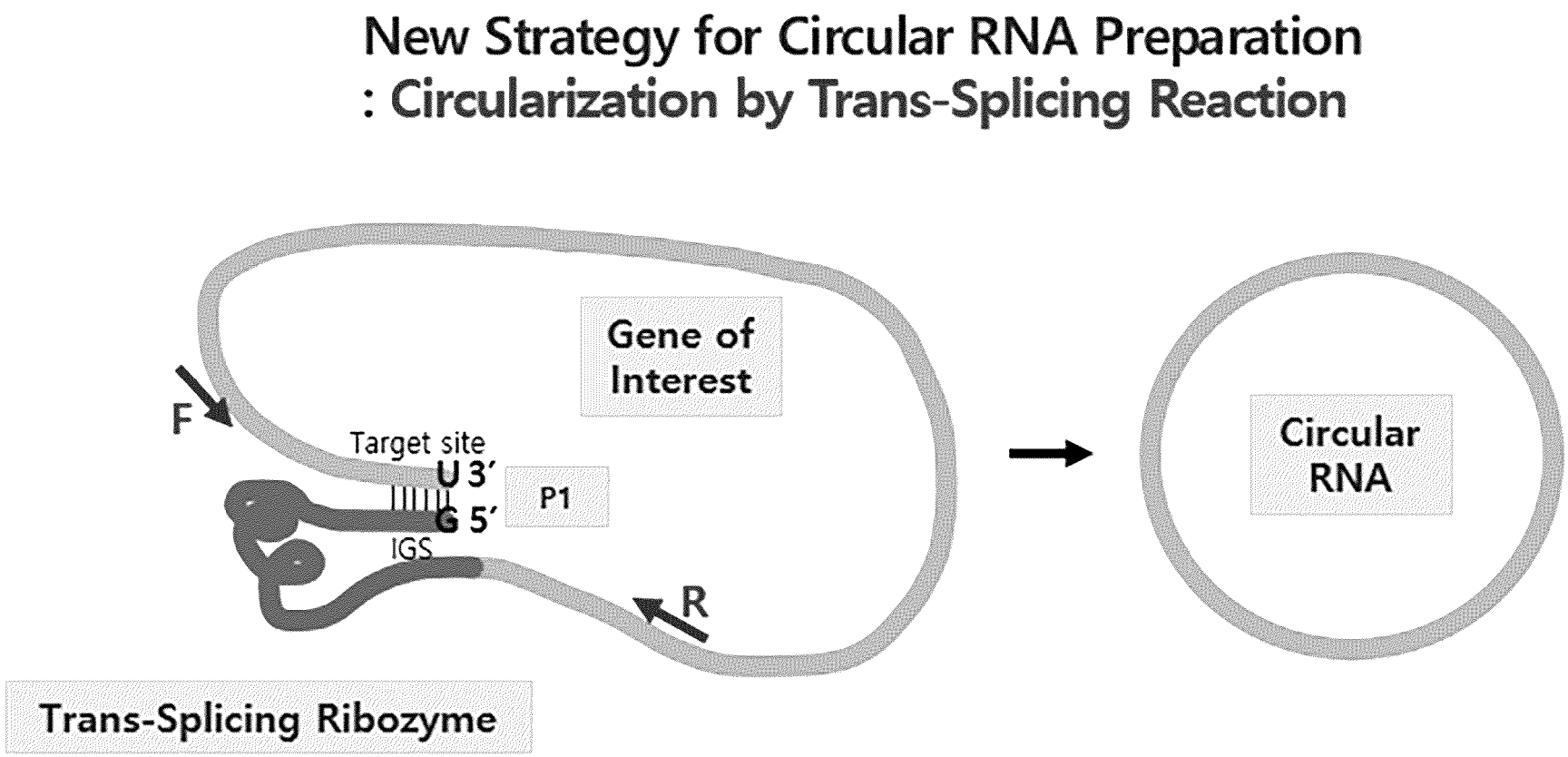

New Strategy for Circular RNA Preparation
: Circularization by Trans-Splicing Reaction
Gene of Interest
F
Target site
U 3′
G 5′
P1
IGS
R
Circular RNA
Trans-Splicing Ribozyme

FIG. 1A

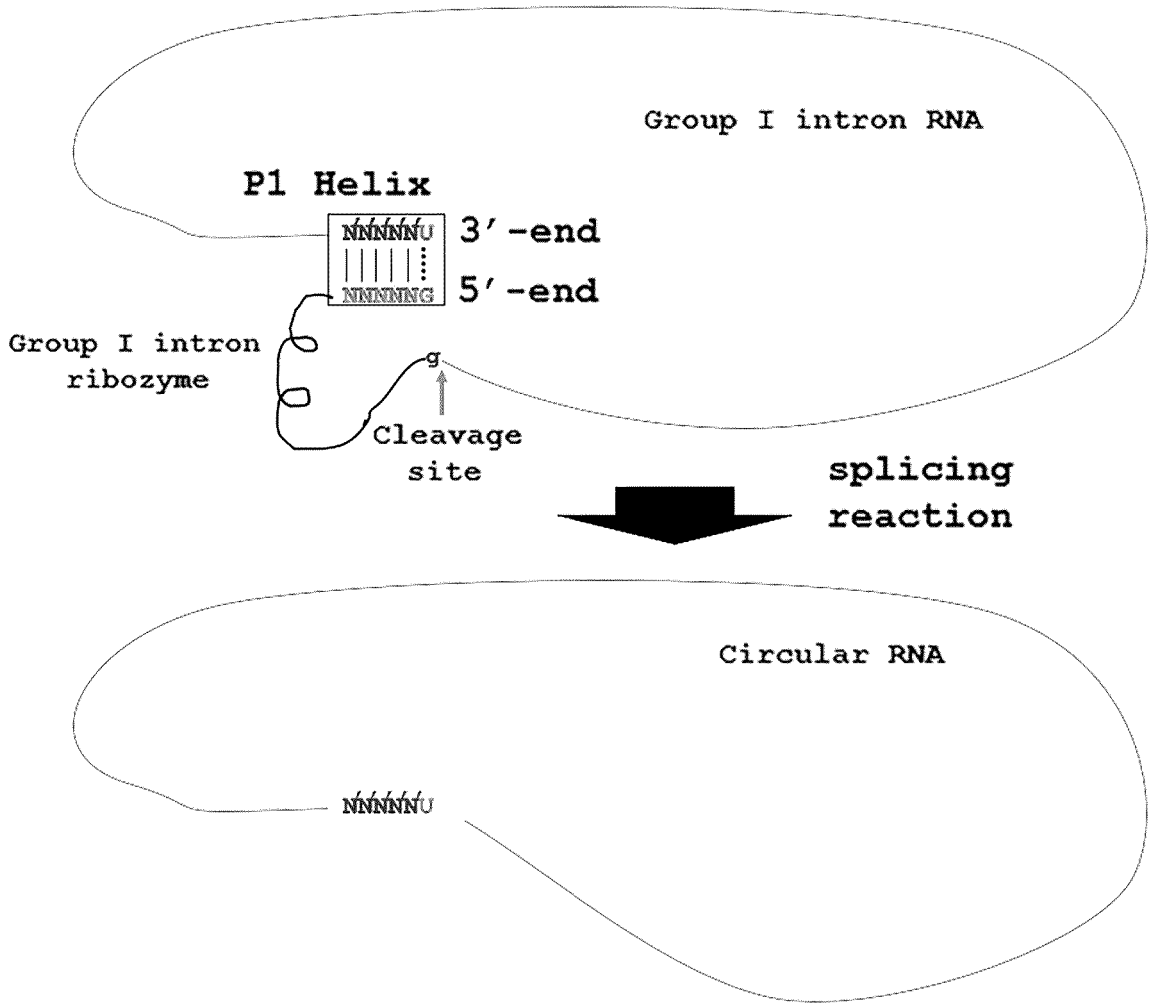

Group I intron RNA
P1 Helix
NNNNNU
NNNNNG
3′-end
5′-end
Group I intron ribozyme
g
Cleavage site
splicing reaction
Circular RNA
NNNNNU

FIG. 1B

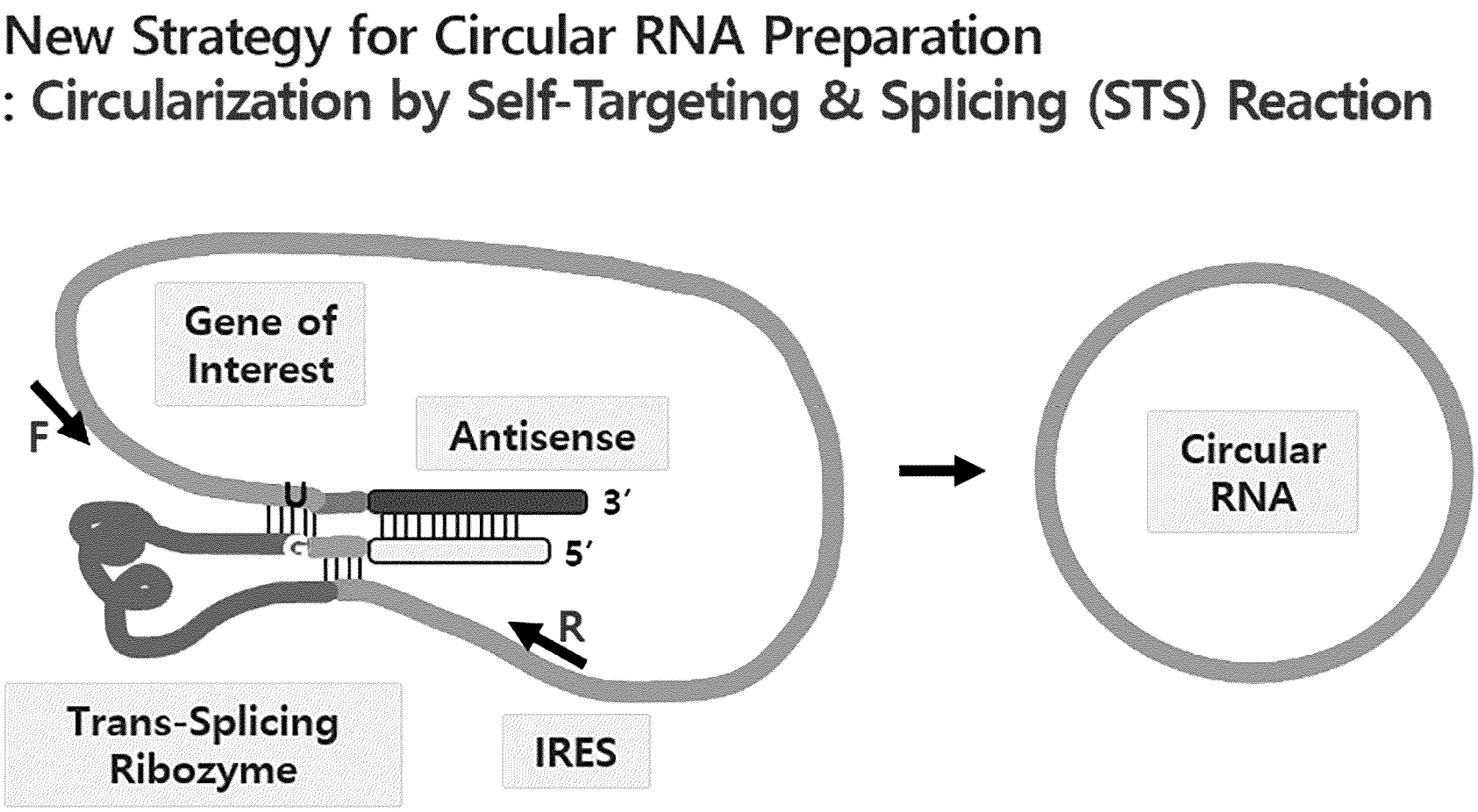

New Strategy for Circular RNA Preparation
: Circularization by Self-Targeting & Splicing (STS) Reaction
Gene of Interest
F
Antisense
U
3′
5′
R
Trans-Splicing Ribozyme
IRES
Circular RNA

FIG. 2A

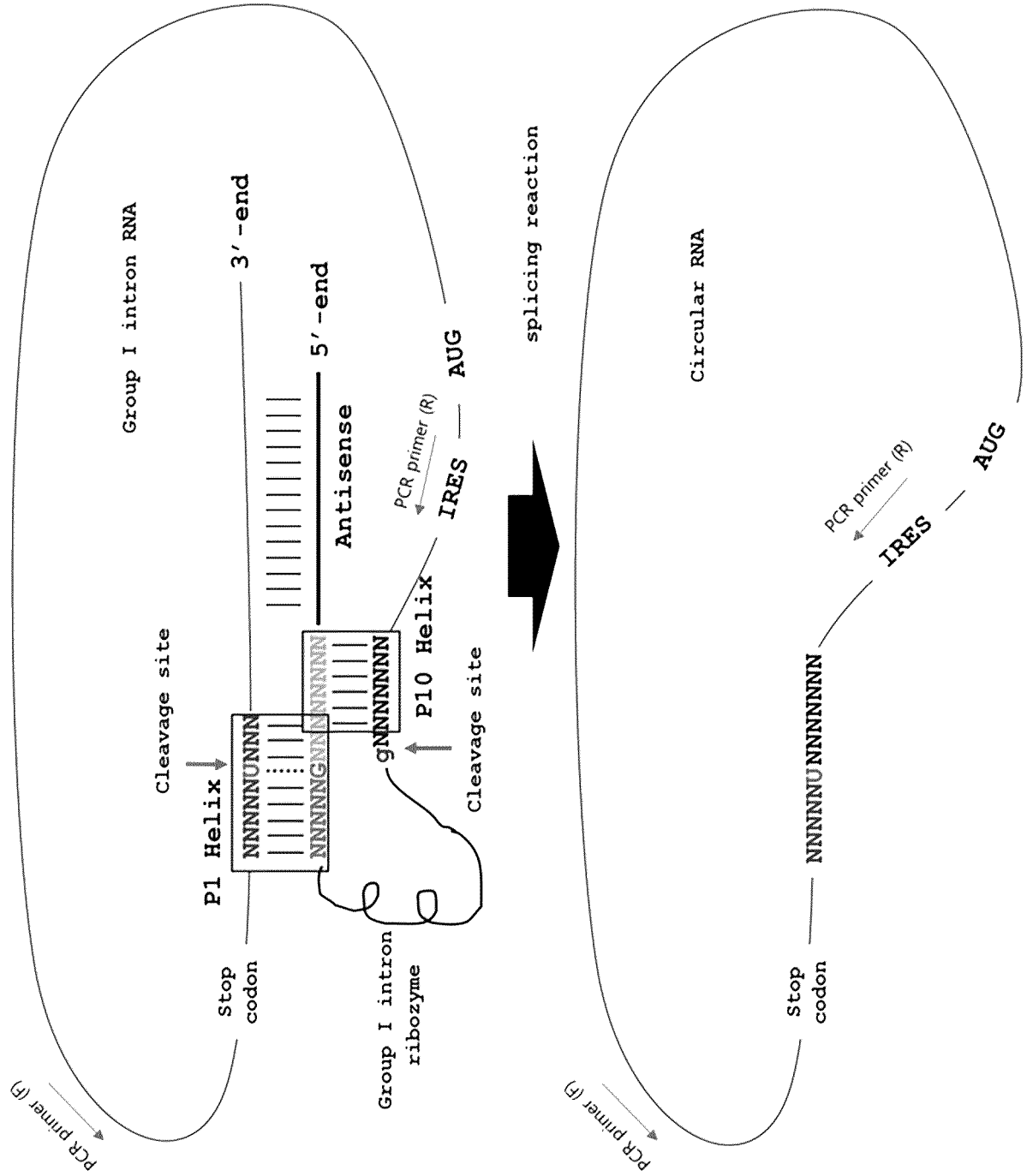

Group I intron RNA
3′-end
5′-end
Antisense
Cleavage site
P1 Helix
NNNNNUNNN
NNNNNGNNNNNN
gNNNNNNNN
P10 Helix
Cleavage site
PCR primer (R)
IRES
AUG
Stop codon
Group I intron ribozyme
PCR primer (F)
splicing reaction
Circular RNA
PCR primer (R)
IRES
AUG
NNNNNUNNNNNNNN
Stop codon
PCR primer (F)

