```
ATCV_Ligase SEQ ID NO:1   MAIQKPLLAASLKKMSVDNLTFPVYATPKLDGIRALKIDGTLVSRTFKPIRNTTISKVLA  60
PBCV_Ligase SEQ ID NO:6   MAITKPLLAATLEN--IEDVQFPCLATPKIDGIRSVKQT-QMLSRTFKPIRNSVMNRLLT  57
                          *** ******:*::  :::: **  ****:****::*    ::**********:.:.::*:

ATCV_Ligase SEQ ID NO:1   SLLPDGSDGEI-LSGKTFQDSTSTVMTTDAGIGSDTTFFWFDYVKDDPDKGYLDRIADMK  119
PBCV_Ligase SEQ ID NO:6   ELLPEGSDGEISIEGATFQDTTSAVMTGHKMYNAKFSYYWFDYVTDDPLKKYIDRVEDMK  117
                          .***:****** :.* ****:**:*** .   .:. :::*****.*** * *:**: ***

ATCV_Ligase SEQ ID NO:1   TFVDQHPEILKDSCVTIVPLFPKKIDTPEELHVFEKWCLDQGFEGVMVRTAGGKYKFGRS  179
PBCV_Ligase SEQ ID NO:6   NYITVHPHILEHAQVKIIPLIPVEINNITELLQYERDVLSKGFEGVMIRKPDGKYKFGRS  177
                          .::  **.**:.: *.*:**:* :*:.  **  :*:  *.:******:*. .********

ATCV_Ligase SEQ ID NO:1   TEKEQILVKIKQFEDDEAVVIGVSALQTNTNDKKLNQLGEMRRTSHQDGKVDLDMLGALD  239
PBCV_Ligase SEQ ID NO:6   TLKEGILLKMKQFKDAEATIISMTALFKNTNTKTKDNFGYSKRSTHKSGKVEEDVMGSIE  237
                          * ** **:*:***:* **.:*.::** .*** *. :::*  :*::*:.***: *::*:::

ATCV_Ligase SEQ ID NO:1   VDWNGIRFSIGTGFDKDTREDLWKQRDSIVGKVVKFKYFSQGIKTAPRFPVFLGFRDEND  299
PBCV_Ligase SEQ ID NO:6   VDYDGVVFSIGTGFDADQRRDFWQNKESYIGKMVKFKYFEMGSKDCPRFPVFIGIRHEED  297

                          **::*: ******** * *.*:*::::* :**:******. * * .******:*:*.*:*
ATCV_Ligase SEQ ID NO:1   M     300
PBCV_Ligase SEQ ID NO:6   R     298
```

FIG. 1

STRUCTURAL MODEL OF ATCV-1 ENZYME

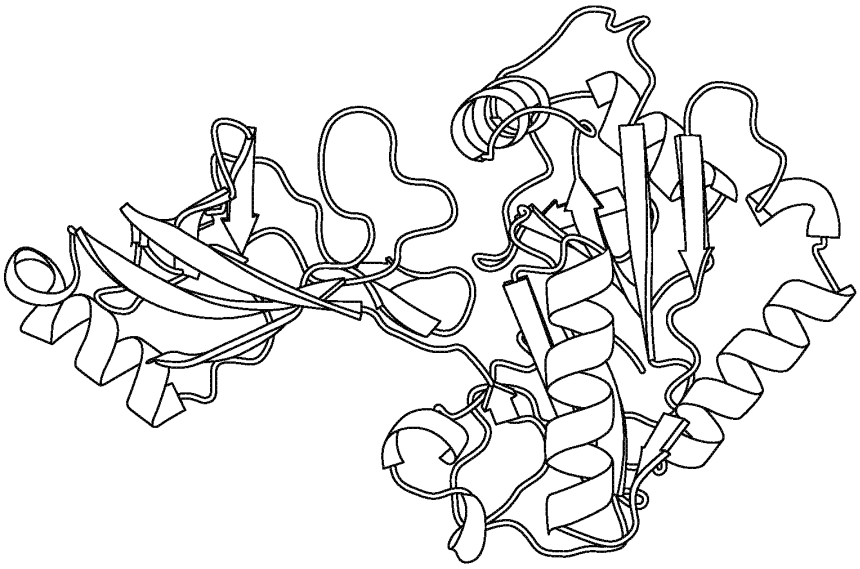


FIG. 2A

ATCV-1 MODEL SUPERIMPOSED OF PBCB-1 CRYSTAL STRUCTURE

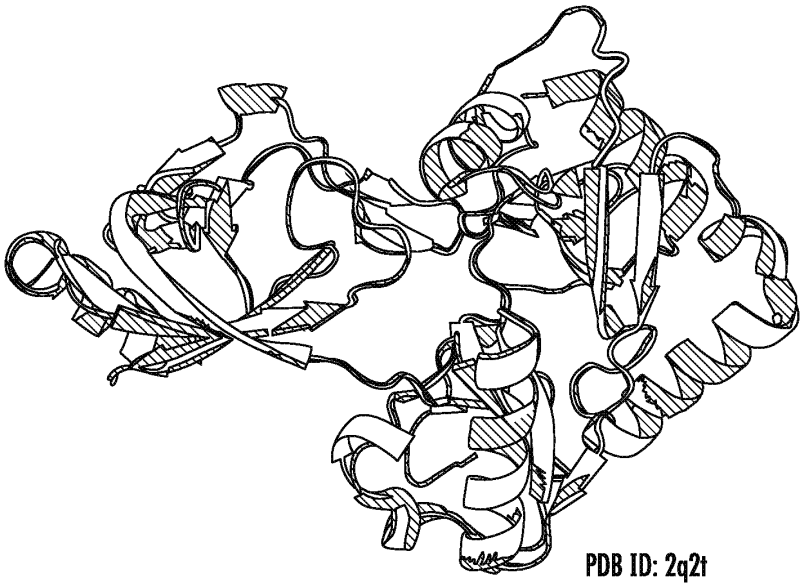
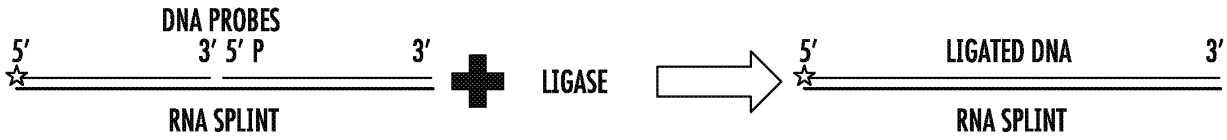


FIG. 2B



EXAMPLE OF RNA AND DNA SEQUENCES EMPLOYED IN ASSAYING THE LIGASE ACTIVITY

Seq ID 2: 5′CTAAGCTCTATAGTCGGACGTAACGAGTAAGACCGTCGAGTCGCCTAGT 3′

Seq ID 3: **FAM**-TACTAGGCGACTCGACGGTCTTAC-OH 3′ (24 nucleotides)

Seq ID 4: **5Phos**-TCGTTACGTCCGACTATAGAGCTTAG-OH 3′ (26 nucleotides)

Seq ID 5: **5Phos**-TCGTTACGTCCGACTATAGAGCTTAG-Atto565 (26 nucleotides)

FIG. 3

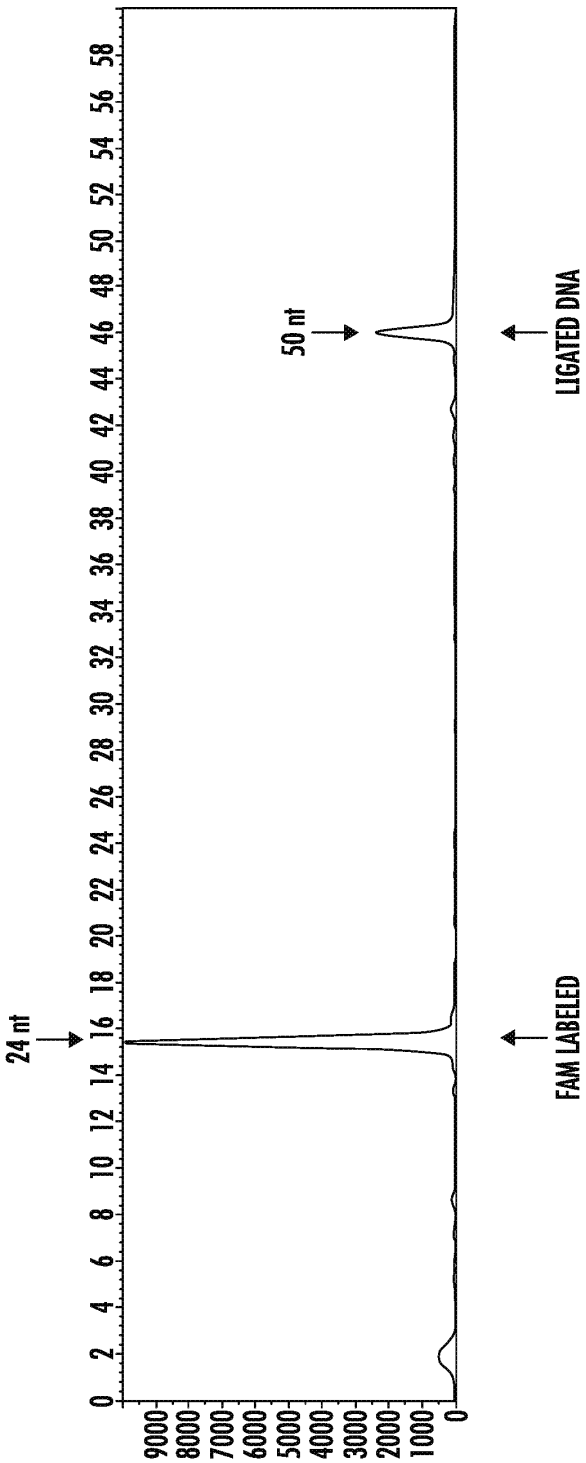

24 nt
50 nt
FAM LABELED ACCEPTOR
LIGATED DNA
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58
0 1000 2000 3000 4000 5000 6000 7000 8000 9000

FIG. 4

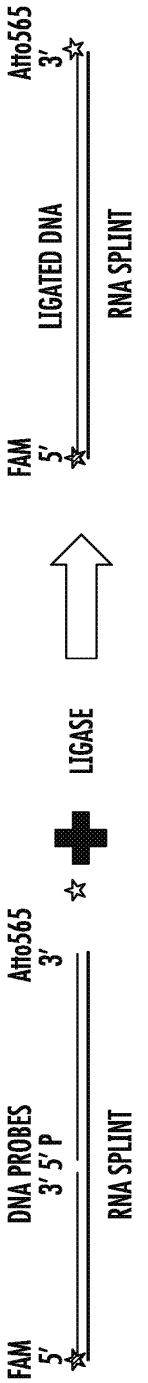

FAM
5'
DNA PROBES
3' 5' P
RNA SPLINT
Atto565
3'
LIGASE
FAM
5'
LIGATED DNA
RNA SPLINT
Atto565
3'

