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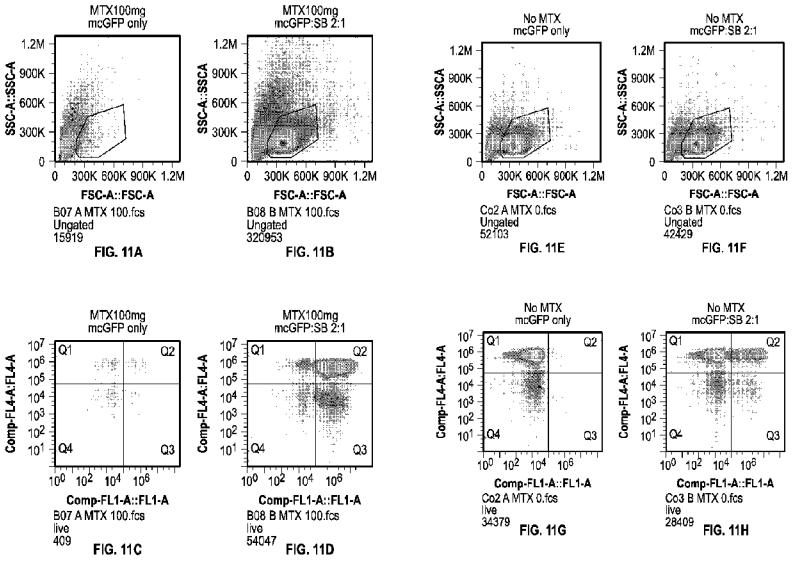
[Continued on next page]

(54) Title: PRODUCTION OF ENGINEERED T-CELLS BY SLEEPING BEAUTY TRANSPOSON COUPLED WITH METHOTREXATE SELECTION



(57) Abstract: Aspects of the invention described herein include methods of treating, inhibiting, ameliorating and/or eliminating a virus or cancer cells in a subject utilizing genetically engineered human T-cells having receptors for a molecule presented by the virus or the cancer cells, wherein the genetically engineered T cells are isolated utilizing a two-stage MTX selection that employs increasing concentrations of MTX.

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— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

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PRODUCTION OF ENGINEERED T-CELLS BY SLEEPING BEAUTY TRANSPOSON COUPLED WITH METHOTREXATE SELECTION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims the benefit of priority to U.S. Provisional Patent Application No. 62/058,973, filed October 2, 2014, U.S. Provisional Patent Application No. 61/977,751, filed April 10, 2014, U.S. Provisional Patent Application No. 61/986,479, filed April 30, 2014, U.S. Provisional Patent Application No. 62/089,730 filed December 9, 2014, U.S. Provisional Patent Application No. 62/090845, filed December 11, 2014, and U.S. Provisional Patent Application No. 62/088,363, filed December 5, 2014. The entire disclosures of the aforementioned applications are hereby expressly incorporated by reference in their entireties.

REFERENCE TO SEQUENCE LISTING, TABLE, OR COMPUTER PROGRAM LISTING

[0002] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is provided as a file entitled SCRI.077PR.TXT, created March 20, 2015, which is 4 kb in size. The information is the electronic format of the Sequence Listing is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0003] Aspects of the invention described herein include methods of treating, inhibiting, ameliorating and/or eliminating a virus or cancer cells in a subject utilizing genetically engineered human T-cells having receptors for a molecule presented by the virus or the cancer cells.

BACKGROUND OF THE INVENTION

[0004] Engineered human T-cells are a promising therapeutic route for cancer immunotherapy and viral therapy. T-cells expressing chimeric antigen receptors combined with additional genes to enhance T-cell proliferation, survival, or tumor homing can further improve efficacy but require multiple stable gene transfer events. Accordingly, methods are needed to increase production efficiency for multiplexed engineered cells. Efficient, stable transduction of T-cells can be achieved using a Sleeping Beauty transposon system in minicircles that are introduced by nucleofection. Rapid selection of transduced cells with methotrexate (MTX) for cells expressing a mutant dihydrofolate reductase (DHFRdm) resistant to metabolic inhibition can also be achieved.

SUMMARY OF THE INVENTION

[0005] Described herein are approaches for the preferential amplification of T cells expressing multiple transgenes, preferably encoding receptors or chimeric receptors specific for a molecule presented by a virus or a cancer cell. In some alternatives, selection pressure on transformed T cells is applied in a two-stage MTX selection utilizing increasing concentrations of MTX.

[0006] In one alternative, a gene delivery polynucleotide for stable insertion of a nucleic acid into an oligonucleotide is provided, wherein the nucleic acid for insertion is flanked by inverted terminal repeat gene sequences in the gene delivery polynucleotide and wherein the gene delivery polynucleotide is selectable is provided, wherein the gene delivery polynucleotide comprises a first sequence, wherein the first sequence comprises a first inverted terminal repeat gene sequence, a second sequence, wherein the second sequence comprises a second inverted terminal repeat gene sequence, a third sequence, wherein the third sequence comprises a promoter region sequence, a fourth sequence, wherein the fourth sequence comprises at least one gene encodes a protein or encodes a sequence for mRNA transcription, and wherein the fourth sequence is optimized, a fifth sequence, wherein the fifth sequence comprises at least one selectable marker cassette encoding a double mutant of dihydrofolate reductase, wherein the double mutant of dihydrofolate reductase has a 15,000 fold or about 15,000 fold reduced affinity for methotrexate, wherein the methotrexate can be used to select for cells transduced with the gene delivery polynucleotide to enhance the ratio

of cells expressing the at least one gene and wherein the fifth sequence is optimized, a sixth sequence, wherein the sixth sequence comprises a first attachment site (attP) and a seventh sequence, wherein the seventh sequence comprises a second attachment site (attB) wherein each of the first sequence, second sequence, third sequence, fourth sequence, fifth sequence, sixth sequence, and seventh sequence have a 5' terminus and a 3 ' terminus, and wherein the 3' terminus of the first sequence comprising the first inverted terminal repeat gene sequence is adjacent to the 5' terminus of the third sequence, the 3' terminus of the third sequence is adjacent to the 5' terminus of the fourth sequence, the 3' terminus of the fourth sequence is adjacent to the 5' terminus of the fifth sequence and the 3' terminus of the fifth sequence is adjacent to the 5' terminus of the second sequence comprising a second inverted terminal repeat. In some alternatives, the gene encoding the double mutant of human dihydrofolate reductase comprises the DNA sequence: ATGGTTGGTTCGCTAAACTGCATCGTCGCTGTGTCCCAGAACATGGGCATCGGCA AGAACGGGGACTTCCCCTGGCCACCGCTCAGGAATGAATCCAGATATTTCCAGA GAATGACCACAACCTCTTCAGTAGAAGGTAAACAGAATCTGGTGATTATGGGTA AGAAGACCTGGTTCTCCATTCCTGAGAAGAATCGACCTTTAAAGGGTAGAATTA ATTTAGTTCTCAGCAGAGAACTCAAGGAACCTCCACAAGGAGCTCATTTTCTTTC CAGAAGTCTAGATGATGCCTTAAAACTTACTGAACAACCAGAATTAGCAAATAA AGTAGACATGGTCTGGATAGTTGGTGGCAGTTCTGTTTATAAGGAAGCCATGAAT CACCCAGGCCATCTTAAACTATTTGTGACAAGGATCATGCAAGACTTTGAAAGTG ACACGTTTTTTCCAGAAATTGATTTGGAGAAATATAAACTTCTGCCAGAATACCC AGGTGTTCTCTCTGATGTCCAGGAGGAGAAAGGCATTAAGTACAAATTTGAAGT ATATGAGAAGAATGATTAA (SEQ ID NO: 2). In some alternatives, the double mutant of human dihydrofolate reductase comprises the protein sequence: MVGSLNCIVA VSQNMGIGKN GDFPWPPLRN ESRYFQRMTT TSSVEGKQNL VIMGKKTWFS IPEKNRPLKG RINLVLSREL KEPPQGAHFL SRSLDDALKL TEQPELANKV DMVWIVGGSS VYKEAMNHPG HLKLFVTRIM QDFESDTFFP EIDLEKYKLL PEYPGVLSDV QEEKGIKYKF EVYEKND (SEQ ID NO: 3). In some alternatives, the gene delivery polynucleotide is circular. In some alternatives, the gene delivery polynucleotide is at least 1kB to 5kB. In some alternatives, the gene delivery polynucleotide is a minicircle. In some alternatives, the promoter region comprises an EF1 promoter sequence. In some

alternatives, the fourth sequence comprises one, two, three, four, or five genes that encode proteins. In some alternatives, the fourth sequence is codon optimized to reduce the total GC/AT ratio of the fourth sequence. In some alternatives, the fourth sequence is optimized by codon optimization for expression in humans. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins, such as a plurality of antibody binding domains, which are specific for the same epitope. In some alternatives, the plurality of related proteins comprise a plurality of antibody binding domains, wherein the plurality of antibody binding domains are specific for the same epitope. In some alternatives, the fifth sequence is codon optimized for expression in humans and/or to reduce the total GC/AT ratio of the fifth sequence. In preferred alternatives, the fifth sequence is optimized by codon optimization for expression in humans. In some alternatives, the protein is a protein for therapy. In some alternatives, the codon optimization and/or a consensus sequence is generated by comparing the variability of sequence and/or nucleobases utilized in a plurality of related sequences. In some alternatives, the protein comprises an antibody or a portion thereof, which may be humanized. In some alternatives, the double mutant of dihydrofolate reductase comprises amino acid mutations of L22F and F31S. In some alternatives, the T cells are precursor T cells. In some alternatives, the precursor T cells are hematopoietic stem cells.

