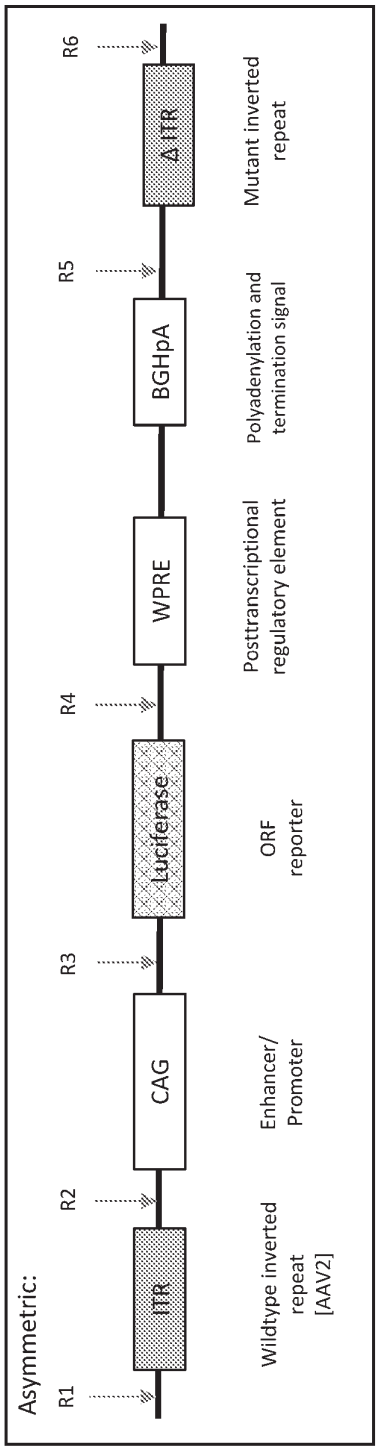


FIG. 1A

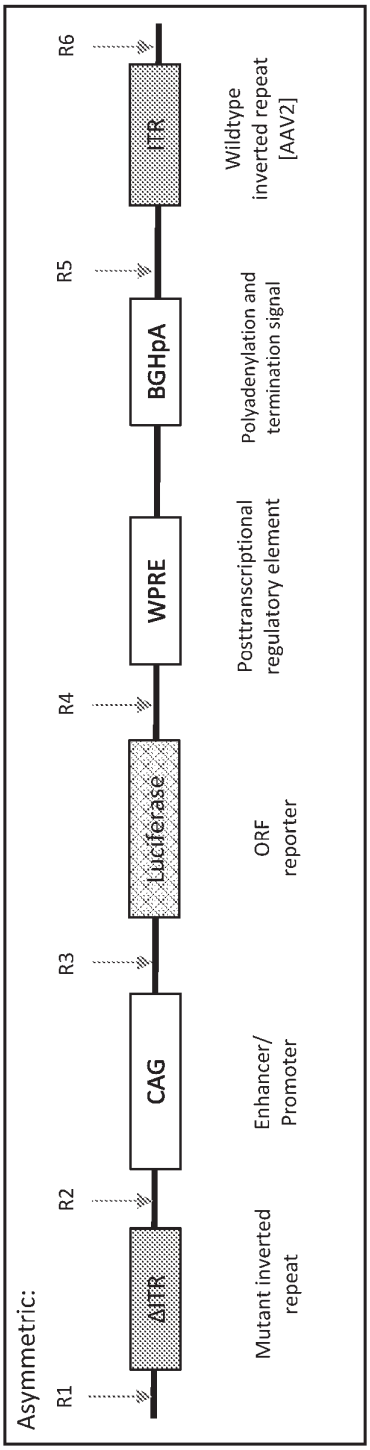


FIG. 1B

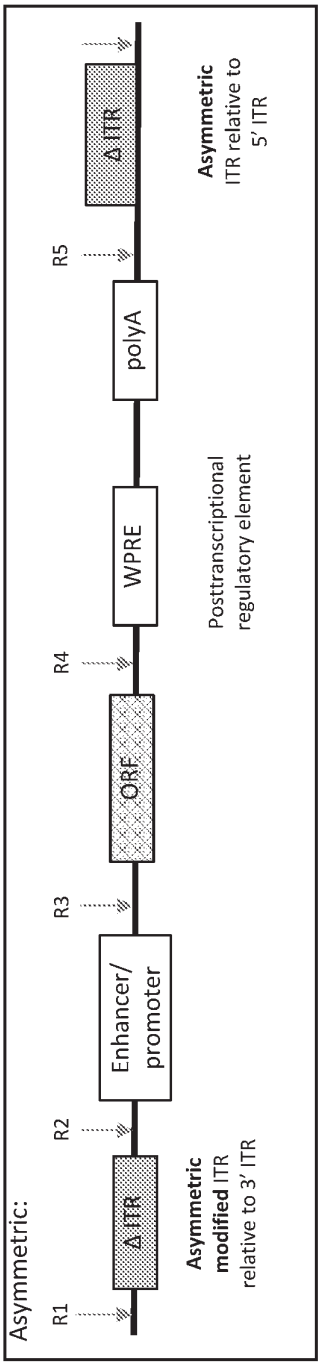


FIG. 1C

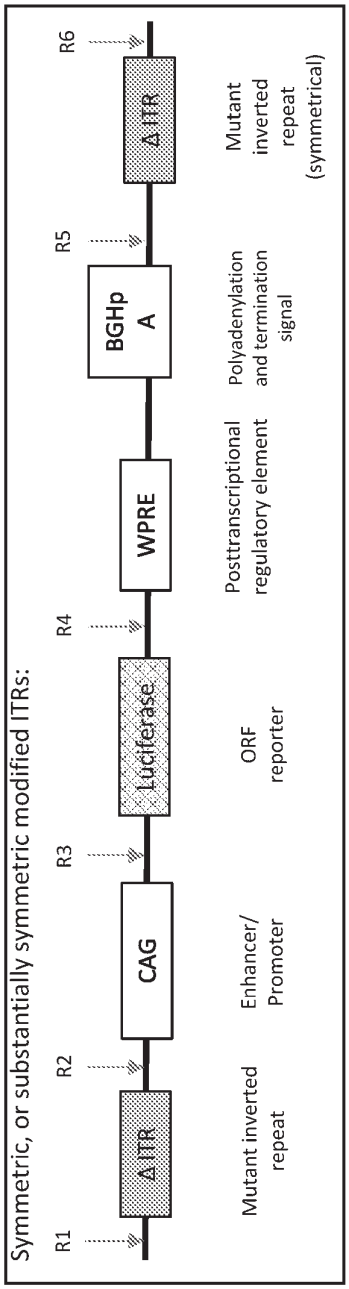

Symmetric, or substantially symmetric modified ITRs:
R1
R2
R3
R4
R5
R6
Δ ITR
CAG
Luciferase
WPRE
BGHp A
Δ ITR
Mutant inverted repeat
Enhancer/ Promoter
ORF reporter
Posttranscriptional regulatory element
Polyadenylation and termination signal
Mutant inverted repeat (symmetrical)

FIG. 1D

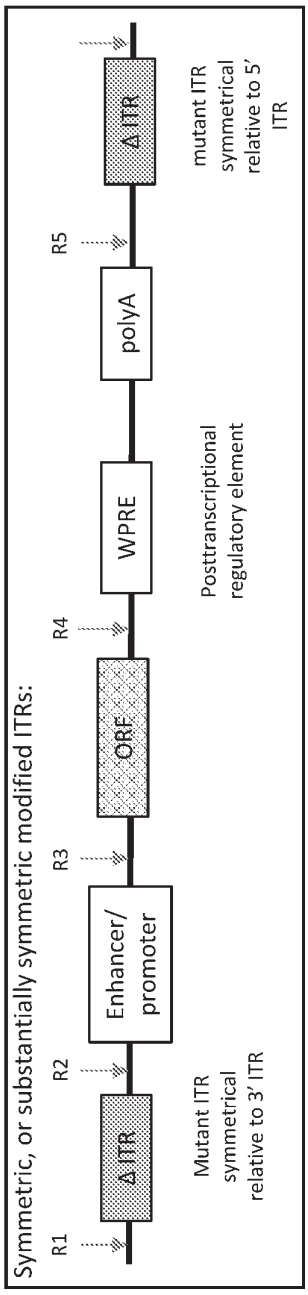

Symmetric, or substantially symmetric modified ITRs:
R1
R2
R3
R4
R5
Δ ITR
Enhancer/ promoter
ORF
WPRE
polyA
Δ ITR
Mutant ITR symmetrical relative to 3' ITR
Posttranscriptional regulatory element
mutant ITR symmetrical relative to 5' ITR

FIG. 1E

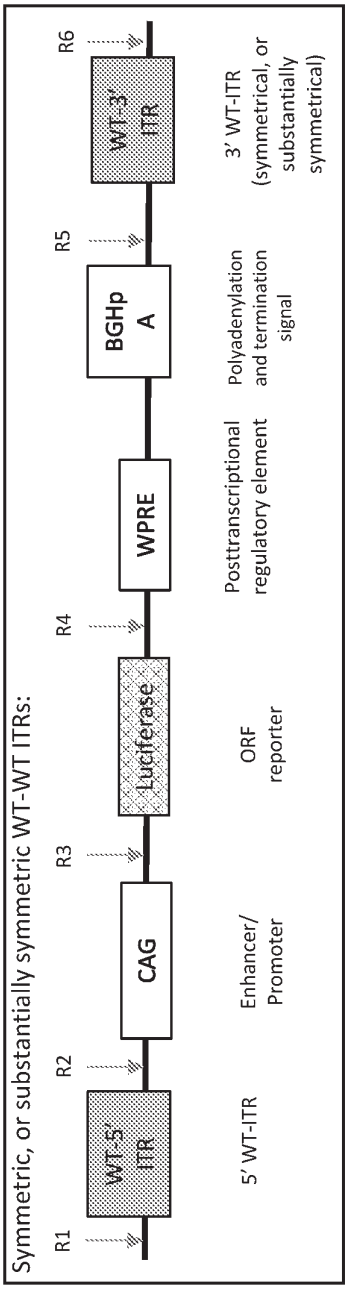

Symmetric, or substantially symmetric WT-WT ITRs:
R1
R2
R3
R4
R5
R6
WT-5' ITR
CAG
Luciferase
WPRE
BGHp A
WT-3' ITR
5' WT-ITR
Enhancer/ Promoter
ORF reporter
Posttranscriptional regulatory element
Polyadenylation and termination signal
3' WT-ITR (symmetrical, or substantially symmetrical)

FIG. 1F

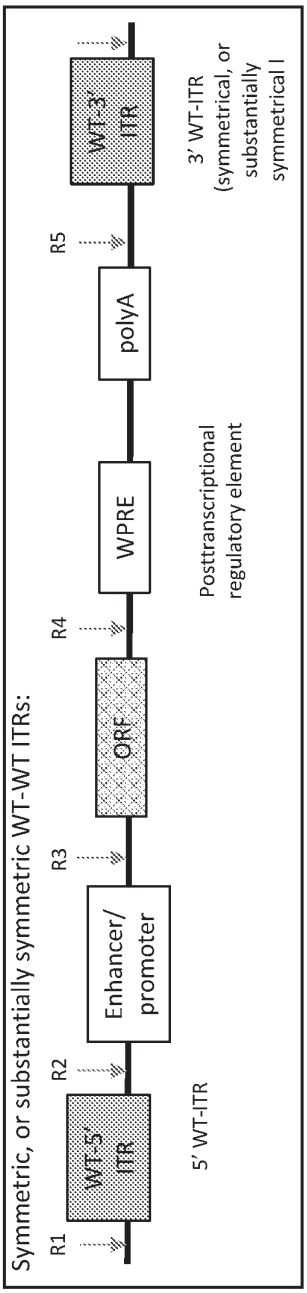

Symmetric, or substantially symmetric WT-WT ITRs:
R1
R2
R3
R4
R5
WT-5' ITR
Enhancer/ promoter
ORF
WPRE
polyA
WT-3' ITR
5' WT-ITR
Posttranscriptional regulatory element
3' WT-ITR (symmetrical, or substantially symmetrical I

FIG. 1G

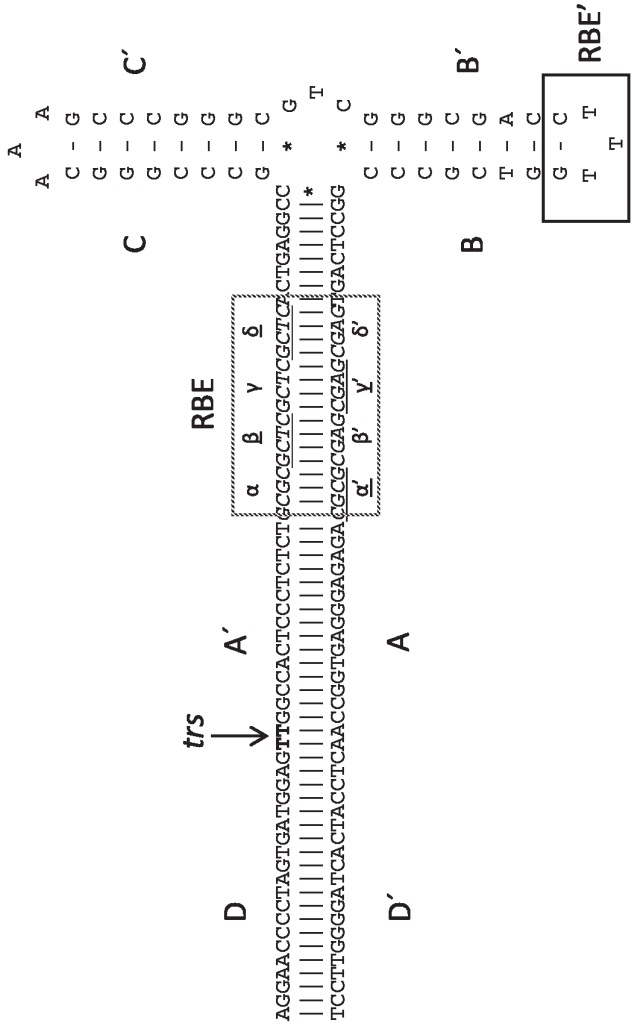


FIG. 2A

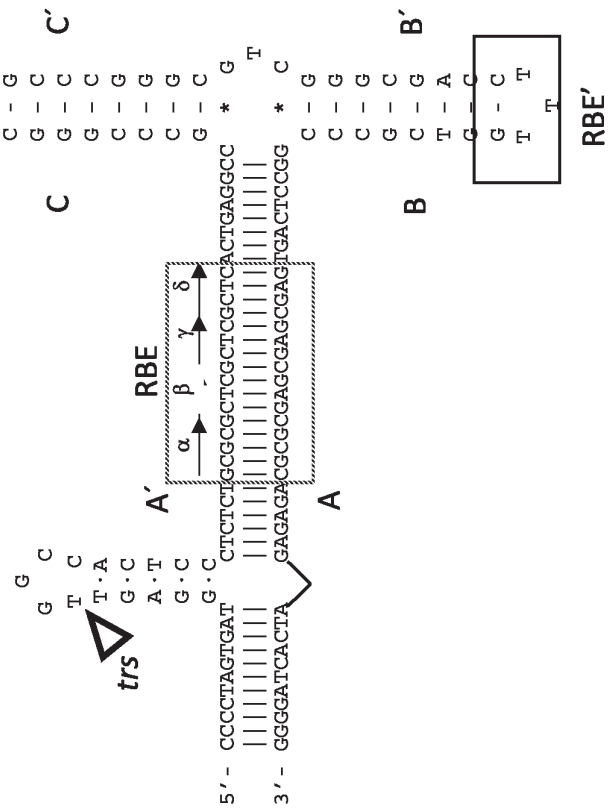

trs
RBE
A′
A
C
C′
B
B′
RBE′
α
β
γ
δ
5′- CCCCTAGTGAT
3′- GGGGATCACTA
CTCTCTGGCGCTCGCTCGCTCACTGAGGCC
GAGAGACCGCGCGAGCGAGCGAGTGACTCCGG

FIG. 2B

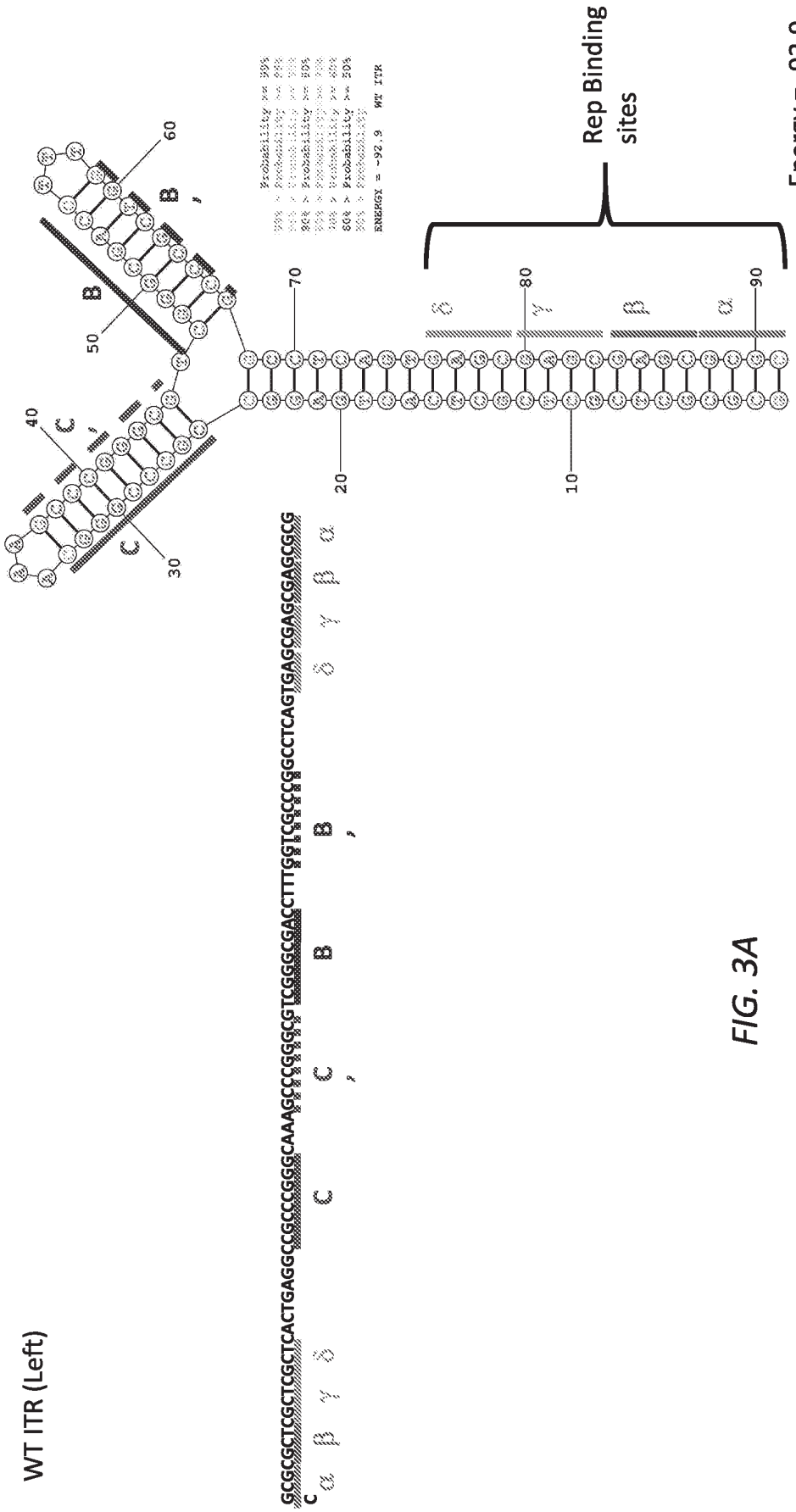
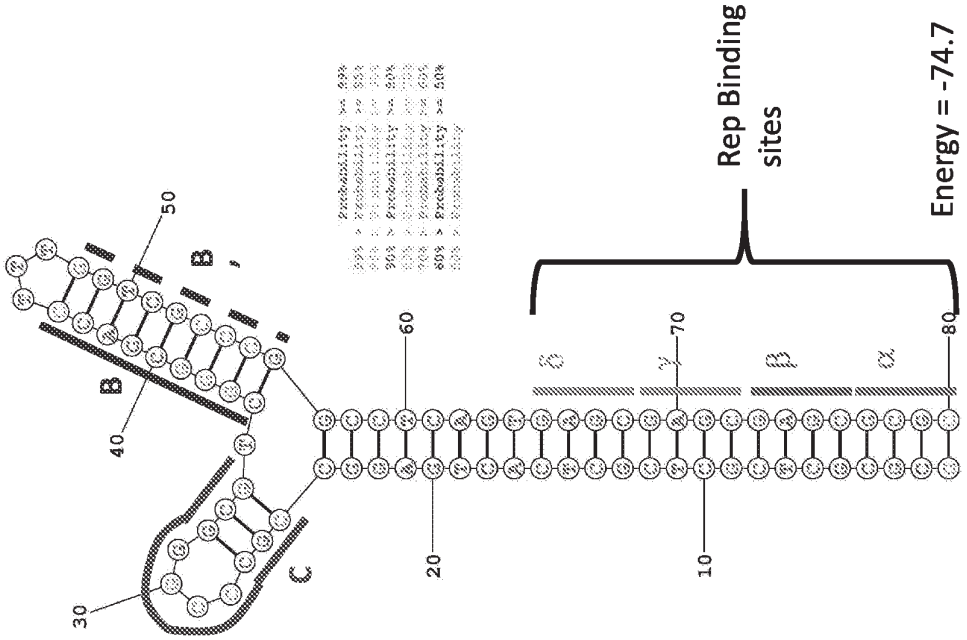