FIG. 2B

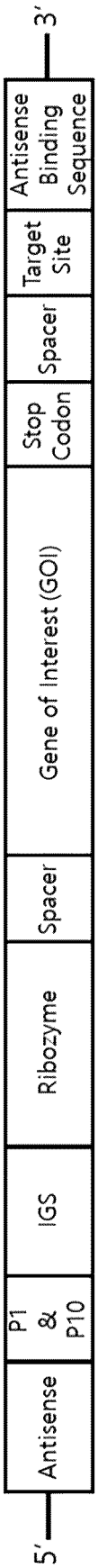

5′
Antisense
P1 & P10
IGS
Ribozyme
Spacer
Gene of Interest (GOI)
Stop Codon
Spacer
Target Site
Antisense Binding Sequence
3′

FIG. 2C

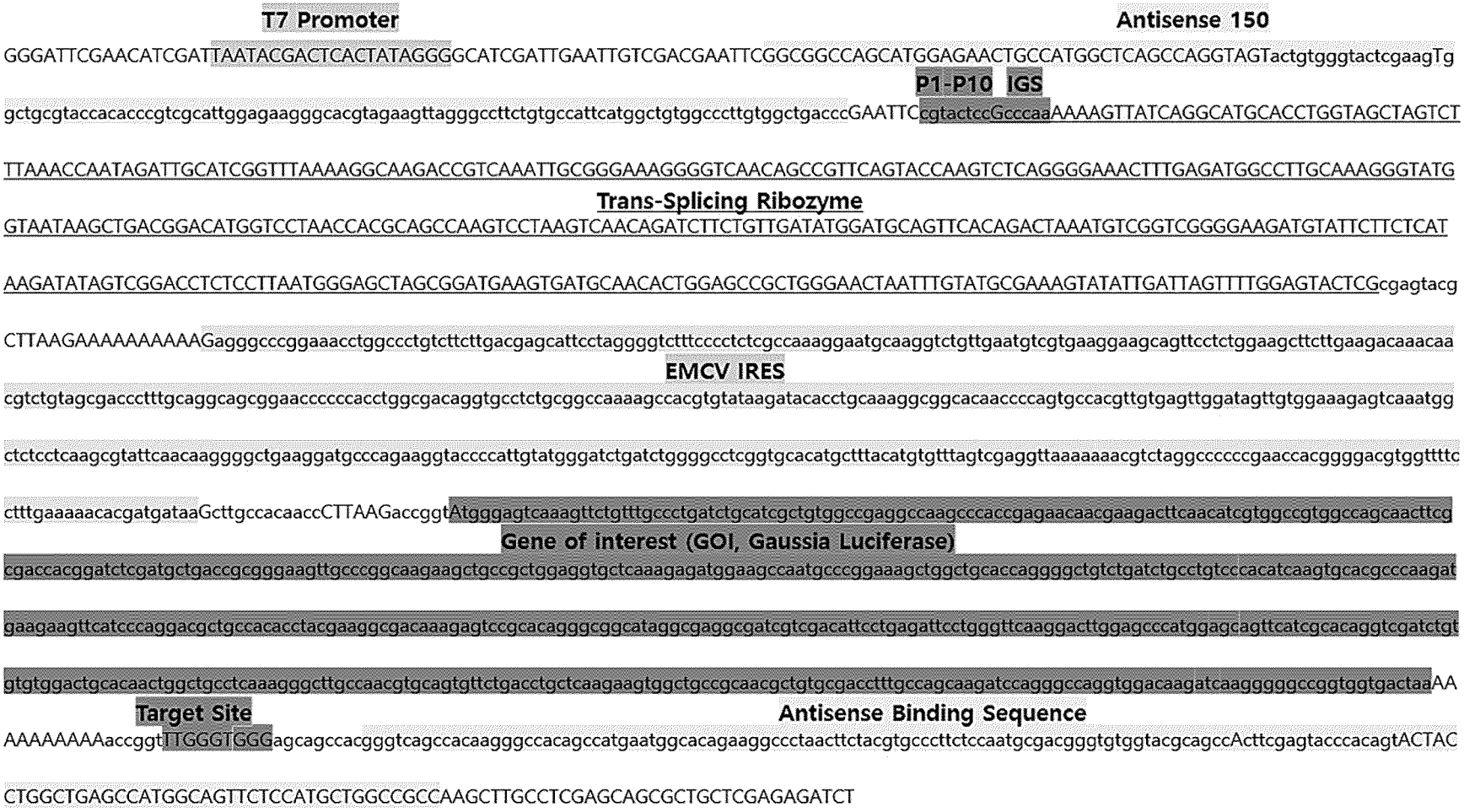

T7 Promoter
Antisense 150
GGGATTCGAACATCGATTAATACGACTCACTATAGGGGCATCGATTGAATTGTCGACGAATTCGGCGGCCAGCATGGAGAACTGCCATGGCTCAGCCAGGTAGTactgtgggtactcgaagTg
P1-P10
IGS
gctgcgtaccacacccgtcgcattggagaagggcacgtagaagttagggccttctgtgccattcatggctgtggcccttgtggctgacccGAATTCcgtactccGcccaaAAAAGTTATCAGGCATGCACCTGGTAGCTAGTCT
TTAAACCAATAGATTGCATCGGTTTAAAAGGCAAGACCGTCAAATTGCGGGAAAGGGGTCAACAGCCGTTCAGTACCAAGTCTCAGGGGAAACTTTGAGATGGCCTTGCAAAGGGTATG
Trans-Splicing Ribozyme
GTAATAAGCTGACGGACATGGTCCTAACCACGCAGCCAAGTCCTAAGTCAACAGATCTTCTGTTGATATGGATGCAGTTCACAGACTAAATGTCGGTCGGGGAAGATGTATTCTTCTCAT
AAGATATAGTCGGACCTCTCCTTAATGGGAGCTAGCGGATGAAGTGATGCAACACTGGAGCCGCTGGGAACTAATTTGTATGCGAAAGTATATTGATTAGTTTTGGAGTACTCGcgagtacg
CTTAAGAAAAAAAAAAAGagggcccggaaacctggccctgtcttcttgacgagcattcctaggggtctttcccctctcgccaaaggaatgcaaggtctgttgaatgtcgtgaaggaagcagttcctctggaagcttcttgaagacaaacaa
EMCV IRES
cgtctgtagcgacccttgcaggcagcggaacccccacctggcgacaggtgcctctgcggccaaaagccacgtgtataagatacacctgcaaaggcggcacaaccccagtgccacgttgtgagttggatagttgtggaaagagtcaaatgg
ctctcctcaagcgtattcaacaaggggctgaaggatgcccagaaggtaccccattgtatgggatctgatctggggcctcggtgcacatgctttacatgtgtttagtcgaggttaaaaaaacgtctaggccccccgaaccacggggacgtggttttc
ctttgaaaaacacgatgataaGcttgccacaaccCTTAAGaccggtAtgggagtcaaagttctgtttgccctgatctgcatcgctgtggccgaggccaagcccaccgagaacaacgaagacttcaacatcgtggccgtggccagcaacttcg
Gene of interest (GOI, Gaussia Luciferase)
cgaccacggatctcgatgctgaccgcgggaagttgcccggcaagaagctgccgctggaggtgctcaaagagatggaagccaatgcccggaaagctggctgcaccaggggctgtctgatctgcctgtcccacatcaagtgcacgcccaagat
gaagaagttcatcccaggacgctgccacacctacgaaggcgacaaagagtccgcacagggcggcataggcgaggcgatcgtcgacattcctgagattcctgggttcaaggacttggagcccatggagcagttcatcgcacaggtcgatctgt
gtgtggactgcacaactggctgcctcaaagggcttgccaacgtgcagtgttctgacctgctcaagaagtggctgccgcaacgctgtgcgacctttgccagcaagatccagggccaggtggacaagatcaaggggccggtggtgactaaAA
Target Site
Antisense Binding Sequence
AAAAAAAAaccggtTTGGGTGGGagcagccacgggtcagccacaagggccacagccatgaatggcacagaaggccctaacttctacgtgcccttctccaatgcgacgggtgtggtacgcagccActtcgagtacccacagtACTAC
CTGGCTGAGCCATGGCAGTTCTCCATGCTGGCCGCCAAGCTTGCCTCGAGCAGCGCTGCTCGAGAGATCT

FIG. 2D

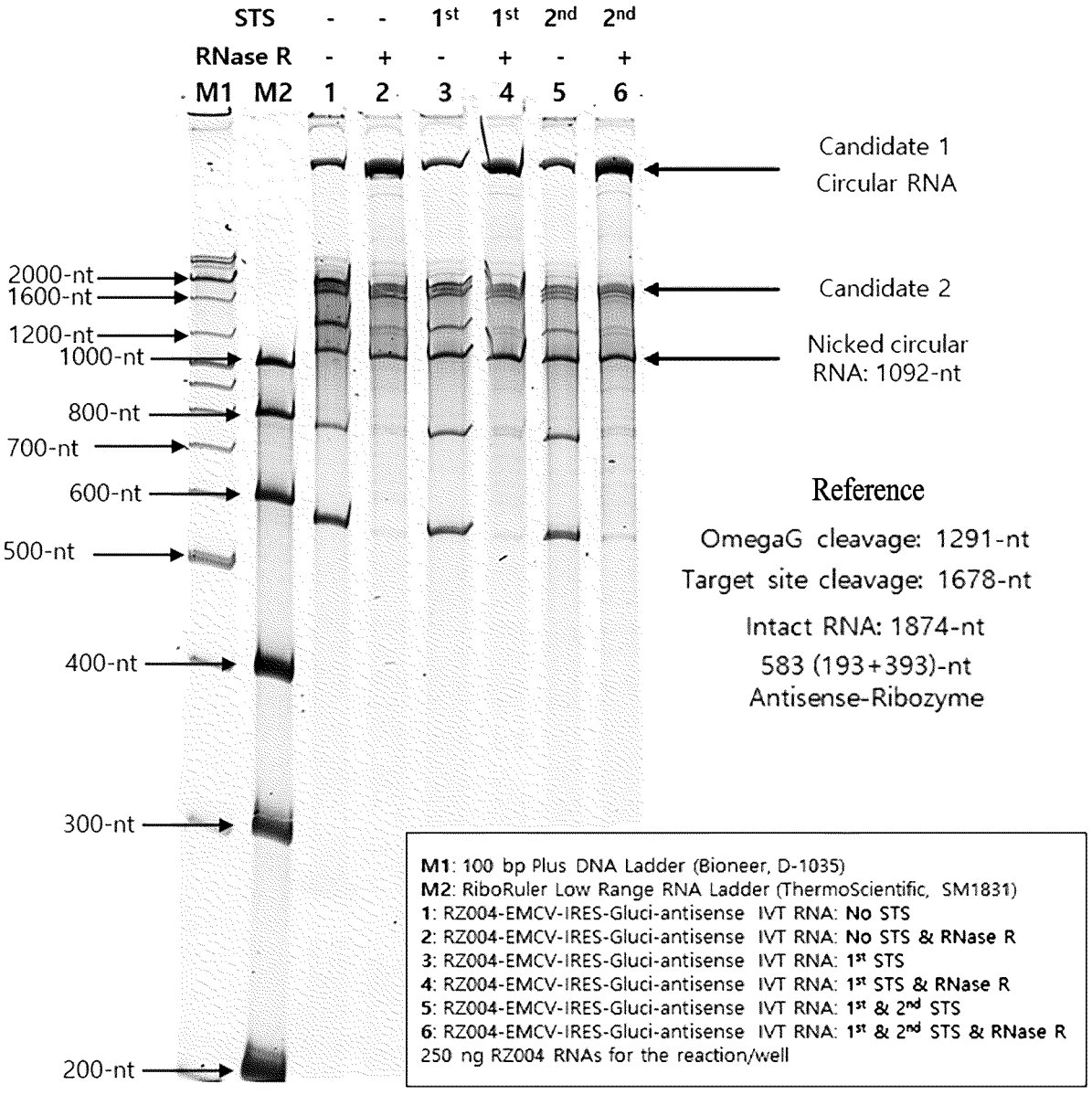
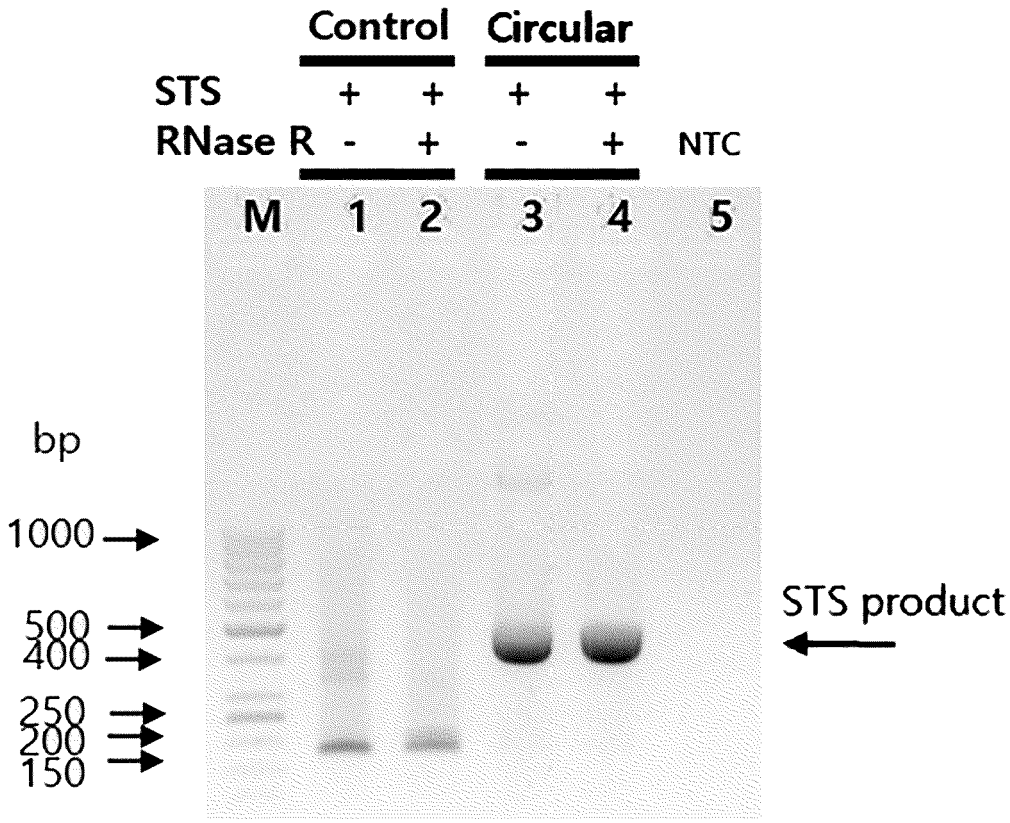

