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(54) Titre : MEGANUCLEASES MODIFIEES AVEC DES SEQUENCES DE RECONNAISSANCES RENCONTREES
DANS LE GENE DE LA REGION CONSTANTE ALPHA DU RECEPTOR DES LYMPHOCYTES T HUMAINS

(54) Title: ENGINEERED MEGANUCLEASES WITH RECOGNITION SEQUENCES FOUND IN THE HUMAN T CELL
RECEPTOR ALPHA CONSTANT REGION GENE

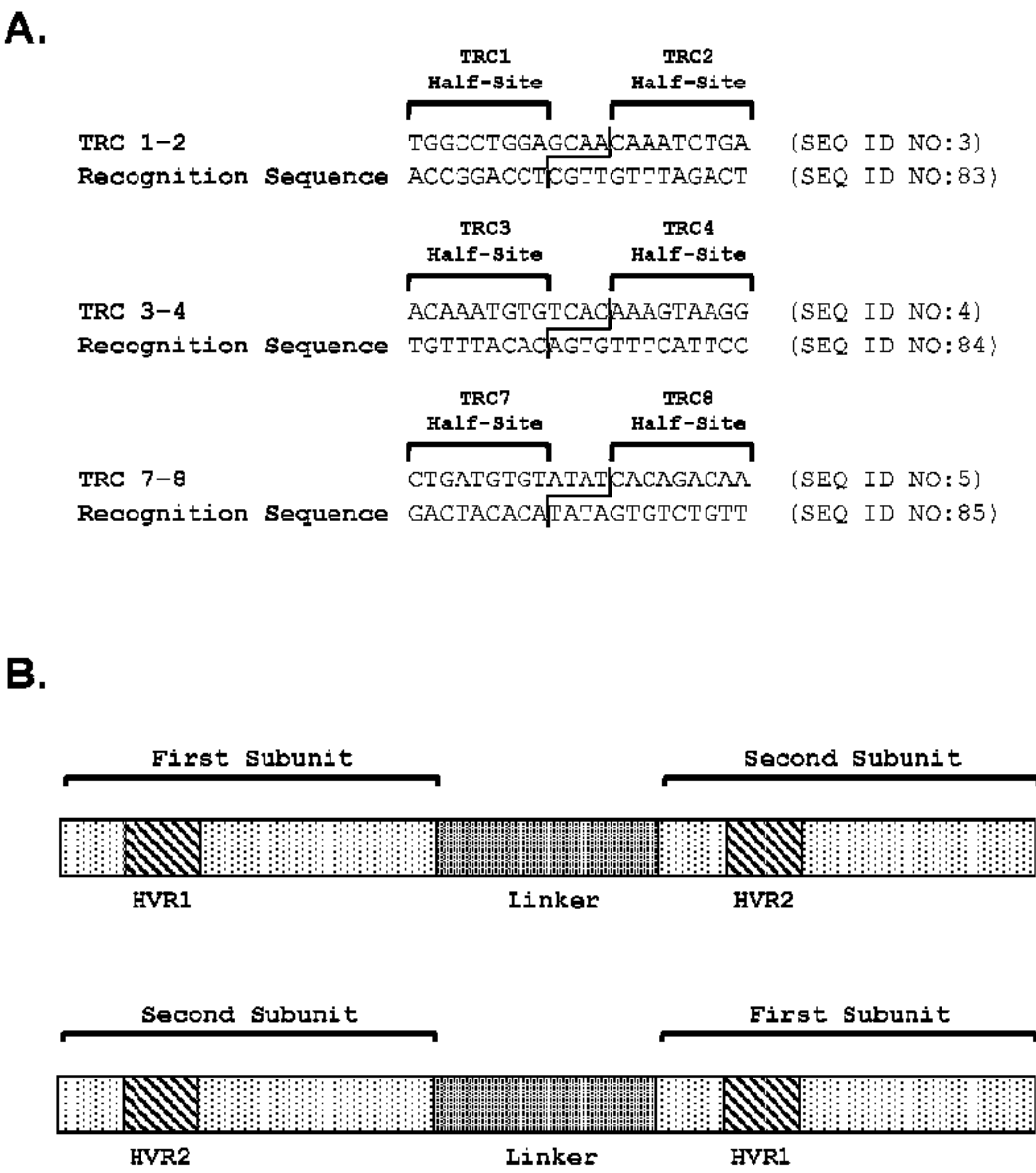


FIGURE 1

(57) **Abrégé/Abstract:**
Disclosed herein are recombinant meganucleases engineered to recognize and cleave a recognition sequence present in the human T cell receptor alpha constant region gene. The present disclosure further relates to the use of such recombinant meganucleases in methods for producing genetically-modified eukaryotic cells.

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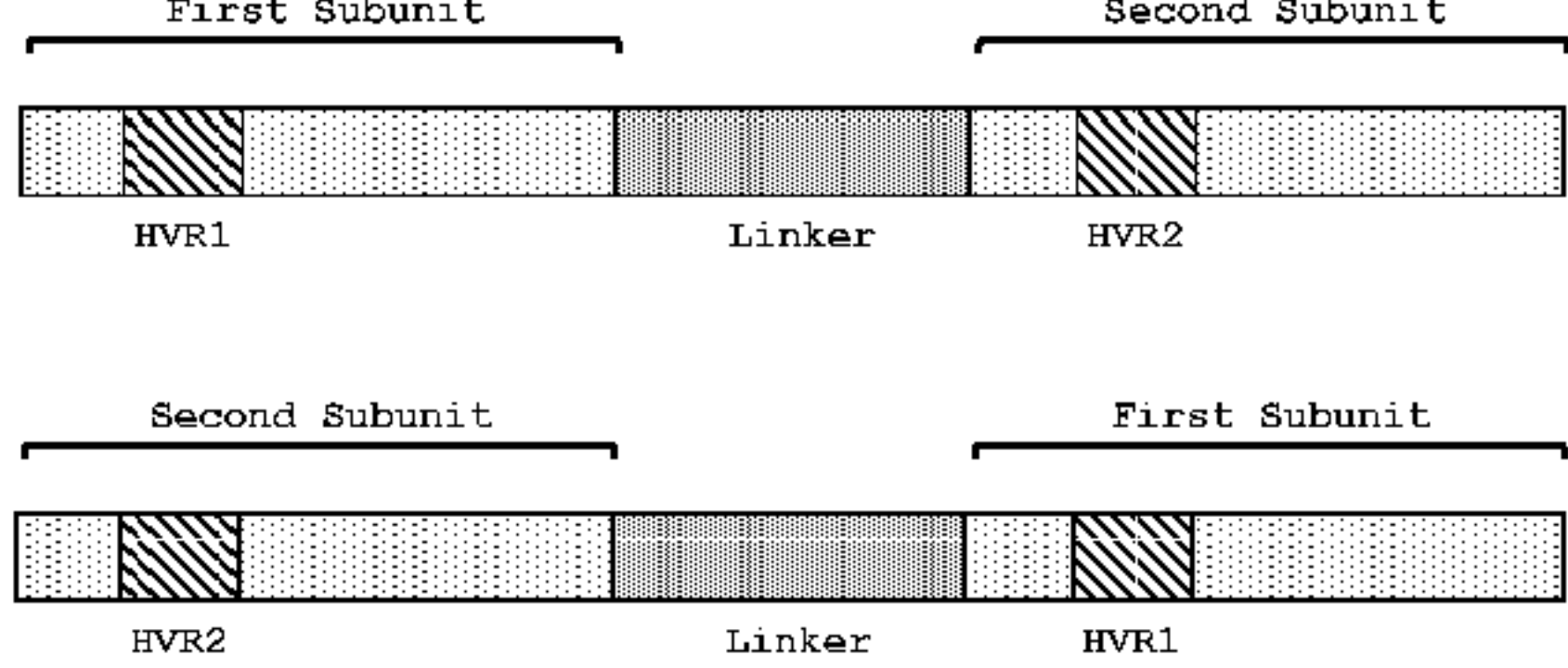
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- (54) Title: ENGINEERED MEGANUCLEASES WITH RECOGNITION SEQUENCES FOUND IN THE HUMAN T CELL RECEPTOR ALPHA CONSTANT REGION GENE
- (57) Abstract: Disclosed herein are recombinant meganucleases engineered to recognize and cleave a recognition sequence present in the human T cell receptor alpha constant region gene. The present disclosure further relates to the use of such recombinant meganucleases in methods for producing genetically-modified eukaryotic cells.
- A.

	TRC1 Half-Site	TRC2 Half-Site	
TRC 1-2	TGGCTGGAGCA	CAATCTGA	(SEQ ID NO:3)
Recognition Sequence	ACCGCACCTCTCTTACACT		(SEQ ID NO:33)
	TRC3 Half-Site	TRC4 Half-Site	
TRC 3-4	ACAAATGCTCA	CAANTAAGG	(SEQ ID NO:4)
Recognition Sequence	TCTTACACCTCTTCAITCC		(SEQ ID NO:34)
	TRC7 Half-Site	TRC8 Half-Site	
TRC 7-8	CTGATGTGTAAT	CACAGACAA	(SEQ ID NO:5)
Recognition Sequence	GACACACATATAGGCTCTGTT		(SEQ ID NO:35)

B.


- FIGURE 1
- WO 2017/062439 A1

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- LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK,
SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,
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**ENGINEERED MEGANUCLEASES WITH RECOGNITION SEQUENCES FOUND
IN THE HUMAN T CELL RECEPTOR ALPHA CONSTANT REGION GENE**

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No. 62/297,445, entitled "Engineered Meganucleases With Recognition Sequences Found In The Human T Cell Receptor Alpha Constant Region Gene," filed February 19, 2016, and U.S. Provisional Application No. 62/237,382, entitled "Engineered Meganucleases With Recognition Sequences Found In The Human T Cell Receptor Alpha Constant Region Gene," filed October 5, 2015, the disclosures of which are hereby incorporated by reference in their entireties.

FIELD OF THE INVENTION

The invention relates to the fields of oncology, cancer immunotherapy, molecular biology and recombinant nucleic acid technology. In particular, the invention relates to recombinant meganucleases engineered to recognize and cleave a recognition sequence found in the human T cell receptor alpha constant region gene. The invention further relates to the use of such recombinant meganucleases in methods for producing genetically-modified eukaryotic cells.

REFERENCE TO A SEQUENCE LISTING SUBMITTED AS

A TEXT FILE VIA EFS-WEB

The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on October 3, 2016, is named 2000706_00179WO1.txt, and is 264,046 bytes in size.

BACKGROUND OF THE INVENTION

T cell adoptive immunotherapy is a promising approach for cancer treatment. This strategy utilizes isolated human T cells that have been genetically-modified to enhance their specificity for a specific tumor associated antigen. Genetic modification may involve the expression of a chimeric antigen receptor (CAR) or an exogenous T cell receptor to graft antigen specificity onto the T cell. By contrast to exogenous T cell receptors, chimeric

antigen receptors derive their specificity from the variable domains of a monoclonal antibody. Thus, T cells expressing chimeric antigen receptors (CAR T cells) induce tumor immunoreactivity in a major histocompatibility complex non-restricted manner. To date, T cell adoptive immunotherapy has been utilized as a clinical therapy for a number of cancers, including B cell malignancies (*e.g.*, acute lymphoblastic leukemia (ALL), B cell non-Hodgkin lymphoma (NHL), and chronic lymphocytic leukemia), multiple myeloma, neuroblastoma, glioblastoma, advanced gliomas, ovarian cancer, mesothelioma, melanoma, and pancreatic cancer.

Despite its potential usefulness as a cancer treatment, adoptive immunotherapy with CAR T cells has been limited, in part, by expression of the endogenous T cell receptor on the cell surface. CAR T cells expressing an endogenous T cell receptor may recognize major and minor histocompatibility antigens following administration to an allogeneic patient, which can lead to the development of graft-versus-host-disease (GVHD). As a result, clinical trials have largely focused on the use of autologous CAR T cells, wherein a patient's T cells are isolated, genetically-modified to incorporate a chimeric antigen receptor, and then re-infused into the same patient. An autologous approach provides immune tolerance to the administered CAR T cells; however, this approach is constrained by both the time and expense necessary to produce patient-specific CAR T cells after a patient's cancer has been diagnosed.

Thus, it would be advantageous to develop "off the shelf" CAR T cells, prepared using T cells from a third party donor, that have reduced expression of the endogenous T cell receptor and do not initiate GVHD upon administration. Such products could be generated and validated in advance of diagnosis, and could be made available to patients as soon as necessary. Therefore, a need exists for the development of allogeneic CAR T cells that lack an endogenous T cell receptor in order to prevent the occurrence of GVHD.

Genetic modification of genomic DNA can be performed using site-specific, rare-cutting, homing endonucleases (also called "meganucleases") that are engineered to recognize specific DNA sequences in a locus of interest. Homing endonucleases are a group of naturally-occurring nucleases that recognize 15-40 base pair cleavage sites commonly found in the genomes of plants and fungi. They are frequently associated with parasitic DNA elements, such as group 1 self-splicing introns and inteins. They naturally promote homologous recombination or gene insertion at specific locations in the host genome by producing a double-stranded break in the chromosome, which recruits the cellular DNA-

repair machinery (Stoddard (2006), *Q. Rev. Biophys.* 38:49-95). Homing endonucleases are commonly grouped into four families: the LAGLIDADG (SEQ ID NO:7) family, the GIY-YIG family, the His-Cys box family and the HNH family. These families are characterized by structural motifs, which affect catalytic activity and recognition sequence. For instance, members of the LAGLIDADG (SEQ ID NO:7) family are characterized by having either one or two copies of the conserved LAGLIDADG (SEQ ID NO:7) motif (Chevalier *et al.* (2001), *Nucleic Acids Res.* 29(18): 3757-3774). The LAGLIDADG (SEQ ID NO:7) homing endonucleases with a single copy of the LAGLIDADG (SEQ ID NO:7) motif form homodimers, whereas members with two copies of the LAGLIDADG (SEQ ID NO:7) motif are found as monomers.

Methods for producing engineered, site-specific recombinant meganucleases are known in the art. I-CreI (SEQ ID NO:6) is a member of the LAGLIDADG (SEQ ID NO:7) family of homing endonucleases that recognizes and cuts a 22 base pair recognition sequence in the chloroplast chromosome of the algae *Chlamydomonas reinhardtii*. Genetic selection techniques have been used to modify the wild-type I-CreI cleavage site preference (Sussman *et al.* (2004), *J. Mol. Biol.* 342: 31-41; Chames *et al.* (2005), *Nucleic Acids Res.* 33: e178; Seligman *et al.* (2002), *Nucleic Acids Res.* 30: 3870-9, Arnould *et al.* (2006), *J. Mol. Biol.* 355: 443-58). More recently, a method of rationally-designing mono-LAGLIDADG (SEQ ID NO:7) homing endonucleases was described that is capable of comprehensively redesigning I-CreI and other homing endonucleases to target widely-divergent DNA sites, including sites in mammalian, yeast, plant, bacterial, and viral genomes (WO 2007/047859).

As first described in WO 2009/059195, I-CreI and its engineered derivatives are normally dimeric but can be fused into a single polypeptide using a short peptide linker that joins the C-terminus of a first subunit to the N-terminus of a second subunit (Li *et al.* (2009), *Nucleic Acids Res.* 37:1650-62; Grizot *et al.* (2009), *Nucleic Acids Res.* 37:5405-19.) Thus, a functional “single-chain” meganuclease can be expressed from a single transcript. Such engineered meganucleases exhibit an extremely low frequency of off-target cutting. By delivering a gene encoding a single-chain meganuclease to a cell, it is possible to specifically and preferentially target, cleave, and disrupt the TCR alpha constant region gene.

The use of engineered meganucleases for cleaving DNA targets in the human T cell receptor alpha constant region was previously disclosed in International Publication No. WO 2014/191527. The '527 publication discloses variants of the I-OnuI meganuclease that are engineered to target a recognition sequence (SEQ ID NO:3 of the '527 publication) within

exon 1 of the TCR alpha constant region gene. Although the '527 publication discusses that a chimeric antigen receptor can be expressed in TCR knockout cells, the authors do not disclose the insertion of the chimeric antigen receptor coding sequence into the meganuclease cleavage site in the TCR alpha constant region gene.

5 The use of other nucleases and mechanisms for disrupting expression of the endogenous TCR have also been disclosed. For example, the use of zinc finger nucleases for disrupting TCR genes in human T cells was described by U.S. Patent No. 8,95,828 and by U.S. Patent Application Publication No. US2014/034902. U.S. Patent Application Publication No. US2014/0301990 describes the use of zinc finger nucleases and
10 transcription-activator like effector nucleases (TALENs), and a CRISPR/Cas system with an engineered single guide RNA for targeting TCR genes in an isolated T cell. U.S. Patent Application Publication No. US2012/0321667 discloses the use of small-hairpin RNAs that target nucleic acids encoding specific TCRs and/or CD3 chains in T cells.

 However, the present invention improves upon the teachings of the prior art. The
15 present inventors are the first to teach genetically-modified cells that comprise an exogenous polynucleotide sequence (*e.g.*, a chimeric antigen receptor or exogenous TCR coding sequence) inserted into the human TCR alpha constant region gene, which simultaneously disrupts expression of the endogenous T cell receptor at the cell surface. Further, the prior art does not teach the meganucleases or the recognition sequences described herein, or their use
20 for producing such genetically-modified cells.

SUMMARY OF THE INVENTION

 The present invention provides recombinant meganucleases that are engineered to recognize and cleave recognition sequences found within residues 93-208 of the human T cell
25 receptor (TCR) alpha constant region gene (SEQ ID NO:1). Such meganuclease are useful for disrupting the TCR alpha constant region gene and, consequently, disrupting the expression and/or function of the cell surface TCR. Therefore, the meganucleases of the invention have utility in immunotherapy, including cancer immunotherapy. Meganuclease cleavage can disrupt gene function either by the mutagenic action of non-homologous end
30 joining or by promoting the introduction of an exogenous polynucleotide into the gene via homologous recombination. In some embodiments, the introduced exogenous polynucleotide comprises a nucleic acid sequence encoding a chimeric antigen receptor, such that the

meganuclease is useful for generating an allogeneic CAR T cell that lacks an endogenous TCR.

The present invention further provides methods comprising the delivery of a recombinant meganuclease protein, or genes encoding a recombinant meganuclease, to a eukaryotic cell in order to produce a genetically-modified eukaryotic cell.

Thus, in one aspect, the invention provides a recombinant meganuclease that recognizes and cleaves a recognition sequence within residues 93-208 of the human T cell receptor alpha constant region gene (SEQ ID NO:1). Such a recombinant meganuclease comprises a first subunit and a second subunit, wherein the first subunit binds to a first recognition half-site of the recognition sequence and comprises a first hypervariable (HVR1) region, and wherein the second subunit binds to a second recognition half-site of the recognition sequence and comprises a second hypervariable (HVR2) region.

In one embodiment, the recognition sequence comprises SEQ ID NO:3 (*i.e.*, the TRC 1-2 recognition sequence).

In one such embodiment, the first meganuclease subunit comprises an amino acid sequence having at least 80%, at least 85%, at least 90%, or at least 95% sequence identity to residues 198-344 of any one of SEQ ID NOs:8-18 or residues 7-153 of any one of SEQ ID NOs:19-27, and the second meganuclease subunit comprises an amino acid sequence having at least 80%, at least 85%, at least 90%, or at least 95% sequence identity to residues 7-153 of any one of SEQ ID NOs:8-18 or residues 198-344 of any one of SEQ ID NOs:19-27.

In another such embodiment, the HVR1 region comprises Y at a position corresponding to: (a) position 215 of any one of SEQ ID NOs:8-18; or (b) position 24 of any one of SEQ ID NOs:19-27. In another such embodiment, the HVR1 region comprises G at a position corresponding to: (a) position 233 of any one of SEQ ID NOs:8-18; or (b) position 42 of any one of SEQ ID NOs:19-27. In another such embodiment, the HVR1 region comprises one or more of Y and G at positions corresponding to (a) positions 215 and 233, respectively, of any one of SEQ ID NOs:8-18; or (b) positions 24 and 42, respectively, of any one of SEQ ID NOs:19-27.

In another such embodiment, the HVR2 region comprises T at a position corresponding to: (a) position 26 of any one of SEQ ID NOs:8-18; or (b) position 217 of any one of SEQ ID NOs:19-27. In another such embodiment, the HVR2 region comprises F or Y at a position corresponding to: (a) position 28 of any one of SEQ ID NOs:8-18; or (b) position 219 of any one of SEQ ID NOs:19-27. In another such embodiment, the HVR2

region comprises F at a position corresponding to: (a) position 38 of any one of SEQ ID NOs:8-18; or (b) position 229 of any one of SEQ ID NOs:19-27. In another such embodiment, the HVR2 region comprises S at a position corresponding to: (a) position 44 of any one of SEQ ID NOs:8-18; or (b) position 235 of any one of SEQ ID NOs:19-27. In
5 another such embodiment, the HVR2 region comprises F or Y at a position corresponding to: (a) position 46 of any one of SEQ ID NOs:8-18; or (b) position 237 of any one of SEQ ID NOs:19-27. In another such embodiment, the HVR2 region comprises one or more of T, F or Y, F, S, and F or Y, and R at positions corresponding to: (a) positions 26, 28, 38, 44, and 46, respectively, of any one of SEQ ID NOs:8-18; or (b) positions 217, 219, 229, 235, and 237,
10 respectively, of any one of SEQ ID NOs:19-27.

In another such embodiment, the HVR1 region comprises residues 215-270 of any one of SEQ ID NOs:8-18 or residues 24-79 of any one of SEQ ID NOs:19-27. In another such embodiment, the HVR2 region comprises residues 24-79 of any one of SEQ ID NOs:8-18 or residues 215-270 of any one of SEQ ID NOs:19-27.

15 In another such embodiment, the first meganuclease subunit comprises residues 198-344 of any one of SEQ ID NOs:8-18 or residues 7-153 of any one of SEQ ID NOs:19-27. In another such embodiment, the second meganuclease subunit comprises residues 7-153 of any one of SEQ ID NOs:8-18 or residues 198-344 of any one of SEQ ID NOs:19-27.

In another such embodiment, the recombinant meganuclease is a single-chain
20 meganuclease comprising a linker, wherein the linker covalently joins the first subunit and the second subunit.

In another such embodiment, the recombinant meganuclease comprises the amino acid sequence of any one of SEQ ID NOs:8-27.

In a further embodiment, the recognition sequence comprises SEQ ID NO:4 (*i.e.*, the
25 TRC 3-4 recognition sequence).

In one such embodiment, the first meganuclease subunit comprises an amino acid sequence having at least 80%, at least 85%, at least 90%, or at least 95% sequence identity to residues 7-153 of SEQ ID NO:28 or 29, and the second meganuclease subunit comprises an amino acid sequence having at least 80%, at least 85%, at least 90%, or at least 95% sequence
30 identity to residues 198-344 of SEQ ID NO:28 or 29.

In another such embodiment, the HVR1 region comprises Y at a position corresponding to position 24 of SEQ ID NO:28 or 29. In another such embodiment, the HVR1 region comprises T at a position corresponding to position 26 of SEQ ID NO:28 or 29.

In another such embodiment, the HVR1 region comprises Y at a position corresponding to position 46 of SEQ ID NO:28 or 29. In another such embodiment, the HVR1 region comprises one or more of Y, T, and Y at positions corresponding to positions 24, 26, and 46, respectively, of SEQ ID NO:28 or 29.

5 In another such embodiment, the HVR2 region comprises H at a position corresponding to position 215 of SEQ ID NO:28 or 29. In another such embodiment, the HVR2 region comprises T at a position corresponding to position 266 of SEQ ID NO:28 or 29. In another such embodiment, the HVR2 region comprises C at a position corresponding to position 268 of SEQ ID NO:28 or 29. In another such embodiment, the HVR2 region
10 comprises one or more of H, T, and C at positions corresponding to positions 215, 266, and 268 of SEQ ID NOs:28 or 29.

In another such embodiment, the HVR1 region comprises residues 24-79 of SEQ ID NO:28 or 29. In another such embodiment, the HVR2 region comprises residues 215-270 of SEQ ID NO:28 or 29.

15 In another such embodiment, the first meganuclease subunit comprises residues 7-153 of SEQ ID NO:28 or 29. In another such embodiment, the second meganuclease subunit comprises residues 198-344 of SEQ ID NO:28 or 29.

In another such embodiment, the recombinant meganuclease is a single-chain meganuclease comprising a linker, wherein the linker covalently joins the first subunit and
20 the second subunit.

In another such embodiment, the recombinant meganuclease comprises the amino acid sequence of SEQ ID NO:28 or 29.

In a further embodiment, the recognition sequence comprises SEQ ID NO:5 (*i.e.*, the TRC 7-8 recognition sequence).

25 In one such embodiment, the first meganuclease subunit comprises an amino acid sequence having at least 80%, at least 85%, at least 90%, or at least 95% sequence identity to residues 7-153 of SEQ ID NO:30 or residues 198-344 of SEQ ID NO:31 or 32, and the second meganuclease subunit comprises an amino acid sequence having at least 80%, at least 85%, at least 90%, or at least 95% sequence identity to residues 198-344 of SEQ ID NO:30
30 or residues 7-153 of SEQ ID NO:31 or 32.

In another such embodiment, the HVR1 region comprises Y at a position corresponding to: (a) position 24 of SEQ ID NO:30; or (b) position 215 of SEQ ID NO:31 or 32.

In another such embodiment, the HVR2 region comprises Y or W at a position corresponding to: (a) position 215 of SEQ ID NO:30; or (b) position 24 of SEQ ID NO:31 or 32. In another such embodiment, the HVR2 region comprises M, L, or W at a position corresponding to: (a) position 231 of SEQ ID NO:30; or (b) position 40 of SEQ ID NO:31 or 32. In another such embodiment, the HVR2 region comprises Y at a position corresponding to: (a) position 237 of SEQ ID NO:30; or (b) position 46 of SEQ ID NO:31 or 32. In another such embodiment, the HVR2 region comprises one or more of Y or W, M, L, or W, and Y at positions corresponding to: (a) positions 215, 231, and 237, respectively, of SEQ ID NO:30; or (b) positions 24, 40, and 46, respectively, of SEQ ID NO:31 or 32.

In another such embodiment, the HVR1 region comprises residues 24-79 of SEQ ID NO:30 or residues 215-270 of SEQ ID NO:31 or 32. In another such embodiment, the HVR2 region comprises residues 215-270 of SEQ ID NO:30 or residues 24-79 of SEQ ID NO:31 or 32.

In another such embodiment, the first meganuclease subunit comprises residues 7-153 of SEQ ID NO:30 or residues 198-344 of SEQ ID NO:31 or 32. In another such embodiment, the second meganuclease subunit comprises residues 198-344 of SEQ ID NO:30 or residues 7-153 of SEQ ID NO:31 or 32.

In another such embodiment, the recombinant meganuclease is a single-chain meganuclease comprising a linker, wherein the linker covalently joins the first subunit and the second subunit.

In another such embodiment, the recombinant meganuclease comprises the amino acid sequence of any one of SEQ ID NOs:30-32.

In another aspect, the invention provides an isolated polynucleotide comprising a nucleic acid sequence encoding a recombinant meganuclease described herein.

In the various aspects of the invention, the isolated polynucleotide can be an mRNA. In one embodiment, the mRNA can encode a single recombinant meganuclease described herein. In other embodiments, the mRNA can be a polycistronic mRNA (*e.g.*, bicistronic, tricistronic, etc.) comprising a coding sequence for at least one meganuclease described herein and a coding sequence for at least one additional protein (*e.g.*, a second nuclease). In particular embodiments, a polycistronic mRNA can encode two or more meganucleases described herein that target different recognition sequences within the same gene (*e.g.*, the T cell receptor alpha constant region gene). In other embodiments, a polycistronic mRNA can encode a meganuclease described herein and a second nuclease that recognizes and cleaves a

different recognition sequence within the same gene (*e.g.*, the T cell receptor alpha constant region gene) or, alternatively, recognizes and cleaves a different recognition sequence within another gene of interest in the genome. In such embodiments, genetically-modified cells produced using such polycistronic mRNA can have multiple genes knocked out
5 simultaneously. Further, genetically-modified cells produced using such polycistronic mRNA can have an exogenous sequence of interest inserted into the genome at one or more of the cleavage sites produced by the encoded nucleases. In additional embodiments, a polycistronic mRNA can encode at least one meganuclease described herein and one additional protein that is beneficial to the cell, improves efficiency of insertion of an
10 exogenous sequence of interest into a cleavage site, and/or is beneficial in the treatment of a disease.

In another aspect, the invention provides a recombinant DNA construct comprising an isolated polynucleotide, wherein the isolated polynucleotide comprises a nucleic acid sequence encoding a recombinant meganuclease described herein. In one embodiment, the
15 recombinant DNA construct encodes a viral vector. In one embodiment, the recombinant DNA construct encodes a recombinant AAV vector.

In another aspect, the invention provides a viral vector comprising an isolated polynucleotide, wherein the isolated polynucleotide comprises a nucleic acid sequence encoding a recombinant meganuclease described herein. In another aspect, the invention
20 provides a recombinant AAV vector comprising an isolated polynucleotide, wherein the isolated polynucleotide comprises a nucleic acid sequence encoding a recombinant meganuclease described herein.

In another aspect, the invention provides a method for producing a genetically-modified eukaryotic cell comprising an exogenous sequence of interest inserted into a
25 chromosome of the eukaryotic cell, the method comprising transfecting a eukaryotic cell with one or more nucleic acids including: (a) a first nucleic acid sequence encoding a recombinant meganuclease described herein; and (b) a second nucleic acid sequence including the sequence of interest; wherein the recombinant meganuclease produces a cleavage site in the chromosome at a recognition sequence comprising SEQ ID NO:3, SEQ ID NO:4, or SEQ ID
30 NO:5, and wherein the sequence of interest is inserted into the chromosome at the cleavage site.

In one embodiment of the method, the second nucleic acid further comprises sequences homologous to sequences flanking the cleavage site, and the sequence of interest is inserted at the cleavage site by homologous recombination.

5 In another embodiment of the method, the second nucleic acid lacks substantial homology to the cleavage site, and the sequence of interest is inserted into the chromosome by non-homologous end-joining.

In another embodiment of the method, the eukaryotic cell is a human T cell or a cell derived therefrom.

10 In another embodiment of the method, the sequence of interest encodes a chimeric antigen receptor. In another embodiment of the method, the sequence of interest encodes an exogenous T cell receptor.

In another embodiment of the method, at least the first nucleic acid sequence is introduced into the cell by an mRNA. In some such embodiments, the mRNA can be a polycistronic mRNA encoding at least one recombinant meganuclease described herein and
15 at least one additional protein (*e.g.*, a second nuclease).

In another embodiment of the method, at least the second nucleic acid sequence is introduced into the eukaryotic cell by a viral vector. In another embodiment of the method, the first nucleic acid sequence and the second nucleic acid sequence are introduced into the eukaryotic cell by the same viral vector or, alternatively, by separate viral vectors.

20 In another embodiment of the method, at least the second nucleic acid sequence is introduced into the eukaryotic cell by a recombinant AAV vector. In another embodiment of the method, the first nucleic acid sequence and the second nucleic acid sequence are introduced into the eukaryotic cell by the same recombinant AAV vector or, alternatively, by separate recombinant AAV vectors.

25 In another embodiment of the method, at least the second nucleic acid sequence is introduced into the eukaryotic cell using a single-stranded DNA template.

In a particular embodiment of the method, the first nucleic acid sequence encoding a recombinant meganuclease described herein is introduced into the cell by an mRNA, and the second nucleic acid sequence including the exogenous sequence of interest is introduced into
30 the cell using a viral vector, preferably a recombinant AAV vector, wherein the cell is a human T cell, and wherein the sequence of interest encodes a chimeric antigen receptor. In such an embodiment, the method produces a genetically-modified T cell that comprises a

chimeric antigen receptor and that has reduced cell-surface expression of the endogenous T cell receptor when compared to a control cell.

In another aspect, the invention provides a method for producing a genetically-modified eukaryotic cell comprising an exogenous sequence of interest inserted into a chromosome of the eukaryotic cell, the method comprising: (a) introducing a recombinant meganuclease described herein into a eukaryotic cell; and (b) transfecting the eukaryotic cell with a nucleic acid including the sequence of interest; wherein the recombinant meganuclease produces a cleavage site in the chromosome at a recognition sequence comprising SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5, and wherein the sequence of interest is inserted into the chromosome at the cleavage site.

In one embodiment of the method, the nucleic acid further comprises sequences homologous to sequences flanking the cleavage site, and the sequence of interest is inserted at the cleavage site by homologous recombination.

In another embodiment of the method, the nucleic acid lacks substantial homology to the cleavage site, and the sequence of interest is inserted into the chromosome by non-homologous end-joining.

In another embodiment of the method, the eukaryotic cell is a human T cell or a cell derived therefrom.

In another embodiment of the method, the sequence of interest encodes a chimeric antigen receptor. In another embodiment of the method, the sequence of interest encodes an exogenous T cell receptor.

In another embodiment of the method, the nucleic acid sequence is introduced into the eukaryotic cell by a viral vector.

In another embodiment of the method, the nucleic acid sequence is introduced into the eukaryotic cell by a recombinant AAV vector.

In another embodiment of the method, the nucleic acid sequence is introduced into the eukaryotic cell using a single-stranded DNA template.

In another aspect, the invention provides a method for producing a genetically-modified eukaryotic cell by disrupting a target sequence in a chromosome of the eukaryotic cell, the method comprising: transfecting a eukaryotic cell with a nucleic acid encoding a recombinant meganuclease described herein; wherein the recombinant meganuclease produces a cleavage site in the chromosome at a recognition sequence comprising SEQ ID

NO:3, SEQ ID NO:4, or SEQ ID NO:5, and wherein the target sequence is disrupted by non-homologous end-joining at the cleavage site.

In one embodiment of the method, the eukaryotic cell is a human T cell or a cell derived therefrom.

5 In another embodiment of the method, the nucleic acid encoding the recombinant meganuclease is an mRNA. In some such embodiments, the mRNA can be a polycistronic mRNA encoding at least one recombinant meganuclease described herein and at least one additional protein (*e.g.*, a second nuclease).

10 In another embodiment, the method further comprises transfecting the eukaryotic cell with a second nucleic acid including an exogenous sequence of interest. In one such embodiment of the method, the second nucleic acid further comprises sequences homologous to sequences flanking the cleavage site, and the sequence of interest is inserted at the cleavage site by homologous recombination.

15 In another embodiment of the method, the sequence of interest encodes a chimeric antigen receptor. In another embodiment of the method, the sequence of interest encodes an exogenous T cell receptor.

In another embodiment of the method, at least the second nucleic acid is introduced into the eukaryotic cell by a viral vector.

20 In another embodiment of the method, at least the second nucleic acid is introduced into the eukaryotic cell by a recombinant AAV vector.

In another embodiment of the method, at least the second nucleic acid sequence is introduced into the eukaryotic cell using a single-stranded DNA template.

25 In another aspect, the invention provides a method for producing a genetically-modified eukaryotic cell by disrupting a target sequence in a chromosome of the eukaryotic cell, the method comprising: introducing a recombinant meganuclease described herein into a eukaryotic cell; wherein the meganuclease produces a cleavage site in the chromosome at a recognition sequence comprising SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5, and the target sequence is disrupted by non-homologous end-joining at the cleavage site.

30 In one embodiment of the method, the eukaryotic cell is a human T cell or a cell derived therefrom.

In another embodiment, the method further comprises transfecting the eukaryotic cell with a nucleic acid including an exogenous sequence of interest.

In one such embodiment of the method, the nucleic acid further comprises sequences homologous to sequences flanking the cleavage site, and the sequence of interest is inserted at the cleavage site by homologous recombination.

In another such embodiment of the method, the sequence of interest encodes a chimeric antigen receptor. In another such embodiment of the method, the sequence of interest encodes an exogenous T cell receptor.

In another such embodiment of the method, the nucleic acid is introduced into the eukaryotic cell by a viral vector.

In another such embodiment of the method, the nucleic acid is introduced into the eukaryotic cell by a recombinant AAV vector.

In another embodiment of the method, the nucleic acid sequence is introduced into the eukaryotic cell using a single-stranded DNA template.

In another aspect, the invention provides a method of producing a genetically-modified non-human organism comprising: producing a genetically-modified non-human eukaryotic cell according to the methods described herein; and (b) growing the genetically-modified non-human eukaryotic cell to produce the genetically-modified non-human organism.

In one embodiment of the method, the non-human eukaryotic cell is selected from the group consisting of a gamete, a zygote, a blastocyst cell, an embryonic stem cell, and a protoplast cell.

In another aspect, the invention provides a genetically-modified eukaryotic cell described herein for use as a medicament. The invention further provides the use of a genetically-modified eukaryotic cell described herein in the manufacture of a medicament for treating a disease in a subject in need thereof. In one such aspect, the medicament is useful in the treatment of cancer.

In some embodiments, the CAR comprises an extracellular antigen-binding domain. In some embodiments, the extracellular ligand-binding domain or moiety can be in the form of a single-chain variable fragment (scFv) derived from a monoclonal antibody, which provides specificity for a particular epitope or antigen (*e.g.*, an epitope or antigen preferentially present on the surface of a cell, such as a cancer cell or other disease-causing cell or particle). The scFv can be attached via a linker sequence. The extracellular ligand-binding domain can be specific for any antigen or epitope of interest. In some embodiments, the scFv can be humanized. The extracellular domain of a chimeric antigen receptor can also

comprise an autoantigen (see, Payne *et al.* (2016), *Science* 353 (6295): 179-184), which can be recognized by autoantigen-specific B cell receptors on B lymphocytes, thus directing T cells to specifically target and kill autoreactive B lymphocytes in antibody-mediated autoimmune diseases. Such CARs can be referred to as chimeric autoantibody receptors (CAARs), and their use is encompassed by the invention.

In another aspect, the invention provides a method of immunotherapy for treating cancer in a subject in need thereof comprising administering to the subject a pharmaceutical composition comprising a genetically-modified cell produced by the methods disclosed herein and a pharmaceutically acceptable carrier.

In some embodiments, the cancer is selected from the group consisting of a cancer of carcinoma, lymphoma, sarcoma, blastomas, and leukemia.

In some embodiments, the cancer is selected from the group consisting of a cancer of B-cell origin, breast cancer, gastric cancer, neuroblastoma, osteosarcoma, lung cancer, melanoma, prostate cancer, colon cancer, renal cell carcinoma, ovarian cancer, rhabdomyo sarcoma, leukemia, and Hodgkin's lymphoma. In some embodiments, the cancer of B-cell origin is selected from the group consisting of B-lineage acute lymphoblastic leukemia, B-cell chronic lymphocytic leukemia, and B-cell non-Hodgkin's lymphoma.

The foregoing and other aspects and embodiments of the present invention can be more fully understood by reference to the following detailed description and claims. Certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. All combinations of the embodiments are specifically embraced by the present invention and are disclosed herein just as if each and every combination was individually and explicitly disclosed. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable sub-combination. All sub-combinations of features listed in the embodiments are also specifically embraced by the present invention and are disclosed herein just as if each and every such sub-combination was individually and explicitly disclosed herein. Embodiments of each aspect of the present invention disclosed herein apply to each other aspect of the invention *mutatis mutandis*.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. TRC recognition sequences in the human TRC alpha constant region gene.

A) Each recognition sequence targeted by a recombinant meganuclease of the invention comprises two recognition half-sites. Each recognition half-site comprises 9 base pairs, separated by a 4 base pair central sequence. The TRC 1-2 recognition sequence (SEQ ID NO:3) spans nucleotides 187-208 of the human T cell alpha constant region (SEQ ID NO:1), and comprises two recognition half-sites referred to as TRC1 and TRC2. The TRC 3-4 recognition sequence (SEQ ID NO:4) spans nucleotides 93-114 of the human T cell alpha constant region (SEQ ID NO:1), and comprises two recognition half-sites referred to as TRC3 and TRC4. The TRC 7-8 recognition sequence (SEQ ID NO:5) spans nucleotides 118-139 of the human T cell alpha constant region (SEQ ID NO:1), and comprises two recognition half-sites referred to as TRC7 and TRC8. B) The recombinant meganucleases of the invention comprise two subunits, wherein the first subunit comprising the HVR1 region binds to a first recognition half-site (*e.g.*, TRC1, TRC3, or TRC7) and the second subunit comprising the HVR2 region binds to a second recognition half-site (*e.g.*, TRC2, TRC4, or TRC8). In embodiments where the recombinant meganuclease is a single-chain meganuclease, the first subunit comprising the HVR1 region can be positioned as either the N-terminal or C-terminal subunit. Likewise, the second subunit comprising the HVR2 region can be positioned as either the N-terminal or C-terminal subunit.

Figure 2A-B. Amino acid alignment of TRC1-binding subunits. A-B) Some recombinant meganucleases encompassed by the invention comprise one subunit that binds the 9 base pair TRC1 recognition half-site of SEQ ID NO:3. Amino acid sequence alignments are provided for the TRC1-binding subunits (SEQ ID NOs:33-52) of the recombinant meganucleases set forth in SEQ ID NOs:8-27. As shown, the TRC1-binding subunit of SEQ ID NOs:8-18 comprises residues 198-344, whereas the TRC1-binding subunit of SEQ ID NOs:19-27 comprises residues 7-153. Each TRC1-binding subunit comprises a 56 amino acid hypervariable region as indicated. Variable residues within the hypervariable region are shaded, with the most frequent amino acids at each position further highlighted; the most prevalent residues are bolded, whereas the second most prevalent are bolded and italicized. Residues outside of the hypervariable region are identical in each subunit, with the exception of a Q or E residue at position 80 or position 271 (see, U.S. Patent No. 8,021,867). All TRC1-binding subunits provided in Figure 2 share at least 90% sequence identity to the

TRC1-binding subunit (residues 198-344) of the TRC 1-2x.87 EE meganuclease (SEQ ID NO:33). Residue numbers shown are those of SEQ ID NOs:8-27.

Figure 3A-B. Amino acid alignment of TRC2-binding subunits. A-B) Some recombinant meganucleases encompassed by the invention comprise one subunit that binds the 9 base pair TRC2 recognition half-site of SEQ ID NO:3. Amino acid sequence alignments are provided for the TRC2-binding subunits (SEQ ID NOs:58-77) of the recombinant meganucleases set forth in SEQ ID NOs:8-27. As shown, the TRC2-binding subunit of SEQ ID NOs:8-18 comprises residues 7-153, whereas the TRC2-binding subunit of SEQ ID NOs:19-27 comprises residues 198-344. Each TRC2-binding subunit comprises a 56 amino acid hypervariable region as indicated. Variable residues within the hypervariable region are shaded, with the most frequent amino acids at each position further highlighted; the most prevalent residues are bolded, whereas the second most prevalent are bolded and italicized. Residues outside of the hypervariable region are identical in each subunit, with the exceptions of a Q or E residue at position 80 or position 271 (see, U.S. Patent No. 8,021,867), and an R residue at position 330 of meganucleases TRC 1-2x.87 EE, TRC 1-2x.87 QE, TRC 1-2x.87 EQ, TRC 1-2x.87, and TRC 1-2x.163 (shaded grey and underlined). All TRC2-binding subunits provided in Figure 3 share at least 90% sequence identity to the TRC2-binding subunit (residues 7-153) of the TRC 1-2x.87 EE meganuclease (SEQ ID NO:58). Residue numbers shown are those of SEQ ID NOs:8-27.

Figure 4. Amino acid alignment of TRC3-binding subunits. Some recombinant meganucleases encompassed by the invention comprise one subunit that binds the 9 base pair TRC3 recognition half-site of SEQ ID NO:4. Amino acid sequence alignments are provided for the TRC3-binding subunits (SEQ ID NOs:53 and 54) of the recombinant meganucleases set forth in SEQ ID NOs:28 and 29. As shown, the TRC3-binding subunit of SEQ ID NOs:28 and 29 comprises residues 7-153. Each TRC3-binding subunit comprises a 56 amino acid hypervariable region as indicated. Variable residues within the hypervariable region are shaded. Residues outside of the hypervariable region are identical in each subunit, with the exceptions of a Q or E residue at position 80 (see, U.S. Patent No. 8,021,867). The TRC3-binding subunits of the TRC 3-4x.3 and TRC 3-4x.19 meganucleases share 97% sequence identity. Residue numbers shown are those of SEQ ID NOs:28 and 29.

Figure 5. Amino acid alignment of TRC4-binding subunits. Some recombinant meganucleases encompassed by the invention comprise one subunit that binds the 9 base pair TRC4 recognition half-site of SEQ ID NO:4. Amino acid sequence alignments are provided

for the TRC4-binding subunits (SEQ ID NOs:78 and 79) of the recombinant meganucleases set forth in SEQ ID NOs:28 and 29. As shown, the TRC4-binding subunit of SEQ ID NOs:28 and 29 comprises residues 198-344. Each TRC4-binding subunit comprises a 56 amino acid hypervariable region as indicated. Variable residues within the hypervariable region are shaded. Residues outside of the hypervariable region are identical in each subunit, with the exceptions of a Q or E residue at position 80 (see, U.S. Patent No. 8,021,867). The TRC4-binding subunits of the TRC 3-4x.3 and TRC 3-4x.19 meganucleases share 97% sequence identity. Residue numbers shown are those of SEQ ID NOs:28 and 29.

Figure 6A-B. Amino acid alignment of TRC7-binding subunits. A-B) Some recombinant meganucleases encompassed by the invention comprise one subunit that binds the 9 base pair TRC7 recognition half-site of SEQ ID NO:5. Amino acid sequence alignments are provided for the TRC7-binding subunits (SEQ ID NOs:55-57) of the recombinant meganucleases set forth in SEQ ID NOs:30-32. As shown, the TRC7-binding subunit of SEQ ID NO:30 comprises residues 7-153, whereas the TRC7-binding subunit of SEQ ID NOs:31 and 32 comprises residues 198-344. Each TRC7-binding subunit comprises a 56 amino acid hypervariable region as indicated. Variable residues within the hypervariable region are shaded, with the most frequent amino acids at each position further highlighted; the most prevalent residues are bolded, whereas the second most prevalent are bolded and italicized. Residues outside of the hypervariable region are identical in each subunit, with the exception of a Q or E residue at position 80 or position 271 (see, U.S. Patent No. 8,021,867). All TRC7-binding subunits provided in Figure 6 share at least 90% sequence identity to the TRC7-binding subunit (residues 7-153) of the TRC 7-8x.7 meganuclease (SEQ ID NO:55). Residue numbers shown are those of SEQ ID NOs:30-32.

Figure 7A-B. Amino acid alignment of TRC8-binding subunits. A-B) Some recombinant meganucleases encompassed by the invention comprise one subunit that binds the 9 base pair TRC8 recognition half-site of SEQ ID NO:5. Amino acid sequence alignments are provided for the TRC8-binding subunits (SEQ ID NOs:80-82) of the recombinant meganucleases set forth in SEQ ID NOs:30-32. As shown, the TRC8-binding subunit of SEQ ID NO:30 comprises residues 198-344, whereas the TRC8-binding subunit of SEQ ID NOs:31 and 32 comprises residues 7-153. Each TRC8-binding subunit comprises a 56 amino acid hypervariable region as indicated. Variable residues within the hypervariable region are shaded, with the most frequent amino acids at each position further highlighted; the most prevalent residues are bolded, whereas the second most prevalent are bolded and

italicized. Residues outside of the hypervariable region are identical in each subunit, with the exception of a Q or E residue at position 80 or position 271 (see, U.S. Patent No. 8,021,867). All TRC8-binding subunits provided in Figure 7 share at least 90% sequence identity to the TRC8-binding subunit (residues 198-344) of the TRC 7-8x.7 meganuclease (SEQ ID NO:80).

5 Residue numbers shown are those of SEQ ID NOs:30-32.

Figure 8. Schematic of reporter assay in CHO cells for evaluating recombinant meganucleases targeting recognition sequences found in the T cell receptor alpha constant region (SEQ ID NO:1). For the recombinant meganucleases described herein, a CHO cell line was produced in which a reporter cassette was integrated stably into the genome of the cell. The reporter cassette comprised, in 5' to 3' order: an SV40 Early Promoter; the 5' 2/3 of the GFP gene; the recognition sequence for an engineered meganuclease of the invention (e.g., the TRC 1-2 recognition sequence, the TRC 3-4 recognition sequence, or the TRC 7-8 recognition sequence); the recognition sequence for the CHO-23/24 meganuclease (WO/2012/167192); and the 3' 2/3 of the GFP gene. Cells stably transfected with this cassette did not express GFP in the absence of a DNA break-inducing agent. Meganucleases were introduced by transduction of plasmid DNA or mRNA encoding each meganuclease. When a DNA break was induced at either of the meganuclease recognition sequences, the duplicated regions of the GFP gene recombined with one another to produce a functional GFP gene. The percentage of GFP-expressing cells could then be determined by flow cytometry as an indirect measure of the frequency of genome cleavage by the meganucleases.

Figure 9. Efficiency of recombinant meganucleases for recognizing and cleaving recognition sequences in the human T cell receptor alpha constant region (SEQ ID NO:1) in a CHO cell reporter assay. Each of the recombinant meganucleases set forth in SEQ ID NOs:8-32 were engineered to target the TRC 1-2 recognition sequence (SEQ ID NO:3), the TRC 3-4 recognition sequence (SEQ ID NO:4), or the TRC 7-8 recognition sequence (SEQ ID NO:5), and were screened for efficacy in the CHO cell reporter assay. The results shown provide the percentage of GFP-expressing cells observed in each assay, which indicates the efficacy of each meganuclease for cleaving a TRC target recognition sequence or the CHO-23/24 recognition sequence. A negative control (RHO 1-2 bs) was further included in each assay. A)-C) Meganucleases targeting the TRC 1-2 recognition sequence. D) Meganucleases targeting the TRC 3-4 recognition sequence. E)-F) Meganucleases targeting the TRC 7-8 recognition sequence. G) Variants of the TRC 1-2x.87 meganuclease, wherein the Q at position 271 is substituted with E (TRC 1-2x.87 QE), the Q at position 80 is substituted with

E (TRC 1-2x.87 EQ), or the Q at position 80 and the Q at position 271 are both substituted with E (TRC 1-2x.87 EE).

Figure 10. Time course of recombinant meganuclease efficacy in CHO cell reporter assay. The TRC 1-2x.87 QE, TRC 1-2x.87 EQ, and TRC 1-2x.87 EE meganucleases were evaluated in the CHO reporter assay, with the percentage of GFP-expressing cells determined 1, 4, 6, 8, and 12 days after introduction of meganuclease-encoding mRNA into the CHO reporter cells.

Figure 11. Analysis of Jurkat cell genomic DNA following transfection with TRC 1-2 meganucleases. At 72 hours post-transfection with mRNA encoding TRC 1-2 meganucleases, genomic DNA was harvested and a T7 endonuclease assay was performed to estimate genetic modification at the endogenous TRC 1-2 recognition sequence.

Figure 12. Dose-response of TRC 1-2 meganuclease expression in Jurkat cells on genetic modification at the endogenous TRC 1-2 recognition sequence. Jurkat cells were transfected with either 3 μ g or 1 μ g of a given TRC 1-2 meganuclease mRNA. At 96 hours, genomic DNA was analyzed using a T7 endonuclease assay.

Figure 13. Cleavage of TRC 1-2 recognition sequence in human T cells. A) CD3+ T cells were stimulated with anti-CD3 and anti-CD28 antibodies for 3 days, then electroporated with mRNA encoding the TRC 1-2x.87 EE meganuclease. Genomic DNA was harvested at 3 days and 7 days post-transfection, and analyzed using a T7 endonuclease assay. B) To determine whether mutations at the endogenous TRC 1-2 recognition sequence were sufficient to eliminate surface expression of the T cell receptor, cells were analyzed by flow cytometry using an anti-CD3 antibody. Control cells (transfected with water) and TRC 1-2x.87 EE-transfected cells were analyzed at day 3 and day 7 post-transfection, and the percentage of CD3-positive and CD3-negative T cells was determined.

Figure 14. Nucleic acid sequences of representative deletions that were observed at the TRC 1-2 recognition sequence in human T cells following expression of TRC 1-2 meganucleases.

Figure 15. Diagram illustrating sequence elements of recombinant AAV vectors and their use in combination with an engineered nuclease to insert an exogenous nucleic acid sequence into the endogenous TCR alpha constant region gene.

Figure 16. Map of plasmid used to produce the AAV405 vector.

Figure 17. Map of plasmid used to produce the AAV406 vector.

Figure 18. Determining the timing of meganuclease mRNA transfection and recombinant AAV transduction to enhance AAV transduction efficiency. Human CD3+ T cells were electroporated with mRNA encoding the TRC 1-2x.87 EE meganuclease and at 2, 4, or 8 hours post-transfection, cells were transduced with a recombinant AAV vector encoding GFP (GFP-AAV). T cells were analyzed by flow cytometry for GFP expression at 72 hours post-transduction to determine transduction efficiency.

Figure 19. Analyzing human T cells for insertion of an exogenous nucleic acid sequence using recombinant AAV vectors. CD3+ T cells transfected with TRC 1-2x.87 EE mRNA and subsequently transduced (2 hours post-transfection) with AAV405 or AAV406. Transduction-only controls were mock transfected (with water) and transduced with either AAV405 or AAV406. Meganuclease-only controls were transfected with TRC 1-2x.87 EE and then mock transduced (with water) at 2 hours post-transfection. Genomic DNA was harvested from T cells and the TRC 1-2 locus was amplified by PCR using primers that recognized sequences beyond the region of homology in the AAV vectors. PCR primers outside of the homology regions only allowed for amplification of the T cell genome, not from the AAV vectors. PCR products were purified and digested with EagI. PCR products were then analyzed for cleavage.

Figure 20. Characterization of EagI insertion into the TRC 1-2 recognition sequence of human T cells using AAV405. A) Undigested PCR product generated from previous experiments was cloned into a pCR-blunt vector. Colony PCR was performed using M13 forward and reverse primers and a portion of PCR products from cells transfected with TRC 1-2x.87 EE and AAV405 was analyzed by gel electrophoresis. Analysis shows a mix of full-length PCR products (approximately 1600 bp), smaller inserts, and empty plasmids (approximately 300 bp). B) In parallel, another portion of PCR products were digested with EagI to determine the percent of clones that contain the EagI recognition site inserted into the TRC 1-2 recognition sequence. PCR products cleaved with EagI generated expected fragments of approximately 700 and 800 bp.

Figure 21. Characterization of EagI insertion into the TRC 1-2 recognition sequence of human T cells using AAV406. A) Undigested PCR product generated from previous experiments was cloned into a pCR-blunt vector. Colony PCR was performed using M13 forward and reverse primers and a portion of PCR products from cells transfected with TRC 1-2x.87 EE and AAV406 was analyzed by gel electrophoresis. Analysis shows a mix of full-length PCR products (approximately 1600 bp), smaller inserts, and empty plasmids

(approximately 300 bp). B) In parallel, another portion of PCR products were digested with EagI to determine the percent of clones that contain the EagI recognition site inserted into the TRC 1-2 recognition sequence. PCR products cleaved with EagI generated expected fragments of approximately 700 and 800 bp.

5 Figure 22. A) Nucleic acid sequences of representative deletions and insertions (*i.e.*, indels) that were observed at the TRC 1-2 recognition sequence in human T cells following expression of TRC 1-2 meganucleases. B) Nucleic acid sequence of the TRC 1-2 recognition sequence confirming insertion of the exogenous nucleic acid sequence comprising the EagI restriction site.

10 Figure 23. Enhancement of recombinant AAV transduction efficiency. Transduction efficiency was further analyzed by optimizing the timing of meganuclease mRNA transfection and subsequent AAV transduction. Human CD3⁺ T cells were electroporated with mRNA encoding the TRC 1-2x.87 EE meganuclease and subsequently transduced with GFP-AAV immediately after transfection or 2 hours post-transfection. Additionally, non-
15 stimulated resting T cells were transduced with GFP-AAV. Mock transduced cells were also analyzed. At 72 hours post-transduction, cells were analyzed by flow cytometry for GFP expression to determine AAV transduction efficiency.

 Figure 24. Map of plasmid used to produce the AAV-CAR100 (AAV408) vector.

 Figure 25. Map of plasmid used to produce the AAV-CAR763 (AAV412) vector.

20 Figure 26. Insertion of chimeric antigen receptor coding sequence at TRC 1-2 recognition site in human T cells. A PCR-based assay was developed to determine whether the AAV412 HDR template was utilized to repair double-strand breaks at the TRC 1-2 recognition sequence.

 Figure 27. Insertion of chimeric antigen receptor coding sequence at TRC 1-2
25 recognition site in human T cells. A PCR-based assay was developed to determine whether the AAV408 HDR template was utilized to repair double-strand breaks at the TRC 1-2 recognition sequence. A) PCR products generated using a primer pair that only amplifies a product on the 5' end of the TRC 1-2 recognition sequence locus if the CAR gene has been inserted into that locus. B) PCR products generated using a primer pair that only amplifies a
30 product on the 3' end of the TRC 1-2 recognition sequence locus if the CAR gene has been inserted into that locus.

 Figure 28. Digital PCR. A) Schematic of a digital PCR assay developed to quantitatively determine insertion efficiency of the chimeric antigen receptor coding

sequence into the TRC 1-2 recognition site in human T cells. B) Results of digital PCR on genomic DNA from human T cells electroporated with a TRC 1-2x.87EE meganuclease mRNA and/or increasing amounts of AAV408.

Figure 29. Cell-surface expression of CD19 chimeric antigen receptor on human T cells. The expression level of the anti-CD19 chimeric antigen receptor was determined in cells that had the CAR gene inserted into the TRC 1-2 recognition sequence using AAV408 as the HDR template. Cell-surface expression was analyzed by flow cytometry. A) Cells that were mock electroporated and mock transduced (MOI – 0), and cells that were mock electroporated and transduced with increasing amounts of AAV408. B) Cells that were electroporated with TRC 1-2x.87EE and mock transduced (MOI – 0), and cells that were electroporated with TRC 1-2x.87EE and transduced with increasing amounts of AAV408.

Figure 30. Map of plasmid used to produce the AAV421 vector.

Figure 31. Map of plasmid used to produce the AAV422 vector.

Figure 32. Insertion of chimeric antigen receptor coding sequence. PCR methods were used to determine if the chimeric antigen receptor coding sequence introduced by AAV421 or AAV422 inserted at the TRC 1-2 recognition site cleaved by the TRC 1-2x.87EE meganuclease. A) Analysis of insertion following transduction with AAV421. B) Analysis of insertion following transduction with AAV422.

Figure 33. Cell-surface expression of CD19 chimeric antigen receptor on human T cells. The expression level of the anti-CD19 chimeric antigen receptor was determined in cells that had the CAR gene inserted into the TRC 1-2 recognition sequence using AAV421 as the HDR template. Cell-surface expression was analyzed by flow cytometry. A) Cells that were mock electroporated and mock transduced (MOI – 0), and cells that were mock electroporated and transduced with increasing amounts of AAV421. B) Cells that were electroporated with TRC 1-2x.87EE and mock transduced (MOI – 0), and cells that were electroporated with TRC 1-2x.87EE and transduced with increasing amounts of AAV421.

Figure 34. Expansion of human T cells expressing a cell-surface chimeric antigen receptor. Methods were determined for preferentially expanding and enriching a CD3⁺/CAR⁺ T cell population following electroporation with mRNA for the TRC 1-2x.87EE meganuclease and transduction with AAV421. A) Supplementation with IL-7 (10 ng/mL) and IL-15 (10 ng/mL). B) Supplementation with IL-7 (10 ng/mL) and IL-15 (10 ng/mL), and incubation with mitomycin C-inactivated IM-9 cells. C) Supplementation with IL-7 (10 ng/mL) and IL-15 (10 ng/mL), and two incubations with mitomycin C-inactivated IM-9 cells.

Figure 35. Cell-surface expression of CD19 chimeric antigen receptor on human T cells. The expression level of the anti-CD19 chimeric antigen receptor was determined in cells that had the CAR gene inserted into the TRC 1-2 recognition sequence using AAV422 as the HDR template. Cell-surface expression was analyzed by flow cytometry. A) Cells
 5 that were mock electroporated and mock transduced (MOI = 0), and cells that were mock electroporated and transduced with increasing amounts of AAV422. B) Cells that were electroporated with TRC 1-2x.87EE and mock transduced (MOI = 0), and cells that were electroporated with TRC 1-2x.87EE and transduced with increasing amounts of AAV422.

Figure 36. Expansion of human T cells expressing a cell-surface chimeric antigen
 10 receptor. Methods were determined for preferentially expanding and enriching a CD3⁺/CAR⁺ T cell population following electroporation with mRNA for the TRC 1-2x.87EE meganuclease and transduction with AAV422. A) Supplementation with IL-7 (10 ng/mL) and IL-15 (10 ng/mL). B) Supplementation with IL-7 (10 ng/mL) and IL-15 (10 ng/mL), and incubation with mitomycin C-inactivated IM-9 cells. C) Supplementation with IL-7 (10
 15 ng/mL) and IL-15 (10 ng/mL), and two incubations with mitomycin C-inactivated IM-9 cells.

Figure 37. Meganuclease knockout efficiency using single-strand AAV. Experiments were conducted to examine the knockout efficiency of two meganucleases in human T cells when simultaneously transduced with a single-stranded AAV vector. A) Cells electroporated with mRNA for TRC 1-2x.87EE and transduced with increasing amounts of the single-
 20 stranded AAV412. B) Cells electroporated with mRNA for a meganuclease targeting the beta-2 microglobulin gene and transduced with increasing amounts of the single-stranded AAV412. C) Cells electroporated with mRNA for TRC 1-2x.87EE and transduced with increasing amounts of the single-stranded AAV422.

Figure 38. Functional activity of anti-CD19 CAR T cells. A) IFN-gamma ELISPOT
 25 assay, in which either CD19⁺ Raji cells or CD19⁻ U937 cells were the target population. B) Cell killing assay in which luciferase-labeled CD19⁺ Raji cells were the target.

Figure 39. Expression of chimeric antigen receptors following transduction with linearized DNA donor templates. These experiments generated plasmids that contain an anti-CD19 CAR gene flanked by homology arms that are homologous to the TRC 1-2 recognition
 30 sequence locus. Different promoters were used in some plasmids, and homology arms were either “short” (200 bp on the 5’ homology arm and 180 bp on the 3’ homology arm) or “long” (985 bp on the 5’ homology arm and 763 bp on the 3’ homology arm). CAR donor plasmids were linearized at a restriction site in the vector backbone and gel purified. A) Background

CD3⁺/CAR⁺ staining. B) Cells electroporated with TRC 1-2x.87EE mRNA alone. C) Cells co-electroporated with TRC 1-2x.87EE mRNA and a long homology arm vector with an EF1 α core promoter with an HTLV enhancer. D) Cells co-electroporated with TRC 1-2x.87EE mRNA and a short homology arm vector with EF1 α core promoter (with no enhancer). E) Cells electroporated with a long homology arm vector with an EF1 α core promoter with an HTLV enhancer in the absence of TRC 1-2x.87EE mRNA. F) Cells electroporated with a short homology arm vector with EF1 α core promoter (with no enhancer) in the absence of TRC 1-2x.87EE mRNA. G) Cells electroporated with a long homology arm construct that contains an MND promoter driving expression of the CAR and an intron in the 5' end of the CAR gene, as well as TRC 1-2x.87EE mRNA. H) Cells electroporated with a long homology arm construct that contains an MND promoter driving expression of the CAR and no intron, as well as TRC 1-2x.87EE mRNA. I) Cells electroporated with a short homology arm plasmid with the MND promoter and no intron, as well as TRC 1-2x.87EE mRNA. J) Cells electroporated with a long homology arm construct that contains an MND promoter driving expression of the CAR and an intron in the 5' end of the CAR gene, but no TRC 1-2x.87EE mRNA. K) Cells electroporated with a long homology arm construct that contains an MND promoter driving expression of the CAR and no intron, but no TRC 1-2x.87EE mRNA. L) Cells electroporated with a short homology arm plasmid with the MND promoter and no intron, but no TRC 1-2x.87EE mRNA. M) Cells electroporated with a short homology arm construct that contained a JeT promoter, as well as TRC 1-2x.87EE mRNA. N) Cells electroporated with a long homology arm construct that contained a CMV promoter, as well as TRC 1-2x.87EE mRNA. O) Cells electroporated with a short homology arm construct that contained a JeT promoter, but no TRC 1-2x.87EE mRNA. P) Cells electroporated with a long homology arm construct that contained a CMV promoter, but no TRC 1-2x.87EE mRNA.

Figure 40. PCR analysis to determine whether the chimeric antigen receptor coding region delivered by linearized DNA constructs was inserted into the TRC 1-2 recognition sequence in human T cells.

Figure 41. Map of plasmid used to produce the AAV423 vector.

