Figure 1

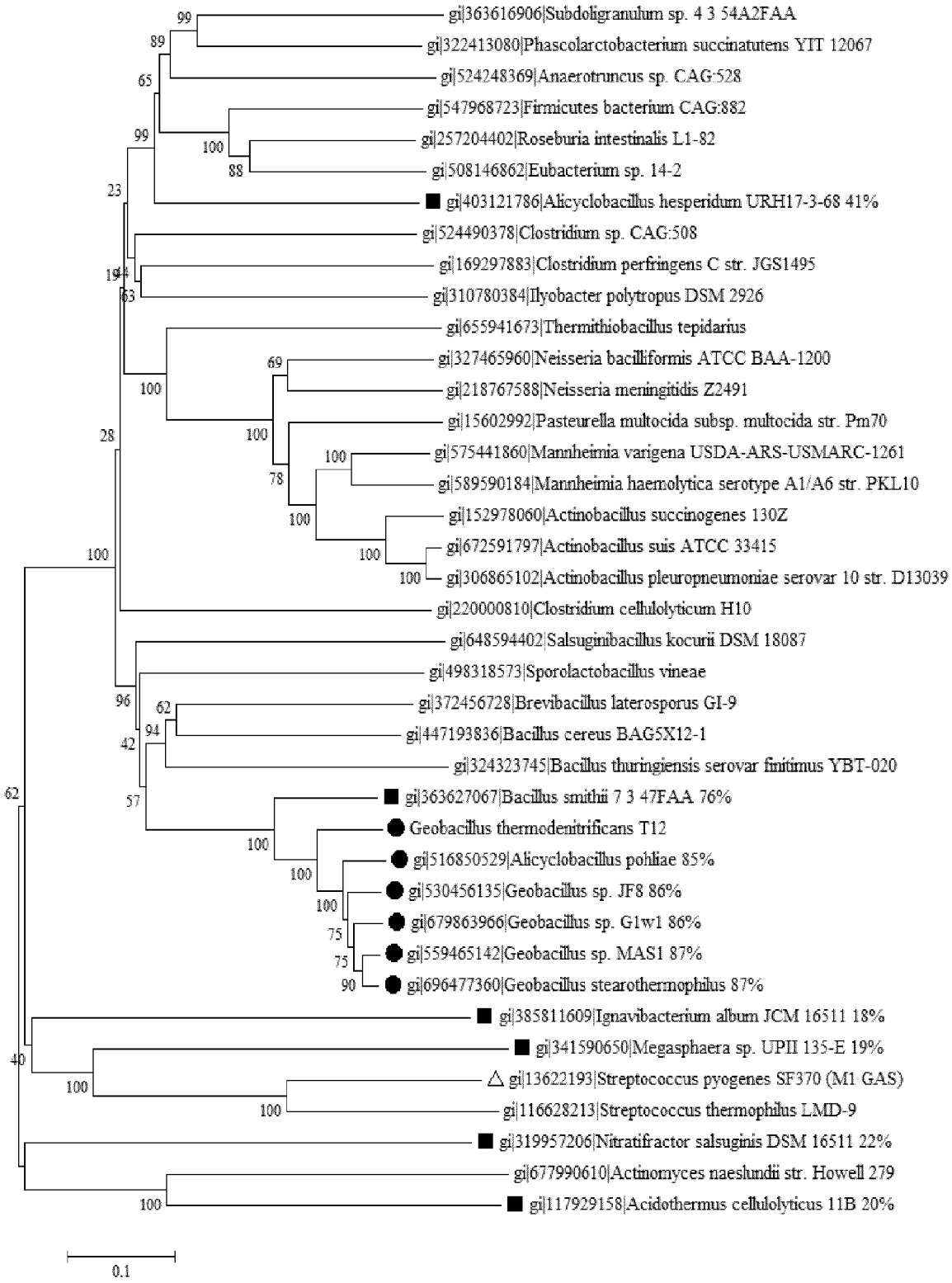

gi|363616906|Subdoligranulum sp. 4 3 54A2FAA
gi|322413080|Phascolarctobacterium succinatutens YIT 12067
gi|524248369|Anaerotruncus sp. CAG:528
gi|547968723|Firmicutes bacterium CAG:882
gi|257204402|Roseburia intestinalis L1-82
gi|508146862|Eubacterium sp. 14-2
gi|403121786|Alicyclobacillus hesperidum URH17-3-68 41%
gi|524490378|Clostridium sp. CAG:508
gi|169297883|Clostridium perfringens C str. JGS1495
gi|310780384|Ilyobacter polytropus DSM 2926
gi|655941673|Thermithiobacillus tepidarius
gi|327465960|Neisseria bacilliformis ATCC BAA-1200
gi|218767588|Neisseria meningitidis Z2491
gi|15602992|Pasteurella multocida subsp. multocida str. Pm70
gi|575441860|Mannheimia varigena USDA-ARS-USMARC-1261
gi|589590184|Mannheimia haemolytica serotype A1/A6 str. PKL10
gi|152978060|Actinobacillus succinogenes 130Z
gi|672591797|Actinobacillus suis ATCC 33415
gi|306865102|Actinobacillus pleuropneumoniae serovar 10 str. D13039
gi|220000810|Clostridium cellulolyticum H10
gi|648594402|Salsuginibacillus kocurii DSM 18087
gi|498318573|Sporolactobacillus vineae
gi|372456728|Brevibacillus laterosporus GI-9
gi|447193836|Bacillus cereus BAG5X12-1
gi|324323745|Bacillus thuringiensis serovar finitimus YBT-020
gi|363627067|Bacillus smithii 7 3 47FAA 76%
Geobacillus thermodenitrificans T12
gi|516850529|Alicyclobacillus pohliae 85%
gi|530456135|Geobacillus sp. JF8 86%
gi|679863966|Geobacillus sp. G1w1 86%
gi|559465142|Geobacillus sp. MAS1 87%
gi|696477360|Geobacillus stearothermophilus 87%
gi|385811609|Ignavibacterium album JCM 16511 18%
gi|341590650|Megasphaera sp. UPII 135-E 19%
gi|13622193|Streptococcus pyogenes SF370 (M1 GAS)
gi|116628213|Streptococcus thermophilus LMD-9
gi|319957206|Nitratifractor salsuginis DSM 16511 22%
gi|677990610|Actinomyces naeslundii str. Howell 279
gi|117929158|Acidothermus cellulolyticus 11B 20%
0.1

Figure 2

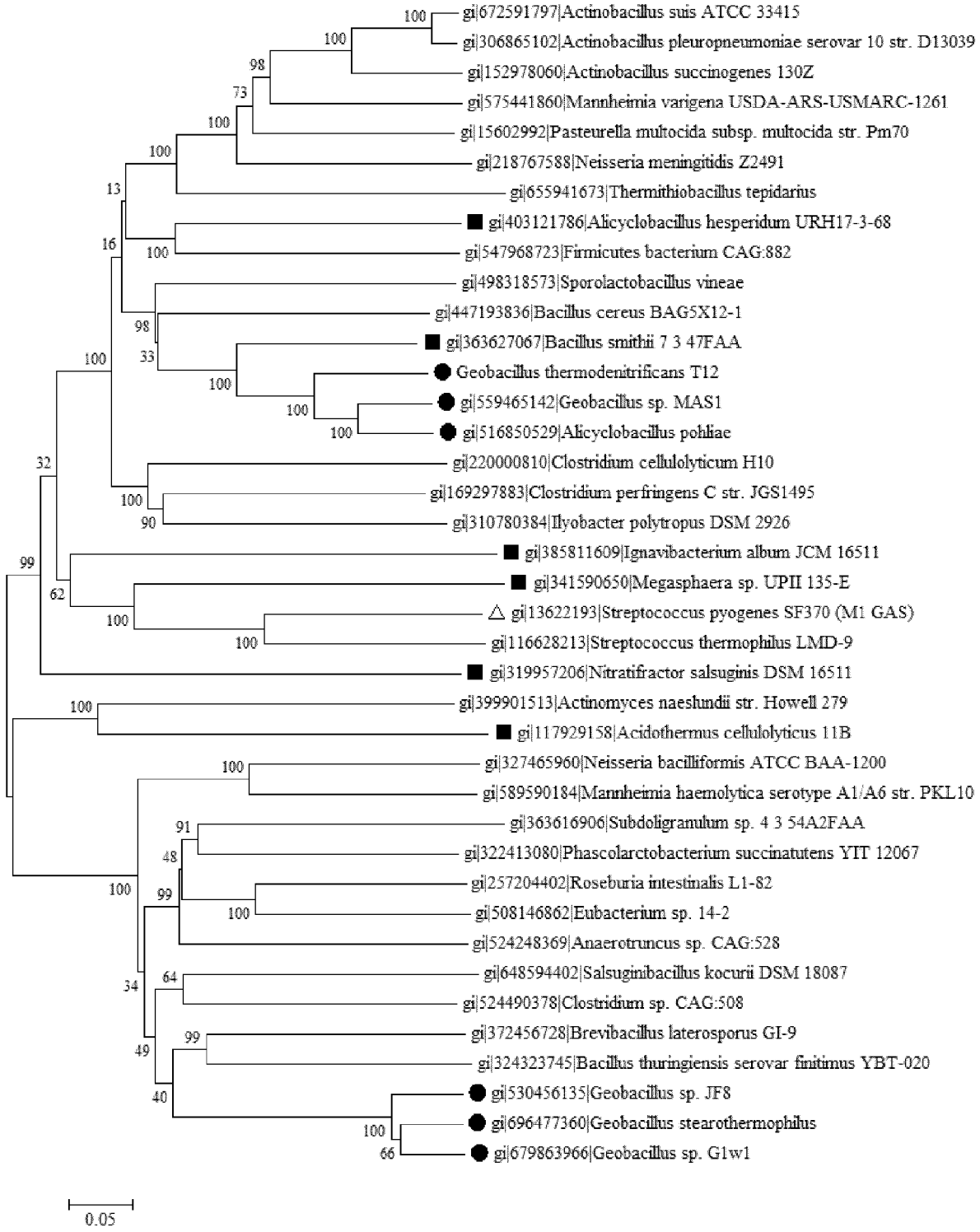

gi|672591797|Actinobacillus suis ATCC 33415
gi|306865102|Actinobacillus pleuropneumoniae serovar 10 str. D13039
gi|152978060|Actinobacillus succinogenes 130Z
gi|575441860|Mannheimia varigena USDA-ARS-USMARC-1261
gi|15602992|Pasteurella multocida subsp. multocida str. Pm70
gi|218767588|Neisseria meningitidis Z2491
gi|655941673|Thermithiobacillus tepidarius
gi|403121786|Alicyclobacillus hesperidum URH17-3-68
gi|547968723|Firmicutes bacterium CAG:882
gi|498318573|Sporolactobacillus vineae
gi|447193836|Bacillus cereus BAG5X12-1
gi|363627067|Bacillus smithii 7 3 47FAA
Geobacillus thermodenitrificans T12
gi|559465142|Geobacillus sp. MAS1
gi|516850529|Alicyclobacillus pohliae
gi|220000810|Clostridium cellulolyticum H10
gi|169297883|Clostridium perfringens C str. JGS1495
gi|310780384|Ilyobacter polytropus DSM 2926
gi|385811609|Ignavibacterium album JCM 16511
gi|341590650|Megasphaera sp. UPII 135-E
gi|13622193|Streptococcus pyogenes SF370 (M1 GAS)
gi|116628213|Streptococcus thermophilus LMD-9
gi|319957206|Nitratifractor salsuginis DSM 16511
gi|399901513|Actinomyces naeslundii str. Howell 279
gi|117929158|Acidothermus cellulolyticus 11B
gi|327465960|Neisseria bacilliformis ATCC BAA-1200
gi|589590184|Mannheimia haemolytica serotype A1/A6 str. PKL10
gi|363616906|Subdoligranulum sp. 4 3 54A2FAA
gi|322413080|Phascolarctobacterium succinatutens YIT 12067
gi|257204402|Roseburia intestinalis L1-82
gi|508146862|Eubacterium sp. 14-2
gi|524248369|Anaerotruncus sp. CAG:528
gi|648594402|Salsuginibacillus kocurii DSM 18087
gi|524490378|Clostridium sp. CAG:508
gi|372456728|Brevibacillus laterosporus GI-9
gi|324323745|Bacillus thuringiensis serovar finitimus YBT-020
gi|530456135|Geobacillus sp. JF8
gi|696477360|Geobacillus stearothermophilus
gi|679863966|Geobacillus sp. G1w1
0.05

Figure 3

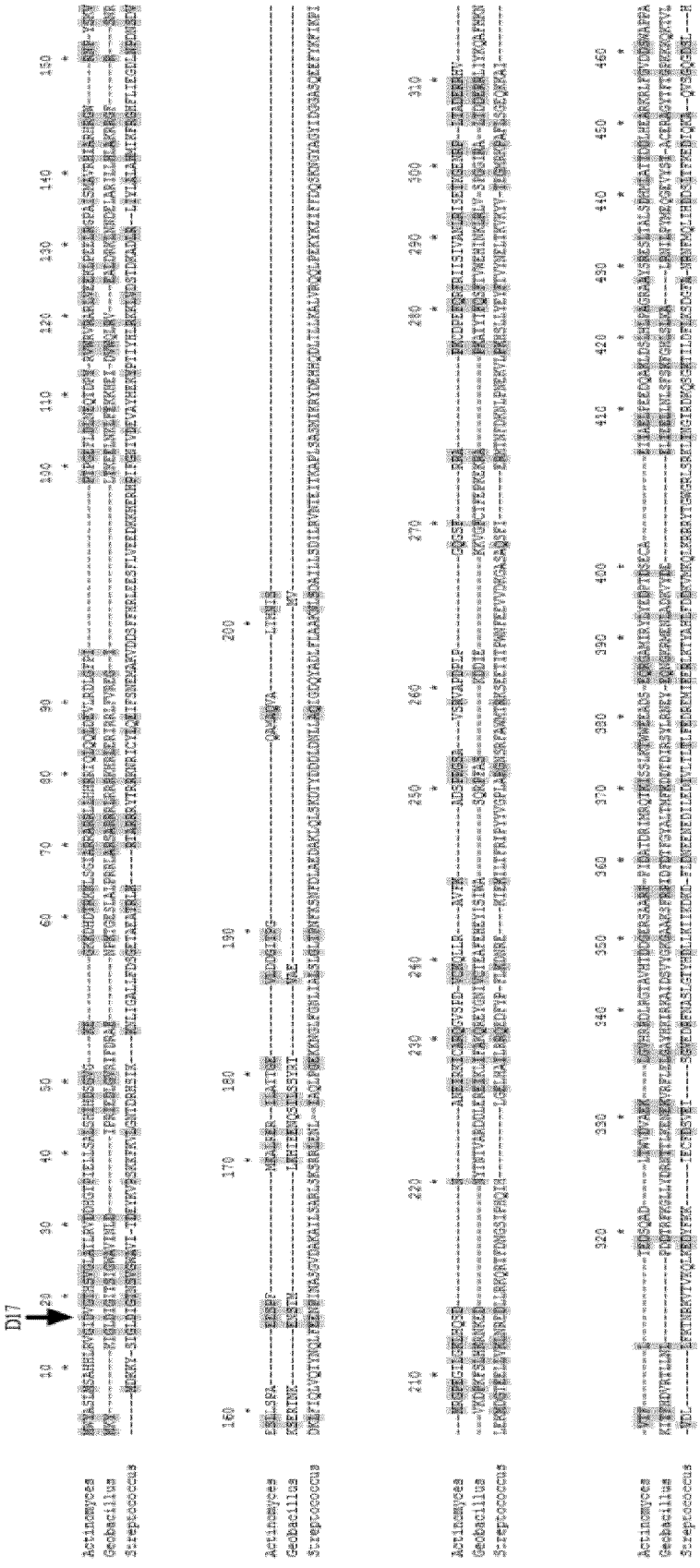


Figure 3 (continued)

