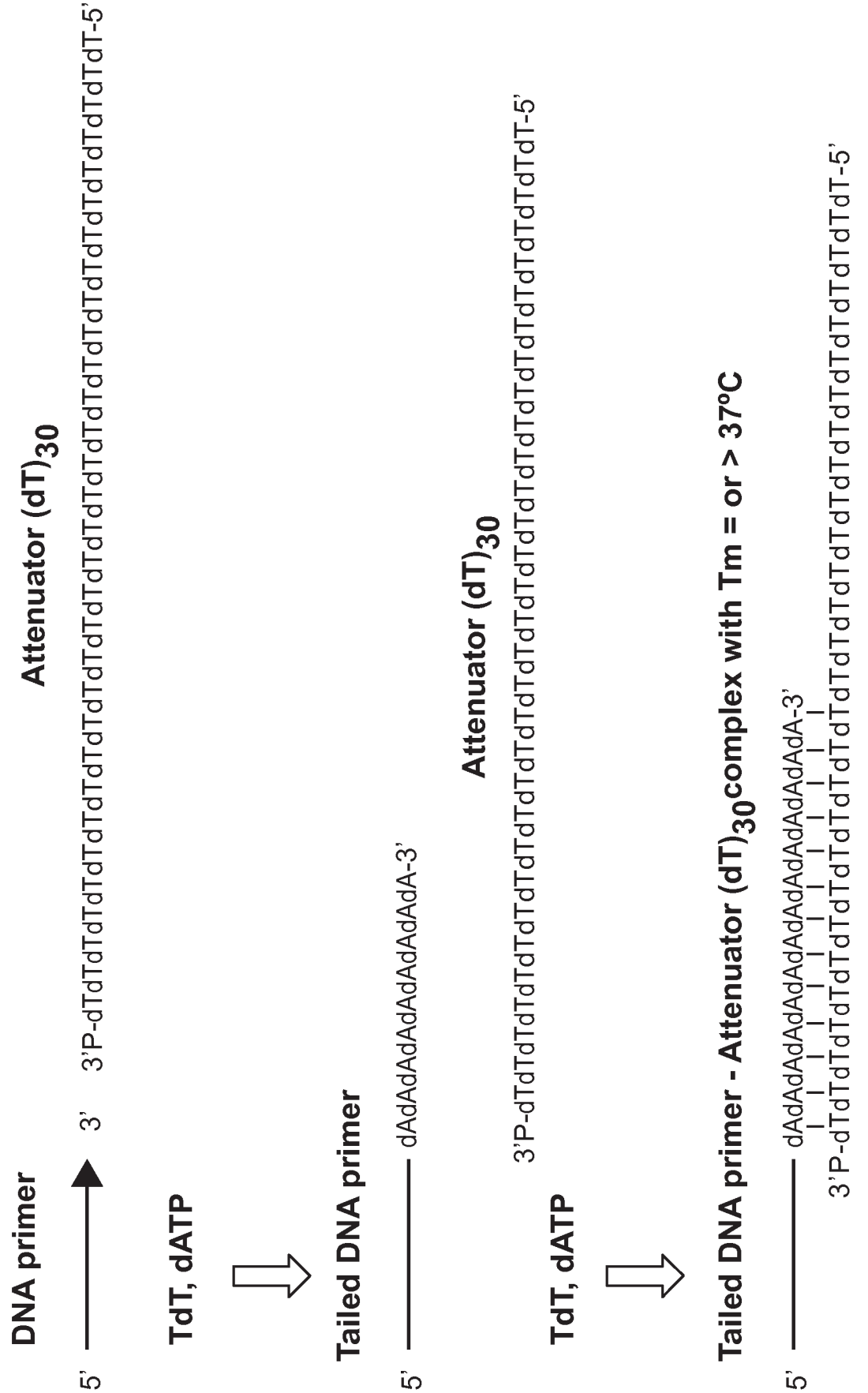

DNA primer
5'
3'
3'P-dTdTdTdTdTdTdTdTdTdTdTdTdTdTdTdTdTdTdTdTdTdTdTdTdT-5'
Attenuator (dT)30
TdT, dATP
Tailed DNA primer
5'
dAdAdAdAdAdAdAdAdA-3'
Attenuator (dT)30
3'P-dTdTdTdTdTdTdTdTdTdTdTdTdTdTdTdTdTdTdTdTdTdTdTdTdT-5'
TdT, dATP
Tailed DNA primer - Attenuator (dT)30complex with Tm = or > 37°C
5'
dAdAdAdAdAdAdAdAdAdAdAdAdAdAdA-3'
3'P-dTdTdTdTdTdTdTdTdTdTdTdTdTdTdTdTdTdTdTdTdTdTdTdTdT-5'

Figure 1

DNA primer
5'
3'
TdT, dATP
Attenuator (dT)13
3'P-dT dT dT dT dT dT dT dT dT dT dT dT dT-5'
Tailed DNA primer
5'
dA dA dA dA dA dA dA dA dA dA dA dA dA-3'
TdT, dATP
Attenuator (dT)13
3'P-dT dT dT dT dT dT dT dT dT dT dT dT dT-5'
Tailed DNA primer - Attenuator (dT)13complex
5'
dA dA dA dA dA dA dA dA dA dA dA dA dA-3'
3'P-dT dT dT dT dT dT dT dT dT dT dT dT dT-5'
TdT, dATP
Tailed DNA primer - Attenuator (dT)13complex with short 3' poly(dA) tail
5'
dA dA dA dA dA dA dA dA dA dA dA dA dA dA -3'
3'P-dT dT dT dT dT dT dT dT dT dT dT dT dT-5'
TdT, dATP
Tailed DNA primer - 2 x Attenuator (dT)13complex
5'
dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA -3'
3'P-dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT-5'
5' 3'

Figure 2

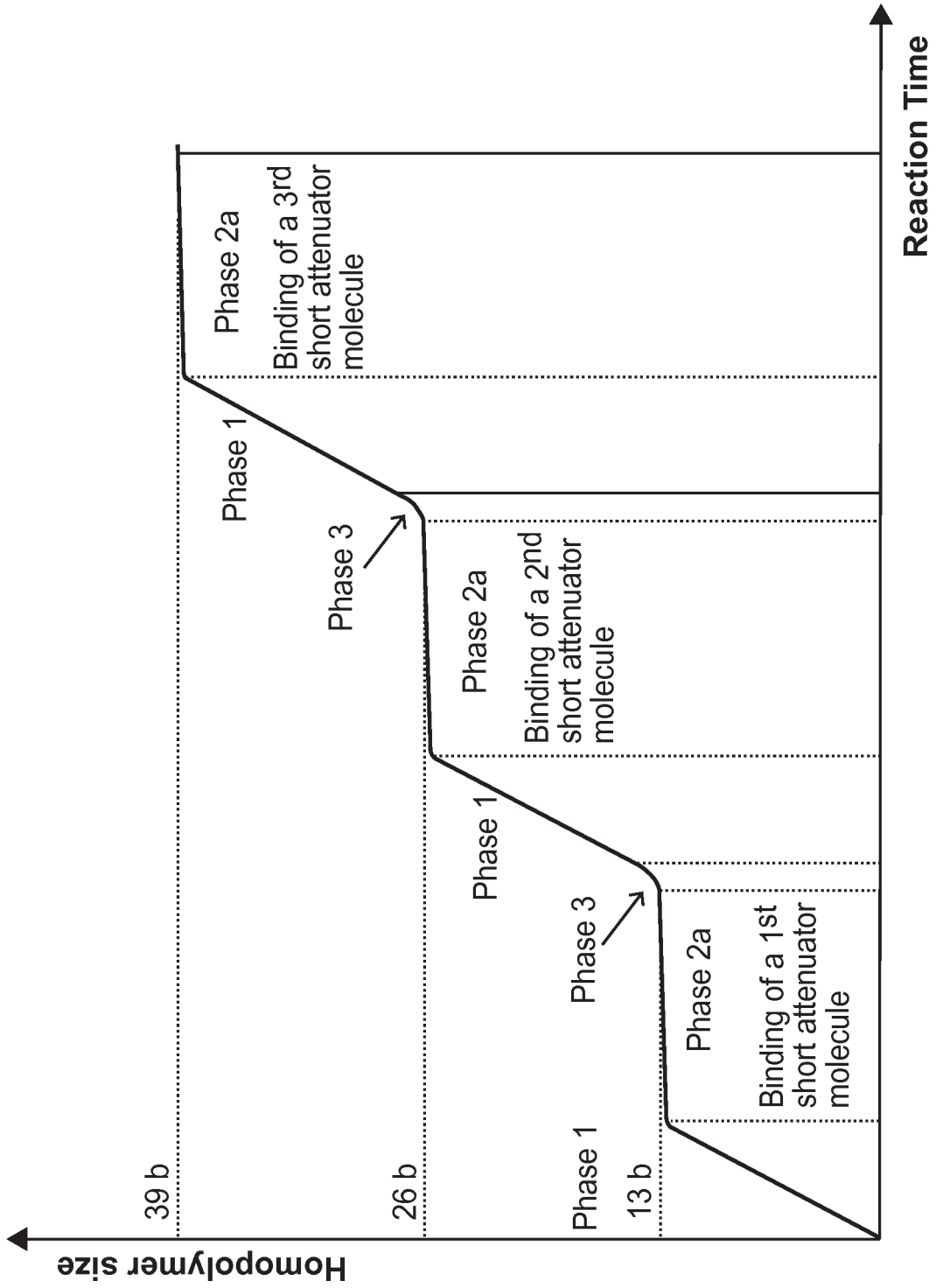

Homopolymer size
Reaction Time
39 b
26 b
13 b
Phase 1
Phase 3
Phase 2a
Binding of a 1st short attenuator molecule
Phase 1
Phase 3
Phase 2a
Binding of a 2nd short attenuator molecule
Phase 1
Phase 2a
Binding of a 3rd short attenuator molecule

Figure 3

SUBSTITUTE SHEET (RULE 26)

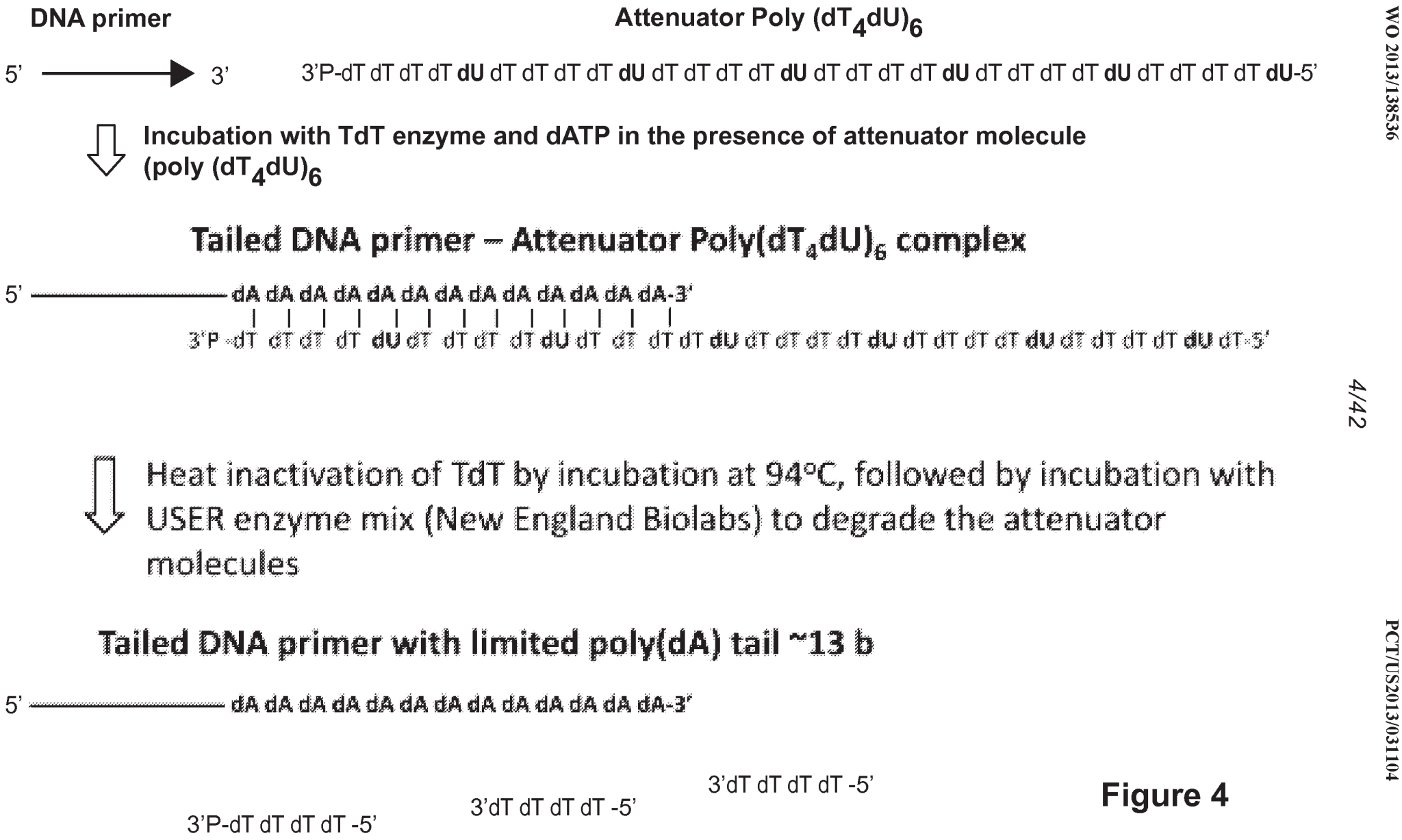



**Figure 4**

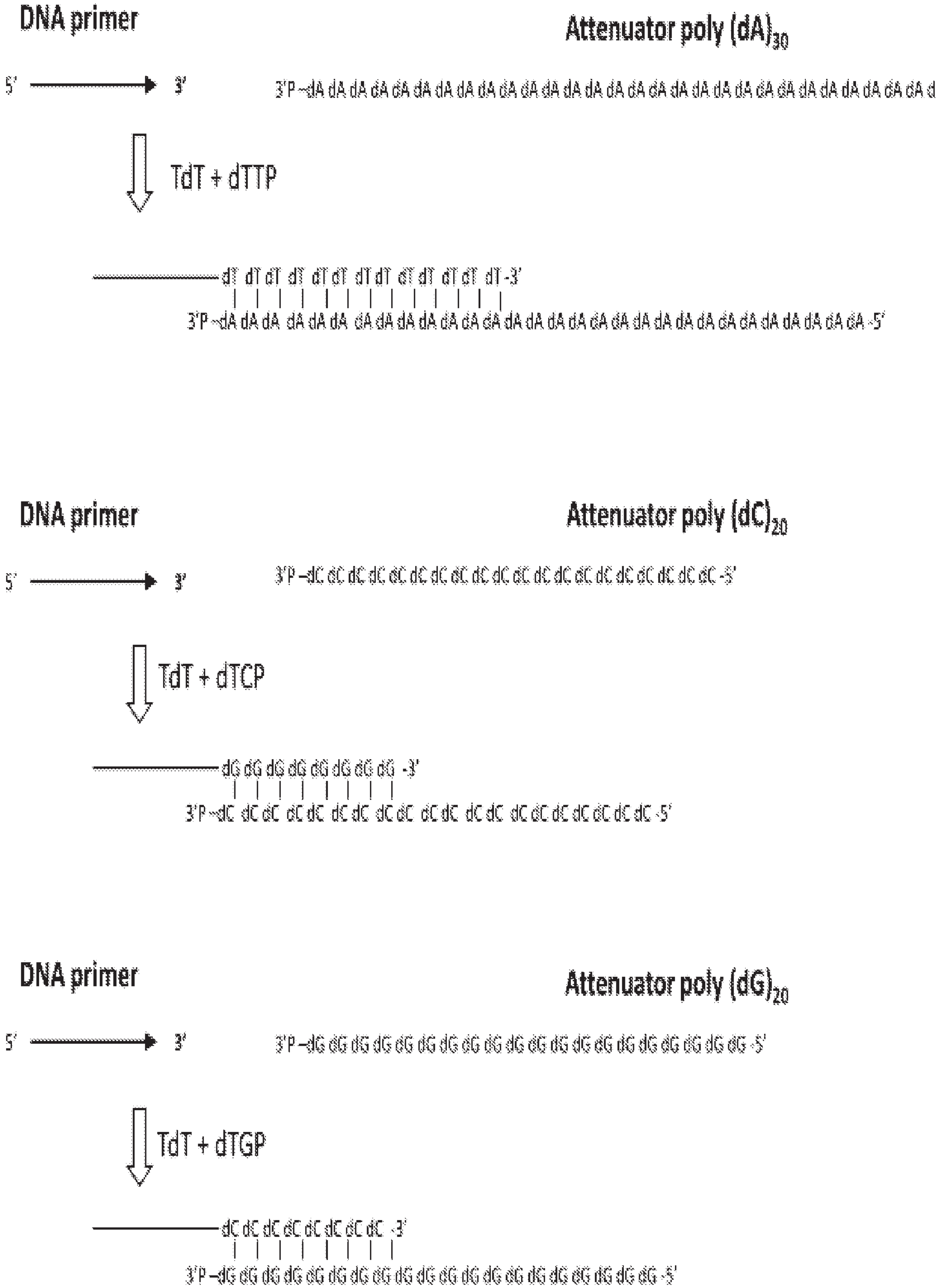

DNA primer
Attenuator poly (dA)$_{30}$
5'
3'
TdT + dTTP
DNA primer
Attenuator poly (dC)$_{20}$
5'
3'
TdT + dTCP
DNA primer
Attenuator poly (dG)$_{20}$
5'
3'
TdT + dTGP

**Figure 5**

DNA primer
5'
3'
3'P -rA rA rA rA rA rA rA rA rA rA rA rArA rA rA rA rA rArA rA rA rA rA rA -5'
Attenuator Poly (rA)30
Incubation with TdT enzyme and dTTP in the presence of attenuator ribo-oligonucleotide molecule poly (A)30
Tailed DNA primer – Attenuator poly (rA)30 complex
dT dT dT dT dT dT dT dT dT dT dT dT -3'
3'P -rA rA rA rA rA rA rA rA rA rA rA rArA rA rA rA rA rArA rA rA rA rA rA -5'
Heat inactivation of TdT by incubation at 94°C, followed by incubation with A ribonuclease (RNase H, RNase A, RNase T1, RNase V1, RNase I, etc, or their cocktails) to degrade the attenuator molecules
Tailed DNA primer with limited poly(dT) tail ~13 b
dT dT dT dT dT dT dT dT dT dT dT dT -3'

