FIG. 1

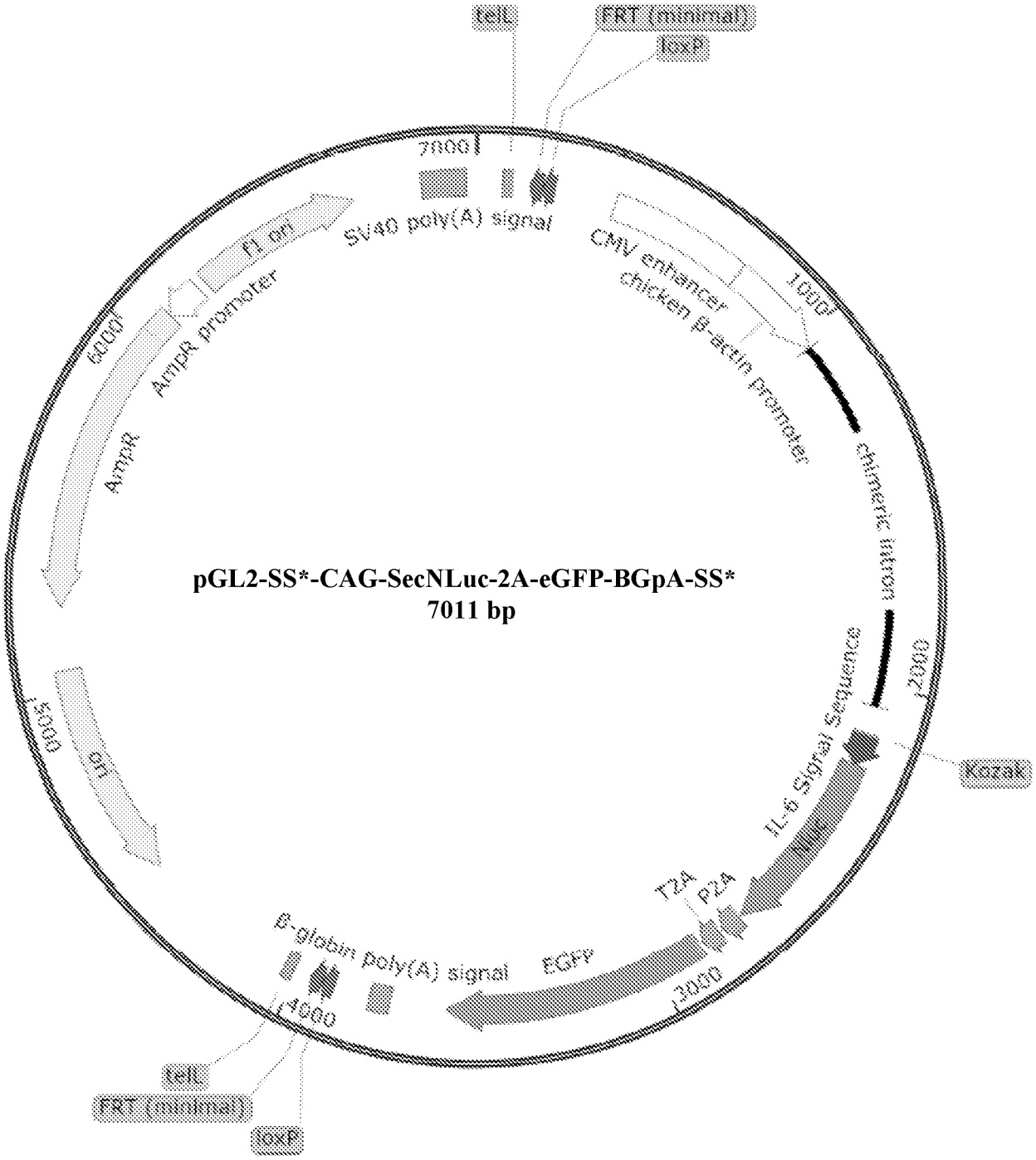

pGL2-SS*-CAG-SecNLuc-2A-eGFP-BGpA-SS*
7011 bp
7000
1000
2000
3000
4000
5000
6000
f1 ori
AmpR promoter
AmpR
ori
SV40 poly(A) signal
CMV enhancer
chicken β-actin promoter
chimeric intron
Kozak
IL-6 Signal Sequence
T2A
P2A
EGFP
β-globin poly(A) signal
loxP
FRT (minimal)
loxP
FRT (minimal)
loxP

FIG. 2

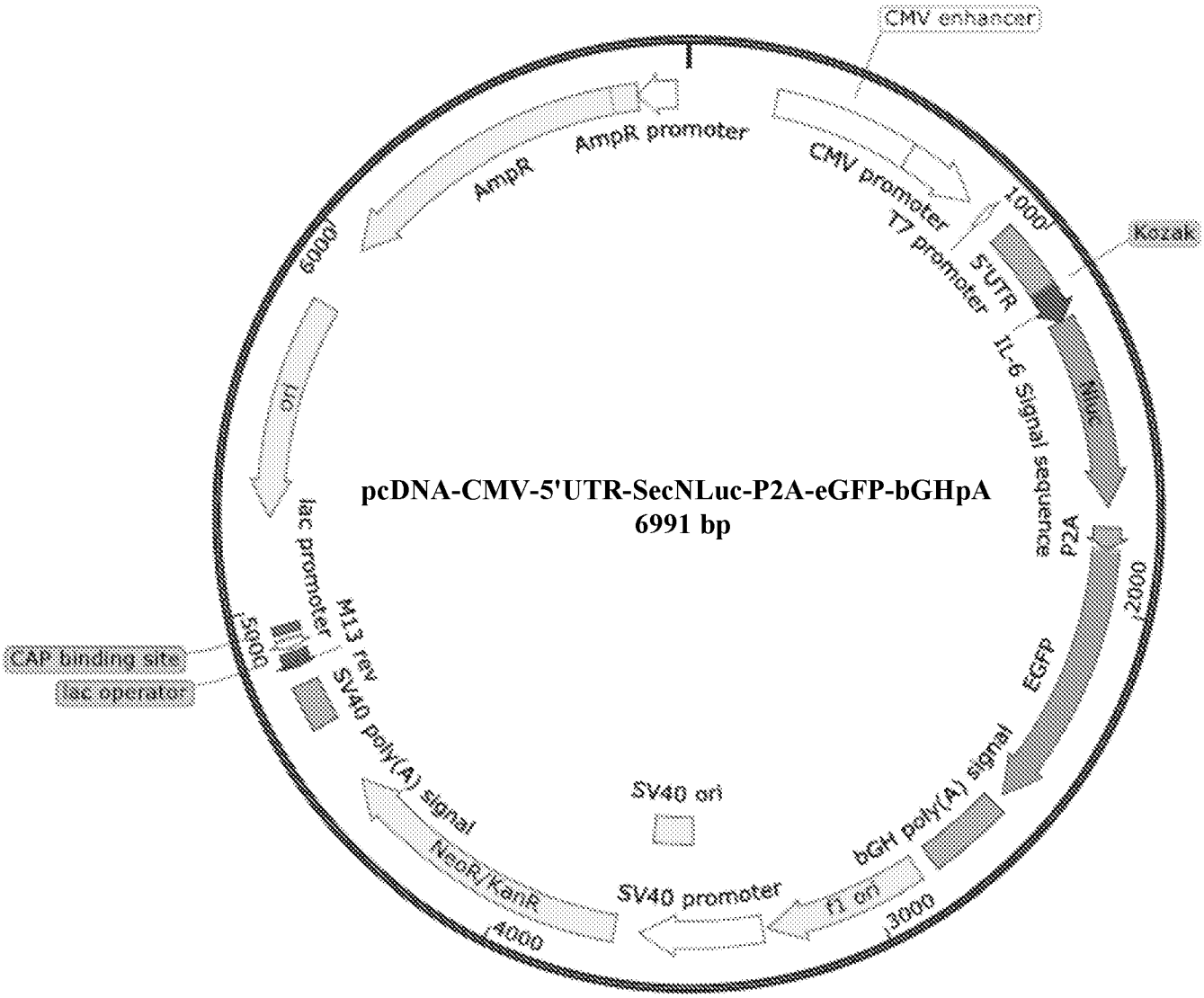

CMV enhancer
AmpR promoter
AmpR
CMV promoter
T7 promoter
1000
5'UTR
Kozak
IL-6 Signal sequence
P2A
2000
eGFP
bGH poly(A) signal
f1 ori
3000
SV40 promoter
NeoR/KanR
4000
SV40 ori
SV40 poly(A) signal
M13 rev
lac promoter
5000
CAP binding site
lac operator
ori
6000
pcDNA-CMV-5'UTR-SecNLuc-P2A-eGFP-bGHpA
6991 bp

FIG. 3

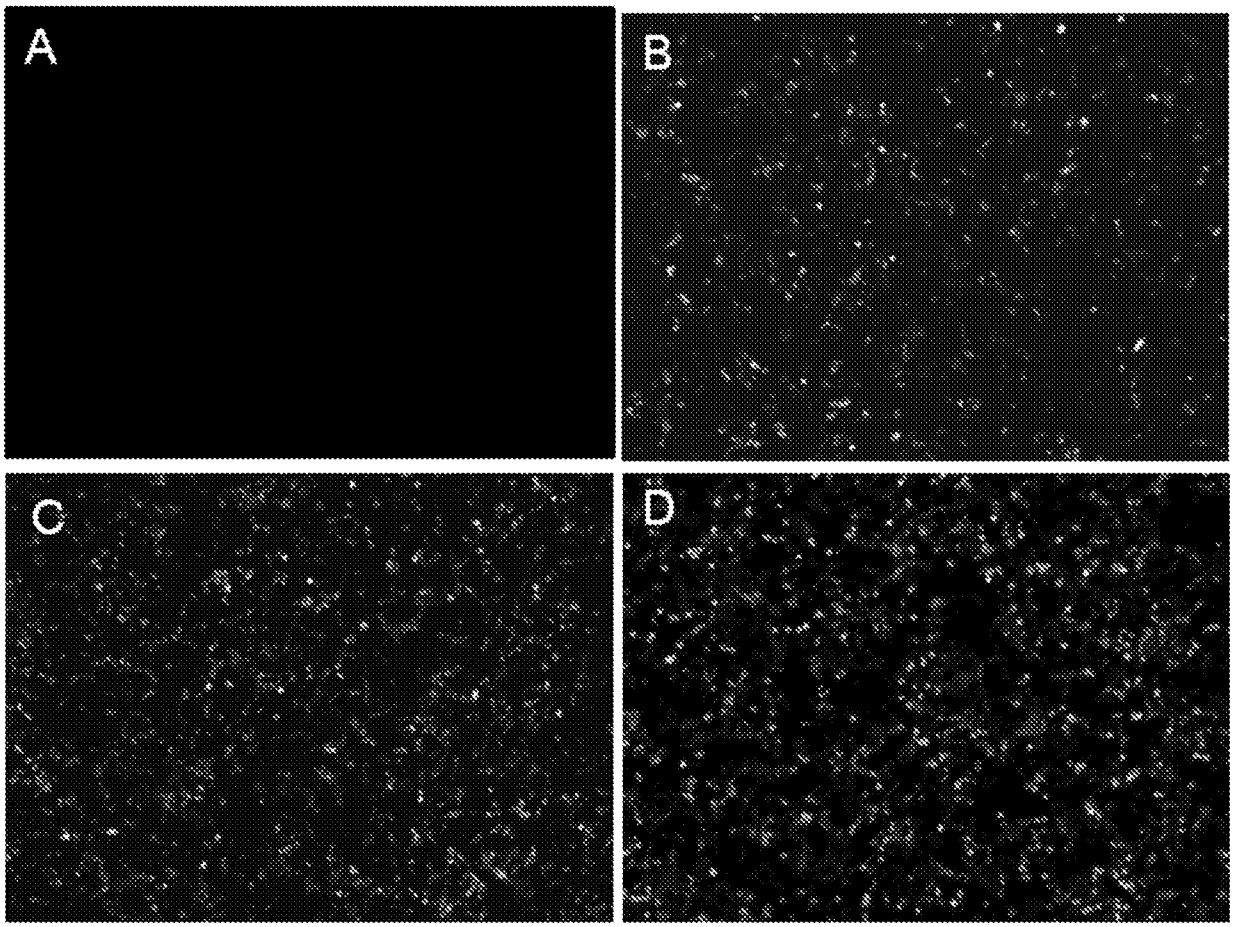


FIG. 4

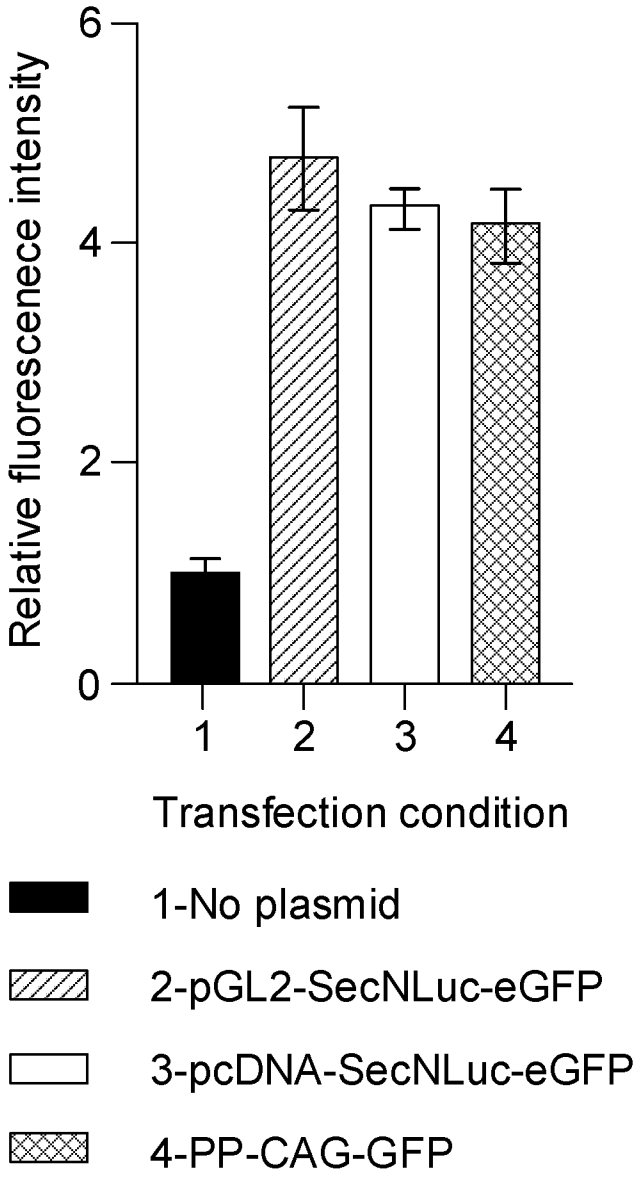

Relative fluorescenece intensity
6
4
2
0
1
2
3
4
Transfection condition
1-No plasmid
2-pGL2-SecNLuc-eGFP
3-pcDNA-SecNLuc-eGFP
4-PP-CAG-GFP

FIG. 5

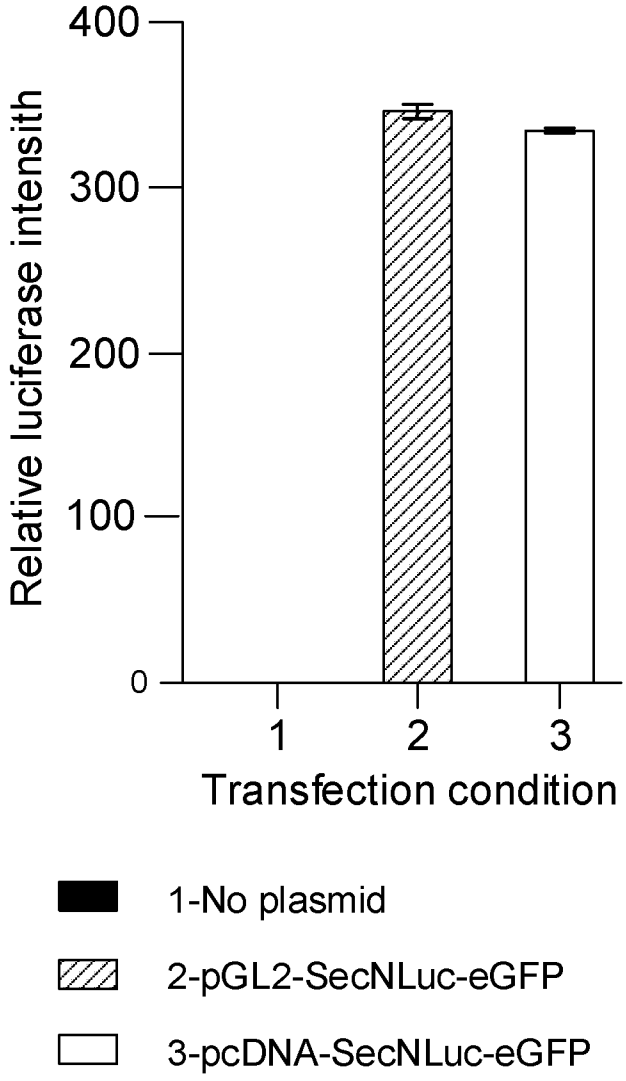

400
300
200
100
0
Relative luciferase intensith
1
2
3
Transfection condition
1-No plasmid
2-pGL2-SecNLuc-eGFP
3-pcDNA-SecNLuc-eGFP

FIG. 6

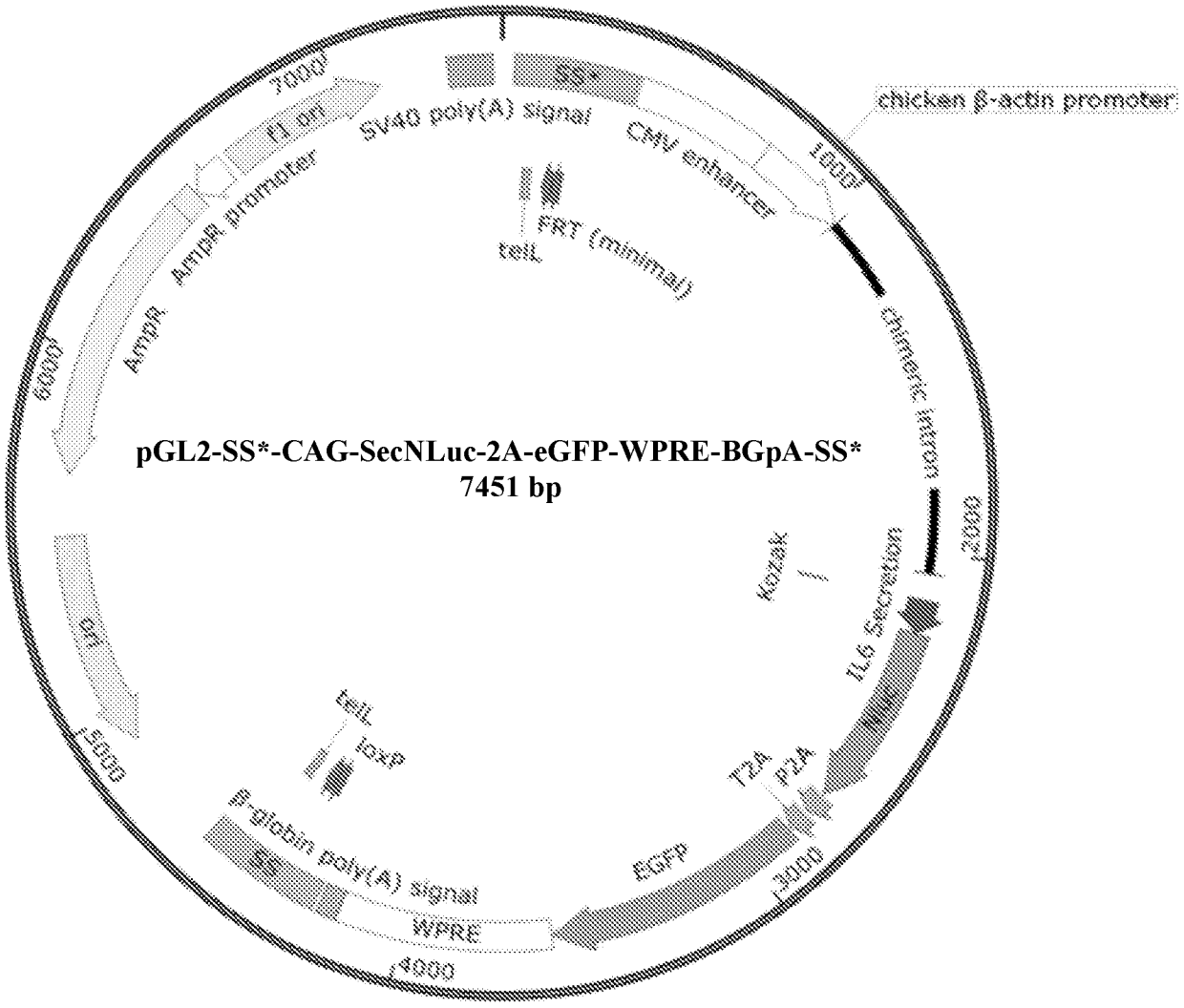

pGL2-SS*-CAG-SecNLuc-2A-eGFP-WPRE-BGpA-SS*
7451 bp
SS*
SV40 poly(A) signal
CMV enhancer
chicken β-actin promoter
chimeric intron
IL6 Secretion
Kozak
T2A
P2A
EGFP
WPRE
β-globin poly(A) signal
SS
loxP
FRT (minimal)
f1 ori
AmpR promoter
AmpR
ori
1000
2000
3000
4000
5000
6000
7000

FIG. 7

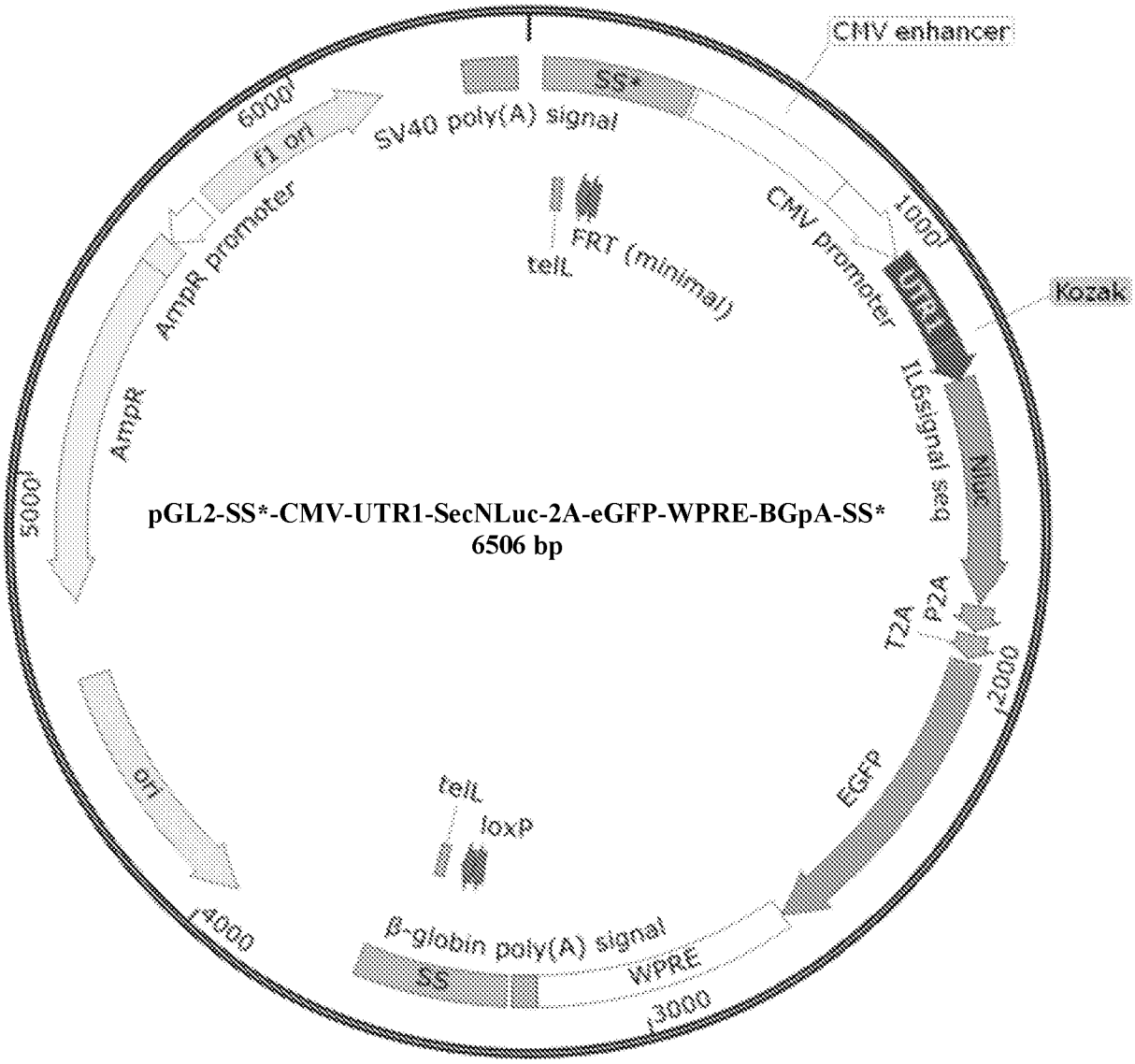

CMV enhancer
SS*
SV40 poly(A) signal
6000
f1 ori
CMV promoter
1000
FRT (minimal)
telL
AmpR promoter
Kozak
AmpR
IL6signal seq
5000
pGL2-SS*-CMV-UTR1-SecNLuc-2A-eGFP-WPRE-BGpA-SS*
6506 bp
T2A
P2A
2000
ori
telL
loxP
EGFP
4000
β-globin poly(A) signal
SS
WPRE
3000

