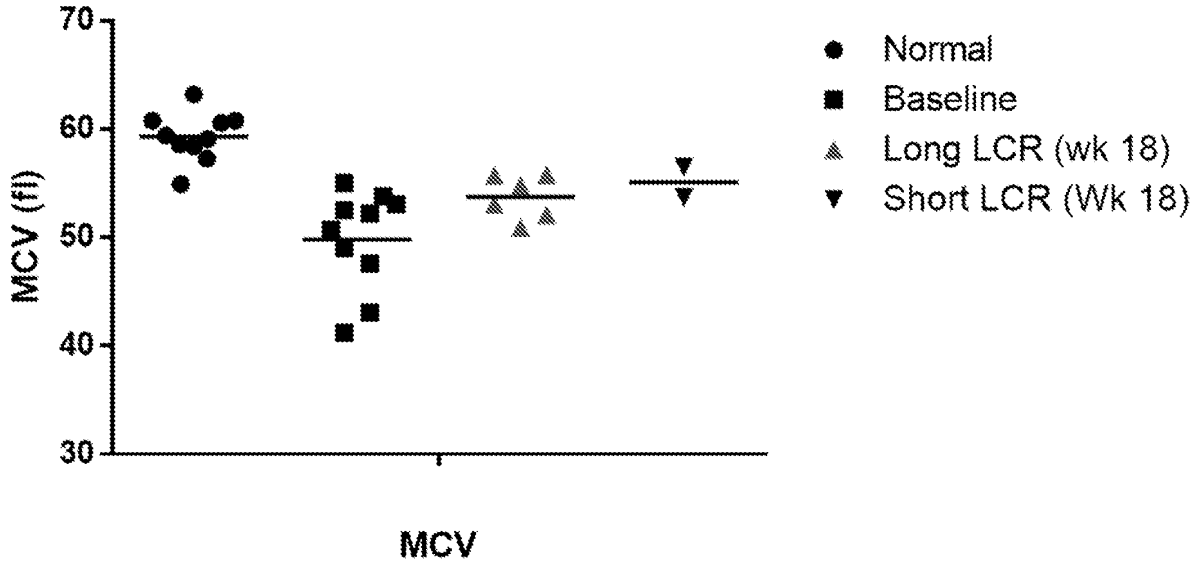


FIG. 39B cont'd



MCH

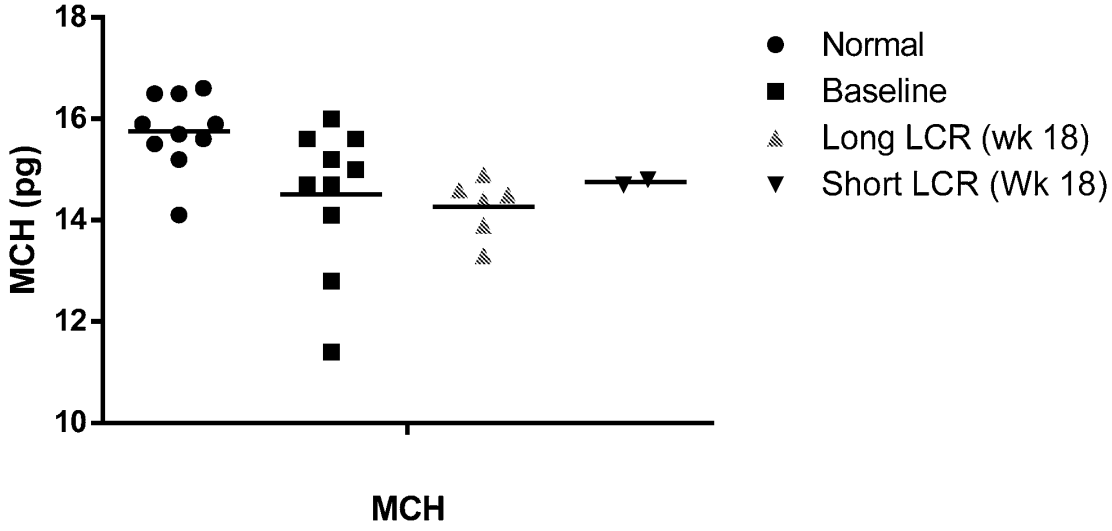
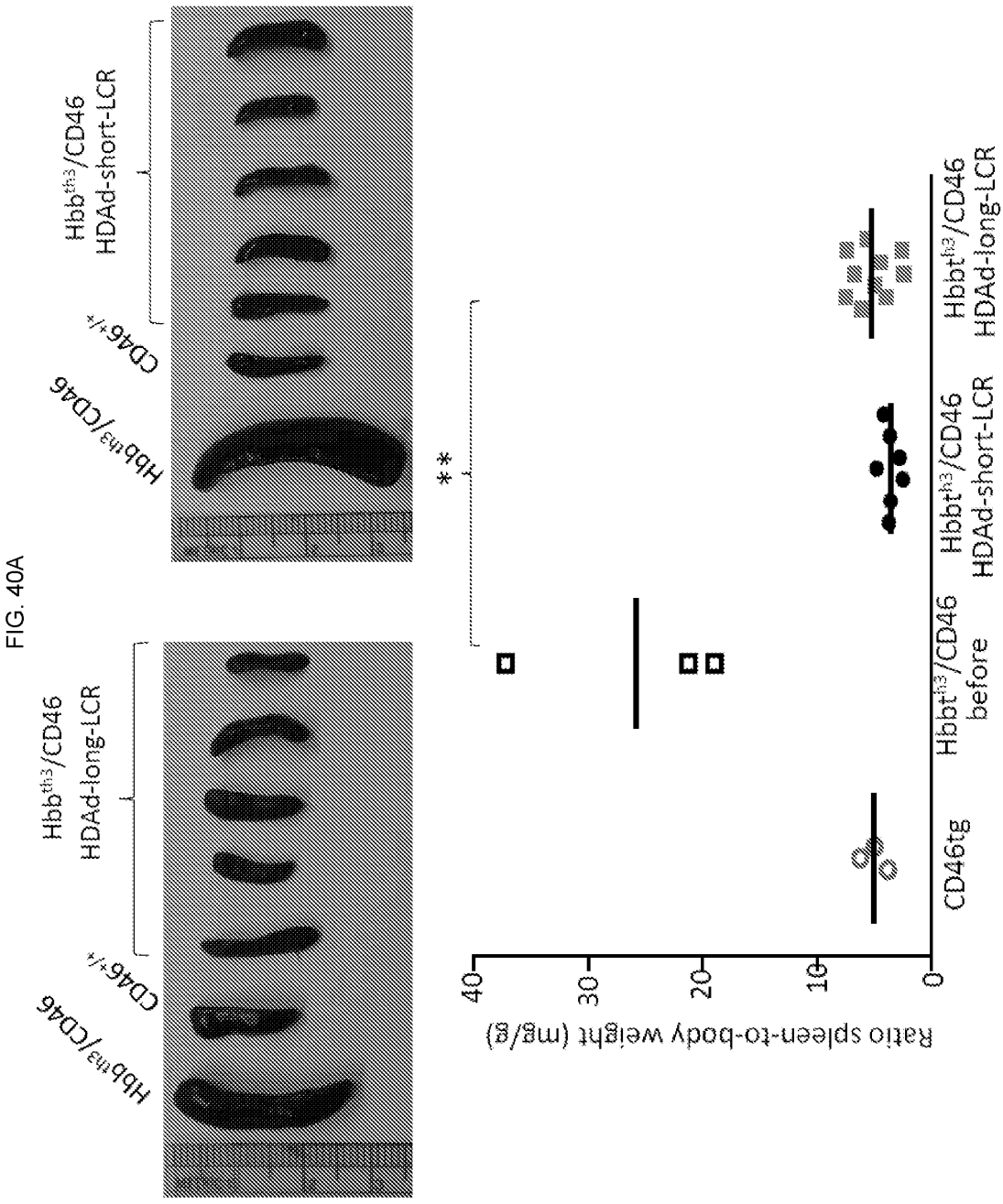


FIG. 40A


Hbb^th3/CD46
HDAd-long-LCR
CD46+/+
Hbb^th3/CD46
Hbb^th3/CD46
HDAd-short-LCR
CD46+/+
Hbb^th3/CD46
**
Ratio spleen-to-body weight (mg/g)
0
10
20
30
40
CD46tg
Hbbt^h3/CD46 before
Hbbt^h3/CD46 HDAd-short-LCR
Hbbt^h3/CD46 HDAd-long-LCR

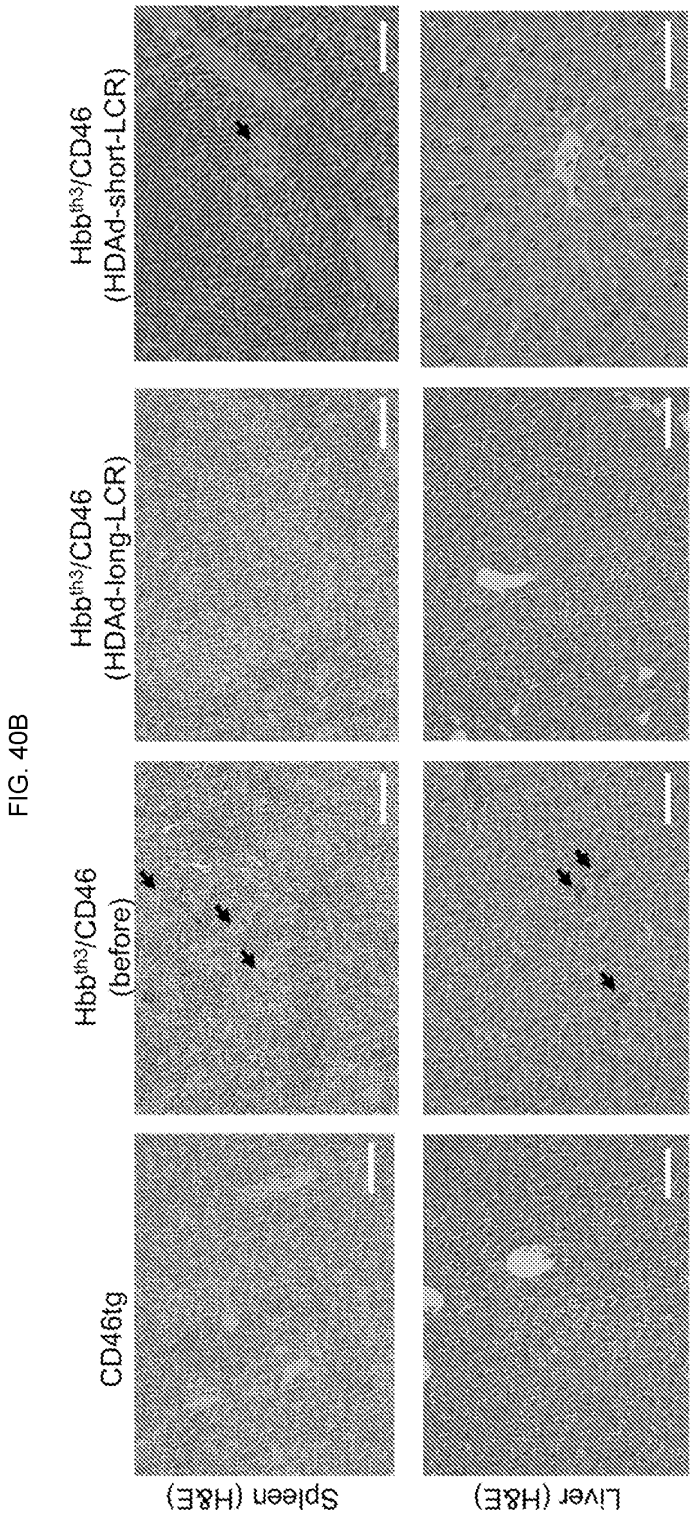

Spleen (H&E)
Liver (H&E)
CD46tg
Hbb^th3/CD46 (before)
Hbb^th3/CD46 (HDAd-long-LCR)
Hbb^th3/CD46 (HDAd-short-LCR)

FIG. 40B

FIG. 41

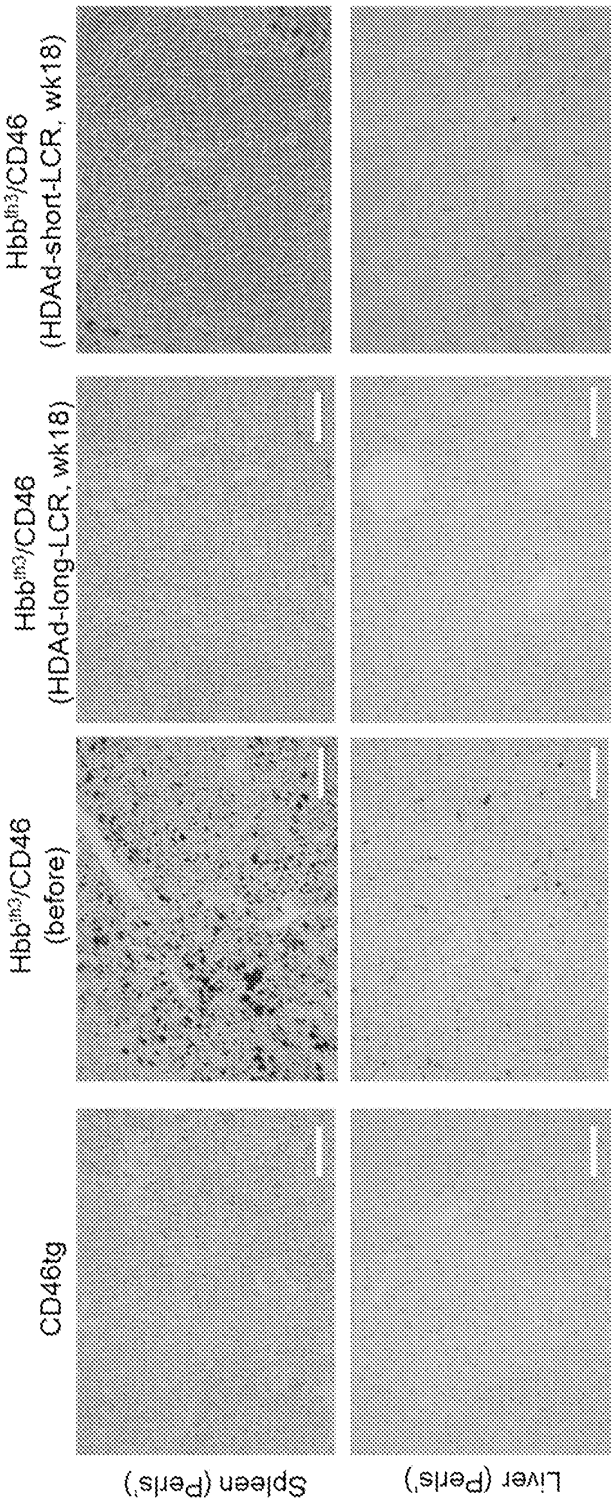

CD46tg
Hbb^tm3/CD46 (before)
Hbb^tm3/CD46 (HDAd-long-LCR, wk18)
Hbb^tm3/CD46 (HDAd-short-LCR, wk18)
Spleen (Perls')
Liver (Perls')

FIG. 42A

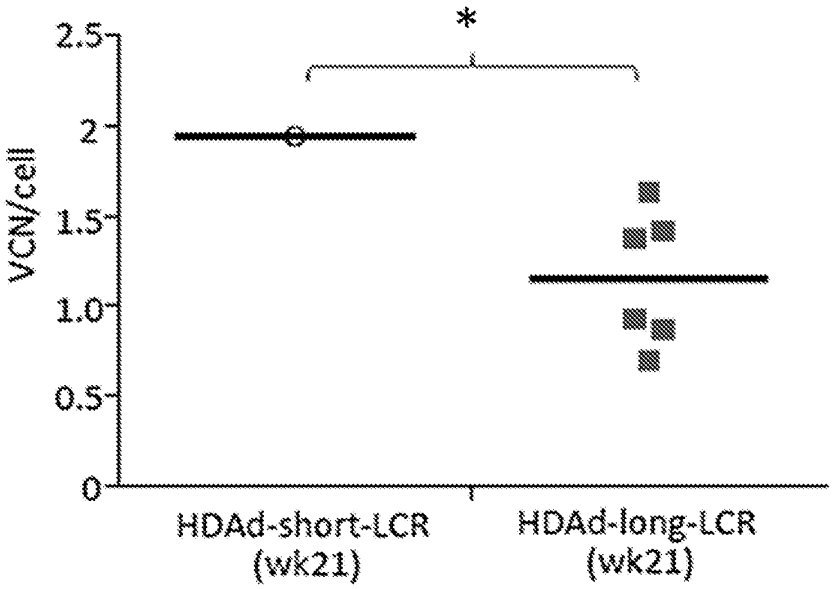


FIG. 42B

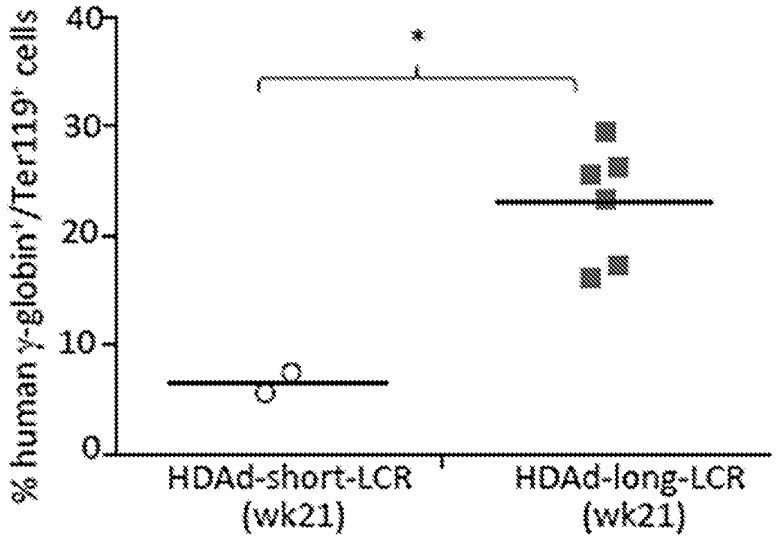


FIG. 42C

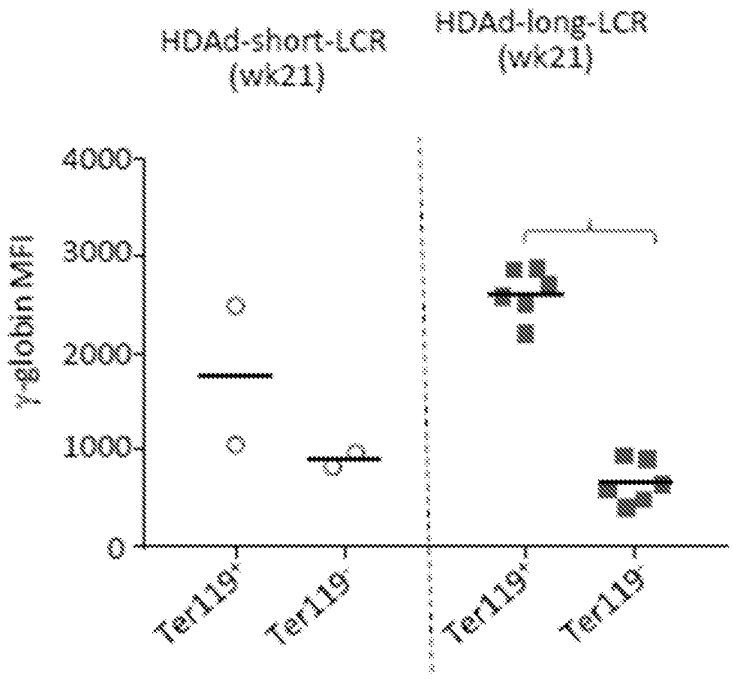

HDAd-short-LCR
(wk21)
HDAd-long-LCR
(wk21)
γ-globin MFI
4000
3000
2000
1000
0
Ter119+
Ter119-
Ter119+
Ter119-

FIG. 43

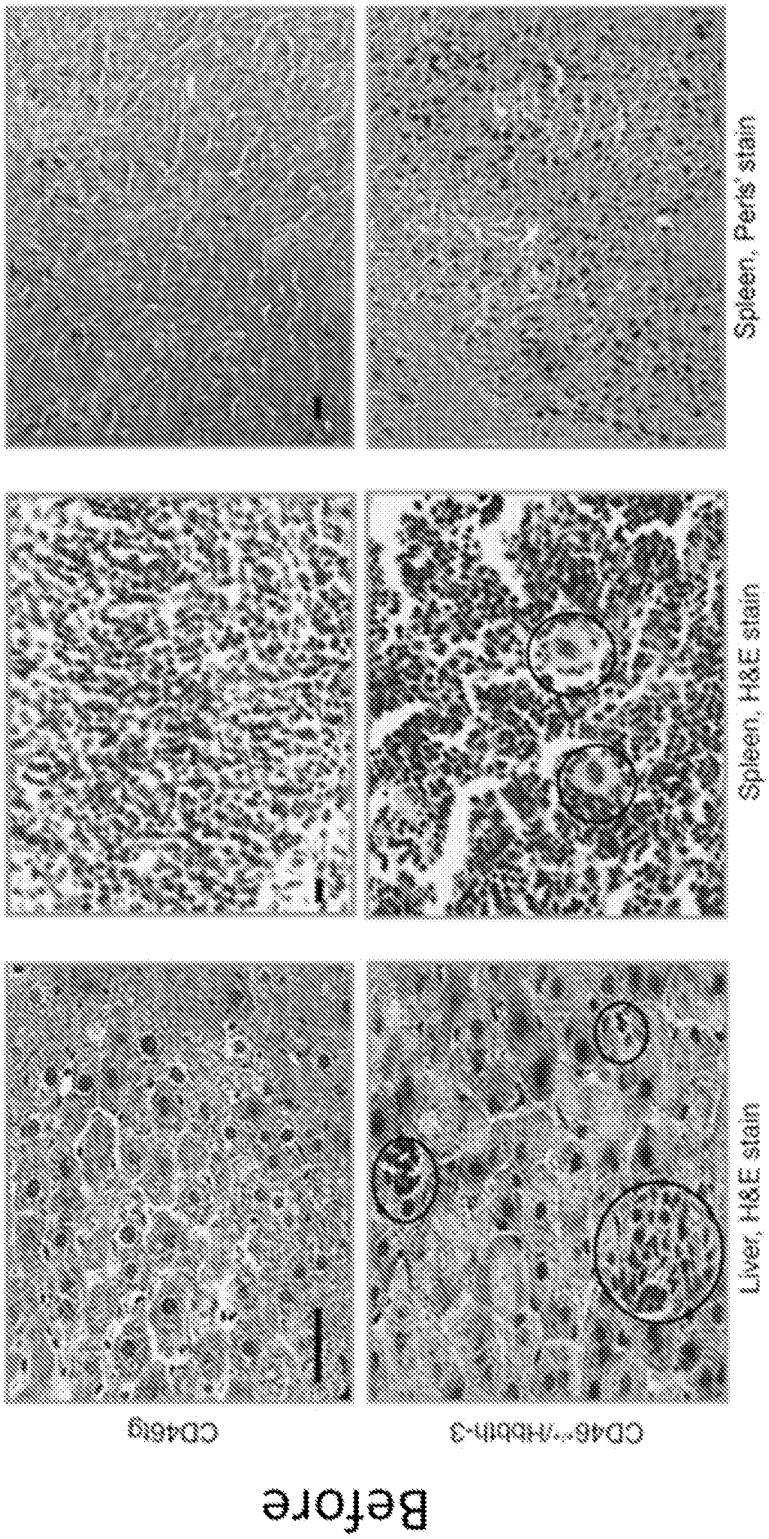

Before
CD46-/Nbbln-3
CD46Ig
Liver, H&E stain
Spleen, H&E stain
Spleen, Perls' stain

FIG. 44A

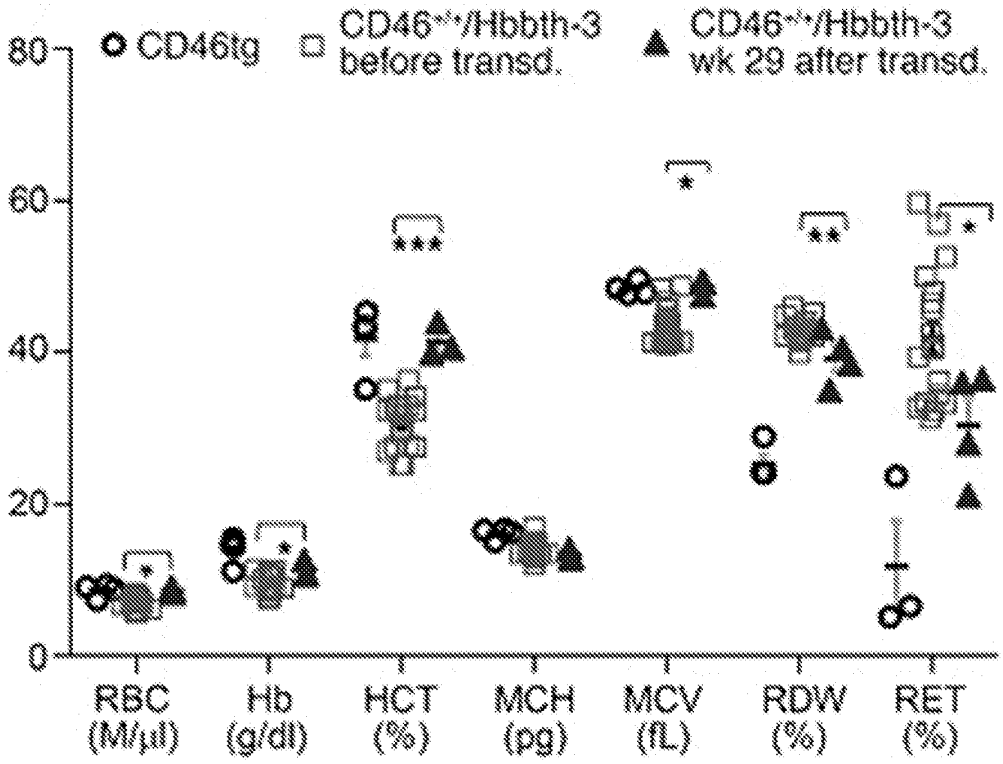


FIG. 44B

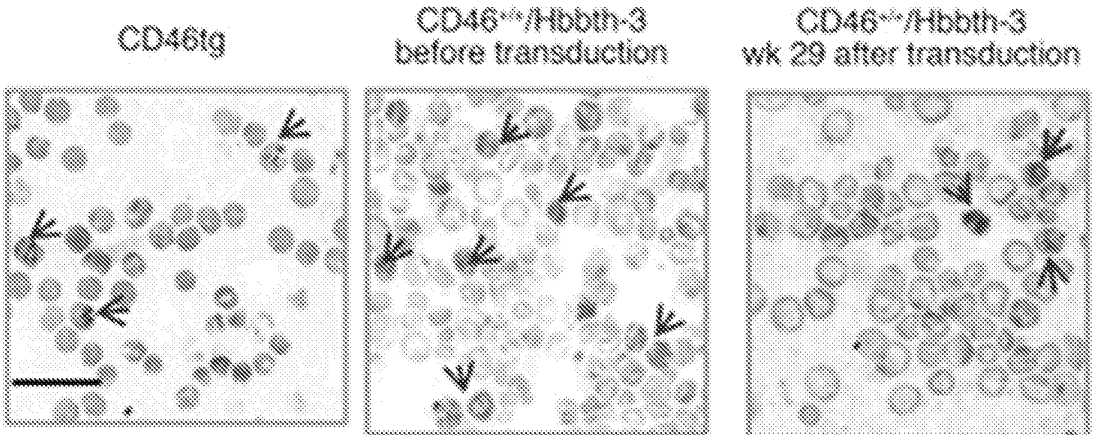


FIG. 44C

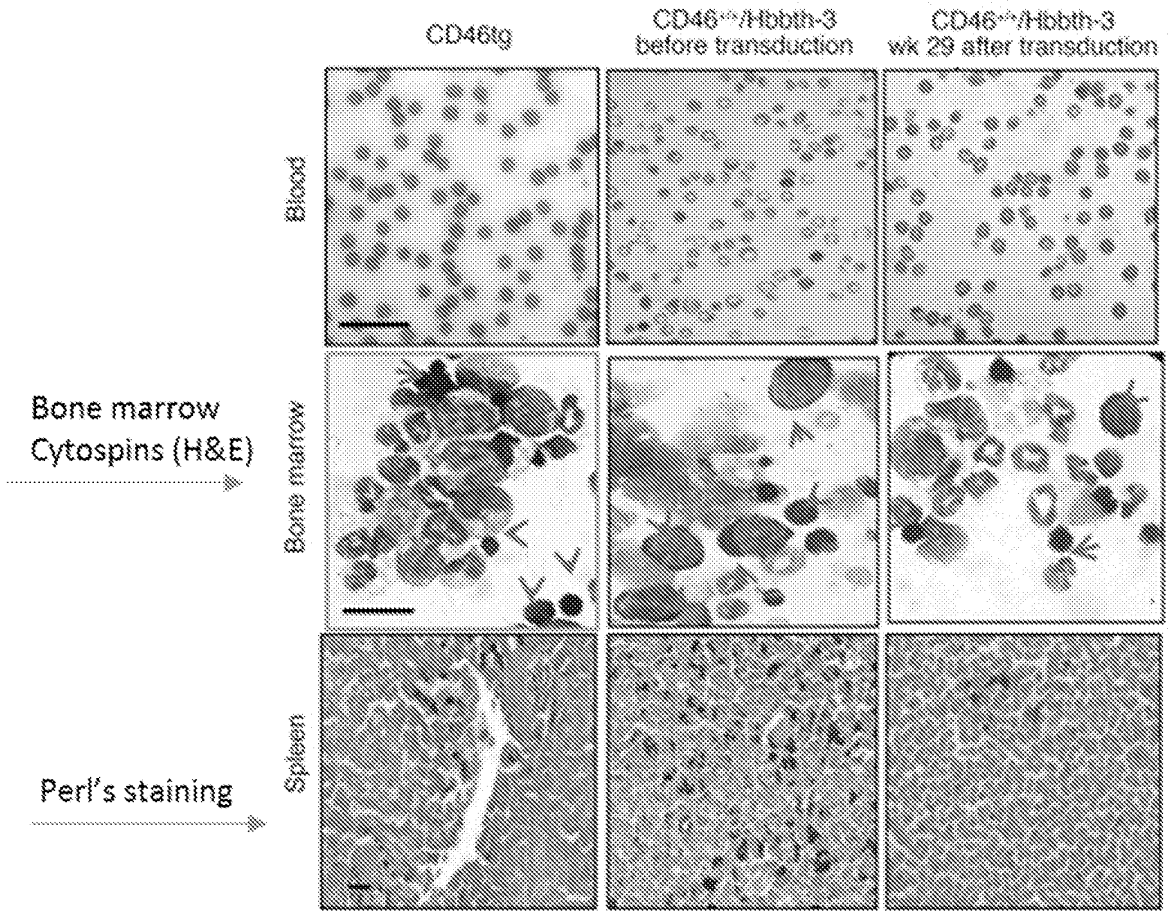

CD46tg
CD46tg/Hbbth-3 before transduction
CD46tg/Hbbth-3 wk 29 after transduction
Blood
Bone marrow
Spleen
Bone marrow Cytospins (H&E)
Perl's staining

FIG. 44D

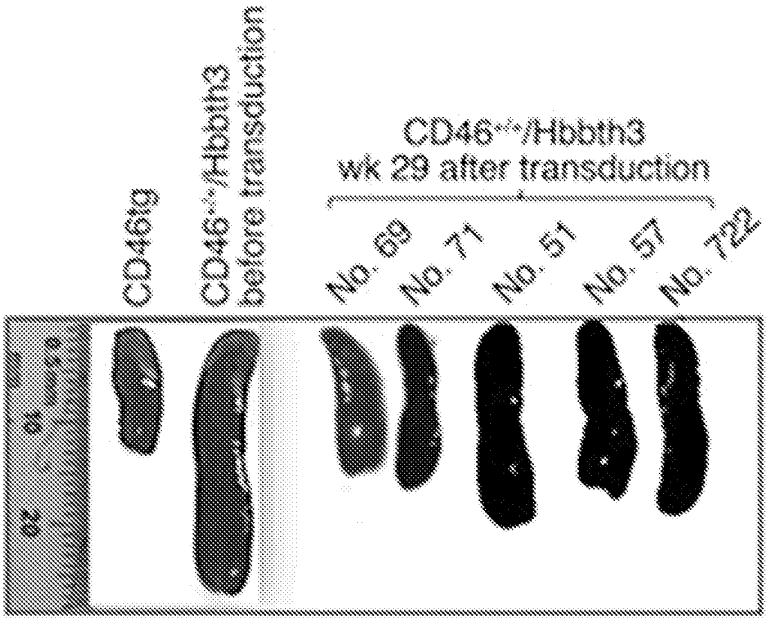


FIG. 44E

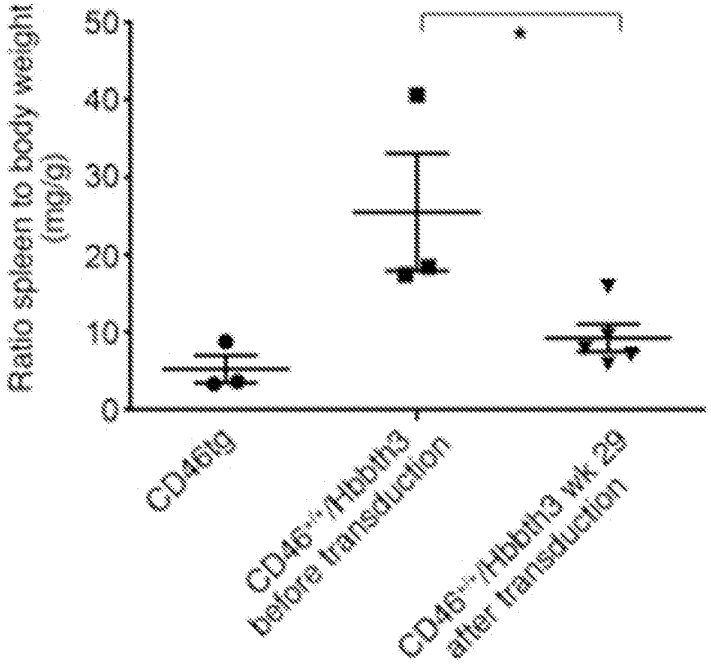


FIG. 45

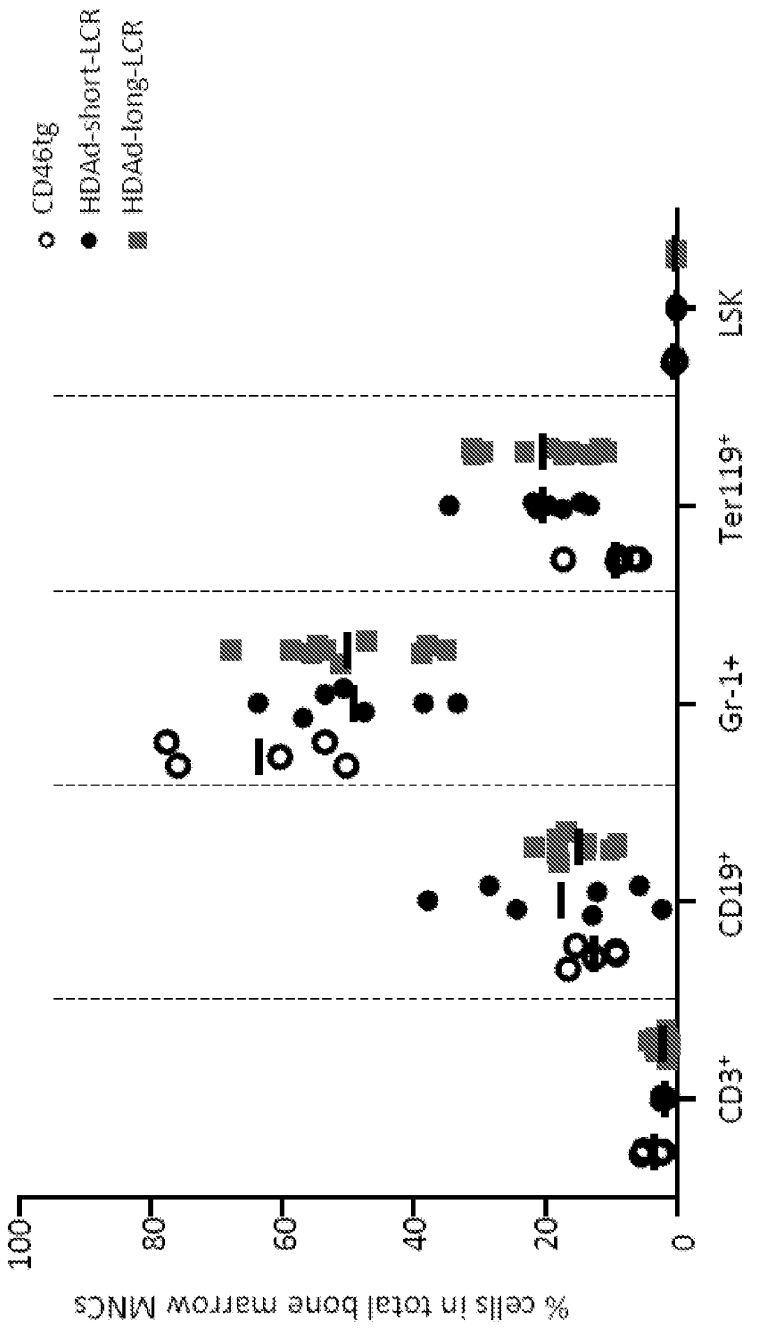

CD46tg
HDAd-short-LCR
HDAd-long-LCR
% cells in total bone marrow MNCs
100
80
60
40
20
0
CD3+
CD19+
Gr-1+
Ter119+
LSK

FIG. 46

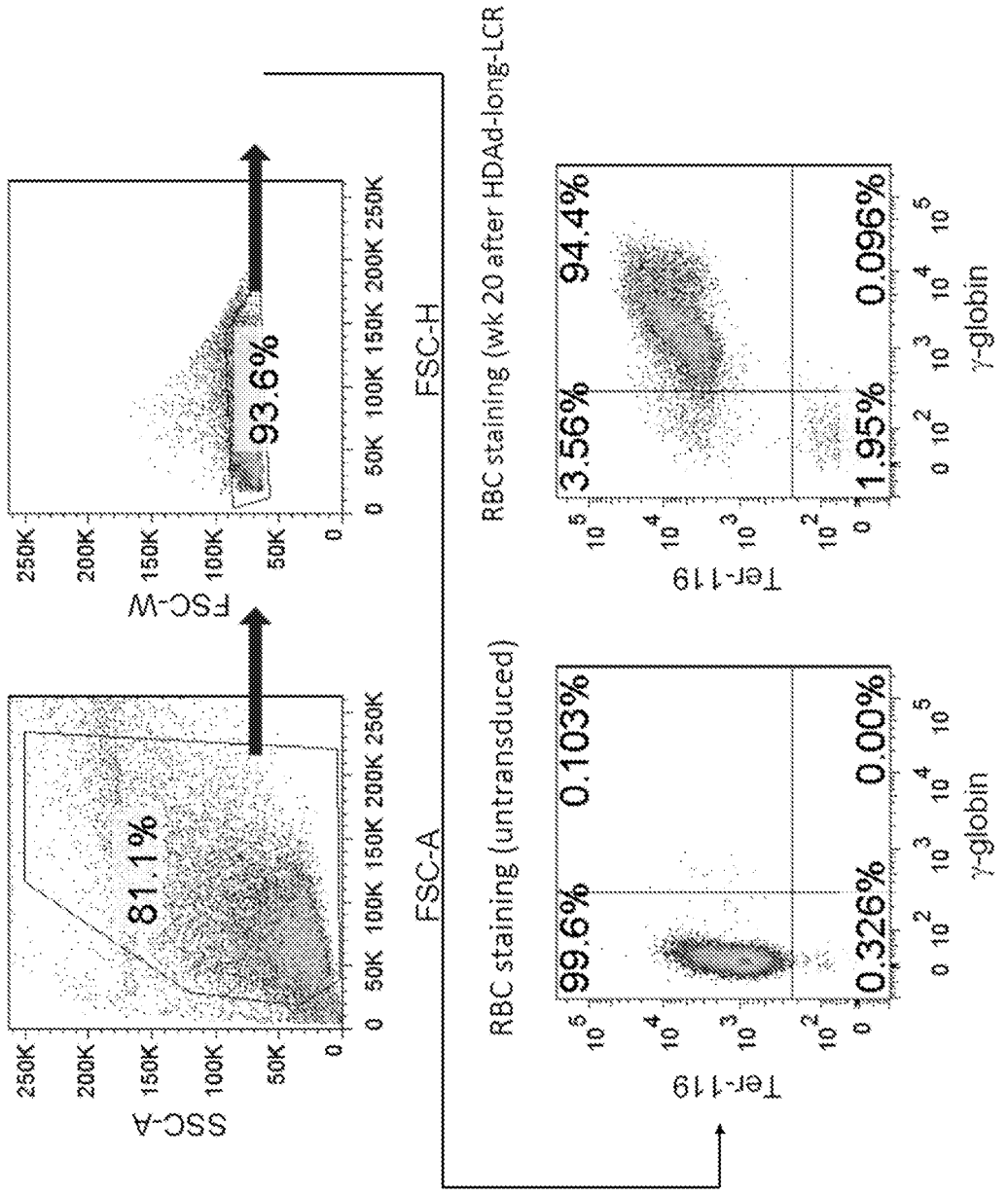

SSC-A
FSC-W
FSC-A
FSC-H
81.1%
93.6%
RBC staining (untransduced)
RBC staining (wk 20 after HDAd-long-LCR
Ter-119
γ-globin
99.6%
0.103%
0.326%
0.00%
3.56%
94.4%
1.95%
0.096%

FIG. 47A

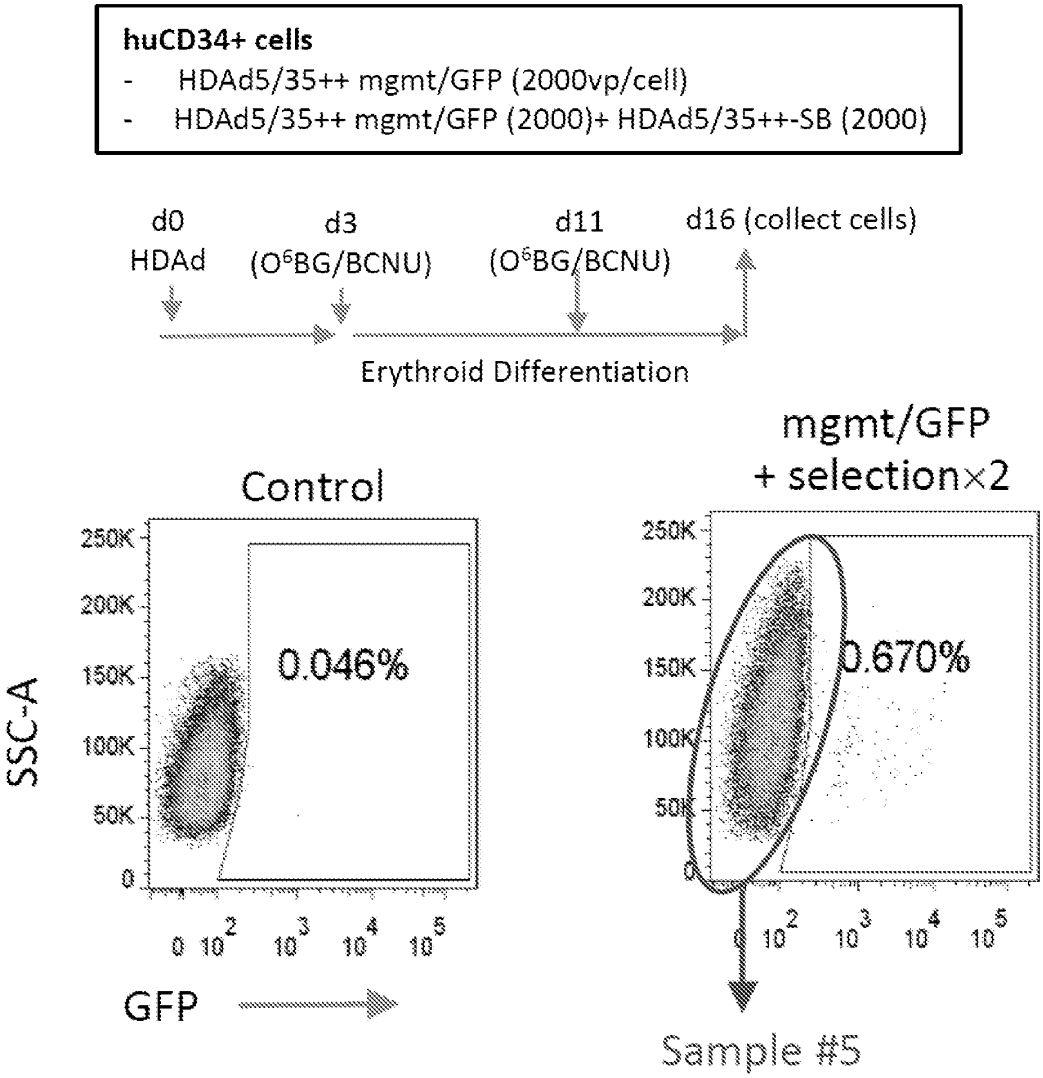

huCD34+ cells
- HDAd5/35++ mgmt/GFP (2000vp/cell)
- HDAd5/35++ mgmt/GFP (2000)+ HDAd5/35++-SB (2000)
d0
HDAd
d3
(O6BG/BCNU)
d11
(O6BG/BCNU)
d16 (collect cells)
Erythroid Differentiation
Control
mgmt/GFP
+ selection×2
SSC-A
GFP
0.046%
0.670%
250K
200K
150K
100K
50K
0
Sample #5

FIG. 47A cont'd

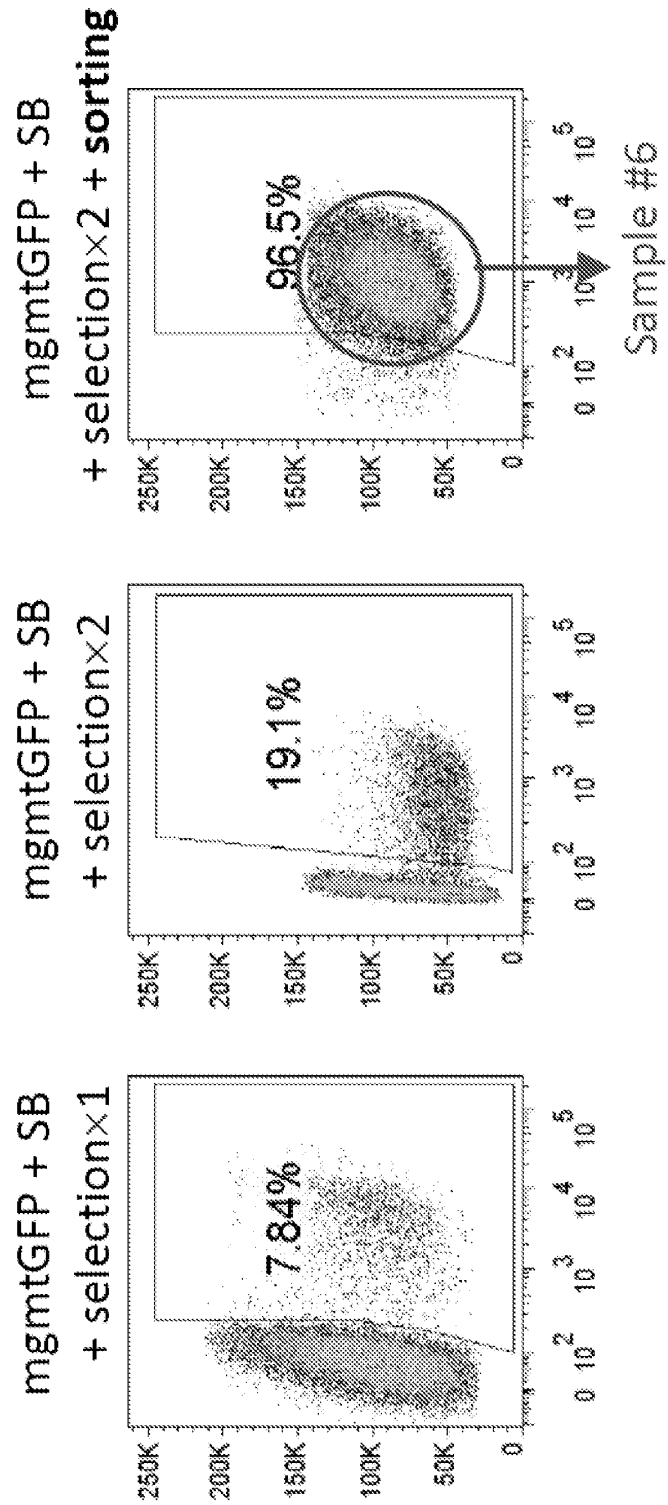


FIG. 47B

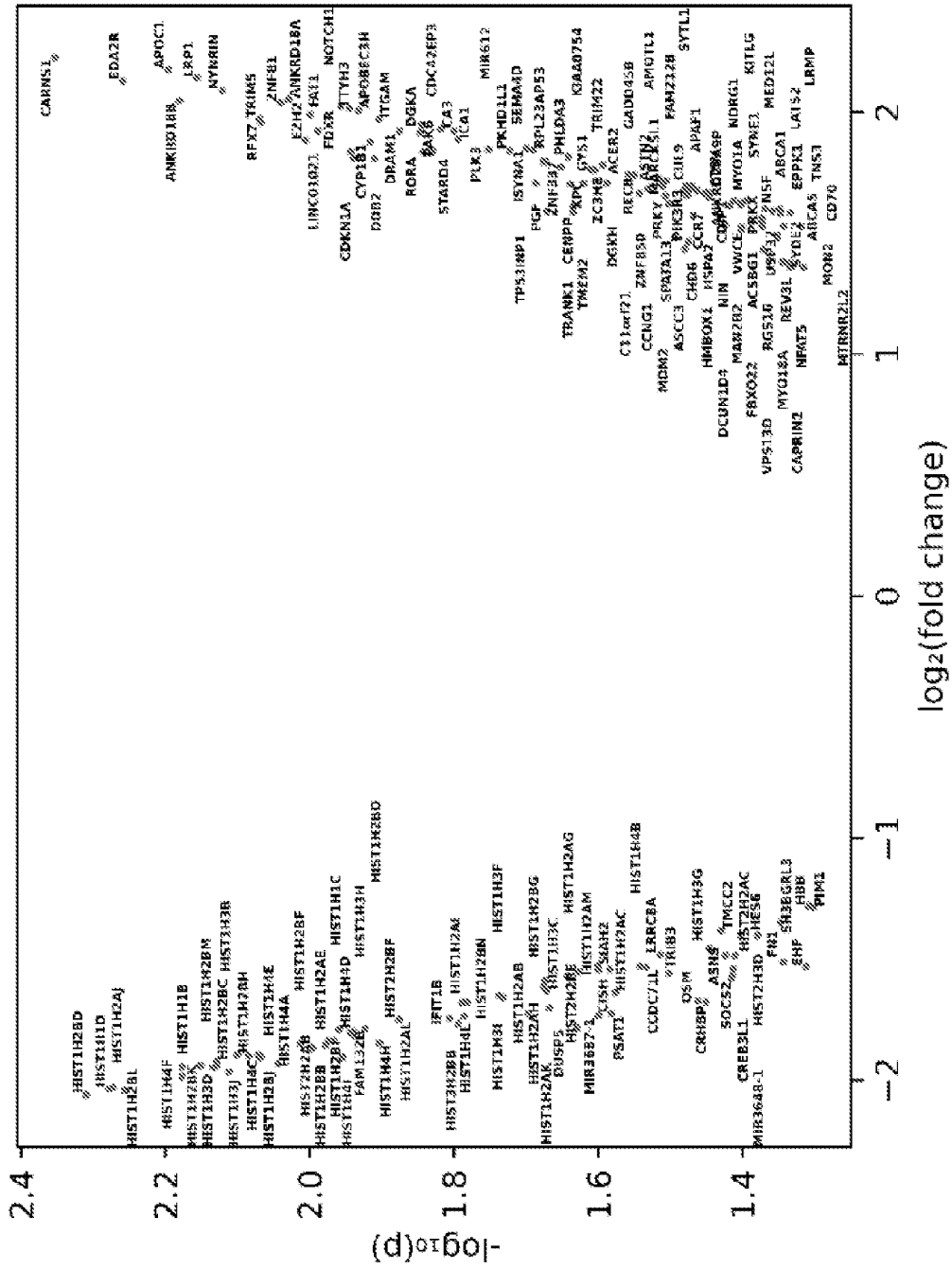


FIG. 48

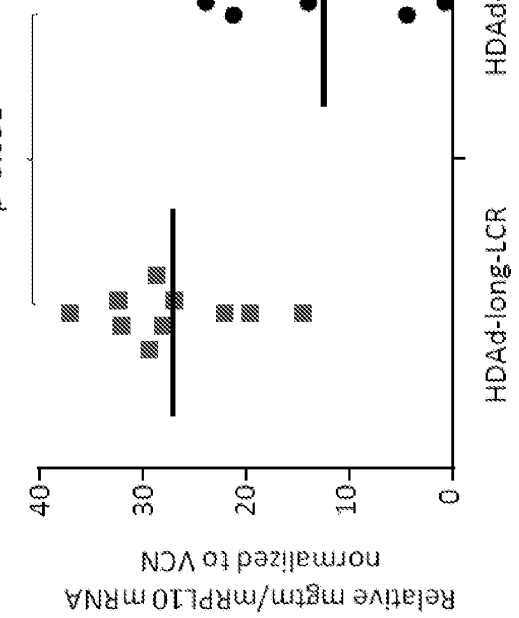
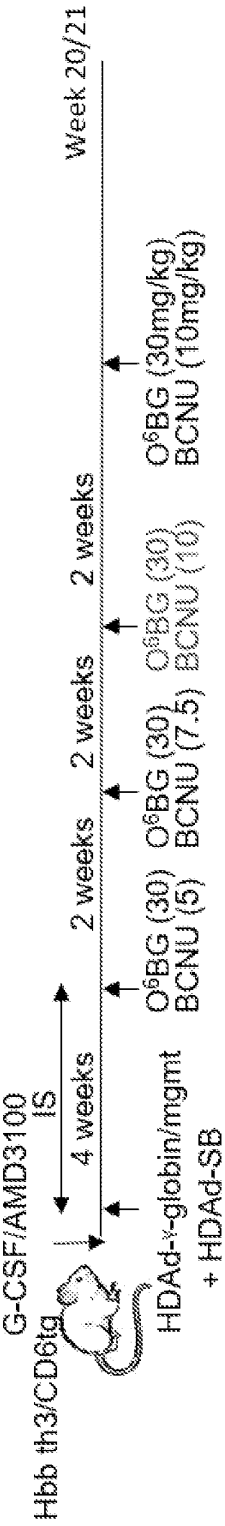

p=0.031
Relative mgtm/mRPL10 mRNA normalized to VCN
40
30
20
10
0
HDAd-long-LCR
HDAd-short-LCR

FIG. 49


Week 20/21
2 weeks
2 weeks
2 weeks
4 weeks
G-CSF/AMD3100
IS
Hbb th3/CD6tg
HDAd-γ-globin/mgmt
+ HDAd-SB
O6BG (30)
BCNU (5)
O6BG (30)
BCNU (7.5)
O6BG (30)
BCNU (10)
O6BG (30mg/kg)
BCNU (10mg/kg)

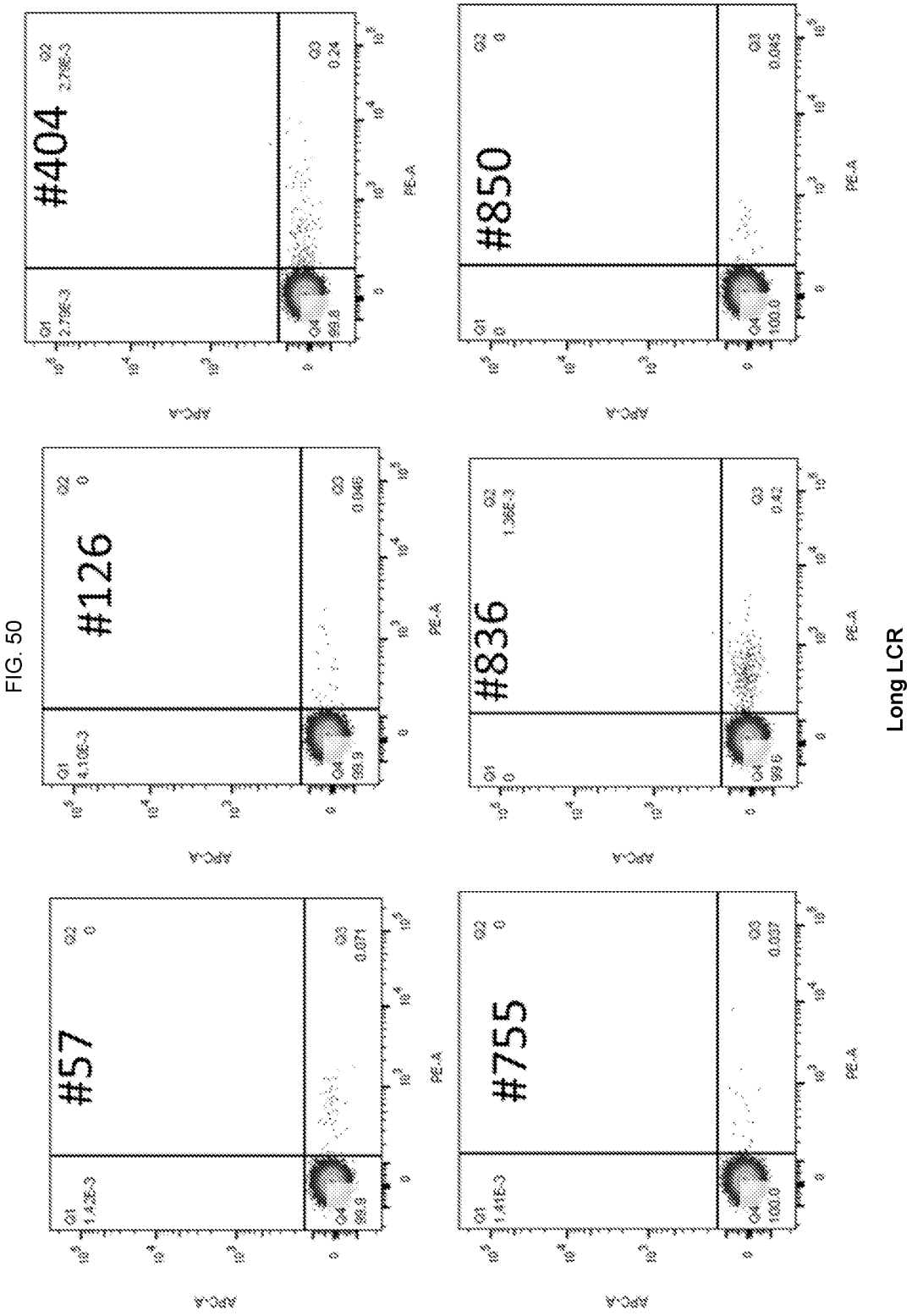


FIG. 50

FIG. 50 cont'd

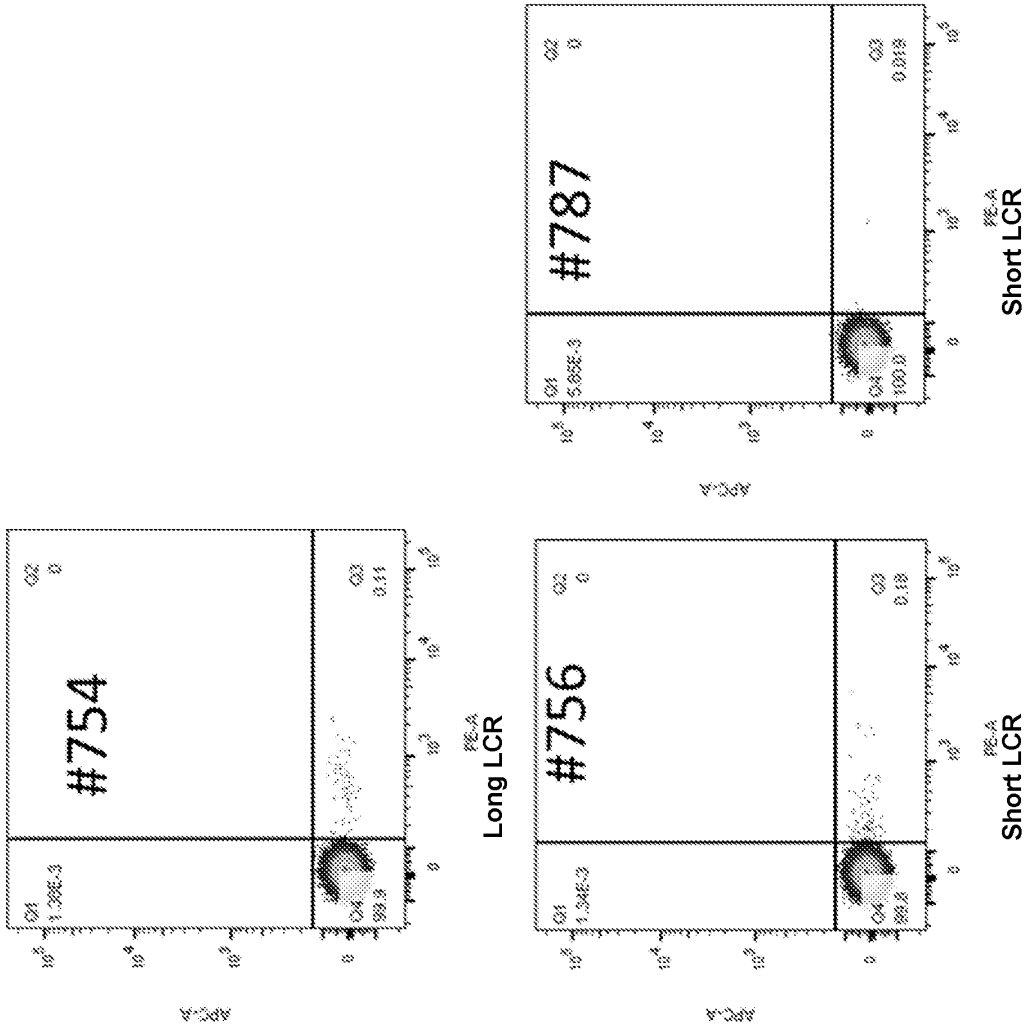


FIG. 51

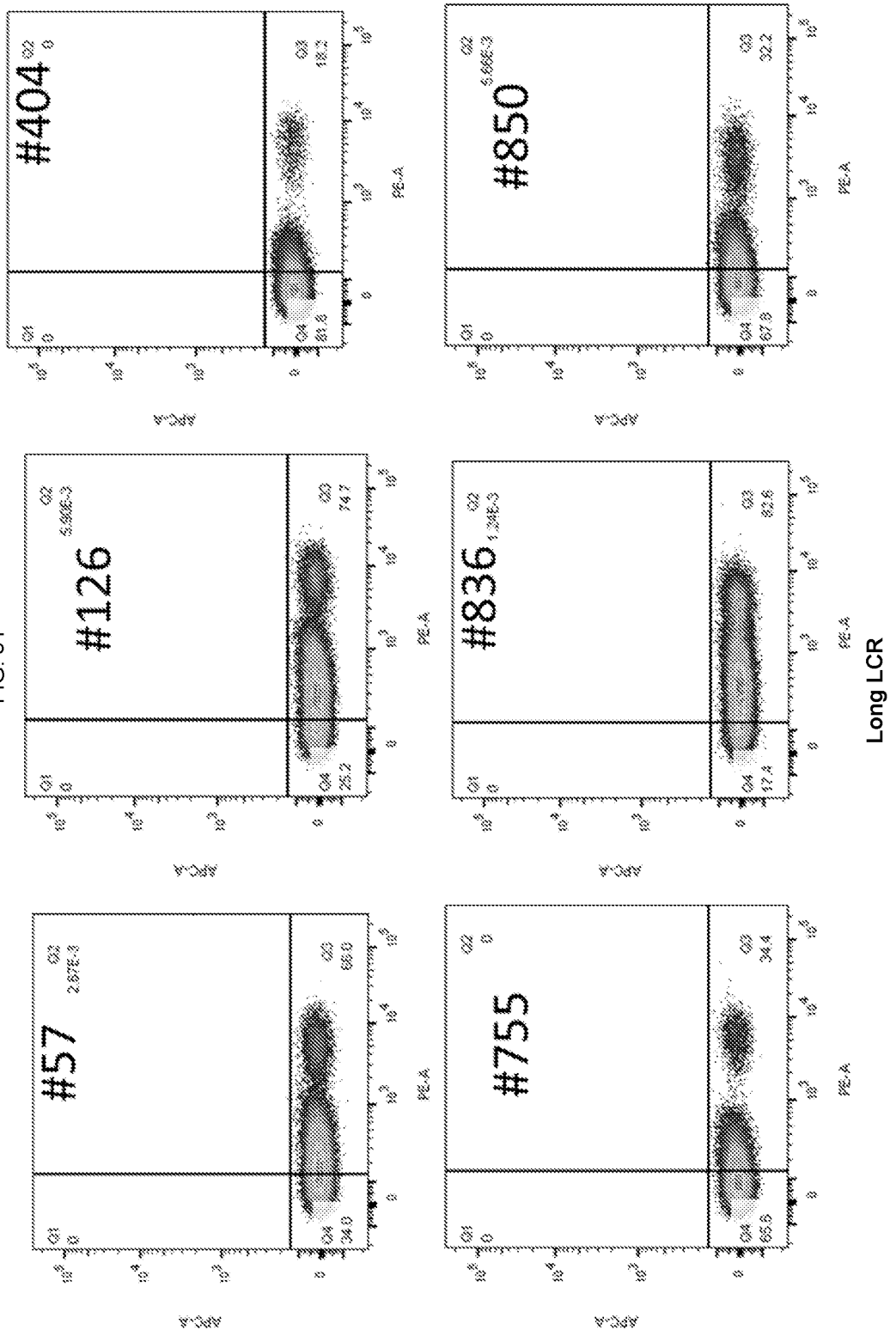


FIG. 51 cont'd

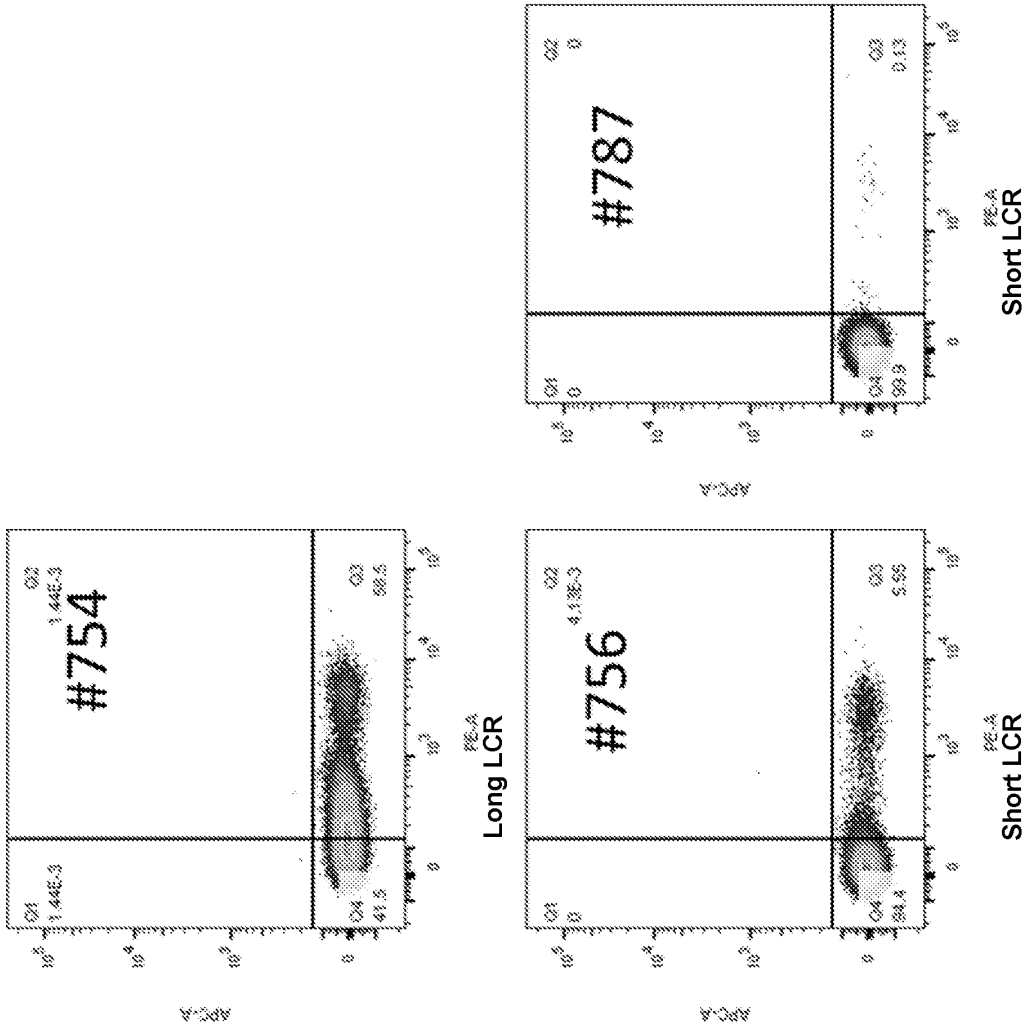


FIG. 52

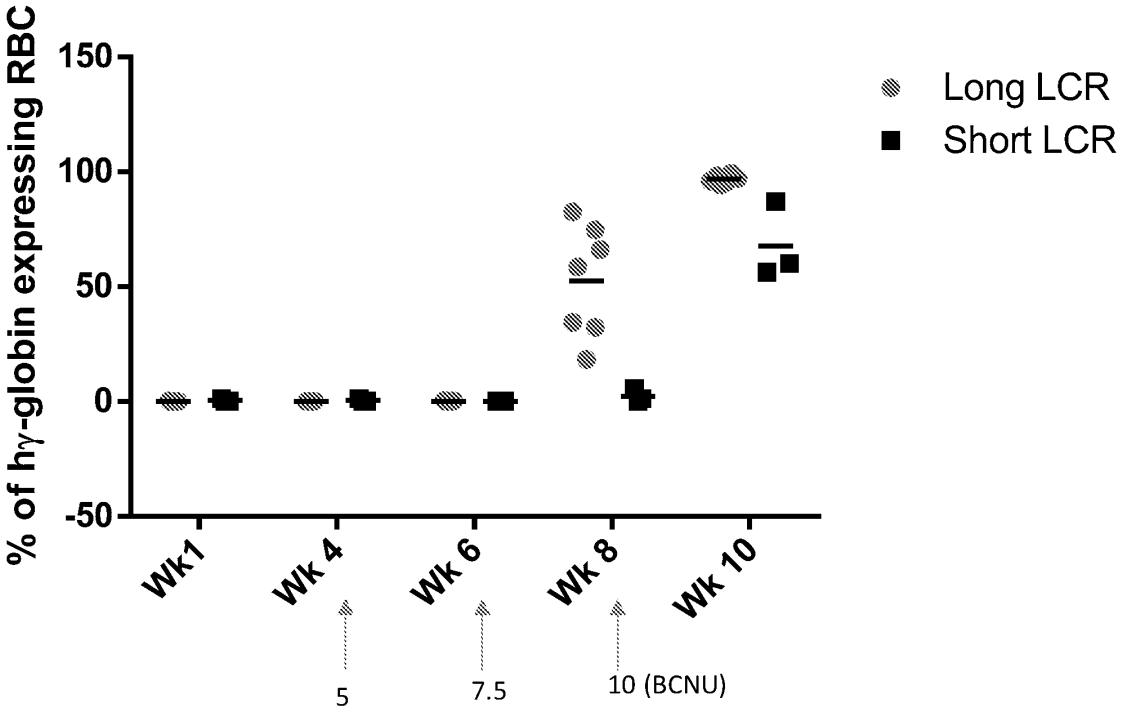

% of hγ-globin expressing RBC
150
100
50
0
-50
Wk1
Wk 4
Wk 6
Wk 8
Wk 10
5
7.5
10 (BCNU)
Long LCR
Short LCR

FIG. 53

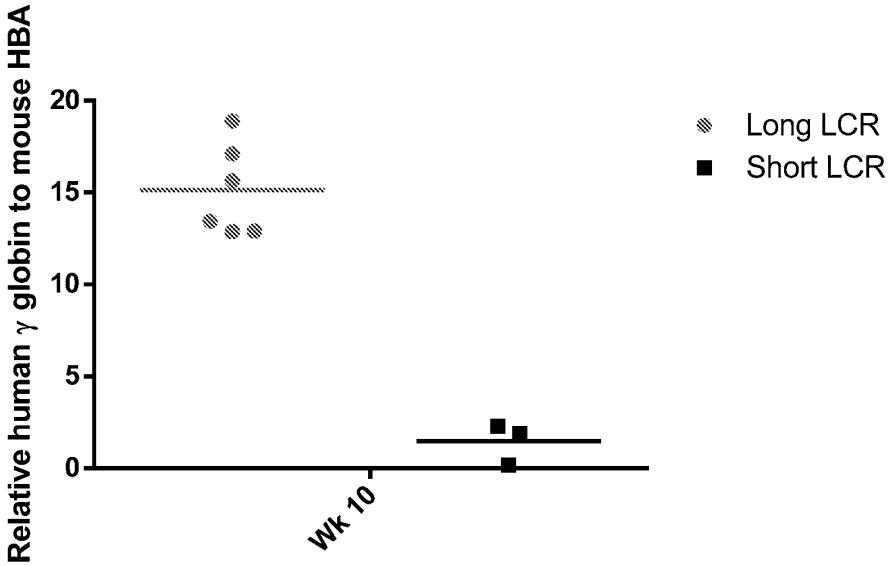

Relative human γ globin to mouse HBA
20
15
10
5
0
Wk 10
Long LCR
Short LCR

FIG. 54

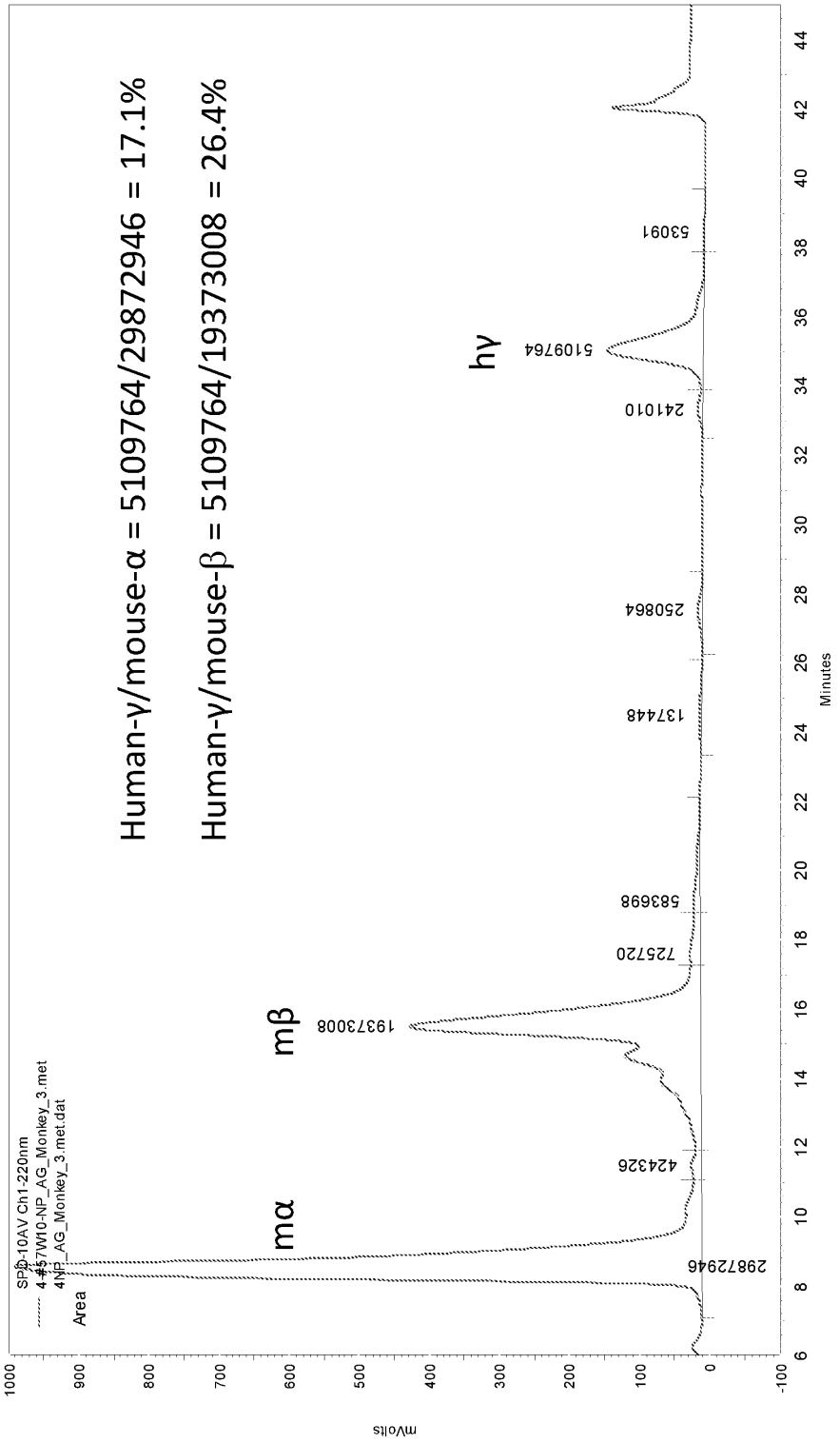

Human-γ/mouse-α = 5109764/29872946 = 17.1%
Human-γ/mouse-β = 5109764/19373008 = 26.4%
mα
mβ
hγ
SP2-10AV Ch1-220nm
4 #57W10-NP_AG_Monkey_3.met
4 NP_AG_Monkey_3.met.dat
Area
mVolts
Minutes
29872946
424326
19373008
725720
583698
137448
250864
241010
5109764
53091

FIG. 55A

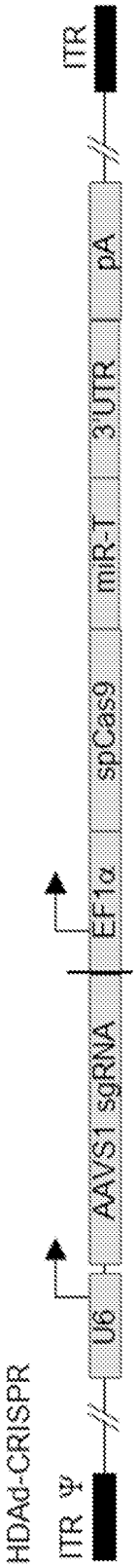


FIG. 55B

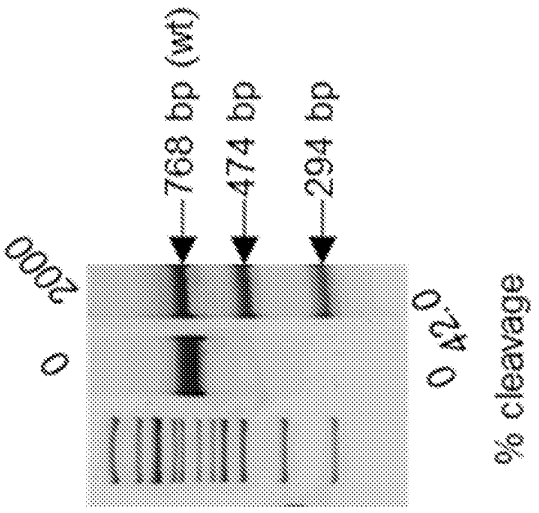
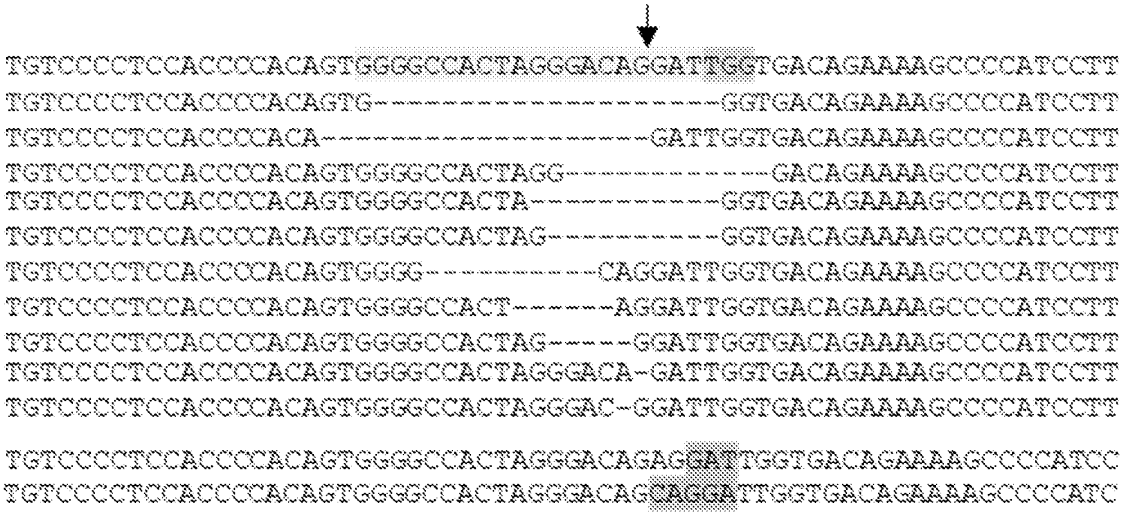


FIG. 55C



(SEQ ID NOs: 6-18)

FIG. 55D

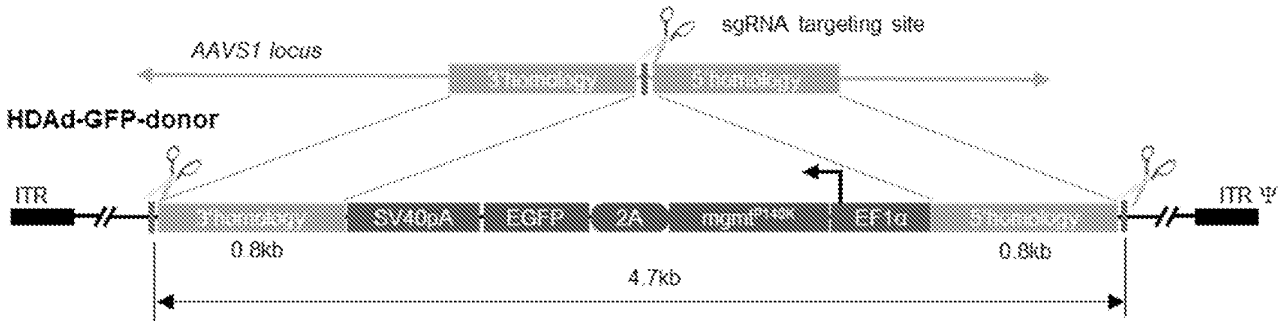


FIG. 55E

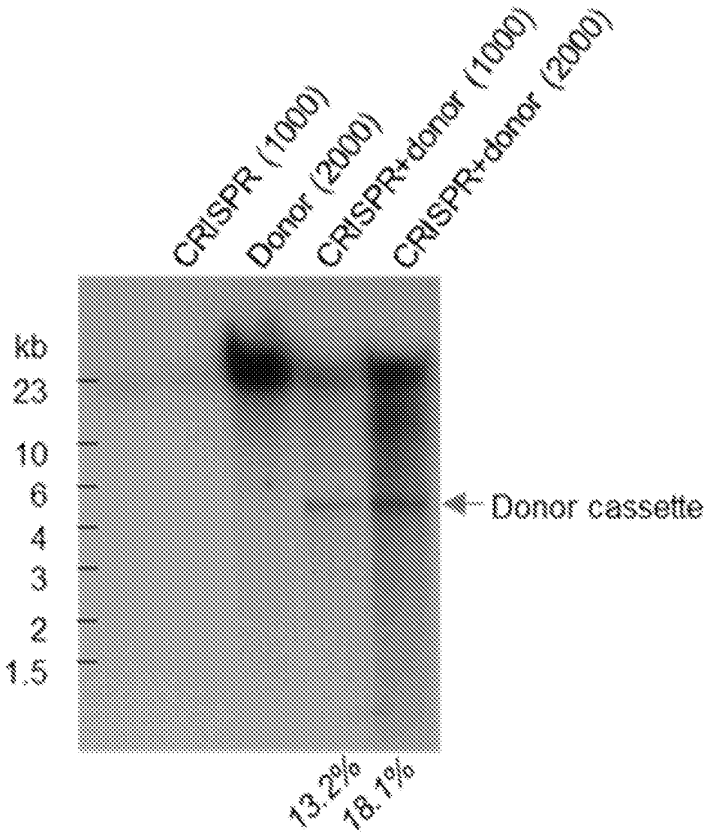


FIG. 56A

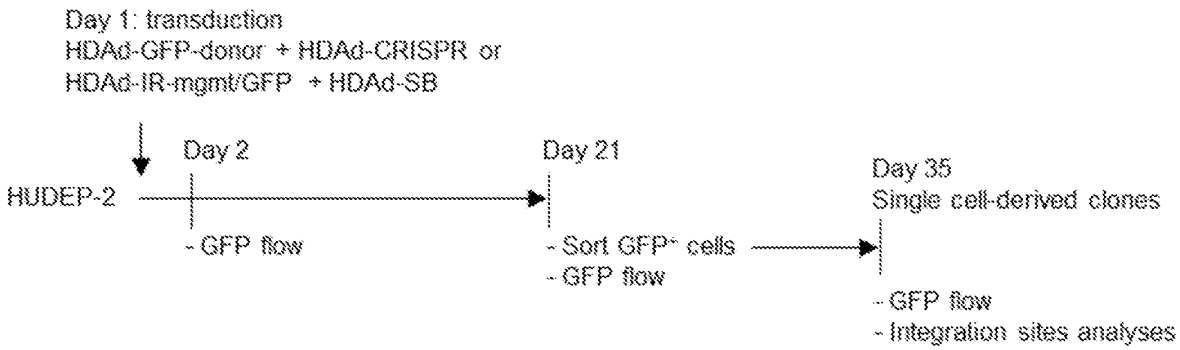


FIG. 56B

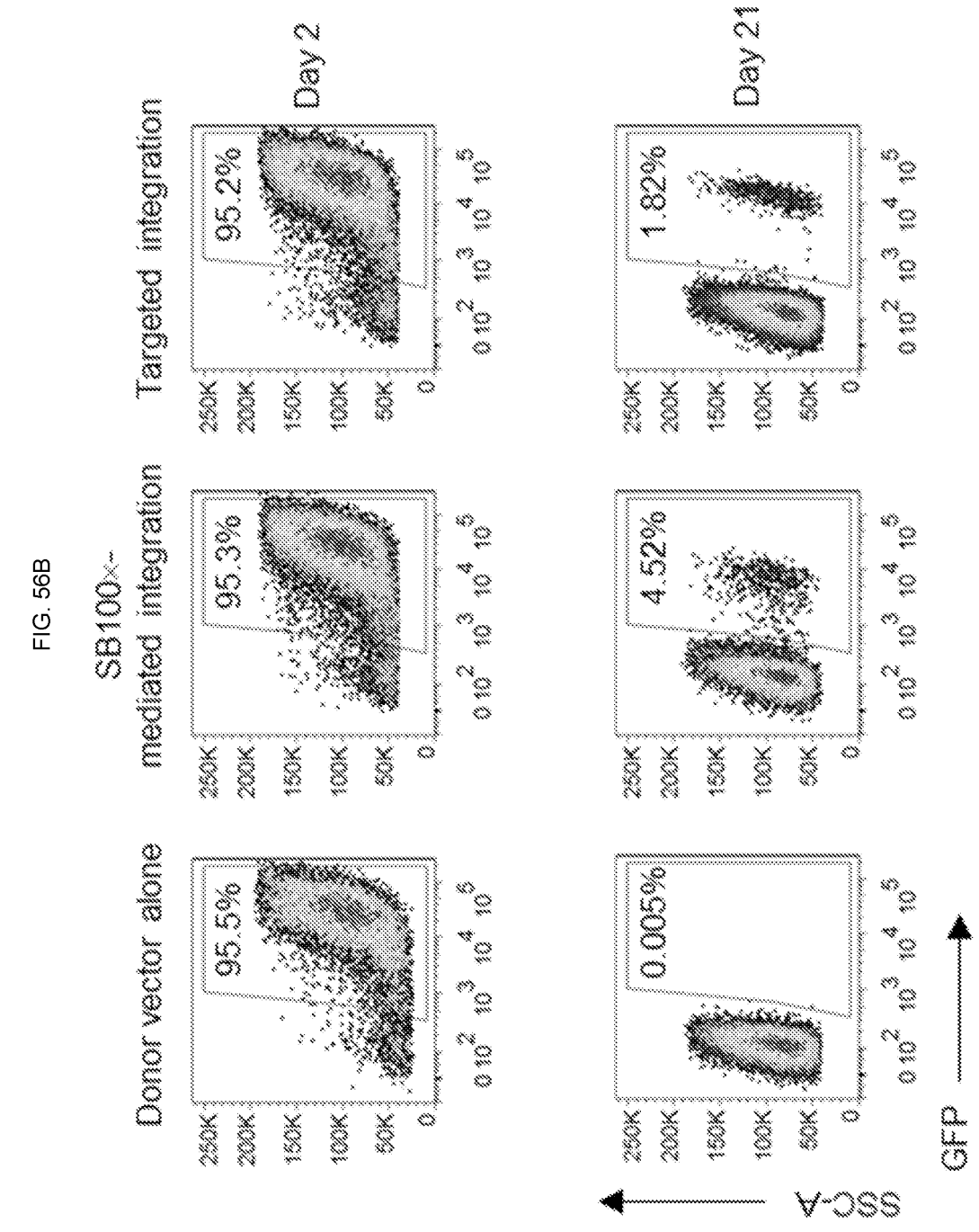

Donor vector alone
SB100x-mediated integration
Targeted integration
Day 2
Day 21
95.5%
95.3%
95.2%
0.005%
4.52%
1.82%
250K
200K
150K
100K
50K
0
SSC-A
GFP

FIG. 56C

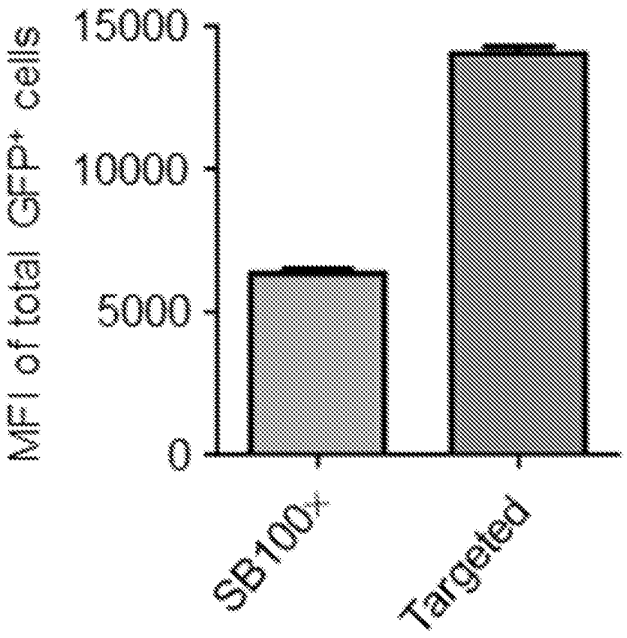


FIG. 56D

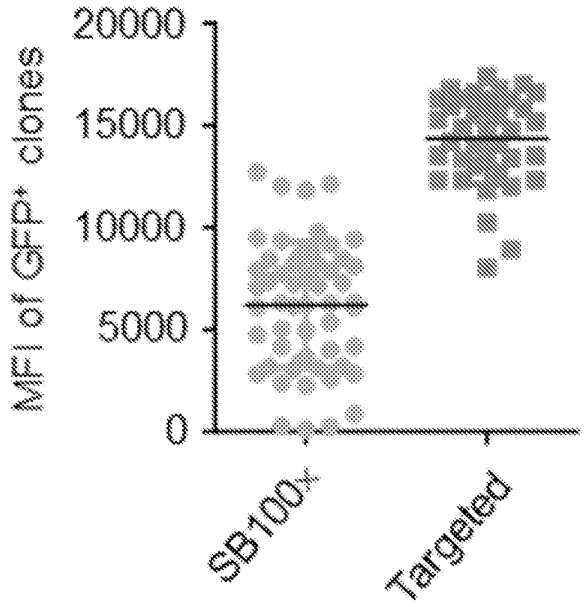


FIG. 56E

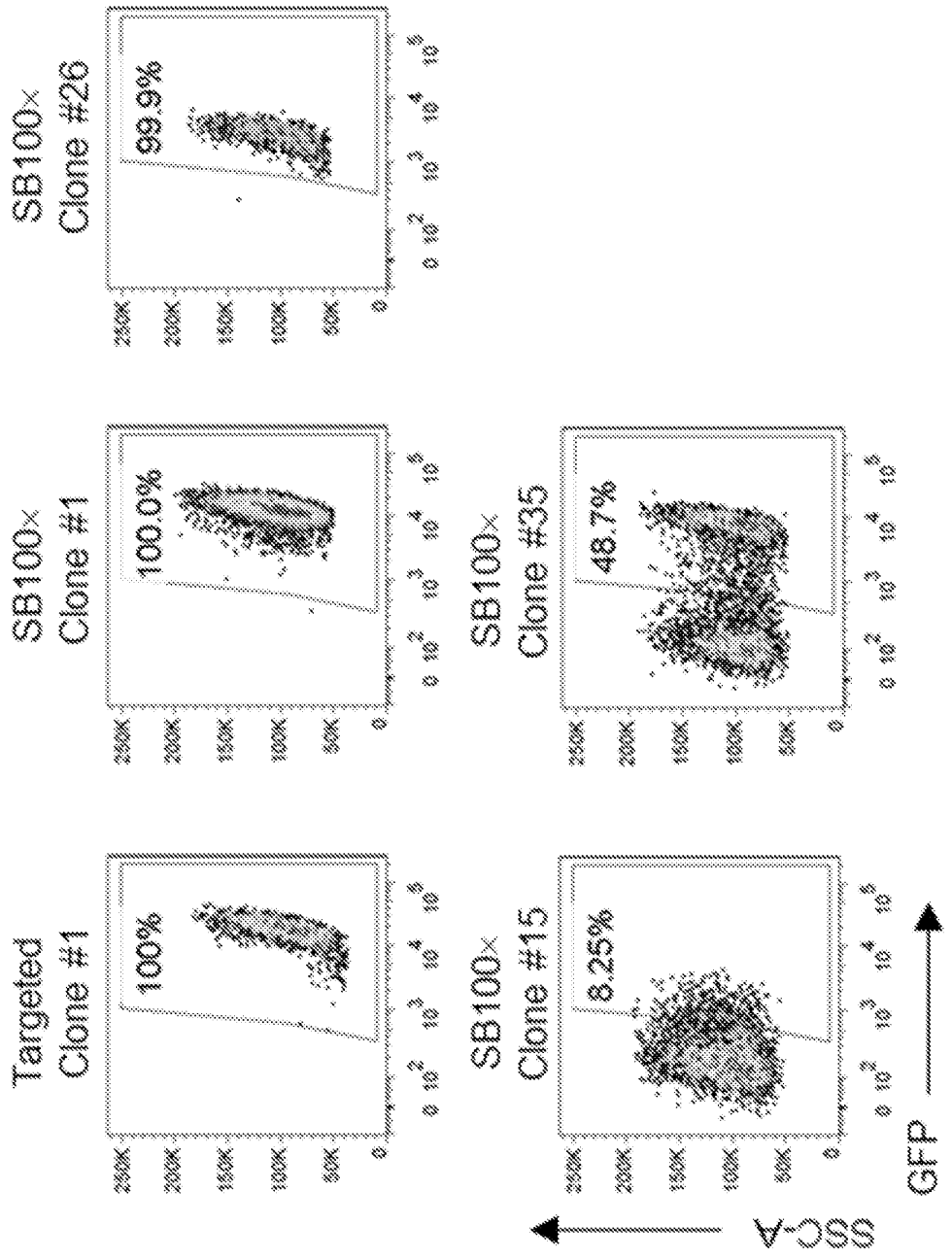

Targeted
Clone #1
100%
SB100×
Clone #1
100.0%
SB100×
Clone #26
99.9%
SB100×
Clone #15
8.25%
SB100×
Clone #35
48.7%
SSC-A
GFP

FIG. 56F

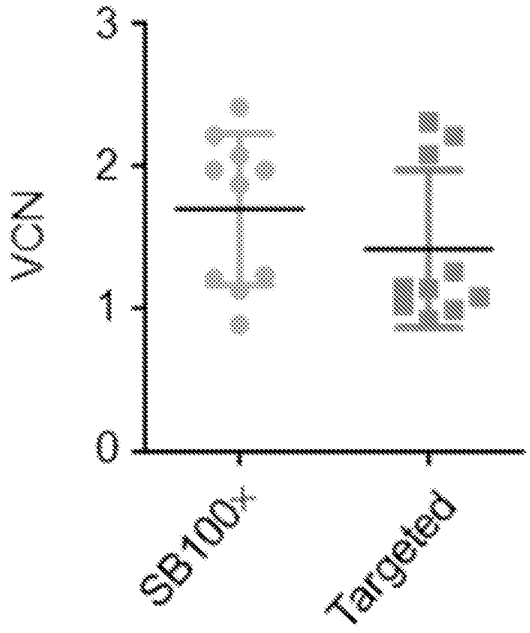


FIG. 57A

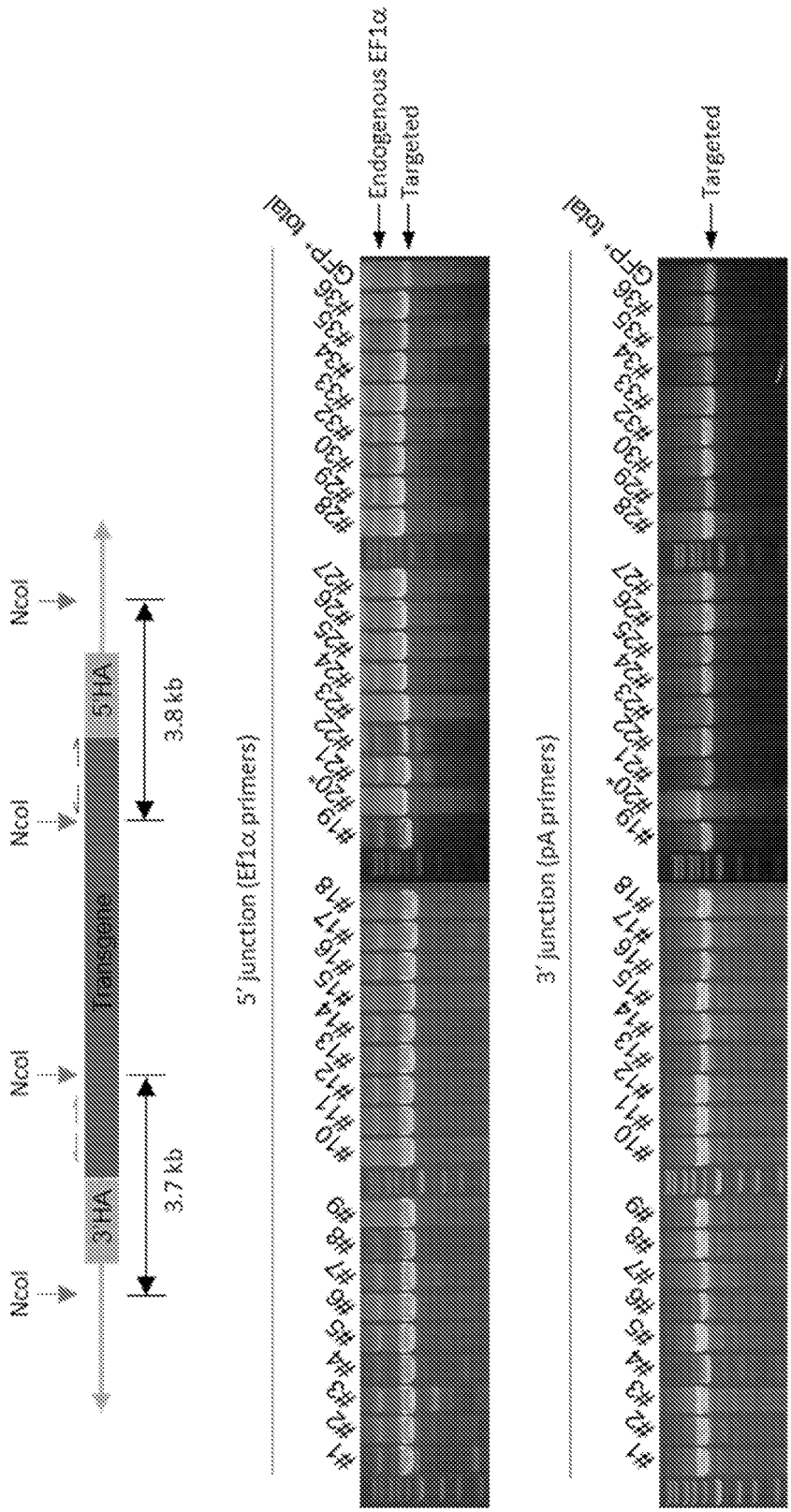

NcoI
NcoI
NcoI
NcoI
5'HA
3'HA
Transgene
3.8 kb
3.7 kb
5' junction (Ef1α primers)
3' junction (pA primers)
Endogenous Ef1α
Targeted
Targeted
total
total

FIG. 57B

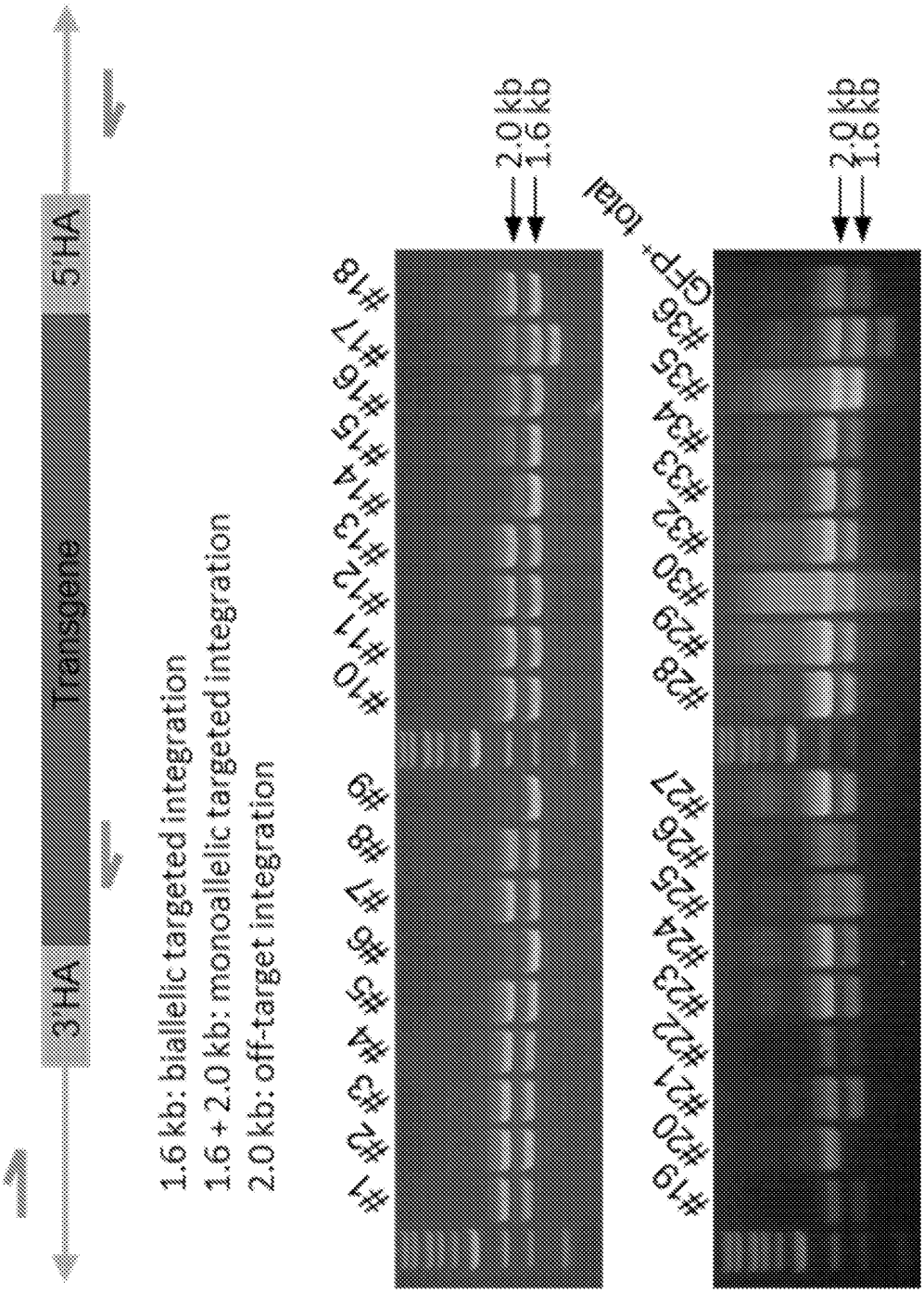

3'HA
Transgene
5'HA
1.6 kb: biallelic targeted integration
1.6 + 2.0 kb: monoallelic targeted integration
2.0 kb: off-target integration
2.0 kb
1.6 kb
2.0 kb
1.6 kb
GFP+ total