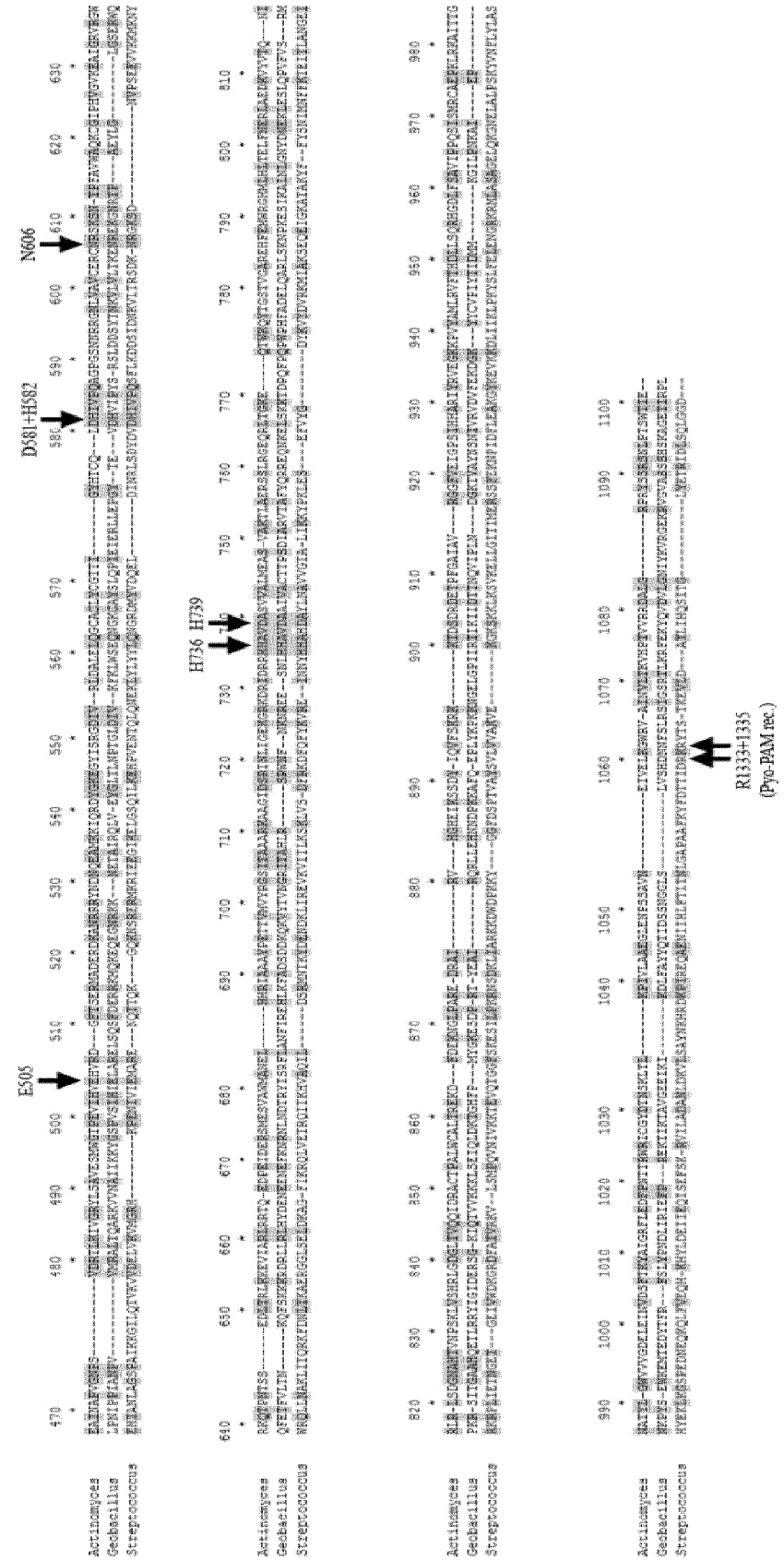


Figure 4

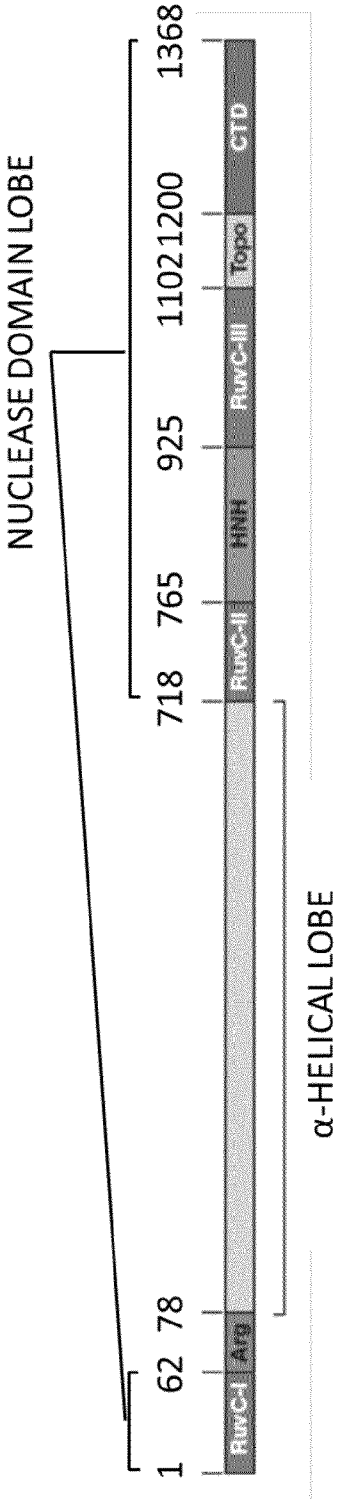

NUCLEASE DOMAIN LOBE
α-HELICAL LOBE
RuvC-I
Arg
RuvC-II
HNH
RuvC-III
Topo
CTD
1
62
78
718
765
925
1102
1200
1368

Figure 5

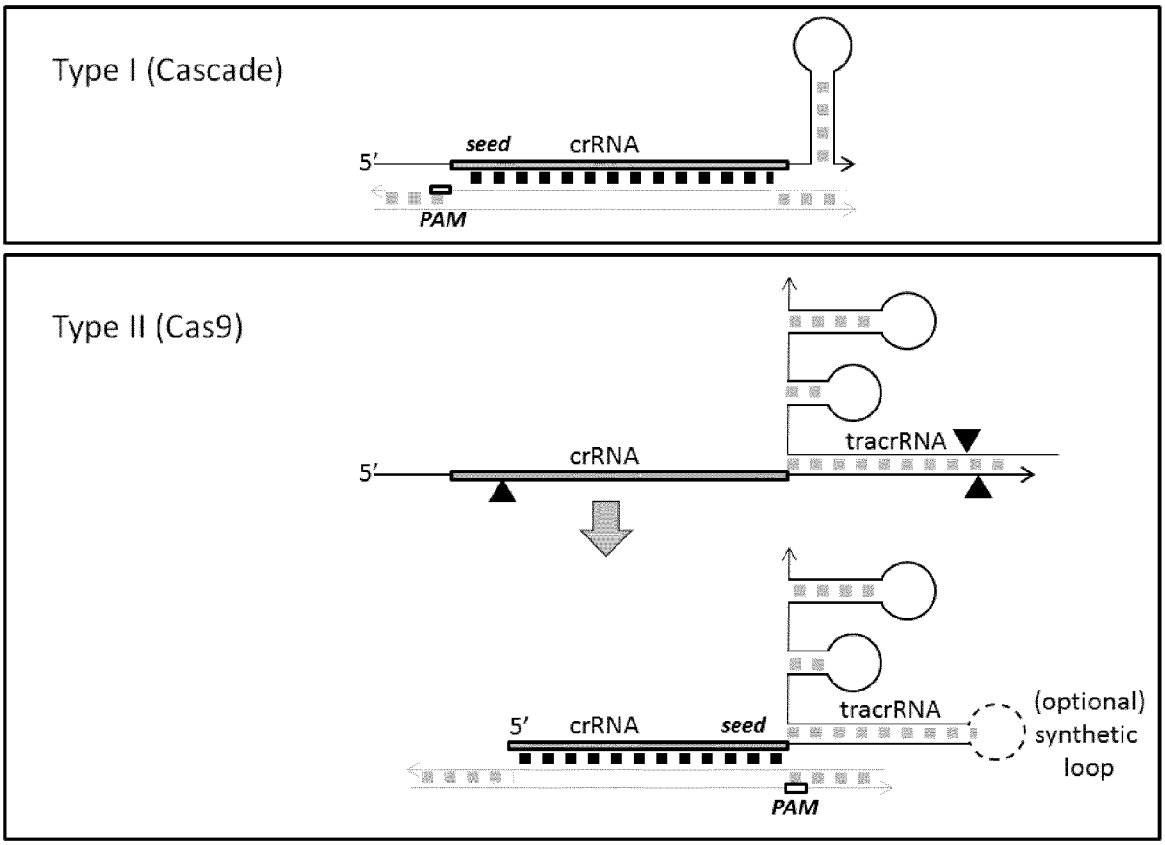

Type I (Cascade)
5′
seed
crRNA
PAM
Type II (Cas9)
5′
crRNA
tracrRNA
5′
crRNA
seed
tracrRNA
(optional)
synthetic
loop
PAM

Figure 6

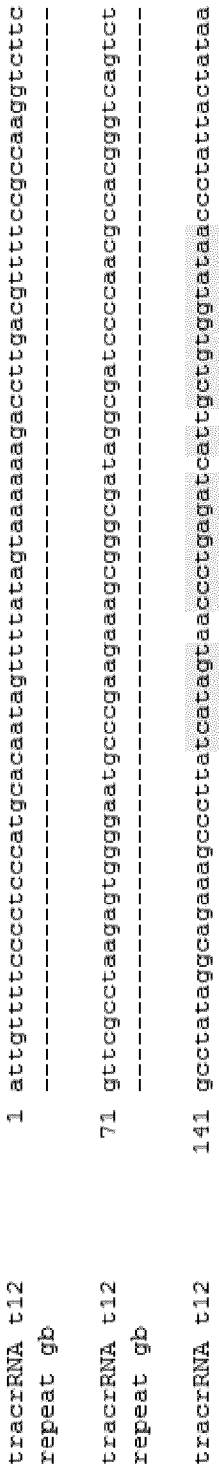

tracrRNA t12
repeat gb
tracrRNA t12
repeat gb
tracrRNA t12
repeat gb
tracrRNA t12
repeat gb
tracrRNA t12
repeat gb
1 attgttttcccctcccatgcacaaatagttttatagtaaaaaagaccttgacgttttccgccaaggtcttc
71 gttcgcctaagagtgggggaatgcccgaagaaagcgggcgataggcgatcccccaacgccacgggtcagtct
141 gcctataggcagaaagccctttatcatagtaacccctgagatcattgctgtggtataaccctattactataa
1 ----------gtcatagttcccctgagatttatcgctgtggtataat----------
211 taatgtttatatttgggaaaatcaagtccttttttctatatttttttatacttttcatttctttcttgcattat
281 gatgatgtgagggaggatagatttctgacaggaggaggtttcacatcg

Figure 7

1. Match to: **DQ453159 DQ453159 Geobacillus virus E2, complete genome**(DQ453159) position: 39980-39951, with: **spacer4** CRISPR No.4 Spacer No.1 (spacer4_4_1) position: 1-30, Strand: +

```
 5' --------UUGGCGGUGCGAAUUCUAACCGUCCCGGAA-------- 3'     <- CRISPR
spacer                                                      RNA
            ||||||||||||||||||||||||||||||
 3' TAGCGTACAACCGCCACGCTTAAGATTGGCAGGGCCTTCGTGGTGT 5'     <-
Protospacer                                                 Sequence
    ||||||||                                ||||||||
 5' ATCGCATGTTGGCGGTGCGAATTCTAACCGTCCCGGAAGCACCACA 3'     <-
[Entrez Nucleotide]
```

Score: 30

2. Match to: **Bacillus alveayuensis strain 24KAM51 LG50_053, whole genome shotgun sequence**(NZ_JYCE01000053) position: 110918-110889, with: **spacer9** CRISPR No.9 Spacer No.1 (spacer9_9_1) position: 1-30, Strand: +

```
 5' --------UUCUACCUCUACUCUCGAUUCACGAAUCGG-------- 3'     <- CRISPR
spacer                                                      RNA
            ||||||||||||||||||||||||||||||
 3' CGACGAGAAAGATGGAGATGAGAGCTAAGTGCTTAGCCAAAAGGTT 5'     <-
Protospacer                                                 Sequence
    ||||||||                                ||||||||
 5' GCTGCTCTTTCTACCTCTACTCTCGATTCACGAATCGGTTTTCCAA 3'     <-
[Entrez Nucleotide]
```

Score: 30

3. Match to: **Anoxybacillus flavithermus WK1, complete genome**(CP000922) position: 701422-701450, with: **spacer5** CRISPR No.5 Spacer No.1 (spacer5_5_1) position: 1-29, Strand: -

```
 5' --------UCACGGAGCUUUACACAAAUAAAGCCGGA-------- 3'     <-  CRISPR
spacer                                                      RNA
            ||||||||||||||||||||||||||||||
 3' GATGTTCTAGTGCCTCGAAATGTGTTTATTTCGGCCTCGCTGCTT 5'     <-
Protospacer                                                 Sequence
    ||||||||                                ||||||||
 5' CTACAAGATCACGGAGCTTTACACAAATAAAGCCGGAGCGACGAA 3'     <- [Entrez
Nucleotide]
```

Score: 29

4. Match to: **Geobacillus kaustophilus strain Et23 LG51_086, whole genome shotgun sequence**(NZ_JYCF01000086) position: 15565-15537, with: **spacer5** CRISPR No.5 Spacer No.1 (spacer5_5_1) position: 1-29, Strand: +

```
 5' --------UCACGGAGCUUUACACAAAUAAAGCCGGA-------- 3'     <-  CRISPR
spacer                                                      RNA
            ||||||||||||||||||||||||||||||
 3' CATGTTCTAGTGCCTCGAAATGTGTTTATTTCGGCCTCGTTGCTT 5'     <-
Protospacer                                                 Sequence
    ||||||||                                ||||||||
```

Figure 7 (continued)

```
 5' GTACAAGATCACGGAGCTTTACACAAATAAAGCCGGAGCAACGAA 3'    <- [Entrez
Nucleotide]
```

