Bcl11a miR1 oligonucleotides:

Sense (SEQ. ID. NO: 1) ACGCT**CGCACAGAACACTCATGGATT***ctccatgtggtagag***AATCCATGAGTGTTCTGTGCG**AG

Anti-sense (SEQ. ID. NO: 2) CGCACT**CGCACAGAACACTCATGGATT***ctctaccacatggag***AATCCATGAGTGTTCTGTGCG**A

Bcl11a miR2 oligonucleotides:

Sense (SEQ. ID. NO: 3) ACGCT**CCAGAGGATGACGATTGTTTA***ctccatgtggtagag***TAAACAATCGTCATCCTCTGG**ag

Anti-sense (SEQ. ID. NO: 4) CGCAct**CCAGAGGATGACGATTGTTTA***ctctaccacatggag***TAAACAATCGTCATCCTCTGG**a

Italicized lower bases = shRNA loop
Bold and upper case bases = BCL11A targeting sequence for forming a shRNA
Underlined bases = Cloning ends (synthetic)

FIG. 1

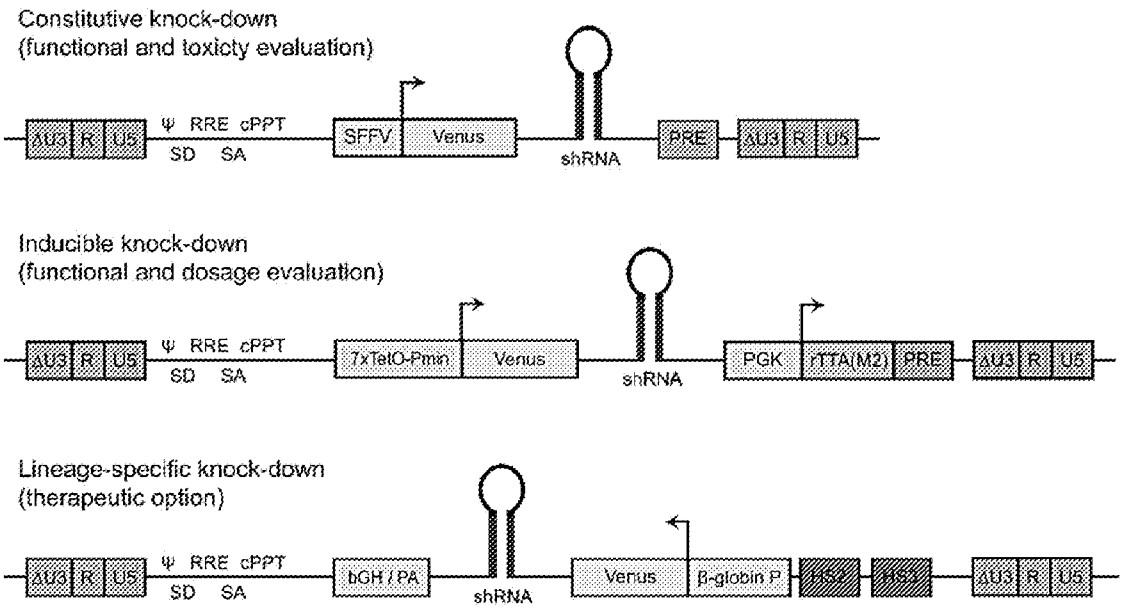
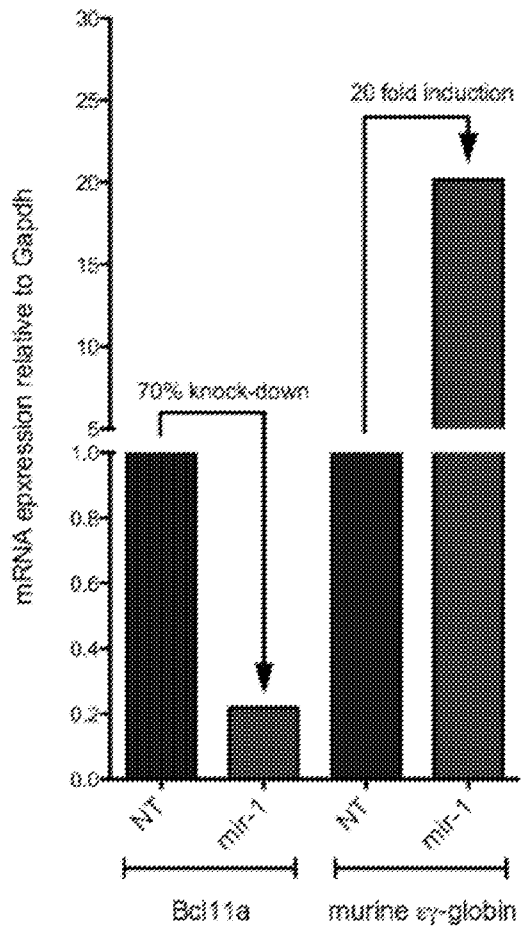


FIG. 2

SFFV-LV efficiently knocks-down Bcl11a and induces εγ-globin expression

in vitro: transduced murine erythroleukemia cells



in vivo: transplanted transduced LSK-HSC

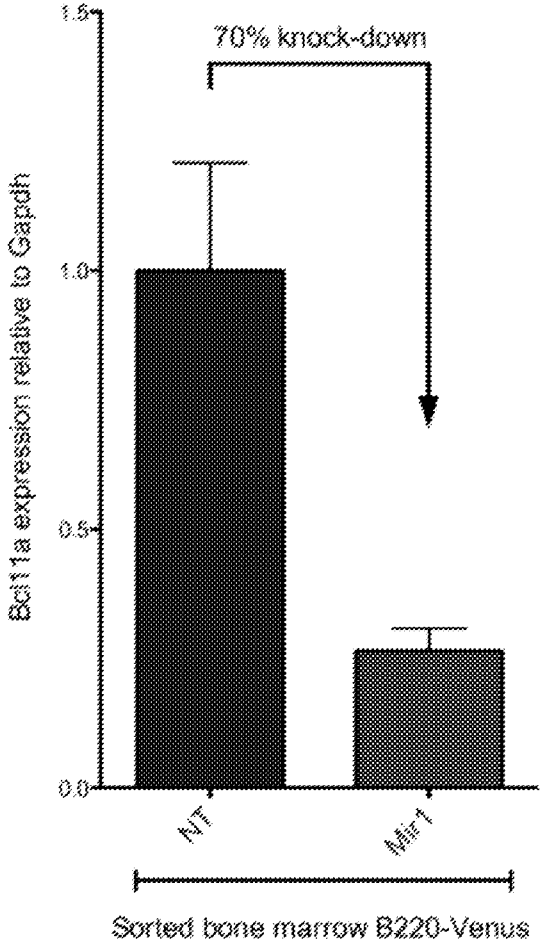
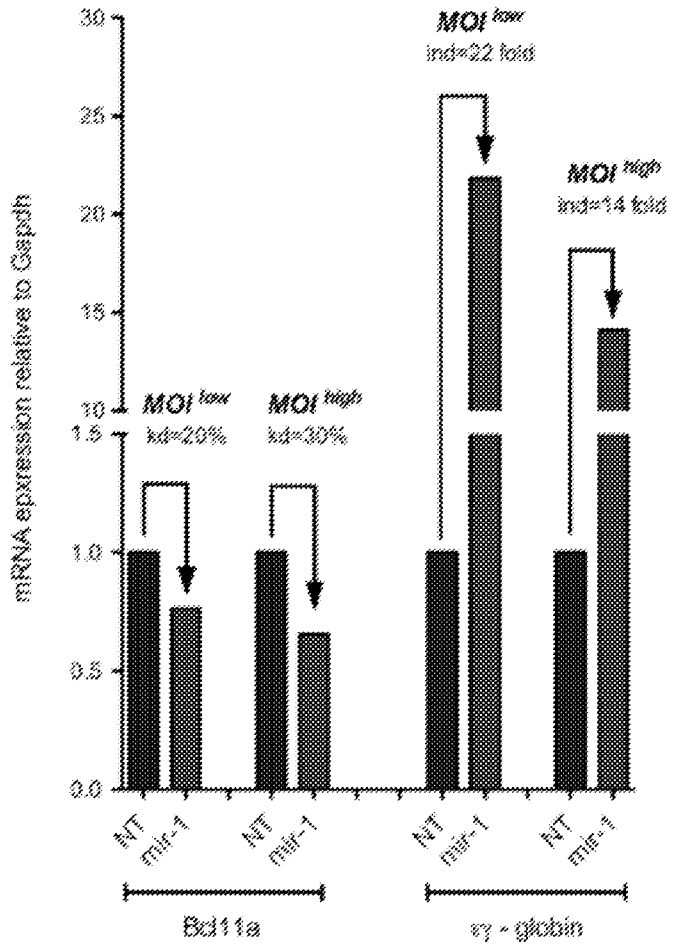


FIG. 3

LCR/TET-LV efficiently knock-down Bcl11a and induce εγ-globin expression

LCR-LV : effects of higher MOI transduction



TET-LV : doxycycline-induction dose-dependence

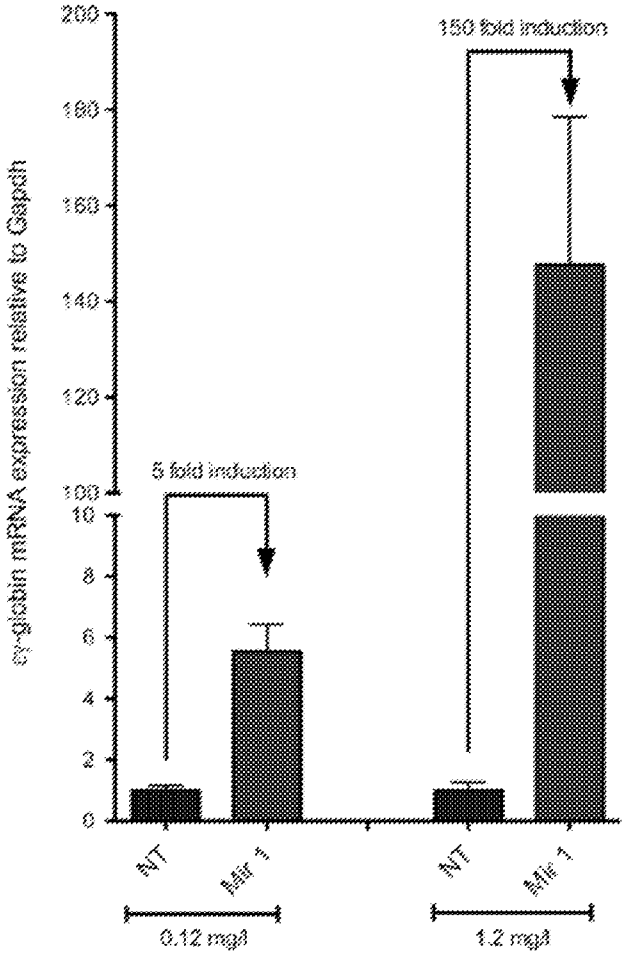
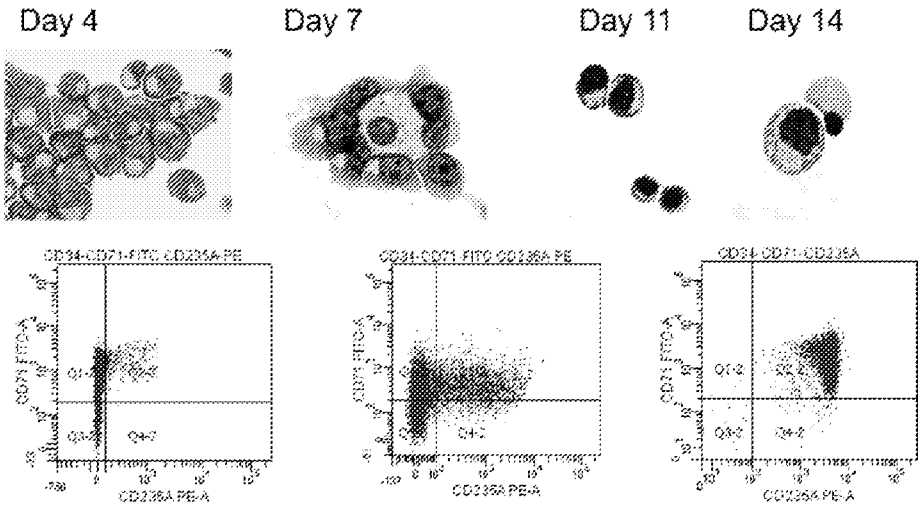
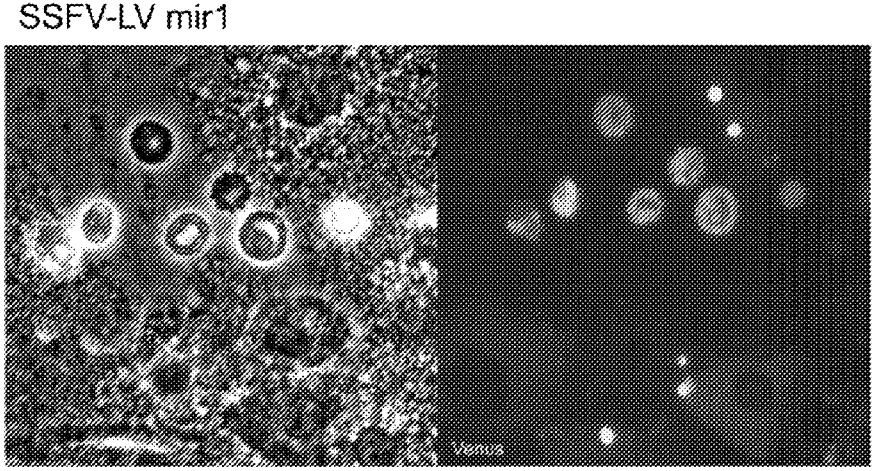
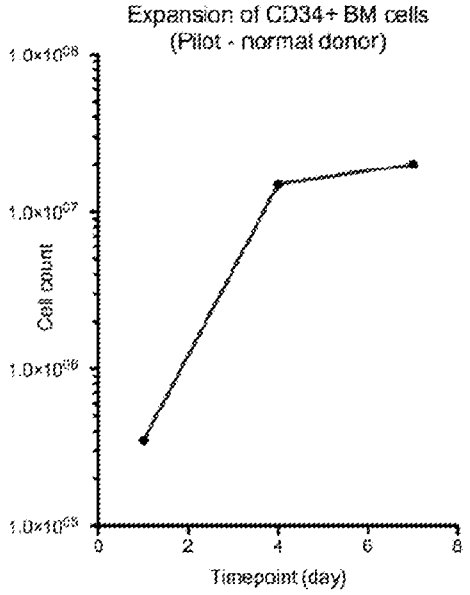


FIG. 4

Transduced CD34+ HSC differentiate ex-vivo into erythrocytes and express HbF







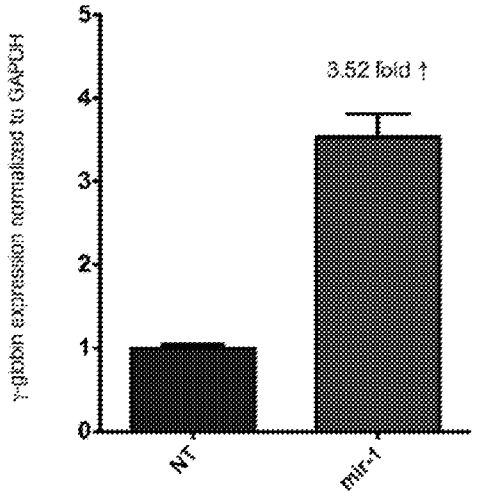


FIG. 5

LCR-LV transduced CD34+ HSCs from patients with SCD transplanted into NSG

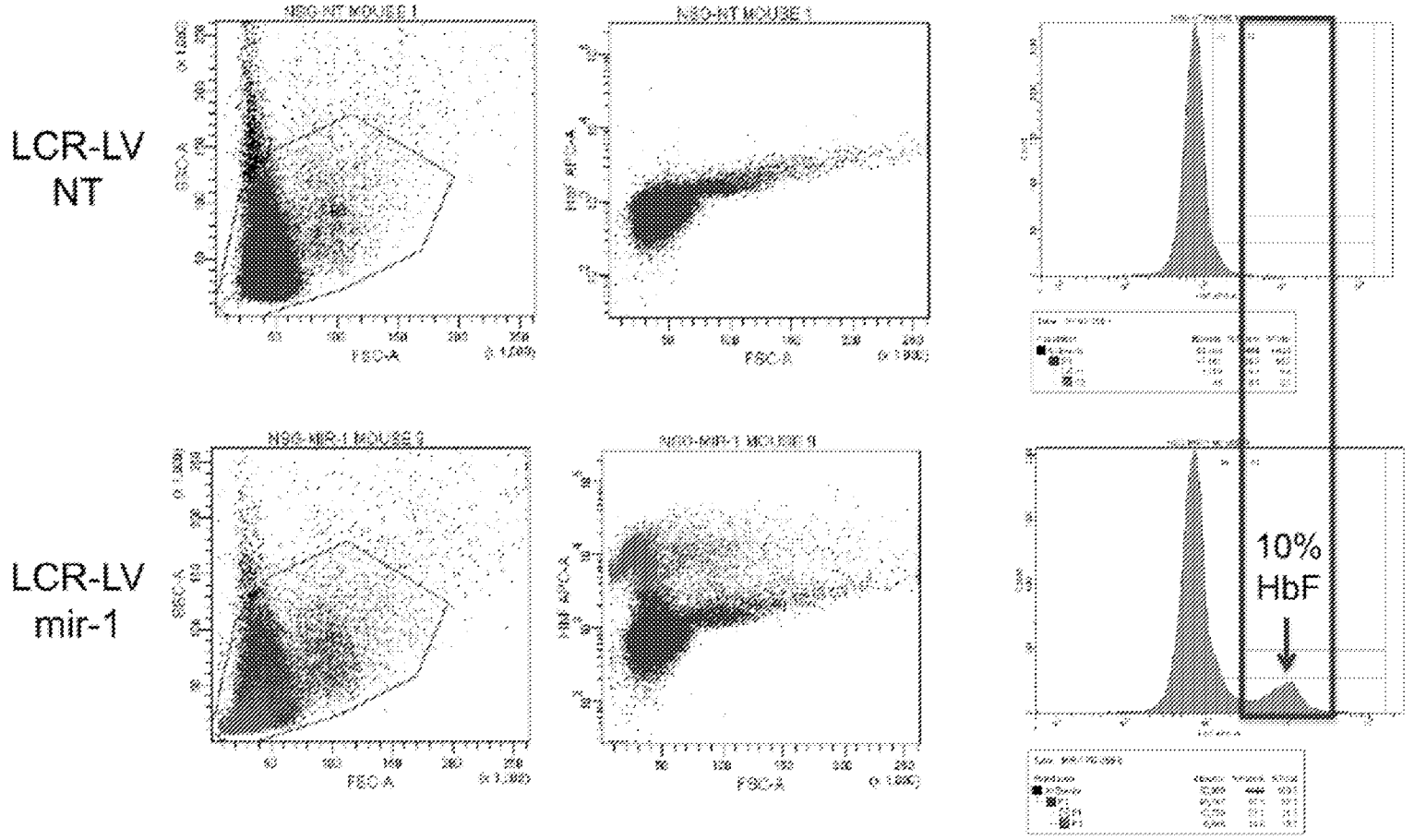
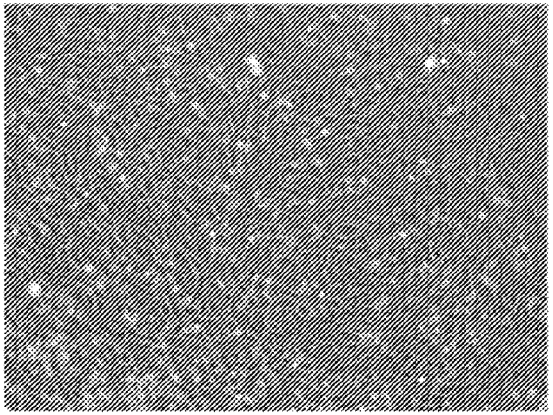
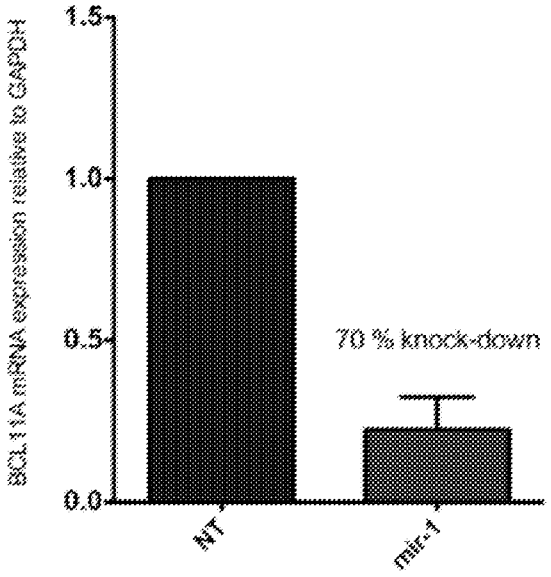


