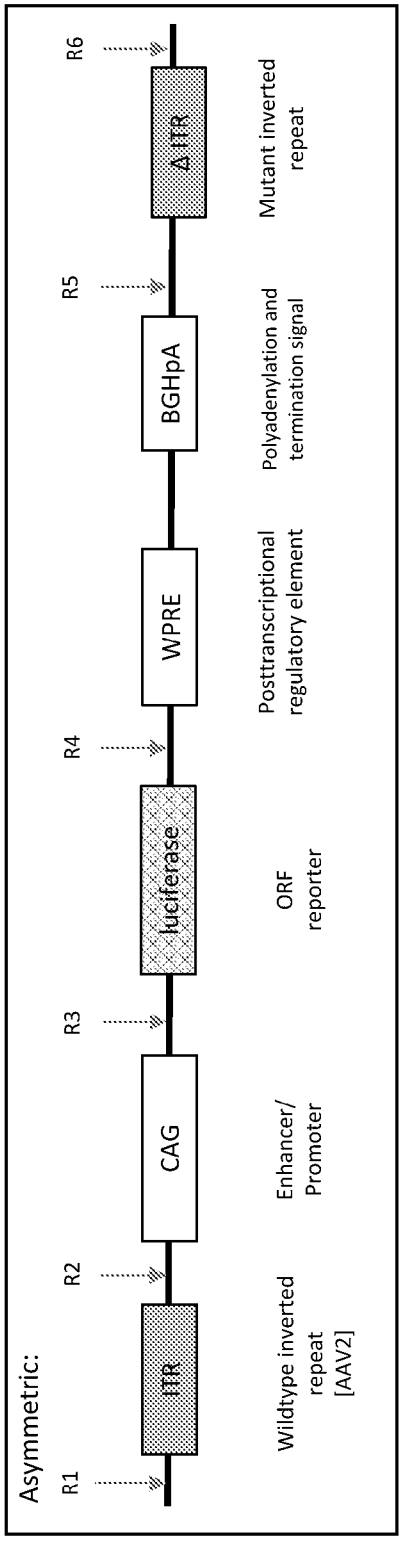


FIG. 1A

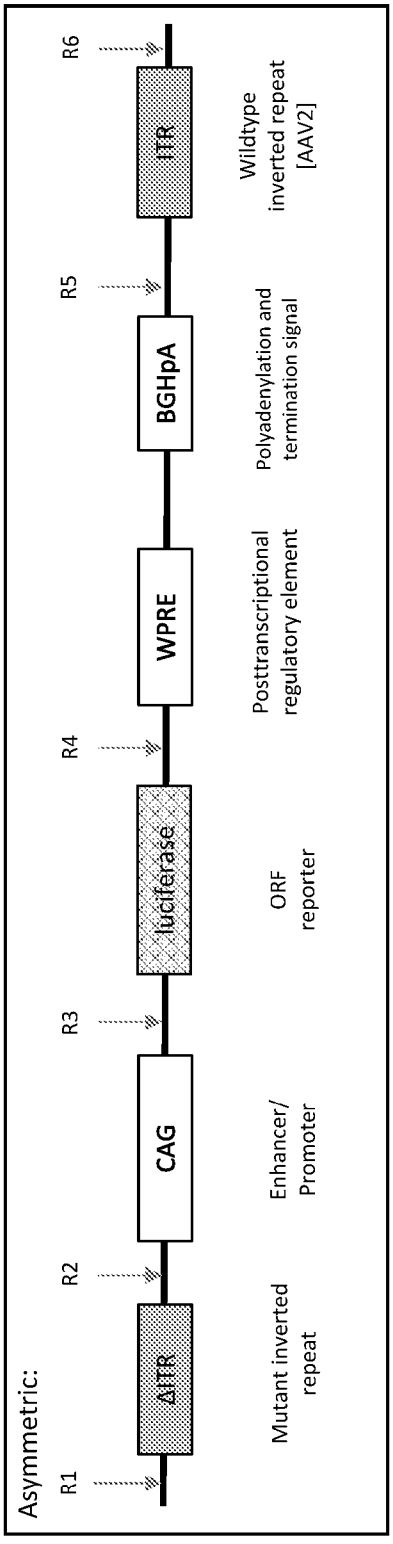


FIG. 1B

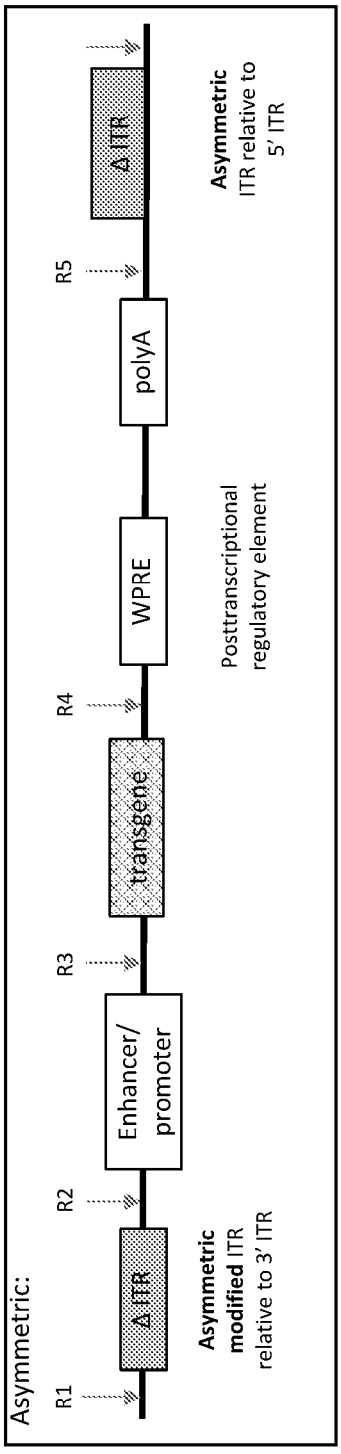


FIG. 1C

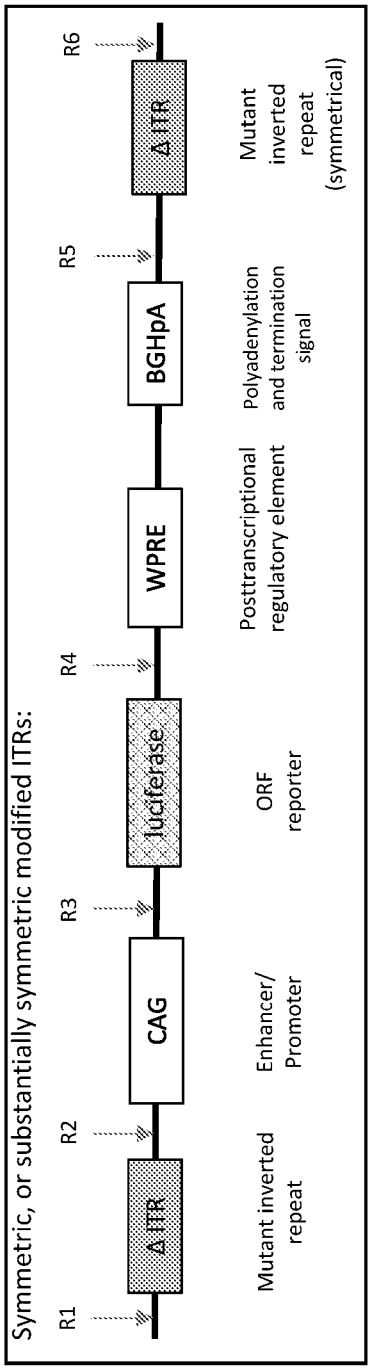

Symmetric, or substantially symmetric modified ITRs:
R1
R2
R3
R4
R5
R6
Δ ITR
CAG
luciferase
WPRE
BGHpA
Δ ITR
Mutant inverted repeat
Enhancer/ Promoter
ORF reporter
Posttranscriptional regulatory element
Polyadenylation and termination signal
Mutant inverted repeat (symmetrical)

FIG. 1D

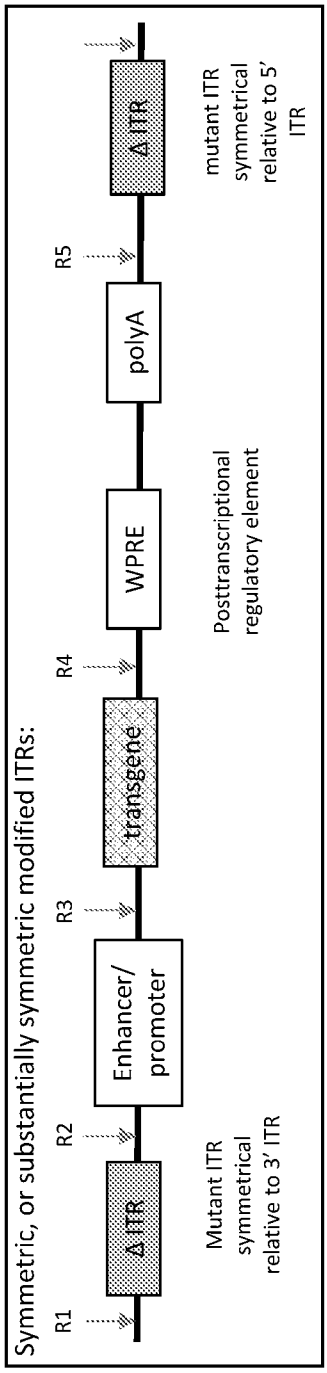

Symmetric, or substantially symmetric modified ITRs:
R1
R2
R3
R4
R5
Δ ITR
Enhancer/ promoter
transgene
WPRE
polyA
Δ ITR
Mutant ITR symmetrical relative to 3' ITR
Posttranscriptional regulatory element
mutant ITR symmetrical relative to 5' ITR

FIG. 1E

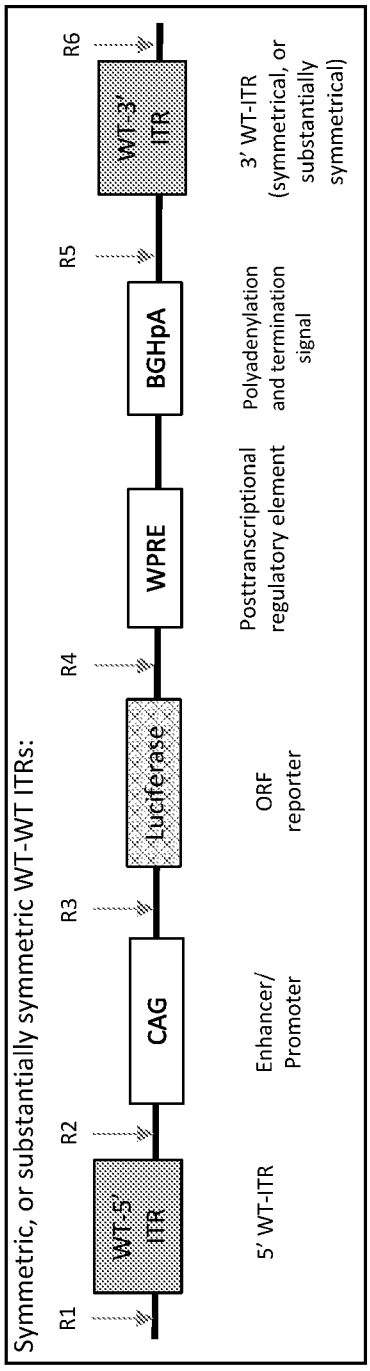

Symmetric, or substantially symmetric WT-WT ITRs:
R1
R2
R3
R4
R5
R6
WT-5' ITR
CAG
Luciferase
WPRE
BGHpA
WT-3' ITR
5' WT-ITR
Enhancer/ Promoter
ORF reporter
Posttranscriptional regulatory element
Polyadenylation and termination signal
3' WT-ITR (symmetrical, or substantially symmetrical)

FIG. 1F

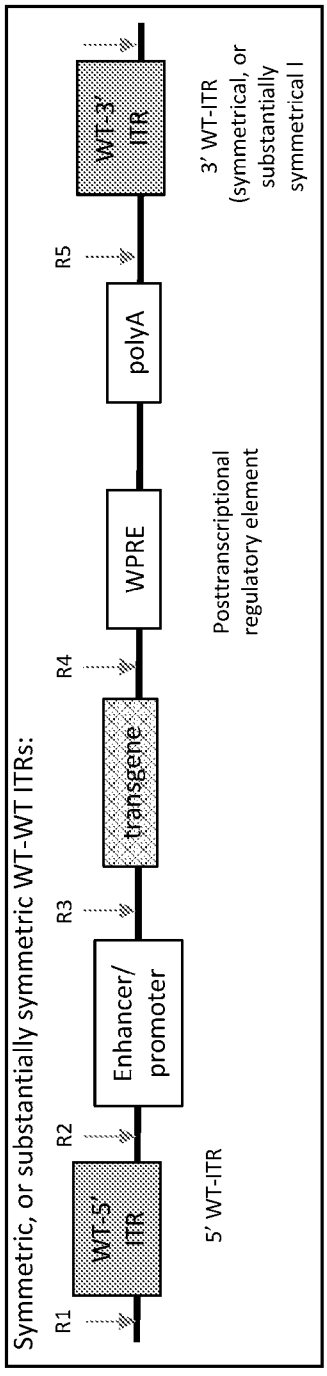

Symmetric, or substantially symmetric WT-WT ITRs:
R1
R2
R3
R4
R5
WT-5' ITR
Enhancer/ promoter
transgene
WPRE
polyA
WT-3' ITR
5' WT-ITR
Posttranscriptional regulatory element
3' WT-ITR (symmetrical, or substantially symmetrical I

FIG. 1G

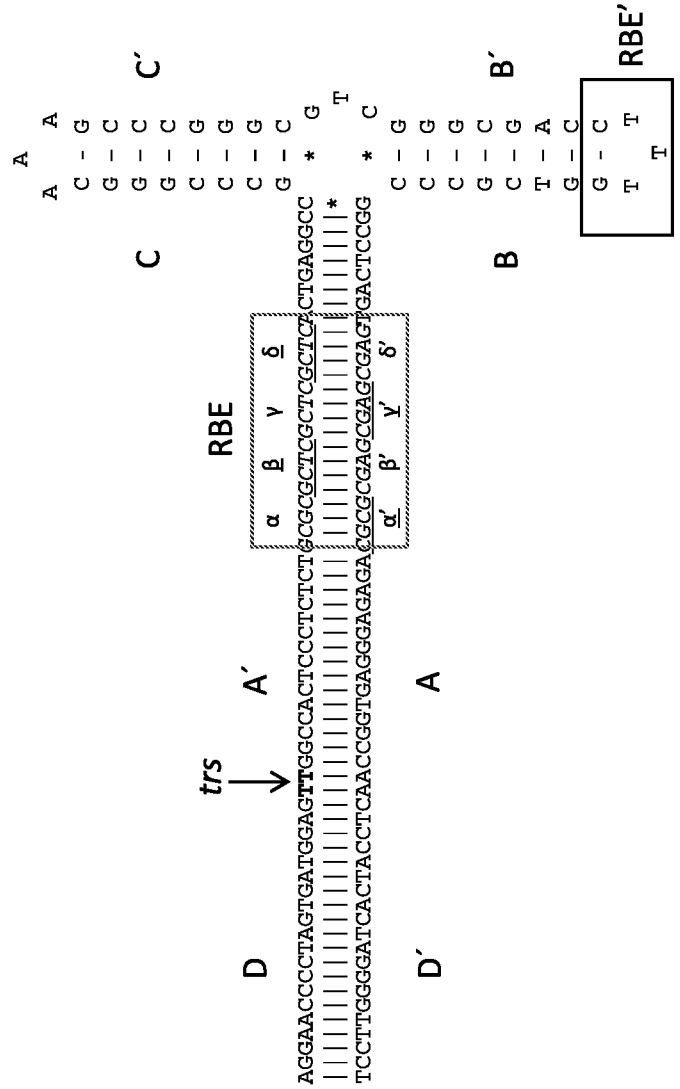


FIG. 2A

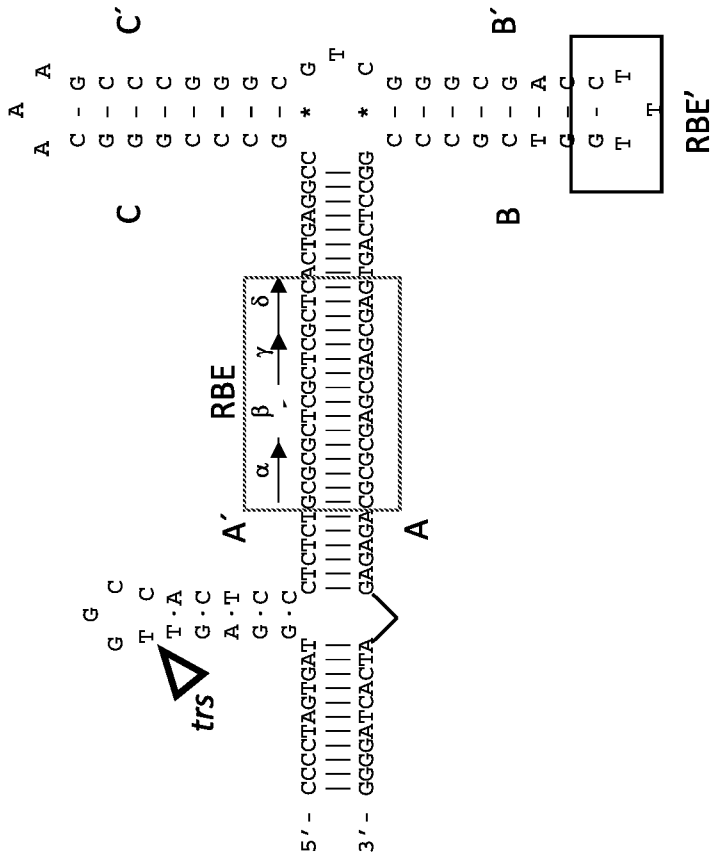


FIG. 2B

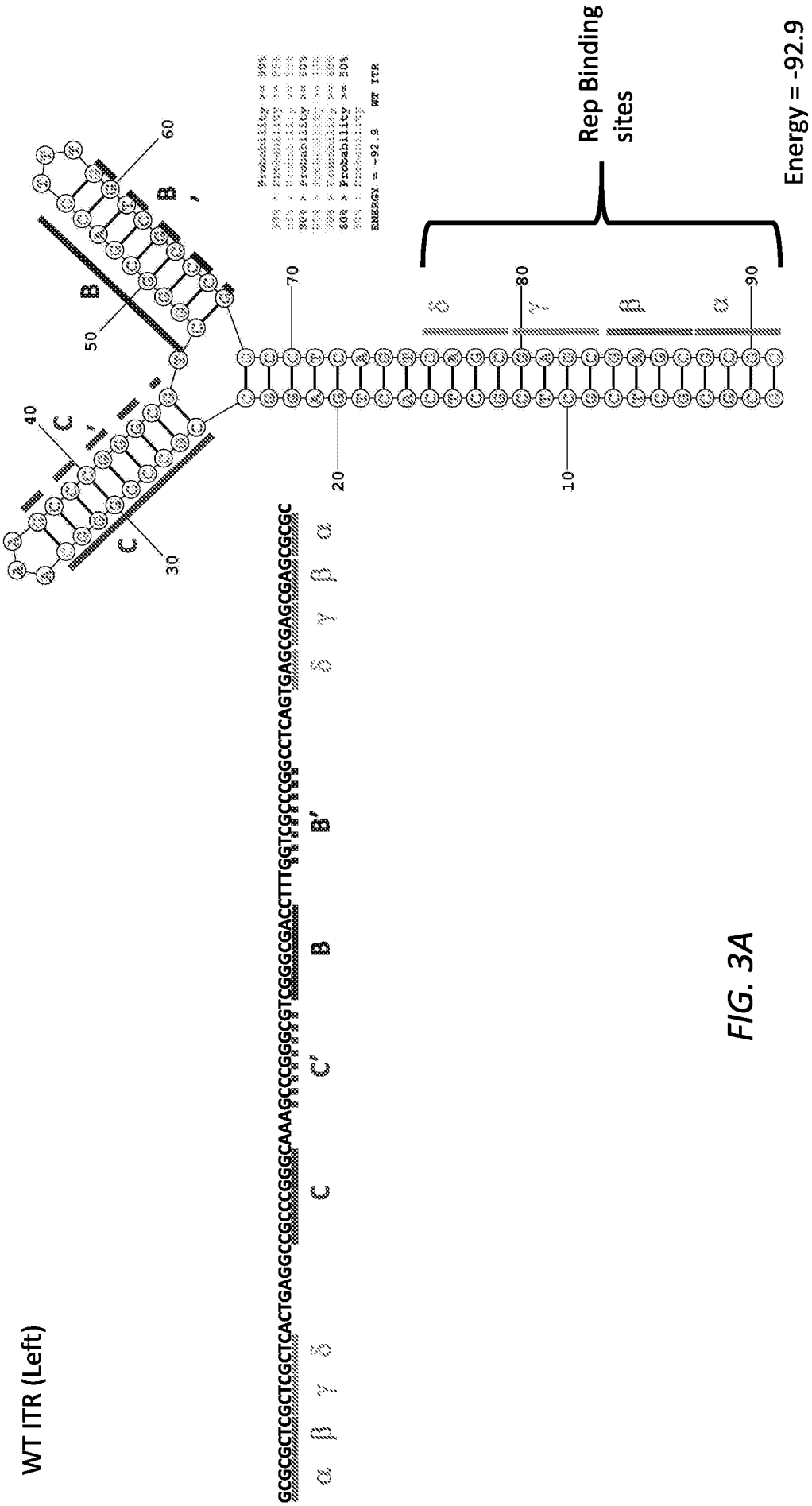
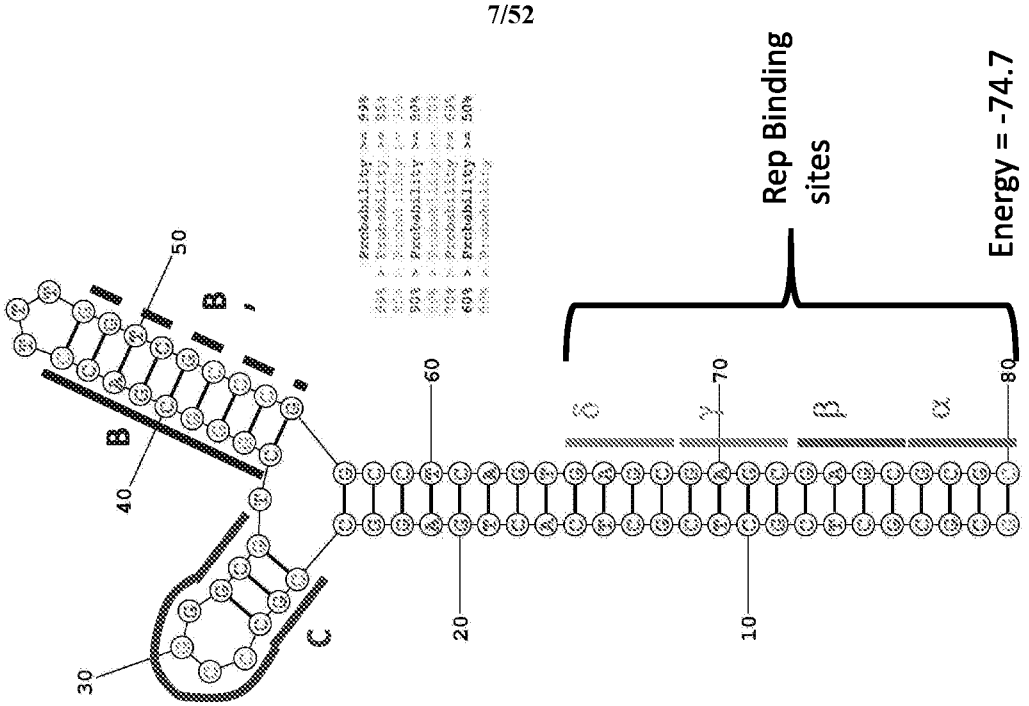