WT ITR (Left)
GCGCGCTCGCTCGCTCACTGAGGCCGCCCGGGCAAAGCCCGGGCGTCGGGCGACCTTTGGTCGCCCGGCCTCAGTGAGCGAGCGAGCGCG
c α β γ δ C B B' C' B B' δ γ β α
Rep Binding sites
Energy = -92.9

FIG. 3A



ITR-1, Left

C α β γ δ C B B' GCGCGCTCGCTCGCTCACTGAGGCCGCCCGGGCGTCGGGCGACCTTTGGTCGCCCGGCCTCAGTGAGCGAGCGAGCGCG δ γ β α

FIG. 3B

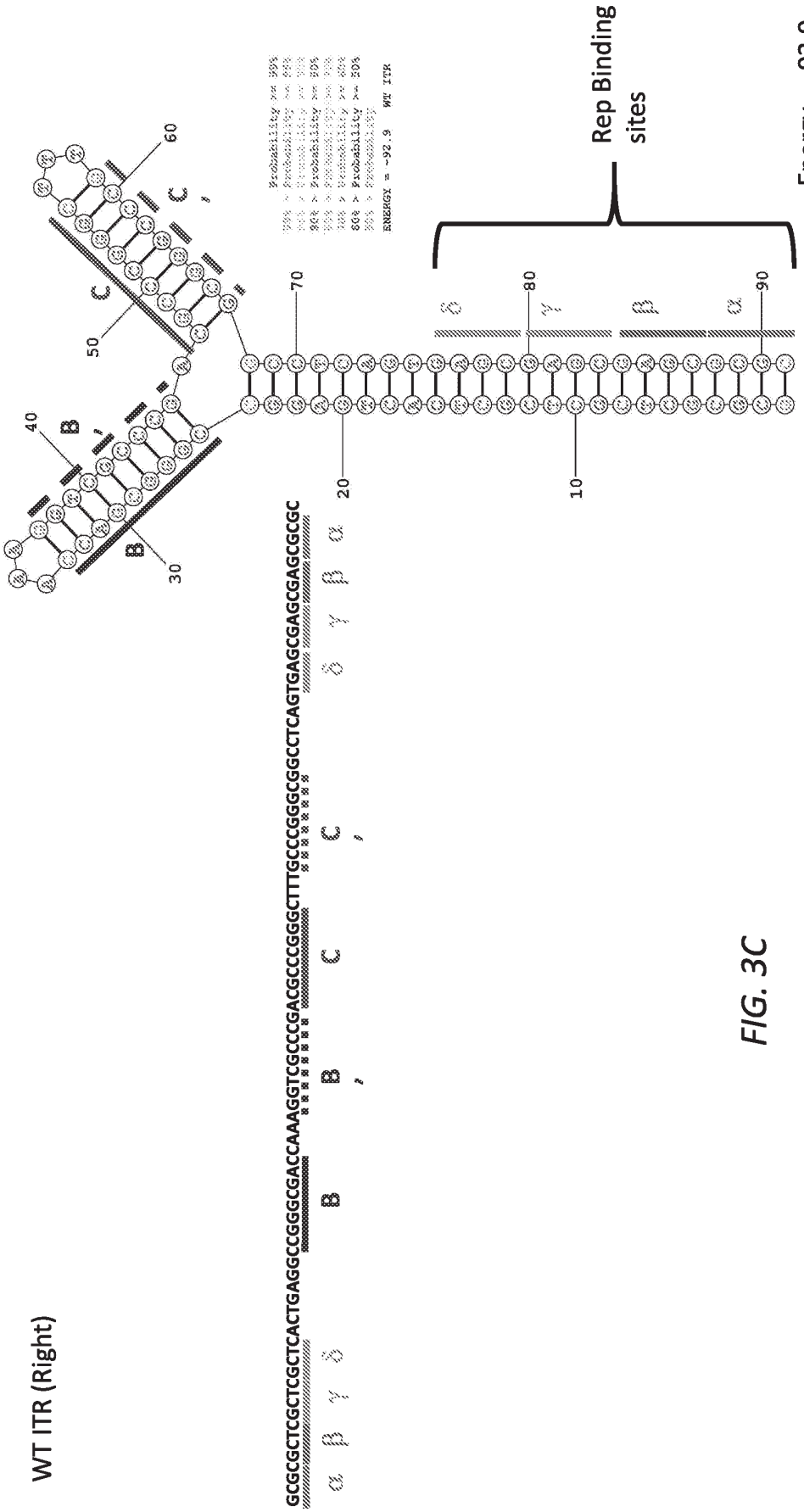
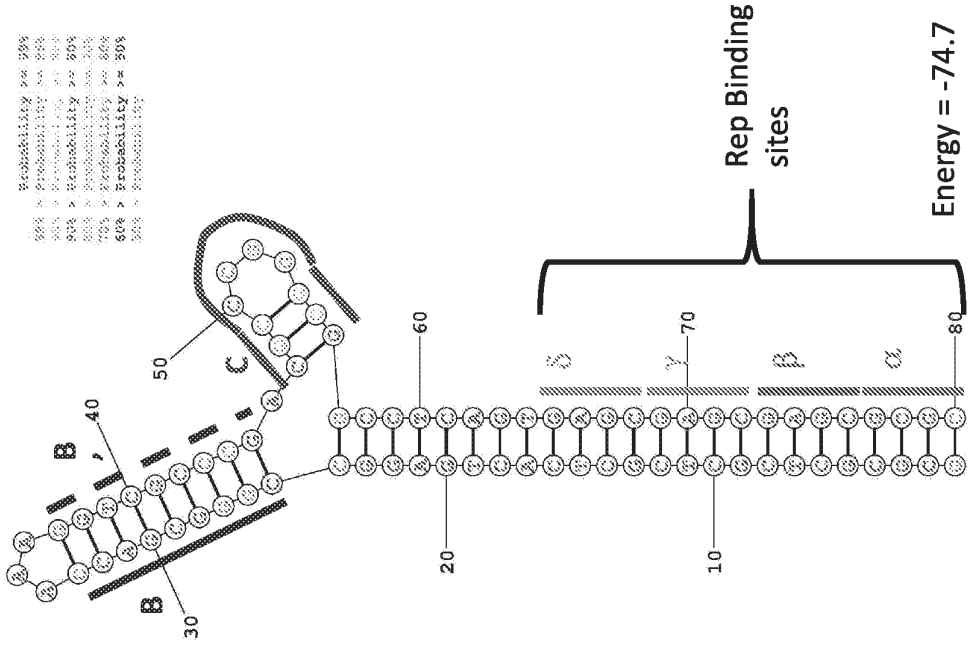

WT ITR (Right)
GCGCGCTCGCTCGCTCACTGAGGCCGGGCGACCAAAGGTCGCCCGACGCCCGGGCTTTGCCCGGGCGGCCTCAGTGAGCGAGCGAGCGCGC
α β γ δ B B C C' B' B'
Rep Binding sites
Energy = -92.9
ENERGY = -92.9 WT ITR
10
20
30
40
50
60
70
80
90

FIG. 3C



ITR-1, Right

GCGCGCTCGCTCGCTCACTGAGGCCGGGCGACCAAAGGTCGCCCGACGCCCGGGCGGCCTCAGTGAGCGAGCGAGCGCGC

α β γ δ B B' C δ γ β α

FIG. 3D

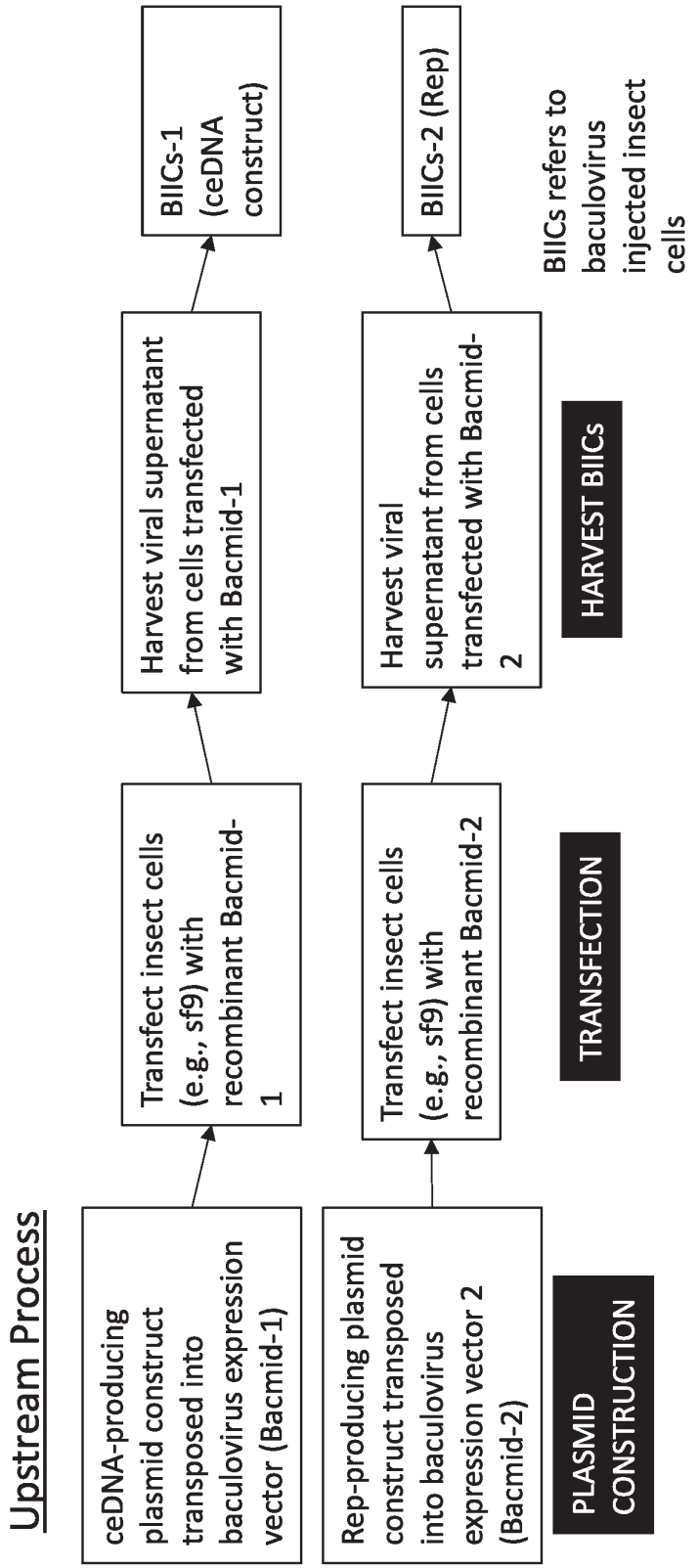

ceDNA Production
Upstream Process
ceDNA-producing plasmid construct transposed into baculovirus expression vector (Bacmid-1)
Rep-producing plasmid construct transposed into baculovirus expression vector 2 (Bacmid-2)
PLASMID CONSTRUCTION
Transfect insect cells (e.g., sf9) with recombinant Bacmid-1
Transfect insect cells (e.g., sf9) with recombinant Bacmid-2
TRANSFECTION
Harvest viral supernatant from cells transfected with Bacmid-1
Harvest viral supernatant from cells transfected with Bacmid-2
HARVEST BIICs
BIICs-1 (ceDNA construct)
BIICs-2 (Rep)
BIICs refers to baculovirus injected insect cells

FIG. 4A

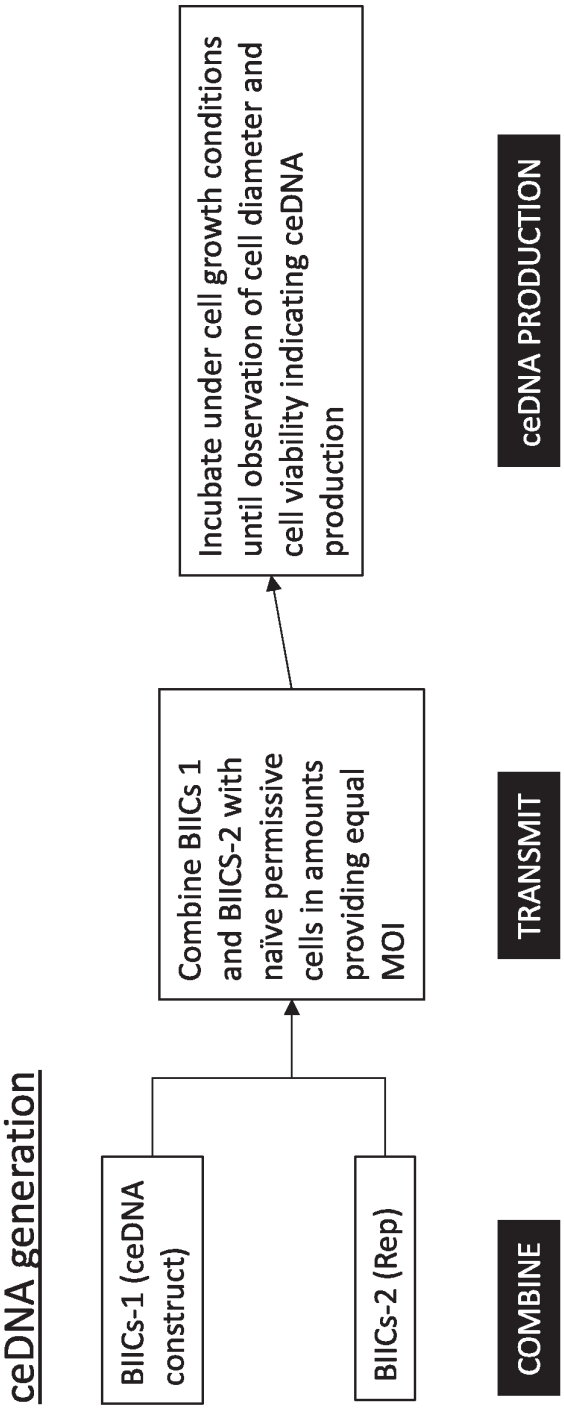

ceDNA Production
ceDNA generation
BIICs-1 (ceDNA construct)
BIICs-2 (Rep)
COMBINE
Combine BIICs 1 and BIICS-2 with naïve permissive cells in amounts providing equal MOI
TRANSMIT
Incubate under cell growth conditions until observation of cell diameter and cell viability indicating ceDNA production
ceDNA PRODUCTION

FIG. 4B

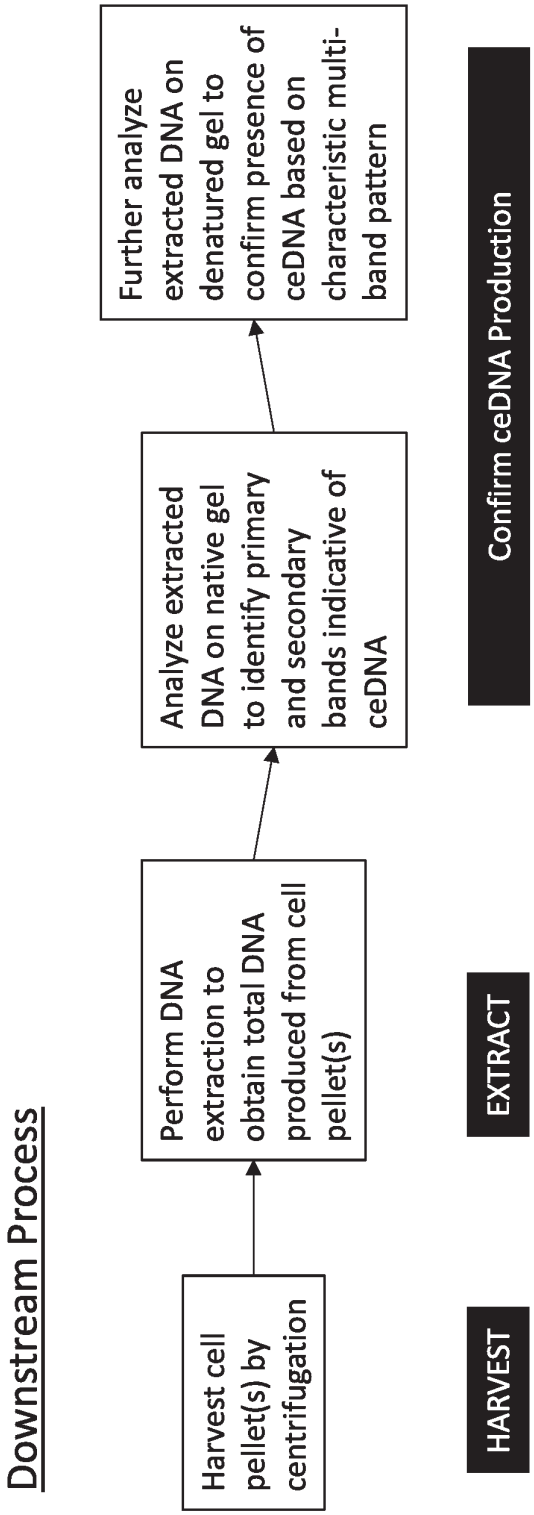

ceDNA Production
Downstream Process
Harvest cell pellet(s) by centrifugation
Perform DNA extraction to obtain total DNA produced from cell pellet(s)
Analyze extracted DNA on native gel to identify primary and secondary bands indicative of ceDNA
Further analyze extracted DNA on denatured gel to confirm presence of ceDNA based on characteristic multi-band pattern
HARVEST
EXTRACT
Confirm ceDNA Production