Score: 29

5. Match to: **DQ453159 DQ453159 Geobacillus virus E2, complete genome**(DQ453159) position: 6492-6463, with: **spacer6** CRISPR No.6 Spacer No.1 (spacer6_6_1) position: 1-30, Strand: +

```
 5' --------CAACACCUUCCGCGCUGUCUCGUCUACUUU-------- 3'    <- CRISPR
spacer                                                      RNA
            ||||||||||||||||||||||||||||||
 3' CGAAGAAAGTTGTGGAAGGCACGACAGAGTAGATGAAAAAATGCTT 5'    <-
Protospacer                                                 Sequence
    ||||||||                                    ||||||||
 5' GCTTCTTTCAACACCTTCCGTGCTGTCTCATCTACTTTTTTACGAA 3'    <-
[Entrez Nucleotide]
```

Score: 26

6. Match to: **Pasteurella bettyae CCUG 2042 contig00003, whole genome shotgun sequence**(NZ_AJSX01000041) position: 137780-137808, with: **spacer7** CRISPR No.7 Spacer No.1 (spacer7_7_1) position: 1-30, Strand: -

```
 5' --------UUGAUUAGCAAUUUGACUUGGGAAUUUAGC-------- 3'    <- CRISPR
spacer                                                      RNA
            ||||||||||||||||||||||||||||||
 3' TAATTGTAAACTAATCGTTAAACTGACGCCTTAAATCATTACGGTT 5'    <-
Protospacer                                                 Sequence
    ||||||||                                    ||||||||
 5' ATTAACATTTGATTAGCAATTTGACTGCGGAATTTAGTAATGCCAA 3'    <-
[Entrez Nucleotide]
```

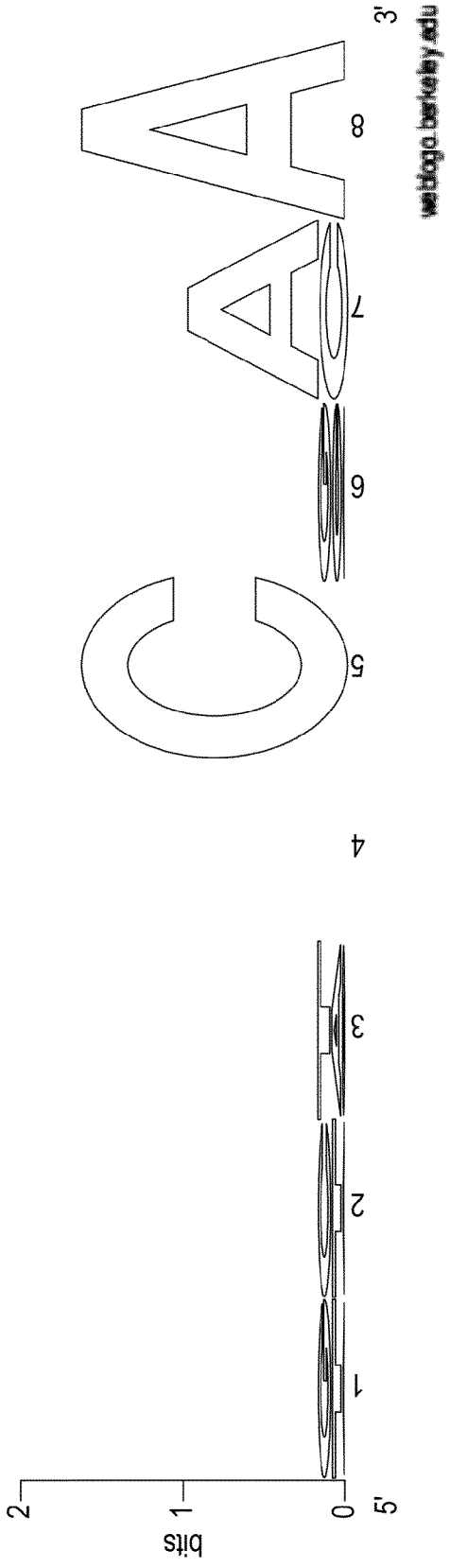


Figure 8

Figure 9

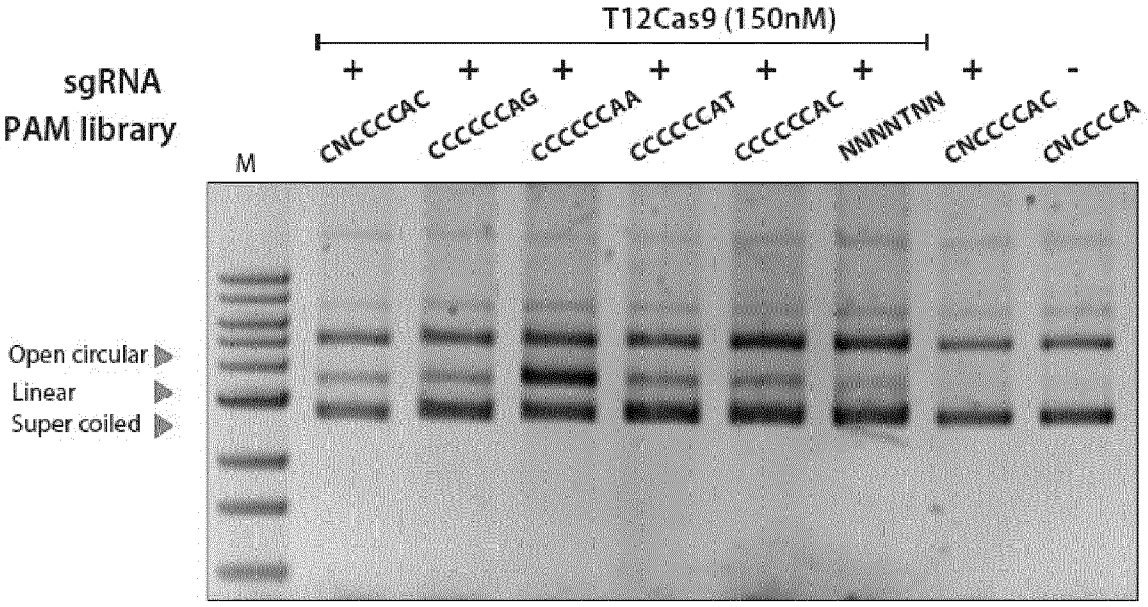

T12Cas9 (150nM)
sgRNA
PAM library
+ + + + + + + -
CNCCCCAC
CCCCCCAG
CCCCCCAA
CCCCCCAT
CCCCCCAC
NNNNTNN
CNCCCCAC
CNCCCCA
M
Open circular
Linear
Super coiled

Figure 10

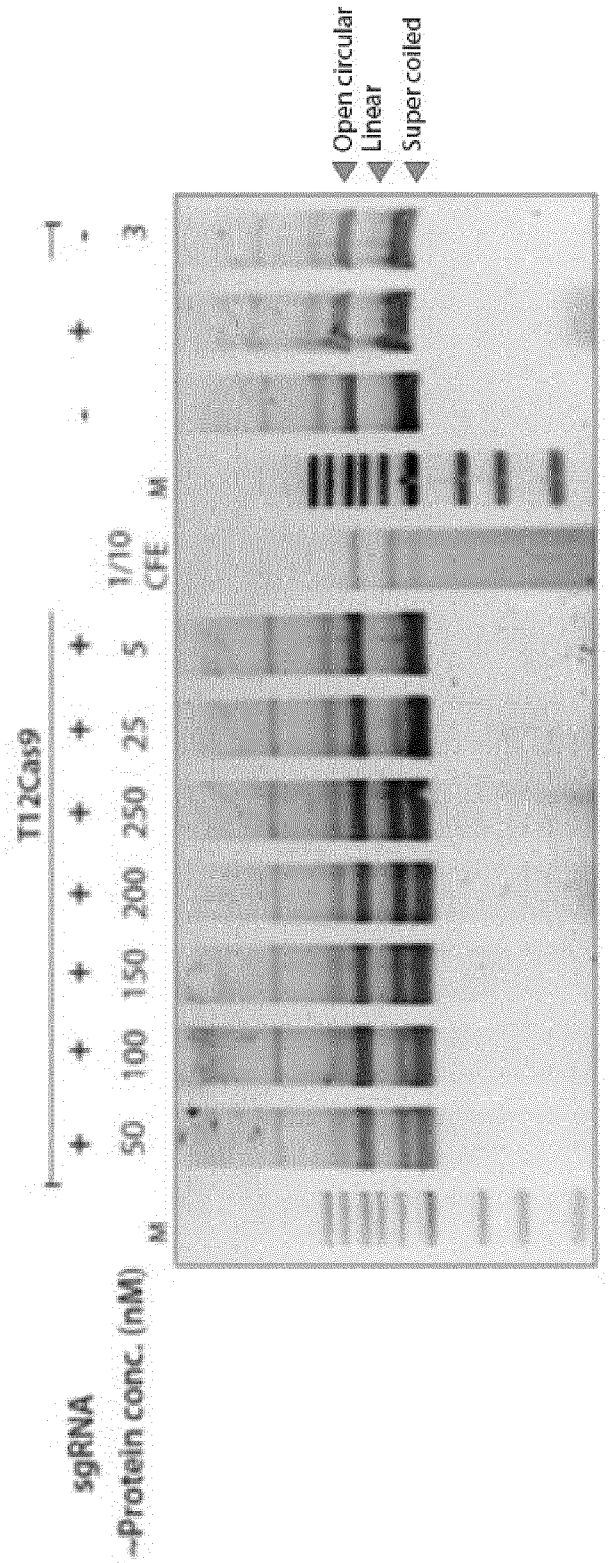

Open circular
Linear
Super coiled
sgRNA
-Protein conc. (nM)
M
50
100
150
200
250
25
5
T12Cas9
1/10 CFE
M
3

Figure 11

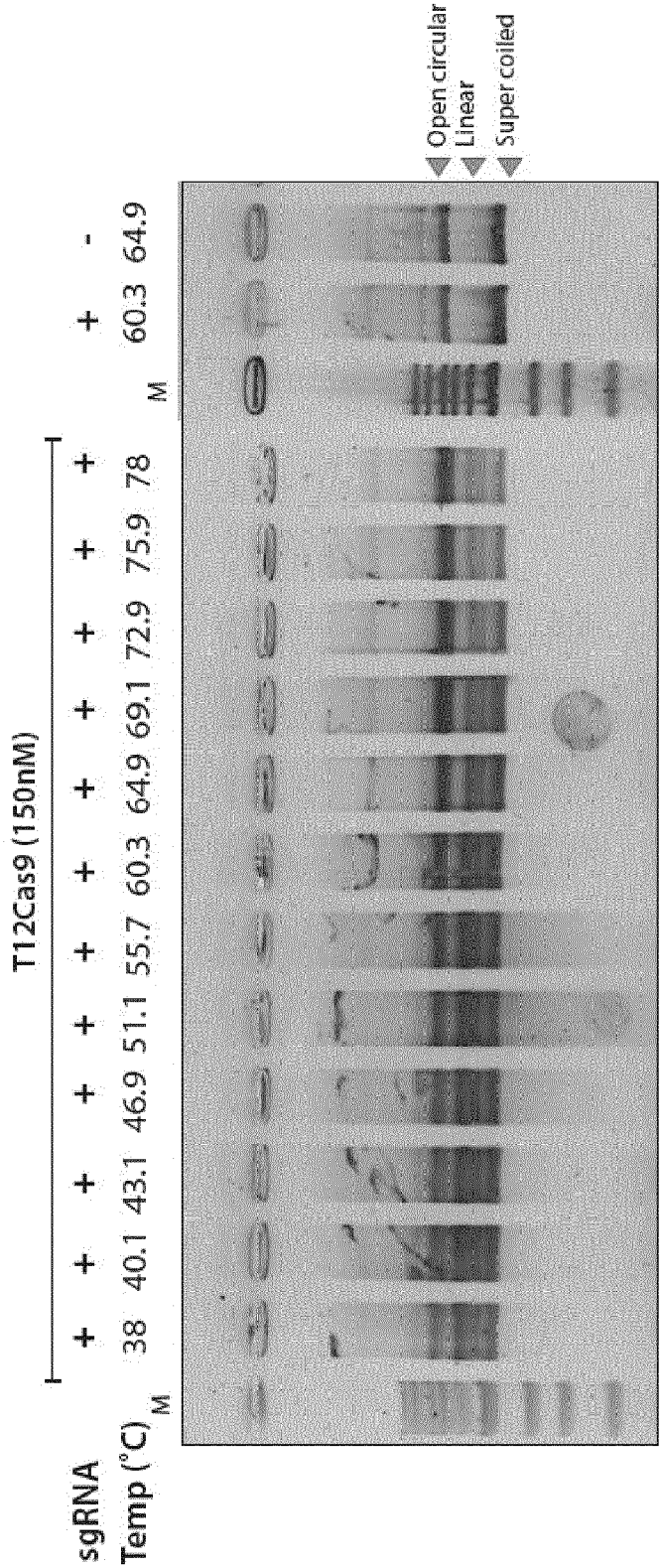
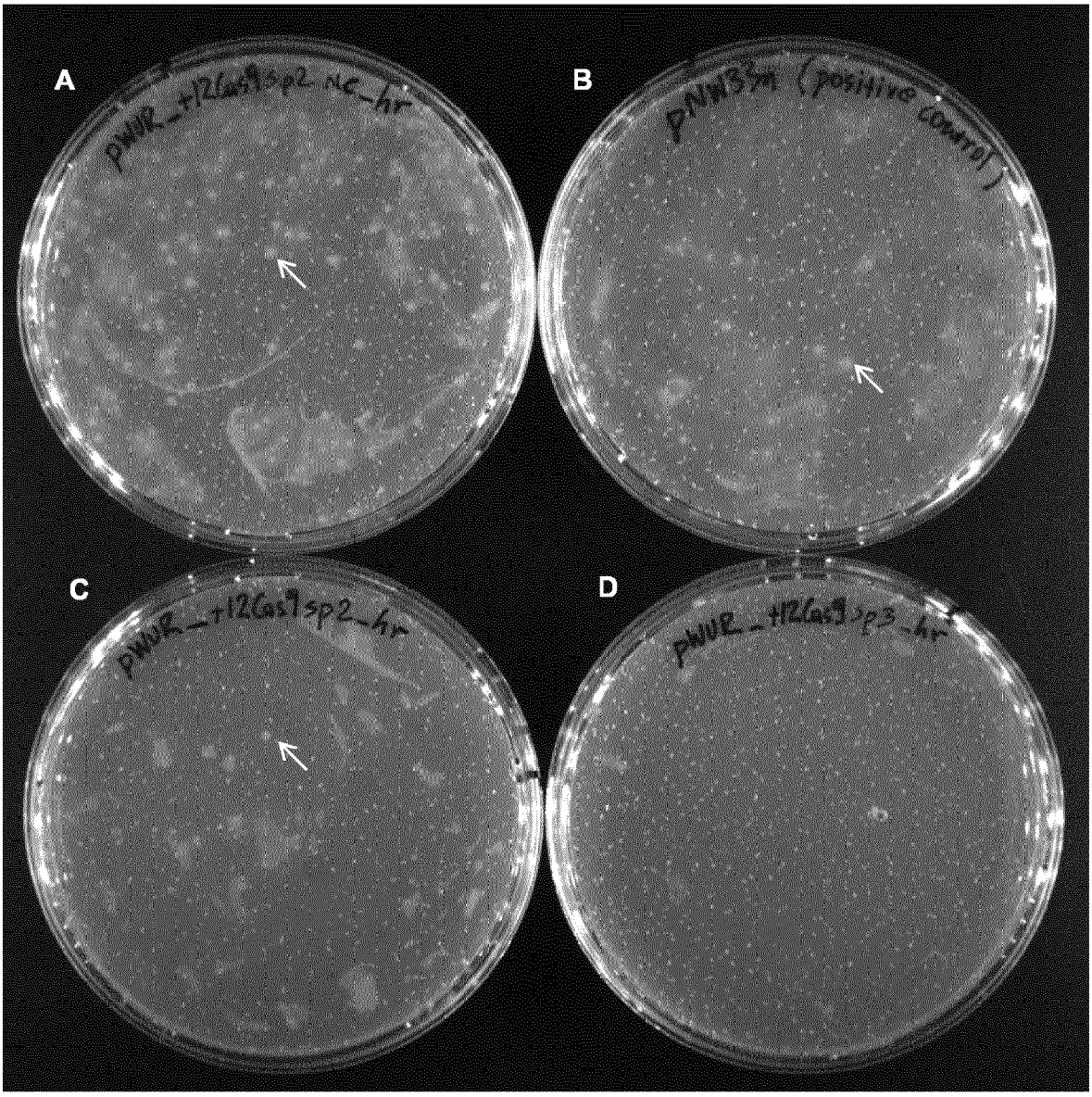

Open circular
Linear
Super coiled
T12Cas9 (150nM)
sgRNA
Temp (°C)
M
38
40.1
43.1
46.9
51.1
55.7
60.3
64.9
69.1
72.9
75.9
78
M
60.3
64.9

Figure 12



Colonies are indicated with arrows.