FIG. 58A

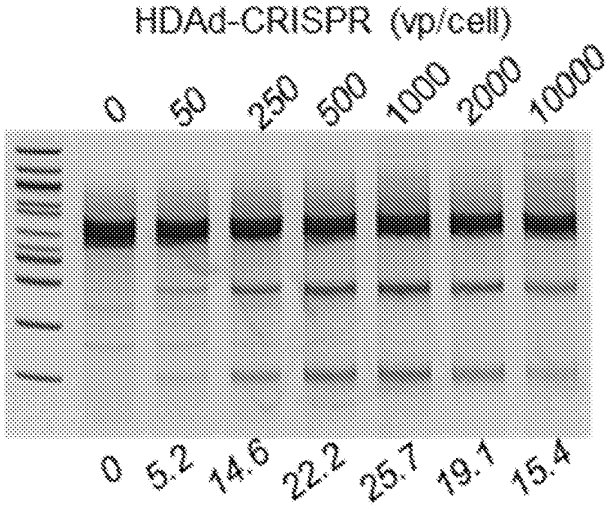


FIG. 58B

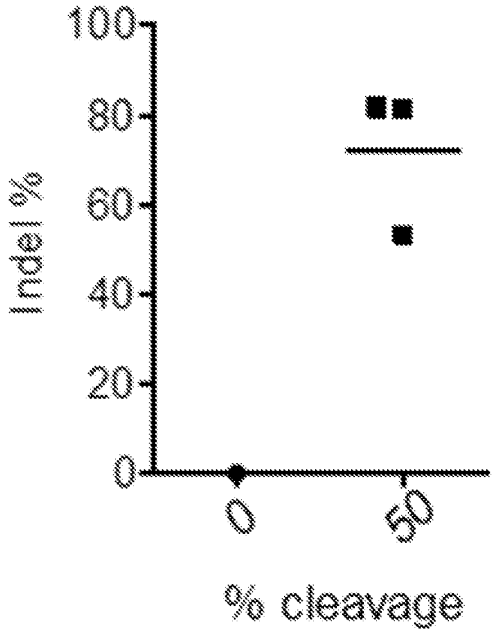


FIG. 58C

(SEQ ID NOs: 19-23, 21, 21, 26-30, 27, 32, 28, 34-47)

FIG. 59A

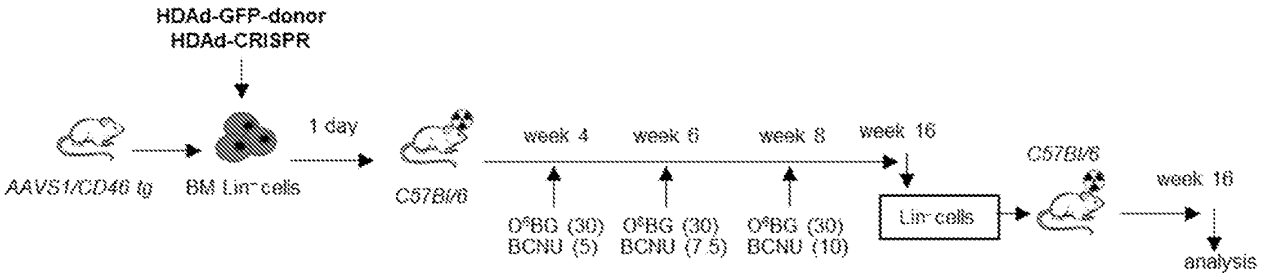


FIG. 59B

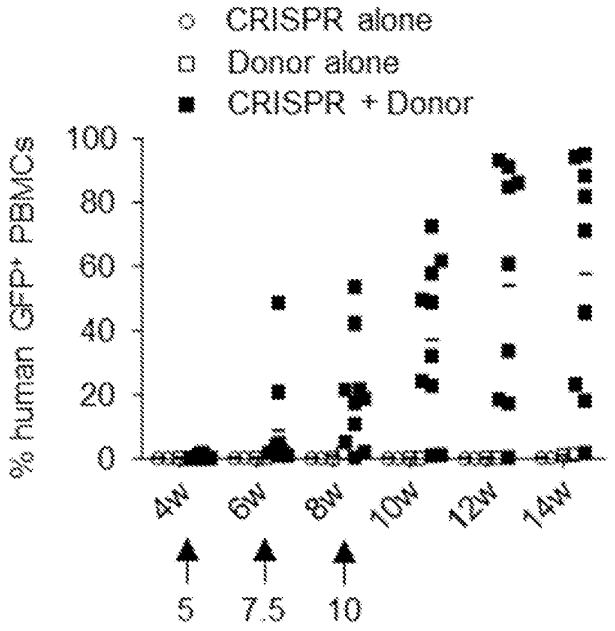


FIG. 59C

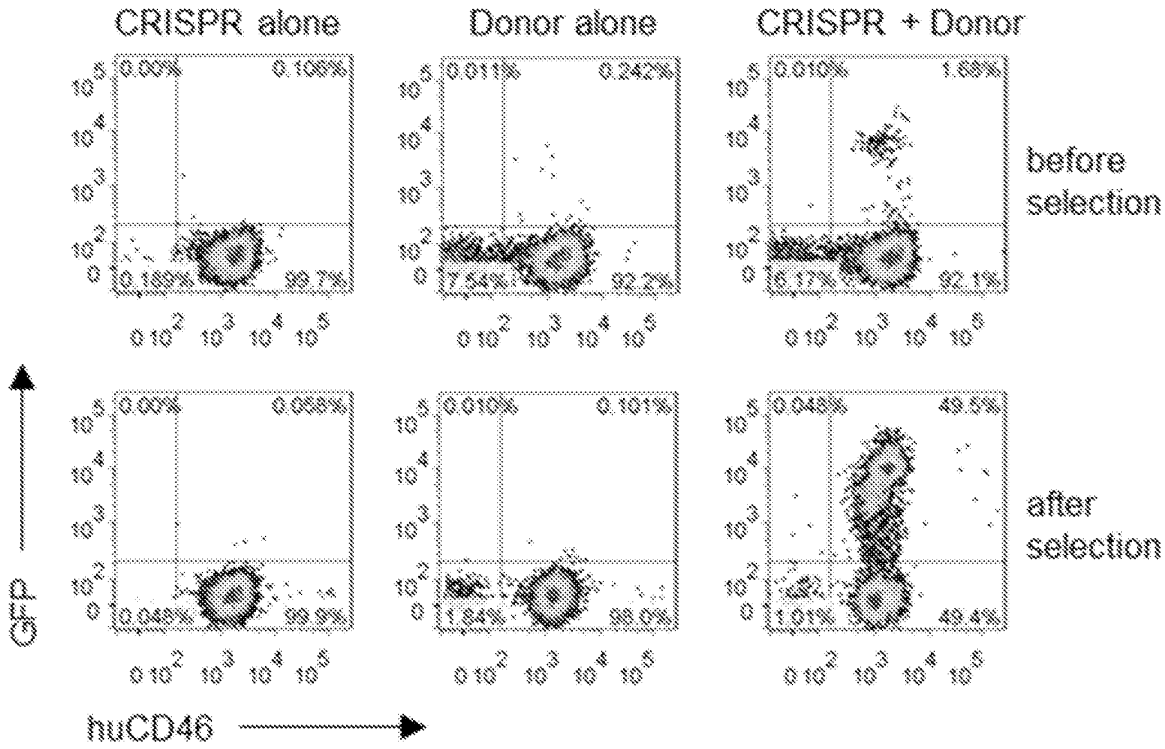


FIG. 59D

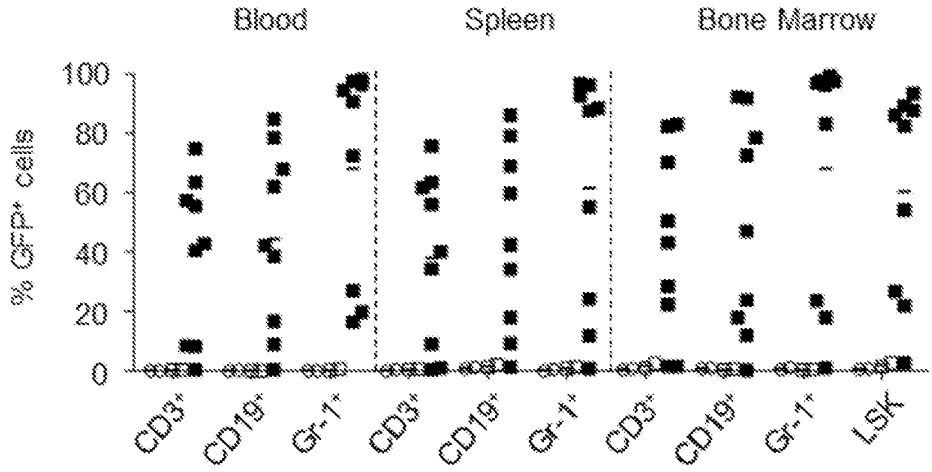


FIG. 60A

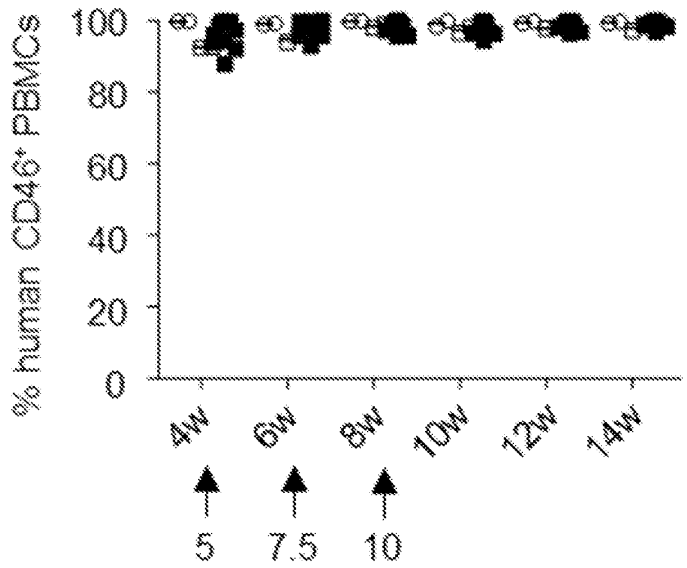


FIG. 60B

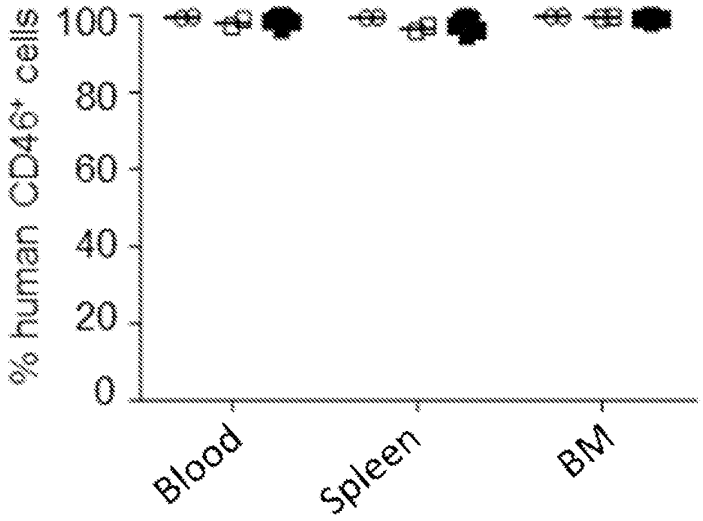


FIG. 60C

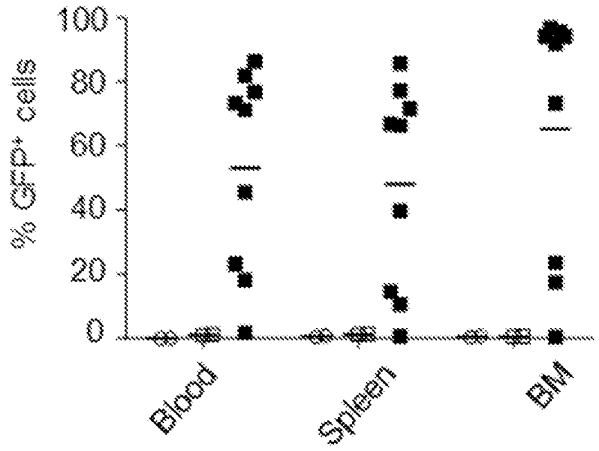


FIG. 60D

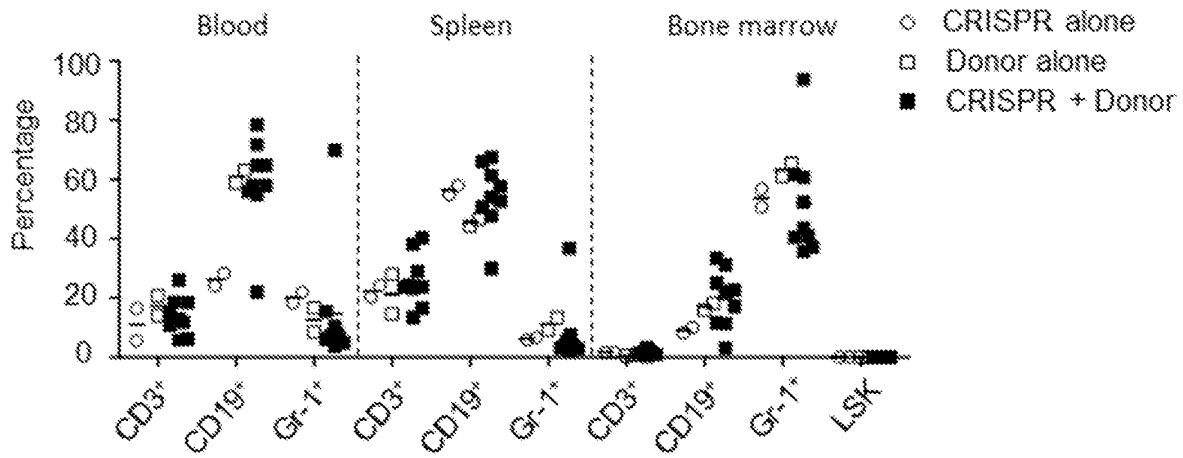


FIG. 60E

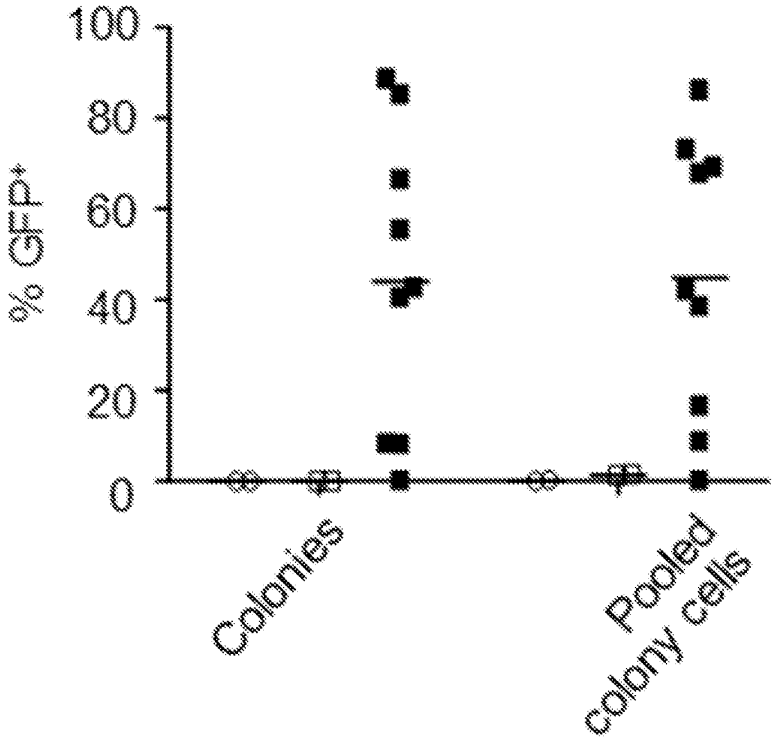


FIG. 61A

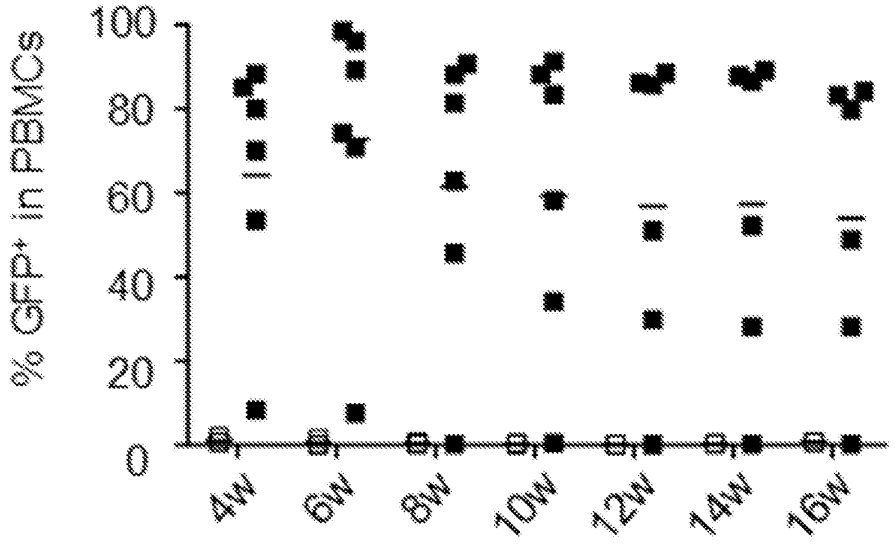


FIG. 61B

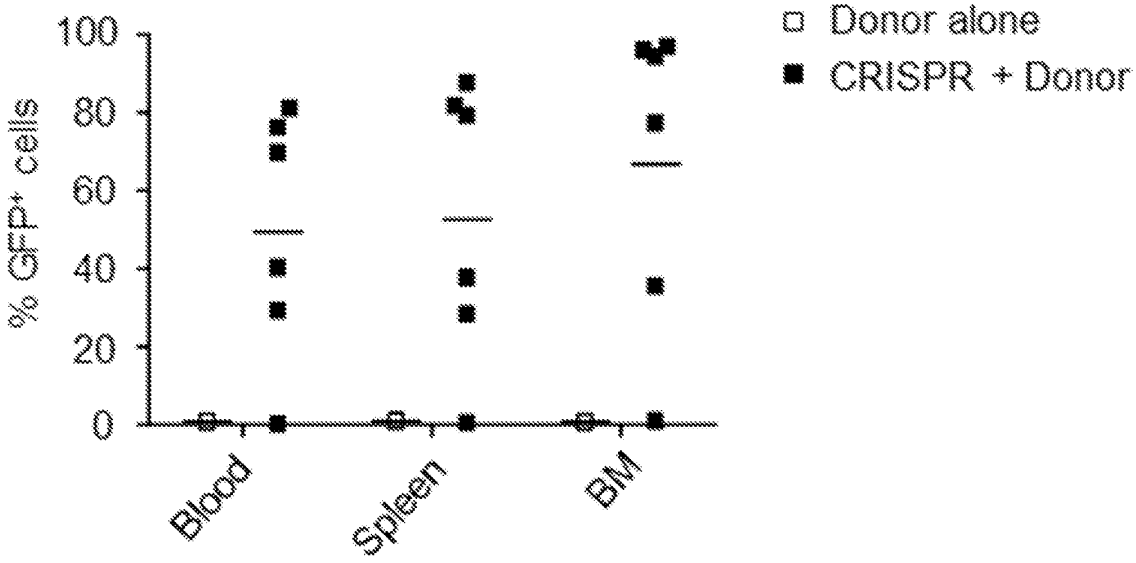


FIG. 61C

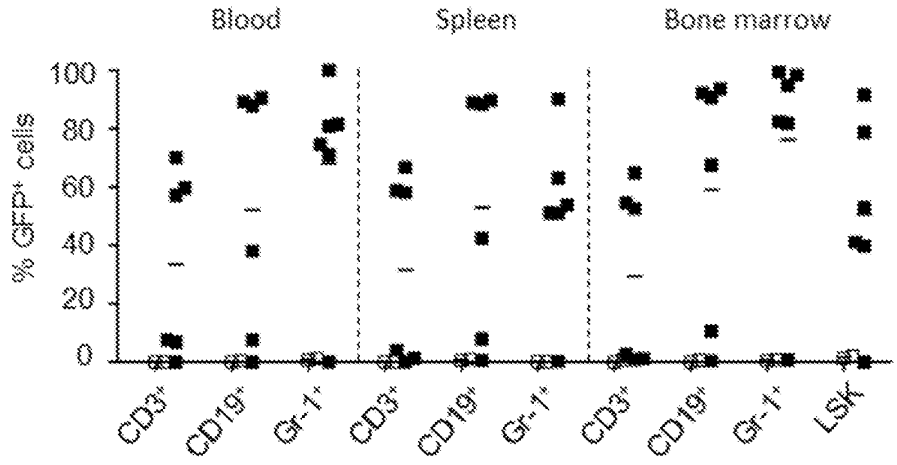


FIG. 61D

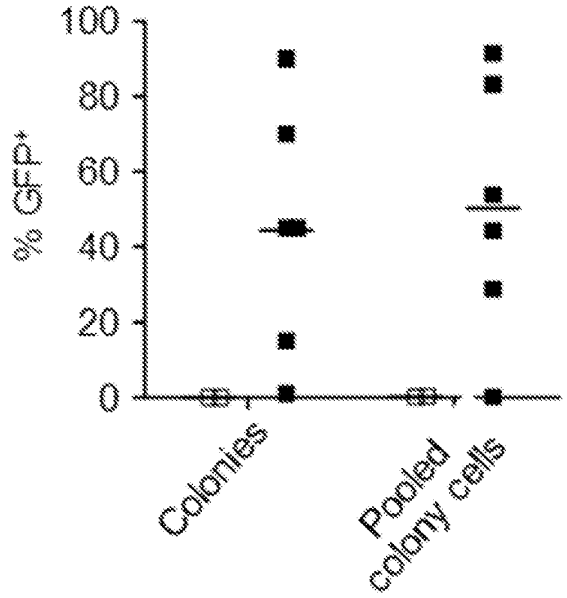


FIG. 61E

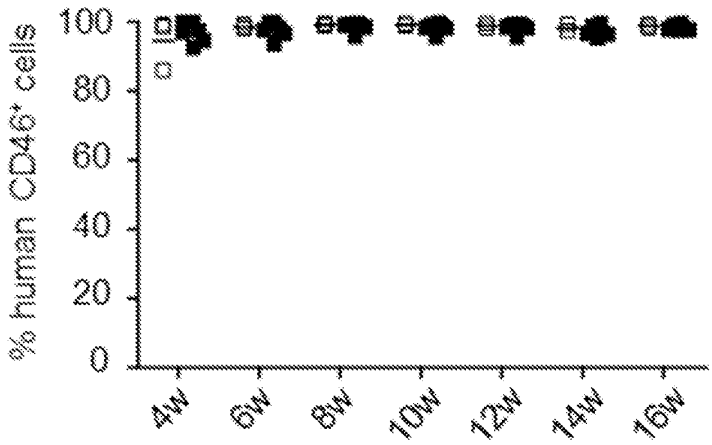


FIG. 61F

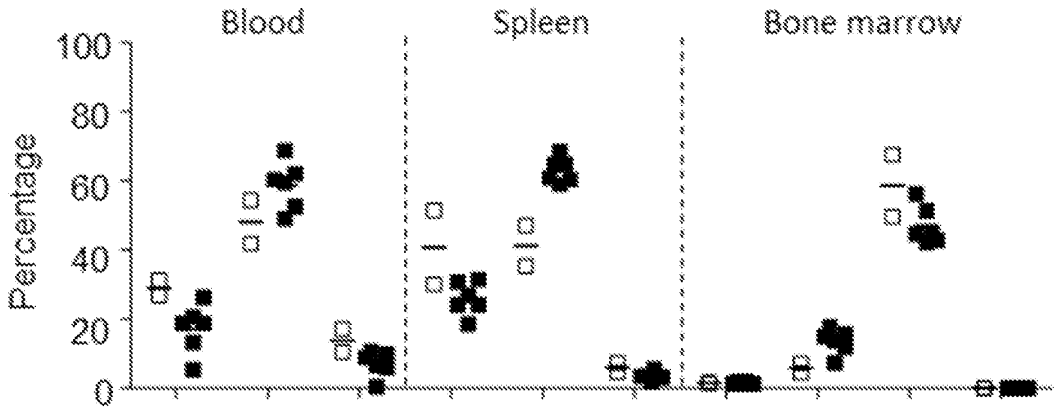


FIG. 62A

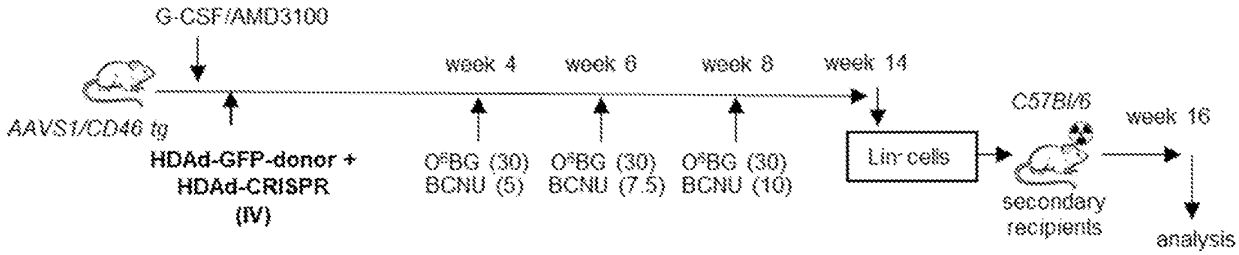


FIG. 62B

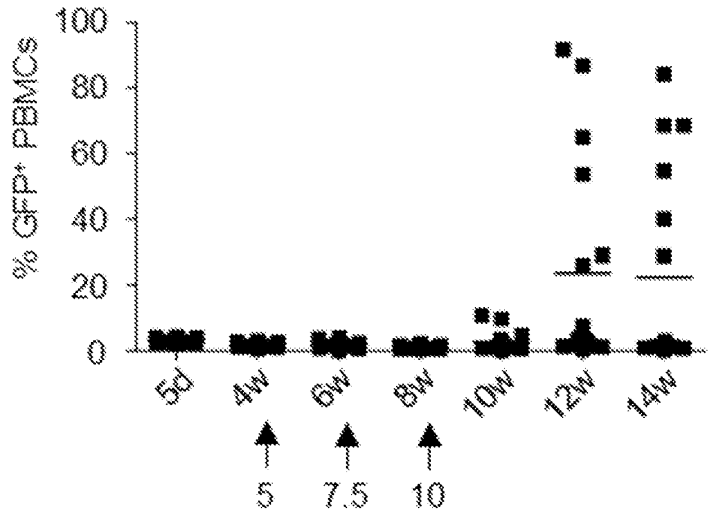


FIG. 62C

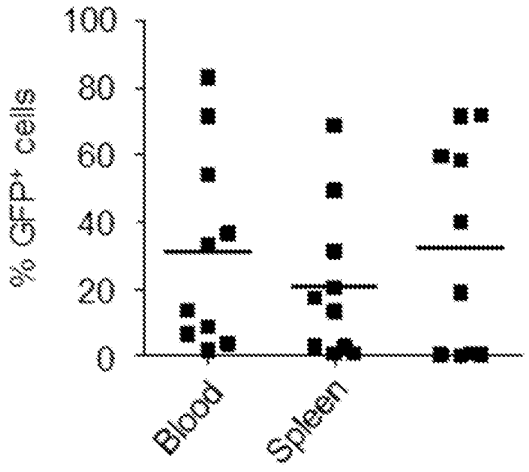


FIG. 62D

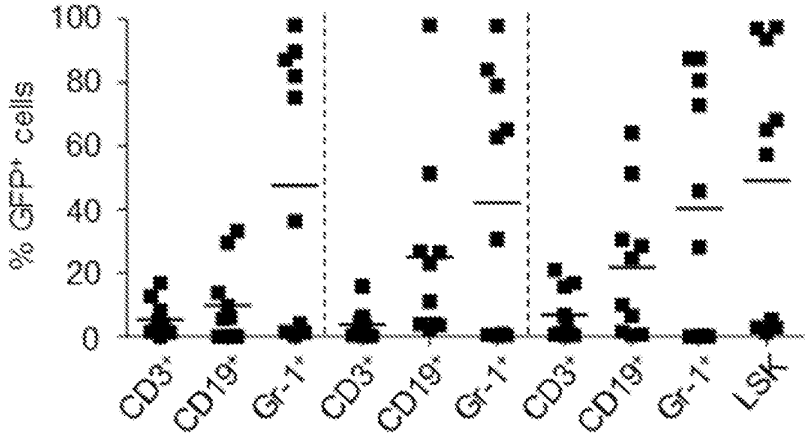


FIG. 62E

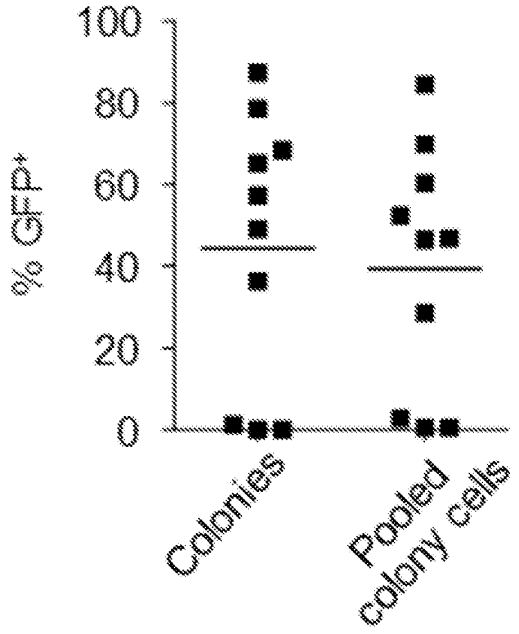


FIG. 62F

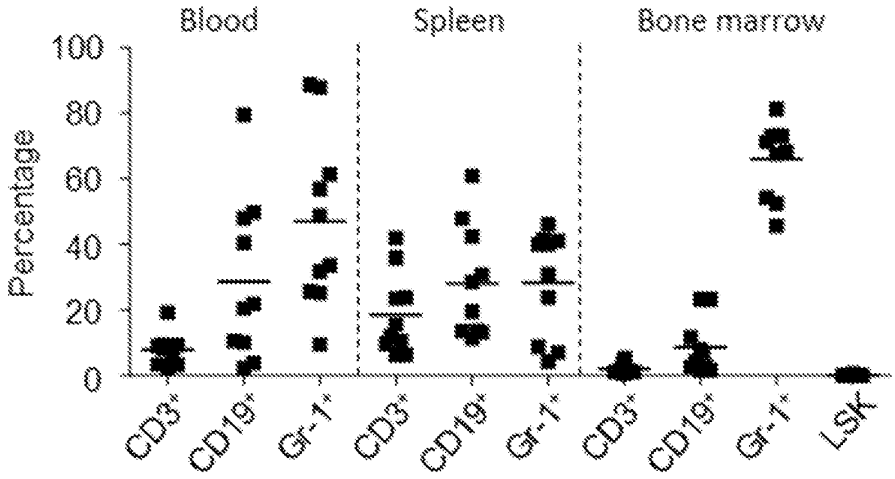


FIG. 63A

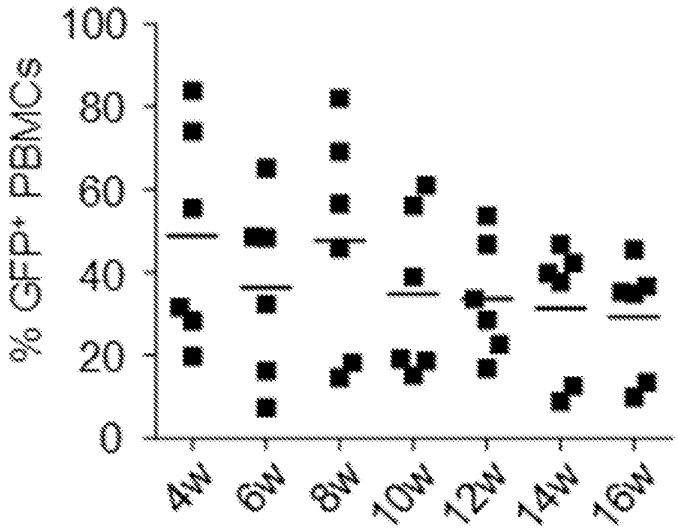


FIG. 63B

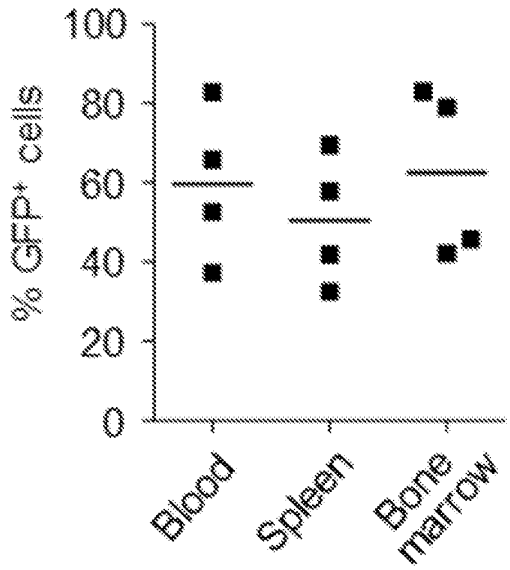


FIG. 63C

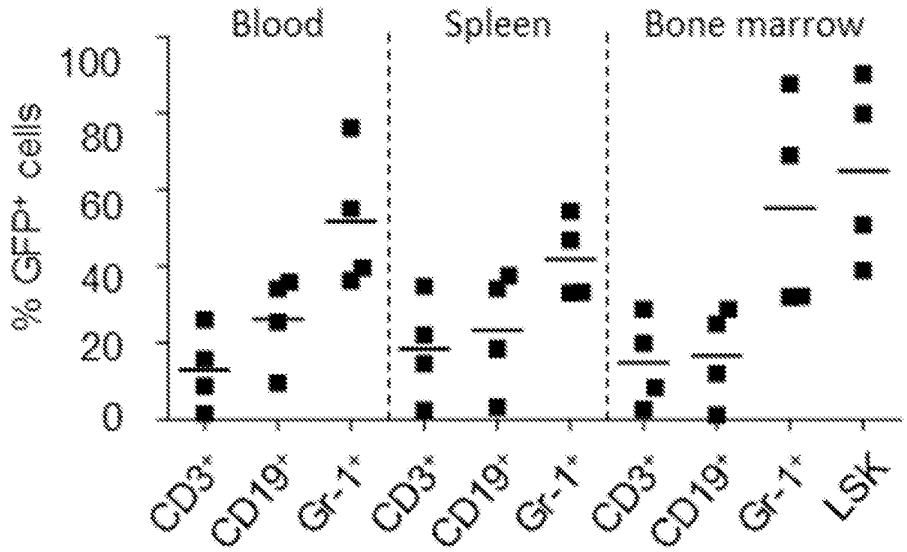


FIG. 63D

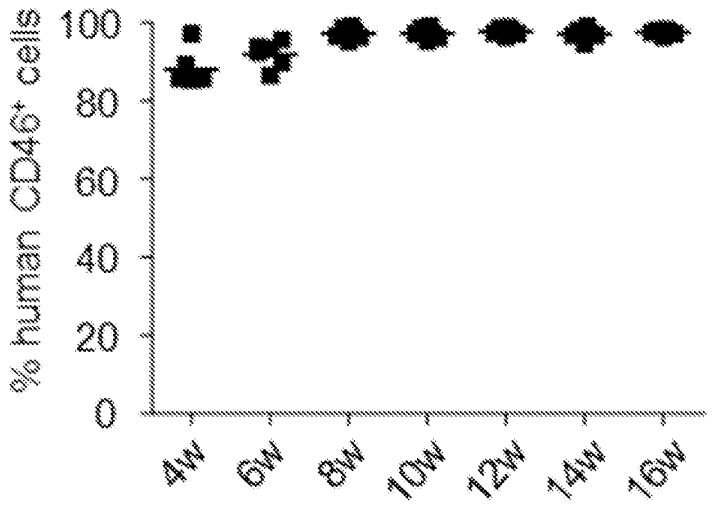


FIG. 63E

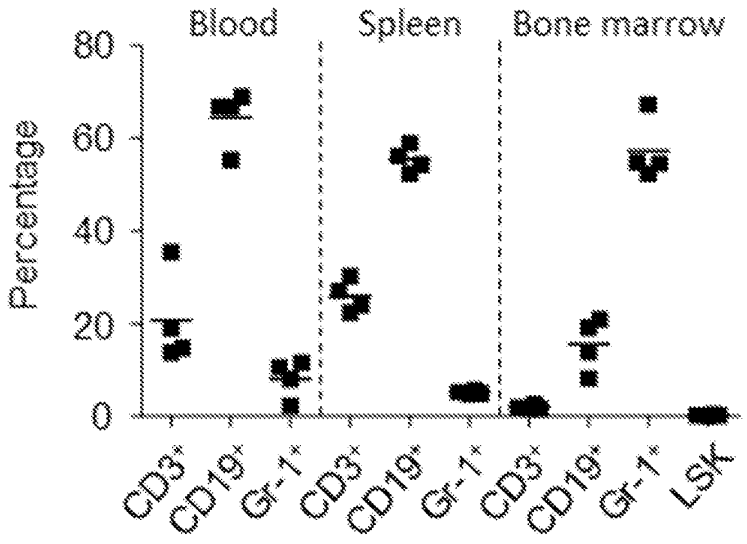


FIG. 64A

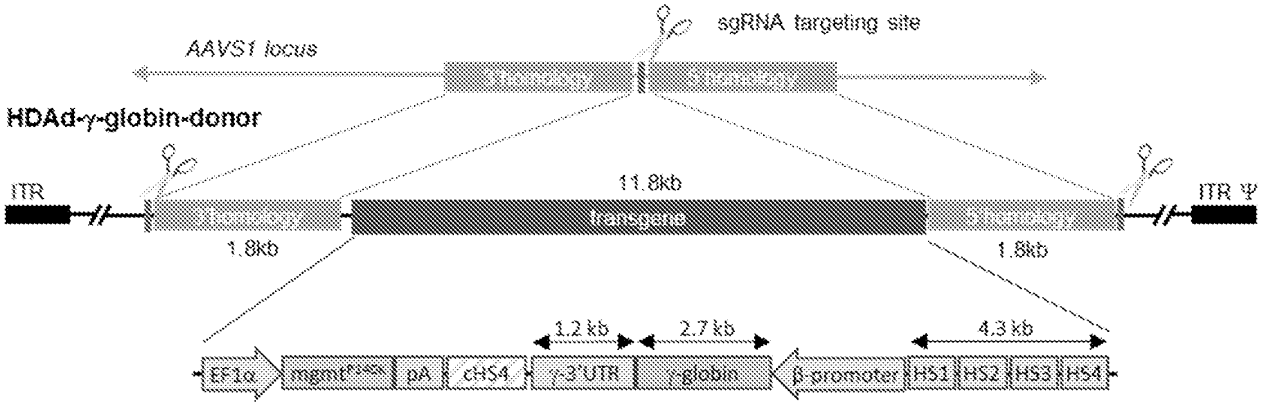


FIG. 64B

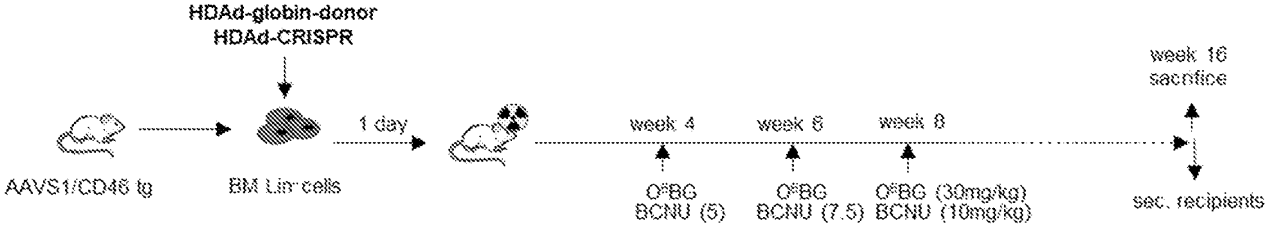


FIG. 64C

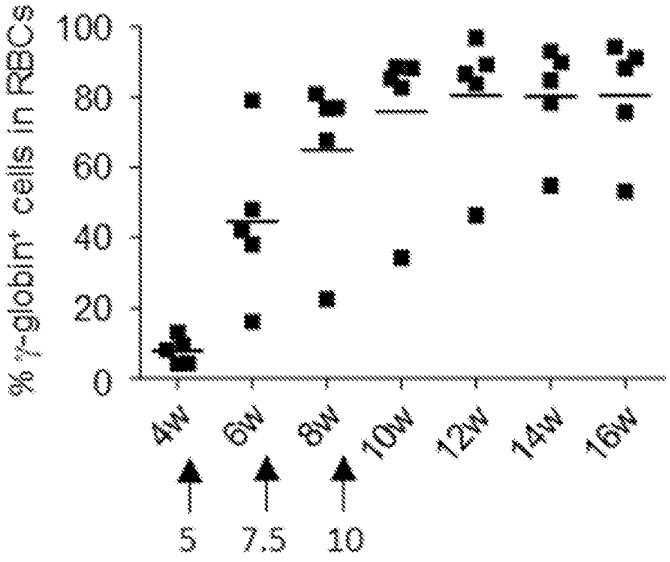


FIG. 64D

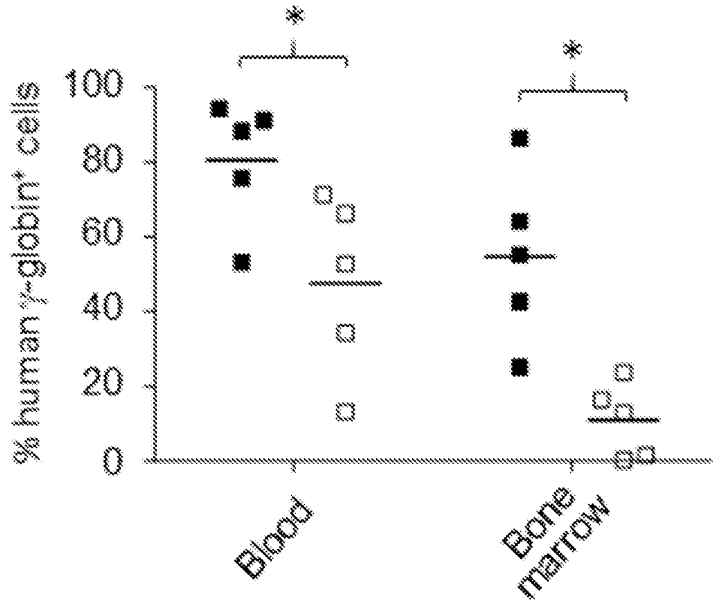


FIG. 64E

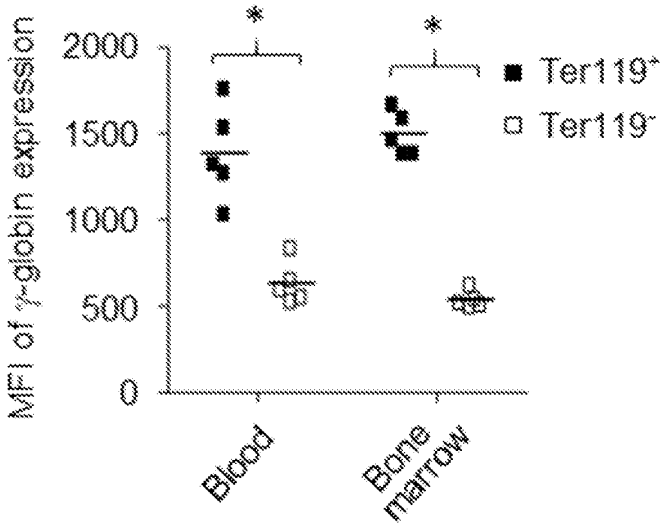


FIG. 64F

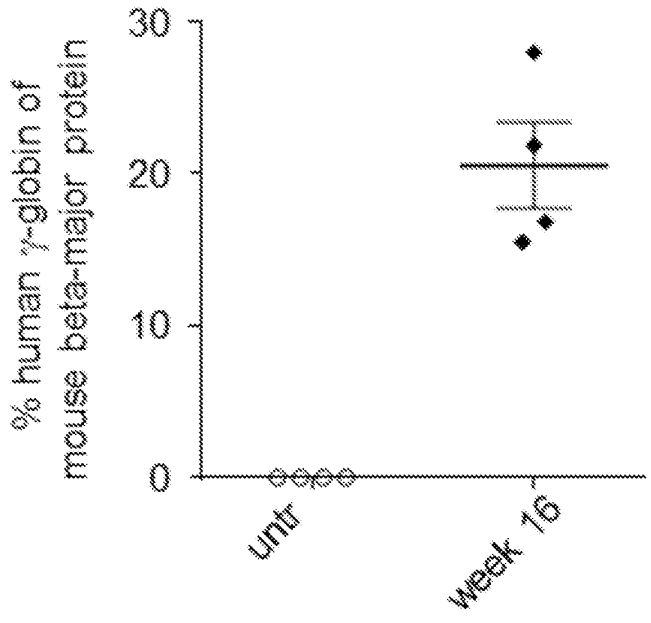


FIG. 64G

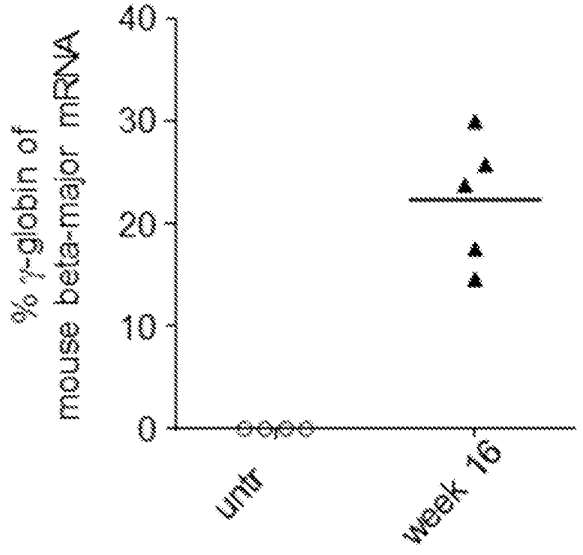


FIG. 64H

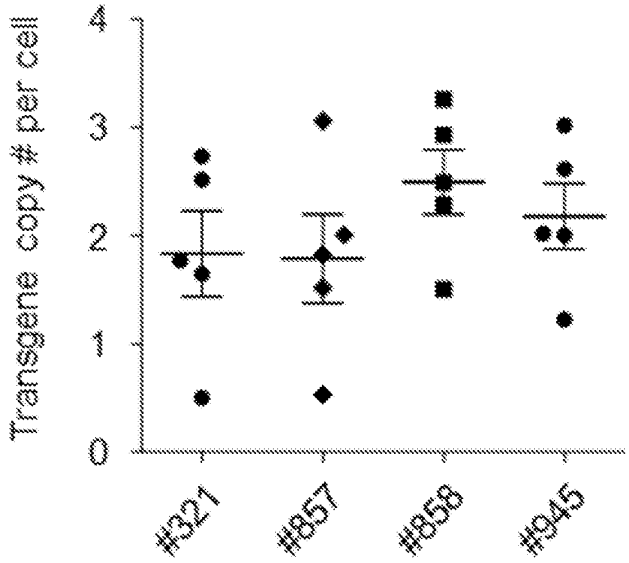


FIG. 65A

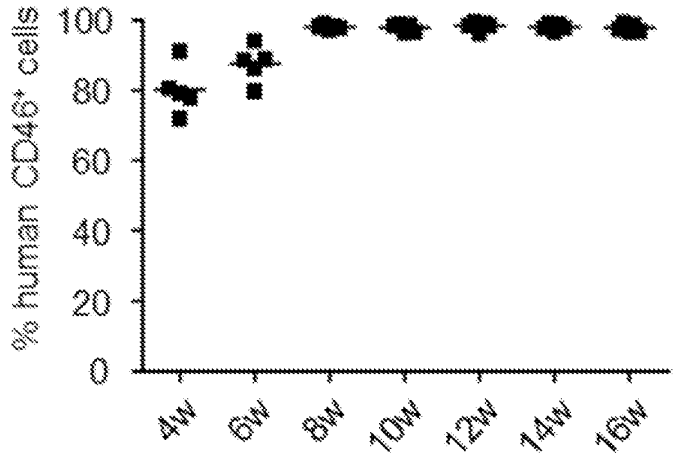


FIG. 65B

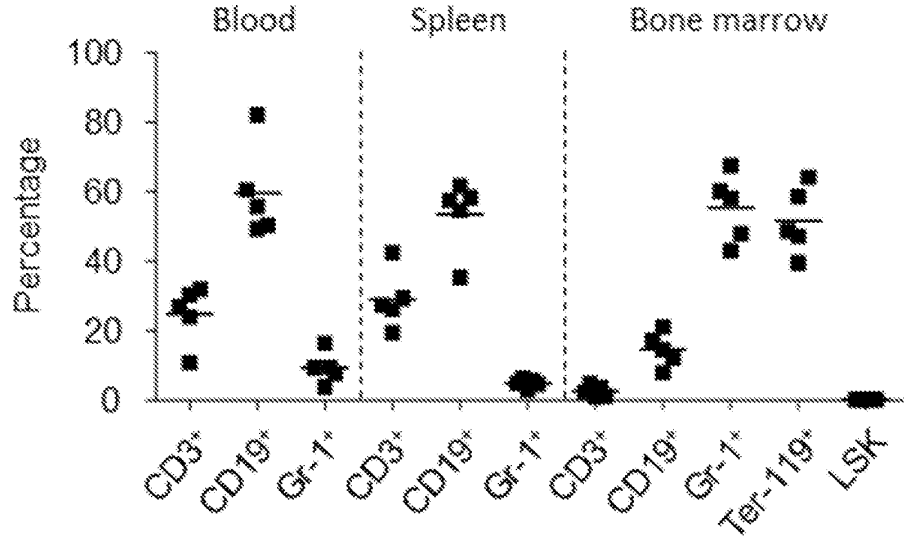


FIG. 66A

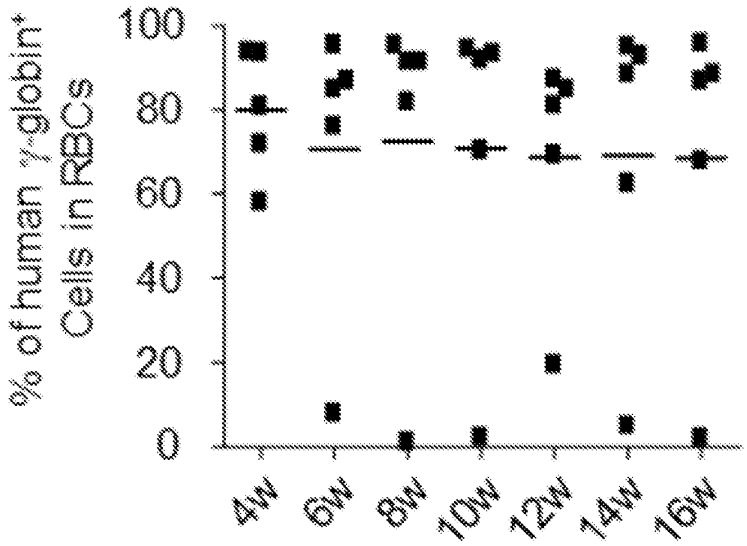


FIG. 66B

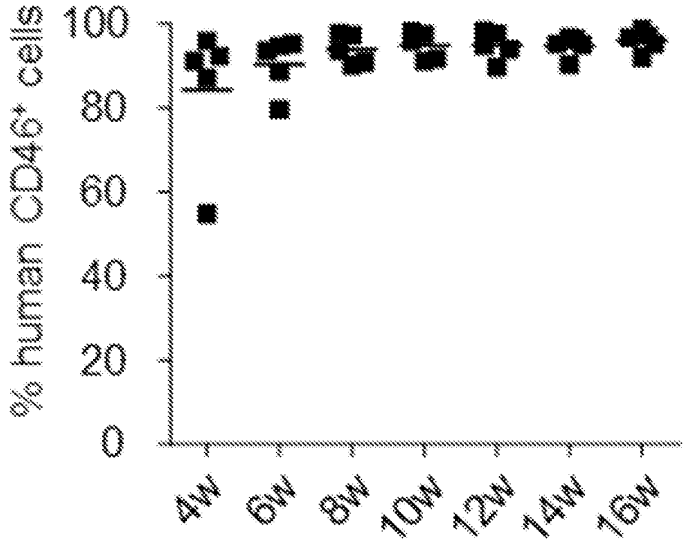


FIG. 66C

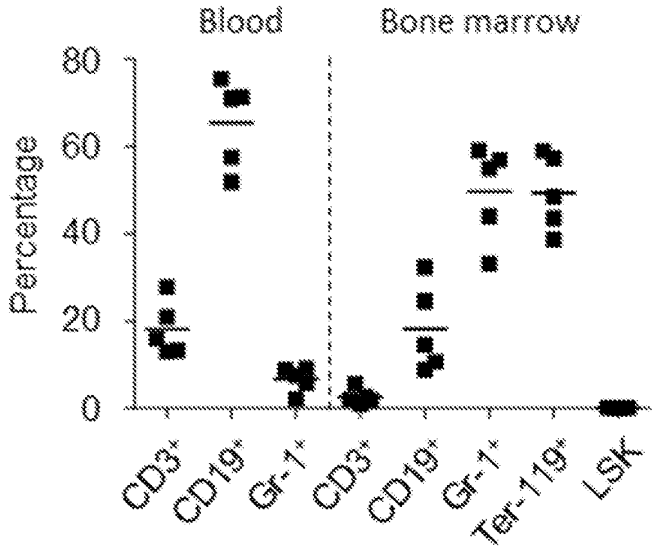

Blood
Bone marrow
Percentage
80
60
40
20
0
CD3+
CD19+
Gr-1+
CD3+
CD19+
Gr-1+
Ter-119+
LSK

FIG. 67A

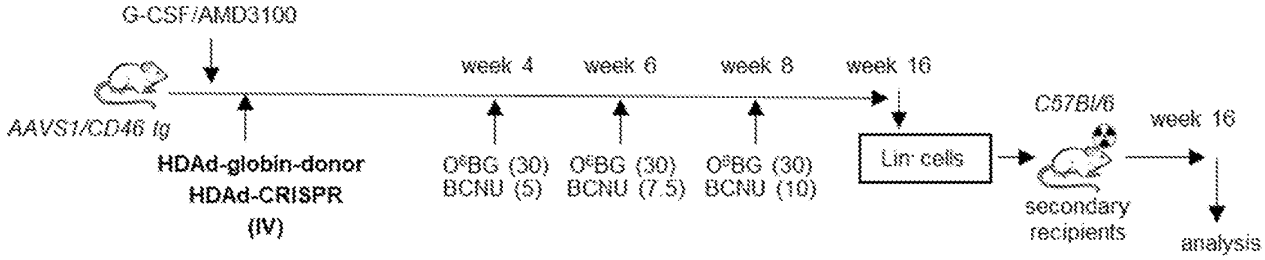


FIG. 67B

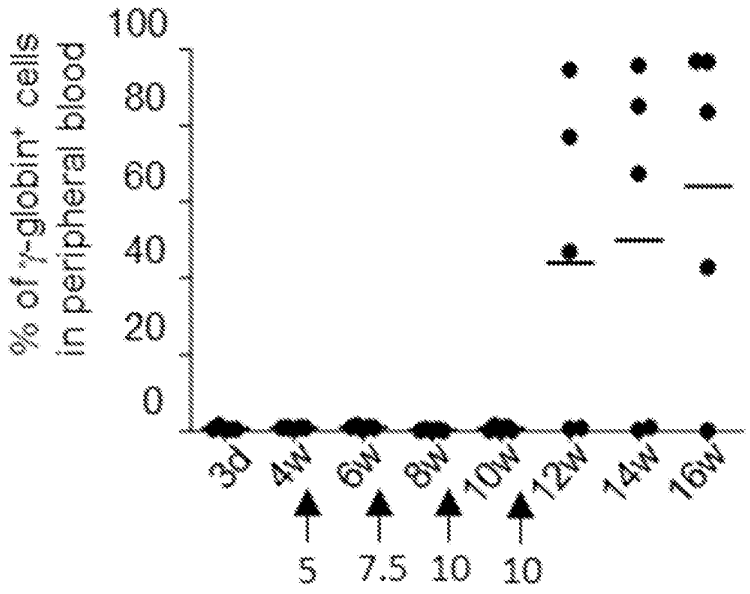


FIG. 67C

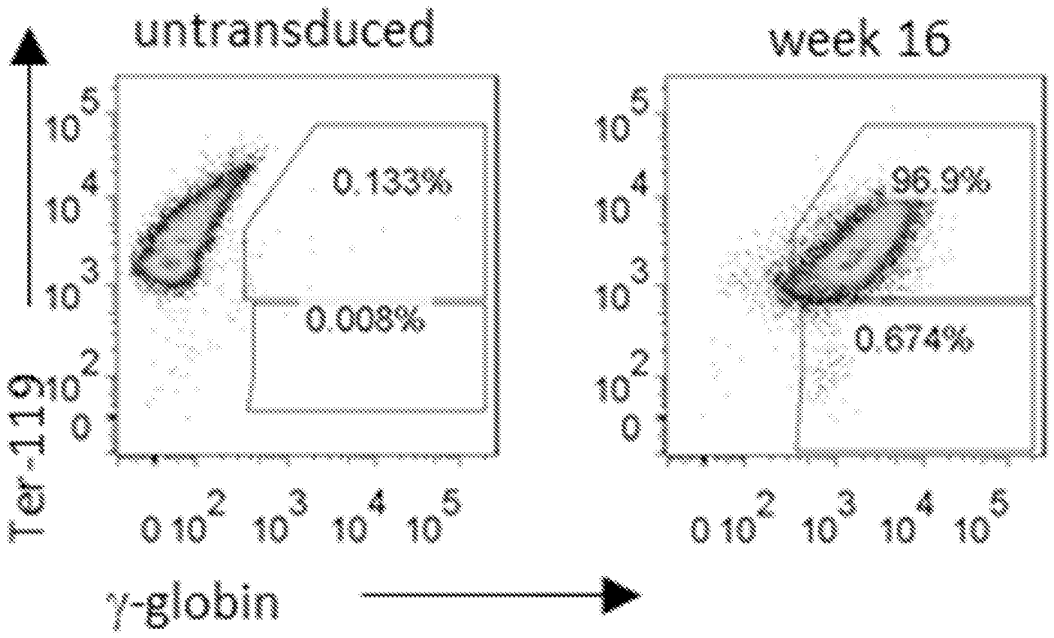


FIG. 67D

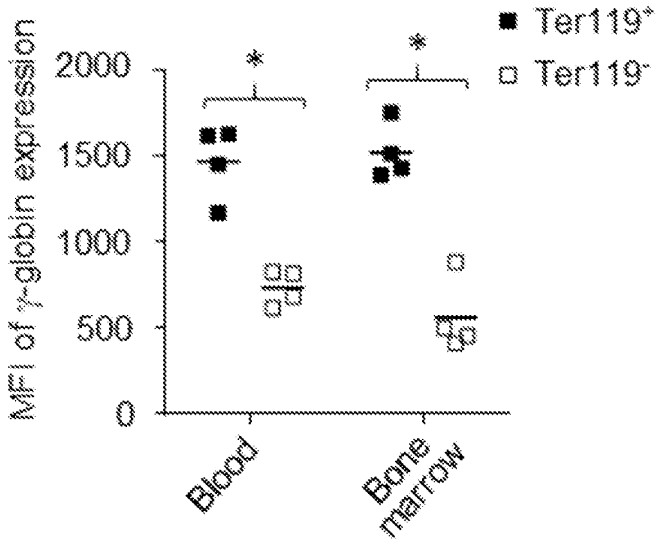


FIG. 67E

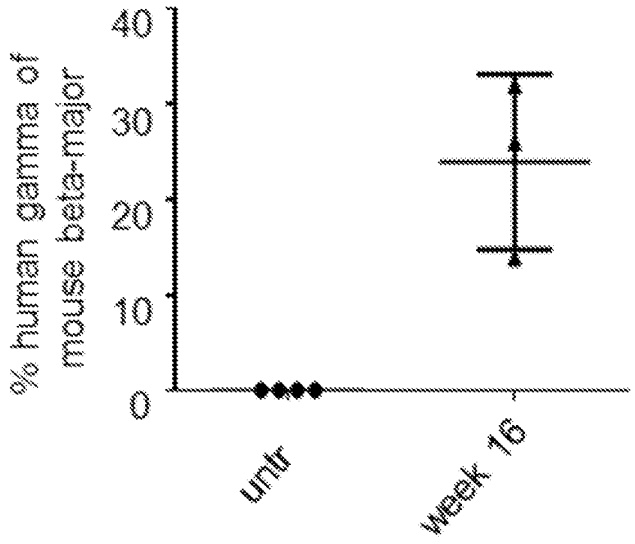


FIG. 67F

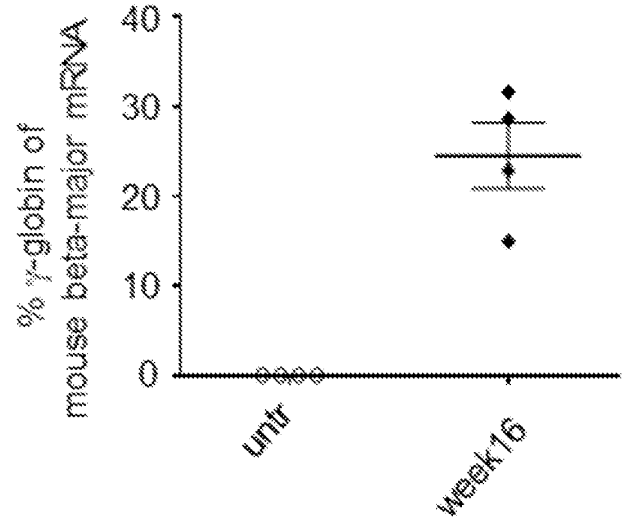


FIG. 67G

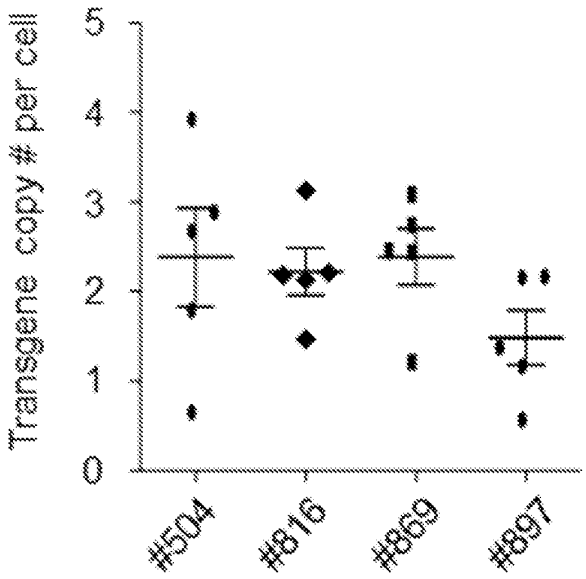


FIG. 67H

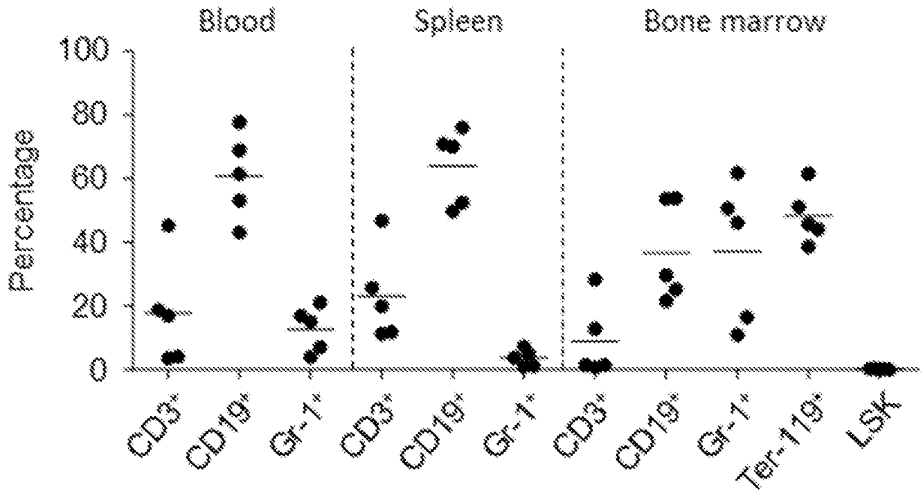


FIG. 68A

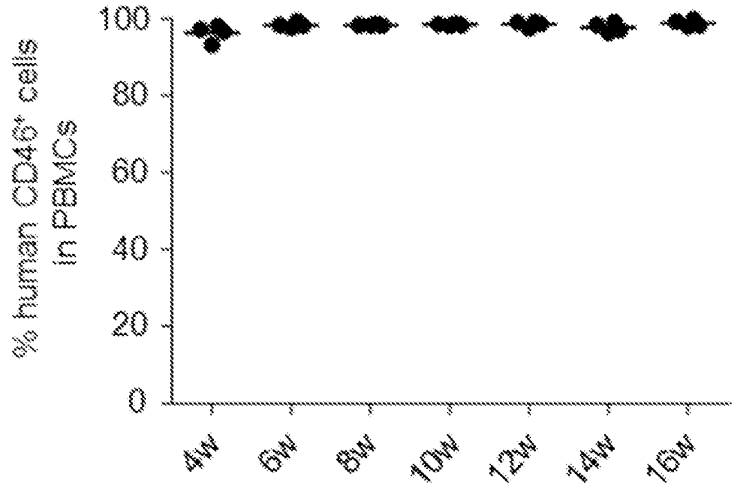


FIG. 68B

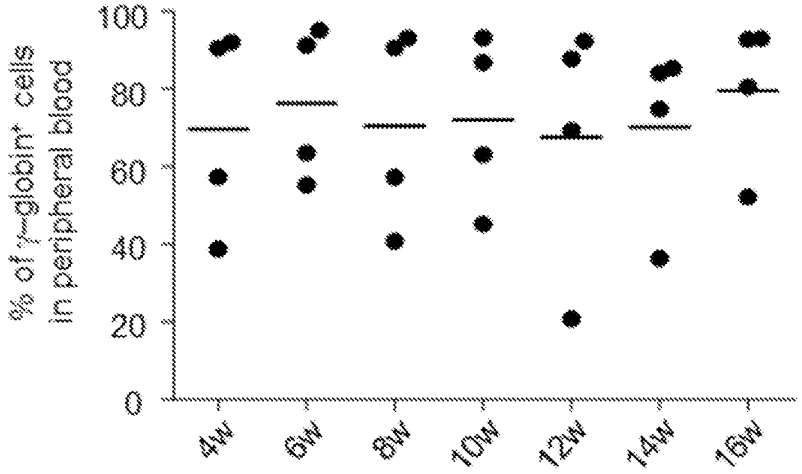


FIG. 68C

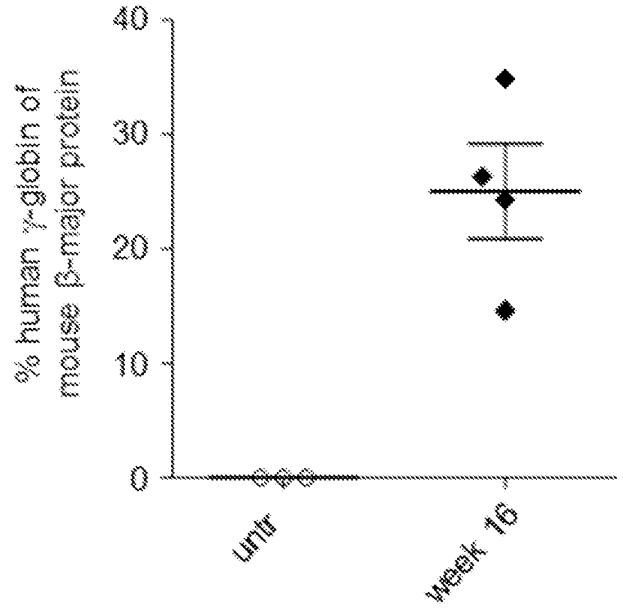


FIG. 68D

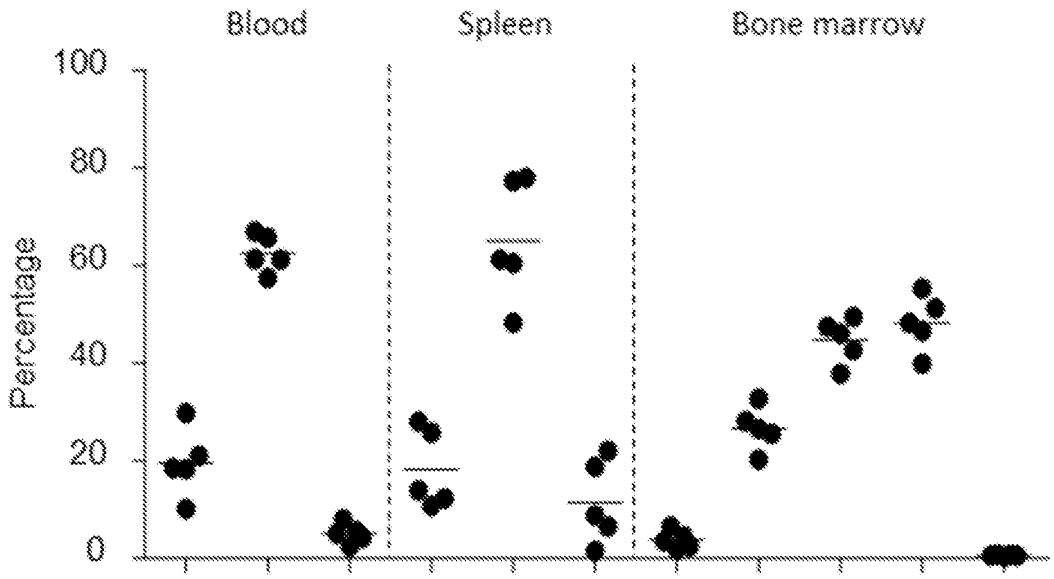


FIG. 69A

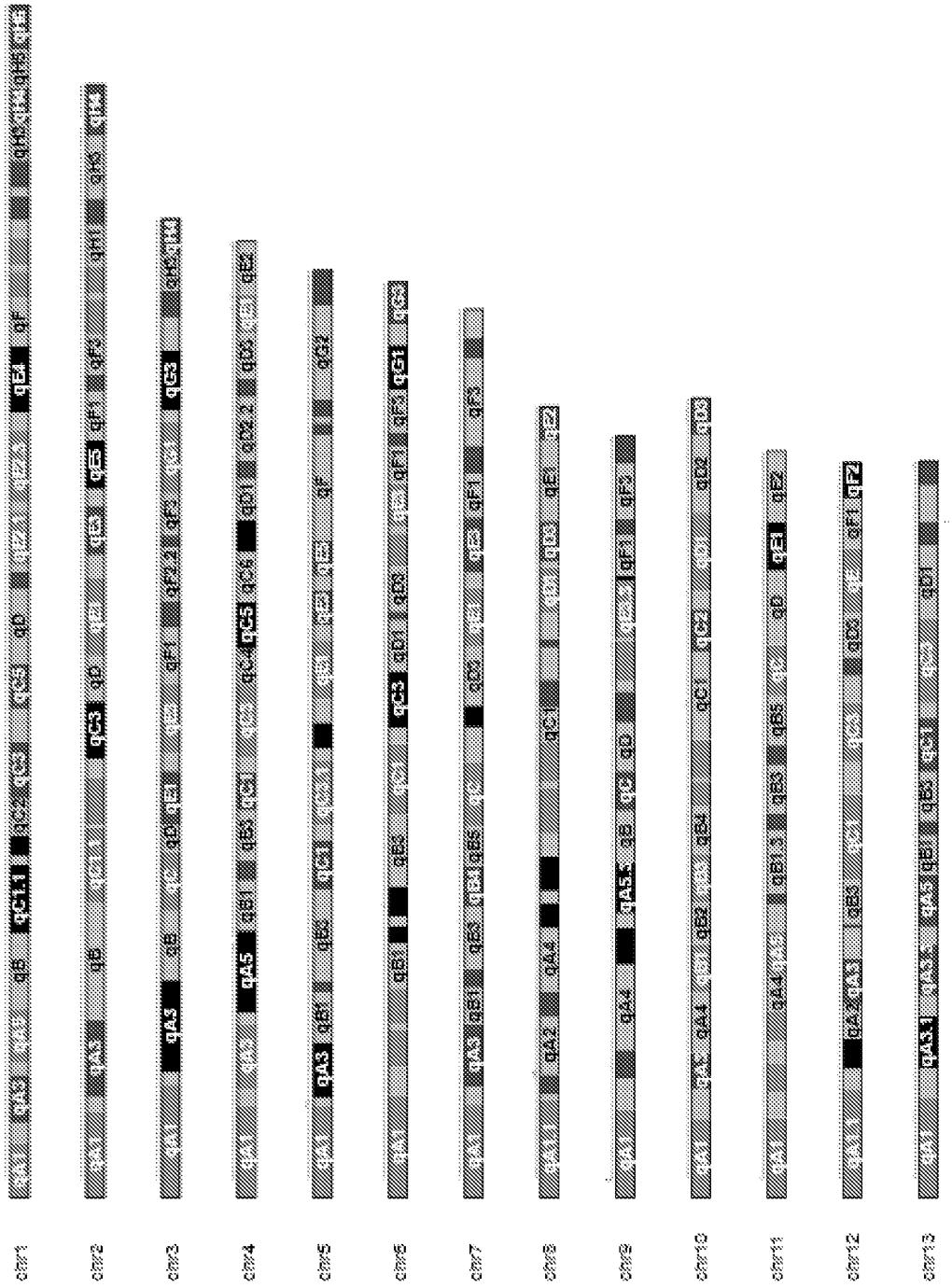

chr1
chr2
chr3
chr4
chr5
chr6
chr7
chr8
chr9
chr10
chr11
chr12
chr13

FIG. 69B

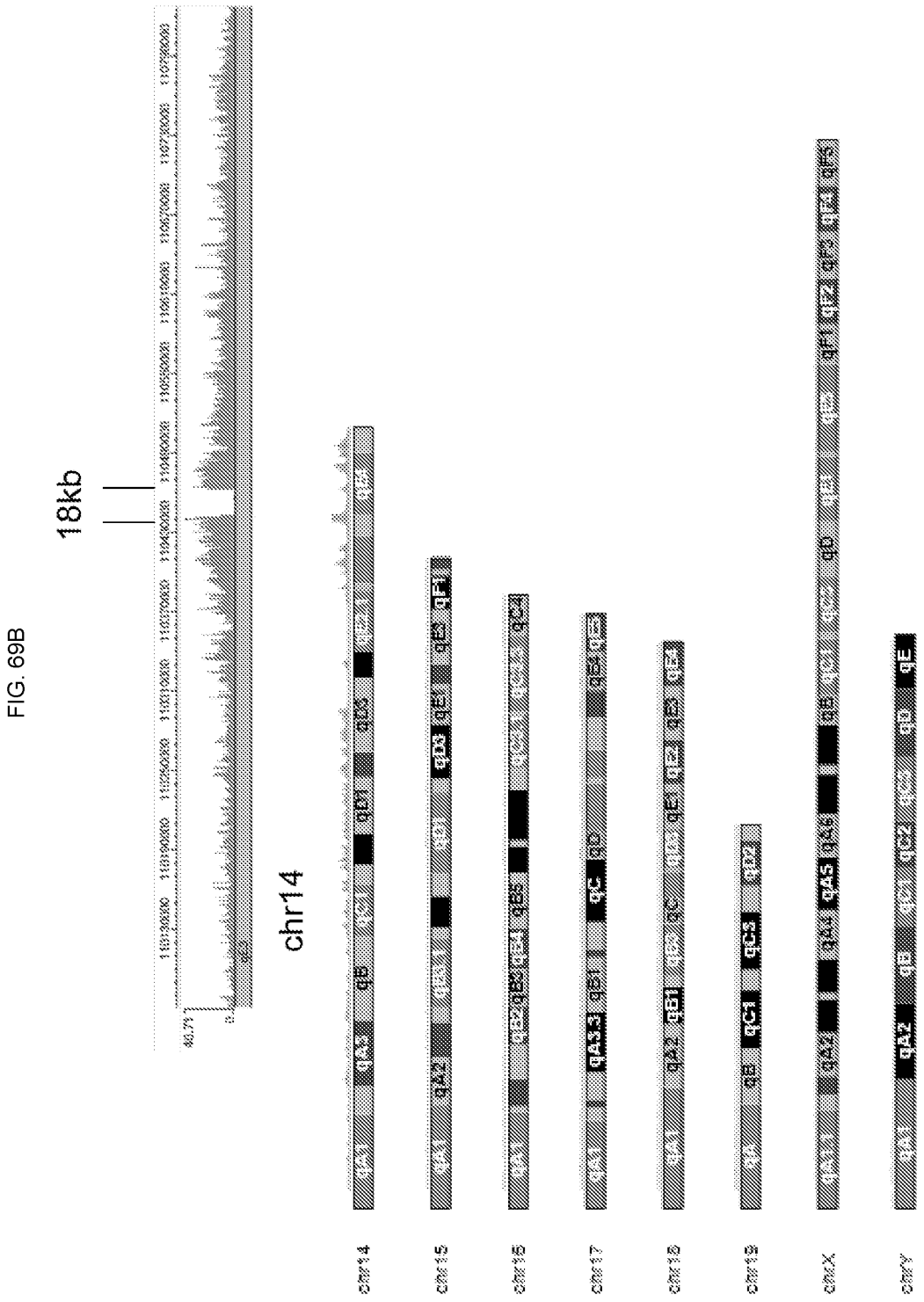


FIG. 70

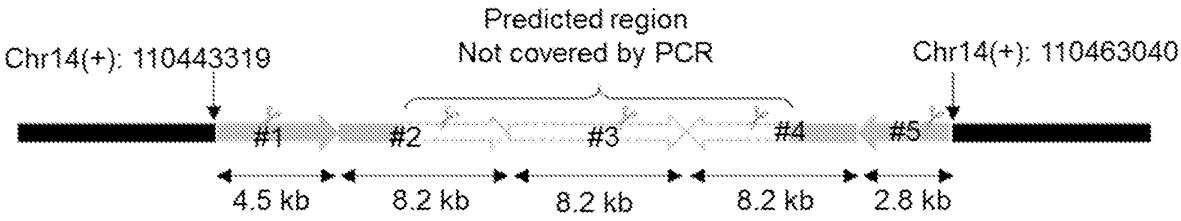


FIG. 71A

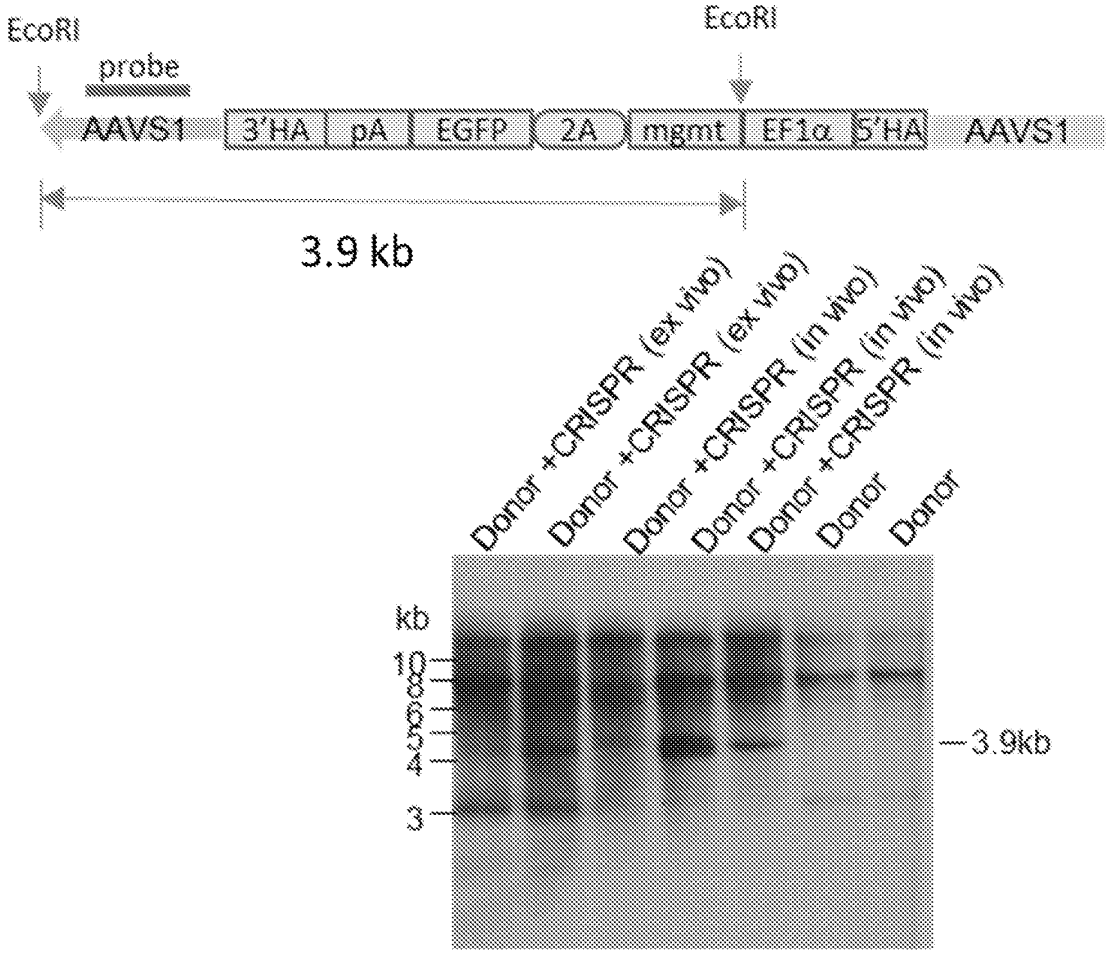


FIG. 71B

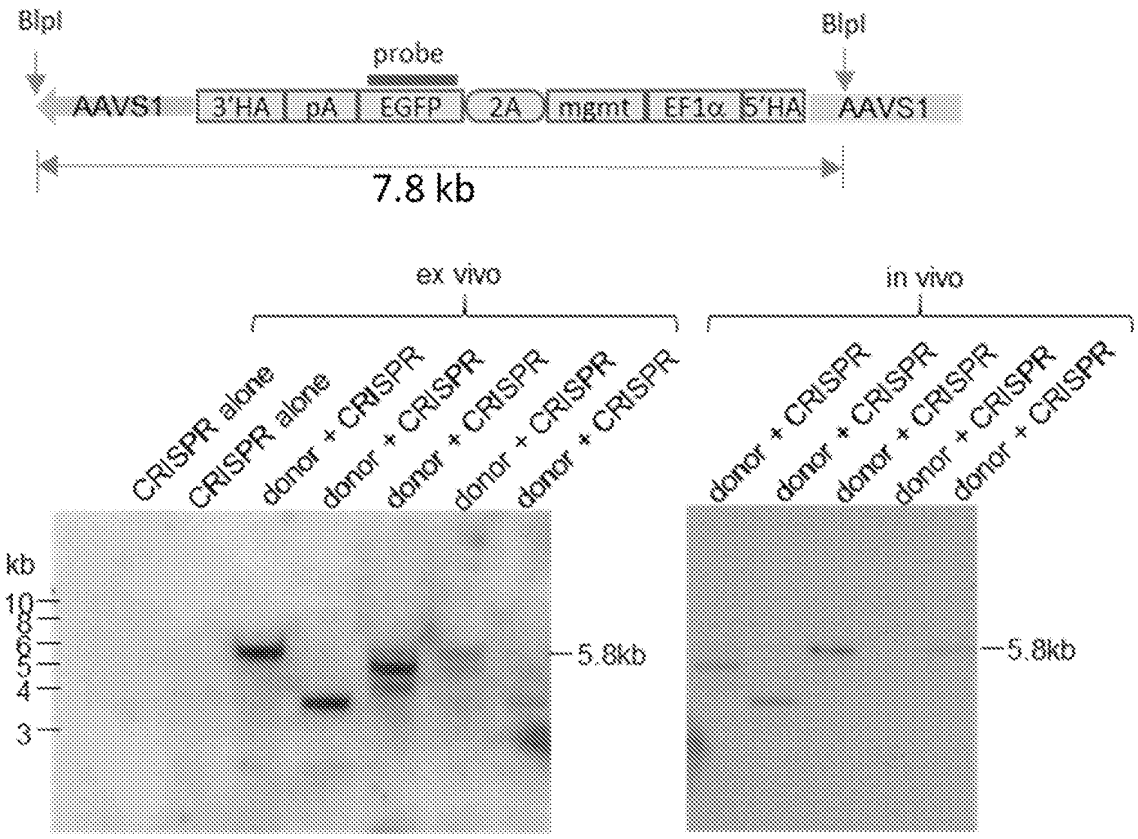

BlpI
probe
BlpI
AAVS1
3'HA
pA
EGFP
2A
mgmt
EF1α
5'HA
AAVS1
7.8 kb
ex vivo
in vivo
CRISPR alone
CRISPR alone
donor + CRISPR
donor + CRISPR
donor + CRISPR
donor + CRISPR
donor + CRISPR
donor + CRISPR
donor + CRISPR
donor + CRISPR
donor + CRISPR
kb
10
8
5
4
3
5.8kb
5.8kb

FIG. 72A

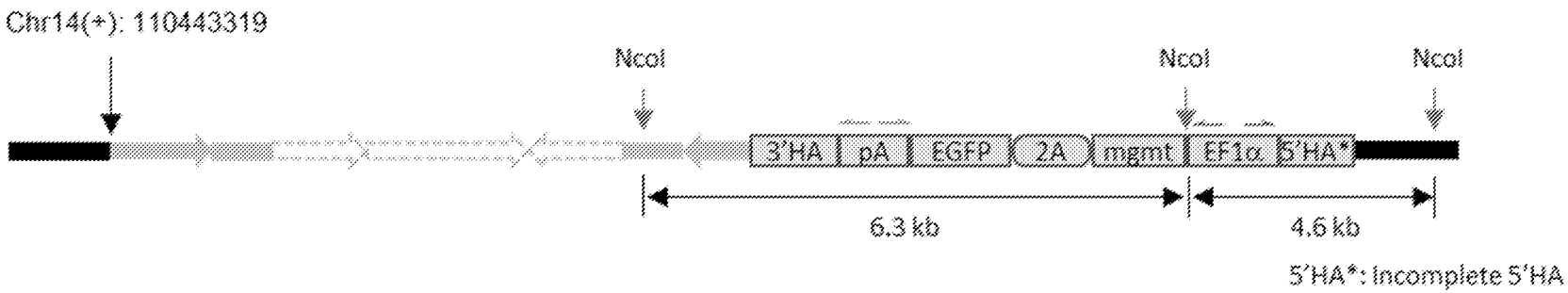


FIG. 72B

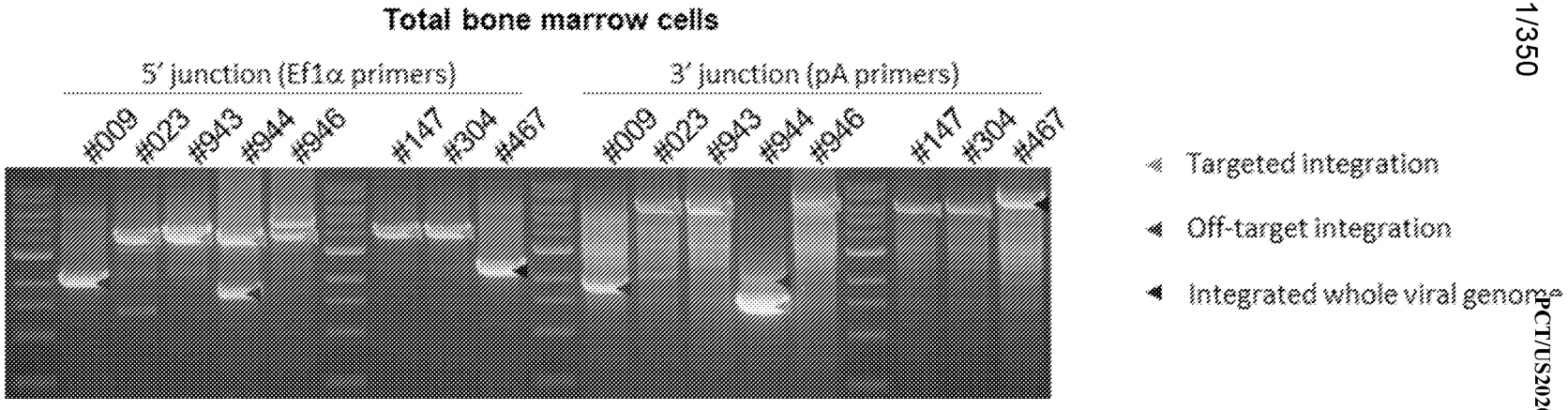


FIG. 72C

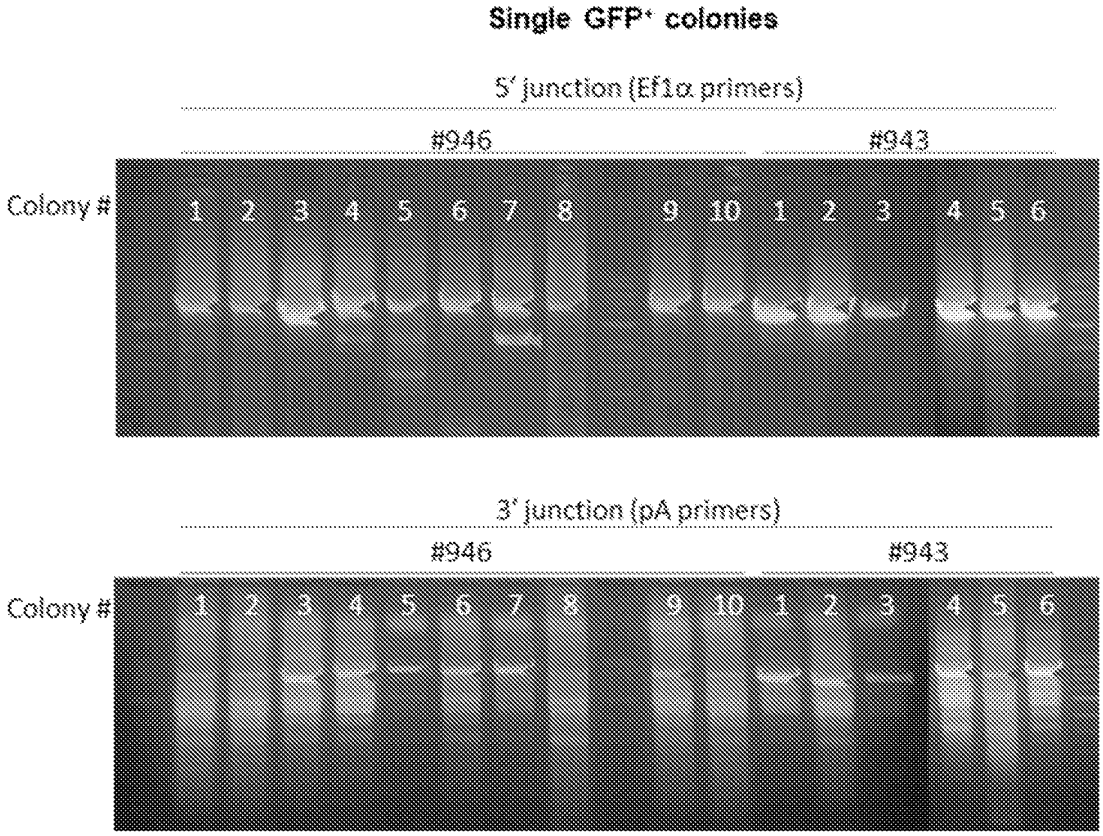

Single GFP+ colonies
5′ junction (Ef1α primers)
#946
#943
Colony #
1 2 3 4 5 6 7 8 9 10 1 2 3 4 5 6
3′ junction (pA primers)
#946
#943
Colony #
1 2 3 4 5 6 7 8 9 10 1 2 3 4 5 6

FIG. 73A

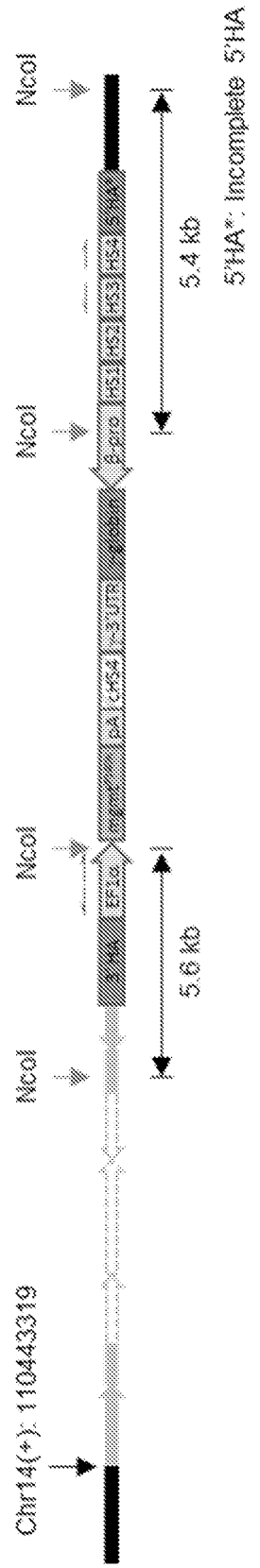


FIG. 73B

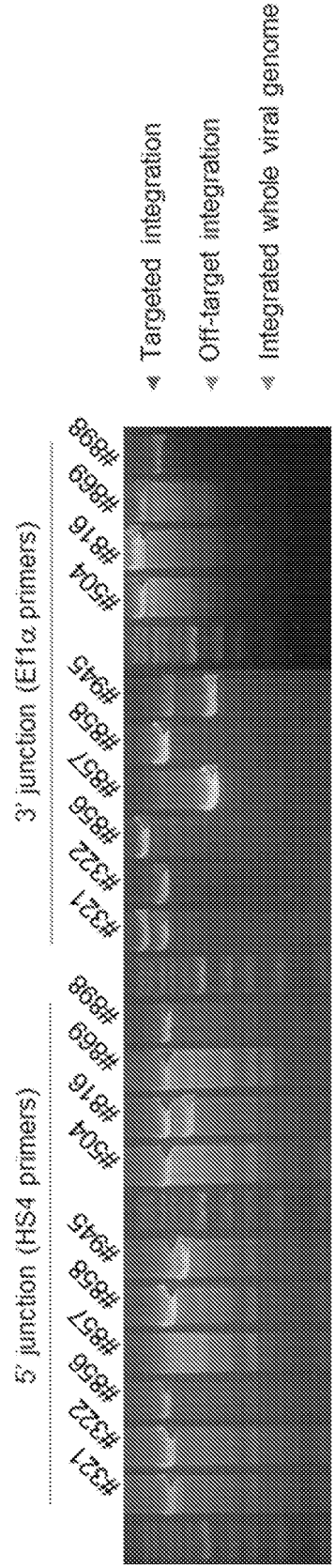


FIG. 74A

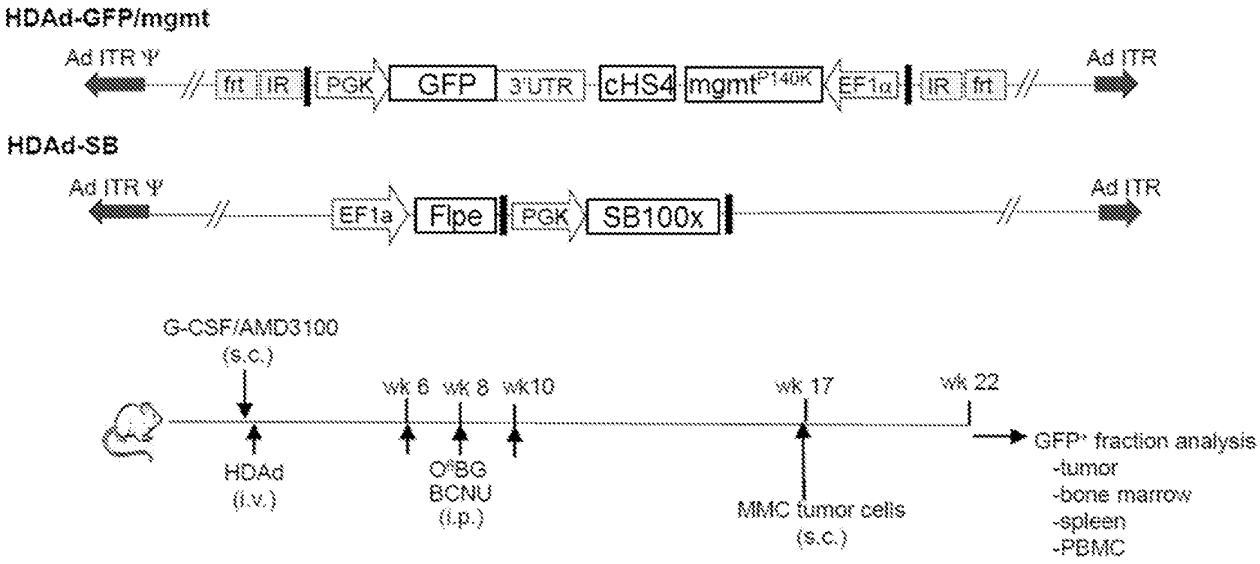


FIG. 74B

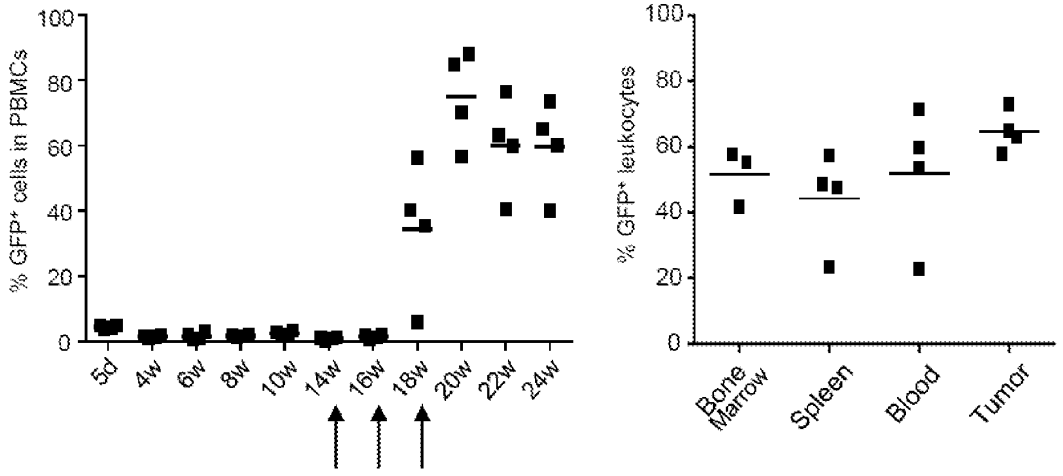
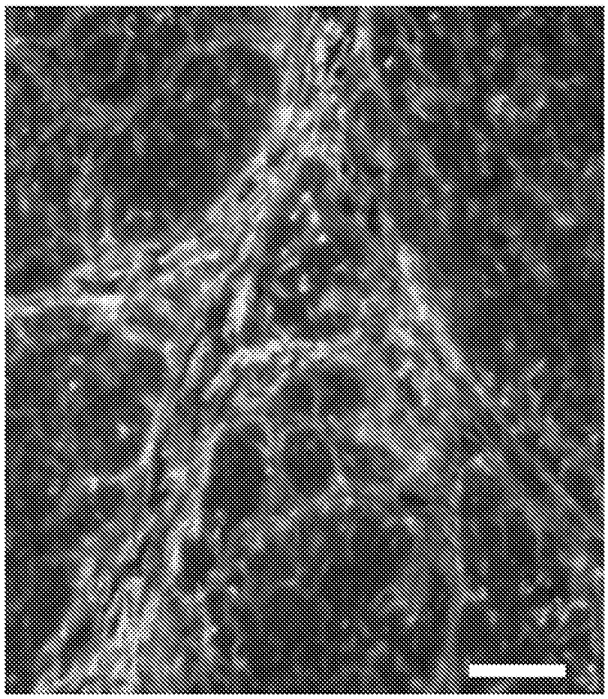


FIG. 74C



GFP/laminin

FIG. 74D

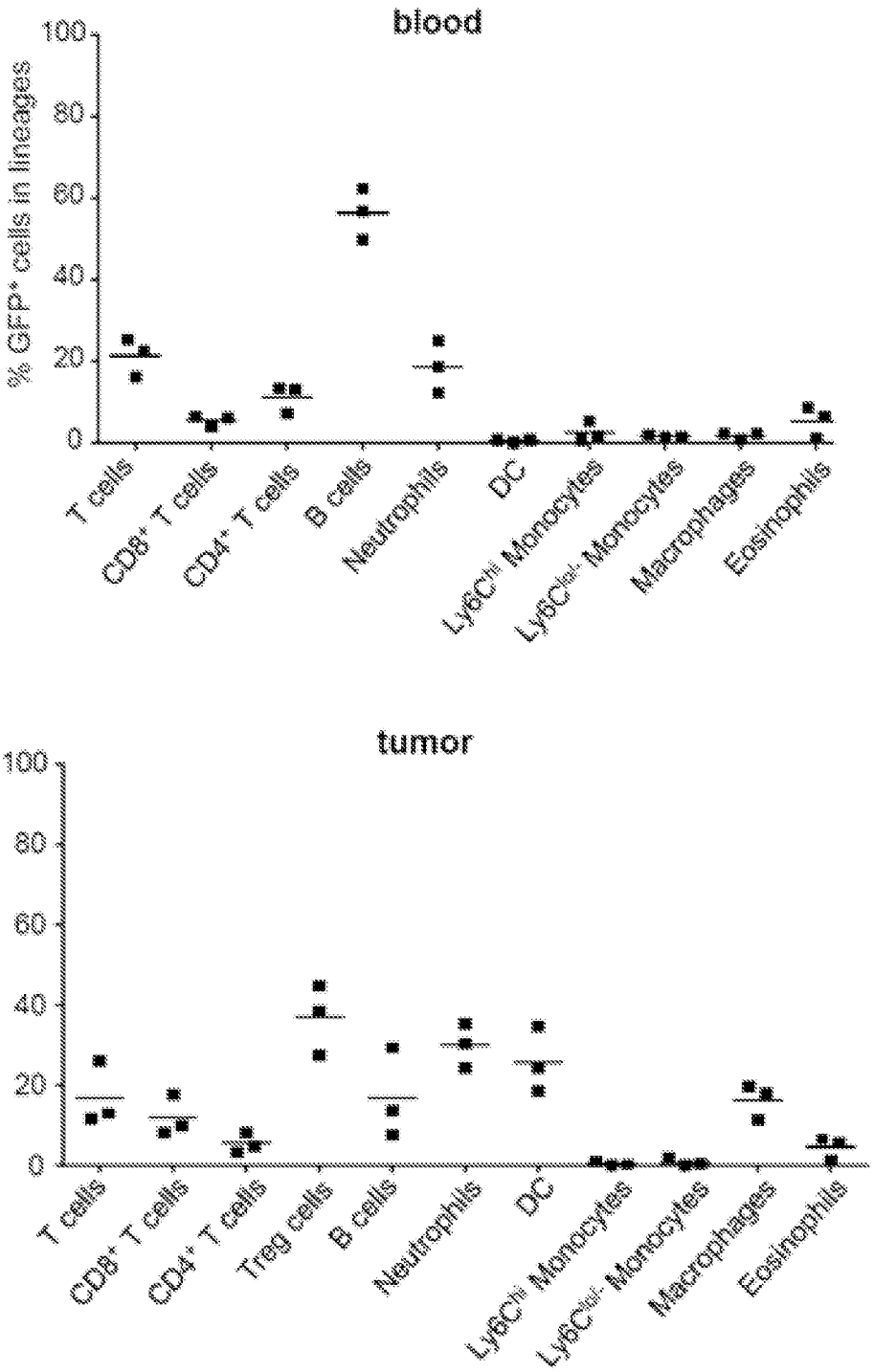

blood
% GFP+ cells in lineages
100
80
60
40
20
0
T cells
CD8+ T cells
CD4+ T cells
B cells
Neutrophils
DC
Ly6Chi Monocytes
Ly6Clo/- Monocytes
Macrophages
Eosinophils
tumor
100
80
60
40
20
0
T cells
CD8+ T cells
CD4+ T cells
Treg cells
B cells
Neutrophils
DC
Ly6Chi Monocytes
Ly6Clo/- Monocytes
Macrophages
Eosinophils