FIG. 4C

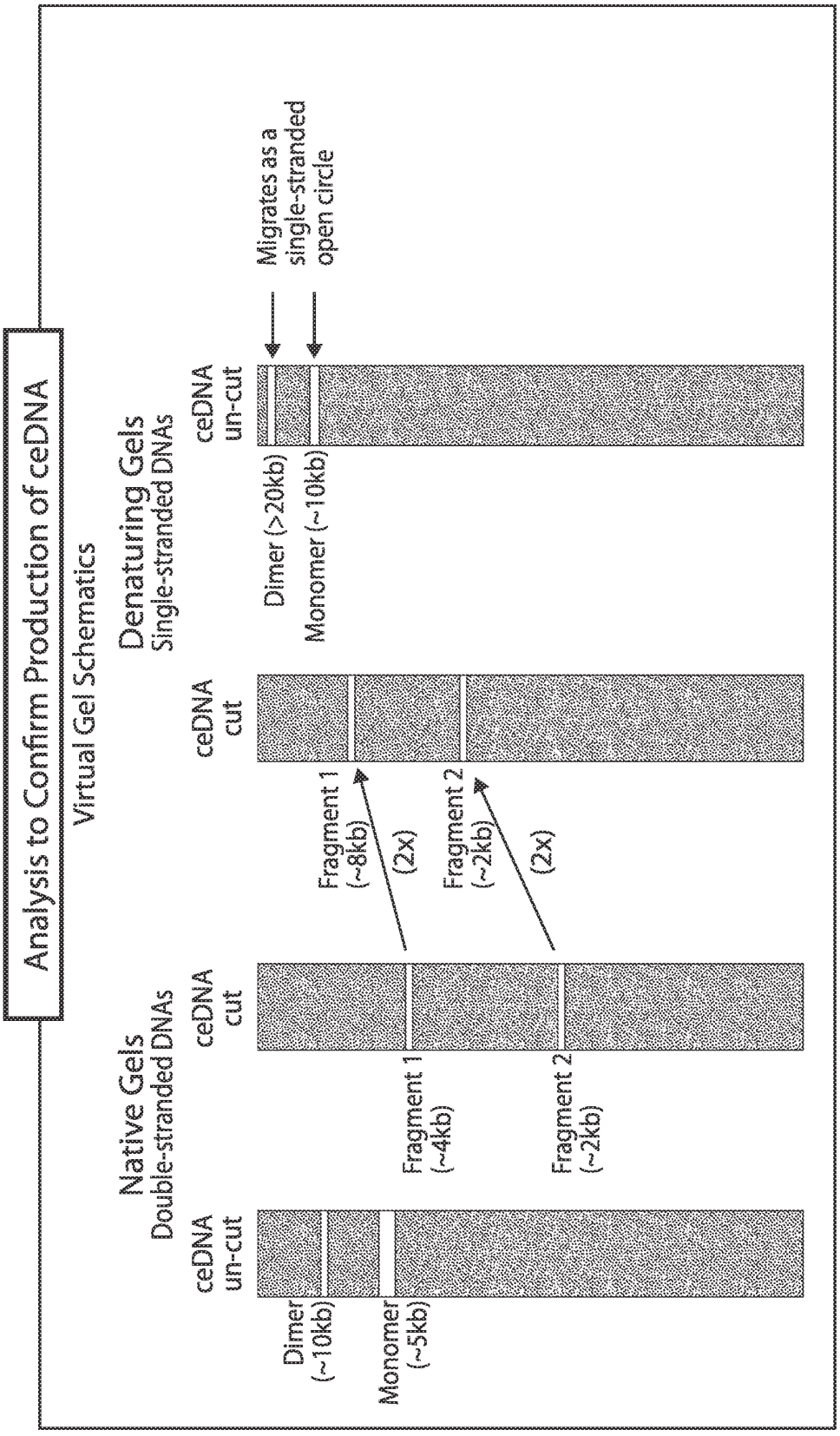

Analysis to Confirm Production of ceDNA
Virtual Gel Schematics
Native Gels
Double-stranded DNAs
ceDNA un-cut
ceDNA cut
Dimer (~10kb)
Monomer (~5kb)
Fragment 1 (~4kb)
Fragment 2 (~2kb)
Fragment 1 (~8kb)
(2x)
Fragment 2 (~2kb)
(2x)
Denaturing Gels
Single-stranded DNAs
ceDNA un-cut
ceDNA cut
Dimer (>20kb)
Monomer (~10kb)
Migrates as a single-stranded open circle

FIG. 4D

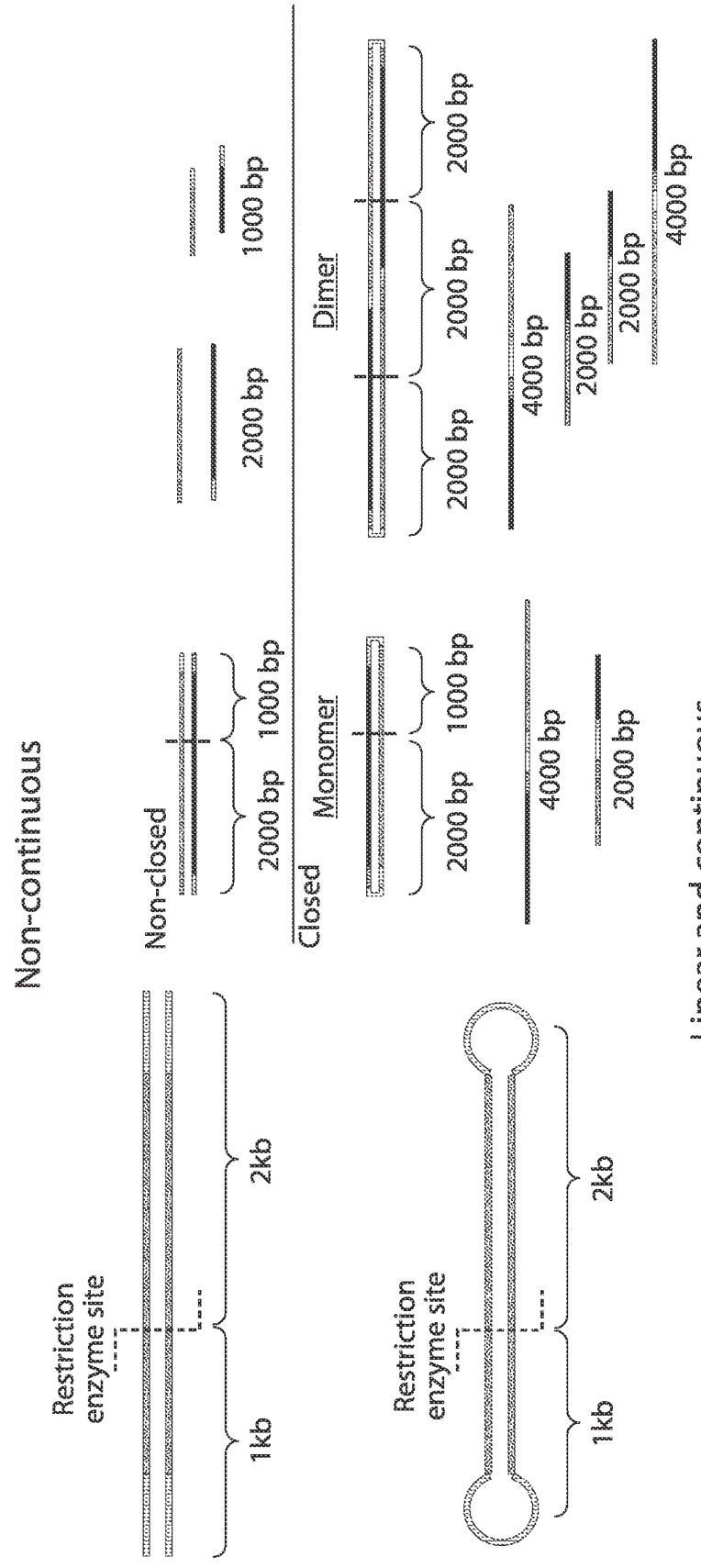

Non-continuous
Restriction enzyme site
1kb
2kb
Non-closed
2000 bp
1000 bp
Closed
Monomer
2000 bp
1000 bp
Dimer
2000 bp
2000 bp
2000 bp
Linear and continuous
Restriction enzyme site
1kb
2kb
4000 bp
2000 bp
4000 bp
2000 bp
2000 bp
4000 bp

FIG. 4E

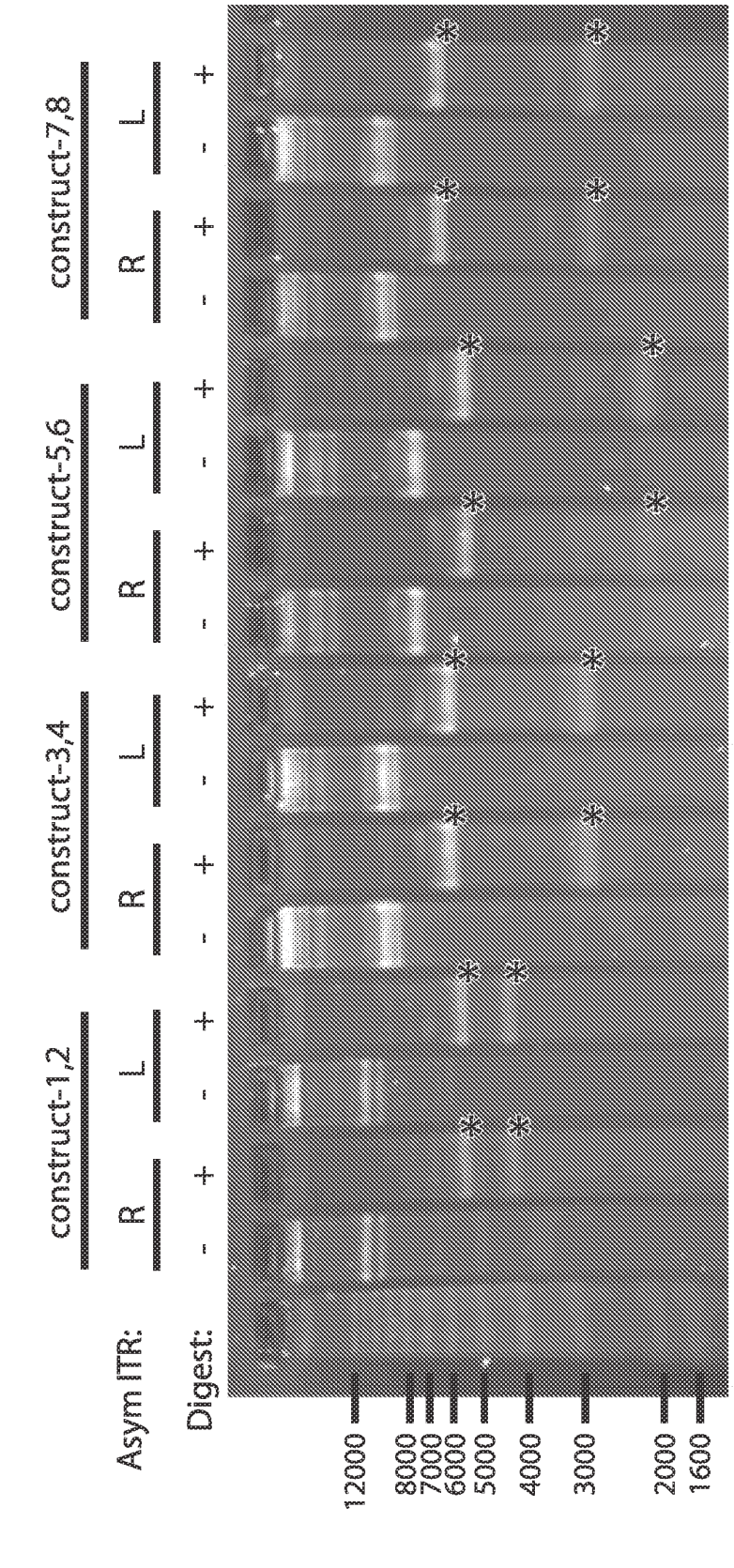

construct-1,2
construct-3,4
construct-5,6
construct-7,8
Asym ITR:
R L R L R L R L
Digest: - + - + - + - + - + - + - + - +
12000
8000
7000
6000
5000
4000
3000
2000
1600
ceDNA fragment sizes:
EcoRI 4172/5506
BamHI 2600/5776
SpeI 1790/5136
XhoI 2376/5800

FIG. 5

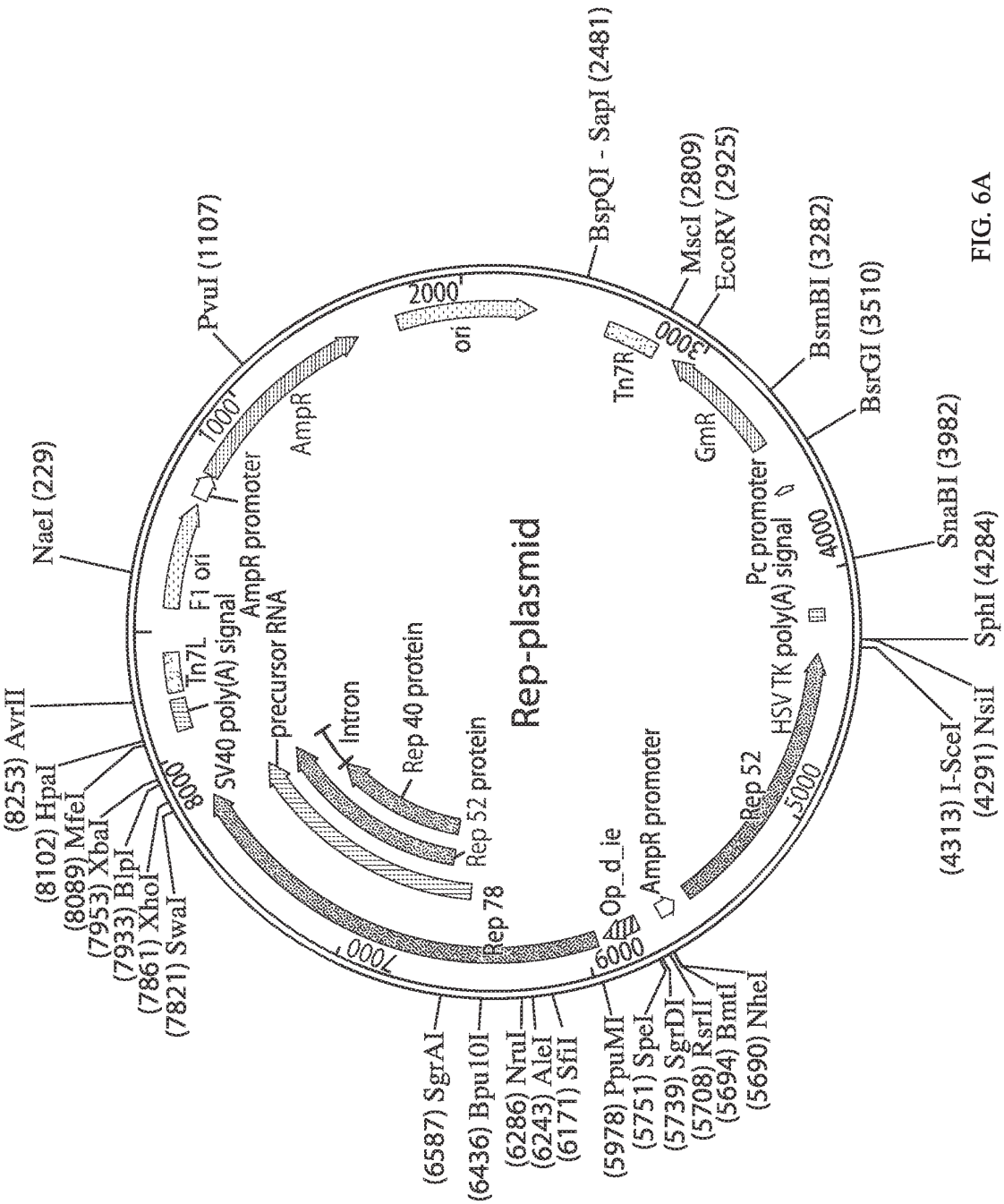

Rep-plasmid
1000
2000
3000
4000
5000
6000
7000
8000
ori
Tn7R
GmR
Pc promoter
HSV TK poly(A) signal
Rep 52
AmpR promoter
Op_d_ie
Rep 78
Rep 52 protein
Rep 40 protein
Intron
precursor RNA
AmpR
AmpR promoter
SV40 poly(A) signal
Tn7L
F1 ori
PvuI (1107)
BspQI - SapI (2481)
MscI (2809)
EcoRV (2925)
BsmBI (3282)
BsrGI (3510)
SnaBI (3982)
SphI (4284)
NsiI (4291)
I-SceI (4313)
NaeI (229)
AvrII (8253)
HpaI (8102)
MfeI (8089)
XbaI (7953)
BlpI (7933)
XhoI (7861)
SwaI (7821)
SgrAI (6587)
Bpu10I (6436)
NruI (6286)
AleI (6243)
SfiI (6171)
PpuMI (5978)
SpeI (5751)
SgrDI (5739)
RsrII (5708)
BmtI (5694)
NheI (5690)

FIG. 6A

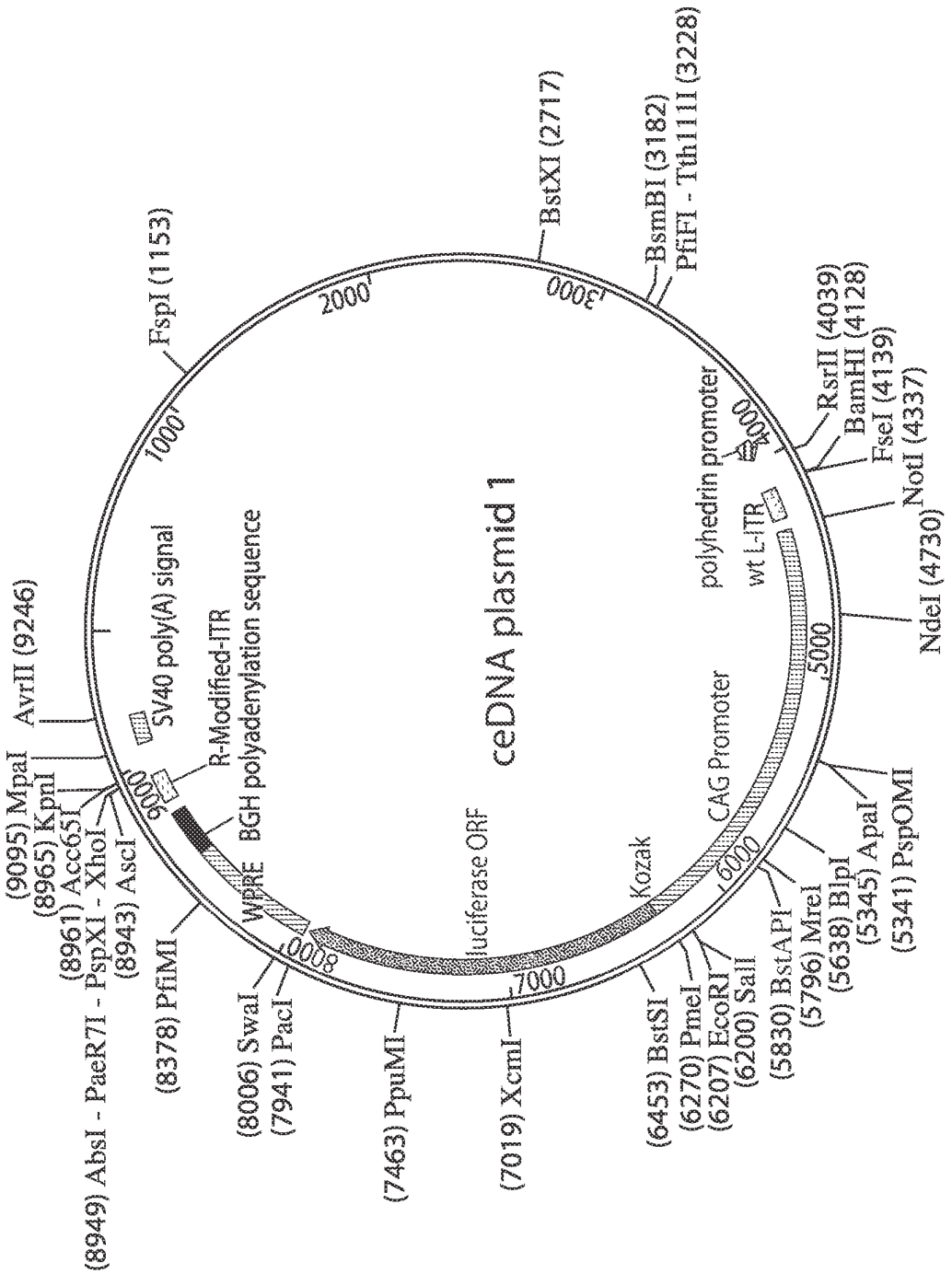

ceDNA plasmid 1
1000
2000
3000
4000
5000
6000
7000
8000
9000
FspI (1153)
BstXI (2717)
BsmBI (3182)
PfiFI - Tth111I (3228)
RsrII (4039)
BamHI (4128)
FseI (4139)
NotI (4337)
NdeI (4730)
(5341) PspOMI
(5345) ApaI
(5638) BlpI
(5796) MreI
(5830) BstAPI
(6200) SalI
(6207) EcoRI
(6270) PmeI
(6453) BstSI
(7019) XcmI
(7463) PpuMI
(7941) PacI
(8006) SwaI
(8378) PfiMI
(8943) AscI
(8949) AbsI - PaeR7I - PspXI - XhoI
(8961) Acc65I
(8965) KpnI
(9095) MpaI
AvrII (9246)
SV40 poly(A) signal
R-Modified-ITR
BGH polyadenylation sequence
WPRE
luciferase ORF
Kozak
CAG Promoter
polyhedrin promoter
wt L-ITR