Figure 13

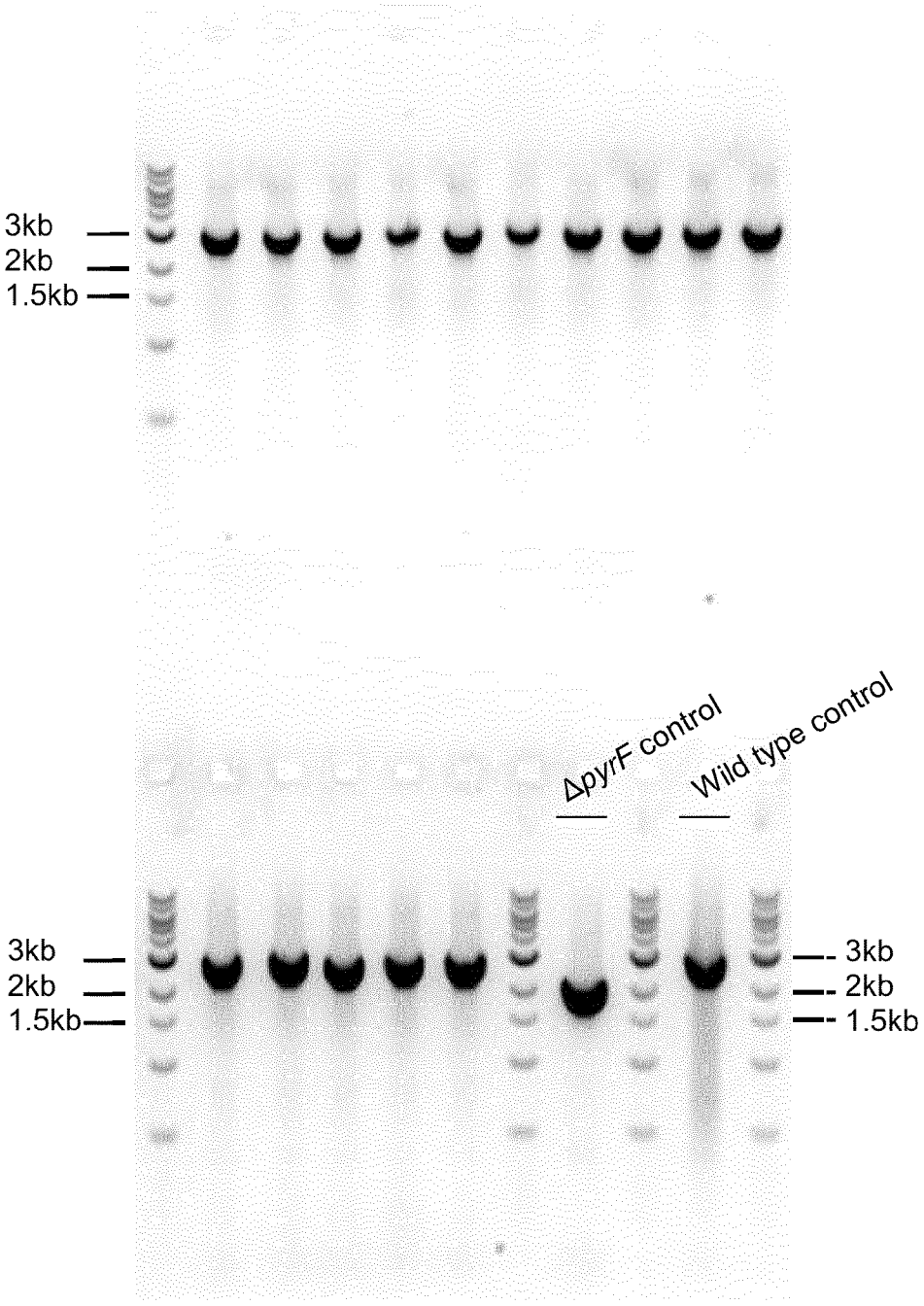
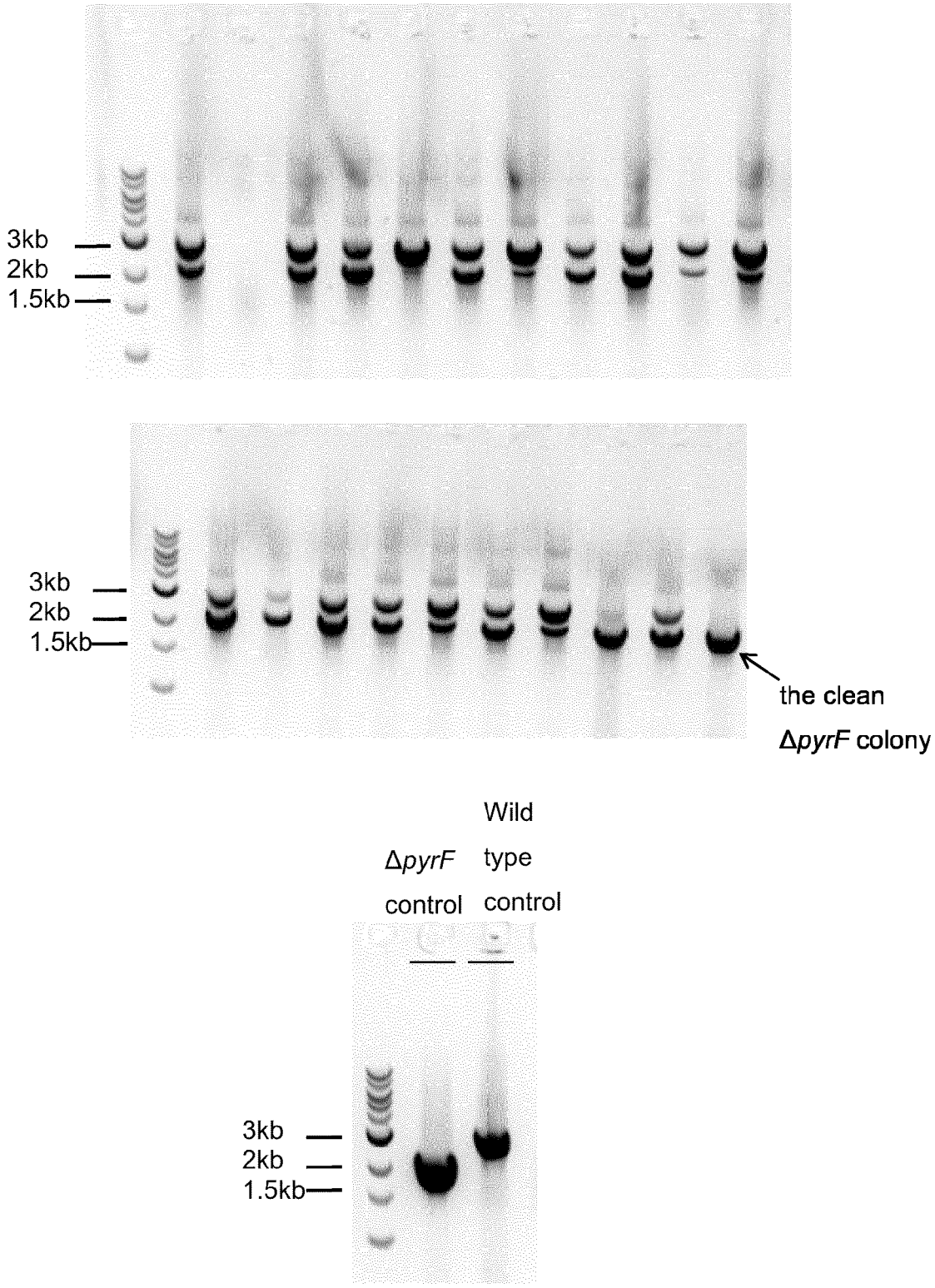

3kb
2kb
1.5kb
ΔpyrF control
Wild type control
3kb
2kb
1.5kb
3kb
2kb
1.5kb

Figure 14


3kb
2kb
1.5kb
3kb
2kb
1.5kb
the clean
ΔpyrF colony
ΔpyrF control
Wild type control
3kb
2kb
1.5kb

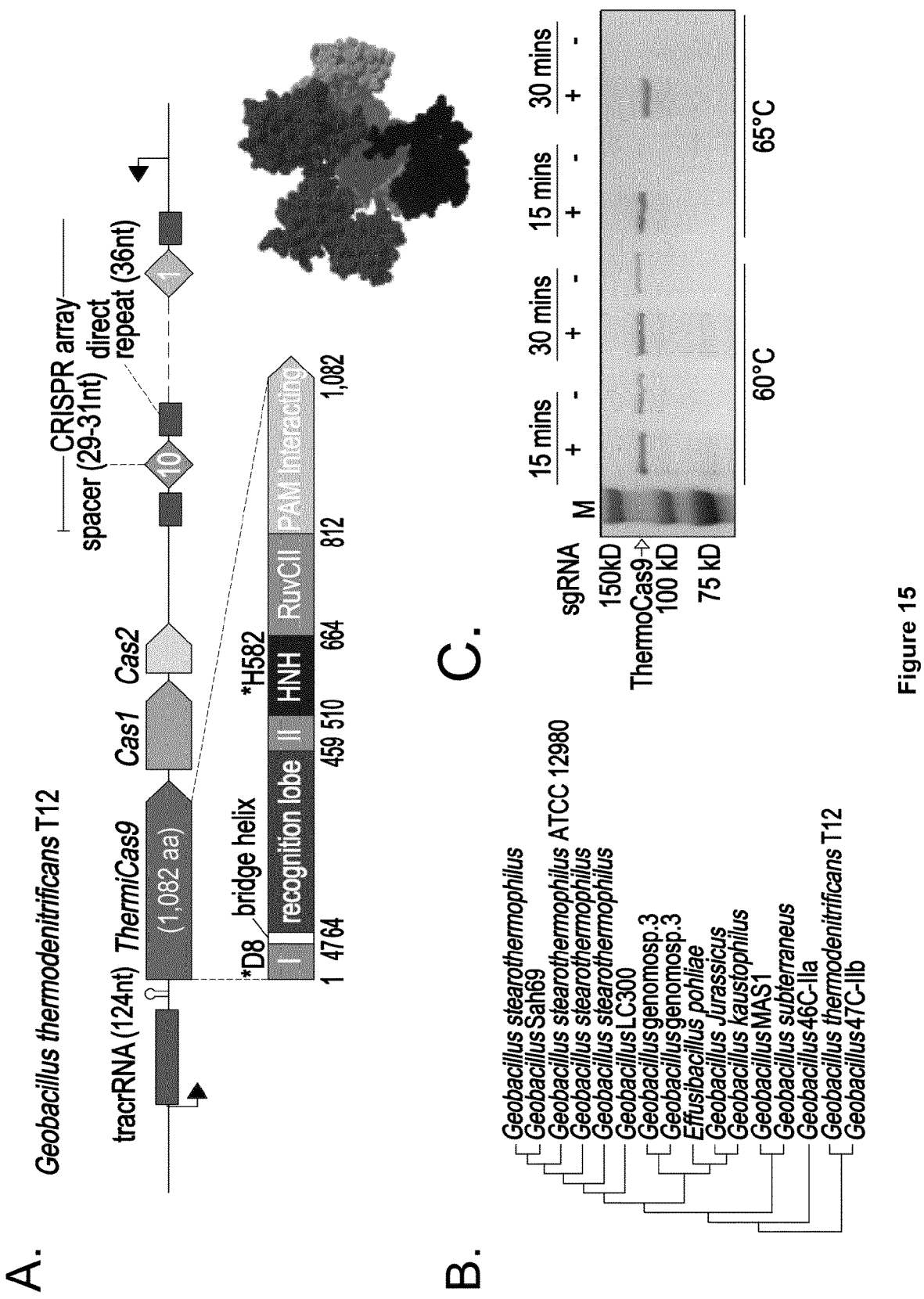

A.
Geobacillus thermodenitrificans T12
tracrRNA (124nt)
ThermiCas9
(1,082 aa)
Cas1
Cas2
CRISPR array
spacer (29-31nt)
direct repeat (36nt)
1
10
1
*D8
bridge helix
*H582
I
recognition lobe
II
HNH
RuvCII
PAM Interacting
1
4
764
459
510
664
812
1,082
B.
Geobacillus stearothermophilus
Geobacillus Sah69
Geobacillus stearothermophilus ATCC 12980
Geobacillus stearothermophilus
Geobacillus stearothermophilus
Geobacillus LC300
Geobacillus genomosp.3
Geobacillus genomosp.3
Effusibacillus pohliae
Geobacillus Jurassicus
Geobacillus kaustophilus
Geobacillus MAS1
Geobacillus subterraneus
Geobacillus 46C-IIa
Geobacillus thermodenitrificans T12
Geobacillus 47C-IIb
C.
sgRNA
M
15 mins
30 mins
15 mins
30 mins
+
-
+
-
+
-
+
-
60°C
65°C
150kD
ThermoCas9
100 kD
75 kD