[0007] In some alternatives, a method of generating engineered multiplexed T-cells for adoptive T-cell immunotherapy is provided, wherein the method comprises providing a gene delivery polynucleotide, introducing the gene delivery polynucleotide into a T-cell, providing a vector encoding a Sleeping Beauty transposase, introducing the vector encoding the Sleeping Beauty transposase into the T-cell, selecting the cells comprising the gene delivery polynucleotide wherein selecting comprises a first round of selection and a second round of selection, wherein the first round of selection comprises adding a selection reagent at a first concentration range and the second round of selection comprises adding the selection reagent at a second concentration range, wherein the second concentration range is higher than the first concentration range and, wherein the second concentration range is at least 1.5 fold higher than that of the first concentration range and isolating the T-cells

expressing a phenotype under selective pressure. In some alternatives, the gene delivery polynucleotide comprises a first sequence, wherein the first sequence comprises a first inverted terminal repeat gene sequence, a second sequence, wherein the second sequence comprises a second inverted terminal repeat gene sequence, a third sequence, wherein the third sequence comprises a promoter region sequence, a fourth sequence, wherein the fourth sequence comprises at least one gene encoding a protein, and wherein the fourth sequence is optimized, a fifth sequence, wherein the fifth sequence comprises at least one selectable marker cassette encoding a double mutant of dihydrofolate reductase, wherein the double mutant of dihydrofolate reductase has a 15,000 fold or about 15,000 fold reduced affinity for methotrexate, wherein the methotrexate can be used as a selection mechanism to selectively amplify cells transduced with the gene delivery polynucleotide and wherein the fifth sequence is optimized, a sixth sequence, wherein the sixth sequence comprises a first attachment site (attP) and a seventh sequence, wherein the seventh sequence comprises a second attachment site (attB) wherein each of the first sequence, second sequence, third sequence, fourth sequence, fifth sequence, sixth sequence, and seventh sequence have a 5' terminus and a 3 ' terminus, and wherein the 3' terminus of the first sequence comprising the first inverted terminal repeat gene sequence is adjacent to the 5' terminus of the third sequence, the 3' terminus of the third sequence is adjacent to the 5' terminus of the fourth sequence, the 3' terminus of the fourth sequence is adjacent to the 5' terminus of the fifth sequence and the 3' terminus of the fifth sequence is adjacent to the 5' terminus of the second sequence comprising a second inverted terminal repeat. In some alternatives, the gene encoding the double mutant of human dihydrofolate reductase comprises the DNA sequence:

ATGGTTGGTTCGCTAAACTGCATCGTCGCTGTGTCCCAGAACATGGGCATCGGCA
AGAACGGGGACTTCCCCTGGCCACCGCTCAGGAATGAATCCAGATATTTCCAGA
GAATGACCACAACCTCTTCAGTAGAAGGTAAACAGAATCTGGTGATTATGGGTA
AGAAGACCTGGTTCTCCATTCCTGAGAAGAATCGACCTTTAAAGGGTAGAATTA
ATTTAGTTCTCAGCAGAGAACTCAAGGAACCTCCACAAGGAGCTCATTTTCTTTC
CAGAAGTCTAGATGATGCCTTAAAACTTACTGAACAACCAGAATTAGCAAATAA
AGTAGACATGGTCTGGATAGTTGGTGGCAGTTCTGTTTATAAGGAAGCCATGAAT
CACCCAGGCCATCTTAAACTATTTGTGACAAGGATCATGCAAGACTTTGAAAGTG
ACACGTTTTTTCCAGAAATTGATTTGGAGAAATATAAACTTCTGCCAGAATACCC

AGGTGTTCTCTCTGATGTCCAGGAGGAGAAAGGCATTAAGTACAAATTTGAAGT ATATGAGAAGAATGATTAA (SEQ ID NO: 2). In some alternatives, the double mutant of human dihydrofolate reductase comprises the protein sequence: MVGSLNCIVA VSQNMGIGKN GDFPWPPLRN ESRYFQRMTT TSSVEGKQNL VIMGKKTWFS IPEKNRPLKG RINLVLSREL KEPPQGAHFL SRSLDDALKL TEQPELANKV DMVWIVGGSS VYKEAMNHPG HLKLFVTRIM QDFESDTFFP EIDLEKYKLL PEYPGVLSDV QEEKGIKYKF EVYEKND (SEQ ID NO: 3). In some alternatives, the gene delivery polynucleotide is circular. In some alternatives, the gene delivery polynucleotide is at least 1kB to 5kB. In some alternatives, the gene delivery polynucleotide is a minicircle. In some alternatives, the promoter region comprises an EF1 promoter sequence. In some alternatives, the fourth sequence comprises one, two, three, four, or five genes that encode proteins. In some alternatives, the fourth sequence is codon optimized to reduce the total GC/AT ratio of the fourth sequence. In some alternatives, the fourth sequence is optimized by codon optimization for expression in humans. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins, such as a plurality of antibody binding domains, which are specific for the same epitope. In some alternatives, the plurality of related proteins comprise a plurality of antibody binding domains, wherein the plurality of antibody binding domains are specific for the same epitope. In some alternatives, the fifth sequence is codon optimized to reduce the total GC/AT ratio of the fifth sequence. In some alternatives, the fifth sequence is optimized by codon optimization for expression in humans. In some alternatives, the protein is a protein for therapy. In some alternatives, the codon optimization and/or consensus sequence is generated by comparing the variability of sequence and/or nucleobases utilized in a plurality of related sequences. In some alternatives, the protein comprises an antibody or a portion thereof, which may be humanized. In some alternatives, the double mutant of dihydrofolate reductase comprises amino acid mutations of L22F and F31S. In some alternatives, the double mutant of dihydrofolate reductase comprises amino acid mutations of L22F and F31S. In some alternatives, the introducing is performed by electroporation. In some alternatives, the selecting is performed by increasing selective pressure through the selective marker

cassette. In some alternatives, the selection reagent comprises an agent for selection. In some alternatives, the agent for selection is methotrexate. In some alternatives, the first concentration range is at least 50nM – 100nM and the second concentration range is at least 75 to 150nM. In some alternatives, the first concentration is 50 nM, 60 nM, 70 nM, 80 nM, 90 nM, or 100nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 75 nM, 80 nM, 90 nM, 100 nM, 110 nM, 120 nM, 130 nM, 140 nM, or 150 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first concentration range is at least 75 nM – 150 nM and the second concentration range is at least 112.5 nM to 225 nM. In some alternatives, the first concentration is 75 nM, 85 nM, 95 nM, 105 nM, 115 nM, 125 nM, 135 nM, 145 nM, or 150 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 112 nM, 122 nM, 132 nM, 142 nM, 152 nM, 162 nM, 172 nM, 182 nM, 192 nM, 202 nM, 212 nM, or 225 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first concentration range is at least 300 nM – 675 nM and the first concentration range is at least 450 nM to 1012 nM. In some alternatives, the first concentration is 300 nM, 350 nM, 400 nM, 450 nM, 500 nM, 550 nM, 600 nM, 650 nM, or 675 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 450 nM, 500 nM, 550 nM, 600 nM, 650 nM, 700 nM, 750 nM, 800 nM, 850 nM, 900 nM, 1000 nM, or 1012 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first round of selection comprises exposing the T-cells to the selection agent for 2, 3, 4, 5, 6 or 7 days before the second round of selection. In some alternatives, the second round of selection comprises exposing the T-cells to the selection agent for at least 2, 3, 4, 5, 6,7, 8, 9, 10, 11, 12, 13, or 14 days or any time that is between a range of times defined by any two of the aforementioned time points before isolation. In some alternatives, the T cells are precursor T cells. In some alternatives, the precursor T cells are hematopoietic stem cells.

[0008] In some alternatives, a method of increasing protein production in a T-cell is provided, wherein the method comprises providing a polynucleotide of, introducing the

polynucleotide into a cell, providing a vector encoding a Sleeping Beauty transposase, introducing the vector encoding the Sleeping Beauty transposase into the T-cell, selecting the cells comprising the gene delivery polynucleotide wherein selecting comprises a first round of selection and a second round of selection, wherein the first round of selection comprises adding a selection reagent at a first concentration range and the second round of selection comprises adding the selection reagent at a second concentration range, wherein the second concentration range is higher than the first concentration range and, wherein the second concentration range is at least 1.5 fold higher than that of the first concentration range and isolating the cells expressing a phenotype under selective pressure. In some alternatives, the gene delivery polynucleotide comprises a first sequence, wherein the first sequence comprises a first inverted terminal repeat gene sequence, a second sequence, wherein the second sequence comprises a second inverted terminal repeat gene sequence, a third sequence, wherein the third sequence comprises a promoter region sequence, a fourth sequence, wherein the fourth sequence comprises at least one gene encoding a protein, and wherein the fourth sequence is optimized, a fifth sequence, wherein the fifth sequence comprises at least one selectable marker cassette encoding a double mutant of dihydrofolate reductase, wherein the double mutant of dihydrofolate reductase has a 15,000 fold or about 15,000 fold reduced affinity for methotrexate, wherein the methotrexate can be used as a selection mechanism to selectively amplify cells transduced with the gene delivery polynucleotide and wherein the fifth sequence is optimized, a sixth sequence, wherein the sixth sequence comprises a first attachment site (attP) and a seventh sequence, wherein the seventh sequence comprises a second attachment site (attB) wherein each of the first sequence, second sequence, third sequence, fourth sequence, fifth sequence, sixth sequence, and seventh sequence have a 5' terminus and a 3 ' terminus, and wherein the 3' terminus of the first sequence comprising the first inverted terminal repeat gene sequence is adjacent to the 5' terminus of the third sequence, the 3' terminus of the third sequence is adjacent to the 5' terminus of the fourth sequence, the 3' terminus of the fourth sequence is adjacent to the 5' terminus of the fifth sequence and the 3' terminus of the fifth sequence is adjacent to the 5' terminus of the second sequence comprising a second inverted terminal repeat. In some alternatives, the gene encoding the double mutant of human dihydrofolate reductase comprises the DNA sequence:

ATGGTTGGTTCGCTAAACTGCATCGTCGCTGTGTCCCAGAACATGGGCATCGGCA
AGAACGGGGACTTCCCCTGGCCACCGCTCAGGAATGAATCCAGATATTTCCAGA
GAATGACCACAACCTCTTCAGTAGAAGGTAAACAGAATCTGGTGATTATGGGTA
AGAAGACCTGGTTCTCCATTCCTGAGAAGAATCGACCTTTAAAGGGTAGAATTA
ATTTAGTTCTCAGCAGAGAACTCAAGGAACCTCCACAAGGAGCTCATTTTCTTTC
CAGAAGTCTAGATGATGCCTTAAAACTTACTGAACAACCAGAATTAGCAAATAA
AGTAGACATGGTCTGGATAGTTGGTGGCAGTTCTGTTTATAAGGAAGCCATGAAT
CACCCAGGCCATCTTAAACTATTTGTGACAAGGATCATGCAAGACTTTGAAAGTG
ACACGTTTTTTCCAGAAATTGATTTGGAGAAATATAAACTTCTGCCAGAATACCC
AGGTGTTCTCTCTGATGTCCAGGAGGAGAAAGGCATTAAGTACAAATTTGAAGT
ATATGAGAAGAATGATTAA (SEQ ID NO: 2). In some alternatives, the double mutant of human dihydrofolate reductase comprises the protein sequence: MVGSLNCIVA VSQNMGIGKN GDFPWPPLRN ESRYFQRMTT TSSVEGKQNL VIMGKKTWFS IPEKNRPLKG RINLVLSREL KEPPQGAHFL SRSLDDALKL TEQPELANKV DMVWIVGGSS VYKEAMNHPG HLKLFVTRIM QDFESDTFFP EIDLEKYKLL PEYPGVLSDV QEEKGIKYKF EVYEKND (SEQ ID NO: 3). In some alternatives, the gene delivery polynucleotide is circular. In some alternatives, the gene delivery polynucleotide is at least 1kB to 5kB. In some alternatives, the gene delivery polynucleotide is a minicircle. In some alternatives, the promoter region comprises an EF1 promoter sequence. In some alternatives, the fourth sequence comprises one, two, three, four, or five genes that encode proteins. In some alternatives, the fourth sequence is codon optimized to reduce the total GC/AT ratio of the fourth sequence. In some alternatives, the fourth sequence is optimized by codon optimization for expression in humans. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins, such as a plurality of antibody binding domains, which are specific for the same epitope. In some alternatives, the plurality of related proteins comprise a plurality of antibody binding domains, wherein the plurality of antibody binding domains are specific for the same epitope. In some alternatives, the fifth sequence is codon optimized to reduce the total GC/AT ratio of the fifth sequence. In some alternatives, the fifth sequence is optimized by codon optimization for