Figure 42. Cell-surface expression of CD19 chimeric antigen receptor on human T cells. The expression level of the anti-CD19 chimeric antigen receptor was determined in cells that had the CAR gene inserted into the TRC 1-2 recognition sequence using AAV423 as the HDR template. Cell-surface expression was analyzed by flow cytometry. A) Cells

that were mock electroporated and mock transduced (MOI – 0), and cells that were mock electroporated and transduced with increasing amounts of AAV423. B) Cells that were electroporated with TRC 1-2x.87EE and mock transduced (MOI – 0), and cells that were electroporated with TRC 1-2x.87EE and transduced with increasing amounts of AAV423.

5 Figure 43. Insertion of chimeric antigen receptor coding sequence. PCR methods were used to determine if the chimeric antigen receptor coding sequence introduced by AAV423 inserted at the TRC 1-2 recognition site cleaved by the TRC 1-2x.87EE meganuclease.

10 Figure 44. Phenotype analysis of anti-CD19 CAR T cells. A) Activated T cells were electroporated with TRC 1-2x.87 EE mRNA, then transduced with an AAV6 vector comprising an anti-CD19 CAR expression cassette driven by a JeT promoter and flanked by homology arms. Following 5 days of culture with IL-2 (10 ng/mL), cells were analyzed for cell-surface CD3 and anti-CD19 CAR expression by flow cytometry. B) CD3⁻ cells were enriched by depleting CD3⁺ cells using anti-CD3 magnetic beads. Depleted cells were then
15 cultured for 3 days in IL-15 (10 ng/mL) and IL-21 (10 ng/mL) and re-analyzed for cell-surface expression of CD3 and anti-CD19 CAR. C) The purified population of CD3⁻ CD19-CAR T cells was analyzed by flow cytometry to determine the percentage of cells that were CD4⁺ and CD8⁺. D) The purified population of CD3⁻ CD19-CAR T cells was further analyzed by flow cytometry to determine whether they were central memory T cells,
20 transitional memory T cells, or effector memory T cells by staining for CD62L and CD45RO.

Figure 45. Raji disseminated lymphoma model. Raji cells stably expressing firefly luciferase (ffLuc)⁴⁴ were injected i.v. into 5-6 week old female NSG mice on Day 1, at a dose of 2.0×10^5 cells per mouse. On Day 4 mice were injected i.v. with PBS or PBS containing gene edited control TCR KO T cells prepared from the same healthy donor PBMC or PBS
25 containing the indicated doses of CAR T cells prepared from the same donor. On the indicated days, live mice were injected i.p. with Luciferin substrate (150mg/kg in saline), anesthetized, and Luciferase activity measured after 7 minutes using IVIS SpectrumCT® (Perkin Elmer, Waltham, MA). Data was analyzed and exported using Living Image software 4.5.1 (Perkin Elmer, Waltham, MA). Luminescence signal intensity is represented
30 by radiance in p/sec/cm²/sr.

BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO: 1 sets forth the nucleotide sequence of the human T cell receptor alpha constant region gene (NCBI Gene ID NO. 28755).

5 SEQ ID NO: 2 sets forth the amino acid sequence encoded by the human T cell receptor alpha constant region.

SEQ ID NO: 3 sets forth the amino acid sequence of the TRC 1-2 recognition sequence.

SEQ ID NO: 4 sets forth the nucleotide sequence of the TRC 3-4 recognition sequence.

10 SEQ ID NO: 5 sets forth the nucleotide sequence of the TRC 7-8 recognition sequence.

SEQ ID NO: 6 sets forth the amino acid sequence of I-CreI.

SEQ ID NO: 7 sets forth the amino acid sequence of the LAGLIDADG motif.

15 SEQ ID NO: 8 sets forth the amino acid sequence of the TRC 1-2x.87 EE meganuclease.

SEQ ID NO: 9 sets forth the amino acid sequence of the TRC 1-2x.87 QE meganuclease.

SEQ ID NO: 10 sets forth the amino acid sequence of the TRC 1-2x.87 EQ meganuclease.

20 SEQ ID NO: 11 sets forth the amino acid sequence of the TRC 1-2x.87 meganuclease.

SEQ ID NO: 12 sets forth the amino acid sequence of the TRC 1-2x.6 meganuclease.

SEQ ID NO: 13 sets forth the amino acid sequence of the TRC 1-2x.20 meganuclease.

SEQ ID NO: 14 sets forth the amino acid sequence of the TRC 1-2x.55 meganuclease.

SEQ ID NO: 15 sets forth the amino acid sequence of the TRC 1-2x.60 meganuclease.

25 SEQ ID NO: 16 sets forth the amino acid sequence of the TRC 1-2x.105 meganuclease.

SEQ ID NO: 17 sets forth the amino acid sequence of the TRC 1-2x.163 meganuclease.

30 SEQ ID NO: 18 sets forth the amino acid sequence of the TRC 1-2x.113_3 meganuclease.

SEQ ID NO: 19 sets forth the amino acid sequence of the TRC 1-2x.5 meganuclease.

SEQ ID NO: 20 sets forth the amino acid sequence of the TRC 1-2x.8 meganuclease.

SEQ ID NO: 21 sets forth the amino acid sequence of the TRC 1-2x.25 meganuclease.

- SEQ ID NO: 22 sets forth the amino acid sequence of the TRC 1-2x.72 meganuclease.
 SEQ ID NO: 23 sets forth the amino acid sequence of the TRC 1-2x.80 meganuclease.
 SEQ ID NO: 24 sets forth the amino acid sequence of the TRC 1-2x.84 meganuclease.
 SEQ ID NO: 25 sets forth the amino acid sequence of the TRC 1-2x.120
- 5 meganuclease.
 SEQ ID NO: 26 sets forth the amino acid sequence of the TRC 1-2x.113_1
 meganuclease.
 SEQ ID NO: 27 sets forth the amino acid sequence of the TRC 1-2x.113_2
 meganuclease.
- 10 SEQ ID NO: 28 sets forth the amino acid sequence of the TRC 3-4x.3 meganuclease.
 SEQ ID NO: 29 sets forth the amino acid sequence of the TRC 3-4x.19 meganuclease.
 SEQ ID NO: 30 sets forth the amino acid sequence of the TRC 7-8x.7 meganuclease.
 SEQ ID NO: 31 sets forth the amino acid sequence of the TRC 7-8x.9 meganuclease.
 SEQ ID NO: 32 sets forth the amino acid sequence of the TRC 7-8x.14 meganuclease.
- 15 SEQ ID NO: 33 sets forth residues 198-344 of the TRC 1-2x.87 EE meganuclease.
 SEQ ID NO: 34 sets forth residues 198-344 of the TRC 1-2x.87 QE meganuclease.
 SEQ ID NO: 35 sets forth residues 198-344 of the TRC 1-2x.87 EQ meganuclease.
 SEQ ID NO: 36 sets forth residues 198-344 of the TRC 1-2x.87 meganuclease.
 SEQ ID NO: 37 sets forth residues 198-344 of the TRC 1-2x.6 meganuclease.
- 20 SEQ ID NO: 38 sets forth residues 198-344 of the TRC 1-2x.20 meganuclease.
 SEQ ID NO: 39 sets forth residues 198-344 of the TRC 1-2x.55 meganuclease.
 SEQ ID NO: 40 sets forth residues 198-344 of the TRC 1-2x.60 meganuclease.
 SEQ ID NO: 41 sets forth residues 198-344 of the TRC 1-2x.105 meganuclease.
 SEQ ID NO: 42 sets forth residues 198-344 of the TRC 1-2x.163 meganuclease.
- 25 SEQ ID NO: 43 sets forth residues 198-344 of the TRC 1-2x.113_3 meganuclease.
 SEQ ID NO: 44 sets forth residues 7-153 of the TRC 1-2x.5 meganuclease.
 SEQ ID NO: 45 sets forth residues 7-153 of the TRC 1-2x.8 meganuclease.
 SEQ ID NO: 46 sets forth residues 7-153 of the TRC 1-2x.25 meganuclease.
 SEQ ID NO: 47 sets forth residues 7-153 of the TRC 1-2x.72 meganuclease.
- 30 SEQ ID NO: 48 sets forth residues 7-153 of the TRC 1-2x.80 meganuclease.
 SEQ ID NO: 49 sets forth residues 7-153 of the TRC 1-2x.84 meganuclease.
 SEQ ID NO: 50 sets forth residues 7-153 of the TRC 1-2x.120 meganuclease.
 SEQ ID NO: 51 sets forth residues 7-153 of the TRC 1-2x.113_1 meganuclease.

- SEQ ID NO: 52 sets forth residues 7-153 of the TRC 1-2x.113_2 meganuclease.
- SEQ ID NO: 53 sets forth residues 7-153 of the TRC 3-4x.3 meganuclease.
- SEQ ID NO: 54 sets forth residues 7-153 of the TRC 3-4x.19 meganuclease.
- SEQ ID NO: 55 sets forth residues 7-153 of the TRC 7-8x.7 meganuclease.
- 5 SEQ ID NO: 56 sets forth residues 198-344 of the TRC 7-8x.9 meganuclease.
- SEQ ID NO: 57 sets forth residues 198-344 of the TRC 7-8x.14 meganuclease.
- SEQ ID NO: 58 sets forth residues 7-153 of the TRC 1-2x.87 EE meganuclease.
- SEQ ID NO: 59 sets forth residues 7-153 of the TRC 1-2x.87 QE meganuclease.
- SEQ ID NO: 60 sets forth residues 7-153 of the TRC 1-2x.87 EQ meganuclease.
- 10 SEQ ID NO: 61 sets forth residues 7-153 of the TRC 1-2x.87 meganuclease.
- SEQ ID NO: 62 sets forth residues 7-153 of the TRC 1-2x.6 meganuclease.
- SEQ ID NO: 63 sets forth residues 7-153 of the TRC 1-2x.20 meganuclease.
- SEQ ID NO: 64 sets forth residues 7-153 of the TRC 1-2x.55 meganuclease.
- SEQ ID NO: 65 sets forth residues 7-153 of the TRC 1-2x.60 meganuclease.
- 15 SEQ ID NO: 66 sets forth residues 7-153 of the TRC 1-2x.105 meganuclease.
- SEQ ID NO: 67 sets forth residues 7-153 of the TRC 1-2x.163 meganuclease.
- SEQ ID NO: 68 sets forth residues 7-153 of the TRC 1-2x.113_3 meganuclease.
- SEQ ID NO: 69 sets forth residues 198-344 of the TRC 1-2x.5 meganuclease.
- SEQ ID NO: 70 sets forth residues 198-344 of the TRC 1-2x.8 meganuclease.
- 20 SEQ ID NO: 71 sets forth residues 198-344 of the TRC 1-2x.25 meganuclease.
- SEQ ID NO: 72 sets forth residues 198-344 of the TRC 1-2x.72 meganuclease.
- SEQ ID NO: 73 sets forth residues 198-344 of the TRC 1-2x.80 meganuclease.
- SEQ ID NO: 74 sets forth residues 198-344 of the TRC 1-2x.84 meganuclease.
- SEQ ID NO: 75 sets forth residues 198-344 of the TRC 1-2x.120 meganuclease.
- 25 SEQ ID NO: 76 sets forth residues 198-344 of the TRC 1-2x.113_1 meganuclease.
- SEQ ID NO: 77 sets forth residues 198-344 of the TRC 1-2x.113_2 meganuclease.
- SEQ ID NO: 78 sets forth residues 198-344 of the TRC 3-4x.3 meganuclease.
- SEQ ID NO: 79 sets forth residues 198-344 of the TRC 3-4x.19 meganuclease.
- SEQ ID NO: 80 sets forth residues 198-344 of the TRC 7-8x.7 meganuclease.
- 30 SEQ ID NO: 81 sets forth residues 7-153 of the TRC 7-8x.9 meganuclease.
- SEQ ID NO: 82 sets forth residues 7-153 of the TRC 7-8x.14 meganuclease.
- SEQ ID NO: 83 sets forth the nucleotide sequence of the antisense strand of the TRC
1-2 recognition sequence.

SEQ ID NO: 84 sets forth the nucleotide sequence of the antisense strand of the TRC 3-4 recognition sequence.

SEQ ID NO: 85 sets forth the nucleotide sequence of the antisense strand of the TRC 7-8 recognition sequence.

5 SEQ ID NO: 86 sets forth nucleotides 162-233 of SEQ ID NO:1.

SEQ ID NO: 87 sets forth nucleotides 162-233 of SEQ ID NO:1 comprising a deletion resulting from cleavage and NHEJ.

SEQ ID NO: 88 sets forth nucleotides 162-233 of SEQ ID NO:1 comprising a deletion resulting from cleavage and NHEJ.

10 SEQ ID NO: 89 sets forth nucleotides 162-233 of SEQ ID NO:1 comprising a deletion resulting from cleavage and NHEJ.

SEQ ID NO: 90 sets forth nucleotides 162-233 of SEQ ID NO:1 comprising a deletion resulting from cleavage and NHEJ.

15 SEQ ID NO: 91 sets forth nucleotides 162-233 of SEQ ID NO:1 comprising a deletion resulting from cleavage and NHEJ.

SEQ ID NO: 92 sets forth nucleotides 162-233 of SEQ ID NO:1 comprising a deletion resulting from cleavage and NHEJ.

SEQ ID NO: 93 sets forth nucleotides 162-233 of SEQ ID NO:1 comprising a deletion resulting from cleavage and NHEJ.

20 SEQ ID NO: 94 sets forth nucleotides 162-233 of SEQ ID NO:1 comprising an insertion resulting from cleavage and NHEJ.

SEQ ID NO: 95 sets forth nucleotides 162-233 of SEQ ID NO:1 comprising an insertion resulting from cleavage and NHEJ.

25 SEQ ID NO: 96 sets forth nucleotides 162-233 of SEQ ID NO:1 comprising a deletion resulting from cleavage and NHEJ.

SEQ ID NO: 97 sets forth nucleotides 162-233 of SEQ ID NO:1 comprising a deletion resulting from cleavage and NHEJ.

SEQ ID NO: 98 sets forth nucleotides 162-233 of SEQ ID NO:1 comprising a deletion resulting from cleavage and NHEJ.

30 SEQ ID NO: 99 sets forth nucleotides 162-233 of SEQ ID NO:1 comprising a deletion resulting from cleavage and NHEJ.

SEQ ID NO: 100 sets forth nucleotides 162-233 of SEQ ID NO:1 comprising a deletion resulting from cleavage and NHEJ.

SEQ ID NO: 101 sets forth nucleotides 162-233 of SEQ ID NO:1 comprising a deletion resulting from cleavage and NHEJ.

SEQ ID NO: 102 sets forth nucleotides 162-233 of SEQ ID NO:1 comprising a deletion resulting from cleavage and NHEJ.

5 SEQ ID NO: 103 sets forth nucleotides 162-233 of SEQ ID NO:1 comprising a deletion resulting from cleavage and NHEJ.

SEQ ID NO: 104 sets forth nucleotides 162-233 of SEQ ID NO:1 comprising a deletion resulting from cleavage and NHEJ.

SEQ ID NO: 105 sets forth nucleotides 181-214 of SEQ ID NO:1.

10 SEQ ID NO: 106 sets forth nucleotides 181-214 of SEQ ID NO:1 comprising an exogenous nucleic acid sequence inserted via homologous recombination.

SEQ ID NO: 107 sets forth the nucleotide sequence of a plasmid used to generate the AAV405 vector.

15 SEQ ID NO: 108 sets forth the nucleotide sequence of a plasmid used to generate the AAV406 vector.

SEQ ID NO: 109 sets forth the nucleotide sequence of a plasmid used to generate the AAV-CAR100 (AAV408) vector.

SEQ ID NO: 110 sets forth the nucleotide sequence of a plasmid used to generate the AAV-CAR763 (AAV412) vector.

20 SEQ ID NO: 111 sets forth the amino acid sequence of an anti-CD19 chimeric antigen receptor.

SEQ ID NO: 112 sets forth the amino acid sequence of an anti-CD19 extracellular ligand-binding domain.

25 SEQ ID NO: 113 sets forth the amino acid sequence of a chimeric antigen receptor intracellular cytoplasmic signaling domain.

SEQ ID NO: 114 sets forth the amino acid sequence of a chimeric antigen receptor intracellular co-stimulatory domain.

SEQ ID NO: 115 sets forth the amino acid sequence of a chimeric antigen receptor signal peptide domain.

30 SEQ ID NO: 116 sets forth the amino acid sequence of a chimeric antigen receptor hinge region.

SEQ ID NO: 117 sets forth the amino acid sequence of a chimeric antigen receptor transmembrane domain.

SEQ ID NO: 118 sets forth the nucleotide sequence of an EF-1 alpha core promoter.

SEQ ID NO: 119 sets forth the nucleotide sequence of an exogenous polynucleotide insert.

5 SEQ ID NO: 120 sets forth the nucleotide sequence of the human TCR alpha constant region gene comprising an exogenous nucleic acid sequence inserted within the TRC 1-2 recognition sequence.

SEQ ID NO: 121 sets forth the nucleotide sequence of the human TCR alpha constant region gene comprising an exogenous nucleic acid sequence inserted within the TRC 3-4 recognition sequence.

10 SEQ ID NO: 122 sets forth the nucleotide sequence of the human TCR alpha constant region gene comprising an exogenous nucleic acid sequence inserted within the TRC 7-8 recognition sequence.

SEQ ID NO: 123 sets forth the nucleic acid sequence of a plasmid used to generate the AAV421 vector.

15 SEQ ID NO: 124 sets forth the nucleic acid sequence of a plasmid used to generate the AAV422 vector.

SEQ ID NO: 125 sets forth the nucleic acid sequence of a plasmid used to generate the AAV423 vector.

20 DETAILED DESCRIPTION OF THE INVENTION

1.1 References and Definitions

The patent and scientific literature referred to herein establishes knowledge that is available to those of skill in the art. The issued US patents, allowed applications, published foreign applications, and references, including GenBank database sequences, which are cited
25 herein are hereby incorporated by reference to the same extent as if each was specifically and individually indicated to be incorporated by reference.

The present invention can be embodied in different forms and should not be construed as limited to the embodiments set forth herein. Rather, these embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the
30 invention to those skilled in the art. For example, features illustrated with respect to one embodiment can be incorporated into other embodiments, and features illustrated with respect to a particular embodiment can be deleted from that embodiment. In addition, numerous variations and additions to the embodiments suggested herein will be apparent to those

skilled in the art in light of the instant disclosure, which do not depart from the instant invention.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention
5 belongs. The terminology used in the description of the invention herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention.

All publications, patent applications, patents, and other references mentioned herein are incorporated by reference herein in their entirety.

As used herein, “a,” “an,” or “the” can mean one or more than one. For example, “a”
10 cell can mean a single cell or a multiplicity of cells.

As used herein, unless specifically indicated otherwise, the word “or” is used in the inclusive sense of “and/or” and not the exclusive sense of “either/or.”

As used herein, the term “meganuclease” refers to an endonuclease that binds double-stranded DNA at a recognition sequence that is greater than 12 base pairs. Preferably, the
15 recognition sequence for a meganuclease of the invention is 22 base pairs. A meganuclease can be an endonuclease that is derived from I-CreI, and can refer to an engineered variant of I-CreI that has been modified relative to natural I-CreI with respect to, for example, DNA-binding specificity, DNA cleavage activity, DNA-binding affinity, or dimerization properties. Methods for producing such modified variants of I-CreI are known in the art (*e.g.*
20 WO 2007/047859). A meganuclease as used herein binds to double-stranded DNA as a heterodimer or as a “single-chain meganuclease” in which a pair of DNA-binding domains are joined into a single polypeptide using a peptide linker. The term “homing endonuclease” is synonymous with the term “meganuclease.” Meganucleases of the invention are substantially non-toxic when expressed in cells, particularly in human T cells, such that cells
25 can be transfected and maintained at 37°C without observing deleterious effects on cell viability or significant reductions in meganuclease cleavage activity when measured using the methods described herein.

As used herein, the term “single-chain meganuclease” refers to a polypeptide comprising a pair of meganuclease subunits joined by a linker. A single-chain meganuclease
30 has the organization: N-terminal subunit – Linker – C-terminal subunit. The two meganuclease subunits will generally be non-identical in amino acid sequence and will recognize non-identical DNA sequences. Thus, single-chain meganucleases typically cleave pseudo-palindromic or non-palindromic recognition sequences. A single-chain meganuclease

may be referred to as a “single-chain heterodimer” or “single-chain heterodimeric meganuclease” although it is not, in fact, dimeric. For clarity, unless otherwise specified, the term “meganuclease” can refer to a dimeric or single-chain meganuclease.

As used herein, the term “linker” refers to an exogenous peptide sequence used to join two meganuclease subunits into a single polypeptide. A linker may have a sequence that is found in natural proteins, or may be an artificial sequence that is not found in any natural protein. A linker may be flexible and lacking in secondary structure or may have a propensity to form a specific three-dimensional structure under physiological conditions. A linker can include, without limitation, those encompassed by U.S. Patent No. 8,445,251. In some embodiments, a linker may have an amino acid sequence comprising residues 154-195 of any one of SEQ ID NOs:8-32.

As used herein, with respect to a protein, the term “recombinant” means having an altered amino acid sequence as a result of the application of genetic engineering techniques to nucleic acids that encode the protein, and cells or organisms that express the protein. With respect to a nucleic acid, the term “recombinant” means having an altered nucleic acid sequence as a result of the application of genetic engineering techniques. Genetic engineering techniques include, but are not limited to, PCR and DNA cloning technologies; transfection, transformation and other gene transfer technologies; homologous recombination; site-directed mutagenesis; and gene fusion. In accordance with this definition, a protein having an amino acid sequence identical to a naturally-occurring protein, but produced by cloning and expression in a heterologous host, is not considered recombinant.

As used herein, the term “wild-type” refers to the most common naturally occurring allele (*i.e.*, polynucleotide sequence) in the allele population of the same type of gene, wherein a polypeptide encoded by the wild-type allele has its original functions. The term “wild-type” also refers a polypeptide encoded by a wild-type allele. Wild-type alleles (*i.e.*, polynucleotides) and polypeptides are distinguishable from mutant or variant alleles and polypeptides, which comprise one or more mutations and/or substitutions relative to the wild-type sequence(s). Whereas a wild-type allele or polypeptide can confer a normal phenotype in an organism, a mutant or variant allele or polypeptide can, in some instances, confer an altered phenotype. Wild-type homing endonucleases are distinguishable from recombinant or non-naturally-occurring meganucleases.

As used herein with respect to recombinant proteins, the term “modification” means any insertion, deletion or substitution of an amino acid residue in the recombinant sequence relative to a reference sequence (*e.g.*, a wild-type or a native sequence).

As used herein, the term “recognition sequence” refers to a DNA sequence that is bound and cleaved by a meganuclease. In the case of a recombinant meganuclease of the invention, a recognition sequence comprises a pair of inverted, 9 base pair “half-sites” or “recognition half-sites” that are separated by four base pairs. In the case of a single-chain meganuclease, the N-terminal subunit of the protein contacts a first half-site and the C-terminal subunit of the protein contacts a second half-site. Cleavage by a meganuclease produces four base pair 3’ “overhangs”. “Overhangs”, or “sticky ends” are short, single-stranded DNA segments that can be produced by meganuclease cleavage of a double-stranded DNA sequence. In the case of meganucleases of the present invention, the overhang comprises bases 10-13 of the 22 base pair recognition sequence.

As used herein, the term “target site” or “target sequence” refers to a region of the chromosomal DNA of a cell comprising a recognition sequence for a meganuclease.

As used herein, the term “DNA-binding affinity” or “binding affinity” means the tendency of a meganuclease to non-covalently associate with a reference DNA molecule (*e.g.*, a recognition sequence or an arbitrary sequence). Binding affinity is measured by a dissociation constant, K_d . As used herein, a meganuclease has “altered” binding affinity if the K_d of the recombinant meganuclease for a reference recognition sequence is increased or decreased by a statistically significant percent change relative to a reference meganuclease.

As used herein, the term “homologous recombination” or “HR” refers to the natural, cellular process in which a double-stranded DNA-break is repaired using a homologous DNA sequence as the repair template (see, *e.g.*, Cahill *et al.* (2006), *Front. Biosci.* 11:1958-1976). The homologous DNA sequence may be an endogenous chromosomal sequence or an exogenous nucleic acid that was delivered to the cell.

As used herein, the term “non-homologous end-joining” or “NHEJ” refers to the natural, cellular process in which a double-stranded DNA-break is repaired by the direct joining of two non-homologous DNA segments (see, *e.g.*, Cahill *et al.* (2006), *Front. Biosci.* 11:1958-1976). DNA repair by non-homologous end-joining is error-prone and frequently results in the untemplated addition or deletion of DNA sequences at the site of repair. In some instances, cleavage at a target recognition sequence results in NHEJ at a target recognition site. Nuclease-induced cleavage of a target site in the coding sequence of a gene

followed by DNA repair by NHEJ can introduce mutations into the coding sequence, such as frameshift mutations, that disrupt gene function. Thus, engineered nucleases such as meganucleases can be used to effectively knock-out a gene in a population of cells.

As used herein, a “chimeric antigen receptor” or “CAR” refers to an engineered
 5 receptor that confers or grafts specificity for an antigen onto an immune effector cell (*e.g.*, a human T cell). A chimeric antigen receptor typically comprises an extracellular ligand-binding domain or moiety and an intracellular domain that comprises one or more stimulatory domains that transduce the signals necessary for T cell activation. In some embodiments, the extracellular ligand-binding domain or moiety can be in the form of single-chain variable
 10 fragments (scFvs) derived from a monoclonal antibody, which provide specificity for a particular epitope or antigen (*e.g.*, an epitope or antigen preferentially present on the surface of a cancer cell or other disease-causing cell or particle). The extracellular ligand-binding domain can be specific for any antigen or epitope of interest. In a particular embodiment, the ligand-binding domain is specific for CD19. In other embodiments, the scFv can be
 15 humanized.

The extracellular domain of a chimeric antigen receptor can also comprise an autoantigen (see, Payne *et al.* (2016), *Science* 353(6295):179-184), which can be recognized by autoantigen-specific B cell receptors on B lymphocytes, thus directing T cells to specifically target and kill autoreactive B lymphocytes in antibody-mediated autoimmune
 20 diseases. Such CARs can be referred to as chimeric autoantibody receptors (CAARs), and their use is encompassed by the invention.

The scFvs can be attached via a linker sequence. The intracellular stimulatory domain can include one or more cytoplasmic signaling domains that transmit an activation signal to the immune effector cell following antigen binding. Such cytoplasmic signaling domains can
 25 include, without limitation, CD3-zeta. The intracellular stimulatory domain can also include one or more intracellular co-stimulatory domains that transmit a proliferative and/or cell-survival signal after ligand binding. Such intracellular co-stimulatory domains can include, without limitation, a CD28 domain, a 4-1BB domain, an OX40 domain, or a combination thereof. A chimeric antigen receptor can further include additional structural elements,
 30 including a transmembrane domain that is attached to the extracellular ligand-binding domain via a hinge or spacer sequence.

As used herein, an “exogenous T cell receptor” or “exogenous TCR” refers to a TCR whose sequence is introduced into the genome of an immune effector cell (*e.g.*, a human T

cell) that may or may not endogenously express the TCR. Expression of an exogenous TCR on an immune effector cell can confer specificity for a specific epitope or antigen (*e.g.*, an epitope or antigen preferentially present on the surface of a cancer cell or other disease-causing cell or particle). Such exogenous T cell receptors can comprise alpha and beta chains or, alternatively, may comprise gamma and delta chains. Exogenous TCRs useful in the invention may have specificity to any antigen or epitope of interest.

As used herein, the term “reduced expression” refers to any reduction in the expression of the endogenous T cell receptor at the cell surface of a genetically-modified cell when compared to a control cell. The term reduced can also refer to a reduction in the percentage of cells in a population of cells that express an endogenous polypeptide (*i.e.*, an endogenous T cell receptor) at the cell surface when compared to a population of control cells. Such a reduction may be up to 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or up to 100%. Accordingly, the term “reduced” encompasses both a partial knockdown and a complete knockdown of the endogenous T cell receptor.

As used herein with respect to both amino acid sequences and nucleic acid sequences, the terms “percent identity,” “sequence identity,” “percentage similarity,” “sequence similarity” and the like refer to a measure of the degree of similarity of two sequences based upon an alignment of the sequences that maximizes similarity between aligned amino acid residues or nucleotides, and that is a function of the number of identical or similar residues or nucleotides, the number of total residues or nucleotides, and the presence and length of gaps in the sequence alignment. A variety of algorithms and computer programs are available for determining sequence similarity using standard parameters. As used herein, sequence similarity is measured using the BLASTp program for amino acid sequences and the BLASTn program for nucleic acid sequences, both of which are available through the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/), and are described in, for example, Altschul *et al.* (1990), *J. Mol. Biol.* 215:403-410; Gish and States (1993), *Nature Genet.* 3:266-272; Madden *et al.* (1996), *Meth. Enzymol.* 266:131-141; Altschul *et al.* (1997), *Nucleic Acids Res.* 25:33 89-3402); Zhang *et al.* (2000), *J. Comput. Biol.* 7(1-2):203-14. As used herein, percent similarity of two amino acid sequences is the score based upon the following parameters for the BLASTp algorithm: word size=3; gap opening penalty=-11; gap extension penalty=-1; and scoring matrix=BLOSUM62. As used herein, percent similarity of two nucleic acid sequences is the score based upon the following

parameters for the BLASTn algorithm: word size=11; gap opening penalty=-5; gap extension penalty=-2; match reward=1; and mismatch penalty=-3.

As used herein with respect to modifications of two proteins or amino acid sequences, the term “corresponding to” is used to indicate that a specified modification in the first
 5 protein is a substitution of the same amino acid residue as in the modification in the second protein, and that the amino acid position of the modification in the first proteins corresponds to or aligns with the amino acid position of the modification in the second protein when the two proteins are subjected to standard sequence alignments (*e.g.*, using the BLASTp program). Thus, the modification of residue “X” to amino acid “A” in the first protein will
 10 correspond to the modification of residue “Y” to amino acid “A” in the second protein if residues X and Y correspond to each other in a sequence alignment, and despite the fact that X and Y may be different numbers.

As used herein, the term “recognition half-site,” “recognition sequence half-site,” or simply “half-site” means a nucleic acid sequence in a double-stranded DNA molecule that is
 15 recognized by a monomer of a homodimeric or heterodimeric meganuclease, or by one subunit of a single-chain meganuclease.

As used herein, the term “hypervariable region” refers to a localized sequence within a meganuclease monomer or subunit that comprises amino acids with relatively high variability. A hypervariable region can comprise about 50-60 contiguous residues, about 53-
 20 57 contiguous residues, or preferably about 56 residues. In some embodiments, the residues of a hypervariable region may correspond to positions 24-79 or positions 215-270 of any one of SEQ ID NOs:8-32. A hypervariable region can comprise one or more residues that contact DNA bases in a recognition sequence and can be modified to alter base preference of the monomer or subunit. A hypervariable region can also comprise one or more residues that
 25 bind to the DNA backbone when the meganuclease associates with a double-stranded DNA recognition sequence. Such residues can be modified to alter the binding affinity of the meganuclease for the DNA backbone and the target recognition sequence. In different embodiments of the invention, a hypervariable region may comprise between 1-20 residues that exhibit variability and can be modified to influence base preference and/or DNA-binding
 30 affinity. In particular embodiments, a hypervariable region comprises between about 15-18 residues that exhibit variability and can be modified to influence base preference and/or DNA-binding affinity. In some embodiments, variable residues within a hypervariable region correspond to one or more of positions 24, 26, 28, 29, 30, 32, 33, 38, 40, 42, 44, 46,

66, 68, 70, 72, 73, 75, and 77 of any one of SEQ ID NOs:8-32. In other embodiments, variable residues within a hypervariable region correspond to one or more of positions 215, 217, 219, 221, 223, 224, 229, 231, 233, 235, 237, 248, 257, 259, 261, 263, 264, 266, and 268 of any one of SEQ ID NOs:8-32.

5 As used herein, the terms “T cell receptor alpha constant region gene” and “TCR alpha constant region gene” are used interchangeably and refer to the human gene identified by NCBI Gen ID NO. 28755 (SEQ ID NO:1).

 The terms “recombinant DNA construct,” “recombinant construct,” “expression cassette,” “expression construct,” “chimeric construct,” “construct,” and “recombinant DNA
10 fragment” are used interchangeably herein and are single or double-stranded polynucleotides. A recombinant construct comprises an artificial combination of single or double-stranded polynucleotides, including, without limitation, regulatory and coding sequences that are not found together in nature. For example, a recombinant DNA construct may comprise regulatory sequences and coding sequences that are derived from different sources, or
15 regulatory sequences and coding sequences derived from the same source and arranged in a manner different than that found in nature. Such a construct may be used by itself or may be used in conjunction with a vector.

 As used herein, a “vector” or “recombinant DNA vector” may be a construct that includes a replication system and sequences that are capable of transcription and translation
20 of a polypeptide-encoding sequence in a given host cell. If a vector is used then the choice of vector is dependent upon the method that will be used to transform host cells as is well known to those skilled in the art. Vectors can include, without limitation, plasmid vectors and recombinant AAV vectors, or any other vector known in that art suitable for delivering a gene encoding a meganuclease of the invention to a target cell. The skilled artisan is well
25 aware of the genetic elements that must be present on the vector in order to successfully transform, select and propagate host cells comprising any of the isolated nucleotides or nucleic acid sequences of the invention.

 As used herein, a “vector” can also refer to a viral vector. Viral vectors can include, without limitation, retroviral vectors, lentiviral vectors, adenoviral vectors, and adeno-
30 associated viral vectors (AAV).

 As used herein, a “polycistronic” mRNA refers to a single messenger RNA that comprises two or more coding sequences (*i.e.*, cistrons) and encodes more than one protein. A polycistronic mRNA can comprise any element known in the art to allow for the

translation of two or more genes from the same mRNA molecule including, but not limited to, an IRES element, a T2A element, a P2A element, an E2A element, and an F2A element.

As used herein, a “human T cell” or “T cell” refers to a T cell isolated from a human donor. Human T cells, and cells derived therefrom, include isolated T cells that have not
5 been passaged in culture, T cells that have been passaged and maintained under cell culture conditions without immortalization, and T cells that have been immortalized and can be maintained under cell culture conditions indefinitely.

As used herein, a “control” or “control cell” refers to a cell that provides a reference point for measuring changes in genotype or phenotype of a genetically-modified cell. A
10 control cell may comprise, for example: (a) a wild-type cell, *i.e.*, of the same genotype as the starting material for the genetic alteration that resulted in the genetically-modified cell; (b) a cell of the same genotype as the genetically-modified cell but that has been transformed with a null construct (*i.e.*, with a construct that has no known effect on the trait of interest); or, (c)
15 a cell genetically identical to the genetically-modified cell but that is not exposed to conditions or stimuli or further genetic modifications that would induce expression of altered genotype or phenotype.

As used herein, the recitation of a numerical range for a variable is intended to convey that the invention may be practiced with the variable equal to any of the values within that range. Thus, for a variable that is inherently discrete, the variable can be equal to any integer
20 value within the numerical range, including the end-points of the range. Similarly, for a variable that is inherently continuous, the variable can be equal to any real value within the numerical range, including the end-points of the range. As an example, and without limitation, a variable that is described as having values between 0 and 2 can take the values 0,
1 or 2 if the variable is inherently discrete, and can take the values 0.0, 0.1, 0.01, 0.001, or
25 any other real values ≥ 0 and ≤ 2 if the variable is inherently continuous.

2.1 Principle of the Invention

The present invention is based, in part, on the discovery that recombinant meganucleases can be engineered to recognize and cleave recognition sequences found
30 within the human TCR alpha constant region gene (SEQ ID NO:1), particularly within residues 93-208. Cleavage at such recognition sequences can allow for NHEJ at the cleavage site and disrupted expression of the human T cell receptor alpha chain subunit, leading to reduced expression and/or function of the T cell receptor at the cell surface.

Additionally, cleavage at such recognition sequences can further allow for homologous recombination of exogenous nucleic acid sequences directly into the TCR alpha constant region gene. Such exogenous nucleic acid sequences can comprise a sequence of interest, such as a sequence encoding a chimeric antigen receptor, an exogenous TCR
5 receptor, or any other polypeptide of interest. Thus, the present invention allows for both the knockout of the endogenous T cell receptor and the expression of an exogenous nucleic acid sequence (*e.g.*, a chimeric antigen receptor or exogenous TCR) by targeting a single recognition site with a single engineered nuclease. Such cells can exhibit reduced or no induction of graft-versus-host-disease (GVHD) when administered to an allogeneic subject.

10

2.2 Meganucleases for Recognizing and Cleaving Recognition Sequences Within the T Cell Receptor Alpha Constant Region Gene

It is known in the art that it is possible to use a site-specific nuclease to make a DNA break in the genome of a living cell, and that such a DNA break can result in permanent
15 modification of the genome via mutagenic NHEJ repair or via homologous recombination with a transgenic DNA sequence. NHEJ can produce mutagenesis at the cleavage site, resulting in inactivation of the allele. NHEJ-associated mutagenesis may inactivate an allele via generation of early stop codons, frameshift mutations producing aberrant non-functional proteins, or could trigger mechanisms such as nonsense-mediated mRNA decay. The use of
20 meganucleases to induce mutagenesis via NHEJ can be used to target a specific mutation or a sequence present in a wild-type allele. The use of meganucleases to induce a double-strand break in a target locus is known to stimulate homologous recombination, particularly of transgenic DNA sequences flanked by sequences that are homologous to the genomic target. In this manner, exogenous nucleic acid sequences can be inserted into a target locus. Such
25 exogenous nucleic acids can encode, for example, a chimeric antigen receptor, an exogenous TCR, or any sequence or polypeptide of interest.

In preferred embodiments, the nucleases used to practice the invention are single-chain meganucleases. A single-chain meganuclease comprises an N-terminal subunit and a C-terminal subunit joined by a linker peptide. Each of the two domains recognizes half of the
30 recognition sequence (*i.e.*, a recognition half-site) and the site of DNA cleavage is at the middle of the recognition sequence near the interface of the two subunits. DNA strand breaks are offset by four base pairs such that DNA cleavage by a meganuclease generates a pair of four base pair, 3' single-strand overhangs.

In some examples, recombinant meganucleases of the invention have been engineered to recognize and cleave the TRC 1-2 recognition sequence (SEQ ID NO:3). Such recombinant meganucleases are collectively referred to herein as “TRC 1-2 meganucleases.” Exemplary TRC 1-2 meganucleases are provided in SEQ ID NOs:8-27.

5 In additional examples, recombinant meganucleases of the invention have been engineered to recognize and cleave the TRC 3-4 recognition sequence (SEQ ID NO:4). Such recombinant meganucleases are collectively referred to herein as “TRC 3-4 meganucleases.” Exemplary TRC 3-4 meganucleases are provided in SEQ ID NOs:28 and 29.

10 In further examples, recombinant meganucleases of the invention have been engineered to recognize and cleave the TRC 7-8 recognition sequence (SEQ ID NO:5). Such recombinant meganucleases are collectively referred to herein as “TRC 7-8 meganucleases.” Exemplary TRC 7-8 meganucleases are provided in SEQ ID NOs:30-32.

15 Recombinant meganucleases of the invention comprise a first subunit, comprising a first hypervariable (HVR1) region, and a second subunit, comprising a second hypervariable (HVR2) region. Further, the first subunit binds to a first recognition half-site in the recognition sequence (*e.g.*, the TRC1, TRC3, or TRC7 half-site), and the second subunit binds to a second recognition half-site in the recognition sequence (*e.g.*, the TRC2, TRC4, or TRC8 half-site). In embodiments where the recombinant meganuclease is a single-chain
20 meganuclease, the first and second subunits can be oriented such that the first subunit, which comprises the HVR1 region and binds the first half-site, is positioned as the N-terminal subunit, and the second subunit, which comprises the HVR2 region and binds the second half-site, is positioned as the C-terminal subunit. In alternative embodiments, the first and second subunits can be oriented such that the first subunit, which comprises the HVR1 region and binds the first half-site, is positioned as the C-terminal subunit, and the second subunit,
25 which comprises the HVR2 region and binds the second half-site, is positioned as the N-terminal subunit. Exemplary TRC 1-2 meganucleases of the invention are provided in Table 1. Exemplary TRC 3-4 meganucleases of the invention are provided in Table 2. Exemplary TRC 7-8 meganucleases of the invention are provided in Table 3.

Table 1. Exemplary recombinant meganucleases engineered to recognize and cleave the TRC 1-2 recognition sequence (SEQ ID NO:3)

Meganuclease	AA SEQ ID	TRC1 Subunit Residues	TRC1 Subunit SEQ ID	*TRC1 Subunit %	TRC2 Subunit Residues	TRC2 Subunit SEQ ID	*TRC2 Subunit %
TRC 1-2x.87 EE	8	198-344	33	100	7-153	58	100
TRC 1-2x.87 QE	9	198-344	34	100	7-153	59	99.3
TRC 1-2x.87 EQ	10	198-344	35	99.3	7-153	60	100
TRC 1-2x.87	11	198-344	36	99.3	7-153	61	99.3
TRC 1-2x.6	12	198-344	37	99.3	7-153	62	94.6
TRC 1-2x.20	13	198-344	38	99.3	7-153	63	91.2
TRC 1-2x.55	14	198-344	39	95.9	7-153	64	91.8
TRC 1-2x.60	15	198-344	40	91.8	7-153	65	91.2
TRC 1-2x.105	16	198-344	41	95.2	7-153	66	95.2
TRC 1-2x.163	17	198-344	42	99.3	7-153	67	99.3
TRC 1-2x.113_3	18	198-344	43	99.3	7-153	68	91.2
TRC 1-2x.5	19	7-153	44	99.3	198-344	69	93.2
TRC 1-2x.8	20	7-153	45	92.5	198-344	70	92.5
TRC 1-2x.25	21	7-153	46	99.3	198-344	71	98.6
TRC 1-2x.72	22	7-153	47	99.3	198-344	72	92.5
TRC 1-2x.80	23	7-153	48	99.3	198-344	73	92.5
TRC 1-2x.84	24	7-153	49	95.2	198-344	74	98.6
TRC 1-2x.120	25	7-153	50	99.3	198-344	75	92.5
TRC 1-2x.113_1	26	7-153	51	100	198-344	76	92.5
TRC 1-2x.113_2	27	7-153	52	99.3	198-344	77	92.5

*“TRC1 Subunit %” and “TRC2 Subunit %” represent the amino acid sequence identity between the TRC1-binding and TRC2-binding subunit regions of each meganuclease and the TRC1-binding and TRC2-binding subunit regions, respectively, of the TRC 1-2x.87 EE meganuclease.

Table 2. Exemplary recombinant meganucleases engineered to recognize and cleave the TRC 3-4 recognition sequence (SEQ ID NO:4)

Meganuclease	AA SEQ ID	TRC3 Subunit Residues	TRC3 Subunit SEQ ID	*TRC3 Subunit %	TRC4 Subunit Residues	TRC4 Subunit SEQ ID	*TRC4 Subunit %
TRC 3-4x.3	28	7-153	53	100	198-344	78	100
TRC 3-4x.19	29	7-153	54	96.6	198-344	79	96.6

*“TRC3 Subunit %” and “TRC4 Subunit %” represent the amino acid sequence identity between the TRC3-binding and TRC4-binding subunit regions of each meganuclease and the TRC3-binding and TRC4-binding subunit regions, respectively, of the TRC 3-4x.3 meganuclease.

Table 3. Exemplary recombinant meganucleases engineered to recognize and cleave the TRC 7-8 recognition sequence (SEQ ID NO:5)

Meganuclease	AA SEQ ID	TRC7 Subunit Residues	TRC7 Subunit SEQ ID	*TRC7 Subunit %	TRC8 Subunit Residues	TRC8 Subunit SEQ ID	*TRC8 Subunit %
TRC 7-8x.7	30	7-153	55	100	198-344	80	100
TRC 7-8x.9	31	198-344	56	97.3	7-153	81	91.2
TRC 7-8x.14	32	198-344	57	97.9	7-153	82	90.5

*“TRC7 Subunit %” and “TRC8 Subunit %” represent the amino acid sequence identity between the TRC7-binding and TRC8-binding subunit regions of each meganuclease and the TRC7-binding and TRC8-binding subunit regions, respectively, of the TRC 7-8x.7 meganuclease.

2.3 Methods for Producing Genetically-Modified Cells

The invention provides methods for producing genetically-modified cells using recombinant meganucleases that recognize and cleave recognition sequences found within the human TCR alpha constant region gene (SEQ ID NO:1). Cleavage at such recognition sequences can allow for NHEJ at the cleavage site and disrupted expression of the human T cell receptor alpha chain subunit, leading to reduced expression and/or function of the T cell receptor at the cell surface. Additionally, cleavage at such recognition sequences can further allow for homologous recombination of exogenous nucleic acid sequences directly into the TCR alpha constant region gene.

Recombinant meganucleases of the invention can be delivered into a cell in the form of protein or, preferably, as a nucleic acid encoding the recombinant meganuclease. Such nucleic acid can be DNA (*e.g.*, circular or linearized plasmid DNA or PCR products) or RNA.

For embodiments in which the recombinant meganuclease coding sequence is delivered in DNA form, it should be operably linked to a promoter to facilitate transcription of the meganuclease gene. Mammalian promoters suitable for the invention include constitutive promoters such as the cytomegalovirus early (CMV) promoter (Thomsen *et al.* (1984), *Proc Natl Acad Sci USA*. 81(3):659-63) or the SV40 early promoter (Benoist and Chambon (1981), *Nature*. 290(5804):304-10) as well as inducible promoters such as the tetracycline-inducible promoter (Dingermann *et al.* (1992), *Mol Cell Biol*. 12(9):4038-45).

In some embodiments, mRNA encoding the engineered nuclease is delivered to the cell because this reduces the likelihood that the gene encoding the engineered nuclease will integrate into the genome of the cell. Such mRNA encoding an engineered nuclease can be produced using methods known in the art such as *in vitro* transcription. In some embodiments,

the mRNA is capped using 7-methyl-guanosine. In some embodiments, the mRNA may be polyadenylated.

In particular embodiments, an mRNA encoding an engineered nuclease of the invention can be a polycistronic mRNA encoding two or more nucleases that are simultaneously expressed in the cell. A polycistronic mRNA can encode two or more nucleases of the invention that target different recognition sequences in the same target gene. Alternatively, a polycistronic mRNA can encode at least one nuclease described herein and at least one additional nuclease targeting a separate recognition sequence positioned in the same gene, or targeting a second recognition sequence positioned in a second gene such that cleavage sites are produced in both genes. A polycistronic mRNA can comprise any element known in the art to allow for the translation of two or more genes (*i.e.*, cistrons) from the same mRNA molecule including, but not limited to, an IRES element, a T2A element, a P2A element, an E2A element, and an F2A element.

Purified recombinant meganuclease proteins can be delivered into cells to cleave genomic DNA, which allows for homologous recombination or non-homologous end-joining at the cleavage site with a sequence of interest, by a variety of different mechanisms known in the art.

In some embodiments, recombinant meganuclease proteins, or DNA/mRNA encoding recombinant meganucleases, are coupled to a cell penetrating peptide or targeting ligand to facilitate cellular uptake. Examples of cell penetrating peptides known in the art include poly-arginine (Jearawiriyapaisarn, *et al.* (2008) *Mol Ther.* 16:1624-9), TAT peptide from the HIV virus (Hudecz *et al.* (2005), *Med. Res. Rev.* 25: 679-736), MPG (Simeoni, *et al.* (2003) *Nucleic Acids Res.* 31:2717-2724), Pep-1 (Deshayes *et al.* (2004) *Biochemistry* 43: 7698-7706, and HSV-1 VP-22 (Deshayes *et al.* (2005) *Cell Mol Life Sci.* 62:1839-49. In an alternative embodiment, recombinant meganucleases, or DNA/mRNA encoding recombinant meganucleases, are coupled covalently or non-covalently to an antibody that recognizes a specific cell-surface receptor expressed on target cells such that the meganuclease protein/DNA/mRNA binds to and is internalized by the target cells. Alternatively, recombinant meganuclease protein/DNA/mRNA can be coupled covalently or non-covalently to the natural ligand (or a portion of the natural ligand) for such a cell-surface receptor. (McCall, *et al.* (2014) *Tissue Barriers.* 2(4):e944449; Dinda, *et al.* (2013) *Curr Pharm Biotechnol.* 14:1264-74; Kang, *et al.* (2014) *Curr Pharm Biotechnol.* 15(3):220-30; Qian *et al.* (2014) *Expert Opin Drug Metab Toxicol.* 10(11):1491-508).

In some embodiments, recombinant meganuclease proteins, or DNA/mRNA encoding recombinant meganucleases, are coupled covalently or, preferably, non-covalently to a nanoparticle or encapsulated within such a nanoparticle using methods known in the art (Sharma, *et al.* (2014) *Biomed Res Int.* 2014). A nanoparticle is a nanoscale delivery system whose length scale is $<1\ \mu\text{m}$, preferably $<100\ \text{nm}$. Such nanoparticles may be designed using a core composed of metal, lipid, polymer, or biological macromolecule, and multiple copies of the recombinant meganuclease proteins, mRNA, or DNA can be attached to or encapsulated with the nanoparticle core. This increases the copy number of the protein/mRNA/DNA that is delivered to each cell and, so, increases the intracellular expression of each recombinant meganuclease to maximize the likelihood that the target recognition sequences will be cut. The surface of such nanoparticles may be further modified with polymers or lipids (*e.g.*, chitosan, cationic polymers, or cationic lipids) to form a core-shell nanoparticle whose surface confers additional functionalities to enhance cellular delivery and uptake of the payload (Jian *et al.* (2012) *Biomaterials.* 33(30): 7621-30).

Nanoparticles may additionally be advantageously coupled to targeting molecules to direct the nanoparticle to the appropriate cell type and/or increase the likelihood of cellular uptake. Examples of such targeting molecules include antibodies specific for cell-surface receptors and the natural ligands (or portions of the natural ligands) for cell surface receptors.

In some embodiments, the meganuclease proteins or DNA/mRNA encoding the meganucleases are encapsulated within liposomes or complexed using cationic lipids (*see, e.g.*, Lipofectamine™, Life Technologies Corp., Carlsbad, CA; Zuris *et al.* (2015) *Nat Biotechnol.* 33: 73-80; Mishra *et al.* (2011) *J Drug Deliv.* 2011:863734). The liposome and lipoplex formulations can protect the payload from degradation, and facilitate cellular uptake and delivery efficiency through fusion with and/or disruption of the cellular membranes of the cells.

In some embodiments, recombinant meganuclease proteins, or DNA/mRNA encoding recombinant meganucleases, are encapsulated within polymeric scaffolds (*e.g.*, PLGA) or complexed using cationic polymers (*e.g.*, PEI, PLL) (Tamboli *et al.* (2011) *Ther Deliv.* 2(4): 523-536).

In some embodiments, recombinant meganuclease proteins, or DNA/mRNA encoding recombinant meganucleases, are combined with amphiphilic molecules that self-assemble into micelles (Tong *et al.* (2007) *J Gene Med.* 9(11): 956-66). Polymeric micelles may include a micellar shell formed with a hydrophilic polymer (*e.g.*, polyethyleneglycol) that can

prevent aggregation, mask charge interactions, and reduce nonspecific interactions outside of the cell.

In some embodiments, recombinant meganuclease proteins, or DNA/mRNA encoding recombinant meganucleases, are formulated into an emulsion or a nanoemulsion (*i.e.*, having
5 an average particle diameter of $< 1\text{nm}$) for delivery to the cell. The term “emulsion” refers to, without limitation, any oil-in-water, water-in-oil, water-in-oil-in-water, or oil-in-water-in-oil dispersions or droplets, including lipid structures that can form as a result of hydrophobic forces that drive apolar residues (e.g., long hydrocarbon chains) away from water and polar head groups toward water, when a water immiscible phase is mixed with an aqueous phase.
10 These other lipid structures include, but are not limited to, unilamellar, paucilamellar, and multilamellar lipid vesicles, micelles, and lamellar phases. Emulsions are composed of an aqueous phase and a lipophilic phase (typically containing an oil and an organic solvent). Emulsions also frequently contain one or more surfactants. Nanoemulsion formulations are well known, *e.g.*, as described in US Patent Application Nos. 2002/0045667 and
15 2004/0043041, and US Pat. Nos. 6,015,832, 6,506,803, 6,635,676, and 6,559,189, each of which is incorporated herein by reference in its entirety.

In some embodiments, recombinant meganuclease proteins, or DNA/mRNA encoding recombinant meganucleases, are covalently attached to, or non-covalently associated with, multifunctional polymer conjugates, DNA dendrimers, and polymeric dendrimers
20 (Mastorakos *et al.* (2015) *Nanoscale*. 7(9): 3845-56; Cheng *et al.* (2008) *J Pharm Sci.* 97(1): 123-43). The dendrimer generation can control the payload capacity and size, and can provide a high payload capacity. Moreover, display of multiple surface groups can be leveraged to improve stability and reduce nonspecific interactions.

In some embodiments, genes encoding a recombinant meganuclease are introduced
25 into a cell using a viral vector. Such vectors are known in the art and include lentiviral vectors, adenoviral vectors, and adeno-associated virus (AAV) vectors (reviewed in Vannucci, *et al.* (2013) *New Microbiol.* 36:1-22). Recombinant AAV vectors useful in the invention can have any serotype that allows for transduction of the virus into the cell and insertion of the meganuclease gene into the cell genome. In particular embodiments,
30 recombinant AAV vectors have a serotype of AAV2 or AAV6. Recombinant AAV vectors can also be self-complementary such that they do not require second-strand DNA synthesis in the host cell (McCarty, *et al.* (2001) *Gene Ther.* 8:1248-54).

If the recombinant meganuclease genes are delivered in DNA form (*e.g.* plasmid) and/or via a viral vector (*e.g.* AAV) they must be operably linked to a promoter. In some embodiments, this can be a viral promoter such as endogenous promoters from the viral vector (*e.g.* the LTR of a lentiviral vector) or the well-known cytomegalovirus- or SV40 virus-early promoters. In a preferred embodiment, meganuclease genes are operably linked to a promoter that drives gene expression preferentially in the target cell (*e.g.*, a human T cell).

The invention further provides for the introduction of an exogenous nucleic acid into the cell, such that the exogenous nucleic acid sequence is inserted into the TRC alpha constant region gene at a nuclease cleavage site. In some embodiments, the exogenous nucleic acid comprises a 5' homology arm and a 3' homology arm to promote recombination of the nucleic acid sequence into the cell genome at the nuclease cleavage site.

Exogenous nucleic acids of the invention may be introduced into the cell by any of the means previously discussed. In a particular embodiment, exogenous nucleic acids are introduced by way of a viral vector, such as a lentivirus, retrovirus, adenovirus, or preferably a recombinant AAV vector. Recombinant AAV vectors useful for introducing an exogenous nucleic acid can have any serotype that allows for transduction of the virus into the cell and insertion of the exogenous nucleic acid sequence into the cell genome. In particular embodiments, the recombinant AAV vectors have a serotype of AAV2 or AAV6. The recombinant AAV vectors can also be self-complementary such that they do not require second-strand DNA synthesis in the host cell.

In another particular embodiment, an exogenous nucleic acid can be introduced into the cell using a single-stranded DNA template. The single-stranded DNA can comprise the exogenous nucleic acid and, in preferred embodiments, can comprise 5' and 3' homology arms to promote insertion of the nucleic acid sequence into the nuclease cleavage site by homologous recombination. The single-stranded DNA can further comprise a 5' AAV inverted terminal repeat (ITR) sequence 5' upstream of the 5' homology arm, and a 3' AAV ITR sequence 3' downstream of the 3' homology arm.

In another particular embodiment, genes encoding a recombinant meganuclease of the invention and/or an exogenous nucleic acid sequence of the invention can be introduced into the cell by transfection with a linearized DNA template. In some examples, a plasmid DNA encoding a recombinant meganuclease and/or an exogenous nucleic acid sequence can be

digested by one or more restriction enzymes such that the circular plasmid DNA is linearized prior to transfection into the cell.

When delivered to a cell, an exogenous nucleic acid of the invention can be operably linked to any promoter suitable for expression of the encoded polypeptide in the cell, including those mammalian promoters and inducible promoters previously discussed. An exogenous nucleic acid of the invention can also be operably linked to a synthetic promoter. Synthetic promoters can include, without limitation, the JeT promoter (WO 2002/012514).

In examples where the genetically-modified cells of the invention are human T cells, or cells derived therefrom, such cells may require activation prior to introduction of a meganuclease and/or an exogenous nucleic acid sequence. For example, T cells can be contacted with anti-CD3 and anti-CD28 antibodies that are soluble or conjugated to a support (*i.e.*, beads) for a period of time sufficient to activate the cells.

Genetically-modified cells of the invention can be further modified to express one or more inducible suicide genes, the induction of which provokes cell death and allows for selective destruction of the cells *in vitro* or *in vivo*. In some examples, a suicide gene can encode a cytotoxic polypeptide, a polypeptide that has the ability to convert a non-toxic pro-drug into a cytotoxic drug, and/or a polypeptide that activates a cytotoxic gene pathway within the cell. That is, a suicide gene is a nucleic acid that encodes a product that causes cell death by itself or in the presence of other compounds. A representative example of such a suicide gene is one that encodes thymidine kinase of herpes simplex virus. Additional examples are genes that encode thymidine kinase of varicella zoster virus and the bacterial gene cytosine deaminase that can convert 5-fluorocytosine to the highly toxic compound 5-fluorouracil. Suicide genes also include as non-limiting examples genes that encode caspase-9, caspase-8, or cytosine deaminase. In some examples, caspase-9 can be activated using a specific chemical inducer of dimerization (CID). A suicide gene can also encode a polypeptide that is expressed at the surface of the cell that makes the cells sensitive to therapeutic and/or cytotoxic monoclonal antibodies. In further examples, a suicide gene can encode recombinant antigenic polypeptide comprising an antigenic motif recognized by the anti-CD20 mAb Rituximab and an epitope that allows for selection of cells expressing the suicide gene. See, for example, the RQR8 polypeptide described in WO2013153391, which comprises two Rituximab-binding epitopes and a QBEnd10-binding epitope. For such a gene, Rituximab can be administered to a subject to induce cell depletion when needed.

2.4 Pharmaceutical Compositions

In some embodiments, the invention provides a pharmaceutical composition comprising a genetically-modified cell of the invention, or a population of genetically-modified cells of the invention, and a pharmaceutical carrier. Such pharmaceutical compositions can be prepared in accordance with known techniques. See, *e.g.*, Remington, *The Science And Practice of Pharmacy* (21st ed. 2005). In the manufacture of a pharmaceutical formulation according to the invention, cells are typically admixed with a pharmaceutically acceptable carrier and the resulting composition is administered to a subject. The carrier must, of course, be acceptable in the sense of being compatible with any other ingredients in the formulation and must not be deleterious to the subject. In some embodiments, pharmaceutical compositions of the invention can further comprise one or more additional agents useful in the treatment of a disease in the subject. In additional embodiments, where the genetically-modified cell is a genetically-modified human T cell (or a cell derived therefrom), pharmaceutical compositions of the invention can further include biological molecules, such as cytokines (*e.g.*, IL-2, IL-7, IL-15, and/or IL-21), which promote *in vivo* cell proliferation and engraftment. Pharmaceutical compositions comprising genetically-modified cells of the invention can be administered in the same composition as an additional agent or biological molecule or, alternatively, can be co-administered in separate compositions.

Pharmaceutical compositions of the invention can be useful for treating any disease state that can be targeted by T cell adoptive immunotherapy. In a particular embodiment, the pharmaceutical compositions of the invention are useful in the treatment of cancer. Such cancers can include, without limitation, carcinoma, lymphoma, sarcoma, blastomas, leukemia, cancers of B-cell origin, breast cancer, gastric cancer, neuroblastoma, osteosarcoma, lung cancer, melanoma, prostate cancer, colon cancer, renal cell carcinoma, ovarian cancer, rhabdomyo sarcoma, leukemia, and Hodgkin's lymphoma. In certain embodiments, cancers of B-cell origin include, without limitation, B-lineage acute lymphoblastic leukemia, B-cell chronic lymphocytic leukemia, and B-cell non-Hodgkin's lymphoma.

2.5 Methods for Producing Recombinant AAV Vectors

In some embodiments, the invention provides recombinant AAV vectors for use in the methods of the invention. Recombinant AAV vectors are typically produced in mammalian cell lines such as HEK-293. Because the viral *cap* and *rep* genes are removed from the vector to prevent its self-replication to make room for the therapeutic gene(s) to be delivered (e.g. the endonuclease gene), it is necessary to provide these *in trans* in the packaging cell line. In addition, it is necessary to provide the “helper” (e.g. adenoviral) components necessary to support replication (Cots D, Bosch A, Chillon M (2013) *Curr. Gene Ther.* 13(5): 370-81). Frequently, recombinant AAV vectors are produced using a triple-transfection in which a cell line is transfected with a first plasmid encoding the “helper” components, a second plasmid comprising the *cap* and *rep* genes, and a third plasmid comprising the viral ITRs containing the intervening DNA sequence to be packaged into the virus. Viral particles comprising a genome (ITRs and intervening gene(s) of interest) encased in a capsid are then isolated from cells by freeze-thaw cycles, sonication, detergent, or other means known in the art. Particles are then purified using cesium-chloride density gradient centrifugation or affinity chromatography and subsequently delivered to the gene(s) of interest to cells, tissues, or an organism such as a human patient.

Because recombinant AAV particles are typically produced (manufactured) in cells, precautions must be taken in practicing the current invention to ensure that the site-specific endonuclease is NOT expressed in the packaging cells. Because the viral genomes of the invention comprise a recognition sequence for the endonuclease, any endonuclease expressed in the packaging cell line will be capable of cleaving the viral genome before it can be packaged into viral particles. This will result in reduced packaging efficiency and/or the packaging of fragmented genomes. Several approaches can be used to prevent endonuclease expression in the packaging cells, including:

1. The endonuclease can be placed under the control of a tissue-specific promoter that is not active in the packaging cells. For example, if a viral vector is developed for delivery of (an) endonuclease gene(s) to muscle tissue, a muscle-specific promoter can be used. Examples of muscle-specific promoters include C5-12 (Liu, *et al.* (2004) *Hum Gene Ther.* 15:783-92), the muscle-specific creatine kinase (MCK) promoter (Yuasa, *et al.* (2002) *Gene Ther.* 9:1576-88), or the smooth muscle 22 (SM22) promoter (Haase, *et al.* (2013) *BMC Biotechnol.* 13:49-54). Examples of CNS (neuron)-specific promoters include the NSE, Synapsin,

and MeCP2 promoters (Lentz, *et al.* (2012) *Neurobiol Dis.* 48:179-88). Examples of liver-specific promoters include albumin promoters (such as Palb), human α 1-antitrypsin (such as PalAT), and hemopexin (such as Phpx) (Kramer, MG *et al.*, (2003) *Mol. Therapy* 7:375-85). Examples of eye-specific promoters include opsin, and corneal epithelium-specific K12 promoters (Martin KRG, Klein RL, and Quigley HA (2002) *Methods* (28): 267-75) (Tong Y, *et al.*, (2007) *J Gene Med*, 9:956-66). These promoters, or other tissue-specific promoters known in the art, are not highly-active in HEK-293 cells and, thus, will not expected to yield significant levels of endonuclease gene expression in packaging cells when incorporated into viral vectors of the present invention. Similarly, the viral vectors of the present invention contemplate the use of other cell lines with the use of incompatible tissue specific promoters (*i.e.*, the well-known HeLa cell line (human epithelial cell) and using the liver-specific hemopexin promoter). Other examples of tissue specific promoters include: synovial sarcomas PDZD4 (cerebellum), C6 (liver), ASB5 (muscle), PPP1R12B (heart), SLC5A12 (kidney), cholesterol regulation APOM (liver), ADPRHL1 (heart), and monogenic malformation syndromes TP73L (muscle). (Jacox E, *et al.*, (2010) *PLoS One* v.5(8):e12274).

2. Alternatively, the vector can be packaged in cells from a different species in which the endonuclease is not likely to be expressed. For example, viral particles can be produced in microbial, insect, or plant cells using mammalian promoters, such as the well-known cytomegalovirus- or SV40 virus-early promoters, which are not active in the non-mammalian packaging cells. In a preferred embodiment, viral particles are produced in insect cells using the baculovirus system as described by Gao, *et al.* (Gao, H., *et al.* (2007) *J. Biotechnol.* 131(2):138-43). An endonuclease under the control of a mammalian promoter is unlikely to be expressed in these cells (Airenne, KJ, *et al.* (2013) *Mol. Ther.* 21(4):739-49). Moreover, insect cells utilize different mRNA splicing motifs than mammalian cells. Thus, it is possible to incorporate a mammalian intron, such as the human growth hormone (HGH) intron or the SV40 large T antigen intron, into the coding sequence of an endonuclease. Because these introns are not spliced efficiently from pre-mRNA transcripts in insect cells, insect cells will not express a functional endonuclease and will package the full-length genome. In contrast,

mammalian cells to which the resulting recombinant AAV particles are delivered will properly splice the pre-mRNA and will express functional endonuclease protein. Haifeng Chen has reported the use of the HGH and SV40 large T antigen introns to attenuate expression of the toxic proteins barnase and diphtheria toxin fragment A in insect packaging cells, enabling the production of recombinant AAV vectors carrying these toxin genes (Chen, H (2012) *Mol Ther Nucleic Acids*. 1(11): e57).

