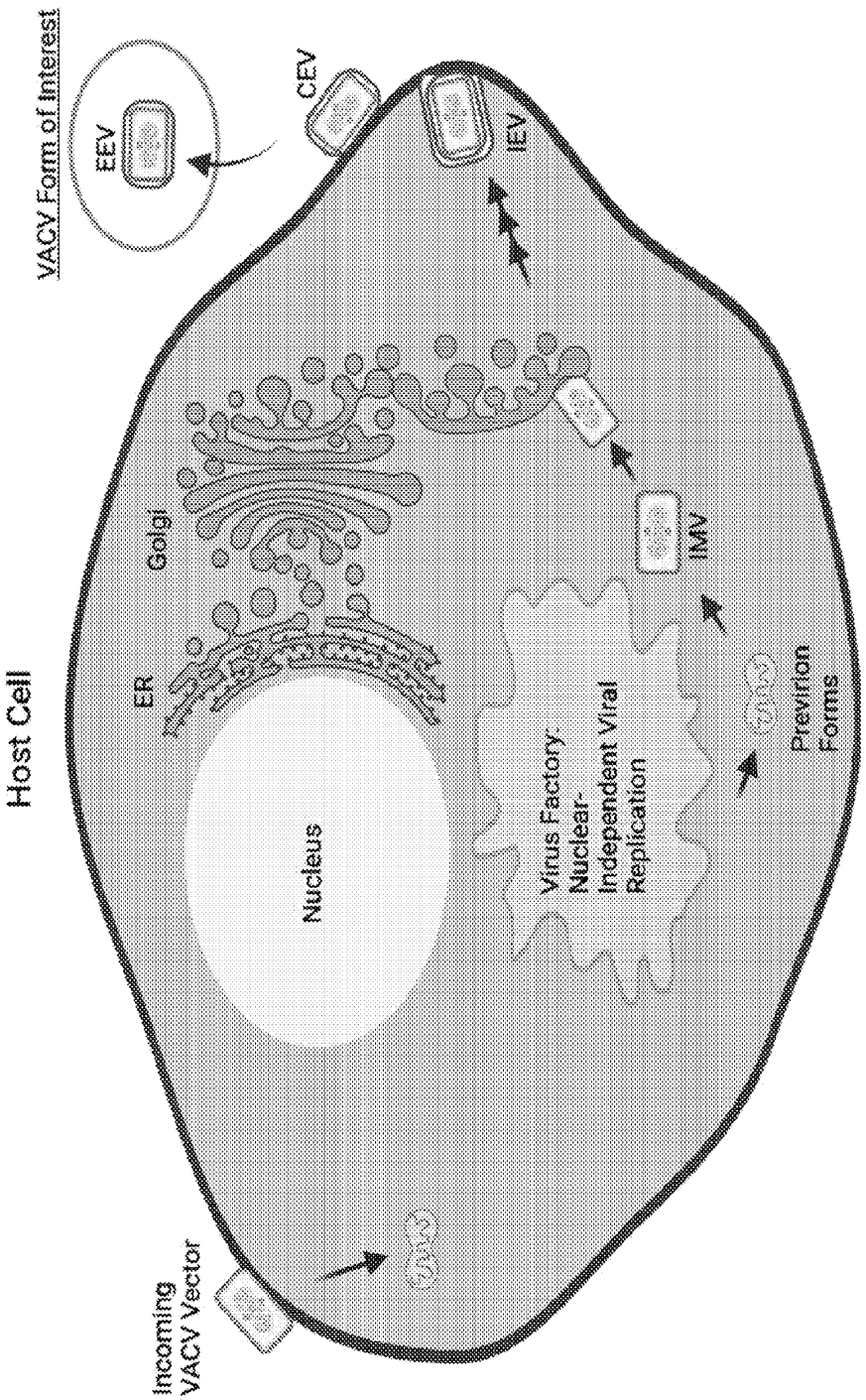

VACV Form of Interest
EEV
CEV
IEV
Host Cell
Golgi
ER
Nucleus
Virus Factory:
Nuclear-
Independent Viral
Replication
IMV
Previrion
Forms
Incoming
VACV Vector

FIG. 1A

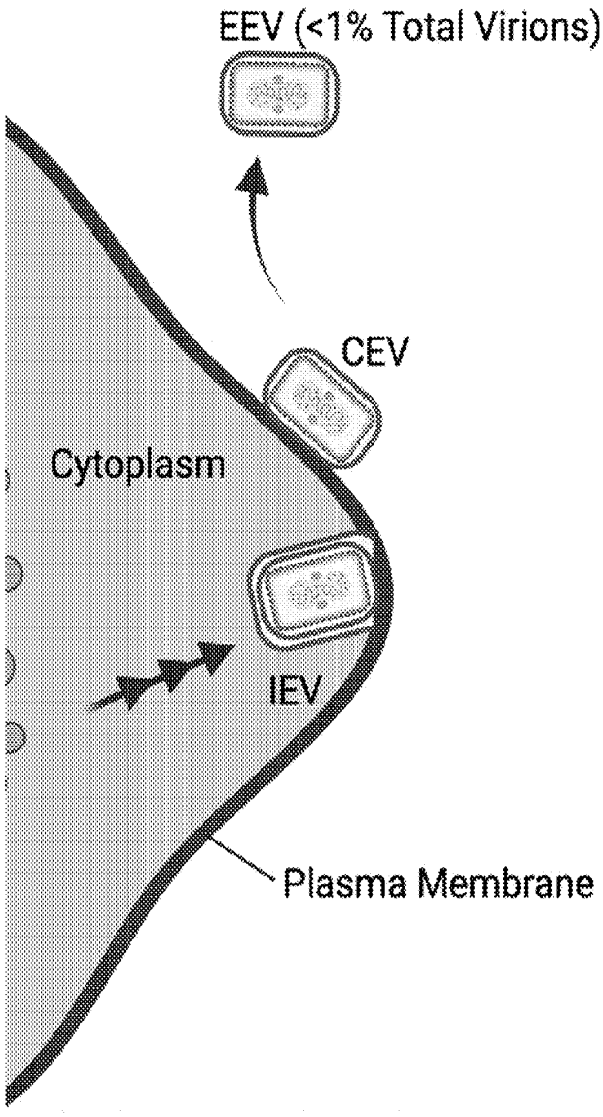


FIG. 1B

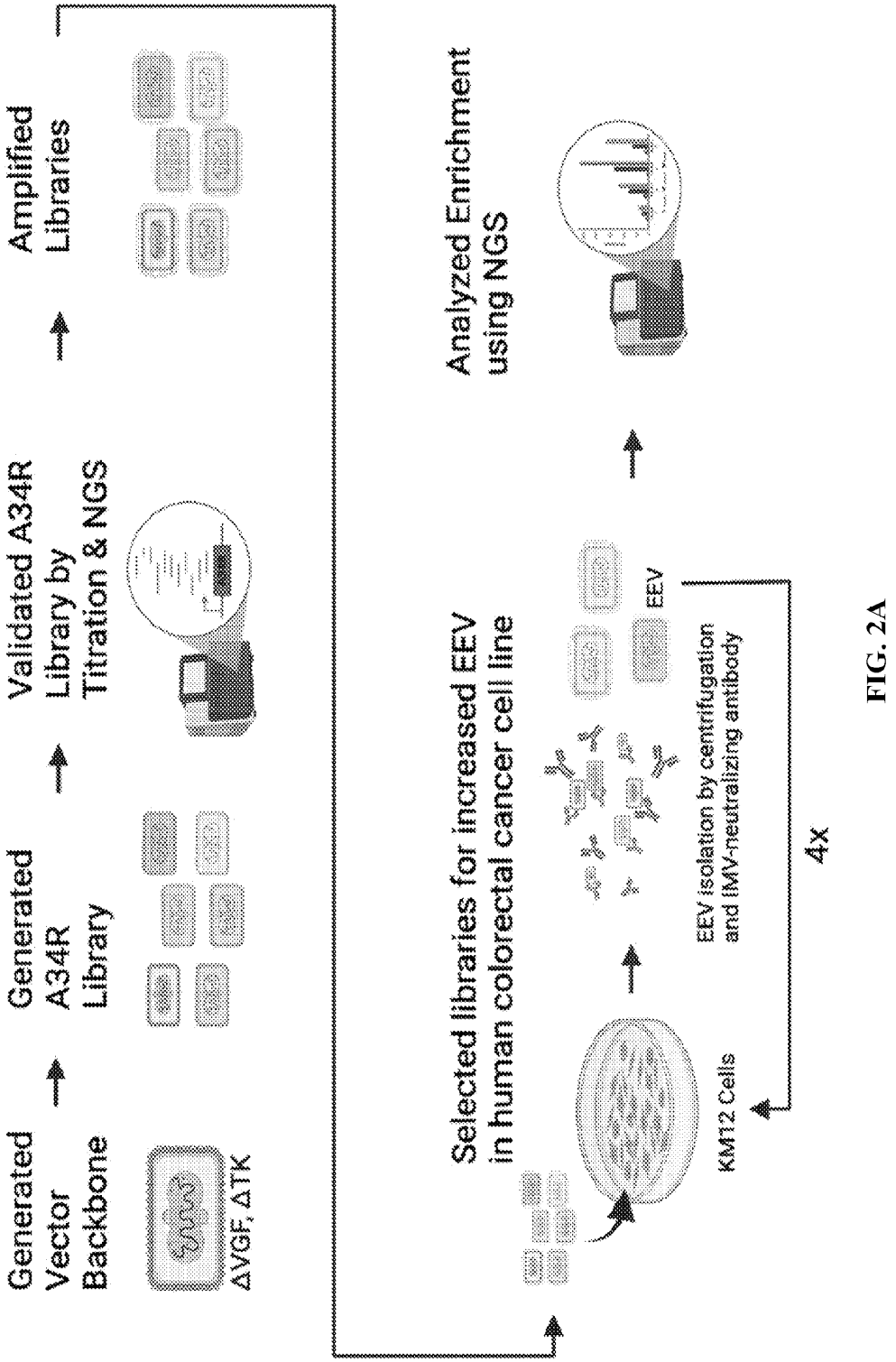

Generated Vector Backbone
ΔVGF, ΔTK
Generated A34R Library
Validated A34R Library by Titration & NGS
Amplified Libraries
Selected libraries for increased EEV in human colorectal cancer cell line
KM12 Cells
EEV isolation by centrifugation and IMV-neutralizing antibody
EEV
4x
Analyzed Enrichment using NGS