WT ITR (Left)
GCGCGCTCGCTCGCTCACTGAGGCCGCCCGGGCAAAGCCCGGGCGTCGGGCGACCTTTGGTCGCCCGGCCTCAGTGAGCGAGCGAGCGCGC
α β γ δ C C' B B' δ γ β α
Rep Binding sites
Energy = -92.9
ENERGY = -92.9 WT ITR

FIG. 3A



ITR-1, Left

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

FIG. 3B

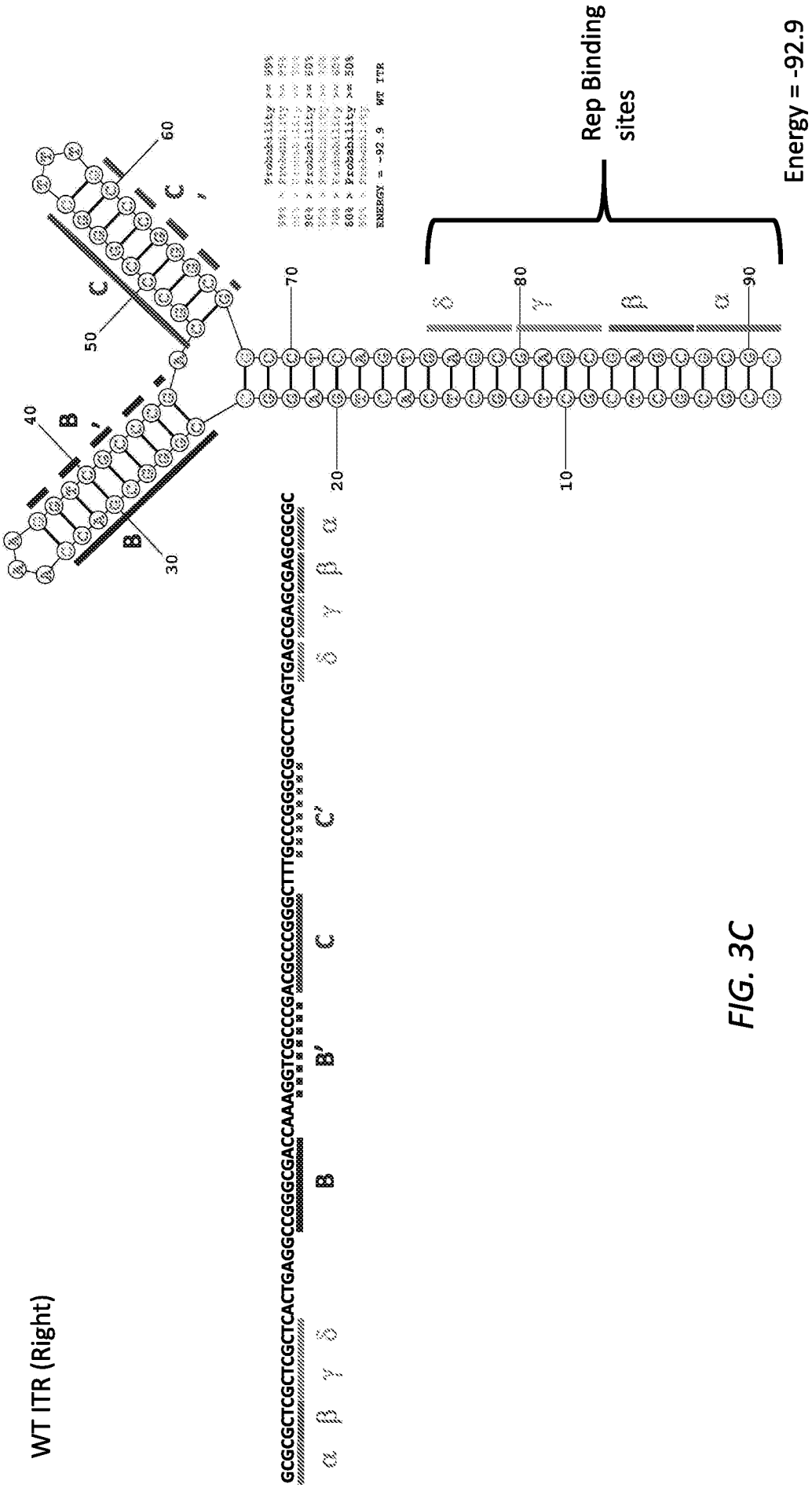
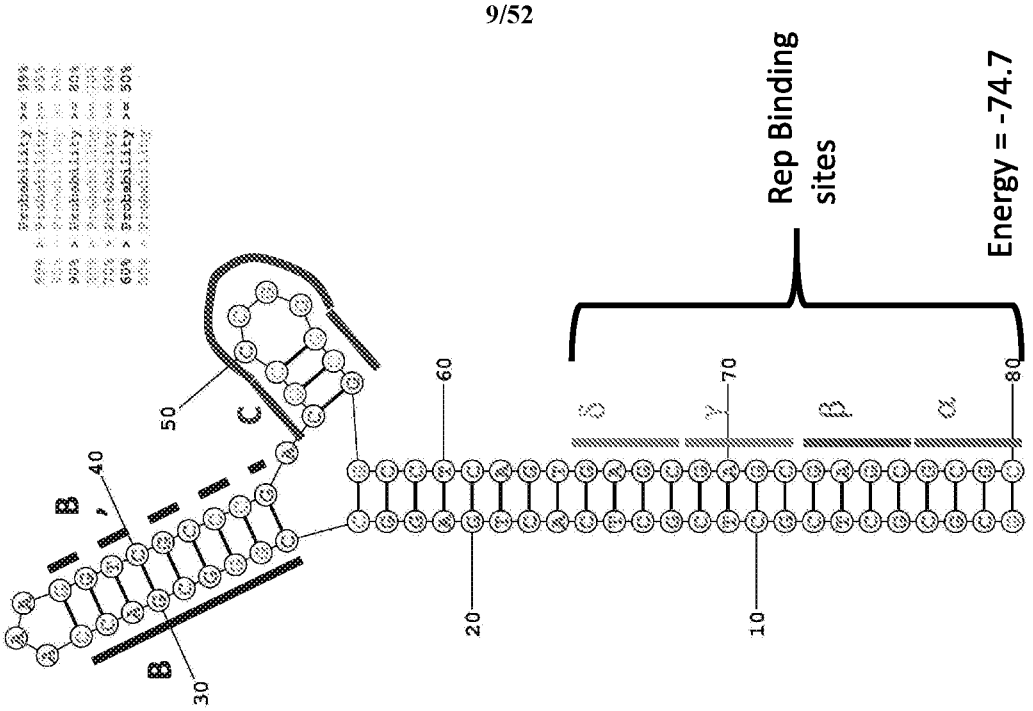

WT ITR (Right)
GCGCGCTCGCTCGCTCACTGAGGCCGGGCGACCAAAGGTCGCCCGACGCCCGGGCTTTGCCCGGGCGGCCTCAGTGAGCGAGCGAGCGCGC
α β γ δ
B
B'
C
C'
δ γ β α
Rep Binding sites
Energy = -92.9
ENERGY = -92.9 WT ITR
10
20
30
40
50
60
70
80
90

FIG. 3C



ITR-1, Right

GCGCGCTCGCTCGCTCACTGAGGCCGGGCGACCAAAGGTCGCCCGACGCCCGGGCGGCCTCAGTGAGCGAGCGAGCGCGC

α β γ δ B B' C δ γ β α

FIG. 3D

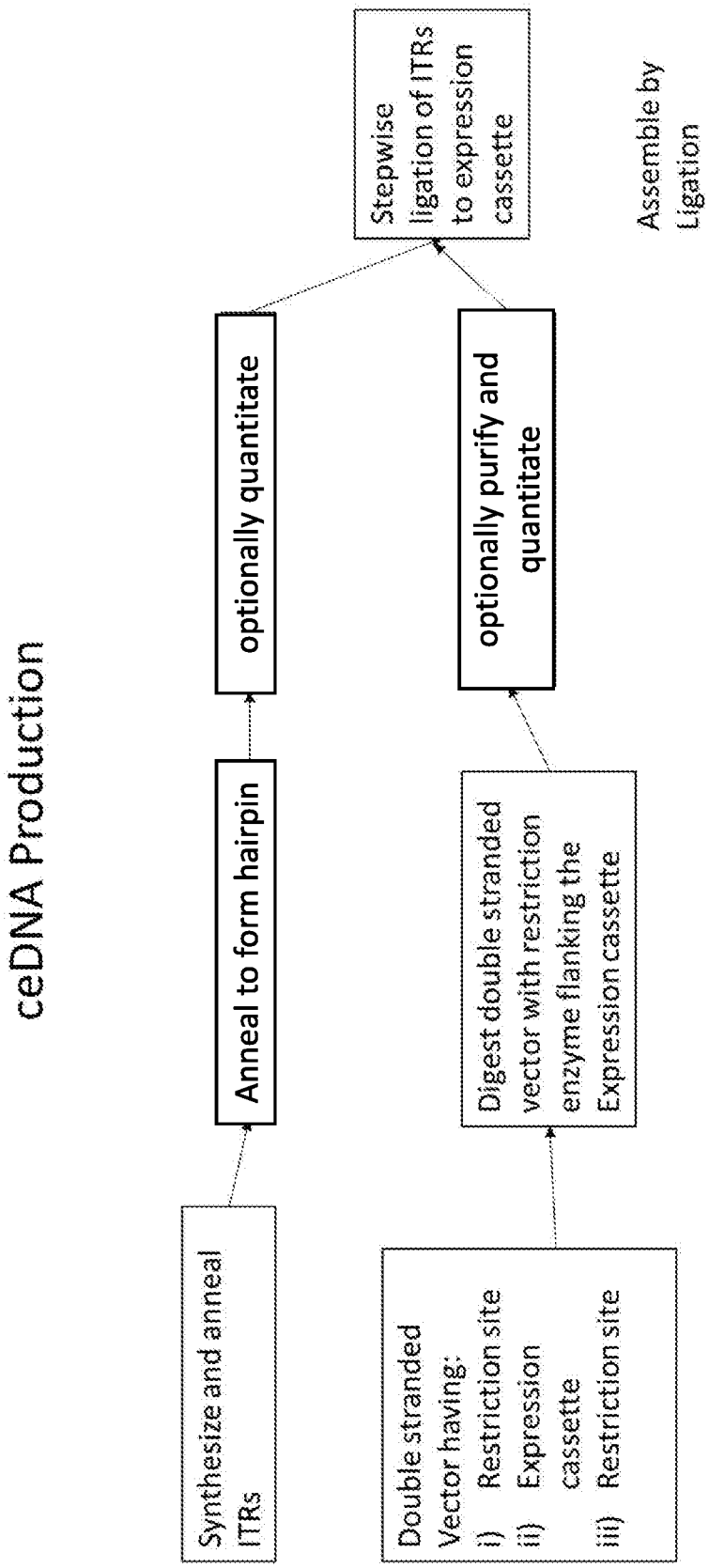

ceDNA Production
Synthesize and anneal ITRs
Anneal to form hairpin
optionally quantitate
Double stranded Vector having:
i) Restriction site
ii) Expression cassette
iii) Restriction site
Digest double stranded vector with restriction enzyme flanking the Expression cassette
optionally purify and quantitate
Stepwise ligation of ITRs to expression cassette
Assemble by Ligation

FIG. 4A

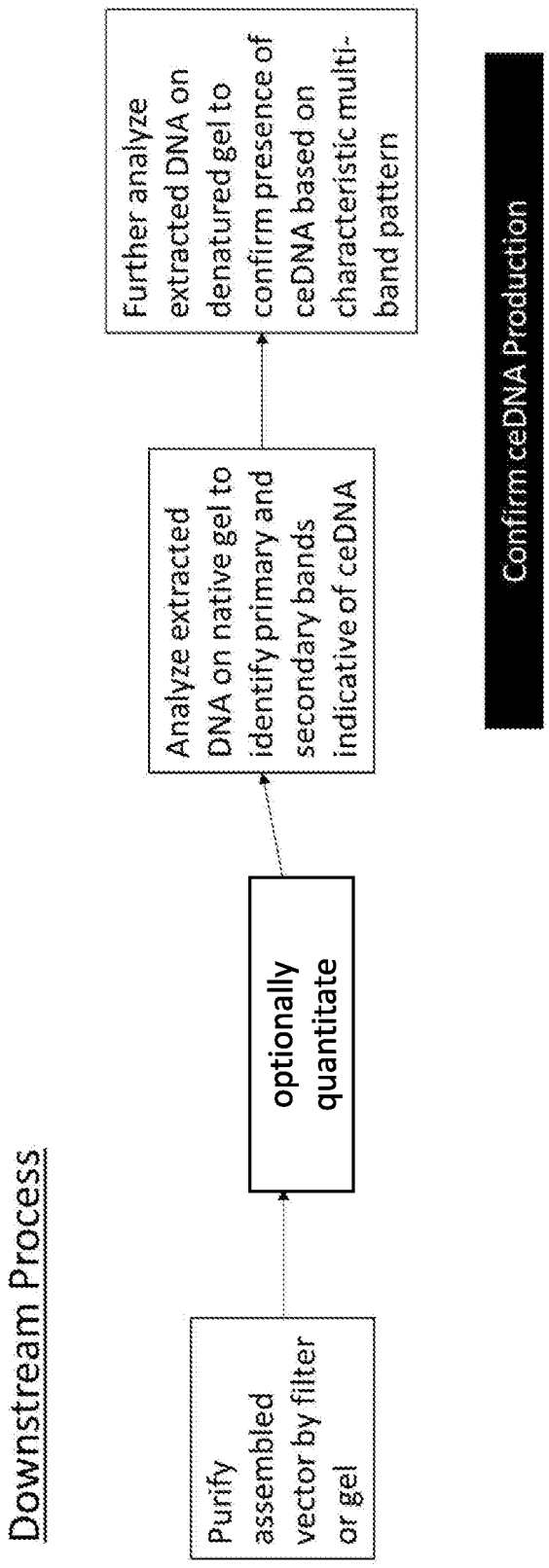

ceDNA Production
Downstream Process
Purify assembled vector by filter or gel
optionally quantitate
Analyze extracted DNA on native gel to identify primary and secondary bands indicative of ceDNA
Further analyze extracted DNA on denatured gel to confirm presence of ceDNA based on characteristic multi-band pattern
Confirm ceDNA Production

FIG. 4B

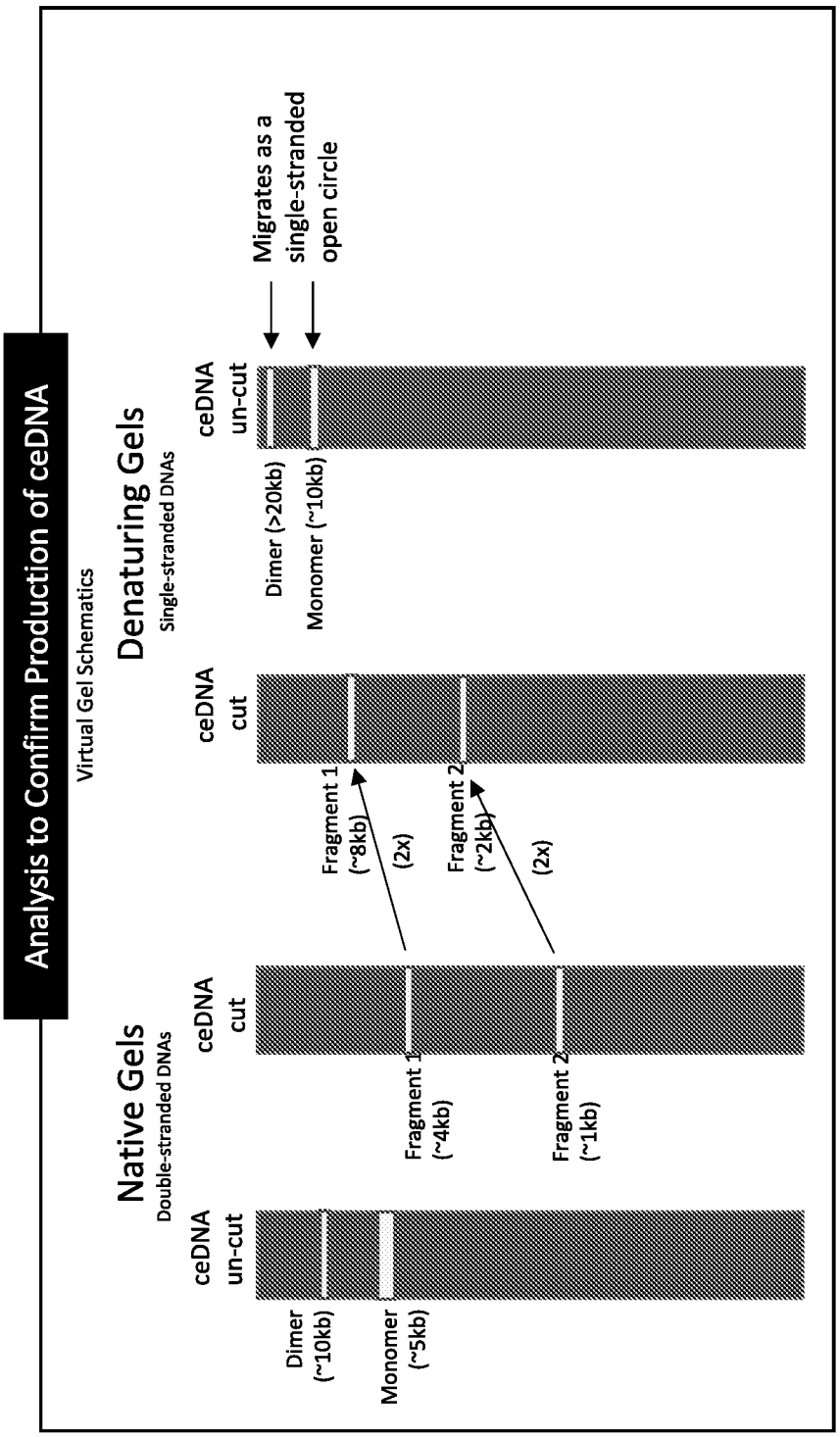

Analysis to Confirm Production of ceDNA
Virtual Gel Schematics
Native Gels
Double-stranded DNAs
ceDNA un-cut
Dimer (~10kb)
Monomer (~5kb)
ceDNA cut
Fragment 1 (~4kb)
Fragment 2 (~1kb)
Fragment 1 (~8kb)
(2x)
Fragment 2 (~2kb)
(2x)
Denaturing Gels
Single-stranded DNAs
ceDNA cut
ceDNA un-cut
Dimer (>20kb)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