3. The endonuclease gene can be operably linked to an inducible promoter such that a small-molecule inducer is required for endonuclease expression. Examples of inducible promoters include the Tet-On system (Clontech; Chen H., *et al.*, (2015) *BMC Biotechnol.* 15(1):4)) and the RheoSwitch system (Intrexon; Sowa G., *et al.*, (2011) *Spine*, 36(10): E623-8). Both systems, as well as similar systems known in the art, rely on ligand-inducible transcription factors (variants of the Tet Repressor and Ecdysone receptor, respectively) that activate transcription in response to a small-molecule activator (Doxycycline or Ecdysone, respectively). Practicing the current invention using such ligand-inducible transcription activators includes: 1) placing the endonuclease gene under the control of a promoter that responds to the corresponding transcription factor, the endonuclease gene having (a) binding site(s) for the transcription factor; and 2) including the gene encoding the transcription factor in the packaged viral genome. The latter step is necessary because the endonuclease will not be expressed in the target cells or tissues following recombinant AAV delivery if the transcription activator is not also provided to the same cells. The transcription activator then induces endonuclease gene expression only in cells or tissues that are treated with the cognate small-molecule activator. This approach is advantageous because it enables endonuclease gene expression to be regulated in a spatio-temporal manner by selecting when and to which tissues the small-molecule inducer is delivered. However, the requirement to include the inducer in the viral genome, which has significantly limited carrying capacity, creates a drawback to this approach.
4. In another preferred embodiment, recombinant AAV particles are produced in a mammalian cell line that expresses a transcription repressor that prevents expression of the endonuclease. Transcription repressors are known in the art and include the Tet-Repressor, the Lac-Repressor, the Cro repressor, and the Lambda-

repressor. Many nuclear hormone receptors such as the ecdysone receptor also act as transcription repressors in the absence of their cognate hormone ligand. To practice the current invention, packaging cells are transfected/transduced with a vector encoding a transcription repressor and the endonuclease gene in the viral genome (packaging vector) is operably linked to a promoter that is modified to comprise binding sites for the repressor such that the repressor silences the promoter. The gene encoding the transcription repressor can be placed in a variety of positions. It can be encoded on a separate vector; it can be incorporated into the packaging vector outside of the ITR sequences; it can be incorporated into the cap/rep vector or the adenoviral helper vector; or, most preferably, it can be stably integrated into the genome of the packaging cell such that it is expressed constitutively. Methods to modify common mammalian promoters to incorporate transcription repressor sites are known in the art. For example, Chang and Roninson modified the strong, constitutive CMV and RSV promoters to comprise operators for the Lac repressor and showed that gene expression from the modified promoters was greatly attenuated in cells expressing the repressor (Chang BD, and Roninson IB (1996) *Gene* 183:137-42). The use of a non-human transcription repressor ensures that transcription of the endonuclease gene will be repressed only in the packaging cells expressing the repressor and not in target cells or tissues transduced with the resulting recombinant AAV vector.

2.6 Recombinant Meganuclease Variants

Embodiments of the invention encompass the recombinant meganucleases described herein, and variants thereof. Further embodiments of the invention encompass isolated polynucleotides comprising a nucleic acid sequence encoding the recombinant meganucleases described herein, and variants of such polynucleotides.

As used herein, “variants” is intended to mean substantially similar sequences. A “variant” polypeptide is intended to mean a polypeptide derived from the “native” polypeptide by deletion or addition of one or more amino acids at one or more internal sites in the native protein and/or substitution of one or more amino acids at one or more sites in the native polypeptide. As used herein, a “native” polynucleotide or polypeptide comprises a parental sequence from which variants are derived. Variant polypeptides encompassed by the embodiments are biologically active. That is, they continue to possess the desired biological

activity of the native protein; *i.e.*, the ability to recognize and cleave recognition sequences found in the human T cell receptor alpha constant region (SEQ ID NO:1), including the TRC 1-2 recognition sequence (SEQ ID NO:3), the TRC 3-4 recognition sequence (SEQ ID NO:4), and the TRC 7-8 recognition sequence (SEQ ID NO:5). Such variants may result, for example, from human manipulation. Biologically active variants of a native polypeptide of the embodiments (*e.g.*, SEQ ID NOs:8-32), or biologically active variants of the recognition half-site binding subunits described herein (*e.g.*, SEQ ID NOs:33-82), will have at least about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99%, sequence identity to the amino acid sequence of the native polypeptide or native subunit, as determined by sequence alignment programs and parameters described elsewhere herein. A biologically active variant of a polypeptide or subunit of the embodiments may differ from that polypeptide or subunit by as few as about 1-40 amino acid residues, as few as about 1-20, as few as about 1-10, as few as about 5, as few as 4, 3, 2, or even 1 amino acid residue.

The polypeptides of the embodiments may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Methods for such manipulations are generally known in the art. For example, amino acid sequence variants can be prepared by mutations in the DNA. Methods for mutagenesis and polynucleotide alterations are well known in the art. See, for example, Kunkel (1985) *Proc. Natl. Acad. Sci. USA* 82:488-492; Kunkel *et al.* (1987) *Methods in Enzymol.* 154:367-382; U.S. Pat. No. 4,873,192; Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York) and the references cited therein. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the protein of interest may be found in the model of Dayhoff *et al.* (1978) *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.), herein incorporated by reference. Conservative substitutions, such as exchanging one amino acid with another having similar properties, may be optimal.

A substantial number of amino acid modifications to the DNA recognition domain of the wild-type I-CreI meganuclease have previously been identified (*e.g.*, U.S. 8,021,867) which, singly or in combination, result in recombinant meganucleases with specificities altered at individual bases within the DNA recognition sequence half-site, such that the resulting rationally-designed meganucleases have half-site specificities different from the

wild-type enzyme. Table 4 provides potential substitutions that can be made in a recombinant meganuclease monomer or subunit to enhance specificity based on the base present at each half-site position (-1 through -9) of a recognition half-site.

Table 4.

	Favored Sense-Strand Base										
Posn.	A	C	G	T	A/T	A/C	A/G	C/T	G/T	A/G/T	A/C/G/T
-1	Y75 L75* C75* Y139* C46* A46*	R70* H75* R75* H46* K46* R46*	K70 E70* E75* E46* D46*	Q70* C70 L70 Y75* Q75* H75* H139 Q46* H46*				T46*			G70 A70 S70 G46*
-2	Q70 T44* A44* V44* I44* L44* N44*	E70 D70 K44* R44*	H70 D44* E44*	Q44*	C44*						
-3	Q68 C24* I24*	E68 F68 K24* R24*	R68	M68 C68 L68 F68		H68		Y68	K68		
-4	A26* Q77	E77 K26*	R77 E26*					S77 Q26*			S26*
-5		E42	R42			K28*	C28* Q42				M66 K66
-6	Q40 C28*	E40 R28*	R40	C40 I40 V40 C79 I79 V79 Q28*	A40 A79 A28* H28*						S40 S28*
-7	N30* Q38	E38 K30* R30*	K38 R38 E30*	I38 L38			C38				H38 N38 Q30*
-8	F33 Y33	E33 D33	F33 H33	L33 V33 I33 F33 C33		R32*	R33				
-9		E32	R32	L32				D32			S32

	Favored Sense-Strand Base									
			K32	V32 A32 C32				I32		N32 H32 Q32 T32

For polynucleotides, a “variant” comprises a deletion and/or addition of one or more nucleotides at one or more sites within the native polynucleotide. One of skill in the art will recognize that variants of the nucleic acids of the embodiments will be constructed such that the open reading frame is maintained. For polynucleotides, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of one of the polypeptides of the embodiments. Variant polynucleotides include synthetically derived polynucleotides, such as those generated, for example, by using site-directed mutagenesis but that still encode a recombinant meganuclease of the embodiments.

Generally, variants of a particular polynucleotide of the embodiments will have at least about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99% or more sequence identity to that particular polynucleotide as determined by sequence alignment programs and parameters described elsewhere herein. Variants of a particular polynucleotide of the embodiments (*i.e.*, the reference polynucleotide) can also be evaluated by comparison of the percent sequence identity between the polypeptide encoded by a variant polynucleotide and the polypeptide encoded by the reference polynucleotide.

The deletions, insertions, and substitutions of the protein sequences encompassed herein are not expected to produce radical changes in the characteristics of the polypeptide. However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by screening the polypeptide for its ability to preferentially recognize and cleave recognition sequences found within the human T cell receptor alpha constant region gene (SEQ ID NO:1).

EXAMPLES

This invention is further illustrated by the following examples, which should not be construed as limiting. Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are intended to be encompassed in the scope of the claims that follow the examples below.

EXAMPLE 1

Characterization of Meganucleases That Recognize and Cleave TRC Recognition Sequences

1. Meganucleases that recognize and cleave the TRC 1-2 recognition sequence

Recombinant meganucleases (SEQ ID NOs:8-27), collectively referred to herein as “TRC 1-2 meganucleases,” were engineered to recognize and cleave the TRC 1-2 recognition sequence (SEQ ID NO:3), which is present in the human T cell receptor alpha constant region. Each TRC 1-2 recombinant meganuclease comprises an N-terminal nuclease-localization signal derived from SV40, a first meganuclease subunit, a linker sequence, and a second meganuclease subunit. A first subunit in each TRC 1-2 meganuclease binds to the TRC1 recognition half-site of SEQ ID NO:3, while a second subunit binds to the TRC2 recognition half-site (see, Figure 1A).

As illustrated in Figures 2 and 3, TRC1-binding subunits and TRC2-binding subunits each comprise a 56 base pair hypervariable region, referred to as HVR1 and HVR2, respectively. TRC1-binding subunits are identical outside of the HVR1 region except at position 80 or position 271 (comprising a Q or E residue), and are highly conserved within the HVR1 region. Similarly, TRC2-binding subunits are also identical outside of the HVR2 region except at position 80 or position 271 (comprising a Q or E residue), and at position 330 of meganucleases TRC 1-2x.87 EE, TRC 1-2x.87 QE, TRC 1-2x.87 EQ, TRC 1-2x.87, and TRC 1-2x.163, which comprise an R residue (shaded grey and underlined). Like the HVR1 region, the HVR2 region is also highly conserved.

The TRC1-binding regions of SEQ ID NOs:8-27 are illustrated in Figure 2 and are provided as SEQ ID NOs:33-52, respectively. Each of SEQ ID NOs:33-52 share at least 90% sequence identity to SEQ ID NO:33, which is the TRC1-binding region of the meganuclease TRC 1-2x.87 EE (SEQ ID NO:8). TRC2-binding regions of SEQ ID NOs:8-27 are illustrated in Figure 3 and are provided as SEQ ID NOs:58-77, respectively. Each of SEQ ID NOs:58-

77 share at least 90% sequence identity to SEQ ID NO:58, which is the TRC2-binding region of the meganuclease TRC 1-2x.87 EE (SEQ ID NO:8).

2. Meganucleases that recognize and cleave the TRC 3-4 recognition sequence

5 Recombinant meganucleases (SEQ ID NOs:28 and 29), collectively referred to herein as “TRC 3-4 meganucleases,” were engineered to recognize and cleave the TRC 3-4 recognition sequence (SEQ ID NO:4), which is present in the human T cell receptor alpha constant region. Each TRC 3-4 recombinant meganuclease comprises an N-terminal nuclease-localization signal derived from SV40, a first meganuclease subunit, a linker
10 sequence, and a second meganuclease subunit. A first subunit in each TRC 3-4 meganuclease binds to the TRC3 recognition half-site of SEQ ID NO:4, while a second subunit binds to the TRC4 recognition half-site (see, Figure 1A).

As illustrated in Figures 4 and 5, TRC3-binding subunits and TRC4-binding subunits each comprise a 56 base pair hypervariable region, referred to as HVR1 and HVR2,
15 respectively. TRC3-binding subunits are identical outside of the HVR1 region except at position 80 or position 271 (comprising a Q or E residue), and are highly conserved within the HVR1 region. Similarly, TRC4-binding subunits are also identical outside of the HVR2 region except at position 80 or position 271 (comprising a Q or E residue), and are highly conserved within the HVR2 region.

20 The TRC3-binding regions of SEQ ID NOs:28 and 29 are illustrated in Figure 4 and are provided as SEQ ID NOs:53 and 54, respectively. SEQ ID NOs:53 and 54 share 96.6% sequence identity. TRC4-binding regions of SEQ ID NOs:28 and 29 are illustrated in Figure 5 and are provided as SEQ ID NOs:78 and 79, respectively. SEQ ID NOs:78 and 79 also share 96.6% sequence identity.

25

3. Meganucleases that recognize and cleave the TRC 7-8 recognition sequence

Recombinant meganucleases (SEQ ID NOs:30-32), collectively referred to herein as “TRC 7-8 meganucleases,” were engineered to recognize and cleave the TRC 7-8 recognition sequence (SEQ ID NO:5), which is present in the human T cell receptor alpha constant region. Each TRC 7-8 recombinant meganuclease comprises an N-terminal nuclease-localization signal derived from SV40, a first meganuclease subunit, a linker sequence, and a second meganuclease subunit. A first subunit in each TRC 7-8 meganuclease binds to the TRC7 recognition half-site of SEQ ID NO:5, while a second subunit binds to the TRC8 recognition half-site (see, Figure 1A).

As illustrated in Figures 6 and 7, TRC7-binding subunits and TRC8-binding subunits each comprise a 56 base pair hypervariable region, referred to as HVR1 and HVR2, respectively. TRC7-binding subunits are identical outside of the HVR1 region except at position 80 or position 271 (comprising a Q or E residue), and are highly conserved within the HVR1 region. Similarly, TRC8-binding subunits are also identical outside of the HVR2 region except at position 80 or position 271 (comprising a Q or E residue), and are highly conserved within the HVR2 region.

The TRC7-binding regions of SEQ ID NOs:30-32 are illustrated in Figure 6 and are provided as SEQ ID NOs:55-57, respectively. Each of SEQ ID NOs:55-57 share at least 90% sequence identity to SEQ ID NO:55, which is the TRC7-binding region of the meganuclease TRC 7-8x.7 (SEQ ID NO:30). TRC8-binding regions of SEQ ID NOs:30-32 are illustrated in Figure 7 and are provided as SEQ ID NOs:80-82, respectively. Each of SEQ ID NOs:80-82 share at least 90% sequence identity to SEQ ID NO:80, which is the TRC8-binding region of the meganuclease TRC 7-8x.7 (SEQ ID NO:30).

4. Cleavage of human T cell receptor alpha constant region recognition sequences in a CHO cell reporter assay

To determine whether TRC 1-2, TRC 3-4, and TRC 7-8 meganucleases could recognize and cleave their respective recognition sequences (SEQ ID NOs:3, 4, and 5, respectively), each recombinant meganuclease was evaluated using the CHO cell reporter assay previously described (see, WO/2012/167192 and Figure 8). To perform the assays, CHO cell reporter lines were produced which carried a non-functional Green Fluorescent Protein (GFP) gene expression cassette integrated into the genome of the cells. The GFP gene in each cell line was interrupted by a pair of recognition sequences such that

intracellular cleavage of either recognition sequence by a meganuclease would stimulate a homologous recombination event resulting in a functional GFP gene.

In CHO reporter cell lines developed for this study, one recognition sequence inserted into the GFP gene was the TRC 1-2 recognition sequence (SEQ ID NO:3), the TRC 3-4 recognition sequence (SEQ ID NO:4), or the TRC 7-8 recognition sequence (SEQ ID NO:5). The second recognition sequence inserted into the GFP gene was a CHO-23/24 recognition sequence, which is recognized and cleaved by a control meganuclease called “CHO-23/24”. CHO reporter cells comprising the TRC 1-2 recognition sequence and the CHO-23/24 recognition sequence are referred to herein as “TRC 1-2 cells.” CHO reporter cells comprising the TRC 3-4 recognition sequence and the CHO-23/24 recognition sequence are referred to herein as “TRC 3-4 cells.” CHO reporter cells comprising the TRC 7-8 recognition sequence and the CHO-23/24 recognition sequence are referred to herein as “TRC 7-8 cells.”

CHO reporter cells were transfected with plasmid DNA encoding their corresponding recombinant meganucleases (*e.g.*, TRC 1-2 cells were transfected with plasmid DNA encoding TRC 1-2 meganucleases) or encoding the CHO-23/34 meganuclease. In each assay, 4×10^5 CHO reporter cells were transfected with 50 ng of plasmid DNA in a 96-well plate using Lipofectamine 2000 (ThermoFisher) according to the manufacturer’s instructions. At 48 hours post-transfection, cells were evaluated by flow cytometry to determine the percentage of GFP-positive cells compared to an untransfected negative control (TRC 1-2bs). As shown in Figure 9, all TRC 1-2, TRC 3-4, and TRC 7-8 meganucleases were found to produce GFP-positive cells in cell lines comprising their corresponding recognition sequence at frequencies significantly exceeding the negative control.

The efficacy of the TRC 1-2x.87 QE, TRC 1-2x.87 EQ, and TRC 1-2x.87 EE meganucleases was also determined in a time-dependent manner. In this study, TRC 1-2 cells (1×10^6) were electroporated with 1×10^6 copies of meganuclease mRNA per cell using a BioRad Gene Pulser Xcell according to the manufacturer’s instructions. At 1, 4, 6, 8, and 12 days post-transfection, cells were evaluated by flow cytometry to determine the percentage of GFP-positive cells. As shown in Figure 10, each TRC 1-2 meganuclease exhibited high efficiency at 2 days post-transfection, with greater than 50% GFP-positive cells observed. This effect persisted over the 12 day period, with no evidence of cell toxicity observed.

5. Conclusions

These studies demonstrated that TRC 1-2 meganucleases, TRC 3-4 meganucleases, and TRC 7-8 meganucleases encompassed by the invention can efficiently target and cleave their respective recognition sequences in cells.

5

EXAMPLE 2

Cleavage of TRC Recognition Sequences in T Cells and Suppression of Cell-Surface T Cell Receptor Expression

10 1. Cleavage of the TRC 1-2 recognition sequence in Jurkat Cells

This study demonstrated that TRC 1-2 meganucleases encompassed by the invention could cleave the TRC 1-2 recognition sequence in Jurkat cells (an immortalized human T lymphocyte cell line). $1e^6$ Jurkat cells were electroporated with $8e^6$ copies of a given TRC 1-2 meganuclease mRNA per cell using a BioRad Gene Pulser Xcell according to the
15 manufacturer's instructions. At 72 hours post-transfection, genomic DNA (gDNA) was harvested from cells and a T7 endonuclease I (T7E) assay was performed to estimate genetic modification at the endogenous TRC 1-2 recognition sequence (Figure 11). In the T7E assay, the TRC 1-2 locus is amplified by PCR using primers that flank the TRC 1-2 recognition sequence. If there are indels (random insertions or deletions) within the TRC 1-2 locus, the
20 resulting PCR product will consist of a mix of wild-type alleles and mutant alleles. The PCR product is denatured and allowed to slowly reanneal. Slow reannealing allows for the formation of heteroduplexes consisting of wild-type and mutant alleles, resulting in mismatched bases and/or bulges. The T7E1 enzyme cleaves at mismatch sites, resulting in cleavage products that can be visualized by gel electrophoresis. Figure 11 clearly
25 demonstrates that thirteen different versions of the TRC 1-2 meganucleases generated positive results in the T7E1 assay, indicating effective generation of indels at the endogenous TRC 1-2 recognition sequence.

To further examine the cleavage properties of TRC 1-2 meganucleases, a dose-response experiment was performed in Jurkat cells. $1e^6$ Jurkat cells were electroporated with
30 either 3 μ g or 1 μ g of a given TRC 1-2 meganuclease mRNA per cell using a BioRad Gene Pulser Xcell according to the manufacturer's instructions. At 96-hours post-transfection, gDNA was harvested and the T7E1 assay was performed as described above. As seen in Figure 12, fifteen different TRC 1-2 meganucleases showed cleavage at the endogenous TRC

1-2 recognition site, including three different versions of the TRC 1-2x.87 meganuclease. TRC 1-2x.87 EE worked especially well, generating a strong signal in the T7E1 assay with little to no toxicity in Jurkat cells.

5 2. Cleavage of TRC 1-2 recognition sequence in human T cells

 This study demonstrated that TRC 1-2 meganucleases encompassed by the invention could cleave the TRC 1-2 recognition sequence in human T cells obtained from a donor. CD3+ T cells were stimulated with anti-CD3 and anti-CD28 antibodies for 3 days, then electroporated with mRNA encoding the TRC 1-2x.87 EE meganuclease using the Amaxa
10 4D-Nucleofector (Lonza) according to the manufacturer's instructions. At 3 days and 7 days post-transfection, gDNA was harvested and the T7E1 assay was performed as described above. Figure 13A demonstrates that TRC 1-2x.87 EE effectively introduced mutations in the endogenous TRC 1-2 recognition sequence in human T cells, indicating that the meganuclease recognized and cleaved the TRC 1-2 recognition sequence. The intensity of
15 cleavage products does not appear to change between day 3 and day 7 post-transfection, suggesting little or no toxicity due to the TRC 1-2x.87 EE meganuclease. To determine whether the mutations at the endogenous TRC 1-2 recognition sequence were sufficient to eliminate surface expression of the T cell receptor, cells were analyzed by flow cytometry using an anti-CD3 antibody. Figure 13B shows that approximately 50% of transfected T
20 cells stained negative for CD3, indicating knockout of the T cell receptor. The CD3 negative population did not change significantly between day 3 and day 7 post-transfection, further indicating little or no toxicity associated with the TRC 1-2x.87 EE meganuclease, or the loss of T cell receptor expression.

 To verify that loss of CD3 expression was due to mutations in the TRC 1-2
25 recognition site, gDNA was harvested from transfected T cells and the TRC 1-2 recognition site locus was amplified by PCR. PCR products were cloned into the pCR-blunt vector using the Zero Blunt PCR cloning kit (Thermo Fisher) according to the manufacturer's instructions. Individual colonies were picked and mini-prepped plasmids were sequenced. Figure 14
shows sequences of several representative deletions that were observed at the TRC 1-2
30 recognition sequence. The observed sequences are typical of deletions resulting from the non-homologous end joining repair of DNA double-strand breaks generated by endonucleases.

In addition to TRC 1-2x.87 EE, other TRC 1-2 meganucleases were able to knockout the T cell receptor in human T cells, including TRC 1-2x.55, and TRC 1-2x.72, albeit to a lesser extent than knockout previously observed for TRC 1-2x.87 EE (Tables 5 and 6). TRC 1-2x.72 Q47E carries a mutation in the active site of the meganuclease (amino acid 47) and serves as a negative control.

Table 5.

Meganuclease	% CD3 ⁺ Cells	
	Day 3	Day 6
TRC 1-2x.72 Q47E	0.38	1.1
TRC 1-2x.55	3.11	10.84

Table 6.

Meganuclease	% CD3 ⁺ Cells	
	Day 3	Day 5
TRC 1-2x.72 Q47E	0.29	0.4
TRC 1-2x.72	2.09	4.19

3. Conclusions

These studies demonstrated that TRC 1-2 meganucleases encompassed by the invention can recognize and cleave the TRC 1-2 recognition sequence in both Jurkat cells (an immortalized T lymphocyte cell line) and in T cells obtained from a human donor. Further, these studies demonstrated that NHEJ occurs at the meganuclease cleavage site, as evidenced by the appearance of indels. Moreover, TRC 1-2 meganucleases were shown to reduce cell-surface expression of the T cell receptor on human T cells obtained from a donor.

EXAMPLE 3

Recombinant AAV Vectors For Introducing Exogenous Nucleic Acids into Human T Cells

1. Recombinant AAV vectors

In the present study, two recombinant AAV vectors (referred to as AAV405 and AAV406) were designed to introduce an exogenous nucleic acid sequence, comprising an EagI restriction site, into the genome of human T cells at the TRC 1-2 recognition sequence via homologous recombination. Each recombinant AAV vector was prepared using a triple-transfection protocol, wherein a cell line is transfected with a first plasmid encoding “helper”

components (*e.g.*, adenoviral) necessary to support replication, a second plasmid comprising the *cap* and *rep* genes, and a third plasmid comprising the viral inverted terminal repeats (ITRs) containing the intervening DNA sequence to be packaged into the virus (*e.g.*, the exogenous nucleic acid sequence) (see, Cots D, Bosch A, Chillon M (2013) *Curr. Gene Ther.* 13(5): 370-81). Figure 15 illustrates the general approach for using recombinant AAV vectors to introduce an exogenous nucleic acid sequence into the cell genome at the nuclease cleavage site.

AAV405 was prepared using the plasmid illustrated in Figure 16 (SEQ ID NO:107). As shown, the AAV405 plasmid generally comprises sequences for a 5' ITR, a CMV enhancer and promoter sequence, a 5' homology arm, a nucleic acid sequence comprising the EagI restriction site, an SV40 poly(A) signal sequence, a 3' homology arm, and a 3' ITR. AAV406 was prepared using the plasmid illustrated in Figure 17 (SEQ ID NO:108). As shown, the AAV406 plasmid comprises similar sequences to those of AAV405, but lacks the CMV enhancer and promoter sequences upstream of the 5' homology arm. The present AAV studies further included the use of an AAV vector encoding GFP (GFP-AAV), which was incorporated as a positive control for AAV transduction efficiency.

2. Introducing exogenous nucleic acid sequences into the TRC 1-2 recognition sequence

To test whether AAV templates would be suitable for homology directed repair (HDR) following generation of a double-strand break with TRC 1-2 meganucleases, a series of experiments were performed using human T cells. In the first experiment, the timing of electroporation with TRC 1-2 RNA and transduction with recombinant AAV vectors was determined. Human CD3⁺ T cells were stimulated with anti-CD3 and anti-CD28 antibodies for 3 days, then electroporated with mRNA encoding the TRC 1-2x.87 EE meganuclease (1 μ g) using the Amaxa 4D-Nucleofector (Lonza) according to the manufacturer's instructions. At either 2, 4, or 8 hours post-transfection, cells were transduced with GFP-AAV (1e⁵ viral genomes per cell). Cells were analyzed by flow cytometry for GFP expression at 72 hours post-transduction. As shown in Figure 18, the highest transduction efficiency was observed when cells were transduced at 2 hours post-transfection (88% GFP-positive cells). Transduction efficiency decreased significantly as the time between transfection and transduction increased, with 78% GFP-positive cells at 4 hours and 65% GFP-positive cells at 8 hours.

Having determined that efficient viral transduction occurred when cells were transduced 2 hours post-transfection, the AAV405 and AAV406 vectors were used as HDR templates in human T cells. CD3⁺ T cells were stimulated and transfected with 1 µg TRC 1-2x.87 EE mRNA as described above. At 2 hours post-transfection, cells were either

5 transduced with AAV405 or AAV406 (1e⁵ viral genomes per cell). As transduction-only controls, cells were mock transfected (with water) and transduced with either AAV405 or AAV406 (1e⁵ viral genomes per cell). For a meganuclease-only control, cells were transfected with TRC 1-2x.87 EE and then mock transduced (with water) at 2 hours post-transfection.

10 To determine whether the AAV vectors served as HDR templates, gDNA was harvested from cells and the TRC 1-2 locus was amplified by PCR using primers that recognized sequences beyond the region of homology in the AAV vectors. PCR primers outside of the homology regions only allowed for amplification of the T cell genome, not from the AAV vectors. PCR products were purified and digested with EagI. Figure 19

15 shows cleavage of the PCR products amplified from cells that were transfected with TRC 1-2x.87 EE and transduced with either AAV vector (see arrows), indicating insertion of the EagI site into the TRC 1-2 recognition sequence. The PCR products from all of the control cell populations are not cleaved by EagI, demonstrating that the insertion of the EagI site requires creation of a DNA double-strand break by a TRC 1-2 meganuclease.

20 To further define the insertion of the EagI site into human T cells, individual products from the bulk PCR product were examined. Undigested PCR product generated from the above experiment was cloned into the pCR-blunt vector using the Zero Blunt PCR cloning kit (Thermo Fisher) according to the manufacturer's instructions. Colony PCR was performed using M13 forward and reverse primers (pCR blunt contains M13 forward and reverse

25 priming sites flanking the insert) and a portion of PCR products from cells transfected with TRC 1-2x.87 EE and either AAV405 or AAV406 were analyzed by gel electrophoresis (Figures 20A and 21A, respectively). In both cases, there are a mix of full-length PCR products (approximately 1600 bp), smaller inserts, and some empty plasmids (approximately 300 bp). In this assay, bands smaller than full-length but larger than empty plasmids are

30 often times sequences containing large deletions within the TRC 1-2 recognition sequence. In parallel, another portion of PCR products were digested with EagI to determine the percent of clones that contain the EagI recognition site inserted into the TRC 1-2 recognition sequence. Figures 20B and 21B show that several PCR products were cleaved with EagI

(e.g., Figure 20B, second row, 6 lanes from the left), generating the expected fragments of approximately 700 and 800 bp. These gels allow for the estimation of EagI insertion to be approximately 25% and 6% for AAV405 and AAV406, respectively (adjusted for empty vectors).

5 To confirm observations from gel electrophoresis of uncut PCR products and digest with EagI, the remaining portion of each PCR product was sequenced. Figure 22A shows sequences of several representative deletions and insertions that were observed at the TRC 1-2 recognition sequence. These sequences are typical of sequences resulting from the non-homologous end joining repair of DNA double-strand breaks generated by endonucleases.

10 All PCR products that were cleaved with EagI contained an EagI site inserted into the TRC 1-2 recognition sequence (Figure 22B).

3. Enhanced AAV transduction efficiency

In light of the observation that AAV transduction was more efficient when it was

15 carried out 2 hours post-transfection than when it was carried out later, an experiment was performed to optimize the timing of transfection and transduction. Human CD3⁺ T cells were stimulated with anti-CD3 and anti-CD28 antibodies for 3 days, then electroporated with the TRC 1-2x.87 EE meganuclease (1 μ g) using the Amaxa 4D-Nucleofector (Lonza) according to the manufacturer's instructions. Immediately after transfection or 2 hours post-

20 transfection, cells were transduced with GFP-AAV (1e⁵ viral genomes per cell). Additionally, non-stimulated cells were transduced with GFP-AAV (1e⁵ viral genomes per cell). At 72 hours post-transduction, cells were analyzed by flow cytometry for GFP expression. Figure 23 shows that GFP-AAV transduction performed 2 hours post-transfection resulted in 90% GFP-positive cells, but that transduction immediately after transfection resulted in 98% GFP-

25 positive cells. Resting T cells appeared refractive to AAV transduction, with approximately 0% GFP-positive cells. Non-transduced cells also showed approximately 0% GFP-positive cells.

4. Summary

30 These studies demonstrate that AAV vectors can be used in conjunction with recombinant meganucleases to incorporate an exogenous nucleic acid sequence into a cleavage site in the TCR alpha constant region via homologous recombination.

EXAMPLE 4

Recombinant AAV Vectors For Introducing Exogenous Nucleic Acids Encoding a Chimeric Antigen Receptor in Human T Cells5 1. Recombinant AAV vectors

In the present study, two recombinant AAV vectors (referred to as AAV-CAR100 and AAV-CAR763) were designed to introduce an exogenous nucleic acid sequence, encoding a chimeric antigen receptor, into the genome of human T cells at the TRC 1-2 recognition sequence via homologous recombination. Each recombinant AAV vector was prepared using
10 the triple-transfection protocol described previously.

AAV-CAR100 (also referred to herein as AAV408) was prepared using the plasmid illustrated in Figure 24 (SEQ ID NO:109). As shown, the AAV-CAR100 (AAV408) is designed for producing a self-complementary AAV vector, and generally comprises sequences for a 5' ITR, a 5' homology arm, a nucleic acid sequence encoding an anti-CD19
15 chimeric antigen receptor, an SV40 poly(A) signal sequence, a 3' homology arm, and a 3' ITR. AAV-CAR763 (also referred to herein as AAV412) was prepared using the plasmid illustrated in Figure 25 (SEQ ID NO:110). As shown, the AAV-CAR763 (AAV412) plasmid generally comprises the same sequences as AAV-CAR100 (AAV408), but is designed for producing a single-stranded AAV vector. Because a single-stranded AAV vector can
20 accommodate a larger payload, the 5' homology arm and the 3' homology arm are longer in AAV-CAR763 (AAV412) than in AAV-CAR100 (AAV408). The present AAV studies will further include the use of an AAV vector encoding GFP (GFP-AAV), which will be incorporated as a positive control for AAV transduction efficiency.

25 2. Introducing a chimeric antigen receptor sequence into the TRC 1-2 recognition sequence

Studies will be conducted to determine the efficiency of using recombinant AAV vectors to insert a chimeric antigen receptor sequence into the TCR alpha constant region gene while, simultaneously, knocking out cell-surface expression of the endogenous TCR
30 receptor.

To confirm transduction efficiency, human CD3+ T cells will be obtained and stimulated with anti-CD3 and anti-CD28 antibodies for 3 days, then electroporated with mRNA encoding the TRC 1-2x.87 EE meganuclease (1µg) using the Amaxa 4D-

Nucleofector (Lonza) according to the manufacturer's instructions. Cells will be transduced with GFP-AAV ($1e^5$ viral genomes per cell) immediately after transfection as described above. Cells will be analyzed by flow cytometry for GFP expression at 72 hours post-transduction to determine transduction efficiency.

5 AAV-CAR100 (AAV408) and AAV-CAR763 (AAV412) vectors will then be used as HDR templates in human T cells for the insertion of the anti-CD19 chimeric antigen receptor sequence. Human CD3⁺ T cells will be stimulated and transfected with 1 μ g TRC 1-2x.87 EE mRNA as described above. Cells will then be transduced with AAV-CAR100 (AAV408) or AAV-CAR763 (AAV412) ($1e^5$ viral genomes per cell) either immediately after
10 transfection or within 0-8 hours of transfection. As transduction-only controls, cells will be mock transfected (with water) and transduced with either AAV-CAR100 (AAV408) or AAV-CAR763 (AAV412) ($1e^5$ viral genomes per cell). For a meganuclease-only control, cells will be transfected with mRNA encoding TRC 1-2x.87 EE and then mock transduced (with water) immediately post-transfection.

15 Insertion of the chimeric antigen receptor sequence will be confirmed by sequencing of the cleavage site in the TCR alpha constant region gene. Cell-surface expression of the chimeric antigen receptor will be confirmed by flow cytometry, using an anti-Fab or anti-CD19 antibody. Knockout of the endogenous T cell receptor at the cell surface will be determined by flow cytometry as previously described.

20

EXAMPLE 5

Insertion and Expression of Chimeric Antigen Receptor

1. Insertion of chimeric antigen receptor sequence into the TRC 1-2 recognition 25 sequence

In the present study, we test whether AAV can provide HDR templates that can be used to insert a chimeric antigen receptor sequence into the TCR alpha constant region gene and, simultaneously, knock out cell-surface expression of the endogenous TCR receptor. In the first experiment, human CD3⁺ T cells ($1e^6$ cells) were stimulated and electroporated with
30 mRNA encoding the TRC 1-2x.87 EE meganuclease (2 μ g) as described above, then immediately transduced with AAV412 ($1e^5$ viral genomes/cell). As controls, cells were mock electroporated, then transduced with AAV412 or electroporated with mRNA encoding

TRC 1-2x.87EE, then mock transduced. An additional control of mock electroporated, mock transduced cells was included.

A PCR-based assay was developed to determine whether the AAV HDR template was utilized to repair double-strand breaks at the TRC 1-2 recognition sequence. Three sets of primer pairs were used for PCR analysis. The first set was designed to amplify a region with the homology arms of AAV412. Since this first primer set (referred to as “Inside homolog arms/CAR region” in Table 7) lies within the homology region, it will either amplify the unmodified TRC 1-2 recognition sequence locus of the genome (349 bp), the AAV412 vector input (2603 bp), or the TRC 1-2 recognition sequence into which the CAR gene has been inserted (2603 bp). The second primer set (referred to as “Outside 5’ homology arm” in Table 7) includes one primer that anneals within the CAR region of the AAV412 HDR template, one primer that anneals in the human genome, outside of the 5’ homology arm of the AAV412 HDR template and will amplify an 1872 bp fragment only if the CAR gene was successfully inserted into the TRC 1-2 recognition sequence. The third primer set (referred to as “Outside 3’ homology arm” in Table 7) includes one primer that anneals within the CAR region of the AAV412 HDR template, and one primer that anneals in the human genome, outside of the 3’ homology arm of the AAV412 HDR template. Similarly to the second primer set, the third primer set will amplify an 1107 bp fragment only if the CAR gene was successfully inserted into the TRC 1-2 recognition sequence. Taken together, PCR products from all three primer sets will indicate whether the CAR sequence is present in cells (primer set 1), and whether it has been inserted into the TRC 1-2 recognition sequence (primer sets 2 and 3).

On day 4 post-transduction cells were analyzed using the PCR primer pairs described above. Briefly, approximately 3,000 cells were harvested, pelleted, and lysed and PCR was performed to determine whether the CAR gene was inserted into the TRC 1-2 recognition sequence. PCR products were resolved on an agarose gel, shown in Figure 26 (lane descriptions can be found in Table 7). Lanes 1-3 are PCR products from the sample that was electroporated with mRNA encoding TRC 1-2x.87EE and mock transduced.

As expected, the first primer pair (“Inside homolog arms/CAR region”) amplified the unmodified TRC 1-2 recognition sequence locus, generating a 349 bp band shown in lane 1. Lanes 2 and 3 correspond to primer pairs that only generate a product if the CAR gene has been inserted into the TRC 1-2 recognition sequence, and do not show products. Lanes 7-9 represent samples that were mock electroporated and mock transduced and show the same

bands as the TRC 1-2x.87EE mRNA only control described above. Lanes 4-6 show PCR products from the sample that was electroporated with TRC 1-2x.87EE mRNA and transduced with AAV412. Lane 4 shows two bands generated by the first primer pair (“Inside homolog arms/CAR region”), indicating amplification of the unmodified TRC 1-2 recognition sequence locus of the genome (349 bp) and the AAV412 vector input (2603 bp) or the TRC 1-2 recognition sequence into which the CAR gene has been inserted (2603 bp). Lanes 5 and 6 show products generated by the primer pairs that only amplify products if the CAR nucleic acid sequence has been inserted into the TRC 1-2x.87EE recognition site. Both bands are the predicted size (1872 and 1107 bp, respectively). Lanes 10-12 represent the sample that was mock electroporated and transduced with AAV412. Lane 10 shows two bands generated by the first primer pair (“Inside homolog arms/CAR region”), indicating amplification of the unmodified TRC 1-2 recognition sequence locus of the genome (349 bp) and the AAV412 vector input (2603 bp). Lanes 11 and 12 correspond to primer pairs that only generate a product if the CAR gene has been inserted into the TRC 1-2 recognition sequence, and do not show products. The absence of bands in lanes 11 and 12 (which include primers outside of the homology arm) indicates that the 2603 bp band in lane 10 was generated from amplification of the AAV412 input.

Taken together, the PCR analysis clearly demonstrates that CAR genes are introduced into the TRC 1-2x.87EE recognition site when both TRC 1-2x.87EE mRNA and AAV412 are present in cells. Thus, we conclude that AAV412 serves to produce suitable HDR templates that can be used to insert a CAR gene into the TRC 1-2x.87EE recognition sequence.

In a second experiment, human CD3+ T cells were stimulated and electroporated with mRNA encoding the TRC 1-2x.87 EE meganuclease as described above, then immediately transduced with increasing amounts of AAV408 (0 μ L, 3.125 μ L, 6.25 μ L, 12.5 μ L, or 25 μ L, which corresponds to approximately 0, 3.125×10^3 , 6.25×10^3 , 1.25×10^4 and 2.5×10^4 viral genomes/cell). As controls, cells were mock electroporated, then transduced with increasing amounts of AAV408. Additional controls included cells that were mock electroporated and mock transduced, as well as cells that were electroporated with TRC 1-2x.87EE mRNA then mock transduced. On day 4 post-transduction, cells were harvested and analyzed as described above, but only using the primer pairs that amplified a product only if the CAR gene has been inserted into the TRC 1-2 recognition sequence. PCR products were resolved on agarose gels, shown in Figure 27. Figure 27A shows the PCR products generated using

the primer pair described above (“Outside 5’ homology arm”) which only amplifies a product on the 5’ end of the TRC 1-2 recognition sequence locus if the CAR gene has been inserted into that locus. Figure 27B shows the PCR products generated using the primer pair described above (“Outside 3’ homology arm”) which only amplifies a product on the 3’ end of the TRC 1-2 recognition sequence locus if the CAR gene has been inserted into that locus. Lane descriptions can be found in Table 8. Lanes 1-5 in both Figure 27A and 27B represent the samples that were either mock electroporated or mock electroporated then mock transduced. No PCR products are visible in mock electroporated cells, indicating the HDR templates produced by AAV408 are unable to insert the CAR gene into the TRC 1-2 recognition sequence in the absence of TRC 1-2x.87EE mRNA. Lane 6 represents the sample that was electroporated with TRC 1-2x.87EE mRNA and mock transduced. No PCR products are visible, indicating that the CAR gene had not been inserted into the TRC 1-2 recognition sequence. Lanes 7-10 represent samples that were electroporated with TRC 1-2x.87EE mRNA and transduced with increasing amounts of AAV408. The appropriately sized bands for each PCR are evident, indicating that AAV408 can produce HDR donors for repair of the TRC 1-2 recognition sequence, resulting in insertion of the CAR gene.

Table 7.

Sample	Nucleofection	Virus	PCR	Product Size
		(100k MOI)		
1	TRC1-2x87EE	---	Inside homolog arms/CAR region	Genomic = 349 bp + CD19 = 2603 bp
2	TRC1-2x87EE	---	Outside 5’ homology arm	1872 bp
3	TRC1-2x87EE	---	Outside 3’ homology arm	1107 bp
4	TRC1-2x87EE	AAV412	Inside homolog arms/CAR region	Genomic = 349 bp + CD19 = 2603 bp
5	TRC1-2x87EE	AAV412	Outside 5’ homology arm	1872 bp
6	TRC1-2x87EE	AAV412	Outside 3’ homology arm	1107 bp
7	Mock (Water)	---	Inside homolog arms/CAR region	Genomic = 349 bp + CD19 = 2603 bp
8	Mock (Water)	---	Outside 5’ homology arm	1872 bp
9	Mock (Water)	---	Outside 3’ homology arm	1107 bp
10	Mock (Water)	AAV412	Inside homolog arms/CAR region	Genomic = 349 bp + CD19 = 2603 bp
11	Mock (Water)	AAV412	Outside 5’ homology arm	1872 bp
12	Mock (Water)	AAV412	Outside 3’ homology arm	1107 bp

Table 8.

Sample	Nucleofection	Virus (AAV408)
		μL
1	Mock (Water)	0
2	Mock (Water)	3.125
3	Mock (Water)	6.25
4	Mock (Water)	12.5
5	Mock (Water)	25
6	TRC1-2x87EE	0
7	TRC1-2x87EE	3.125
8	TRC1-2x87EE	6.25
9	TRC1-2x87EE	12.5
10	TRC1-2x87EE	25

The PCR-based assays described above are useful in determining whether the CAR gene had been inserted into the TRC 1-2 recognition sequence, but do not give information on efficiency. To determine the efficiency of CAR insertion, we developed a digital PCR-based assay (schematic shown in Figure 28A). In this assay, two primer sets are used. The first set amplifies an irrelevant gene sequence and serves a reference sequence to control for template number. The second set consists of one primer that anneals within the CAR gene and one primer that anneals outside of the 3' homology arm, such that a product is only amplified if the CAR gene has been inserted into the TRC 1-2 recognition sequence. A VIC-labeled probe anneals within the amplicon generated from the first primer set and FAM-labeled probe anneals within the amplicon generated by the second set of primers. By dividing the number of amplicons detected by the FAM-labeled probe to the number of reference sequence amplicons detected by the VIC-labeled probe, it is possible to accurately quantitate the percent of TRC 1-2 recognition sequence loci that were modified by insertion of the CAR gene.

Figure 28B shows the results of the digital PCR assay for samples that were either mock electroporated then transduced, electroporated with TRC 1-2x.87EE mRNA then mock transduced, or electroporated with TRC 1-2x.87EE mRNA then transduced with increasing amounts of AAV408. Digital PCR was performed using genomic DNA isolated from cells approximately 1 week post-transduction. Consistent with the observations from the PCR described in Figure 27, both control samples (transduction only or electroporation only) were found to have 0% CAR gene inserted into the TRC 1-2x.87EE recognition sequence. Samples that were electroporated with mRNA encoding TRC 1-2x.87EE then transduced

with increasing amounts of AAV408 were found to have between approximately 1.5% and 7%. The assay was performed on two different instruments (labeled QX200 and QS3D) and showed remarkable agreement, demonstrating the sensitivity and precision of this digital PCR-based assay.

5

2. Expression of anti-CD19 chimeric antigen receptor on T cells

In addition to determining whether CAR insertion occurred at the molecular level, we sought to determine the expression level of the anti-CD19 chimeric antigen receptor in cells that had the CAR gene inserted into the TRC 1-2 recognition sequence using AAV408 as the HDR template. Additionally, we examined the efficiency in which insertion of the CAR into the TRC 1-2x.87EE recognition sequence resulted in knockout of the T cell receptor. Samples described above and analyzed in Figures 27 and 28 were also analyzed for CAR and CD3 expression by flow cytometry. Approximately 4 days post-transduction, cells were labeled with antibodies that recognize the anti-CD19 CAR (anti-Fab-Alexa647) or CD3 (CD3⁺BB515) and analyzed by flow cytometry. Figure 29A shows flow cytometry plots, with anti-CAR labeling shown on the Y axis and anti-CD3 labeling shown on the X axis. Cells that were mock electroporated and mock transduced (MOI-0) were overwhelmingly CD3⁺/CAR⁻ (the lower right quadrant, 98.7%). Cells that were mock electroporated then transduced with increasing amounts of AAV408 looked essentially identical to the control cells, with the CD3⁺/CAR⁻ populations at 98.8%, 99, 99%, and 99.1%. Thus we conclude that the AAV408 virus alone is not driving detectable levels of CAR expression, nor is it capable of disrupting expression of the T cell receptor.

Figure 29B shows flow cytometry plots for samples that were either electroporated with mRNA encoding TRC 1-2x.87EE then mock transduced or cells that were electroporated with TRC 1-2x.87EE then transduced with increasing amounts of AAV408. Cells that were electroporated then mock transduced show 47.1% CD3⁻ cells, indicating efficient knockout of the T cell receptor complex. Background labeling with the anti-CD19 CAR was very low, with 0.6% in the CD3⁻ population and 0.78% in the CD3⁺ population. Samples that were electroporated with mRNA encoding TRC 1-2x.87EE then transduced with increasing amounts of AAV408 showed CAR labeling in the CD3⁻ population, ranging from 2.09% to 5.9%. There was also a slight increase in CAR labeling in the CD3⁺ population, ranging from 1.08% to 1.91%. We did not determine the cause of the increase in CAR⁺ cells in the CD3⁺ population, although it is possible that the CAR was inserted into the

non-expressed T cell receptor allele (only one allele of the T cell receptor alpha chain is expressed and incorporated into the T cell receptor complex).

These data correlated well with the quantitative digital PCR-based assay described above. For example, at the highest MOI of AAV408 (2.5×10^4 viral genomes/cell), the digital PCR assay showed approximately 6% CAR insertion, and the flow cytometry assay showed 5.9% CAR⁺/CD3⁺ cells. If one takes into account the CAR⁺/CD3⁺ population, the data are still quite comparable, with the flow cytometry assay showing approximately 7.8% CAR⁺ compared to 6% by digital PCR.

EXAMPLE 6

Characterization of Additional AAV Vectors

1. Insertion of a chimeric antigen receptor sequence into the TRC 1-2 recognition sequence

Having shown that AAV vectors could provide suitable HDR templates to insert CAR genes into the TRC 1-2x.87EE recognition sequence, we sought to optimize the configuration of the AAV vector. We generated a vector that could be used to produce self-complementary AAV genomes that included the CAR gene expression cassette driven by a JeT promoter, flanked by short regions of homology to the TRC 1-2 recognition sequence locus and AAV ITRs. This vector is referred to as AAV421 (Figure 30; SEQ ID NO:123). Short homology arms were necessary due to limited packaging capacity of self-complementary AAV. Additionally, we generated a vector that could be used to produce single-strand AAV genomes that includes the CAR gene expression cassette driven by a CMV promoter, flanked by long homology arms and AAV ITRs. This vector is referred to as AAV422 (Figure 31; SEQ ID NO:124). Since single-strand AAV genomes have a larger cargo capacity, we were able to utilize longer homology arms than in the self-complementary vector.

To test whether AAV421 and AAV422 were useful to target insertion of the CAR gene into the TRC 1-2 recognition sequence, several experiments similar to those described above were carried out in human CD3⁺ T cells. In a first experiment, human CD3⁺ T cells (1×10^6 cells) were either mock electroporated then transduced with increasing amounts of AAV421 or 422, or electroporated with TRC 1-2x.87EE mRNA (2 μ g) then transduced with increasing amounts of AAV421 or AAV422. AAV422 MOIs were significantly higher than AAV421 in this experiment than in the experiments described above (approximate MOIs

were 1.25×10^4 , 2.5×10^4 , 5×10^4 and 1×10^5 viral genomes/cell) because earlier experiments with AAV408 suggested that higher MOIs would result in more efficient CAR insertion. The AAV421 virus stock was not concentrated enough to allow for titers significantly higher than in the experiments described earlier. As controls, cells were electroporated (mock or with TRC 1-2x.87EE mRNA) then mock transduced. As an additional component to this experiment, a “large scale” condition was performed, in which 10×10^6 cells (10 times more than a typical experiment) were electroporated with TRC 1-2x.87EE mRNA then transduced with AAV422 (2.5×10^4 viral genomes/cell). Lastly, we also tested a second virus stock of AAV421 to compare to the primary virus stock.

Insertion of the CAR was determined by PCR as described above, using primer pairs that only amplify products if the CAR gene has been inserted into the TRC 1-2x.87EE recognition sequence. PCR was resolved by agarose gel, shown in Figure 32A and 32B (lane descriptions can be found in Tables 9 and 10). Sample 1 in Figure 32A was mock electroporated then mock transduced, and samples 2-5 were mock electroporated then transduced with AAV421. The gel shows that none of these samples generated PCR products, indicating that AAV421, in the absence of TRC 1-2x.87EE mRNA, is unable to drive insertion of the CAR gene into the TRC 1-2 recognition sequence. Additionally, the control sample that was electroporated with TRC 1-2x.87EE mRNA then mock transduced (sample 6), did not show any PCR products. Samples 7-10 in Figure 32A were electroporated with TRC 1-2x.87EE mRNA, then transduced with increasing amounts of AAV421. The gel shows PCR bands for products extending beyond both the 5' and 3' homology arm (the two bands under each sample number), demonstrating integration of the CAR gene into the TRC 1-2 recognition sequence. Lastly in Figure 32A, lanes 11 and 12 represent samples that were electroporated with TRC 1-2x.87EE mRNA then transduced with AAV422, either starting with 1×10^6 or 10×10^6 cells/sample, respectively. The presence of both PCR bands (larger in the first set, because different primer was used to account for a longer homology arm) indicate successful insertion of the CAR gene into the TRC 1-2 recognition sequence.

Sample 1 in Figure 32B was mock electroporated then mock transduced, and samples 2-5 were mock electroporated then transduced with increasing amounts of AAV422 (Table 10). The gel shows that none of these samples generated PCR products, indicating that AAV422, in the absence of TRC 1-2x.87EE mRNA, is unable to drive insertion of the CAR gene into the TRC 1-2 recognition sequence. Samples 7-10 in Figure 32B were

electroporated with TRC 1-2x.87EE mRNA, then transduced with increasing amounts of AAV422. The gel shows PCR bands for products extending beyond both the 5' and 3' homology arm, demonstrating integration of the CAR gene into the TRC 1-2 recognition sequence. Lastly, sample 11 represents the sample that was electroporated with TRC 1-2x.87EE mRNA then transduced with a AAV421 from a different virus stock than samples shown in Figure 32A. The presence of bands indicate insertion of the CAR gene into the TRC 1-2 recognition sequence and confirms reproducibility between different virus stocks. Taken together, Figure 32 clearly demonstrates that both AAV421 and AAV422 are capable of generating HDR templates suitable for inserting the CAR gene into the TRC 1-2 recognition sequence.

Table 9.

Sample	Nucleofection	AAV	μl	MOI (approximate)
		Virus	AAV	
1	Mock (Water)	421	0	0
2	Mock (Water)	421	3.125	3906
3	Mock (Water)	421	6.25	7813
4	Mock (Water)	421	12.5	15625
5	Mock (Water)	421	25	31250
6	TRC1-2x87EE	421	0	0
7	TRC1-2x87EE	421	3.125	3906
8	TRC1-2x87EE	421	6.25	7813
9	TRC1-2x87EE	421	12.5	15625
10	TRC1-2x87EE	421	25	31250
11	TRC1-2x87EE	422	6.25	25000
12	TRC1-2x87EE	422	62.5	25000
	Large Scale			

Table 10.

Sample	Nucleofection	AAV	μ l	MOI
		Virus	AAV	(approximate)
1	Mock (Water)	422	0	0
2	Mock (Water)	422	3.125	12500
3	Mock (Water)	422	6.25	25000
4	Mock (Water)	422	12.5	50000
5	Mock (Water)	422	25	100000
6	TRC1-2x87EE	422	0	0
7	TRC1-2x87EE	422	3.125	12500
8	TRC1-2x87EE	422	6.25	25000
9	TRC1-2x87EE	422	12.5	50000
10	TRC1-2x87EE	422	25	100000
11	TRC1-2x87EE	421B	25	10000

2. Expression of anti-CD19 chimeric antigen receptor on T cells using AAV421

Here, we sought to determine the expression level of the anti-CD19 chimeric antigen receptor in cells that had the CAR gene inserted into the TRC 1-2 recognition sequence using AAV421. Samples described above and analyzed in Figures 32A were also analyzed for CAR and CD3 expression by flow cytometry. Approximately 4 days post-transduction, cells were labeled with antibodies that recognize the anti-CD19 CAR or CD3 and analyzed by flow cytometry. Figure 33A shows flow cytometry plots for cells that were mock electroporated and transduced with AAV421, along with control cells that were mock electroporated and mock transduced. Cells that were mock electroporated and mock transduced (MOI-0) were overwhelmingly CD3⁺/CAR⁻ (the lower right quadrant, 98.8%). Cells that were mock electroporated then transduced with increasing amounts of AAV421 looked essentially identical to the control cells, with the CD3⁺/CAR⁻ populations at 98.8%, 98.6%, 98.8% and 97.9%. Thus, we conclude that the AAV421 virus alone is not driving detectable levels of CAR expression, nor is it capable of disrupting expression of the T cell receptor.

Figure 33B shows flow cytometry plots for samples that were either electroporated with TRC 1-2x.87EE mRNA then mock transduced or cells that were electroporated with TRC 1-2x.87EE then transduced with increasing amounts of AAV421. Cells that were electroporated then mock transduced show 56.7% CD3⁻ cells, indicating efficient knockout of the T cell receptor complex. Background labeling with the anti-CD19 CAR was very low, with 0.48% in the CD3⁻ population and 0.36% in the CD3⁺ population. Samples that were

electroporated with mRNA encoding TRC 1-2x.87EE then transduced with increasing amounts of AAV412 showed significant amounts of CAR labeling in the CD3⁻ population, ranging from 4.99% to 13.4%. There was also a slight increase in CAR labeling in the CD3⁺ population, ranging from 1.27% to 3.95%. As mentioned above, it is possible that the CAR gene was inserted into the non-expressed T cell receptor allele. Also in contrast to experiments with AAV408, the CAR⁺ population was much better defined, with a higher mean fluorescence intensity, suggesting that the JeT promoter drives higher expression than the eF1 α core promoter.

While evaluating insertion of the CAR gene using AAV421 in conjunction with TRC 1-x.87EE, we sought to determine a method that would allow us to preferentially expand and enrich the CD3⁻/CAR⁺ population. From the experiment described above and shown in Figure 33, we used cells that were electroporated with TRC 1-2x.87EE mRNA (2 μ g) then transduced with AAV421 (3.13e⁴ viral genomes/cell). Control samples were mock electroporated and mock transduced, mock electroporated and transduced with AAV421, or electroporated with TRC 1-2x.87EE and mock transduced taken from the experiment described above and shown in Figure 33. As a control enrichment and expansion process, these cells were incubated for 6 days in complete growth medium supplemented with IL-7 and IL-15 (both at 10 ng/mL). Cells were then labeled with antibodies against the anti-CD19 CAR and CD3 and analyzed by flow cytometry (Figure 34A). Cells that were mock electroporated and mock transduced showed low levels of background staining in the CD3⁻/CAR⁺ quadrant (0.13%). The CD3⁻/CAR⁺ population was essentially the same in samples that were either mock electroporated then transduced with AAV or electroporated with TRC 1-2x.87EE mRNA then mock transduced (0.16% and 0.55%, respectively). Cells that were electroporated with TRC 1-2x.87EE mRNA and mock transduced had a CD3⁻/CAR⁻ population of 53.2%, very close to the amount stained in the first part of this experiment shown in Figure 33B (56.7%). Cells that were electroporated with TRC 1-2x.87EE and transduced with AAV showed 12.6% CD3⁻/CAR⁺ cells, almost identical to the original labeling of these cells shown in Figure 33 (13.4%), demonstrating that mixture of IL-7 and IL-15 is insufficient to enrich or expand the specific CD3⁻/CAR⁺ cell population.

We next sought to enrich for the CD3⁻/CAR⁺ population in an antigen-specific manner by incubating the 4 samples described above with IM-9 cells, which present CD19 on the cell surface. IM-9 cells were inactivated by pre-treatment with mitomycin C and incubated with samples at a 1:1 ratio for 6 days in the presence of IL-7 and IL-15 (10ng/mL).

Cells were then labeled with antibodies against CD3 and the anti-CD19 CAR and analyzed by flow cytometry (Figure 34B). Cells that were mock electroporated and mock transduced showed low levels of background staining in the CD3⁺/CAR⁺ quadrant (0.2%). The CD3⁺/CAR⁺ population was the same in samples that were mock electroporated then transduced with AAV (0.2%) and slightly higher in cells that were electroporated with TRC 1-2x.87EE and mock transduced (1.24%). The increase in CD3⁺/CAR⁺ cells the TRC 1-2x.87EE alone control is considered background since no CAR nucleic acid was ever introduced into the system. Cells that were electroporated with TRC 1-2x.87EE mRNA and mock transduced had a CD3⁺/CAR⁺ population of 42.5%, which is significantly lower than they were prior to expansion (56.7%, Figure 33) suggesting that CD⁺ cells may have a growth advantage in this system. However, cells that were electroporated with TRC 1-2x.87EE and transduced with AAV showed 49.9% CD3⁺/CAR⁺ cells, a dramatic increase compared to the original labeling of these cells shown in Figure 33 (13.4%), demonstrating that incubation of this sample with IM-9 cells in the presence of IL-7 and IL-15 is quite effective in enriching and expanding the CD3⁺/CAR⁺ population. The CD3⁺/CAR⁺ population was also expanded under these conditions, with the mock electroporated/AAV transduced sample and the TRC 1-2x.87EE electroporated/AV transduced sample showing 2.53% and 15.3% CD3⁺/CAR⁺, respectively.

In the cells that were electroporated with TRC 1-2x.87EE then transduced with AAV421, 24.2% of the CD3⁺ population was CAR⁺ prior to expansion (Figure 33B). After incubation in medium supplemented with IL-7 and IL-15, that 25.3% of the CD3⁺ cells were CAR⁺ (Figure 34A) indicating that the ratio of gene knock-in to gene-knockout was unchanged. However, after incubation with IM-9 cells in addition to IL-7 and IL-15, over 80% (80.35%, Figure 34B) of the CD3⁺ cells were CAR⁺, demonstrating that incubation with IM-9 cells resulted in antigen-specific enrichment.

Since mitocmyin C inactivates cells very potently and IM-9 cells were not persisting long in the mixed culture, we reasoned that a second infusion of IM-9 cells might further increase enrichment of CD3⁺/CAR⁺ cells. Some of the cells described above and shown in Figure 34B would mixed with fresh IM-9 cells (pre-treated with mitocmycin C) in medium containing IL-7 and IL-15 and were incubated another 6 days. Cells were then stained for CD3 and anti-CD19 CAR and analyzed by flow cytometry (Figure 34C). The percentage of CD3⁺/CAR⁺ cells in any of the control samples were essentially unchanged compared to the first round of enrichment on IM-9 cells.

However, the cells that were electroporated with TRC 1-2x.87EE and transduced with AAV421 showed a significant enrichment of the CD3⁺/CAR⁺ population, increasing from 49.9% (after the first round of incubation with IM-9 cells, Figure 34B) to 65.7% (Figure 34C). Importantly, 93.75% of the CD3⁺ population was CAR⁺, indicating further antigen-specific expansion.

3. Expression of anti-CD19 chimeric antigen receptor on T cells using AAV422

We also examined expression of the anti-CD19 CAR from cells in which AAV422 was used to provide the HDR template (described above, PCR results shown in Figure 32B). Approximately 4 days post-transduction, cells were labeled with antibodies that recognize the anti-CD19 CAR or CD3 and analyzed by flow cytometry. Figure 35A shows flow cytometry plots for cells that were mock electroporated and transduced with increasing amounts of AAV422, along with control cells that were mock electroporated and mock transduced. Cells that were mock electroporated and mock transduced (MOI-0) were overwhelmingly CD3⁺/CAR⁻ (the lower right quadrant, 98.8%). Cells that were mock electroporated then transduced with increasing amounts of AAV422 looked essentially identical to the control cells, with the CD3⁺/CAR⁻ populations at 98.6%, 98.6%, 98.9% and 98.4%. Thus, the AAV422 vector alone is not driving detectable levels of CAR expression, nor is it capable of disrupting expression of the T cell receptor.

Figure 35B shows flow cytometry plots for samples that were either electroporated with TRC 1-2x.87EE mRNA then mock transduced or cells that were electroporated with TRC 1-2x.87EE then transduced with increasing amounts of AAV422. Cells that were electroporated then mock transduced show 59.3% CD3⁻ cells, indicating efficient knockout of the T cell receptor complex. Background labeling with the anti-CD19 CAR was very low, with 1.47% in the CD3⁻ population and 0.52% in the CD3⁺ population. Samples that were electroporated with mRNA encoding TRC 1-2x.87EE then transduced with increasing amounts of AAV422 showed significant amounts of CAR labeling in the CD3⁻ population, ranging from 14.7% to 20.3%. There was also a slight increase in CAR labeling in the CD3⁺ population, ranging from 2.3% to 2.7%.

Surprisingly, we observed a noticeable increase in T cell receptor knockout efficiency in the presence of AAV422. Overall CD3 knockout efficiency with increasing AAV422 was 71.6%, 74.9%, 77.8% and 74.4% compared to 59.3% in the TRC 1-2x.87EE electroporation alone. In contrast, overall CD3 knockout efficiency with increasing AAV421 was 56.99%,

56.62%, 57.4% and 55.4% compared to 57.18% in the TRC 1-2x.87EE electroporation alone (Figure 33B). Thus, it appears that electroporation with TRC 1-2x.87EE in the presence of single-stranded AAV genomes, but not self-complimentary AAV genomes, results in an increase in the overall knockout efficiency of the TRC 1-2x.87EE nuclease. Because of this increase, the percent of CD3⁺ cells that are CAR⁺ is not significantly different between cells transduced with AAV421 and AAV422 despite the higher numbers of CD3⁺/CAR⁺ cells. The highest percent of CD3⁺ cells that were CAR⁺ using AAV421 was 24.18% (MOI = 3.13e⁴ viral genomes/cell) compared to 26.48% with AAV422 (MOI = 1e⁵ viral genomes/cell). This observation is particularly interesting considering the large difference in MOI between AAV421 and AAV422.

