FIG. 1A

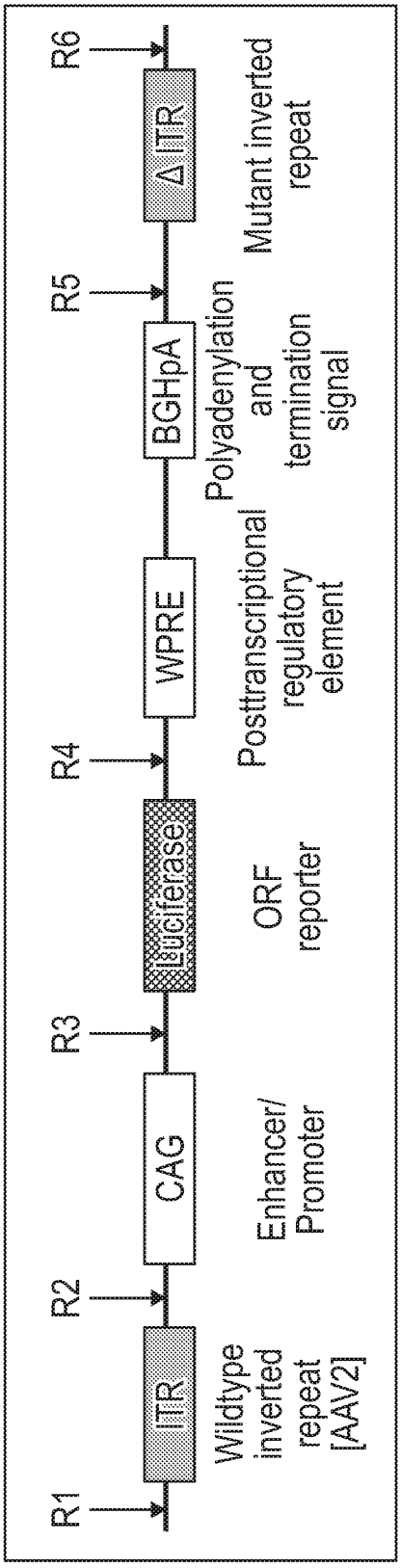

R1
ITR
Wildtype inverted repeat [AAV2]
R2
CAG
Enhancer/ Promoter
R3
Luciferase
ORF reporter
R4
WPRE
Posttranscriptional regulatory element
R5
BGHpA
Polyadenylation and termination signal
R6
ΔITR
Mutant inverted repeat

FIG. 1B

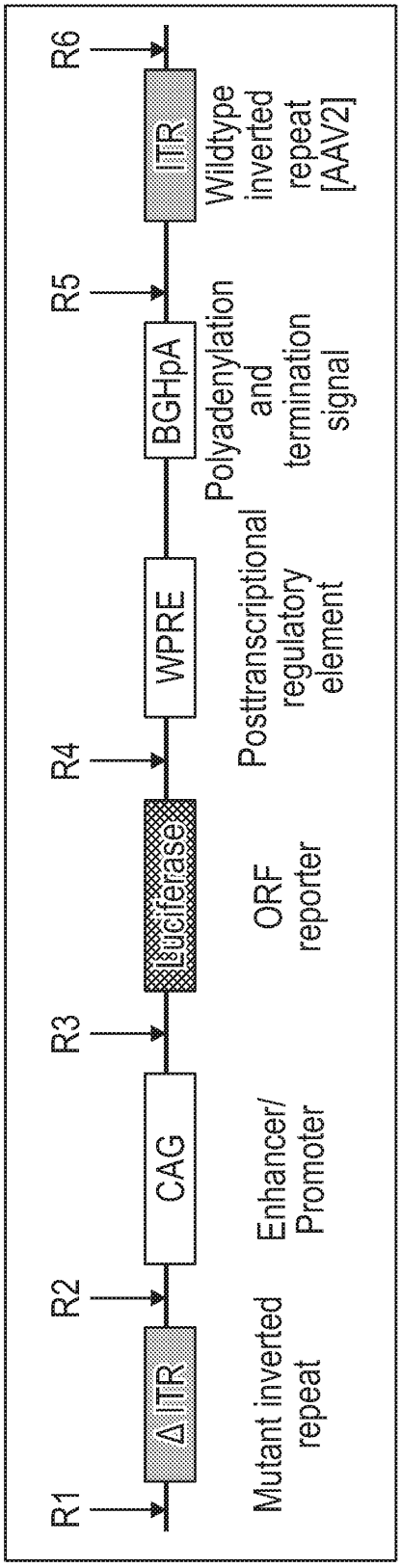
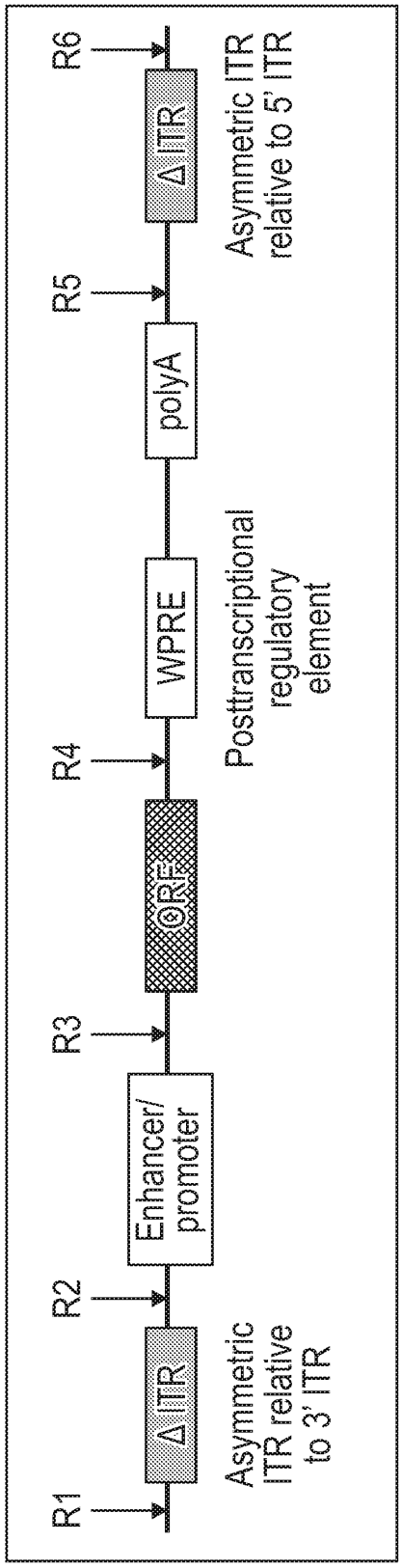

R1
ΔITR
Mutant inverted repeat
R2
CAG
Enhancer/ Promoter
R3
Luciferase
ORF reporter
R4
WPRE
Posttranscriptional regulatory element
R5
BGHpA
Polyadenylation and termination signal
R6
ITR
Wildtype inverted repeat [AAV2]

FIG. 1C


R1
Δ ITR
Asymmetric ITR relative to 3' ITR
R2
Enhancer/ promoter
R3
ORF
R4
WPRE
Posttranscriptional regulatory element
R5
polyA
R6
Δ ITR
Asymmetric ITR relative to 5' ITR

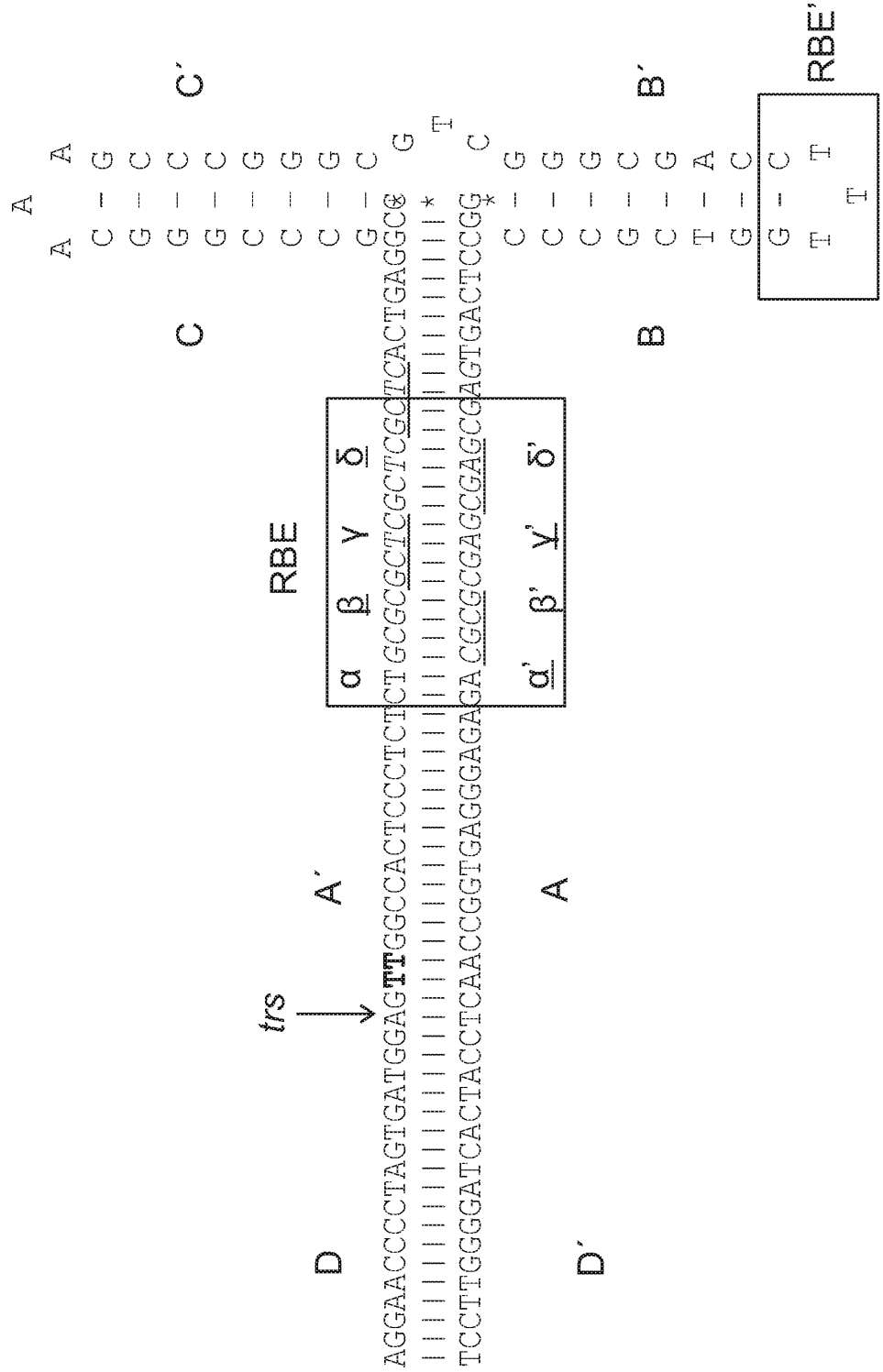



FIG. 2A

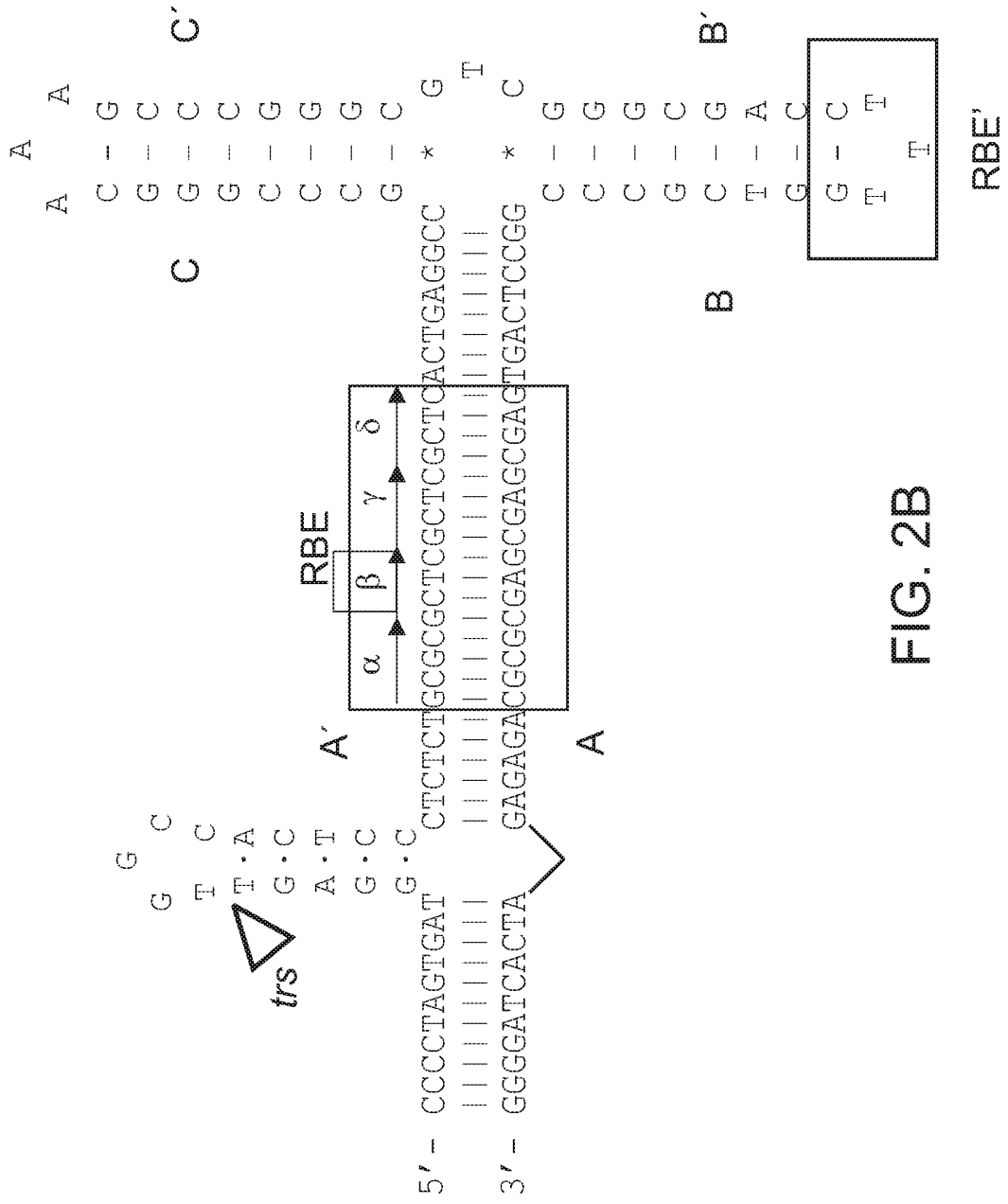
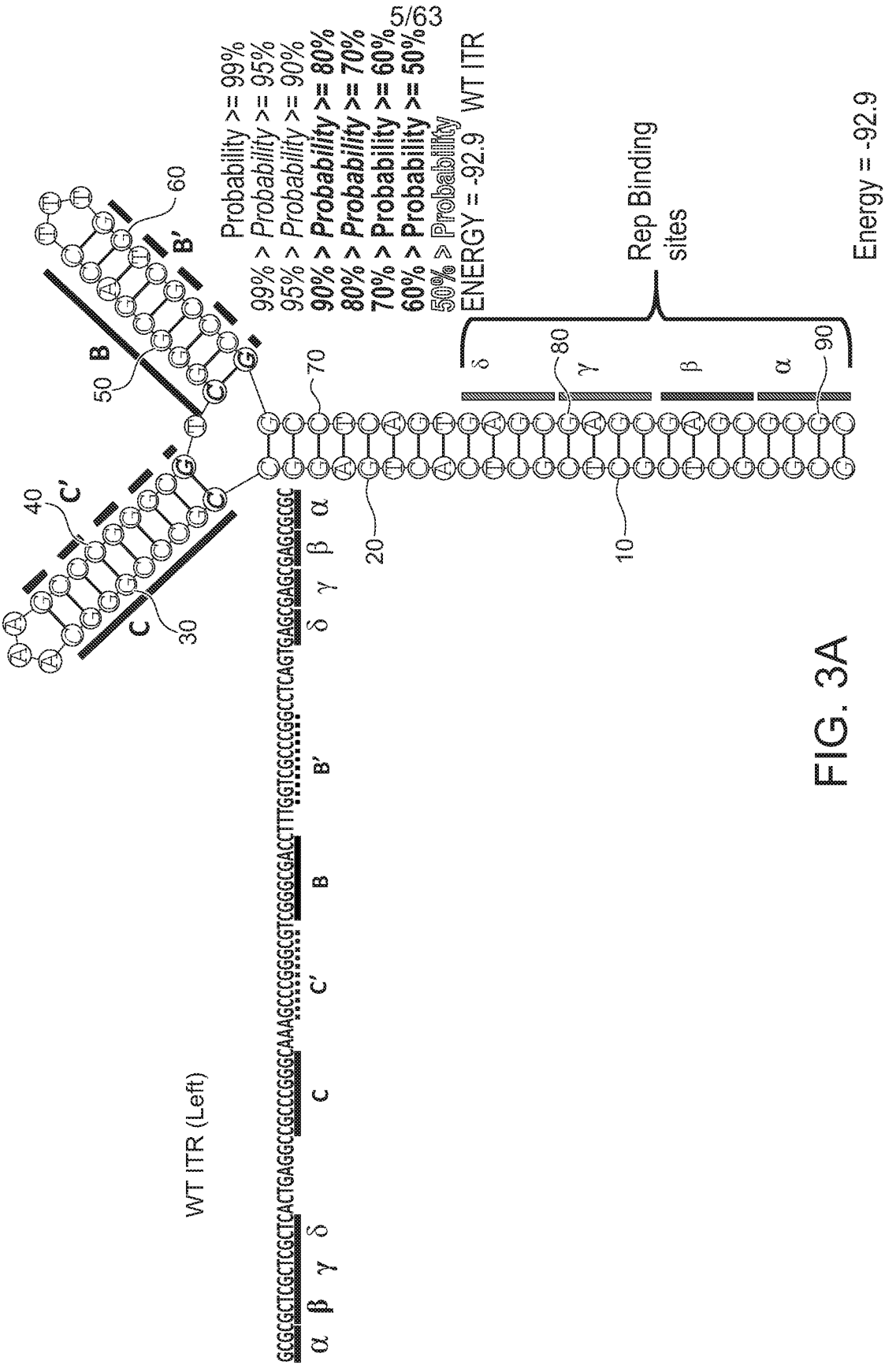

C'
B'
C
B
RBE'
RBE
A'
A
α
β
γ
δ
trs
5′ – CCCCTAGTGAT
3′ – GGGGATCACTA
CTCTCTGCGCGCTCGCTCGCTCACTGAGGCC
GAGAGACGCGCGAGCGAGCGAGTGACTCCGG

FIG. 2B


WT ITR (Left)
GCGCGCTCGCTCGCTCACTGAGGCCGCCCGGGCAAAGCCCGGGCGTCGGGCGACCTTTGGTCGCCCGGCCTCAGTGAGCGAGCGAGCGCGC
α β γ δ C C' B B' δ γ β α
Probability >= 99%
99% > Probability >= 95%
95% > Probability >= 90%
90% > Probability >= 80%
80% > Probability >= 70%
70% > Probability >= 60%
60% > Probability >= 50%
50% > Probability
ENERGY = -92.9 WT ITR
Rep Binding sites
Energy = -92.9
FIG. 3A

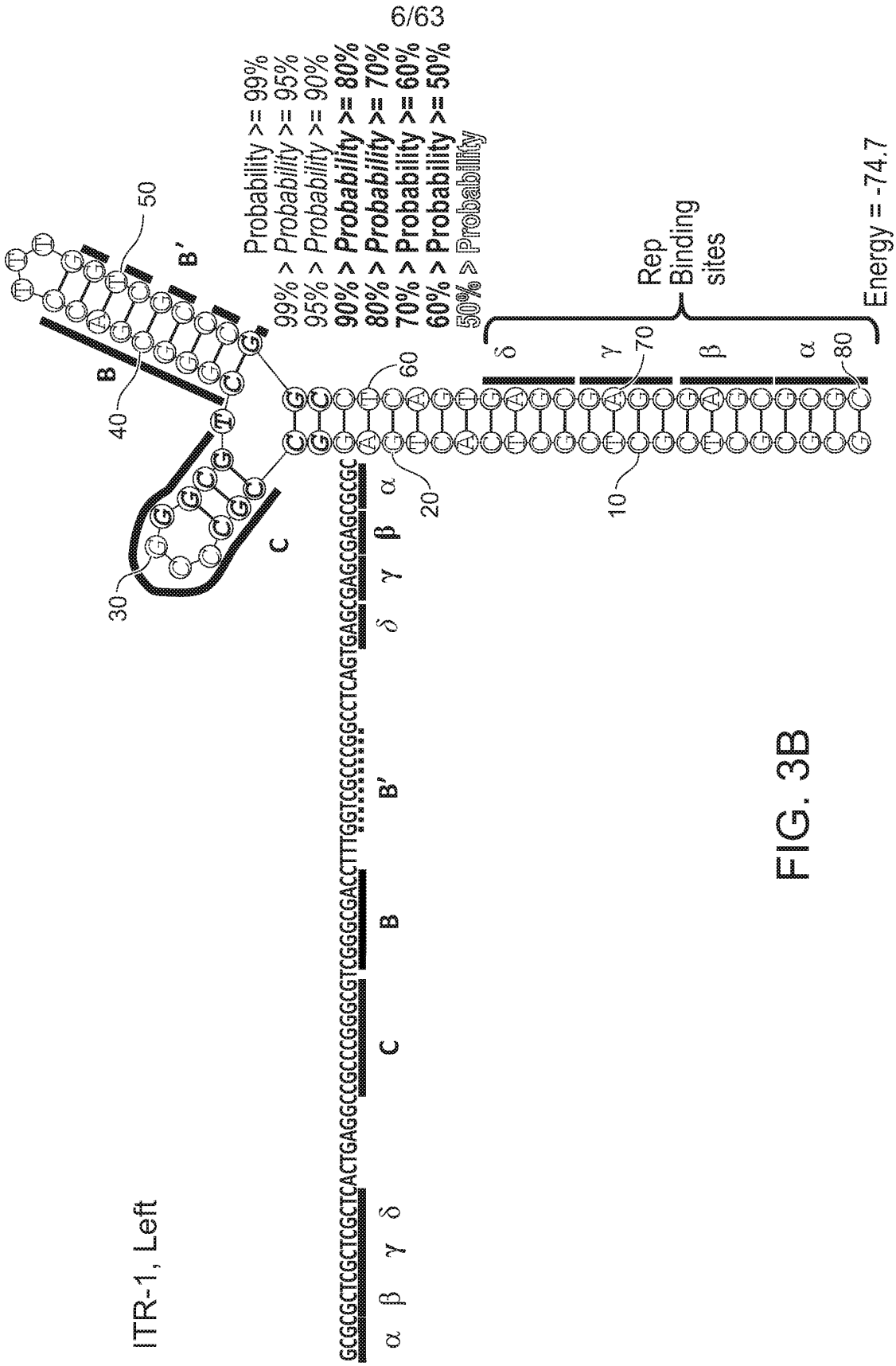

ITR-1, Left
GCGCGCTCGCTCGCTCACTGAGGCCGCCCGGGCAGACCTTTGGTCGCCCGGCCTCAGTGAGCGAGCGAGCGCGC
α β γ δ C B B' δ γ β α
Probability >= 99%
99% > Probability >= 95%
95% > Probability >= 90%
90% > Probability >= 80%
80% > Probability >= 70%
70% > Probability >= 60%
60% > Probability >= 50%
50% > Probability
Rep Binding sites
Energy = -74.7
B
B'
C
10
20
30
40
50
60
70
80

FIG. 3B

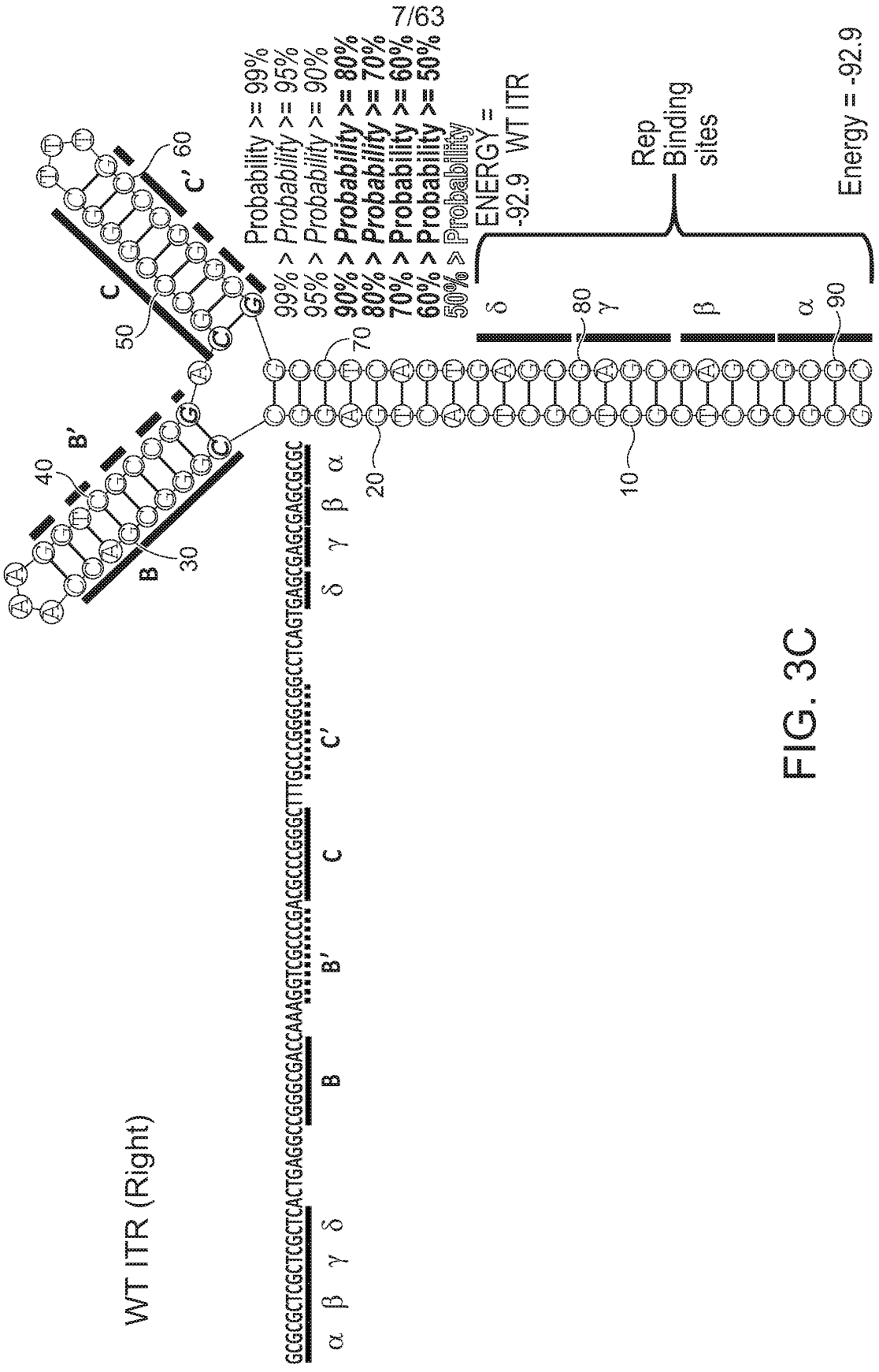

WT ITR (Right)
GCGCGCTCGCTCACTGAGGCCGGGCGACCAAAGGTCGCCCGACGCCCGGGCTTTGCCCGGGCGGCCTCAGTGAGCGAGCGAGCGCGC
α β γ δ
B
B'
C
C'
δ γ β α
Probability >= 99%
99% > Probability >= 95%
95% > Probability >= 90%
90% > Probability >= 80%
80% > Probability >= 70%
70% > Probability >= 60%
60% > Probability >= 50%
50% > Probability
ENERGY = -92.9 WT ITR
Rep Binding sites
Energy = -92.9
δ
γ
β
α
10
20
30
40
50
60
70
80
90

