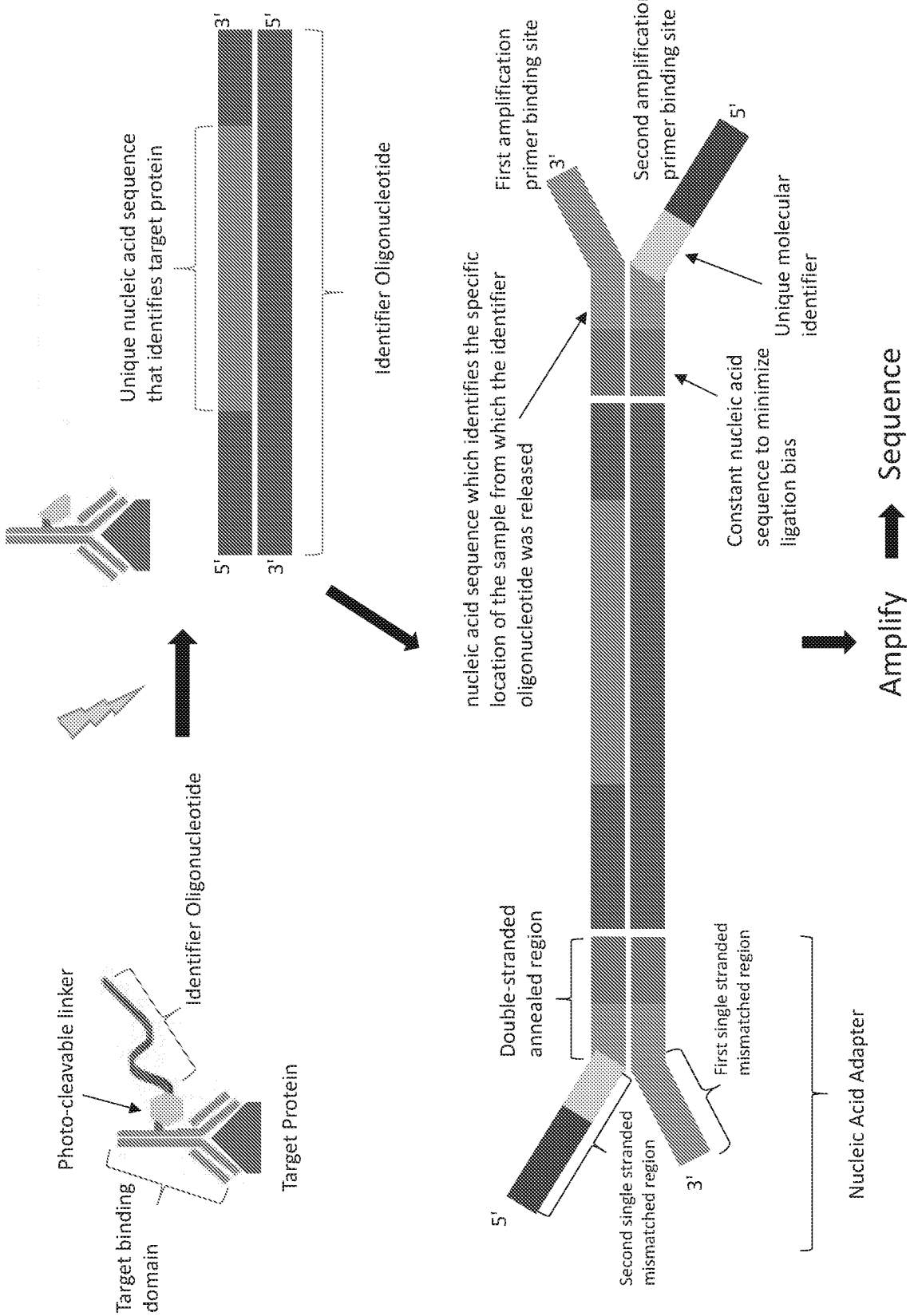


Photo-cleavable linker
Identifier Oligonucleotide
Target binding domain
Target Protein
Unique nucleic acid sequence that identifies target protein
Identifier Oligonucleotide
5'
3'
3'
5'
nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released
First amplification primer binding site
Second amplification primer binding site
Constant nucleic acid sequence to minimize ligation bias
Unique molecular identifier
Double-stranded annealed region
First single stranded mismatched region
Second single stranded mismatched region
Nucleic Acid Adapter
Amplify
Sequence

Figure 1

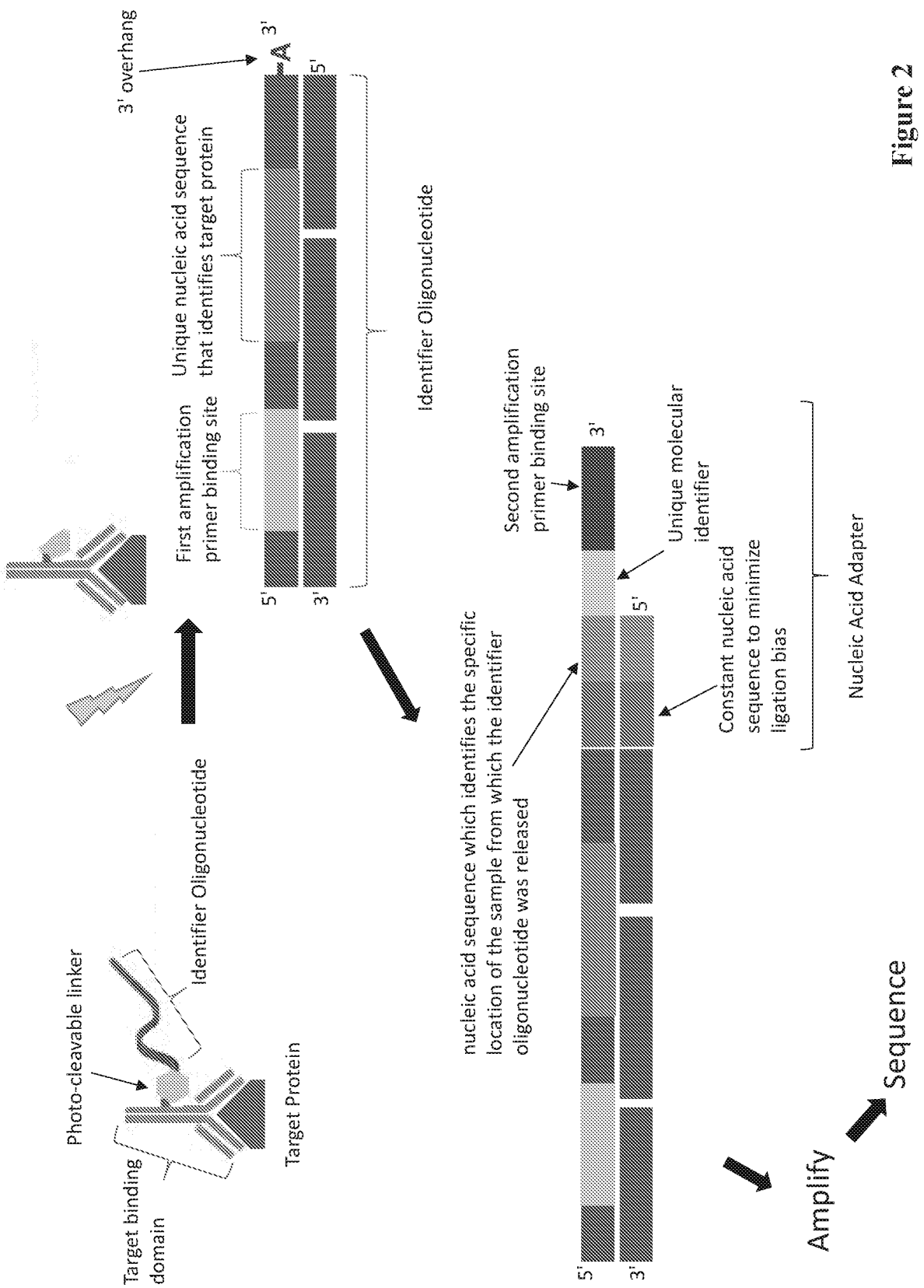

Target binding domain
Photo-cleavable linker
Identifier Oligonucleotide
Target Protein
First amplification primer binding site
Unique nucleic acid sequence that identifies target protein
3' overhang
A
3'
5'
5'
3'
Identifier Oligonucleotide
nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released
Second amplification primer binding site
3'
Unique molecular identifier
5'
Constant nucleic acid sequence to minimize ligation bias
Nucleic Acid Adapter
5'
3'
Amplify
Sequence

Figure 2

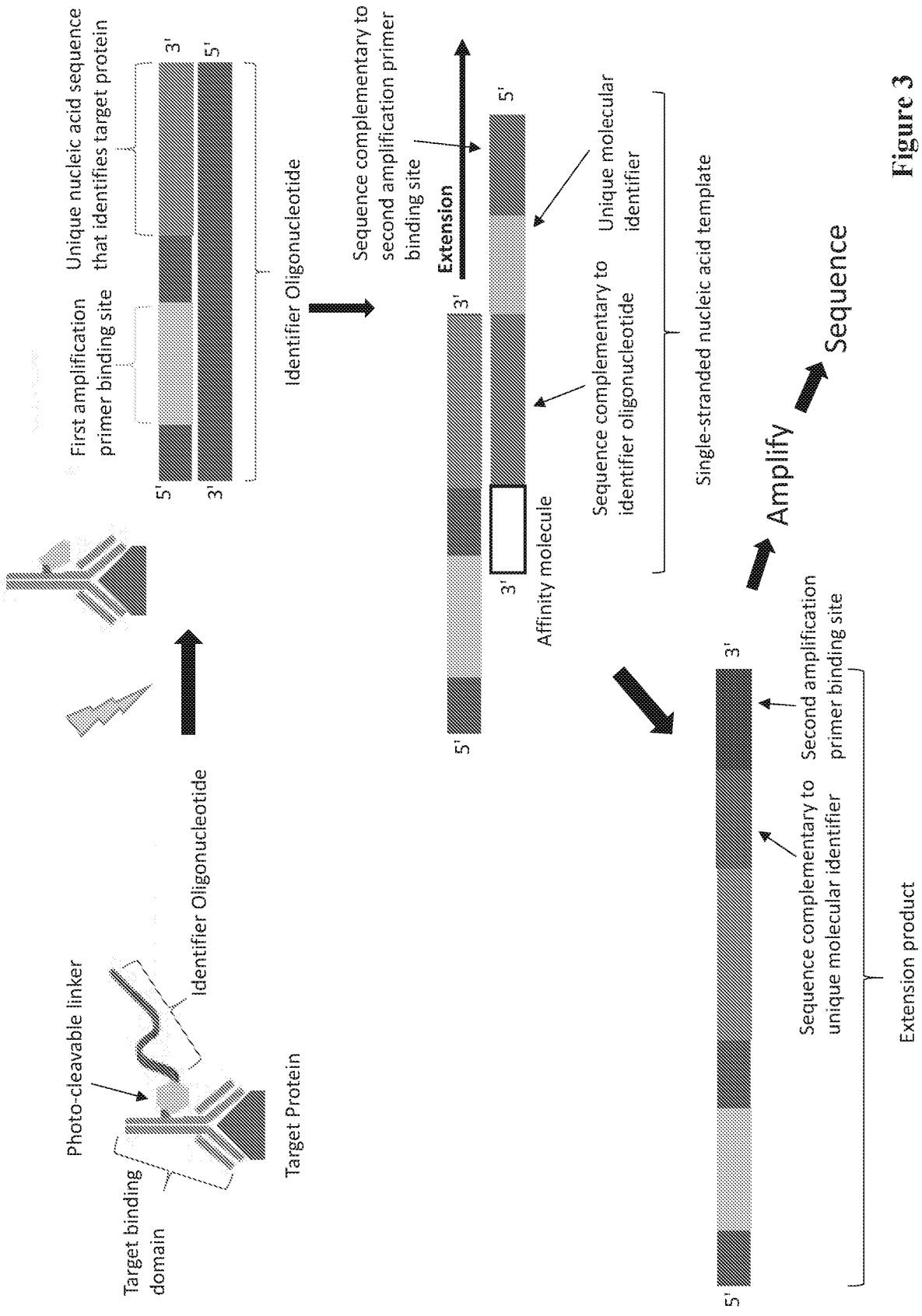


Photo-cleavable linker
Identifier Oligonucleotide
Target binding domain
Target Protein
Identifier Oligonucleotide
First amplification primer binding site
Unique nucleic acid sequence that identifies target protein
5'
3'
3'
5'
Sequence complementary to second amplification primer binding site
Extension
3'
5'
Affinity molecule
Sequence complementary to identifier oligonucleotide
Unique molecular identifier
Single-stranded nucleic acid template
Amplify
Sequence
5'
3'
Sequence complementary to unique molecular identifier
Second amplification primer binding site
Extension product

Figure 3

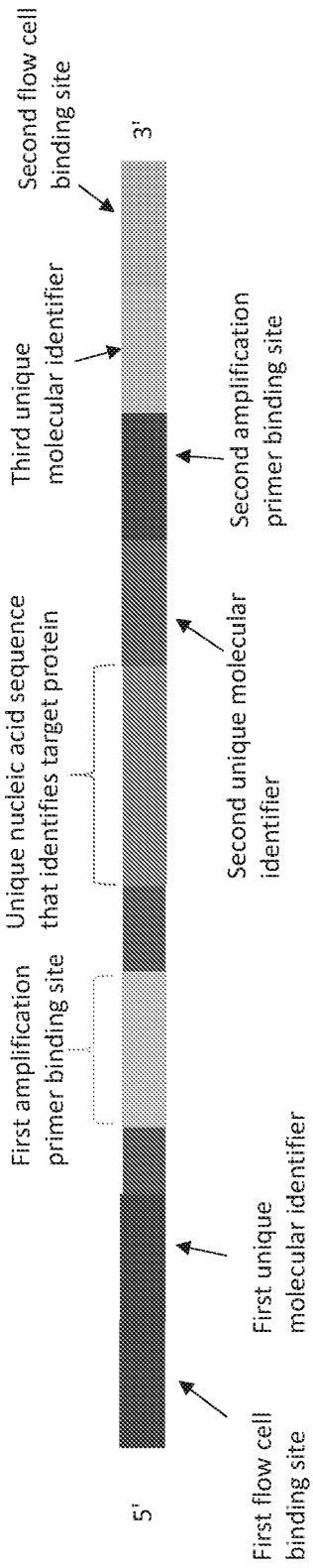

5'
First flow cell binding site
First unique molecular identifier
First amplification primer binding site
Unique nucleic acid sequence that identifies target protein
Second unique molecular identifier
Third unique molecular identifier
Second amplification primer binding site
Second flow cell binding site
3'