The concept of utilizing IM-9 cells to specifically enrich for CD3⁺/CAR⁺ cells was tested using cells from this experiment. Again, rather than testing the entire panel, we only attempted enrichment of either cells mock electroporated then transduced with AAV422 or electroporated with TRC 1-2x.87EE then transduced with AAV422 (2.5e⁴ viral genomes/cell) in a new experiment. Figure 36A shows flow cytometry plots at approximately day 4 post-transduction. Mock electroporated/transduced cells showed background staining of CD3⁺/CAR⁺ cells at 0.13%. In comparison, cells electroporated with TRC 1-2x.87EE the transduced with AAV422 showed 4.44% CD3⁺/CAR⁺ cells. Cells were incubated with IM-9 cells (pre-treated with mitomycin) in the presence of IL-7 and IL-15 for 6 days as described above, then analyzed by flow cytometry. Figure 36B shows that incubation with IM-9 cells dramatically increased the CD3⁺/CAR⁺ population in AAV422 transduced cells to 35.8%. The CAR⁺ cells make up 45.2% of the total CD3⁺ population, compared to 6.69% prior to enrichment (Figure 36A). As above, we also further enriched by a second addition of IM-9 cells (Figure 36C). Two rounds of incubation with IM-9 cells resulted in 65.1% CD3⁺/CAR⁺ cells. The CAR⁺ cells make up 78.25% of the total CD3⁺ population, indicating significant, antigen-dependent enrichment of CD3⁺/CAR⁺ cells.

These data, in conjunction with the data presented above, clearly demonstrate that cells that have had an anti-CD19 CAR gene inserted into the TRC 1-2 recognition sequence can be successfully enriched by incubation with IM-9 cells in the presence of IL-7 and IL-15, and can result in a CD3⁺ population that is over 90% CAR⁺ in as little as 12 days of culture.

4. Increased knockout efficiency observed when using single-strand AAV vectors

In the present study, we followed up on the observation that single-stranded AAV vectors increased knockout efficiency of the TRC 1-2x.87EE nuclease. In a first experiment, cells were electroporated with TRC 1-2x.87EE (2 μ g) and either mock transduced or transduced with increasing amounts of AAV412 (6.25e⁴, 1.25e⁴, 2.5e⁴ or 5e⁴ viral genomes/cell). On day 4 post-transduction, cells were labeled with an antibody against CD3 and analyzed by flow cytometry (Figure 37A). In the mock transduced cells, 20.7% are CD3⁺ compared to 21.6%, 23.7%, 25.5% and 25% with increasing AAV412, indicating that TRC 1-2x.87EE knockout efficiency is up to 23% higher in the presence of AAV412 (25.5% compared to 20.7%).

To determine whether this increase in knockout efficiency was nuclease specific, in an additional experiment, cells were electroporated with mRNA (2 μ g) encoding a nuclease targeting the β 2-microglobulin gene and either mock transduced or transduced with increasing amounts of AAV412. Cells were stained for β 2-microglobulin on day 4 post-transduction and analyzed by flow cytometry (Figure 37B). In the mock transduced cells, β 2-microglobulin knockout efficiency was 64.5% and increased in the transduced cells to 68.6%, 70.7%, 77.2% and 82.5% with increasing amounts of AAV412, demonstrating an increase in knockout efficiency of up to 27.9% (82.5% compared to 64.5%).

In a parallel experiment, cells were electroporated with TRC 1-2x.87EE mRNA and either mock transduced or transduced with AAV422 (using the same MOIs as AAV412). Cells were labeled with an antibody against CD3 and cells were analyzed by flow cytometry (Figure 37C). The mock transduced cells showed 62.2% T cell receptor knockout, and with increasing amounts of AAV, the T cell receptor knockout frequency increased to 72.6%, 75.5%, 78.3% and 75.1%. Here, the presence of AAV422 increases the knockout efficiency of TRC 1-2x.87EE by up to 25.8% (78.3% compared to 62.2%). It is striking that the increase in percent knockout efficiency is almost identical between these three experiments, using two different nucleases and two different AAV vectors. Taken together, these data strongly indicate that transduction of cells with single strand AAV vectors increase the knockout efficiency of our nucleases, irrespective of nuclease or AAV cargo.

5. Activity of T cells expressing anti-CD19 chimeric antigen receptor

The above experiments clearly demonstrate the generation of CAR T cells by electroporating cells with TRC 1-2x.87EE mRNA, then immediately transducing cells with

AAV421, and that these cells can be enriched for a CD3⁺/CAR⁺ population by co-culture with CD19 expressing IM-9 cells. We next examined the activity of these CAR T cells against target cells. In the first experiment, the cells described above and shown in Figure 34C were used in an IFN-gamma ELISPOT assay, in which either CD19⁺ Raji cells or CD19⁻ U937

5 cells were the target population. As shown in Figure 38A, when anti-CD19 CAR T cells were incubated with U937 cells, they did not secrete IFN-gamma regardless of the target:effector ratio. Incubating CAR T cells with Raji cells, however, resulted in high levels of IFN-gamma secretion, in a dose-dependent manner, indicating that secretion of IFN-gamma is antigen-specific.

10 These CAR T cells were also used in a cell killing assay in which luciferase-labeled Raji cells were the target. Briefly, CAR T cells were incubated with luciferase-labeled Raji cells at a ratio of 10:1. At several time points, cells were washed and lysed to measure luciferase activity as a measure of how many cells remained. Control cells showed luciferase activity greater than 5500 arbitrary units (Figure 38B). Co-incubation for 2, 3, 4 and 5 hours

15 resulted in a decrease in luciferase activity to 4598, 3292, 2750 and 1932 arbitrary units, respectively. Thus, within 5 hours of co-incubation, luciferase activity was reduced approximately 65%, indicating strong cytolytic activity of the CAR T cells.

Taken together, these data demonstrate that anti-CD19 CAR T cells generated according to the methods described herein are effective at killing CD19⁺ cells.

20

EXAMPLE 7

Linearized Plasmid DNA

1. Expression of chimeric antigen receptor from linearized plasmid DNA

25 Since HDR templates produced by AAV are linear DNA molecules, we hypothesized that linear DNA from any source may be a suitable HDR template for inserting a CAR gene into the TRC 1-2 recognition sequence. To test this, we generated several plasmids that contain an anti-CD19 CAR gene flanked by homology arms that are homologous to the TRC 1-2 recognition sequence locus. Different promoters were used in some plasmids, and

30 homology arms were either “short” (200 bp on the 5’ homology arm and 180 bp on the 3’ homology arm) to mimic the self-complimentary AAV vectors, or “long” (985 bp on the 5’ homology arm and 763 bp on the 3’ homology arm) to mimic the single strand AAV vectors. Plasmids with short homology arms are labeled “pDS” and those with long homology arms

are labeled “pDI.” Additionally, some plasmid contained an intron upstream of the CAR gene.

The CAR donor plasmids were linearized at a restriction site in the vector backbone and gel purified. Human CD3⁺ T cells were either electroporated with the linearized CAR donor plasmid alone (varying amounts between 500 ng and 1000 ng, depending on the concentration of the purified linearized plasmid), or co-electroporated with TRC 1-2.87EE mRNA (2 µg). As controls, cells were either mock electroporated or electroporated with TRC 1-2x.87EE alone. The graphs in Figure 39 are labelled with descriptions for all electroporations. Approximately 4 days post-electroporation, cells were labelled with antibodies against CD3 and the anti-CD19 CAR and analyzed by flow cytometry (Figure 39). Figure 39A shows background CD3⁻/CAR⁺ staining of 0.15%. It should be noted that the background CD3⁺/CAR⁺ staining was unusually high at 4.31%. Figure 39B shows cells that were electroporated with TRC 1-2x.87EE mRNA alone, demonstrating 60.8% CD3 knockout. Figures 39C and 39D represent samples that were co-electroporated with TRC 1-2x.87EE mRNA and either the long homology arm vector with an EF1α core promoter with an HTLV enhancer or the short homology arm vector with EF1α core promoter (with no enhancer). Interestingly, the linearized CAR donor with the EF1α core promoter alone generated a CD3⁻/CAR⁺ population of 2.38%, while the vector harboring the EF1α core promoter with the HTLV enhancer did not generate a significant percentage of CD3⁻/CAR⁺ cells. Cells that were electroporated with these two vectors in the absence of TRC 1-2x.87EE mRNA showed no significant increase in the CD3⁻/CAR⁺ population (Figure 39E and 39F). The increase in the CD3⁻/CAR⁺ population with the EF1α core promoter vector in the presence of TRC 1-2x.87EE suggested that a linearized plasmid could serve as an HDR template to repair double strand breaks at the TRC 1-2 recognition sequence.

Figures 39G and 39H show two long homology arm constructs that both contain an MND promoter driving expression of the CAR. One of these constructs, shown in Figure 39G, also contains an intron in the 5' end of the CAR gene. Surprisingly, the long homology arm plasmid with an MND promoter and intron showed significant CAR expression (Figure 39G, 4.14% CD3⁻/CAR⁺) while the intron-less construct (Figure 39H) did not show detectable CAR expression when co-electroporated with TRC 1-2x.87EE mRNA. A short homology arm plasmid with the MND promoter, but with no intron, was also tested with TRC 1-2x.87EE mRNA and did not demonstrate any CAR expression (Figure 39I). None of

the MND promoter-containing constructs generated any CAR⁺ cells in the absence of TRC 1-2x.87EE mRNA (Figures 39J, 39K, and 39L).

Lastly in this experiment, we tested a short homology arm construct that contained a JeT promoter driving expression of the CAR and a “long” homology arm construct with a CMV promoter driving expression of the CAR. Alone, neither of these linearized plasmids resulted in significant CAR⁺ cells (Figure 39O and 39P). When cells were co-electroporated with TRC 1-2x.87EE mRNA, the JeT containing construct showed 2.69% CD3⁺CAR⁺ cells and the CMV containing construct yielded 2.7% CD3⁺/CAR⁺ cells.

The flow plots shown in Figure 39 clearly demonstrate that linearized plasmid DNA that encodes the CAR, flanked by homology arms, can serve as HDR templates to repair DNA breaks caused by TRC 1-2x.87EE, resulting in insertion of the CAR nucleic acid. It is clear that promoter strength plays a significant role in expression of the CAR, and some promoters drive more efficient expression when there is an intron in the gene.

To confirm that insertion of the CAR using linearized DNA constructs was specific to the TRC 1-2 recognition sequence locus, we analyzed cells as described above using primers that sat within the CAR and outside of the homology arms (Figure 40, Table 11). Samples 1 and 2 are PCR products from cells that were either mock electroporated or electroporated with only mRNA encoding TRC 1-2x.87EE. Consistent with results shown above, no PCR bands are present indicating the lack of CAR gene in the TRC 1-2 recognition site. Samples 3, 4 and 5 are from cells that were co-electroporated with TRC 1-2x.87EE and a linearized CAR homology plasmid (samples names in Figure 40). Each sample shows two PCR bands of the predicted size indicating insertion of the CAR gene expression cassette into the TRC 1-2 recognition site. Samples 6, 7, and 8 are from cells that were electroporated with the same linearized CAR homology plasmids as samples 3, 4, and 5 but without TRC 1-2x.87EE mRNA. As expected, no PCR bands are present. Samples 9 and 10 are PCR products from cells that were either mock electroporated or electroporated with only mRNA encoding TRC 1-2x.87EE and show no PCR bands. Samples 11, 12, 13 and 14 are from cells that were co-electroporated with TRC 1-2x.87EE and a linearized CAR homology plasmid (samples names in Figure 40). Each sample shows two PCR bands of the predicted size indicating insertion of the CAR gene into the TRC 1-2 recognition site. Samples 15, 16, 17, and 18 are from cells that were electroporated with the same linearized CAR homology plasmids as samples 11, 12, 13, and 14 but without TRC 1-2x.87EE mRNA. As expected, no PCR bands are present.

Figures 39 and 40 clearly demonstrate that co-electroporating human CD3⁺ T cells with mRNA encoding TRC 1-2x.87EE and a linearized CAR homology plasmid is an effective method to insert the CAR gene into the TRC 1-2 recognition sequence.

5 **Table 11.**

Sample	Nucleofection	Linearized plasmid
1	Mock (Water)	---
2	TRC1-2x87EE	---
3	TRC1-2x87EE	pDS EF1- α Core
4	TRC1-2x87EE	pDS 200 MND NC
5	TRC1-2x87EE	pDS 200 JET NC
6	Mock (Water)	pDS EF1- α Core
7	Mock (Water)	pDS 200 MND NC
8	Mock (Water)	pDS 200 JET NC
9	Mock (Water)	---
10	TRC1-2x87EE	---
11	TRC1-2x87EE	pDI EF1- α NC
12	TRC1-2x87EE	pDI MND intron NC
13	TRC1-2x87EE	pDI MND NC
14	TRC1-2x87EE	pDI CMV 985 NC 763
15	Mock (Water)	pDI EF1- α NC
16	Mock (Water)	pDI MND intron NC
17	Mock (Water)	pDI MND NC
18	Mock (Water)	pDI CMV 985 NC 763
19	Mock (Water)	---
20	TRC1-2x87EE	---
21	TRC1-2x87EE	pDS MCS
22	Mock (Water)	PDS MCS

EXAMPLE 8

Characterization of Additional AAV Vectors

10 1. Use of AAV with JeT promoter and long homology arms

Collectively, the data shown above indicate that vectors utilizing the JeT promoter drive high, consistent expression of the CAR and that longer homology arms may increase gene insertion efficiency. We designed and generated the vector shown in Figure 41 (SEQ

ID NO:125), which was used to make single-strand AAV with long homology arms, and a JeT promoter driving expression of the anti-CD19 CAR (referred to herein as AAV423). Human CD3⁺ T cells were electroporated with mRNA encoding TRC 1-2x.87EE and transduced with increasing amounts of AAV423. Since data shown above suggested that higher MOIs may result in increased insertion efficiency, we used titers ranging from 1.875e⁴ to 1.5e⁵. As controls, cells were either electroporated with mRNA encoding TRC 1-2x.87EE then mock transduced or mock electroporated then transduced with increasing amounts of AAV423. On day 6 post-transduction, cells were labeled with antibodies recognizing CD3 or the anti-CD19 CAR and analyzed by flow cytometry. As shown in Figure 42, cells that were mock electroporated then transduced with increasing amounts of AAV423 are overwhelmingly CD3⁺/CAR⁻ (ranging from 96.6% to 98.5%). Cells that were electroporated with mRNA encoding TRC 1-2x.87EE and mock transduced were 39% CD3⁻ indicating efficient knockout of the T cell receptor. In these cells, background CAR staining was very low (around 2%). Cells that were electroporated with mRNA encoding TRC 1-2x.87EE then transduced with increasing amounts of AAV423 showed dramatic CAR staining in conjunction with CD3 knockout. CD3⁻/CAR⁺ populations ranged from 21.6% to 22.7%, while CD3⁺/CAR⁺ populations were around 2%. As described above, the presence of single-strand AAV increased the overall gene modification efficiency at the TRC 1-2 recognition site, with total CD3⁻ populations increasing from 41.44% in the control cells to 57.6%, 59.2%, 58.7%, and 56.1% in cells that were electroporated then transduced with increasing amounts of AV423. The percent of CD3⁻ cells that were CAR⁺ ranged from 37.5% to 39.9% indicating a dramatic increase in insertion efficiency compared to data described above.

To confirm that insertion of the CAR using AAV423 was specific to the TRC 1-2 recognition sequence locus, we analyzed cells as described above using primers that sat within the CAR and outside of the homology arms (Figure 43, Table 12).

Table 12.

Sample	Nucleofection	AAV (μ l)	MOI
1	Mock (Water)	---	---
2	Mock (Water)	---	---
3	Mock (Water)	pDI JET Prep A (3.125)	18750
4	Mock (Water)	pDI JET Prep A (6.25)	37500
5	Mock (Water)	pDI JET Prep A (12.5)	7500
6	Mock (Water)	pDI JET Prep A (25)	150000
7	TRC1-2x87EE	---	---
8	TRC1-2x87EE	pDI JET Prep A (3.125)	18750
9	TRC1-2x87EE	pDI JET Prep A (6.25)	37500
10	TRC1-2x87EE	pDI JET Prep A (12.5)	7500
11	TRC1-2x87EE	pDI JET Prep A (25)	150000

Samples 1 and 2 are PCR products from cells that were mock electroporated. Consistent with results shown above, no PCR bands are present indicating the lack of CAR gene in the TRC 1-2 recognition site. Samples 3-6 are from cells that were mock electroporated then transduced with increasing amounts of AAV423. Consistent with results above, there are no PCR bands present. Sample 7 is from cells electroporated with mRNA encoding TRC 1-2x.87EE then mock transduced, and shows no PCR bands. Samples 8-11 are from cells electroporated with mRNA encoding TRC 1-2x.87EE then transduced with increasing amounts of AAV423, and show the PCR bands expected if the CAR is inserted into the TRC 1-2 recognition sequence.

Given the ability of AAV423 to insert the CAR sequence into the TRC 1-2 recognition site following cleavage, it is further envisioned that the AAV423 plasmid (Figure 41) could be linearized by digestion with a and delivered to the cell by digestion with one or more restriction enzymes, such that the T cells could be transfected with a linearized DNA template which could integrate into the TRC 1-2 recognition site and encode an anti-CD19 CAR.

EXAMPLE 9

In Vivo Efficacy of Anti-CD19 TCR-Negative CAR T cells

1. Murine model of disseminated B cell lymphoma

The efficacy of the gene-edited anti-CD19 CAR T cells was evaluated in a murine model of disseminated B cell lymphoma. Activated T cells were electroporated with TRC 1-

2x.87 EE mRNA as described above, then transduced with an AAV6 vector comprising an anti-CD19 CAR expression cassette driven by a JeT promoter and flanked by homology arms. Following 5 days of culture with IL-2 (10 ng/mL), cells were analyzed for cell-surface CD3 and anti-CD19 CAR expression by flow cytometry as previously described (Figure 44A). CD3⁻ cells were enriched by depleting CD3⁺ cells using anti-CD3 magnetic beads. Depleted cells were then cultured for 3 days in IL-15 (10 ng/mL) and IL-21 (10 ng/mL) and re-analyzed for cell-surface expression of CD3 and anti-CD19 CAR (Figure 44B). Isolation of the CD3⁻ population was quite efficient, yielding 99.9% purity as measured by flow cytometry following depletion of CD3⁺ cells (Figure 44B). The purified CD3⁻ population comprised 56% CD4⁺ and 44% CD8⁺ cells (Figure 44C), and had primarily central memory/transitional memory phenotypes, determined by staining for CD62L and CD45RO (Figure 44D).

Studies utilizing the Raji disseminated lymphoma model were conducted by Charles River Laboratories International Inc. (Morrisville, NC, USA). CD19⁺ Raji cells stably expressing firefly luciferase (ffLuc)⁴⁴ were injected i.v. into 5-6 week old female NSG mice on Day 1, at a dose of 2.0×10^5 cells per mouse. On Day 4 mice were injected i.v. with PBS or PBS containing gene edited control TCR KO T cells prepared from the same healthy donor PBMC or PBS containing the indicated doses of CAR T cells prepared from the same donor. On the indicated days, live mice were injected i.p. with Luciferin substrate (150mg/kg in saline), anesthetized, and Luciferase activity measured after 7 minutes using IVIS SpectrumCT (Perkin Elmer, Waltham, MA). Data was analyzed and exported using Living Image software 4.5.1 (Perkin Elmer, Waltham, MA). Luminescence signal intensity is represented by radiance in p/sec/cm²/sr.

25 2. Results

As shown in Figure 45, growth of CD19⁺ Raji cells was evident in all mice at low levels by day 8, and increased significantly in untreated and TCR⁻ control groups by day 11. In control groups, significant tumor growth was observed by day 15, and by day 18 or 19 all control groups were euthanized. In contrast, all groups of mice treated with anti-CD19 CAR T cells showed no evidence of tumor growth by day 11 and, with the exception of a single mouse in the low dose group, remained tumor-free through day 29 of the study. Tumor re-growth was observed in three mice in the low dose cohort around day 36. One of the three

died at day 42, though imaging revealed only low levels of tumor in this animal, so it is unlikely that death was tumor-related.

3. Conclusions

- 5 These results provide clear evidence for *in vivo* clearance of CD19⁺ tumor cells by gene-edited CD3⁺ CAR T cells and support further preclinical development of this platform for allogeneic CAR T cell therapy.

AMENDED CLAIMS

received by the International Bureau on 17 February 2017 (17.02.2017)

1. A recombinant meganuclease that recognizes and cleaves a recognition sequence comprising SEQ ID NO:3, wherein said recombinant meganuclease comprises a first subunit and a second subunit, wherein said first subunit binds to a first recognition half-site of said recognition sequence and comprises a first hypervariable (HVR1) region, and wherein said second subunit binds to a second recognition half-site of said recognition sequence and comprises a second hypervariable (HVR2) region, wherein said first subunit comprises an amino acid sequence having at least 80% sequence identity to residues 198-344 of any one of SEQ ID NOs:8-18 or residues 7-153 of any one of SEQ ID NOs:19-27, and wherein said second subunit comprises an amino acid sequence having at least 80% sequence identity to residues 7-153 of any one of SEQ ID NOs:8-18 or residues 198-344 of any one of SEQ ID NOs:19-27.
2. The recombinant meganuclease of claim 1, wherein said HVR1 region comprises Y at a position corresponding to:
 - (a) position 215 of any one of SEQ ID NOs:8-18; or
 - (b) position 24 of any one of SEQ ID NOs:19-27.
3. The recombinant meganuclease of claim 1 or claim 2, wherein said HVR1 region comprises residues 215-270 of any one of SEQ ID NOs:8-18 or residues 24-79 of any one of SEQ ID NOs:19-27.
4. The recombinant meganuclease of any one of claims 1-3, wherein said HVR2 region comprises residues 24-79 of any one of SEQ ID NOs:8-18 or residues 215-270 of any one of SEQ ID NOs:19-27.
5. The recombinant meganuclease of any one of claims 1-4, wherein said first subunit comprises residues 198-344 of any one of SEQ ID NOs:8-18 or residues 7-153 of any one of SEQ ID NOs:19-27.
6. The recombinant meganuclease of any one of claims 1-5, wherein said second subunit comprises residues 7-153 of any one of SEQ ID NOs:8-18 or residues 198-344 of any one of SEQ ID NOs:19-27.

7. The recombinant meganuclease of any one of claims 1-6, wherein said recombinant meganuclease is a single-chain meganuclease comprising a linker, wherein said linker covalently joins said first subunit and said second subunit.
8. The recombinant meganuclease of any one of claims 1-7, wherein said recombinant meganuclease comprises the amino acid sequence of any one of SEQ ID NOs:8-27.
9. A recombinant meganuclease that recognizes and cleaves a recognition sequence comprising SEQ ID NO:4, wherein said recombinant meganuclease comprises a first subunit and a second subunit, wherein said first subunit binds to a first recognition half-site of said recognition sequence and comprises a first hypervariable (HVR1) region, and wherein said second subunit binds to a second recognition half-site of said recognition sequence and comprises a second hypervariable (HVR2) region, wherein said first subunit comprises an amino acid sequence having at least 80% sequence identity to residues 7-153 of SEQ ID NO:28 or 29, and wherein said second subunit comprises an amino acid sequence having at least 80% sequence identity to residues 198-344 of SEQ ID NO:28 or 29.
10. The recombinant meganuclease of claim 9, wherein said HVR1 region comprises Y at a position corresponding to position 24 of SEQ ID NO:28 or 29.
11. The recombinant meganuclease of claim 9 or claim 10, wherein said HVR1 region comprises T at a position corresponding to position 26 of SEQ ID NO:28 or 29.
12. The recombinant meganuclease of any one of claims 9-11, wherein said HVR1 region comprises Y at a position corresponding to position 46 of SEQ ID NO:28 or 29.
13. The recombinant meganuclease of any one of claims 9-12, wherein said HVR2 region comprises H at a position corresponding to position 215 of SEQ ID NO:28 or 29.
14. The recombinant meganuclease of any one of claims 9-13, wherein said HVR2 region comprises T at a position corresponding to position 266 of SEQ ID NO:28 or 29.

15. The recombinant meganuclease of any one of claims 9-14, wherein said HVR2 region comprises C at a position corresponding to position 268 of SEQ ID NO:28 or 29.
16. The recombinant meganuclease of any one of claims 9-15, wherein said HVR1 region comprises residues 24-79 of SEQ ID NO:28 or 29.
17. The recombinant meganuclease of any one of claims 9-16, wherein said HVR2 region comprises residues 215-270 of SEQ ID NO:28 or 29.
18. The recombinant meganuclease of any one of claims 9-17, wherein said first subunit comprises residues 7-153 of SEQ ID NO:28 or 29.
19. The recombinant meganuclease of any one of claims 9-18, wherein said second subunit comprises residues 198-344 of SEQ ID NO:28 or 29.
20. The recombinant meganuclease of any one of claims 9-19, wherein said recombinant meganuclease is a single-chain meganuclease comprising a linker, wherein said linker covalently joins said first subunit and said second subunit.
21. The recombinant meganuclease of any one of claims 9-20, wherein said recombinant meganuclease comprises the amino acid sequence of SEQ ID NO:28 or 29.
22. A recombinant meganuclease that recognizes and cleaves a recognition sequence comprising SEQ ID NO:5, wherein said recombinant meganuclease comprises a first subunit and a second subunit, wherein said first subunit binds to a first recognition half-site of said recognition sequence and comprises a first hypervariable (HVR1) region, and wherein said second subunit binds to a second recognition half-site of said recognition sequence and comprises a second hypervariable (HVR2) region, wherein said first subunit comprises an amino acid sequence having at least 80% sequence identity to residues 7-153 of SEQ ID NO:30 or residues 198-344 of SEQ ID NO:31 or 32, and wherein said second subunit comprises an amino acid sequence having at least 80% sequence identity to residues 198-344 of SEQ ID NO:30 or residues 7-153 of SEQ ID NO:31 or 32.

23. The recombinant meganuclease of claim 22, wherein said HVR1 region comprises Y at a position corresponding to:

- (a) position 24 of SEQ ID NO:30; or
- (b) position 215 of SEQ ID NO:31 or 32

24. The recombinant meganuclease of claim 22 or claim 23, wherein said HVR1 region comprises residues 24-79 of SEQ ID NO:30 or residues 215-270 of SEQ ID NO:31 or 32.

25. The recombinant meganuclease of any one of claims 22-24, wherein said HVR2 region comprises residues 215-270 of SEQ ID NO:30 or residues 24-79 of SEQ ID NO:31 or 32.

26. The recombinant meganuclease of any one of claims 22-25, wherein said first subunit comprises residues 7-153 of SEQ ID NO:30 or residues 198-344 of SEQ ID NO:31 or 32.

27. The recombinant meganuclease of any one of claims 22-26, wherein said second subunit comprises residues 198-344 of SEQ ID NO:30 or residues 7-153 of SEQ ID NO:31 or 32.

28. The recombinant meganuclease of any one of claims 22-27, wherein said recombinant meganuclease is a single-chain meganuclease comprising a linker, wherein said linker covalently joins said first subunit and said second subunit.

29. The recombinant meganuclease of any one of claims 22-28, wherein said recombinant meganuclease comprises the amino acid sequence of any one of SEQ ID NOs:30-32.

30. An isolated polynucleotide comprising a nucleic acid sequence encoding said recombinant meganuclease of any one of claims 1-29.

31. A recombinant DNA construct comprising said isolated polynucleotide of claim 30.

32. The recombinant DNA construct of claim 31, wherein said recombinant DNA construct encodes a recombinant adeno-associated virus (AAV) vector.

33. A recombinant AAV vector comprising said isolated polynucleotide of claim 30.
34. A method for producing a genetically-modified eukaryotic cell comprising an exogenous sequence of interest inserted into a chromosome of said eukaryotic cell, said method comprising transfecting a eukaryotic cell with one or more nucleic acids including:
- (a) a first nucleic acid sequence encoding a recombinant meganuclease of any one of claims 1-29; and
 - (b) a second nucleic acid sequence including said sequence of interest;
- wherein said recombinant meganuclease produces a cleavage site in said chromosome at a recognition sequence comprising SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5, and wherein said sequence of interest is inserted into said chromosome at said cleavage site.
35. The method of claim 34, wherein said second nucleic acid further comprises sequences homologous to sequences flanking said cleavage site and said sequence of interest is inserted at said cleavage site by homologous recombination.
36. The method of claim 34 or claim 35, wherein said eukaryotic cell is a human T cell or a cell derived therefrom.
37. The method of any one of claims 34-36, wherein said sequence of interest encodes a chimeric antigen receptor.
38. The method of any one of claims 34-37, wherein at least said second nucleic acid sequence is introduced into said eukaryotic cell by a recombinant AAV vector.
39. A method for producing a genetically-modified eukaryotic cell comprising an exogenous sequence of interest inserted into a chromosome of said eukaryotic cell, said method comprising:
- (a) introducing said recombinant meganuclease of any one of claims 1-29 into a eukaryotic cell; and
 - (b) transfecting said eukaryotic cell with a nucleic acid including said sequence of interest;

wherein said recombinant meganuclease produces a cleavage site in said chromosome at a recognition sequence comprising SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5, and wherein said sequence of interest is inserted into said chromosome at said cleavage site.

40. The method of claim 39, wherein said nucleic acid further comprises sequences homologous to sequences flanking said cleavage site and said sequence of interest is inserted at said cleavage site by homologous recombination.

41. The method of claim 39 or claim 40, wherein said eukaryotic cell is a human T cell or a cell derived therefrom.

42. The method of any one of claims 39-41, wherein said sequence of interest encodes a chimeric antigen receptor.

43. The method of any one of claims 39-42, wherein said nucleic acid sequence is introduced into said eukaryotic cell by a recombinant AAV vector.

44. A method for producing a genetically-modified eukaryotic cell by disrupting a target sequence in a chromosome of said eukaryotic cell, said method comprising:

transfecting a eukaryotic cell with a nucleic acid encoding said recombinant meganuclease of any one of claims 1-29;

wherein said recombinant meganuclease produces a cleavage site in said chromosome at a recognition sequence comprising SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5, and wherein said target sequence is disrupted by non-homologous end-joining at said cleavage site.

45. The method of claim 44, wherein said eukaryotic cell is a human T cell or a cell derived therefrom.

46. The method of claim 44 or claim 45, further comprising transfecting said eukaryotic cell with a second nucleic acid including an exogenous sequence of interest.

47. The method of claim 46, wherein said second nucleic acid further comprises sequences homologous to sequences flanking said cleavage site, and wherein said sequence of interest is inserted at said cleavage site by homologous recombination.

48. The method of claim 46 or claim 47, wherein said sequence of interest encodes a chimeric antigen receptor.

49. The method of any one of claims 46-48, wherein at least said second nucleic acid is introduced into said eukaryotic cell by a recombinant AAV vector.

50. A method for producing a genetically-modified eukaryotic cell by disrupting a target sequence in a chromosome of said eukaryotic cell, comprising:

introducing said recombinant meganuclease of any one of claims 1-29 into a eukaryotic cell;

wherein said meganuclease produces a cleavage site in said chromosome at a recognition sequence comprising SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5, and said target sequence is disrupted by non-homologous end-joining at said cleavage site.

51. The method of claim 50, wherein said eukaryotic cell is a human T cell or a cell derived therefrom.

52. The method of claim 50 or claim 51, further comprising transfecting said eukaryotic cell with a nucleic acid including an exogenous sequence of interest.

53. The method of claim 52, wherein said nucleic acid further comprises sequences homologous to sequences flanking said cleavage site, and wherein said sequence of interest is inserted at said cleavage site by homologous recombination.

54. The method of claim 52 or claim 53, wherein said sequence of interest encodes a chimeric antigen receptor.

55. The method of any one of claims 52-54, wherein said nucleic acid is introduced into said eukaryotic cell by a recombinant AAV vector.

56. A method of immunotherapy for treating cancer in a subject in need thereof, said method comprising administering to said subject a pharmaceutical composition comprising said genetically-modified cell produced by the method of any one of claims 34-55 and a pharmaceutically acceptable carrier.

57. The method of claim 56, wherein said cancer is selected from the group consisting of a cancer of carcinoma, lymphoma, sarcoma, blastomas, and leukemia.

58. The method of claim 56, wherein the cancer is selected from the group consisting of a cancer of B-cell origin, breast cancer, gastric cancer, neuroblastoma, osteosarcoma, lung cancer, melanoma, prostate cancer, colon cancer, renal cell carcinoma, ovarian cancer, rhabdomyo sarcoma, leukemia, and Hodgkin's lymphoma.

59. The method of claim 58, wherein the cancer of B-cell origin is selected from the group consisting of B-lineage acute lymphoblastic leukemia, B-cell chronic lymphocytic leukemia, and B-cell non-Hodgkin's lymphoma.

A.

	TRC1 Half-Site	TRC2 Half-Site	
	┌───────────┐	┌───────────┐	
TRC 1-2	TGGCCTGGAGCAA	CAAATCTGA	(SEQ ID NO:3)
Recognition Sequence	ACCGGACCT	CGTTGTTTAGACT	(SEQ ID NO:83)
	TRC3 Half-Site	TRC4 Half-Site	
	┌───────────┐	┌───────────┐	
TRC 3-4	ACAAATGTGTCAC	AAAGTAAGG	(SEQ ID NO:4)
Recognition Sequence	TGTTTACAC	AGTGTTCATTCC	(SEQ ID NO:84)
	TRC7 Half-Site	TRC8 Half-Site	
	┌───────────┐	┌───────────┐	
TRC 7-8	CTGATGTGTATAT	CACAGACAA	(SEQ ID NO:5)
Recognition Sequence	GACTACACAT	TATAGTGTCTGTT	(SEQ ID NO:85)

B.

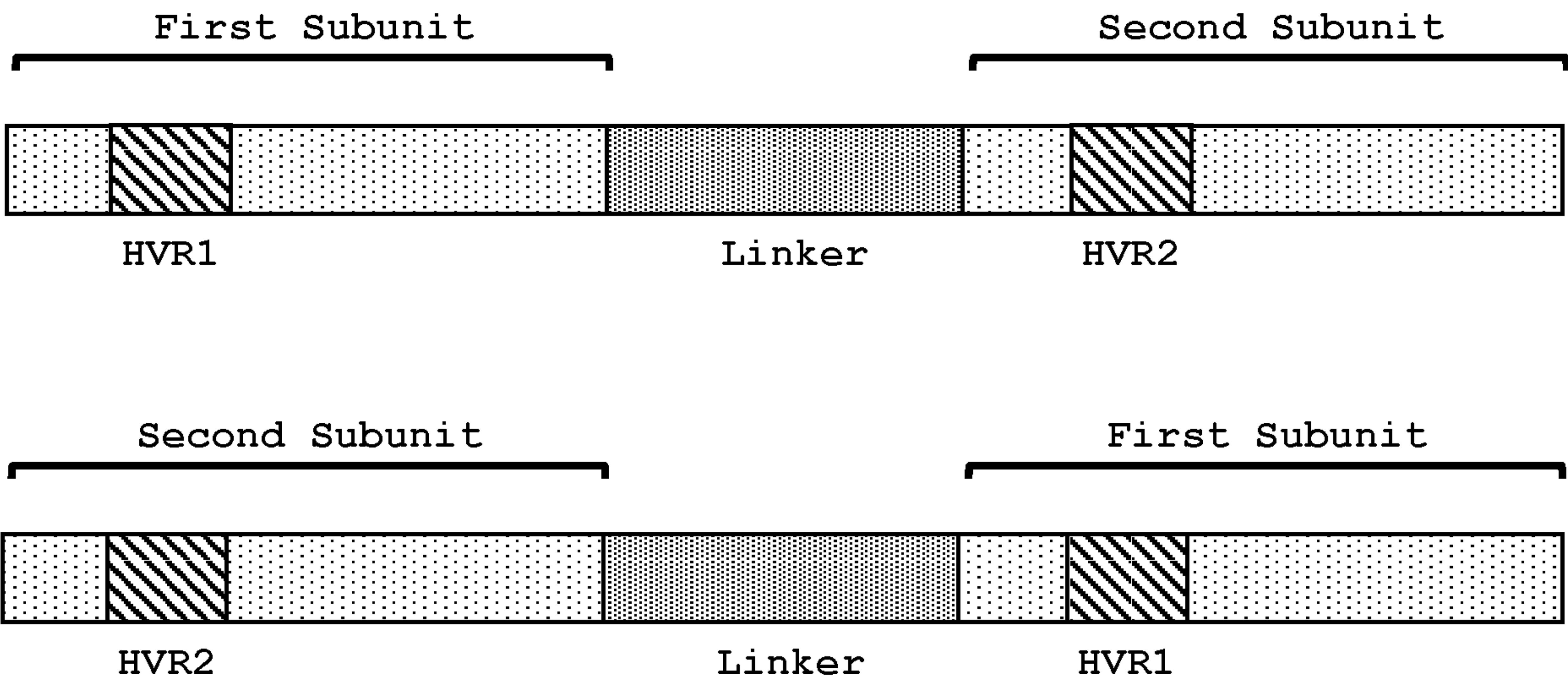


FIGURE 1

SEQ ID		Hypervariable Region 1 (HVR1)		271
NO:				
33	TRC 1-2x.87	EE	KEFLLYLAGFVDGSGSI	YACIAPRQGSKFKHRLKLGFAGVGGKTQRRWFLDKLVDEIGVGYYVYD RGSVSEYYVLS E
34	TRC 1-2x.87	QE	KEFLLYLAGFVDGSGSI	YACIAPRQGSKFKHRLKLGFAGVGGKTQRRWFLDKLVDEIGVGYYVYD RGSVSEYYVLS E
35	TRC 1-2x.87	EQ	KEFLLYLAGFVDGSGSI	YACIAPRQGSKFKHRLKLGFAGVGGKTQRRWFLDKLVDEIGVGYYVYD RGSVSEYYVLS Q
36	TRC 1-2x.87		KEFLLYLAGFVDGSGSI	YACIAPRQGSKFKHRLKLGFAGVGGKTQRRWFLDKLVDEIGVGYYVYD RGSVSEYYVLS Q
37	TRC 1-2x.6		KEFLLYLAGFVDGSGSI	YACIAPRQGSKFKHRLKLGFAGVGGKTQRRWFLDKLVDEIGVGYYVYD RGSVSEYYVLS Q
38	TRC 1-2x.20		KEFLLYLAGFVDGSGSI	YACIAPRQGSKFKHRLKLGFAGVGGKTQRRWFLDKLVDEIGVGYYVYD RGSVSEYYVLS Q
39	TRC 1-2x.55		KEFLLYLAGFVDGSGSI	YATIAPCQ RA KFKHRLKLGF TV GQKTQRRWFLDKLVDEIGVG HVYDRGSVSEYYVLS E
40	TRC 1-2x.60		KEFLLYLAGFVDGSGSI	YAQIKPQQ RA KFKHRL LLAF TV S QKTQRRWFLDKLVDEIGVG YVDDRGGVSEYI LS E
41	TRC 1-2x.105		KEFLLYLAGFVDGSGSI	YASIRPSQ RS KFKHKLGLGFAGVGGKTQRRWFLDKLVDEIGVG YVDRGSVSEYYVLS Q
42	TRC 1-2x.163		KEFLLYLAGFVDGSGSI	YACIAPRQGSKFKHRLKLGFAGVGGKTQRRWFLDKLVDEIGVG YVDRGSVSEYYVLS Q
43	TRC 1-2x.113_3		KEFLLYLAGFVDGSGSI	YACIAPRQGSKFKHRLKLGFAGVGGKTQRRWFLDKLVDEIGVG YVDRGSVSEYYVLS Q

SEQ ID		Hypervariable Region 1 (HVR1)		80
NO:				
44	TRC 1-2x.5		KEFLLYLAGFVDGSGSI	YACIAPRQGSKFKHRLKLGFAGVGGKTQRRWFLDKLVDEIGVGYYVYD RGSVSEYYVLS Q
45	TRC 1-2x.8		KEFLLYLAGFVDGSGSI	YATI RP AQ RA KFKHRLVLGF FE VGGKTQRRWFLDKLVDEIGVG YVDRGSVSKYRL SQ
46	TRC 1-2x.25		KEFLLYLAGFVDGSGSI	YACIAPRQGSKFKHRLKLGFAGVGGKTQRRWFLDKLVDEIGVGYYVYD RGSVSEYYVLS Q
47	TRC 1-2x.72		KEFLLYLAGFVDGSGSI	YACIAPRQGSKFKHRLKLGFAGVGGKTQRRWFLDKLVDEIGVGYYVYD RGSVSEYYVLS Q
48	TRC 1-2x.80		KEFLLYLAGFVDGSGSI	YACIAPRQGSKFKHRLKLGFAGVGGKTQRRWFLDKLVDEIGVGYYVYD RGSVSEYYVLS Q
49	TRC 1-2x.84		KEFLLYLAGFVDGSGSI	YATIAPCQ RA KFKHRLKLGF TV GQKTQRRWFLDKLVDEIGVG HVYDRGSVSEYYVLS Q
50	TRC 1-2x.120		KEFLLYLAGFVDGSGSI	YACIAPRQGSKFKHRLKLGFAGVGGKTQRRWFLDKLVDEIGVGYYVYD RGSVSEYYVLS Q
51	TRC 1-2x.113_1		KEFLLYLAGFVDGSGSI	YACIAPRQGSKFKHRLKLGFAGVGGKTQRRWFLDKLVDEIGVG YVDRGSVSEYYVLS E
52	TRC 1-2x.113_2		KEFLLYLAGFVDGSGSI	YACIAPGG GS KFKHRLKLGFAGVGGKTQRRWFLDKLVDEIGVG YVDRGSVSEYYVLS E

FIGURE 2A

272		344	
TRC 1-2x.87	EE	IKPLHNFLTQLQPPFLKLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDSKTRKTTSETVRAVLD	
TRC 1-2x.87	QE	IKPLHNFLTQLQPPFLKLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDSKTRKTTSETVRAVLD	
TRC 1-2x.87	EQ	IKPLHNFLTQLQPPFLKLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDSKTRKTTSETVRAVLD	
TRC 1-2x.87		IKPLHNFLTQLQPPFLKLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDSKTRKTTSETVRAVLD	
TRC 1-2x.6		IKPLHNFLTQLQPPFLKLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDSKTRKTTSETVRAVLD	
TRC 1-2x.20		IKPLHNFLTQLQPPFLKLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDSKTRKTTSETVRAVLD	
TRC 1-2x.55		IKPLHNFLTQLQPPFLKLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDSKTRKTTSETVRAVLD	
TRC 1-2x.60		IKPLHNFLTQLQPPFLKLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDSKTRKTTSETVRAVLD	
TRC 1-2x.105		IKPLHNFLTQLQPPFLKLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDSKTRKTTSETVRAVLD	
TRC 1-2x.163		IKPLHNFLTQLQPPFLKLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDSKTRKTTSETVRAVLD	
TRC 1-2x.113_3		IKPLHNFLTQLQPPFLKLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDSKTRKTTSETVRAVLD	
81		153	
TRC 1-2x.5		IKPLHNFLTQLQPPFLKLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDSKTRKTTSETVRAVLD	
TRC 1-2x.8		IKPLHNFLTQLQPPFLKLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDSKTRKTTSETVRAVLD	
TRC 1-2x.25		IKPLHNFLTQLQPPFLKLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDSKTRKTTSETVRAVLD	
TRC 1-2x.72		IKPLHNFLTQLQPPFLKLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDSKTRKTTSETVRAVLD	
TRC 1-2x.80		IKPLHNFLTQLQPPFLKLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDSKTRKTTSETVRAVLD	
TRC 1-2x.84		IKPLHNFLTQLQPPFLKLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDSKTRKTTSETVRAVLD	
TRC 1-2x.120		IKPLHNFLTQLQPPFLKLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDSKTRKTTSETVRAVLD	
TRC 1-2x.113_1		IKPLHNFLTQLQPPFLKLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDSKTRKTTSETVRAVLD	
TRC 1-2x.113_2		IKPLHNFLTQLQPPFLKLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDSKTRKTTSETVRAVLD	

FIGURE 2B

198		Hypervariable Region 2 (HVR2)										271																																														
SEQ ID	NO:																																																									
58	TRC 1-2x.87	EE	KEFLLYLAGFVDGDGSI	FAS	Y	P	H	Q	R	A	K	F	K	H	F	L	K	L	T	F	A	V	Y	Q	K	T	Q	R	R	W	F	L	D	K	L	V	D	E	I	G	V	G	Y	V	Y	D	S	G	S	V	S	E	Y	R	L	S	E	
59	TRC 1-2x.87	QE	KEFLLYLAGFVDGDGSI	FAS	Y	P	H	Q	R	A	K	F	K	H	F	L	K	L	T	F	A	V	Y	Q	K	T	Q	R	R	W	F	L	D	K	L	V	D	E	I	G	V	G	Y	V	Y	D	S	G	S	V	S	E	Y	R	L	S	E	
60	TRC 1-2x.87	EQ	KEFLLYLAGFVDGDGSI	FAS	Y	P	H	Q	R	A	K	F	K	H	F	L	K	L	T	F	A	V	Y	Q	K	T	Q	R	R	W	F	L	D	K	L	V	D	E	I	G	V	G	Y	V	Y	D	S	G	S	V	S	E	Y	R	L	S	E	
61	TRC 1-2x.87		KEFLLYLAGFVDGDGSI	FAS	Y	P	H	Q	R	A	K	F	K	H	F	L	K	L	T	F	A	V	Y	Q	K	T	Q	R	R	W	F	L	D	K	L	V	D	E	I	G	V	G	Y	V	Y	D	S	G	S	V	S	E	Y	R	L	S	E	
62	TRC 1-2x.6		KEFLLYLAGFVDGDGSI	YAC	I	Y	P	H	Q	R	A	K	F	K	H	L	L	K	L	V	F	A	V	H	Q	K	T	Q	R	R	W	F	L	D	K	L	V	D	E	I	G	V	G	Y	V	Y	D	A	G	S	V	S	E	Y	R	L	S	E
63	TRC 1-2x.20		KEFLLYLAGFVDGDGSI	YAC	I	Y	P	D	Q	R	T	K	F	K	H	G	L	R	L	N	F	S	V	F	Q	K	T	Q	R	R	W	F	L	D	K	L	V	D	E	I	G	V	G	Y	V	F	D	A	G	S	V	S	E	Y	R	L	S	E
64	TRC 1-2x.55		KEFLLYLAGFVDGDGSI	FAT	I	H	P	D	Q	R	S	K	F	K	H	Y	L	R	L	F	F	S	V	F	Q	K	T	Q	R	R	W	F	L	D	K	L	V	D	E	I	G	V	G	Y	V	Y	D	A	G	S	V	S	E	Y	R	L	S	E
65	TRC 1-2x.60		KEFLLYLAGFVDGDGSI	FAQ	I	K	P	D	Q	K	M	K	F	K	H	Y	L	S	L	H	F	S	V	F	Q	K	T	Q	R	R	W	F	L	D	K	L	V	D	E	I	G	V	G	Y	V	Y	D	G	G	S	V	S	E	Y	R	L	S	E
66	TRC 1-2x.105		KEFLLYLAGFVDGDGSI	FAS	I	N	P	D	Q	R	A	K	F	K	H	S	L	K	L	T	F	S	V	Y	Q	K	T	Q	R	R	W	F	L	D	K	L	V	D	E	I	G	V	G	Y	V	Y	D	T	G	S	V	S	E	Y	R	L	S	E
67	TRC 1-2x.163		KEFLLYLAGFVDGDGSI	FAS	Y	P	H	Q	R	A	K	F	K	H	F	L	K	L	T	F	A	V	Y	Q	K	T	Q	R	R	W	F	L	D	K	L	V	D	E	I	G	V	G	Y	V	Y	D	S	G	S	V	S	E	Y	R	L	S	E	
68	TRC 1-2x.113_3		KEFLLYLAGFVDGDGSI	YAC	I	A	P	D	Q	R	A	K	F	K	H	Y	L	R	L	Q	F	S	V	F	Q	K	T	Q	R	R	W	F	L	D	K	L	V	D	E	I	G	V	G	Y	V	F	D	A	G	S	V	S	E	Y	R	L	S	E

SEQ ID	7	Hypervariable Region 2 (HVR2)										80
NO:												
69	TRC 1-2x.5	KEFLLYLAGFVDG	GSI	Y A T I Y P D Q R A K F K H A L K L I F S V F Q K T Q R R W F L D K L V D E I G V G Y V Y D G S V S E Y R L S E								
70	TRC 1-2x.8	KEFLLYLAGFVDG	GSI	F A T I A P D Q R P K F K H Q L R L I F N V C Q K T Q R R W F L D K L V D E I G V G Y V Y D T G S V S E Y R L S E								
71	TRC 1-2x.25	KEFLLYLAGFVDG	GSI	F A S I Y P H Q R A K F K H F L K L T F A V Y Q K T Q R R W F L D K L V D E I G V G Y V Y D S G S V S E Y R L S E								
72	TRC 1-2x.72	KEFLLYLAGFVDG	GSI	F A T I V P E Q R S K F K H Y L K L T F S V F Q K T Q R R W F L D R L V D E I G V G Y V Y D A G S V S E Y R L S E								
73	TRC 1-2x.80	KEFLLYLAGFVDG	GSI	F A T I F P D Q R M K F K H Q L R L H F C V H Q K T Q R R W F L D K L V D E I G V G Y V Y D S G S V S E Y R L S E								
74	TRC 1-2x.84	KEFLLYLAGFVDG	GSI	F A S I Y P H Q R A K F K H F L K L T F A V Y Q K T Q R R W F L D K L V D E I G V G Y V Y D S G S V S E Y R L S E								
75	TRC 1-2x.120	KEFLLYLAGFVDG	GSI	F A T I F P D Q R M K F K H Q L R L H F C V H Q K T Q R R W F L D K L V D E I G V G Y V Y D S G S V S E Y R L S E								
76	TRC 1-2x.113_1	KEFLLYLAGFVDG	GSI	F A T I F P D Q R M K F K H Q L R L H F C V H Q K T Q R R W F L D K L V D E I G V G Y V Y D S G S V S E Y R L S E								
77	TRC 1-2x.113_2	KEFLLYLAGFVDG	GSI	F A T I F P D Q R M K F K H Q L R L G F A V H Q K T Q R R W F L D K L V D E I G V G Y V Y D R G S V S E Y R L S E								

FIGURE 3A

272		344	
TRC 1-2x.87	EE	IKPLHNFLTQLQPPFLKCLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDS	RTRKTTSETVRAVLD
TRC 1-2x.87	QE	IKPLHNFLTQLQPPFLKCLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDS	RTRKTTSETVRAVLD
TRC 1-2x.87	EQ	IKPLHNFLTQLQPPFLKCLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDS	RTRKTTSETVRAVLD
TRC 1-2x.87		IKPLHNFLTQLQPPFLKCLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDS	RTRKTTSETVRAVLD
TRC 1-2x.6		IKPLHNFLTQLQPPFLKCLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDS	KTRKTTSETVRAVLD
TRC 1-2x.20		IKPLHNFLTQLQPPFLKCLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDS	KTRKTTSETVRAVLD
TRC 1-2x.55		IKPLHNFLTQLQPPFLKCLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDS	KTRKTTSETVRAVLD
TRC 1-2x.60		IKPLHNFLTQLQPPFLKCLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDS	KTRKTTSETVRAVLD
TRC 1-2x.105		IKPLHNFLTQLQPPFLKCLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDS	KTRKTTSETVRAVLD
TRC 1-2x.163		IKPLHNFLTQLQPPFLKCLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDS	RTRKTTSETVRAVLD
TRC 1-2x.113_3		IKPLHNFLTQLQPPFLKCLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDS	KTRKTTSETVRAVLD
81		153	
TRC 1-2x.5		IKPLHNFLTQLQPPFLKCLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDS	KTRKTTSETVRAVLD
TRC 1-2x.8		IKPLHNFLTQLQPPFLKCLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDS	KTRKTTSETVRAVLD
TRC 1-2x.25		IKPLHNFLTQLQPPFLKCLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDS	KTRKTTSETVRAVLD
TRC 1-2x.72		IKPLHNFLTQLQPPFLKCLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDS	KTRKTTSETVRAVLD
TRC 1-2x.80		IKPLHNFLTQLQPPFLKCLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDS	KTRKTTSETVRAVLD
TRC 1-2x.84		IKPLHNFLTQLQPPFLKCLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDS	KTRKTTSETVRAVLD
TRC 1-2x.120		IKPLHNFLTQLQPPFLKCLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDS	KTRKTTSETVRAVLD
TRC 1-2x.113_1		IKPLHNFLTQLQPPFLKCLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDS	KTRKTTSETVRAVLD
TRC 1-2x.113_2		IKPLHNFLTQLQPPFLKCLKQKQANLVLKIIEQLPSAKESPDKFLEVCTWVDQIAALNDS	KTRKTTSETVRAVLD

FIGURE 3B

SEQ ID		Hypervariable Region 1 (HVR1)	
NO:		7	80
53	TRC 3-4x.3	KEFLLYLAGFVDGDGSI	YATICPCQTLKFKHYLTLSFSVYQKTQRRWFLDKLVDEIGVGYYDDQGSVSCYRLSQ
54	TRC 3-4x.19	KEFLLYLAGFVDGDGSI	YATICPDQALKFKHYLSLTFAVYQKTQRRWFLDKLVDEIGVGYYDDQGSVSCYRLSQ
81			153
TRC 3-4x.3		IKPLHNFLTQLQPFLKQKQANLVLKIIIEQLPSAKESPDKFLEVCTWVDQIAALNDSKTRKTTSETVRAVLD	
TRC 3-4x.19		IKPLHNFLTQLQPFLKQKQANLVLKIIIEQLPSAKESPDKFLEVCTWVDQIAALNDSKTRKTTSETVRAVLD	

FIGURE 4

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SEQ ID	7	Hypervariable Region 1 (HVR1)	80
NO:			
55	TRC 7-8x.7	KEFLLYLAGFVDGDSIIYACITPQQDMKF ~~KHR~~ LQ ~~LR~~ FC ~~TV~~ QKTQRRWF ~~LD~~ KLVD ~~E~~ IGVGYV ~~QD~~ CSVSEYRLSE	

SEQ ID	NO:	198	Hypervariable Region 1 (HVR1)	271
56	TRC 7-8x.9	KEFLLYLAGFVDGDSI	YACITPQQDMKF ~~KHRLQ~~ LRFCVTQKTQRRWF ~~L~~ DKLVDEIGVGYYVQD	HGGA ~~SEY~~ R ~~L~~ SE
57	TRC 7-8x.14	KEFLLYLAGFVDGDSI	YACITPQQDMKF ~~KHRLQ~~ LRFCVTQKTQRRWF ~~L~~ DKLVDEIGVGYYVQD	KGSA ~~SEY~~ R ~~L~~ SE

FIGURE 6A

		9/62	
TRC 7-8x.7	81	153	
	IKPLHNFLTQLQPFLLKQKQANLVLKIIIEQLPSAKESPDKFLFVCTWVDQIAALNDSKTRKTTSETVRAVLD		
TRC 7-8x.9 TRC 7-8x.14	272	344	
	IKPLHNFLTQLQPFLLKQKQANLVLKIIIEQLPSAKESPDKFLFVCTWVDQIAALNDSKTRKTTSETVRAVLD IKPLHNFLTQLQPFLLKQKQANLVLKIIIEQLPSAKESPDKFLFVCTWVDQIAALNDSKTRKTTSETVRAVLD		

FIGURE 6B

SEQ ID		198	Hypervariable Region 2 (HVR2)	271
NO:				
80	TRC 7-8x.7	KEFLLYLAGFVDGDSI	VASIKPQQVAKFKHRLMLLEFFVYQKTQRRWFLDKLVDEIGVGYYDLGGASRYVLSQ	
SEQ ID		7	Hypervariable Region 2 (HVR2)	80
NO:				
81	TRC 7-8x.9	KEFLLYLAGFVDGDSI	YACIKPDQAAKFKHRLLLLEFTVCQKTQRRWFLDKLVDEIGVGYYVDQGSVSEYRLSQ	
82	TRC 7-8x.14	KEFLLYLAGFVDGDSI	WASIRPTQLAKFKHALWLGFAVYQKTQRRWFLDKLVDEIGVGYYVDSGSVSKYTLSE	

FIGURE 7A

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344

IKP LHNFLTQLQPF LKLKQKQANLV LKIIIEQLPSAKESPDKFLEVCTWVDQIAALNDSKTRKTTSETVRAVLD

153

81

IKP LHNFLTQLQPF LKLKQKQANLV LKIIIEQLPSAKESPDKFLEVCTWVDQIAALNDSKTRKTTSETVRAVLD
IKP LHNFLTQLQPF LKLKQKQANLV LKIIIEQLPSAKESPDKFLEVCTWVDQIAALNDSKTRKTTSETVRAVLD

TRC 7-8x.7

TRC 7-8x.9
TRC 7-8x.14

FIGURE 7B

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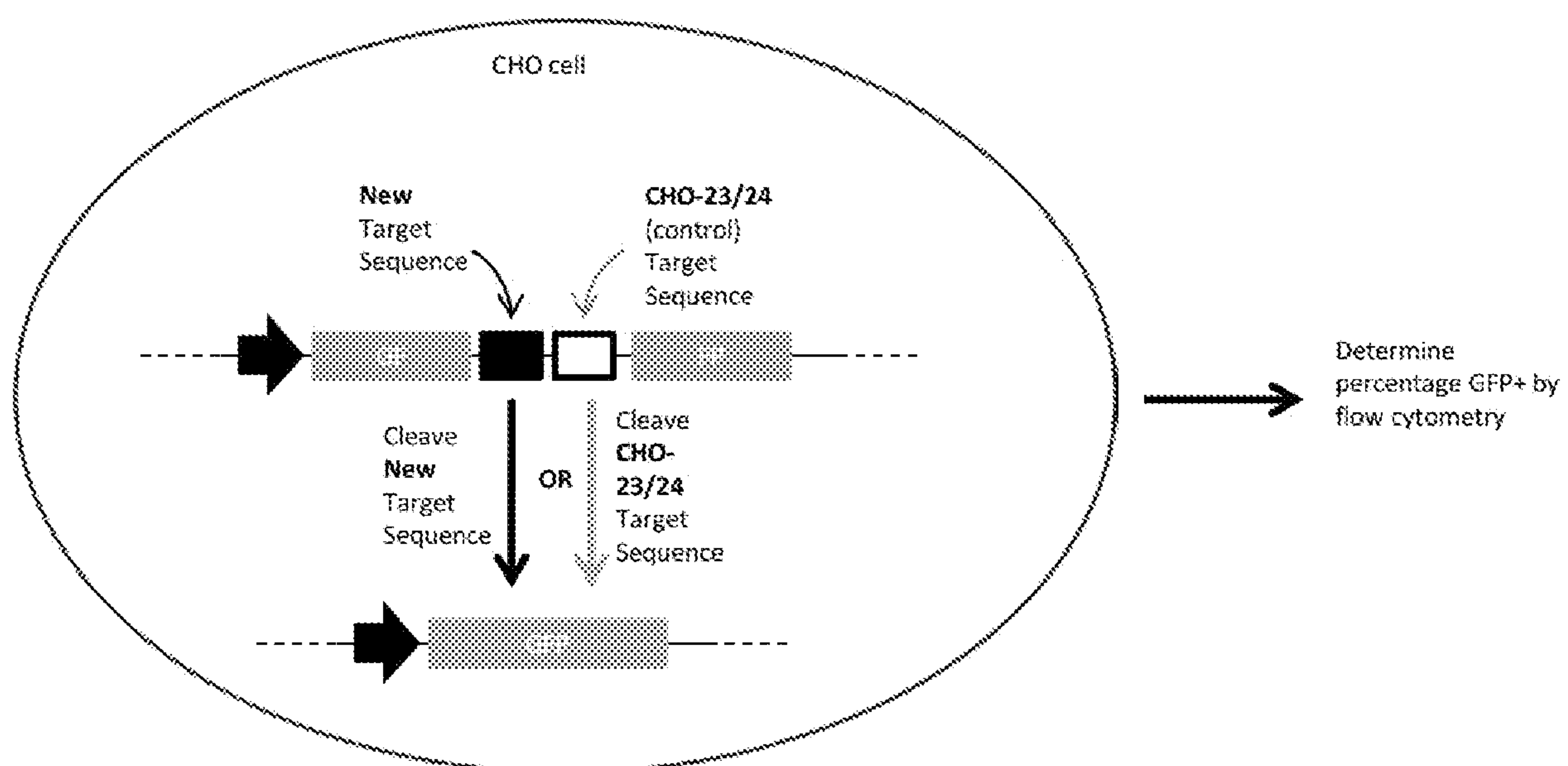
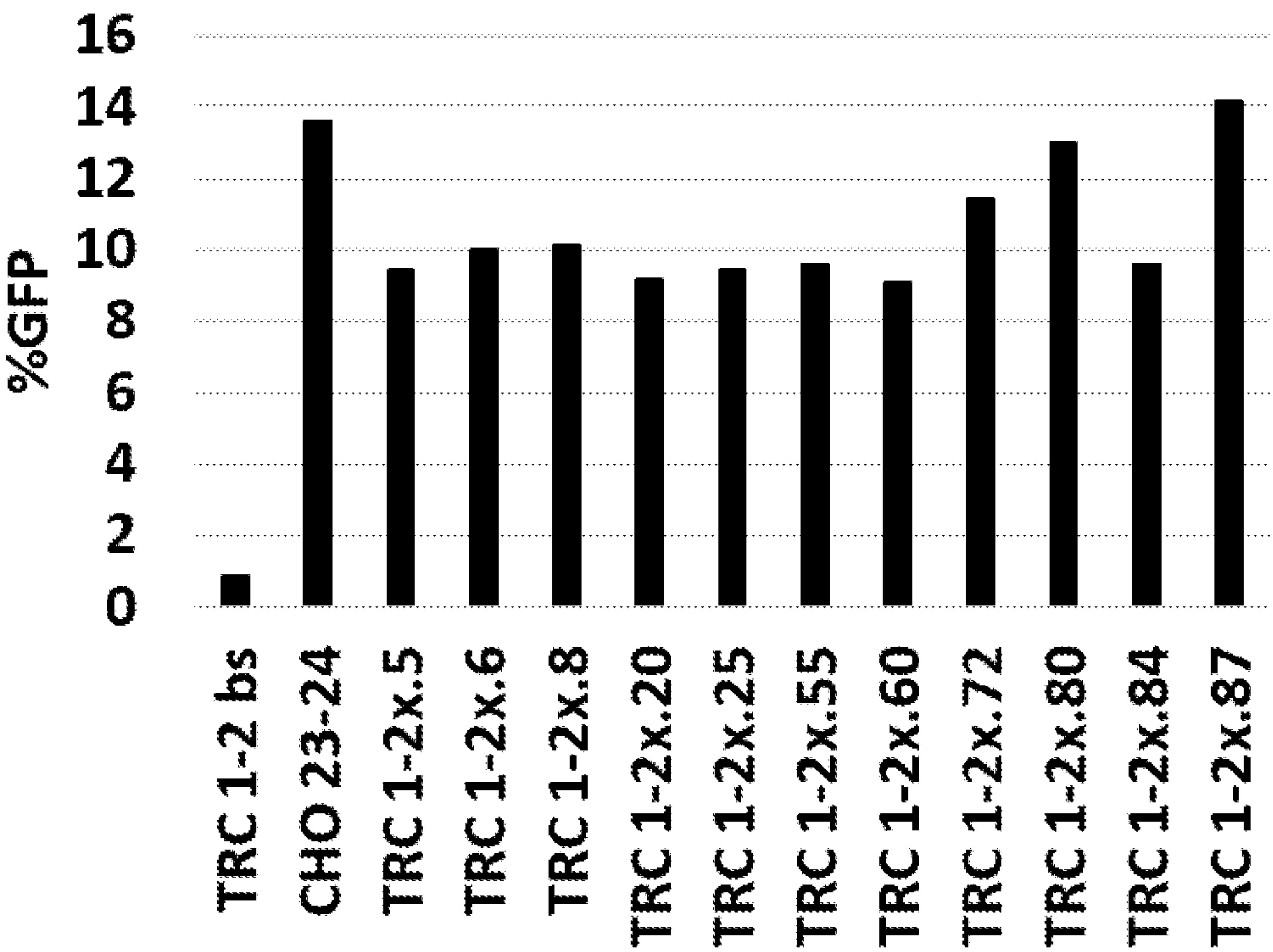


FIGURE 8

A.



B.