FIG. 8

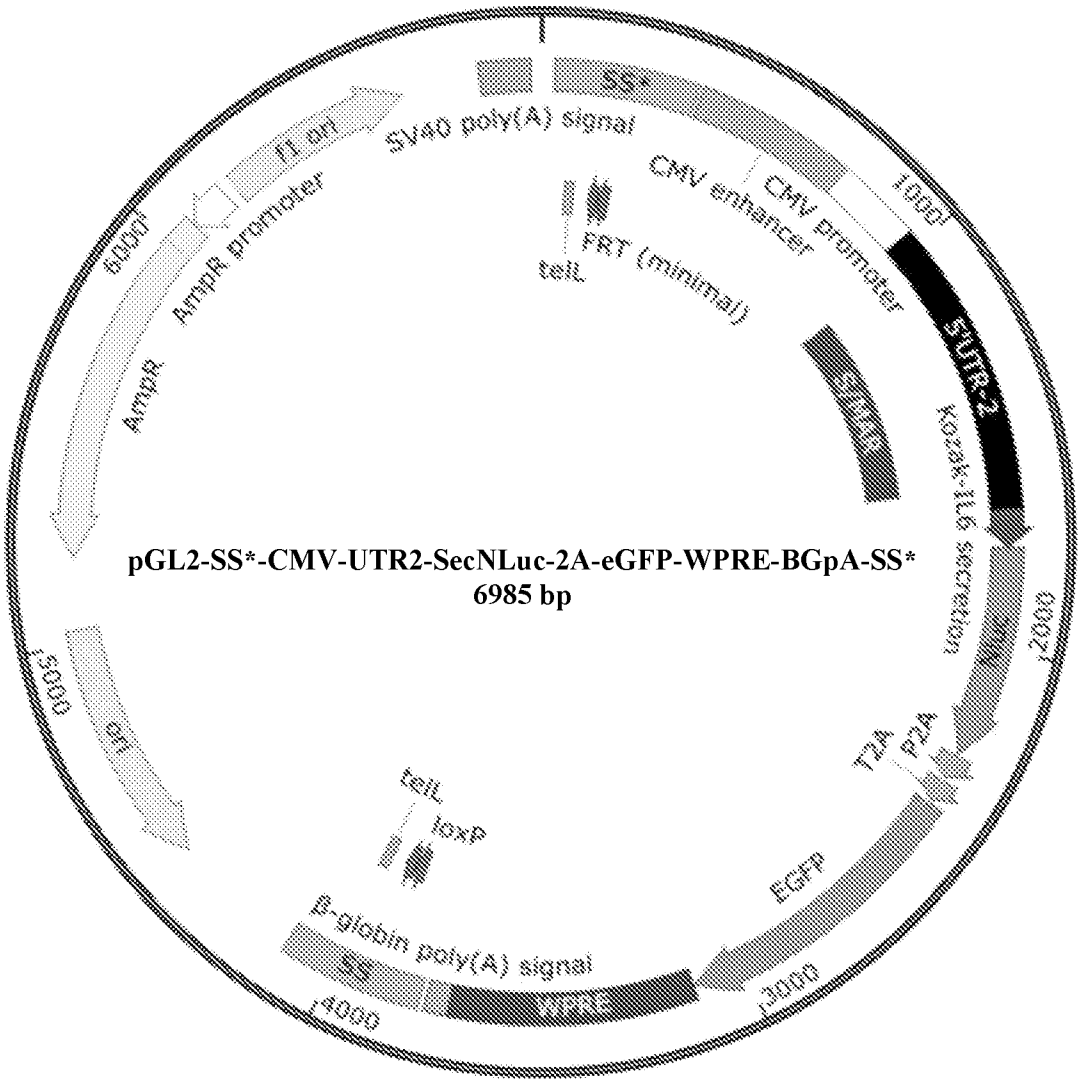

SS*
SV40 poly(A) signal
f1 ori
CMV enhancer
CMV promoter
1000
5'UTR-2
Kozak-IL6 secretion
2000
T2A
P2A
EGFP
3000
WPRE
4000
β-globin poly(A) signal
SS*
5000
ori
loxP
FRT (minimal)
6000
AmpR promoter
AmpR
pGL2-SS*-CMV-UTR2-SecNLuc-2A-eGFP-WPRE-BGpA-SS*
6985 bp

FIG. 9

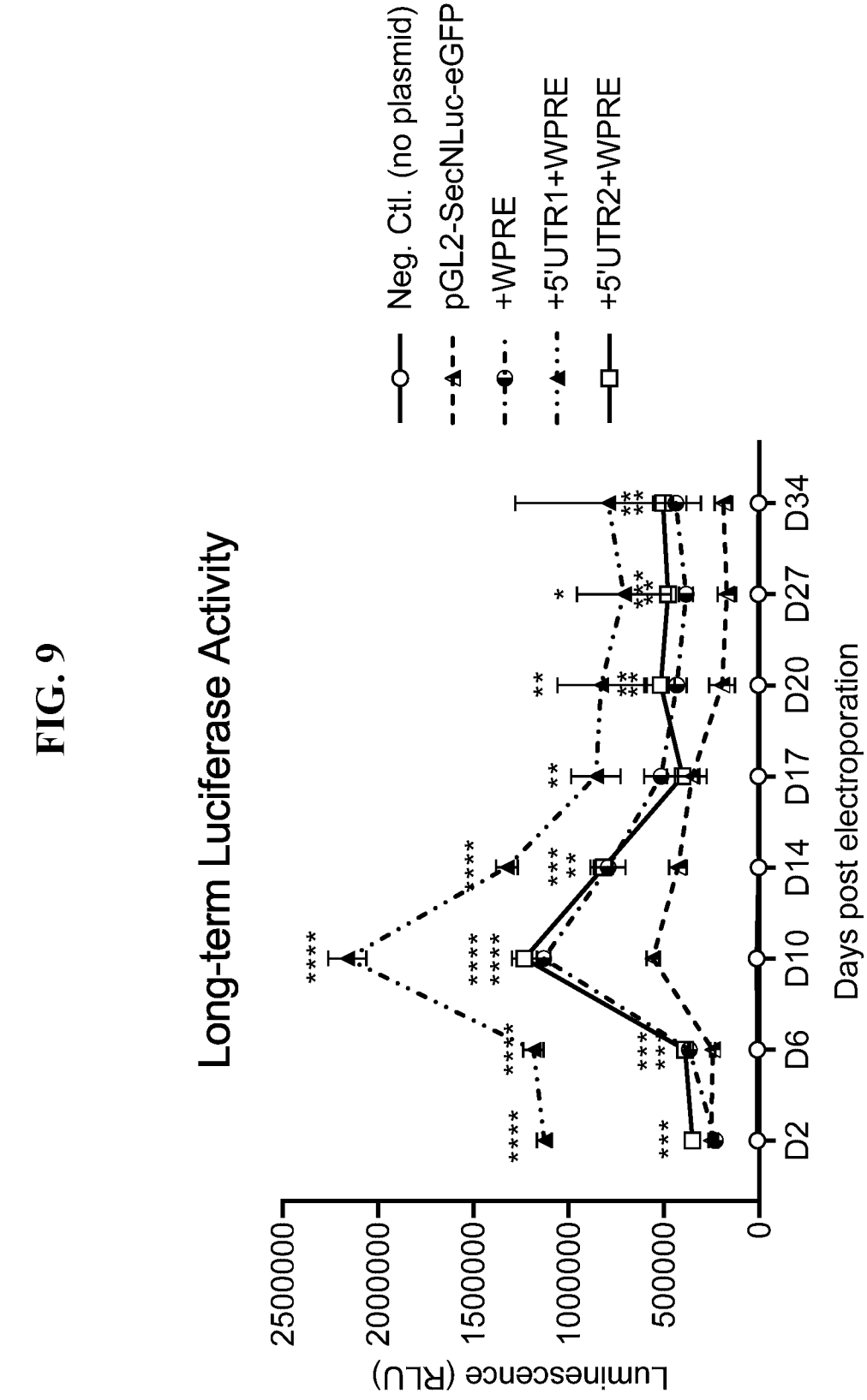

Long-term Luciferase Activity
Neg. Ctl. (no plasmid)
pGL2-SecNLuc-eGFP
+WPRE
+5'UTR1+WPRE
+5'UTR2+WPRE
Luminescence (RLU)
2500000
2000000
1500000
1000000
500000
0
D2
D6
D10
D14
D17
D20
D27
D34
Days post electroporation

FIG. 10

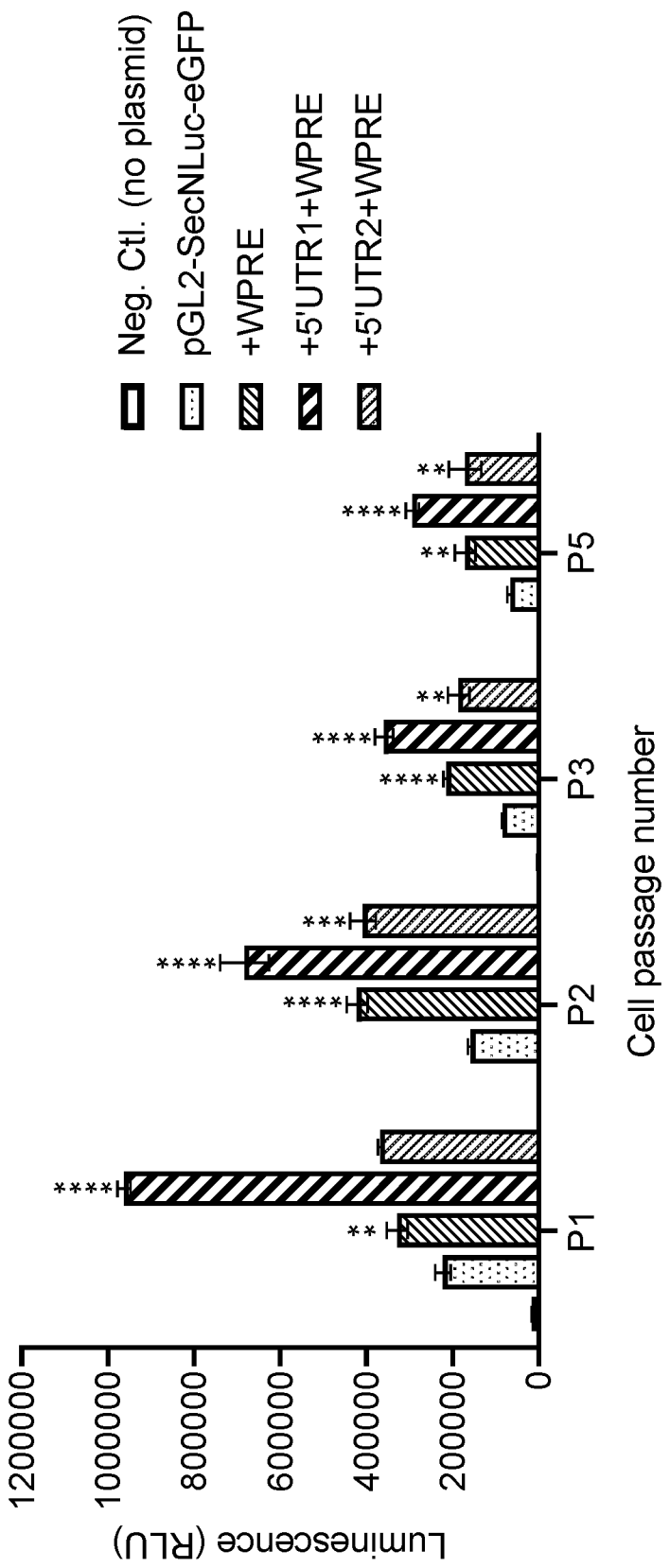

Neg. Ctl. (no plasmid)
pGL2-SecNLuc-eGFP
+WPRE
+5'UTR1+WPRE
+5'UTR2+WPRE
Luminescence (RLU)
0
200000
400000
600000
800000
1000000
1200000
P1
P2
P3
P5
Cell passage number
**
****
***

FIG. 11

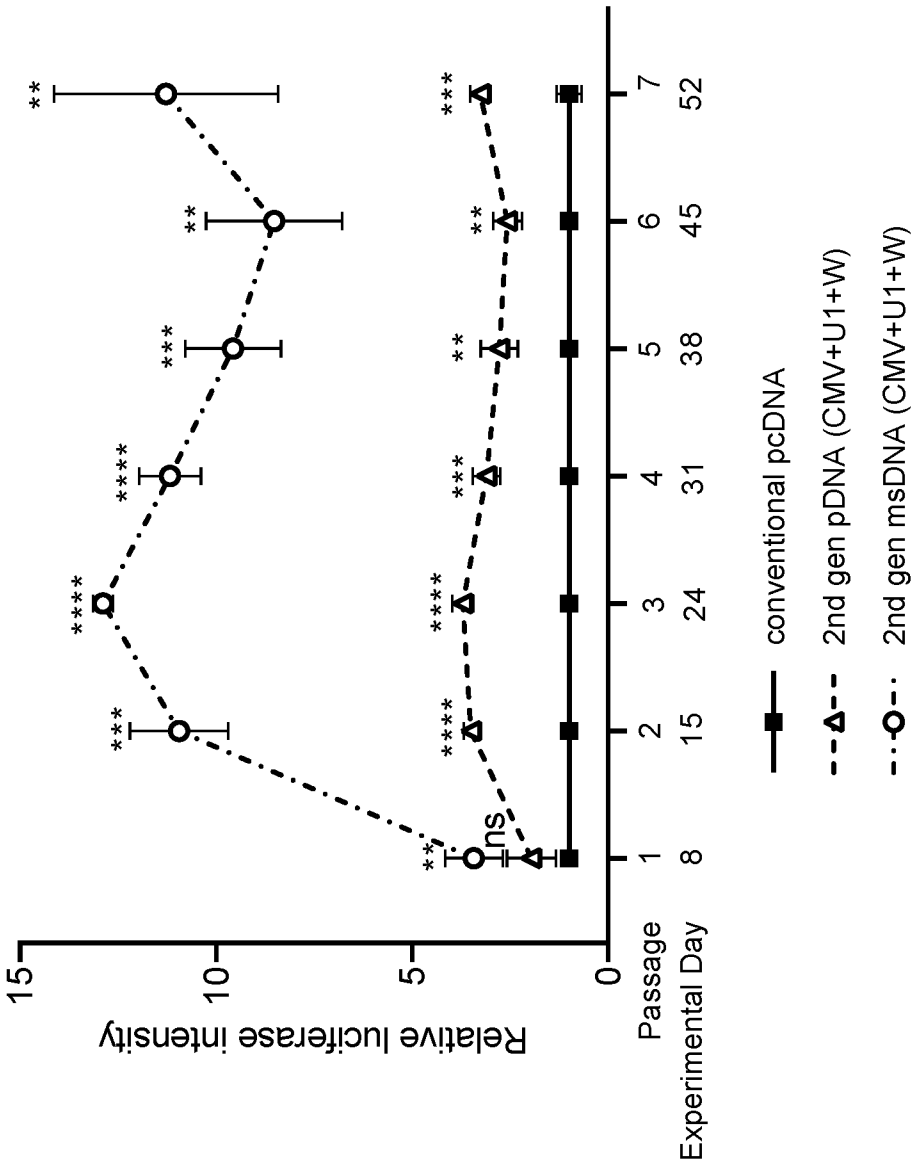

Relative luciferase intensity
15
10
5
0
Passage
Experimental Day
1
2
3
4
5
6
7
8
15
24
31
38
45
52
conventional pcDNA
2nd gen pDNA (CMV+U1+W)
2nd gen msDNA (CMV+U1+W)
ns
**
***
****

FIG. 12

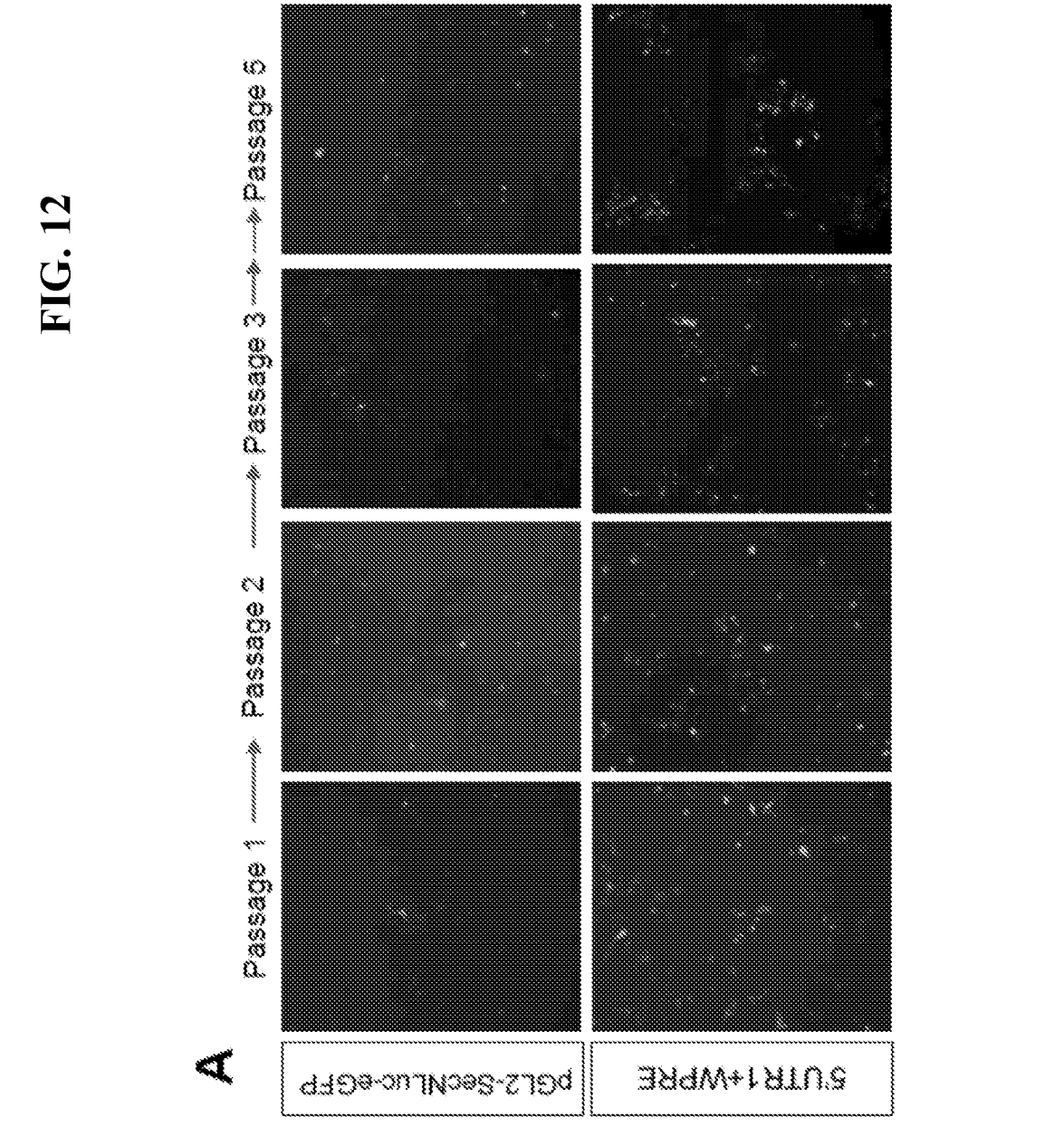

A
Passage 1
Passage 2
Passage 3
Passage 6
pGL2-SecNLuc-eGFP
5'UTR1+WPRE
B
GFP+ cells in field of view
400
300
200
100
0
ns
ns
ns
P1
P2
P3
P5
Cell Passage Number
pGL2-SecNLuc-eGFP
5'UTR1+WPRE
C
Mean Fluorescence Intensity
50000
40000
30000
20000
10000
0
P5
pGL2-SecNLuc-eGFP
5'UTR1+WPRE

FIG. 13

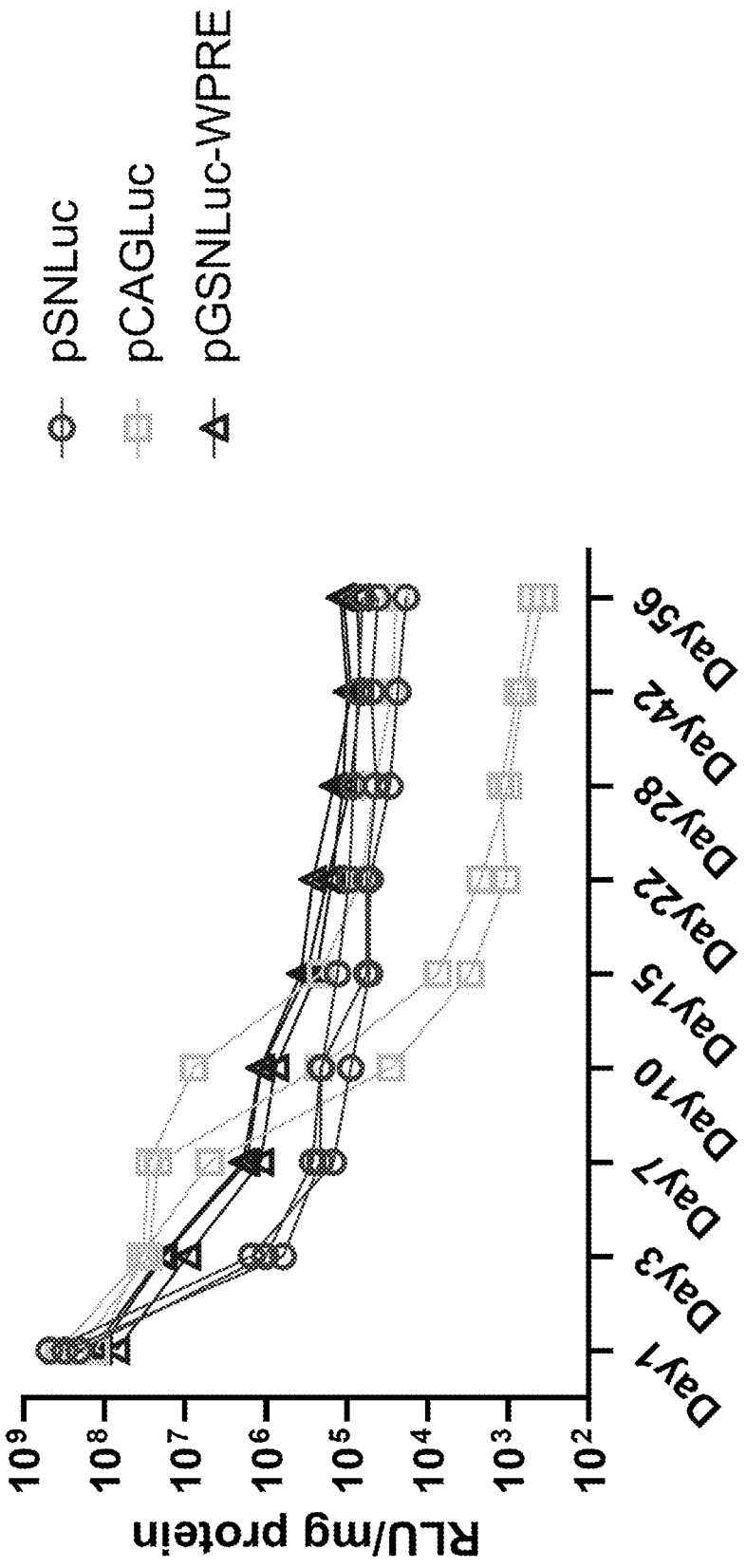

pSNLuc
pCAGLuc
pGSNLuc-WPRE
RLU/mg protein
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
Day1
Day3
Day7
Day10
Day15
Day22
Day28
Day42
Day56