FIG. 2A

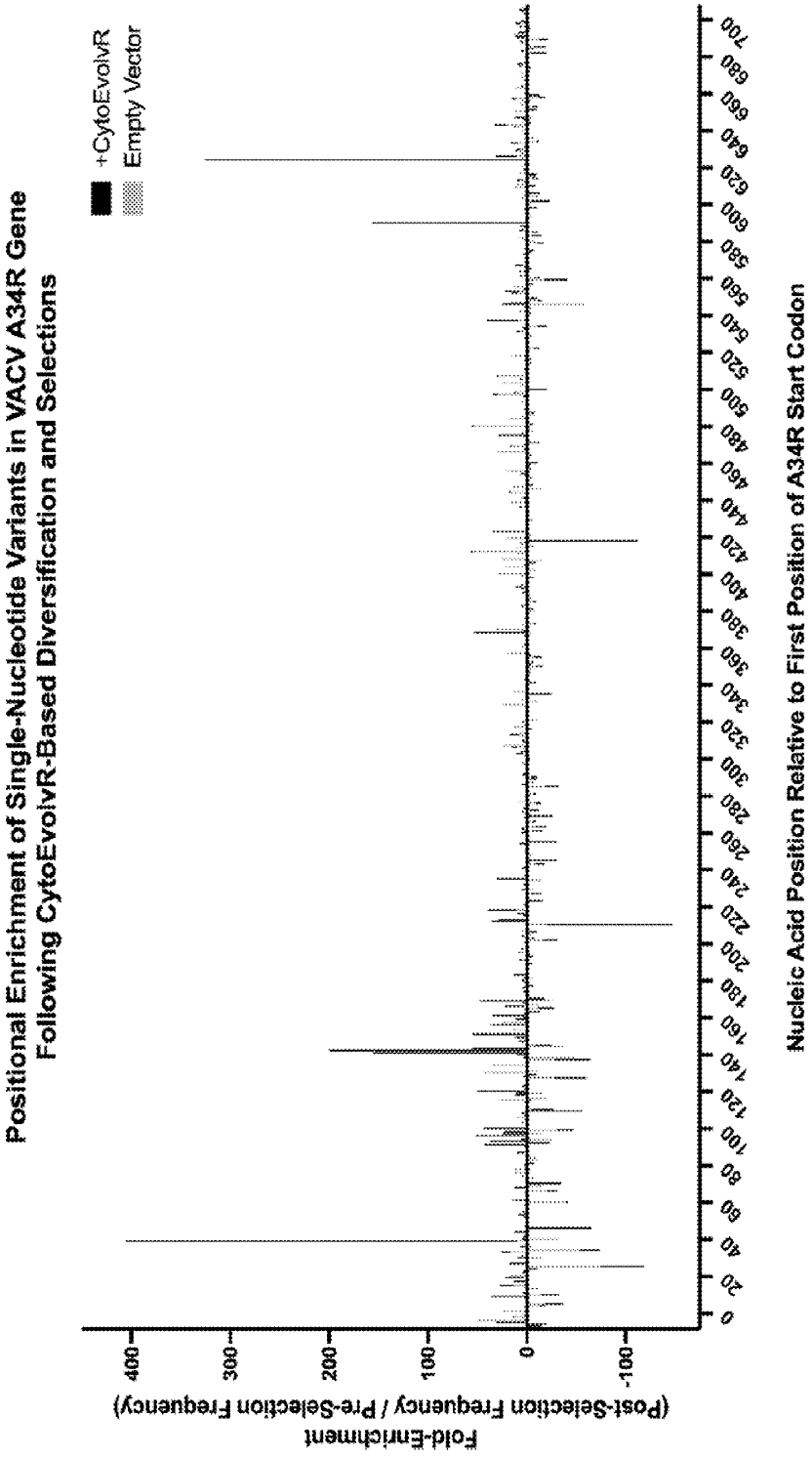

Positional Enrichment of Single-Nucleotide Variants in VACV A34R Gene
Following CytoEvolvR-Based Diversification and Selections
+CytoEvolvR
Empty Vector
Fold-Enrichment
(Post-Selection Frequency / Pre-Selection Frequency)
400
300
200
100
0
-100
Nucleic Acid Position Relative to First Position of A34R Start Codon
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
420
440
460
480
500
520
540
560
580
600
620
640
660
680
700

FIG. 2B

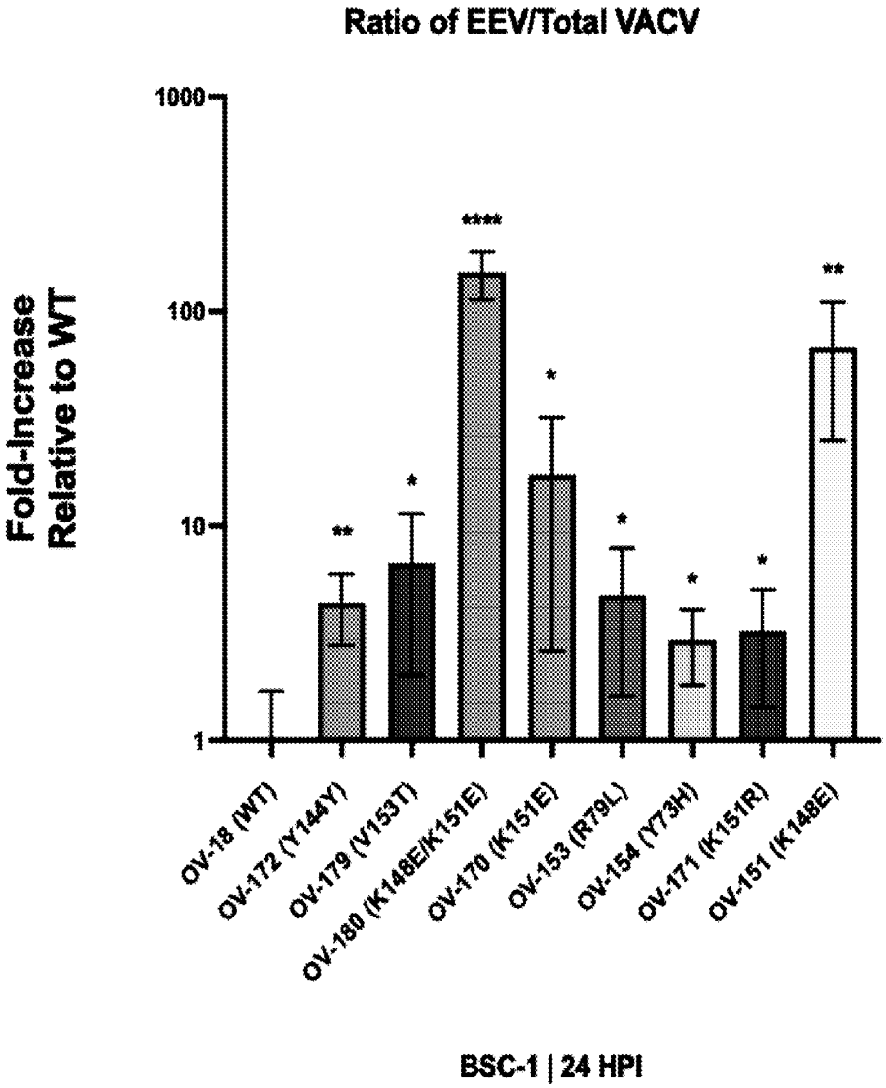

Ratio of EEV/Total VACV
Fold-Increase Relative to WT
1000
100
10
1
****
**
*
*
*
*
*
**
OV-18 (WT)
OV-172 (Y144Y)
OV-179 (V153T)
OV-180 (K148E/K151E)
OV-170 (K151E)
OV-153 (R79L)
OV-154 (Y73H)
OV-171 (K151R)
OV-151 (K148E)
BSC-1 | 24 HPI

FIG. 3A

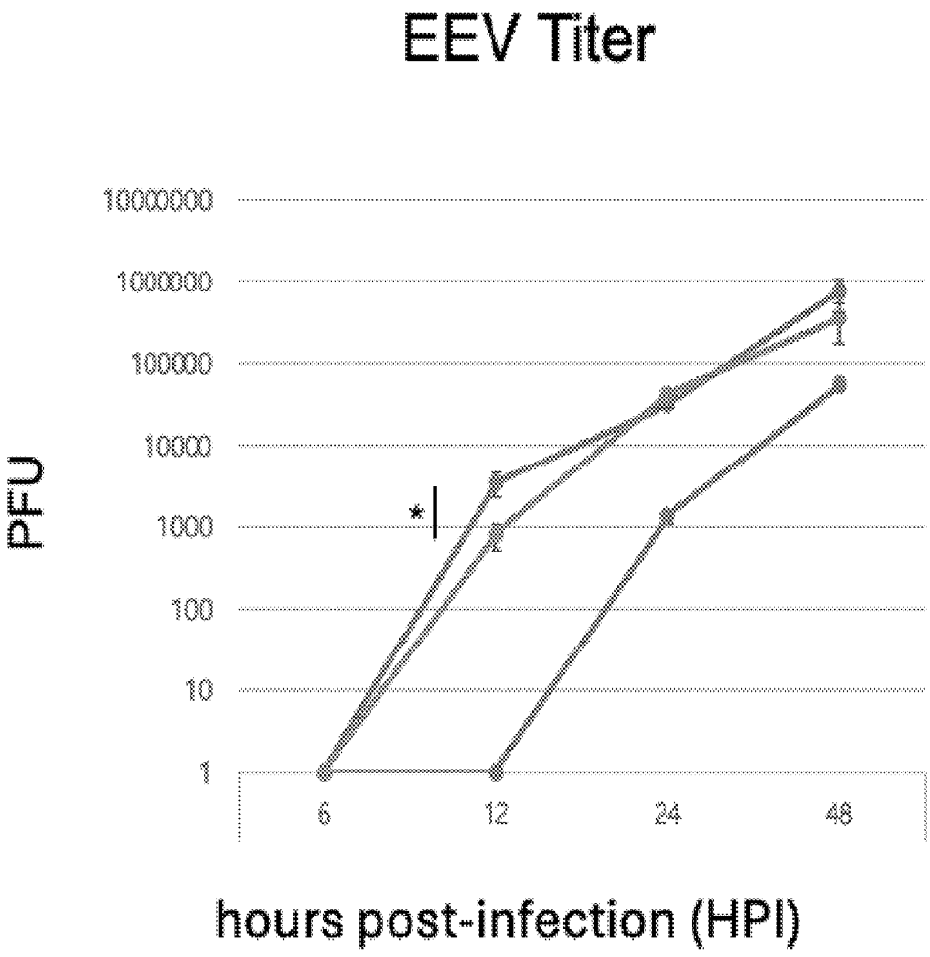

EEV Titer
10000000
1000000
100000
10000
1000
100
10
1
PFU
*
6
12
24
48
hours post-infection (HPI)