Figure 4

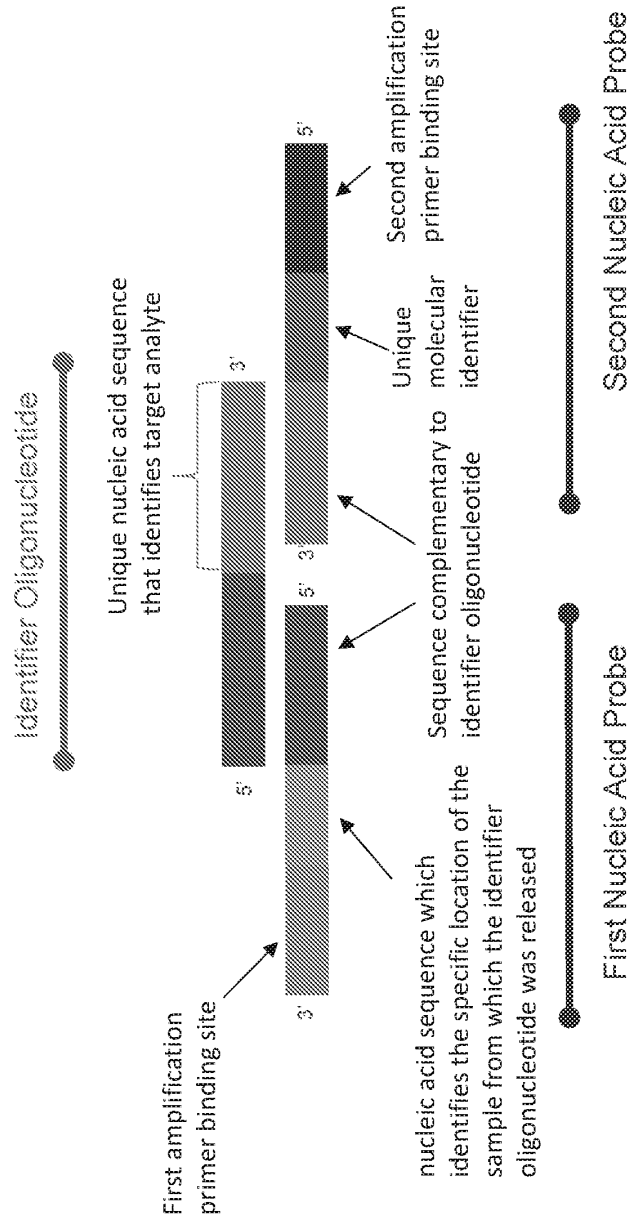

Identifier Oligonucleotide
Unique nucleic acid sequence that identifies target analyte
3'
5'
5'
3'
3'
5'
First amplification primer binding site
nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released
Sequence complementary to identifier oligonucleotide
Unique molecular identifier
Second amplification primer binding site
First Nucleic Acid Probe
Second Nucleic Acid Probe

Figure 5

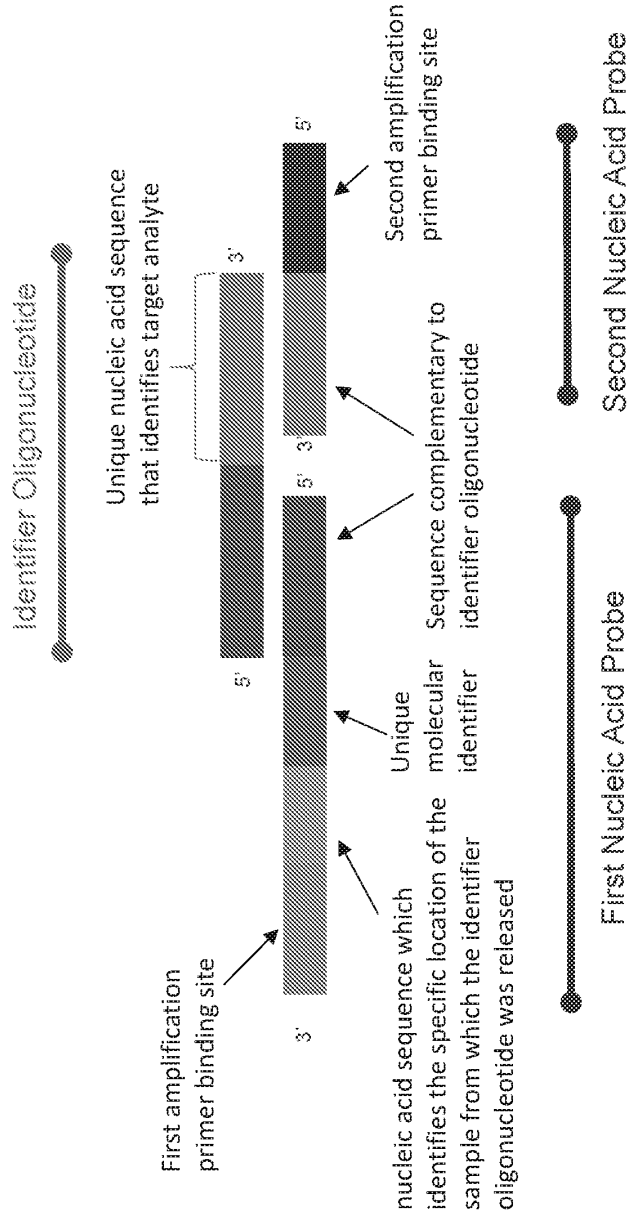

Identifier Oligonucleotide
Unique nucleic acid sequence that identifies target analyte
First amplification primer binding site
nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released
Unique molecular identifier
Sequence complementary to identifier oligonucleotide
Second amplification primer binding site
First Nucleic Acid Probe
Second Nucleic Acid Probe
5'
3'

Figure 6

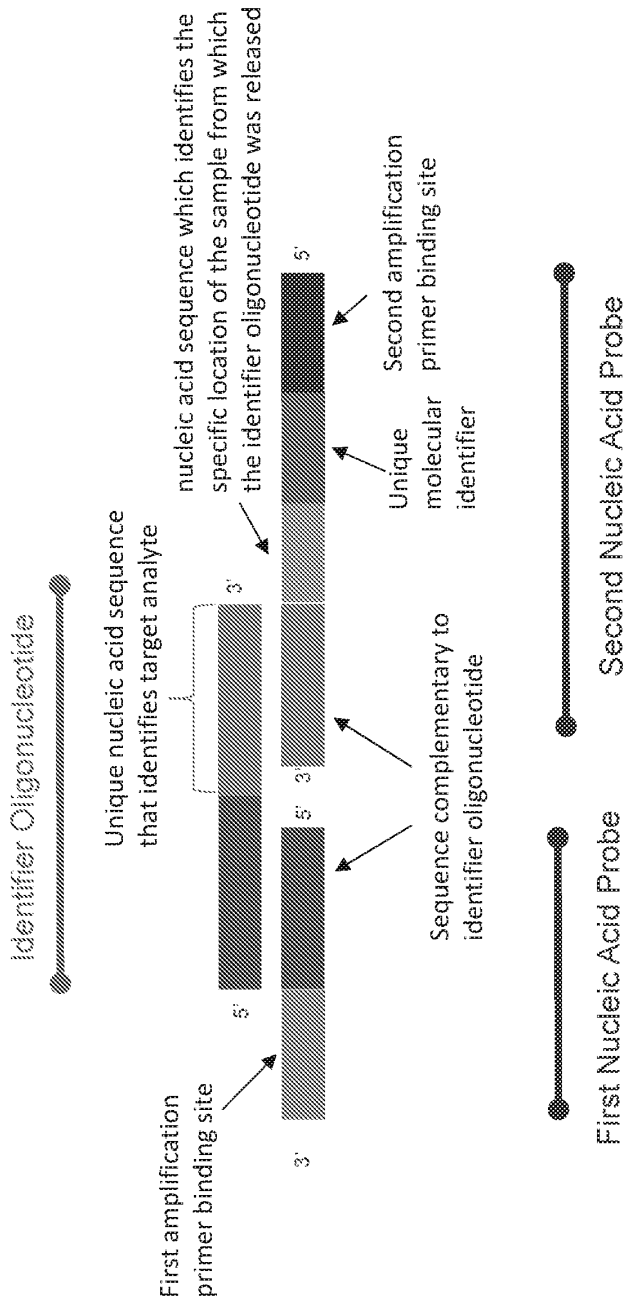

Identifier Oligonucleotide
Unique nucleic acid sequence that identifies target analyte
nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released
First amplification primer binding site
Sequence complementary to identifier oligonucleotide
Unique molecular identifier
Second amplification primer binding site
5'
3'
First Nucleic Acid Probe
Second Nucleic Acid Probe

Figure 7

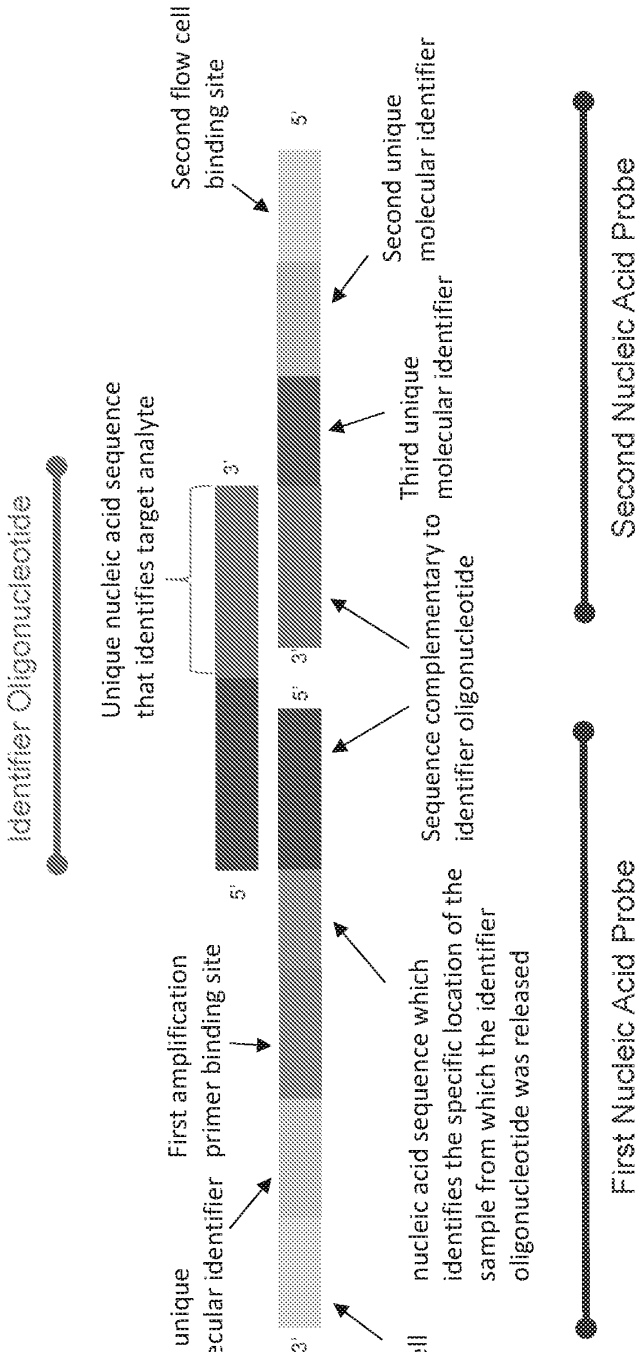

Identifier Oligonucleotide
Unique nucleic acid sequence that identifies target analyte
Second flow cell binding site
Second unique molecular identifier
Third unique molecular identifier
Sequence complementary to identifier oligonucleotide
First amplification primer binding site
First unique molecular identifier
nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released
First flow cell binding site
First Nucleic Acid Probe
Second Nucleic Acid Probe
5'
3'

Figure 8

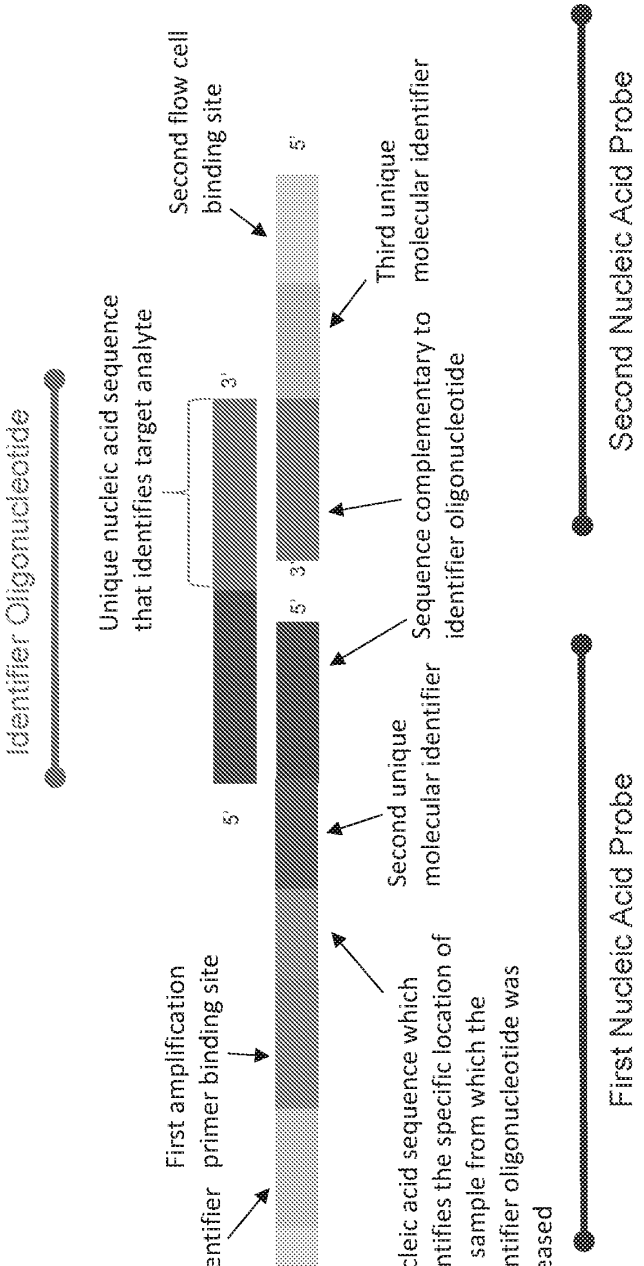

Identifier Oligonucleotide
Unique nucleic acid sequence that identifies target analyte
Second flow cell binding site
Third unique molecular identifier
Sequence complementary to identifier oligonucleotide
Second unique molecular identifier
First amplification primer binding site
First unique molecular identifier
Nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released
First flow cell binding site
First Nucleic Acid Probe
Second Nucleic Acid Probe
5'
3'

Figure 9

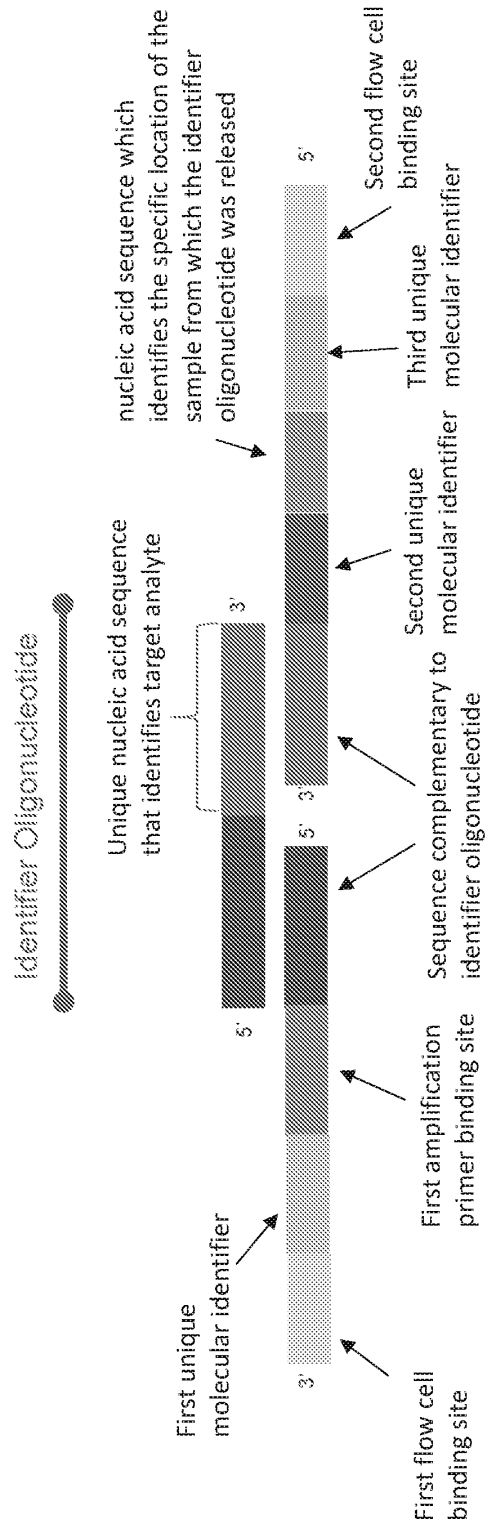

Identifier Oligonucleotide
Unique nucleic acid sequence that identifies target analyte
nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released
Second flow cell binding site
Third unique molecular identifier
Second unique molecular identifier
Sequence complementary to identifier oligonucleotide
First amplification primer binding site
First unique molecular identifier
First flow cell binding site
5'
3'
First Nucleic Acid Probe
Second Nucleic Acid Probe