FIG. 6

Investigating potential toxicity of Bcl11a role in lymphoid development



Venus-sorted hCD34 HSC on MS-5, 100x



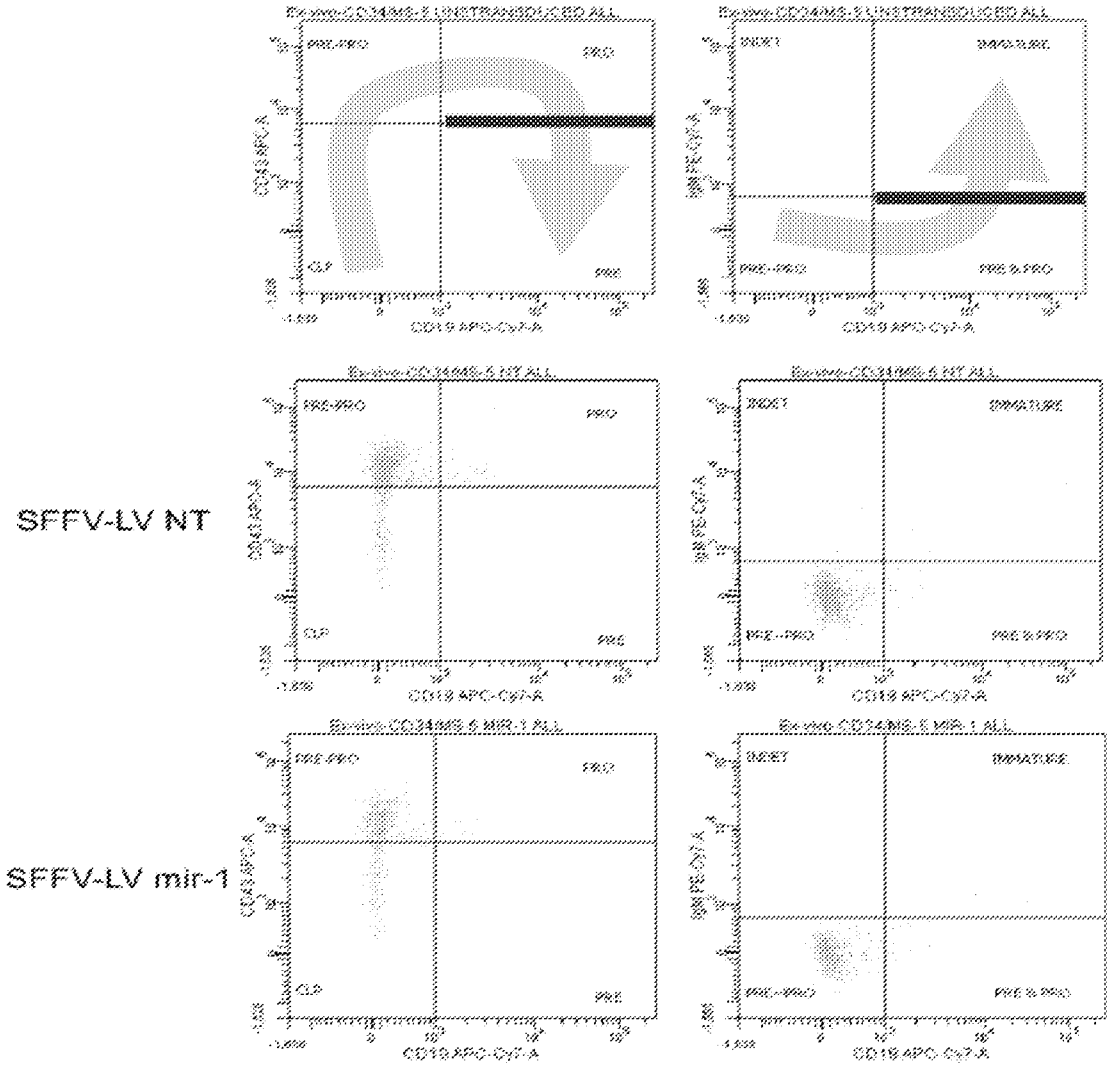


FIG. 7

shRNAs vs microRNA adapted shRNAs

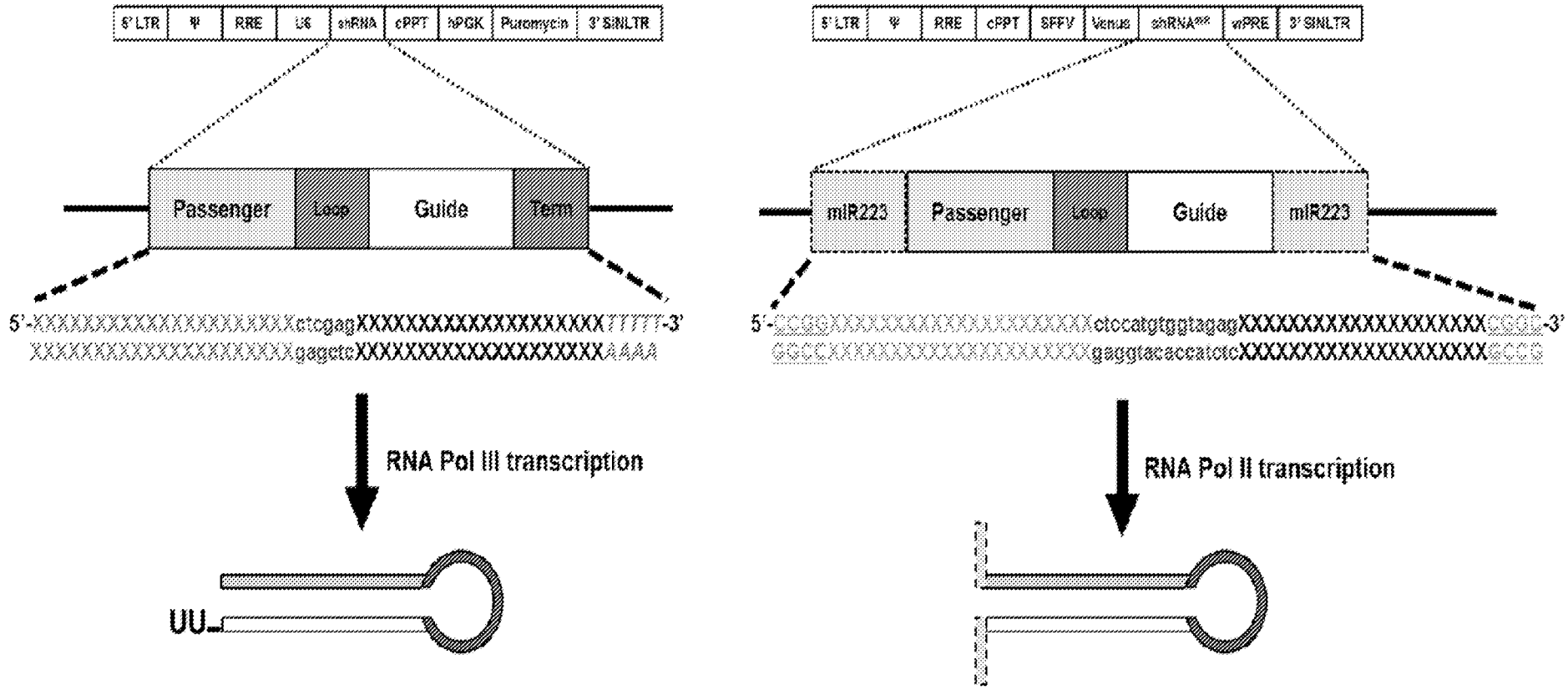


FIG. 8A

shRNA screen targeting Bcl11A using pLKO vector

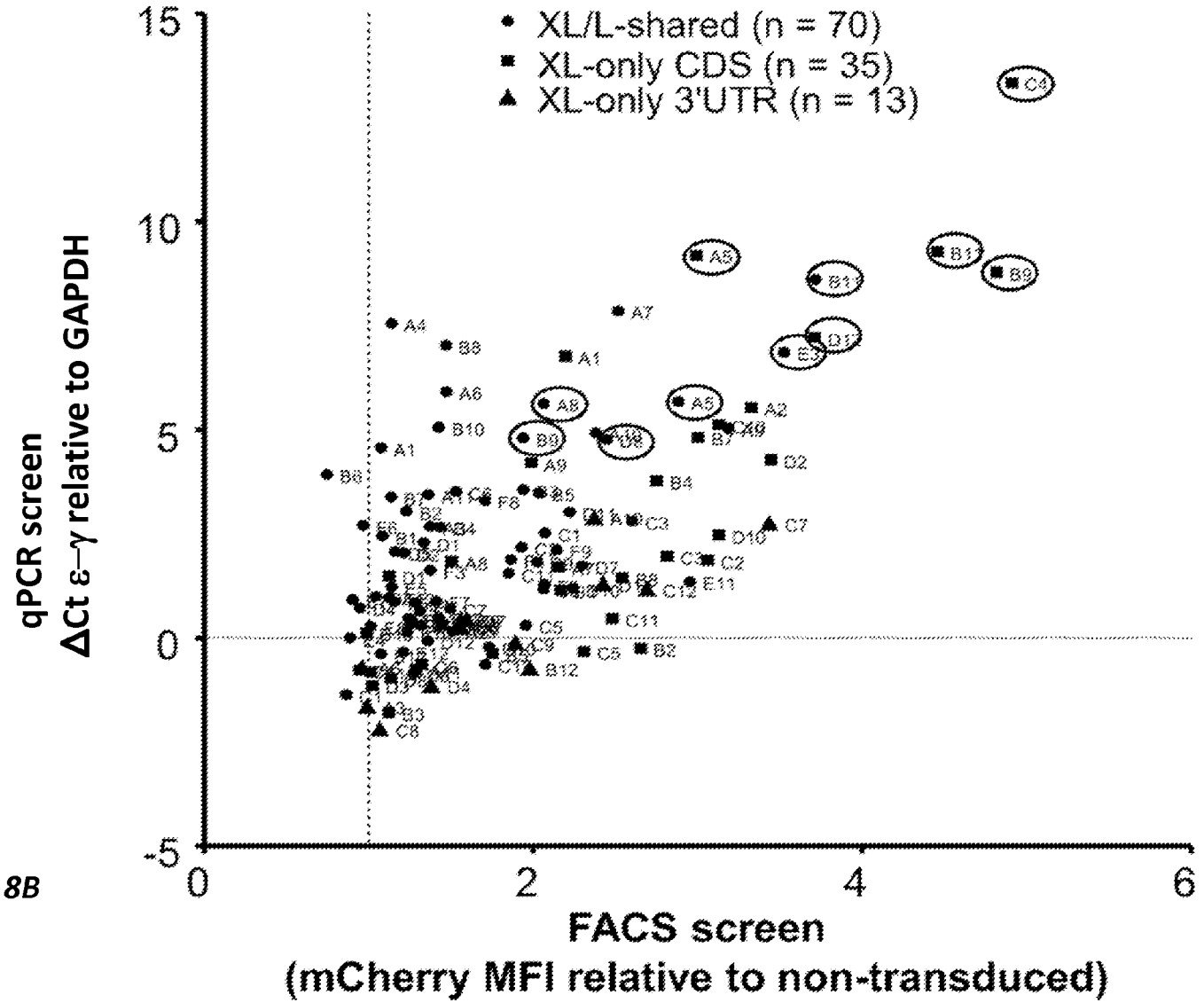


FIG. 8B

BCL11A knockdown and ε-γ induction in pLKO and LEGO back bones

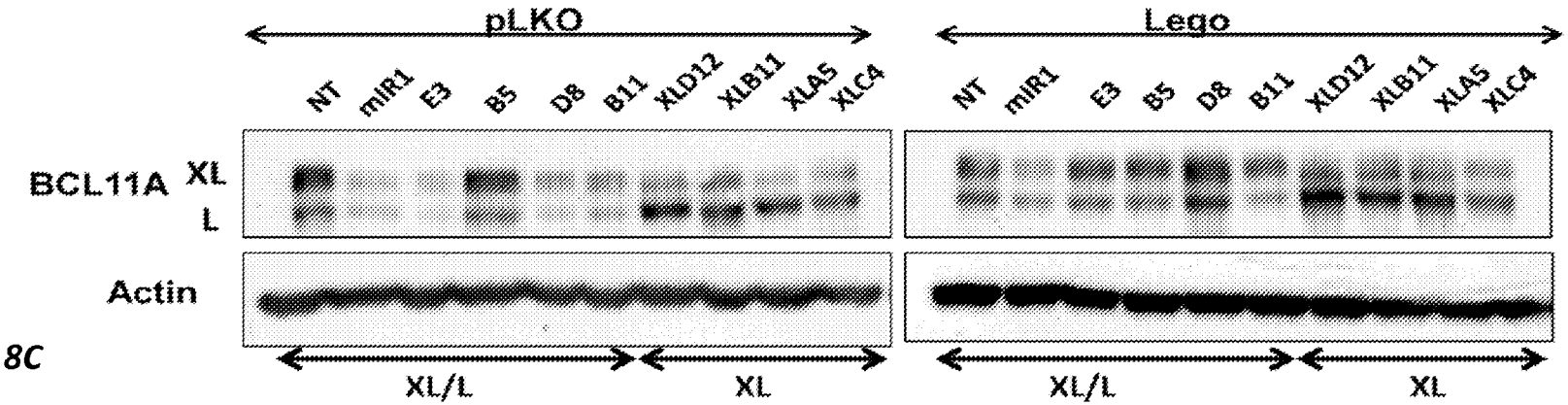


FIG. 8C

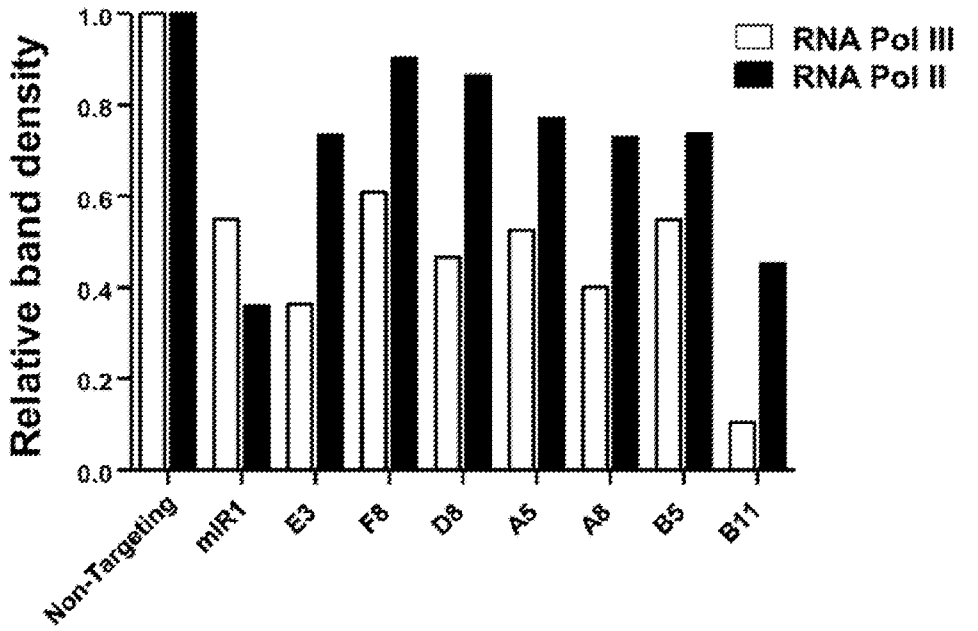


FIG. 8D

Relative ε–γ induction / GAPDH
100000
10000
1000
100
10
1
PLKO
LEGO
N=3
Mean ± SD
*p<0.05
NT PLKO
NT LEGO
miR-1 PLKO
miR-1 LEGO
E3 PLKO
E3 LEGO
E5 PLKO
E5 LEGO
B5 PLKO
B5 LEGO
DB PLKO
DB LEGO
B-1 PLKO
B-1 LEGO
50D-12 PLKO
50D-12 LEGO
50D-1 PLKO
50D-1 LEGO
50A5 PLKO
50A5 LEGO
50C4 PLKO
50C4 LEGO