FIG. 6B

FIG 7A -1

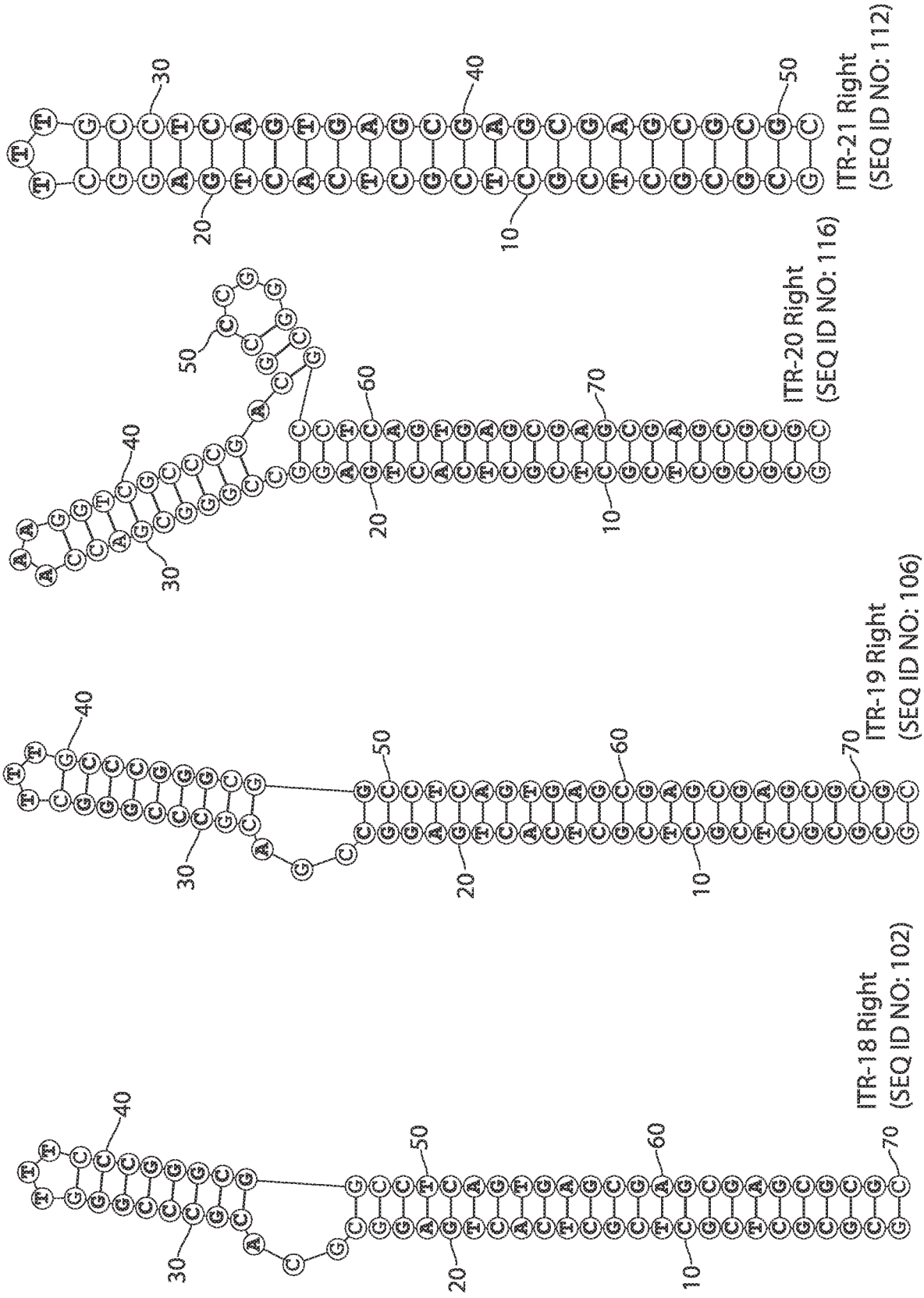
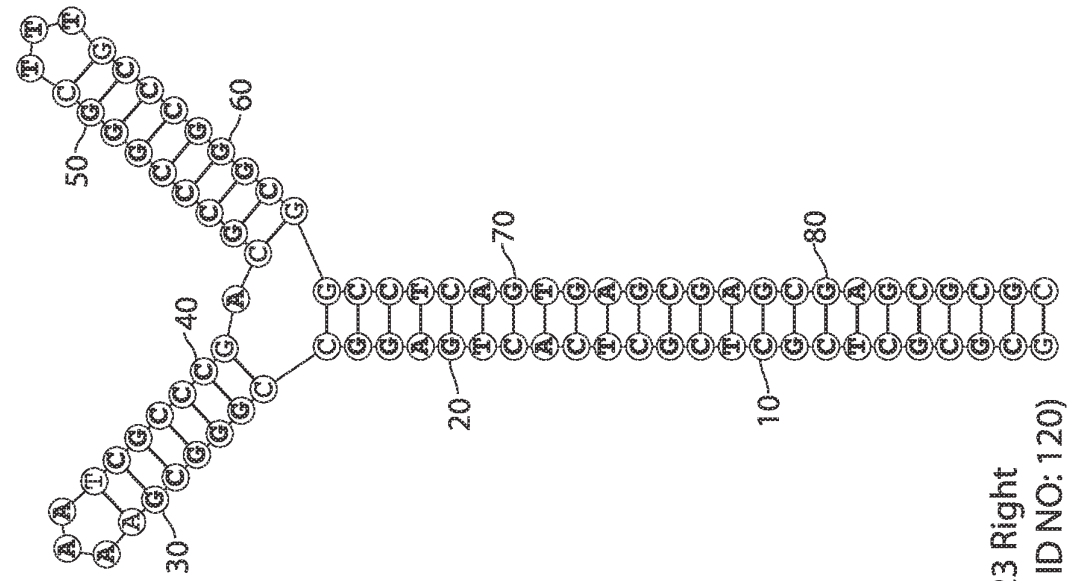

ITR-21 Right
(SEQ ID NO: 112)
ITR-20 Right
(SEQ ID NO: 116)
ITR-19 Right
(SEQ ID NO: 106)
ITR-18 Right
(SEQ ID NO: 102)

FIG. 7A-2


ITR-23 Right
(SEQ ID NO: 120)
10
20
30
40
50
60
70
80

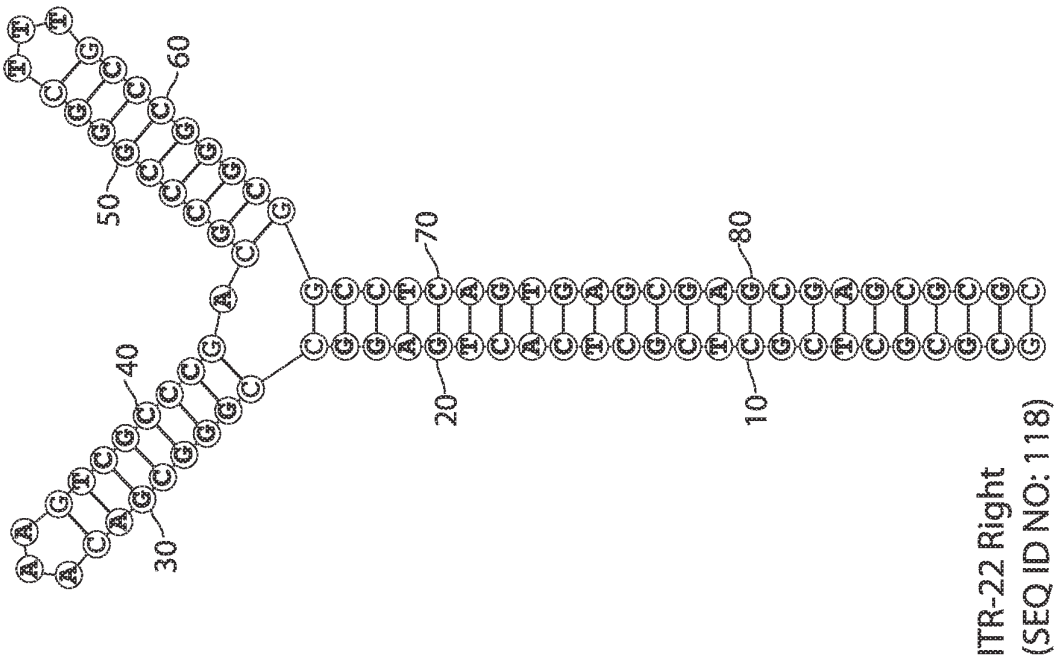
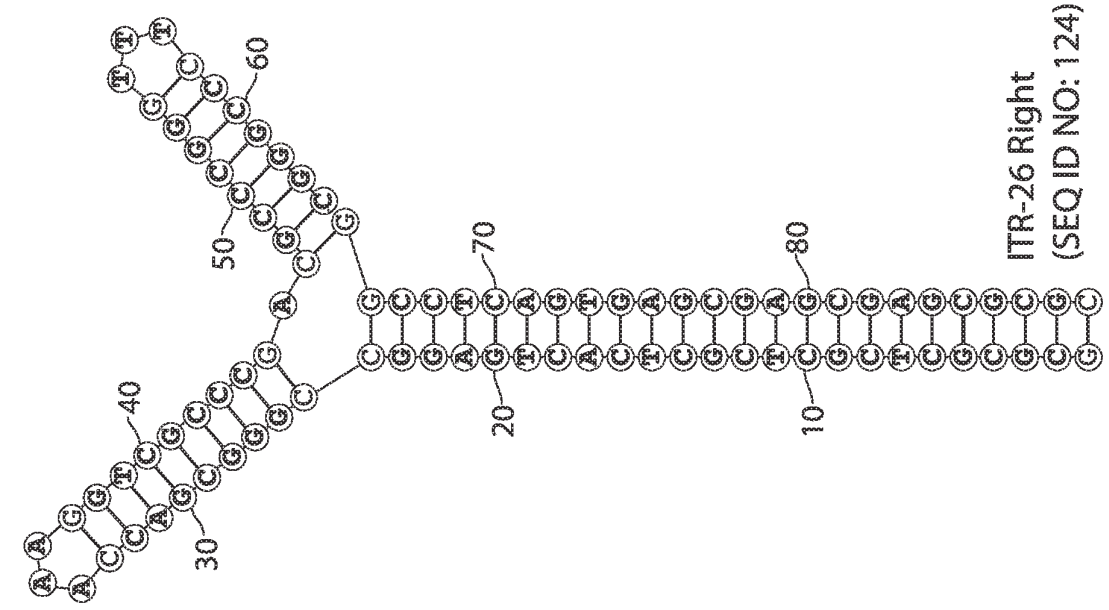

ITR-22 Right
(SEQ ID NO: 118)
10
20
30
40
50
60
70
80

FIG. 7A-3


ITR-26 Right
(SEQ ID NO: 124)
10
20
30
40
50
60
70
80

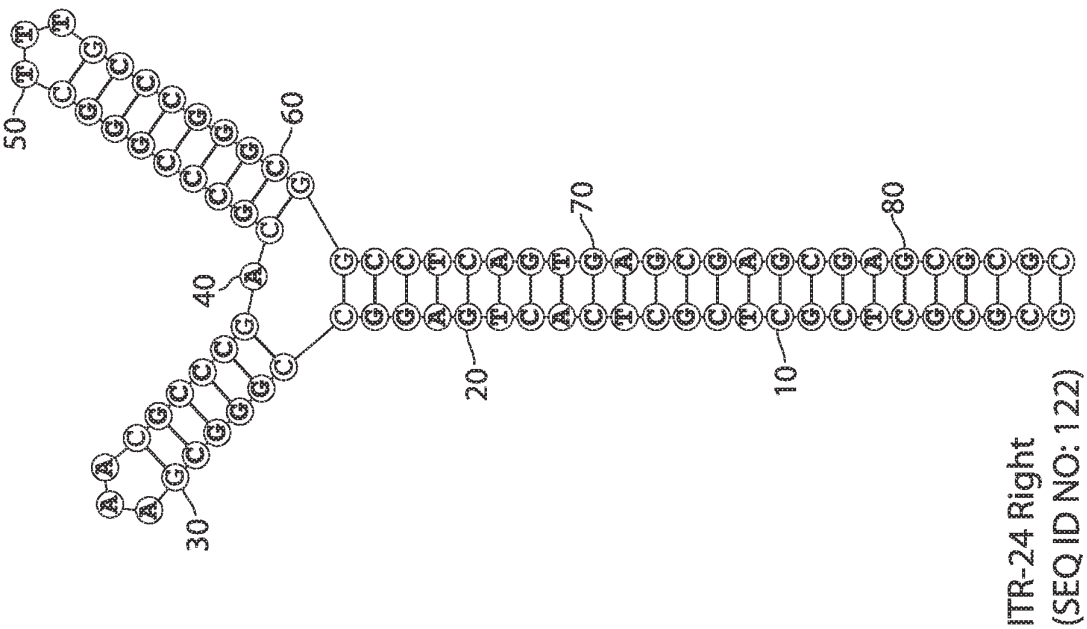
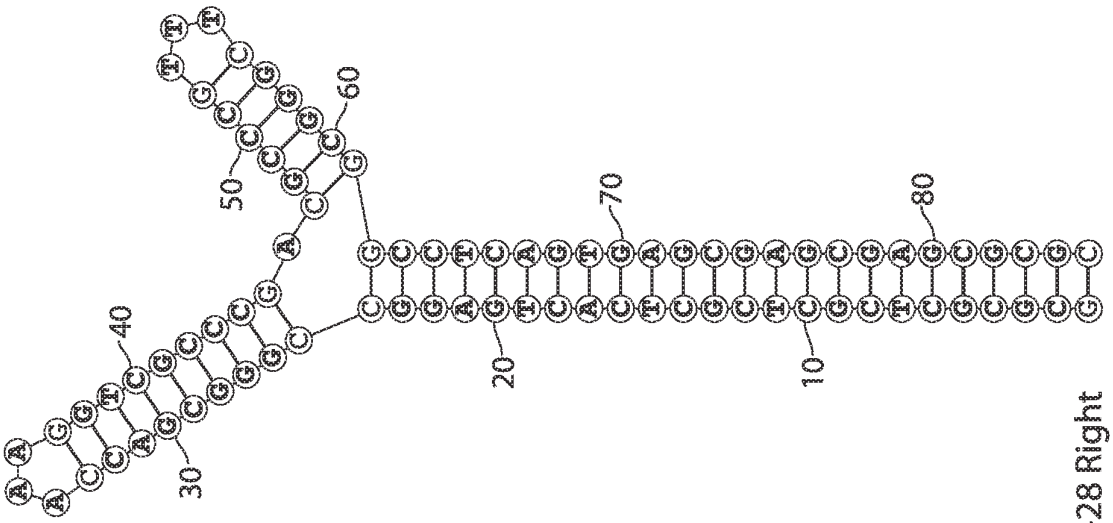
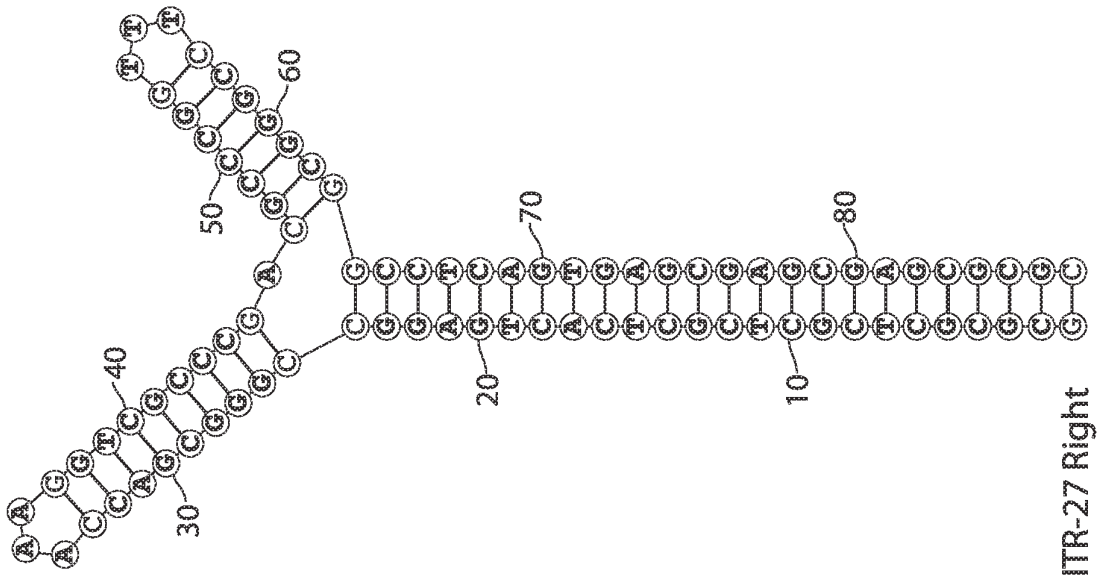