expression in humans. In some alternatives, the protein is a protein for therapy. In some alternatives, the codon optimization and/or consensus sequence is generated by comparing the variability of sequence and/or nucleobases utilized in a plurality of related sequences. In some alternatives, the protein comprises an antibody or a portion thereof, which may be humanized. In some alternatives, the double mutant of dihydrofolate reductase comprises amino acid mutations of L22F and F31S. In some alternatives, the double mutant of dihydrofolate reductase comprises amino acid mutations of L22F and F31S. In some alternatives, the introducing is performed by electroporation. In some alternatives, the selecting is performed by increasing selective pressure through the selective marker cassette. In some alternatives, the selection reagent comprises an agent for selection. In some alternatives, the agent for selection is methotrexate. In some alternatives, the first concentration range is at least 50nM – 100nM and the second concentration range is at least 75 to 150nM. In some alternatives, the first concentration is 50 nM, 60 nM, 70 nM, 80 nM, 90 nM, or 100nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 75 nM, 80 nM, 90 nM, 100 nM, 110 nM, 120 nM, 130 nM, 140 nM, or 150 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first concentration range is at least 75 nM – 150 nM and the second concentration range is at least 112.5 nM to 225 nM. In some alternatives, the first concentration is 75 nM, 85 nM, 95 nM, 105 nM, 115 nM, 125 nM, 135 nM, 145 nM, 150 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 112 nM, 122 nM, 132 nM, 142 nM, 152 nM, 162 nM, 172 nM, 182 nM, 192 nM, 202 nM, 212 nM, or 225 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first concentration range is at least 300 nM – 675 nM and the first concentration range is at least 450 nM to 1012 nM. In some alternatives, the first concentration is 300 nM, 350 nM, 400 nM, 450 nM, 500 nM, 550 nM, 600 nM, 650 nM, or 675 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 450 nM, 500 nM, 550 nM, 600 nM, 650 nM, 700 nM, 750 nM, 800 nM, 850 nM, 900 nM, 1000 nM, or 1012 nM or any concentration that is between a range of concentrations defined

by any two of the aforementioned concentrations. In some alternatives, the first round of selection comprises exposing the T-cells to the selection agent for 2, 3, 4, 5, 6 or 7 days before the second round of selection. In some alternatives, the second round of selection comprises exposing the T-cells to the selection agent for at least 2, 3, 4, 5, 6,7, 8, 9, 10, 11, 12, 13, or 14 days or any time that is between a range of times defined by any two of the aforementioned time points before isolation. In some alternatives, the T cells are precursor T cells. In some alternatives, the precursor T cells are hematopoietic stem cells.

[0009] In some alternatives, an engineered multiplexed T-cell for adoptive T-cell immunotherapy generated by any one of the methods of is provided. In some alternatives, the engineered multiplexed T-cells for adoptive T-cell immunotherapy is generated by a method, wherein the method comprises providing a gene delivery polynucleotide, introducing the gene delivery polynucleotide into a T-cell, providing a vector encoding a Sleeping Beauty transposase, introducing the vector encoding the Sleeping Beauty transposase into the T-cell, selecting the cells comprising the gene delivery polynucleotide wherein selecting comprises a first round of selection and a second round of selection, wherein the first round of selection comprises adding a selection reagent at a first concentration range and the second round of selection comprises adding the selection reagent at a second concentration range, wherein the second concentration range is higher than the first concentration range and, wherein the second concentration range is at least 1.5 fold higher than that of the first concentration range and isolating the T-cells expressing a phenotype under selective pressure. In some alternatives, the gene delivery polynucleotide comprises a first sequence, wherein the first sequence comprises a first inverted terminal repeat gene sequence, a second sequence, wherein the second sequence comprises a second inverted terminal repeat gene sequence, a third sequence, wherein the third sequence comprises a promoter region sequence, a fourth sequence, wherein the fourth sequence comprises at least one gene encoding a protein, and wherein the fourth sequence is optimized, a fifth sequence, wherein the fifth sequence comprises at least one selectable marker cassette encoding a double mutant of dihydrofolate reductase, wherein the double mutant of dihydrofolate reductase has a 15,000 fold or about 15,000 fold reduced affinity for methotrexate, wherein the methotrexate can be used as a selection mechanism to selectively amplify cells transduced with the gene delivery polynucleotide and wherein the fifth sequence is optimized, a sixth sequence, wherein the

sixth sequence comprises a first attachment site (attP) and a seventh sequence, wherein the seventh sequence comprises a second attachment site (attB) wherein each of the first sequence, second sequence, third sequence, fourth sequence, fifth sequence, sixth sequence, and seventh sequence have a 5' terminus and a 3 ' terminus, and wherein the 3' terminus of the first sequence comprising the first inverted terminal repeat gene sequence is adjacent to the 5' terminus of the third sequence, the 3' terminus of the third sequence is adjacent to the 5' terminus of the fourth sequence, the 3' terminus of the fourth sequence is adjacent to the 5' terminus of the fifth sequence and the 3' terminus of the fifth sequence is adjacent to the 5' terminus of the second sequence comprising a second inverted terminal repeat. In some alternatives, the gene encoding the double mutant of human dihydrofolate reductase comprises the DNA sequence: ATGGTTGGTTCGCTAAACTGCATCGTCGCTGTGTCCCAGAACATGGGCATCGGCA AGAACGGGGACTTCCCCTGGCCACCGCTCAGGAATGAATCCAGATATTTCCAGA GAATGACCACAACCTCTTCAGTAGAAGGTAAACAGAATCTGGTGATTATGGGTA AGAAGACCTGGTTCTCCATTCCTGAGAAGAATCGACCTTTAAAGGGTAGAATTA ATTTAGTTCTCAGCAGAGAACTCAAGGAACCTCCACAAGGAGCTCATTTTCTTTC CAGAAGTCTAGATGATGCCTTAAAACTTACTGAACAACCAGAATTAGCAAATAA AGTAGACATGGTCTGGATAGTTGGTGGCAGTTCTGTTTATAAGGAAGCCATGAAT CACCCAGGCCATCTTAAACTATTTGTGACAAGGATCATGCAAGACTTTGAAAGTG ACACGTTTTTTCCAGAAATTGATTTGGAGAAATATAAACTTCTGCCAGAATACCC AGGTGTTCTCTCTGATGTCCAGGAGGAGAAAGGCATTAAGTACAAATTTGAAGT ATATGAGAAGAATGATTAA (SEQ ID NO: 2). In some alternatives, the double mutant of human dihydrofolate reductase comprises the protein sequence: MVGSLNCIVA VSQNMGIGKN GDFPWPPLRN ESRYFQRMTT TSSVEGKQNL VIMGKKTWFS IPEKNRPLKG RINLVLSREL KEPPQGAHFL SRSLDDALKL TEQPELANKV DMVWIVGGSS VYKEAMNHPG HLKLFVTRIM QDFESDTFFP EIDLEKYKLL PEYPGVLSDV QEEKGIKYKF EVYEKND (SEQ ID NO: 3). In some alternatives, the gene delivery polynucleotide is circular. In some alternatives, the gene delivery polynucleotide is at least 1kB to 5kB. In some alternatives, the gene delivery polynucleotide is a minicircle. In some alternatives, the promoter region comprises an EF1 promoter sequence. In some alternatives, the fourth sequence comprises one, two, three, four, or five genes that encode

proteins. In some alternatives, the fourth sequence is codon optimized to reduce the total GC/AT ratio of the fourth sequence. In some alternatives, the fourth sequence is optimized by codon optimization for expression in humans. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins, such as a plurality of antibody binding domains, which are specific for the same epitope. In some alternatives, the plurality of related proteins comprise a plurality of antibody binding domains, wherein the plurality of antibody binding domains are specific for the same epitope. In some alternatives, the fifth sequence is codon optimized to reduce the total GC/AT ratio of the fifth sequence. In some alternatives, the fifth sequence is optimized by codon optimization for expression in humans. In some alternatives, the protein is a protein for therapy. In some alternatives, the codon optimization and/or consensus sequence is generated by comparing the variability of sequence and/or nucleobases utilized in a plurality of related sequences. In some alternatives, the protein comprises an antibody or a portion thereof, which may be humanized. In some alternatives, the double mutant of dihydrofolate reductase comprises amino acid mutations of L22F and F31S. In some alternatives, the double mutant of dihydrofolate reductase comprises amino acid mutations of L22F and F31S. In some alternatives, the introducing is performed by electroporation. In some alternatives, the selecting is performed by increasing selective pressure through the selective marker cassette. In some alternatives, the selection reagent comprises an agent for selection. In some alternatives, the agent for selection is methotrexate. In some alternatives, the first concentration range is at least 50nM – 100nM and the second concentration range is at least 75 to 150nM. In some alternatives, the first concentration is 50 nM, 60 nM, 70 nM, 80 nM, 90 nM, or 100nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 75 nM, 80 nM, 90 nM, 100 nM, 110 nM, 120 nM, 130 nM, 140 nM, or 150 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first concentration range is at least 75 nM – 150 nM and the second concentration range is at least 112.5 nM to 225 nM. In some alternatives, the first concentration is 75 nM, 85 nM, 95 nM, 105 nM, 115 nM, 125 nM, 135 nM, 145 nM, or

150 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 112 nM, 122 nM, 132 nM, 142 nM, 152 nM, 162 nM, 172 nM, 182 nM, 192 nM, 202 nM, 212 nM, or 225 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first concentration range is at least 300 nM – 675 nM and the first concentration range is at least 450 nM to 1012 nM. In some alternatives, the first concentration is 300 nM, 350 nM, 400 nM, 450 nM, 500 nM, 550 nM, 600 nM, 650 nM, or 675 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 450 nM, 500 nM, 550 nM, 600 nM, 650 nM, 700 nM, 750 nM, 800 nM, 850 nM, 900 nM, 1000 nM, or 1012 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first round of selection comprises exposing the T-cells to the selection agent for 2, 3, 4, 5, 6 or 7 days before the second round of selection. In some alternatives, the second round of selection comprises exposing the T-cells to the selection agent for at least 2, 3, 4, 5, 6,7, 8, 9, 10, 11, 12, 13, or 14 days or any time that is between a range of times defined by any two of the aforementioned time points before isolation. In some alternatives, the gene delivery polynucleotide comprises a first sequence, wherein the first sequence comprises a first inverted terminal repeat gene sequence, a second sequence, wherein the second sequence comprises a second inverted terminal repeat gene sequence, a third sequence, wherein the third sequence comprises a promoter region sequence, a fourth sequence, wherein the fourth sequence comprises at least one gene encoding a protein, and wherein the fourth sequence is optimized, a fifth sequence, wherein the fifth sequence comprises at least one selectable marker cassette encoding a double mutant of dihydrofolate reductase, wherein the double mutant of dihydrofolate reductase has a 15,000 fold or about 15,000 fold reduced affinity for methotrexate, wherein the methotrexate can be used as a selection mechanism to selectively amplify cells transduced with the gene delivery polynucleotide and wherein the fifth sequence is optimized, a sixth sequence, wherein the sixth sequence comprises a first attachment site (attP) and a seventh sequence, wherein the seventh sequence comprises a second attachment site (attB) wherein each of the first sequence, second sequence, third sequence, fourth sequence, fifth sequence, sixth sequence, and seventh sequence have a 5'