FIG. 75

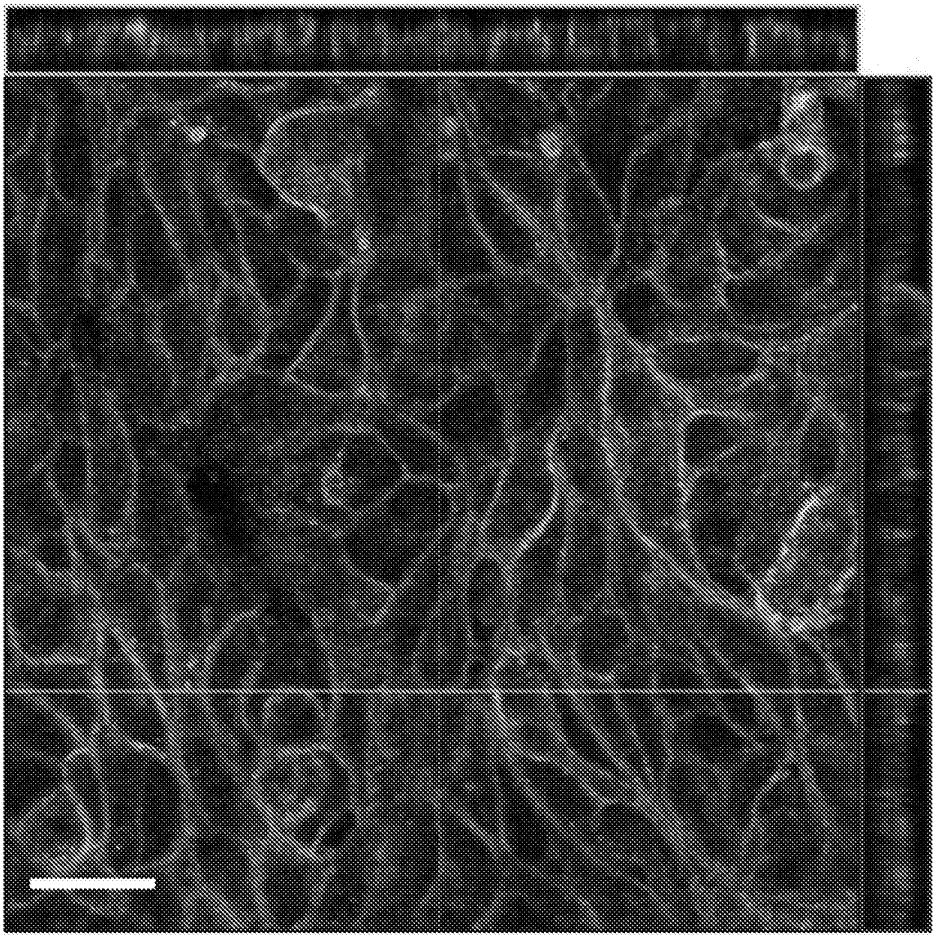


FIG. 76

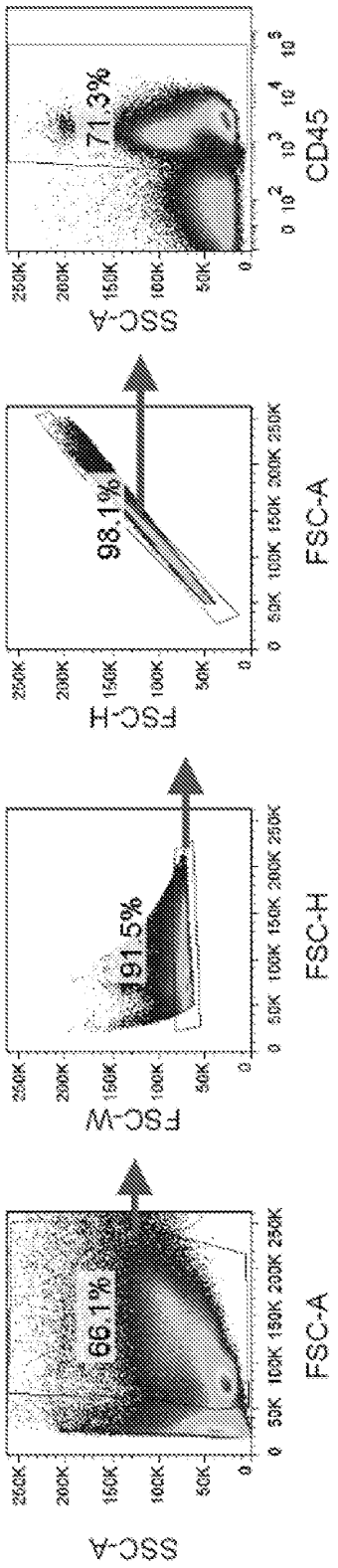


FIG. 76 cont'd

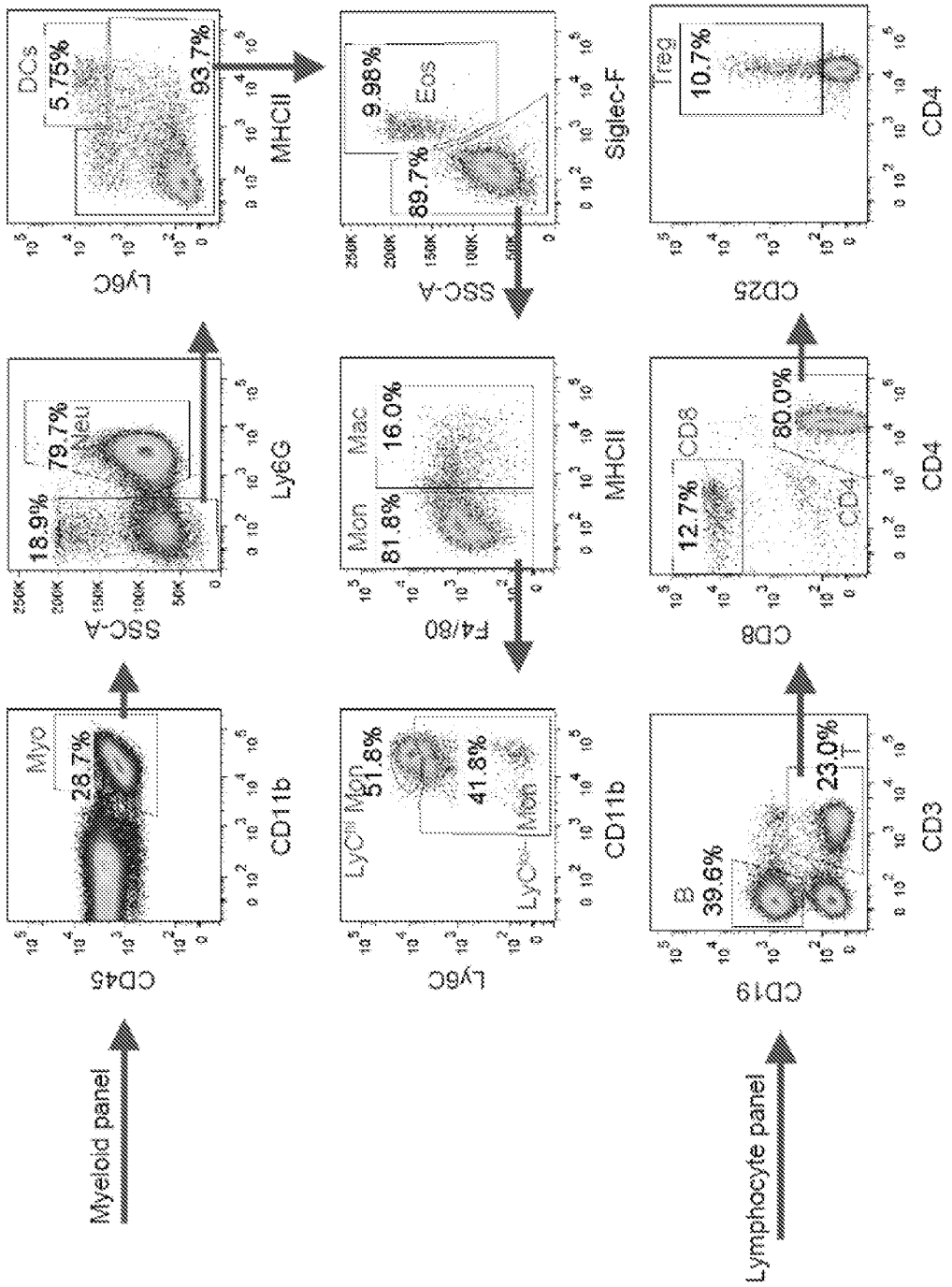

Myeloid panel
Lymphocyte panel
CD45
CD11b
Myo
28.7%
SSC-A
Ly6G
18.9%
79.7%
Neu
Ly6C
DCs
5.75%
93.7%
MHCII
Ly6C
CD11b
Ly6C^hi Mon
51.8%
41.8%
Ly6C^lo Mon
F4/80
MHCII
Mon
81.8%
Mac
16.0%
SSC-A
Siglec-F
9.98%
Eos
89.7%
CD19
CD3
B
39.6%
T
23.0%
CD8
CD4
12.7%
CD8
CD4
80.0%
CD25
CD4
Treg
10.7%

FIG. 77

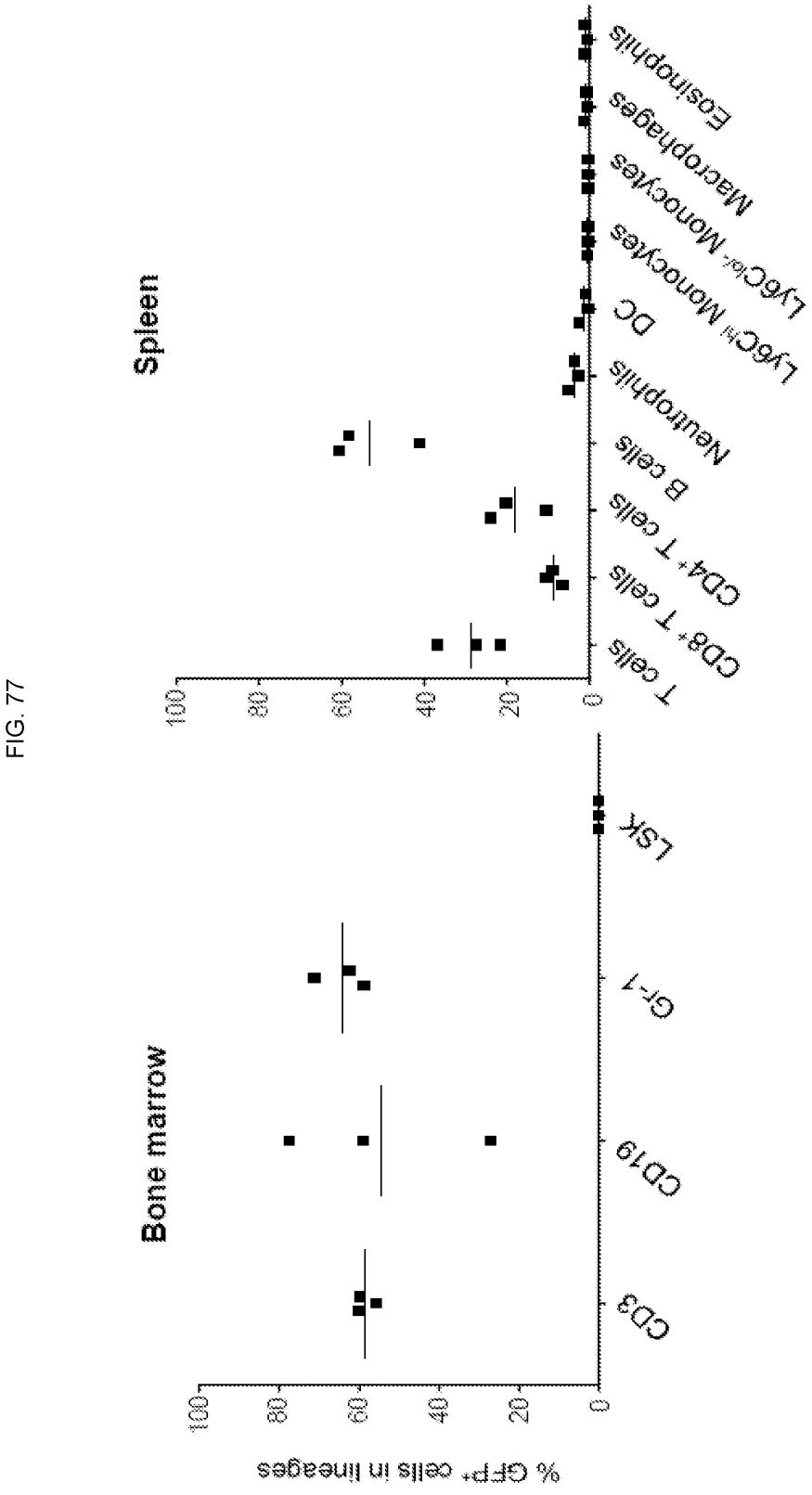

% GFP+ cells in lineages
Bone marrow
CD3
CD19
Gr-1
LSK
Spleen
T cells
CD8+ T cells
CD4+ T cells
B cells
Neutrophils
DC
Ly6Chi Monocytes
Ly6Clo Monocytes
Macrophages
Eosinophils
0
20
40
60
80
100

FIG. 78A

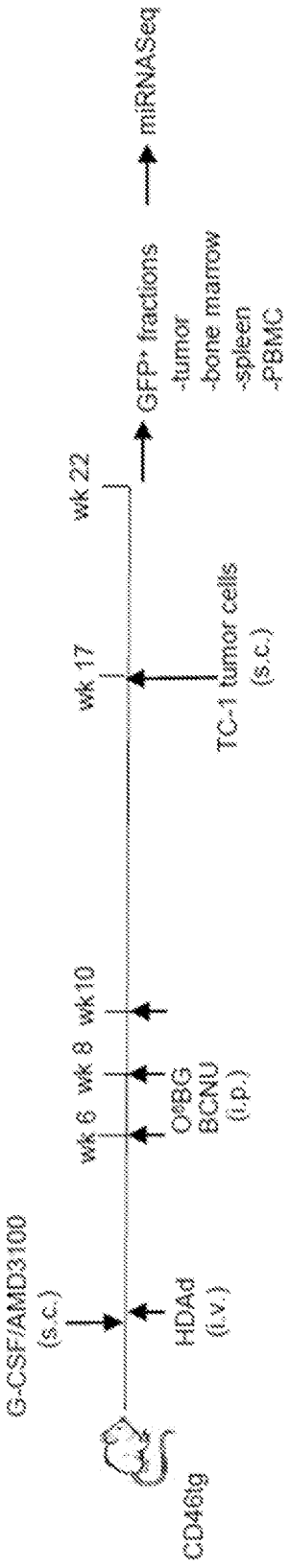


FIG. 78B

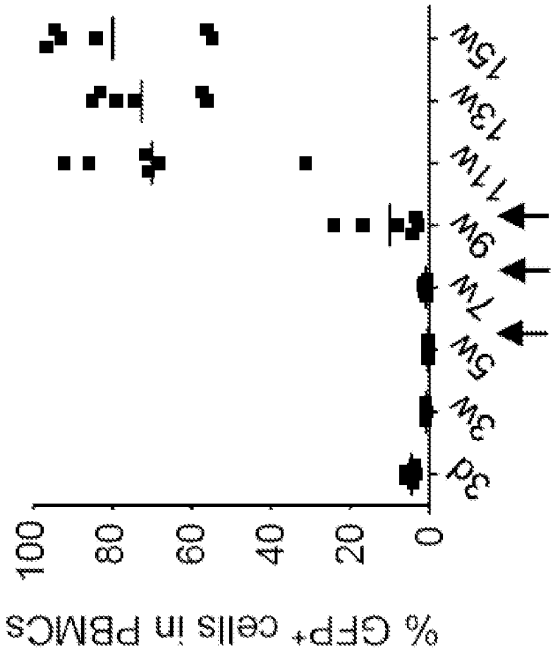


FIG. 78C

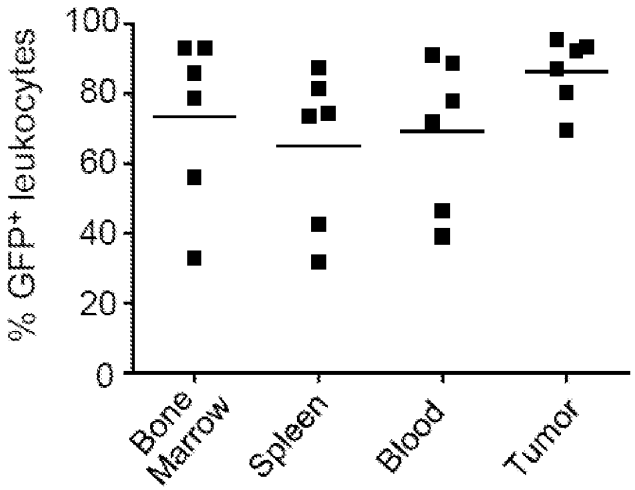


FIG. 78D

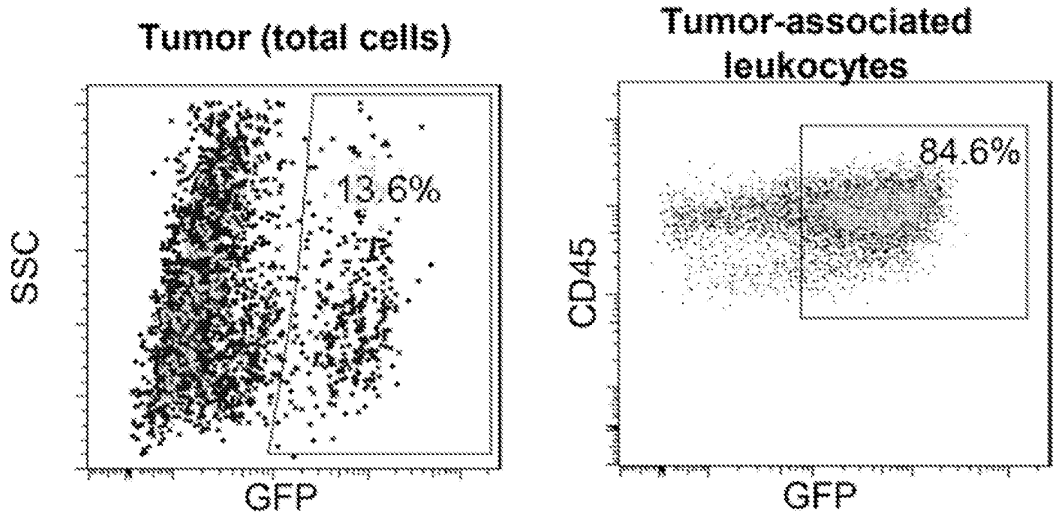


FIG. 78E

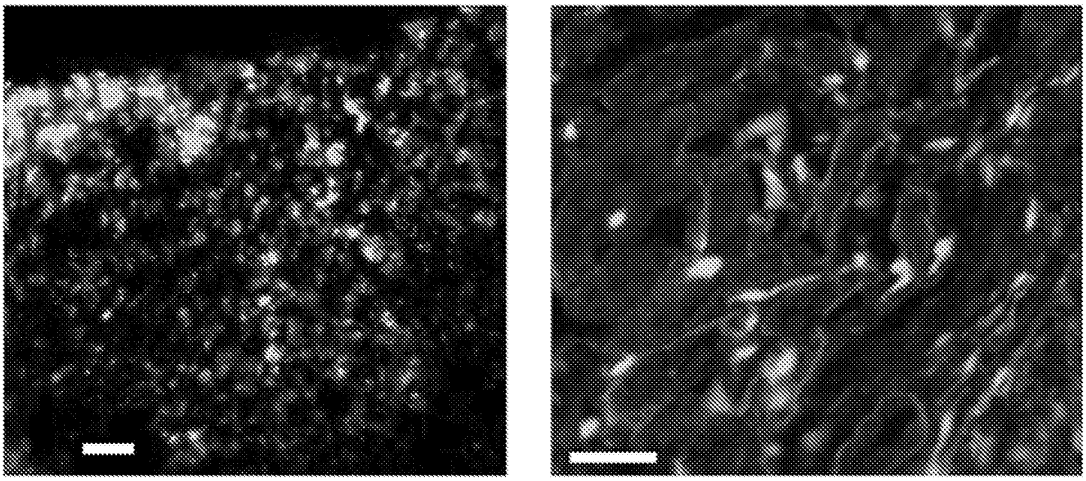


FIG. 78F

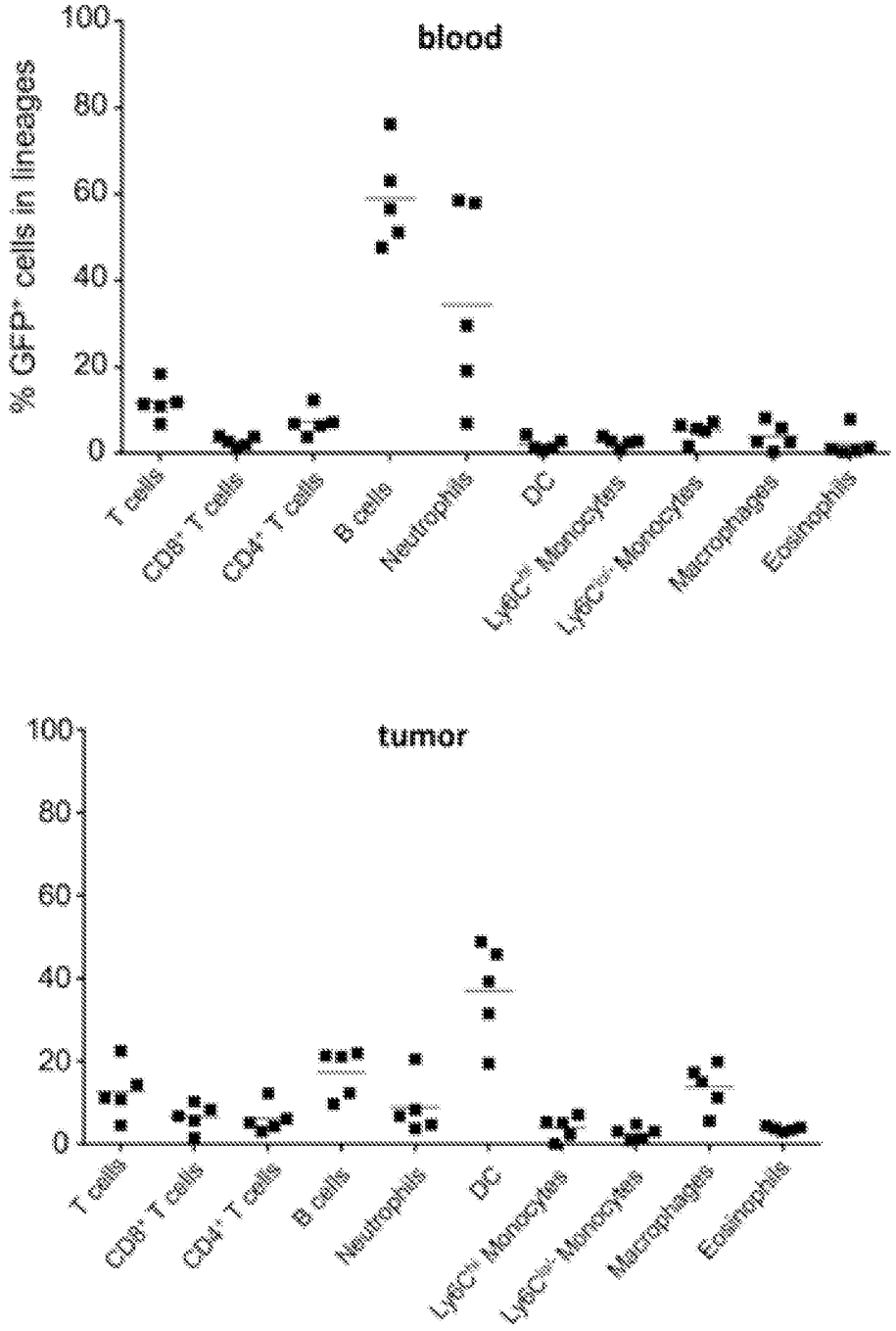

blood
% GFP+ cells in lineages
100
80
60
40
20
0
T cells
CD8+ T cells
CD4+ T cells
B cells
Neutrophils
DC
Ly6C+ Monocytes
Ly6C- Monocytes
Macrophages
Eosinophils
tumor
100
80
60
40
20
0
T cells
CD8+ T cells
CD4+ T cells
B cells
Neutrophils
DC
Ly6C+ Monocytes
Ly6C- Monocytes
Macrophages
Eosinophils

FIG. 79A

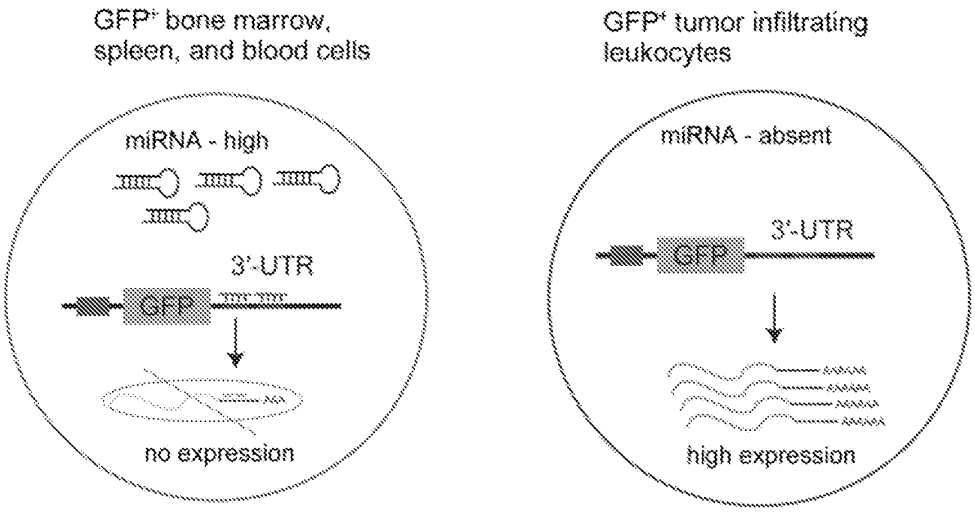


FIG. 79B

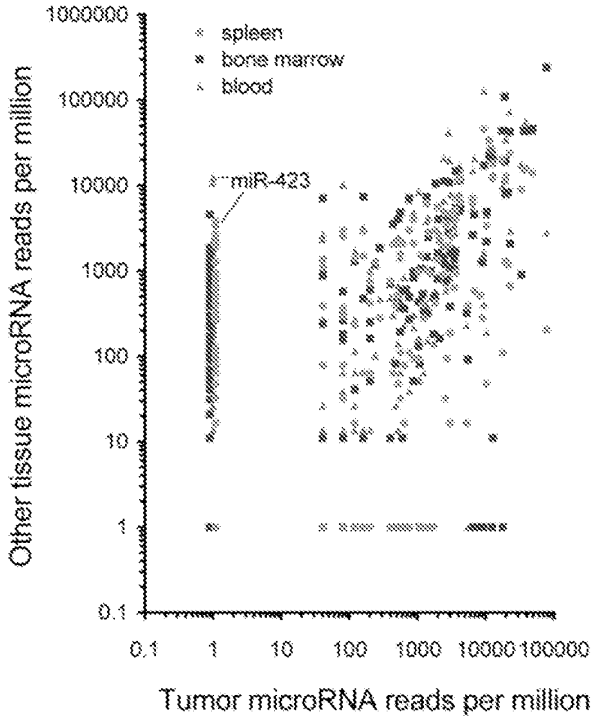


FIG. 79C

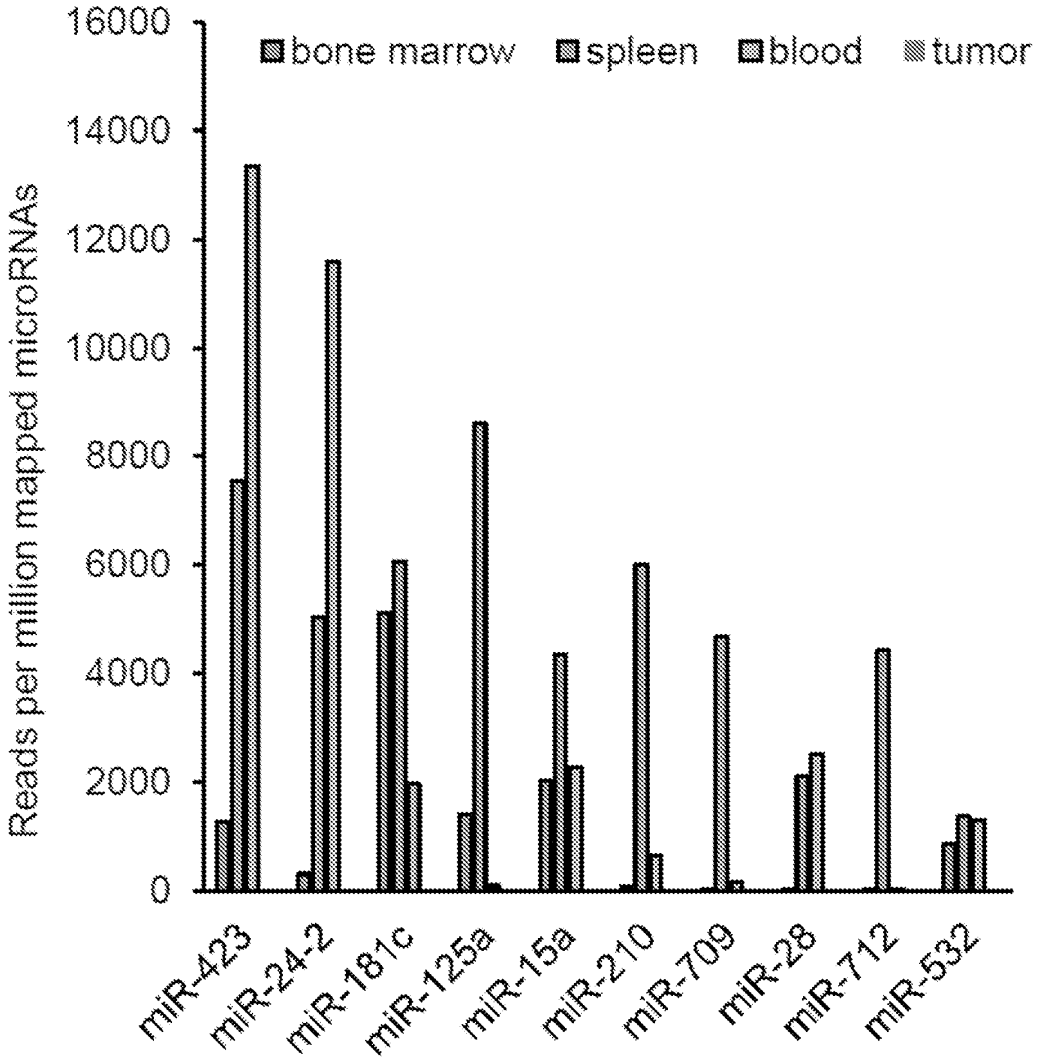

16000
14000
12000
10000
8000
6000
4000
2000
0
Reads per million mapped microRNAs
bone marrow
spleen
blood
tumor
miR-423
miR-24-2
miR-181c
miR-125a
miR-15a
miR-210
miR-709
miR-28
miR-712
miR-532

FIG. 80A

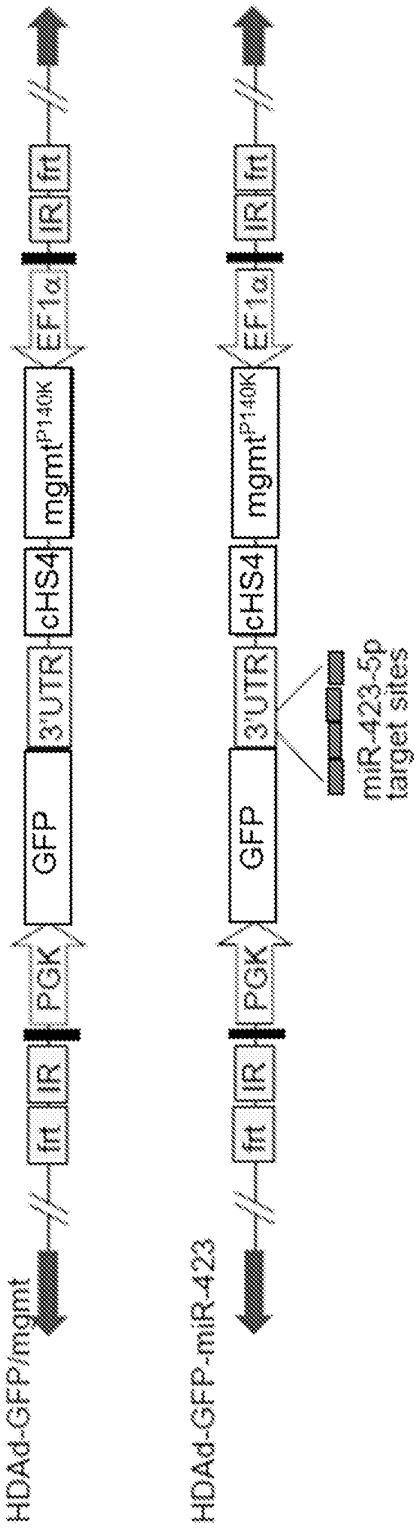

HDAd-GFP/mgmt
ITR
IR
PGK
GFP
3'UTR
cHS4
mgmt^{P140K}
EF1α
IR
ITR
HDAd-GFP-miR-423
ITR
IR
PGK
GFP
3'UTR
miR-423-5p target sites
cHS4
mgmt^{P140K}
EF1α
IR
ITR

FIG. 80B

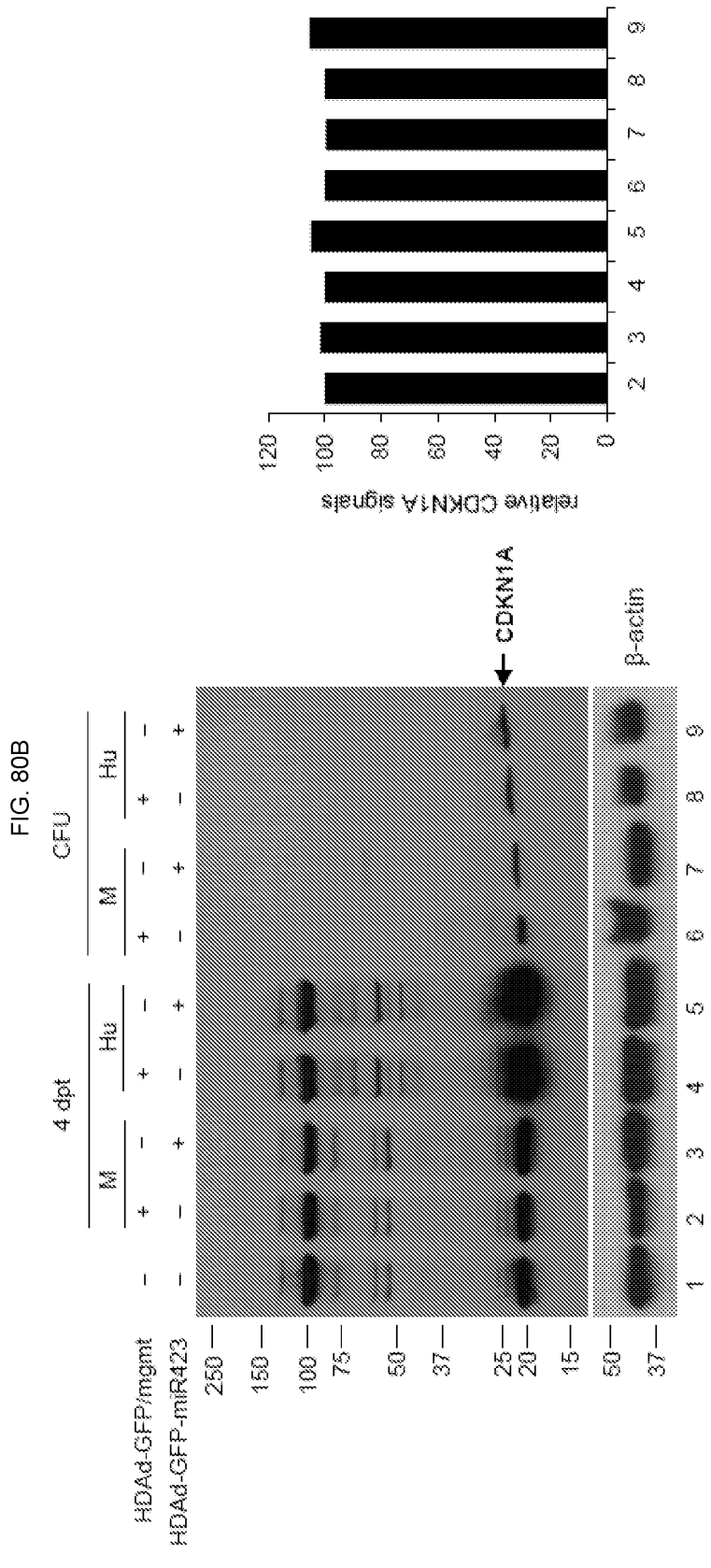

4 dpt
CFU
M
Hu
M
Hu
HDAd-GFP/mgmt
HDAd-GFP-miR423
250
150
100
75
50
37
25
20
15
50
37
CDKN1A
β-actin
1 2 3 4 5 6 7 8 9
relative CDKN1A signals
120
100
80
60
40
20
0
2 3 4 5 6 7 8 9

FIG. 80C

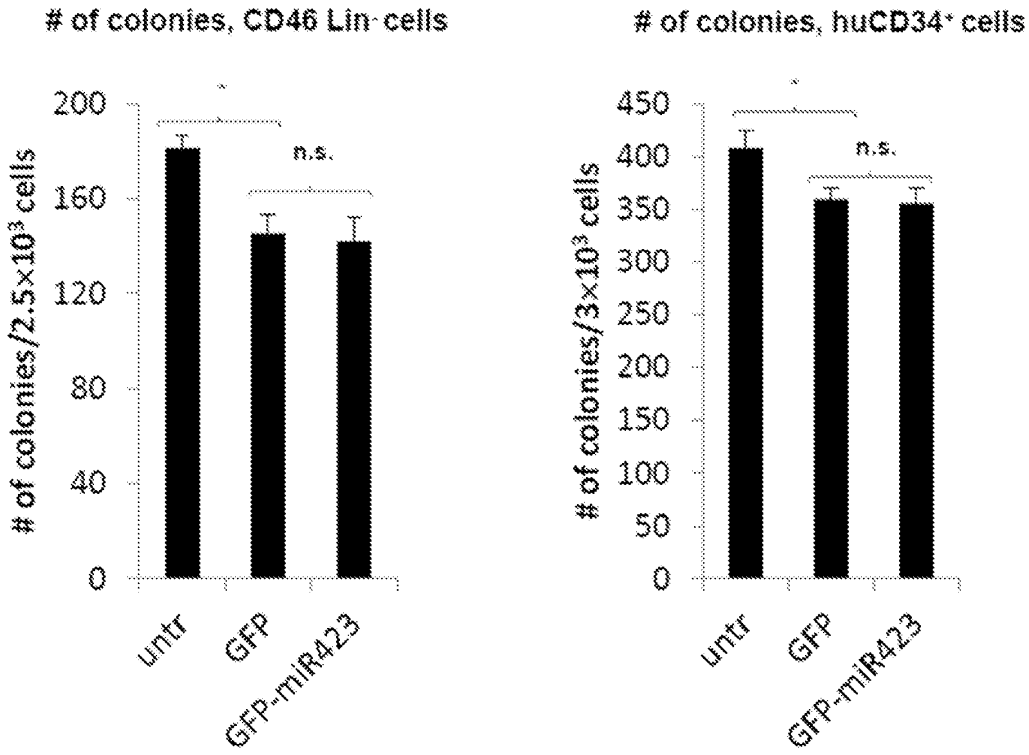


FIG. 81

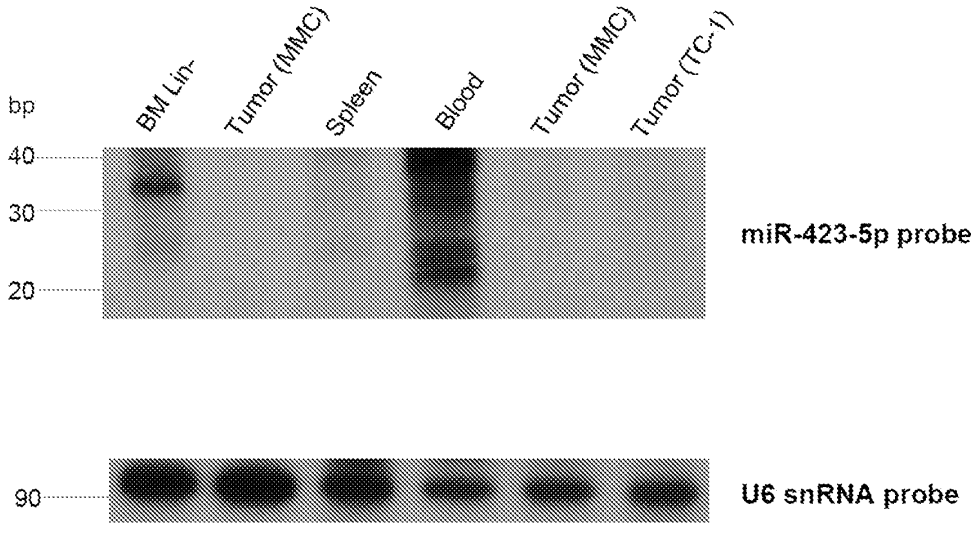


FIG. 82A

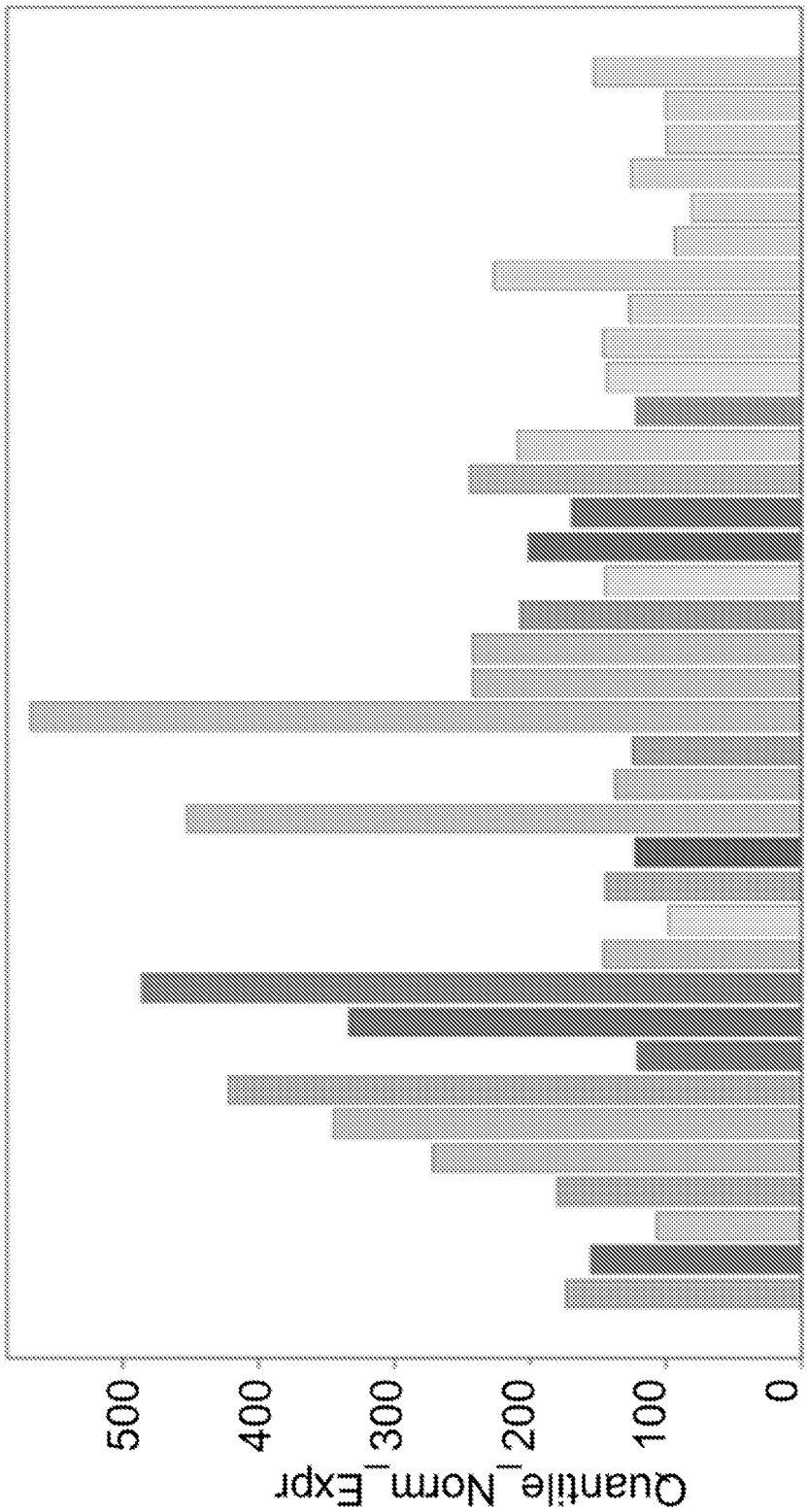


FIG. 82B

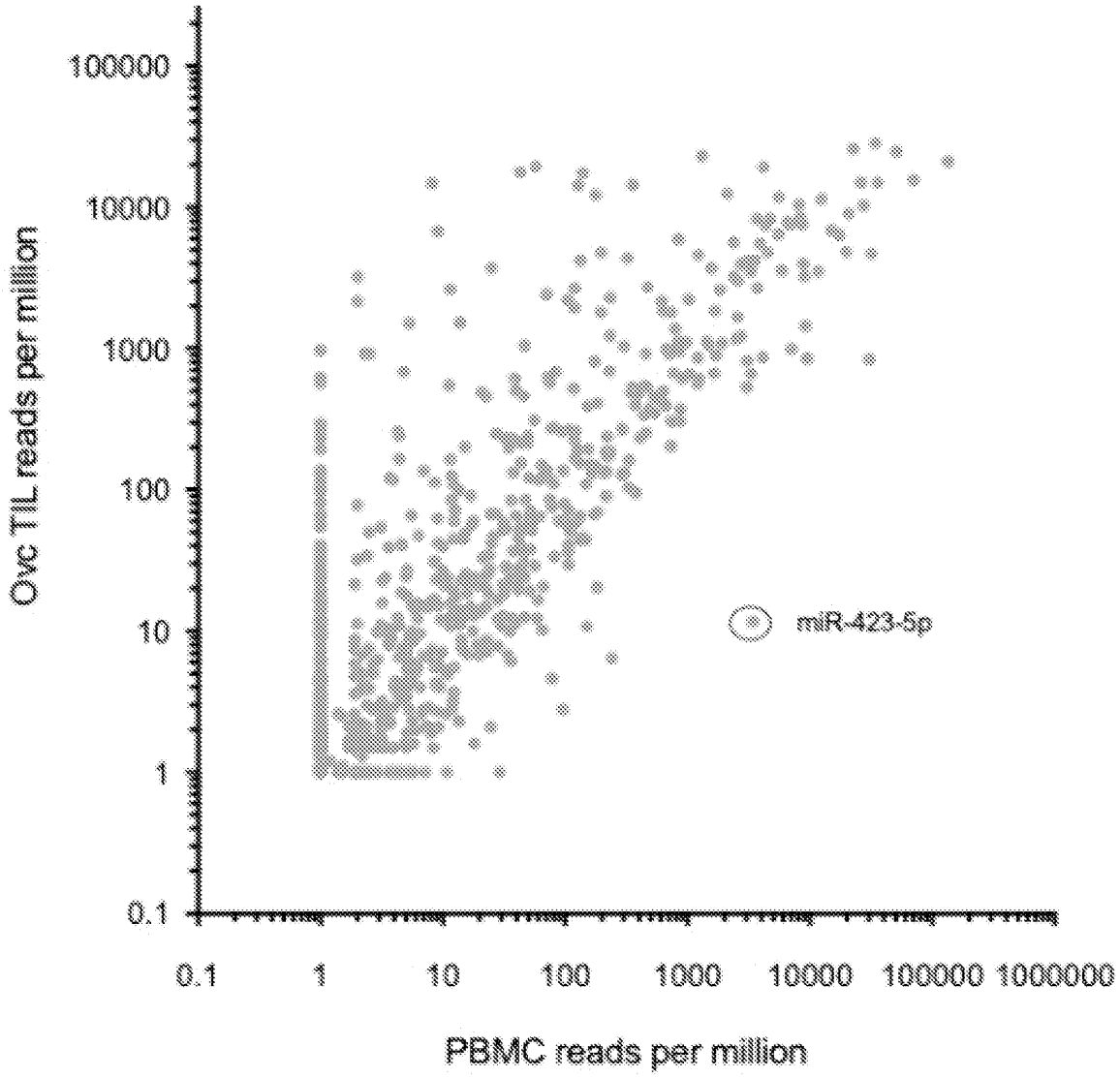

Ovc TIL reads per million
100000
10000
1000
100
10
1
0.1
0.1
1
10
100
1000
10000
100000
1000000
PBMC reads per million
miR-423-5p

FIG. 83A

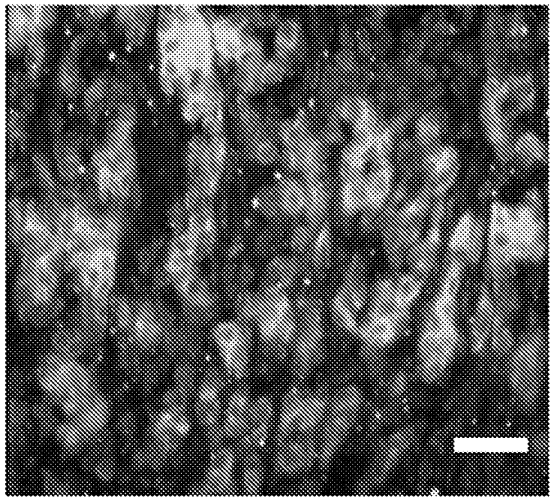


FIG. 83B

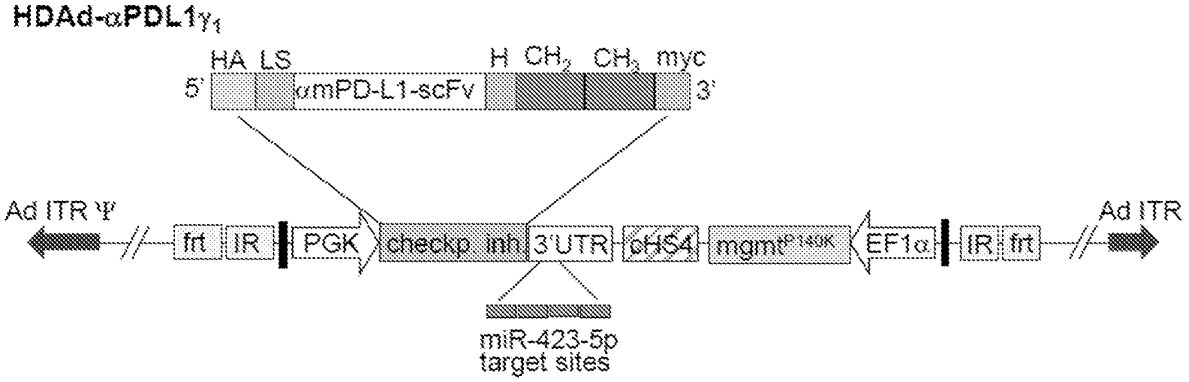


FIG. 83C

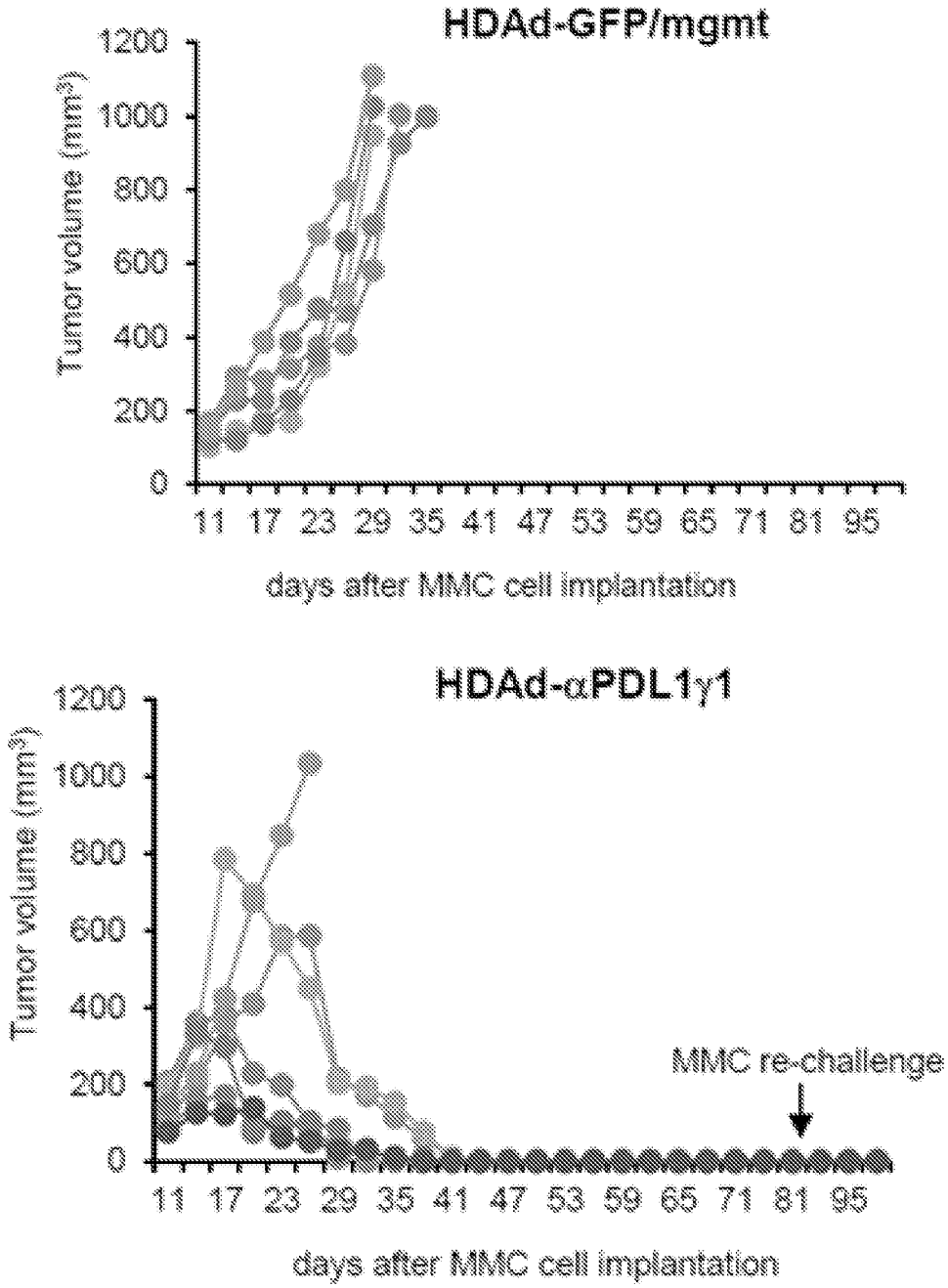

HDAd-GFP/mgmt
Tumor volume (mm3)
1200
1000
800
600
400
200
0
11 17 23 29 35 41 47 53 59 65 71 81 95
days after MMC cell implantation
HDAd-αPDL1γ1
Tumor volume (mm3)
1200
1000
800
600
400
200
0
MMC re-challenge
11 17 23 29 35 41 47 53 59 65 71 81 95
days after MMC cell implantation

FIG. 83D

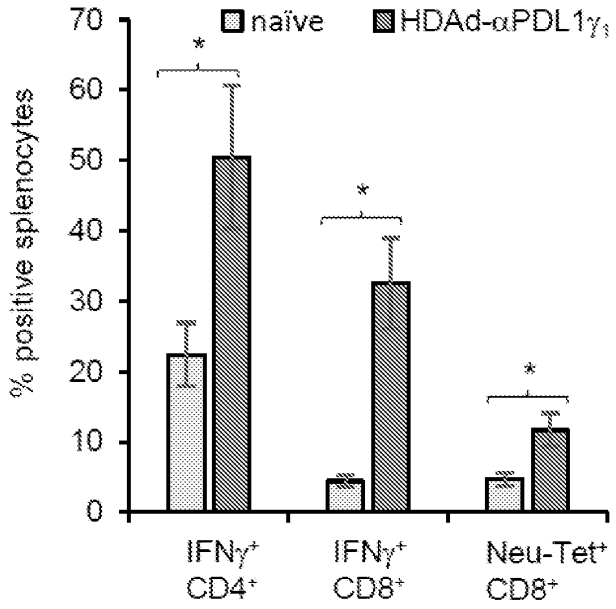


FIG. 83E

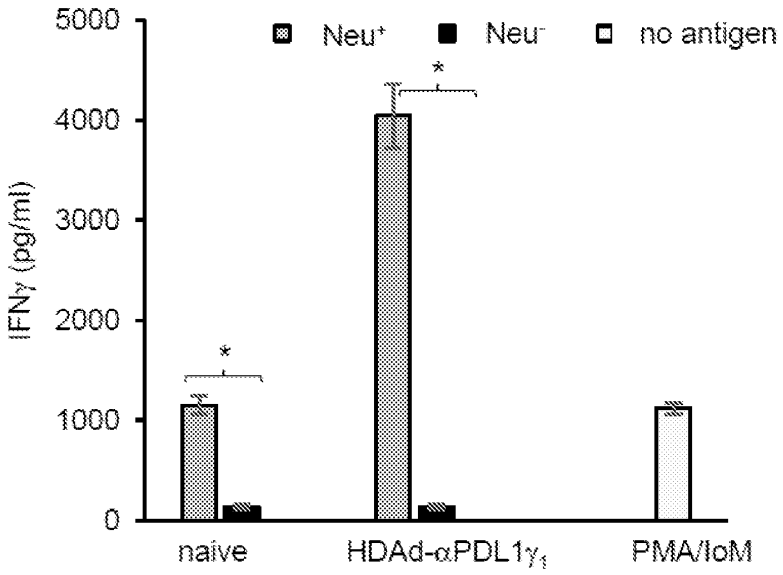
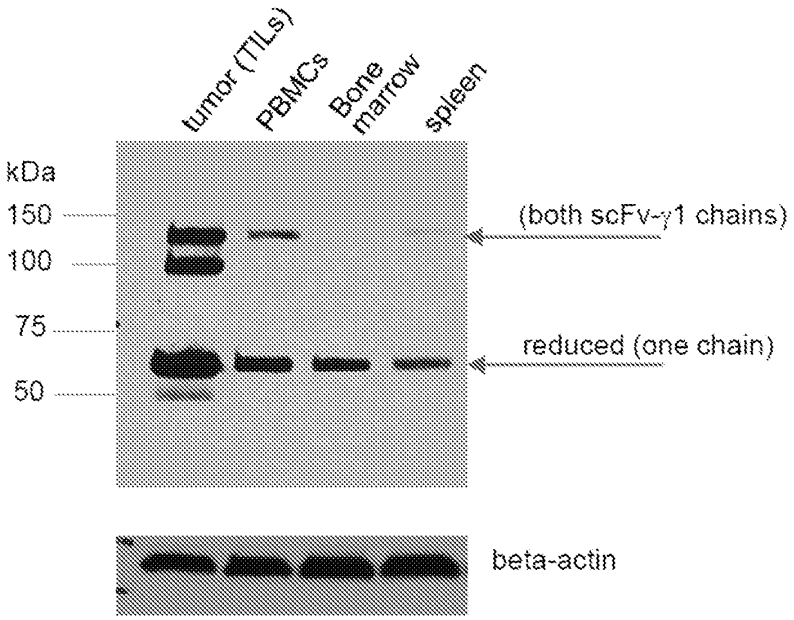


FIG. 84A


tumor (TILs)
PBMCs
Bone marrow
spleen
kDa
150
100
75
50
(both scFv-γ1 chains)
reduced (one chain)
beta-actin

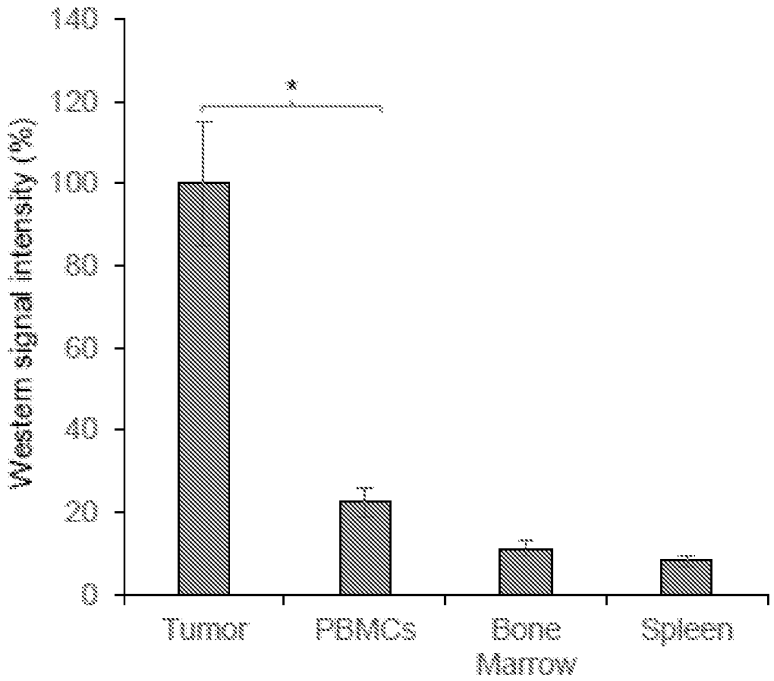

Western signal intensity (%)
140
120
100
80
60
40
20
0
*
Tumor
PBMCs
Bone Marrow
Spleen

FIG. 84B

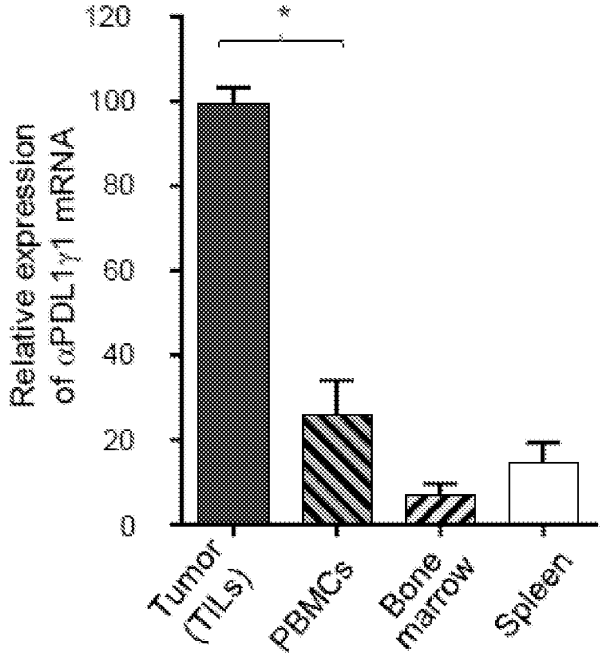


FIG. 84C

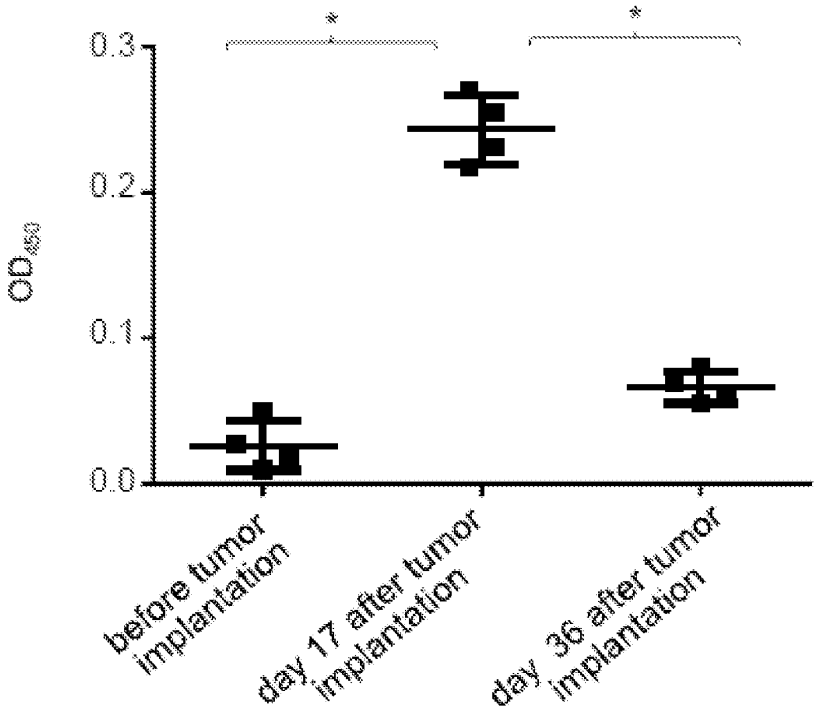


FIG. 85A

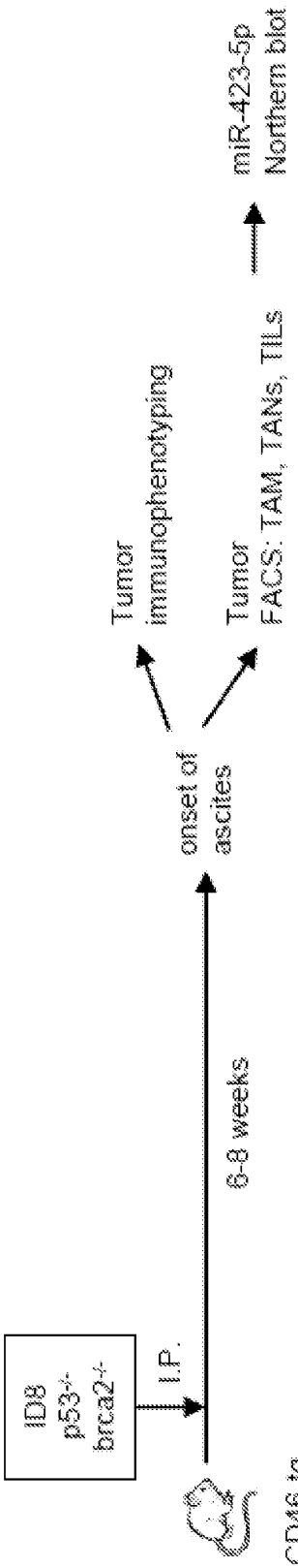

ID8
p53-/-
brca2-/-
I.P.
6-8 weeks
onset of
ascites
CD46-tg
Tumor
immunophenotyping
Tumor
FACS: TAM, TANs, TILs
miR-423-5p
Northern blot

FIG. 85B

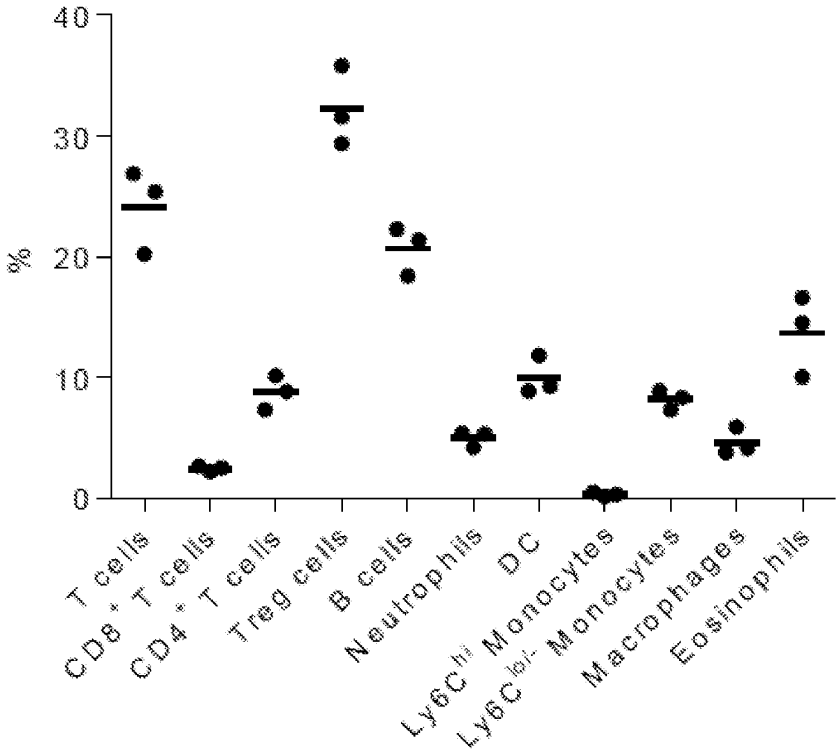

40
30
20
10
0
%
T cells
CD8+ T cells
CD4+ T cells
Treg cells
B cells
Neutrophils
DC
Ly6C^hi Monocytes
Ly6C^lo/- Monocytes
Macrophages
Eosinophils

FIG. 85C

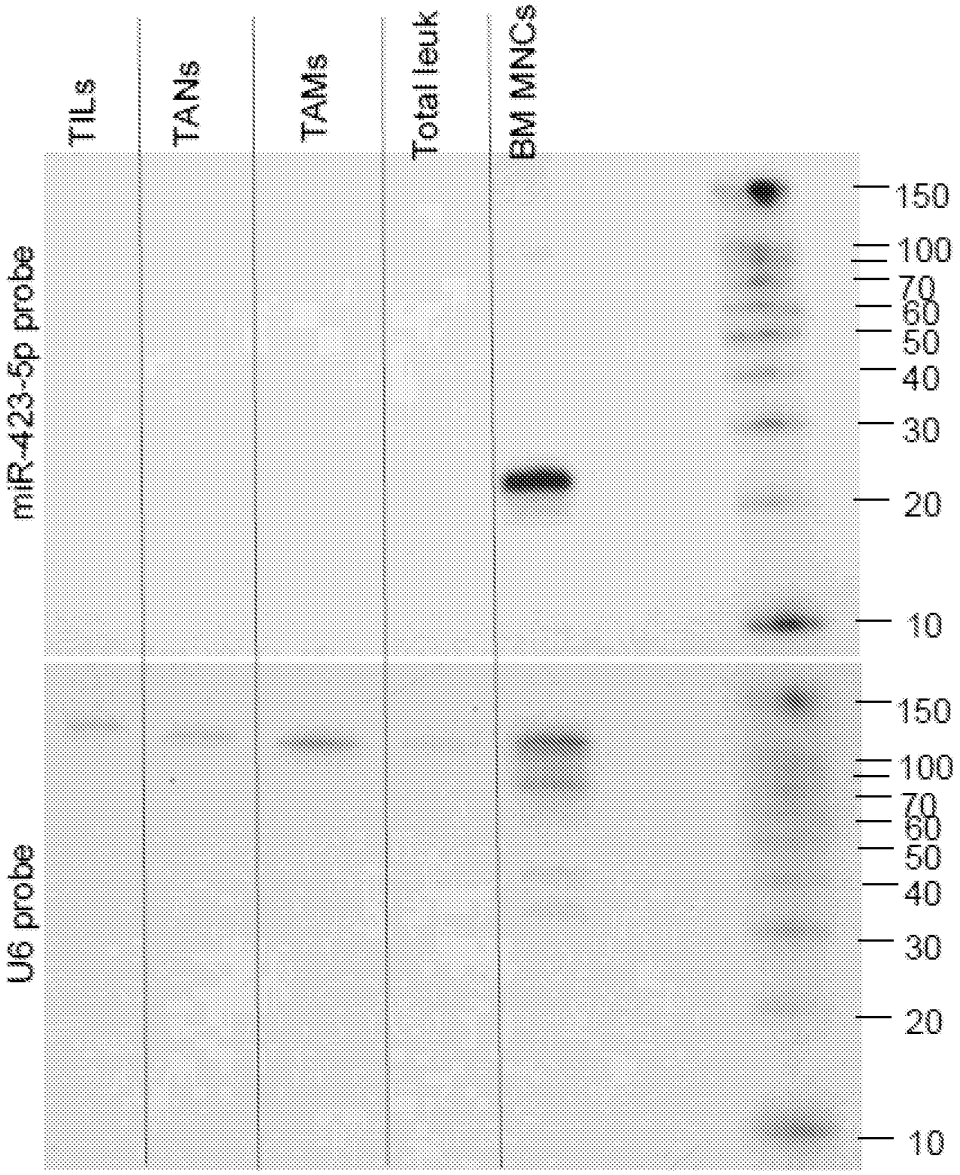

TILs
TANs
TAMs
Total leuk
BM MNCs
miR-423-5p probe
U6 probe
150
100
70
60
50
40
30
20
10

FIG. 85D

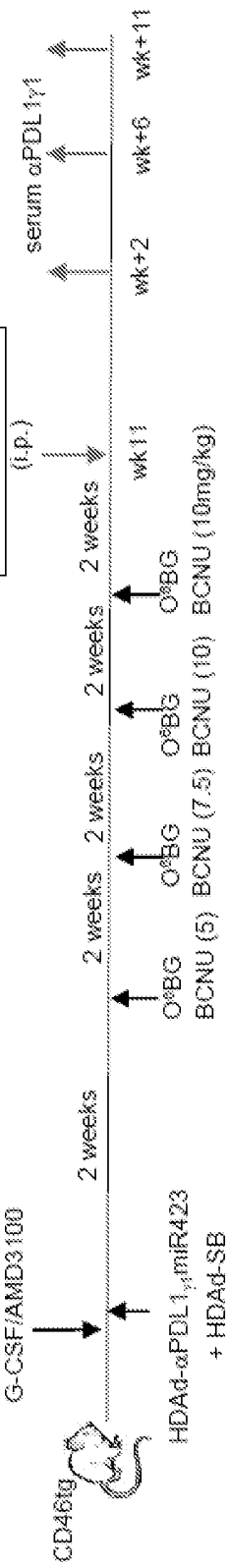


FIG. 85E

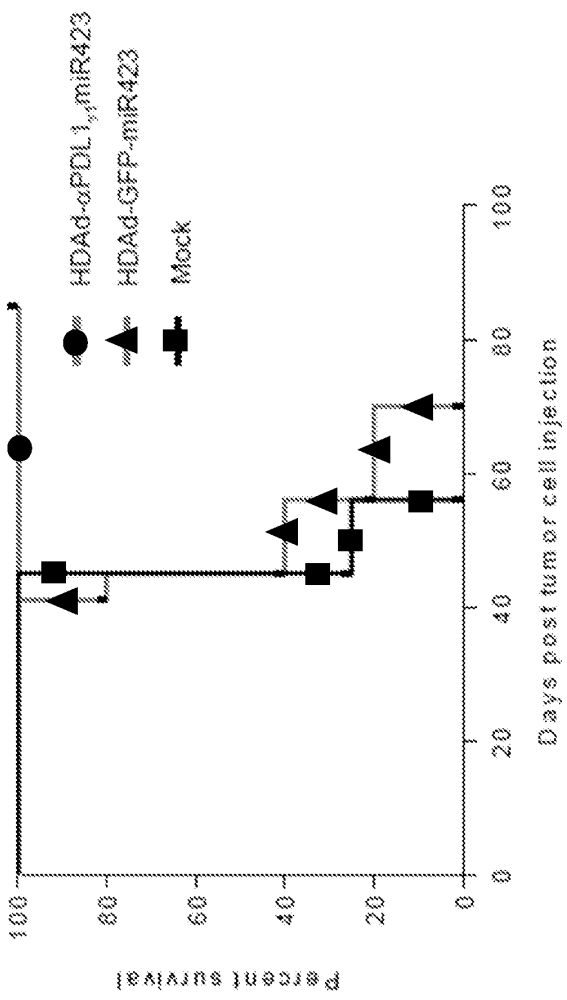


FIG. 85F

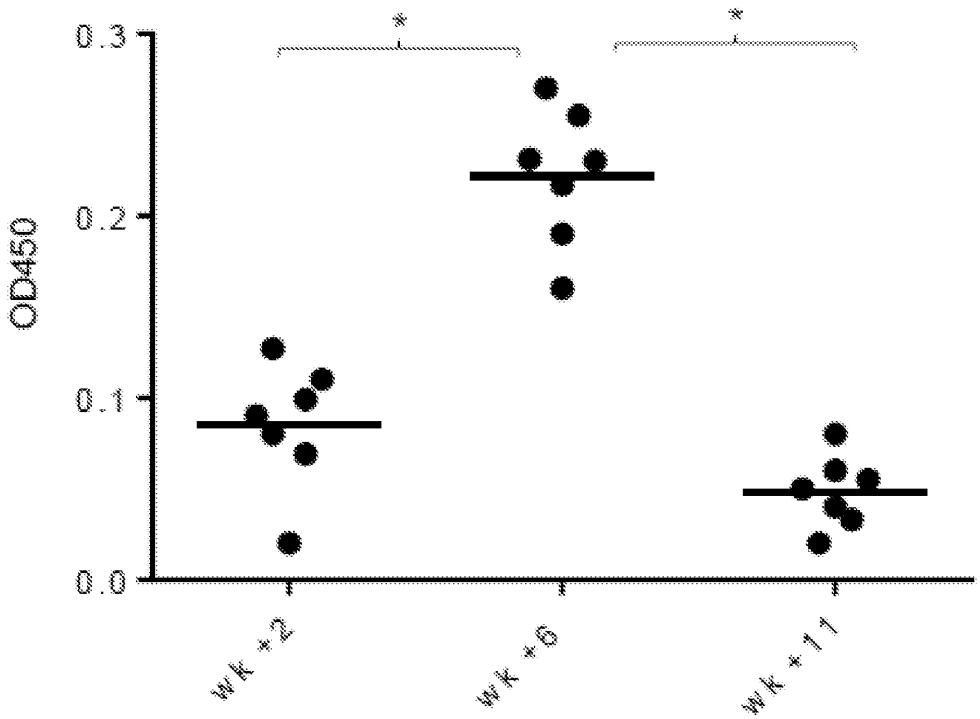


FIG. 86A

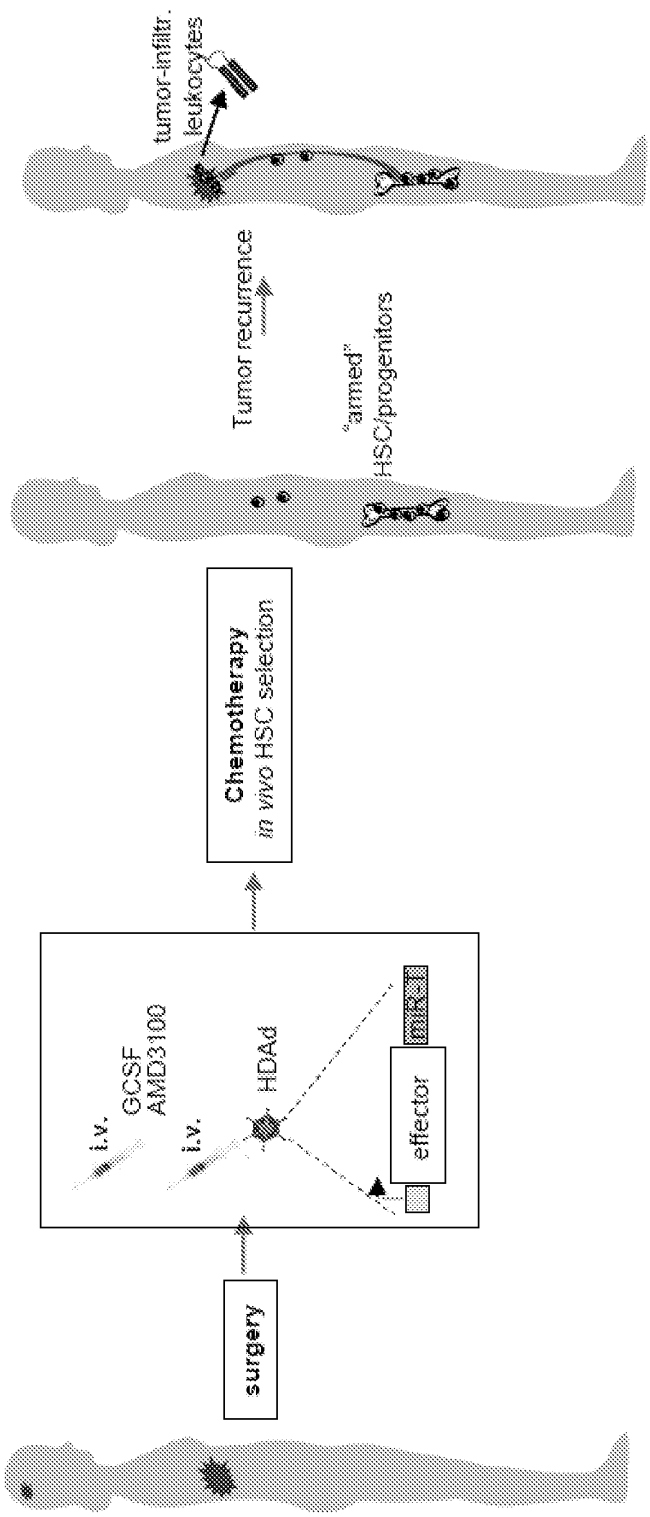

surgery
i.v.
GCSF
AMD3100
i.v.
HDAd
miR-1
effector
Chemotherapy
in vivo HSC selection
"armed"
HSC/progenitors
Tumor recurrence
tumor-infiltr.
leukocytes

FIG. 86B

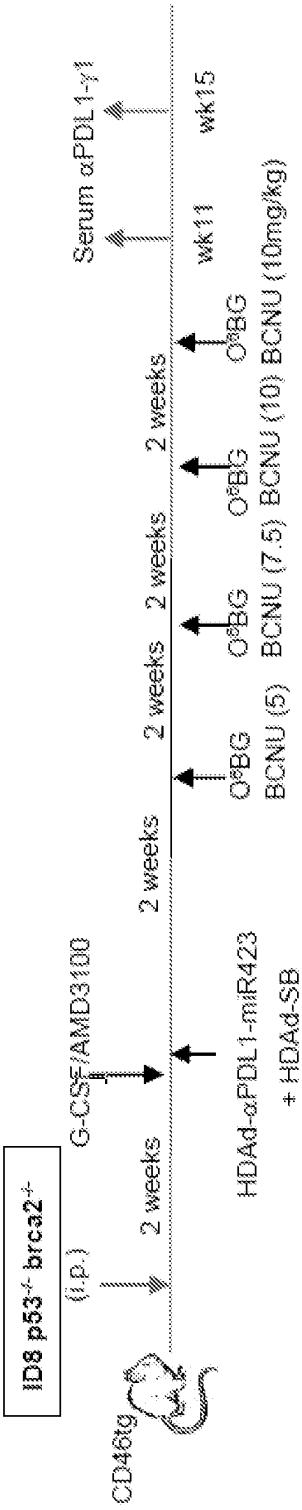

ID8 p53-/- brca2-/-
(i.p.)
CD46tg
2 weeks
G-CSF/AMD3100
HDAd-αPDL1-miR423
+ HDAd-SB
2 weeks
O6BG
BCNU (5)
2 weeks
O6BG
BCNU (7.5)
2 weeks
O6BG
BCNU (10)
2 weeks
O6BG
BCNU (10mg/kg)
wk11
Serum αPDL1-γ1
wk15

FIG. 86C

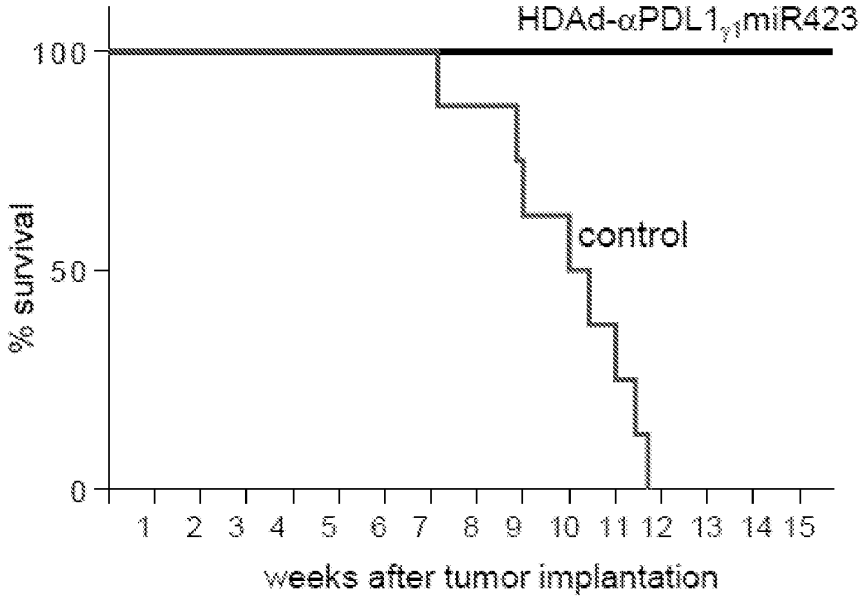


FIG. 86D

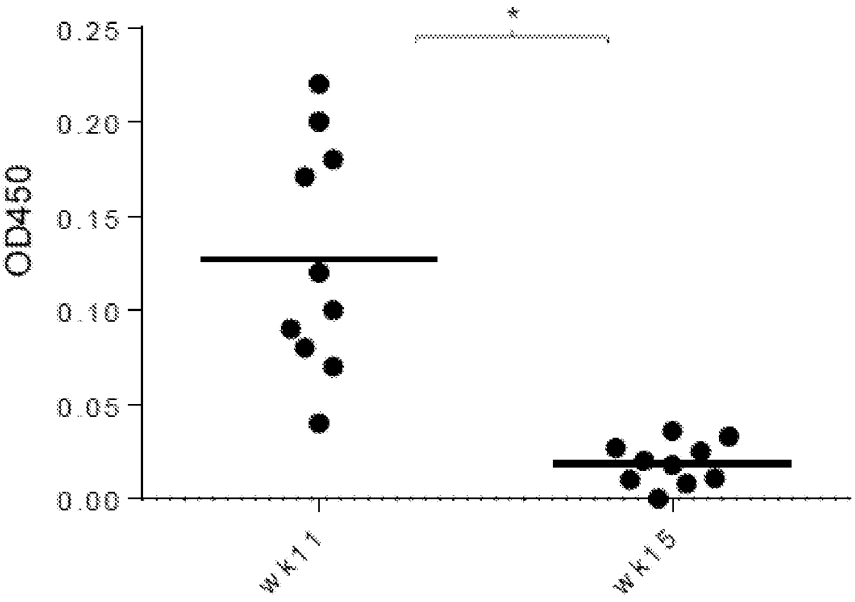


FIG. 87A

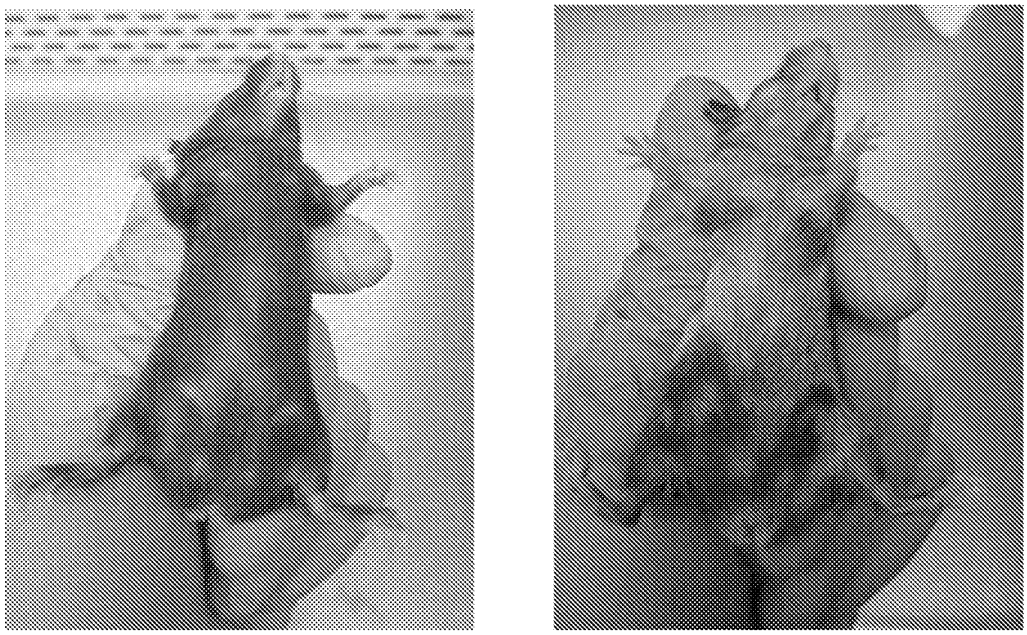


FIG. 87B

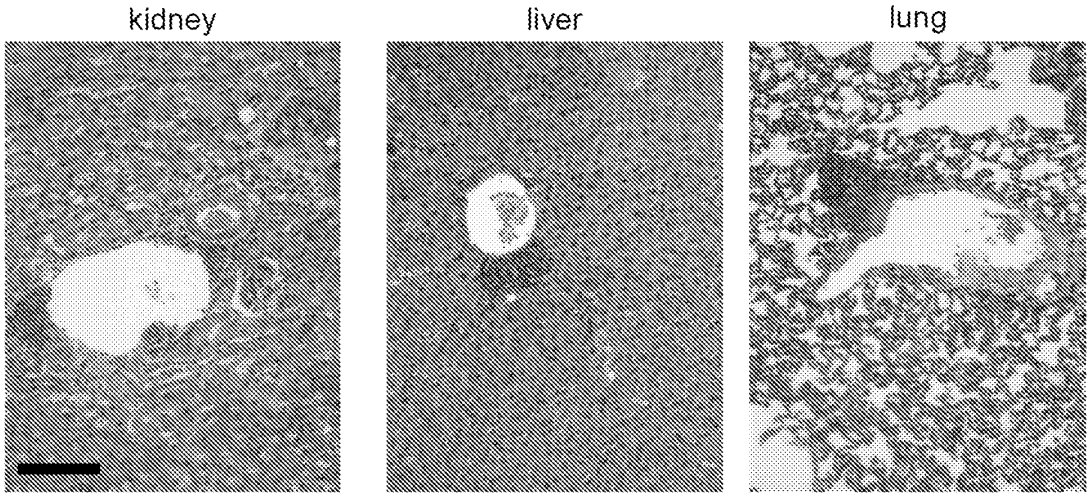


FIG. 88A

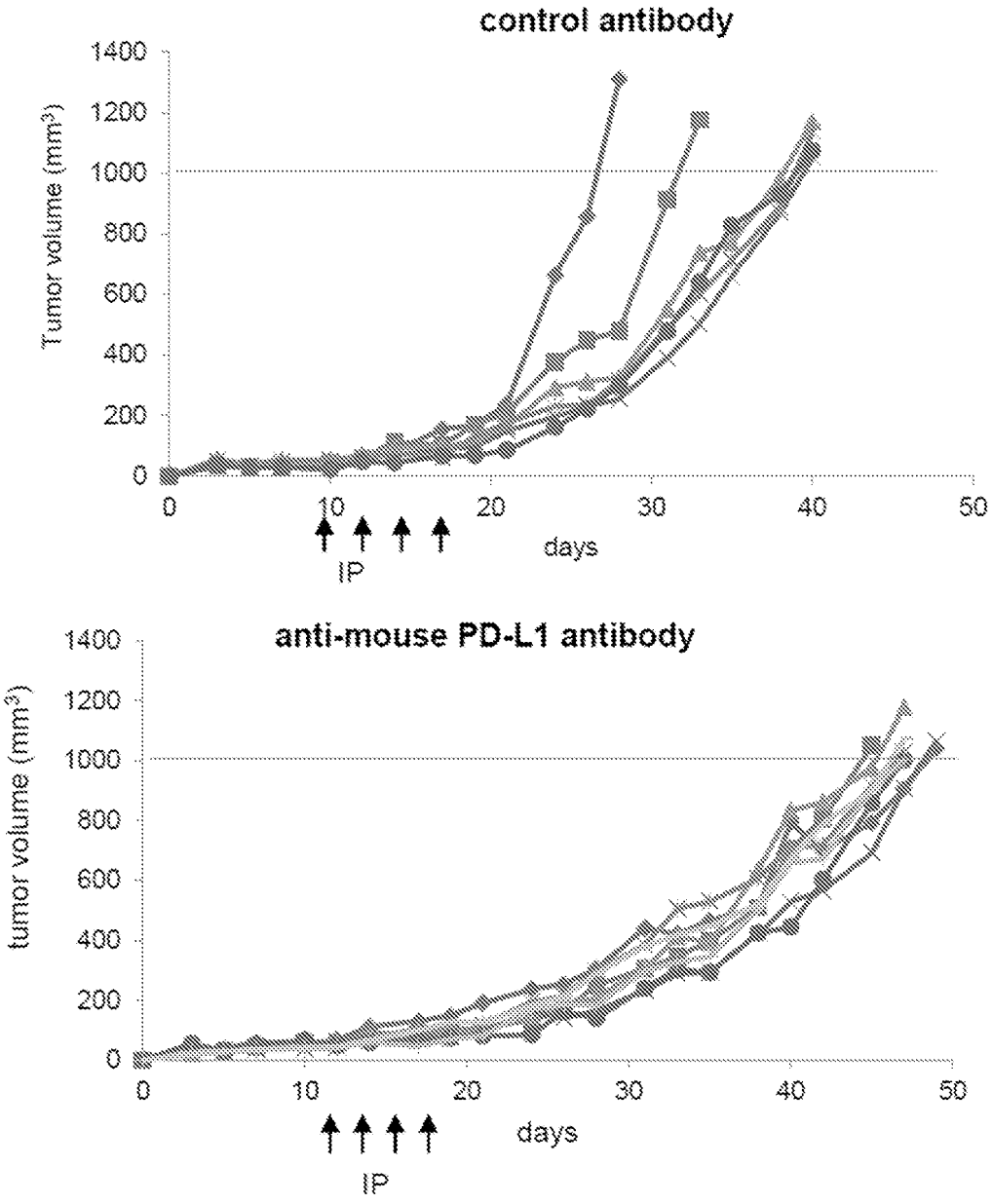

control antibody
Tumor volume (mm3)
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
50
days
IP
anti-mouse PD-L1 antibody
tumor volume (mm3)
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
50
days
IP

FIG. 88B

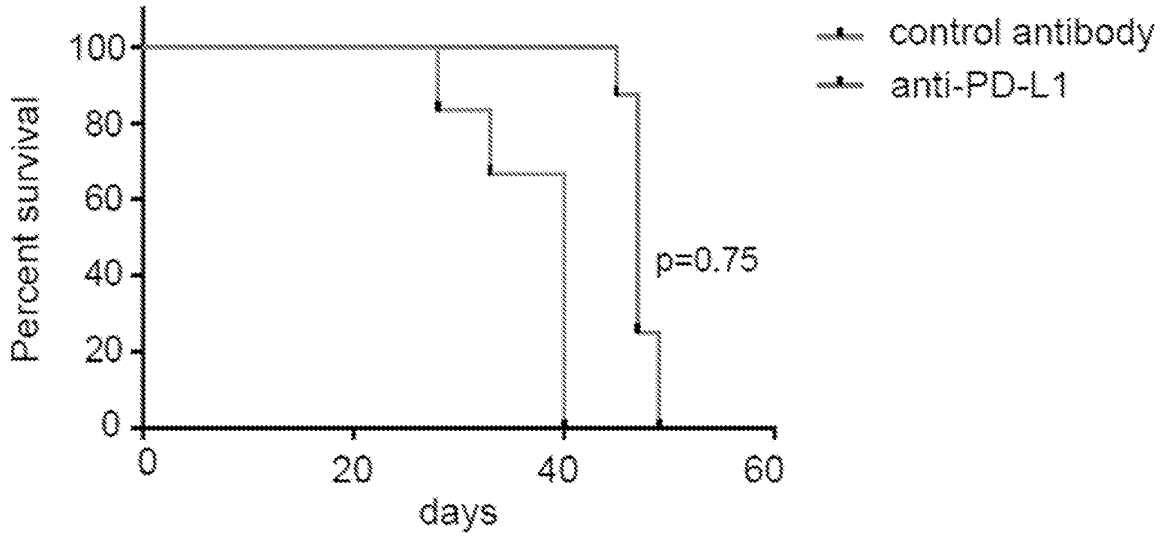

control antibody
anti-PD-L1
Percent survival
100
80
60
40
20
0
0
20
40
60
days
p=0.75

FIG. 88C

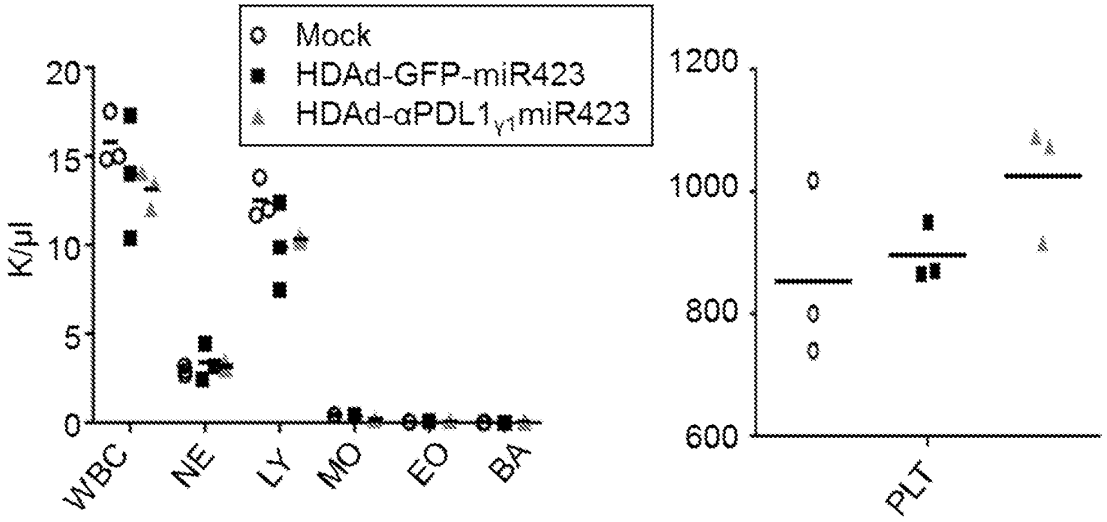


FIG. 88D

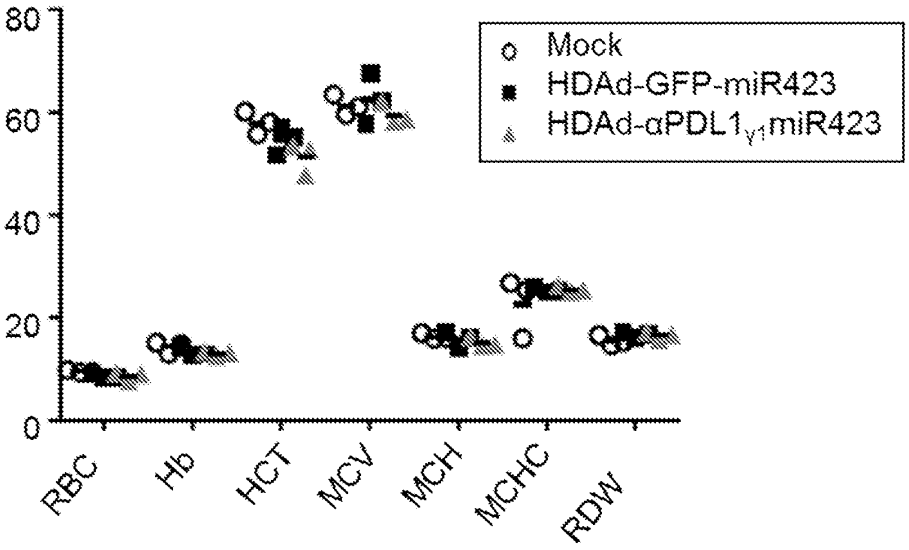


FIG. 88E

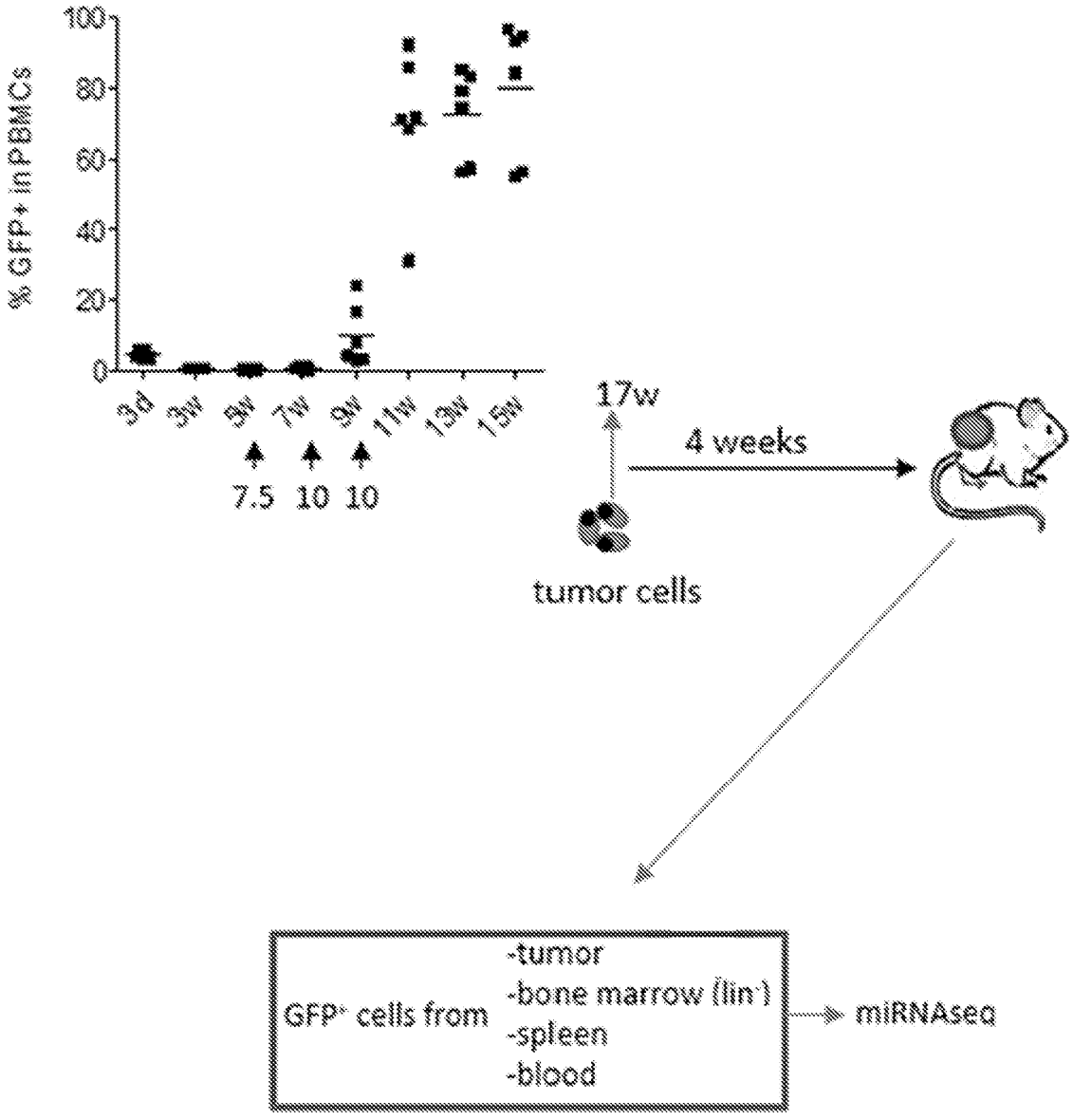
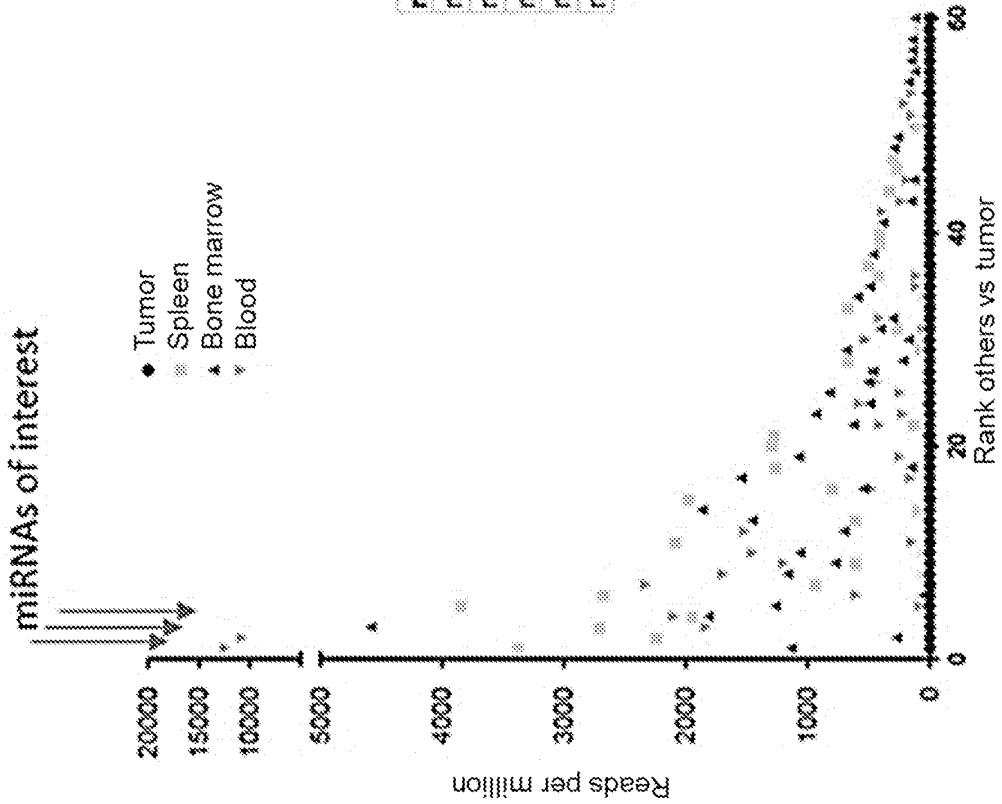

% GFP+ in PBMCs
100
80
60
40
20
0
3d
3w
5w
7w
9w
11w
13w
15w
7.5
10
10
17w
4 weeks
tumor cells
GFP+ cells from
-tumor
-bone marrow (lin-)
-spleen
-blood
miRNAseq