FIG. 3B

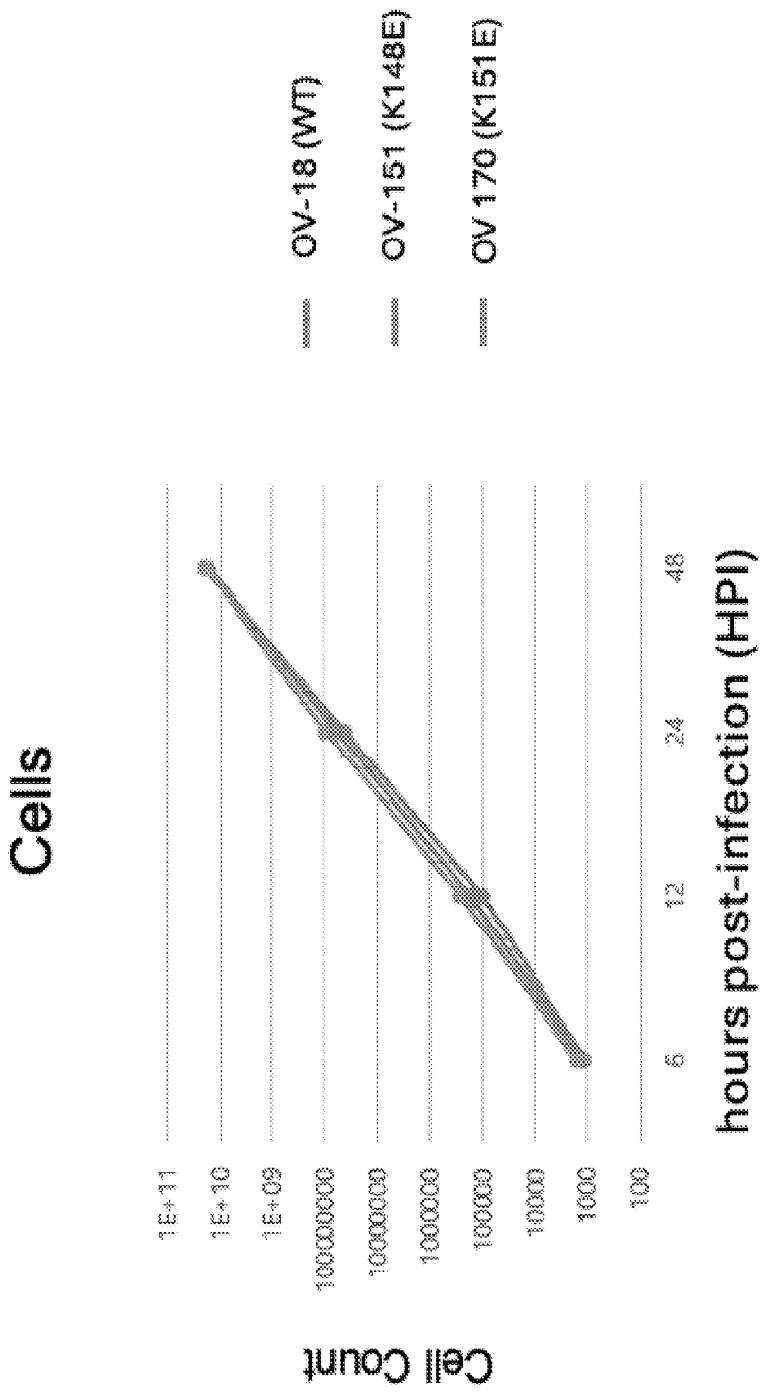

Cells
OV-18 (WT)
OV-151 (K148E)
OV 170 (K151E)
Cell Count
1E+11
1E+10
1E+09
100000000
10000000
1000000
100000
10000
1000
100
6
12
24
48
hours post-infection (HPI)

FIG. 3C

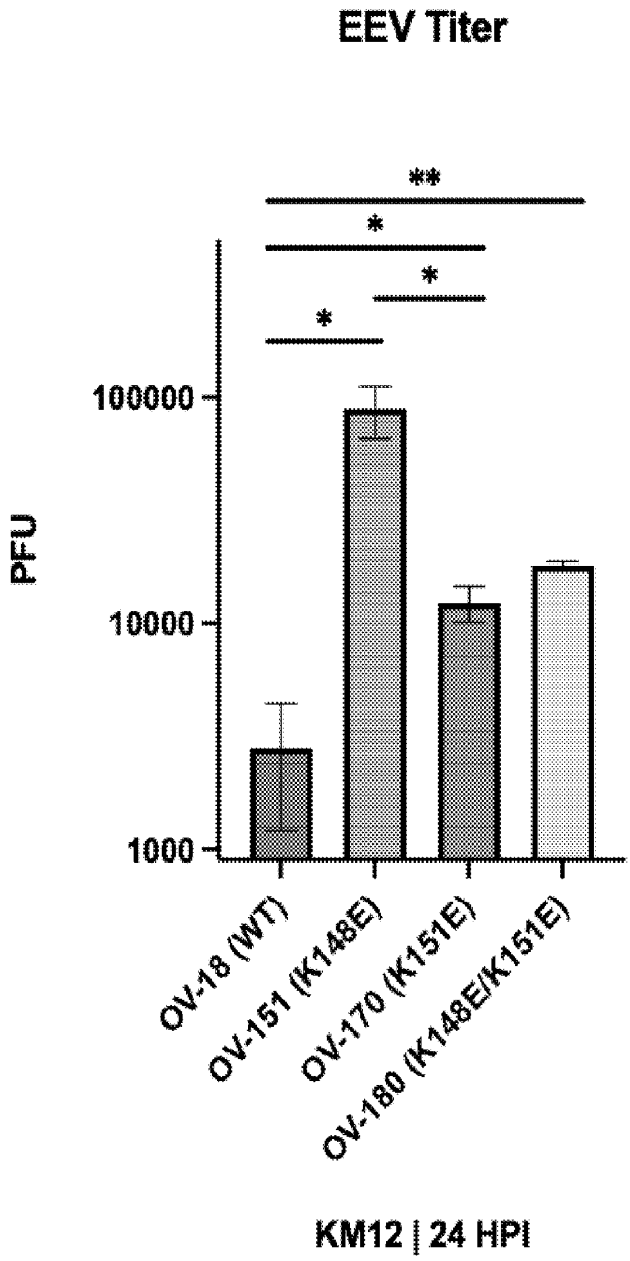

EEV Titer
**
*
*
*
100000
10000
1000
PFU
OV-18 (WT)
OV-151 (K148E)
OV-170 (K151E)
OV-180 (K148E/K151E)
KM12 | 24 HPI

FIG. 4A

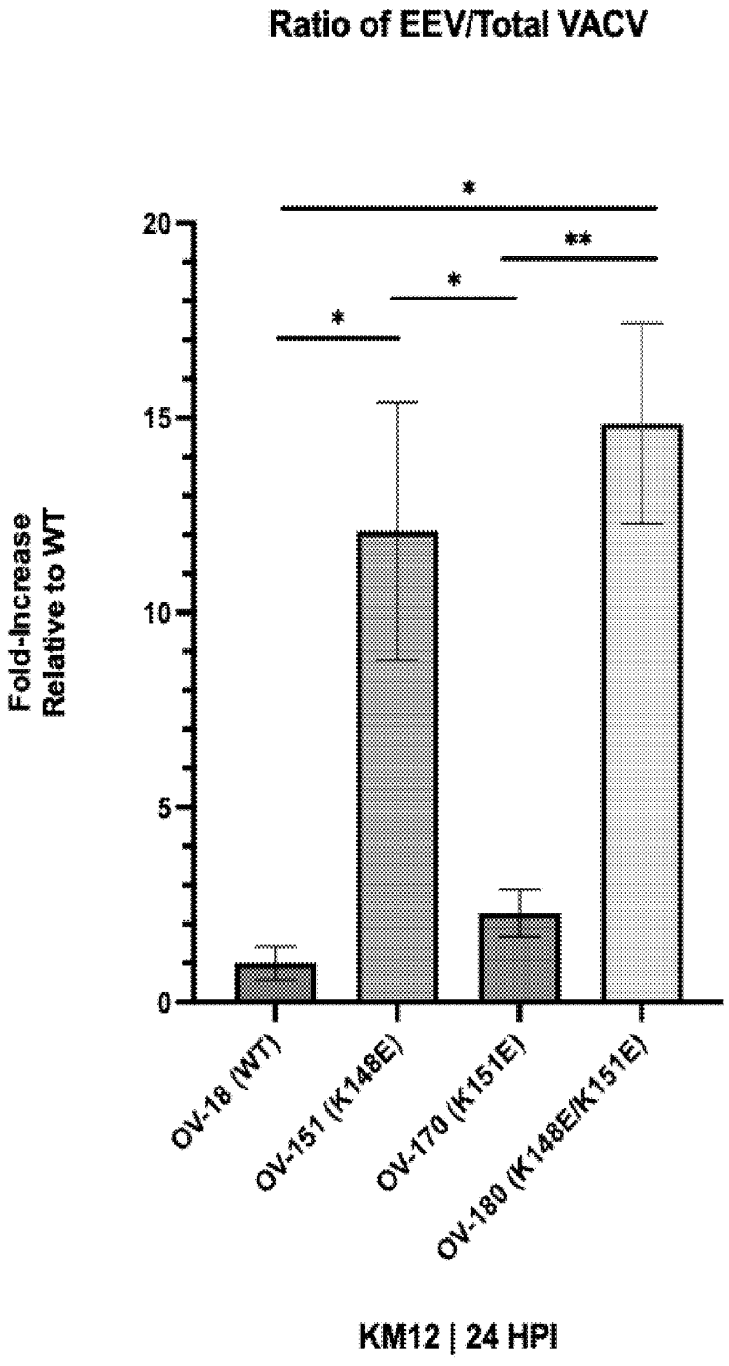

Ratio of EEV/Total VACV
Fold-Increase Relative to WT
0
5
10
15
20
*
*
*
**
OV-18 (WT)
OV-151 (K148E)
OV-170 (K151E)
OV-180 (K148E/K151E)
KM12 | 24 HPI