FIG. 8E

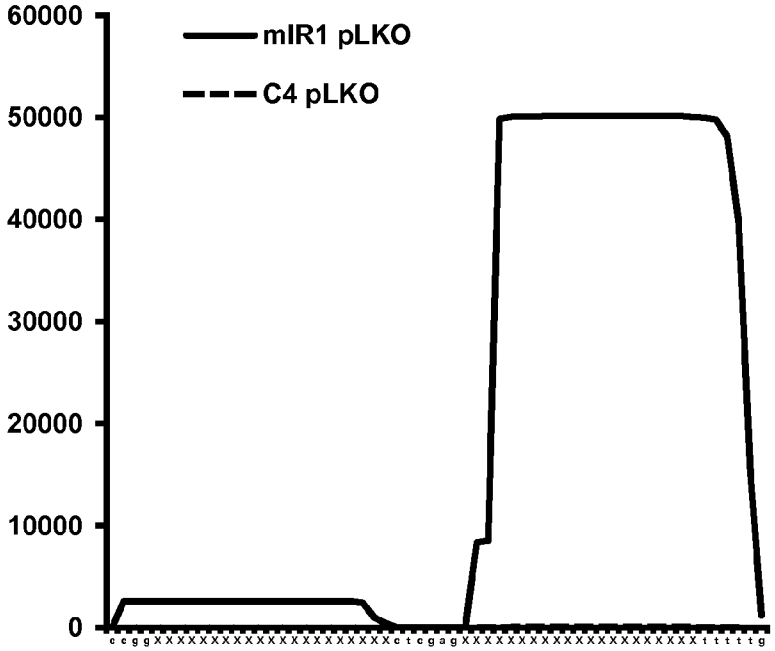

60000
50000
40000
30000
20000
10000
0
mIR1 pLKO
C4 pLKO

FIG. 9A

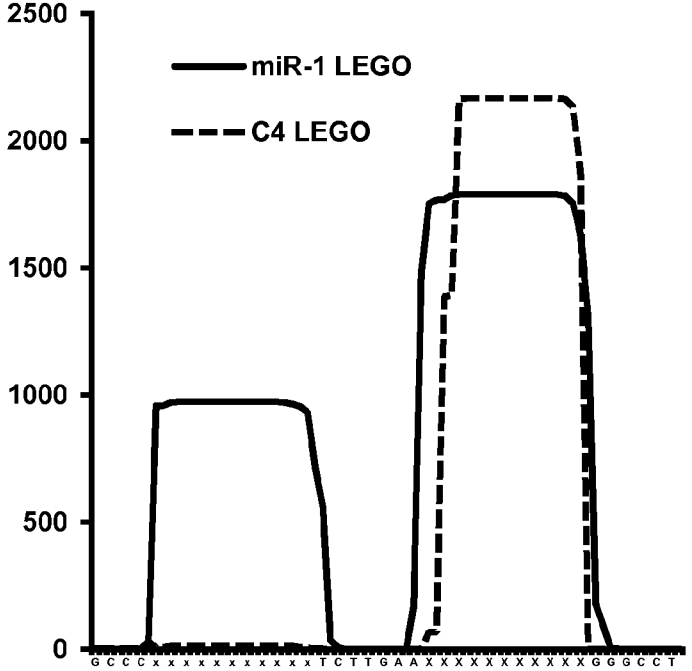

2500
2000
1500
1000
500
0
miR-1 LEGO
C4 LEGO

FIG. 9B

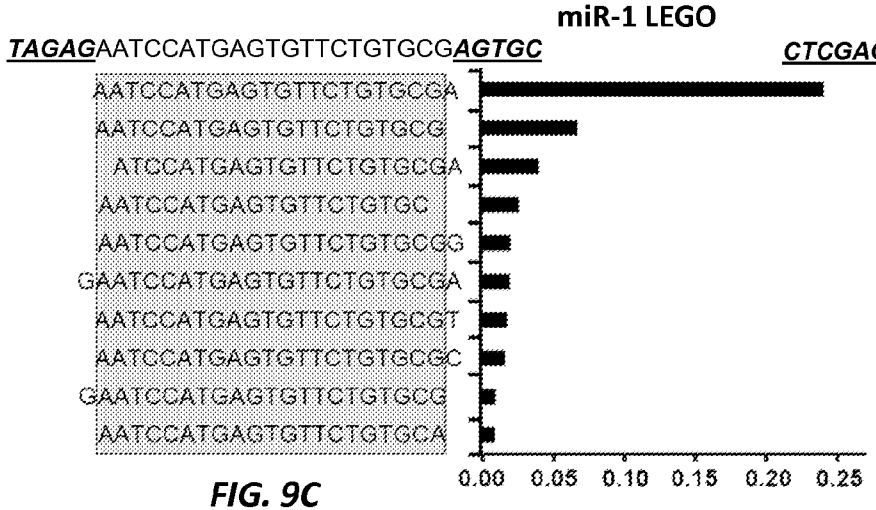


FIG. 9C

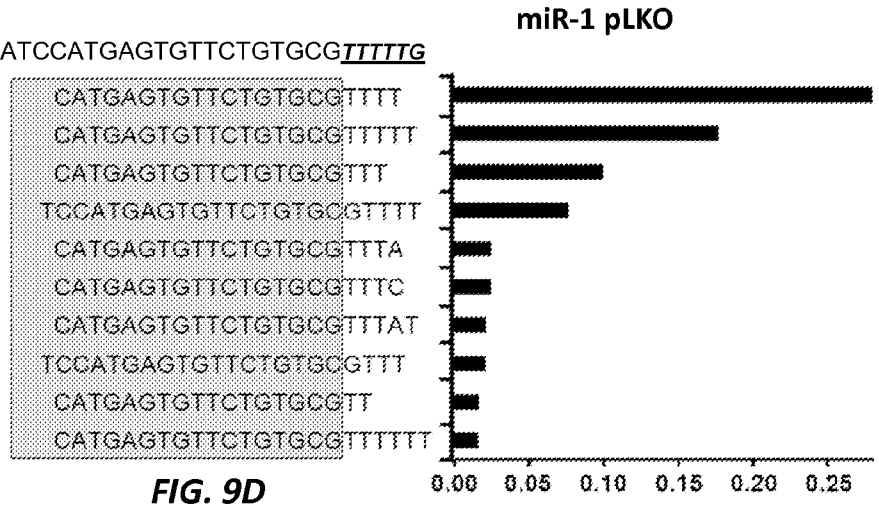


FIG. 9D

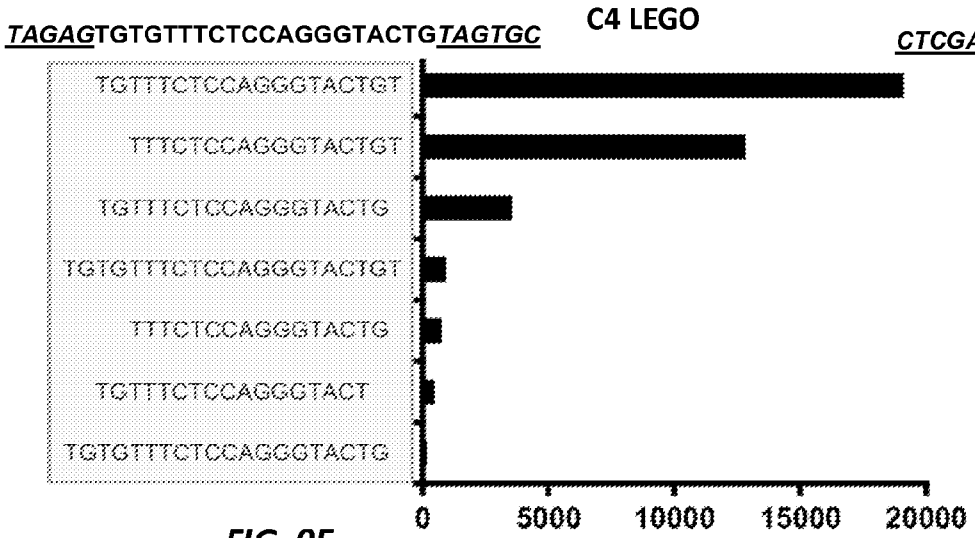


FIG. 9E

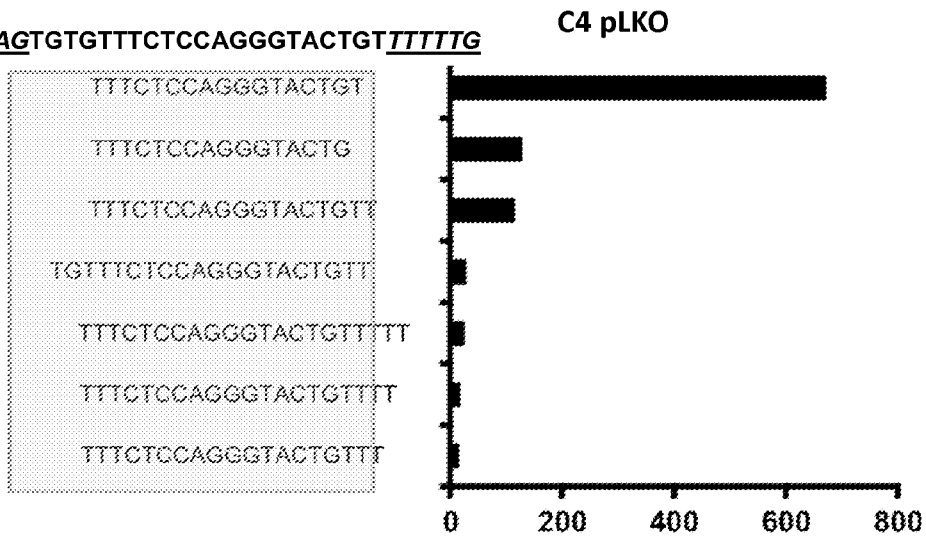


FIG. 9F

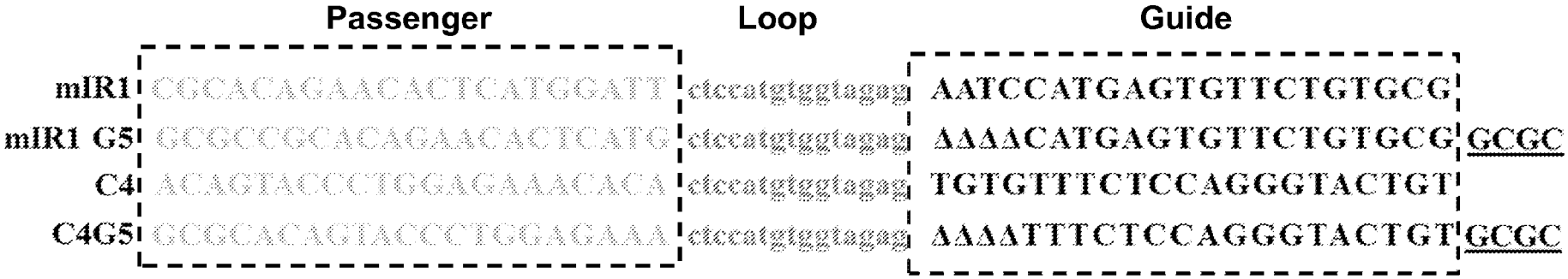


FIG. 10A

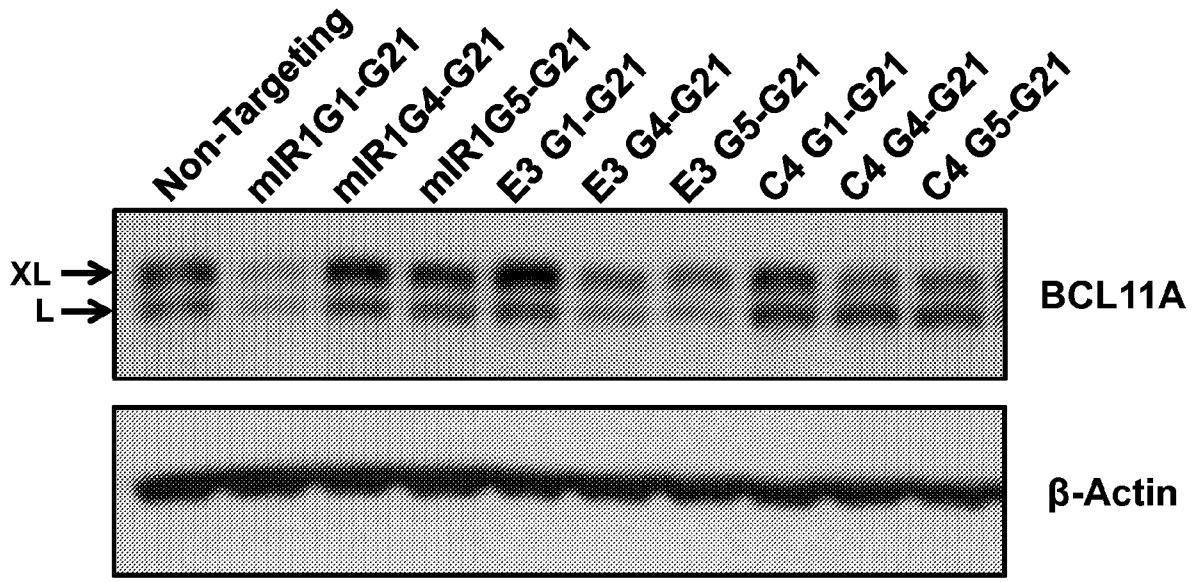


FIG. 10B

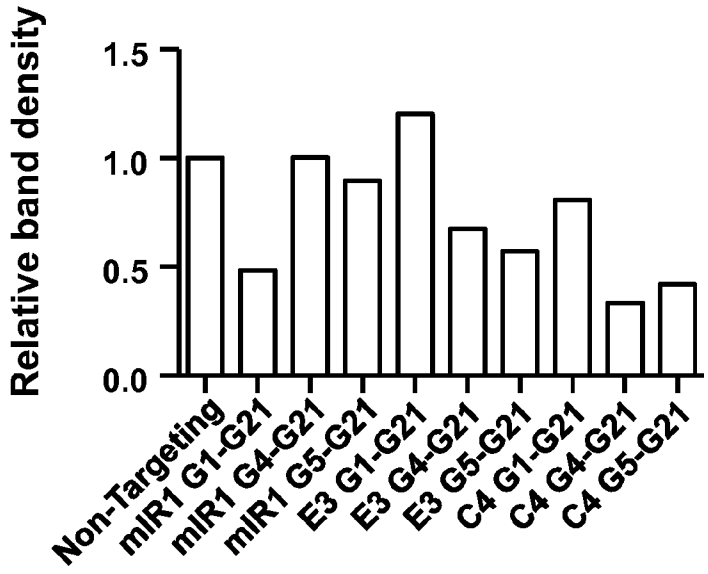


FIG. 10C

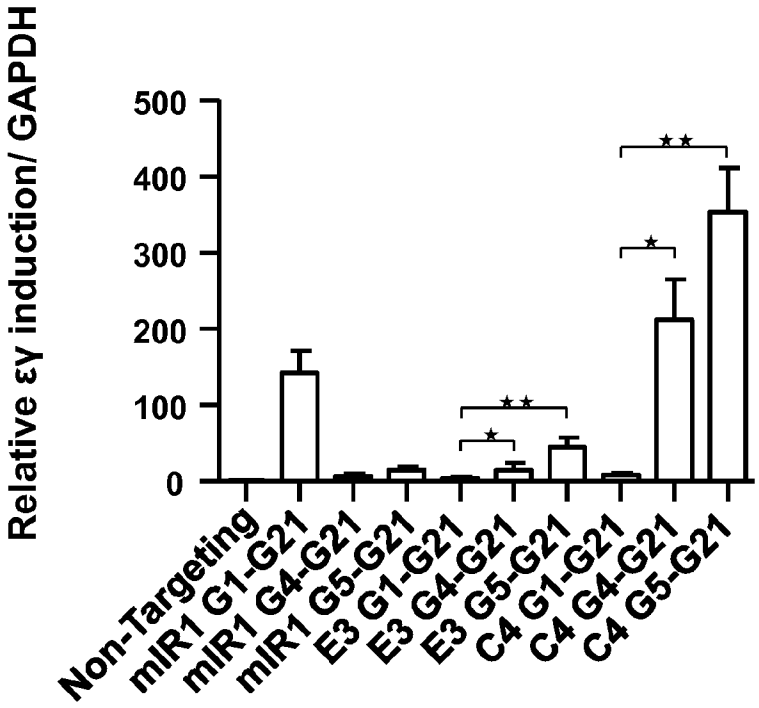


FIG. 10D

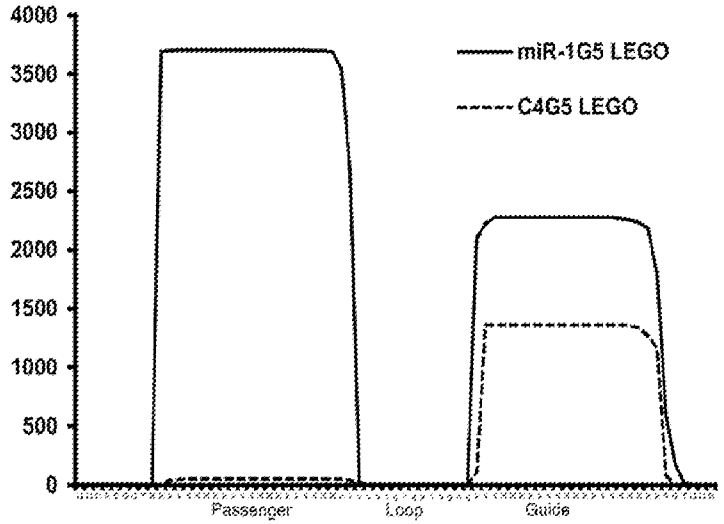


FIG. 11A

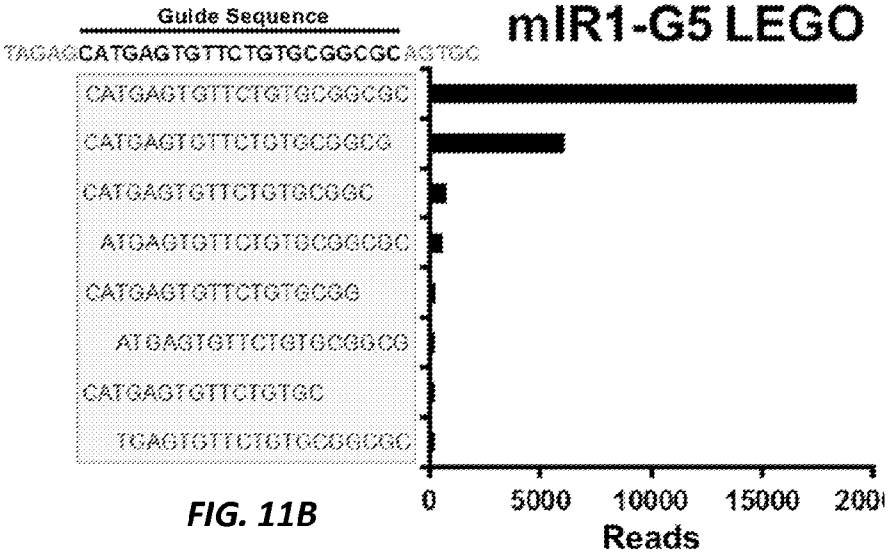


FIG. 11B

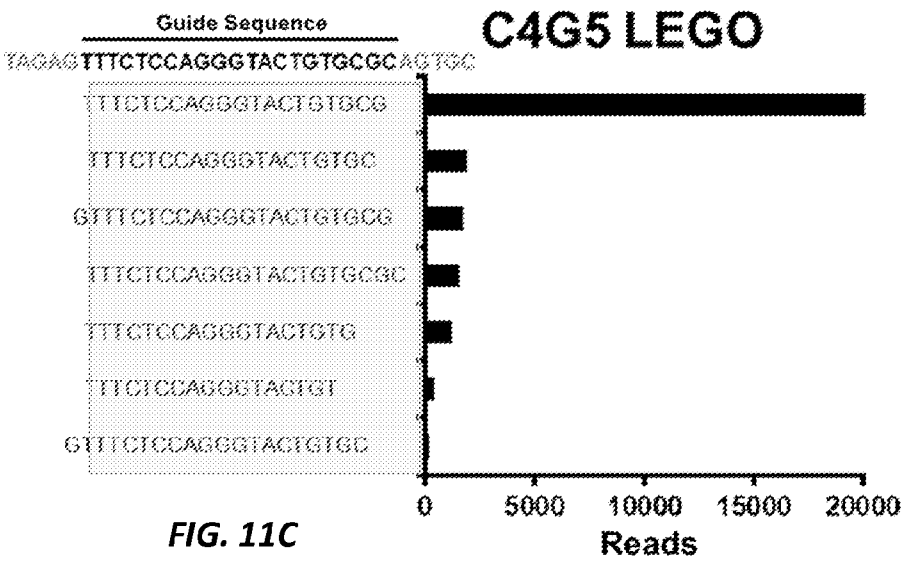


FIG. 11C

Candidates from the shRNA screen targeting Bcl11A using pLKO vector

	Passenger	Loop	Guide	
mIR1	CGCACAGAACACTCATGGATT	ctccatgtggtagag	AATCCATGAGTGTTCTGTGCG	SEQ. ID. NO: 25
E3	TCGGAGACTCCAGACAATCGC	ctccatgtggtagag	GCGATTGTCTGGAGTCTCCGA	SEQ. ID. NO: 26
B5	CCTCCAGGCAGCTCAAAGATC	ctccatgtggtagag	GATCTTTGAGCTGCCTGGAGG	SEQ. ID. NO: 27
D8	TTCTCTTGCAACACGCACAGA	ctccatgtggtagag	TCTGTGCGTGTTGCAAGAGAA	SEQ. ID. NO: 28
B11	TCAGGACTAGGTGCAGAATGT	ctccatgtggtagag	ACATTCTGCACCTAGTCCTGA	SEQ. ID. NO: 29
50D12	GATCGAGTGTTGAATAATGAT	ctccatgtggtagag	ATCATTATTCAACACTCGATC	SEQ. ID. NO: 30
50B11	CACTGTCCACAGGAGAAGCCA	ctccatgtggtagag	TGGCTTCTCCTGTGGACAGTG	SEQ. ID. NO: 32
50A5	CAGTACCCTGGAGAAACACAT	ctccatgtggtagag	ATGTGTTTCTCCAGGGTACTG	SEQ. ID. NO: 31
50C4	ACAGTACCCTGGAGAAACACA	ctccatgtggtagag	TGTGTTTCTCCAGGGTACTGT	SEQ. ID. NO: 33