FIG. 14

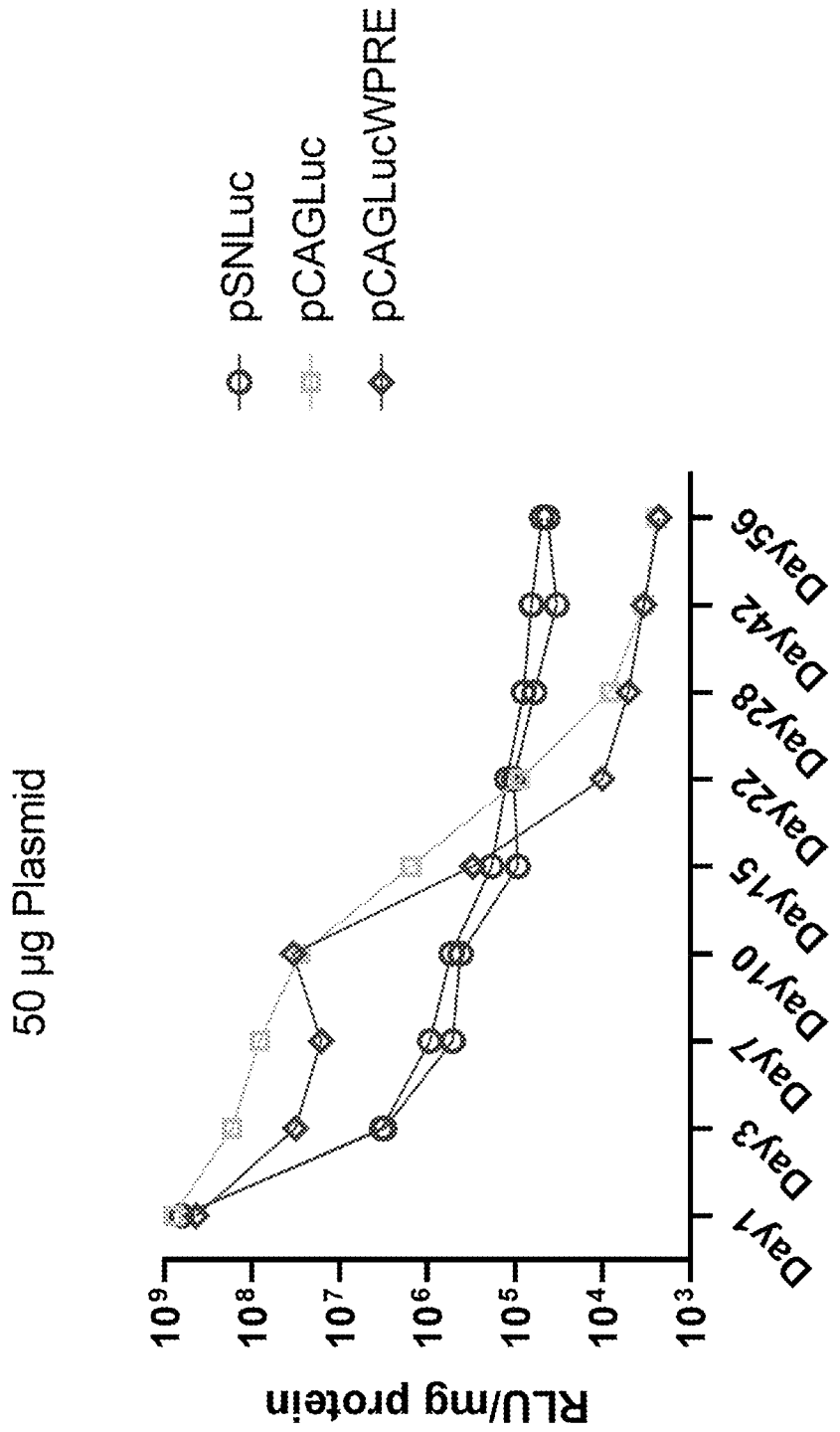

50 µg Plasmid
pSNLuc
pCAGLuc
pCAGLucWPRE
RLU/mg protein
10^9
10^8
10^7
10^6
10^5
10^4
10^3
Day1
Day3
Day7
Day10
Day15
Day22
Day28
Day42
Day56

FIG. 15

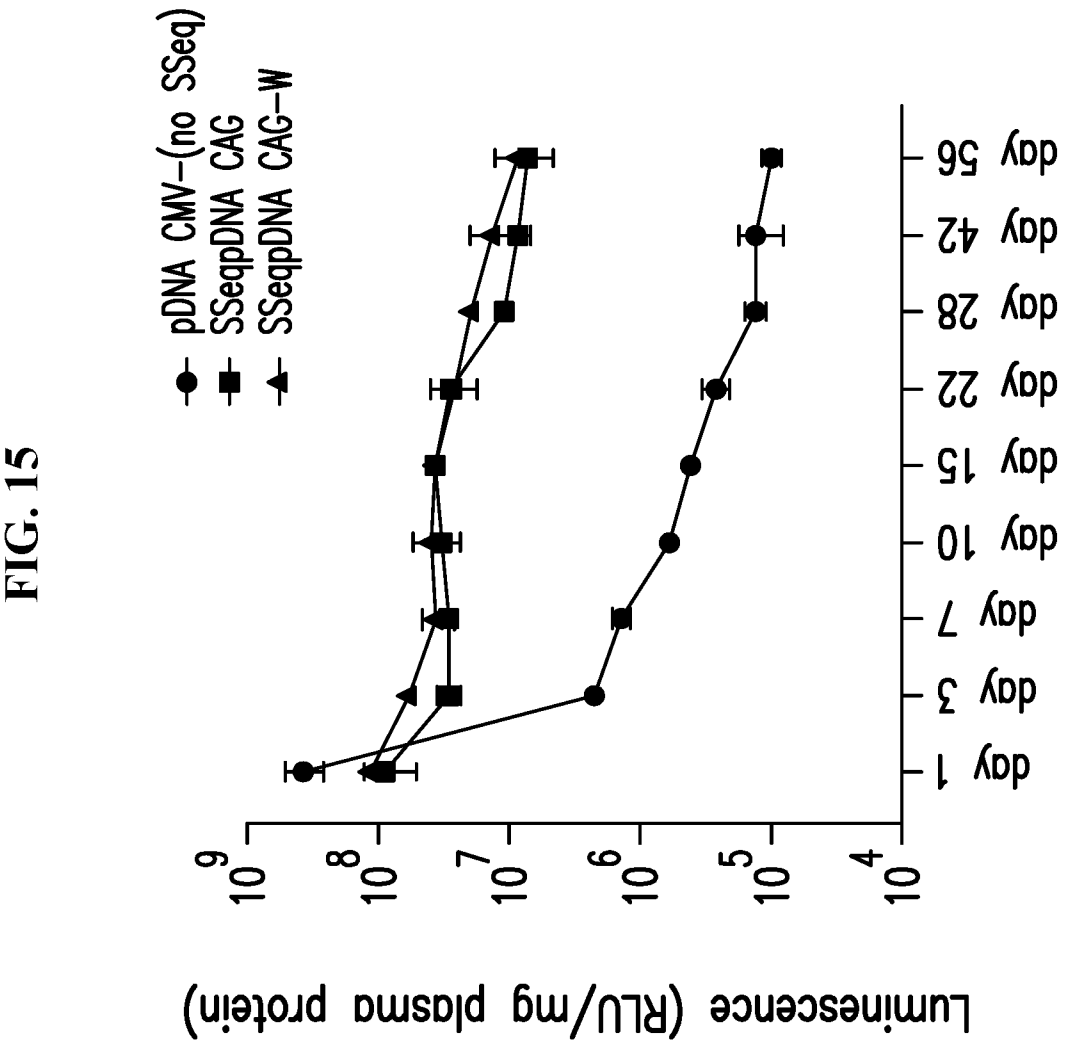

pDNA CMV-(no SSeq)
SSeqpDNA CAG
SSeqpDNA CAG-W
Luminescence (RLU/mg plasma protein)
10^9
10^8
10^7
10^6
10^5
10^4
day 1
day 3
day 7
day 10
day 15
day 22
day 28
day 42
day 56

FIG. 16

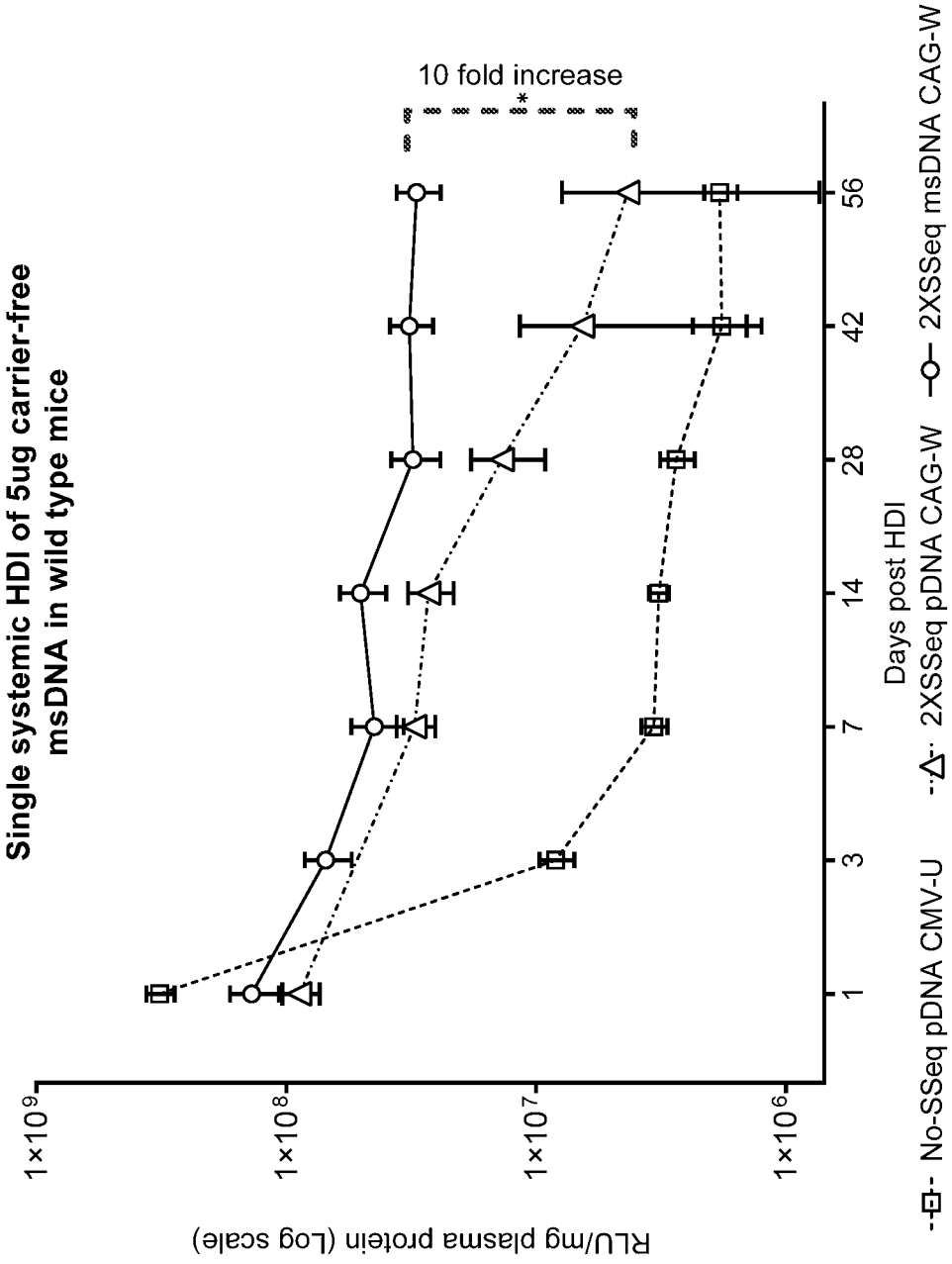

Single systemic HDI of 5ug carrier-free msDNA in wild type mice
10 fold increase
*
RLU/mg plasma protein (Log scale)
1×10^9
1×10^8
1×10^7
1×10^6
1
3
7
14
28
42
56
Days post HDI
No-SSeq pDNA CMV-U
2XSSeq pDNA CAG-W
2XSSeq msDNA CAG-W

FIG. 17

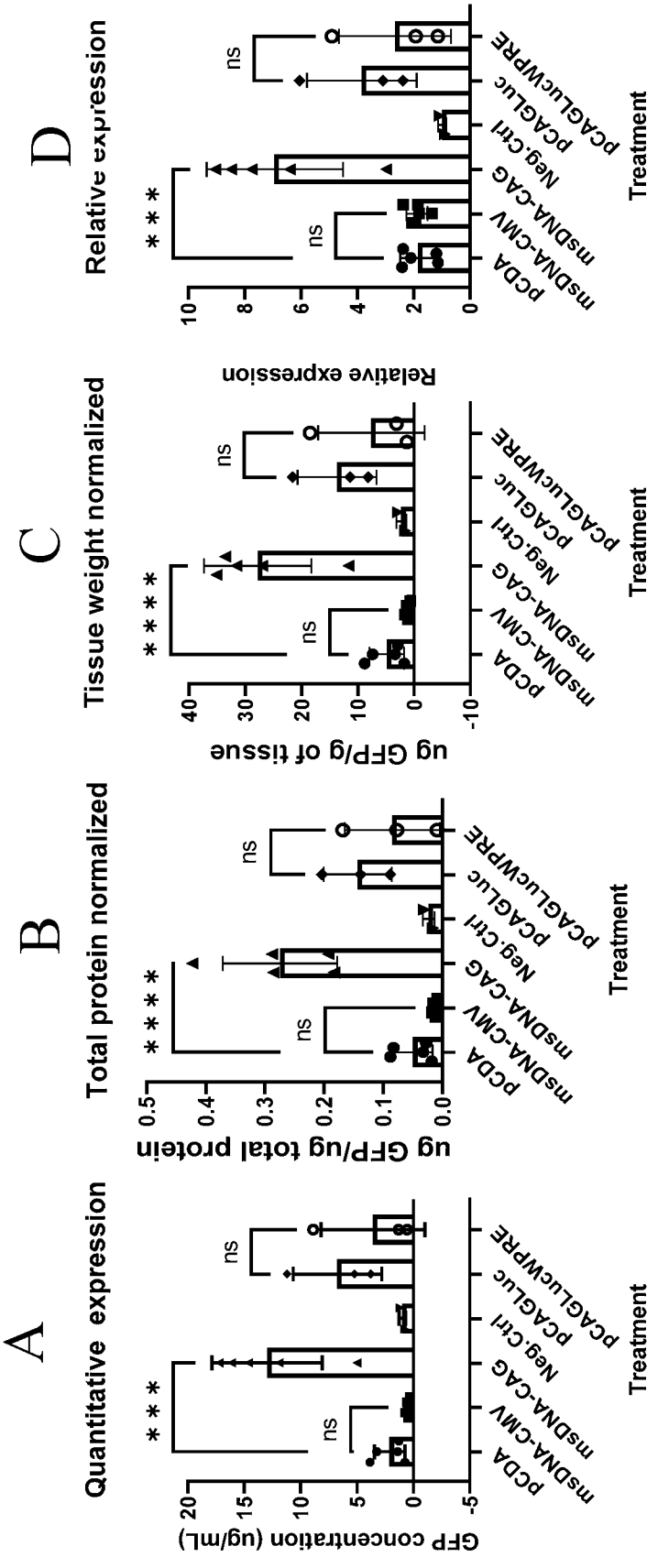

A
Quantitative expression
GFP concentration (ug/mL)
20
15
10
5
0
-5
****
ns
ns
pCDA
msDNA-CMV
msDNA-CAG
Neg.Ctrl
pCAGLuc
pCAGLucWPRE
Treatment
B
Total protein normalized
ug GFP/ug total protein
0.5
0.4
0.3
0.2
0.1
0.0
****
ns
ns
pCDA
msDNA-CMV
msDNA-CAG
Neg.Ctrl
pCAGLuc
pCAGLucWPRE
Treatment
C
Tissue weight normalized
Relative expression
ug GFP/g of tissue
40
30
20
10
0
-10
****
ns
ns
pCDA
msDNA-CMV
msDNA-CAG
Neg.Ctrl
pCAGLuc
pCAGLucWPRE
Treatment
D
Relative expression
10
8
6
4
2
0
****
ns
ns
pCDA
msDNA-CMV
msDNA-CAG
Neg.Ctrl
pCAGLuc
pCAGLucWPRE
Treatment

FIG. 18

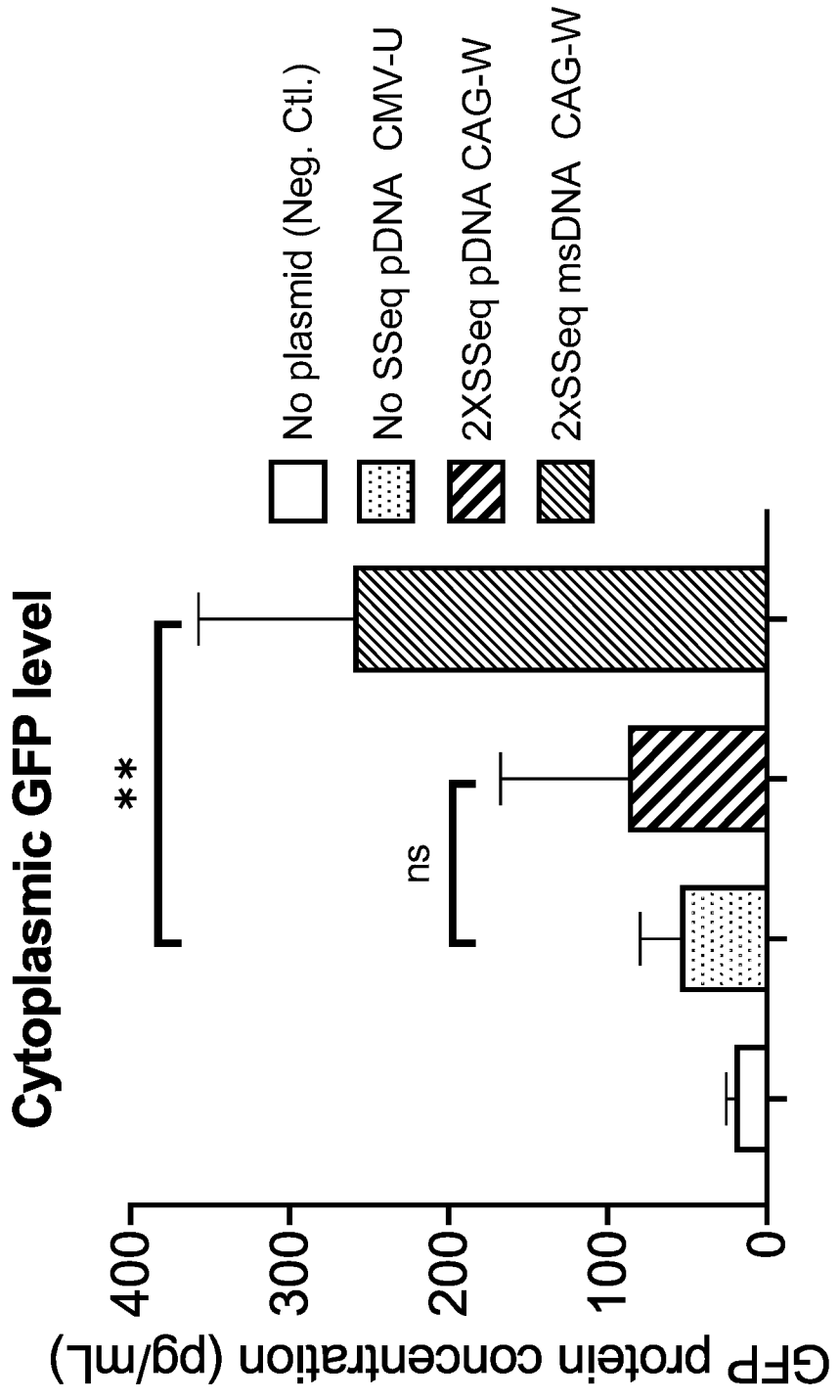

Cytoplasmic GFP level
GFP protein concentration (pg/mL)
0
100
200
300
400
**
ns
No plasmid (Neg. Ctl.)
No SSeq pDNA CMV-U
2XSSeq pDNA CAG-W
2xSSeq msDNA CAG-W

FIG. 19

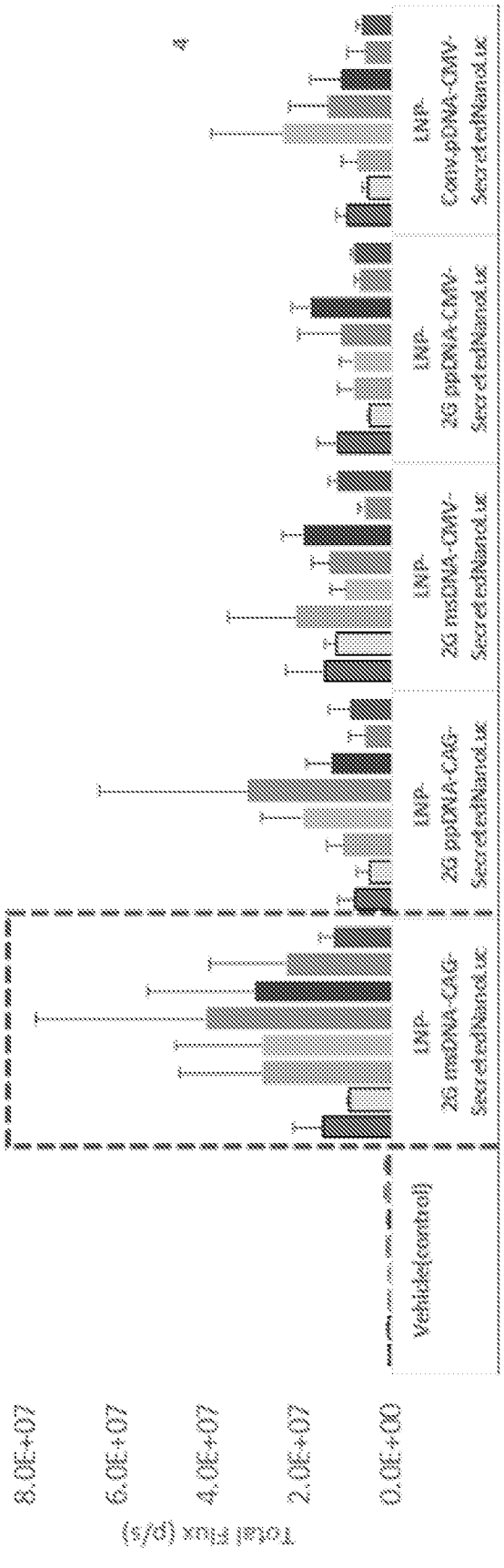

Total Flux (p/s)
8.0E+07
6.0E+07
4.0E+07
2.0E+07
0.0E+00
Vehicle(control)
LNP-2G msDNA-CAG-SecretedNanoLuc
LNP-2G ppDNA-CAG-SecretedNanoLuc
LNP-2G msDNA-CMV-SecretedNanoLuc
LNP-2G ppDNA-CMV-SecretedNanoLuc
LNP-Conv.pDNA-CMV-SecretedNanoLuc
4