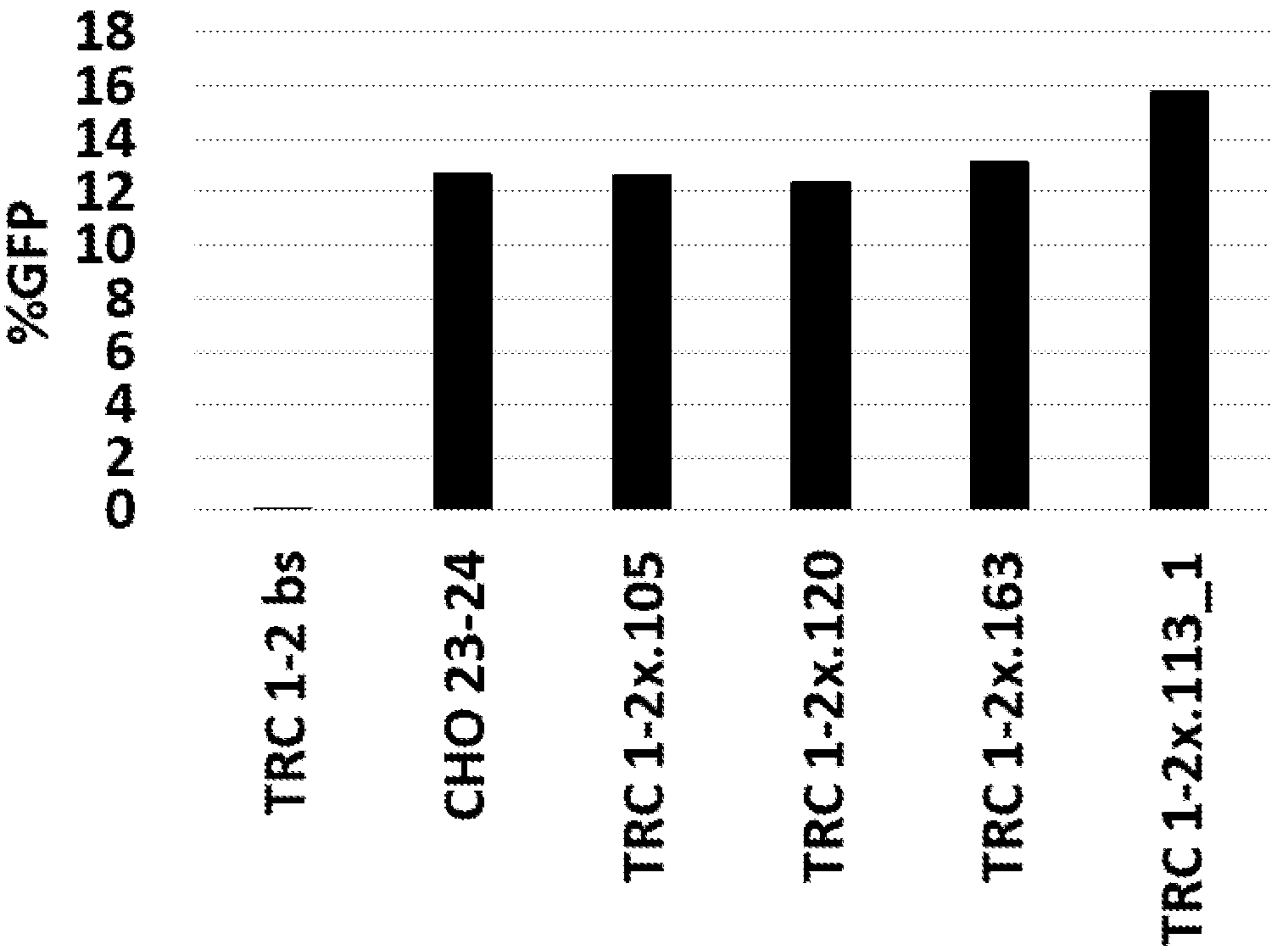
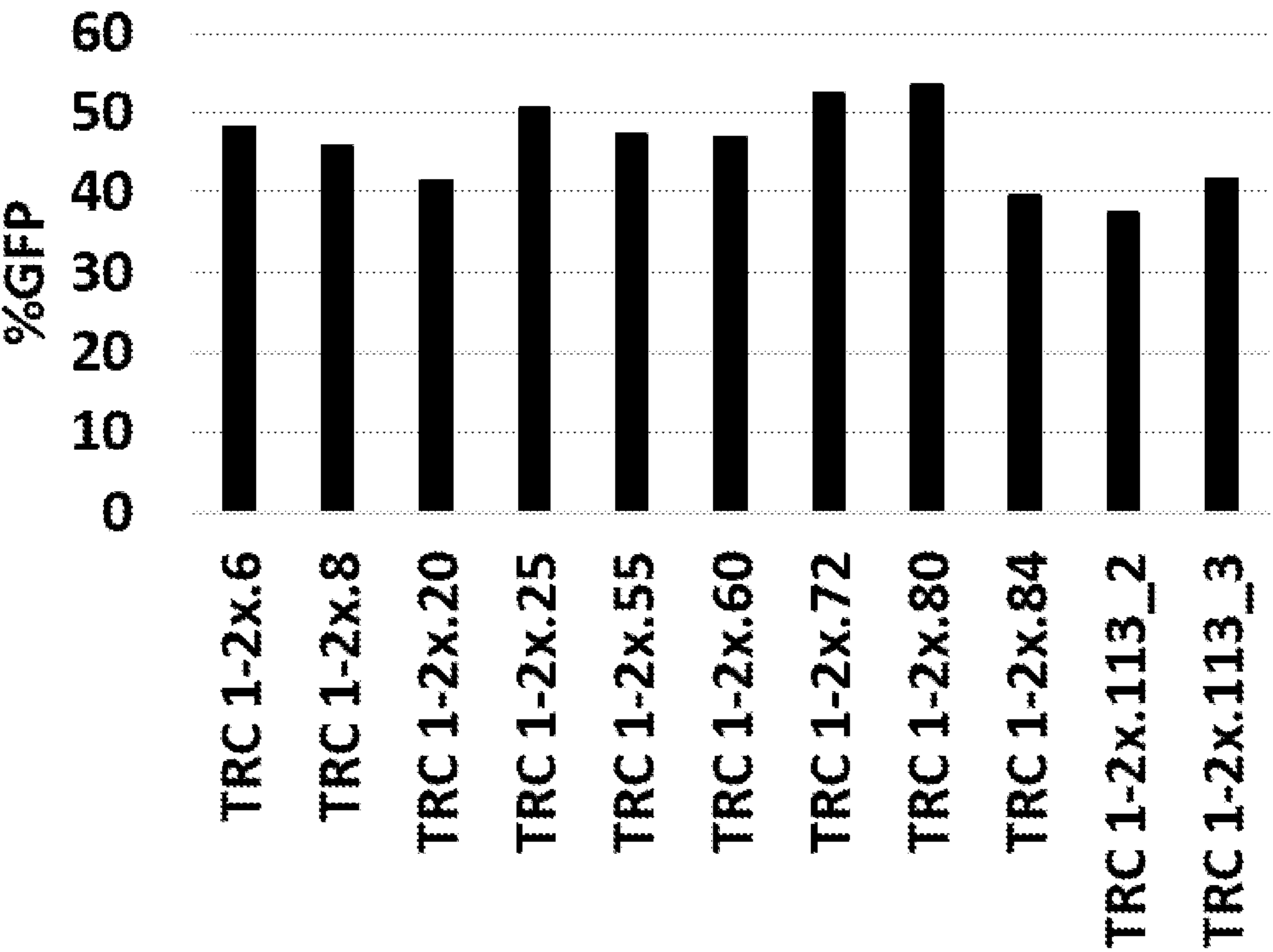


FIGURE 9

C.



D.

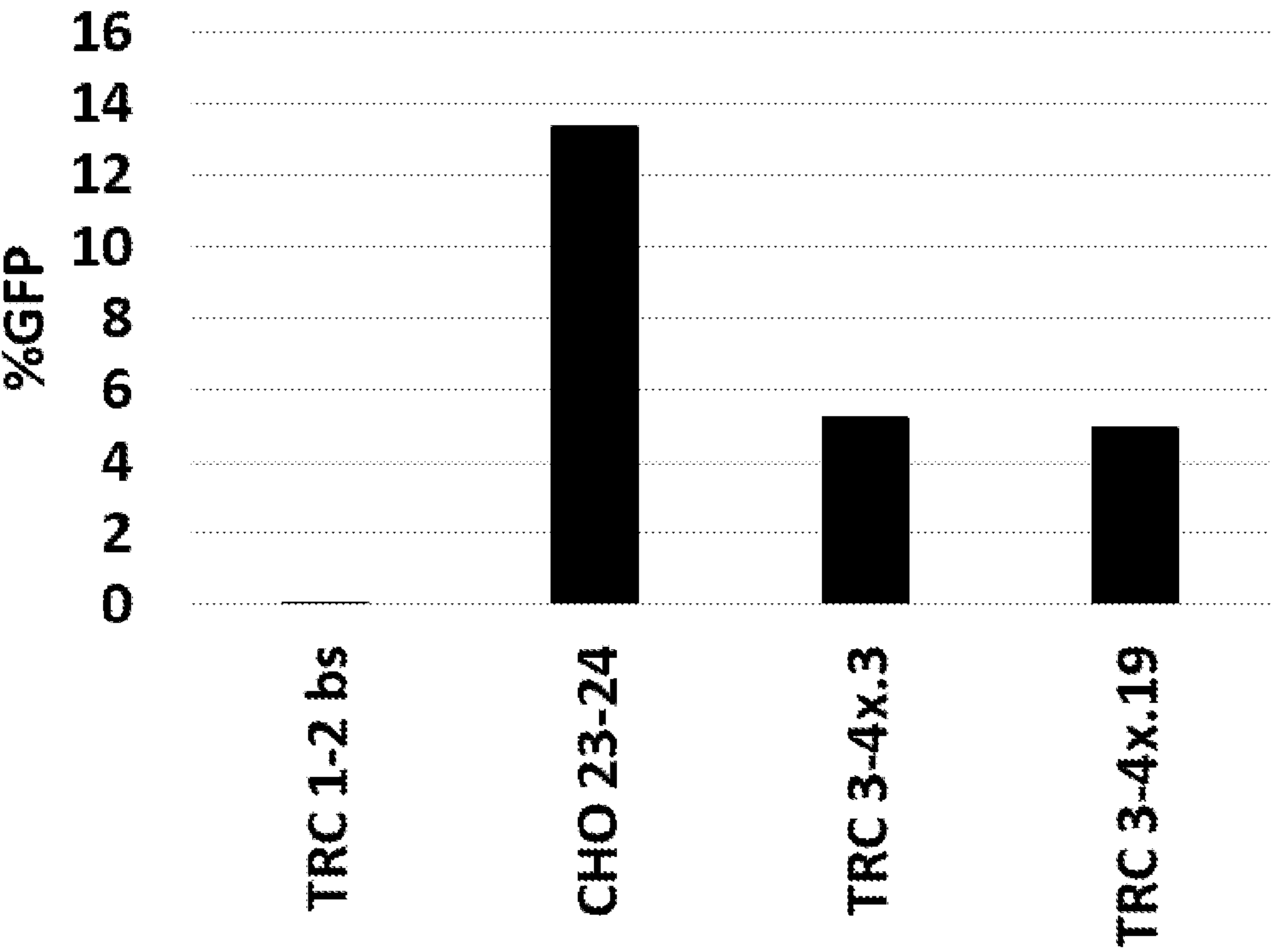
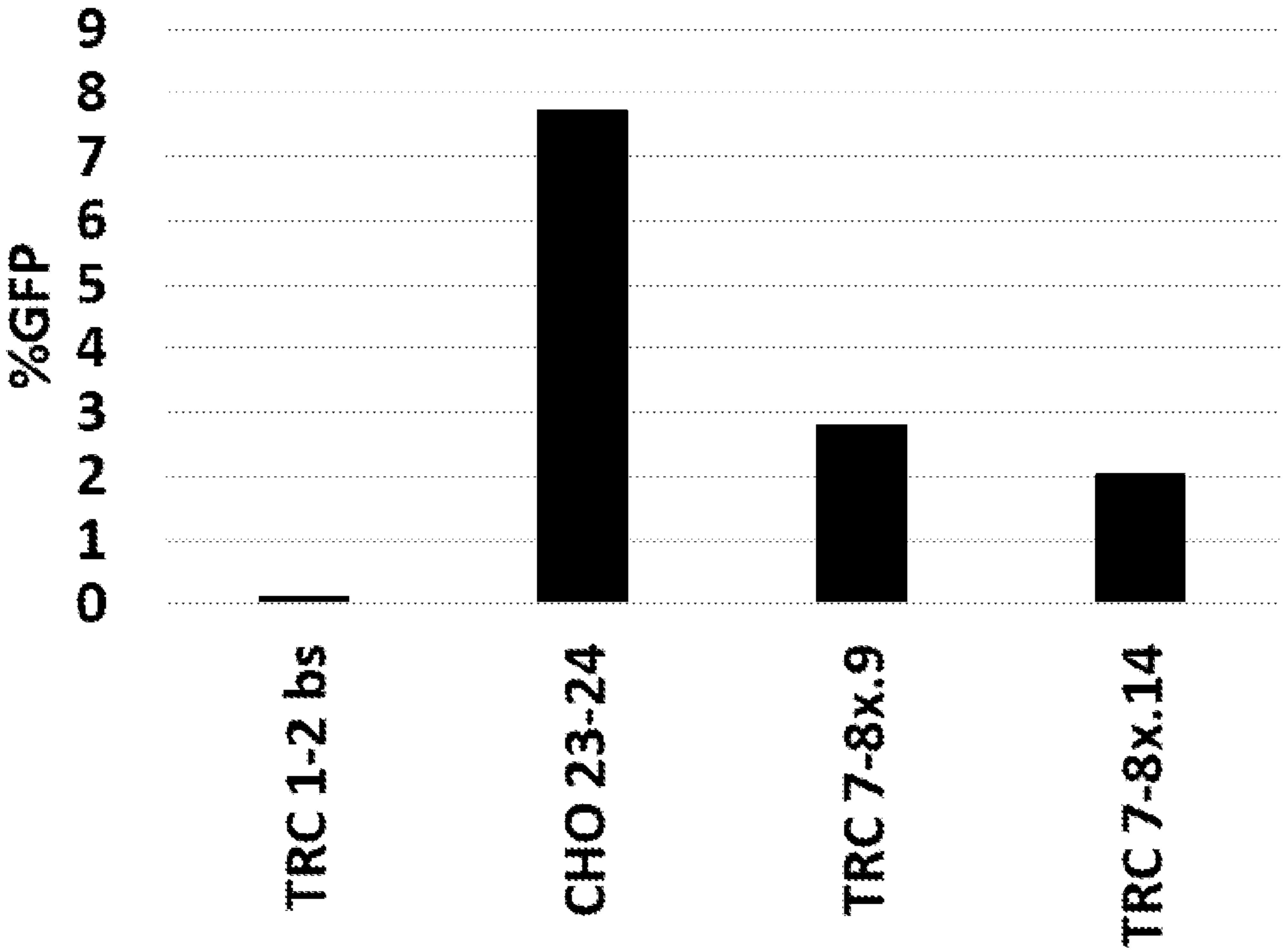


FIGURE 9 (cont.)

E.



F.

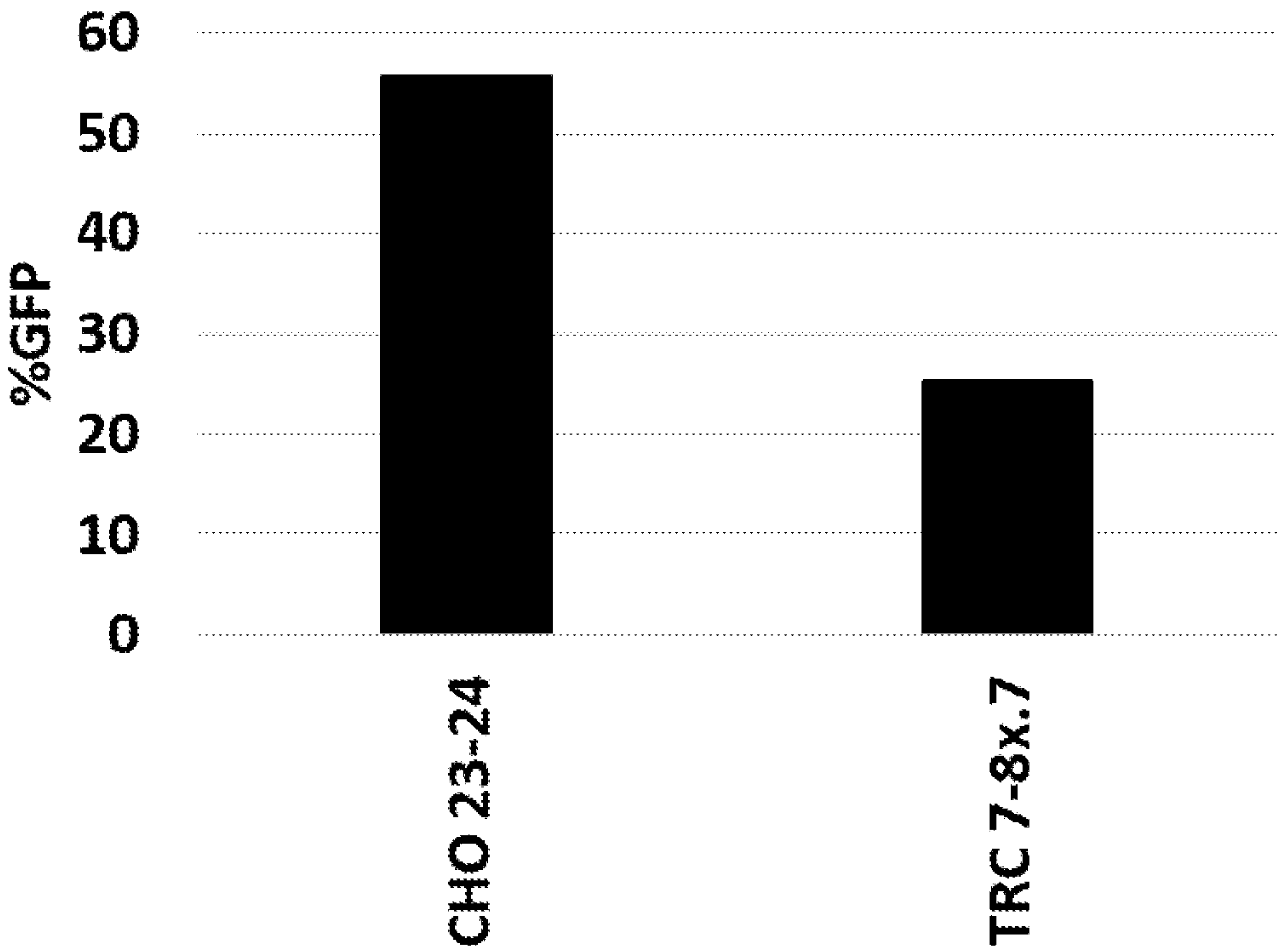


FIGURE 9 (cont.)

G.

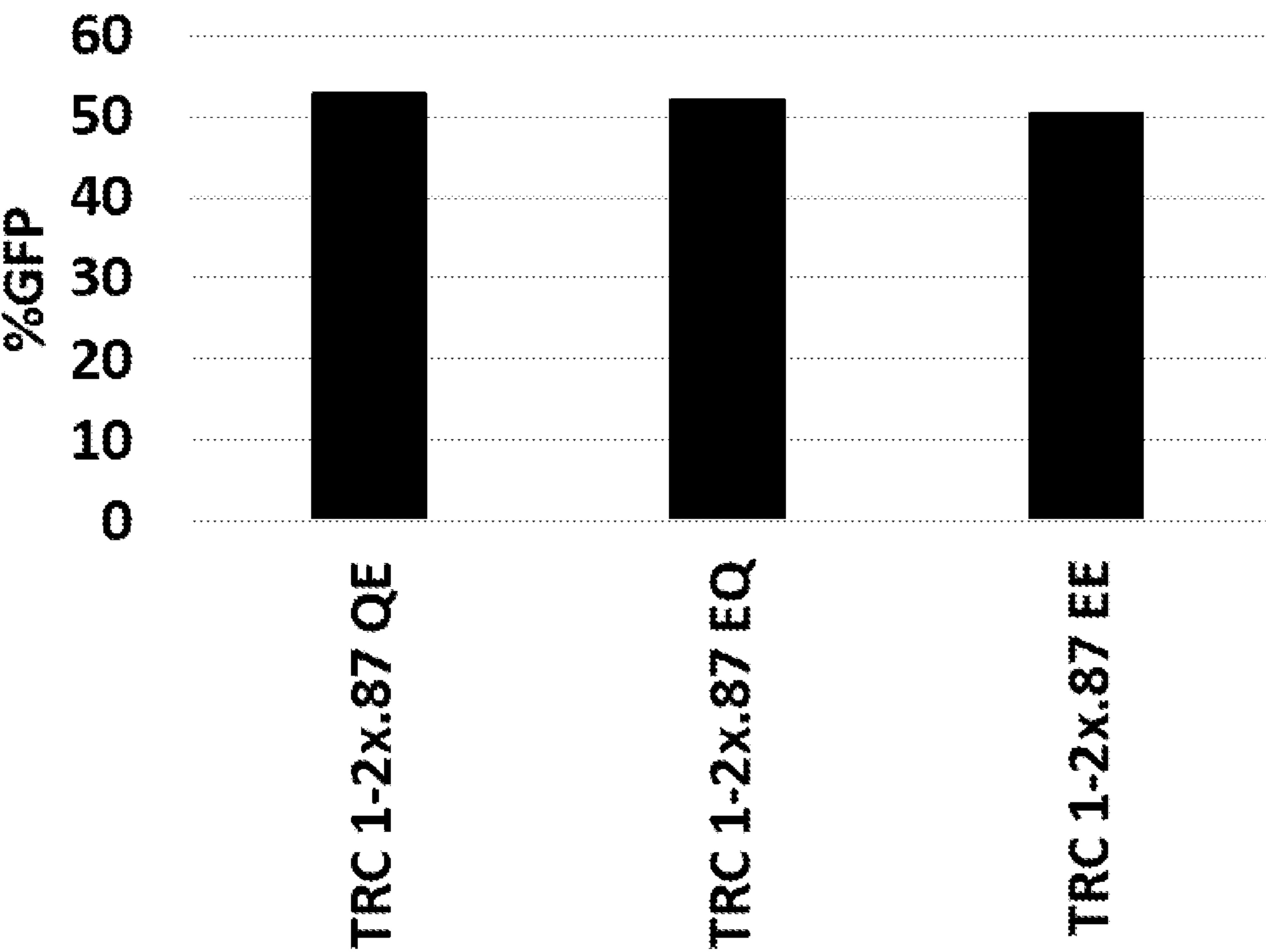


FIGURE 9 (cont.)

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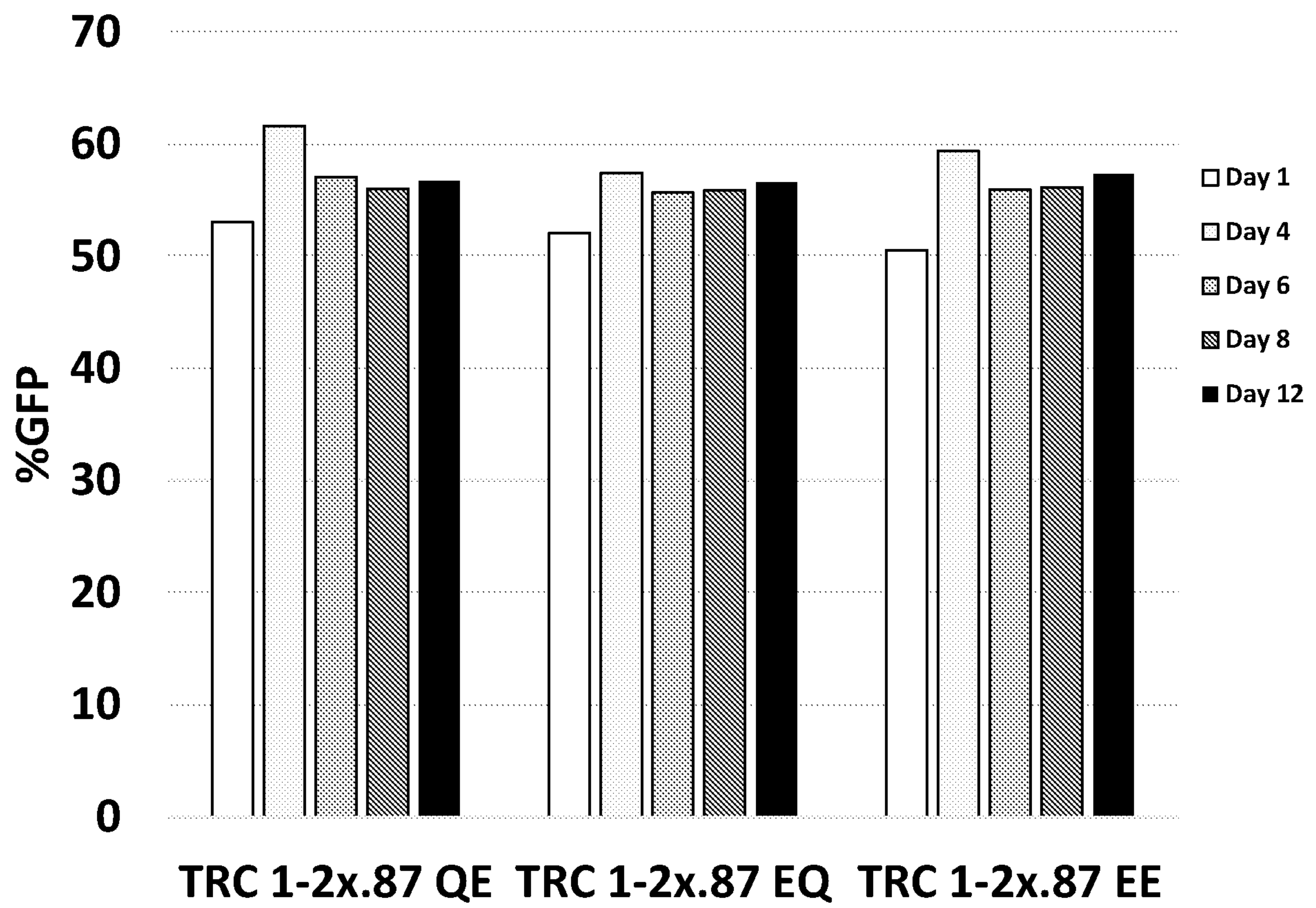


FIGURE 10

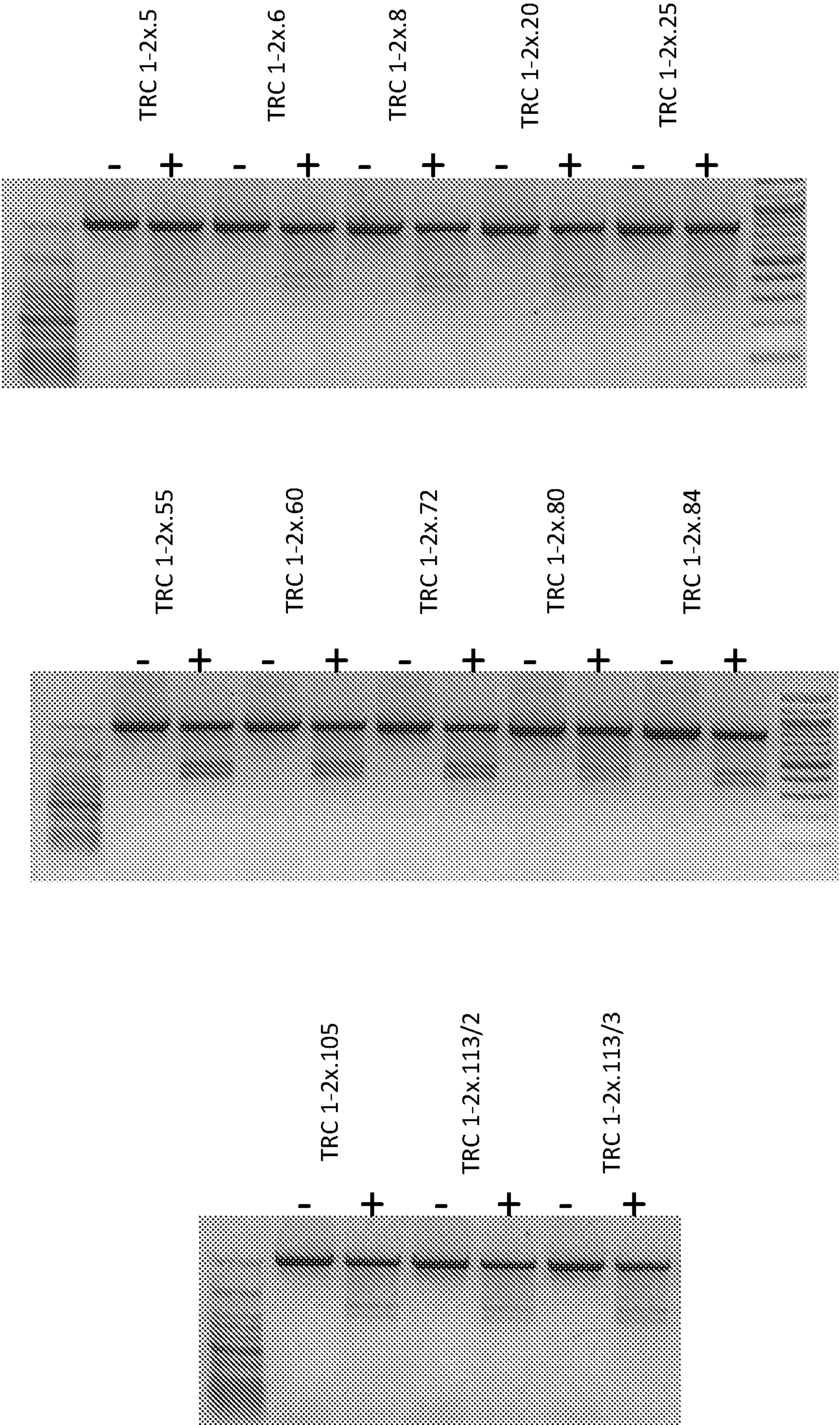


FIGURE 11

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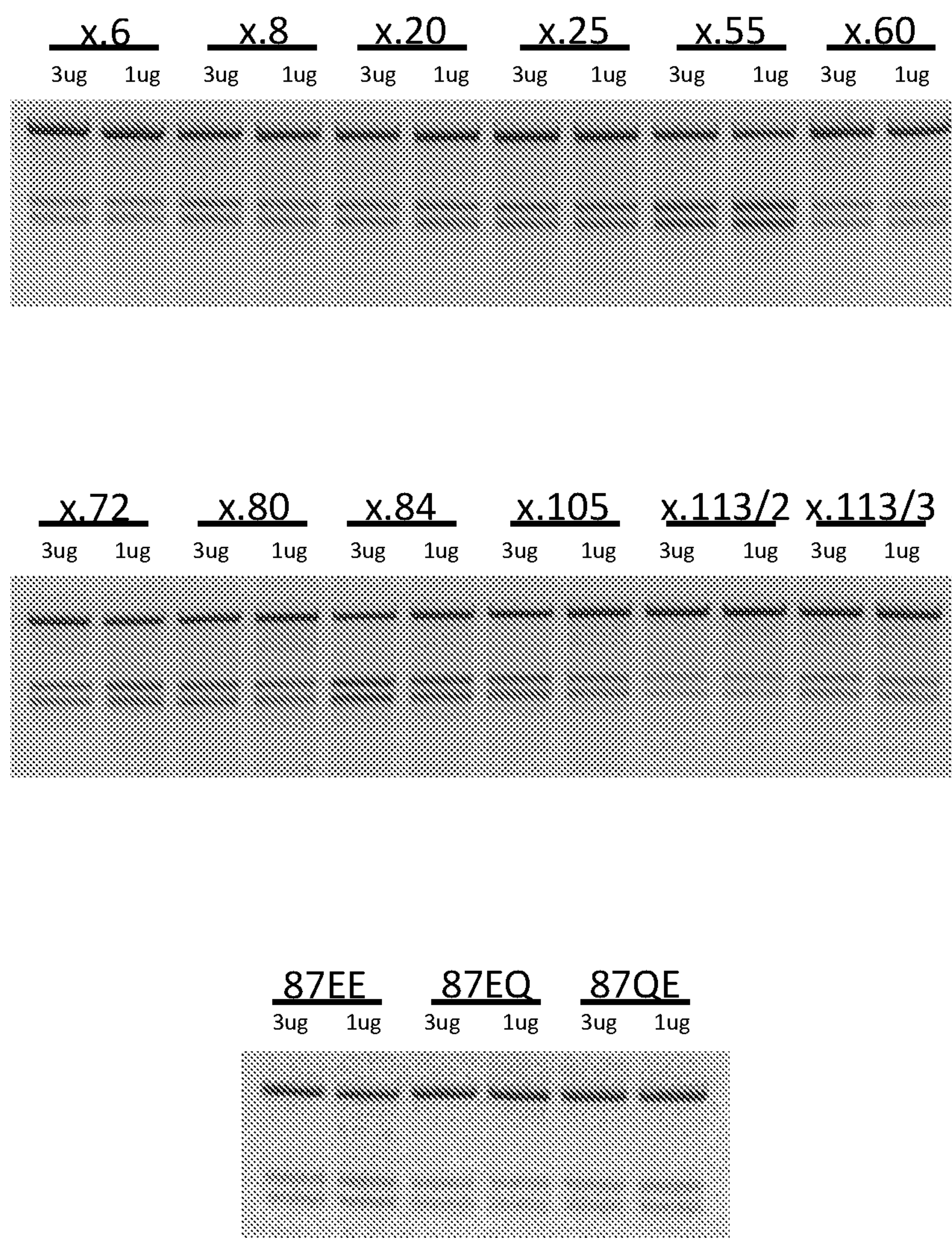
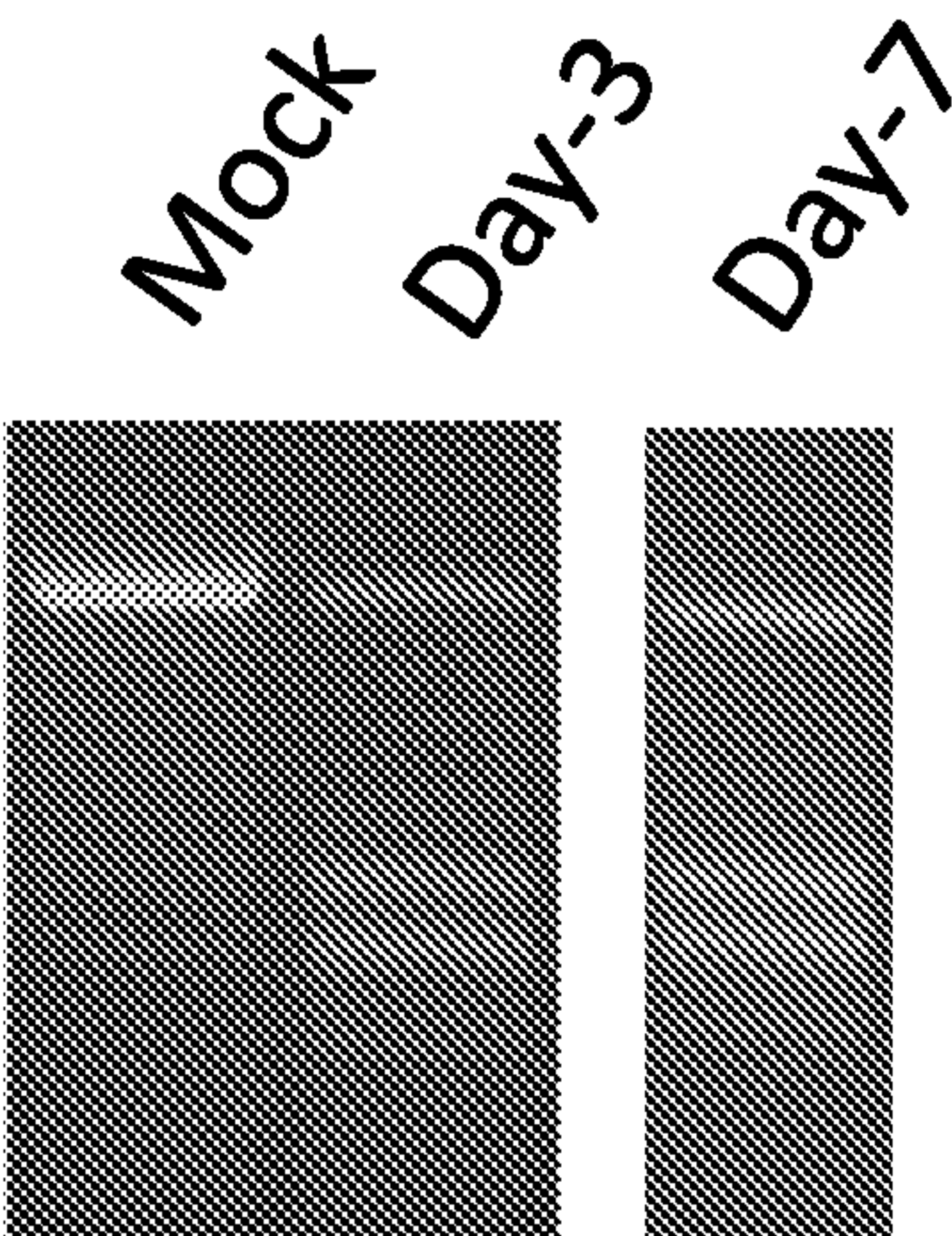


FIGURE 12

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A.



B.

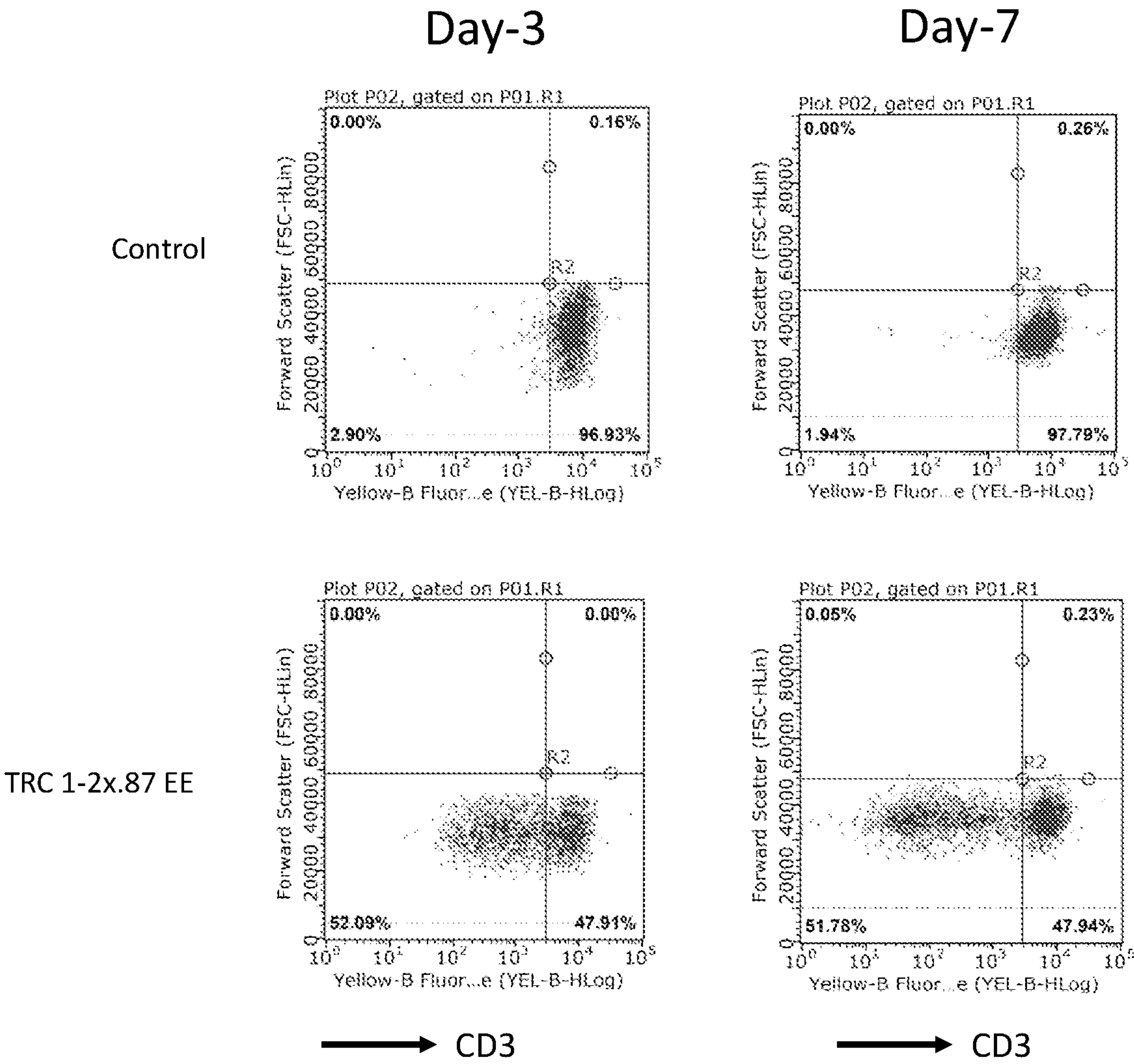
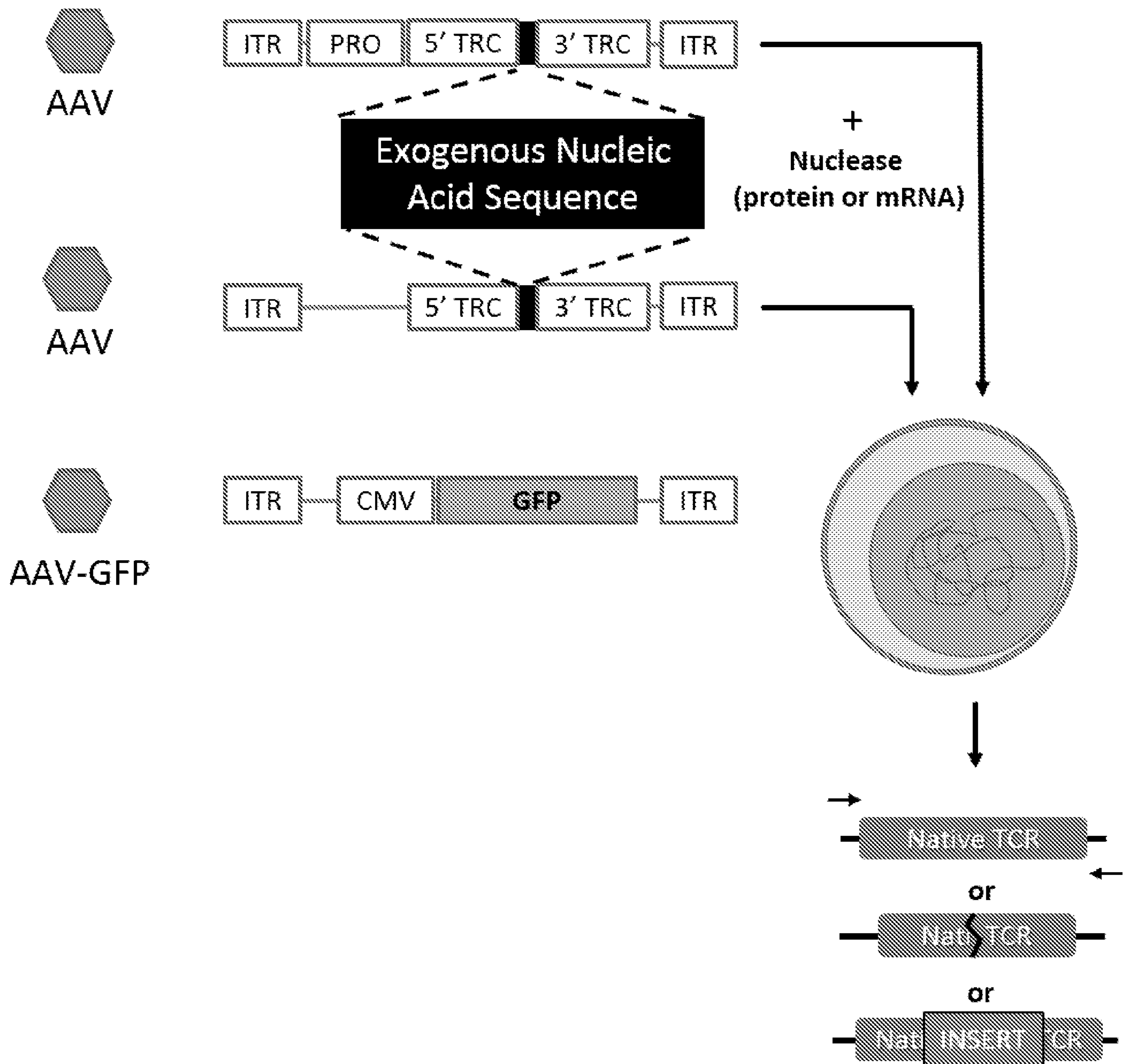


FIGURE 13

SEQ ID		162	233
NO:			
86	Wild-Type	ATGGACTTCAAGAGCAACAGTGCTGTGGCCCTGGAGCAACAATCTGA	CTTTGCATGTGCAAAACGCCCTTCAAC
87	Indel	ATGGACTTCAAGAGCAACAGTGCTGTGGCCCTGGAG--CAAAATCTGACTTTGCA	TGCAAAACGCCCTTCAAC
88	Indel	ATGGACTTCAAGAGCAACA-----AACAAATCTGACTTTGCA	TGCAAAACGCCCTTCAAC
89	Indel	ATGGACTTCAAGAGCAACAGTGCTGTGGCCCTGGAG-----AATCTGACTTTGCA	TGCAAAACGCCCTTCAAC
90	Indel	ATGGACTTCAAGAGCAACAGTGCTGTGGCCCTGGAG-----TCTGACTTTGCA	TGCAAAACGCCCTTCAAC
91	Indel	ATGGACTTCAAGAGCAA-----ACAAATCTGACTTTGCA	TGCAAAACGCCCTTCAAC
92	Indel	ATGGACTTCAAGAGCAACAGTGCTGTGGCCCTGGAG--ACAAATCTGACTTTGCA	TGCAAAACGCCCTTCAAC
93	Indel	ATGGACTTCAAGAGCAACAGTGCTGTGGCCCTGGAGCAA---TCTGACTTTGCA	TGCAAAACGCCCTTCAAC
94	Indel	ATGGACTTCAAGAGCAACAGTGCTGTGGCCCTGGAGCAACGGCAACA	AAATCTGACTTTGCAATGTGCAAAACGCCCTTCAAC
95	Indel	ATGGACTTCAAGAGCAACAGTGCTGTGGCCCTGGAGCAAAAGAA	CAAAATCTGACTTTGCAATGTGCAAAACGCCCTTCAAC

FIGURE 14

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**FIGURE 15**

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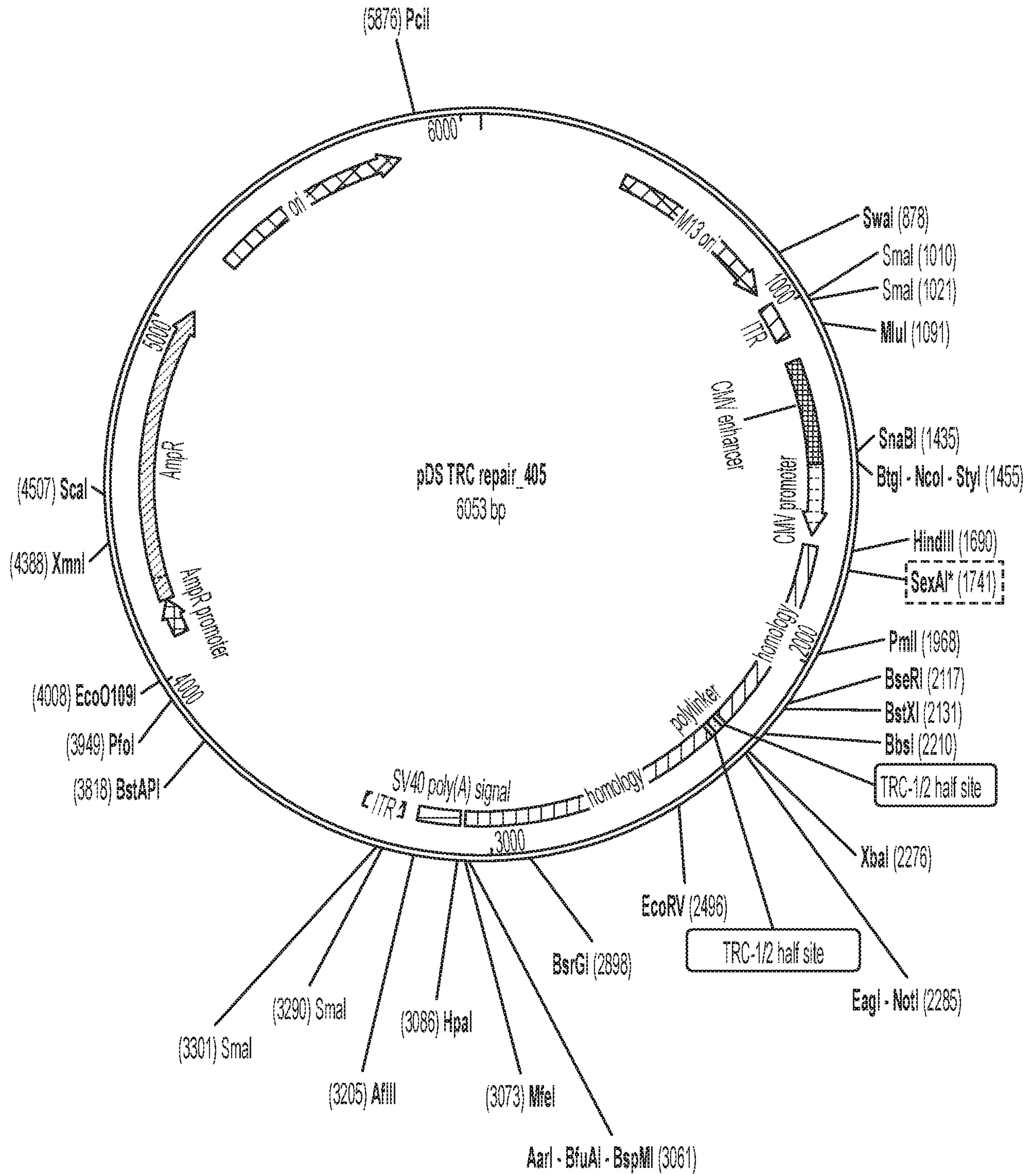


FIGURE 16

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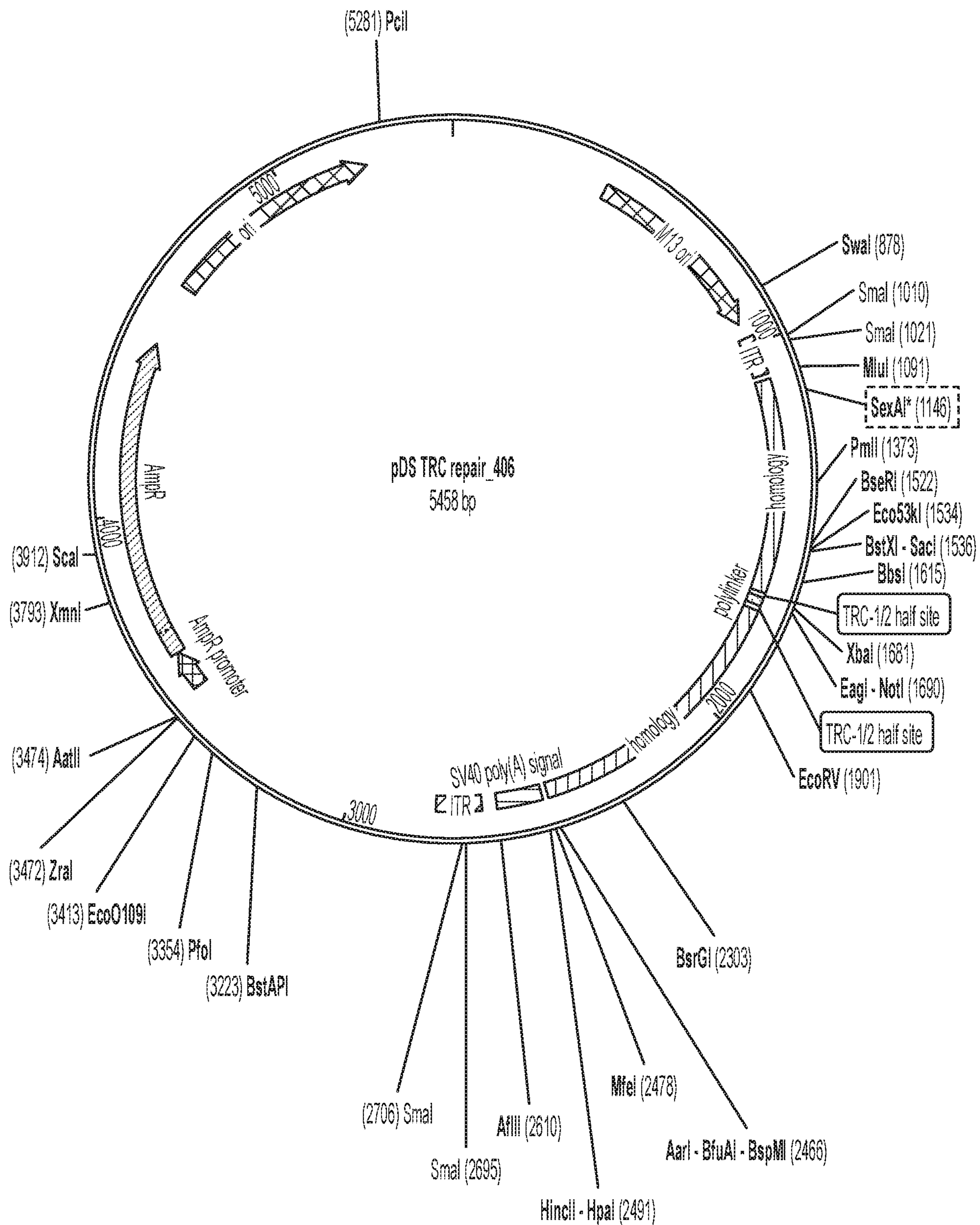
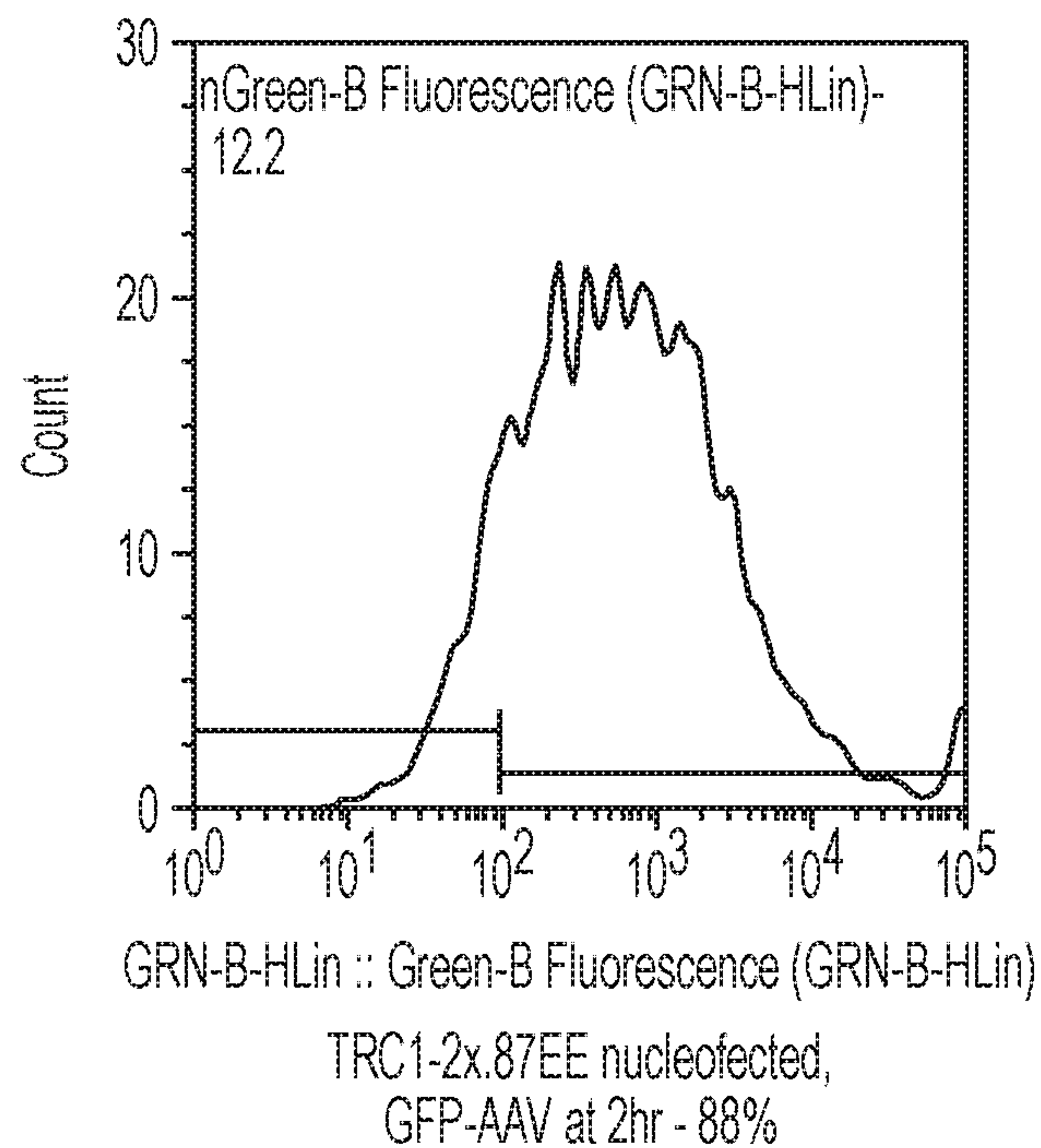


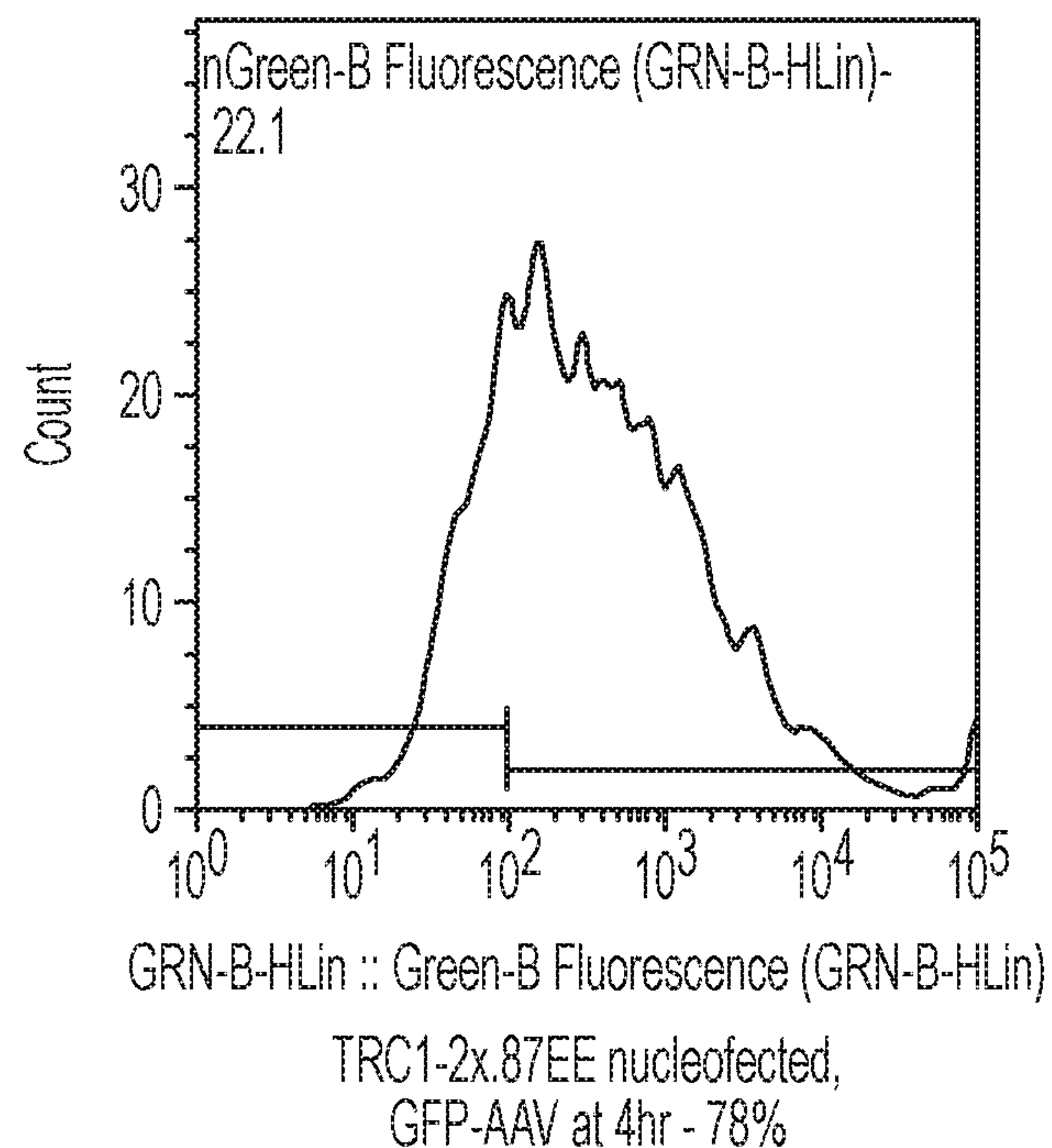
FIGURE 17

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A.



B.



C.

