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(54) Title: METHODS TO INCREASE IMMUNOGENICITY OF RDRP

(57) Abstract: Disclosed are recombinant vectors comprising one or more polynucleotides encoding at least one modified ribonucleic acid (RNA) dependent RNA polymerase (RdRp), as well as pharmaceutical compositions and methods of their use.

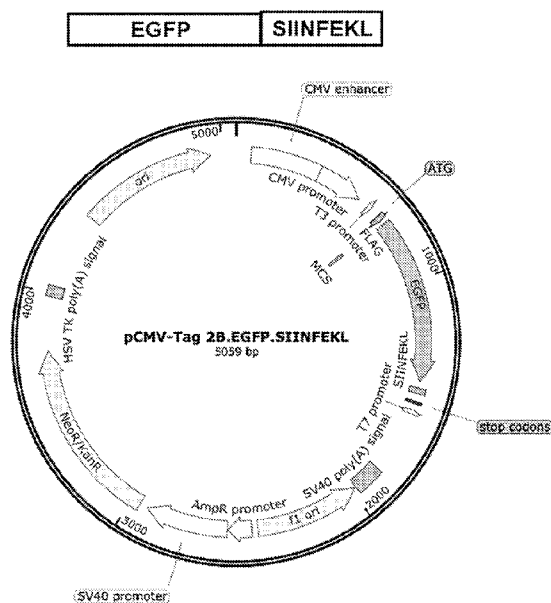


FIG. 1A

Published:

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METHODS TO INCREASE IMMUNOGENICITY OF RDRP

CROSS REFERENCE TO RELATED APPLICATION

[0001] This patent application claims the benefit of U.S. Provisional Patent Application No. 63/339,186, filed May 6, 2022, the disclosure of which is incorporated by reference in its entirety herein.

INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED ELECTRONICALLY

[0002] Incorporated by reference in its entirety herein is a computer-readable nucleotide/amino acid sequence listing submitted concurrently herewith and identified as follows: One 36,109 Byte XML file named “766903.XML,” created May 4, 2023.

BACKGROUND OF THE INVENTION

[0003] Vaccines have proven to be effective mitigators of viral damage. Although there have been many recent advances in vaccine technology, viral infections continue to plague society and cause significant morbidity and mortality. There is also a lack of effective treatments available for many viral infections. Accordingly, there is a need for improved vaccines.

BRIEF SUMMARY OF THE INVENTION

[0004] Aspects of the invention provide recombinant vectors comprising one or more polynucleotides encoding at least one modified viral ribonucleic acid (RNA) dependent RNA polymerase (RdRp), wherein the modified RdRp comprises: (a) at least one fragment of RdRp; (b) RdRp and a regulatory protein that enhances proteasomal degradation, wherein the RdRp is conjugated or fused to the regulatory protein directly or indirectly via a linker; (c) RdRp and a peptide sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T) (PEST sequence) that enhances proteasomal degradation; or (d) a combination of two or more of (a)-(c).

[0005] An aspect of the invention provides pharmaceutical compositions comprising the vectors described herein and a pharmaceutically acceptable carrier.

[0006] An aspect of the invention provides methods of destabilizing SARS-CoV-2 RdRp in a cell, the methods comprising contacting a cell infected with SARS-CoV-2 virus vectors described herein.

[0007] An aspect of the invention provides methods of increasing immune response to SARS-CoV-2 virus in a subject, the methods comprising administering the vectors or pharmaceutical compositions described herein to the subject.

[0008] An aspect of the invention provides methods of increasing cytotoxic T lymphocyte (CTL) response to SARS-CoV-2 virus in a subject, the methods comprising administering the vectors or pharmaceutical compositions described herein to the subject.

[0009] An aspect of the invention provides methods of increasing Major Histocompatibility complex (MHC) class I immune response to SARS-CoV-2 virus in a subject, the methods comprising administering the vectors or pharmaceutical compositions described herein to the subject.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING(S)

[0010] Figure 1A includes schematic showing a 5,059 base pair circular plasmid named “pCMV-Tag 2B-EGFP-SIINFELK.” pCMV-Tag 2B-EGFP-SIINFELK contains EGFP to test expression via fluorescence and SIINFELK (OVA257-264 peptide, SEQ ID NO: 15) to test MHC class I peptide presentation. pCMV-Tag 2B-EGFP-SIINFELK also contains a human cytomegalovirus (CMV) enhancer, a CMV promoter, a T3 promoter, a multiple cloning site (MCS), ATG start codon, a FLAG-tag, stop codons, a T7 promoter, a SV40 poly(A) signal, an f1 origin of replication (f1 ori), an AmpR promoter, a NeoR/KanR, a HSV TK poly(A) signal, and origin of replication (ori) sequences. pCMV-Tag 2B-EGFP-SIINFELK was transfected into 293H2Kb cells for protein expression. The top of Figure 1A shows a schematic of EGFP fused to SIINFELK (SEQ ID NO: 15).

[0011] Figure 1B includes a schematic showing a 7,821 base pair circular plasmid named “pCMVTag2B-RdRp-EGFP-SIINFELK.” pCMVTag2B-RdRp-EGFP-SIINFELK contains EGFP, SIINFELK (SEQ ID NO: 15), and SarS-CoV-2 RdRp (non-structural protein subunit 12 (nsp12), codon-optimized). pCMVTag2B-RdRp-EGFP-SIINFELK also contains a human CMV enhancer, a CMV promoter, a T3 promoter, a Kozak consensus, stop codons, a T7 promoter, a SV40 poly(A) signal, an f1 ori, an AmpR promoter, an SV40 promoter, a NeoR/KanR, a HSV TK poly(A) signal, and ori sequences. The SarS-CoV-2 RdRp sequence contains Fbw7 degron, APCC1, anaphase-promoting complex or cyclosome destruction box

(APC/C 2 (D Box)), APCC3 (D Box), and APCC4 (ABBAyCDC20). The top of Figure 1B shows a schematic of RdRp fused to EGFP and EGFP fused to SIINFEKL (SEQ ID NO: 15).

[0012] Figure 2 is an exemplary gel following a Western blot analysis that screened for protein expressions. pCMVTag2B-EGFP was a positive control (vector plus florescent protein), pCMVTag2B-EGFP-SIINFEKL was the positive control for MHC class I presentation, pCMVTag2B-RdRp-EGFP-SIINFEKL was the target gene, pCMVTag2B-EGFP-LANA-SIINFEKL was the negative control for MHC class I presentation, pCMV-Tag2B was the negative control, i.e., the empty vector, pCMVTag2B-d1EGFP-SIINFEKL and pCMVTag2B-RdRp-d1EGFP-SIINFEKL contained a PEST sequence. Beta-Tubulin (Tubulin) was used as loading control. LANA refers to Kaposi's sarcoma-associated herpesvirus (KSHV) latency-associated nuclear antigen 1 (LANA1, SEQ ID NO: 17).

[0013] Figure 3 is a set of images showing EGFP expression of vectors of aspects of the present invention on a fluorescence microscope.

[0014] Figures 4A-4C are sets of flow cytometry plots showing the EGFP levels versus SIINFEKL (SEQ ID NO: 15) presentation level of exemplary samples. In Figure 4A, the percentages at the top of each plot (i.e., 2%, 6%, 10%, 20%, 40%, and 100%) refer to the gradient of vector concentration EGFP-SIINFEKL (SEQ ID NO: 15), whereas 100% indicates 2µg of plasmid transfected to 40,000 cells in a 6-well plate by 2µl of lipofectamine 2000 (Invitrogen). In Figure 4B, the percentages at the top of each plot (i.e., 6% and 15%) refers to the gradient of vector concentration EGFP-LANA1-SIINFEKL (SEQ ID NO: 15). In Figure 4C, the percentages at the top of each plot (i.e., 100%) refers to the gradient of vector concentration RdRp-EGFP-SIINFEKL (SEQ ID NO: 15). This analysis plot was run in duplicate.

[0015] Figure 5A is a set of schematics showing aspects of the present invention. From top to bottom, the schematics show: (1) ubiquitin fused to EGFP fused to SIINFEKL (SEQ ID NO: 15); (2) ubiquitin fused to RdRp fused to EGFP fused to SIINFEKL (SEQ ID NO: 15); (3) ubiquitin fused to an A-R linker fused to EGFP fused to SIINFEKL (SEQ ID NO: 15); and (4) ubiquitin fused to an A-R linker fused to RdRp fused to EGFP fused to SIINFEKL (SEQ ID NO: 15).

[0016] Figure 5B is schematic showing an aspect of the present invention. Specifically, it shows a fusion protein with RdRp fused to SIINFEKL (SEQ ID NO: 15) fused to EGFP fused to a PEST sequence.

[0017] Figure 5C is a schematic showing an aspect of the present invention. Specifically, it shows two fusion proteins that were used together (SEQ ID NOs: 10 and 12) fused to EGFP fused to SIINFEKL (SEQ ID NO: 15), and the second (bottom) fusion protein with amino acids 366-581 of the full length RdRp (SEQ ID NO: 13) fused to EGFP fused to SIINFEKL (SEQ ID NO: 15).

[0018] Figure 6A is a set of schematics showing aspects of the present invention by adding a ubiquitin to RdRp N-terminus.

[0019] Figure 6B is a set of flow cytometry plots showing MHC class I presentation of SIINFEKL (SEQ ID NO: 15) peptide in ubiquitin containing RdRp fusion proteins of Figure 6A. SIINFEKL (SEQ ID NO: 15) presentation increased from 1.13% to 2.83% by ubiquitin tag.

[0020] Figure 7A is a set of schematics showing aspects of the present invention by adding a ubiquitin as well as an Alanine-Arginine linker.

[0021] Figure 7B is a set of flow cytometry plots showing MHC class I presentation of SIINFEKL (SEQ ID NO: 15) peptide in ubiquitin containing RdRp fusion proteins of Figure 7A. The ubiquitin-A-R increased SIINFEKL (SEQ ID NO: 15) presentation from 1.13% to 4.88%.

[0022] Figure 8A is a set of schematics showing aspects of the present invention by addition of a PEST sequence.

[0023] Figure 8B is a set of flow cytometry plots showing the MHC class I presentation of SIINFEKL (SEQ ID NO: 15) peptide of the PEST containing RdRp fusion proteins of Figure 8A. The PEST sequence increased SIINFEKL (SEQ ID NO: 15) presentation from 5.11% to 7.97%.

[0024] Figure 9 is a schematic showing the layout of RdRp fragment constructs of aspects of the present invention that were inserted in the N-terminal of EGFP-SIINFEKL (SEQ ID NO: 15).

[0025] Figure 10 is an exemplary gel following a Western blot analysis that screened for expression of RdRp fragment constructs of aspects of the present invention.

[0026] Figure 11 is a set of flow cytometry plots showing EGFP level and SIINFEKL (SEQ ID NO: 15) presentation of fragmented RdRp-EGFP-SIINFEKL constructs.

[0027] Figure 12 is a set of flow cytometry plots showing EGFP level and SIINFEKL (SEQ ID NO: 15) presentation of fragmented RdRp-EGFP-SIINFEKL constructs. Compared with Figure 11, most fragmented RdRp constructs showed increased SIINFEKL (SEQ ID

NO: 15) presentation. SIINFEKL (SEQ ID NO: 15) presentation was increased from 11% for RdRp (FL) (SEQ ID NO: 13) up to 24.6% for RdRp.aa.1-130 construct (SEQ ID NO: 1).

[0028] Figure 13 is a set of flow cytometry plots showing EGFP level and SIINFEKL (SEQ ID NO: 15) presentation of fragmented RdRp-EGFP-SIINFEKL constructs. It further shows that all small fragments (<200bp) could dramatically increase SIINFEKL (SEQ ID NO: 15) presentation, up to 30.1% for RdRp.aa.366-581 construct (SEQ ID NO: 10).

[0029] Figure 14 is a graph comparing the EGFP level and SIINFEKL (SEQ ID NO: 15) presentation of fragmented RdRp-EGFP-SIINFEKL constructs and full length RdRp-EGFP-SIINFEKL. The majority of fragments showed increased SIINFEKL (SEQ ID NO: 15) presentation.

[0030] Figure 15A is a flow cytometry plot showing RdRp fragments used in combination for SIINFEKL (SEQ ID NO: 15) presentation. All fragment combinations showed increased SIINFEKL (SEQ ID NO: 15) presentation, even when the protein expression level is comparable.

[0031] Figure 15B is a flow cytometry plot showing RdRp fragments used in combination for SIINFEKL (SEQ ID NO: 15) presentation. All fragment combinations showed increased SIINFEKL (SEQ ID NO: 15) presentation, even when the protein expression level is comparable.

[0032] Figure 15C is a flow cytometry plot showing RdRp fragments used in combination for SIINFEKL (SEQ ID NO: 15) presentation. All fragment combinations showed increased SIINFEKL (SEQ ID NO: 15) presentation, even when the protein expression level is comparable.

[0033] Figure 16A is a flow cytometry plot showing EGFP-SIINFEKL and EGFP-LANA1 as a control for RdRp-EGFP-SIINFEKL.

[0034] Figure 16B is a flow cytometry plot showing EGFP-SIINFEKL and EGFP-LANA1 as a control for RdRp-EGFP-SIINFEKL.

[0035] Figure 17A is a flow cytometry plot showing Hsp70-EGFP-SIINFEKL as a control for RdRp-EGFP-SIINFEKL.

[0036] Figure 17B is a flow cytometry plot showing Hsp70-EGFP-SIINFEKL as a control for RdRp-EGFP-SIINFEKL.

[0037] Figure 18 is a graph showing GFP versus SIINFEKL (SEQ ID NO: 15) level and fitting lines for the shown constructs. The higher slope of the fitting line indicates higher SIINFEKL (SEQ ID NO: 15) presentation at the same GFP level (protein expression level).

[0038] Figure 19A is a flow cytometry plot showing the results of analysis of constructs of aspects of the present invention at gradient (x3) concentrations.

[0039] Figure 19B is a flow cytometry plot showing the results of analysis of constructs of aspects of the present invention at gradient (x3) concentrations.

[0040] Figure 19C is a flow cytometry plot showing the results of analysis of constructs of aspects of the present invention at gradient (x3) concentrations.

[0041] Figure 19D is a flow cytometry plot showing the results of analysis of constructs of aspects of the present invention at gradient (x3) concentrations.

[0042] Figure 20A is a flow cytometry plot showing the results of analysis of constructs of aspects of the present invention at gradient (x3) concentrations.

[0043] Figure 20B is a flow cytometry plot showing the results of analysis of constructs of aspects of the present invention at gradient (x3) concentrations.

[0044] Figure 20C is a flow cytometry plot showing the results of analysis of constructs of aspects of the present invention at gradient (x3) concentrations.

[0045] Figure 20D is a flow cytometry plot showing the results of analysis of constructs of aspects of the present invention at gradient (x3) concentrations.

[0046] Figure 20E is a flow cytometry plot showing the results of analysis of constructs of aspects of the present invention at gradient (x3) concentrations.

[0047] Figure 20F is a flow cytometry plot showing the results of analysis of constructs of aspects of the present invention at gradient (x3) concentrations.

[0048] Figure 21 is a graph showing GFP versus SIINFEKL (SEQ ID NO: 15) level and fitting lines for the shown constructs (1st). The higher slope of the fitting line indicates higher SIINFEKL (SEQ ID NO: 15) presentation at the same GFP level (protein expression level). This graph is similar to Figure 18 but at a lower overall dose.

[0049] Figure 22A is a flow cytometry plot showing the results of analysis of constructs of aspects of the present invention at gradient (x3) concentrations.

[0050] Figure 22B is a flow cytometry plot showing the results of analysis of constructs of aspects of the present invention at gradient (x3) concentrations.

[0051] Figure 22C is a flow cytometry plot showing the results of analysis of constructs of aspects of the present invention at gradient (x3) concentrations.

[0052] Figure 22D is a flow cytometry plot showing the results of analysis of constructs of aspects of the present invention at gradient (x3) concentrations.

[0053] Figure 23A is a flow cytometry plot showing the results of analysis of constructs of aspects of the present invention at gradient (x3) concentrations.

[0054] Figure 23B is a flow cytometry plot showing the results of analysis of constructs of aspects of the present invention at gradient (x3) concentrations.

[0055] Figure 23C is a flow cytometry plot showing the results of analysis of constructs of aspects of the present invention at gradient (x3) concentrations.

[0056] Figure 24A is a flow cytometry plot showing the results of analysis of constructs of aspects of the present invention at gradient (x3) concentrations.

[0057] Figure 24B is a flow cytometry plot showing the results of analysis of constructs of aspects of the present invention at gradient (x3) concentrations.

[0058] Figure 25 is a graph showing GFP versus SIINFEKL (SEQ ID NO: 15) level and fitting lines for the shown constructs (2nd).

[0059] Figure 26A is a flow cytometry plot showing GFP versus SIINFEKL (SEQ ID NO: 15) level expression of constructs of aspects of the present invention transfected into B16 melanoma cells (human).

[0060] Figure 26B is a flow cytometry plot showing GFP versus SIINFEKL (SEQ ID NO: 15) level expression of constructs of aspects of the present invention transfected into B16 melanoma cells (human).

[0061] Figure 26C is a flow cytometry plot showing GFP versus SIINFEKL (SEQ ID NO: 15) level expression of constructs of aspects of the present invention transfected into B16 melanoma cells (human).

[0062] Figure 26D is a flow cytometry plot showing GFP versus SIINFEKL (SEQ ID NO: 15) level expression of constructs of aspects of the present invention transfected into B16 melanoma cells (human).

[0063] Figure 26E is a flow cytometry plot showing GFP versus SIINFEKL (SEQ ID NO: 15) level expression of constructs of aspects of the present invention transfected into B16 melanoma cells (human).

DETAILED DESCRIPTION OF THE INVENTION

[0064] The majority of viruses infecting humans and other animals have RNA genomes. These genomes may be double-stranded (ds) or single-stranded (ss). The RNA-dependent RNA polymerase (RdRp) of all known single-stranded RNA viruses is located within the viral particle and is responsible for the transcription and replication of the viral genome.

Presenting destabilized RdRp in a recombinant vector to a viral host has been found to increase the MHC class I presentation which indicates promising use of destabilized RdRp to increase the protection of the host from the virus. The RdRp can be destabilized by modifying the RdRp in several ways.

[0065] Specifically, an aspect of the invention provides recombinant vectors comprising one or more polynucleotides encoding at least one modified RdRp, wherein the modified RdRp comprises: (a) at least one fragment of RdRp; (b) RdRp and a regulatory protein that enhances proteasomal degradation, wherein the RdRp is conjugated or fused to the regulatory protein directly or indirectly via a linker; (c) RdRp and a peptide sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T) (PEST sequence) that enhances proteasomal degradation; or (d) a combination of two or more of (a)-(c) (i.e., two or three of (a)-(c)).

[0066] The RdRp can be from any single-stranded RNA virus. A “single-stranded RNA virus” comprises a single stranded RNA genome. Corona viruses are single-stranded RNA viruses.

[0067] In some aspects, the RdRp is a corona virus RdRp. In at least one aspect, the RdRp is a SARS-CoV-2 RdRp. In an aspect, the corona virus is Middle East Respiratory Syndrome (MERS)-CoV. In an aspect, the corona virus is severe acute respiratory syndrome (SARS)-CoV. In an aspect, the corona virus is an alpha, beta, gamma, delta, or omicron type of corona virus. In an aspect, the corona virus is 229E (alpha coronavirus), NL63 (alpha coronavirus), OC43 (beta coronavirus), or HKU1 (beta coronavirus). In an aspect, the corona virus is SARS-CoV-1 or SARS-CoV-2. In an aspect, the corona virus is SARS-CoV-2. In an aspect, the corona virus is SARS-CoV-2 variant alpha (B.1.1.7 and Q lineages), beta (B.1.351 and descendent lineages), gamma (P.1 and descendent lineages), epsilon (B.1.427 and B.1.429), eta (B.1.525), iota (B.1.526), kappa (B.1.617.1), 1.617.3, mu (B.1.621, B.1.621.1), zeta (P.2), delta (B.1.617.2 and AY lineages), or omicron (B.1.1.529 and BA lineages). In an aspect, the corona virus is historically a non-human animal corona virus that evolves, mutates, or is modified to cause infection in a human.

[0068] The RdRp used can be a full-length or a fragment of a full-length RdRp. In an aspect, the at least one fragment of RdRp comprises at least about 100 amino acids (e.g., comprises at least about 105 amino acids, at least about 110 amino acids, at least about 115 amino acids, at least about 120 amino acids, at least about 125 amino acids, at least about 130 amino acids, at least about 135 amino acids, at least about 140 amino acids, at least about 145 amino acids). In another aspect, the at least one fragment of RdRp comprises no more than

about 950 amino acids (e.g., comprises no more than about 960 amino acids, no more than about 970 amino acids, no more than about 980 amino acids, no more than about 990 amino acids, no more than about 1,000 amino acids, or no more than about 1,100 amino acids). In an aspect, the at least one fragment of RdRp comprises from about 100 amino acids to about 950 amino acids, from about 125 amino acids to about 900 amino acids, from about 150 amino acids to about 850 amino acids, from about 175 amino acids to about 800 amino acids, or from about 200 amino acids to about 750 amino acids.

[0069] In an additional aspect, the at least one fragment of RdRp comprises at least one of SEQ ID NOs: 1-12. In a further aspect, the vector of an aspect of the invention comprises: (a) SEQ ID NOs: 1, 8, and 9; (b) SEQ ID NOs: 2 and 6; (c) SEQ ID NOs: 4 and 7; (d) SEQ ID NOs: 10 and 12; (e) SEQ ID NOs: 1, 7, 8, 9, and 10; or (f) a combination of two or more (a)-(e) (i.e., two, three, four, or five of (a)-(e)). Figure 9 shows how exemplary fragments of RdRp present in the full length RdRp of Sars-Cov-2. Table 1 below provides the RdRp fragment number, SEQ ID NO, size, and corresponding amino acids of the full length RdRp of Sars-Cov-2.

Table 1

SEQ ID NO	RdRp Fragment Number	Portion of RdRp	Size (kbp)
13	WT	aa 1-932 (full length)	2.8
1	1	RdRp aa 1-130	0.4
2	3	RdRp aa 1-276	0.8
3	4	RdRp aa 1-365	1.1
4	5	RdRp aa 1-581	1.7
5	7	RdRp aa 250-932	2.1
6	8	RdRp aa 277-932	2
7	10	RdRp aa 582-932	1.1
8	11	RdRp aa 131-249	0.4
9	12	RdRp aa 250-365	0.3
10	13	RdRp aa 366-581	0.6
11	14	Full length RdRp with aa 251-365 deleted	2.5
12	15	Full length RdRp	2.2

SEQ ID NO	RdRp Fragment Number	Portion of RdRp	Size (kbp)
		with aa 366-581 deleted	

[0070] In an aspect, more than one fragment of a RdRp is in a single vector. In a further aspect, two fragments of a RdRp are used, wherein the two portions together provide the amino acid sequences of the full length RdRp. For example, RdRp fragment 14 of Sars-Cov-2 is full length RdRp with amino acids 251-365 deleted (SEQ ID NO: 11) and fragment 12 of Sars-Cov-2 is 250-365 amino acids of Sars-Cov-2 full length RdRp (SEQ ID NO: 9).

Together, SEQ ID NOS: 9 and 11 provide the amino acid sequences of the full length RdRp.

[0071] In a further aspect, the vector comprises a regulatory protein. In an aspect, the regulatory protein is ubiquitin or a ubiquitin-like regulatory protein (e.g., ISG15, NEDD8, and SUMO). In an aspect, the regulatory protein is ubiquitin. In an aspect, the regulatory protein modifies the function of RdRp when the regulatory protein is conjugated or fused to RdRp via a linker by enhancing proteasomal degradation of RdRp.

[0072] In an aspect, the regulatory protein is conjugated to the N-terminus of RdRp. In an aspect, the regulatory protein is directly conjugated to the N-terminus of RdRp. In an aspect, the regulatory protein is indirectly conjugated to the N-terminus of RdRp via a linker. In an aspect, the regulatory protein is fused to the N-terminus of RdRp. In an aspect, the regulatory protein is directly fused to the N-terminus of RdRp. In an aspect, the regulatory protein is indirectly fused to the N-terminus of RdRp via a linker.

[0073] In an aspect, the regulatory protein is conjugated to the C-terminus of RdRp. In an aspect, the regulatory protein is directly conjugated to the C-terminus of RdRp. In an aspect, the regulatory protein is indirectly conjugated to the C-terminus of RdRp via a linker. In an aspect, the regulatory protein is fused to the C-terminus of RdRp. In an aspect, the regulatory protein is directly fused to the C-terminus of RdRp. In an aspect, the regulatory protein is indirectly fused to the C-terminus of RdRp via a linker.

[0074] In an aspect, the linker that is conjugated or fused to RdRp is an Alanine-Arginine linker. An "Alanine-Arginine linker," "A-R linker," and "A-R," refer to a linker that comprises at least one alanine and at least one arginine. The linker can be two or more amino acids in length and a suitable linker will be long enough to prevent misfolding of RdRp protein and the regulatory protein. In an aspect, the Alanine-Arginine linker comprises from 2 to 8 amino acids, from 2 to 6 amino acids, or from 2 to 4 amino acids.

[0075] In a further aspect, the vectors comprise a peptide sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T) (PEST sequence) that enhances proteasomal degradation.

[0076] In an aspect, the PEST sequence is a mutated version of residues 422-461 of mouse ornithine decarboxylase (residue numbers refer to the full-length sequence of the mouse ornithine decarboxylase). See, e.g., Li, et al., *J. of Biological Chem.*, 273(52): 34970-34975 (1998); Kitsera, et al., *Biotechniques*, 43(2): 222-227 (2007); and Kwun, et al., *Virology*, 412(2): 357-365 (2011).

[0077] In an aspect, the PEST sequence comprises at least 10 (e.g., at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 25, or at least 30) amino acids. In a further aspect, at least 50% (e.g., at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95%) of the total amino acids in the PEST sequence comprise P, E, S, or T.