FIG. 4C

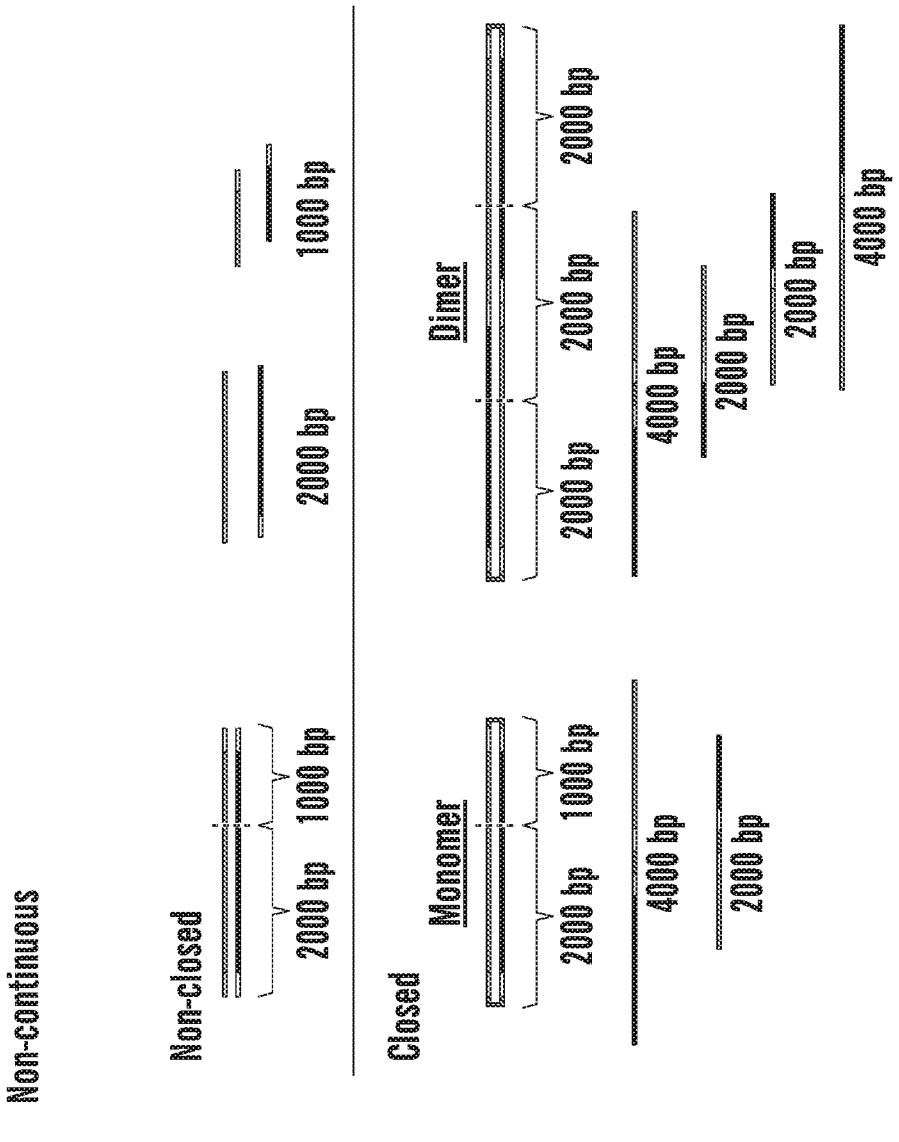

Non-continuous
Non-closed
2000 bp
1000 bp
Closed
Monomer
2000 bp
1000 bp
4000 bp
2000 bp
Dimer
2000 bp
2000 bp
2000 bp
4000 bp
2000 bp
2000 bp
4000 bp
Linear and continuous
Restriction enzyme site
1kb
2kb
Restriction enzyme site
1kb
2kb

FIG. 4D

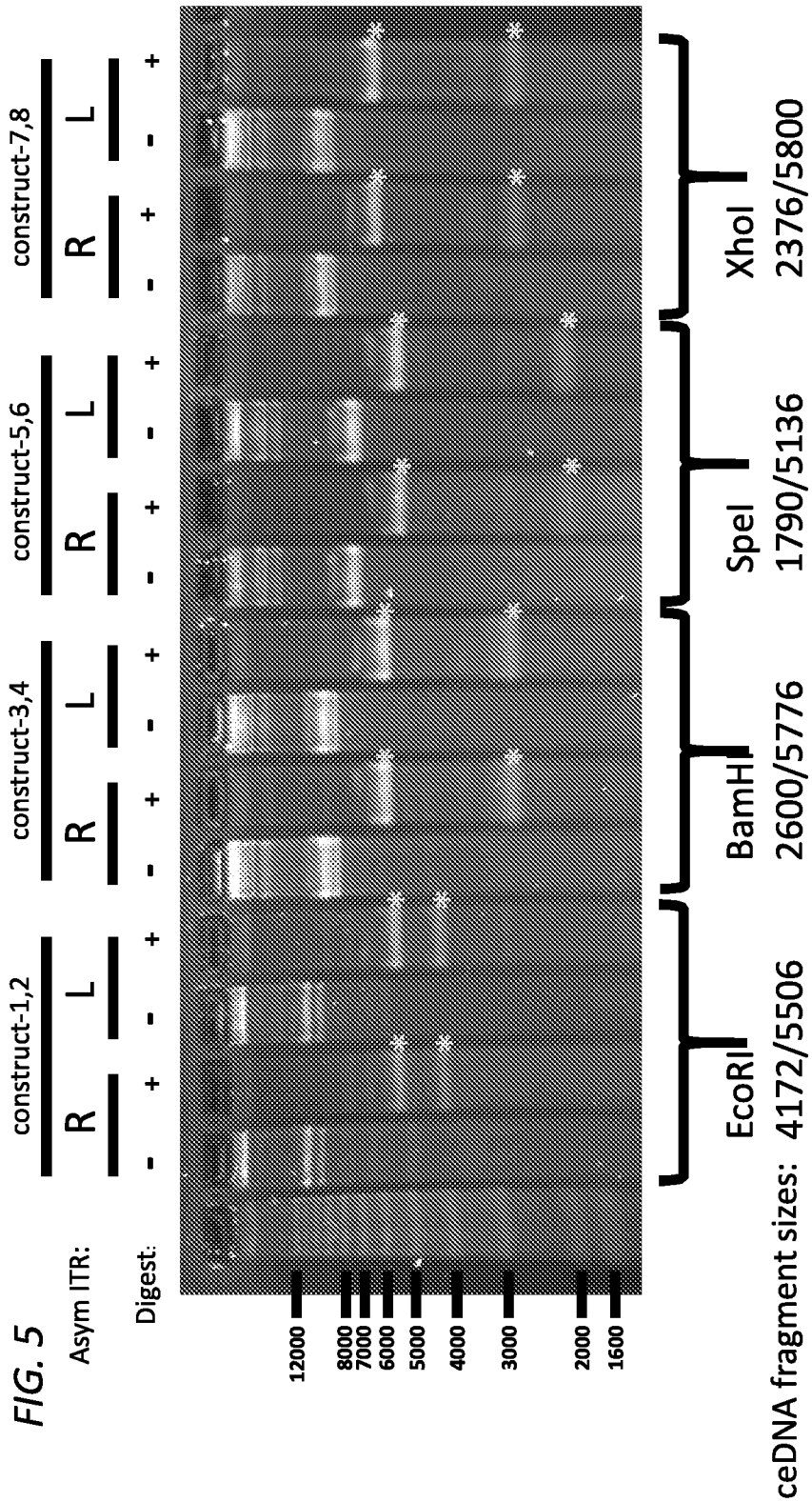


FIG. 5
Asym ITR:
Digest:
construct-1,2
construct-3,4
construct-5,6
construct-7,8
R L
- + - +
12000
8000
7000
6000
5000
4000
3000
2000
1600
EcoRI
BamH
SpeI
XhoI
ceDNA fragment sizes: 4172/5506 2600/5776 1790/5136 2376/5800

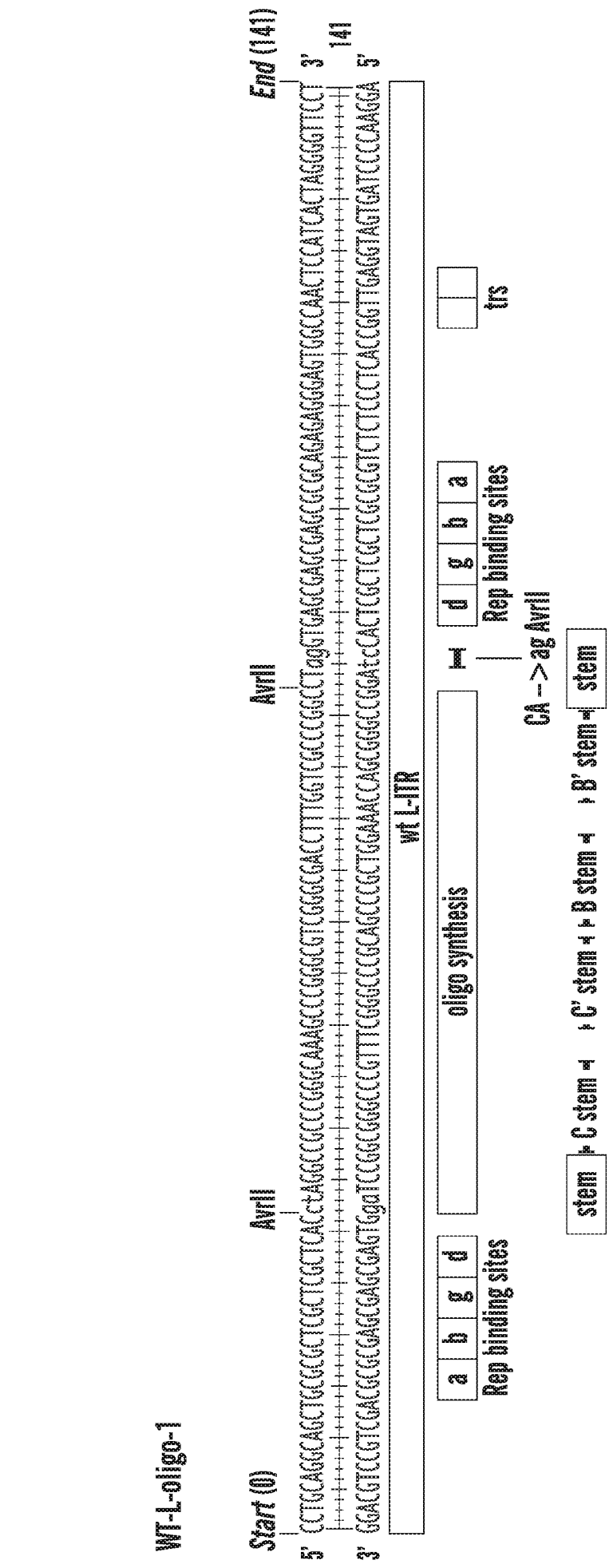

WT-L-oligo-1
Start (0)
AvrII
AvrII
End (141)
5' CCTGCAGGCAGCTGCGCGCTCGCTCGCTCACTGAGGCCGCCCGGGCAAAGCCCGGGCGTCGGGCGACCTTTGGTCGCCCGGCCTCAGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGCCAACTCCATCACTAGGGGTTCCT 3'
3' GGACGTCCGTCGACGCGAGCGCGAGTGACTCCGGGCGGGTTCCGAAACCAGCGGGCCGGAGTCACTCGCTCGCGCTCTCTCCCTCACCGGTTGAGGTAGTGATCCCCAAGGA 5'
141
wt L-ITR
a b g d
Rep binding sites
oligo synthesis
d g b a
Rep binding sites
trs
CA --> ag AvrII
stem
C stem
C' stem
B stem
B' stem
stem

FIG. 6A

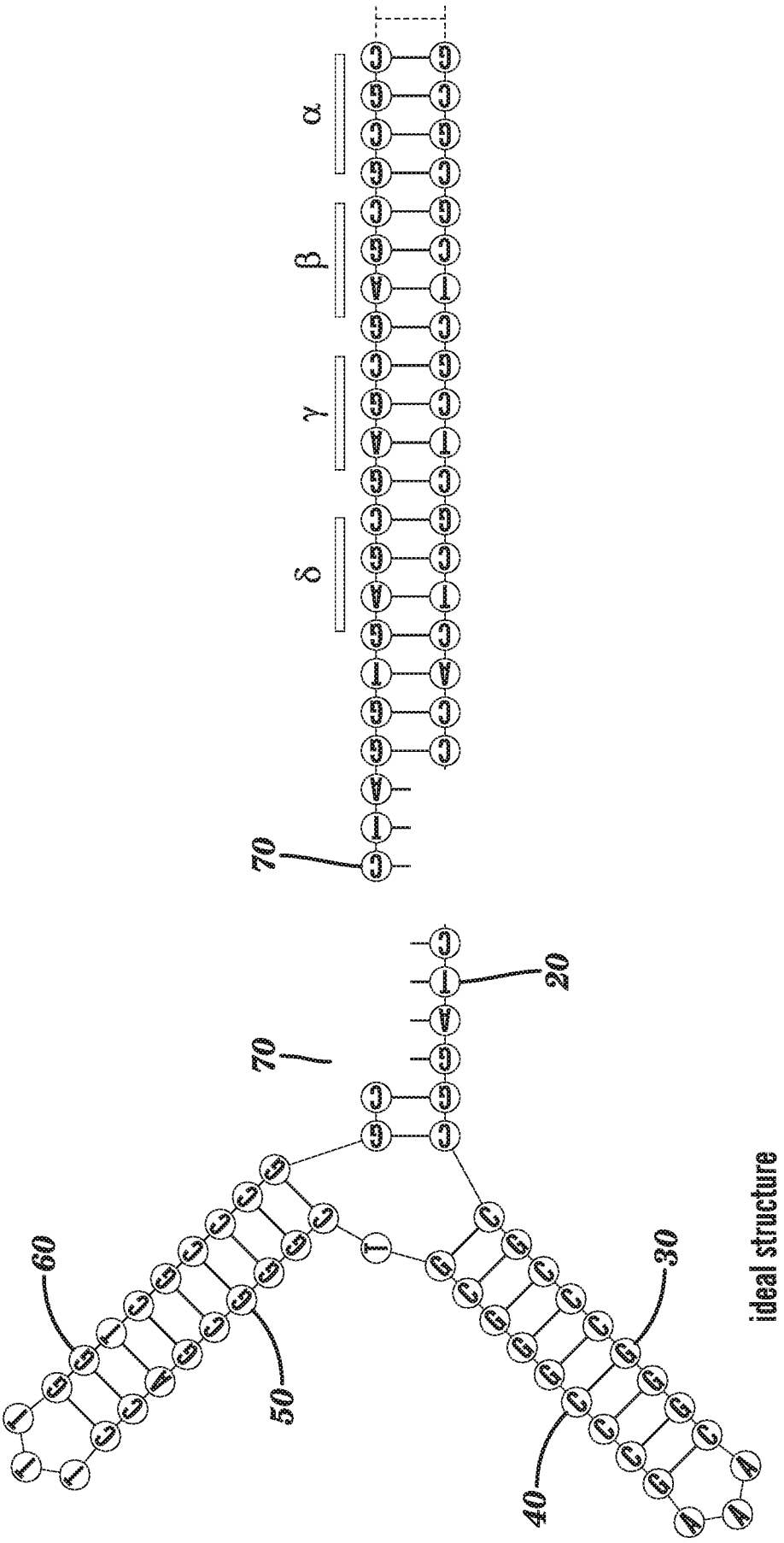


FIG. 6A (cont.)

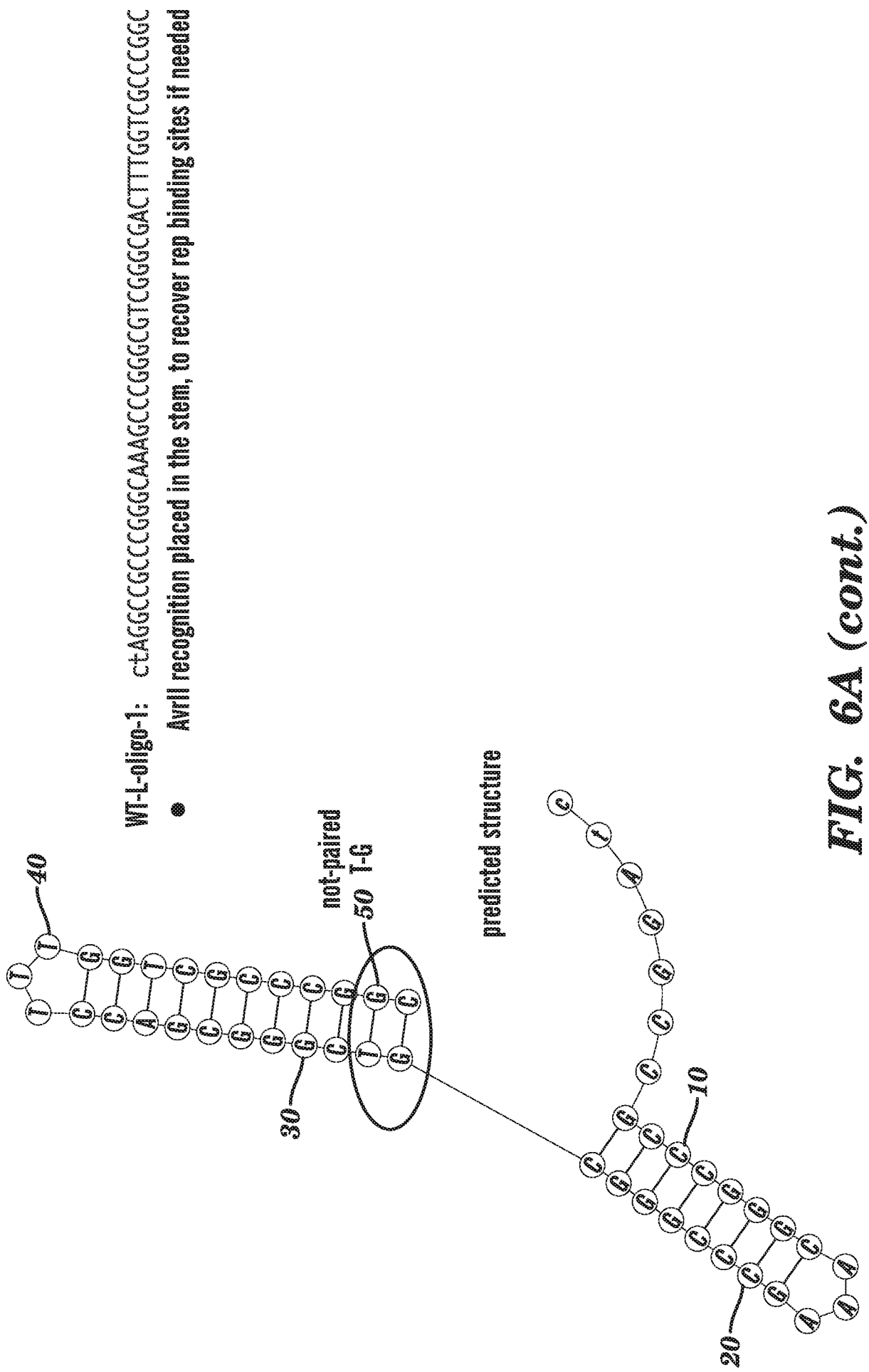

WT-L-oligo-1: ctAGGCCGCCCCGGGCAAAGCCCGGGCGTCGGGCGACTTTGGTCGCCCGGC
● AvrII recognition placed in the stem, to recover rep binding sites if needed
predicted structure
not-paired
T-G
40
50
30
10
20

FIG. 6A (cont.)

