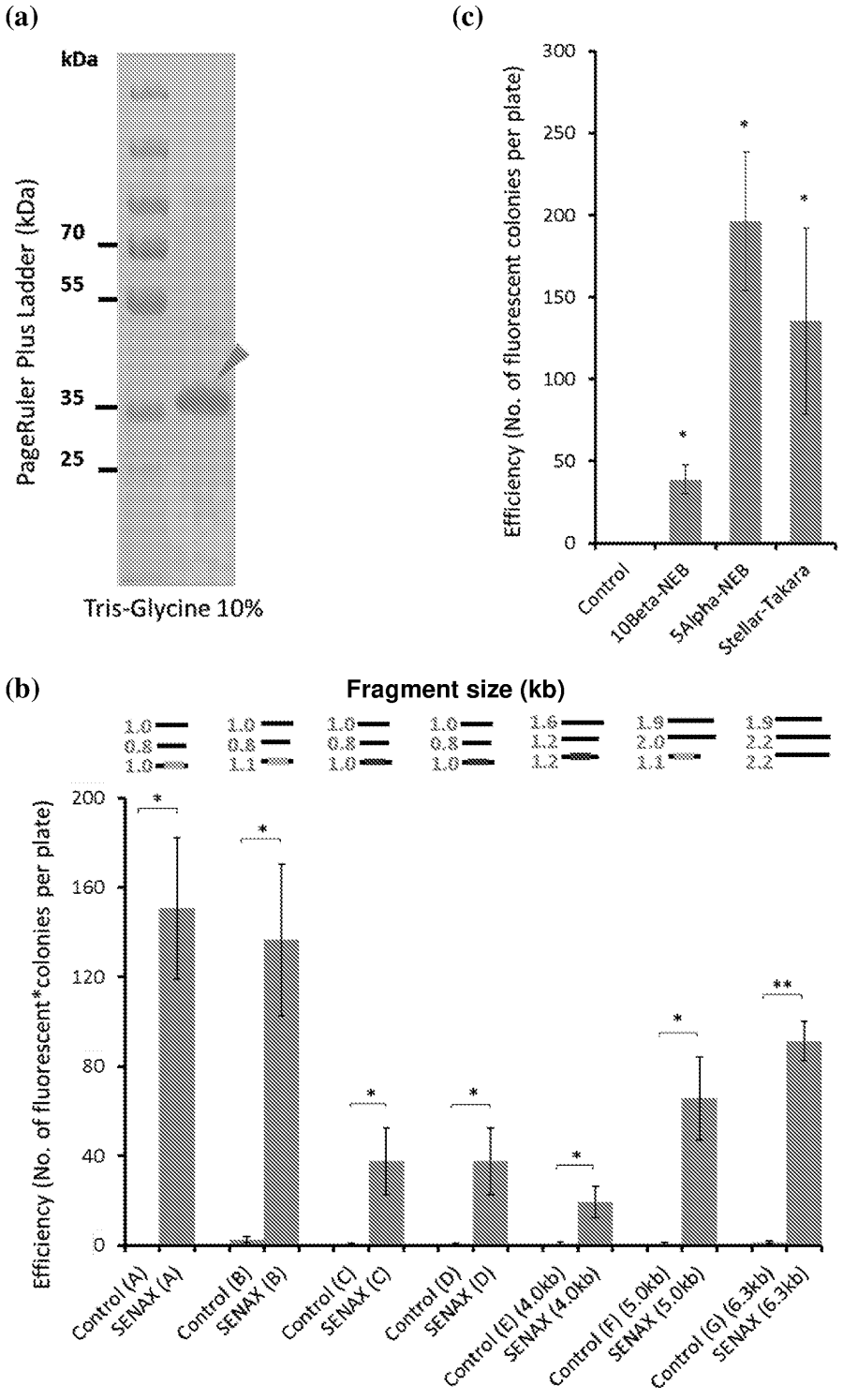

(a)
kDa
PageRuler Plus Ladder (kDa)
70
55
35
25
Tris-Glycine 10%
(c)
Efficiency (No. of fluorescent colonies per plate)
300
250
200
150
100
50
0
Control
10Beta-NEB
5Alpha-NEB
Stellar-Takara
(b)
Fragment size (kb)
Efficiency (No. of fluorescent*colonies per plate)
200
160
120
80
40
0
Control (A)
SENAX (A)
Control (B)
SENAX (B)
Control (C)
SENAX (C)
Control (D)
SENAX (D)
Control (E) (4.0kb)
SENAX (4.0kb)
Control (F) (5.0kb)
SENAX (5.0kb)
Control (G) (6.3kb)
SENAX (6.3kb)

Fig. 1

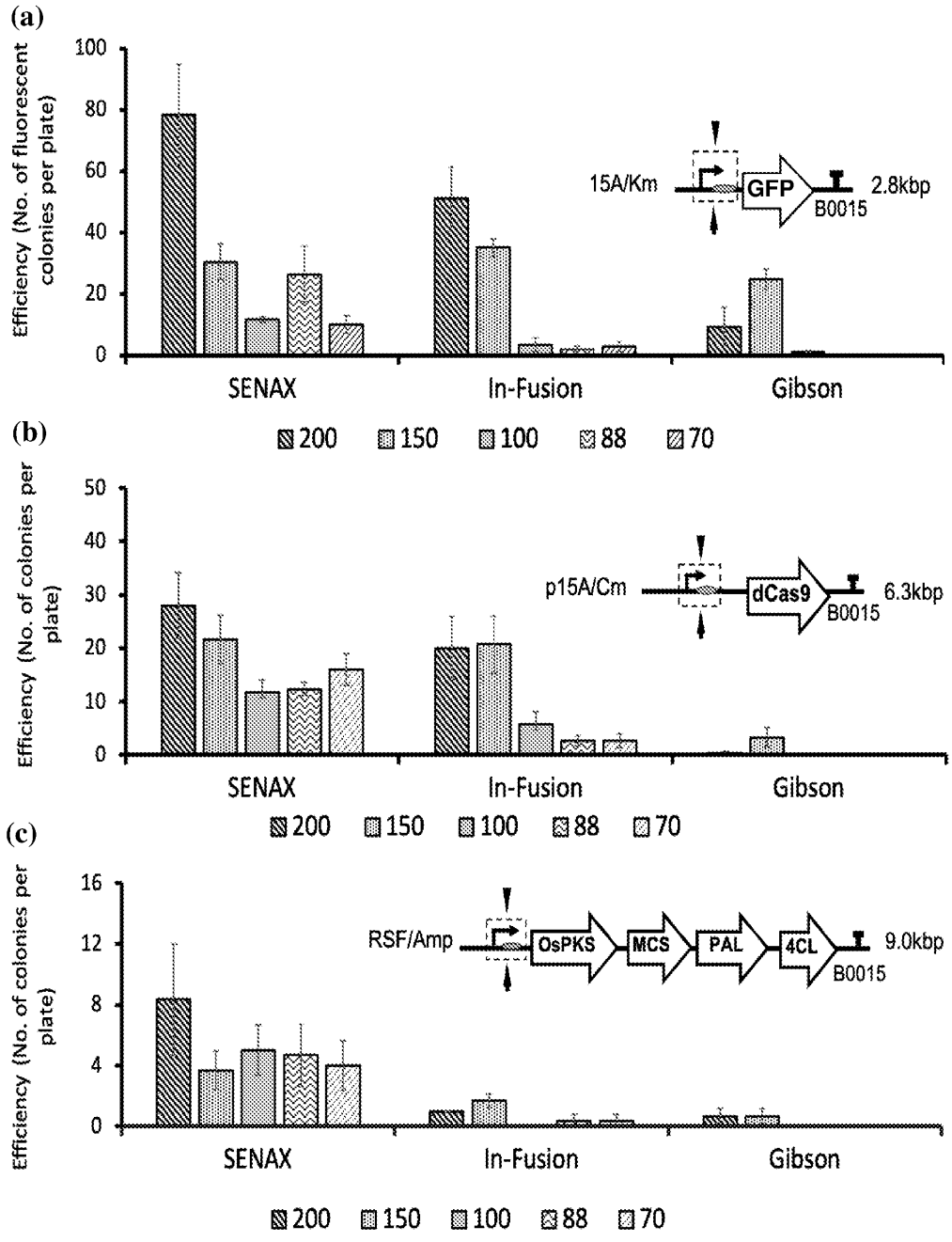
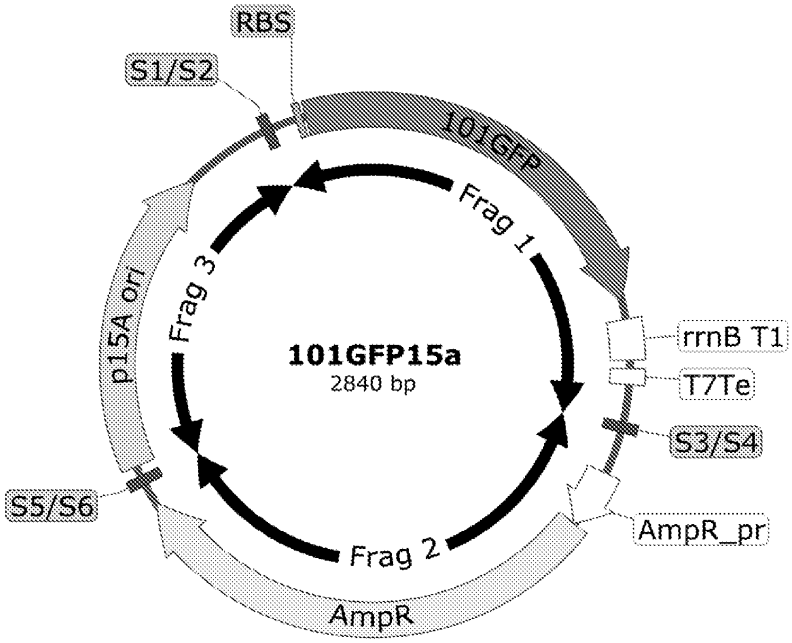

(a)
Efficiency (No. of fluorescent colonies per plate)
100
80
60
40
20
0
15A/Km
GFP
B0015
2.8kbp
SENAX
In-Fusion
Gibson
200
150
100
88
70
(b)
Efficiency (No. of colonies per plate)
50
40
30
20
10
0
p15A/Cm
dCas9
B0015
6.3kbp
SENAX
In-Fusion
Gibson
200
150
100
88
70
(c)
Efficiency (No. of colonies per plate)
16
12
8
4
0
RSF/Amp
OsPKS
MCS
PAL
4CL
B0015
9.0kbp
SENAX
In-Fusion
Gibson
200
150
100
88
70

Fig. 2

(a) 3-fragment



4-fragment

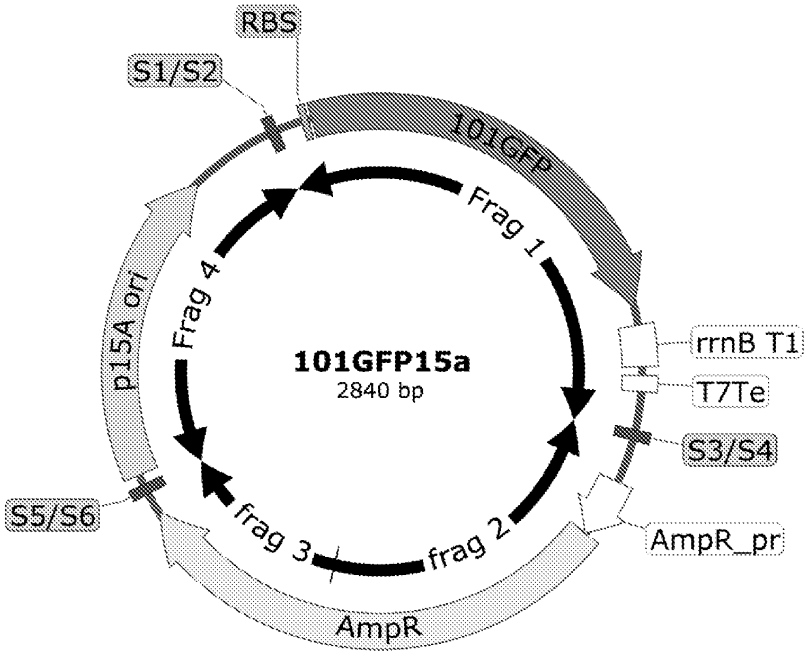
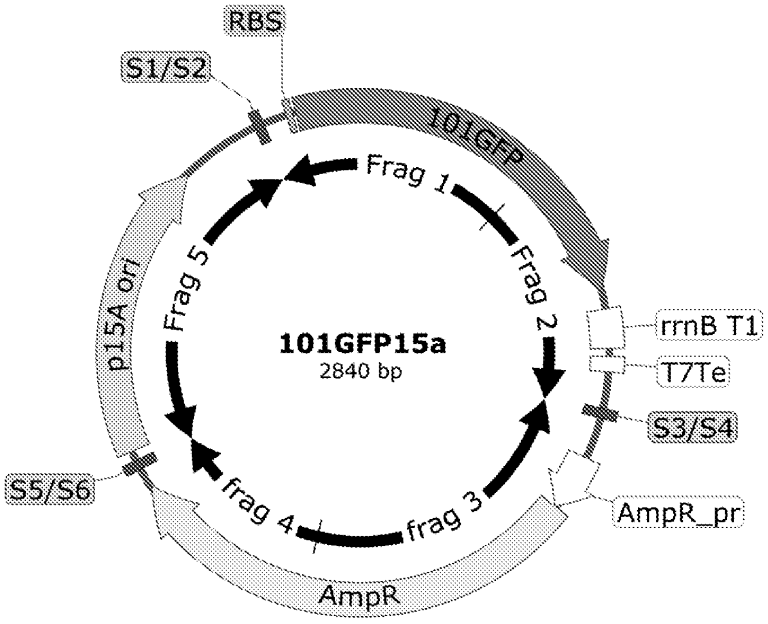


Fig. 3

5-fragment



6-fragment

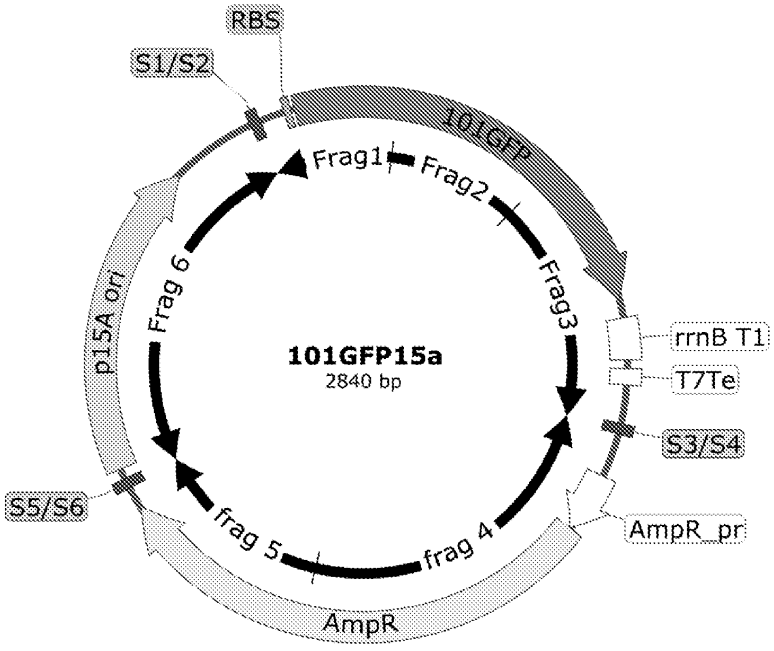
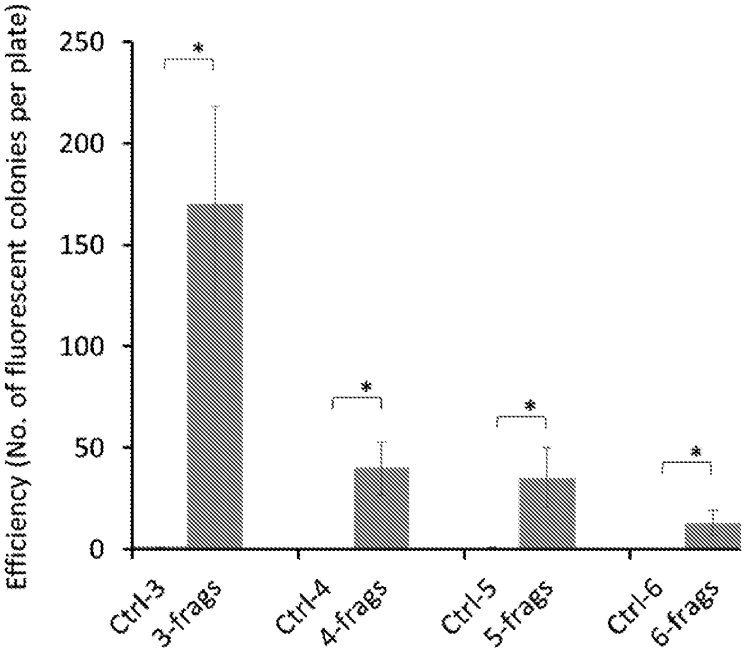


Fig. 3a (cont.)

(a) (cont.)



(b)

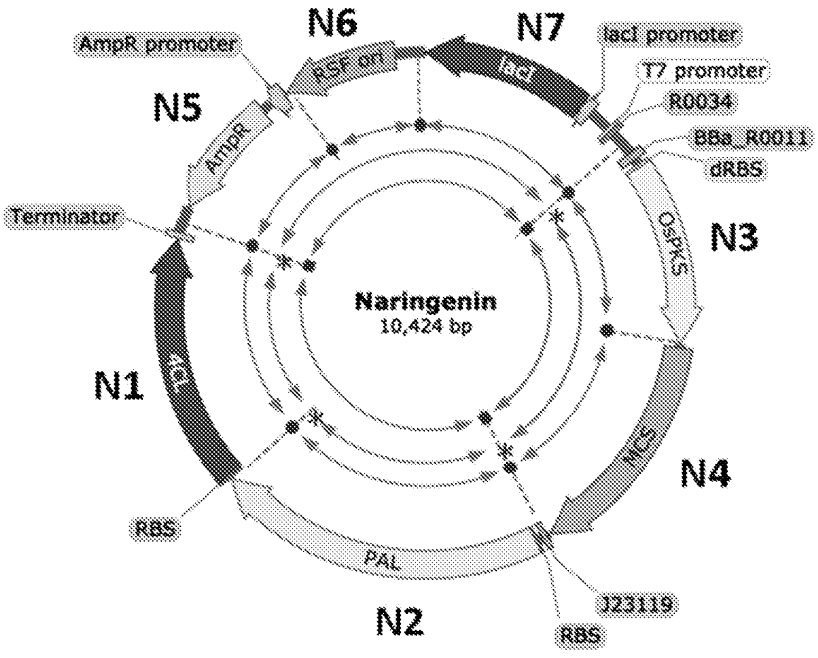


Fig. 3 (cont.)

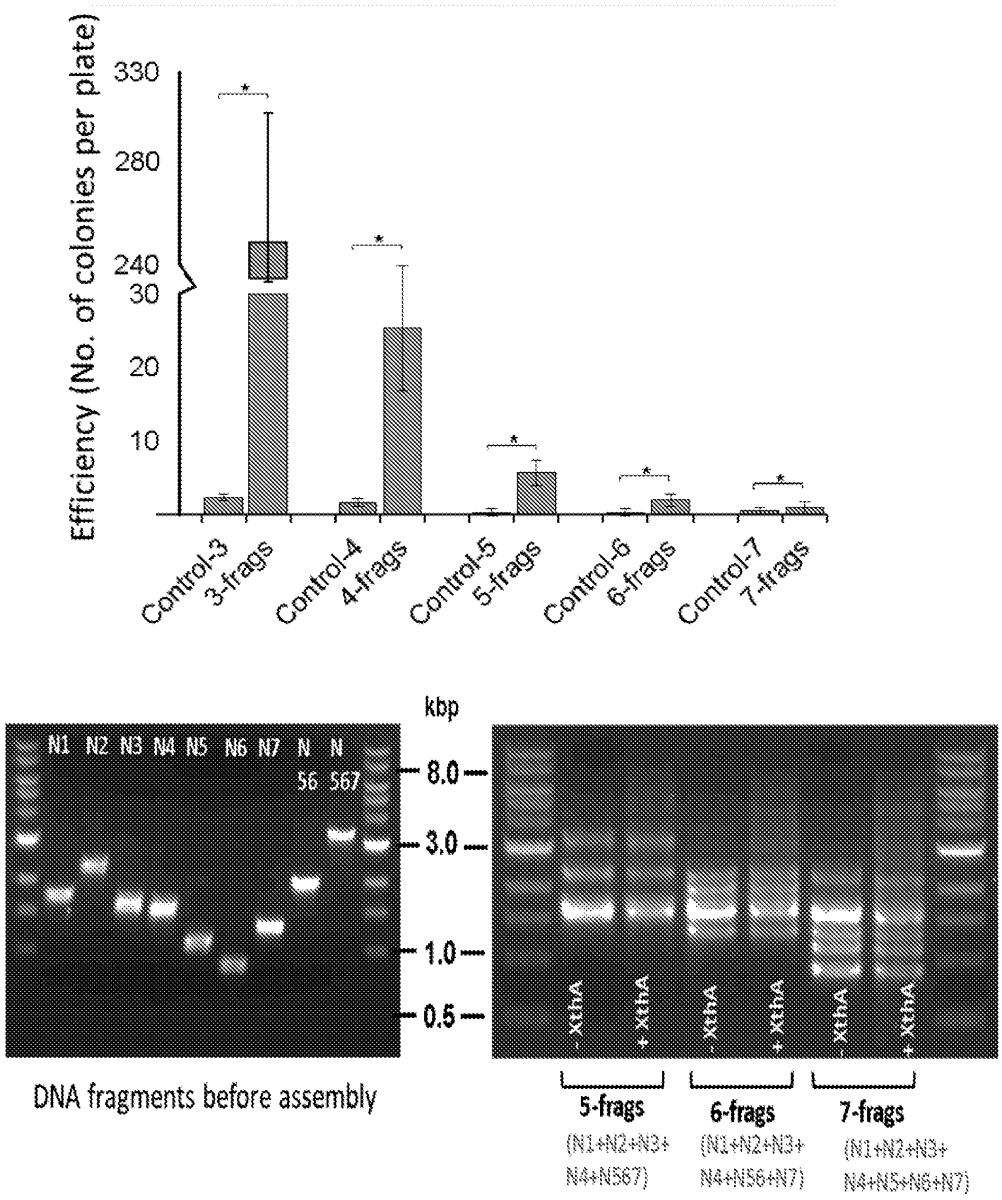

Efficiency (No. of colonies per plate)
330
280
240
30
20
10
Control-3 3-frags
Control-4 4-frags
Control-5 5-frags
Control-6 6-frags
Control-7 7-frags
kbp
N1 N2 N3 N4 N5 N6 N7 N N
56 567
8.0
3.0
1.0
0.5
- XthA
+ XthA
DNA fragments before assembly
5-frags
(N1+N2+N3+ N4+N567)
6-frags
(N1+N2+N3+ N4+N56+N7)
7-frags
(N1+N2+N3+ N4+N5+N6+N7)

Fig. 3b (cont.)