Figure 10

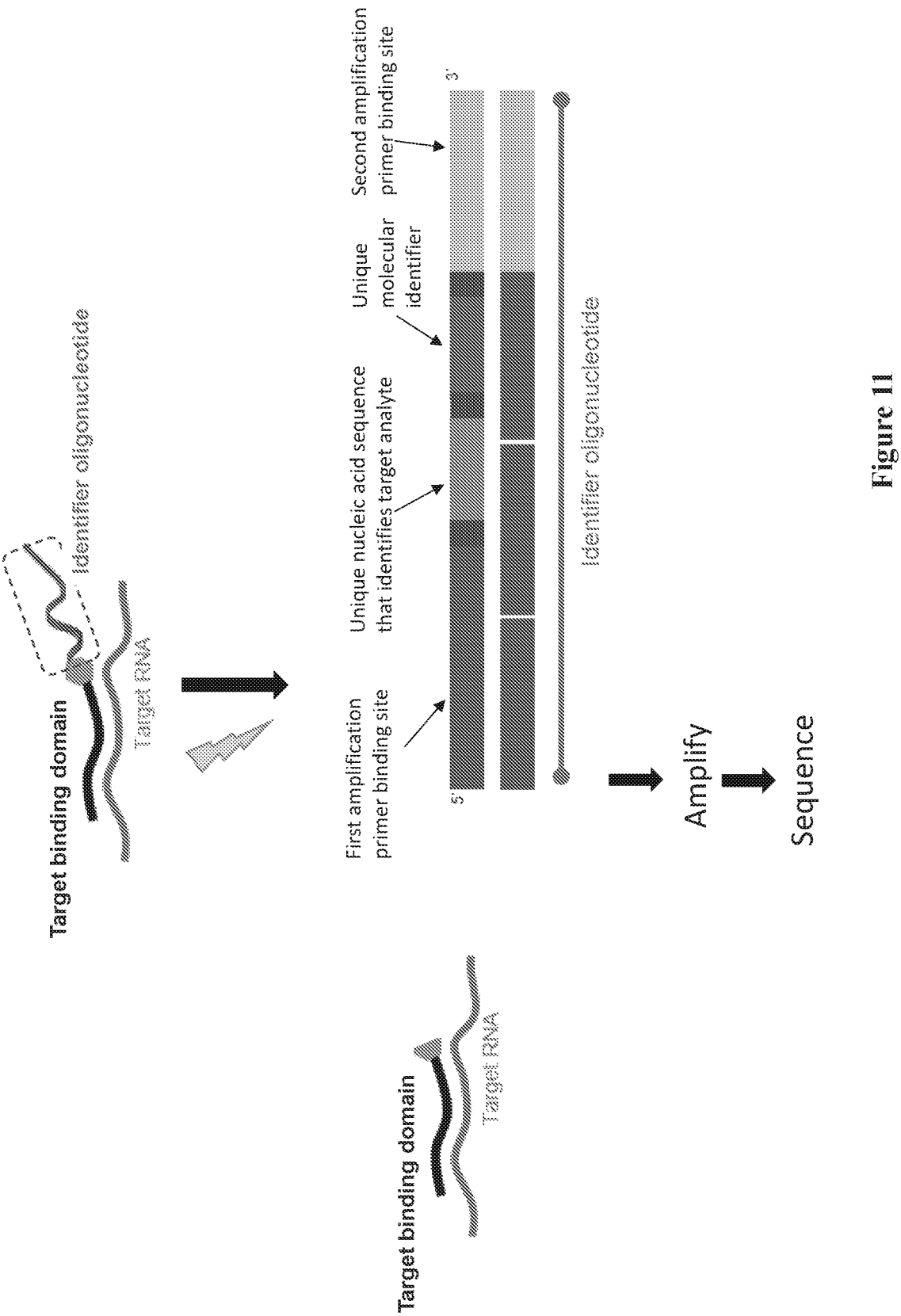

Target binding domain
Identifier oligonucleotide
Target RNA
First amplification primer binding site
Unique nucleic acid sequence that identifies target analyte
Unique molecular identifier
Second amplification primer binding site
5'
3'
Identifier oligonucleotide
Amplify
Sequence
Target binding domain
Target RNA

Figure 11

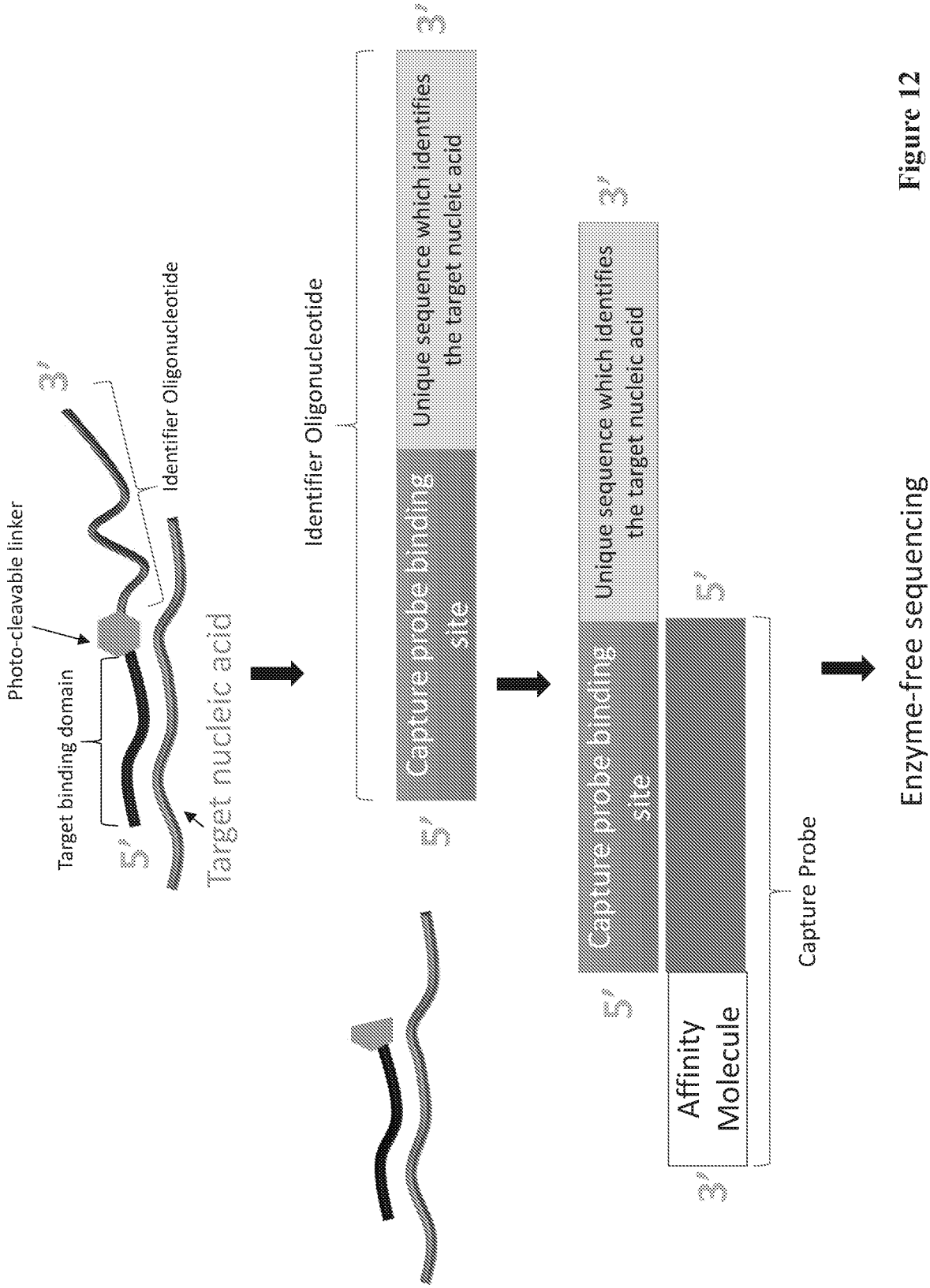


Photo-cleavable linker
Target binding domain
3'
5'
Identifier Oligonucleotide
Target nucleic acid
Identifier Oligonucleotide
Capture probe binding site
Unique sequence which identifies the target nucleic acid
3'
5'
Capture probe binding site
Unique sequence which identifies the target nucleic acid
3'
5'
5'
Affinity Molecule
3'
Capture Probe
Enzyme-free sequencing

Figure 12

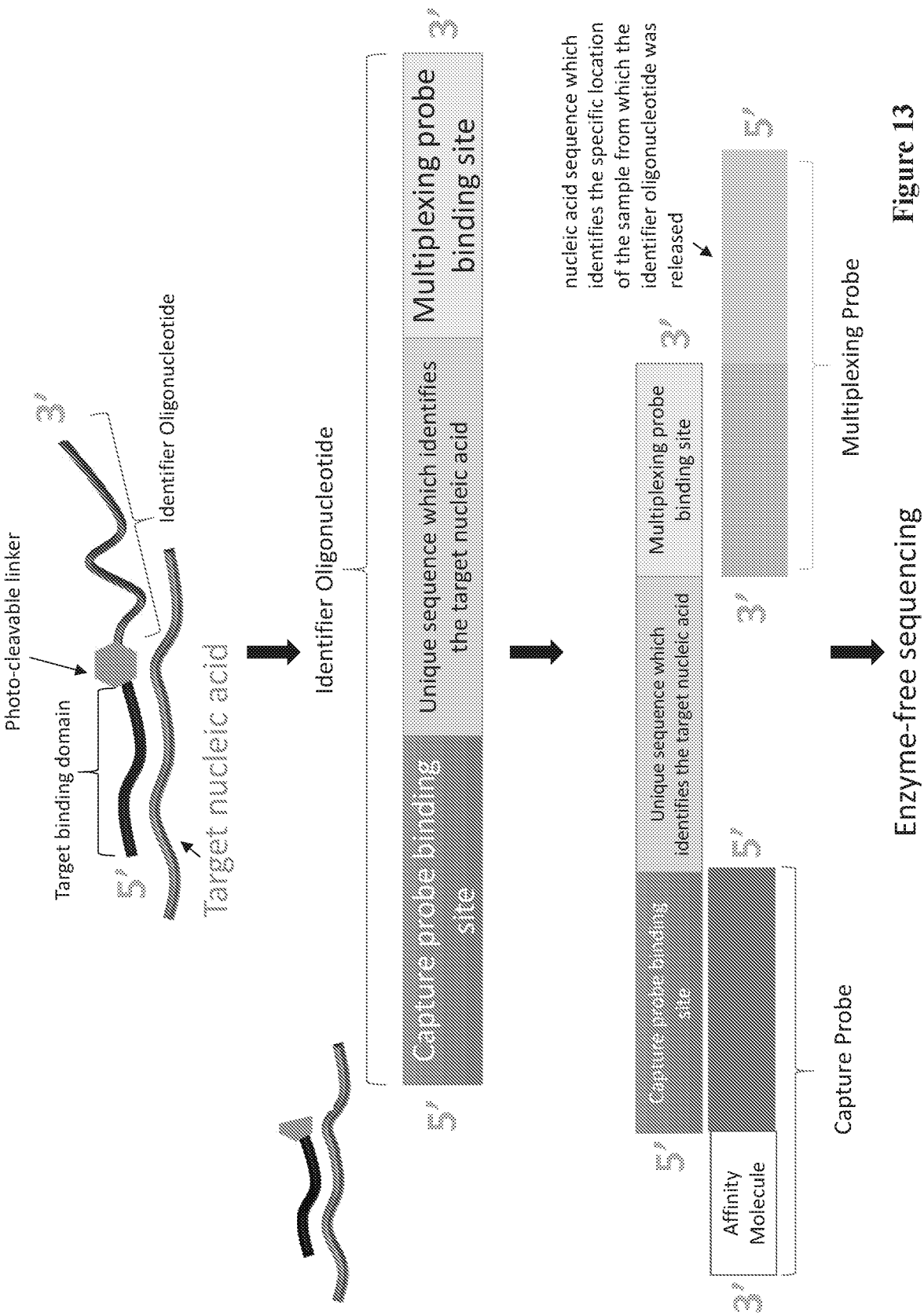


Photo-cleavable linker
Target binding domain
5'
3'
Identifier Oligonucleotide
Target nucleic acid
Identifier Oligonucleotide
5'
Capture probe binding site
Unique sequence which identifies the target nucleic acid
Multiplexing probe binding site
3'
nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released
Affinity Molecule
Capture probe binding site
Unique sequence which identifies the target nucleic acid
Multiplexing probe binding site
Capture Probe
Multiplexing Probe
Enzyme-free sequencing

Figure 13

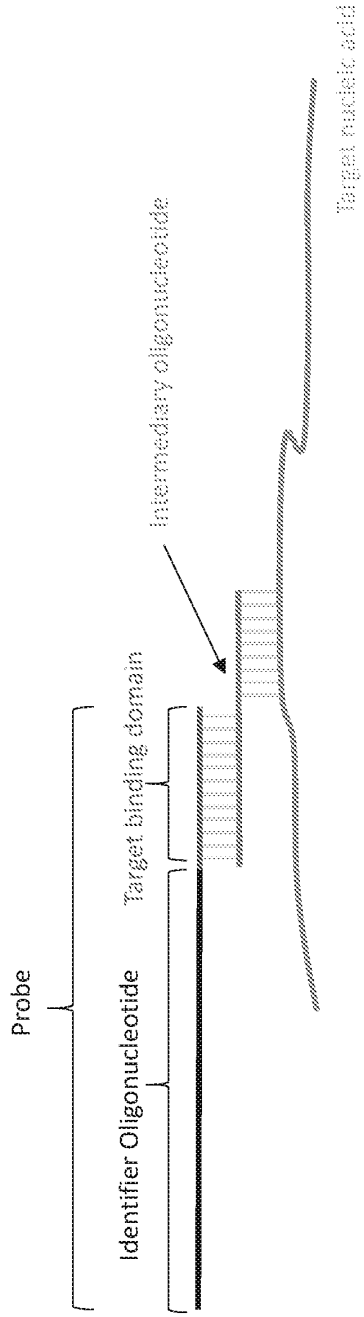

Target nucleic acid
Intermediary oligonucleotide
Target binding domain
Probe
Identifier Oligonucleotide