FIG. 5

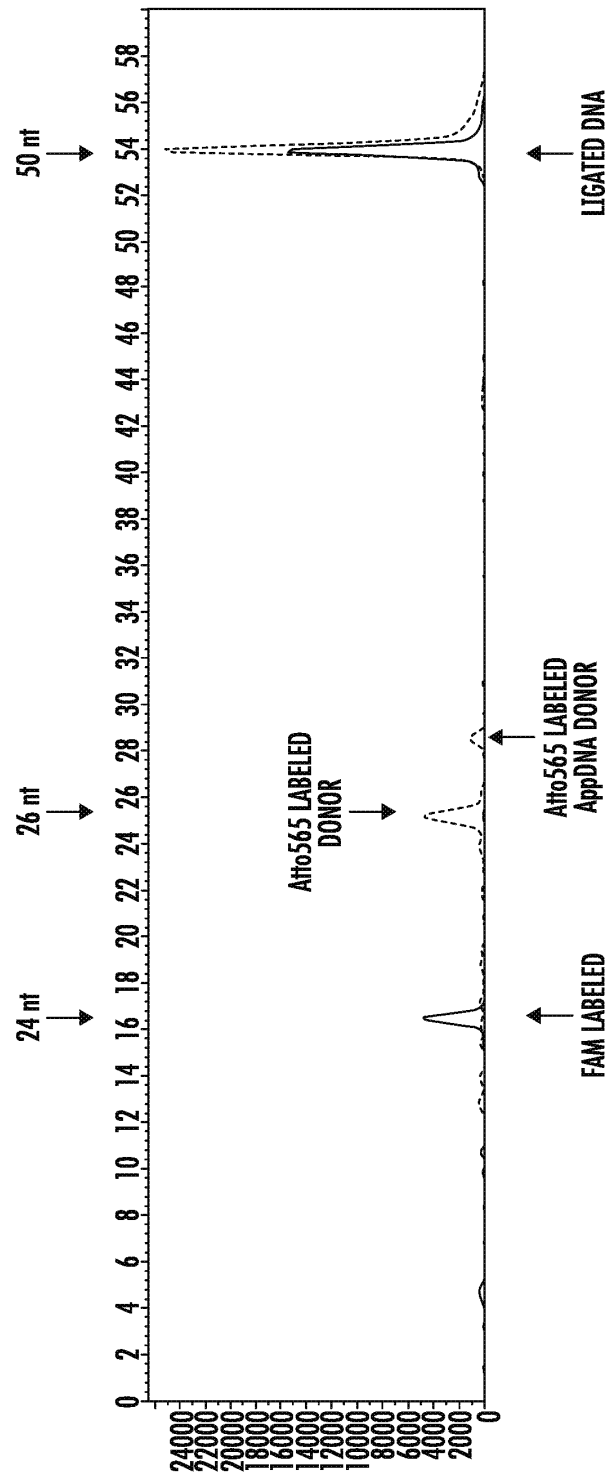

50 nt
26 nt
24 nt
Atto565 LABELED DONOR
Atto565 LABELED AppDNA DONOR
LIGATED DNA
FAM LABELED ACCEPTOR
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58
24000 22000 20000 18000 16000 14000 12000 10000 8000 6000 4000 2000 0

FIG. 6

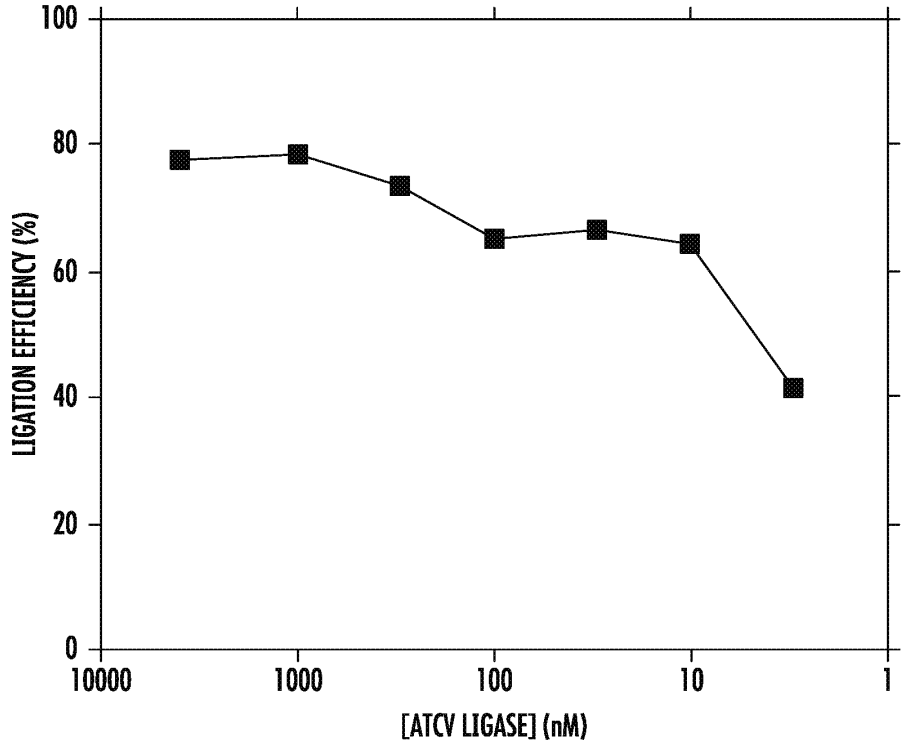

100
80
60
40
20
0
LIGATION EFFICIENCY (%)
10000
1000
100
10
1
[ATCV LIGASE] (nM)

FIG. 7

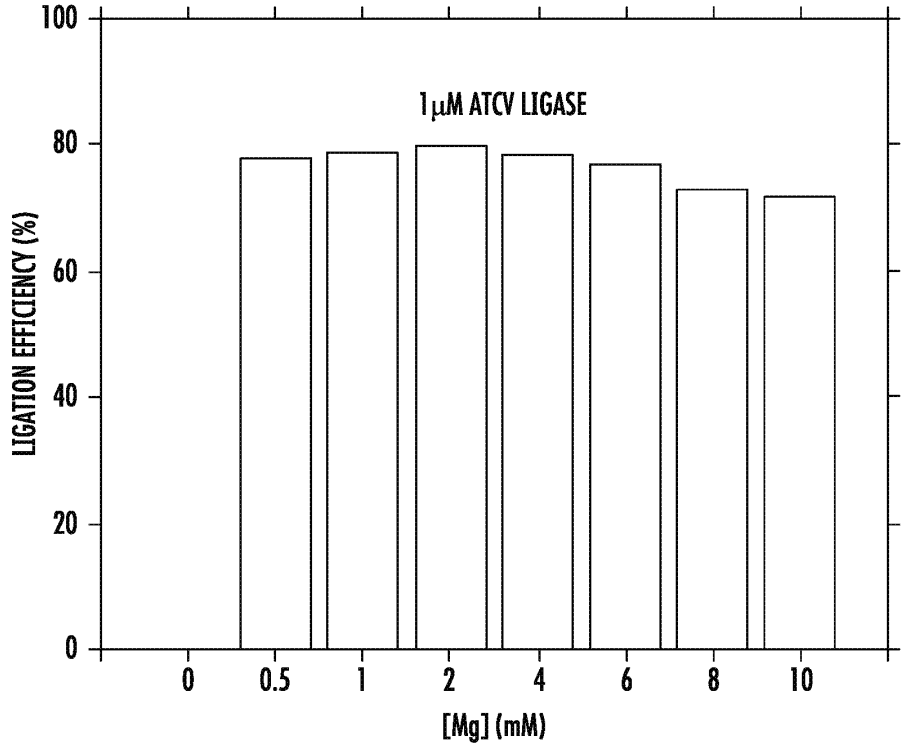

100
80
60
40
20
0
LIGATION EFFICIENCY (%)
1μM ATCV LIGASE
0
0.5
1
2
4
6
8
10
[Mg] (mM)

FIG. 8

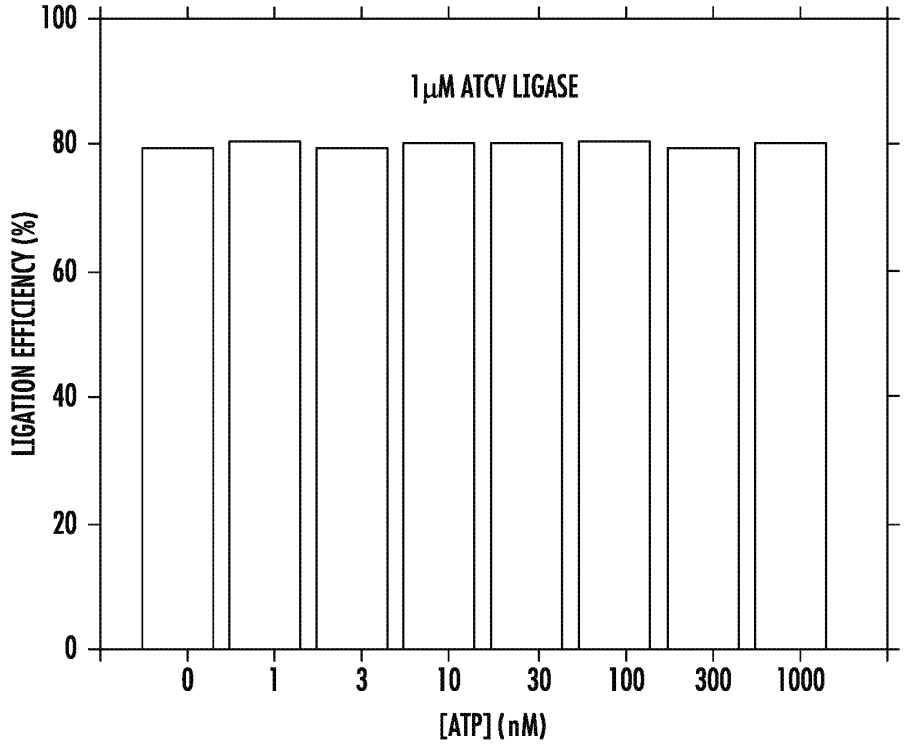

1 μM ATCV LIGASE
LIGATION EFFICIENCY (%)
100
80
60
40
20
0
0
1
3
10
30
100
300
1000
[ATP] (nM)