Figure 6

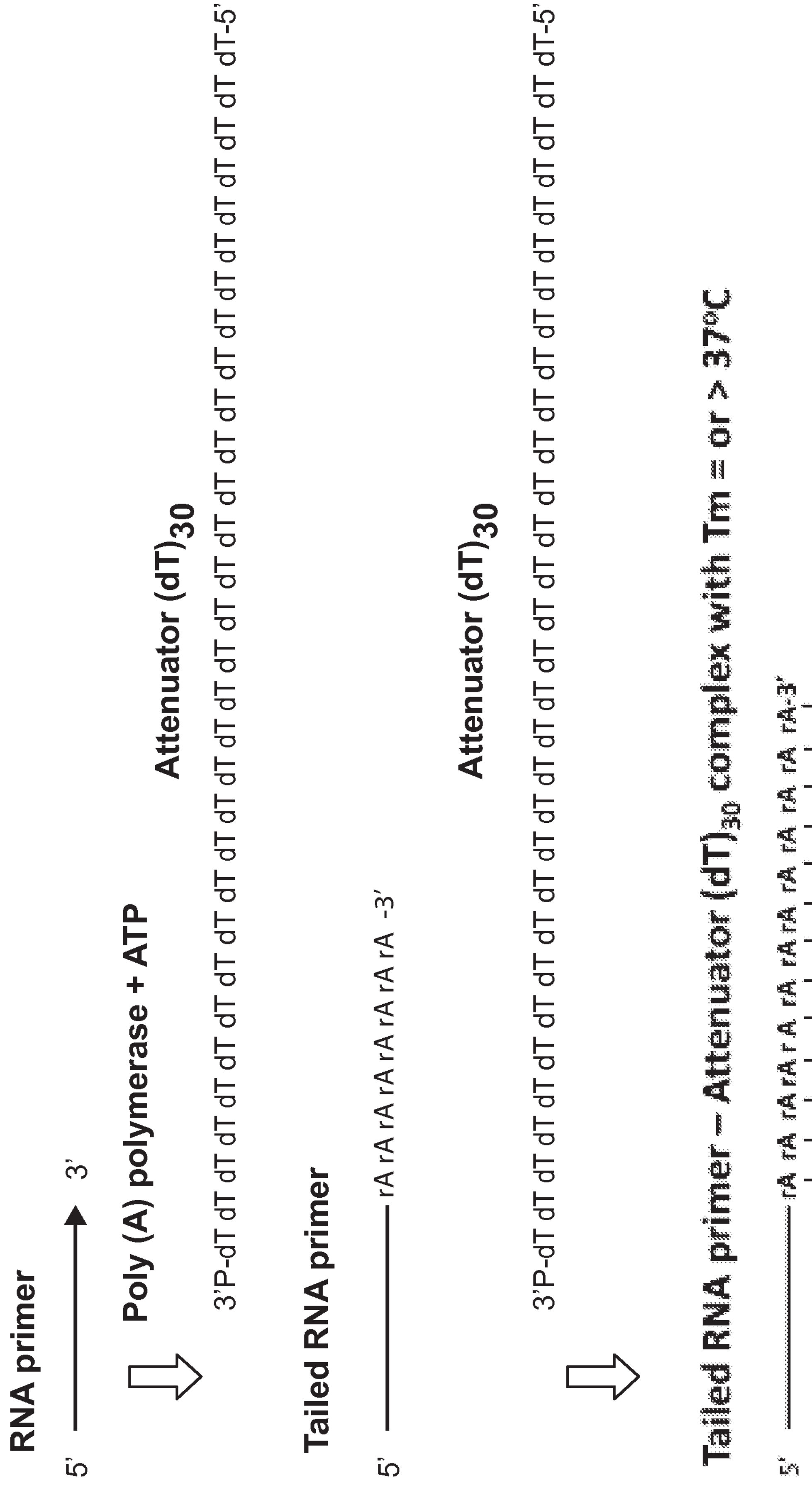

RNA primer
5'
3'
Poly (A) polymerase + ATP
3'P-dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT-5'
Attenuator $(dT)_{30}$
Tailed RNA primer
5'
rA rA rA rA rA rA rA rA -3'
Attenuator $(dT)_{30}$
3'P-dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT dT-5'
Tailed RNA primer – Attenuator $(dT)_{30}$ complex with Tm = or > 37°C

Figure 7

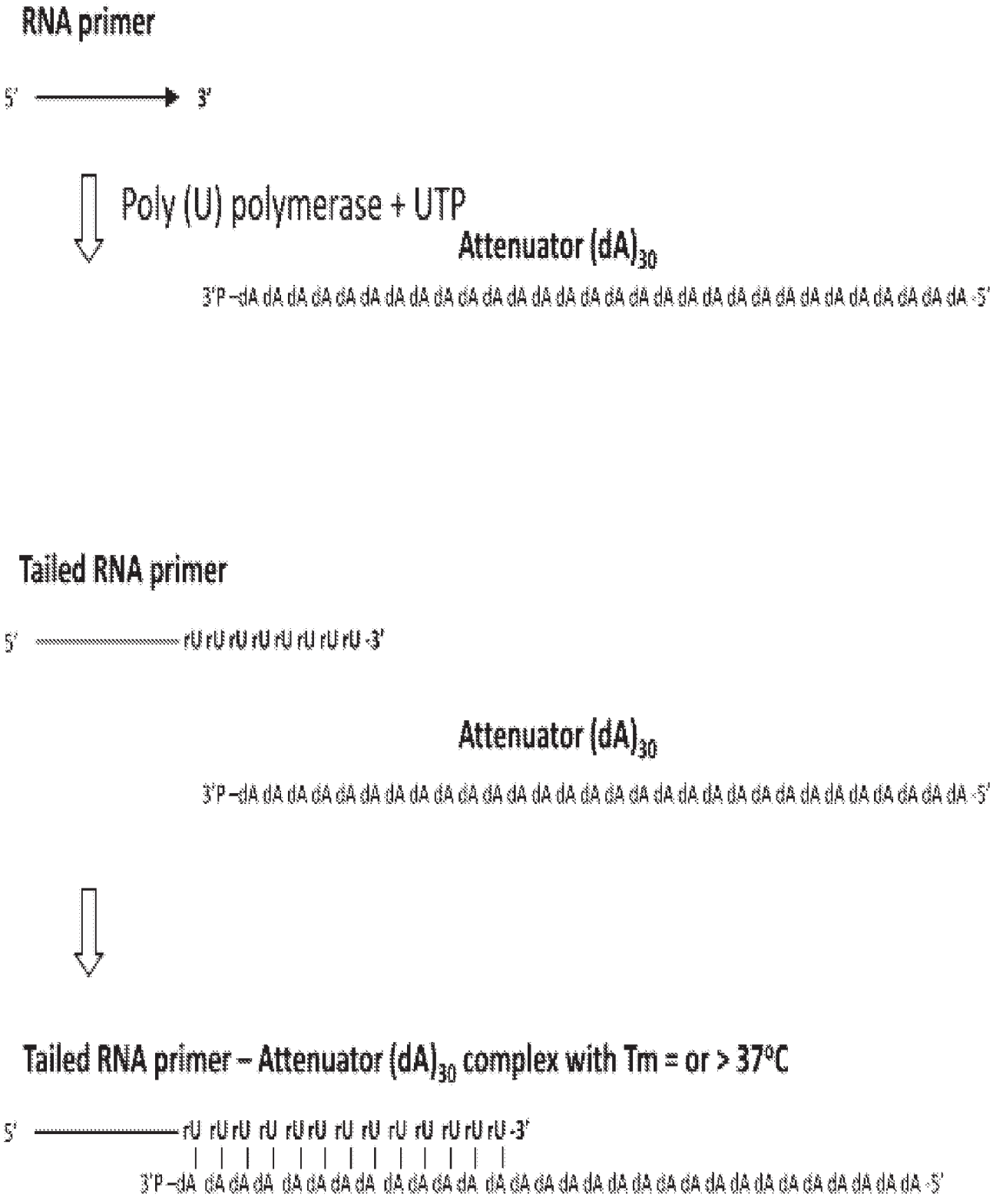

RNA primer
5'
3'
Poly (U) polymerase + UTP
Attenuator (dA)30
3'P –dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA -5'
Tailed RNA primer
5'
rU rU rU rU rU rU rU rU -3'
Attenuator (dA)30
3'P –dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA -5'
Tailed RNA primer – Attenuator (dA)30 complex with Tm = or > 37°C
5'
rU rU rU rU rU rU rU rU rU rU rU rU rU -3'
3'P –dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA dA -5'

**Figure 8**

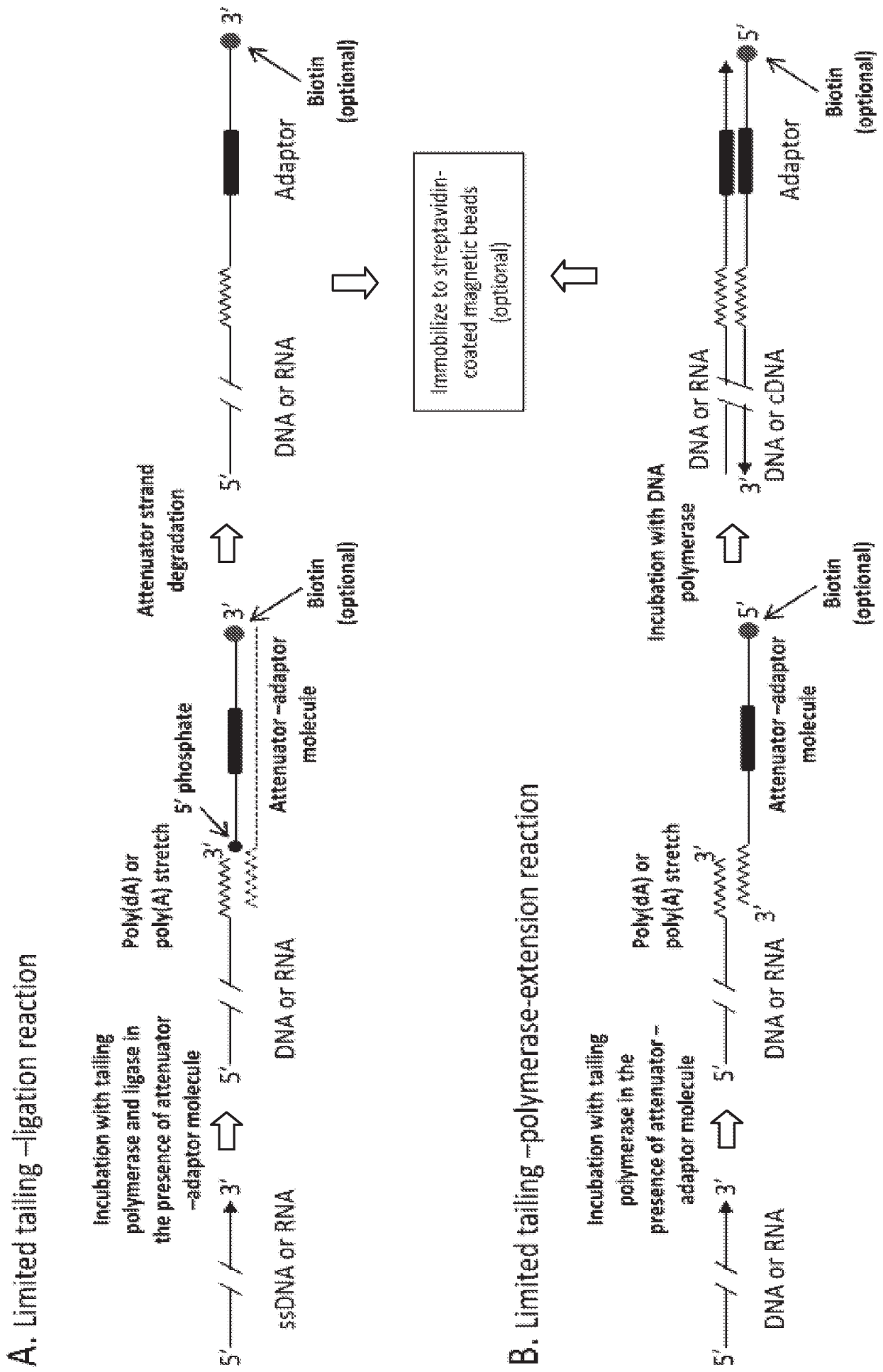

A. Limited tailing –ligation reaction
Incubation with tailing polymerase and ligase in the presence of attenuator –adaptor molecule
Poly(dA) or poly(A) stretch
5' phosphate
Attenuator strand degradation
ssDNA or RNA
DNA or RNA
Attenuator –adaptor molecule
Biotin (optional)
DNA or RNA
Adaptor
Biotin (optional)
Immobilize to streptavidin-coated magnetic beads (optional)
B. Limited tailing –polymerase-extension reaction
Incubation with tailing polymerase in the presence of attenuator – adaptor molecule
Poly(dA) or poly(A) stretch
DNA or RNA
Attenuator –adaptor molecule
Biotin (optional)
Incubation with DNA polymerase
DNA or RNA
DNA or cDNA
Adaptor
Biotin (optional)

Figure 9

SUBSTITUTE SHEET (RULE 26)

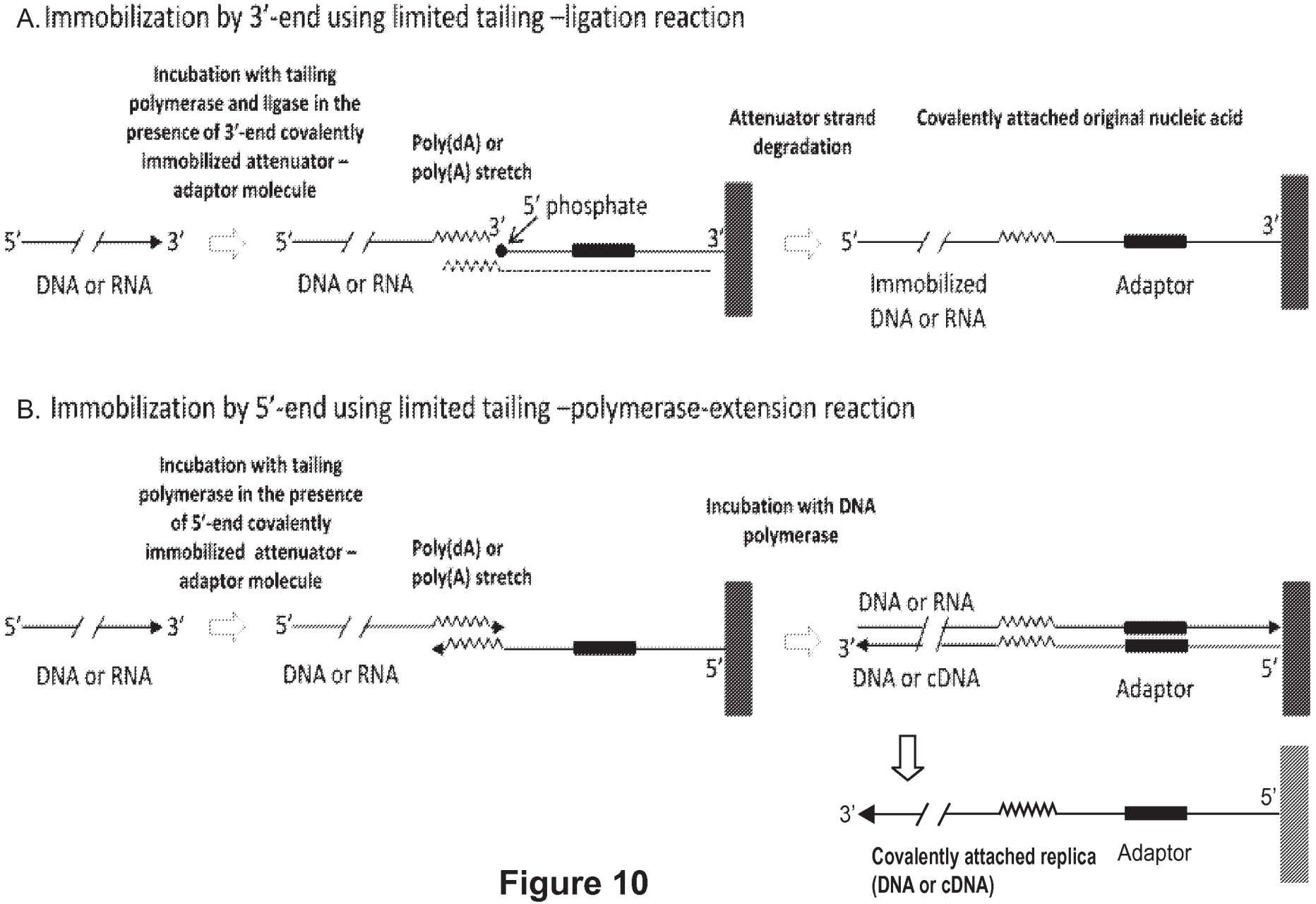



**Figure 10**

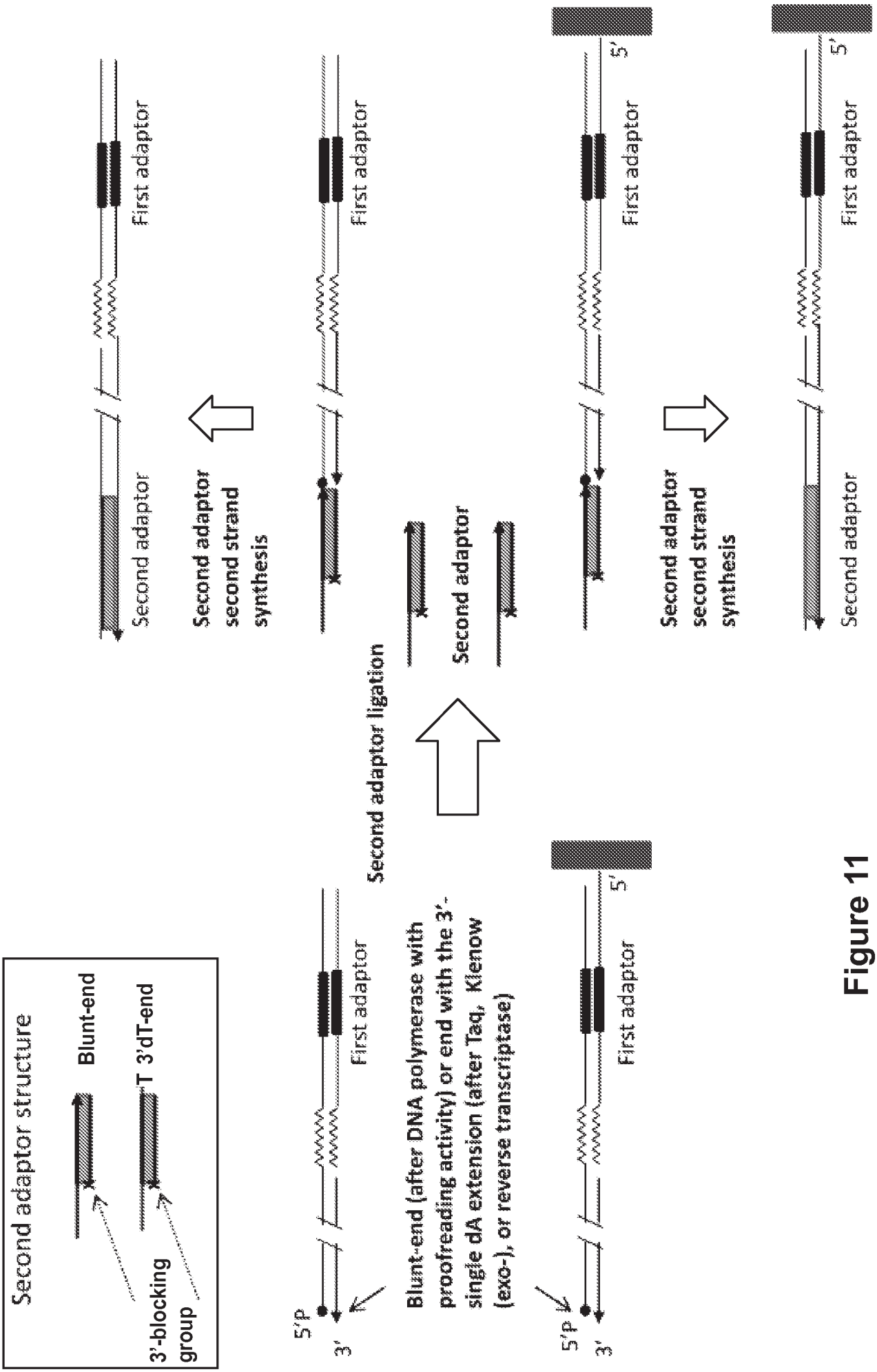

Second adaptor structure
Blunt-end
T 3'dT-end
3'-blocking group
5'P
3'
First adaptor
Blunt-end (after DNA polymerase with proofreading activity) or end with the 3'-single dA extension (after Taq, Klenow (exo-), or reverse transcriptase)
Second adaptor ligation
Second adaptor
Second adaptor second strand synthesis
5'
First adaptor
Second adaptor
Second adaptor second strand synthesis
5'
First adaptor
Second adaptor