terminus and a 3 ‘ terminus, and wherein the 3’ terminus of the first sequence comprising the first inverted terminal repeat gene sequence is adjacent to the 5’ terminus of the third sequence, the 3’ terminus of the third sequence is adjacent to the 5’ terminus of the fourth sequence, the 3’ terminus of the fourth sequence is adjacent to the 5’ terminus of the fifth sequence and the 3’ terminus of the fifth sequence is adjacent to the 5’ terminus of the second sequence comprising a second inverted terminal repeat. In some alternatives, the gene encoding the double mutant of human dihydrofolate reductase comprises the DNA sequence: ATGGTTGGTTCGCTAAACTGCATCGTCGCTGTGTCCCAGAACATGGGCATCGGCA AGAACGGGGACTTCCCCTGGCCACCGCTCAGGAATGAATCCAGATATTTCCAGA GAATGACCACAACCTCTTCAGTAGAAGGTAAACAGAATCTGGTGATTATGGGTA AGAAGACCTGGTTCTCCATTCCTGAGAAGAATCGACCTTTAAAGGGTAGAATTA ATTTAGTTCTCAGCAGAGAACTCAAGGAACCTCCACAAGGAGCTCATTTTCTTTC CAGAAGTCTAGATGATGCCTTAAAACTTACTGAACAACCAGAATTAGCAAATAA AGTAGACATGGTCTGGATAGTTGGTGGCAGTTCTGTTTATAAGGAAGCCATGAAT CACCCAGGCCATCTTAAACTATTTGTGACAAGGATCATGCAAGACTTTGAAAGTG ACACGTTTTTTCCAGAAATTGATTTGGAGAAATATAAACTTCTGCCAGAATACCC AGGTGTTCTCTCTGATGTCCAGGAGGAGAAAGGCATTAAGTACAAATTTGAAGT ATATGAGAAGAATGATTAA (SEQ ID NO: 2). In some alternatives, the double mutant of human dihydrofolate reductase comprises the protein sequence: MVGSLNCIVA VSQNMGIGKN GDFPWPPLRN ESRYFQRMTT TSSVEGKQNL VIMGKKTWFS IPEKNRPLKG RINLVLSREL KEPPQGAHFL SRSLDDALKL TEQPELANKV DMVWIVGGSS VYKEAMNHPG HLKLFVTRIM QDFESDTFFP EIDLEKYKLL PEYPGVLSDV QEEKGIKYKF EVYEKND (SEQ ID NO: 3). In some alternatives, the gene delivery polynucleotide is circular. In some alternatives, the gene delivery polynucleotide is at least 1kB to 5kB. In some alternatives, the gene delivery polynucleotide is a minicircle. In some alternatives, the promoter region comprises an EF1 promoter sequence. In some alternatives, the fourth sequence comprises one, two, three, four, or five genes that encode proteins. In some alternatives, the fourth sequence is codon optimized to reduce the total GC/AT ratio of the fourth sequence. In some alternatives, the fourth sequence is optimized by codon optimization for expression in humans. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of

related proteins. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins, such as a plurality of antibody binding domains, which are specific for the same epitope. In some alternatives, the plurality of related proteins comprise a plurality of antibody binding domains, wherein the plurality of antibody binding domains are specific for the same epitope. In some alternatives, the fifth sequence is codon optimized to reduce the total GC/AT ratio of the fifth sequence. In some alternatives, the fifth sequence is optimized by codon optimization for expression in humans. In some alternatives, the protein is a protein for therapy. In some alternatives, the codon optimization and/or consensus sequence is generated by comparing the variability of sequence and/or nucleobases utilized in a plurality of related sequences. In some alternatives, the protein comprises an antibody or a portion thereof, which may be humanized. In some alternatives, the double mutant of dihydrofolate reductase comprises amino acid mutations of L22F and F31S. In some alternatives, the introducing is performed by electroporation. In some alternatives, the selecting is performed by increasing selective pressure through the selective marker cassette. In some alternatives, the selection reagent comprises an agent for selection. In some alternatives, the agent for selection is methotrexate. In some alternatives, the first concentration range is at least 50nM – 100nM and the second concentration range is at least 75 to 150nM. In some alternatives, the first concentration is 50 nM, 60 nM, 70 nM, 80 nM, 90 nM, or 100nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 75 nM, 80 nM, 90 nM, 100 nM, 110 nM, 120 nM, 130 nM, 140 nM, or 150 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first concentration range is at least 75 nM – 150 nM and the second concentration range is at least 112.5 nM to 225 nM. In some alternatives, the first concentration is 75 nM, 85 nM, 95 nM, 105 nM, 115 nM, 125 nM, 135 nM, 145 nM, or 150 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 112 nM, 122 nM, 132 nM, 142 nM, 152 nM, 162 nM, 172 nM, 182 nM, 192 nM, 202 nM, 212 nM, or 225 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first concentration range is at least 300 nM – 675 nM and the first

concentration range is at least 450 nM to 1012 nM. In some alternatives, the first concentration is 300 nM, 350 nM, 400 nM, 450 nM, 500 nM, 550 nM, 600 nM, 650 nM, or 675 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 450 nM, 500 nM, 550 nM, 600 nM, 650 nM, 700 nM, 750 nM, 800 nM, 850 nM, 900 nM, 1000 nM, or 1012 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first round of selection comprises exposing the T-cells to the selection agent for 2, 3, 4, 5, 6 or 7 days before the second round of selection. In some alternatives, the second round of selection comprises exposing the T-cells to the selection agent for at least 2, 3, 4, 5, 6,7, 8, 9, 10, 11, 12, 13, or 14 days or any time that is between a range of times defined by any two of the aforementioned time points before isolation. In some alternatives, the T cells are precursor T cells. In some alternatives, the precursor T cells are hematopoietic stem cells.

[0010] In some alternatives, a method of treating, inhibiting, or ameliorating cancer or a disease in a subject is provided, wherein the method comprises administering to the subject the modified or engineered multiplexed T-cell generated as described below. In some alternatives, the engineered multiplexed T-cells for adoptive T-cell immunotherapy is generated by a method, wherein the method comprises providing a gene delivery polynucleotide, introducing the gene delivery polynucleotide into a T-cell, providing a vector encoding a Sleeping Beauty transposase, introducing the vector encoding the Sleeping Beauty transposase into the T-cell, selecting the cells comprising the gene delivery polynucleotide wherein selecting comprises a first round of selection and a second round of selection, wherein the first round of selection comprises adding a selection reagent at a first concentration range and the second round of selection comprises adding the selection reagent at a second concentration range, wherein the second concentration range is higher than the first concentration range and, wherein the second concentration range is at least 1.5 fold higher than that of the first concentration range and isolating the T-cells expressing a phenotype under selective pressure. In some alternatives, the gene delivery polynucleotide comprises a first sequence, wherein the first sequence comprises a first inverted terminal repeat gene sequence, a second sequence, wherein the second sequence comprises a second inverted terminal repeat gene sequence, a third sequence, wherein the third sequence

comprises a promoter region sequence, a fourth sequence, wherein the fourth sequence comprises at least one gene encoding a protein, and wherein the fourth sequence is optimized, a fifth sequence, wherein the fifth sequence comprises at least one selectable marker cassette encoding a double mutant of dihydrofolate reductase, wherein the double mutant of dihydrofolate reductase has a 15,000 fold or about 15,000 fold reduced affinity for methotrexate, wherein the methotrexate can be used as a selection mechanism to selectively amplify cells transduced with the gene delivery polynucleotide and wherein the fifth sequence is optimized, a sixth sequence, wherein the sixth sequence comprises a first attachment site (attP) and a seventh sequence, wherein the seventh sequence comprises a second attachment site (attB) wherein each of the first sequence, second sequence, third sequence, fourth sequence, fifth sequence, sixth sequence, and seventh sequence have a 5' terminus and a 3 ' terminus, and wherein the 3' terminus of the first sequence comprising the first inverted terminal repeat gene sequence is adjacent to the 5' terminus of the third sequence, the 3' terminus of the third sequence is adjacent to the 5' terminus of the fourth sequence, the 3' terminus of the fourth sequence is adjacent to the 5' terminus of the fifth sequence and the 3' terminus of the fifth sequence is adjacent to the 5' terminus of the second sequence comprising a second inverted terminal repeat. In some alternatives, the gene encoding the double mutant of human dihydrofolate reductase comprises the DNA sequence: ATGGTTGGTTCGCTAAACTGCATCGTCGCTGTGTCCCAGAACATGGGCATCGGCA AGAACGGGGACTTCCCCTGGCCACCGCTCAGGAATGAATCCAGATATTTCCAGA GAATGACCACAACCTCTTCAGTAGAAGGTAAACAGAATCTGGTGATTATGGGTA AGAAGACCTGGTTCTCCATTCCTGAGAAGAATCGACCTTTAAAGGGTAGAATTA ATTTAGTTCTCAGCAGAGAACTCAAGGAACCTCCACAAGGAGCTCATTTTCTTTC CAGAAGTCTAGATGATGCCTTAAAACTTACTGAACAACCAGAATTAGCAAATAA AGTAGACATGGTCTGGATAGTTGGTGGCAGTTCTGTTTATAAGGAAGCCATGAAT CACCCAGGCCATCTTAAACTATTTGTGACAAGGATCATGCAAGACTTTGAAAGTG ACACGTTTTTTCCAGAAATTGATTTGGAGAAATATAAACTTCTGCCAGAATACCC AGGTGTTCTCTCTGATGTCCAGGAGGAGAAAGGCATTAAGTACAAATTTGAAGT ATATGAGAAGAATGATTAA (SEQ ID NO: 2). In some alternatives, the double mutant of human dihydrofolate reductase comprises the protein sequence: MVGSLNCIVA VSQNMGIGKN GDFPWPPLRN ESRYFQRMTT TSSVEGKQNL VIMGKKTWFS