FIG. 9

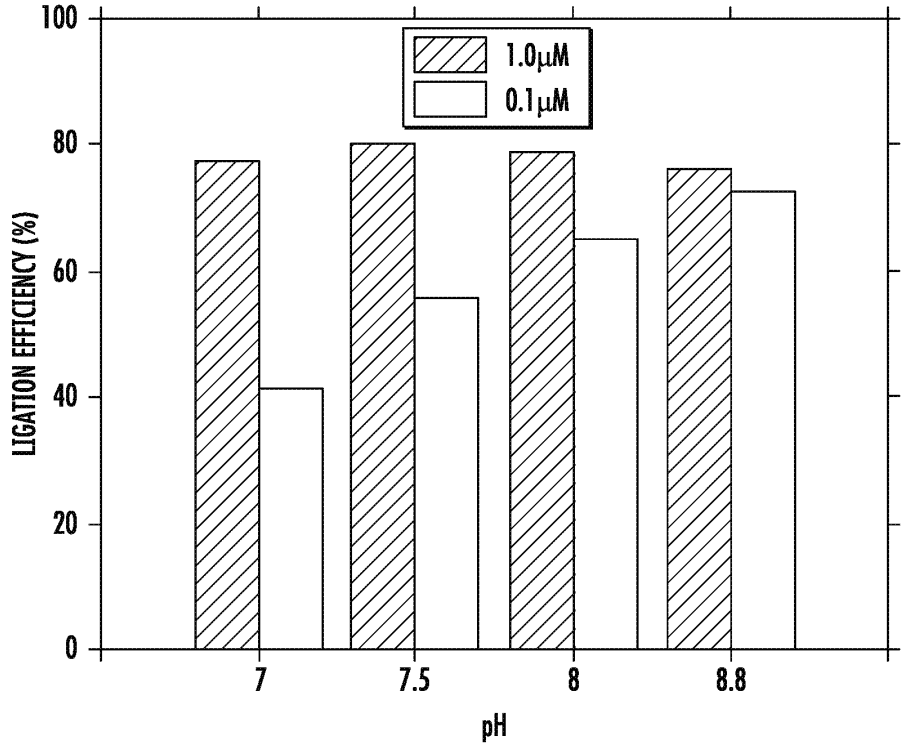


FIG. 10

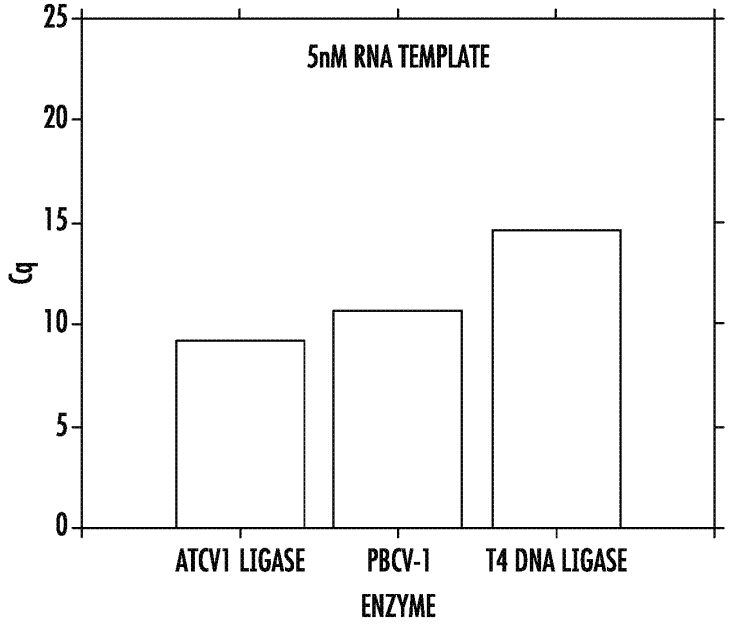


FIG. 11A

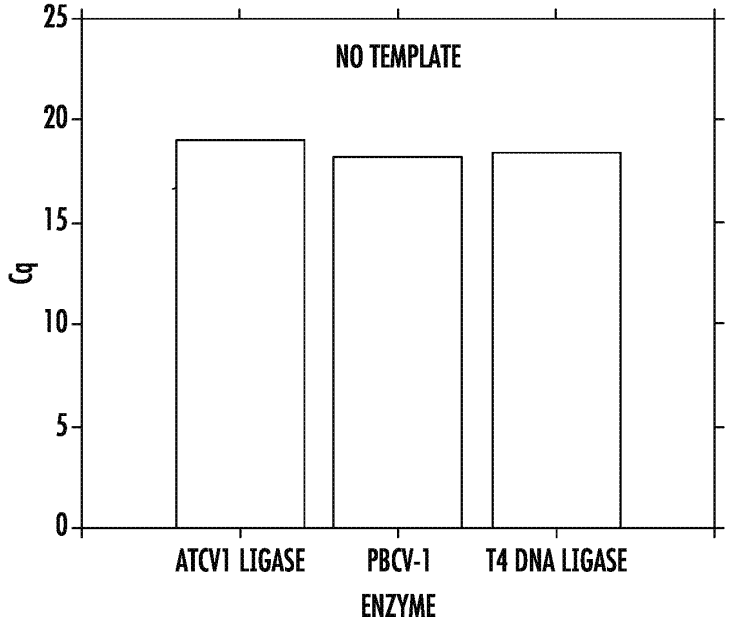


FIG. 11B

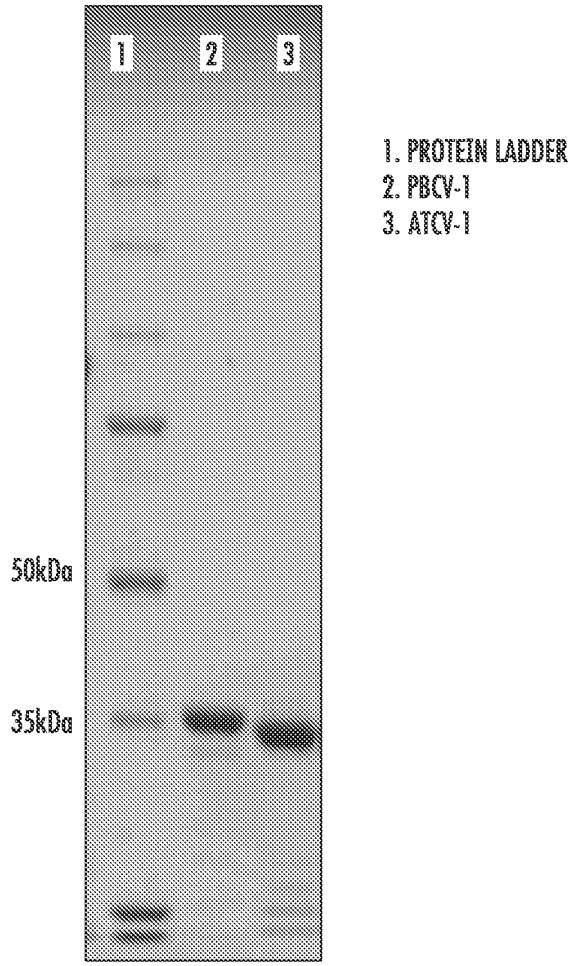


FIG. 12

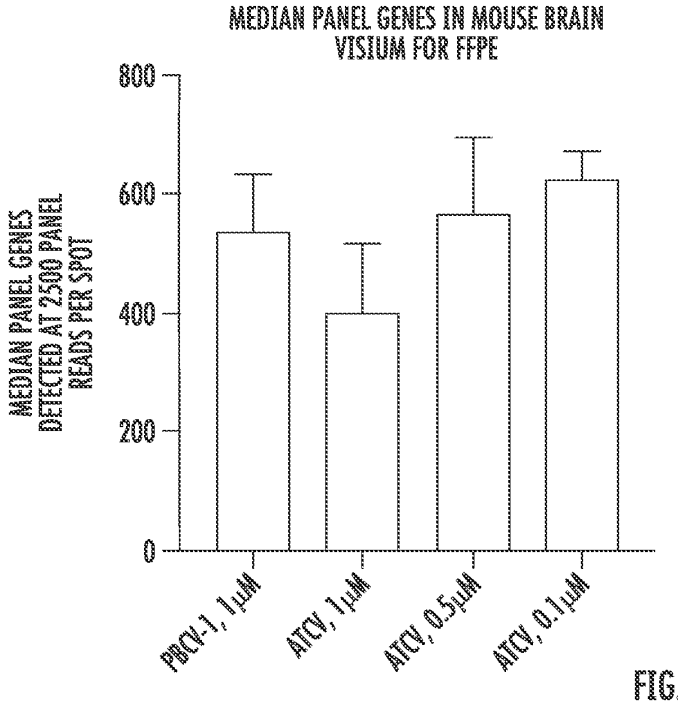


FIG. 13A

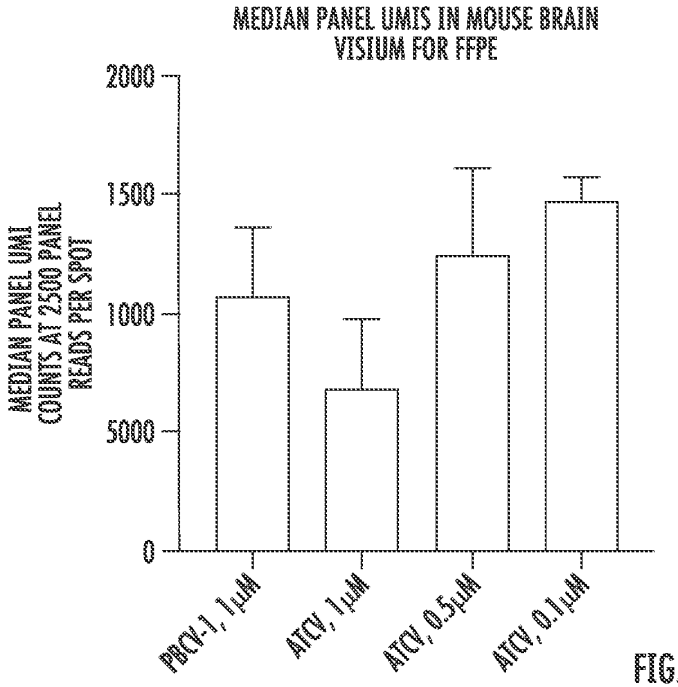


FIG. 13B

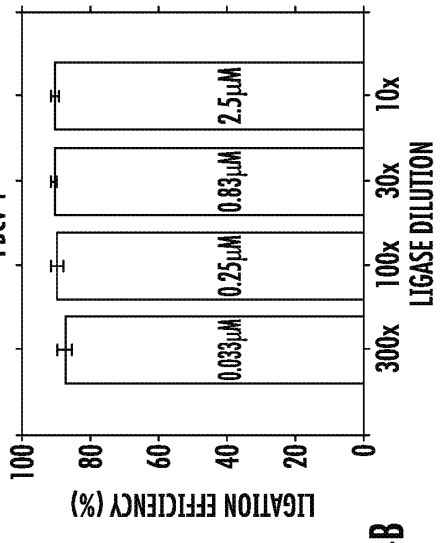


FIG. 14B

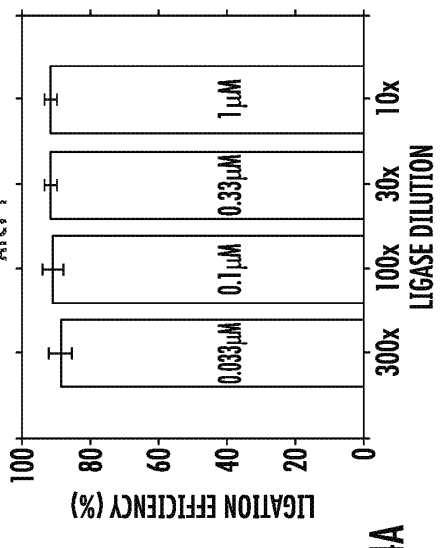


FIG. 14A

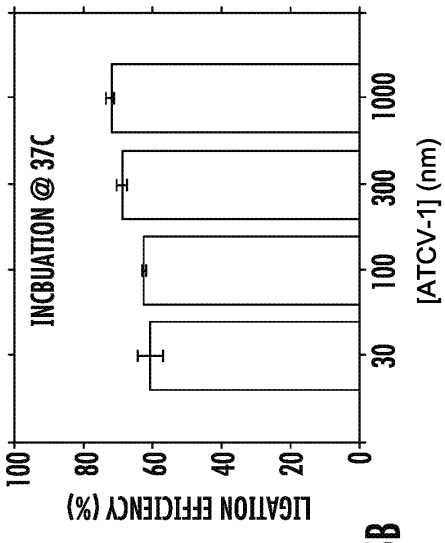


FIG. 15B

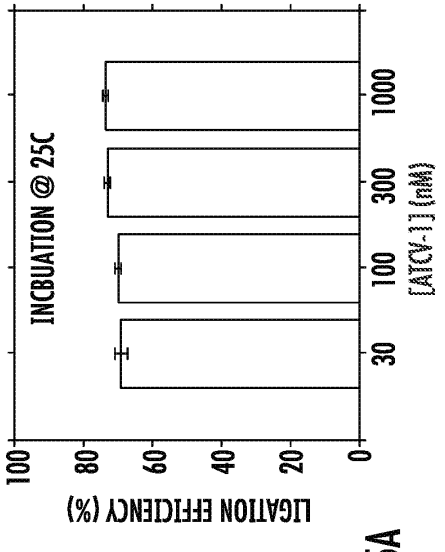


FIG. 15A

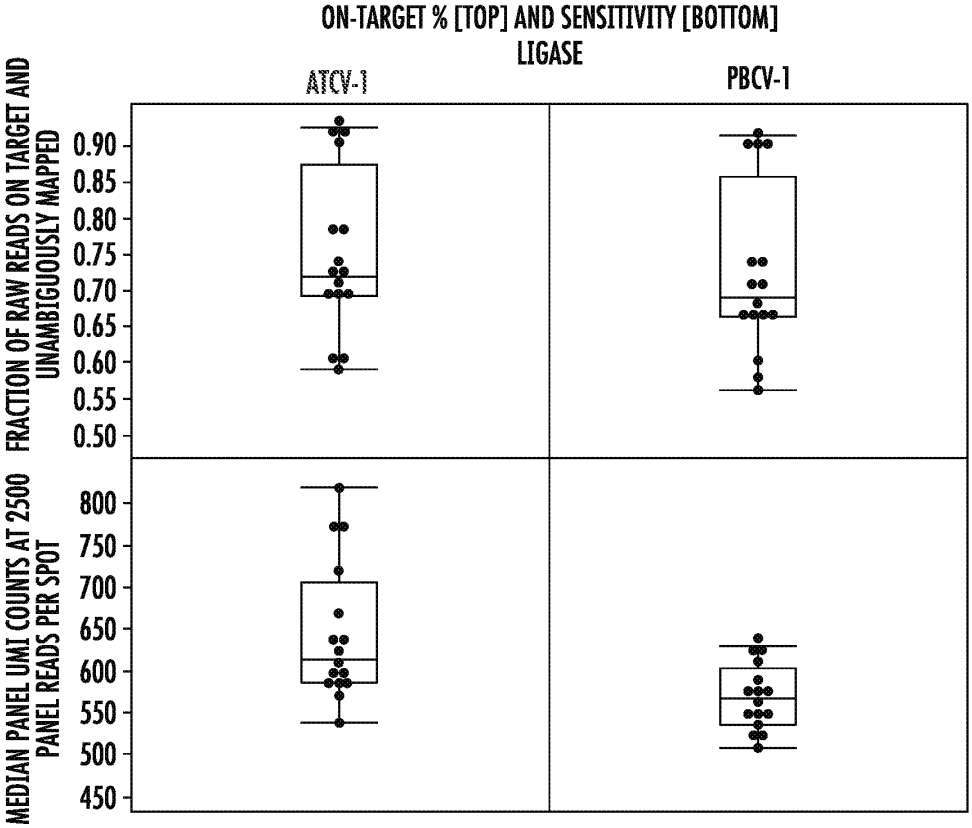

ON-TARGET % [TOP] AND SENSITIVITY [BOTTOM]
LIGASE
ATCV-1
PBCV-1
FRACTION OF RAW READS ON TARGET AND UNAMBIGUOUSLY MAPPED