FIG. 12A

Guide strand sequence composition and distribution in PLKO

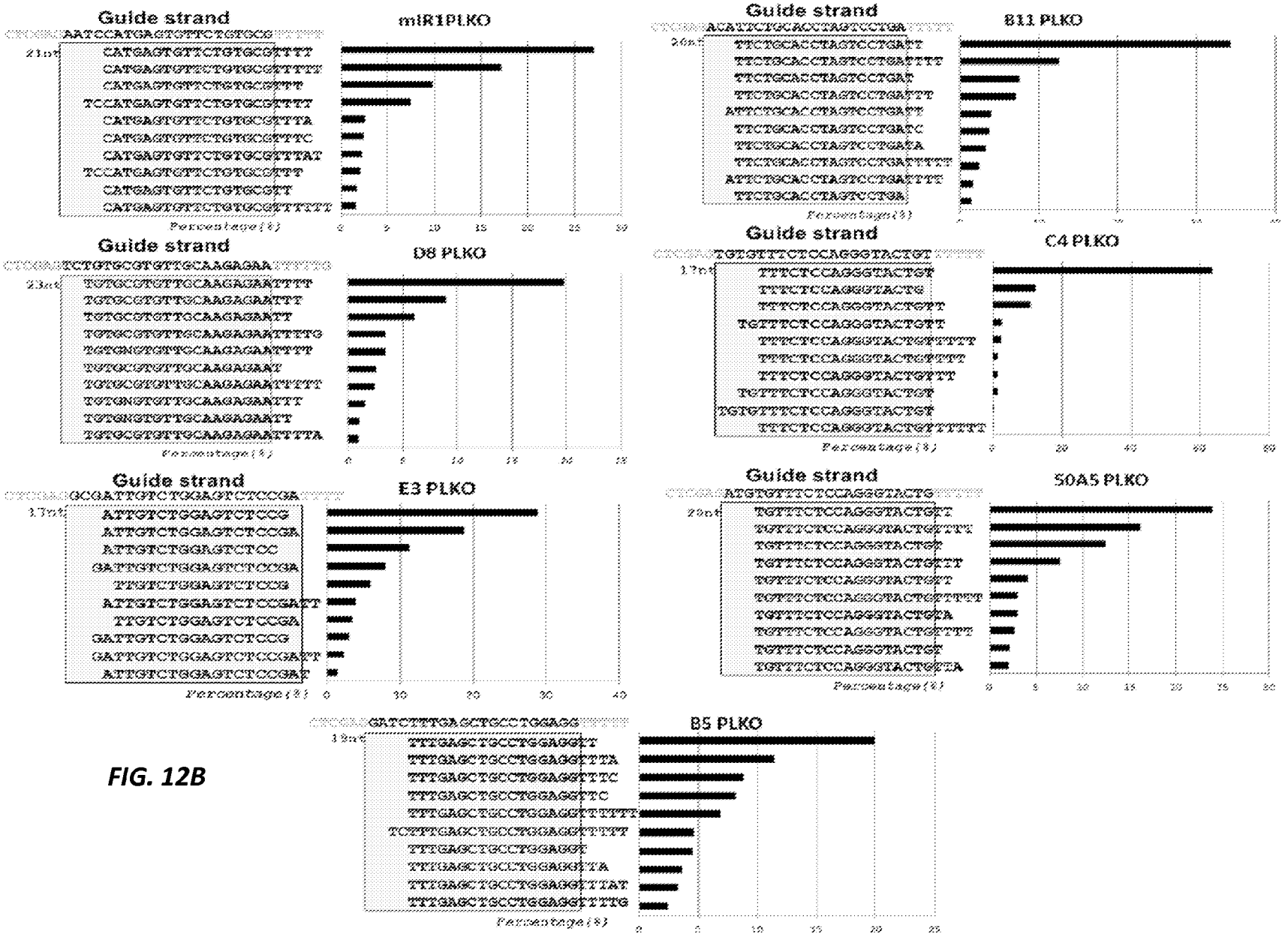


FIG. 12B

Guide strand sequence composition and distribution in LEGO

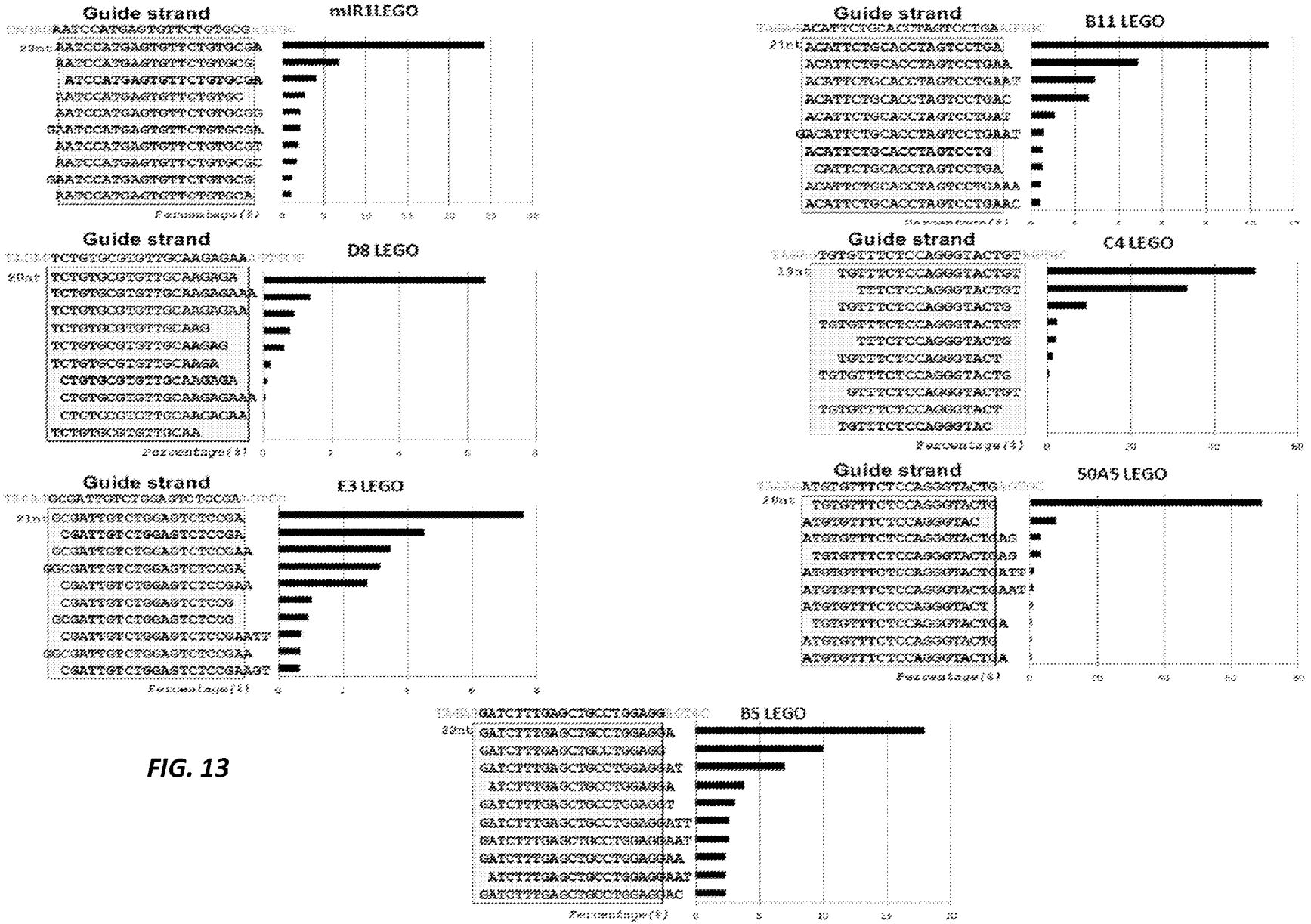


FIG. 13

Design of new shRNAs to mimic mature guide strands produced in pLKO vector

	Passenger	Loop	Guide	
mIR1G5	gcgcCGCACAGAACACTCATGxxxxx	ctccatgtggtagag	xxxxxCATGAGTGTTCTGTGCGgcgc	SEQ. ID. NO: 34
E3G5	gcgcTCGGAGACTCCAGACAAxxxxx	ctccatgtggtagag	xxxxxTTGTCTGGAGTCTCCGAgcgc	SEQ. ID. NO: 35
B5G5	gcgcCCTCCAGGCAGCTCAAAxxxxx	ctccatgtggtagag	xxxxxTTTGAGCTGCCTGGAGGgcgc	SEQ. ID. NO: 36
D8G5	gcgcTTCTCTTGCAACACGCAxxxxx	ctccatgtggtagag	xxxxxTGCGTGTTGCAAGAGAAgcgc	SEQ. ID. NO: 37
B11G5	gcgcTCAGGACTAGGTGCAGAxxxxx	ctccatgtggtagag	xxxxxTCTGCACCTAGTCCTGAgcgc	SEQ. ID. NO: 38
50D12G5	gcgcGATCGAGTGTTGAATAAxxxxx	ctccatgtggtagag	xxxxxTTATTCAACACTCGATCgcgc	SEQ. ID. NO: 39
50B11G5	gcgcCACTGTCCACAGGAGAAxxxxx	ctccatgtggtagag	xxxxxTTCTCCTGTGGACAGTGgcgc	SEQ. ID. NO: 41
50A5G5	GCGCCAGTACCCTGGAGAAACxxxxx	ctccatgtggtagag	xxxxxGTTTCTCCAGGGTACTGgcgc	SEQ. ID. NO: 40
50C4G5	gcgcACAGTACCCTGGAGAAAxxxxx	ctccatgtggtagag	xxxxxTTTCTCCAGGGTACTGTgcgc	SEQ. ID. NO: 42

FIG. 14A

Guide strand sequence composition and distribution in MODIFIED LEGO

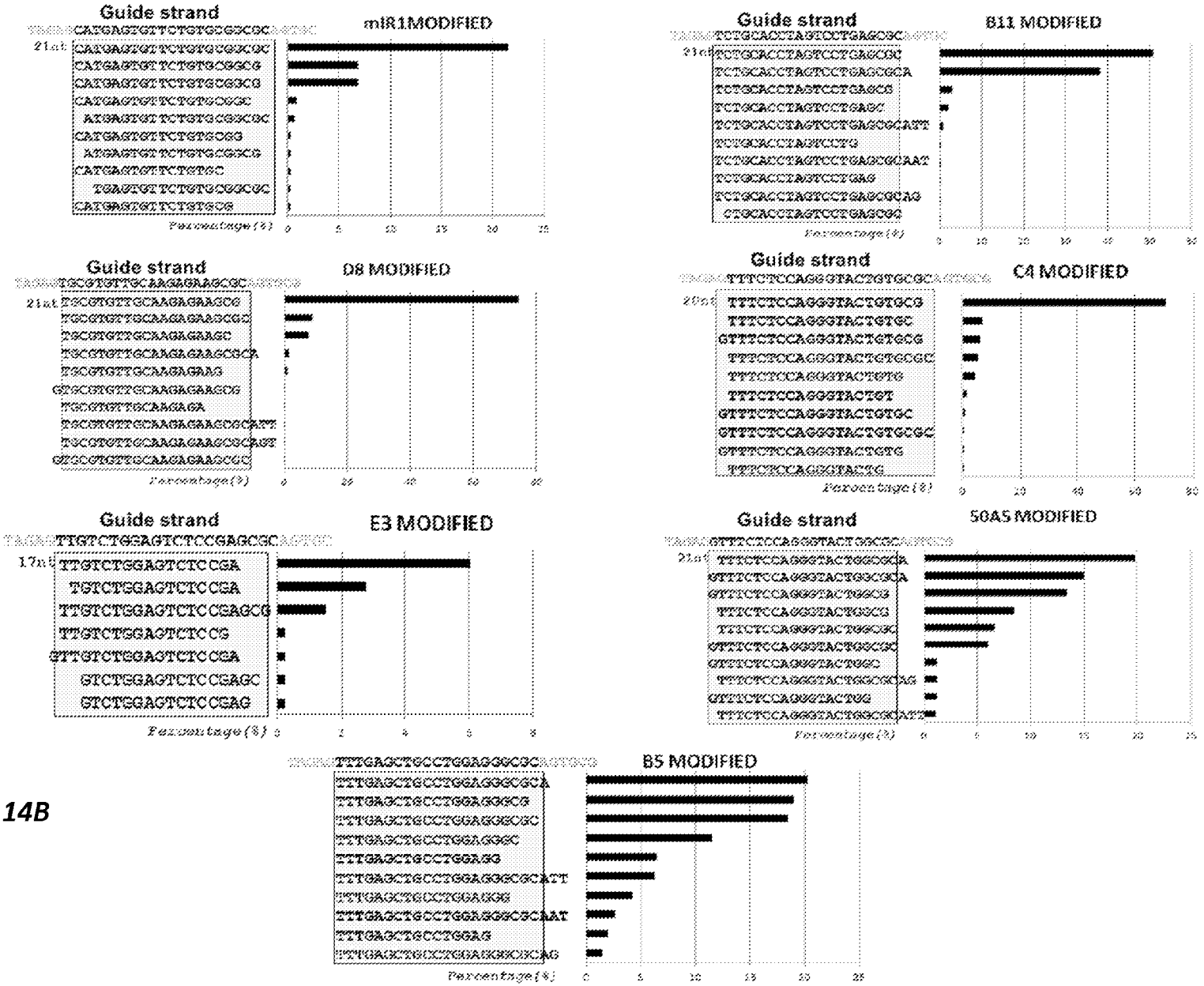


FIG.14B

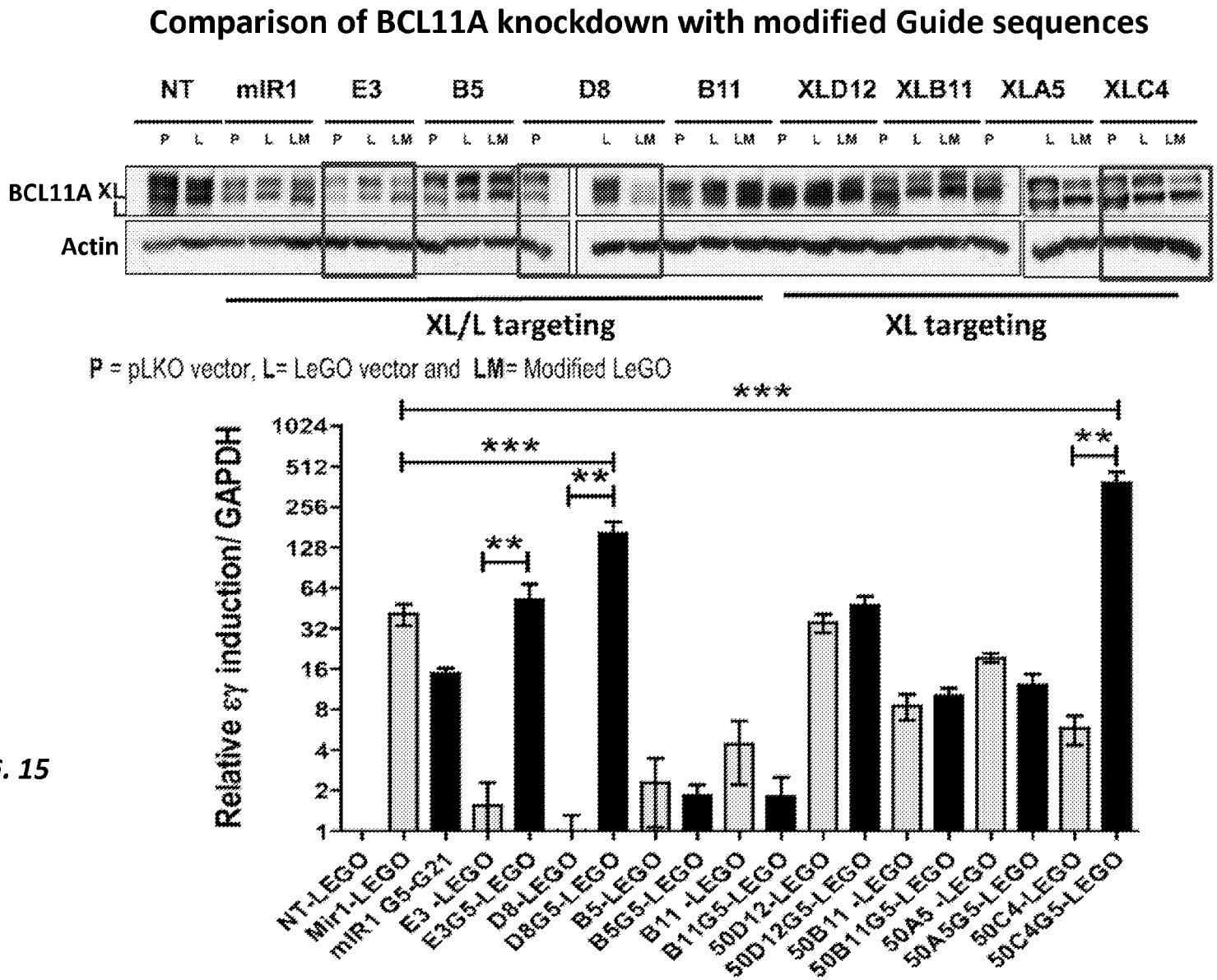

Comparison of BCL11A knockdown with modified Guide sequences
NT
mIR1
E3
B5
D8
B11
XLD12
XLB11
XLA5
XLC4
P L P L LM P L LM P L LM P L LM P L LM P L LM P L LM P L LM P L LM
BCL11A XL
Actin
XL/L targeting
XL targeting
P = pLKO vector, L= LeGO vector and LM= Modified LeGO
Relative εγ induction/ GAPDH
1024
512
256
128
64
32
16
8
4
2
1
***
***
**
**
**
NT-LEGO
Mir1-LEGO
mIR1 G5-G21
E3 -LEGO
E3G5-LEGO
D8-LEGO
D8G5-LEGO
B5-LEGO
B5G5-LEGO
B11 -LEGO
B11G5-LEGO
50D12-LEGO
50D12G5-LEGO
50B11 -LEGO
50B11G5-LEGO
50A5 -LEGO
50A5G5-LEGO
50C4-LEGO
50C4G5-LEGO

FIG. 15

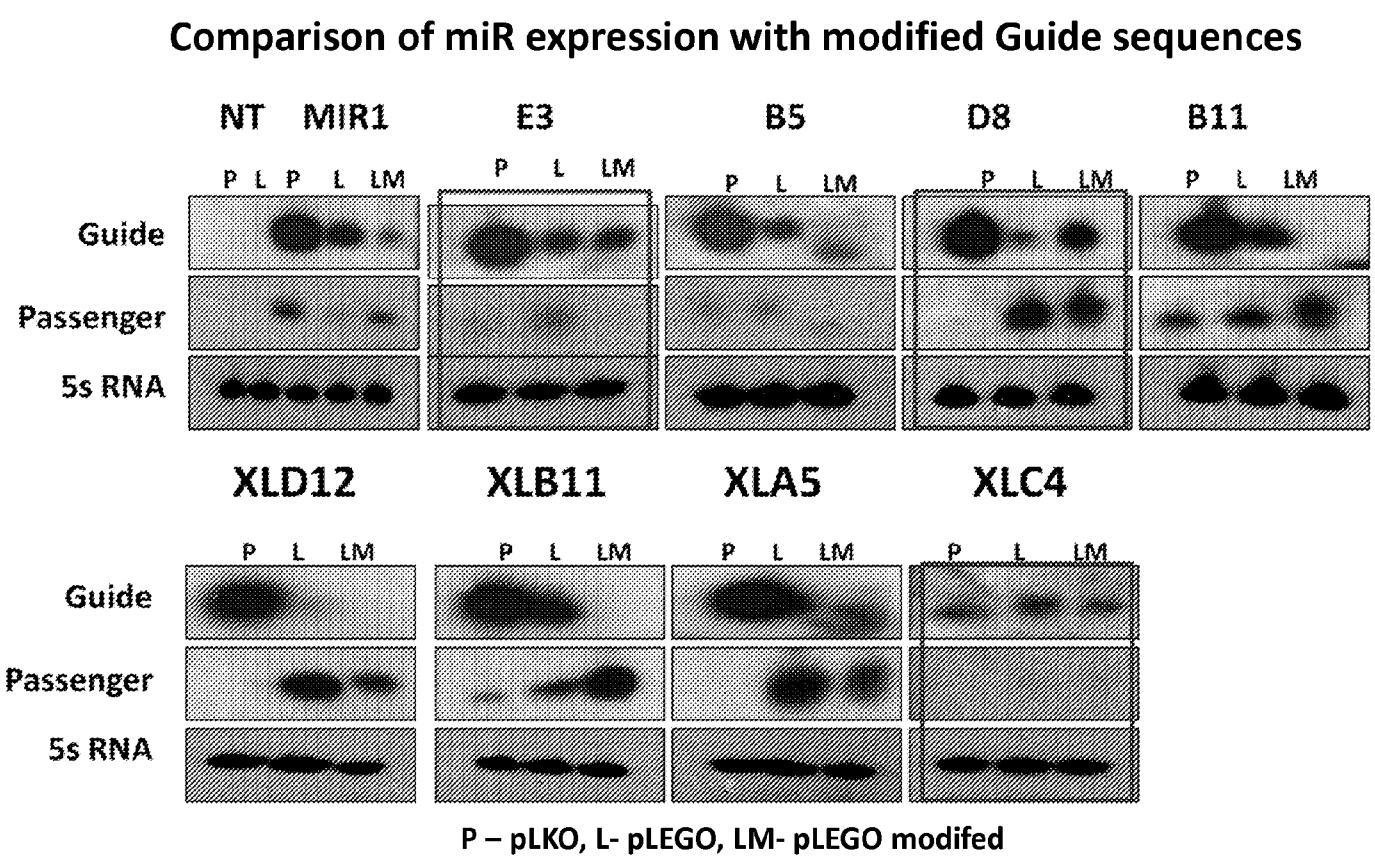
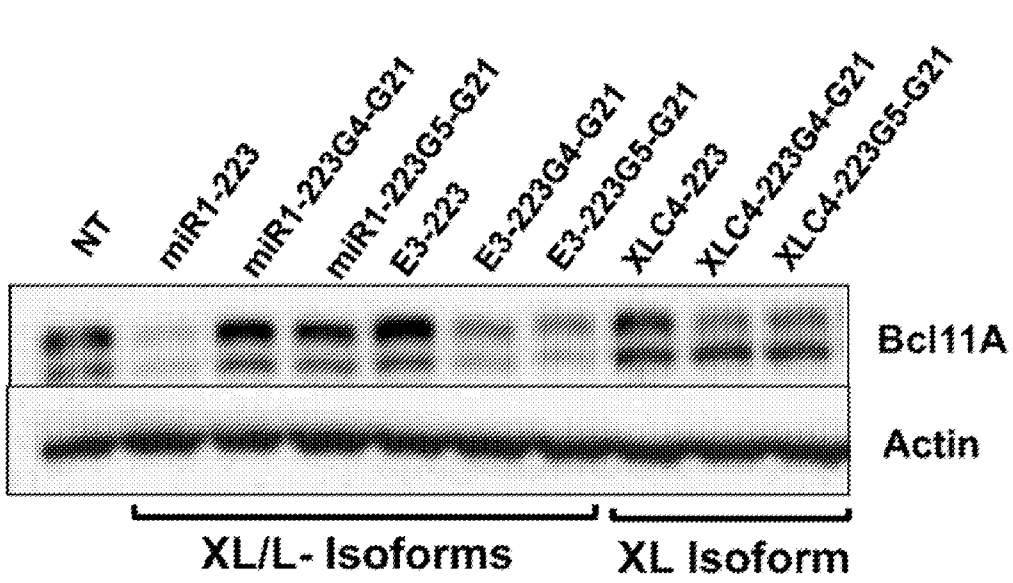
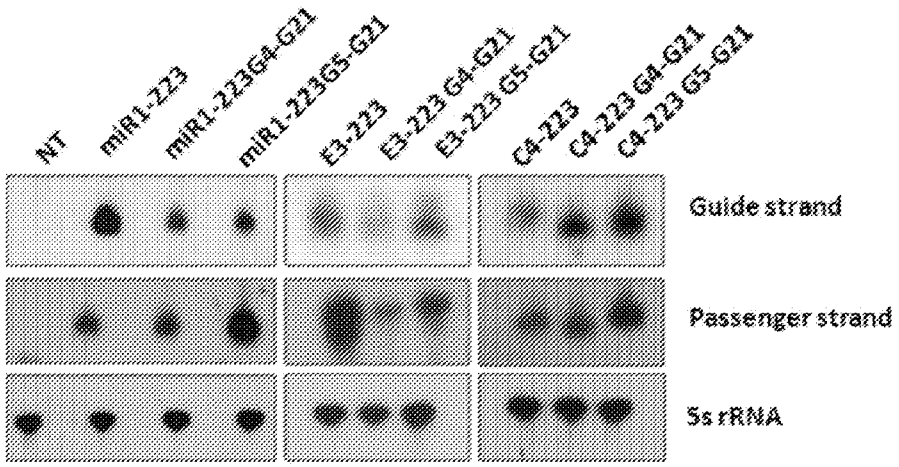

Comparison of miR expression with modified Guide sequences
NT
MIR1
E3
B5
D8
B11
P L P L LM
P L LM
Guide
Passenger
5s RNA
XLD12
XLB11
XLA5
XLC4
P L LM
P – pLKO, L- pLEGO, LM- pLEGO modifed

FIG. 16

BCL11a knockdown efficiency and εY induction with LEGO vectors





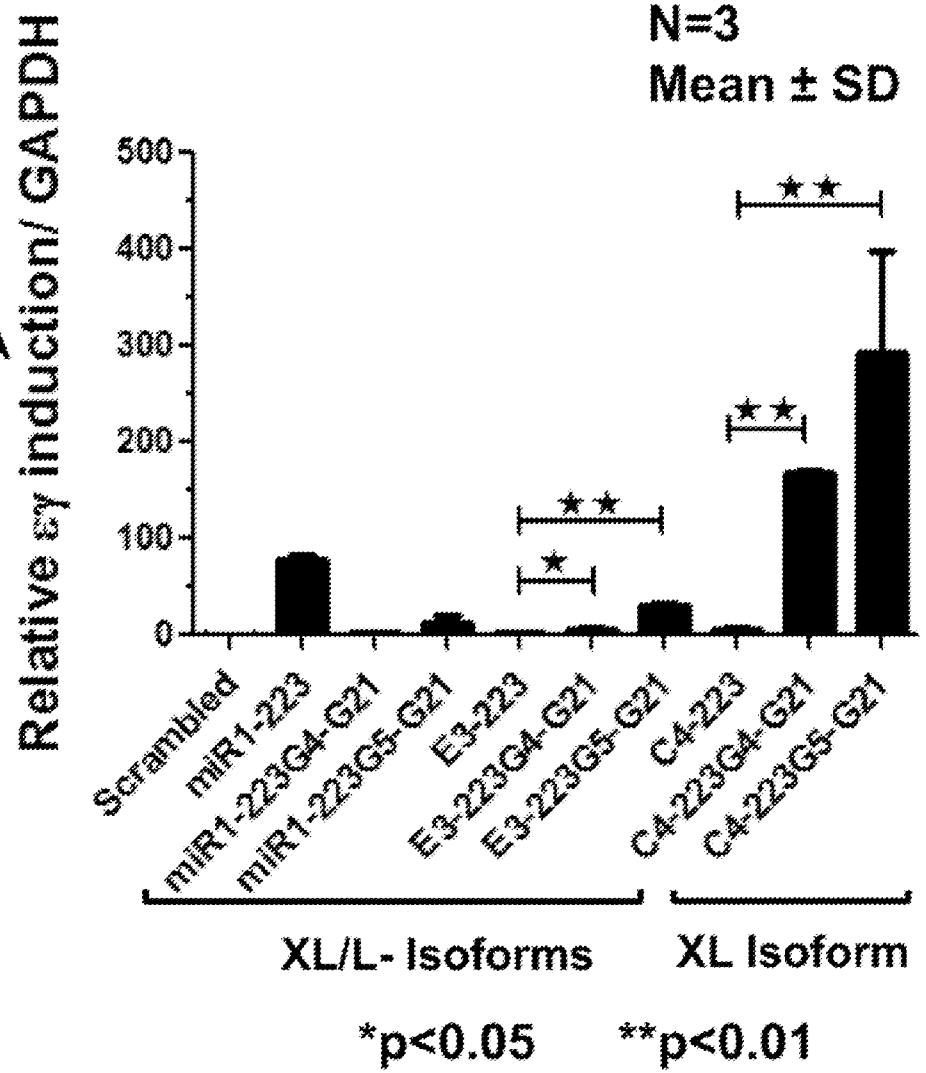


FIG. 17

Differential processing in pol-III shRNA vectors and pol-II microRNA adapted shRNA vectors