[0078] In an aspect, the PEST sequence has at least about 90% sequence identity to SEQ ID NO: 14 (e.g., at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity to SEQ ID NO: 14). In an aspect, the PEST sequence consists essentially of or consists of 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to SEQ ID NO: 14.

[0079] In an aspect, the PEST sequence is conjugated to the N-terminus of RdRp. In an aspect, the PEST sequence is directly conjugated to the N-terminus of RdRp. In an aspect, the PEST sequence is indirectly conjugated to the N-terminus of RdRp via a linker. In an aspect, the PEST sequence is fused to the N-terminus of RdRp. In an aspect, the PEST sequence is directly fused to the N-terminus of RdRp. In an aspect, the PEST sequence is indirectly fused to the N-terminus of RdRp via a linker.

[0080] In an aspect, the PEST sequence is conjugated to the C-terminus of RdRp. In an aspect, the PEST sequence is directly conjugated to the C-terminus of RdRp. In an aspect, the PEST sequence is indirectly conjugated to the C-terminus of RdRp via a linker. In an aspect, the PEST sequence is fused to the C-terminus of RdRp. In an aspect, the PEST sequence is directly fused to the C-terminus of RdRp. In an aspect, the PEST sequence is indirectly fused to the C-terminus of RdRp via a linker.

[0081] In an aspect, there is a "Glycine-Serine" linker between RdRp and EGFP in a construct. In an aspect, the Glycine-Serine linker may contain various quantities of amino

acid residues, e.g., GGGGSGGGGSGGGGSGGGGS (SEQ ID NO: 23) or GGGGSGGGGSGGGGS (SEQ ID NO: 24). In an aspect, the Glycine-Serine linker may have GGGGS (SEQ ID NO: 25) or GGGGA repeats (SEQ ID NO: 26). In some aspects the Glycine-Serine linker comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 GGGGS (SEQ ID NO: 25) or GGGGA repeats (SEQ ID NO: 26).

[0082] As aspect of the invention also relates to pharmaceutical compositions comprising the vectors of the present invention and a pharmaceutically acceptable carrier. The pharmaceutically acceptable carrier (or excipient) is preferably one that is chemically inert to the vector and one that has no detrimental side effects or toxicity under the conditions of use. Such pharmaceutically acceptable carriers include, but are not limited to, water, saline, Cremophor EL (Sigma Chemical Co., St. Louis, MO), propylene glycol, polyethylene glycol, alcohol, and combinations thereof. The choice of carrier will be determined in part by the particular vector, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of the composition. Methods for preparing administrable compositions are known or apparent to those skilled in the art and are described in more detail in, for example, *Remington: The Science and Practice of Pharmacy*, 22nd Ed., Pharmaceutical Press (2012). In an aspect, the pharmaceutical composition is formulated for subcutaneous injection.

[0083] An aspect of the invention also relates to methods of destabilizing SARS-CoV-2 RdRp in a cell, the methods comprising contacting a cell with the vectors of the present invention.

[0084] An aspect of the invention also relates to methods of increasing immune response to SARS-CoV-2 virus in a subject, the methods comprising administering the vectors or the pharmaceutical compositions of the present invention to the subject. The increase in immune response (e.g., CTL response, etc.) refers to a comparison between subjects that have and have not been administered the vectors or the pharmaceutical compositions of the present invention.

[0085] An aspect of the invention also relates to methods of increasing CTL response to SARS-CoV-2 virus in a subject, the methods comprising administering the vectors or the pharmaceutical compositions of the present invention to the subject. The immune system of the subject responds to the vectors by stimulating an immune response comprising the production of CTLs. The increase in CTL response refers to a comparison between subjects

that have and have not been administered the vectors or the pharmaceutical compositions of the present invention.

[0086] An aspect of the invention also relates to methods of increasing MHC class I immune response to SARS-CoV-2 virus in a subject, the methods comprising administering the vectors or the pharmaceutical compositions of the present invention to the subject. The increase in MHC class I immune response refers to a comparison between subjects that have and have not been administered the vectors or the pharmaceutical compositions of the present invention.

[0087] T cells are antigen specific immune cells that function in response to specific antigen signals. T cells do not respond to antigens in a free or soluble form. For a T cell to respond to an antigen, it requires the antigen to be bound to a MHC.

[0088] MHC proteins provide the means by which T cells differentiate native or “self” cells from foreign cells. There are two types of MHC, class I MHC and class II MHC. Cytolytic T cells (CD8+) predominately interact with class I MHC proteins. Both MHC complexes are transmembrane proteins with a majority of their structure on the external surface of the cell. Additionally, both classes of MHC have a peptide binding cleft on their external portions. It is in this cleft that small fragments of proteins, native or foreign, are bound and presented to the extracellular environment. T cells specific for the peptide bound to a recognizable MHC complex bind to these MHC-peptide complexes and proceed to the next stages of the immune response.

[0089] The vector, or pharmaceutical composition thereof, enhances the immunogenicity (particularly the CTL immunogenicity) for a broad spectrum vaccine, e.g., a SARS-CoV-2 vaccine.

[0090] The vector, or pharmaceutical composition thereof, can be administered to a subject by any suitable route including, but not limited to, parental (subcutaneous, intramuscular, intradermal, intraperitoneal, intrathecal, intravenous, and intratumoral), topical, oral, or local administration. In an aspect, the vector is administered subcutaneously.

[0091] In an aspect, the vector, or pharmaceutical composition thereof, is administered one time to the subject. In another aspect, the vector is administered more than once to the subject (i.e., multiple times, two, three, four, or more times to the subject).

[0092] In another aspect, more than one vector is administered to the subject. For example, one vector may comprise polynucleotides encoding RdRp fragment 5 (SEQ ID NO:

4) and a second vector may comprise polynucleotides encoding RdRp fragment 10 (SEQ ID NO: 7).

[0093] In an aspect of the invention, the subject is a mammal, such as a non-human mammal including a mouse, rat, guinea pig, hamster, rabbit, cat, dog, pig, cow, horse, a non-human primate, or a human. In an aspect, the subject is a human.

[0094] An aspect of the invention provides circular plasmids. Exemplary plasmids are provided in Figures 1A and 1B. pCMV-Tag 2B.EGFP.SIINFEKL is a 5,059 base pair circular plasmid and contains EGFP and SIINFEKL (OVA257-264 peptide, SEQ ID NO: 15) to test MHC class I peptide presentation. Specifically, pCMV-Tag 2B.EGFP.SIINFEKL contains, in the following order, a CMV enhancer, a CMV promoter, a T3 promoter, MCS, ATG start codon, a FLAG-tag, EGFP, SIINFEKL (SEQ ID NO: 15), stop codons, a T7 promoter, a SV40 poly(A) signal, an f1 ori, an AmpR promoter, a NeoR/KanR, a HSV TK poly(A) signal, and ori sequences.

[0095] pCMVTag2B-RdRp-EGFP-SIINFEKL is a 7,821 base pair circular plasmid and contains EGFP, SIINKEKL (SEQ ID NO: 15), and SarS-CoV-2 RdRp (non-structural protein subunit 12 (nsp12), codon-optimized). Specifically, the pCMVTag2B-RdRp-EGFP-SIINFEKL contains, in the following order, a human CMV enhancer, a CMV promoter, a T3 promoter, a Kozak consensus, SarS-CoV-2 RdRp, MCS, EGFP, SIINKEKL (SEQ ID NO: 15), stop codons, a T7 promoter, a SV40 poly(A) signal, an f1 ori, an AmpR promoter, an SV40 promoter, a NeoR/KanR, a HSV TK poly(A) signal, and ori sequences. The SarS-CoV-2 RdRp sequence contains Fbw7 degron, APCC1, APC/C 2 (D Box), APCC3 (D Box), and APCC4 (ABBAyCDC20). “D box” refers to sequence RxxL (SEQ ID NO: 16), wherein the two middle amino acids (i.e., the “x”s) can be any amino acid.

[0096] The modified RdRp proteins can be created using any suitable means. The plasmids described herein are merely exemplary plasmids used to determine RdRp expression via fluorescence and MHC class I expression. The recombinant vectors of an aspect of the invention can be co-administered with any vaccine. For example, the recombinant vectors of an aspect of the invention can be co-administered with a SARS-CoV-2 vaccine. The recombinant vectors of an aspect of the invention can be co-administered with an mRNA vaccine. The recombinant vectors of an aspect of the invention can be co-administered with BNT162b2 (COMIRNATY), JNJ-78436735, or mRNA-1273.

[0097] The vectors of an aspect of the invention can deliver the RdRp to the cells by any suitable means. For example, in an aspect of the invention, the RdRp can be delivered by transfection (e.g., mRNA transfection) or by introduction to the cells using a viral carrier.

[0098] Any suitable viral carrier can be used in an aspect of the invention, for example, an adenovirus, a poxvirus, a retrovirus, a herpes simplex virus, or a baculovirus. In an aspect of the invention, the viral carrier is an adenovirus.

[0099] A further aspect of the invention provides a polypeptide comprising an amino acid sequence encoded by the recombinant vectors of an aspect of the invention.

[0100] Aspects, including embodiments, of the subject matter described herein may be beneficial alone or in combination, with one or more other aspects or embodiments. Without limiting the foregoing description, certain non-limiting aspects of the disclosure numbered 1-24 are provided below. As will be apparent to those of skill in the art upon reading this disclosure, each of the individually numbered aspects may be used or combined with any of the preceding or following individually numbered aspects. This is intended to provide support for all such combinations of aspects and is not limited to combinations of aspects explicitly provided below:

[0101] (1) A recombinant vector comprising one or more polynucleotides encoding at least one modified ribonucleic acid (RNA) dependent RNA polymerase (RdRp), wherein the modified RdRp comprises:

- (a) at least one fragment of RdRp;
- (b) RdRp and a regulatory protein that enhances proteasomal degradation, wherein the RdRp is conjugated or fused to the regulatory protein directly or indirectly via a linker;
- (c) RdRp and a peptide sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T) (PEST sequence) that enhances proteasomal degradation; or
- (d) a combination of two or more of (a)-(c).

[0102] (2) The vector of aspect 1, wherein the RdRp of (a), (b), or (c) is a corona virus RdRp.

[0103] (3) The vector of aspect 1, wherein the RdRp of (a), (b), or (c) is SARS-CoV-2 RdRp.

[0104] (4) The vector of any one of aspects 1-3, wherein the at least one fragment of RdRp comprises at least 100 amino acids.

[0105] (5) The vector of any one of aspects 1-4, wherein the at least one fragment of RdRp comprises no more than 950 amino acids.

[0106] (6) The vector of any one of aspects 1-5, wherein the at least one fragment of RdRp comprises at least one of SEQ ID NOs: 1-12.

[0107] (7) The vector of any one of aspects 1-6, wherein the vector comprises:

- (a) SEQ ID NOs: 1, 8, and 9;
- (b) SEQ ID NOs: 2 and 6;
- (c) SEQ ID NOs: 4 and 7;
- (d) SEQ ID NOs: 10 and 12;
- (e) SEQ ID NOs: 1, 7, 8, 9, and 10; or
- (f) a combination of two or more of (a)-(e).

[0108] (8) The vector of any one of aspects 1-3, wherein the regulatory protein is ubiquitin.

[0109] (9) The vector of aspect 1 or 8, wherein the regulatory protein is conjugated or fused to the N-terminus of RdRp.

[0110] (10) The vector of aspect 1 or 8, wherein the regulatory protein is conjugated or fused to the C-terminus of RdRp.

[0111] (11) The vector of any one of aspects 1-3 or 8-10, wherein the linker is an Alanine-Arginine linker.

[0112] (12) The vector of any one of aspects 1-3, wherein the PEST sequence comprises at least 10 amino acids and wherein at least 50% of the total amino acids in the PEST sequence comprise P, E, S, or T.

[0113] (13) The vector of any one of aspects 1-3, wherein the PEST sequence has at least 90% sequence identity to SEQ ID NO: 14.

[0114] (14) The vector of any one of aspects 1-3, 12, or 13, wherein the PEST sequence is conjugated or fused to the N-terminus of RdRp.

[0115] (15) The vector of any one of aspects 1-3, 12, or 13, wherein the PEST sequence is conjugated or fused to the C-terminus of RdRp.

[0116] (16) A pharmaceutical composition comprising the vector of any one of aspects 1-15 and a pharmaceutically acceptable carrier.

[0117] (17) The pharmaceutical composition of aspect 16, wherein the pharmaceutical composition is formulated for subcutaneous injection.

[0118] (18) A method of destabilizing SARS-CoV-2 RdRp in a cell, the method comprising contacting a cell with the vector according to any one of aspects 1-15.

[0119] (19) A method of increasing immune response to SARS-CoV-2 virus in a subject, the method comprising administering the vector according to any one of aspects 1-15 or the pharmaceutical composition of aspect 16 or 17 to the subject.

[0120] (20) A method of increasing cytotoxic T lymphocyte (CTL) response to SARS-CoV-2 virus in a subject, the method comprising administering the vector according to any one of aspects 1-15 or the pharmaceutical composition of aspect 16 or 17 to the subject.

[0121] (21) A method of increasing Major Histocompatibility Complex (MHC) class I immune response to SARS-CoV-2 virus in a subject, the method comprising administering the vector according to any one of aspects 1-15 or the pharmaceutical composition of aspect 16 or 17 to the subject.

[0122] (22) The method of any one of aspects 19-21, wherein the vector is administered subcutaneously.

[0123] (23) The method of any one of aspects 19-22, wherein the vector is administered more than once to the subject.

[0124] (24) The method of any one of aspects 19-23, wherein the subject is a human.

[0125] The following example further illustrates the invention but, of course, should not be construed as in any way limiting its scope.

EXAMPLE 1

[0126] This example demonstrates the production and desirable immunity response of vectors of the present invention.

[0127] *Materials and Methods*

[0128] Plasmids

[0129] Constructs of the present invention were generated by PCR as described previously (Kwun, et al., *J. Virol.* 81: 8225-8235 (2007)). Figures 1A and 1B show schematics of plasmids of aspects of the invention. As there are currently no standard ways of determining RdRp expression, EGFP was used to test fluorescence expression level and the chicken ovalalbumin epitope SIINFEKL (OVA257-264 peptide, SEQ ID NO: 15) was used to test MHC class I peptide presentation. For SarS-CoV-2 RdRp, non-structural protein subunit 12 (nsp12) was used. The SarS-CoV-2 RdRp sequence contains Fbw7 degron, APCC1, APC/C 2 (D Box), APCC3 (D Box), and APCC4 (ABBAyCDC20). A destabilized

EGFP was amplified by PCR using primers (pd1EGFP-sense, GGA TCC GCC ACC ATG GTG AGC AAG GGC GAG GAG CTG (SEQ ID NO: 19); pd1EGFP-antisense, GAA TTC CAC ATT GAT CCT AGC AGA AGC ACA (SEQ ID NO: 20)) and inserted into BamHI/EcoRI sites in the N-terminus of the constructs. SIINFEKL (SEQ ID NO: 15) was introduced into HindIII/XhoI sites using direct ligation of following primers: SIIN-Forward 5'-AAG CTT AGC ATA ATT AAT TTC GAA AAG CTC TAA GCG GCC GCG CTC GAG-3' (SEQ ID NO: 21); SIIN-Reverse 5'-CTC GAG CGC GGC CGC TTA GAG CTT TTC GAA ATT AAT TAT GCT AAG CTT-3' (SEQ ID NO: 22). Correct insert sequences were determined by DNA sequencing for all plasmids.

[0130] Analysis of GFP Fluorescence (Protein Turnover)

[0131] HEK293 cells were grown in Dulbeccos' Modified Eagle Medium (DMEM) supplemented with 10% FBS and transfected with Lipofectamine-2000 (Invitrogen), with the constructs. To examine inhibition of GFP turnover, pd1EGFP-N1 (Kwun, et al., (2007)) encoding a destabilized EGFP with an estimated half-life ($t_{1/2}$) of 1 h was used for constructing fusions to CR subdomains or constructs at the N-terminus of EGFP. Samples were harvested and lysed in buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% SDS, 3 mM EDTA, 1% Triton X-100, 1 mM NaF, and 1 mM Na orthovanadate) supplemented with proteinase inhibitors.

[0132] Flow Cytometry Analysis for Antigen Presentation

[0133] HEK293 cells stably expressing the mouse class I allele H-2Kb were grown in DMEM with 10% FBS supplemented with 0.5 mg/ml G418 (HyClone Laboratories, Inc.). For flow cytometry analysis, 293KbC2 cells were transfected with 1 μ g of the constructs and harvested and washed with PBS 24 hrs after transfection and stained with allophycocyanin (APC) or phycoerytherin (PE) anti-mouse MHC class I Kb-SIINFEKL (25-D1.16, SEQ ID NO: 15, eBioscience) for 1h at 4 degrees C. Cells were washed twice with PBS and analyzed using a BD LSRFORTESSA™ Cell Analyzer. For each experiment, gating was performed for positive EGFP fluorescence, and 30,000 events were collected and analyzed. All experiments were repeated at least three times for reproducibility, with representative experiments shown. Similar procedures were used for the B16 melanoma cells.

[0134] Fluorescence Microscopy

[0135] Cells were analyzed using a Nikon TS100 with Spot insight digital camera or an Olympus AX70 epifluorescence microscope equipped with a Spot RT digital camera.

[0136] Statistical Analysis

[0137] Data were compared by analysis of variance with paired student's t-test using Prism software (GraphPad). Values were considered significant at $p < 0.05$.

[0138] *Discussion*

[0139] RdRp was modified in three general ways: (1) fusion with ubiquitin at N-terminal; (2) fusion with a PEST sequence; and (3) fragmentation of RdRp. With these modifications, the increment of MHC class I immune response of RdRp (tested *in vitro* through SIINFEKL (SEQ ID NO: 15) peptide presentation) was determined. In summary, the modified RdRp resulted in destabilized RdRp which generated higher level of MHC class I peptide presentation and therefore will serve as antigens for vaccines, e.g., mRNA-based vaccines. The destabilized RdRp also is expected to increase the CTL response, which correlates to better vaccine protection. The data provide methods to modify RdRp to increase CTL immune response. The modified RdRp provides higher efficacy for vaccines, e.g., mRNA vaccines.

[0140] Figures 2 (Western Blot) and 3 (fluorescence images) are included as examples of the protein expression levels. Figures 4A-4C are sets of flow cytometry plots showing the EGFP levels versus SIINFEKL (SEQ ID NO: 15) presentation level of exemplary samples. At a similar GFP level, RdRp showed significantly lower MHC class I presentation. Without being bound to any particular theory, this could be due to the nature of virus to escape immune response. EGFP expression and SIINFEKL (SEQ ID NO: 15) presentation is saturated at around 20% for EGFP-SIINFEKL. Comparing Figures 4A and 4C, the GFP level is similar 156% EGFP-SIINFEKL vs. 100% RdRp-EGFP-SIINFEKL.

[0141] Figures 5A-5C, 6A, 7A, and 8A exemplary schematics showing aspects of the present invention.

[0142] Figures 6B, 7B, and 8B are sets of flow cytometry plots showing MHC class I presentation of SIINFEKL (SEQ ID NO: 15).

[0143] Figure 10 is an exemplary gel following a Western blot analysis that screened for expression of RdRp fragment constructs.

[0144] Figures 11-13 are sets of flow cytometry plots showing EGFP level and SIINFEKL (SEQ ID NO: 15) presentation of fragmented RdRp-EGFP-SIINFEKL constructs.

[0145] Figure 14 is a graph comparing the EGFP level and SIINFEKL (SEQ ID NO: 15) presentation of fragmented RdRp-EGFP-SIINFEKL constructs to full length RdRp-EGFP-SIINFEKL.

[0146] Figures 15A-15C are flow cytometry plots showing RdRp fragment combined (add up to a full-length RdRp) for SIINFEKL (SEQ ID NO: 15) presentation.

[0147] Figures 16A, 16B, 17A, and 17B are flow cytometry plots showing EGFP-SIINFEKL (SEQ ID NO: 15) and EGFP-LANA1 as a control for RdRp-EGFP-SIINFEKL. Hsp70-EGFP-SIINFEKL has similar GFP level but lower SIINFEKL (SEQ ID NO: 15) presentation compared with RdRp-EGFP-SIINFEKL, which indicates that RdRp could actively suppress its peptide presentation.

[0148] Figure 18 is a graph showing GFP versus SIINFEKL (SEQ ID NO: 15) level and fitting lines for the shown constructs. The higher slope of the fitting line indicates higher SIINFEKL (SEQ ID NO: 15) presentation at the same GFP level (protein expression level). Ubiquitin-RdRp-EGFP-SIINFEKL shows higher SIINFEKL (SEQ ID NO: 15) presentation.

[0149] Figures 19A-19D, 20A-20F, 22A-22D, 23A-23C, and 24A-24B are flow cytometry plots showing for constructs at gradient (x3) concentrations. Combining Figures 19A-19D and Figures 20A-20F, fragmented RdRp combinations and ubiquitin increased SIINFEKL (SEQ ID NO: 15) presentation compared to full length RdRp.

[0150] Figure 21 is a graph showing GFP versus SIINFEKL (SEQ ID NO: 15) level and fitting lines for the shown constructs (1st). Higher slope of the fitting line indicates higher SIINFEKL (SEQ ID NO: 15) presentation at the same GFP level (protein expression level). This graph is similar to Figure 18 but at a lower overall dose. Compared to RES (FL RdRp), all modified constructs except RSD presented higher SIINFEKL (SEQ ID NO: 15) peptides. Again, the SIINFEKL (SEQ ID NO: 15) presentation was increased in fragmented RdRp and Ubiquitin-RdRp constructs. Figure 25 is a graph showing GFP versus SIINFEKL (SEQ ID NO: 15) level and fitting lines for the shown constructs (2nd).

[0151] Figures 26A-26E are flow cytometry plots showing GFP versus SIINFEKL (SEQ ID NO: 15) level of constructs transfected into B16 melanoma cells (human). Fragmented RdRp showed higher SIINFEKL (SEQ ID NO: 15) presentation, in agreement with 293H2Kb cells. Similar to 293 cells, fragmented RdRp combination constructs showed higher SIINFEKL (SEQ ID NO: 15) presentation compared to full length RdRp, indicating a cell type independent effect.

[0152] All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were

individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

[0153] The use of the terms “a” and “an” and “the” and “at least one” and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The use of the term “at least one” followed by a list of one or more items (for example, “at least one of A and B”) is to be construed to mean one item selected from the listed items (A or B) or any combination of two or more of the listed items (A and B), unless otherwise indicated herein or clearly contradicted by context. The terms “comprising,” “having,” “including,” and “containing” are to be construed as open-ended terms (i.e., meaning “including, but not limited to,”) unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., “such as”) provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0154] Preferred embodiments and aspects of this invention are described herein. Variations of those preferred embodiments and aspects may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

CLAIMS:

1. A recombinant vector comprising one or more polynucleotides encoding at least one modified ribonucleic acid (RNA) dependent RNA polymerase (RdRp), wherein the modified RdRp comprises:

(a) at least one fragment of RdRp;

(b) RdRp and a regulatory protein that enhances proteasomal degradation, wherein the RdRp is conjugated or fused to the regulatory protein directly or indirectly via a linker;

(c) RdRp and a peptide sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T) (PEST sequence) that enhances proteasomal degradation; or

(d) a combination of two or more of (a)-(c).

2. The vector of claim 1, wherein the RdRp of (a), (b), or (c) is a corona virus RdRp.

3. The vector of claim 1, wherein the RdRp of (a), (b), or (c) is SARS-CoV-2 RdRp.

4. The vector of any one of claims 1-3, wherein the at least one fragment of RdRp comprises at least 100 amino acids.

5. The vector of any one of claims 1-3, wherein the at least one fragment of RdRp comprises no more than 950 amino acids.

6. The vector of any one of claims 1-3, wherein the at least one fragment of RdRp comprises at least one of SEQ ID NOs: 1-12.

7. The vector of any one of claims 1-3, wherein the vector comprises:

(a) SEQ ID NOs: 1, 8, and 9;

(b) SEQ ID NOs: 2 and 6;

(c) SEQ ID NOs: 4 and 7;

(d) SEQ ID NOs: 10 and 12;

(e) SEQ ID NOs: 1, 7, 8, 9, and 10; or

(f) a combination of two or more of (a)-(e).

8. The vector of any one of claims 1-3, wherein the regulatory protein is ubiquitin.

9. The vector of claim 1, wherein the regulatory protein is conjugated or fused to the N-terminus of RdRp.

10. The vector of claim 1, wherein the regulatory protein is conjugated or fused to the C-terminus of RdRp.

11. The vector of any one of claims 1-3, 9, or 10, wherein the linker is an Alanine-Arginine linker.

12. The vector of any one of claims 1-3, wherein the PEST sequence comprises at least 10 amino acids and wherein at least 50% of the total amino acids in the PEST sequence comprise P, E, S, or T.

13. The vector of any one of claims 1-3, wherein the PEST sequence has at least 90% sequence identity to SEQ ID NO: 14.

14. The vector of any one of claims 1-3, wherein the PEST sequence is conjugated or fused to the N-terminus of RdRp.

15. The vector of any one of claims 1-3, wherein the PEST sequence is conjugated or fused to the C-terminus of RdRp.

16. A pharmaceutical composition comprising the vector of any one of claims 1-3 and a pharmaceutically acceptable carrier.

17. The pharmaceutical composition of claim 16, wherein the pharmaceutical composition is formulated for subcutaneous injection.

18. A method of destabilizing SARS-CoV-2 RdRp in a cell, the method comprising contacting a cell with the vector according to any one of claims 1-3.

19. A method of increasing immune response to SARS-CoV-2 virus in a subject, the method comprising administering the vector according to any one of claims 1-3 to the subject.

20. A method of increasing cytotoxic T lymphocyte (CTL) response to SARS-CoV-2 virus in a subject, the method comprising administering the vector according to any one of claims 1-3 to the subject.

21. A method of increasing Major Histocompatibility Complex (MHC) class I immune response to SARS-CoV-2 virus in a subject, the method comprising administering the vector according to any one of claims 1-3 to the subject.