STS - - 1st 1st 2nd 2nd
RNase R - + - + - +
M1 M2 1 2 3 4 5 6
2000-nt
1600-nt
1200-nt
1000-nt
800-nt
700-nt
600-nt
500-nt
400-nt
300-nt
200-nt
Candidate 1
Circular RNA
Candidate 2
Nicked circular
RNA: 1092-nt
Reference
OmegaG cleavage: 1291-nt
Target site cleavage: 1678-nt
Intact RNA: 1874-nt
583 (193+393)-nt
Antisense-Ribozyme
M1: 100 bp Plus DNA Ladder (Bioneer, D-1035)
M2: RiboRuler Low Range RNA Ladder (ThermoScientific, SM1831)
1: RZ004-EMCV-IRES-Gluci-antisense IVT RNA: No STS
2: RZ004-EMCV-IRES-Gluci-antisense IVT RNA: No STS & RNase R
3: RZ004-EMCV-IRES-Gluci-antisense IVT RNA: 1st STS
4: RZ004-EMCV-IRES-Gluci-antisense IVT RNA: 1st STS & RNase R
5: RZ004-EMCV-IRES-Gluci-antisense IVT RNA: 1st & 2nd STS
6: RZ004-EMCV-IRES-Gluci-antisense IVT RNA: 1st & 2nd STS & RNase R
250 ng RZ004 RNAs for the reaction/well

FIG. 3



M: GenRuler 50 bp DNA Ladder (Therrmo Scientific, SM0373)
1: EMCV-IRES-Gluci-antisense IVT RNA Control + STS
2: EMCV-IRES-Gluci-antisense IVT RNA Control + STS & RNase R
3: RZ004-EMCV-IRES-Gluci-antisense IVT RNA: Direct STS
4: RZ004-EMCV-IRES-Gluci-antisense IVT RNA: Direct STS & RNase R
5: NTC

FIG. 4A

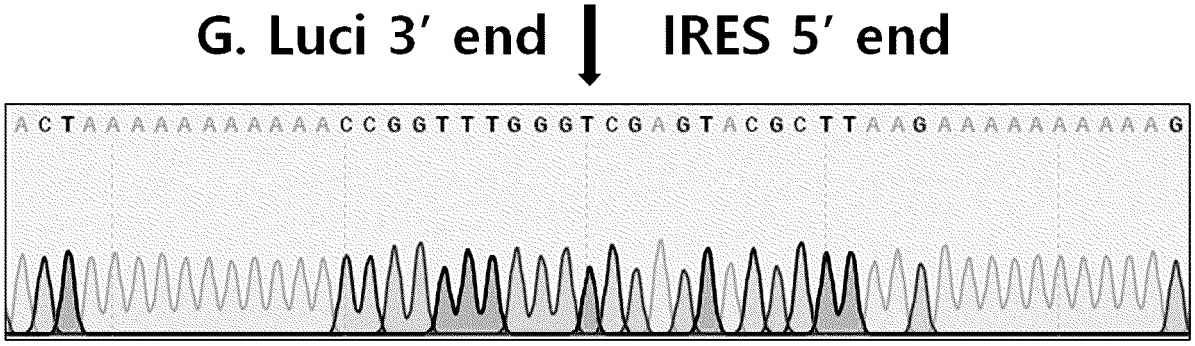
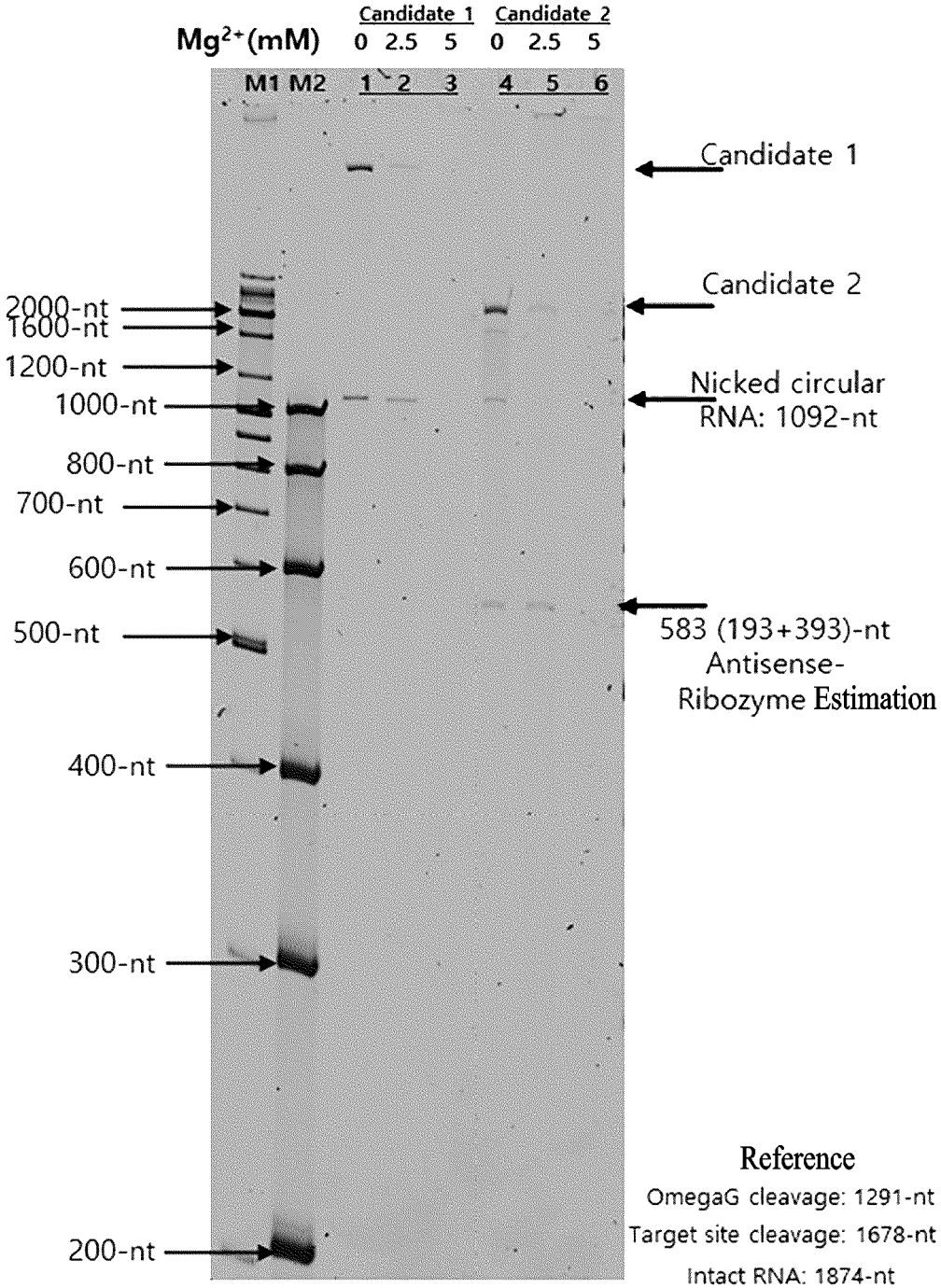

G. Luci 3′ end
IRES 5′ end
ACTAAAAAAAAAAACCGGTTTGGGTCGAGTACGCTTAAGAAAAAAAAAAAG

FIG. 4B



M1: 100 bp Plus DNA Ladder (Bioneer, D-1035)
M2: RiboRuler Low Range RNA Ladder (ThermoScientific, SM1831)
1: Circular RNA candidate 1 elu
2: Circular RNA candidate 1 elu (**2.5 mM $MgCl_2$, 65°C 30 min**)
3: Circular RNA candidate 1 elu (**5 mM $MgCl_2$, 65°C 30 min**)
4: Circular RNA candidate 2 elu
5: Circular RNA candidate 2 elu (**2.5 mM $MgCl_2$, 65°C 30 min**)
6: Circular RNA candidate 2 elu (**5 mM $MgCl_2$, 65°C 30 min**)
100 ng RNA for the reaction/well

FIG. 5

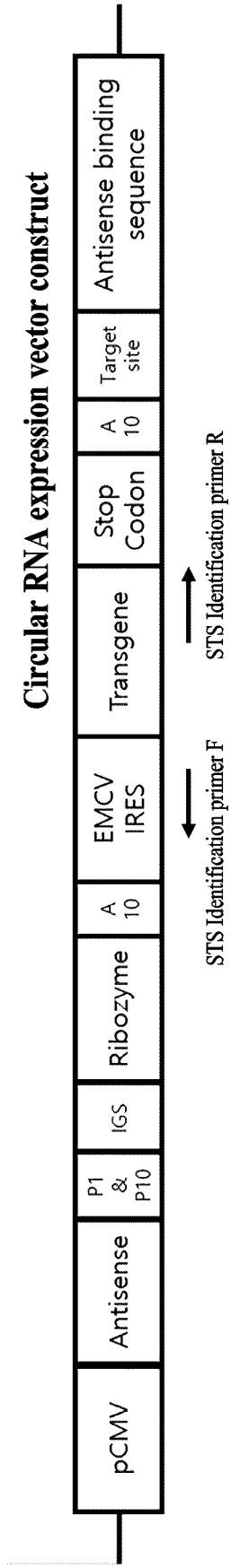

Circular RNA expression vector construct
pCMV
Antisense
P1 & P10
IGS
Ribozyme
A 10
EMCV IRES
Transgene
Stop Codon
A 10
Target site
Antisense binding sequence
STS Identification primer F
STS Identification primer R

FIG. 6A

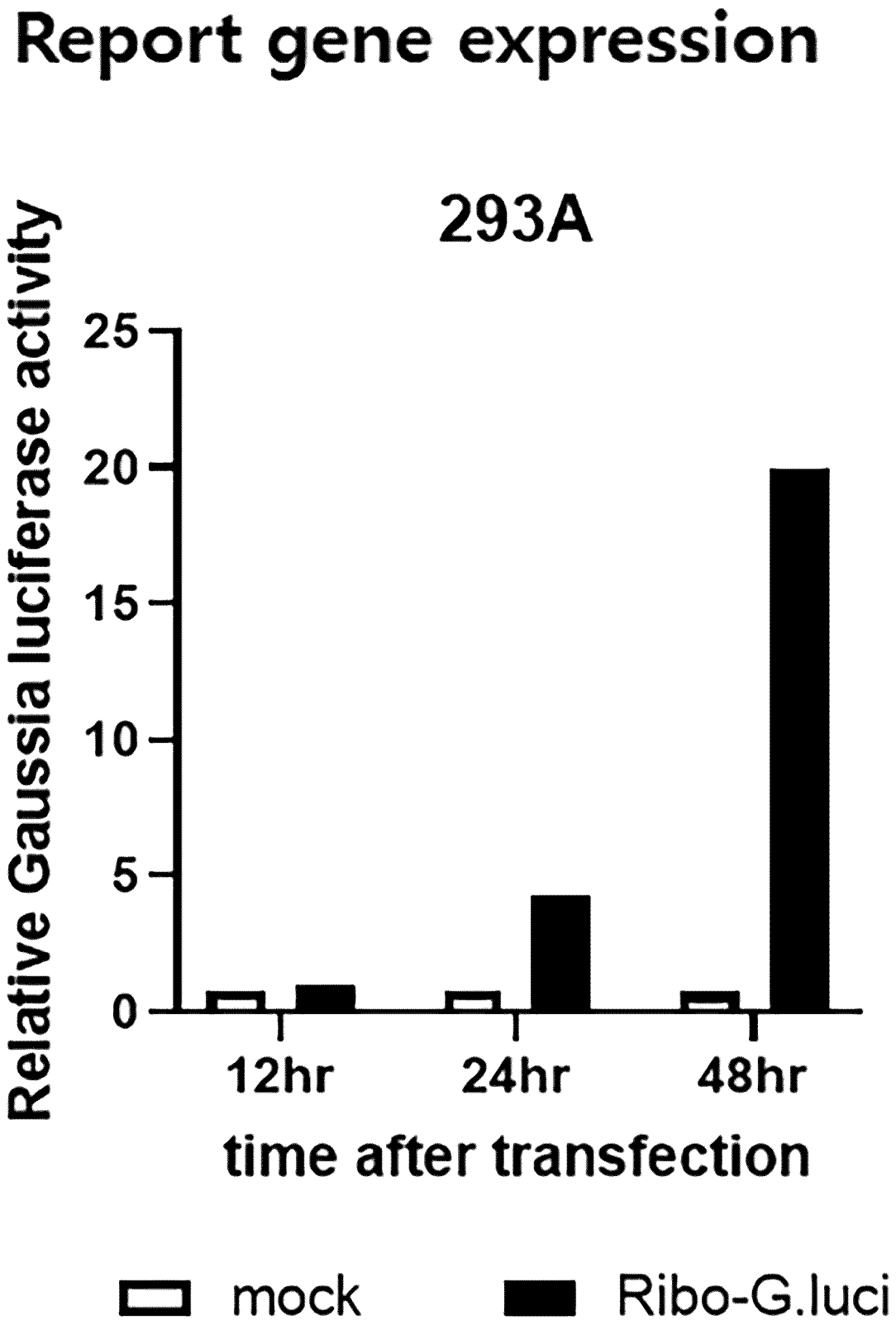

Report gene expression
293A
Relative Gaussia luciferase activity
25
20
15
10
5
0
12hr
24hr
48hr
time after transfection
mock
Ribo-G.luci