ITR-24 Right
(SEQ ID NO: 122)
10
20
30
40
50
60
70
80

FIG. 7A-4

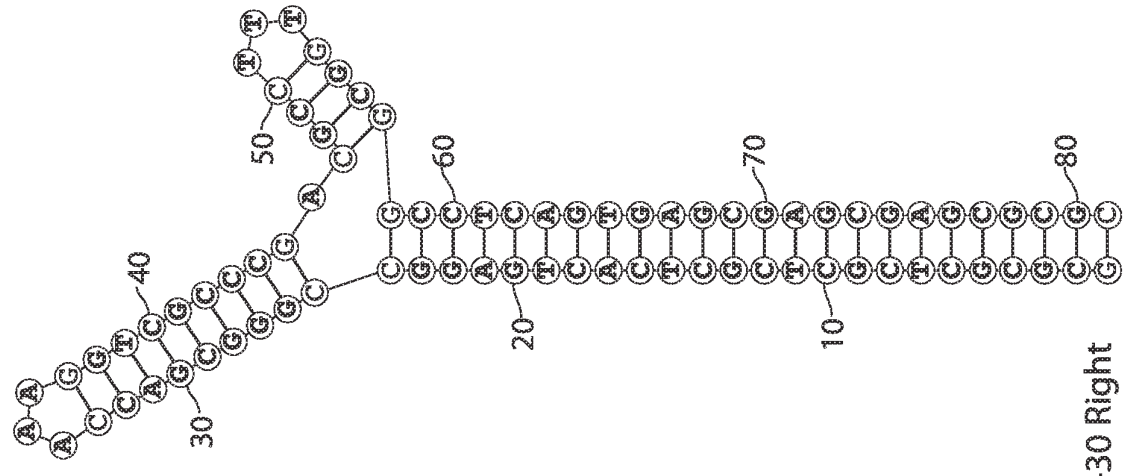


ITR-28 Right
(SEQ ID NO: 128)

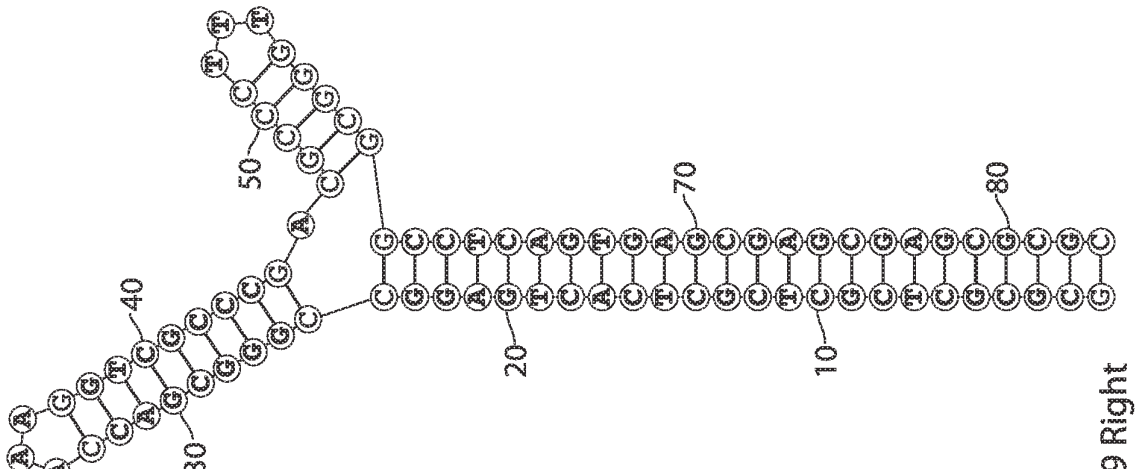


ITR-27 Right
(SEQ ID NO: 126)

FIG. 7A-5

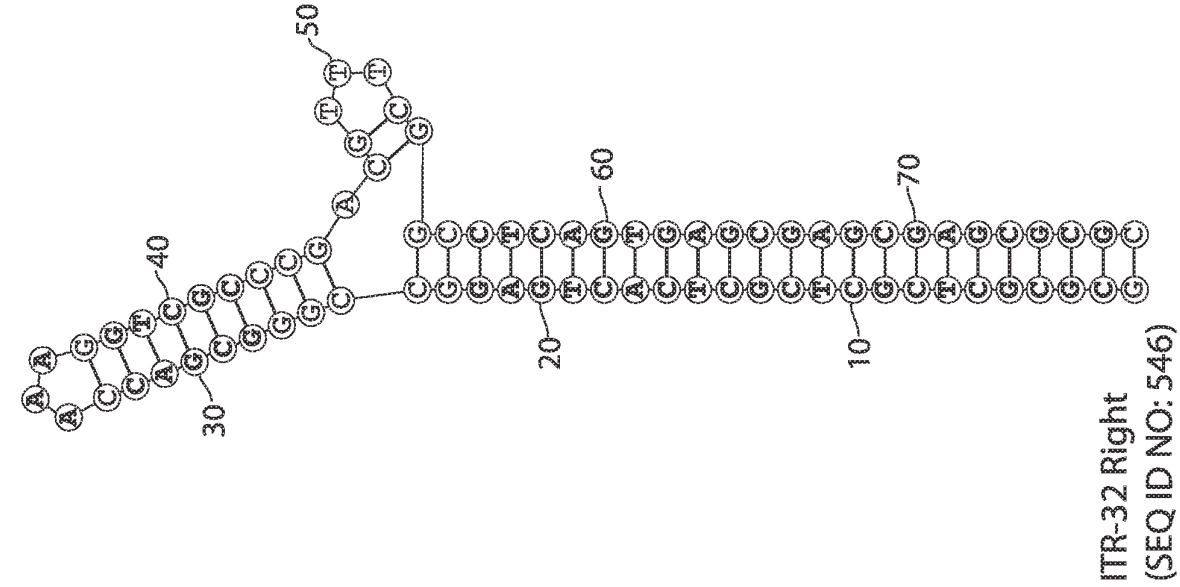


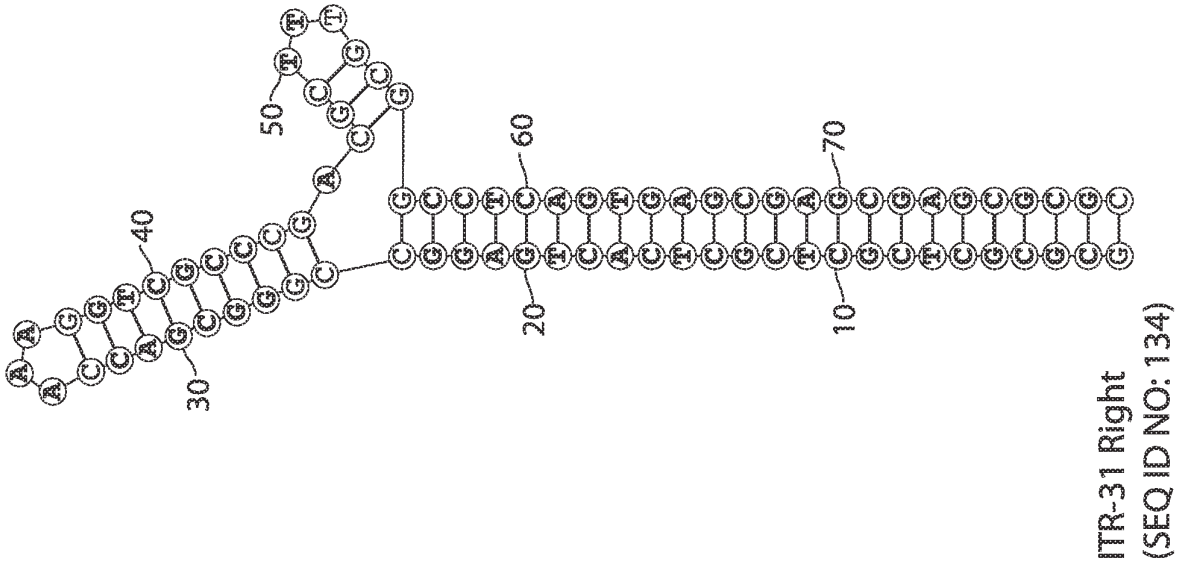
ITR-30 Right
(SEQ ID NO: 132)



ITR-29 Right
(SEQ ID NO: 130)

FIG. 7A-6





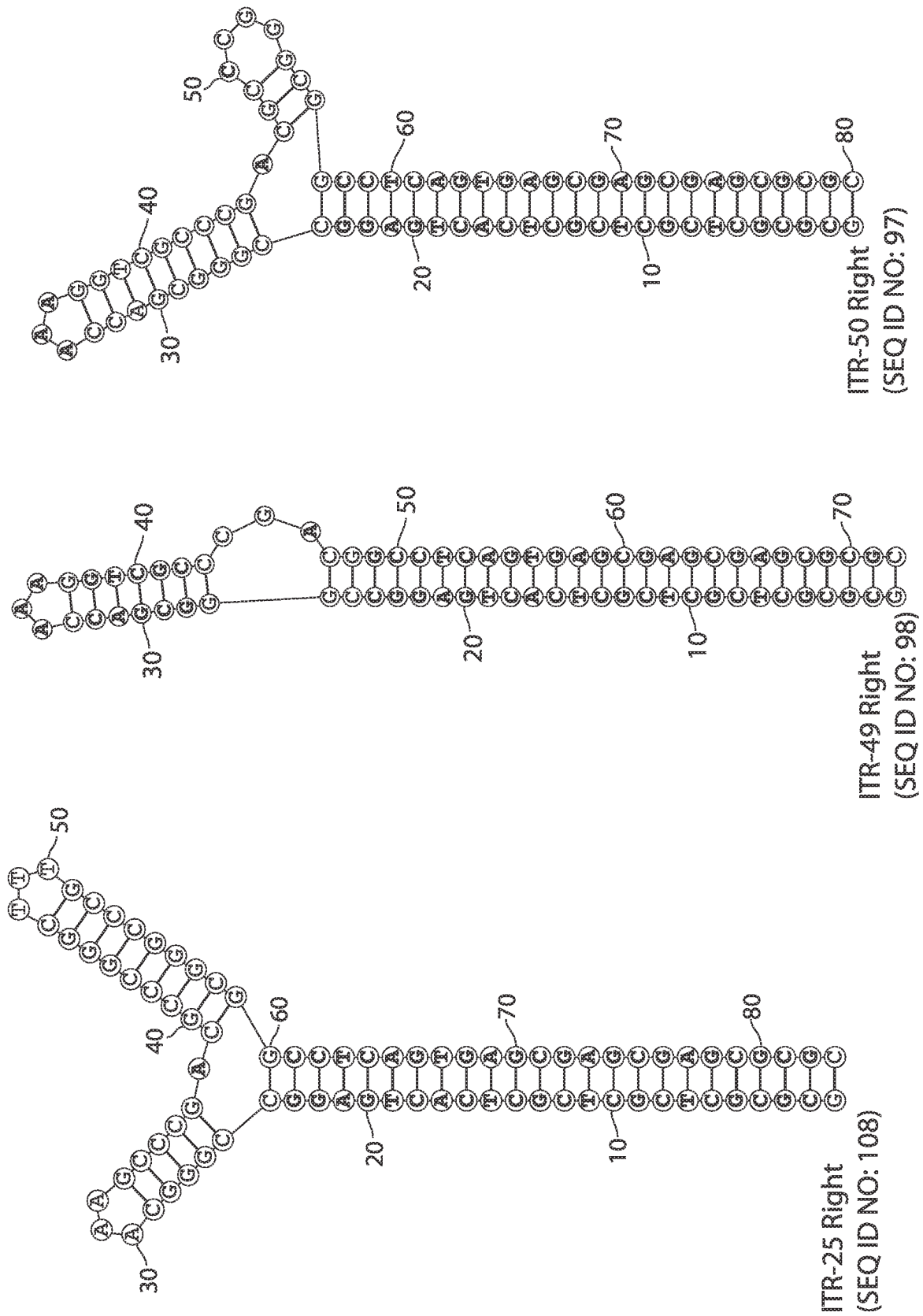
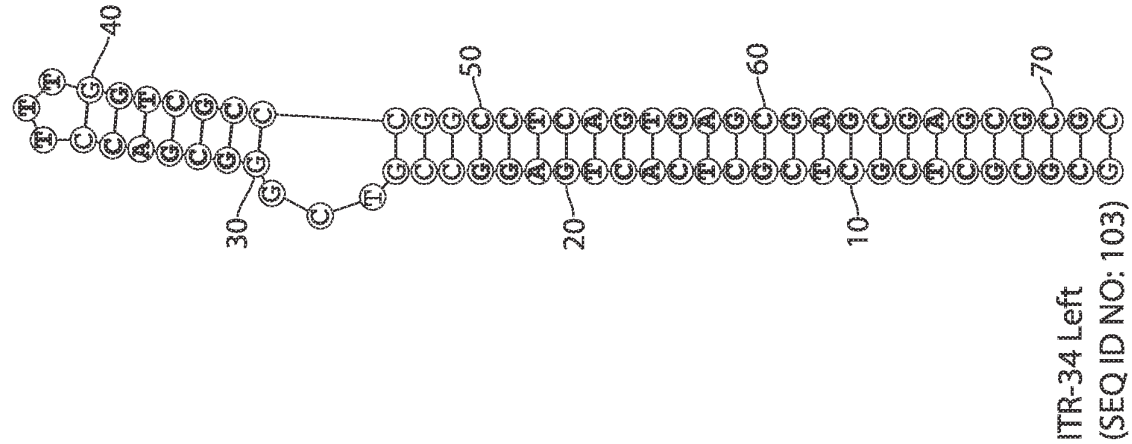
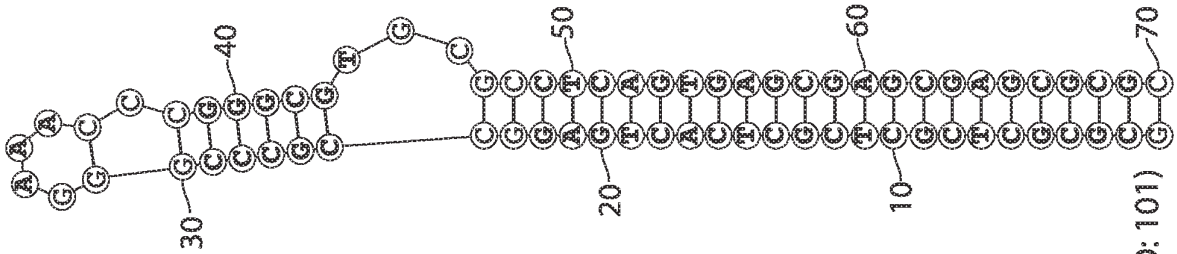

ITR-50 Right
(SEQ ID NO: 97)
ITR-49 Right
(SEQ ID NO: 98)
ITR-25 Right
(SEQ ID NO: 108)

FIG. 7A-7

FIG. 7B-1



ITR-34 Left
(SEQ ID NO: 103)



ITR-18 Left
(SEQ ID NO: 101)

FIG. 7B-2

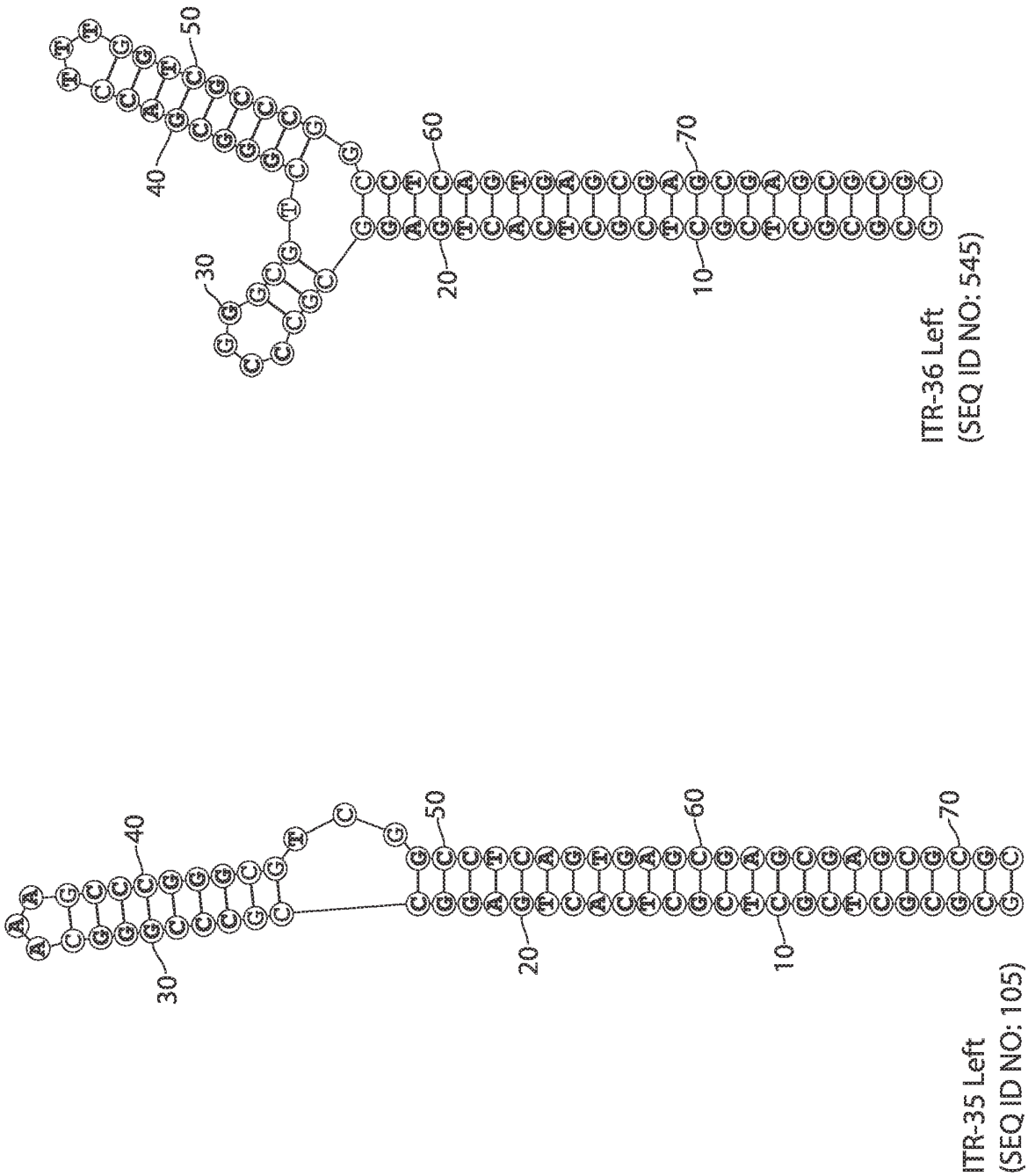
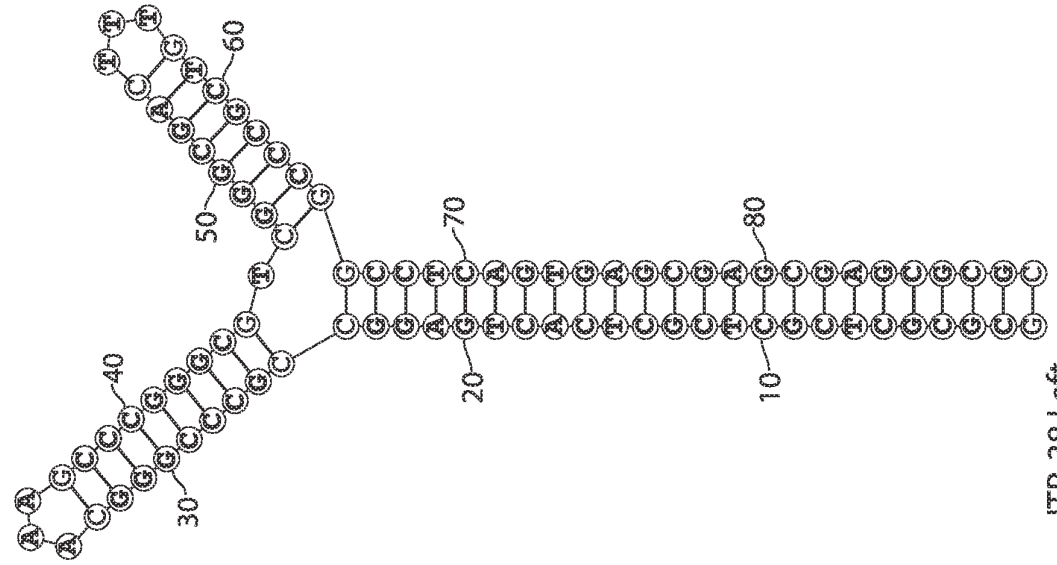