FIG. 4B

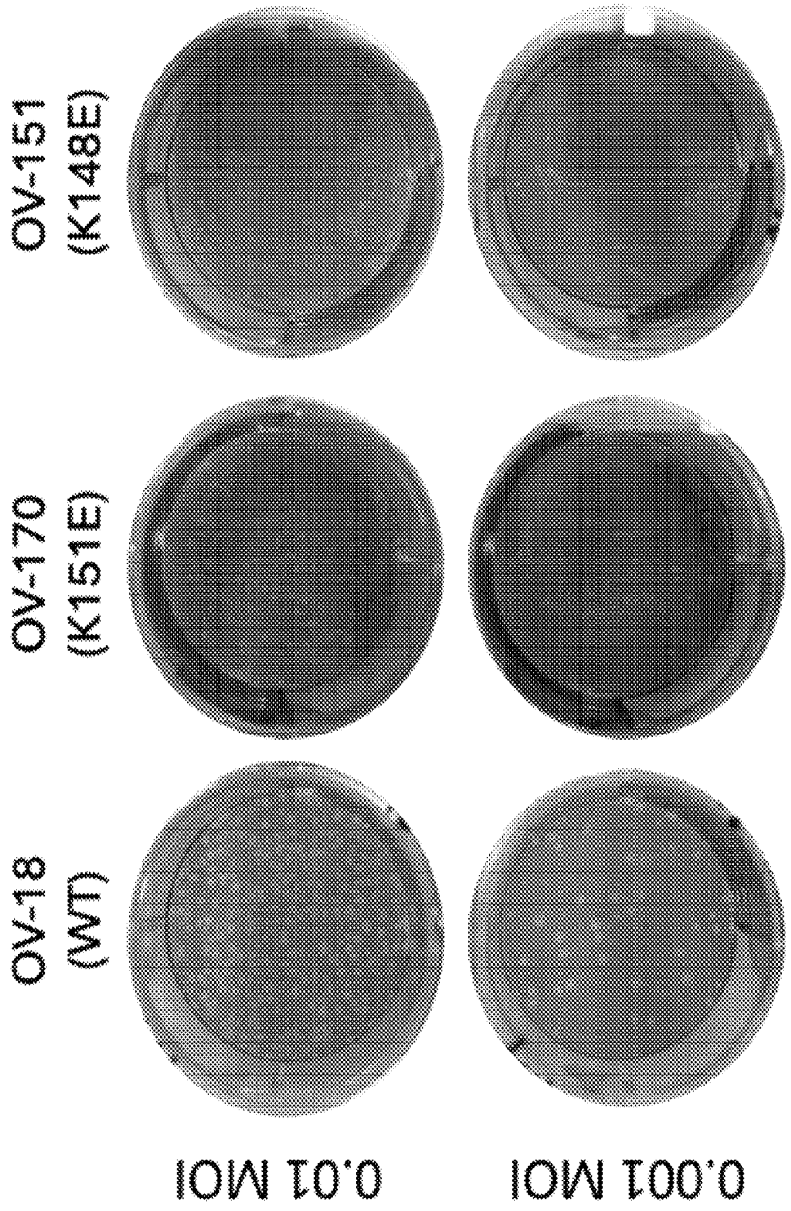


FIG. 5A

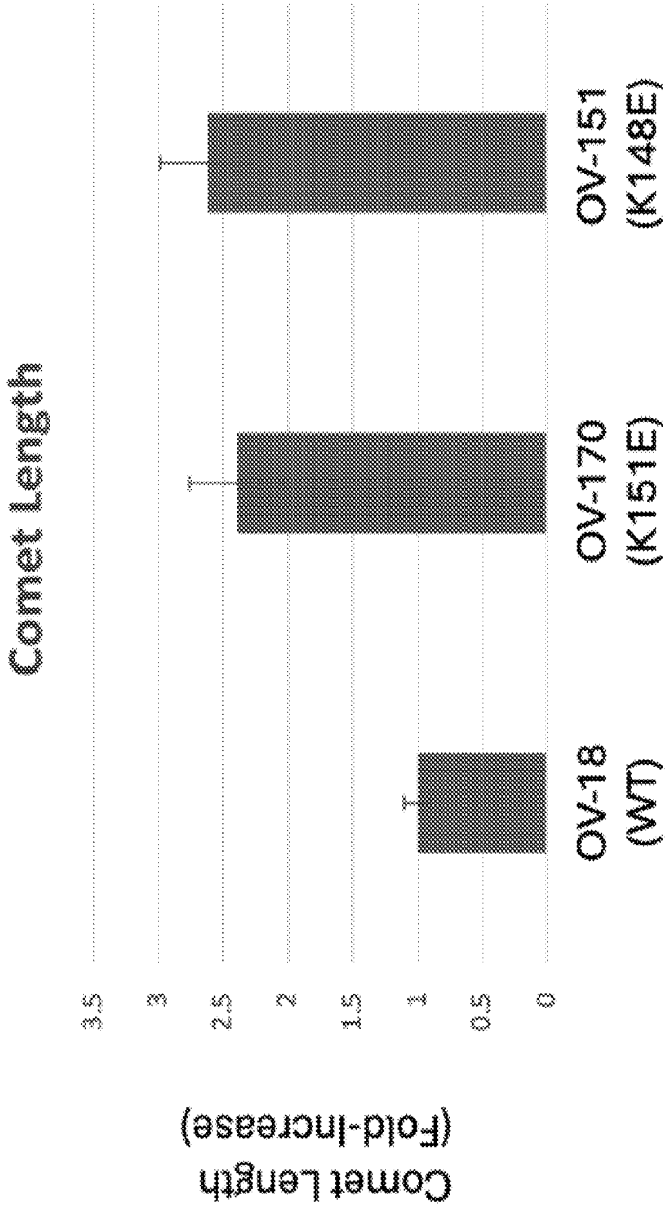

Comet Length
(Fold-Increase)
3.5
3
2.5
2
1.5
1
0.5
0
OV-18
(WT)
OV-170
(K151E)
OV-151
(K148E)

FIG. 5B

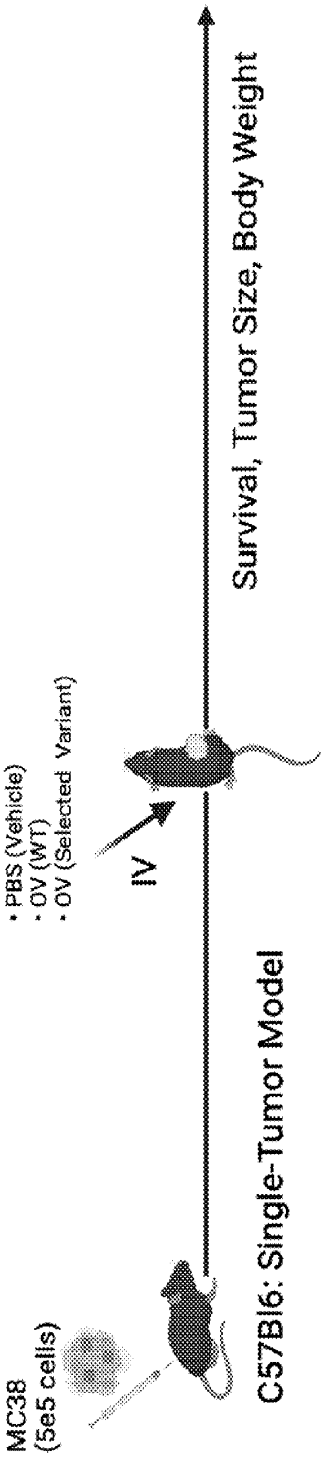

MC38
(5e5 cells)
C57Bl6: Single-Tumor Model
IV
• PBS (Vehicle)
• OV (WT)
• OV (Selected Variant)
Survival, Tumor Size, Body Weight

FIG. 6A


PBS (-) Control
OV Encoding Wild-Type A34R Protein + IL-2v
OV Encoding A34R Protein K148E Variant + IL-2v
Percent Survival
100
50
0
0
10
20
30
Days after tumor implantation

FIG. 6B

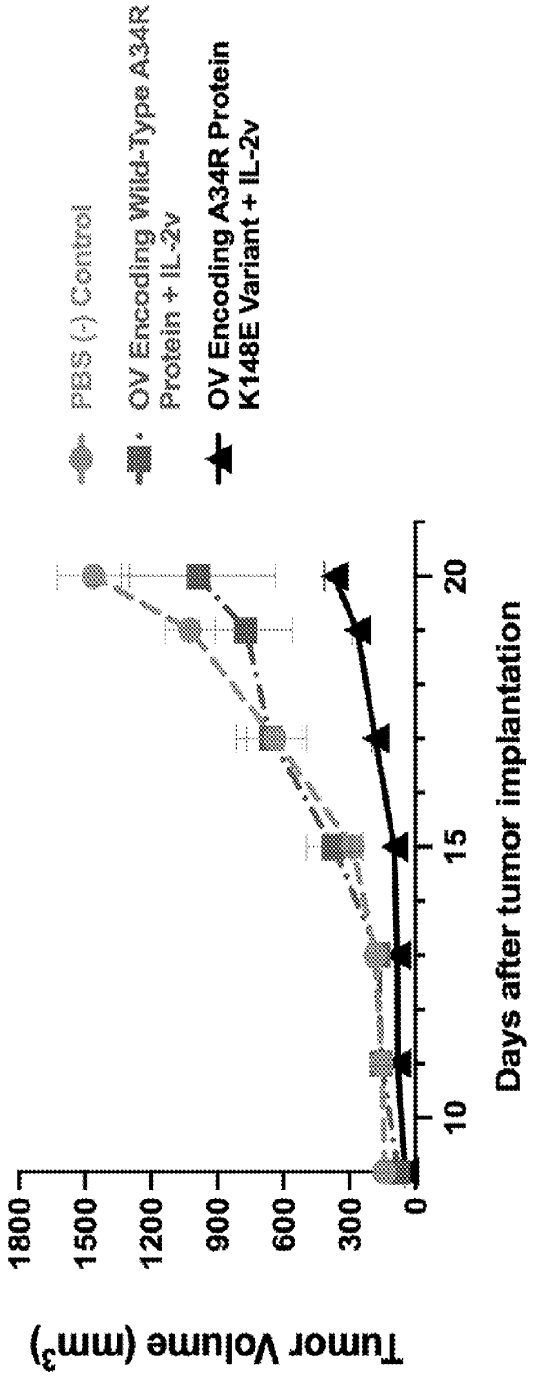

PBS (-) Control
OV Encoding Wild-Type A34R Protein + IL-2v
OV Encoding A34R Protein K148E Variant + IL-2v
Tumor Volume (mm³)
1800
1500
1200
900
600
300
0
10
15
20
Days after tumor implantation

FIG. 6C

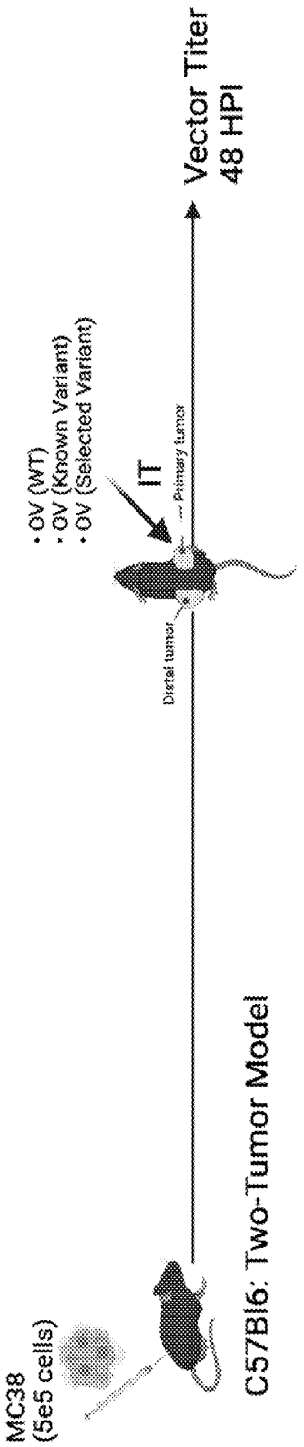

Vector Titer
48 HPI
IT
Primary tumor
• OV (WT)
• OV (Known Variant)
• OV (Selected Variant)
Distal tumor
C57Bl6: Two-Tumor Model
MC38
(5e5 cells)

FIG. 7A

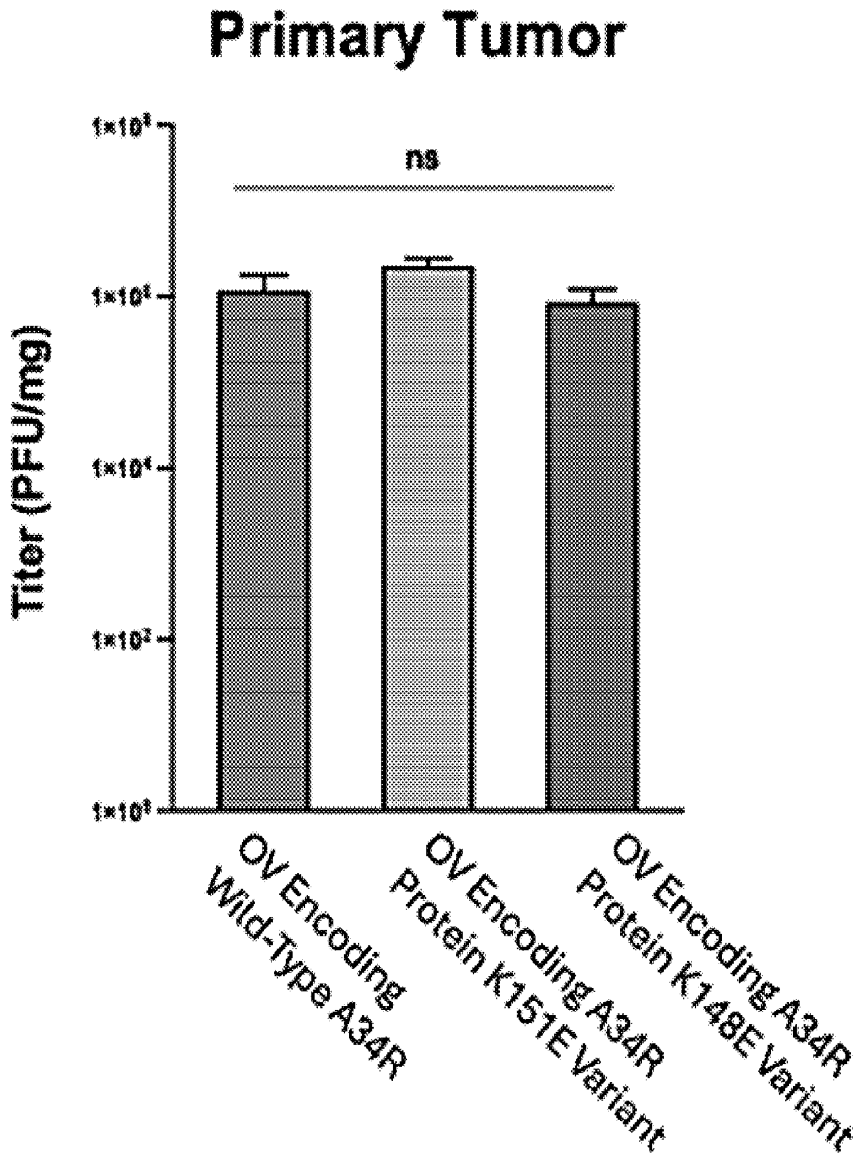

Primary Tumor
ns
Titer (PFU/mg)
1×10⁵
1×10⁴
1×10³
1×10²
1×10¹
OV Encoding Wild-Type A34R
OV Encoding A34R Protein K151E Variant
OV Encoding A34R Protein K148E Variant