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
MEDIAN PANEL UMI COUNTS AT 2500 PANEL READS PER SPOT
800
750
700
650
600
550
500
450

FIG. 16

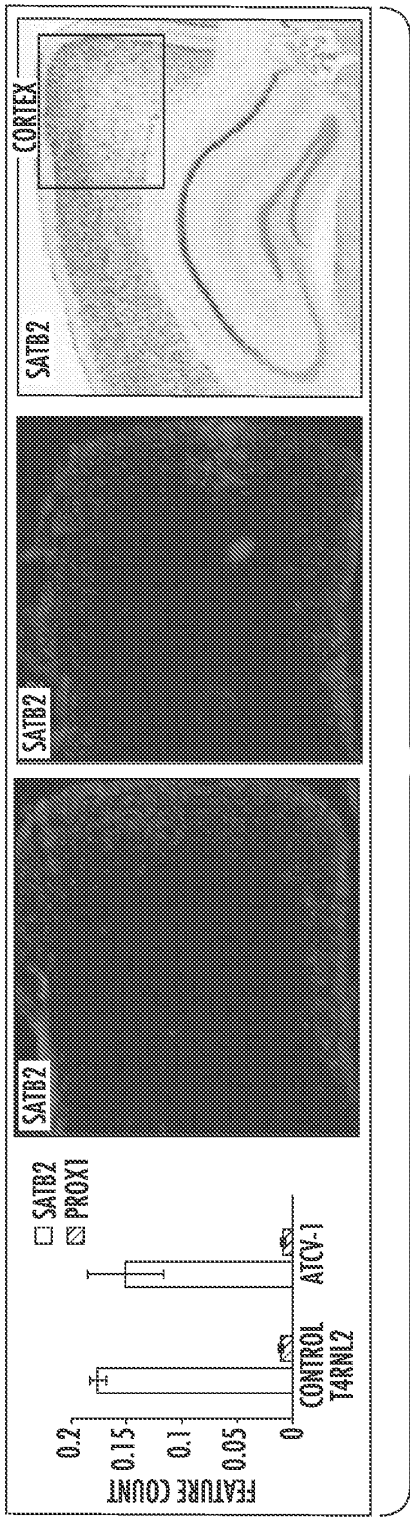


FIG. 17A

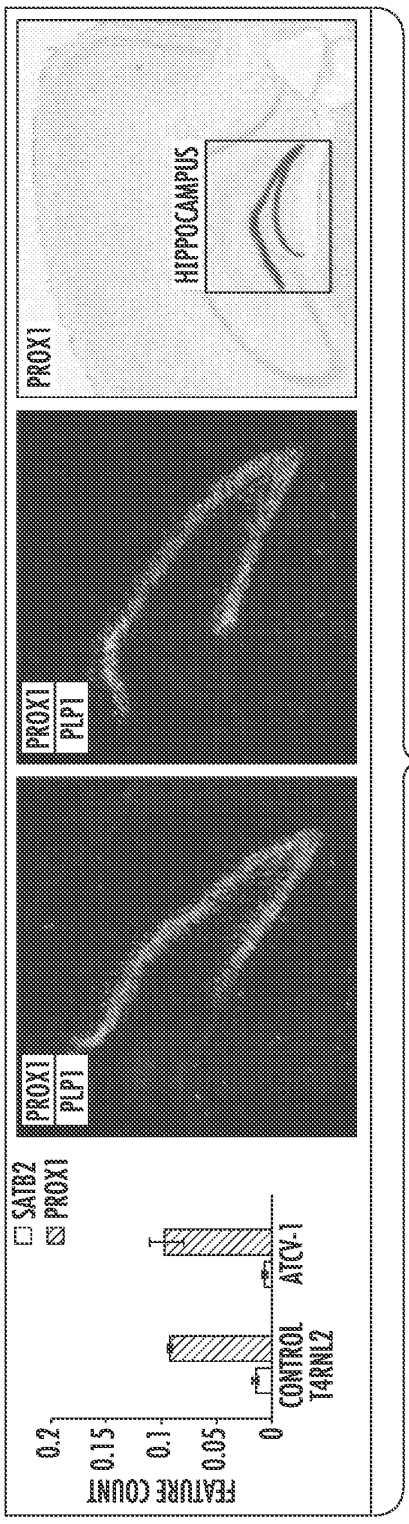


FIG. 17B

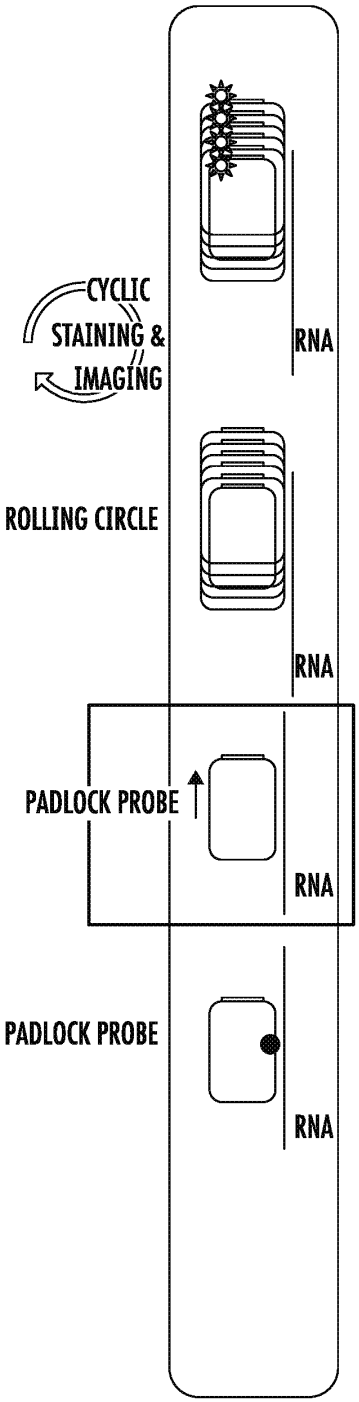

CYCLIC
STAINING &
IMAGING
RNA
ROLLING CIRCLE
RNA
PADLOCK PROBE
RNA
PADLOCK PROBE
RNA

FIG. 18

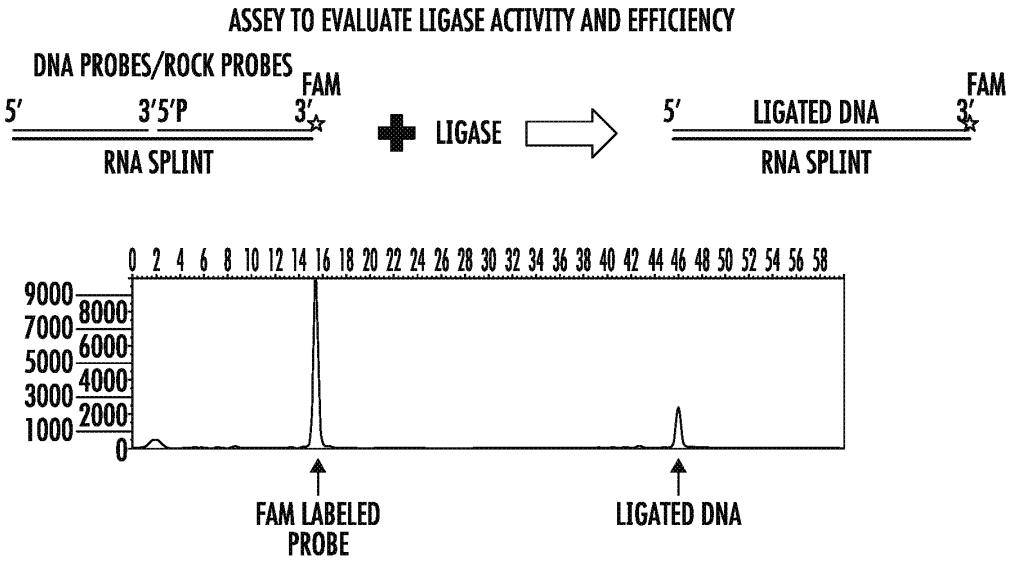

ASSEY TO EVALUATE LIGASE ACTIVITY AND EFFICIENCY
DNA PROBES/ROCK PROBES
FAM
5′
3′5′P
3′
RNA SPLINT
LIGASE
5′
LIGATED DNA
FAM
3′
RNA SPLINT
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
FAM LABELED PROBE
LIGATED DNA