Figure 15

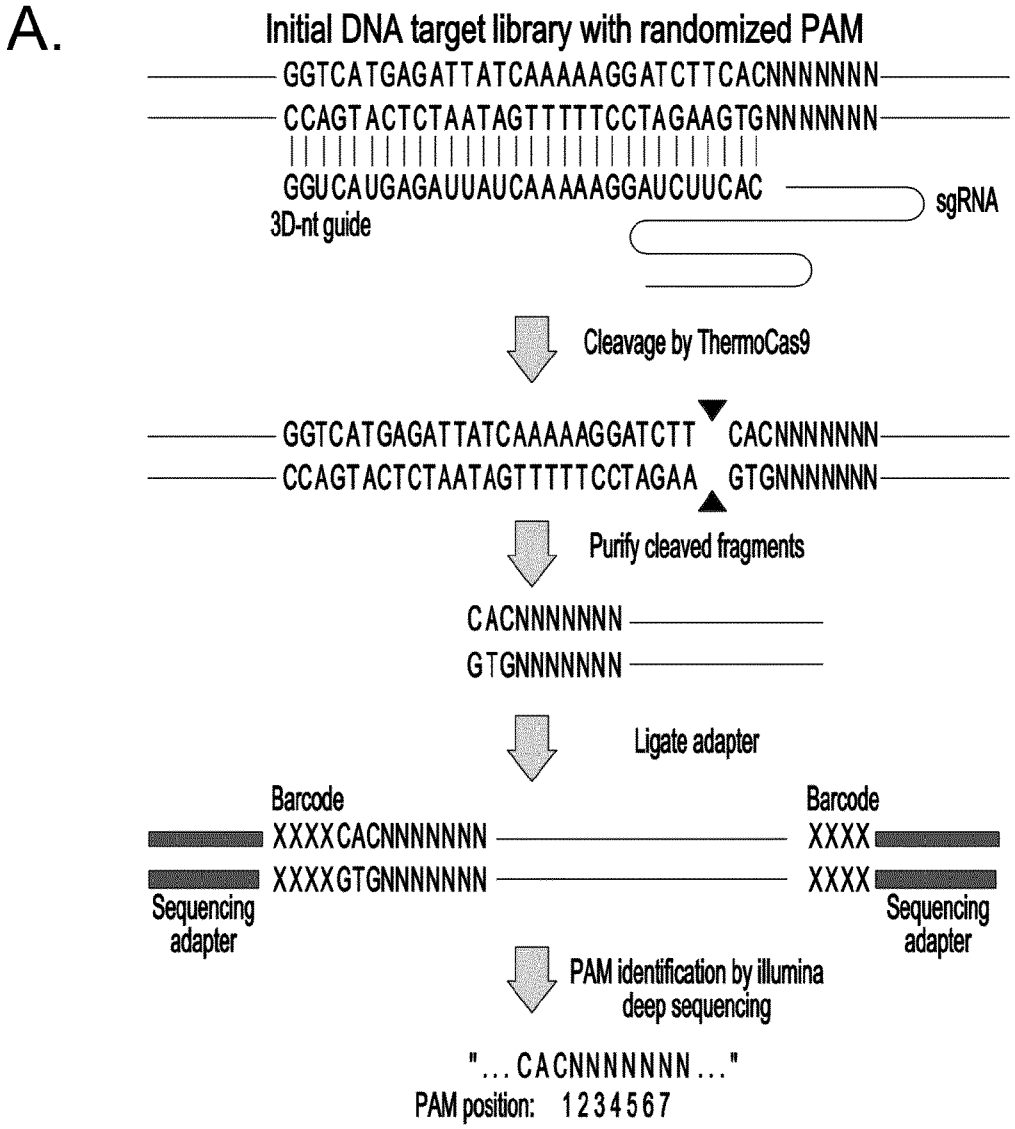
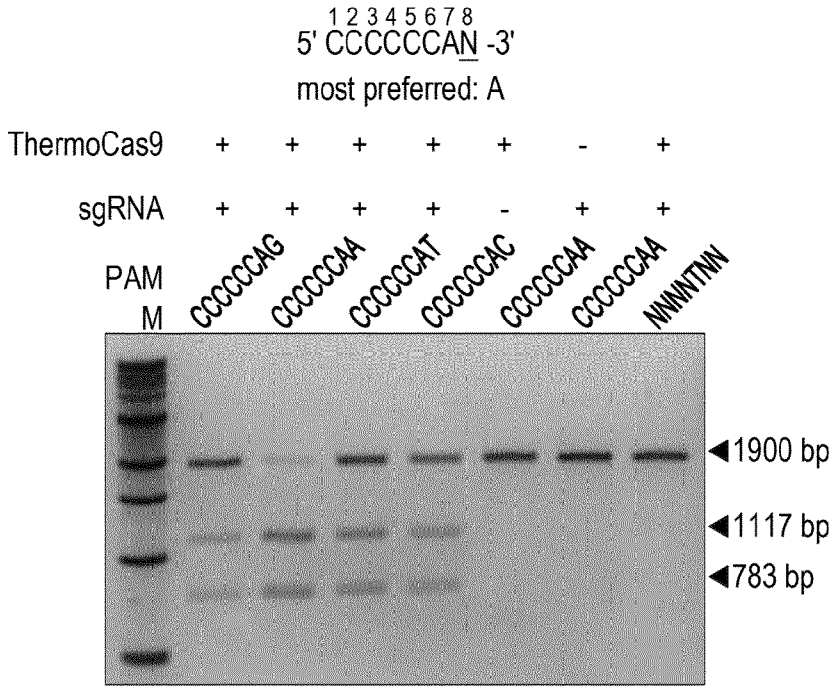

A.
Initial DNA target library with randomized PAM
GGTCATGAGATTATCAAAAAGGATCTTCACNNNNNNN
CCAGTACTCTAATAGTTTTTCCTAGAAGTGNNNNNNN
GGUCAUGAGAUUAUCAAAAAGGAUCUUCAC
3D-nt guide
sgRNA
Cleavage by ThermoCas9
GGTCATGAGATTATCAAAAAGGATCTT CACNNNNNNN
CCAGTACTCTAATAGTTTTTCCTAGAA GTGNNNNNNN
Purify cleaved fragments
CACNNNNNNN
GTGNNNNNNN
Ligate adapter
Barcode
XXXXCACNNNNNNN
XXXXGTGNNNNNNN
Sequencing adapter
Barcode
XXXX
XXXX
Sequencing adapter
PAM identification by illumina deep sequencing
"...CACNNNNNNN..."
PAM position: 1234567
B.
bits
2
1
0
5'
1
2
3
4
5
6
7
3'
7
5' PAM position

Figure 16

C.


1 2 3 4 5 6 7 8
5' CCCCCCAN -3'
most preferred: A
ThermoCas9 + + + + + - +
sgRNA + + + + - + +
PAM
M
CCCCCAG
CCCCCAA
CCCCCAT
CCCCCAC
CCCCCAA
CCCCCAA
NNNNTNN
1900 bp
1117 bp
783 bp

D.

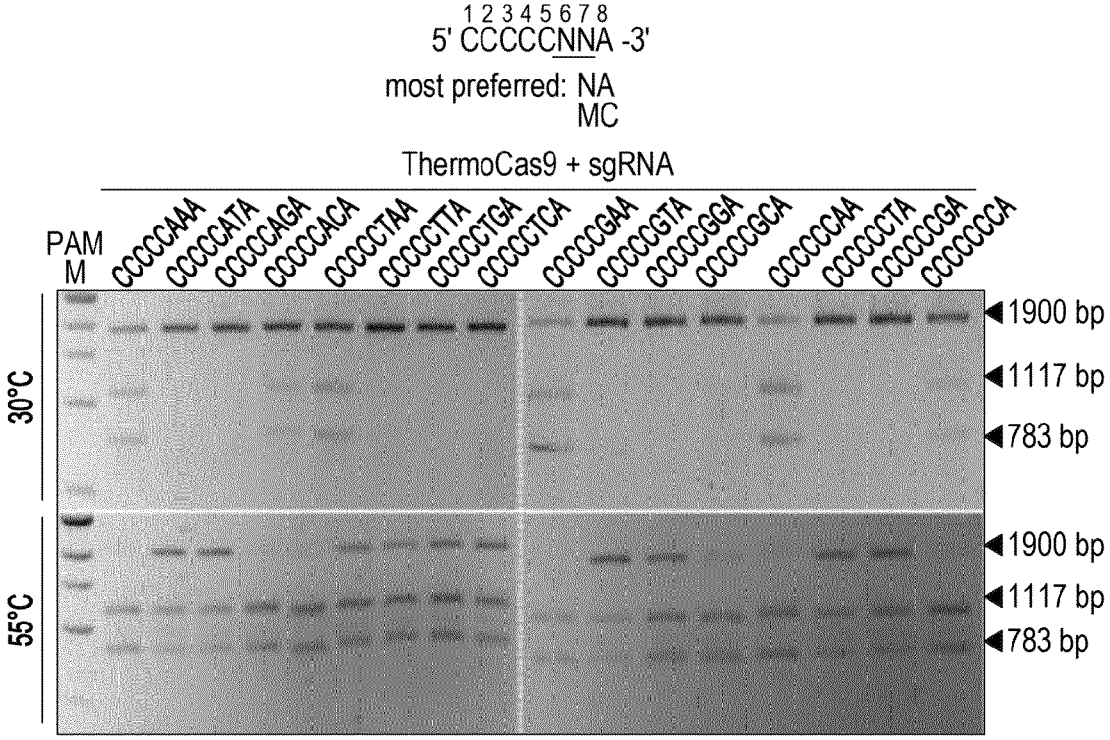
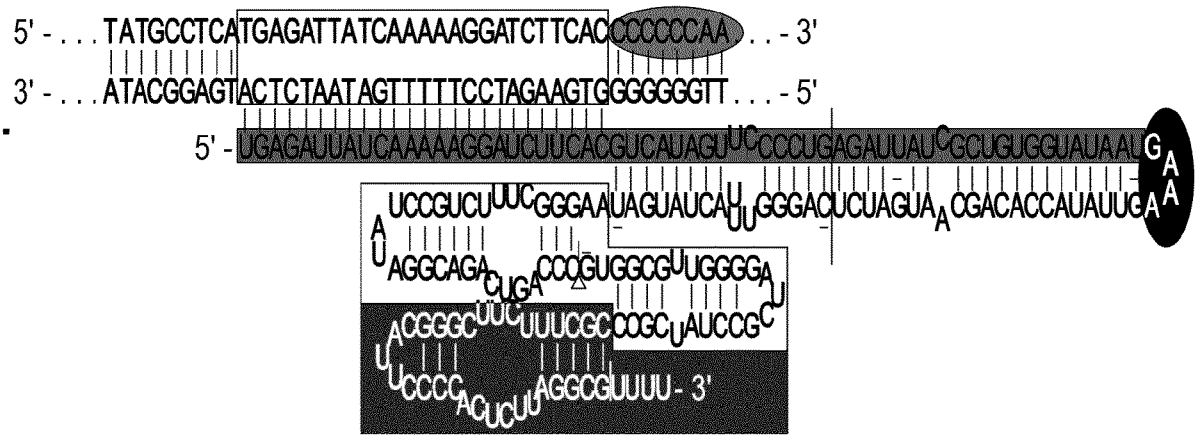

1 2 3 4 5 6 7 8
5' CCCCCNNA -3'
most preferred: NA
MC
ThermoCas9 + sgRNA
PAM
M
CCCCCAA
CCCCCATA
CCCCCAGA
CCCCCACA
CCCCCTAA
CCCCCTTA
CCCCCTGA
CCCCCTCA
CCCCCGAA
CCCCCGTA
CCCCCGGA
CCCCCGCA
CCCCCCAA
CCCCCCTA
CCCCCCGA
CCCCCCCA
30°C
55°C
1900 bp
1117 bp
783 bp

Figure 16

A.


5' - . . . TATGCCTCATGAGATTATCAAAAAGGATCTTCACCCCCCCAA . . - 3'
3' - . . . ATACGGAGTACTCTAATAGTTTTTCCTAGAAGTGGGGGGGTT . . . - 5'
5' -
- 3'

B.

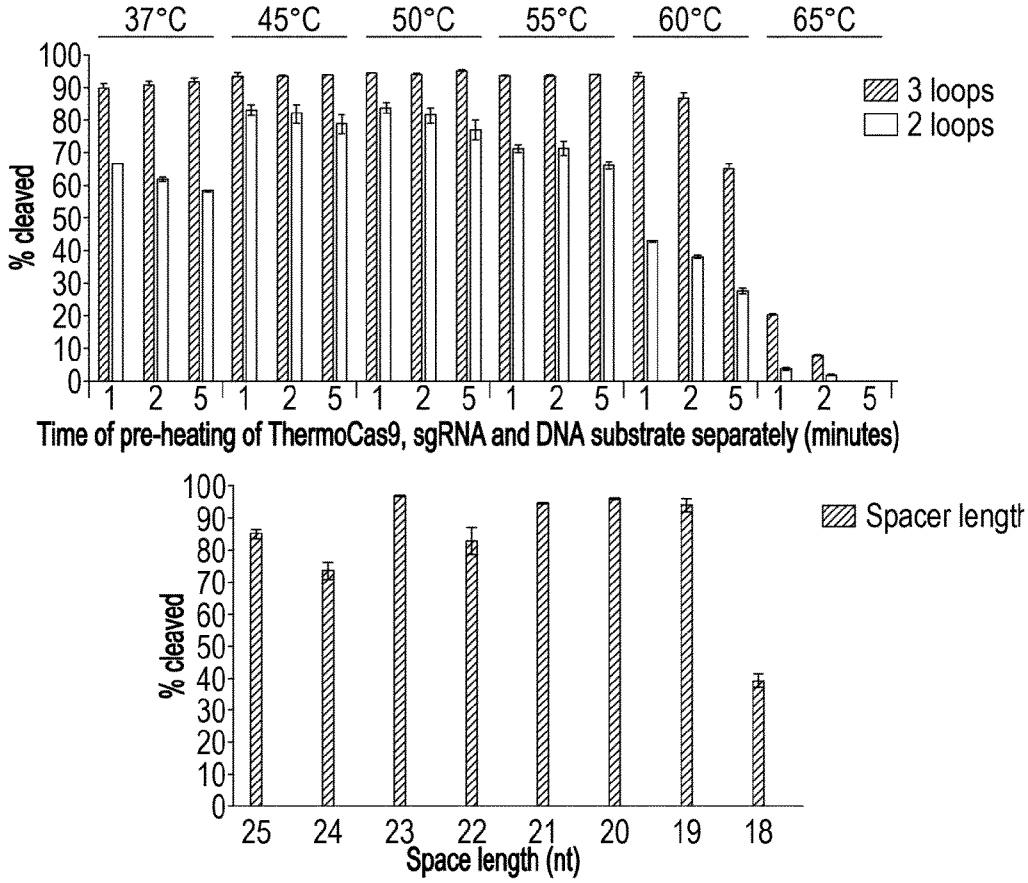
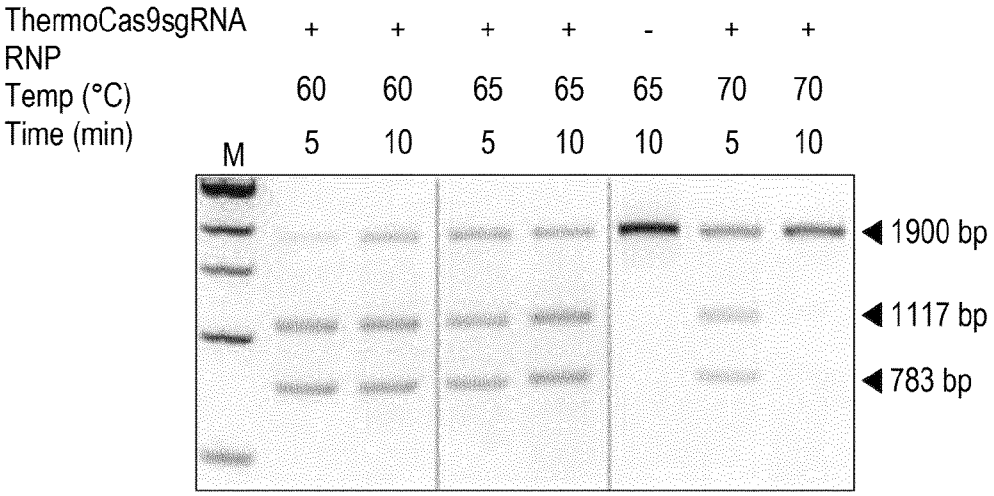

37°C
45°C
50°C
55°C
60°C
65°C
% cleaved
3 loops
2 loops
Time of pre-heating of ThermoCas9, sgRNA and DNA substrate separately (minutes)
Spacer length
Space length (nt)

Figure 17

C.