FIG. 7B

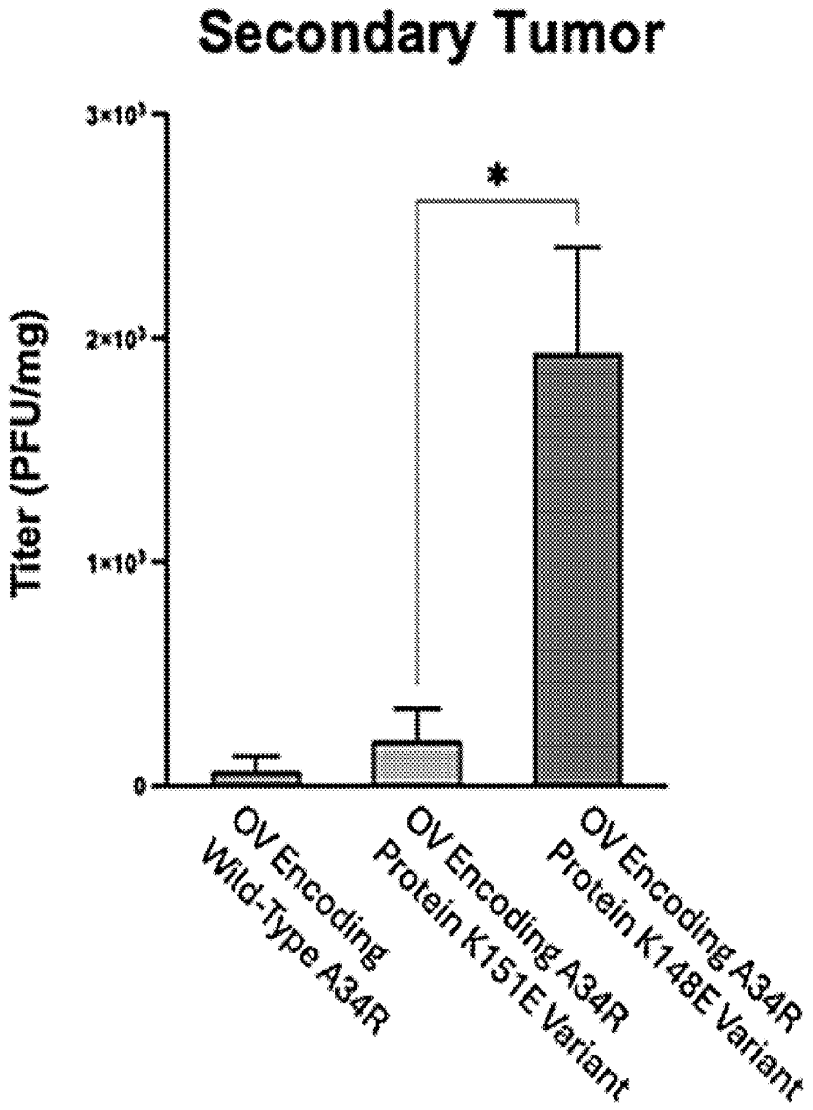

Secondary Tumor
Titer (PFU/mg)
3×10⁷
2×10⁷
1×10⁷
0
*
OV Encoding Wild-Type A34R
OV Encoding A34R Protein K151E Variant
OV Encoding A34R Protein K148E Variant

FIG. 7C

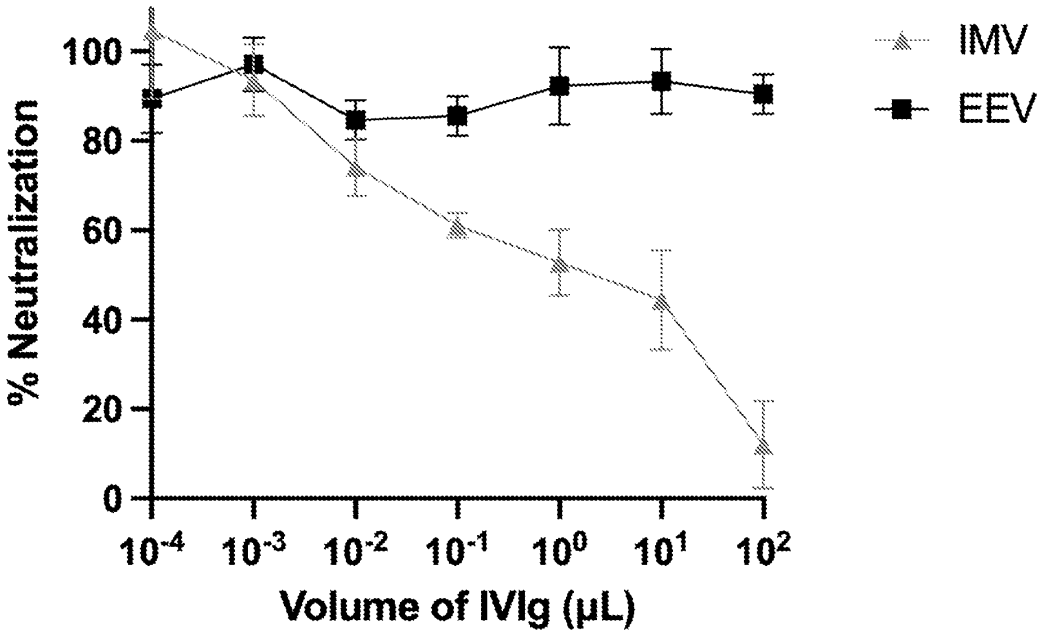

% Neutralization
100
80
60
40
20
0
10-4
10-3
10-2
10-1
100
101
102
Volume of IVIg (µL)
IMV
EEV

FIG. 8

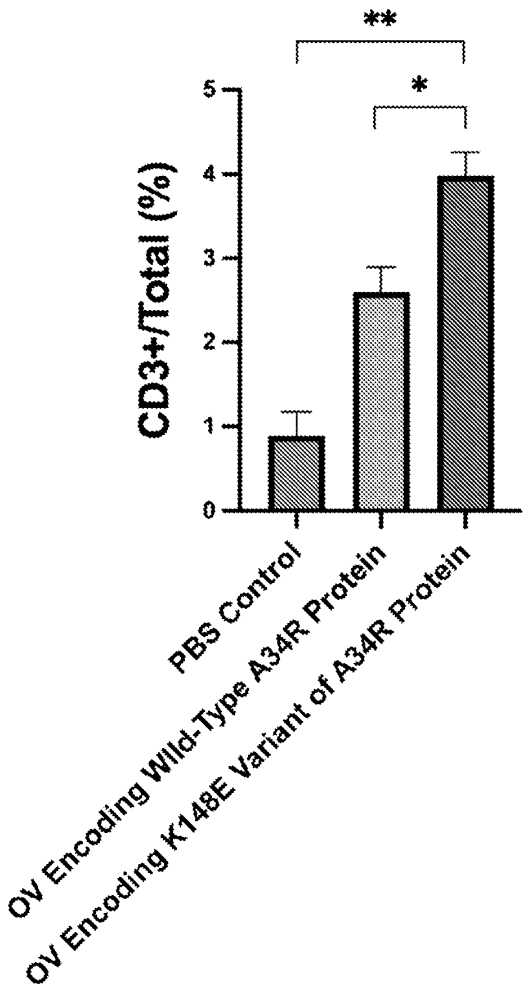

**
*
5
4
3
2
1
0
CD3+/Total (%)
PBS Control
OV Encoding Wild-Type A34R Protein
OV Encoding K148E Variant of A34R Protein

FIG. 9A

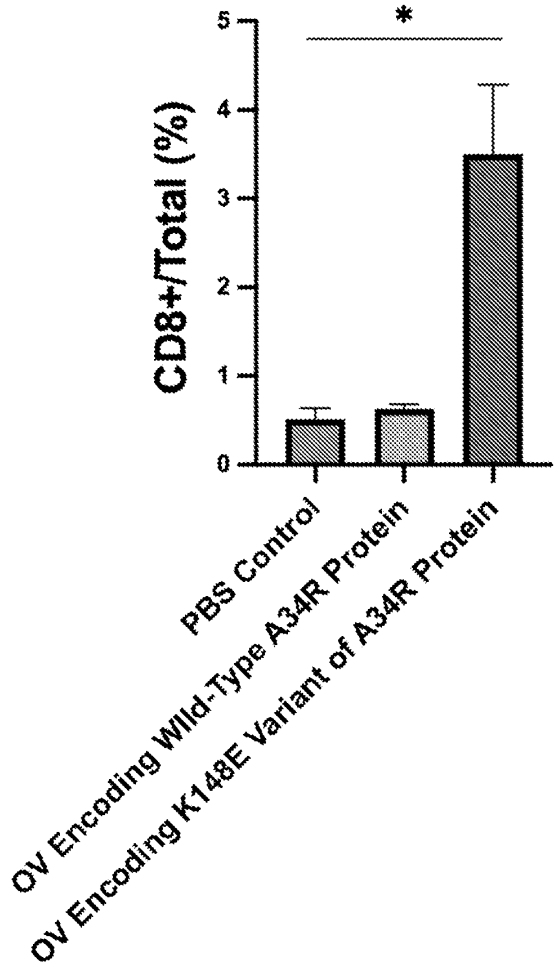

*
5
4
3
2
1
0
CD8+/Total (%)
PBS Control
OV Encoding Wild-Type A34R Protein
OV Encoding K148E Variant of A34R Protein