FIG. 19

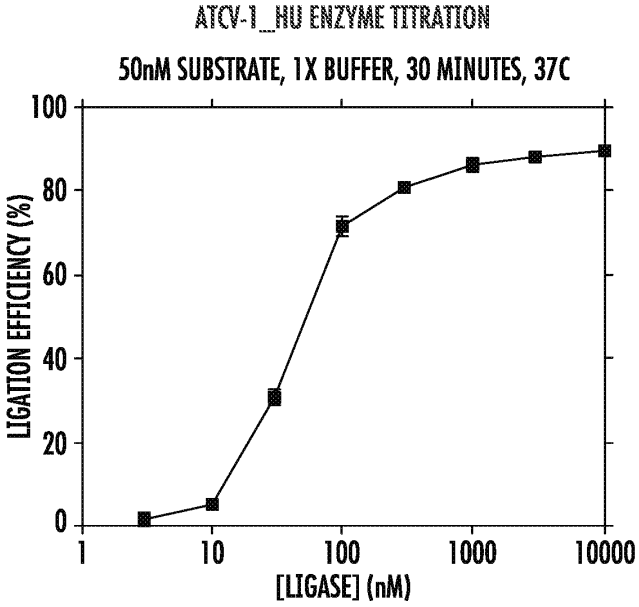

ATCV-1_HU ENZYME TITRATION
50nM SUBSTRATE, 1X BUFFER, 30 MINUTES, 37C
LIGATION EFFICIENCY (%)
100
80
60
40
20
0
1
10
100
1000
10000
[LIGASE] (nM)

FIG. 20

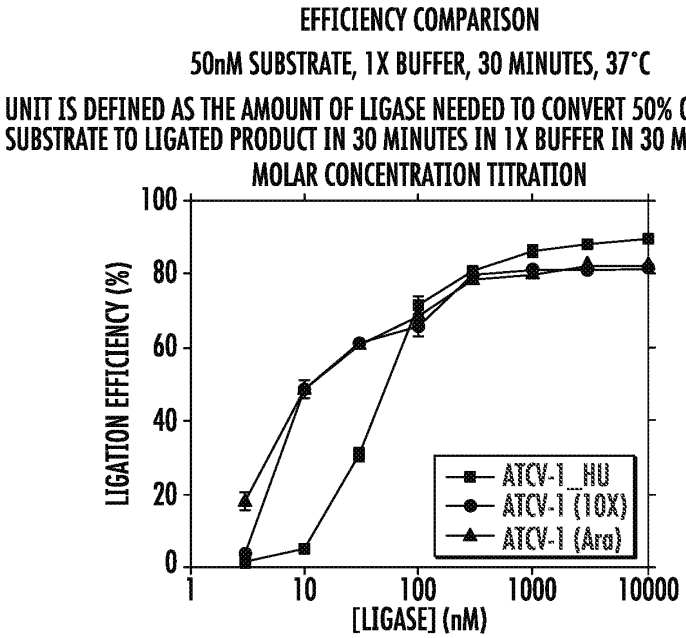

EFFICIENCY COMPARISON
50nM SUBSTRATE, 1X BUFFER, 30 MINUTES, 37°C
UNIT IS DEFINED AS THE AMOUNT OF LIGASE NEEDED TO CONVERT 50% OF A TRIPARTITE SUBSTRATE TO LIGATED PRODUCT IN 30 MINUTES IN 1X BUFFER IN 30 MINUTES AT 37°C
MOLAR CONCENTRATION TITRATION
LIGATION EFFICIENCY (%)
100
80
60
40
20
0
1
10
100
1000
10000
[LIGASE] (nM)
ATCV-1_HU
ATCV-1 (10X)
ATCV-1 (Ara)

FIG. 21

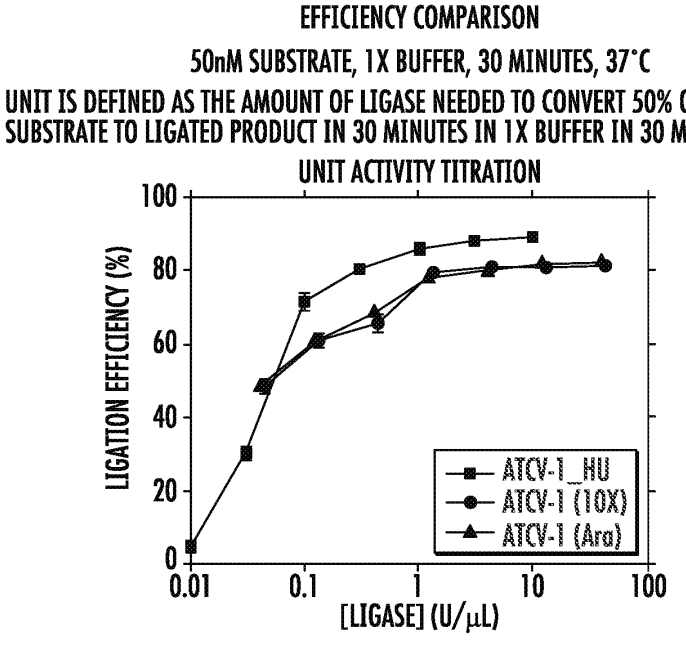

EFFICIENCY COMPARISON
50nM SUBSTRATE, 1X BUFFER, 30 MINUTES, 37°C
UNIT IS DEFINED AS THE AMOUNT OF LIGASE NEEDED TO CONVERT 50% OF A TRIPARTITE SUBSTRATE TO LIGATED PRODUCT IN 30 MINUTES IN 1X BUFFER IN 30 MINUTES AT 37°C
UNIT ACTIVITY TITRATION
LIGATION EFFICIENCY (%)
100
80
60
40
20
0
0.01
0.1
1
10
100
[LIGASE] (U/μL)
ATCV-1_HU
ATCV-1 (10X)
ATCV-1 (Ara)

FIG. 22

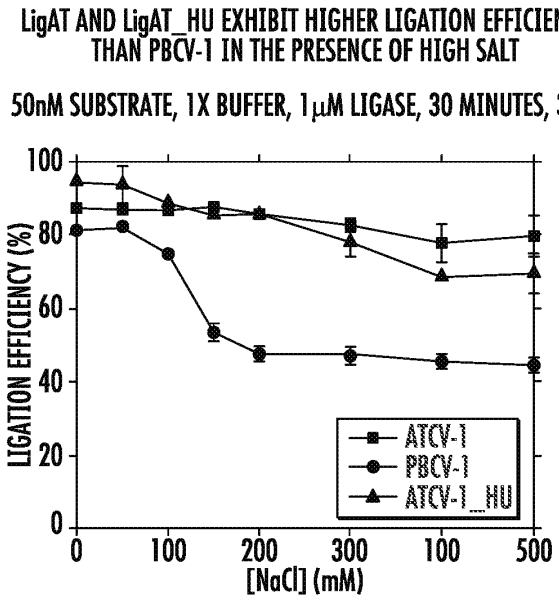

LigAT AND LigAT_HU EXHIBIT HIGHER LIGATION EFFICIENCY THAN PBCV-1 IN THE PRESENCE OF HIGH SALT
50nM SUBSTRATE, 1X BUFFER, 1μM LIGASE, 30 MINUTES, 37°C
LIGATION EFFICIENCY (%)
100
80
60
40
20
0
0
100
200
300
100
500
[NaCl] (mM)
ATCV-1
PBCV-1
ATCV-1_HU

FIG. 23

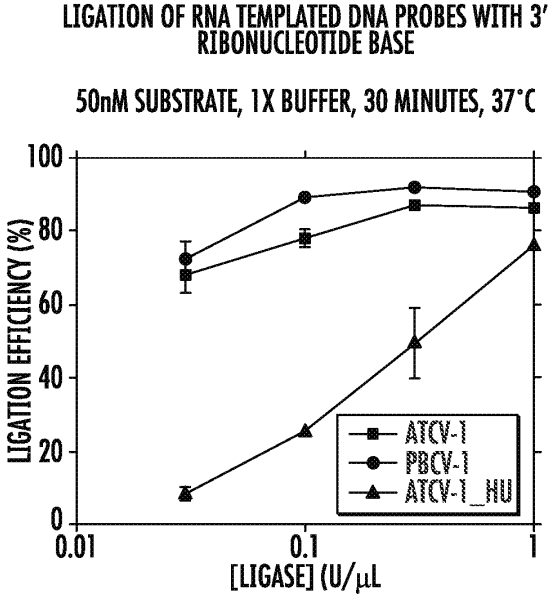

LIGATION OF RNA TEMPLATED DNA PROBES WITH 3' RIBONUCLEOTIDE BASE
50nM SUBSTRATE, 1X BUFFER, 30 MINUTES, 37°C
LIGATION EFFICIENCY (%)
100
80
60
40
20
0
0.01
0.1
1
[LIGASE] (U/μL
ATCV-1
PBCV-1
ATCV-1_HU