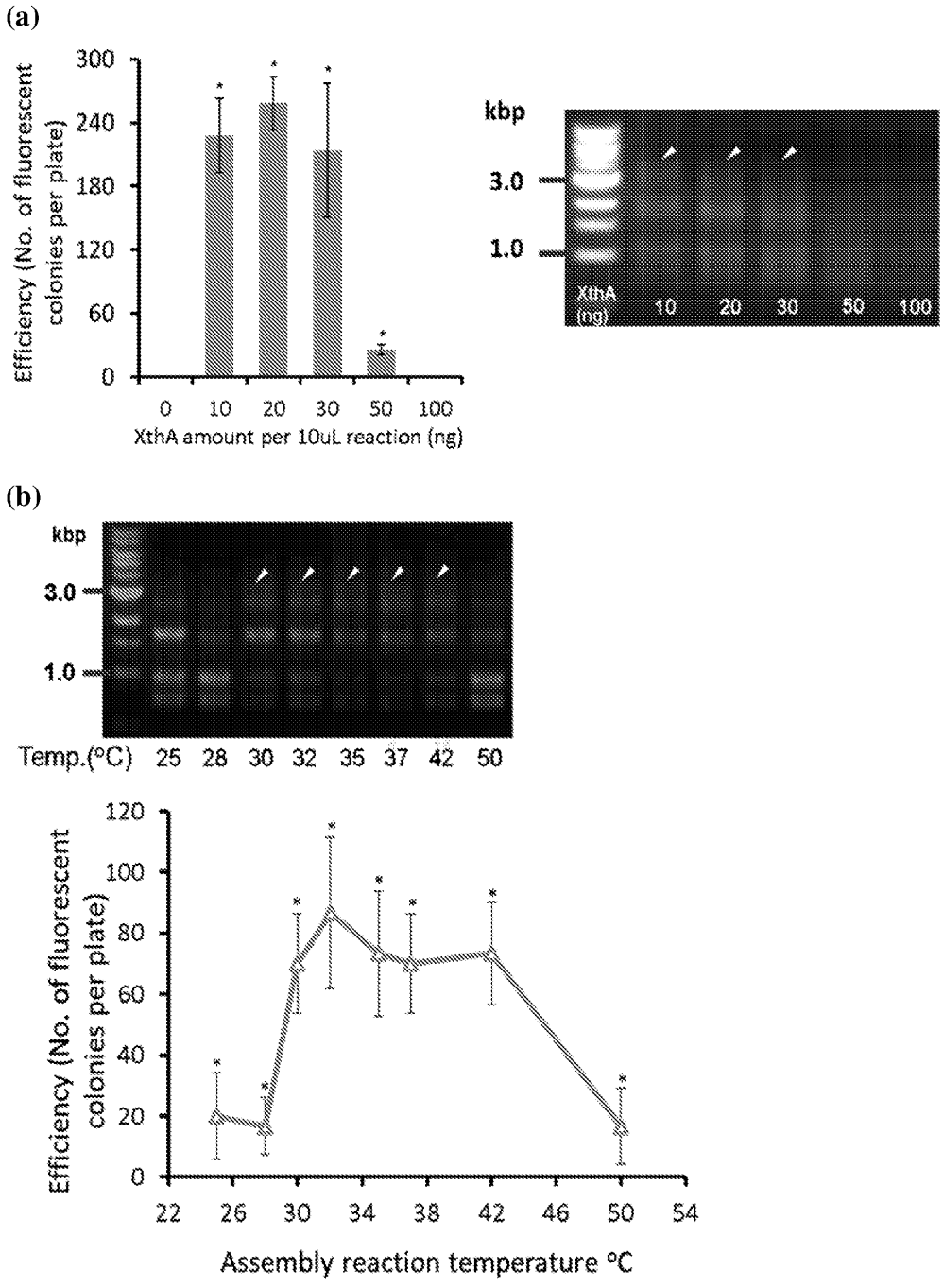

(a)
Efficiency (No. of fluorescent colonies per plate)
300
240
180
120
60
0
0 10 20 30 50 100
XthA amount per 10uL reaction (ng)
kbp
3.0
1.0
XthA (ng)
10 20 30 50 100
(b)
kbp
3.0
1.0
Temp.(°C) 25 28 30 32 35 37 42 50
Efficiency (No. of fluorescent colonies per plate)
120
100
80
60
40
20
0
22 26 30 34 38 42 46 50 54
Assembly reaction temperature °C

Fig. 4

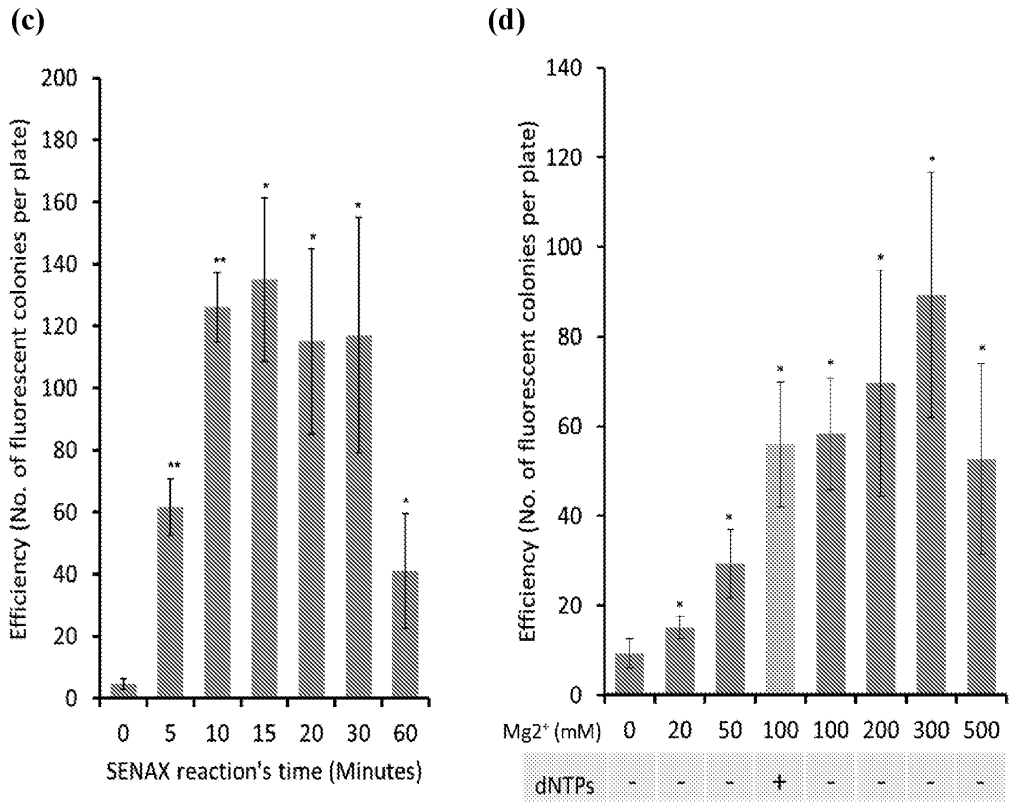

(c)
Efficiency (No. of fluorescent colonies per plate)
200
180
160
140
120
100
80
60
40
20
0
0 5 10 15 20 30 60
SENAX reaction's time (Minutes)
(d)
Efficiency (No. of fluorescent colonies per plate)
140
120
100
80
60
40
20
0
Mg2+ (mM) 0 20 50 100 100 200 300 500
dNTPs - - - + - - - -

Fig. 4 (cont.)

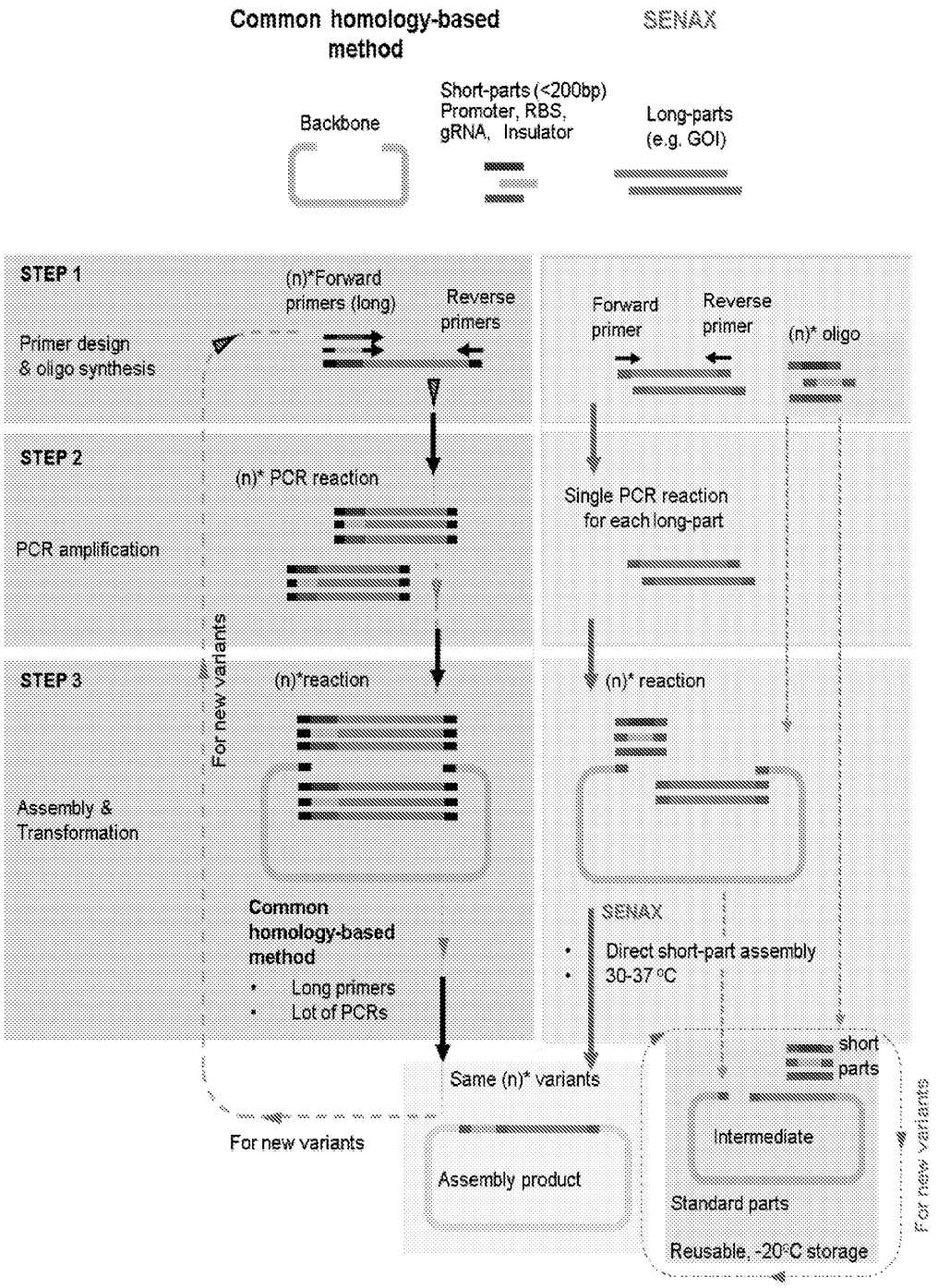

Common homology-based method
SENAX
Backbone
Short-parts (<200bp) Promoter, RBS, gRNA, Insulator
Long-parts (e.g. GOI)
STEP 1
Primer design & oligo synthesis
(n)*Forward primers (long)
Reverse primers
Forward primer
Reverse primer
(n)* oligo
STEP 2
PCR amplification
(n)* PCR reaction
Single PCR reaction for each long-part
STEP 3
Assembly & Transformation
(n)*reaction
(n)* reaction
For new variants
Common homology-based method
• Long primers
• Lot of PCRs
SENAX
• Direct short-part assembly
• 30-37 °C
short parts
Same (n)* variants
For new variants
Intermediate
Assembly product
Standard parts
Reusable, -20°C storage
For new variants

Fig. 5

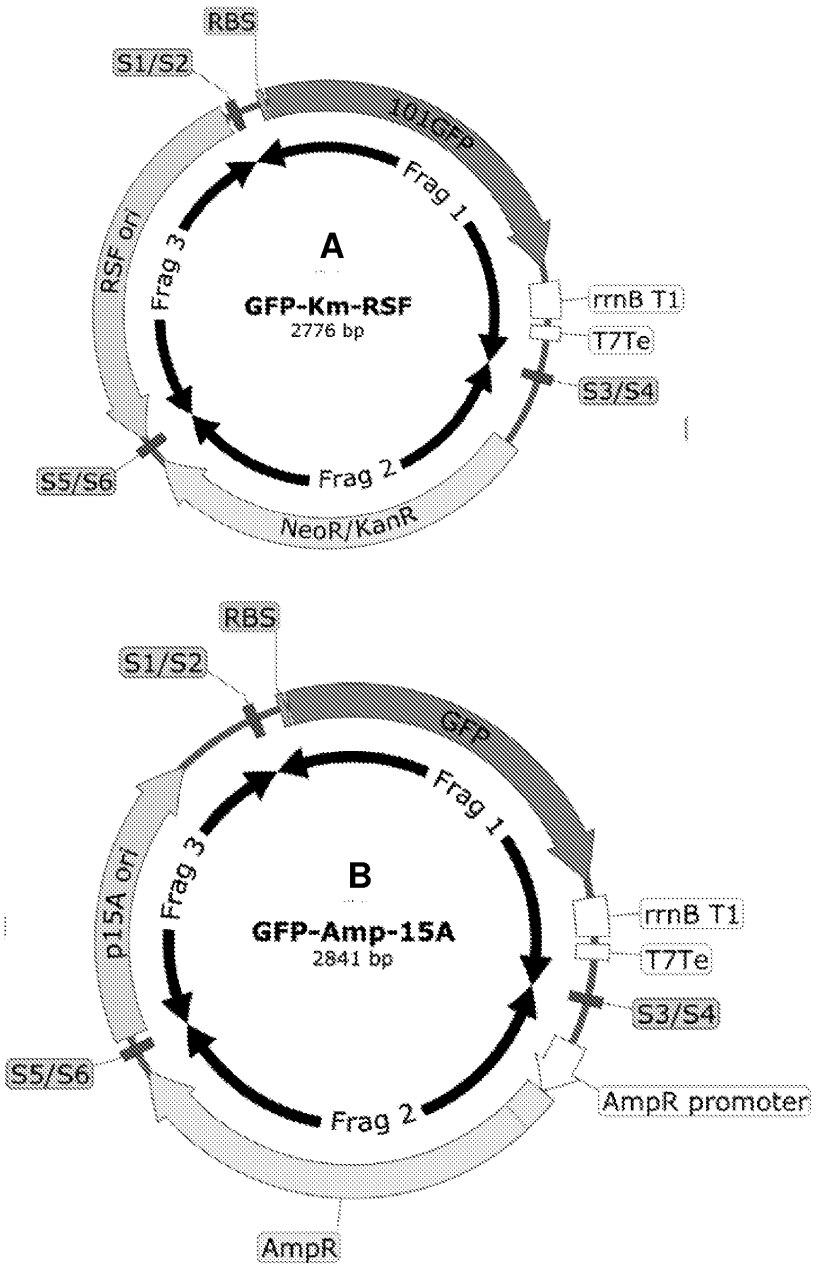

RBS
S1/S2
101GFP
Frag 1
A
GFP-Km-RSF
2776 bp
RSF ori
Frag 3
rrnB T1
T7Te
S3/S4
S5/S6
Frag 2
NeoR/KanR
RBS
S1/S2
GFP
Frag 1
B
GFP-Amp-15A
2841 bp
p15A ori
Frag 3
rrnB T1
T7Te
S3/S4
S5/S6
Frag 2
AmpR promoter
AmpR

Fig. 6

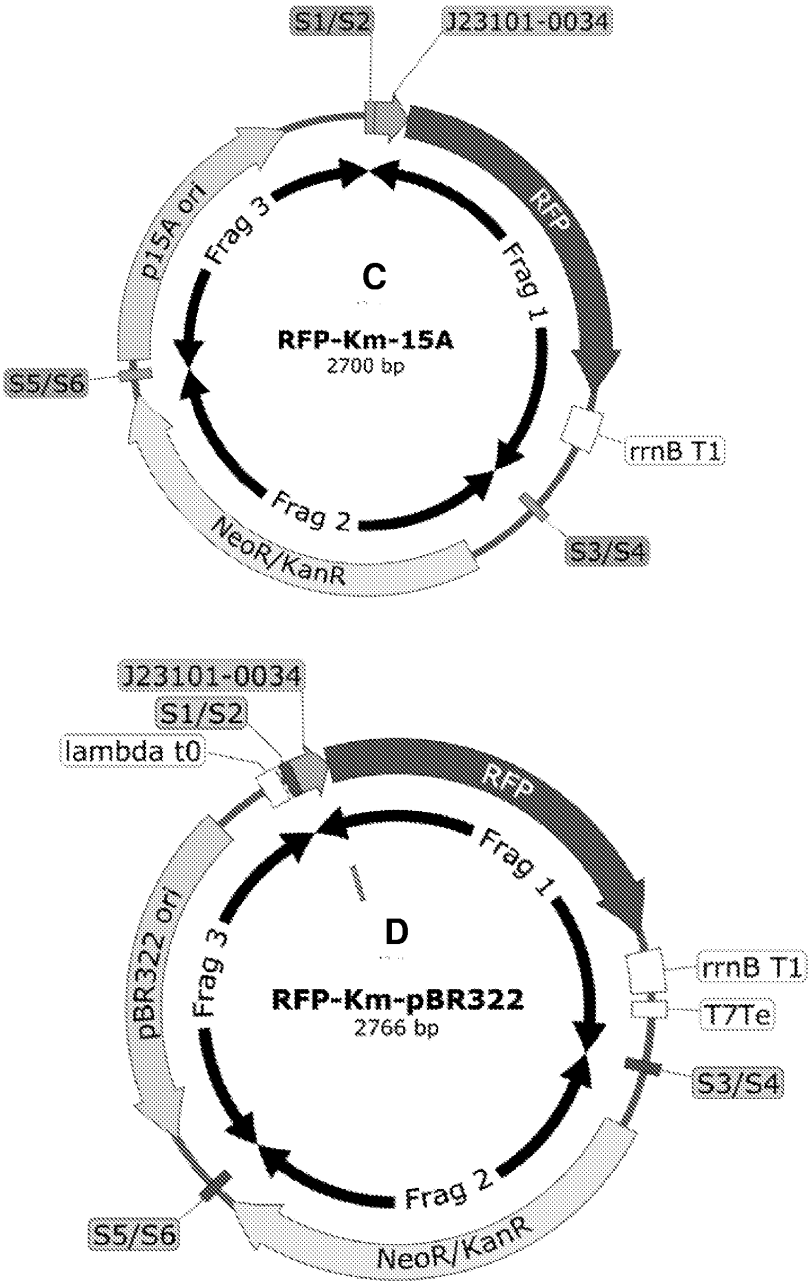

S1/S2
J23101-0034
RFP
Frag 1
rrnB T1
S3/S4
NeoR/KanR
Frag 2
S5/S6
p15A ori
Frag 3
C
RFP-Km-15A
2700 bp
J23101-0034
S1/S2
lambda t0
RFP
Frag 1
rrnB T1
T7Te
S3/S4
NeoR/KanR
Frag 2
S5/S6
pBR322 ori
Frag 3
D
RFP-Km-pBR322
2766 bp

Fig. 6 (cont.)

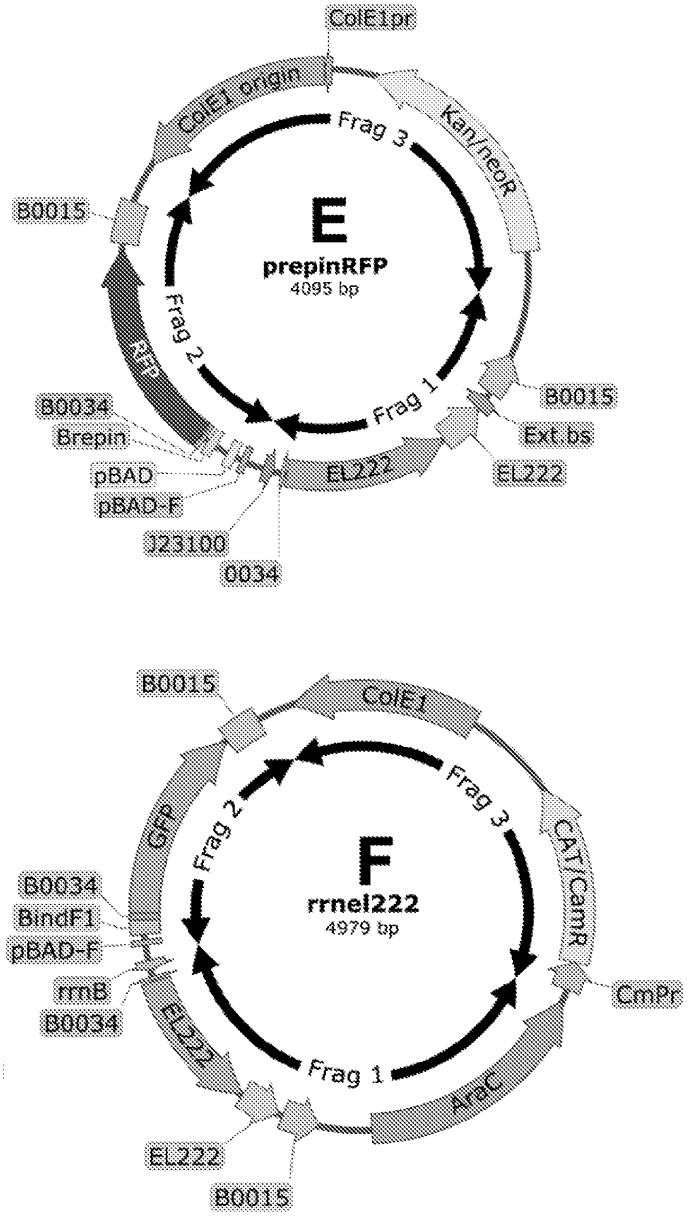

ColE1pr
ColE1 origin
Kan/neoR
Frag 3
E
prepinRFP
4095 bp
B0015
Frag 2
RFP
Frag 1
B0015
Ext.bs
EL222
EL222
B0034
Brepin
pBAD
pBAD-F
J23100
0034
B0015
ColE1
Frag 3
CAT/CamR
CmPr
Frag 2
GFP
F
rrnel222
4979 bp
B0034
BindF1
pBAD-F
rrnB
B0034
EL222
Frag 1
AraC
EL222
B0015

Fig. 6 (cont.)