22. The method of claim 19, wherein the vector is administered subcutaneously.

23. The method of claim 19, wherein the vector is administered more than once to the subject.

24. The method of claim 19, wherein the subject is a human.

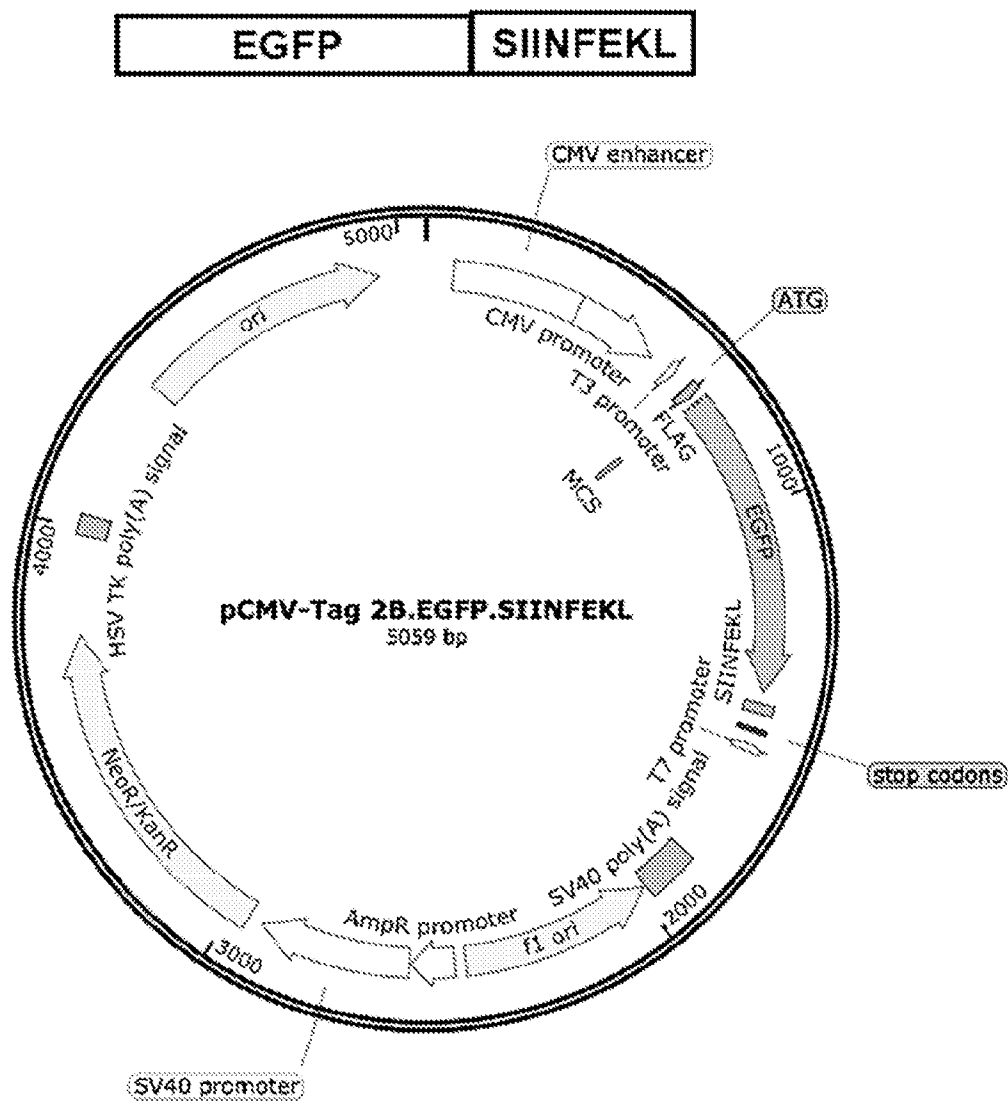


FIG. 1A

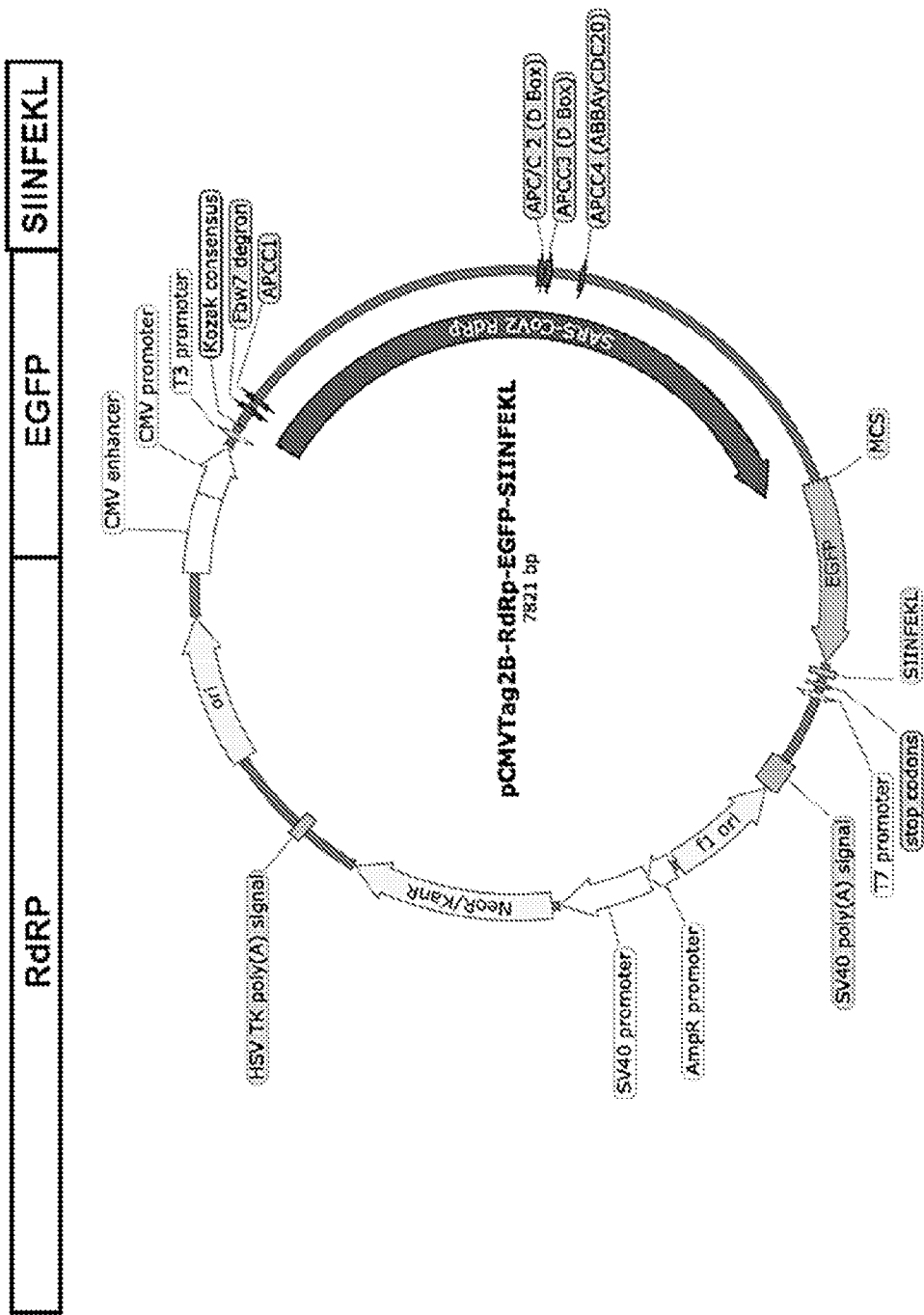


FIG. 1B

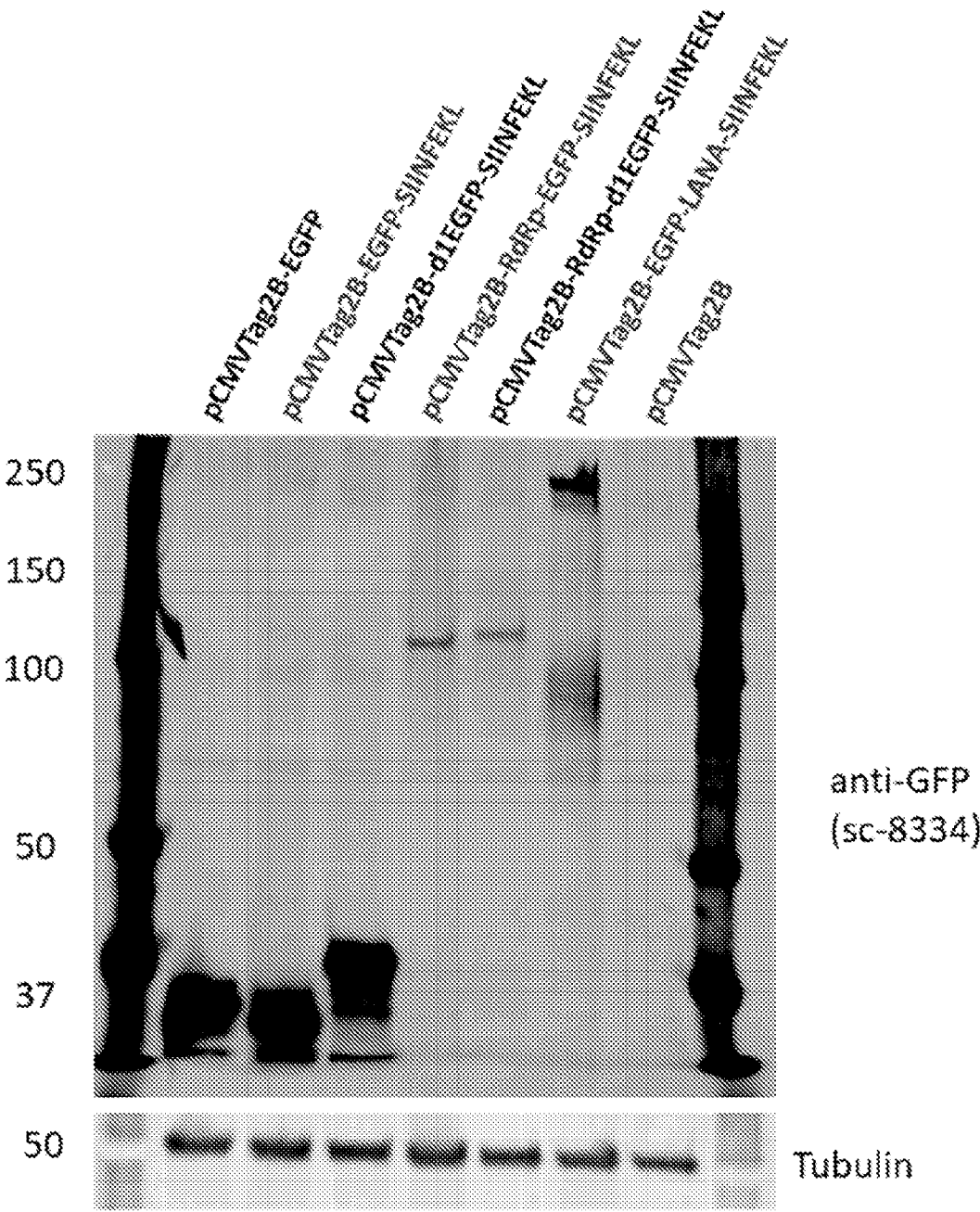


FIG. 2

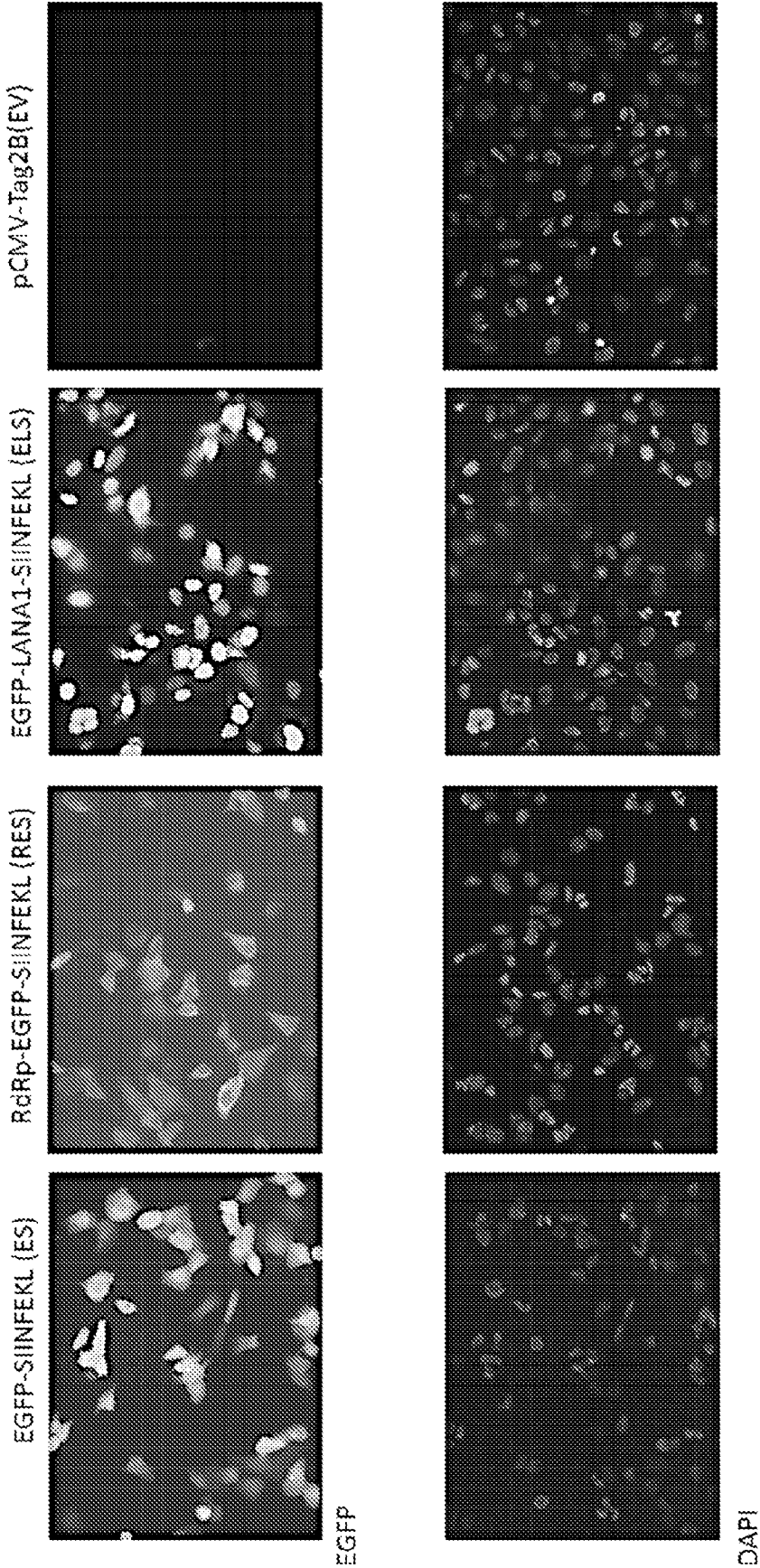


FIG. 3

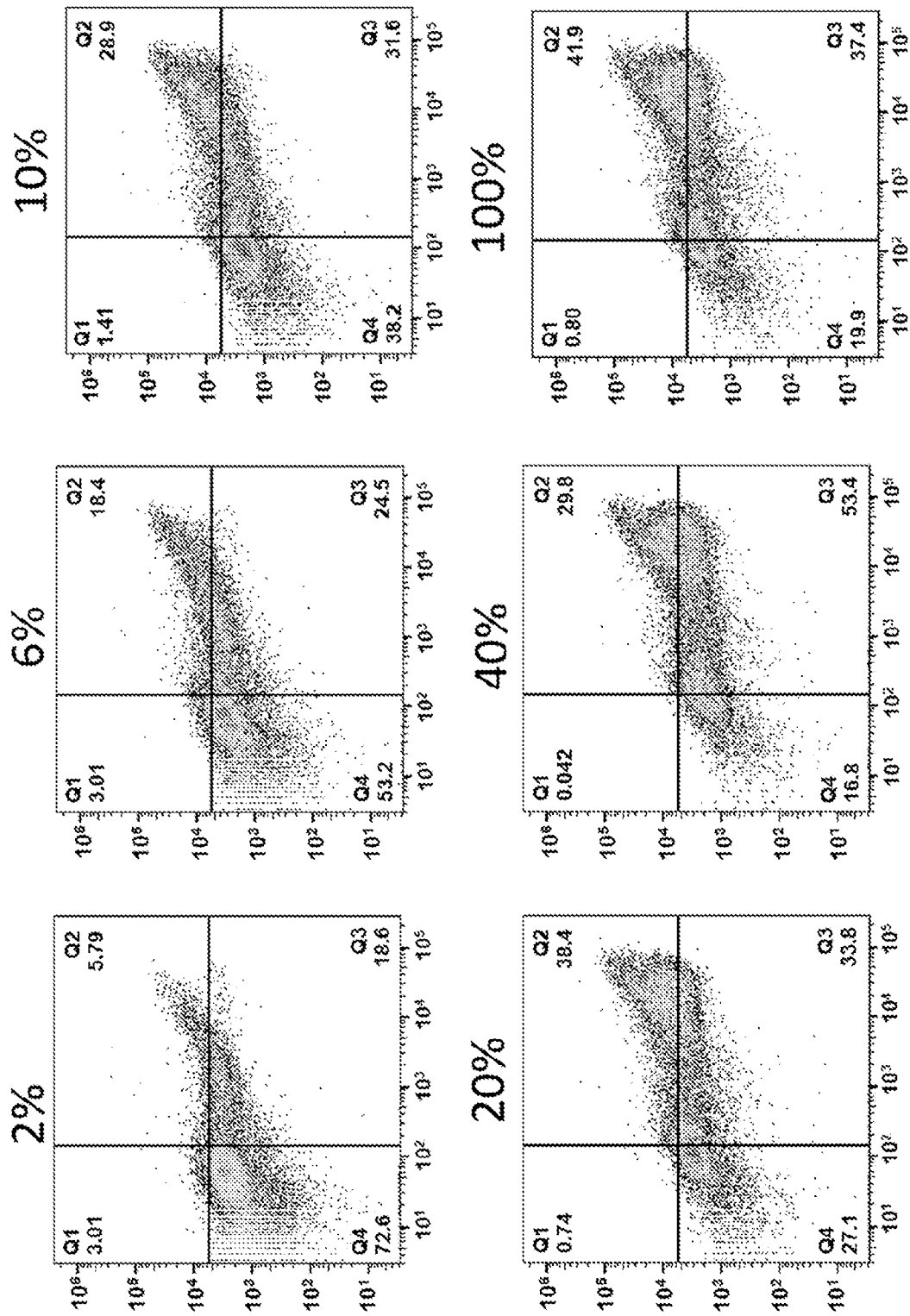


FIG. 4A

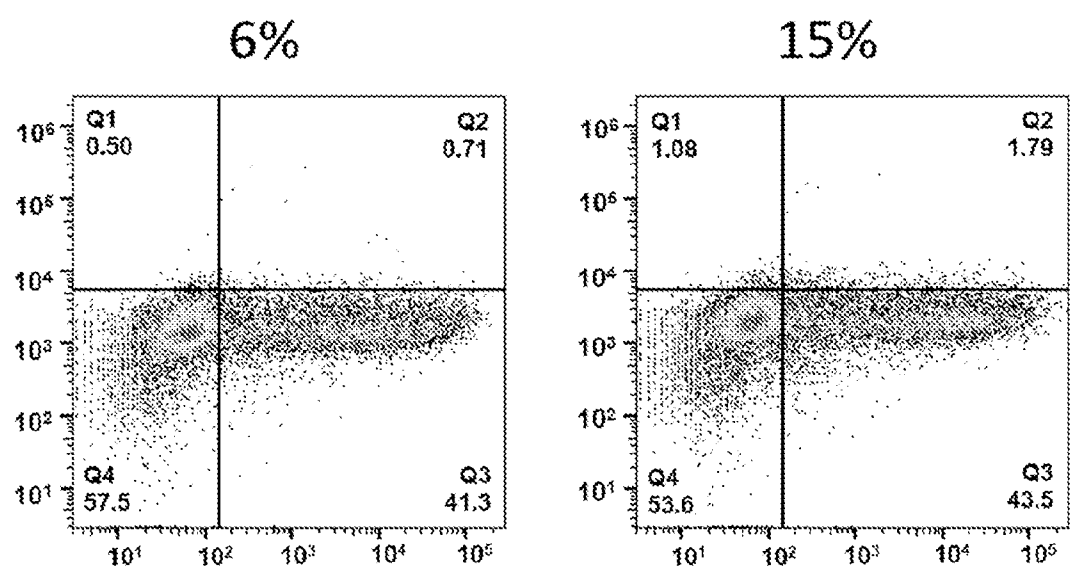


FIG. 4B

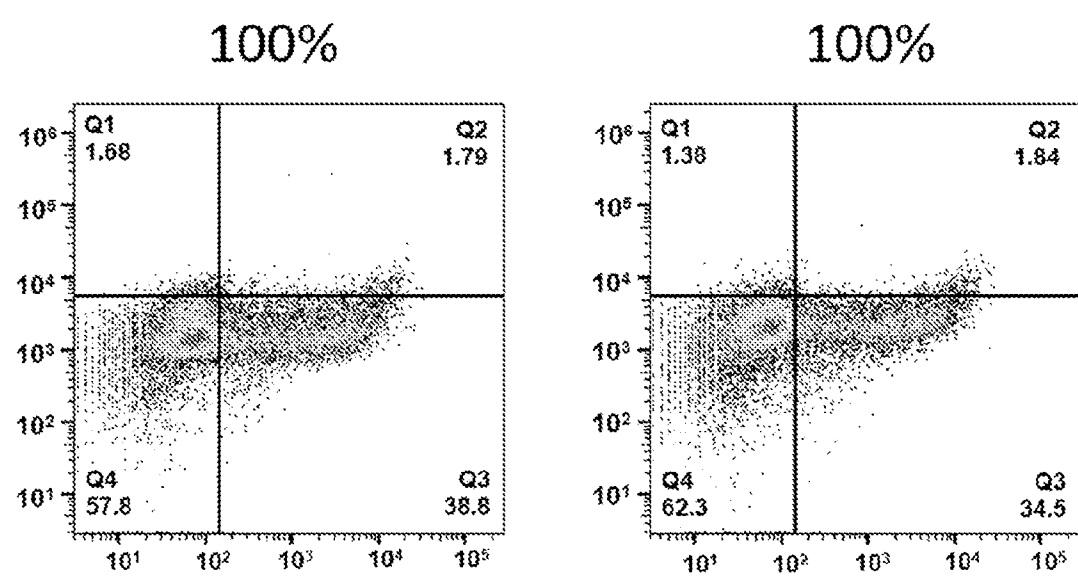


FIG. 4C

Modified RdRp form 1: Ubiquitin to enhance proteasomal degradation

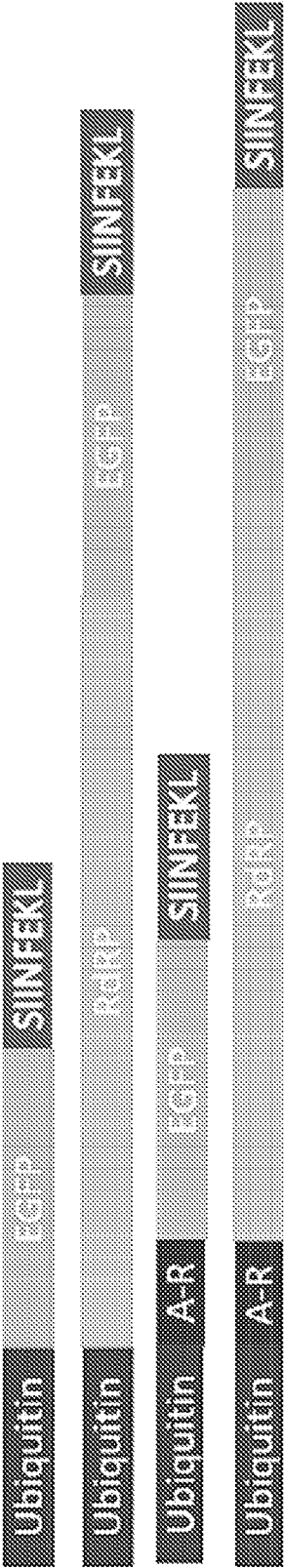


FIG. 5A

Modified RdRp form 2: PEST sequence to enhance proteasomal degradation