FIG. 3C

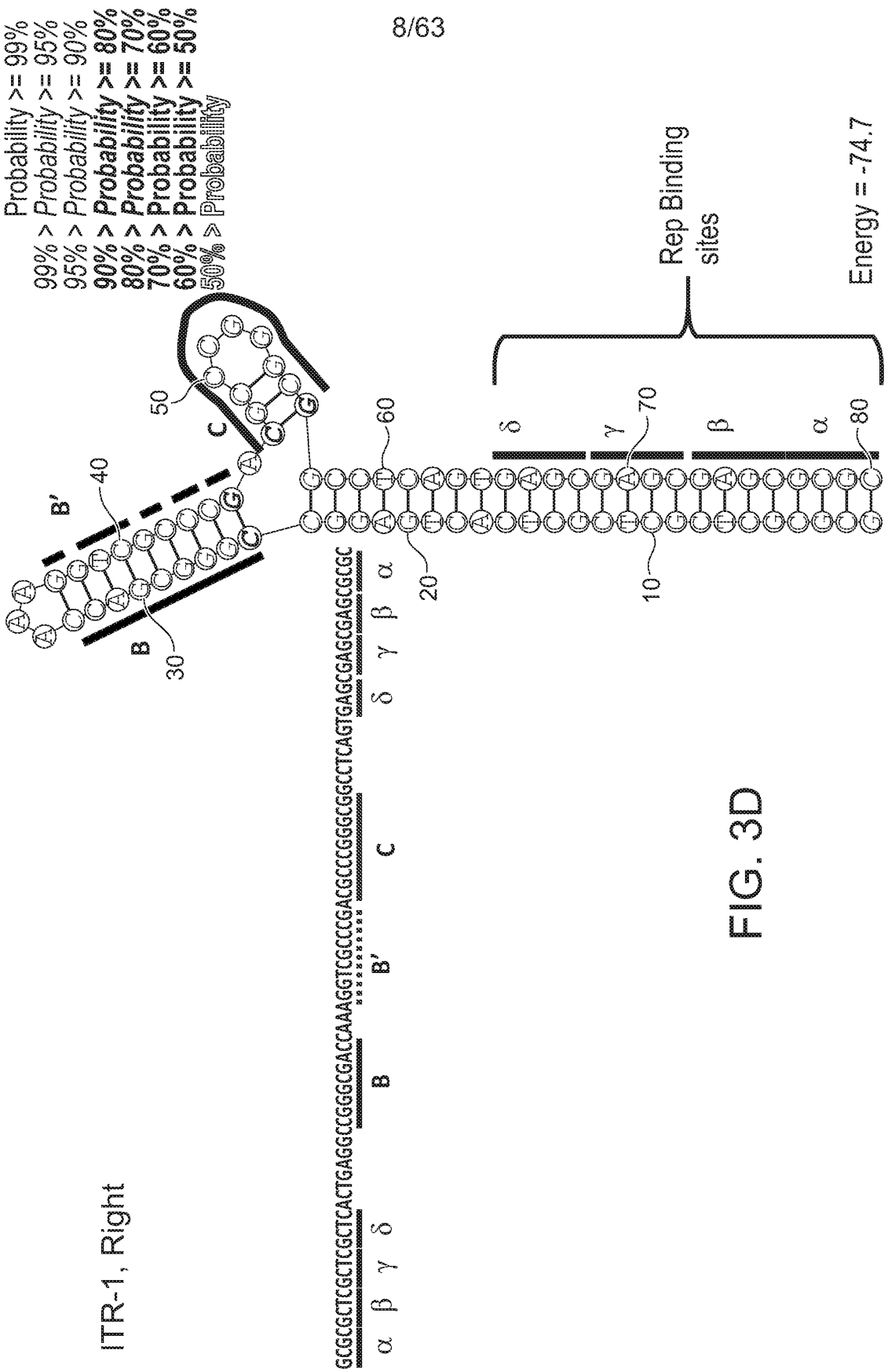

Probability >= 99%
99% > Probability >= 95%
95% > Probability >= 90%
90% > Probability >= 80%
80% > Probability >= 70%
70% > Probability >= 60%
60% > Probability >= 50%
50% > Probability
Rep Binding sites
Energy = -74.7
ITR-1, Right
GCGCGCTCGCTCGCTCACTGAGGCCGGGCGACCAAAGGTCGCCCGACGCCCGGGCGGCCTCAGTGAGCGAGCGAGCGCGC
α β γ δ
B
B'
C
δ γ β α
10
20
30
40
50
60
70
80

FIG. 3D

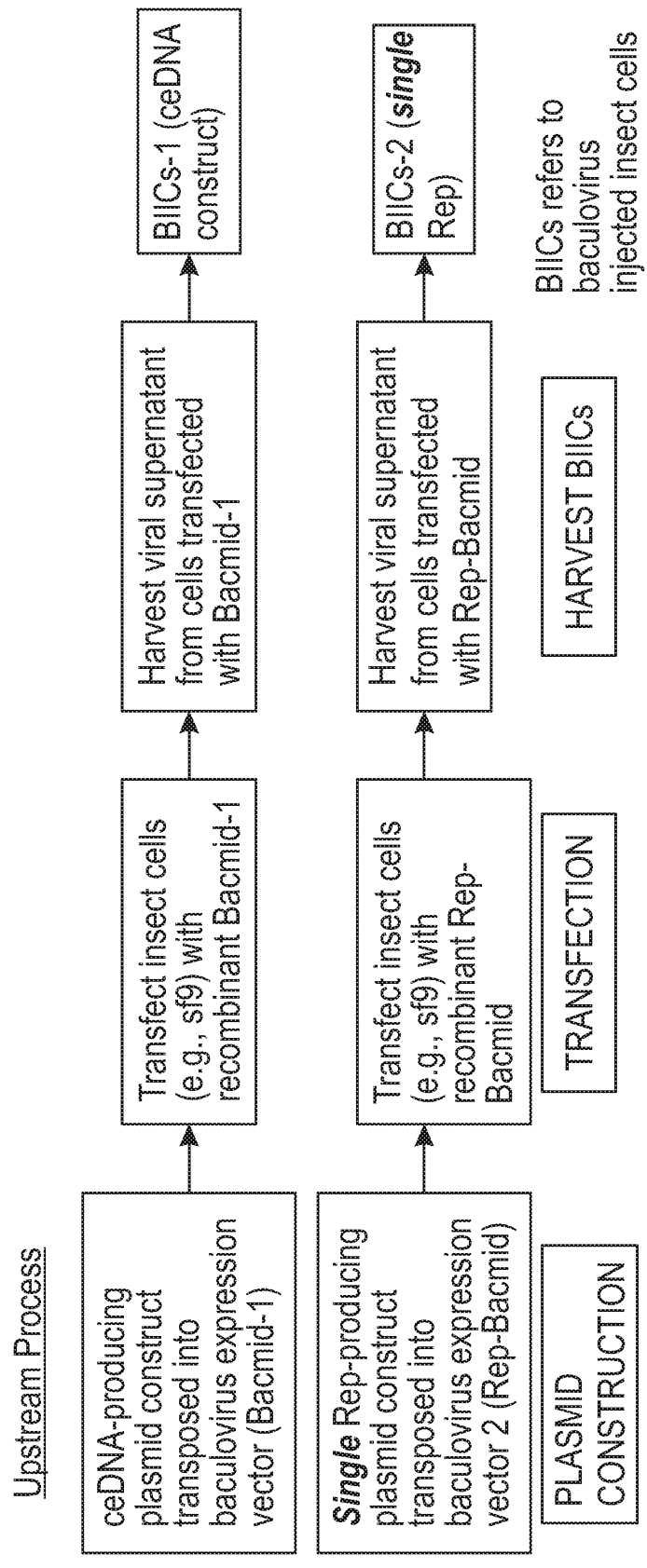

ceDNA Production
Upstream Process
ceDNA-producing plasmid construct transposed into baculovirus expression vector (Bacmid-1)
Single Rep-producing plasmid construct transposed into baculovirus expression vector 2 (Rep-Bacmid)
PLASMID CONSTRUCTION
Transfect insect cells (e.g., sf9) with recombinant Bacmid-1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

FIG. 4A

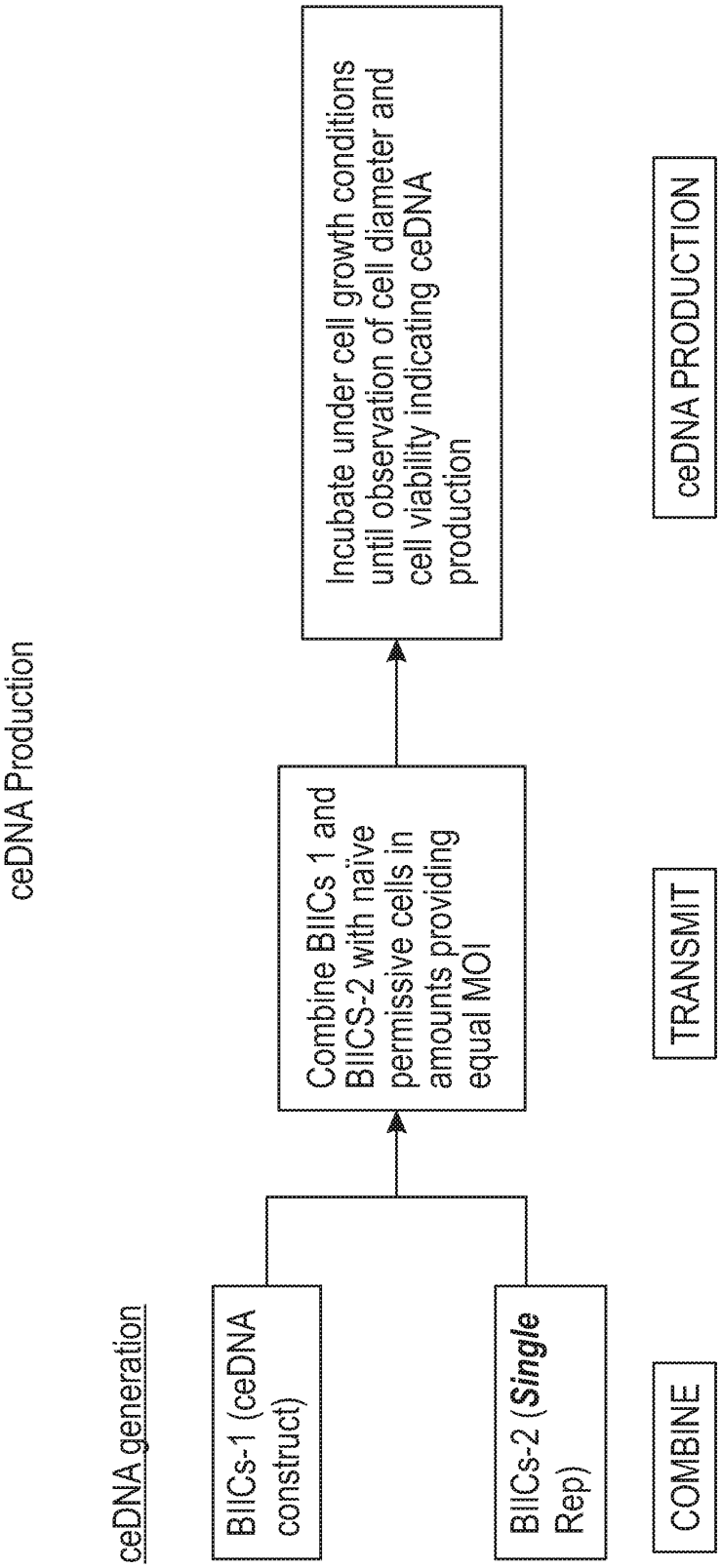

ceDNA generation
BIICs-1 (ceDNA construct)
BIICs-2 (Single Rep)
COMBINE
Combine BIICs 1 and BIICS-2 with naïve permissive cells in amounts providing equal MOI
TRANSMIT
Incubate under cell growth conditions until observation of cell diameter and cell viability indicating ceDNA production
ceDNA PRODUCTION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

FIG. 4B

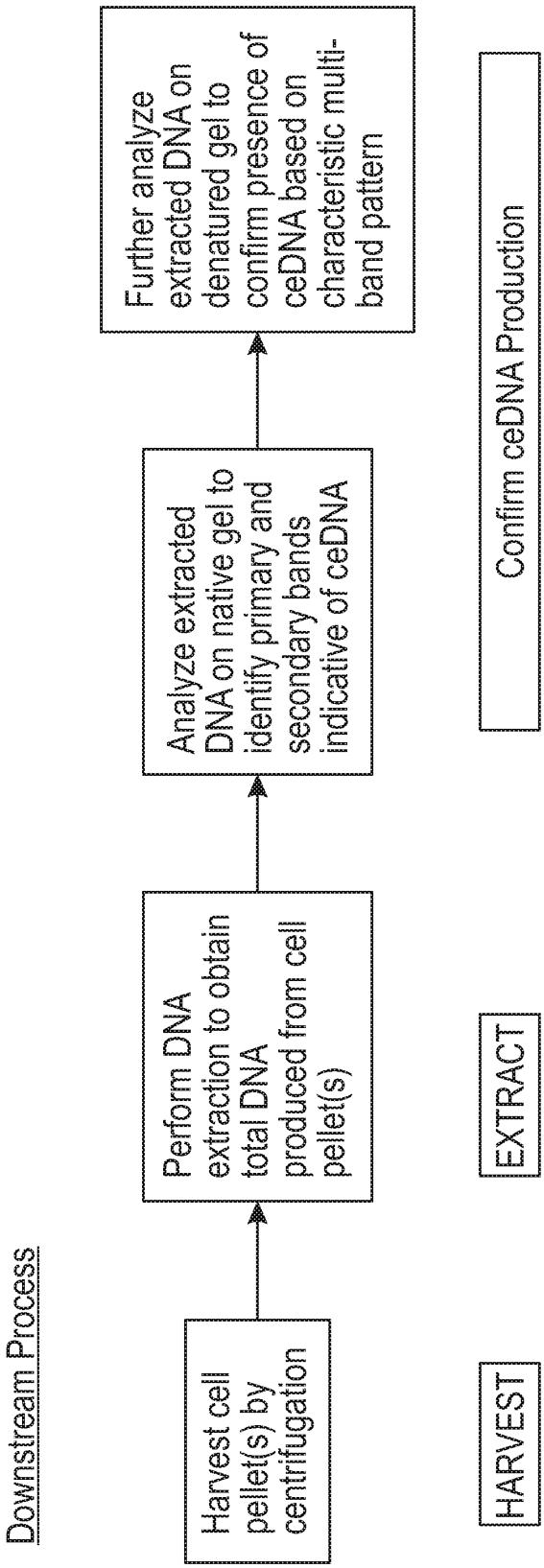

ceDNA Production
Downstream Process
Harvest cell pellet(s) by centrifugation
Perform DNA extraction to obtain total DNA produced from cell pellet(s)
Analyze extracted DNA on native gel to identify primary and secondary bands indicative of ceDNA
Further analyze extracted DNA on denatured gel to confirm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

FIG. 4C

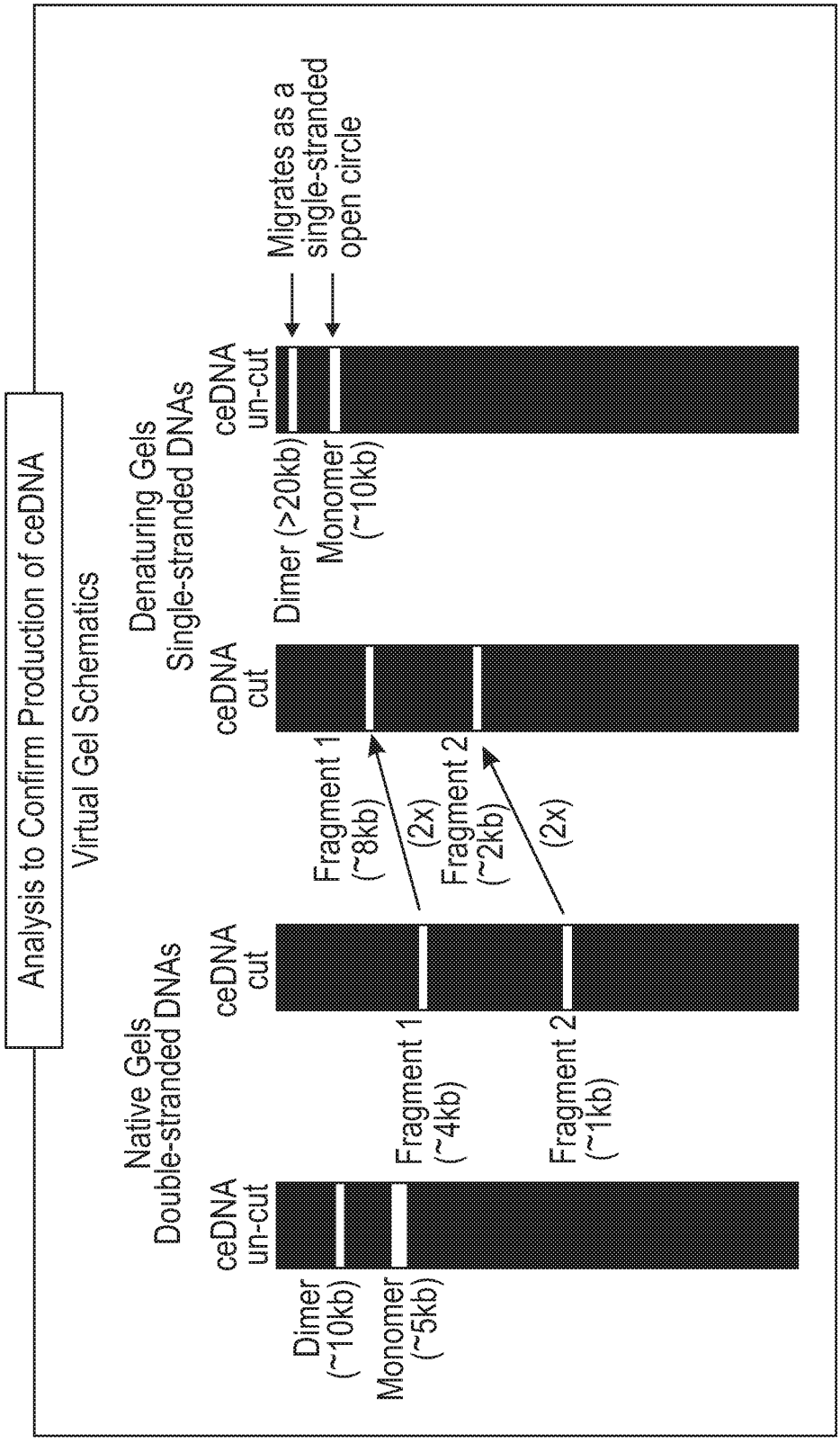

Analysis to Confirm Production of ceDNA
Virtual Gel Schematics
Native Gels
Double-stranded DNAs
ceDNA un-cut
Dimer (~10kb)
Monomer (~5kb)
ceDNA cut
Fragment 1 (~4kb)
Fragment 2 (~1kb)
Denaturing Gels
Single-stranded DNAs
ceDNA cut
Fragment 1 (~8kb)
(2x)
Fragment 2 (~2kb)
(2x)
ceDNA un-cut
Dimer (>20kb)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

FIG. 4D

FIG. 4E

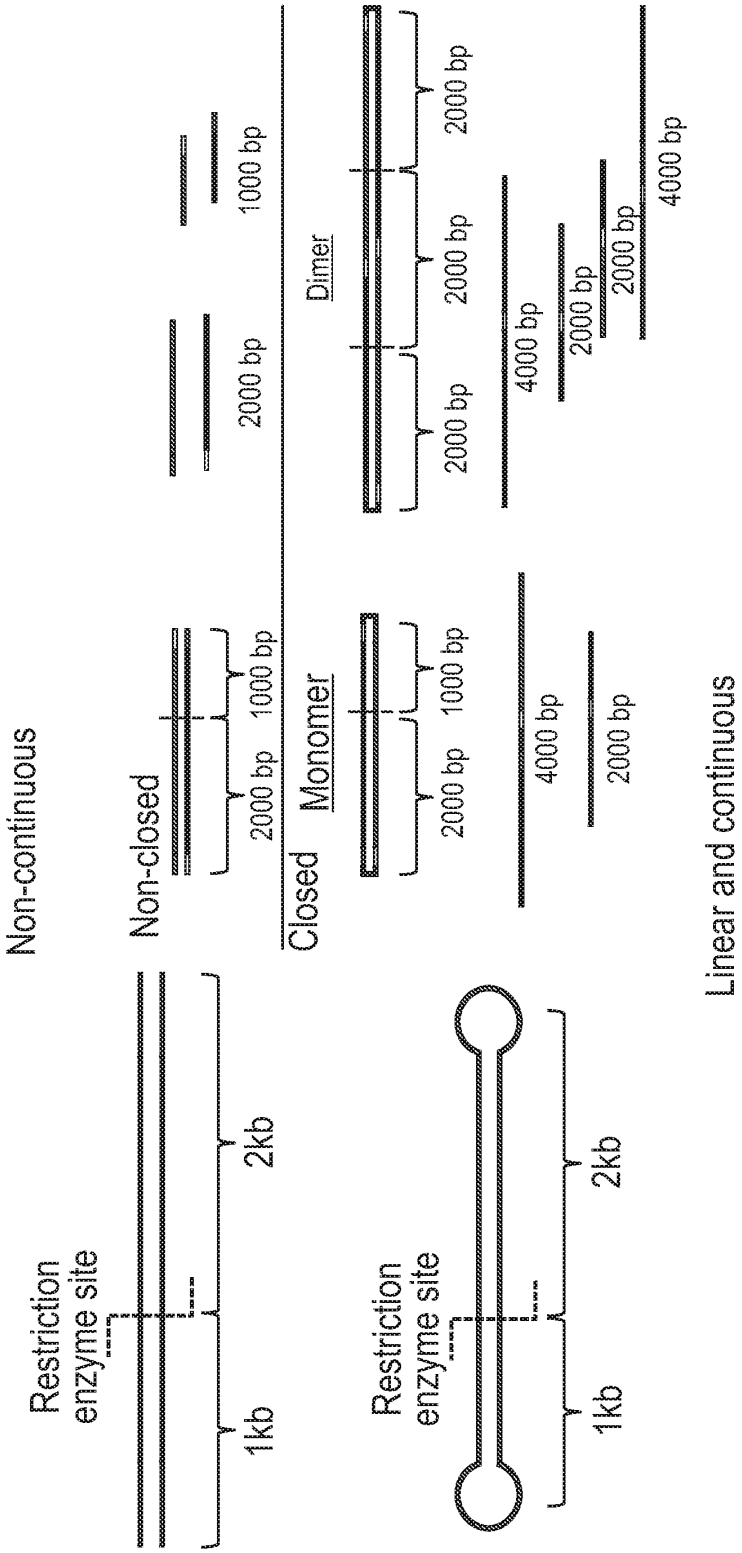
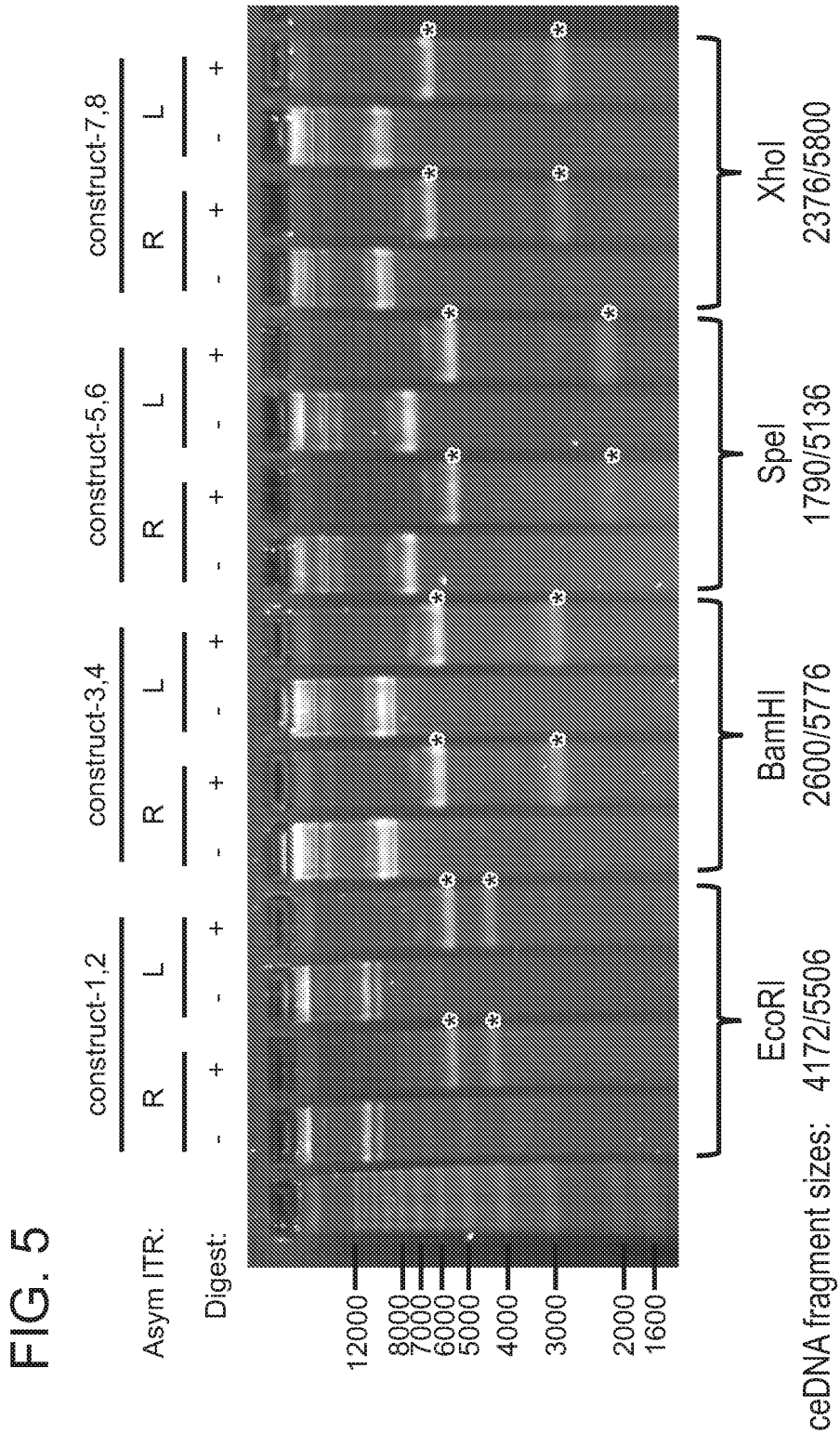

Non-continuous
Non-closed
Closed
Monomer
Dimer
Linear and continuous
Restriction enzyme site
1kb
2kb
2000 bp
1000 bp
4000 bp

FIG. 5


construct-1,2
construct-3,4
construct-5,6
construct-7,8
Asym ITR:
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

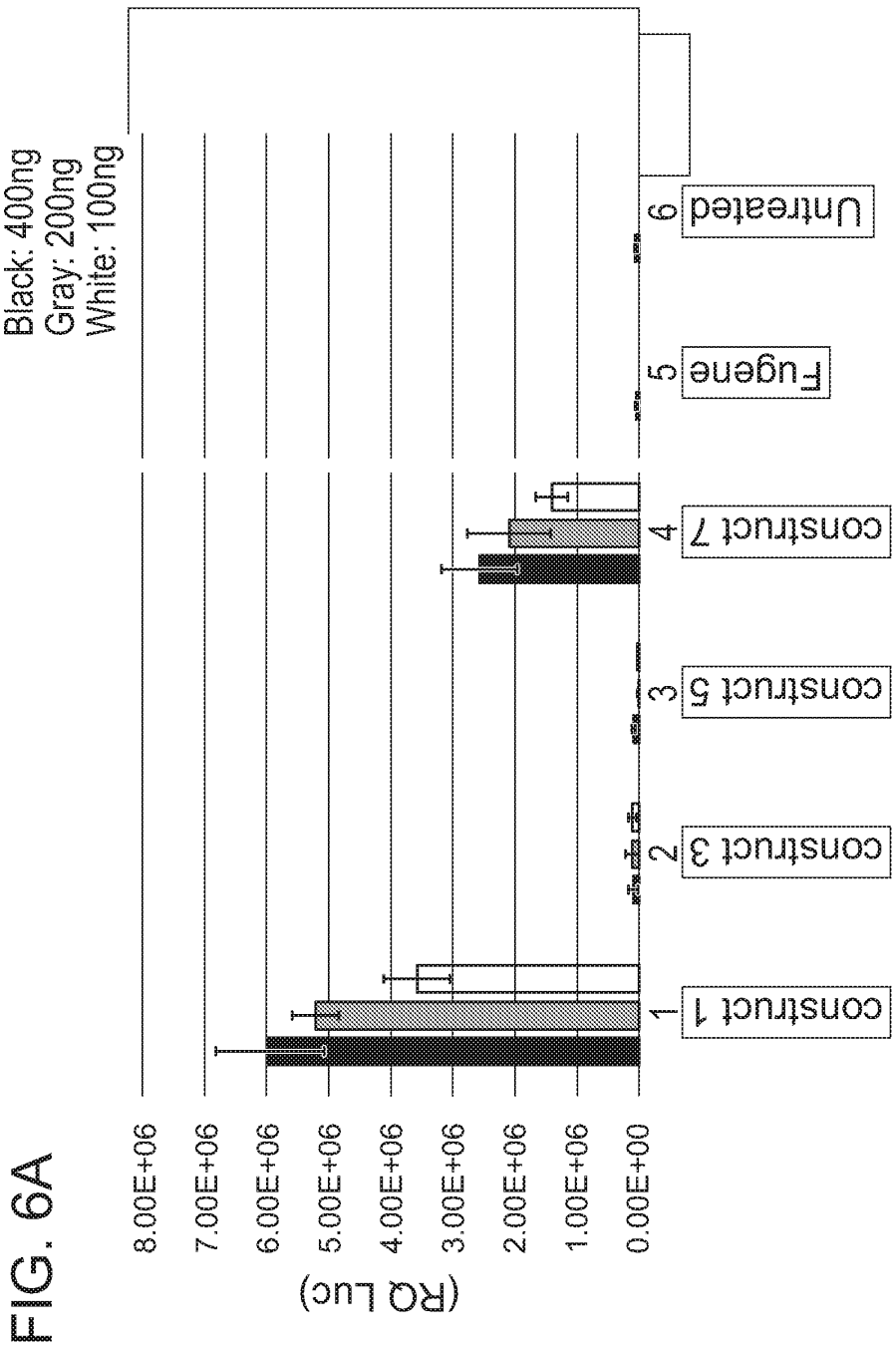

Black: 400ng
Gray: 200ng
White: 100ng
(RQ Luc)
8.00E+06
7.00E+06
6.00E+06
5.00E+06
4.00E+06
3.00E+06
2.00E+06
1.00E+06
0.00E+00
1
construct 1
2
construct 3
3
construct 5
4
construct 7
5
TFugene
6
Untreated