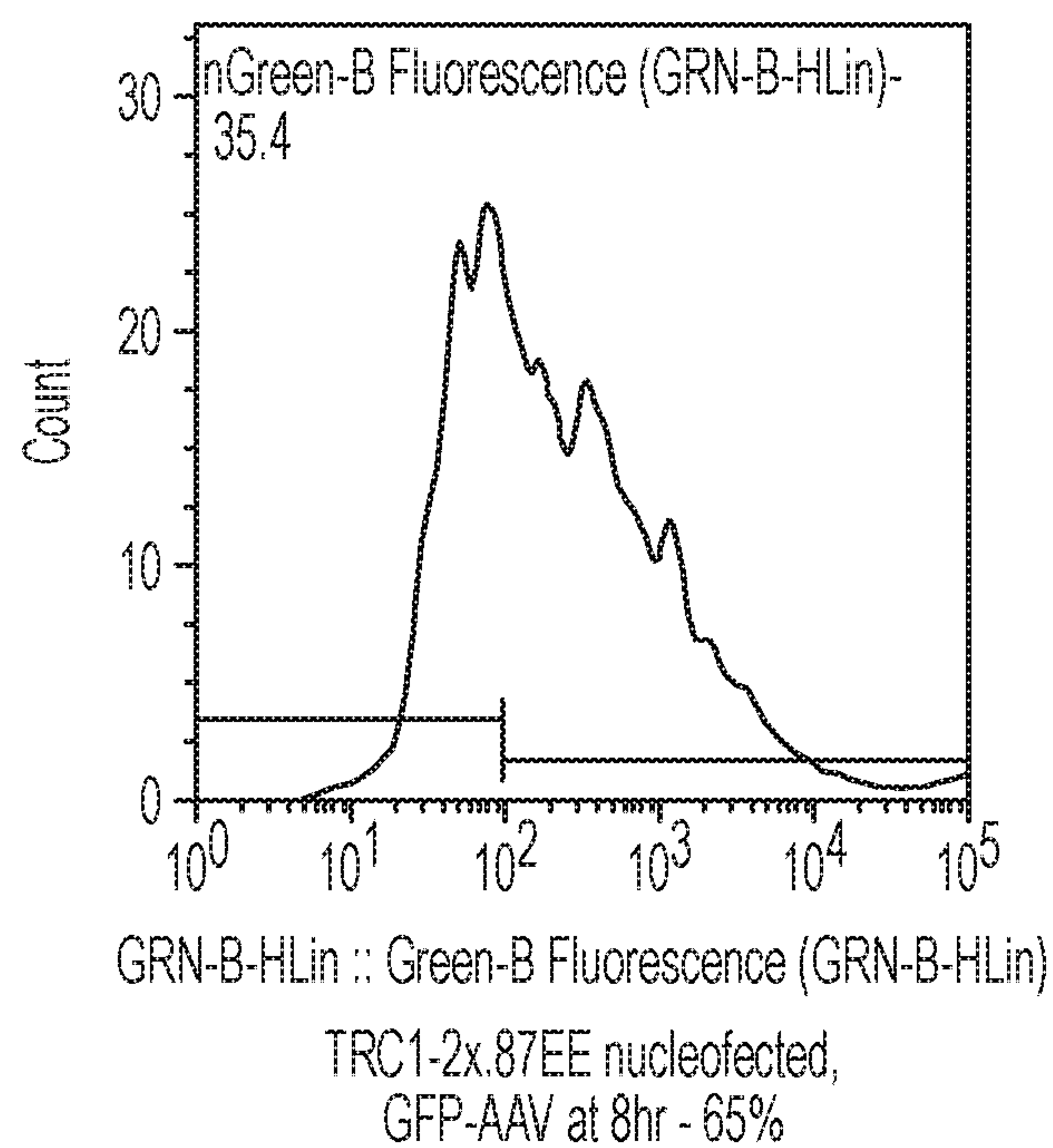


FIGURE 18

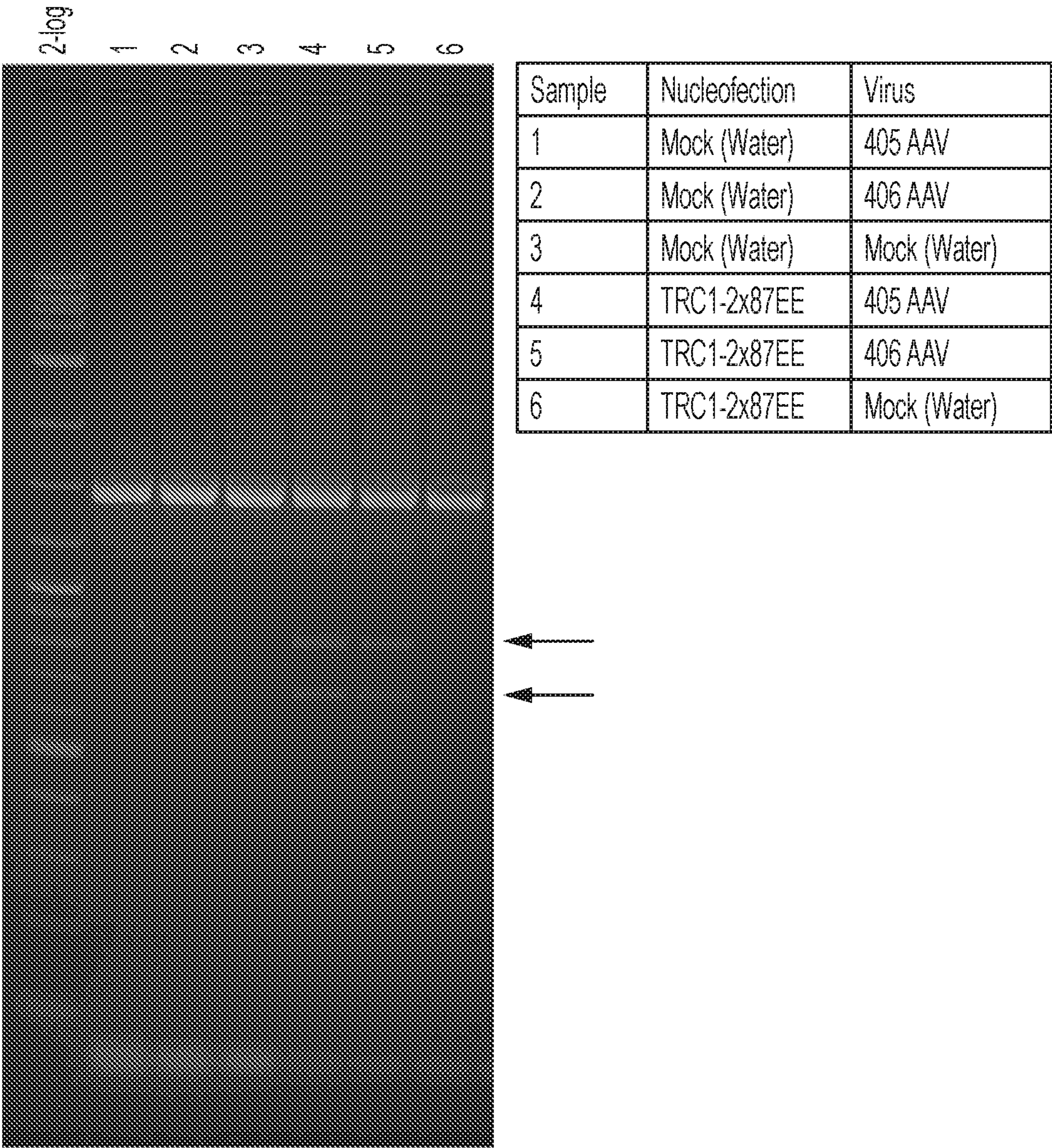
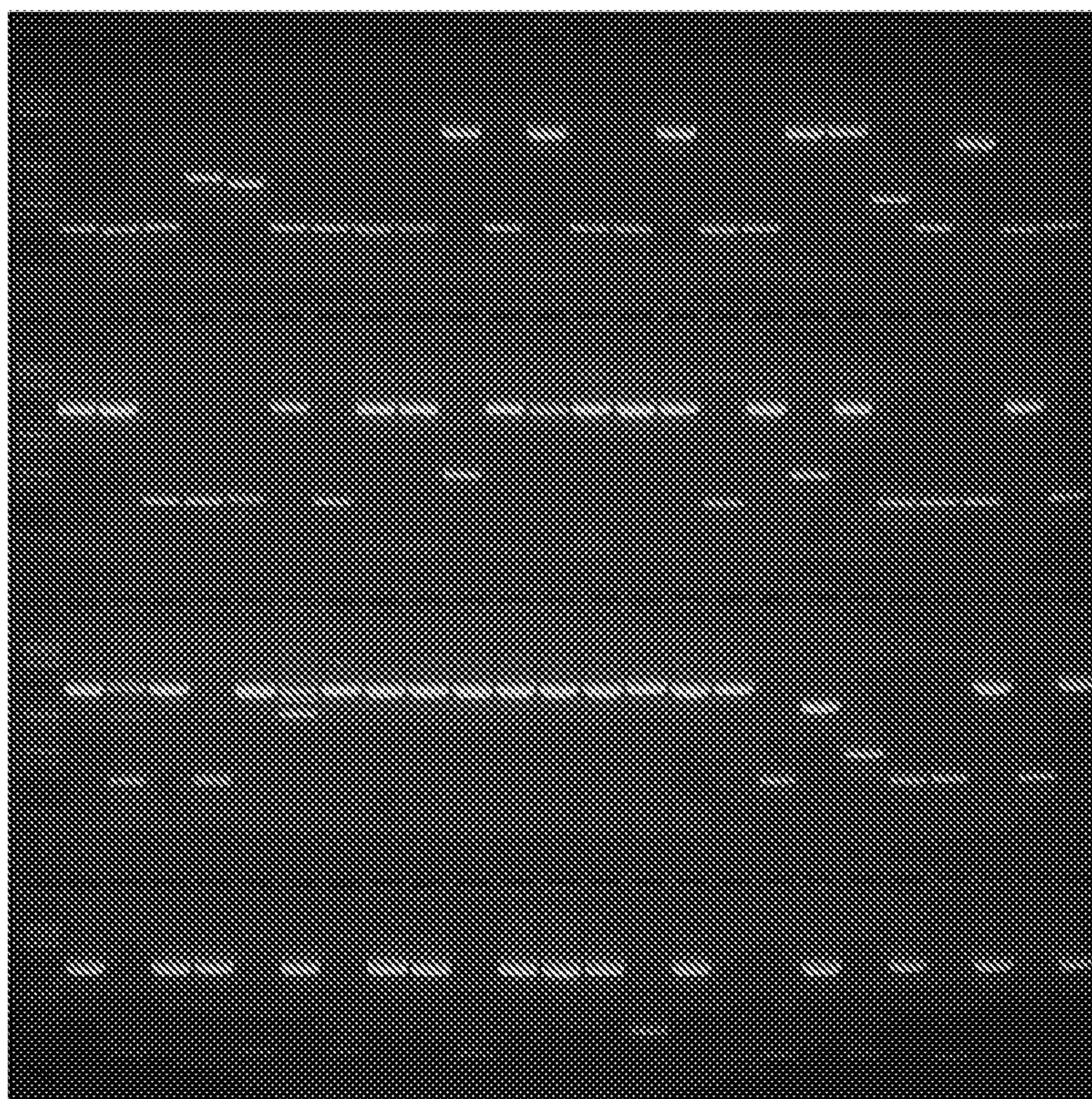


FIGURE 19

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A.



B.

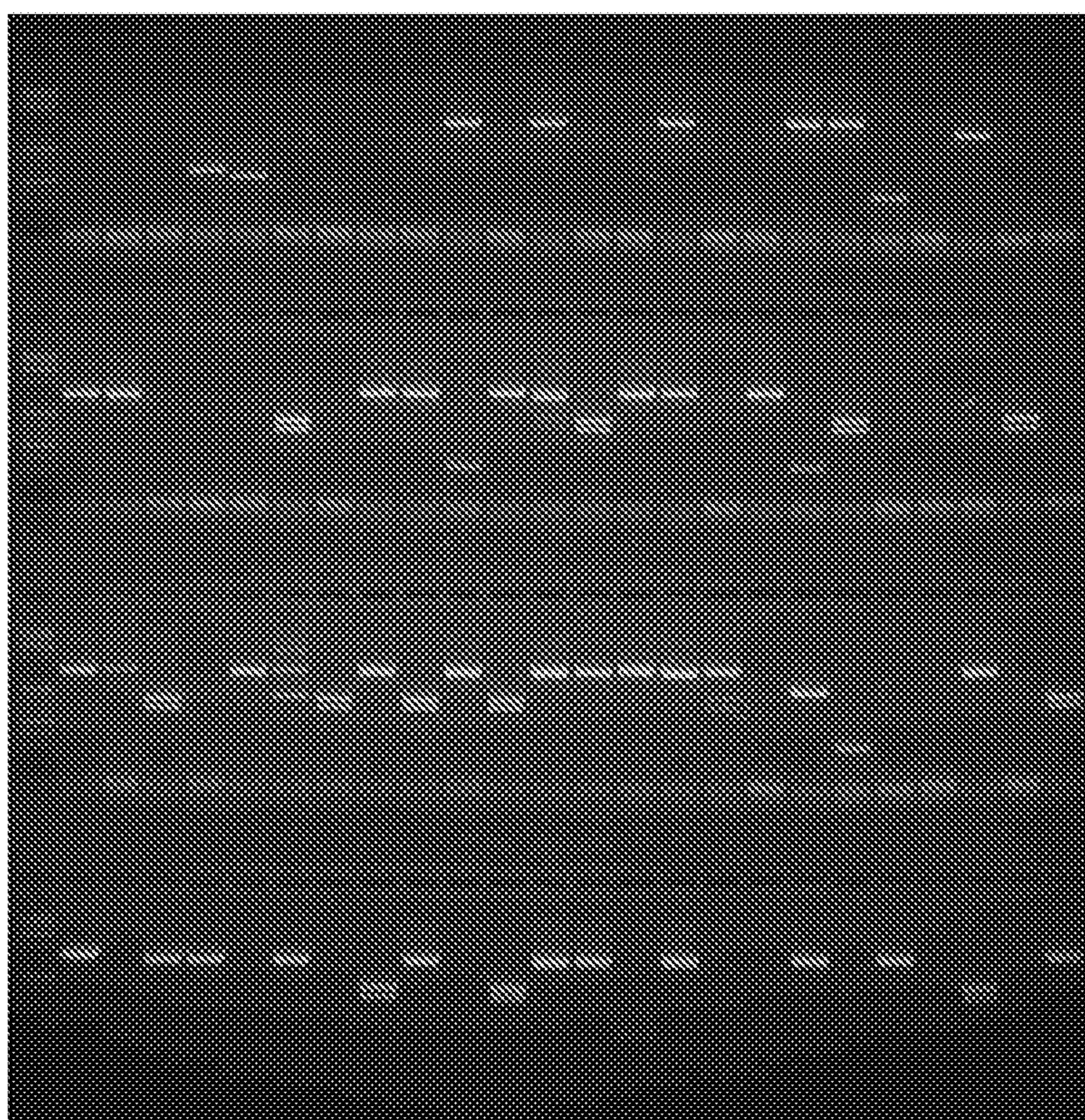
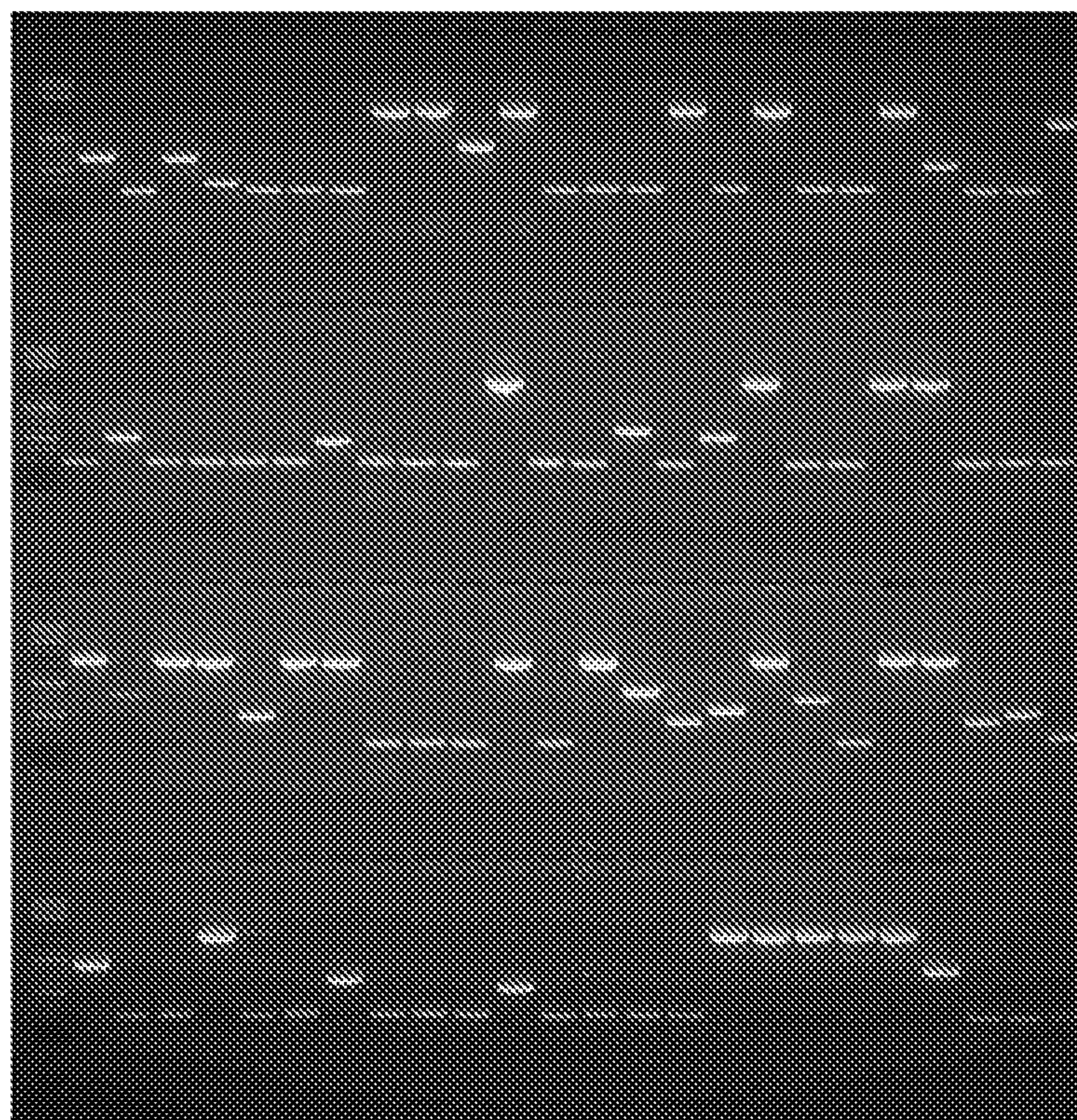


FIGURE 20

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A.



B.

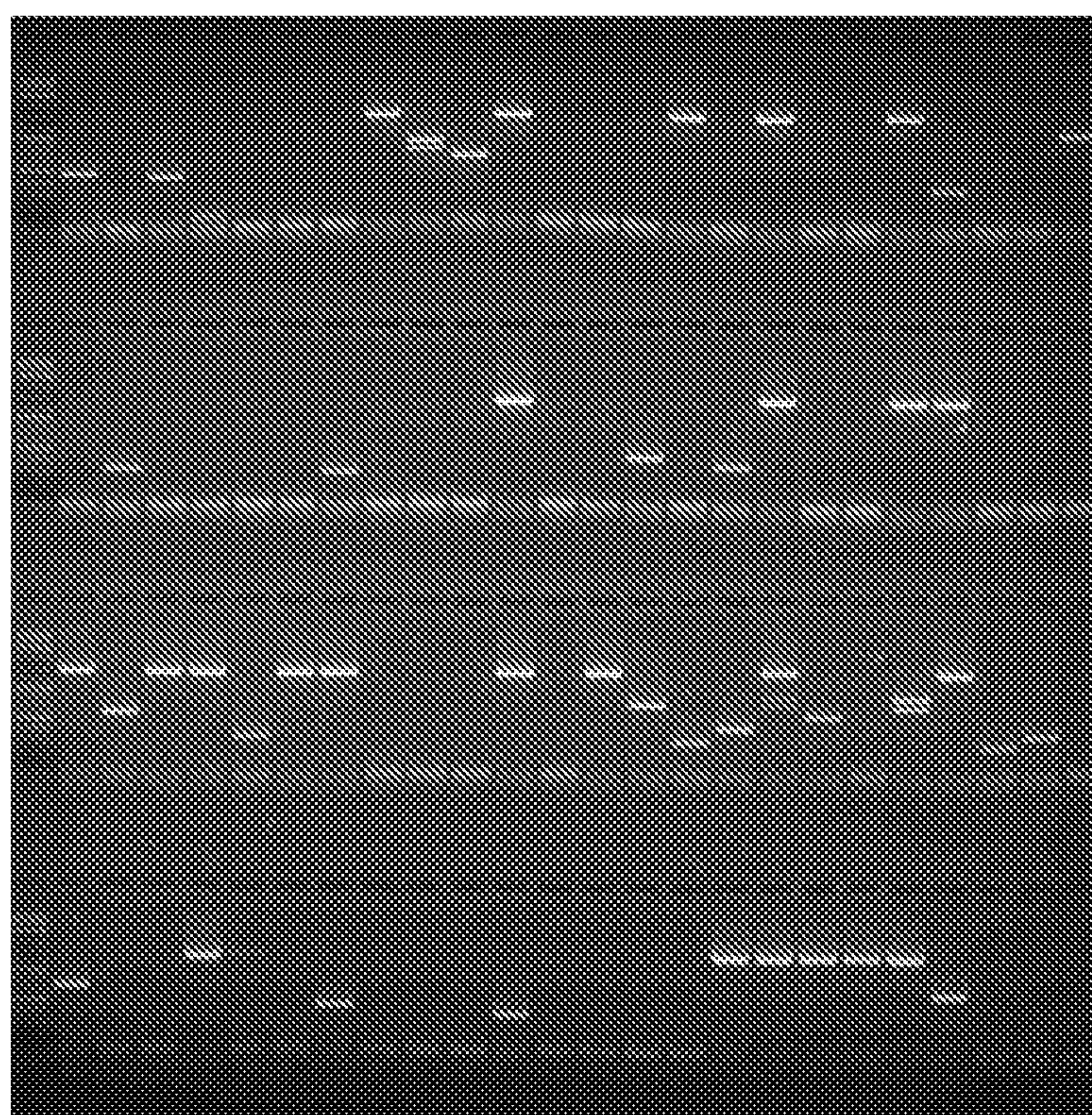


FIGURE 21

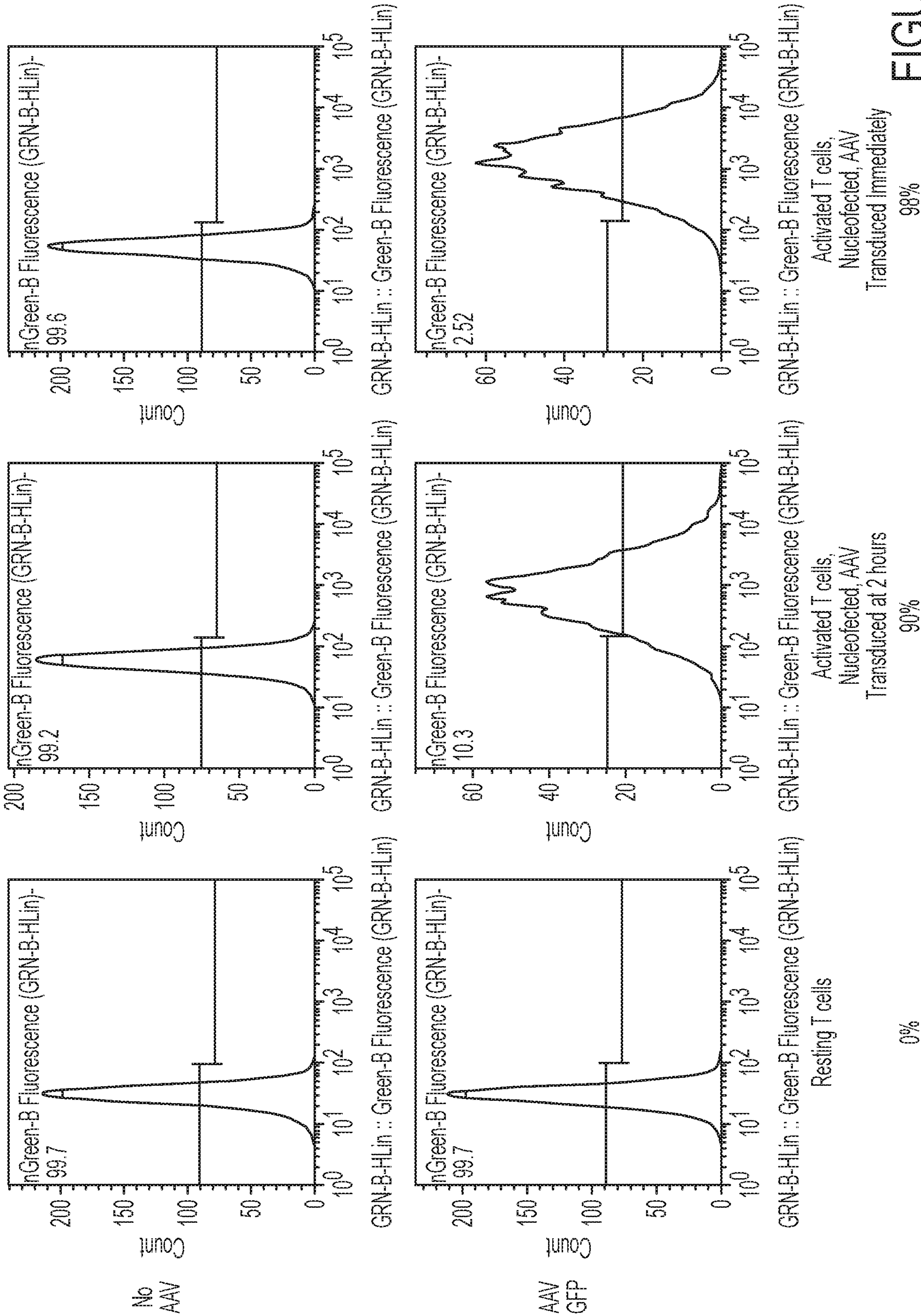


FIGURE 23

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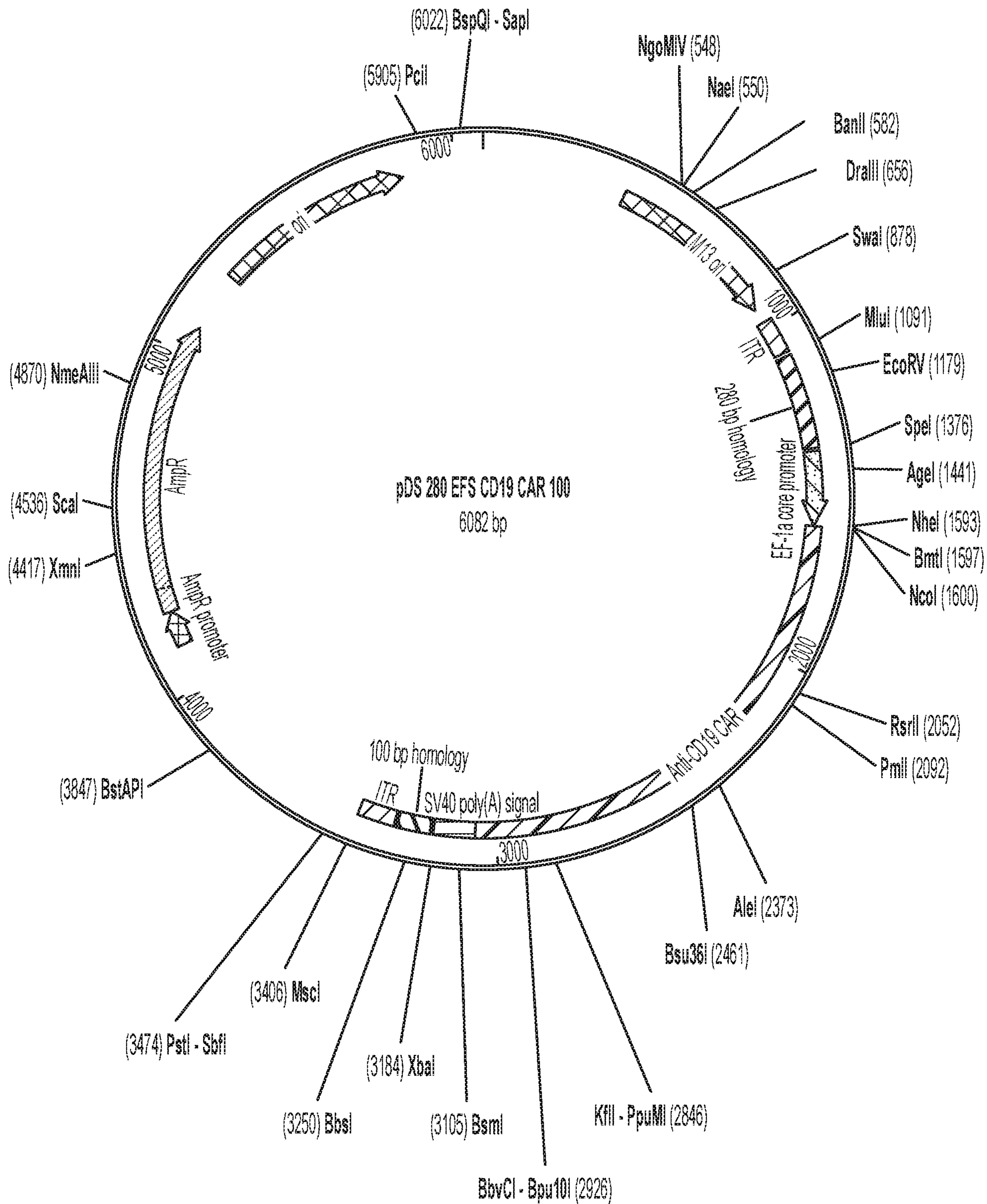


FIGURE 24

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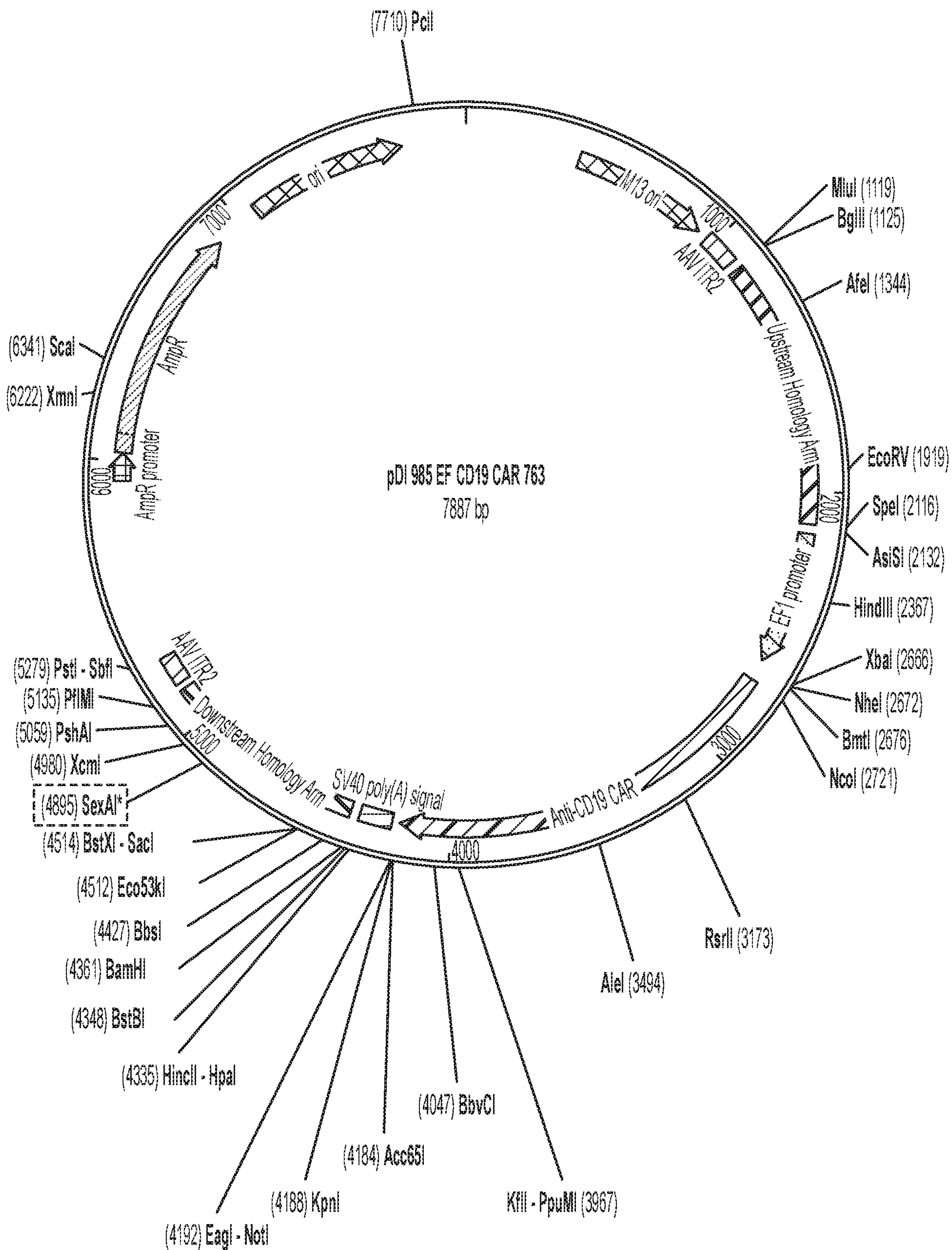


FIGURE 25

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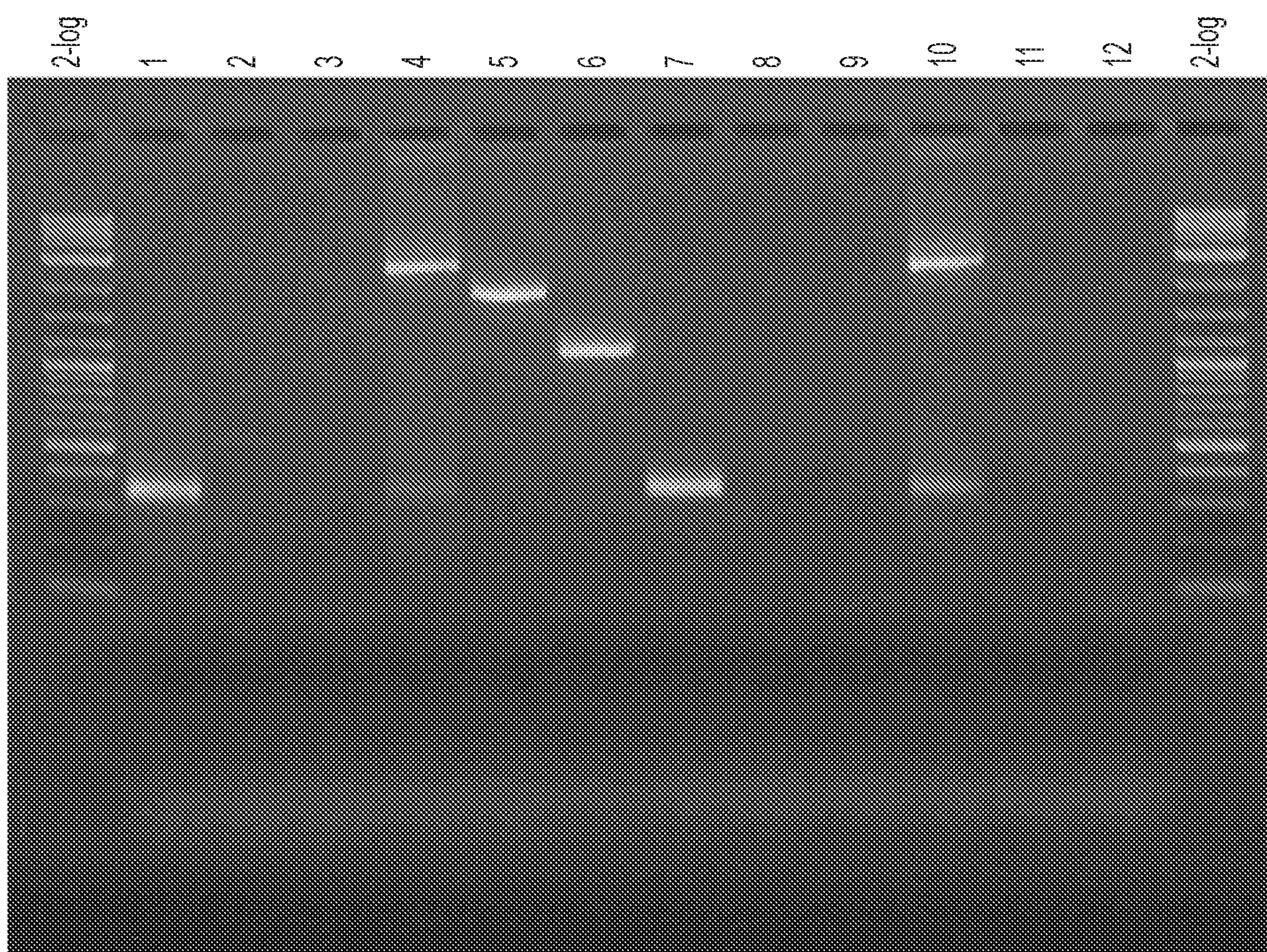
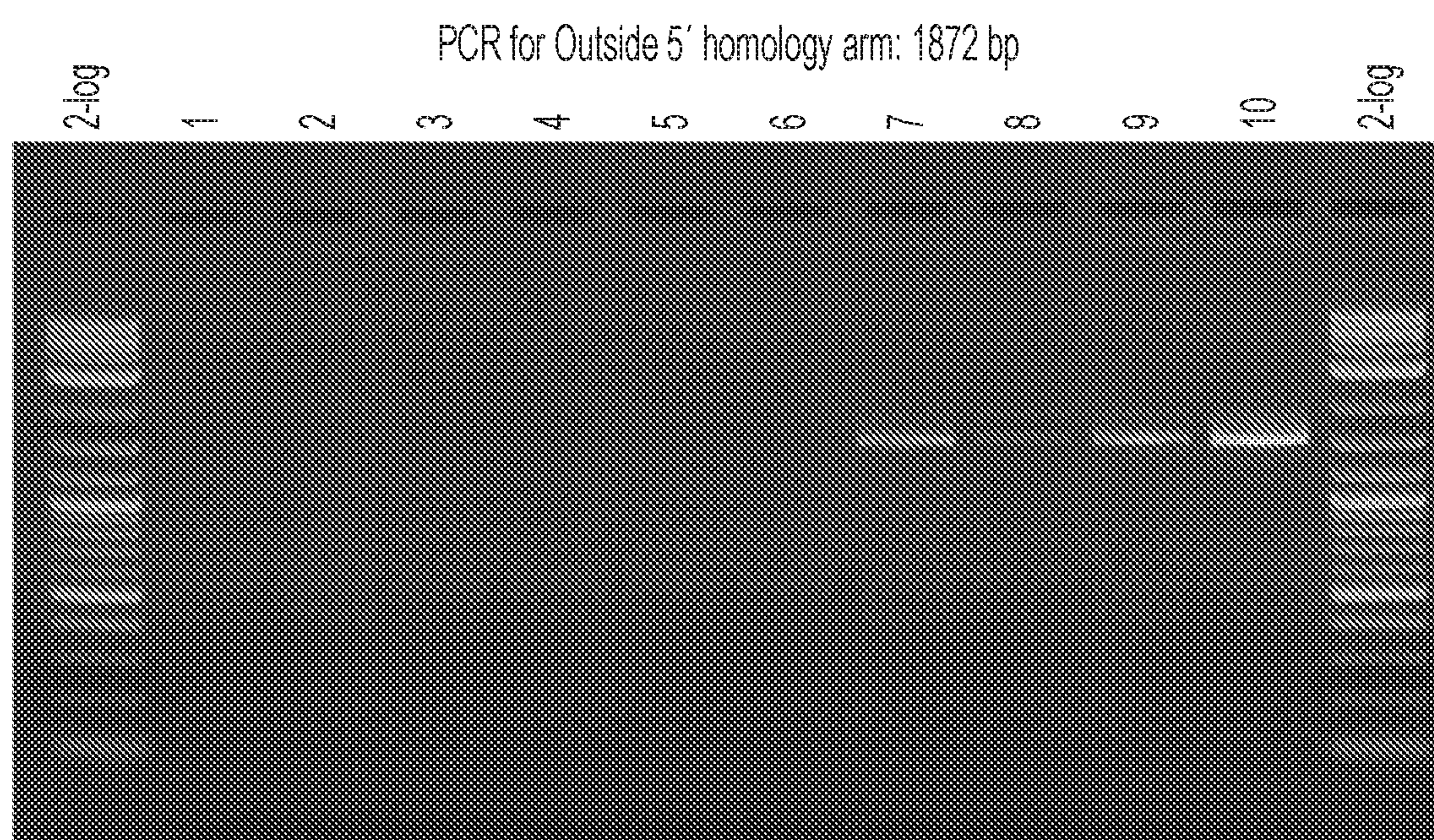


FIGURE 26

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A.



B.

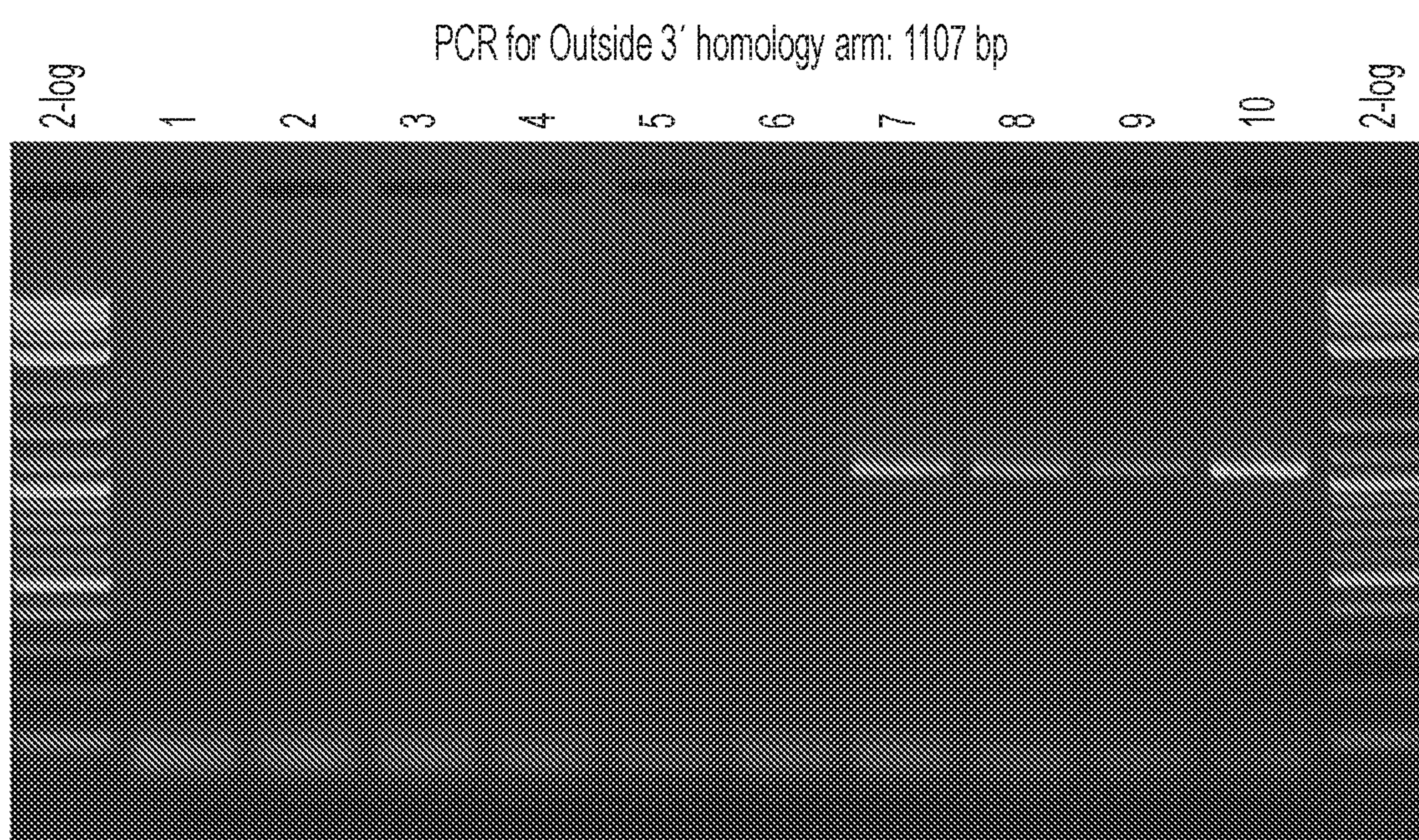
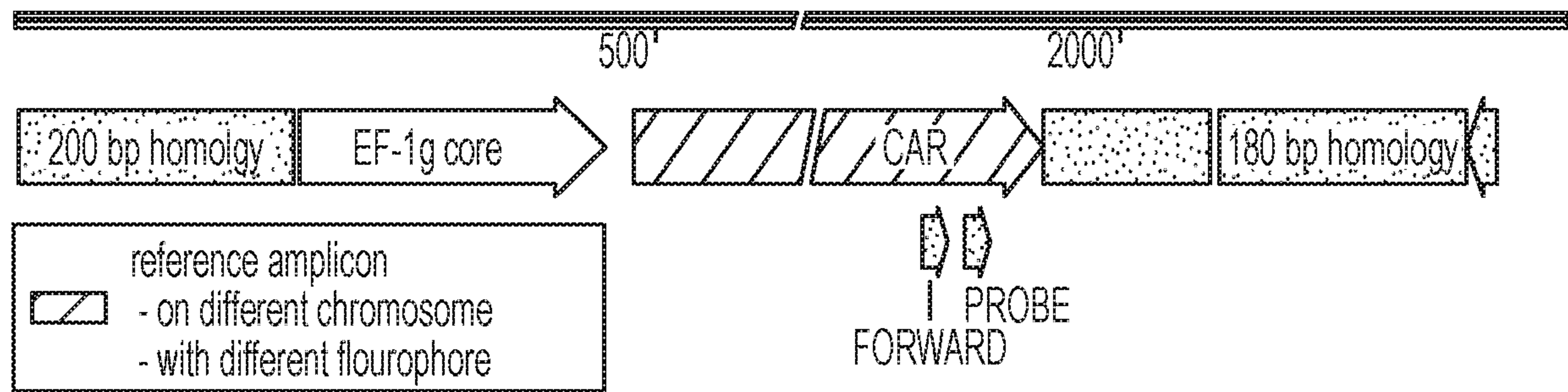


FIGURE 27

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A.



B.

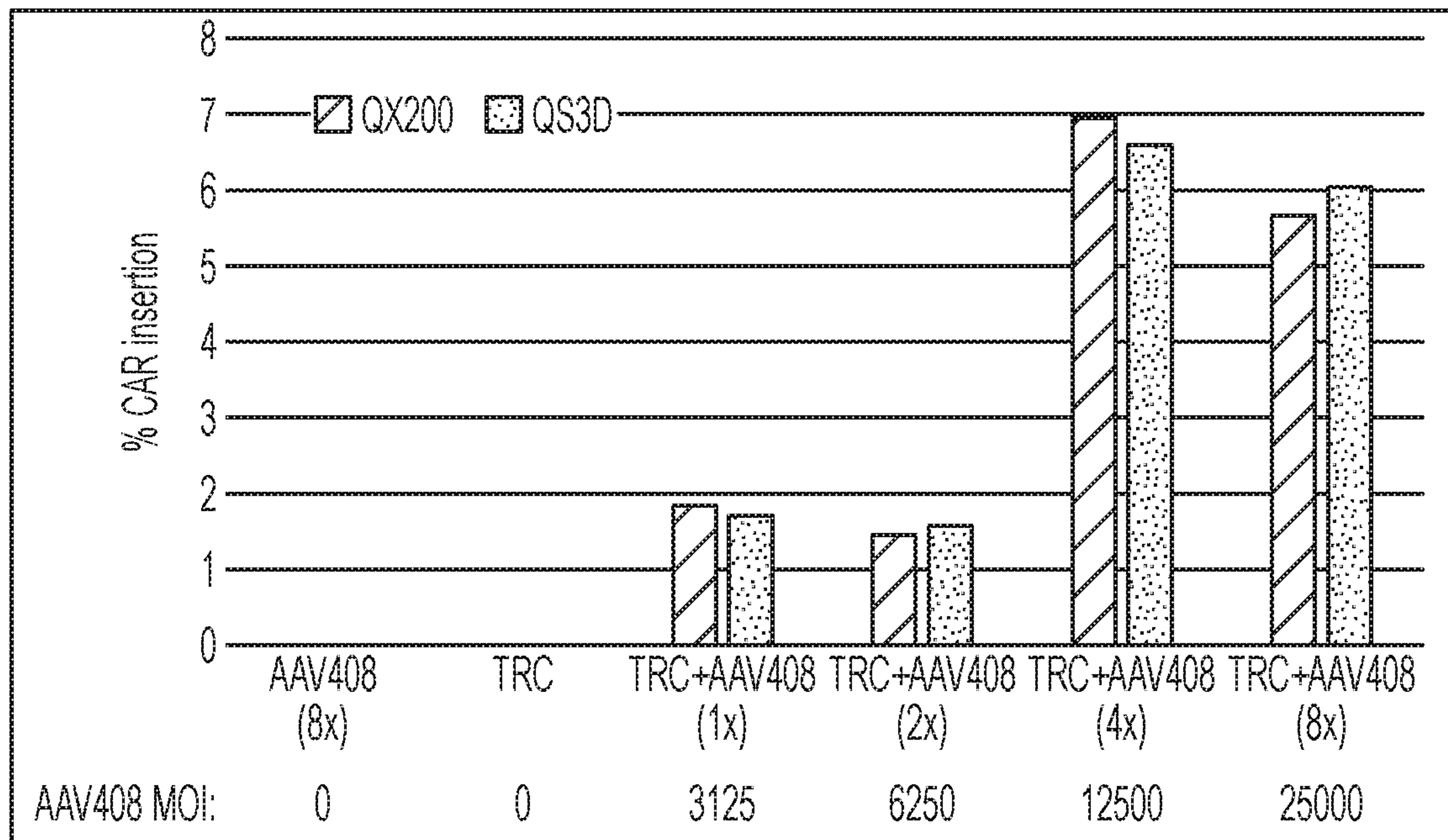


FIGURE 28

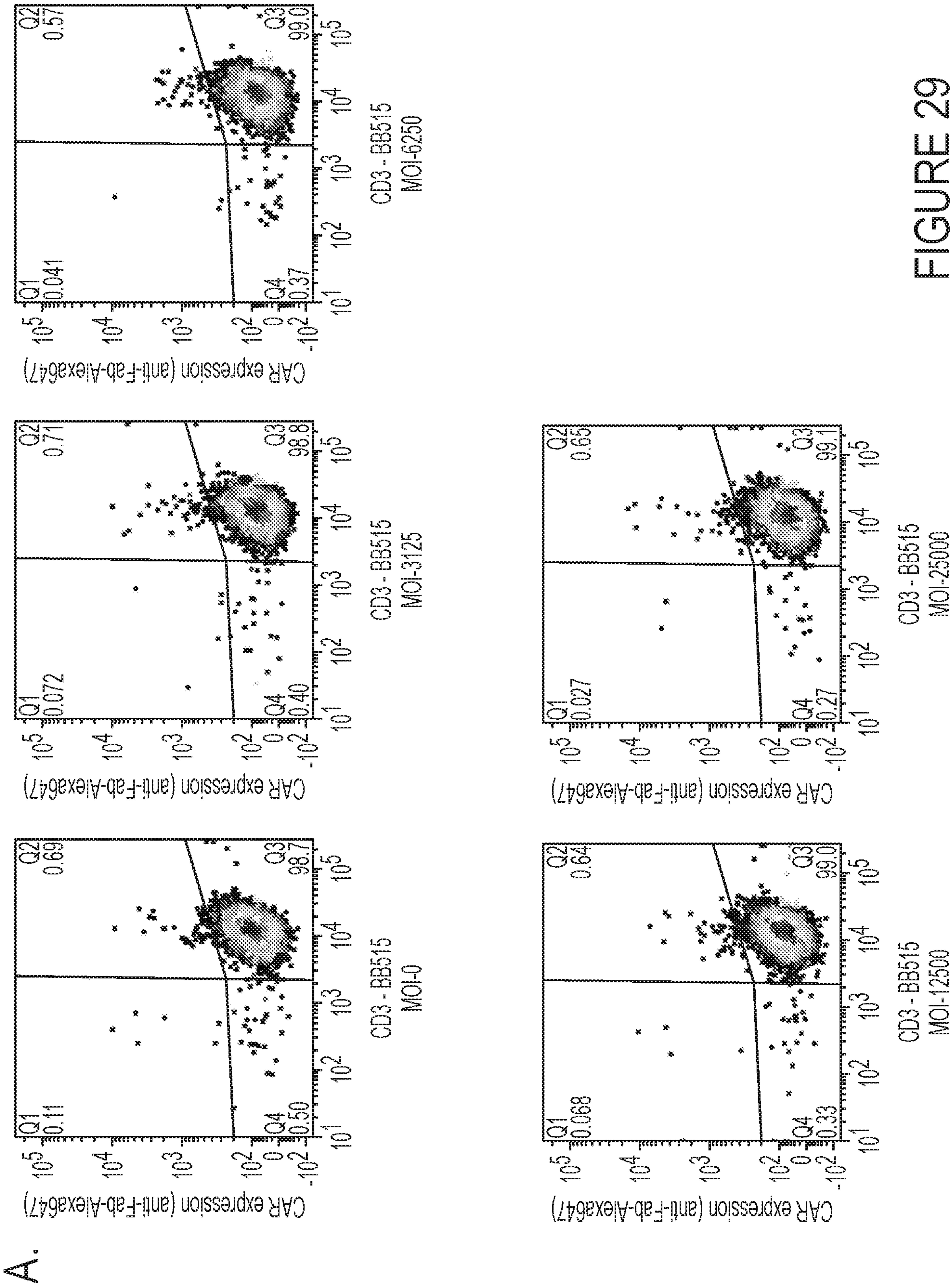


FIGURE 29

B.

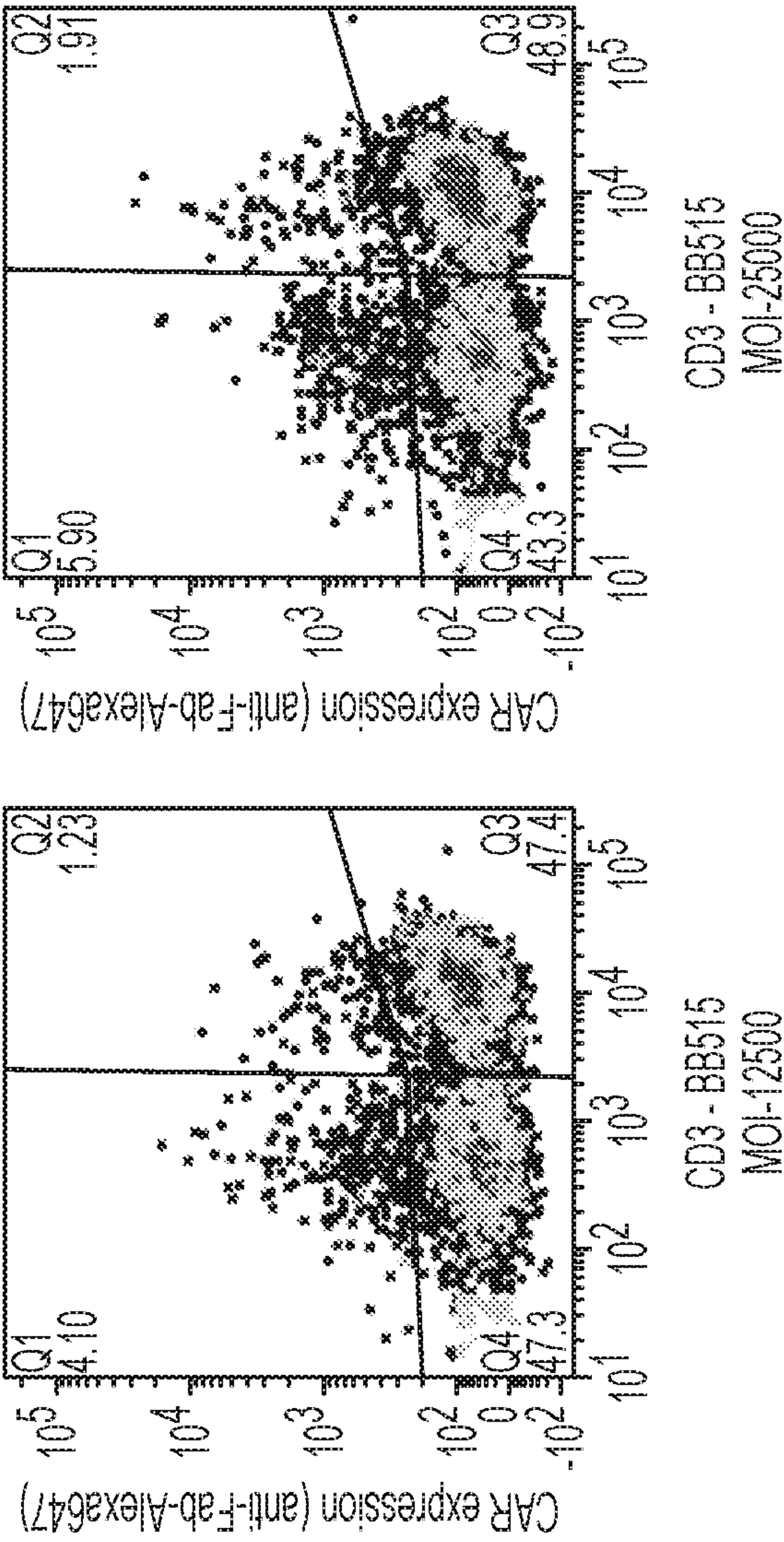
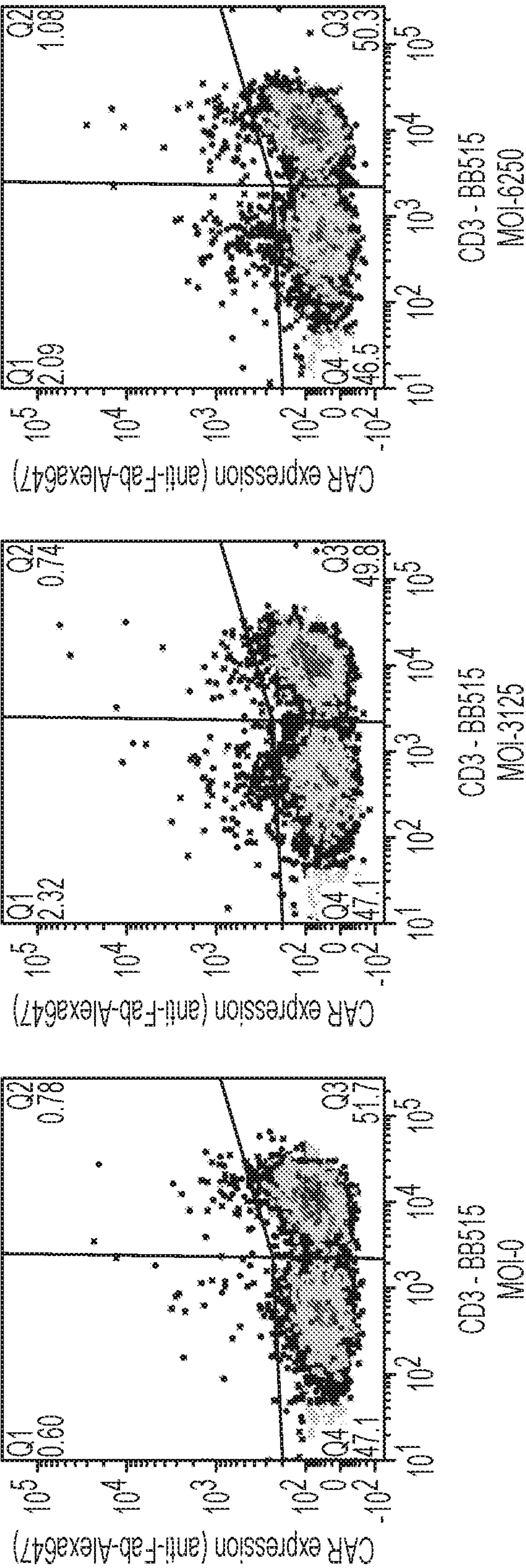


FIGURE 29
CONTINUED

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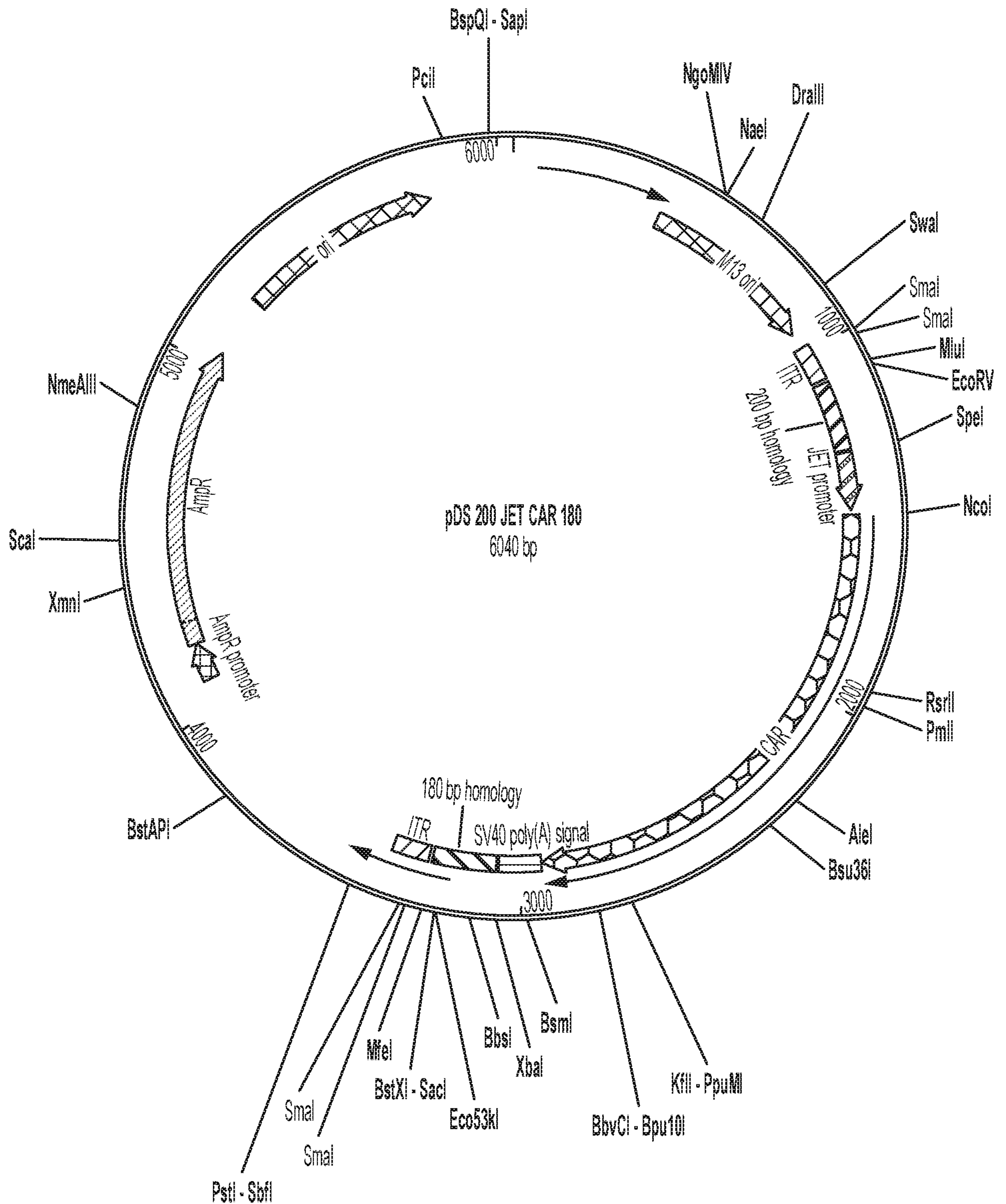


FIGURE 30

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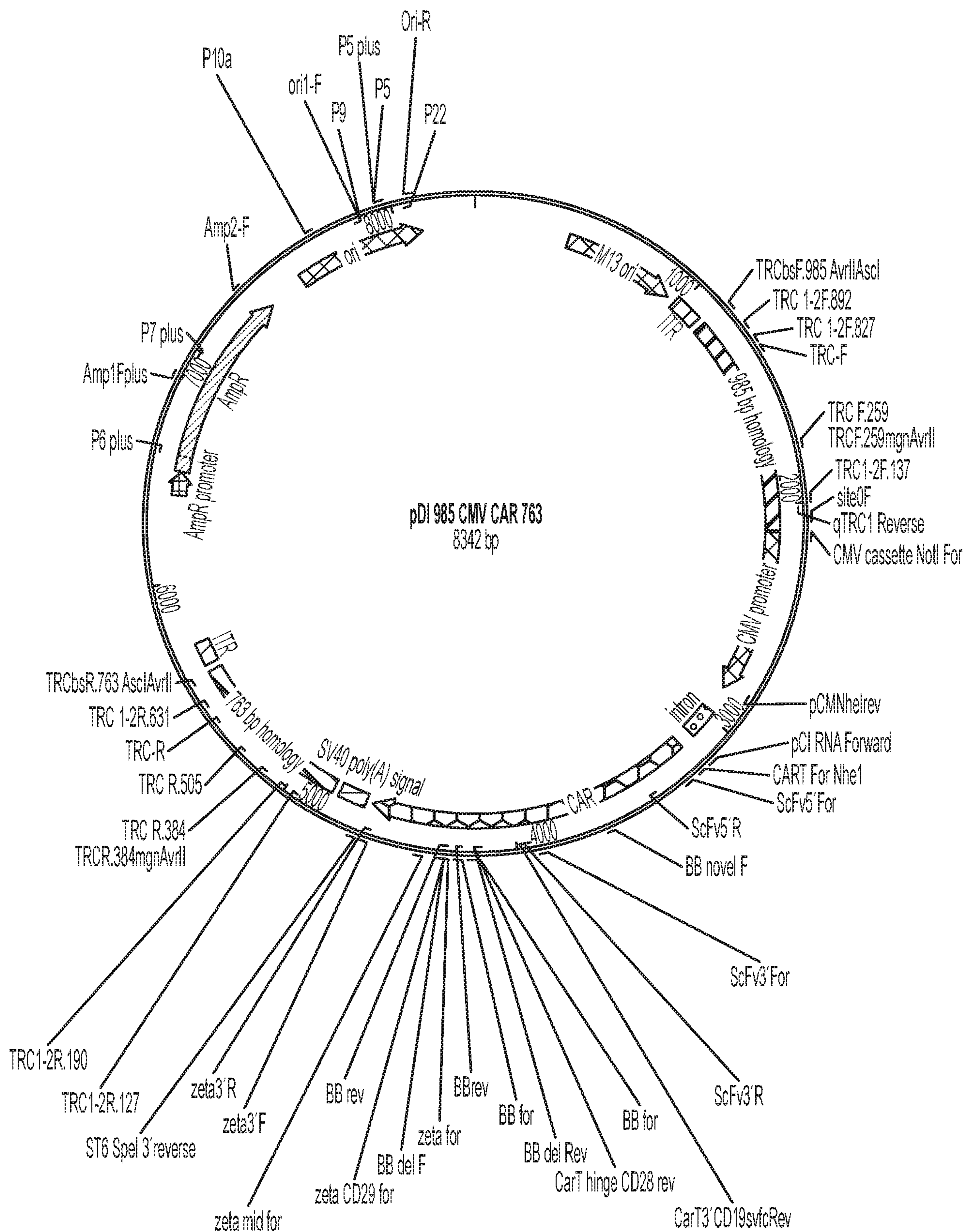


FIGURE 31

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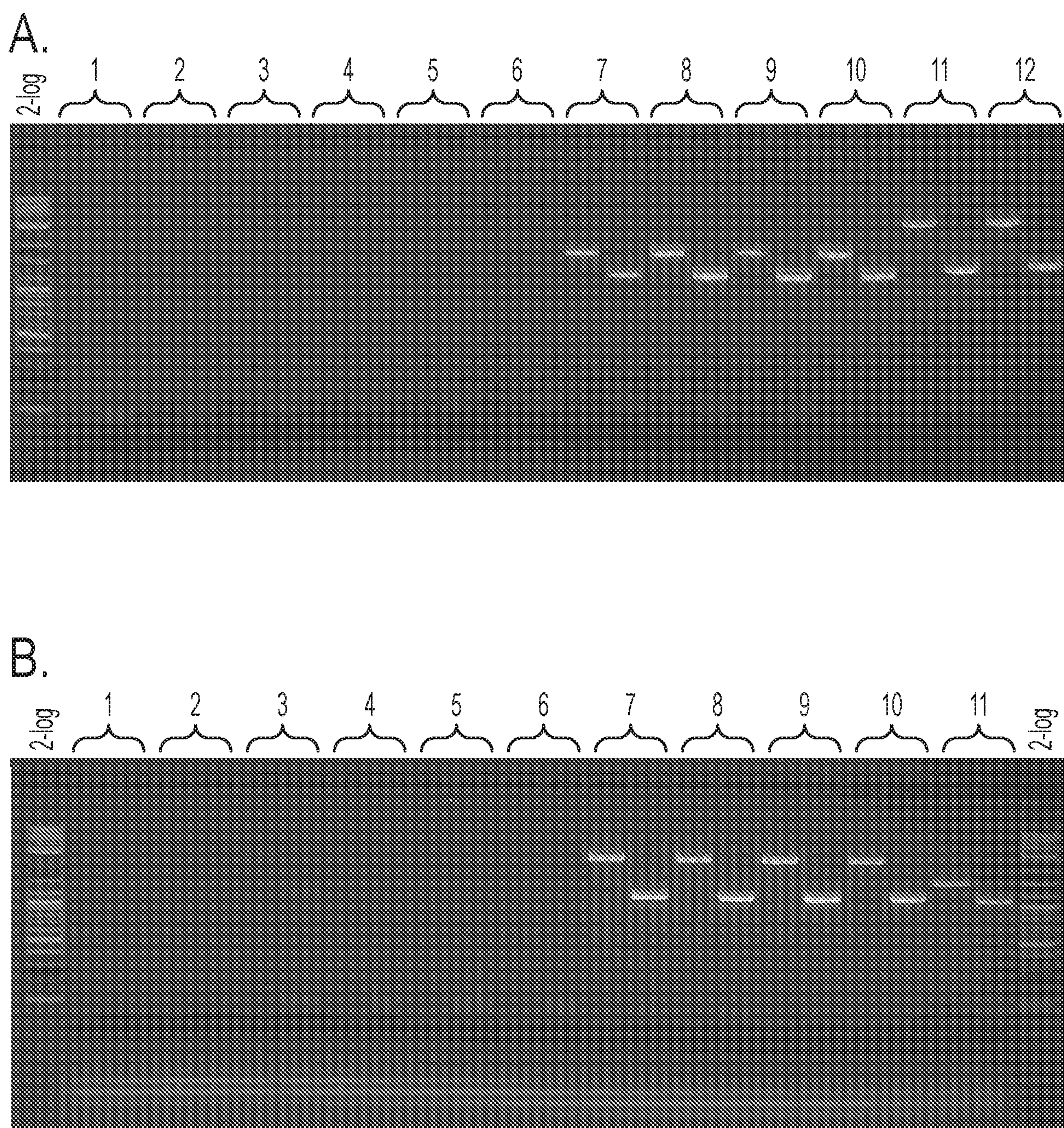


FIGURE 32

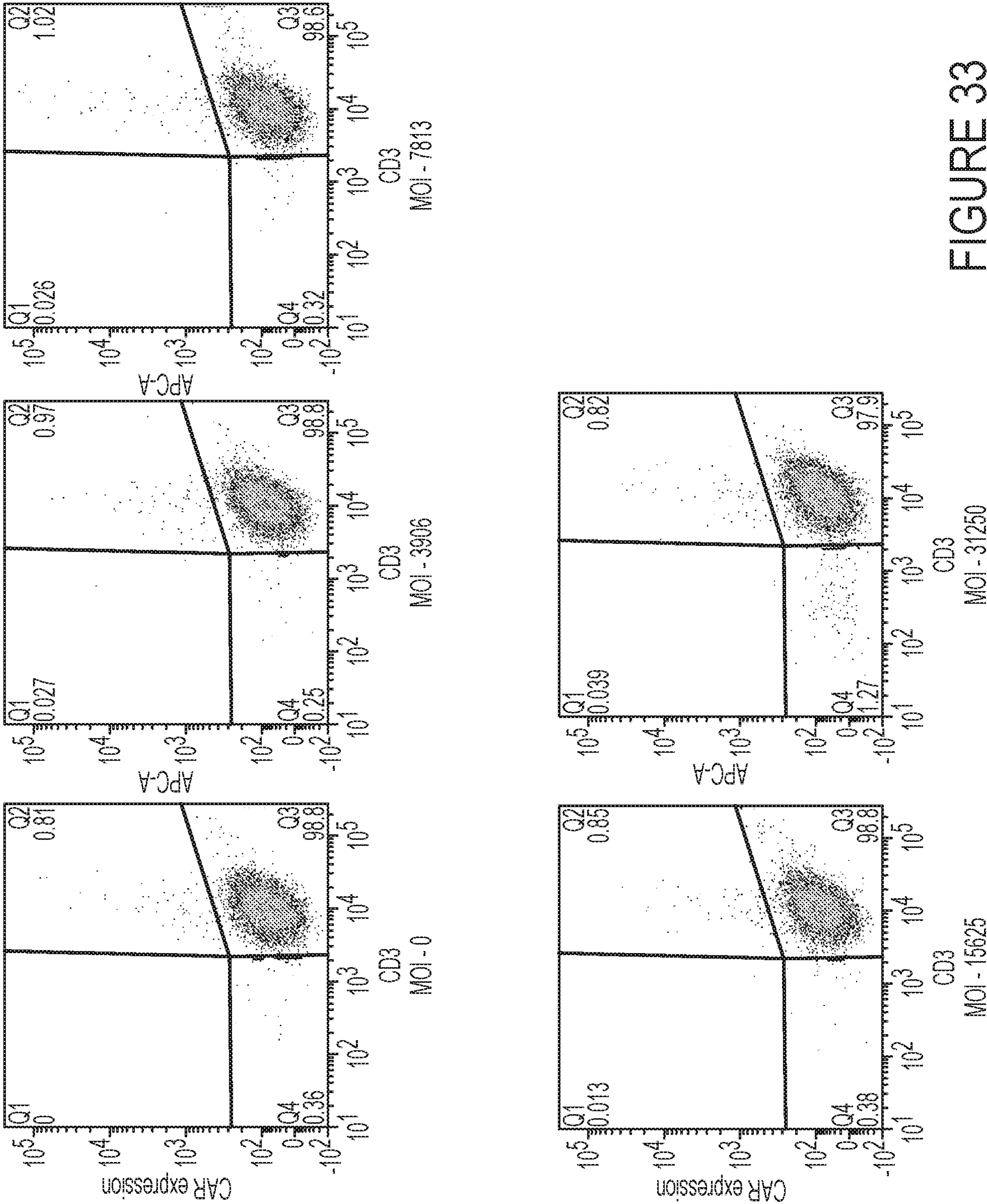


FIGURE 33

B.