FIG. 5B

Modified RdRp form 3: fragmented RdRp in combination

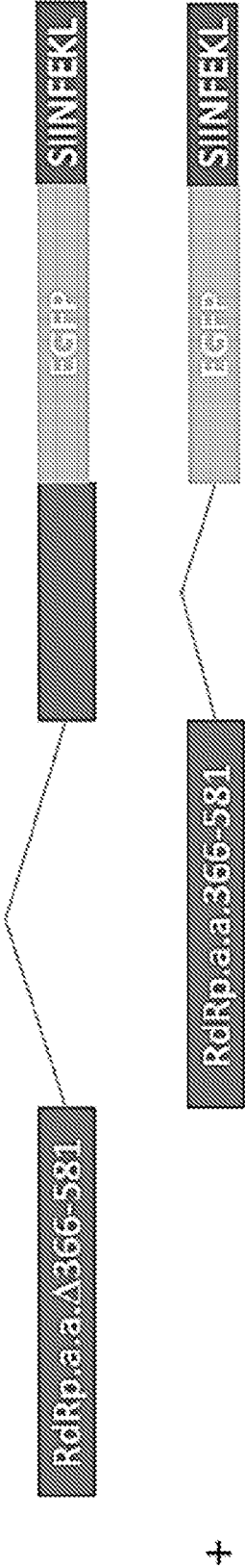


FIG. 5C



FIG. 6A

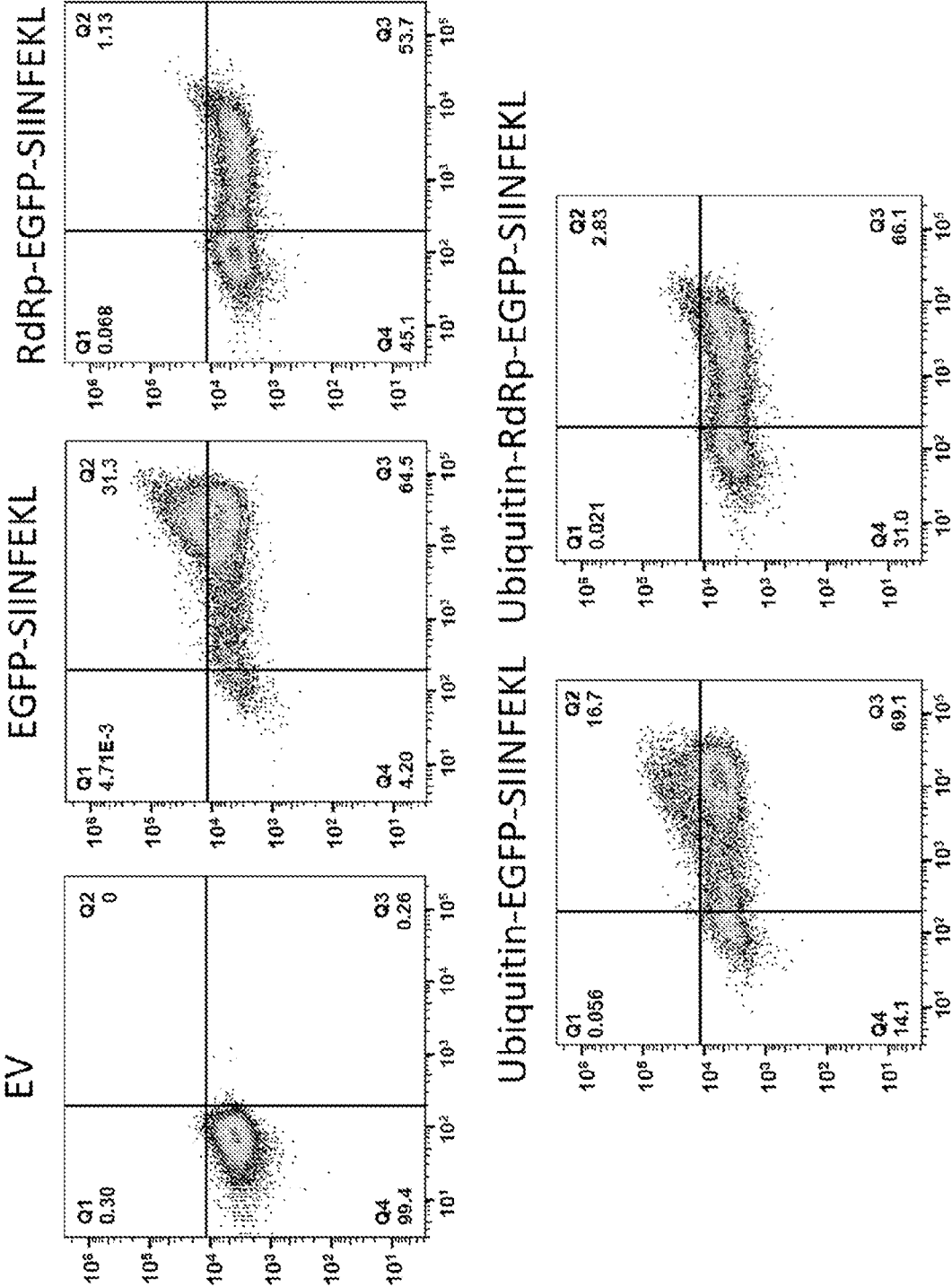


FIG. 6B

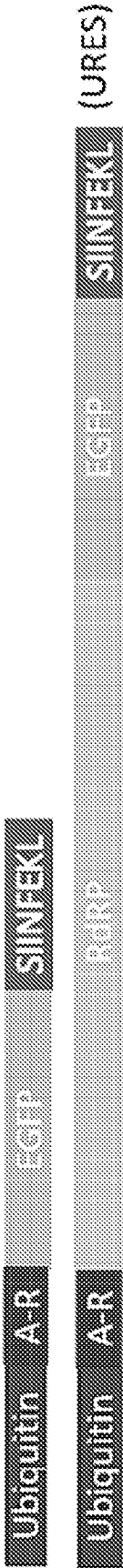


FIG. 7A

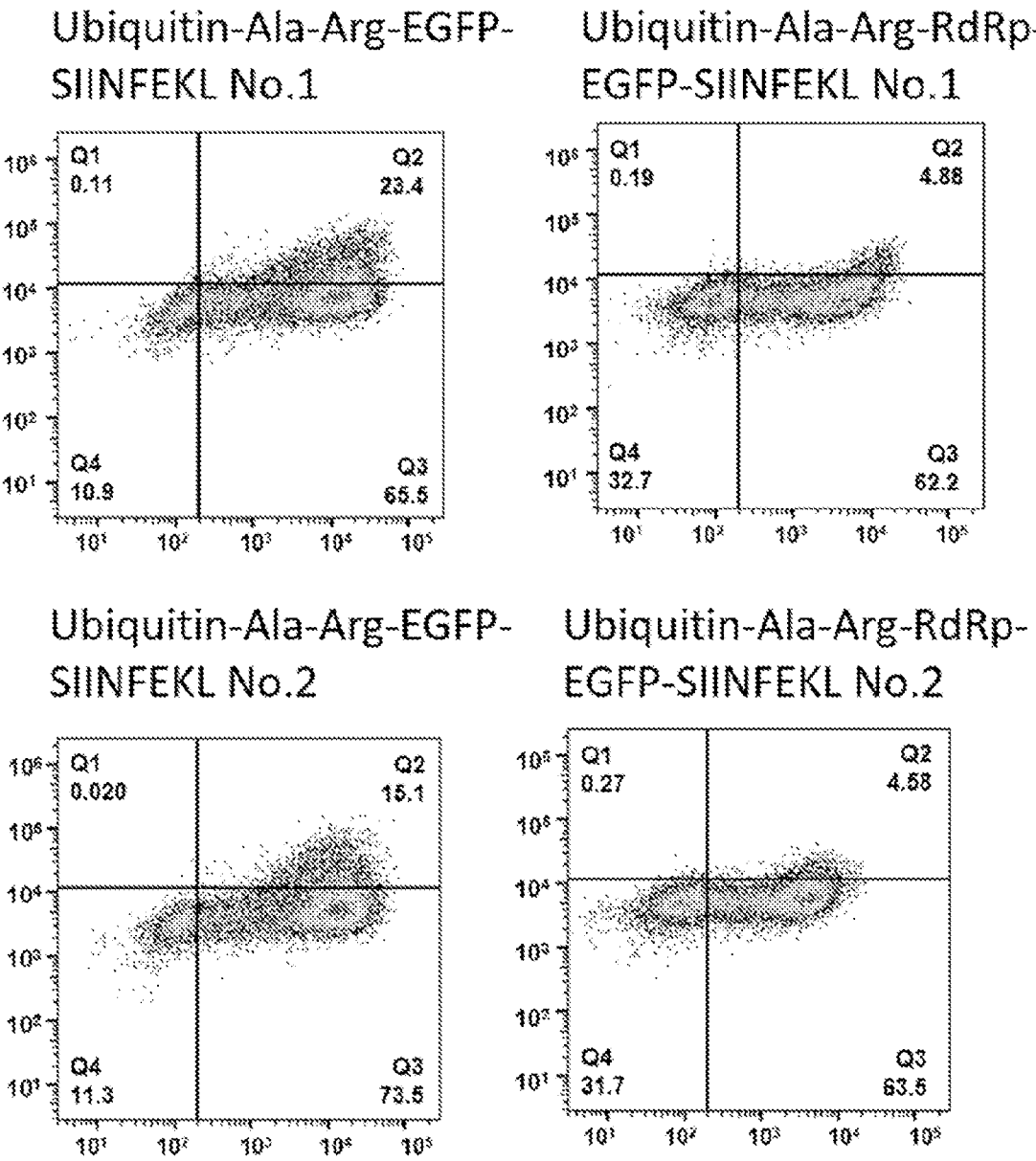


FIG. 7B

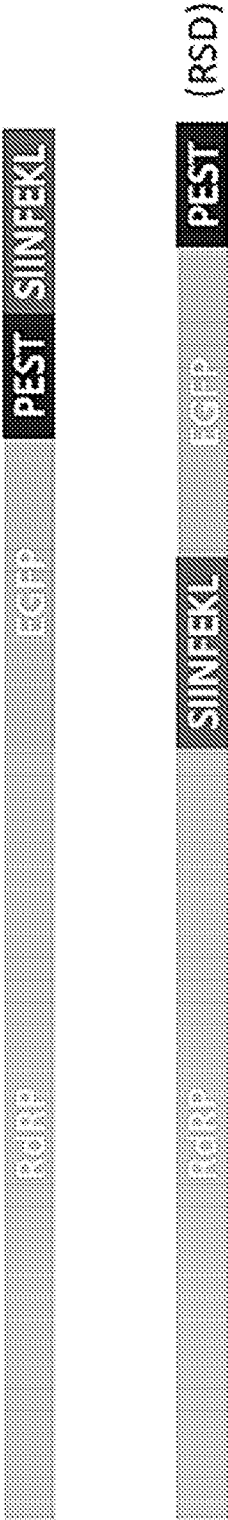


FIG. 8A

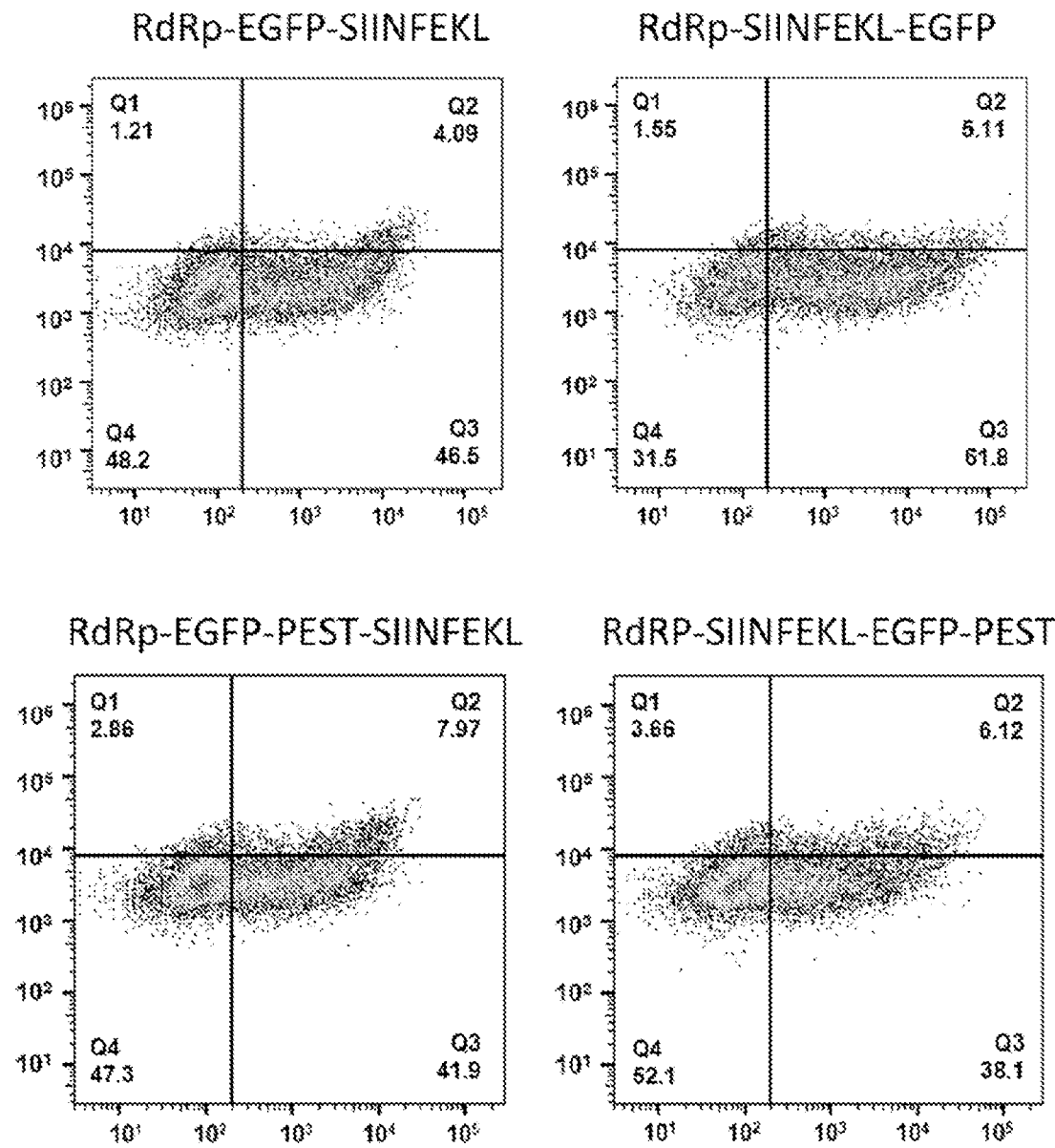


FIG. 8B

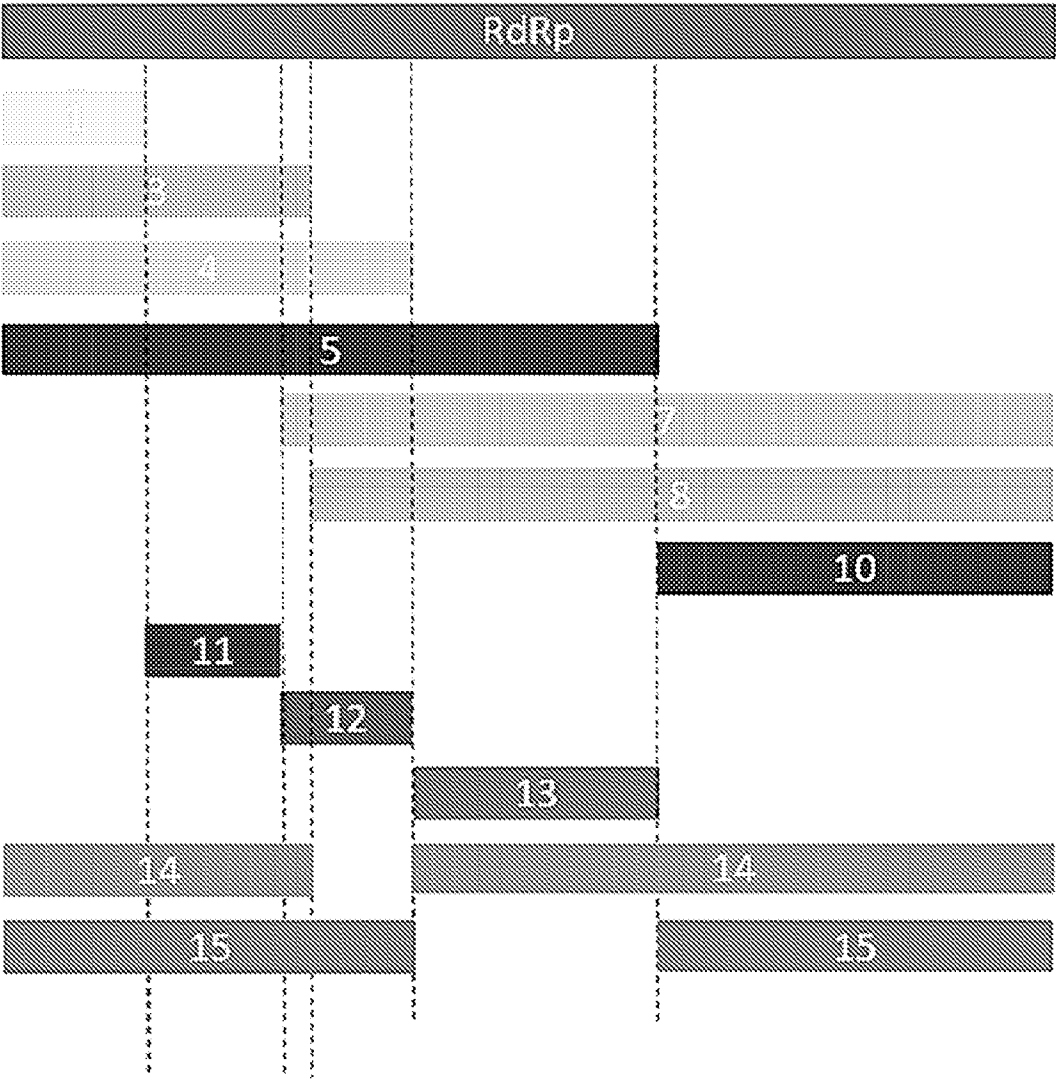


FIG. 9

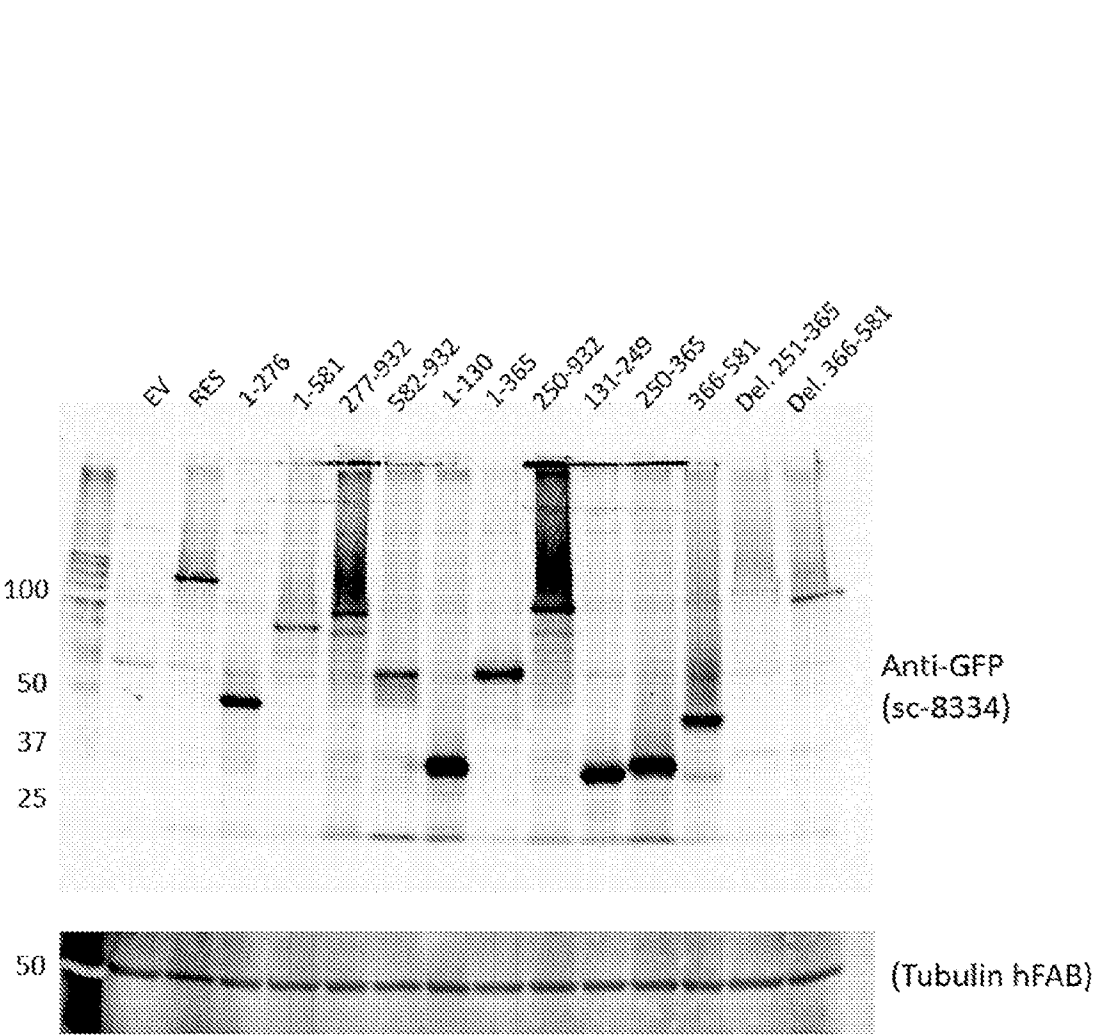


FIG. 10

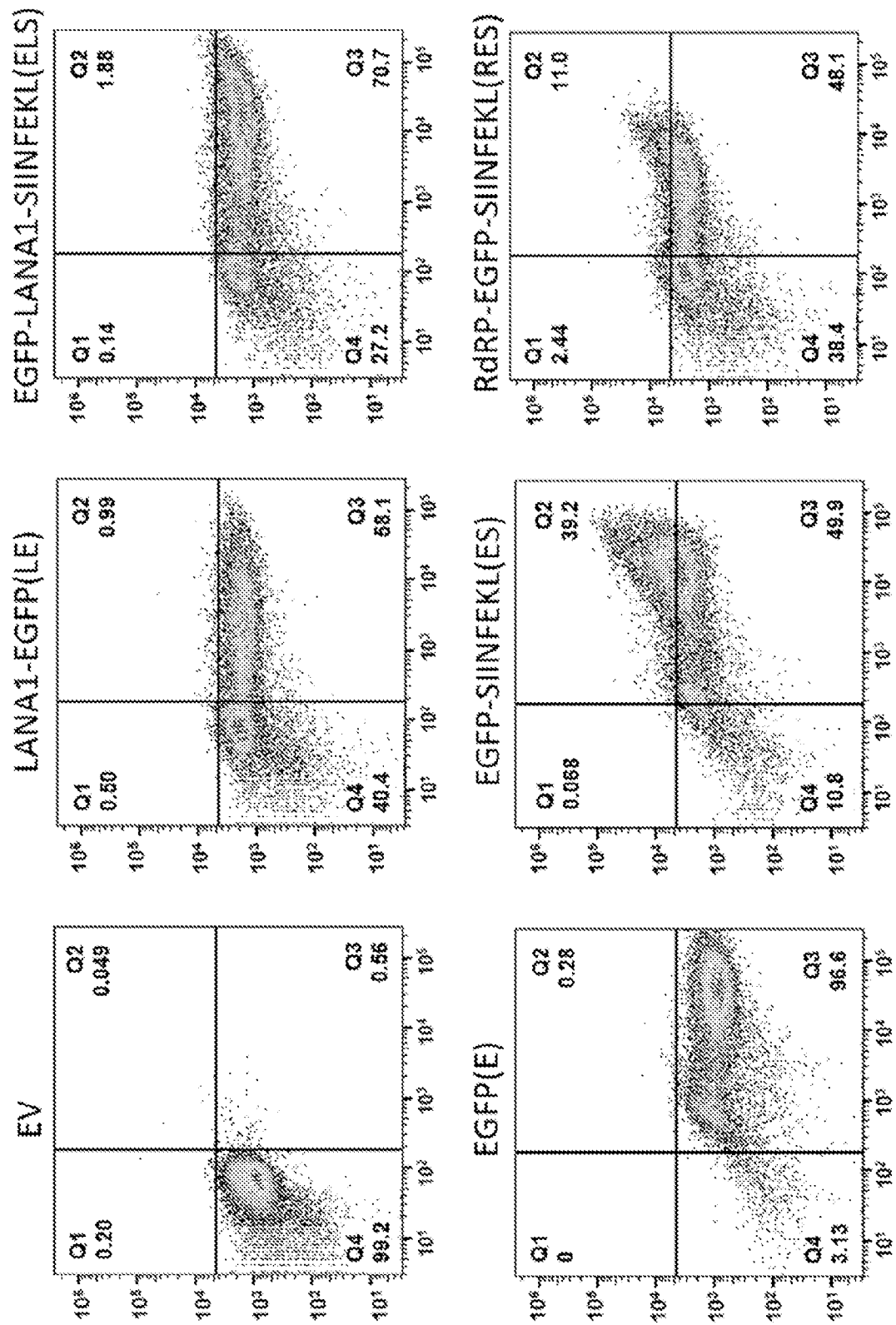


FIG. 11

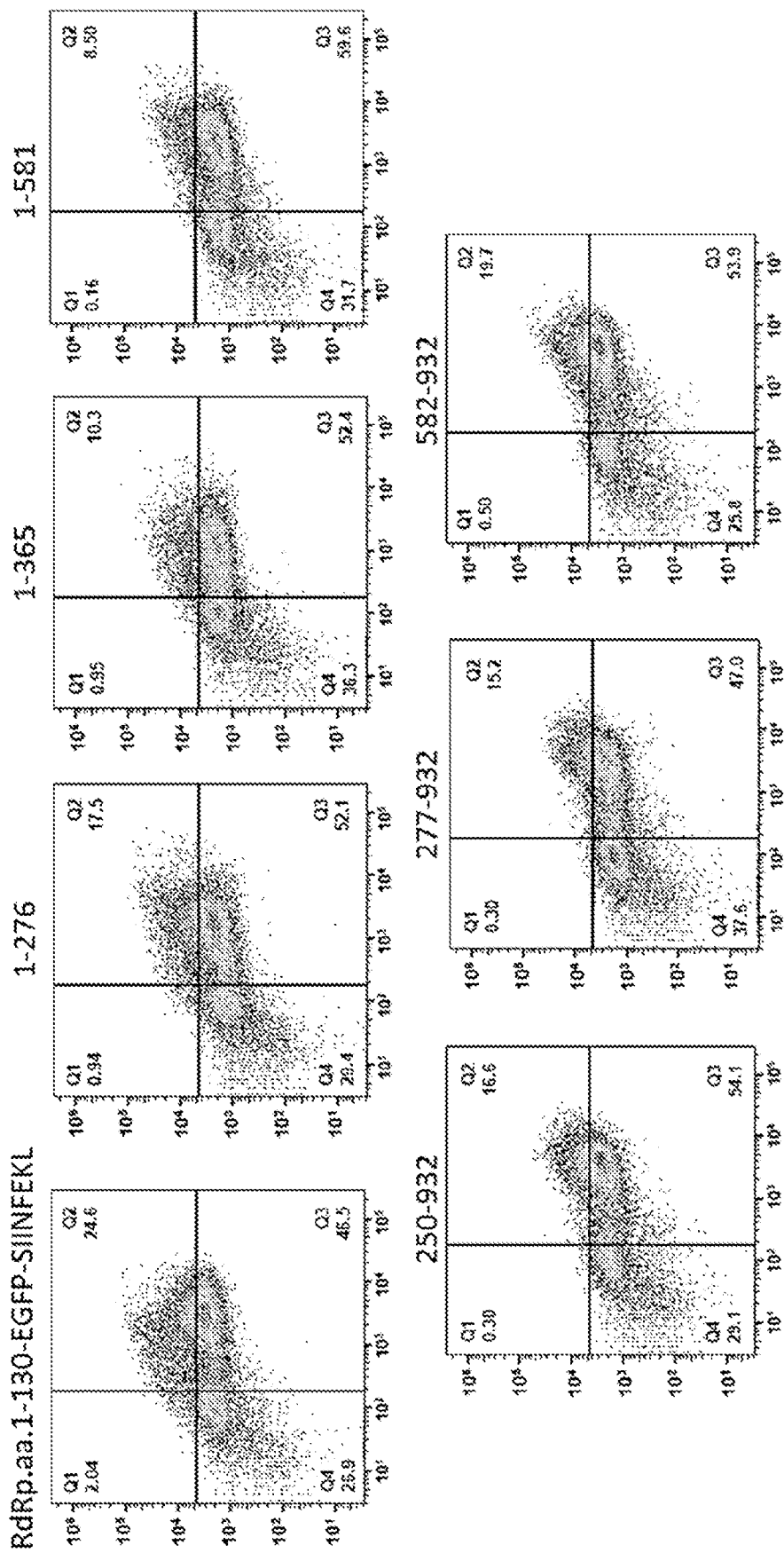


FIG. 12

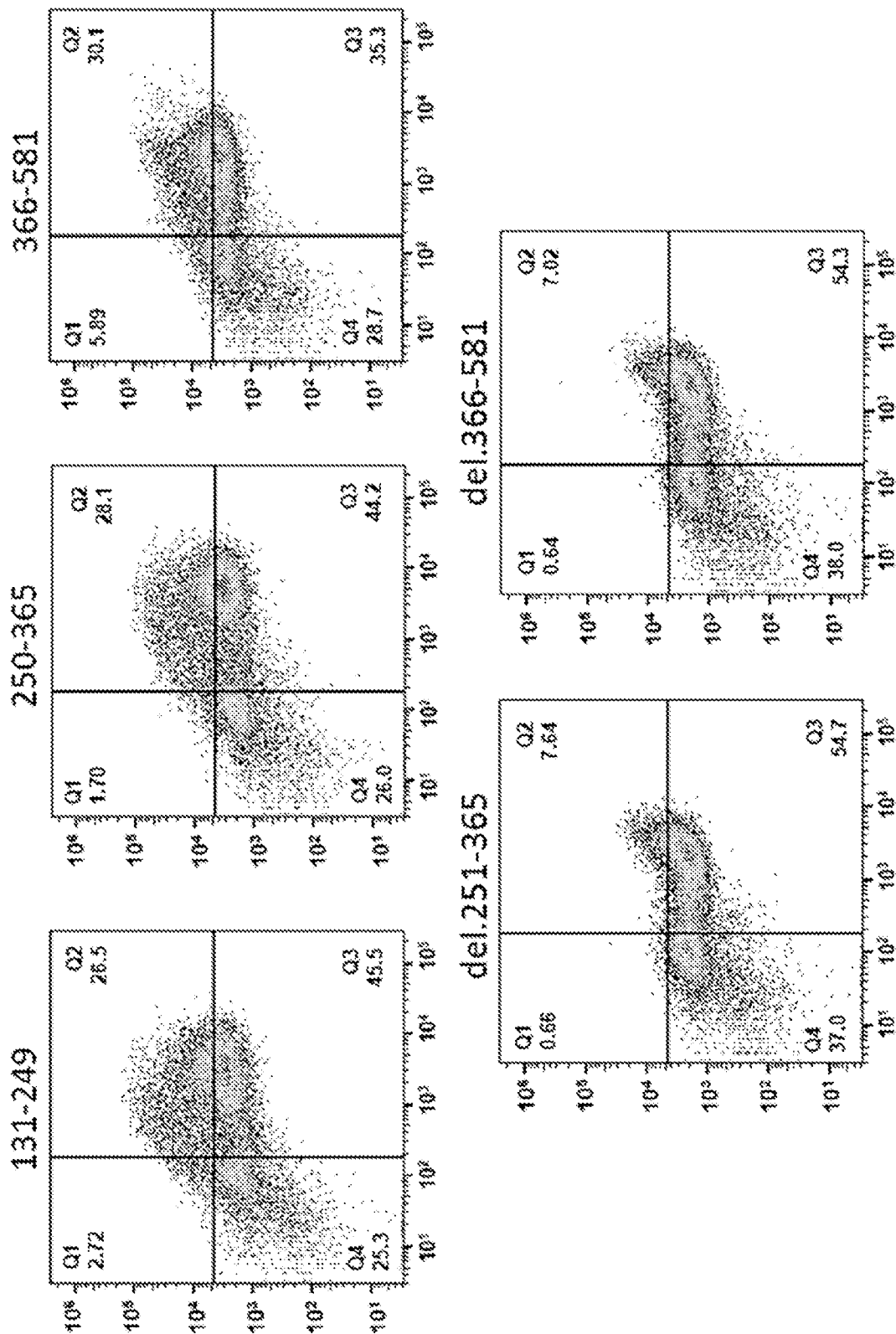


FIG. 13

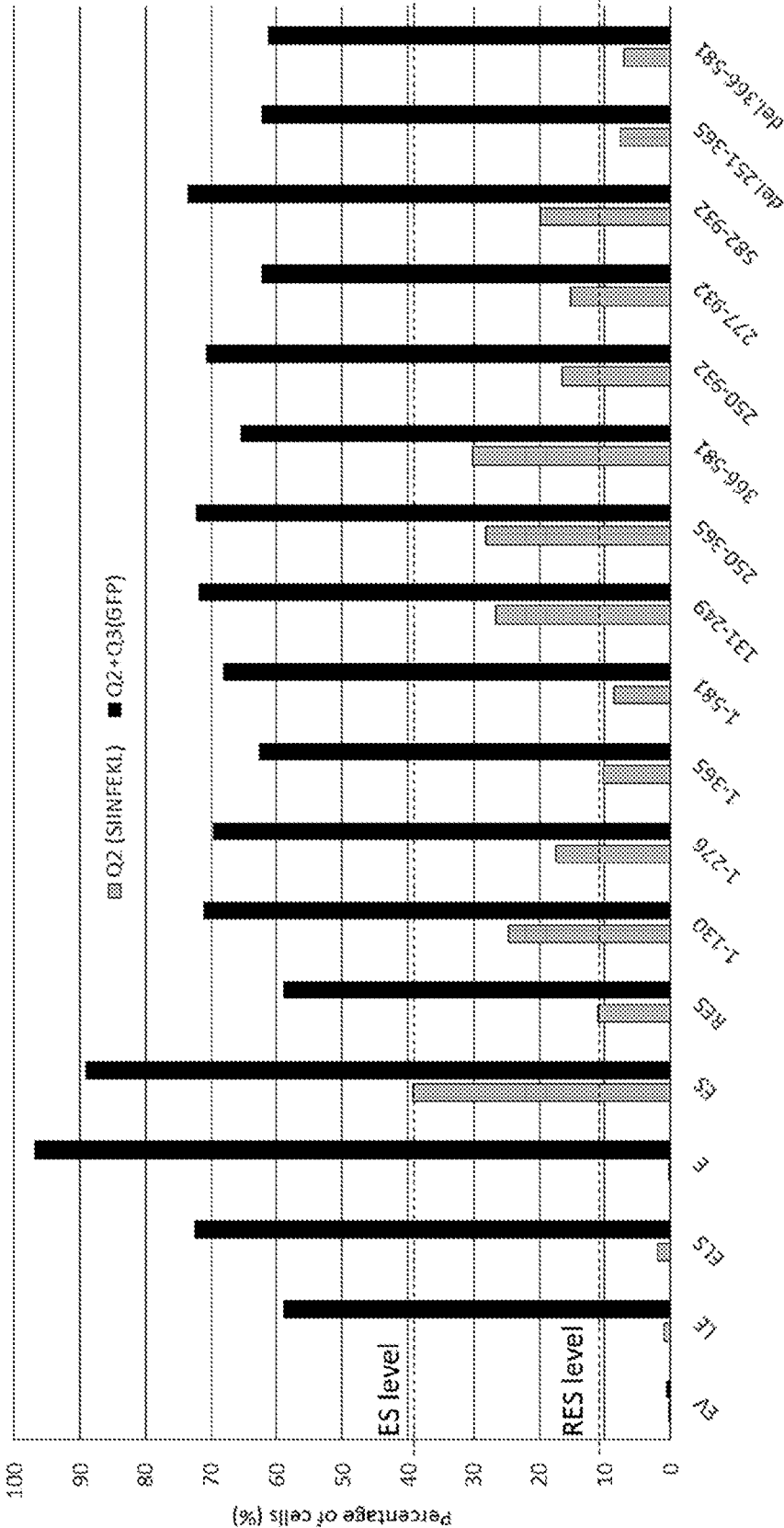


FIG. 14

RdRp-EGFP-SIINF EKL(RES)