Figure 14

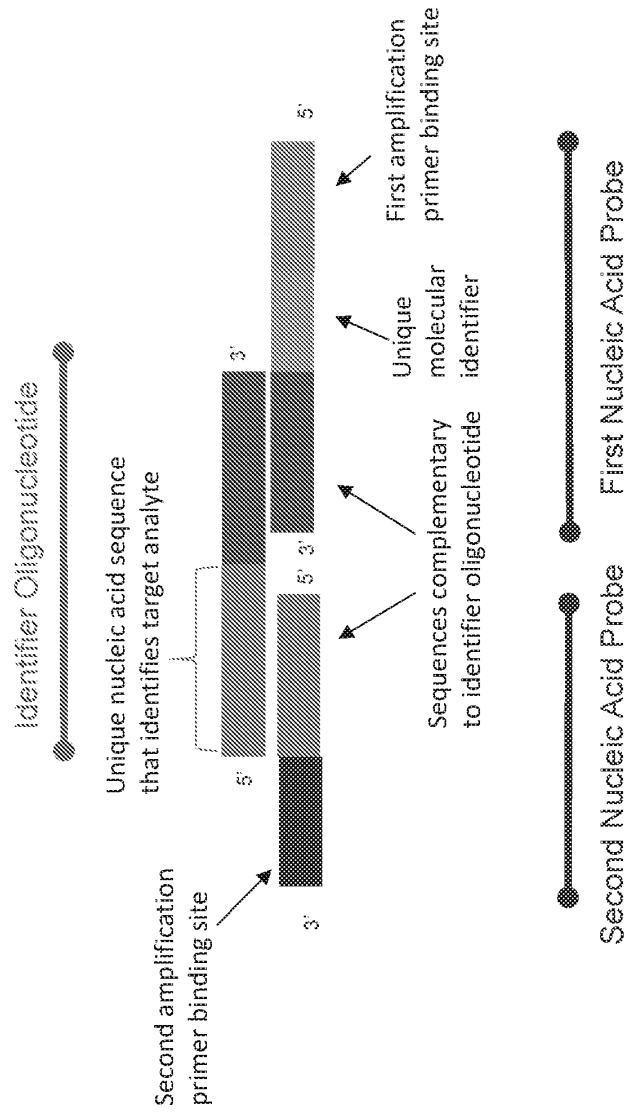

Identifier Oligonucleotide
Unique nucleic acid sequence
that identifies target analyte
Second amplification
primer binding site
Sequences complementary
to identifier oligonucleotide
Unique
molecular
identifier
First amplification
primer binding site
Second Nucleic Acid Probe
First Nucleic Acid Probe
5'
3'
5'
3'
3'
5'

Figure 15

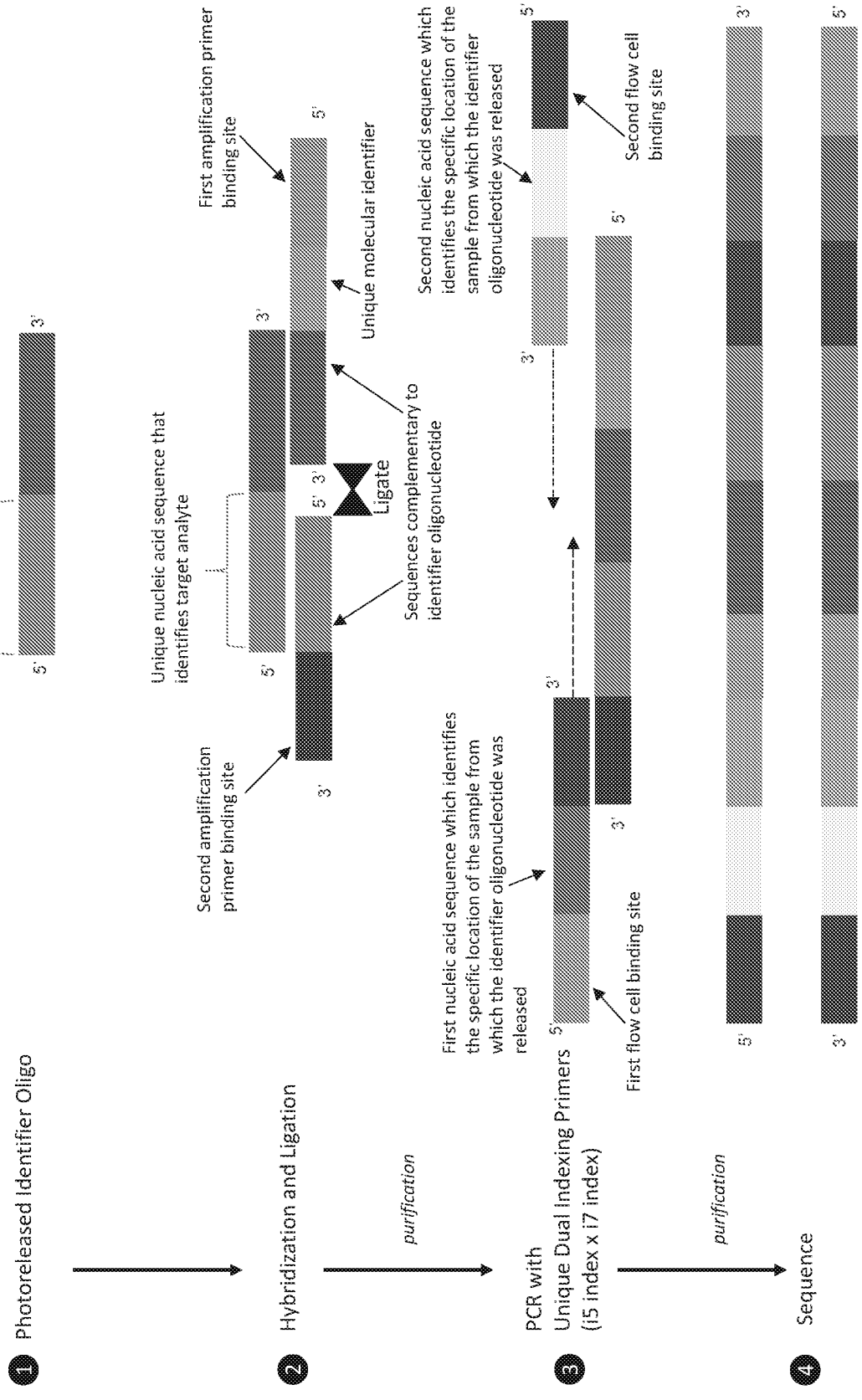

1 Photoreleased Identifier Oligo
Unique nucleic acid sequence that identifies target analyte
5'
3'
2 Hybridization and Ligation
Unique nucleic acid sequence that identifies target analyte
Second amplification primer binding site
Sequences complementary to identifier oligonucleotide
Ligate
First amplification primer binding site
Unique molecular identifier
purification
3 PCR with Unique Dual Indexing Primers (i5 index x i7 index)
First nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released
Second nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released
First flow cell binding site
Second flow cell binding site
purification
4 Sequence

Figure 16

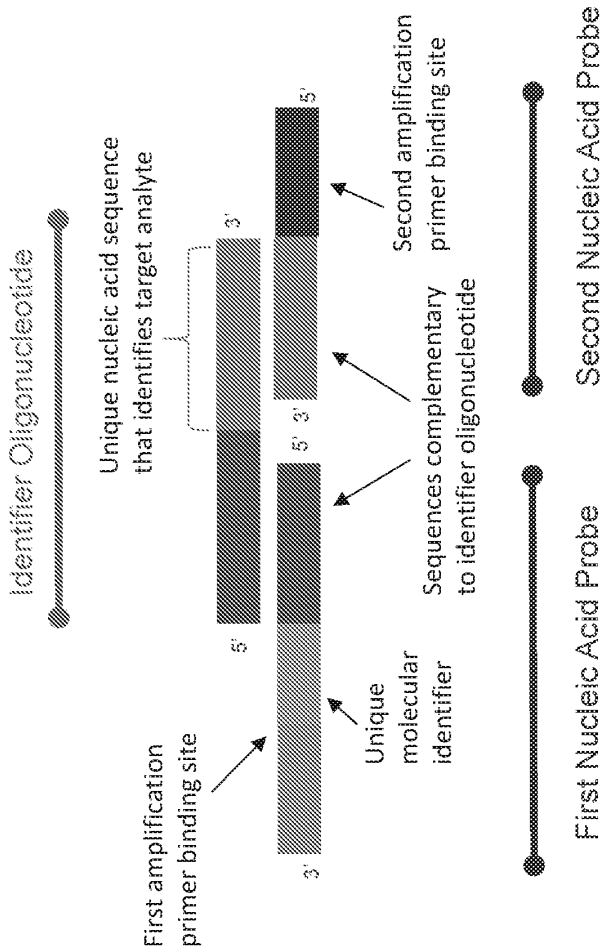

Identifier Oligonucleotide
Unique nucleic acid sequence
that identifies target analyte
First amplification
primer binding site
Unique
molecular
identifier
Sequences complementary
to identifier oligonucleotide
Second amplification
primer binding site
5'
3'
5'
3'
5'
3'
First Nucleic Acid Probe
Second Nucleic Acid Probe

Figure 17

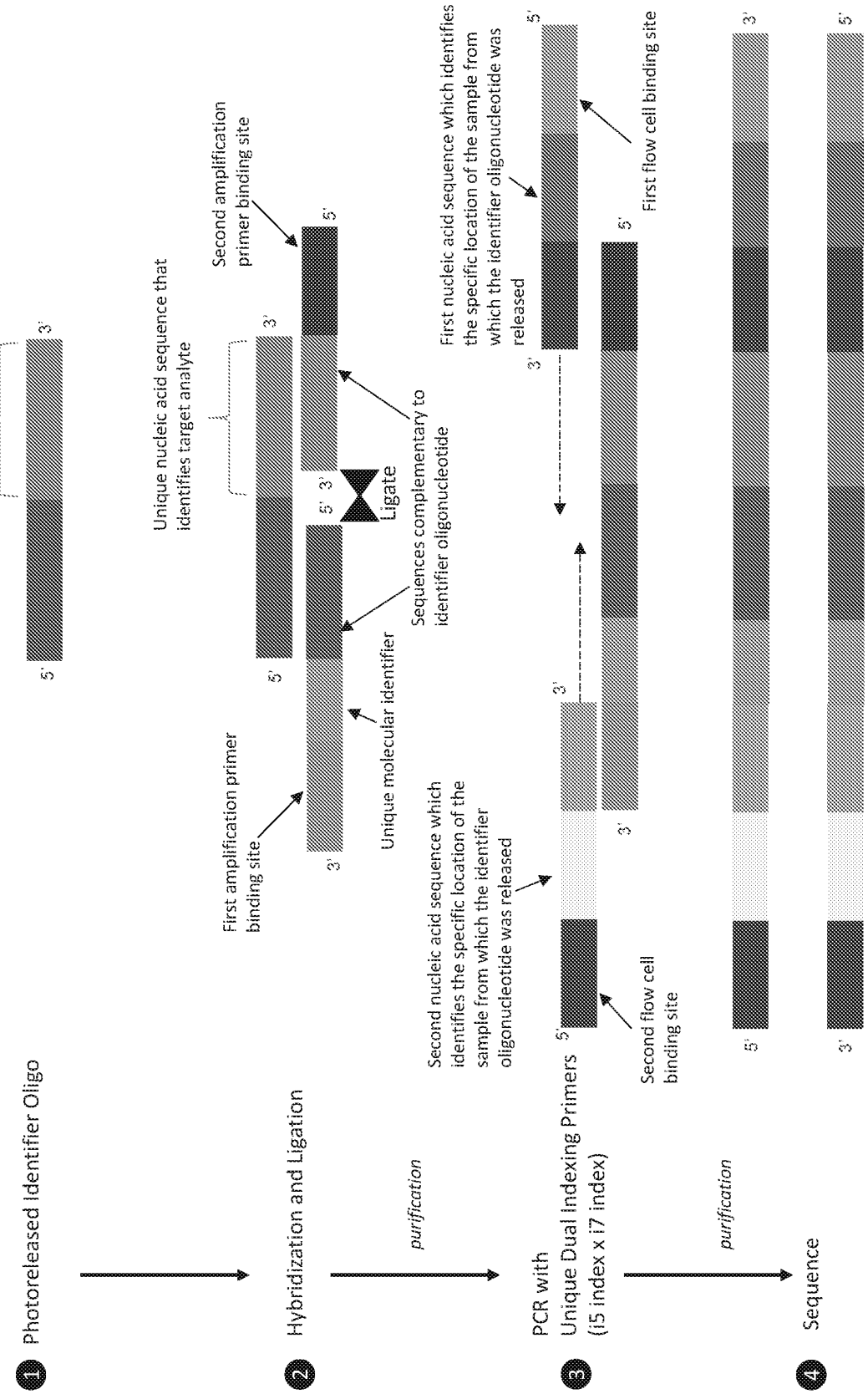

1 Photoreleased Identifier Oligo
Unique nucleic acid sequence that identifies target analyte
2 Hybridization and Ligation
Unique nucleic acid sequence that identifies target analyte
Second amplification primer binding site
First amplification primer binding site
Unique molecular identifier
Sequences complementary to identifier oligonucleotide
Ligate
purification
3 PCR with Unique Dual Indexing Primers (i5 index x i7 index)
Second nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released
First nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released
Second flow cell binding site
First flow cell binding site
purification
4 Sequence

Figure 18

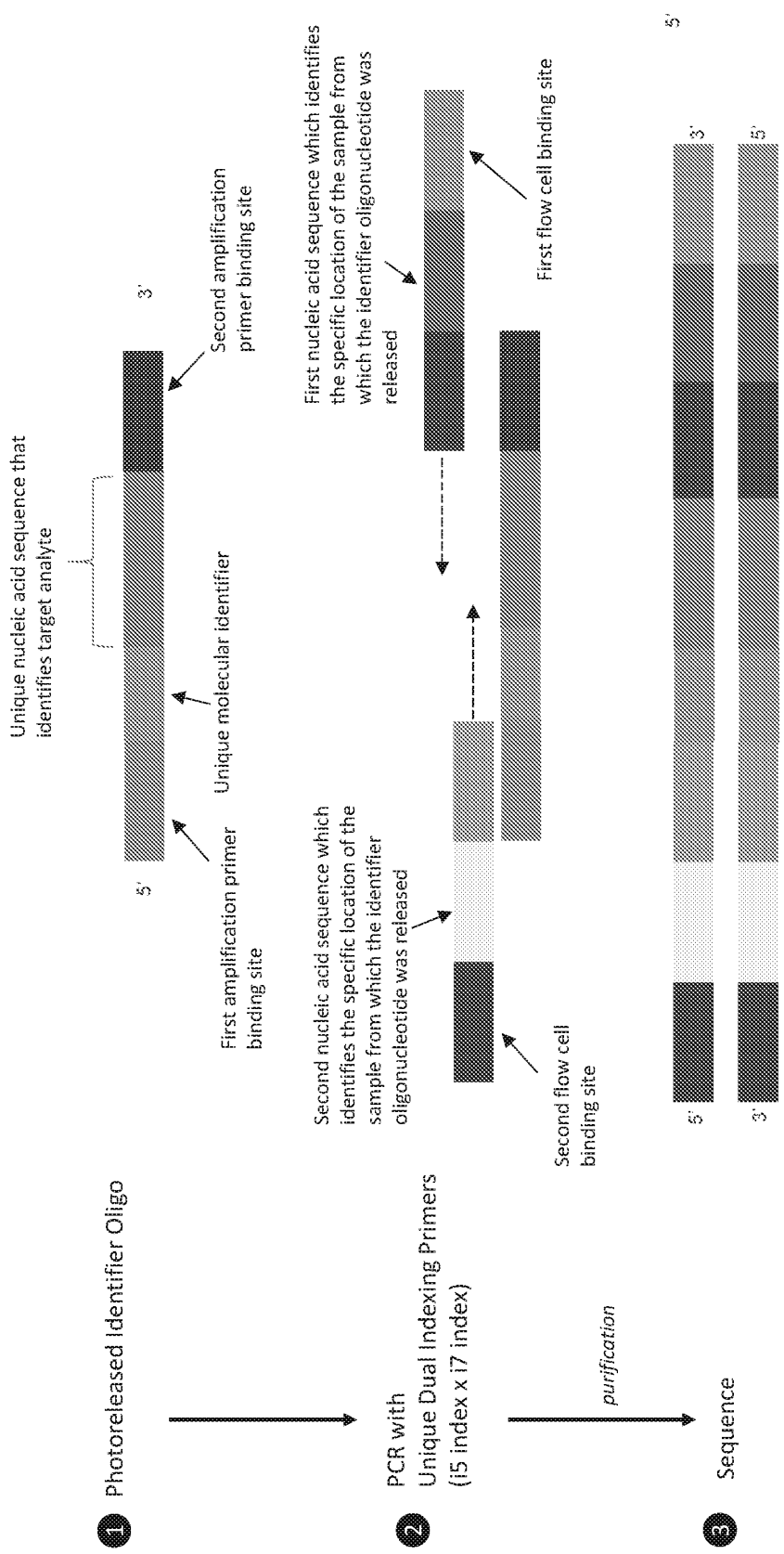

1 Photoreleased Identifier Oligo
Unique nucleic acid sequence that identifies target analyte
5'
3'
First amplification primer binding site
Unique molecular identifier
Second amplification primer binding site
2 PCR with Unique Dual Indexing Primers (i5 index x i7 index)
Second nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released
First nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released
Second flow cell binding site
First flow cell binding site
purification
3 Sequence
5'
3'
3'
5'