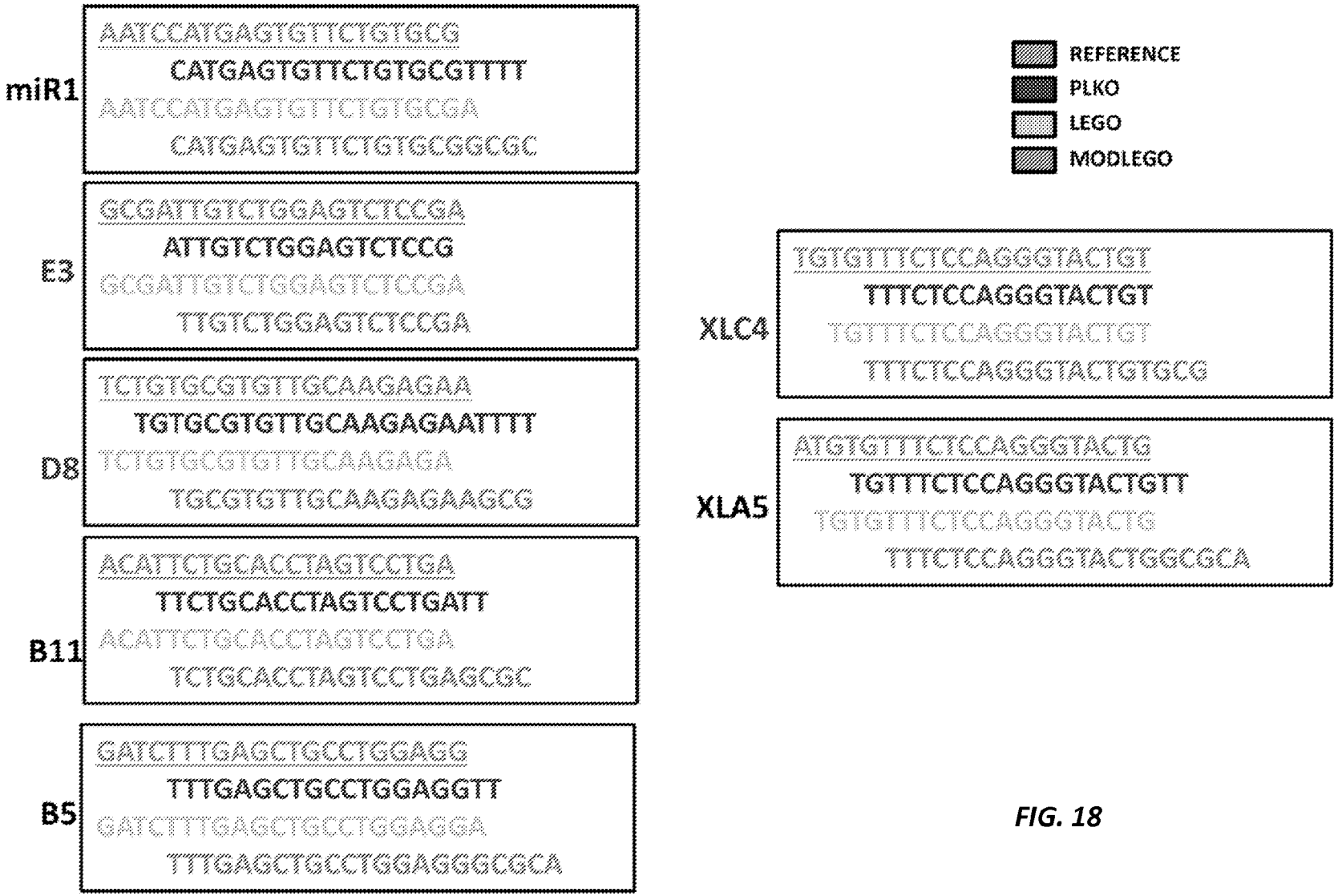


FIG. 18

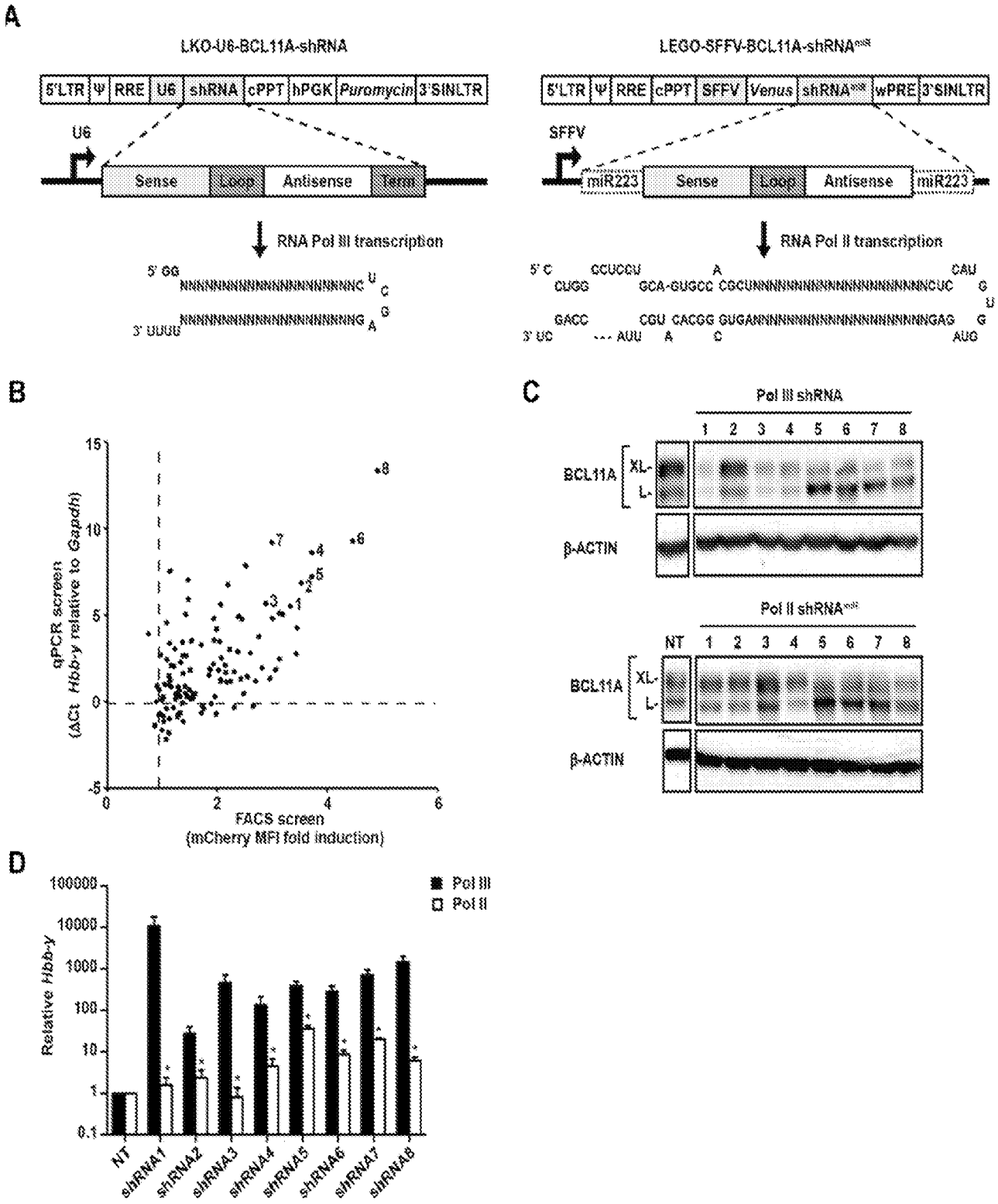

A
LKO-U6-BCL11A-shRNA
5'LTR
Ψ
RRE
U6
shRNA
cPPT
hPGK
Puromycin
3'SINLTR
U6
Sense
Loop
Antisense
Term
RNA Pol III transcription
LEGO-SFFV-BCL11A-shRNAmiR
5'LTR
Ψ
RRE
cPPT
SFFV
Venus
shRNAmiR
wPRE
3'SINLTR
SFFV
miR223
Sense
Loop
Antisense
miR223
RNA Pol II transcription
B
qPCR screen
(ΔCt Hbb-y relative to Gapdh)
FACS screen
(mCherry MFI fold induction)
C
Pol III shRNA
1 2 3 4 5 6 7 8
BCL11A
XL
L
β-ACTIN
Pol II shRNAmiR
NT 1 2 3 4 5 6 7 8
BCL11A
XL
L
β-ACTIN
D
Pol III
Pol II
Relative Hbb-y
NT
shRNA1
shRNA2
shRNA3
shRNA4
shRNA5
shRNA6
shRNA7
shRNA8

FIG. 19

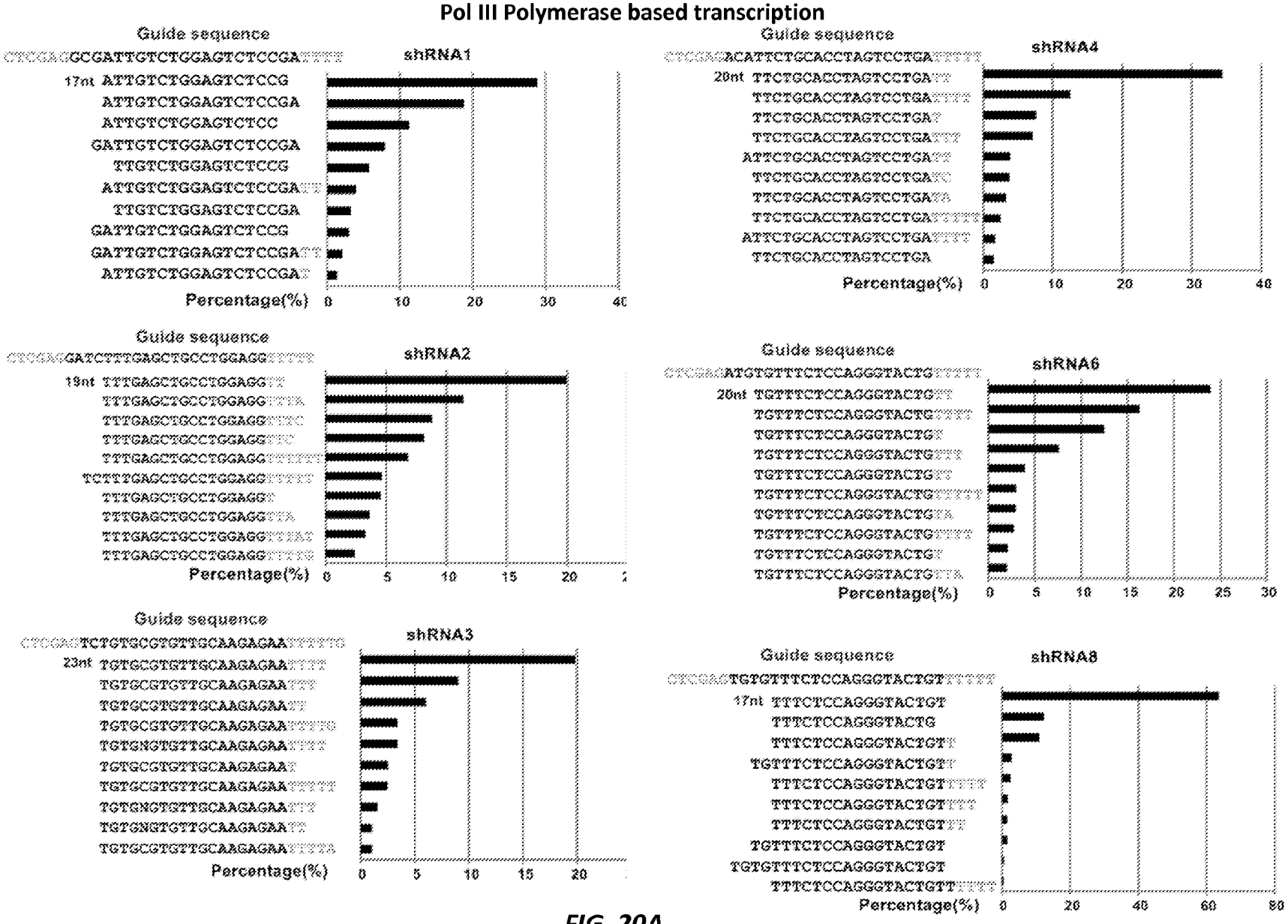

Pol III Polymerase based transcription
Guide sequence
shRNA1
17nt ATTGTCTGGAGTCTCCG
ATTGTCTGGAGTCTCCGA
ATTGTCTGGAGTCTCC
GATTGTCTGGAGTCTCCGA
TTGTCTGGAGTCTCCG
ATTGTCTGGAGTCTCCGA
TTGTCTGGAGTCTCCGA
GATTGTCTGGAGTCTCCG
GATTGTCTGGAGTCTCCGA
ATTGTCTGGAGTCTCCGA
Percentage(%) 0 10 20 30 40
Guide sequence
shRNA4
20nt TTCTGCACCTAGTCCTGA
TTCTGCACCTAGTCCTGA
TTCTGCACCTAGTCCTGA
TTCTGCACCTAGTCCTGA
ATTCTGCACCTAGTCCTGA
TTCTGCACCTAGTCCTGA
TTCTGCACCTAGTCCTGA
TTCTGCACCTAGTCCTGA
ATTCTGCACCTAGTCCTGA
TTCTGCACCTAGTCCTGA
Percentage(%) 0 10 20 30 40
Guide sequence
shRNA2
19nt TTTGAGCTGCCTGGAGG
TTTGAGCTGCCTGGAGG
TTTGAGCTGCCTGGAGG
TTTGAGCTGCCTGGAGG
TTTGAGCTGCCTGGAGG
TCTTTGAGCTGCCTGGAGG
TTTGAGCTGCCTGGAGG
TTTGAGCTGCCTGGAGG
TTTGAGCTGCCTGGAGG
TTTGAGCTGCCTGGAGG
Percentage(%) 0 5 10 15 20
Guide sequence
shRNA6
20nt TGTTTCTCCAGGGTACTG
TGTTTCTCCAGGGTACTG
TGTTTCTCCAGGGTACTG
TGTTTCTCCAGGGTACTG
TGTTTCTCCAGGGTACTG
TGTTTCTCCAGGGTACTG
TGTTTCTCCAGGGTACTG
TGTTTCTCCAGGGTACTG
TGTTTCTCCAGGGTACTG
TGTTTCTCCAGGGTACTG
Percentage(%) 0 5 10 15 20 25 30
Guide sequence
shRNA3
23nt TGTGCGTGTTGCAAGAGAA
TGTGCGTGTTGCAAGAGAA
TGTGCGTGTTGCAAGAGAA
TGTGCGTGTTGCAAGAGAA
TGTGNGTGTTGCAAGAGAA
TGTGCGTGTTGCAAGAGAA
TGTGCGTGTTGCAAGAGAA
TGTGNGTGTTGCAAGAGAA
TGTGNGTGTTGCAAGAGAA
TGTGCGTGTTGCAAGAGAA
Percentage(%) 0 5 10 15 20
Guide sequence
shRNA8
17nt TTTCTCCAGGGTACTGT
TTTCTCCAGGGTACTG
TTTCTCCAGGGTACTGT
TGTTTCTCCAGGGTACTGT
TTTCTCCAGGGTACTGT
TTTCTCCAGGGTACTGT
TTTCTCCAGGGTACTGT
TGTTTCTCCAGGGTACTGT
TGTGTTTCTCCAGGGTACTGT
TTTCTCCAGGGTACTGTT
Percentage(%) 0 20 40 60 80

FIG. 20A

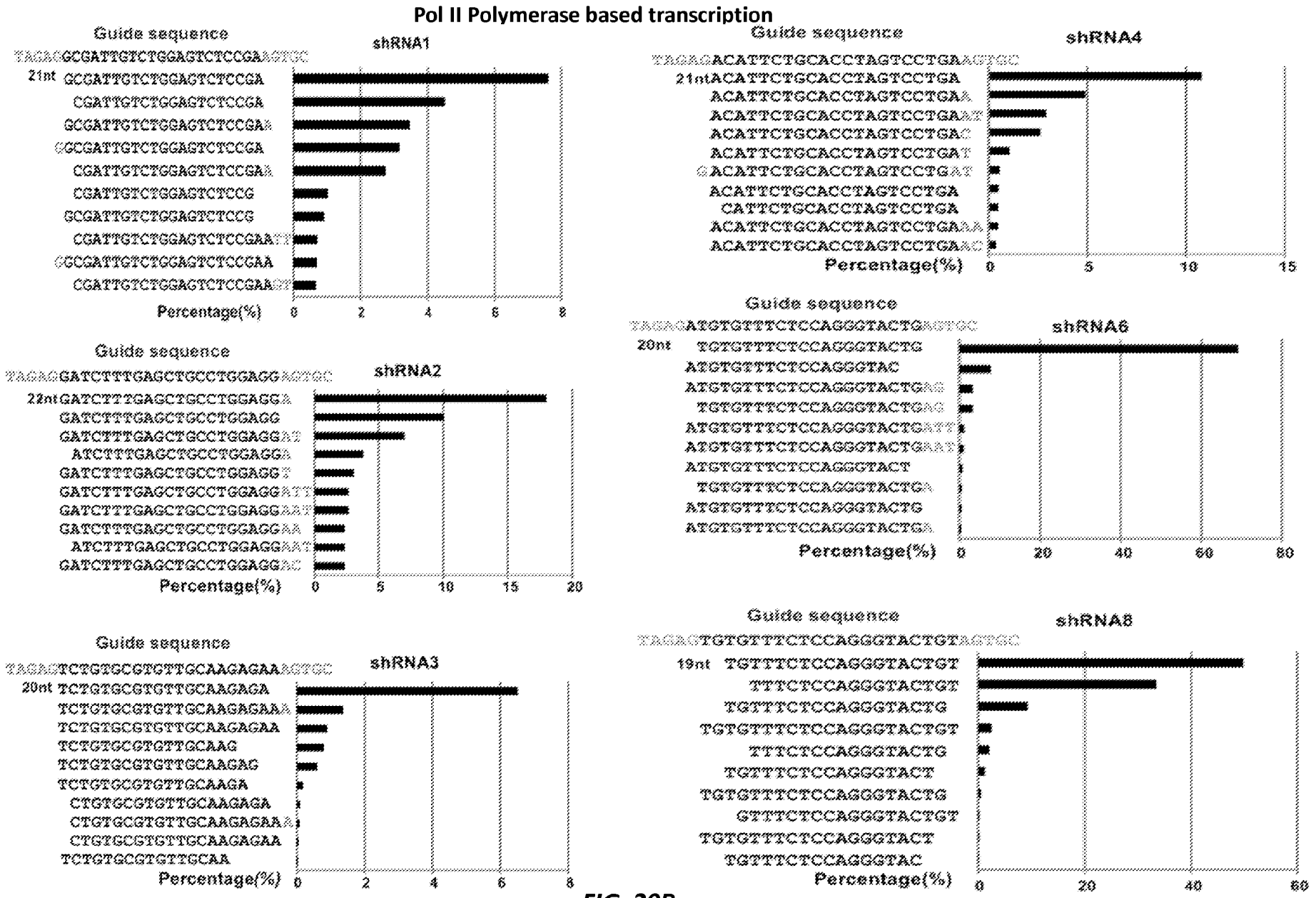

Pol II Polymerase based transcription
Guide sequence
shRNA1
21nt GCGATTGTCTGGAGTCTCCGA
Percentage(%)
shRNA2
22nt GATCTTTGAGCTGCCTGGAGGA
Percentage(%)
shRNA3
20nt TCTGTGCGTGTTGCAAGAGA
Percentage(%)
shRNA4
21nt ACATTCTGCACCTAGTCCTGA
Percentage(%)
shRNA6
20nt TGTGTTTCTCCAGGGTACTG
Percentage(%)
shRNA8
19nt TGTTTCTCCAGGGTACTGT
Percentage(%)