Figure 11

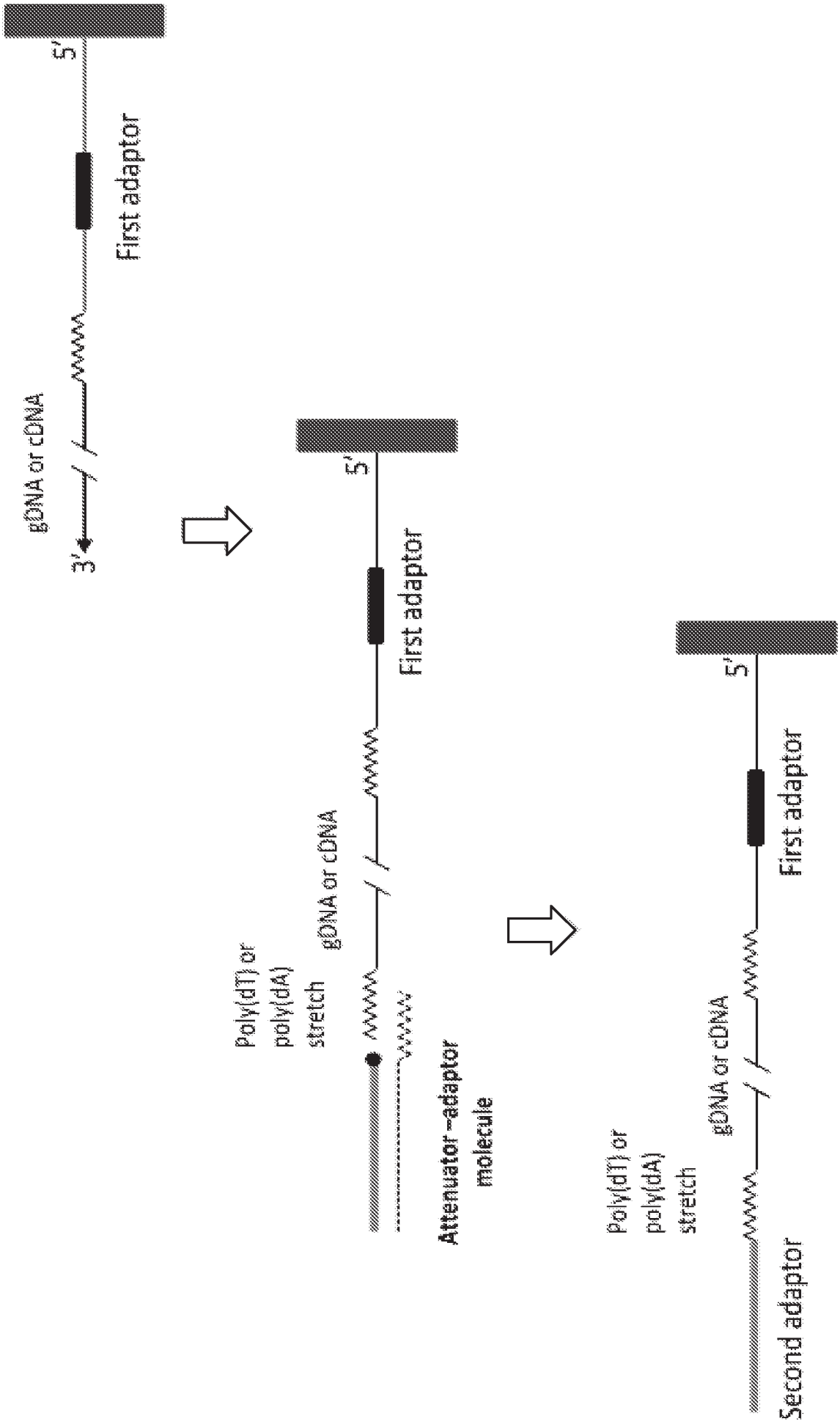

5'
First adaptor
gDNA or cDNA
3'
5'
First adaptor
Poly(dT) or
poly(dA)
stretch
gDNA or cDNA
Attenuator –adaptor
molecule
5'
First adaptor
Poly(dT) or
poly(dA)
stretch
gDNA or cDNA
Second adaptor

Figure 12

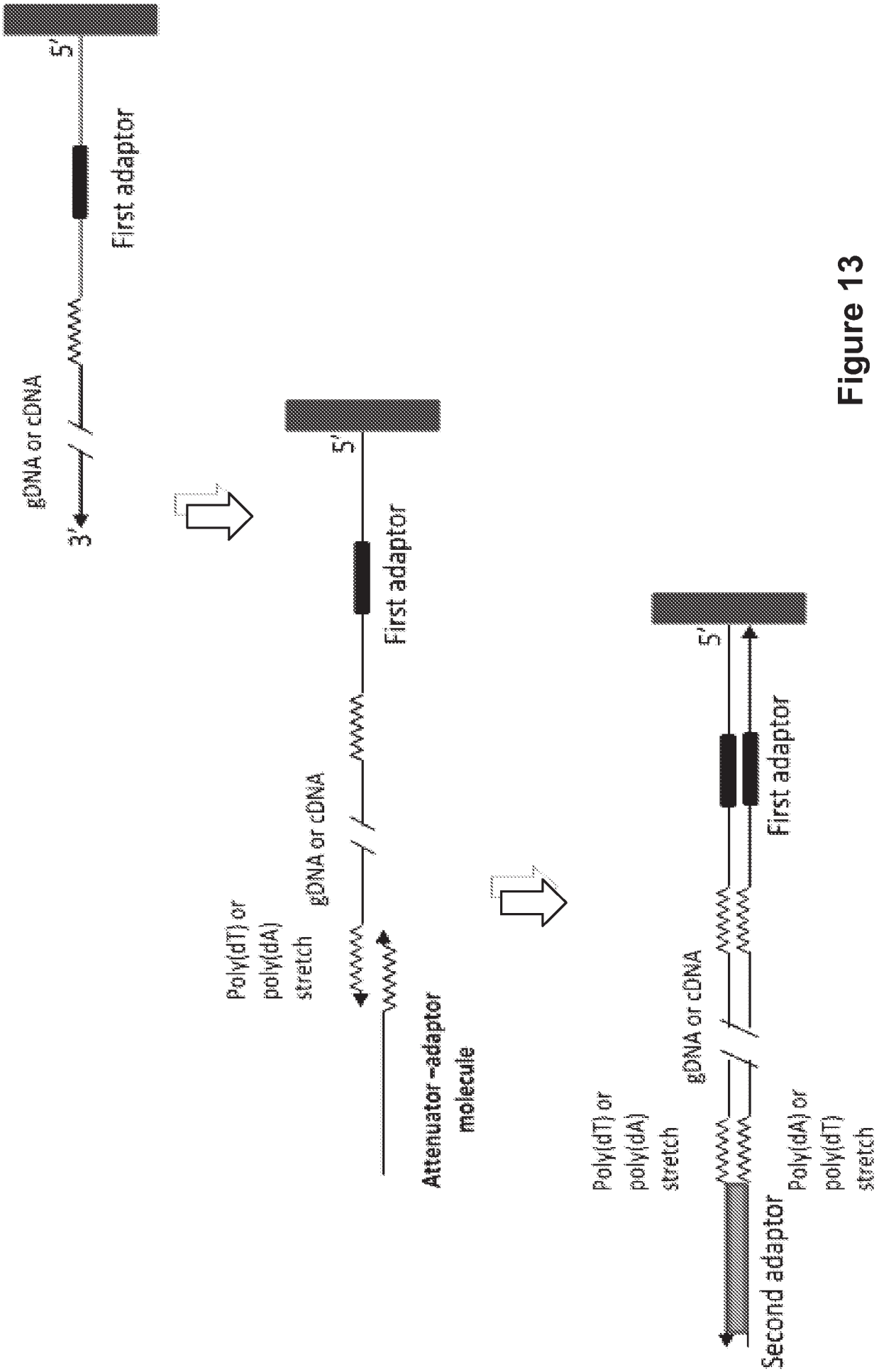

5'
First adaptor
gDNA or cDNA
3'
5'
First adaptor
Poly(dT) or poly(dA) stretch
gDNA or cDNA
Attenuator –adaptor molecule
5'
First adaptor
gDNA or cDNA
Poly(dT) or poly(dA) stretch
Poly(dA) or poly(dT) stretch
Second adaptor

Figure 13

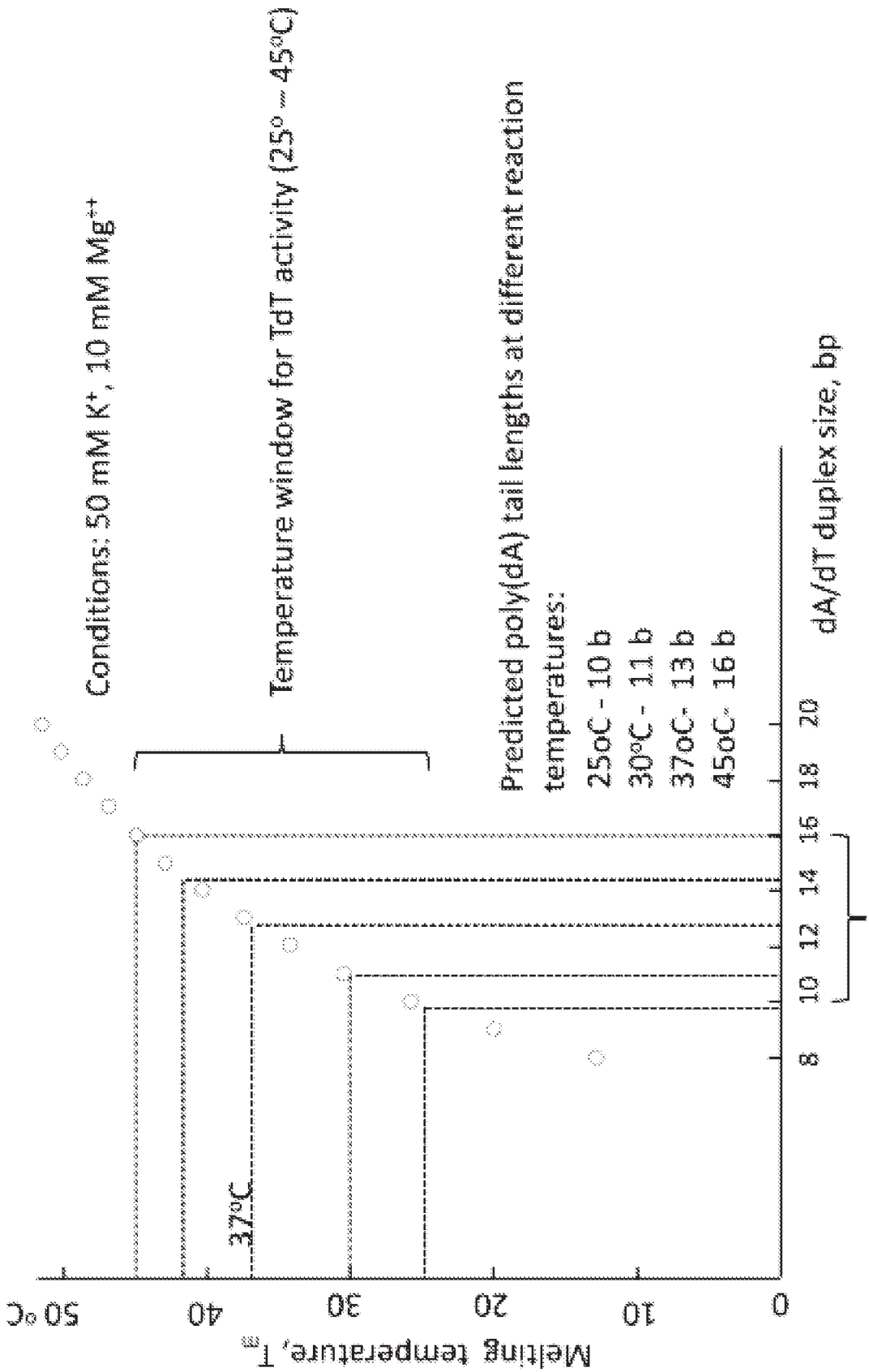

Conditions: 50 mM K+, 10 mM Mg++
Temperature window for TdT activity (25° – 45°C)
Predicted poly(dA) tail lengths at different reaction temperatures:
25oC - 10 b
30°C - 11 b
37oC- 13 b
45oC- 16 b
37°C
Melting temperature, Tm
0
10
20
30
40
50 °C
8
10
12
14
16
18
20
dA/dT duplex size, bp
Poly(dA) tail size range

Figure 14

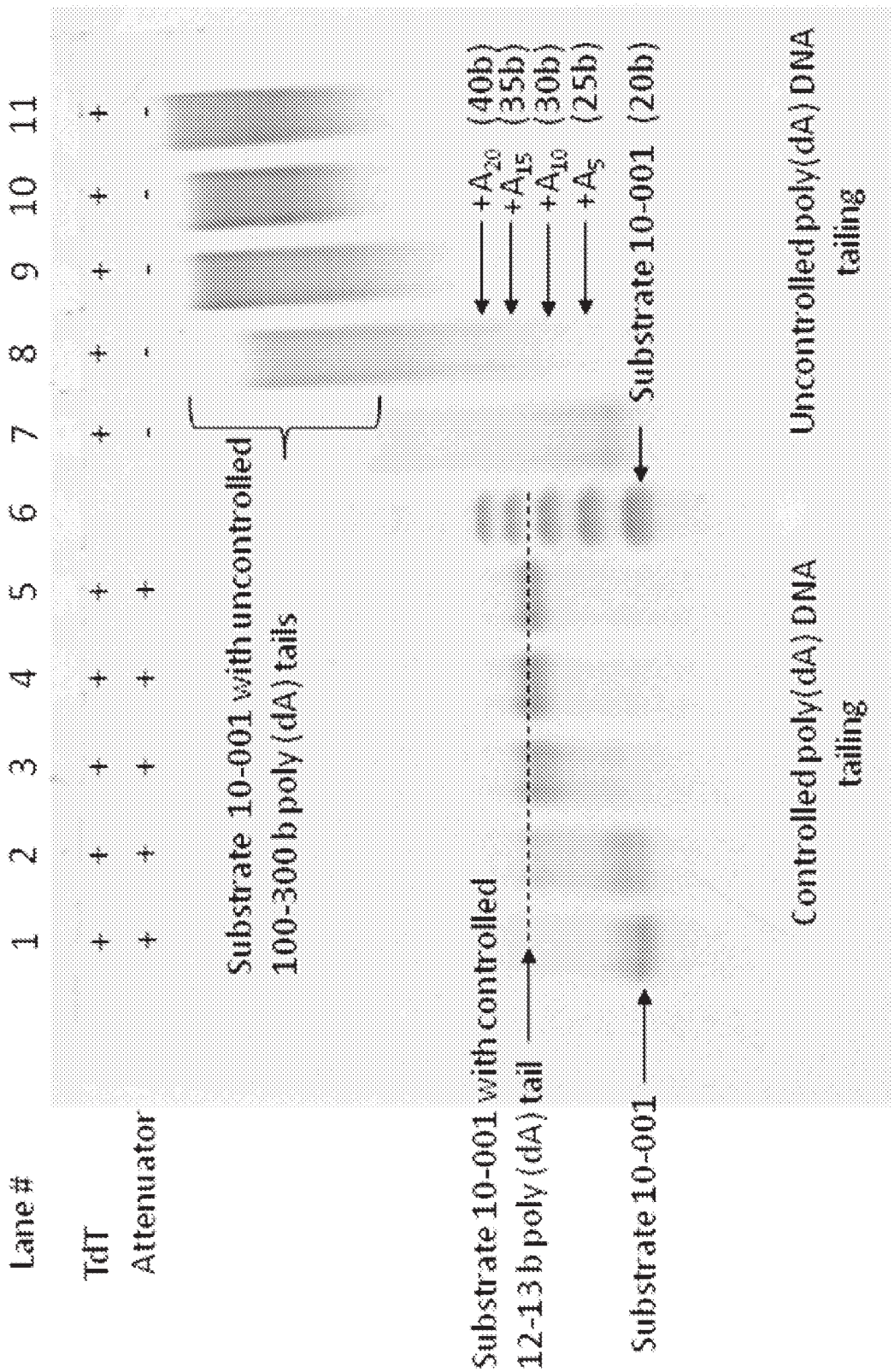

Lane # 1 2 3 4 5 6 7 8 9 10 11
TdT + + + + + + + + + + +
Attenuator + + + + + - - - - - -
Substrate 10-001 with uncontrolled 100-300 b poly (dA) tails
Substrate 10-001 with controlled 12-13 b poly (dA) tail
Substrate 10-001
Controlled poly(dA) DNA tailing
Uncontrolled poly(dA) DNA tailing
+A20 (40b)
+A15 (35b)
+A10 (30b)
+A5 (25b)
Substrate 10-001 (20b)

Figure 15

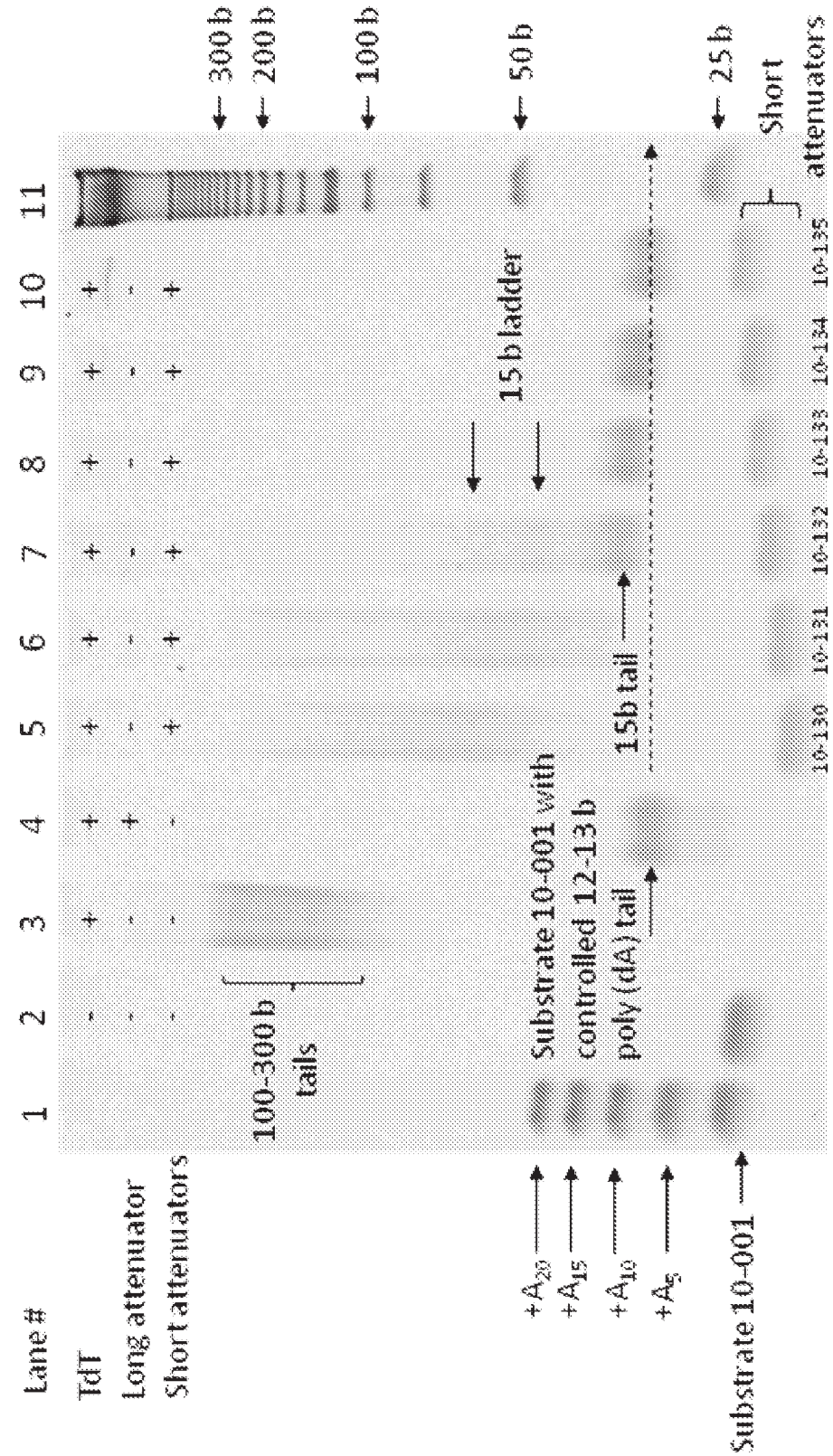

Lane #
1
2
3
4
5
6
7
8
9
10
11
TdT
Long attenuator
Short attenuators
100-300 b tails
300 b
200 b
100 b
50 b
25 b
15 b ladder
Substrate 10-001 with controlled 12-13 b poly (dA) tail
15b tail
Short attenuators
10-130
10-131
10-132
10-133
10-134
10-135
+A20
+A15
+A10
+A5
Substrate 10-001