Figure 19

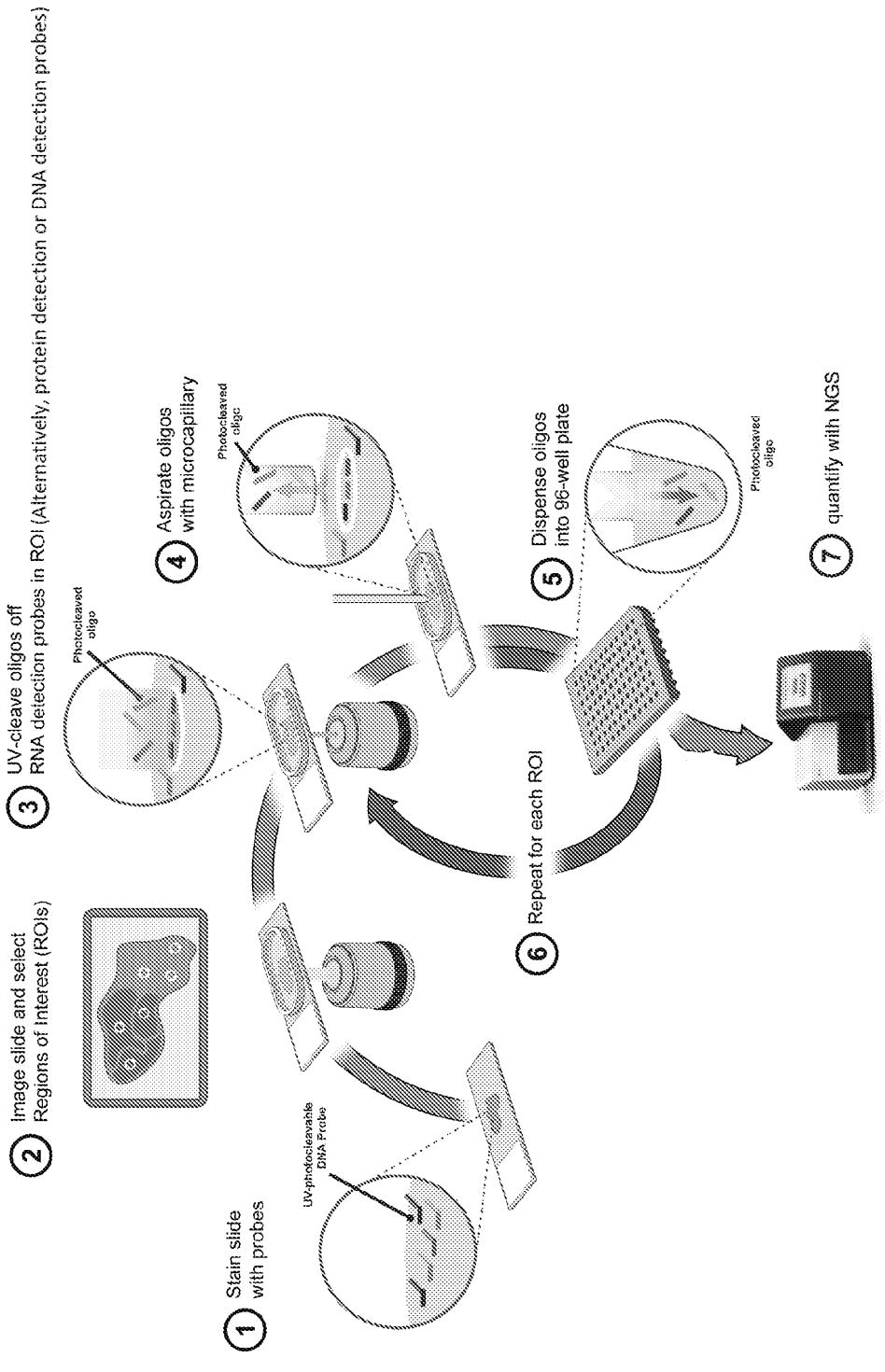

1 Stain slide with probes
UV-photocleavable DNA Probe
2 Image slide and select Regions of Interest (ROIs)
3 UV-cleave oligos off RNA detection probes in ROI (Alternatively, protein detection or DNA detection probes)
Photocleaved oligo
4 Aspirate oligos with microcapillary
Photocleaved oligo
5 Dispense oligos into 96-well plate
Photocleaved oligo
6 Repeat for each ROI
7 quantify with NGS

Figure 20

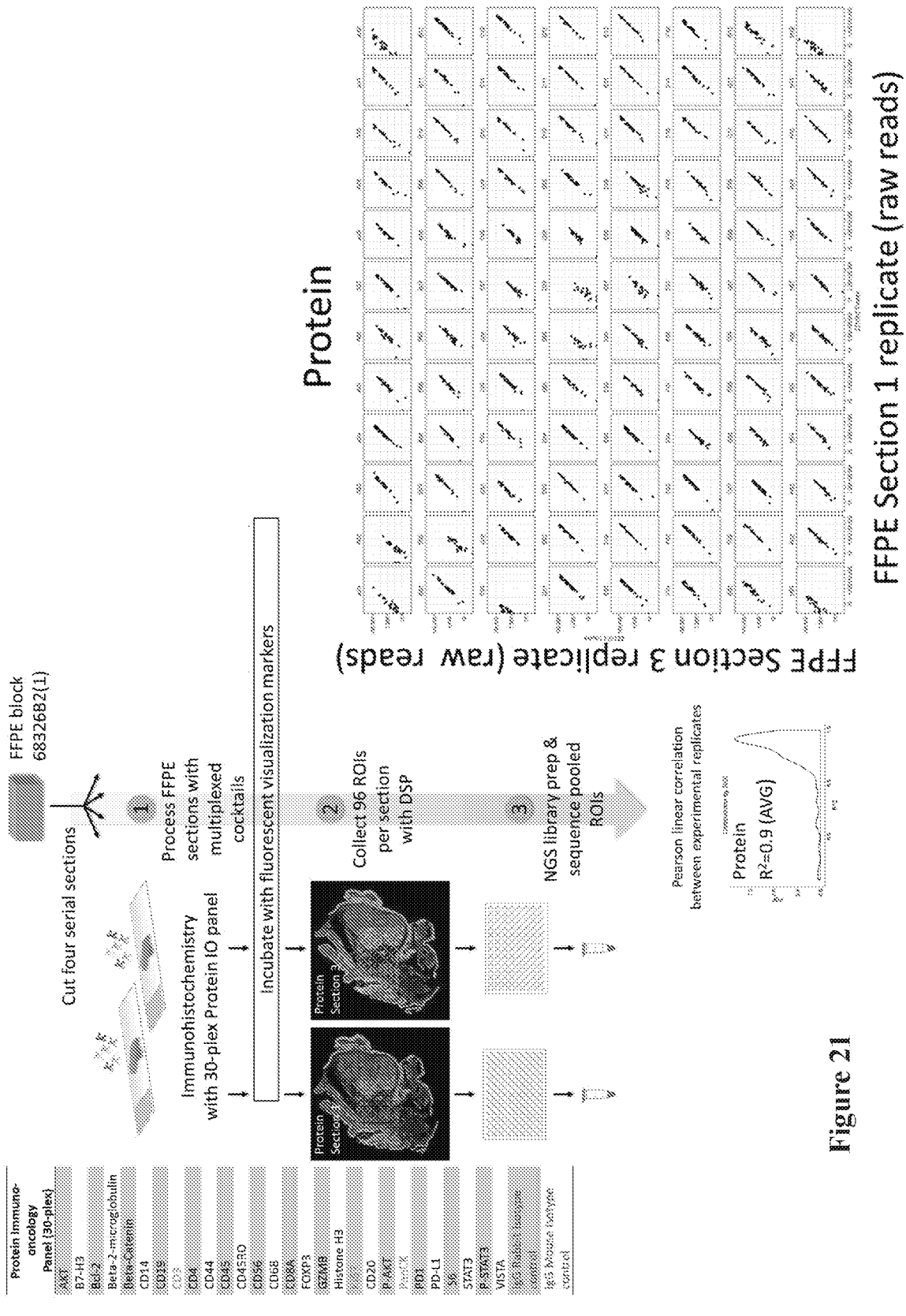

Protein Immuno-oncology Panel (30-plex)
AKT
B7-H3
Bcl-2
Beta-2-microglobulin
Beta-Catenin
CD14
CD19
CD3
CD4
CD44
CD45
CD45RO
CD56
CD68
CD8A
FOXP3
GZMB
Histone H3
IDO1
CD20
P-AKT
PanCK
PD1
PD-L1
S6
STAT3
P-STAT3
VISTA
IgG Rabbit Isotype control
IgG Mouse Isotype control
FFPE block 68326B2(1)
Cut four serial sections
1
Process FFPE sections with multiplexed cocktails
Immunohistochemistry with 30-plex Protein IO panel
Incubate with fluorescent visualization markers
2
Collect 96 ROIs per section with DSP
3
NGS library prep & sequence pooled ROIs
Protein Section 1
Protein Section 2
Pearson linear correlation between experimental replicates
Protein
R²=0.9 (AVG)
FFPE Section 3 replicate (raw reads)
Protein
FFPE Section 1 replicate (raw reads)

Figure 21

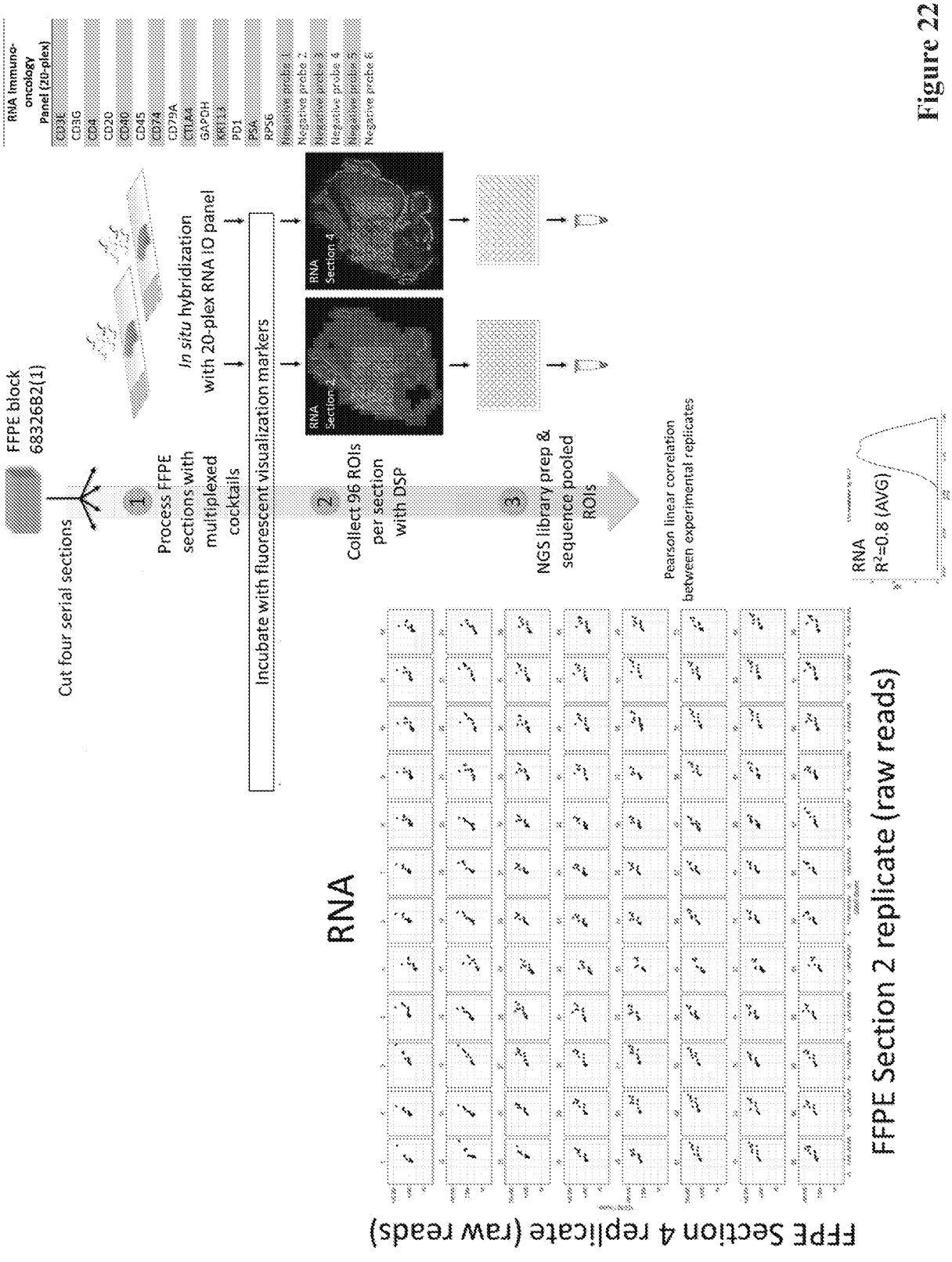

RNA Immuno-oncology Panel (20-plex)
CD3E
CD3G
CD4
CD20
CD40
CD45
CD74
CD79A
CTLA4
GAPDH
KRT13
PD1
PSK
RPS6
Negative probe 1
Negative probe 2
Negative probe 3
Negative probe 4
Negative probe 5
Negative probe 6
FFPE block 68326B2(1)
Cut four serial sections
1 Process FFPE sections with multiplexed cocktails
In situ hybridization with 20-plex RNA IO panel
Incubate with fluorescent visualization markers
2 Collect 96 ROIs per section with DSP
RNA Section 2
RNA Section 4
3 NGS library prep & sequence pooled ROIs
Pearson linear correlation between experimental replicates
RNA
R²=0.8 (AVG)
RNA
FFPE Section 4 replicate (raw reads)
FFPE Section 2 replicate (raw reads)