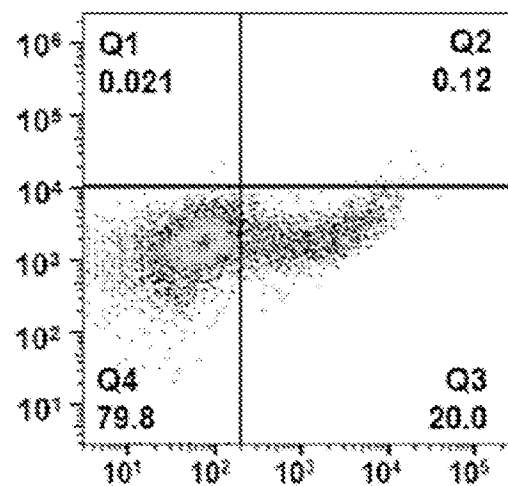


FIG. 15A

Fragment combinations

1-130 & 131-249 & 250-932 1-276 & 277-932

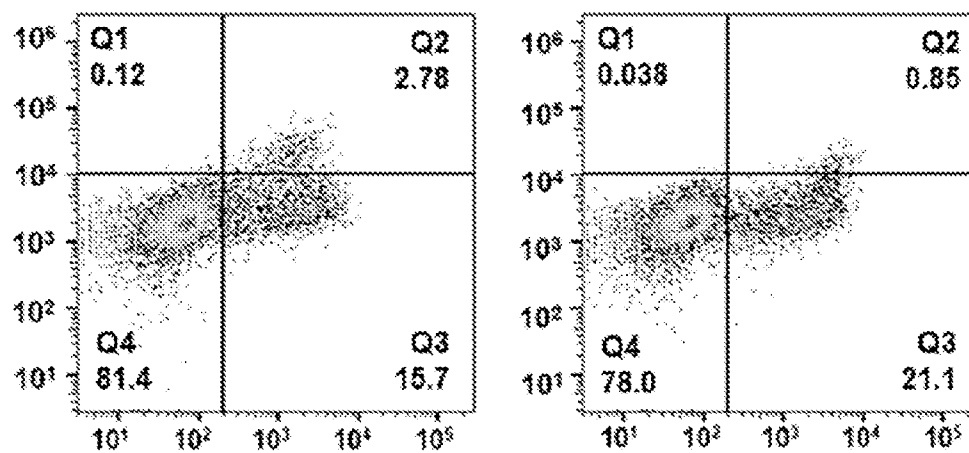


FIG. 15B

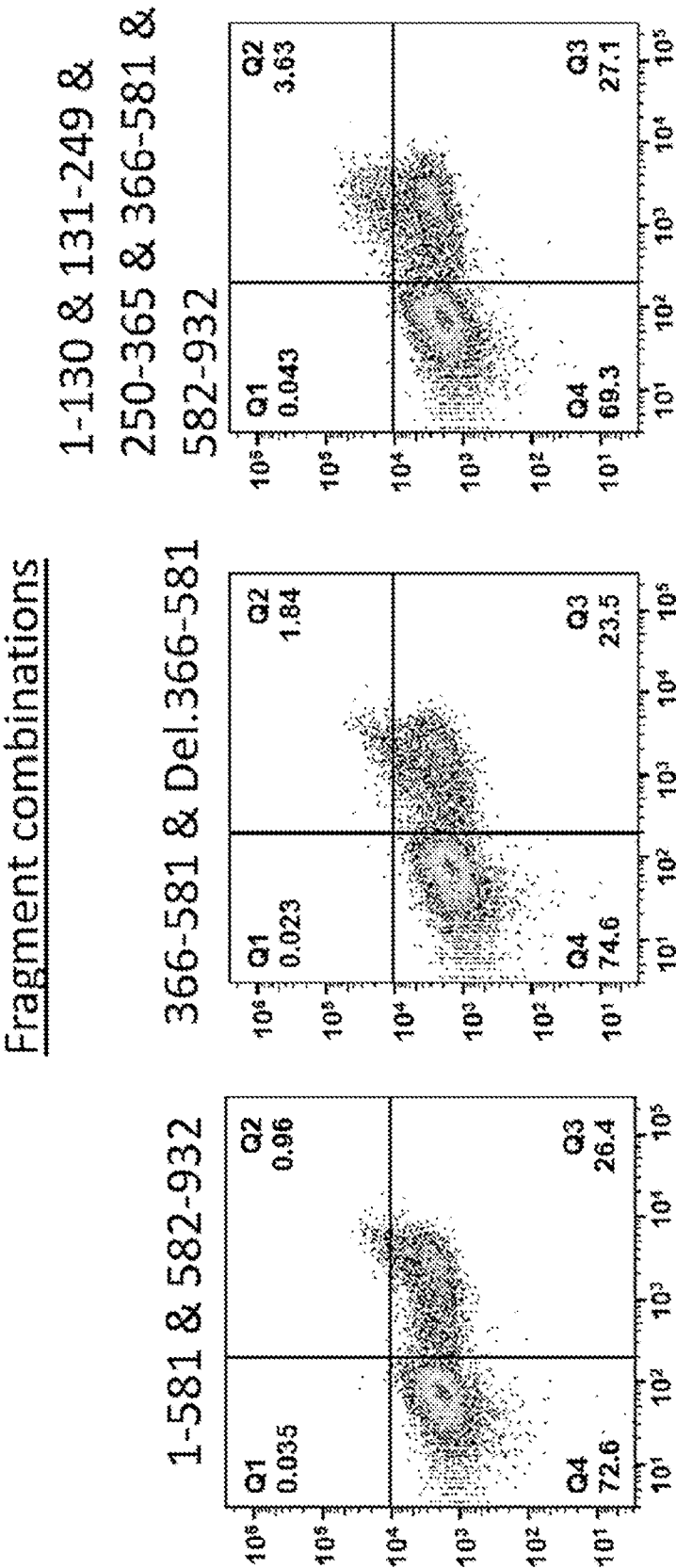


FIG. 15C

ES gradients

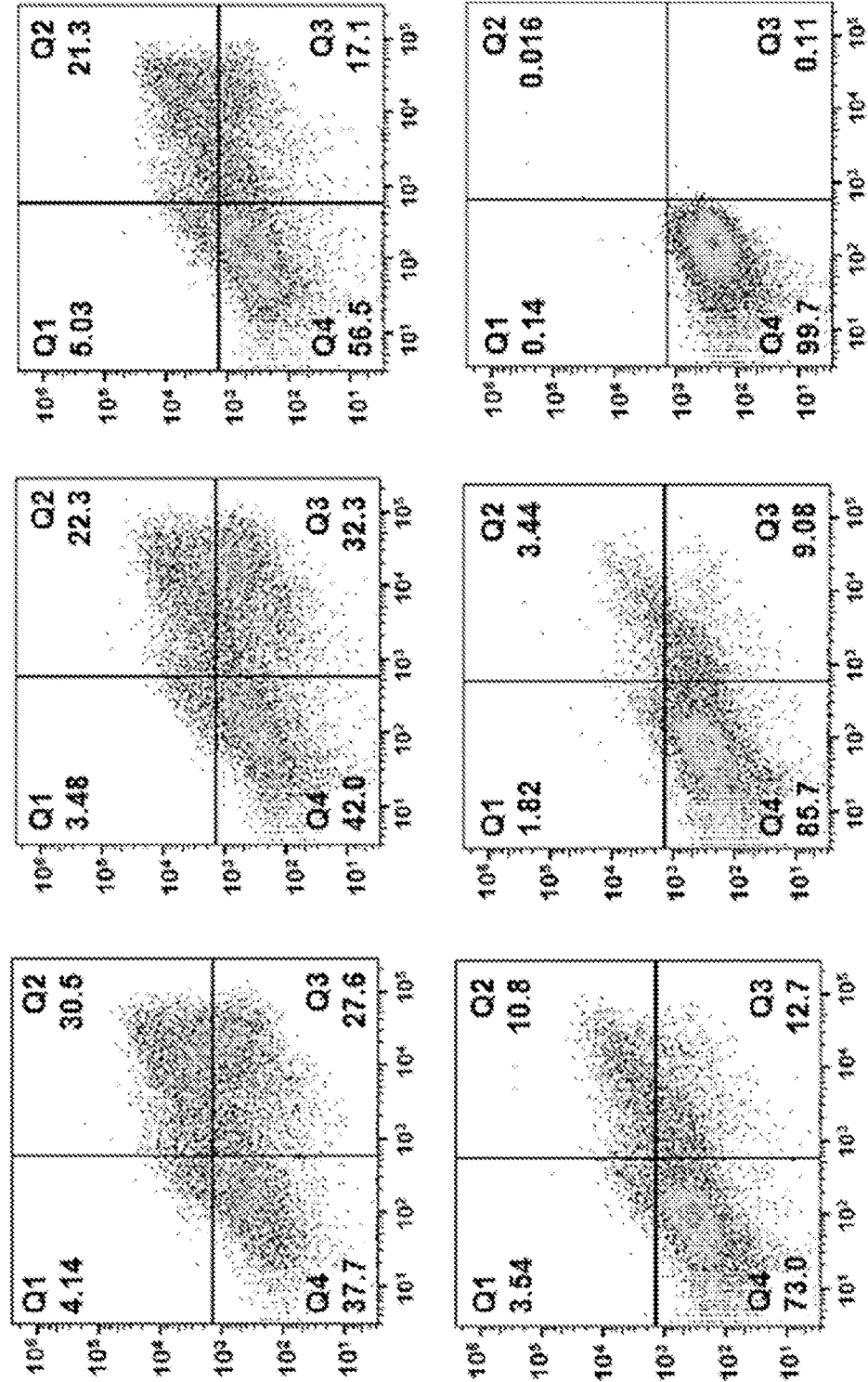


FIG. 16A

ELS gradients

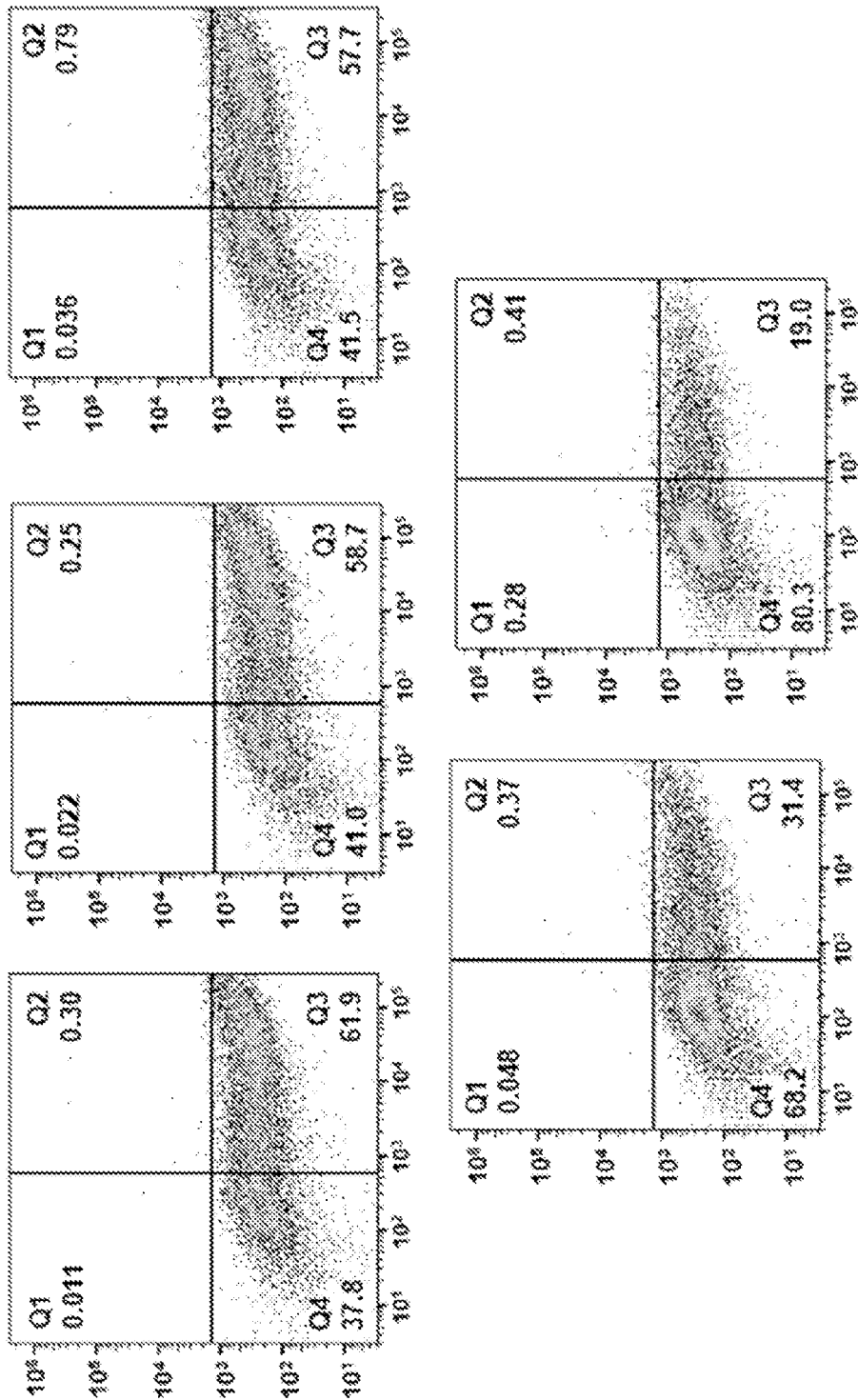


FIG. 16B

Hsp70-ES gradients

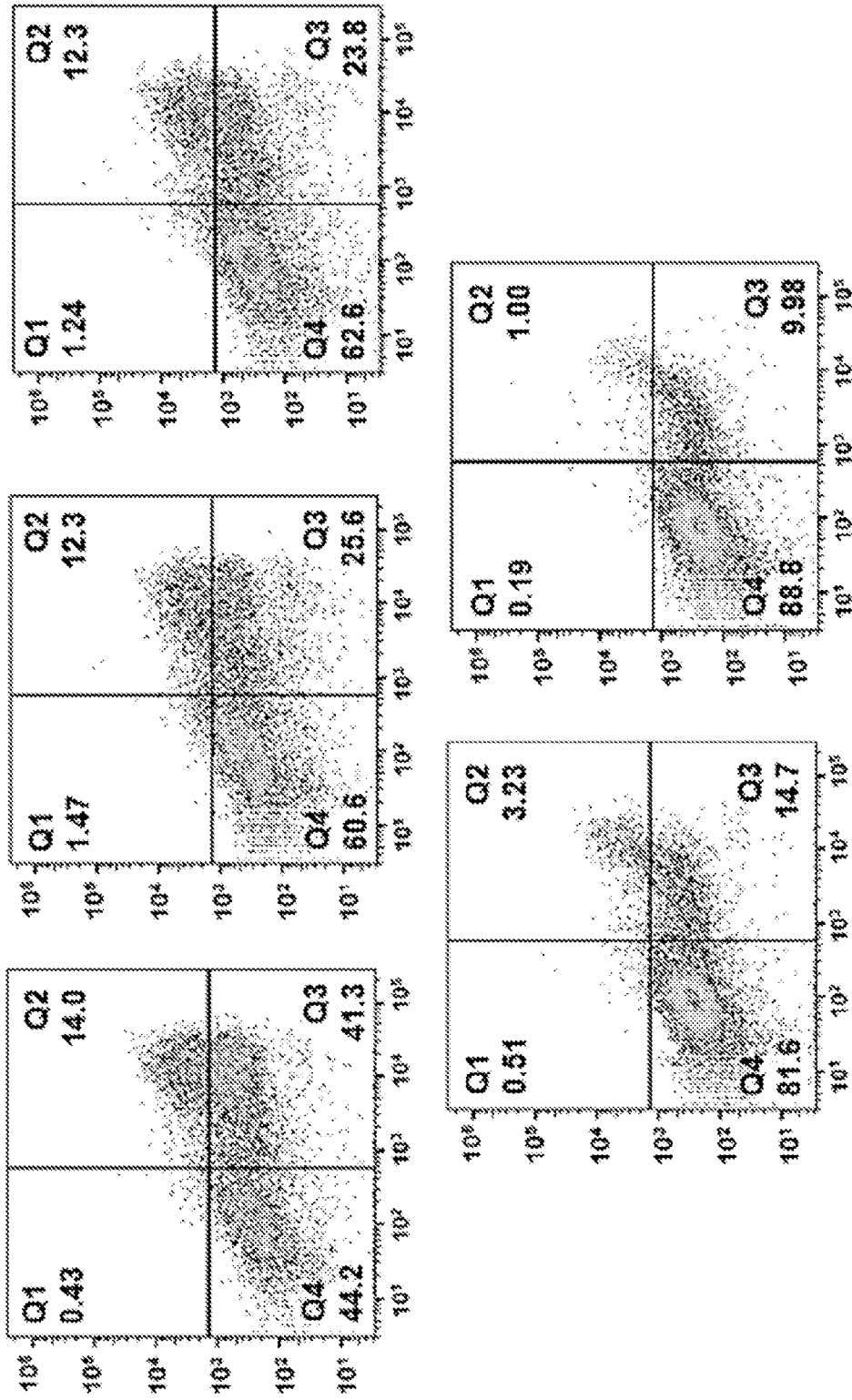
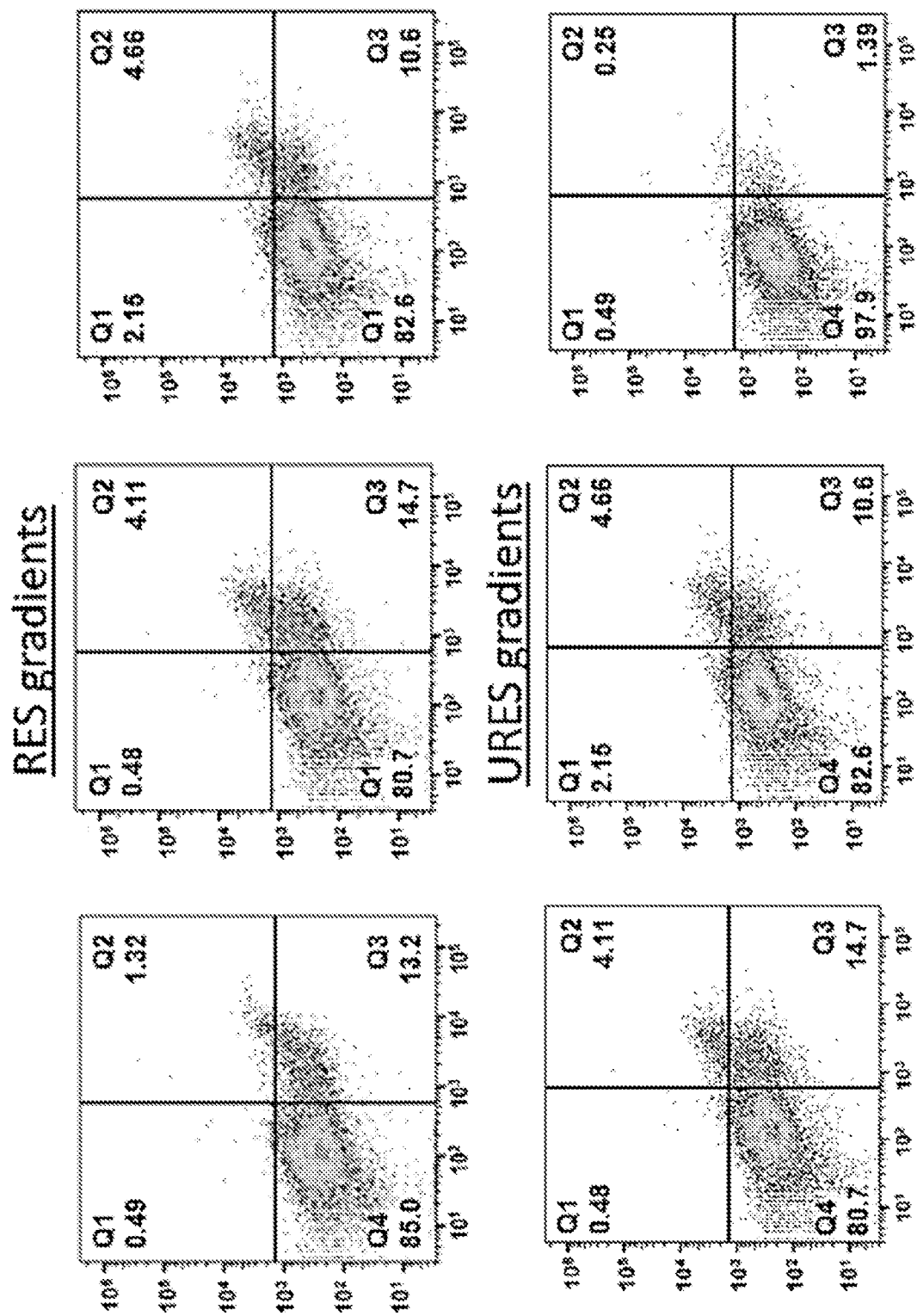