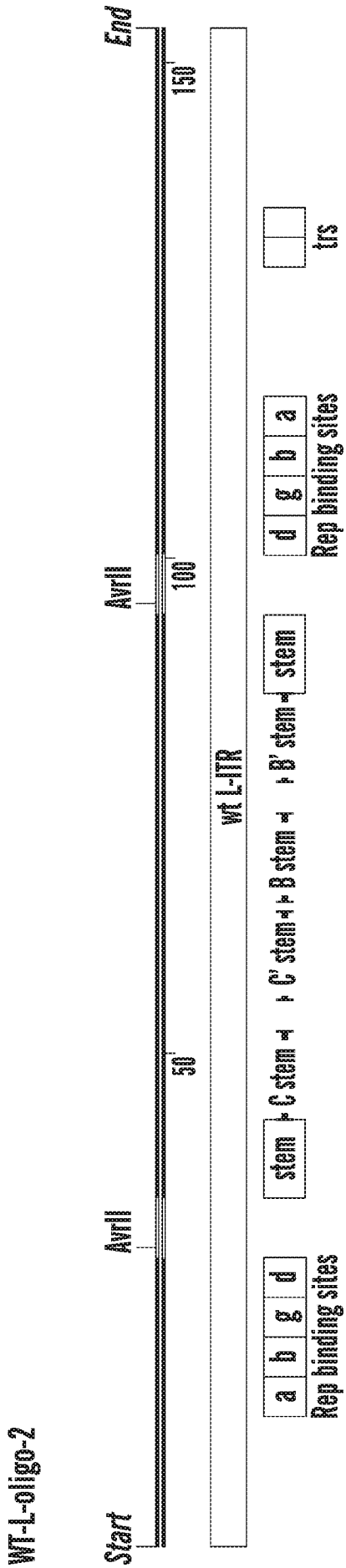

WT-L-oligo-2
Start
AvrII
AvrII
End
50
100
150
wt L-ITR
a
b
g
d
Rep binding sites
stem
C stem
C' stem
B stem
B' stem
stem
d
g
b
a
Rep binding sites
trs
wt L-ITR avrII
153 bp

FIG. 6B

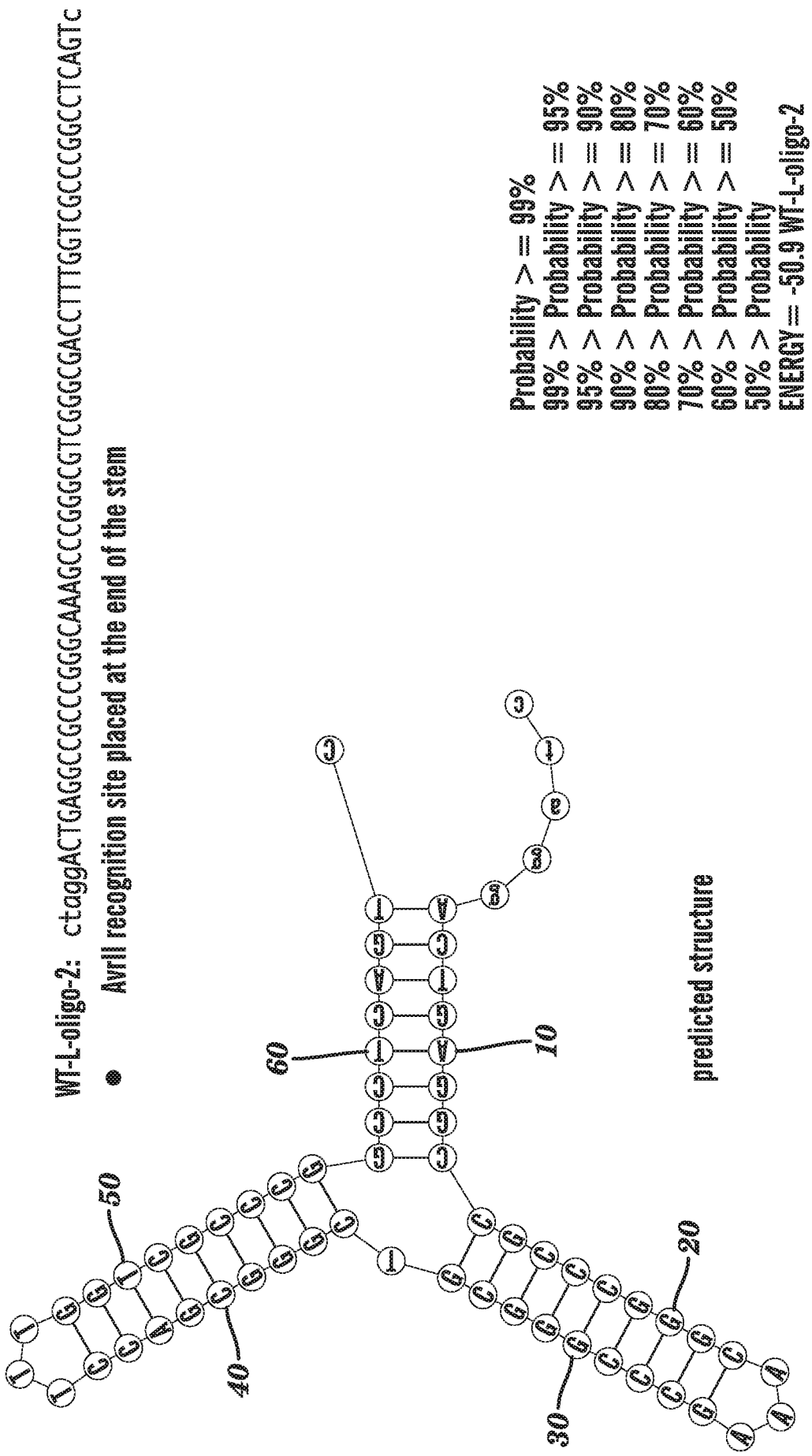

WT-L-oligo-2: ctaggACTGAGGCCGCCCGGGCAAAGCCCGGGCGTCGGGCGACCTTTGGTCGCCCGGCCTCAGTc
● AvrII recognition site placed at the end of the stem
Probability > == 99%
99% > Probability > == 95%
95% > Probability > == 90%
90% > Probability > == 80%
80% > Probability > == 70%
70% > Probability > == 60%
60% > Probability > == 50%
50% > Probability
ENERGY = -50.9 WT-L-oligo-2
predicted structure
10
20
30
40
50
60

FIG. 6B (cont.)

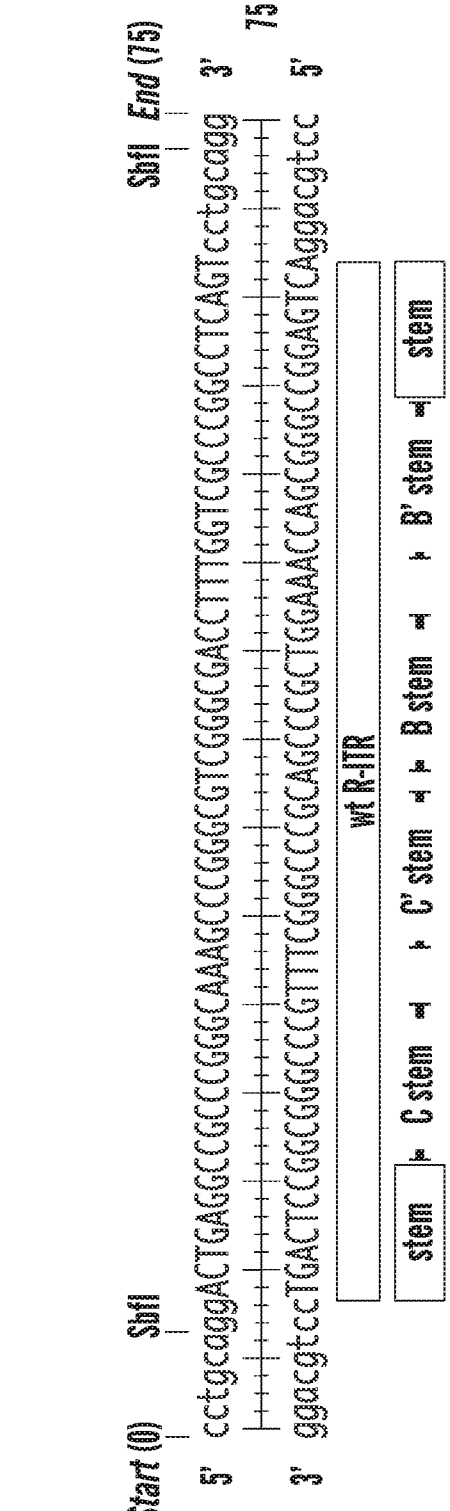

WT-R-oligo-3
Start (0)
SbfI
SbfI
End (75)
5'
3'
3'
5'
75
cctgcaggACTGAGCCCGCCGGGCAAAGCCCGGGCGTCGGGCGACCTTTGGTCGCCCGGCCTCAGTcctgcagg
ggacgtccTGACTCCGGGCGGCCCGTTTCGGGCCCGCAGCCCGCTGGAAACCAGCGGGCCGGAGTCAggacgtcc
wt R-ITR
stem
C stem
C' stem
B stem
B' stem
stem

FIG. 6C

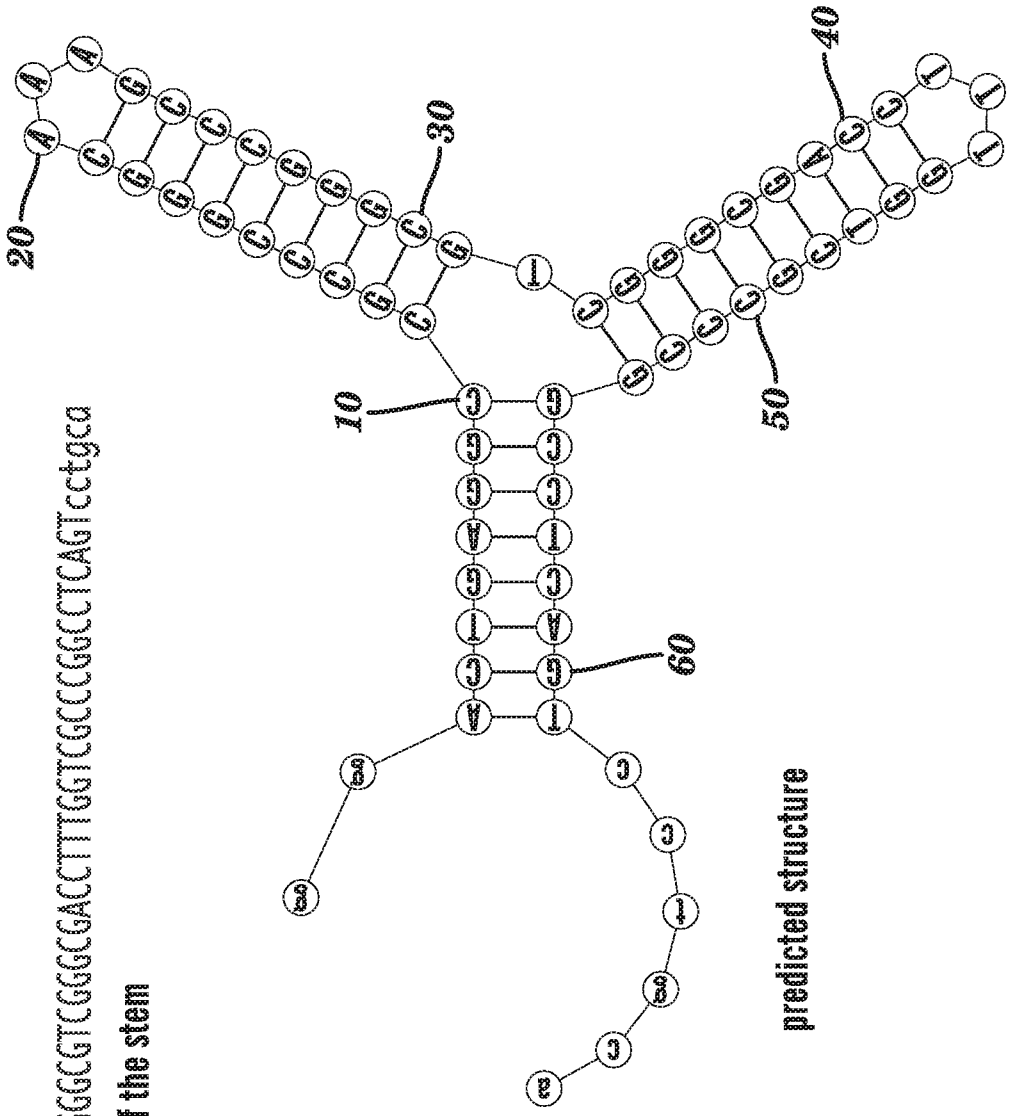

WT-R-oligo-3: ggACTGAGGCCGCCCGGGCAAAGCCCGGGCGTCGGGCGACCTTTGGTCGCCCGGCCTCAGTcctgca
Sbf1 recognition site placed at the end of the stem
Probability > = 99%
99% > Probability > = 95%
95% > Probability > = 90%
90% > Probability > = 80%
80% > Probability > = 70%
70% > Probability > = 60%
60% > Probability > = 50%
50% > Probability
ENERGY = -50.9 WT-R-oligo-3
predicted structure
10
20
30
40
50
60

FIG. 6C (cont.)

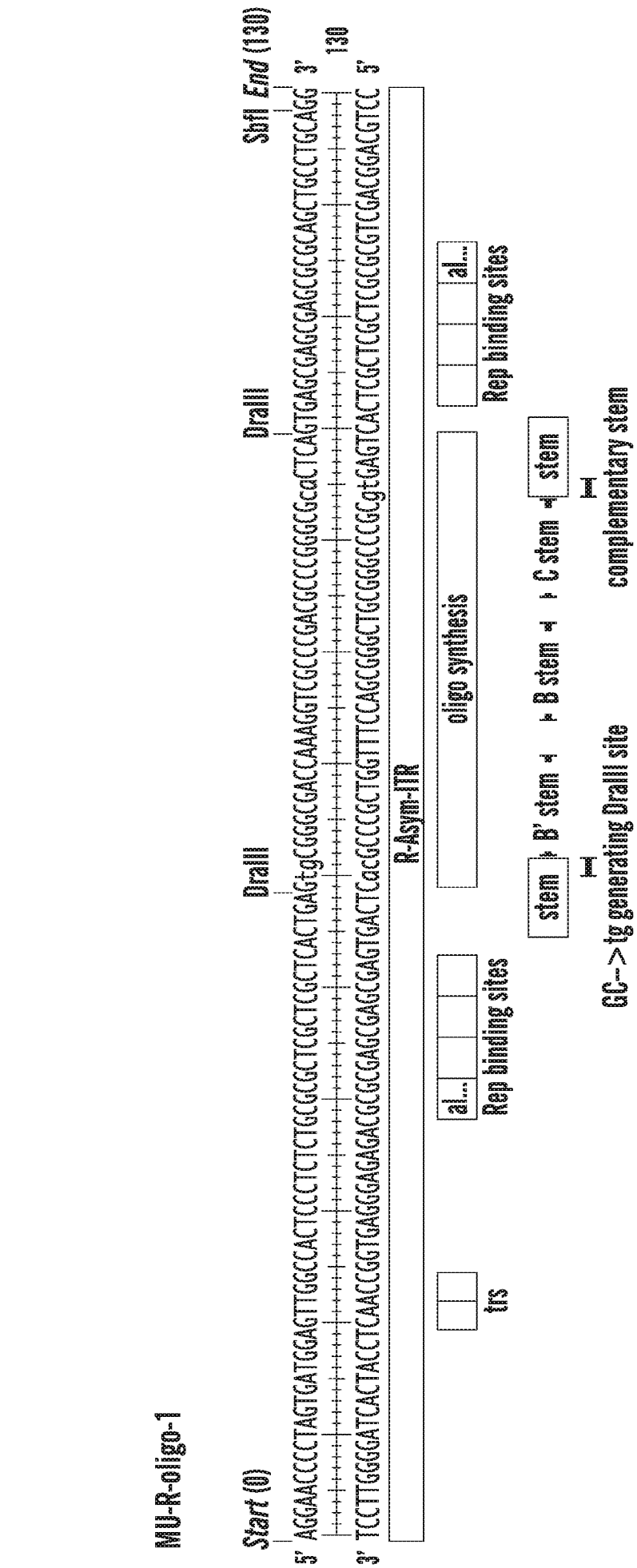

MU-R-oligo-1
Start (0)
DraIII
DraIII
SbfI End (130)
5' AGGAACCCCTAGTGATGGAGTTGGCCACTCCCTCTCTGCGCGCTCGCTCGCTCACTGAGtgCGGGCGACCAAAGGTCGCCCGACGCCCGGGCGGCaaCTCAGTGAGCGAGCGAGCGCGCAGCTGCCTGCAGG 3'
3' TCCTTGGGGATCACTACCTCAACCGGTGAGGGAGAGACGCGCGAGCGAGTCGACTCacGCCCGCTGGTTTCCAGCGGGCTGCGGGGCCCGCgtGAGTCACTCGCTCGCGCGTCGACGGAGCGTCC 5'
130
R-Asym-ITR
trs
al...
Rep binding sites
oligo synthesis
al...
Rep binding sites
stem
B' stem
B stem
C stem
stem
GC-->tg generating DraIII site
complementary stem

FIG. 6D

MU-R-oligo-1: GtgCGGGCGACCAAAGGTCGCCCGACGCCCCGGGCGCaCTCA
● DraIII recognition placed in the stem, to recover rep binding sites if needed
Probability > = 99%
99% > Probability > = 95%
95% > Probability > = 90%
90% > Probability > = 80%
80% > Probability > = 70%
70% > Probability > = 60%
60% > Probability > = 50%
50% > Probability
ENERGY = -22.0 MU-R-oligo-1
10
20
30
40
predicted structure

FIG. 6D (cont.)

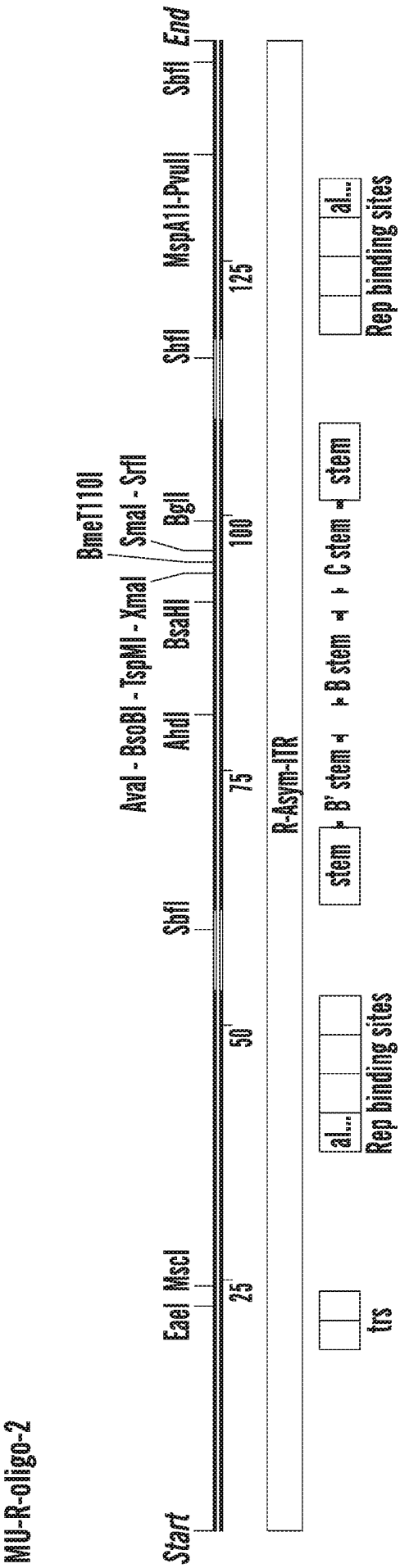
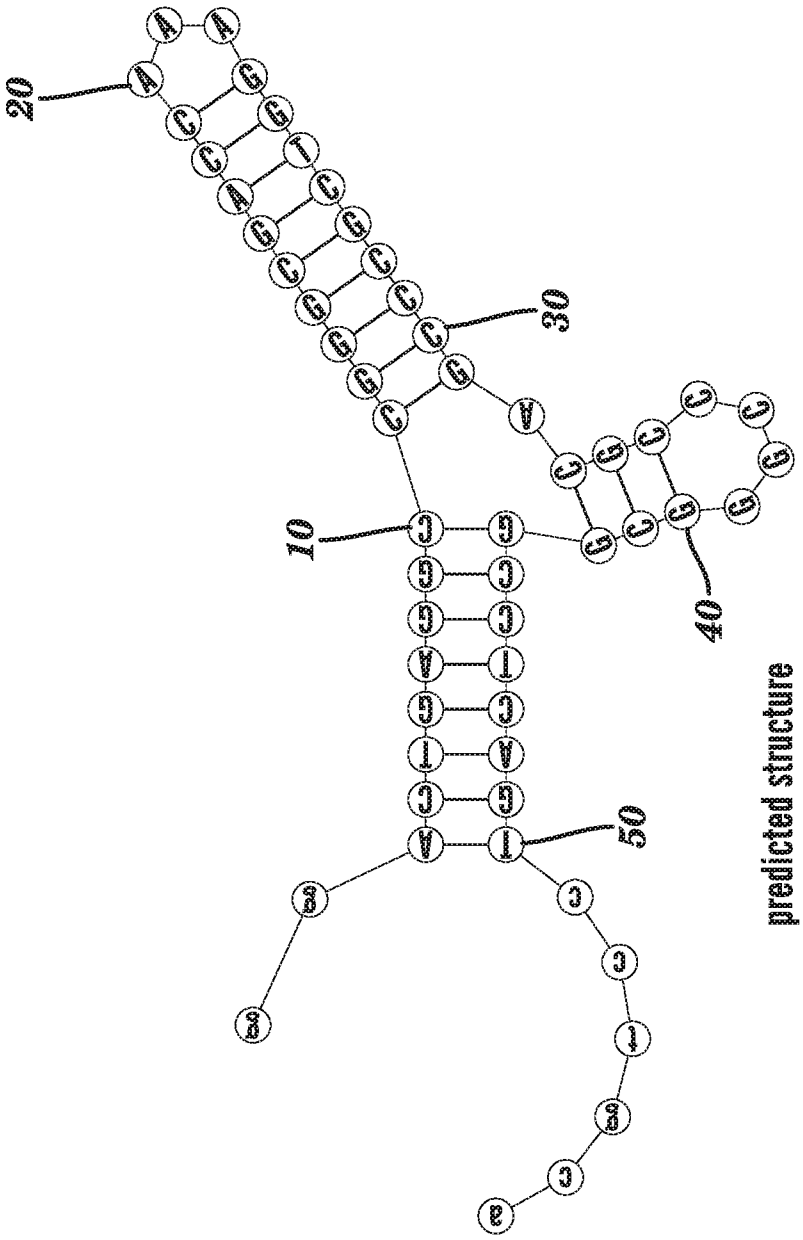

MU-R-oligo-2
Start
EaeI MscI
SbfI
AhdI
BsaHI
AvaI - BsoBI - TspMI - XmaI
SmaI - SrfI
BmeT110I
BglII
SbfI
MspA1I-PvuII
SbfI End
25
50
75
100
125
R-Asym-ITR
trs
al...
Rep binding sites
stem
B' stem
B stem
C stem
stem
al...
Rep binding sites
R-Asym-ITR SbfI
146 bp

FIG. 7A

MU-R-oligo-2: ggACTGAGGCCGGGCGACCAAAGGTCGCCCGACGCCCGGGCGGCCTCAGTcctgca

- Sbf1 recognition site placed at end of the stem



predicted structure

Probability > = 99%
99% > Probability > = 95%
95% > Probability > = 90%
90% > Probability > = 80%
80% > Probability > = 70%
70% > Probability > = 60%
60% > Probability > = 50%
50% > Probability
ENERGY = -33.2 MU-R-oligo-2

FIG. 7A (cont.)