Figure 22

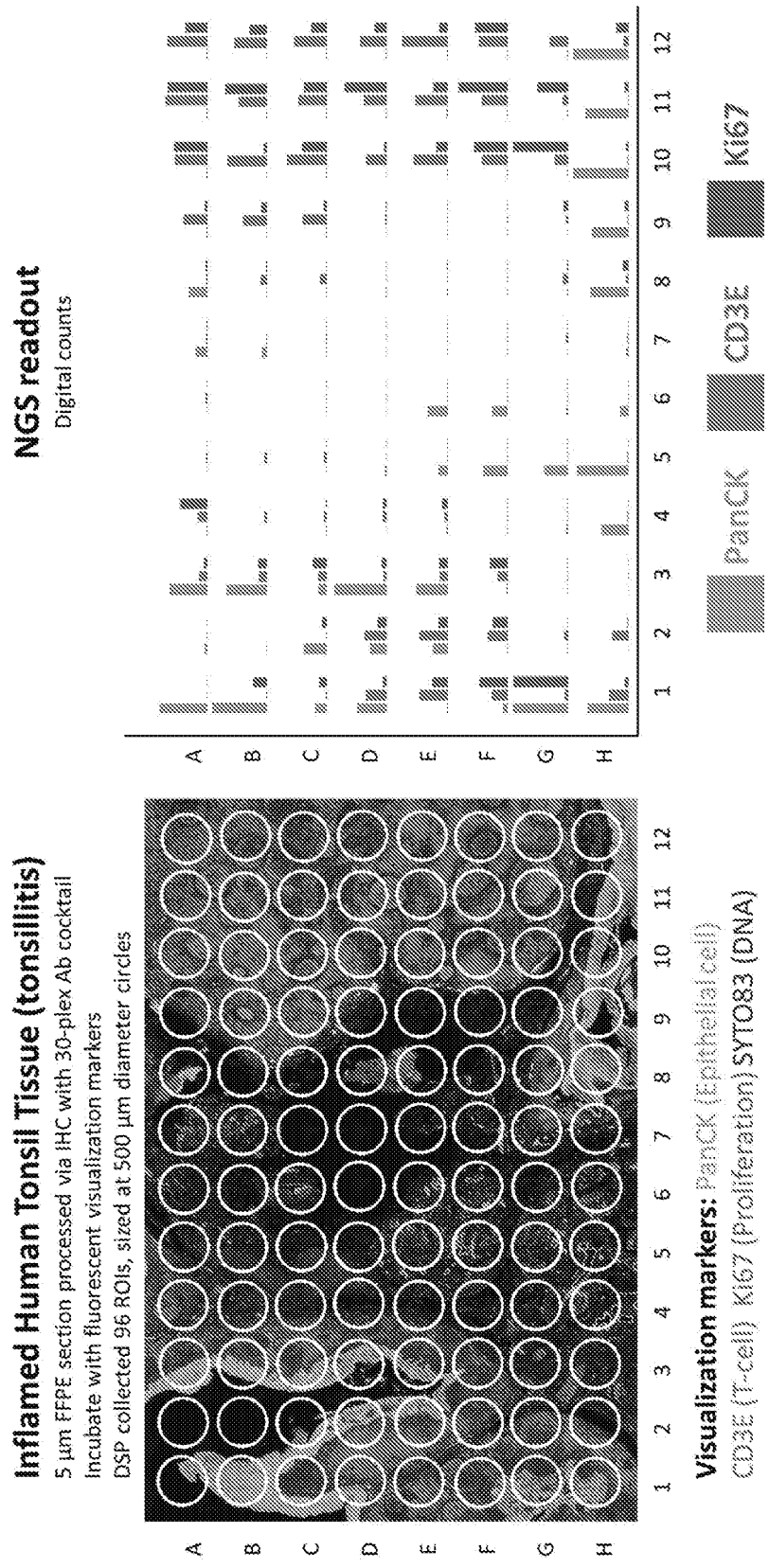

Inflamed Human Tonsil Tissue (tonsillitis)
5 µm FFPE section processed via IHC with 30-plex Ab cocktail
Incubate with fluorescent visualization markers
DSP collected 96 ROIs, sized at 500 µm diameter circles
Visualization markers: PanCK (Epithelial cell)
CD3E (T-cell) Ki67 (Proliferation) SYTO83 (DNA)
NGS readout
Digital counts
PanCK
CD3E
Ki67
A
B
C
D
E
F
G
H
1 2 3 4 5 6 7 8 9 10 11 12

Figure 23

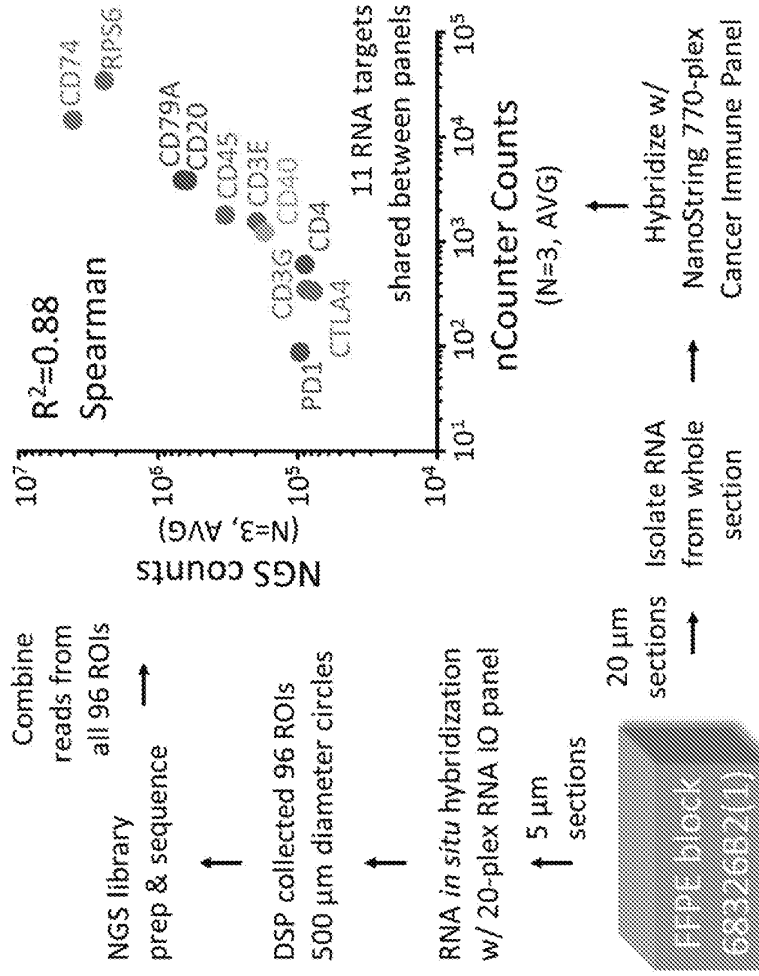

FFPE block
(632266132(1))
20 µm sections
Isolate RNA from whole section
Hybridize w/ NanoString 770-plex Cancer Immune Panel
5 µm sections
RNA in situ hybridization w/ 20-plex RNA IO panel
DSP collected 96 ROIs 500 µm diameter circles
NGS library prep & sequence
Combine reads from all 96 ROIs
NGS counts (N=3, AVG)
nCounter Counts (N=3, AVG)
R2=0.88
Spearman
11 RNA targets shared between panels
CD74
RPS6
CD79A
CD20
CD45
CD3E
CD3G
CD40
CD4
PD1
CTLA4

Figure 24

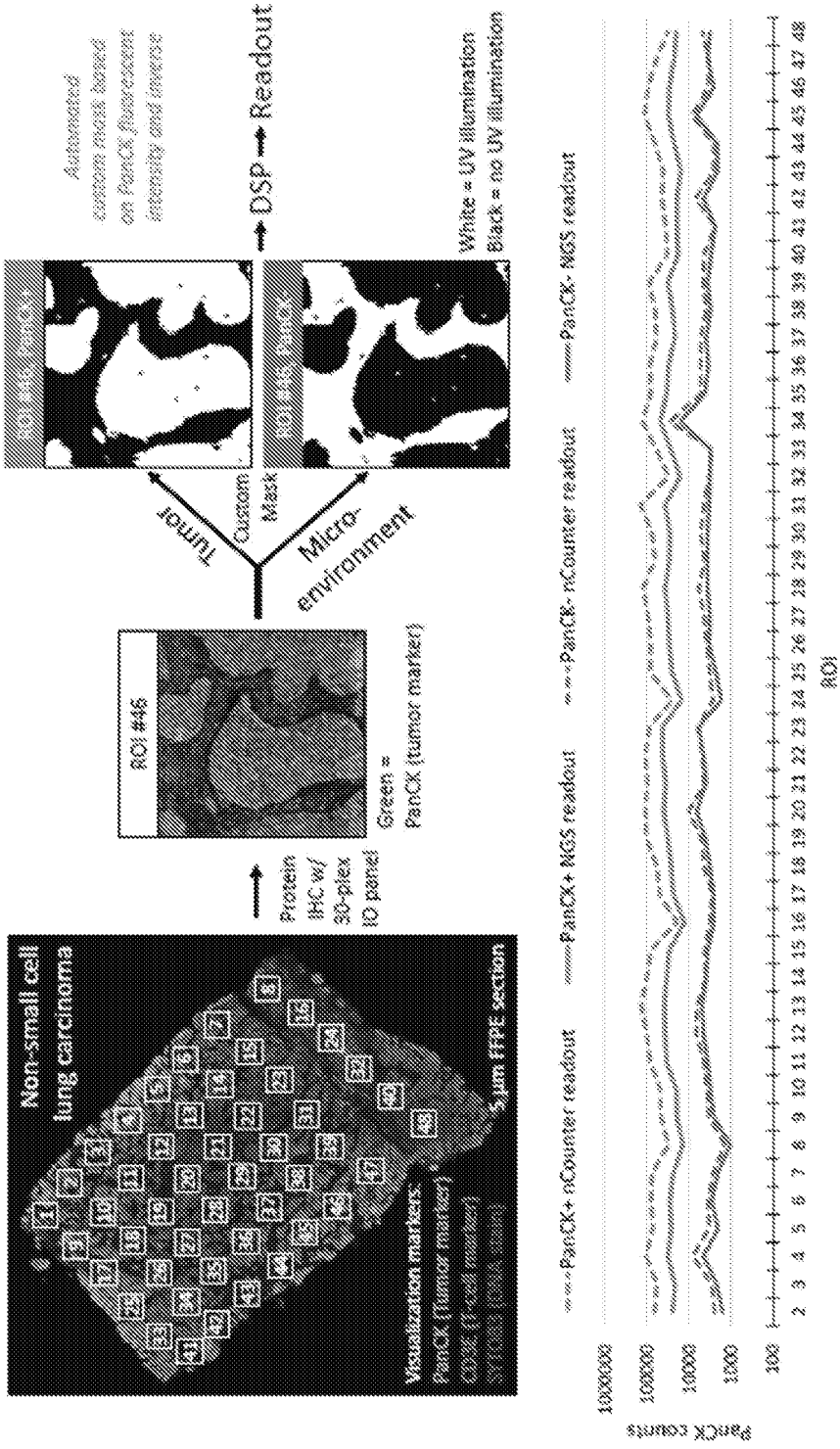
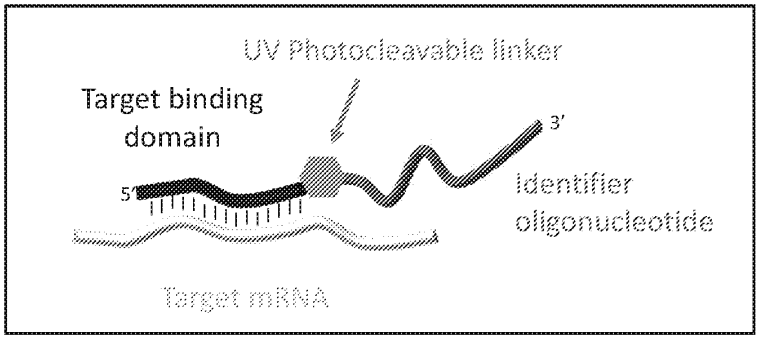

Non-small cell lung carcinoma
5 µm FFPE section
Visualization markers:
PanCK (tumor marker)
Protein IHC w/ 30-plex IO panel
ROI #46
Green = PanCK (tumor marker)
Tumor
Custom Mask
Micro-environment
Automated custom mask based on PanCK fluorescent intensity and morphology
DSP → Readout
White = UV illumination
Black = no UV illumination
PanCK+ nCounter readout
PanCK+ NGS readout
PanCK- nCounter readout
PanCK- NGS readout
PanCK counts
10000000
1000000
100000
10000
1000
100
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48
ROI

Figure 25


UV Photocleavable linker
Target binding domain
3'
5'
Identifier oligonucleotide
Target mRNA

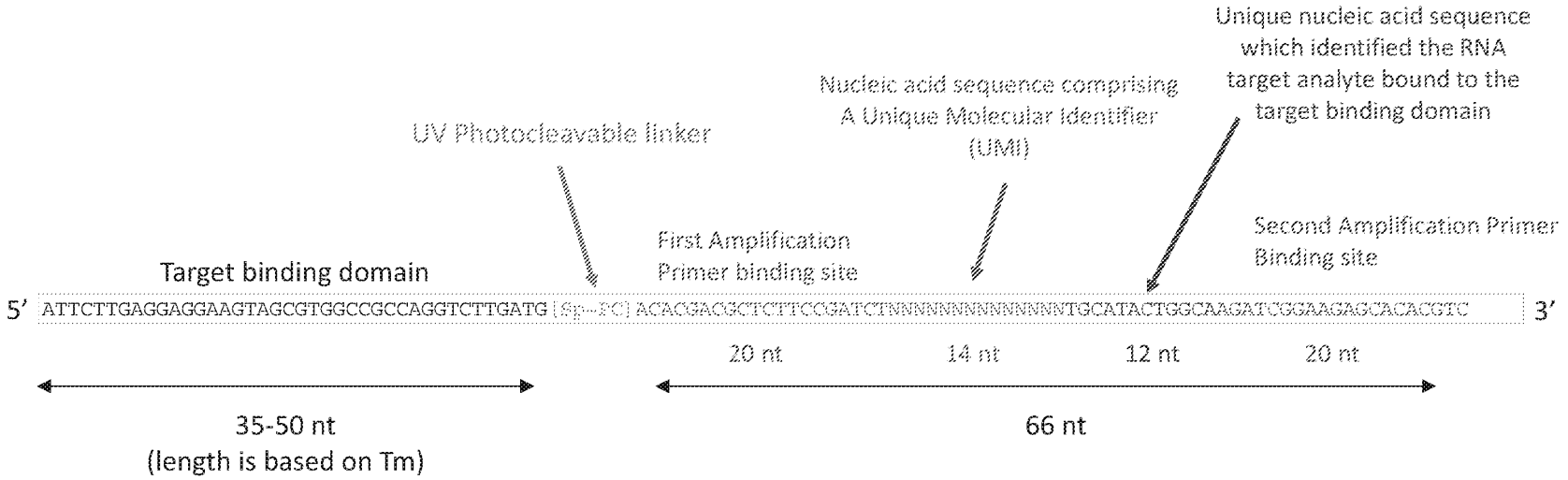

Unique nucleic acid sequence which identified the RNA target analyte bound to the target binding domain
Nucleic acid sequence comprising A Unique Molecular Identifier (UMI)
UV Photocleavable linker
Second Amplification Primer Binding site
First Amplification Primer binding site
Target binding domain
5'
ATTCTTGAGGAGGAAGTAGCGTGGCCGCCAGGTCTTGATG
[Sp—FC]
ACACGACGCTCTTCCGATCTNNNNNNNNNNNNNNNNTGCATACTGGCAAGATCGGAAGAGCACACGTC
3'
20 nt
14 nt
12 nt
20 nt
35-50 nt
(length is based on Tm)
66 nt