FIG. 17A



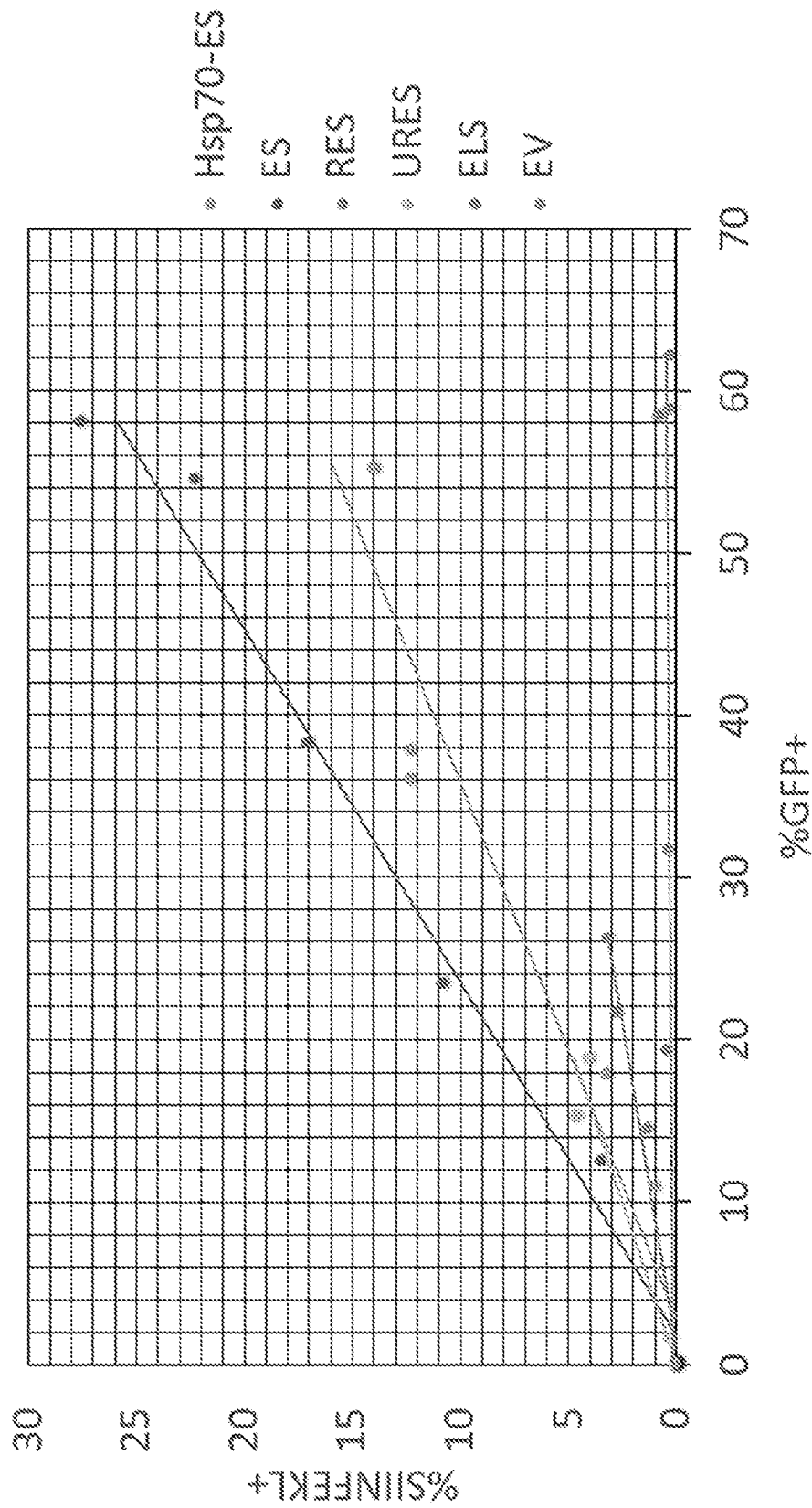


FIG. 18

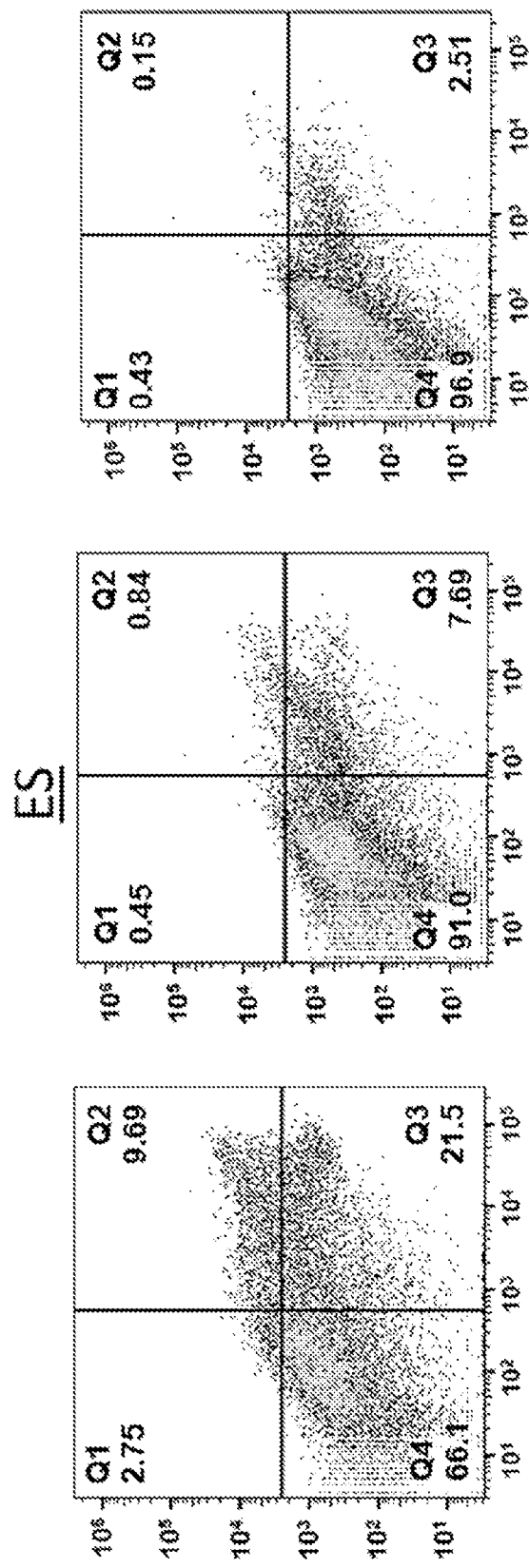


FIG. 19A

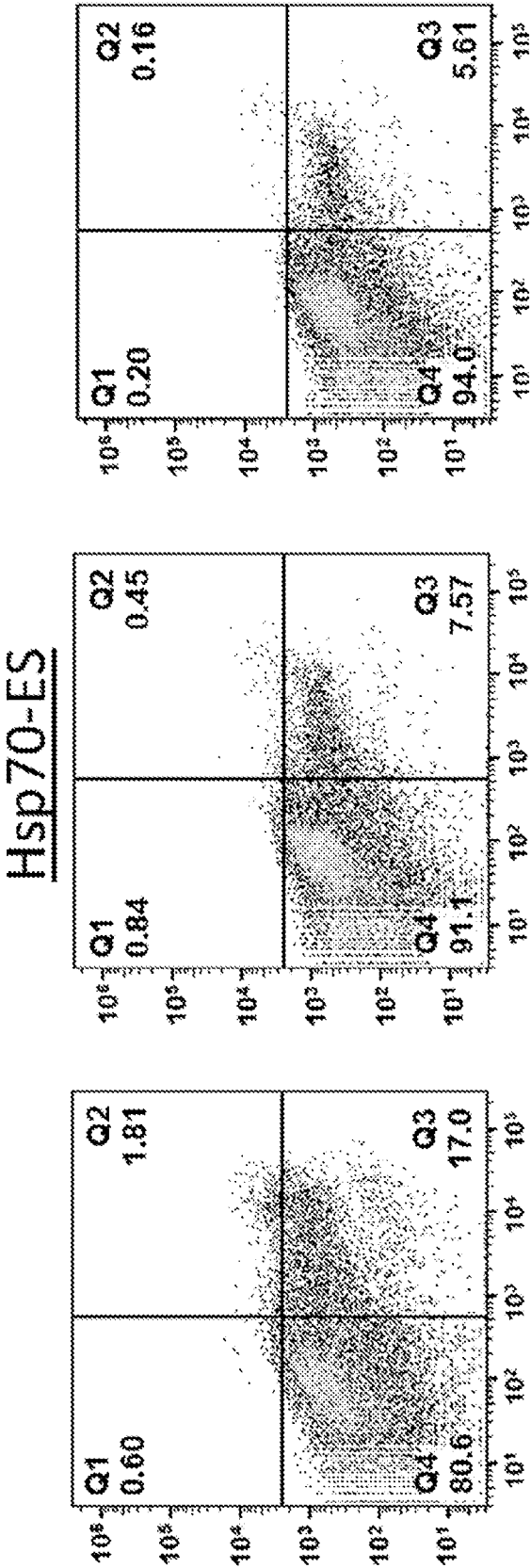


FIG. 19B

$\frac{1}{2}$ RdRp.aa.1-581-ES + $\frac{1}{2}$ RdRp.aa.582-932-ES

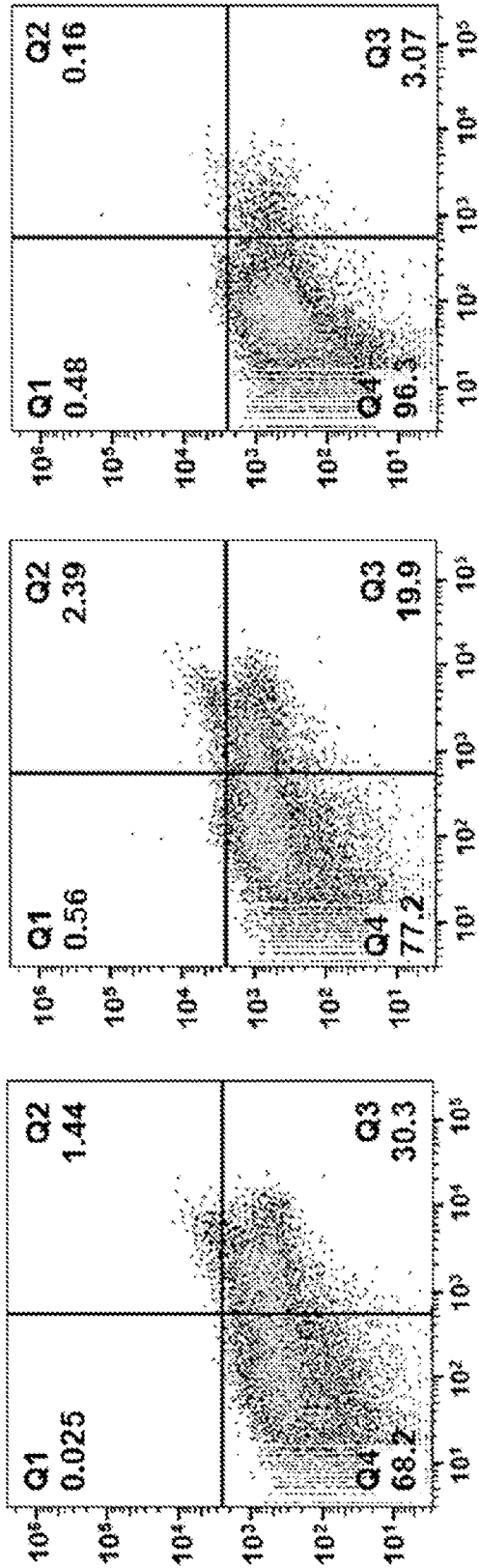


FIG. 19C

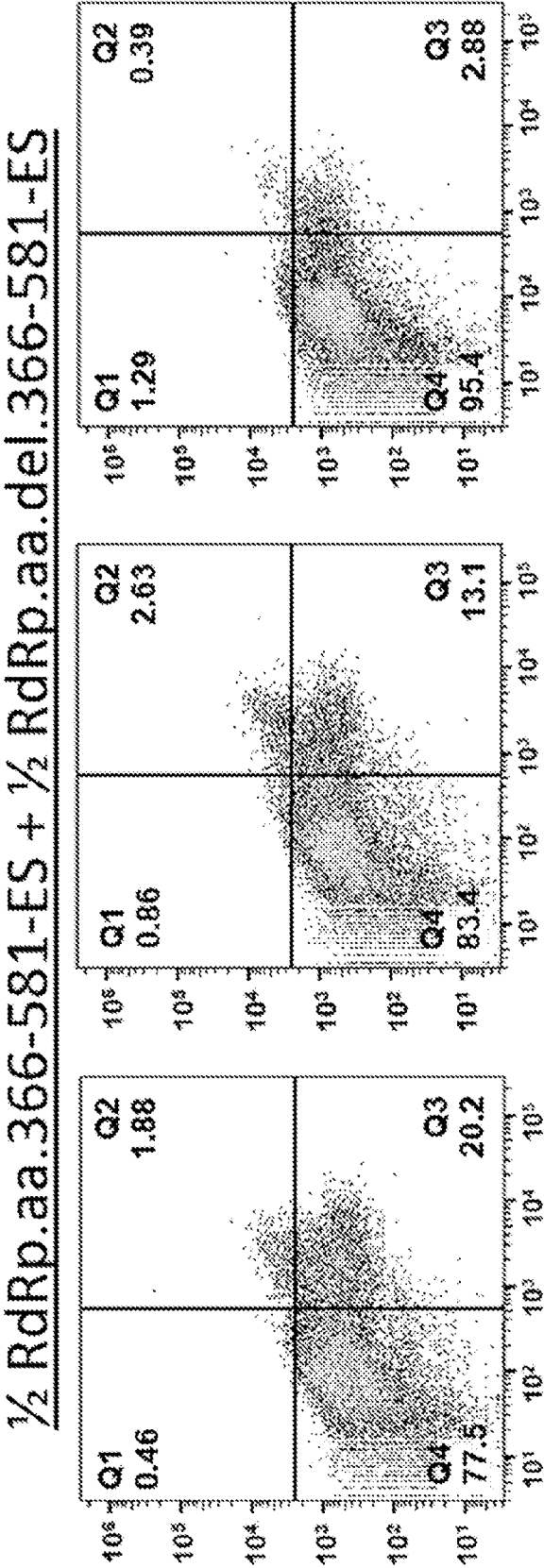


FIG. 19D

RES

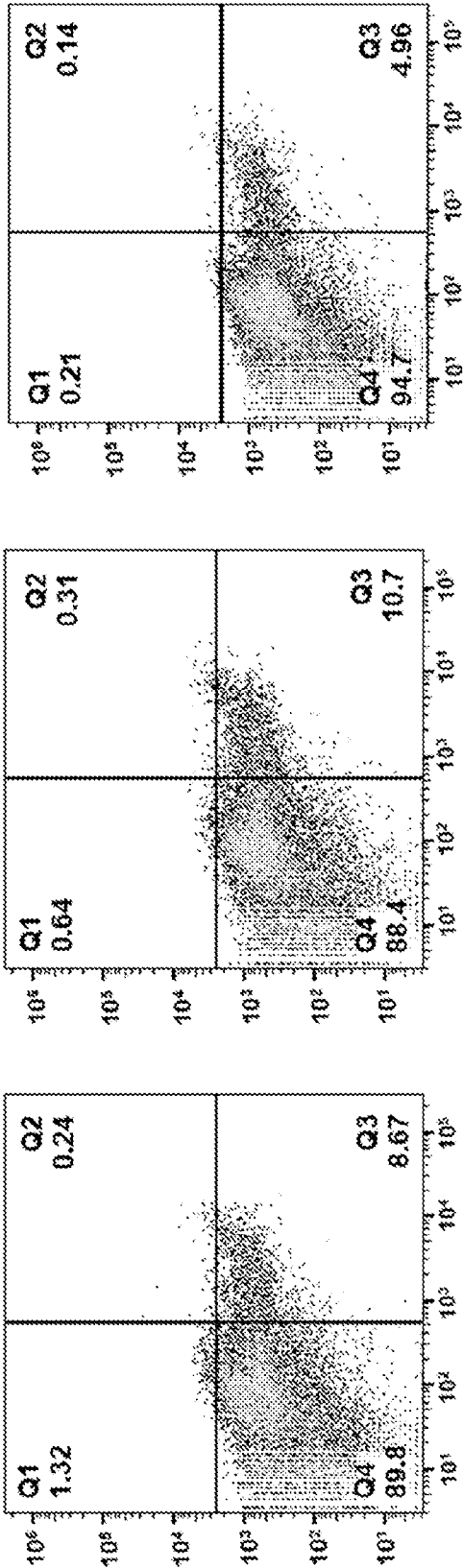


FIG. 20A

URES

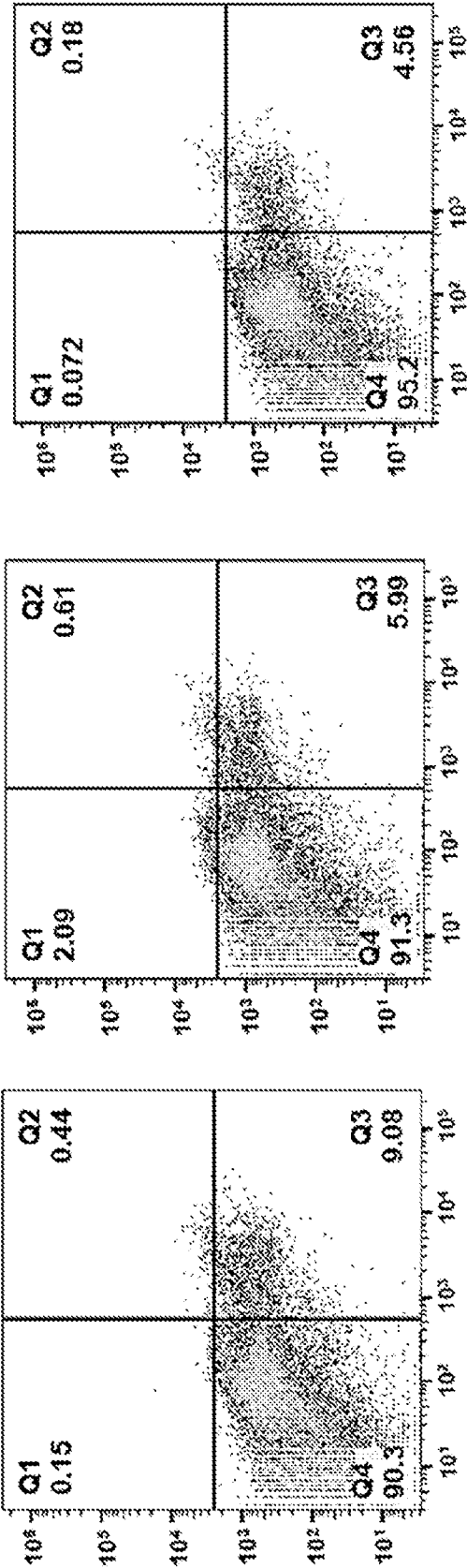


FIG. 20B

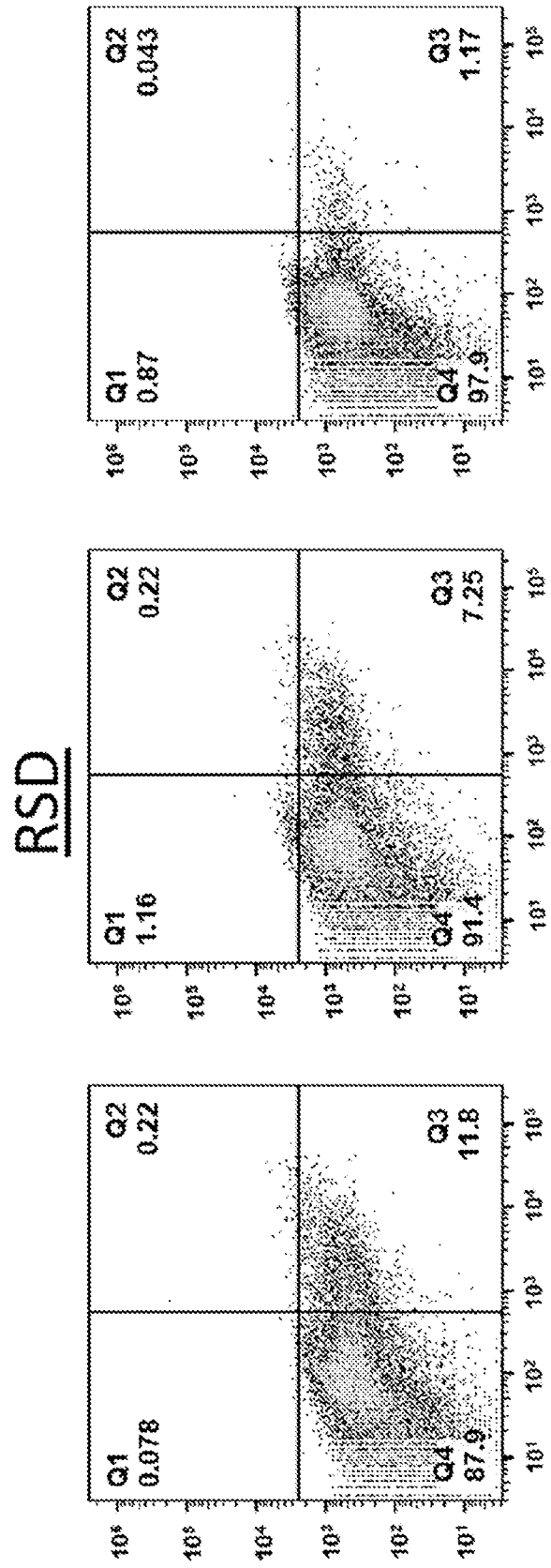


FIG. 20C

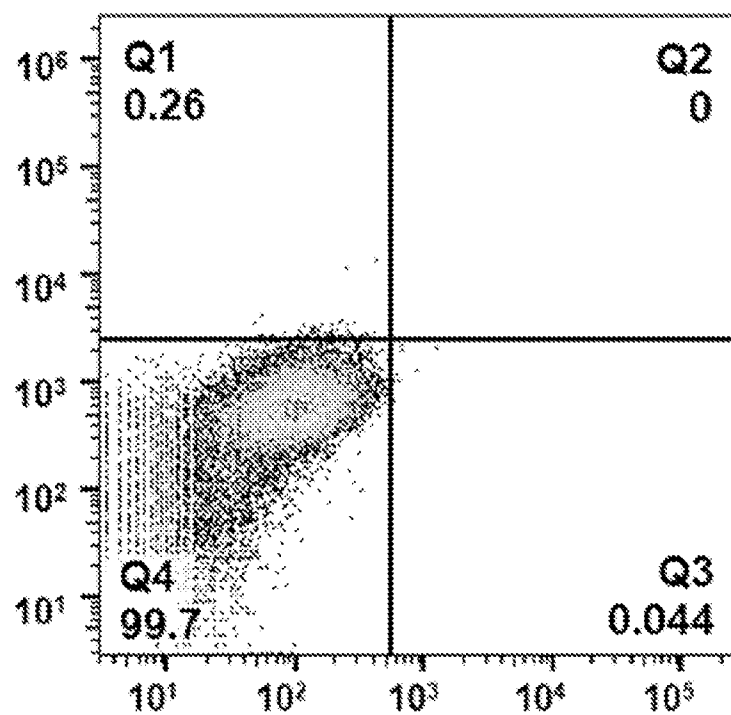
EV

FIG. 20D

ELS

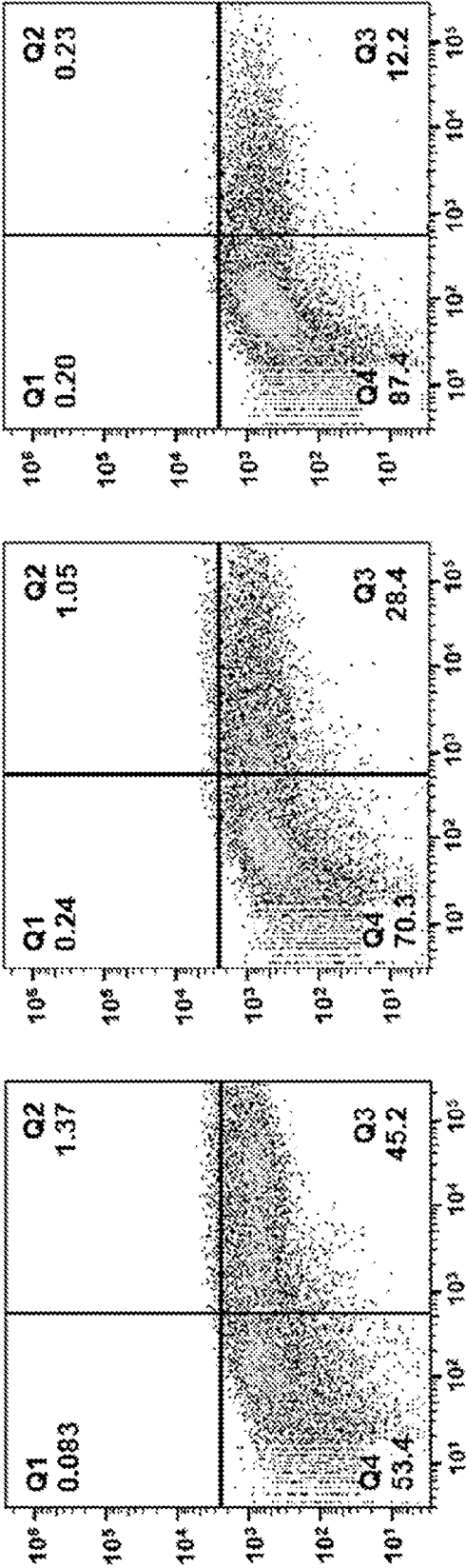


FIG. 20E

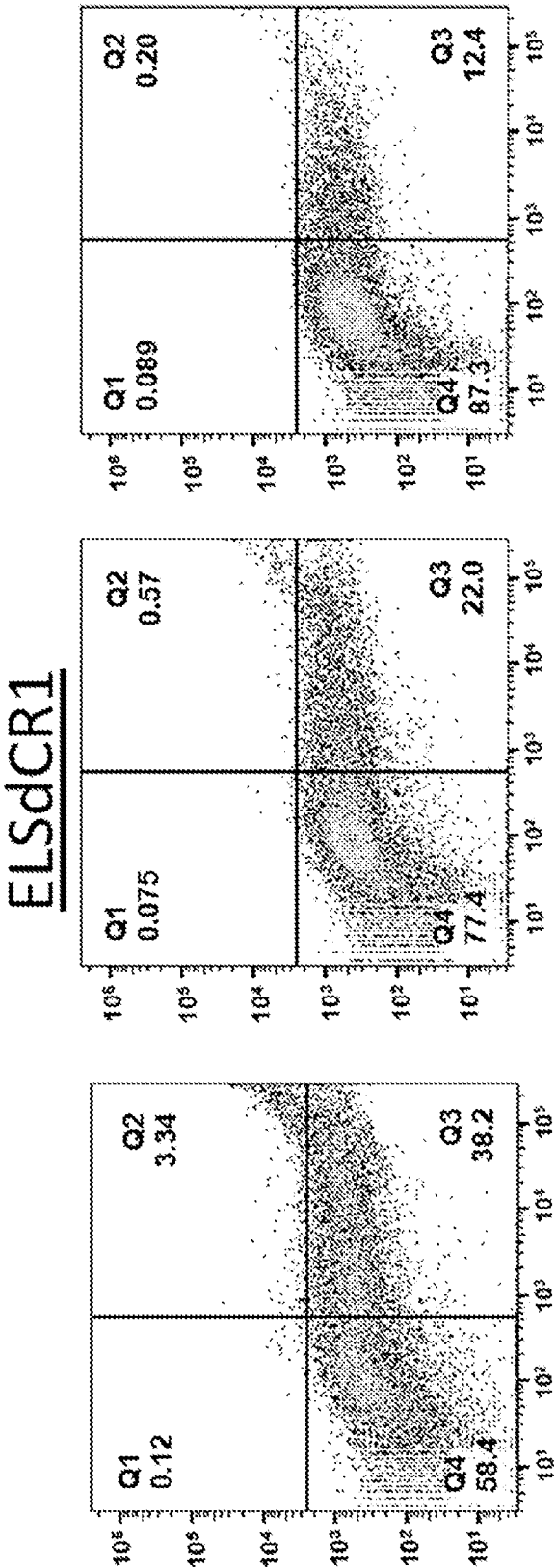


FIG. 20F

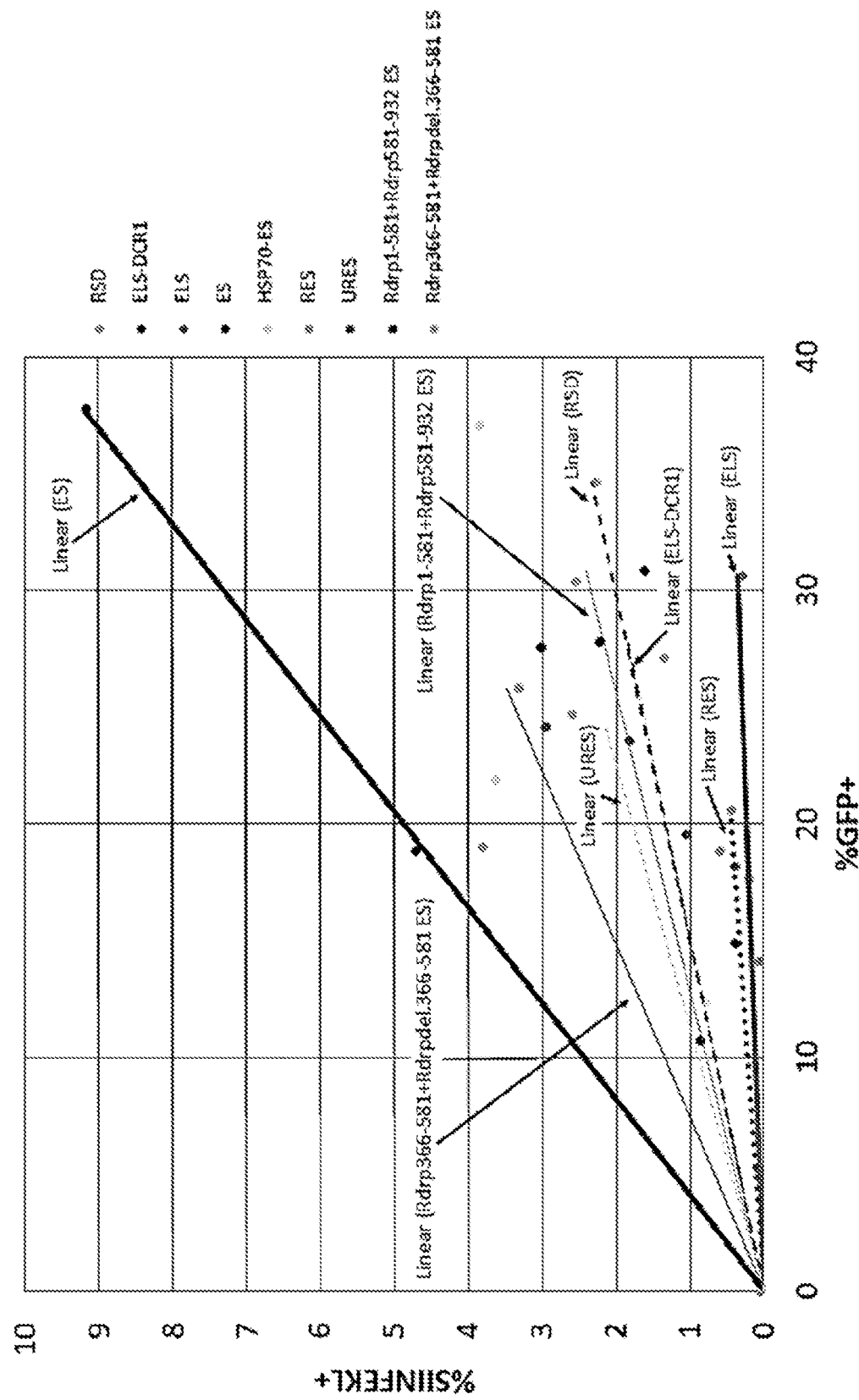


FIG. 21

ES

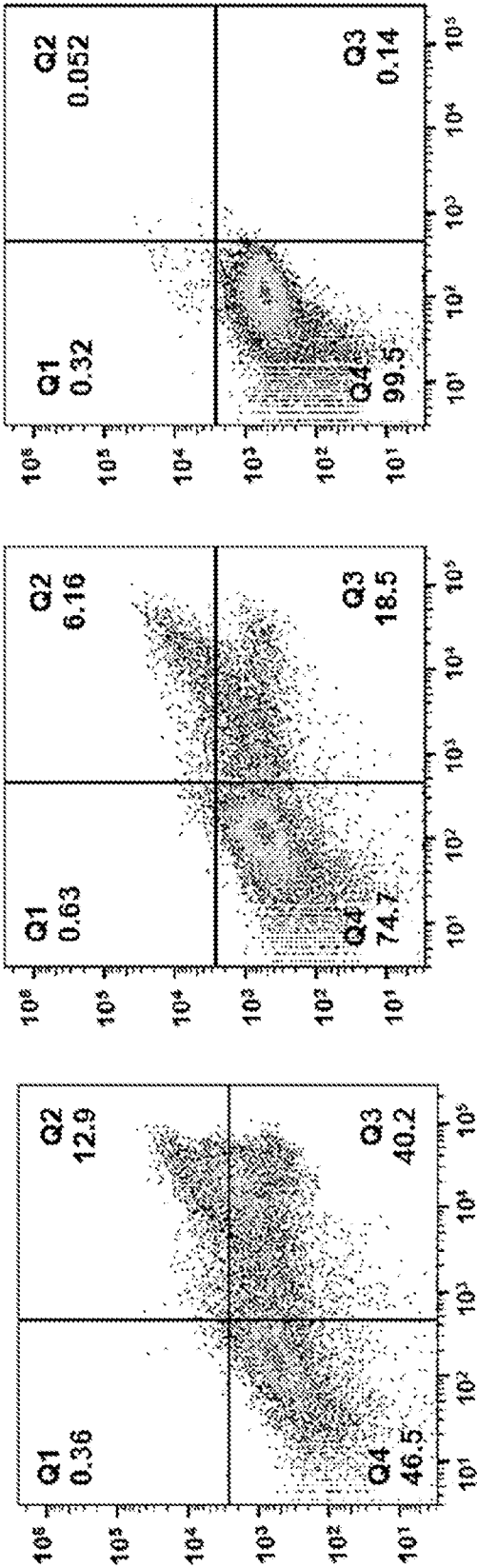


FIG. 22A

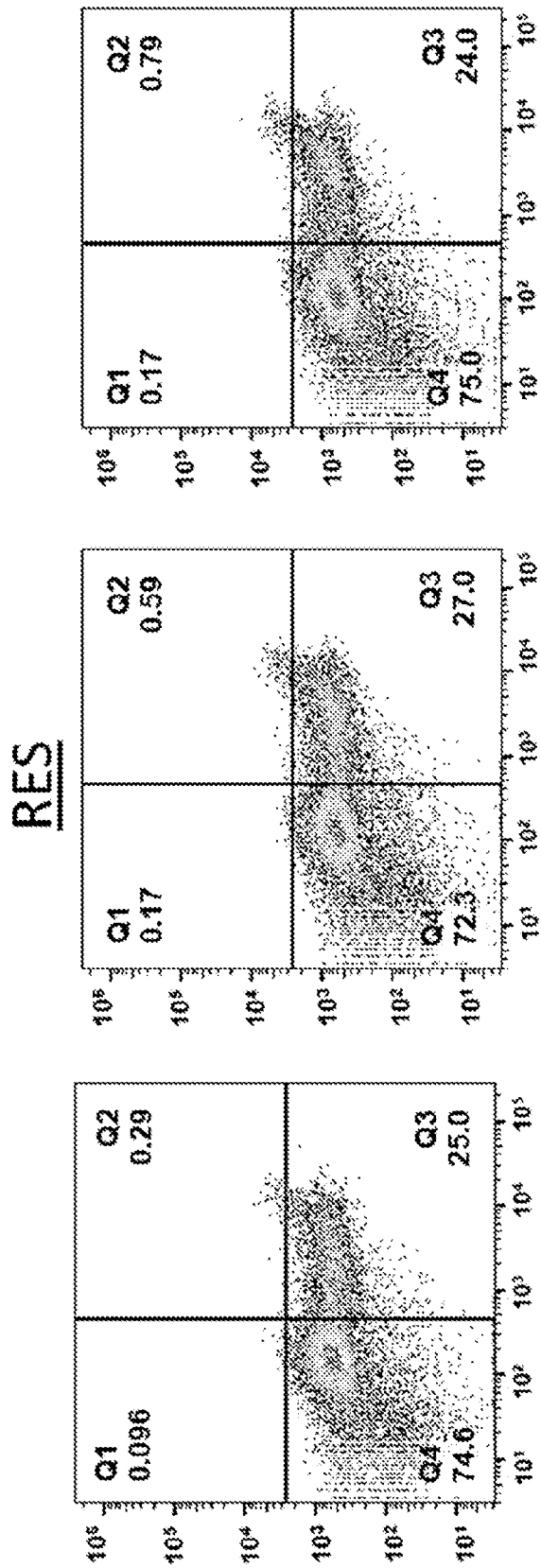


FIG. 22B

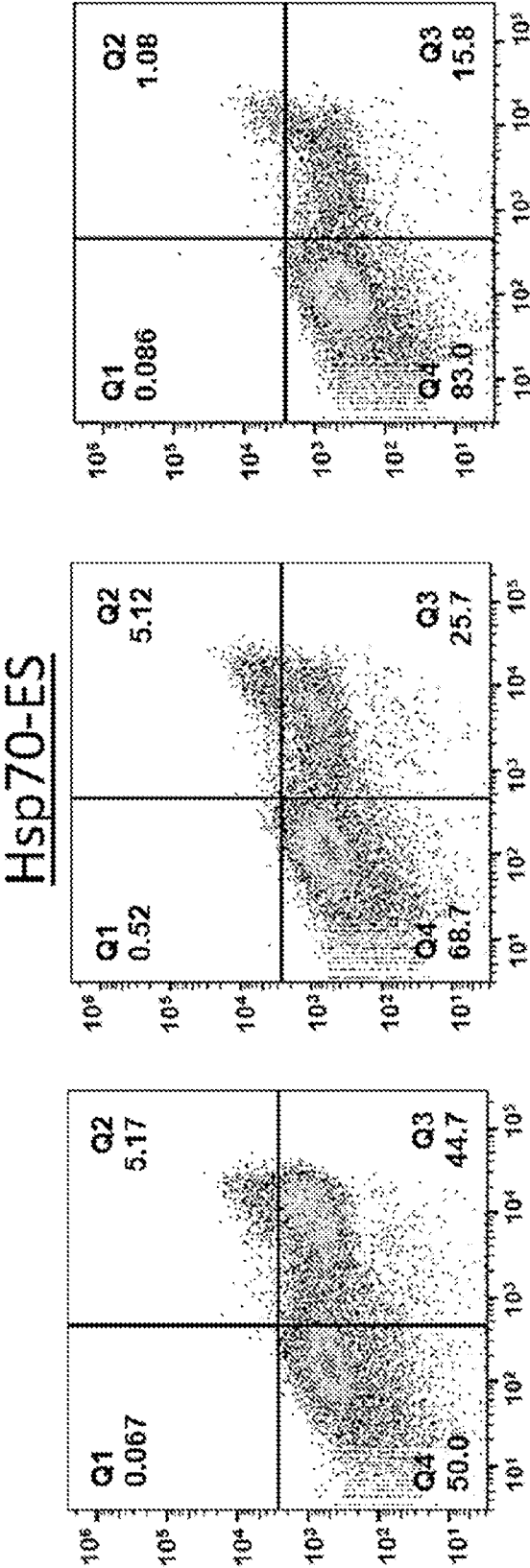


FIG. 22C

URES

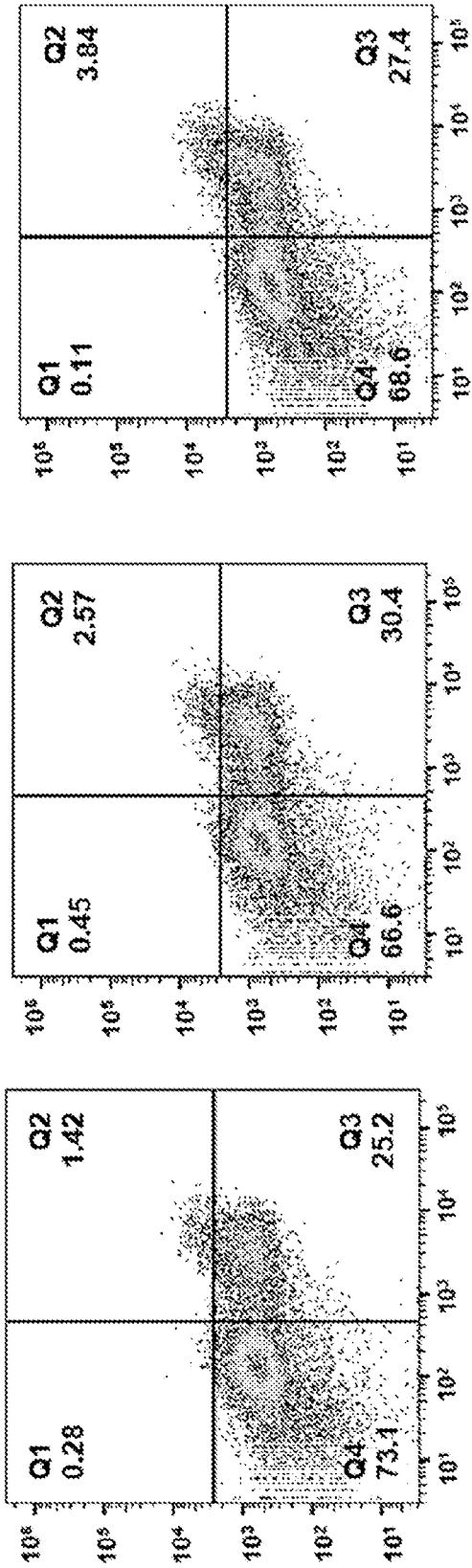


FIG. 22D

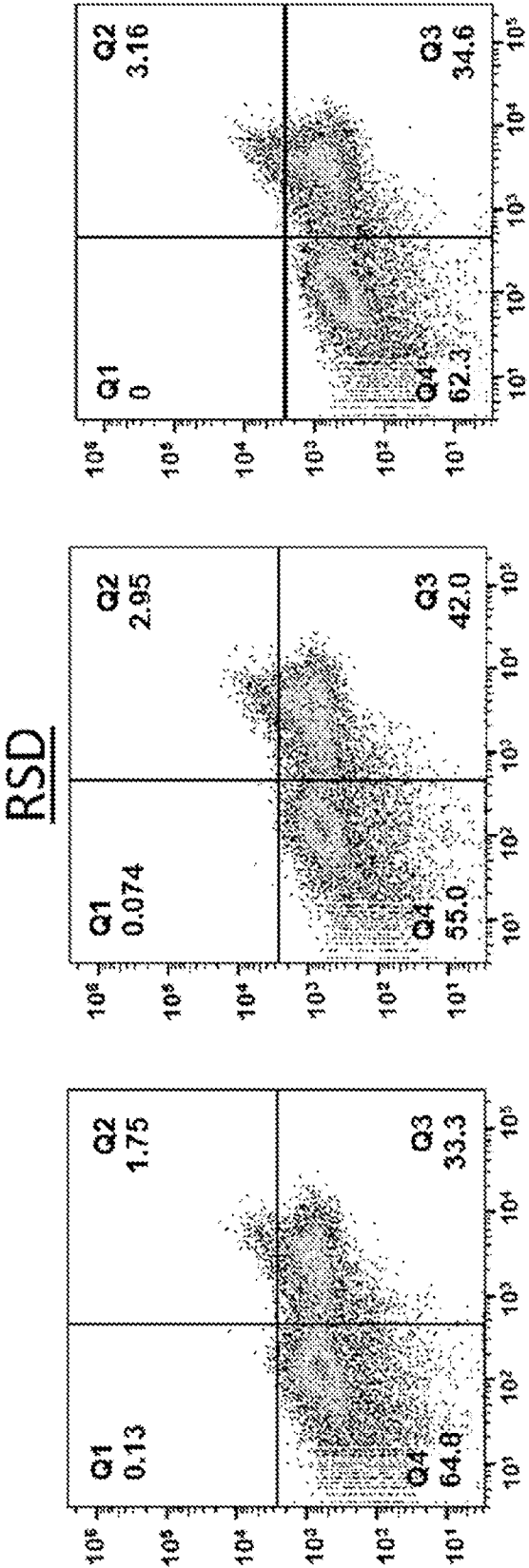


FIG. 23A

$\frac{1}{2}$ RdRp.aa.1-581-ES + $\frac{1}{2}$ RdRp.aa.582-932-ES

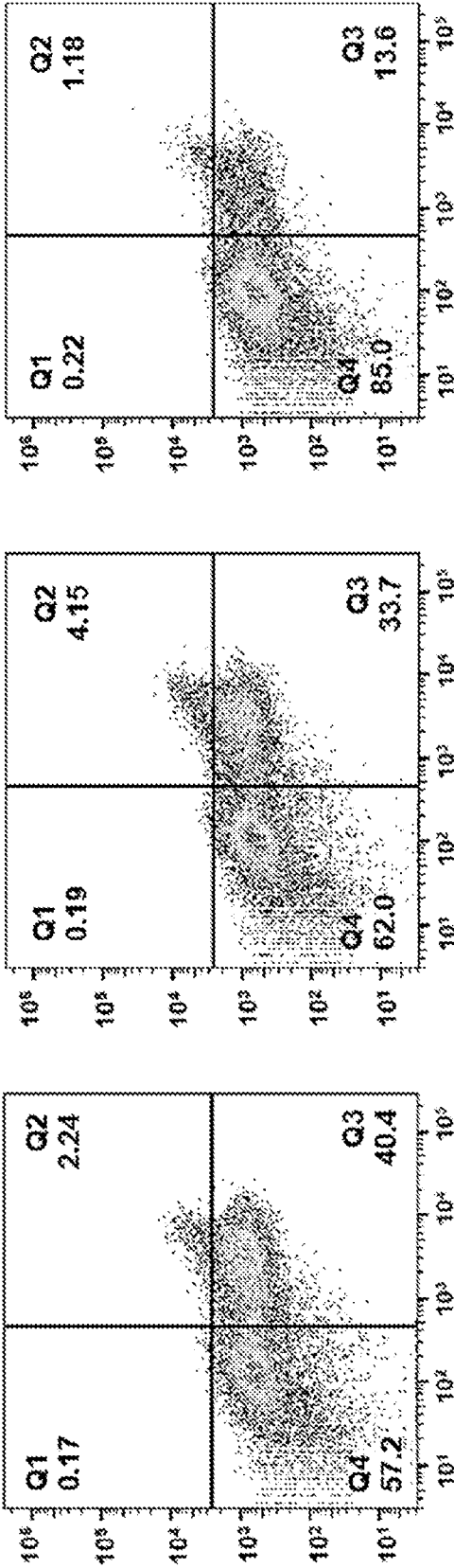


FIG. 23B

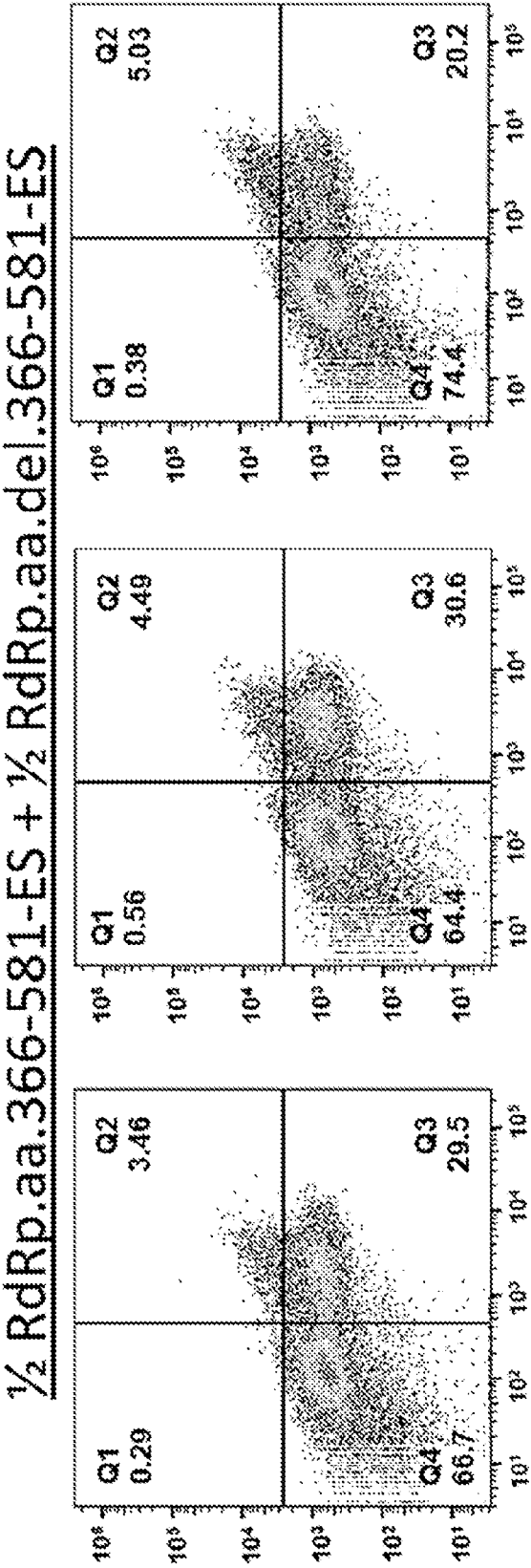


FIG. 23C

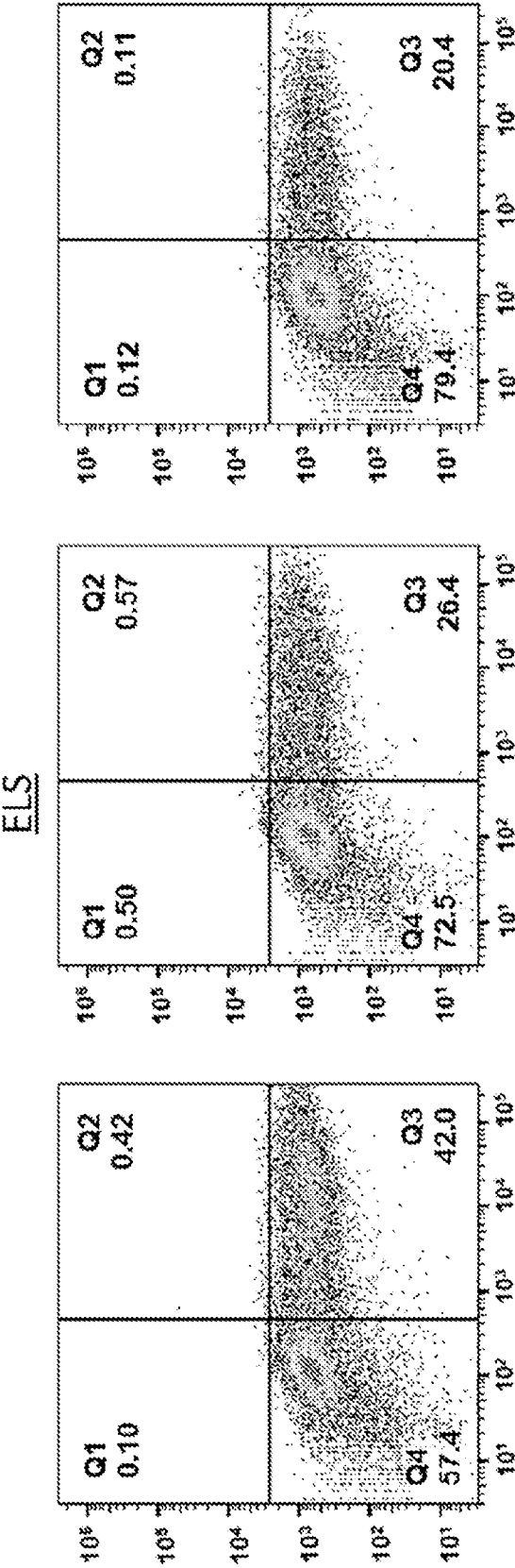


FIG. 24A

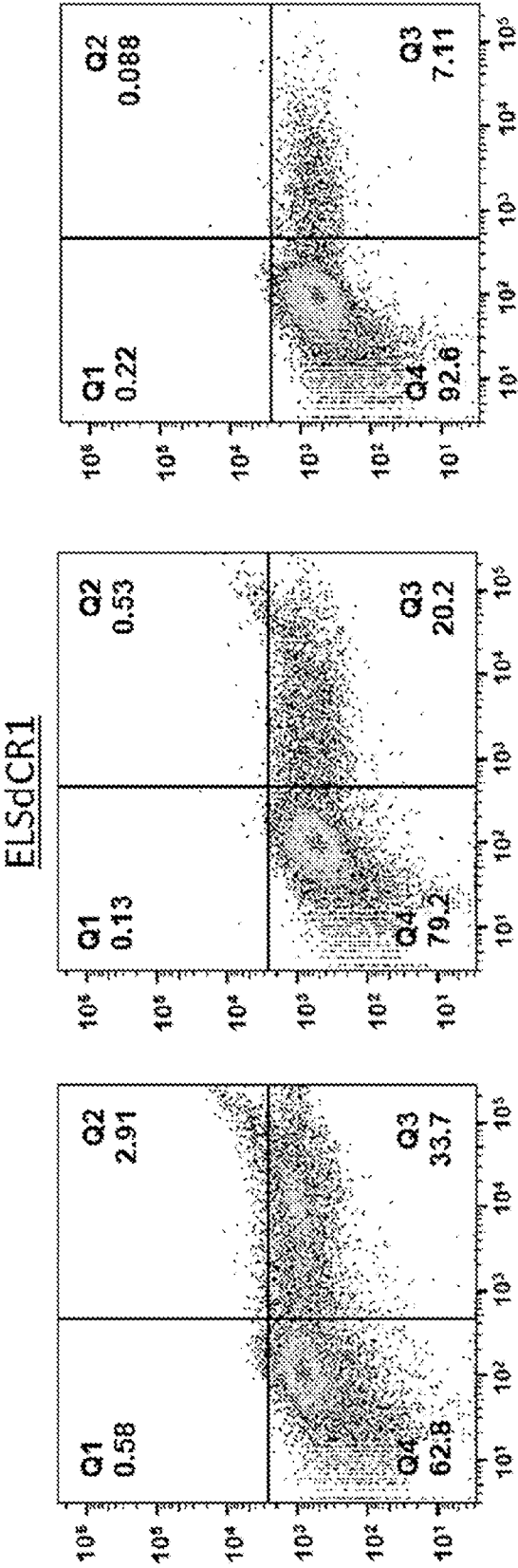


FIG. 24B

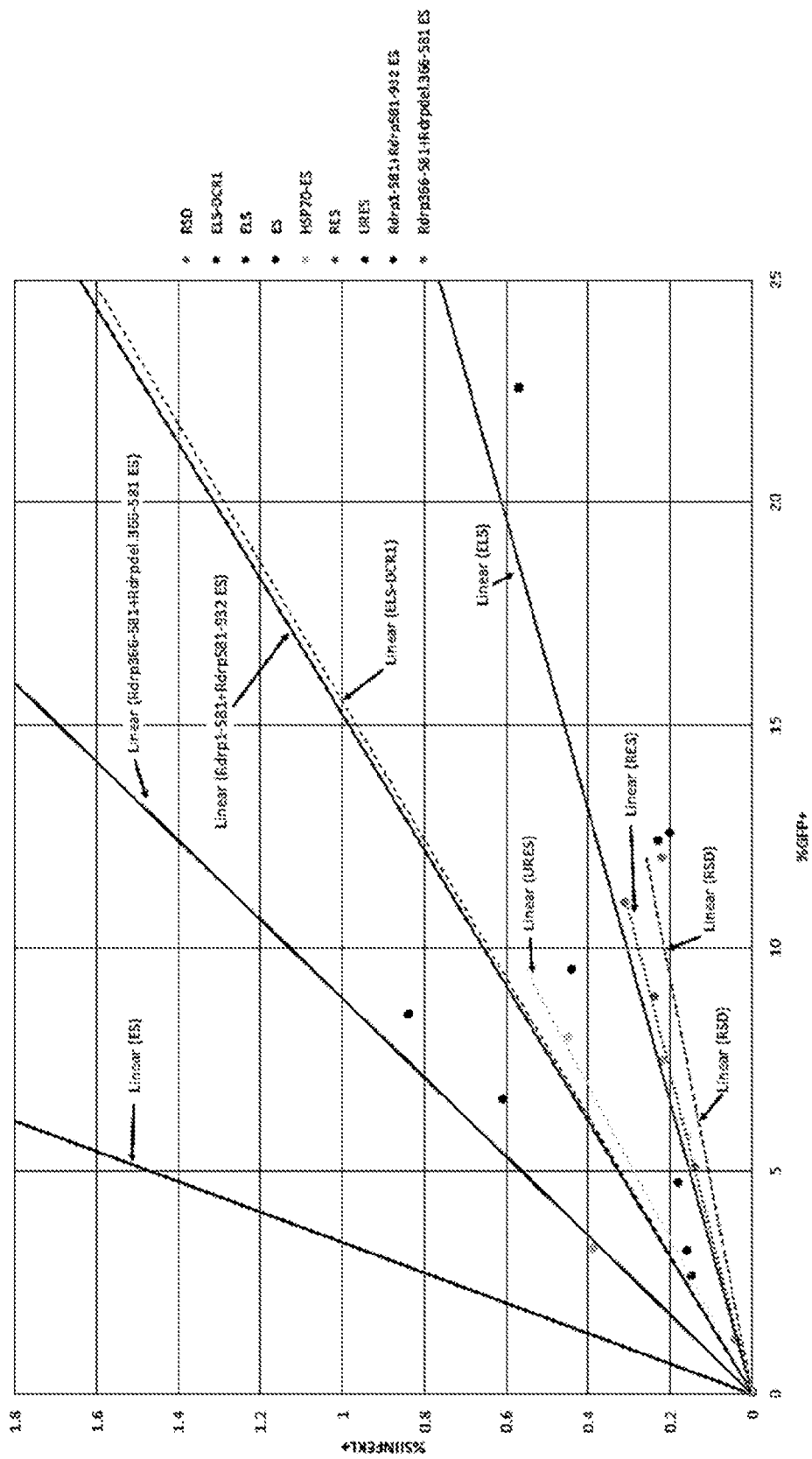


FIG. 25

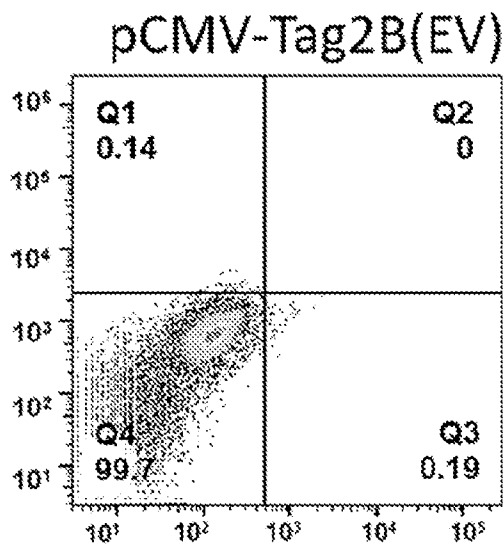


FIG. 26A

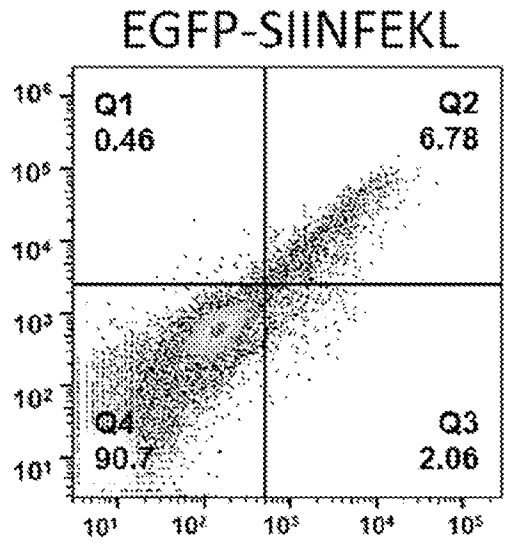


FIG. 26B

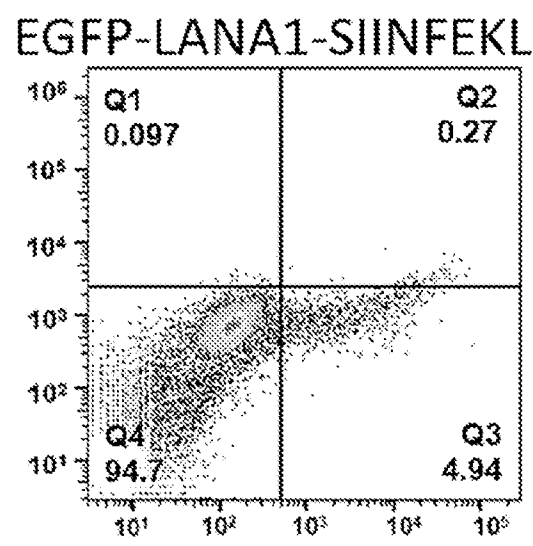


FIG. 26C

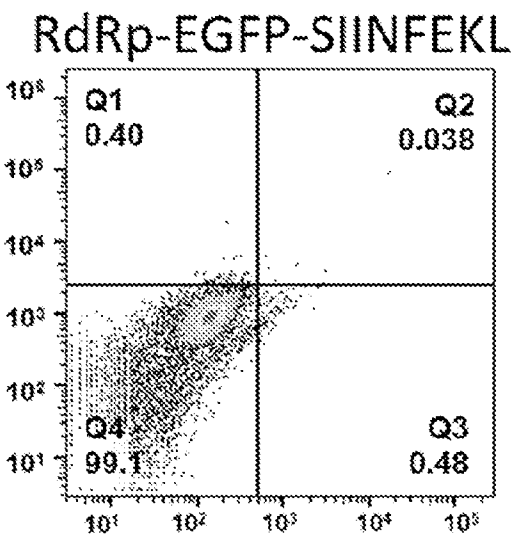


FIG. 26D

½ Rdrp.aa.366-581-EGFP-SIINFELK+
½ RdRp.aa.del.366-581-EGFP-SIINFELK

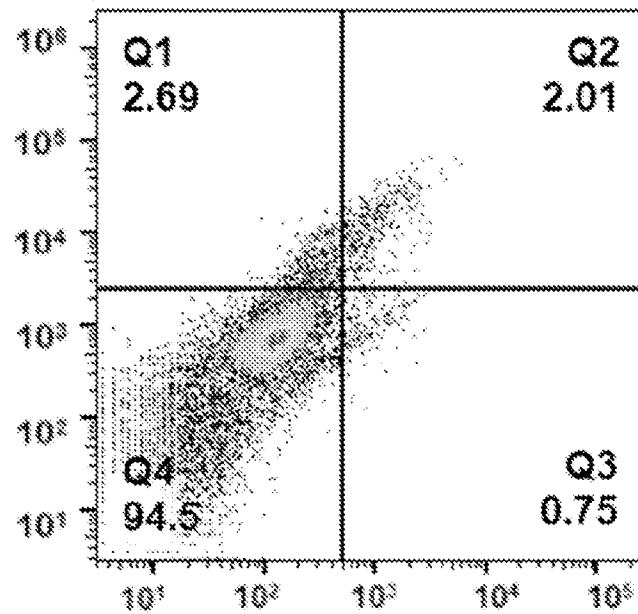


FIG. 26E

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2023/066604

A. CLASSIFICATION OF SUBJECT MATTER IPC(8) - INV. - C12N 15/85; A61K 38/16; A61P 31/14; C07K 14/165; C12N 9/12 (2023.01) ADD. CPC - INV. - C12N 15/85; A61K 38/162; A61P 31/14; C07K 14/165; C12N 9/127; C12Y 207/07048 (2023.08) ADD. - C12N 2770/20022; C12N 2770/20034 (2023.08) According to International Patent Classification (IPC) or to both national classification and IPC																	
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) See Search History document Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched See Search History document Electronic database consulted during the international search (name of database and, where practicable, search terms used) See Search History document																	
C. DOCUMENTS CONSIDERED TO BE RELEVANT <table border="1"> <thead> <tr> <th>Category*</th> <th>Citation of document, with indication, where appropriate, of the relevant passages</th> <th>Relevant to claim No.</th> </tr> </thead> <tbody> <tr> <td>A</td> <td>WO 2022/076903 A1 (ATEA PHARMACEUTICALS INC.) 14 April 2022 (14.04.2022) entire document</td> <td>1-6, 16-24</td> </tr> <tr> <td>A</td> <td>US 2021/0315988 A1 (E-MO BIOLOGY INC.) 14 October 2021 (14.10.2021) entire document</td> <td>1-6, 16-24</td> </tr> <tr> <td>A</td> <td>WO 2021/163398 A1 (EPIVAX INC.) 19 August 2021 (19.08.2021) entire document</td> <td>1-6, 16-24</td> </tr> <tr> <td>A</td> <td>WO 2022/066823 A1 (MERCK SHARP & DOHME CORP.) 31 March 2022 (31.03.2022) entire document</td> <td>1-6, 16-24</td> </tr> </tbody> </table>			Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.	A	WO 2022/076903 A1 (ATEA PHARMACEUTICALS INC.) 14 April 2022 (14.04.2022) entire document	1-6, 16-24	A	US 2021/0315988 A1 (E-MO BIOLOGY INC.) 14 October 2021 (14.10.2021) entire document	1-6, 16-24	A	WO 2021/163398 A1 (EPIVAX INC.) 19 August 2021 (19.08.2021) entire document	1-6, 16-24	A	WO 2022/066823 A1 (MERCK SHARP & DOHME CORP.) 31 March 2022 (31.03.2022) entire document	1-6, 16-24
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.															
A	WO 2022/076903 A1 (ATEA PHARMACEUTICALS INC.) 14 April 2022 (14.04.2022) entire document	1-6, 16-24															
A	US 2021/0315988 A1 (E-MO BIOLOGY INC.) 14 October 2021 (14.10.2021) entire document	1-6, 16-24															
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A	WO 2022/066823 A1 (MERCK SHARP & DOHME CORP.) 31 March 2022 (31.03.2022) entire document	1-6, 16-24															
☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.																	