FIG. 6A

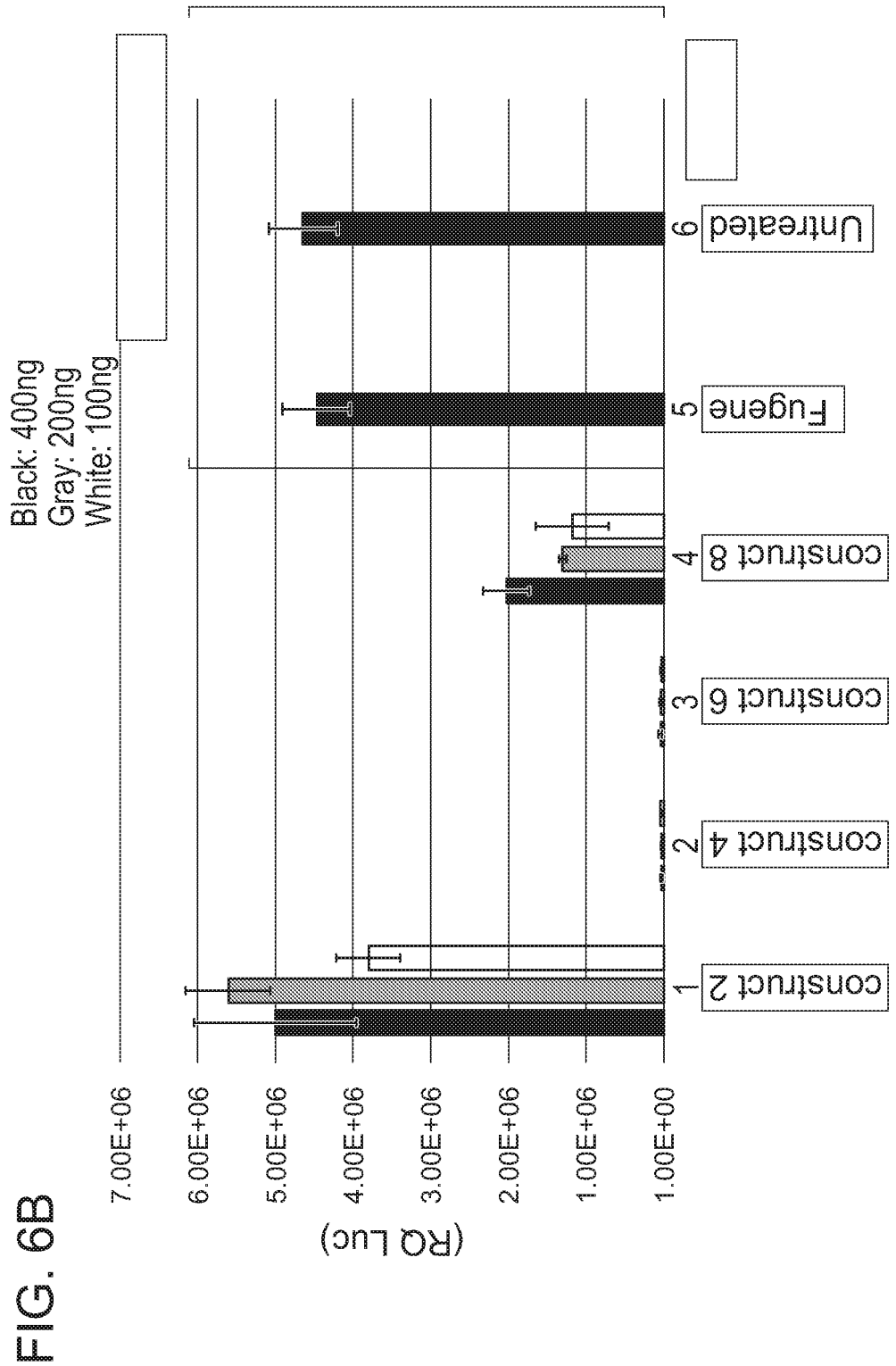

Black: 400ng
Gray: 200ng
White: 100ng
(RQ Luc)
7.00E+06
6.00E+06
5.00E+06
4.00E+06
3.00E+06
2.00E+06
1.00E+06
1.00E+00
1 construct 2
2 construct 4
3 construct 6
4 construct 8
5 Fugene
6 Untreated

FIG. 6B

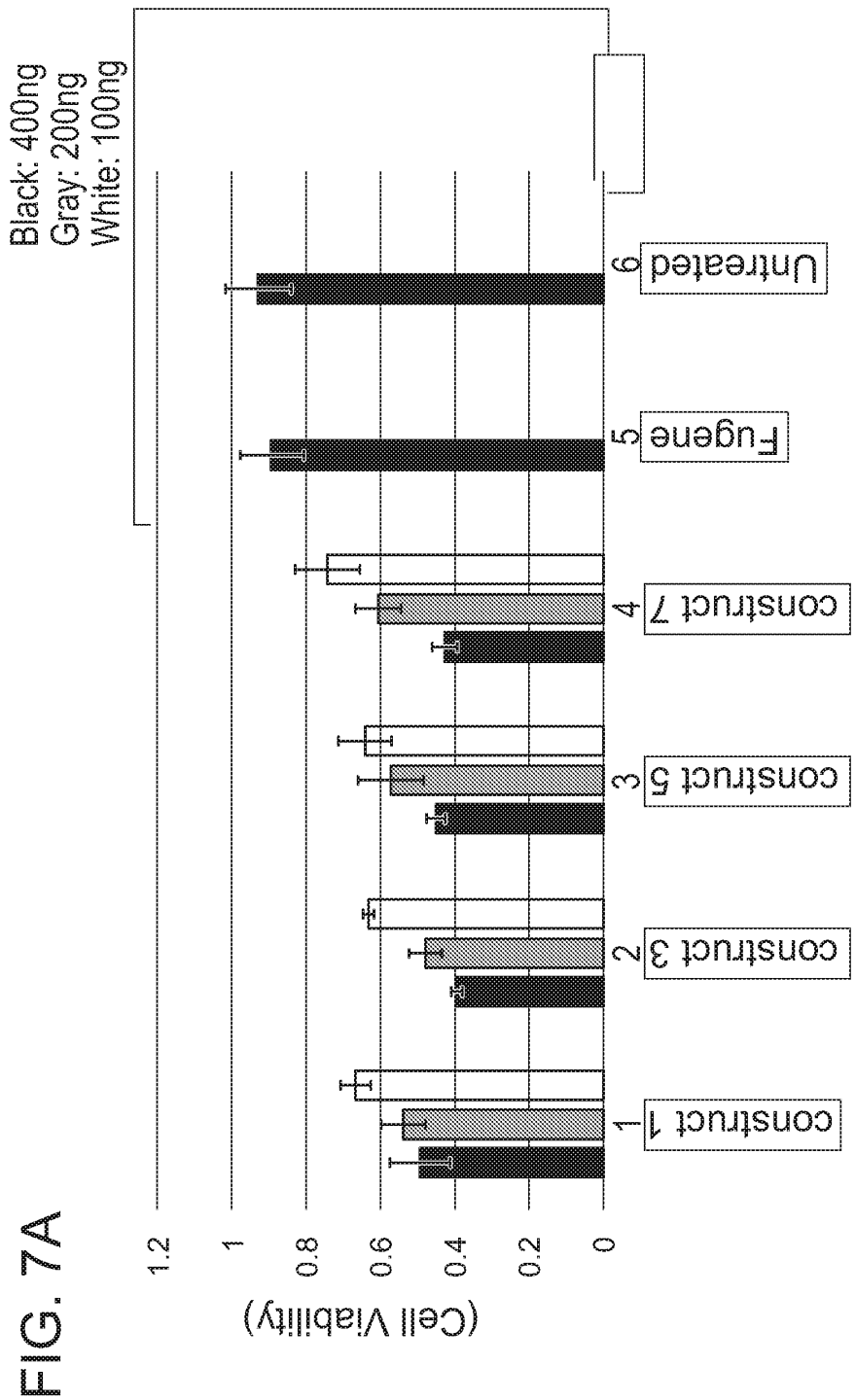

Black: 400ng
Gray: 200ng
White: 100ng
(Cell Viability)
1.2
1
0.8
0.6
0.4
0.2
0
construct 1
construct 3
construct 5
construct 7
Fugene
Untreated
1
2
3
4
5
6

FIG. 7A

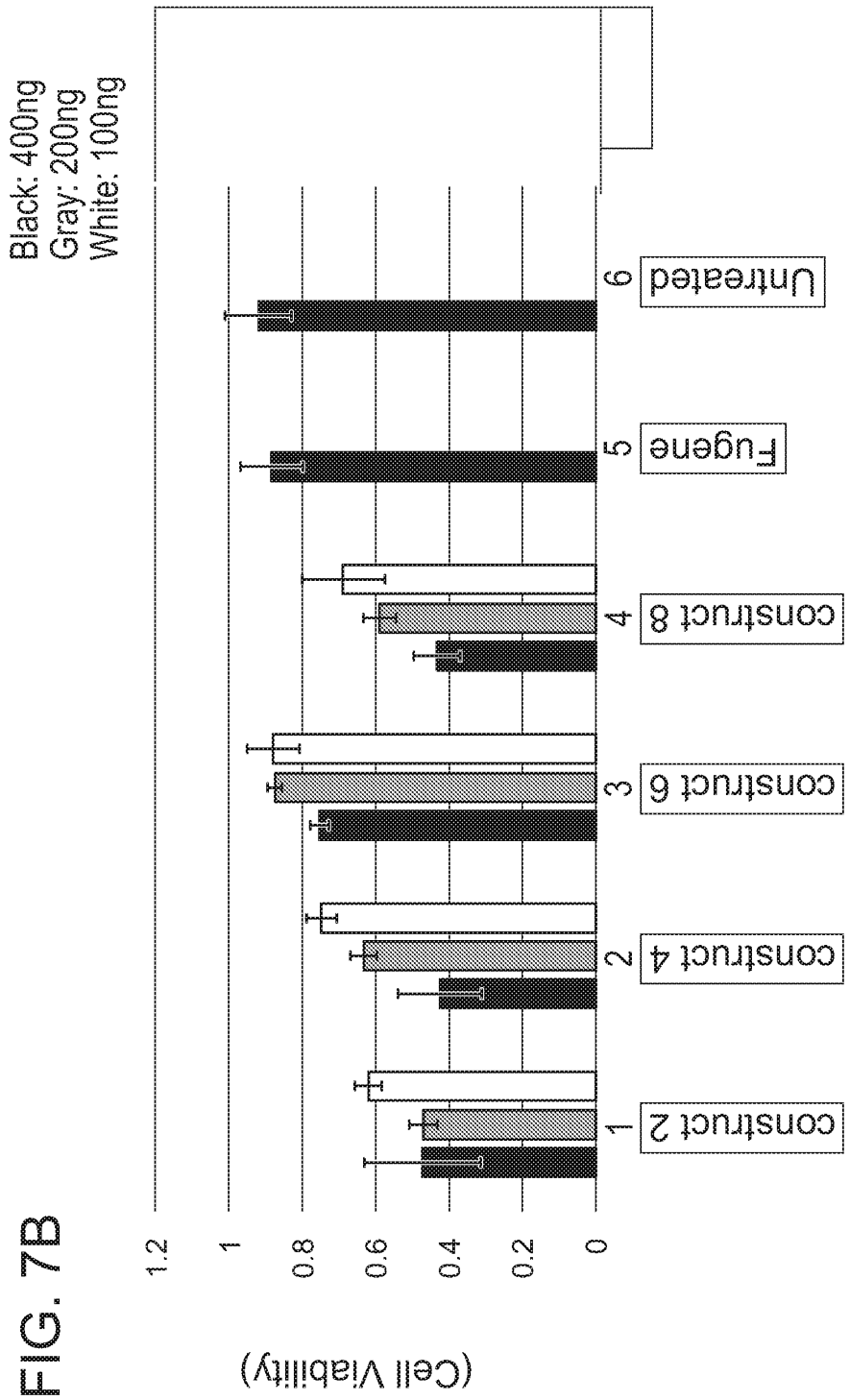

Black: 400ng
Gray: 200ng
White: 100ng
(Cell Viability)
1.2
1
0.8
0.6
0.4
0.2
0
1
construct 2
2
construct 4
3
construct 6
4
construct 8
5
Fugene
6
Untreated

FIG. 7B

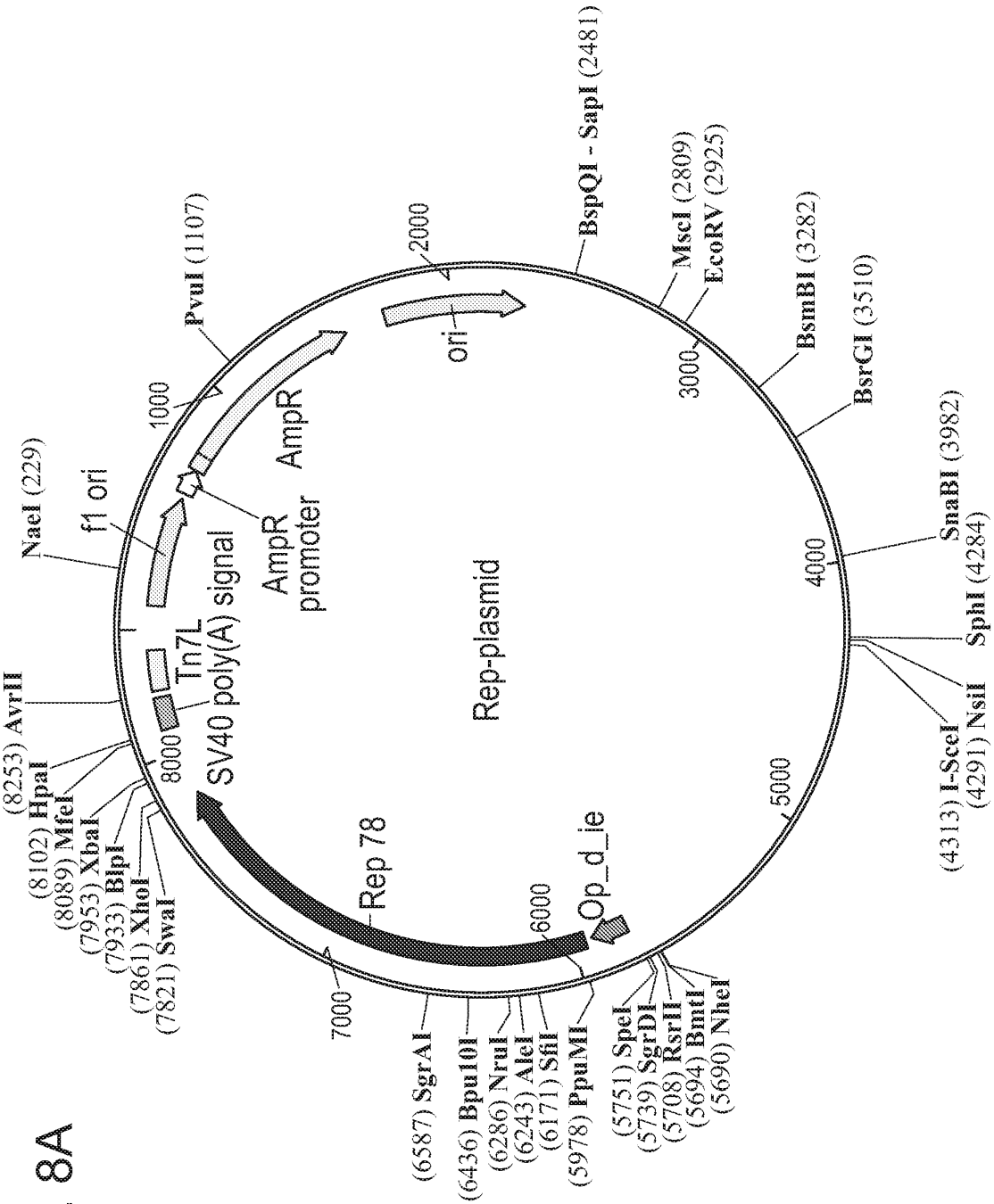

Rep-plasmid
ori
AmpR
AmpR promoter
SV40 poly(A) signal
Tn7L
f1 ori
Rep 78
Op_d_ie
1000
2000
3000
4000
5000
6000
7000
8000
PvuI (1107)
BspQI - SapI (2481)
MscI (2809)
EcoRV (2925)
BsmBI (3282)
BsrGI (3510)
SnaBI (3982)
SphI (4284)
NsiI (4291)
I-SceI (4313)
NaeI (229)
AvrII (8253)
HpaI (8102)
MfeI (8089)
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. 8A

FIG. 8B

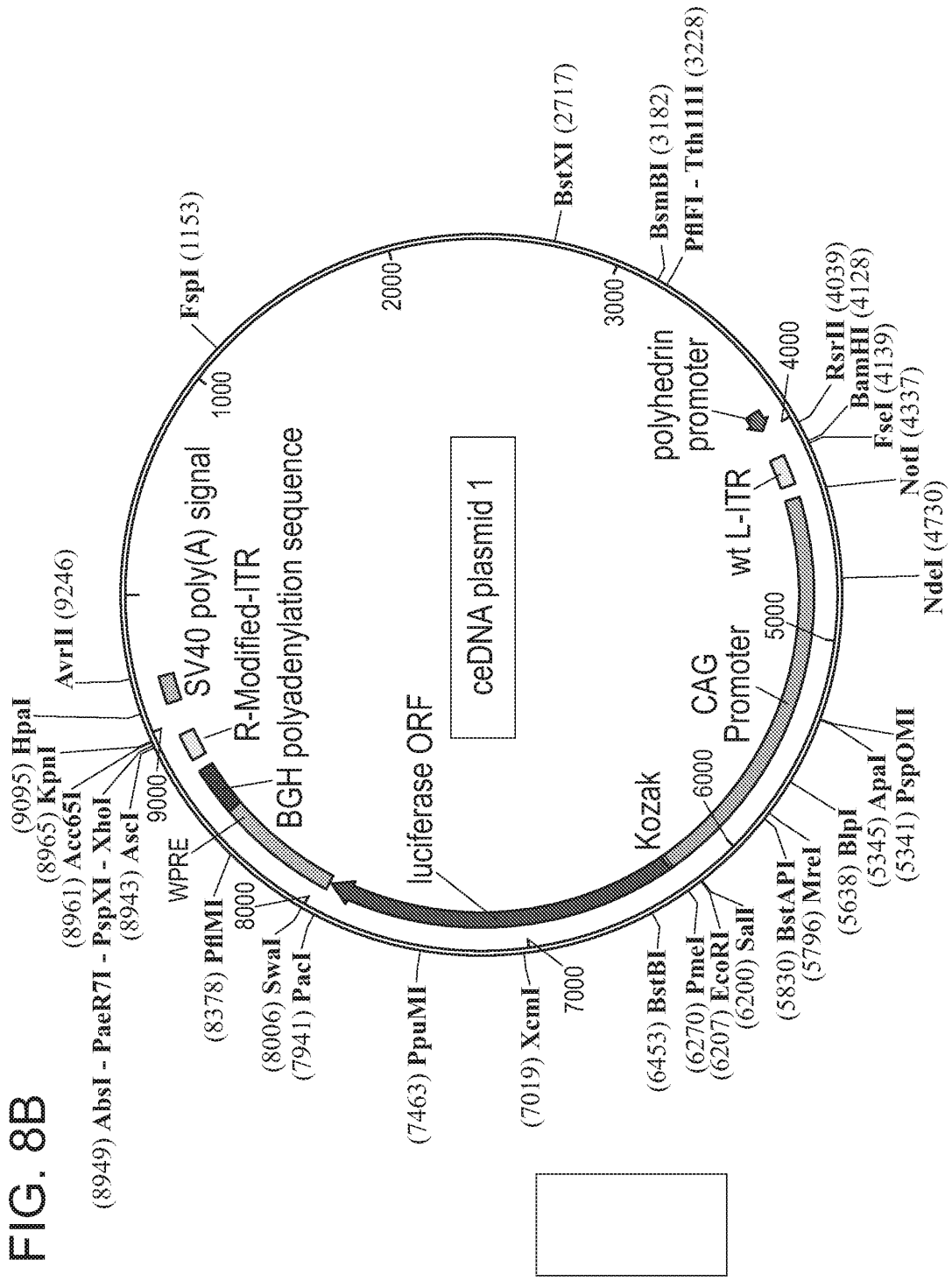

ceDNA plasmid 1
1000
2000
3000
4000
5000
6000
7000
9000
8000
SV40 poly(A) signal
R-Modified-ITR
BGH polyadenylation sequence
luciferase ORF
Kozak
CAG Promoter
wt L-ITR
polyhedrin promoter
WPRE
FspI (1153)
BstXI (2717)
BsmBI (3182)
PflFI - Tth111I (3228)
RsrII (4039)
BamHI (4128)
FseI (4139)
NotI (4337)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

FIG. 9A

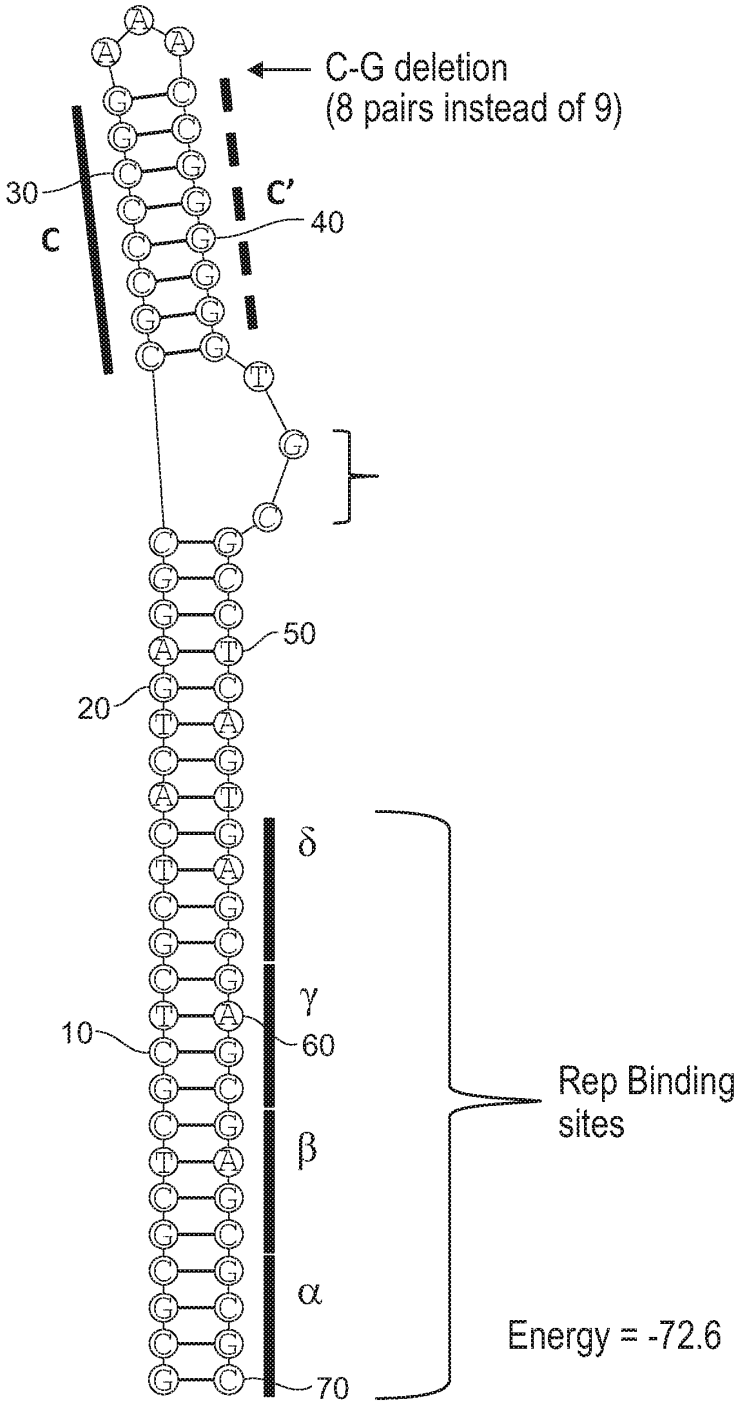

C-G deletion
(8 pairs instead of 9)
30
C
C'
40
50
20
δ
γ
10
60
β
α
70
Rep Binding sites
ITR-2, Left
Energy = -72.6

FIG. 9B

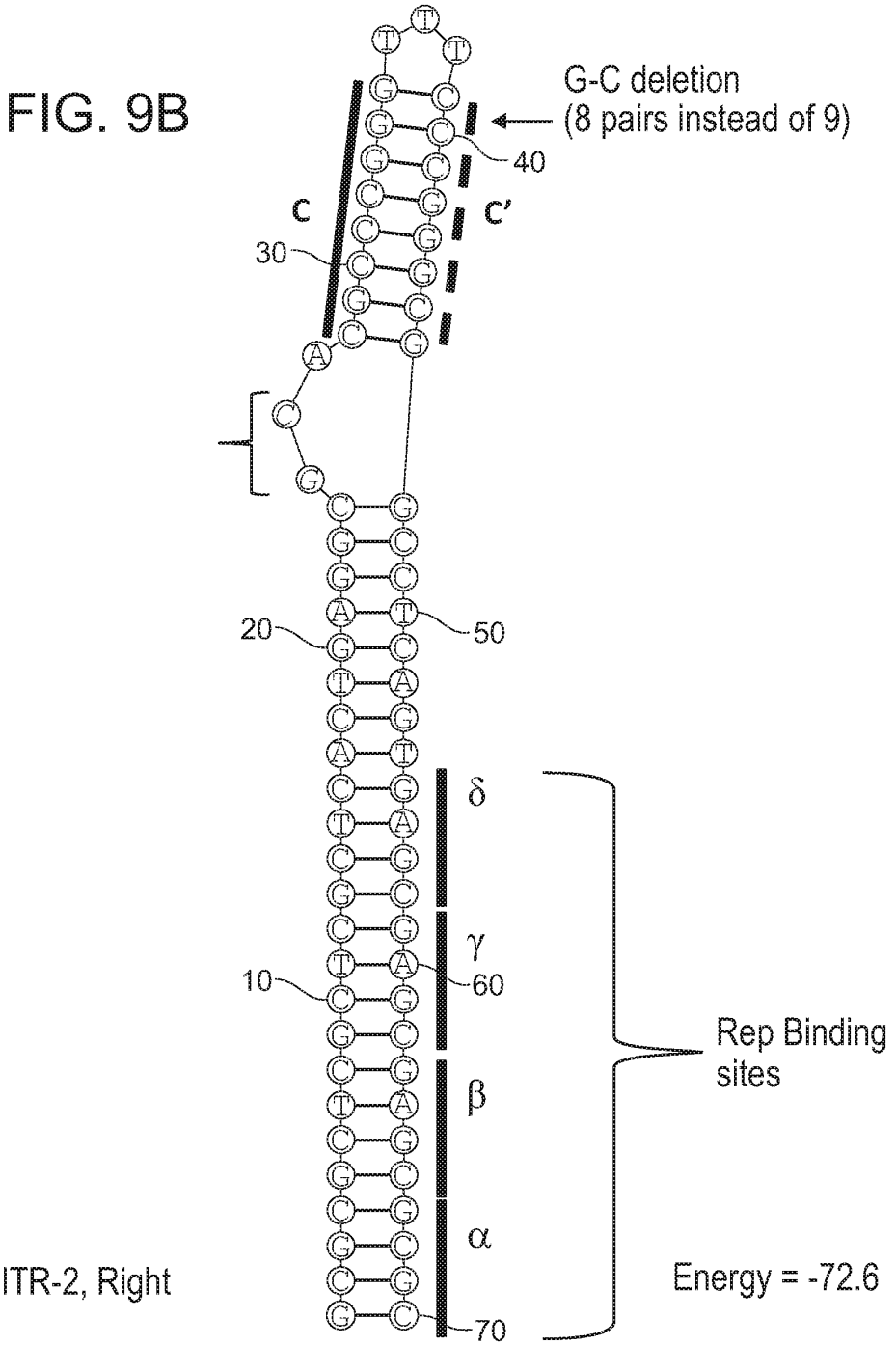

G-C deletion
(8 pairs instead of 9)
40
C
C'
30
20
50
δ
γ
10
60
β
α
70
Rep Binding
sites
ITR-2, Right
Energy = -72.6