FIG. 7B-3



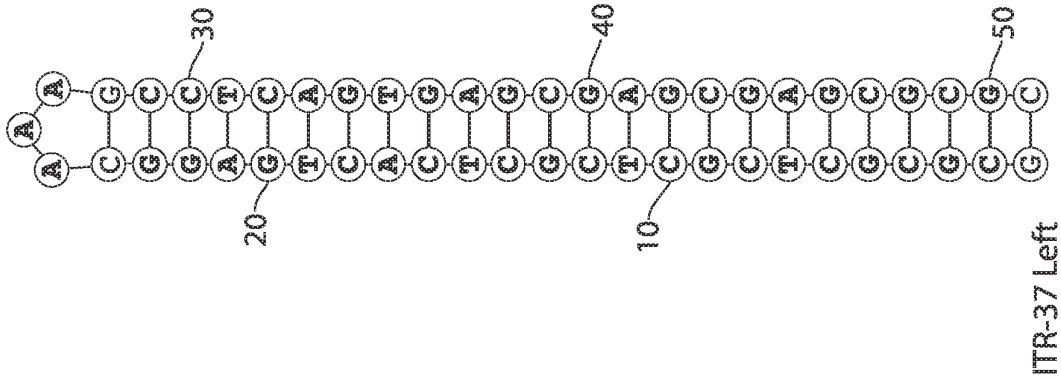


FIG. 7B-4

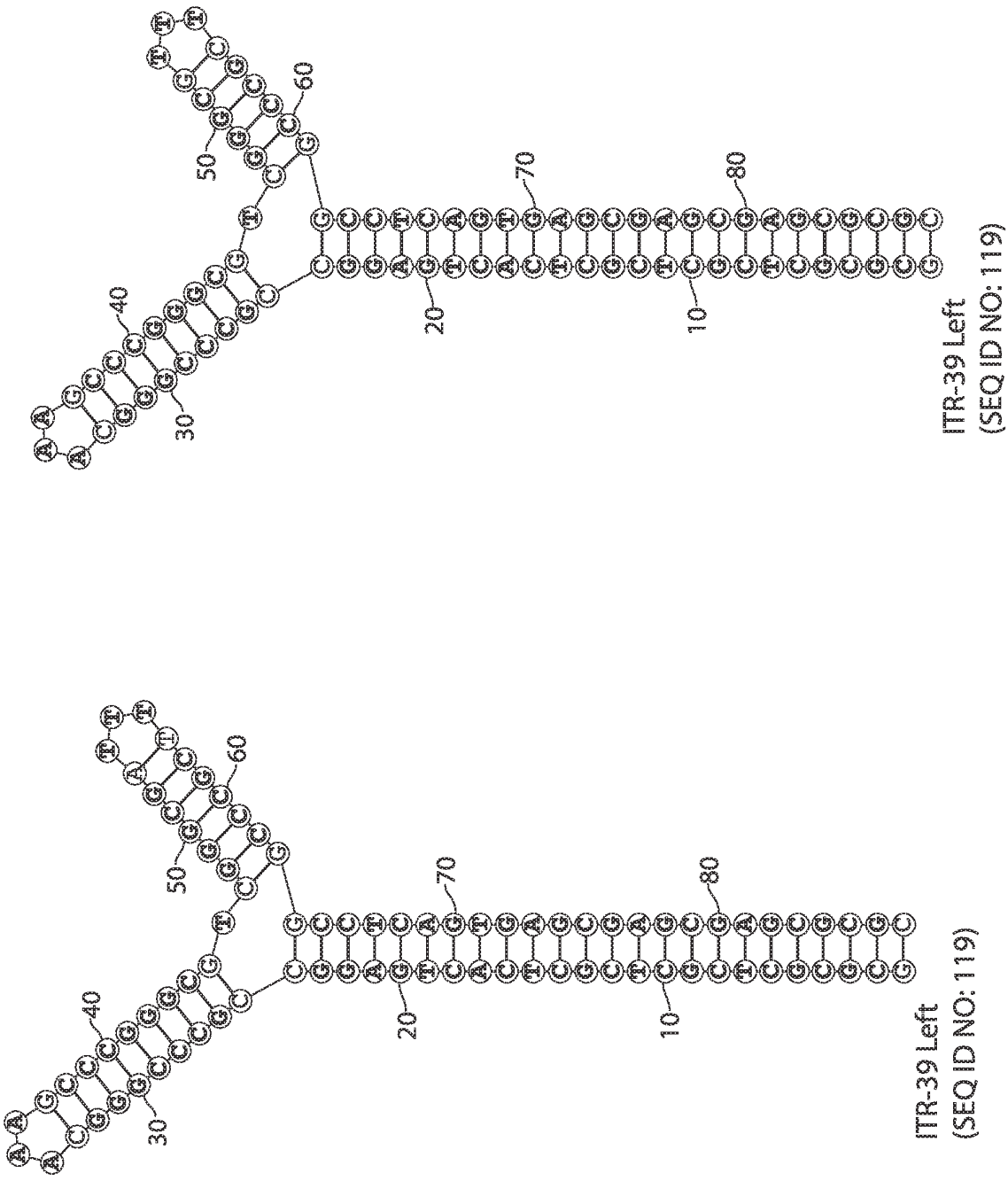
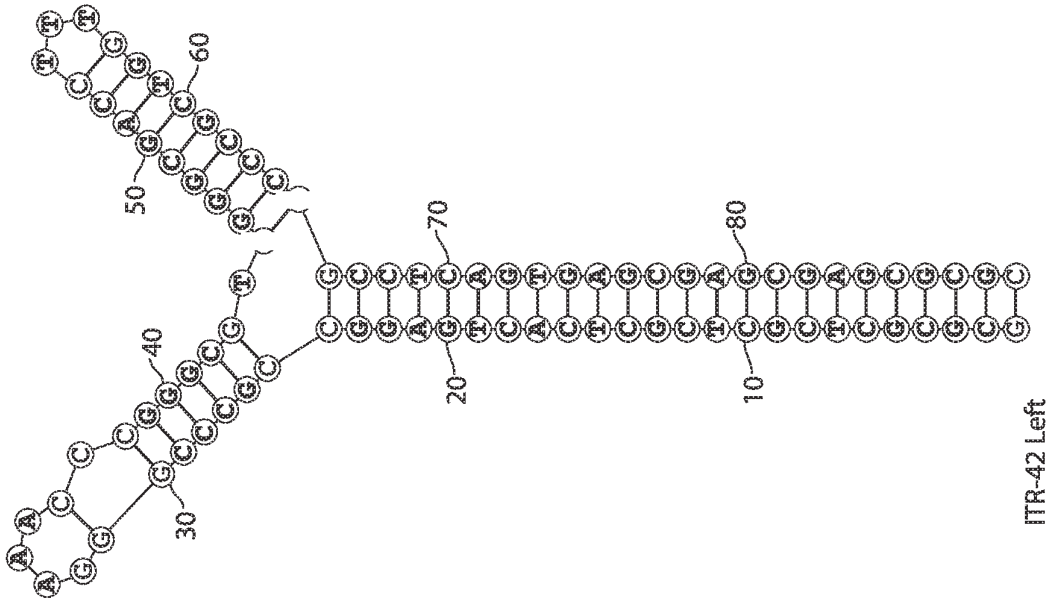
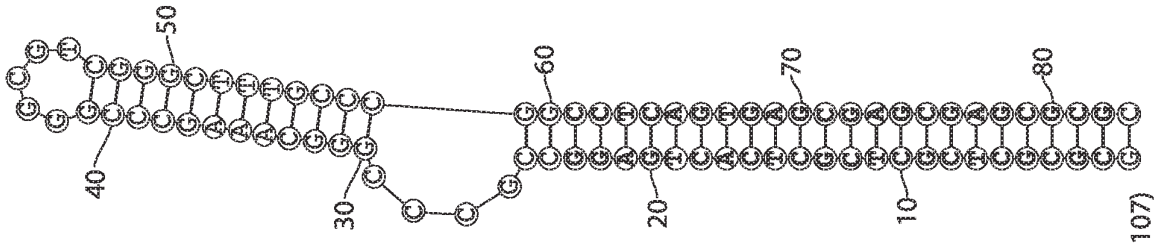


FIG. 7B-5



ITR-42 Left
(SEQ ID NO: 123)



ITR-41 Left
(SEQ ID NO: 107)

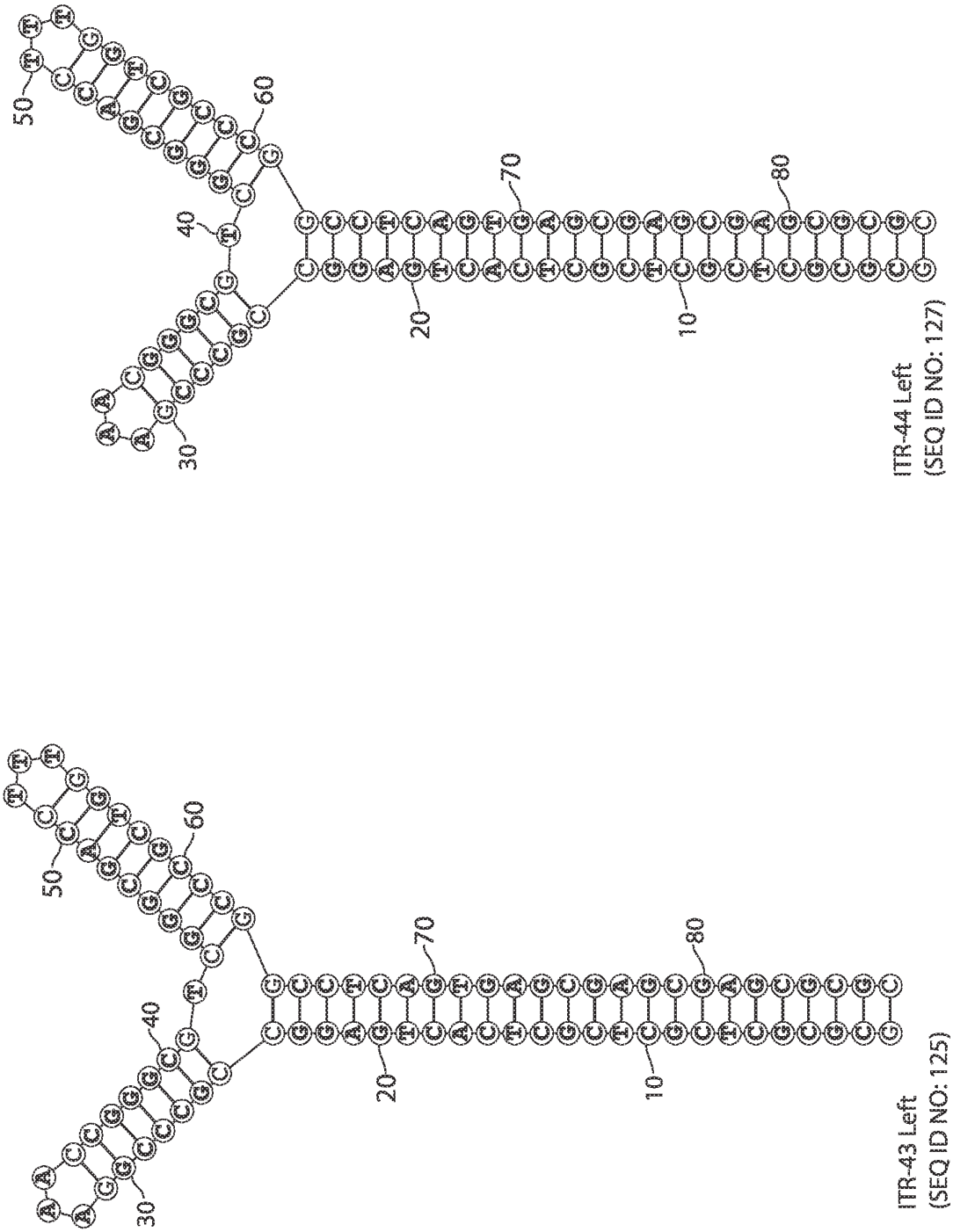
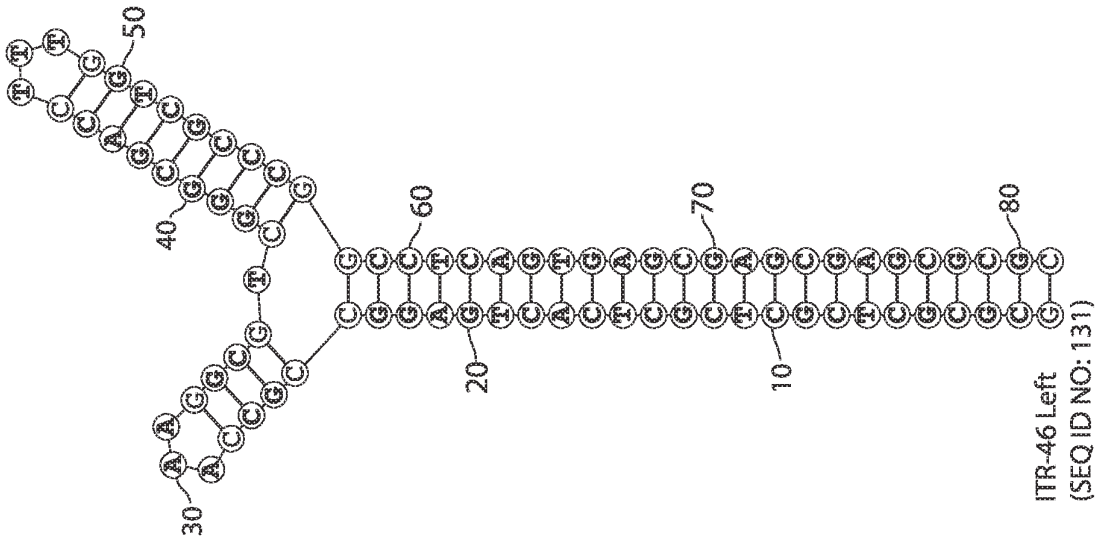
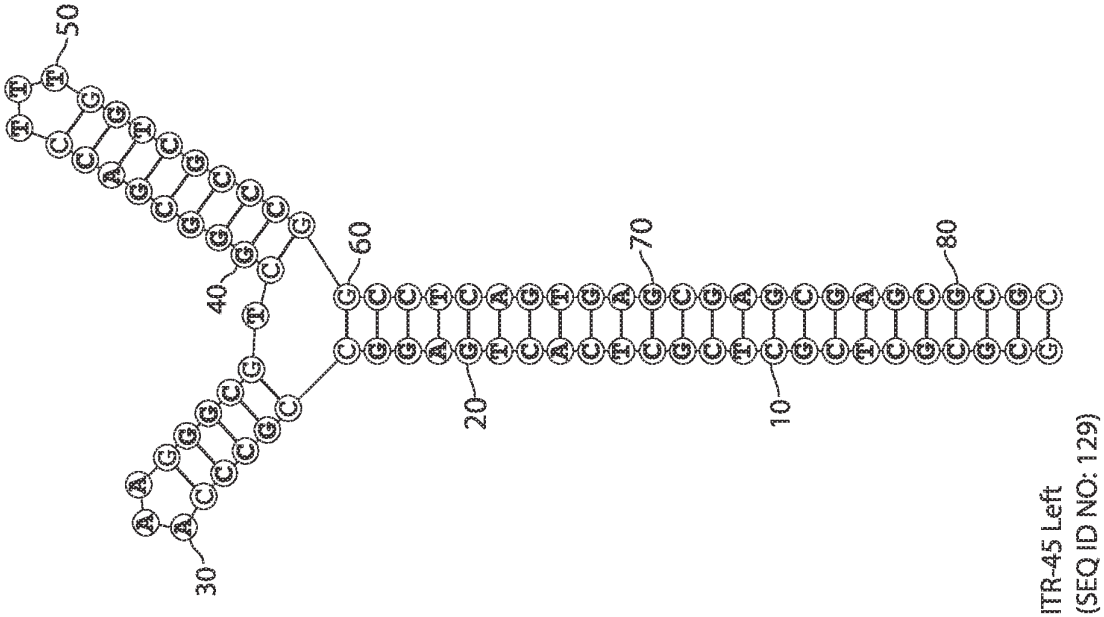

50
60
40
70
80
30
20
10
ITR-44 Left
(SEQ ID NO: 127)
50
60
40
70
80
30
20
10
ITR-43 Left
(SEQ ID NO: 125)

FIG. 7B-6

FIG. 7B-7

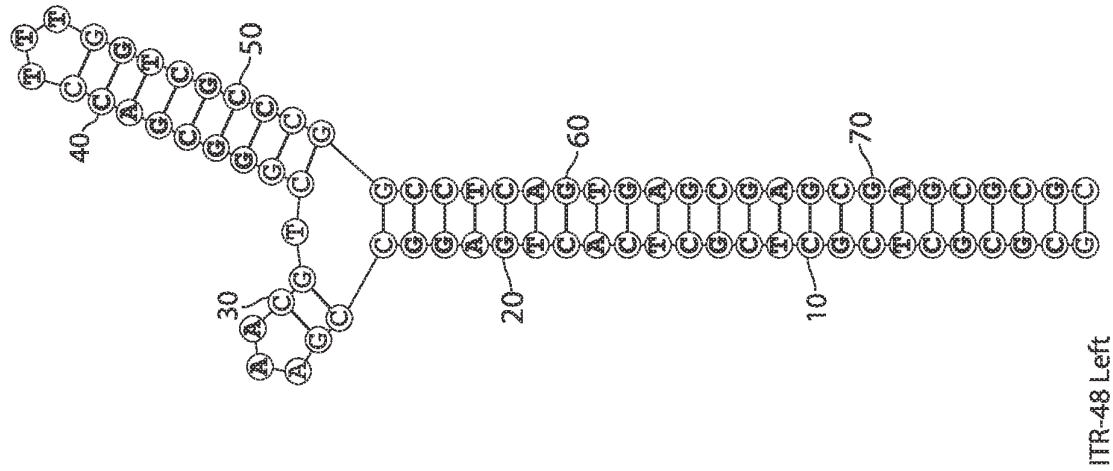


ITR-46 Left
(SEQ ID NO: 131)

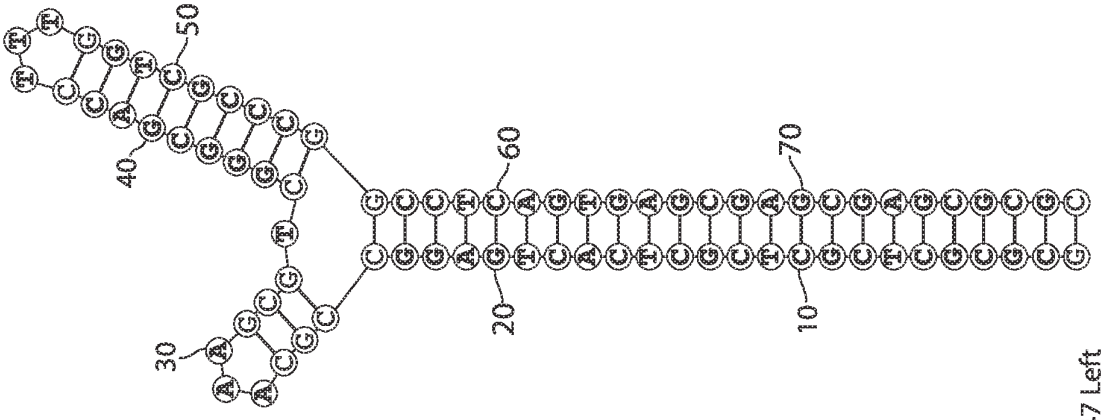


ITR-45 Left
(SEQ ID NO: 129)