<table border="0"> <tr> <td> * Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "D" document cited by the applicant in the international application "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed </td> <td> "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family </td> </tr> </table>			* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "D" document cited by the applicant in the international application "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family													
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "D" document cited by the applicant in the international application "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family																
Date of the actual completion of the international search 05 September 2023		Date of mailing of the international search report OCT 04 2023															
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300		Authorized officer Taina Matos Telephone No. PCT Helpdesk: 571-272-4300															

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2023/066604

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed.
 - b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13ter.1(a)),
☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2023/066604

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
See extra sheet(s).

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-6, 16-24

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2023/066604

Continued from Box No. III Observations where unity of invention is lacking

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees need to be paid.

Group I+: claims 1-24 are drawn to a recombinant vectors, pharmaceutical compositions comprising the same, methods of destabilizing SARS-CoV-2 RdRp in a cell comprising the same, methods of increasing immune response to SARS-CoV-2 virus in a subject comprising the same, methods of increasing cytotoxic T lymphocyte (CTL) response to SARSCoV-2 virus in a subject, and methods of increasing Major Histocompatibility Complex (MHC) class I immune response to SARS-CoV-2 virus in a subject.

The first invention of Group I+ is restricted to a recombinant vector comprising one or more polynucleotides encoding at least one modified ribonucleic acid (RNA) dependent RNA polymerase (RdRp), wherein the modified RdRp comprises: (a) at least one fragment of RdRp selected to consist of SEQ ID NO:1, pharmaceutical compositions comprising the same, methods of destabilizing SARS-CoV-2 RdRp in a cell comprising the same, methods of increasing immune response to SARS-CoV-2 virus in a subject comprising the same, methods of increasing cytotoxic T lymphocyte (CTL) response to SARSCoV-2 virus in a subject, and methods of increasing Major Histocompatibility Complex (MHC) class I immune response to SARS-CoV-2 virus in a subject. It is believed that claims 1-6 and 16-24 read on this first named invention and thus these claims will be searched without fee to the extent that they read on SEQ ID NO:1.

Applicant is invited to elect additional recombinant vectors comprising RdRp fragments and their respective, corresponding SEQ ID NOs to be searched by paying an additional fee for each election. An exemplary election would be a recombinant vector comprising one or more polynucleotides encoding at least one modified ribonucleic acid (RNA) dependent RNA polymerase (RdRp), wherein the modified RdRp comprises: (a) at least one fragment of RdRp selected to consist of SEQ ID NO:2, pharmaceutical compositions comprising the same, methods of destabilizing SARS-CoV-2 RdRp in a cell comprising the same, methods of increasing immune response to SARS-CoV-2 virus in a subject comprising the same, methods of increasing cytotoxic T lymphocyte (CTL) response to SARSCoV-2 virus in a subject, and methods of increasing Major Histocompatibility Complex (MHC) class I immune response to SARS-CoV-2 virus in a subject. Additional recombinant vectors and their respective, corresponding SEQ ID NOs will be searched upon the payment of additional fees. Applicants must specify the claims that read on any additional elected inventions. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined.

The inventions listed in Groups I+ do not relate to a single general inventive concept under PCT Rule 13.1, because under PCT Rule 13.2 they lack the same or corresponding special technical features for the following reasons:

The Groups I+ formulas do not share a significant structural element responsible for " encoding at least one modified ribonucleic acid (RNA) dependent RNA polymerase (RdRp), wherein the modified RdRp comprises: (a) at least one fragment of RdRp" " wherein the at least one fragment of RdRp comprises at least one of SEQ ID NOs: 1-12."