FIG. 9B

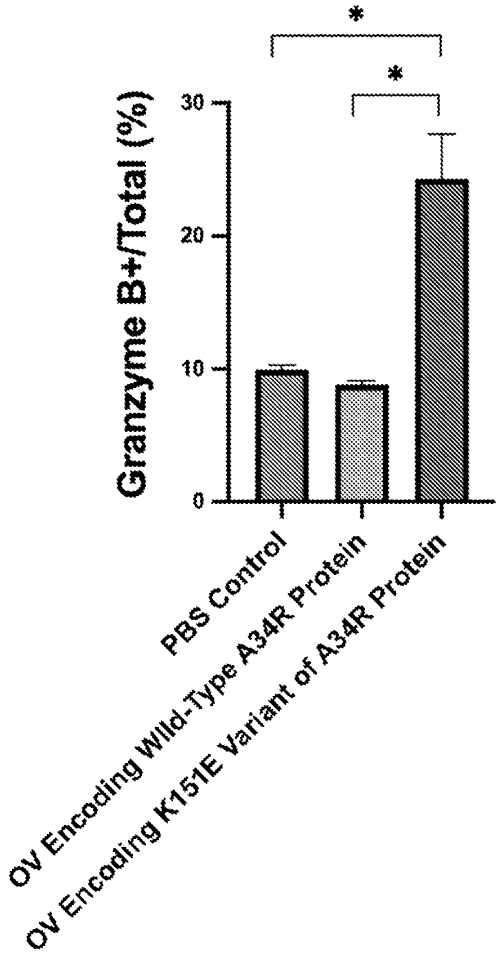

*
*
Granzyme B+/Total (%)
30
20
10
0
PBS Control
OV Encoding Wild-Type A34R Protein
OV Encoding K151E Variant of A34R Protein

FIG. 9C

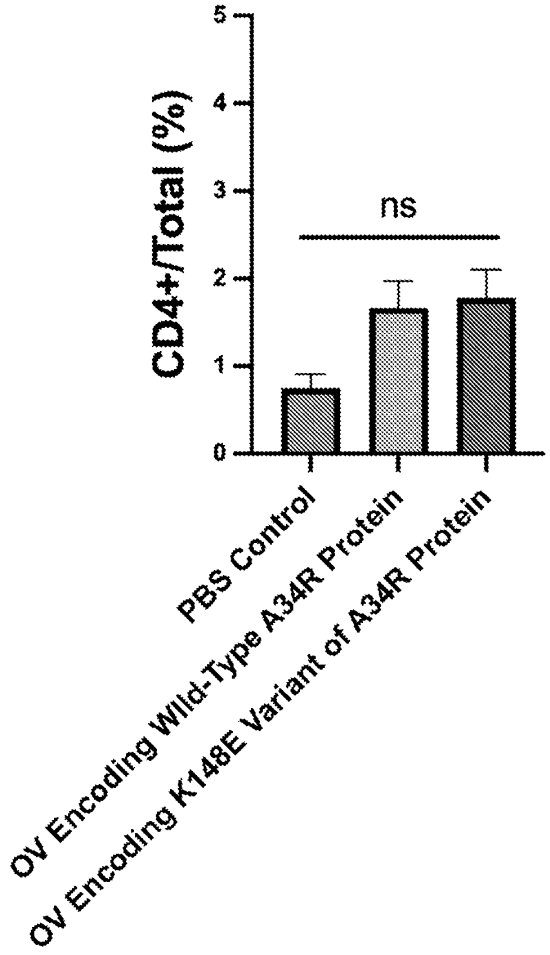

CD4+/Total (%)
5
4
3
2
1
0
ns
PBS Control
OV Encoding Wild-Type A34R Protein
OV Encoding K148E Variant of A34R Protein

FIG. 9D

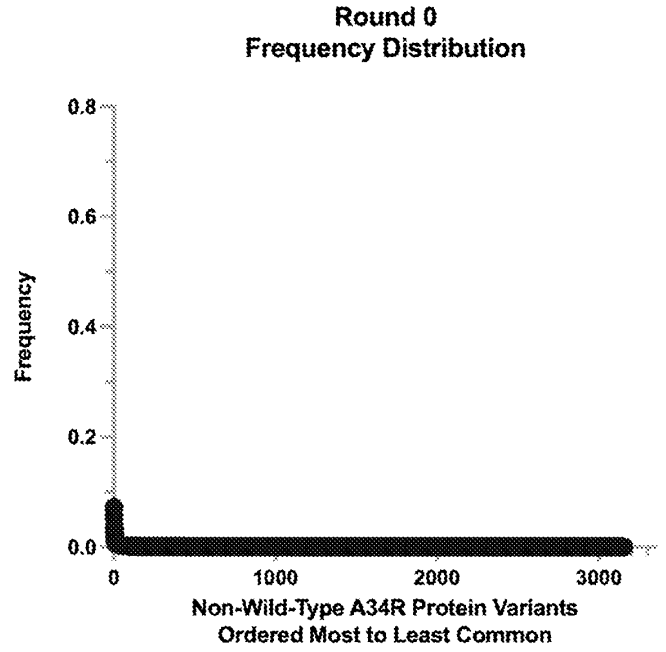


FIG. 10A

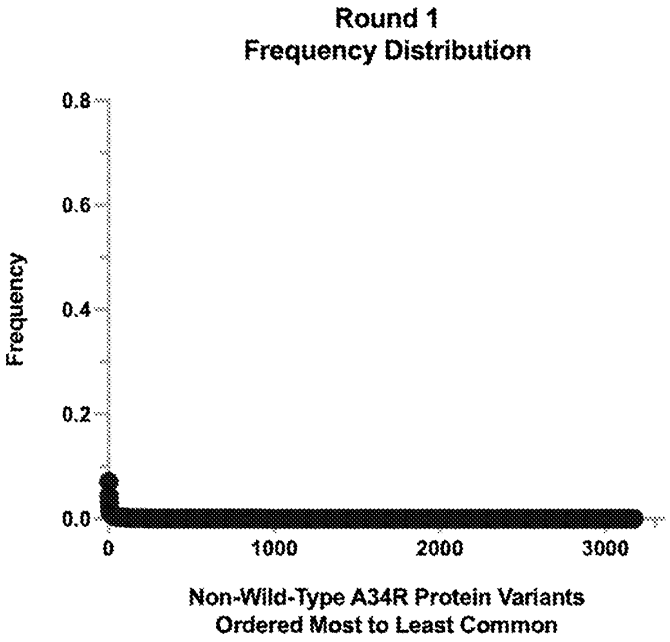


FIG. 10B

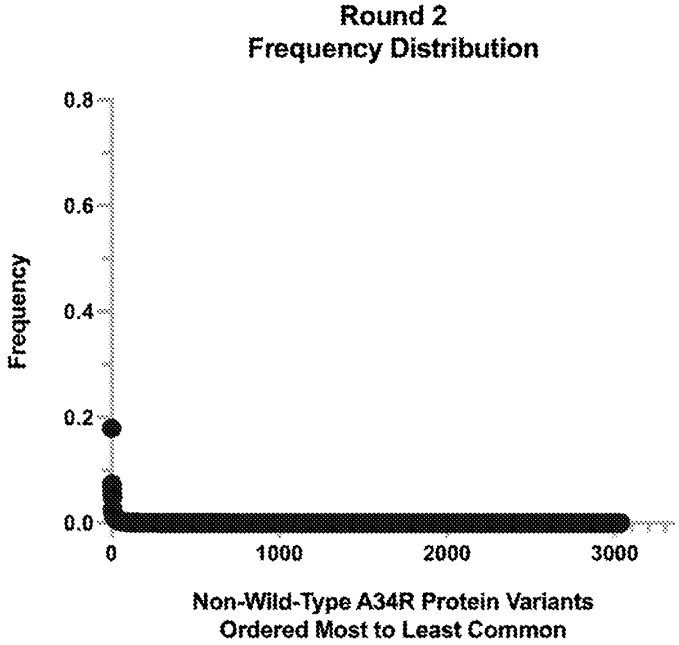


FIG. 10C

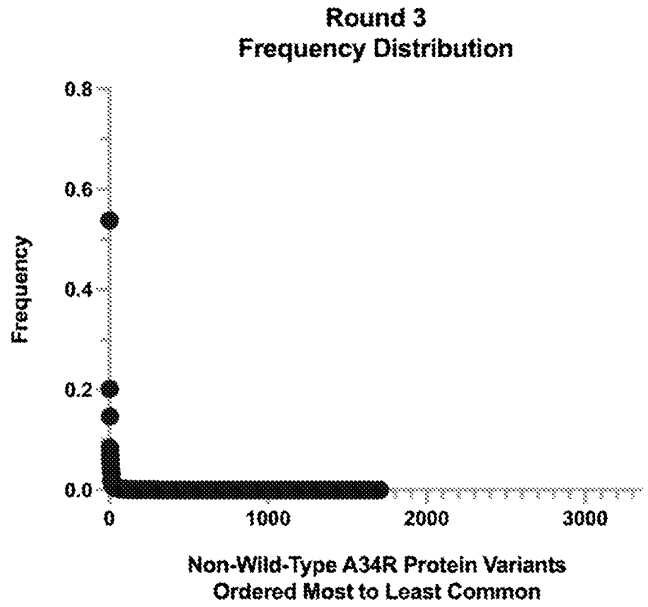


FIG. 10D

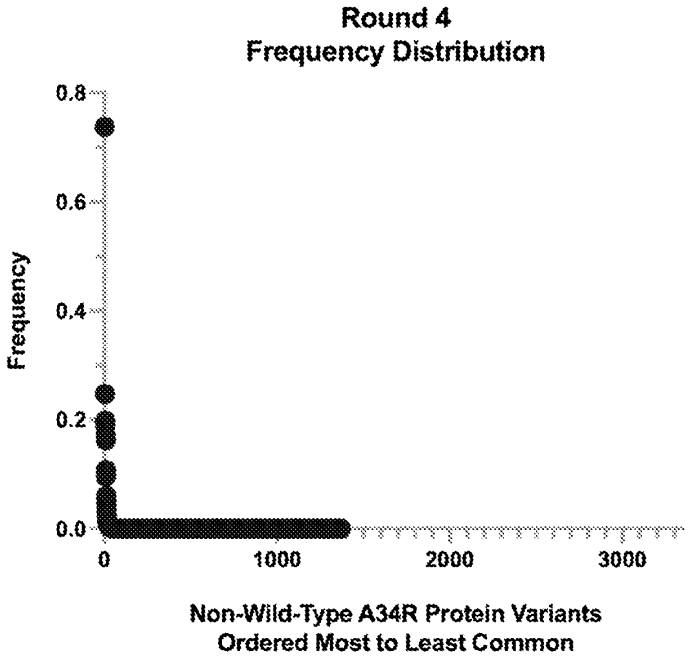

Round 4
Frequency Distribution
0.8
0.6
0.4
0.2
0.0
Frequency
0
1000
2000
3000
Non-Wild-Type A34R Protein Variants
Ordered Most to Least Common

FIG. 10E

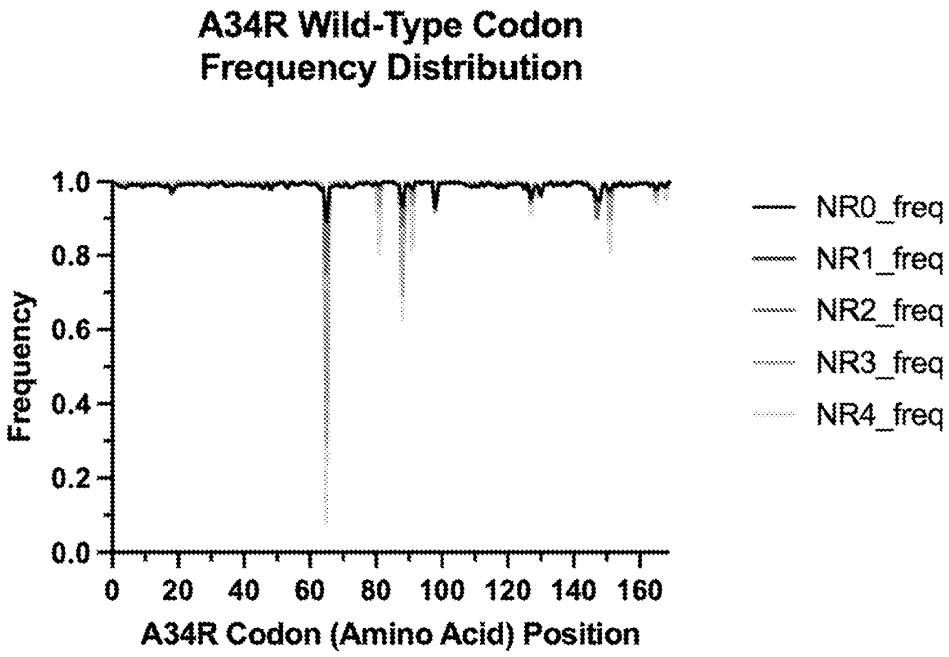

A34R Wild-Type Codon
Frequency Distribution
Frequency
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
120
140
160
A34R Codon (Amino Acid) Position
NR0_freq
NR1_freq
NR2_freq
NR3_freq
NR4_freq