FIG. 20B

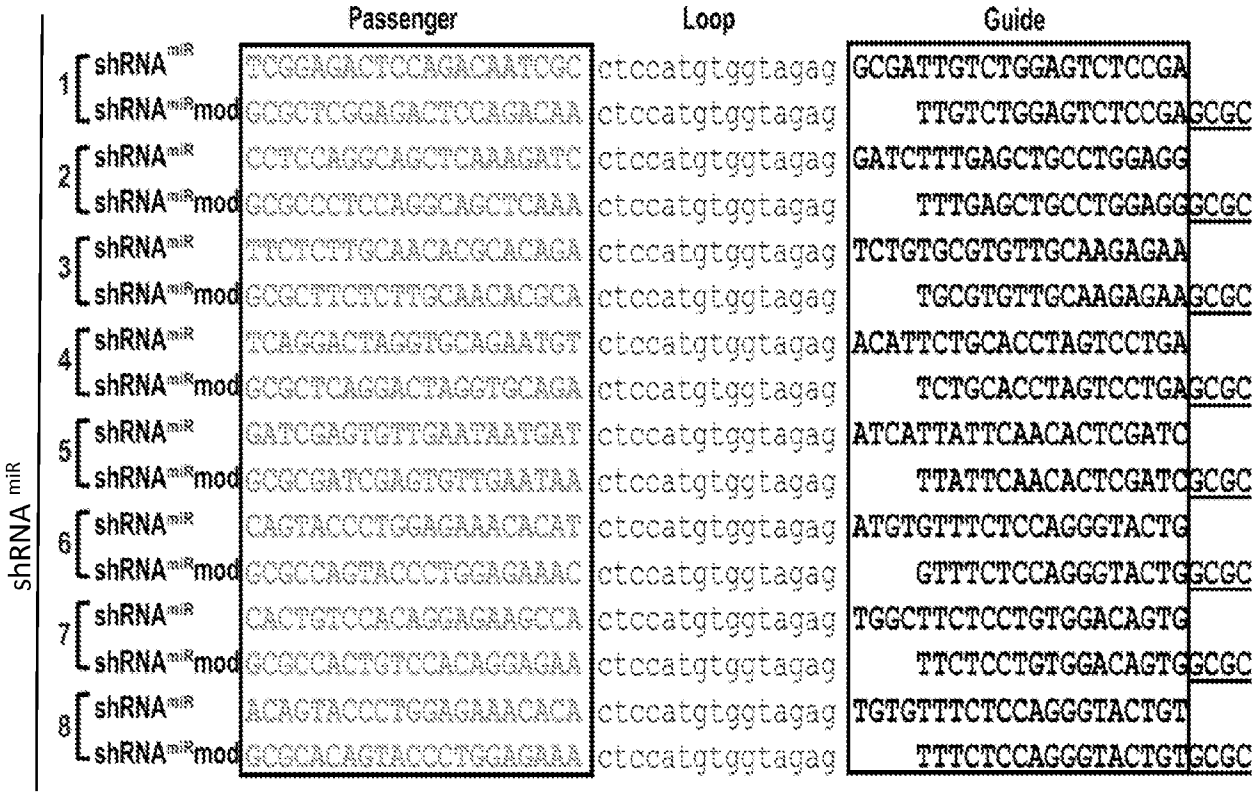


FIG. 21A

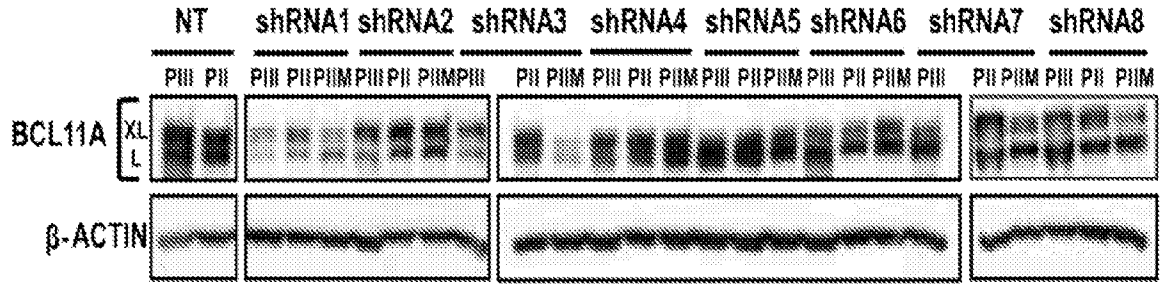


FIG. 21B

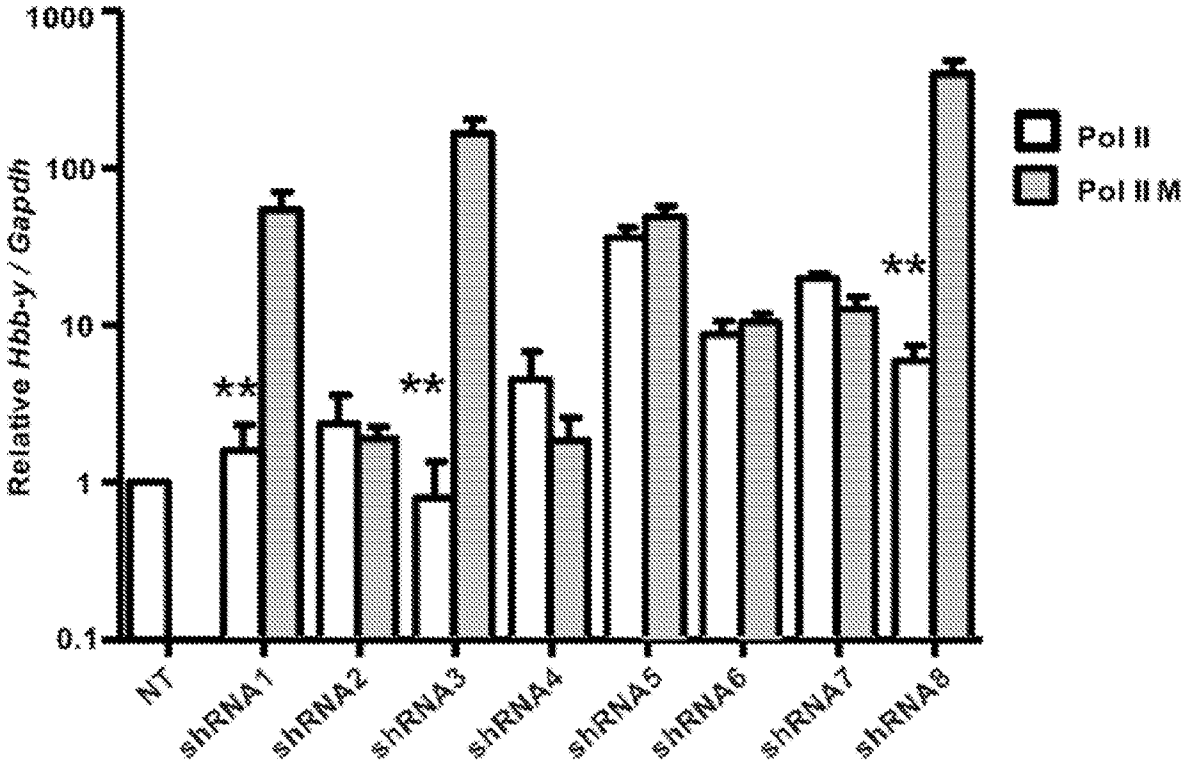


FIG. 21C

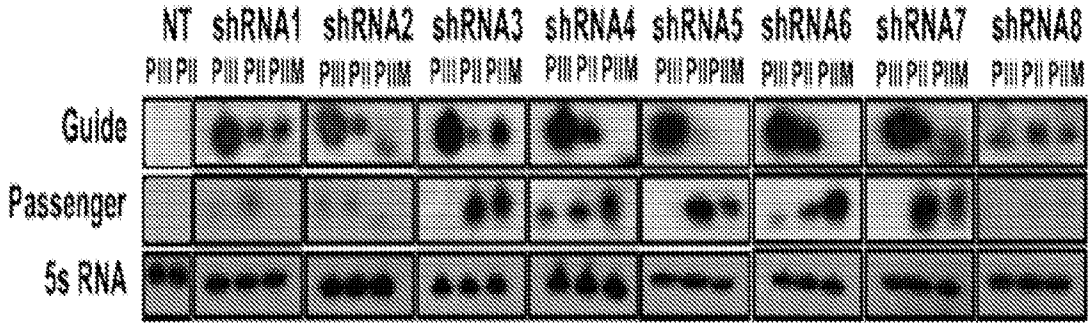


FIG. 21D

Modified for Pol II Polymerase based transcription

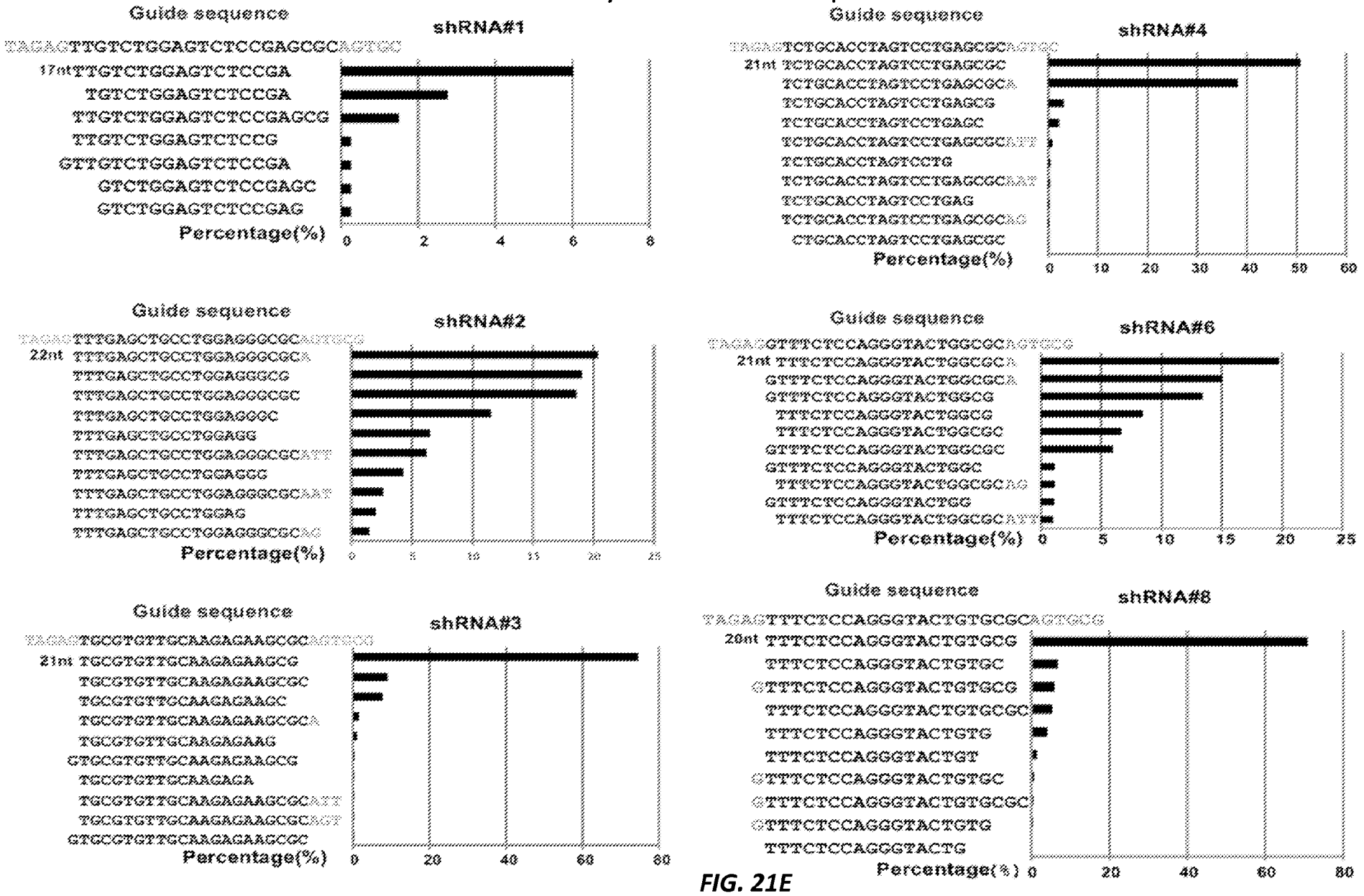


FIG. 21E

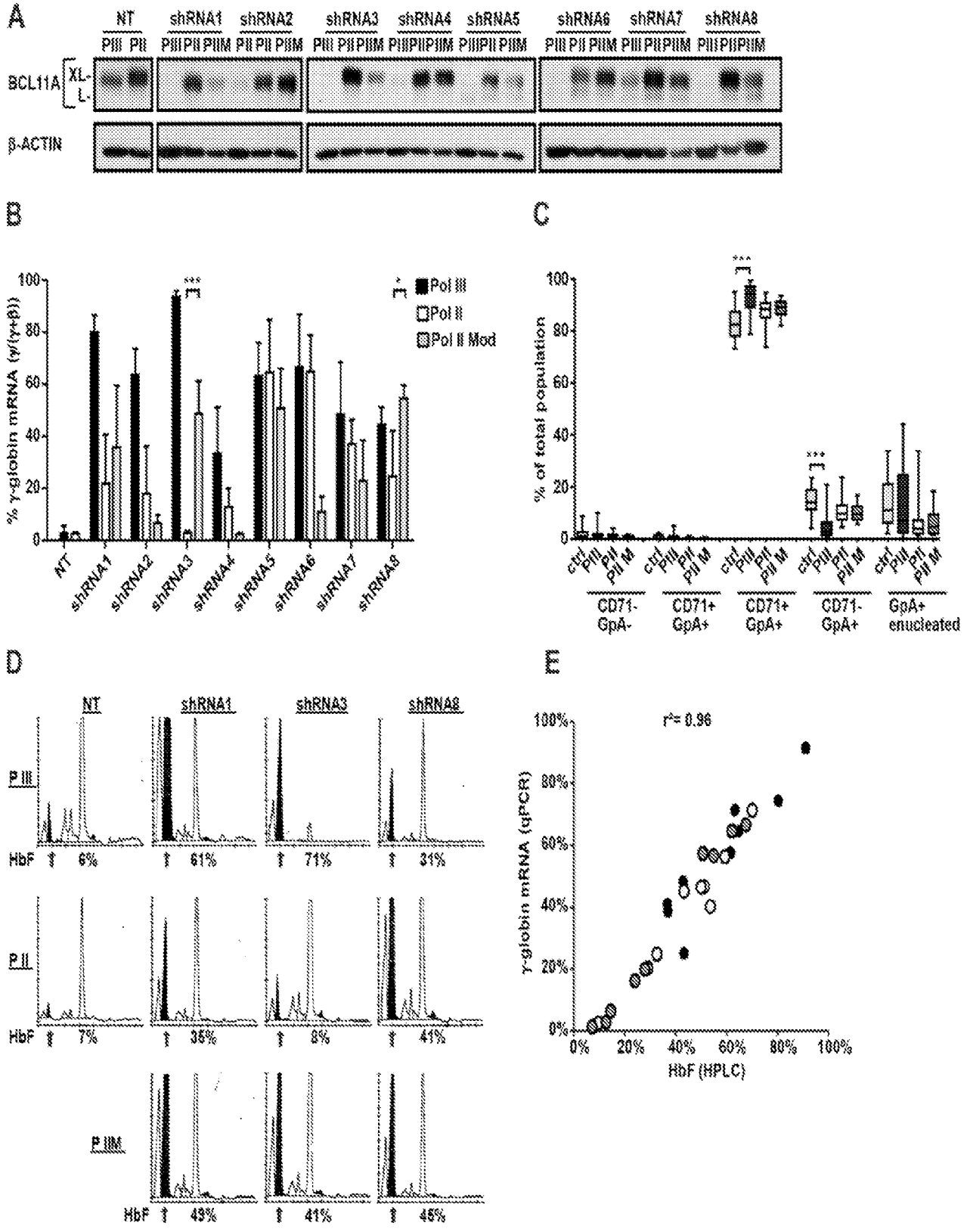

A
NT
shRNA1
shRNA2
shRNA3
shRNA4
shRNA5
shRNA6
shRNA7
shRNA8
PIII PII
PIII PII PIIM
PII PII PIIM
PIII PII PIIM
PIII PII PIIM
PIII PII PIIM
PIII PII PIIM
PIII PII PIIM
PIII PII PIIM
BCL11A
XL-
L-
β-ACTIN
B
% γ-globin mRNA (γ/(γ+β))
Pol III
Pol II
Pol II Mod
NT
shRNA1
shRNA2
shRNA3
shRNA4
shRNA5
shRNA6
shRNA7
shRNA8
C
% of total population
ctrl
PIII
PII
PII M
CD71- GpA-
CD71+ GpA+
CD71+ GpA+
CD71- GpA+
GpA+ enucleated
D
NT
shRNA1
shRNA3
shRNA8
P III
HbF
6%
61%
71%
31%
P II
HbF
7%
35%
8%
41%
P IIM
HbF
43%
41%
45%
E
r²= 0.96
γ-globin mRNA (qPCR)
HbF (HPLC)
0%
20%
40%
60%
80%
100%

FIG. 22

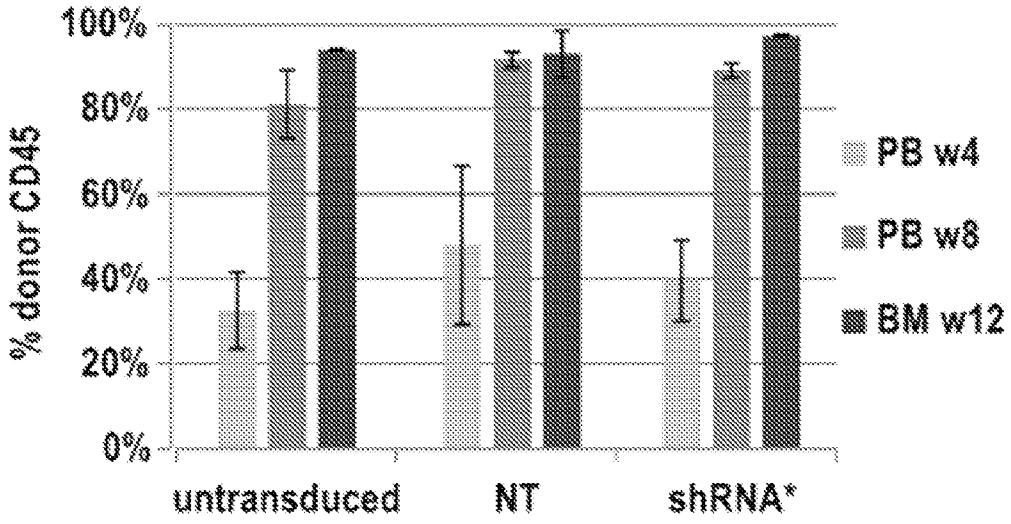


FIG. 23A

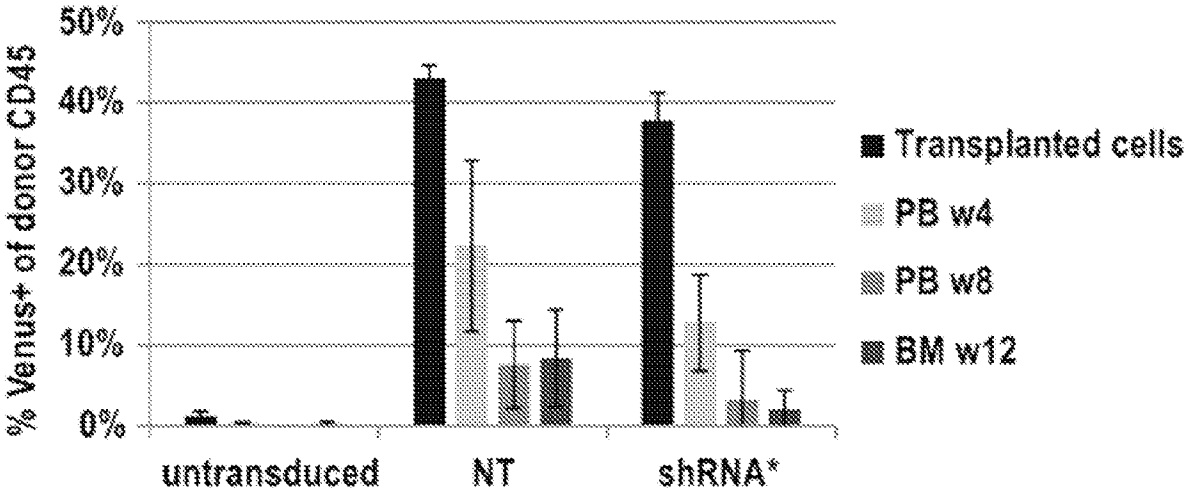


FIG. 23B

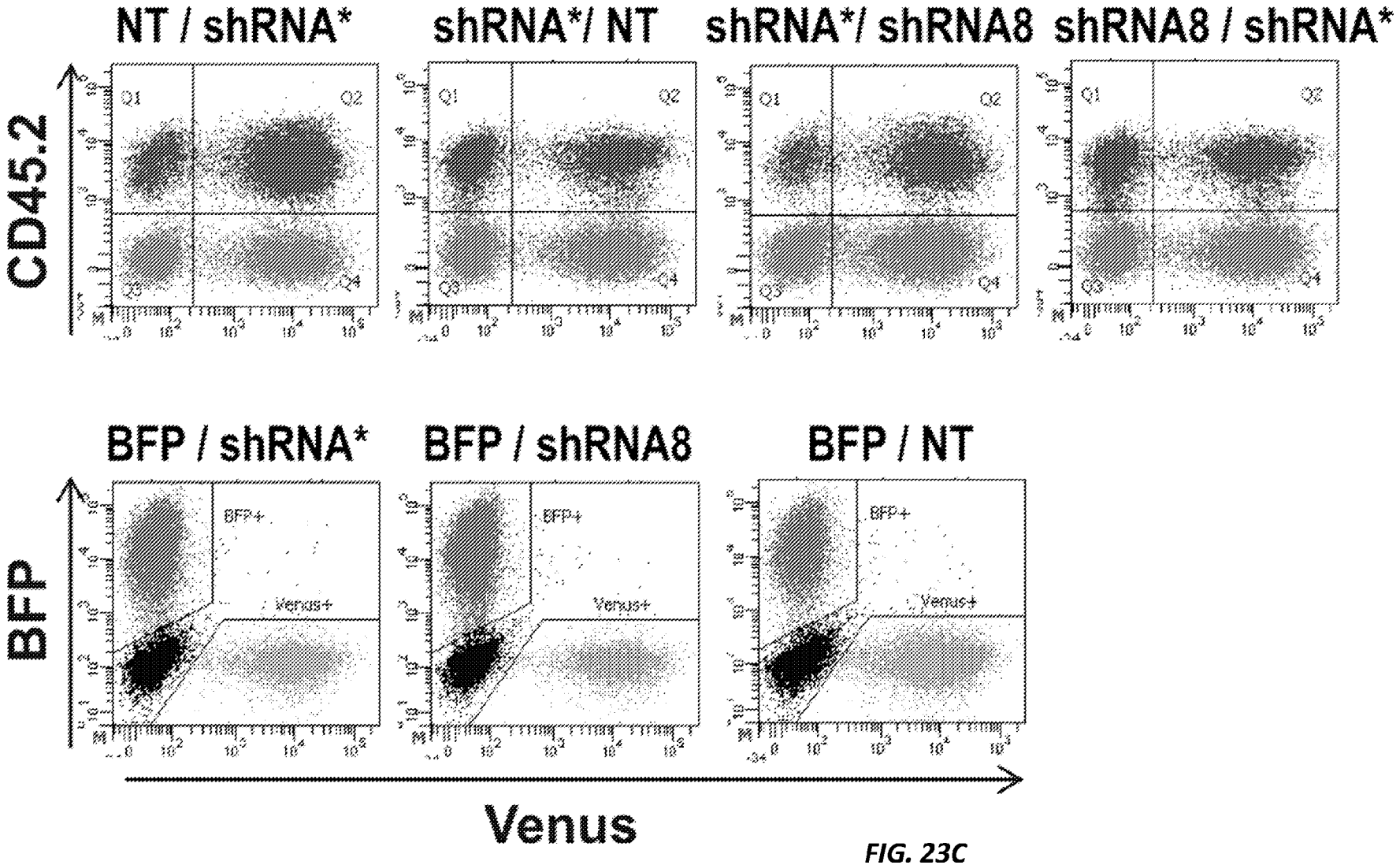

NT / shRNA*
shRNA*/ NT
shRNA*/ shRNA8
shRNA8 / shRNA*
CD45.2
Q1
Q2
Q3
Q4
BFP / shRNA*
BFP / shRNA8
BFP / NT
BFP
BFP+
Venus+
Venus