FIG. 6B

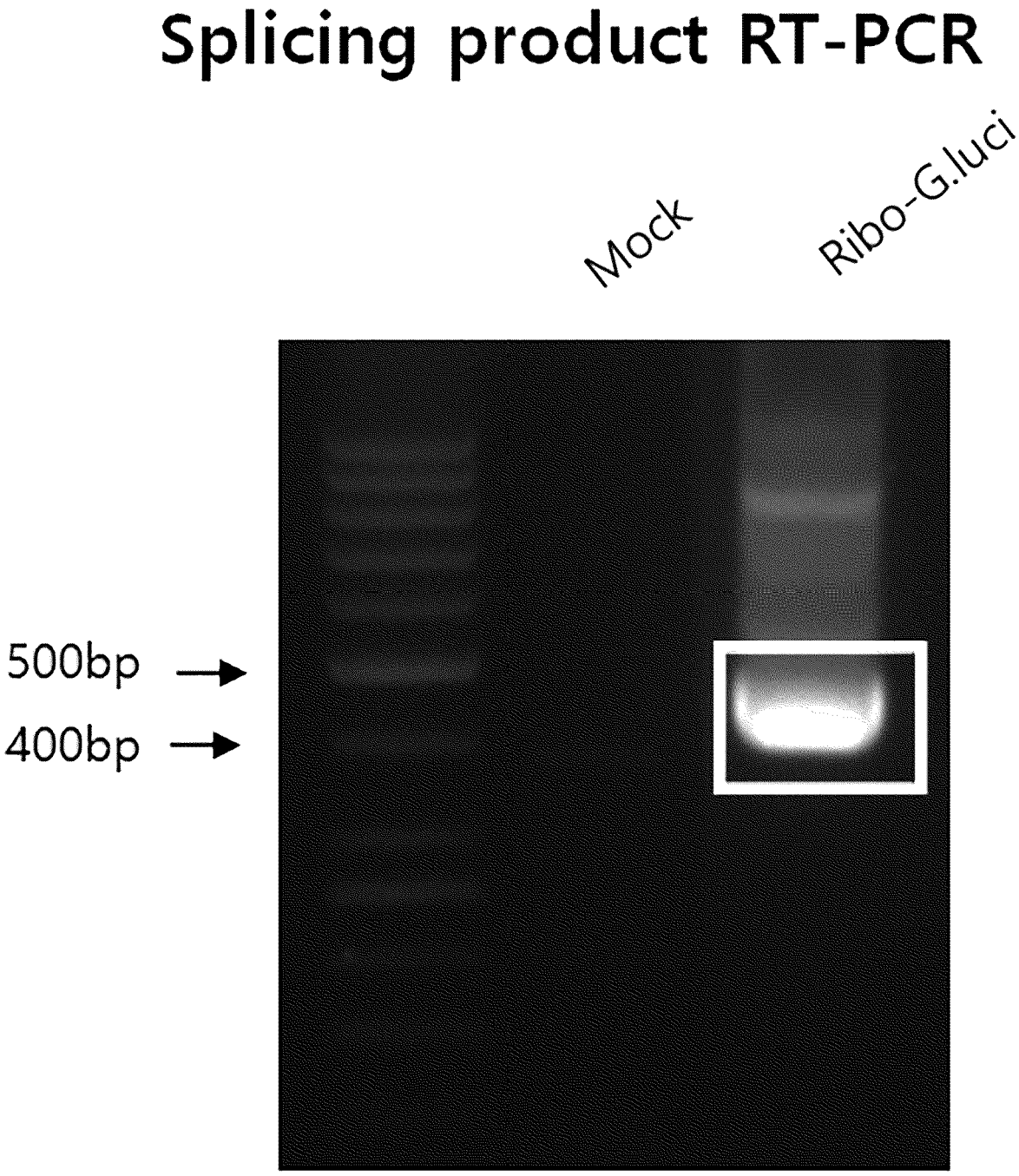


FIG. 6C

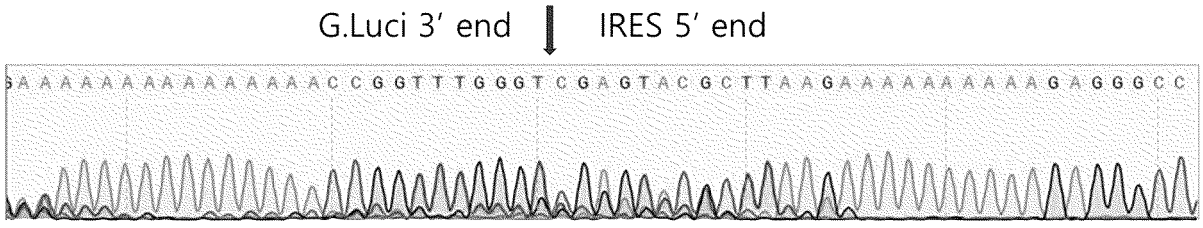

G.Luci 3′ end
IRES 5′ end
GAAAAAAAAAAAAAAACCGGTTTGGGTCGAGTACGCTTAAGAAAAAAAAAAAGAGGGCC

FIG. 6D

Analysis condition

✓ HPLC system: Agilent 1290 Infinity II Bio UHPLC
✓ Analytical column: Agilent PLRP-S 4000A 8um, 2.1 x 50mm
✓ Injection volume: 2 µL (Prep 10 – 20uL)
✓ Flow rate: 0.5 mL/min
✓ Column oven temperature: 60 ℃
✓ Detector: UV 260nm
✓ Mobile phase A: 100mM Hexylamine acetate pH7.0
✓ Mobile phase B: 100mM Hexylamine acetate in acetonitrile
✓ Gradient

- Fraction collection

Time(min)	%A	%B
0	70	30
2	70	30
15	45	55
16	10	90
17	10	90
17.1	70	30
20	70	30

- Analytical

Time(min)	%A	%B
0	80	20
2	80	20
50	40	60
51	10	90
53	10	90
53.1	80	20
56	80	20

✓ Collected from 96-well plate (PP, 250 uL) by increasing 2 position/6port valve position at interval of 0.1 minutes in range of fraction collection 9 minutes-12 minutes

FIG. 7A

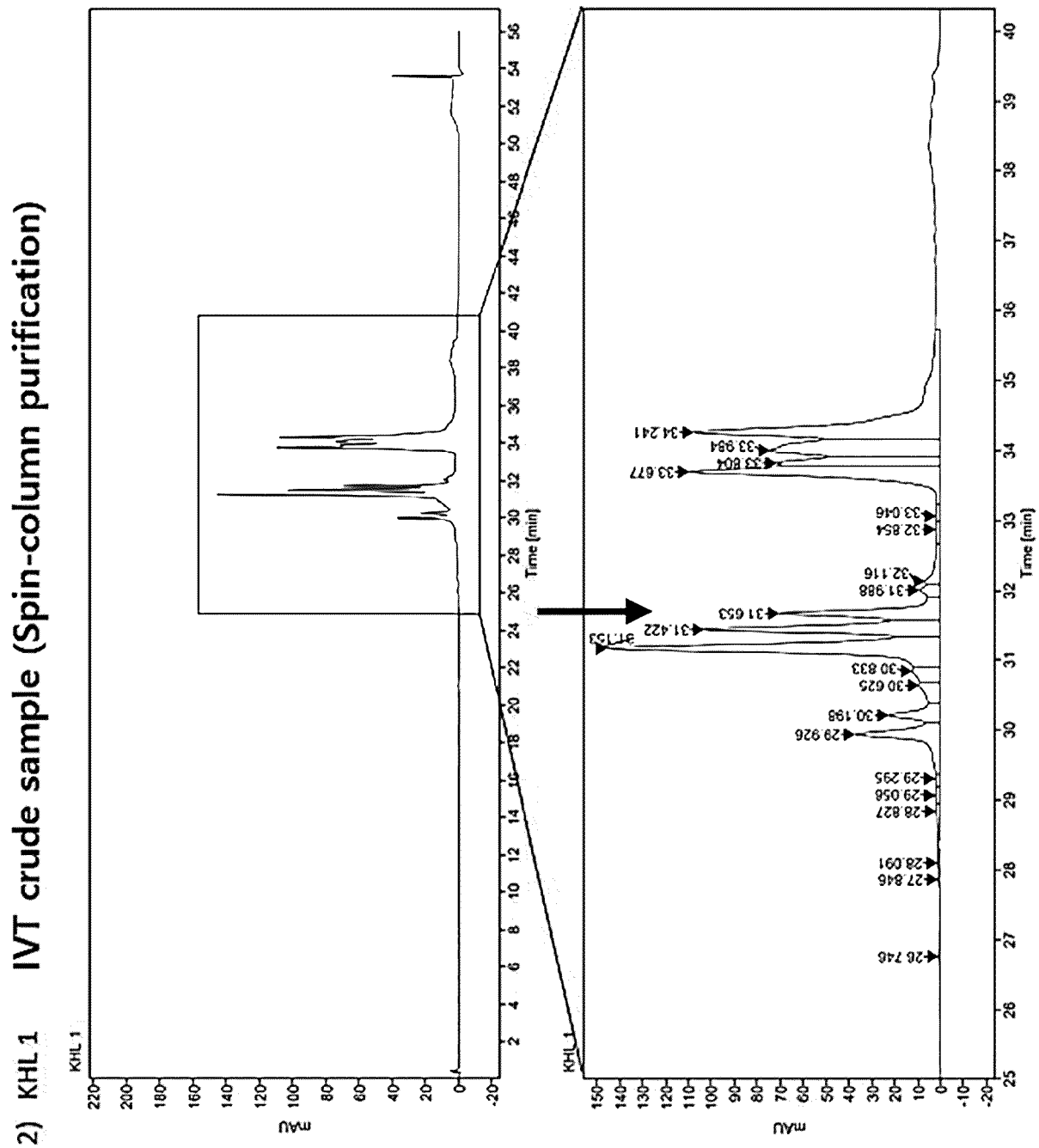

2) KHL 1 IVT crude sample (Spin-column purification)
KHL 1
mAU
Time (min)
34.241
33.984
33.804
33.677
33.046
32.854
32.116
31.988
31.653
31.422
31.153
30.833
30.625
30.198
29.926
29.295
29.058
28.827
28.091
27.846
26.746

FIG. 7B

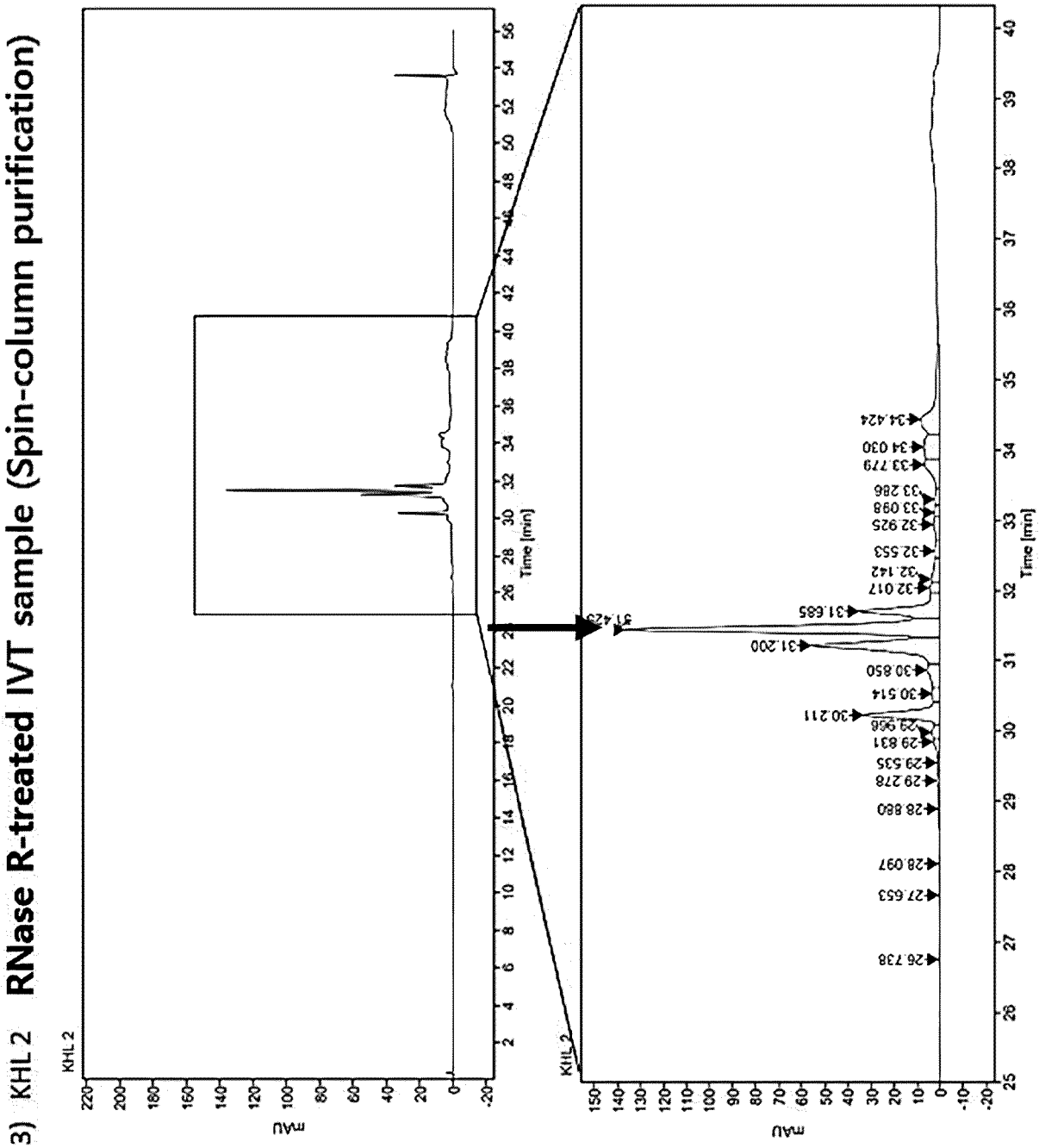

3) KHL2 RNase R-treated IVT sample (Spin-column purification)
KHL2
mAU
Time [min]
31.428
31.685
31.200
30.850
30.514
30.211
29.958
29.831
29.535
29.278
28.880
28.097
27.653
26.738
32.017
32.142
32.553
32.925
33.098
33.286
33.779
34.030
34.424