FIG. 7B-8



ITR-48 Left
(SEQ ID NO: 547)



ITR-47 Left
(SEQ ID NO: 133)

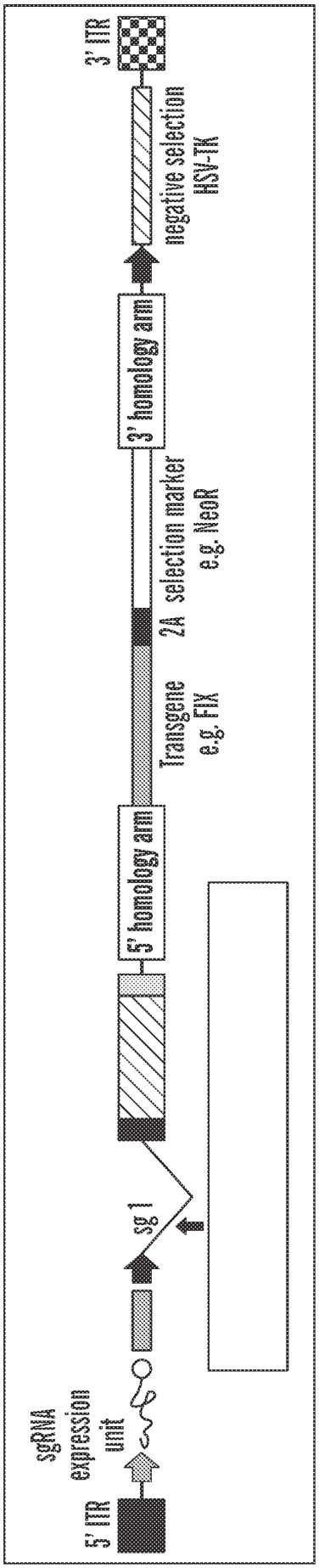

5' ITR
sgRNA expression unit
sg 1
5' homology arm
Transgene e.g. FIX
2A selection marker e.g. NeoR
3' homology arm
negative selection HSV-TK
3' ITR

FIG. 8

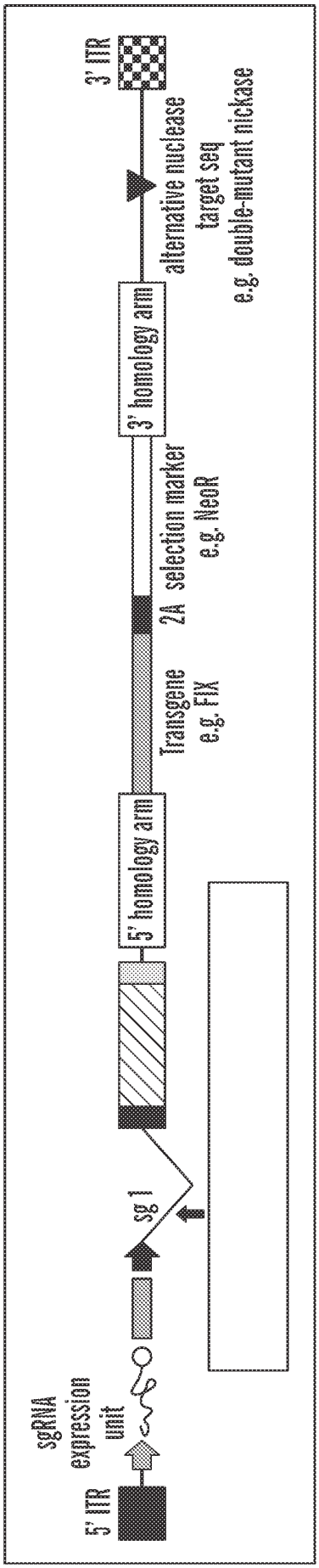
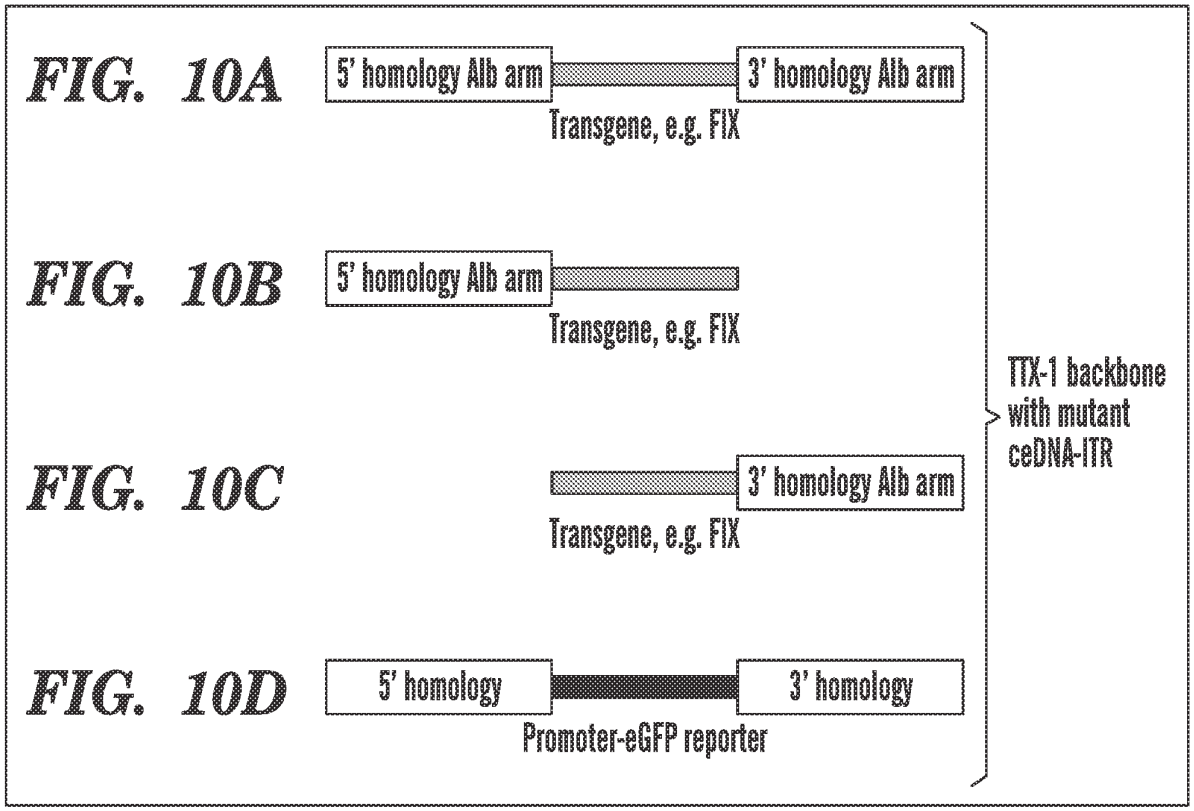
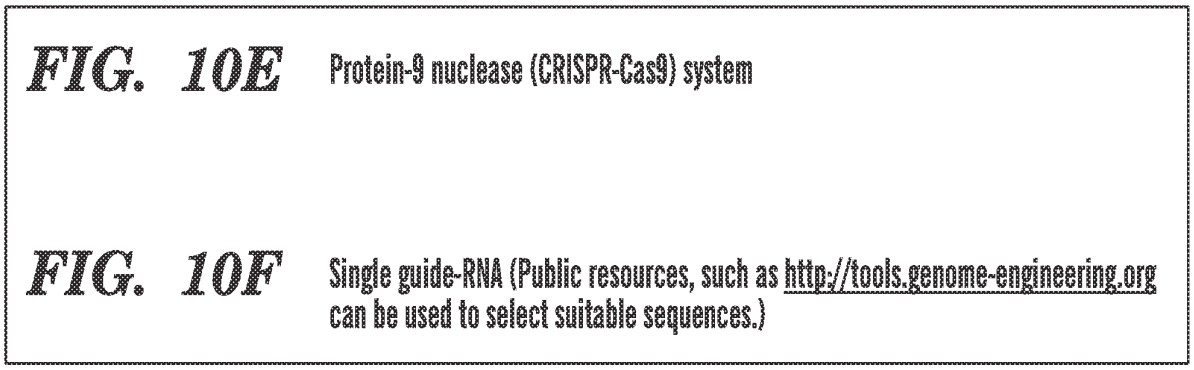

5' ITR
sgRNA expression unit
sg 1
5' homology arm
Transgene e.g. FIX
2A selection marker e.g. NeoR
3' homology arm
alternative nuclease target seq e.g. double-mutant nickase
3' ITR

FIG. 9


FIG. 10A
5' homology Alb arm
3' homology Alb arm
Transgene, e.g. FIX
FIG. 10B
5' homology Alb arm
Transgene, e.g. FIX
FIG. 10C
3' homology Alb arm
Transgene, e.g. FIX
FIG. 10D
5' homology
3' homology
Promoter-eGFP reporter
TTX-1 backbone with mutant ceDNA-ITR


FIG. 10E
Protein-9 nuclease (CRISPR-Cas9) system
FIG. 10F
Single guide-RNA (Public resources, such as http://tools.genome-engineering.org can be used to select suitable sequences.)

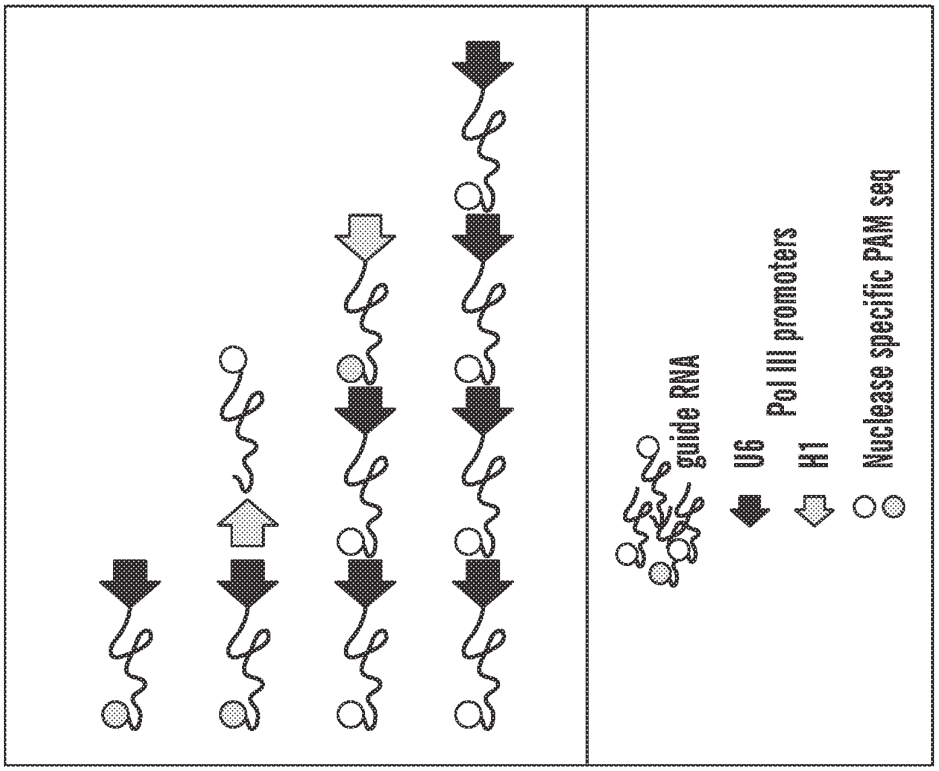

guide RNA
U6
Pol III promoters
H1
Nuclease specific PAM seq

FIG. 12

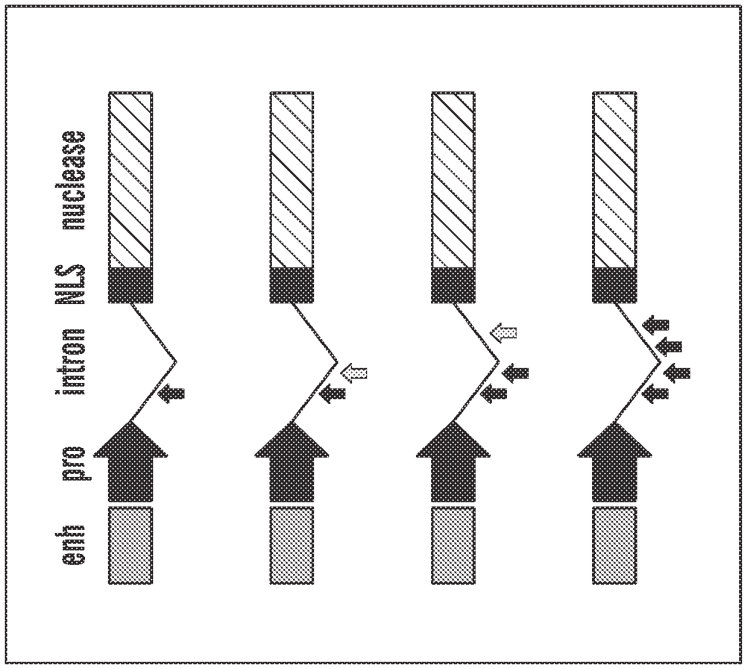

enh
pro
intron
NLS
nuclease

FIG. 11

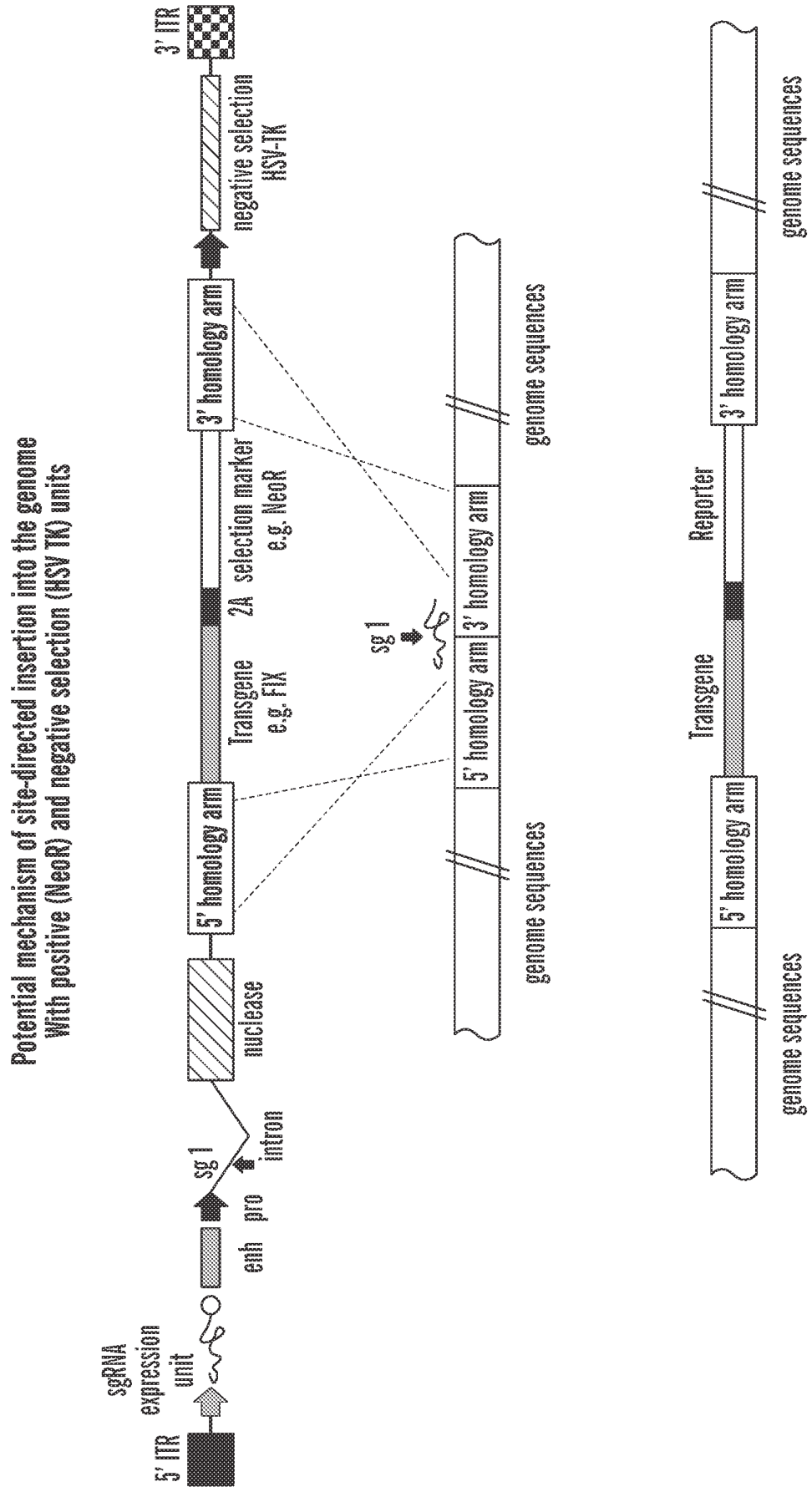

Potential mechanism of site-directed insertion into the genome
With positive (NeoR) and negative selection (HSV TK) units
5' ITR
sgRNA expression unit
enh
pro
sg 1
intron
nuclease
5' homology arm
Transgene e.g. FIX
2A selection marker e.g. NeoR
3' homology arm
negative selection HSV-TK
3' ITR
genome sequences
sg 1
5' homology arm
3' homology arm
genome sequences
5' homology arm
Transgene
Reporter
3' homology arm
genome sequences

FIG. 13

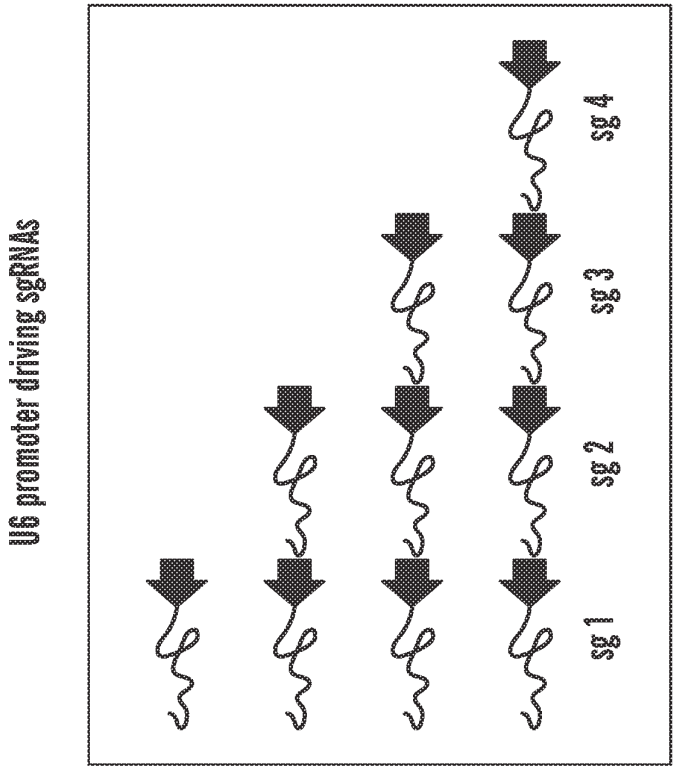


FIG. 14

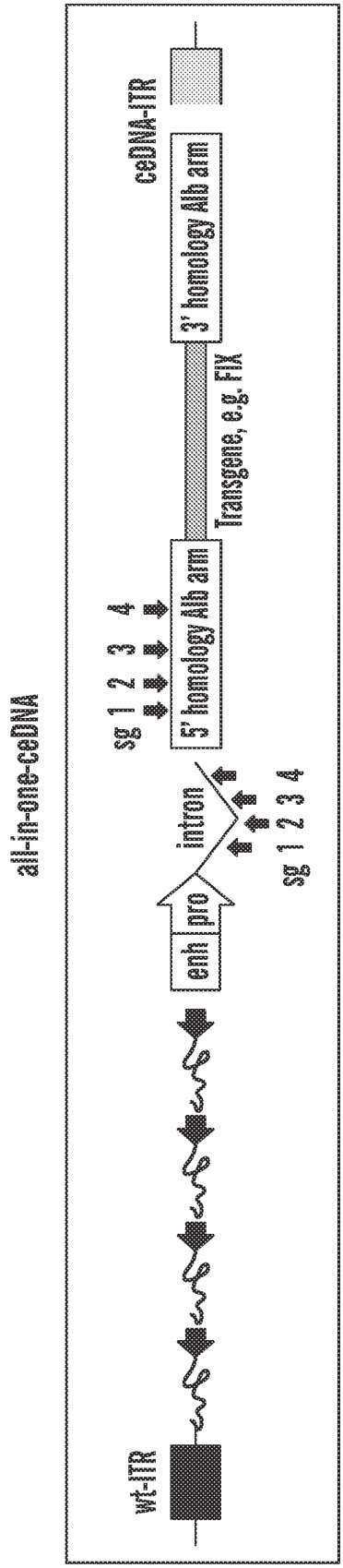

all-in-one-ceDNA
wt-ITR
enh
pro
intron
sg 1 2 3 4
5' homology Alb arm
sg 1 2 3 4
Transgene, e.g. FIX
3' homology Alb arm
ceDNA-ITR