Figure 16A

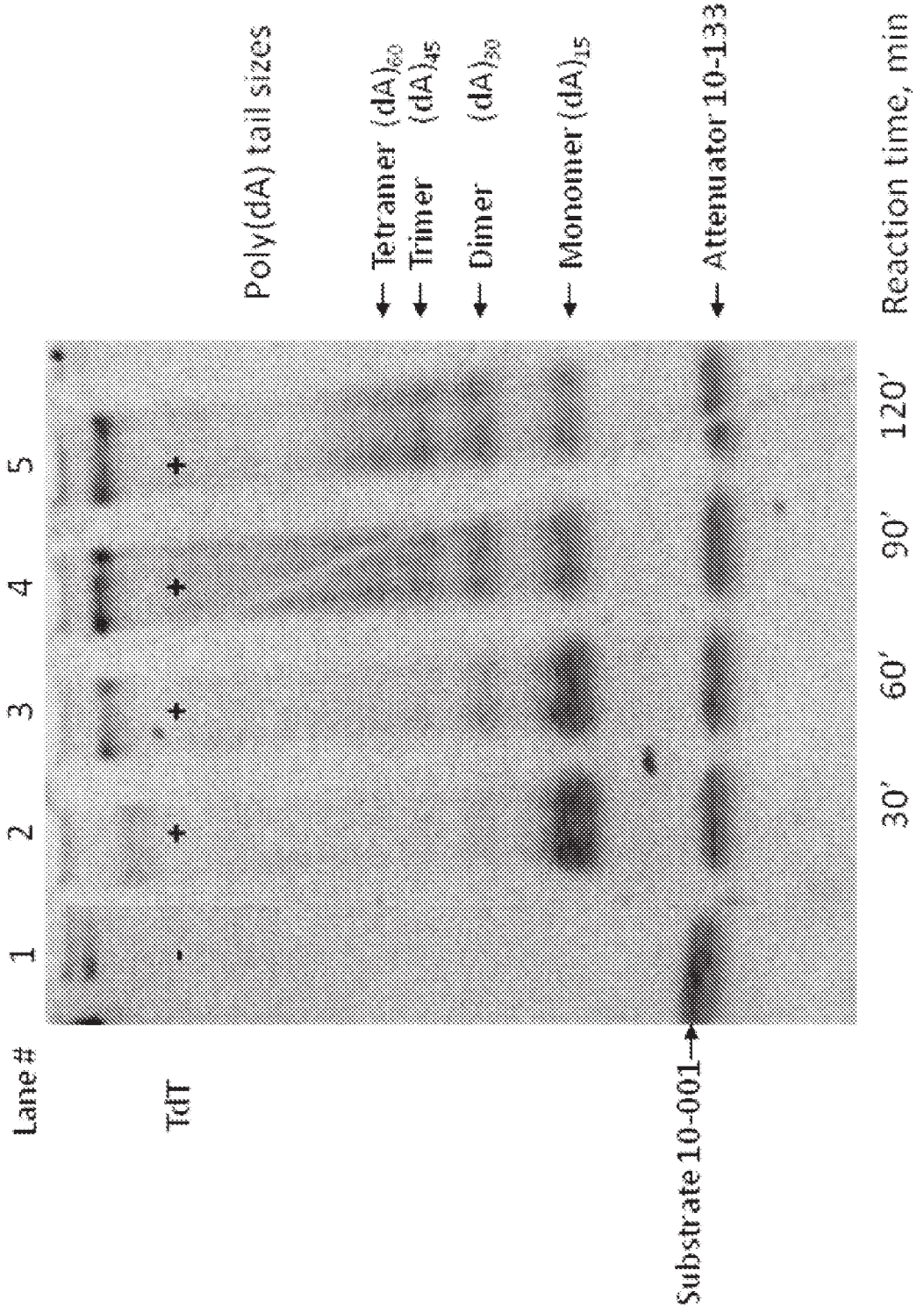

Lane #
1
2
3
4
5
TdT
-
+
+
+
+
Poly(dA) tail sizes
← Tetramer (dA)60
← Trimer (dA)45
← Dimer (dA)30
← Monomer (dA)15
← Attenuator 10-133
Substrate 10-001→
30'
60'
90'
120'
Reaction time, min

Figure 16B

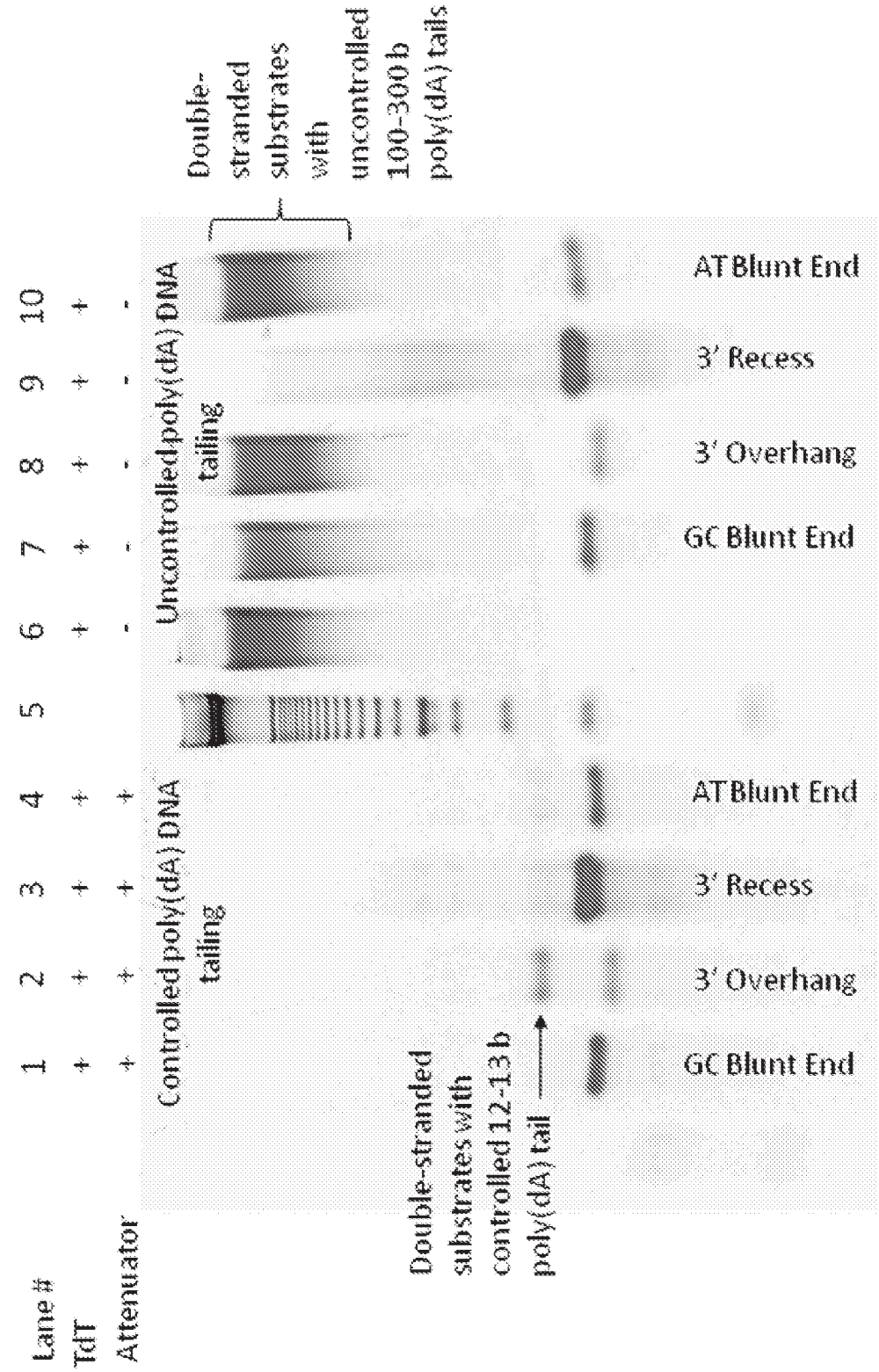

Lane #
TdT
Attenuator
1 2 3 4 5 6 7 8 9 10
+ + + + + + + + + +
+ + + + + - - - - -
Controlled poly(dA) DNA tailing
Uncontrolled poly(dA) DNA tailing
Double-stranded substrates with uncontrolled 100-300 b poly(dA) tails
Double-stranded substrates with controlled 12-13 b poly(dA) tail
AT Blunt End
3' Recess
3' Overhang
GC Blunt End
AT Blunt End
3' Recess
3' Overhang
GC Blunt End

Figure 17

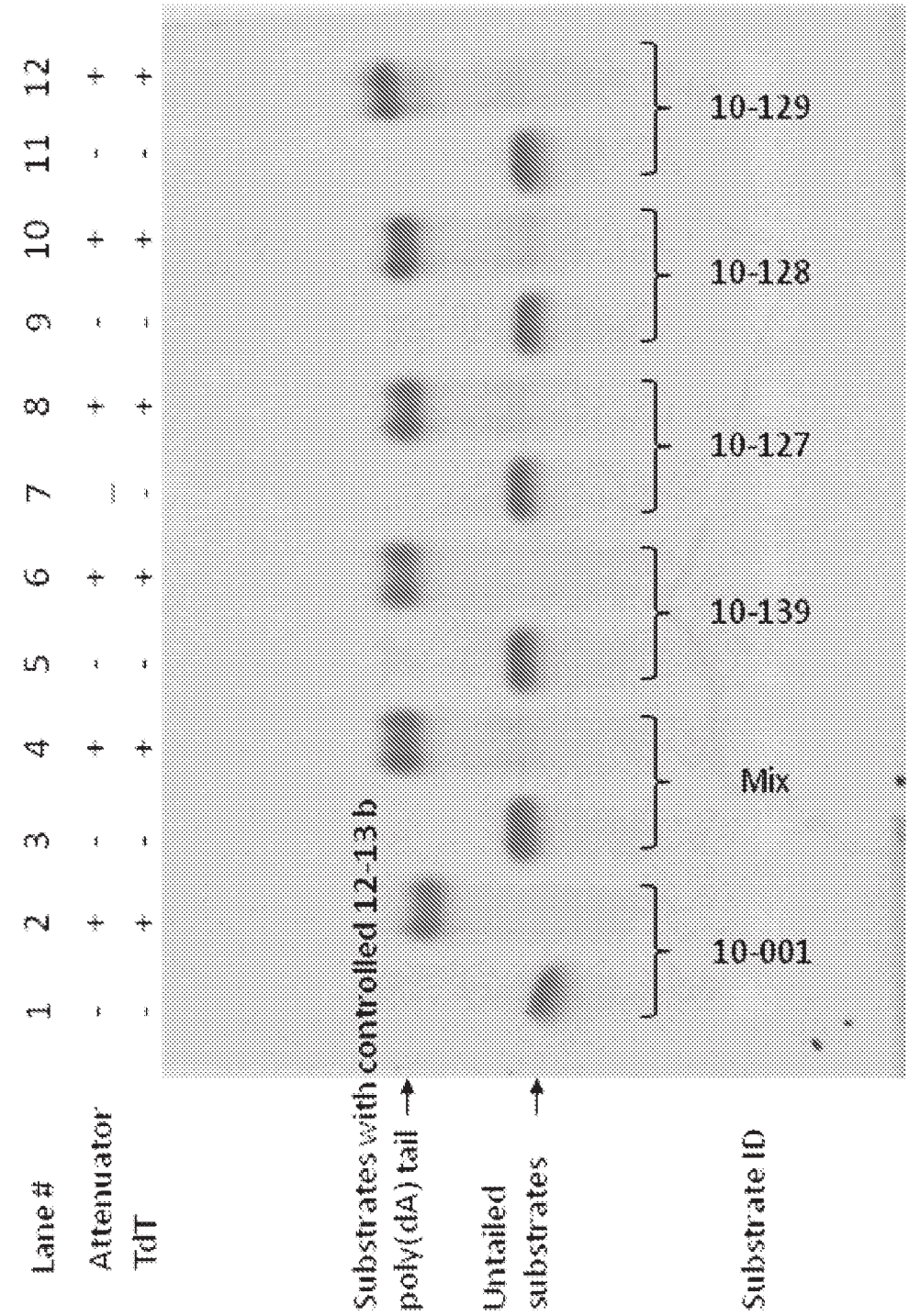

Lane #
1 2 3 4 5 6 7 8 9 10 11 12
Attenuator - + - + - + - + - + - +
TdT - + - + - + - + - + - +
Substrates with controlled 12-13 b poly(dA) tail
Untailed substrates
10-001
Mix
10-139
10-127
10-128
10-129
Substrate ID

Figure 18

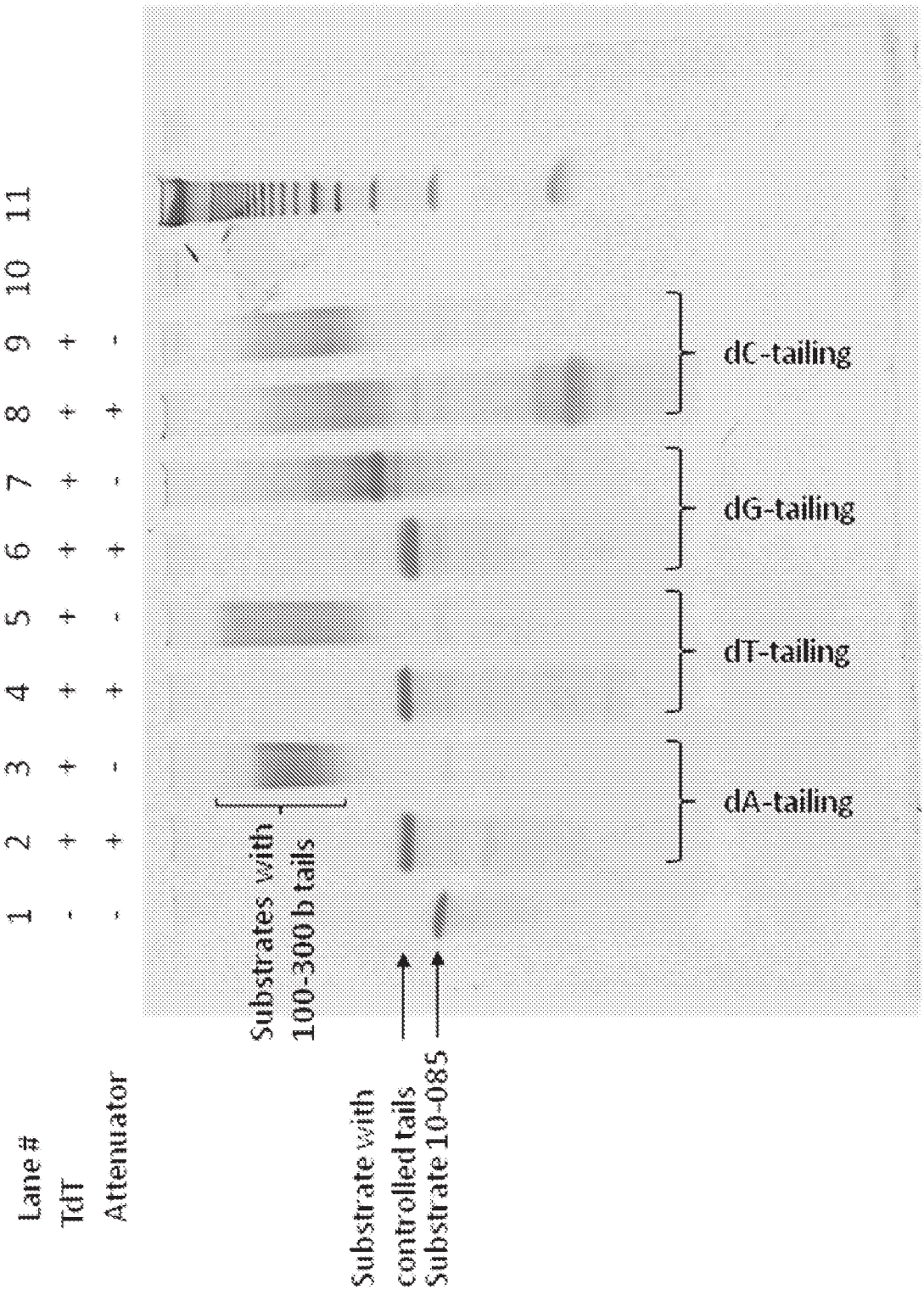

Lane # 1 2 3 4 5 6 7 8 9 10 11
TdT - + + + + + + + + + -
Attenuator - + - + - + - + - + -
Substrates with 100-300 b tails
Substrate with controlled tails
Substrate 10-085
dA-tailing
dT-tailing
dG-tailing
dC-tailing

Figure 19

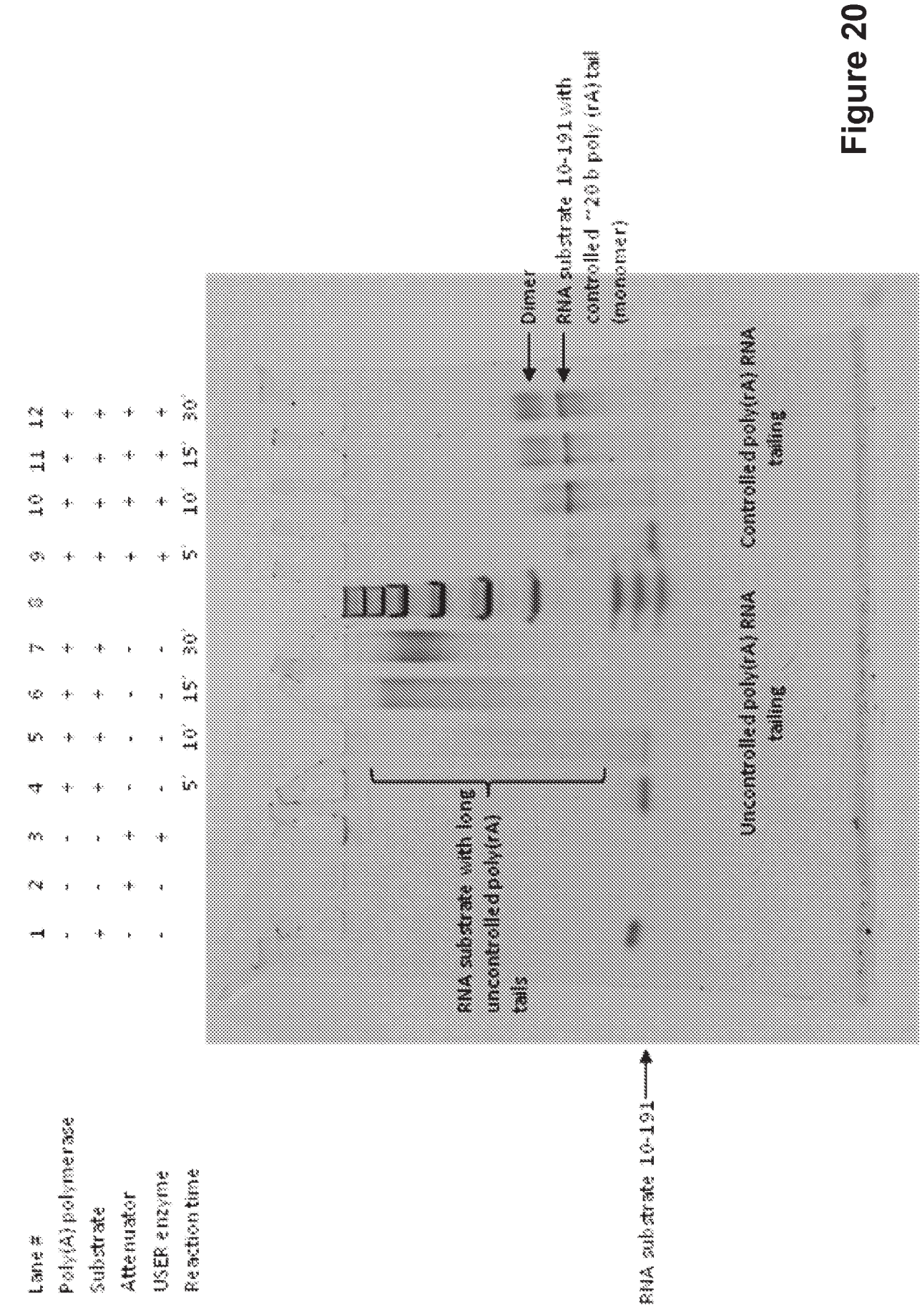

Lane # 1 2 3 4 5 6 7 8 9 10 11 12
Poly(A) polymerase - - - + + + + + + + + +
Substrate + + + + + + + + + + + +
Attenuator - + + - - - - + + + + +
USER enzyme - - + - - - - + + + + +
Reaction time 5' 10' 15' 30' 5' 10' 15' 30'
RNA substrate with long uncontrolled poly(rA) tails
Dimer
RNA substrate 10-191 with controlled ~20 b poly (rA) tail (monomer)
RNA substrate 10-191
Uncontrolled poly(rA) RNA tailing
Controlled poly(rA) RNA tailing