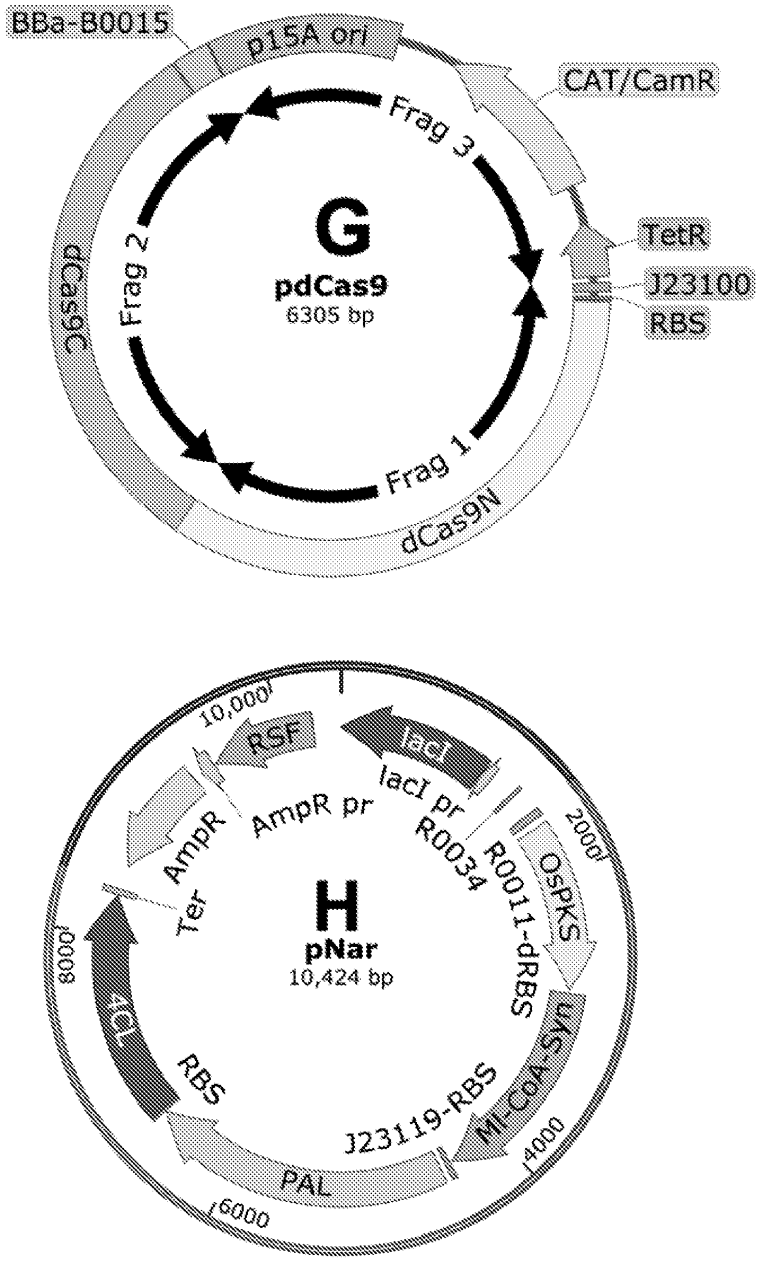
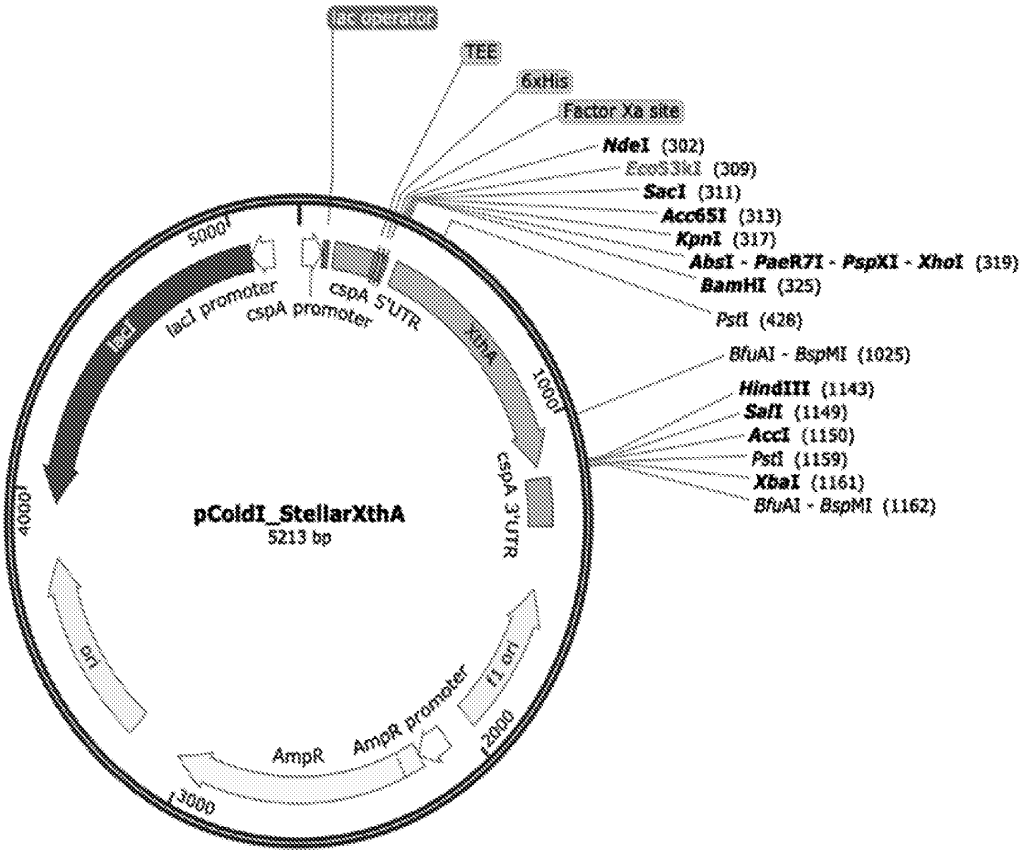

BBa-B0015
p15A ori
CAT/CamR
Frag 3
G
pdCas9
6305 bp
TetR
J23100
RBS
dCas9C
Frag 2
Frag 1
dCas9N
10,000
RSF
lacI
lacI pr
AmpR pr
AmpR
R0034
R0011-dRBS
OsPKS
2000
Ter
H
pNar
10,424 bp
8000
4CL
RBS
J23119-RBS
MI-CoA-SYN
4000
PAL
6000

Fig. 6 (cont.)




```
  1 MKFVSFNING LRARPHQLEA IVEKHQPDVI GLQETKVHDD MFPLEEVAKL
 51 GYNVFYHGQK GHYGVALLTK ETPIAVRRGF PGDDEEAQRR IIMAEIPSPL
101 GNVTVINGYF PQGESRDHPI KFPAKAQFYQ NLQNYLETEL KRENPVLIMG
151 DMNISPGDLD IGIGEENRKR WLRTGKCSFL PEEREWMERL MSWGLVDTFR
201 HANPQTADRF SWFDYRSKGF DDNRGLRIDL LLASQPLAEC CVETGIDYEI
251 RSMEKPSDHA PVWATFRR (SEQ ID NO: 2)
```

Fig. 7

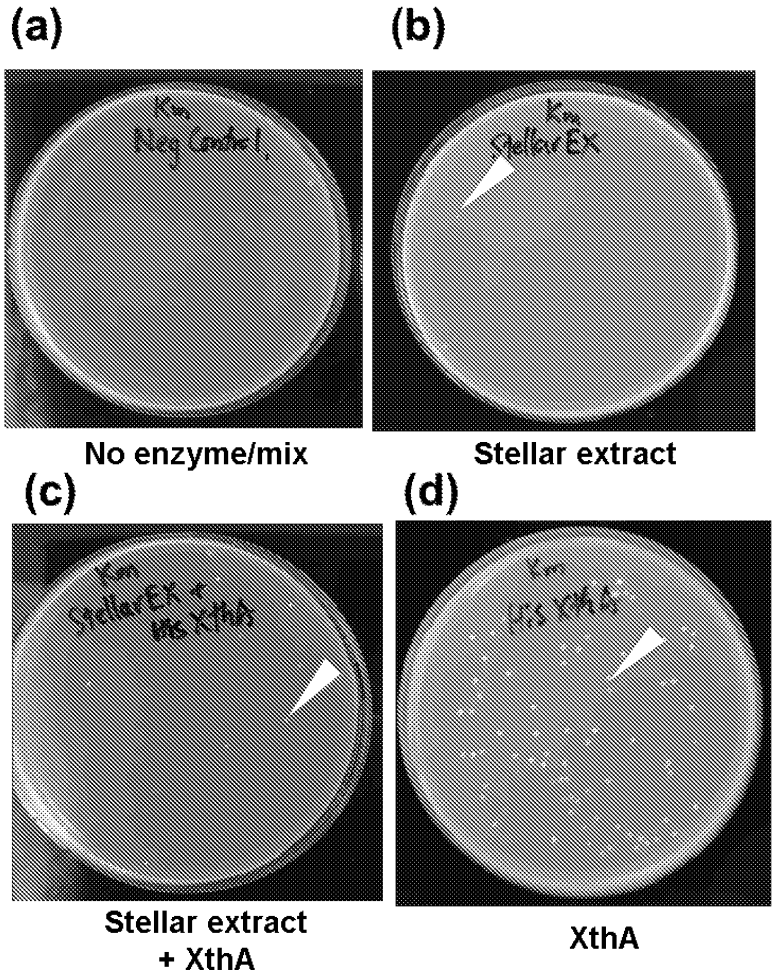

(a)
(b)
No enzyme/mix
Stellar extract
(c)
(d)
Stellar extract
+ XthA
XthA

Fig. 8

(a)

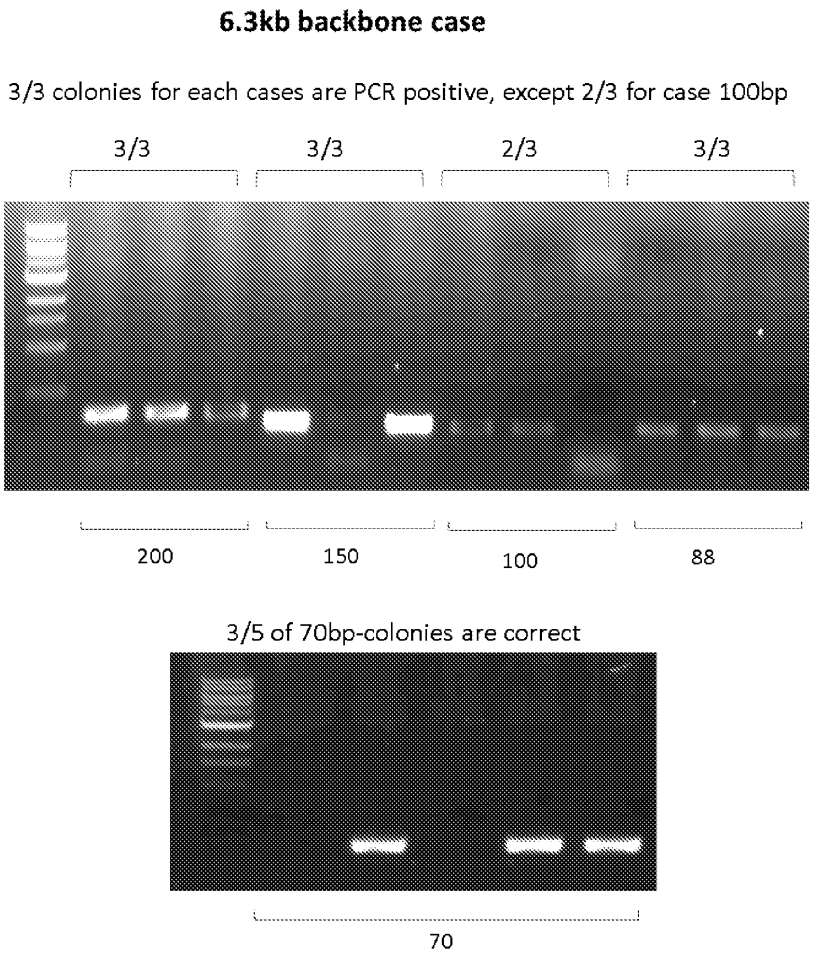

6.3kb backbone case
3/3 colonies for each cases are PCR positive, except 2/3 for case 100bp
3/3
3/3
2/3
3/3
200
150
100
88
3/5 of 70bp-colonies are correct
70

Fig. 9

(b)

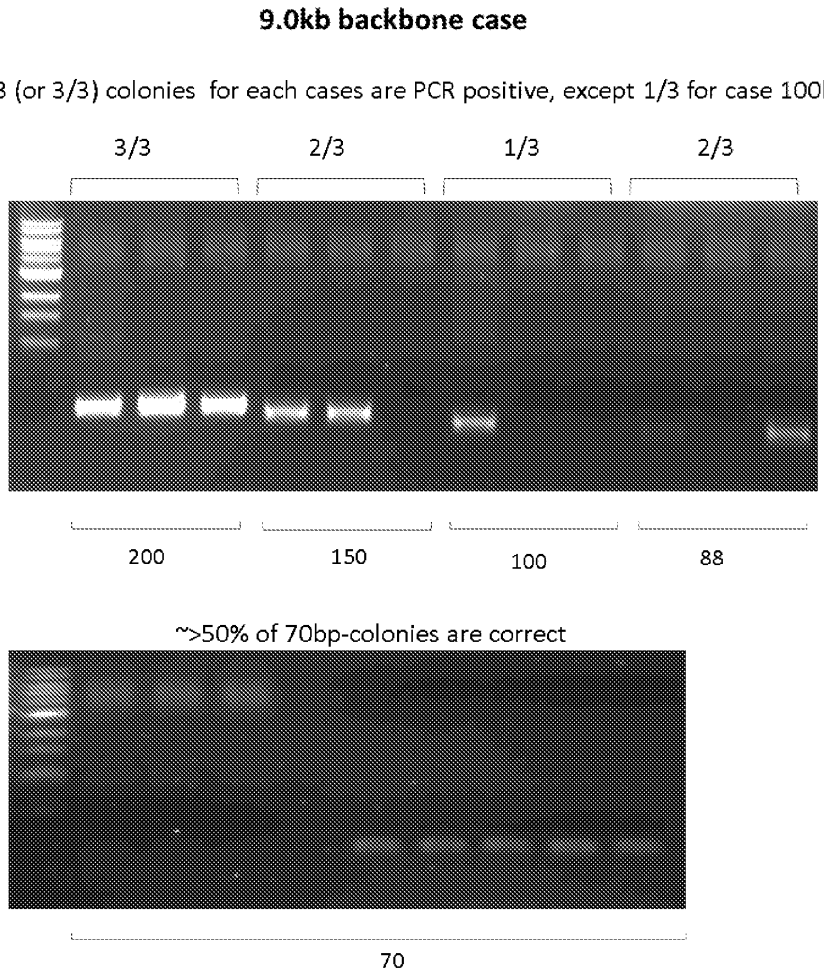

9.0kb backbone case
2/3 (or 3/3) colonies for each cases are PCR positive, except 1/3 for case 100bp
3/3
2/3
1/3
2/3
200
150
100
88
~>50% of 70bp-colonies are correct
70

Fig. 9 (cont.)

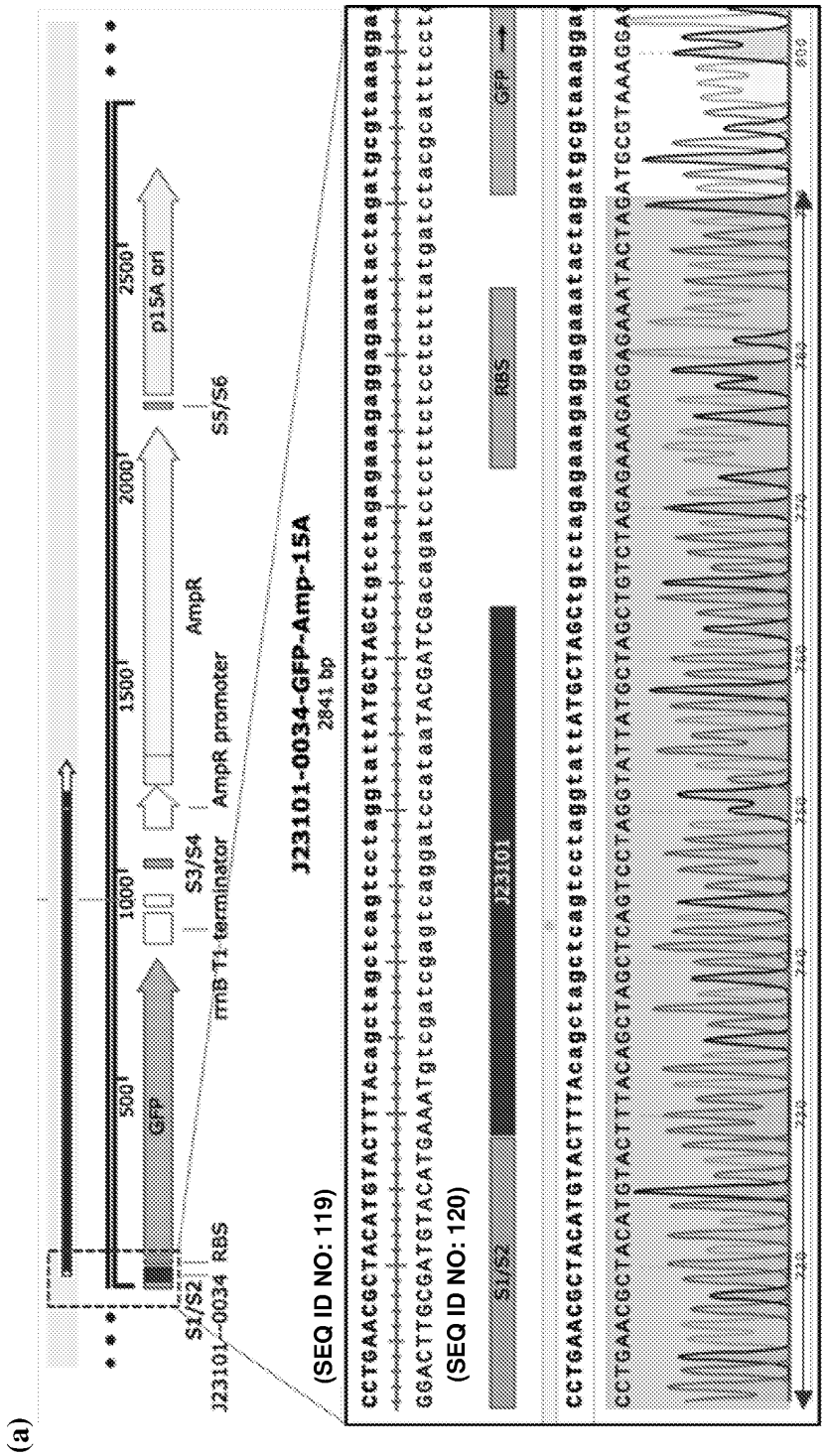

(a)
500
1000
1500
2000
2500
GFP
p15A ori
S1/S2
J23101_0034 RBS
rrnB T1 terminator
S3/S4
AmpR promoter
AmpR
S5/S6
J23101-0034-GFP-Amp-15A
2841 bp
(SEQ ID NO: 119)
CCTGAACGCTACATGTACTTTACagctagctcagtcctaggtattATGCTAGctgtctagagaaagaggagaaatactagatgcgtaaagga
GGACTTGCGATGTACATGAAATgtcgatcgagtccaggatccataaTACGATCGacagatctctttctcctctttatgatctacgcatttcct
(SEQ ID NO: 120)
S1/S2
J23101
RBS
GFP
CCTGAACGCTACATGTACTTTACagctagctcagtcctaggtattATGCTAGctgtctagagaaagaggagaaatactagatgcgtaaagga
CCTGAACGCTACATGTACTTTACAGCTAGCTCAGTCCTAGGTATTATGCTAGCTGTCTAGAGAAAGAGGAGAAATACTAGATGCGTAAAGGA
88bp

Fig. 10

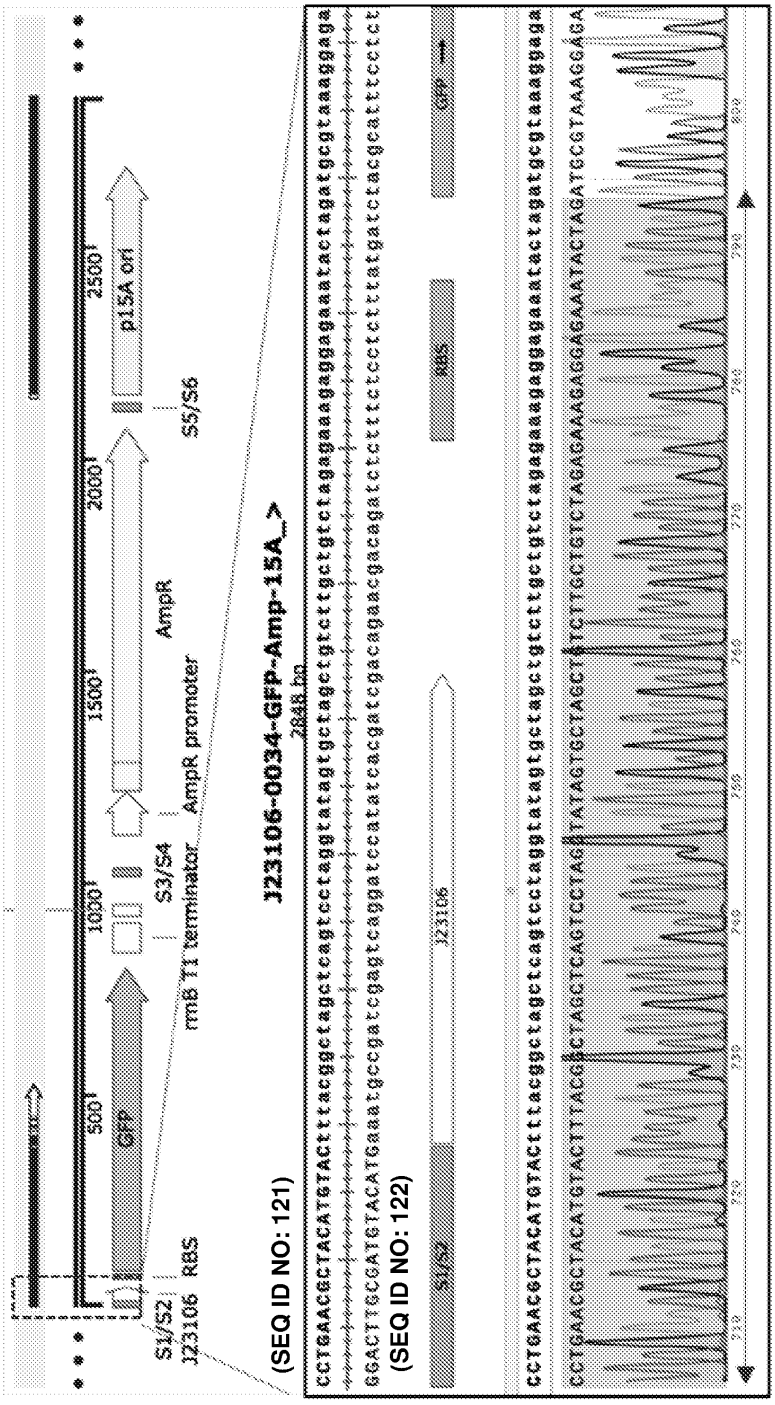

(b)
J23106-0034-GFP-Amp-15A_>
2848 bp
(SEQ ID NO: 121)
(SEQ ID NO: 122)
S1/S2
J23106
RBS
GFP
rrnB T1 terminator
S3/S4
AmpR promoter
AmpR
p15A ori
S5/S6
88bp

Fig. 10 (cont.)