FIG. 20

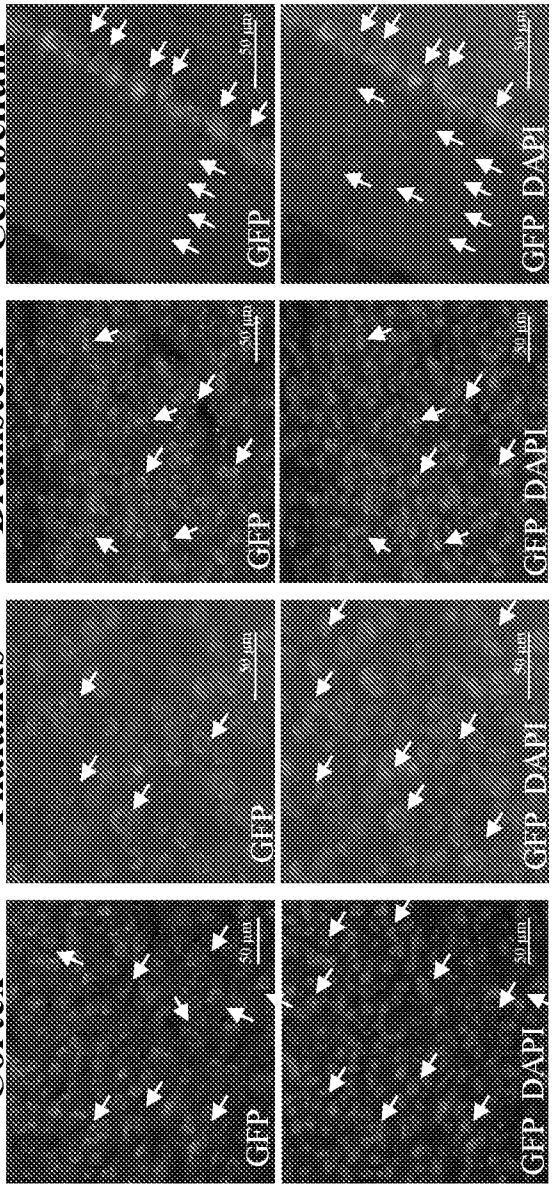


FIG. 21

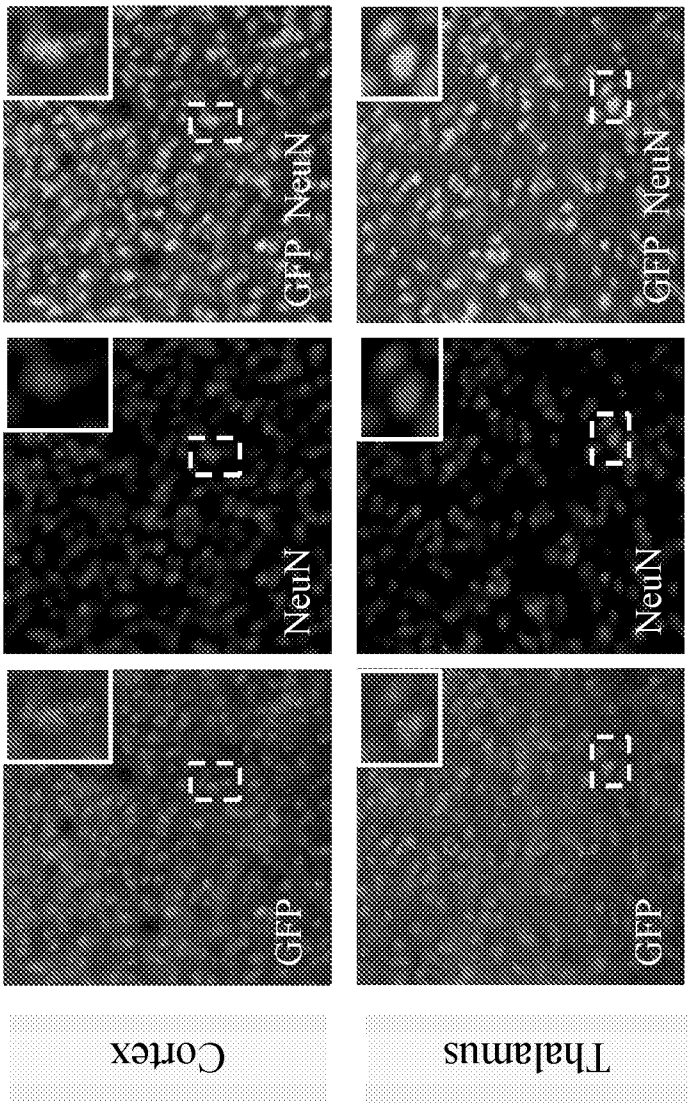


FIG. 22

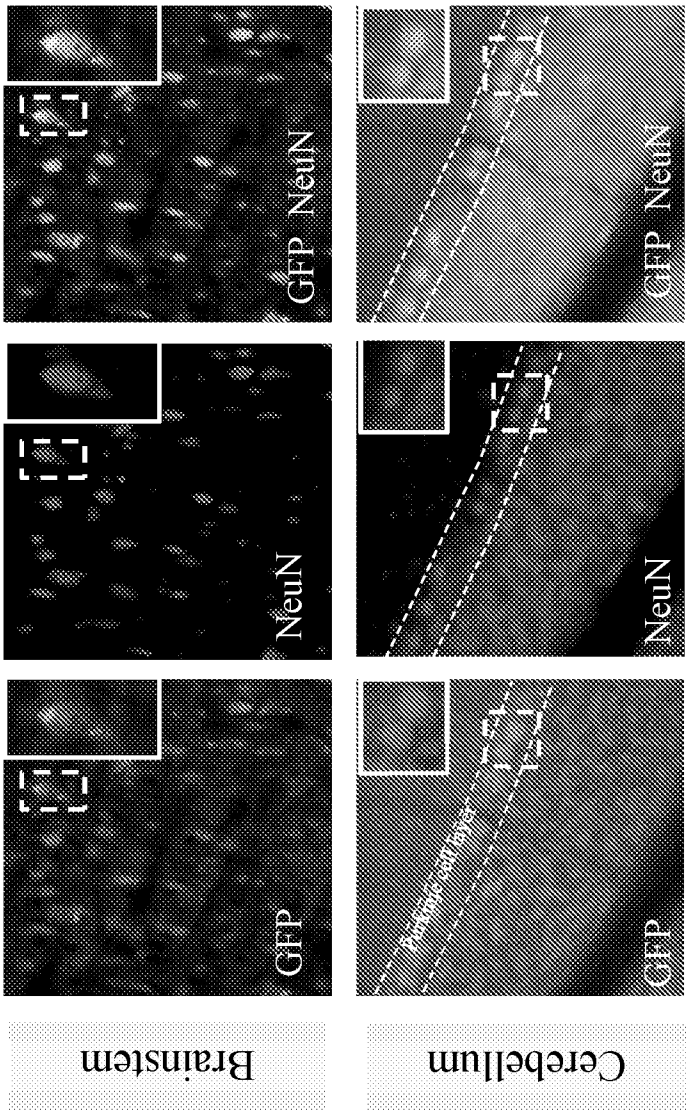

Brainstem
Cerebellum
GFP
NeuN
GFP NeuN
GFP
NeuN
GFP NeuN
Purkinje cell layer

FIG. 23

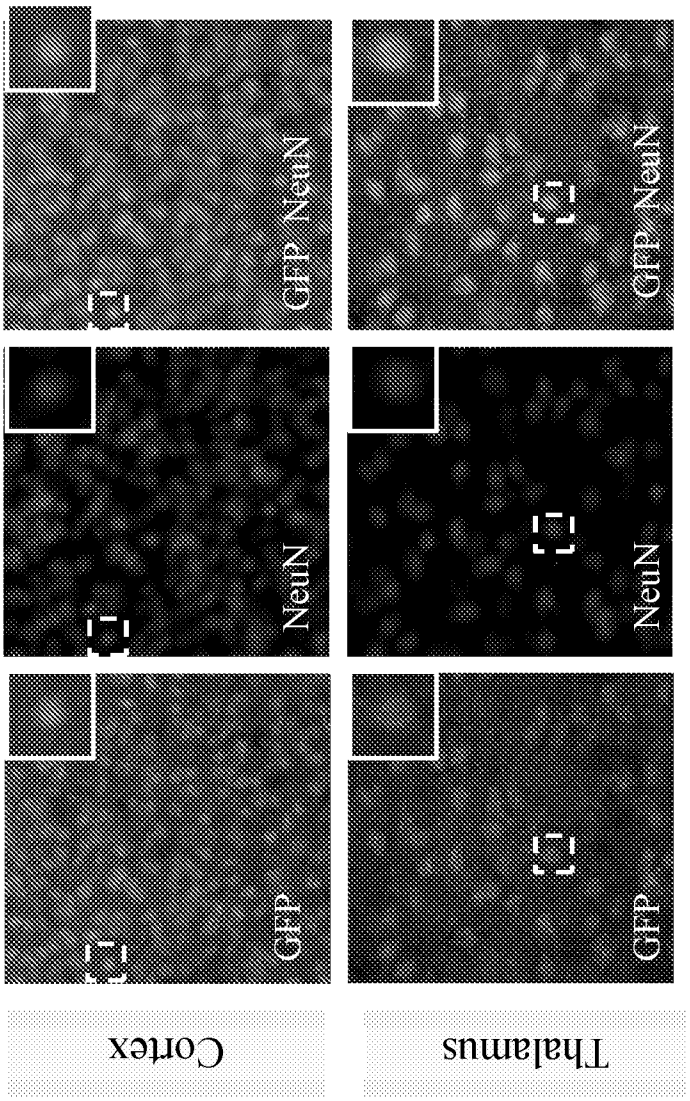


FIG. 24

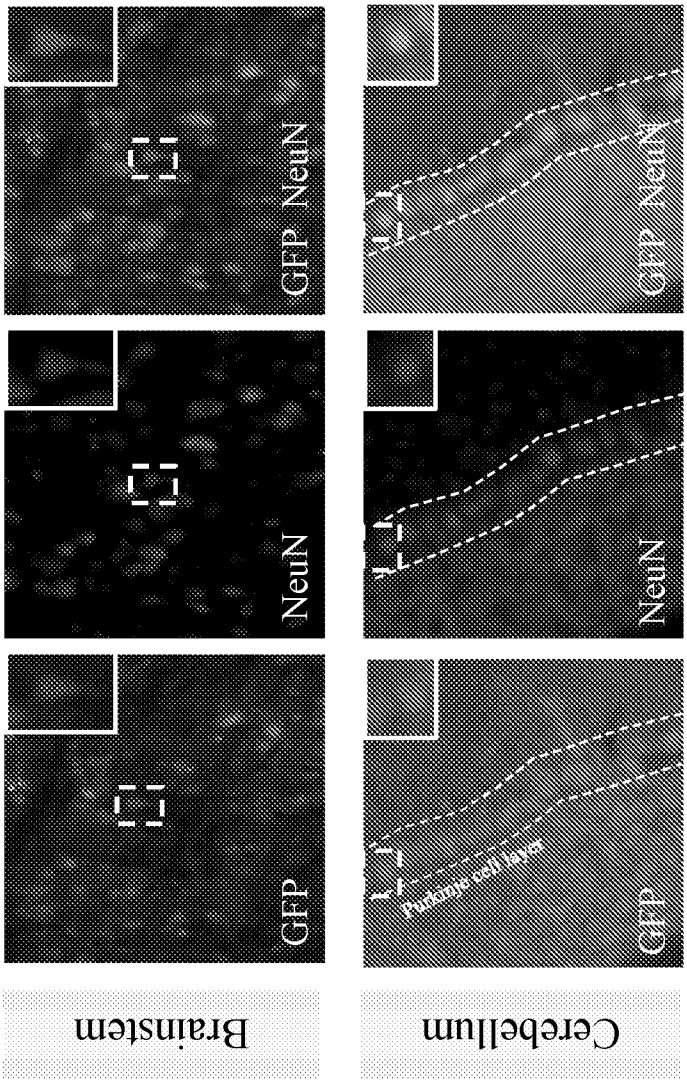

Brainstem
Cerebellum
GFP
NeuN
GFP NeuN
Purkinje cell layer

FIG. 25

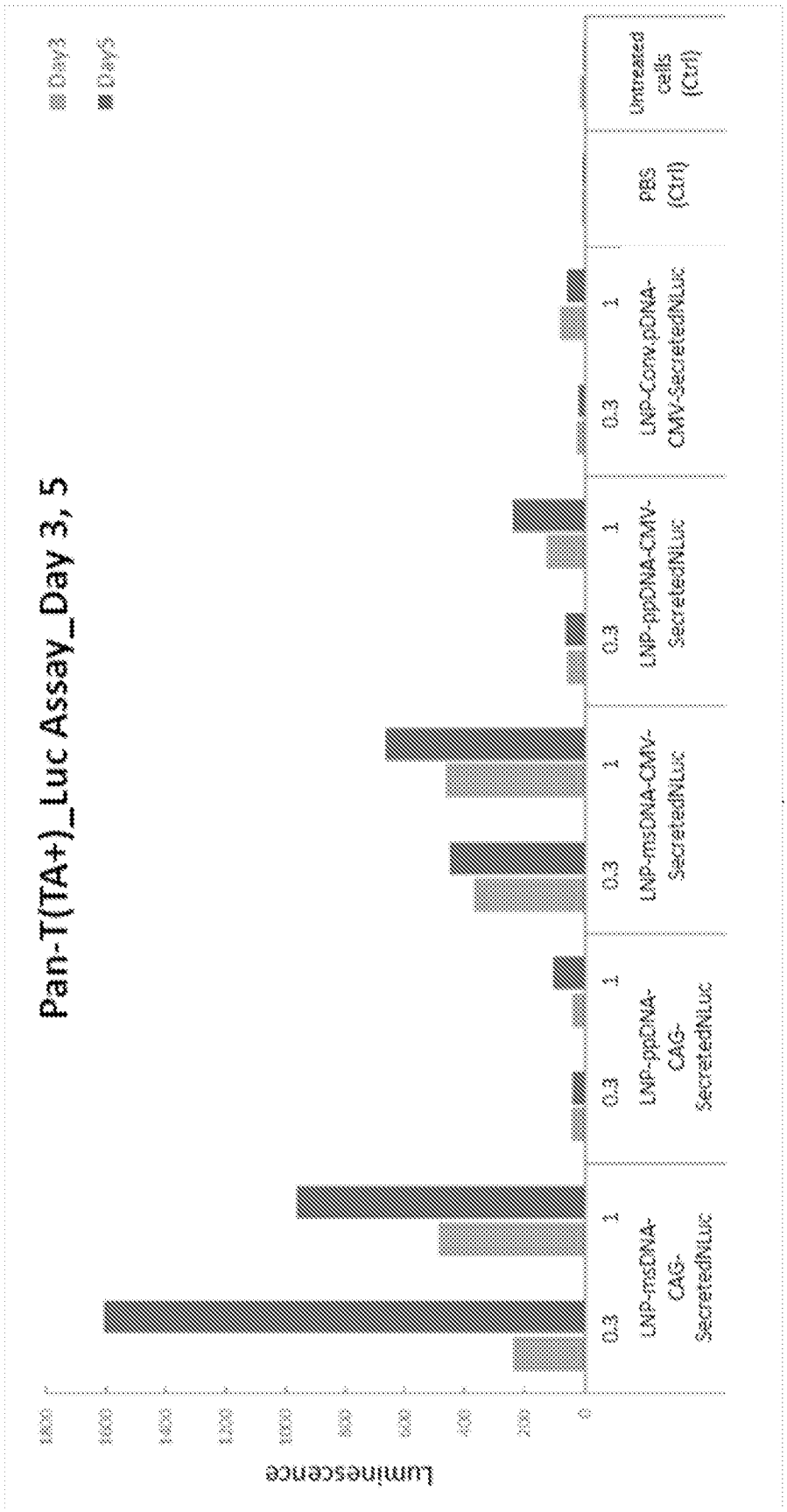

Pan-T(TA+)_Luc Assay_Day 3, 5
Day3
Day5
luminescence
0
200
400
600
800
1000
1200
1400
1600
1800
0.3
1
LNP-msDNA-CAG-SecretedNLuc
LNP-ppDNA-CAG-SecretedNLuc
LNP-msDNA-CMV-SecretedNLuc
LNP-ppDNA-CMV-SecretedNLuc
LNP-Conv.pDNA-CMV-SecretedNLuc
PBS (Ctrl)
Untreated cells (Ctrl)

FIG. 26

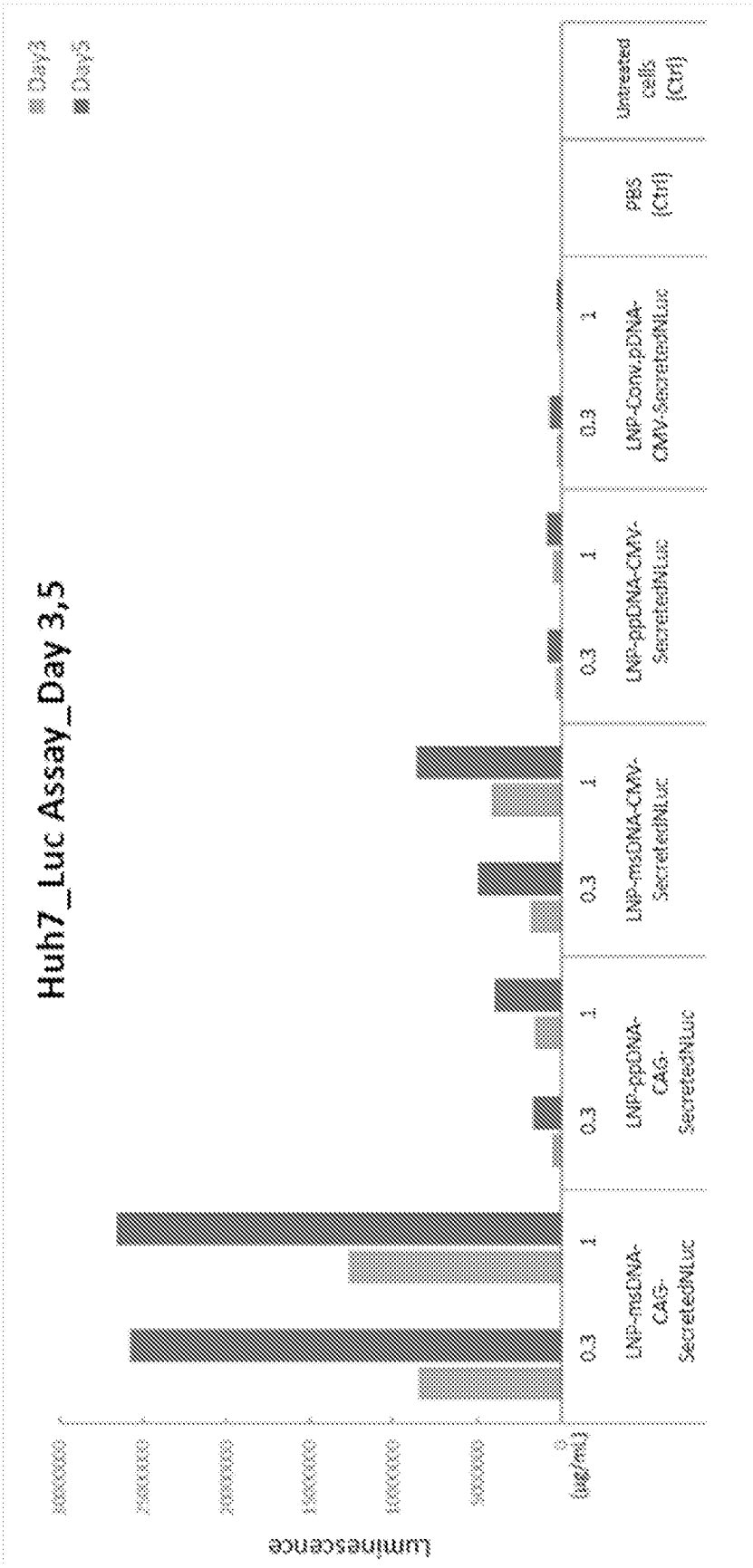
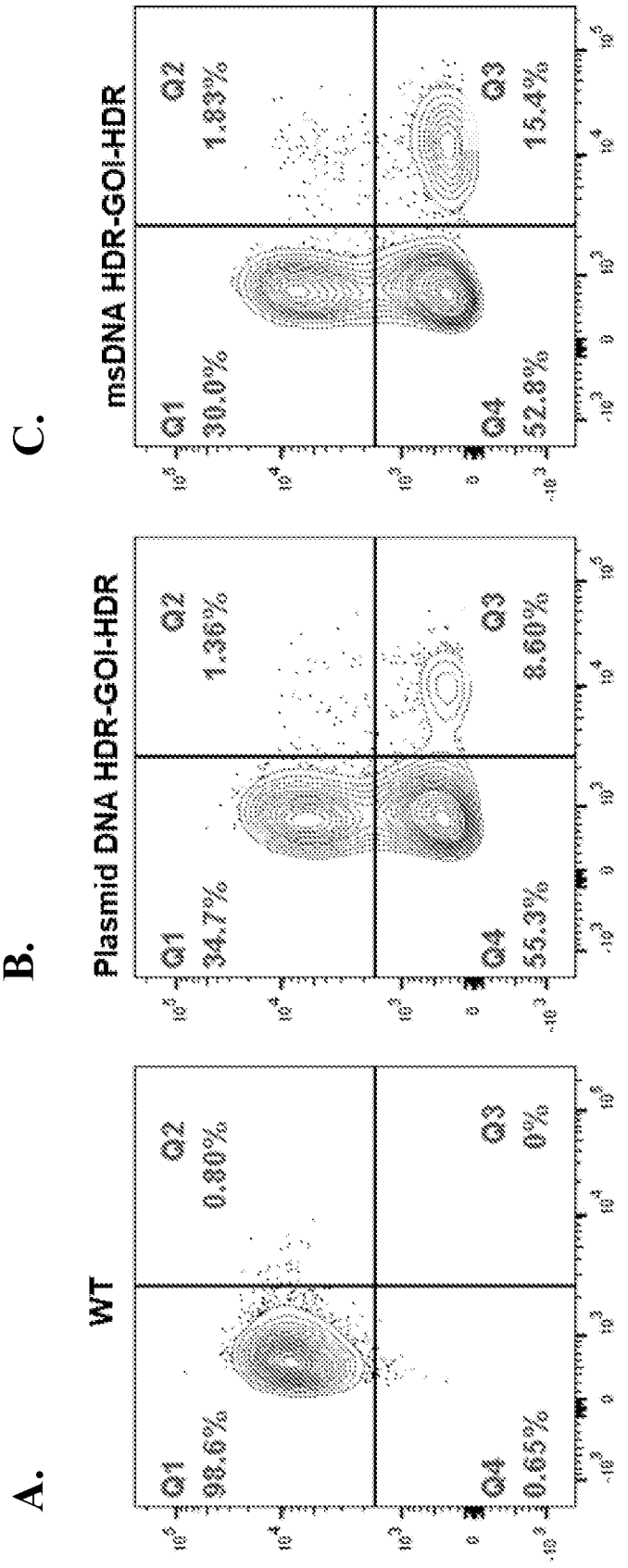
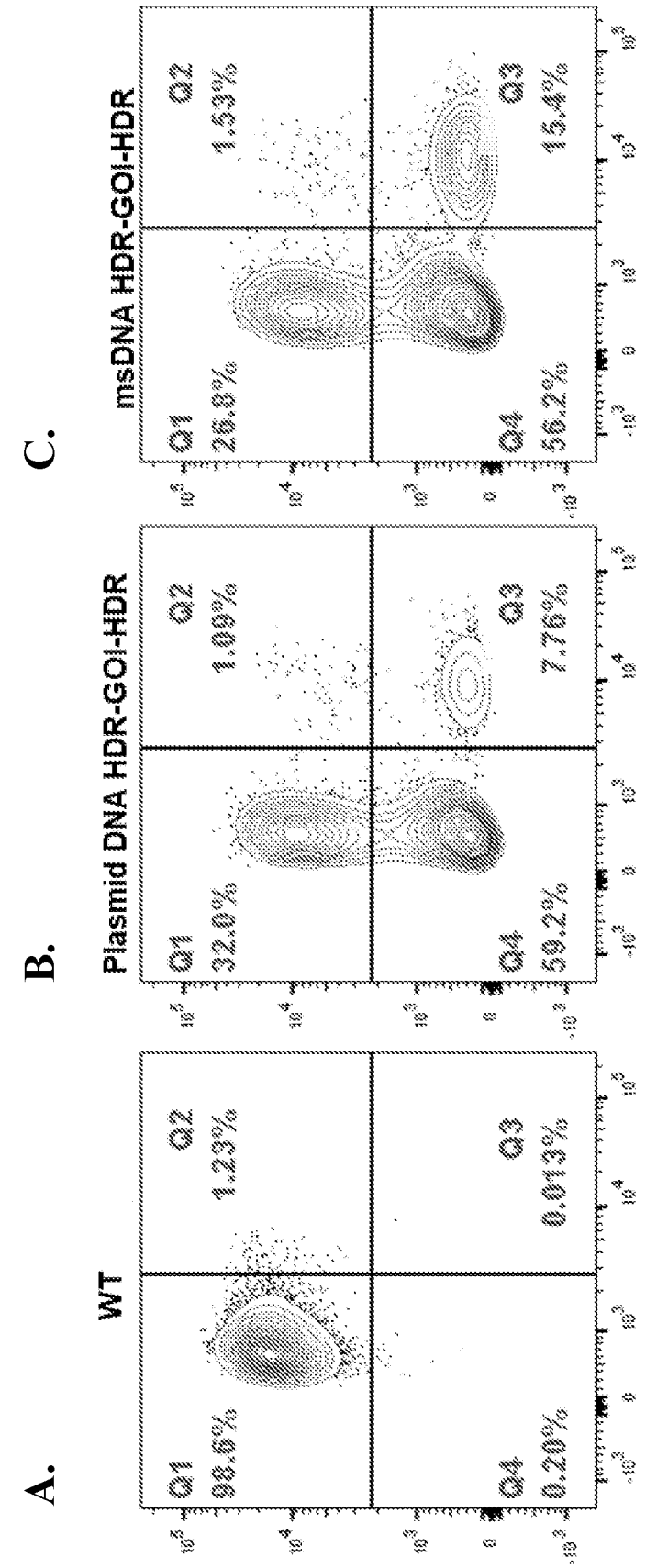