ThermoCas9sgRNA + + + + - + +
RNP
Temp (°C) 60 60 65 65 65 70 70
Time (min) 5 10 5 10 10 5 10
M
1900 bp
1117 bp
783 bp

D.

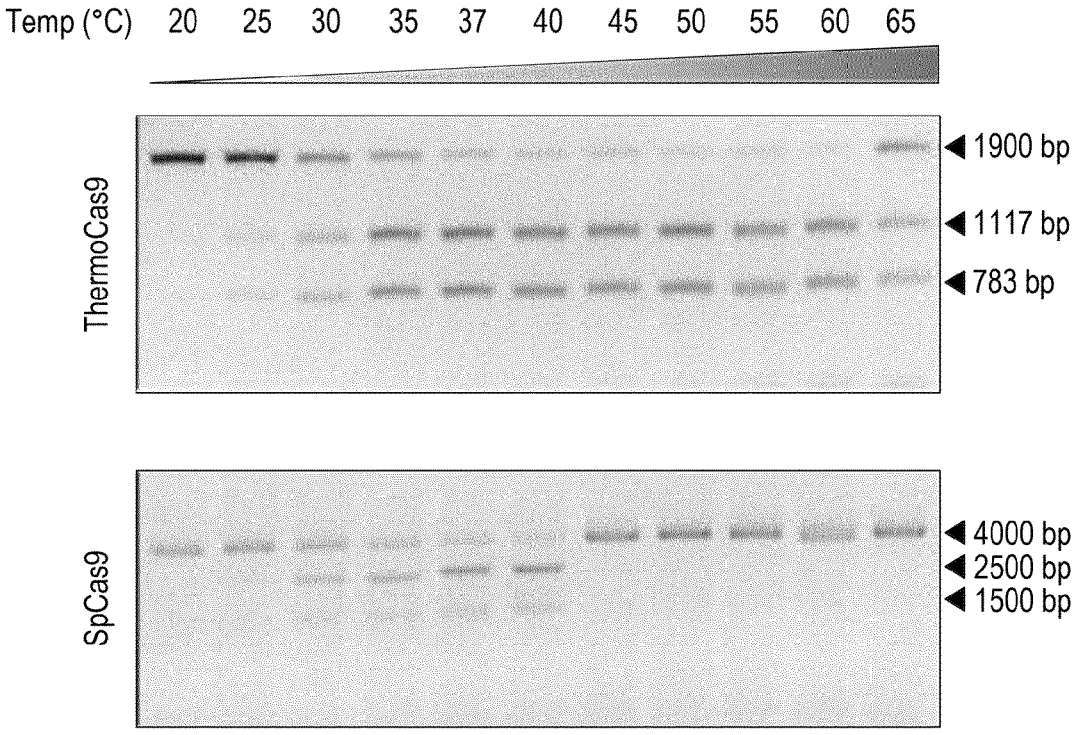

Temp (°C) 20 25 30 35 37 40 45 50 55 60 65
ThermoCas9
1900 bp
1117 bp
783 bp
SpCas9
4000 bp
2500 bp
1500 bp

Figure 17

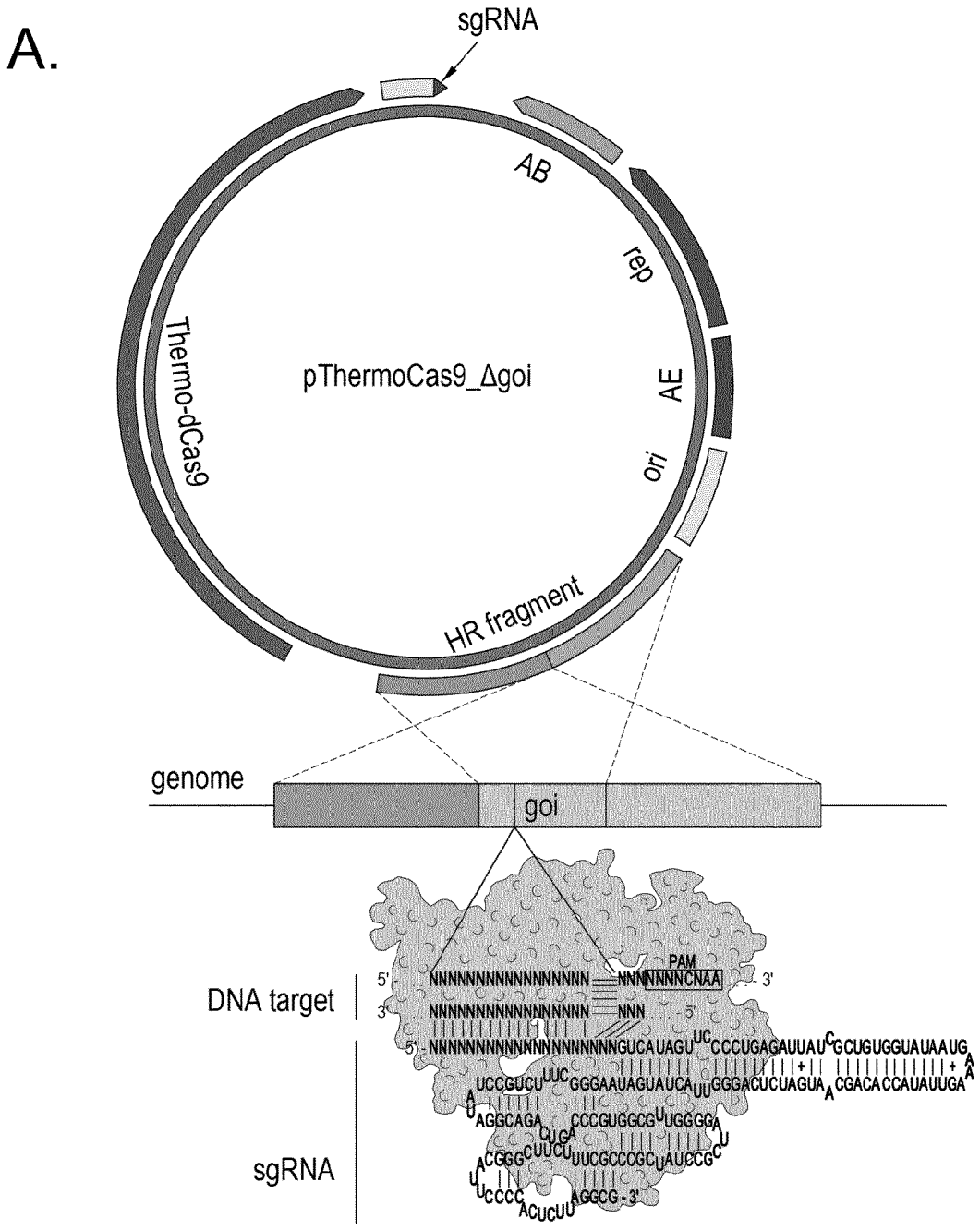

A.
sgRNA
AB
rep
AE
ori
HR fragment
Thermo-dCas9
pThermoCas9_Δgoi
genome
goi
DNA target
sgRNA
PAM
B.
Colomy PCR: Bacillus smith II ΔpyrF
Mix ΔpyrF/WT
ΔpyrF
M
2900 bp
2165 bp

Figure 18

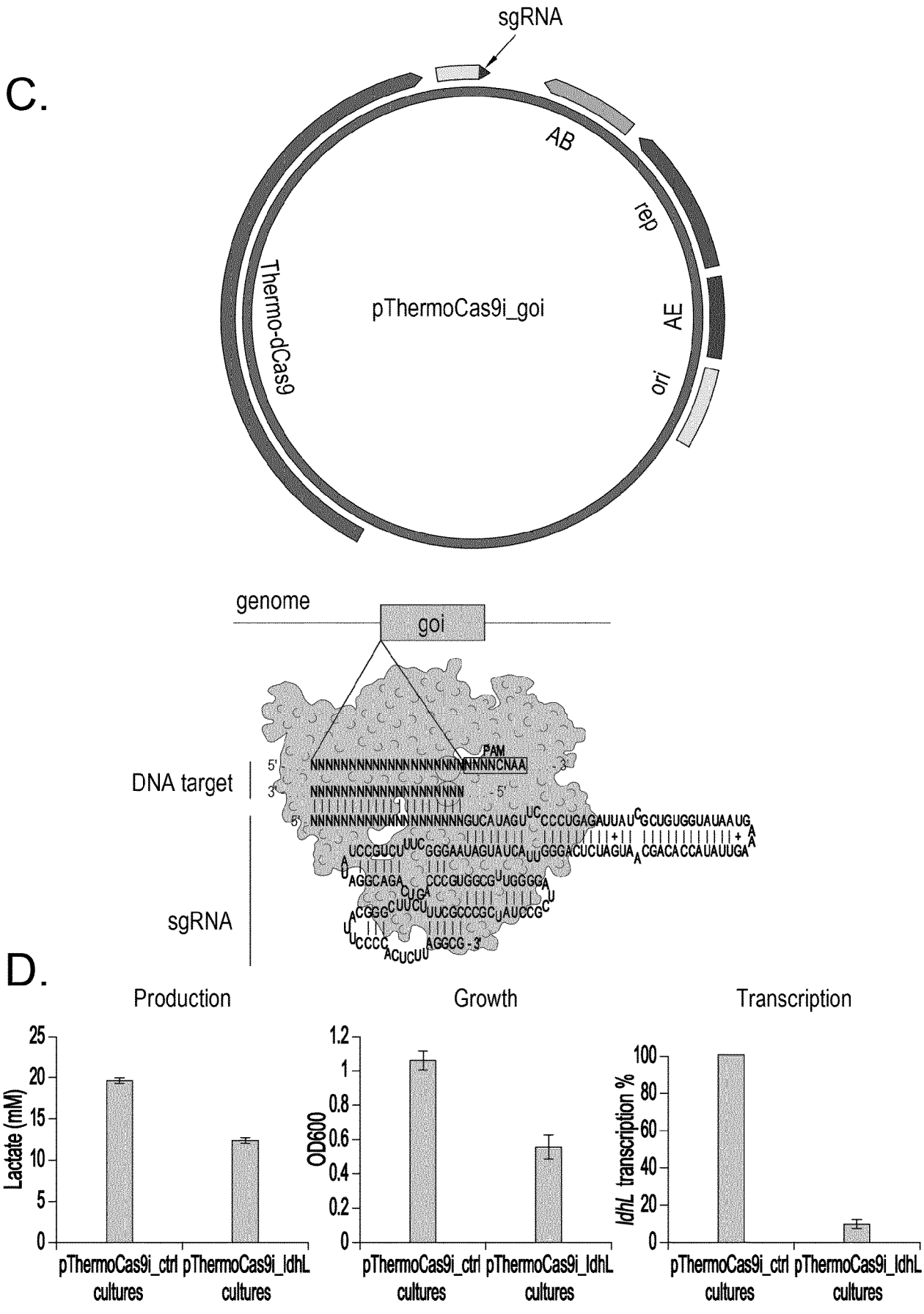

C.
sgRNA
AB
rep
AE
ori
Thermo-dCas9
pThermoCas9i_goi
genome
goi
PAM
DNA target
sgRNA
D.
Production
Growth
Transcription
Lactate (mM)
OD600
ldhL transcription %
pThermoCas9i_ctrl cultures
pThermoCas9i_ldhL cultures

Figure 18

Figure 19

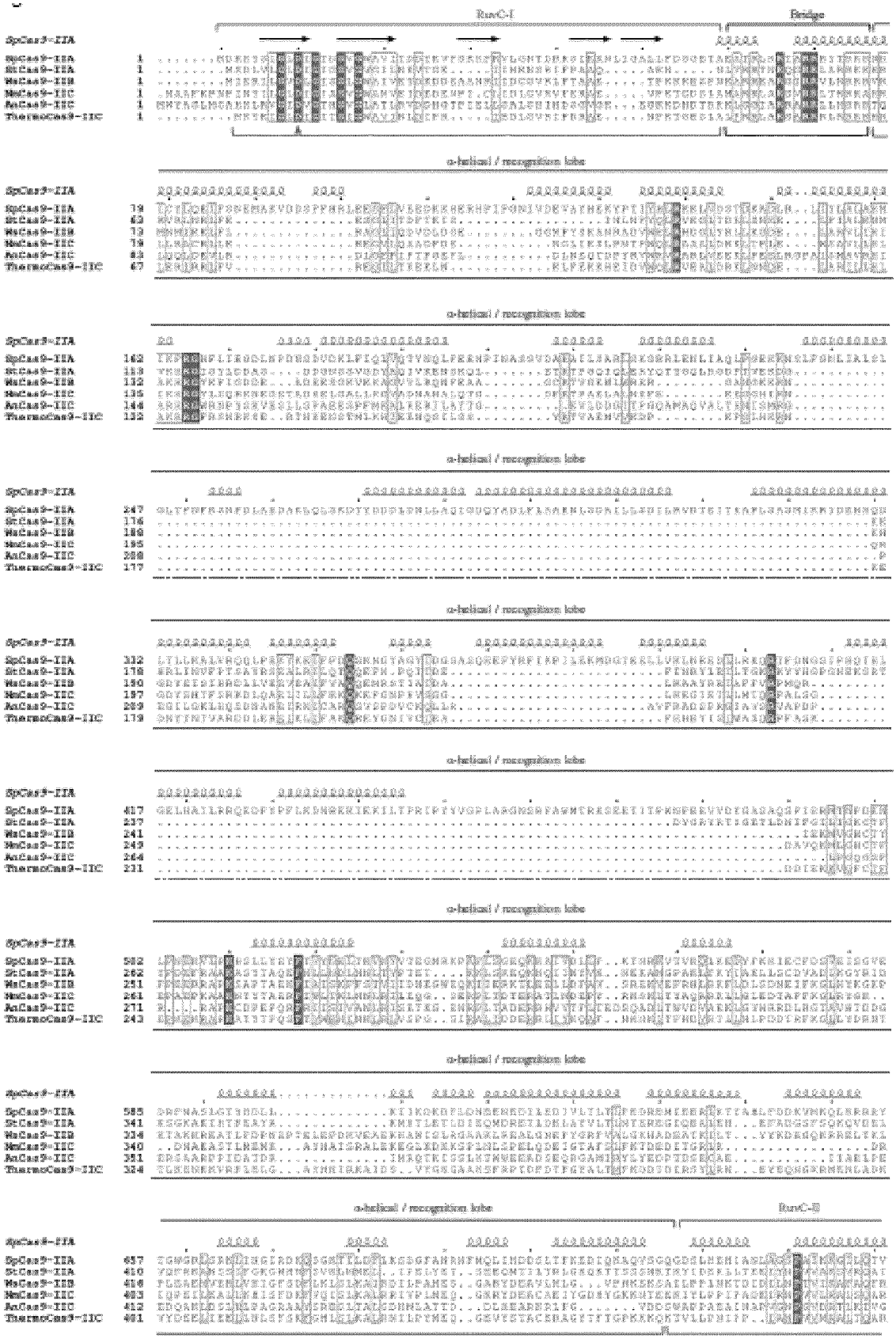

RuvC-I
Bridge
α-helical / recognition lobe
α-helical / recognition lobe
α-helical / recognition lobe
α-helical / recognition lobe
α-helical / recognition lobe
α-helical / recognition lobe
α-helical / recognition lobe
RuvC-II
SpCas9-IIA
StCas9-IIA
WsCas9-IIB
NmCas9-IIC
AsCas9-IIC
ThermoCas9-IIC