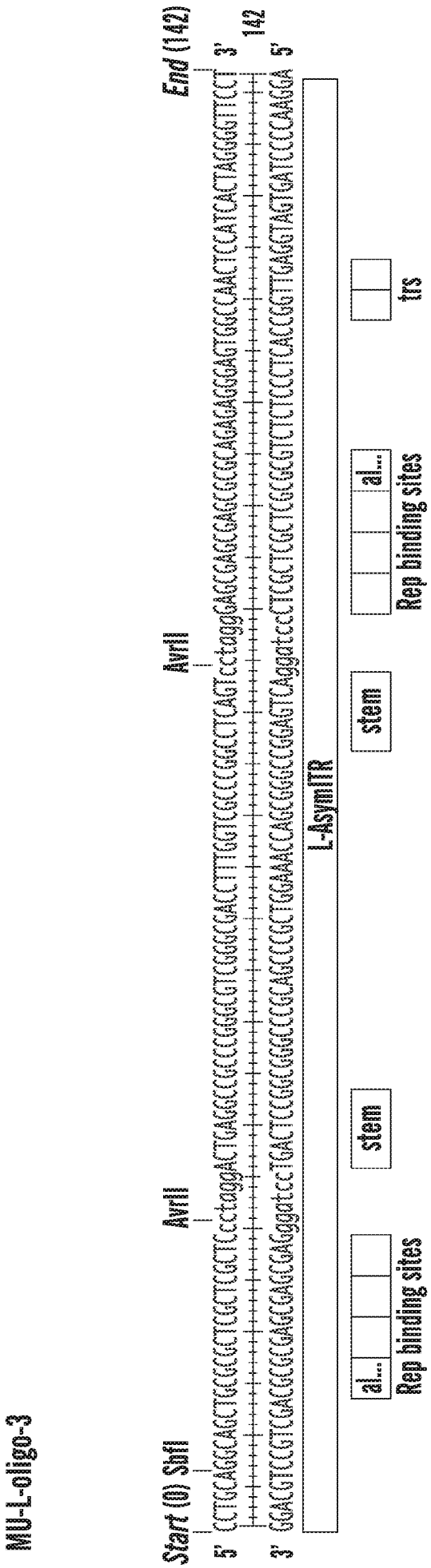

MU-L-oligo-3
Start (0) SbfI
AvrII
AvrII
End (142)
5'
3'
142
al...
Rep binding sites
stem
stem
L-AsymITR
al...
Rep binding sites
trs

FIG. 7B

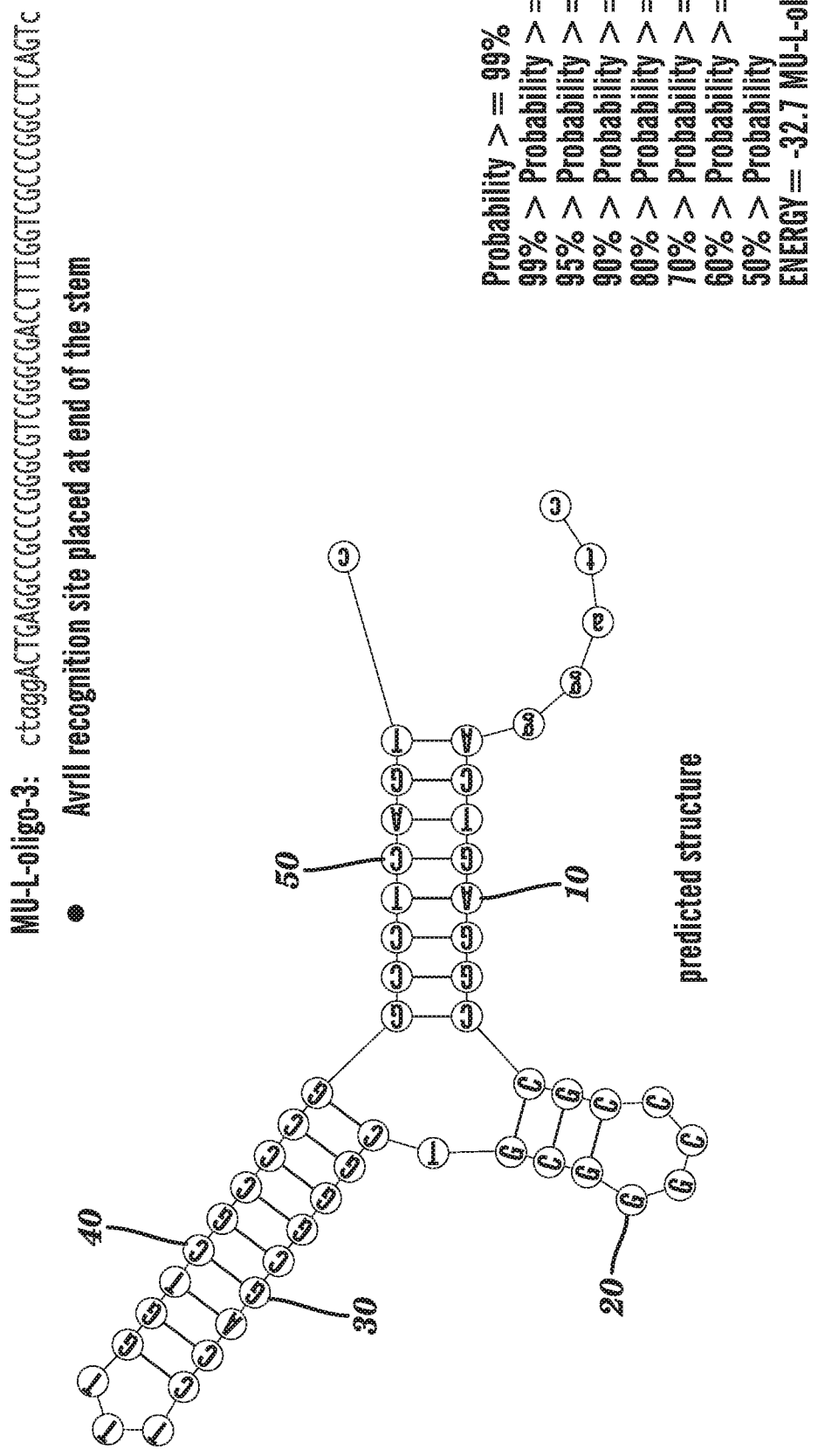

MU-L-oligo-3: ctaggACTGAGCCGCCCGGGCGTCGGGCGACCTTTGGTCGCCCGGCCTCAGTc
● AvrII recognition site placed at end of the stem
Probability > = 99%
99% > Probability > = 95%
95% > Probability > = 90%
90% > Probability > = 80%
80% > Probability > = 70%
70% > Probability > = 60%
60% > Probability > = 50%
50% > Probability
ENERGY = -32.7 MU-L-oligo-3
predicted structure
10
20
30
40
50

FIG. 7B (cont.)

MU-R-oligo-4
Start
SbfI
AhdI
SbfI End
10
20
30
40
50
dITR R
56 bp
MU-R-oligo-4: ggACTGAGGCCGGGCGACCAAAGGTCGCCCGACGCCCTCAGTcctgca
● SbfI recognition site placed at end of the stem
Probability > = 99%
99% > Probability > = 95%
95% > Probability > = 90%
90% > Probability > = 80%
80% > Probability > = 70%
70% > Probability > = 60%
60% > Probability > = 50%
50% > Probability
ENERGY = -32.8 MU-L-oligo-4
predicted structure

FIG. 7C

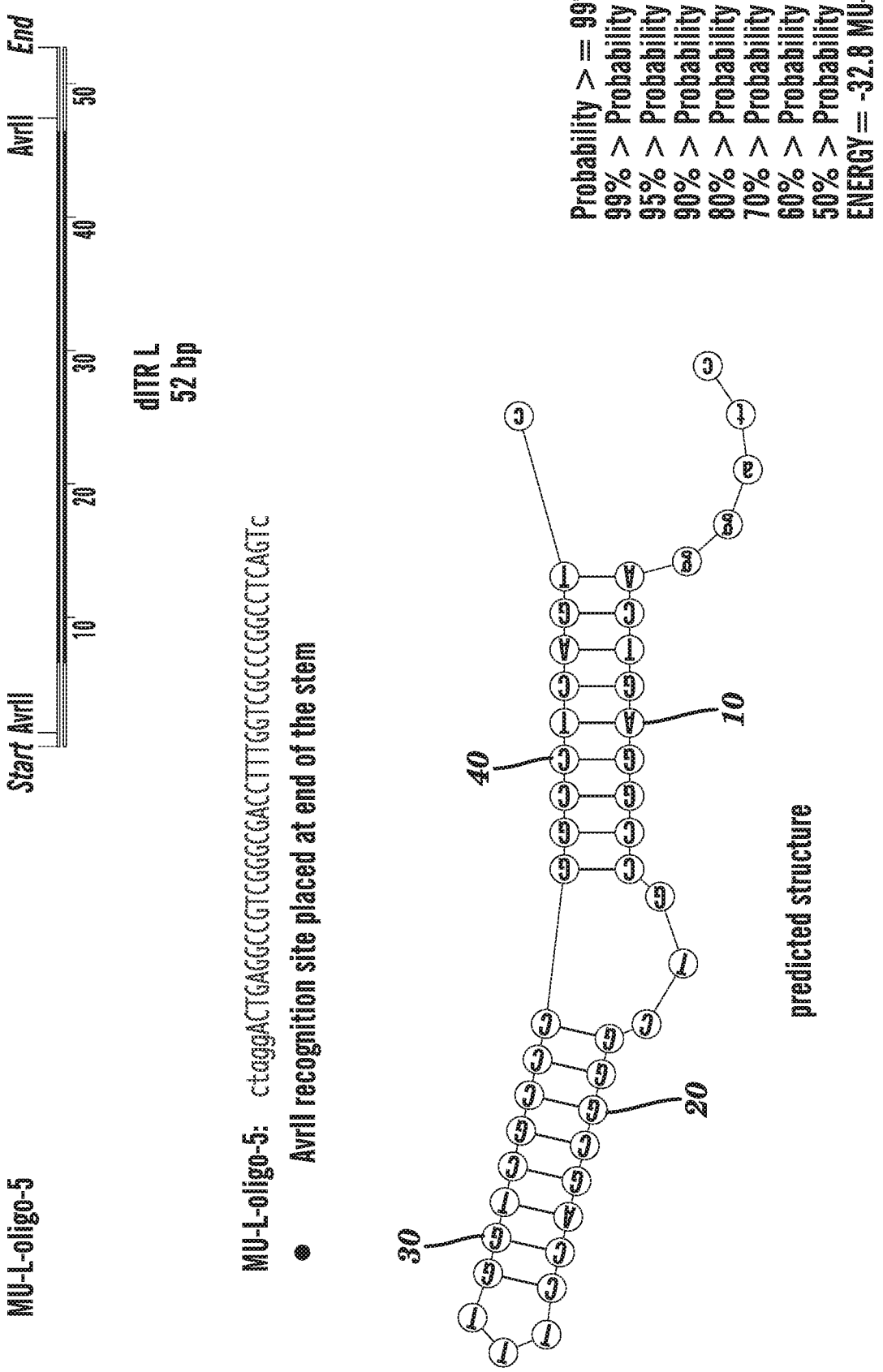

MU-L-oligo-5
Start AvrII
AvrII End
10
20
30
40
50
dITR L
52 bp
MU-L-oligo-5: ctaggACTGAGGCCGTCGGGCGACCTTTGGTCGCCCGGCCTCAGTc
● AvrII recognition site placed at end of the stem
predicted structure
Probability > = 99%
99% > Probability > = 95%
95% > Probability > = 90%
90% > Probability > = 80%
80% > Probability > = 70%
70% > Probability > = 60%
60% > Probability > = 50%
50% > Probability
ENERGY = -32.8 MU-L-oligo-5

FIG. 7D

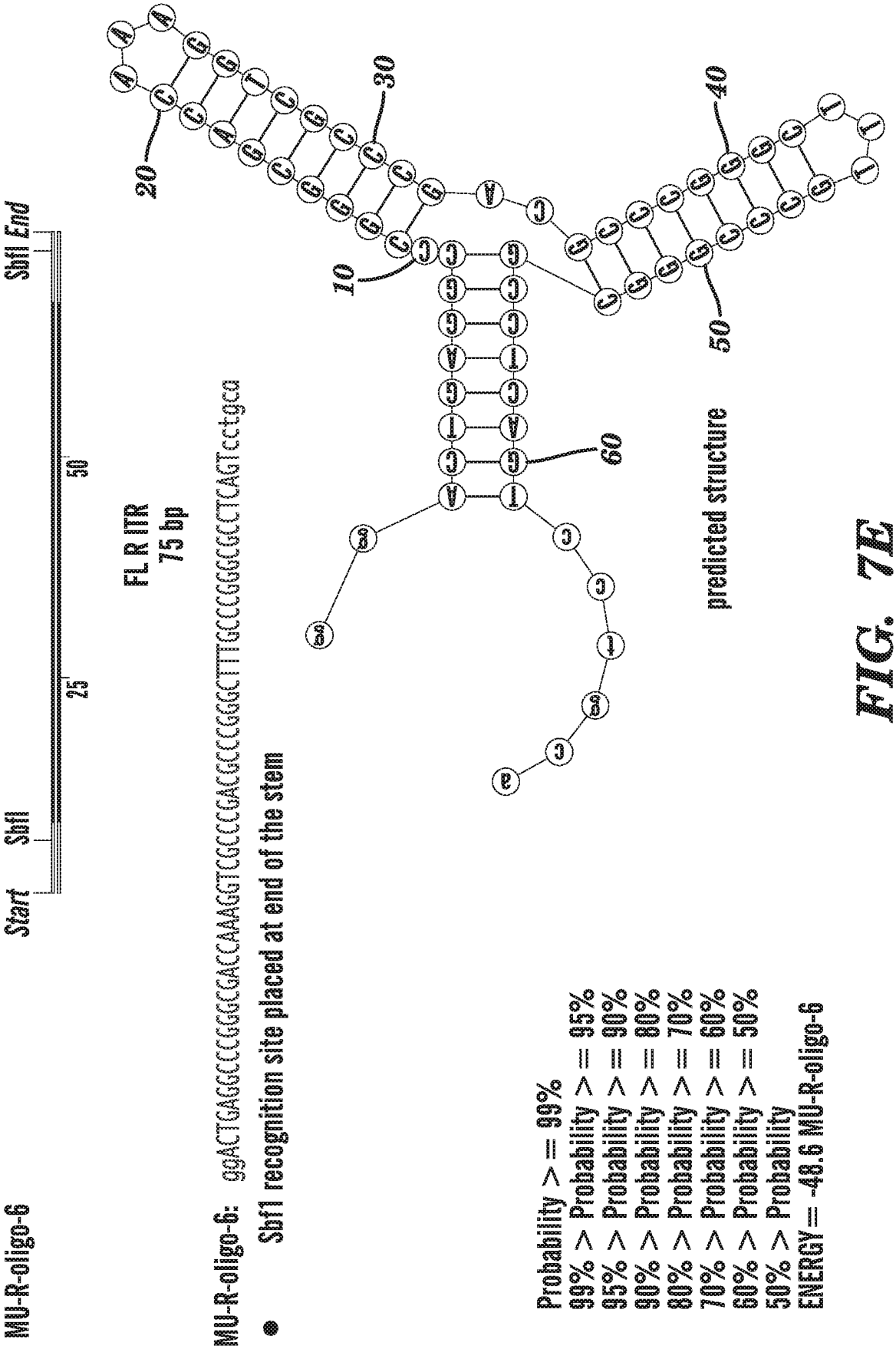

MU-R-oligo-6
Start
SbfI
SbfI End
25
50
FL R ITR
75 bp
MU-R-oligo-6: ggACTGAGGCCCGGGCGACCAAAGGTCGCCCGACGCCCGGGCTTTGCCCGGGCGCCTCAGTcctgca
Sbf1 recognition site placed at end of the stem
predicted structure
10
20
30
40
50
60
Probability > = 99%
99% > Probability > = 95%
95% > Probability > = 90%
90% > Probability > = 80%
80% > Probability > = 70%
70% > Probability > = 60%
60% > Probability > = 50%
50% > Probability
ENERGY = -48.6 MU-R-oligo-6

FIG. 7E

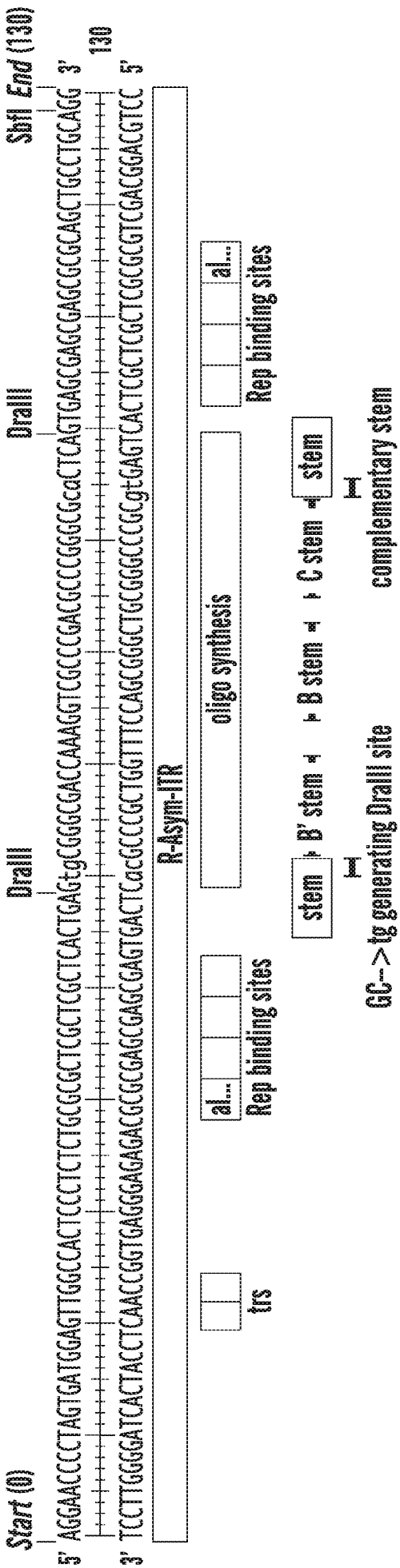
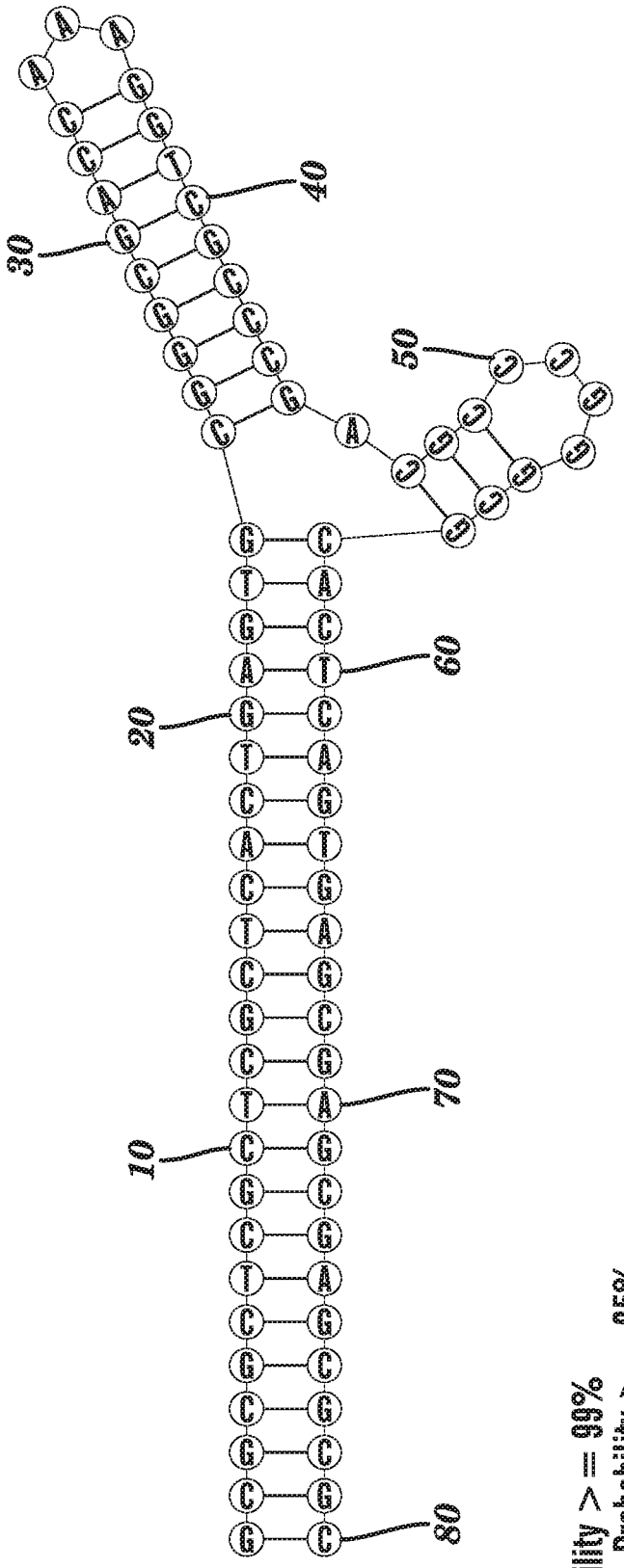