(c)

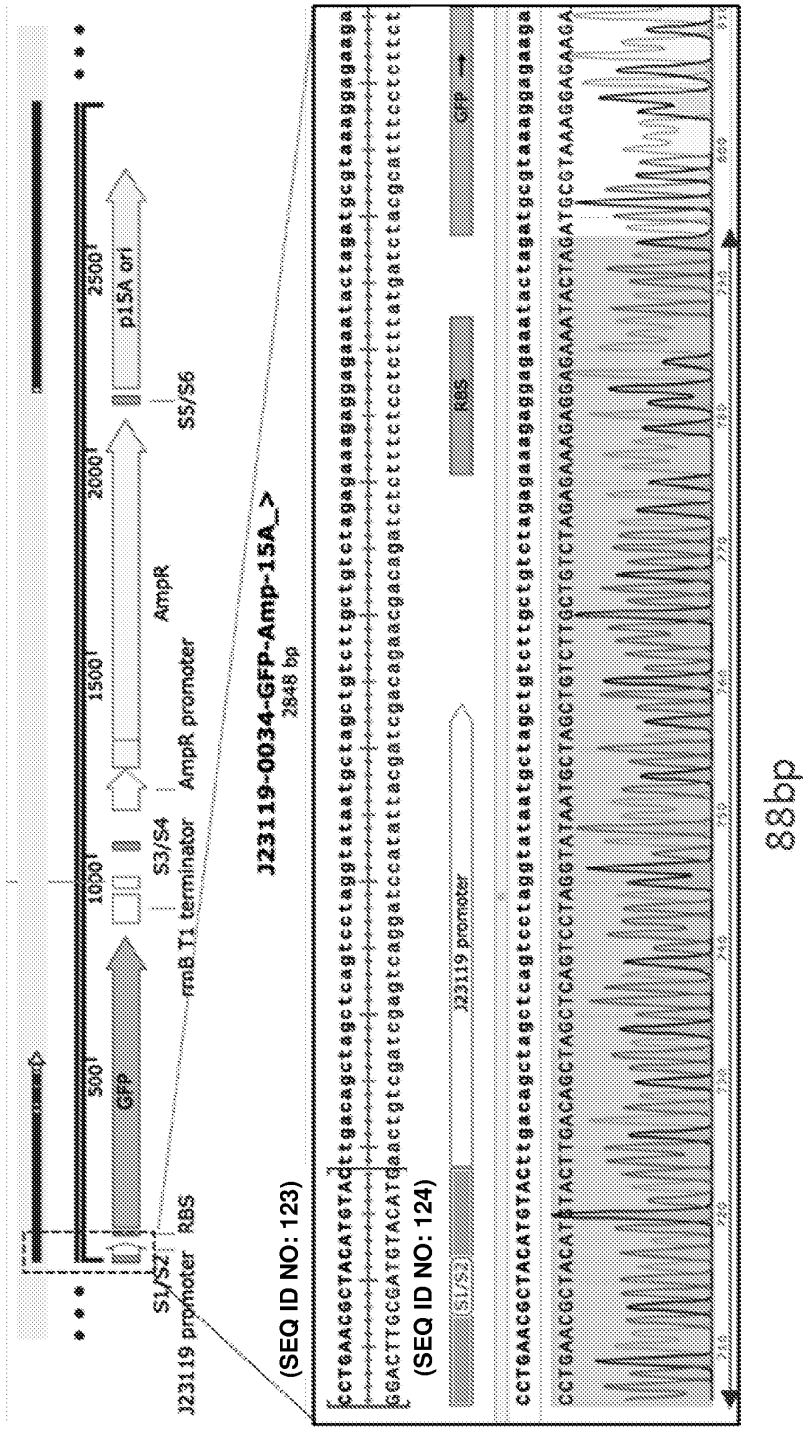

J23119-0034-GFP-Amp-15A_>
2848 bp
(SEQ ID NO: 123)
(SEQ ID NO: 124)
J23119 promoter
S1/S2
RBS
GFP
500
1000
1500
2000
2500
rrnB T1 terminator
S3/S4
AmpR
AmpR promoter
p15A ori
S5/S6
88bp

Fig. 10 (cont.)

(d)

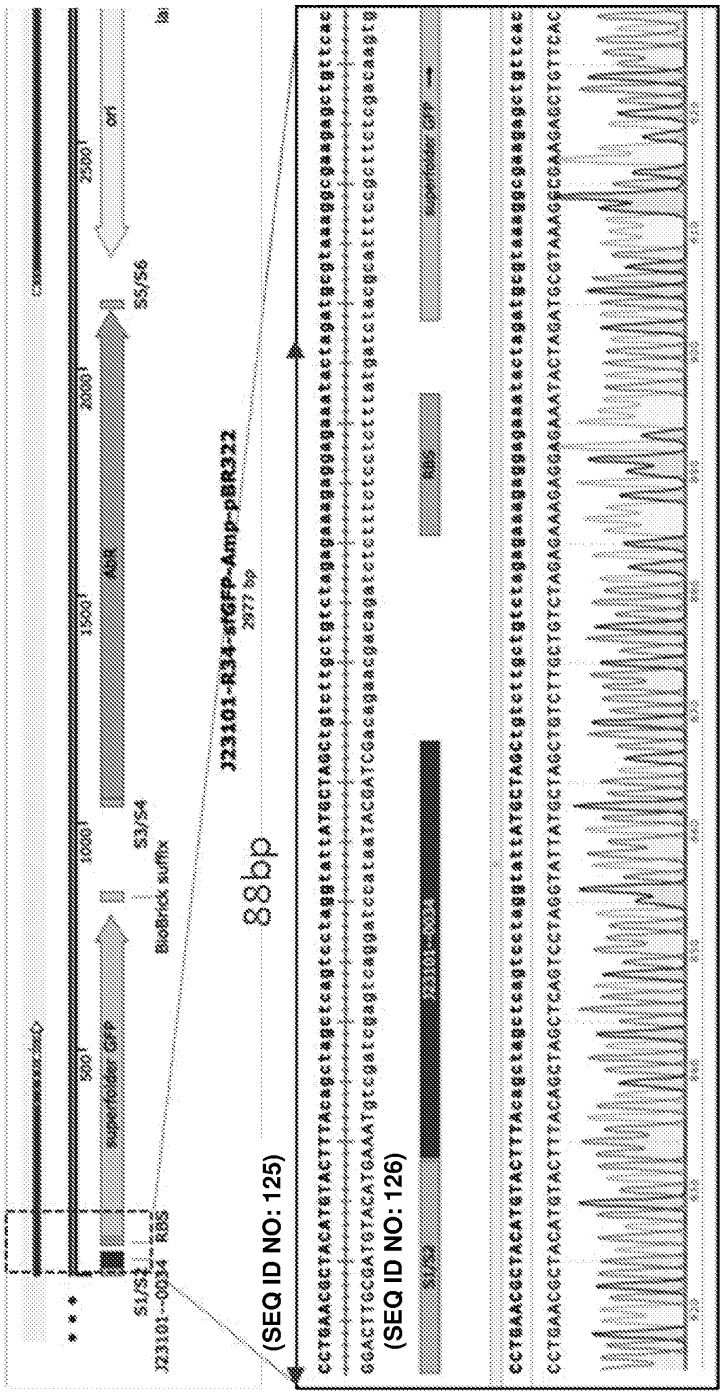

J23101-R34-sfGFP-Amp-pBR322
2977 bp
88bp
(SEQ ID NO: 125)
(SEQ ID NO: 126)
superfolder GFP
BioBrick suffix
S1/S2
S3/S4
S5/S6
RBS
J23101--0034
500
1000
1500
2000
2500

Fig. 10 (cont.)

(e)

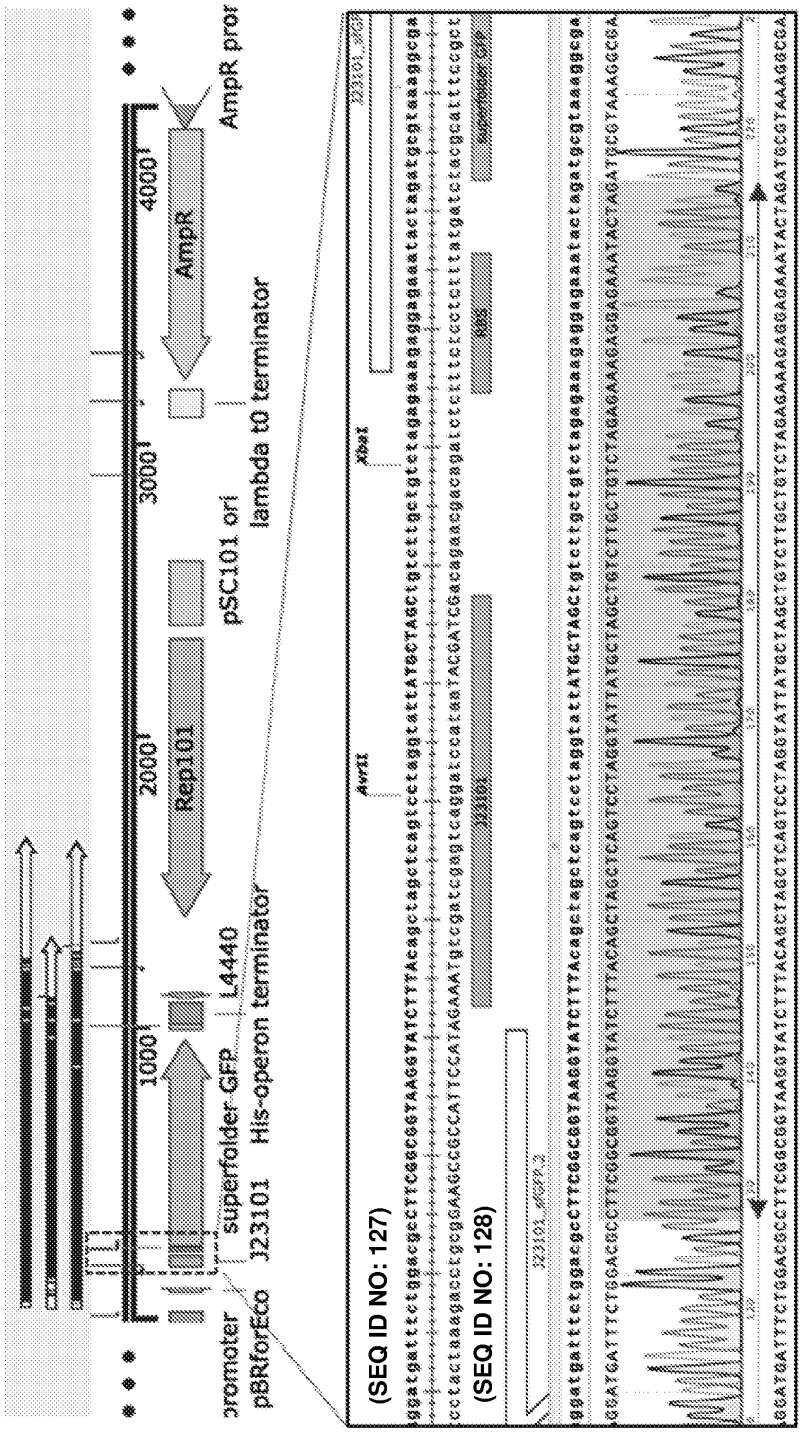
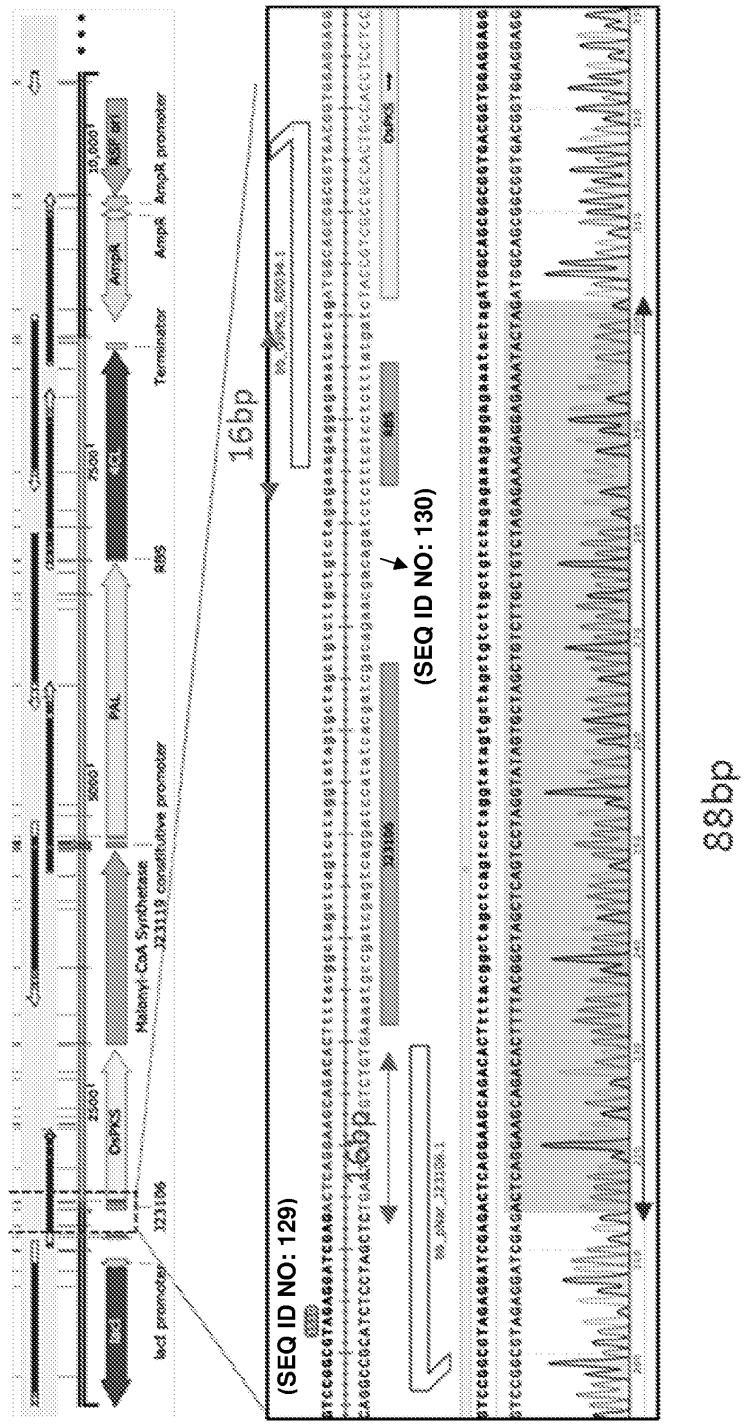
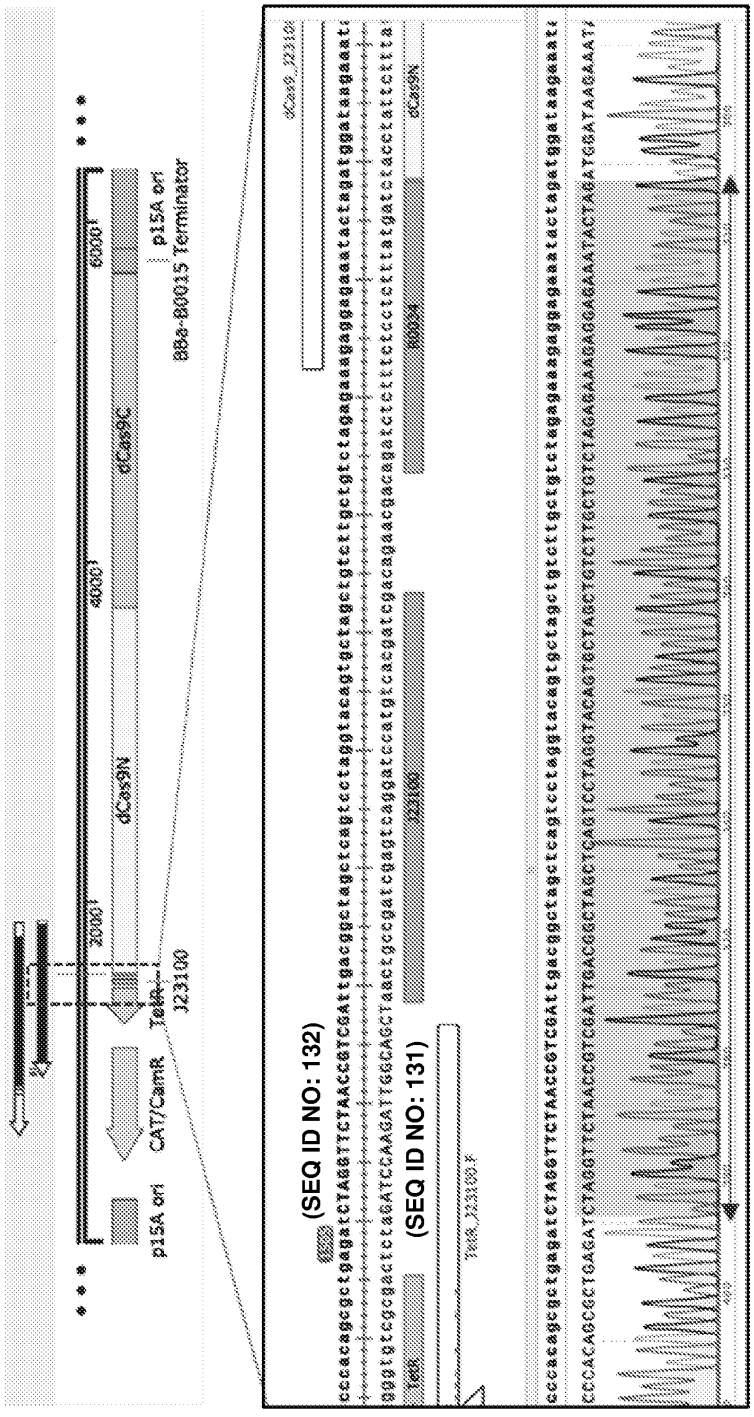

1000
2000
3000
4000
AmpR
AmpR pror
Rep101
pSC101 ori
lambda t0 terminator
superfolder GFP
L4440
His-operon terminator
pBRforEco
J23101
(SEQ ID NO: 127)
(SEQ ID NO: 128)
AvrII
XbaI
88bp

Fig. 10 (cont.)


(SEQ ID NO: 129)
(SEQ ID NO: 130)
16bp
16bp
88bp
lacI promoter
CoPICS
Malonyl-CoA Synthetase
PAL
RBS
Terminator
AmpR
AmpR promoter

(f)

Fig. 10 (cont.)


p15A ori
CAT/CamR
TetR
J23100
dCas9N
dCas9C
p15A ori
B0015 Terminator
(SEQ ID NO: 132)
(SEQ ID NO: 131)
88bp

(g)

Fig. 10 (cont.)