FIG. 10A

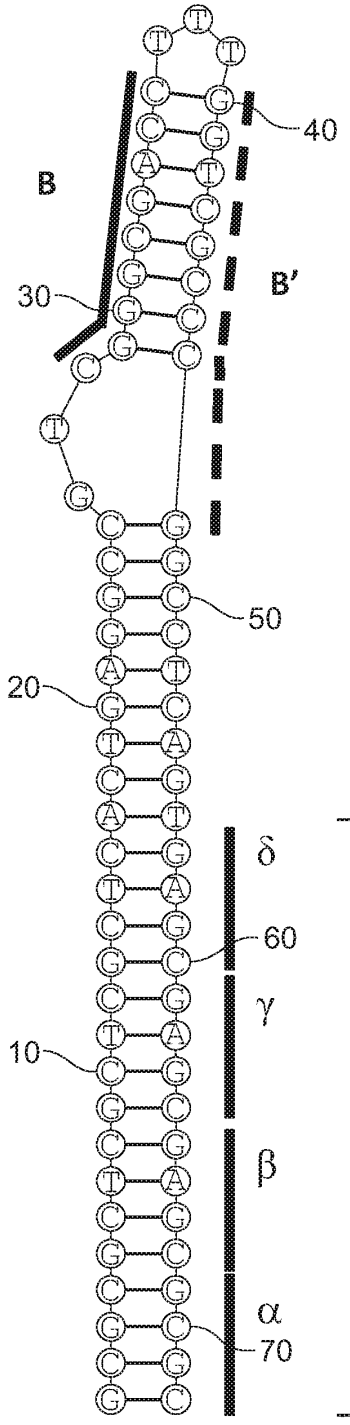

B
B'
30
40
50
20
δ
60
γ
10
β
α
70
Rep Binding sites
ITR-3, Left
Energy = -74.8

FIG. 10B

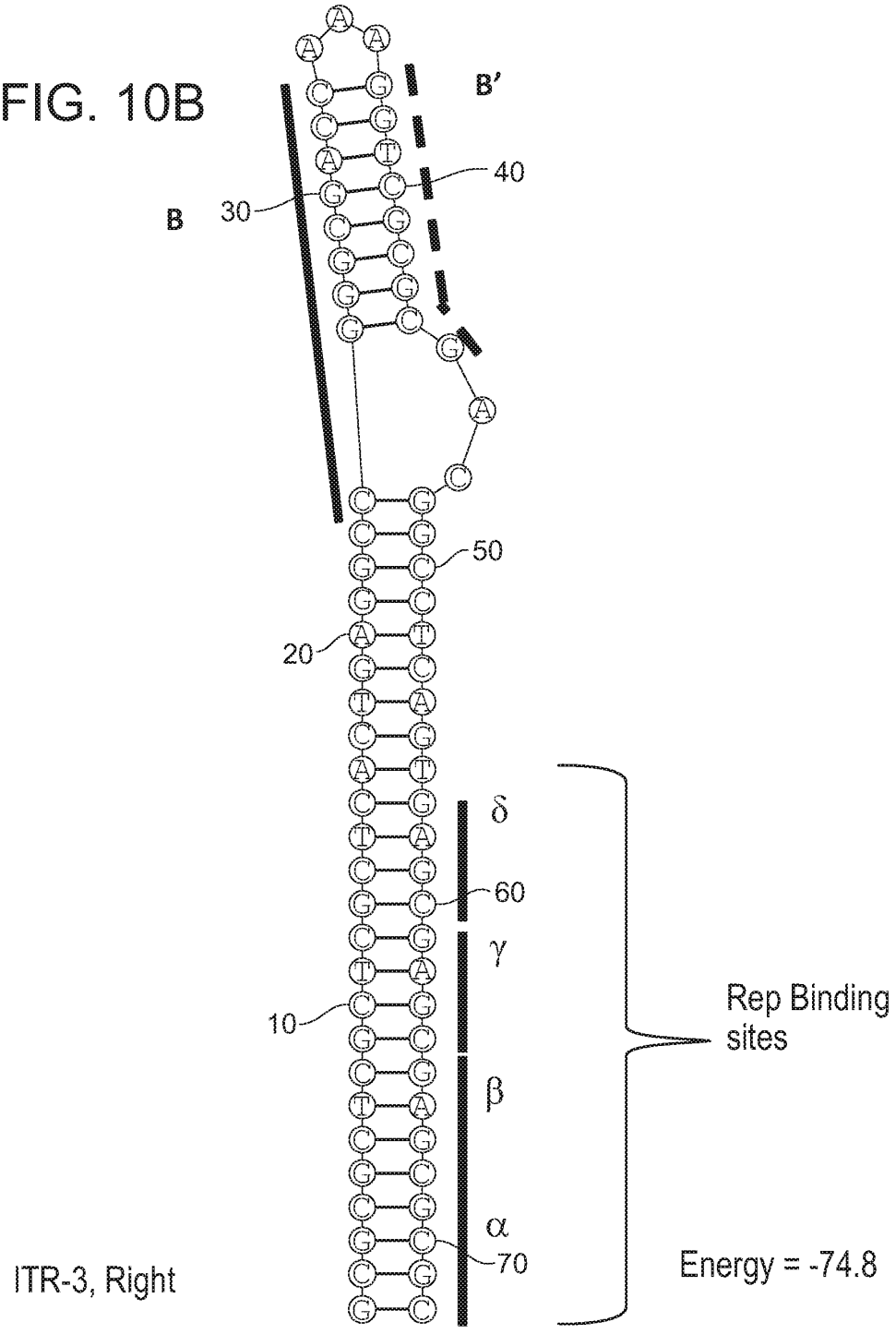

B'
B
30
40
50
20
δ
60
γ
10
β
α
70
Rep Binding sites
ITR-3, Right
Energy = -74.8

FIG. 11A

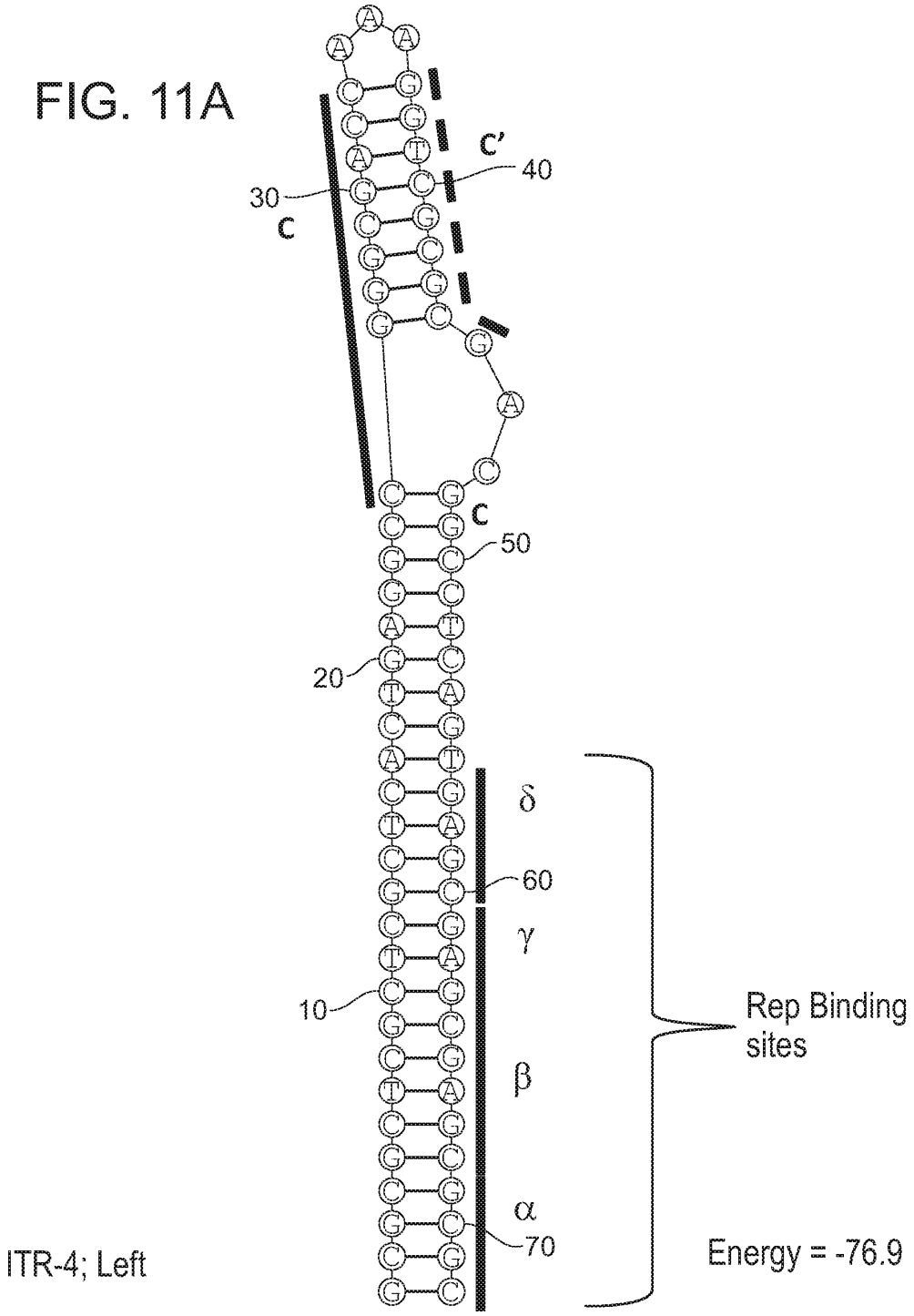

C'
40
30
C
C
50
20
δ
60
γ
10
Rep Binding sites
β
α
70
ITR-4; Left
Energy = -76.9

FIG. 11B

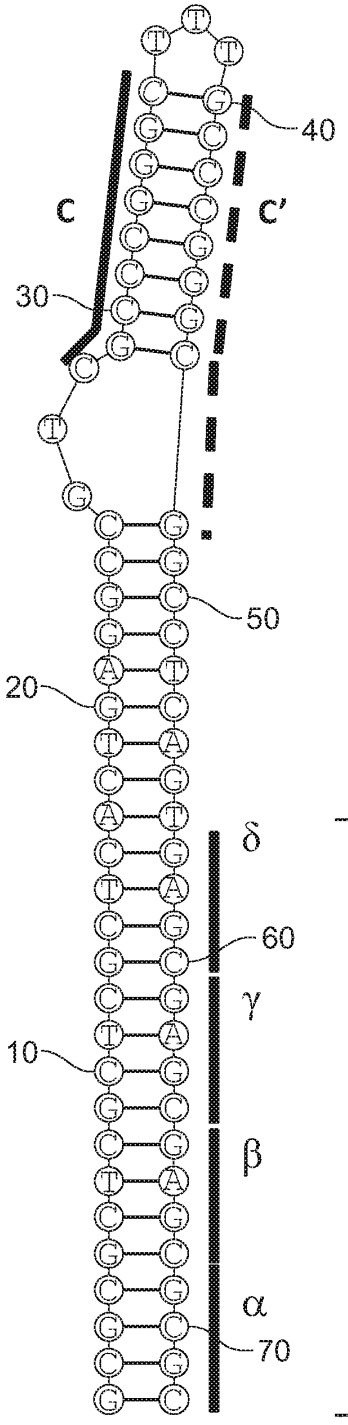
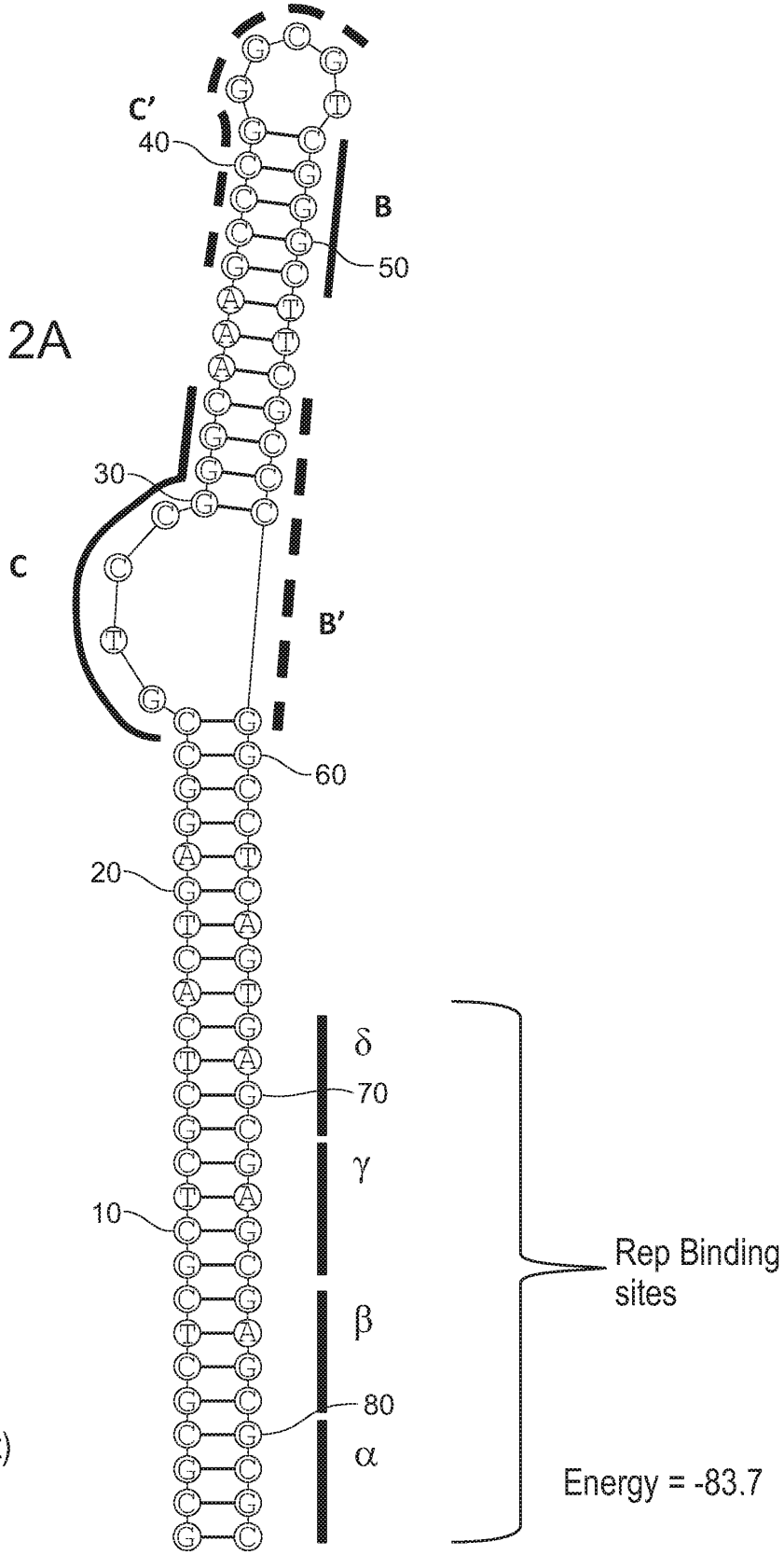

40
C
C'
30
50
20
δ
60
γ
10
β
α
70
Rep Binding sites
ITR-4, Right
Energy = -76.9

FIG. 12A




ITR-10, (Left)

FIG. 12B

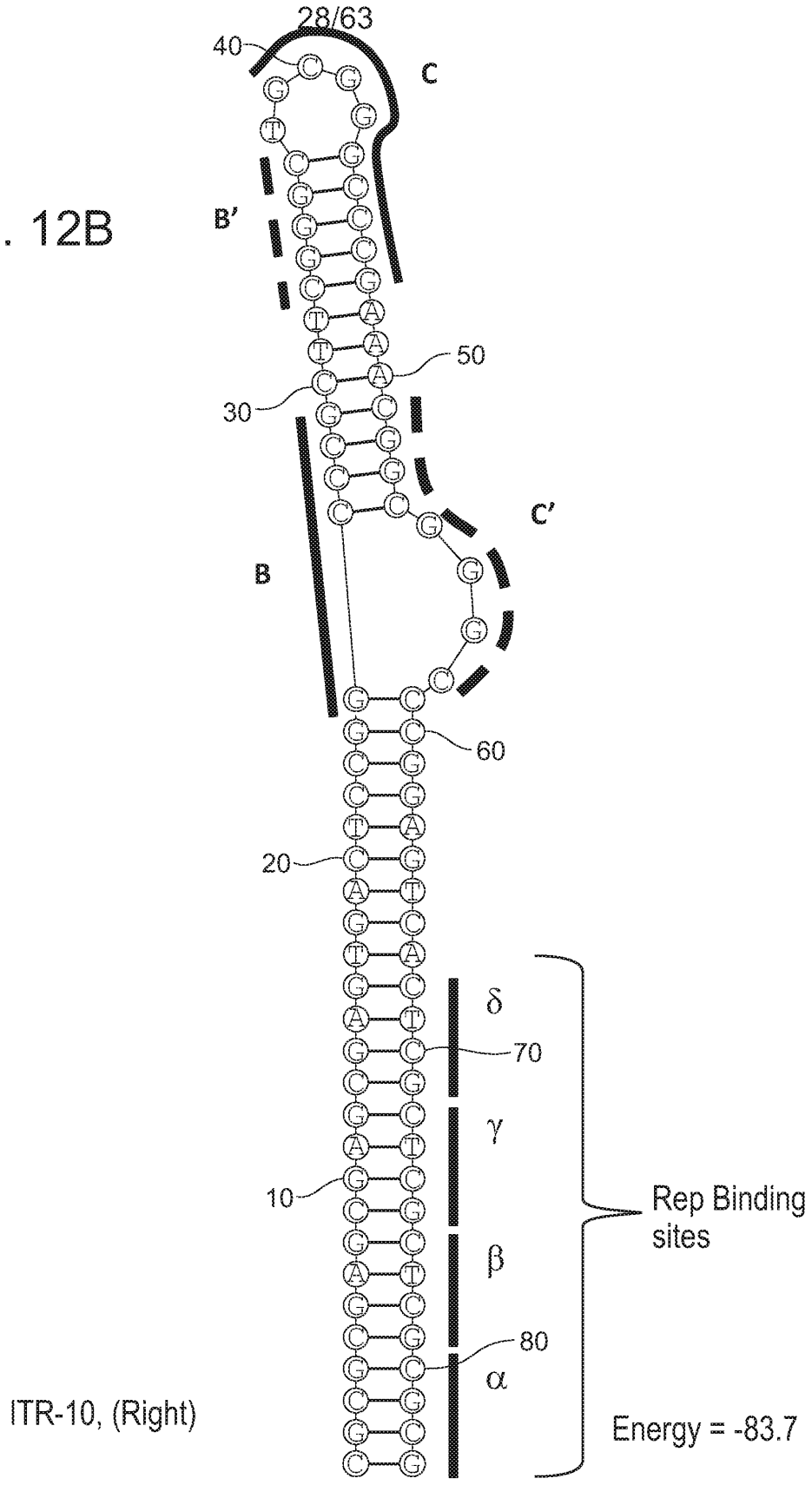

40
C
B'
50
30
C'
B
60
20
δ
70
γ
10
Rep Binding sites
β
80
α
ITR-10, (Right)
Energy = -83.7

FIG. 13A

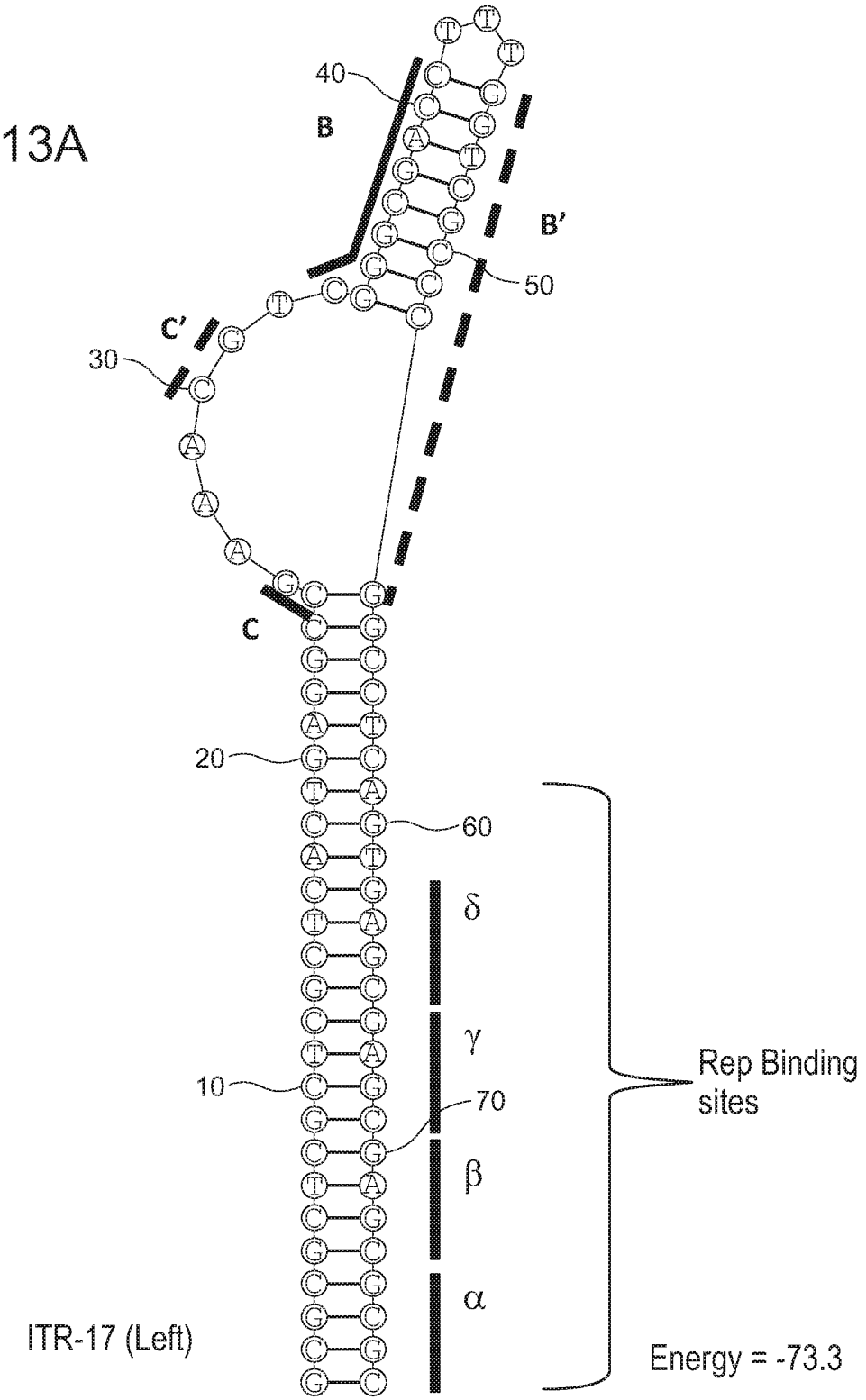
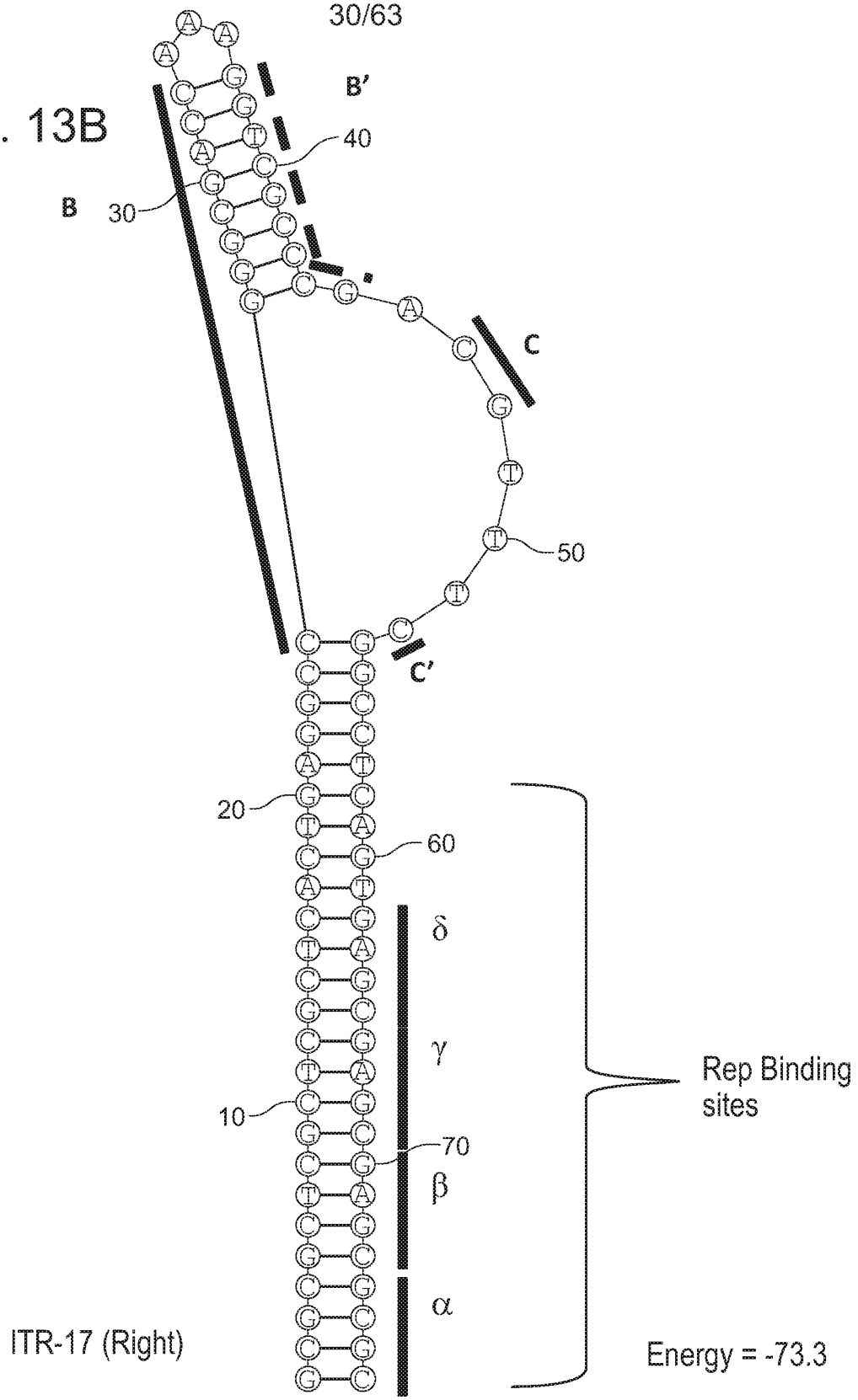

40
B
B'
50
C'
30
C
20
60
δ
γ
10
70
β
α
Rep Binding sites
ITR-17 (Left)
Energy = -73.3

FIG. 13B


B'
40
B
30
C
50
C'
20
60
δ
γ
10
70
β
α
Rep Binding sites
ITR-17 (Right)
Energy = -73.3