Huh7_Luc Assay_Day 3,5
Day3
Day5
luminescence
(ug/mL)
0.3
1
LNP-msDNA-CAG-SecretedNLuc
0.3
1
LNP-ppDNA-CAG-SecretedNLuc
0.3
1
LNP-msDNA-CMV-SecretedNLuc
0.3
1
LNP-ppDNA-CMV-SecretedNLuc
0.3
1
LNP-Conv.pDNA-CMV-SecretedNLuc
PBS (Ctrl)
Untreated cells (Ctrl)

FIG. 27



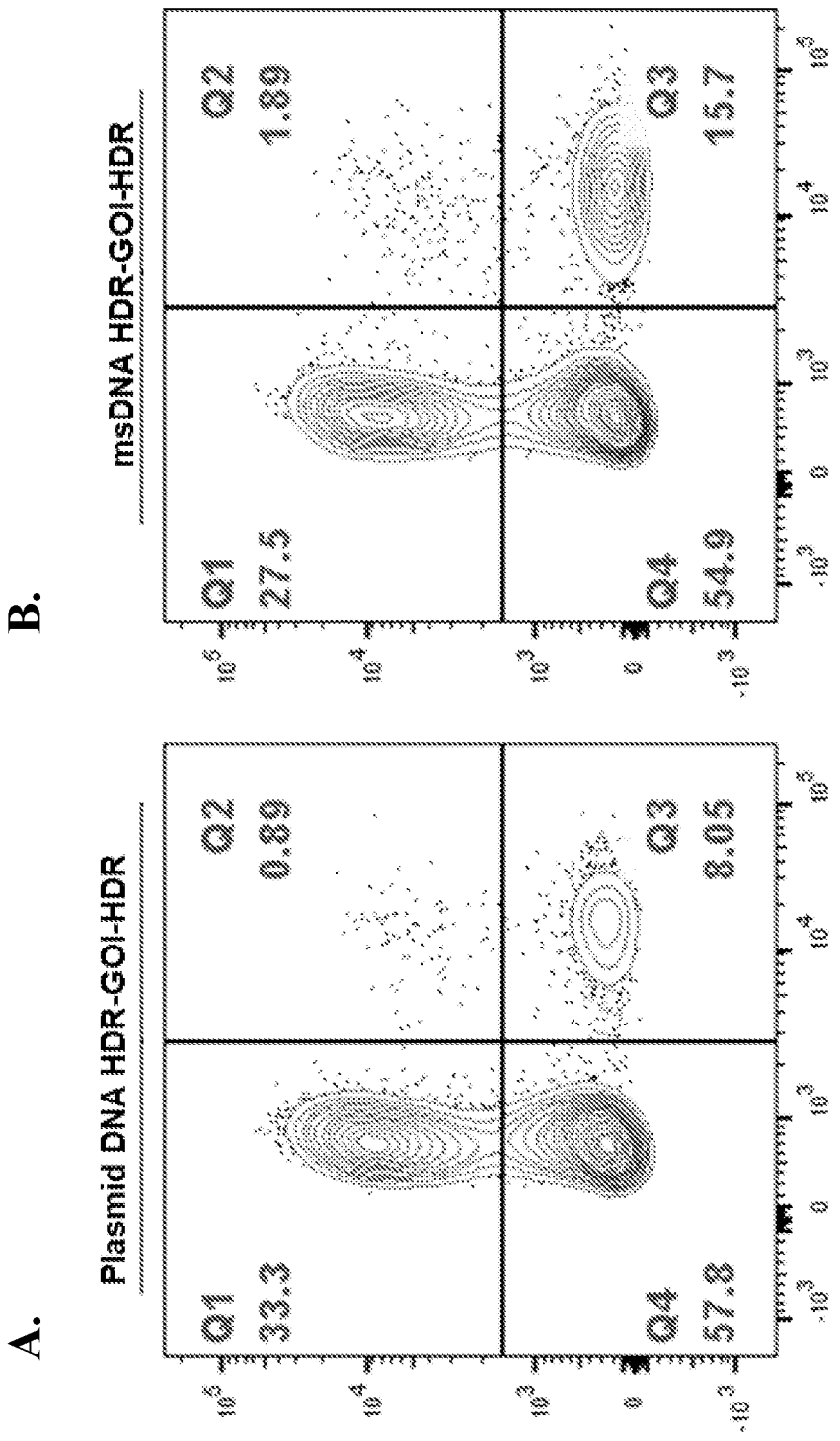
3 Days Post HDR Knock-In Efficiency (Q3)

FIG. 28



7 Days Post HDR Knock-In Efficiency (Q3)

FIG. 29



15 Days Post HDR Knock-In Efficiency (Q3)

FIG. 30

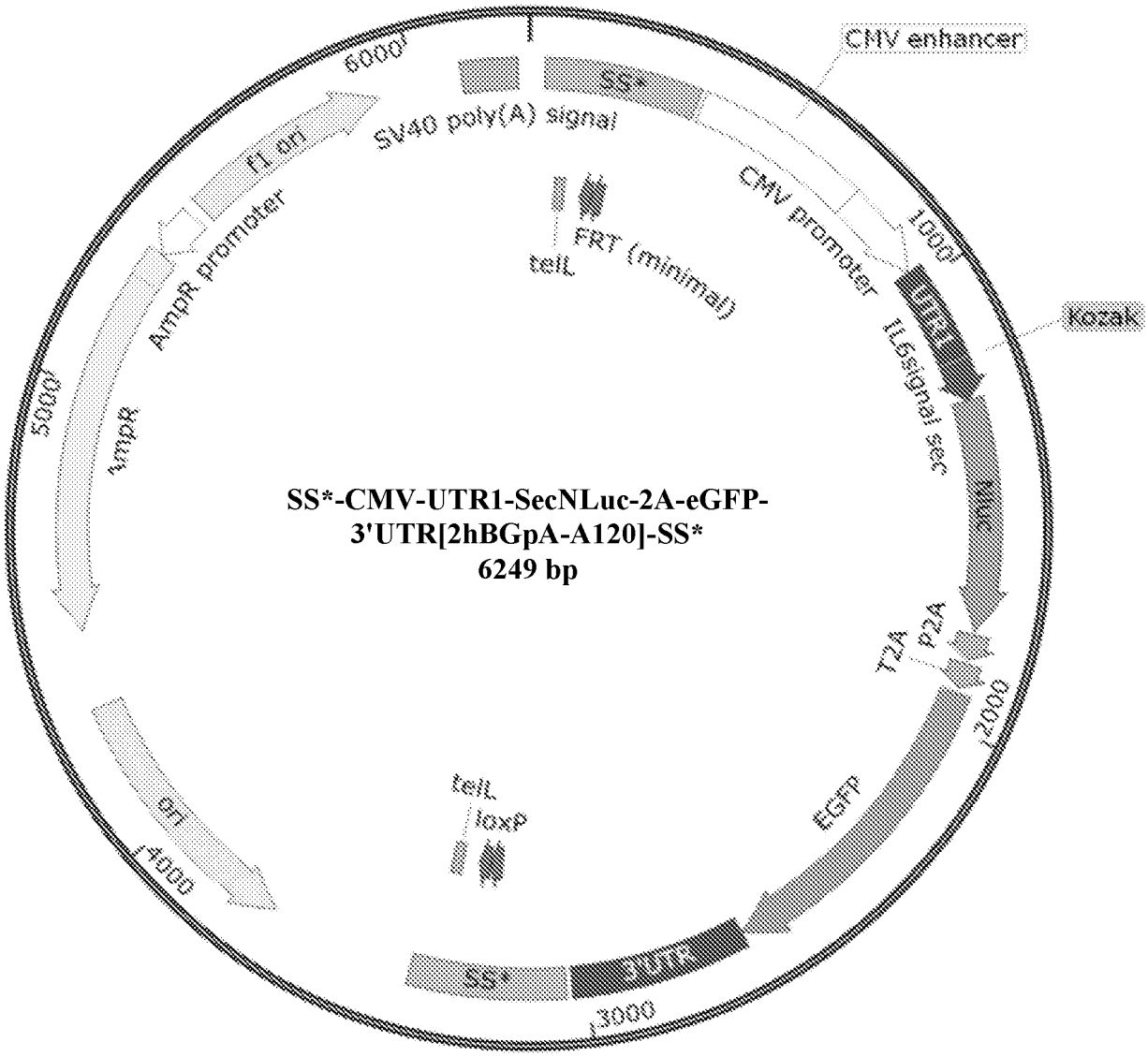

CMV enhancer
SS*
SV40 poly(A) signal
f1 ori
CMV promoter
6000
1000
FRT (minimal)
telL
AmpR promoter
UTR1
Kozak
IL6signal sec
5000
ampR
SecNLuc
T2A
p2A
2000
telL
loxP
ori
4000
EGFP
SS*
3UTR
3000
SS*-CMV-UTR1-SecNLuc-2A-eGFP-
3'UTR[2hBGpA-A120]-SS*
6249 bp

FIG. 31

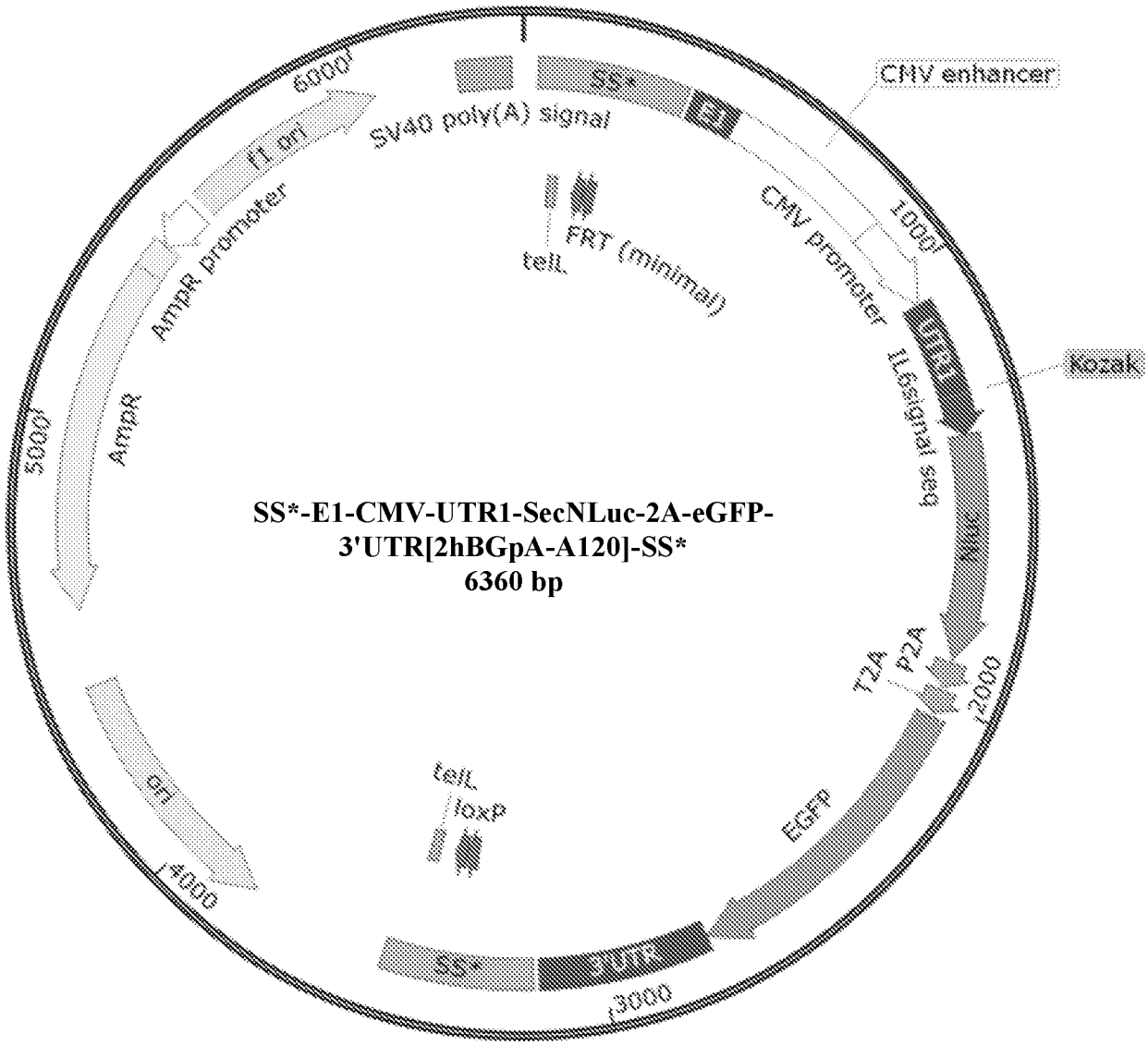

SS*-E1-CMV-UTR1-SecNLuc-2A-eGFP-
3'UTR[2hBGpA-A120]-SS*
6360 bp
6000
SS*
E1
CMV enhancer
SV40 poly(A) signal
f1 ori
CMV promoter
1000
FRT (minimal)
tetL
AmpR promoter
UTR1
IL6signal seq
Kozak
5000
AmpR
SecNLuc
T2A
P2A
2000
ori
tetL
loxP
EGFP
4000
SS*
3'UTR
3000

FIG. 32

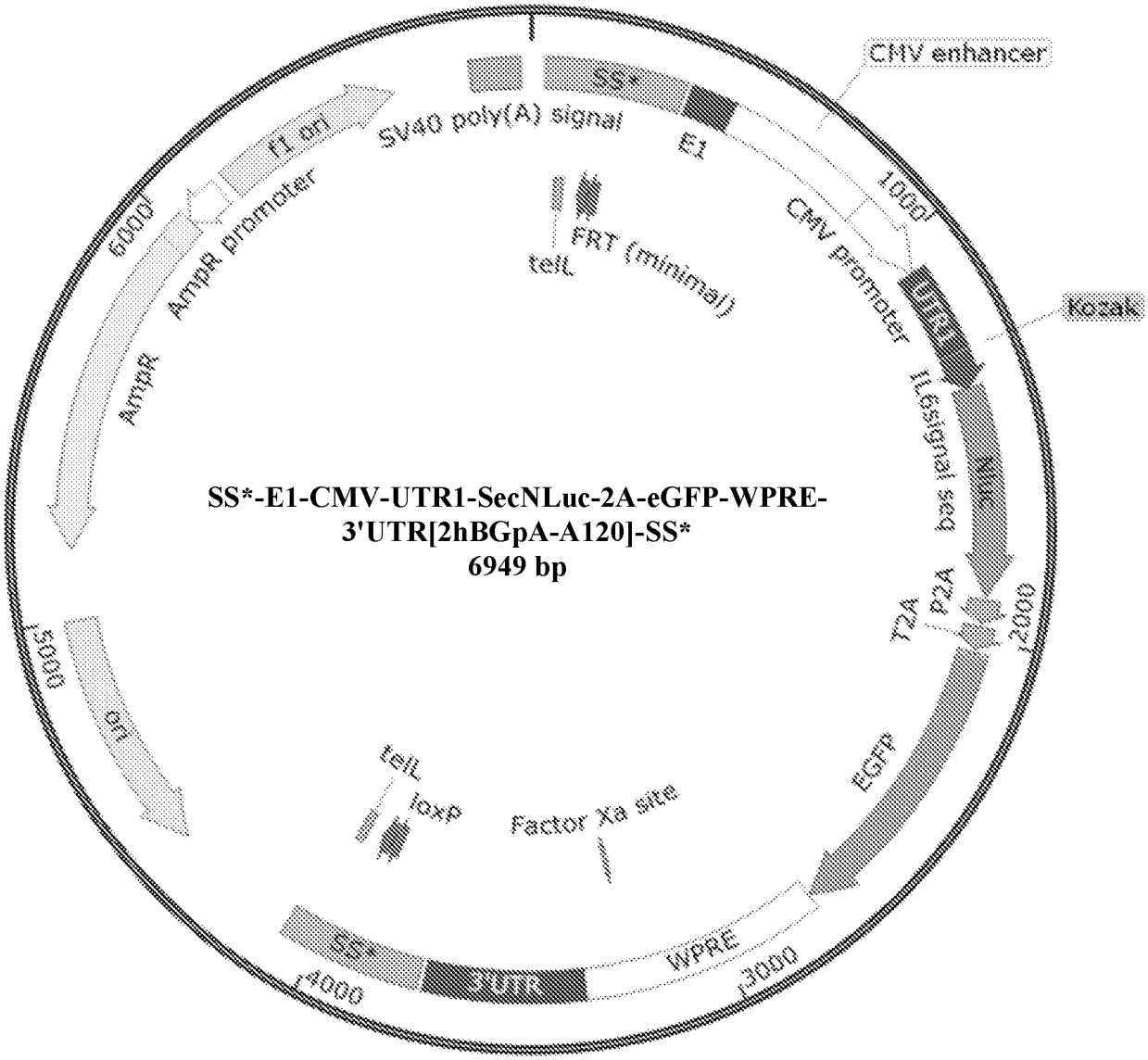

SS*-E1-CMV-UTR1-SecNLuc-2A-eGFP-WPRE-
3'UTR[2hBGpA-A120]-SS*
6949 bp
CMV enhancer
SS*
SV40 poly(A) signal
E1
f1 ori
6000
AmpR promoter
AmpR
FRT (minimal)
teiL
CMV promoter
10000
UTR1
Kozak
IL6signal seq
NLuc
P2A
T2A
2000
EGFP
5000
ori
teiL
loxp
Factor Xa site
SS*
4000
3'UTR
WPRE
3000

FIG. 33

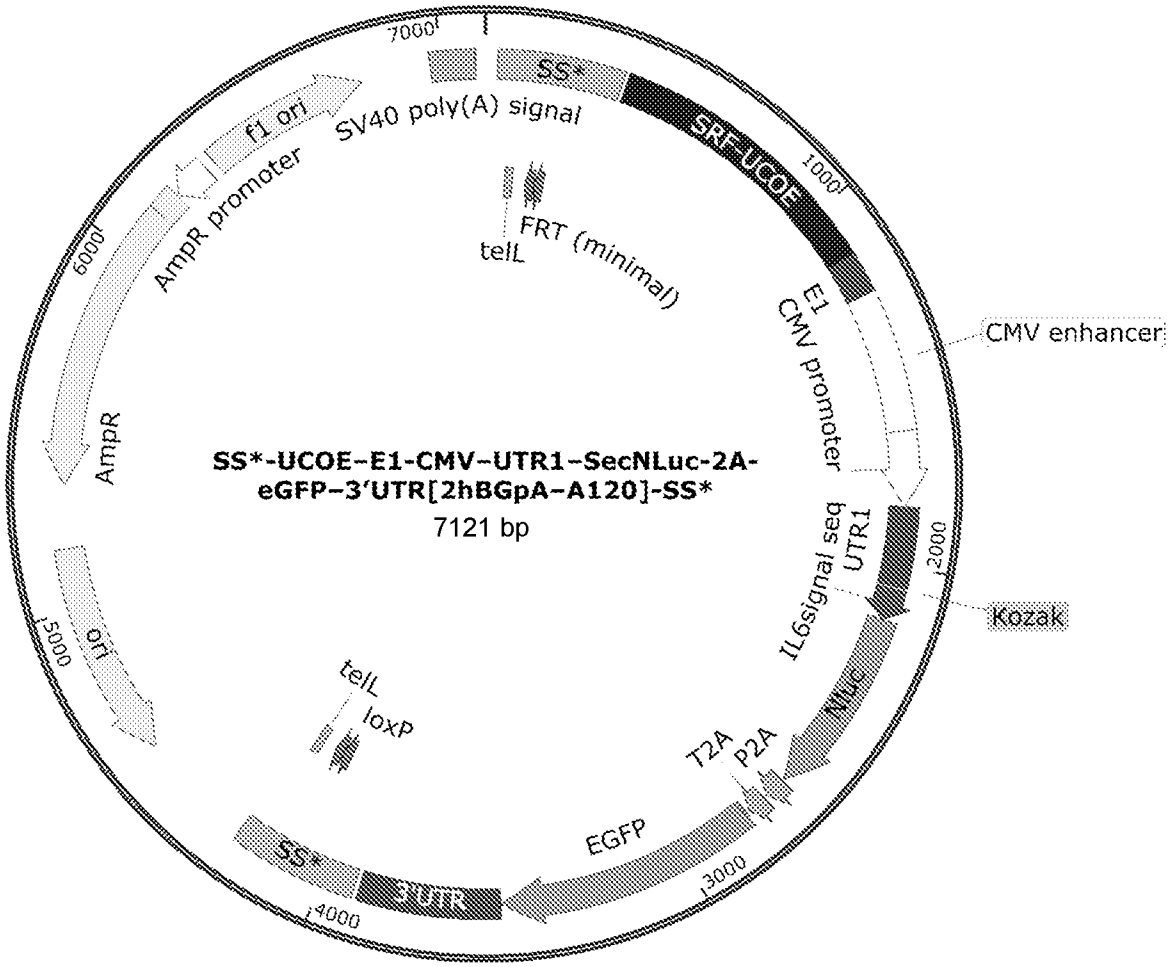

7000
SS*
SRF UCOE
1000
E1
CMV promoter
CMV enhancer
IL6signal seq
UTR1
2000
Kozak
NLuc
T2A
P2A
EGFP
3000
SS*
3'UTR
4000
tetL
loxP
5000
ori
AmpR
6000
AmpR promoter
f1 ori
SV40 poly(A) signal
tetL
FRT (minimal)
SS*-UCOE-E1-CMV-UTR1-SecNLuc-2A-
eGFP-3'UTR[2hBGpA-A120]-SS*
7121 bp