(h)

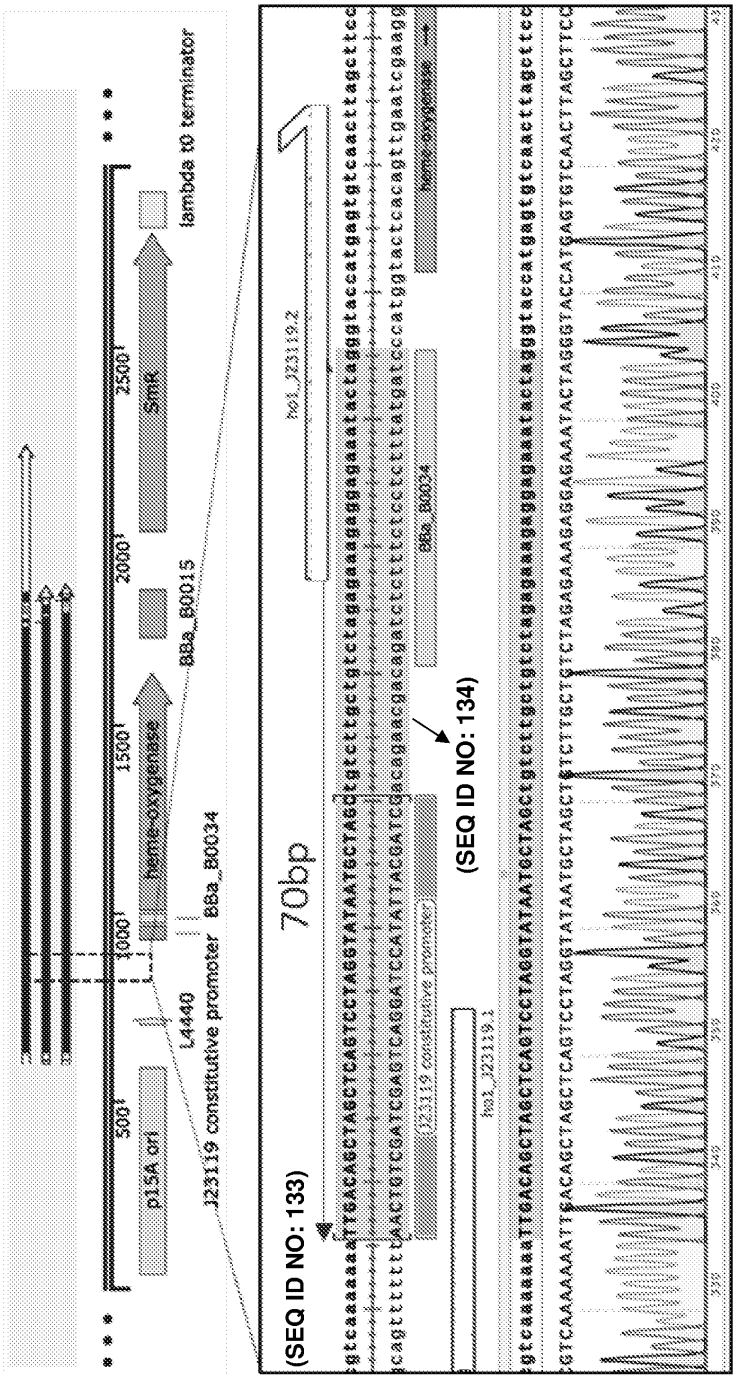
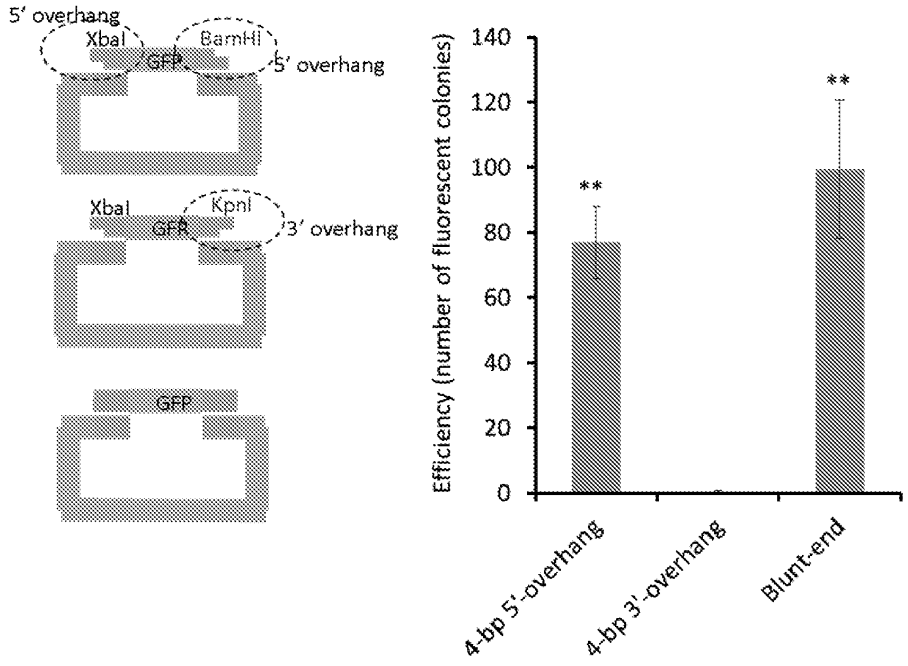

p15A ori
500
1000
1500
2000
2500
L4440
J23119 constitutive promoter
BBa_B0034
heme-oxygenase
BBa_B0015
SmR
lambda t0 terminator
(SEQ ID NO: 133)
70bp
hs1_J23119.2
(SEQ ID NO: 134)
BBa_B0034
heme-oxygenase
hs1_J23119.1

Fig. 10 (cont.)

(a)

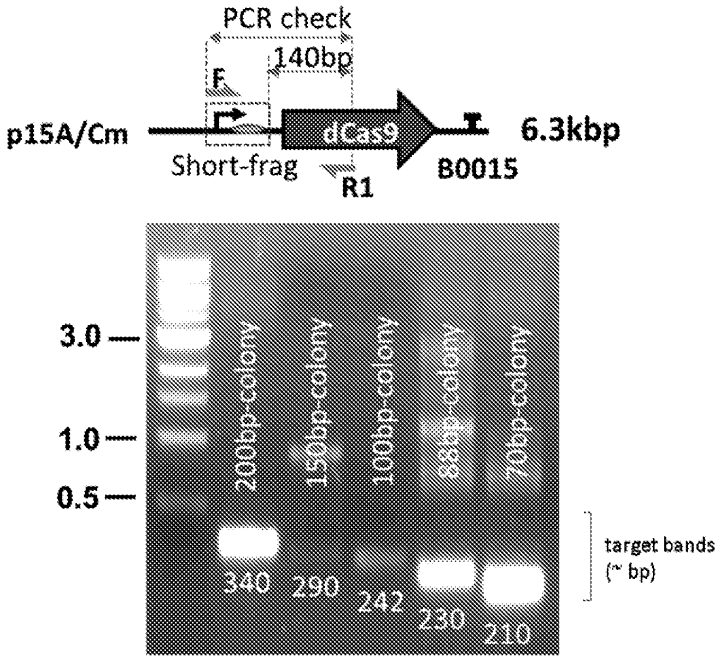

5' overhang
XbaI
BamHI
GFP
5' overhang
XbaI
KpnI
GFP
3' overhang
GFP
Efficiency (number of fluorescent colonies)
140
120
100
80
60
40
20
0
**
**
4-bp 5'-overhang
4-bp 3'-overhang
Blunt-end

(b)


(SEQ ID NO: 115)
15bp
(SEQ ID NO: 117)
15bp
(SEQ ID NO: 118)
(SEQ ID NO: 116)
Blunt-end
(SEQ ID NO: 61)
(SEQ ID NO: 62)
5'-prime overhang
(SEQ ID NO: 53)
(SEQ ID NO: 54)
3'-prime overhang
(SEQ ID NO: 55)
(SEQ ID NO: 56)
Backbone (3kb)

Fig. 11

(a)


PCR check
140bp
F
p15A/Cm
dCas9
6.3kbp
Short-frag
R1
B0015
3.0
1.0
0.5
200bp-colony
150bp-colony
100bp-colony
88bp-colony
70bp-colony
target bands
(~ bp)
340
290
242
230
210

(b)

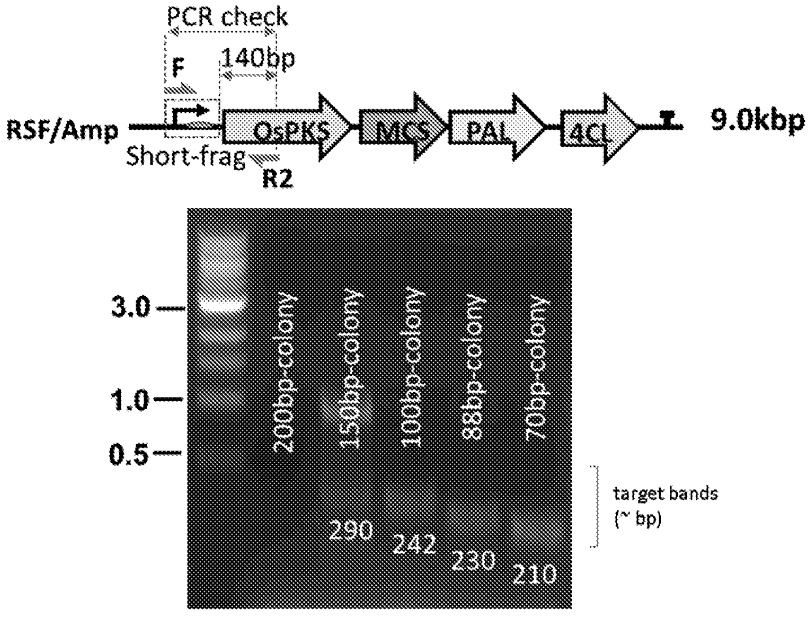

PCR check
F
140bp
RSF/Amp
OsPKS
MCS
PAL
4CL
9.0kbp
Short-frag
R2
3.0
1.0
0.5
200bp-colony
150bp-colony
100bp-colony
88bp-colony
70bp-colony
target bands
(~ bp)
290
242
230
210

Fig. 12

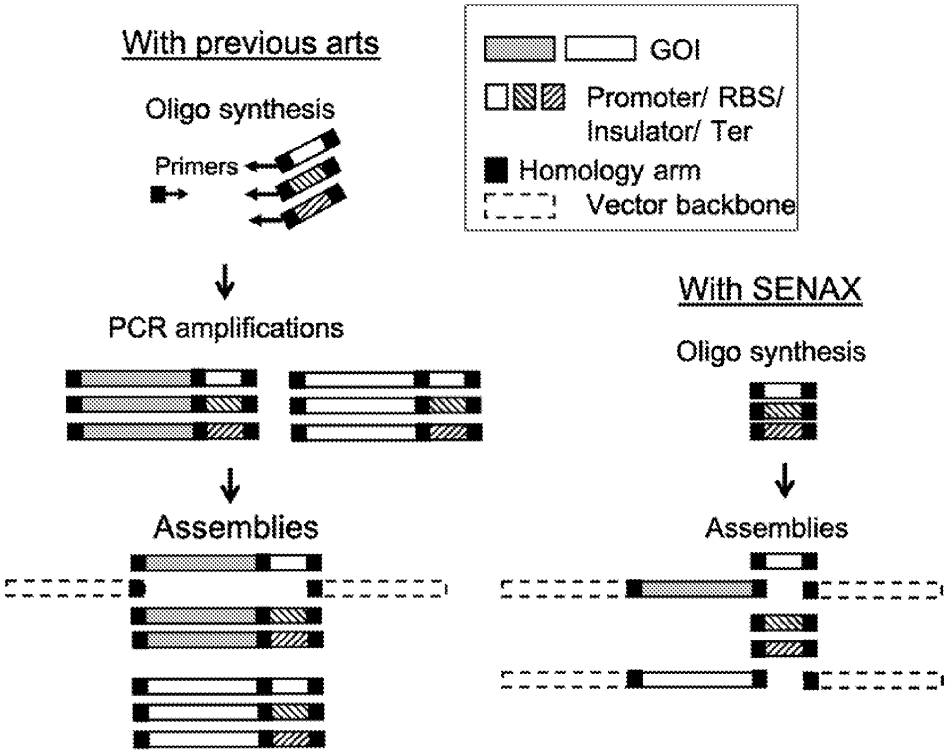

With previous arts
Oligo synthesis
Primers
PCR amplifications
Assemblies
GOI
Promoter/ RBS/ Insulator/ Ter
Homology arm
Vector backbone
With SENAX
Oligo synthesis
Assemblies

Fig. 13

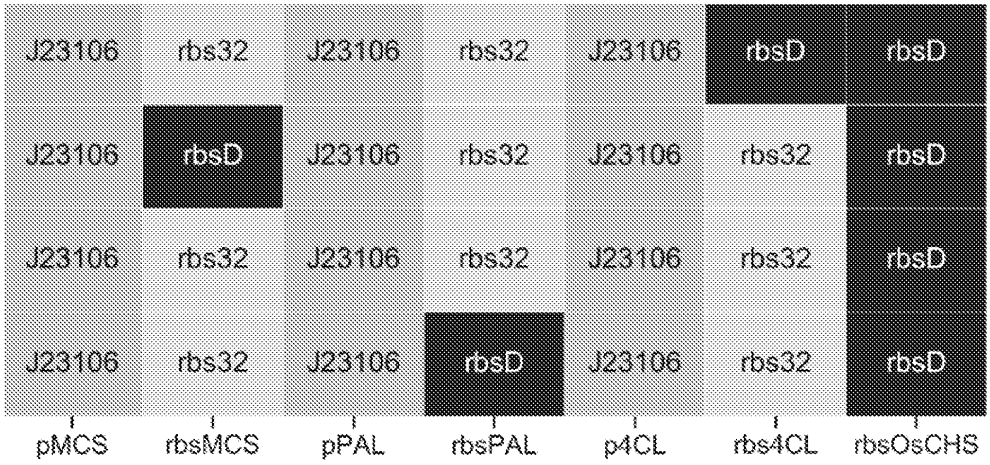

J23106
rbs32
rbsD
pMCS
rbsMCS
pPAL
rbsPAL
p4CL
rbs4CL
rbsOsCHS

Fig. 14

(a)

S/N	Short-fragment	Size (bp)	BackBone	Final Size	Function
1	promoter-RBS pLac-0034	114	RSF origin KanR	4.2 kb	Drive OsPKS gene's expression
2	promoter-RBS pLac-0032	115	RSF origin KanR	4.2 kb	Drive OsPKS gene's expression
3	promoter-RBS pLac-0029	117	RSF origin KanR	4.2 kb	Drive OsPKS gene's expression
4	promoter-RBS J23114-0034	88	RSF origin KanR	3.6 kb	Drive MCS gene's expression
5	promoter-RBS J23102-0034	88	RSF origin KanR	3.6 kb	Drive MCS gene's expression
6	promoter-RBS J23114-0029	89	RSF origin KanR	3.6 kb	Drive MCS gene's expression
7	promoter-RBS J23106-0029	89	RSF origin KanR	3.6 kb	Drive MCS gene's expression
8	promoter-RBS J23106-0034	88	RSF origin KanR	3.6 kb	Drive 4CL gene's expression
9	promoter-RBS J23101-0032	88	RSF origin KanR	3.6 kb	Drive 4CL gene's expression
10	promoter-RBS J23101-0029	89	RSF origin KanR	3.6 kb	Drive 4CL gene's expression
11	promoter-RBS J23102-0034	88	RSF origin KanR	3.6 kb	Drive 4CL gene's expression
12	promoter-RBS J23102-0034	88	15A origin SmR	4.4 kb	Drive gRNA expression
13	promoter-RBS J23106-0034	88	15A origin SmR	4.4 kb	Drive gRNA expression
14	promoter-RBS J23100-0034	88	15A origin CamR	6.4 kb	Drive a dCas9 domain's expression
15	promoter-RBS J23100-0034	88	15A origin CamR	6.9 kb	Drive a dCas9 domain's expression
16	promoter-RBS J23100-0034	88	15A origin CamR	9.1 kb	Drive a fusion protein expression
17	promoter-RBS J23106-0034	88	15A origin CamR	9.1 kb	Drive a fusion protein expression
18	promoter-RBS J23114-0034	88	15A origin CamR	9.1 kb	Drive a fusion protein expression

Fig. 15

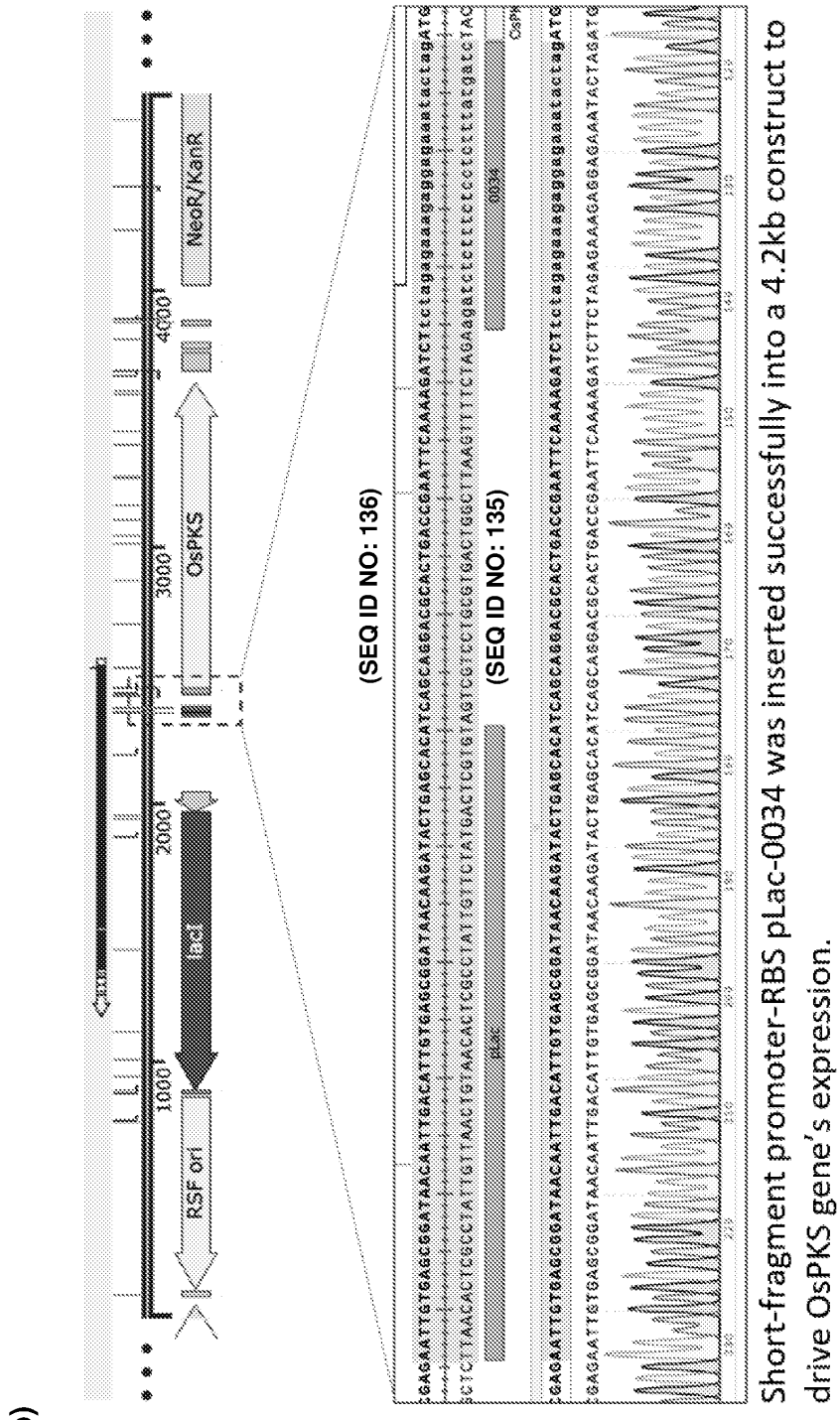

(b)
RSF ori
lacI
OsPKS
NeoR/KanR
1000
2000
3000
4000
(SEQ ID NO: 136)
(SEQ ID NO: 135)
Short-fragment promoter-RBS pLac-0034 was inserted successfully into a 4.2kb construct to drive OsPKS gene's expression.

Fig. 15 (cont.)

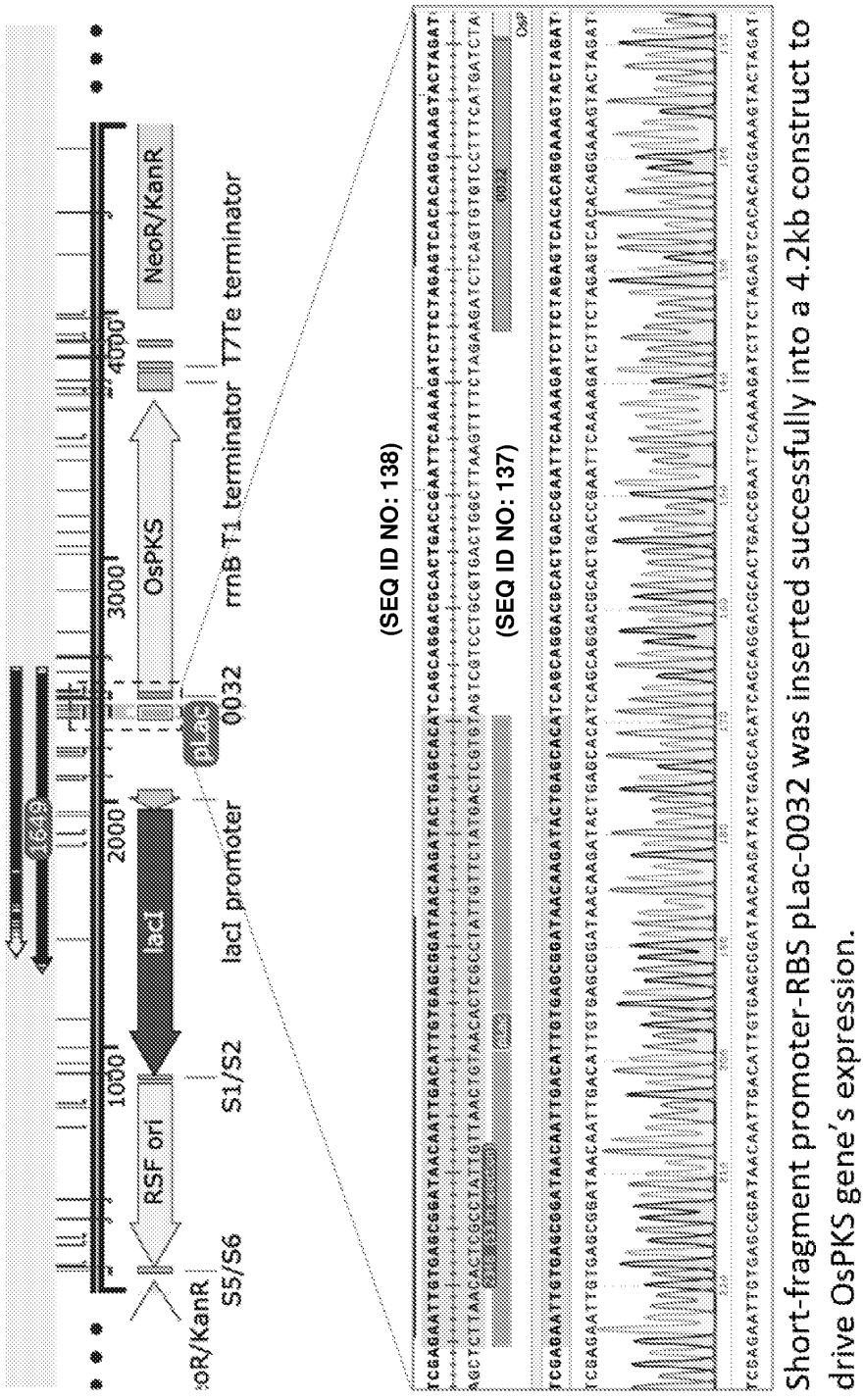

RSF ori
S1/S2
lacI promoter
0032
rrnB T1 terminator
T7Te terminator
NeoR/KanR
S5/S6
OsPKS
(SEQ ID NO: 138)
(SEQ ID NO: 137)
Short-fragment promoter-RBS pLac-0032 was inserted successfully into a 4.2kb construct to drive OsPKS gene's expression.

Fig. 15b (cont.)