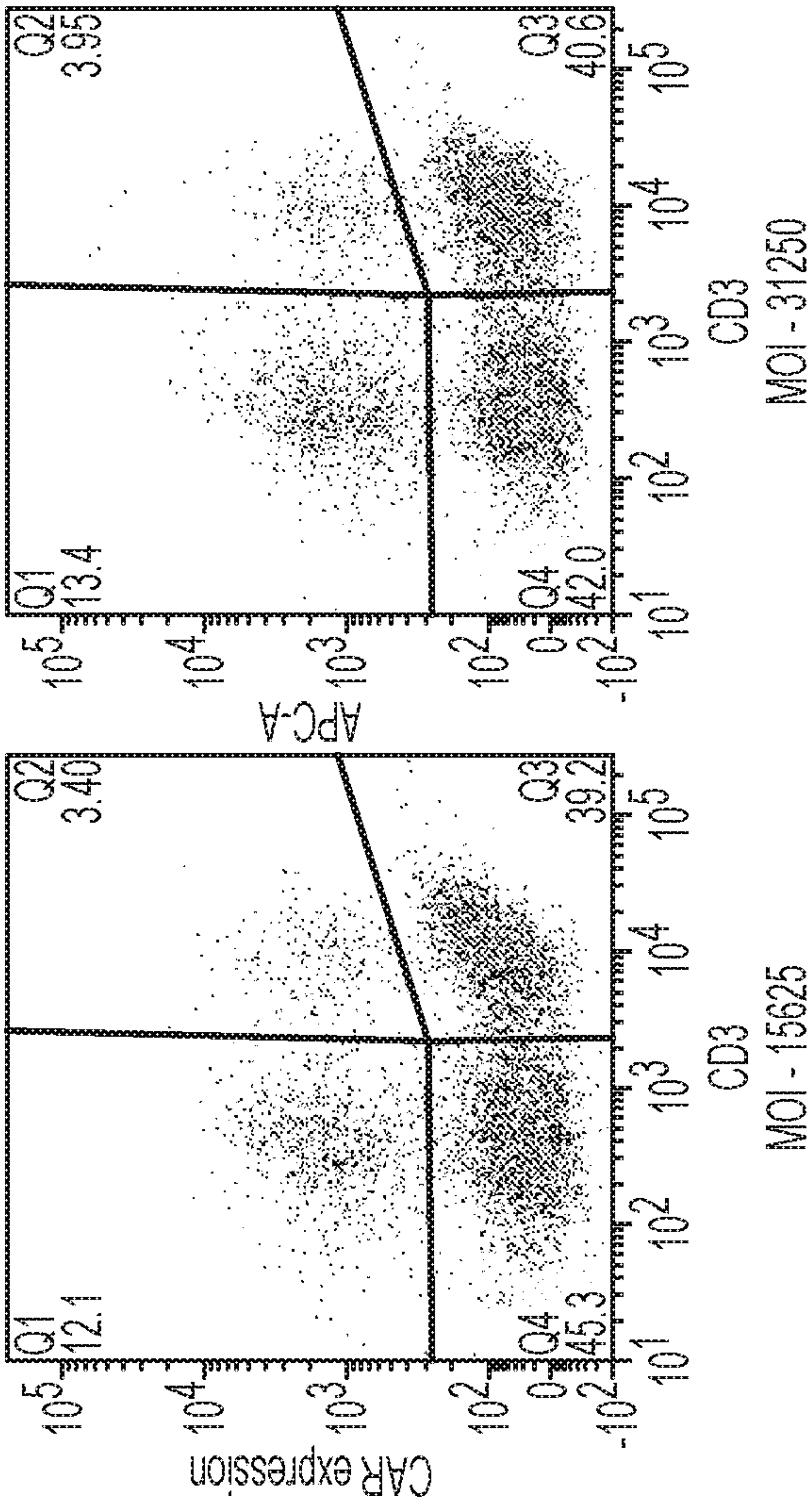
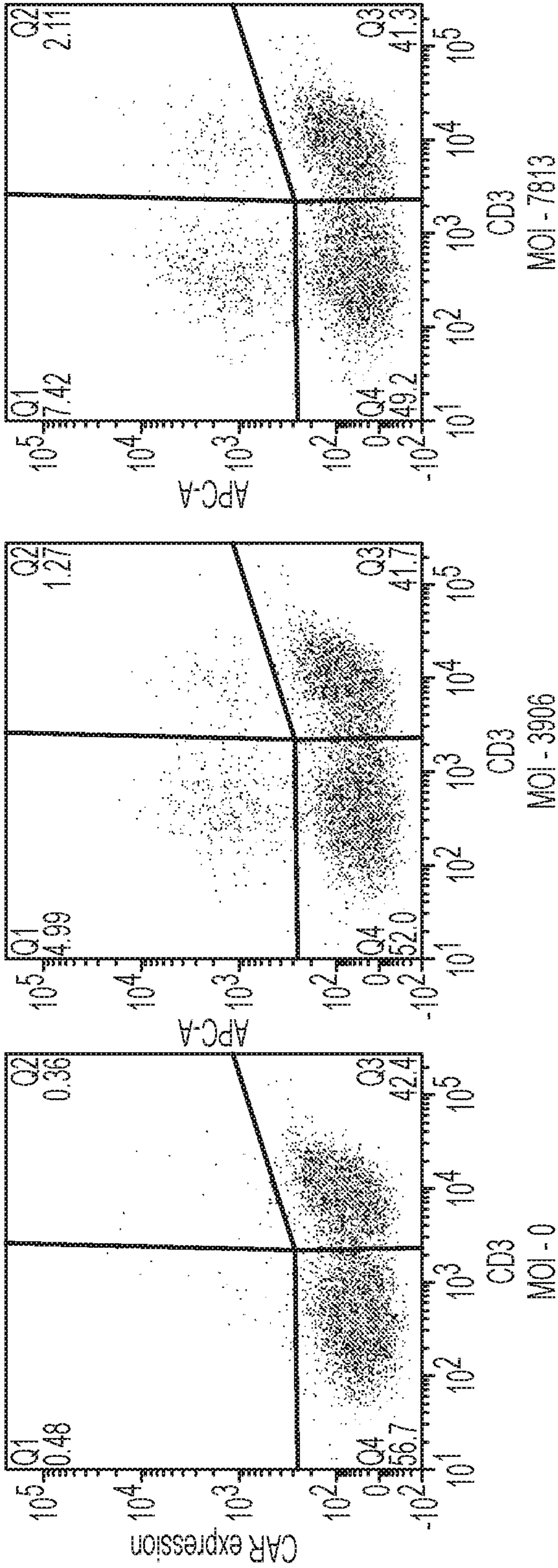


FIGURE 33
CONTINUED

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A.

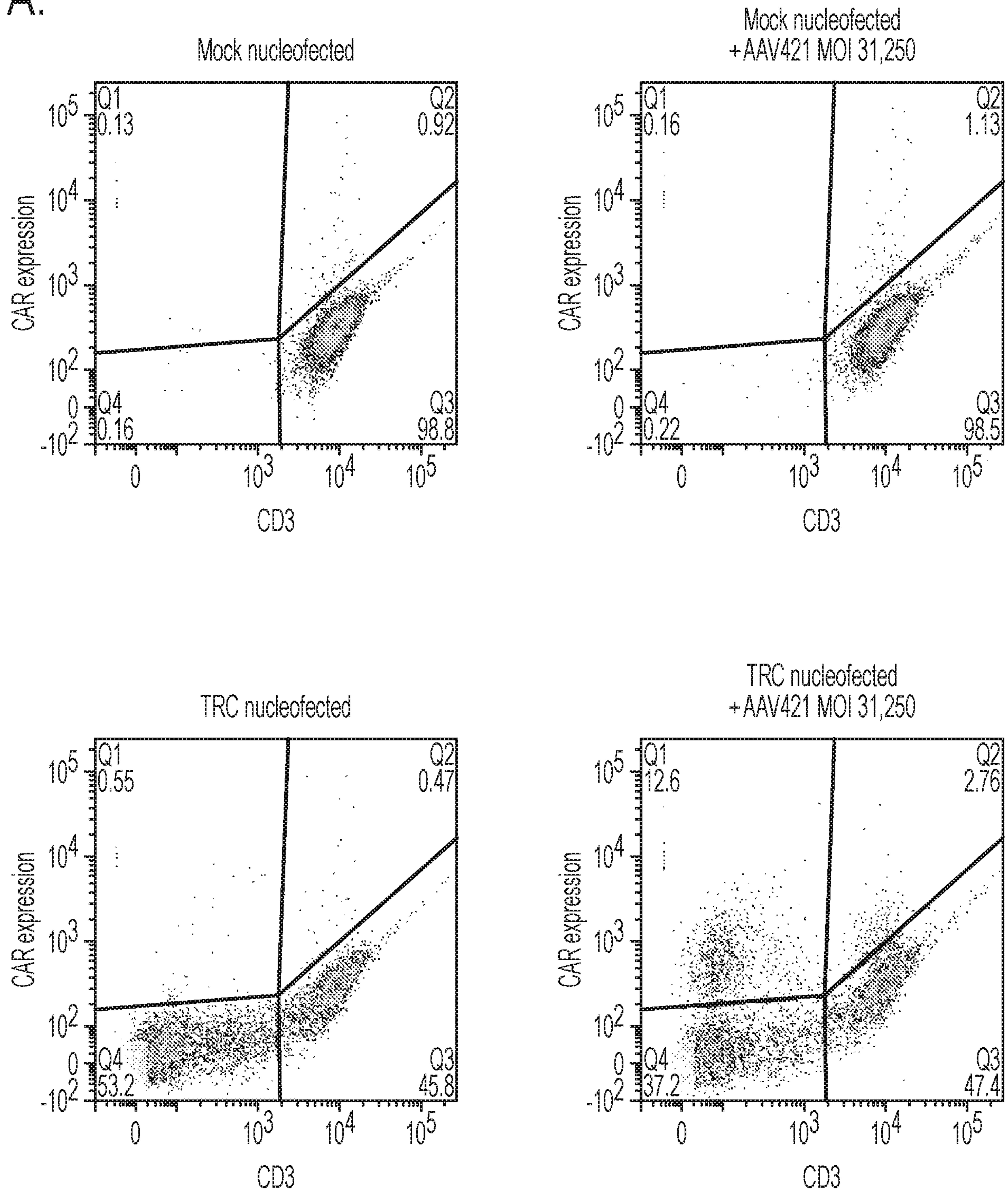
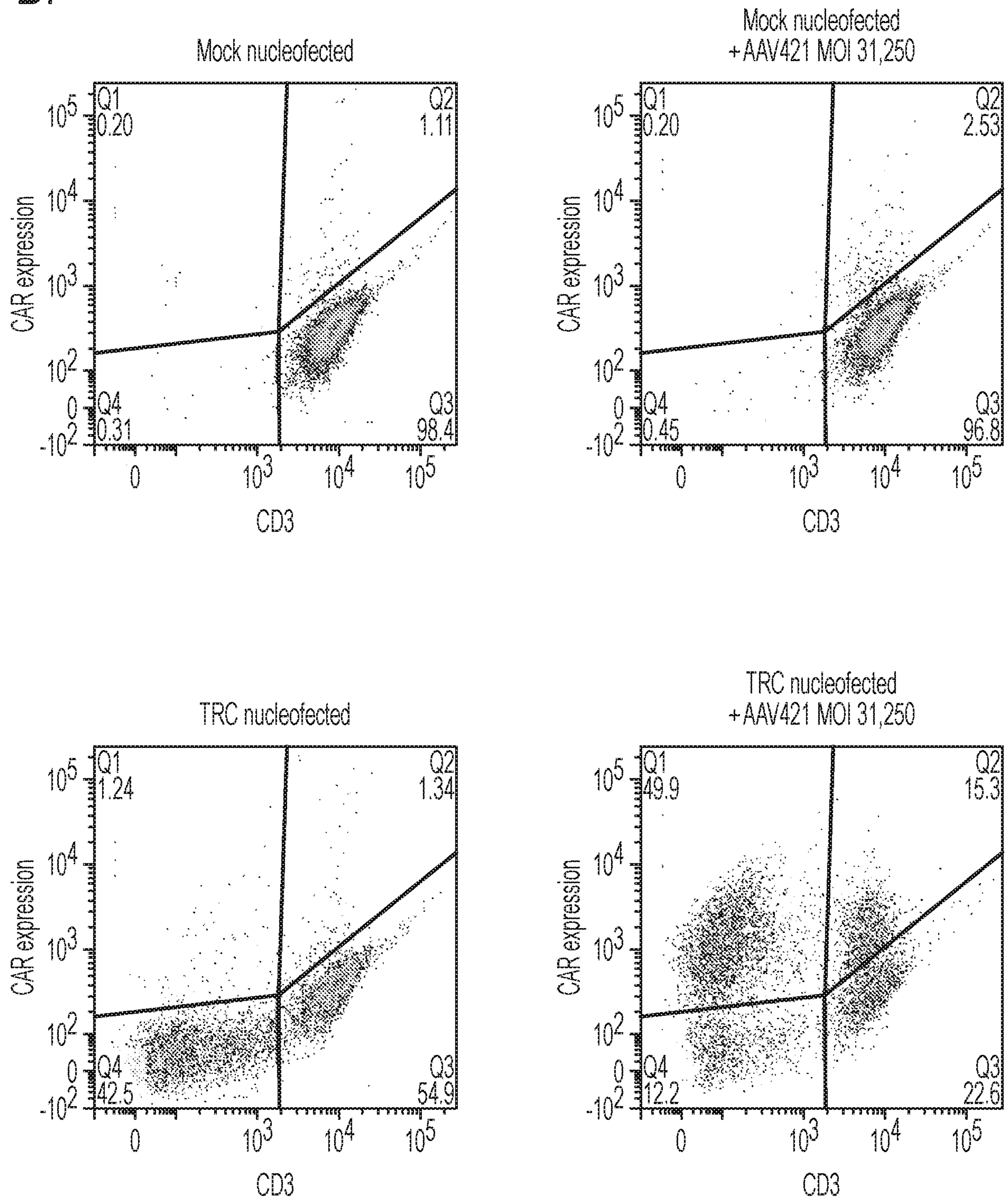


FIGURE 34

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B.

FIGURE 34
CONTINUED

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C.

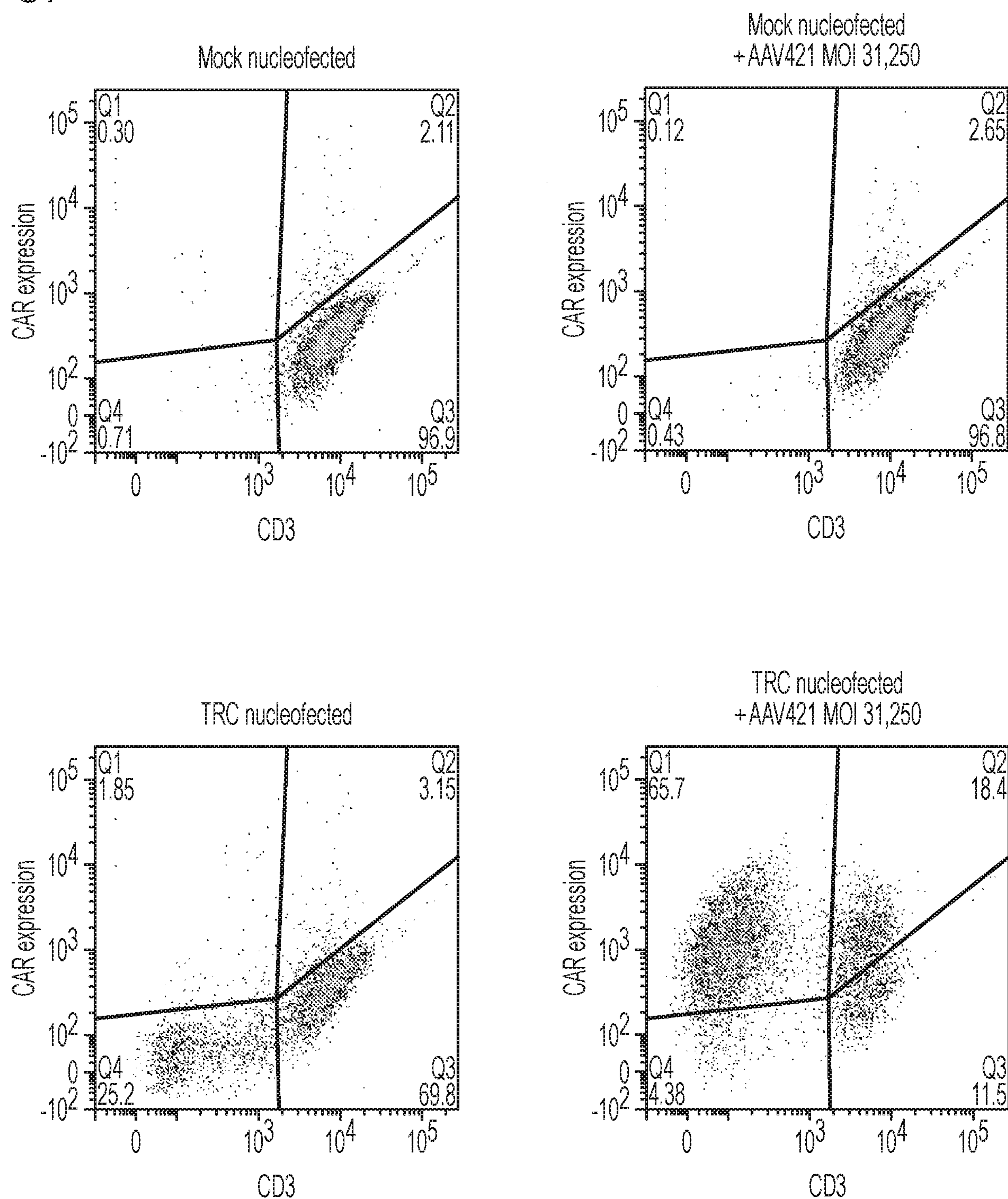


FIGURE 34
CONTINUED

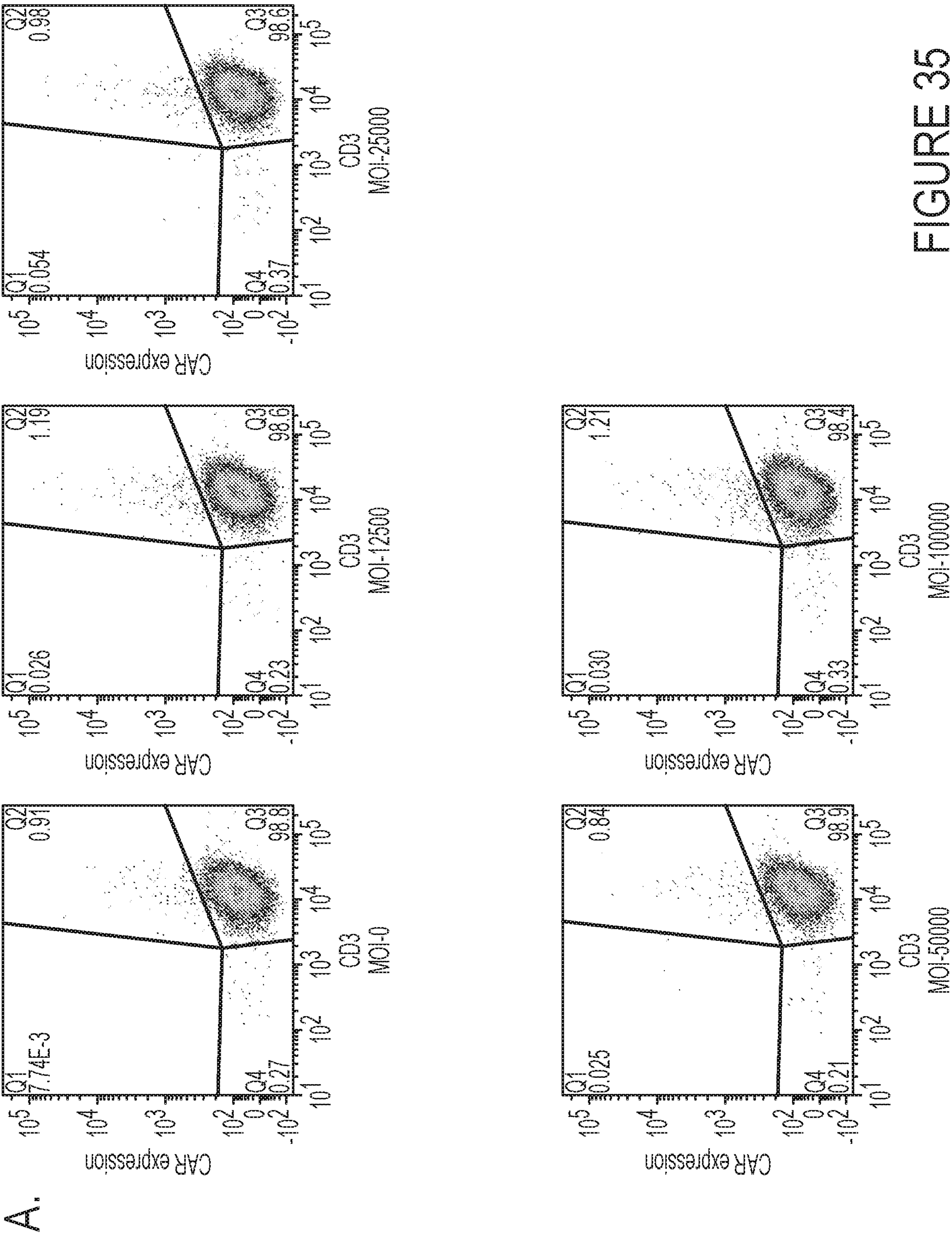


FIGURE 35

B.

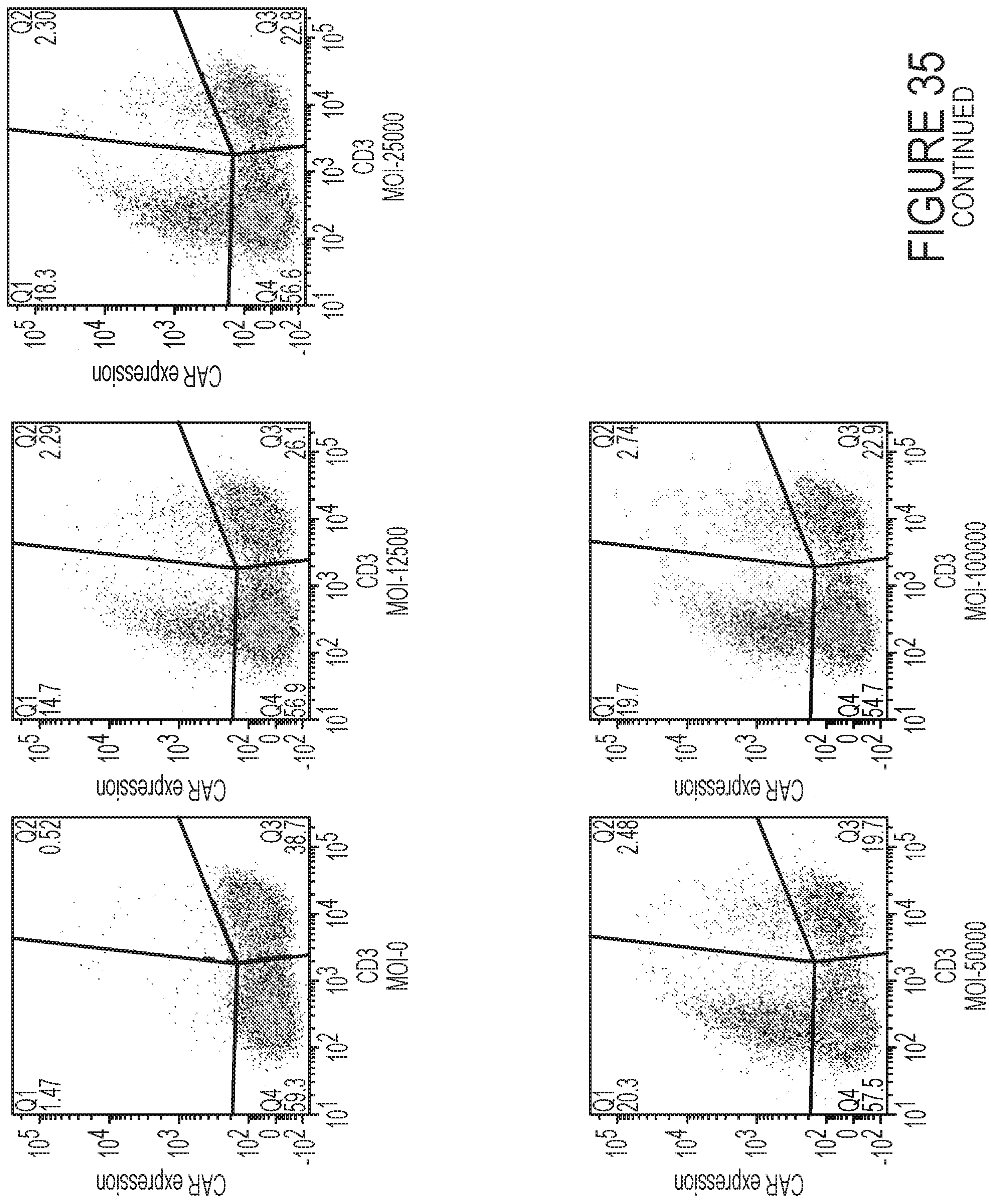
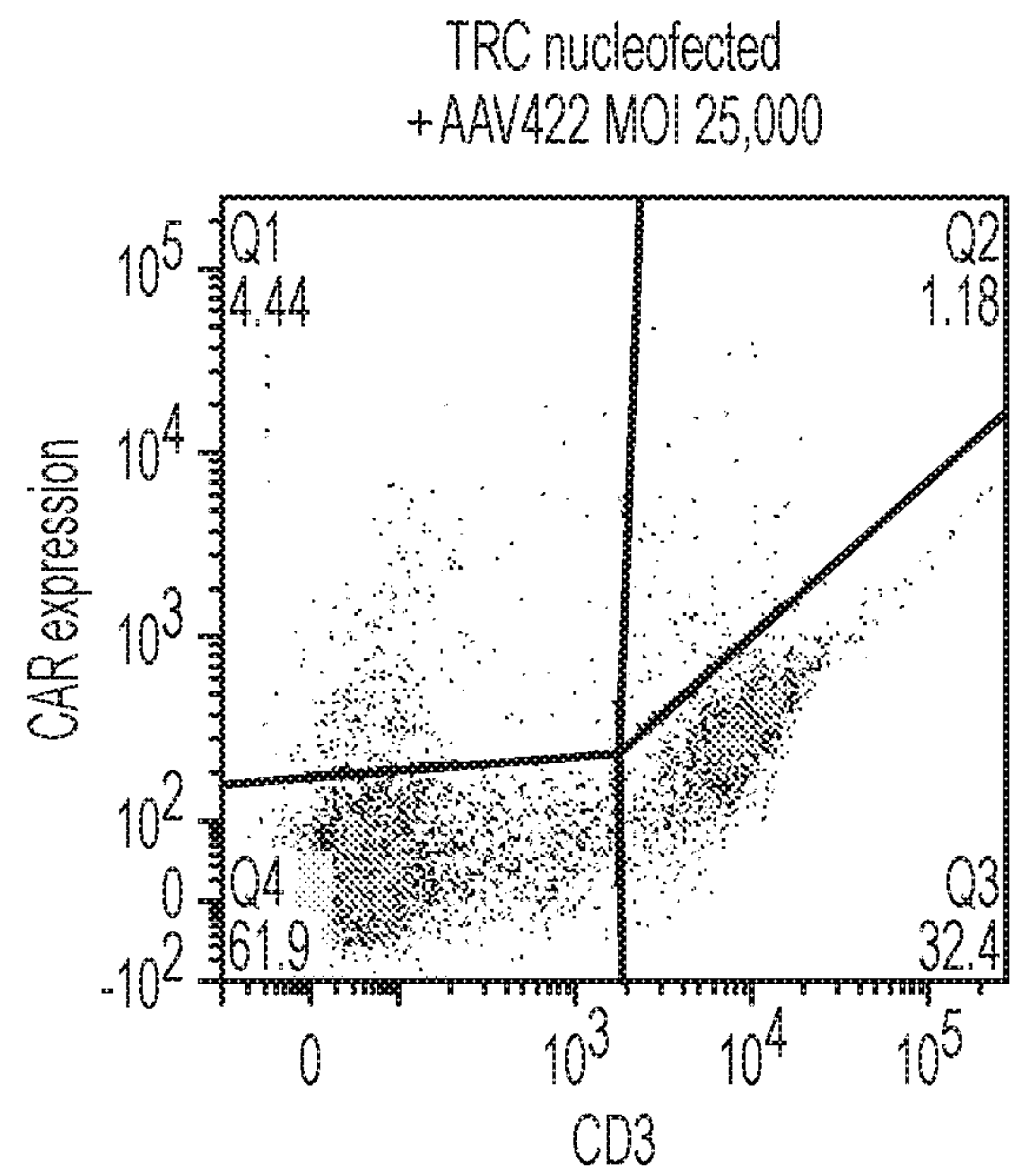
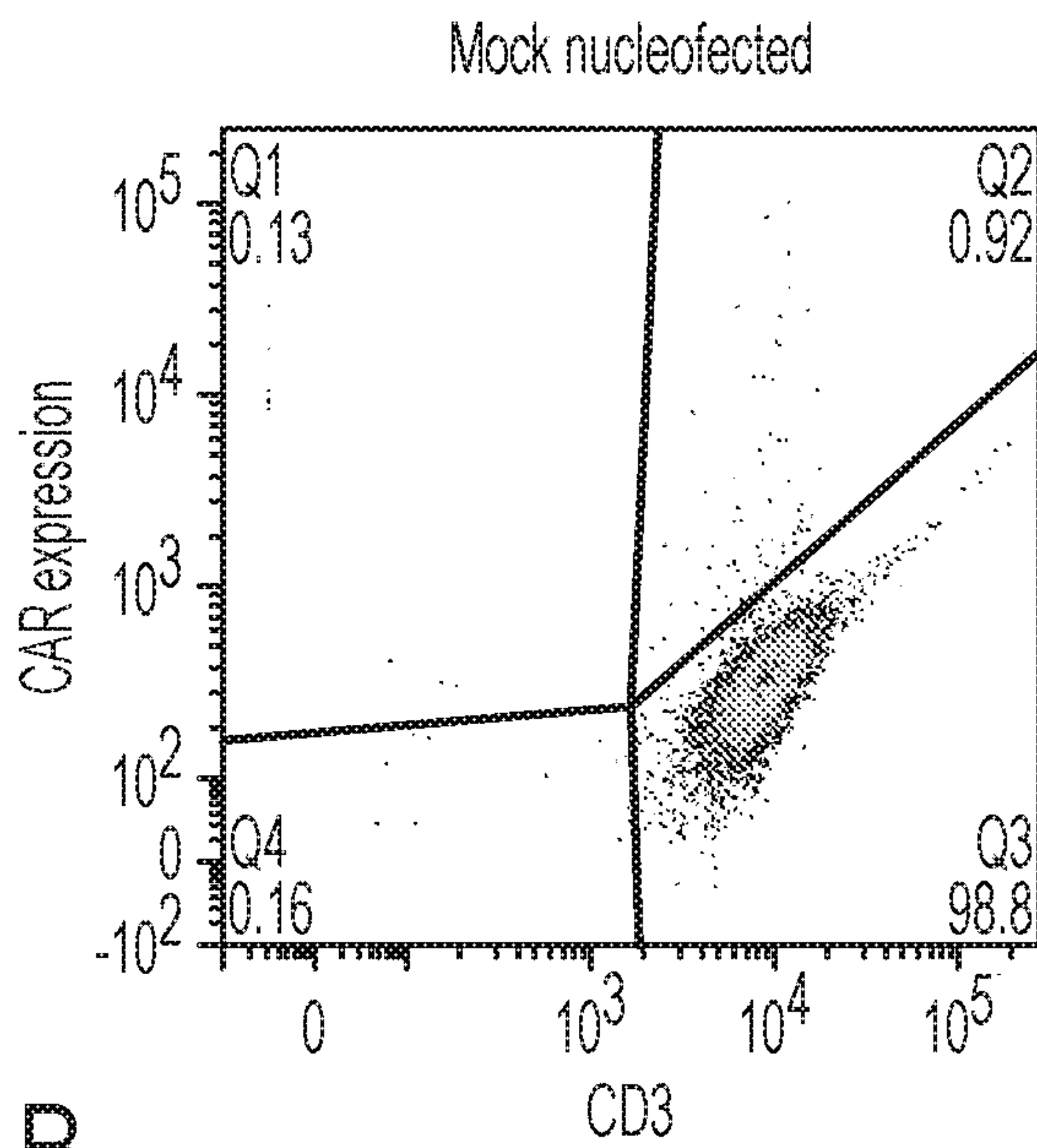


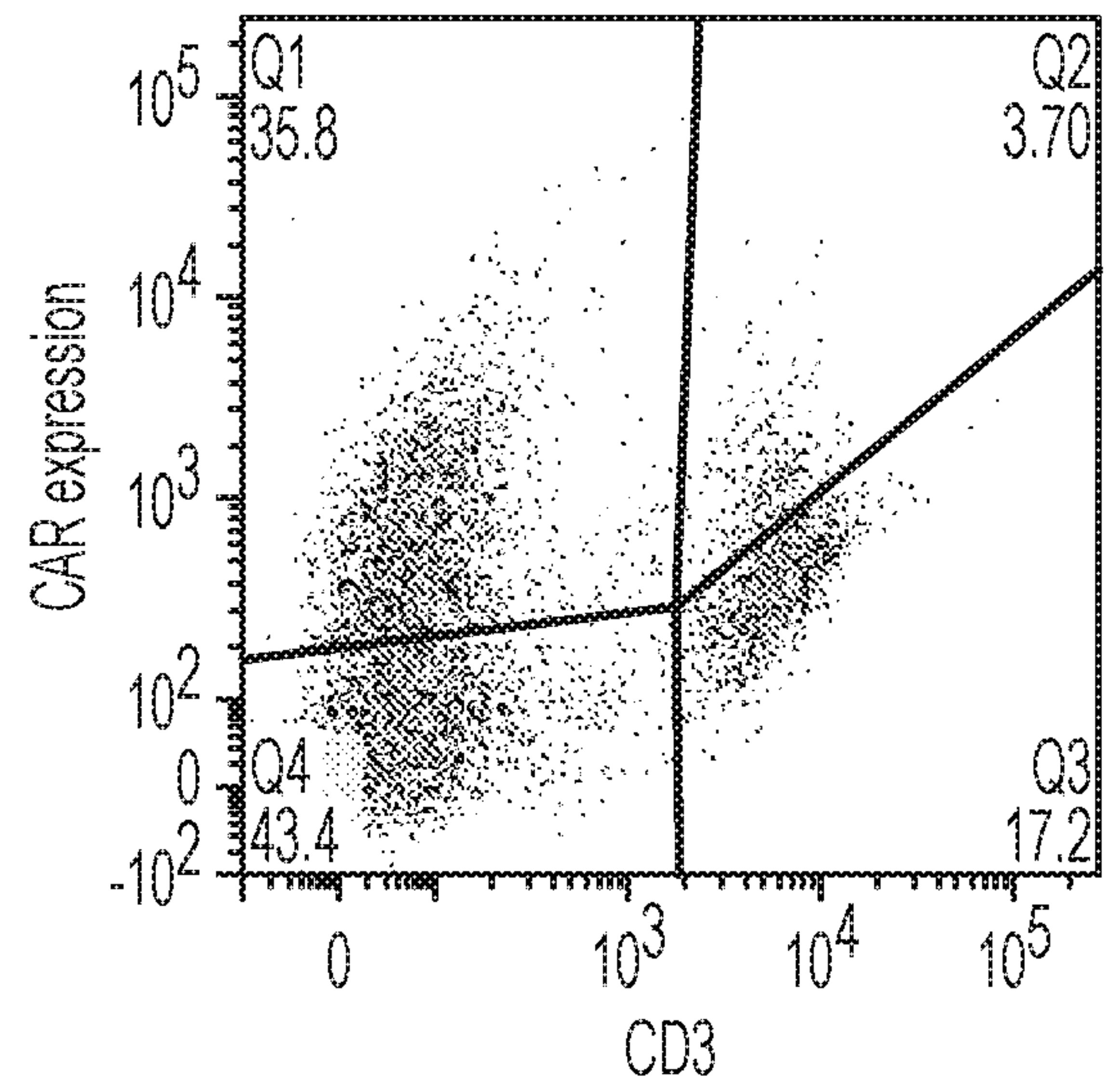
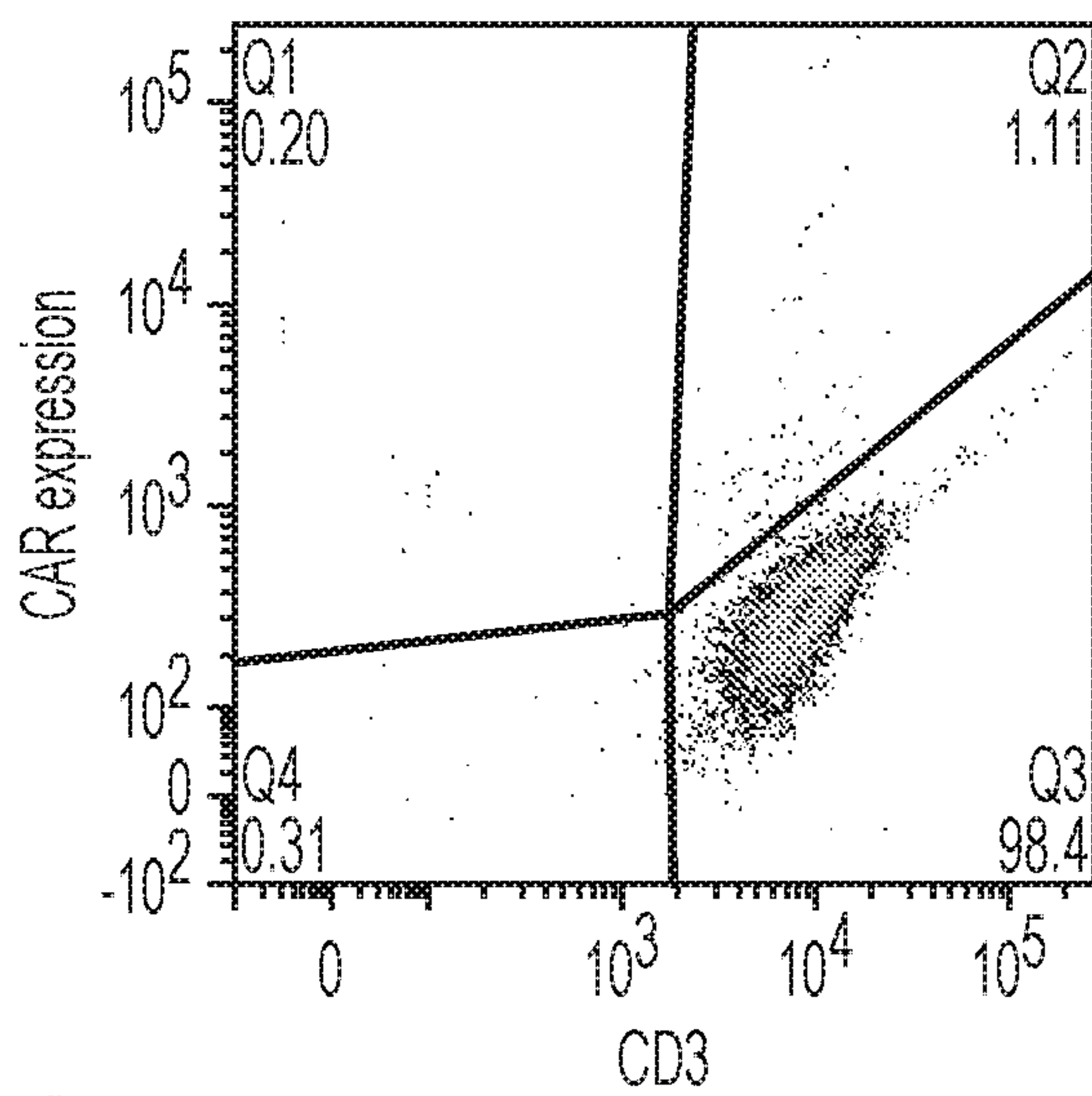
FIGURE 35
CONTINUED

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A.



B.



C.

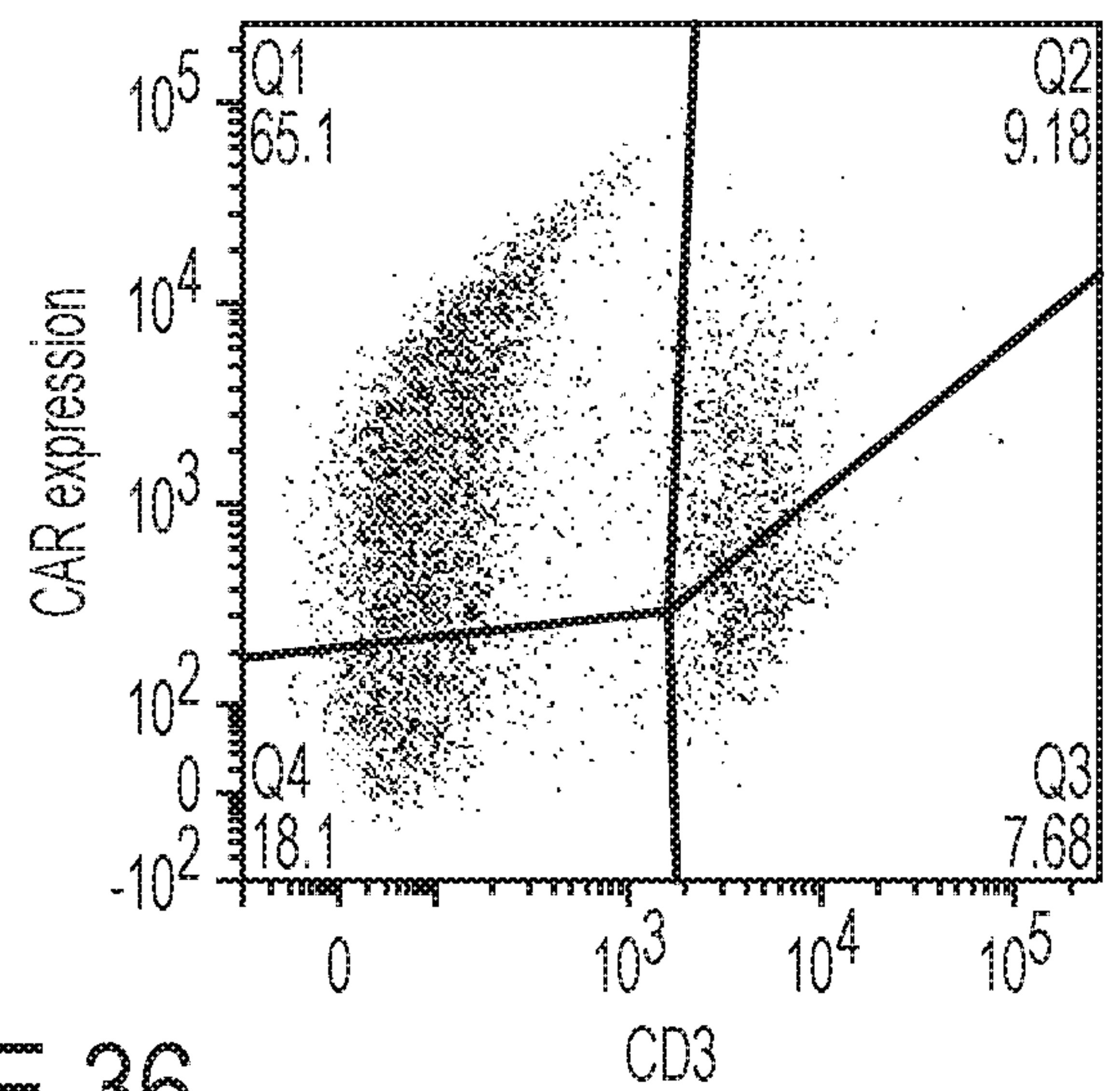
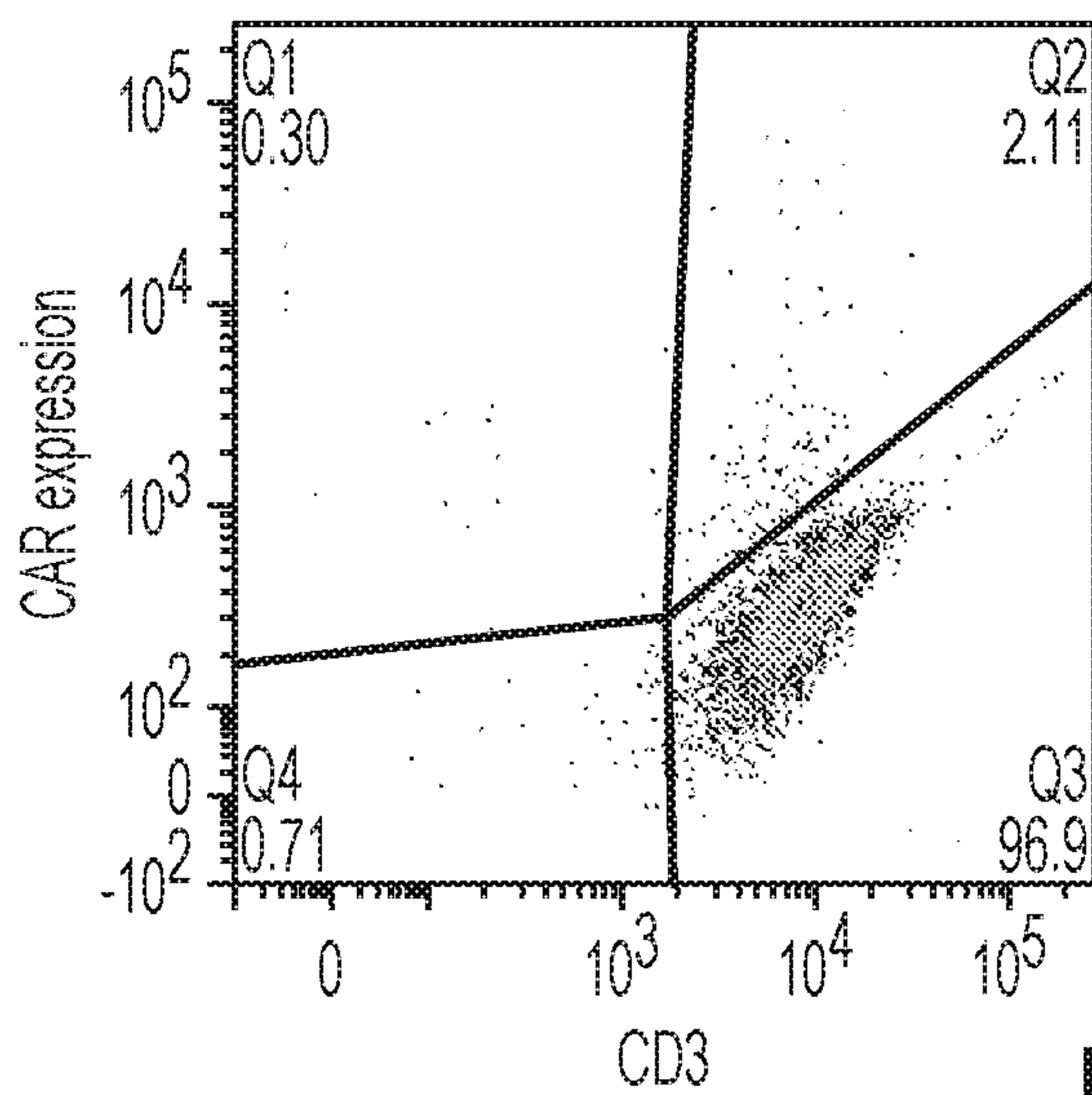


FIGURE 36

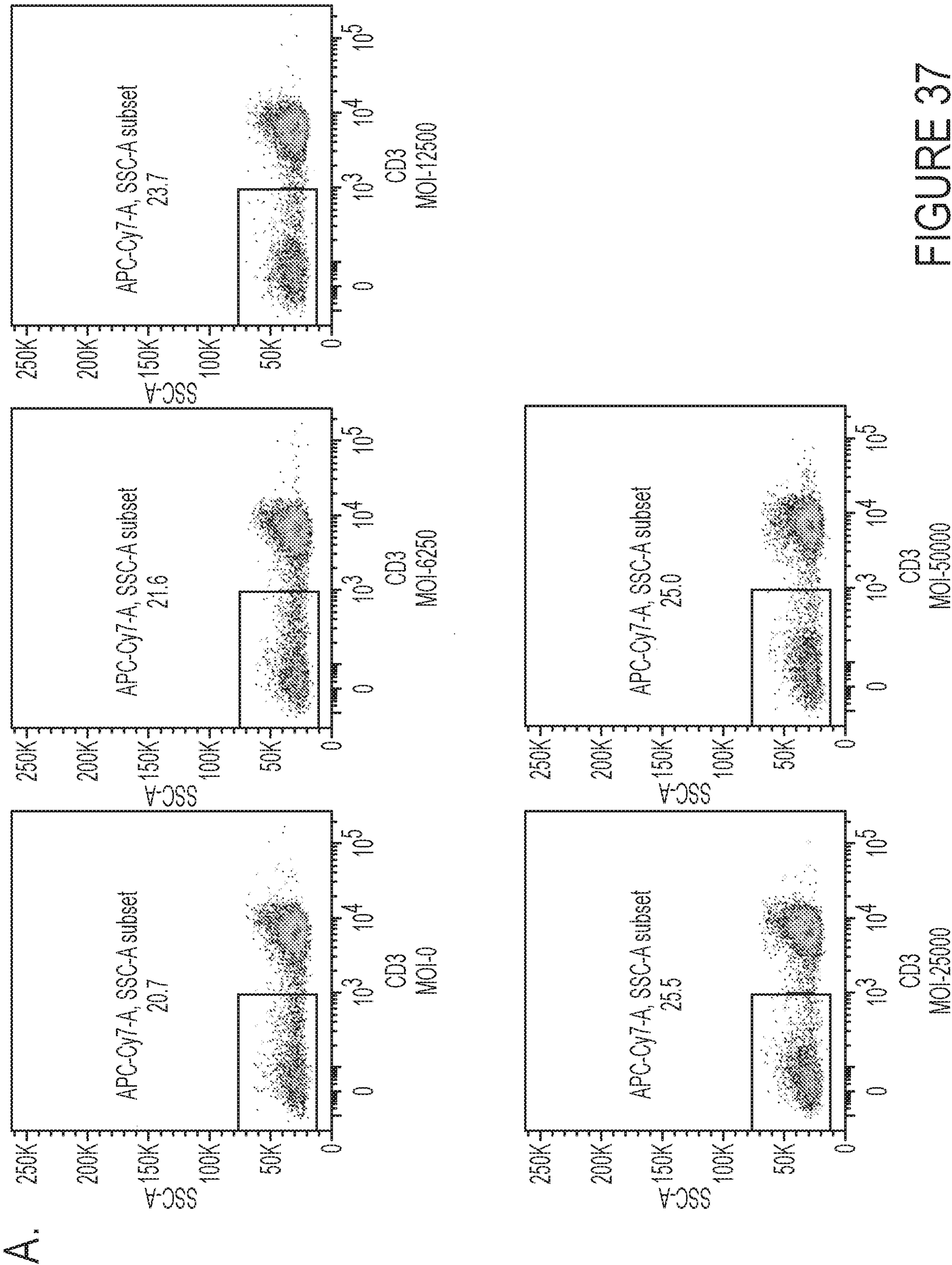


FIGURE 37

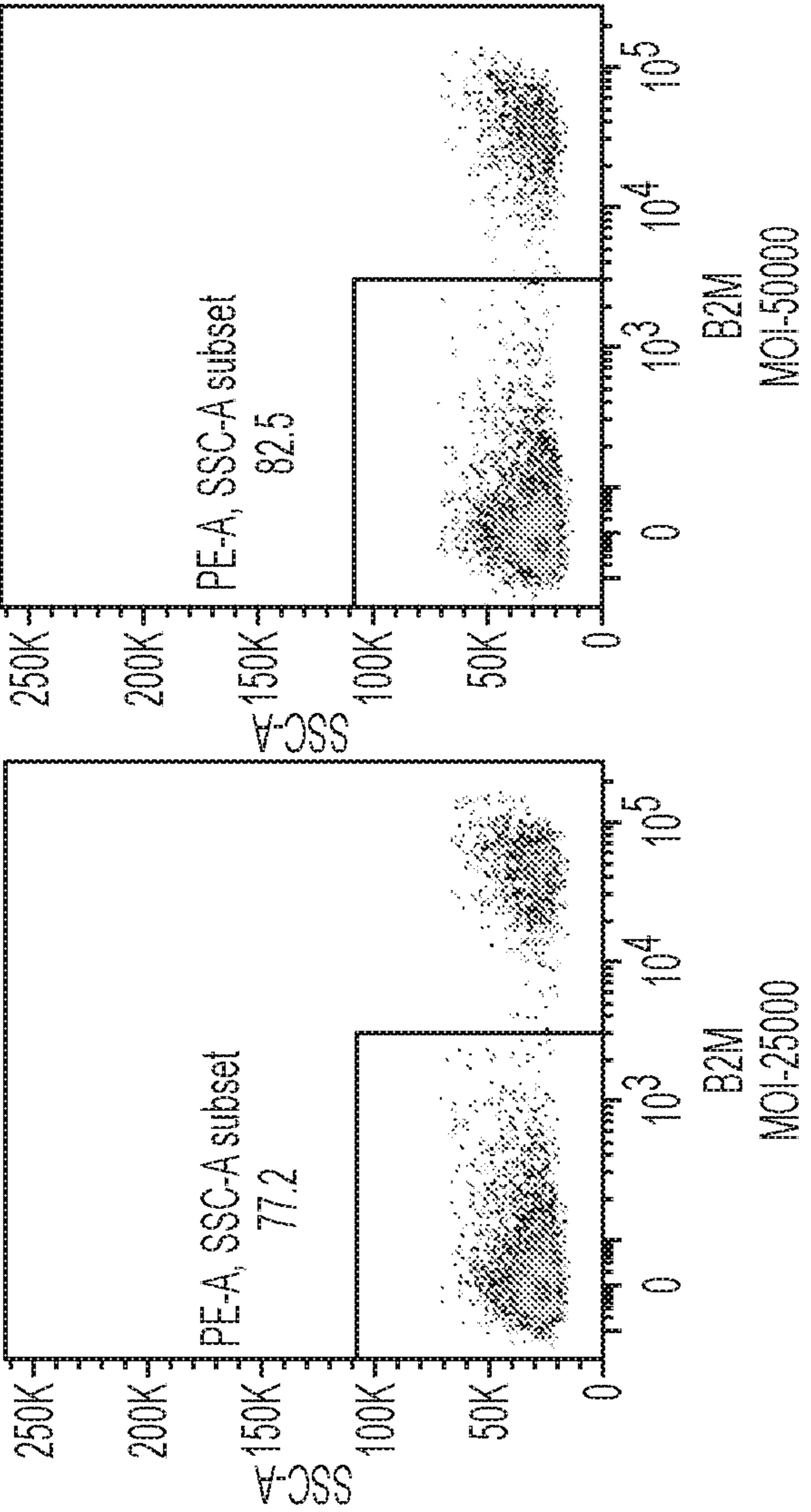
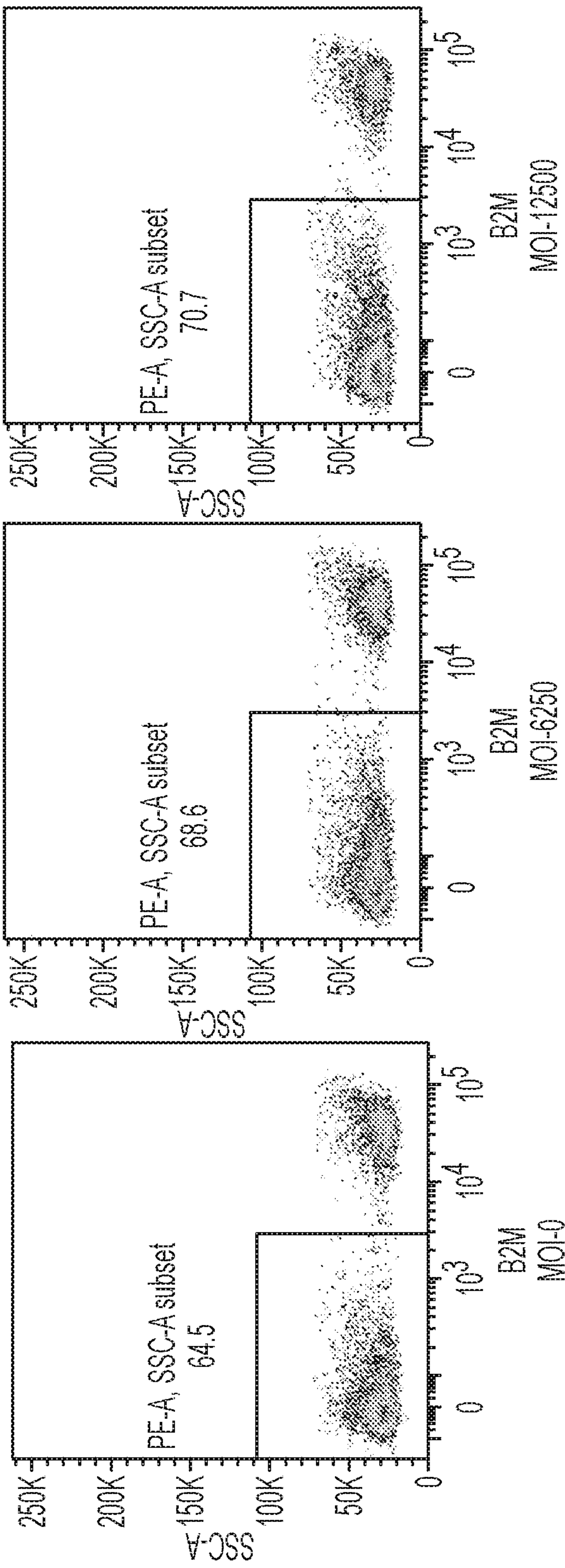


FIGURE 37
CONTINUED

C.