FIG. 23C

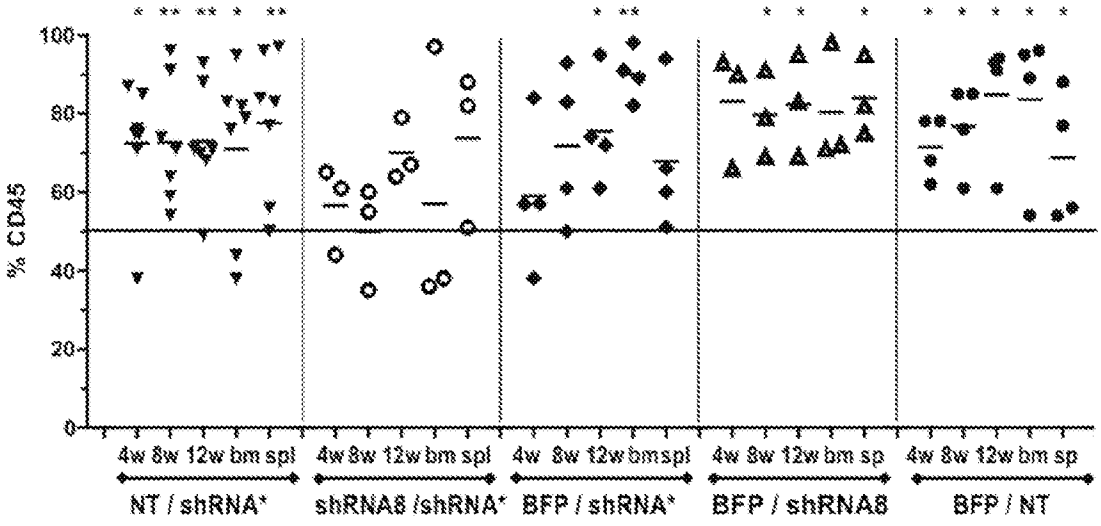


FIG. 23D

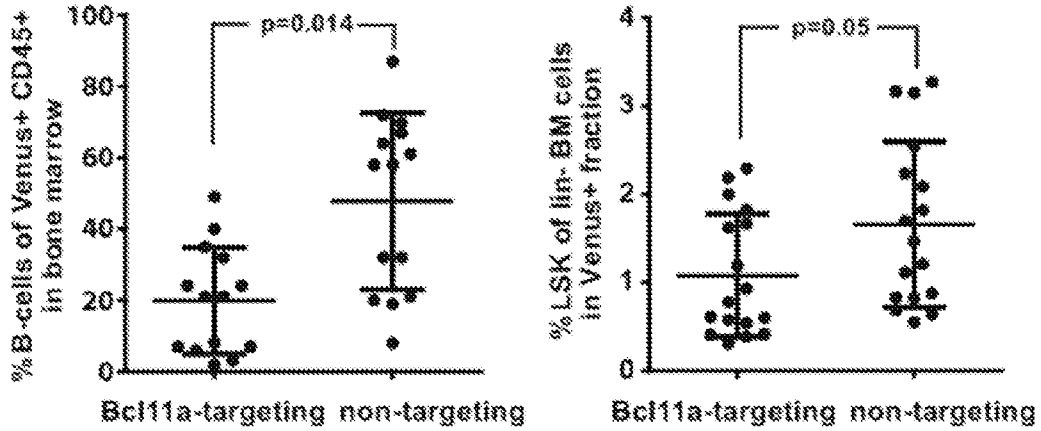


FIG. 23E

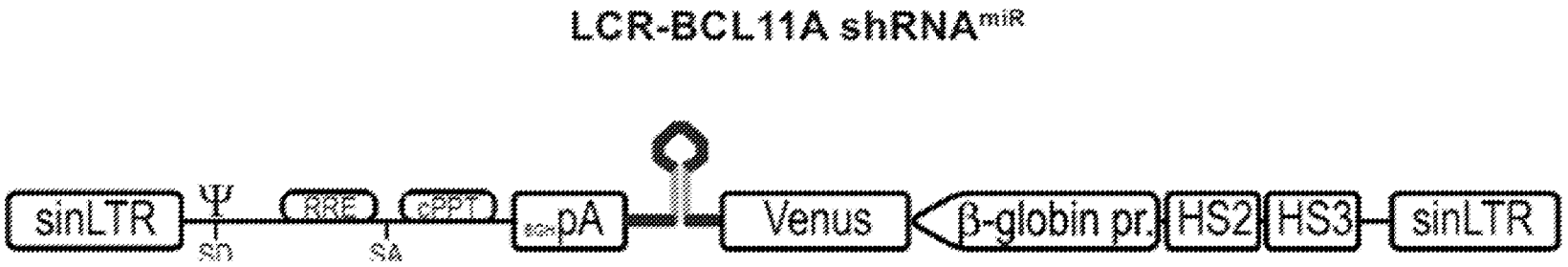


FIG. 23F

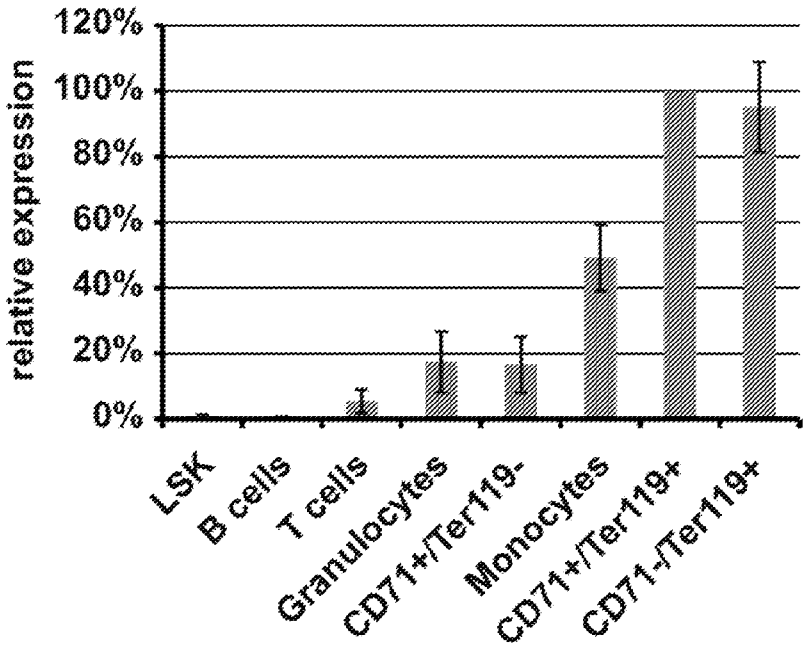


FIG. 23G

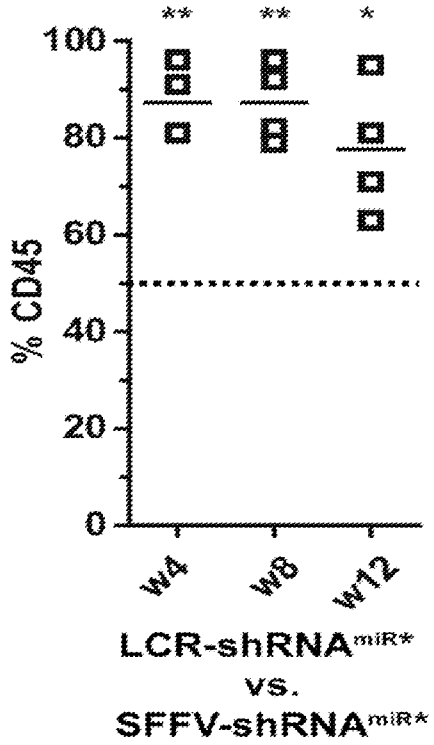


FIG. 23H

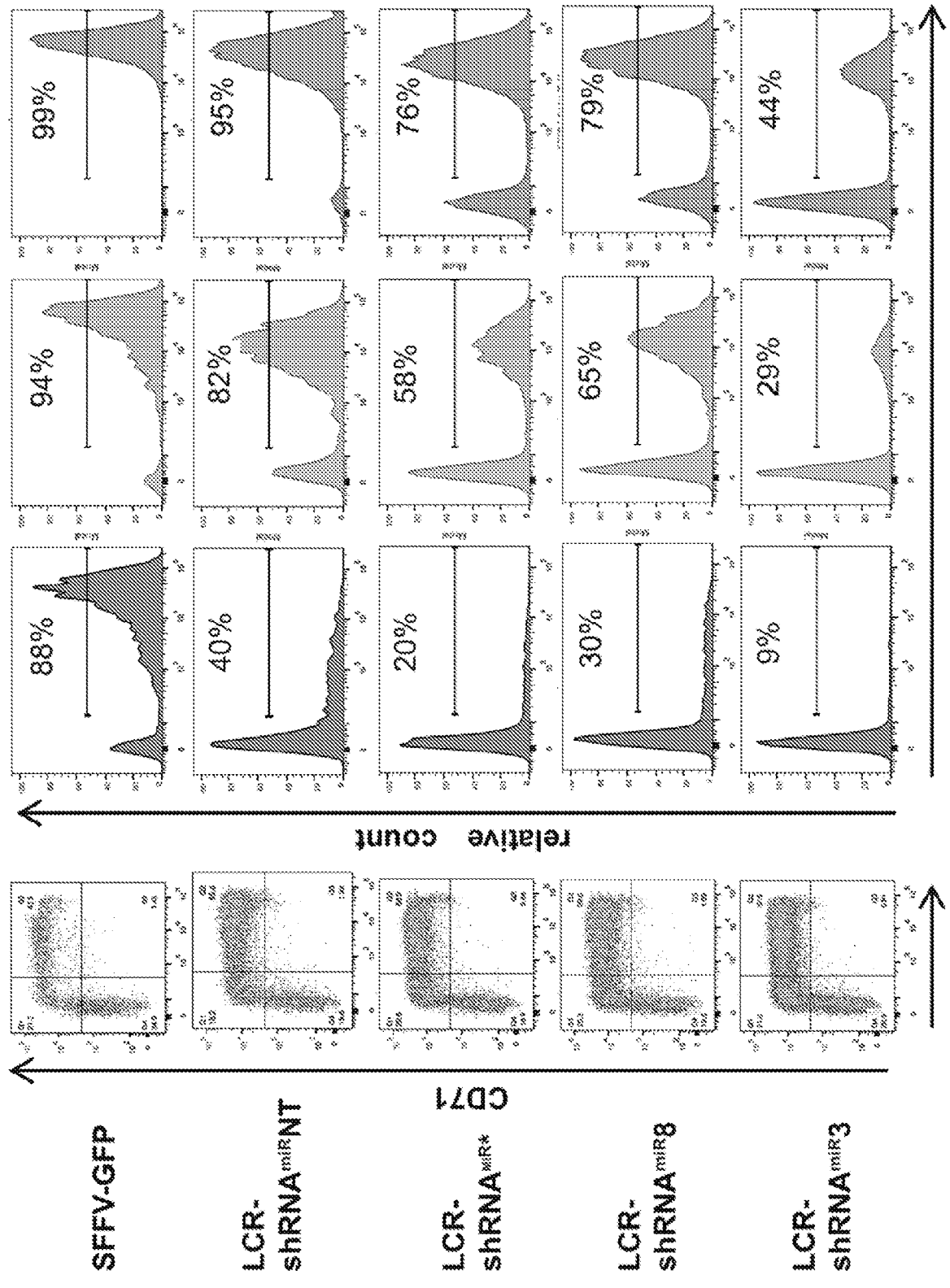

CD71- / GpA-
CD71+ / GpA-
CD71+/GpA+
88%
94%
99%
40%
82%
95%
20%
58%
76%
30%
65%
79%
9%
29%
44%
relative count
Venus
GpA
CD71
SFFV-GFP
LCR-shRNA^miRNT
LCR-shRNA^miR*
LCR-shRNA^miR8
LCR-shRNA^miR3

FIG. 23I

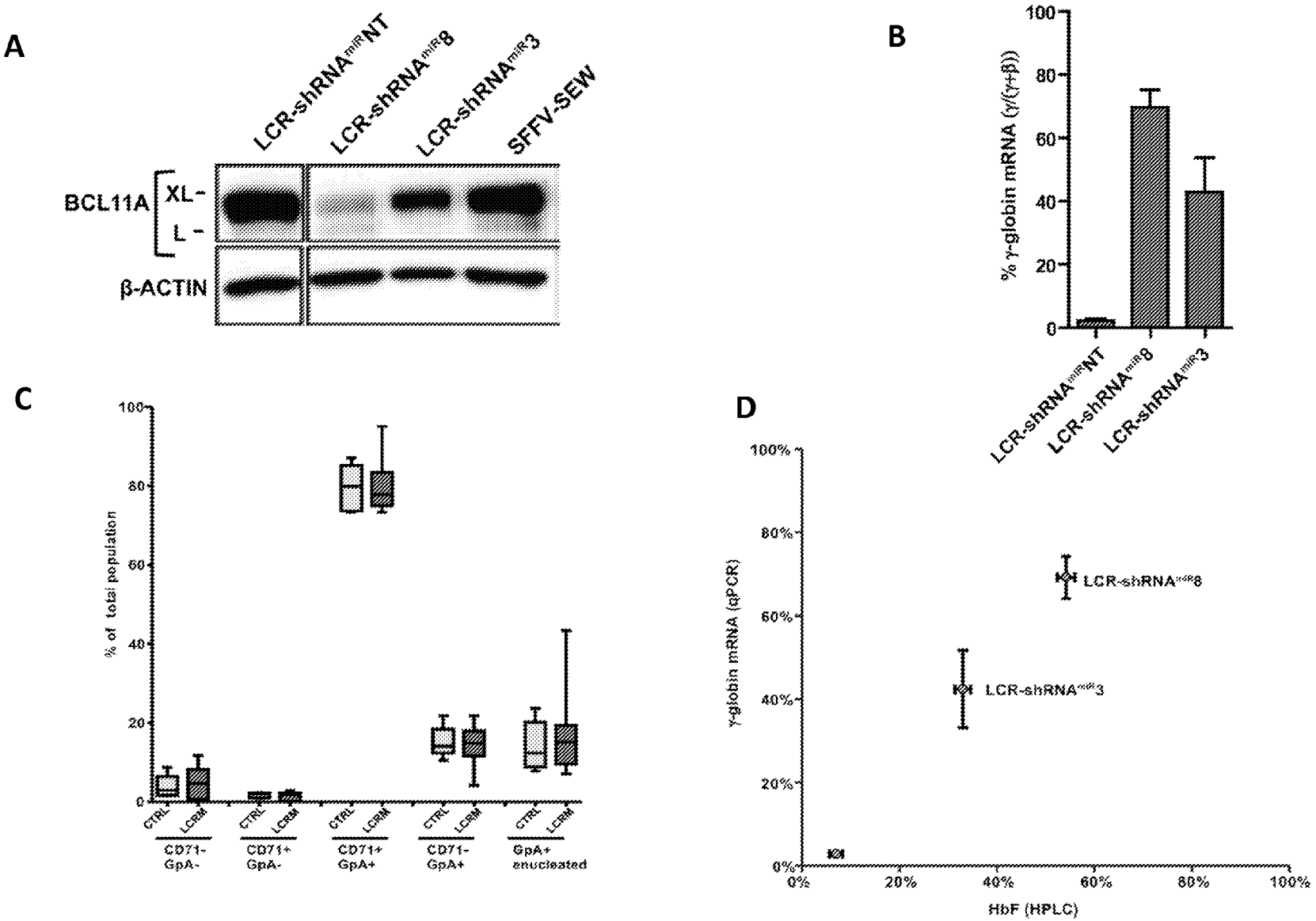
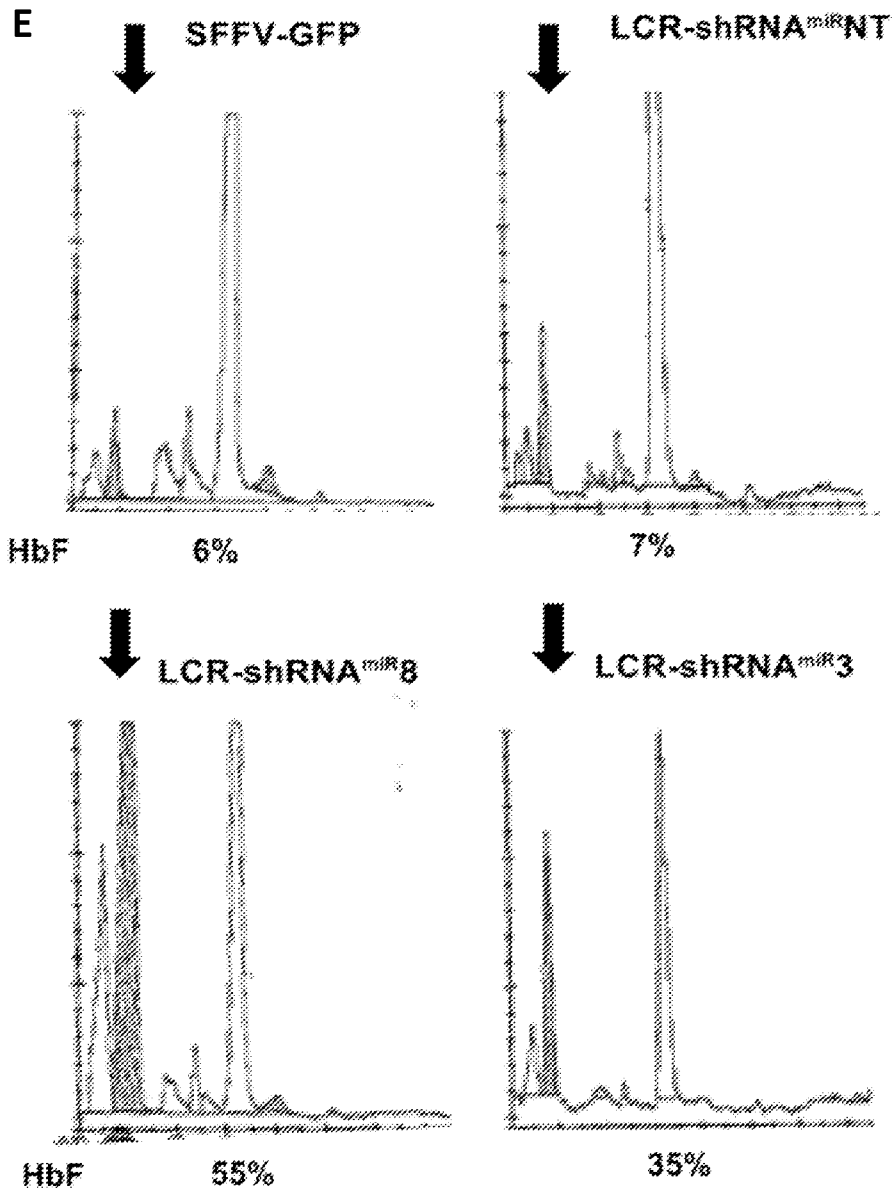

A
LCR-shRNA miRNT
LCR-shRNA miR8
LCR-shRNA miR3
SFFV-SEW
BCL11A XL-
L-
β-ACTIN
B
% γ-globin mRNA (γ/(γ+β))
100
80
60
40
20
0
LCR-shRNA miRNT
LCR-shRNA miR8
LCR-shRNA miR3
C
% of total population
100
80
60
40
20
0
CTRL
LCRM
CD71- GpA-
CD71+ GpA-
CD71+ GpA+
CD71- GpA+
GpA+ enucleated
D
γ-globin mRNA (qPCR)
100%
80%
60%
40%
20%
0%
LCR-shRNA miR8
LCR-shRNA miR3
0%
20%
40%
60%
80%
100%
HbF (HPLC)

FIG. 24


E
SFFV-GFP
LCR-shRNA^{miR}NT
HbF
6%
7%
LCR-shRNA^{miR}8
LCR-shRNA^{miR}3
HbF
55%
35%