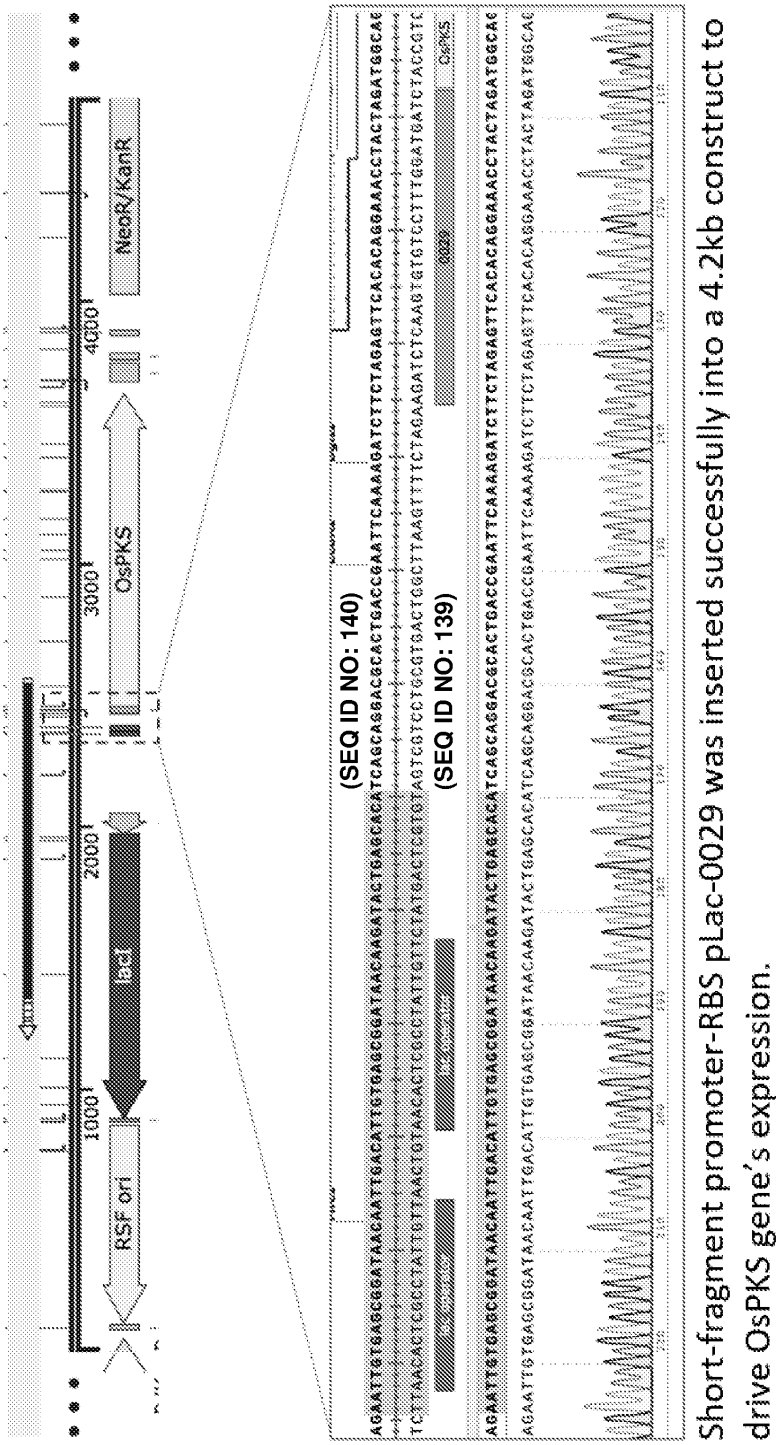

RSF ori
Pcl
OsPKS
NeoR/KanR
1000
2000
3000
4000
(SEQ ID NO: 140)
(SEQ ID NO: 139)
Short-fragment promoter-RBS pLac-0029 was inserted successfully into a 4.2kb construct to drive OsPKS gene's expression.

Fig. 15b (cont.)

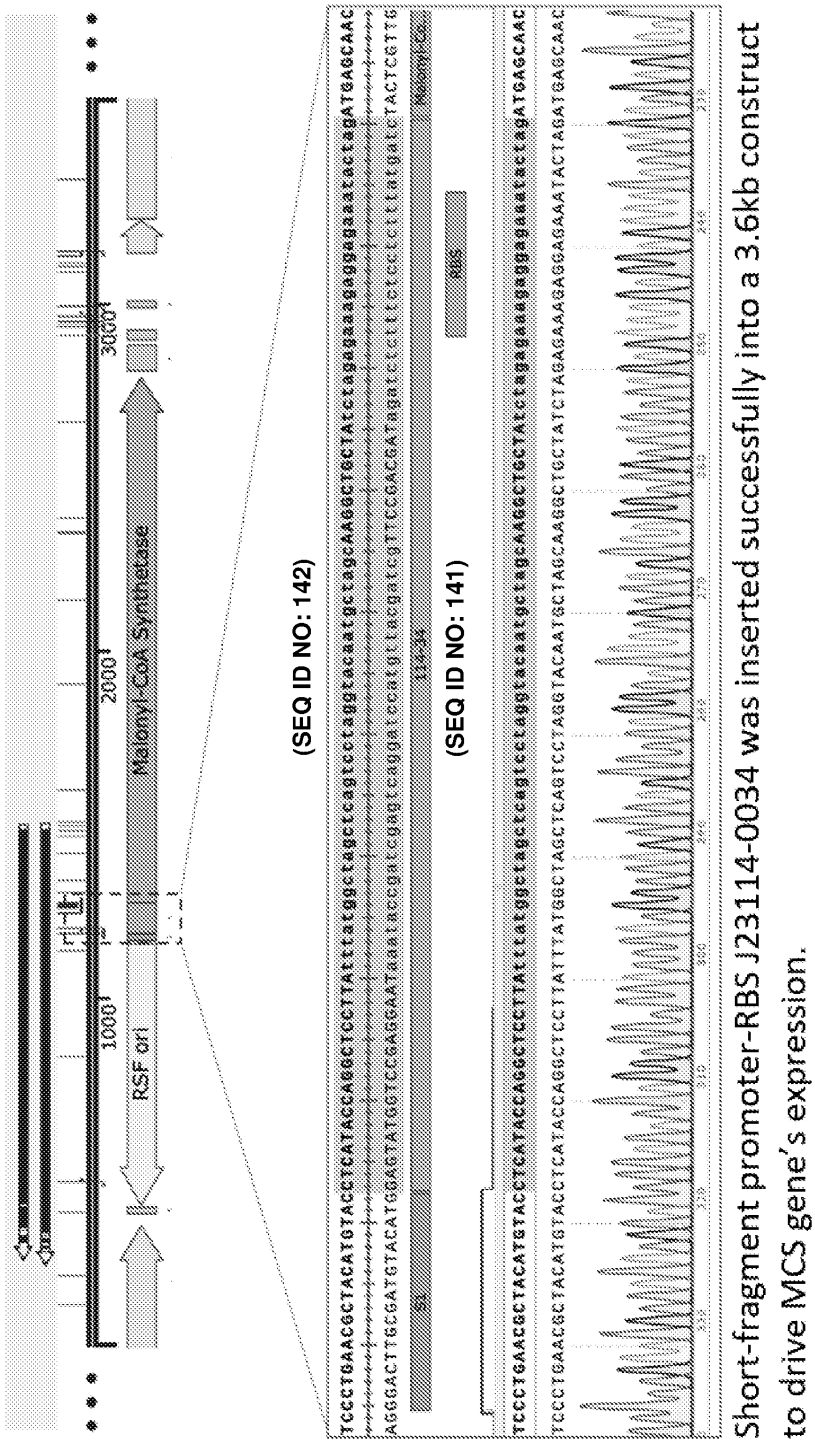

RSF ori
Malonyl-CoA Synthetase
(SEQ ID NO: 142)
(SEQ ID NO: 141)
Short-fragment promoter-RBS J23114-0034 was inserted successfully into a 3.6kb construct to drive MCS gene's expression.

Fig. 15b (cont.)

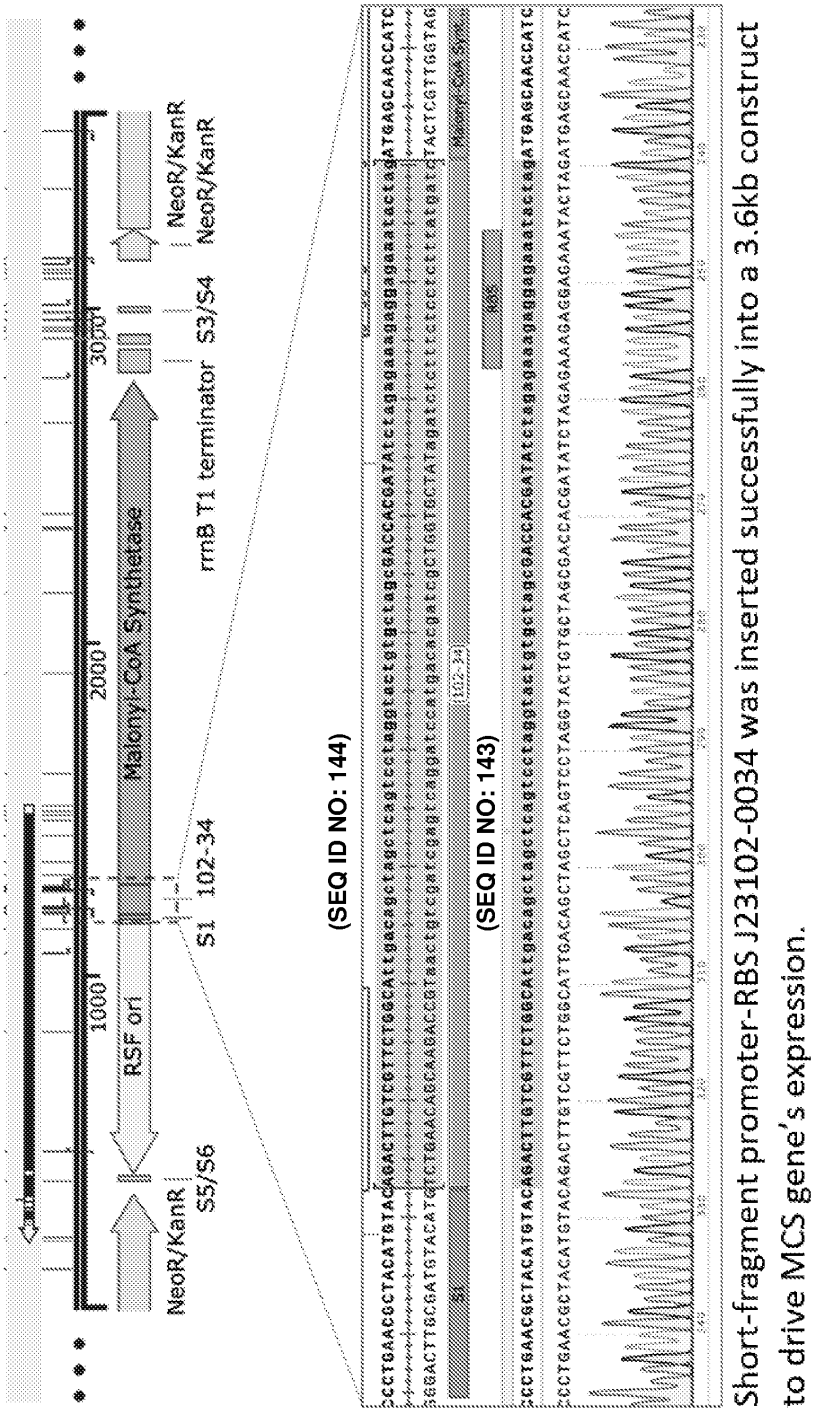

NeoR/KanR
S5/S6
RSF ori
1000
Malonyl-CoA Synthetase
2000
3000
rrnB T1 terminator
S3/S4
NeoR/KanR
S1 102-34
(SEQ ID NO: 144)
(SEQ ID NO: 143)
Short-fragment promoter-RBS J23102-0034 was inserted successfully into a 3.6kb construct to drive MCS gene's expression.

Fig. 15b (cont.)

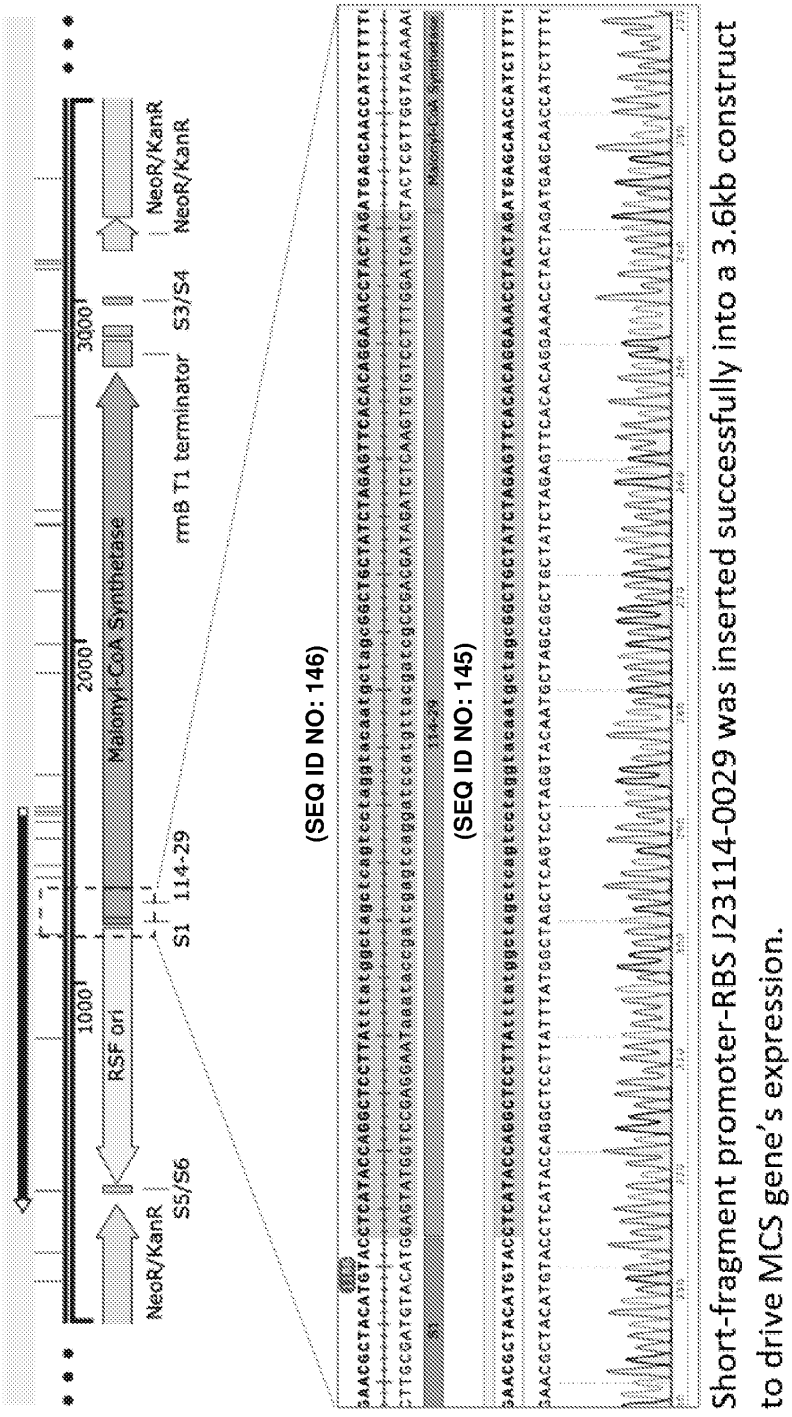

1000
2000
3000
RSF ori
Malonyl-CoA Synthetase
NeoR/KanR
S5/S6
S1
114-29
rrnB T1 terminator
S3/S4
NeoR/KanR
(SEQ ID NO: 146)
(SEQ ID NO: 145)
Short-fragment promoter-RBS J23114-0029 was inserted successfully into a 3.6kb construct to drive MCS gene's expression.

Fig. 15b (cont.)

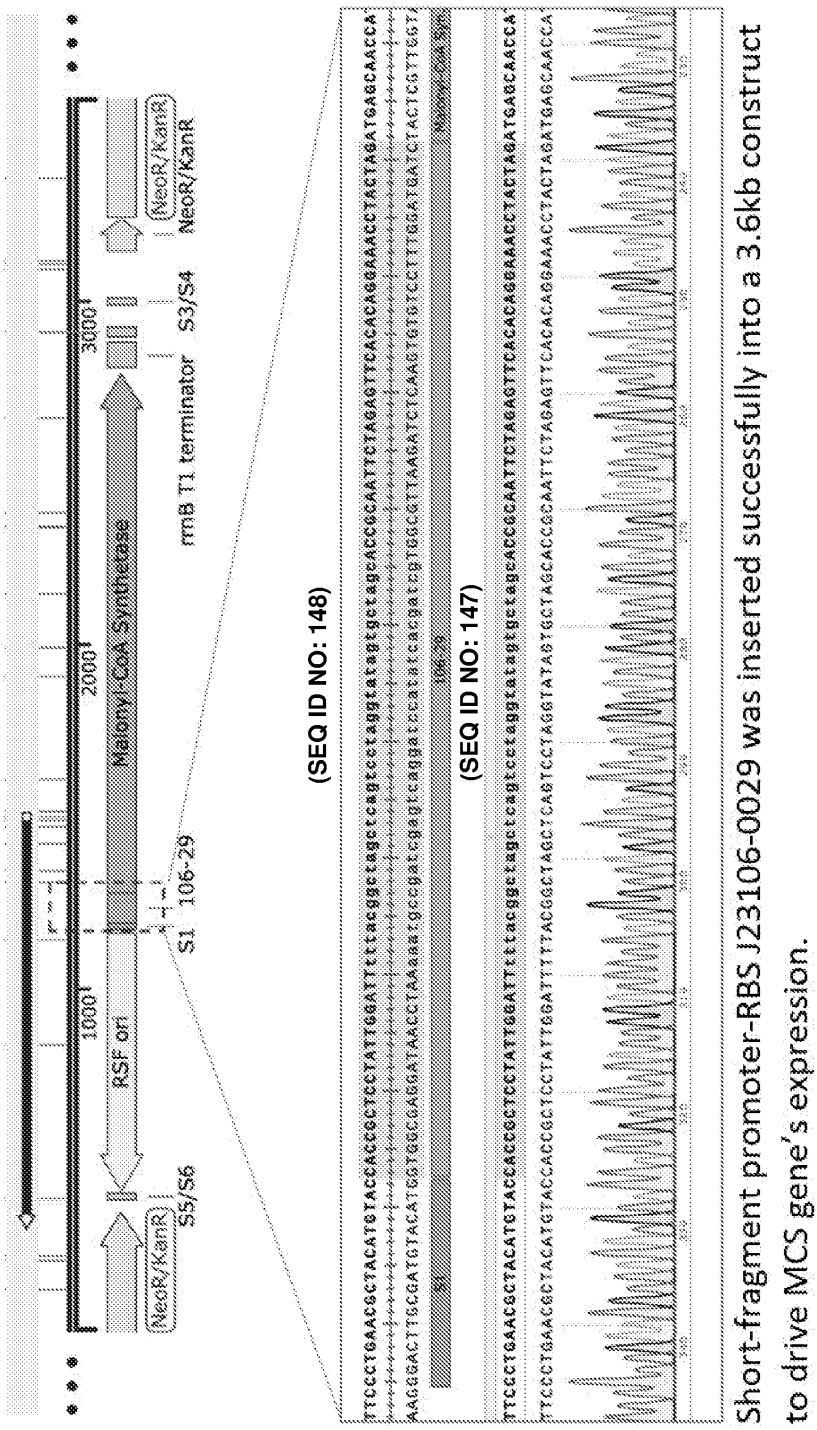

RSF ori
Malonyl-CoA Synthetase
rrnB T1 terminator
NeoR/KanR
S5/S6
S1
106-29
S3/S4
(SEQ ID NO: 148)
(SEQ ID NO: 147)
Short-fragment promoter-RBS J23106-0029 was inserted successfully into a 3.6kb construct to drive MCS gene's expression.

Fig. 15b (cont.)

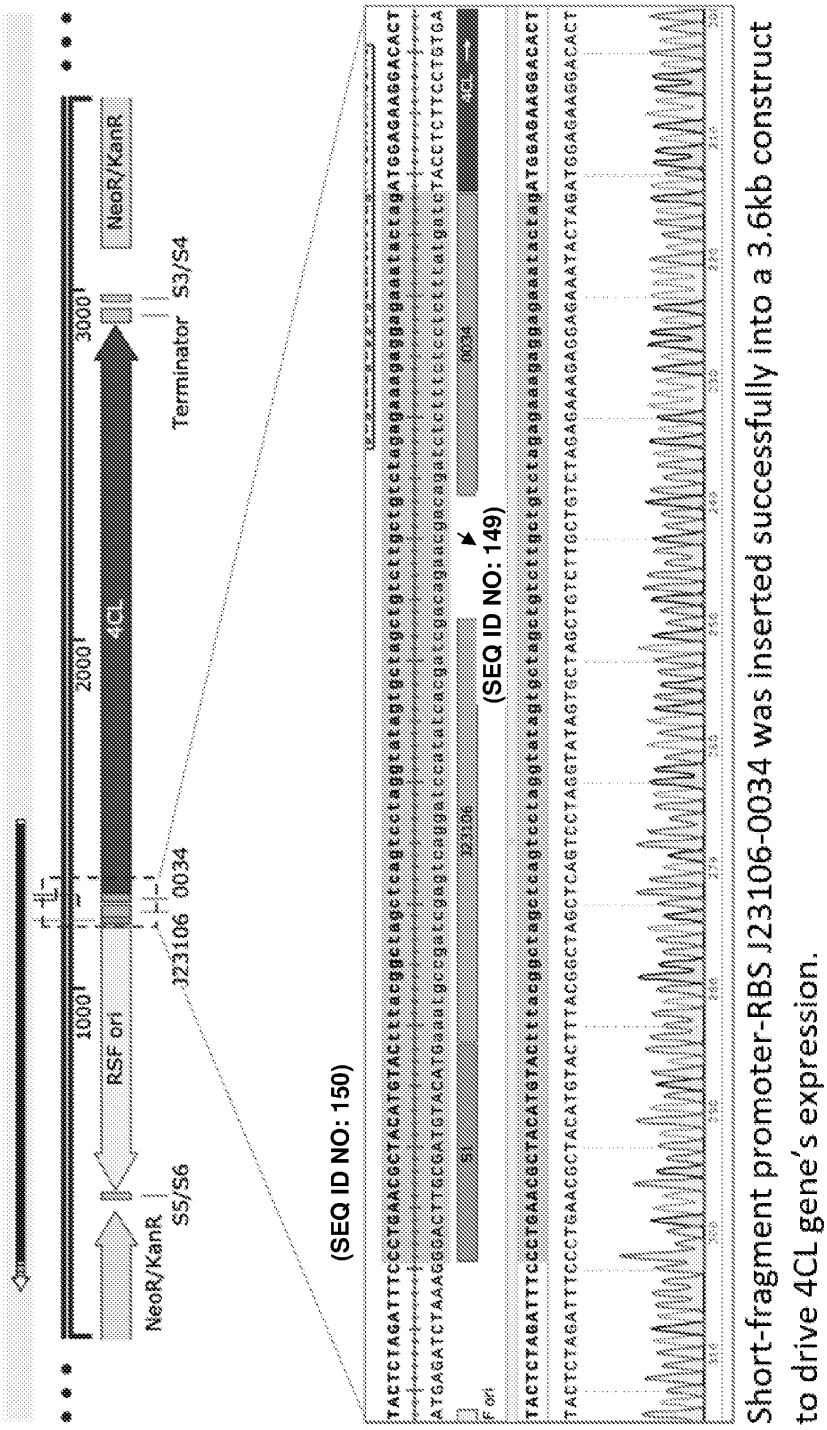

RSF ori
NeoR/KanR
S5/S6
J23106
0034
4CL
Terminator
S3/S4
(SEQ ID NO: 150)
(SEQ ID NO: 149)
Short-fragment promoter-RBS J23106-0034 was inserted successfully into a 3.6kb construct to drive 4CL gene's expression.