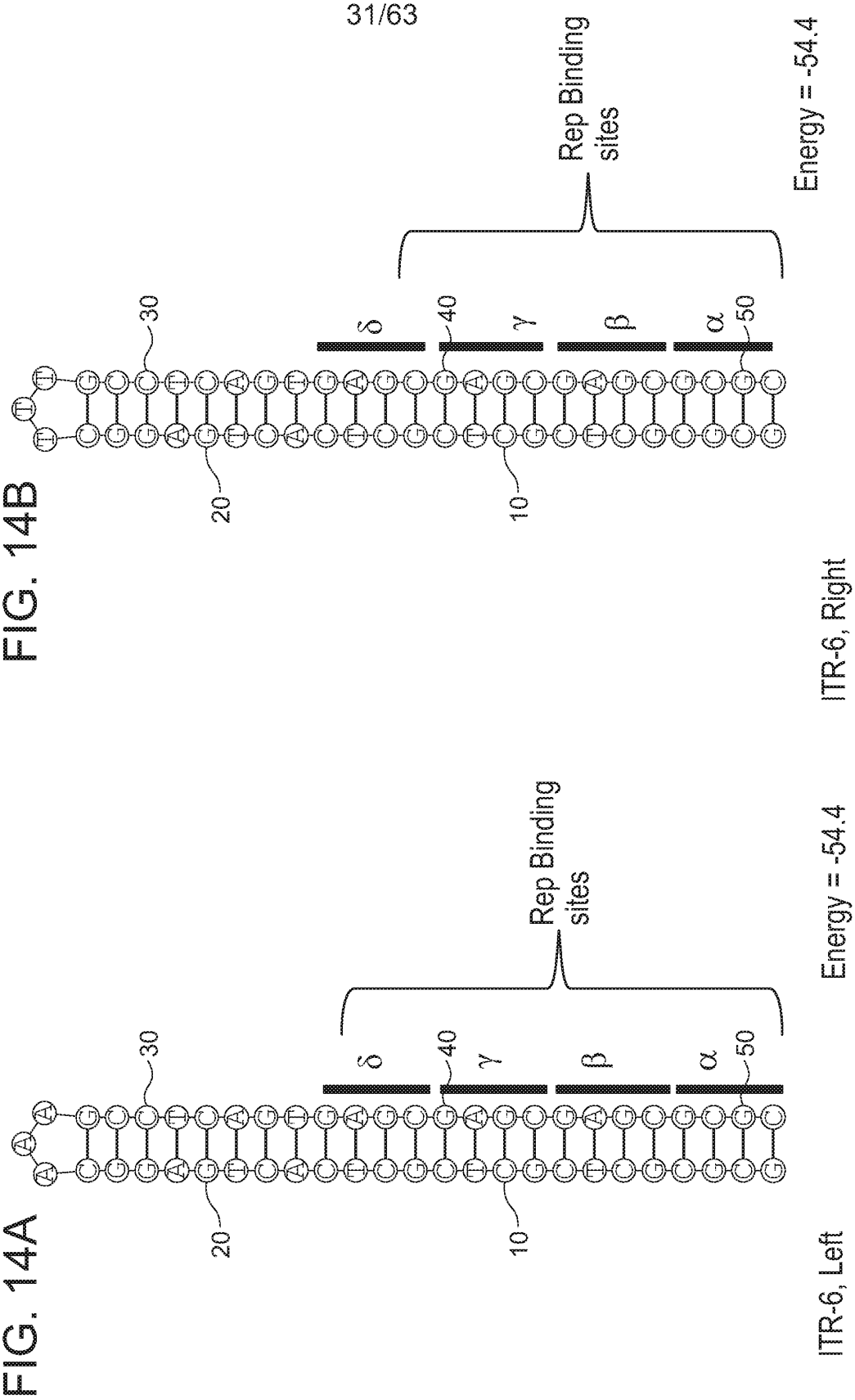



FIG. 14B

FIG. 14A

FIG. 15A

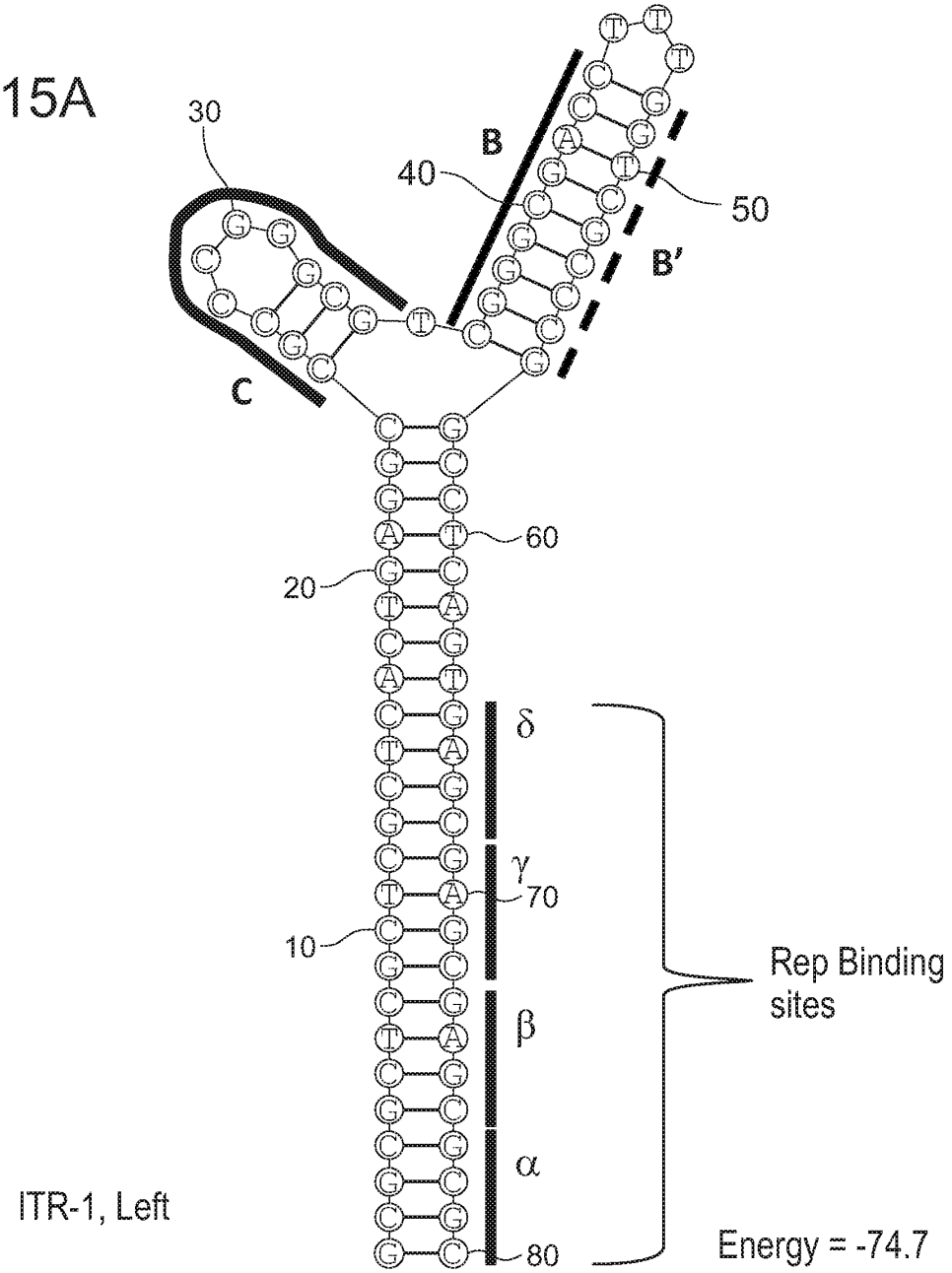

30
B
40
50
B'
C
60
20
δ
γ
70
10
Rep Binding sites
β
α
ITR-1, Left
80
Energy = -74.7

FIG. 15B

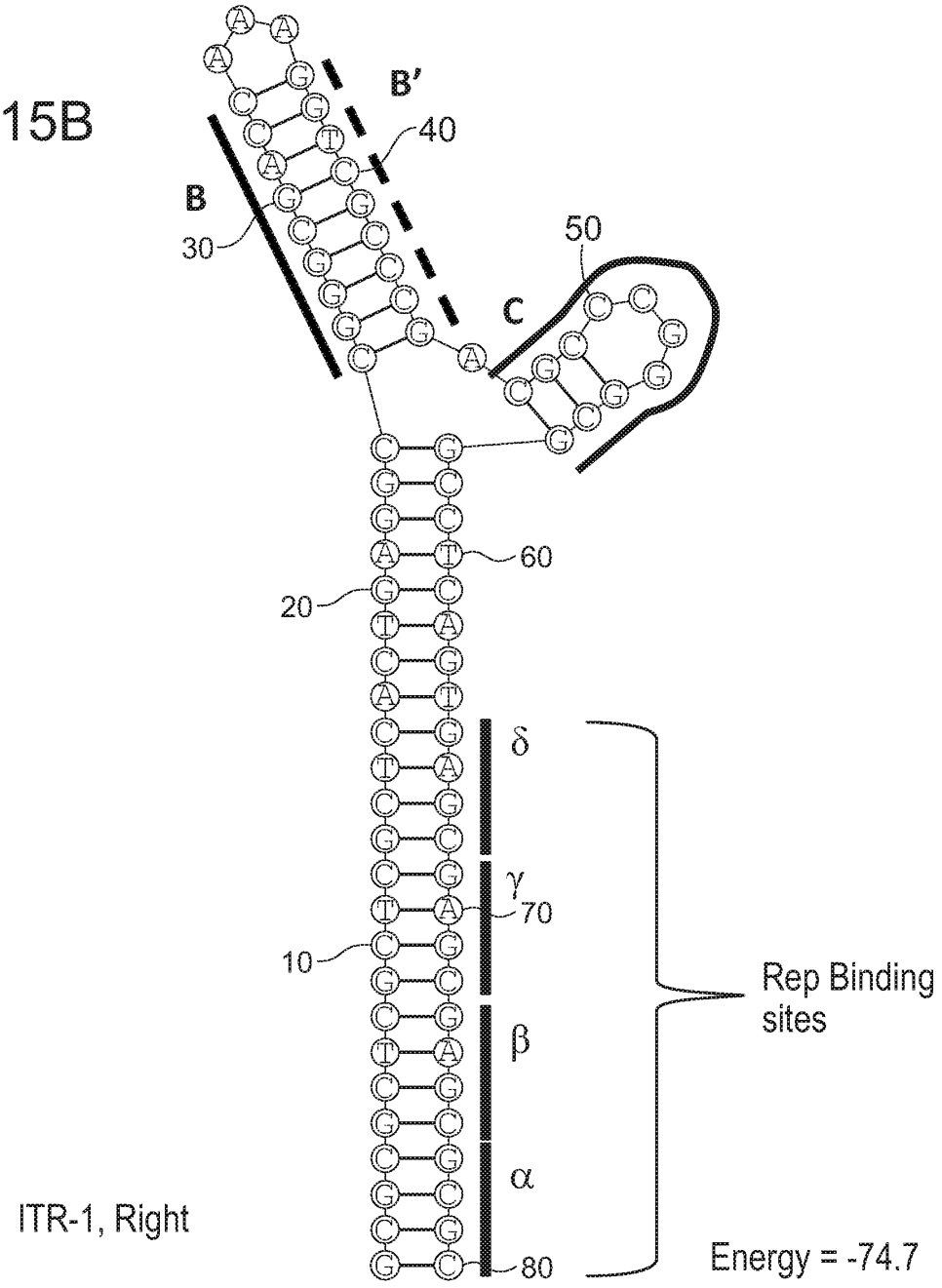

B'
40
B
30
50
C
60
20
δ
γ
70
10
Rep Binding sites
β
α
80
ITR-1, Right
Energy = -74.7

FIG. 16A

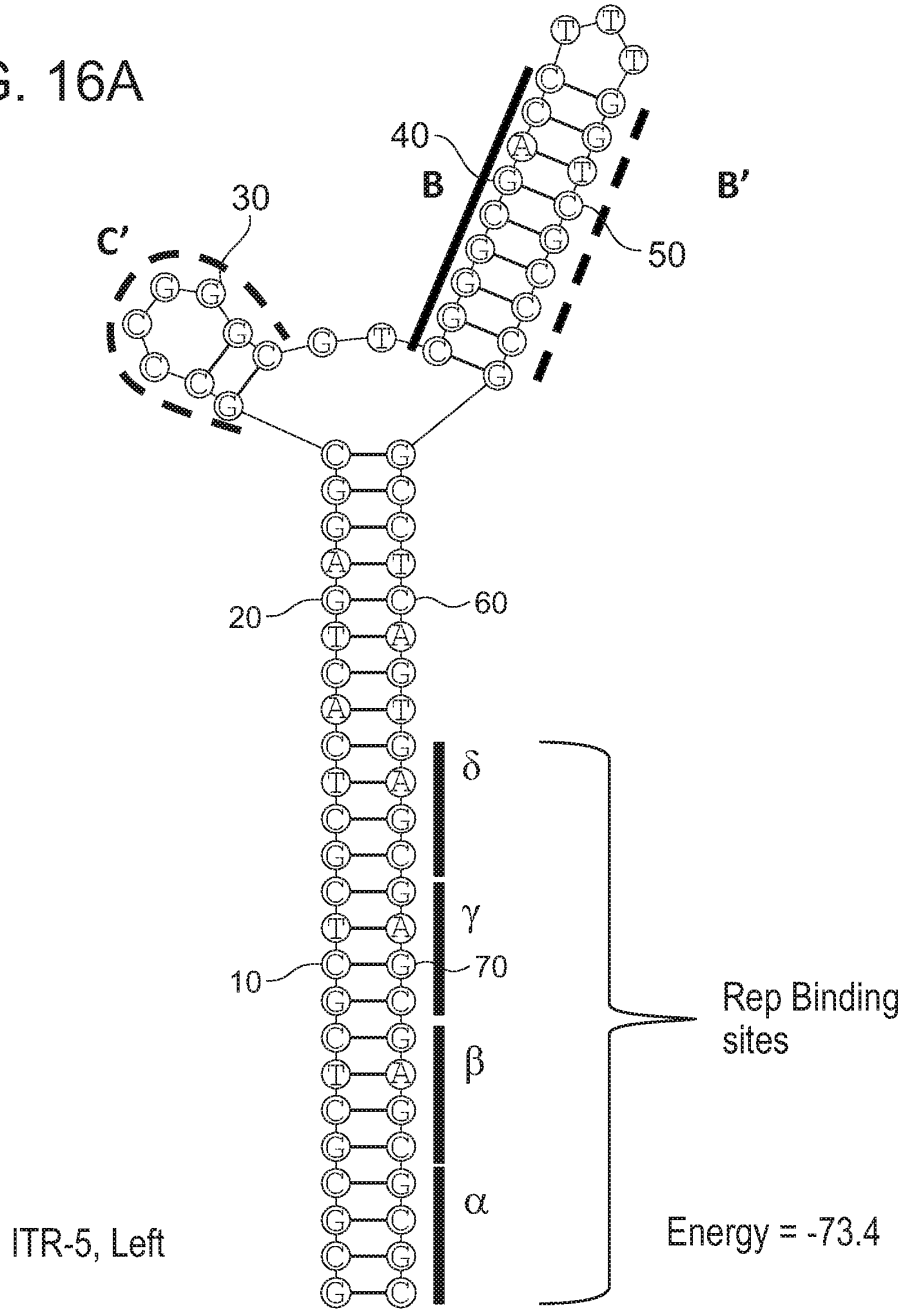
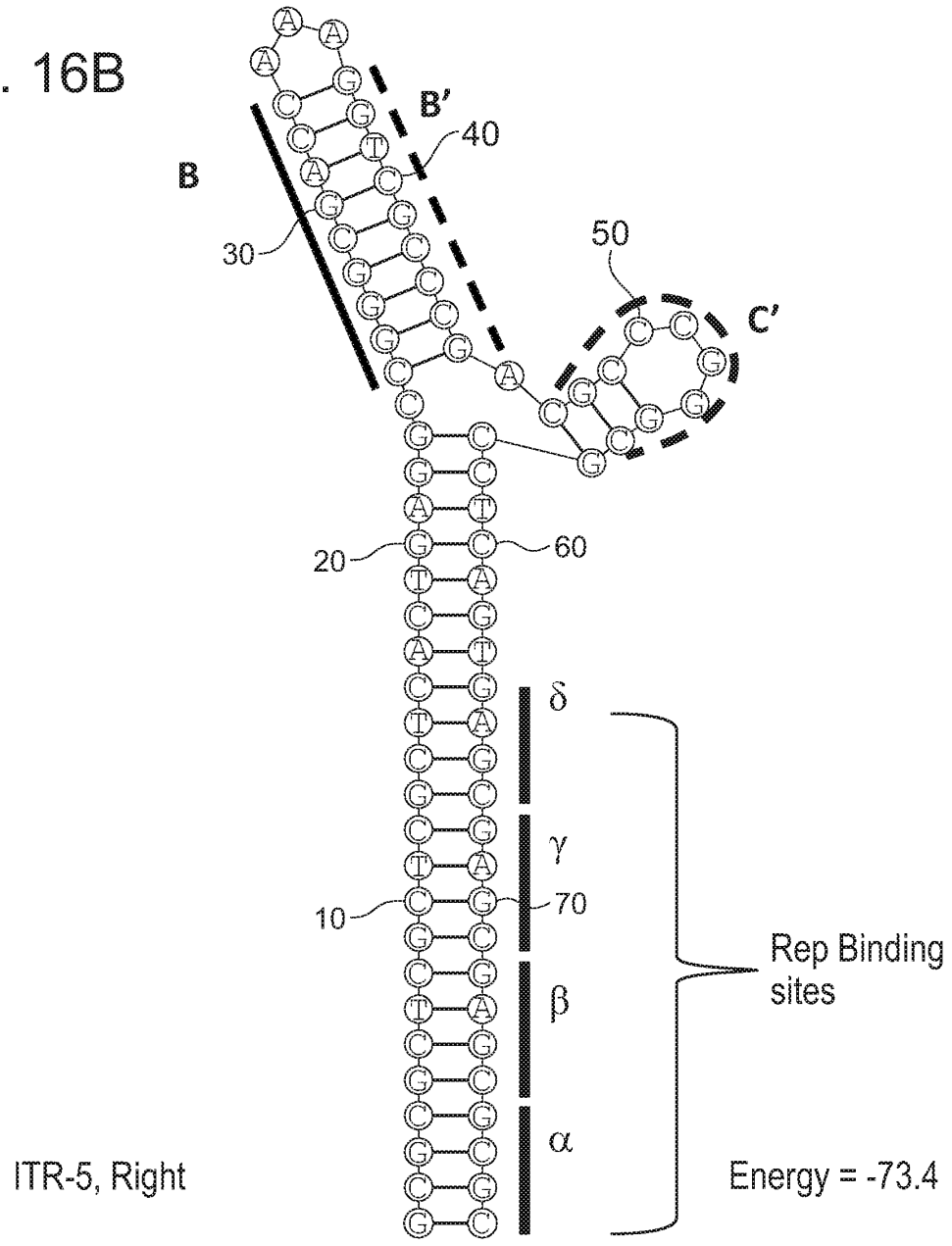

40
B
B'
30
C'
50
20
60
10
70
δ
γ
β
α
Rep Binding sites
ITR-5, Left
Energy = -73.4

FIG. 16B


B
B'
C'
30
40
50
60
20
10
70
δ
γ
β
α
Rep Binding sites
ITR-5, Right
Energy = -73.4