Start (0)
SbfI End (130)
5' AGGAACCCCTAGTGATGGAGTTGGCCACTCCCTCTCTGCGCGCTCGCTCGCTCACTGAGtgCGGGCGACCAAAGGTCGCCCGACGCCCCGGGCGCaaCTCAGTGAGCGAGCGAGCGCGCAGCTGCCTGCAGG 3'
3' TCCTTGGGGATCACTACCTCAACCGGTGAGGGAGAGACGCGCGAGCGAGTGACTCacGCCCCGCTGGTTCCAGCGGGCTGCGGGGCCCGCgt+GAGTCACTCGCTCGCGCTCGACGGAACGTCC 5'
DraIII
DraIII
130
R-Asym-ITR
trs
al...
Rep binding sites
oligo synthesis
al...
Rep binding sites
stem
B' stem
B stem
C stem
stem
GC-->tg generating DraIII site
complementary stem

FIG. 8



Probability > = 99%
99% > Probability > = 95%
95% > Probability > = 90%
90% > Probability > = 80%
80% > Probability > = 70%
70% > Probability > = 60%
60% > Probability > = 50%
50% > Probability
ENERGY = -72.9 WT oligo

FIG. 8 (cont.)

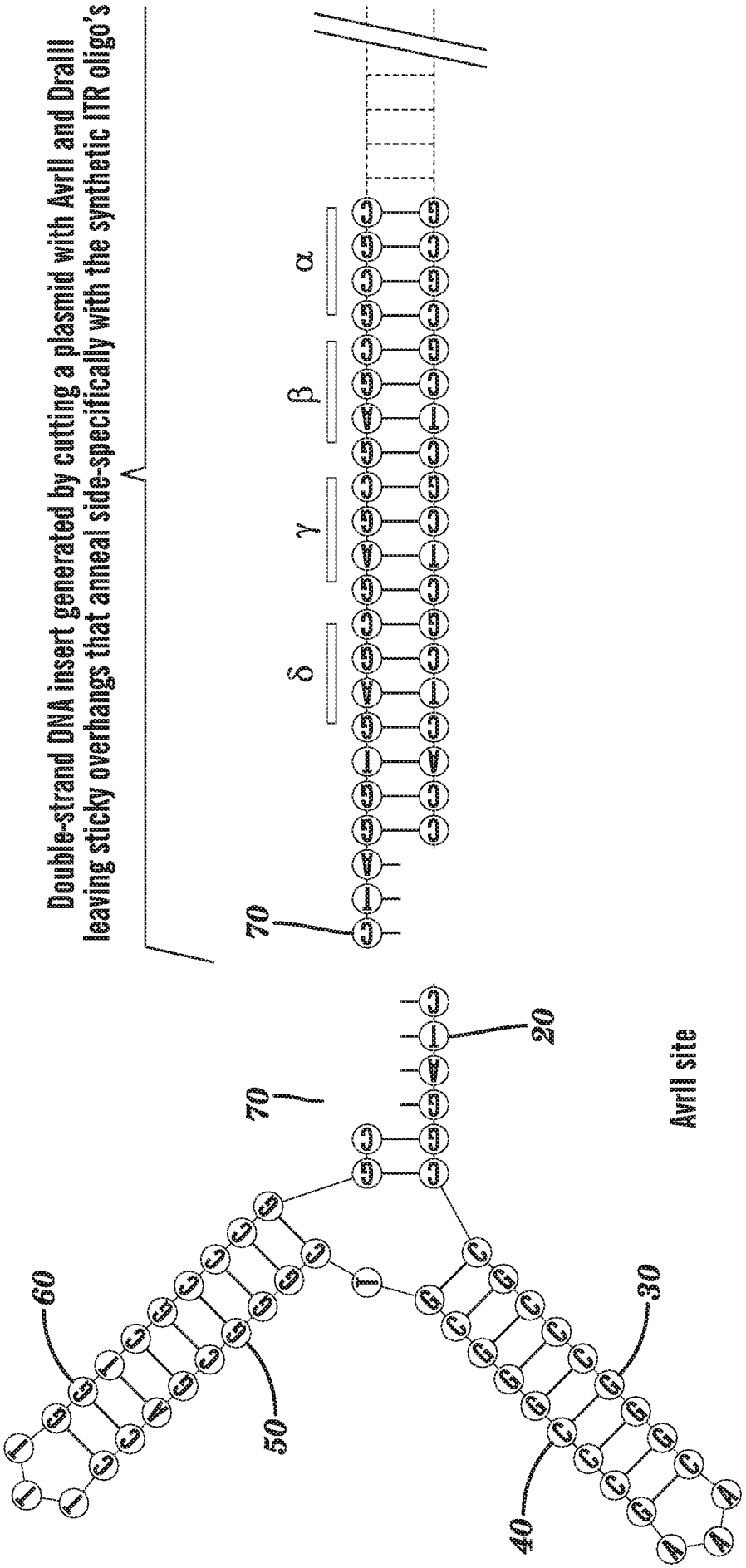

Double-strand DNA insert generated by cutting a plasmid with AvrII and DraIII
leaving sticky overhangs that anneal side-specifically with the synthetic ITR oligo's
δ
γ
β
α
70
70
20
AvrII site
60
50
40
30

FIG. 9

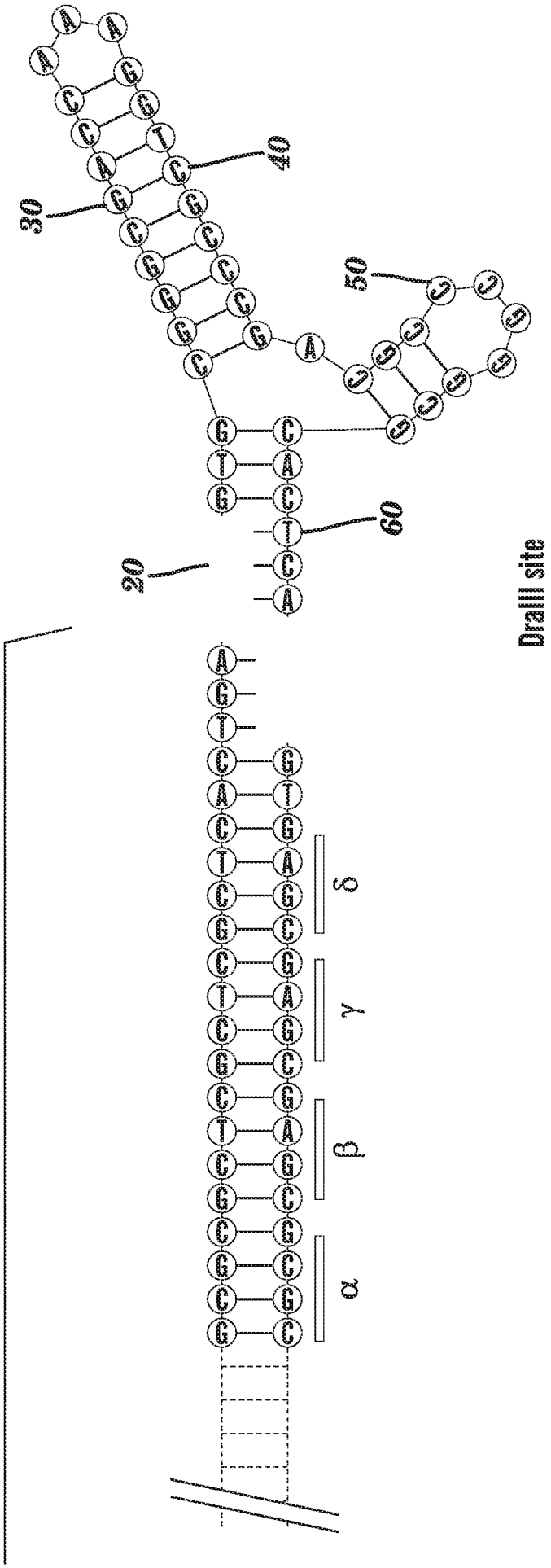


FIG. 9 (cont.)

FIG. 10B

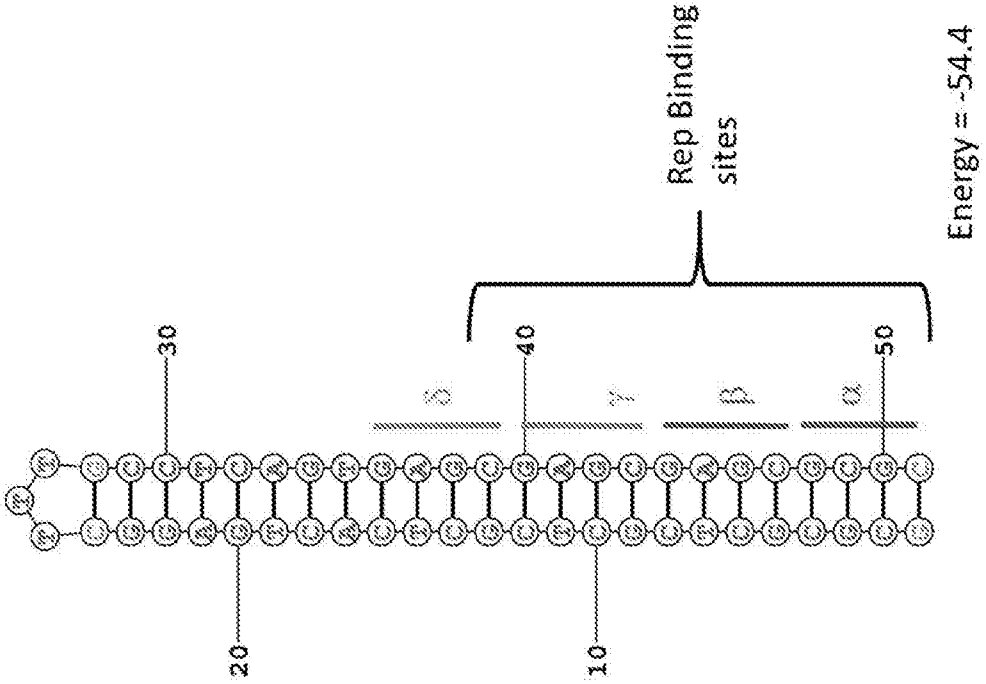
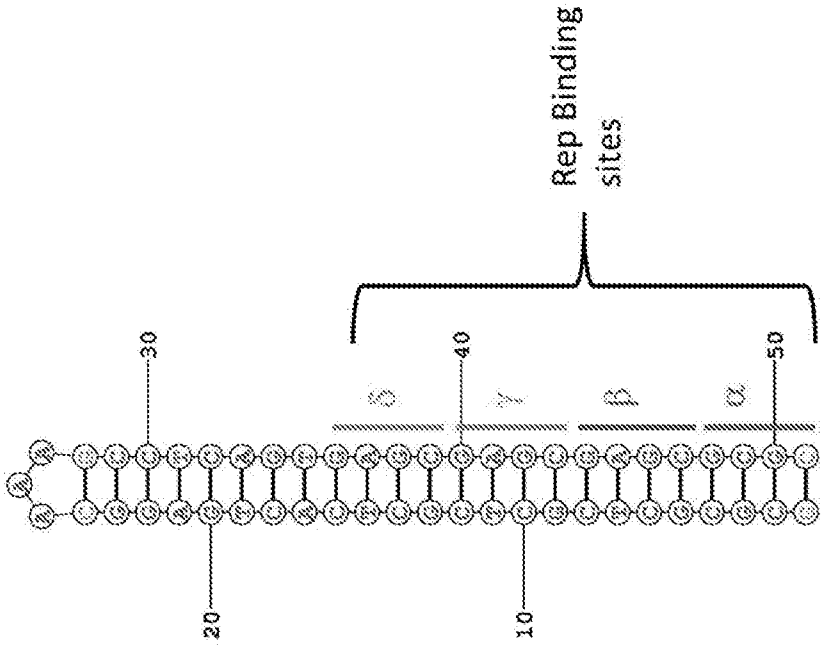


FIG. 10A



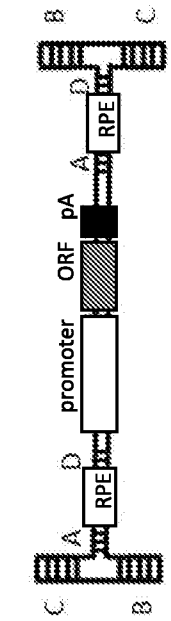


FIG. 11A

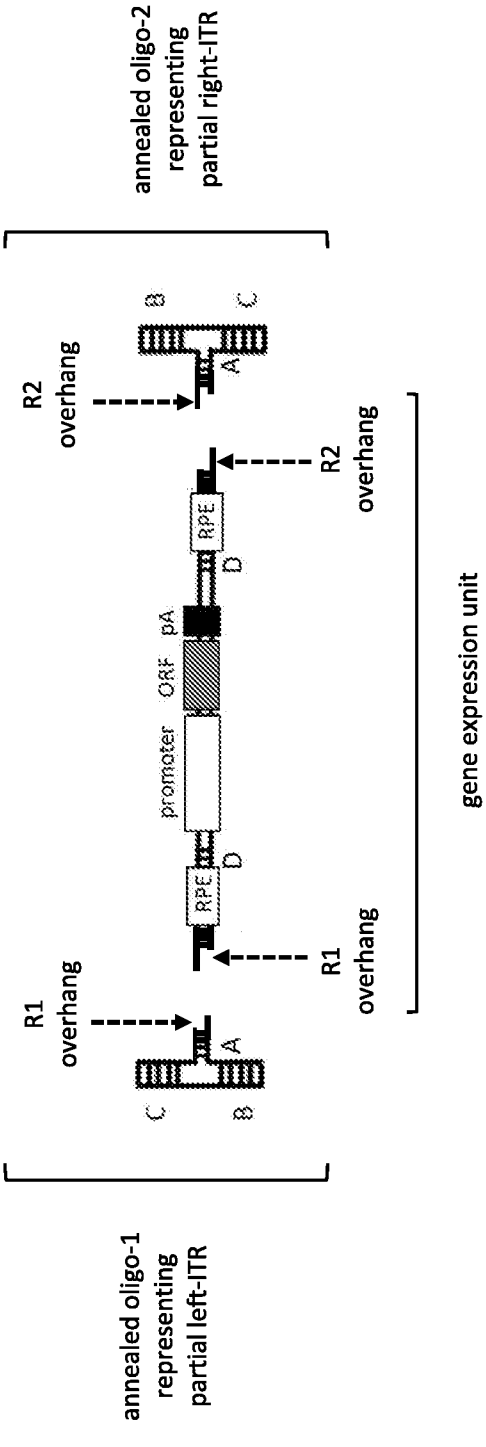
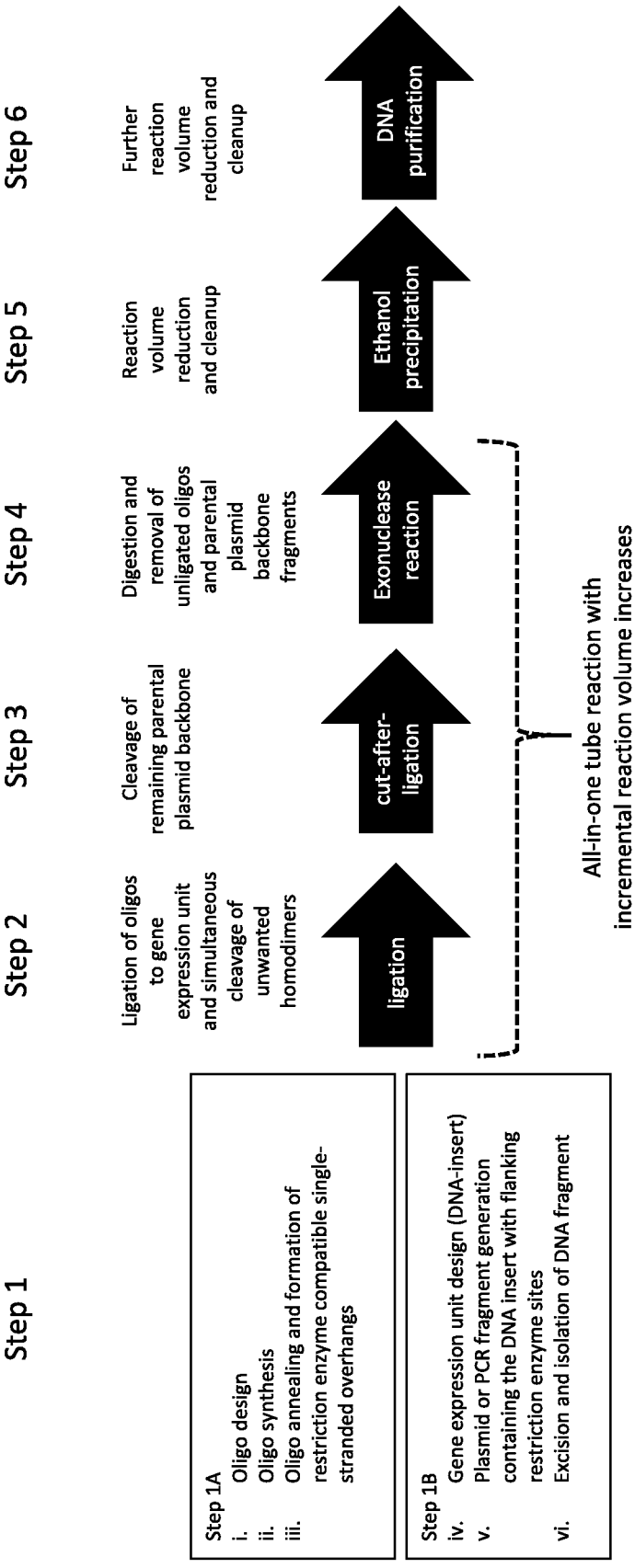


FIG. 11B

FIG. 12


Step 1
Step 2
Step 3
Step 4
Step 5
Step 6
Step 1A
i. Oligo design
ii. Oligo synthesis
iii. Oligo annealing and formation of restriction enzyme compatible single-stranded overhangs
Step 1B
iv. Gene expression unit design (DNA-insert)
v. Plasmid or PCR fragment generation containing the DNA insert with flanking restriction enzyme sites
vi. Excision and isolation of DNA fragment
ligation
Ligation of oligos to gene expression unit and simultaneous cleavage of unwanted homodimers
cut-after-ligation
Cleavage of remaining parental plasmid backbone
Exonuclease reaction
Digestion and removal of unligated oligos and parental plasmid backbone fragments
Ethanol precipitation
Reaction volume reduction and cleanup
DNA purification
Further reaction volume reduction and cleanup
All-in-one tube reaction with incremental reaction volume increases