FIG. 7C

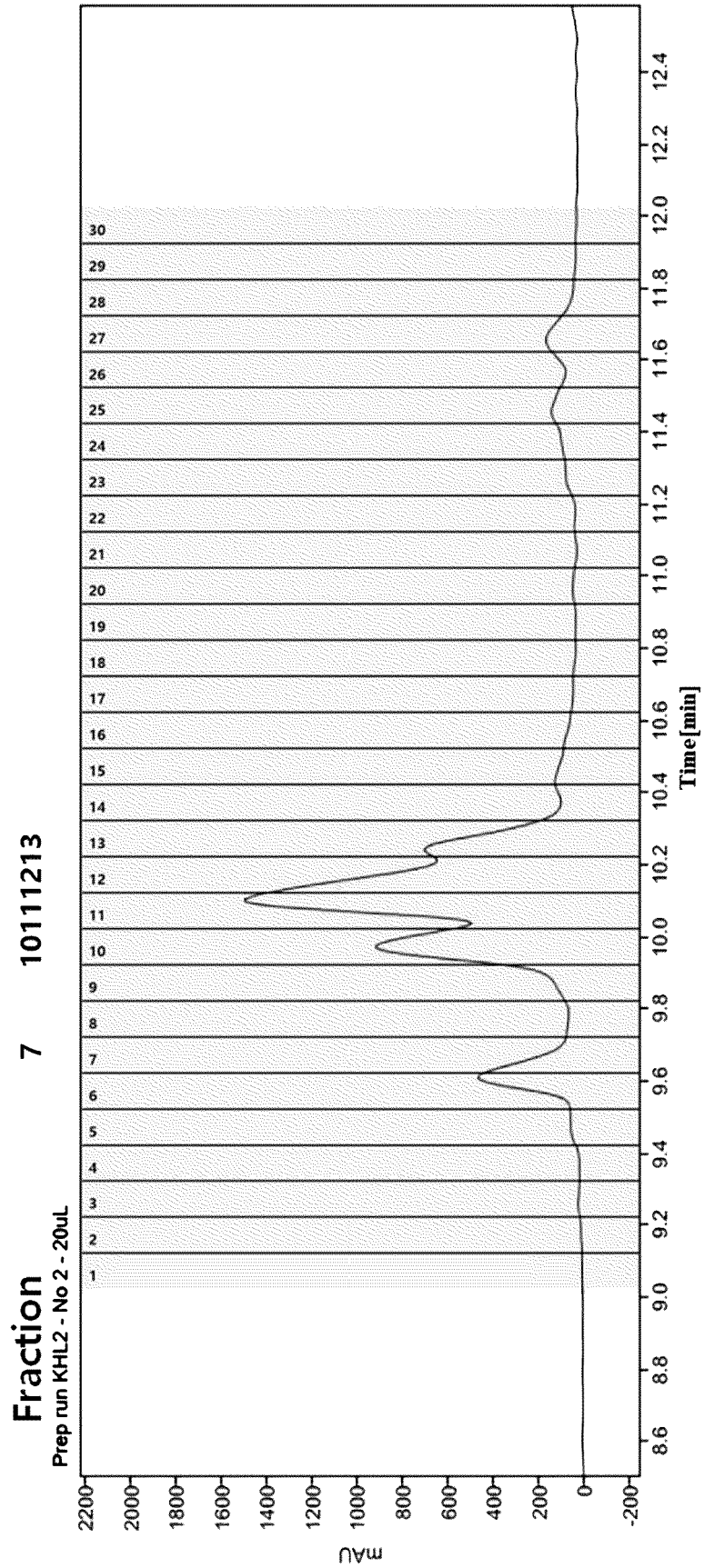
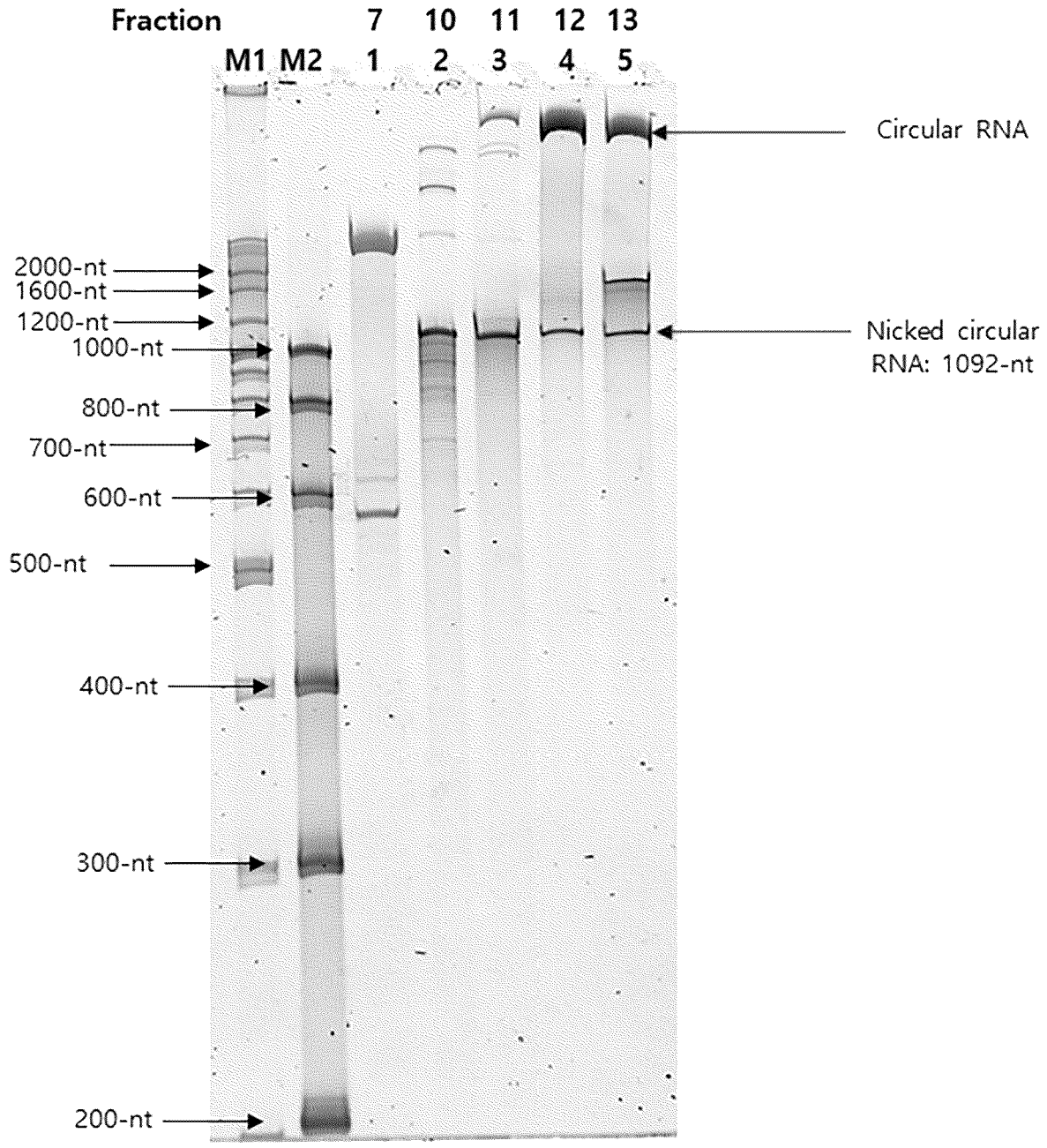

Fraction
Prep run KHL2 - No 2 - 20uL
7
10111213
mAU
Time[min]
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
-200
8.6
8.8
9.0
9.2
9.4
9.6
9.8
10.0
10.2
10.4
10.6
10.8
11.0
11.2
11.4
11.6
11.8
12.0
12.2
12.4
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30

FIG. 8A



M1: 100 bp Plus DNA Ladder (Bioneer, D-1035)
M2: RiboRuler Low Range RNA Ladder (ThermoScientific, SM1831)
1: RZ004-EMCV-IRES-Gluci-antisense IVT RNA: Fraction 7
2: RZ004-EMCV-IRES-Gluci-antisense IVT RNA: Fraction 10
3: RZ004-EMCV-IRES-Gluci-antisense IVT RNA: Fraction 11
4: RZ004-EMCV-IRES-Gluci-antisense IVT RNA: Fraction 12
5: RZ004-EMCV-IRES-Gluci-antisense IVT RNA: Fraction 13
200 ng/well

FIG. 8B

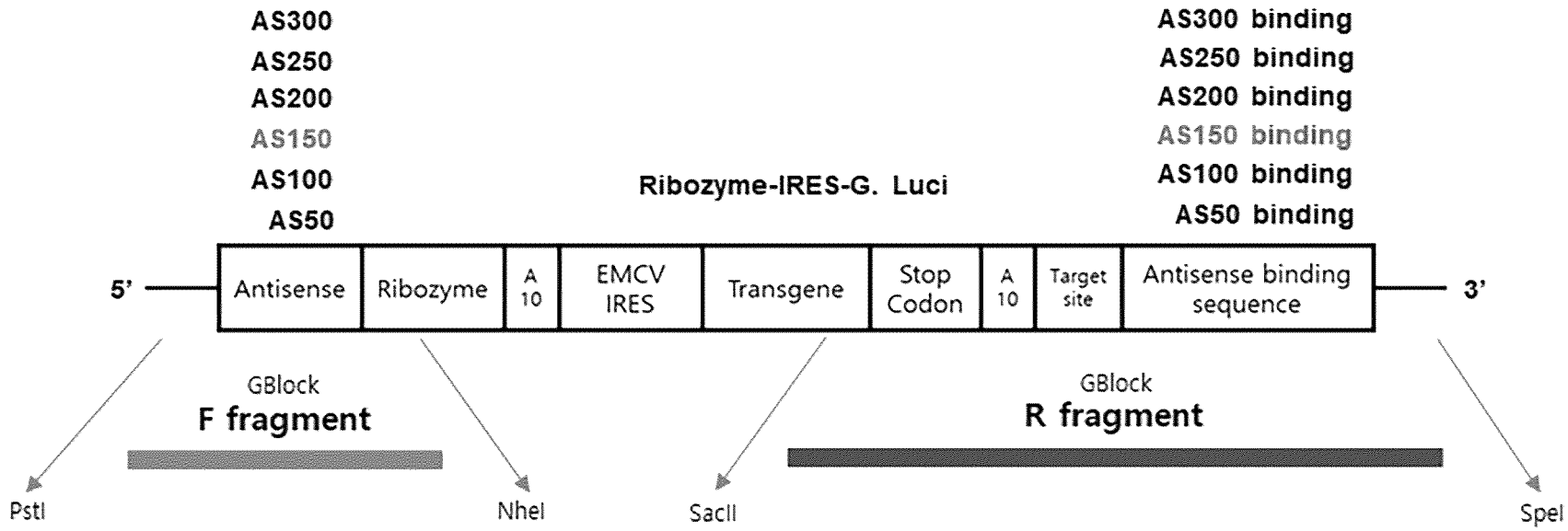

AS300
AS250
AS200
AS150
AS100
AS50
Ribozyme-IRES-G. Luci
AS300 binding
AS250 binding
AS200 binding
AS150 binding
AS100 binding
AS50 binding
5'
Antisense
Ribozyme
A 10
EMCV IRES
Transgene
Stop Codon
A 10
Target site
Antisense binding sequence
3'
GBlock
F fragment
GBlock
R fragment
PstI
NheI
SacII
SpeI
Backbone plasmid: pTOP blunt-RZ-004-EMCV IRES-Gluci-antisense binding sequence_v07_T7 promoter (reverse)

FIG. 9

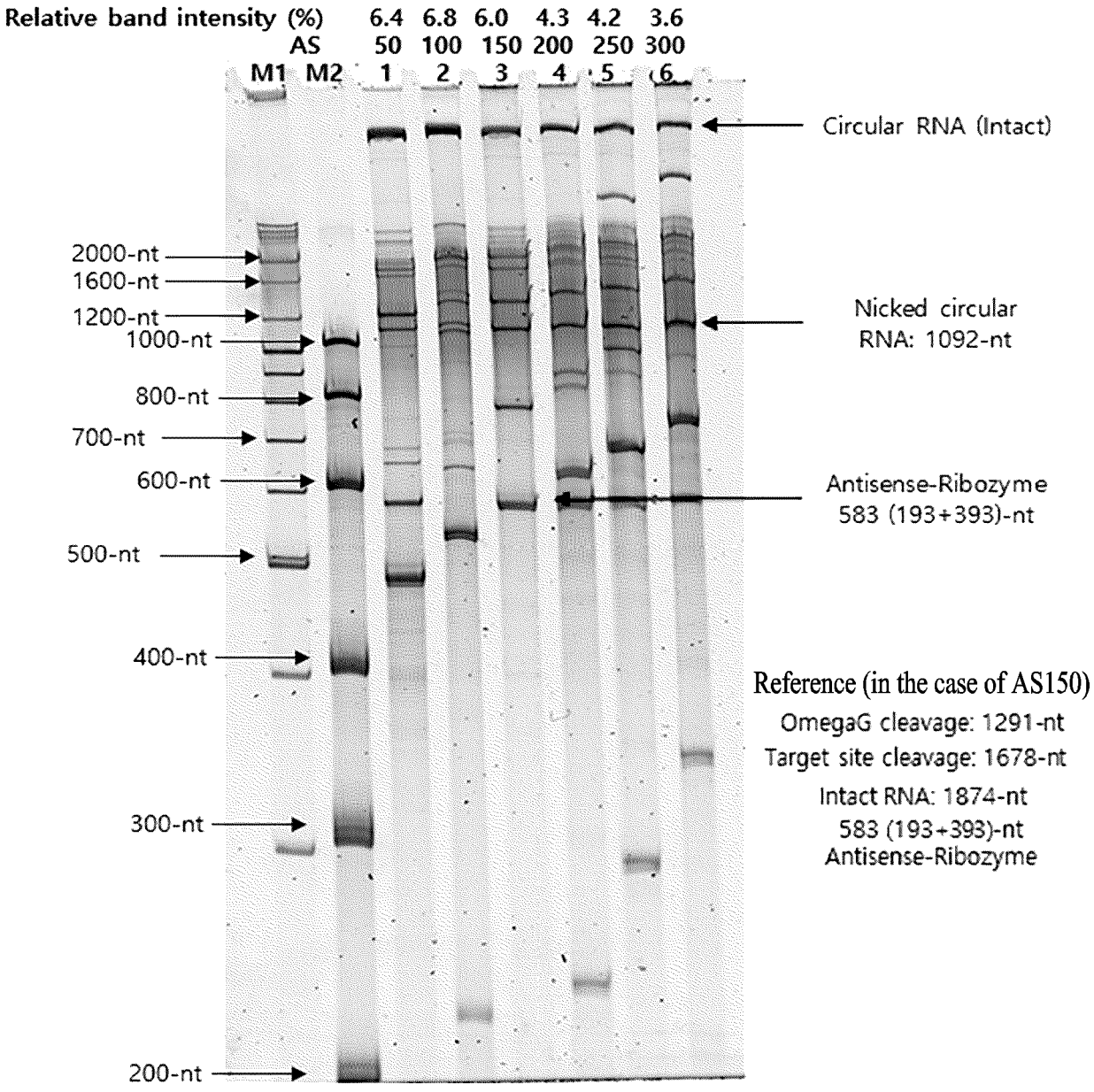

Relative band intensity (%)
6.4 6.8 6.0 4.3 4.2 3.6
AS 50 100 150 200 250 300
M1 M2 1 2 3 4 5 6
Circular RNA (Intact)
2000-nt
1600-nt
1200-nt
1000-nt
800-nt
700-nt
600-nt
500-nt
400-nt
300-nt
200-nt
Nicked circular RNA: 1092-nt
Antisense-Ribozyme 583 (193+393)-nt
Reference (in the case of AS150)
OmegaG cleavage: 1291-nt
Target site cleavage: 1678-nt
Intact RNA: 1874-nt
583 (193+393)-nt Antisense-Ribozyme

FIG. 10

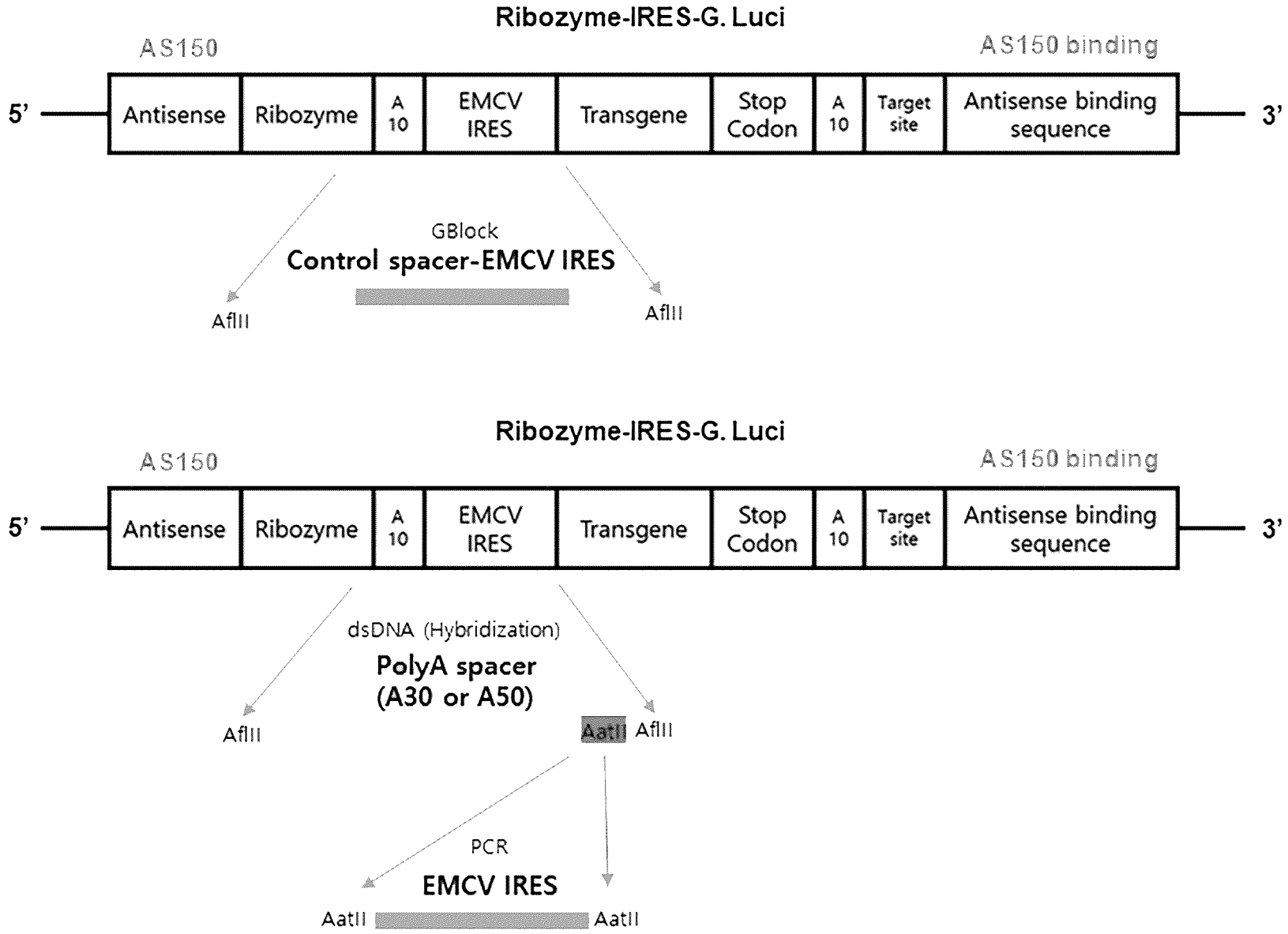

Ribozyme-IRES-G. Luci
AS150
AS150 binding
5'
Antisense
Ribozyme
A 10
EMCV IRES
Transgene
Stop Codon
A 10
Target site
Antisense binding sequence
3'
GBlock
Control spacer-EMCV IRES
AflII
AflII
Ribozyme-IRES-G. Luci
AS150
AS150 binding
5'
Antisense
Ribozyme
A 10
EMCV IRES
Transgene
Stop Codon
A 10
Target site
Antisense binding sequence
3'
dsDNA (Hybridization)
PolyA spacer
(A30 or A50)
AflII
AatII
AflII
PCR
EMCV IRES
AatII
AatII