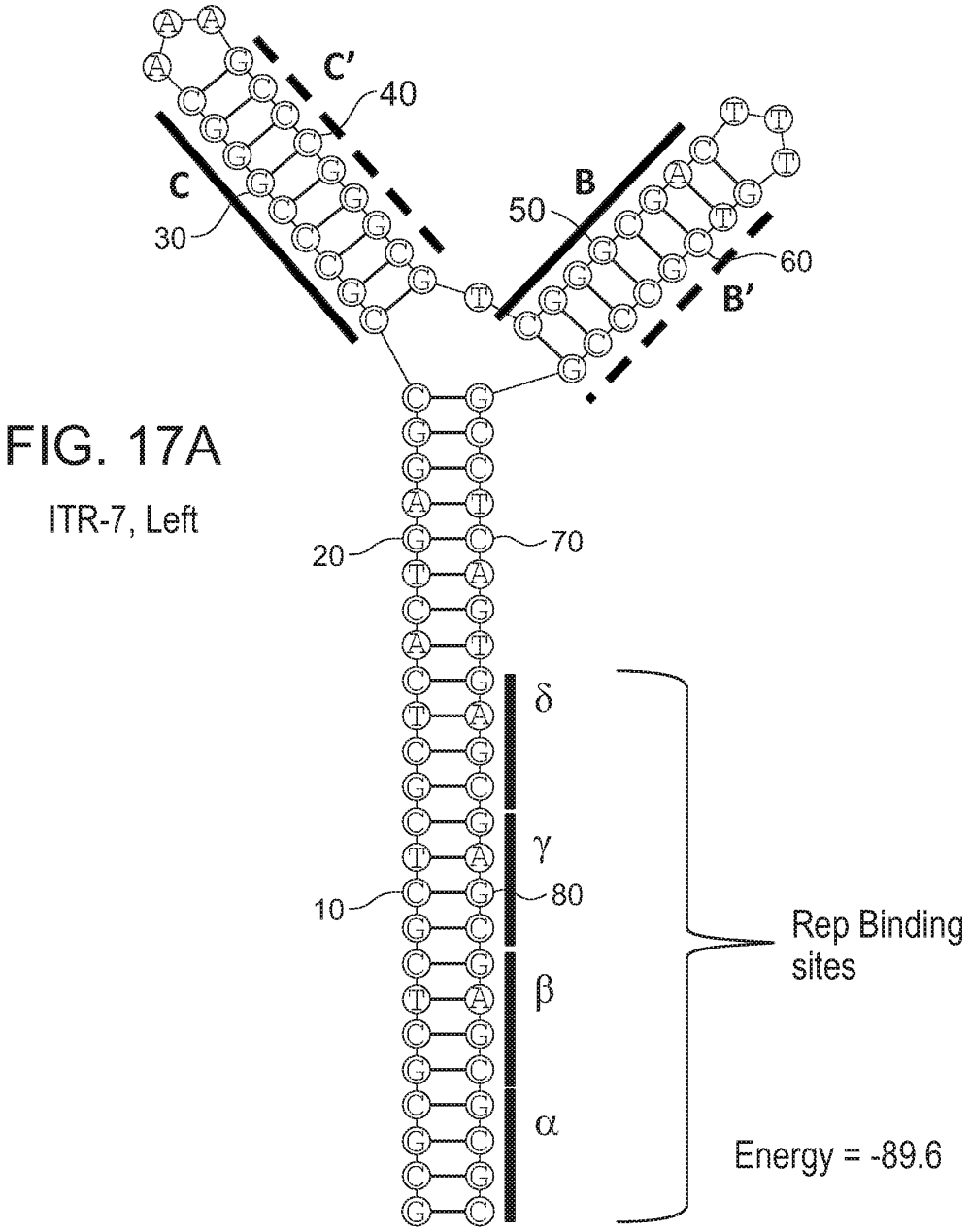



FIG. 17A

ITR-7, Left

FIG. 17B

ITR-7, Right

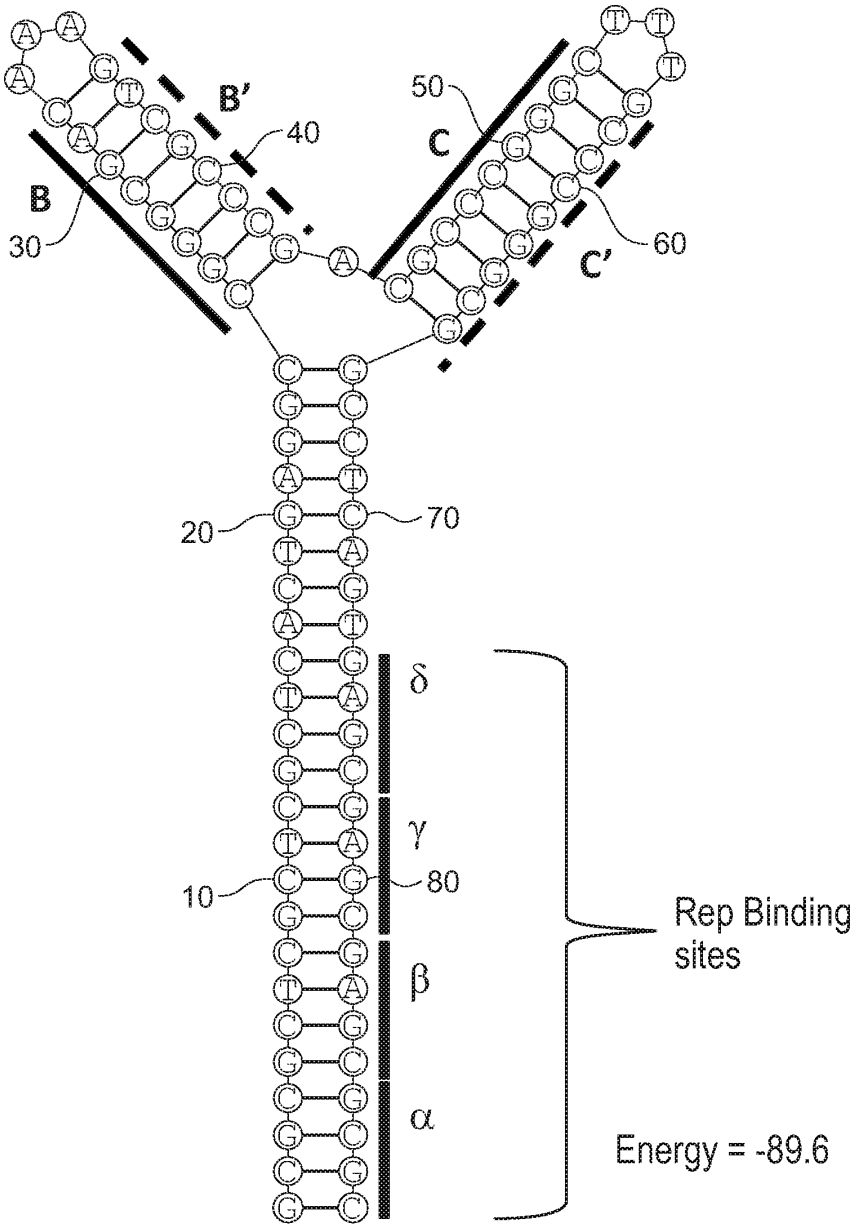


FIG. 18A

ITR-8, Left

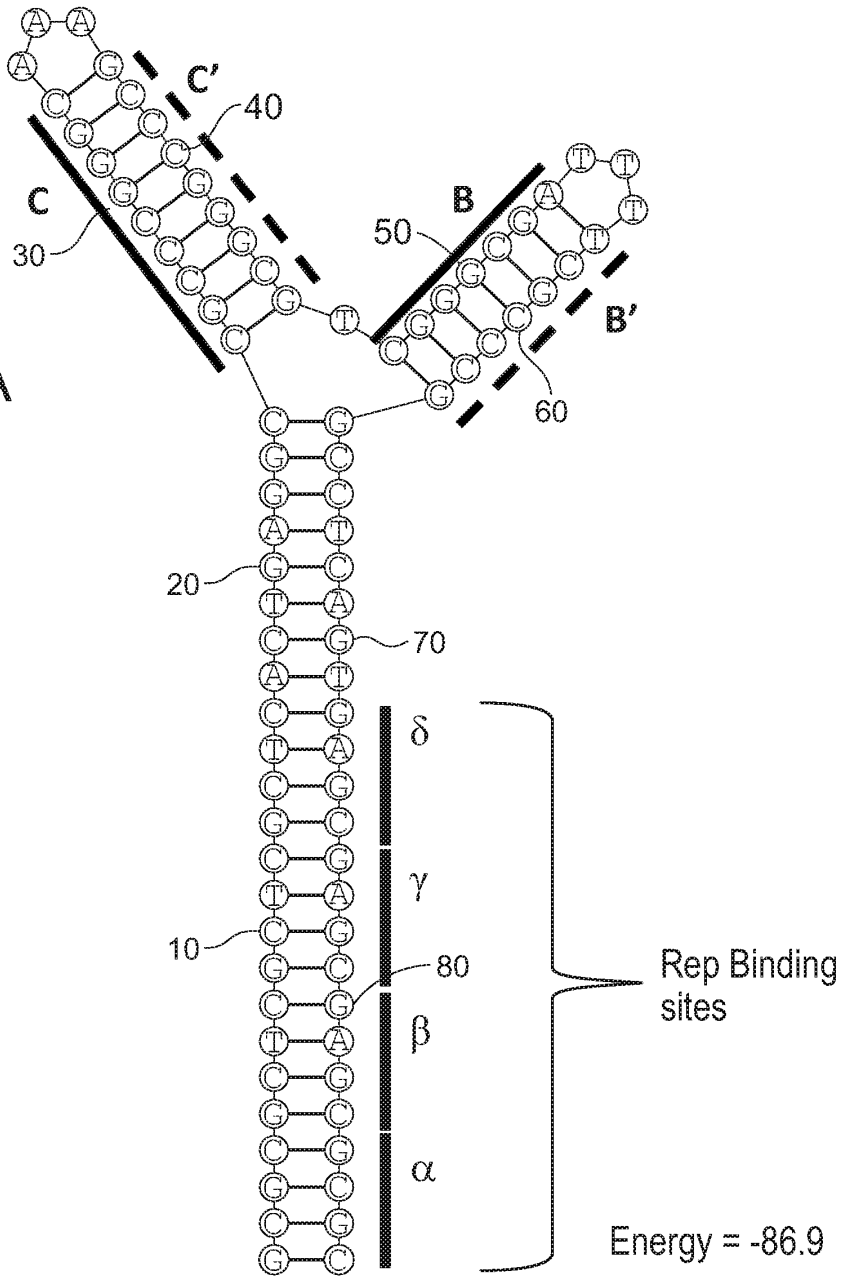


FIG. 18B

ITR-8, Right

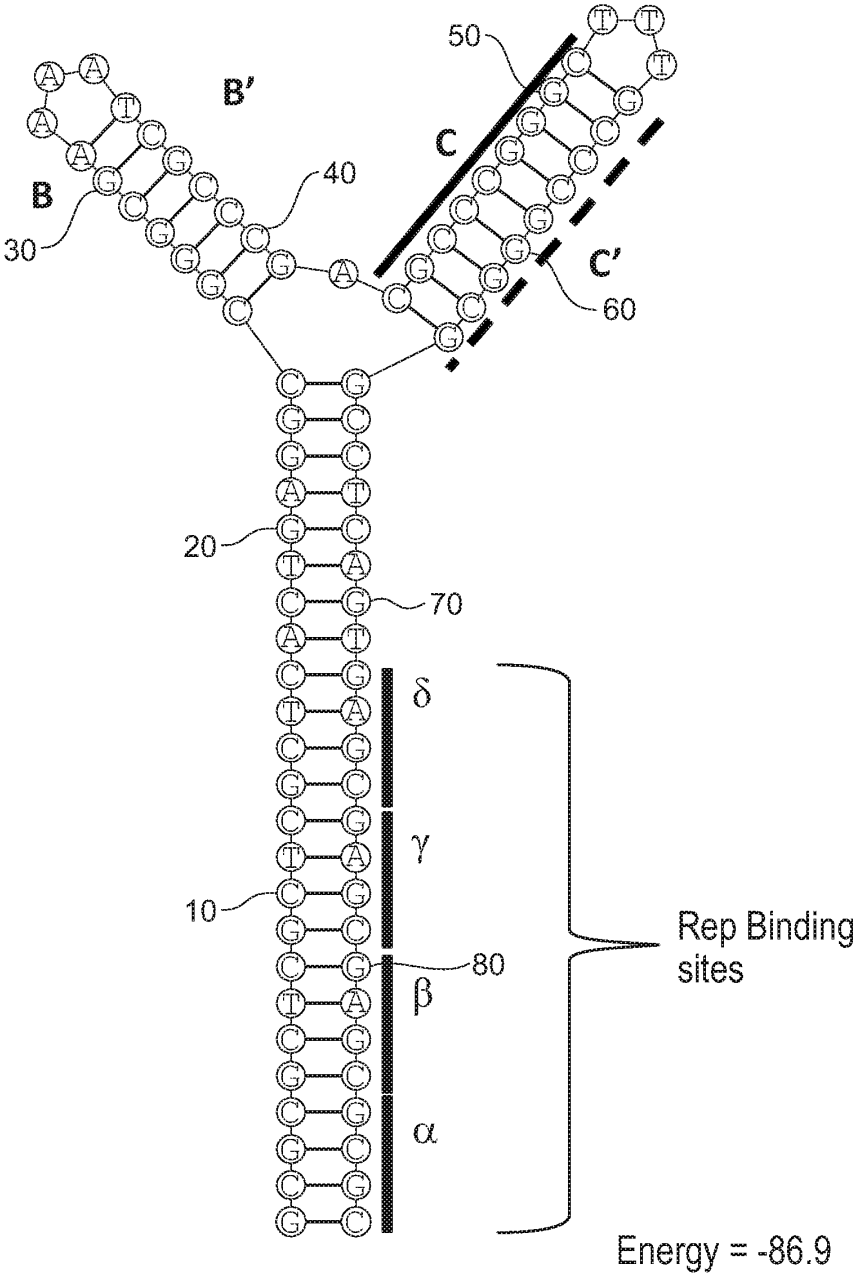


FIG. 19A

ITR-9, Left

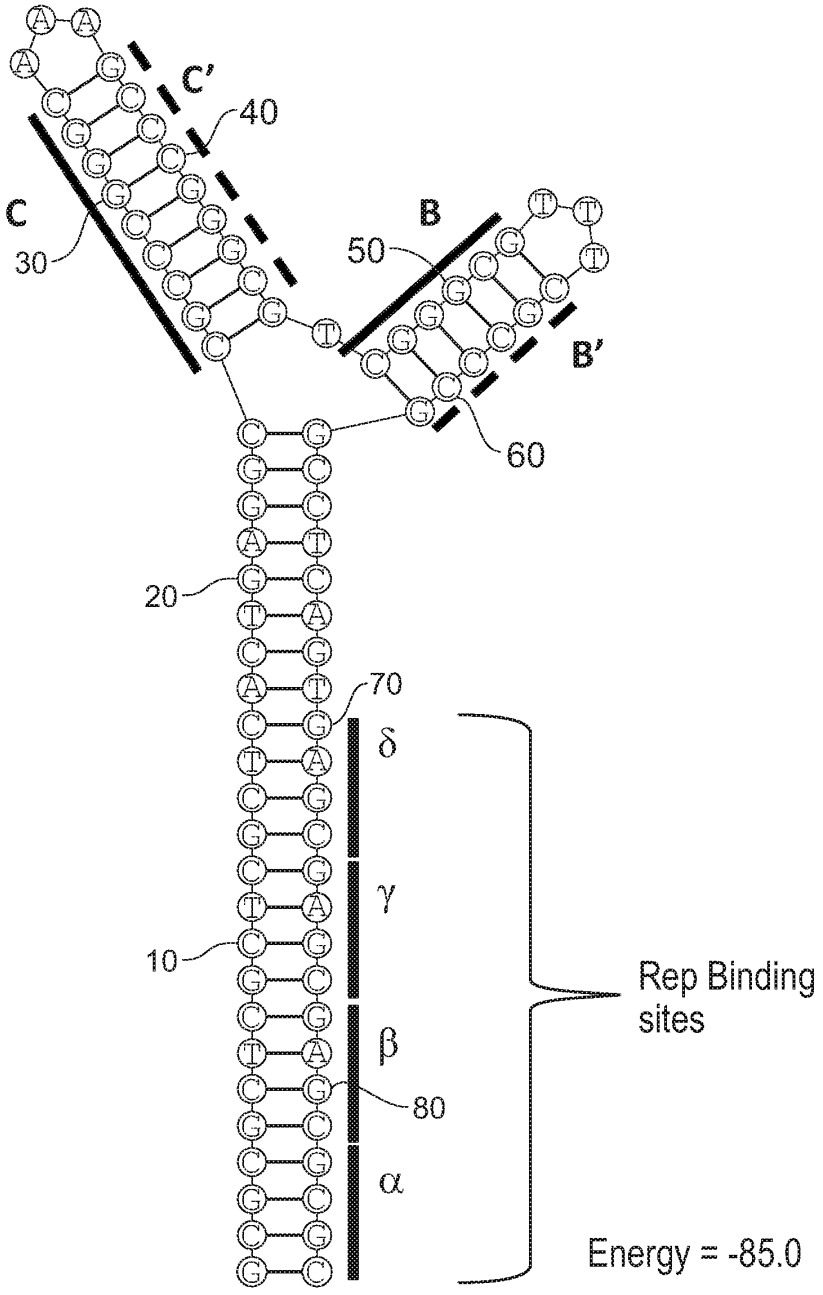


FIG. 19B

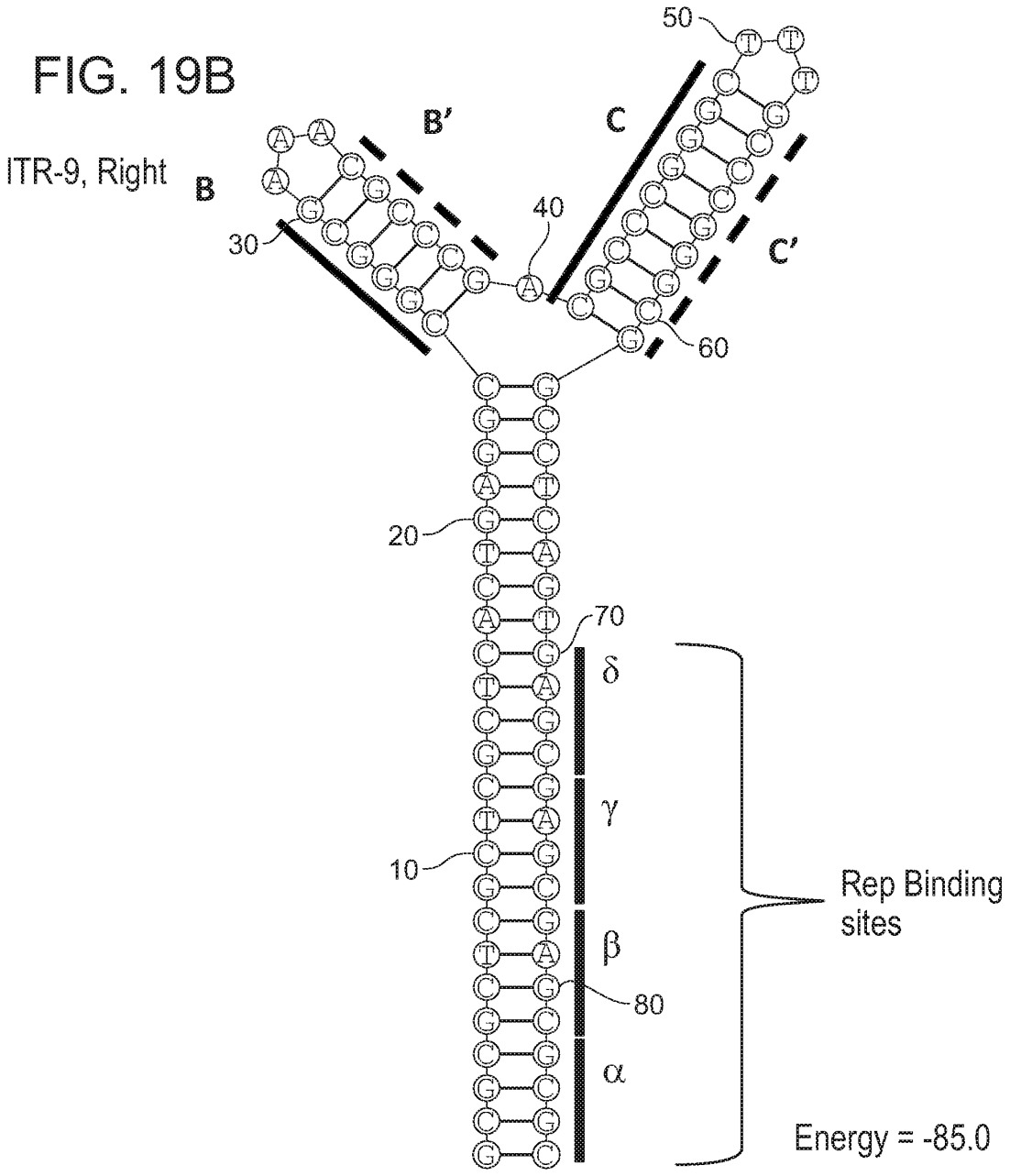

ITR-9, Right
B
B'
C
C'
50
40
30
60
20
70
10
80
δ
γ
β
α
Rep Binding sites
Energy = -85.0

FIG. 20A

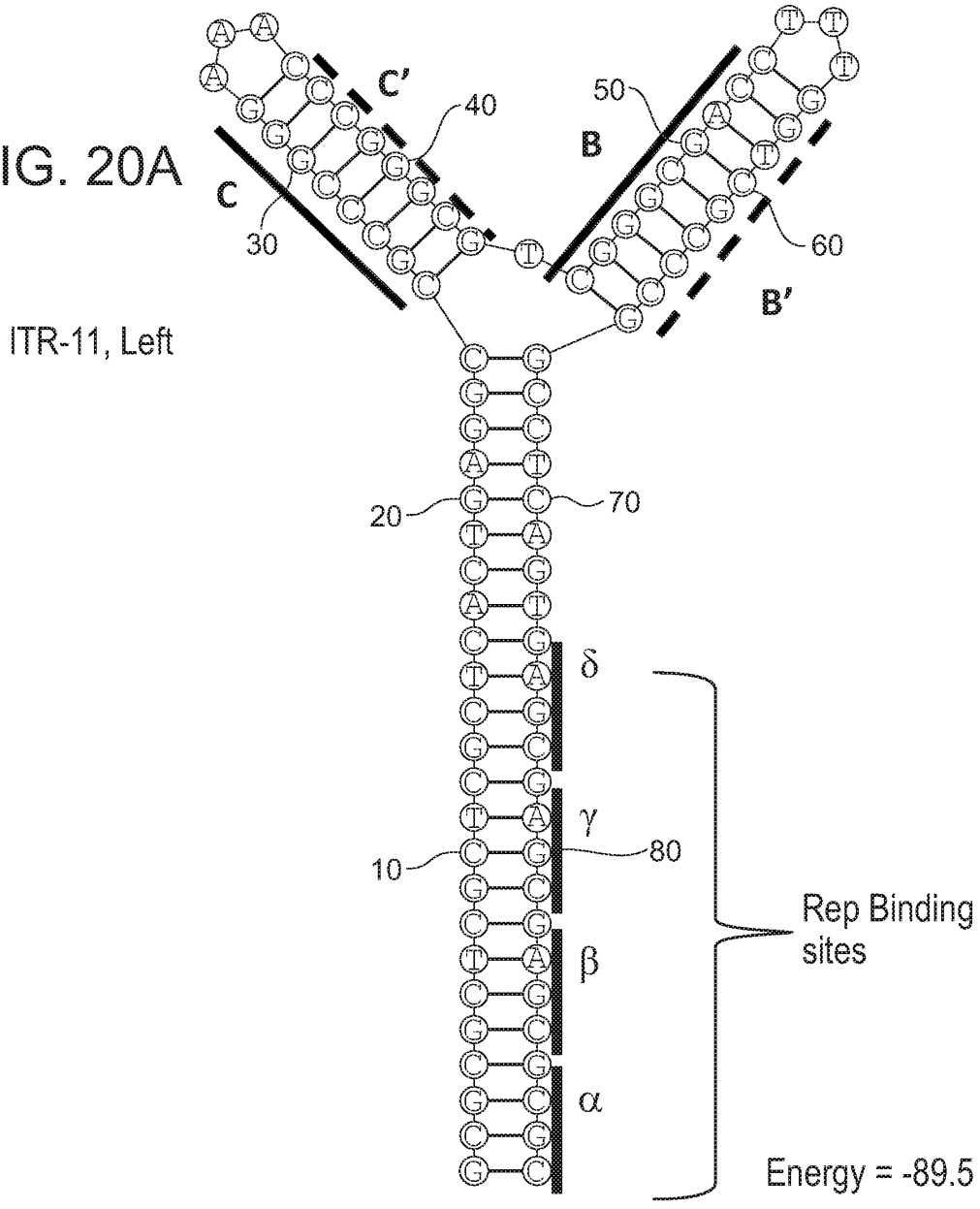
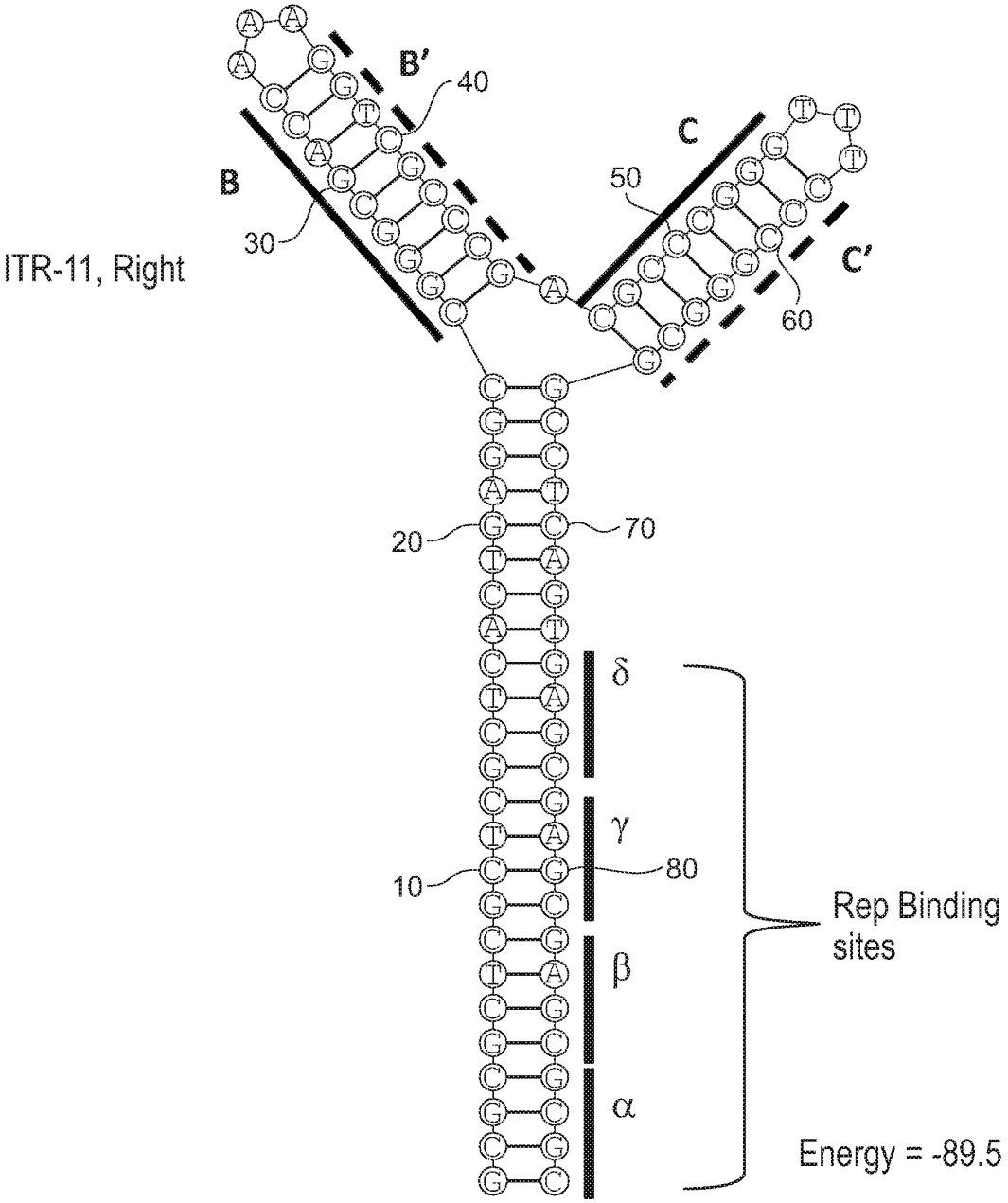

ITR-11, Left
C'
C
B
B'
30
40
50
60
20
70
10
80
δ
γ
β
α
Rep Binding sites
Energy = -89.5

FIG. 20B


ITR-11, Right
B'
B
C
C'
40
30
50
60
20
70
10
80
δ
γ
β
α
Rep Binding sites
Energy = -89.5

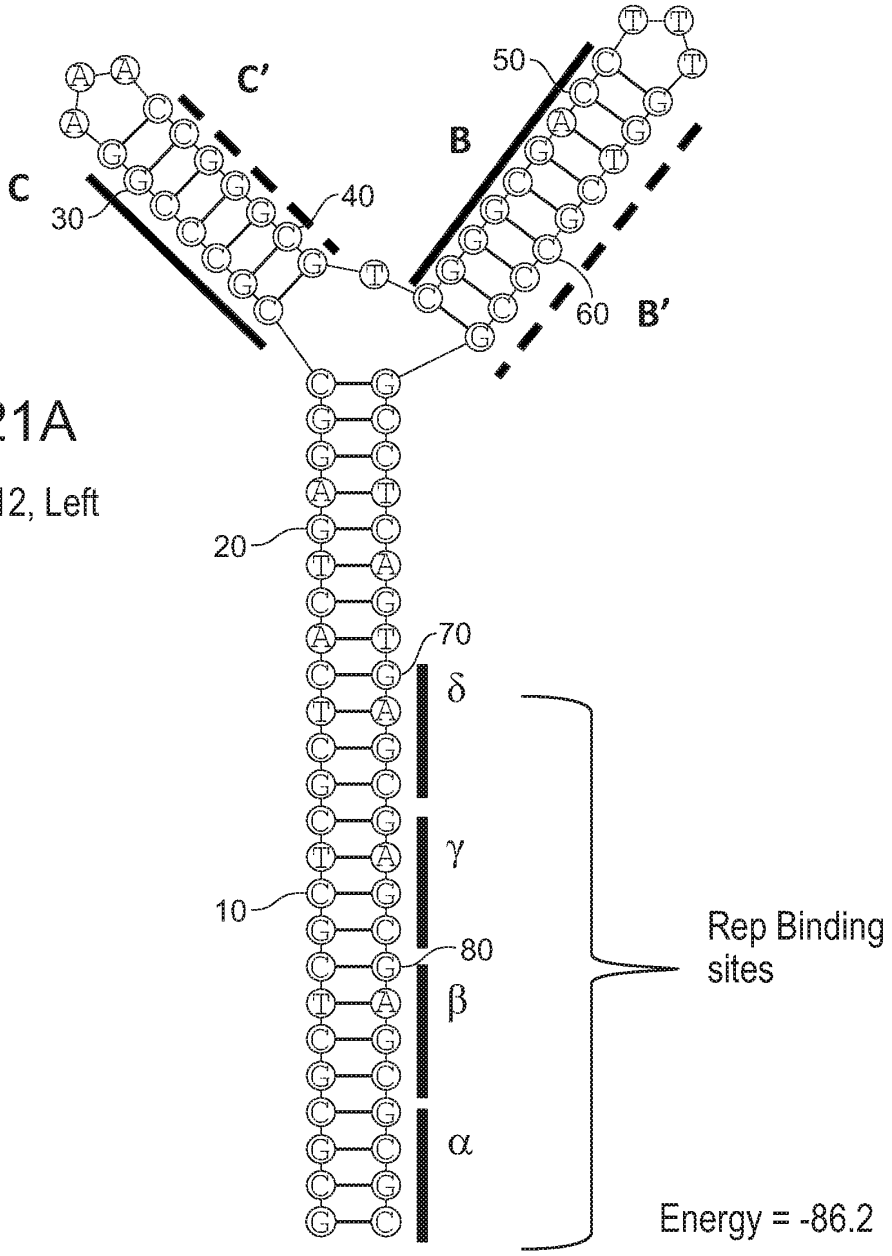



FIG. 21A

ITR-12, Left

FIG. 21B

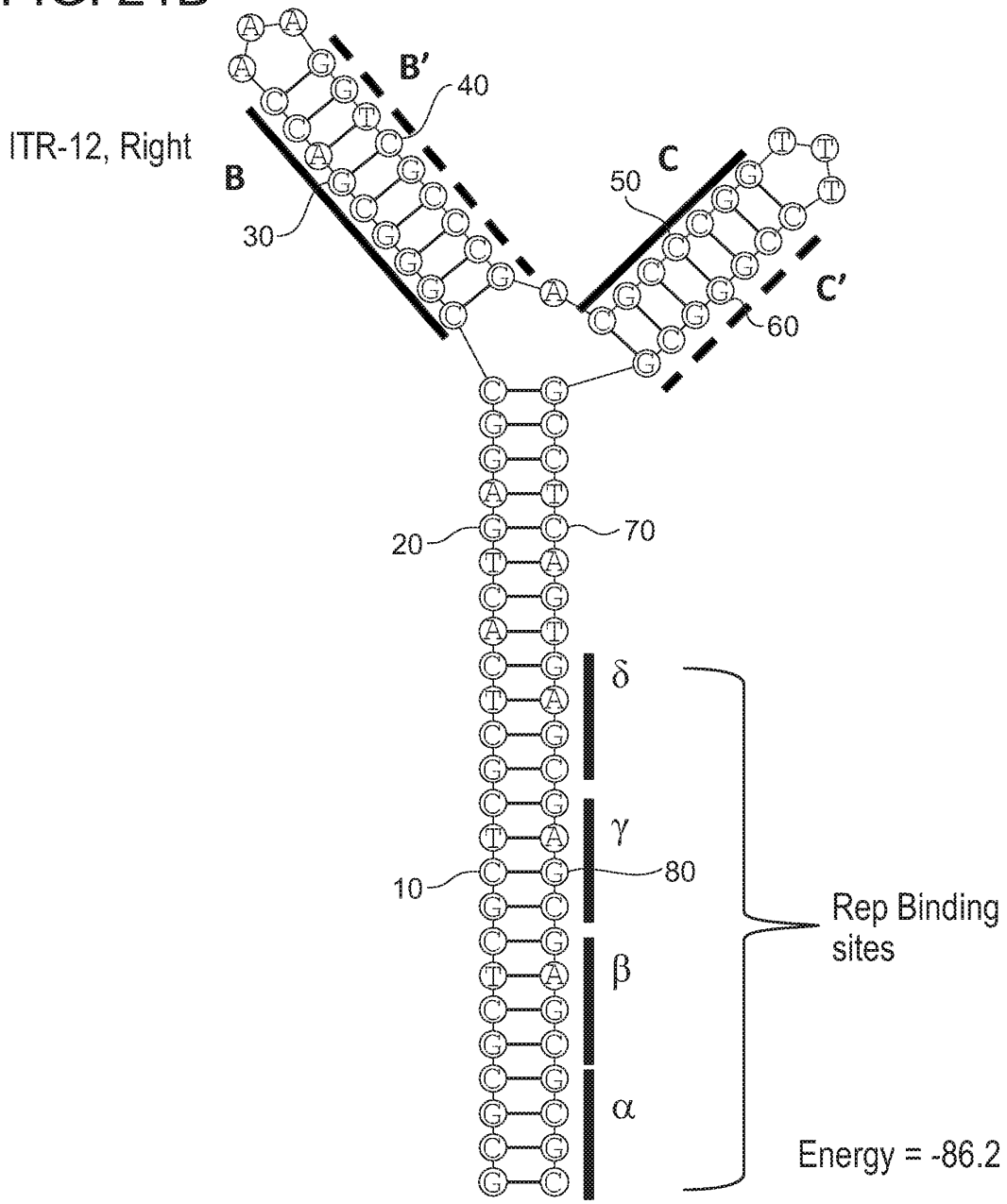

ITR-12, Right
B
B'
C
C'
30
40
50
60
20
70
10
80
δ
γ
β
α
Rep Binding sites
Energy = -86.2