IPEKNRPLKG RINLVLSREL KEPPQGAHFL SRSLDDALKL TEQPELANKV DMVWIVGGSS VYKEAMNHPG HLKLFVTRIM QDFESDTFFP EIDLEKYKLL PEYPGVLSDV QEEKGIKYKF EVYEKND (SEQ ID NO: 3). In some alternatives, the gene delivery polynucleotide is circular. In some alternatives, the gene delivery polynucleotide is a minicircle. In some alternatives, the gene delivery polynucleotide is at least 1kB to 5kB. In some alternatives, the promoter region comprises an EF1 promoter sequence. In some alternatives, the fourth sequence comprises one, two, three, four, or five genes that encode proteins. In some alternatives, the fourth sequence is codon optimized to reduce the total GC/AT ratio of the fourth sequence. In some alternatives, the fourth sequence is optimized by codon optimization for expression in humans. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins, such as a plurality of antibody binding domains, which are specific for the same epitope. In some alternatives, the plurality of related proteins comprise a plurality of antibody binding domains, wherein the plurality of antibody binding domains are specific for the same epitope. In some alternatives, the fifth sequence is codon optimized to reduce the total GC/AT ratio of the fifth sequence. In some alternatives, the fifth sequence is optimized by codon optimization for expression in humans. In some alternatives, the protein is a protein for therapy. In some alternatives, the codon optimization and/or consensus sequence is generated by comparing the variability of sequence and/or nucleobases utilized in a plurality of related sequences. In some alternatives, the protein comprises an antibody or a portion thereof, which may be humanized. In some alternatives, the double mutant of dihydrofolate reductase comprises amino acid mutations of L22F and F31S. In some alternatives, the double mutant of dihydrofolate reductase comprises amino acid mutations of L22F and F31S. In some alternatives, the T cells are precursor T cells. In some alternatives, the precursor T cells are hematopoietic stem cells. In some alternatives, the introducing is performed by electroporation. In some alternatives, the selecting is performed by increasing selective pressure through the selective marker cassette. In some alternatives, the selection reagent comprises an agent for selection. In some alternatives, the agent for selection is methotrexate. In some alternatives, the first concentration range is at least 50nM – 100nM and the second

concentration range is at least 75 to 150nM. In some alternatives, the first concentration is 50 nM, 60 nM, 70 nM, 80 nM, 90 nM, or 100nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 75 nM, 80 nM, 90 nM, 100 nM, 110 nM, 120 nM, 130 nM, 140 nM, or 150 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first concentration range is at least 75 nM – 150 nM and the second concentration range is at least 112.5 nM to 225 nM. In some alternatives, the first concentration is 75 nM, 85 nM, 95 nM, 105 nM, 115 nM, 125 nM, 135 nM, 145 nM, or 150 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 112 nM, 122 nM, 132 nM, 142 nM, 152 nM, 162 nM, 172 nM, 182 nM, 192 nM, 202 nM, 212 nM, or 225 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first concentration range is at least 300 nM – 675 nM and the first concentration range is at least 450 nM to 1012 nM. In some alternatives, the first concentration is 300 nM, 350 nM, 400 nM, 450 nM, 500 nM, 550 nM, 600 nM, 650 nM, or 675 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 450 nM, 500 nM, 550 nM, 600 nM, 650 nM, 700 nM, 750 nM, 800 nM, 850 nM, 900 nM, 1000 nM, or 1012 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first round of selection comprises exposing the T-cells to the selection agent for 2, 3, 4, 5, 6 or 7 days before the second round of selection. In some alternatives, the second round of selection comprises exposing the T-cells to the selection agent for at least 2, 3, 4, 5, 6,7, 8, 9, 10, 11, 12, 13, or 14 days or any time that is between a range of times defined by any two of the aforementioned time points before isolation. In some alternatives, the subject is human.

[0011] In some alternatives, a method of generating engineered multiplexed T-cells for adoptive T-cell immunotherapy is provided, wherein the method comprises providing a gene delivery polynucleotide, introducing the gene delivery polynucleotide into a T-cell, providing a vector encoding a Sleeping Beauty transposase, introducing the vector encoding the Sleeping Beauty transposase into the T-cell, selecting the cells comprising the

gene delivery polynucleotide wherein selecting comprises a first round of selection and a second round of selection, wherein the first round of selection comprises adding a selection reagent at a first concentration range and the second round of selection comprises adding the selection reagent at a second concentration range, wherein the second concentration range is higher than the first concentration range and, wherein the second concentration range is at least 1.5 fold higher than that of the first concentration range and isolating the T-cells expressing a phenotype under selective pressure. In some alternatives, the gene delivery polynucleotide comprises a first sequence, wherein the first sequence comprises a first inverted terminal repeat gene sequence, a second sequence, wherein the second sequence comprises a second inverted terminal repeat gene sequence, a third sequence, wherein the third sequence comprises a promoter region sequence, a fourth sequence, wherein the fourth sequence comprises at least one gene encoding a protein, and wherein the fourth sequence is optimized, a fifth sequence, wherein the fifth sequence comprises at least one selectable marker cassette encoding a double mutant of dihydrofolate reductase, wherein the double mutant of dihydrofolate reductase has a 15,000 fold or about 15,000 fold reduced affinity for methotrexate, wherein the methotrexate can be used as a selection mechanism to selectively amplify cells transduced with the gene delivery polynucleotide and wherein the fifth sequence is optimized, a sixth sequence, wherein the sixth sequence comprises a first attachment site (attP) and a seventh sequence, wherein the seventh sequence comprises a second attachment site (attB) wherein each of the first sequence, second sequence, third sequence, fourth sequence, fifth sequence, sixth sequence, and seventh sequence have a 5' terminus and a 3 ' terminus, and wherein the 3' terminus of the first sequence comprising the first inverted terminal repeat gene sequence is adjacent to the 5' terminus of the third sequence, the 3' terminus of the third sequence is adjacent to the 5' terminus of the fourth sequence, the 3' terminus of the fourth sequence is adjacent to the 5' terminus of the fifth sequence and the 3' terminus of the fifth sequence is adjacent to the 5' terminus of the second sequence comprising a second inverted terminal repeat. In some alternatives, the gene encoding the double mutant of human dihydrofolate reductase comprises the DNA sequence:

ATGGTTGGTTCGCTAAACTGCATCGTCGCTGTGTCCCAGAACATGGGCATCGGCA
AGAACGGGGACTTCCCCTGGCCACCGCTCAGGAATGAATCCAGATATTTCCAGA
GAATGACCACAACCTCTTCAGTAGAAGGTAAACAGAATCTGGTGATTATGGGTA

AGAAGACCTGGTTCTCCATTCCTGAGAAGAATCGACCTTTAAAGGGTAGAATTA
ATTTAGTTCTCAGCAGAGAACTCAAGGAACCTCCACAAGGAGCTCATTTTCTTTC
CAGAAGTCTAGATGATGCCTTAAAACTTACTGAACAACCAGAATTAGCAAATAA
AGTAGACATGGTCTGGATAGTTGGTGGCAGTTCTGTTTATAAGGAAGCCATGAAT
CACCCAGGCCATCTTAAACTATTTGTGACAAGGATCATGCAAGACTTTGAAAGTG
ACACGTTTTTTCCAGAAATTGATTTGGAGAAATATAAACTTCTGCCAGAATACCC
AGGTGTTCTCTCTGATGTCCAGGAGGAGAAAGGCATTAAGTACAAATTTGAAGT
ATATGAGAAGAATGATTAA (SEQ ID NO: 2). In some alternatives, the double mutant of human dihydrofolate reductase comprises the protein sequence: MVGSLNCIVA VSQNMGIGKN GDFPWPPLRN ESRYFQRMTT TSSVEGKQNL VIMGKKTWFS IPEKNRPLKG RINLVLSREL KEPPQGAHFL SRSLDDALKL TEQPELANKV DMVWIVGGSS VYKEAMNHPG HLKLFVTRIM QDFESDTFFP EIDLEKYKLL PEYPGVLSDV QEEKGIKYKF EVYEKND (SEQ ID NO: 3). In some alternatives, the gene delivery polynucleotide is circular. In some alternatives, the gene delivery polynucleotide is at least 1kB to 5kB. In some alternatives, the gene delivery polynucleotide is a minicircle. In some alternatives, the promoter region comprises an EF1 promoter sequence. In some alternatives, the fourth sequence comprises one, two, three, four, or five genes that encode proteins. In some alternatives, the fourth sequence is codon optimized to reduce the total GC/AT ratio of the fourth sequence. In some alternatives, the fourth sequence is optimized by codon optimization for expression in humans. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins, such as a plurality of antibody binding domains, which are specific for the same epitope. In some alternatives, the plurality of related proteins comprise a plurality of antibody binding domains, wherein the plurality of antibody binding domains are specific for the same epitope. In some alternatives, the fifth sequence is codon optimized to reduce the total GC/AT ratio of the fifth sequence. In some alternatives, the fifth sequence is optimized by codon optimization for expression in humans. In some alternatives, the protein is a protein for therapy. In some alternatives, the codon optimization and/or consensus sequence is generated by comparing the variability of sequence and/or nucleobases utilized in a plurality

of related sequences. In some alternatives, the protein comprises an antibody or a portion thereof, which may be humanized. In some alternatives, the double mutant of dihydrofolate reductase comprises amino acid mutations of L22F and F31S. In some alternatives, the double mutant of dihydrofolate reductase comprises amino acid mutations of L22F and F31S. In some alternatives, the introducing is performed by electroporation. In some alternatives, the selecting is performed by increasing selective pressure through the selective marker cassette. In some alternatives, the selection reagent comprises an agent for selection. In some alternatives, the agent for selection is methotrexate. In some alternatives, the first concentration range is at least 50nM – 100nM and the second concentration range is at least 75 to 150nM. In some alternatives, the first concentration is 50 nM, 60 nM, 70 nM, 80 nM, 90 nM, or 100nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 75 nM, 80 nM, 90 nM, 100 nM, 110 nM, 120 nM, 130 nM, 140 nM, or 150 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first concentration range is at least 75 nM – 150 nM and the second concentration range is at least 112.5 nM to 225 nM. In some alternatives, the first concentration is 75 nM, 85 nM, 95 nM, 105 nM, 115 nM, 125 nM, 135 nM, 145 nM, or 150 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 112 nM, 122 nM, 132 nM, 142 nM, 152 nM, 162 nM, 172 nM, 182 nM, 192 nM, 202 nM, 212 nM, or 225 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first concentration range is at least 300 nM – 675 nM and the first concentration range is at least 450 nM to 1012 nM. In some alternatives, the first concentration is 300 nM, 350 nM, 400 nM, 450 nM, 500 nM, 550 nM, 600 nM, 650 nM, or 675 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 450 nM, 500 nM, 550 nM, 600 nM, 650 nM, 700 nM, 750 nM, 800 nM, 850 nM, 900 nM, 1000 nM, or 1012 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first round of selection comprises exposing the T-cells to the selection agent for 2, 3, 4, 5, 6 or 7 days before the second round of selection. In some alternatives, the second round of selection

comprises exposing the T-cells to the selection agent for at least 2, 3, 4, 5, 6,7, 8, 9, 10, 11, 12, 13, or 14 days or any time that is between a range of times defined by any two of the aforementioned time points before isolation. In some alternatives, the T cells comprise precursor T cells. In some alternatives, the precursor T cells are hematopoietic stem cells.