Fig. 15b (cont.)

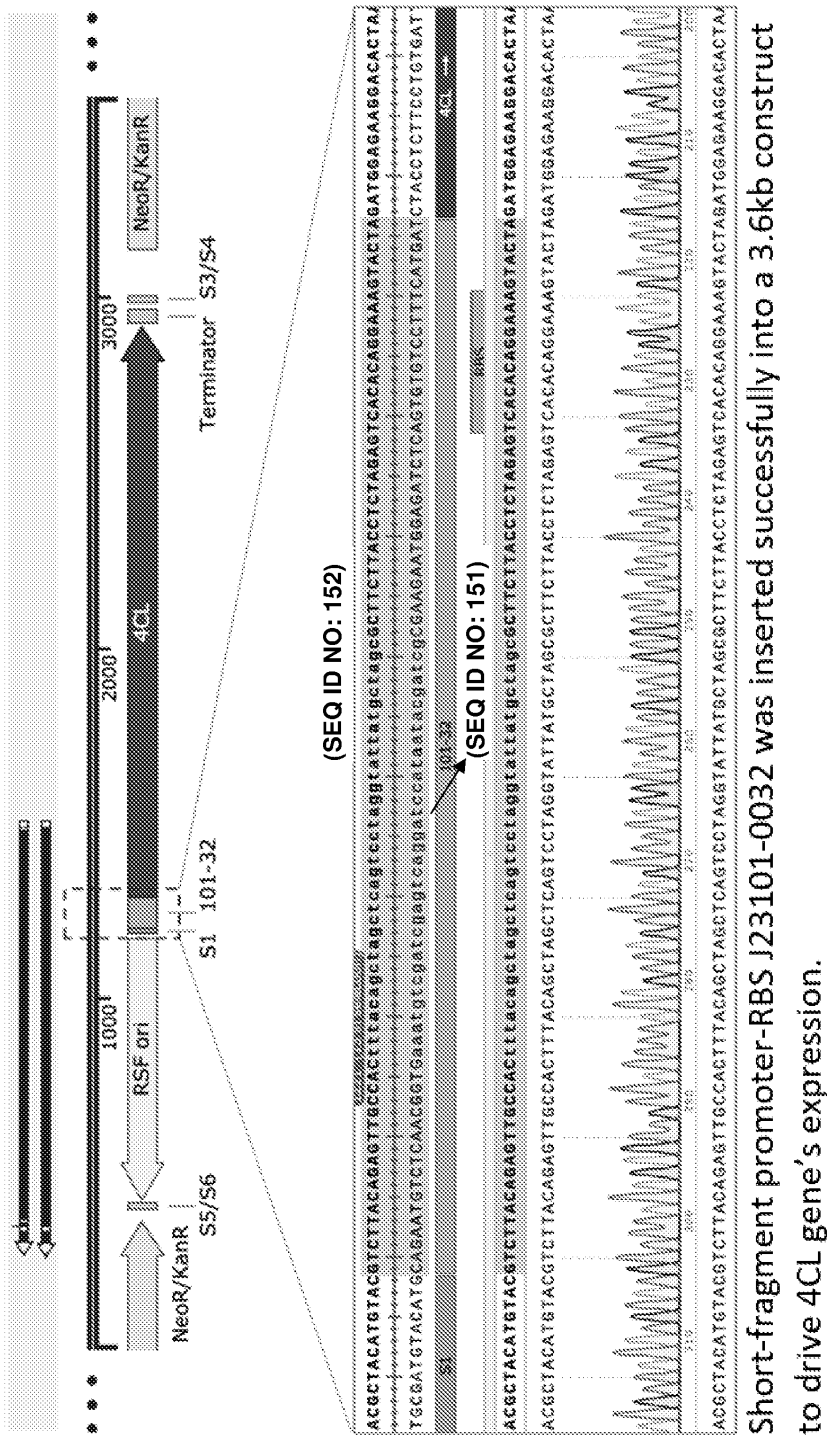

NeoR/KanR
S5/S6
RSF ori
1000
2000
3000
4CL
S1
101-32
Terminator
S3/S4
NeoR/KanR
(SEQ ID NO: 152)
(SEQ ID NO: 151)
Short-fragment promoter-RBS J23101-0032 was inserted successfully into a 3.6kb construct to drive 4CL gene's expression.

Fig. 15b (cont.)

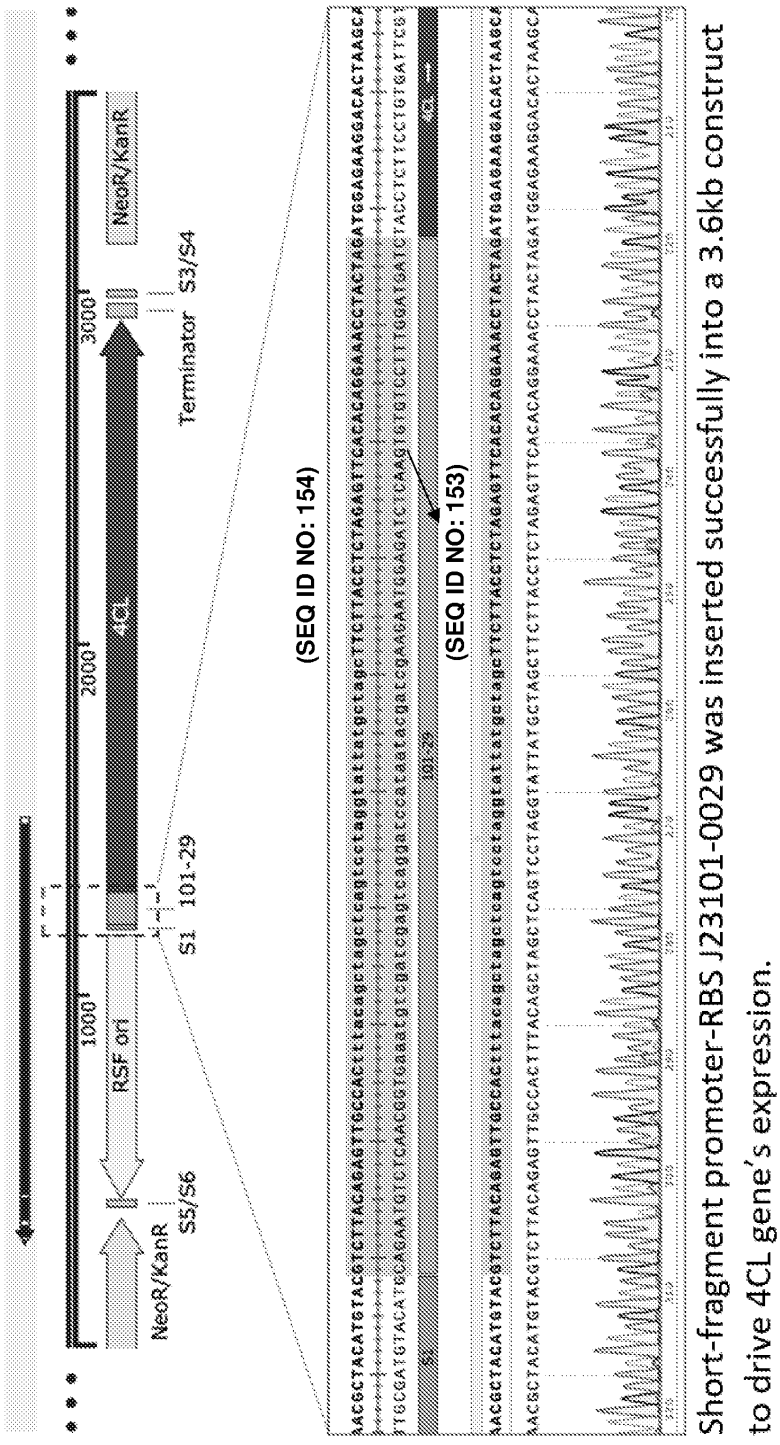

1000
2000
3000
RSF ori
4CL
NeoR/KanR
NeoR/KanR
S5/S6
S1
101-29
Terminator
S3/S4
(SEQ ID NO: 154)
(SEQ ID NO: 153)
Short-fragment promoter-RBS J23101-0029 was inserted successfully into a 3.6kb construct to drive 4CL gene's expression.

Fig. 15b (cont.)

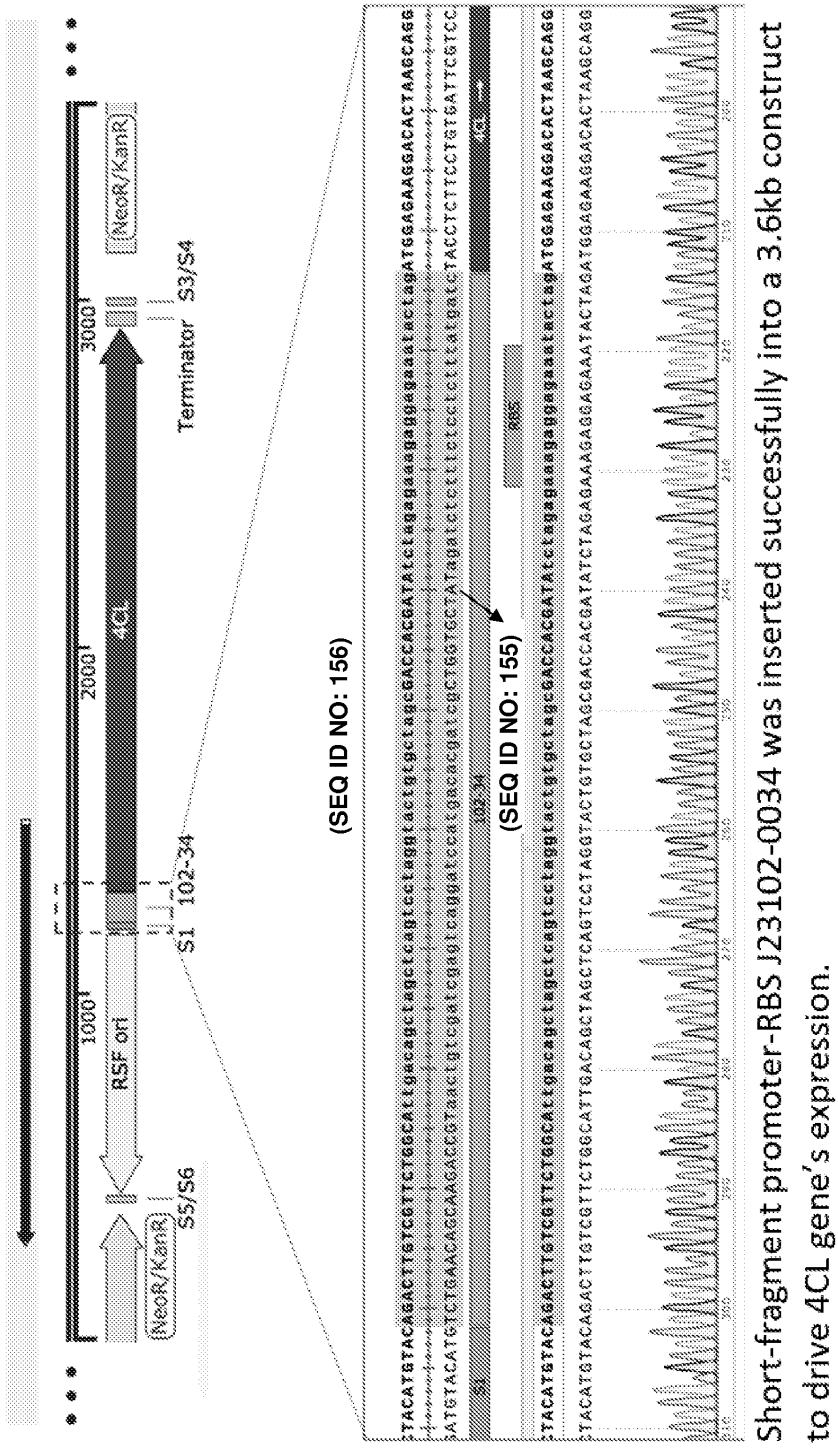

1000
RSF ori
NeoR/KanR
S5/S6
2000
4CL
3000
NeoR/KanR
Terminator
S3/S4
S1
102-34
(SEQ ID NO: 156)
(SEQ ID NO: 155)
Short-fragment promoter-RBS J23102-0034 was inserted successfully into a 3.6kb construct to drive 4CL gene's expression.

Fig. 15b (cont.)

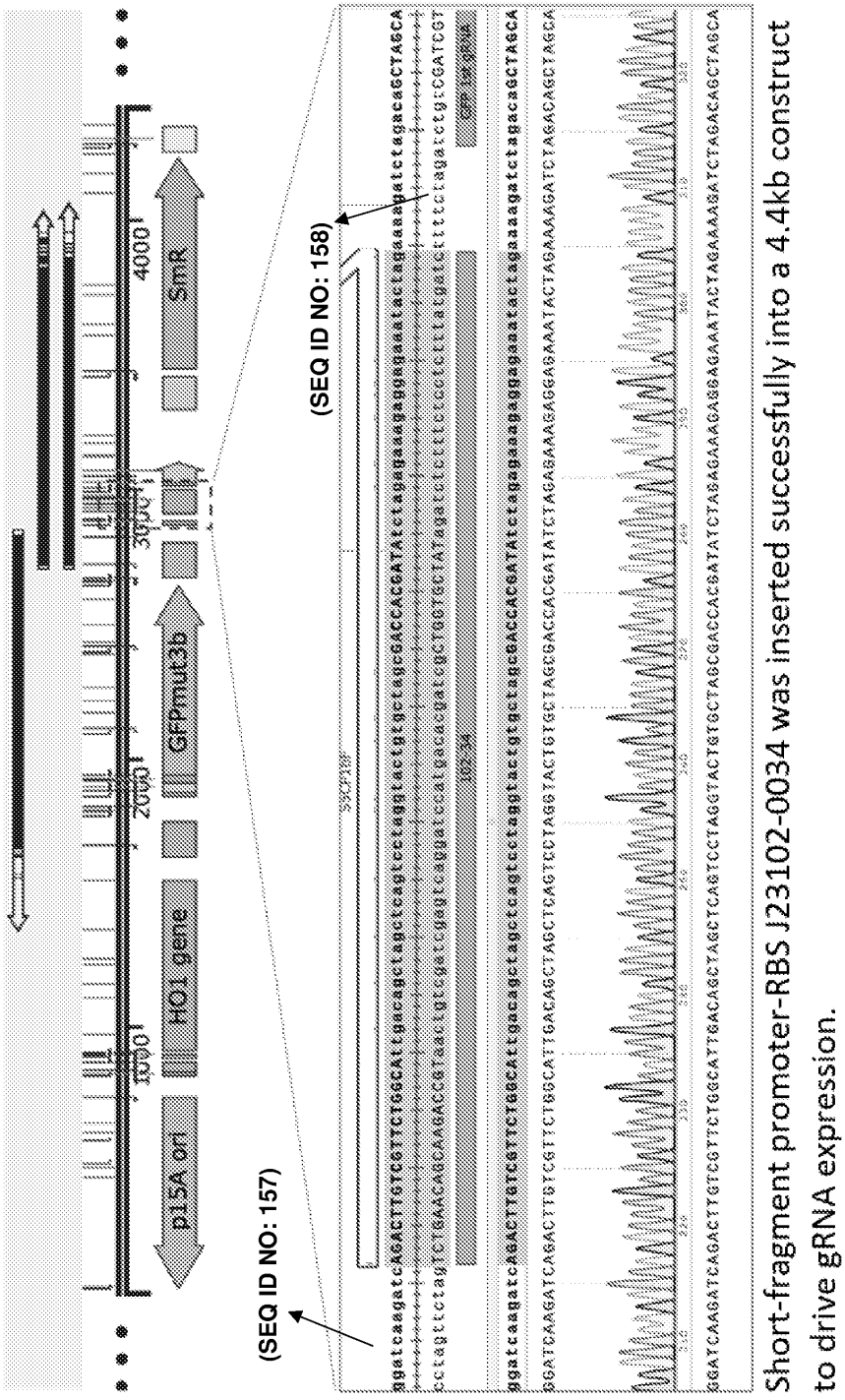

p15A ori
HO1 gene
GFPmut3b
SmR
1000
2000
3000
4000
(SEQ ID NO: 157)
(SEQ ID NO: 158)
Short-fragment promoter-RBS J23102-0034 was inserted successfully into a 4.4kb construct to drive gRNA expression.

Fig. 15b (cont.)

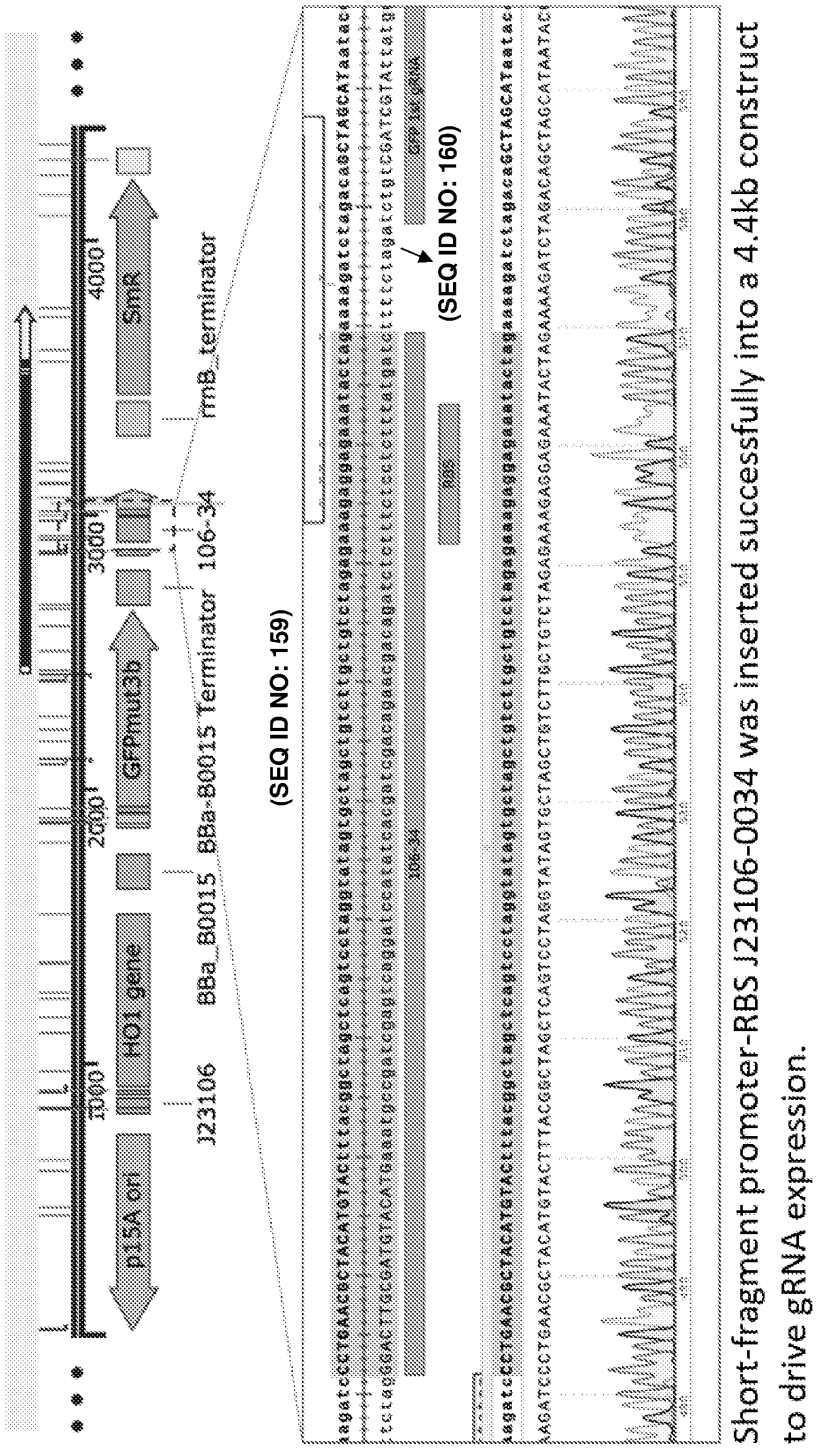

p15A ori
HO1 gene
GFPmut3b
SmR
1000
2000
3000
4000
J23106
BBa_B0015
BBa-B0015 Terminator
106-34
rrnB_terminator
(SEQ ID NO: 159)
(SEQ ID NO: 160)
Short-fragment promoter-RBS J23106-0034 was inserted successfully into a 4.4kb construct
to drive gRNA expression.

Fig. 15b (cont.)