Figure 19

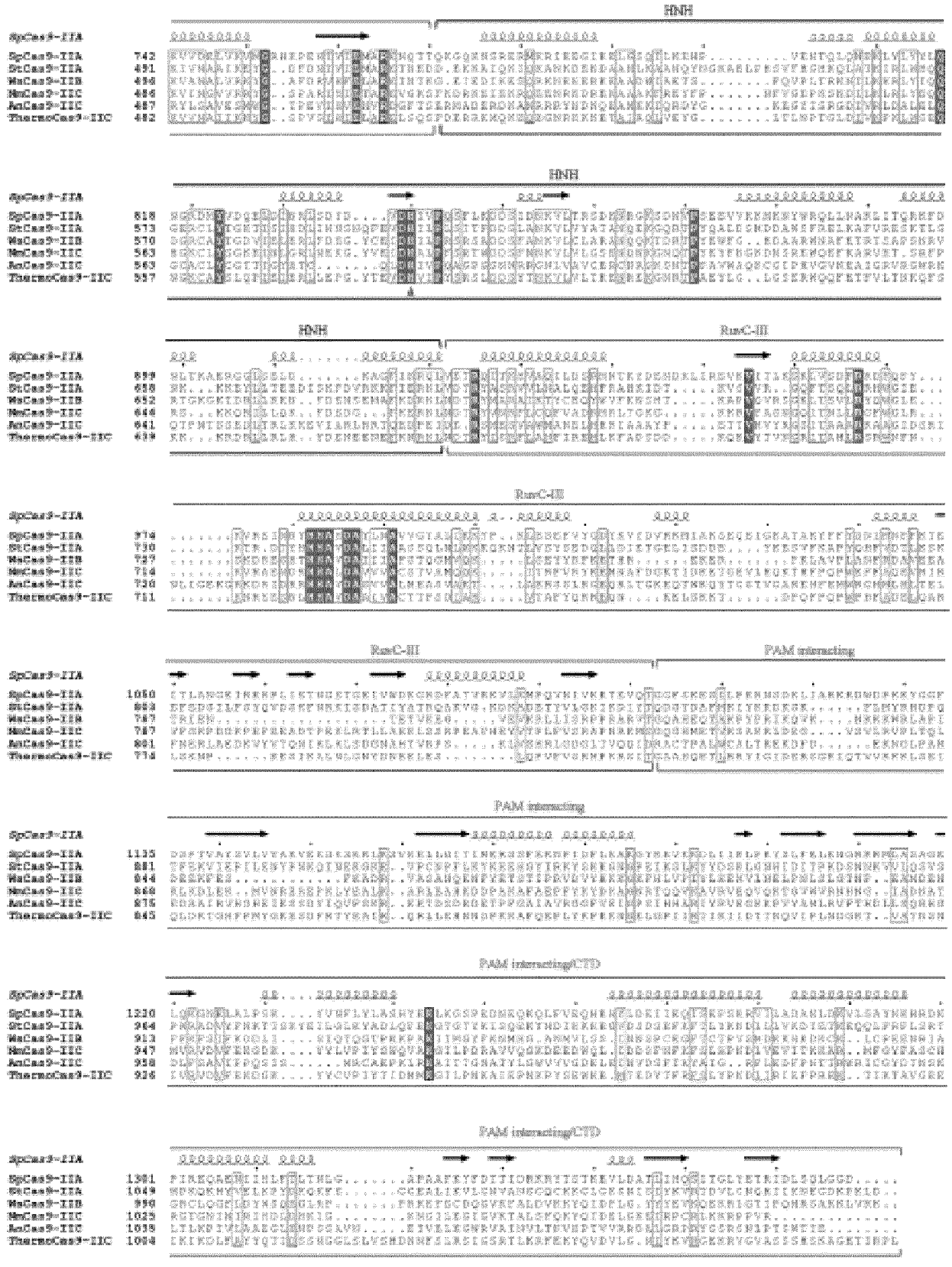

HNH
RuvC-III
PAM interacting
PAM interacting/CTD
SpCas9-IIA
SpCas9-IIA
StCas9-IIA
NmCas9-IIB
NmCas9-IIC
AnCas9-IIC
ThermoCas9-IIC

Figure 20

A.

Match to: **DQ453159 DQ453159 Geobacillus virus E2, complete genome**(DQ453159) position: 39980-39951, with: **spacer4** CRISPR No.4 Spacer No.1 (spacer4_4_1) position: 1-30, Strand: +

```
 5' --------UUGGCGGUGCGAAUUCUAACCGUCCCGGAA-------- 3'     <- CRISPR
spacer                                                      RNA
              ||||||||||||||||||||||||||||||
 3' TAGCGTACAACCGCCACGCTTAAGATTGGCAGGGCCTTCGTGGTGT 5'     <-
Protospacer                                                 Sequence
    ||||||||                                  ||||||||
 5' ATCGCATGTTGGCGGTGCGAATTCTAACCGTCCCGGAAGCACCACA 3'     <-
[Entrez Nucleotide]
```

Match to: **DQ453159 DQ453159 Geobacillus virus E2, complete genome**(DQ453159) position: 6492-6463, with: **spacer6** CRISPR No.6 Spacer No.1 (spacer6_6_1) position: 1-30, Strand: +

```
 5' --------CAACACCUUCCGCGCUGUCUCGUCUACUUU-------- 3'     <- CRISPR
spacer                                                      RNA
              |||||||||||| |||||||| |||||||
 3' CGAAGAAAGTTGTGGAAGGCACGACAGAGTAGATGAAAAAATGCTT 5'     <-
Protospacer                                                 Sequence
    ||||||||                                  ||||||||
 5' GCTTCTTTCAACACCTTCCGTGCTGTCTCATCTACTTTTTACGAA 3'     <-
[Entrez Nucleotide]
```

B.

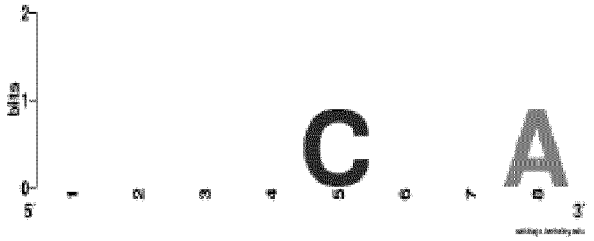


Figure 21

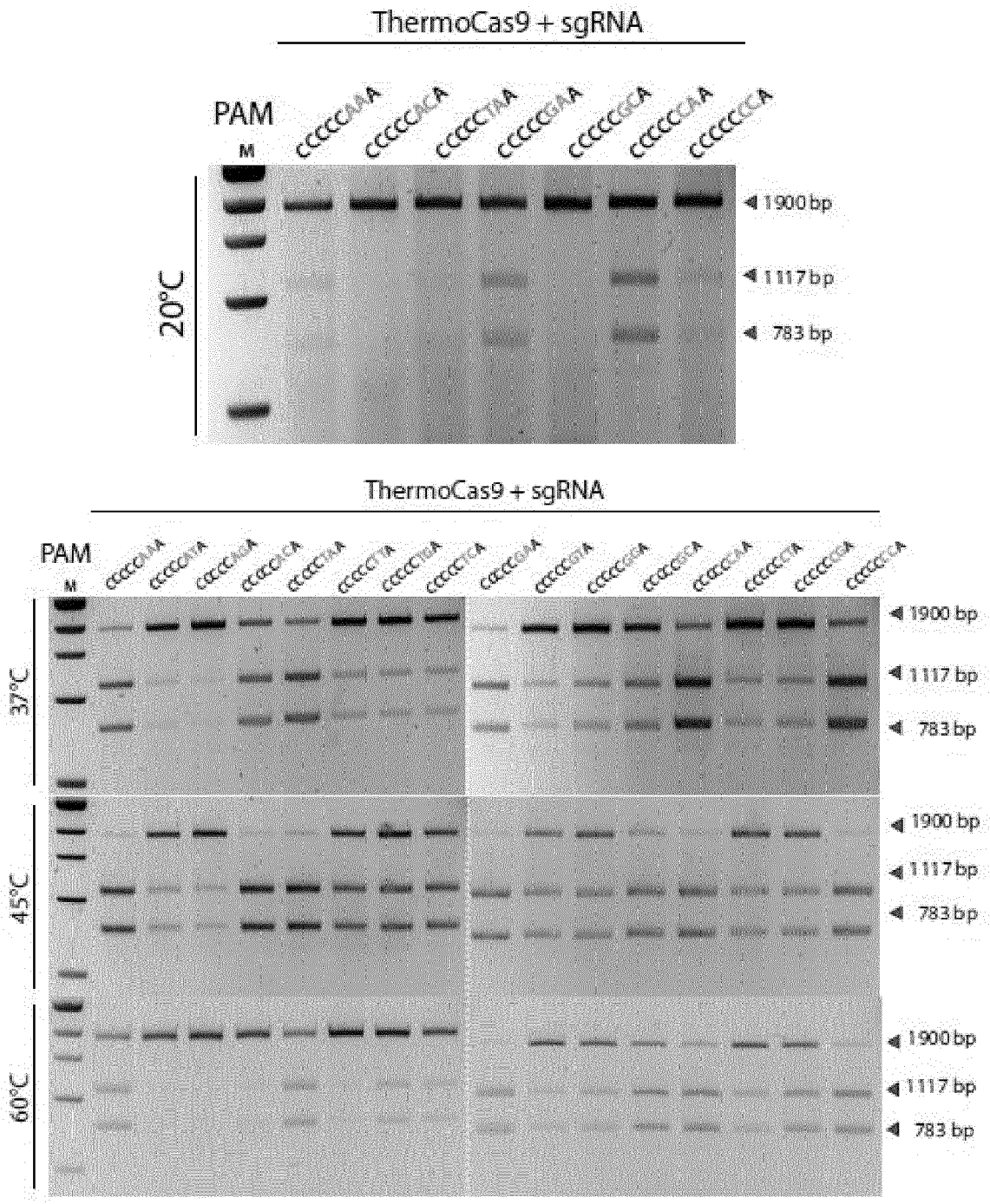

ThermoCas9 + sgRNA
PAM
M
20°C
1900 bp
1117 bp
783 bp
ThermoCas9 + sgRNA
PAM
M
37°C
45°C
60°C
1900 bp
1117 bp
783 bp

Figure 22

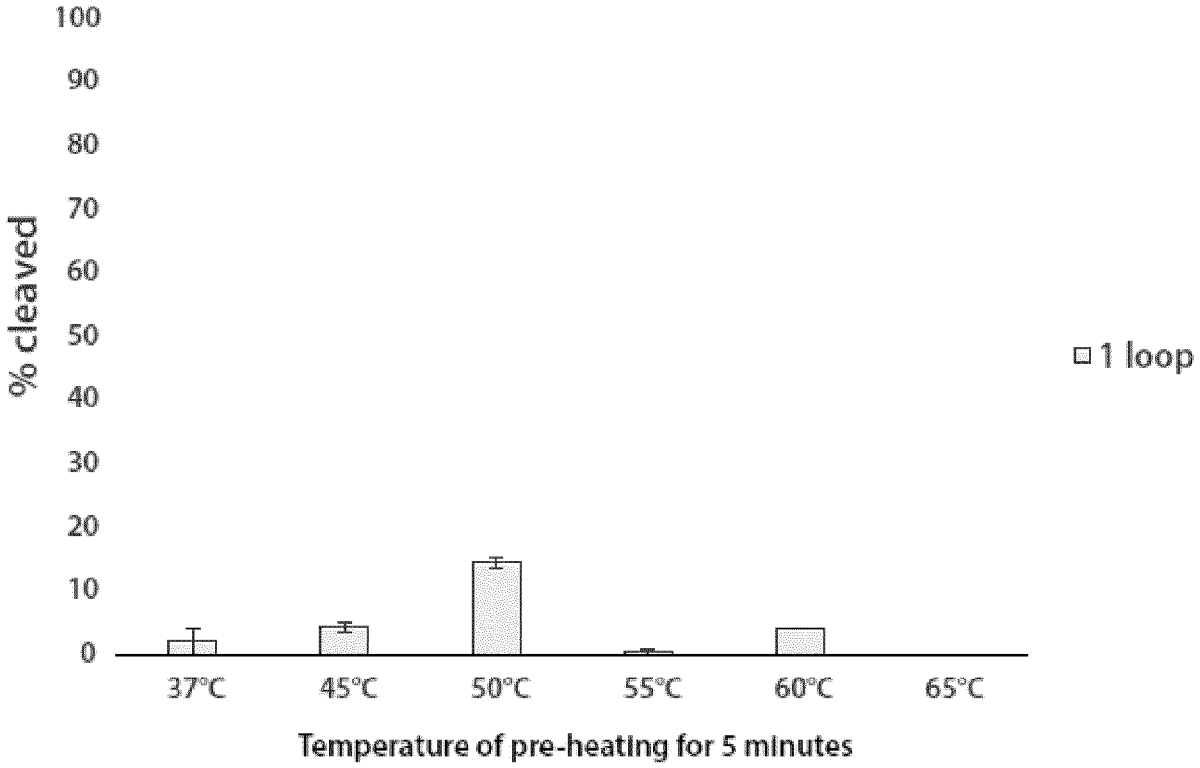

100
90
80
70
60
50
40
30
20
10
0
% cleaved
37°C
45°C
50°C
55°C
60°C
65°C
Temperature of pre-heating for 5 minutes
1 loop