Additionally, even if Groups I+ were considered to share the technical features of A recombinant vector comprising one or more polynucleotides encoding at least one modified ribonucleic acid (RNA) dependent RNA polymerase (RdRp), wherein the modified RdRp comprises: (a) at least one fragment of RdRp; (b) RdRp and a regulatory protein that enhances proteasomal degradation, wherein the RdRp is conjugated or fused to the regulatory protein directly or indirectly via a linker; (c) RdRp and a peptide sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T) (PEST sequence) that enhances proteasomal degradation; or (d) a combination of two or more of (a)-(c); A pharmaceutical composition comprising the vector and a pharmaceutically acceptable carrier; A method of destabilizing SARS-CoV-2 RdRp in a cell, the method comprising contacting a cell with the vector; A method of increasing immune response to SARS-CoV-2 virus in a subject, the method comprising administering the vector; A method of increasing cytotoxic T lymphocyte (CTL) response to SARSCoV-2 virus in a subject, the method comprising administering the vector; and A method of increasing Major Histocompatibility Complex (MHC) class I immune response to SARS-CoV-2 virus in a subject, the method comprising administering the vector, these shared technical features do not represent a contribution over the prior art.

Specifically, WO 2021/163398 A1 to Epivax Inc. teaches a recombinant vector comprising one or more polynucleotides encoding at least one modified ribonucleic acid (RNA) dependent RNA polymerase (RdRp) (the present disclosure provides a novel class of T-cell epitopes (which may be isolated, synthetic, or recombinant) and II-key constructs, which comprise a peptide or polypeptide chain derived from SARS-CoV-2 proteins (e.g., encoded proteins from a SARS-CoV-2 genome), including the... ORF1ab RNA-dependent RNA polymerase, Pg. 30, Lns. 21-29; a nucleic acid molecule encoding the polypeptide is cloned into an expression cassette or expression vector, Pg. 89, Lns. 25-27); A pharmaceutical composition comprising the vector and a pharmaceutically acceptable carrier (a pharmaceutical composition, the pharmaceutical composition comprising a T-cell epitope compound or composition of the instant disclosure (e.g.,... expression vectors,... as disclosed herein) and a pharmaceutically acceptable carrier, Pg. 12, Lns. 20-28); A method of destabilizing SARS-CoV-2 RdRp in a cell, the method comprising contacting a cell with the vector (methods of immunizing or inducing an immune response in a subject, said method comprising administering to said subject... expression vectors,... as described herein, Pg. 13, Lns. 19-27; the term " immune response" refers to the concerted action of lymphocytes, antigen presenting cells, phagocytic cells, granulocytes, and soluble macromolecules produced by the above cells or the liver (including antibodies, cytokines, and complement) that results in selective damage to, destruction of, or elimination from the human body of... invading pathogens (including a virus), Pg. 21, Lns. 12-17); A method of increasing immune response to SARS-CoV-2 virus in a subject, the method comprising administering the vector (methods of immunizing or inducing an immune response in a subject, said method comprising administering to said subject... expression vectors,... as described herein, Pg. 13, Lns. 19-27; It is demonstrated herein that certain naturally occurring T-cells (in aspects, including CD4+ and CD8+ T-cells), can be engaged, activated, and/or applied to induce immunity or induce an immune response against pathogens such as SARS-CoV-2, Pg. 20, Lns. 10-12); and A method of increasing Major Histocompatibility Complex (MHC) class I immune response to SARS-CoV-2 virus in a subject, the method comprising administering the vector (methods of immunizing or inducing an immune response in a subject, said method comprising administering to said subject... expression vectors,... as described herein, Pg. 13, Lns. 19-27; The molecules of MHC class I present endogenously synthesized antigens, for example viral proteins, Pg. 25, Lns. 8-10).

The inventions listed in Groups I+ therefore lack unity under Rule 13 because they do not share a same or corresponding special technical features.