FIG. 88E cont'd



miRNA	Tumor	Spleen	BM	Blood
mir-423	1.00	7563.82	1263.47	1350.56
mir-24-2	1.00	5019.65	302.65	11586.12
mir-181c	1.00	6065.20	5106.75	1970.61
mir-125a	1.00	8609.37	1408.71	83.07
mir-15a	1.00	4357.46	2023.19	2257.84

FIG. 88F

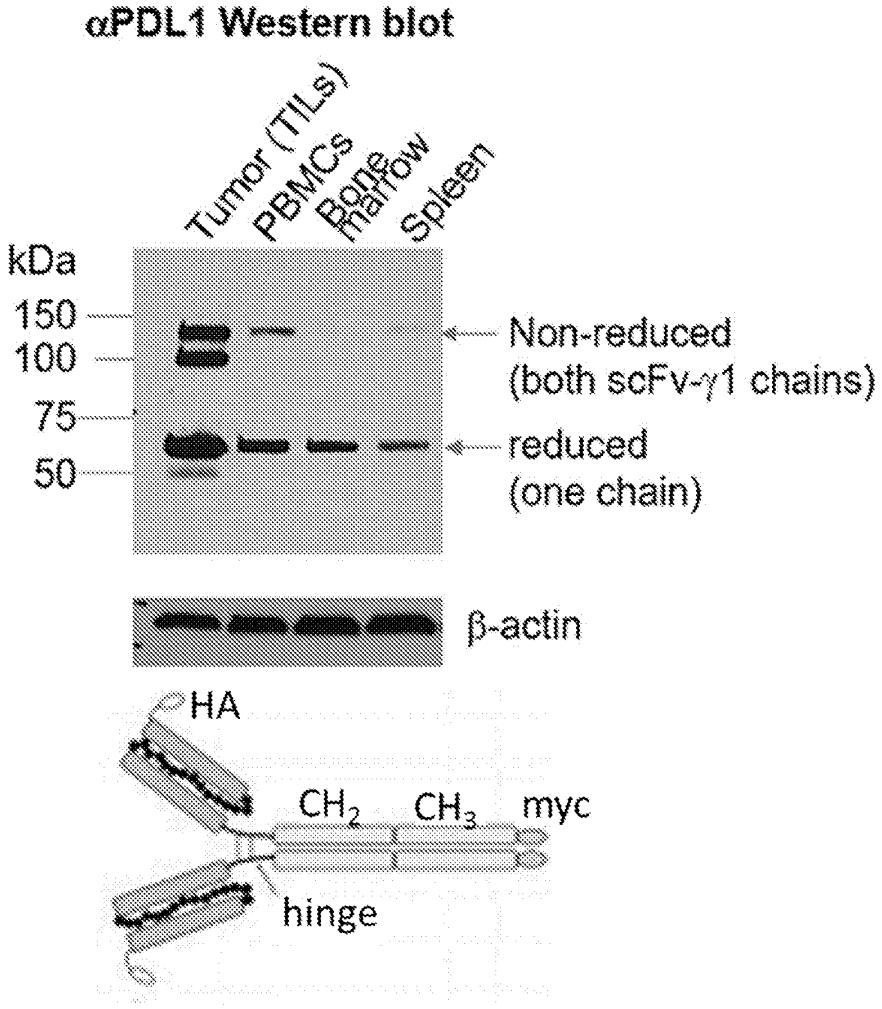

αPDL1 Western blot
Tumor (TILs)
PBMCs
Bone marrow
Spleen
kDa
150
100
75
50
Non-reduced
(both scFv-γ1 chains)
reduced
(one chain)
β-actin
HA
CH2
CH3
myc
hinge

FIG. 88F cont'd

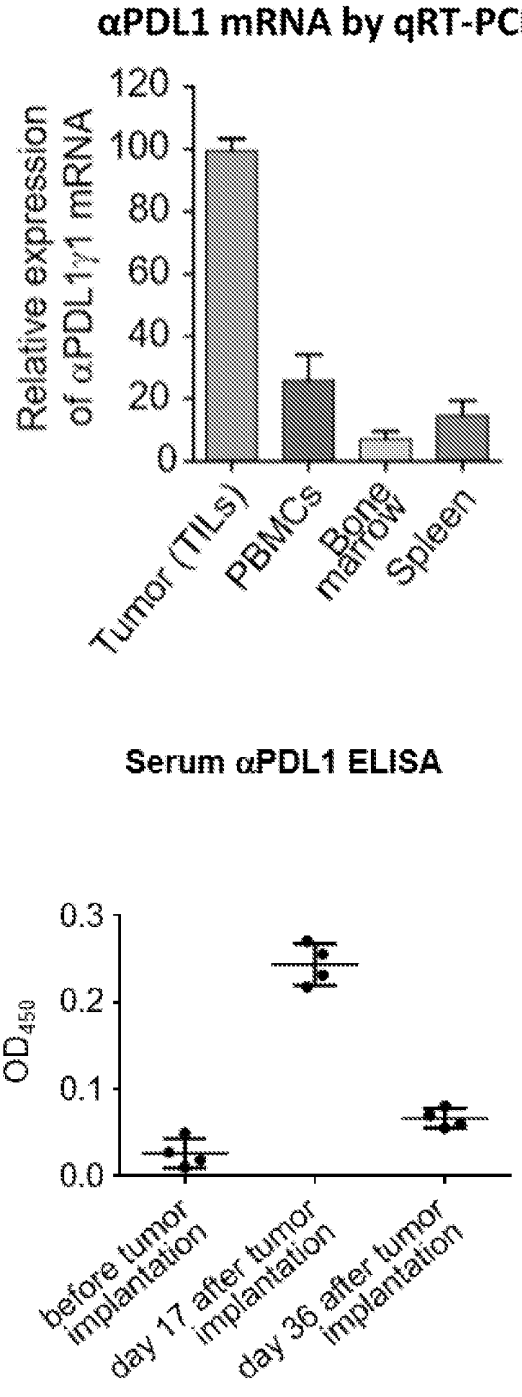


FIG. 88G

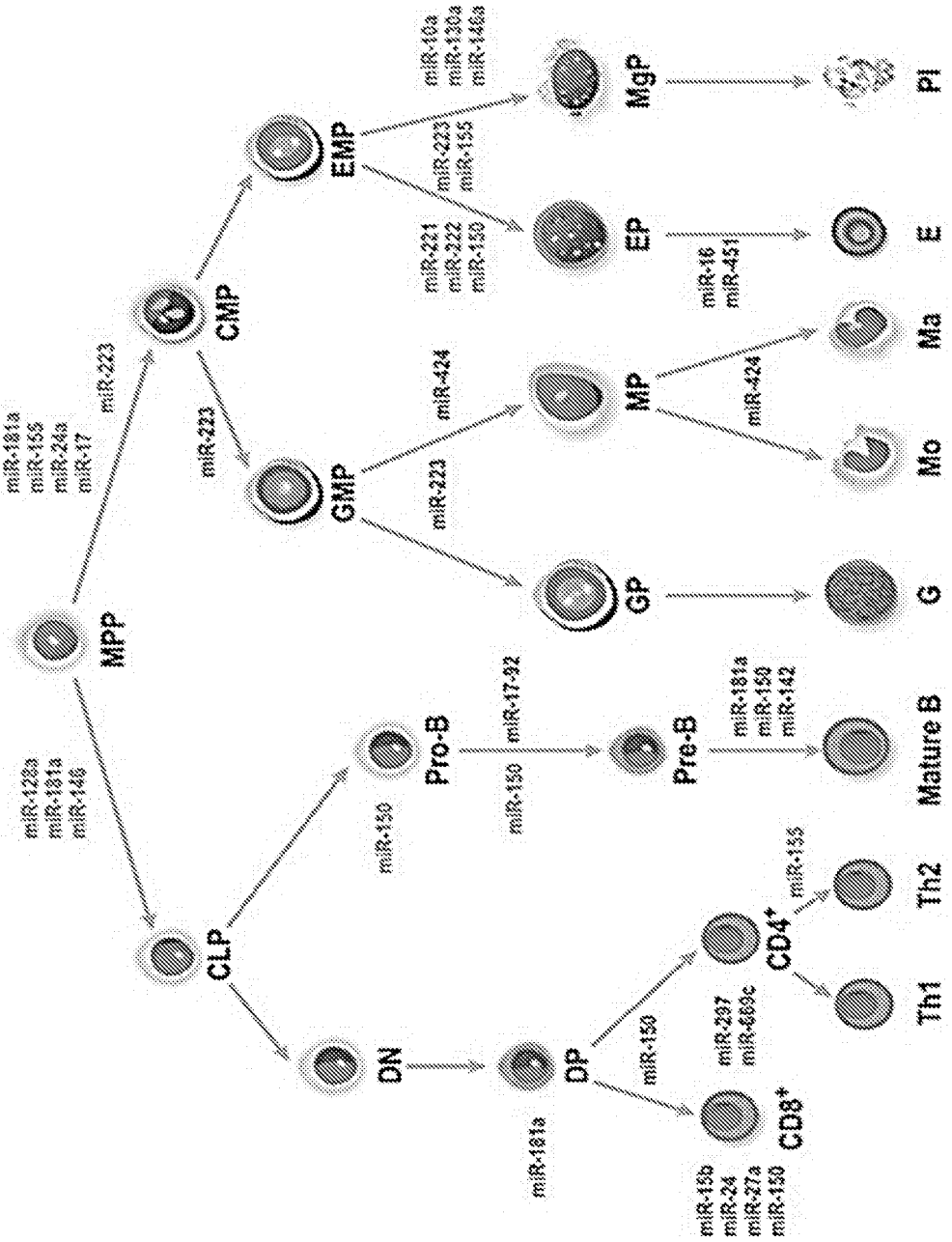

MPP
CMP
CLP
EMP
GMP
MgP
EP
MP
GP
Pro-B
Pre-B
DN
DP
CD4+
CD8+
Pl
E
Ma
Mo
G
Mature B
Th2
Th1
miR-128a
miR-181a
miR-155
miR-24a
miR-17
miR-223
miR-128a
miR-181a
miR-146
miR-223
miR-223
miR-424
miR-223
miR-10a
miR-130a
miR-146a
miR-223
miR-155
miR-221
miR-222
miR-150
miR-16
miR-451
miR-424
miR-150
miR-17-92
miR-150
miR-181a
miR-150
miR-142
miR-150
miR-181a
miR-150
miR-297
miR-669c
miR-135
miR-15b
miR-24
miR-27a
miR-150

FIG. 88G cont'd

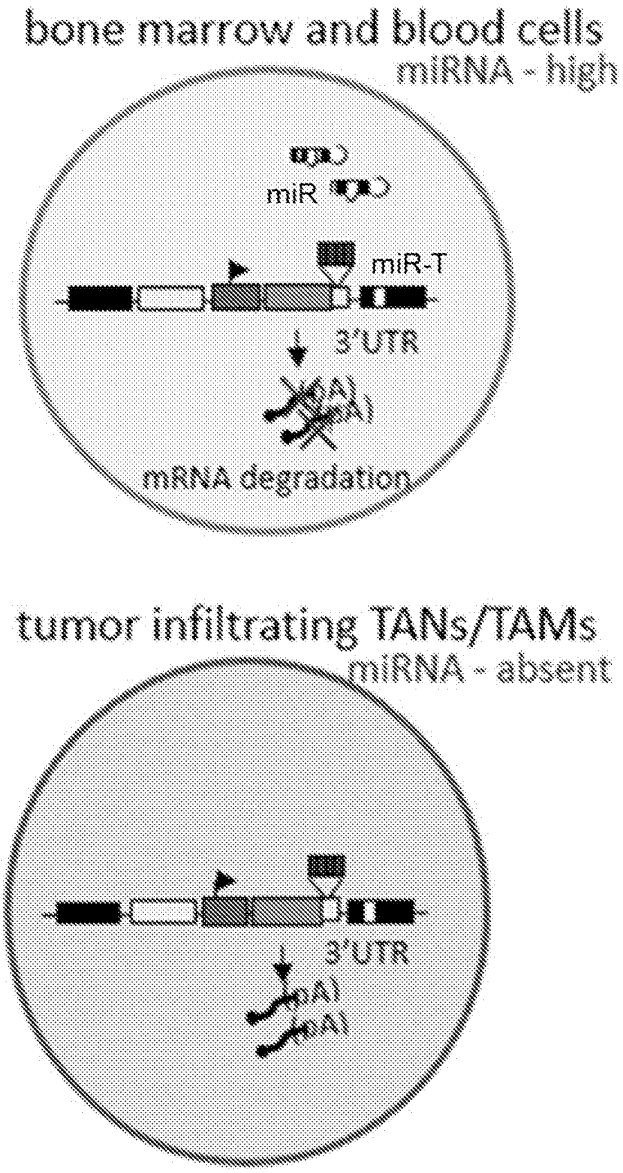


FIG. 88H

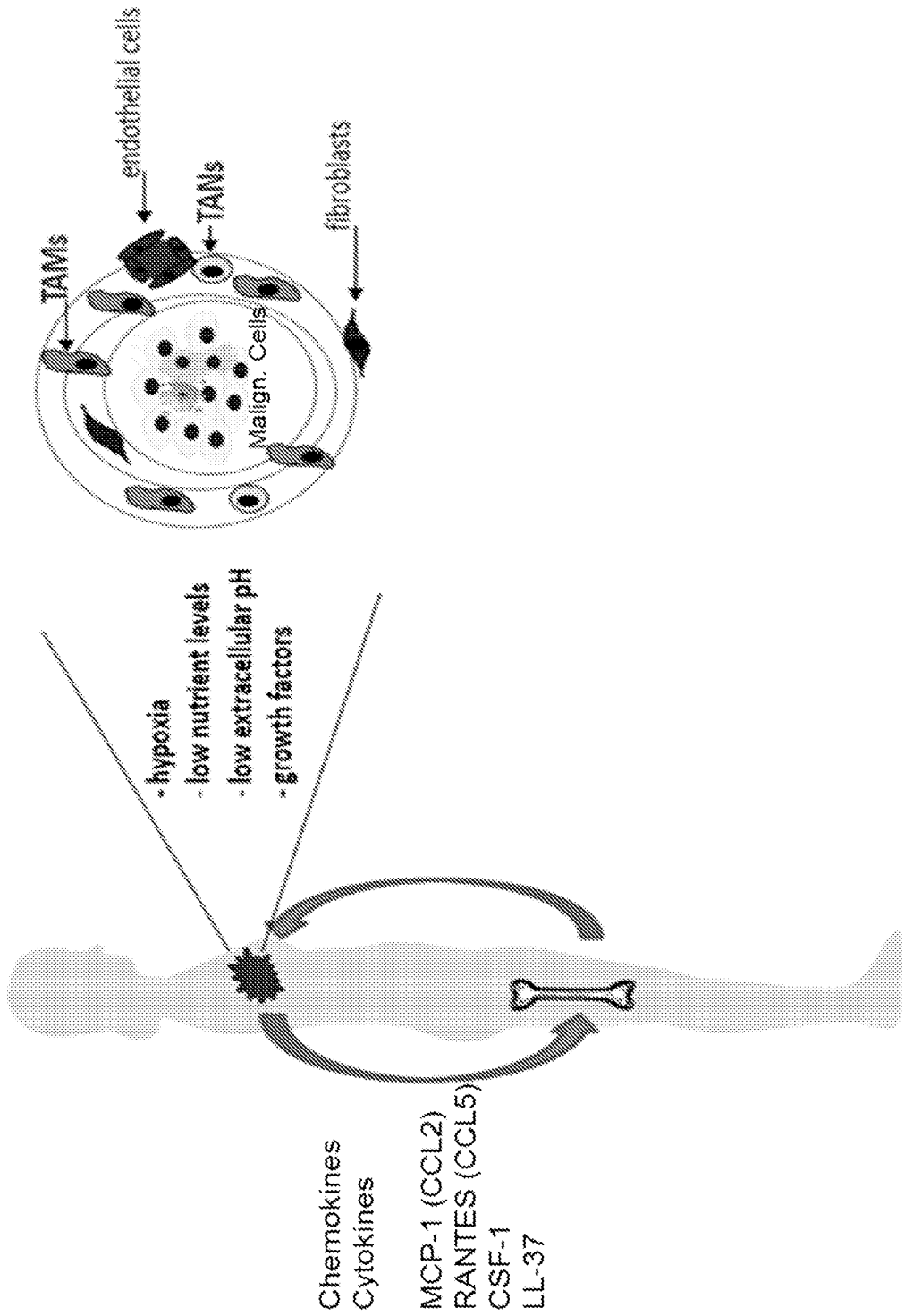

endothelial cells
TANs
fibroblasts
TAMs
Malign. Cells
- hypoxia
- low nutrient levels
- low extracellular pH
- growth factors
Chemokines
Cytokines
MCP-1 (CCL2)
RANTES (CCL5)
CSF-1
LL-37

FIG. 88H cont'd

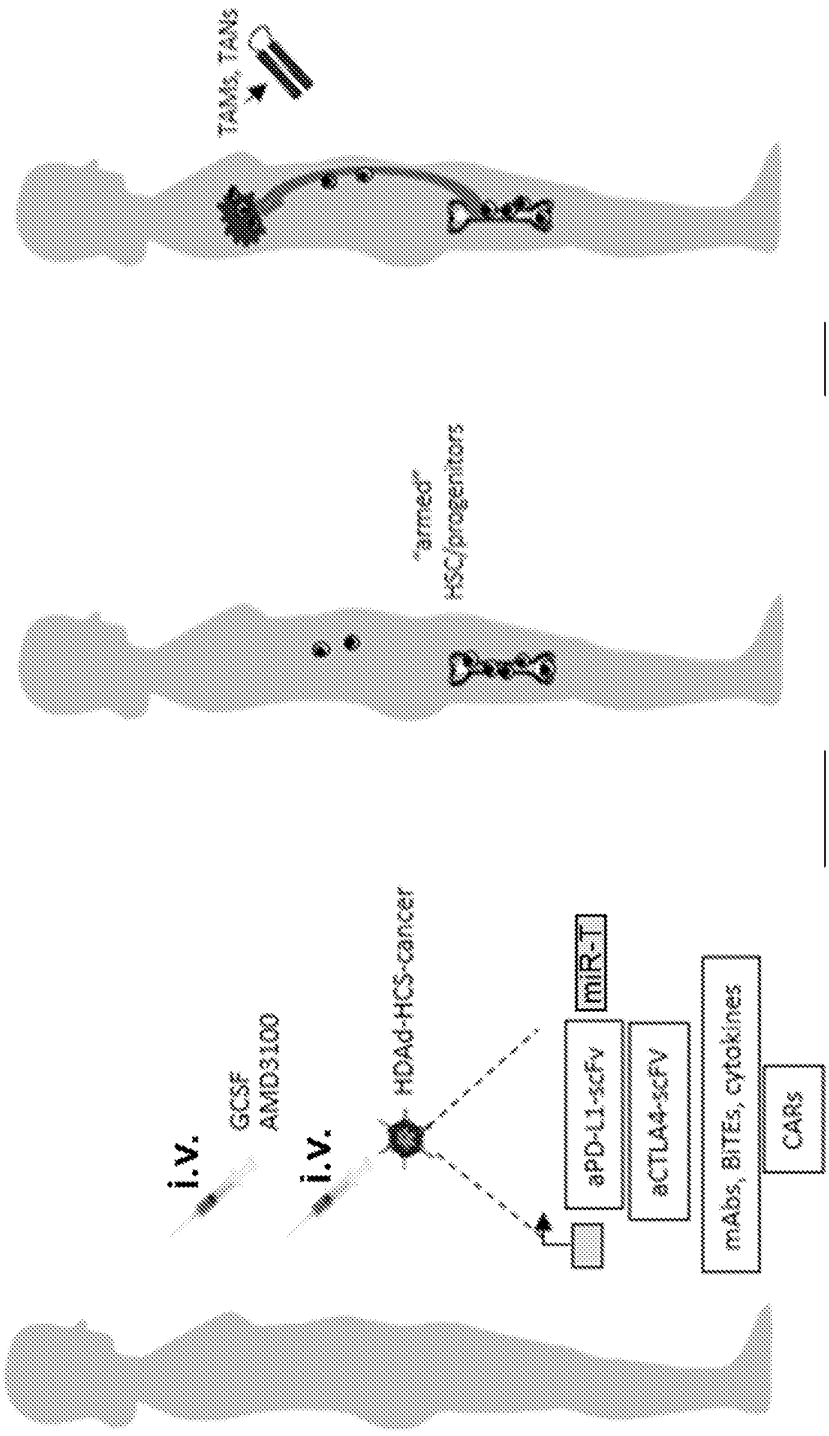
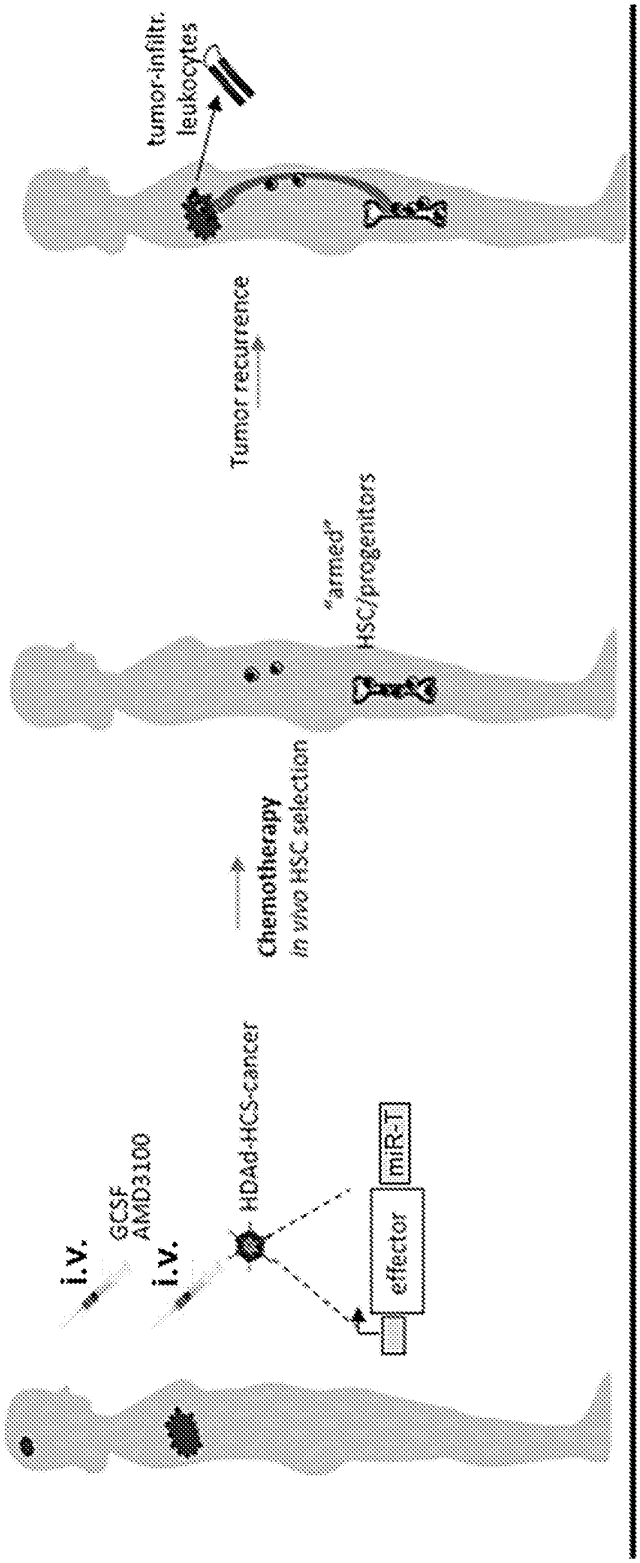


FIG. 88H cont'd



Biopsies:
→immunophenotype known→ select optimal immune-effector gene
→neo-antigens known→ personalized CARs/BiTEs

-difficult to treat full-fledged tumors

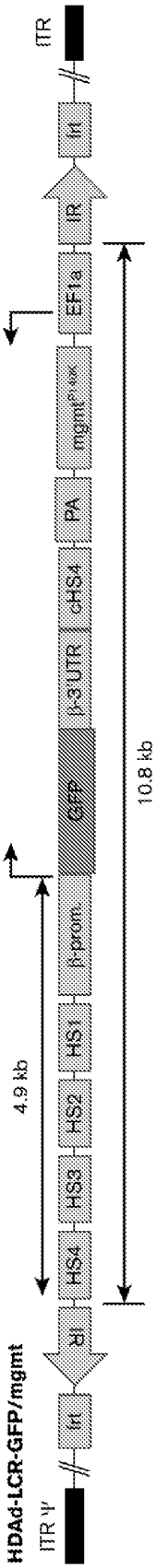


FIG. 89A

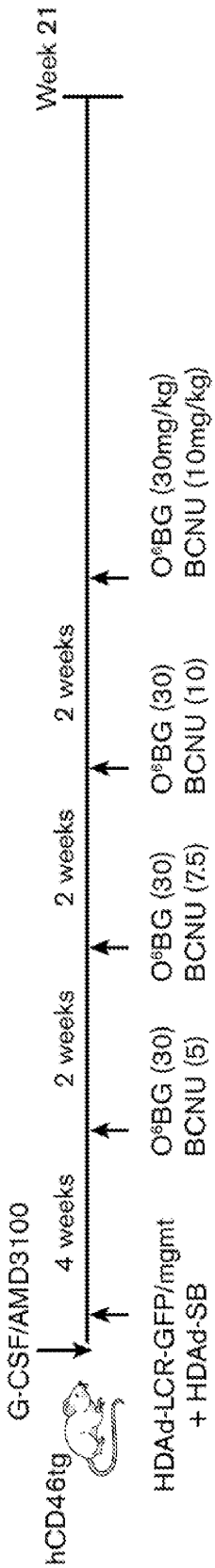


FIG. 89B

FIG. 89C

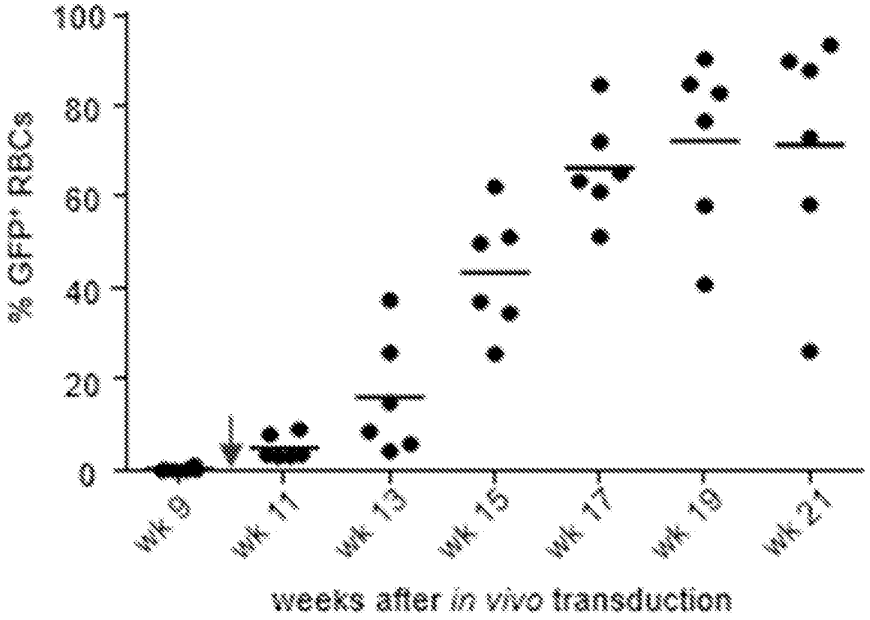


FIG. 89D

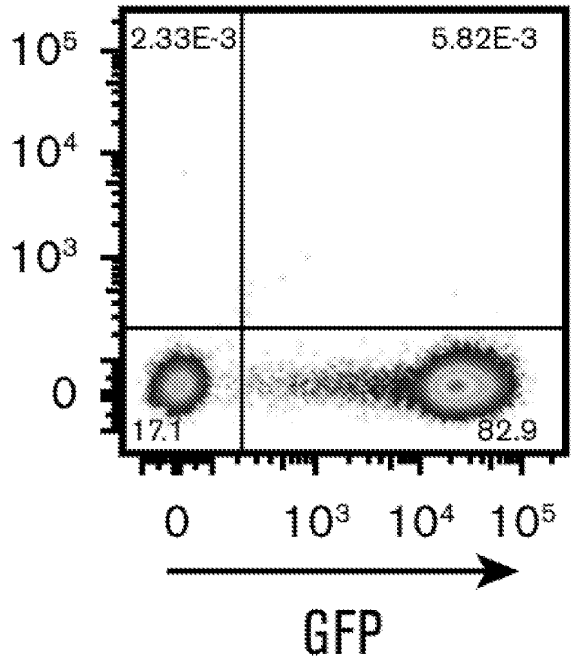


FIG. 89E

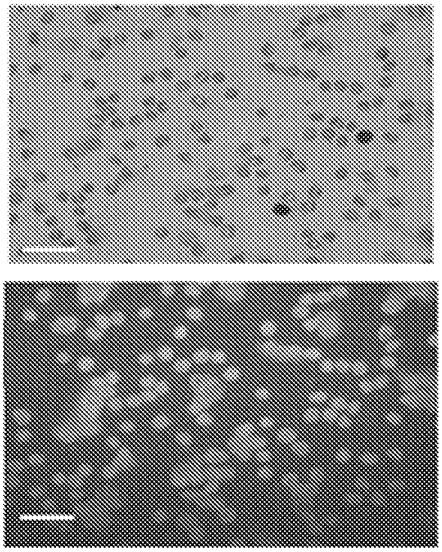


FIG. 89F

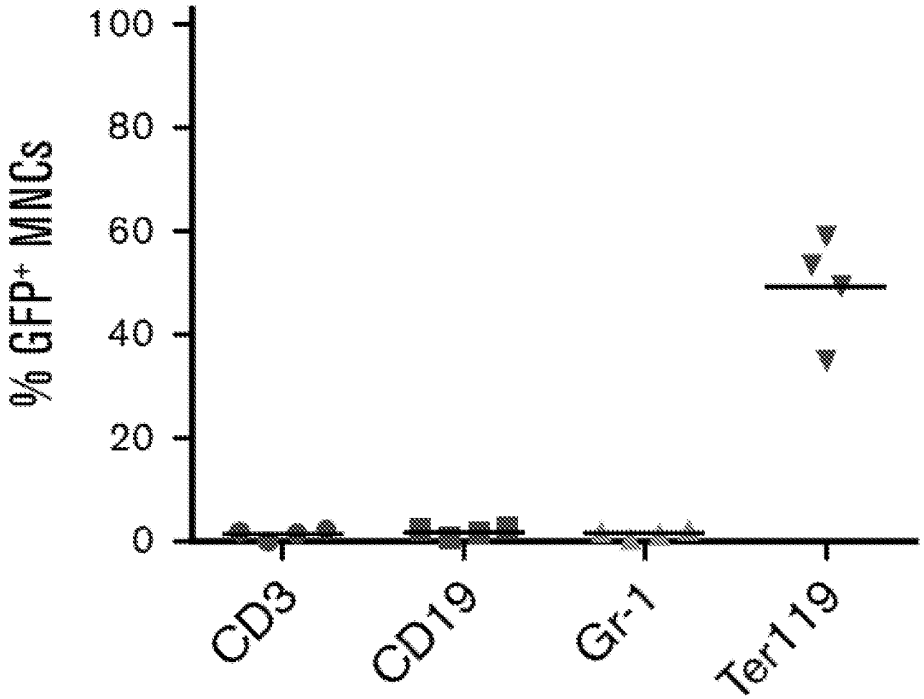


FIG. 89G

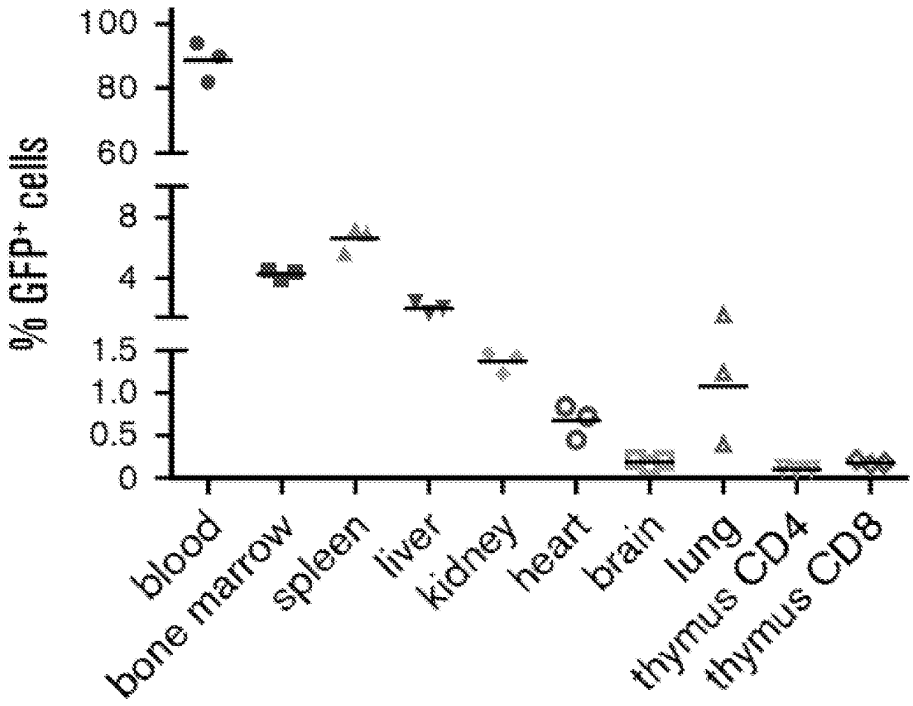


FIG. 89H

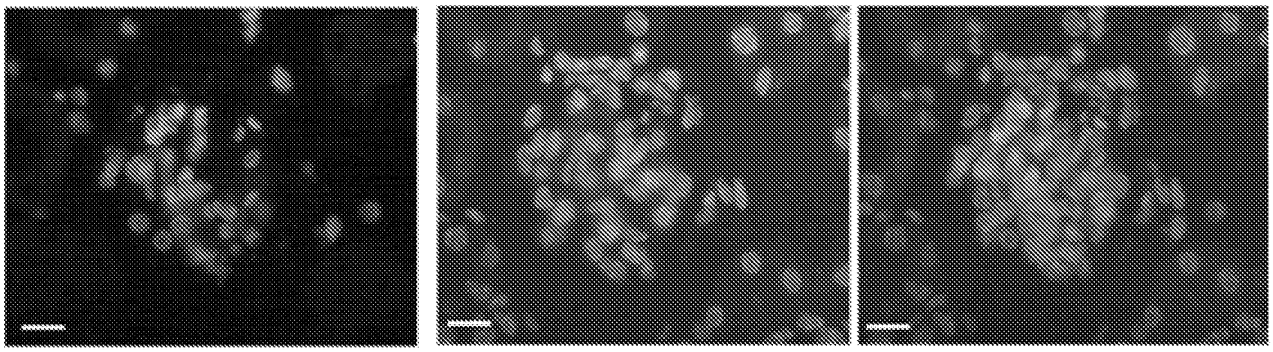


FIG. 90A

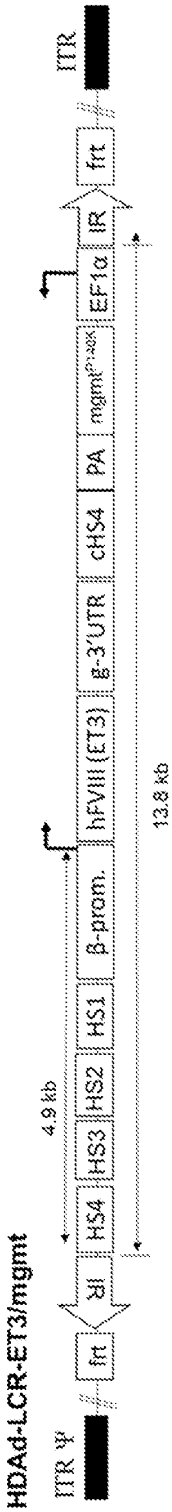


FIG. 90B

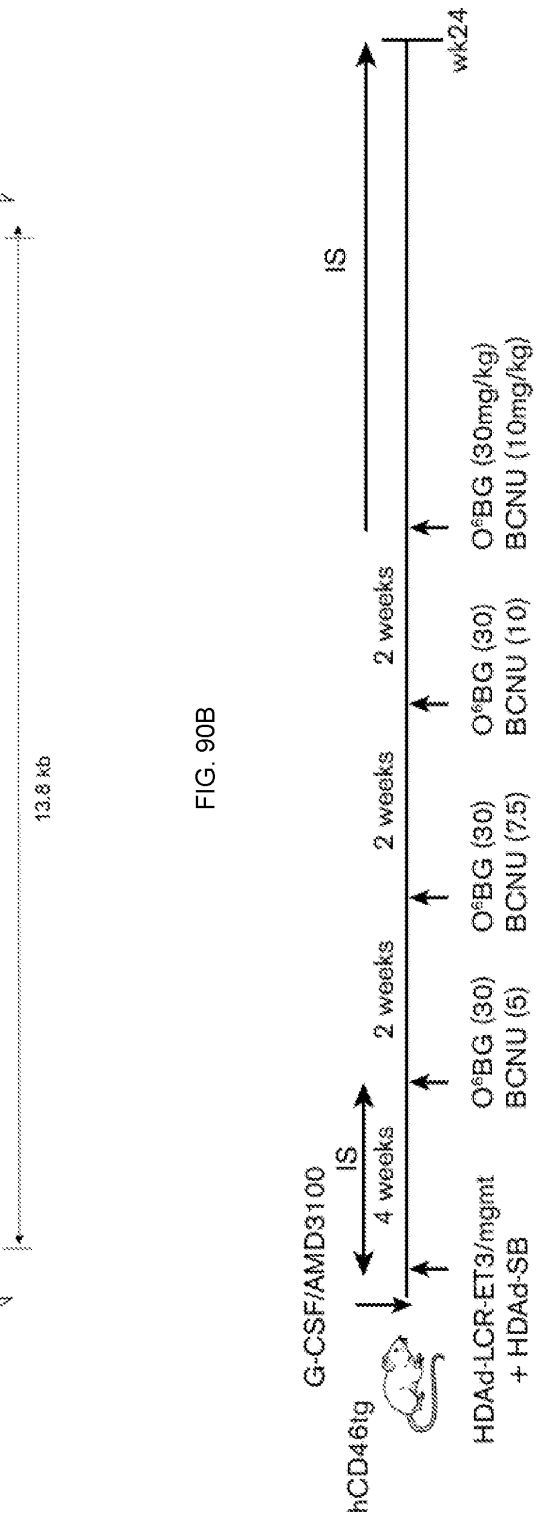


FIG. 90C

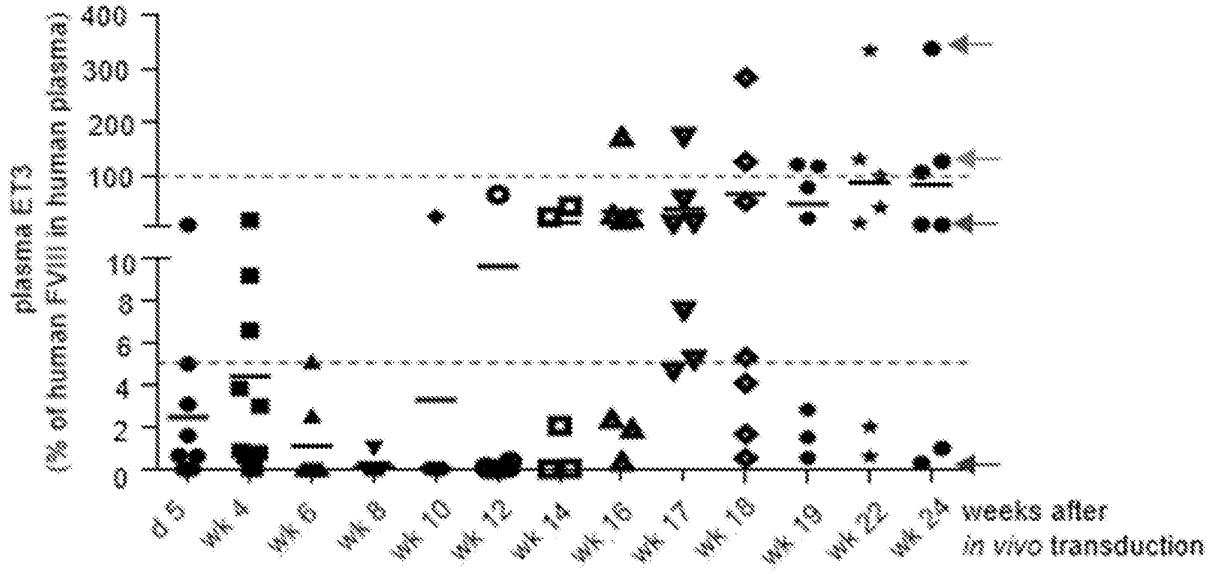


FIG. 90D

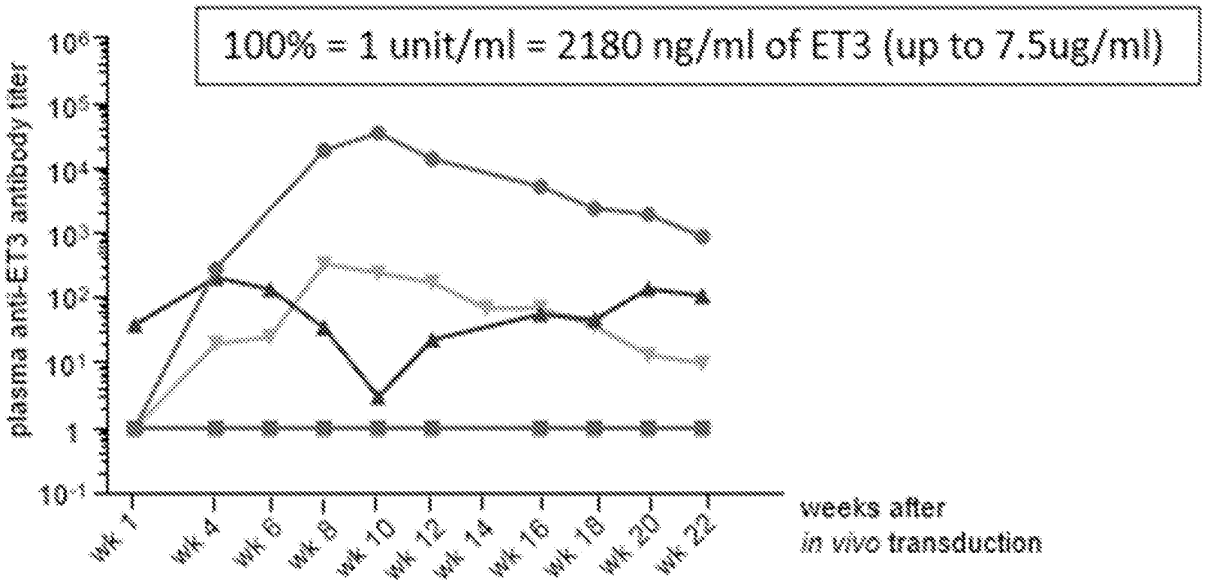


FIG. 90E

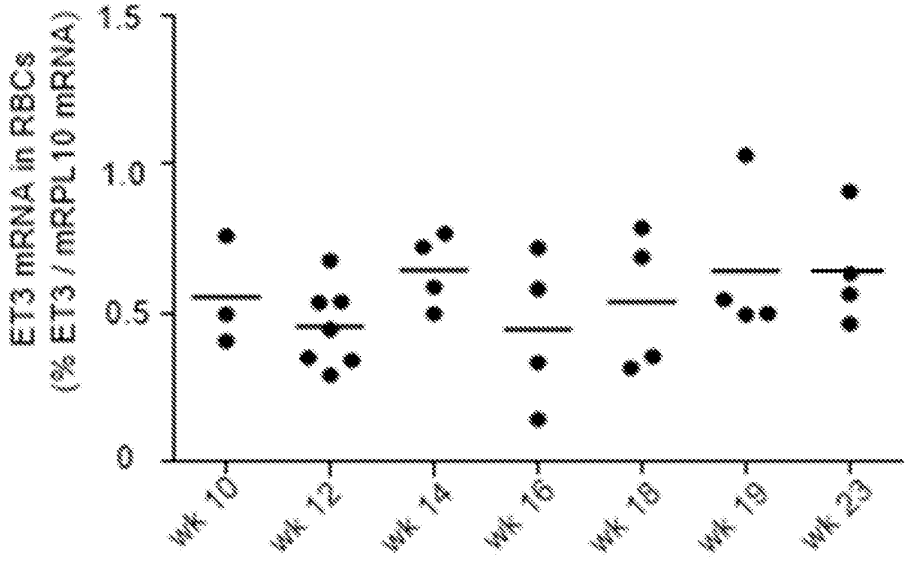


FIG. 90F

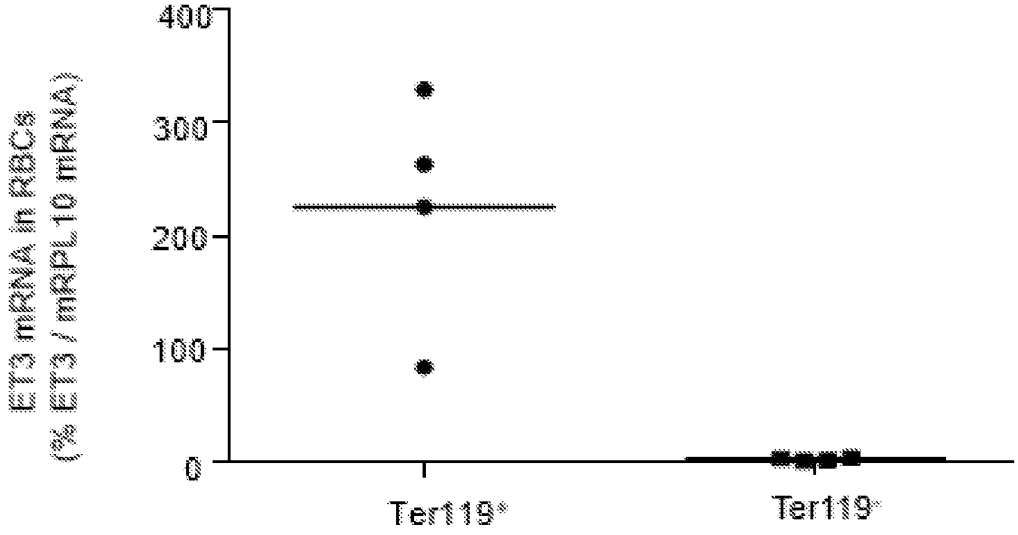


FIG. 90G

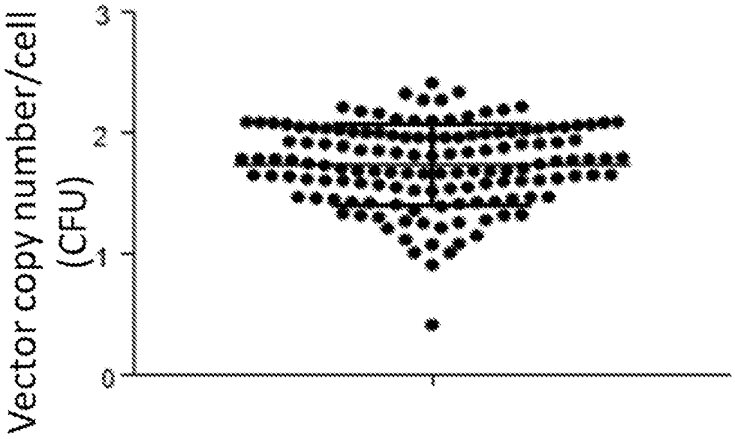


FIG. 90H

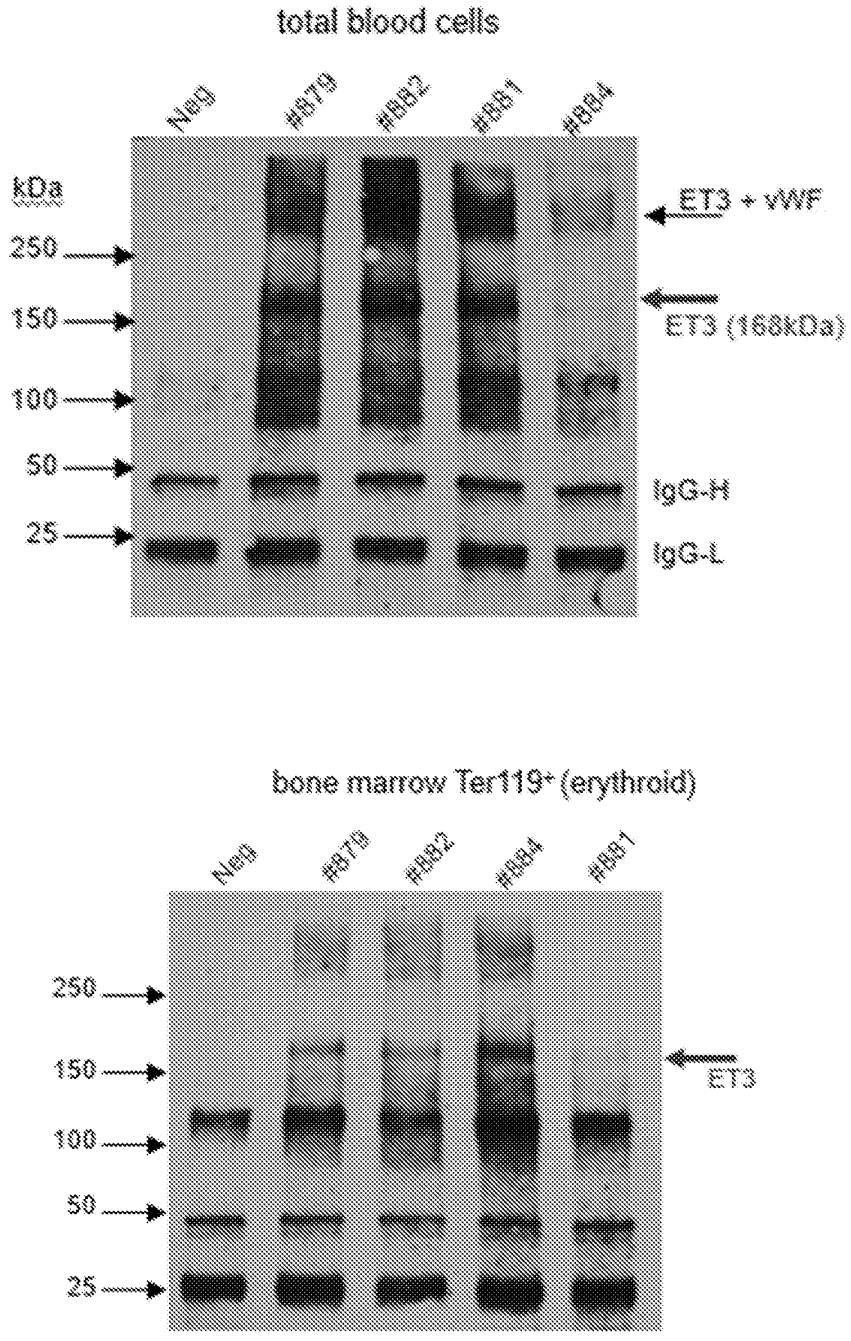

total blood cells
Neg
#879
#882
#881
#884
kDa
250
150
100
50
25
ET3 + vWF
ET3 (168kDa)
IgG-H
IgG-L
bone marrow Ter119+ (erythroid)
Neg
#879
#882
#884
#881
250
150
100
50
25
ET3

FIG. 90H cont'd

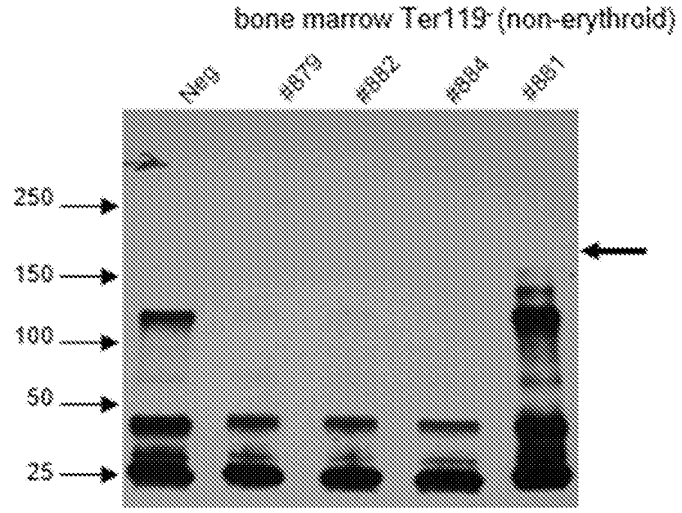


FIG. 90I

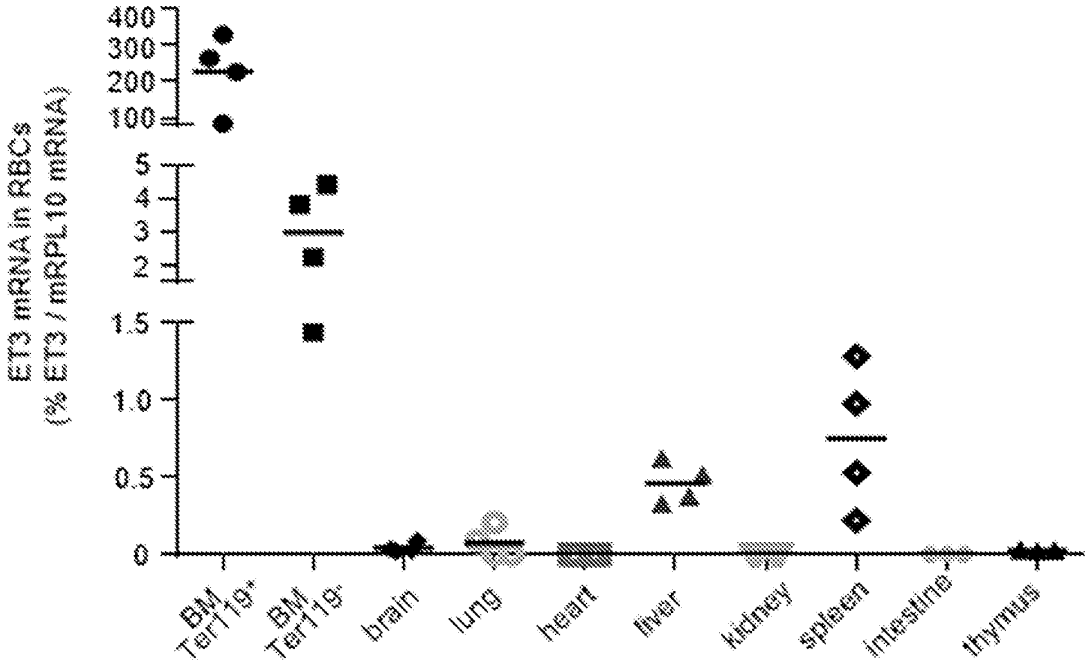


FIG. 91A

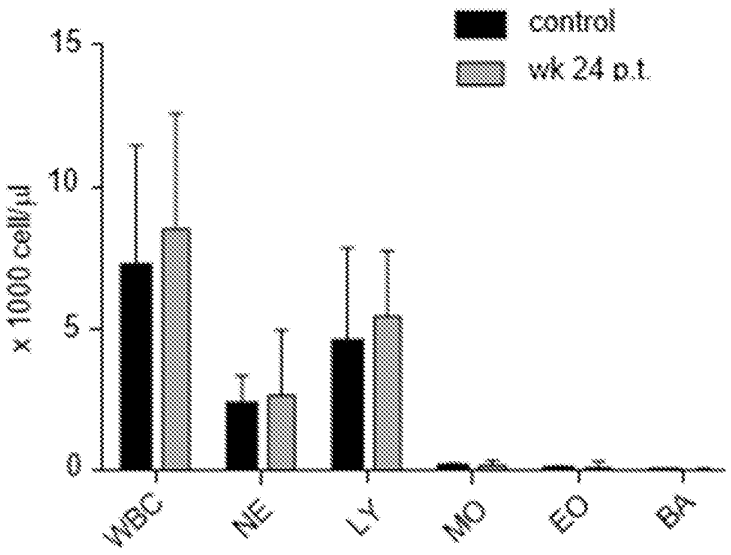


FIG. 91B

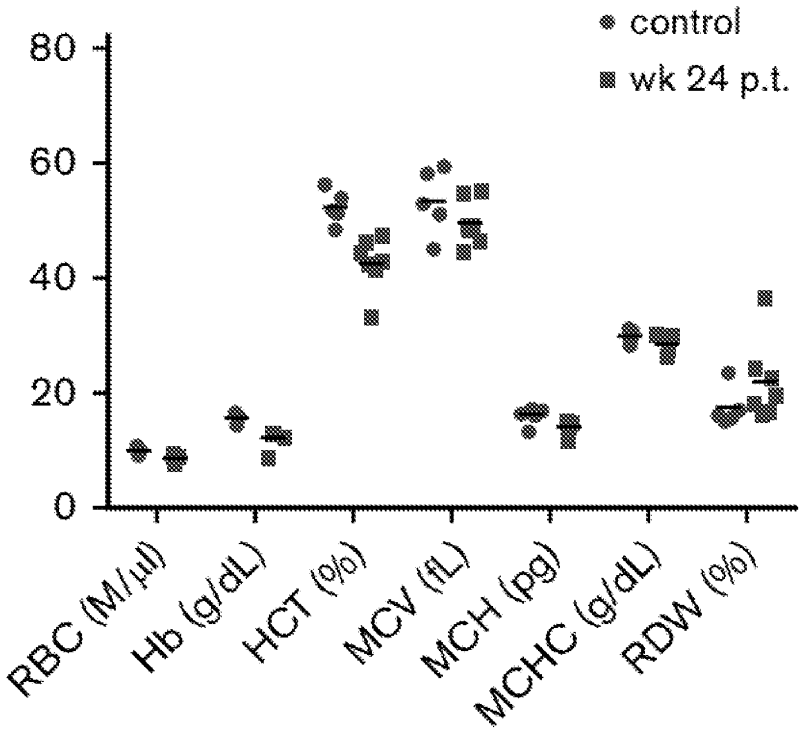


FIG. 91C

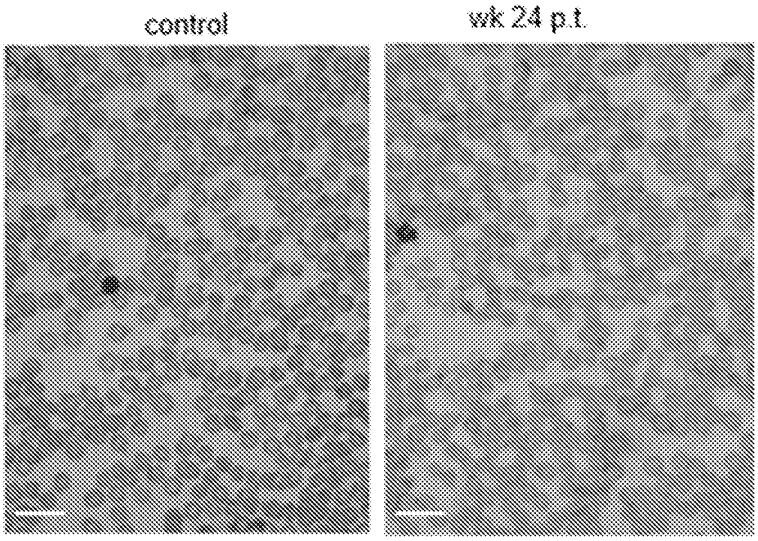


FIG. 91D

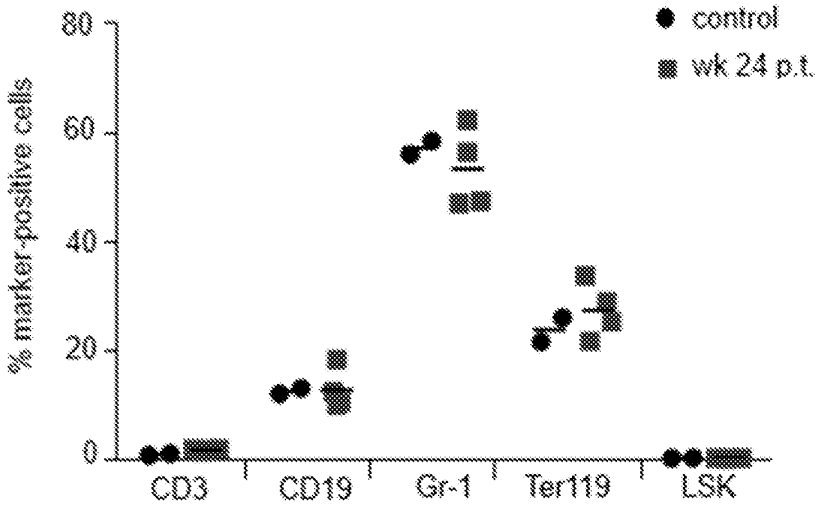


FIG. 92A

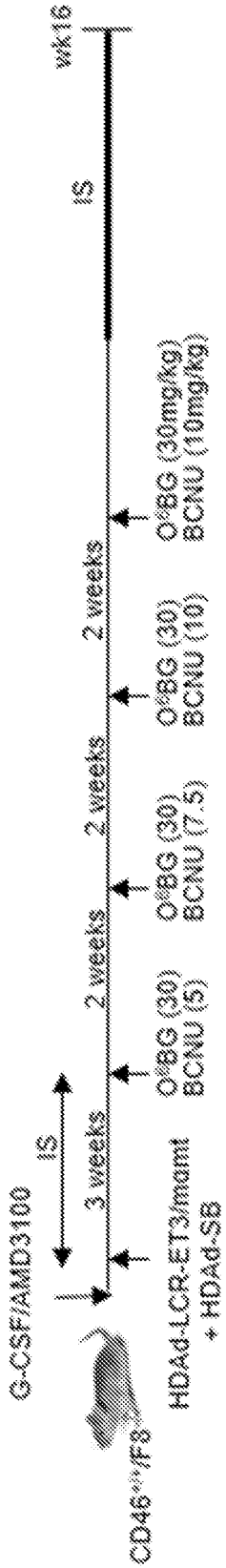


FIG. 92B

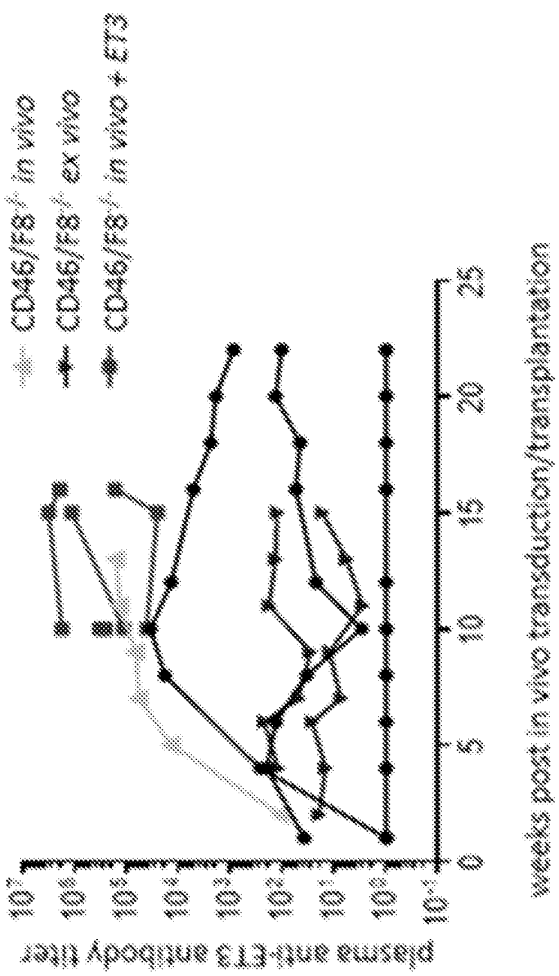


FIG. 92C

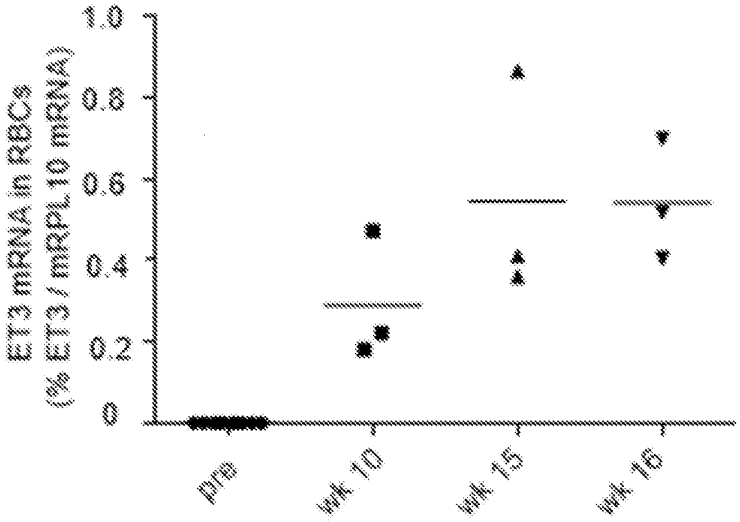


FIG. 92D

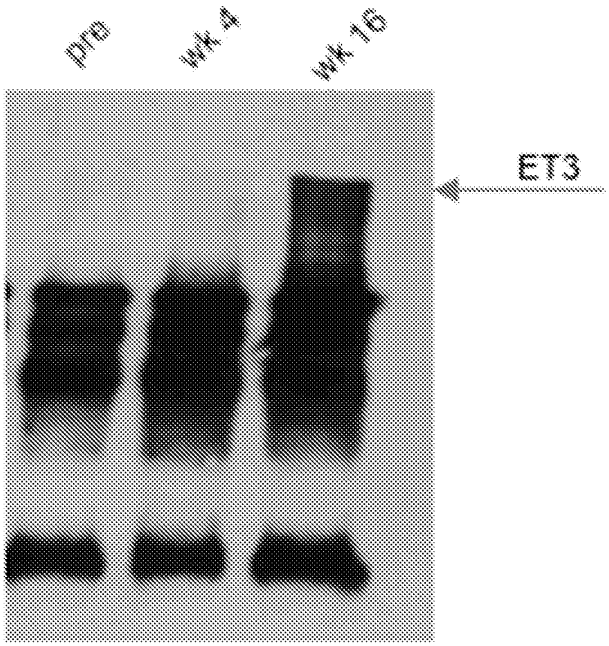


FIG. 92E

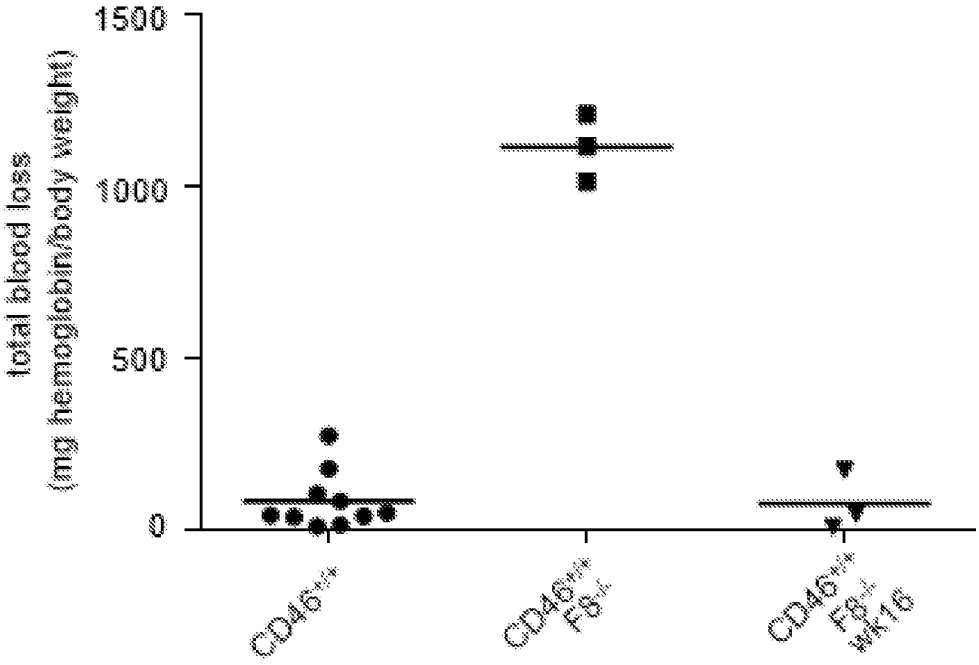


FIG. 92F

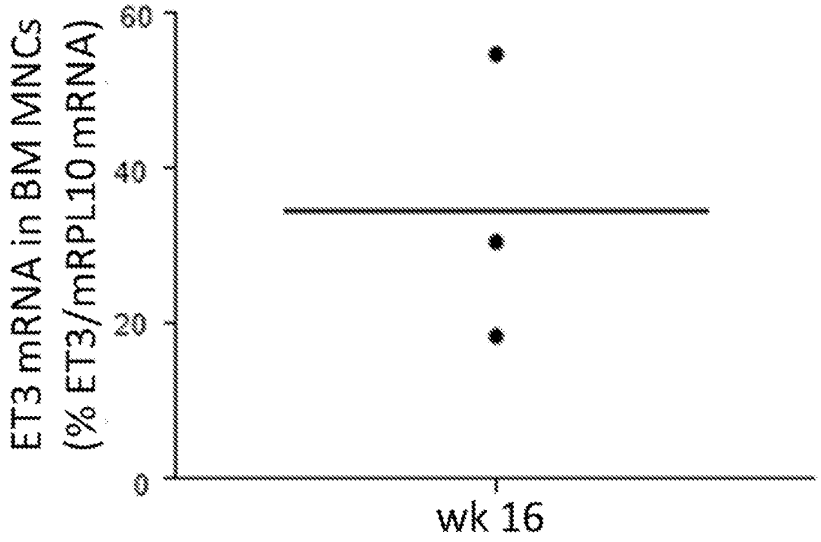


FIG. 92G

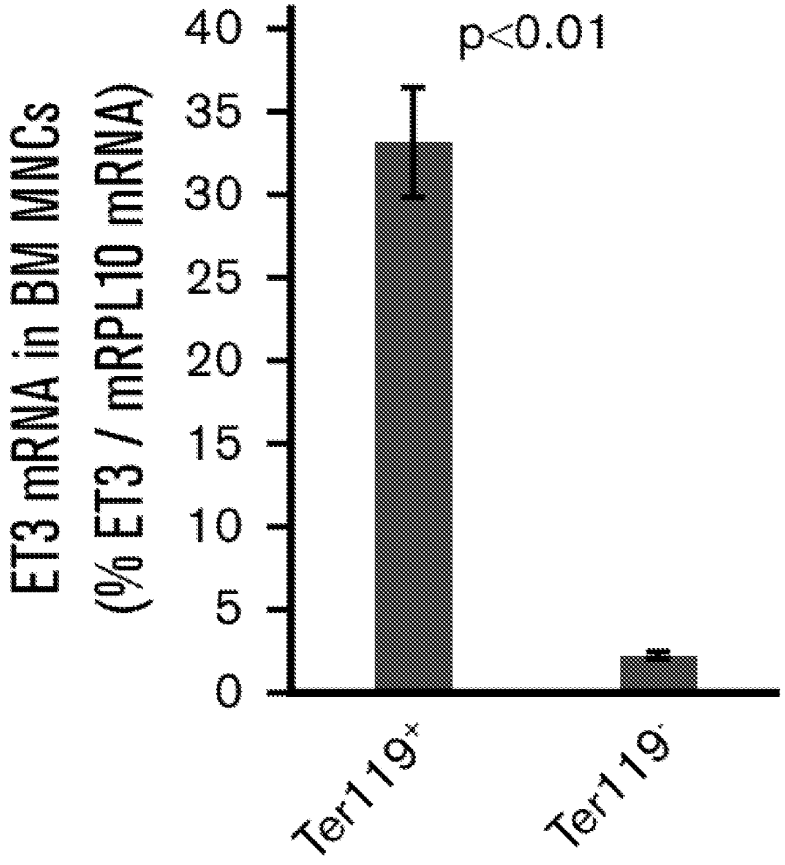

ET3 mRNA in BM MNCs
(% ET3 / mRPL10 mRNA)
40
35
30
25
20
15
10
5
0
p<0.01
Ter119+
Ter119-

FIG. 93A

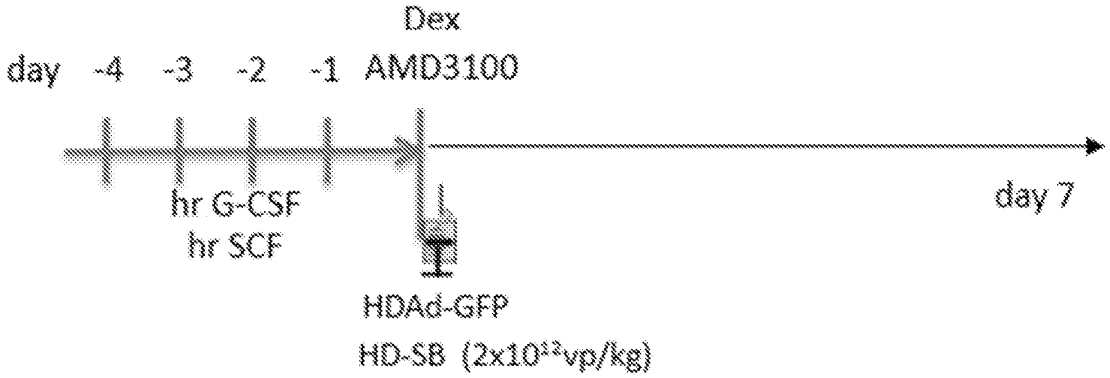


FIG. 93B

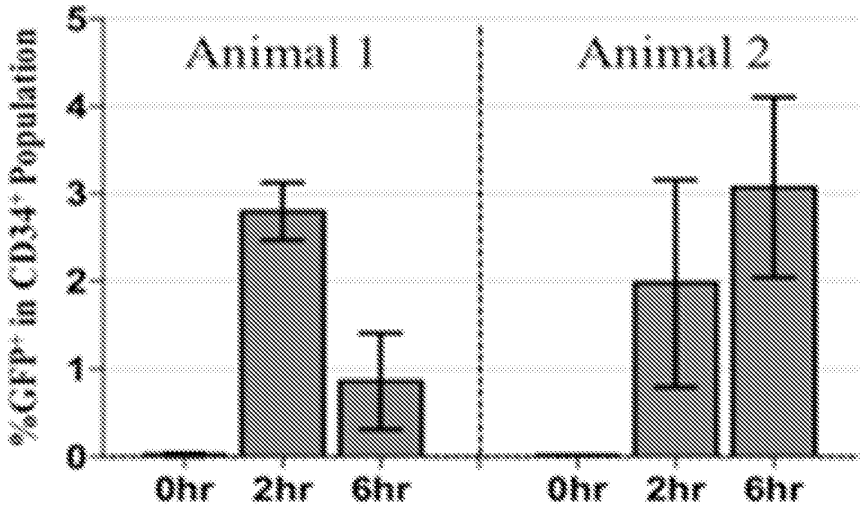


FIG. 93C

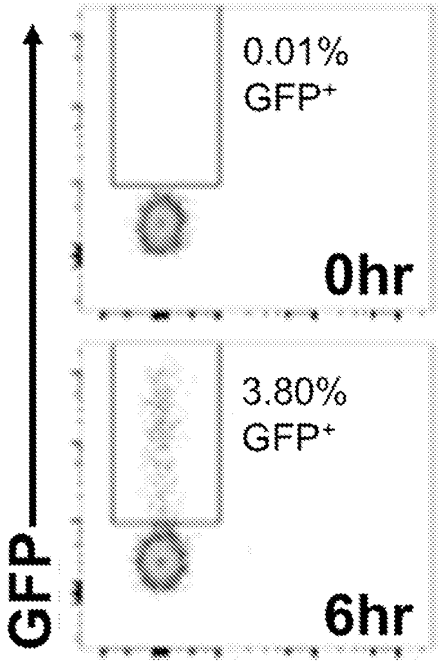


FIG. 93D

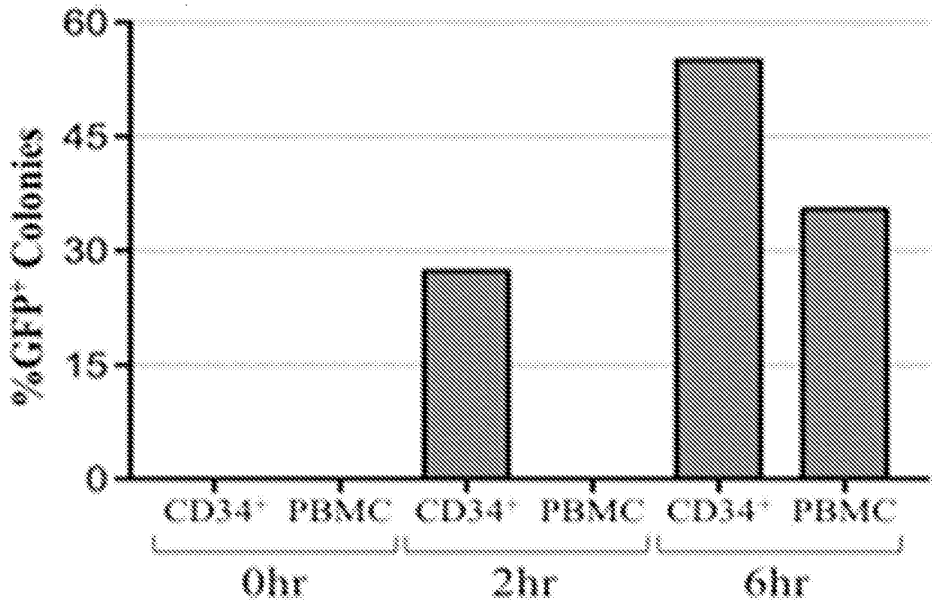


FIG. 93E

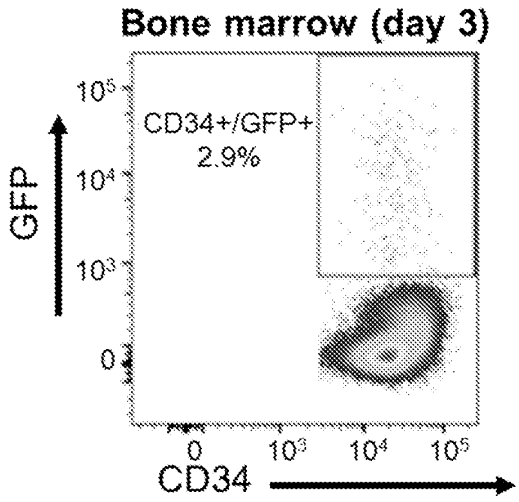

Bone marrow (day 3)
CD34+/GFP+
2.9%
GFP
10^5
10^4
10^3
0
0
10^3
10^4
10^5
CD34

FIG. 94A

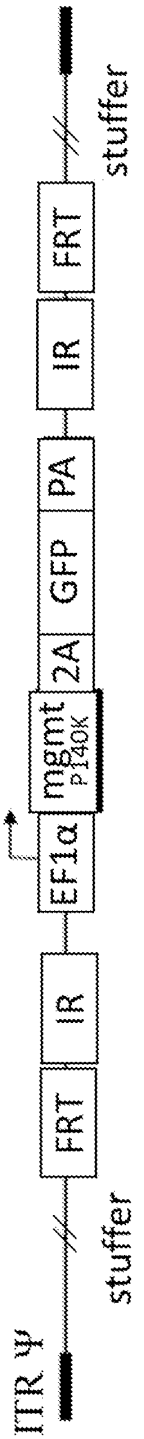


FIG. 94B

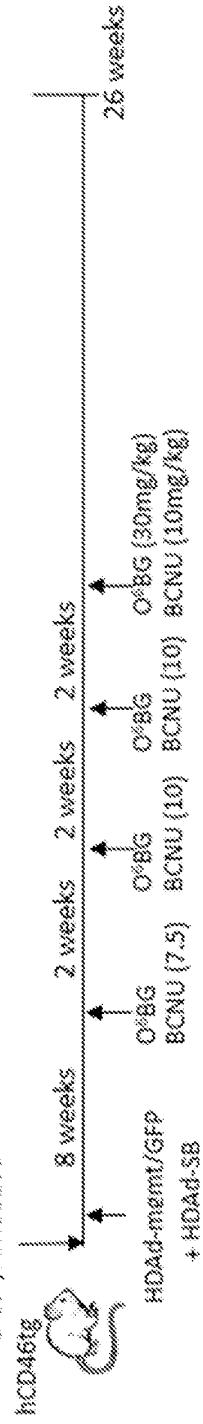


FIG. 94C

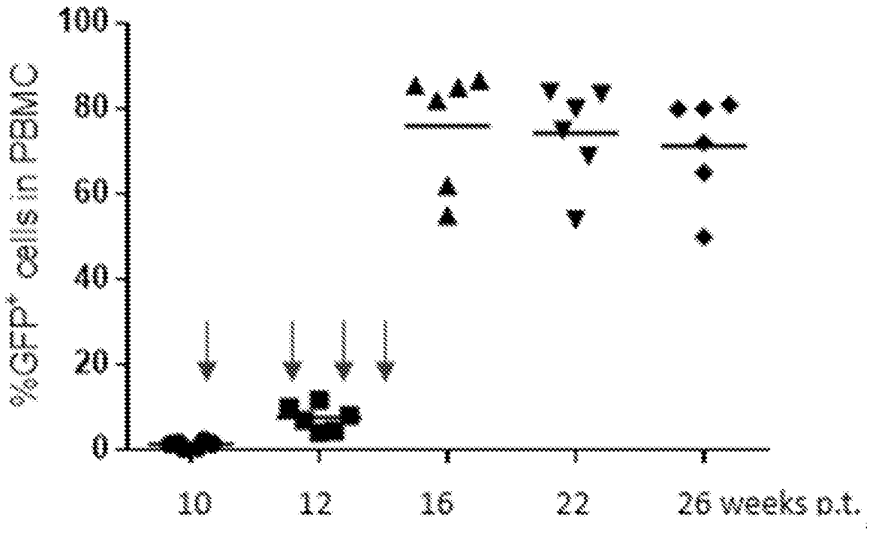


FIG. 94D

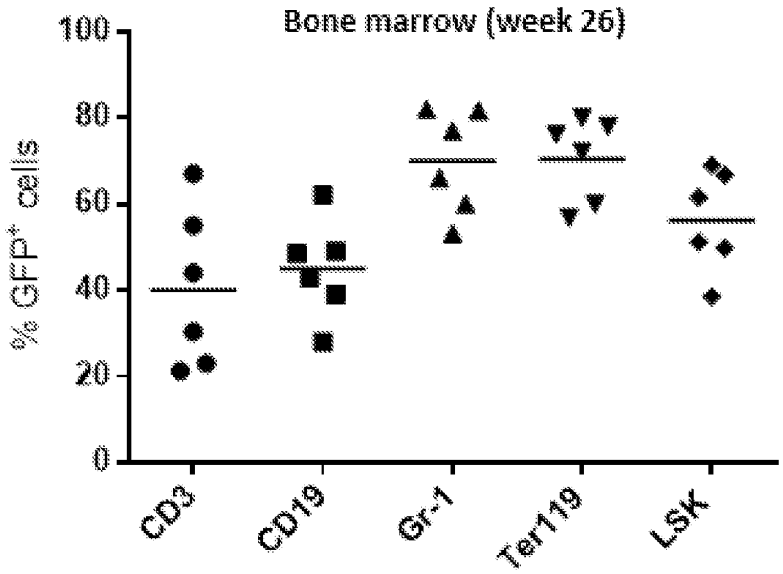


FIG. 94E

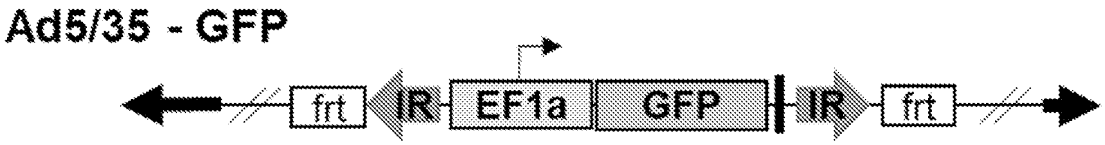


FIG. 94F

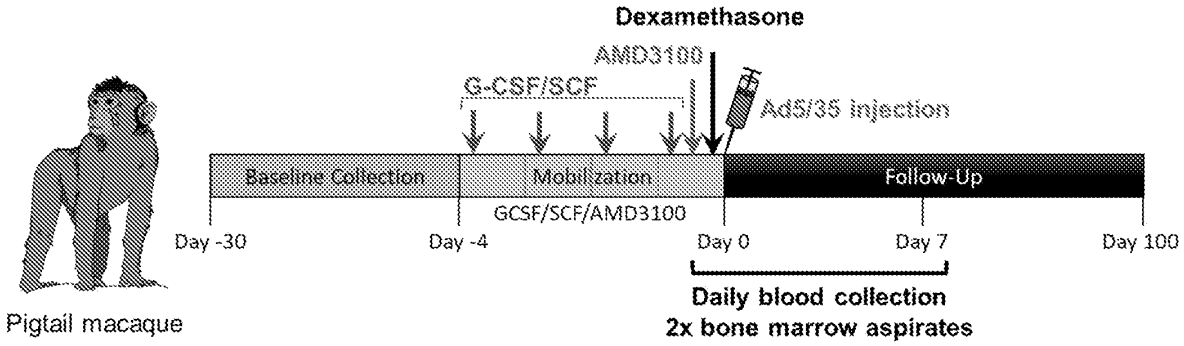


FIG. 94G

Animal ID	G-CSF	SCF	AMD3100	Dose
Z12406	50µg/kg	50µg/kg	-	$2x10^{12}$vp/kg
Z13126	50µg/kg	50µg/kg	4mg/kg	$4x10^{12}$vp/kg
Z15263	50µg/kg	50µg/kg	4mg/kg	$3x10^{12}$vp/kg

FIG. 94H

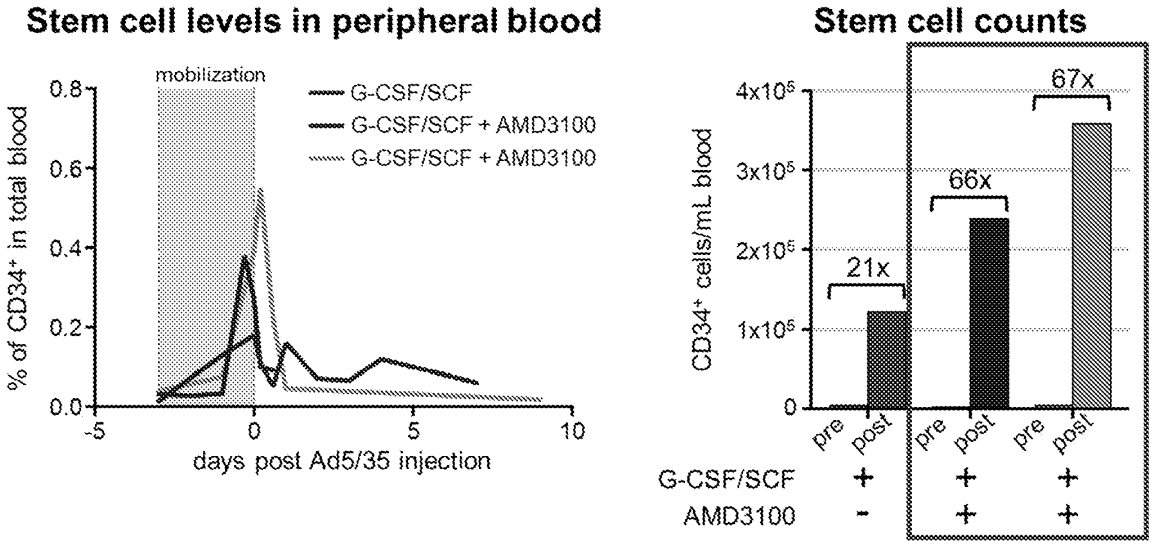


FIG. 94I

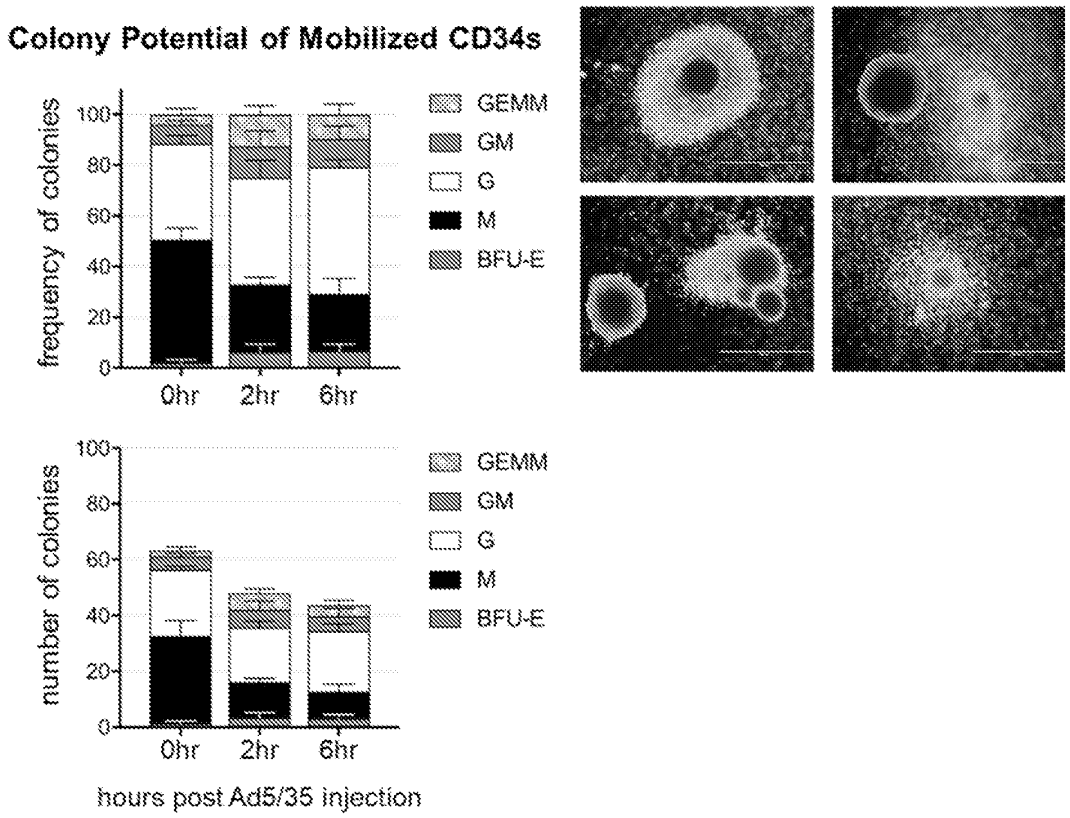


FIG. 94J

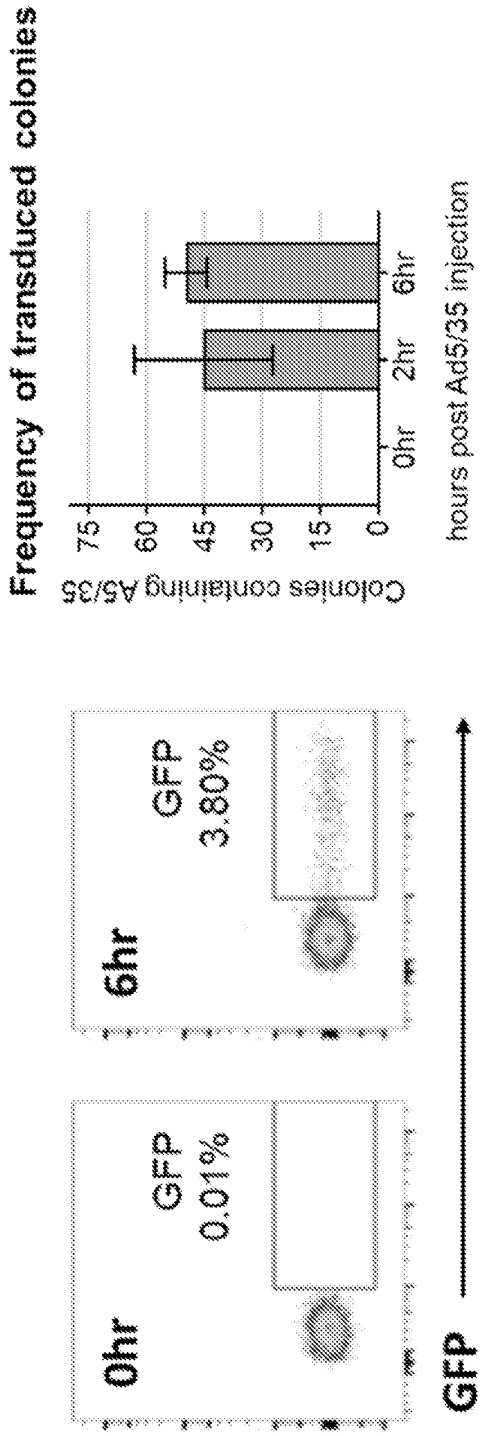


FIG. 94K

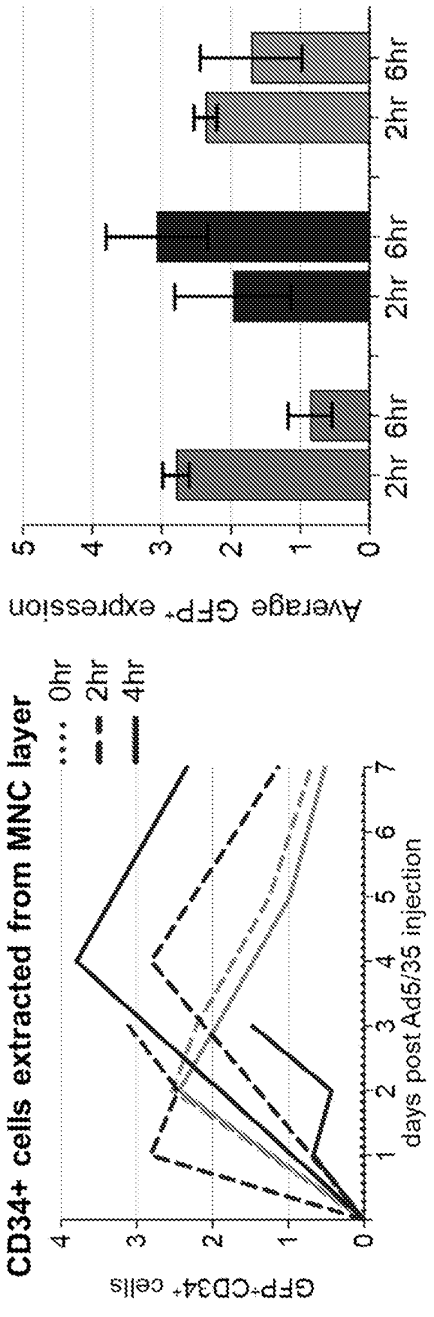


FIG. 94L

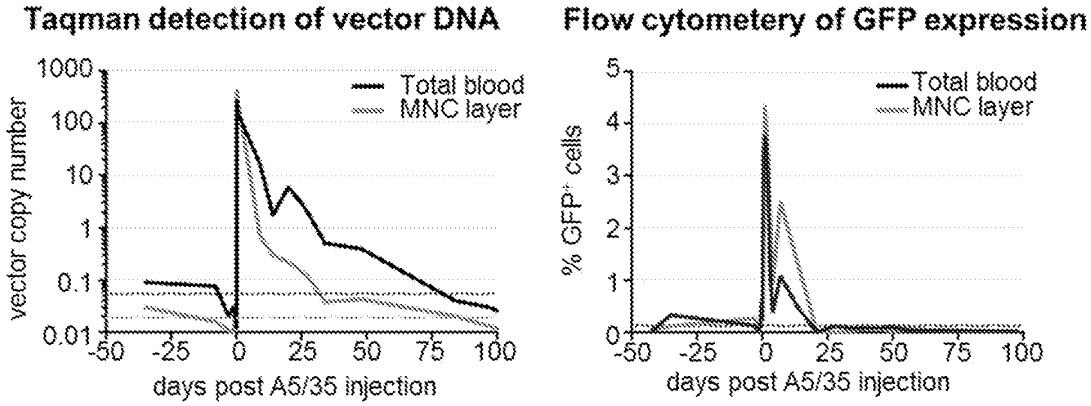


FIG. 94M

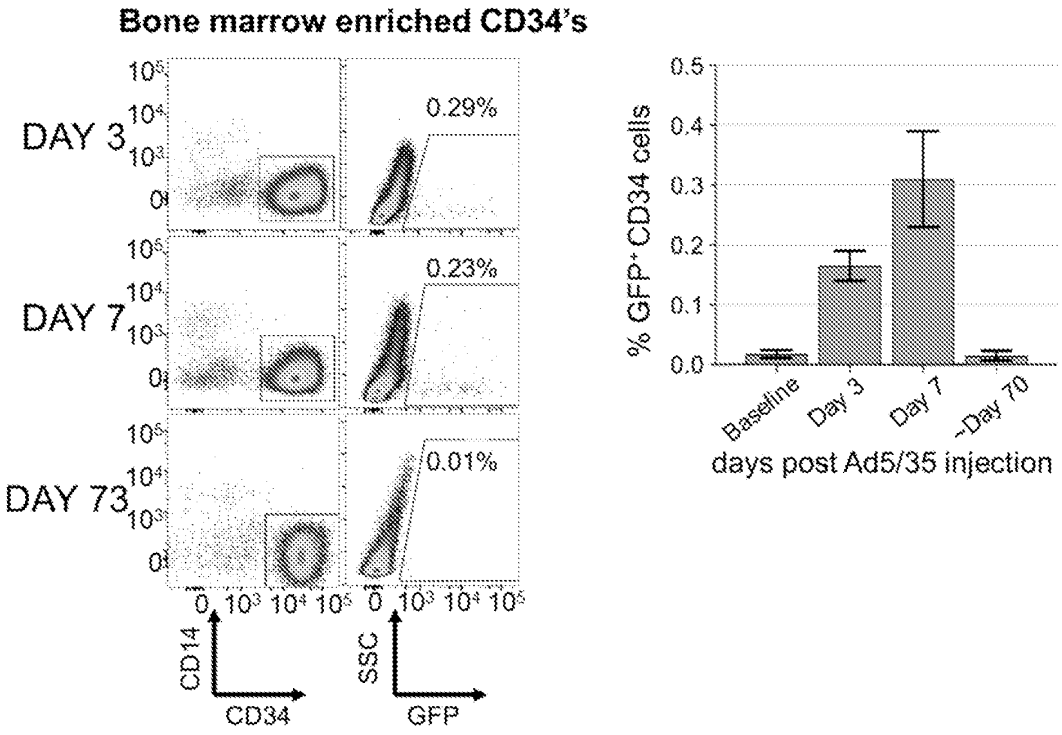


FIG. 95

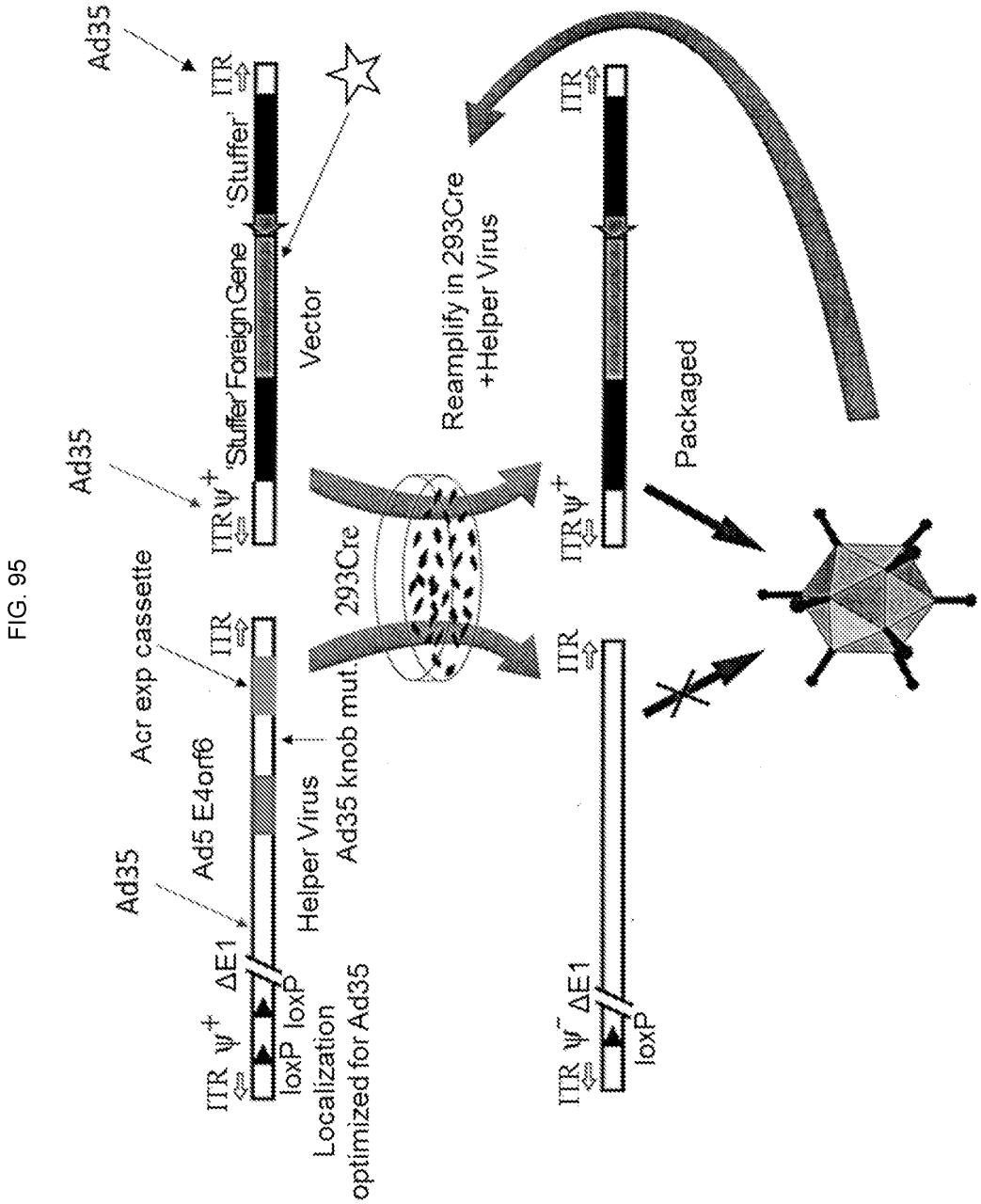

Ad35
ITR
'Stuffer'
Foreign Gene
Vector
Ad35
ITR ψ+
Acr exp cassette
Ad5 E4orf6
Ad35
ITR ψ+ ΔE1
loxP loxP
Helper Virus
Ad35 knob mut.
Localization optimized for Ad35
293Cre
Reamplify in 293Cre +Helper Virus
Packaged
ITR
ITR ψ+
ITR
ITR ψ- ΔE1
loxP

FIG. 96

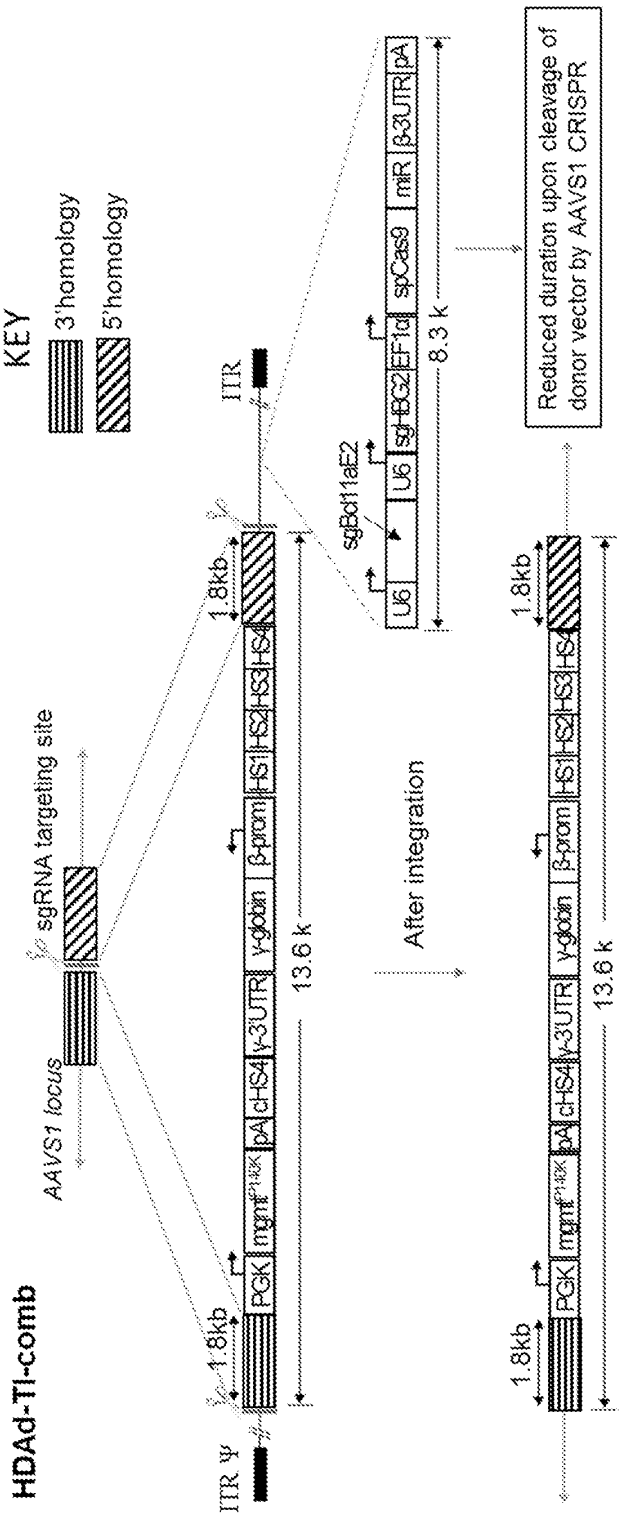

KEY
3'homology
5'homology
HDAd-TI-comb
AAVS1 locus
sgRNA targeting site
ITR Ψ
1.8kb
PGK
mgmt^P140K
pA
cHS4
γ-3'UTR
γ-globin
β-prom
HS1
HS2
HS3
HS4
13.6 k
ITR
sgBcl11aE2
U6
sg-BG2
EF1α
spCas9
miR
β-3UTR
pA
8.3 k
After integration
Reduced duration upon cleavage of donor vector by AAVS1 CRISPR