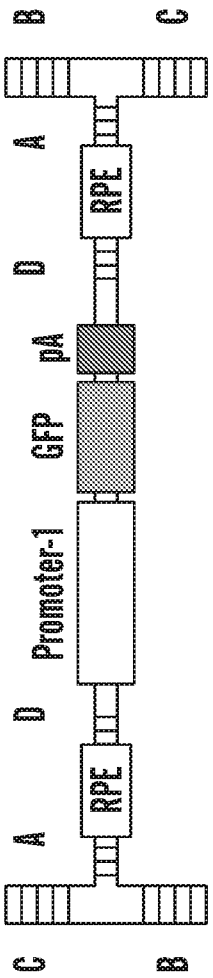


FIG. 13A

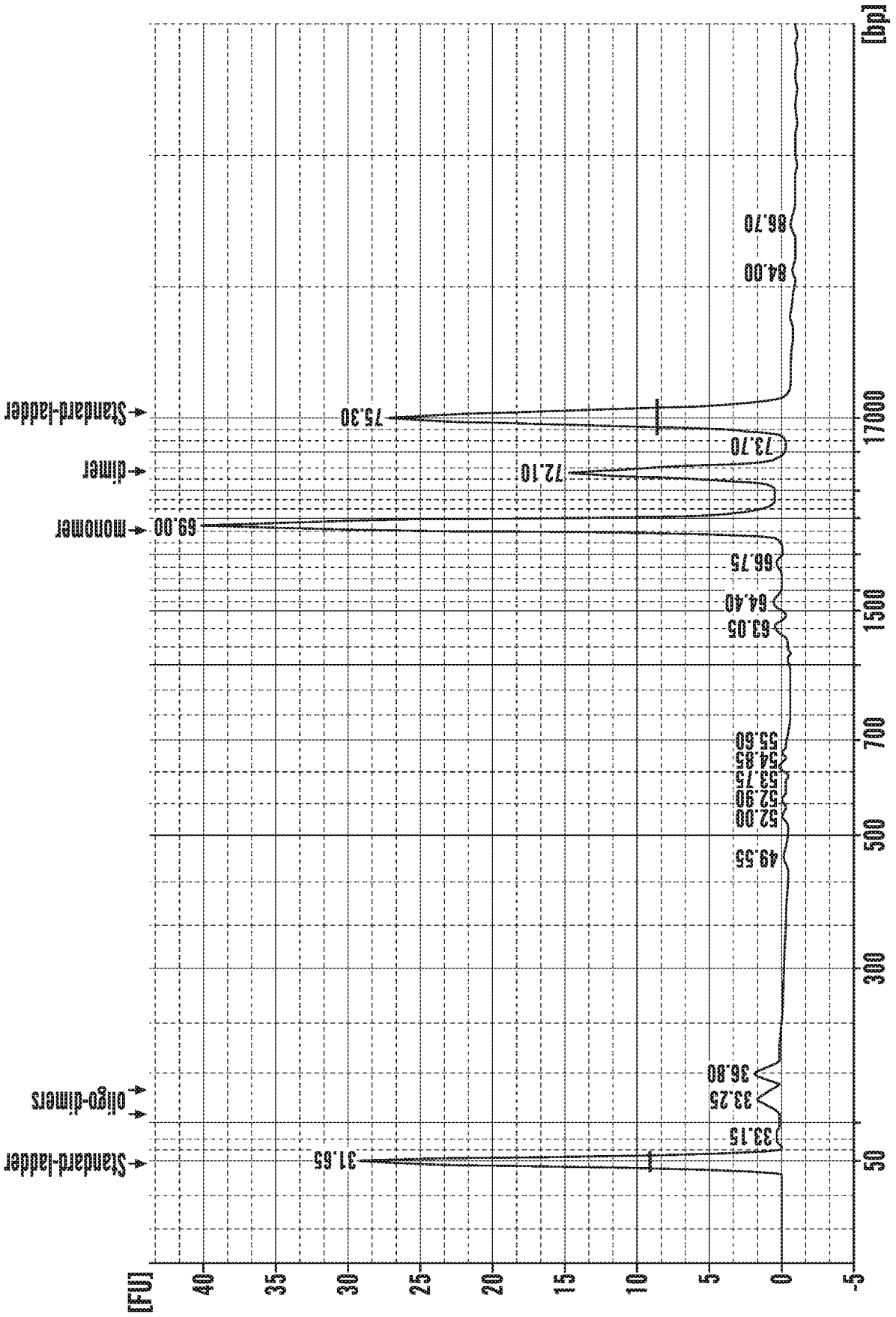

[bp]
[FU]
Standard-ladder
oligo-dimers
Standard-ladder
dimer
monomer
Standard-ladder
31.65
33.15
33.25
36.80
49.55
52.00
52.90
53.75
54.85
55.60
63.05
64.40
66.75
69.00
72.10
73.70
75.30
84.00
86.70

FIG. 13B

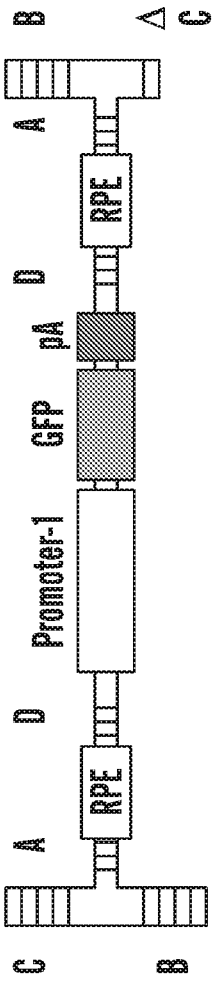


FIG. 14A

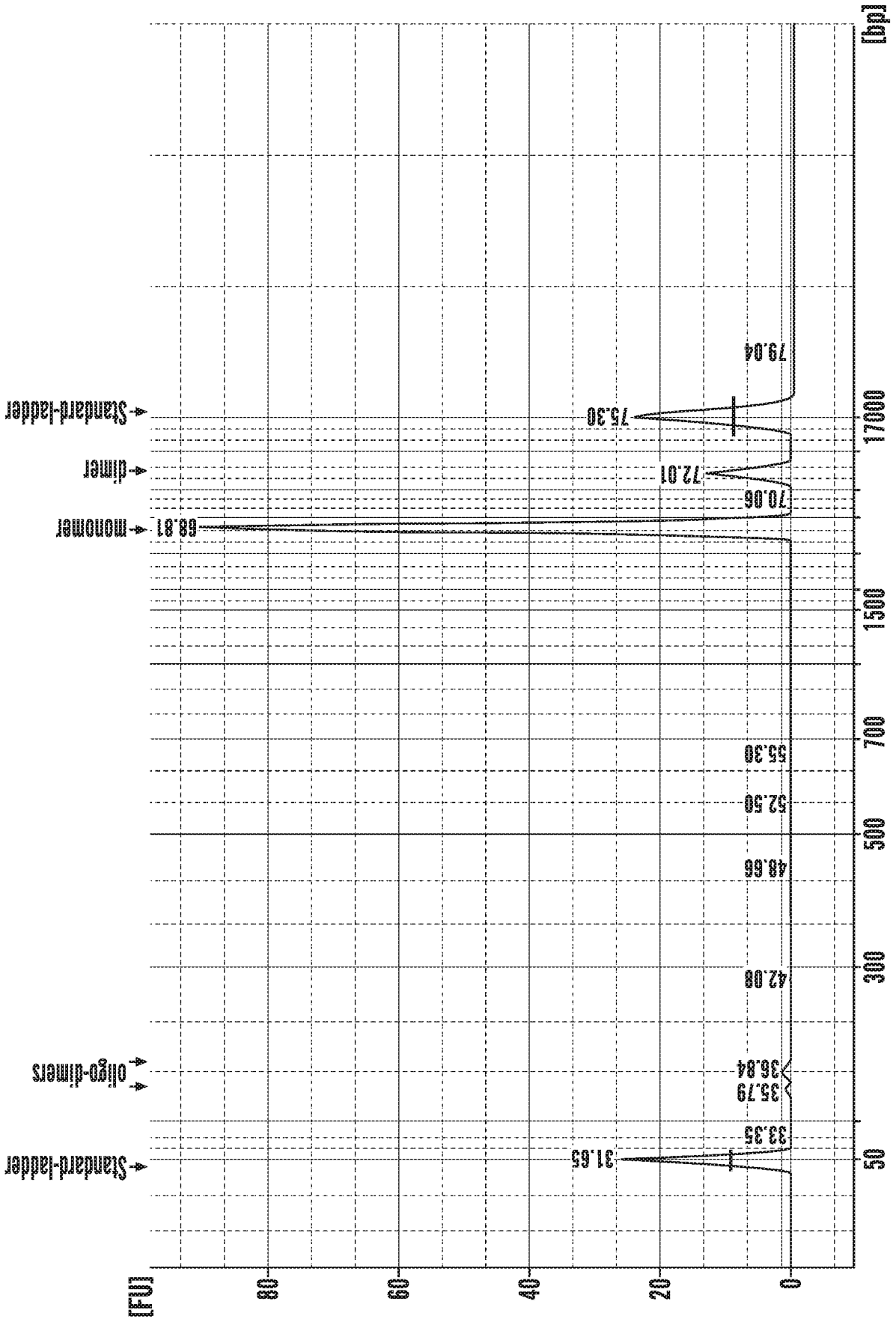

[FU]
0
20
40
60
80
[bp]
50
300
500
700
1500
17000
Standard-ladder
oligo-dimers
Standard-ladder
monomer
dimer
Standard-ladder
31.65
33.35
35.79
36.84
42.08
48.66
52.50
55.30
68.81
70.06
72.01
75.30
79.04

FIG. 14B

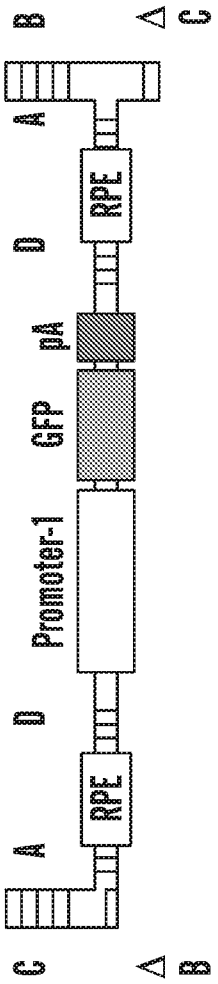


FIG. 15A

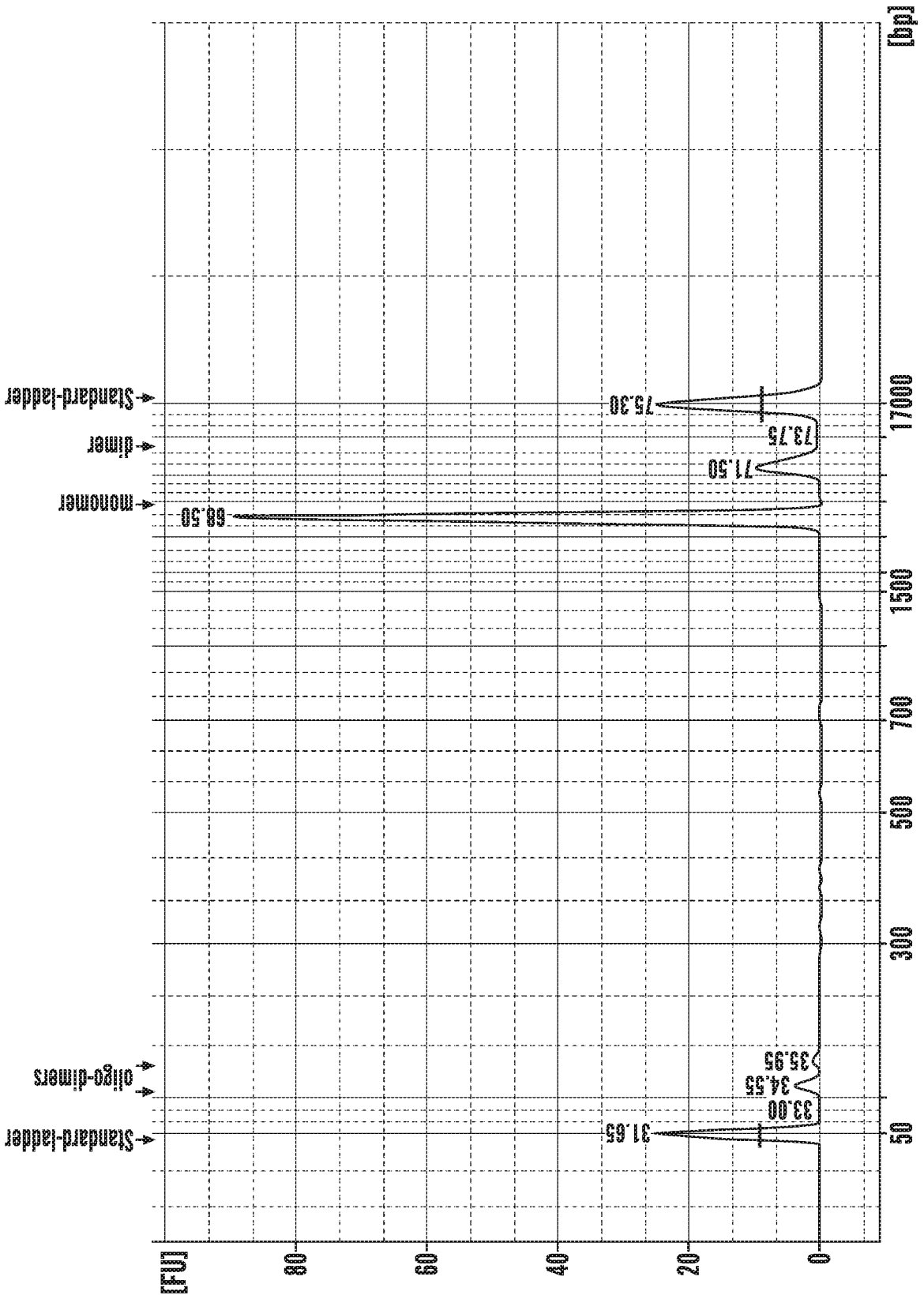

[FU]
0
20
40
60
80
[bp]
50
300
500
700
1500
17000
Standard-ladder
oligo-dimers
Standard-ladder
dimer
monomer
31.65
33.00
34.55
35.95
68.50
71.50
73.75
75.30

FIG. 15B

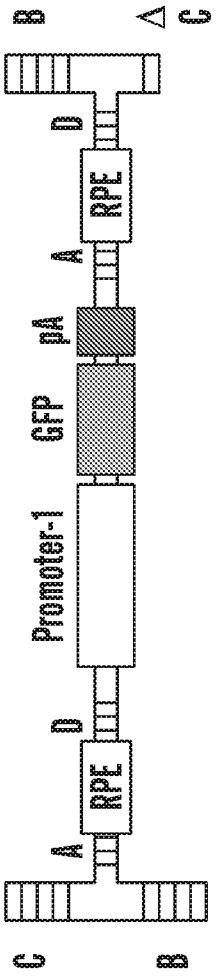


FIG. 16A

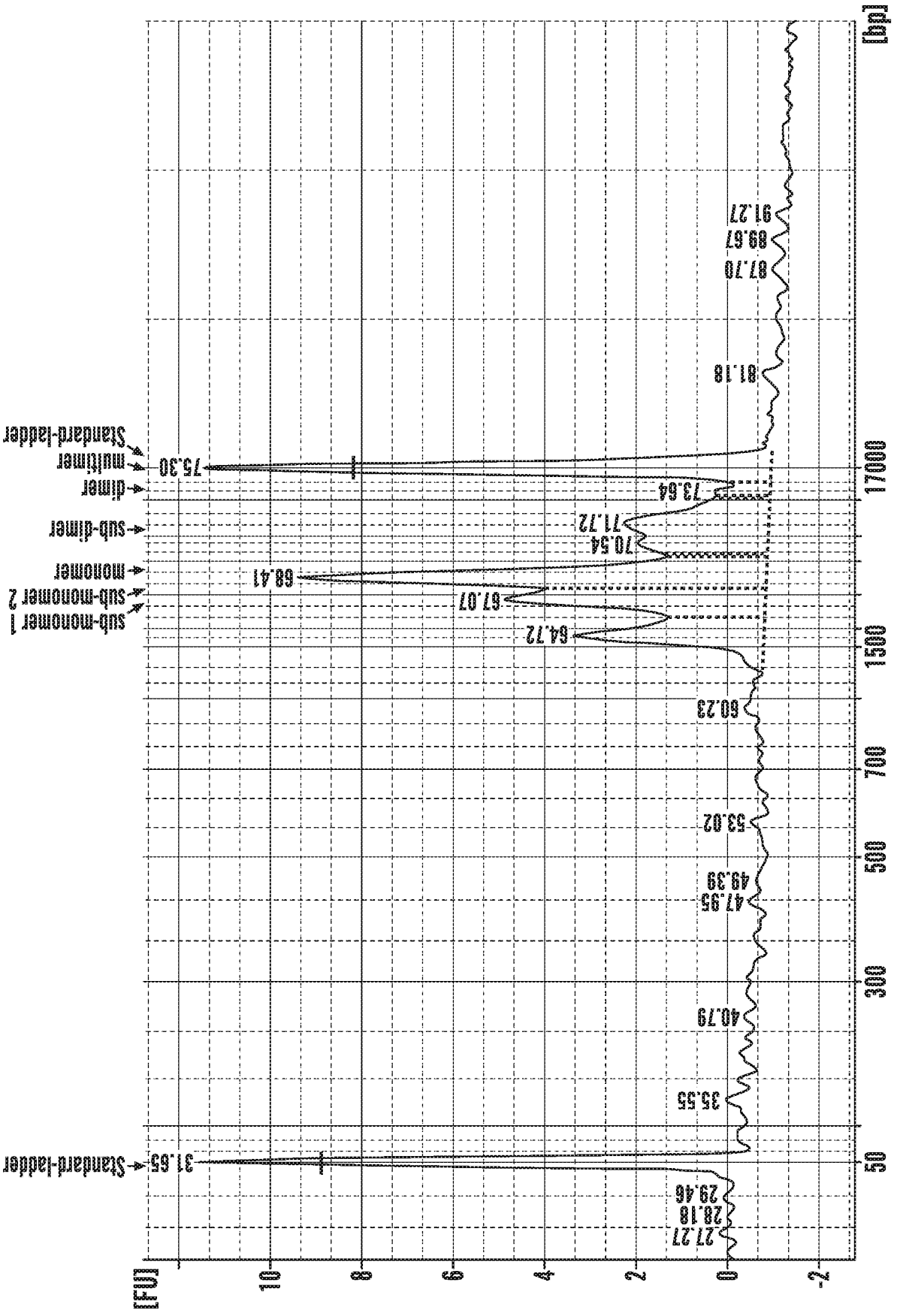
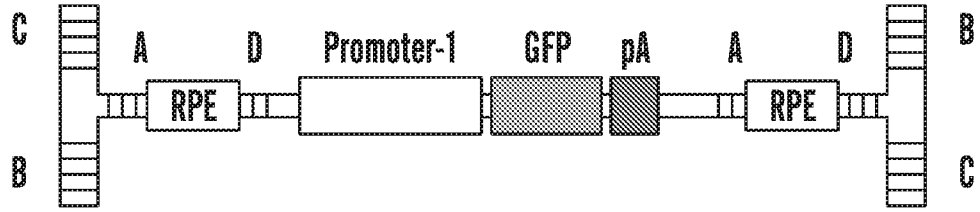

[FAU]
10
8
6
4
2
0
-2
50
300
500
700
1500
17000
[bp]
27.27
28.18
29.46
31.65
35.55
40.79
47.95
49.39
53.02
60.23
64.72
67.07
68.41
70.54
71.72
73.64
75.30
81.18
87.70
89.57
91.27
Standard-ladder
dimer
multimer
Standard-ladder
sub-dimer
monomer
sub-monomer 1
sub-monomer 2

FIG. 16B



Synthetic eGFP-ceDNA [wt/wt]