FIG. 11

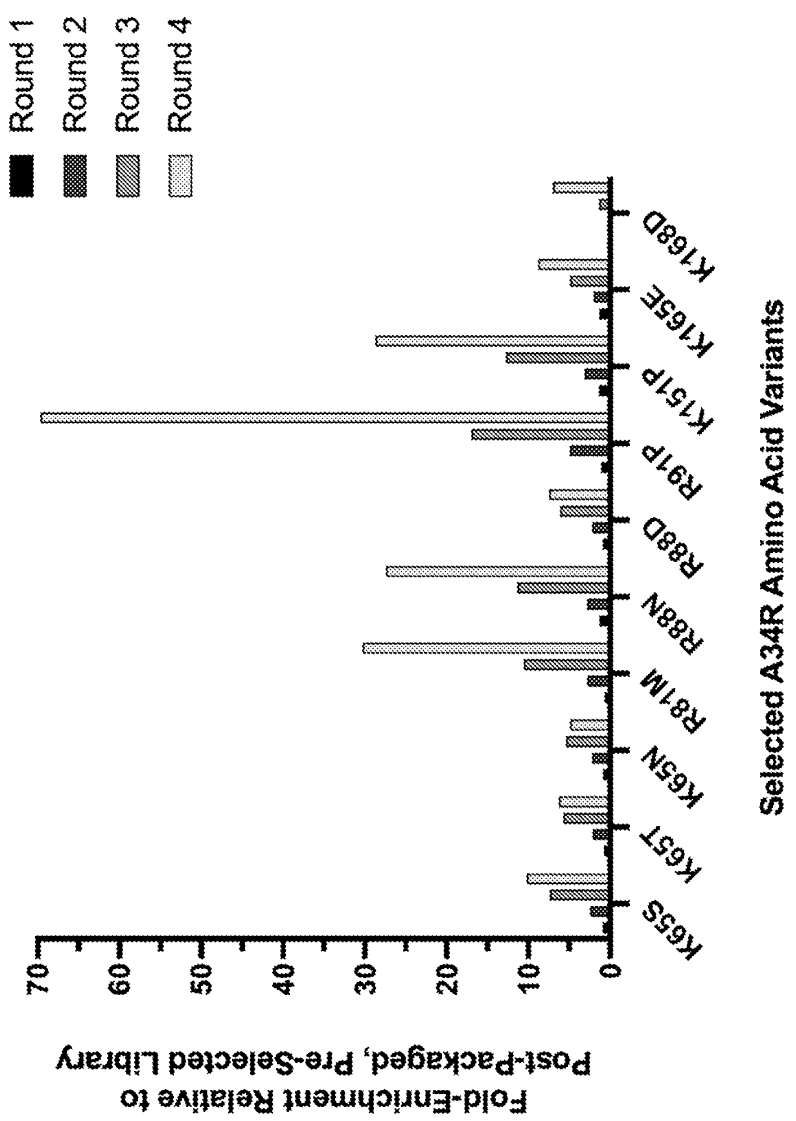
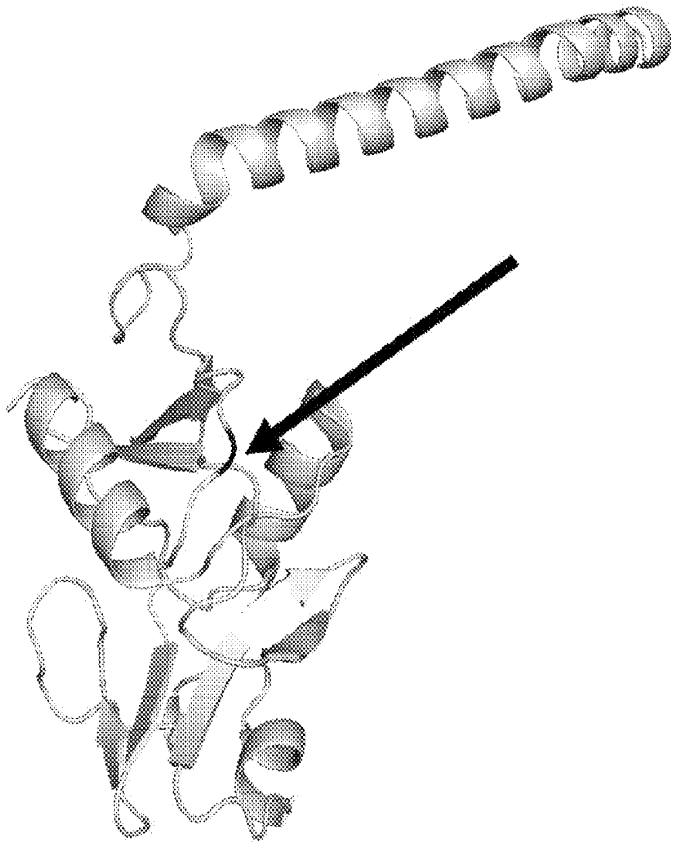
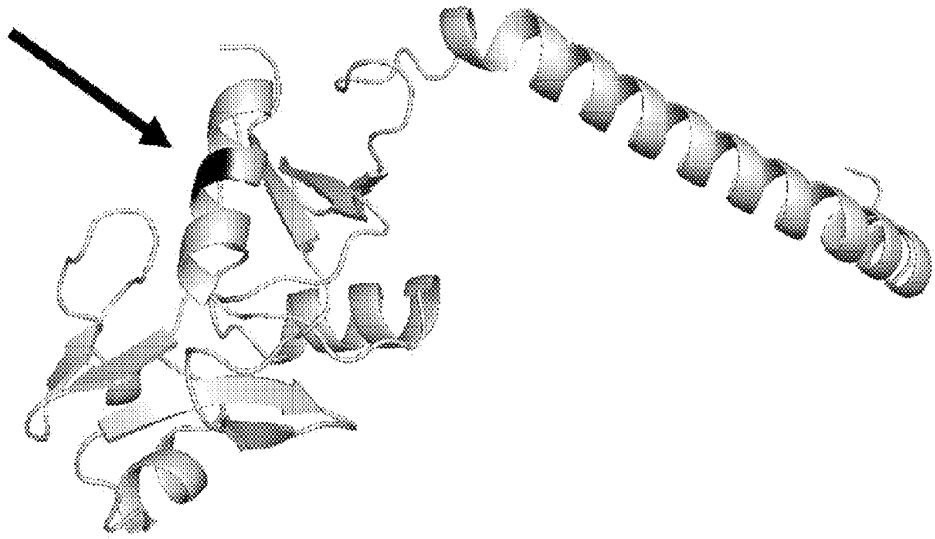

Round 1
Round 2
Round 3
Round 4
Fold-Enrichment Relative to Post-Packaged, Pre-Selected Library
0
10
20
30
40
50
60
70
K65S
K65T
K65N
R81M
R88N
R88D
R91P
K151P
K165E
K168D
Selected A34R Amino Acid Variants