FIG. 97A

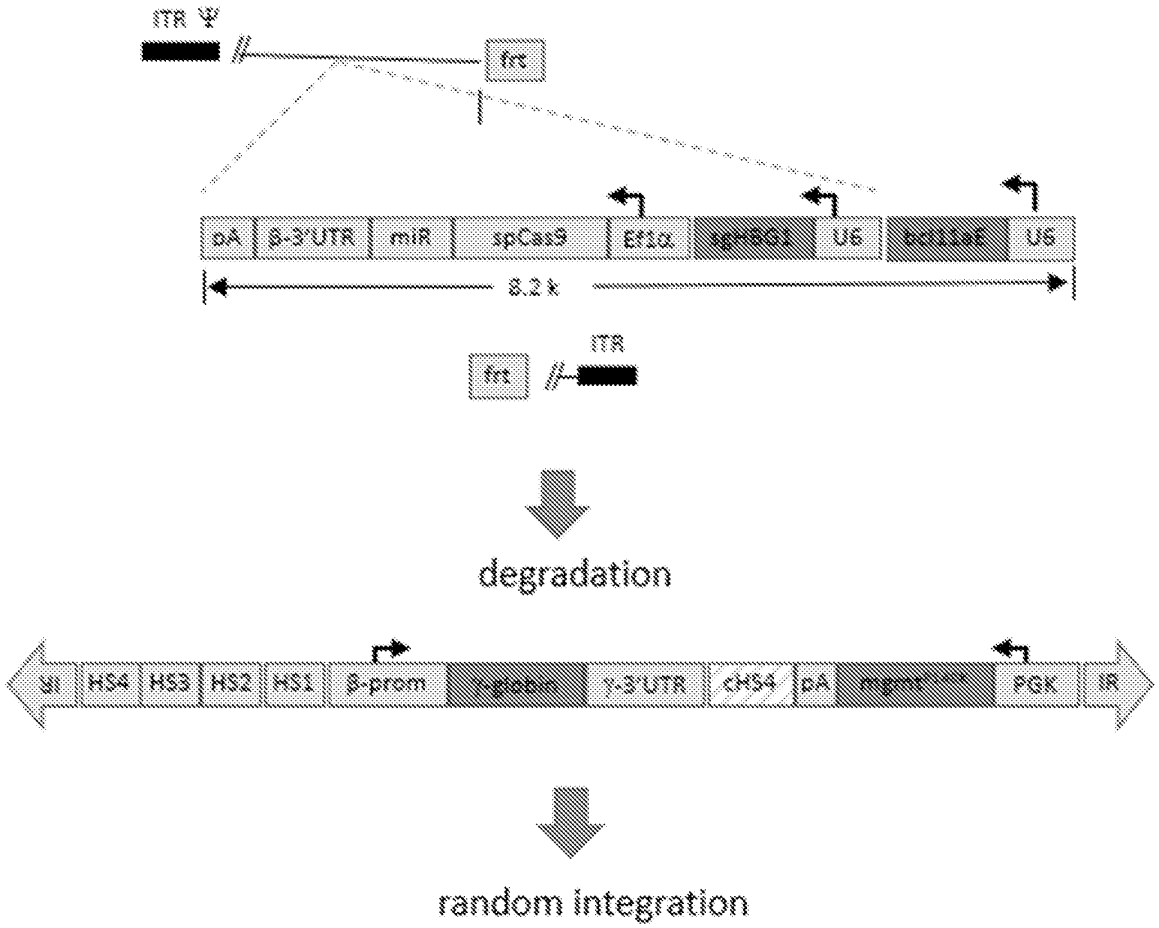

ITR
frt
pA
β-3'UTR
miR
spCas9
Ef1α
sgHSG1
U6
sgLLaE
U6
8.2 k
frt
ITR
degradation
5'
HS4
HS3
HS2
HS1
β-prom
γ-globin
γ-3'UTR
cHS4
pA
mgmt P140K
PGK
3'
random integration

FIG. 97B

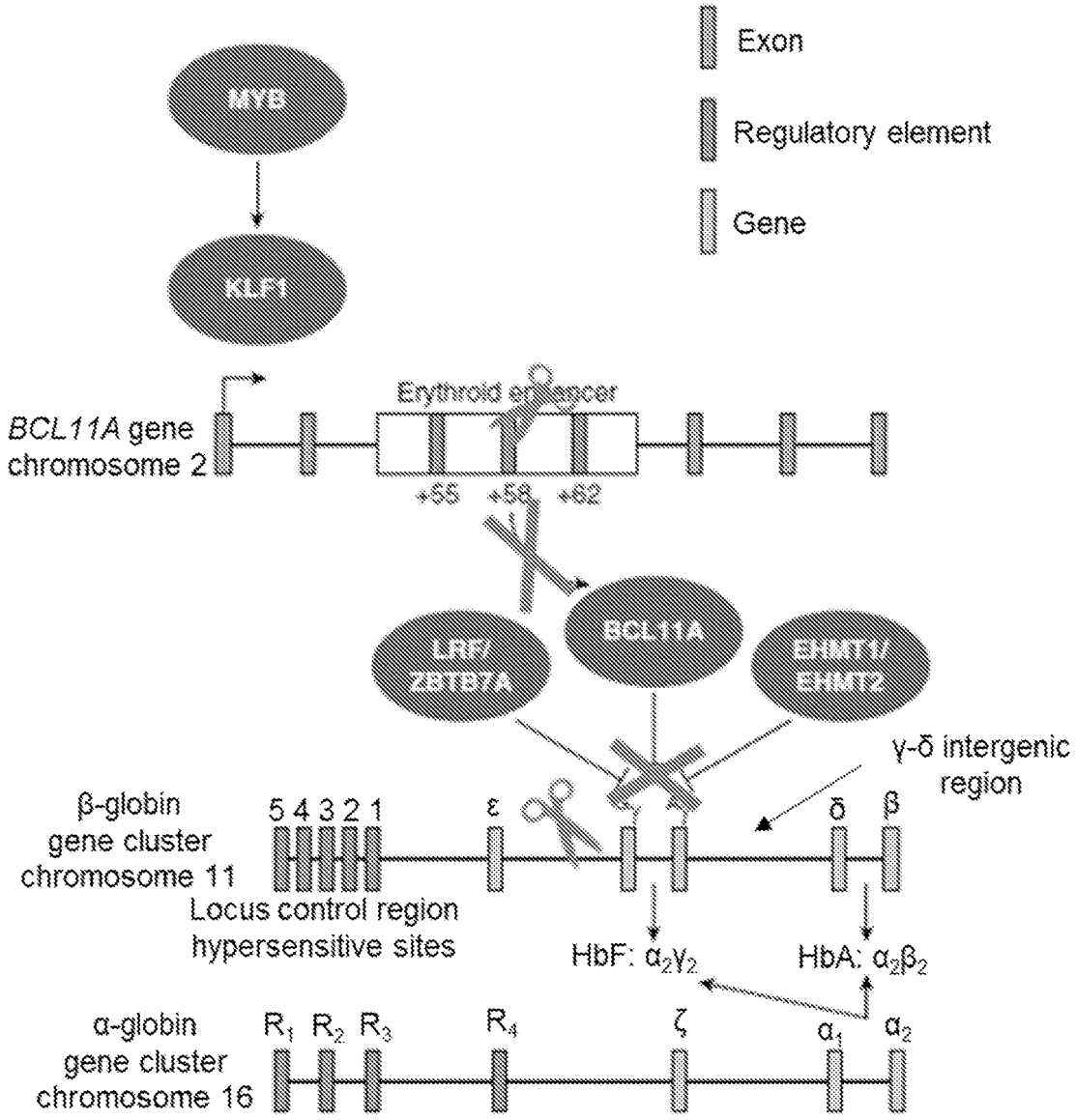

Exon
Regulatory element
Gene
MYB
KLF1
BCL11A gene
chromosome 2
Erythroid enhancer
+55
+58
+62
LRF/
ZBTB7A
BCL11A
EHMT1/
EHMT2
γ-δ intergenic
region
β-globin
gene cluster
chromosome 11
5 4 3 2 1
ε
δ
β
Locus control region
hypersensitive sites
HbF: α2γ2
HbA: α2β2
α-globin
gene cluster
chromosome 16
R1 R2 R3
R4
ζ
α1
α2

FIG. 97C

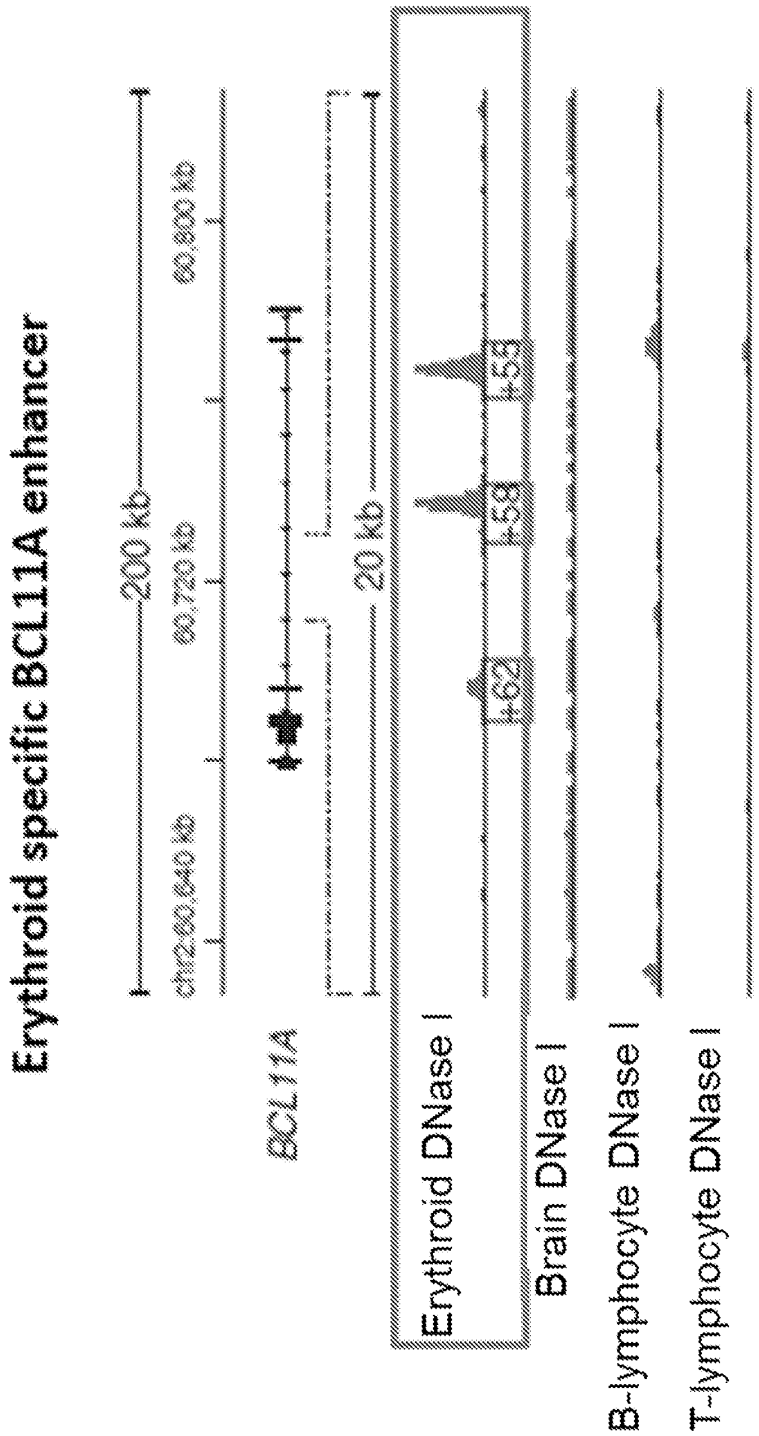

Erythroid specific BCL11A enhancer
200 kb
chr2:60,640 kb
60,720 kb
60,800 kb
BCL11A
20 kb
+62
+58
+33
Erythroid DNase I
Brain DNase I
B-lymphocyte DNase I
T-lymphocyte DNase I

FIG. 97D

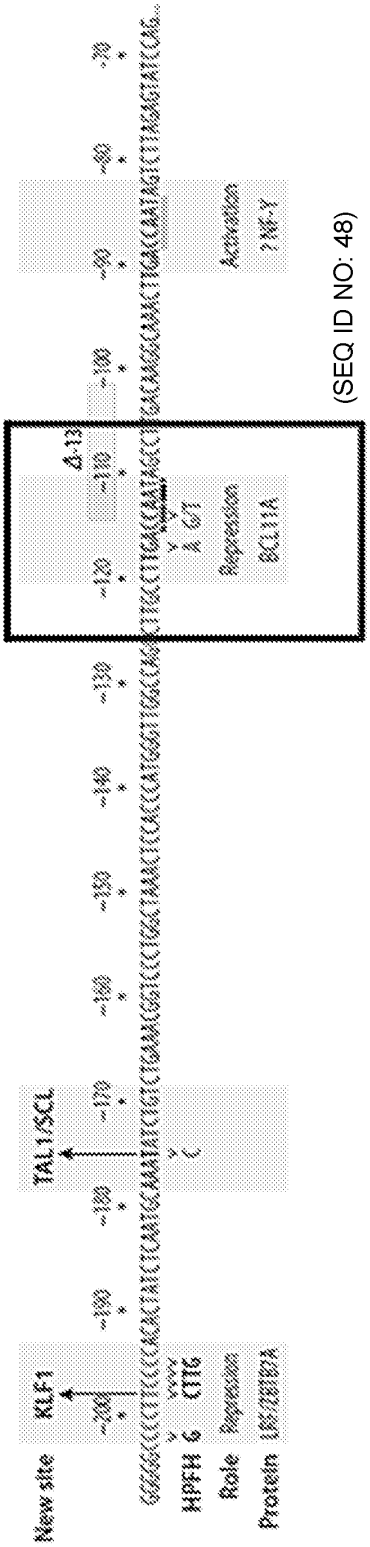


FIG. 98A

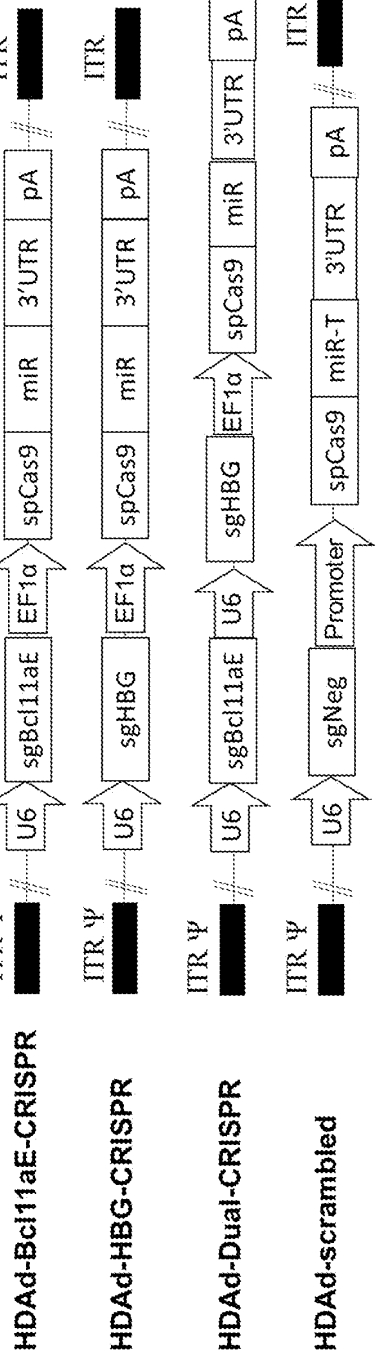


FIG. 98B

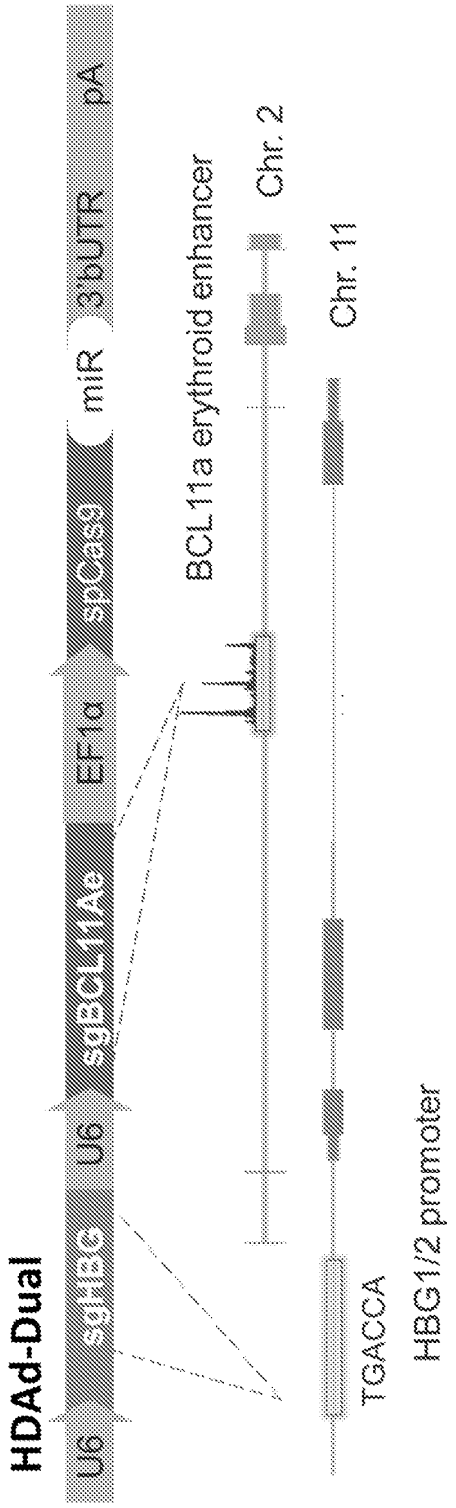


FIG. 98C

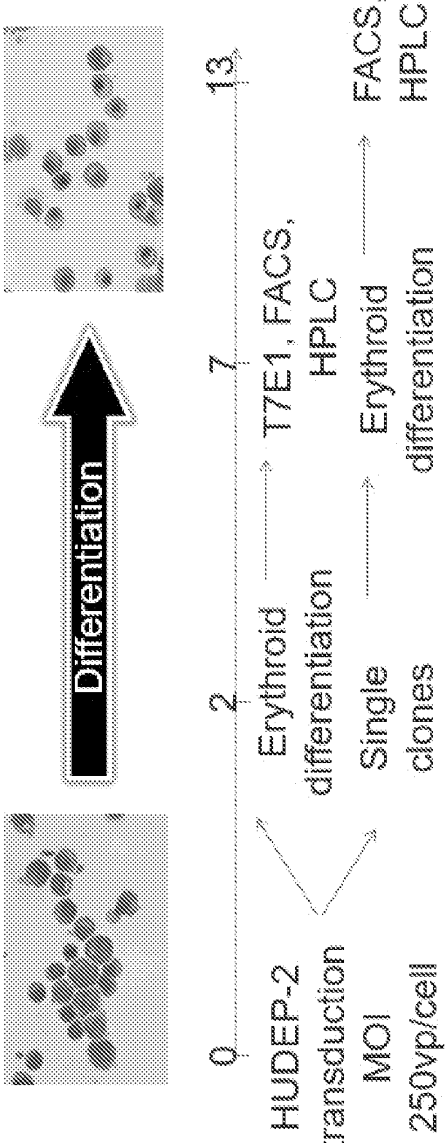


FIG. 98D

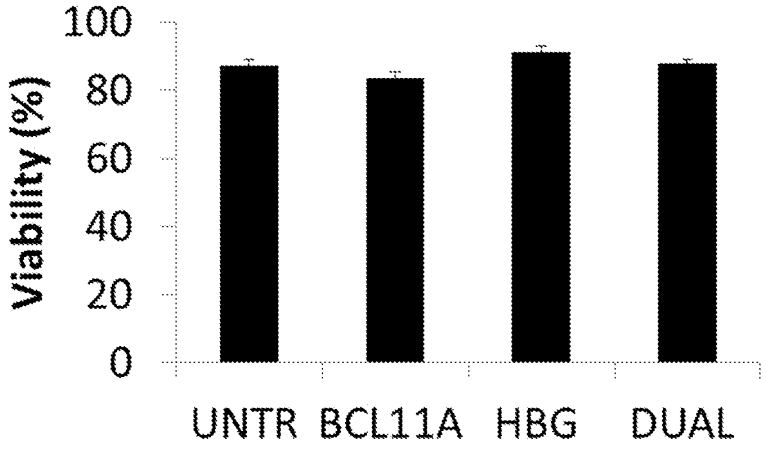


FIG. 98E

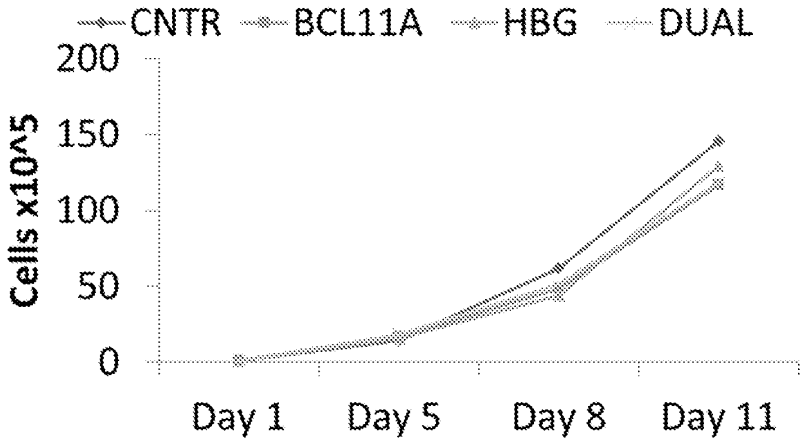


FIG. 98F

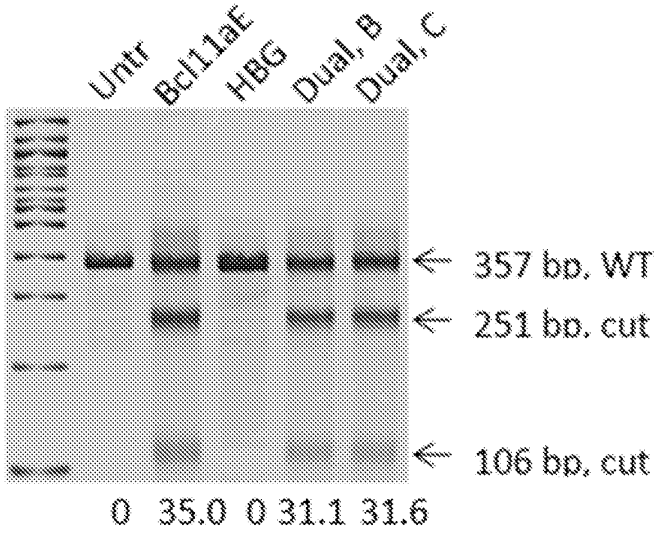


FIG. 98G

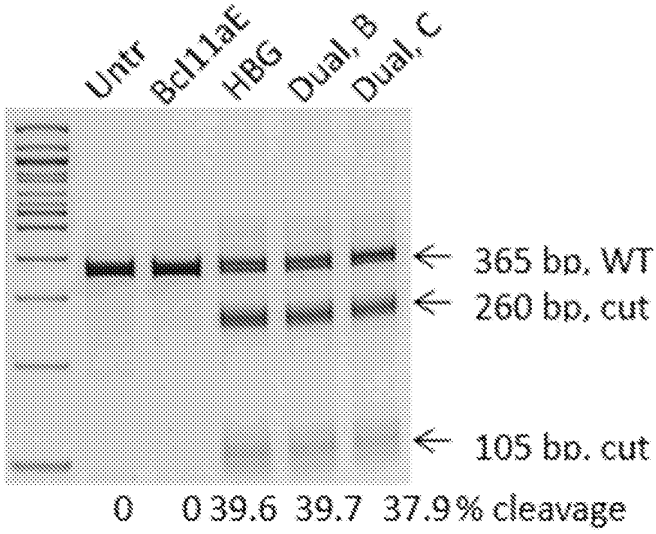


FIG. 98H

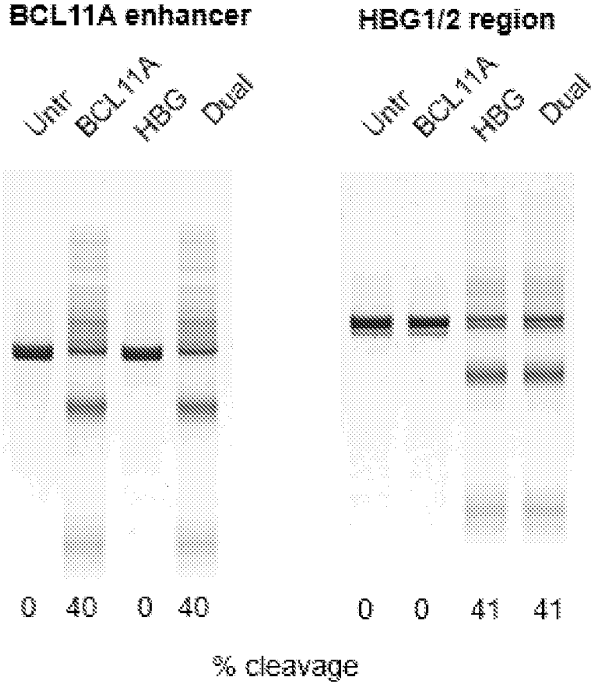
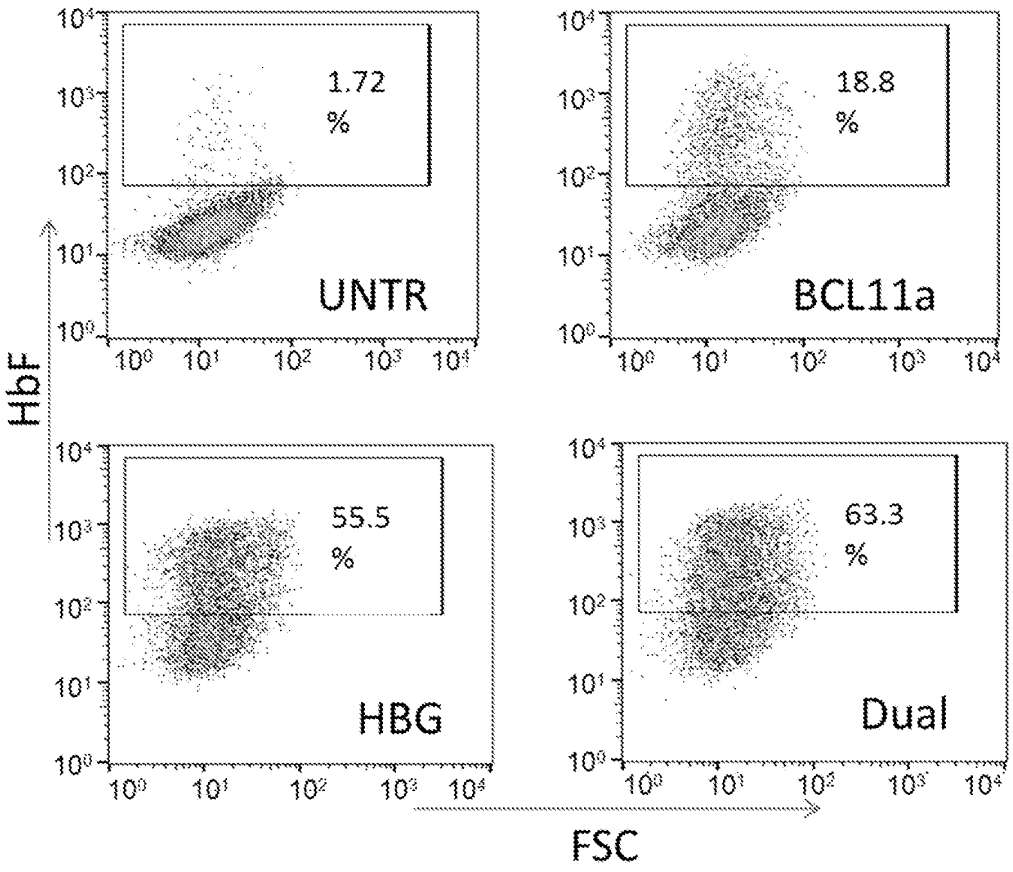

BCL11A enhancer
HBG1/2 region
Untr
BCL11A
HBG
Dual
Untr
BCL11A
HBG
Dual
0 40 0 40
0 0 41 41
% cleavage

FIG. 98I


1.72 %
UNTR
18.8 %
BCL11a
55.5 %
HBG
63.3 %
Dual
HbF
FSC

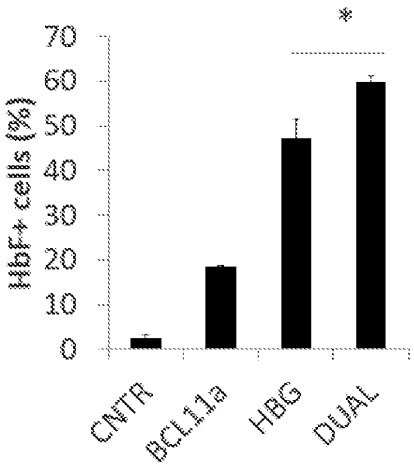

HbF+ cells (%)
70
60
50
40
30
20
10
0
*
CNTR
BCL11a
HBG
DUAL

FIG. 98J

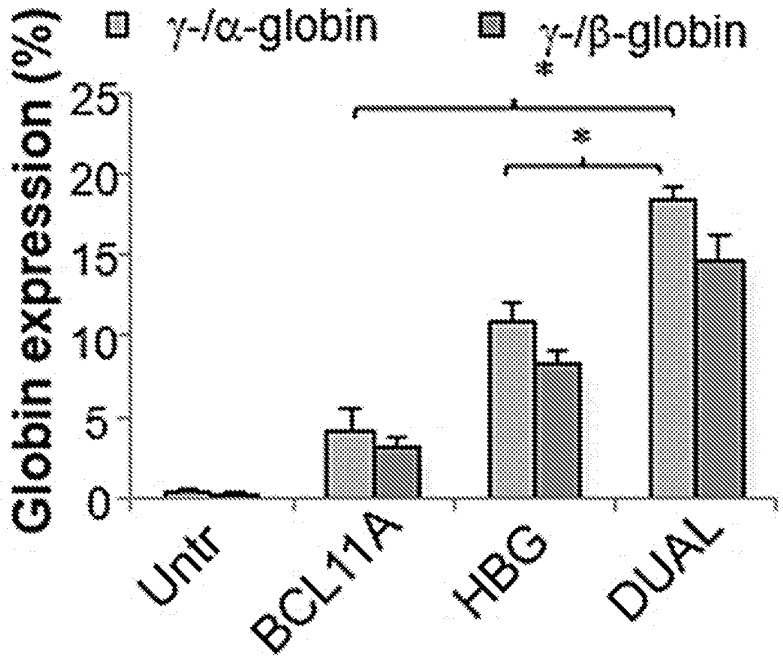

γ-/α-globin
γ-/β-globin
Globin expression (%)
25
20
15
10
5
0
*
*
Untr
BCL11A
HBG
DUAL

FIG. 98K

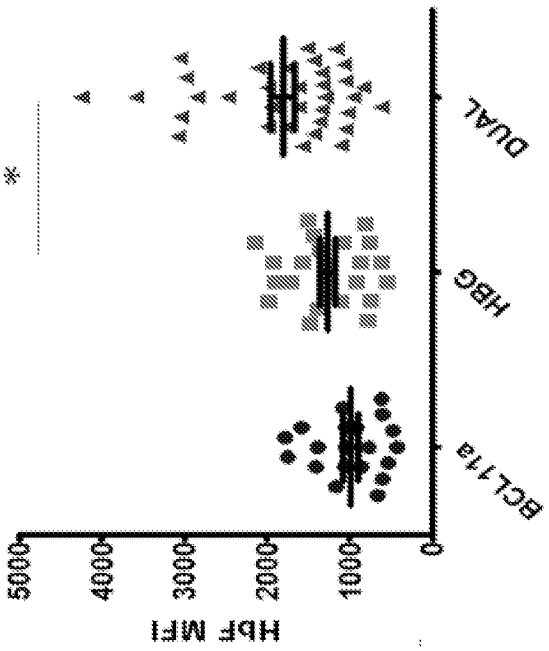


FIG. 98L

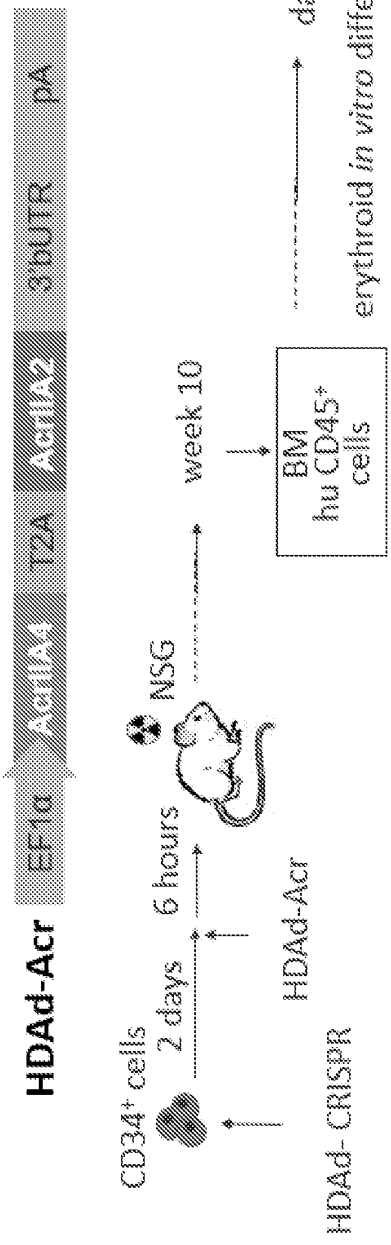


FIG. 98M

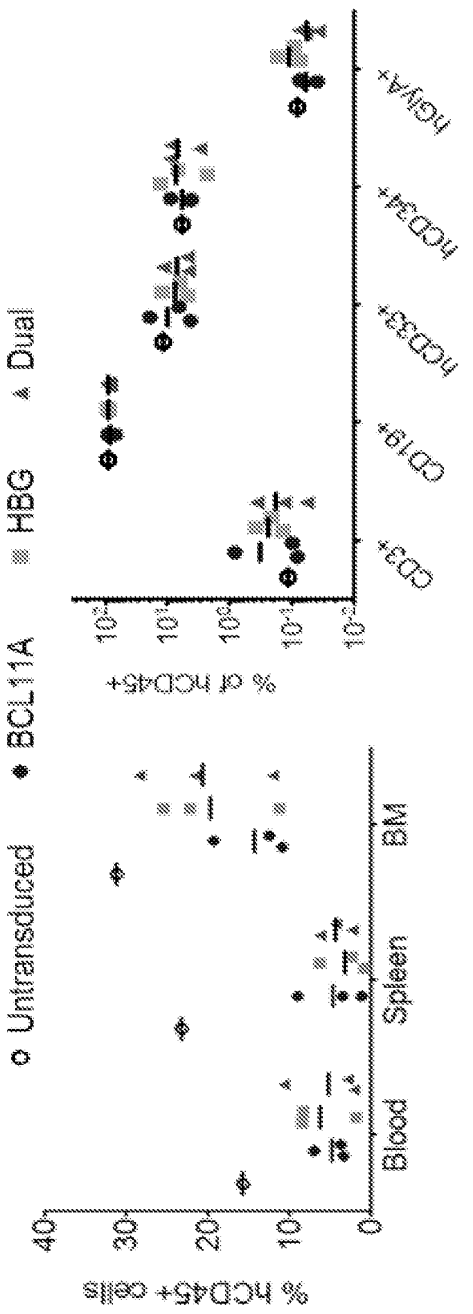


FIG. 98N



FIG. 99A

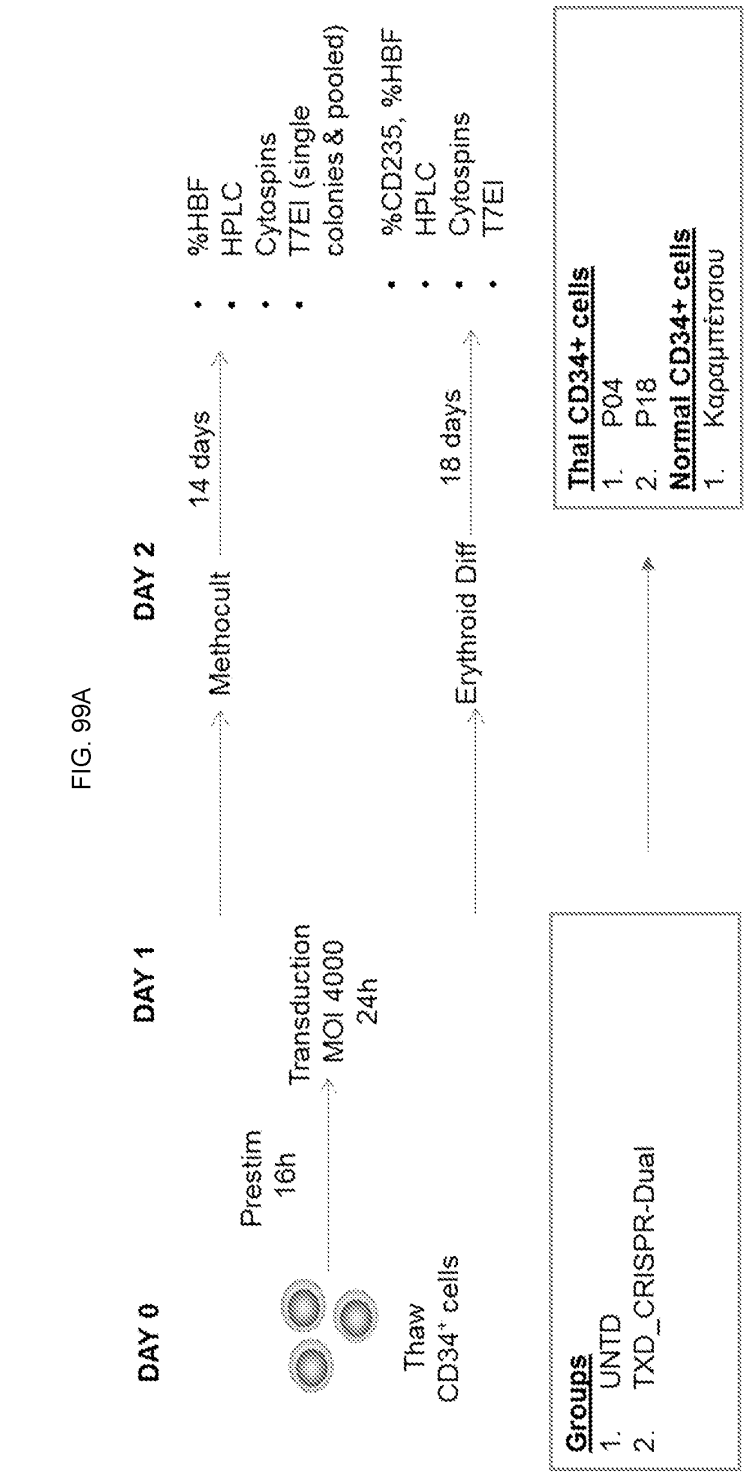

DAY 0
DAY 1
DAY 2
Thaw CD34+ cells
Prestim 16h
Transduction MOI 4000 24h
Methocult
14 days
Erythroid Diff
18 days
%HBF
HPLC
Cytospins
T7EI (single colonies & pooled)
%CD235, %HBF
HPLC
Cytospins
T7EI
Groups
1. UNTD
2. TXD_CRISPR-Dual
Thal CD34+ cells
1. P04
2. P18
Normal CD34+ cells
1. Καραμπέτσιου

FIG. 99B

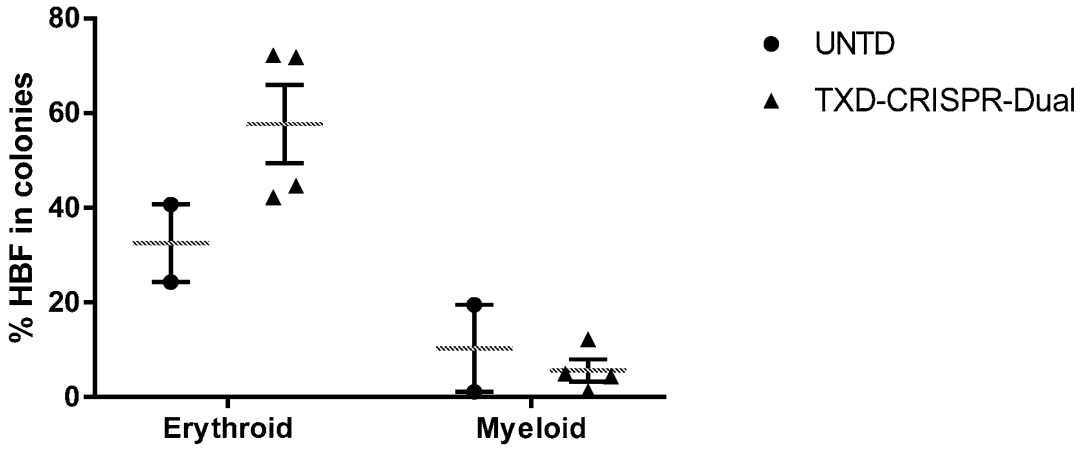


FIG. 99C

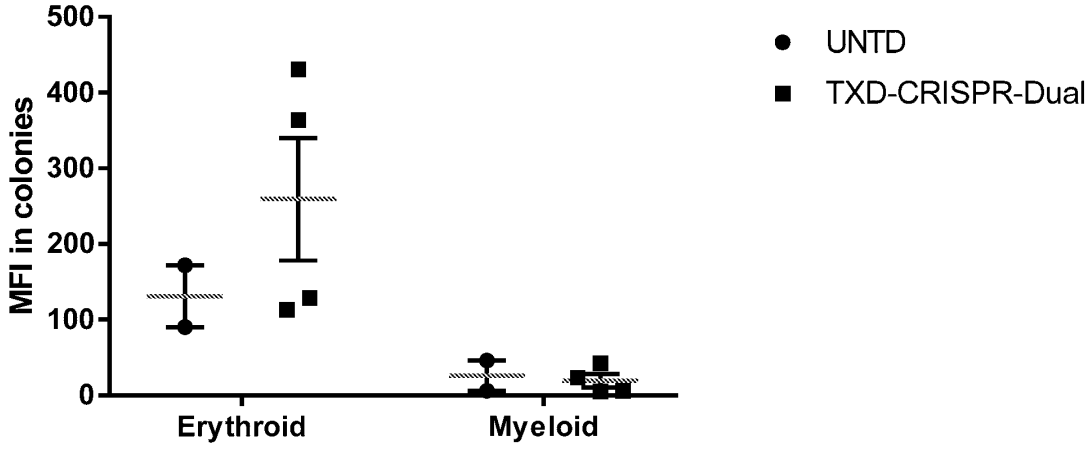


FIG. 99D

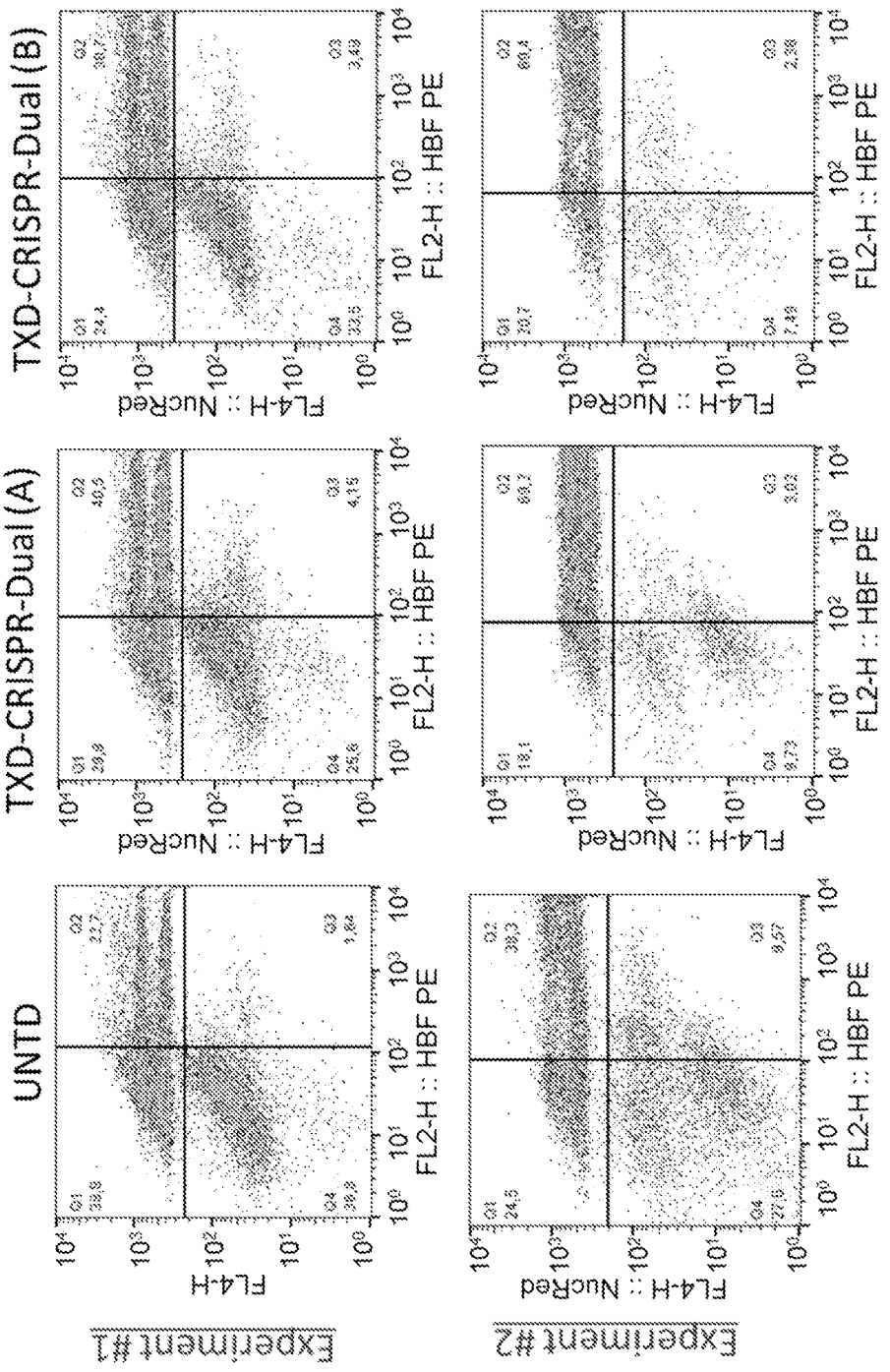

UNTD
TXD-CRISPR-Dual (A)
TXD-CRISPR-Dual (B)
Experiment #1
Experiment #2
FL4-H
FL4-H :: NucRed
FL2-H :: HBF PE

FIG. 99E

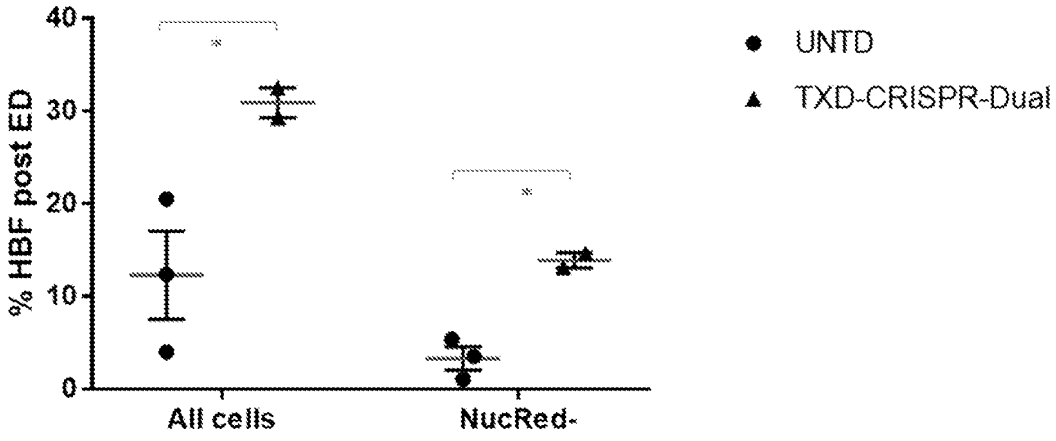


FIG. 99F

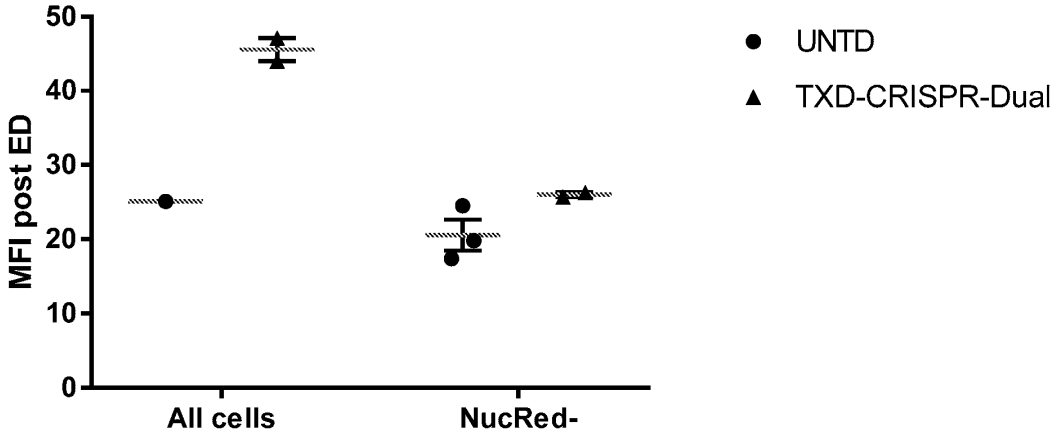


FIG. 99G

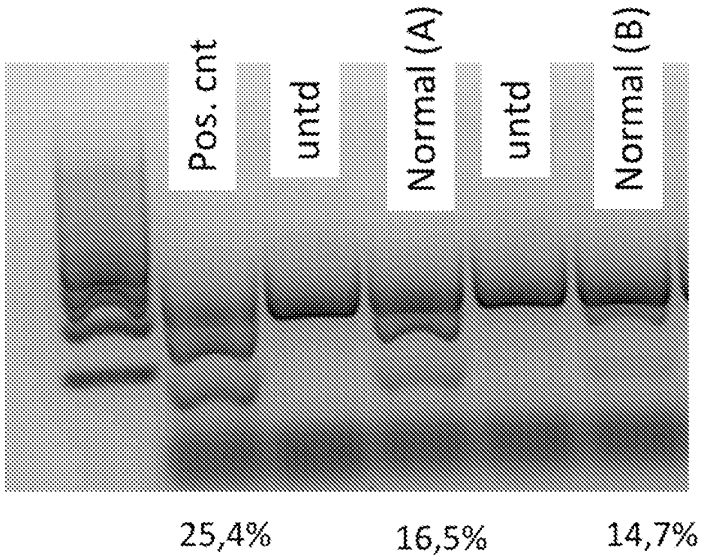


FIG. 99H

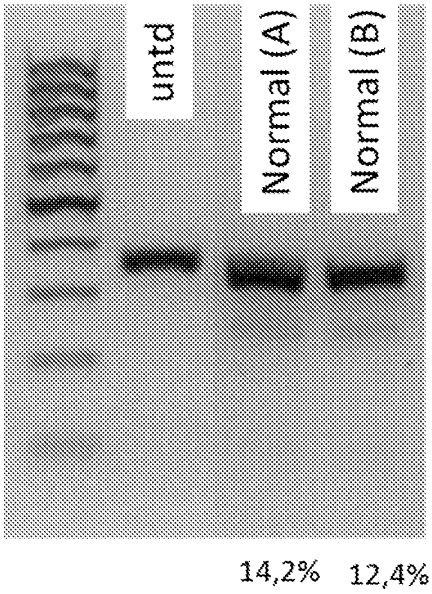


FIG. 99I

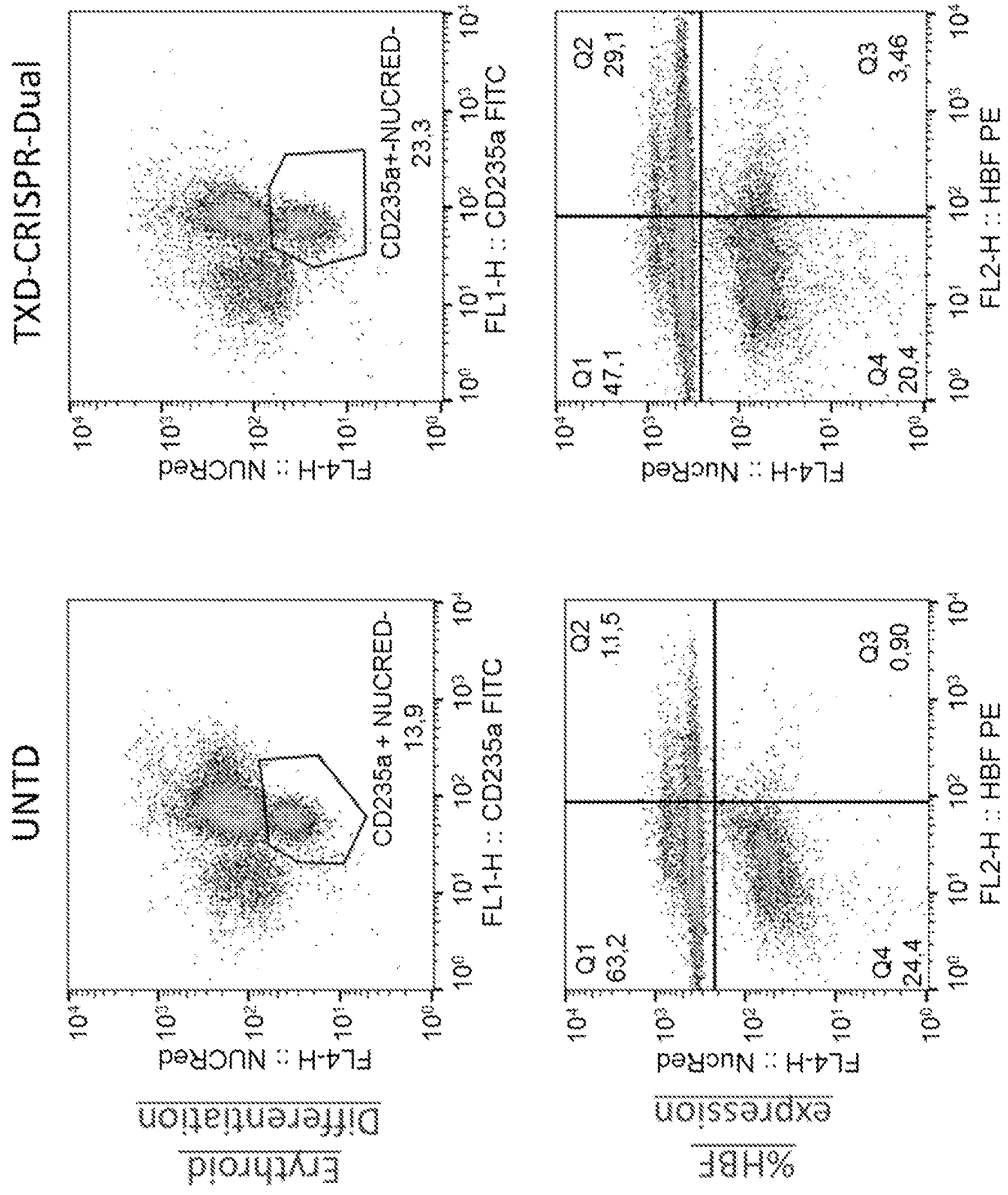

UNTD
TXD-CRISPR-Dual
Erythroid Differentiation
%HBF expression
FL4-H :: NUCRed
FL1-H :: CD235a FITC
FL2-H :: HBF PE
FL4-H :: NucRed
CD235a + NUCRED- 13,9
CD235a+-NUCRED- 23,3
Q1 63,2
Q2 11,5
Q3 0,90
Q4 24,4
Q1 47,1
Q2 29,1
Q3 3,46
Q4 20,4

FIG. 99J

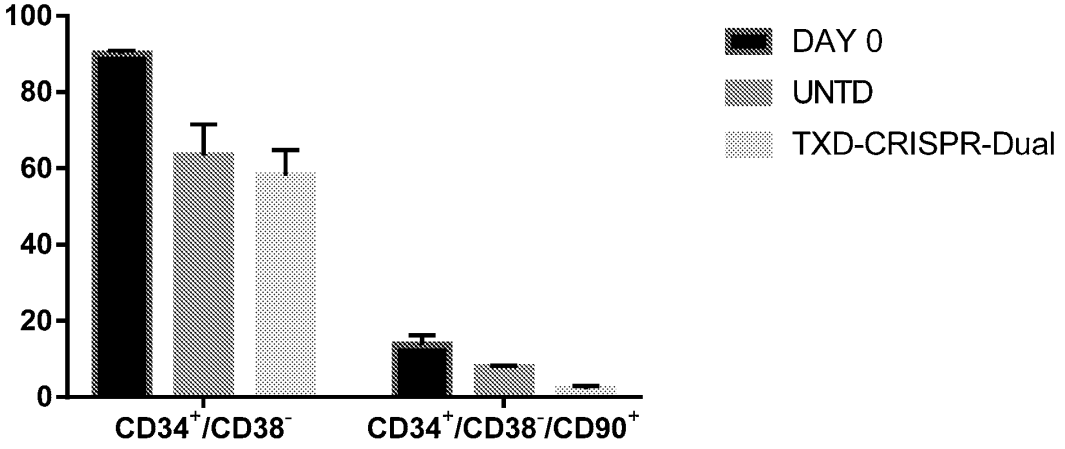


FIG. 99 K

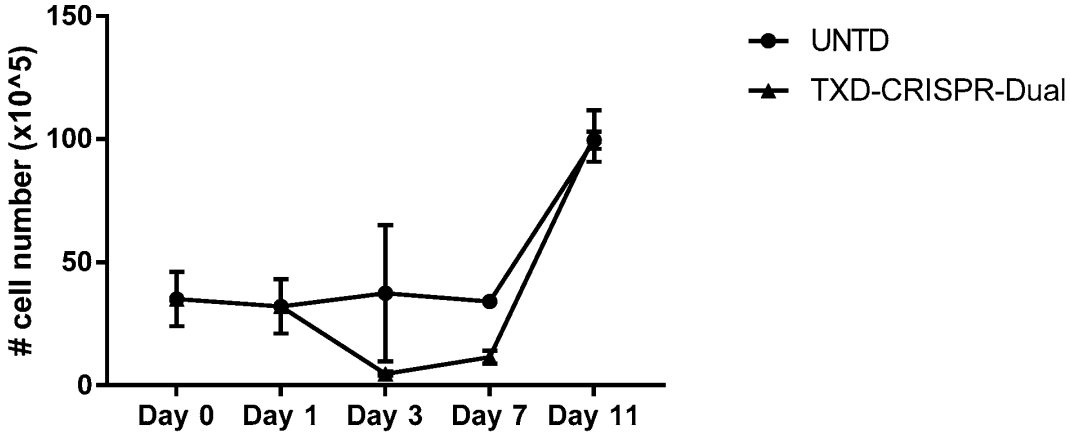


FIG. 99L

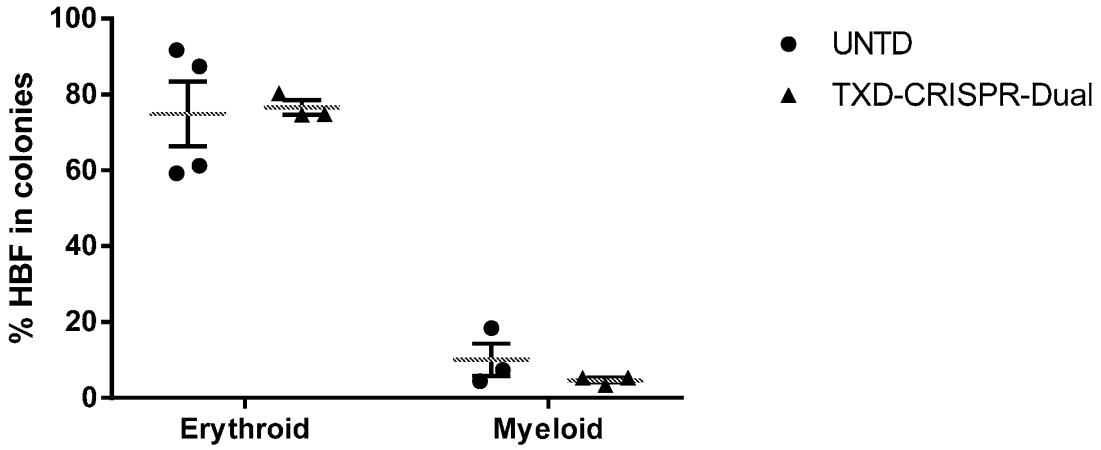


FIG. 99M



FIG. 99N

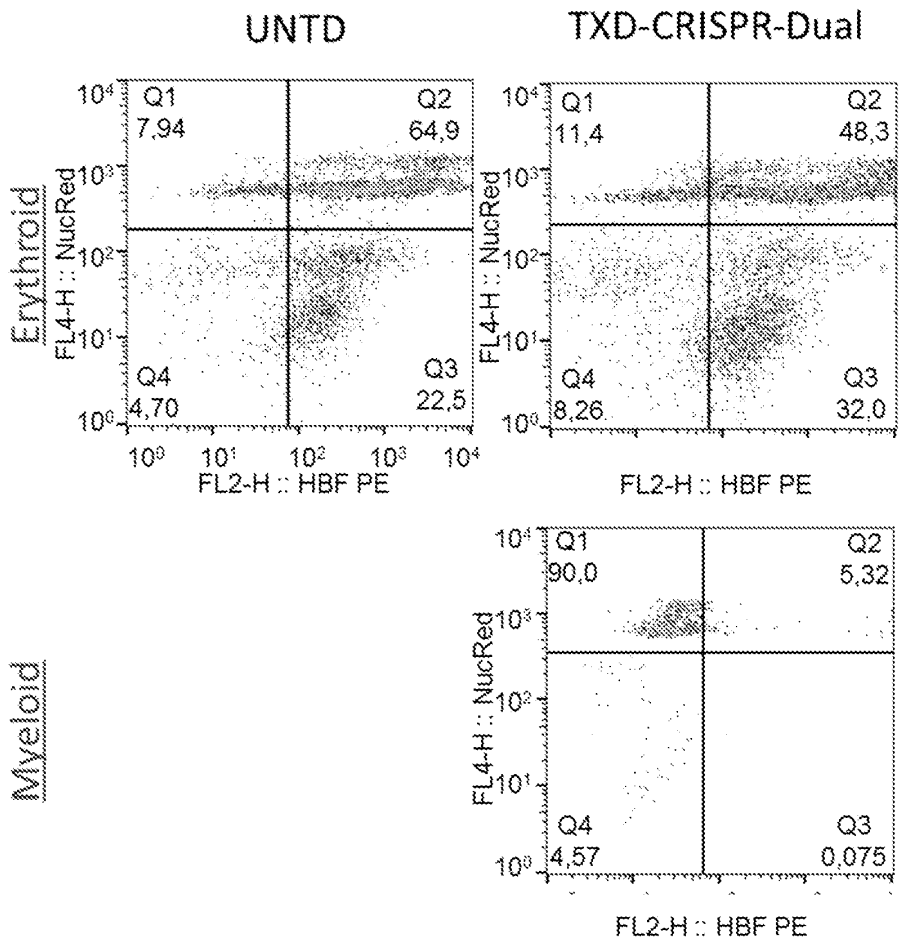

UNTD
TXD-CRISPR-Dual
Erythroid
Myeloid
FL4-H :: NucRed
FL2-H :: HBF PE
Q1
7,94
Q2
64,9
Q4
4,70
Q3
22,5
Q1
11,4
Q2
48,3
Q4
8,26
Q3
32,0
Q1
90,0
Q2
5,32
Q4
4,57
Q3
0,075

FIG. 99O

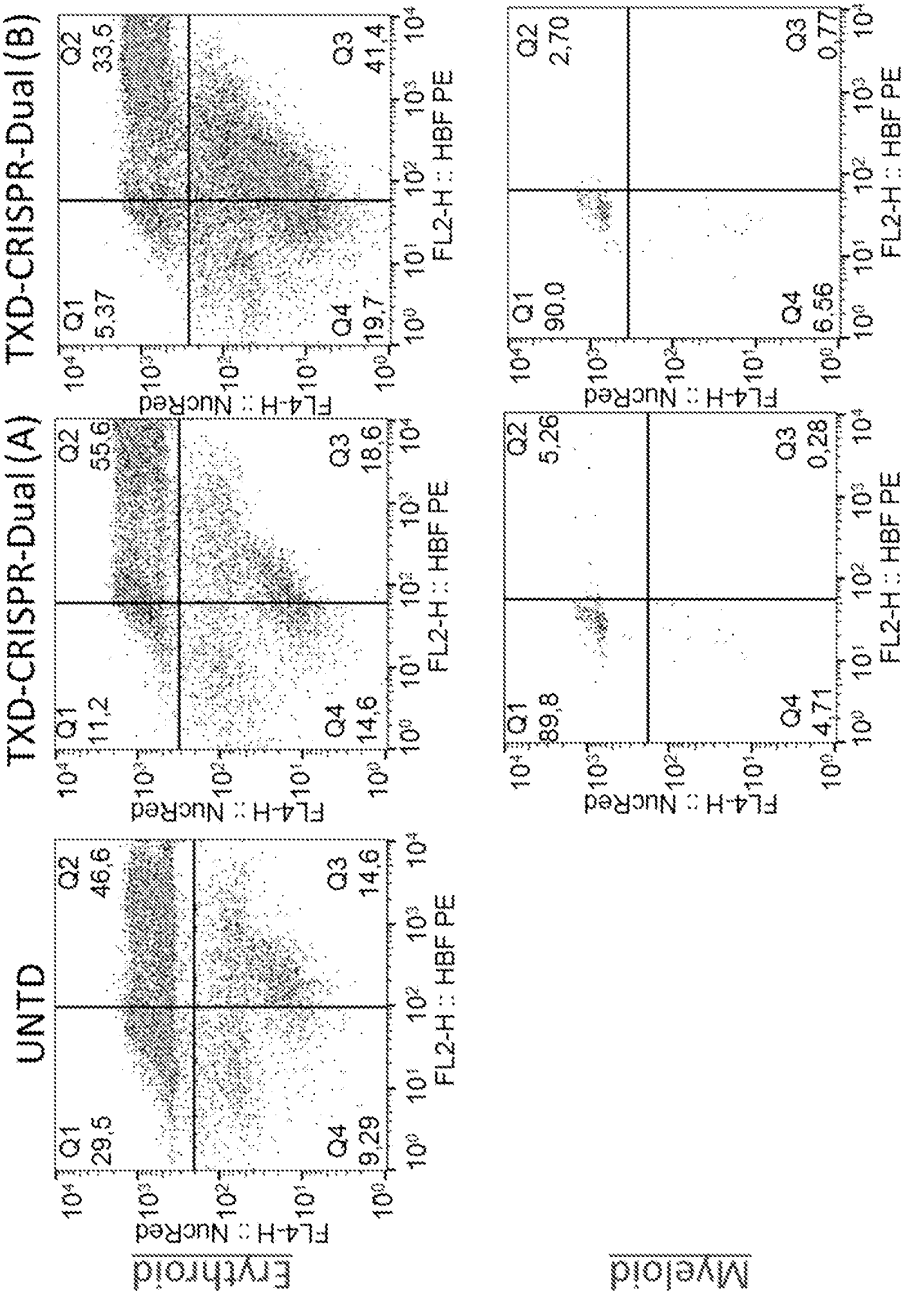

UNTD
TXD-CRISPR-Dual (A)
TXD-CRISPR-Dual (B)
Erythroid
Myeloid
FL4-H :: NucRed
FL2-H :: HBF PE
Q1 29,5
Q2 46,6
Q3 14,6
Q4 9,29
Q1 11,2
Q2 55,6
Q3 18,6
Q4 14,6
Q1 5,37
Q2 33,5
Q3 41,4
Q4 19,7
Q1 89,8
Q2 5,26
Q3 0,28
Q4 4,71
Q1 90,0
Q2 2,70
Q3 0,77
Q4 6,56

FIG. 99P

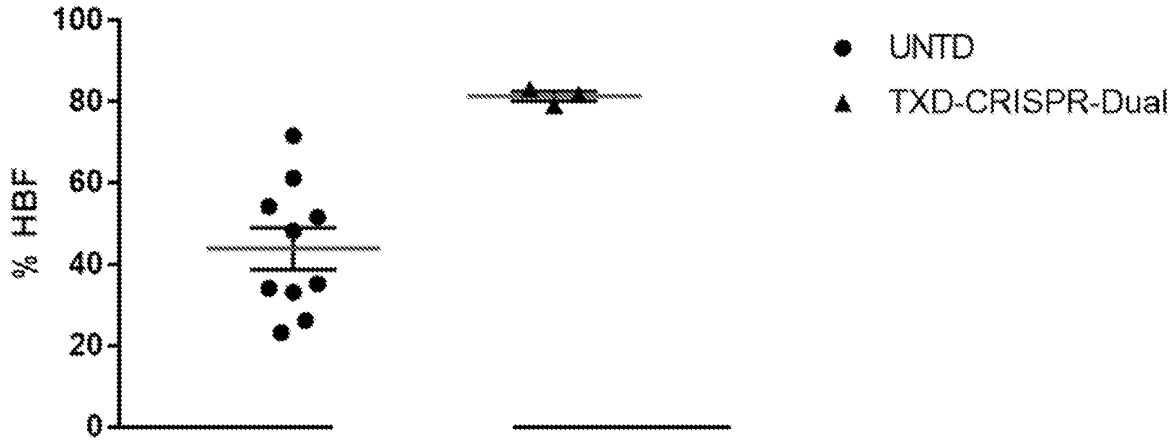


FIG. 99Q

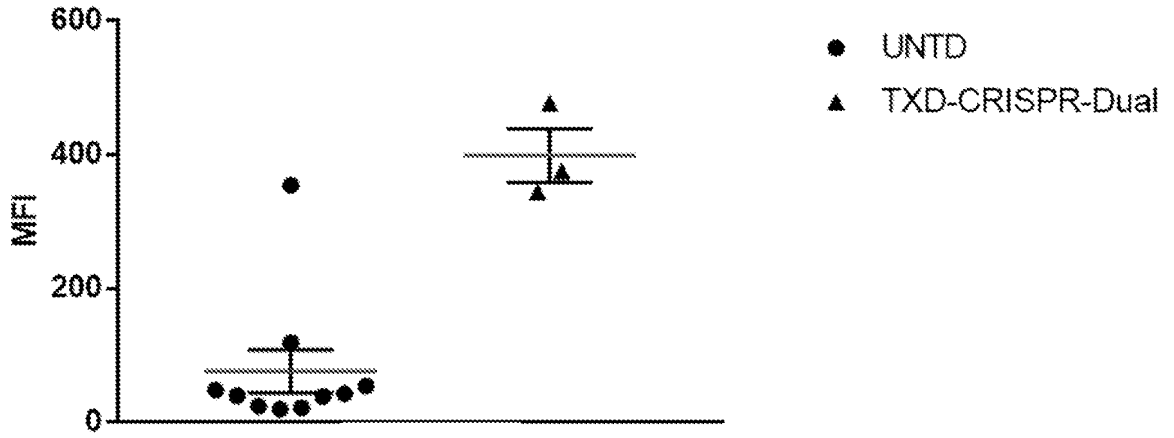


FIG. 99R

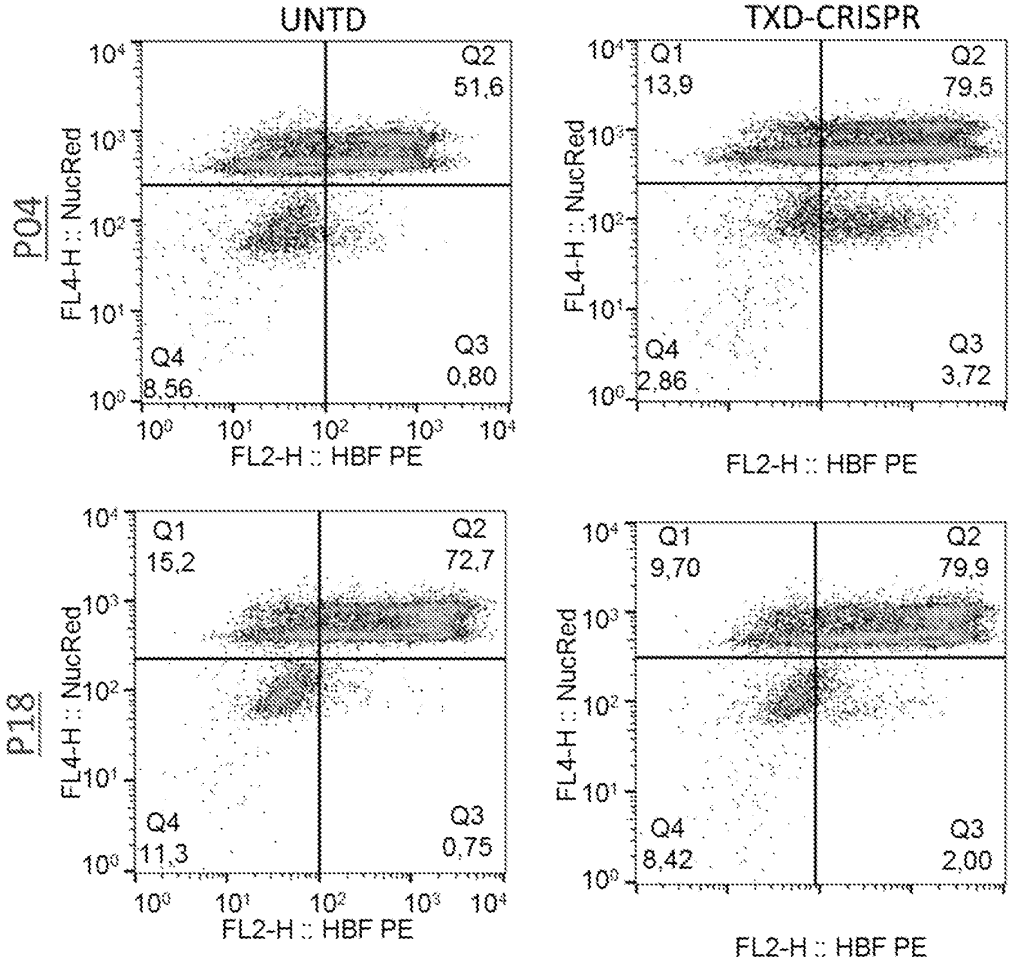
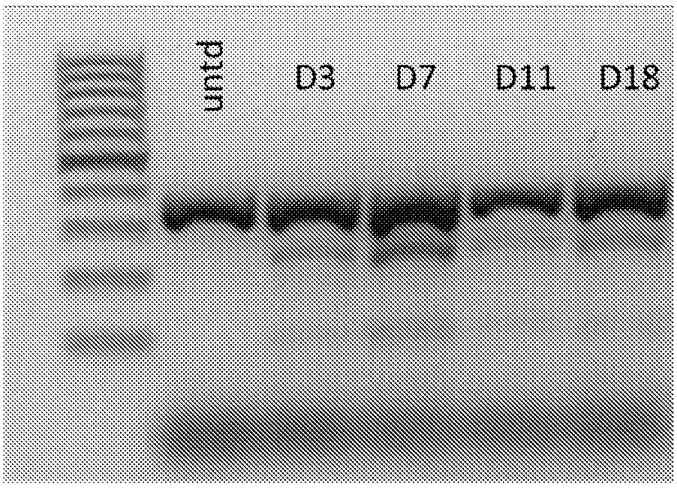

UNTD
TXD-CRISPR
P04
P18
FL4-H :: NucRed
FL2-H :: HBF PE
Q1
Q2
Q3
Q4
51,6
0,80
8,56
13,9
79,5
2,86
3,72
15,2
72,7
11,3
0,75
9,70
79,9
8,42
2,00

FIG. 99S

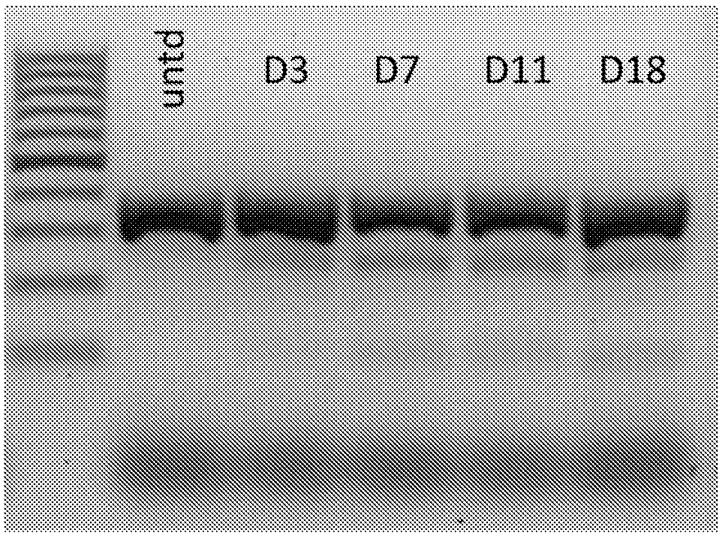
p04



12,2% 19,5% 13,6% 15,5%

FIG. 99T

p18



11,4% 15,9% 14,3% 15,7%

FIG. 99U

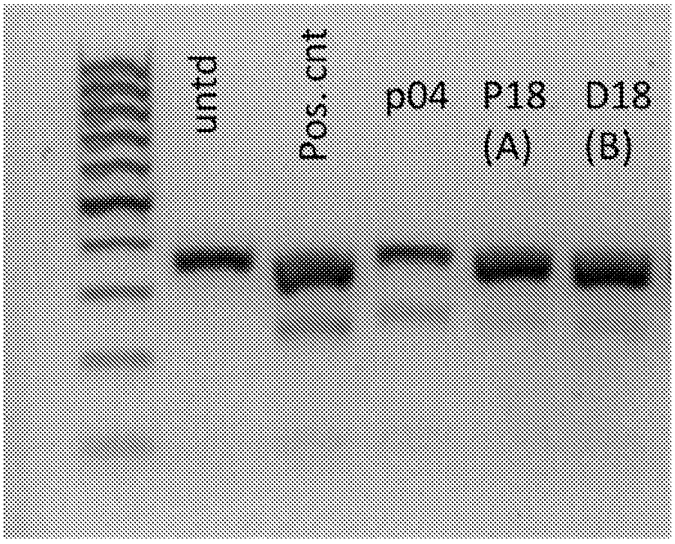


FIG. 100

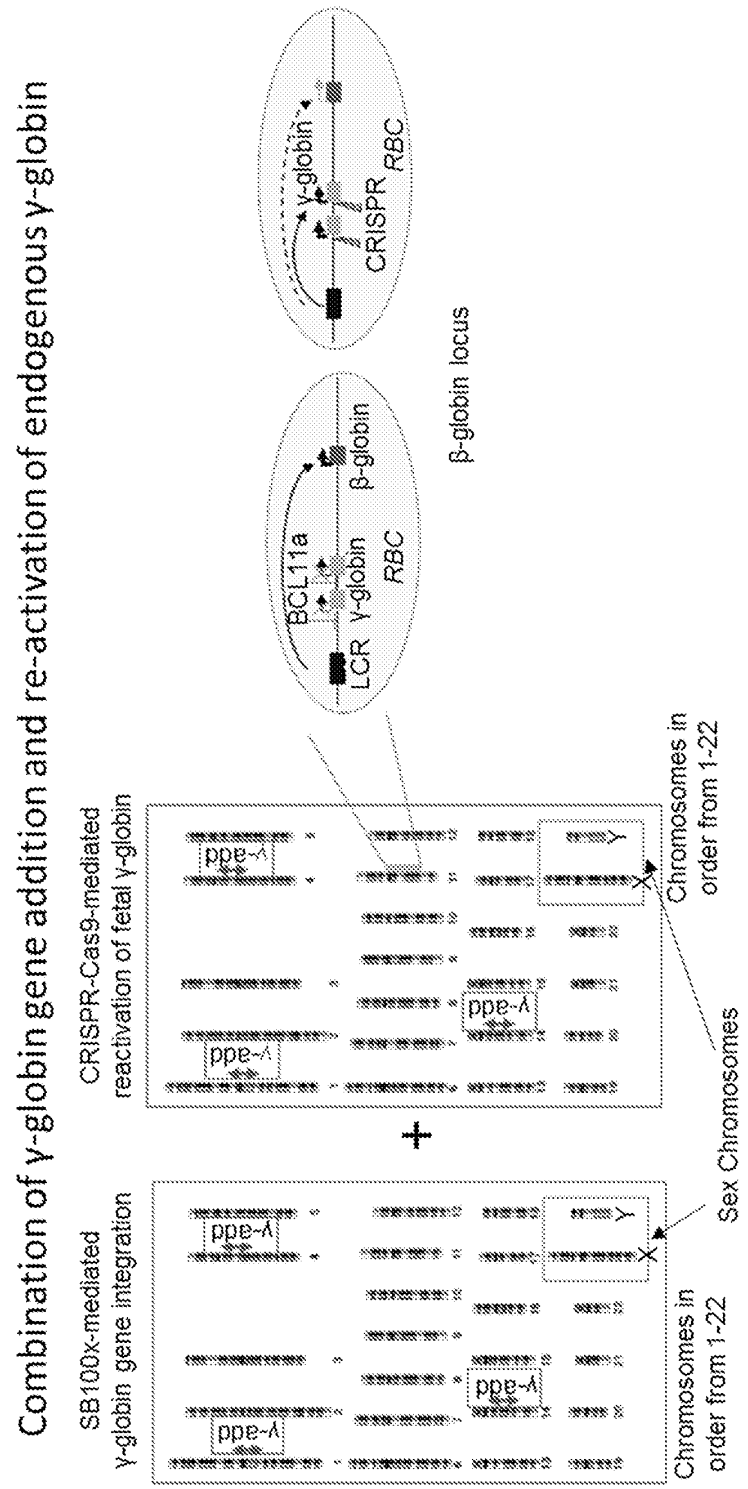

Combination of γ-globin gene addition and re-activation of endogenous γ-globin
SB100x-mediated γ-globin gene integration
CRISPR-Cas9-mediated reactivation of fetal γ-globin
γ-globin
β-globin
BCL11a
LCR
γ-globin
RBC
β-globin locus
γ-globin
CRISPR
RBC
Chromosomes in order from 1-22
Sex Chromosomes
Chromosomes in order from 1-22

FIG. 101

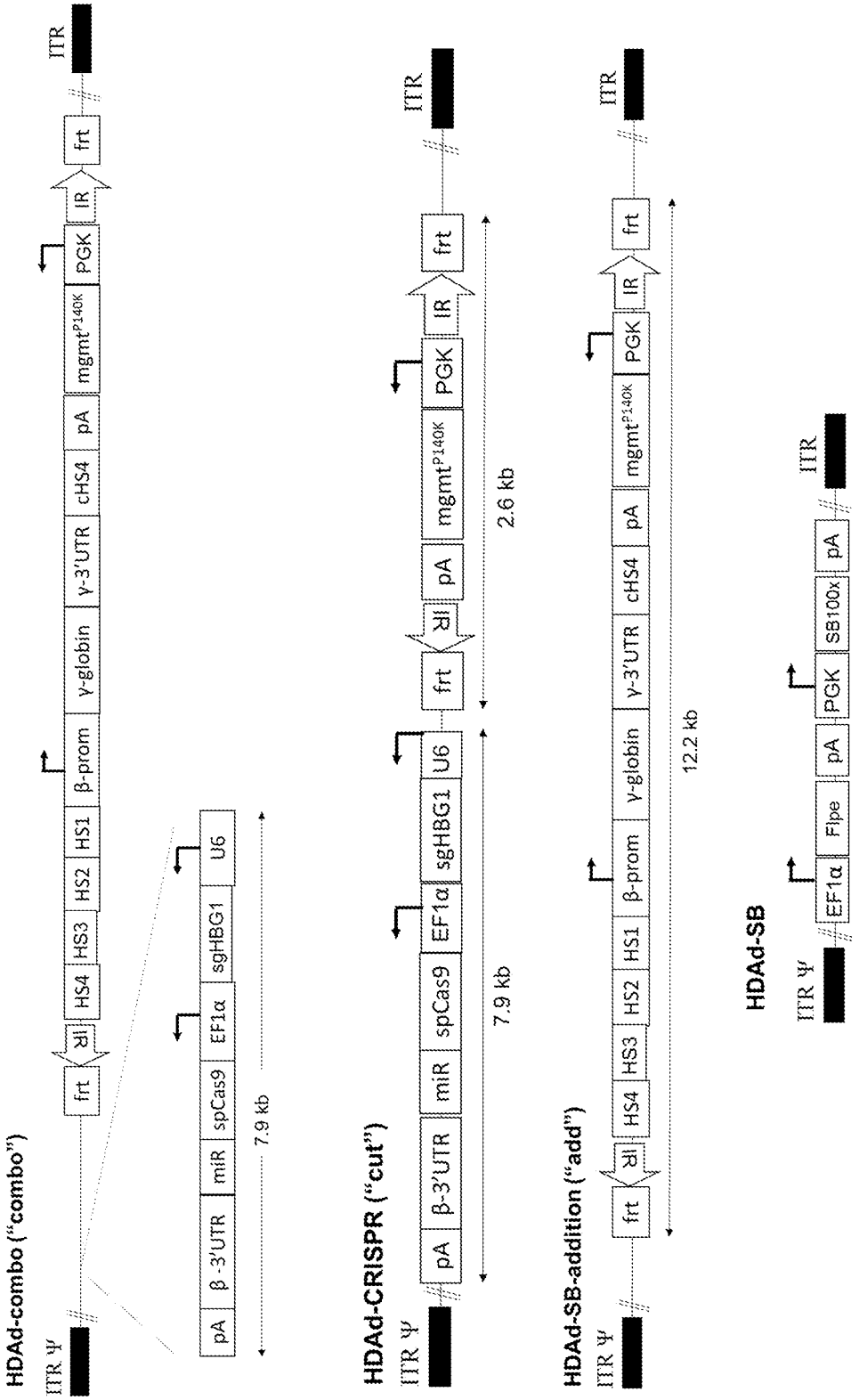

HDAd-combo ("combo")
ITR Ψ
frt
IR
HS4
HS3
HS2
HS1
β-prom
γ-globin
γ-3'UTR
cHS4
pA
mgmt^{P140K}
PGK
IR
frt
ITR
pA
β-3'UTR
miR
spCas9
EF1α
sgHBG1
U6
7.9 kb
HDAd-CRISPR ("cut")
ITR Ψ
pA
β-3'UTR
miR
spCas9
EF1α
sgHBG1
U6
7.9 kb
frt
IR
pA
mgmt^{P140K}
PGK
IR
frt
ITR
2.6 kb
HDAd-SB-addition ("add")
ITR Ψ
frt
IR
HS4
HS3
HS2
HS1
β-prom
γ-globin
γ-3'UTR
cHS4
pA
mgmt^{P140K}
PGK
IR
frt
ITR
12.2 kb
HDAd-SB
ITR Ψ
EF1α
Flpe
pA
PGK
SB100x
pA
ITR

FIG. 102

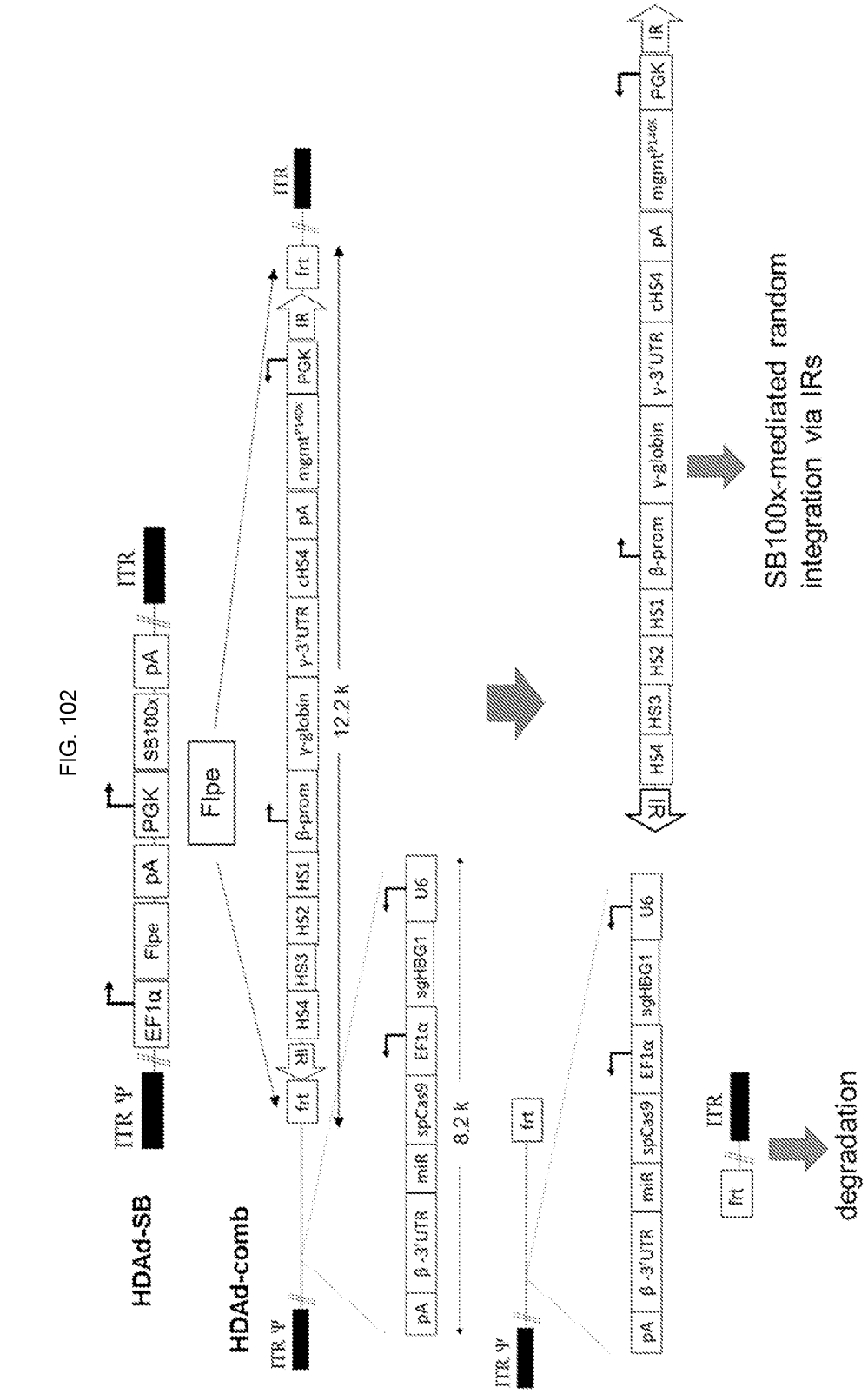

HDAd-SB
ITR Ψ
EF1α
Flpe
pA
PGK
SB100x
pA
ITR
HDAd-comb
Flpe
ITR Ψ
frt
IR
HS4
HS3
HS2
HS1
β-prom
γ-globin
γ-3'UTR
cHS4
pA
mgmt^P140K
PGK
IR
frt
ITR
12.2 k
pA
β-3'UTR
miR
spCas9
EF1α
sgHBG1
U6
8.2 k
frt
ITR Ψ
SB100x-mediated random integration via IRs
IR
HS4
HS3
HS2
HS1
β-prom
γ-globin
γ-3'UTR
cHS4
pA
mgmt^P140K
PGK
IR
frt
ITR
degradation

FIG. 103A

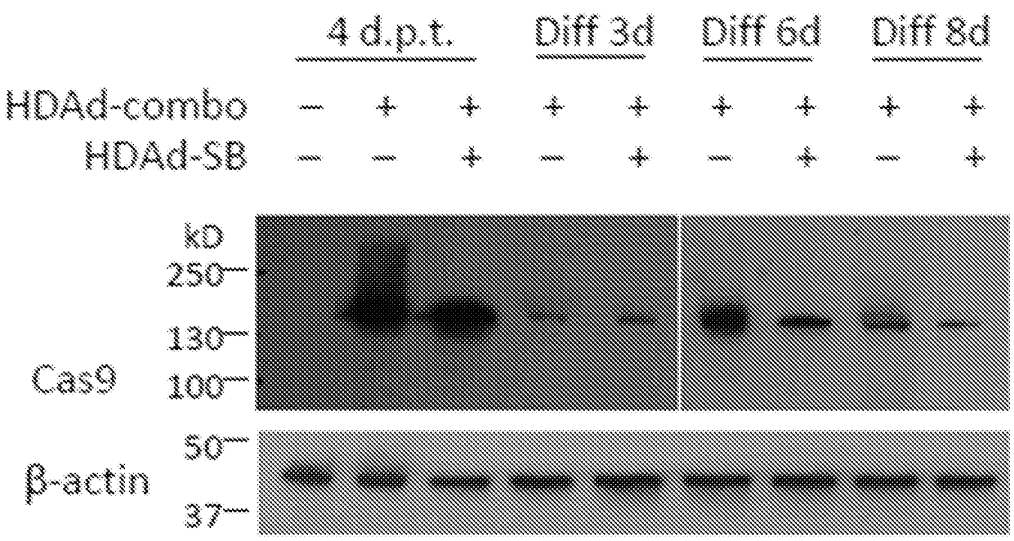


FIG. 103B

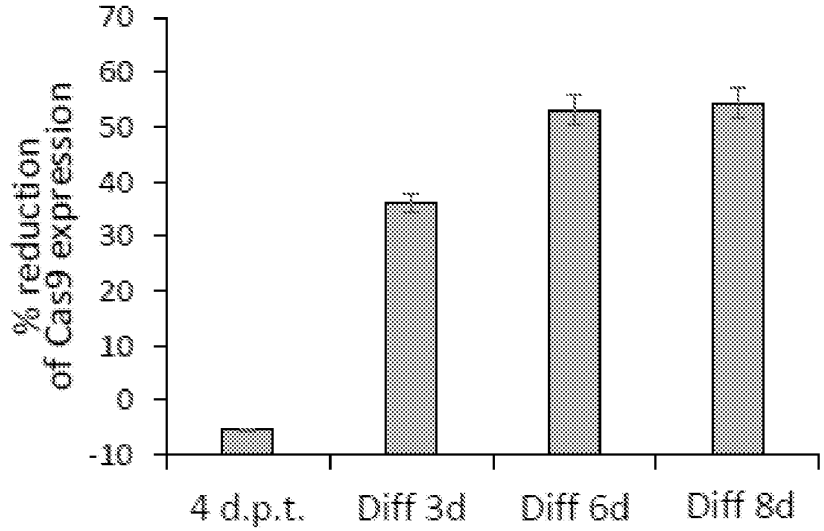


FIG. 103C

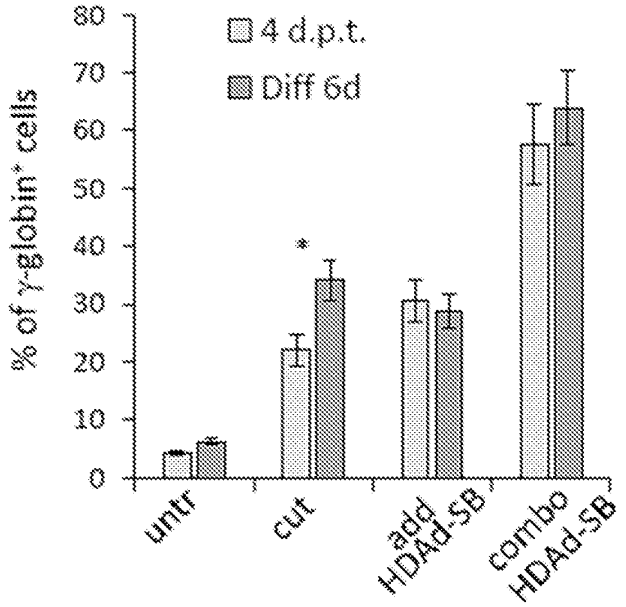


FIG. 103D

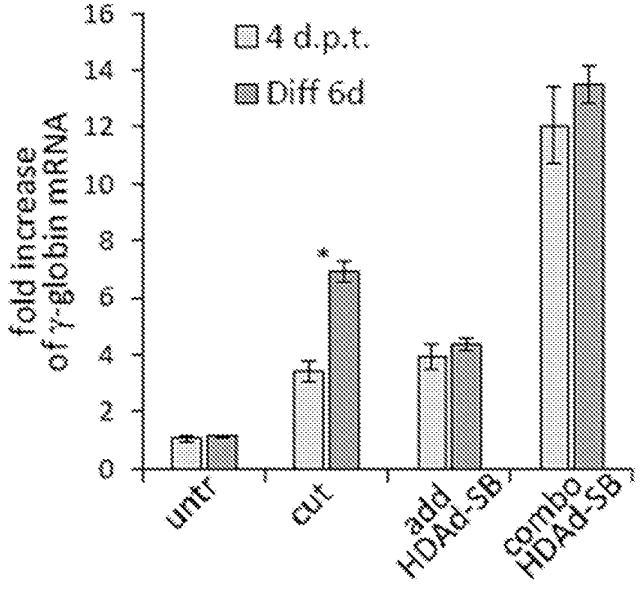


FIG. 104A

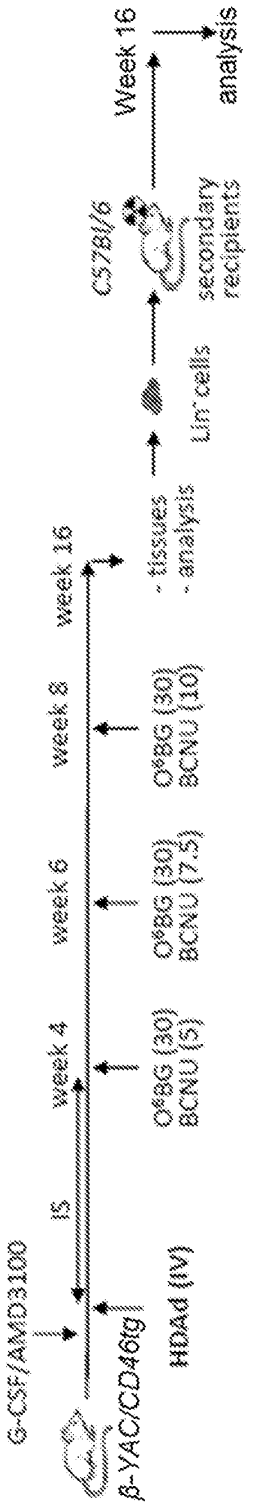


FIG. 104B

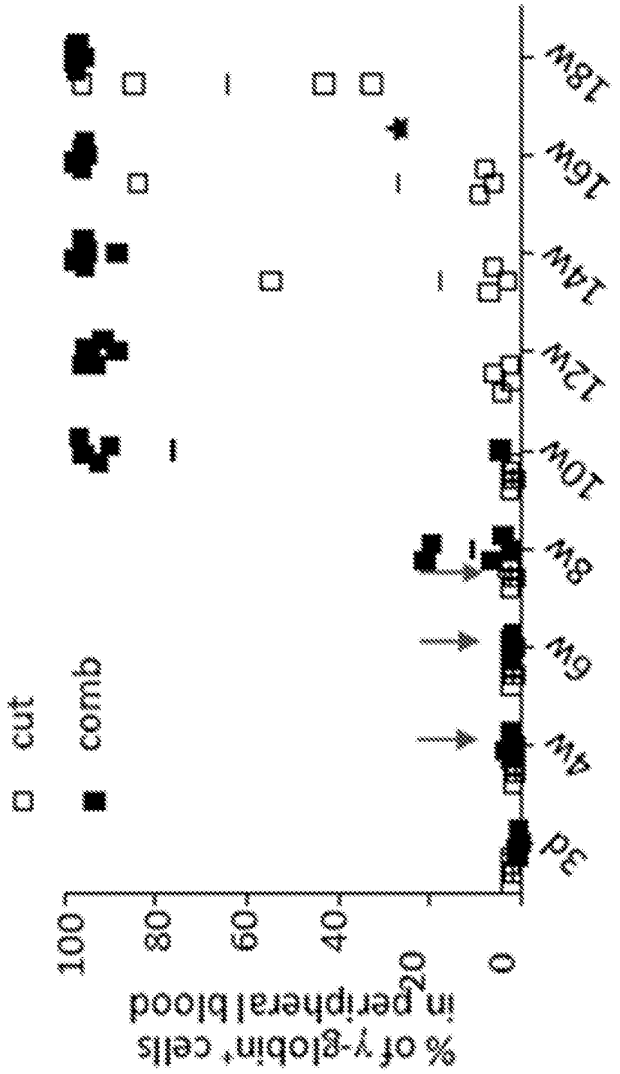


FIG. 104C

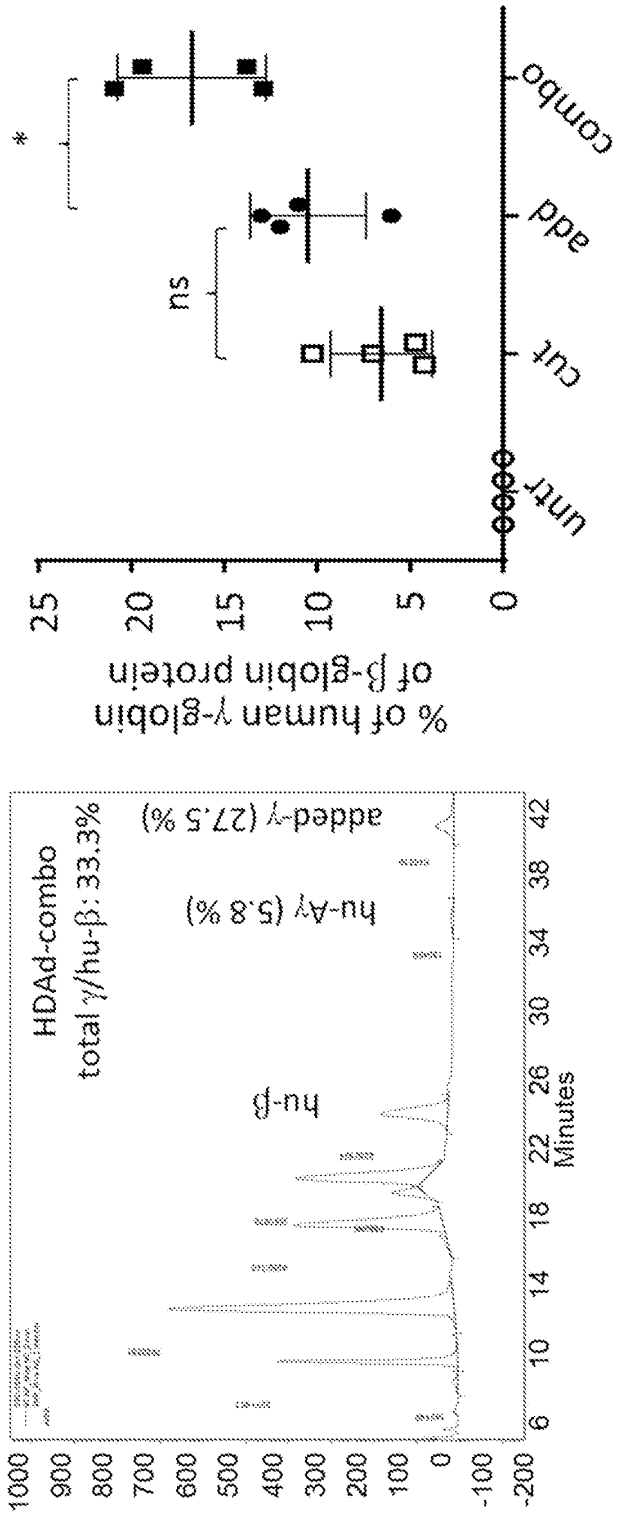

% of human γ-globin of β-globin protein
25
20
15
10
5
0
untr
cut
add
combo
ns
*
HDAd-combo
total γ/hu-β: 33.3%
hu-β
hu-Aγ (5.8 %)
added-γ (27.5 %)
1000
900
800
700
600
500
400
300
200
100
0
-100
-200
6
10
14
18
22
26
30
34
38
42
Minutes