FIG. 24

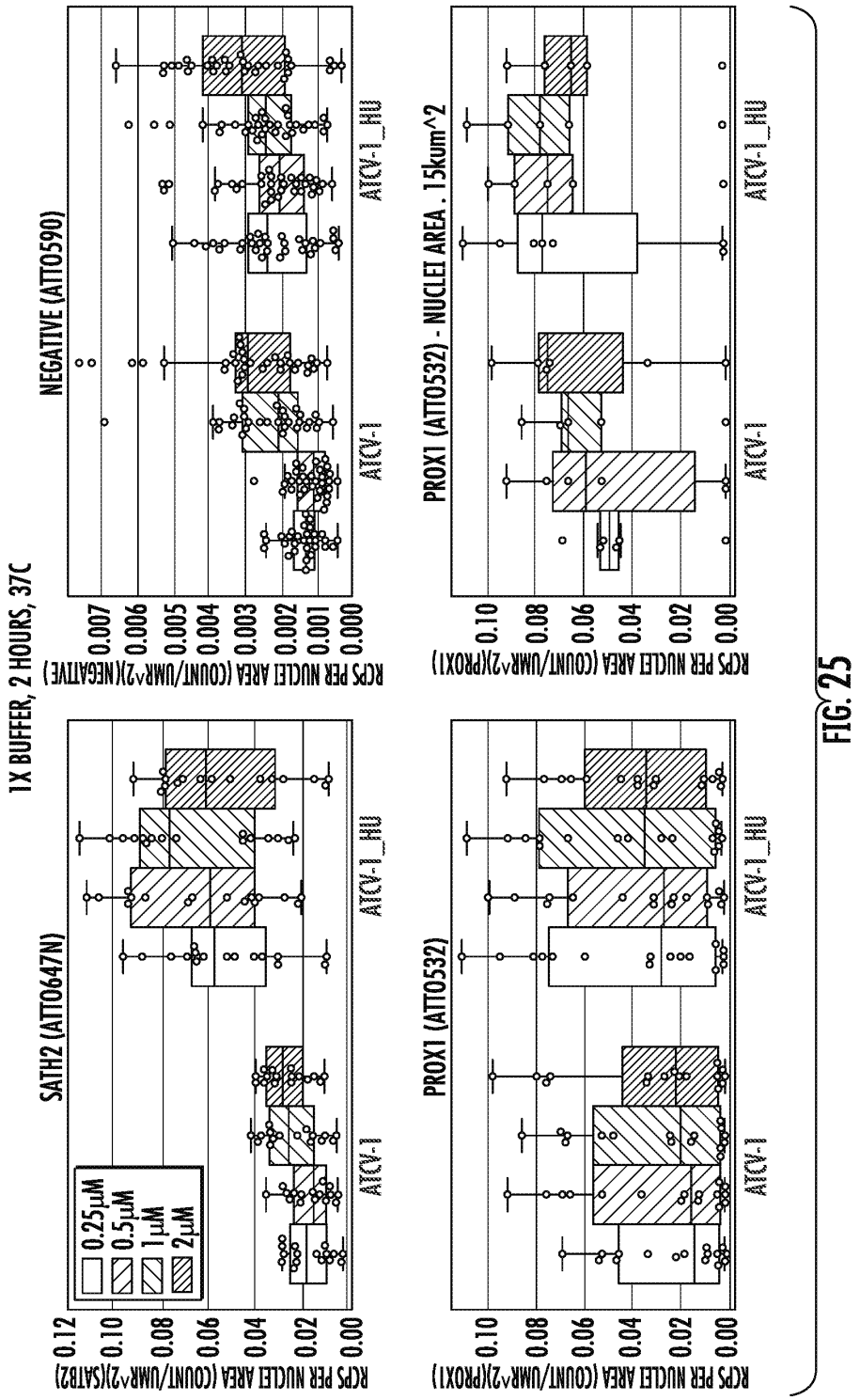
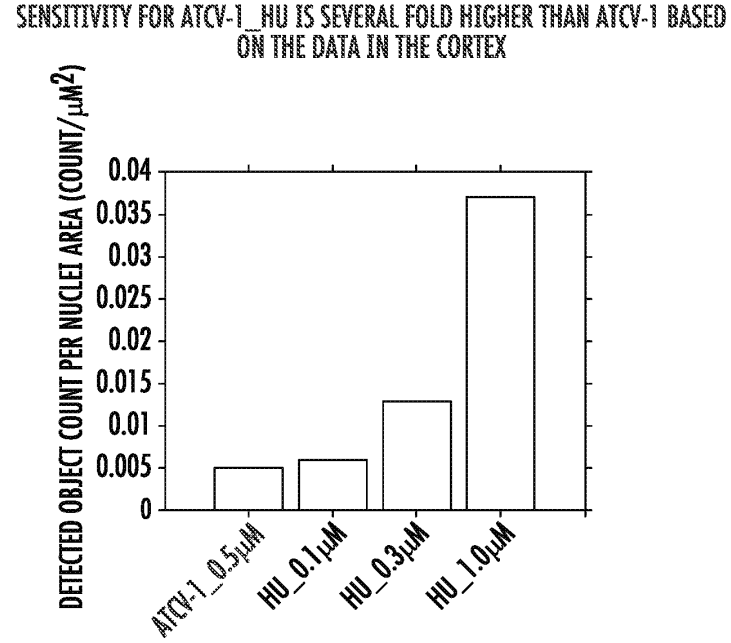

1X BUFFER, 2 HOURS, 37C
SATH2 (ATTO647N)
PROX1 (ATTO532)
NEGATIVE (ATTO590)
PROX1 (ATTO532) - NUCLEI AREA . 15kum^2
0.25µM
0.5µM
1 µM
2µM
ATCV-1
ATCV-1_HU
RCPS PER NUCLEI AREA (COUNT/UMR^2)(SATB2)
RCPS PER NUCLEI AREA (COUNT/UMR^2)(PROX1)
RCPS PER NUCLEI AREA (COUNT/UMR^2)(NEGATIVE)
RCPS PER NUCLEI AREA (COUNT/UMR^2)(PROX1)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0.000

FIG. 25



ATCV-1_HU @ 1.0μM HAS 8X HIGHER SENSITIVITY THAN LigAT @ 0.5μM.
ATCV-1_HU @ 0.3μM HAS 2.5X HIGHER SENSITIVITY THAN LigAT @ 0.5μM.

FIG. 26

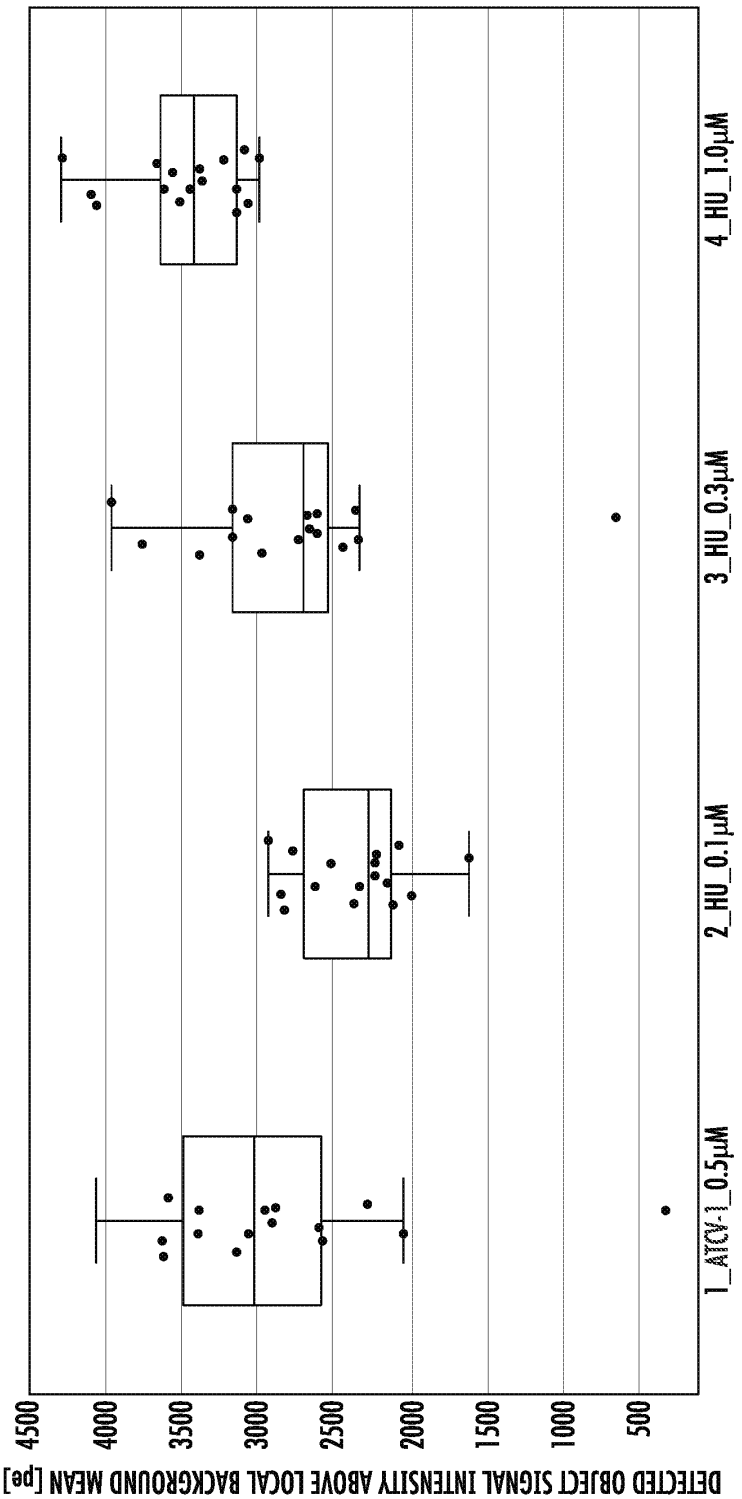

DETECTED OBJECT SIGNAL INTENSITY ABOVE LOCAL BACKGROUND MEAN [pe]
4500
4000
3500
3000
2500
2000
1500
1000
500
1_ATCV-1_0.5µM
2_HU_0.1µM
3_HU_0.3µM
4_HU_1.0µM

FIG. 27

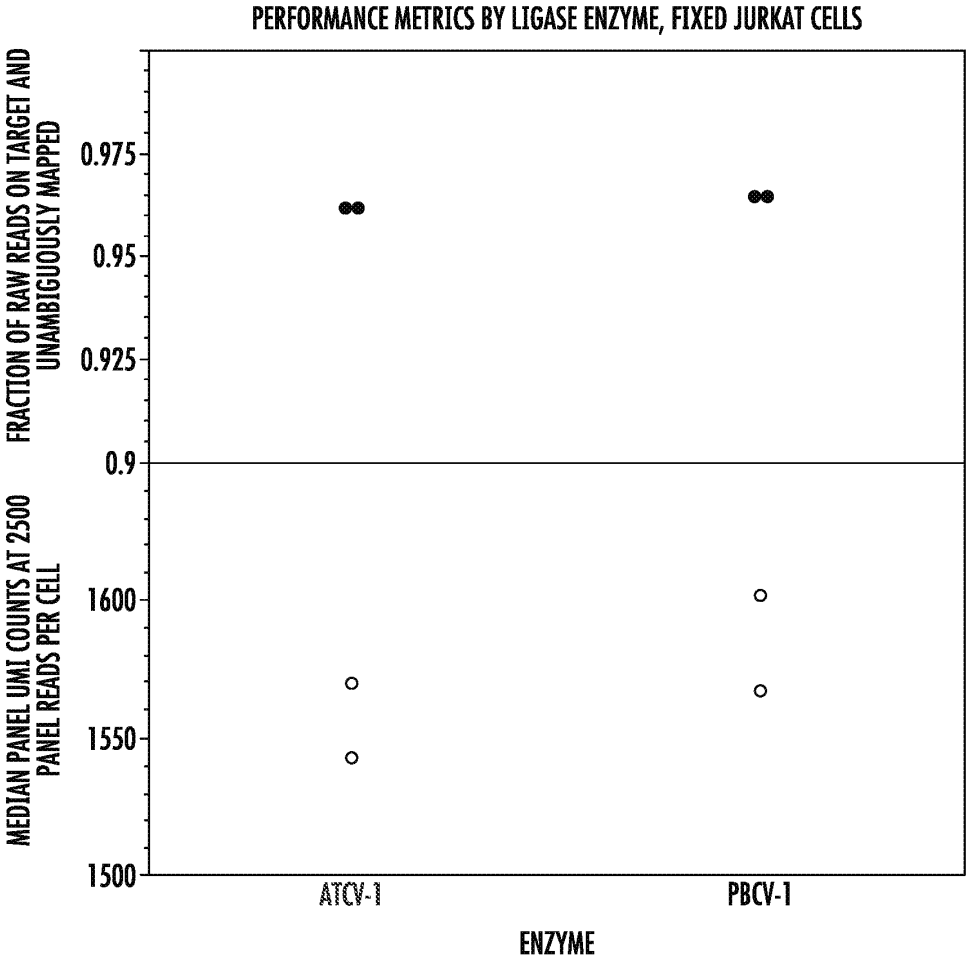

PERFORMANCE METRICS BY LIGASE ENZYME, FIXED JURKAT CELLS
FRACTION OF RAW READS ON TARGET AND UNAMBIGUOUSLY MAPPED
0.975
0.95
0.925
0.9
MEDIAN PANEL UMI COUNTS AT 2500 PANEL READS PER CELL
1600
1550
1500
ATCV-1
PBCV-1
ENZYME