Figure 23

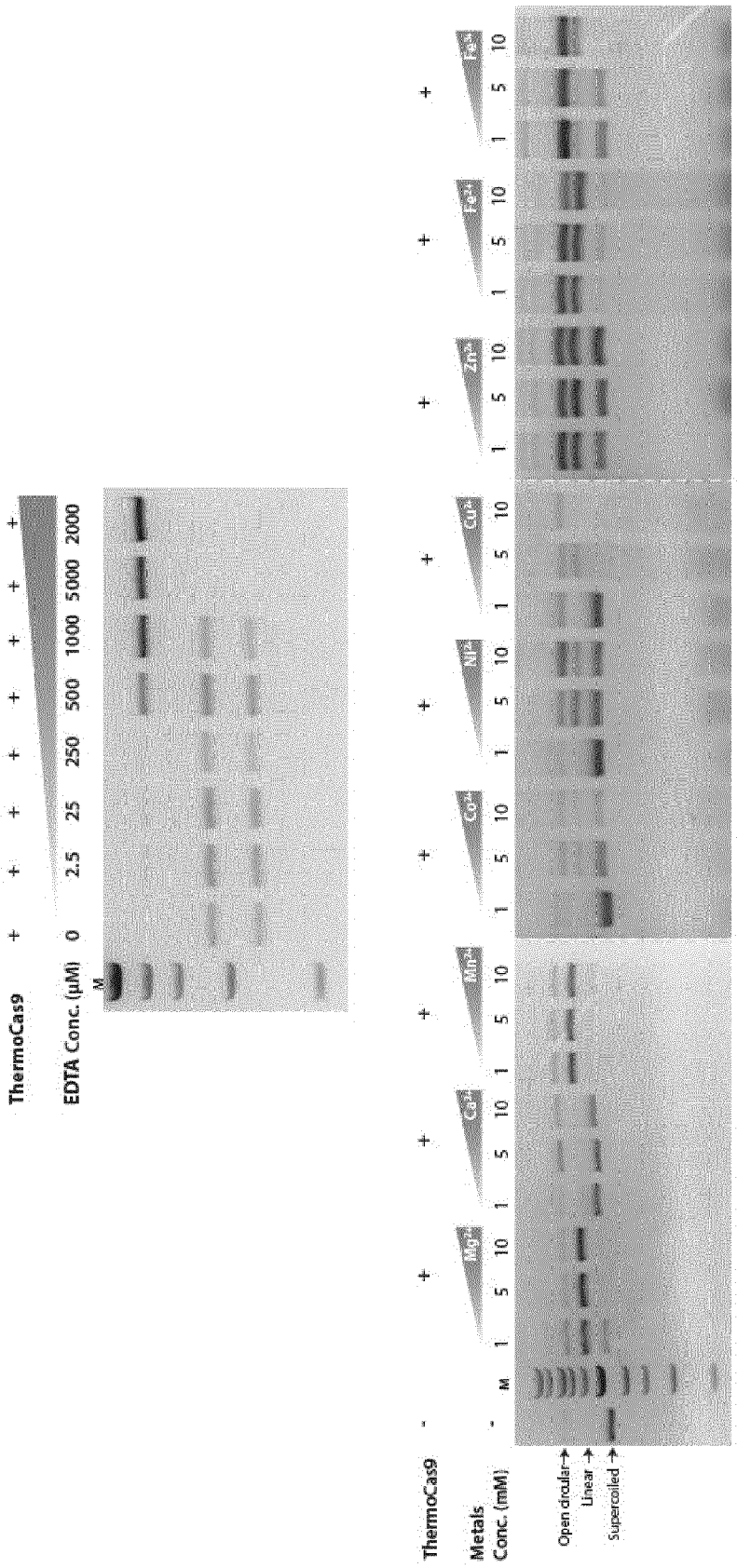

ThermoCas9
EDTA Conc. (μM)
M
0
2.5
25
250
500
1000
5000
2000
ThermoCas9
Metals
Conc. (mM)
Open circular→
Linear →
Supercoiled →
Mg2+
Ca2+
Mn2+
Co2+
Ni2+
Cu2+
Zn2+
Fe2+
Fe3+

Figure 23

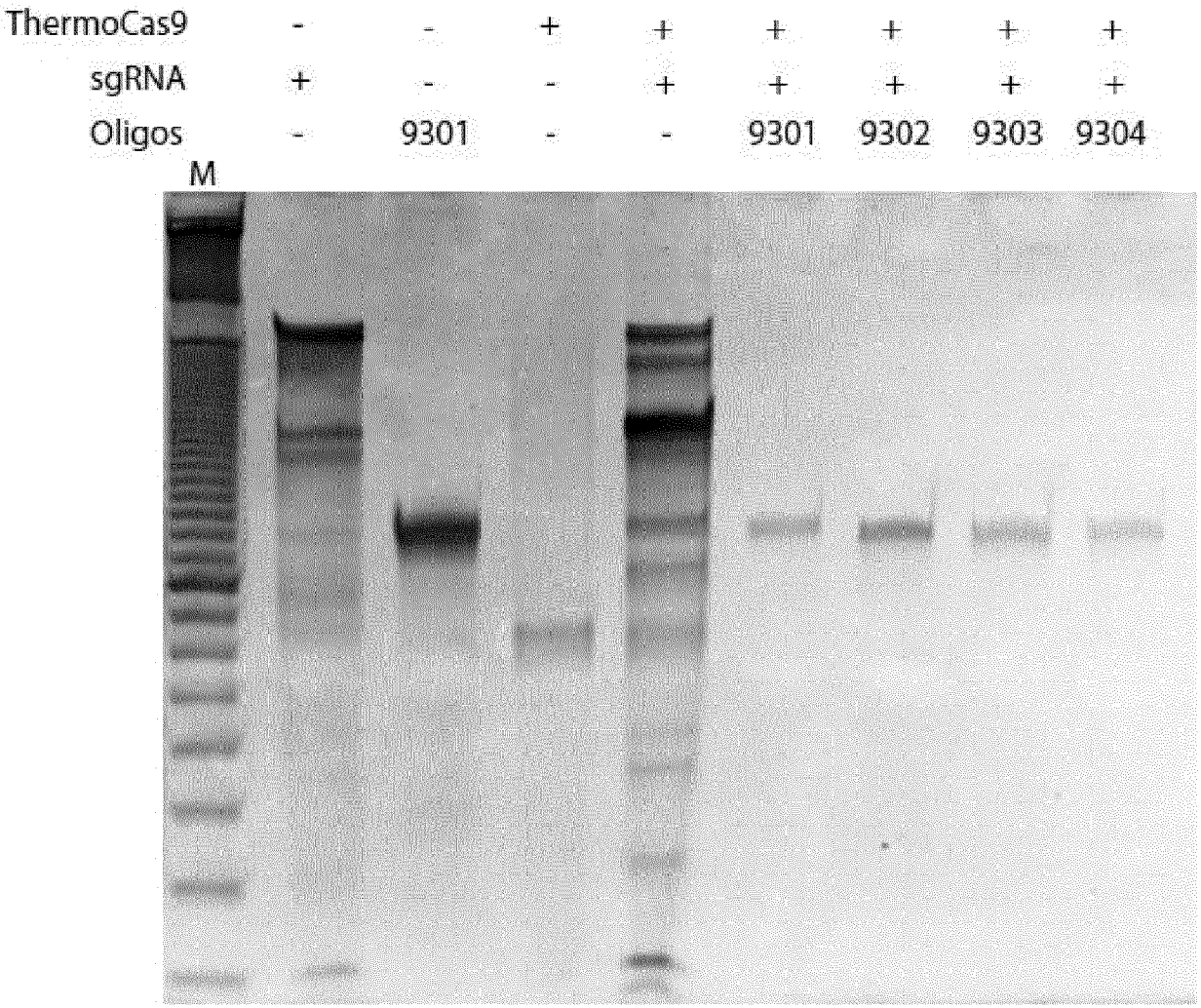


Figure 24

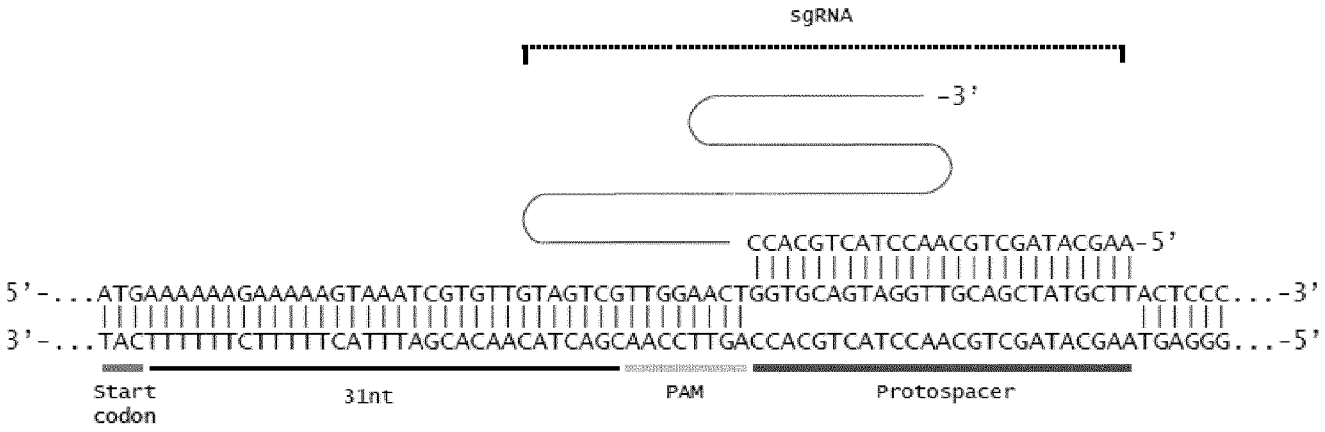
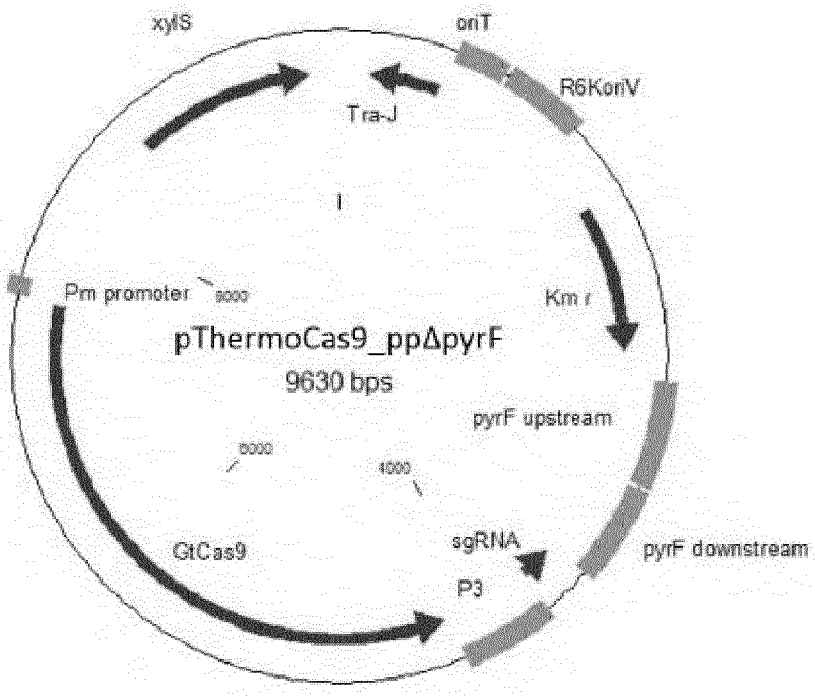


Figure 25



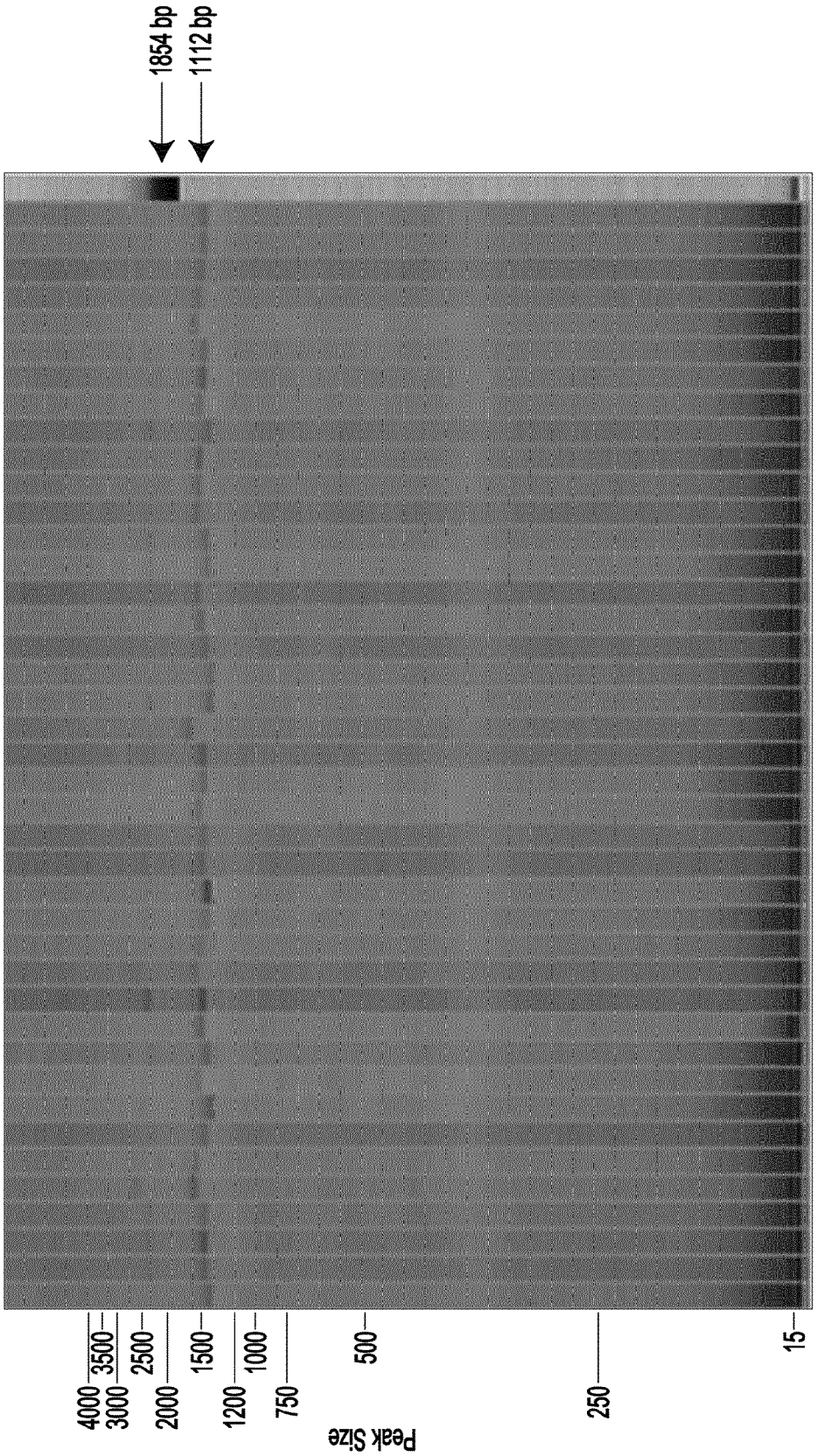

1854 bp
1112 bp
4000
3500
3000
2500
2000
1500
1200
1000
750
500
250
15
Peak Size

Figure 26