FIG. 34

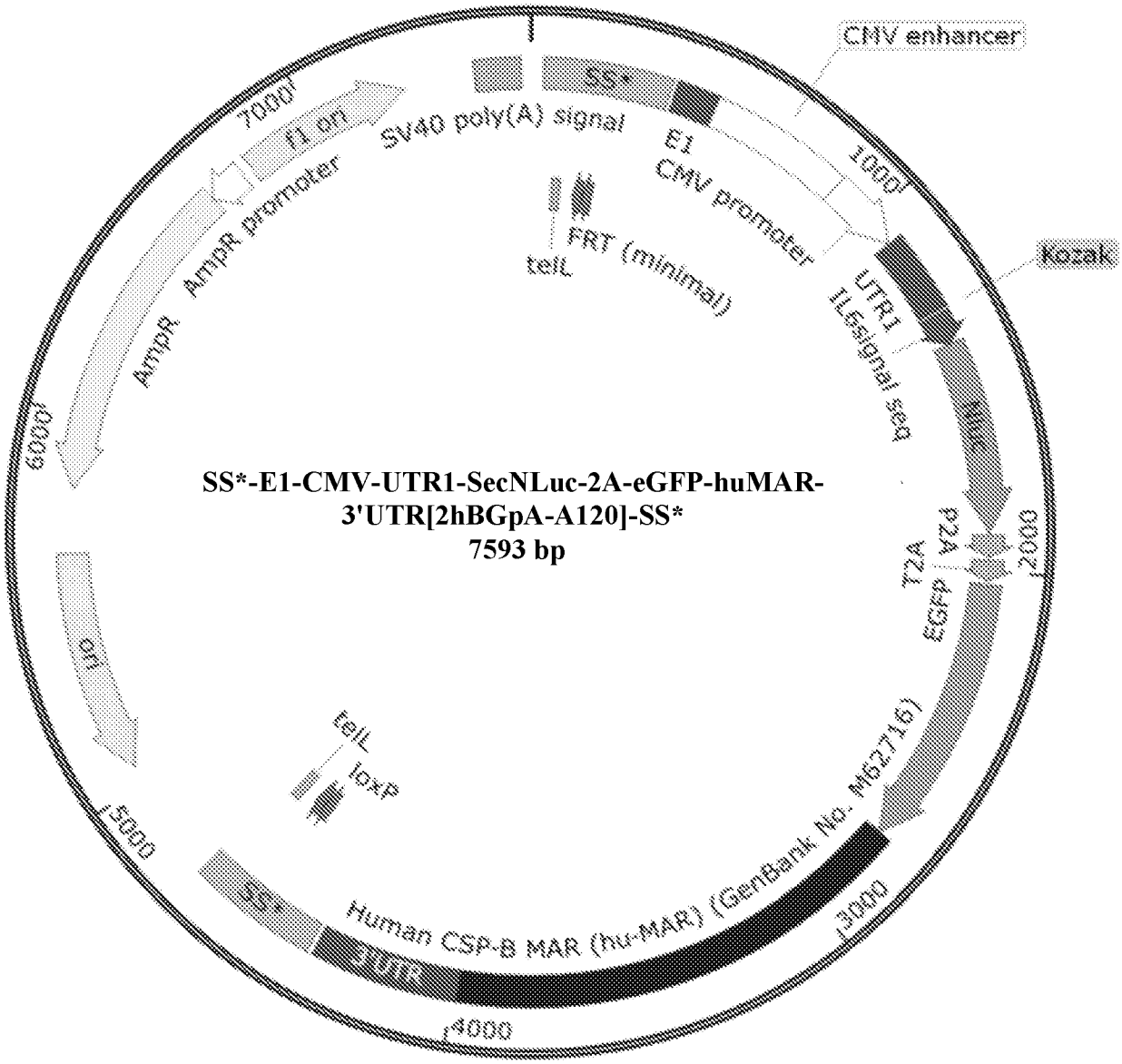

CMV enhancer
SS*
7000
f1 ori
SV40 poly(A) signal
E1
CMV promoter
1000
FRT (minimal)
tetL
UTR1
IL6signal seq
Kozak
AmpR
AmpR promoter
6000
SS*-E1-CMV-UTR1-SecNLuc-2A-eGFP-huMAR-
3'UTR[2hBGpA-A120]-SS*
7593 bp
T2A
EGFP
P2A
2000
ori
tetL
loxP
5000
SS*
Human CSP-B MAR (hu-MAR) (GenBank No. M62716)
3000
4000

FIG. 35

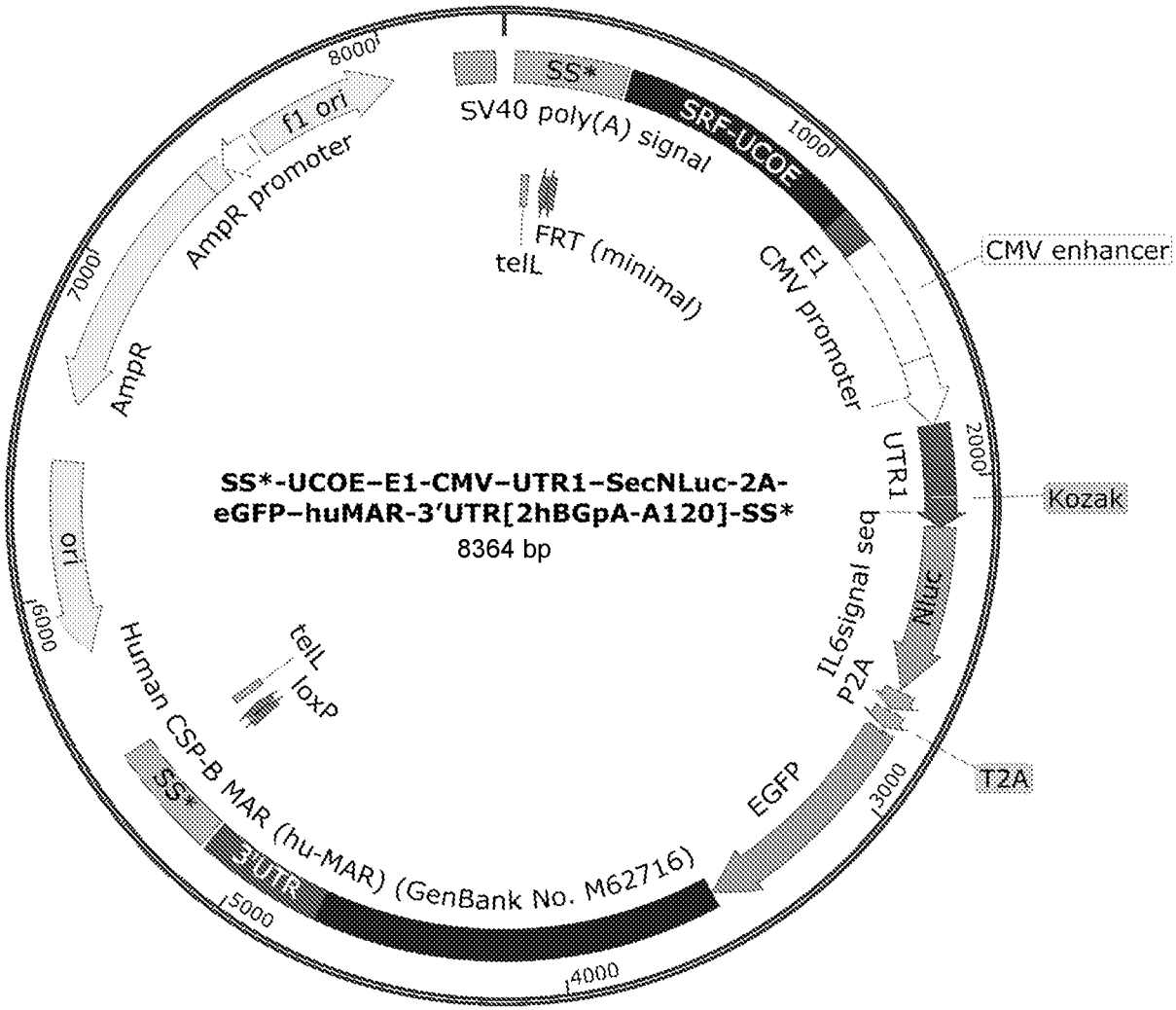

SS*-UCOE-E1-CMV-UTR1-SecNLuc-2A-eGFP-huMAR-3'UTR[2hBGpA-A120]-SS*
8364 bp
SS*
SV40 poly(A) signal
SRF-UCOE
E1
CMV promoter
CMV enhancer
UTR1
Kozak
NLuc
IL6signal seq
P2A
T2A
EGFP
Human CSP-B MAR (hu-MAR) (GenBank No. M62716)
SS*
3'UTR
loxP
tetL
FRT (minimal)
tetL
AmpR promoter
AmpR
f1 ori
ori
1000
2000
3000
4000
5000
6000
7000
8000

FIG. 36

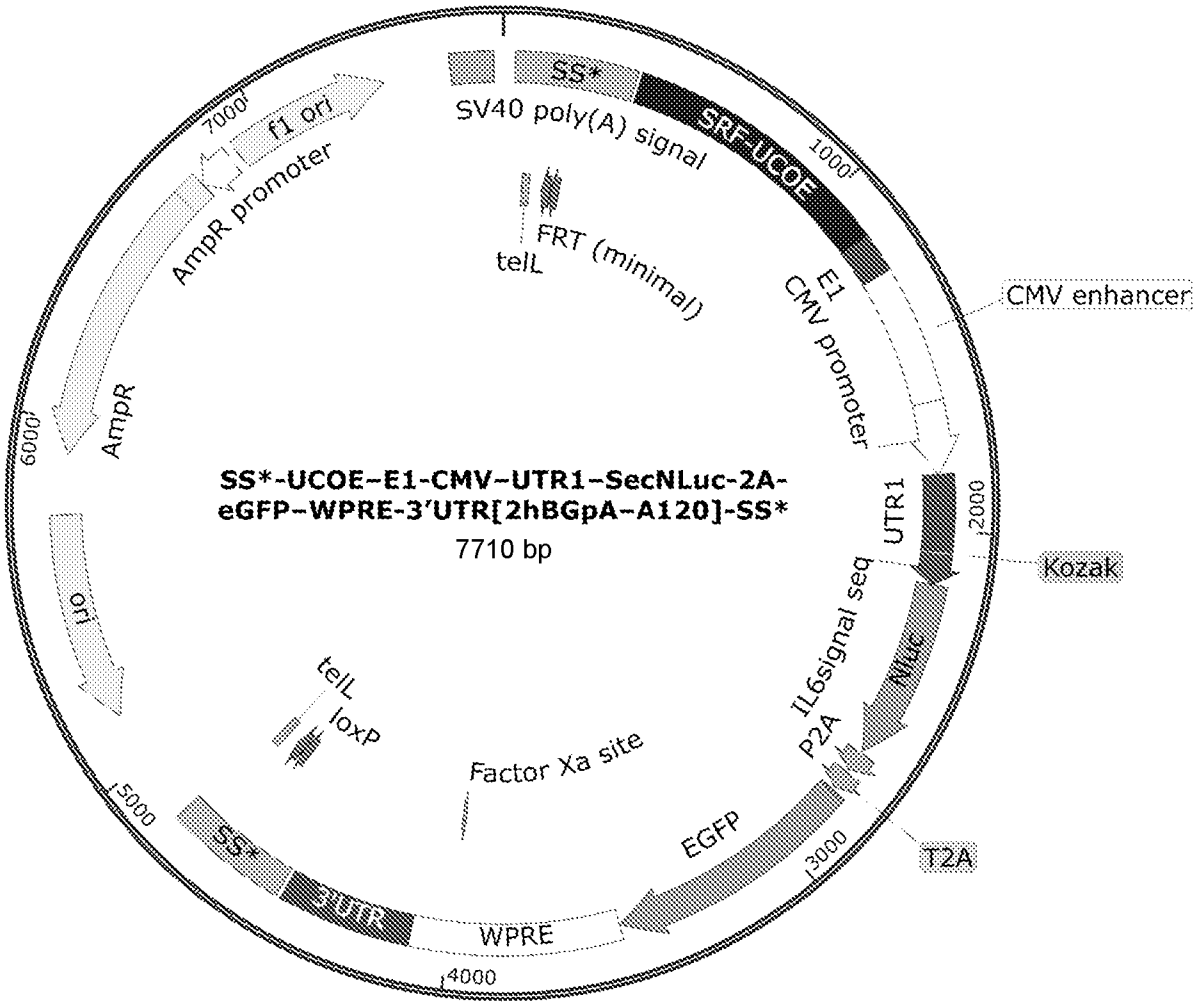

SS*
SV40 poly(A) signal
SRF UCOE
1000
CMV enhancer
E1
CMV promoter
FRT (minimal)
telL
f1 ori
7000
AmpR promoter
AmpR
6000
SS*-UCOE-E1-CMV-UTR1-SecNLuc-2A-
eGFP-WPRE-3'UTR[2hBGpA-A120]-SS*
7710 bp
UTR1
2000
Kozak
IL6signal seq
NLuc
P2A
ori
telL
loxP
Factor Xa site
5000
SS*
3'UTR
WPRE
EGFP
3000
T2A
4000

FIG. 37

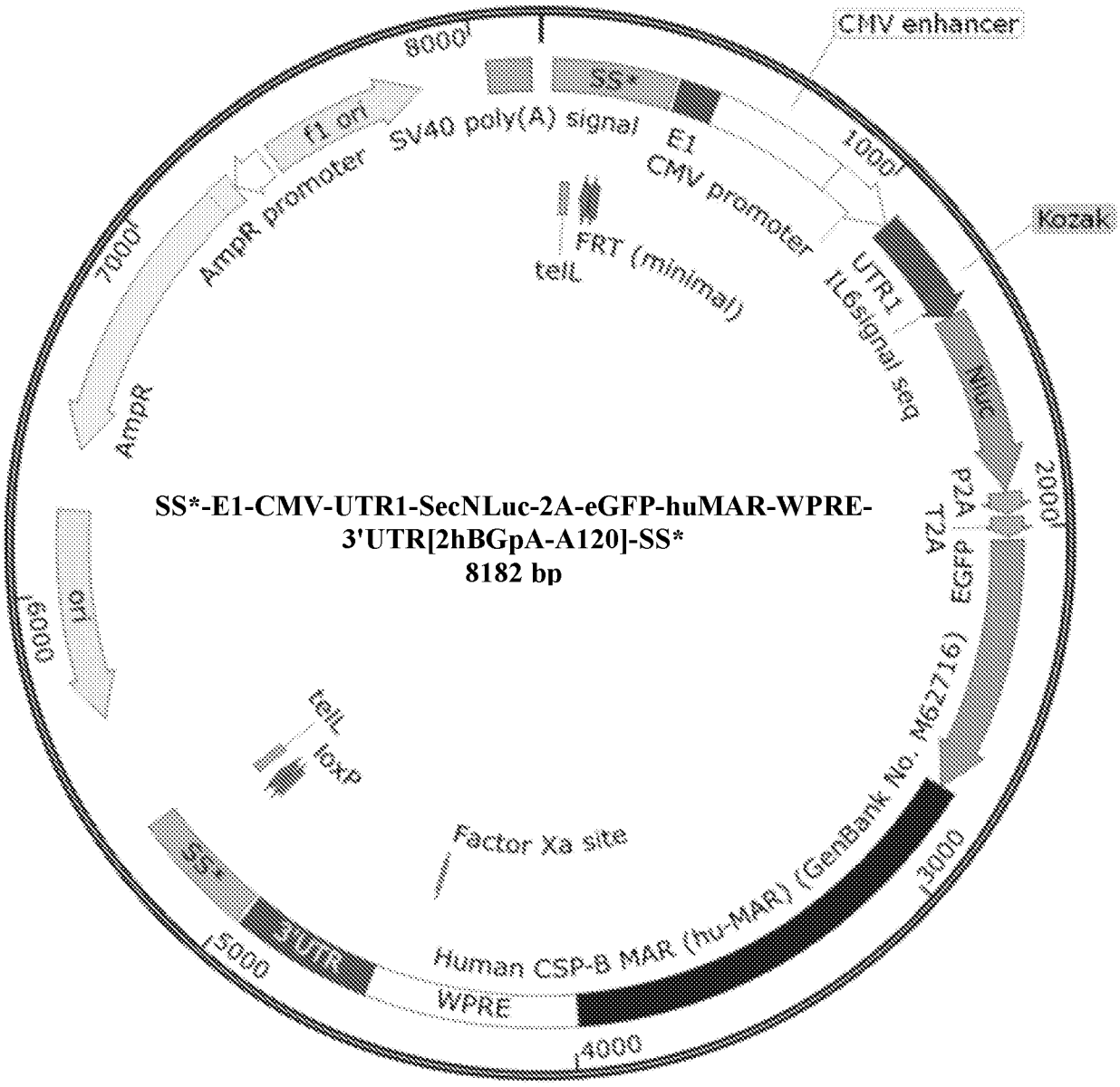

CMV enhancer
8000
SS*
f1 ori
SV40 poly(A) signal
E1
1000
CMV promoter
AmpR promoter
7000
FRT (minimal)
tetL
UTR1
Kozak
IL6signal seq
AmpR
NLuc
2000
P2A
T2A
EGFP
ori
6000
tetL
loxP
Factor Xa site
SS*
3000
UTR2
Human CSP-B MAR (hu-MAR) (GenBank No. M62716)
5000
WPRE
4000
SS*-E1-CMV-UTR1-SecNLuc-2A-eGFP-huMAR-WPRE-
3'UTR[2hBGpA-A120]-SS*
8182 bp

FIG. 38

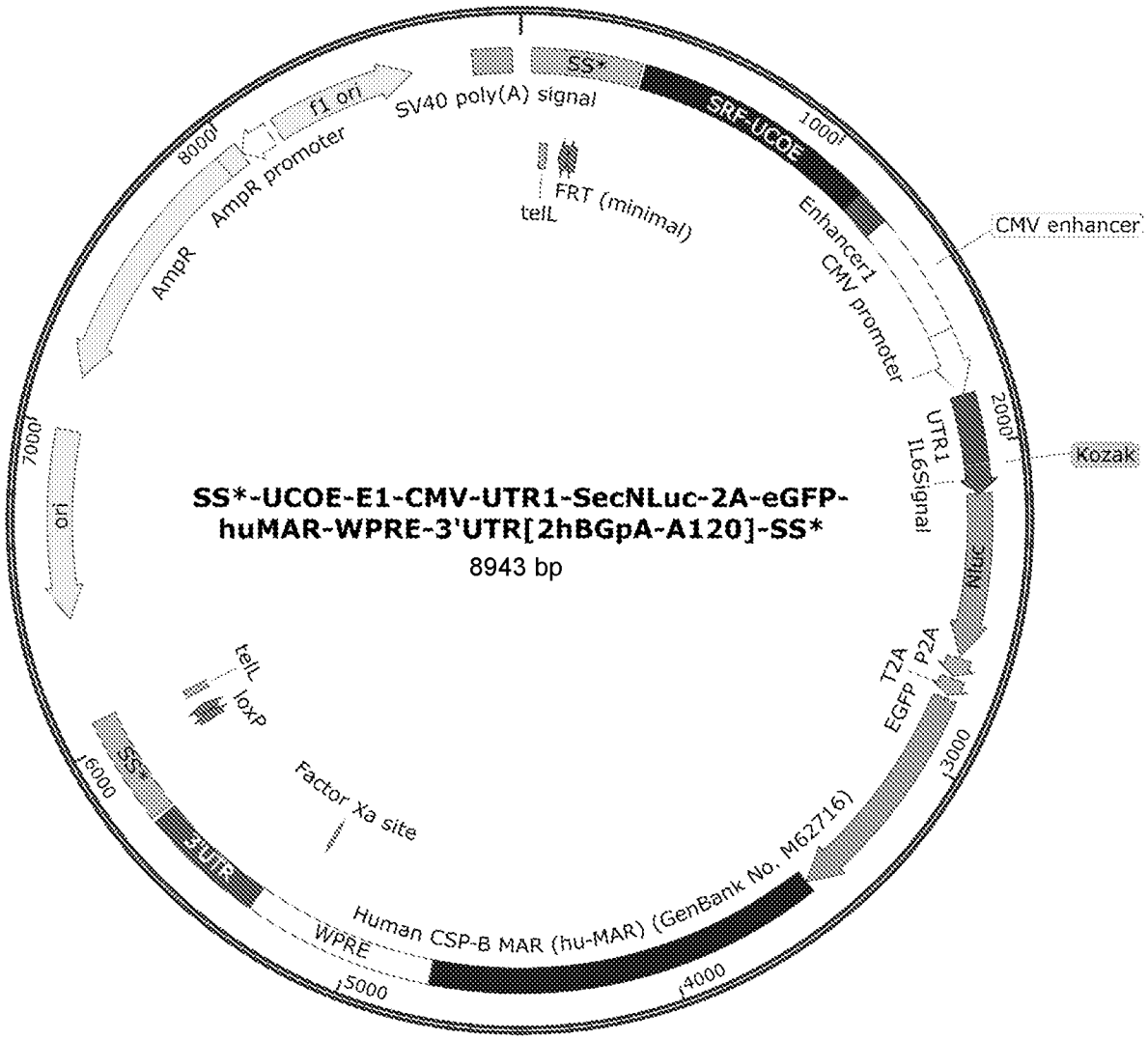

SS*-UCOE-E1-CMV-UTR1-SecNLuc-2A-eGFP-huMAR-WPRE-3'UTR[2hBGpA-A120]-SS*
8943 bp
SS*
SRF-UCOE
Enhancer1
CMV promoter
CMV enhancer
UTR1
IL6Signal
Kozak
NLuc
P2A
T2A
EGFP
Human CSP-B MAR (hu-MAR) (GenBank No. M62716)
WPRE
SS*
Factor Xa site
loxP
tetL
ori
AmpR
AmpR promoter
f1 ori
SV40 poly(A) signal
FRT (minimal)
tetL
1000
2000
3000
4000
5000
6000
7000
8000