Figure 26

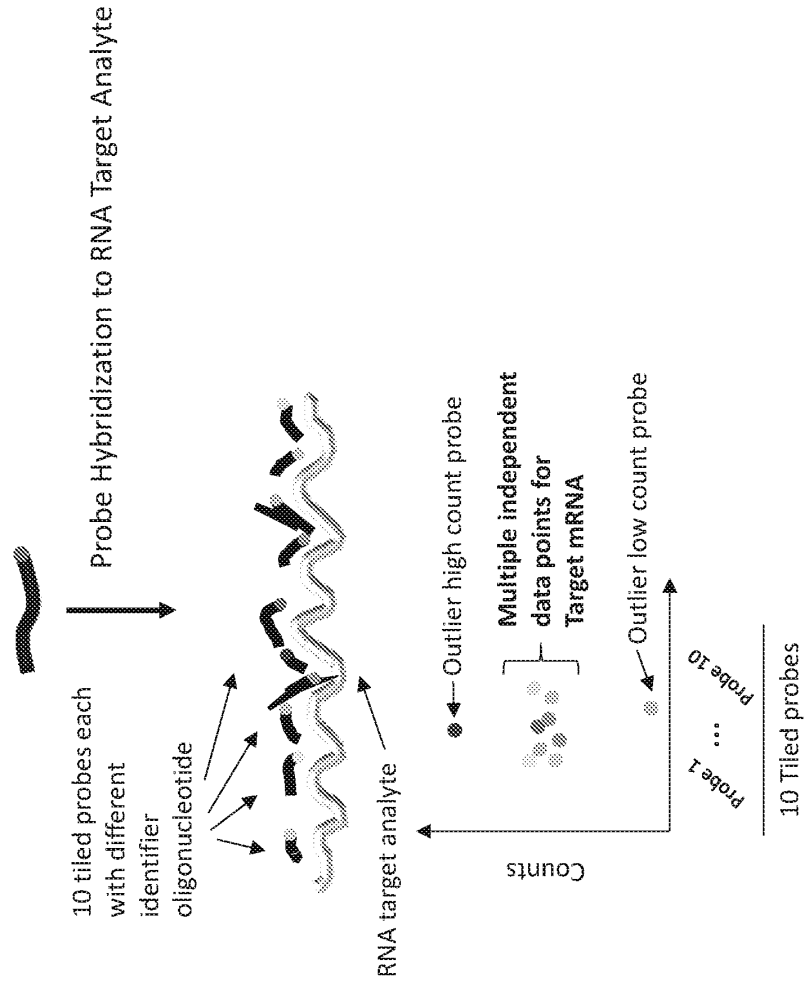

Probe Hybridization to RNA Target Analyte
10 tiled probes each with different identifier oligonucleotide
RNA target analyte
Outlier high count probe
Multiple independent data points for Target mRNA
Outlier low count probe
Counts
probe 1
...
probe 10
10 Tiled probes

Figure 27

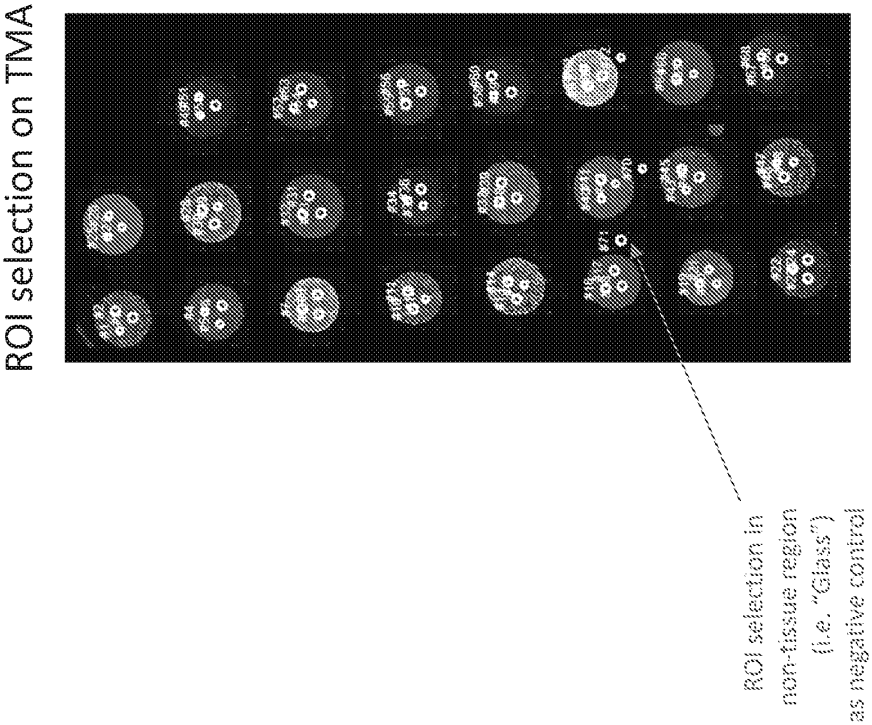

ROI selection on TMA
ROI selection in non-tissue region (i.e. "Glass") as negative control

Figure 28

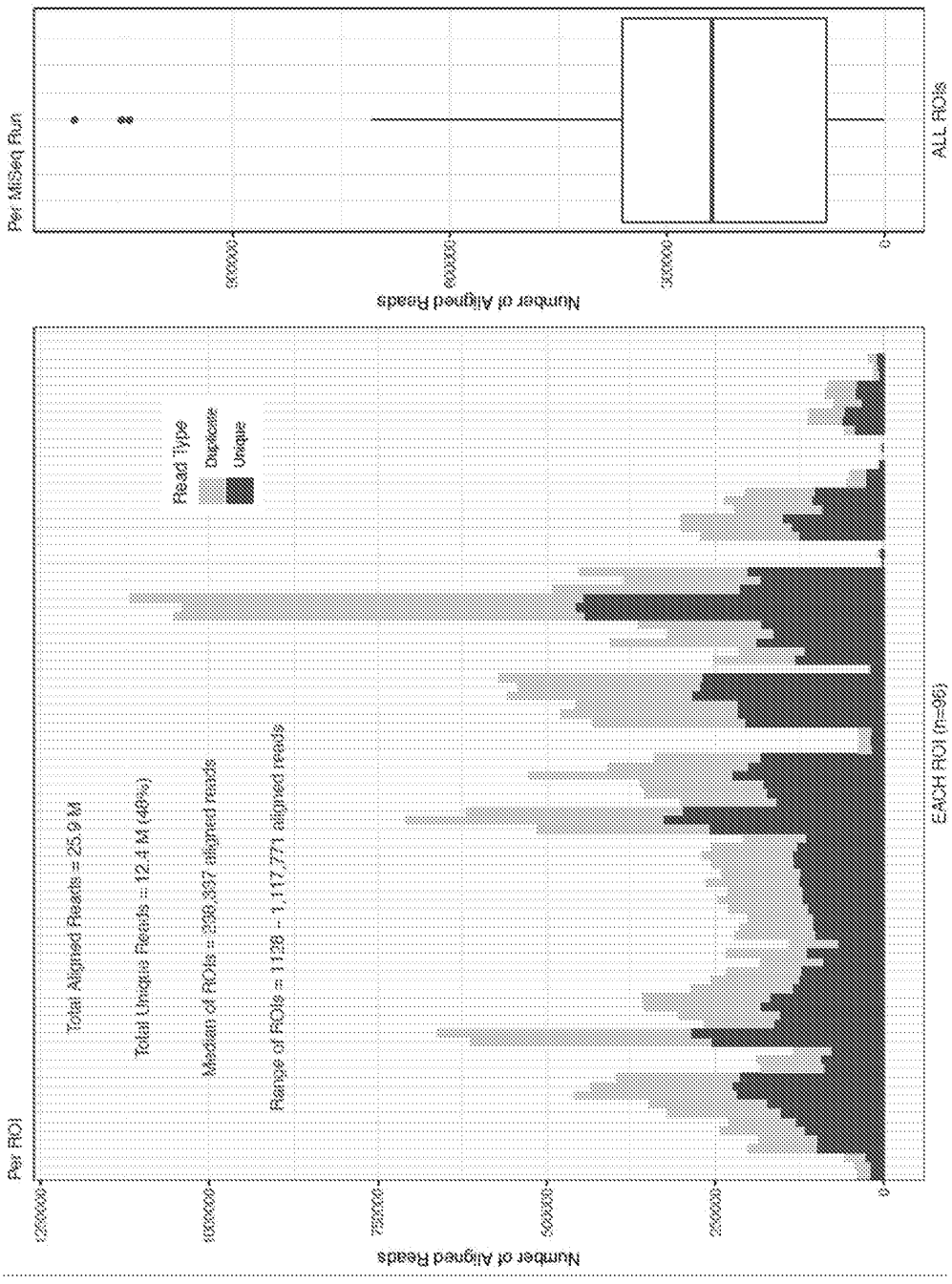

Per MiSeq Run
Number of Aligned Reads
ALL ROIs
Per ROI
Total Aligned Reads = 25.9 M
Total Unique Reads = 12.4 M (48%)
Median of ROIs = 230,337 aligned reads
Range of ROIs = 1138 - 1,117,771 aligned reads
Read Type
Duplicate
Unique
EACH ROI (n=96)

Figure 29

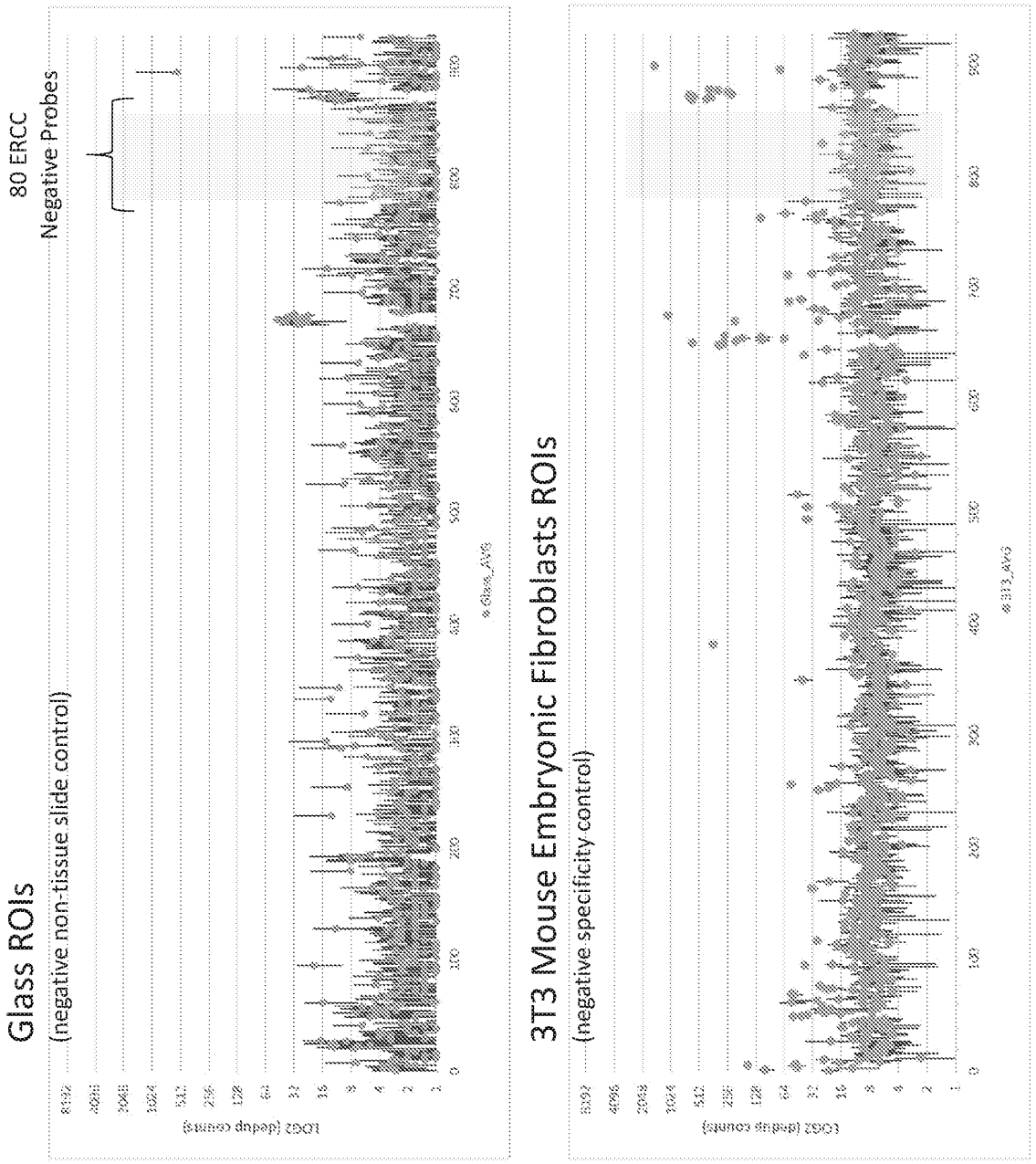

Glass ROIs
(negative non-tissue slide control)
80 ERCC
Negative Probes
LOG2 (dedup counts)
Glass_AOI
3T3 Mouse Embryonic Fibroblasts ROIs
(negative specificity control)
LOG2 (dedup counts)
3T3_AOI