FIG. 11

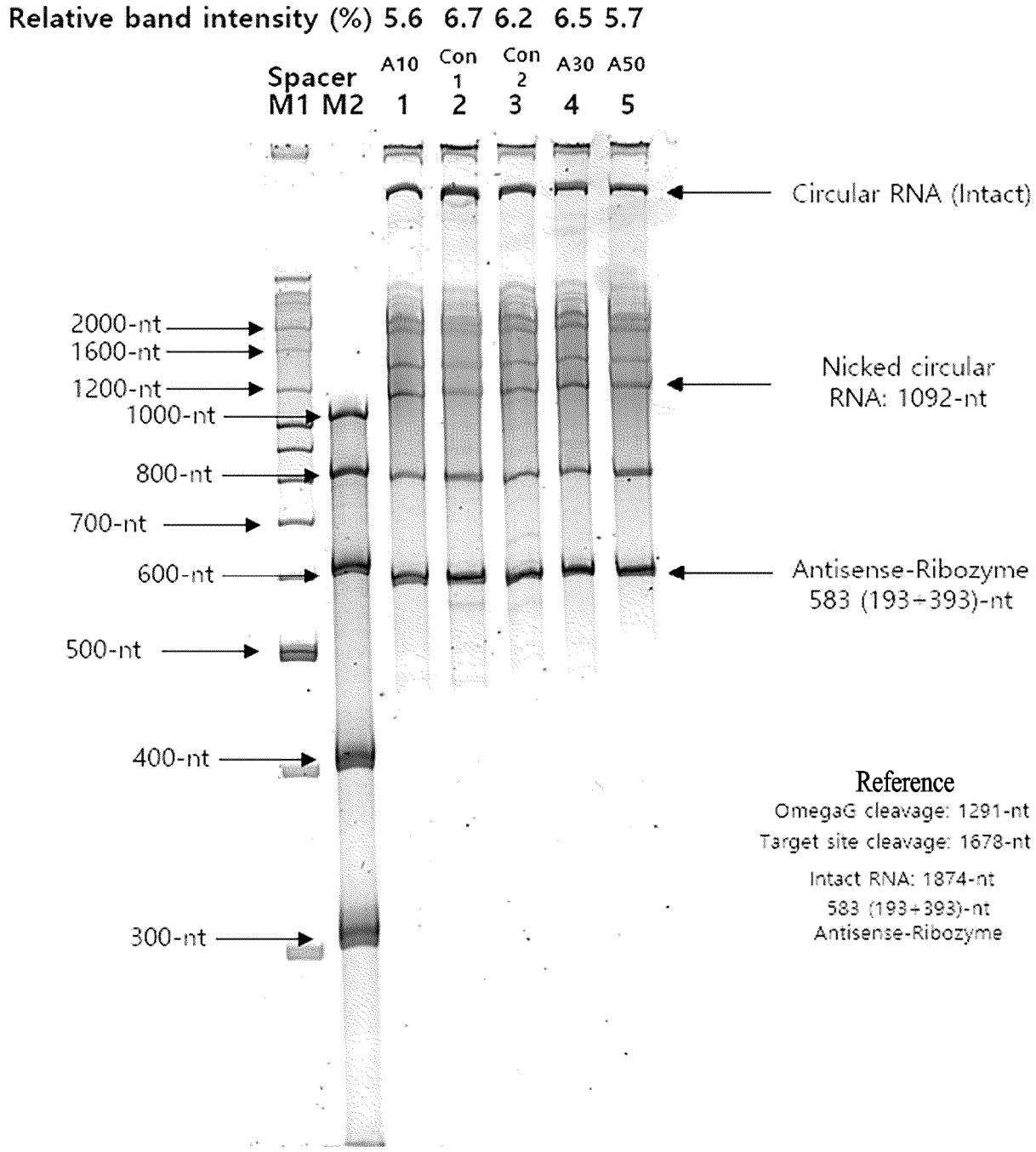

Relative band intensity (%) 5.6 6.7 6.2 6.5 5.7
Spacer
M1 M2
A10
1
Con 1
2
Con 2
3
A30
4
A50
5
Circular RNA (Intact)
2000-nt
1600-nt
1200-nt
1000-nt
800-nt
700-nt
600-nt
500-nt
400-nt
300-nt
Nicked circular RNA: 1092-nt
Antisense-Ribozyme 583 (193+393)-nt
Reference
OmegaG cleavage: 1291-nt
Target site cleavage: 1678-nt
Intact RNA: 1874-nt
583 (193+393)-nt Antisense-Ribozyme

FIG. 12

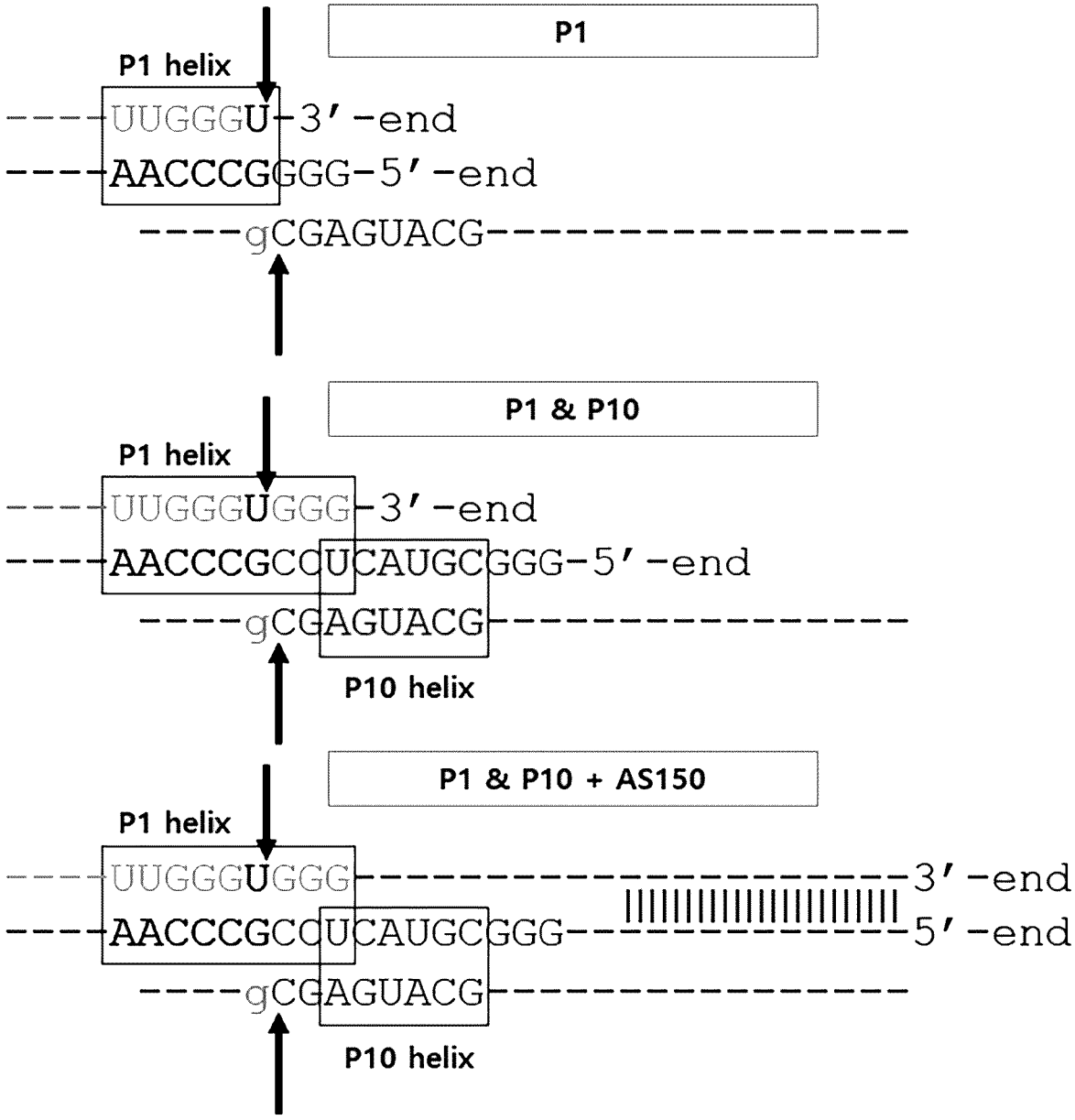

P1
P1 helix
UUGGGU-3′-end
AACCCGGGG-5′-end
gCGAGUACG
P1 & P10
P1 helix
UUGGGUGGG-3′-end
AACCCGCCUCAUGCGGG-5′-end
gCGAGUACG
P10 helix
P1 & P10 + AS150
P1 helix
UUGGGUGGG
3′-end
AACCCGCCUCAUGCGGG
5′-end
gCGAGUACG
P10 helix

FIG. 13

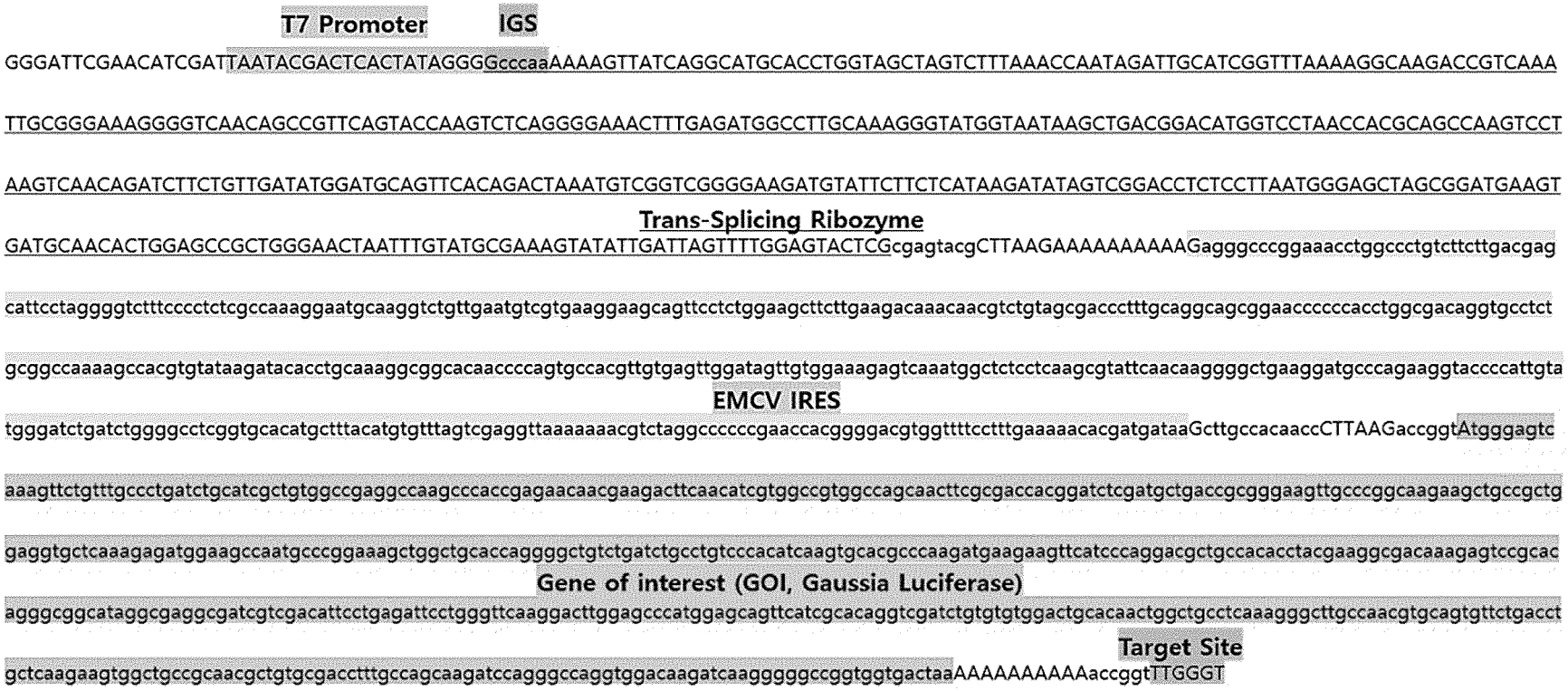

T7 Promoter
IGS
GGGATTCGAACATCGATTAATACGACTCACTATAGGGGcccaaAAAAGTTATCAGGCATGCACCTGGTAGCTAGTCTTTAAACCAATAGATTGCATCGGTTTAAAAGGCAAGACCGTCAAA
TTGCGGGAAAGGGGTCAACAGCCGTTCAGTACCAAGTCTCAGGGGAAACTTTGAGATGGCCTTGCAAAGGGTATGGTAATAAGCTGACGGACATGGTCCTAACCACGCAGCCAAGTCCT
AAGTCAACAGATCTTCTGTTGATATGGATGCAGTTCACAGACTAAATGTCGGTCGGGGAAGATGTATTCTTCTCATAAGATATAGTCGGACCTCTCCTTAATGGGAGCTAGCGGATGAAGT
Trans-Splicing Ribozyme
GATGCAACACTGGAGCCGCTGGGAACTAATTTGTATGCGAAAGTATATTGATTAGTTTTGGAGTACTCGcgagtacgCTTAAGAAAAAAAAAAAGagggcccggaaacctggccctgtcttcttgacgag
cattcctaggggtctttcccctctcgccaaaggaatgcaaggtctgttgaatgtcgtgaaggaagcagttcctctggaagcttcttgaagacaaacaacgtctgtagcgacccctttgcaggcagcggaacccccccacctggcgacaggtgcctct
gcggccaaaagccacgtgtataagatacacctgcaaaggcggcacaaccccagtgccacgttgtgagttggatagttgtggaaagagtcaaatggctctcctcaagcgtattcaacaaggggctgaaggatgcccagaaggtaccccattgta
EMCV IRES
tgggatctgatctggggcctcggtgcacatgctttacatgtgtttagtcgaggttaaaaaaacgtctaggccccccgaaccacggggacgtggttttcctttgaaaaacacgatgataaGcttgccacaaccCTTAAGaccggtAtgggagtc
aaagttctgtttgccctgatctgcatcgctgtggccgaggccaagcccaccgagaacaacgaagacttcaacatcgtggccgtggccagcaacttcgcgaccacggatctcgatgctgaccgcgggaagttgcccggcaagaagctgccgctg
gaggtgctcaaagagatggaagccaatgcccggaaagctggctgcaccaggggctgtctgatctgcctgtcccacatcaagtgcacgcccaagatgaagaagttcatcccaggacgctgccacacctacgaaggcgacaaagagtccgcac
Gene of interest (GOI, Gaussia Luciferase)
agggcggcataggcgaggcgatcgtcgacattcctgagattcctgggttcaaggacttggagcccatggagcagttcatcgcacaggtcgatctgtgtgtggactgcacaactggctgcctcaaagggcttgccaacgtgcagtgttctgacct
Target Site
gctcaagaagtggctgccgcaacgctgtgcgacctttgccagcaagatccagggccaggtggacaagatcaaggggccggtggtgactaaAAAAAAAAAAaccggtTTGGGT