Figure 20

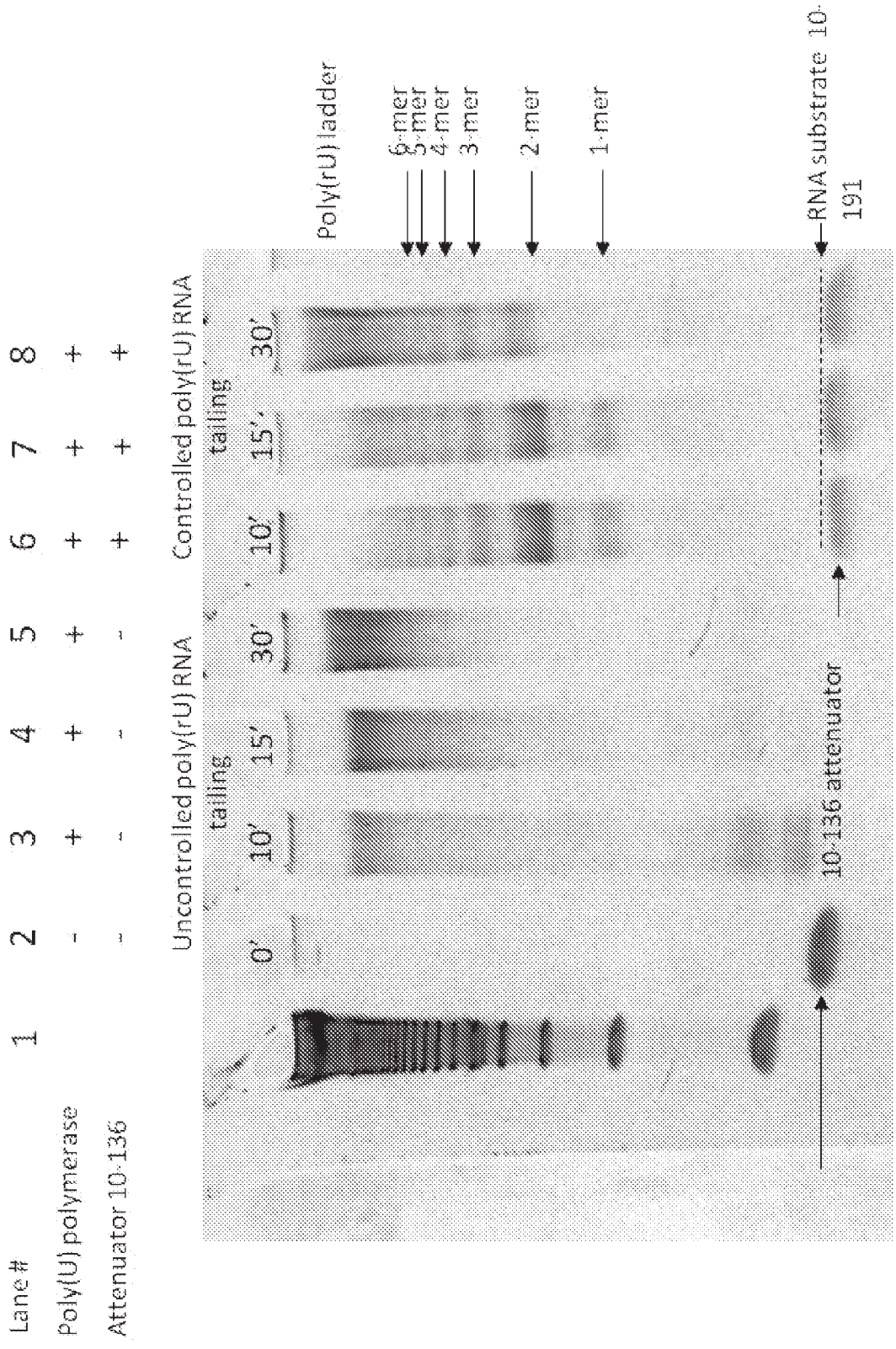

Lane # 1 2 3 4 5 6 7 8
Poly(U) polymerase - + + + + + +
Attenuator 10-136 - - - - + + + +
Uncontrolled poly(rU) RNA tailing
0′ 10′ 15′ 30′
Controlled poly(rU) RNA tailing
10′ 15′ 30′
Poly(rU) ladder
6-mer
5-mer
4-mer
3-mer
2-mer
1-mer
RNA substrate 10-191
10-136 attenuator

Figure 21a

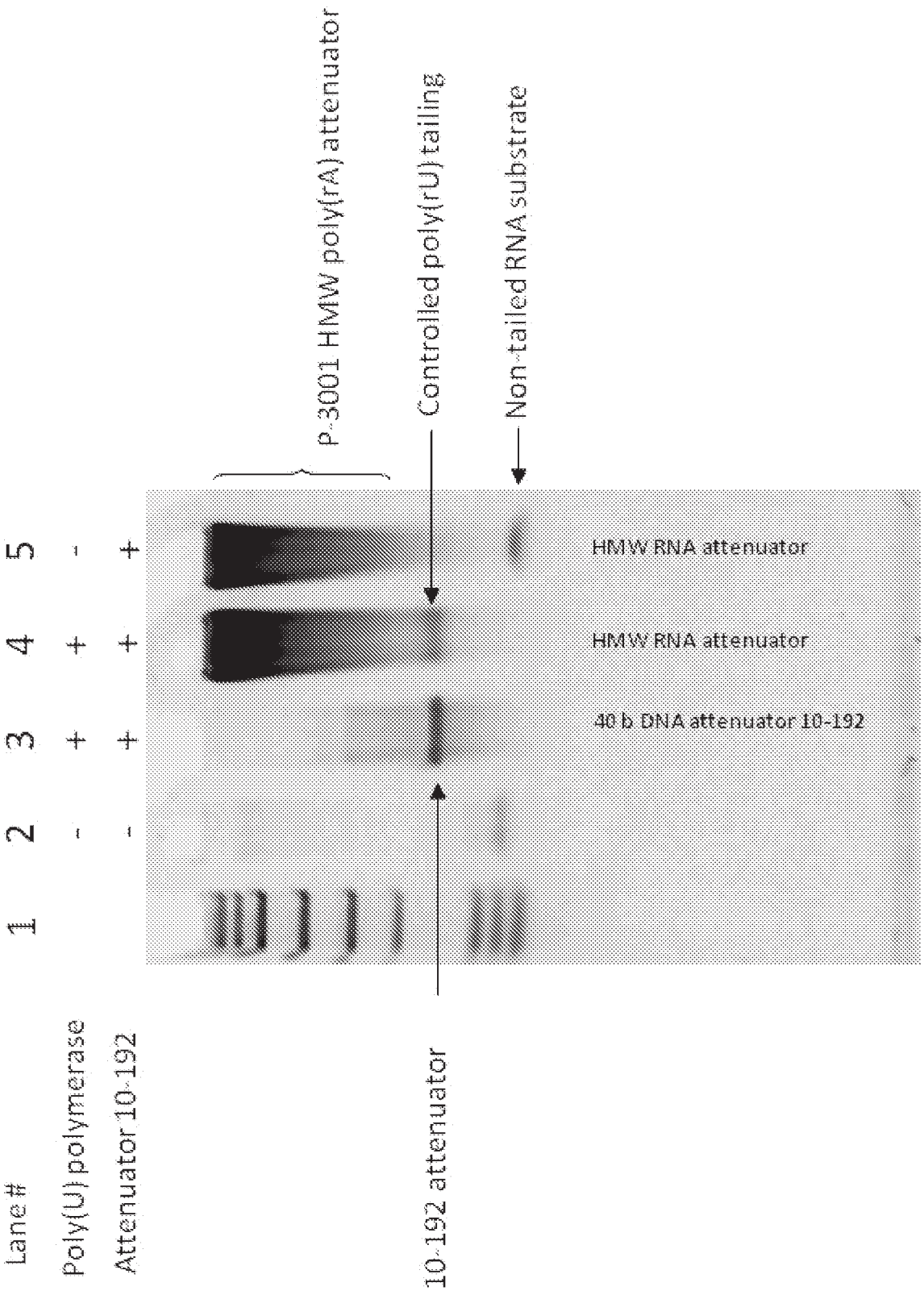

Lane #
1 2 3 4 5
Poly(U) polymerase - + + + +
Attenuator 10-192 - + + + -
10-192 attenuator
P-3001 HMW poly(rA) attenuator
Controlled poly(rU) tailing
Non-tailed RNA substrate
HMW RNA attenuator
HMW RNA attenuator
40 b DNA attenuator 10-192

Figure 21b

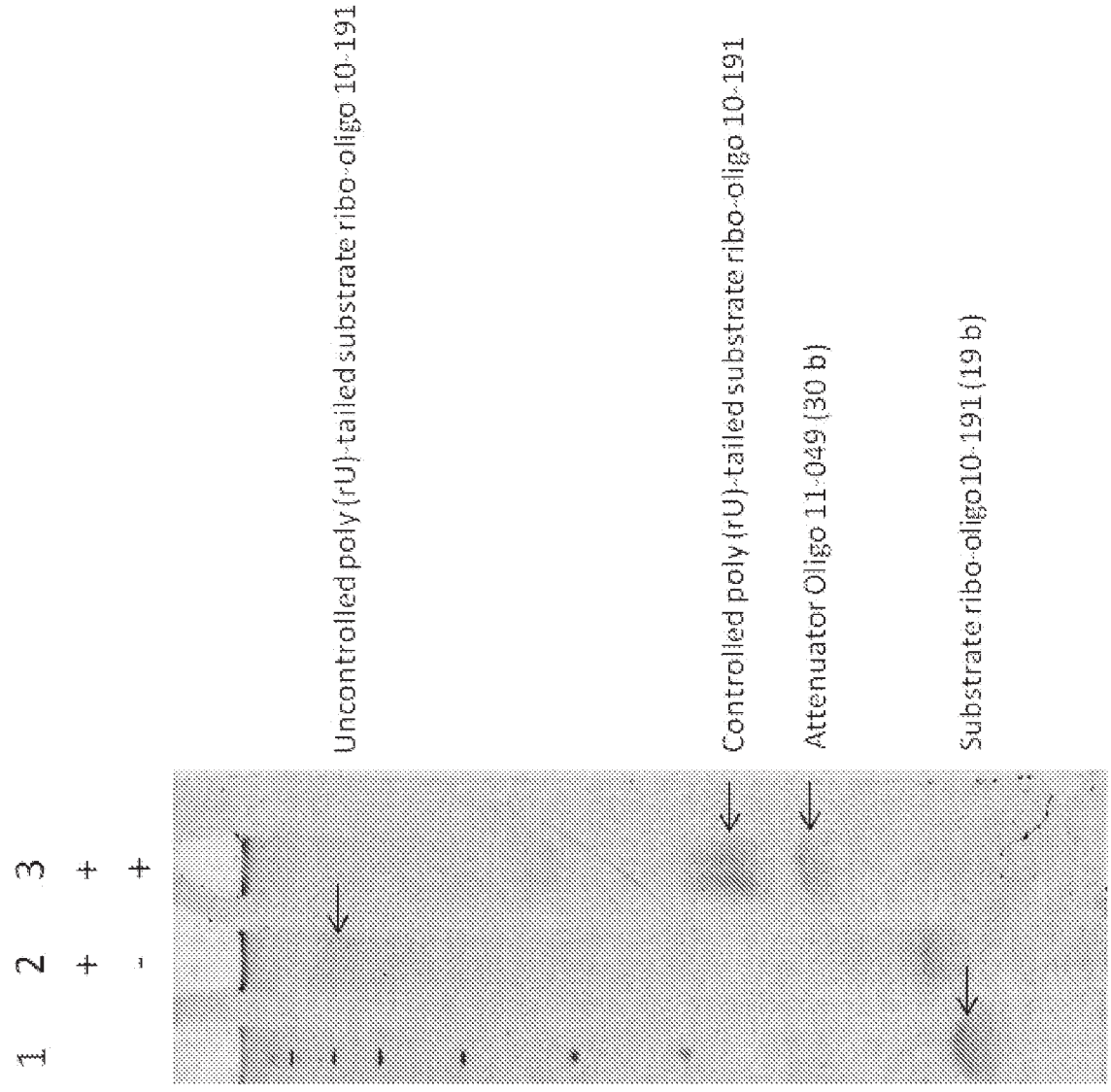

Lane #
1
2
3
Poly(U) polymerase
+
+
Attenuator 11-049
-
+
BP
1,000
500
300
150
80
50
Uncontrolled poly(rU)-tailed substrate ribo-oligo 10-191
Controlled poly(rU)-tailed substrate ribo-oligo 10-191
Attenuator Oligo 11-049 (30 b)
Substrate ribo-oligo10-191 (19 b)

**Figure 21c**

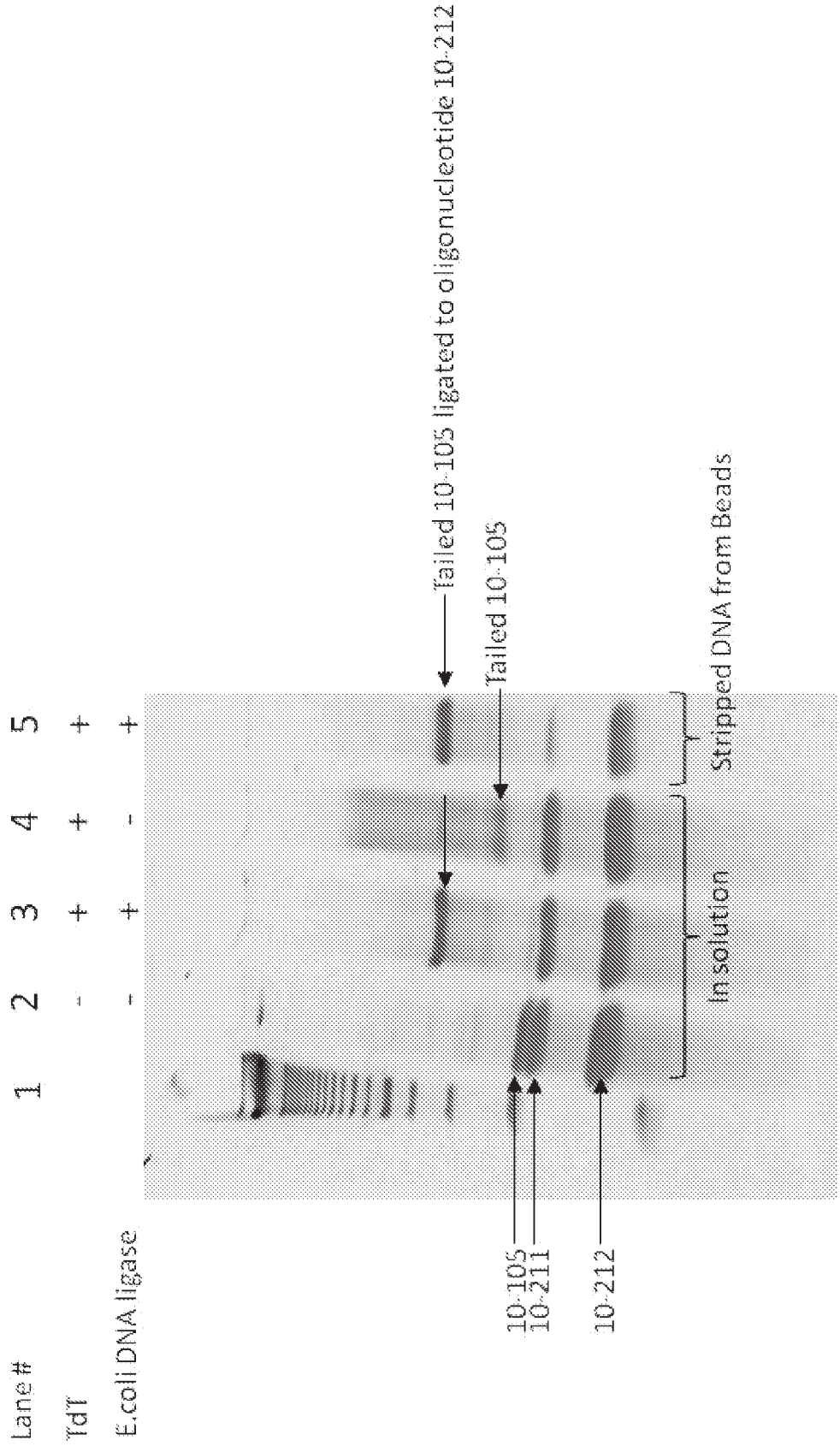

Lane#
1 2 3 4 5
TdT - + + + +
E.coli DNA ligase - + + - +
Tailed 10-105 ligated to oligonucleotide 10-212
Tailed 10-105
Stripped DNA from Beads
In solution
10-105
10-211
10-212

Figure 22

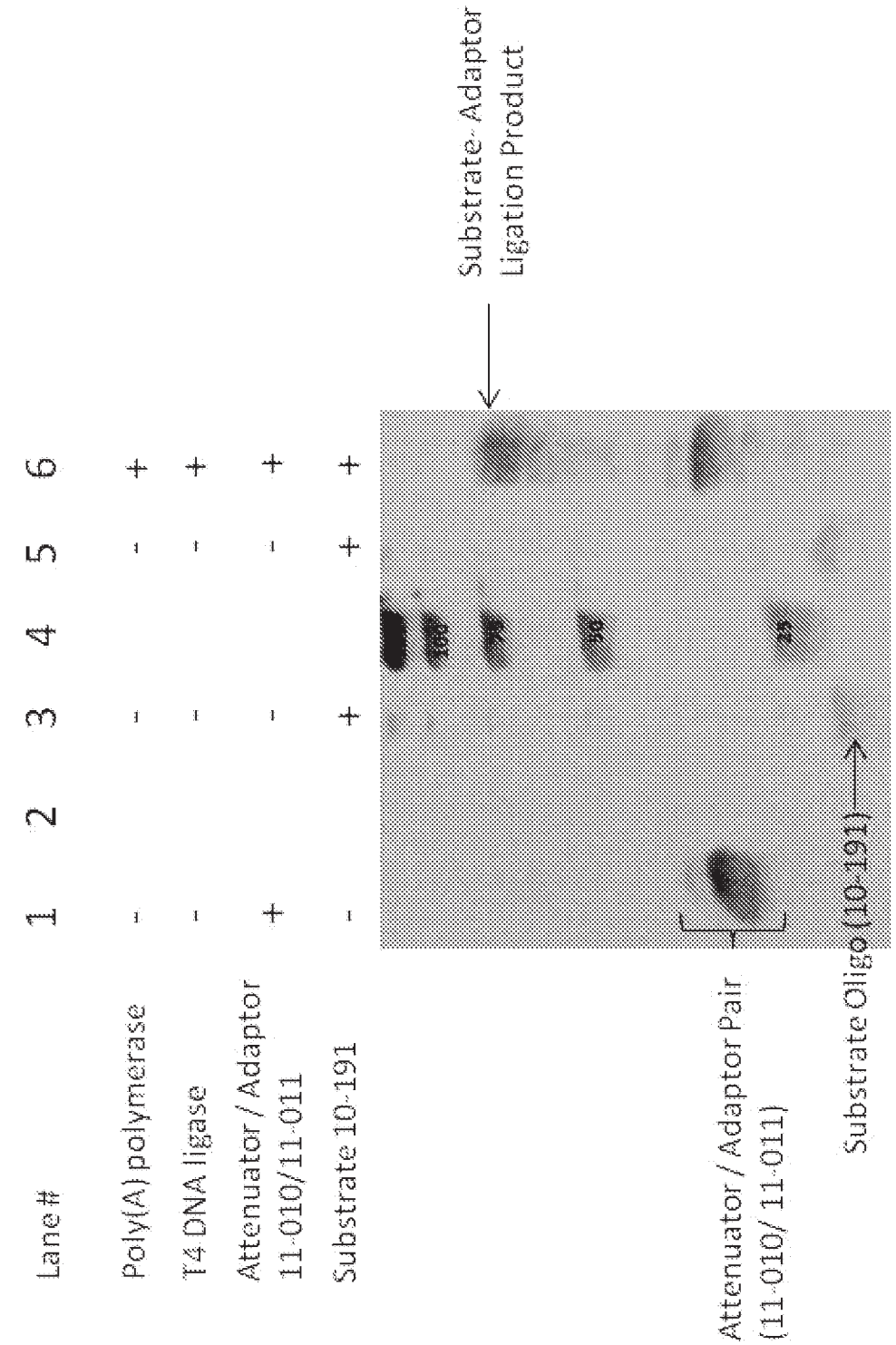

Lane #
1
2
3
4
5
6
Poly(A) polymerase
-
-
-
-
+
+
T4 DNA ligase
-
-
-
-
+
+
Attenuator / Adaptor 11-010/11-011
+
-
-
-
+
+
Substrate 10-191
-
-
+
+
-
+
Substrate-Adaptor Ligation Product
Attenuator / Adaptor Pair (11-010/ 11-011)
Substrate Oligo (10-191)