FIG. 28

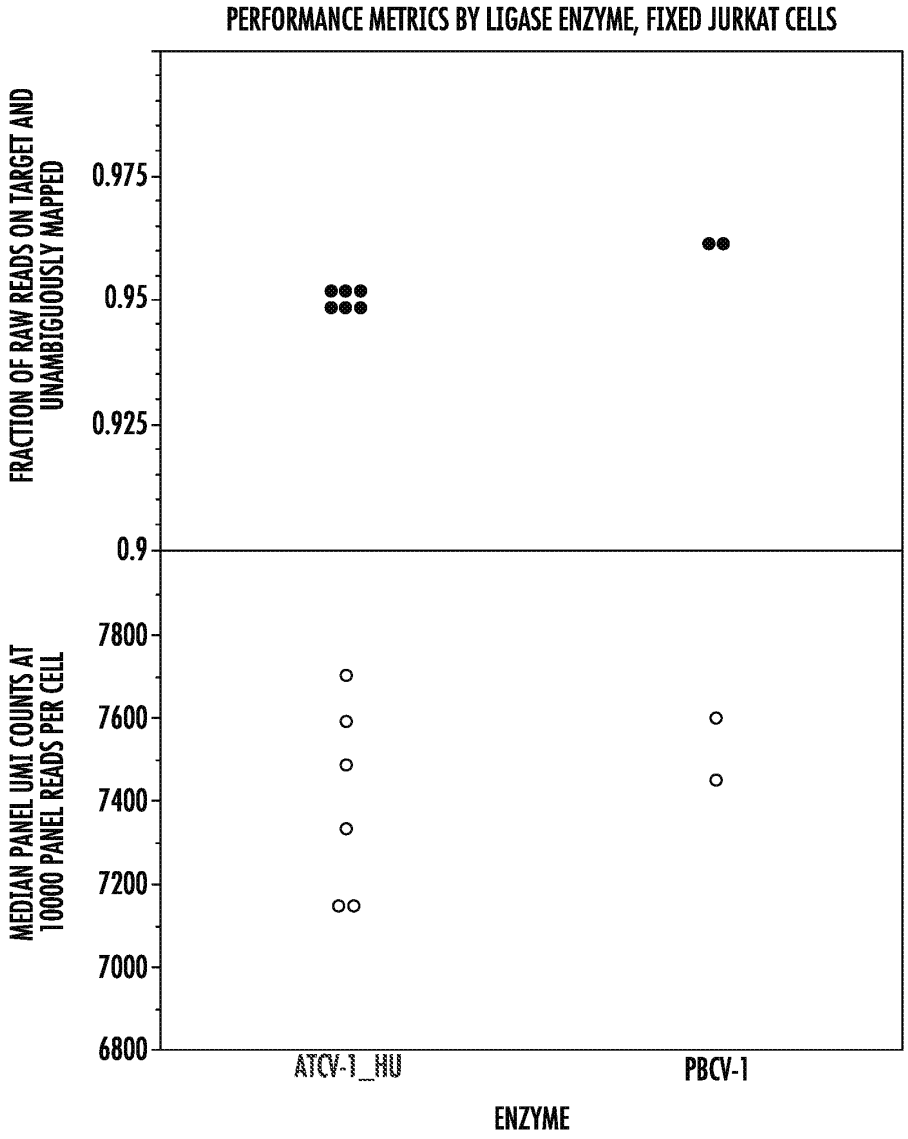

PERFORMANCE METRICS BY LIGASE ENZYME, FIXED JURKAT CELLS
FRACTION OF RAW READS ON TARGET AND UNAMBIGUOUSLY MAPPED
0.975
0.95
0.925
0.9
MEDIAN PANEL UMI COUNTS AT 10000 PANEL READS PER CELL
7800
7600
7400
7200
7000
6800
ATCV-1_HU
PBCV-1
ENZYME

FIG. 29

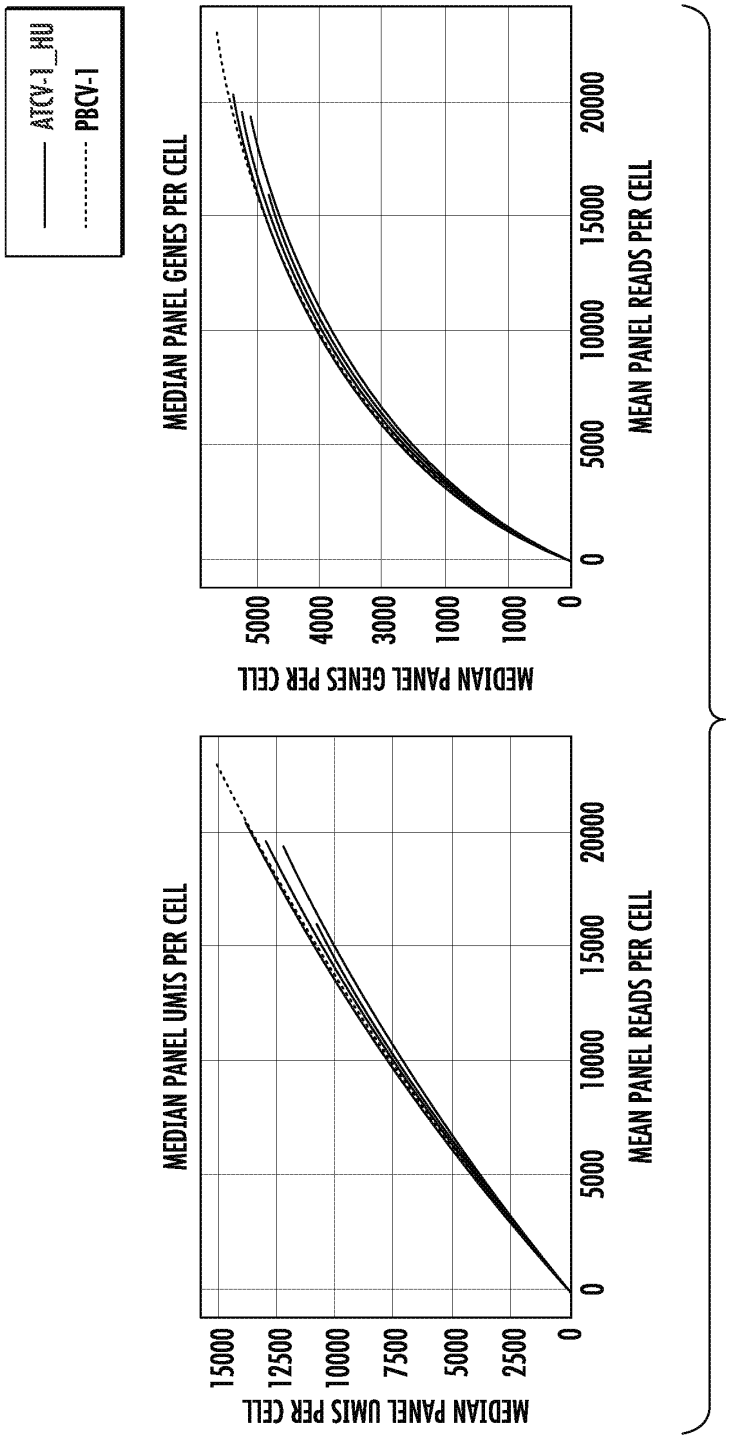

ATCV-1_HU
PBCV-1
MEDIAN PANEL GENES PER CELL
MEAN PANEL READS PER CELL
MEDIAN PANEL UMIS PER CELL
MEAN PANEL READS PER CELL

FIG. 30

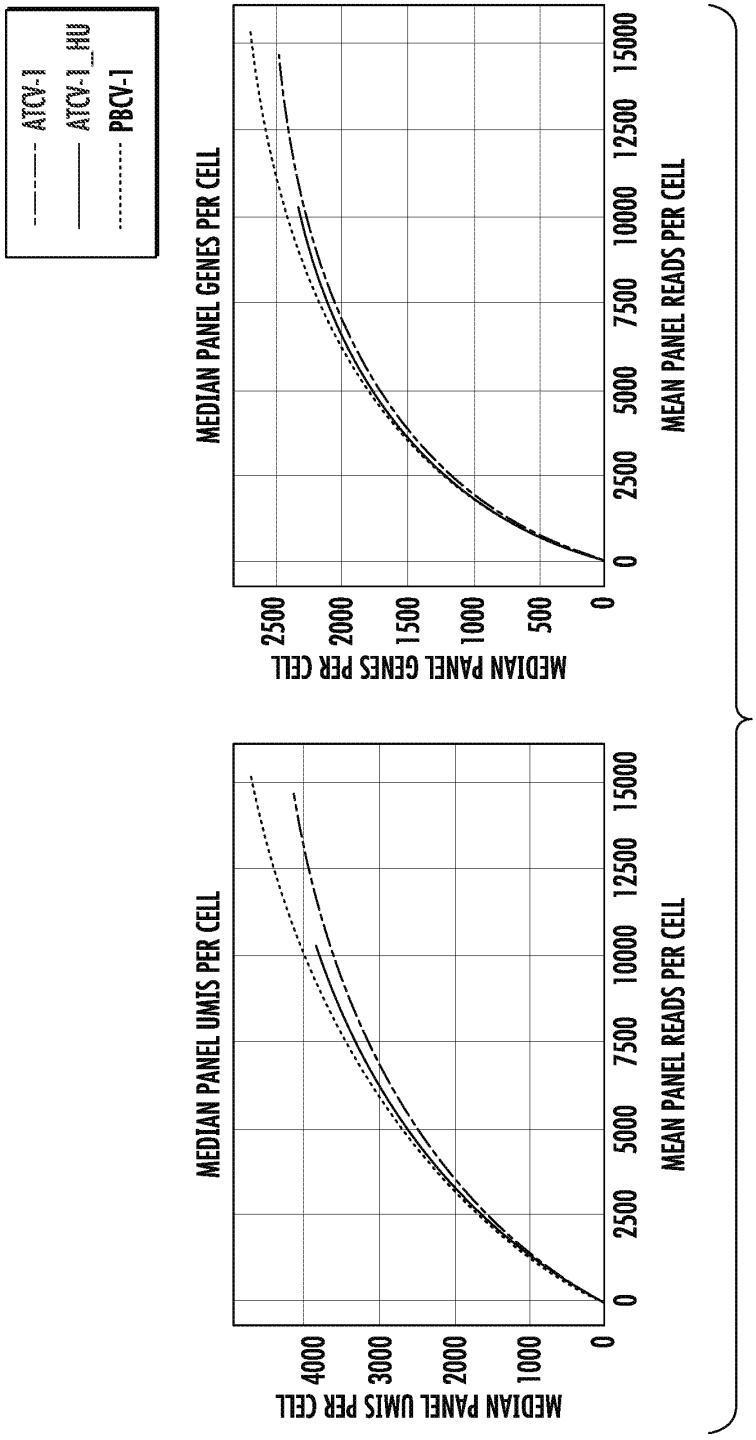

ATCV-1
ATCV-1_HIU
PBCV-1
MEDIAN PANEL GENES PER CELL
0
500
1000
1500
2000
2500
0
2500
5000
7500
10000
12500
15000
MEAN PANEL READS PER CELL
MEDIAN PANEL UMIS PER CELL
0
1000
2000
3000
4000
0
2500
5000
7500
10000
12500
15000
MEAN PANEL READS PER CELL