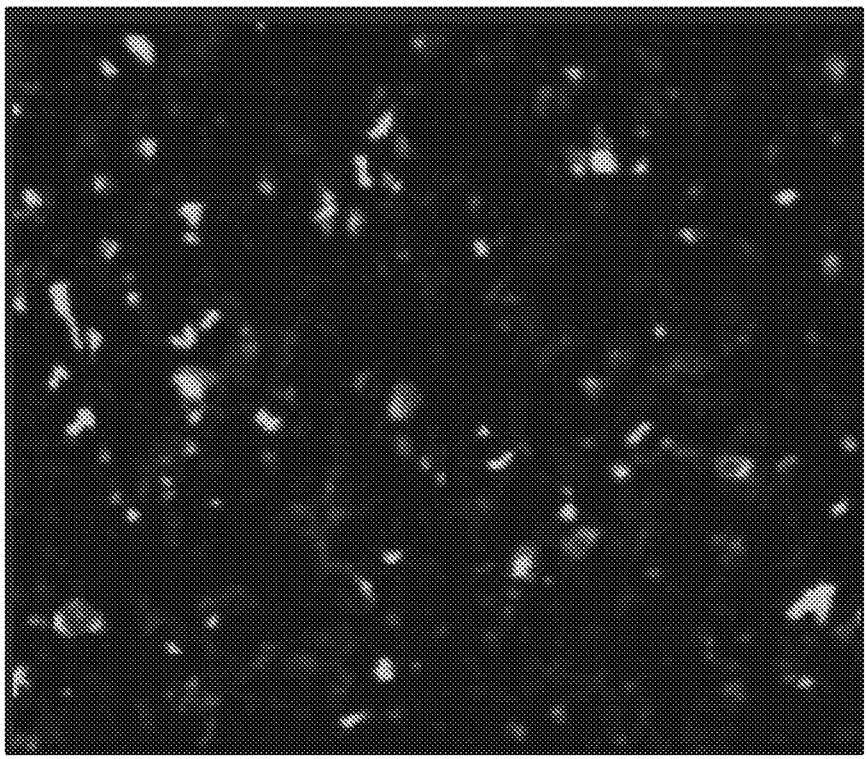
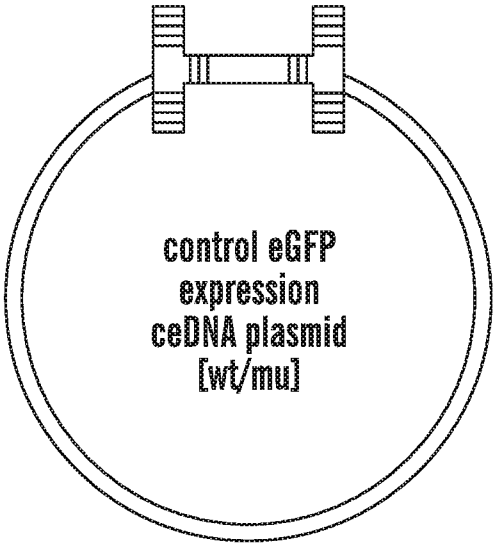


FIG. 17



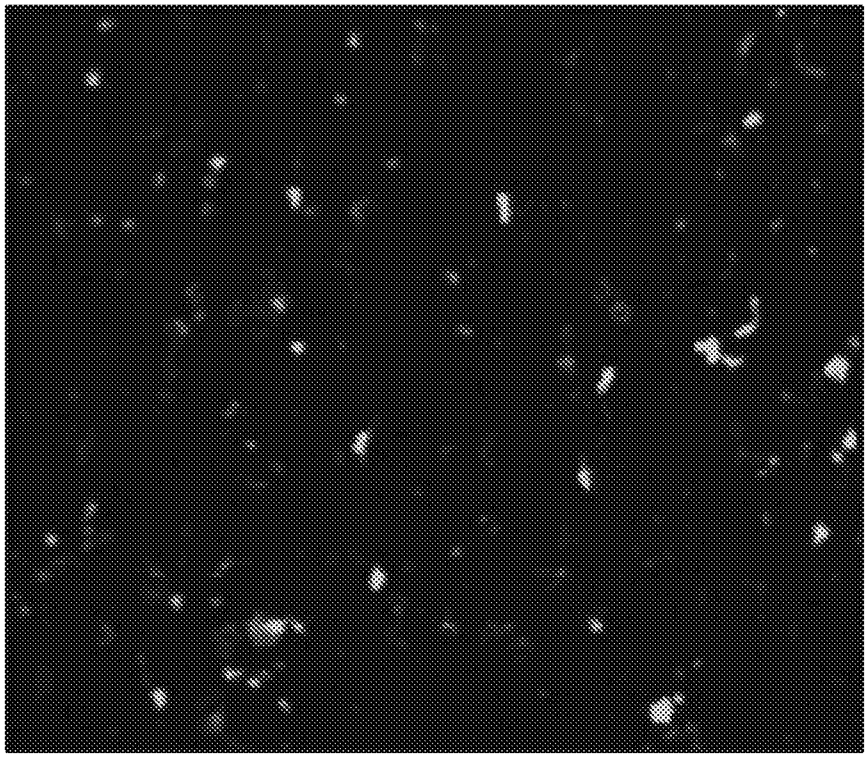
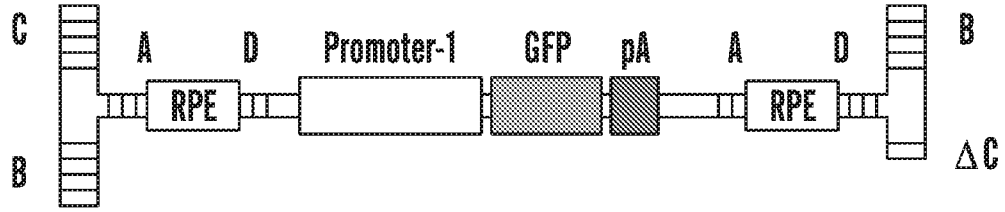


FIG. 17 (cont.)


C
A
D
Promoter-1
GFP
pA
A
D
B
RPE
RPE
B
ΔC

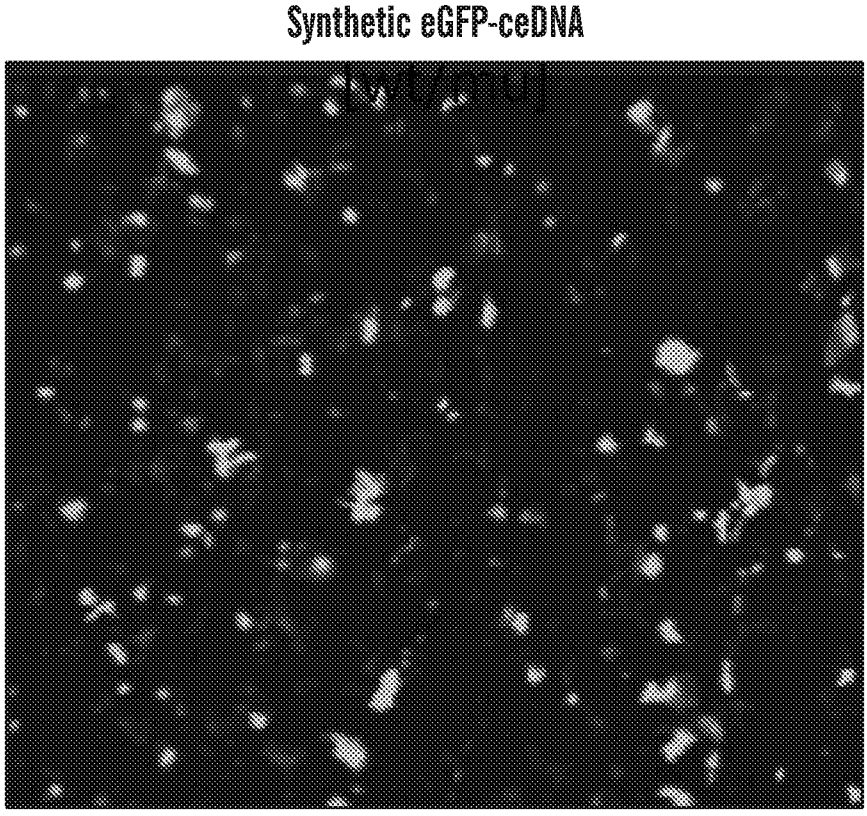
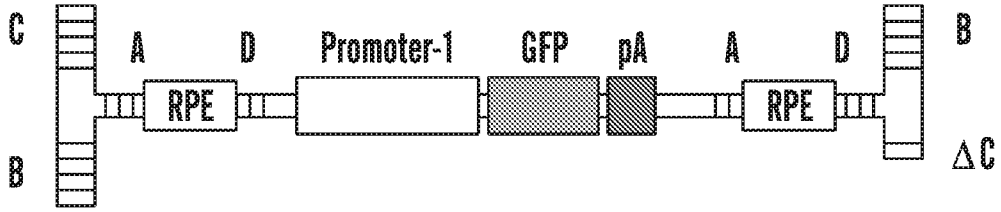

Synthetic eGFP-ceDNA
[WT/ITR]

FIG. 17 (cont.)



Control SF9-derived eGFP-ceDNA [wt/mu]

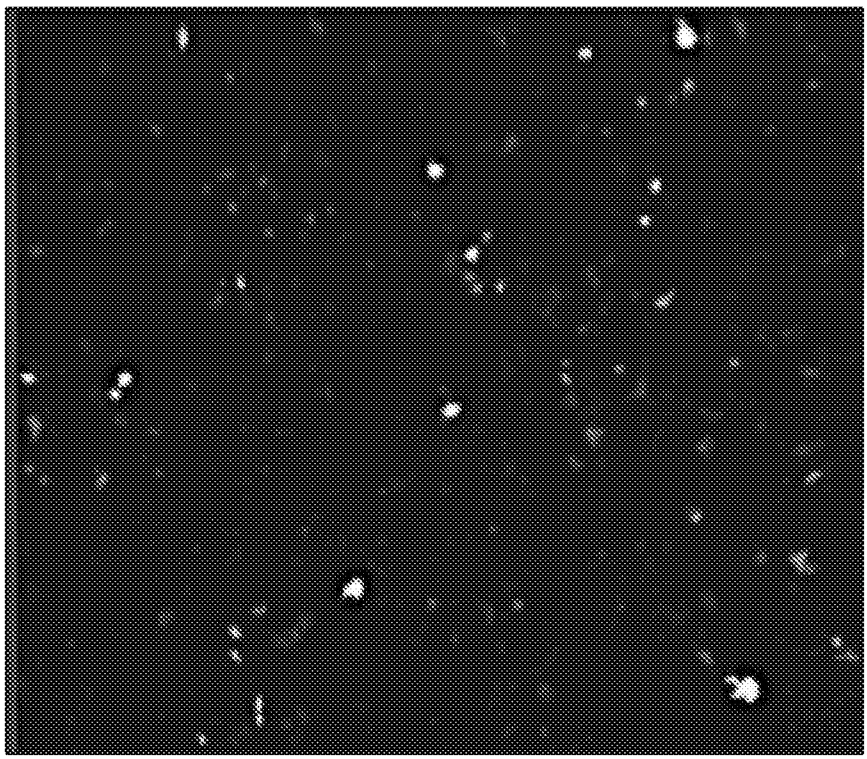
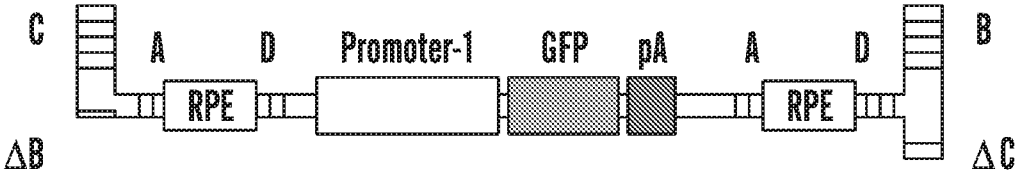


FIG. 17 (cont.)



Synthetic eGFP-ceDNA [mu/mu]

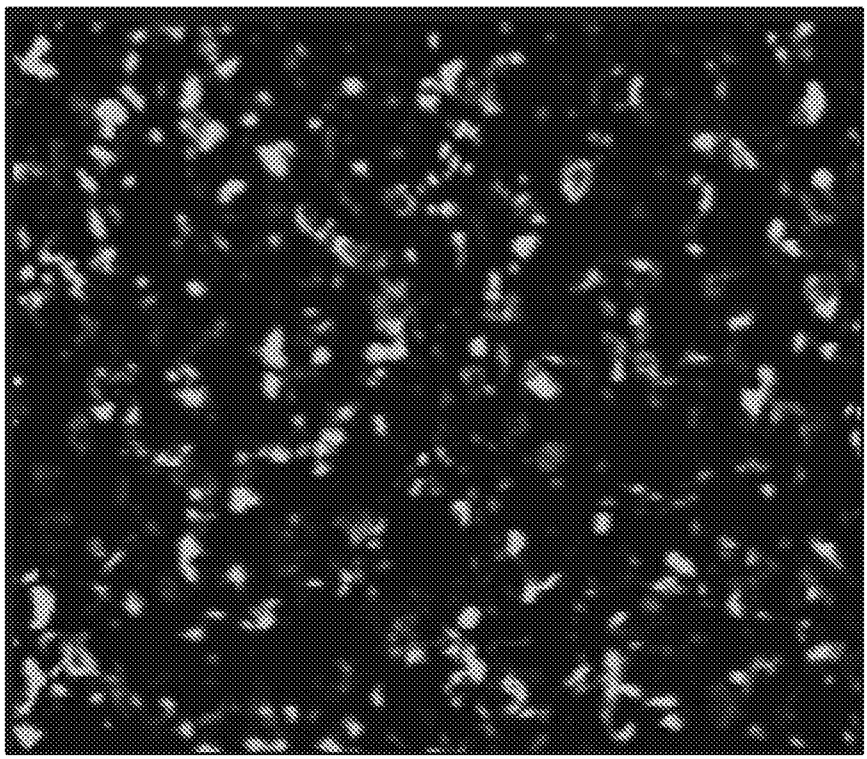


FIG. 17 (cont.)

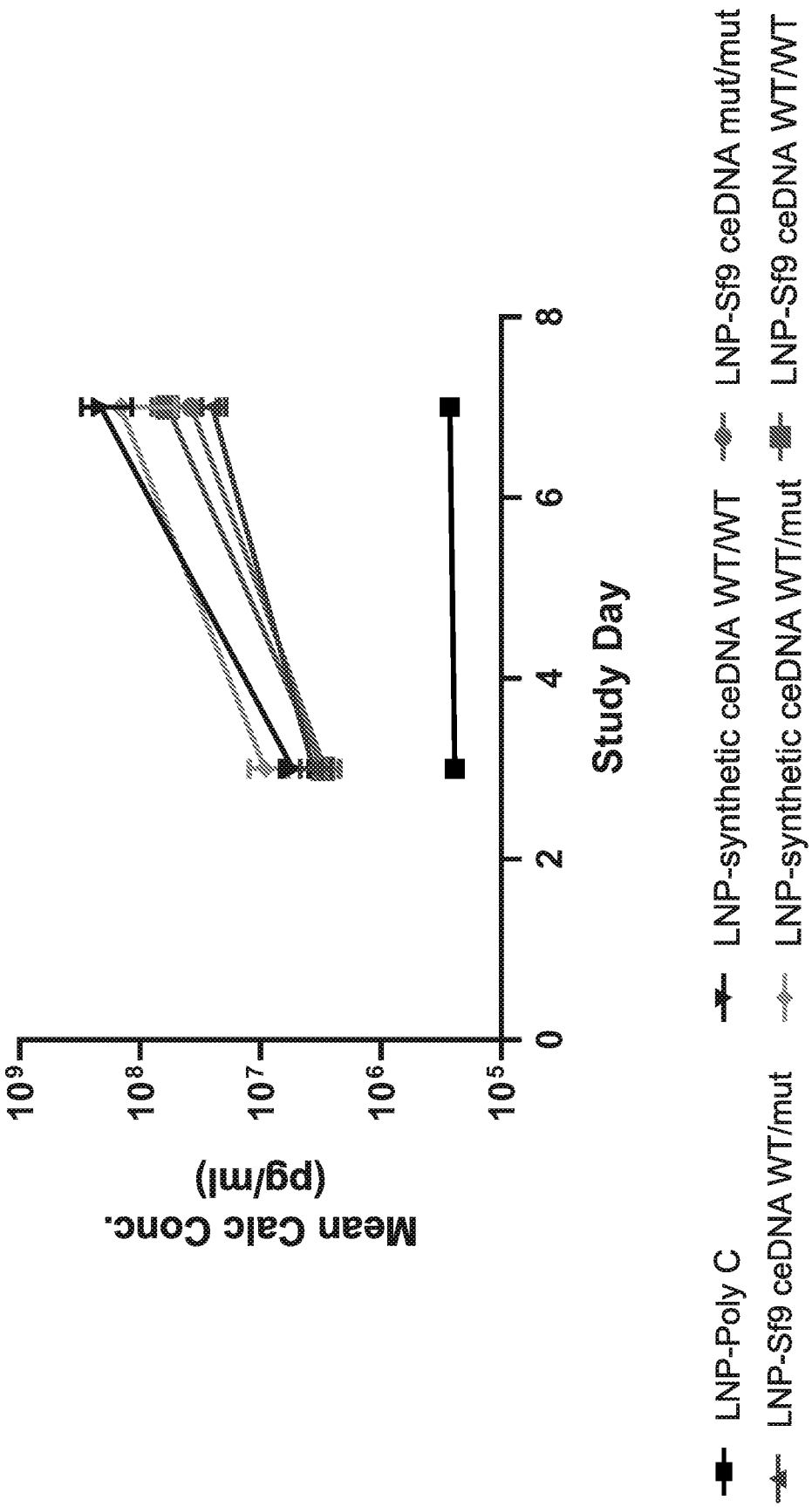

Mean Calc Conc. (pg/ml)
10^9
10^8
10^7
10^6
10^5
0
2
4
6
8
Study Day
LNP-Poly C
LNP-Sf9 ceDNA WT/mut
LNP-synthetic ceDNA WT/WT
LNP-synthetic ceDNA WT/mut
LNP-Sf9 ceDNA mut/mut
LNP-Sf9 ceDNA WT/WT

FIG. 18A

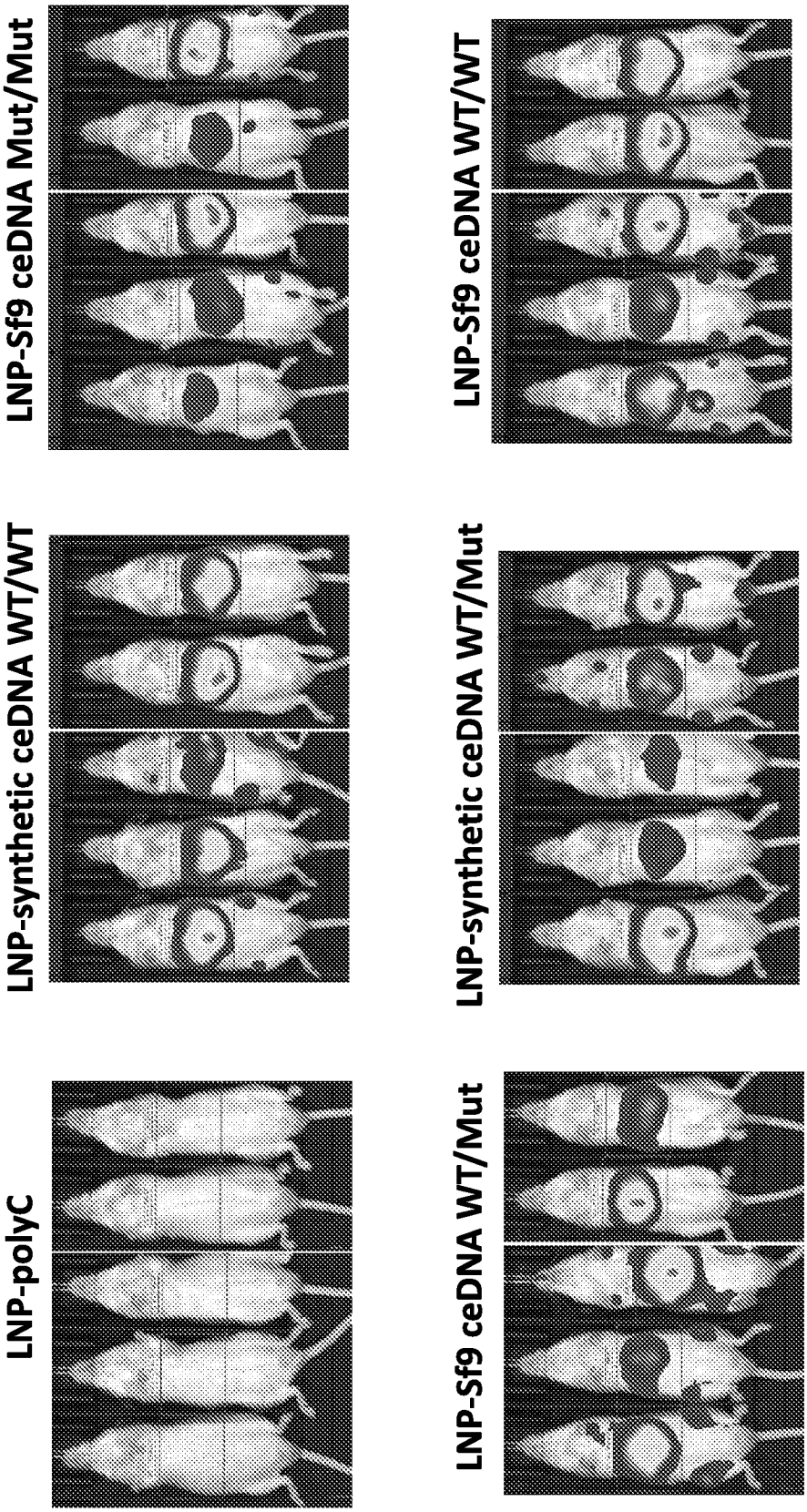

LNP-polyC
LNP-synthetic ceDNA WT/WT
LNP-Sf9 ceDNA Mut/Mut
LNP-Sf9 ceDNA WT/Mut
LNP-synthetic ceDNA WT/Mut
LNP-Sf9 ceDNA WT/WT

FIG. 18B