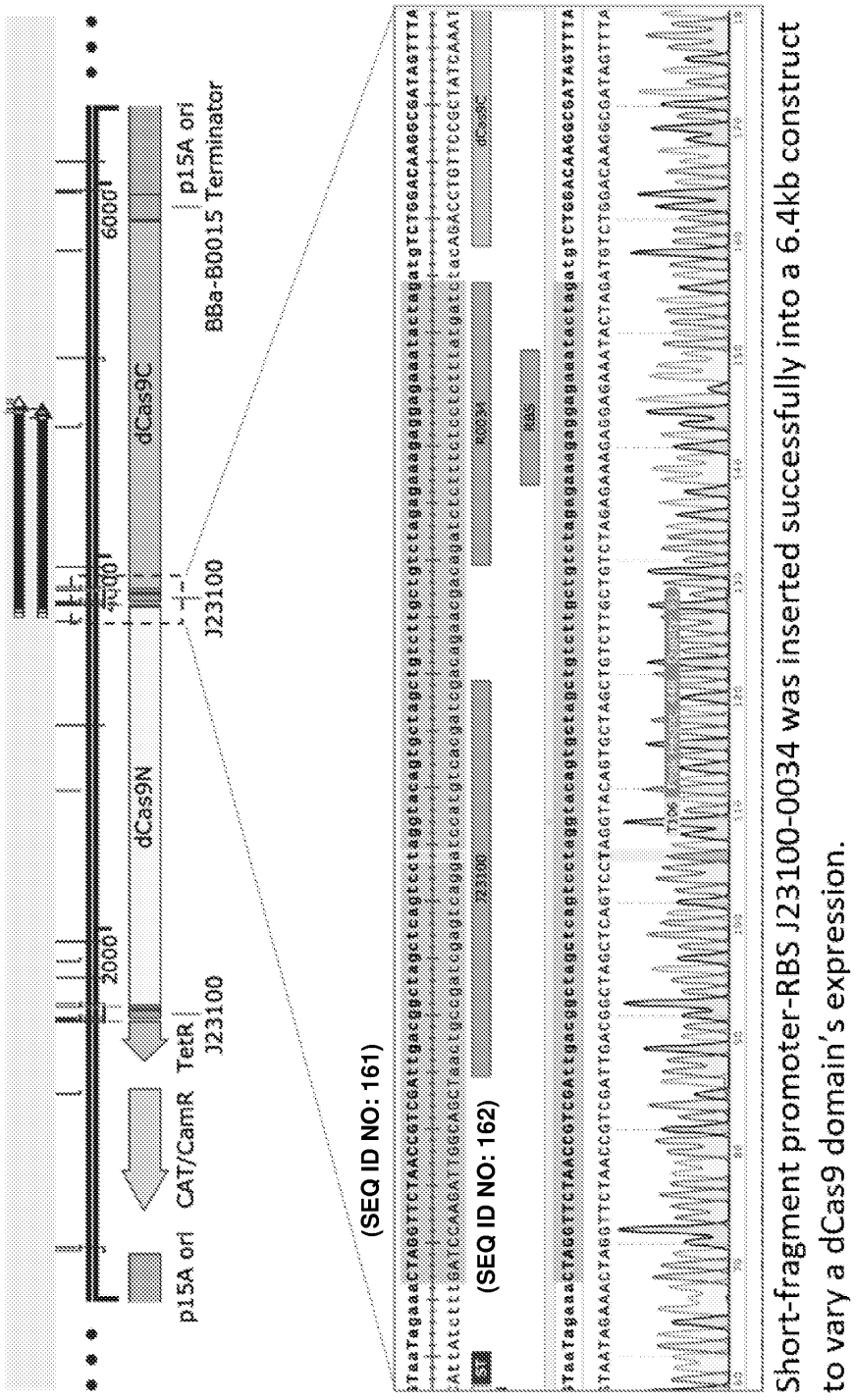

p15A ori
CAT/CamR
TetR
J23100
dCas9N
dCas9C
p15A ori
BBa-B0015 Terminator
J23100
(SEQ ID NO: 161)
(SEQ ID NO: 162)
Short-fragment promoter-RBS J23100-0034 was inserted successfully into a 6.4kb construct to vary a dCas9 domain's expression.

Fig. 15b (cont.)

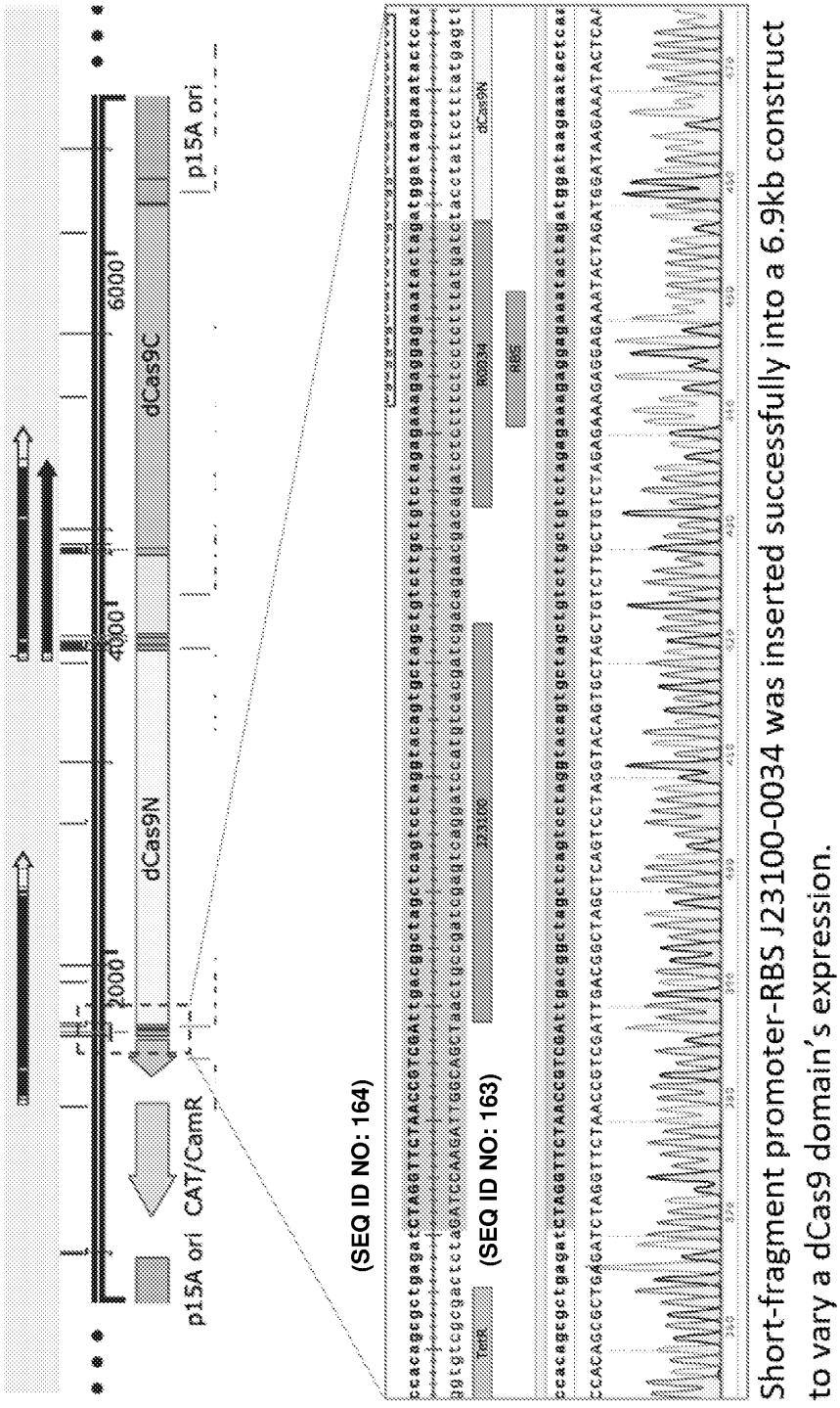

2000
4000
6000
dCas9N
dCas9C
p15A ori
CAT/CamR
p15A ori
(SEQ ID NO: 164)
(SEQ ID NO: 163)
Short-fragment promoter-RBS J23100-0034 was inserted successfully into a 6.9kb construct to vary a dCas9 domain's expression.

Fig. 15b (cont.)

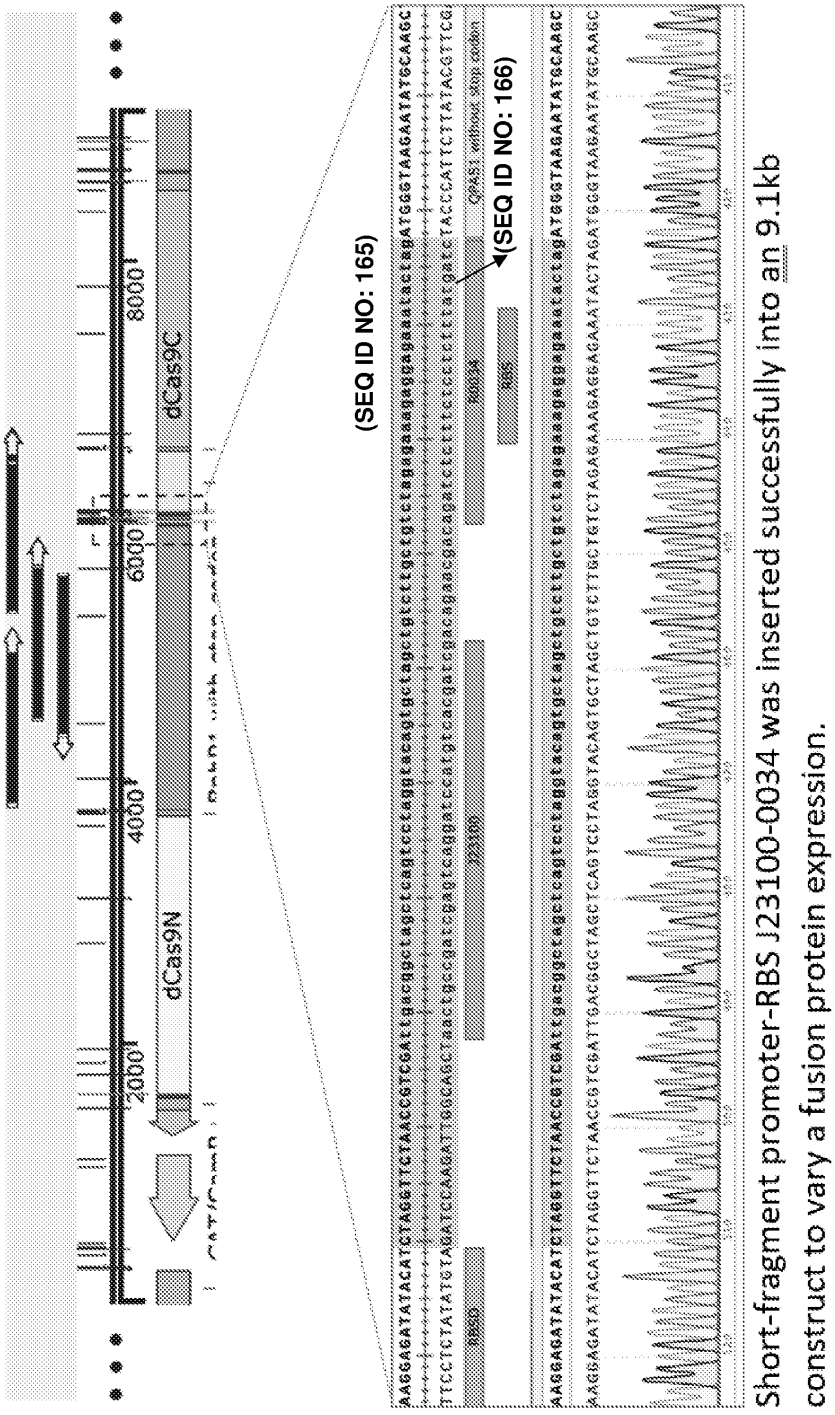

2000
4000
6000
8000
dCas9N
dCas9C
(SEQ ID NO: 165)
(SEQ ID NO: 166)
Short-fragment promoter-RBS J23100-0034 was inserted successfully into an 9.1kb construct to vary a fusion protein expression.

Fig. 15b (cont.)

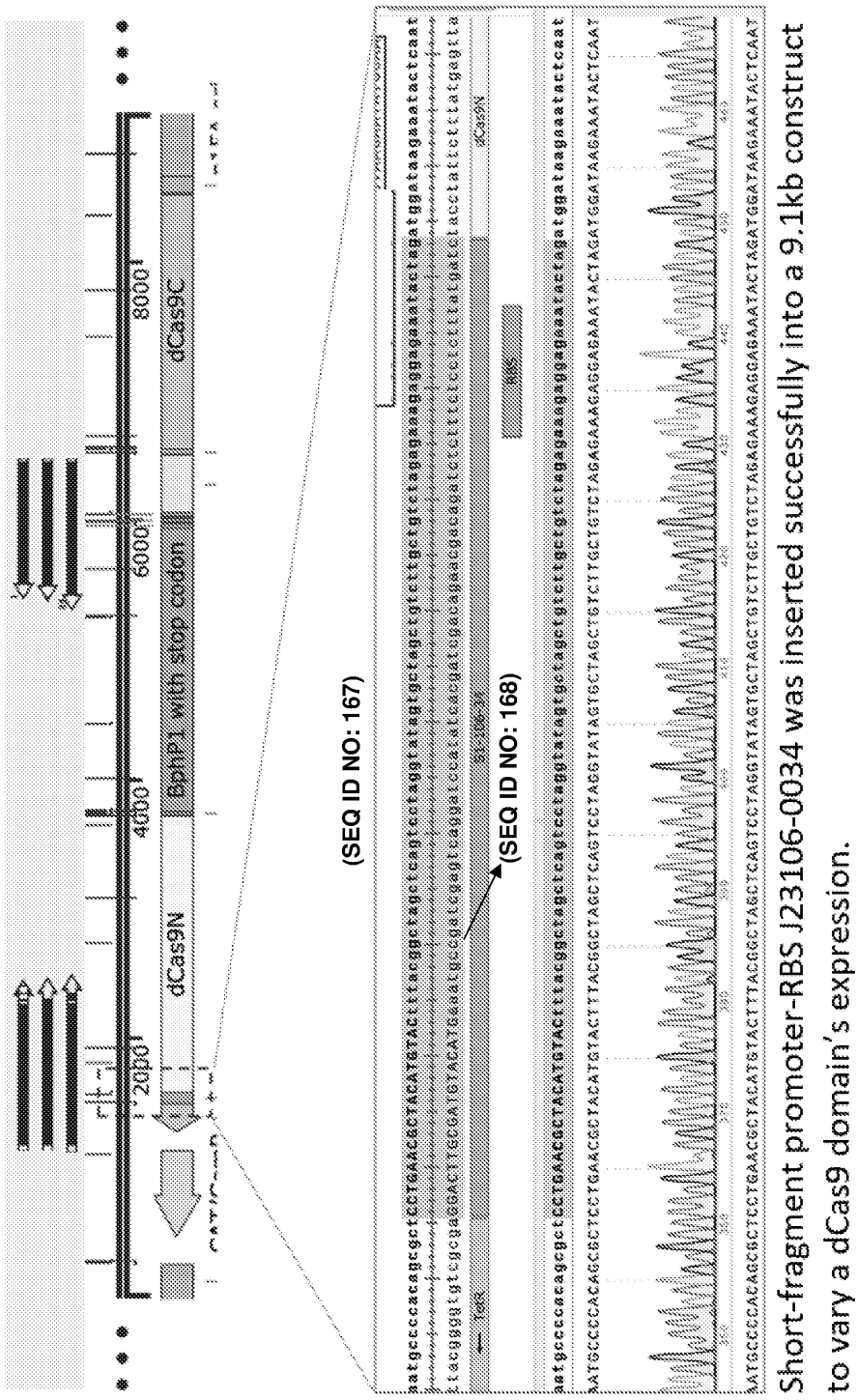

dCas9N
BpnP1 with stop codon
dCas9C
2000
4000
6000
8000
(SEQ ID NO: 167)
(SEQ ID NO: 168)
Short-fragment promoter-RBS J23106-0034 was inserted successfully into a 9.1kb construct to vary a dCas9 domain's expression.

Fig. 15b (cont.)

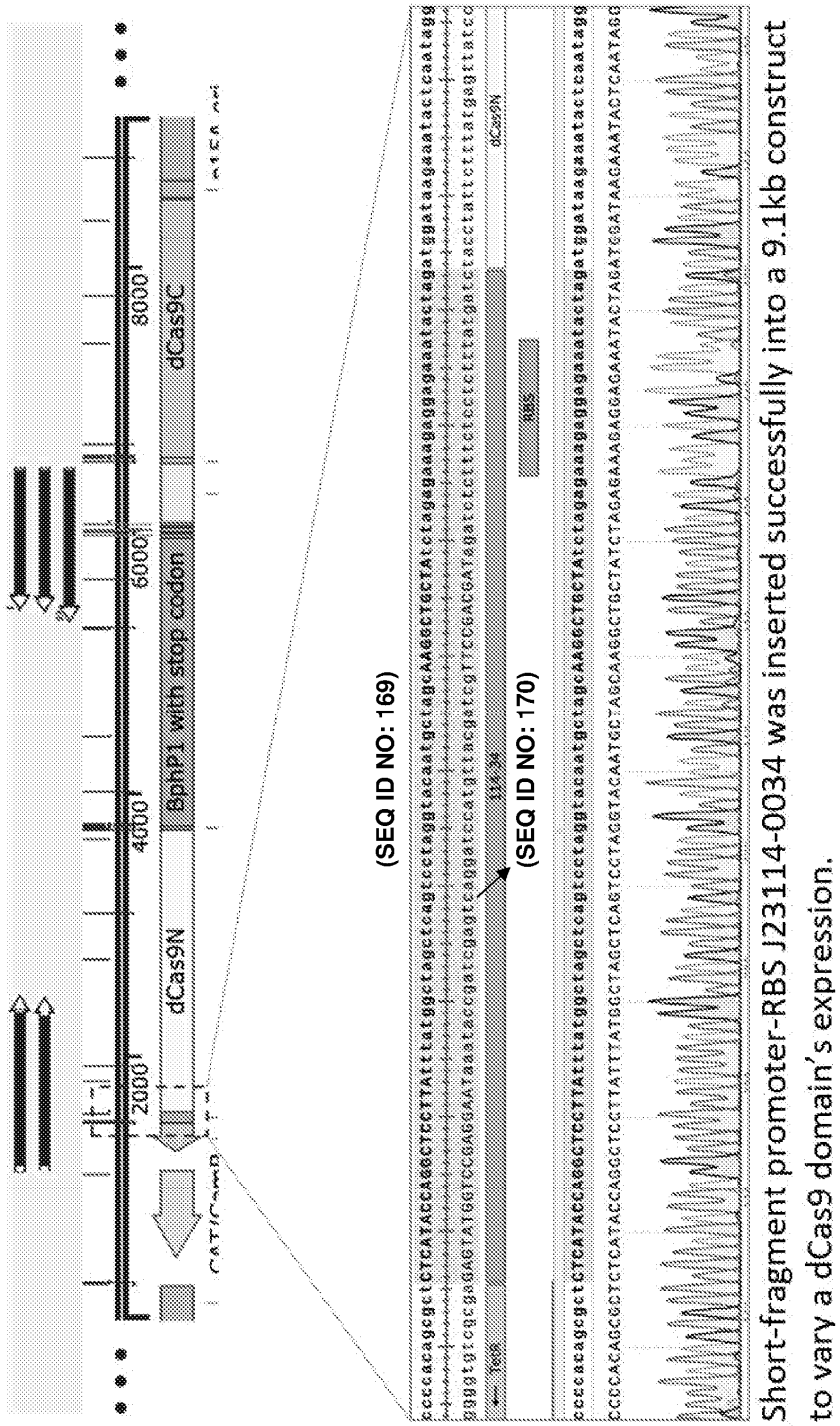
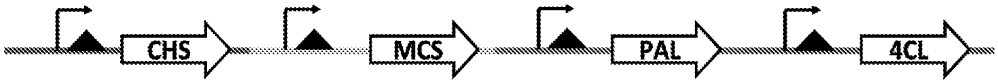

2000
4000
6000
8000
dCas9N
BphP1 with stop codon
dCas9C
(SEQ ID NO: 169)
(SEQ ID NO: 170)
Short-fragment promoter-RBS J23114-0034 was inserted successfully into a 9.1kb construct to vary a dCas9 domain's expression.

Fig. 15b (cont.)



Promoter-RBS	Gene	Promoter-RBS	Gene	Promoter-RBS	Gene	Promoter-RBS	Gene
pLac-dRBS	CHS	106-dRBS	MCS	106-32	PAL	106-32	4CL
pLac-dRBS	CHS	106-32	MCS	106-32	PAL	106-dRBS	4CL
pLac-dRBS	CHS	106-32	MCS	106-dRBS	PAL	106-32	4CL
pLac-dRBS	CHS	-	MCS	106-32	PAL	106-32	4CL
pLac-dRBS	CHS	-	MCS	106-32	PAL	106-dRBS	4CL
pLac-dRBS	CHS	-	MCS	106-dRBS	PAL	106-32	4CL
pLac-dRBS	CHS	114-34	MCS	106-32	PAL	101-29	4CL
pLac-dRBS	CHS	102-34	MCS	106-32	PAL	101-29	4CL
pLac-dRBS	CHS	114-34	MCS	106-32	PAL	101-d	4CL
pLac-dRBS	CHS	34	MCS	106-29	PAL	101-32	4CL

Fig. 16

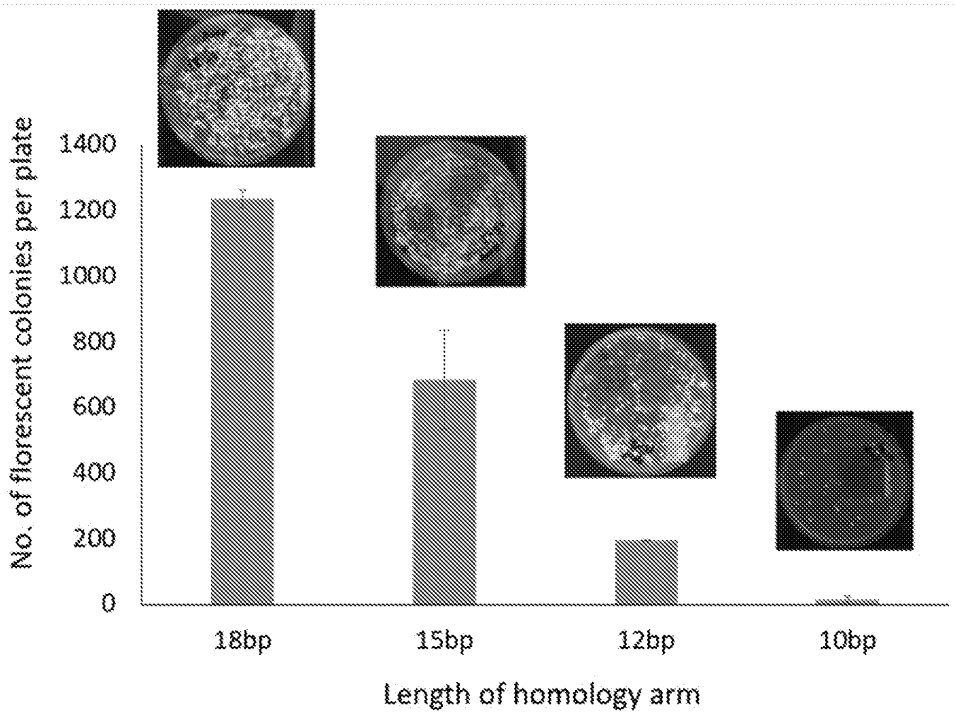


Fig. 17