FIG. 12



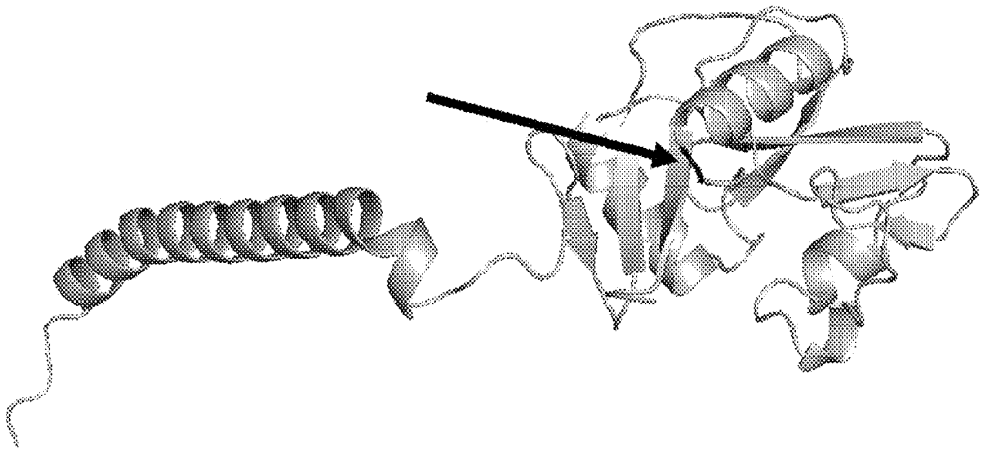
A34R Protein K65 Position

FIG. 13A



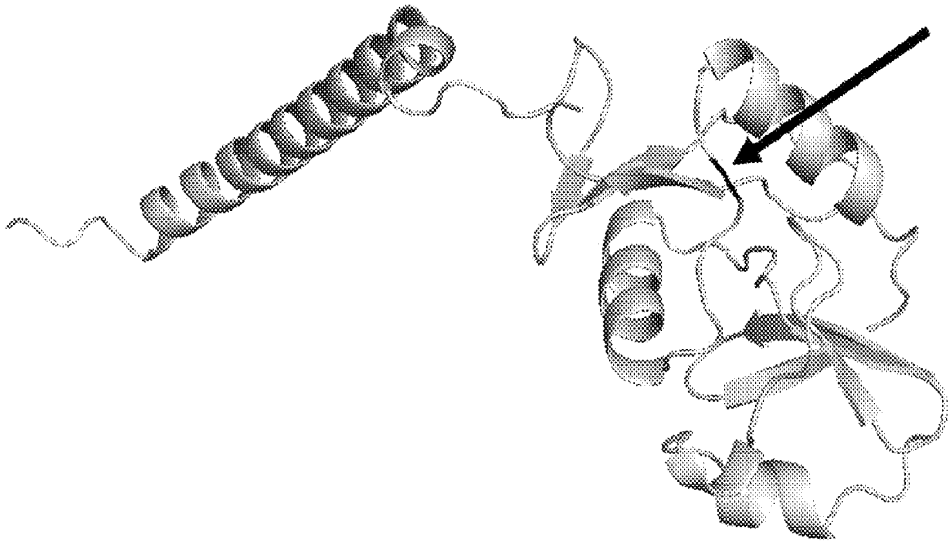
A34R Protein Y73 Position

FIG. 13B



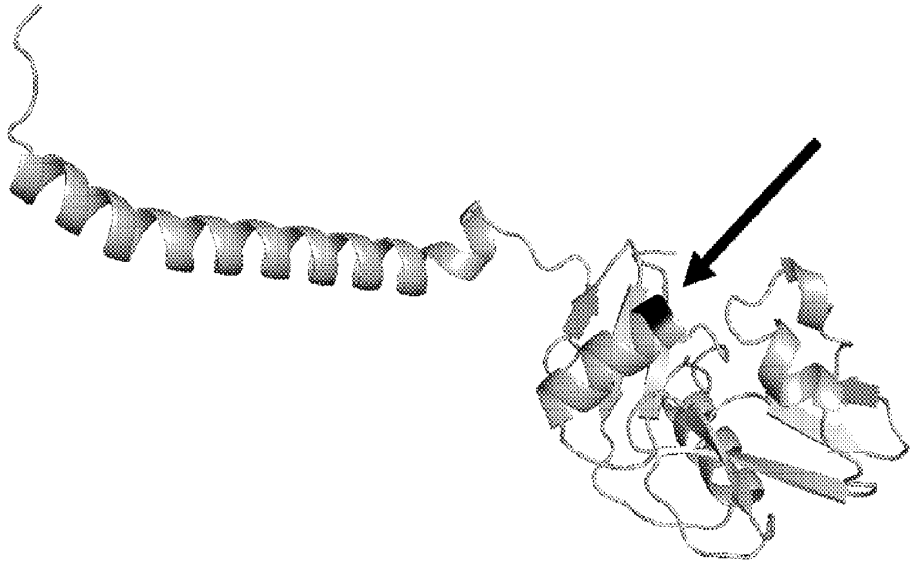
A34R Protein R79 Position

FIG. 13C



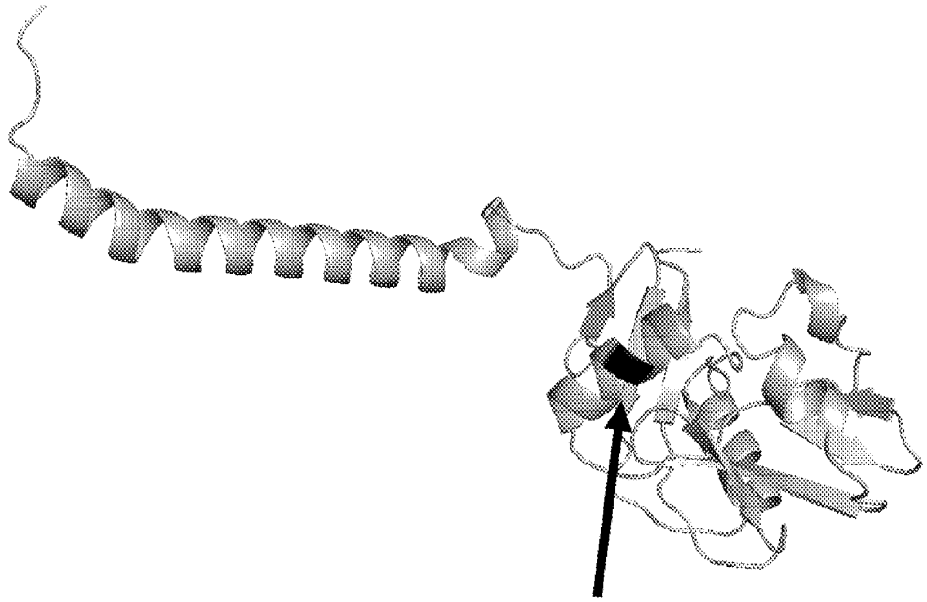
A34R Protein R81 Position

FIG. 13D



A34R Protein R88 Position

FIG. 13E



A34R Protein R91 Position

FIG. 13F

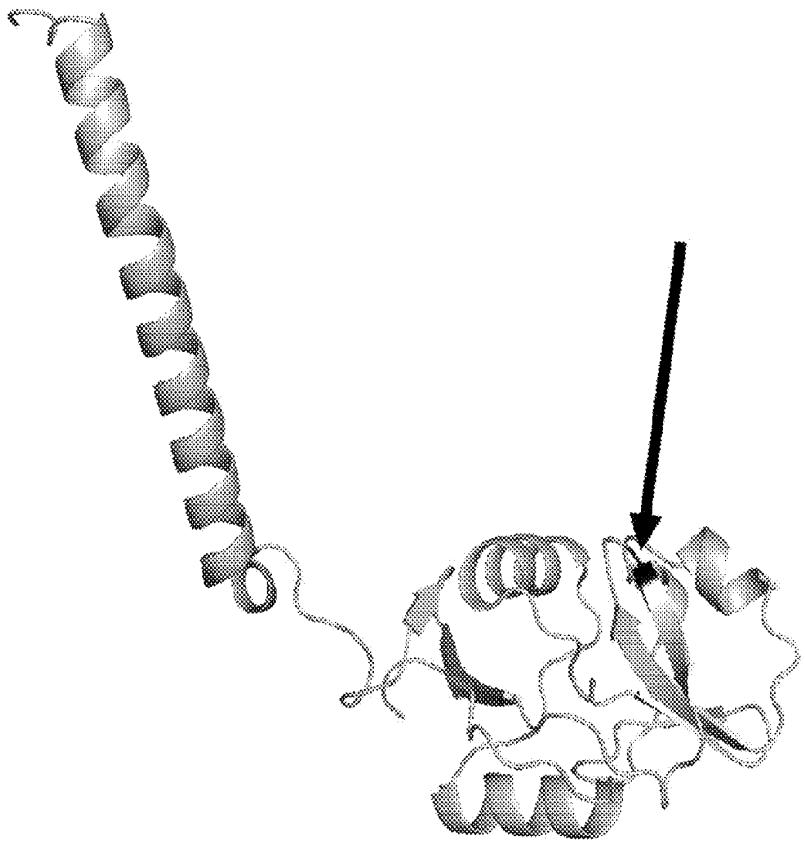


FIG. 13G

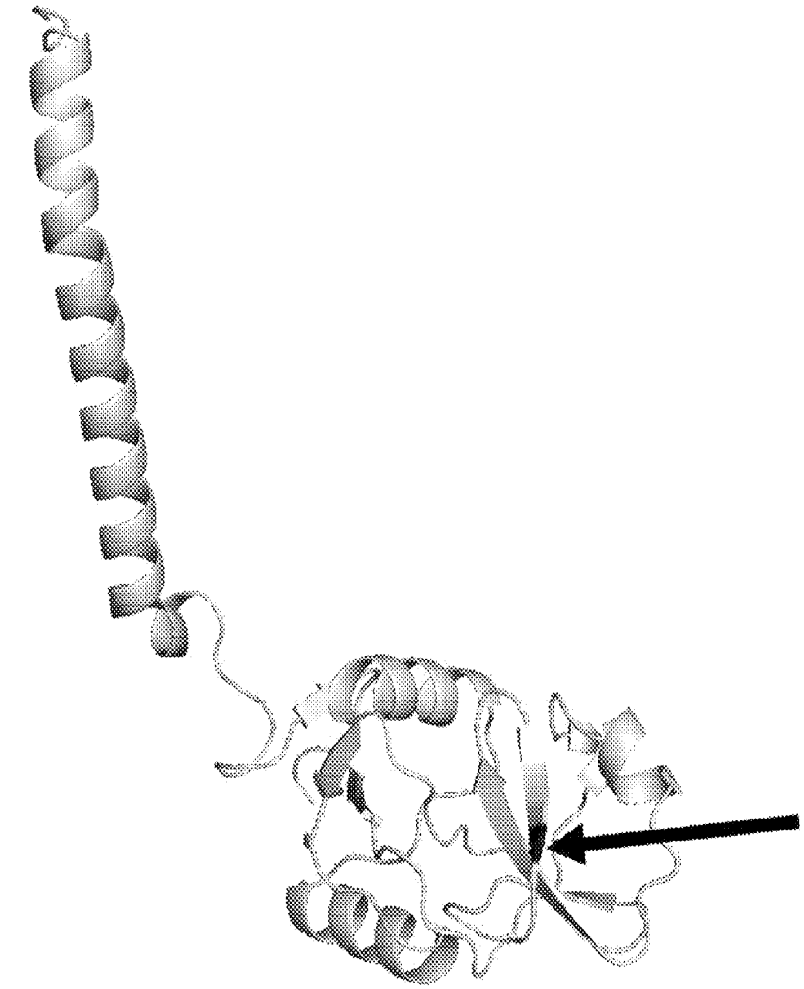
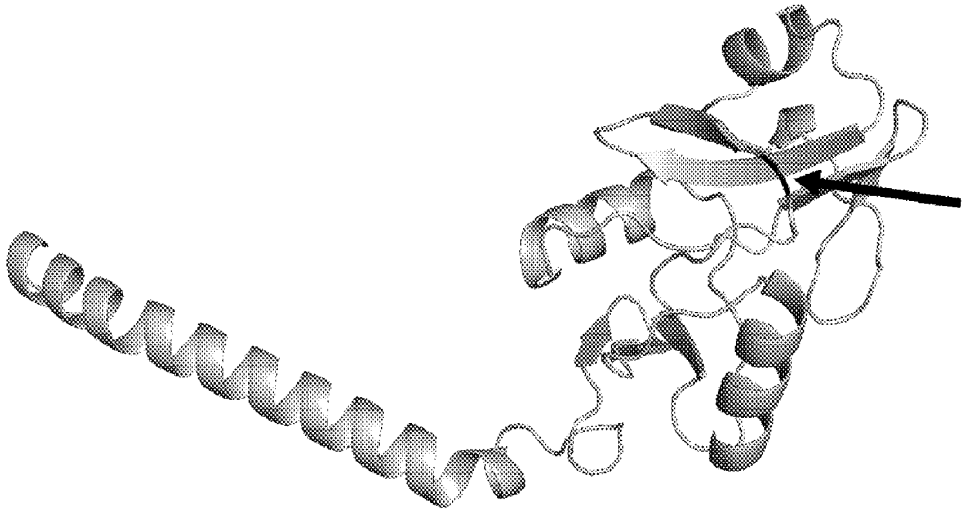
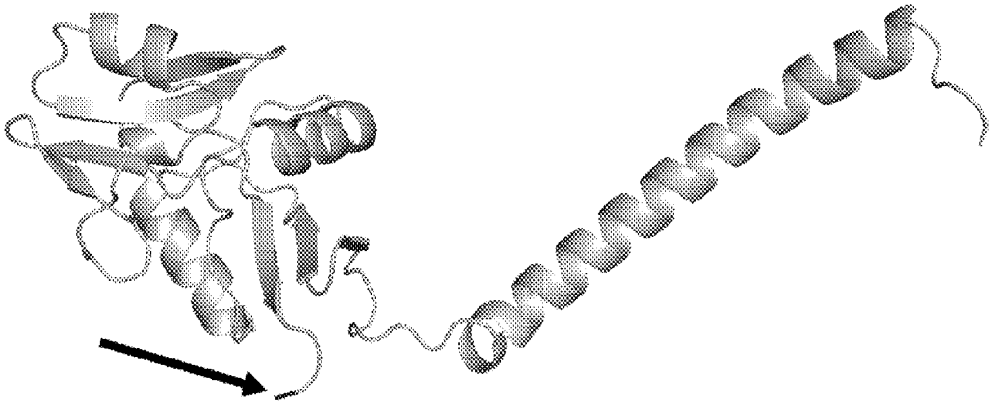


FIG. 13H



A34R Protein V153 Position

FIG. 13I



A34R Protein K165 Position

FIG. 13J



A34R Protein K168 Position

FIG. 13K