FIG. 22A

ITR-13, Left

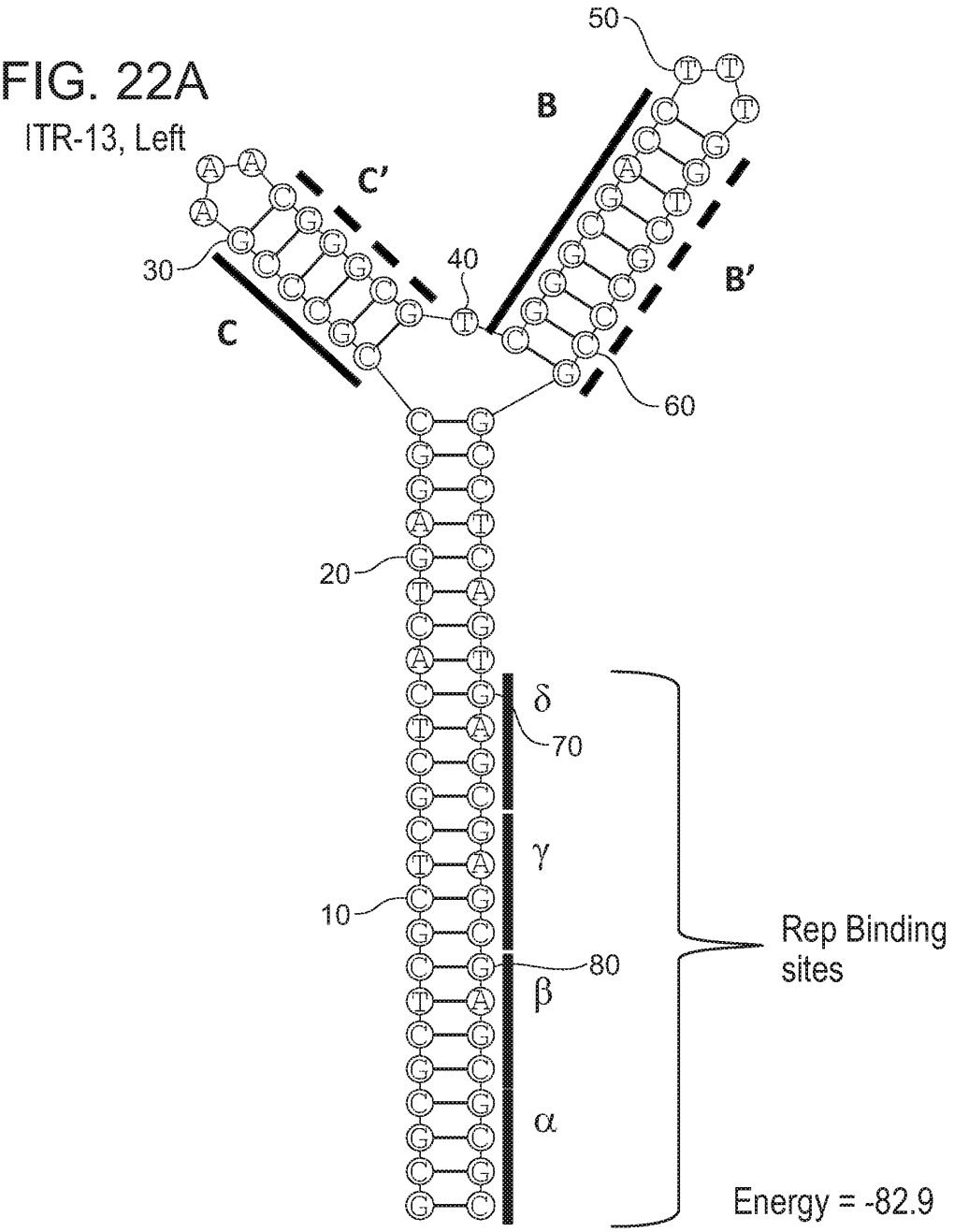


FIG. 22B

ITR-13, Right

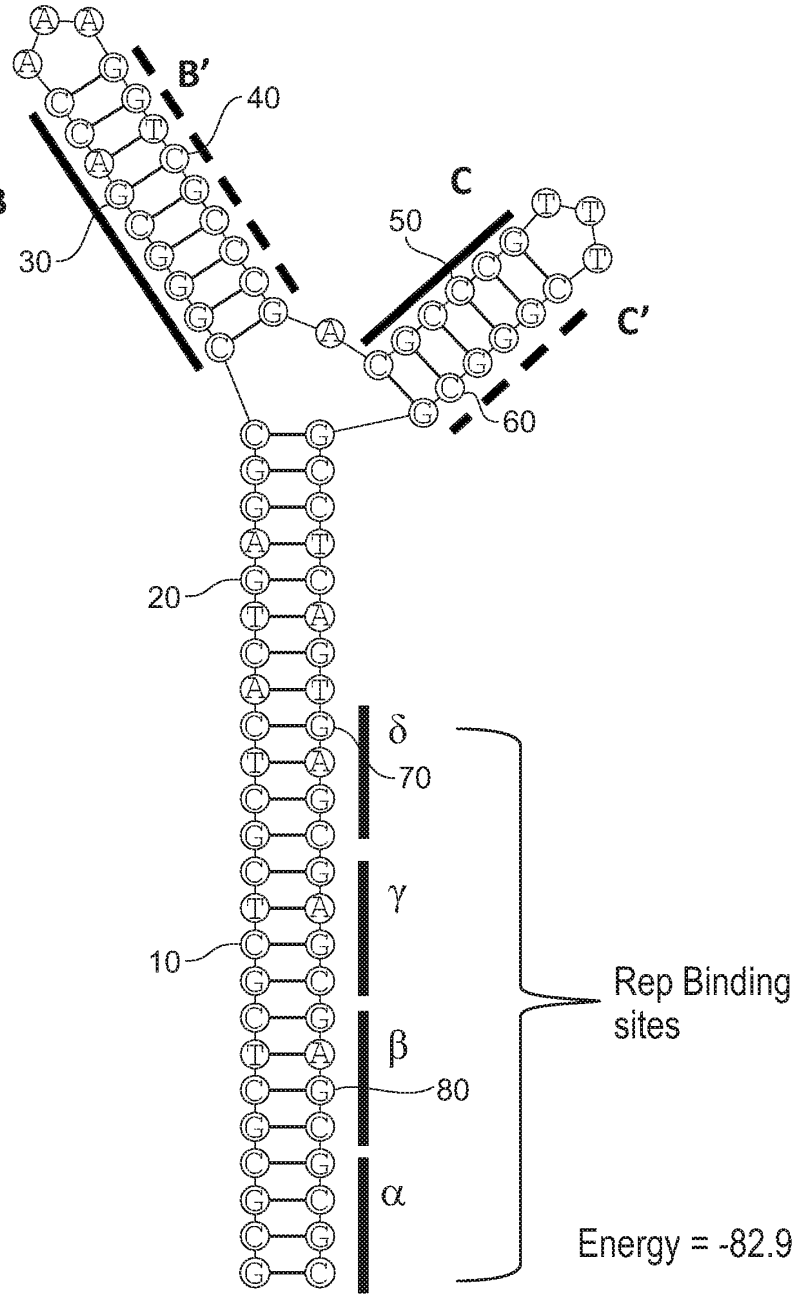


FIG. 23A

ITR-14, Left

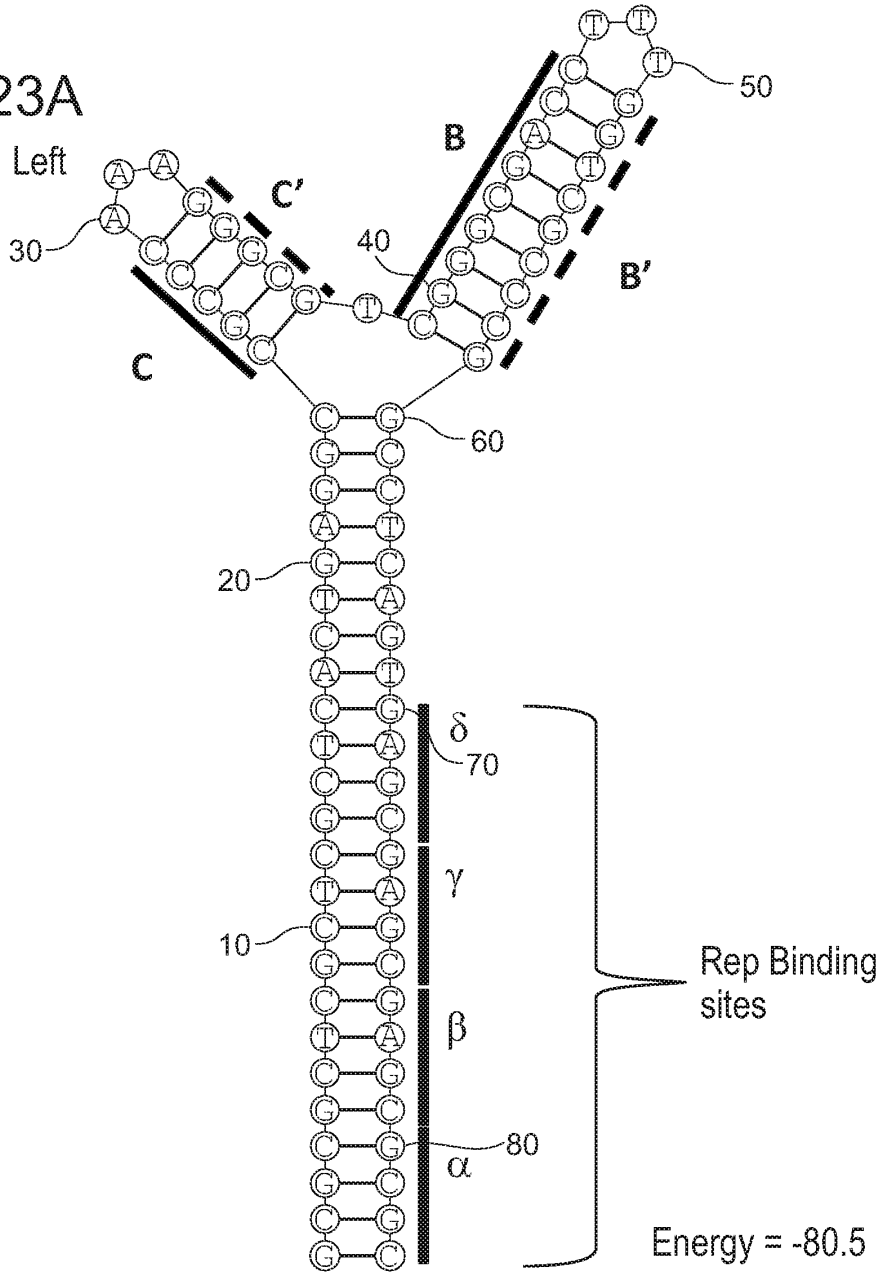


FIG. 23B

ITR-14, Right

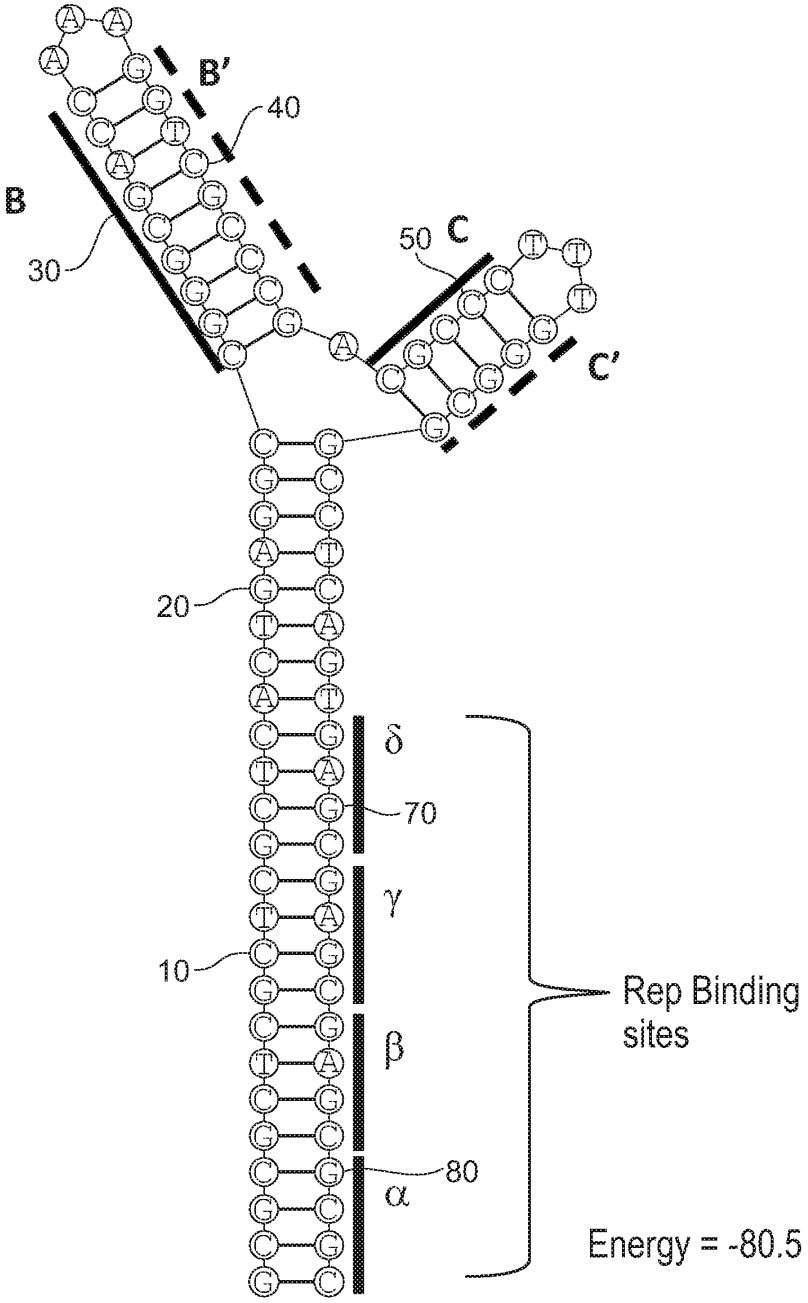


FIG. 24A

ITR-15, Left

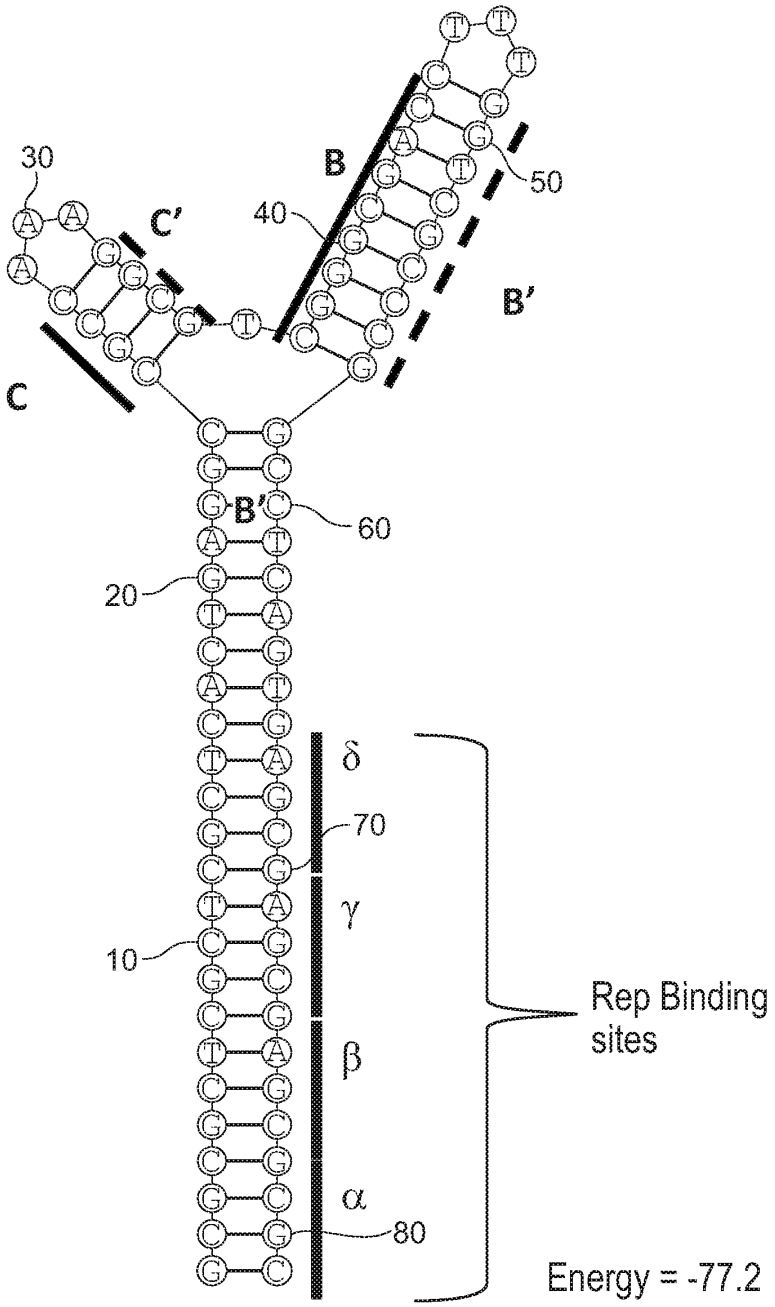


FIG. 24B

ITR-15, Right

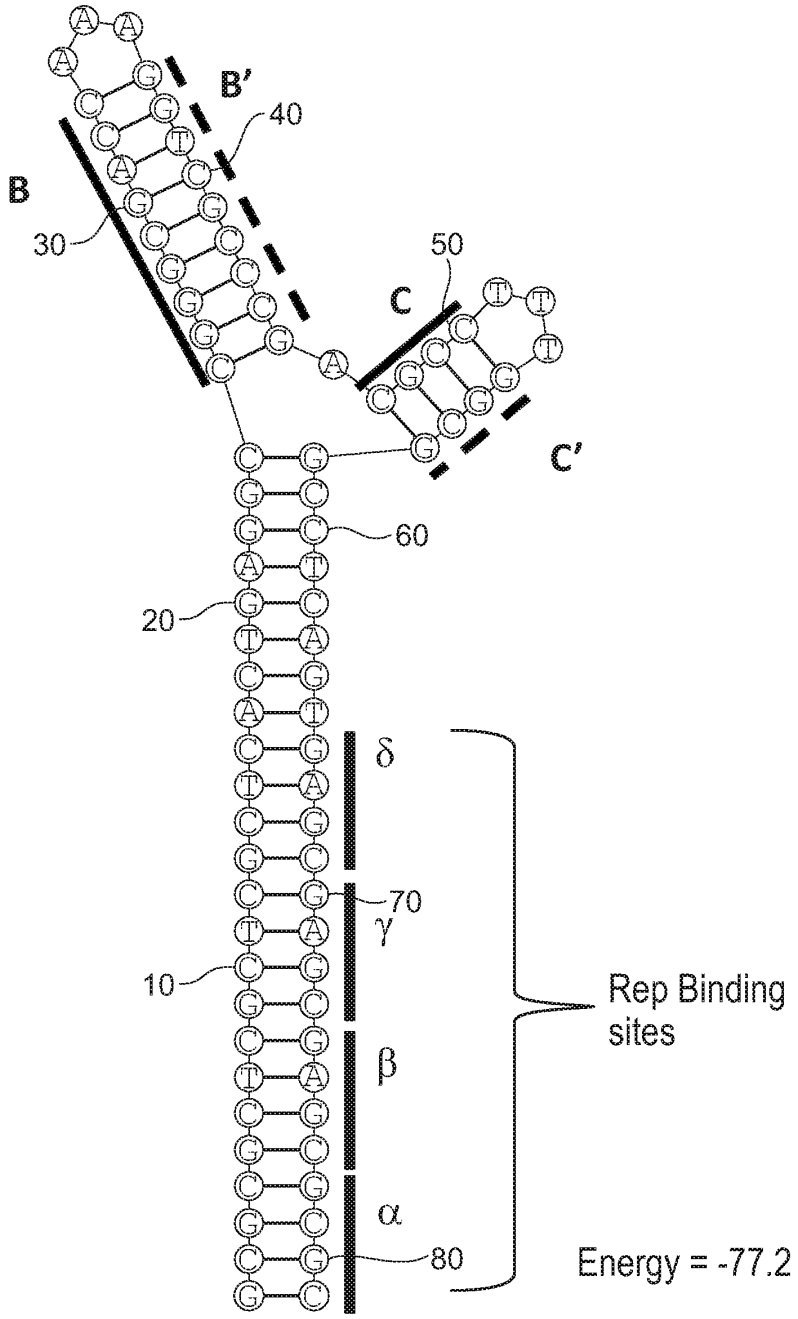


FIG. 25A

ITR-16, Left

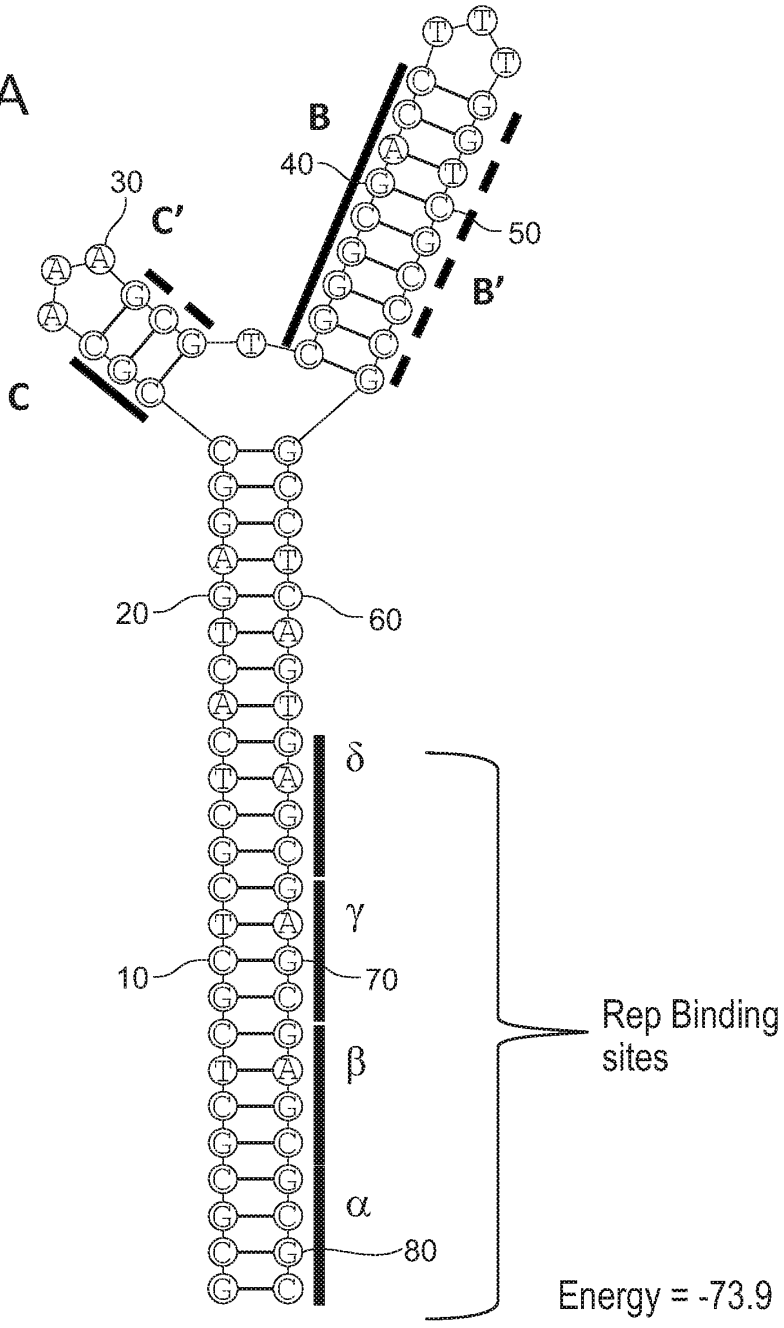


FIG. 25B

ITR-16, Right

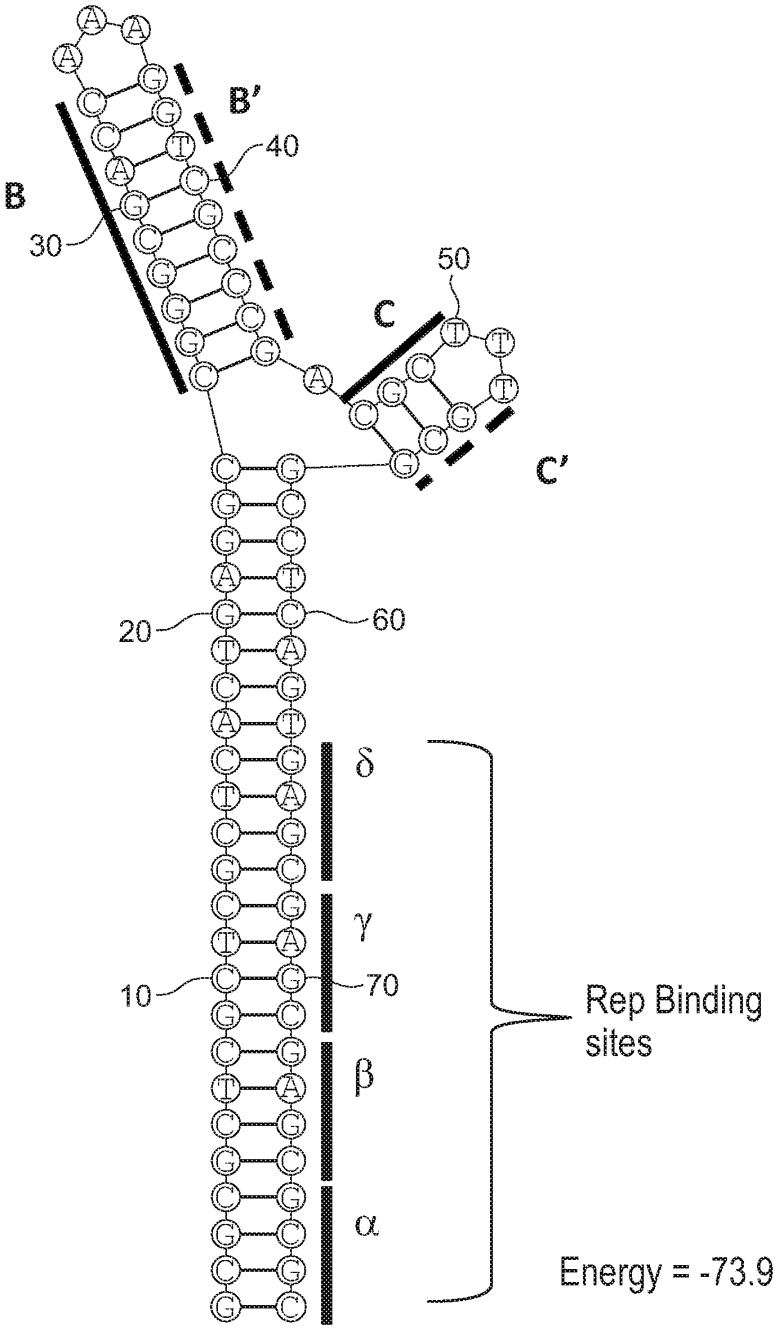


FIG. 26A

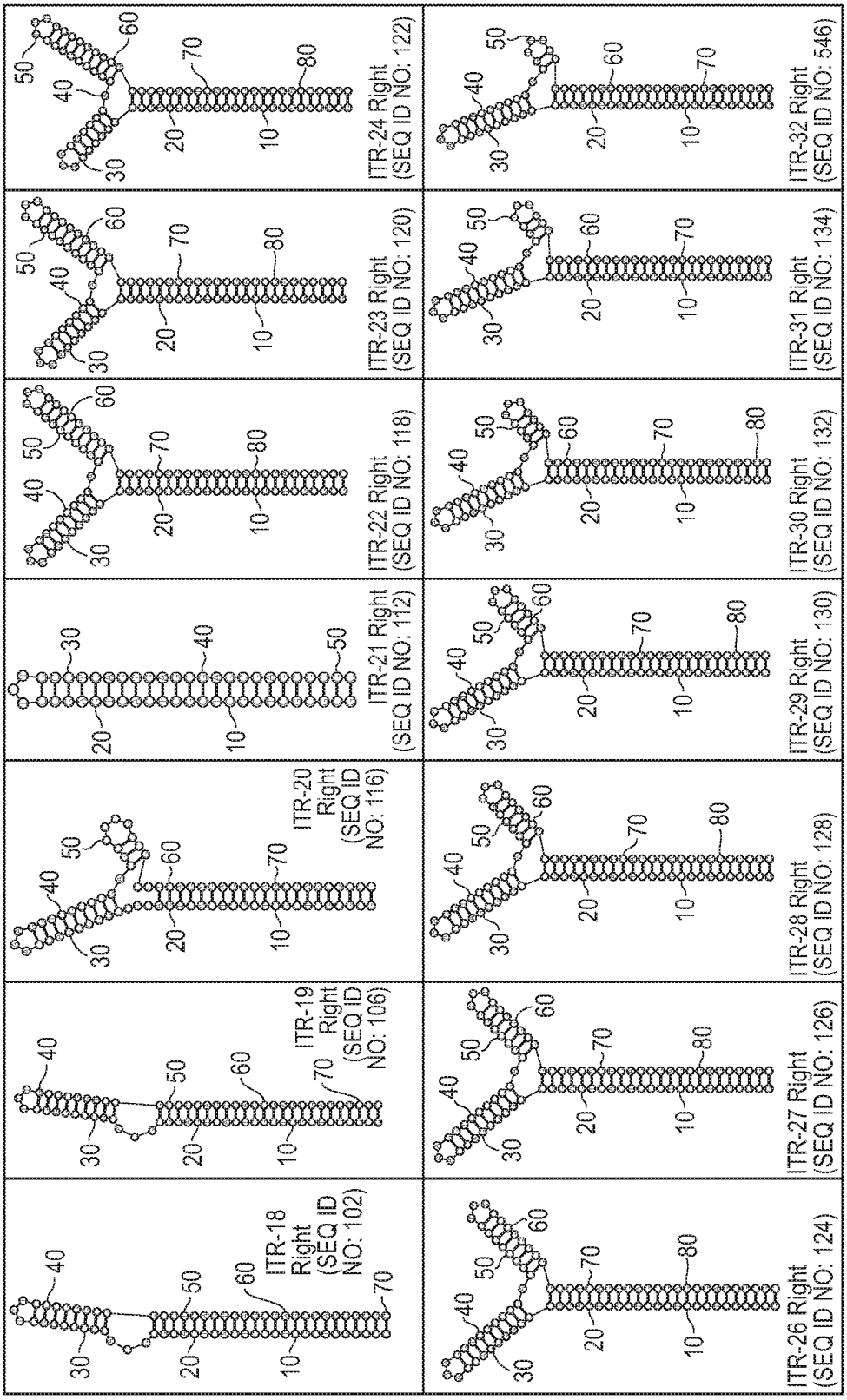

ITR-18 Right (SEQ ID NO: 102)
ITR-19 Right (SEQ ID NO: 106)
ITR-20 Right (SEQ ID NO: 116)
ITR-21 Right (SEQ ID NO: 112)
ITR-22 Right (SEQ ID NO: 118)
ITR-23 Right (SEQ ID NO: 120)
ITR-24 Right (SEQ ID NO: 122)
ITR-26 Right (SEQ ID NO: 124)
ITR-27 Right (SEQ ID NO: 126)
ITR-28 Right (SEQ ID NO: 128)
ITR-29 Right (SEQ ID NO: 130)
ITR-30 Right (SEQ ID NO: 132)
ITR-31 Right (SEQ ID NO: 134)
ITR-32 Right (SEQ ID NO: 546)

FIG. 26A (Cont.)