Figure 30

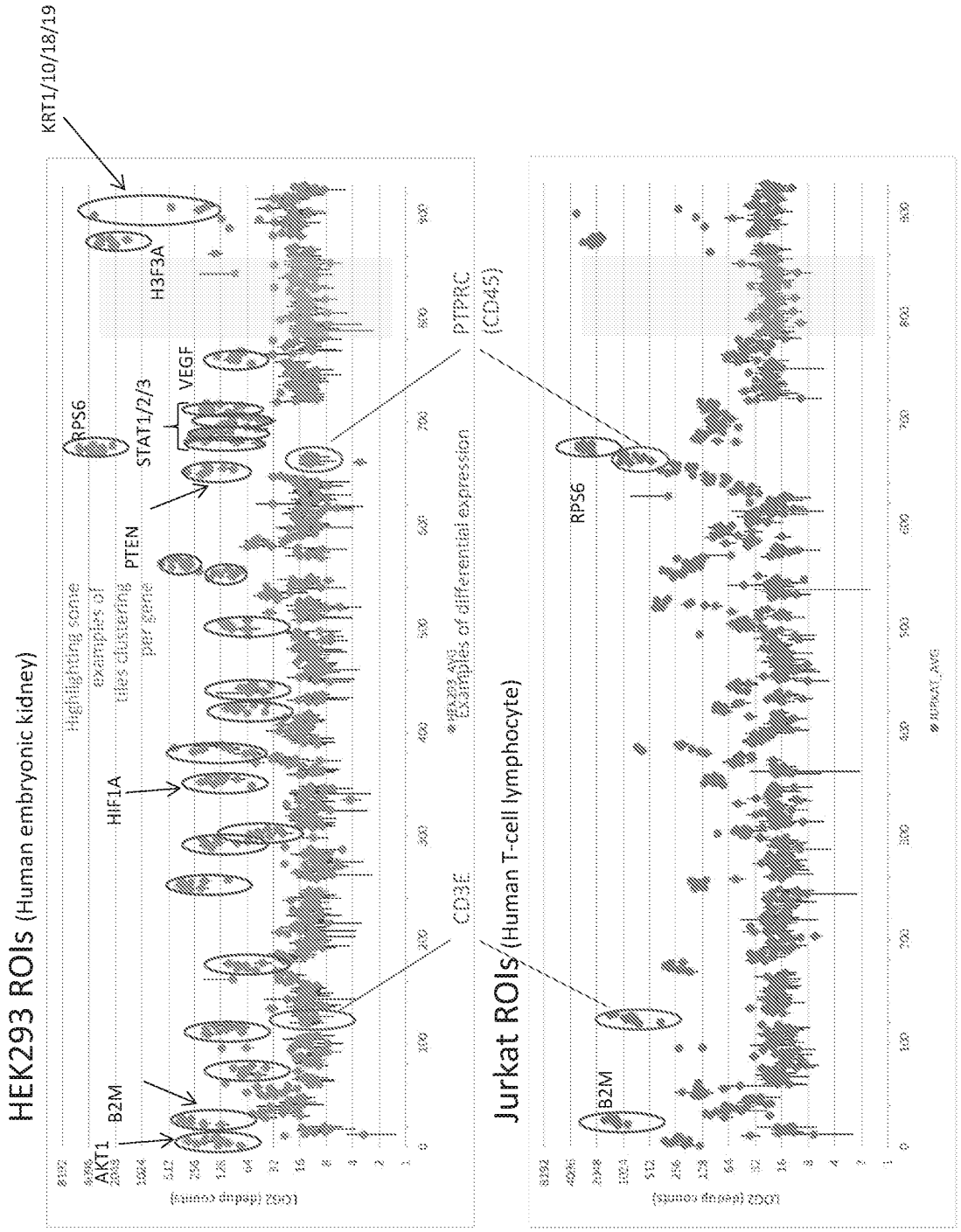

HEK293 ROIs (Human embryonic kidney)
Highlighting some examples of tiles clustering per gene
KRT1/10/18/19
RPS6
H3F3A
STAT1/2/3
VEGF
PTEN
HIF1A
B2M
AKT1
CD3E
PTPRC (CD45)
Jurkat ROIs (Human T-cell lymphocyte)
RPS6
B2M

Figure 31

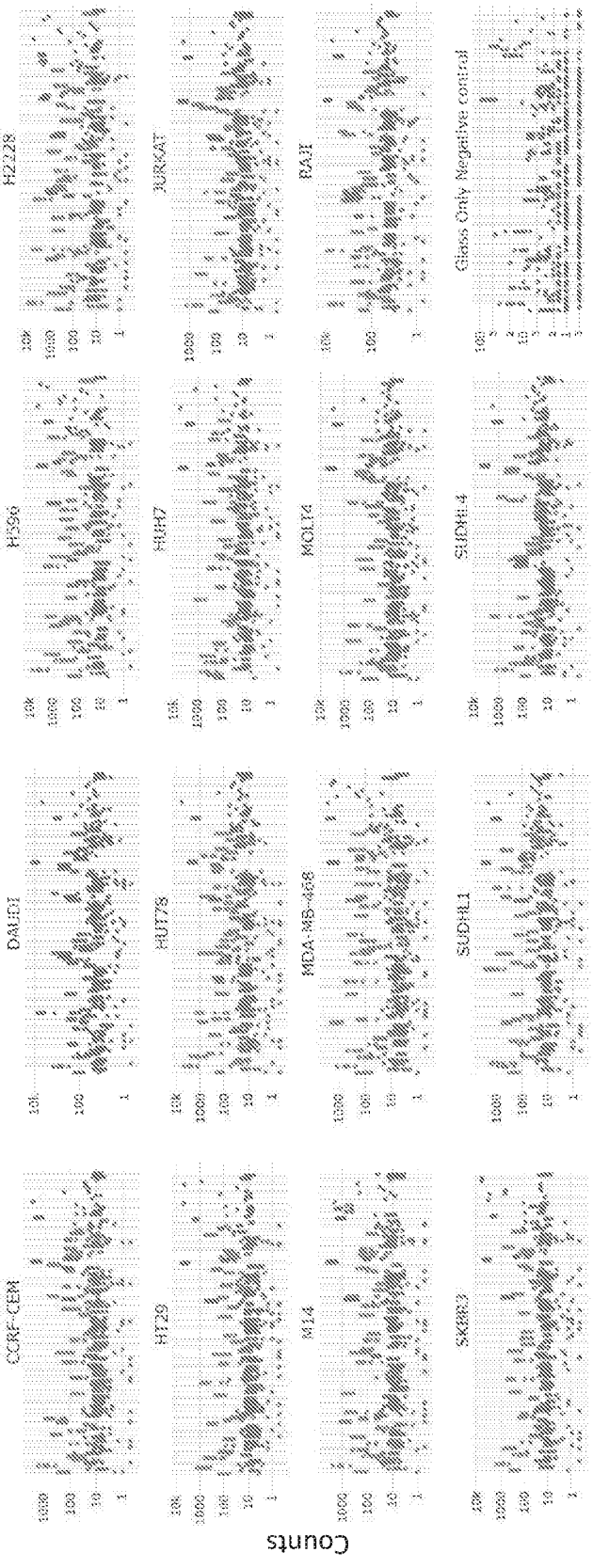

Counts
Target Analyte (96 RNA targets)
IO360 Expression from nCounter
Expressed (>200 counts)
Not Expressed (<10 Counts)
Not determined
CCRF-CEM
DAUDI
H596
H2228
HT29
HUT78
HUH7
JURKAT
M14
MDA-MB-468
MOLT4
RAJI
SKBR3
SUDHL1
SUDHL4
Glass Only Negative control

Figure 32

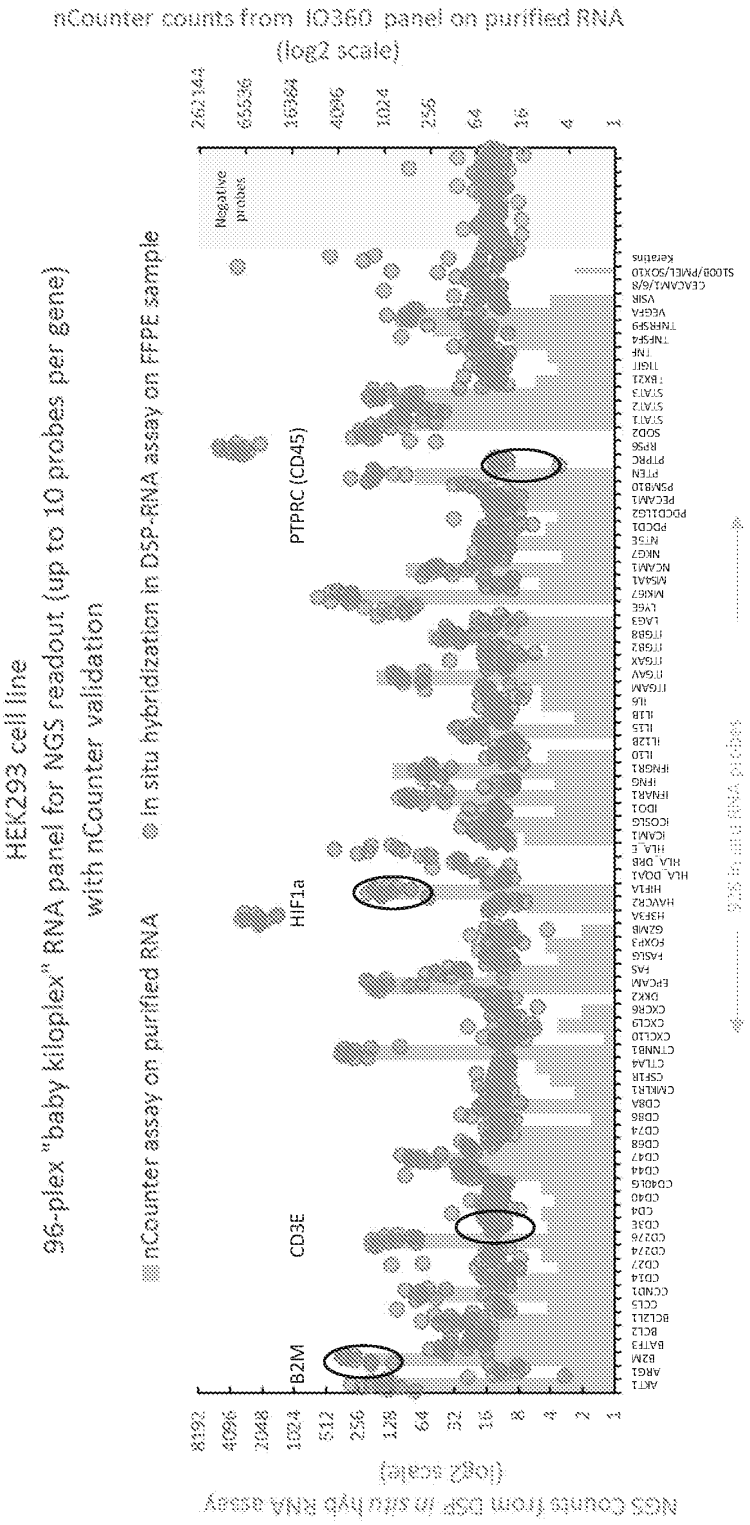

HEK293 cell line
96-plex "baby kiloplex" RNA panel for NGS readout (up to 10 probes per gene)
with nCounter validation
nCounter assay on purified RNA
in situ hybridization in DSP-RNA assay on FFPE sample
nCounter counts from IO360 panel on purified RNA
(log2 scale)
NGS Counts from DSP in situ hyb RNA assay
(log2 scale)
Negative probes
B2M
CD3E
HIF1a
PTPRC (CD45)
262144
65536
16384
4096
1024
256
64
16
4
1
8192
4096
2048
1024
512
256
128
64
32
16
8
4
2
1
ARG1
B2M
BATF3
BCL2
BCL2L1
CCL5
CCND1
CD14
CD27
CD274
CD276
CD3E
CD4
CD40
CD40LG
CD44
CD47
CD68
CD74
CD86
CD8A
CAMLR1
CSF1R
CTLA4
CTNNB1
CXCL10
CXCL9
CXCR6
DKK2
EPCAM
FAS
FASLG
FOXP3
GZMB
H3F3A
HAVCR2
HIF1A
HLA_DOA1
HLA_DRB
HLA_E
ICAM1
ICOSLG
IDO1
IFNAR1
IFNG
IFNGR1
IL10
IL12B
IL15
IL18
ITGAM
ITGAV
ITGAX
ITGB2
ITGB8
LAG3
LYVE
MKI67
MS4A1
NCAM1
NKG7
NT5E
PDCD1
PDCD1LG2
PECAM1
PSMB10
PTEN
PTPRC
RPS6
SOD2
STAT1
STAT2
STAT3
TBX21
TIGIT
TNF
TNFSF4
TNFRSF9
VEGFA
VSIR
CEACAM1/6/8
S100B/PMEL/SOX10
Keratins
B2M in situ RNA probes

Figure 33

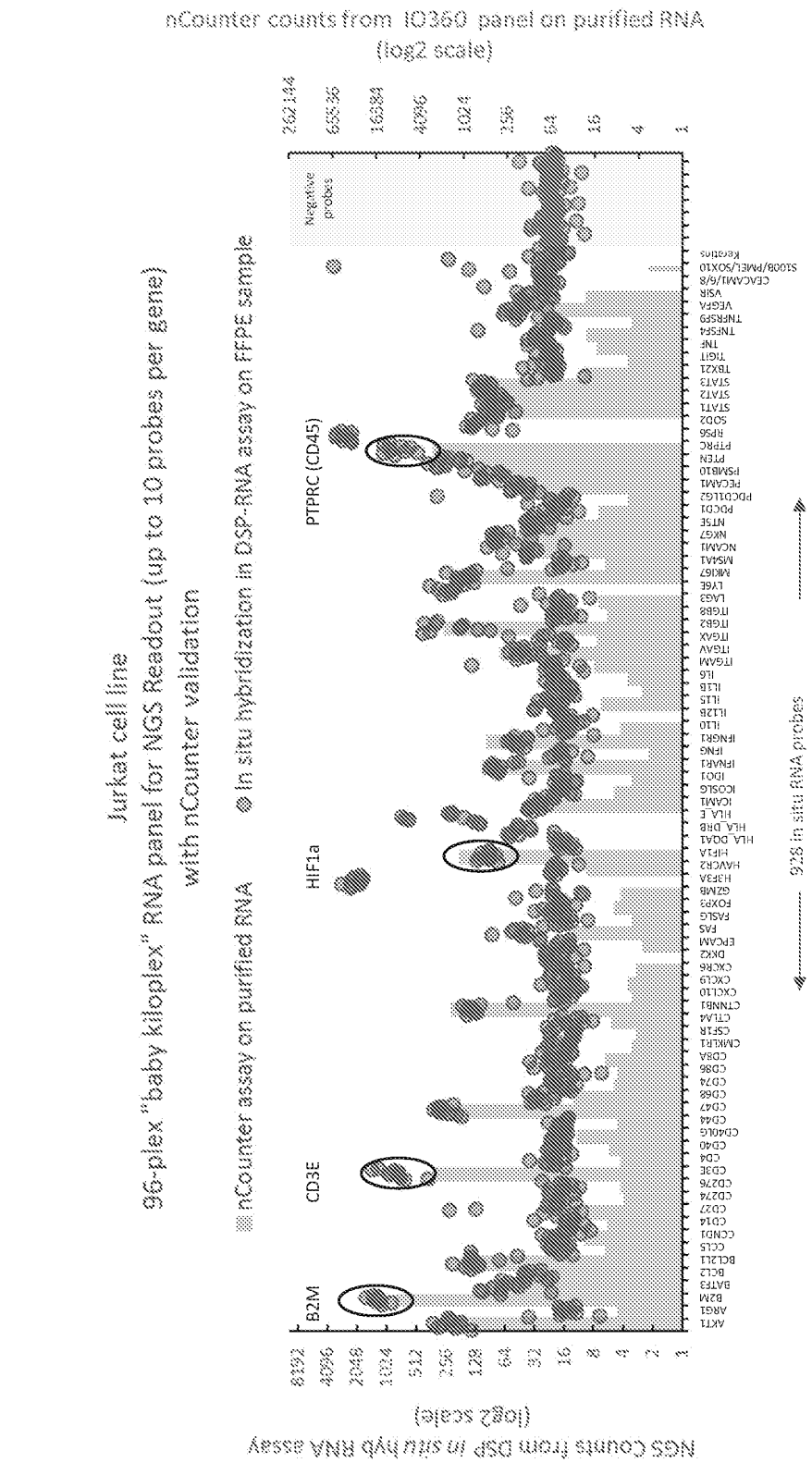

nCounter counts from IO360 panel on purified RNA
(log2 scale)
Jurkat cell line
96-plex "baby kiloplex" RNA panel for NGS Readout (up to 10 probes per gene)
with nCounter validation
nCounter assay on purified RNA
In situ hybridization in OSP-RNA assay on FFPE sample
B2M
CD3E
HIF1a
PTPRC (CD45)
Negative probes
926 in situ RNA probes
NGS Counts from OSP in situ hyb RNA assay
(log2 scale)

Figure 34