FIG. 31

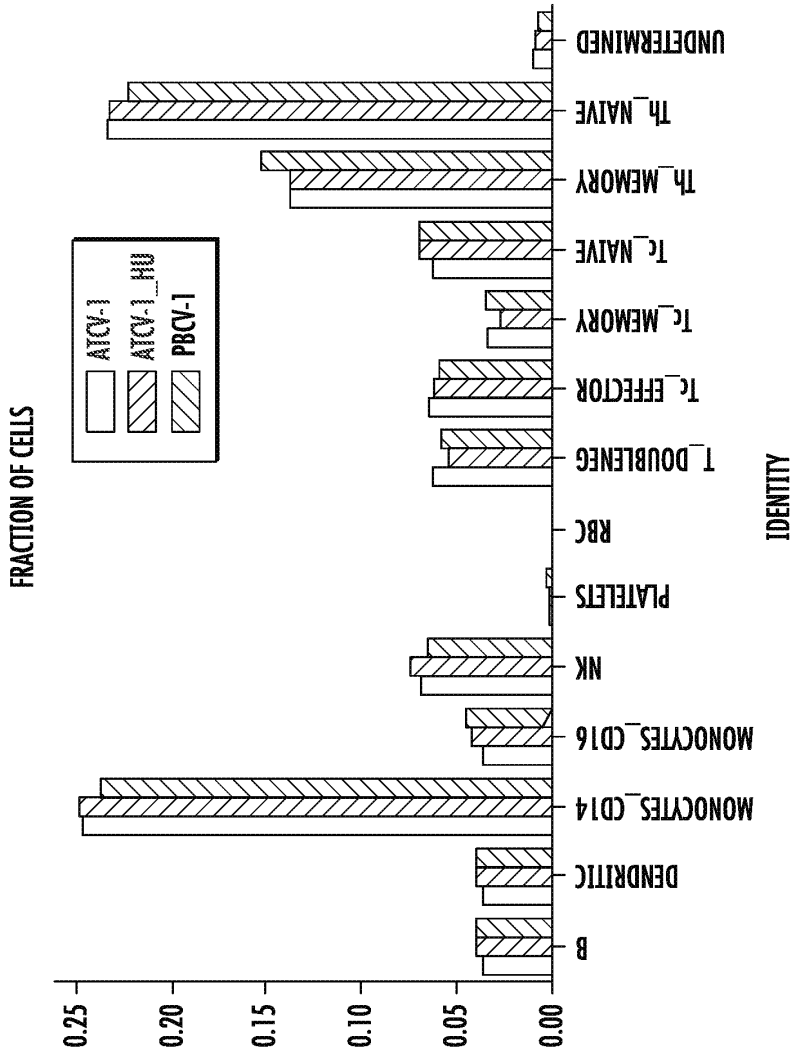

FRACTION OF CELLS
0.25
0.20
0.15
0.10
0.05
0.00
ATCV-1
ATCV-1_HIB
PBCV-1
B
DENDRITIC
MONOCYTES_CD14
MONOCYTES_CD16
NK
PLATELETS
RBC
T_DOUBLENEG
Tc_EFFECTOR
Tc_MEMORY
Tc_NAIVE
Th_MEMORY
Th_NAIVE
UNDETERMINED
IDENTITY

FIG. 32

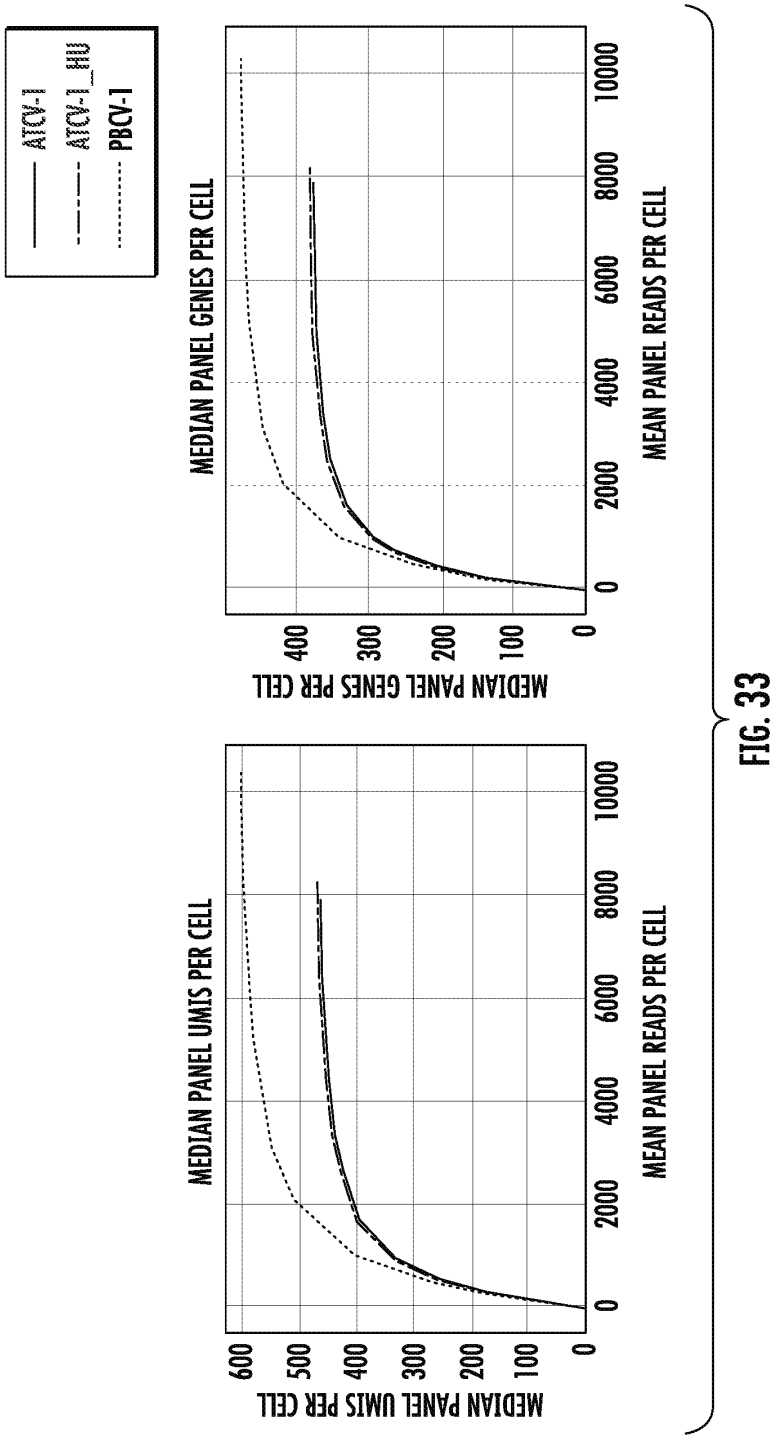

ATCV-1
ATCV-1_HU
PBCV-1
MEDIAN PANEL GENES PER CELL
MEDIAN PANEL GENES PER CELL
0
100
200
300
400
0
2000
4000
6000
8000
10000
MEAN PANEL READS PER CELL
MEDIAN PANEL UMIS PER CELL
MEDIAN PANEL UMIS PER CELL
0
100
200
300
400
500
600
0
2000
4000
6000
8000
10000
MEAN PANEL READS PER CELL

FIG. 33

	5'	3'	5'	3'
CTL SEQ ID NO:14	/56-FAM/TACTAGGCGACTCGACGGTCTTAC		/5Phos/TCGTTACGTCCGACTATAGAGCTTAG	
3PMM1 SEQ ID NO:15	/56-FAM/TACTAGGCGACTCGACGGTCTTAA		/5Phos/TCGTTACGTCCGACTATAGAGCTTAG	
3PMM2 SEQ ID NO:16	/56-FAM/TACTAGGCGACTCGACGGTCTTCC		/5Phos/TCGTTACGTCCGACTATAGAGCTTAG	
3PMM1 SEQ ID NO:17	/56-FAM/TACTAGGCGACTCGACGGTCTAAC		/5Phos/TCGTTACGTCCGACTATAGAGCTTAG	
5PMM1 SEQ ID NO:18	/56-FAM/TACTAGGCGACTCGACGGTCTTAC		/5Phos/GCGTTACGTCCGACTATAGAGCTTAG	
5PMM2 SEQ ID NO:19	/56-FAM/TACTAGGCGACTCGACGGTCTTAC		/5Phos/TAGTTACGTCCGACTATAGAGCTTAG	
5PMM3 SEQ ID NO:20	/56-FAM/TACTAGGCGACTCGACGGTCTTAC		/5Phos/TCGTTACGTCCGACTATAGAGCTTAG	

FIG. 34A

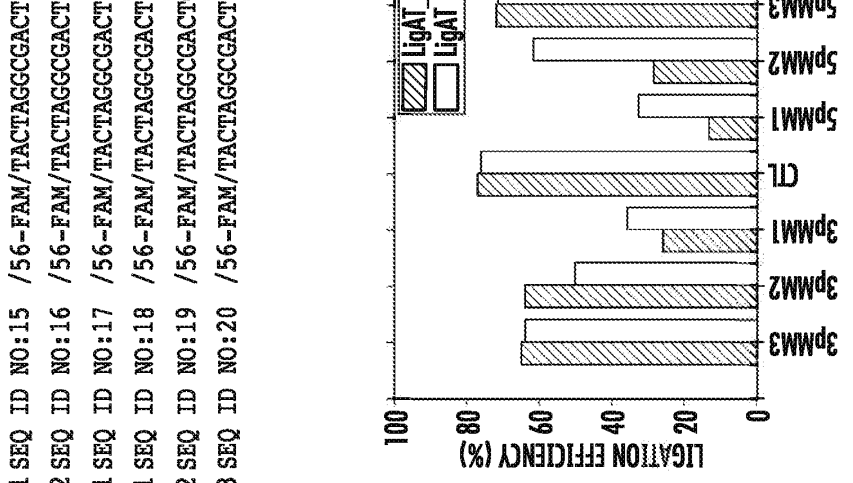


FIG. 34B

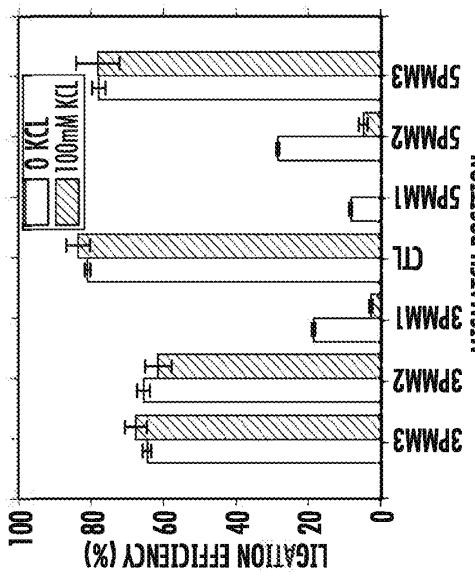


FIG. 34C

```
TEMPLATE. SEQ ID NO:21   ATGATCCGCTGAGCTGCCAGAATG----------AGCAATGCAGGCTGATATCTCGAATC
5CTL SEQ ID NO:22        /56-FAM/TACTAGGCGACTCGACGGTCTTAC  /5Phos/TCGTTACGTCCGACTATAGAGCTTAG
5PMM1_A SEQ ID NO:23     /56-FAM/TACTAGGCGACTCGACGGTCTTAC  /5Phos/ACGTTACGTCCGACTATAGAGCTTAG
5PMM1_C SEQ ID NO:24     /56-FAM/TACTAGGCGACTCGACGGTCTTAC  /5Phos/CCGTTACGTCCGACTATAGAGCTTAG
5PMM1_G SEQ ID NO:25     /56-FAM/TACTAGGCGACTCGACGGTCTTAC  /5Phos/GCGTTACGTCCGACTATAGAGCTTAG
```

FIG. 35A

FIG. 35B