Figure 23

Substrate 10-105
5'-TAG TTG CTG CAA CCT AGT CTA GAT CTA TAA ACG CAC CTT TGG ACA CGG GG
Attenuator-adaptor
P-GATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTA-P
rUrUTTTTTTTTTTCTAGCC TTCT CGCAGCACATC C CTTT CTCACAT – B
TdT +dATP
5'-TAG TTG CTG CAA CCT AGT CTA GAT CTA TAA ACG CAC CTT TGG ACA CGG GG AAAAAAAAAAAA
P-GATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTA-P
rUrUTTTTTTTTTTCTAGCC TTCT CGCAGCACATC C CTTT CTCACAT – B
E.coli ligase + NAD
Substrate 10-105 with attached poly(dA)12 linker and adaptor 10-212 / 10-211
5'-TAG TTG CTG CAA CCT AGT CTA GAT CTA TAA ACG CAC CTT TGG ACA CGG GG AAAAAAAAAAAAAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTA-P
rUrUTTTTTTTTTTCTAGCC TTCT CGCAGCACATC C CTTT CTCACAT – B

**Figure 24a**

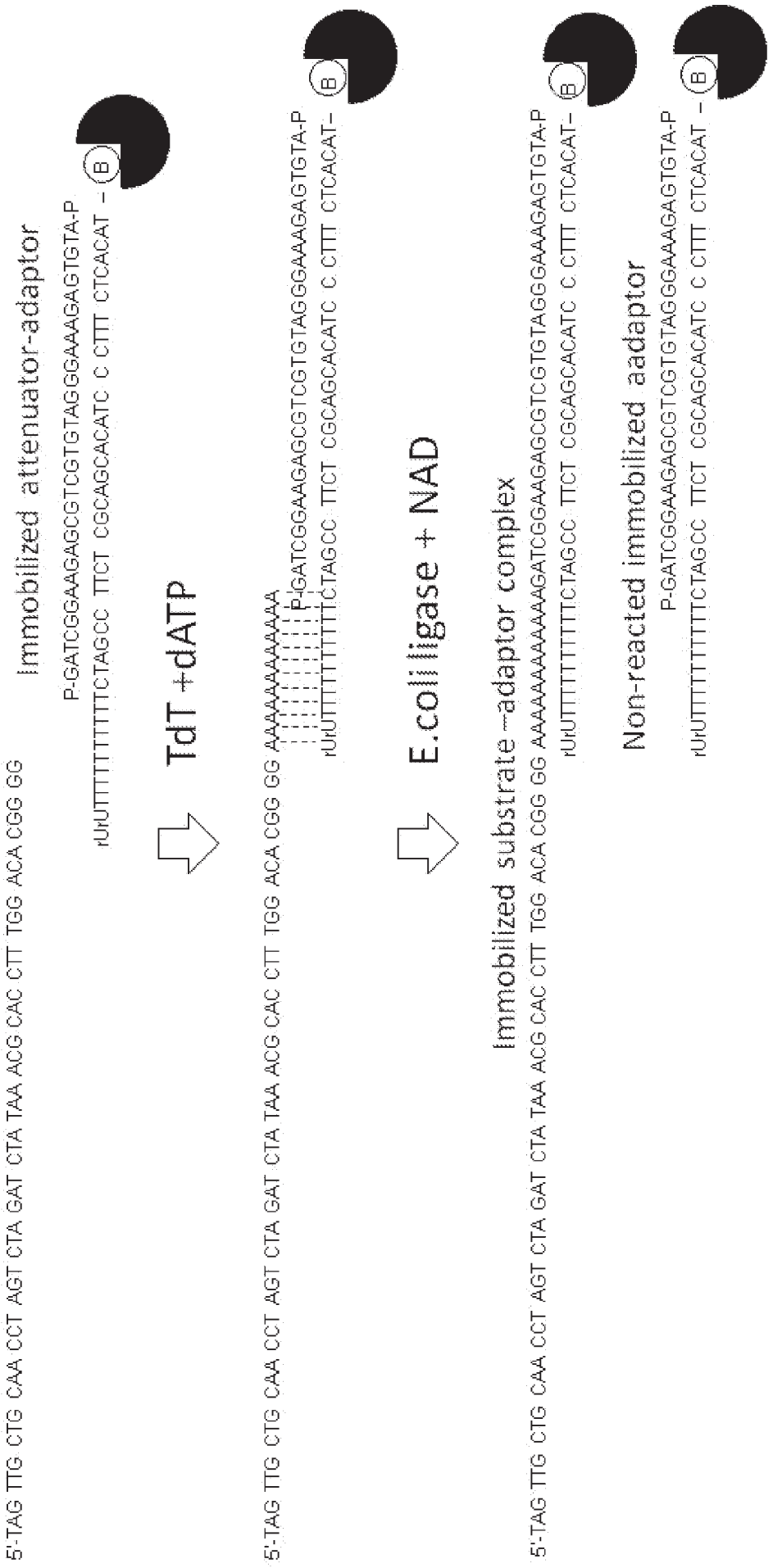

Substrate 10-105
5'-TAG TTG CTG CAA CCT AGT CTA GAT CTA TAA ACG CAC CTT TGG ACA CGG GG
Immobilized attenuator-adaptor
P-GATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTA-P
rUrUTTTTTTTTTTCTAGCC TTCT CGCAGCACATC C CTTT CTCACAT -
B
TdT +dATP
5'-TAG TTG CTG CAA CCT AGT CTA GAT CTA TAA ACG CAC CTT TGG ACA CGG GG AAAAAAAAAAAA
P-GATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTA-P
rUrUTTTTTTTTTTCTAGCC TTCT CGCAGCACATC C CTTT CTCACAT-
B
E.coliligase + NAD
Immobilized substrate –adaptor complex
5'-TAG TTG CTG CAA CCT AGT CTA GAT CTA TAA ACG CAC CTT TGG ACA CGG GG AAAAAAAAAAAAAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTA-P
rUrUTTTTTTTTTTCTAGCC TTCT CGCAGCACATC C CTTT CTCACAT-
B
Non-reacted immobilized aadaptor
P-GATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTA-P
rUrUTTTTTTTTTTCTAGCC TTCT CGCAGCACATC C CTTT CTCACAT -
B

**Figure 24b**

RNA substrate 10-191
5'-rGrGrCrCrUrUrGrUrUrCrCrUrGrUrCrCrCrCrA-3'
Attenuator-adaptor
5'P-CTTATTGCTGTGGTTGGTTCCTGTGCTGTTTT-3'P
3'P-TTTTUTTTTUTTTTUTTTUGAAUAACGACACCAACC-5'
Poly(A) polymerase + dATP
5'-rGrGrCrCrUrUrGrUrUrCrCrUrGrUrCrCrCrArArArArArArArArArArArArA
3'P-TTTT U TTTT U TTTT U TTTT U GAAUAACGACACCAACC-5'
5'P-CTTATTGCTGTGGTTGGTTCCTGTGCTGTTTT-3'P
T4 DNA ligase + dATP
RNA substrate 10-191 with attached poly(rA)20 linker and DNA adaptor 11-010 / 11-011
5'-rGrGrCrCrUrUrGrUrUrCrCrUrGrUrCrCrCrArArArArArArArArArArArArArArArArArA CTTATTGCTGTGGTTGGTTCCTGTGCTGTTTT-3'P
3'P-TTTTUTTTTUTTTTUTTTUGAAUAACGACACCAACC-5'

**Figure 24c**

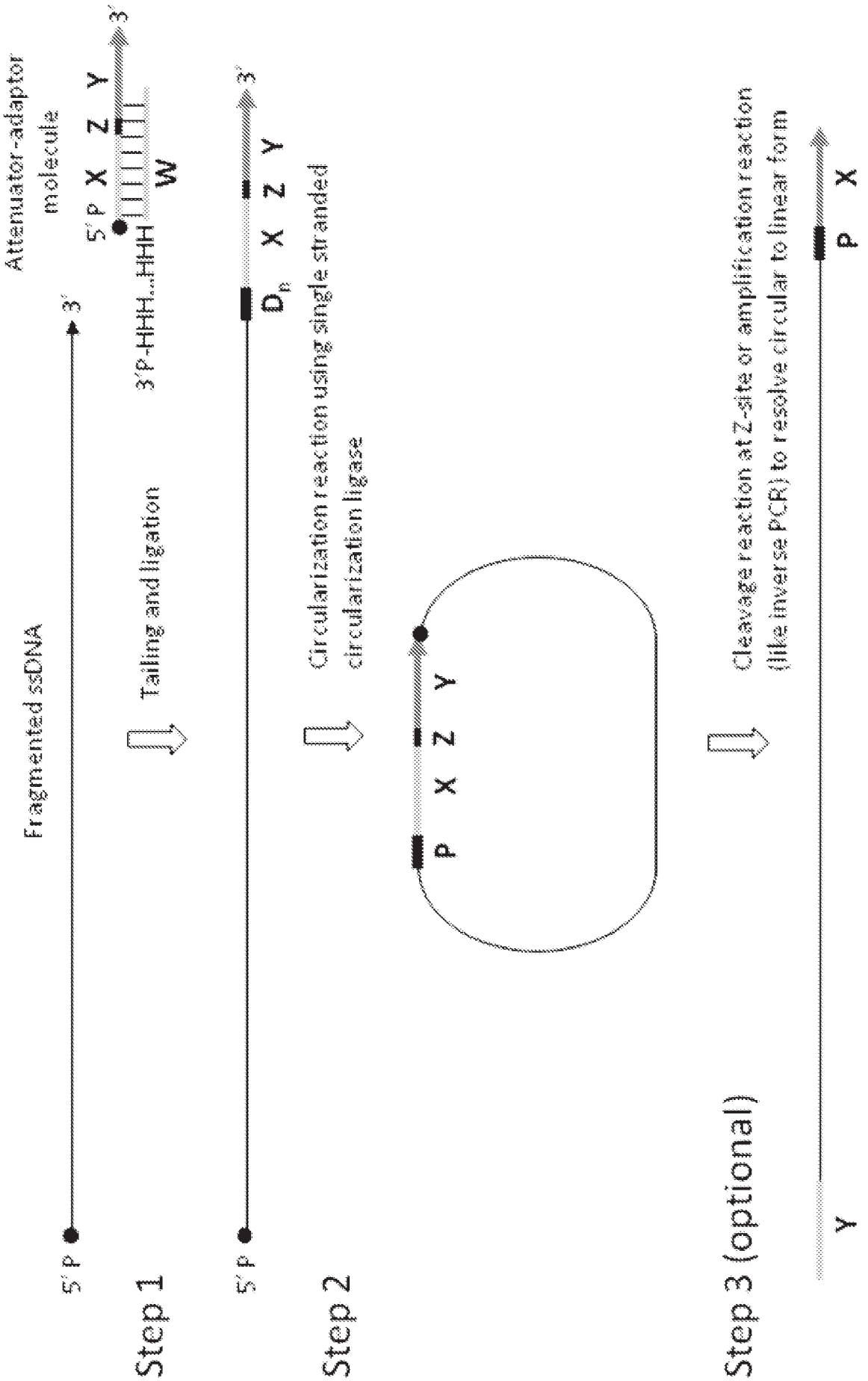

Attenuator-adaptor molecule
5' P X Z Y 3'
3'P-HHH...HHH W
Fragmented ssDNA
5' P 3'
Step 1
Tailing and ligation
5' P
$D_n$ X Z Y 3'
Step 2
Circularization reaction using single stranded circularization ligase
P X Z Y
Step 3 (optional)
Cleavage reaction at Z-site or amplification reaction (like inverse PCR) to resolve circular to linear form
P X
Y

Figure 25

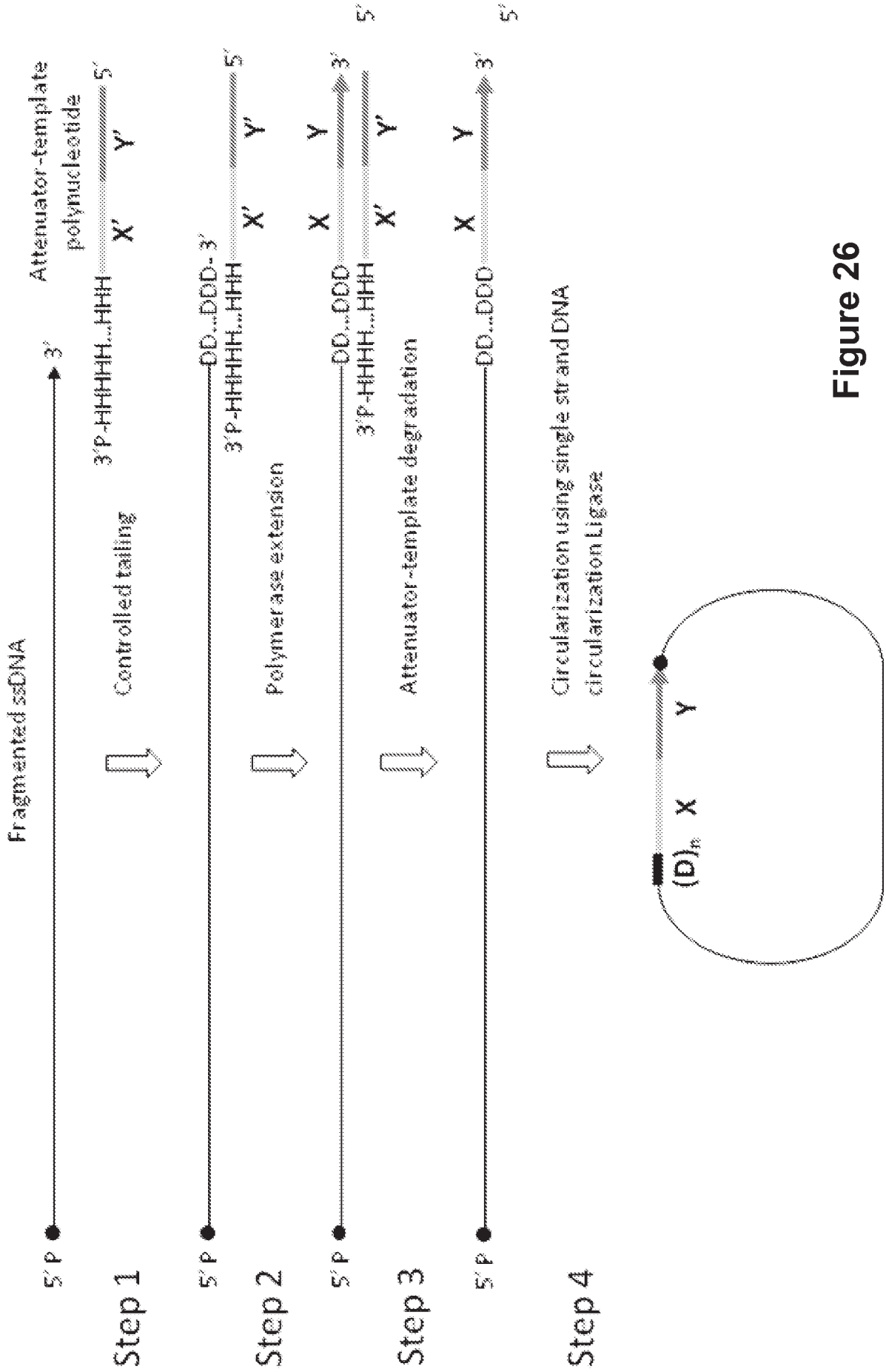

Fragmented ssDNA
5' P
3'
Step 1
Controlled tailing
Attenuator-template polynucleotide
3'P-HHHHH...HHH
X'
Y'
5'
Step 2
Polymerase extension
DD...DDD- 3'
3'P-HHHHH...HHH
X'
Y'
5'
Step 3
Attenuator-template degradation
DD...DDD
X
Y
3'
Step 4
Circularization using single strand DNA circularization Ligase
(D)n
X
Y

Figure 26

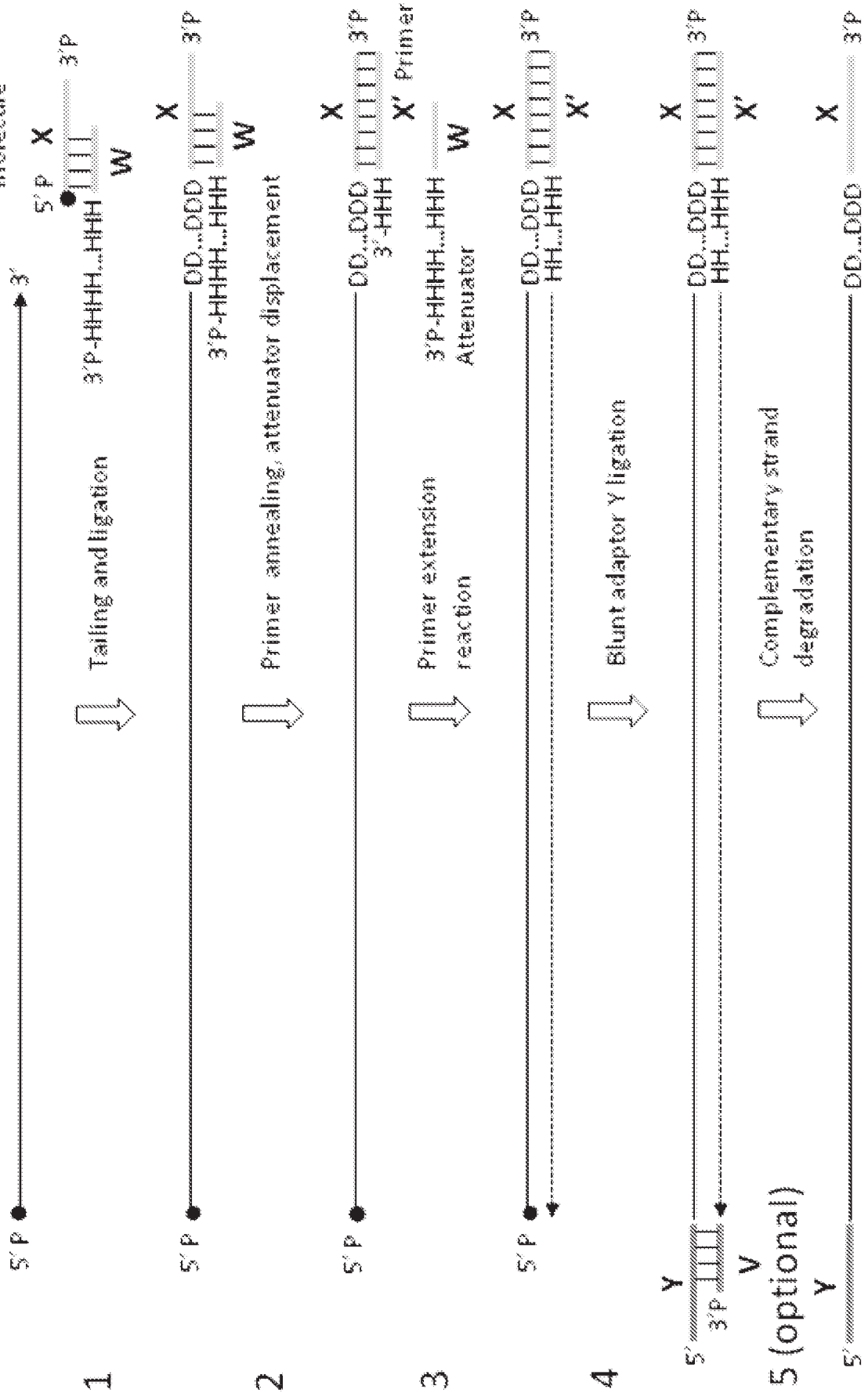

Fragmented ssDNA
Attenuator-adaptor molecule
5'P
3'
5'P X
3'P
3'P-HHHH...HHH
W
Tailing and ligation
OD...DOD
X
3'P
3'P-HHHH...HHH
W
Primer annealing, attenuator displacement
3'-HHH
X'
Primer
Primer extension reaction
Attenuator
Blunt adaptor Y ligation
HH...HHH
Y
V
Complementary strand degradation
Step 1
Step 2
Step 3
Step 4
Step 5 (optional)