[0012] In some alternatives, a method of generating engineered cells for adoptive T-cell immunotherapy comprising, providing a gene delivery polynucleotide, introducing the gene delivery polynucleotide into a precursor T cell, providing a vector encoding a Sleeping Beauty transposase, introducing the vector encoding the Sleeping Beauty transposase into the precursor T cell, selecting the precursor T cells comprising the gene delivery polynucleotide; wherein selecting comprises a first round of selection and a second round of selection, wherein the first round of selection comprises adding a selection reagent at a first concentration range and the second round of selection comprises adding the selection reagent at a second concentration range, wherein the second concentration range is higher than the first concentration range and, wherein the second concentration range is at least 1.5 fold higher than that of the first concentration range and isolating the precursor T-cells expressing a phenotype under selective pressure. In some alternatives, the gene delivery polynucleotide is for stable insertion of a nucleic acid into an oligonucleotide wherein the nucleic acid for insertion is flanked by inverted terminal repeat gene sequences in the gene delivery polynucleotide and wherein the gene delivery polynucleotide is selectable, wherein the gene delivery polynucleotide comprises a first sequence, wherein the first sequence comprises a first inverted terminal repeat gene sequence, a second sequence, wherein the second sequence comprises a second inverted terminal repeat gene sequence, a third sequence, wherein the third sequence comprises a promoter region sequence, a fourth sequence, wherein the fourth sequence comprises at least one gene, wherein the at least one gene encodes a protein or encodes a sequence for mRNA transcription, and wherein the fourth sequence is optimized, a fifth sequence, wherein the fifth sequence comprises at least one selectable marker cassette encoding a double mutant of dihydrofolate reductase, wherein the double mutant of dihydrofolate reductase has a 15,000 fold or about 15,000 fold reduced affinity for methotrexate, wherein the methotrexate can be used to select for cells transduced with the gene delivery polynucleotide, to enhance the ratio of cells expressing the at least one gene and wherein the fifth sequence is optimized, a sixth sequence, wherein the sixth

sequence comprises a first attachment site (attP) and a seventh sequence, wherein the seventh sequence comprises a second attachment site (attB); wherein each of the first sequence, second sequence, third sequence, fourth sequence, fifth sequence, sixth sequence, and seventh sequence have a 5' terminus and a 3 ' terminus, and wherein the 3' terminus of the first sequence comprising the first inverted terminal repeat gene sequence is adjacent to the 5' terminus of the third sequence, the 3' terminus of the third sequence is adjacent to the 5' terminus of the fourth sequence, the 3' terminus of the fourth sequence is adjacent to the 5' terminus of the fifth sequence and the 3' terminus of the fifth sequence is adjacent to the 5' terminus of the second sequence comprising a second inverted terminal repeat. In some alternatives, the gene delivery polynucleotide is circular. In some alternatives, the gene delivery polynucleotide is at least 1kB to 5kB. In some alternatives, the promoter region comprises an EF1 promoter sequence. In some alternatives, the fourth sequence comprises one, two, three, four, or five genes that encode proteins. In some alternatives, the fourth sequence is codon optimized to reduce the total GC/AT ratio of the fourth sequence. In some alternatives, the fourth sequence is optimized by codon optimization for expression in humans. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins, such as a plurality of antibody binding domains, which are specific for the same epitope. In some alternatives, the plurality of related proteins comprise a plurality of antibody binding domains, wherein the plurality of antibody binding domains are specific for the same epitope. In some alternatives, the fifth sequence is codon optimized to reduce the total GC/AT ratio of the fifth sequence. In some alternatives, the fifth sequence is optimized by codon optimization for expression in humans. In some alternatives, the codon optimization and/or consensus sequence is generated by comparing the variability of sequence and/or nucleobases utilized in a plurality of related sequences. In some alternatives, the protein is a protein for therapy. In some alternatives, the protein comprises an antibody or a portion thereof, which may be humanized. In some alternatives, the double mutant of dihydrofolate reductase comprises amino acid mutations of L22F and F31S. In some alternatives, the gene delivery polynucleotide is a minicircle. In some alternatives, the introducing is performed by electroporation. In some alternatives, the selecting is performed

by increasing selective pressure through the selective marker cassette. In some alternatives, the selection reagent comprises an agent for selection. In some alternatives, the agent for selection is methotrexate. In some alternatives, the first concentration range is at least 50nM – 100nM and the second concentration range is at least 75 to 150nM. In some alternatives, the first concentration range is at least 75 nM – 150 nM and the second concentration range is at least 112.5 nM to 225 nM. In some alternatives, the first concentration range is at least 300 nM – 675 nM and the first concentration range is at least 450 nM to 1012 nM. In some alternatives, the first round of selection comprises exposing the T-cells to the selection agent for 2, 3, 4, 5, 6 or 7 days before the second round of selection. In some alternatives, the second round of selection comprises exposing the T-cells to the selection agent for at least 2, 3, 4, 5, 6,7, 8, 9, 10, 11, 12, 13, or 14 days or any time that is between a range of times defined by any two of the aforementioned time points before isolation. In some alternatives, the T cell precursor is a hematopoietic stem cell.

[0013] In some alternatives, a method of increasing protein production in a precursor T-cell is provided wherein the method comprises providing a polynucleotide, introducing the polynucleotide into a cell, providing a vector encoding a Sleeping Beauty transposase; introducing the vector encoding the Sleeping Beauty transposase into the precursor T-cell, selecting the precursor T cells comprising the gene delivery polynucleotide, wherein selecting comprises a first round of selection and a second round of selection, wherein the first round of selection comprises adding a selection reagent at a first concentration range and the second round of selection comprises adding the selection reagent at a second concentration range, wherein the second concentration range is higher than the first concentration range and, wherein the second concentration range is at least 1.5 fold higher than that of the first concentration range and isolating the precursor T cells expressing a phenotype under selective pressure. In some alternatives, the gene delivery polynucleotide is for stable insertion of a nucleic acid into an oligonucleotide wherein the nucleic acid for insertion is flanked by inverted terminal repeat gene sequences in the gene delivery polynucleotide and wherein the gene delivery polynucleotide is selectable, wherein the gene delivery polynucleotide comprises a first sequence, wherein the first sequence comprises a first inverted terminal repeat gene sequence, a second sequence, wherein the second sequence comprises a second inverted terminal repeat gene sequence, a third sequence,

wherein the third sequence comprises a promoter region sequence, a fourth sequence, wherein the fourth sequence comprises at least one gene, wherein the at least one gene encodes a protein or encodes a sequence for mRNA transcription, and wherein the fourth sequence is optimized, a fifth sequence, wherein the fifth sequence comprises at least one selectable marker cassette encoding a double mutant of dihydrofolate reductase, wherein the double mutant of dihydrofolate reductase has a 15,000 fold or about 15,000 fold reduced affinity for methotrexate, wherein the methotrexate can be used to select for cells transduced with the gene delivery polynucleotide, to enhance the ratio of cells expressing the at least one gene and wherein the fifth sequence is optimized, a sixth sequence, wherein the sixth sequence comprises a first attachment site (attP) and a seventh sequence, wherein the seventh sequence comprises a second attachment site (attB); wherein each of the first sequence, second sequence, third sequence, fourth sequence, fifth sequence, sixth sequence, and seventh sequence have a 5' terminus and a 3 ' terminus, and wherein the 3' terminus of the first sequence comprising the first inverted terminal repeat gene sequence is adjacent to the 5' terminus of the third sequence, the 3' terminus of the third sequence is adjacent to the 5' terminus of the fourth sequence, the 3' terminus of the fourth sequence is adjacent to the 5' terminus of the fifth sequence and the 3' terminus of the fifth sequence is adjacent to the 5' terminus of the second sequence comprising a second inverted terminal repeat. In some alternatives, the gene delivery polynucleotide is circular. In some alternatives, the gene delivery polynucleotide is at least 1kB to 5kB. In some alternatives, the promoter region comprises an EF1 promoter sequence. In some alternatives, the fourth sequence comprises one, two, three, four, or five genes that encode proteins. In some alternatives, the fourth sequence is codon optimized to reduce the total GC/AT ratio of the fourth sequence. In some alternatives, the fourth sequence is optimized by codon optimization for expression in humans. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins, such as a plurality of antibody binding domains, which are specific for the same epitope. In some alternatives, the plurality of related proteins comprise a plurality of antibody binding domains, wherein the plurality of antibody binding domains are specific for the same epitope. In some alternatives, the fifth sequence is codon

optimized to reduce the total GC/AT ratio of the fifth sequence. In some alternatives, the fifth sequence is optimized by codon optimization for expression in humans. In some alternatives, the codon optimization and/or consensus sequence is generated by comparing the variability of sequence and/or nucleobases utilized in a plurality of related sequences. In some alternatives, the protein is a protein for therapy. In some alternatives, the protein comprises an antibody or a portion thereof, which may be humanized. In some alternatives, the double mutant of dihydrofolate reductase comprises amino acid mutations of L22F and F31S. In some alternatives, the gene delivery polynucleotide is a minicircle. In some alternatives, the introducing is performed by electroporation. In some alternatives, the selecting is performed by increasing selective pressure through the selective marker cassette. In some alternatives, the selection reagent comprises an agent for selection. In some alternatives, the agent for selection is methotrexate.

[0014] In some alternatives, wherein the first concentration range is at least 50nM – 100 nM and the second concentration range is at least 75 to 150 nM. In some alternatives, the first concentration range is at least 75nM – 150nM and the second concentration range is at least 112.5 nM to 225 nM. In some alternatives, the first concentration range is at least 300 nM – 675 nM and the second concentration range is at least 450 nM to 1012 nM. In some alternatives, the first round of selection comprises exposing the cells to the selection agent for 2, 3, 4, 5, 6 or 7 days before the second round of selection. In some alternatives, the second round of selection comprises exposing the cells to the selection agent for at least 2, 3, 4, 5, 6, or 7 days before isolation. In some alternatives, the precursor T cells are hematopoietic stem cells.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] **Figure 1** shows an overall schematic of the gene delivery minicircle producer plasmid, MC_T3/FP-DHFRdm. The minicircle with T3 generation of Sleeping Beauty transposon comprises an EF1a promoter, a fusion of fluorescent protein (FP; maxGFP, mCherry, or Blue Fluorescent protein (BFP)), *Thosea asigna* virus 2A peptide (T2A), and double mutant of dihydrofolate reductase (DHFRdm) insensitive to methotrexate (MTX), positioned between inverted terminal repeats (ITRs, arrows). Recombination at attB/attP sites generates a minicircle while the remaining bacterial backbone is enzymatically degraded.

[0016] **Figure 2** shows a series of bar graphs that demonstrate the optimization of the transposon:transposase DNA ratio. H9 cells were nucleofected with 2 μg of MC_T3/eGFP-T2A-DHFRdm DNA (transposon) and increasing amounts of MC_SB100X (transposase) DNA (0.5, 1, 2, 4, 8 ug). Flow cytometry was performed at 24 hours (striped bars) and at 7 days (black bars) after nucleofection to assess transient and stable transfection efficiency. Numbers above the bars indicate integration efficiency, which is calculated as percent of stable over transient GFP expression.

[0017] **Figure 3** shows a series of bar graphs, which demonstrate the effect of MTX concentration during the selection process. Flow cytometric analysis of H9 cell populations stably transfected with T3/GFP-T2A-DHFRdm transposon DNA grown in the presence of increasing concentrations of MTX (0, 50, 100, and 200 nM) at 3 days (white bars), 5 days (horizontal stripes), 7 days (vertical stripes), and 10 days (black bars) was performed. *Panel A* of **Figure 3** shows the percent GFP+/PI− and *Panel B* of **Figure 3** shows the mean GFP relative fluorescence units (RFU).

[0018] **Figure 4** shows a series of bar graphs that demonstrate the transgene persistence after MTX withdrawal. As shown is the flow cytometric analysis of H9 cell populations that were stably transfected with T3/GFP-T2A-DHFRdm transposon grown in media supplemented with different concentrations of MTX (50, 100, and 200 nM) for 2 weeks (black bars), after which MTX selection was withdrawn and data collected at different time points afterwards: 1 week (horizontal stripes), 2 weeks (vertical stripes), 3 weeks (checked bars), and 4 weeks (white bars). *Panel A* of **Figure 4** shows the percent GFP+/PI−; *Panel B* of **Figure 4** shows the mean GFP relative fluorescence units (RFU).

[0019] **Figure 5A** shows the transposon copy number per human haploid genome. Genomic DNA was isolated from populations of H9 cells stably transfected with T3/GFP-T2A-DHFRdm transposon DNA before and after selection with different concentrations of MTX (50, 100, and 200 nM). The average transposon copy number was determined by quantitative PCR. The "Gold standard" was generated by the limiting dilution method. The "Sorted" population was created by sorting the original H9 population (8% of integrated transposon) to 100% GFP positive cells. The asterisk (*) above the bracketed bar graphs indicates the difference between 200 nM MTX and sorted population was significantly different according to a Student's T-test (P=0.04).