FIG. 14

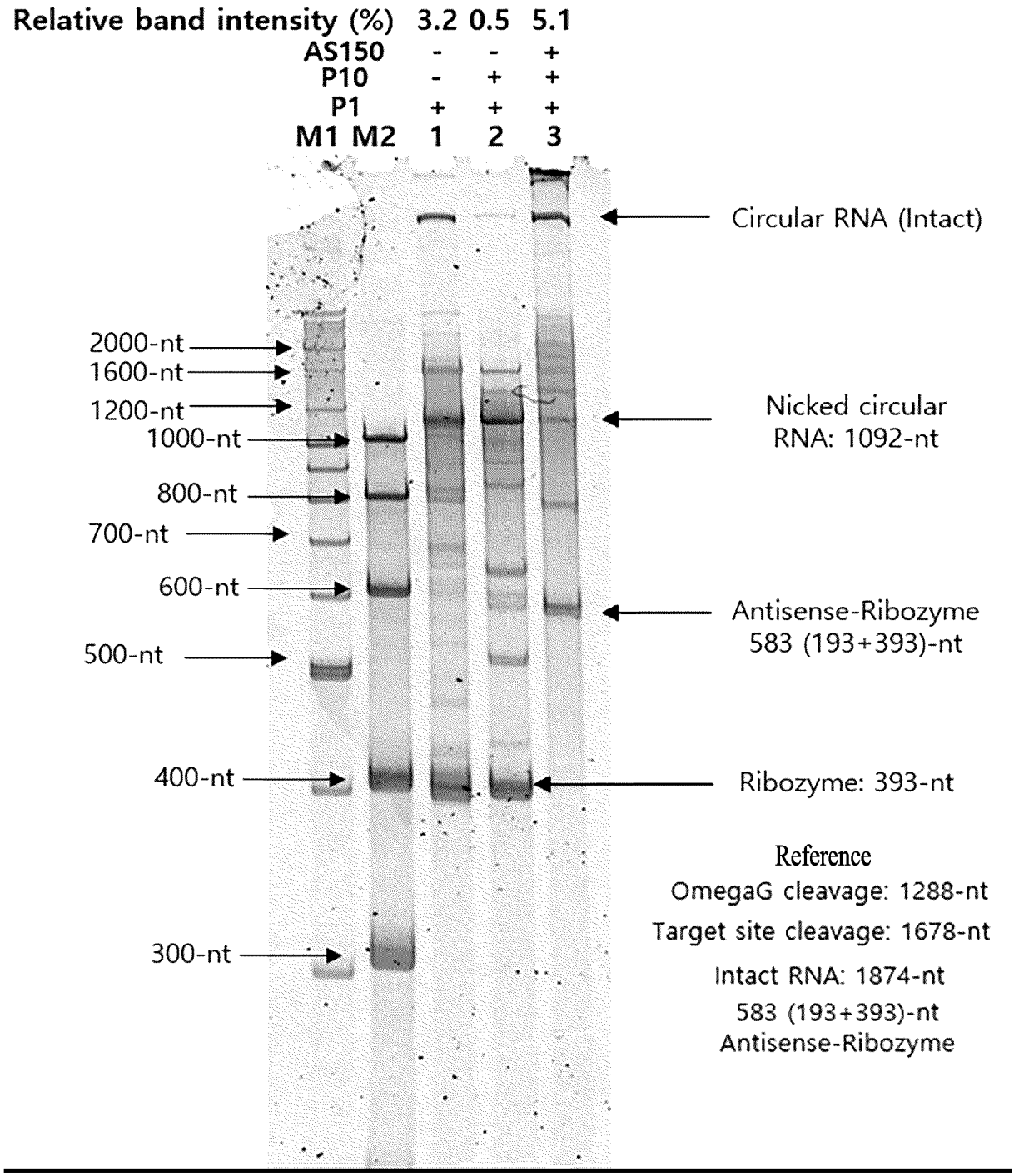

Relative band intensity (%) 3.2 0.5 5.1
AS150 - - +
P10 - + +
P1 + + +
M1 M2 1 2 3
Circular RNA (Intact)
2000-nt
1600-nt
1200-nt
1000-nt
800-nt
700-nt
600-nt
500-nt
400-nt
300-nt
Nicked circular RNA: 1092-nt
Antisense-Ribozyme 583 (193+393)-nt
Ribozyme: 393-nt
Reference
OmegaG cleavage: 1288-nt
Target site cleavage: 1678-nt
Intact RNA: 1874-nt
583 (193+393)-nt Antisense-Ribozyme

FIG. 15

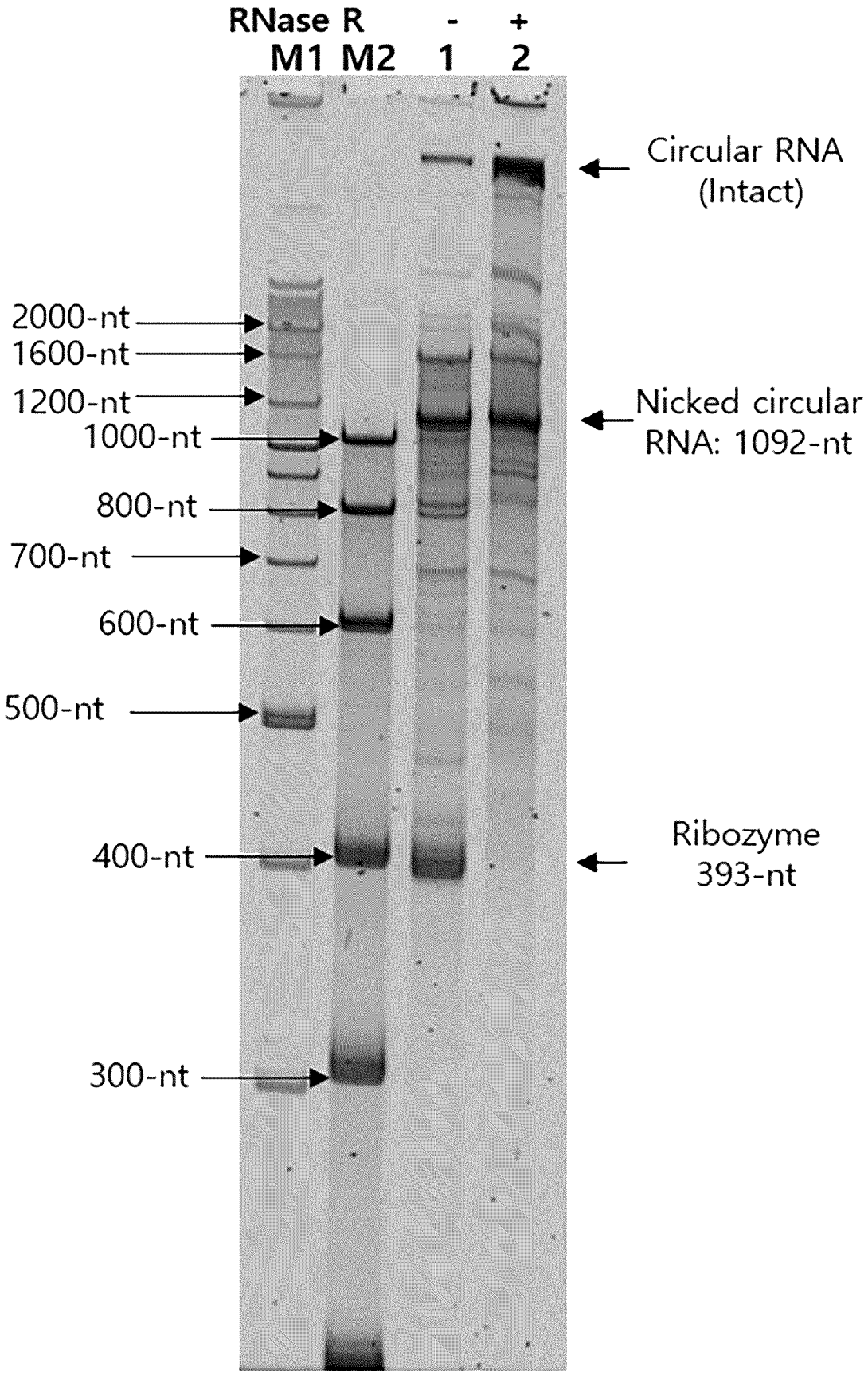

RNase R
-
+
M1
M2
1
2
Circular RNA
(Intact)
2000-nt
1600-nt
1200-nt
1000-nt
800-nt
700-nt
600-nt
500-nt
Nicked circular
RNA: 1092-nt
400-nt
Ribozyme
393-nt
300-nt