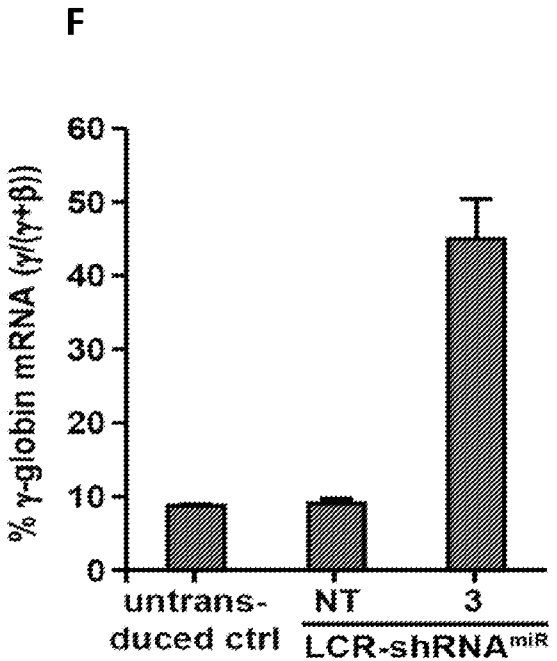

F
% γ-globin mRNA (γ/(γ+β))
60
50
40
30
20
10
0
untrans-duced ctrl
NT
3
LCR-shRNA^{miR}

FIG. 24

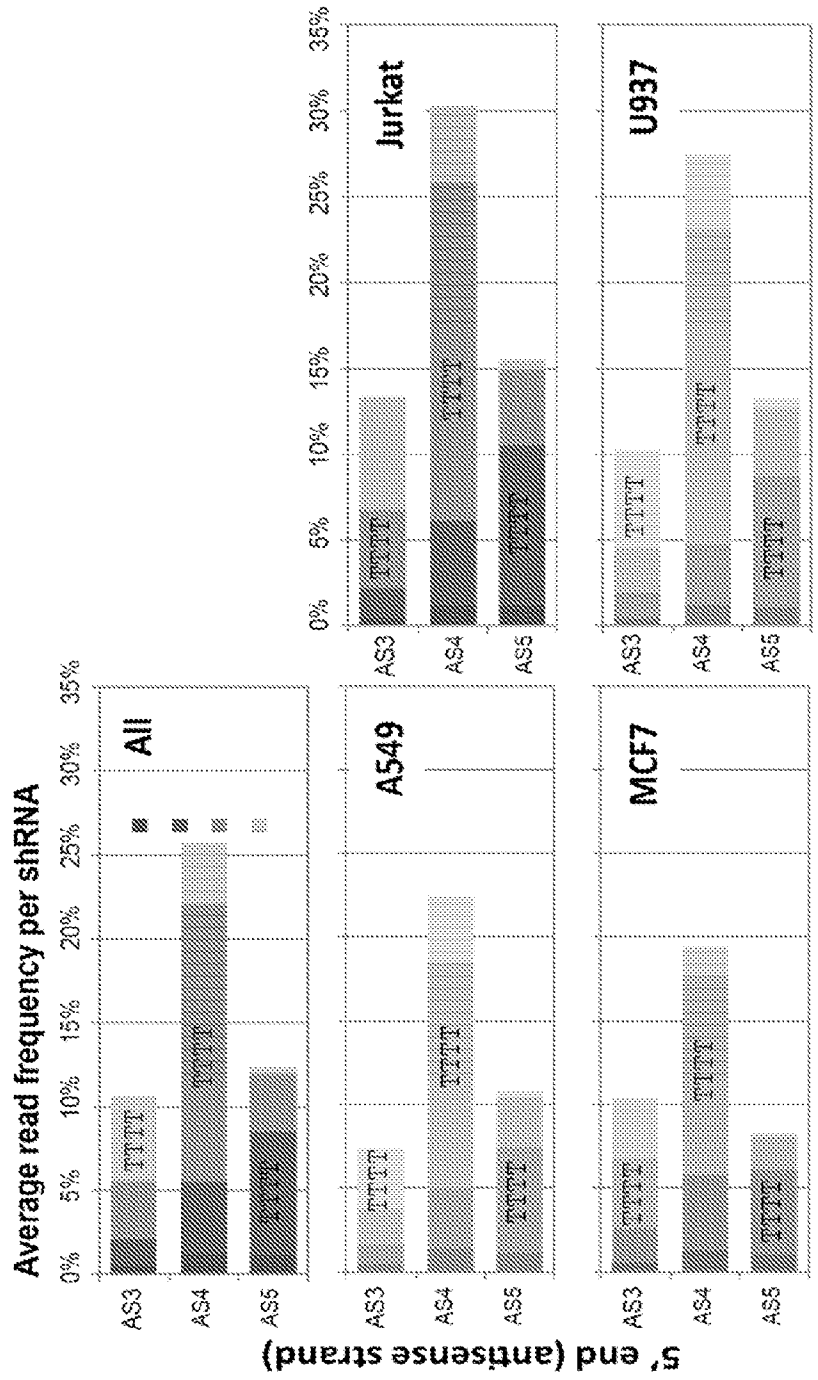

Average read frequency per shRNA
0%
5%
10%
15%
20%
25%
30%
35%
All
A549
MCF7
Jurkat
U937
AS3
AS4
AS5
TTTT
5' end (antisense strand)

FIG. 25

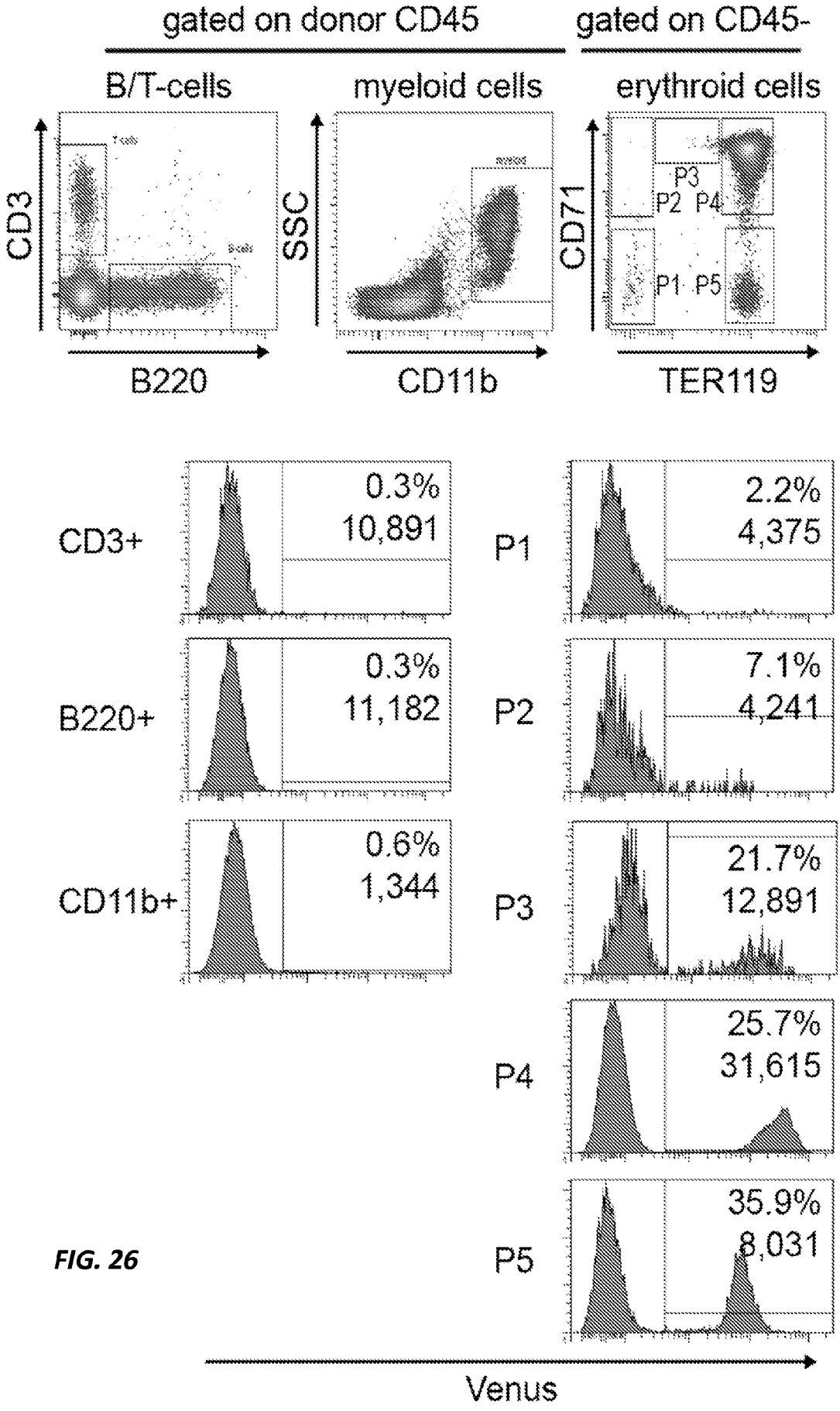

gated on donor CD45
gated on CD45-
B/T-cells
myeloid cells
erythroid cells
CD3
B220
SSC
CD11b
CD71
TER119
P1
P2
P3
P4
P5
CD3+
0.3%
10,891
B220+
0.3%
11,182
CD11b+
0.6%
1,344
2.2%
4,375
7.1%
4,241
21.7%
12,891
25.7%
31,615
35.9%
8,031
Venus

FIG. 26

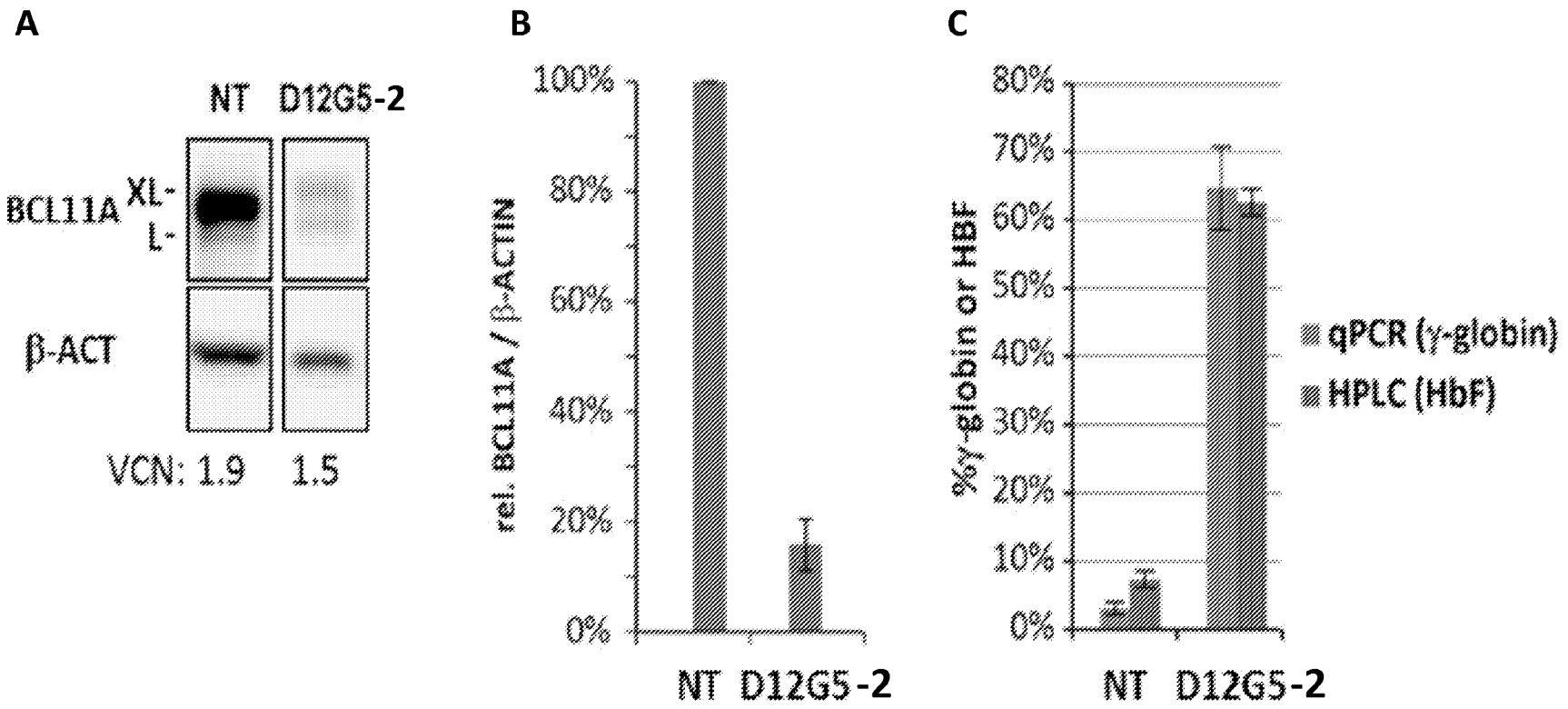

A
NT
D12G5-2
BCL11A XL-
L-
β-ACT
VCN: 1.9
1.5
B
rel. BCL11A / β-ACTIN
100%
80%
60%
40%
20%
0%
NT
D12G5-2
C
%γ-globin or HBF
80%
70%
60%
50%
40%
30%
20%
10%
0%
qPCR (γ-globin)
HPLC (HbF)
NT
D12G5-2

FIG. 27

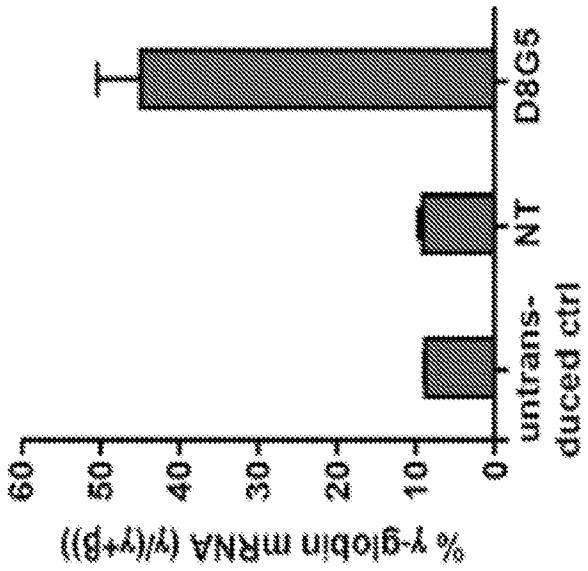


FIG. 28

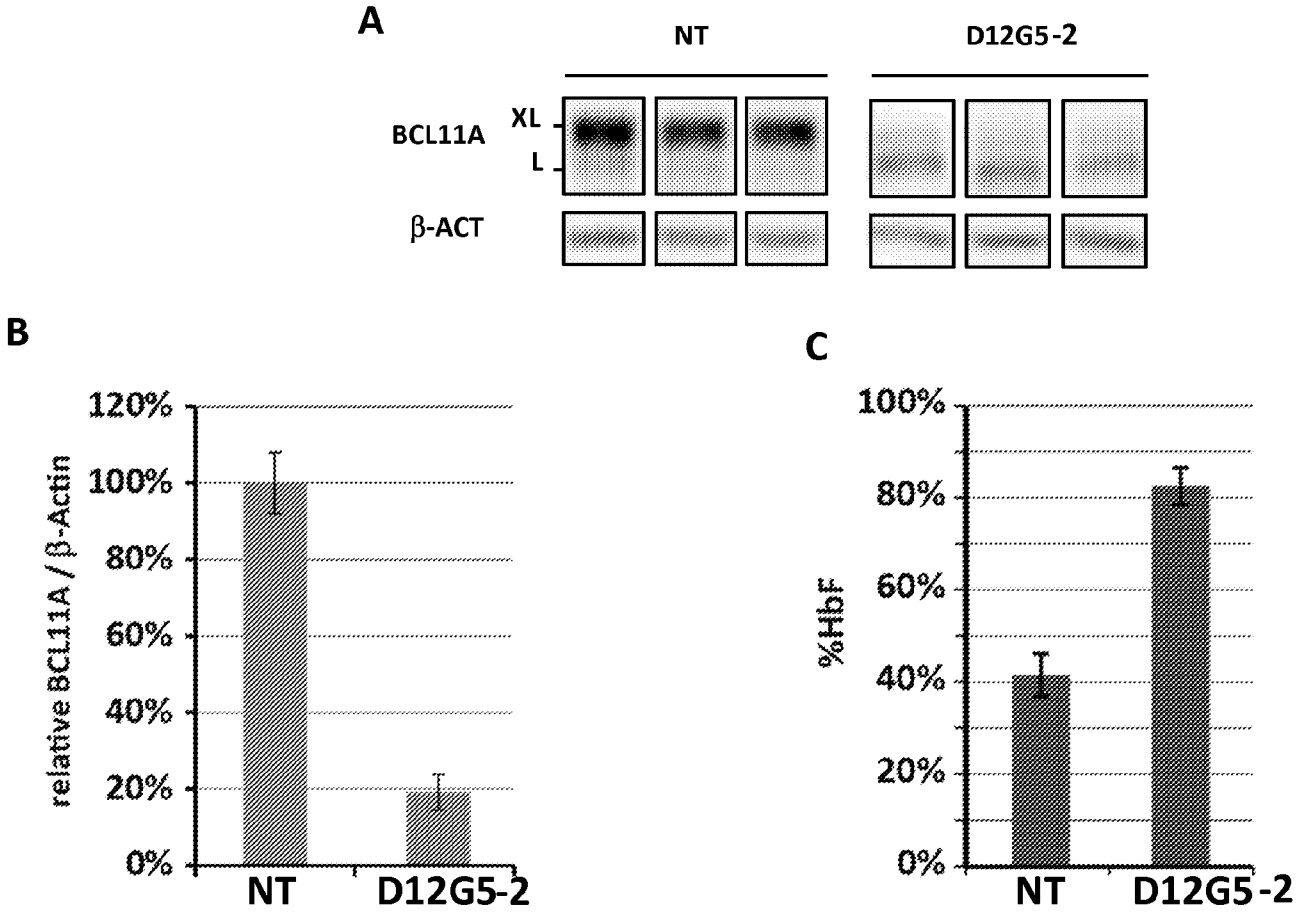

A
NT
D12G5-2
BCL11A
XL
L
β-ACT
B
relative BCL11A / β-Actin
120%
100%
80%
60%
40%
20%
0%
NT
D12G5-2
C
%HbF
100%
80%
60%
40%
20%
0%
NT
D12G5-2

FIG. 29

BCL11A knockdown with sequences in both SFFV and LCR backbones in CD34 differentiated erythroid cells.

ShRNA	SEQUENCE	
Mir1	CGCACAGAACACTCATGGATTctccatgtggtagagAATCCATGAGTGTTCTGTGCG	SEQ. ID. NO: 25
E3G5	GCGCTCGGAGACTCCAGACAActccatgtggtagagTTGTCTGGAGTCTCCGAgcgc	SEQ. ID. NO: 35
D8G5	GCGCTTCTCTTGCAACACGCActccatgtggtagagTGCGTGTTGCAAGAGAAgcgc	SEQ. ID. NO: 37
C4G5	GCGCACAGTACCCTGGAGAAActccatgtggtagagTTTCTCCAGGGTACTGTgcgc	SEQ. ID. NO: 42
D12G5	GCGCGATCGAGTGTTGAATAActccatgtggtagagTTATTCAACACTCGATCgcgc	SEQ. ID. NO: 39
D12	GATCGAGTGTTGAATAATGATctccatgtggtagagATCATTATTCAACACTCGATC	SEQ. ID. NO: 30
A5	CAGTACCCTGGAGAAACACATctccatgtggtagagATGTGTTTCTCCAGGGTACTG	SEQ. ID. NO: 31
NT	CAACAAGATGAAGAGCACCAActccatgtggtagagTTGGTGCTCTTCATCTTGTTG	SEQ. ID. NO: 45

FIG. 30

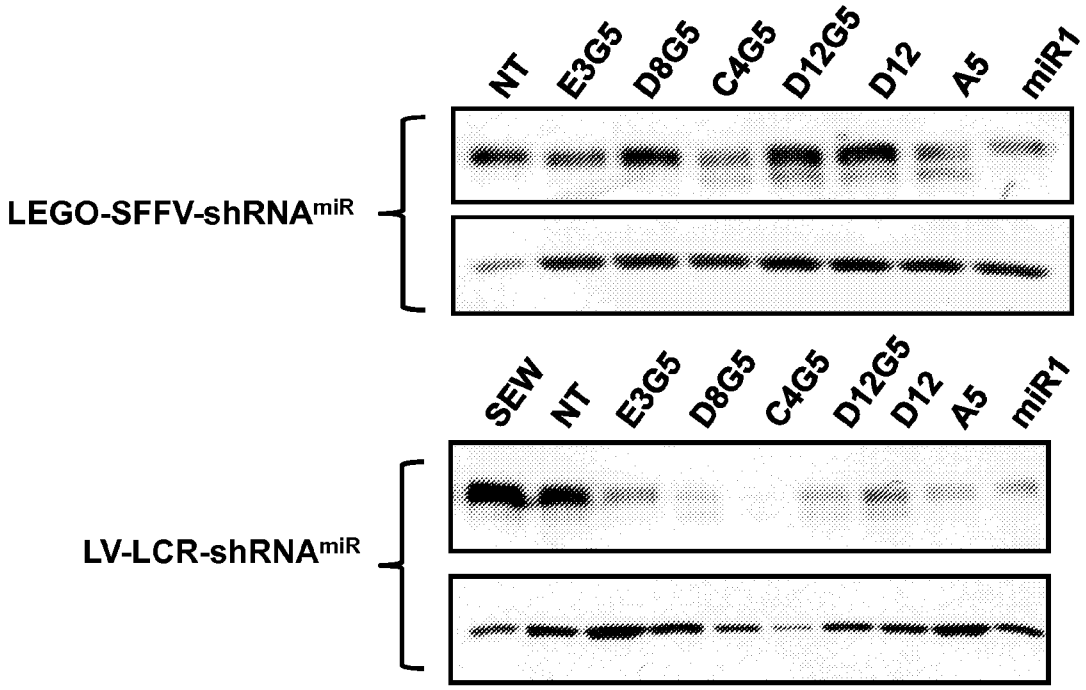


FIG. 31