FIG. 104D

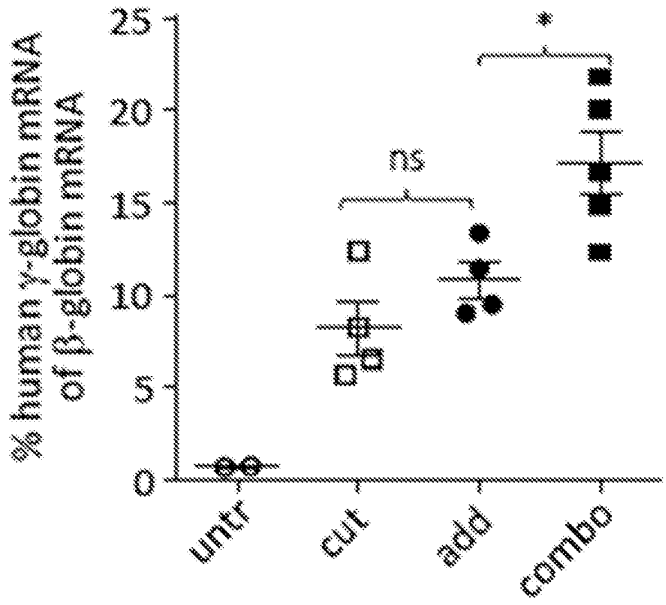


FIG. 104E

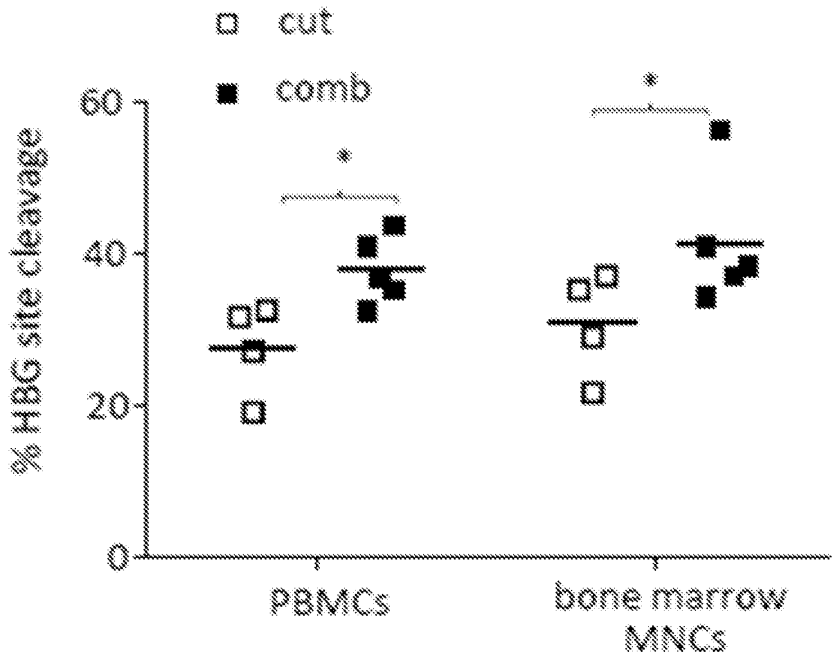


FIG. 104F

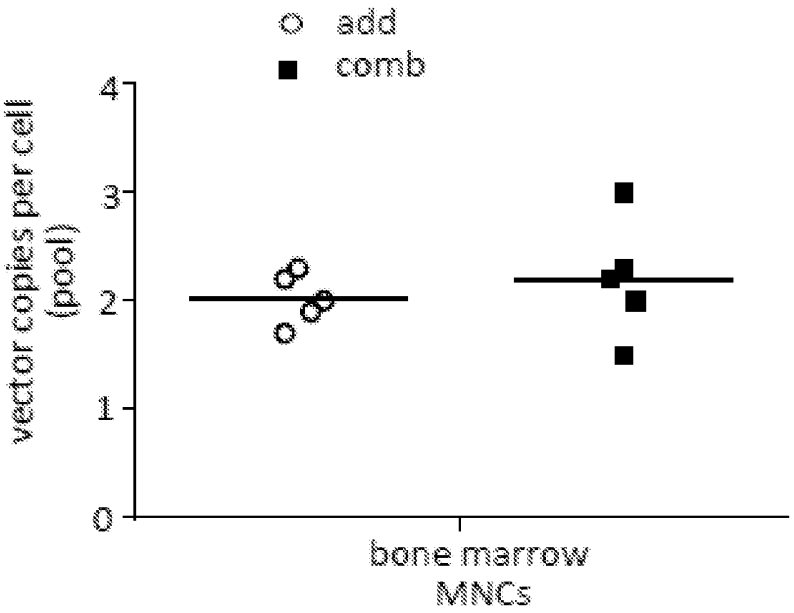


FIG. 104G

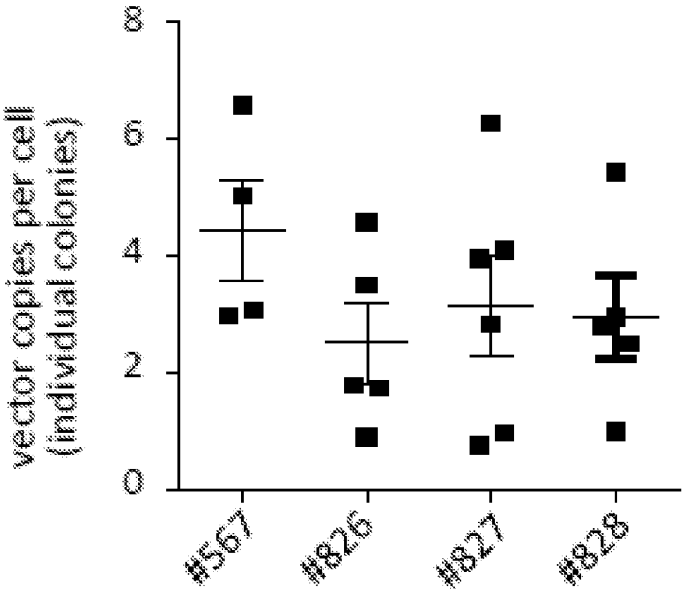


FIG. 104H

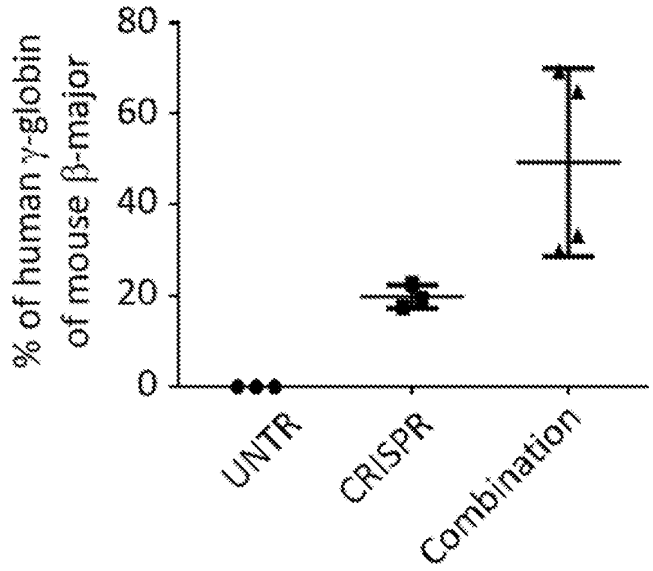


FIG. 105

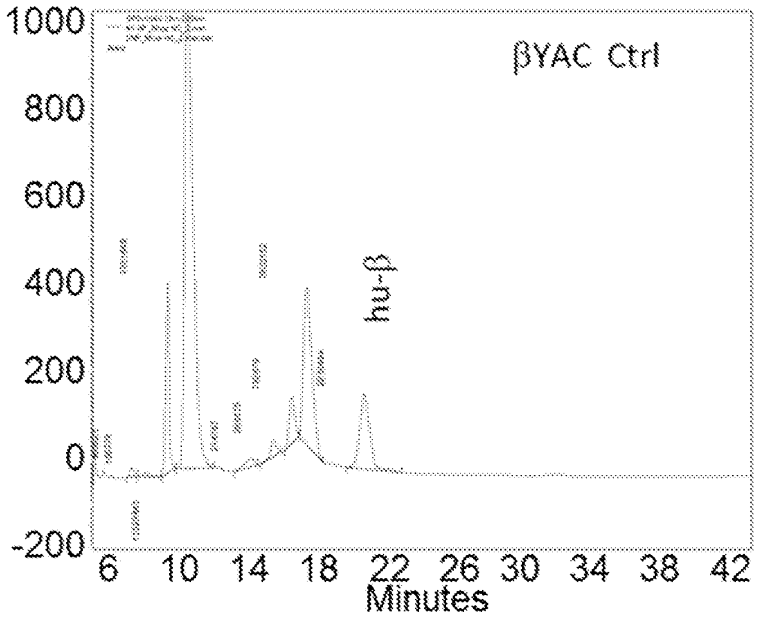


FIG. 105 cont'd

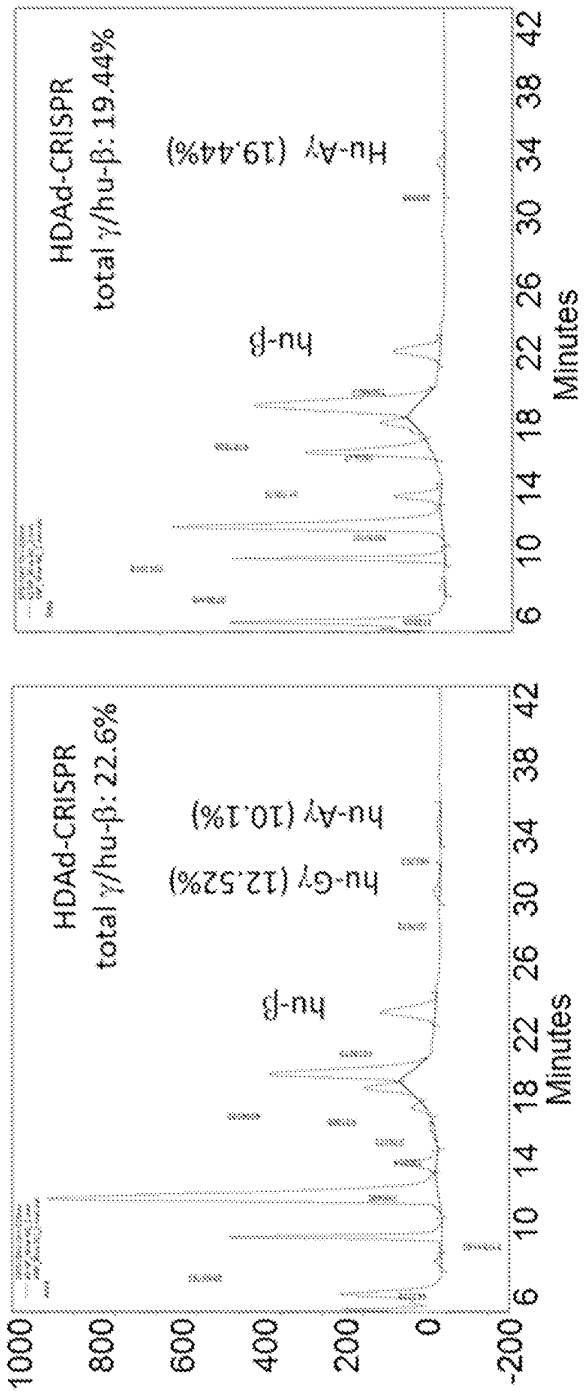


FIG. 105 cont'd

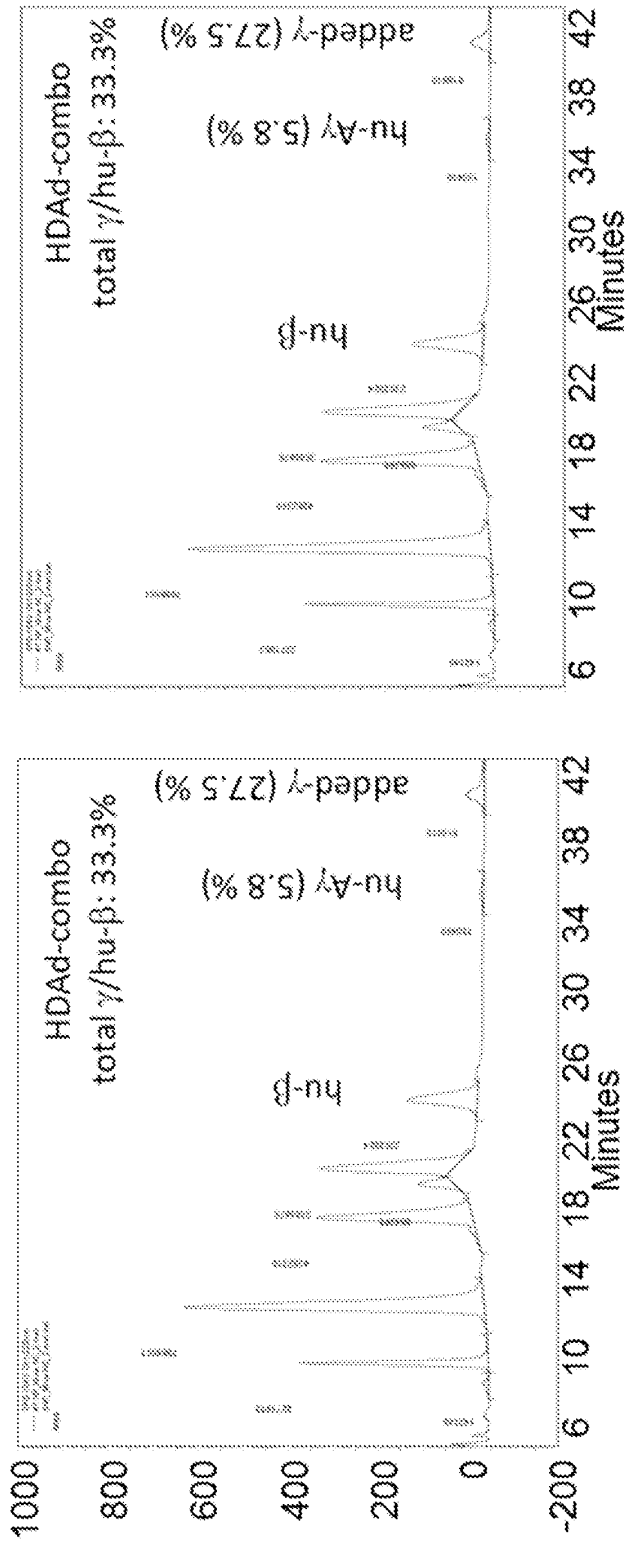
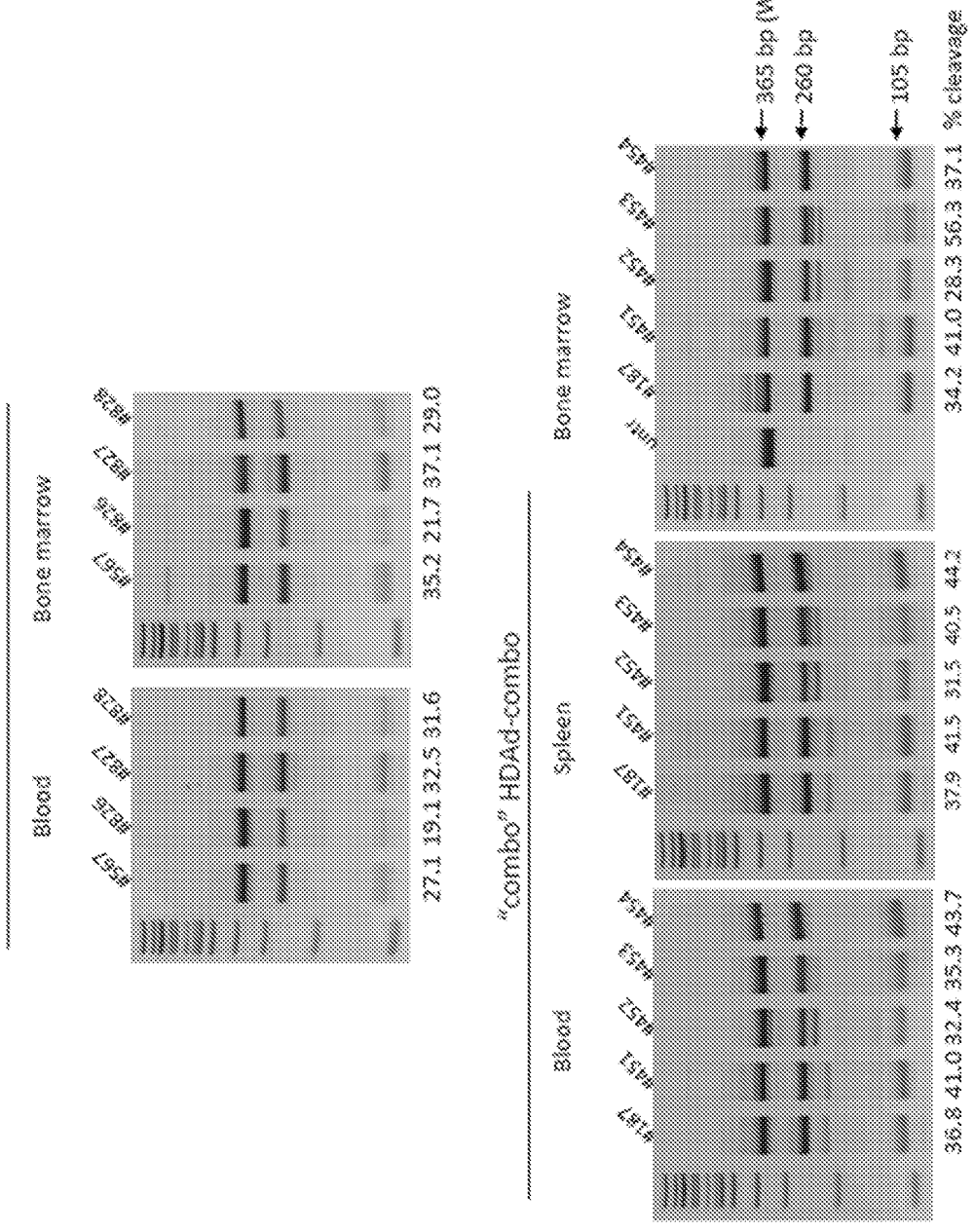


FIG. 106


"cut" HDAd-CRISPR
Blood
Bone marrow
#253
#254
#255
#256
27.1 19.1 32.5 31.6
35.2 21.7 37.1 29.0
"combo" HDAd-combo
Blood
Spleen
Bone marrow
#147
#151
#152
#153
#154
#187
#188
#189
#190
#191
36.8 41.0 32.4 35.3 43.7
37.9 41.5 31.5 40.5 44.2
34.2 41.0 28.3 56.3 37.1
365 bp (WT)
260 bp
105 bp
% cleavage

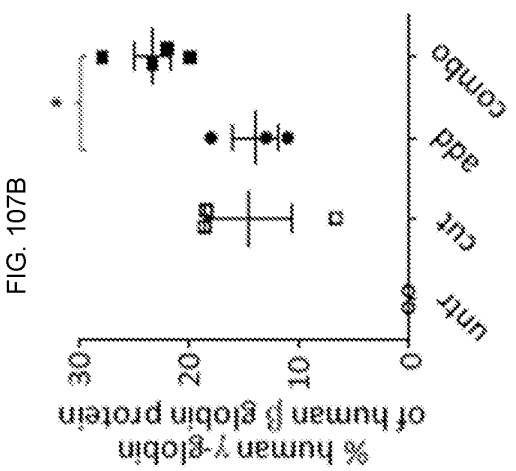


FIG. 107B

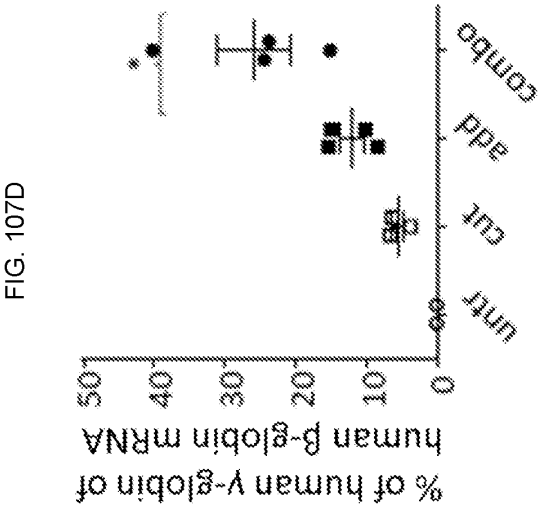


FIG. 107D

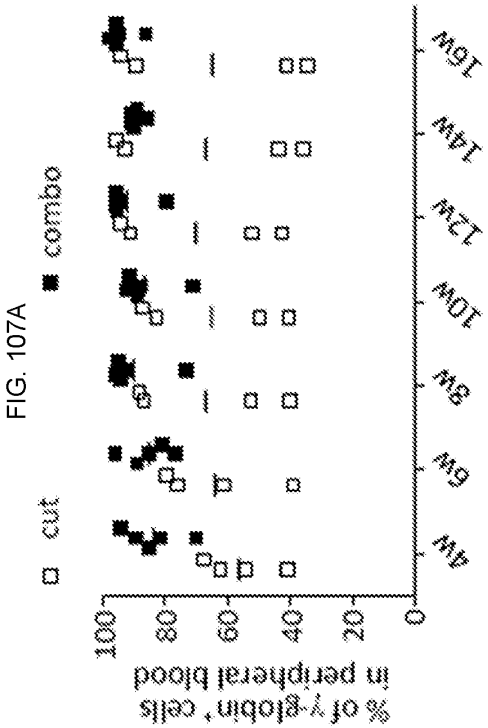


FIG. 107A

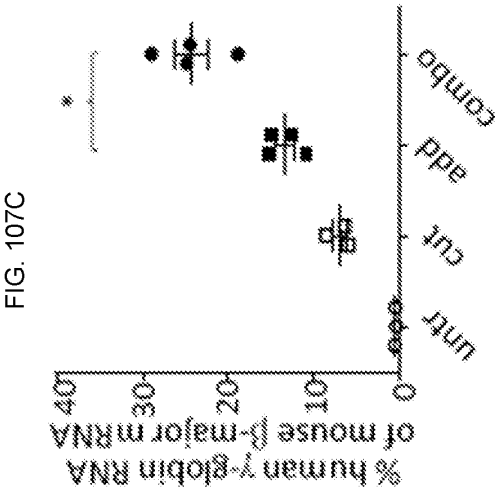


FIG. 107C

FIG. 107E

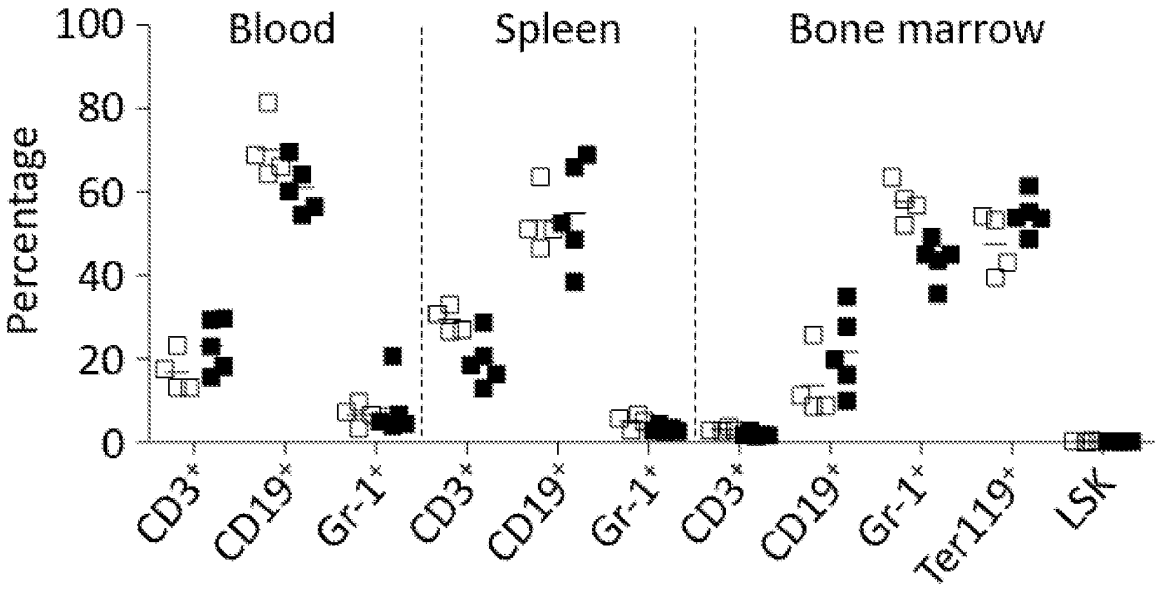


FIG. 107F

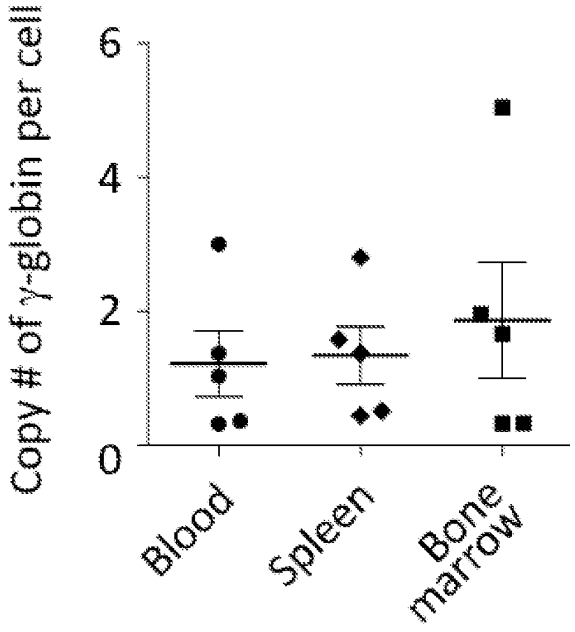


FIG. 108A

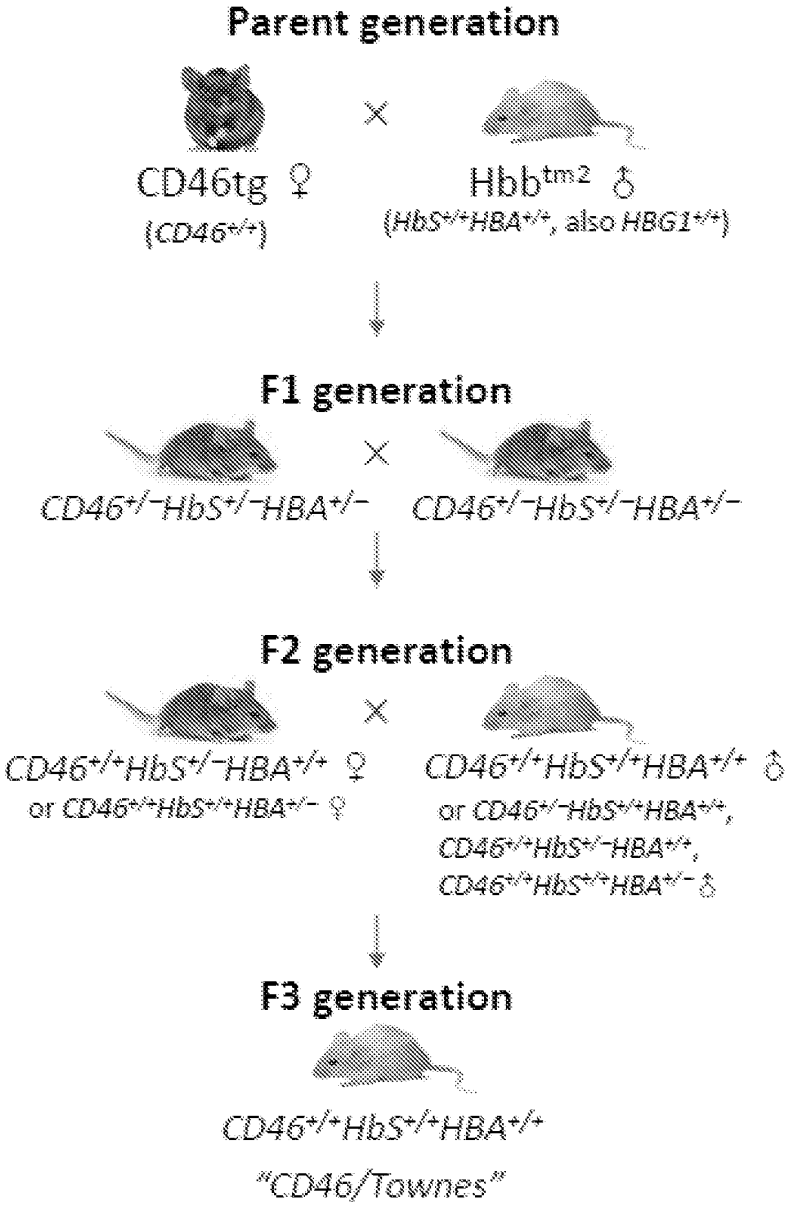

Parent generation
CD46tg ♀
(CD46+/+)
×
Hbbtm2 ♂
(HbS+/+HBA+/+, also HBG1+/+)
F1 generation
CD46+/−HbS+/−HBA+/−
×
CD46+/−HbS+/−HBA+/−
F2 generation
CD46+/+HbS+/−HBA+/+ ♀
or CD46+/+HbS+/+HBA+/− ♀
×
CD46+/+HbS+/+HBA+/+ ♂
or CD46+/−HbS+/+HBA+/+,
CD46+/+HbS+/−HBA+/+,
CD46+/+HbS+/+HBA+/− ♂
F3 generation
CD46+/+HbS+/+HBA+/+
"CD46/Townes"

FIG. 108B

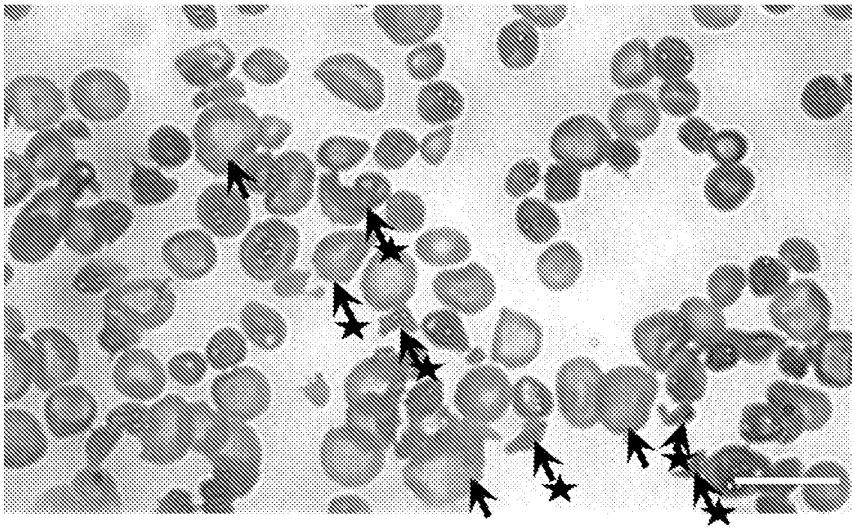


FIG. 108C

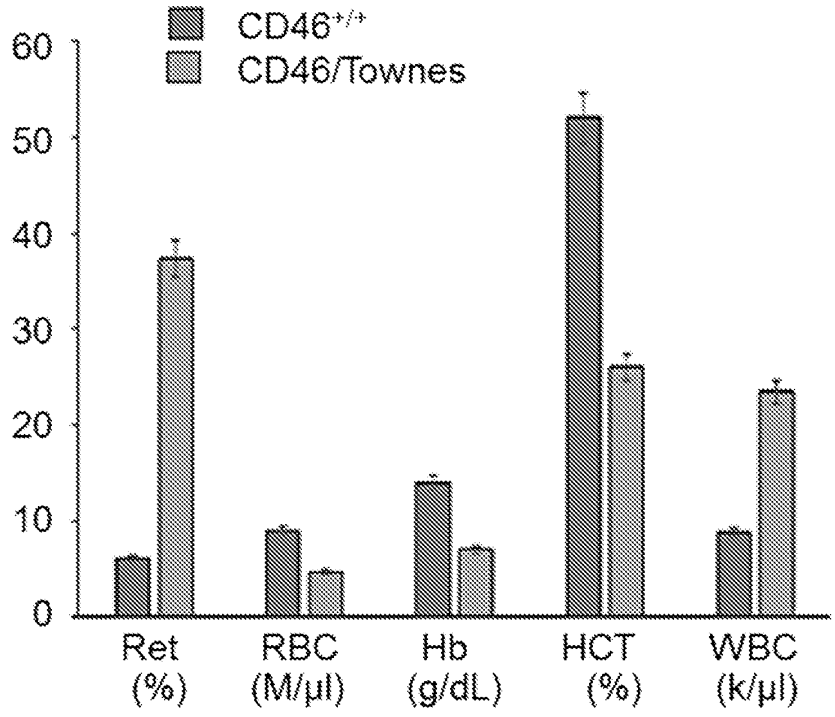


FIG. 108D

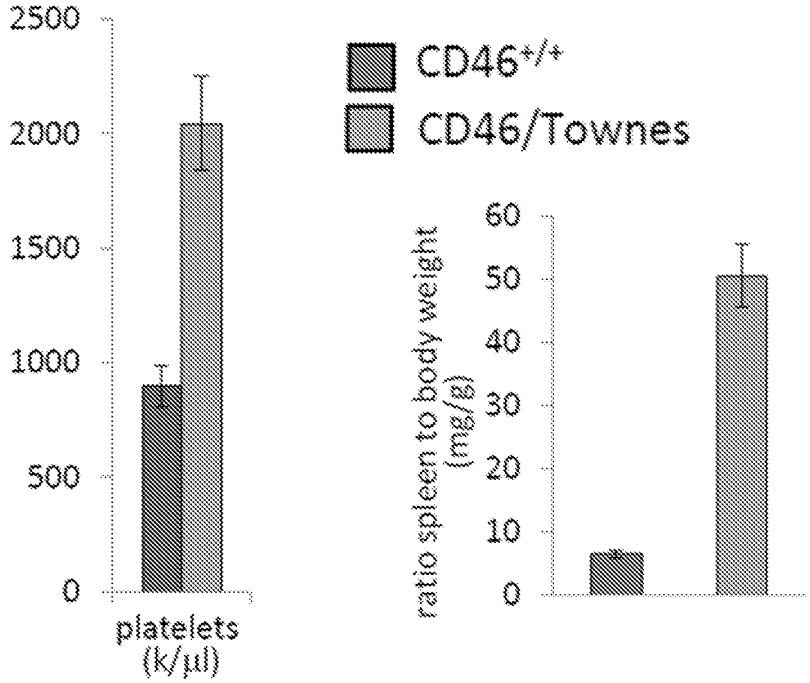


FIG. 109A

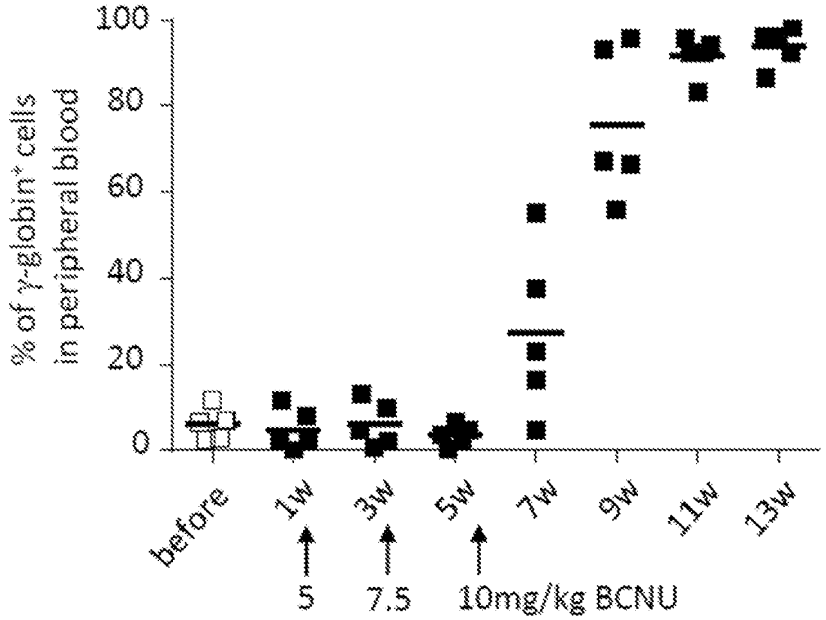


FIG. 109B

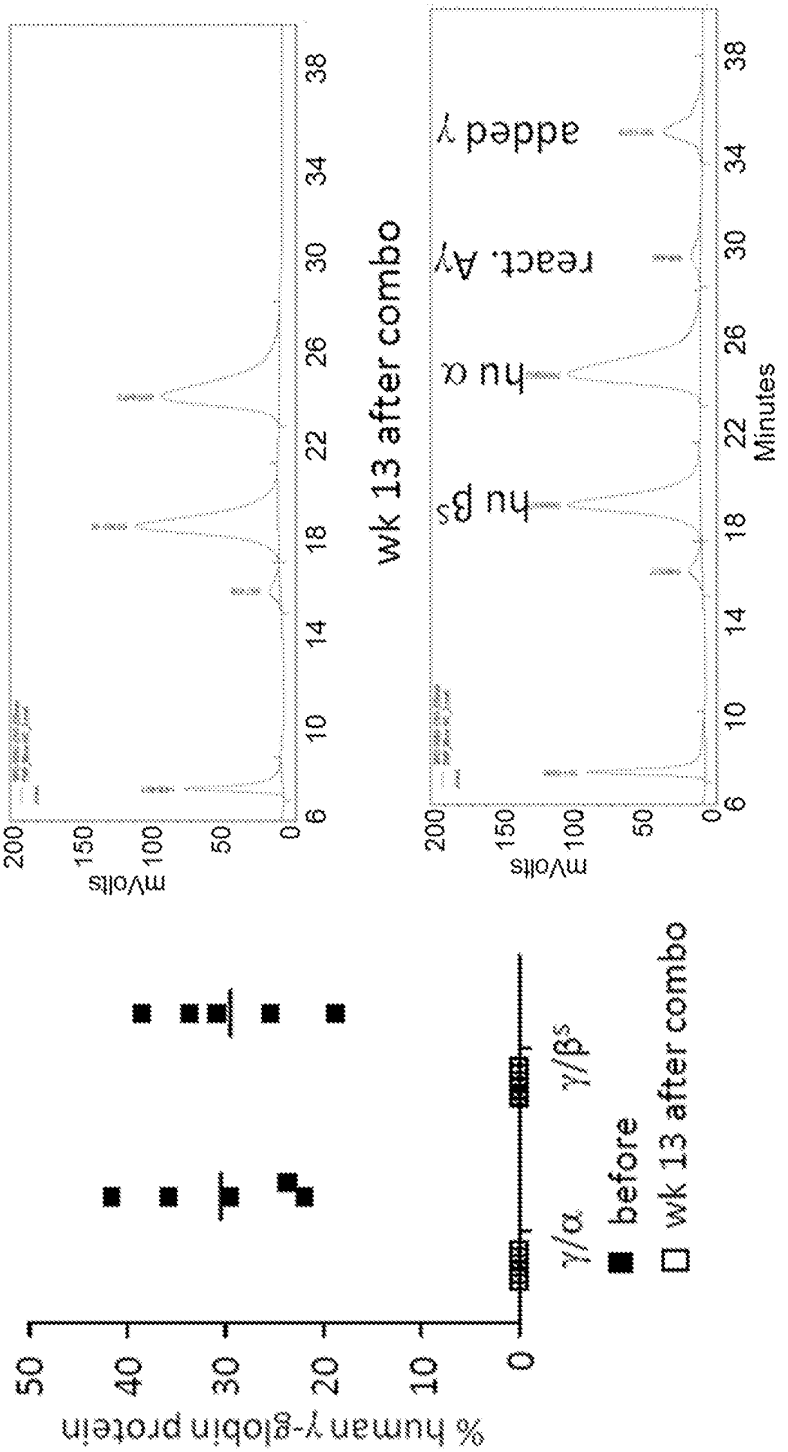

before
wk 13 after combo
mVolts
Minutes
hu βs
hu α
react. Ay
added γ
% human γ-globin protein
γ/α
γ/βs
before
wk 13 after combo

FIG. 109C

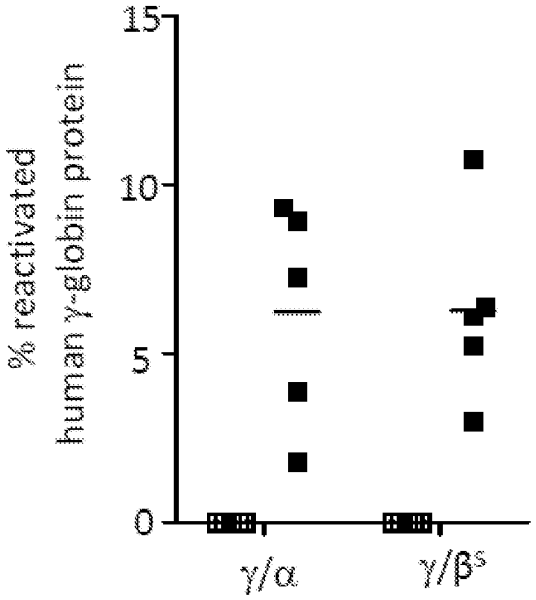


FIG. 109D

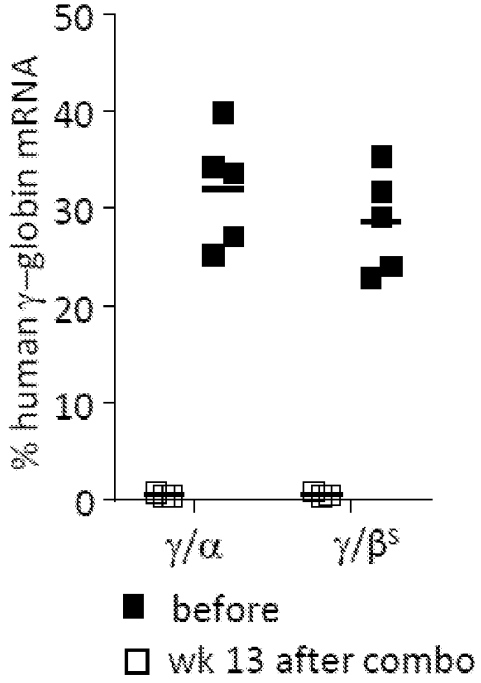


FIG. 109E

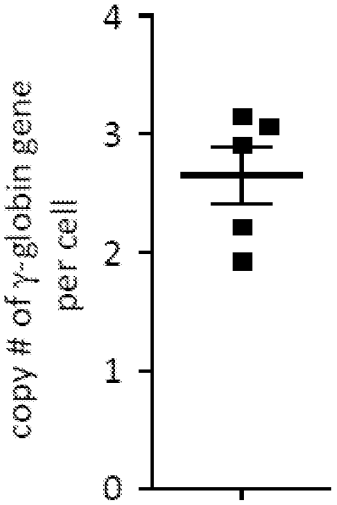


FIG. 109F

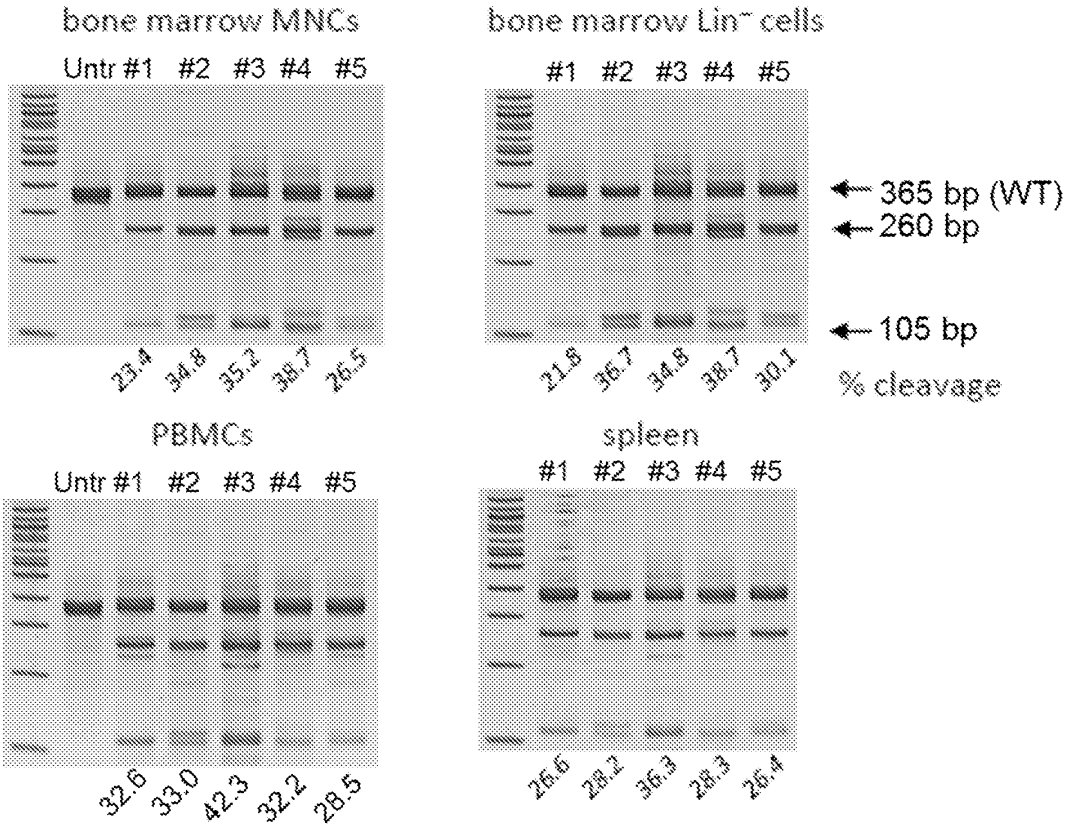


FIG. 110A

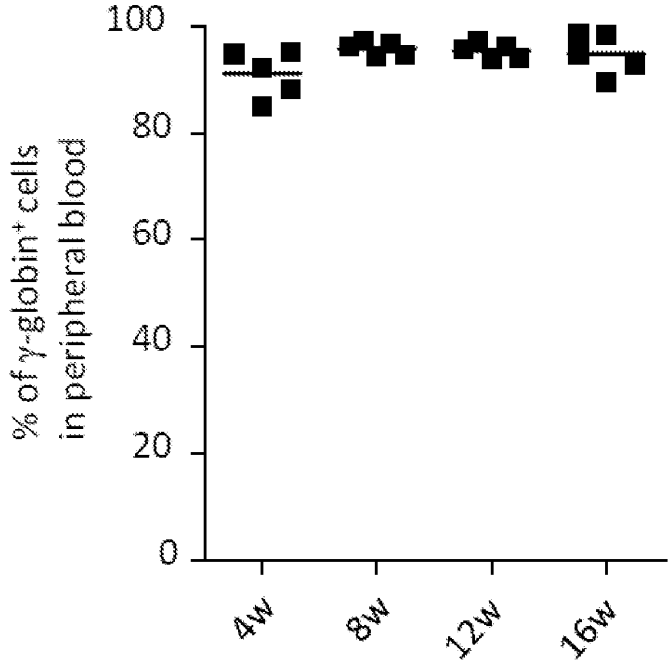


FIG. 110B

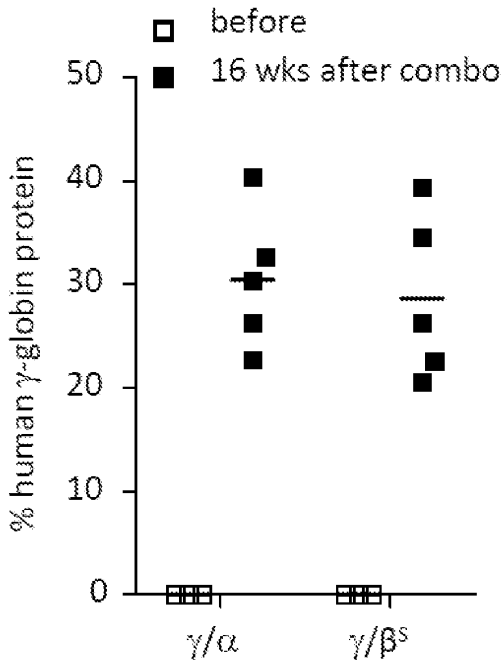


FIG. 111A

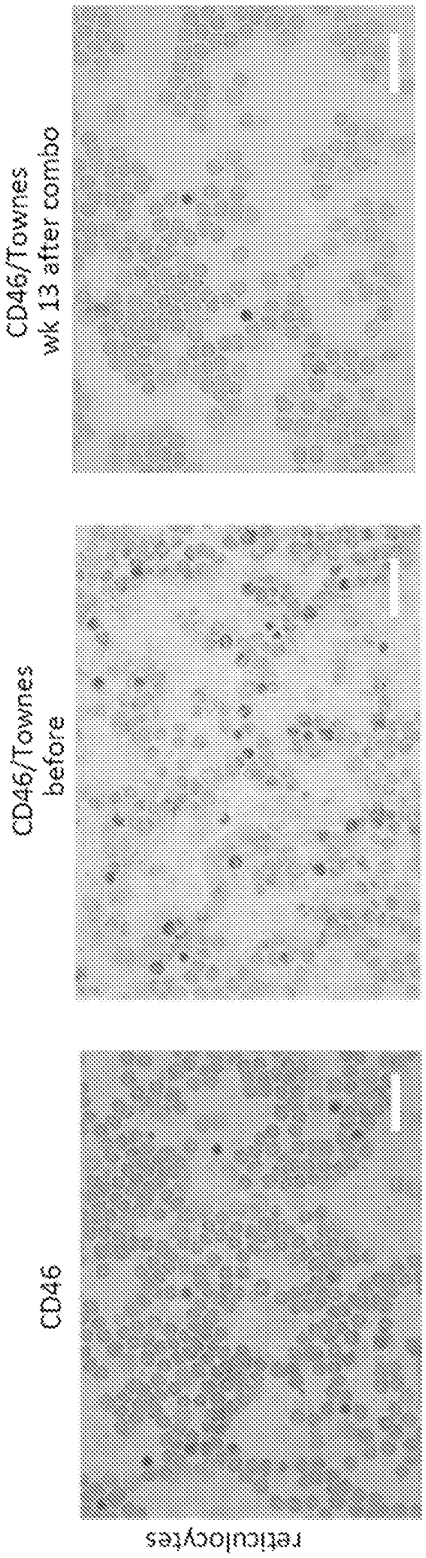


FIG. 111B

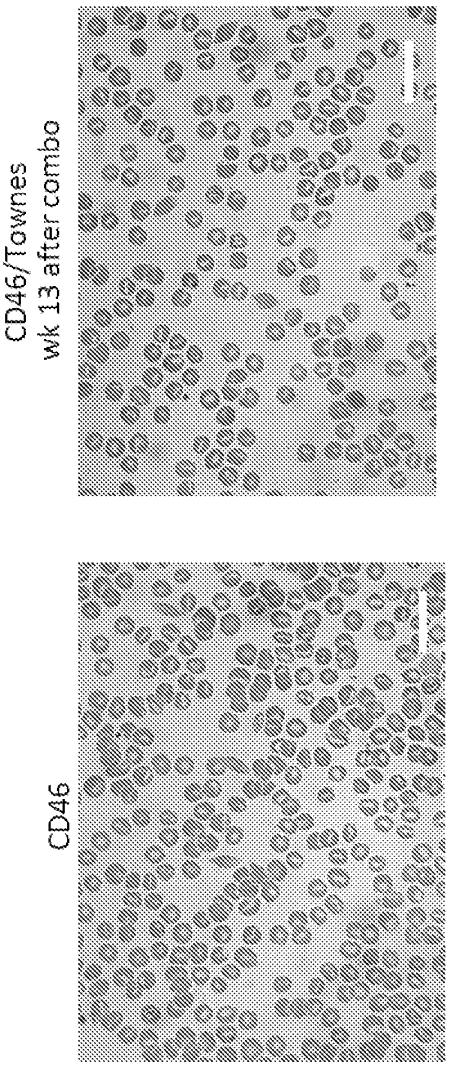


FIG. 111C

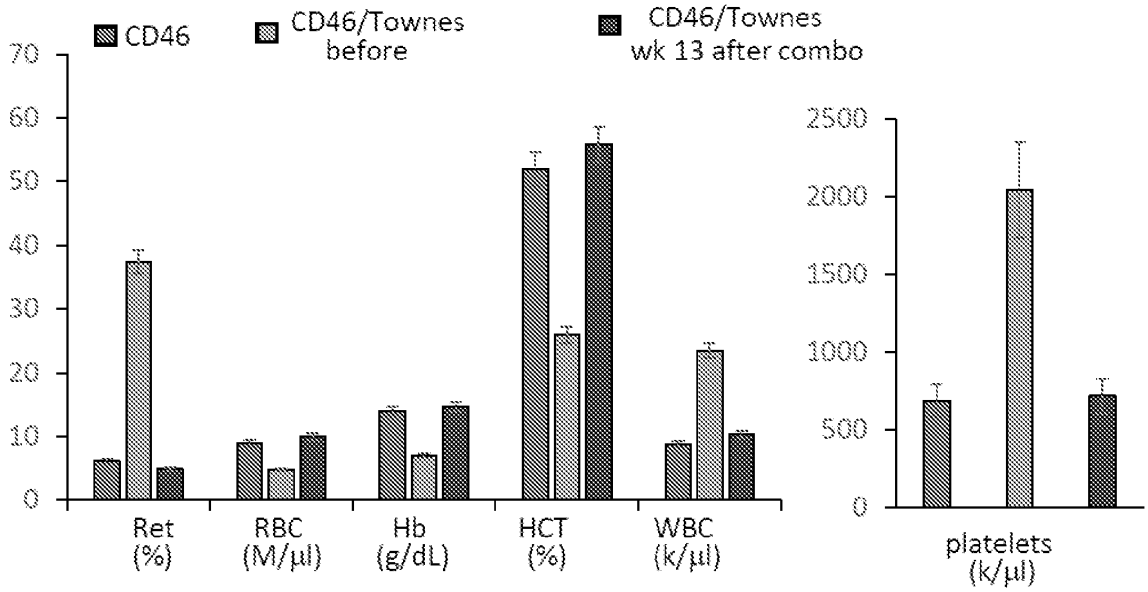

CD46
CD46/Townes before
CD46/Townes wk 13 after combo
70
60
50
40
30
20
10
0
Ret (%)
RBC (M/µl)
Hb (g/dL)
HCT (%)
WBC (k/µl)
2500
2000
1500
1000
500
0
platelets (k/µl)

FIG. 112A

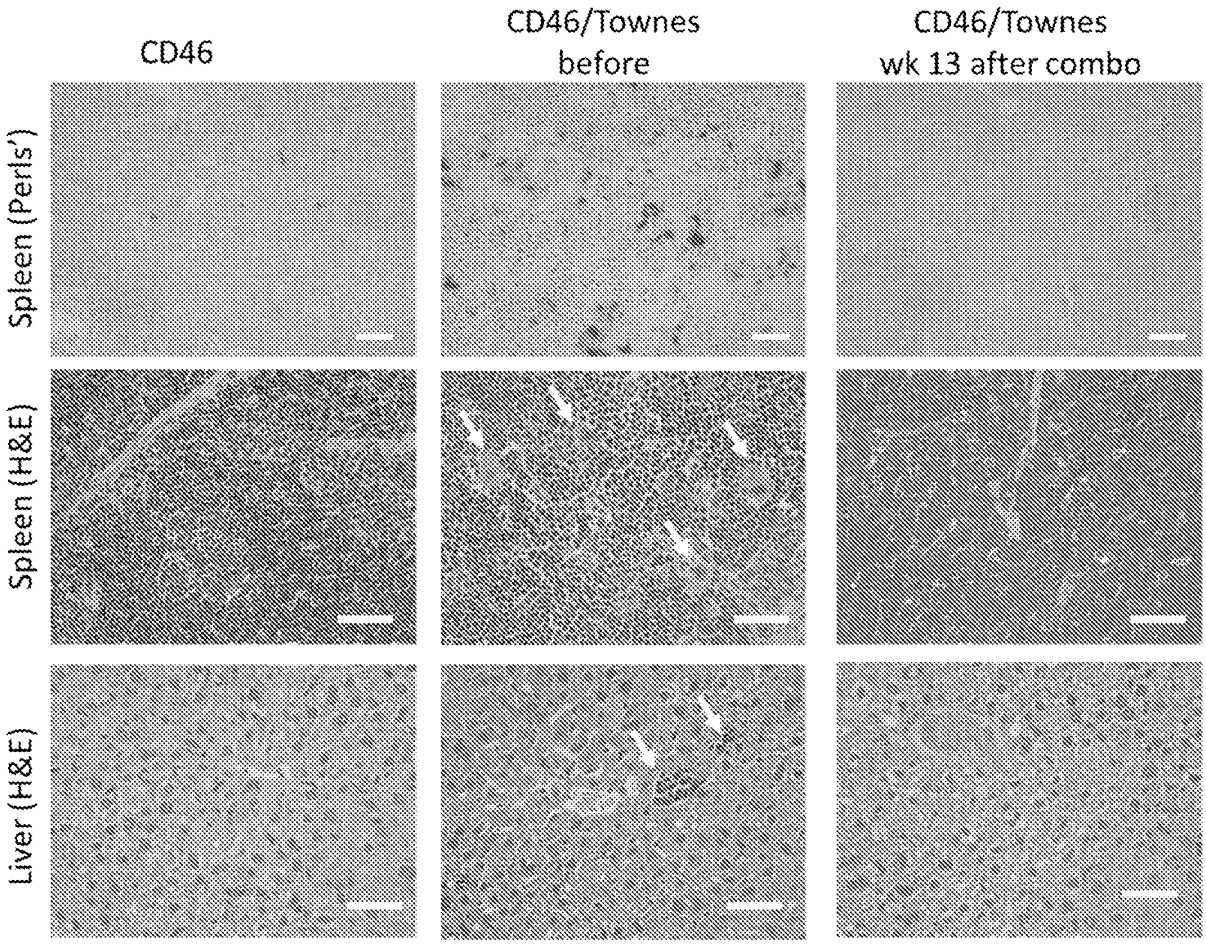
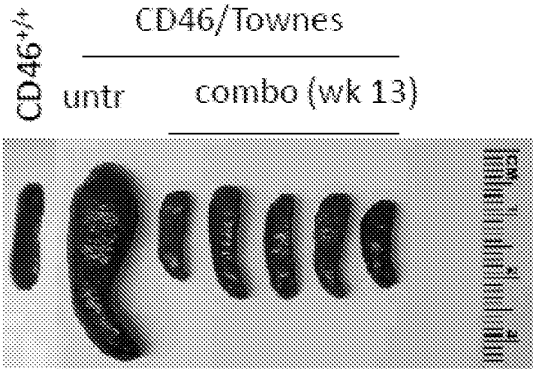


FIG. 112B



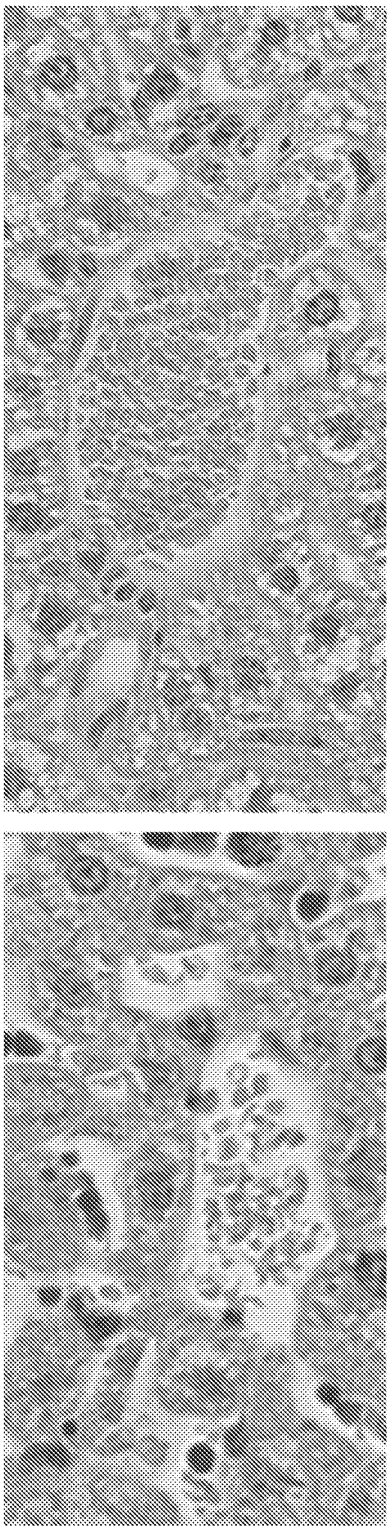
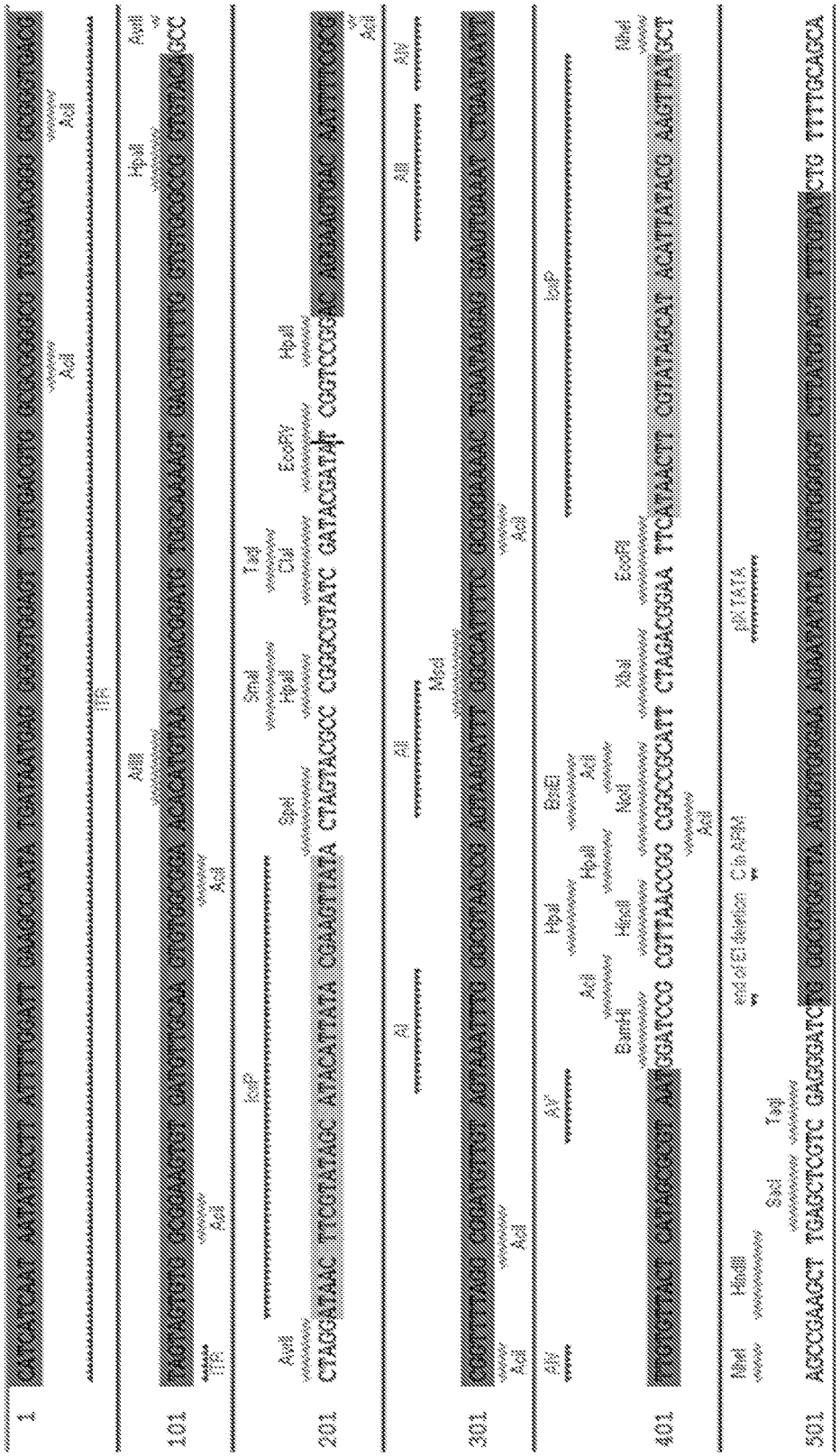


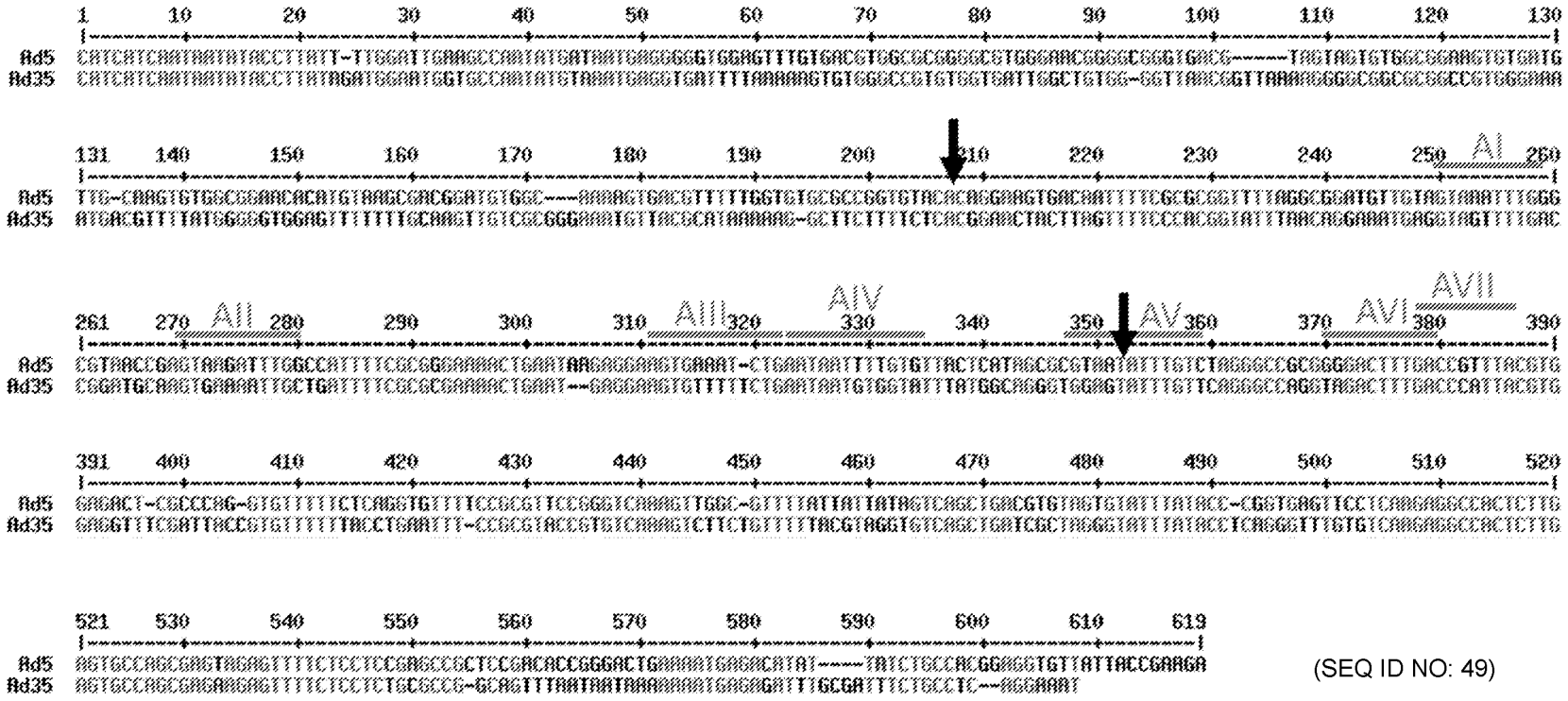
FIG. 112C

FIG. 113



SEQ ID NO: 262

FIG. 114



(SEQ ID NO: 49)

(SEQ ID NO: 50)

FIG. 115

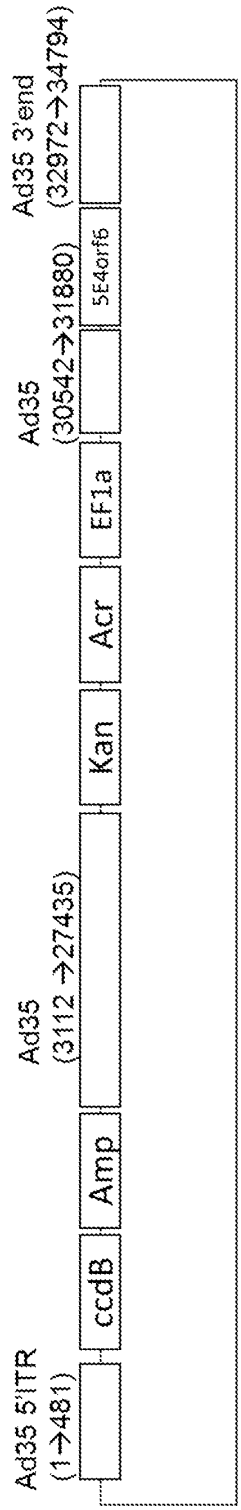


FIG. 116

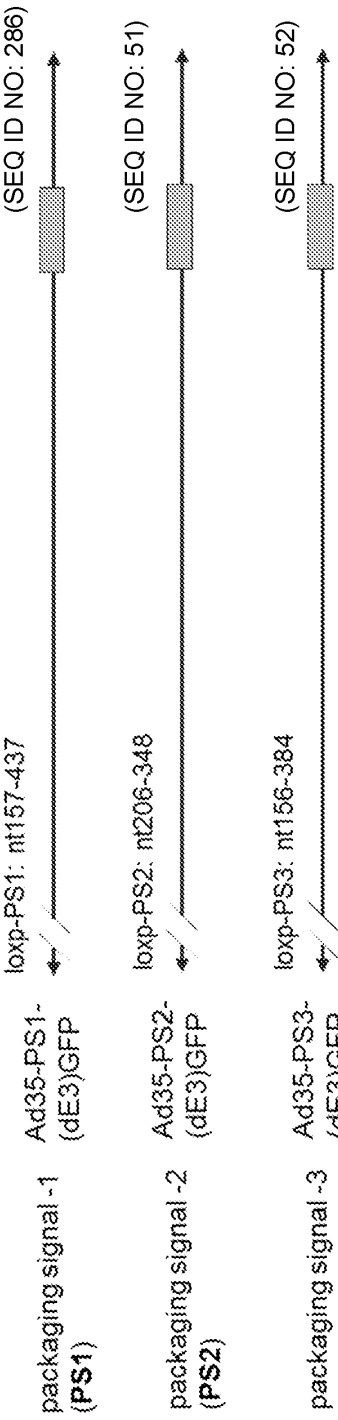


FIG. 117

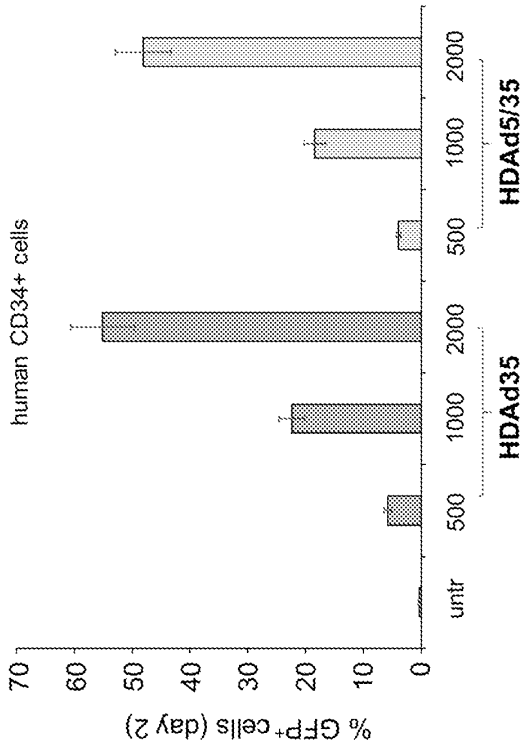


FIG. 118

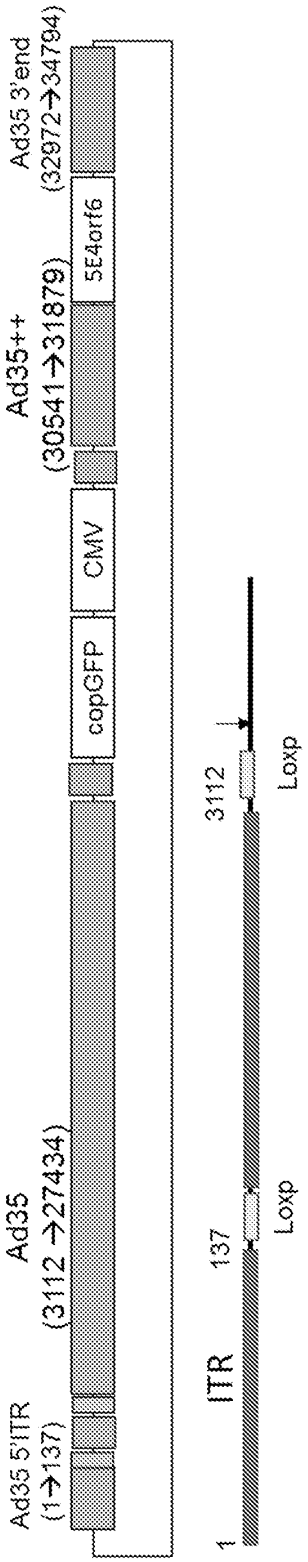


FIG. 119

CATCATCAATAATATACCTTATAGATGGAATGGTGCCAATATGTAAATGAGGTGATTTTAAAAAGTGTGGGCCGTGTGGTGATTGG
CTGTGGGGTTAACGGTTAAAAGGGGCGGCGCGGCCGTGGGAAAATGACGTT**ATTTAAAT**ATAACTTCGTATAGCATACATTATA
CGAAGTTATGAGGTAGTTTTGTTCAGGGGCAAGTGAAAATTGACCCATTACGCGCGAAAACTGAATGAGGAAGTGTTTTTCTGAA
TAATGTGGTATTTATGGCAGGGTGGAGTATTTGACCGGATCCAGGTAGACTTTGCTGATTTTCGTGGAGGTTT*ATAACTTCGTATA*
*GCATACATTATACGAAGTTAT***ATTTAAAT** (SEQ ID NO: 264)

Ad35 ITR 1→137bp: (positions 1-137 of SEQ ID NO: 264)

CATCATCAATAATATACCTTATAGATGGAATGGTGCCAATATGTAAATGAGGTGATTTTAAAAAGTGTGGGCCGTGT
GGTGATTGGCTGTGGGGTTAACGGTTAAAAGGGGCGGCGCGGCCGTGGGAAAATGACGTT

SwaI site: (positions 146-145 and 363-370 of SEQ ID NO: 264) ATTTAAAT

LoxP site: (positions 138-179 and 329-362 of SEQ ID NO: 264) ATAACTTCGTATAGCATACATTATACGAAGTTAT

Mutated packaging signal: (positions 138-145 of SEQ ID NO: 264)

GAGGTAGTTTTGTTCAGGGGCAAGTGAAAATTGACCCATTACGCGCGAAAACTGAATGAGGAAGTGTTTTTCTGAAT
AATGTGGTATTTATGGCAGGGTGGAGTATTTGACCGGATCCAGGTAGACTTTGCTGATTTTCGTGGAGGTTT

FIG. 120A

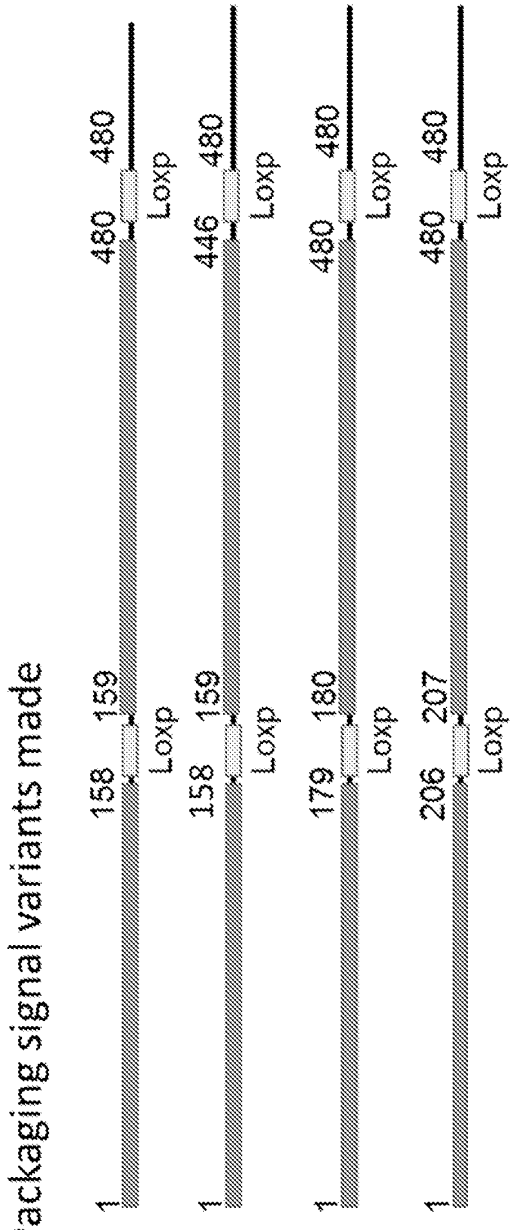

Packaging signal variants made
1
158
159
Loxp
480
480
Loxp
1
158
159
Loxp
446
480
Loxp
1
179
180
Loxp
480
480
Loxp
1
206
207
Loxp
480
480
Loxp

FIG. 120B

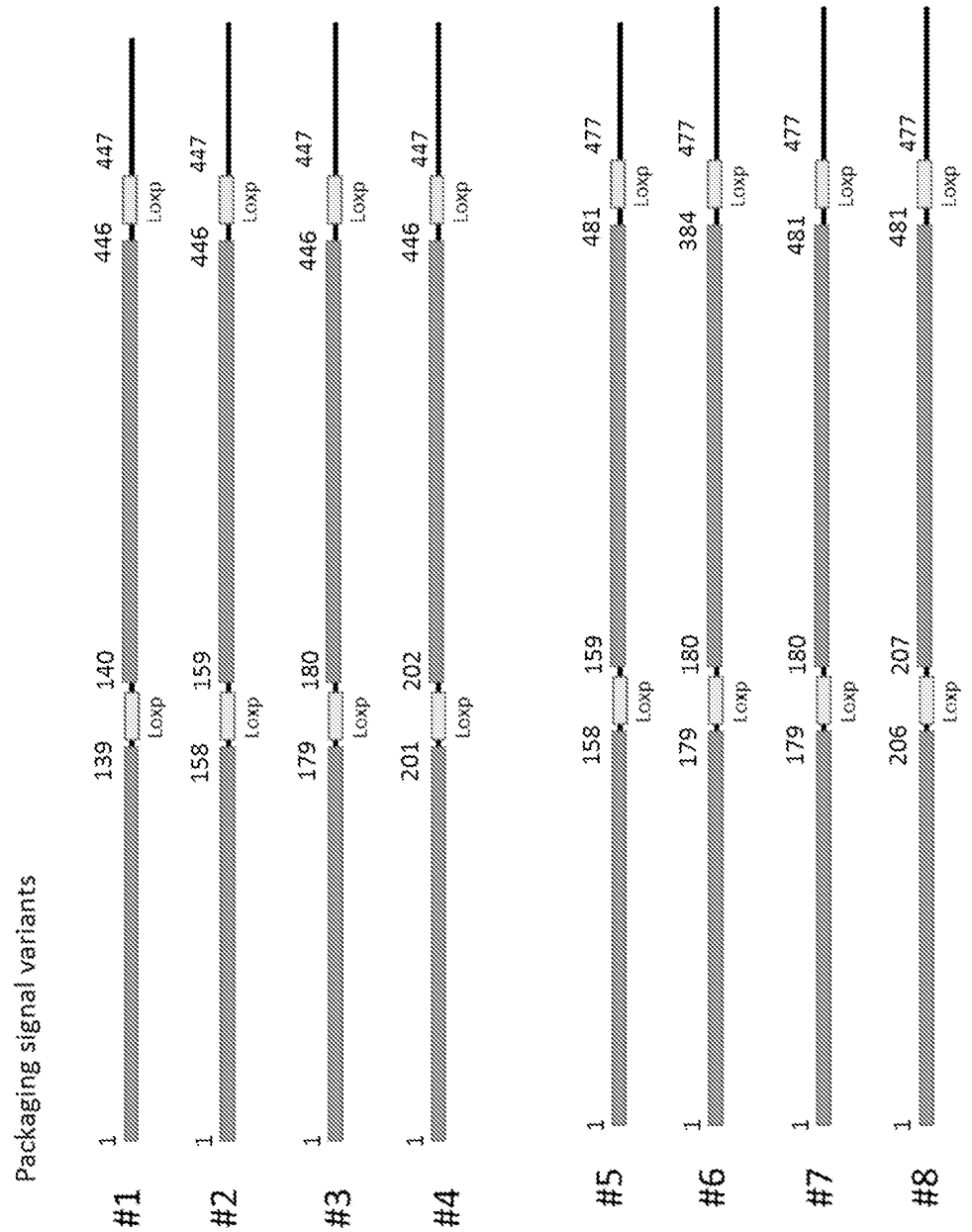

Packaging signal variants
#1
1
139
140
446
447
LoxP
#2
1
158
159
446
447
LoxP
#3
1
179
180
446
447
LoxP
#4
1
201
202
446
447
LoxP
#5
1
158
159
481
477
LoxP
#6
1
179
180
384
477
LoxP
#7
1
179
180
481
477
LoxP
#8
1
206
207
481
477
LoxP

FIG. 121

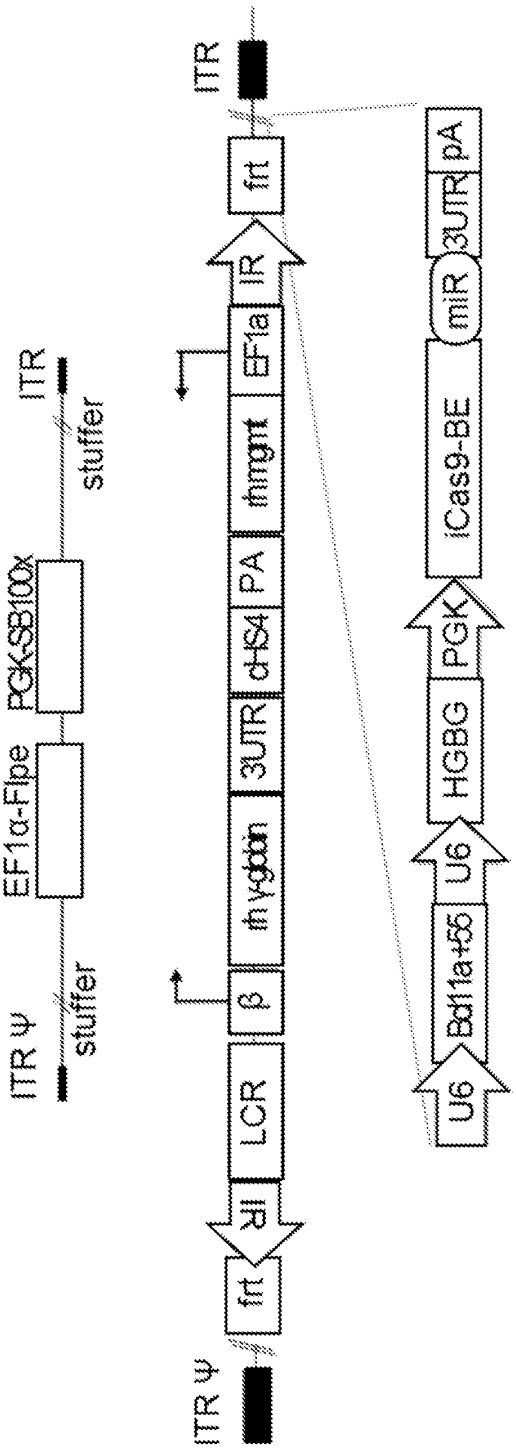

ITR Ψ
stuffer
EF1α-Flpe
PGK-SB100x
ITR
stuffer
ITR Ψ
frt
IR
LCR
β
hygro
3UTR
cHS4
PA
mini
EF1a
IR
frt
ITR
U6
Bcl11a+55
U6
HGBG
PGK
iCas9-BE
miR
3UTR
pA

FIG. 122

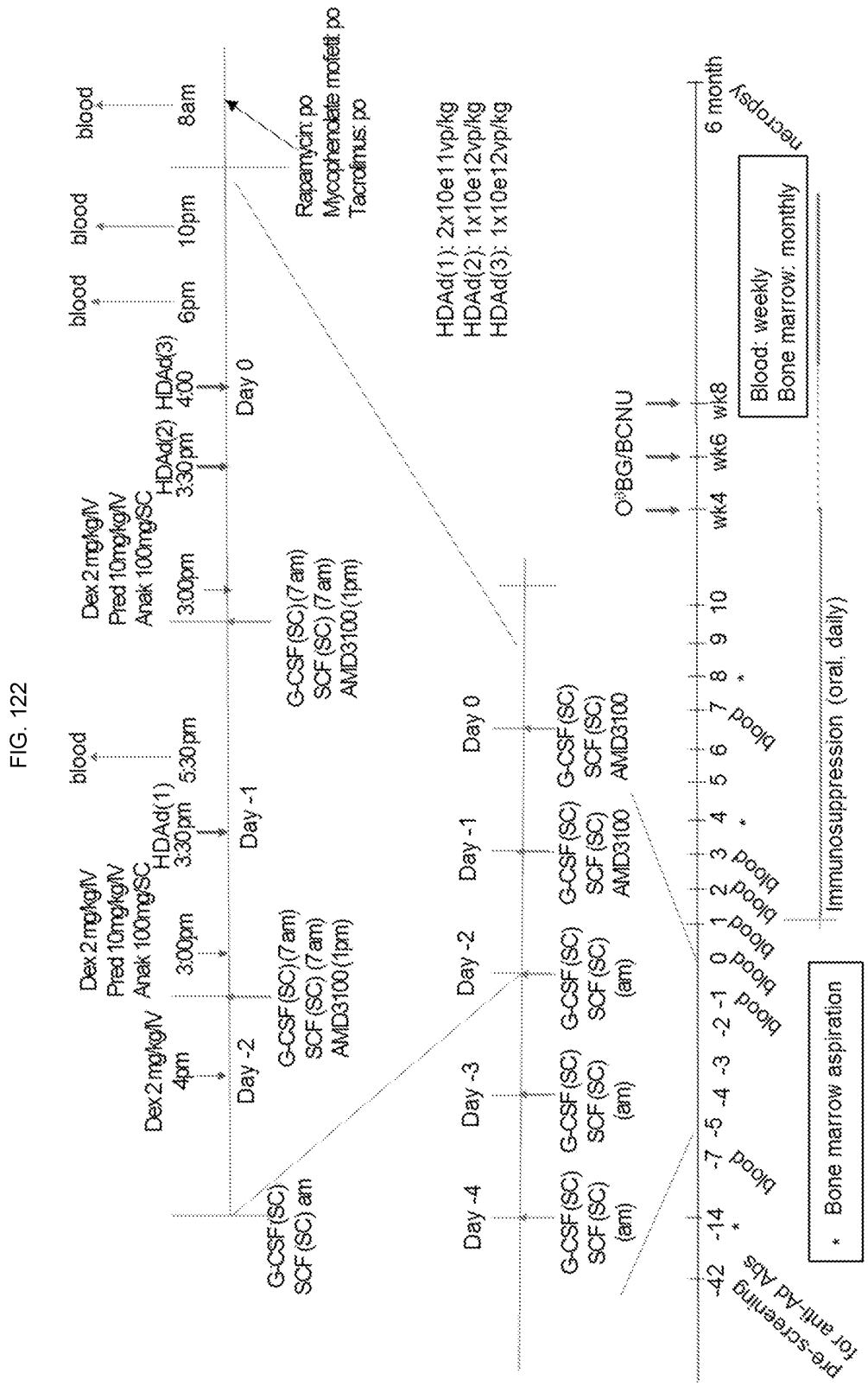

Dex 2 mg/kg/IV
4pm
Day -2
G-CSF (SC)
SCF (SC) am
Day -4
G-CSF (SC)
SCF (SC)
(am)
Day -3
G-CSF (SC)
SCF (SC)
(am)
Day -2
G-CSF (SC)
SCF (SC)
(am)
Dex 2 mg/kg/IV
Pred 10mg/kg/IV
Anak 100mg/SC
3:00pm
G-CSF (SC)(7 am)
SCF (SC) (7 am)
AMD3100 (1pm)
HDAd(1)
3:30pm
Day -1
G-CSF (SC)
SCF (SC)
AMD3100
blood
5:30pm
Day -1
G-CSF (SC)
SCF (SC)
AMD3100
Day 0
Dex 2 mg/kg/IV
Pred 10mg/kg/IV
Anak 100mg/SC
3:00pm
G-CSF (SC)(7 am)
SCF (SC) (7 am)
AMD3100 (1pm)
HDAd(2)
3:30pm
HDAd(3)
4:00
Day 0
blood
6pm
blood
10pm
blood
8am
Rapamycin po
Mycophenolate mofetil po
Tacrolimus po
HDAd(1): 2x10e11vp/kg
HDAd(2): 1x10e12vp/kg
HDAd(3): 1x10e12vp/kg
O6BG/BCNU
-42
-14
*
-7
blood
-5
-4
-3
-2
-1
0
blood
1
blood
2
blood
3
blood
4
*
5
6
blood
7
8
9
10
wk4
wk6
wk8
6 month
necropsy
Blood: weekly
Bone marrow: monthly
Immunosuppression (oral, daily)
pre-screening for anti-Ad Abs
* Bone marrow aspiration

FIG. 123

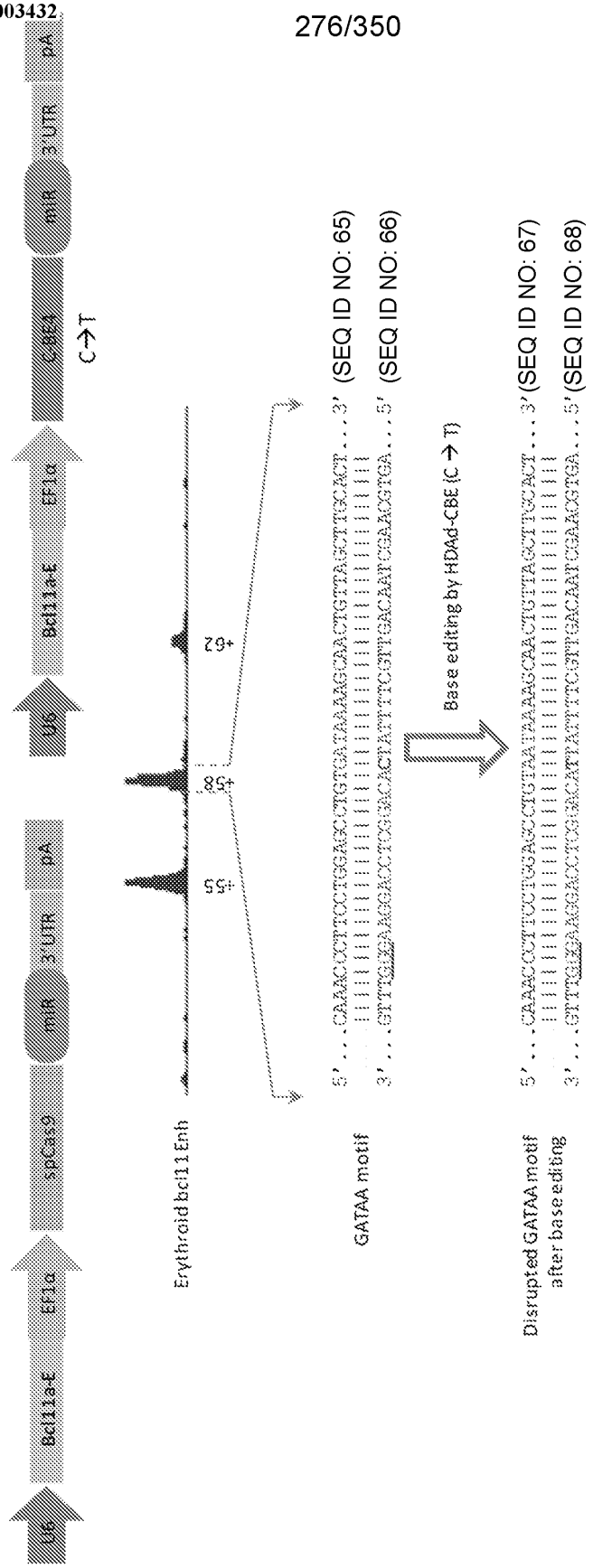

HDAd-wtCRISPR
U6
Bcl11a-E
EF1α
spCas9
miR
3'UTR
pA
HDAd-C-BE
U6
Bcl11a-E
EF1α
C-BEA
miR
3'UTR
pA
C→T
Erythroid bcl11 Enh
+55
+58
+62
GATAA motif
5'...CAAACCTTCCTGGAAGCTGTGATAAAAGCAACTGTTAGCTTGCACT...3' (SEQ ID NO: 65)
3'...GTTTGGAAGGACCTTCGACACTATTTCGTTGACAATCGAACGTGA...5' (SEQ ID NO: 66)
Base editing by HDAd-CBE (C → T)
Disrupted GATAA motif after base editing
5'...CAAACCTTCCTGGAGCCTGTAATAAAAGCAACTGTTAGCTTGCACT...3' (SEQ ID NO: 67)
3'...GTTTGGAAGGACCTCGGACATTATTTCGTTGACAATCGAACGTGA...5' (SEQ ID NO: 68)

FIG. 124A

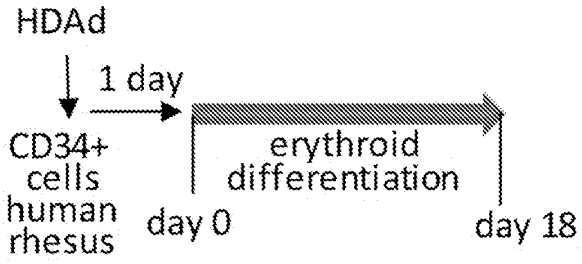


FIG. 124B

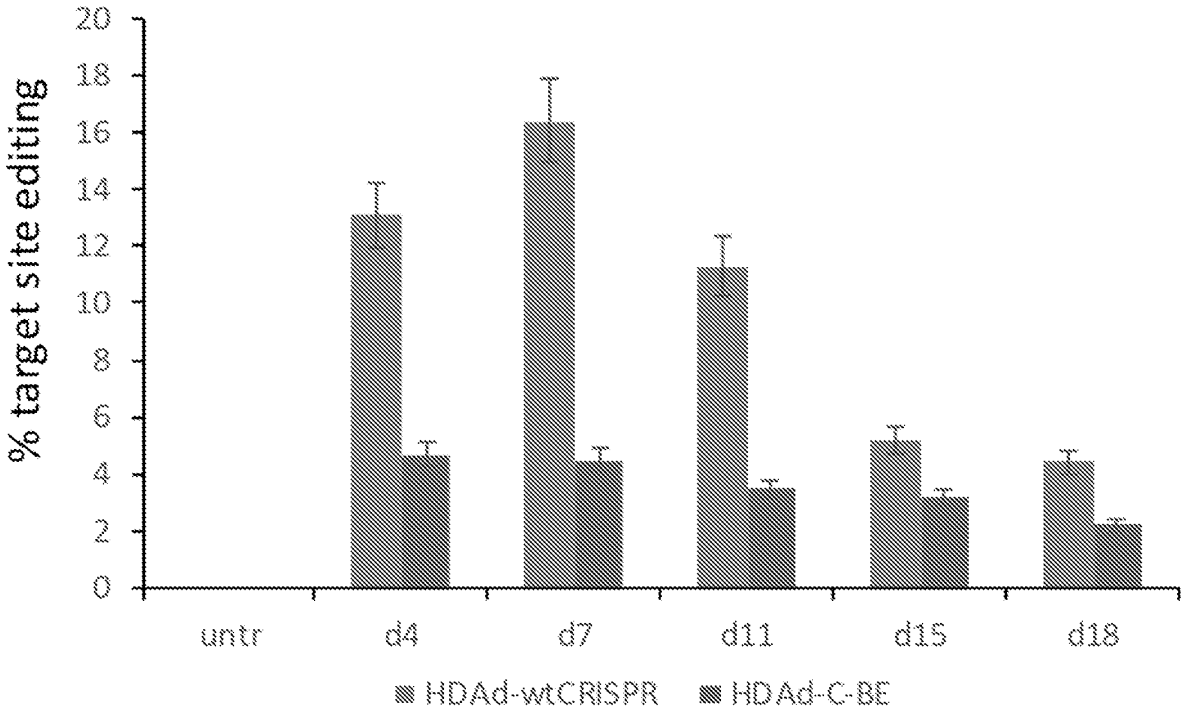


FIG. 124C

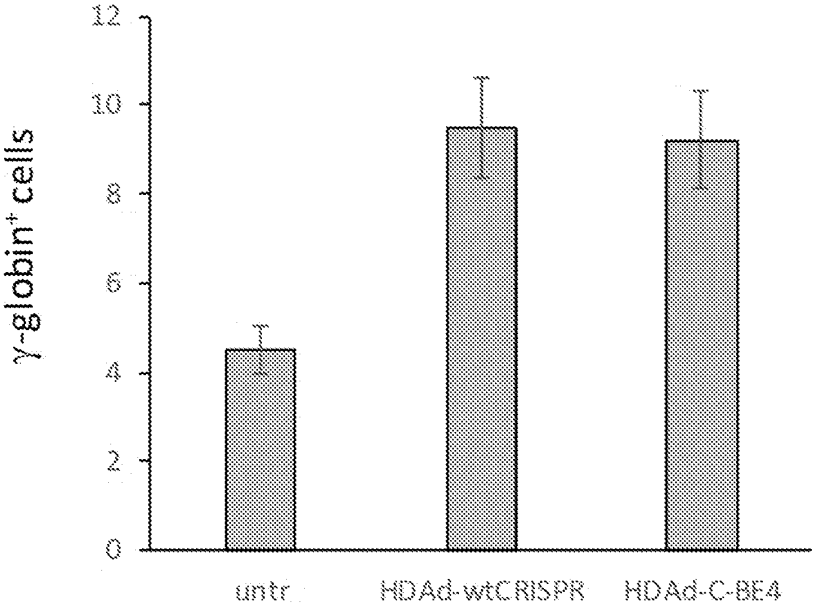


FIG. 125

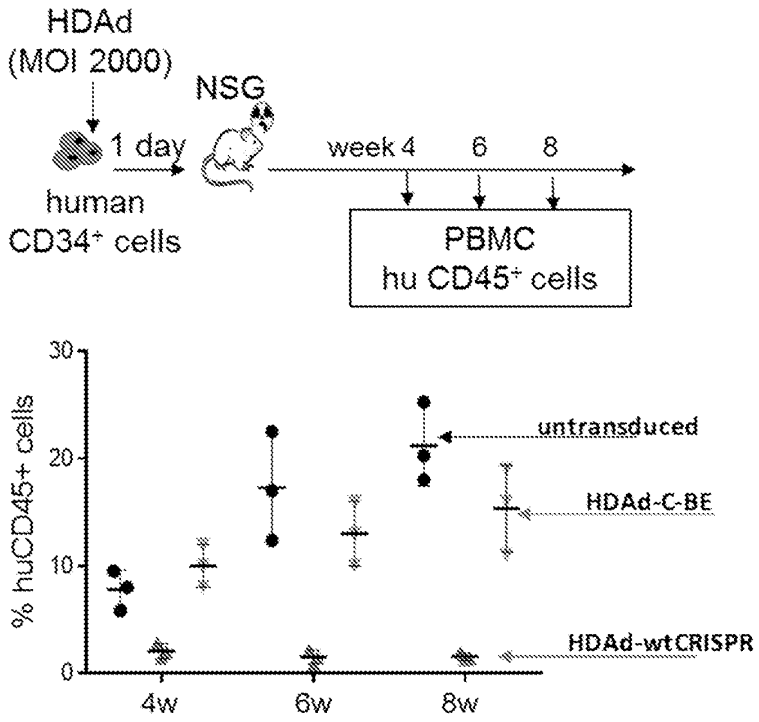


FIG. 126

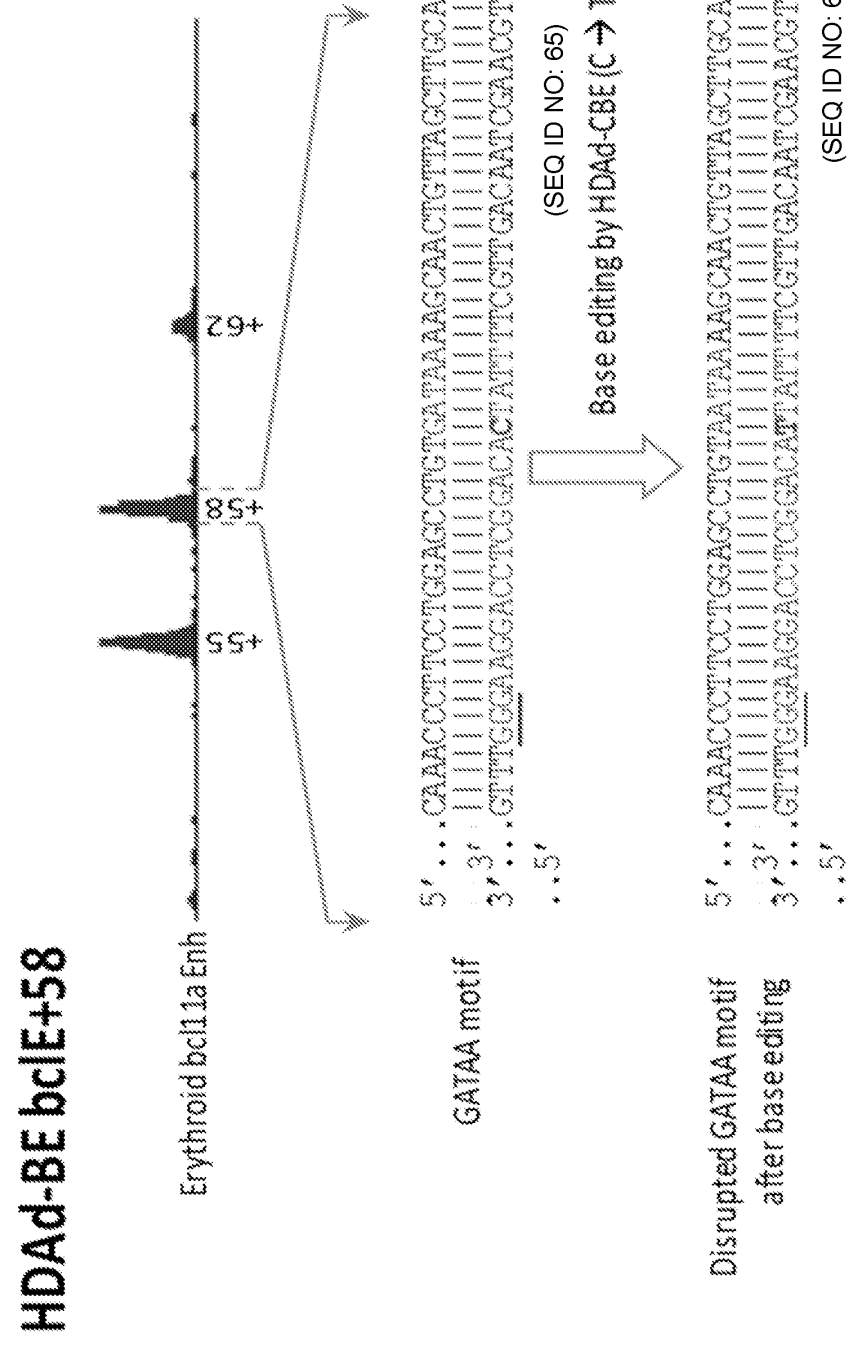

HDAd-BE bcl E+58
Erythroid bcl11a Enh
+55
+85
+62
GATAA motif
5'...CAAACCCTTCCTGGAGCCTGTGATAAAAGCAACTGTTAGCTTGCACT
3'...GTTTGGGAAGGACCTCGGACACTATTTTCGTTGACAATCGAACGTGA
5'
(SEQ ID NO: 65)
Base editing by HDAd-CBE (C → T)
Disrupted GATAA motif after base editing
5'...CAAACCCTTCCTGGAGCCTGTGATTAAAAGCAACTGTTAGCTTGCACT
3'...GTTTGGGAAGGACCTCGGACACTATTTTCGTTGACAATCGAACGTGA
5'
(SEQ ID NO: 67)