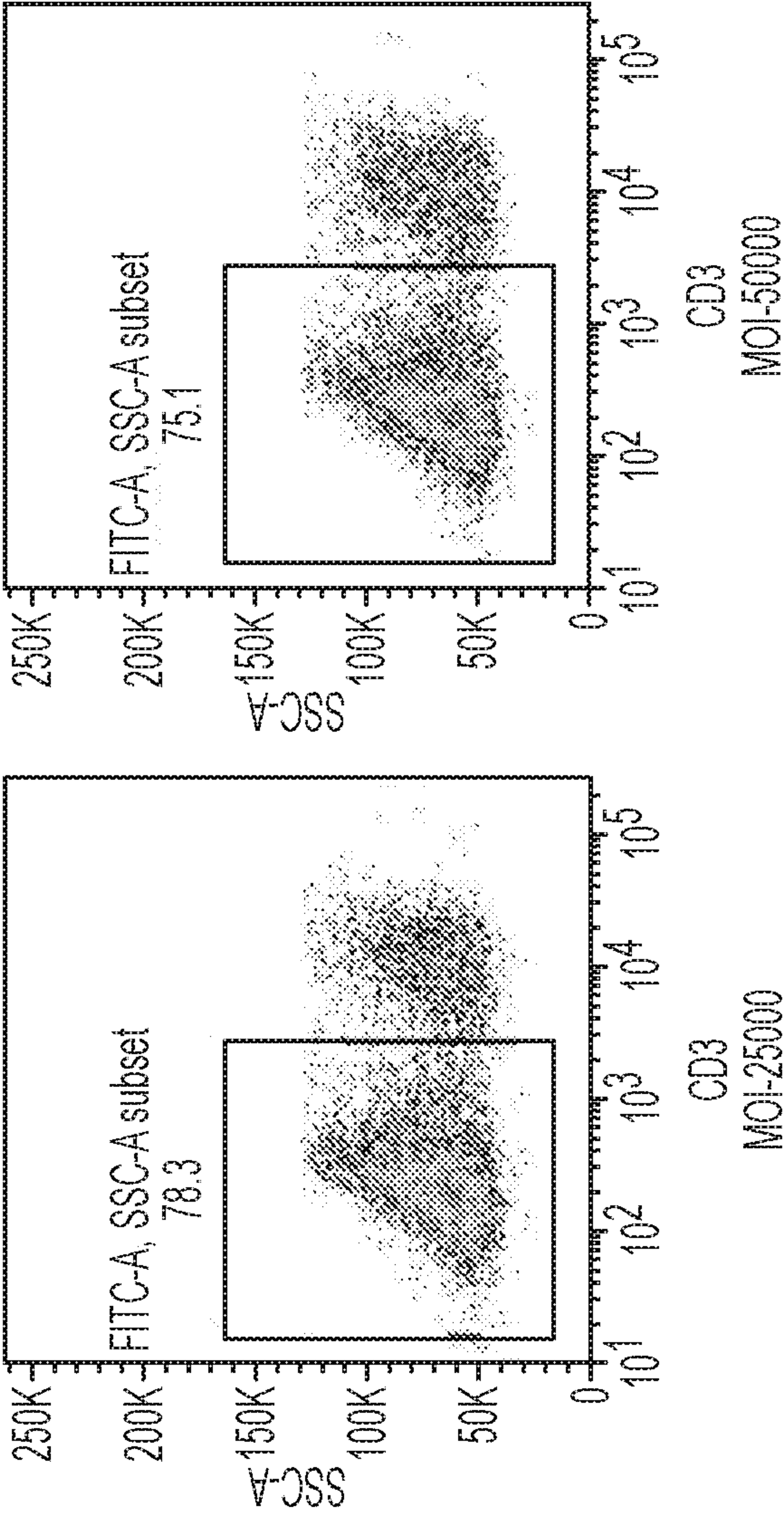
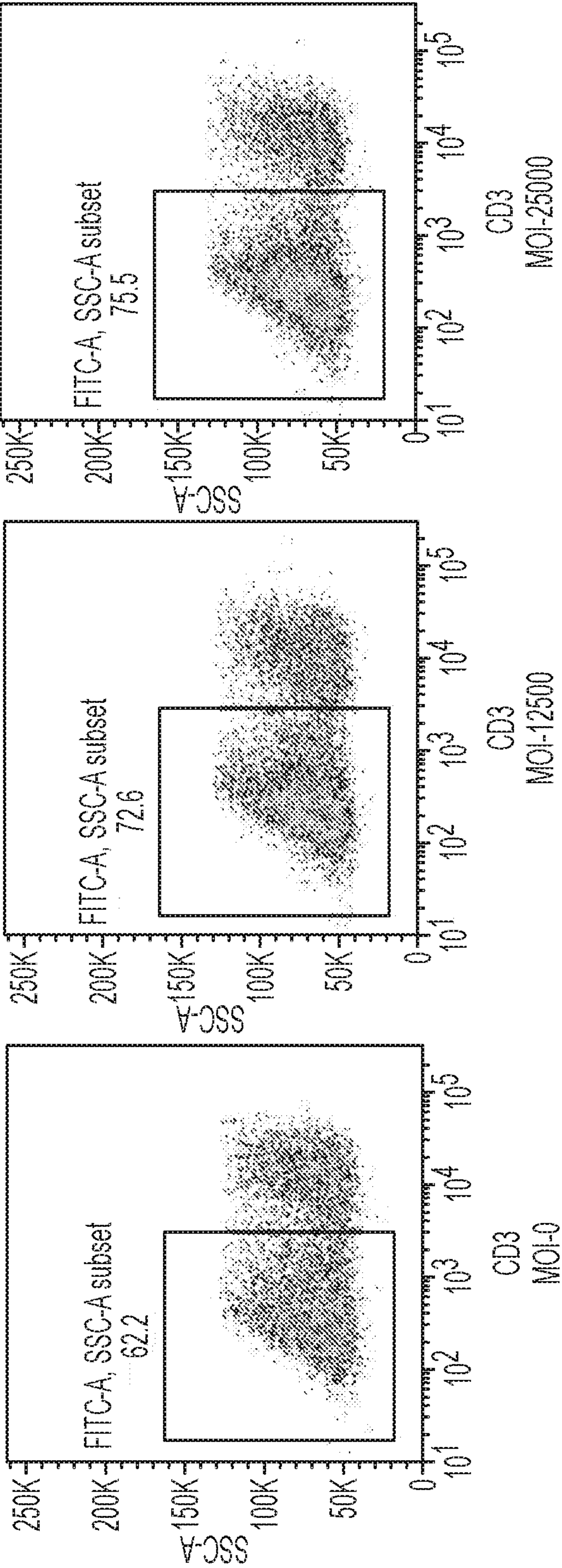
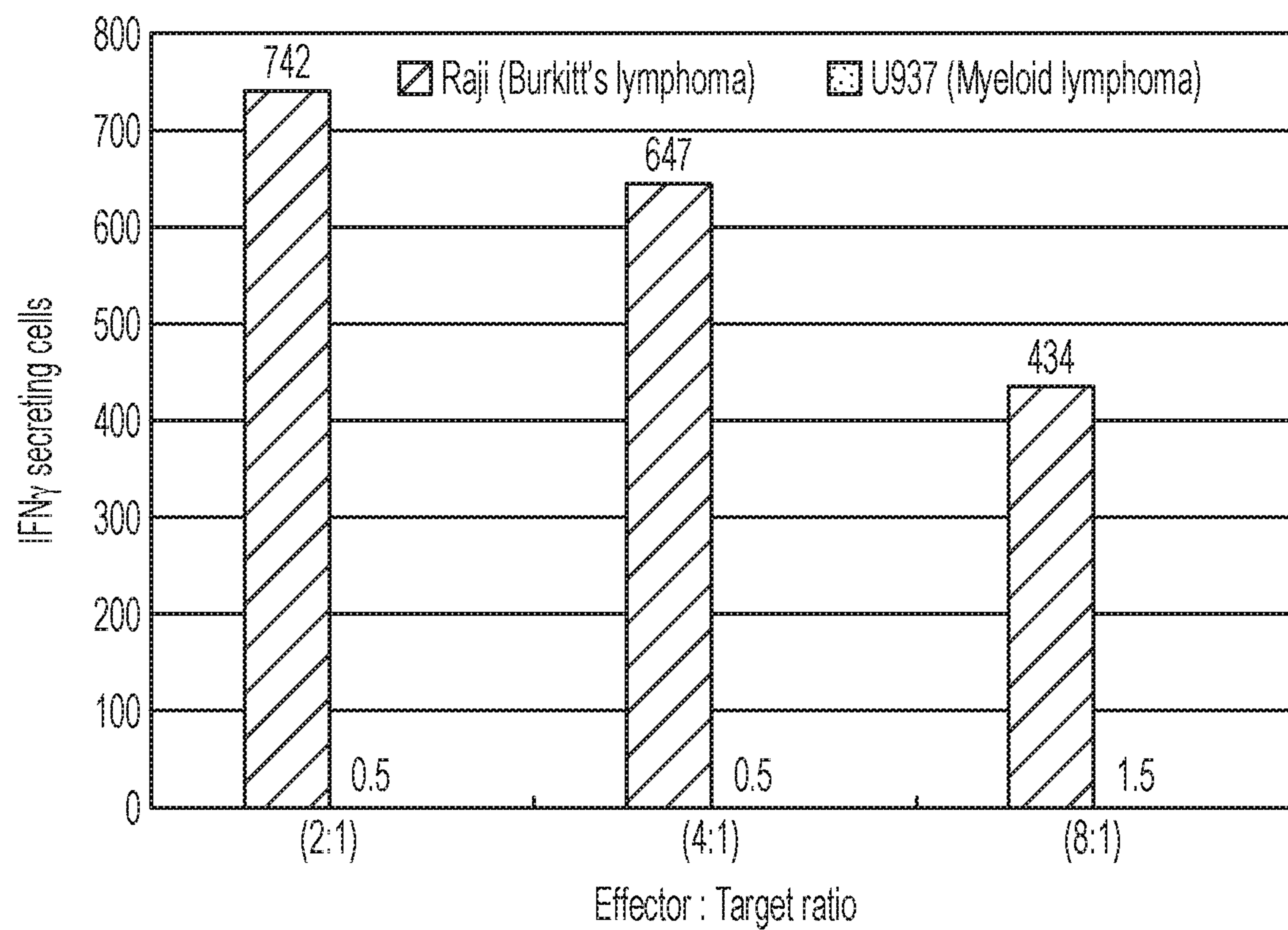


FIGURE 37
CONTINUED

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A.



B.

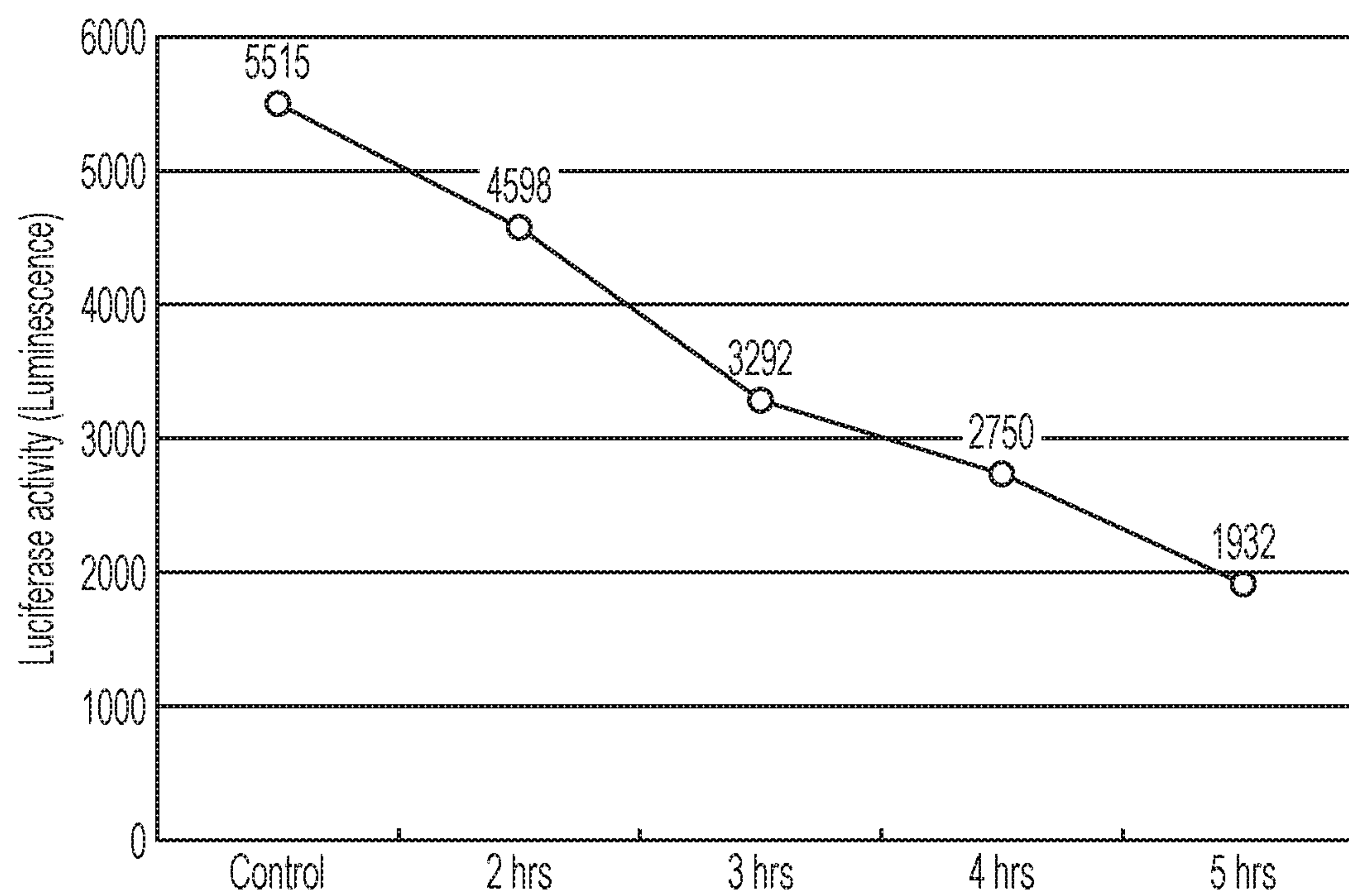


FIGURE 38

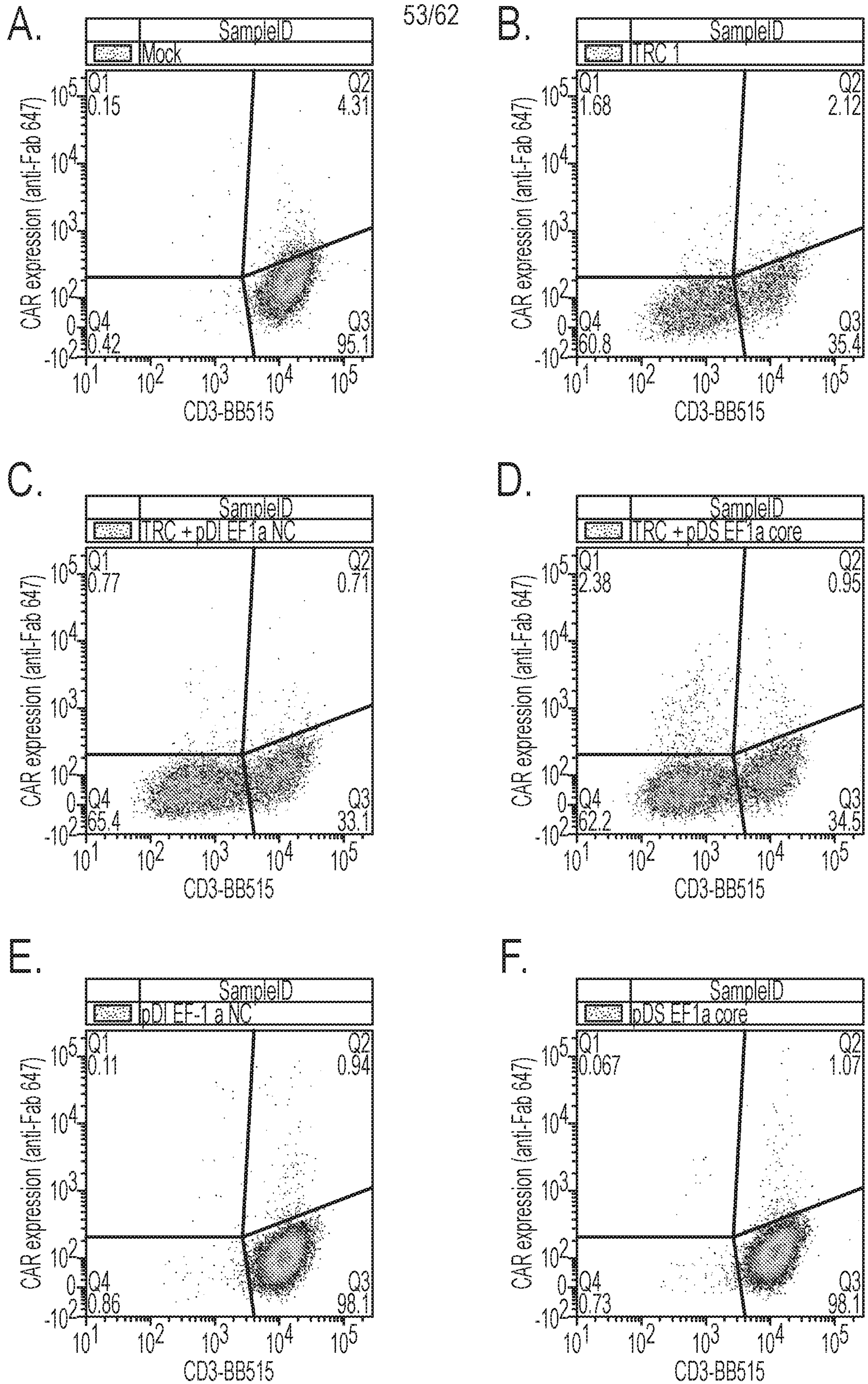


FIGURE 39

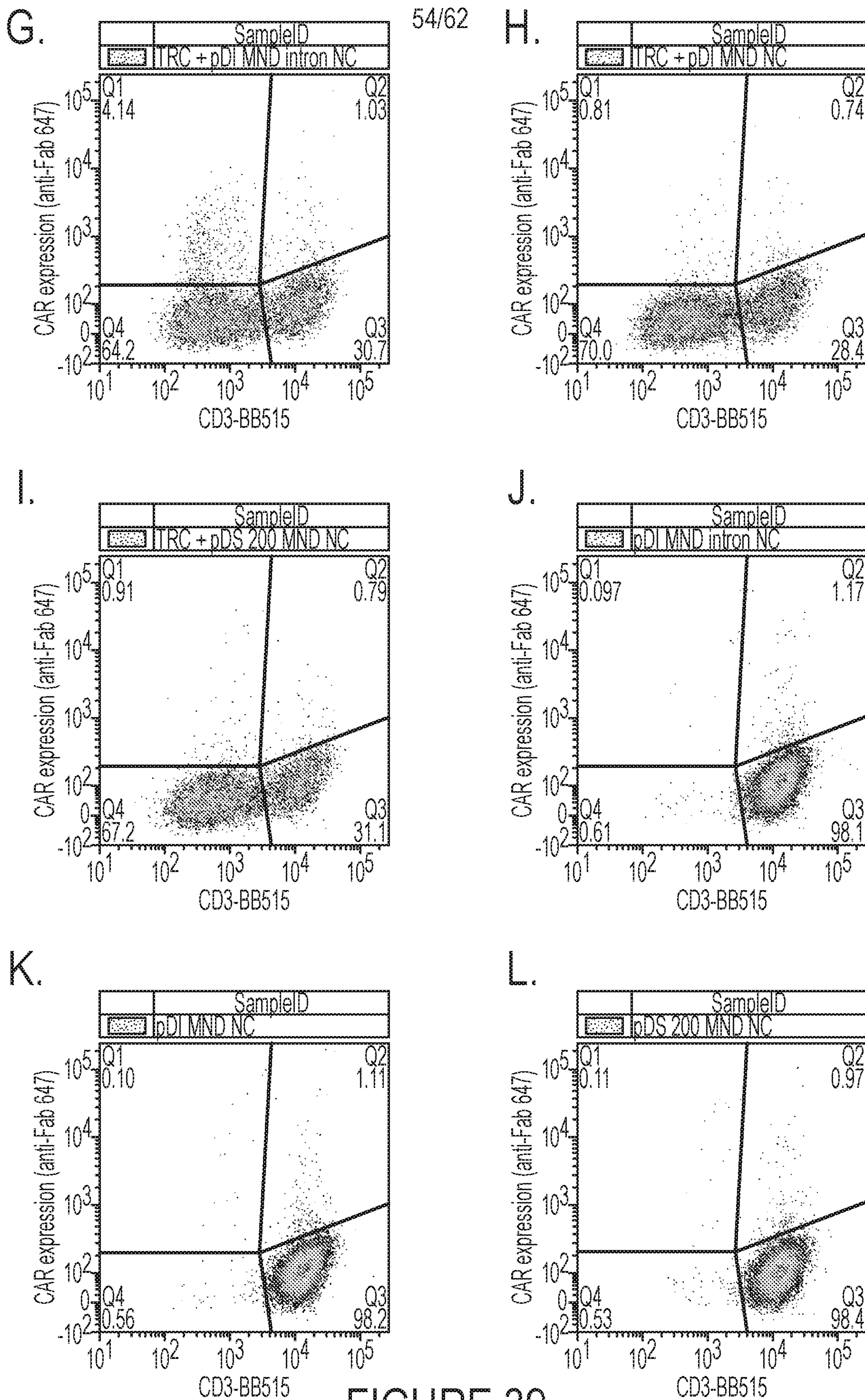
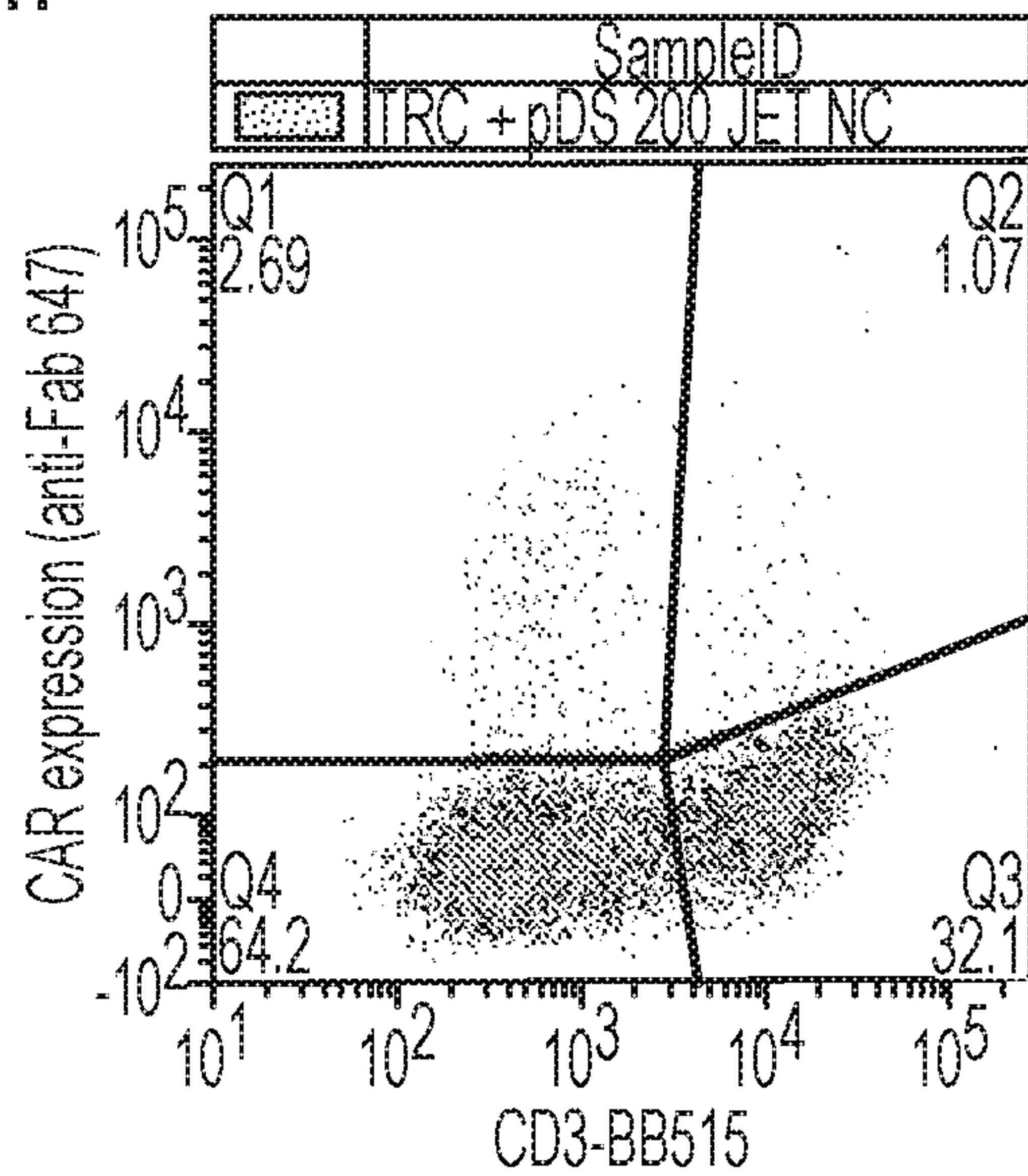


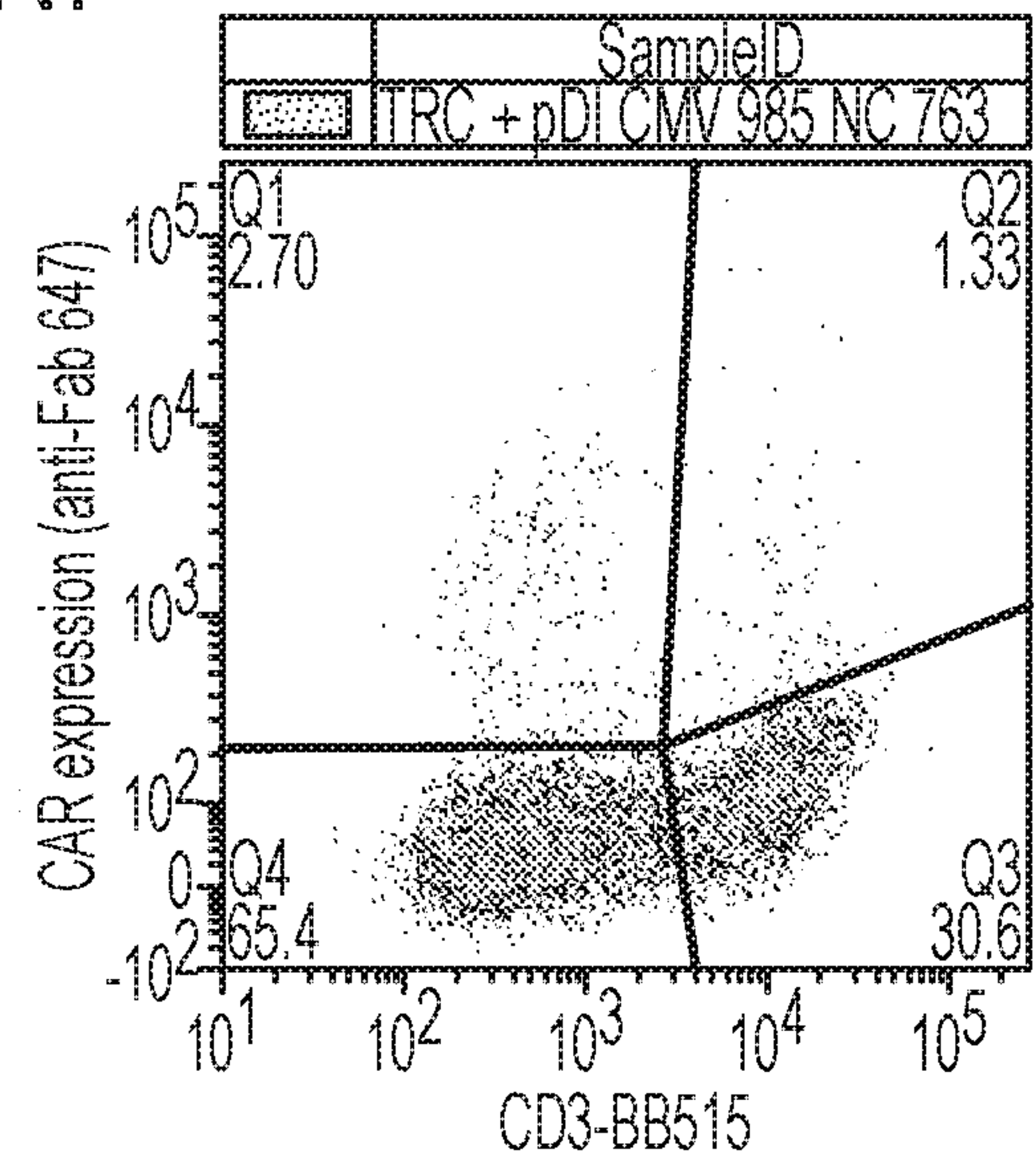
FIGURE 39
CONTINUED

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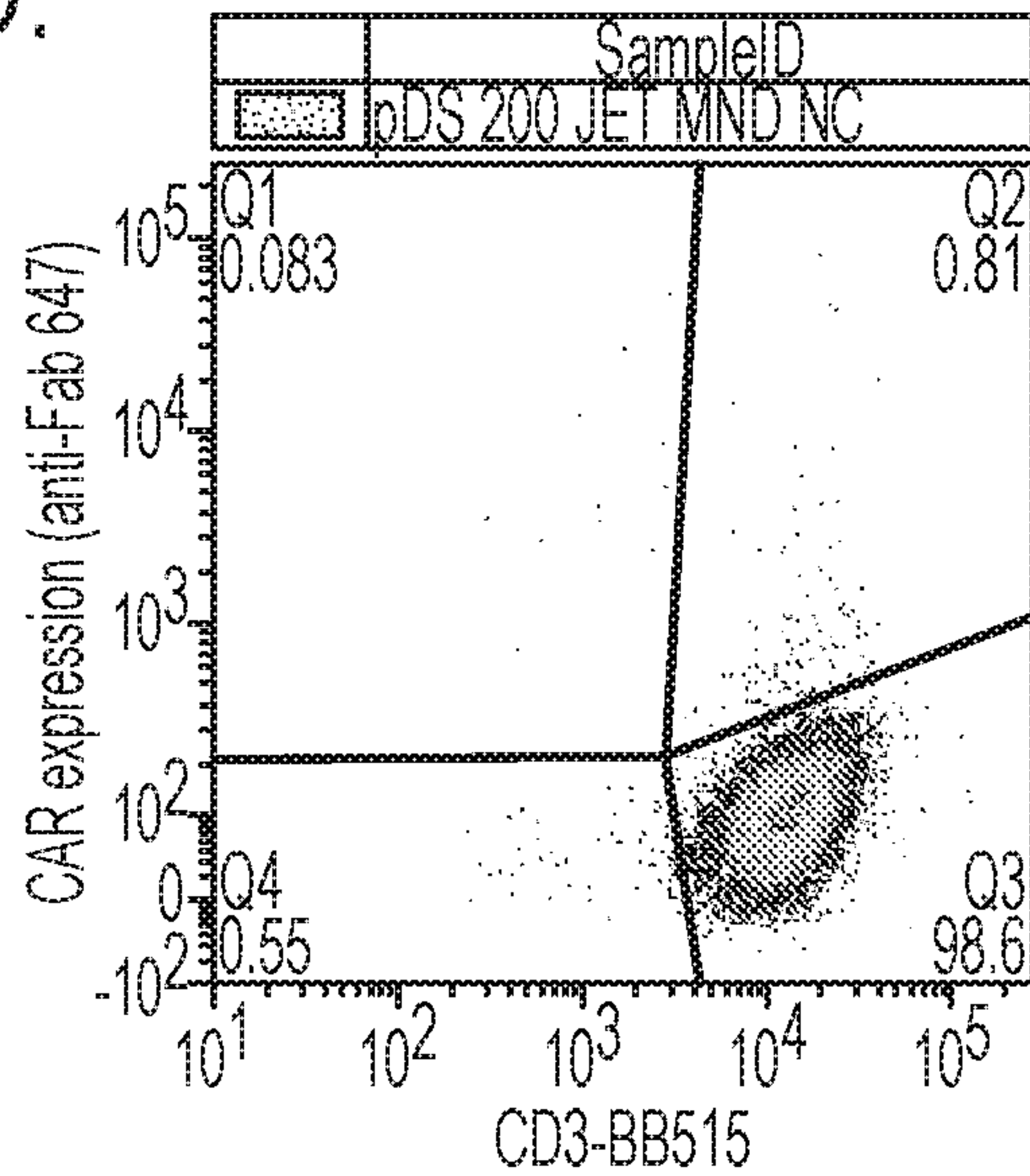
M.



N.



O.



P.

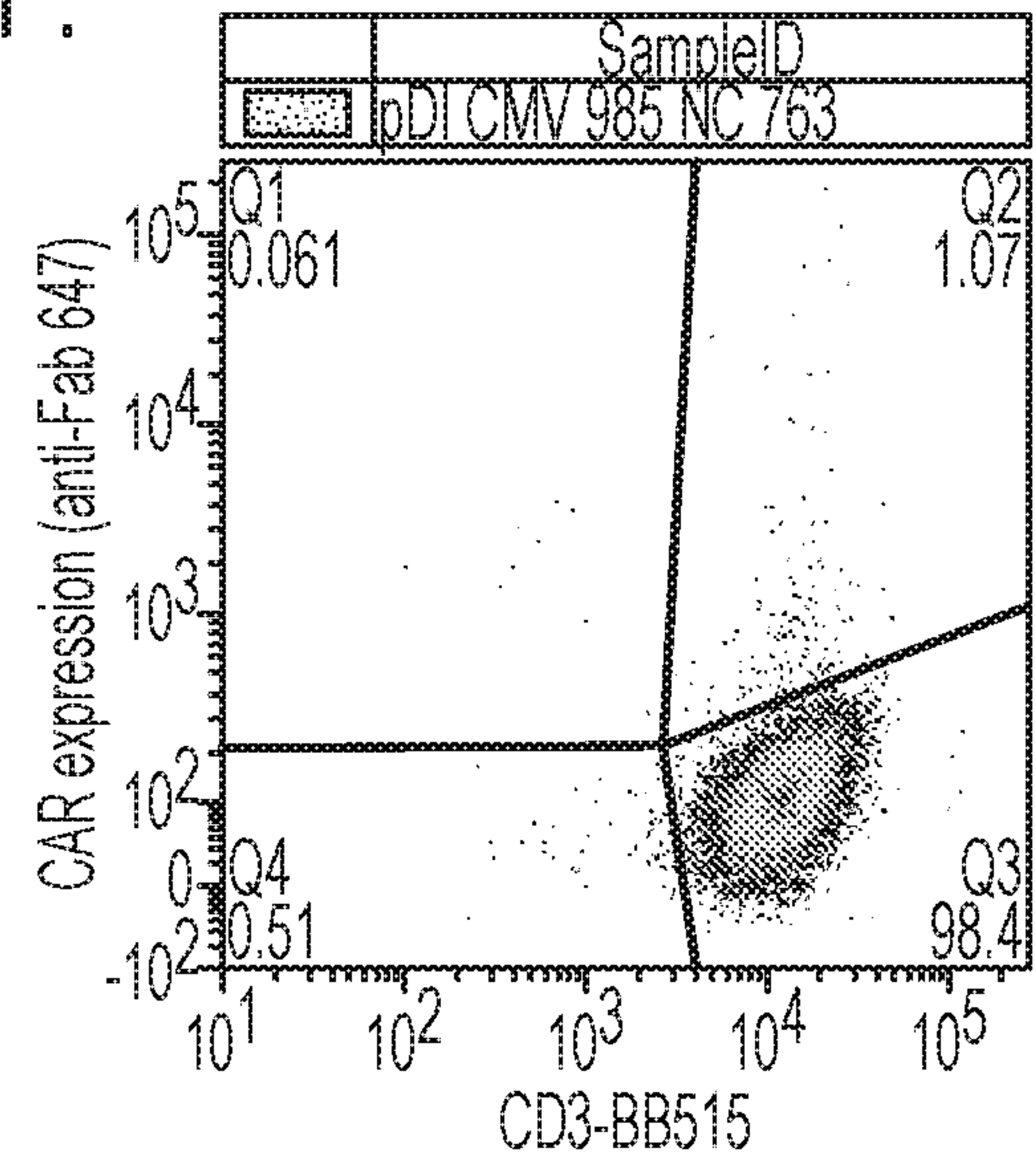


FIGURE 39
CONTINUED

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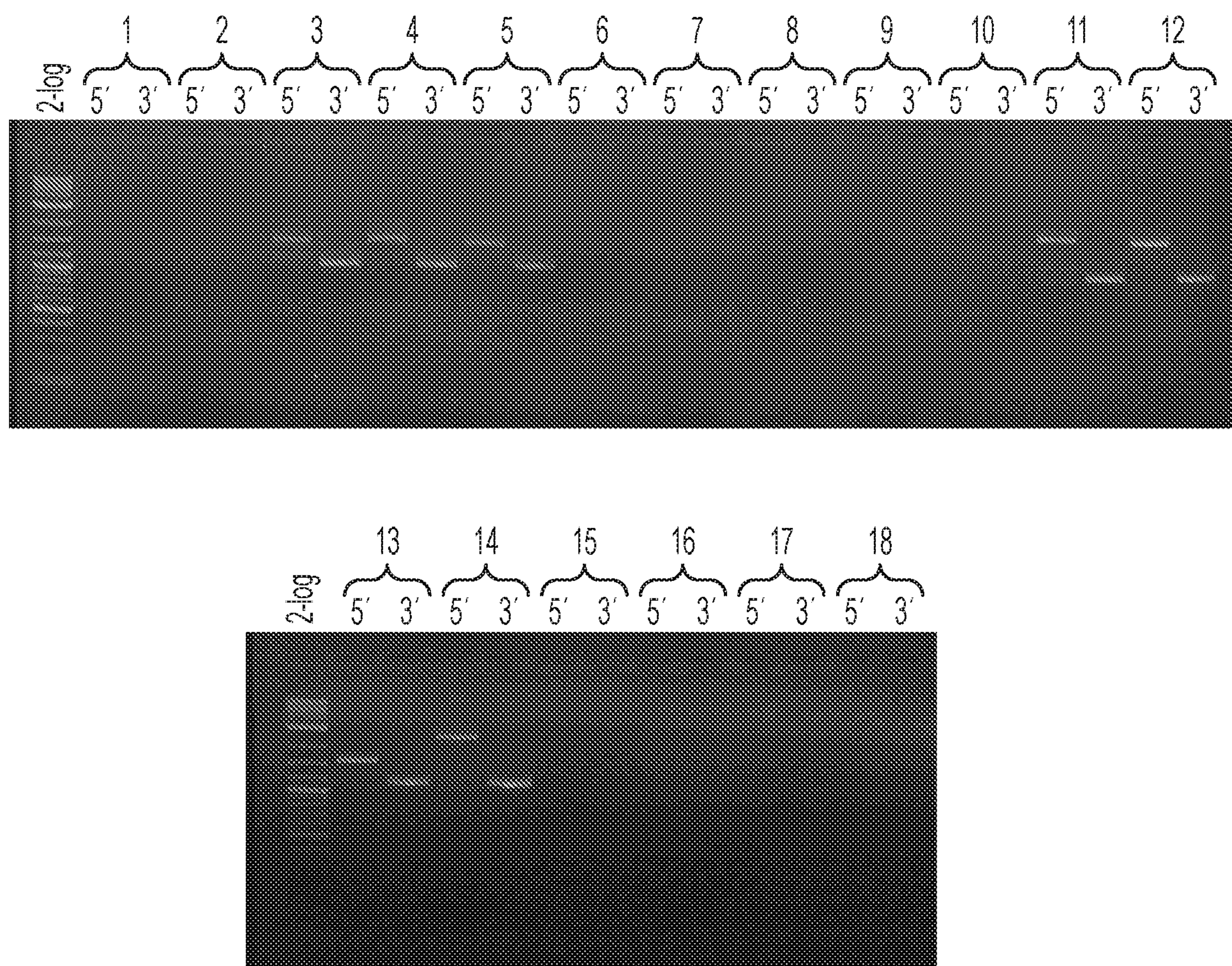


FIGURE 40

pDI 985 JET CAR 763
7464 bp

The circular map displays various restriction enzyme sites around the perimeter. Key features include:

- M13 ori**: Multiple cloning site at approximately 1000 bp.
- JET promoter**: Located near the M13 ori.
- SV40 poly(A) signal**: Located near the bottom center.
- CAR**: Coding sequence for the chimeric antigen receptor, located between the poly(A) signal and the AmpR promoter.
- Amp^r promoter**: Located near the top left.
- ori**: Origin of replication, located near the top left.
- IR**: Inverted repeats, located near the bottom left.
- SexAI***: A specific restriction site highlighted in a dashed box on the left side.

Restriction enzymes shown include: BclI, PciI, PvuI, FspI, EcoRI, NaeI, BtgZI, DraIII, AclI, SwaI, NdeI, AscI, MluI, BglII, AarI, Afel, AarI, EcoRV, SpeI, BspEI, NcoI, BtgZI, EcoNI, RsrII, PmlI, AleI, Bsu36I, SacII, KpnI, SfiI, NarI, PsrI, PfoI, SfiI, KasI, PluTI, KffI - PpuMI, AatII, BsgI, ZraI, EcoNI, Acc65I, HincII - HpaI, BstBI, BamHI, BbsI, Eco53kI, BstXI - SacI, NmeAIII, PmlI, AjuI, NgoMIV, NaeI, BsmI, SexAI*, DraIII, AlfI, XcmI, PshAI, MfeI, PflMI, AscI, PstI - SbfI, EcoRI, NdeI, BstAPI, PfoI, ZraI, AatII, XmnI, Scal, PvuI.

FIGURE 41

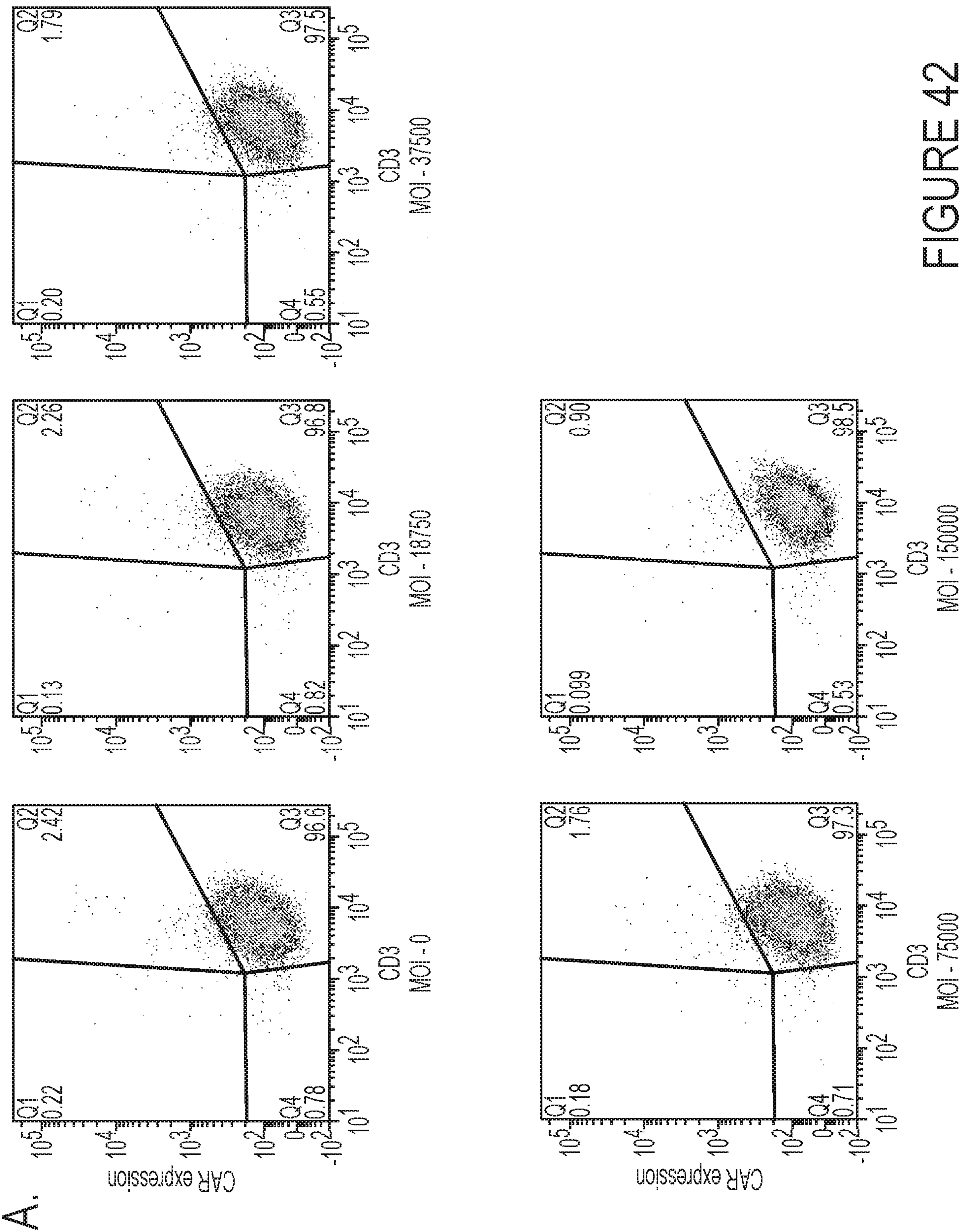


FIGURE 42

B.

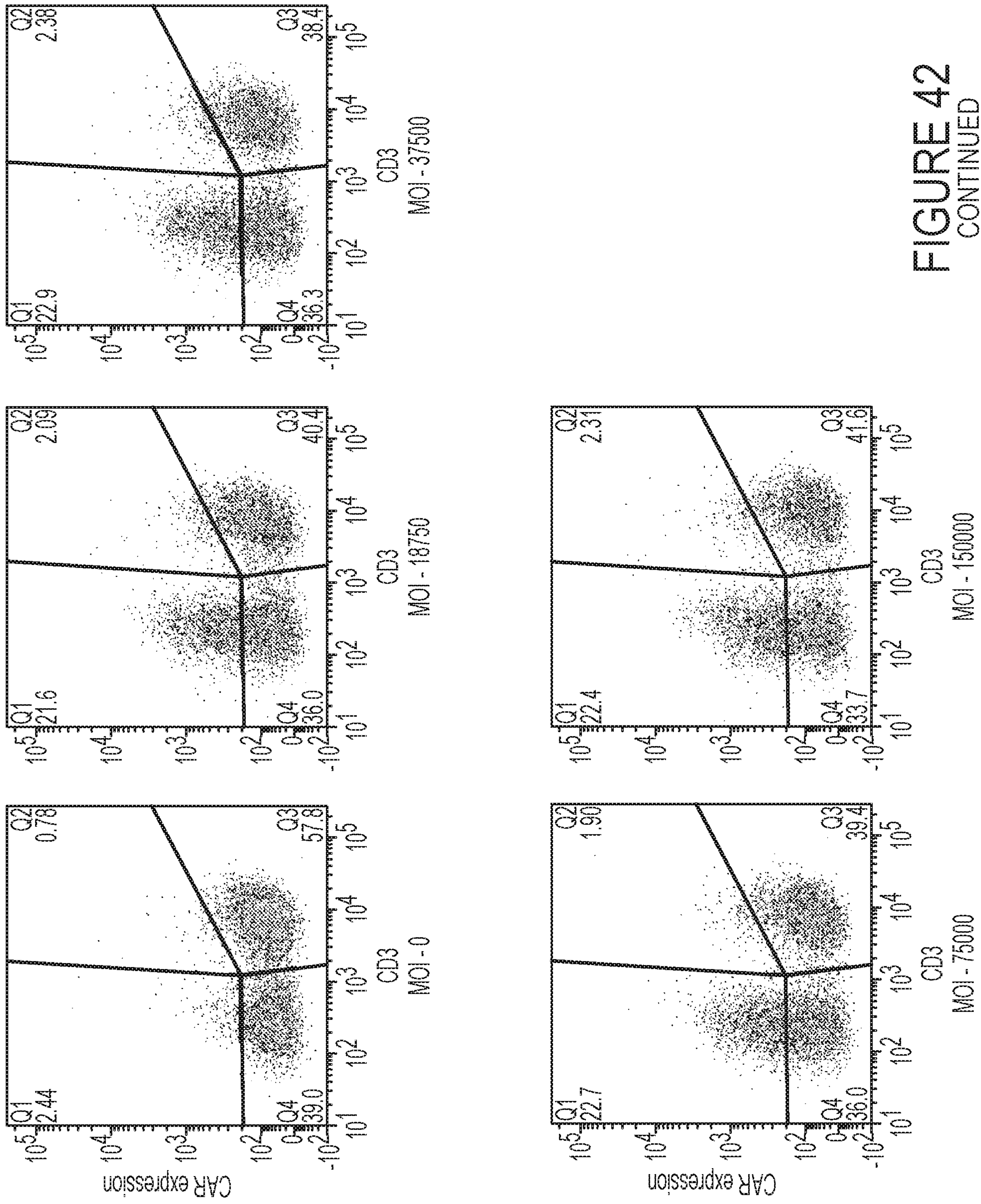


FIGURE 42
CONTINUED

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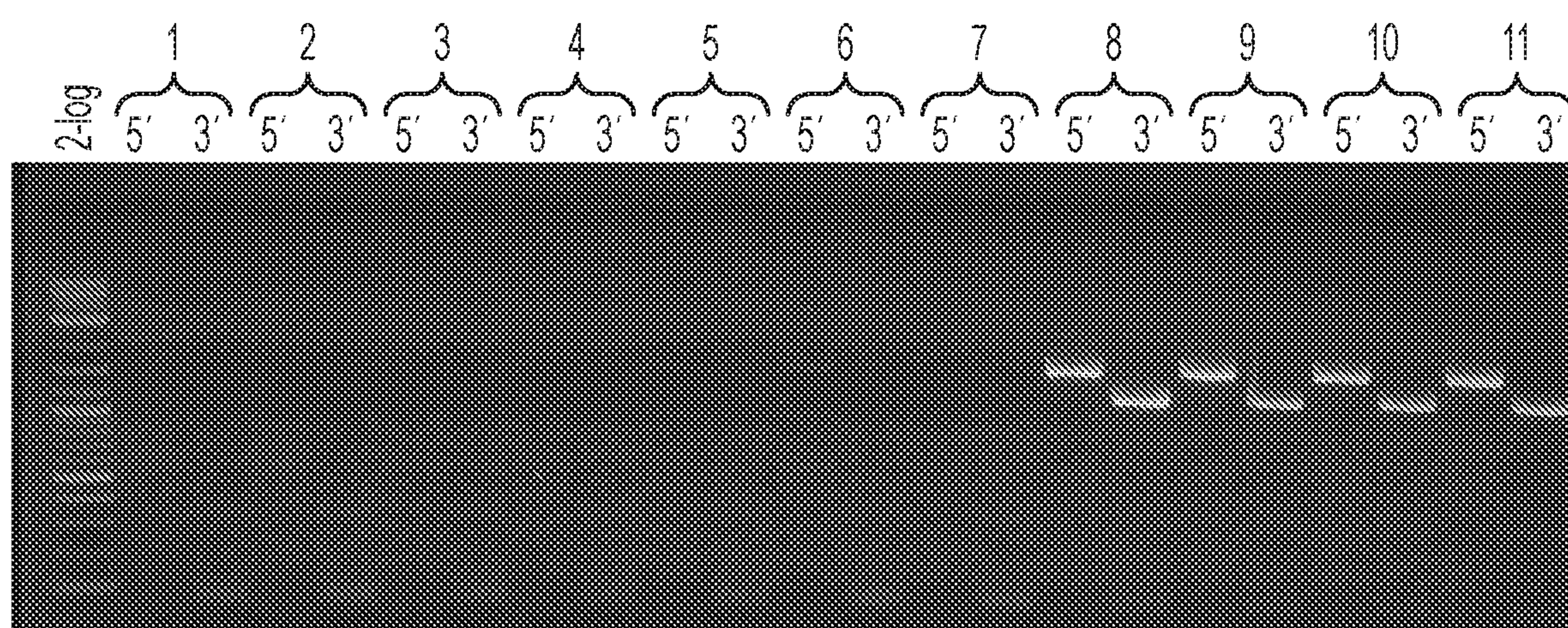


FIGURE 43

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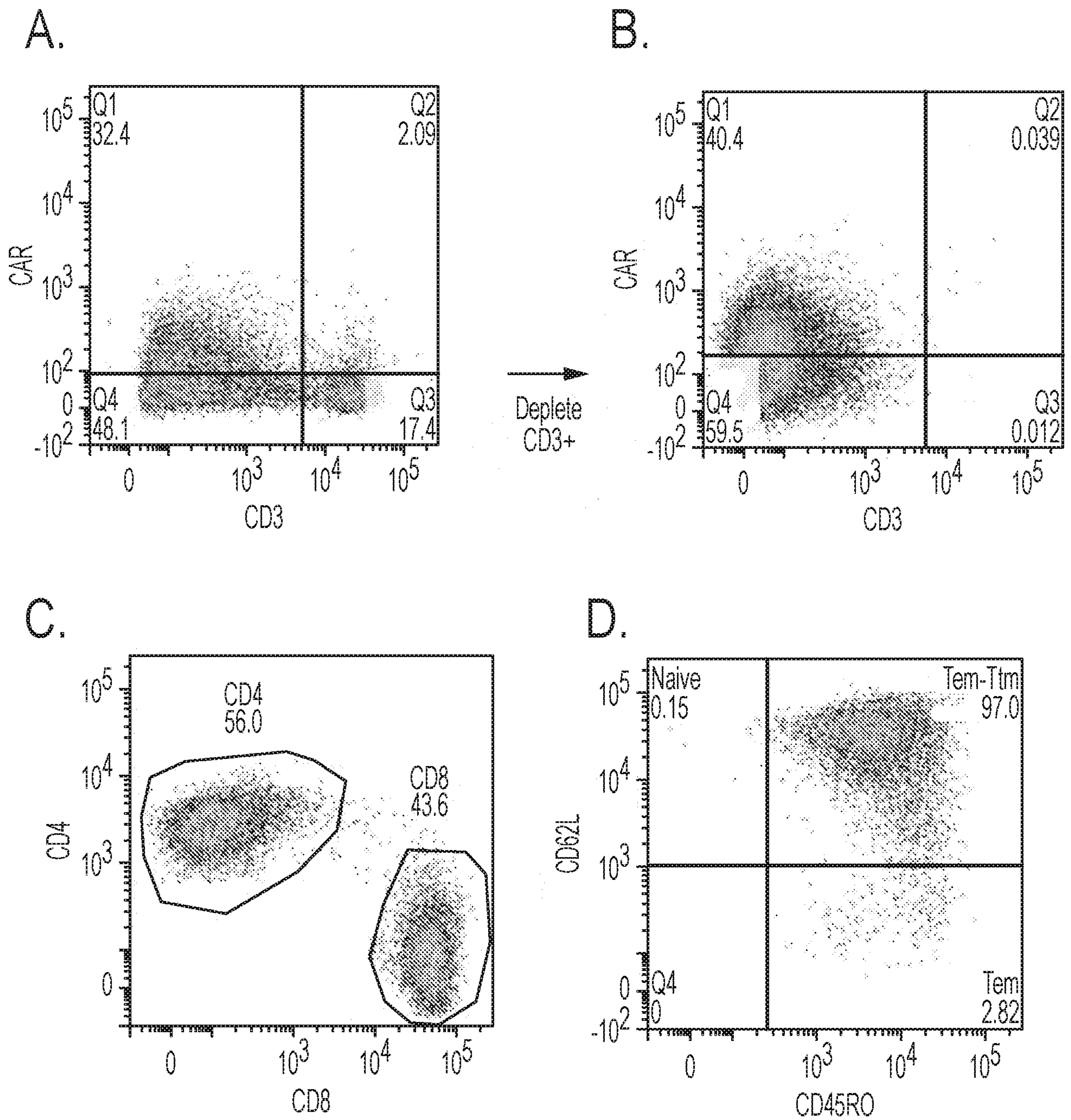


FIGURE 44

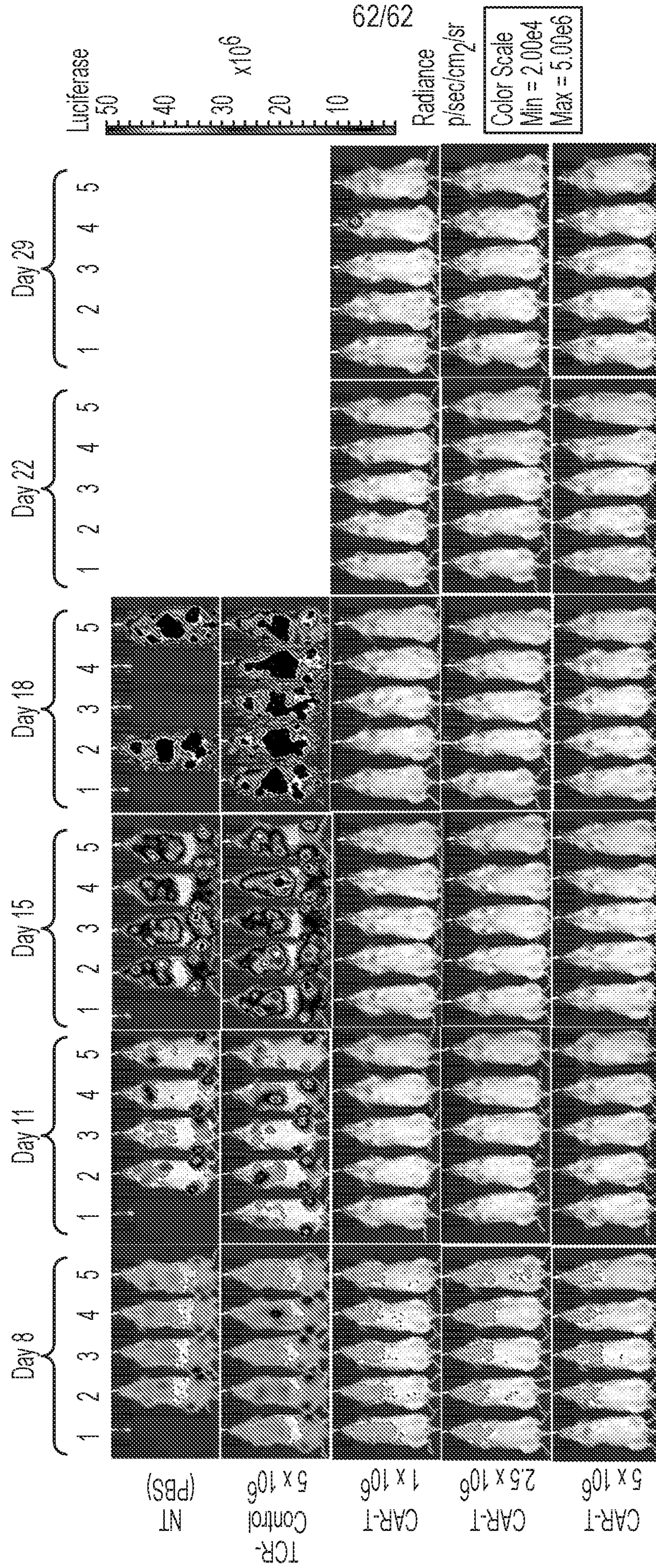


FIGURE 45

A.

	TRC1 Half-Site	TRC2 Half-Site	
	┌───────────┐	┌───────────┐	
TRC 1-2	TGGCCTGGAGCAA	CAAATCTGA	(SEQ ID NO:3)
Recognition Sequence	ACCGGACCT	CGTTGTTTAGACT	(SEQ ID NO:83)
	TRC3 Half-Site	TRC4 Half-Site	
	┌───────────┐	┌───────────┐	
TRC 3-4	ACAAATGTGTCAC	AAAGTAAGG	(SEQ ID NO:4)
Recognition Sequence	TGTTTACAC	AGTGTTTCATTCC	(SEQ ID NO:84)
	TRC7 Half-Site	TRC8 Half-Site	
	┌───────────┐	┌───────────┐	
TRC 7-8	CTGATGTGTATAT	CACAGACAA	(SEQ ID NO:5)
Recognition Sequence	GACTACACAT	TATAGTGTCTGTT	(SEQ ID NO:85)

B.

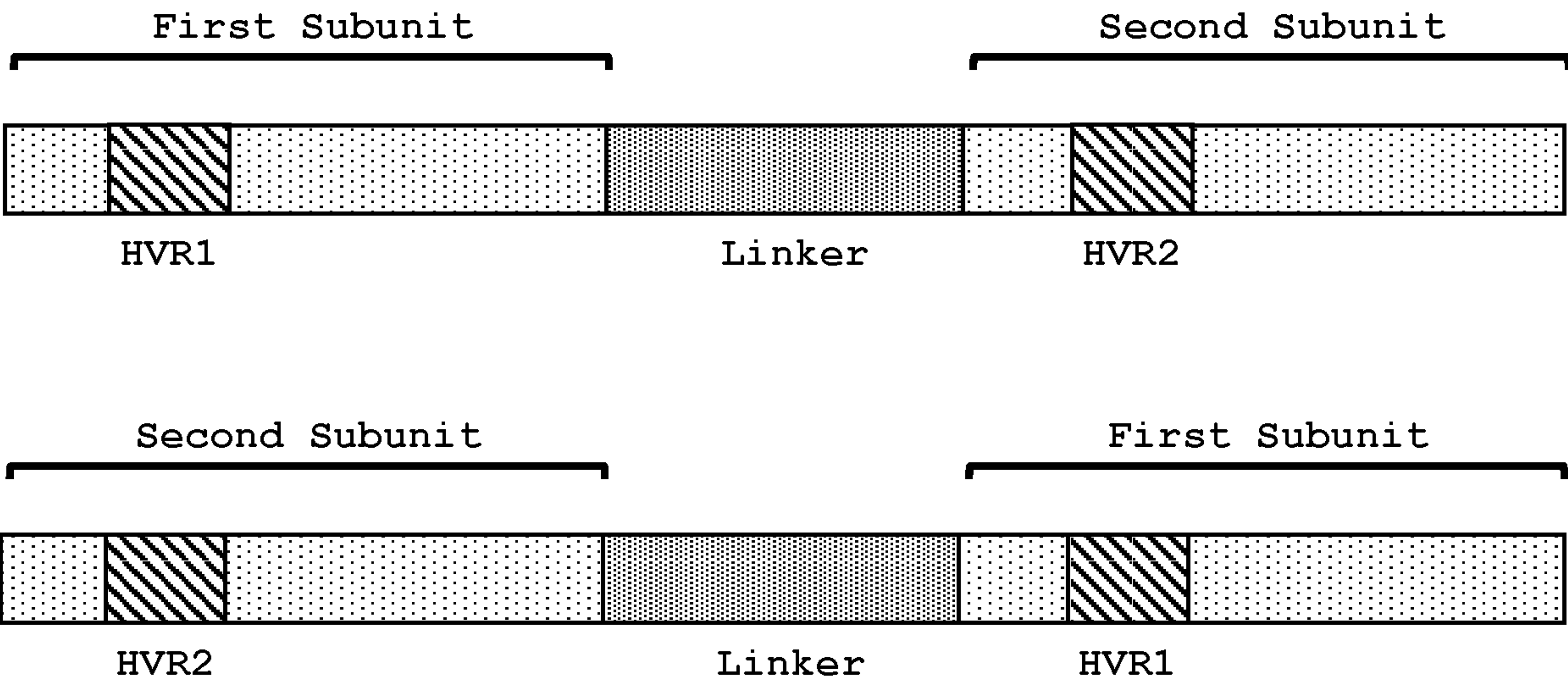


FIGURE 1