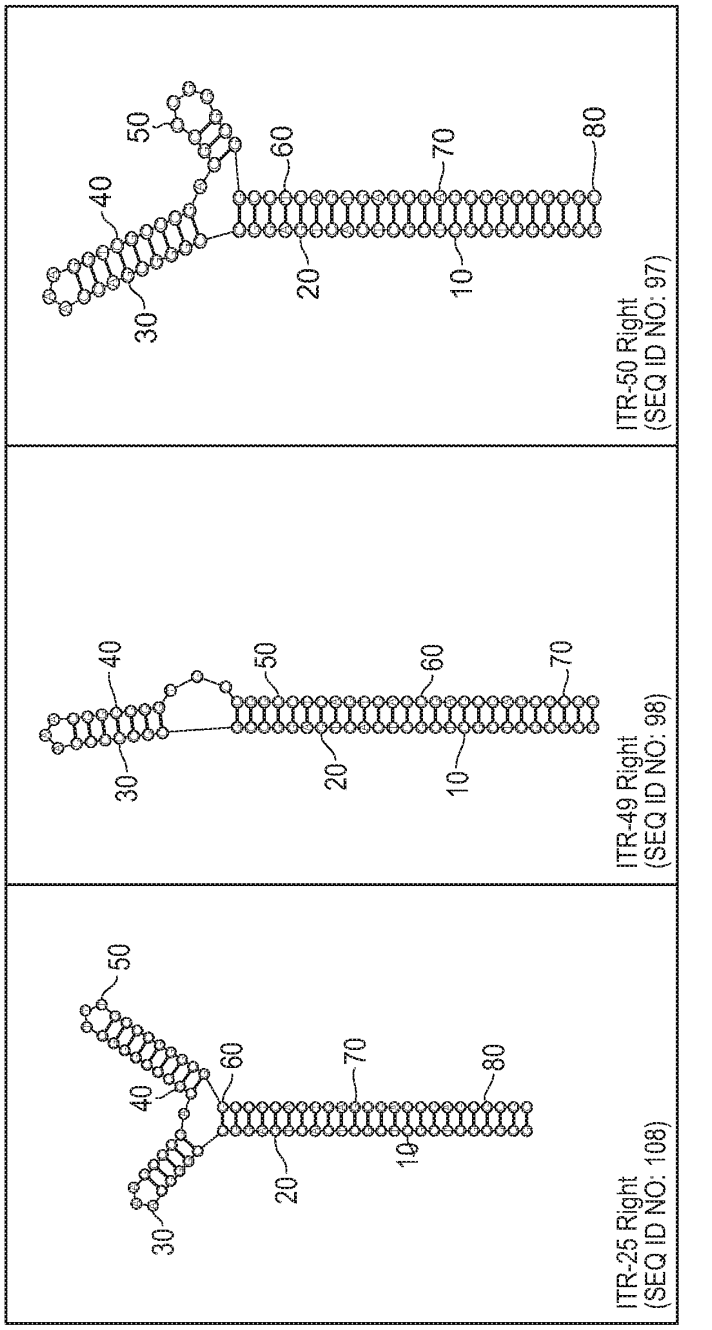

Probability >= 99%
99% > Probability >= 95%
95% > Probability >= 90%
90% > Probability >= 80%
80% > Probability >= 70%
70% > Probability >= 60%
60% > Probability >= 50%
50% > Probability
ITR-50 Right
(SEQ ID NO: 97)
ITR-49 Right
(SEQ ID NO: 98)
ITR-25 Right
(SEQ ID NO: 108)

FIG. 26B

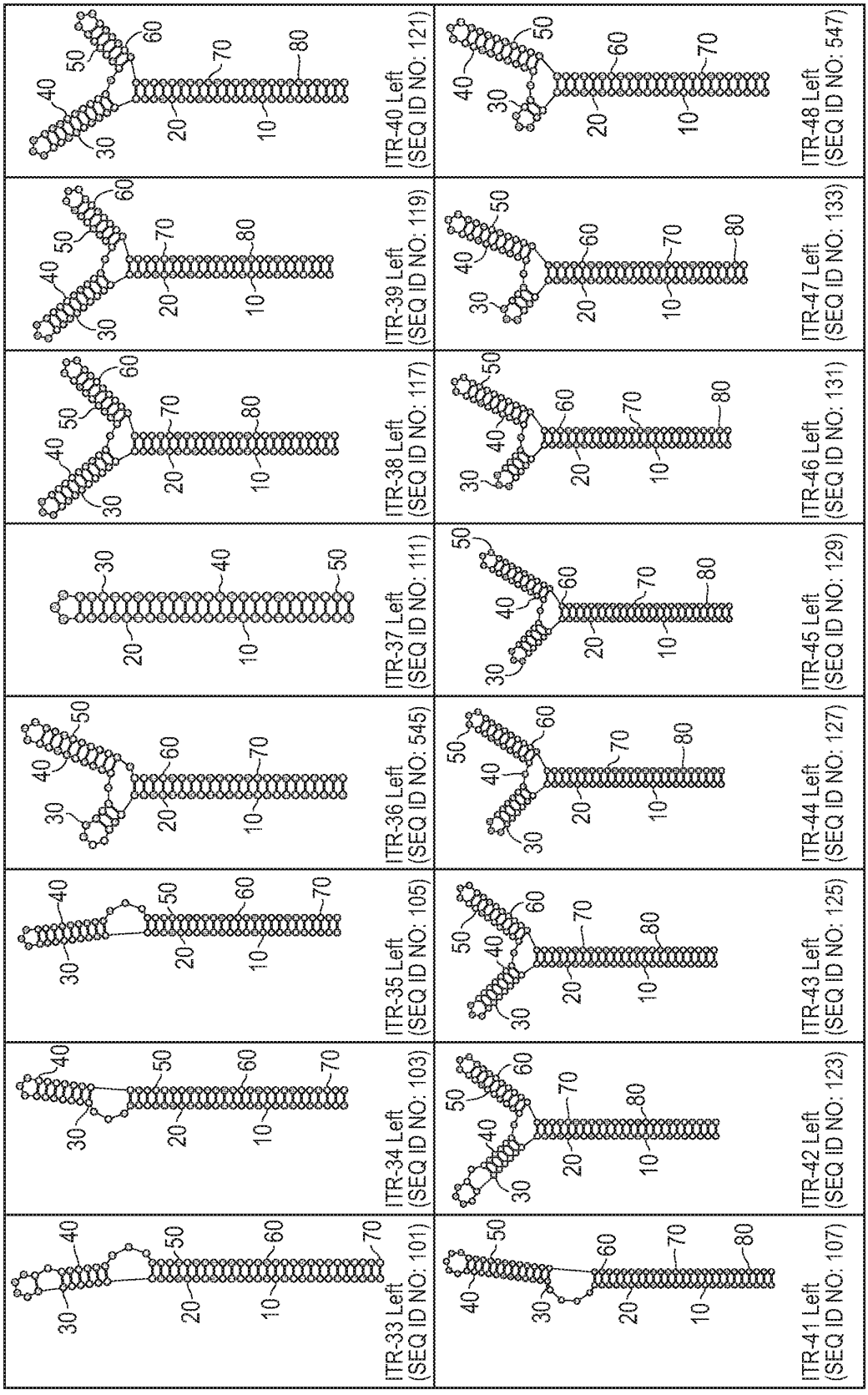
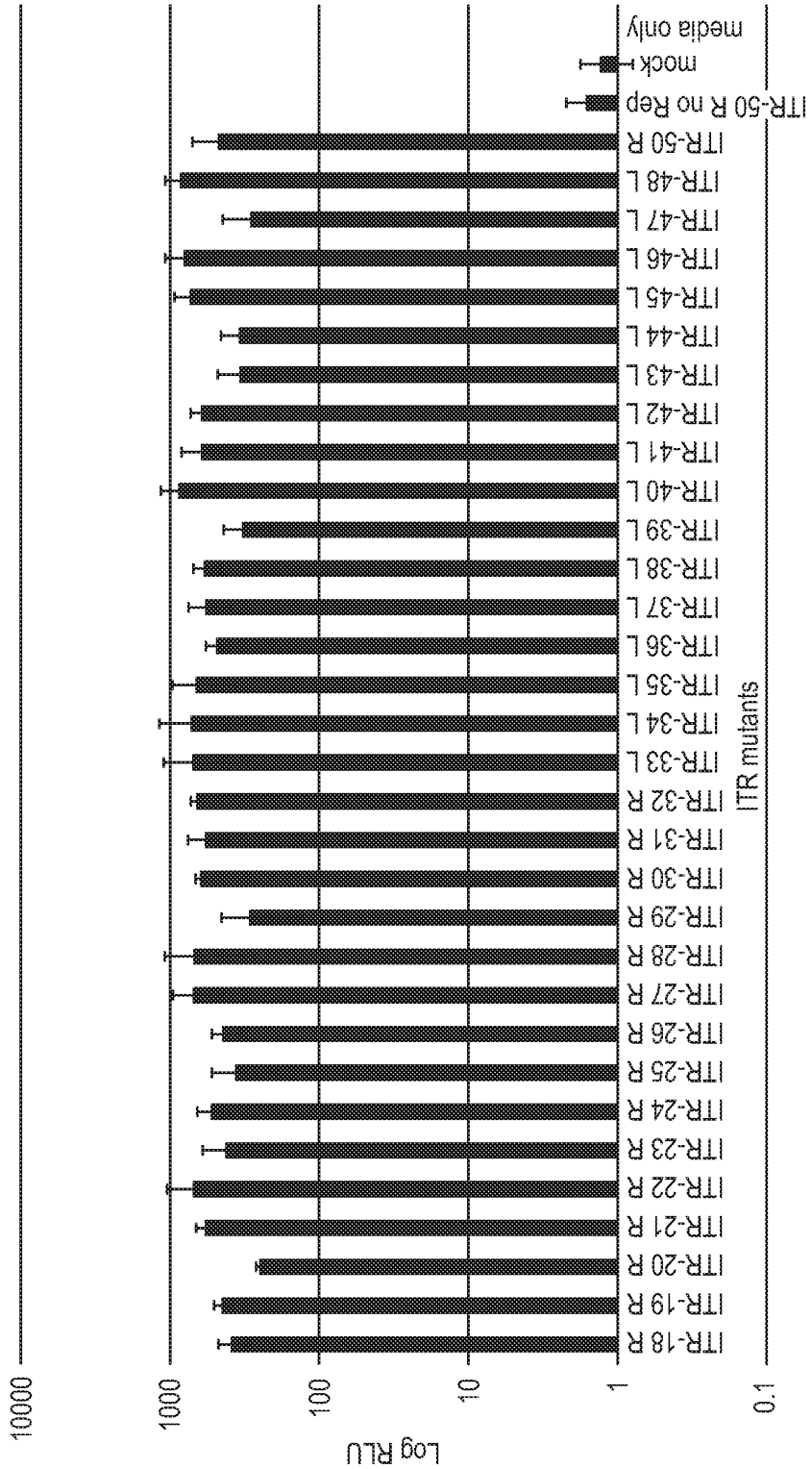

ITR-33 Left (SEQ ID NO: 101)
ITR-34 Left (SEQ ID NO: 103)
ITR-35 Left (SEQ ID NO: 105)
ITR-36 Left (SEQ ID NO: 545)
ITR-37 Left (SEQ ID NO: 111)
ITR-38 Left (SEQ ID NO: 117)
ITR-39 Left (SEQ ID NO: 119)
ITR-40 Left (SEQ ID NO: 121)
ITR-41 Left (SEQ ID NO: 107)
ITR-42 Left (SEQ ID NO: 123)
ITR-43 Left (SEQ ID NO: 125)
ITR-44 Left (SEQ ID NO: 127)
ITR-45 Left (SEQ ID NO: 129)
ITR-46 Left (SEQ ID NO: 131)
ITR-47 Left (SEQ ID NO: 133)
ITR-48 Left (SEQ ID NO: 547)

FIG. 27


Luciferase Activity of ITR mutant constructs
Log RLU
10000
1000
100
10
1
0.1
ITR mutants
ITR-18 R
ITR-19 R
ITR-20 R
ITR-21 R
ITR-22 R
ITR-23 R
ITR-24 R
ITR-25 R
ITR-26 R
ITR-27 R
ITR-28 R
ITR-29 R
ITR-30 R
ITR-31 R
ITR-32 R
ITR-33 L
ITR-34 L
ITR-35 L
ITR-36 L
ITR-37 L
ITR-38 L
ITR-39 L
ITR-40 L
ITR-41 L
ITR-42 L
ITR-43 L
ITR-44 L
ITR-45 L
ITR-46 L
ITR-47 L
ITR-48 L
ITR-50 R
ITR-50 R no Rep
mock
media only

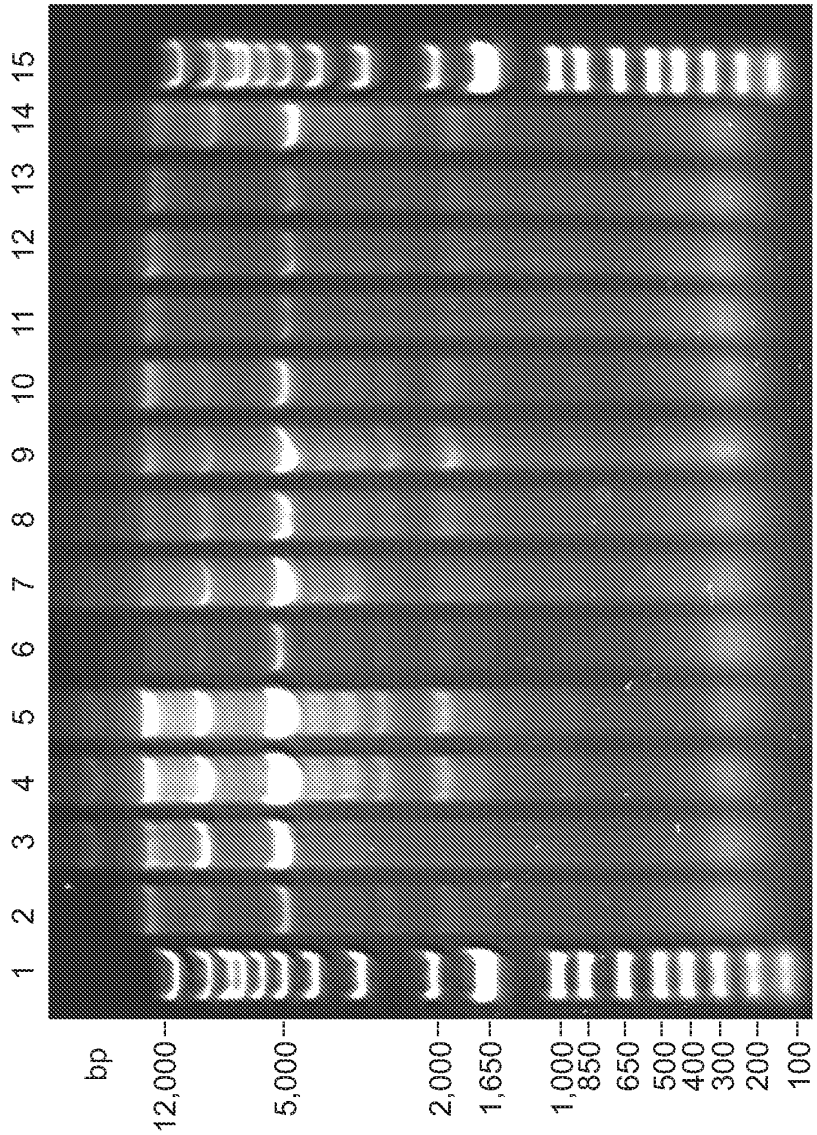

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
bp
12,000
5,000
2,000
1,650
1,000
850
650
500
400
300
200
100

FIG. 28

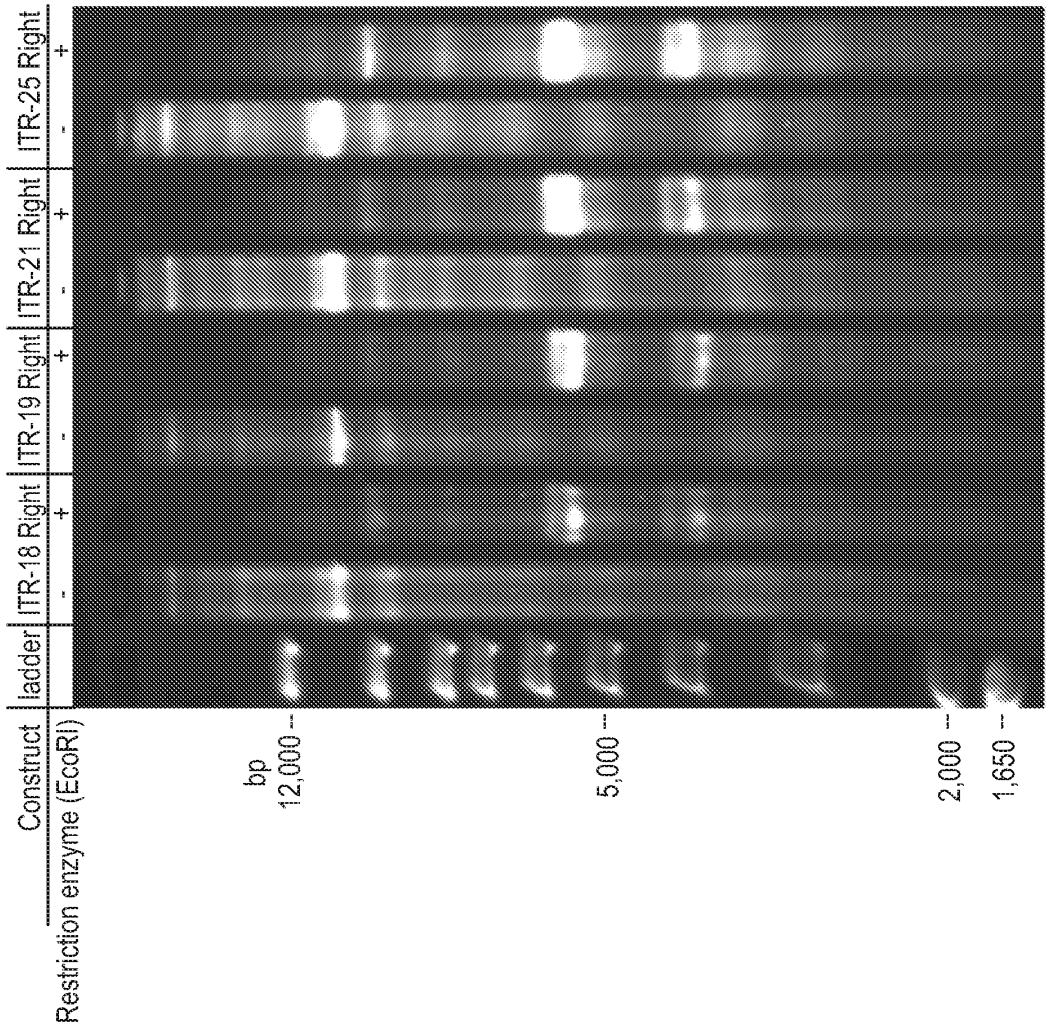
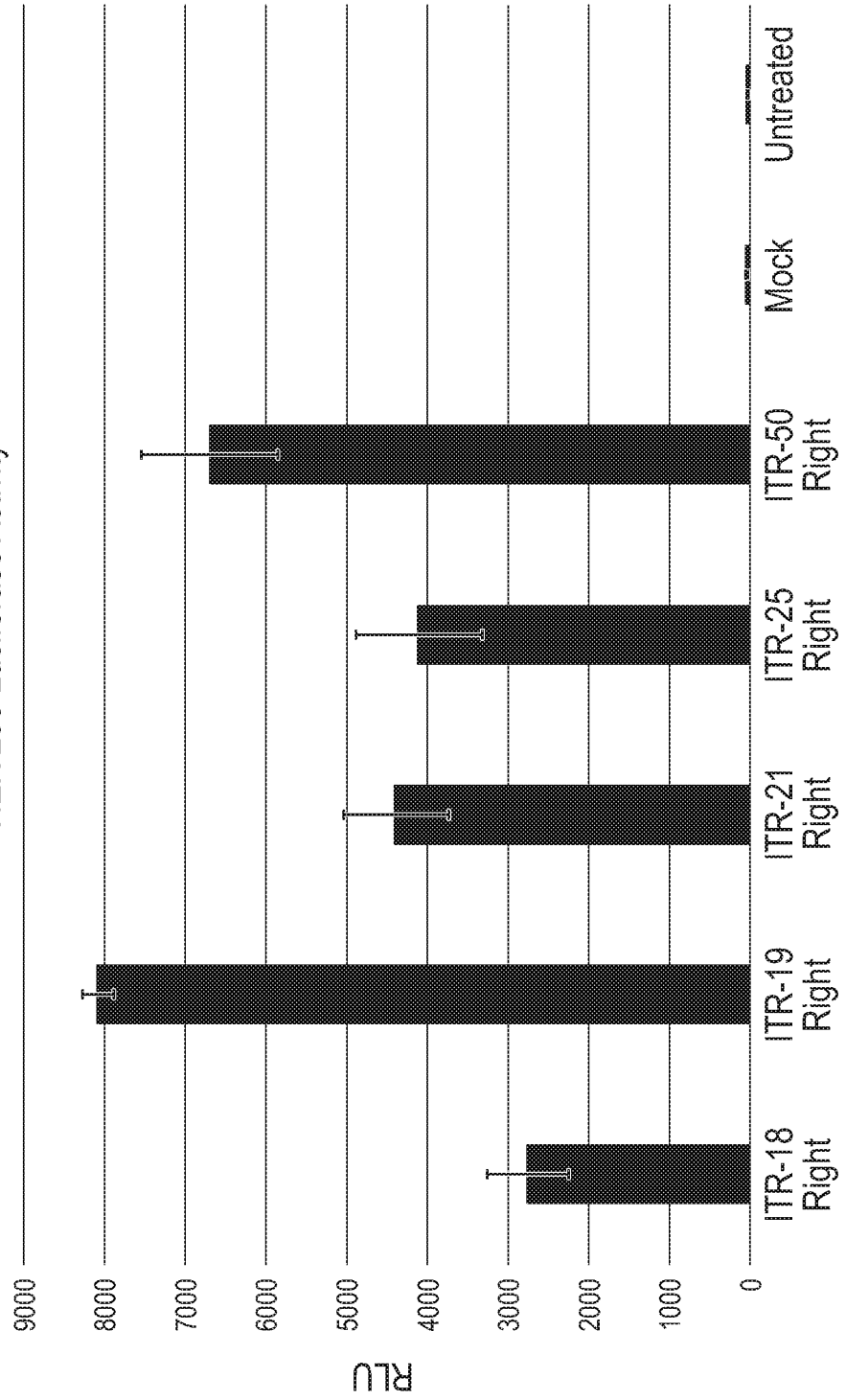

Construct
Restriction enzyme (EcoRI)
ladder
ITR-18 Right
ITR-19 Right
ITR-21 Right
ITR-25 Right
-
+
bp
12,000
5,000
2,000
1,650

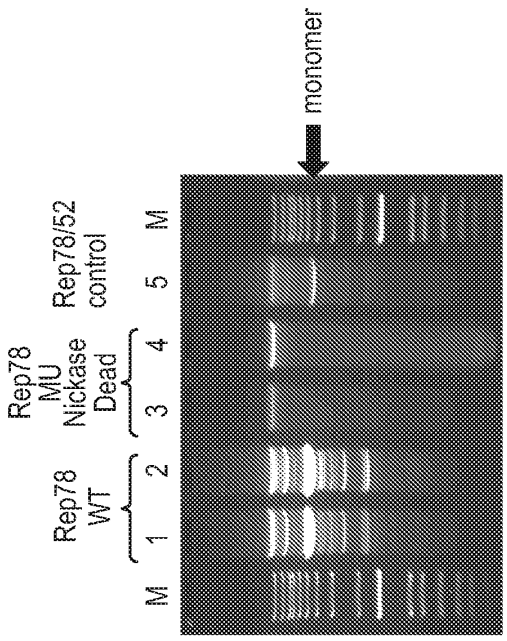
FIG. 29

FIG. 30


HEK 293 Luciferase Activity
RLU
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
ITR-18 Right
ITR-19 Right
ITR-21 Right
ITR-25 Right
ITR-50 Right
Mock
Untreated

| Rep | DNA binding | DNA nicking | Helicase | ATPase | Zn Finger |
|---|---|---|---|---|---|
| 78 | + | + | + | + | + |
| 78-Y156F | + | - | + | + | + |
| 78-K340H | + | + | - | - | + |
| 78-Met-> Gly | + | + | + | + | + |
| 68 | + | + | + | + | - |
| 52 | - | - | + | + | + |
| 40 | - | - | + | + | - |

FIG. 31




| # | 10ul loaded on the gel (3% of production) | Harvest viability | ceDNA |
|---|---|---|---|
| 1. | pIE78 Met-> Gly TTX1 (1550ng) | 86.0 | YYY |
| 2. | pIE78 Met-> Thr TTX1 (1720ng) | 85.5 | YYY |
| 3. | pIE78 Gly Y156F TTX1 (1000ng) | 85.0 | N |
| 4. | pIE78 Thr Y156F TTX1 (2410ng) | 86.0 | N |
| 5. | WT Rep78/52 TTX1 (1420ng) | 75.8 | Y |

FIG. 32A

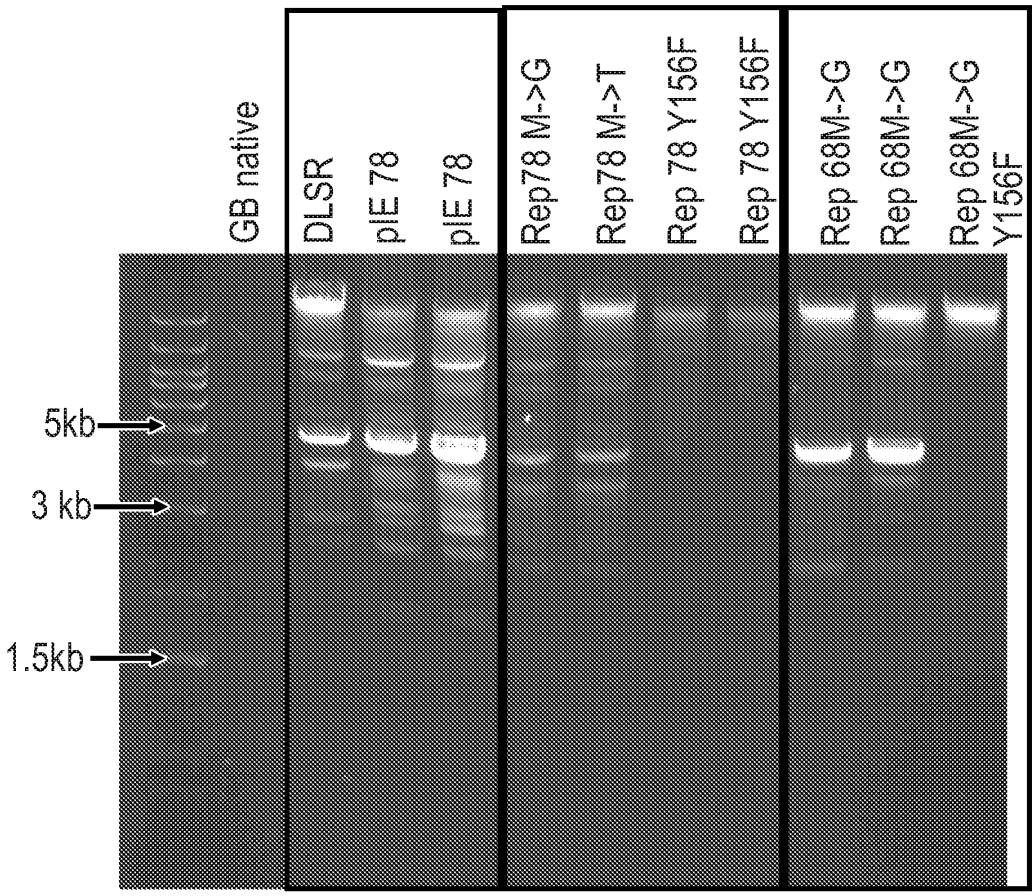

GB native
DLSR
pIE 78
pIE 78
Rep78 M->G
Rep78 M->T
Rep 78 Y156F
Rep 78 Y156F
Rep 68M->G
Rep 68M->G
Rep 68M->G Y156F
5kb
3 kb
1.5kb

FIG. 32B