FIG. 15

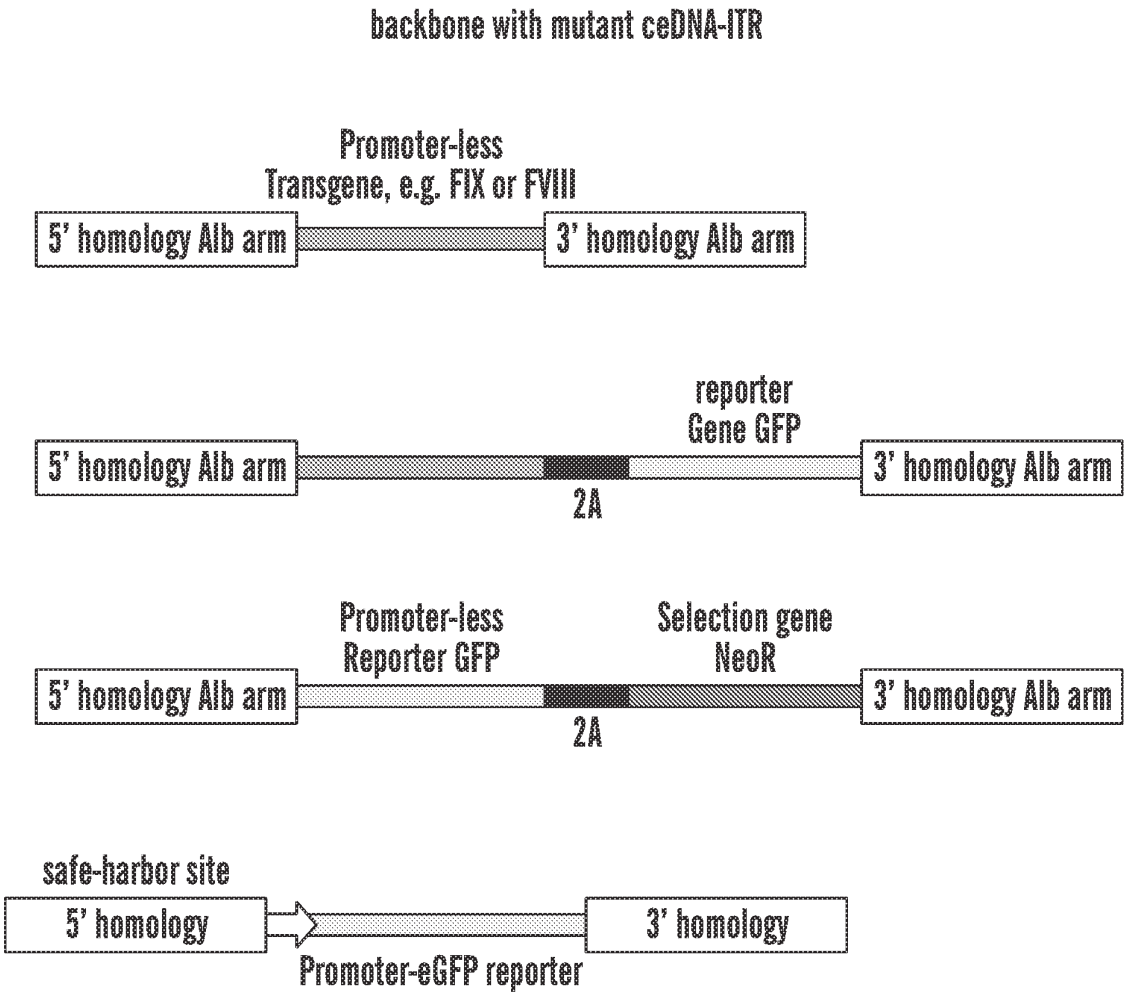

backbone with mutant ceDNA-ITR
Promoter-less
Transgene, e.g. FIX or FVIII
5' homology Alb arm
3' homology Alb arm
reporter
Gene GFP
5' homology Alb arm
3' homology Alb arm
2A
Promoter-less
Reporter GFP
Selection gene
NeoR
5' homology Alb arm
3' homology Alb arm
2A
safe-harbor site
5' homology
3' homology
Promoter-eGFP reporter

FIG. 16

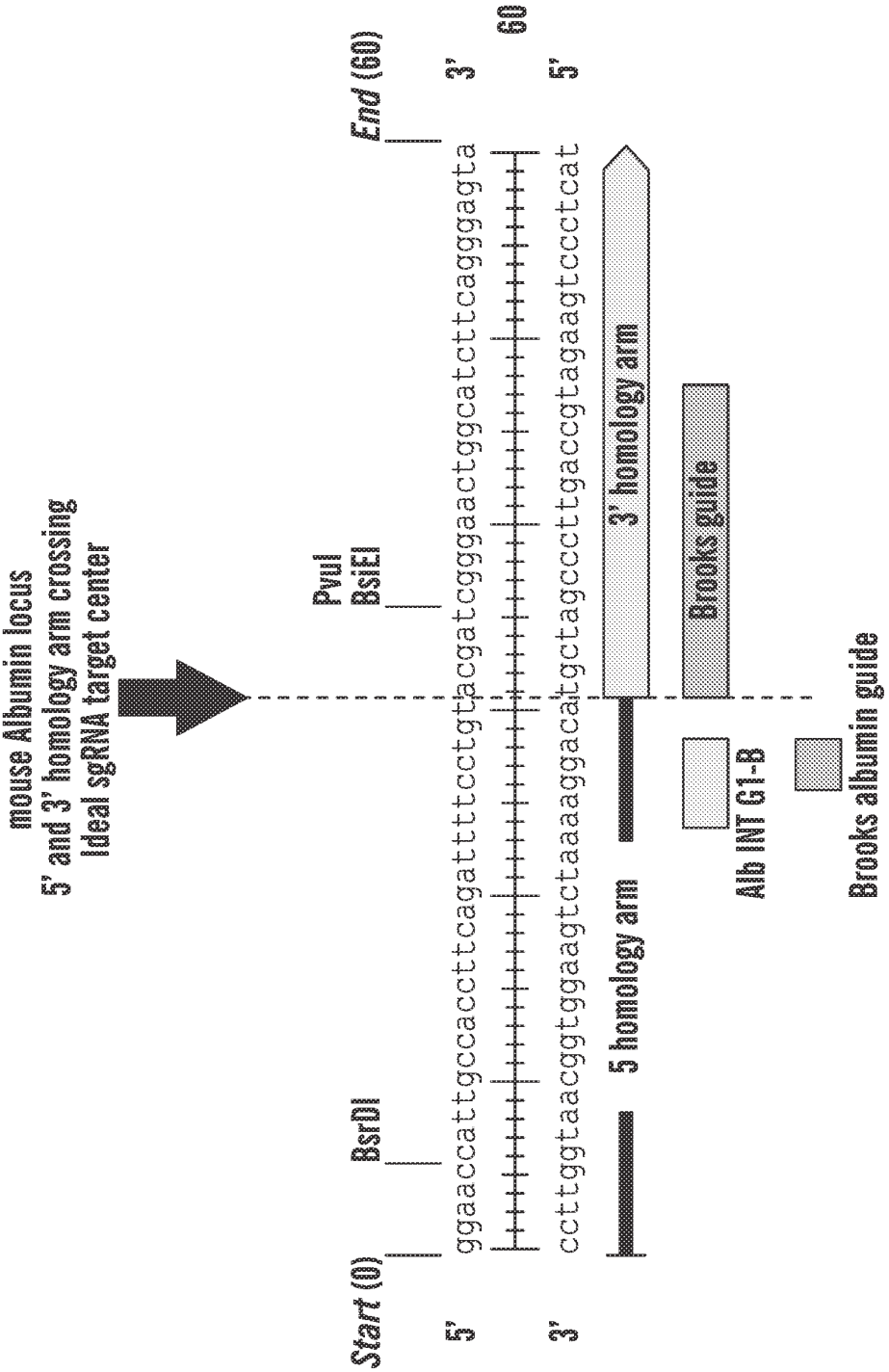

mouse Albumin locus
5' and 3' homology arm crossing
Ideal sgRNA target center
Start (0)
BsrDI
PvuI
BsiEI
End (60)
5'
ggaaccattgccaccttcagatttcctgtacgatcgggaactggcatcttcagggagta
3'
cctttggtaacggtggaagtctaaaaggacatgctagcccttgaccgtagaagtccctcat
3'
60
5'
5 homology arm
3' homology arm
Brooks guide
Alb INT G1-B
Brooks albumin guide

FIG. 17

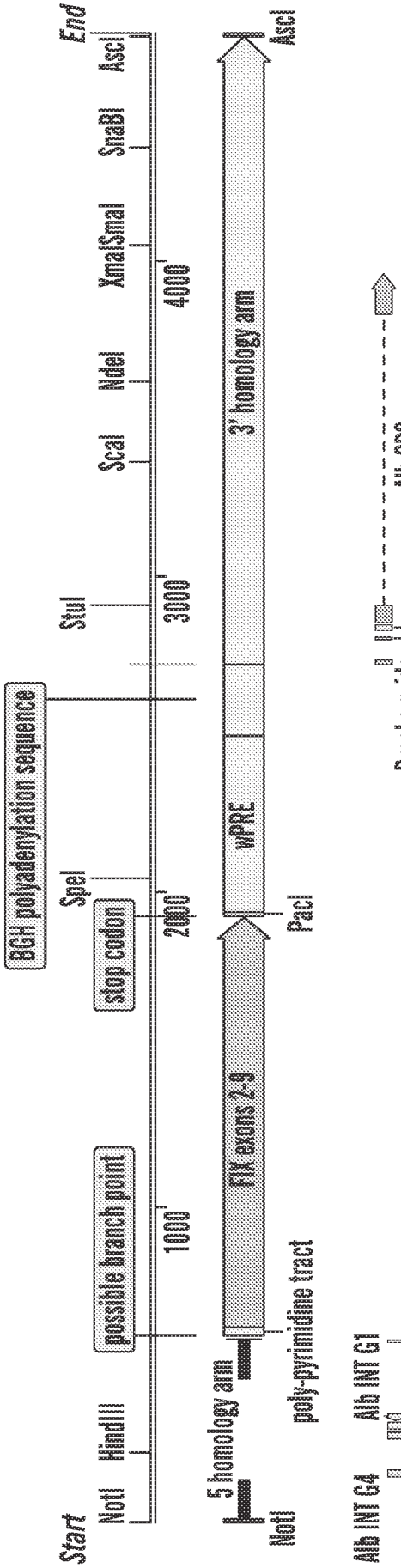

Start
NotI
HindIII
possible branch point
1000
5 homology arm
NotI
poly-pyrimidine tract
FIX exons 2-8
stop codon
SpeI
2000
PacI
wPRE
3' homology arm
AscI
End
AscI
SnaBI
XmaISmaI
4000
NdeI
ScaI
3000
StuI
BGH polyadenylation sequence
Alb INT G4
Alb INT G1
Alb INT G1-B
Alb INT G3
Alb INT G2
Brooks albumin guide
Brooks guide
possible branch point
poly-PY tract
Alb CDS
ORF-FIX-5'3'arm-NotI-AscI fragment
4723 bp

FIG. 17 (cont.)

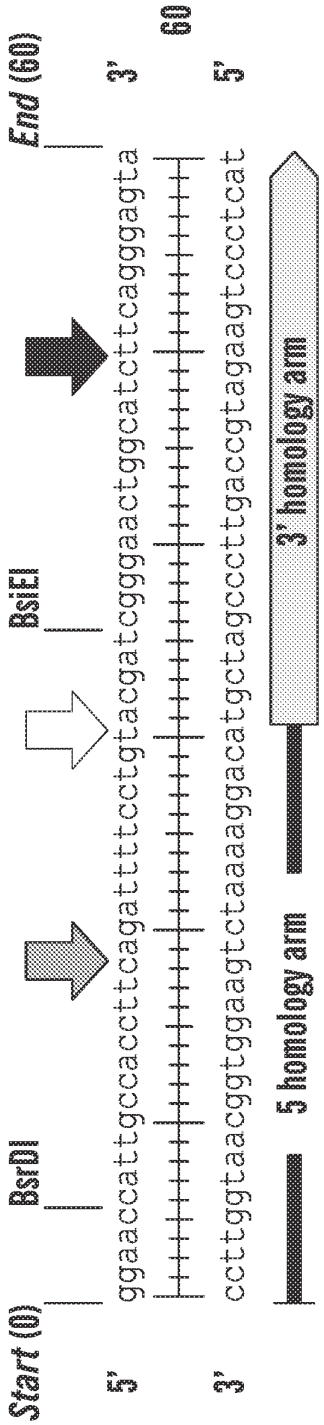

Start (0)
BsrDI
PvuI
BsiEI
End (60)
5'
ggaaccattgccaccttcagatttcctgtacgatcgggaactggcatcttcagggagta
3'
cctttggtaacggtggaagtctaaaaggacatgctagccccttgaccgtagaagtccctcat
60
5'
3'
5 homology arm
3' homology arm
mouse Albumin locus
5' and 3' homology arm crossing
ideal sgRNA target center

FIG. 18A


GATCGGGAACTGGCATCTTC
(+)
AGG
4
recommended
GATCGTACAGGAAAATCTGA
(-)
AGG
7
recommended
ATCGGGAACTGGCATCTTCA
(+)
GGG
6
recommended
CGTACAGGAAAATCTGAAGG
(-)
TGG
9
recommended
TCAGATTTTCCTGTACGATC
(+)
GGG
7
recommended
TTTCCTGTACGATCGGGAAC
(+)
TGG
21

FIG. 18B

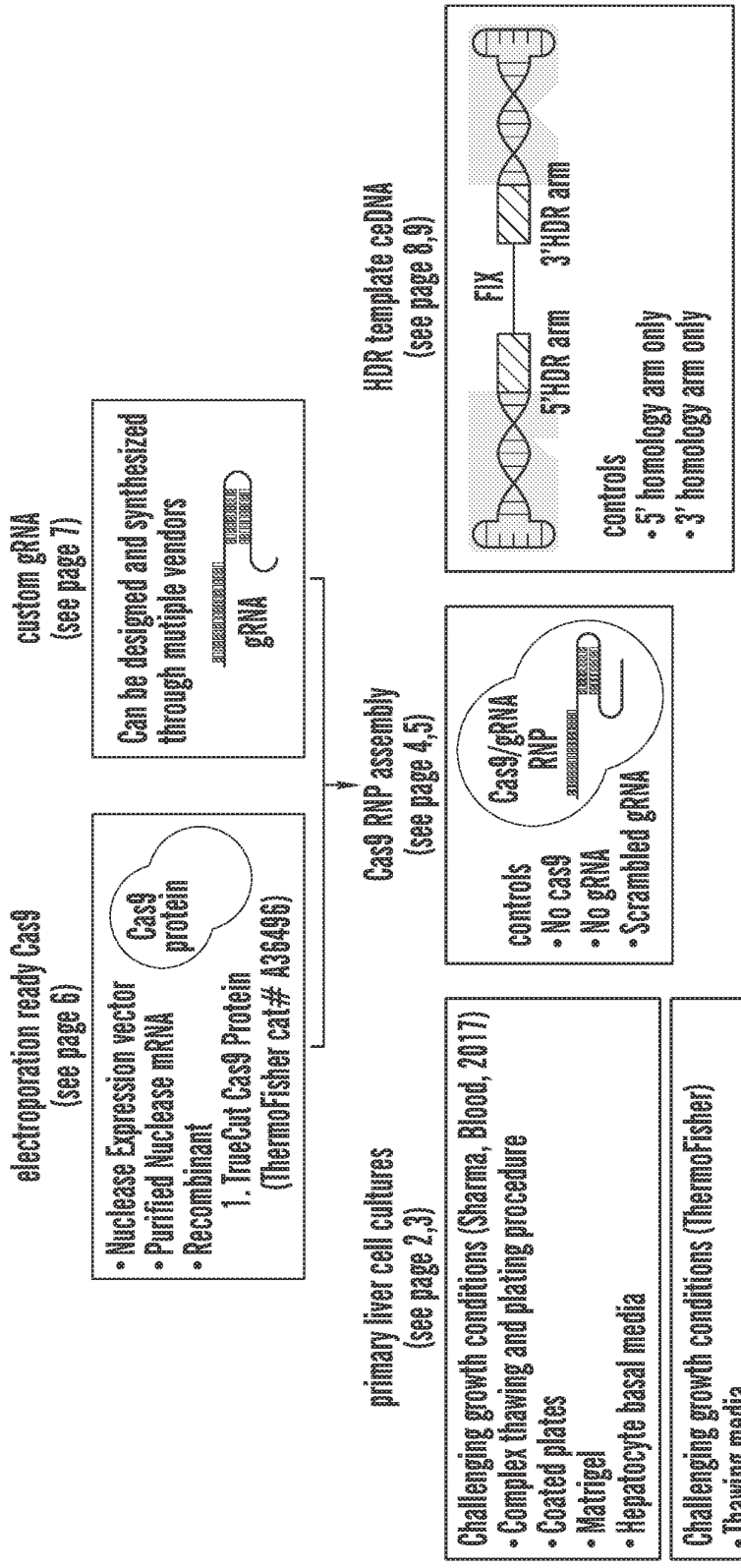

electroporation ready Cas9
(see page 6)
• Nuclease Expression vector
• Purified Nuclease mRNA
• Recombinant
Cas9 protein
1. TrueCut Cas9 Protein
(ThermoFisher cat# A36496)
custom gRNA
(see page 7)
Can be designed and synthesized through mutiple vendors
gRNA
primary liver cell cultures
(see page 2,3)
Challenging growth conditions (Sharma, Blood, 2017)
• Complex thawing and plating procedure
• Coated plates
• Matrigel
• Hepatocyte basal media
Cas9 RNP assembly
(see page 4,5)
controls
• No cas9
• No gRNA
• Scrambled gRNA
Cas9/gRNA RNP
HDR template ceDNA
(see page 8,9)
5'HDR arm
FIX
3'HDR arm
controls
• 5' homology arm only
• 3' homology arm only
NHEJ or HDR pathways
Challenging growth conditions (ThermoFisher)
• Thawing media
• Plating media
• Incubation media
• Matrigel (GelTrex)
Detection of FIX secretion (ELISA)
Deep Sequencing of genomic target DNA

FIG. 19