FIG. 16A

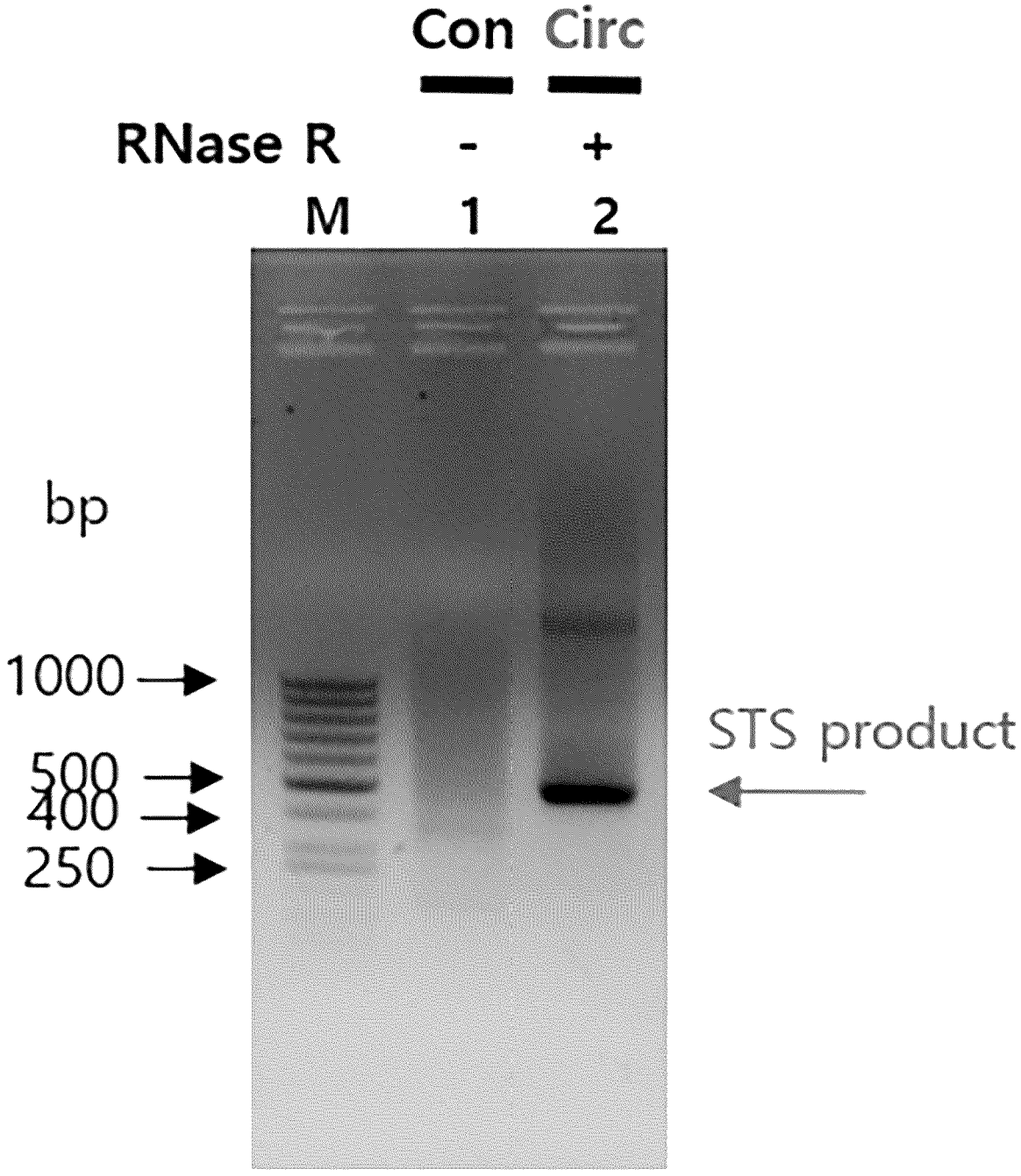


FIG. 16B

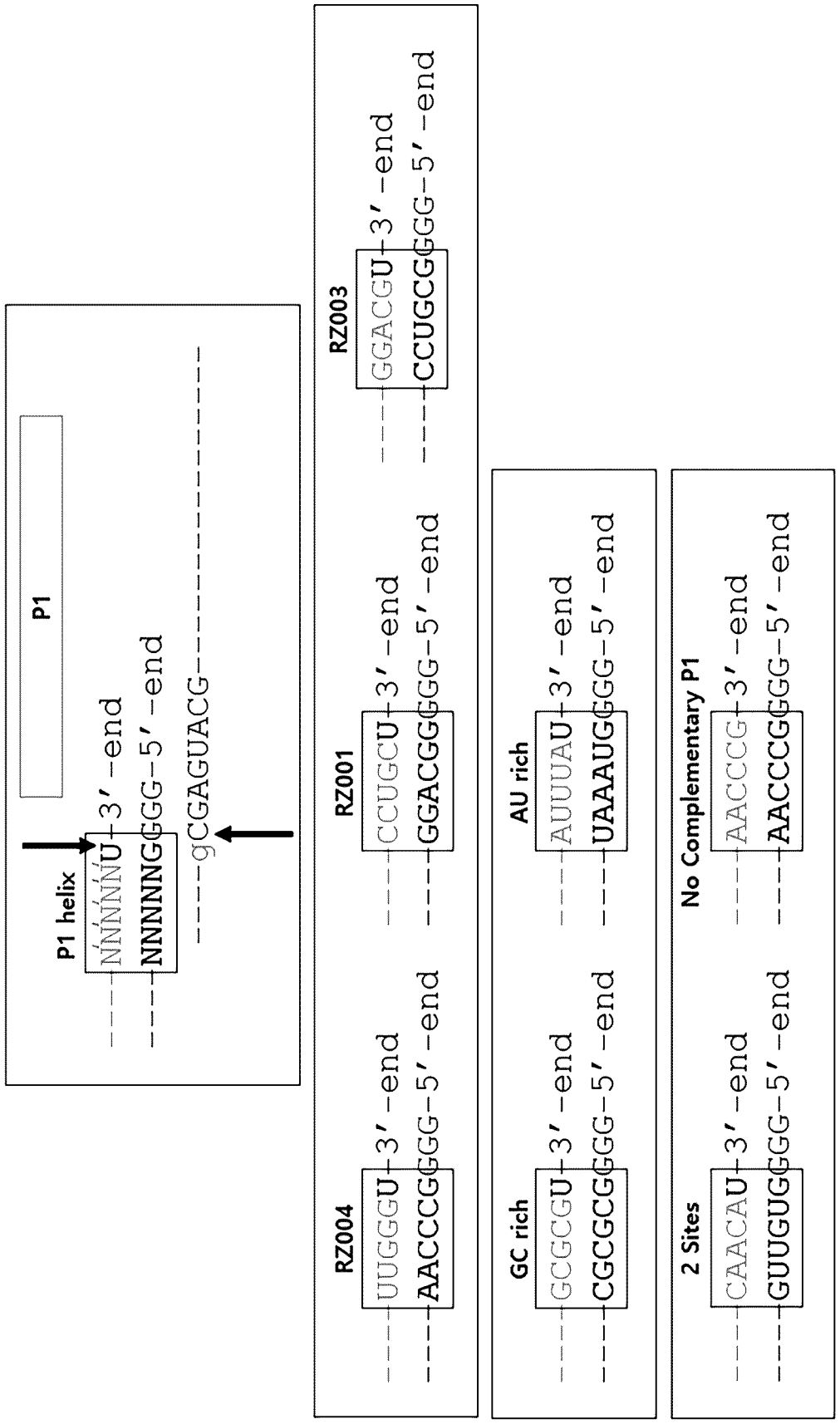
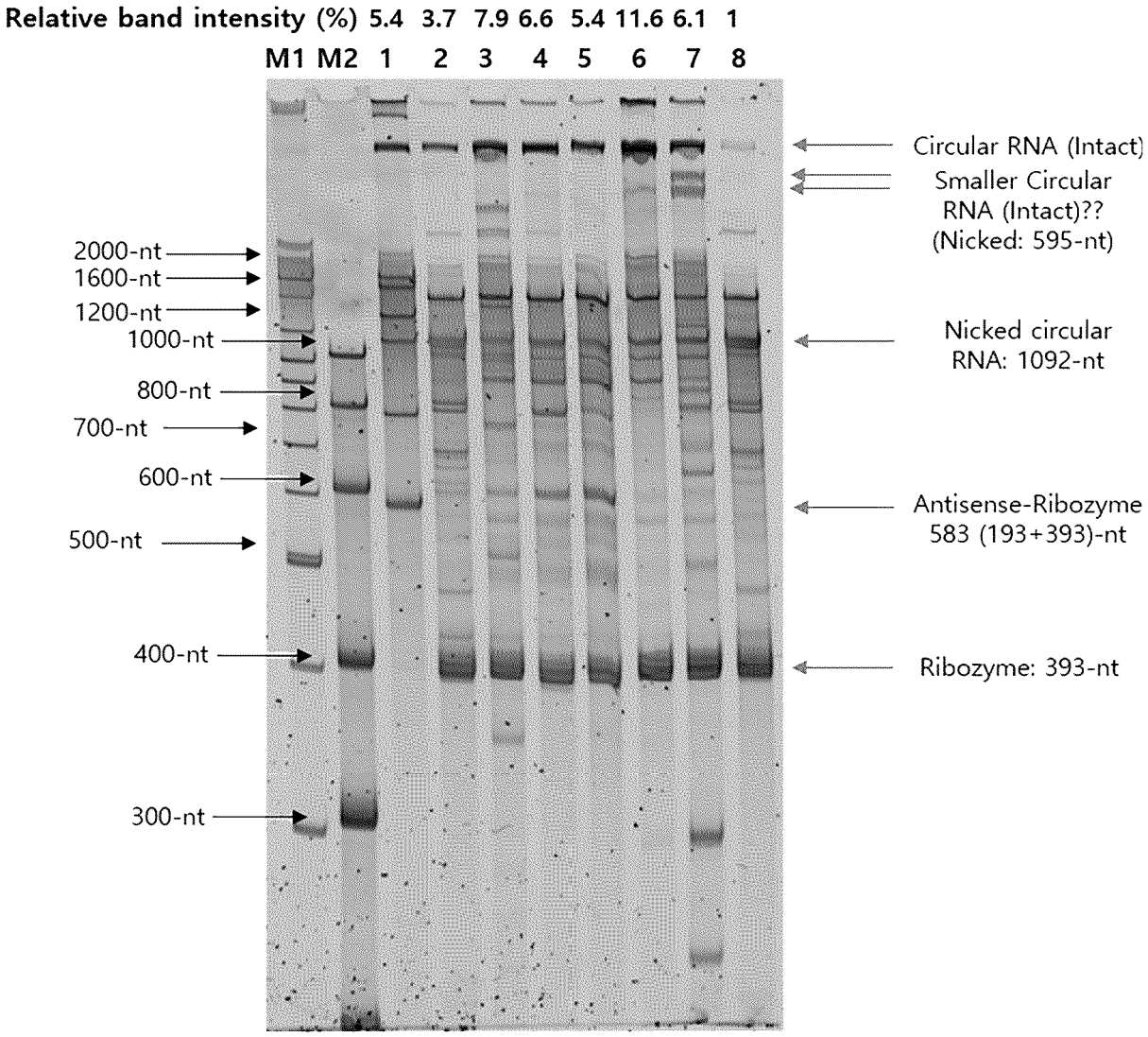

P1
P1 helix
NNNNU
NNNNNGGG
3′-end
5′-end
gCGAGUACG
RZ004
UUGGGU
AACCCGGGG
RZ001
CCUGCU
GGACGGGGG
RZ003
GGACGU
CCUGCGGGG
GC rich
GCGCGU
CGCGCGGGG
AU rich
AUUUAU
UAAAUGGGG
2 Sites
CAACAU
GUUGUGGGG
No Complementary P1
AACCCG
AACCCGGGG

FIG. 17



M1: 100 bp Plus DNA Ladder (Bioneer, D-1035)
M2: RiboRuler Low Range RNA Ladder (ThermoScientific, SM1831)
1: RZ004-**EMCV-IRES-Gluci-AS150** (P1, P10, AS150) **IVT RNA**
2: RZ004-**EMCV-IRES-Gluci** (P1, No AS) **IVT RNA**
3: RZ001-**EMCV-IRES-Gluci** (P1, No AS) **IVT RNA**
4: RZ003-**EMCV-IRES-Gluci** (P1, No AS) **IVT RNA**
5: GC-rich-**EMCV-IRES-Gluci** (P1, No AS) **IVT RNA**
6: AU-rich-**EMCV-IRES-Gluci** (P1, No AS) **IVT RNA**
7: 2 sites-**EMCV-IRES-Gluci** (P1, No AS) **IVT RNA**
8: No complementary-**EMCV-IRES-Gluci** (P1, No AS) **IVT RNA**
250 ng IVT RNAs/well

FIG. 18

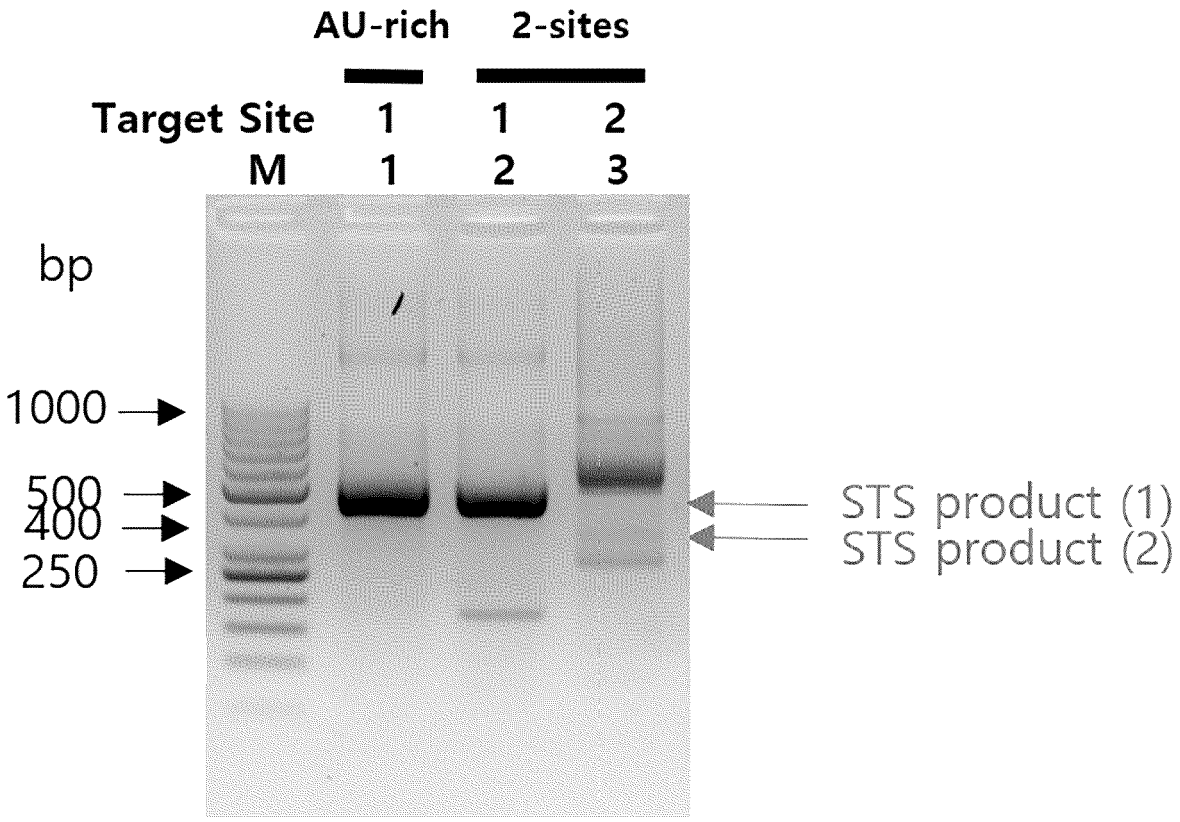


FIG. 19

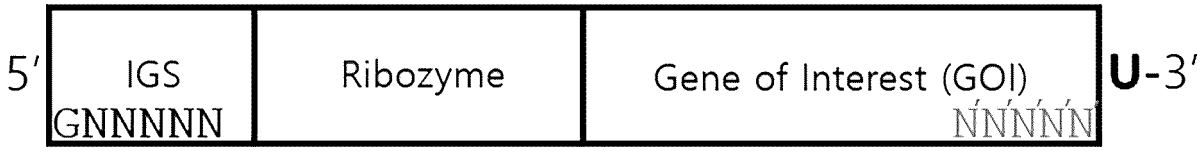


FIG. 20A

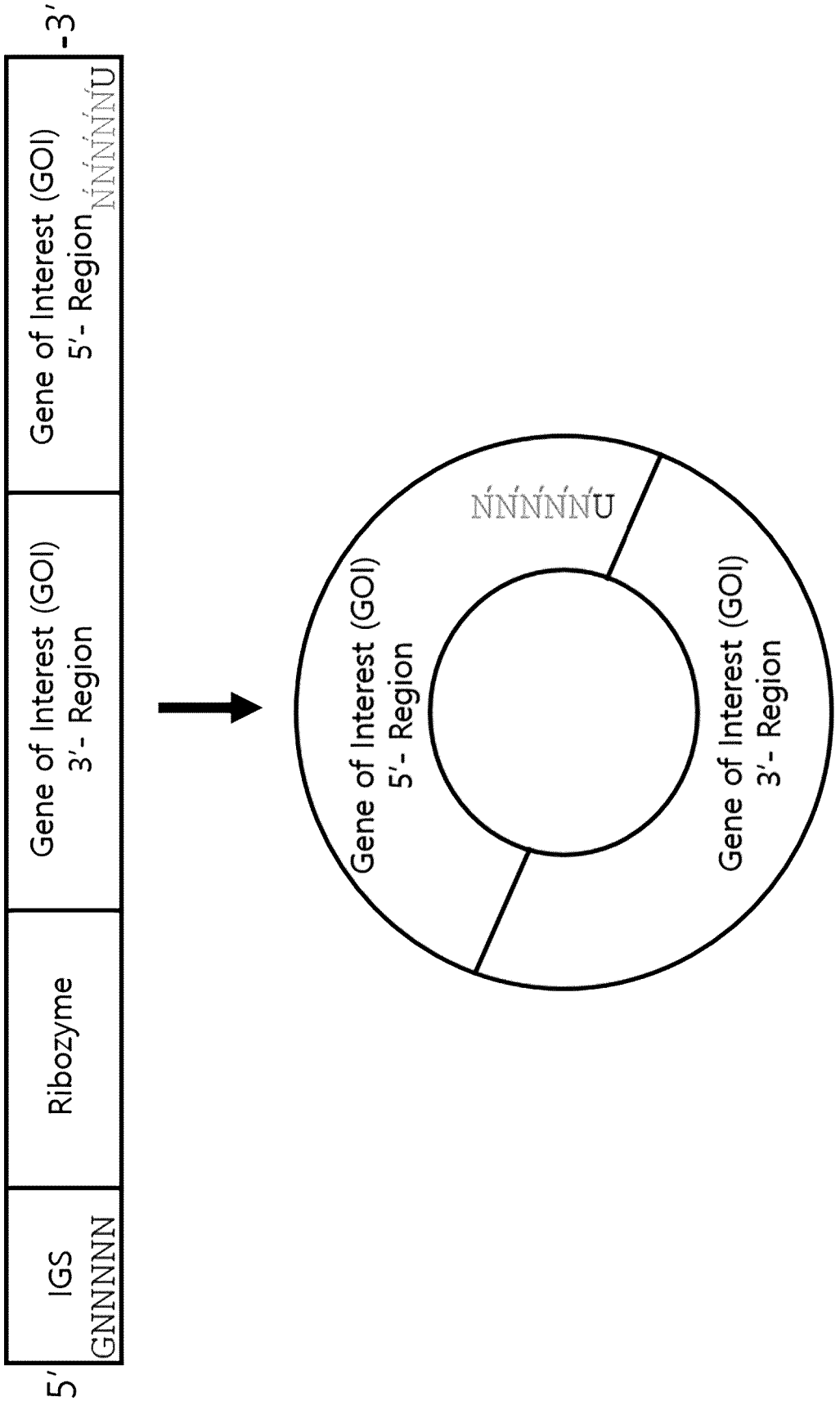

5′
IGS
GNNNNN
Ribozyme
Gene of Interest (GOI)
3′- Region
Gene of Interest (GOI)
5′- Region
NNNNNU
-3′
NNNNNU
Gene of Interest (GOI)
5′- Region
Gene of Interest (GOI)
3′- Region

FIG. 20B