Figure 27

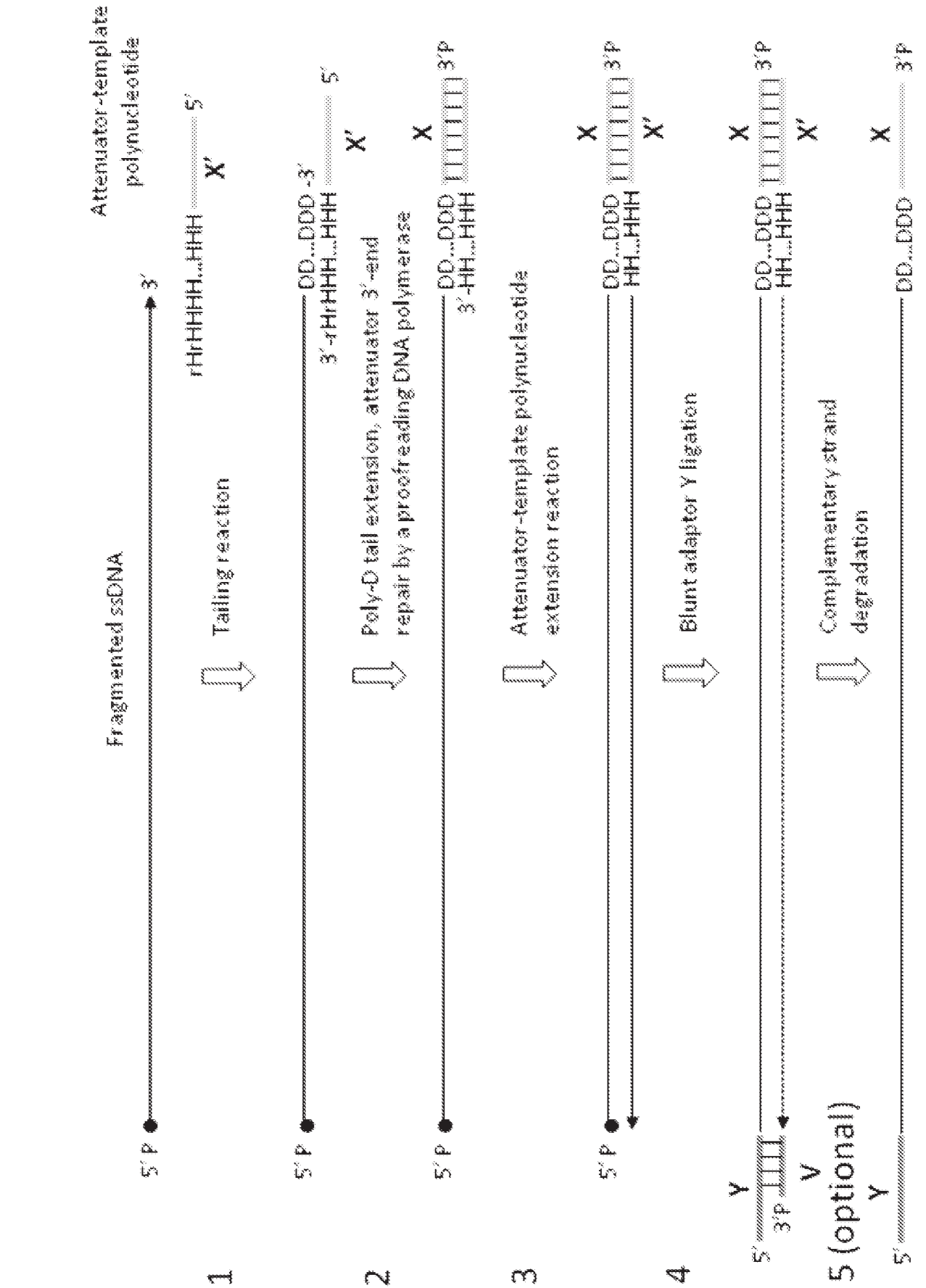

Fragmented ssDNA
Attenuator-template polynucleotide
Step 1
Tailing reaction
Step 2
Poly-D tail extension, attenuator 3'-end repair by a proofreading DNA polymerase
Step 3
Attenuator-template polynucleotide extension reaction
Step 4
Blunt adaptor Y ligation
Step 5 (optional)
Complementary strand degradation

Figure 28

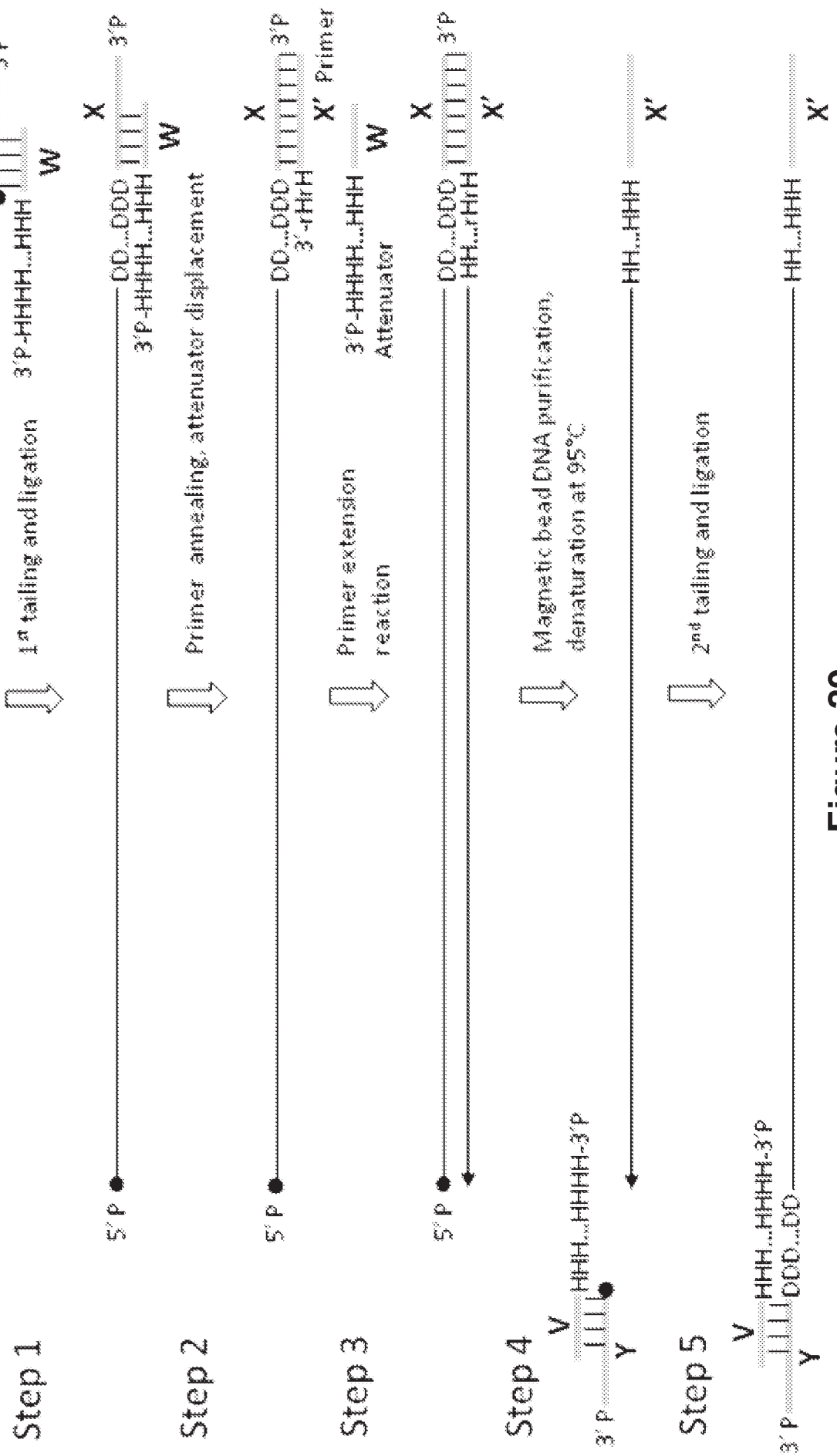

Attenuator-adaptor molecule
Fragmented ssDNA or RNA
5′ P
3′
Step 1
1st tailing and ligation
3′P-HHHH...HHH
5′ P X
3′P
W
Step 2
Primer annealing, attenuator displacement
DD...DDD
3′P-HHHH...HHH
X
3′P
W
Step 3
Primer extension reaction
3′P-HHHH...HHH
Attenuator
DD...DDD
3′-rHrH
X
X′ Primer
W
Step 4
Magnetic bead DNA purification, denaturation at 95°C
HH...rHrH
HH...HHH
V
HHH...HHHH-3′P
3′ P
Y
X′
Step 5
2nd tailing and ligation
DDD...DD
HH...HHH
V
HHH...HHHH-3′P
3′ P
Y
X′

Figure 29

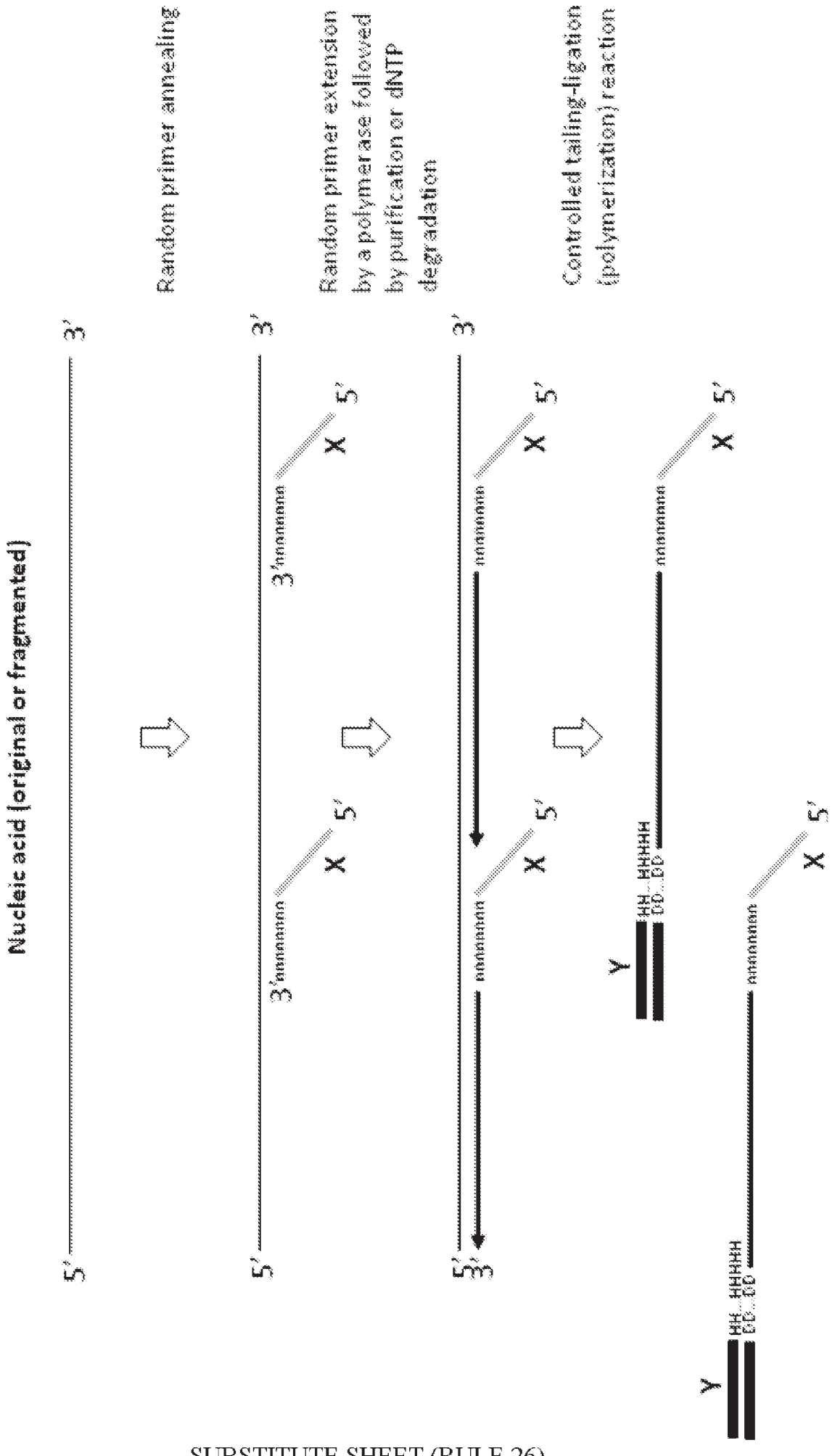

Nucleic acid (original or fragmented)
Random primer annealing
Random primer extension by a polymerase followed by purification or dNTP degradation
Controlled tailing-ligation (polymerization) reaction
3'
5'
X
Y

Figure 30

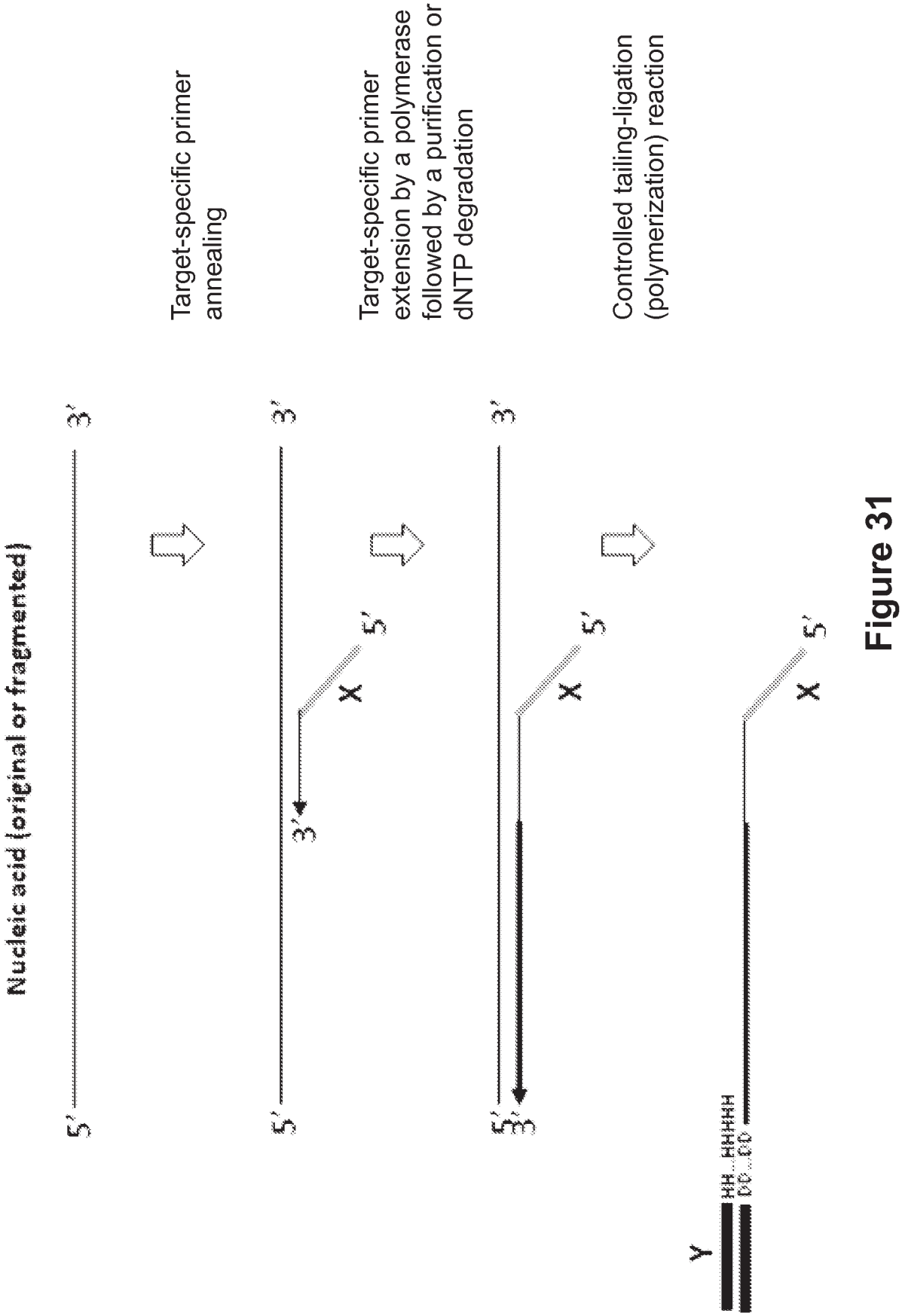

Nucleic acid (original or fragmented)
5'
3'
Target-specific primer annealing
5'
3'
X
5'
3'
Target-specific primer extension by a polymerase followed by a purification or dNTP degradation
5'
3'
X
5'
Controlled tailing-ligation (polymerization) reaction
5'
X
5'
Y

Figure 31

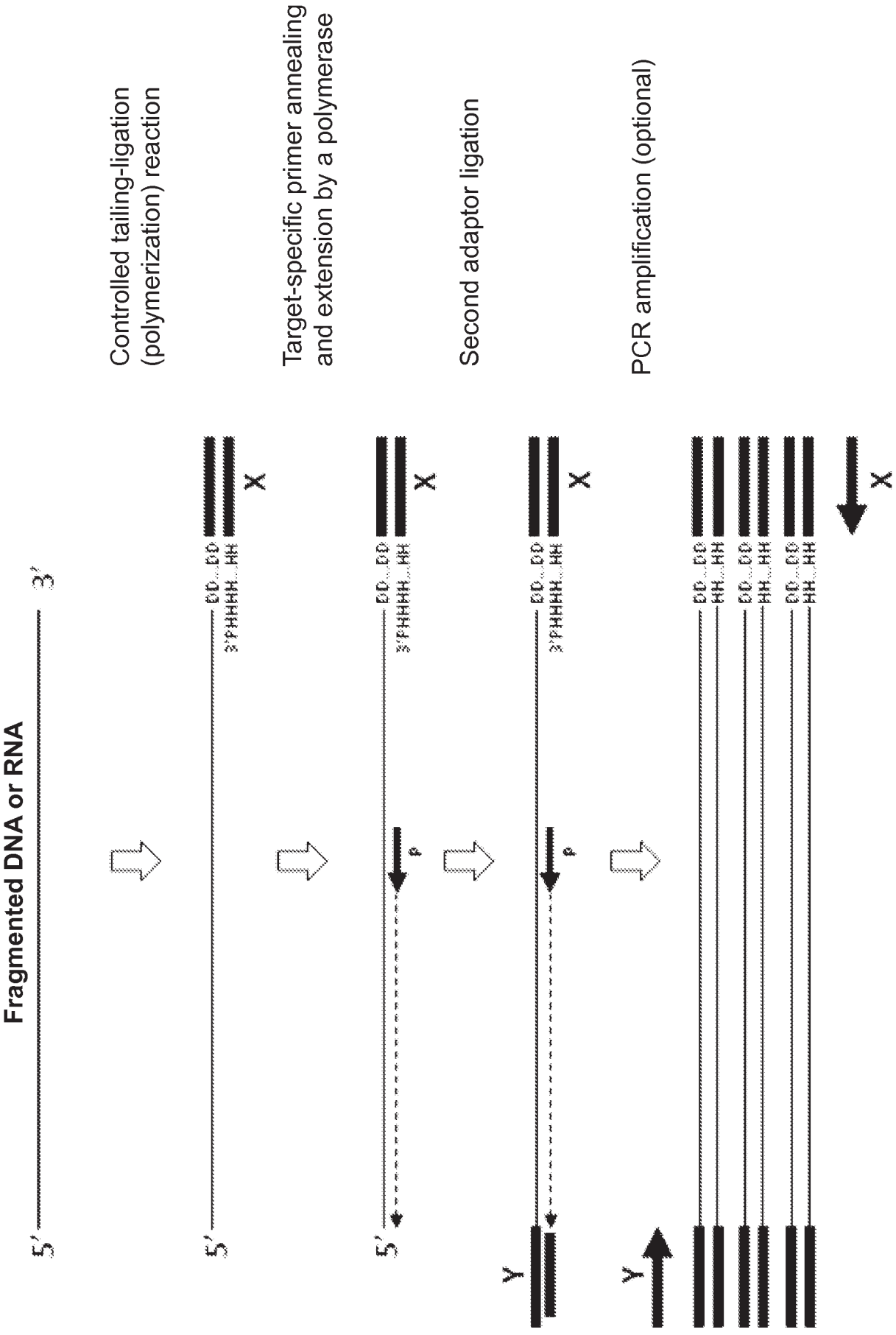

Fragmented DNA or RNA
5'
3'
Controlled tailing-ligation (polymerization) reaction
Target-specific primer annealing and extension by a polymerase
Second adaptor ligation
PCR amplification (optional)
X
Y