FIG. 39

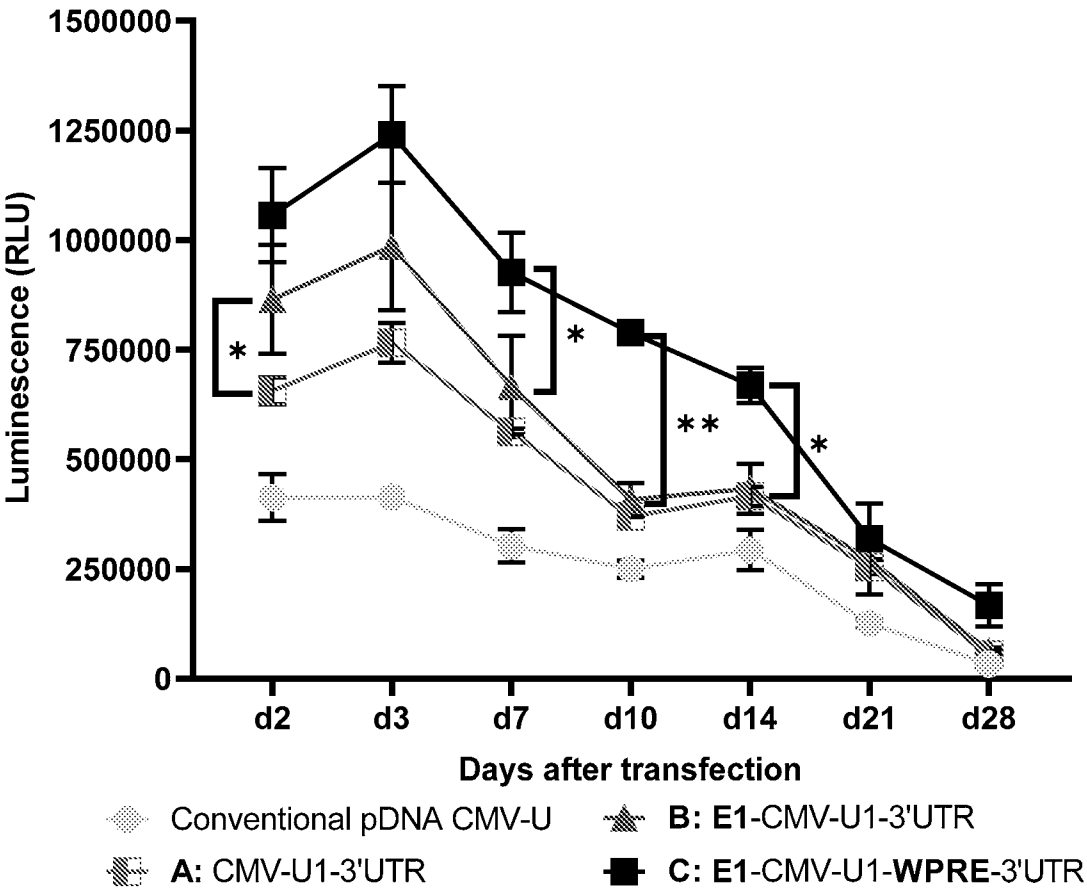

1500000
1250000
1000000
750000
500000
250000
0
Luminescence (RLU)
*
*
**
*
d2
d3
d7
d10
d14
d21
d28
Days after transfection
Conventional pDNA CMV-U
B: E1-CMV-U1-3'UTR
A: CMV-U1-3'UTR
C: E1-CMV-U1-WPRE-3'UTR

FIG. 40

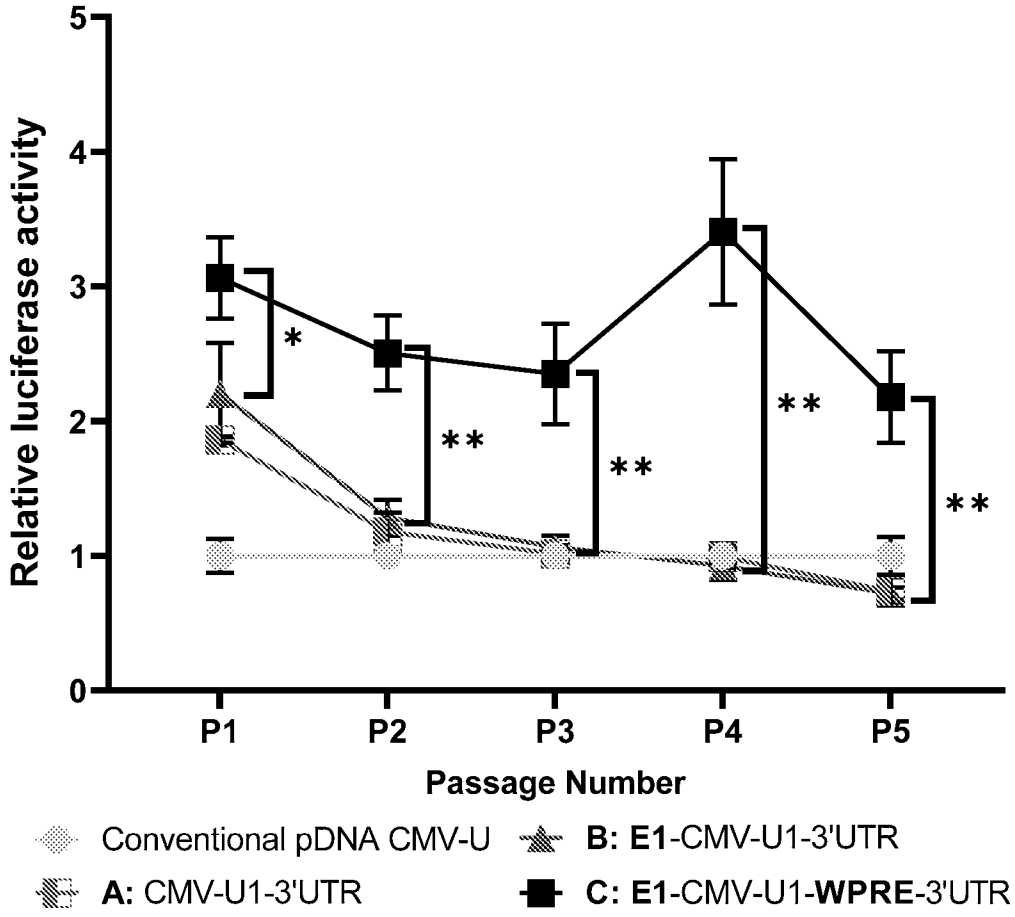

Relative luciferase activity
5
4
3
2
1
0
P1
P2
P3
P4
P5
Passage Number
*
**
**
**
**
Conventional pDNA CMV-U
B: E1-CMV-U1-3'UTR
A: CMV-U1-3'UTR
C: E1-CMV-U1-WPRE-3'UTR

FIG. 41

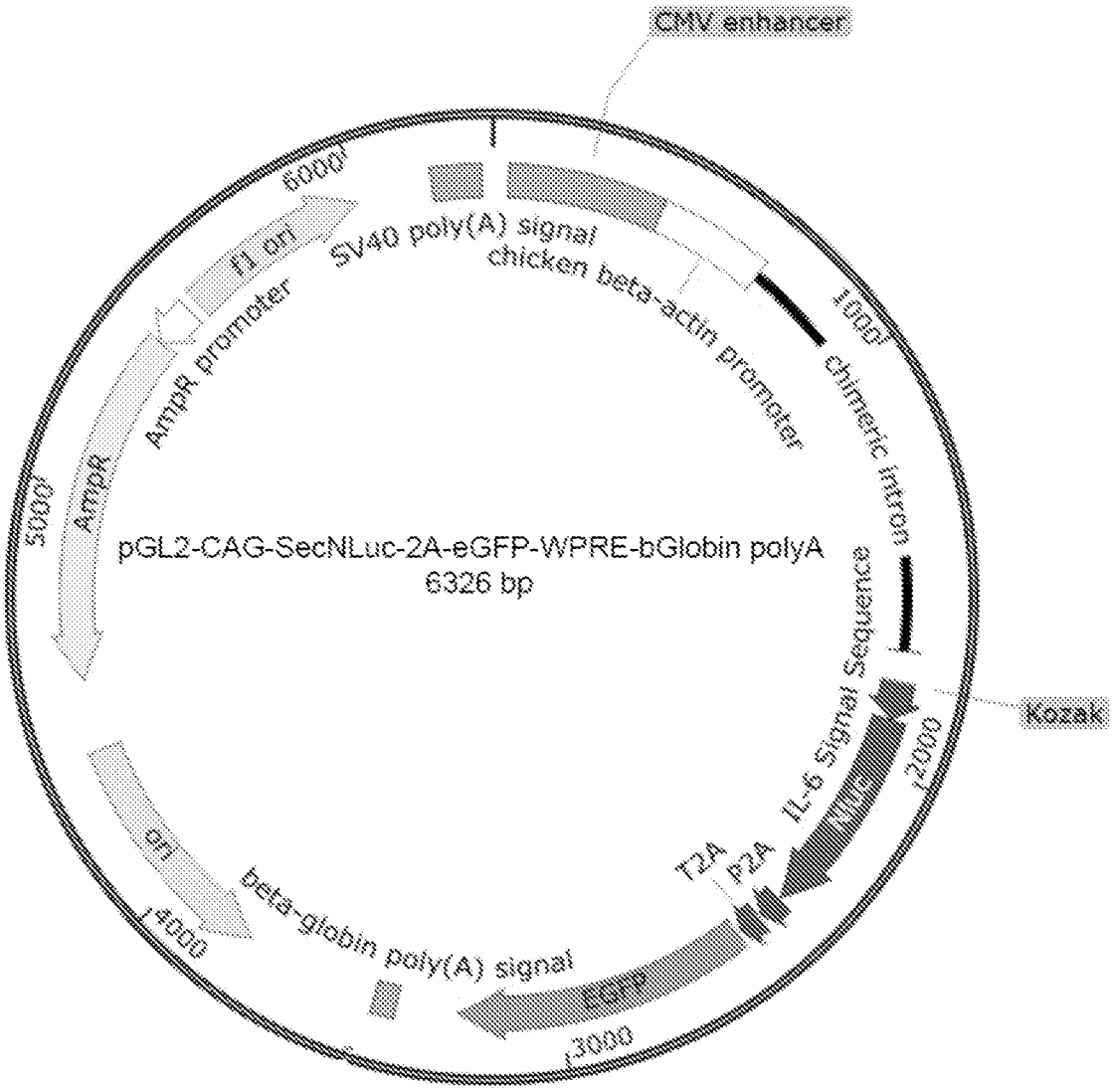

CMV enhancer
6000
f1 ori
SV40 poly(A) signal
chicken beta-actin promoter
1000
chimeric intron
AmpR promoter
AmpR
5000
pGL2-CAG-SecNLuc-2A-eGFP-WPRE-bGlobin polyA
6326 bp
aquamberg
Kozak
2000
IL-6 Signal Sequence
T2A
P2A
ori
beta-globin poly(A) signal
4000
3000

FIG. 42

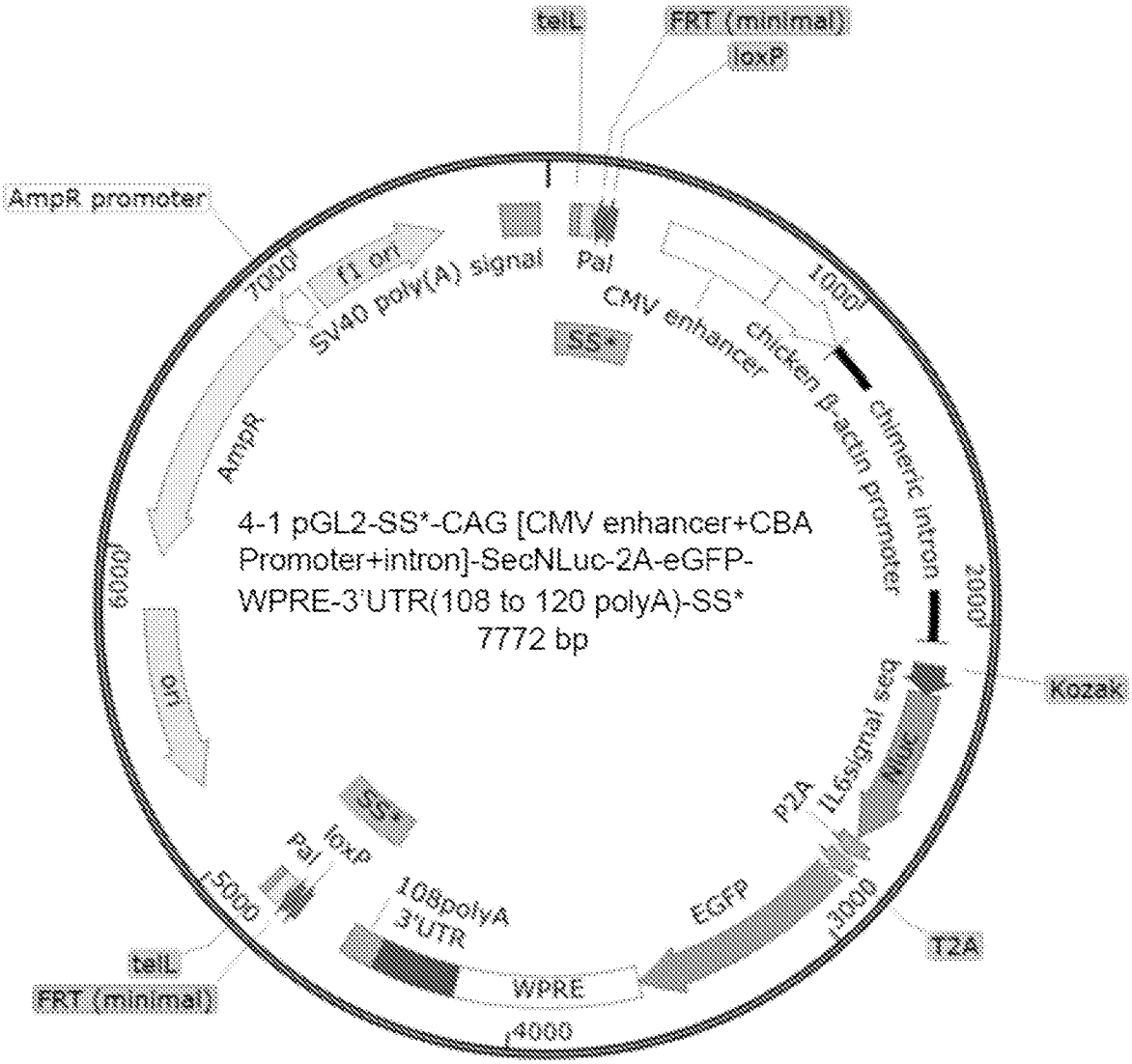

4-1 pGL2-SS*-CAG [CMV enhancer+CBA Promoter+intron]-SecNLuc-2A-eGFP-WPRE-3'UTR(108 to 120 polyA)-SS*
7772 bp
tetL
FRT (minimal)
loxP
AmpR promoter
7000
f1 ori
SV40 poly(A) signal
PsI
CMV enhancer
chicken β-actin promoter
chimeric intron
10000
20000
Kozak
SS*
AmpR
6000
ori
P2A
IL6signal seq
5000
108polyA 3'UTR
EGFP
3000
T2A
WPRE
4000

FIG. 43

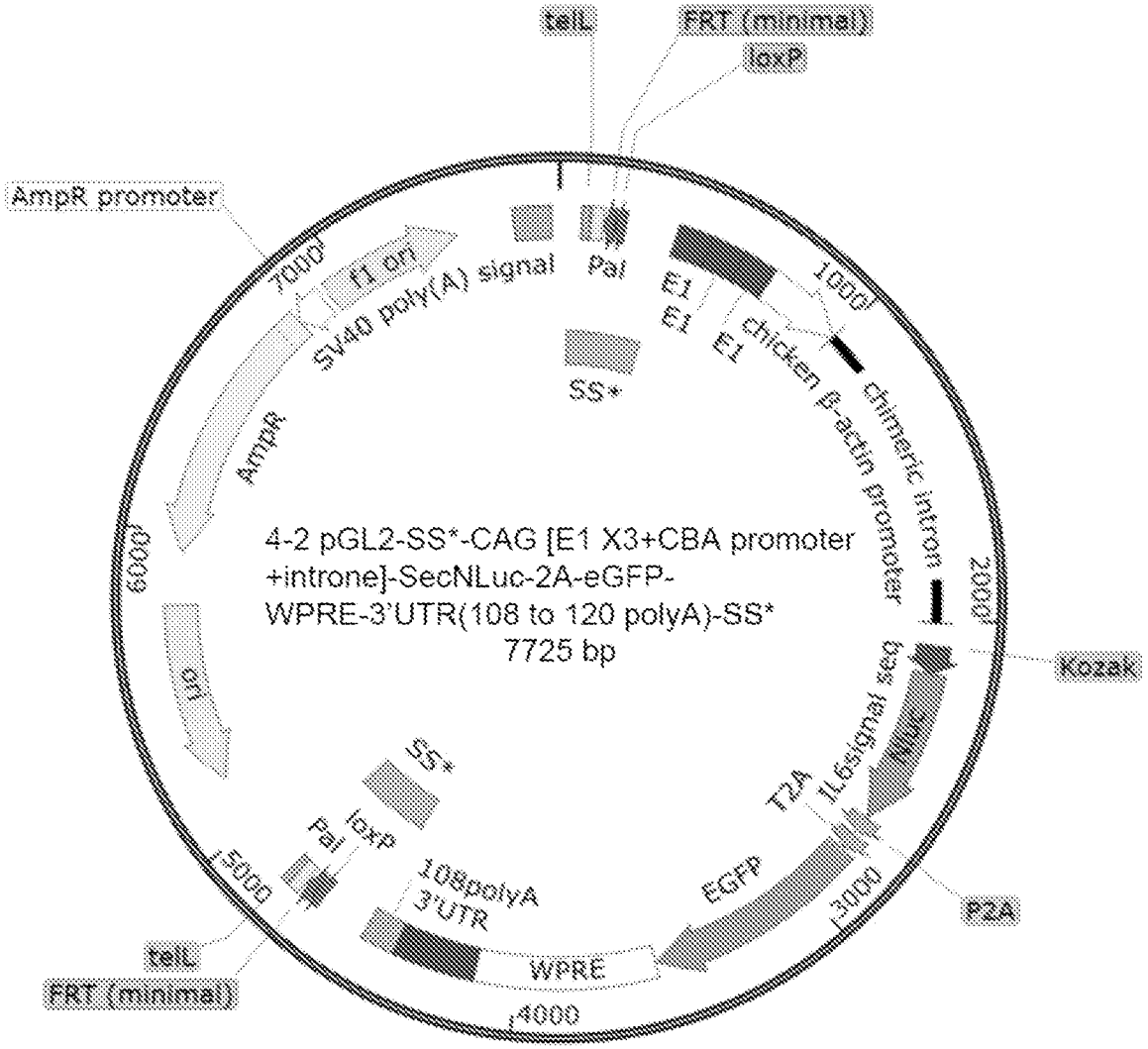

teiL
FRT (minimal)
loxP
AmpR promoter
7000
f1 ori
SV40 poly(A) signal
PaI
E1
E1
E1
E1
1000
chicken β-actin promoter
chimeric intron
2000
SS*
AmpR
6000
4-2 pGL2-SS*-CAG [E1 X3+CBA promoter
+introne]-SecNLuc-2A-eGFP-
WPRE-3'UTR(108 to 120 polyA)-SS*
7725 bp
ori
Kozak
6xHis
IL6signal
T2A
SS*
PaI
loxP
5000
108polyA
3'UTR
EGFP
3000
P2A
teiL
FRT (minimal)
WPRE
4000

FIG. 44

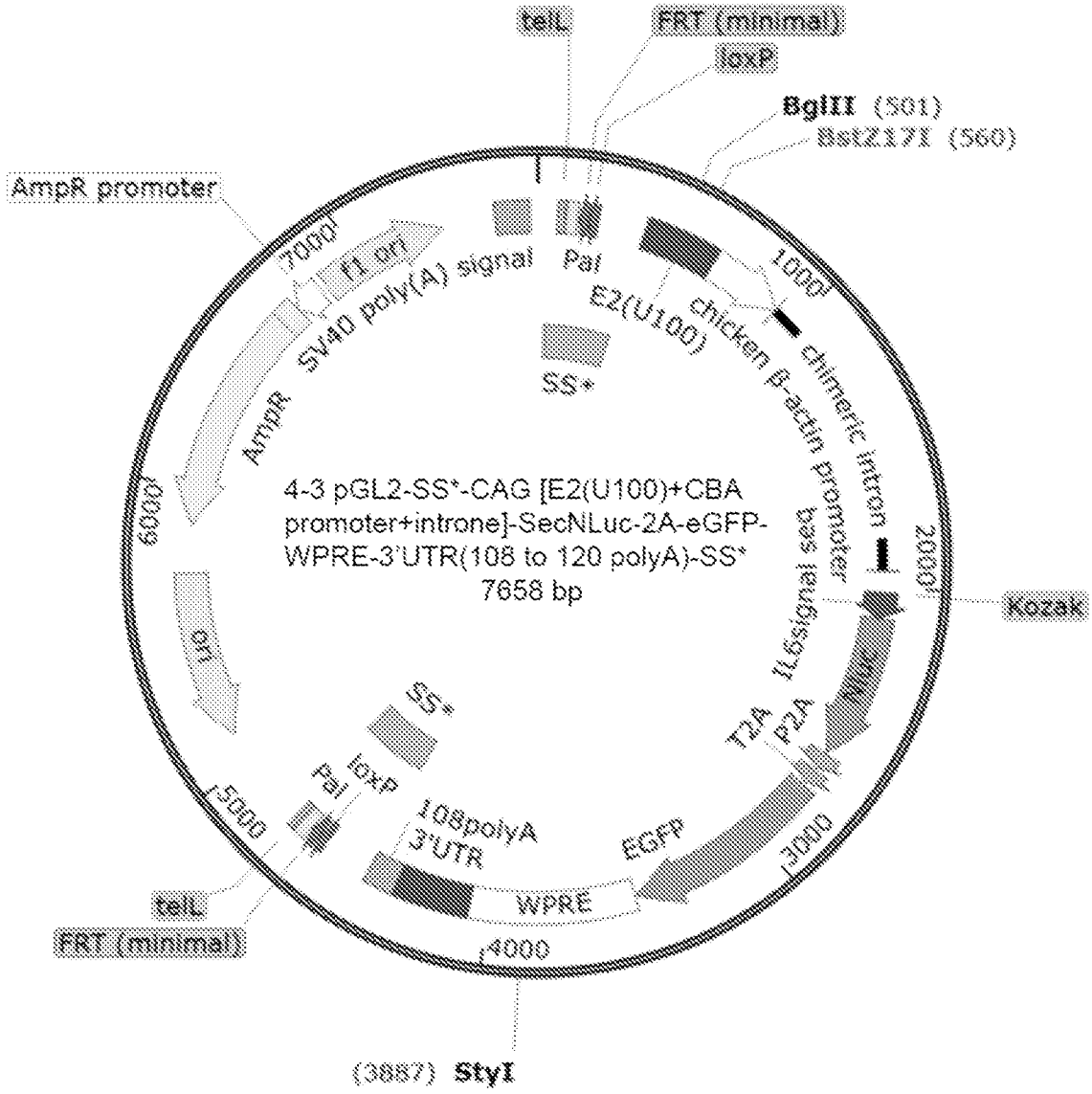

telL
FRT (minimal)
loxP
BglII (501)
Bst2171 (560)
AmpR promoter
7000
f1 ori
SV40 poly(A) signal
PaI
E2(U100)
chicken β-actin promoter
chimeric intron
1000
SS*
AmpR
4-3 pGL2-SS*-CAG [E2(U100)+CBA promoter+introne]-SecNLuc-2A-eGFP-WPRE-3'UTR(108 to 120 polyA)-SS*
7658 bp
6000
ori
SS*
PaI
loxP
5000
108polyA 3'UTR
WPRE
4000
T2A
P2A
EGFP
3000
2000
Kozak
telL
FRT (minimal)
(3887) StyI