[0020] **Figure 5B** shows the distribution of transposon integration events. Sixty clones were isolated by limited dilution method from an H9 population that was previously selected with 200 nM MTX to 100% cells with integrated T3/GFP-T2A-DHFRdm transposon. Genomic DNA was isolated and transposon copy number determined by relative RT-qPCR. Numbers were rounded to the nearest integer value (e.g., 0.5-1.5 was rounded to 1). $N = 60$; mean ± standard deviation = 1.78 ± 0.69. Probabilities of integration events and standard error were calculated from these data (inset table).

[0021] **Figure 6** shows a series of pie graphs representing the analysis of the multiplexing of transposons. As shown in *Panels A-C* are the flow cytometric analysis of H9 cell populations nucleofected with 3 minicircles carrying transposons with different fluorescent proteins (FPs) (MC_T3/GFP-T2A-DHFRdm, MC_T3/ BFP-T2A-DHFRdm, MC_T3/mCherry-T2A-DHFRdm), 2 μg each and 6 μg of MC_SB100X DNA at different time points: (*Panel A*) 24 hours after transfection (transient expression), (*Panel B*) 1 week (stable integration), and (*Panel C*) 1 week of selection with 200 nM of MTX.

[0022] **Figure 7** shows the bar graph analysis of step selection of the distribution of expression of single, double, and triple FPs. H9 cell population stably transfected with three transposons was selected with 200 nM MTX for a week and then was exposed to higher MTX concentrations of 500 and 1000 nM.

[0023] **Figure 8** shows an example of the flow analysis for the stable expression of transposon DNA with Sleeping Beauty in lymphocytes after MTX selection. Freshly thawed PBMC cells were electroporated with minicircle GFP (mcGFP) DNA (MC_T3/GFP-T2A-DHFRdm) and Sleeping Beauty transposase DNA (MC_SB100X), then stimulated with Miltenyi Transact beads which selectively activate T-cells by binding to CD3 and CD28. 1 week after electroporation, samples of the PBMC cells were selected using 25, 50 and 100 nM MTX for 12 days (50 nM shown here). *Panels A, B,* and *C* show the sequential selection for lymphocytes (A), single cells (B), and live cells (C). Shown in *Panel D* are the high levels of GFP expression in both the CD8+ and CD8- populations. Note that for this donor, the majority of lymphocytes after stimulation are CD8+ T cells.

[0024] **Figure 9** shows histograms of the initial expression of transposon DNA with Sleeping Beauty in lymphocytes. PBMC were transfected with either mcGFP DNA alone (10 ug), mcGFP (10 ug) and MC_SB100X DNA (5 ug) at a mcGFP:MC_SB100X ratio

of 2:1, mcGFP (10 ug) and MC_SB100X DNA (10 ug) at a mcGFP:MC_SB100X ratio of 1:1, a pMAXGFP (10 ug) control, or a no DNA control. Shown in *Panel A* are the results for cells in which Transact beads were not added, two days after transfection as an example of the initial electroporation efficiency. Shown in *Panel B* are the results in cells exposed to transact beads after five days. While by day 5 the levels of mcGFP DNA decline to near control levels, the expression of mcGFP in cells co-transfected with transposase remain elevated.

[0025] **Figure 10** shows the expression of GFP transposon DNA and the levels of cell growth in transfected lymphocytes in the week before MTX addition. PBMC were transfected with either mcGFP DNA alone, mcGFP and MC_SB100X DNA at a mcGFP:MC_SB100X ratio of 2:1, mcGFP and MC_SB100X DNA at a mcGFP:MC_SB100X ratio of 1:1, a pMAXGFP control (10 ug), or a no DNA control. *Panel A* shows the decreasing levels of GFP expression from day 2 to day 7. Panel B shows the level of live cells from day 0 to day 7 of the transfected cell samples which had been treated with Miltenyi Transact beads on d0. Panel C shows the level of live cells from day 0 to day 7 of the transfected cell samples in the absence of Transact beads. As shown, there is a slow growth of the cells transfected with mcGFP DNA in the presence of Miltenyi Transact beads.

[0026] **Figure 11** shows the stable expression of transposon DNA with Sleeping Beauty in T-cells following 1 week of MTX selection. Shown are the flow cytometry scattergrams in which GFP production and proliferation of T-cells modified to express GFP after transfection with transposon DNA and Sleeping Beauty transposase DNA were investigated. *Panels A, B, E,* and *F* show the scatter profiles to identify lymphocytes, while *Panels C, D, G,* and *H* show CD8 and GFP expression. *Panels A-D* show the flow cytometry analysis of cells treated with 100 nM MTX. *Panels E-H* shows the flow cytometry analysis of cells that were not treated with MTX. Shown in *Panels A, C, E* and *G*, are samples transfected with mcGFP alone. *Panels B, D, F* and *H* show the flow cytometry results of cells transfected with mcGFP and MC_SB100X (Sleeping Beauty transposase) DNA at 2:1. As demonstrated in *Panel D*, in T-cells (both CD8+ and CD8-) co-transfected with mcGFP and SB100X such that the GFP gene is stably inserted into the cellular genome, about 95% of the cells stably express GFP in the presence of MTX at 100nM while only about 23% express GFP in the absence of MTX.

[0027] **Figure 12** shows the proliferation and the GFP/CD8 expression in transposon-transfected lymphocytes after 14 days of MTX selection. Cell samples were transfected with no DNA (control), mcGFP alone, mcGFP and MC_SB100X DNA at a mcGFP:MC_SB100X ratio of 2:1 ratio, or mcGFP and MC_SB100X DNA at a mcGFP:MC_SB100X ratio of 1:1. After 1 week, the cells were selected using 0 nM MTX (control), 25 nM MTX, 50nM MTX, or 100 nM MTX. The lymphocyte window, shown in the first, third, fifth and seventh columns, demonstrates the survival of only stably transfected cells in the presence of higher concentrations of MTX. The live, single lymphocytes were gated for GFP and CD8 detection in the second, fourth, sixth and eighth columns. For the cell samples transfected with mcGFP alone, GFP expression is lost over time (second column). However cells transfected with both mcGFP and MC_SB100X stably express GFP both with MTX selection (>90%) and without MTX selection (~20%) (columns four and six). As shown in the samples transfected with mcGFP and MC_SB100X DNA, MTX was effective for selection at concentrations of 50 and 100 nM MTX and no significant difference was seen between the ratios 2:1 or 1:1. Note that the majority of lymphocytes are CD8+ T-cells.

[0028] **Figure 13** shows both the lymphocyte window and GFP/CD8 expression in transposon-transfected cells after 19 days of MTX selection. Cell samples were transfected with no DNA (control), mcGFP alone, mcGFP and MC_SB100X DNA at a mcGFP:MC_SB100X ratio of 2:1 ratio, or mcGFP and MC_SB100X DNA at a mcGFP:MC_SB100X ratio of 1:1. The cells were selected using 0 nM MTX (control), 25 nM MTX, 50nM MTX, or 100 nM MTX. The lymphocyte window is shown in the first, third, fifth and seventh columns, showing the survival of only stably transfected cells in the presence of MTX. The live, single lymphocytes were gated for GFP and CD8 detection in the second, fourth, sixth and eighth columns. For the cell samples transfected with mcGFP alone, GFP expression is lost over time (second column). However cells transfected with both mcGFP and MC_SB100X stably express GFP both with MTX selection (>90%) and without MTX selection (~20%) (columns four and six). As shown in the samples transfected with mcGFP and MC_SB100X DNA, MTX was effective for selection at concentrations of 50 and 100 nM MTX, and slightly less for 25 nM. The mcGFP:SB ratios 2:1 or 1:1 were similarly effective.

[0029] **Figure 14** shows the live cell counts of cells that stably express transposon DNA and undergoes MTX selection. Trypan blue cell counts were taken at 7, 14, and 19 days post transfection. PBMC samples were transfected with no DNA (control), mcGFP alone, mcGFP and MC_SB100X DNA at a mcGFP:MC_SB100X ratio of 2:1 ratio, or mcGFP and MC_SB100X DNA at a mcGFP:MC_SB100X ratio of 1:1. The cells were selected on day 7 using 0 nM MTX (control), 25 nM MTX, 50nM MTX, or 100 nM MTX. *Panel A*, shows the level of live cells in the absence of MTX. *Panel B* shows the levels of live cells after exposure to 100nM MTX. *Panel C* shows the levels of live cells after exposure to 50nM. *Panel D* shows the levels of live cells after exposure to 25nM MTX. As MTX slows the growth of cells by inhibiting the metabolism of folic acid, only cells that were transfected with both the mcGFP transposon co-expressing the MTX-resistance gene (DHFRdm) and the MC_SB100X plasmid encoding the Sleeping Beauty Transposase were able to proliferate in the presence of high MTX, due to stable expression of the integrated transposon DNA.

[0030] **Figure 15** shows an analysis of GFP expression by lymphocytes stably expressing GFP transposon DNA with Sleeping Beauty transposase under MTX selection. PBMC samples were transfected with mcGFP alone, mcGFP and MC_SB100X at a mcGFP:MC_SB100X ratio of 2:1, mcGFP and MC_SB100X at a mcGFP:MC_SB100X ratio of 1:1, pMAXGFP (10 ug), and no DNA (control). Cells were exposed to MTX on day 7 after transfection, and GFP expression was measured for live, single lymphocytes. *Panel A* shows the level of GFP expression on days 2, 5, 7, 14, and 19 in the absence of MTX. *Panel B* shows the level of GFP expression from days 7, 14, and 19 of lymphocytes transfected with mcGFP alone under MTX selection at MTX concentrations of 0 nM, 25 nM, 50 nM and 100 nM. *Panel C* shows the GFP expression of T-cells transfected with mcGFP and MC_SB100X at a mcGFP:MC_SB100X ratio of 2: 1 under MTX selection of 0nM, 25nM, 50nM and 100nM. *Panel D* shows the GFP expression of T-cells transfected with mcGFP and MC_SB100X at a mcGFP:MC_SB100X ratio of 1: 1 under control of MTX selection concentrations of 0 nM, 25 nM, 50 nM and 100 nM. As shown, the results from transfecting with mcGFP and MC_SB100X with a 2:1 and a 1:1 ratio were similar, with approximately 75% GFP expression at 25 nM and approximately 90% GFP expression at 50 and 100 nM

after 1 week of MTX. Additionally, there was minimal difference in the GFP expression between the treatment with 50 nM MTX and 100 nM MTX.

[0031] **Figure 16:** Sleeping Beauty Transposons: minicircle constructs. As shown in the figure are the schematics of several sleeping beauty constructs designed for several alternatives described herein.

[0032] **Figure 17:** As shown are several scattergrams of cells transfected with Sleeping Beauty transposons carrying a gene for expression of GFP. As shown are the cells fourteen days after transfection. Cells were electroporated with SB100X or transposons carrying genes for GFP.

[0033] **Figure 18**. Sleeping Beauty Transposons and MTX: GFP transposon. As shown, cells were transfected with different ratios of mcGFP plasmids and the Sleeping Beauty transposon carrying a gene for expression of GFP (McGFP: SB at a 1:1 and 2:1 ratio). As shown, GFP expression was low with no MTX was added after 18 days. With the Sleeping Beauty transposon, it is shown that there is an increase in GFP expression in the presence of MTX.