Figure 32

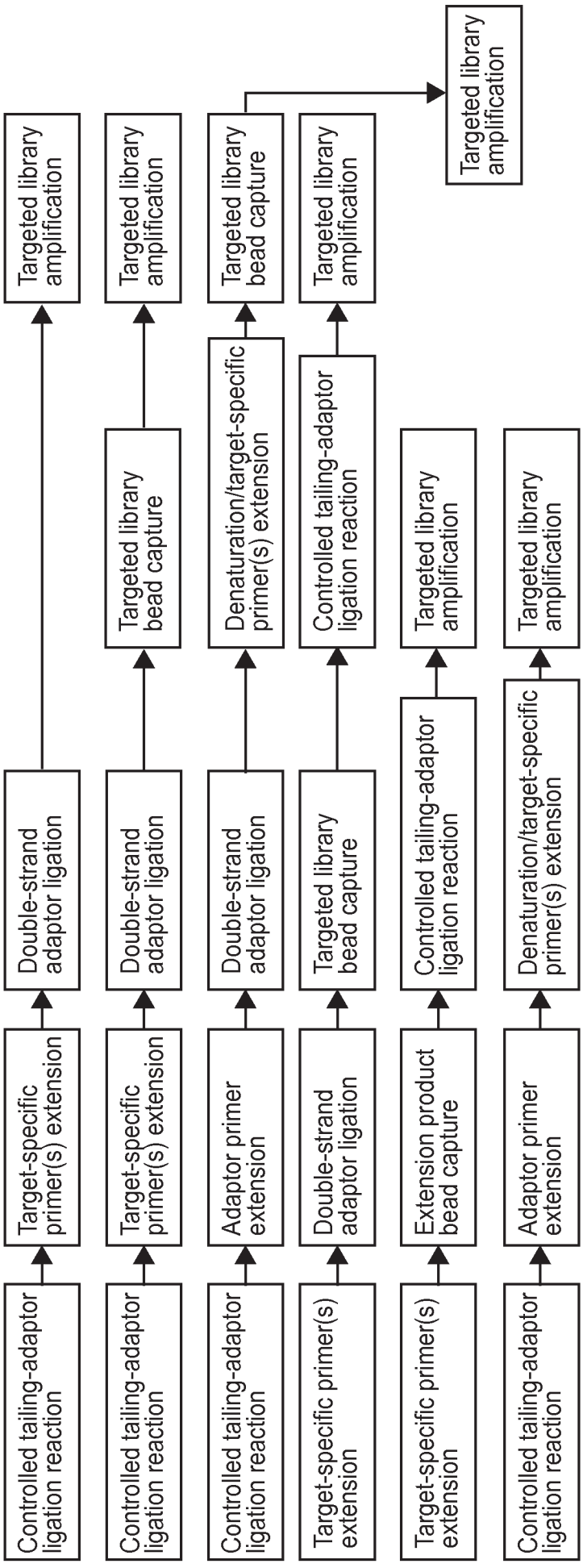

Controlled tailing-adaptor ligation reaction
Target-specific primer(s) extension
Double-strand adaptor ligation
Targeted library amplification
Controlled tailing-adaptor ligation reaction
Target-specific primer(s) extension
Double-strand adaptor ligation
Targeted library bead capture
Targeted library amplification
Controlled tailing-adaptor ligation reaction
Adaptor primer extension
Double-strand adaptor ligation
Denaturation/target-specific primer(s) extension
Targeted library bead capture
Target-specific primer(s) extension
Double-strand adaptor ligation
Targeted library bead capture
Controlled tailing-adaptor ligation reaction
Targeted library amplification
Target-specific primer(s) extension
Extension product bead capture
Controlled tailing-adaptor ligation reaction
Targeted library amplification
Controlled tailing-adaptor ligation reaction
Adaptor primer extension
Denaturation/target-specific primer(s) extension
Targeted library amplification
Targeted library amplification

**Figure 33**

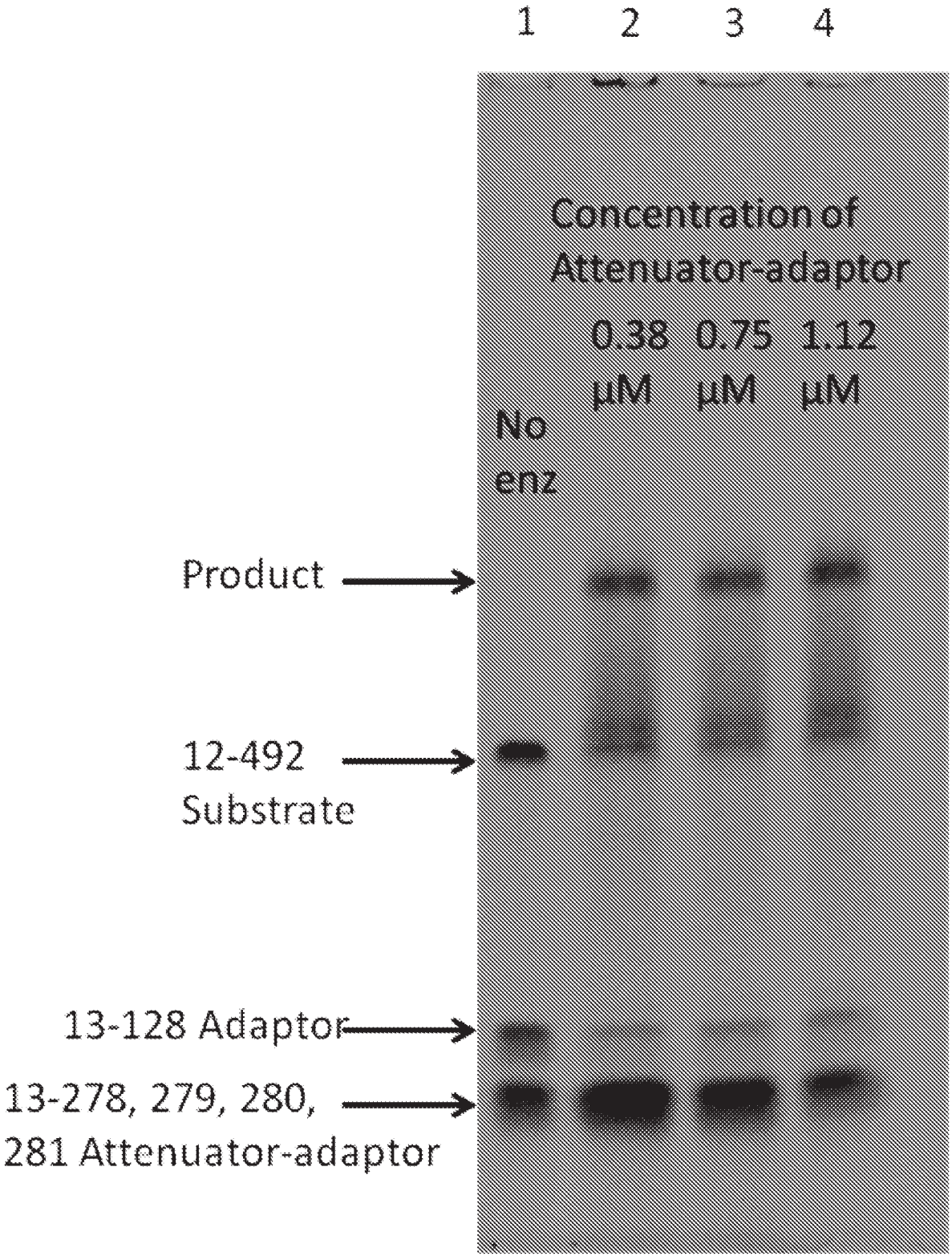

1
2
3
4
Concentration of Attenuator-adaptor
0.38 µM
0.75 µM
1.12 µM
No enz
Product
12-492 Substrate
13-128 Adaptor
13-278, 279, 280, 281 Attenuator-adaptor

**Figure 34**

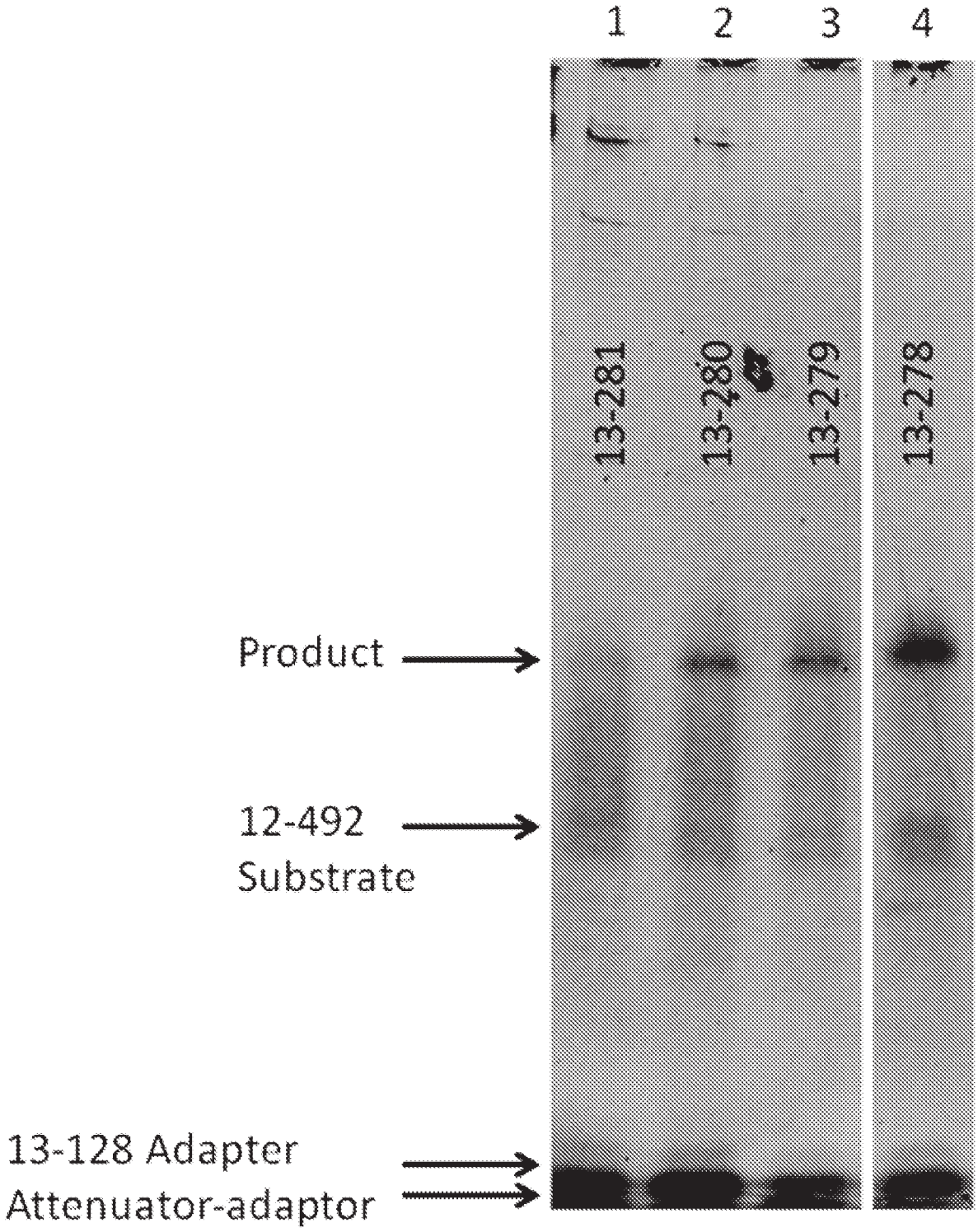

1
2
3
4
13-281
13-280
13-279
13-278
Product
12-492
Substrate
13-128 Adapter
Attenuator-adaptor

**Figure 35**

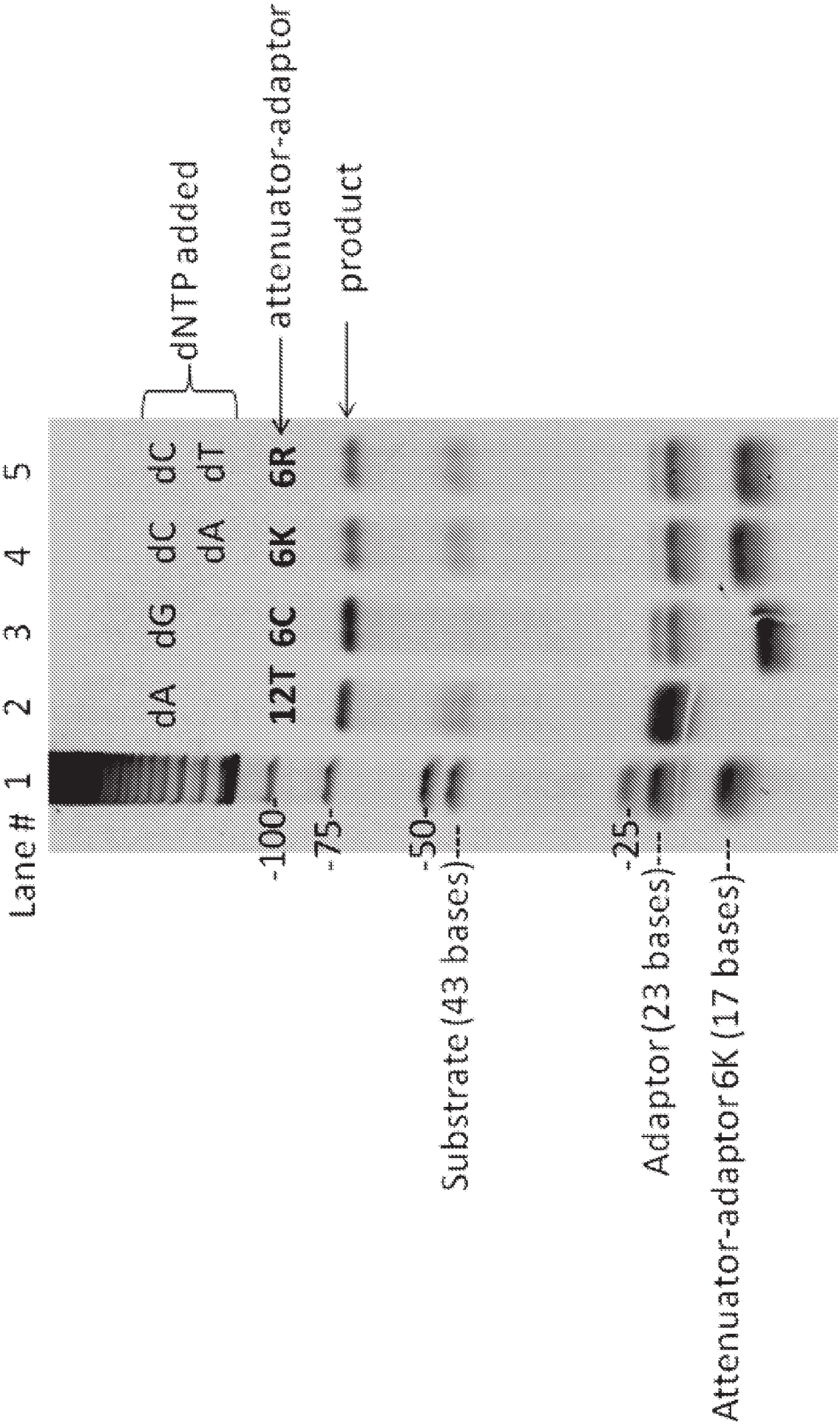

Lane # 1 2 3 4 5
dA dG dC dC
dA dT
dNTP added
12T 6C 6K 6R
attenuator-adaptor
product
100
75
50
25
Substrate (43 bases)
Adaptor (23 bases)
Attenuator-adaptor 6K (17 bases)

Figure 36

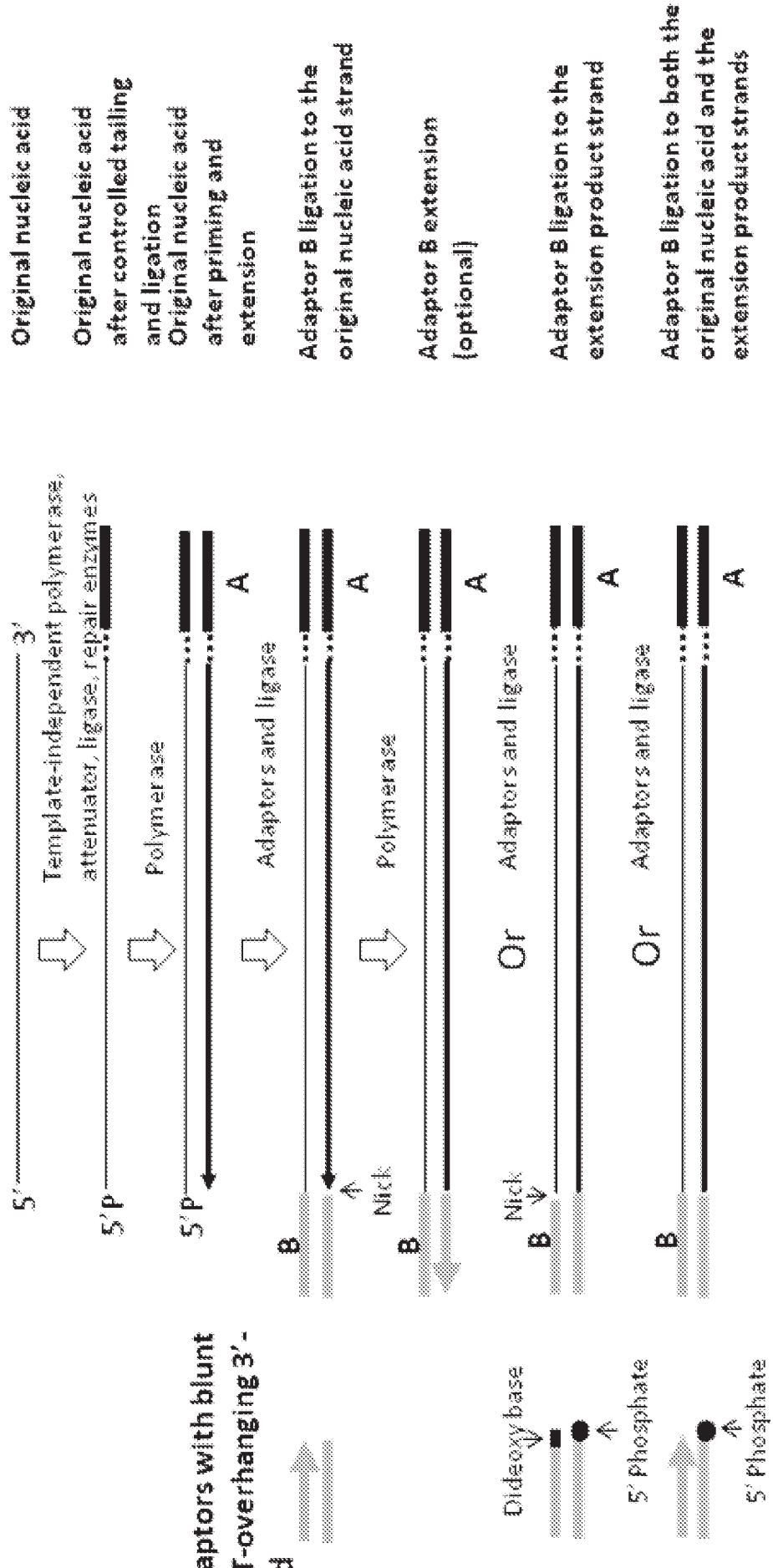

Original nucleic acid
Original nucleic acid after controlled tailing and ligation
Original nucleic acid after priming and extension
Adaptor B ligation to the original nucleic acid strand
Adaptor B extension (optional)
Adaptor B ligation to the extension product strand
Adaptor B ligation to both the original nucleic acid and the extension product strands
5'
3'
Template-independent polymerase, attenuator, ligase, repair enzymes
5' P
Polymerase
5' P
Adaptors and ligase
A
B
Nick
Polymerase
Or
Adaptors and ligase
Nick
Or
Adaptors and ligase
Adaptors with blunt or T-overhanging 3'-end
Dideoxy base
5' Phosphate
5' Phosphate

Figure 37