FIG. 126 cont'd

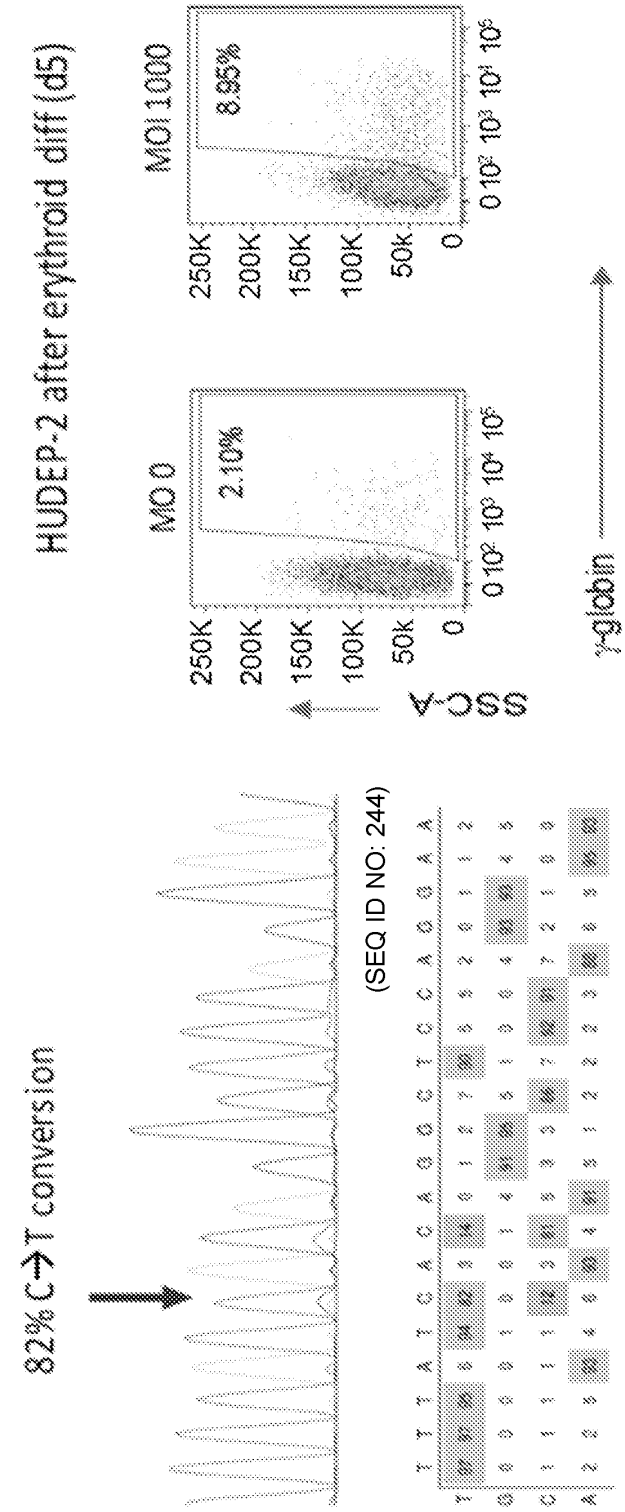


FIG. 126 cont'd

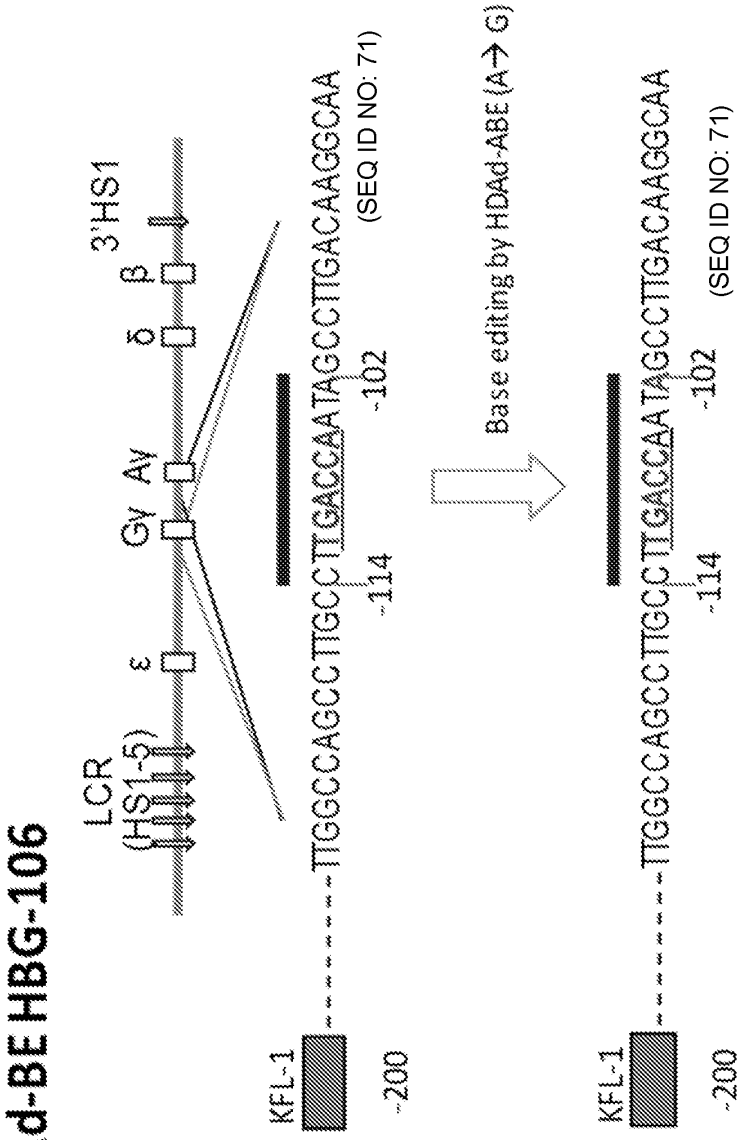


FIG. 126 cont'd

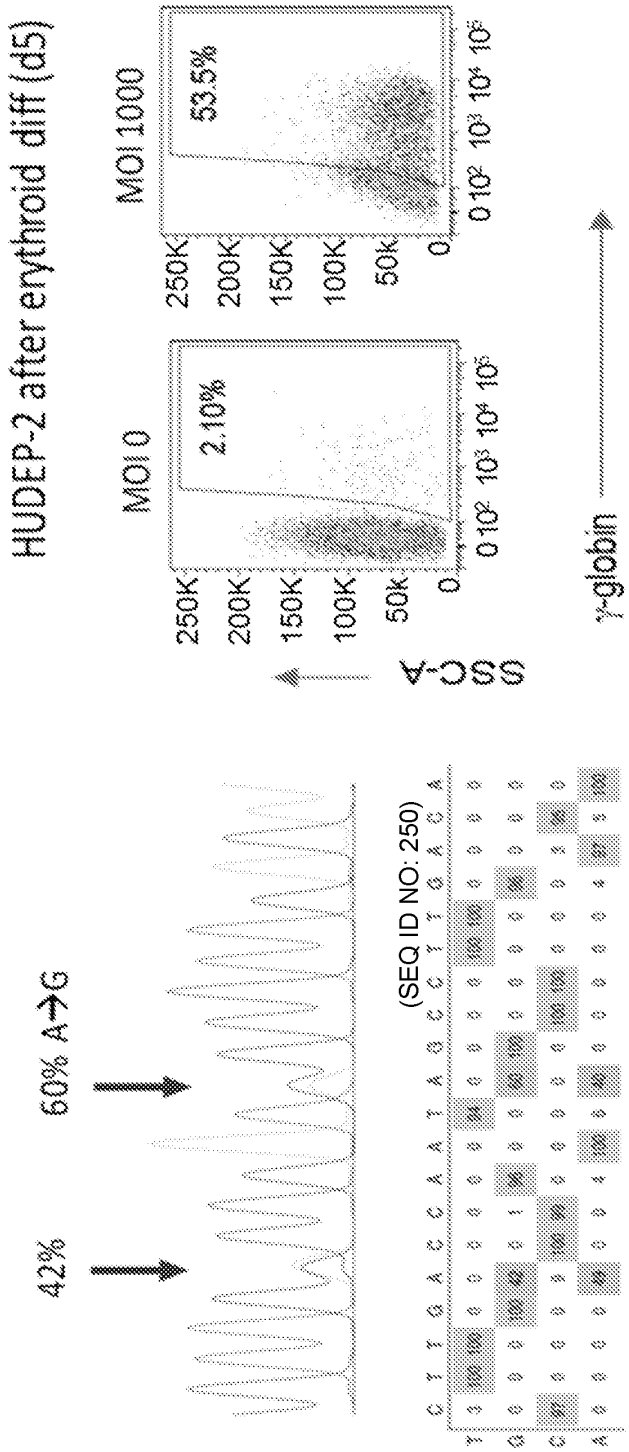


FIG.127

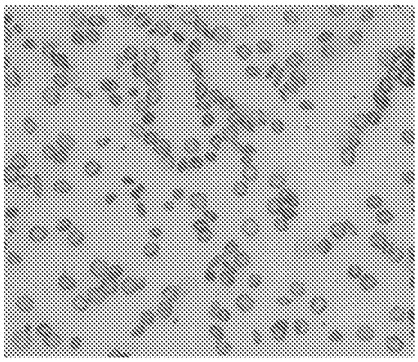


FIG. 127B

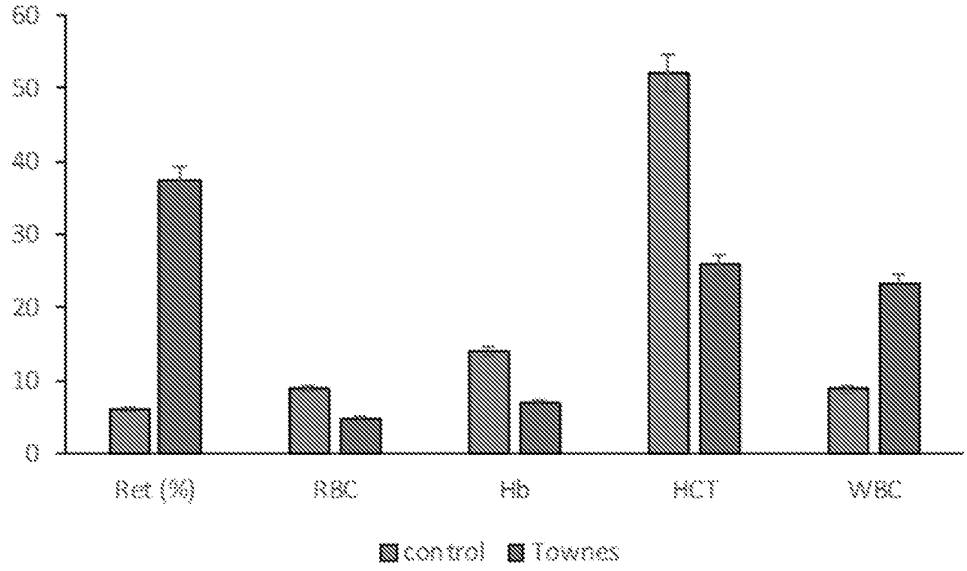


FIG. 128A

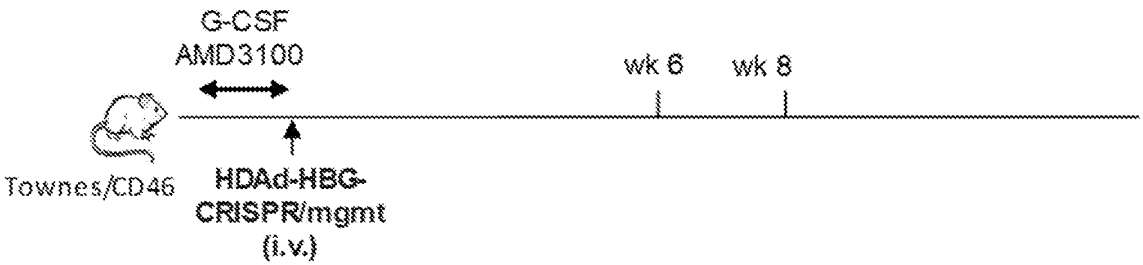


FIG. 128B

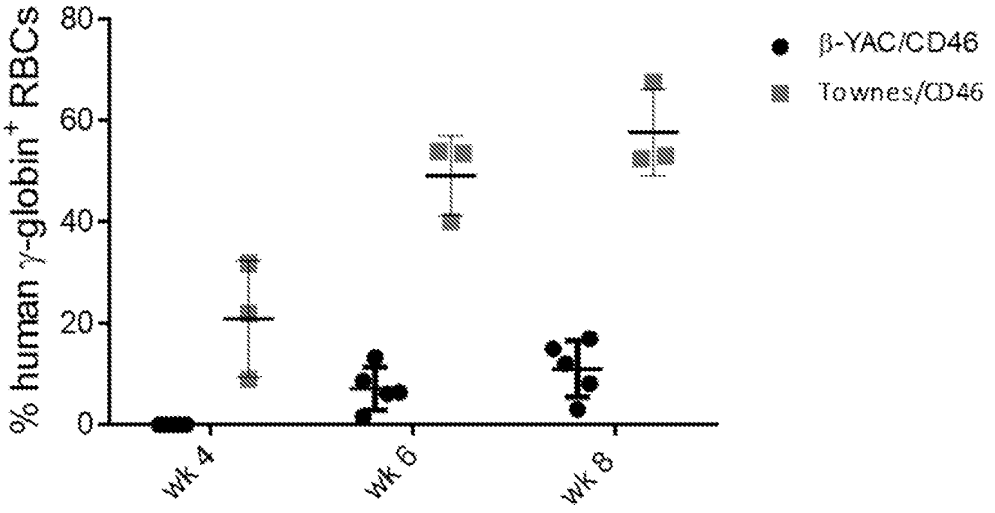


FIG. 128C

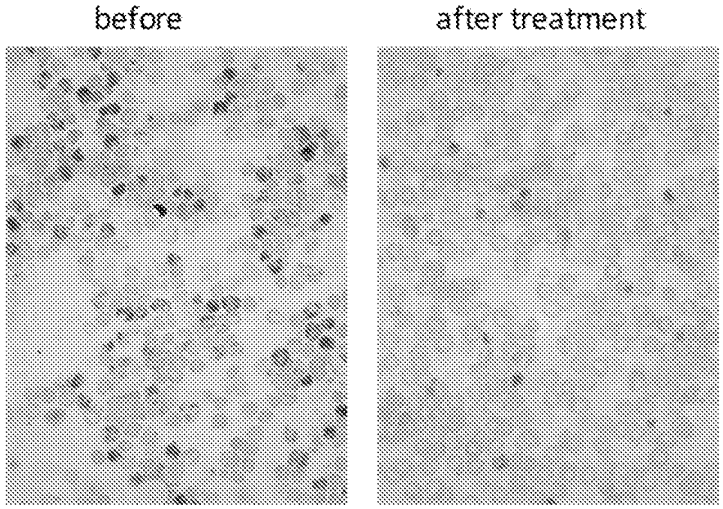


FIG. 129A

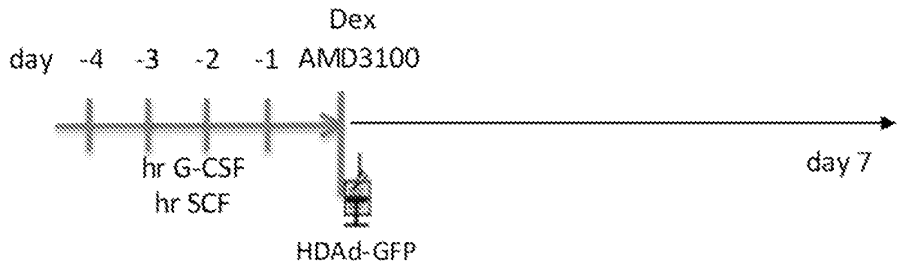


FIG.129B

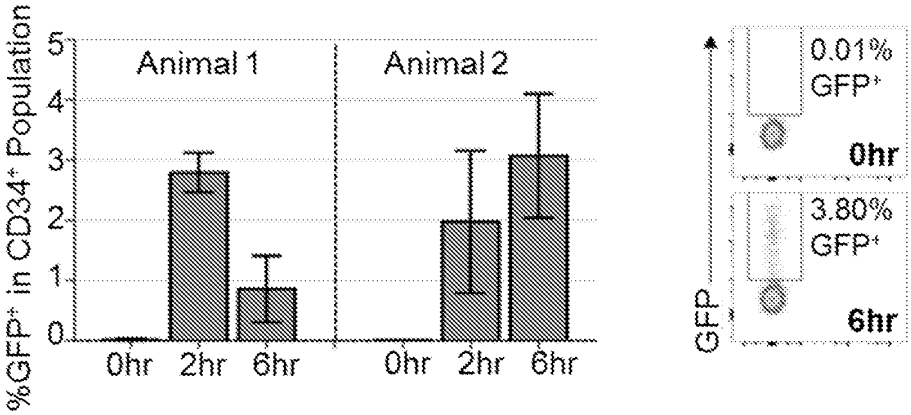


FIG. 129C

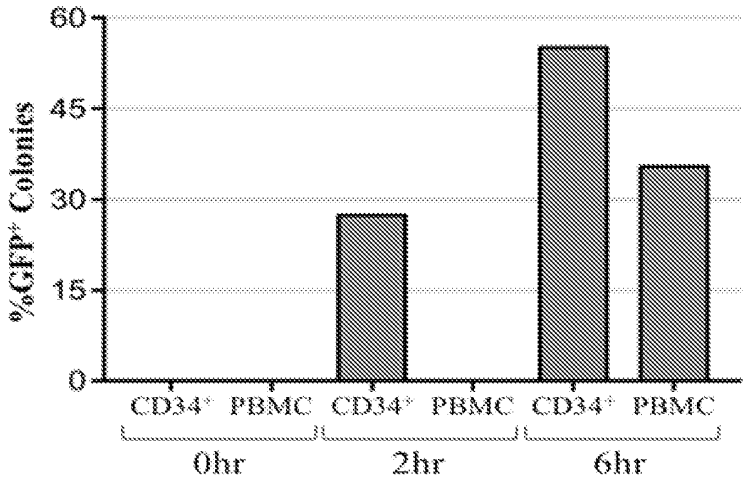


FIG. 129D

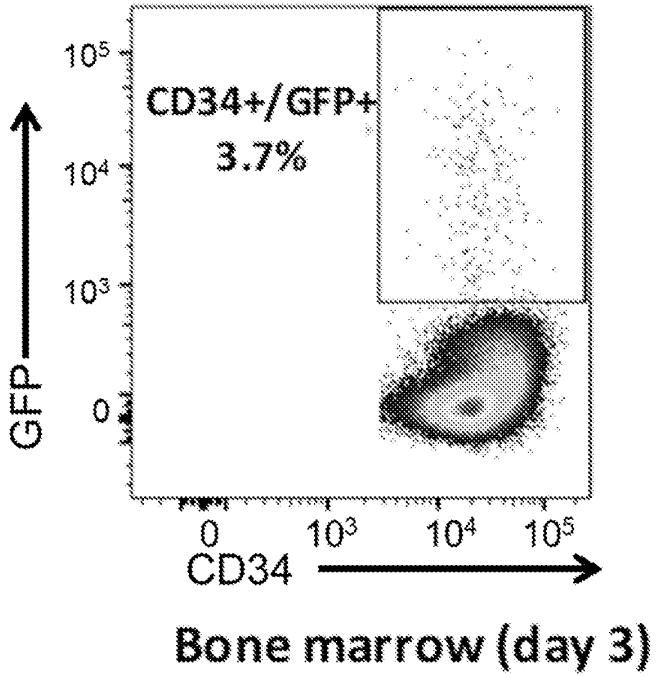


FIG. 130

γ-globin reactivation after transient transfection of in HUDEP-2

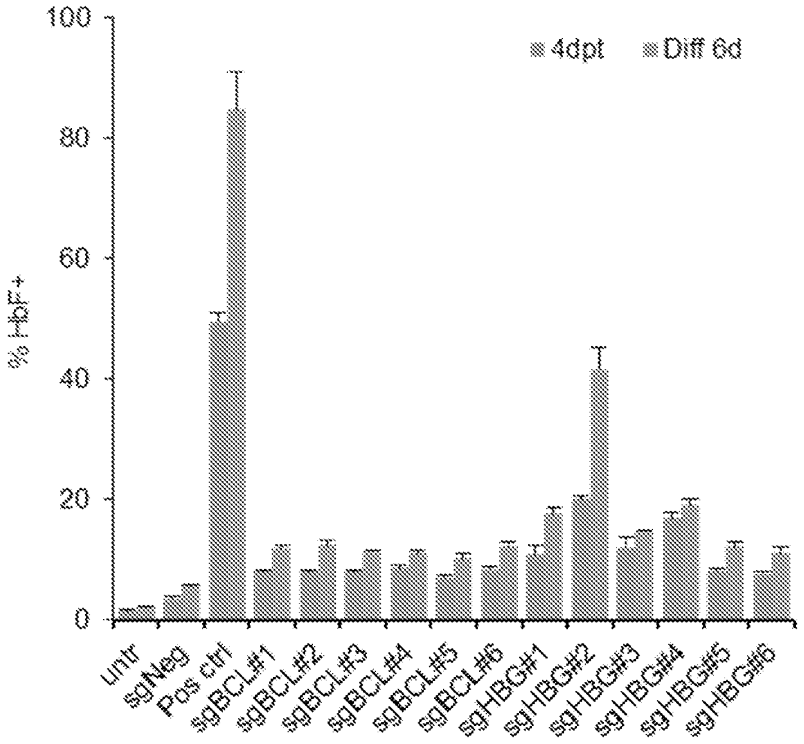


FIG. 131A

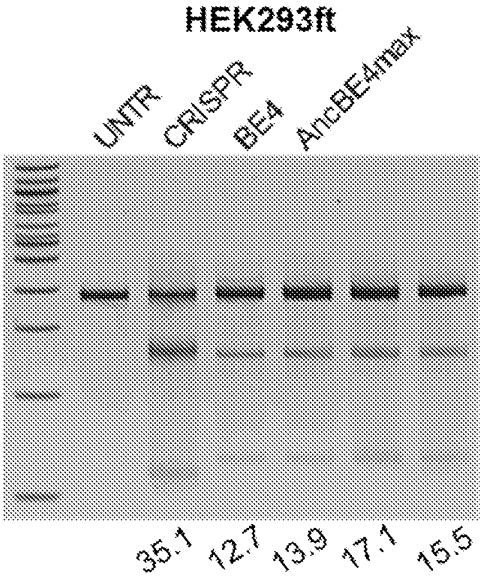


FIG. 131B

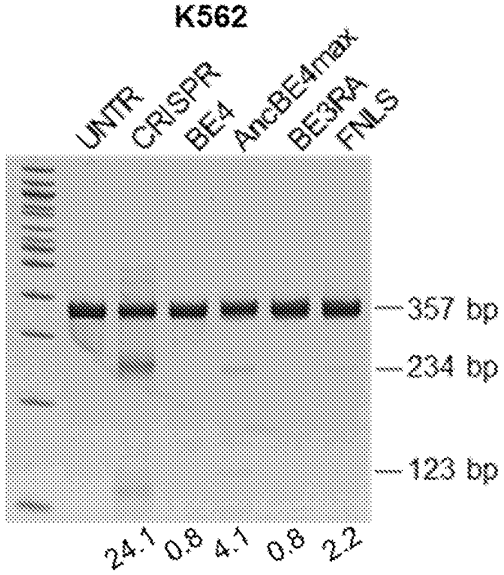


FIG. 132A

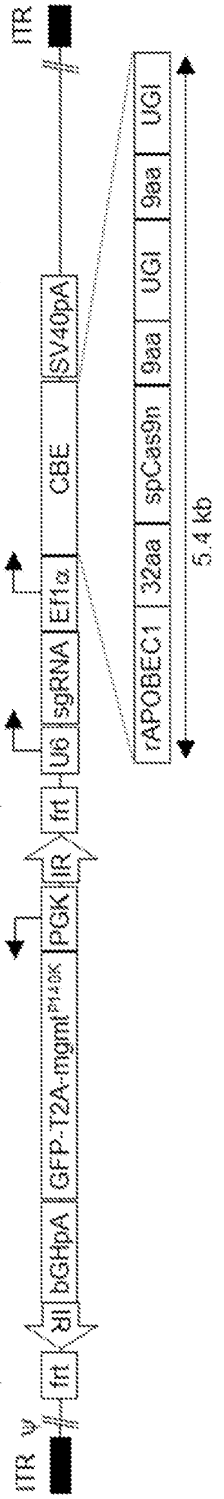


FIG. 132B

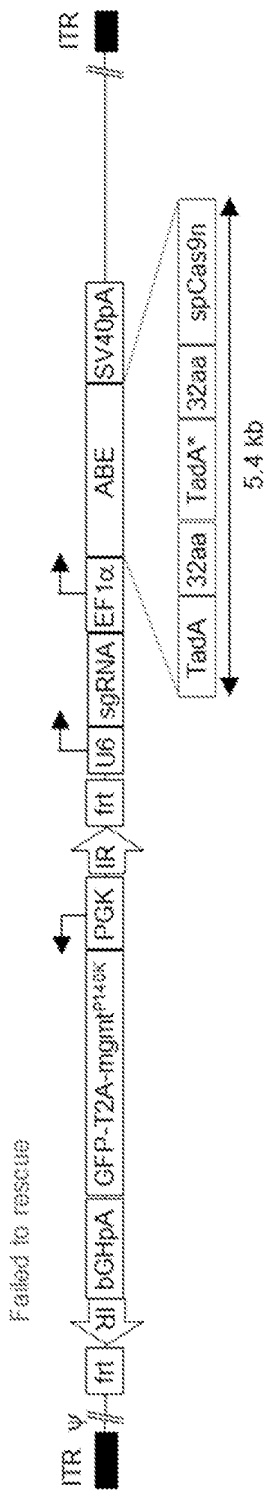


FIG. 132C

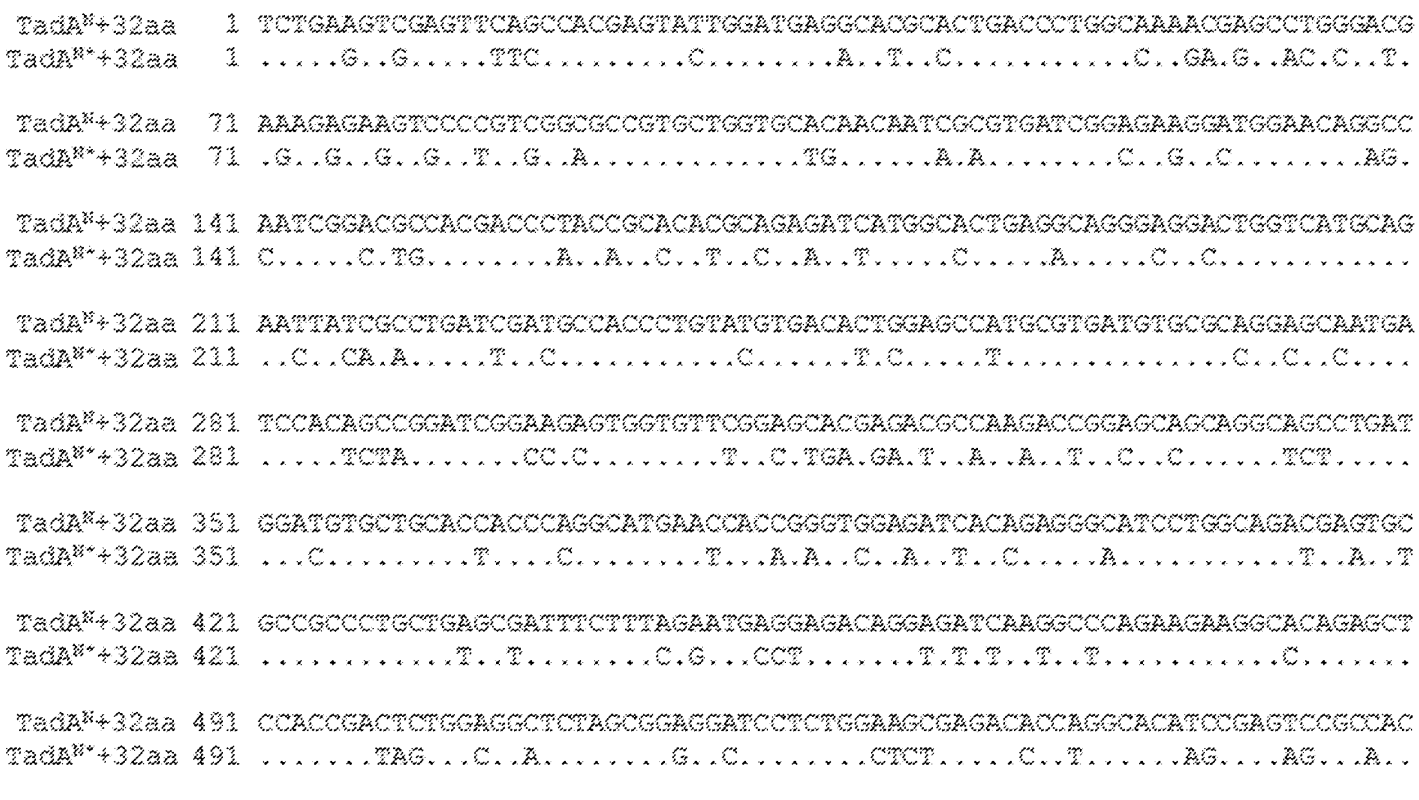

TadA^N+32aa 1 TCTGAAGTCGAGTTCAGCCACGAGTATTGGATGAGGCACGCACTGACCCTGGCAAAACGAGCCTGGGACG
TadA^N*+32aa 1 .....G..G.....TTC.....C.....A..T..C.....C..GA.G..AC.C..T.
TadA^N+32aa 71 AAAGAGAAGTCCCCGTCGGCGCCGTGCTGGTGCACAACAATCGCGTGATCGGAGAAGGATGGAACAGGCC
TadA^N*+32aa 71 .G..G..G..G..T..G..A.....TG.....A.A.....C..G..C.....AG.
TadA^N+32aa 141 AATCGGACGCCACGACCCTACCGCACACGCAGAGATCATGGCACTGAGGCAGGGAGGACTGGTCATGCAG
TadA^N*+32aa 141 C.....C.TG.....A..A..C..T..C..A..T.....C.....A.....C..C.....
TadA^N+32aa 211 AATTATCGCCTGATCGATGCCACCCTGTATGTGACACTGGAGCCATGCGTGATGTGCGCAGGAGCAATGA
TadA^N*+32aa 211 ..C..CA.A....T..C.....C.....T.C....T.....C..C..C....
TadA^N+32aa 281 TCCACAGCCGGATCGGAAGAGTGGTGTTCGGAGCACGACGCCAAGACCGGAGCAGCAGGCAGCCTGAT
TadA^N*+32aa 281 .....TCTA.....CC.C.....T..C.TGA.GA.T..A..A..T..C..C.....TCT.....
TadA^N+32aa 351 GGATGTGCTGCACCACCCAGGCATGAACCACCGGGTGGAGATCACAGAGGGCATCCTGGCAGACGAGTGC
TadA^N*+32aa 351 ...C.....T....C.....T..A.A..C..A..T..C.....A.....T..A..T
TadA^N+32aa 421 GCCGCCCTGCTGAGCGATTTCTTTAGAATGAGGAGACAGGAGATCAAGGCCCAGAAGAAGGCACAGAGCT
TadA^N*+32aa 421 .....T..T.....C.G...CCT.....T.T.T..T..T.....C.....
TadA^N+32aa 491 CCACCGACTCTGGAGGCTCTAGCGGAGGATCCTCTGGAAGCGAGACACCAGGCACATCCGAGTCCGCCAC
TadA^N*+32aa 491 .....TAG...C..A.....G..C.....CTCT.....C..T.....AG....AG...A..
TadA^N+32aa 561 ACCAGAGTCTTCCGGCGGCTCCTCCGGAGGATCC (SEQ ID NO: 260)
TadA^N*+32aa 561 ...T..AAGCAG...G...AG.AG...G..GAG. (SEQ ID NO: 261)

FIG. 133A

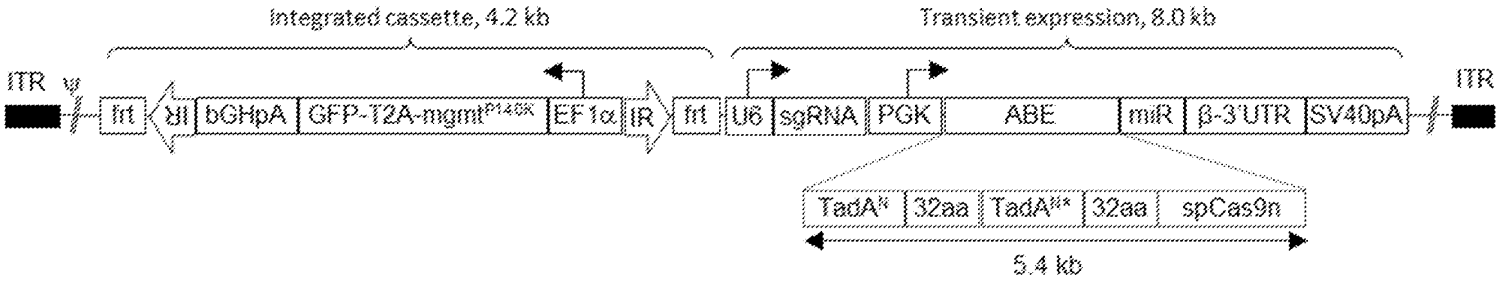


FIG. 133B

Full name	Editor	Guide RNA	Yield (× 10^{12} vp)
HDAd5/35++_BE4max-sgBCL#1-FI-mgmt/GFP	CBE	sgBCL#1	1.2
HDAd5/35++_BE4max-sgHBG#1-FI-mgmt/GFP	CBE	sgHBG#1	0.8
HDAd5/35++_ABEopti-sgHBG#2-FI-mgmt/GFP	ABE	sgHBG#2	3.8
HDAd5/35++_ABEopti-sgHBG#4-FI-mgmt/GFP	ABE	sgHBG#4	2.8
HDAd5/35++_BE4max-Dual#1-FI-mgmt/GFP	CBE	sgBCL#1 + sgHBG#1	0.5
HDAd5/35++_BE4max-sgNeg-FI-mgmt/GFP	CBE	sgNeg	1.5

FIG. 133C

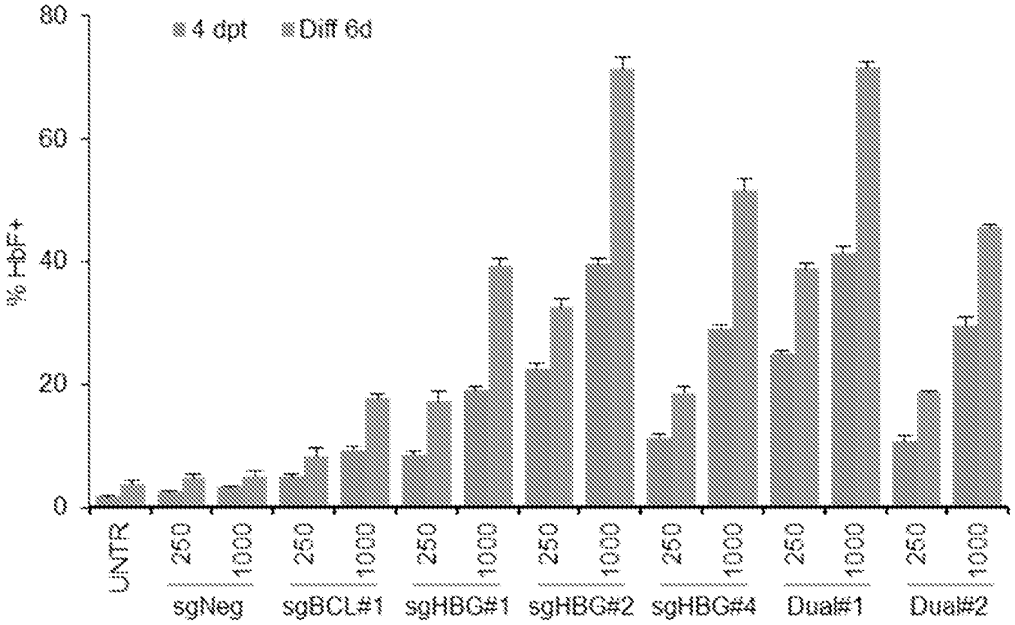
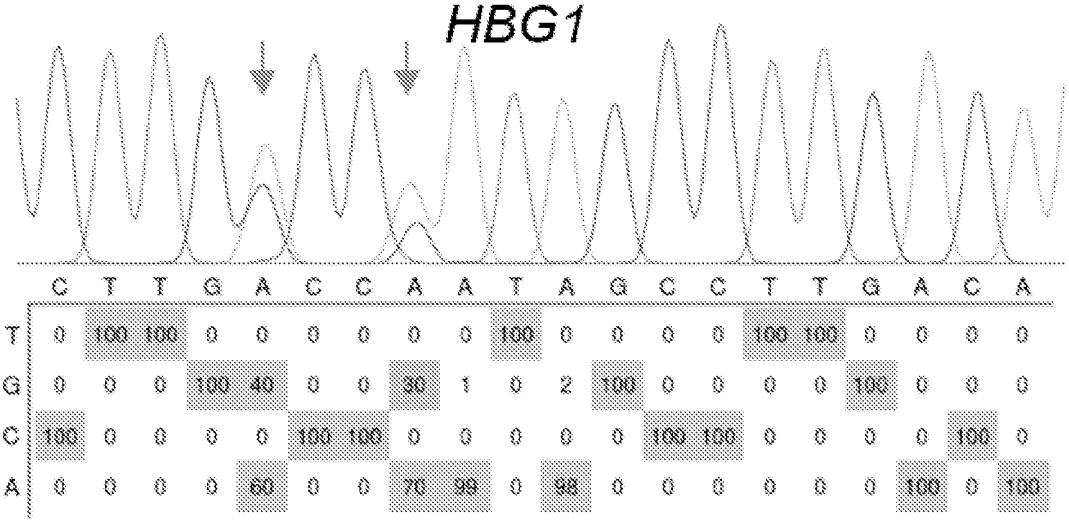

4 dpt
Diff 6d
% HbF+
80
60
40
20
0
UNTR
250
1000
sgNeg
sgBCL#1
sgHBG#1
sgHBG#2
sgHBG#4
Dual#1
Dual#2

FIG. 133D

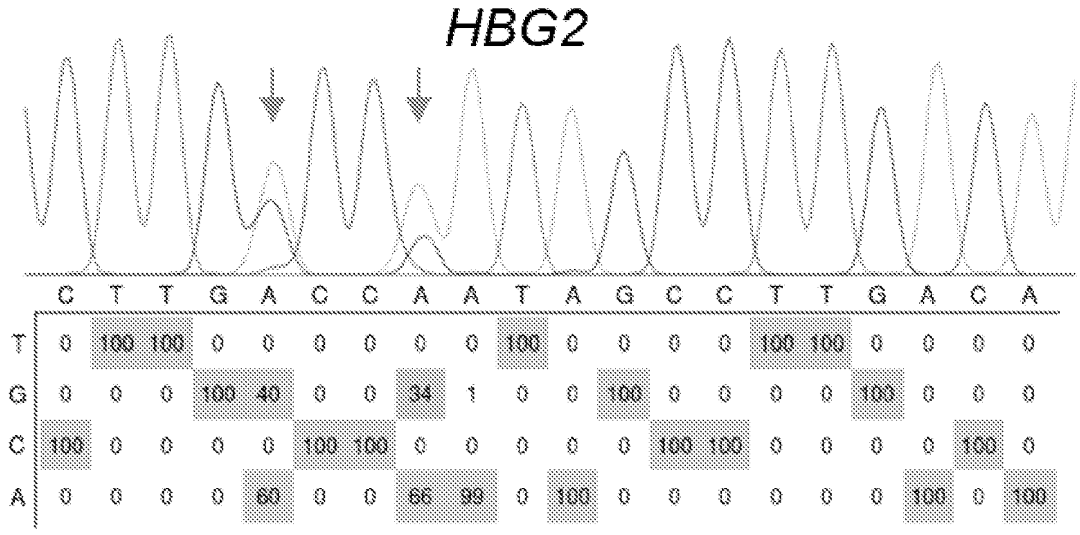
sgHBG#2

HBG1



(SEQ ID NO: 250)

HBG2



(SEQ ID NO: 250)

FIG. 133E

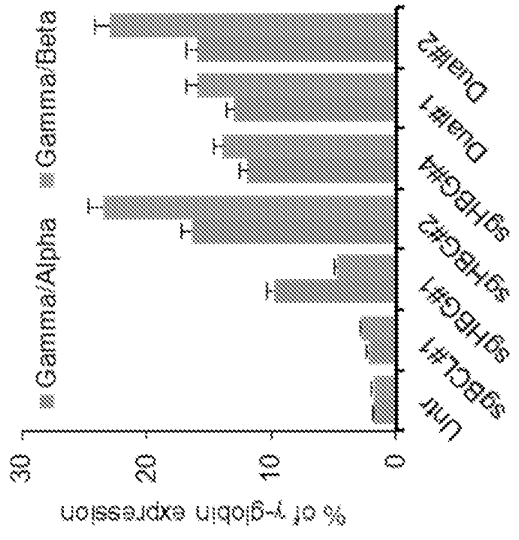


FIG. 133F

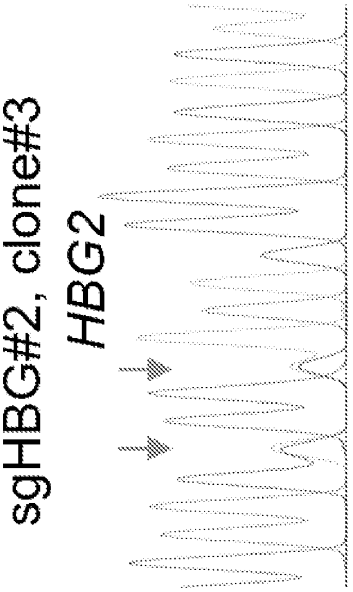


FIG. 133G

FIG. 133H

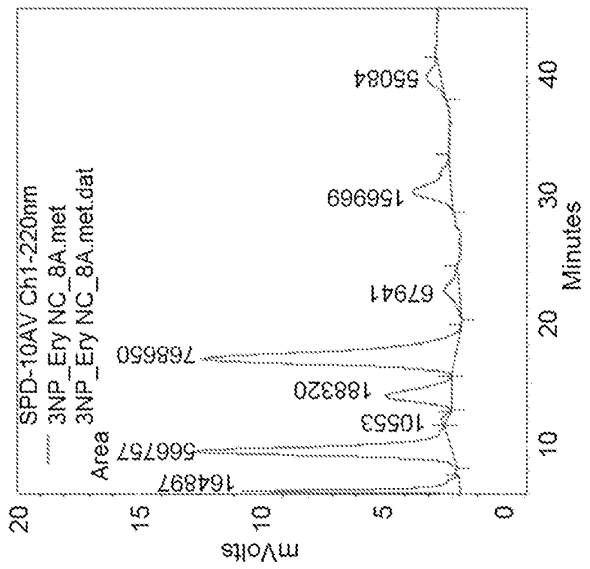


FIG. 134A

sgBCL#1
Bcl11A

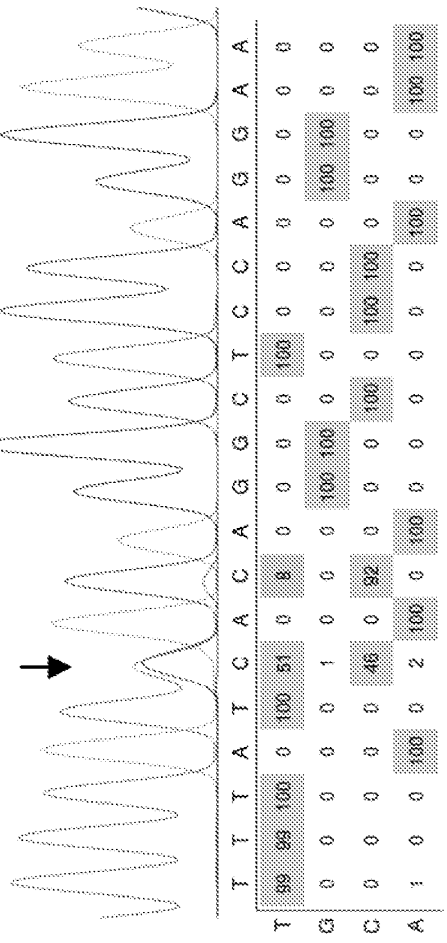


FIG. 134A cont'd

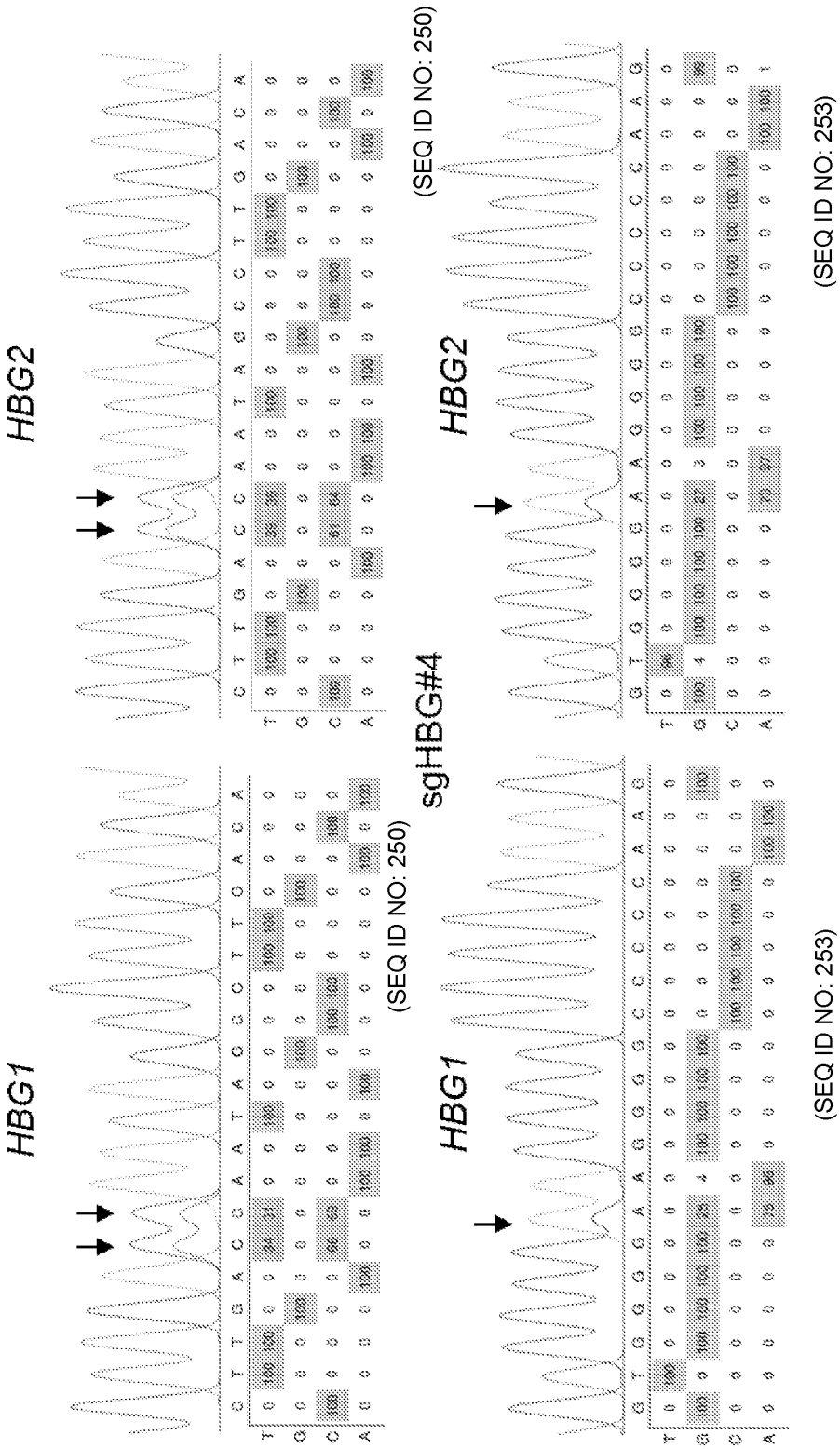


FIG. 134B

sgBCL#1, Clone#5
Bcl11A

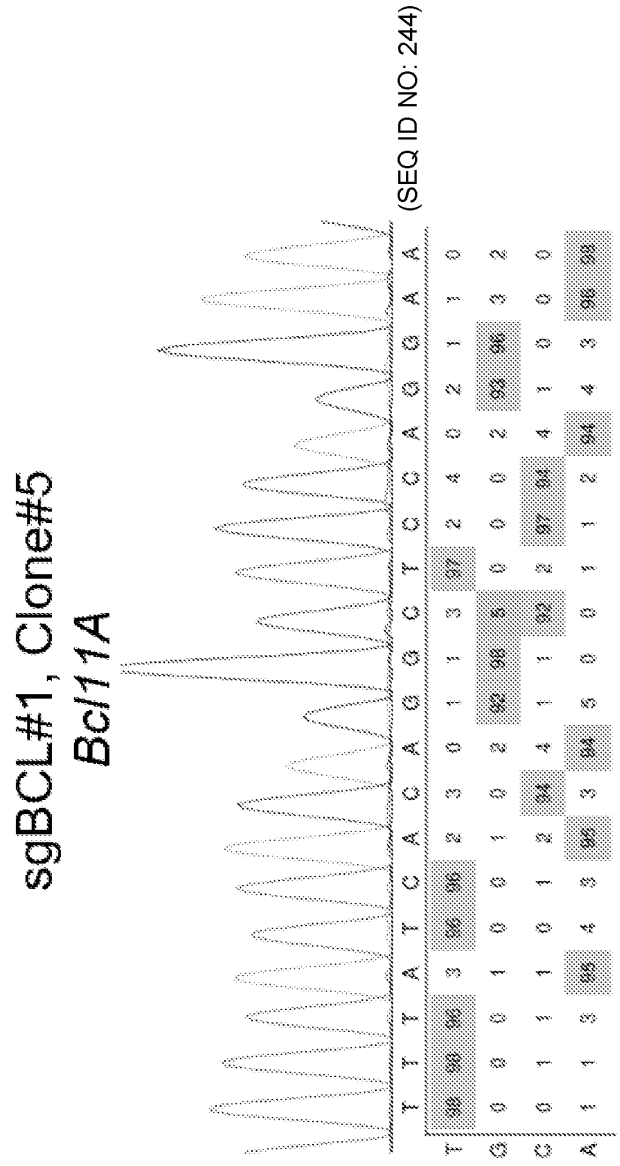


FIG. 134B cont'd

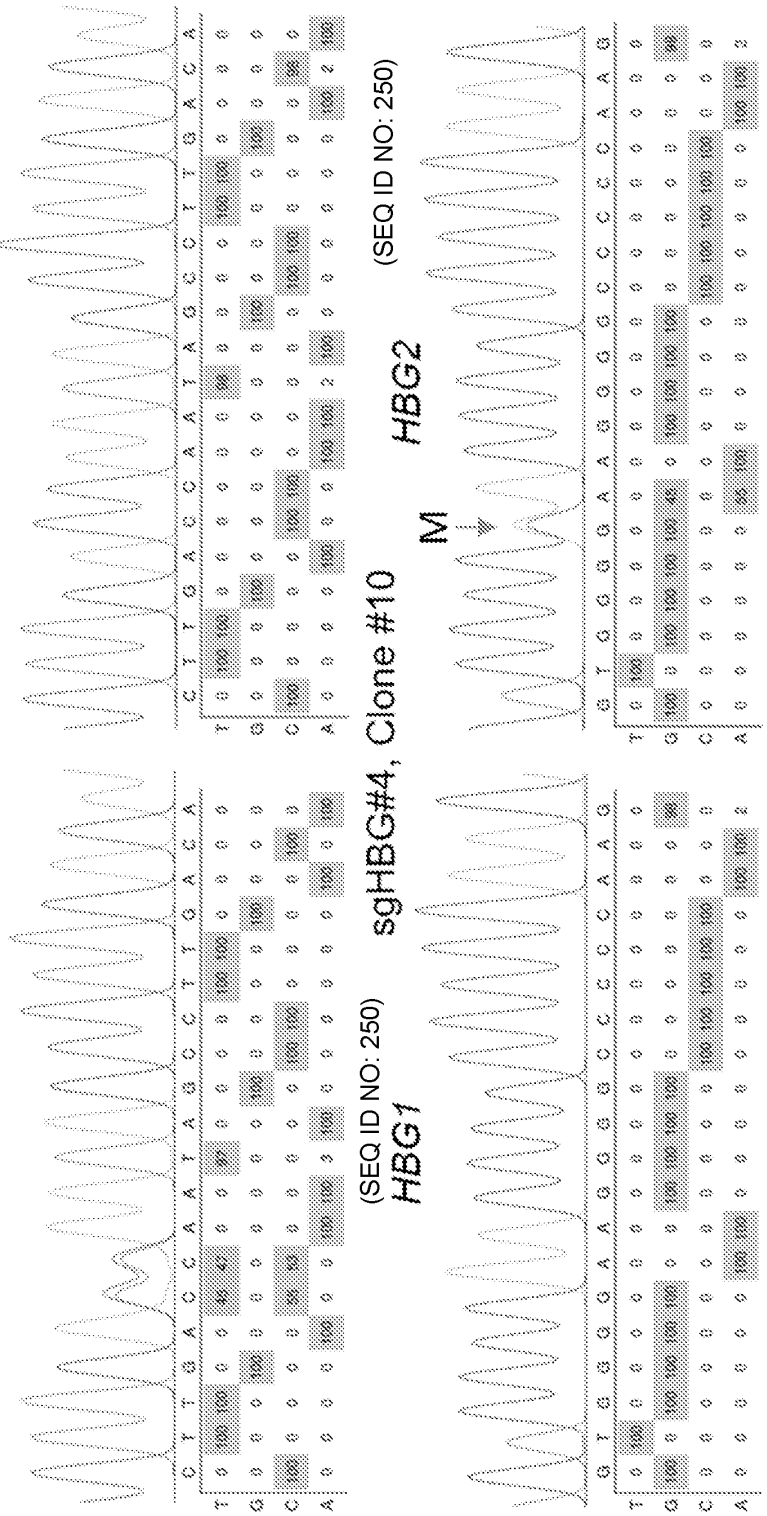


FIG. 134C

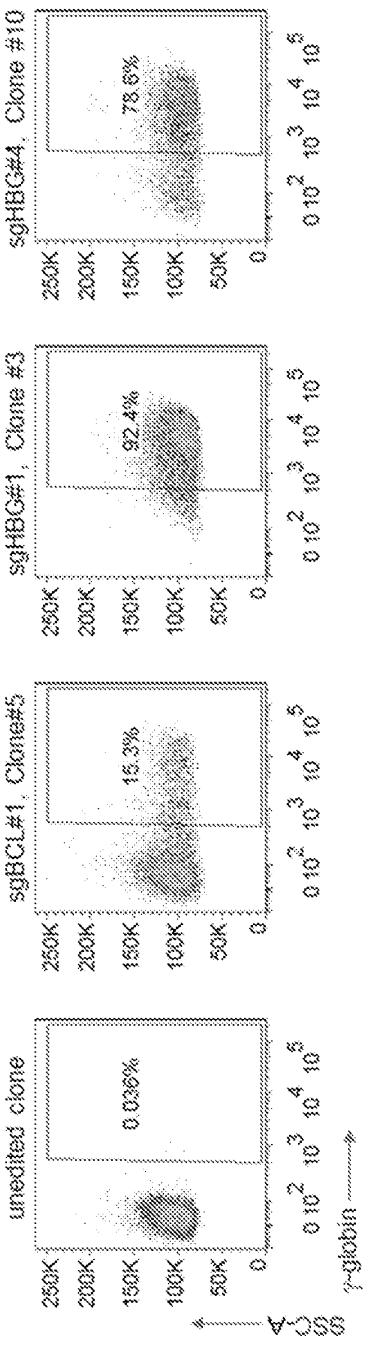


FIG. 135A

FIG. 135B

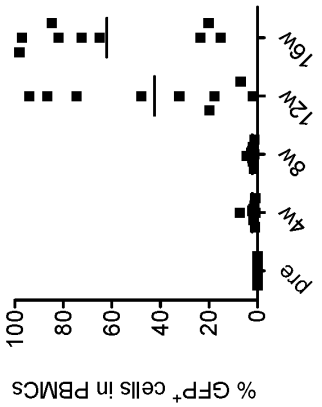


FIG. 135C

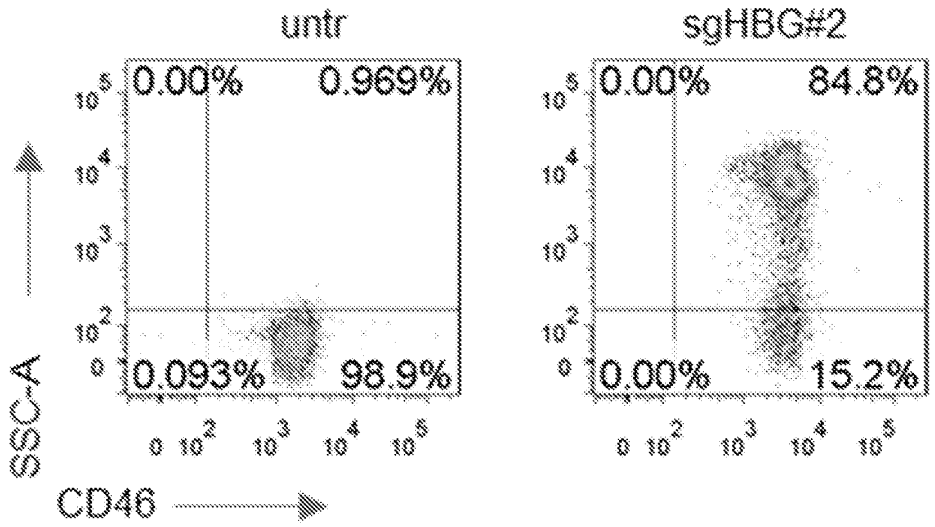


FIG. 135D

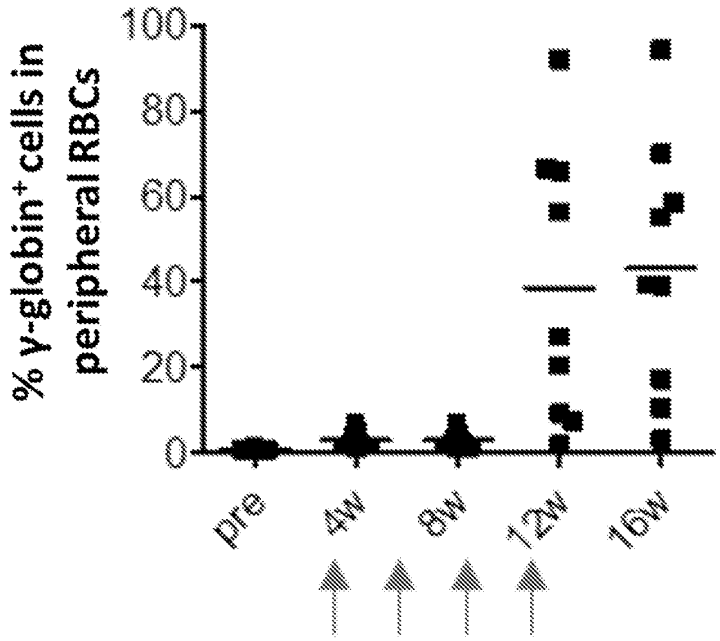


FIG. 135E

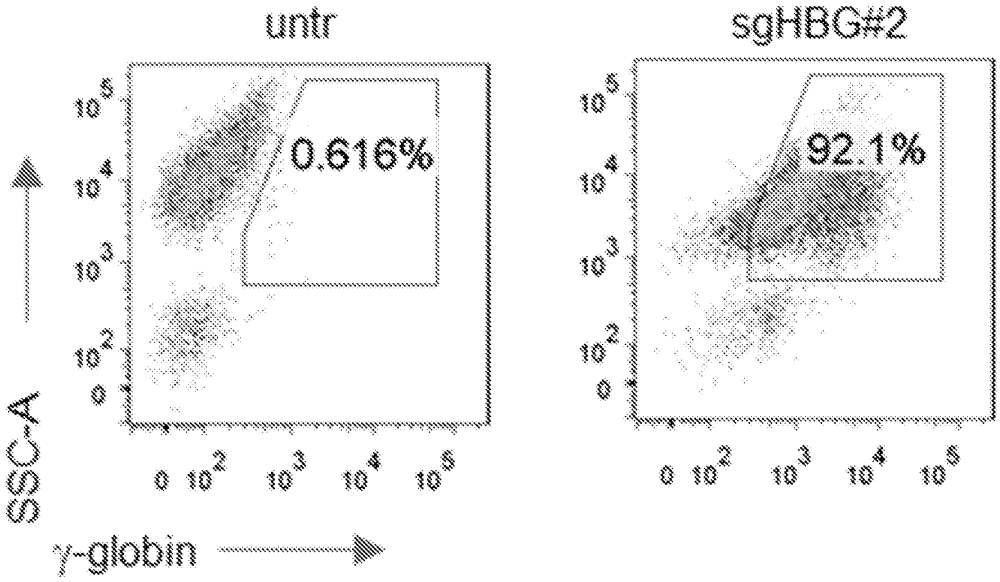


FIG. 135F

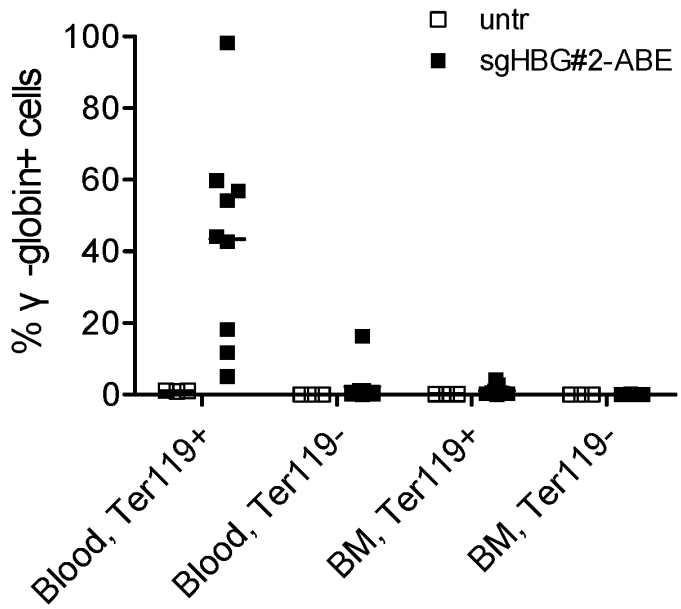


FIG. 135G

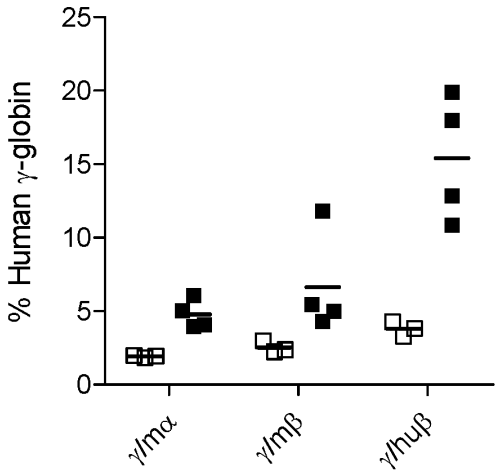


FIG. 135H

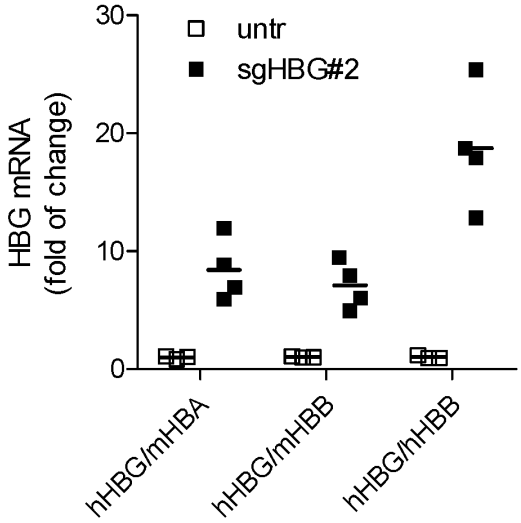


FIG. 135I

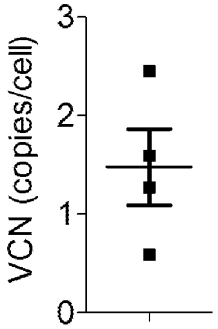


FIG. 136

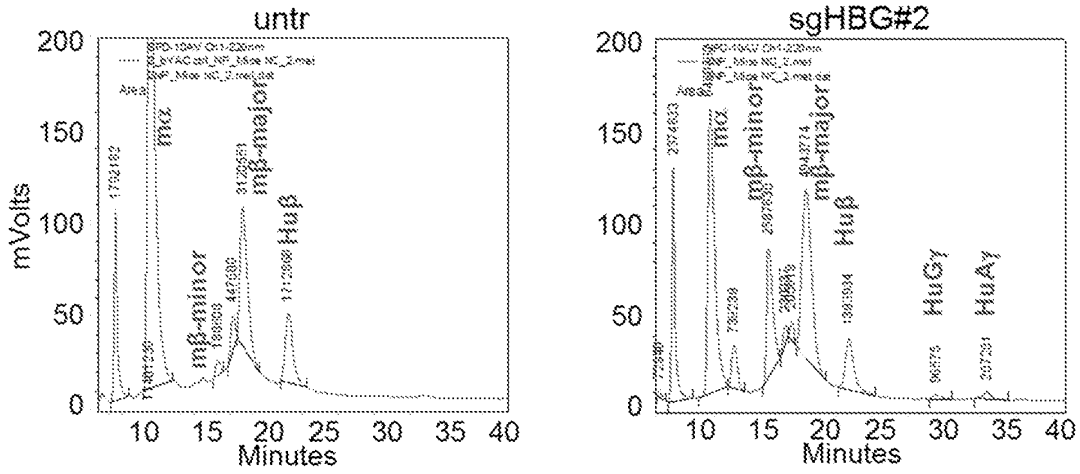
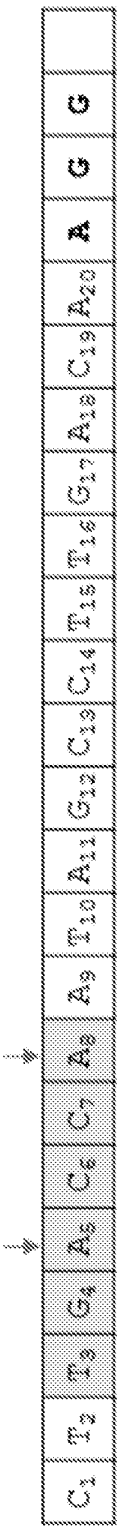

untr
sgHBG#2
mVolts
Minutes
mα
mβ-minor
mβ-major
Huβ
HuGγ
HuAγ

FIG. 137A



(SEQ ID NO: 276)

FIG. 137B

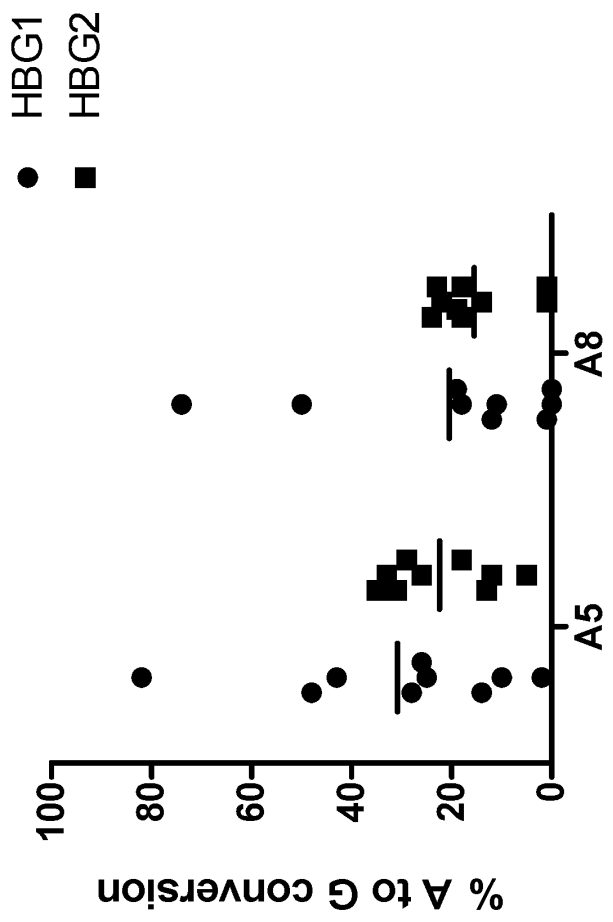


FIG. 137C

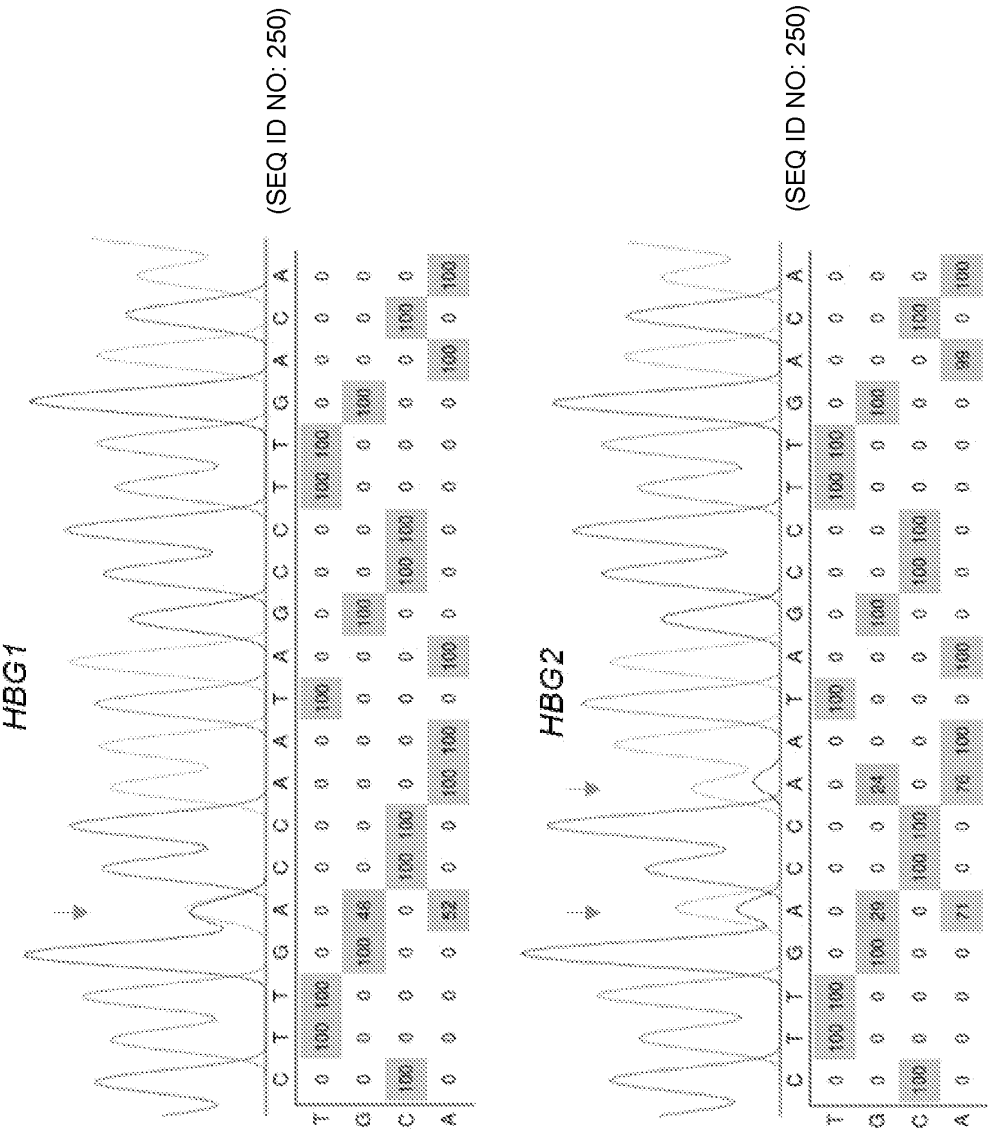


FIG. 137D

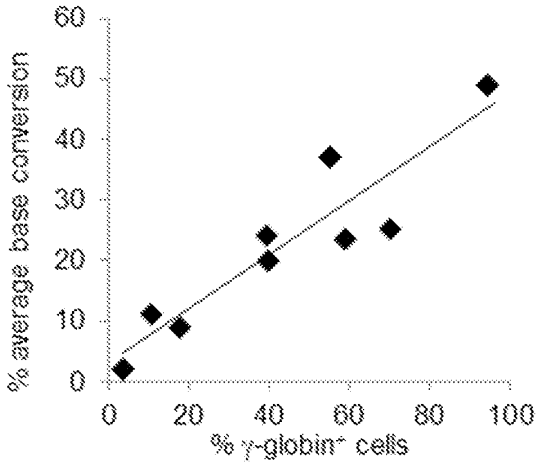


FIG. 137E

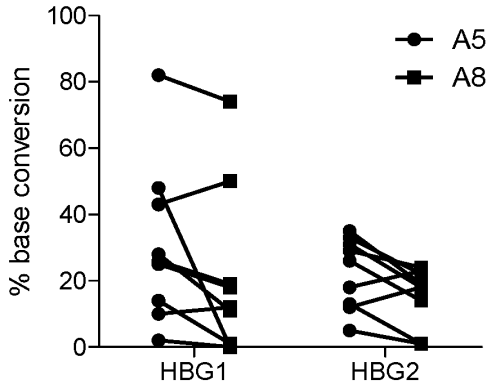


FIG. 137F

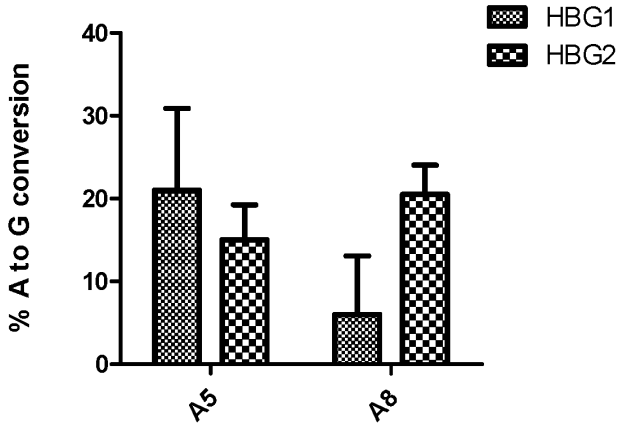


FIG. 137G

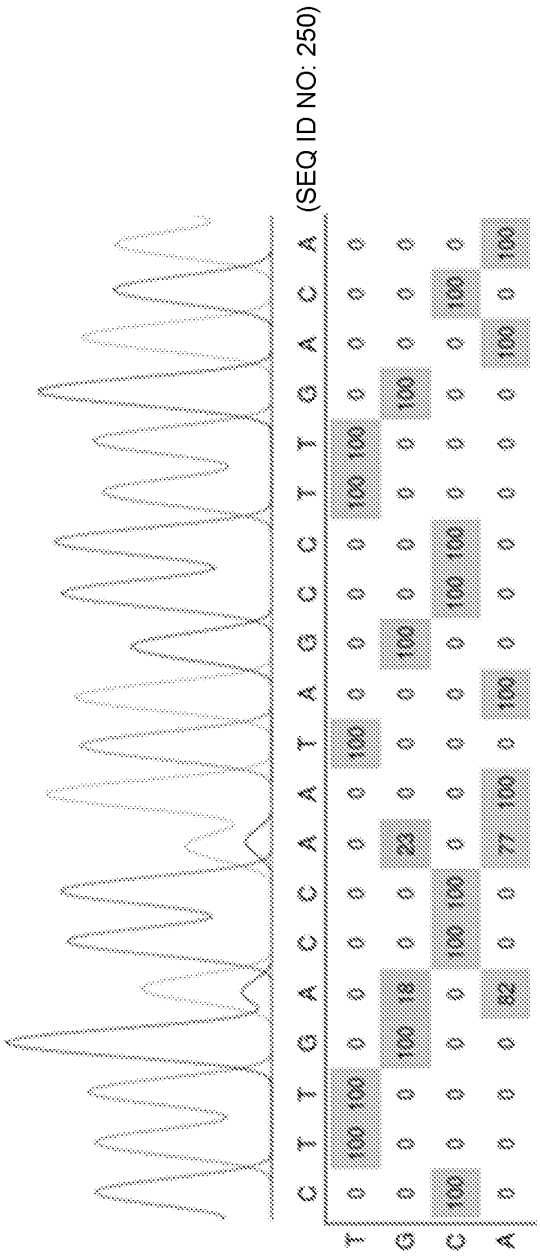

(SEQ ID NO: 250)
C T T G A C C C A A T A G C C C T T G A C A C A
T 0 100 100 0 0 0 0 0 0 100 0 0 0 0 0 0 0 0 0 0
G 0 0 0 100 18 0 0 0 23 0 0 100 0 0 0 0 0 0 0 0
C 100 0 0 0 0 100 100 0 0 0 0 0 100 100 0 0 100 0 0 0
A 0 0 0 0 82 0 0 0 77 100 0 0 0 0 100 100 0 100 0 100

FIG. 138A

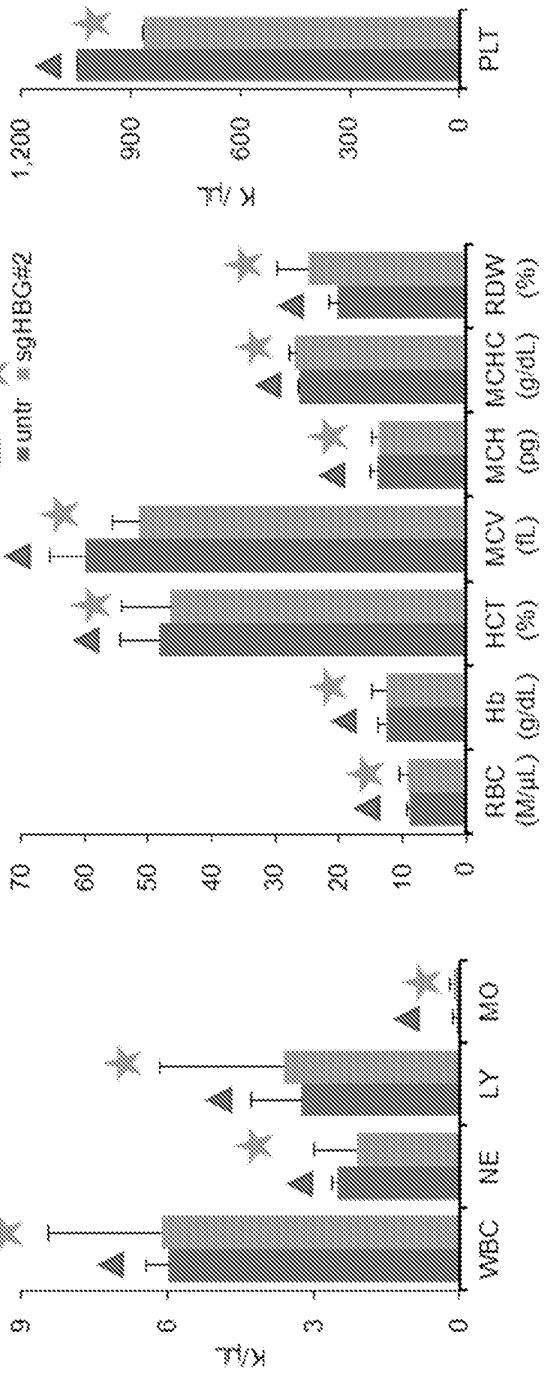

untr
sgHBG#2
K/μL
9
6
3
0
WBC
NE
LY
MO
70
60
50
40
30
20
10
0
RBC (M/μL)
Hb (g/dL)
HCT (%)
MCV (fL)
MCH (pg)
MCHC (g/dL)
RDW (%)
K/μL
1,200
900
600
300
0
PLT

FIG. 138B

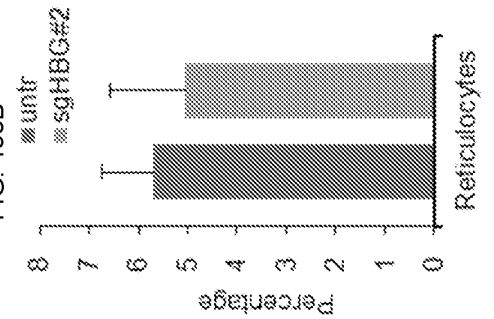


FIG. 138C

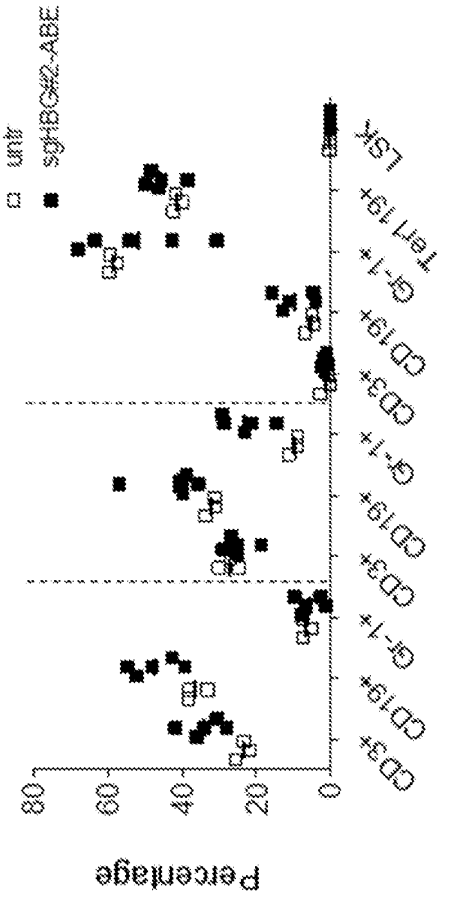
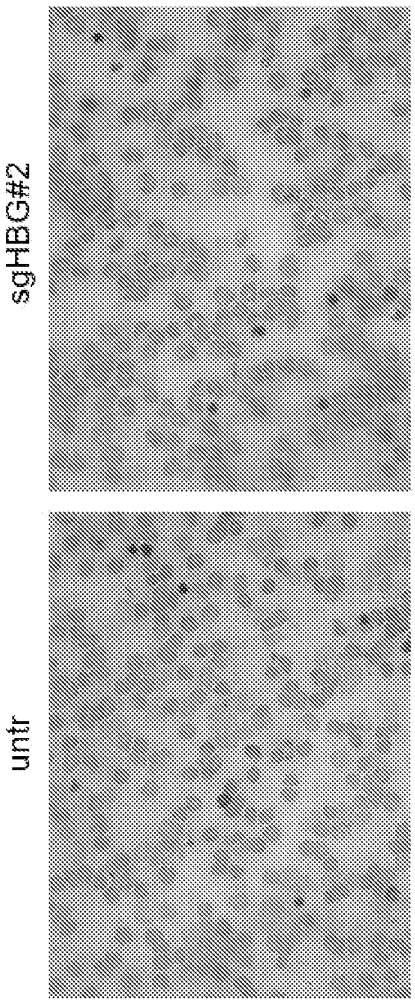


FIG. 138D



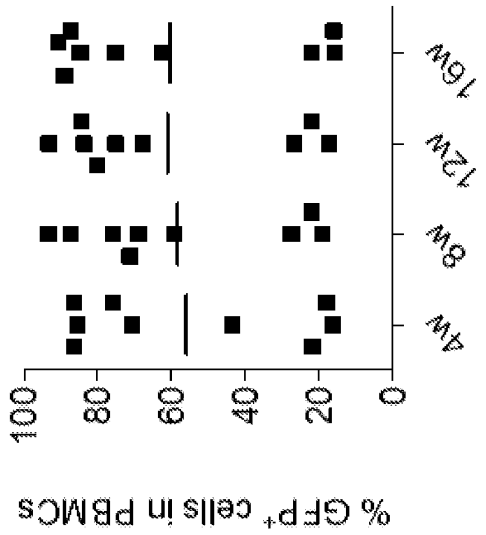


FIG. 139B

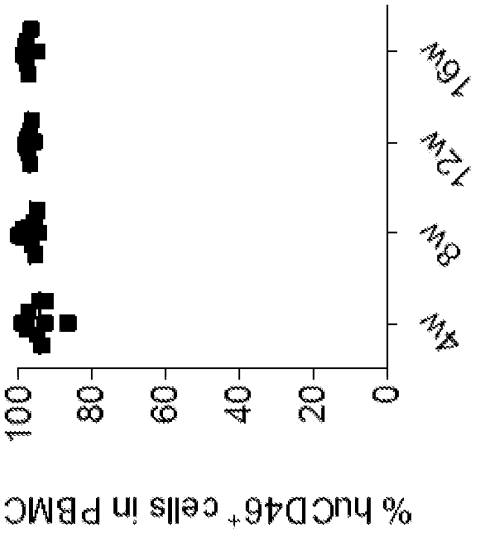


FIG. 139A

FIG. 139C

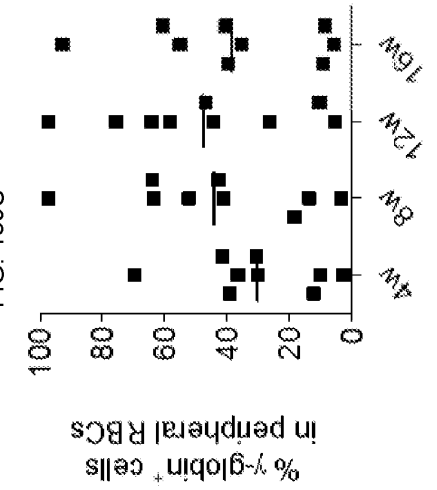


FIG. 140A

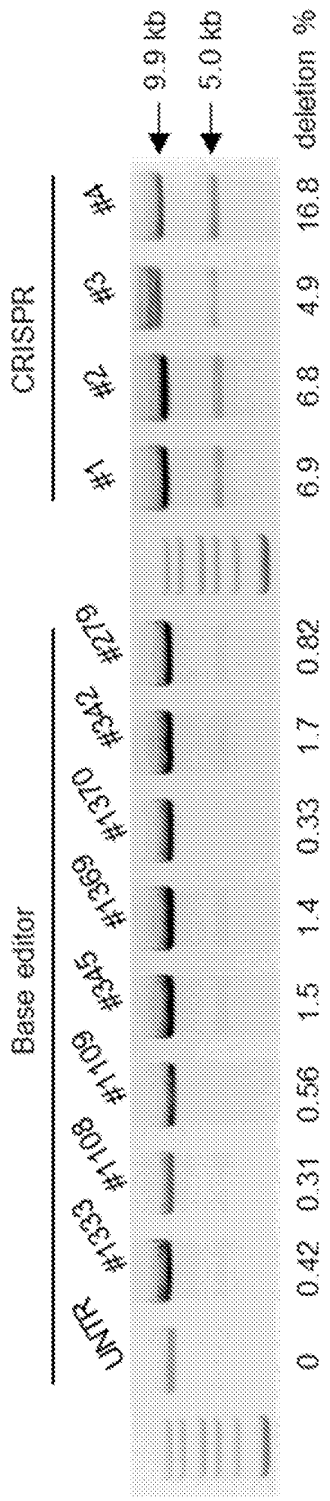


FIG. 140B

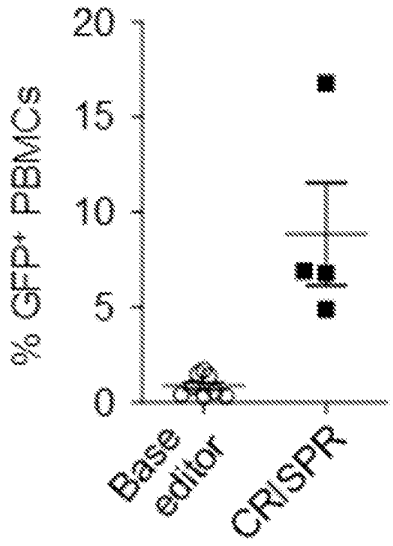


FIG. 141

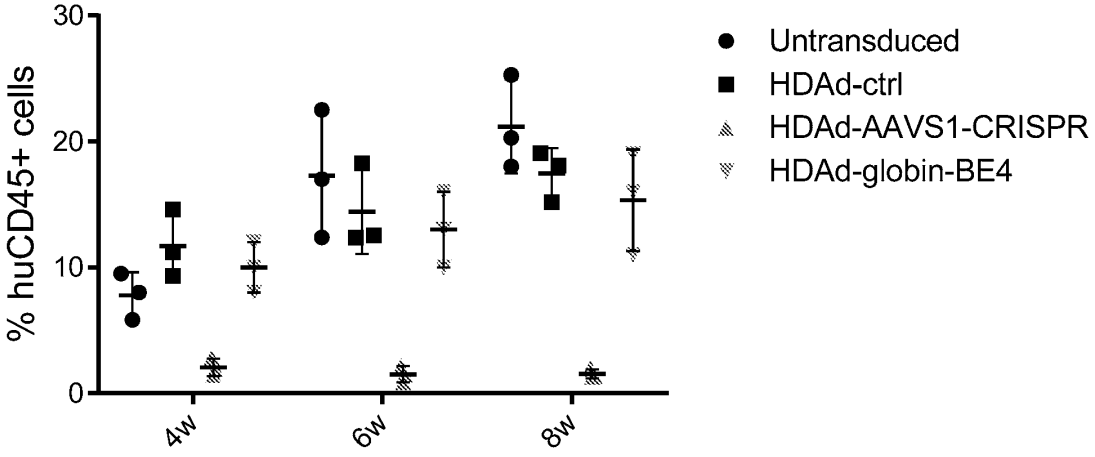


FIG. 142

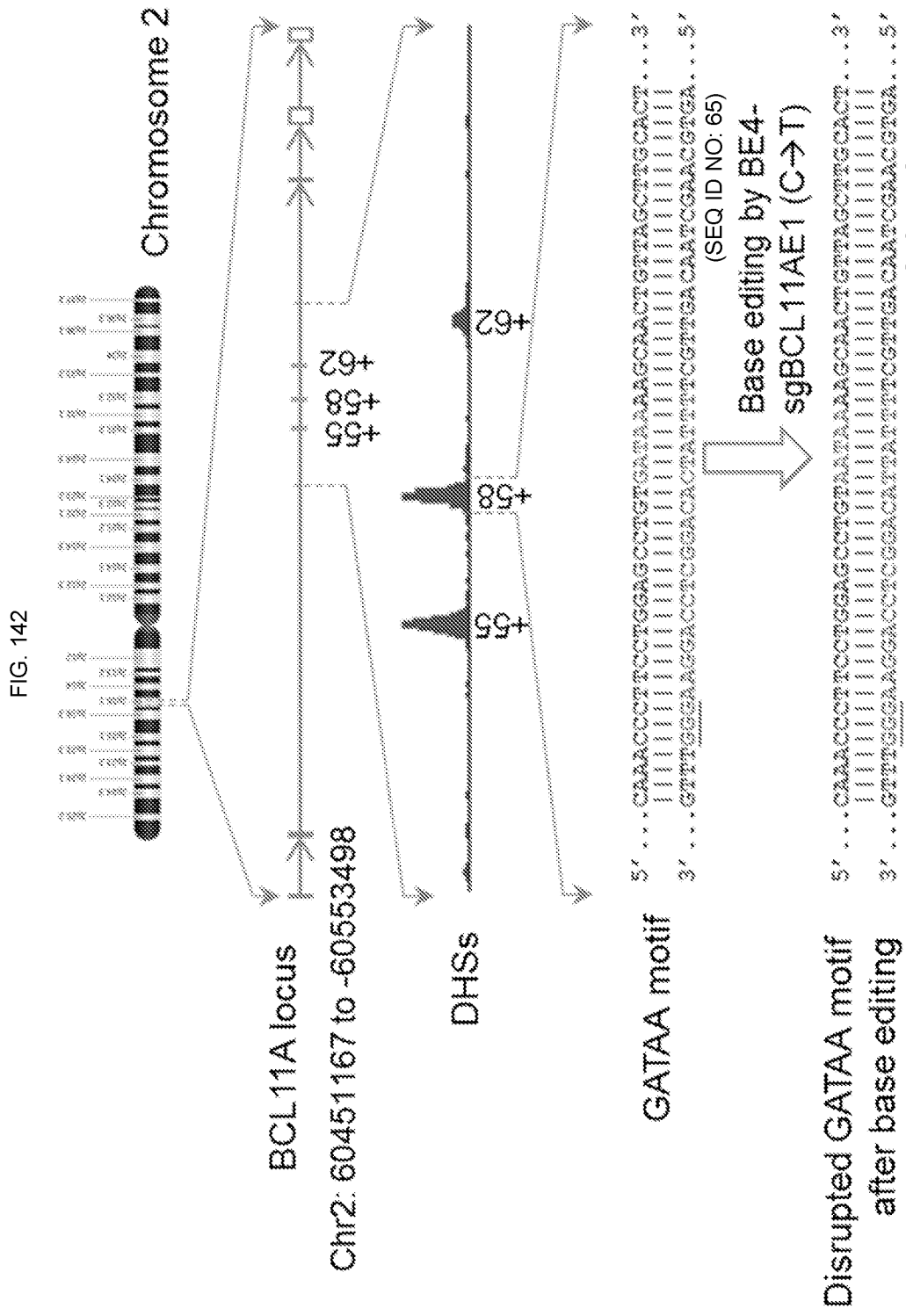

Chromosome 2
BCL11A locus
Chr2: 60451167 to -60553498
+55
+58
+62
DHSs
+55
+58
+62
GATAA motif
5' . . . CAAACCCTTCCTGGAGCCTGTGATAAAAGCAACTGTTAGCTTGCACT . . . 3'
3' . . . GTTTGGGAAGGACCTCGGACACTATTTCGTTGACAATCGAACGTGA . . . 5'
(SEQ ID NO: 65)
Base editing by BE4-
sgBCL11AE1 (C→T)
Disrupted GATAA motif
after base editing
5' . . . CAAACCCTTCCTGGAGCCTGTGTAATAAAAGCAACTGTTAGCTTGCACT . . . 3'
3' . . . GTTTGGGAAGGACCTCGGACACATTATTTCGTTGACAATCGAACGTGA . . . 5'
(SEQ ID NO: 67)

FIG. 143

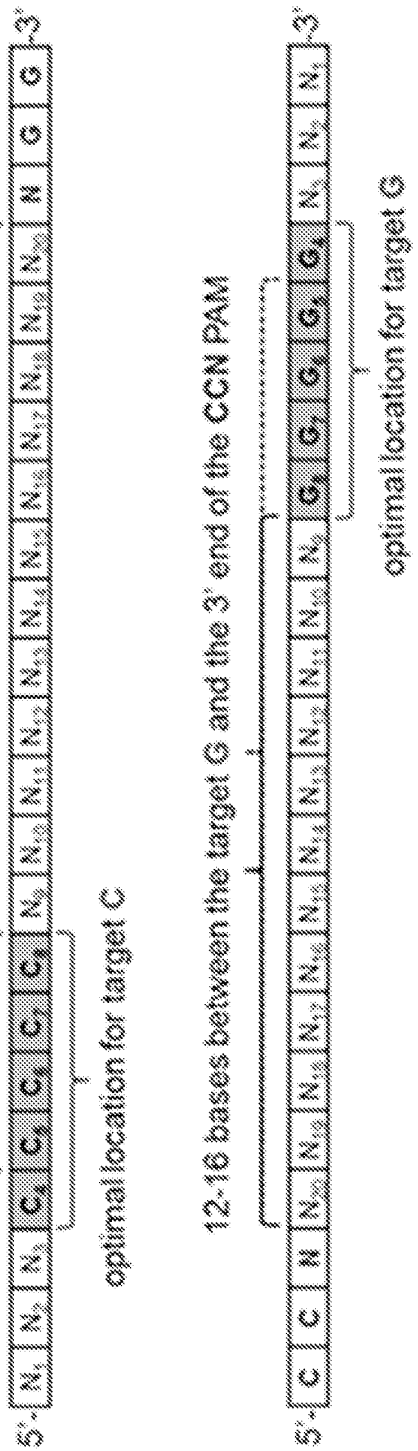

12-16 bases between the target C and the 5' end of the NGG PAM
5'-
3'
optimal location for target C
12-16 bases between the target G and the 3' end of the CCN PAM
5'-
3'
optimal location for target G

FIG. 144

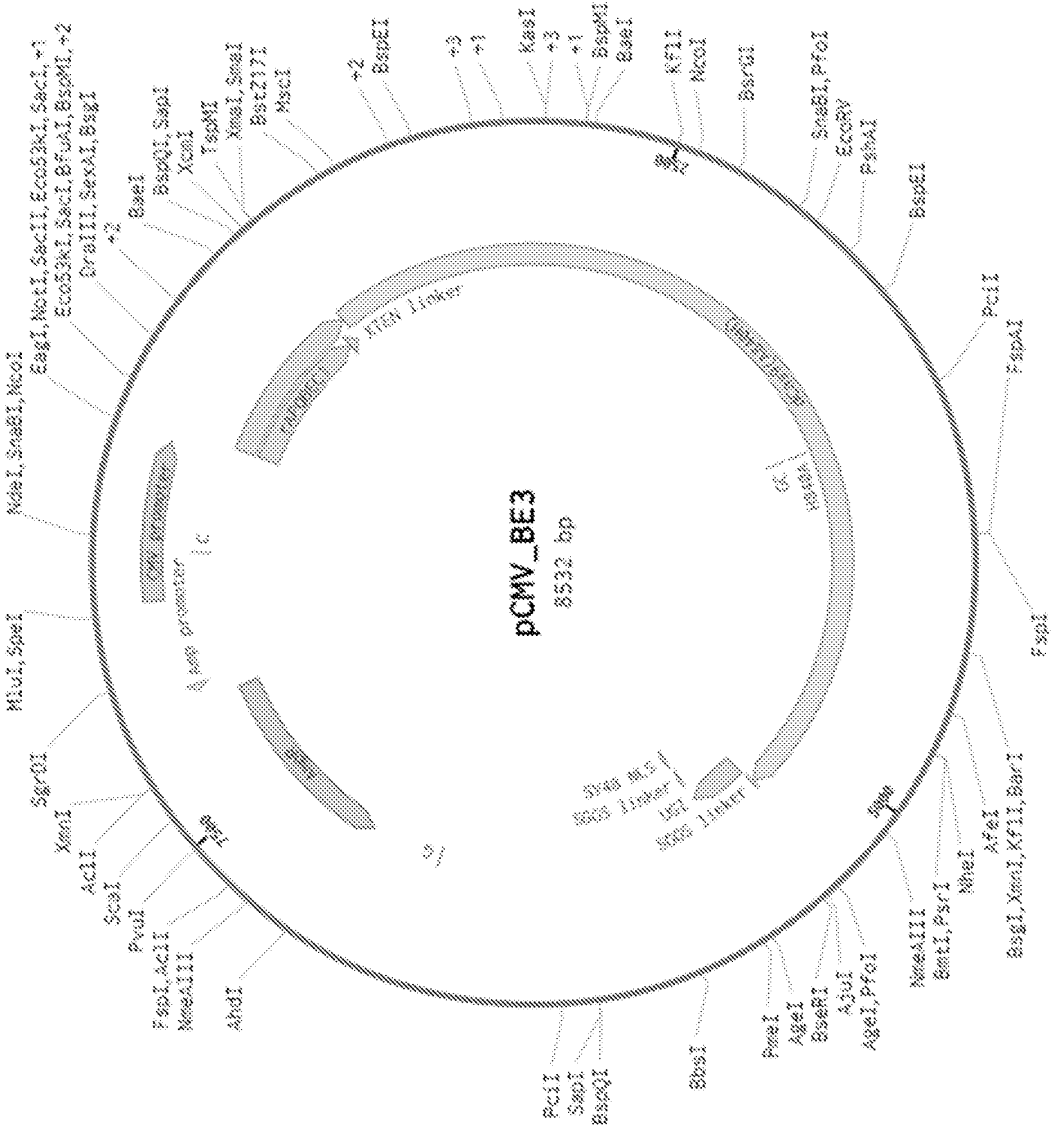

pCMV_BE3
8532 bp
CMV promoter
T2A
rAPOBEC1
UGI
SV40 NLS
nSpCas9
SV40 NLS
UGI
SV40 NLS
nucleoplasmin NLS
rAPOBEC1
XTEN linker
SV40 NLS
nucleoplasmin NLS
SV40 NLS
HA tag
bGH poly(A) signal
f1 ori
pUC ori
Lac promoter
lac
AmpR
MluI, SpeI
NcoI, SnaBI, NdeI
EagI, NotI, SacII, Eco53kI, SacI, +1
Eco53kI, SacI, BfuAI, BspMI, +2
DraIII, SexAI, BsgI
+2
BseYI
BsaXI, SapI
XcmI
TspMI
XmaI, SmaI
Bst2I7I
MscI
+2
BspEI
+3
+1
KasI
+3
+1
BspMI
BsaI
KflI
NcoI
BsrGI
SnaBI, PfoI
EcoRV
PshAI
BssHII
PciI
FspAI
FseI
BsgI, XmaI, KflI, BsrI
AflII
NheI
BmtI, PsrI
MreAIII
AgeI, PfoI
AgeI
BsaBI
AgeI
PmeI
BbsI
PciI
SapI
BsaQI
AhdI
FseI, AclI
MreAIII
PvuI
ScaI
AclI
XmnI
SgrDI

FIG. 145

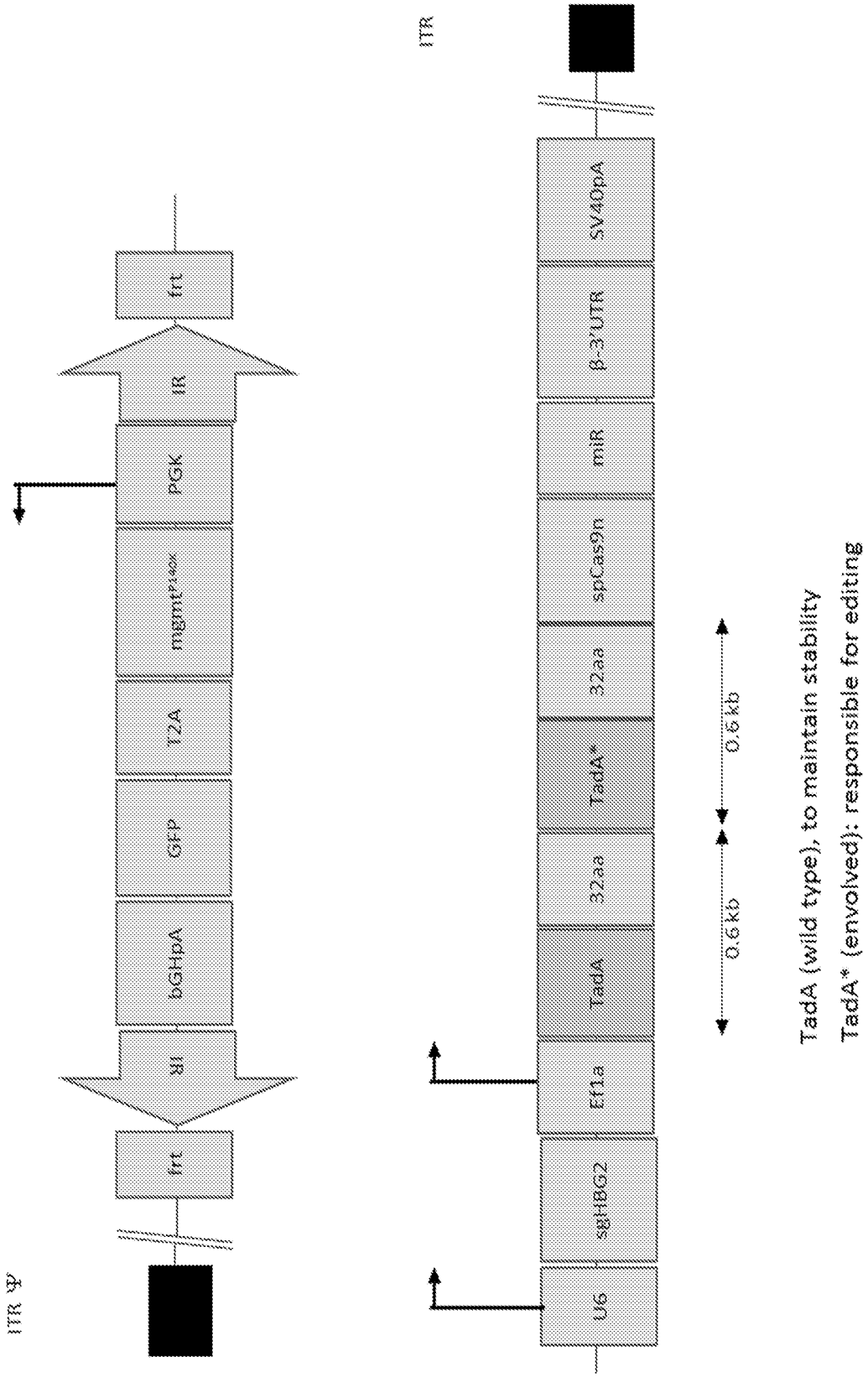

ITR Ψ
frt
IR
PGK
mgmtP140K
T2A
GFP
bGHpA
IR
frt
ITR
U6
sgHBG2
Ef1a
TadA
32aa
TadA*
32aa
spCas9n
miR
β-3'UTR
SV40pA
0.6 kb
0.6 kb
TadA (wild type), to maintain stability
TadA* (envolved): responsible for editing

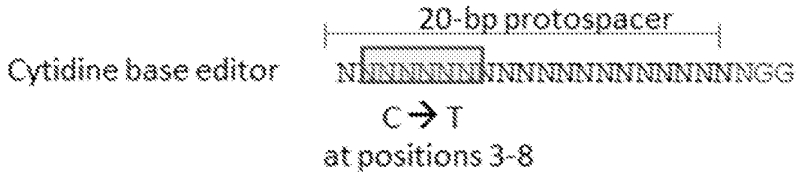
FIG. 146

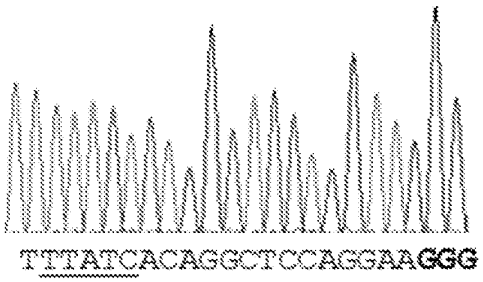
(SEQ ID NO: 265)
(SEQ ID NO: 266)

DNA sequence of two 'TadA + 32aa'

(SEQ ID NO: 267)
(SEQ ID NO: 268)