FIG. 45

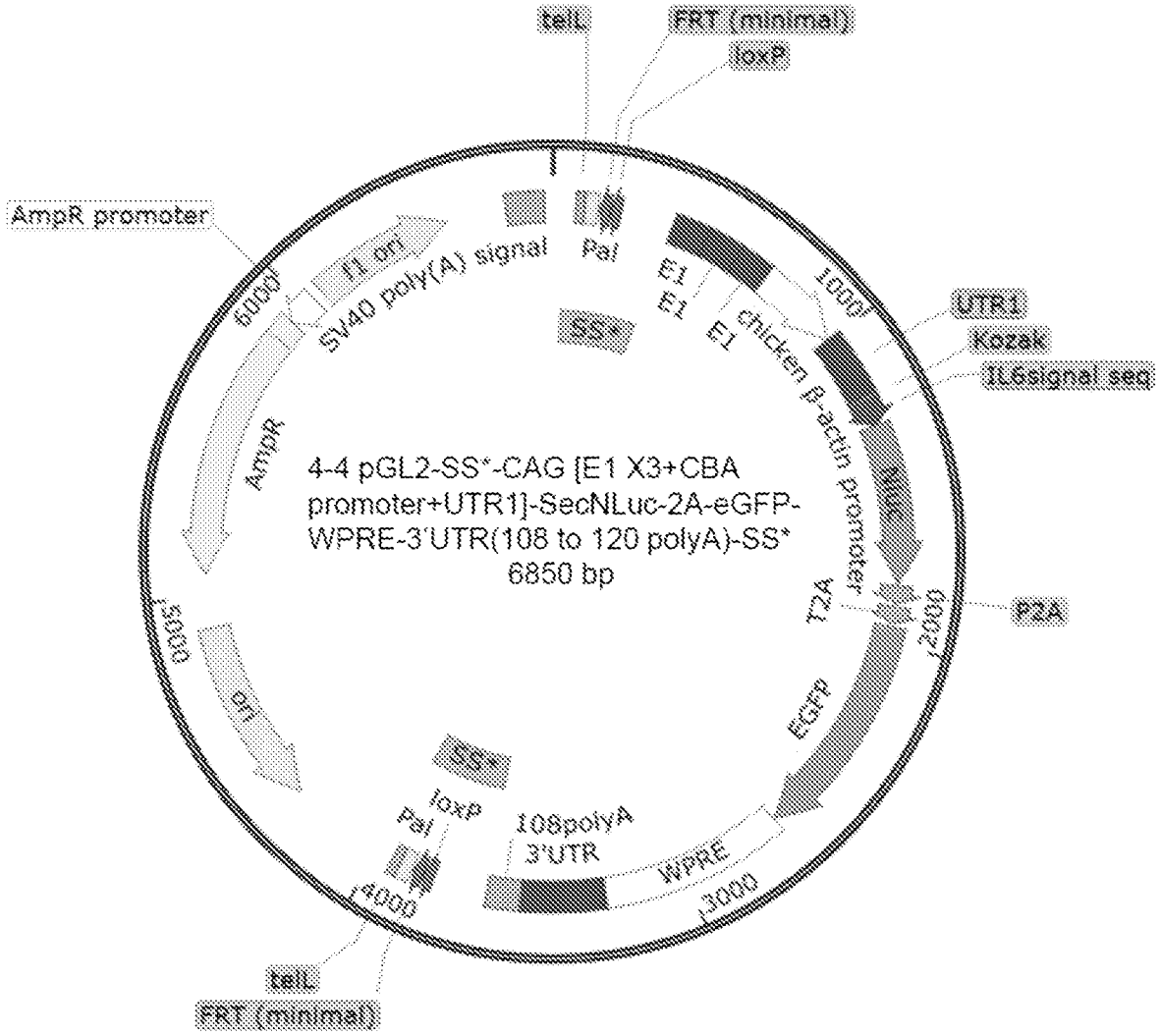

telL
FRT (minimal)
loxP
AmpR promoter
6000
f1 ori
SV40 poly(A) signal
PaI
SS*
E1
E1
E1
chicken β-actin promoter
1000
UTR1
Kozak
IL6signal seq
Nluc
T2A
2000
P2A
AmpR
5000
ori
EGFP
SS*
loxP
PaI
108polyA
3'UTR
WPRE
3000
4000
telL
FRT (minimal)
4-4 pGL2-SS*-CAG {E1 X3+CBA promoter+UTR1}-SecNLuc-2A-eGFP-WPRE-3'UTR(108 to 120 polyA)-SS*
6850 bp

FIG. 46

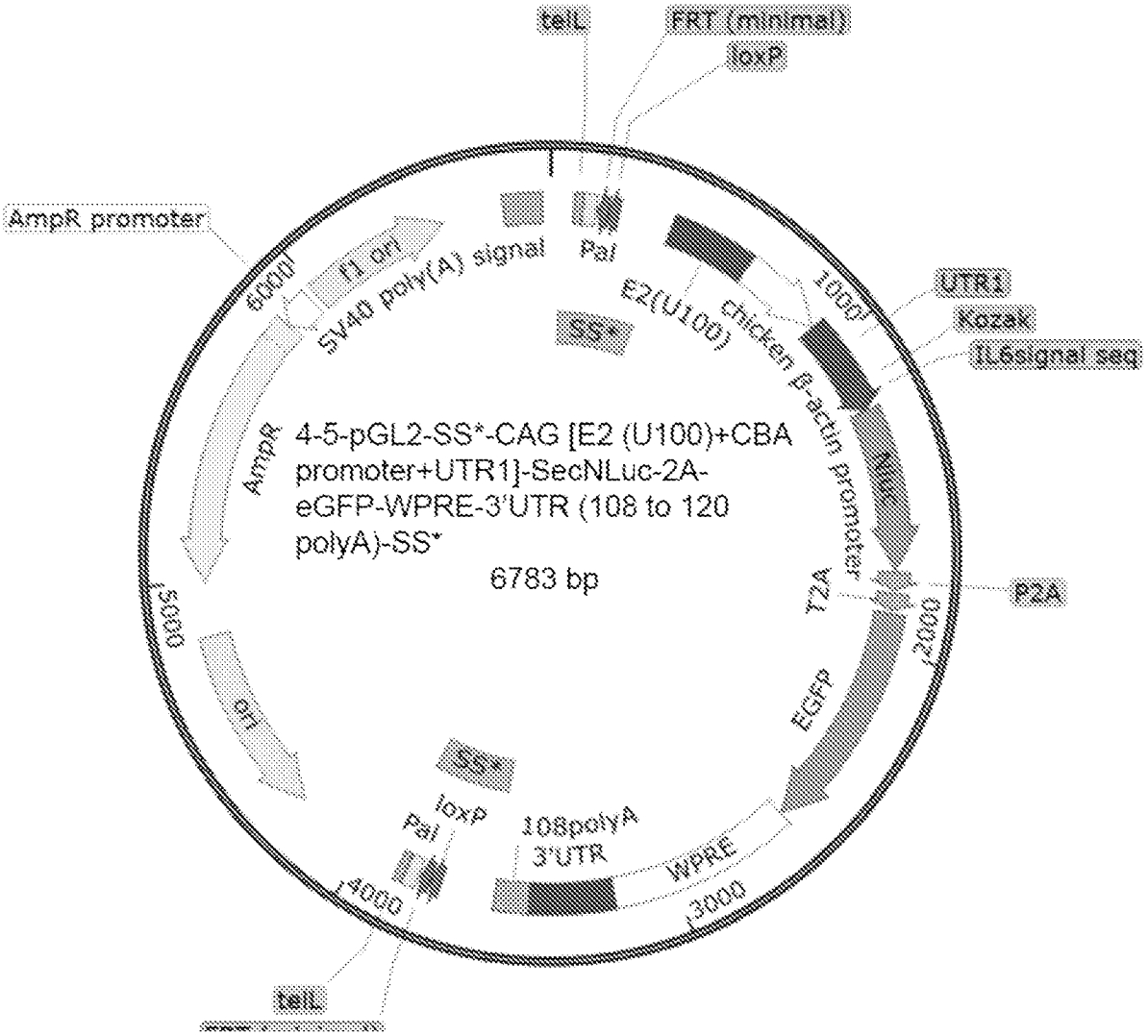

teiL
FRT (minimal)
loxP
AmpR promoter
6000
f1 ori
SV40 poly(A) signal
PaI
SS*
E2(U100)
chicken β-actin promoter
1000
UTR1
Kozak
IL6signal seq
Nluc
T2A
P2A
2000
EGFP
WPRE
3000
108polyA
3'UTR
SS*
loxP
PaI
4000
teiL
ori
5000
AmpR
4-5-pGL2-SS*-CAG [E2 (U100)+CBA promoter+UTR1]-SecNLuc-2A-eGFP-WPRE-3'UTR (108 to 120 polyA)-SS*
6783 bp

FIG. 47

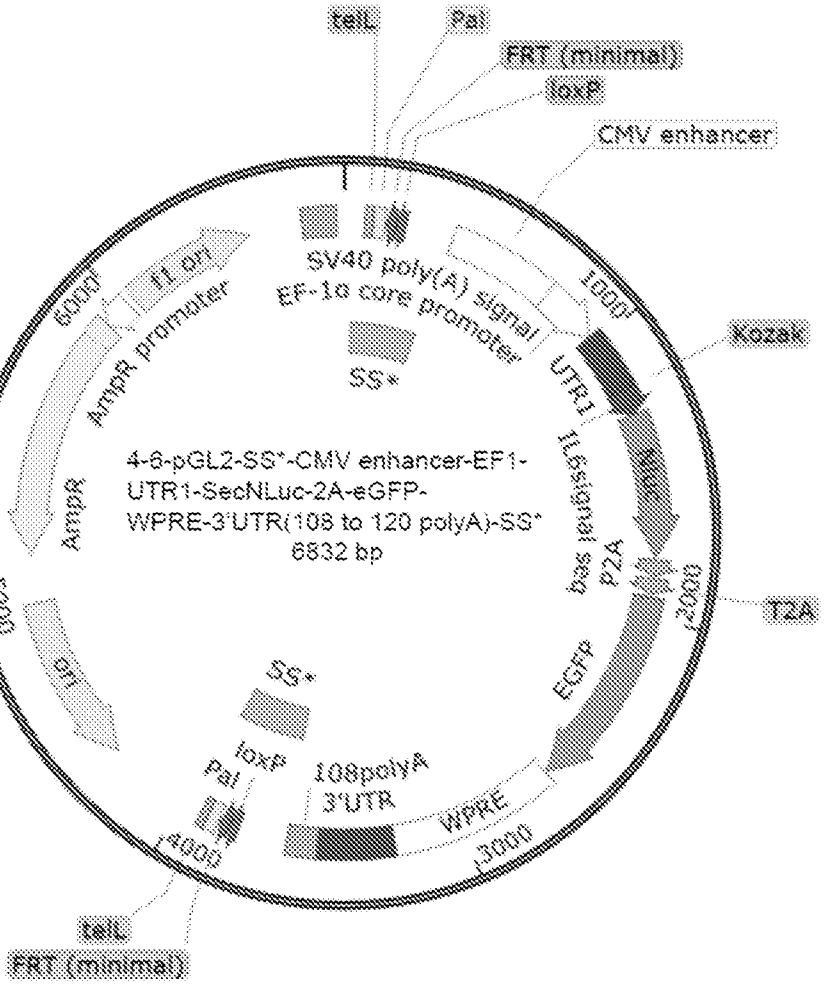

telL
PaI
FRT (minimal)
loxP
CMV enhancer
SV40 poly(A) signal
EF-1α core promoter
SS*
1000
Kozak
UTR1
1Lsignal seq
Nluc
d2A
2000
T2A
EGFP
WPRE
3000
108polyA
3'UTR
SS*
loxP
PaI
4000
telL
FRT (minimal)
3500
ori
AmpR
AmpR promoter
f1 ori
500
4-6-pGL2-SS*-CMV enhancer-EF1-
UTR1-SecNLuc-2A-eGFP-
WPRE-3'UTR(108 to 120 polyA)-SS*
6832 bp

FIG. 48

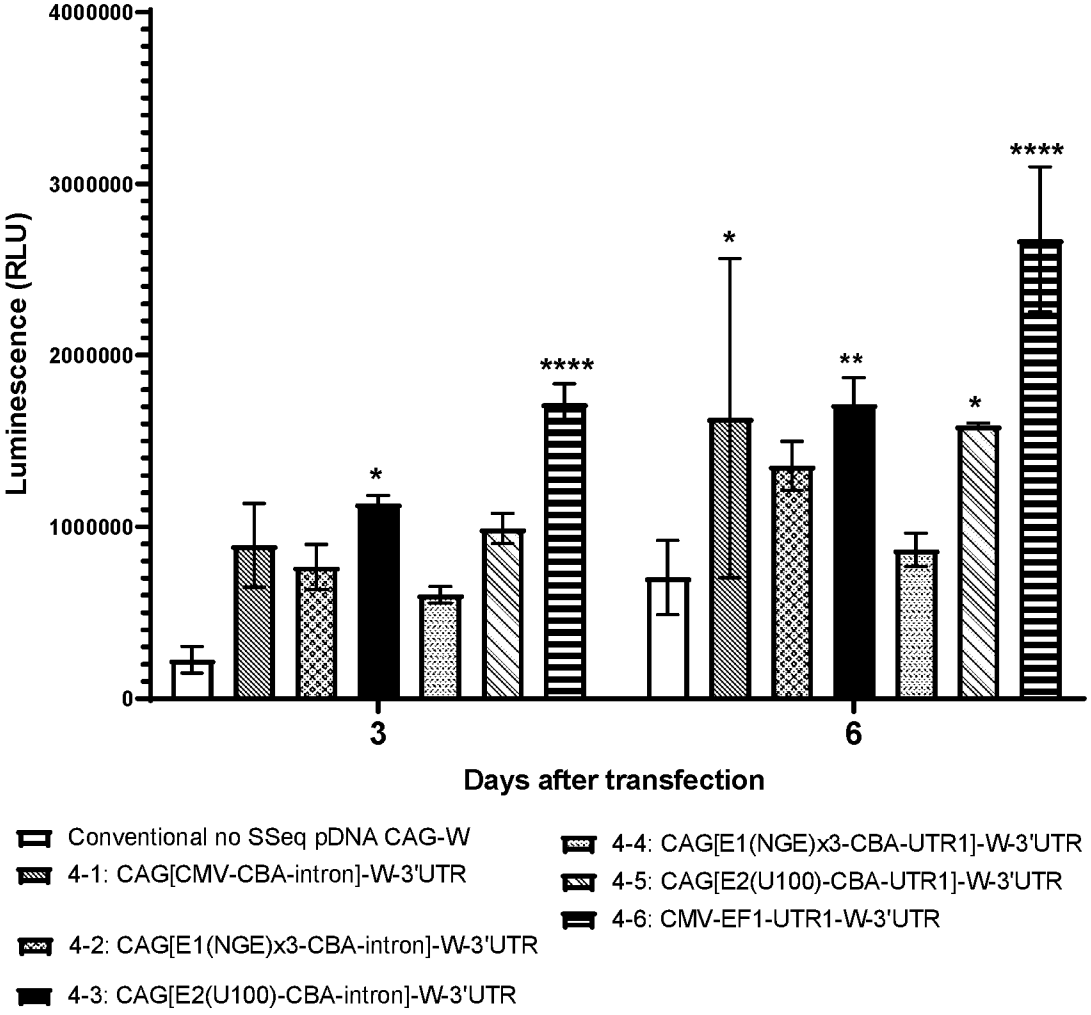

4000000
3000000
2000000
1000000
0
Luminescence (RLU)
*
****
*
**
*
****
3
6
Days after transfection
Conventional no SSeq pDNA CAG-W
4-1: CAG[CMV-CBA-intron]-W-3'UTR
4-2: CAG[E1(NGE)x3-CBA-intron]-W-3'UTR
4-3: CAG[E2(U100)-CBA-intron]-W-3'UTR
4-4: CAG[E1(NGE)x3-CBA-UTR1]-W-3'UTR
4-5: CAG[E2(U100)-CBA-UTR1]-W-3'UTR
4-6: CMV-EF1-UTR1-W-3'UTR

FIG. 49



FIG. 50

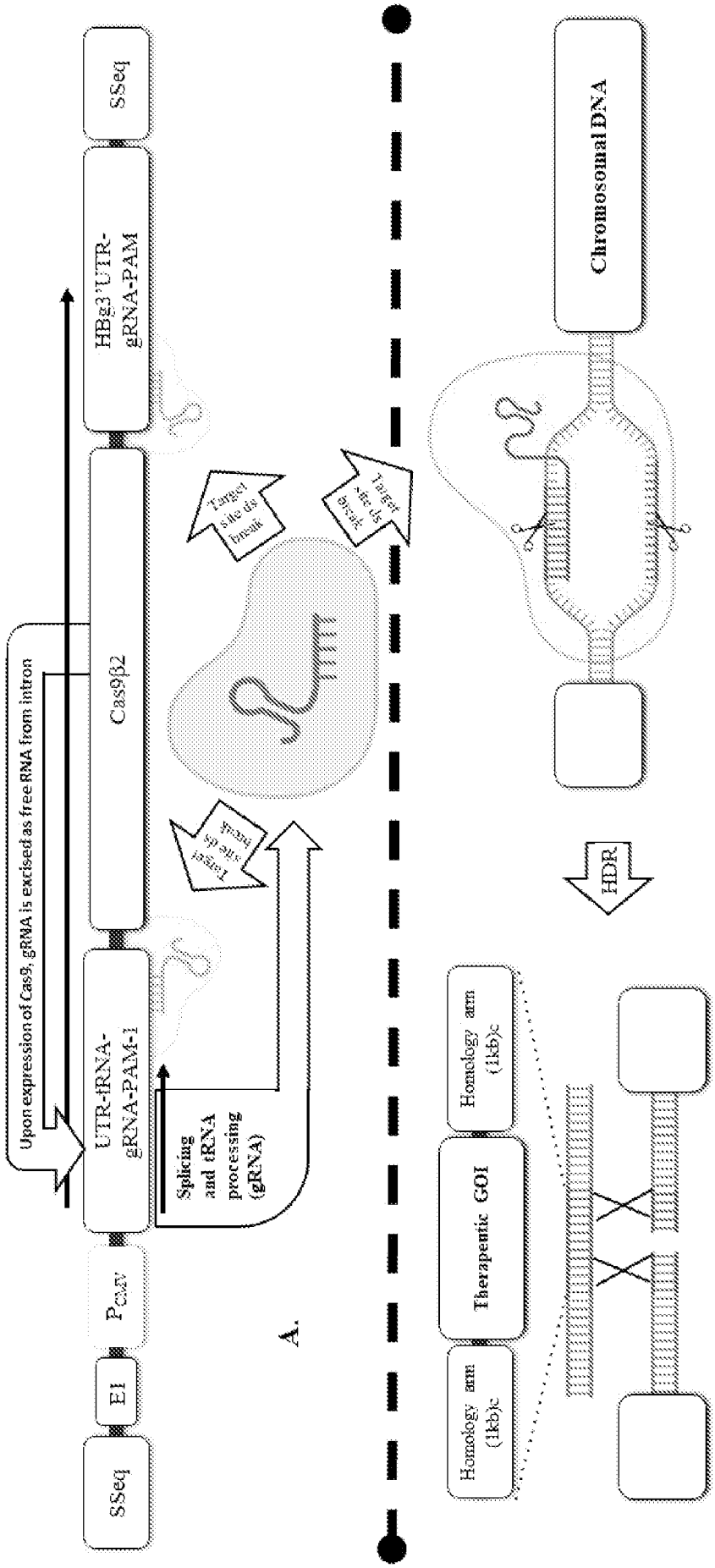
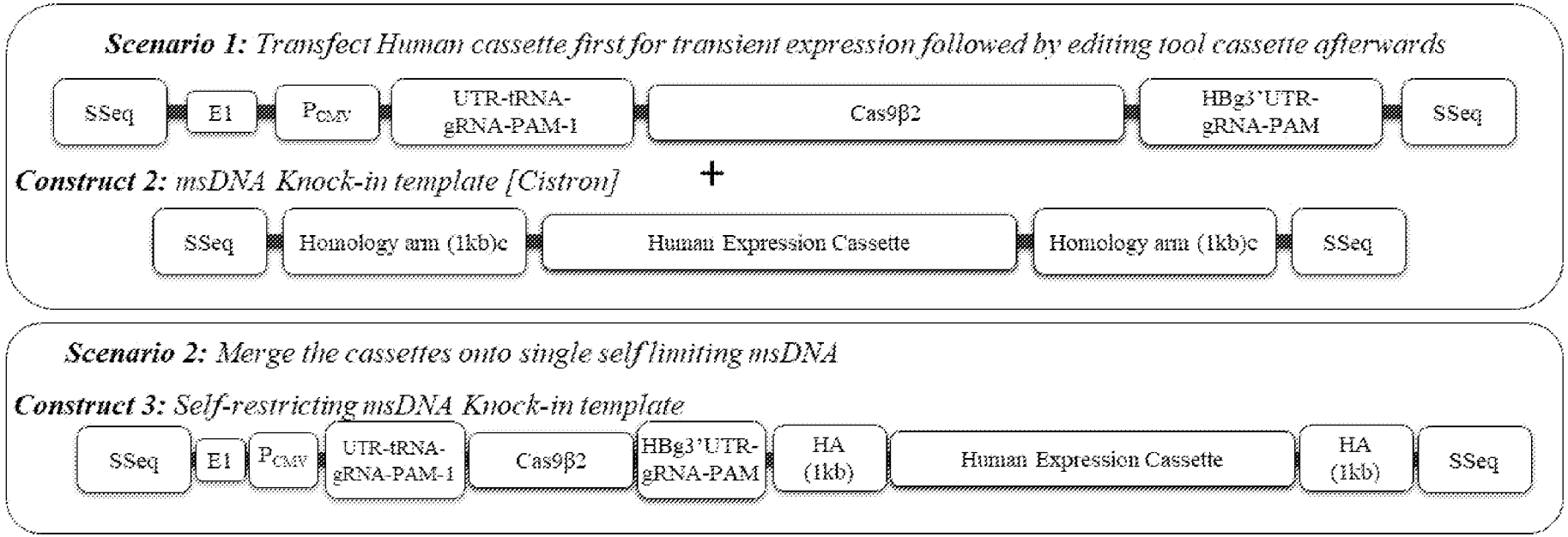

SSeq
E1
P_{CMV}
UTR-tRNA-gRNA-PAM-1
Cas9β2
HBg3'UTR-gRNA-PAM
SSeq
Upon expression of Cas9, gRNA is excised as free RNA from intron
Splicing and tRNA processing (gRNA)
Target site ds break
Target site ds break
TTTT
A.
Homology arm (1kb)c
Therapeutic GOI
Homology arm (1kb)c
HDR
Chromosomal DNA

FIG. 51


Scenario 1: Transfect Human cassette first for transient expression followed by editing tool cassette afterwards
SSeq
E1
P CMV
UTR-tRNA-gRNA-PAM-1
Cas9β2
HBg3'UTR-gRNA-PAM
SSeq
Construct 2: msDNA Knock-in template [Cistron]
+
SSeq
Homology arm (1kb)c
Human Expression Cassette
Homology arm (1kb)c
SSeq
Scenario 2: Merge the cassettes onto single self limiting msDNA
Construct 3: Self-restricting msDNA Knock-in template
SSeq
E1
P CMV
UTR-tRNA-gRNA-PAM-1
Cas9β2
HBg3'UTR-gRNA-PAM
HA (1kb)
Human Expression Cassette
HA (1kb)
SSeq