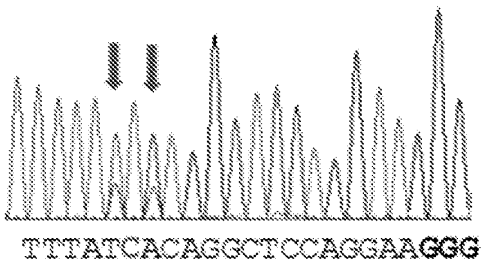
FIG. 147





Wild type sequence
Underlined: GATAA motif
Bold: PAM motif

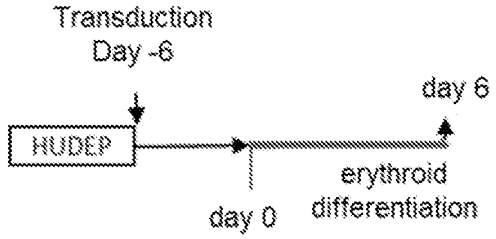
(SEQ ID NO: 269)

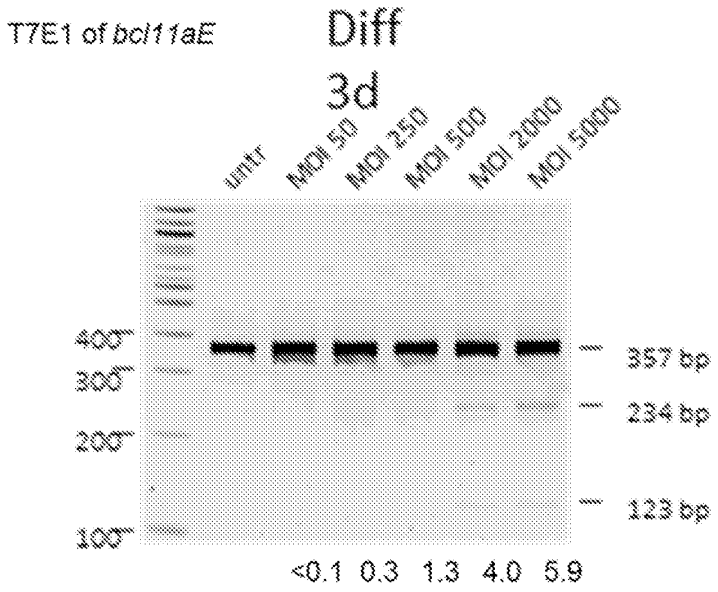


Base edited sequence
Arrows: edited bases
Purple: disrupted base of GATAA motif

(SEQ ID NO: 269)

FIG. 148


Transduction
Day -6
day 6
HUDEP
erythroid
differentiation
day 0


T7E1 of bcl11aE
Diff
3d
untr
MOI 50
MOI 250
MOI 500
MOI 2000
MOI 5000
400
300
200
100
357 bp
234 bp
123 bp
<0.1 0.3 1.3 4.0 5.9

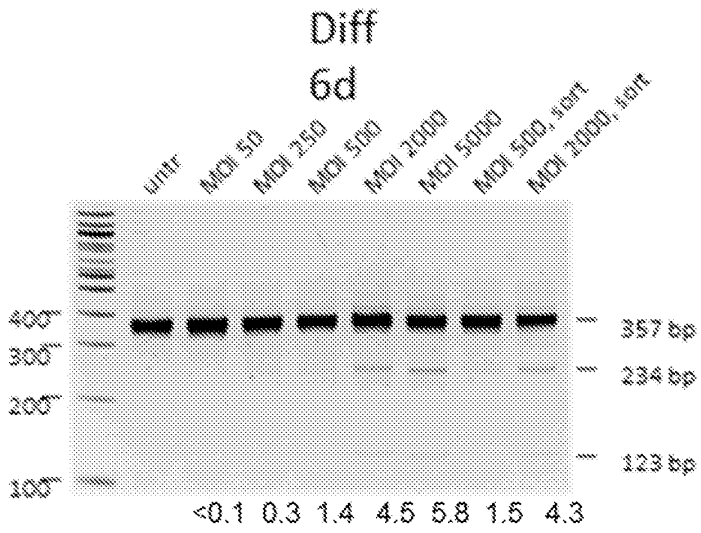

Diff
6d
untr
MOI 50
MOI 250
MOI 500
MOI 2000
MOI 5000
MOI 500, sort
MOI 2000, sort
400
300
200
100
357 bp
234 bp
123 bp
<0.1 0.3 1.4 4.5 5.8 1.5 4.3

FIG. 149

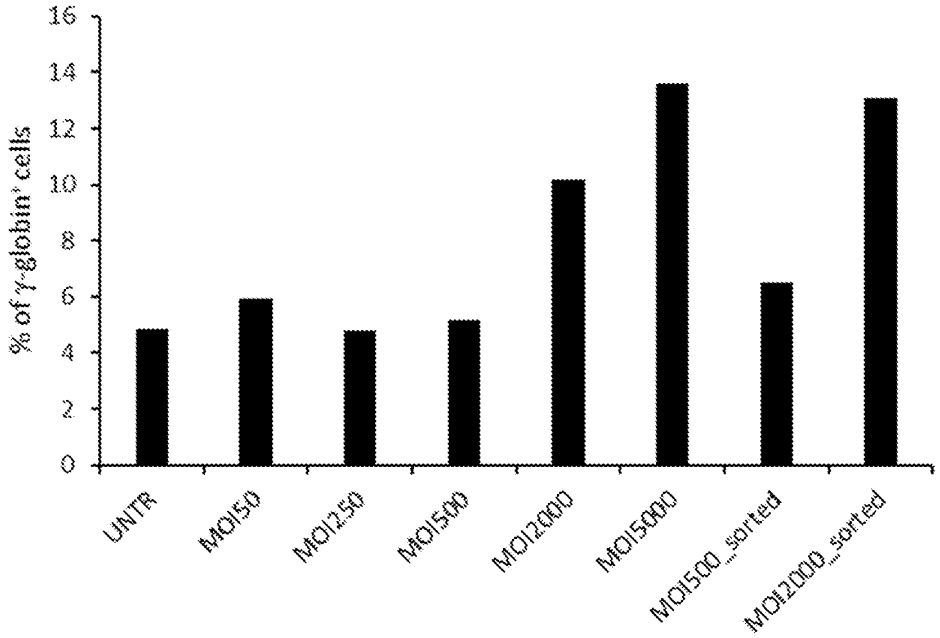


FIG. 150

Reactivation of HbF by Base editing

Cytidine base editor: C → T
Adenine base editor: A → G

Constructed vectors:

pBS-AncBE4max-sgBCL11AE1	+58 GATA motif, G --> A
pBS-AncBE4max-sgBCL11AE2	+58 GATA motif, G --> A
pBS-ABEmax-sgBCL11AE3	+58 GATA motif, T --> C
pBS-ABEmax-sgBCL11AE4	+58 GATA motif, T --> C
pBS-AncBE4max-sgHBG1	13bp deletion, CCAAT box, CC --> TT
pBS-ABEmax-sgHBG2	13bp deletion, CCAAT box, A --> G
pBS-ABEmax-sgHBG3	13bp deletion, CCAAT box, T --> C
pBS-ABEmax-sgHBG4	-198 T --> C
pBS-AncBE4max-sgR5-1	control

FIG. 151

	Date	Full name	Titer	Note
	101918-3	HDAd5/35++_BE4max-sgBCL11Ae1-FI-mgmtGFP	0.5 x 10e12	
→	041619-2	HDAd5/35++_BE4max-sgHBG1-FI-mgmtGFP	very low (use as 0.05 x 10e12)	
→	032819-1	HDAd5/35++_ABEopti-sgHBG2-FI-mgmtGFP	2.8 x 10e12	
→	041819-1	HDAd5/35++_ABEopti-sgHBG4-FI-mgmtGFP	1.52 x 10e12	
→	041619-3	HDAd5/35++_BE4max-Dual1-FI-mgmtGFP	very low (use as 0.05 x 10e12)	sgBcl1 + sgHBG1
	041619-1	HDAd5/35++_ABEopti-Dual-skip-FI-mgmtGFP	2.5 x 10e12	sgHBG2 + sgHBB-Stop
	041819-2	HDAd5/35++_BE4max-sgR5-1-FI-mgmtGFP	0.9 x 10e12	Non-targeting ctrl

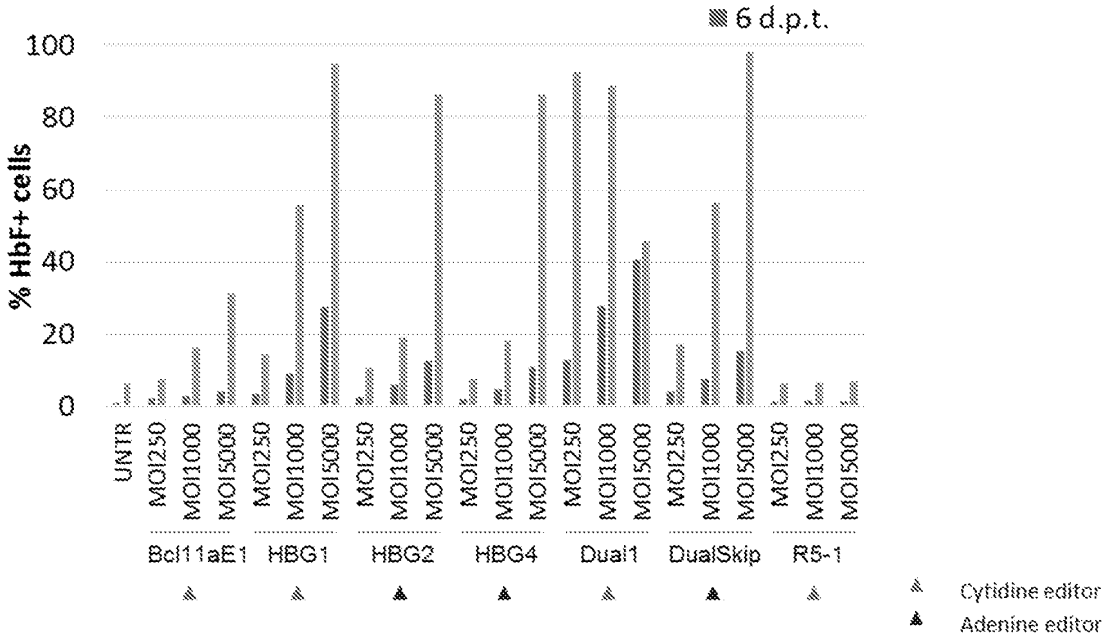


FIG. 152

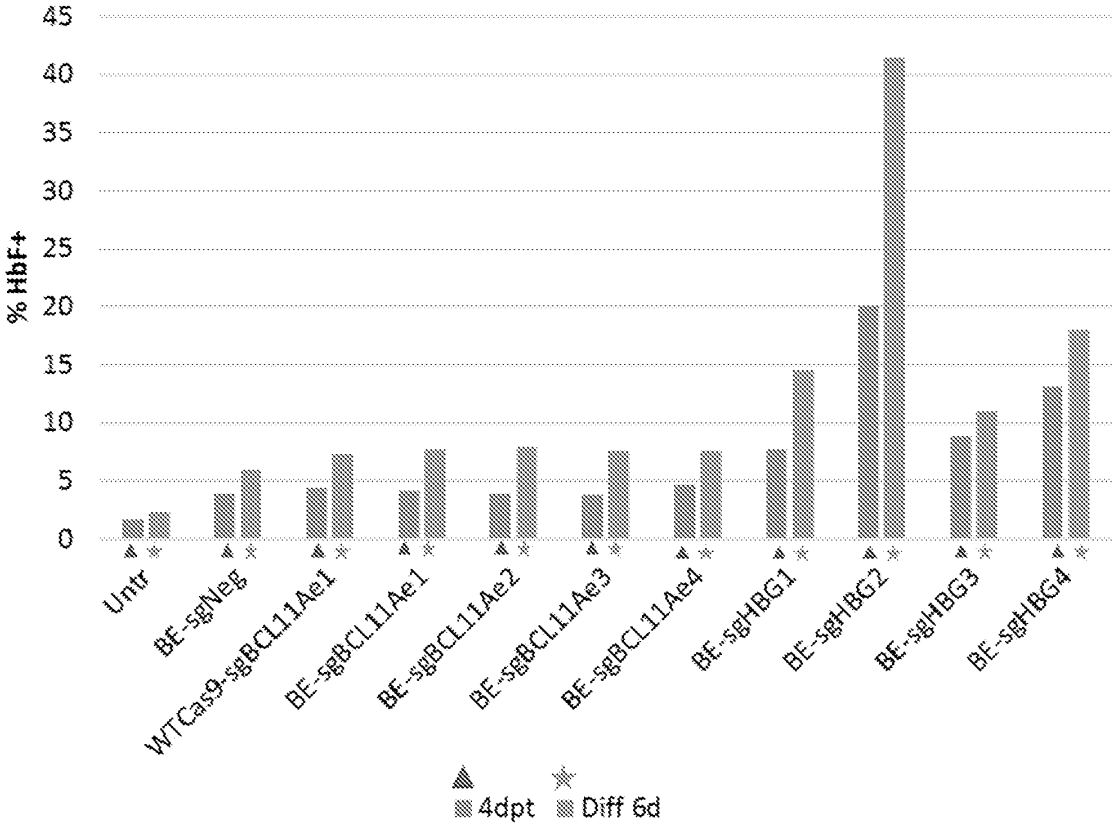

45
40
35
30
25
20
15
10
5
0
% HbF+
Untr
BE-sgNeg
WTCas9-sgBCL11Ae1
BE-sgBCL11Ae1
BE-sgBCL11Ae2
BE-sgBCL11Ae3
BE-sgBCL11Ae4
BE-sgHBG1
BE-sgHBG2
BE-sgHBG3
BE-sgHBG4
4dpt
Diff 6d

FIG. 153

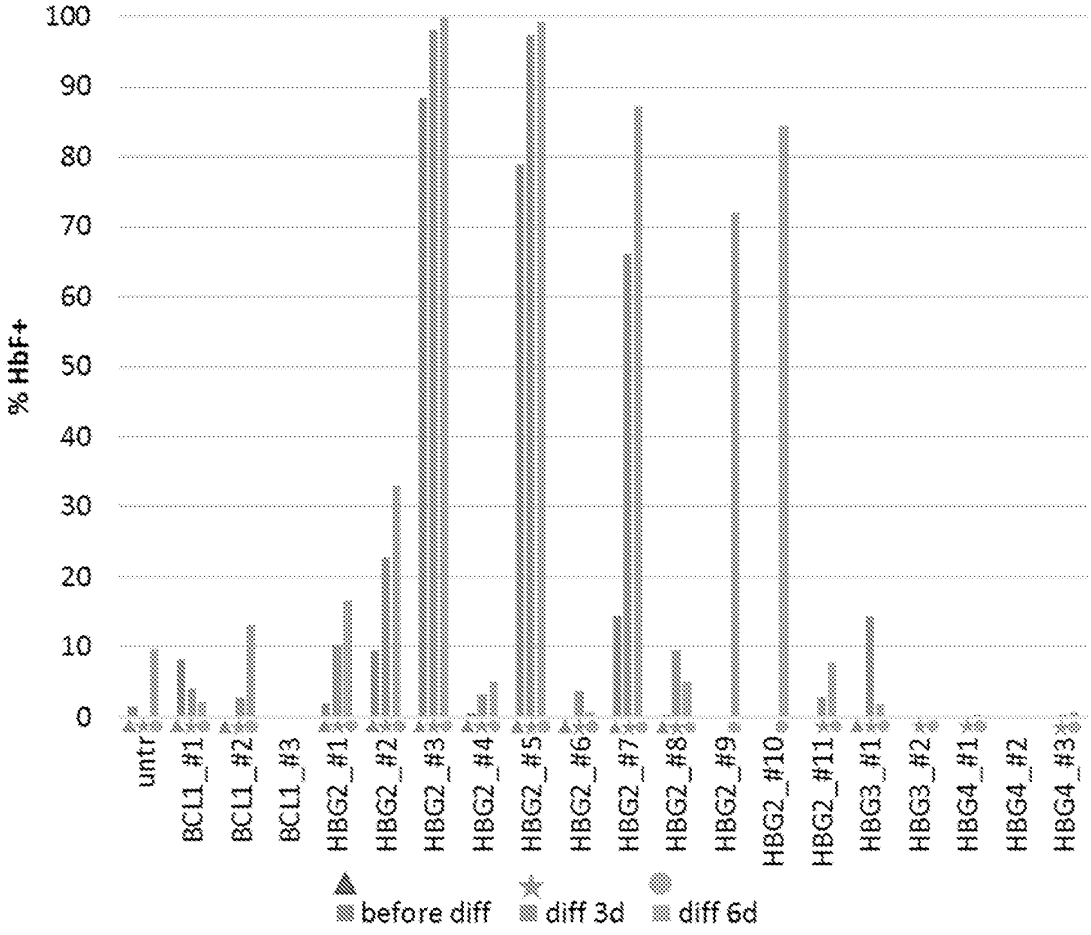

% HbF+
100
90
80
70
60
50
40
30
20
10
0
untr
BCL1_#1
BCL1_#2
BCL1_#3
HBG2_#1
HBG2_#2
HBG2_#3
HBG2_#4
HBG2_#5
HBG2_#6
HBG2_#7
HBG2_#8
HBG2_#9
HBG2_#10
HBG2_#11
HBG3_#1
HBG3_#2
HBG4_#1
HBG4_#2
HBG4_#3
before diff
diff 3d
diff 6d

FIG. 154A

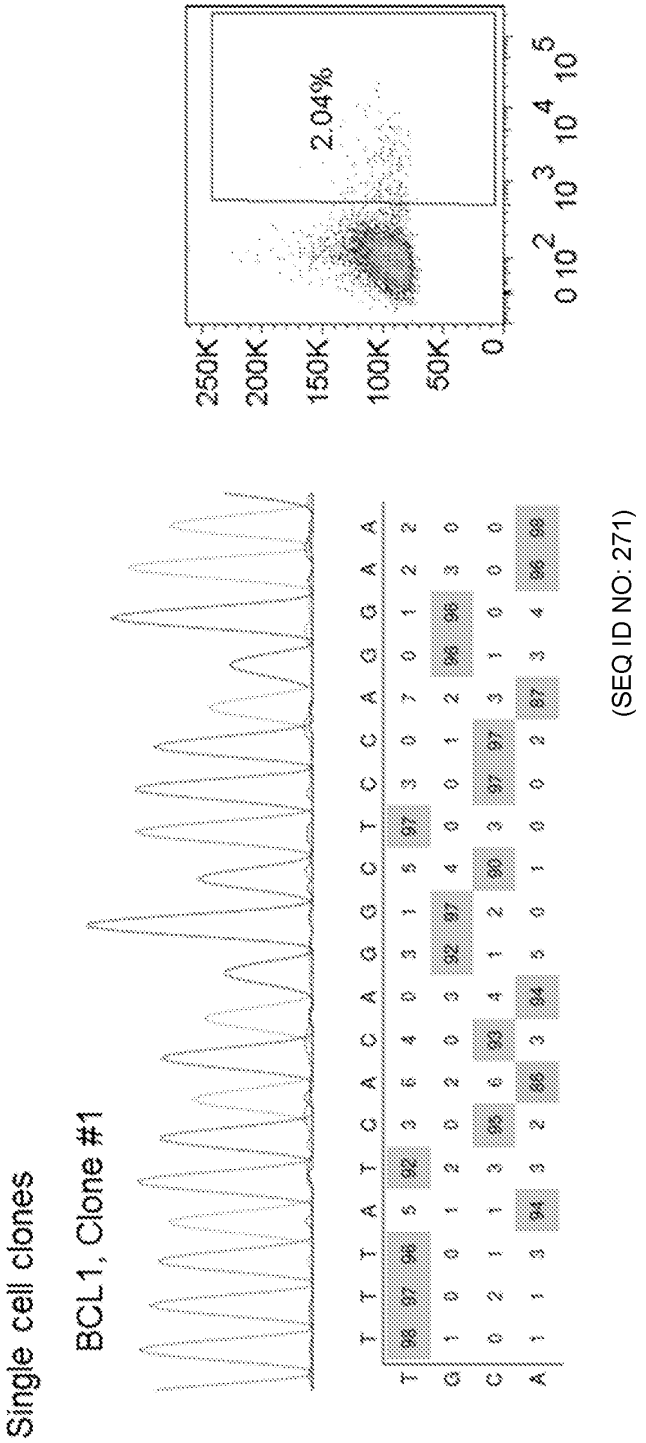

Single cell clones
BCL1, Clone #1
T T T A T C A C A G C C T C C A G G A A
T 96 97 96 5 92 3 6 4 0 3 1 5 97 3 0 7 0 1 2 2
G 1 0 0 1 2 0 2 0 0 3 92 97 4 0 0 1 2 96 96 3 0 0
C 0 2 1 1 3 95 6 93 4 1 2 93 3 97 97 3 1 0 0 0
A 1 1 3 94 3 2 89 3 94 5 0 1 0 0 0 2 97 3 4 96 96
250K
200K
150K
100K
50K
0
0 10^2 10^3 10^4 10^5
2.04%
(SEQ ID NO: 271)

FIG. 154B

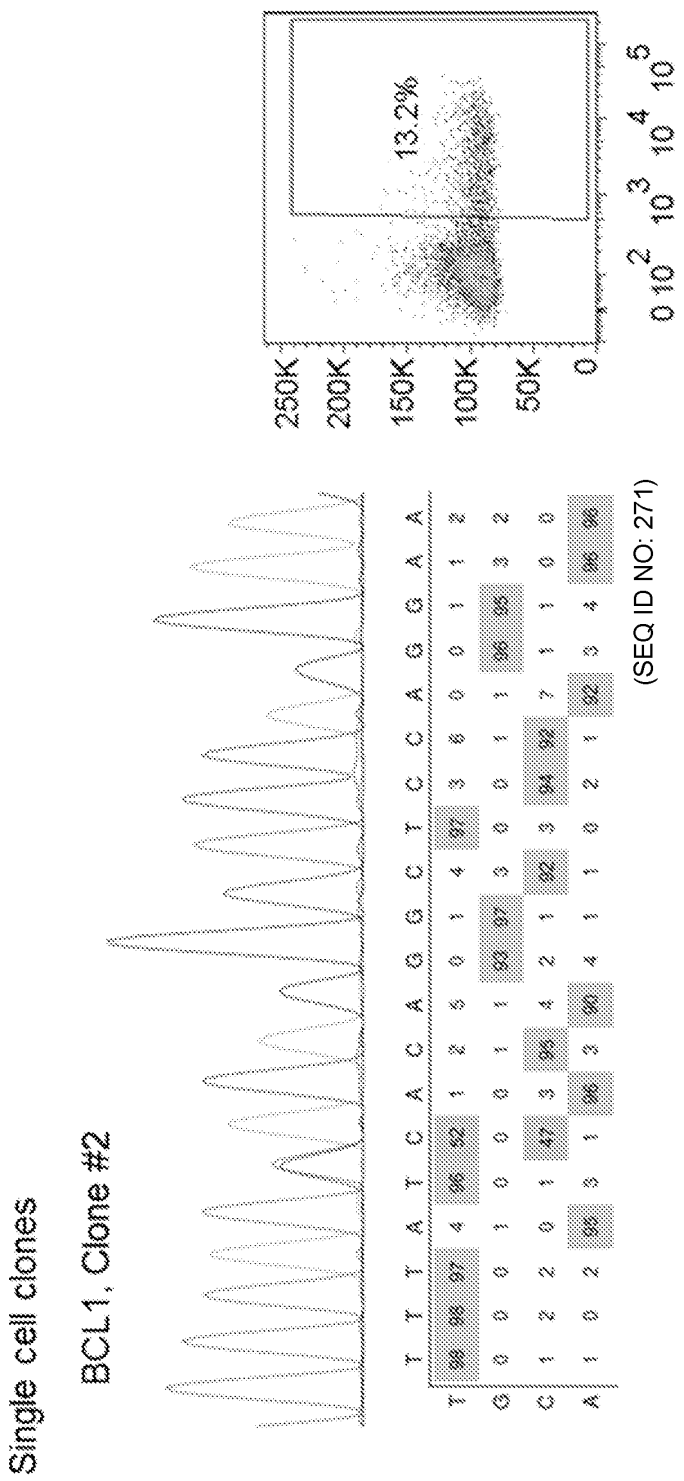
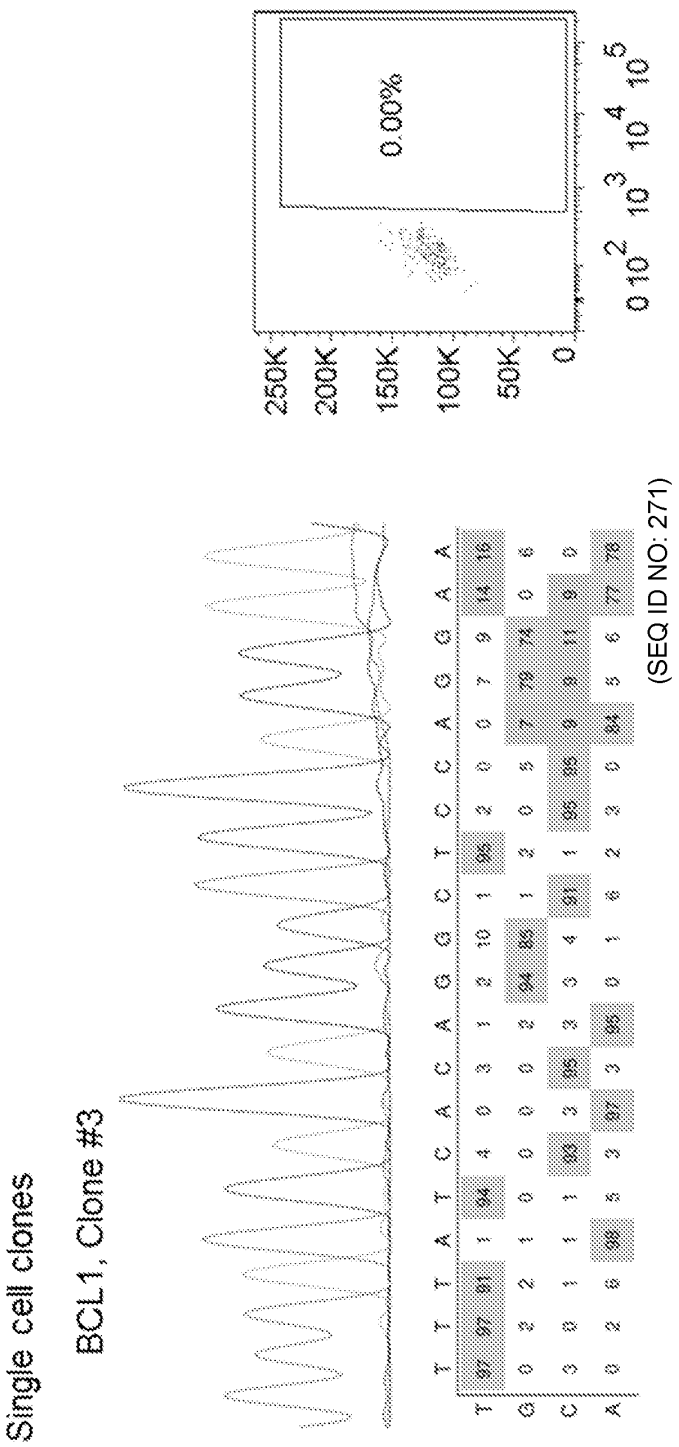

Single cell clones
BCL1, Clone #2
250K
200K
150K
100K
50K
0
0 10^2 10^3 10^4 10^5
13.2%
T T T A T C A C A G G C T C C A G G A A A
T
G
C
A
(SEQ ID NO: 271)

FIG. 154C


Single cell clones
BCL1, Clone #3
0.00%
250K
200K
150K
100K
50K
0
0 10^2 10^3 10^4 10^5
T T T A T C A C A G G G C T C C A G G A A
T 97 97 91 1 94 4 0 3 1 2 10 1 96 2 0 0 5 7 79 74 0 6
G 0 2 2 1 0 0 0 0 2 84 85 1 2 0 5 7 79 74 0 6
C 0 0 1 1 1 83 3 85 3 3 4 91 1 95 95 9 8 11 9 0
A 0 2 6 98 3 3 87 3 95 0 1 6 2 3 0 84 3 6 77 76
(SEQ ID NO: 271)

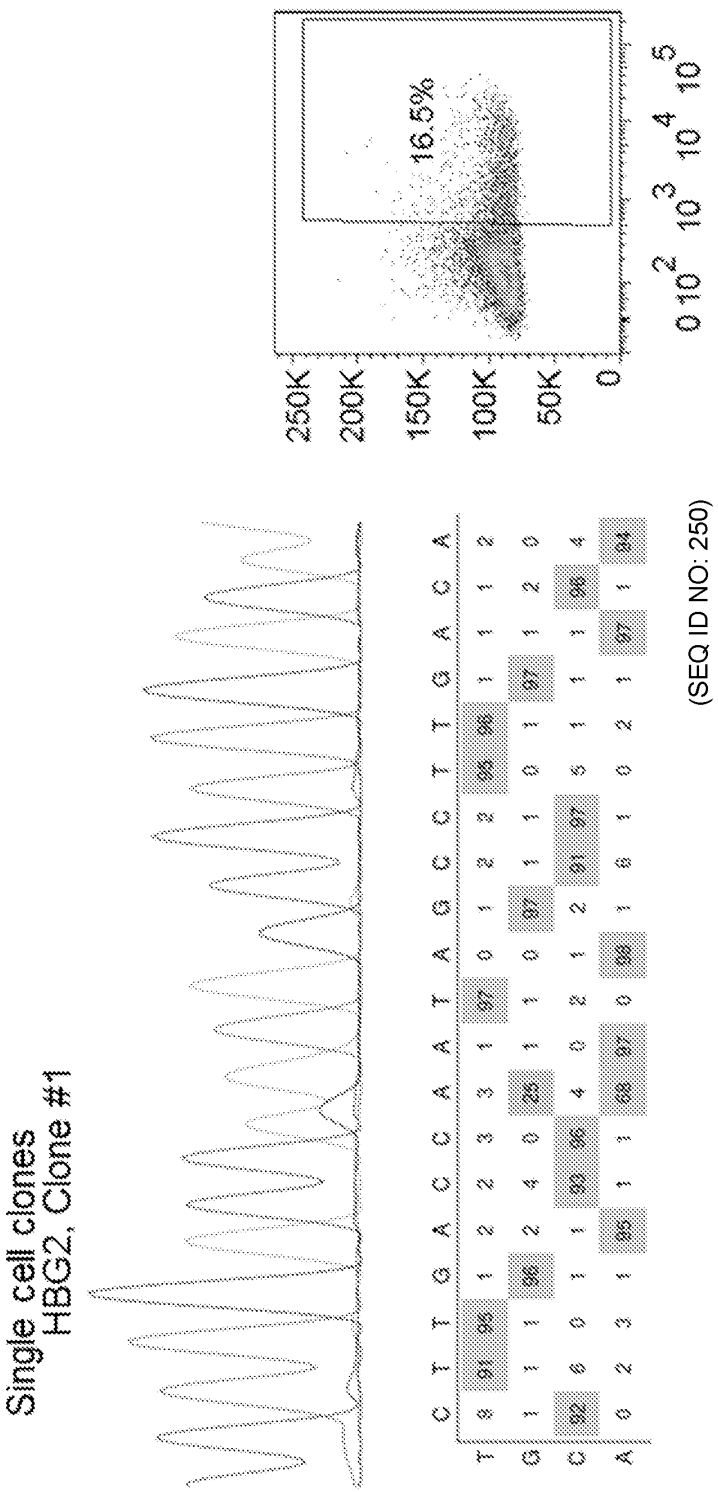

Single cell clones
HBG2, Clone #1
16.5%
250K
200K
150K
100K
50K
0
0 10^2 10^3 10^4 10^5
C T T G A C C C A A T A G G C C T T G A C A
T
G
C
A
(SEQ ID NO: 250)

FIG. 154D

FIG. 154E

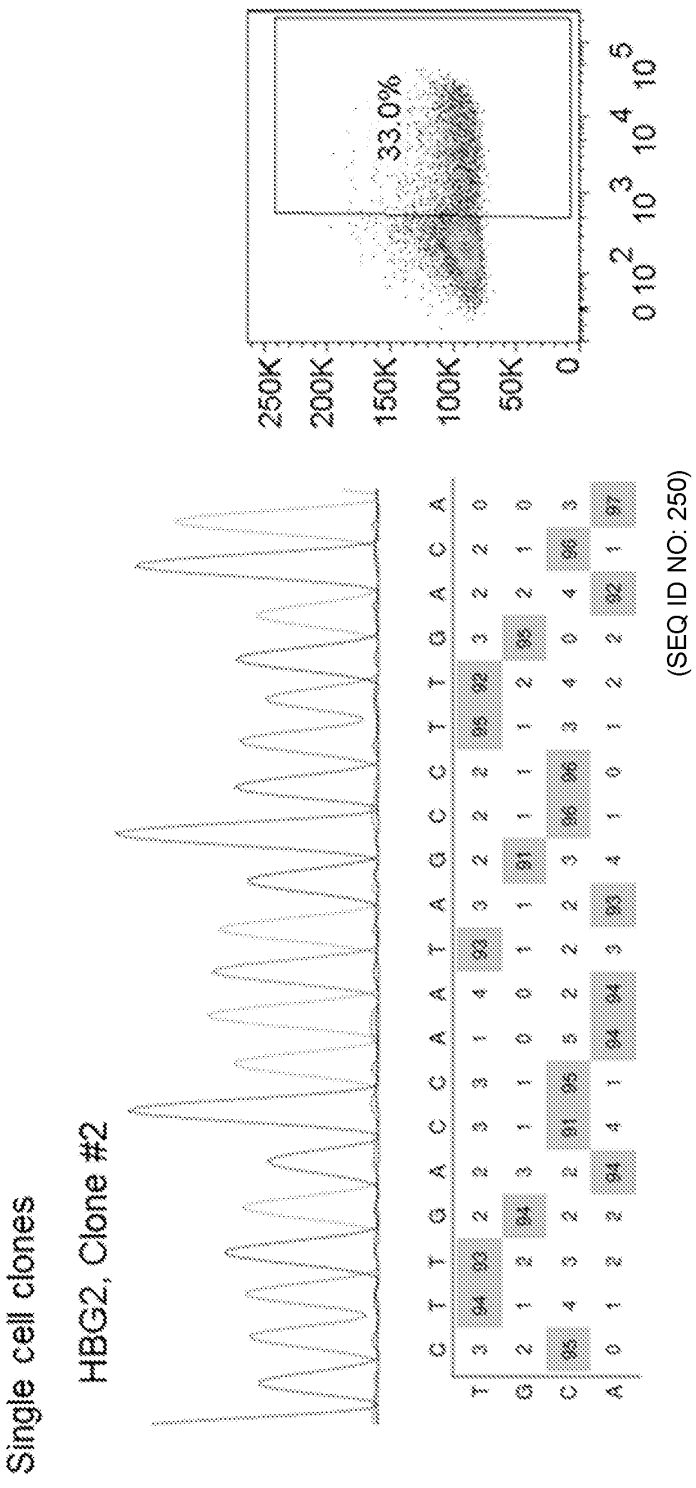
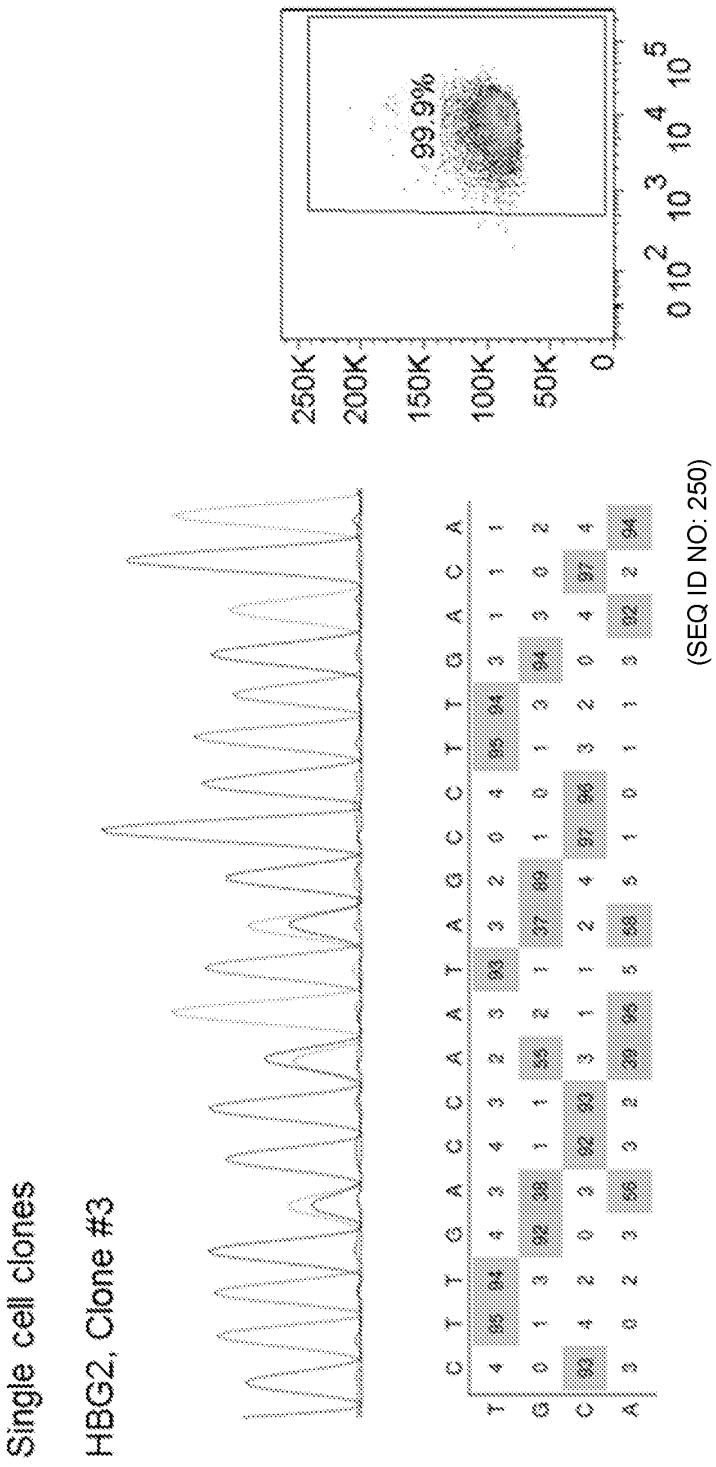
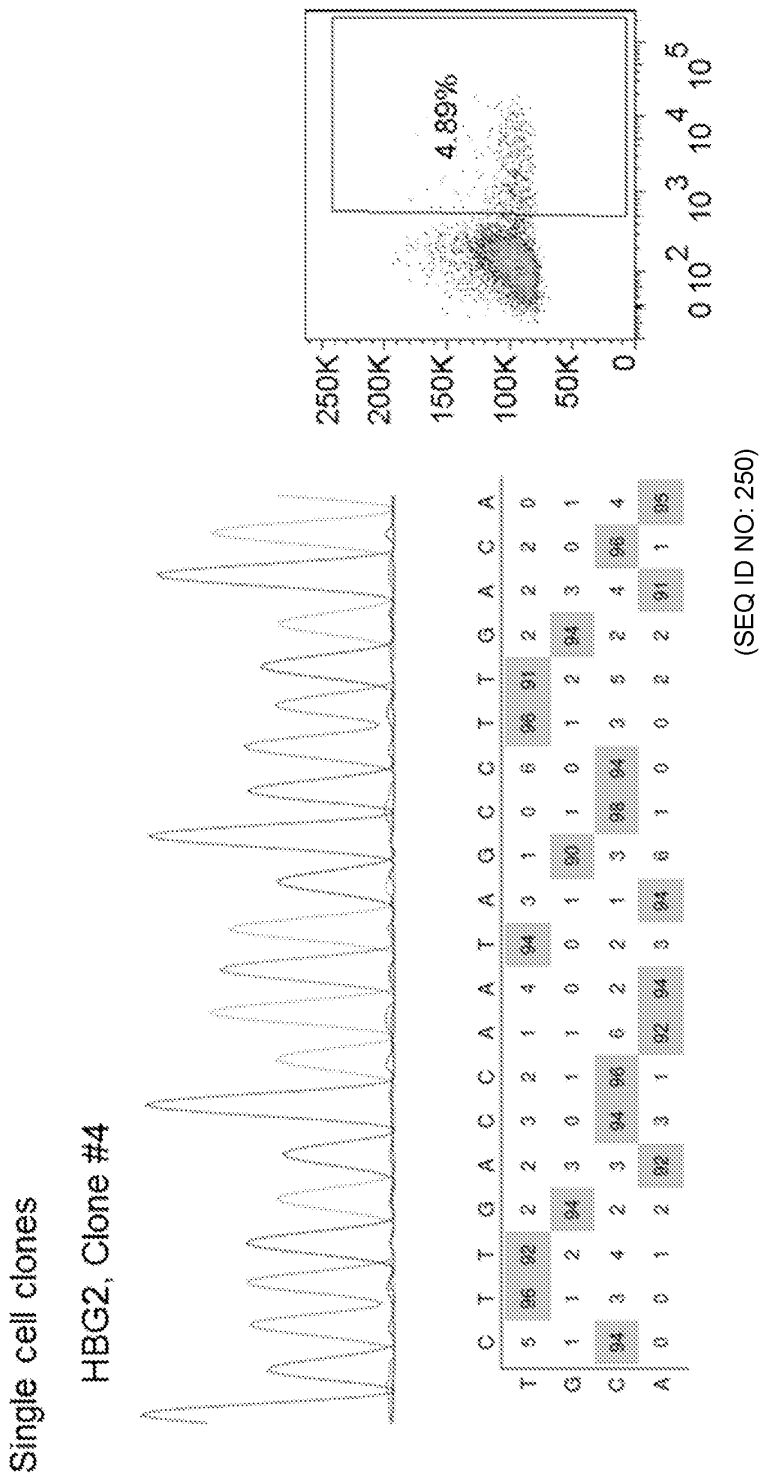

Single cell clones
HBG2, Clone #2
33.0%
250K
200K
150K
100K
50K
0
0 10^2 10^3 10^4 10^5
C T T G A C C A A T A G C C T T G A C A
T
G
C
A
(SEQ ID NO: 250)

FIG. 154F



(SEQ ID NO: 250)

FIG. 154G



(SEQ ID NO: 250)

FIG. 154H

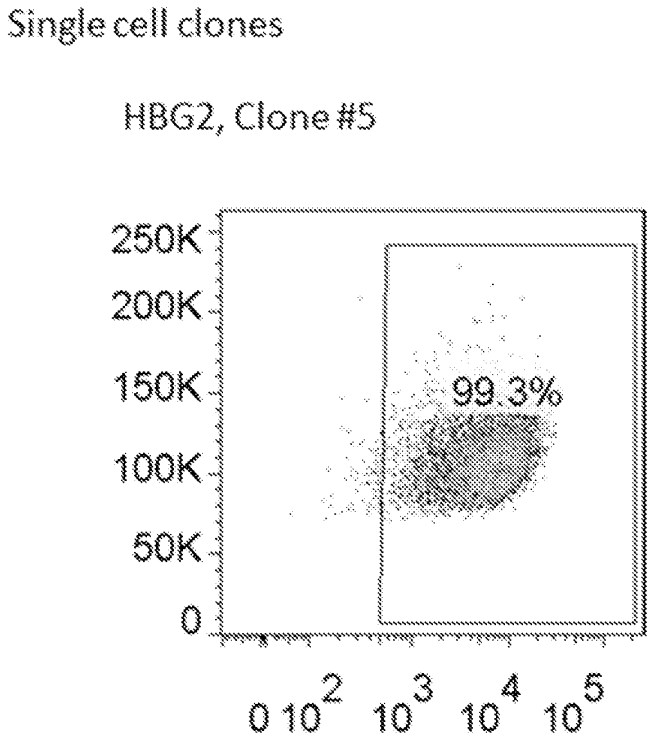

Single cell clones
HBG2, Clone #5
250K
200K
150K
100K
50K
0
99.3%
0 10^2 10^3 10^4 10^5

FIG. 154I

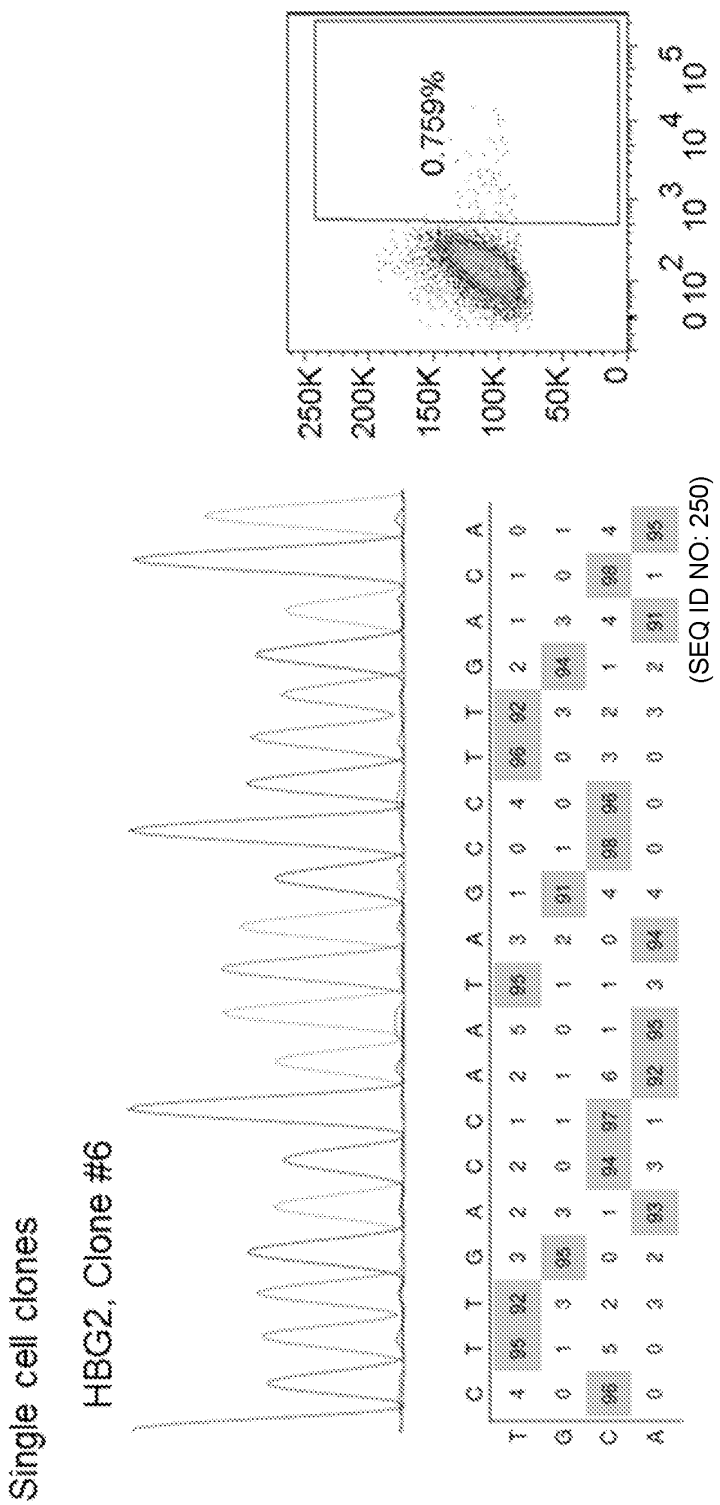
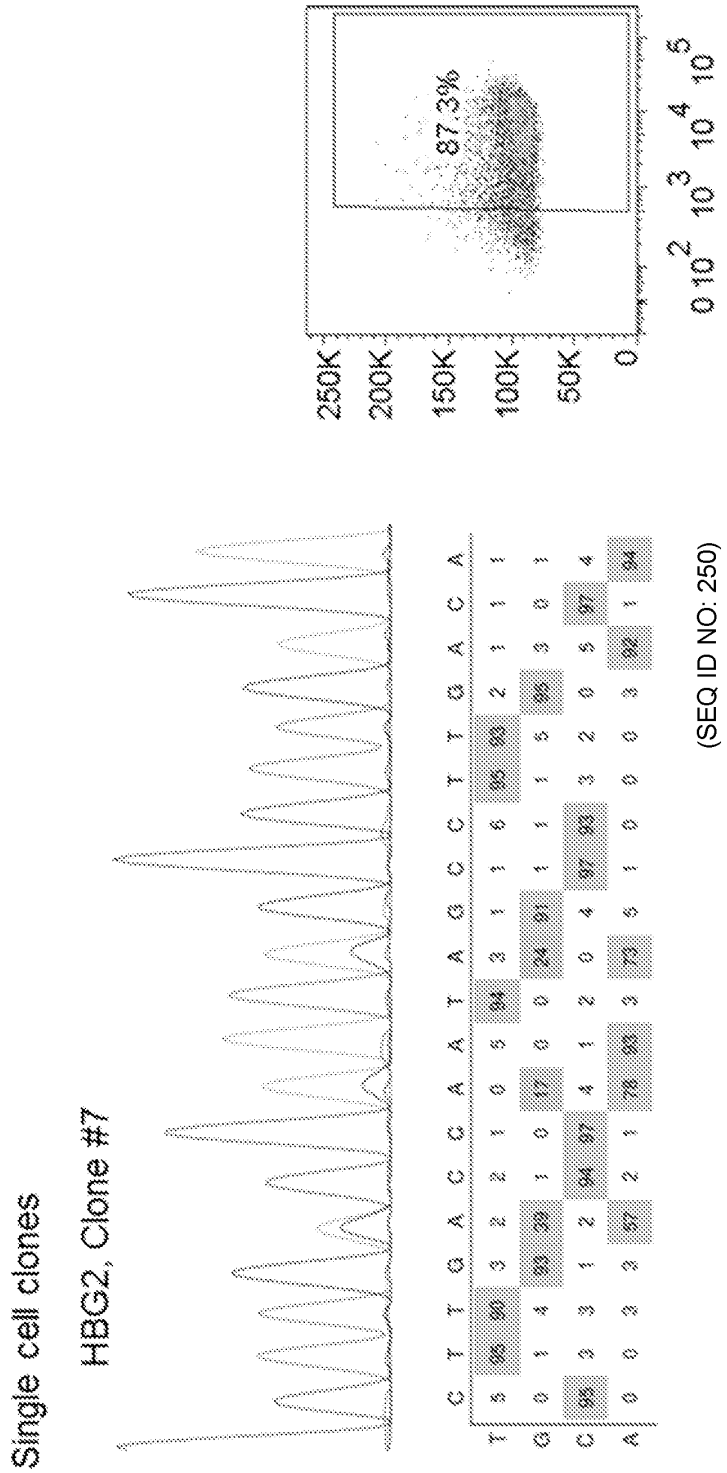
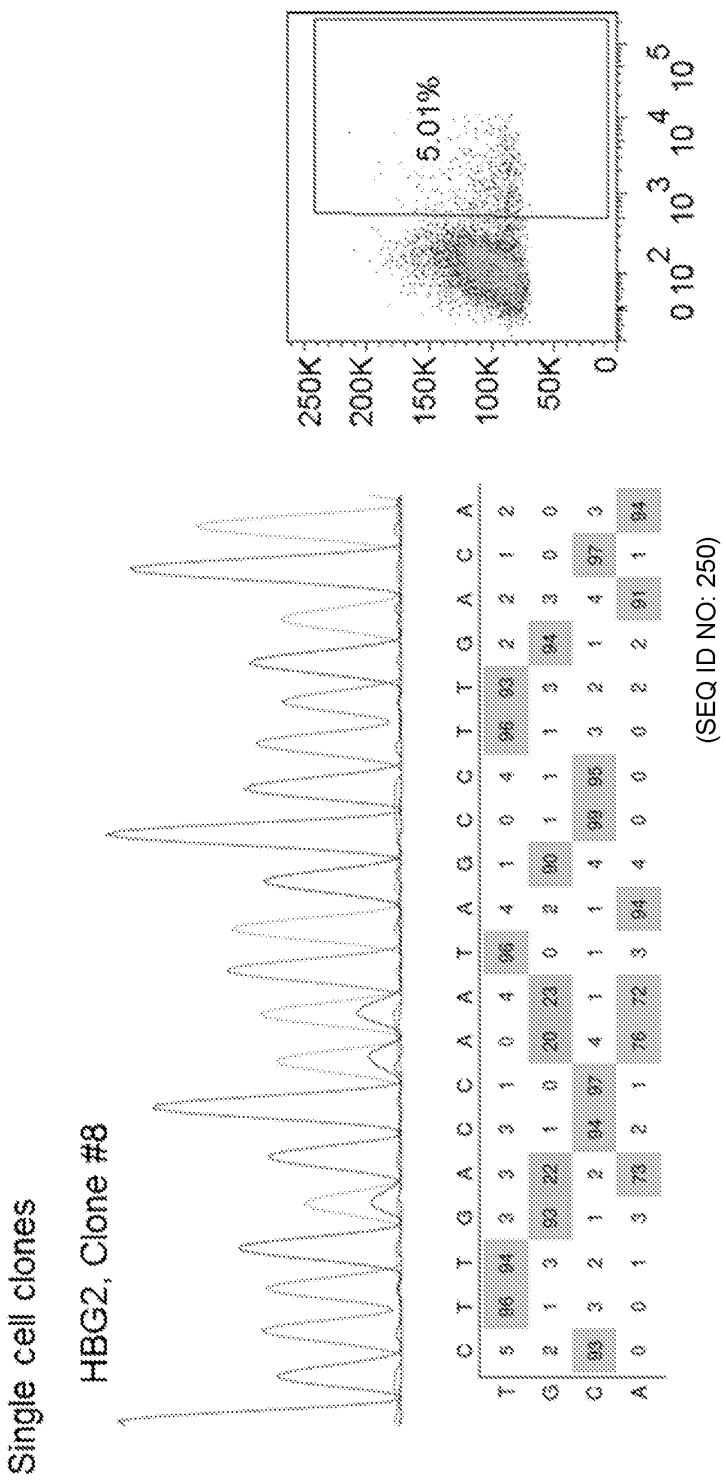

Single cell clones
HBG2, Clone #6
0.759%
250K
200K
150K
100K
50K
0
0 10^2 10^3 10^4 10^5
C T T G A C C A A T A G C C T T G A C A
T
G
C
A
(SEQ ID NO: 250)

FIG. 154J



(SEQ ID NO: 250)

FIG. 154K



(SEQ ID NO: 250)

FIG. 154L

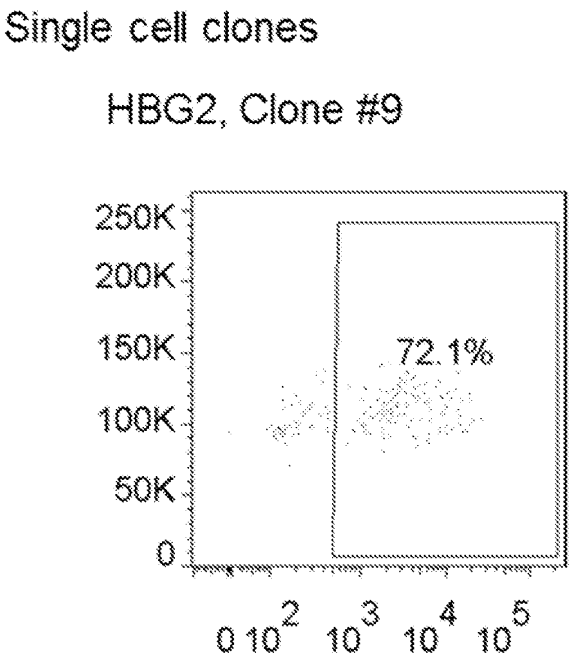


FIG. 154M

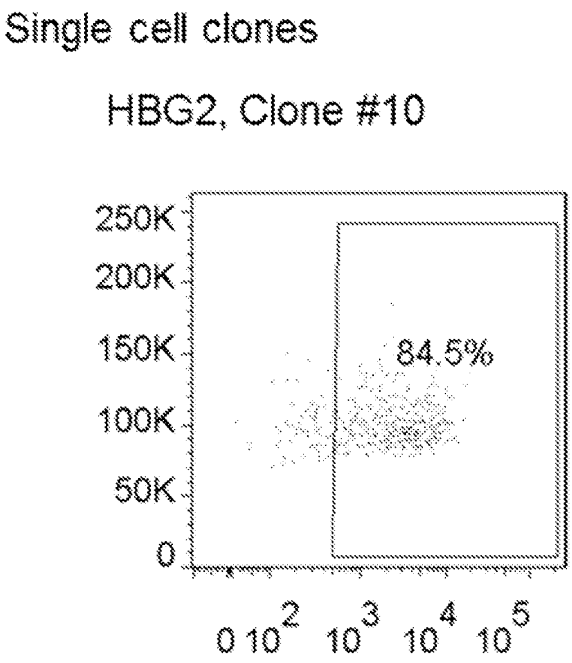


FIG. 154N

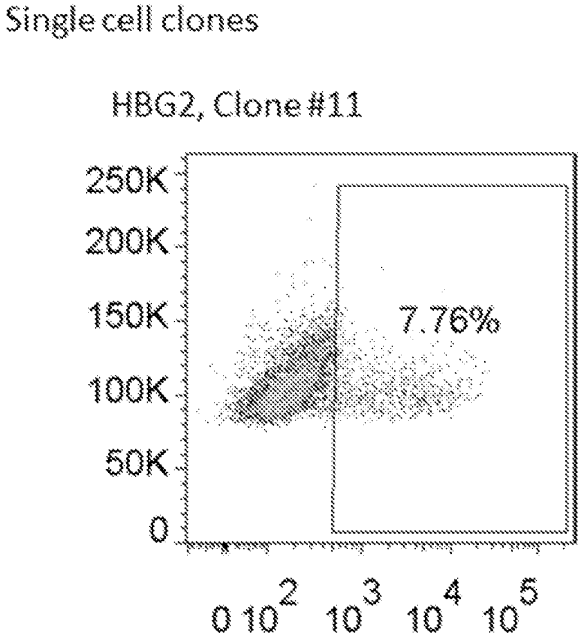
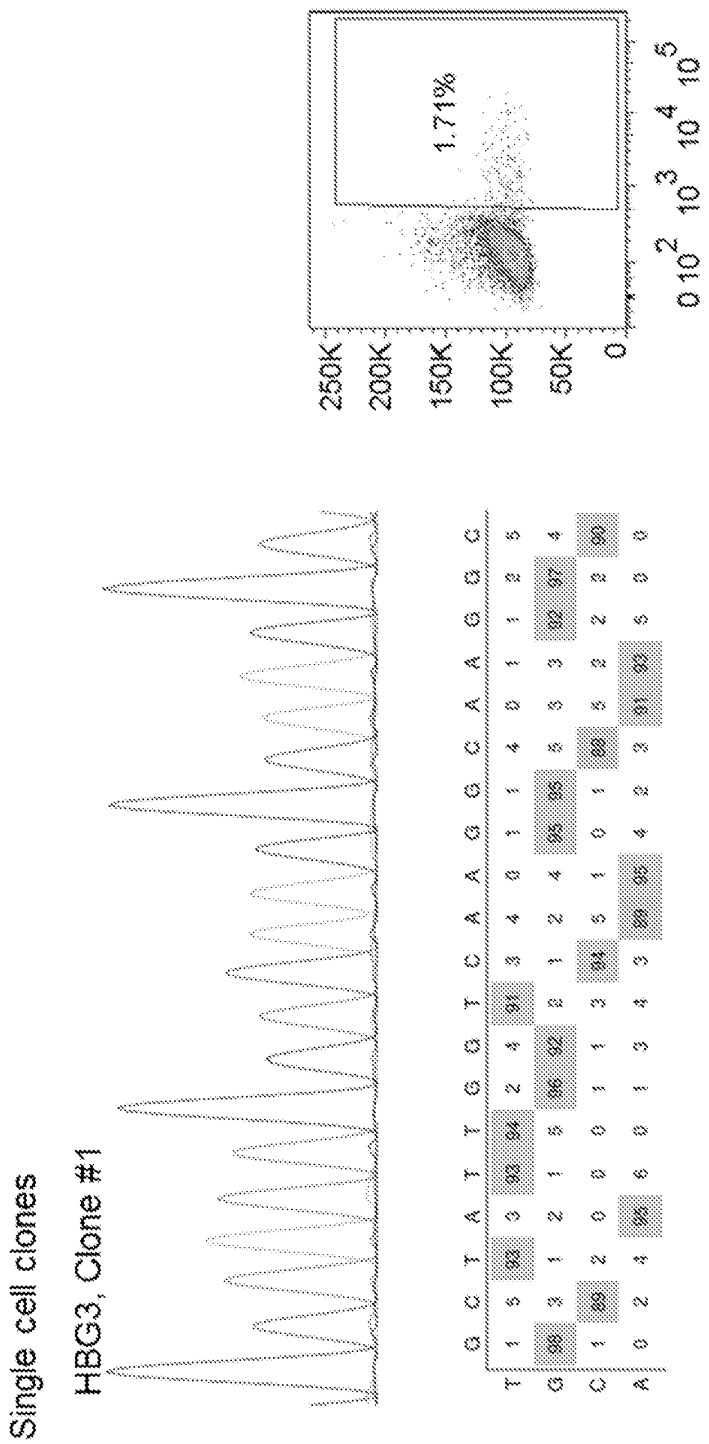

Single cell clones
HBG2, Clone #11
250K
200K
150K
100K
50K
0
7.76%
0
10^2
10^3
10^4
10^5

FIG. 154O



(SEQ ID NO: 252)

FIG. 154P

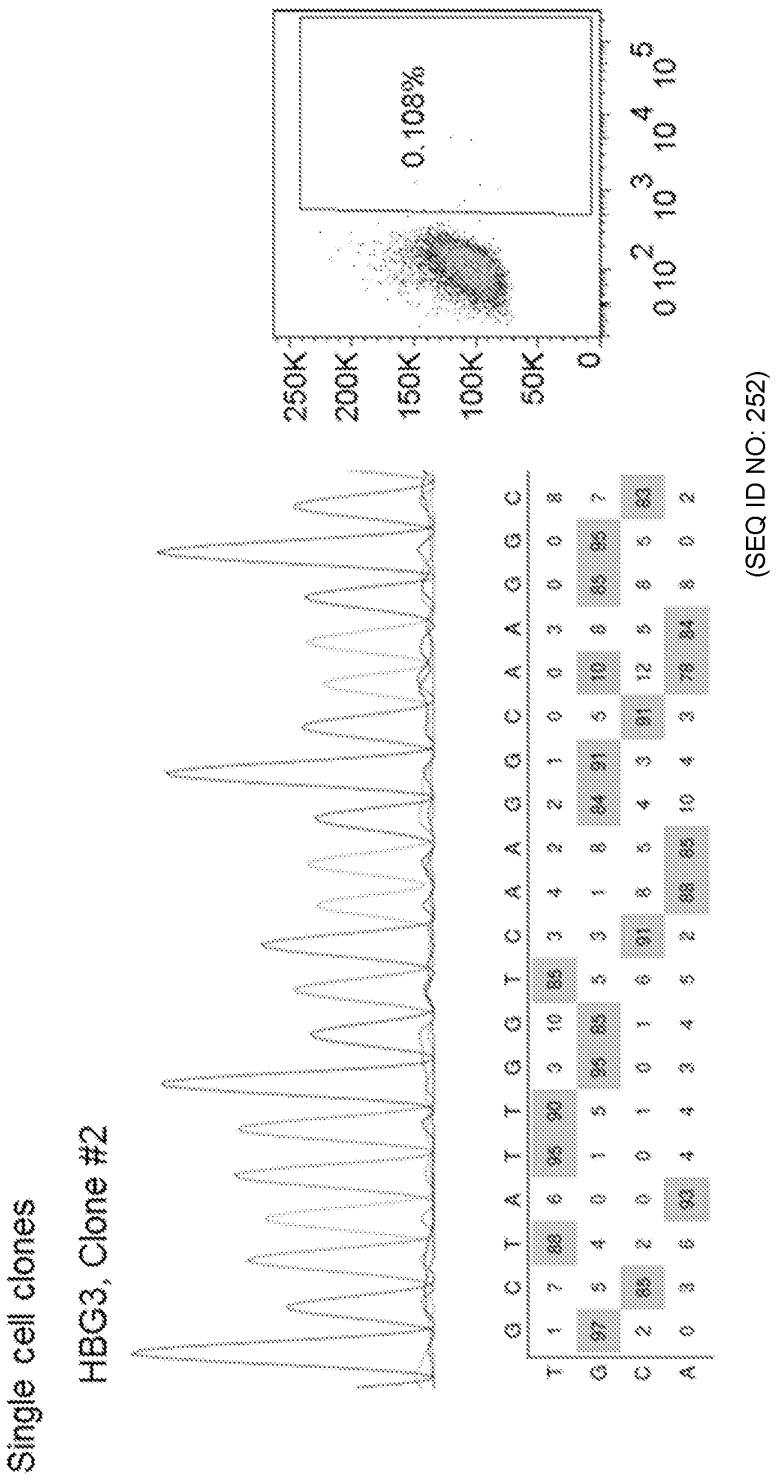

Single cell clones
HBG3, Clone #2
0.108%
250K
200K
150K
100K
50K
0
0 10^2 10^3 10^4 10^5
(SEQ ID NO: 252)

FIG. 154Q

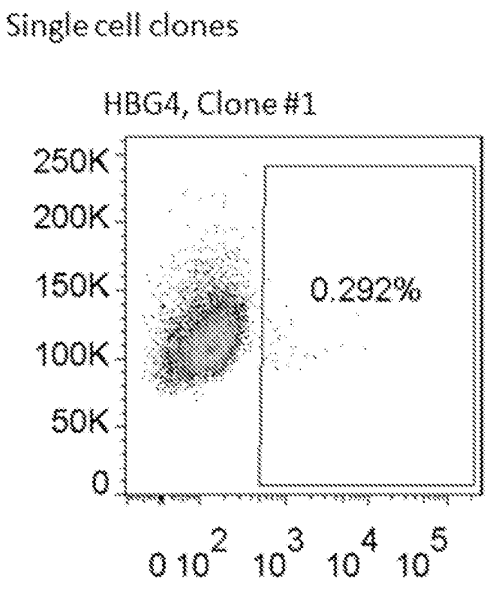


FIG. 154R

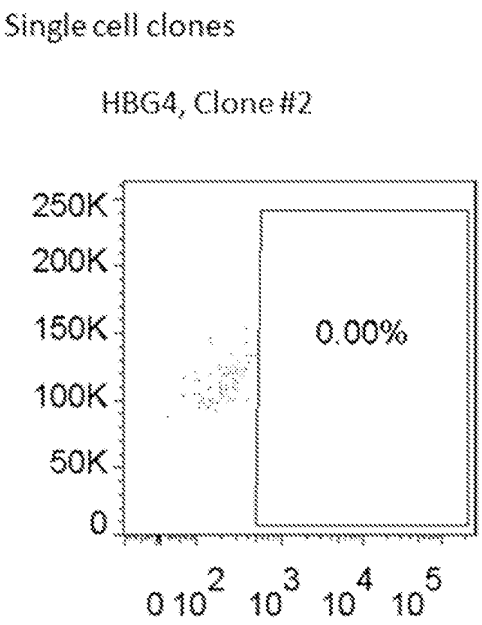
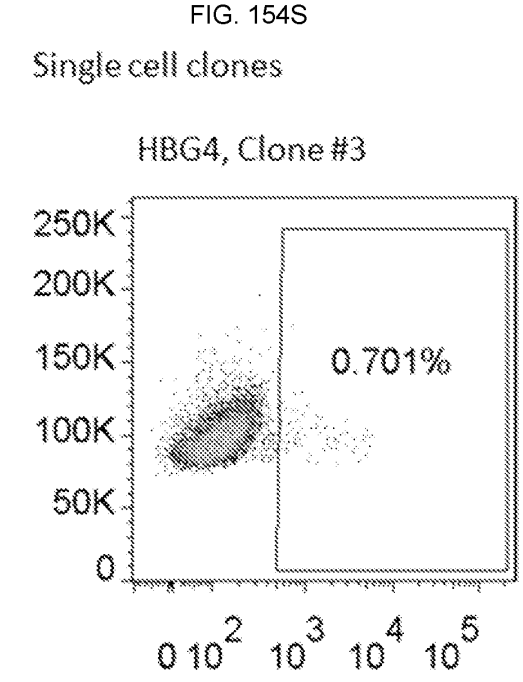
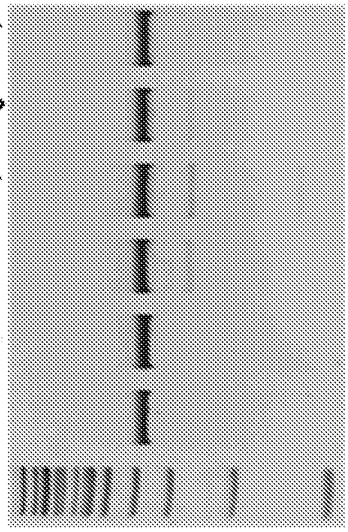


FIG. 154S




UNTR
BE alone
BE-sgHBG1
BE-sgHBG2
BE-sgHBG3
BE-sgHBG4

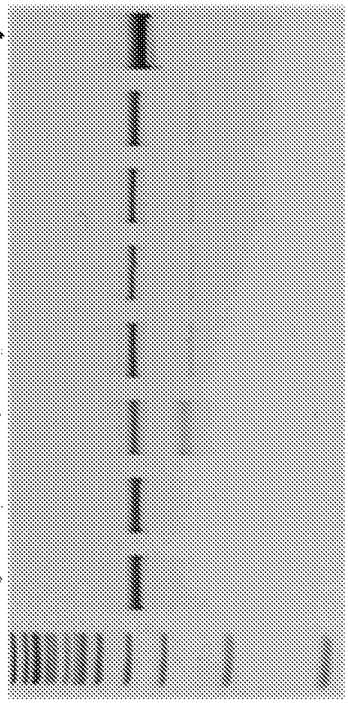

UNTR
BE alone
WTCas9-sgBCL11Ae1
BE + sgBCL11Ae1 (2 vectors)
BE-sgBCL11Ae1
BE-sgBCL11Ae2
BE-sgBCL11Ae3
BE-sgBCL11Ae4

FIG. 155

FIG. 156A

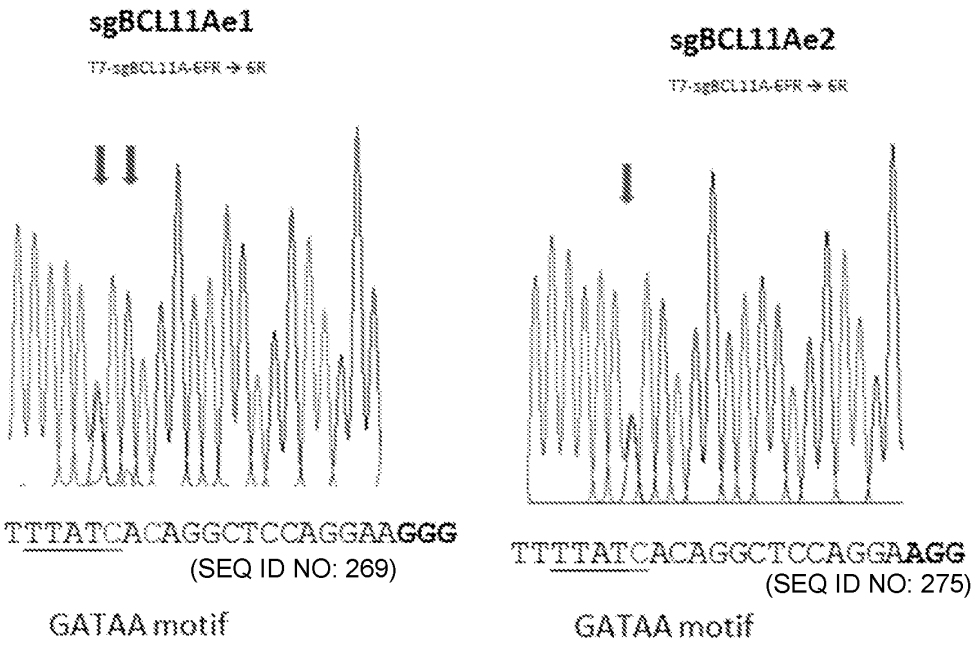


FIG. 156B

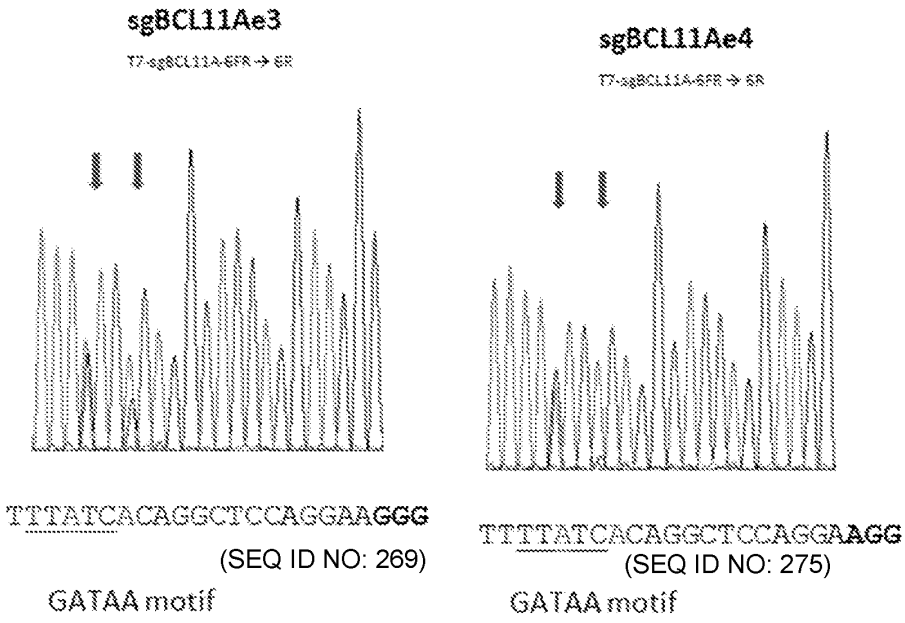


FIG. 156C

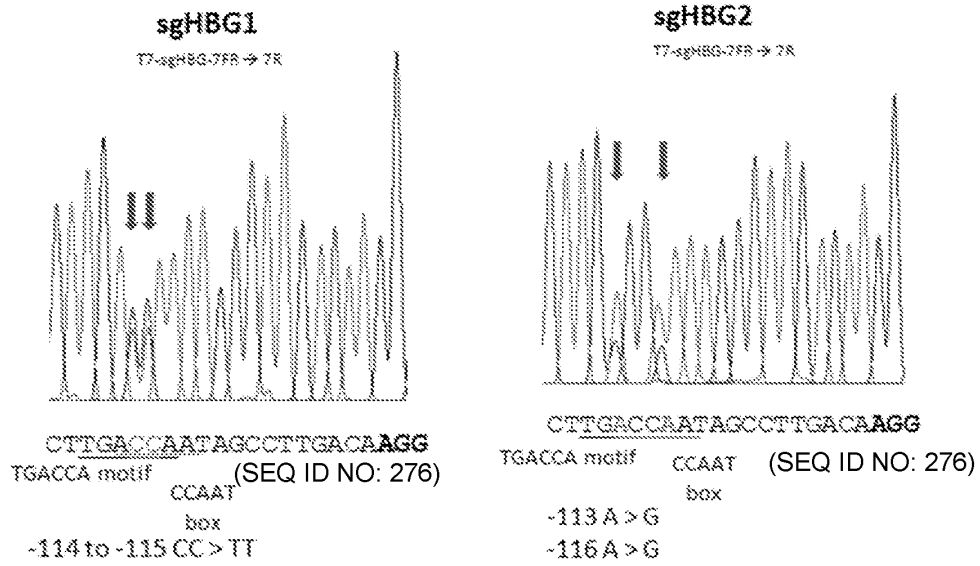


FIG. 156D

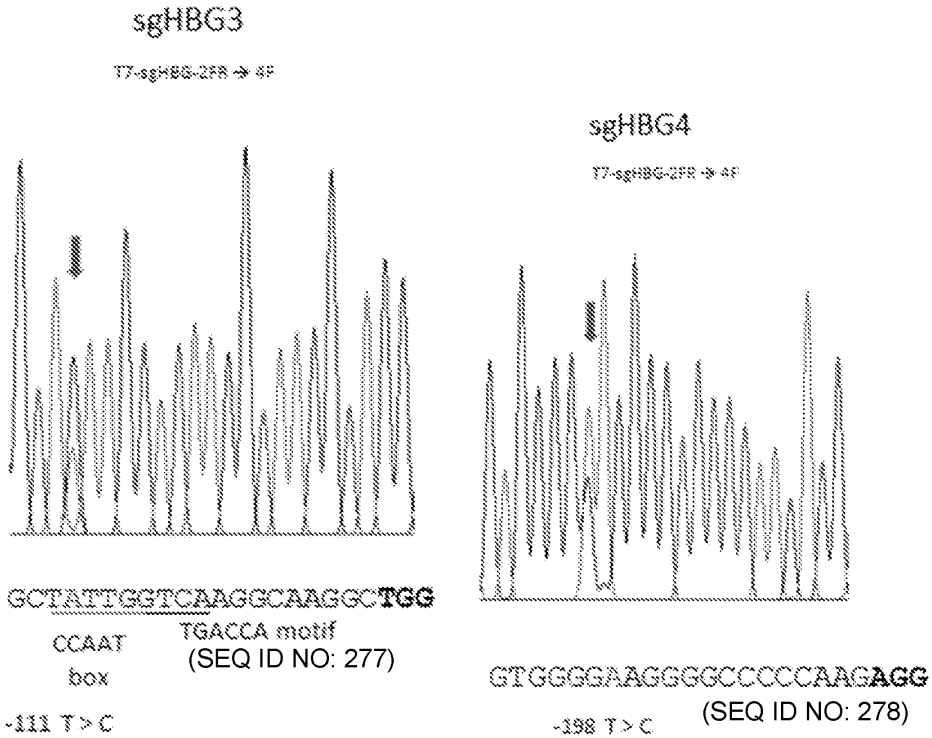


FIG. 157

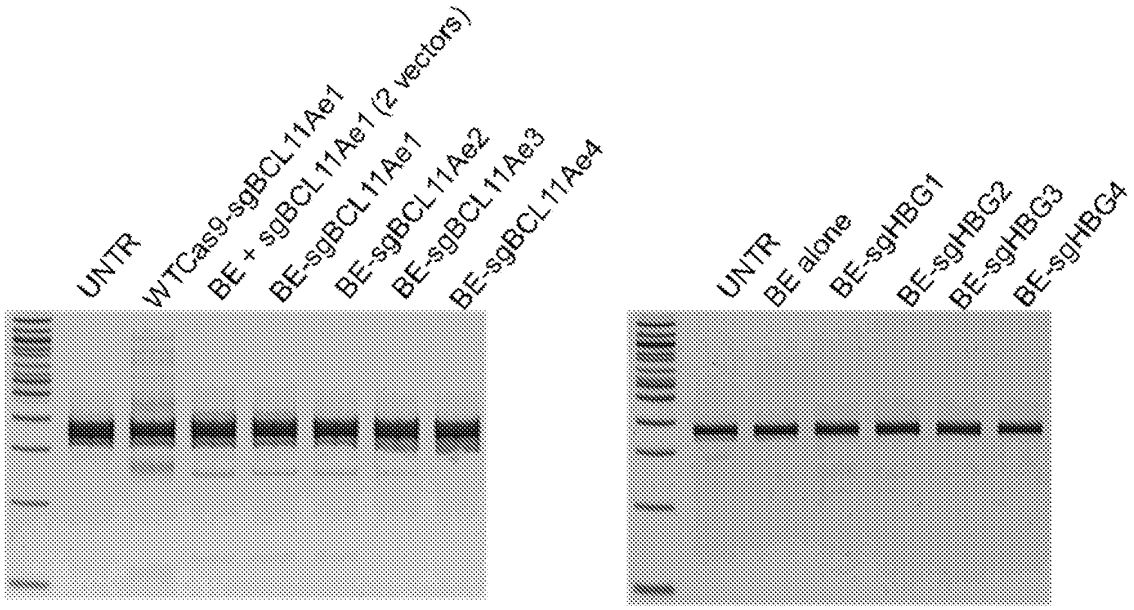


FIG. 158

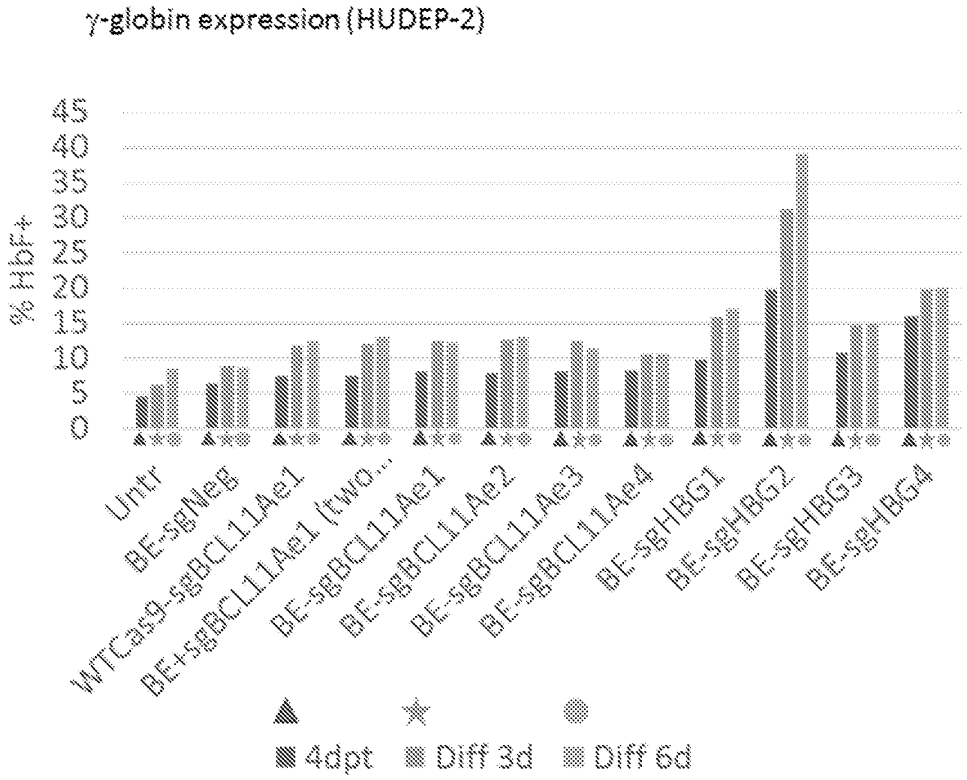


FIG. 159A

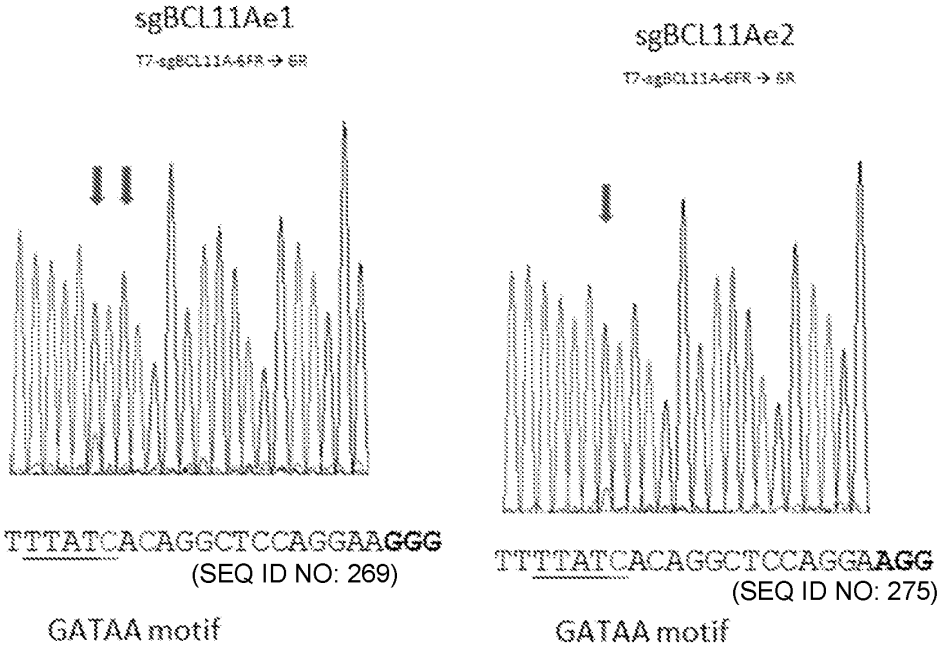


FIG. 159B

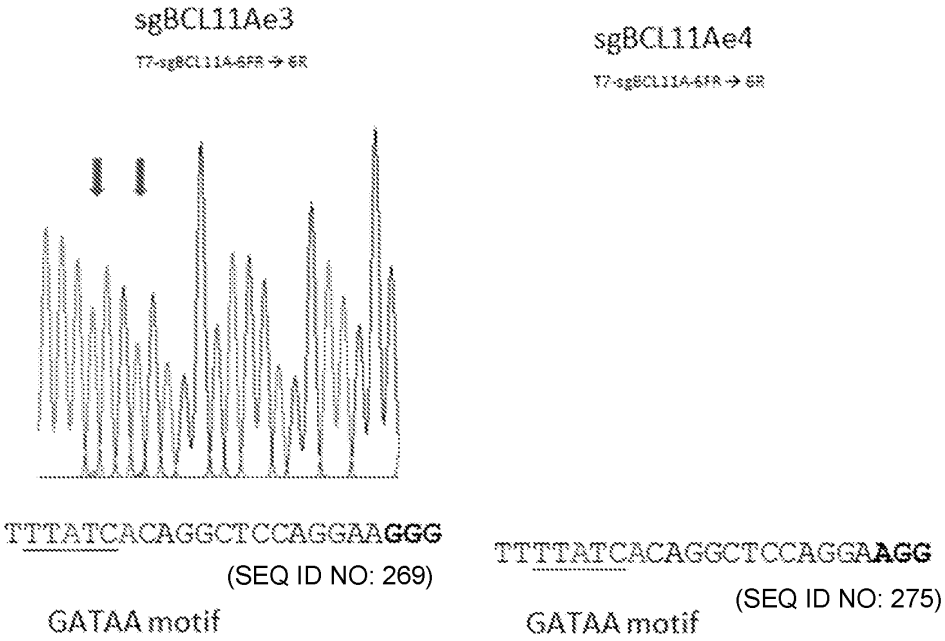


FIG. 159C

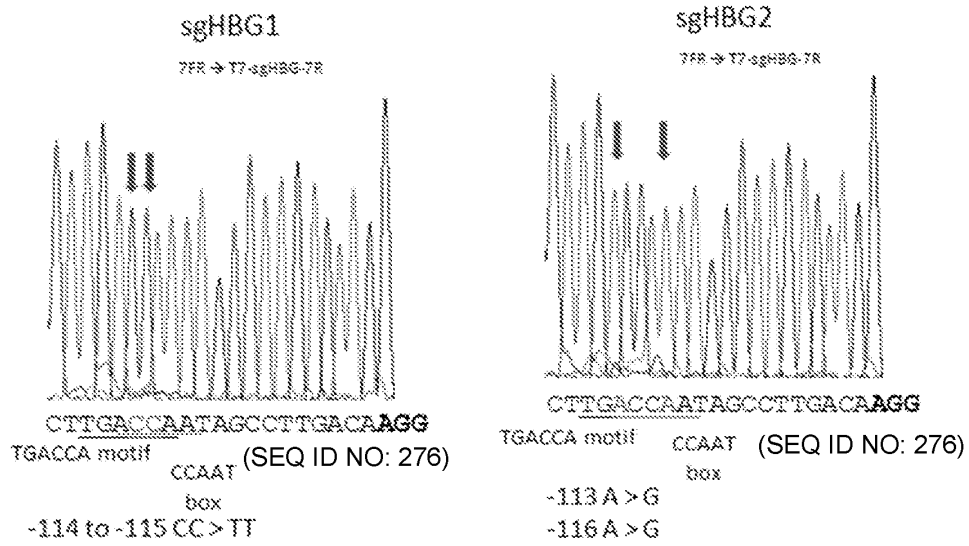


FIG. 159D

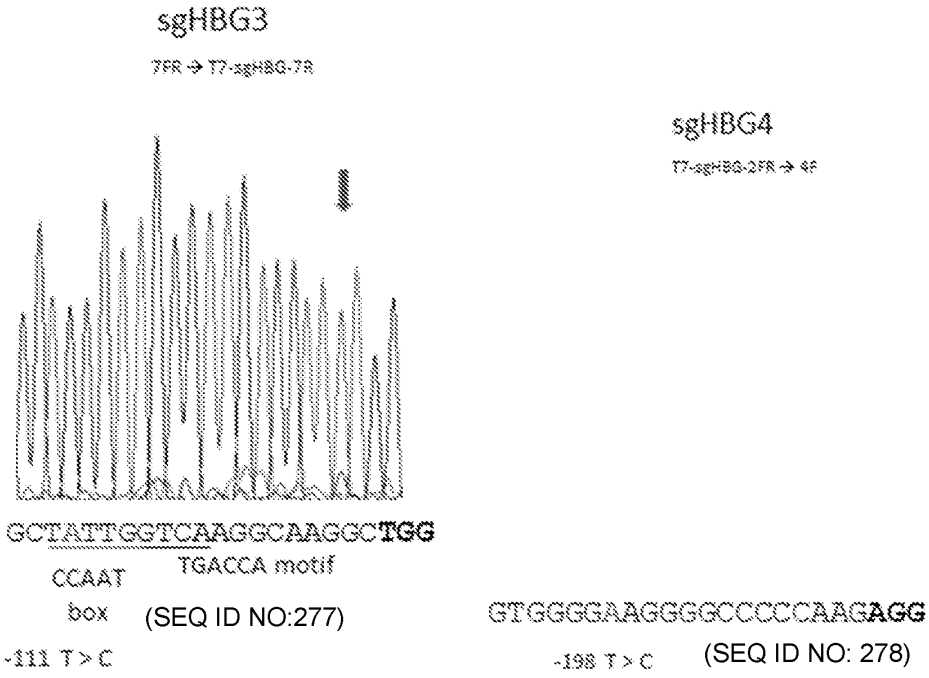


FIG. 160

Selected constructs for HDAd virus production (under Maxi preparation)

Constructed vectors:		Corresponding Ad constructs:
pBS-AncBE4max-sgBCL11AE1	+58 GATA motif, G --> A	pHCA-BE4max-sgBCL11Ae1-FI-mgmt/GFP
pBS-AncBE4max-sgBCL11AE2	+58 GATA motif, G --> A	
pBS-ABEmax-sgBCL11AE3	+58 GATA motif, T --> C	
pBS-ABEmax-sgBCL11AE4	+58 GATA motif, T --> C	
pBS-AncBE4max-sgHBG1-1	13bp deletion, CCAAT box, CC --> TT	
pBS-ABEmax-sgHBG1-2	13bp deletion, CCAAT box, A --> G	pHCA-ABEmax-sgHBG2-FI-mgmt/GFP
pBS-ABEmax-sgHBG1-3	13bp deletion, CCAAT box, T --> C	
pBS-ABEmax-sgHBG1-4	-198 T --> C	pHCA-ABEmax-sgHBG4-FI-mgmt/GFP
pBS-AncBE4max-sgR5-1	control	pHCA-BE4max-sgR5-1-FI-mgmt/GFP (Ctrl)

FIG. 161

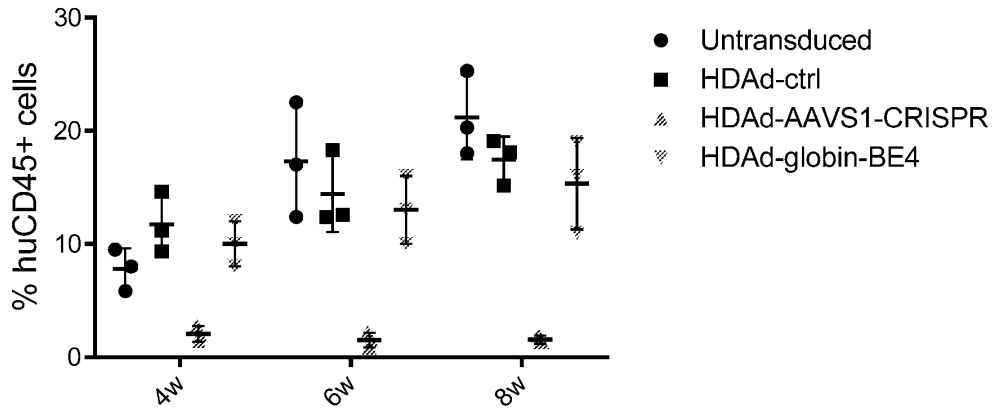
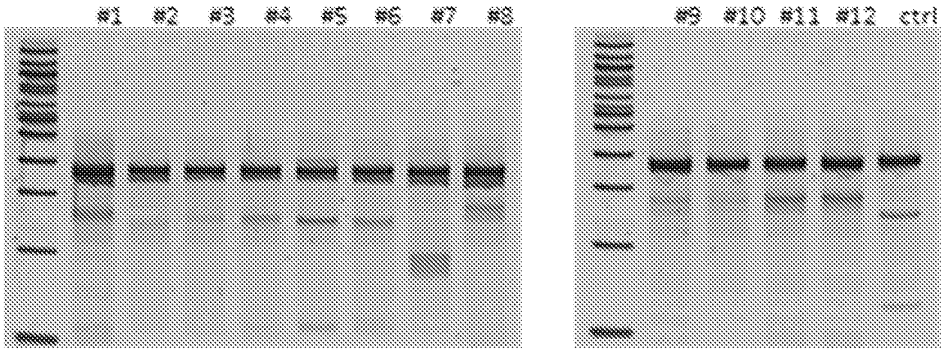


FIG. 162

Transient transfection of HUDEP-2 cells:
(cleavage by T7EI)

Bcl11a enhancer



HBG promoter

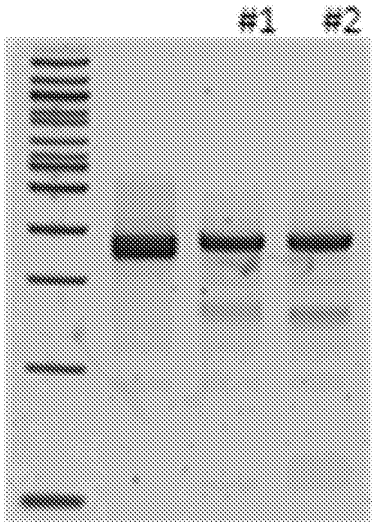


FIG. 163

Dual base editing vector application

Reactivation of γ-globin by disrupting BCL11A binding sites + Termination of mutant HBB by CRISPR-STOP

GAG → GTG sickle mutation

TGG → TAA by cytidine editor

ATGGTGCATCTGACTCCTGAGGAGAAGTCTGCCGTTACTGCCCTGTGGGGCAAGGTGA
ACGTGGATGAAGTTGGTGGTGAGGCCCTGGGCAGGCTGCTGGTGGTCTACCCTTGGAC
CCAGAGGTTCTTTGAGTCCTTTGGGGATCTGTCCACTCCTGATGCTGTTATGGGCAAC
CCTAAGGTGAAGGCTCATGGCAAGAAAGTGCTCGGTGCCTTTAGTGATGGCCTGGCTC
ACCTGGACAACCTCAAGGGCACCTTTGCCACACTGAGTGAGCTGCACTGTGACAAGCT
GCACGTGGATCCTGAGAACTTCAGGCTCCTGGGCAACGTGCTGGTCTGTGTGCTGGCC
CATCACTTTGGCAAAGAATTCACCCCACCAGTGCAGGCTGCCTATCAGAAAGTGGTGG
CTGGTGTGGCTAATGCCCTGGCCCACAAGTATCACTAA

(SEQ ID NO: 279)

CAG → TAG by cytidine editor

FIG. 164

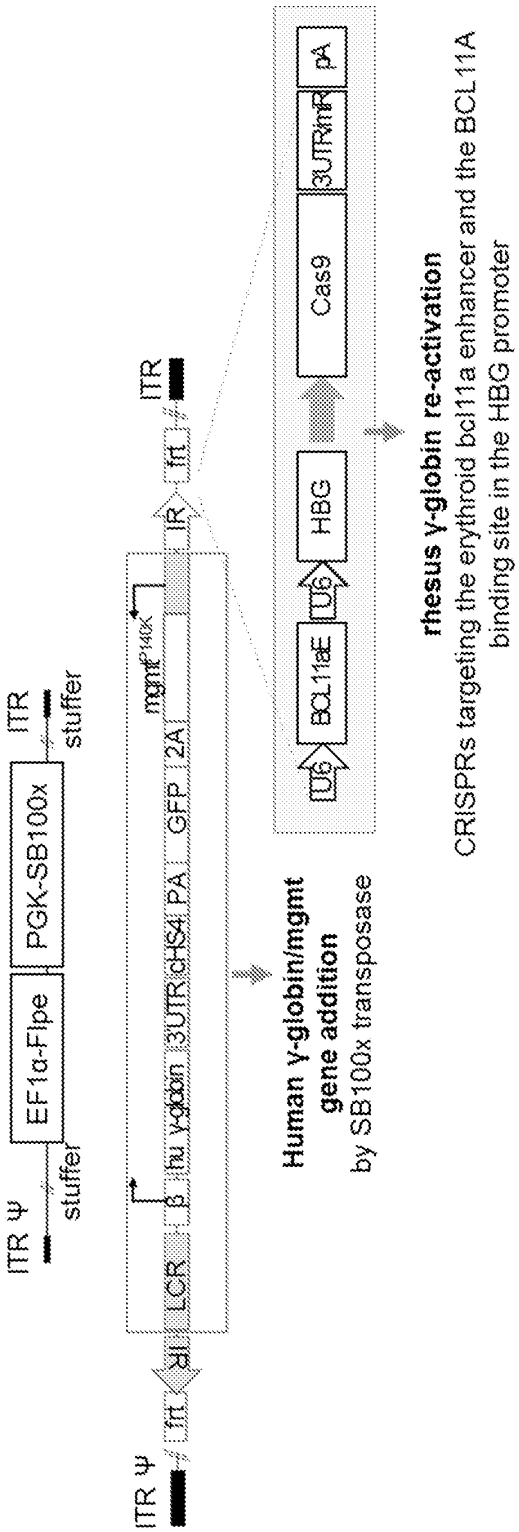

ITR Ψ
stuffer
EF1α-Flpe
PGK-SB100x
ITR
stuffer
ITR Ψ
frt
IR
LCR
β
hu γ-globin
3'UTR cHS4
PA
GFP
2A
mgmt P140K
IR
frt
ITR
Human γ-globin/mgmt gene addition
by SB100x transposase
U6
BCL11aE
U6
HBG
Cas9
3'UTR
mR
pA
rhesus γ-globin re-activation
CRISPRs targeting the erythroid bcl11a enhancer and the BCL11A binding site in the HBG promoter

FIG. 165

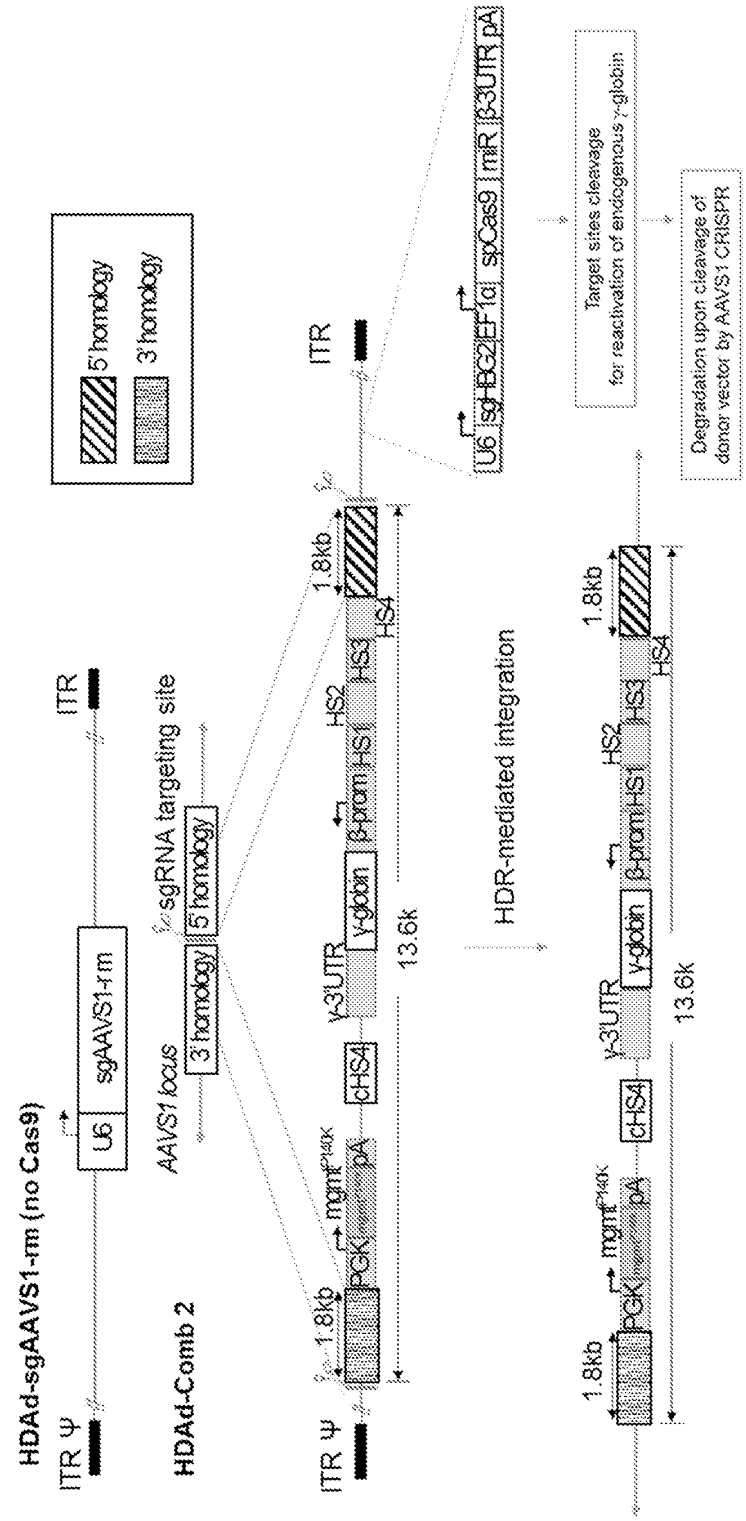
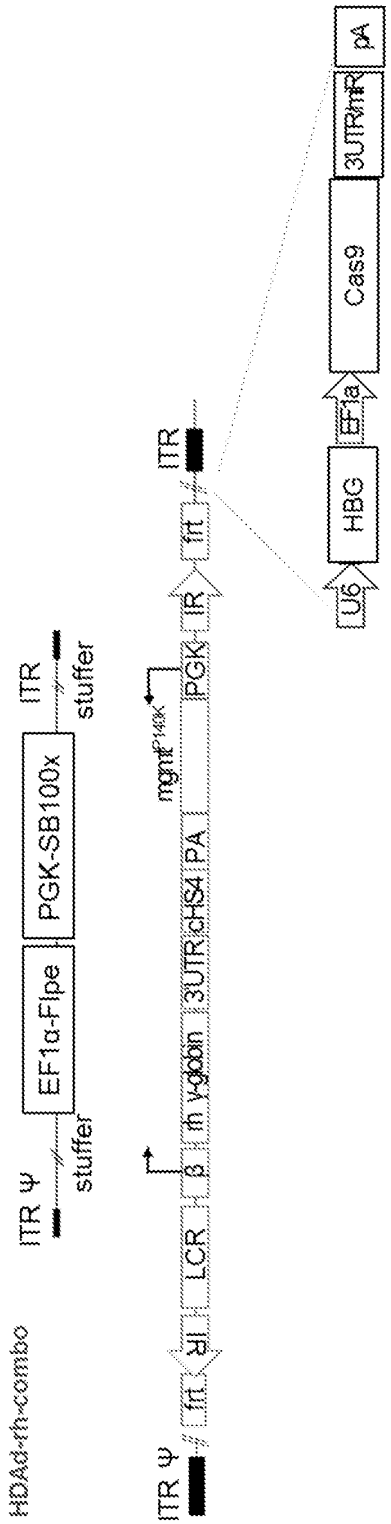

HDAd-sgAAVS1-rm (no Cas9)
ITR Ψ
U6
sgAAVS1-rm
ITR
HDAd-Comb 2
AAVS1 locus
3' homology
sgRNA targeting site
5' homology
5' homology
3' homology
ITR Ψ
1.8kb
PGK
mgmt^P140K
pA
cHS4
γ-3'UTR
γ-globin
β-prom
HS1
HS2
HS3
HS4
13.6k
HDR-mediated integration
ITR
1.8kb
U6
sgHBG2/EF1α
spCas9
miR
β-3'UTR pA
Target sites cleavage
for reactivation of endogenous γ-globin
Degradation upon cleavage of
donor vector by AAVS1 CRISPR
1.8kb
PGK
mgmt^P140K
pA
cHS4
γ-3'UTR
γ-globin
β-prom
HS1
HS2
HS3
HS4
13.6k
1.8kb

FIG. 166


HDAd-rh-combo
ITR Ψ
stuffer
EF1α-Flpe
PGK-SB100x
ITR
stuffer
ITR Ψ
frt
IR
LCR
β
m γ-globin
3'UTR cHS4 PA
mgmt^P140K
PGK
IR
frt
ITR
U6
HBG
EF1a
Cas9
3'UTRmiR
pA