[0034] **Figure 19.** Sleeping Beauty Transposons: minicircle constructs. As shown in the figure are the schematics of several sleeping beauty constructs designed for several alternatives described herein.

[0035] **Figure 20.** Sleeping Beauty Transposons and MTX:GFP transposon—SB100X DNA and RNA. Cells were electroporated with SB100X (DNA or RNA) or transposons carrying genes for GFP, CARs, or GFP/mCherry/BFP.

[0036] **Figure 21.** Sleeping Beauty Transposons and MTX: GFP transposon—SB100X DNA and RNA. As shown in the figure are several scattergrams of the cells that are transfected with GFP gene carrying transposons. Several samples of cells are transfected with DNA comprising a gene for GFP expression (2.5ug and 5ug), mcGFP only, and RNA (1ug and 3ug). The samples are split and grown under the influence of varying concentrations of MTX at 0 uM, 50uM and 100uM.

[0037] **Figure 22.** Sleeping Beauty Transposons and MTX: GFP transposon—SB100X DNA and RNA. Cells were transfected with Sleeping Beauty transposons carrying a gene for GFP expression at different concentrations as seen in the top left panel. MTX was

then added at day 7 after transfection. As shown, cells transfected with 50ug to 100ug can express GFP after day 7 to day 14.

[0038] **Figure 23.** GFP expressing DNA and RNA in the presence of MTX. As shown cells transfected with mcGFP, GFP: SB, and GFP:SB RNA were grown and exposed to MTX seven days after transfection. As a control, cells were grown to fourteen days without exposure to MTX (top left panel).

[0039] **Figure 24.** Expression of GFP in cells transfected with GFP: SB. As shown in the left panel, cells were transfected with varying concentrations of GFP: SB (2.5 ug, 5 ug) and exposed to different concentrations of MTX (50 uM and 100uM). As shown, cells were able to express GFP in the presence of MTX optimally at 50 uM MTX when they were transfected with 5 ug of GFP: SB. This experiment was also performed using RNA, however, DNA has a higher efficiency for leading to expression of the protein.

[0040] **Figure 25.** Sleeping Beauty Transposons: minicircle constructs. As shown in the figure are the schematics of several sleeping beauty constructs designed for several alternatives described herein.

[0041] **Figure 26.** Expression of CD19CAR. A Sleeping Beauty construct carrying a gene for CD19CAR was constructed (SB: CD19CAR). Cells were transfected with either DNA (2.5 ug or 5ug), or RNA (1ug or 3ug). As shown, cells that were transfected with DNA or RNA at both concentrations were able to express the CD19CAR in the presence of 50 uM MTX. This was also shown for cells that were transfected with the RNA at 1ug in the presence of 100uM MTX.

[0042] **Figure 27.** Expression of CD19CAR. A Sleeping Beauty construct carrying a gene for CD19CAR was constructed (SB: CD19CAR). Cells were transfected with either DNA (2.5 ug or 5ug), or RNA (1ug or 3ug). Cells were grown and at day seven after transfection, were exposed to MTX. The CD19CAR also included an EGFRt tag. As shown, detection of the tag correlates to the expression of the CD19CAR. After exposure to MTX, detection of the tag was seen in cells that were transfected with the DNA carrying the Sleeping Beauty construct carrying a gene for CAR19 as well as the cells transfected with the RNA carrying the Sleeping Beauty construct carrying a gene for CAR19.

[0043] **Figure 28.** Sleeping Beauty Transposons and MTX: CD19 CAR: CD8+ cell growth. Expression of CD19CAR. A Sleeping Beauty construct carrying a gene for

CD19CAR was constructed (SB: CD19CAR). Cells were transfected with either DNA (2.5 ug or 5ug), or RNA (1ug or 3ug). Cells were grown and at day seven after transfection, were exposed to MTX. As shown, the CD8+ cells were able to grow when a lower concentration of DNA was transfected. However, with RNA , it was seen that a higher concentration led to better expression, but a lower concentration led to better initial growth of the cells.

[0044] **Figure 29.** Sleeping Beauty Transposons: minicircle constructs. As shown in the figure are the schematics of several sleeping beauty constructs designed for several alternatives described herein.

[0045] **Figure 30.** Sleeping Beauty Transposons and MTX: Multiplex 3 FP's. Cells were electroporated with DNA or mcFP and grown in the presence of MTX. Afterwards, cells were analyzed for expression of mCherry, BFP, and/or GFP as indicated by the scattergrams.

[0046] **Figure 31.** Sleeping Beauty Transposons and MTX: Multiplex 3 FP's.

[0047] **Figure 32.** Sleeping Beauty Transposons: minicircle constructs. As shown in the figure are the schematics of several sleeping beauty constructs designed for several alternatives described herein.

[0048] **Figure 33.** As shown, cells electroporated with DNA comprising Sleeping Beauty transposons were subjected to different concentrations of MTX at the second round of selection.

[0049] **Figure 34.** Expression of Smarker proteins in cells electroporated with DNA comprising Sleeping Beauty transposons in the presence of different concentrations of MTX (2, 100nM, 250nM, and 500nM).

[0050] **Figure 35.** Sleeping Beauty Transposons: minicircle constructs. As shown in the figure are the schematics of several sleeping beauty constructs designed for several alternatives described herein.

DETAILED DESCRIPTION

[0051] The following definitions are provided to facilitate understanding of the embodiments or alternatives of the invention.

[0052] As used herein, "a" or "an" can mean one or more than one.

[0053] As used herein, the term "about" indicates that a value includes the inherent variation of error for the method being employed to determine a value, or the variation that exists among experiments.

[0054] As used herein, "nucleic acid" or "nucleic acid molecule" refers to polynucleotides, such as deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), oligonucleotides, fragments generated by the polymerase chain reaction (PCR), and fragments generated by any of ligation, scission, endonuclease action, and exonuclease action. Nucleic acid molecules can be composed of monomers that are naturally-occurring nucleotides (such as DNA and RNA), or analogs of naturally-occurring nucleotides (e.g., enantiomeric forms of naturally-occurring nucleotides), or a combination of both. Modified nucleotides can have alterations in sugar moieties and/or in pyrimidine or purine base moieties. Sugar modifications include, for example, replacement of one or more hydroxyl groups with halogens, alkyl groups, amines, and azido groups, or sugars can be functionalized as ethers or esters. Moreover, the entire sugar moiety can be replaced with sterically and electronically similar structures, such as aza-sugars and carbocyclic sugar analogs. Examples of modifications in a base moiety include alkylated purines and pyrimidines, acylated purines or pyrimidines, or other well-known heterocyclic substitutes. Nucleic acid monomers can be linked by phosphodiester bonds or analogs of such linkages. Analogs of phosphodiester linkages include phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoranilidate, phosphoramidate, and the like. The term "nucleic acid molecule" also includes so-called "peptide nucleic acids," which comprise naturally-occurring or modified nucleic acid bases attached to a polyamide backbone. Nucleic acids can be either single stranded or double stranded. In some alternatives described herein, a gene delivery polynucleotide for stable insertion of a nucleic acid into a gene is provided. "Oligonucleotide" can be used interchangeable with nucleic acid and can refer to DNA or RNA, either double stranded or a single stranded piece or DNA or RNA.

[0055] A "gene" is the molecular unit of heredity of a living organism, describing some stretches of deoxyribonucleic acids (DNA) and ribonucleic acids (RNA) that code for a polypeptide or for an RNA chain that has a function in the organism, and can be a locatable region in the genome of an organism. In some alternatives described herein, a gene delivery

polynucleotide for stable insertion of a nucleic acid into a gene, wherein the nucleic acid for insertion is flanked by inverted terminal repeat gene sequences in the gene delivery polynucleotide and wherein the gene delivery polynucleotide is selectable, is provided.

[0056] A "chromosome," is a packaged and organized chromatin, a complex of macromolecules found in cells, consisting of DNA, protein and RNA. In some alternatives, a gene delivery polynucleotide for stable insertion of a nucleic acid into a gene, wherein the nucleic acid for insertion is flanked by inverted terminal repeat gene sequences in the gene delivery polynucleotide and wherein the gene delivery polynucleotide is selectable, the gene delivery polynucleotide, is provided. In some alternatives, the nucleic acid is inserted into a gene of a chromosome.

[0057] A "promoter" is a nucleotide sequence that directs the transcription of a structural gene. In some alternatives, a promoter is located in the 5' non-coding region of a gene, proximal to the transcriptional start site of a structural gene. Sequence elements within promoters that function in the initiation of transcription are often characterized by consensus nucleotide sequences. These promoter elements include RNA polymerase binding sites, TATA sequences, CAAT sequences, differentiation-specific elements (DSEs; McGehee et al., Mol. Endocrinol. 7:551 (1993); incorporated by reference in its entirety), cyclic AMP response elements (CREs), serum response elements (SREs; Treisman, Seminars in Cancer Biol. 1:47 (1990); incorporated by reference in its entirety), glucocorticoid response elements (GREs), and binding sites for other transcription factors, such as CRE/ATF (O'Reilly et al., J. Biol. Chem. 267:19938 (1992); incorporated by reference in its entirety), AP2 (Ye et al., J. Biol. Chem. 269:25728 (1994); incorporated by reference in its entirety), SP1, cAMP response element binding protein (CREB; Loeken, Gene Expr. 3:253 (1993); incorporated by reference in its entirety) and octamer factors (see, in general, Watson et al., eds., Molecular Biology of the Gene, 4th ed. (The Benjamin/Cummings Publishing Company, Inc. 1987; incorporated by reference in its entirety)), and Lemaigre and Rousseau, Biochem. J. 303:1 (1994); incorporated by reference in its entirety). As used herein, a promoter can be constitutively active, repressible or inducible. If a promoter is an inducible promoter, then the rate of transcription increases in response to an inducing agent. In contrast, the rate of transcription is not regulated by an inducing agent if the promoter is a constitutive promoter. Repressible promoters are also known. In some alternatives, a gene delivery polynucleotide

is provided. In some alternatives, the gene delivery polynucleotide comprises a promoter sequence.

[0058] "Selectable marker cassette," is a gene introduced into a vector or a cell that confers a trait for artificial selection. A selectable marker cassette can be a screenable marker to allow a researcher to distinguish between wanted and unwanted cells, or to enrich for a specific cell type. In some alternatives, a gene delivery polynucleotide is provided. In some alternatives, the gene delivery polynucleotide comprises a selectable marker cassette.

[0059] "Dihydrofolate reductase", or DHFR, as described herein, is an enzyme that reduces dihydrofolic acid to tetrahydrofolic acid, using NADPH as electron donor, which can be converted to the kinds of tetrahydrofolate cofactors used in 1-carbon transfer chemistry. In some alternatives described herein, a gene delivery polynucleotide is provided. In some alternatives, the gene delivery polynucleotide comprises at least one selectable marker cassette encoding for a double mutant of dihydrofolate reductase.

[0060] "Methotrexate" (MTX), as described herein, is an antimetabolite and antifolate drug. It acts by inhibiting the metabolism of folic acid. In some alternatives, a method of generating engineered multiplexed T-cells for adoptive T-cell immunotherapy is provided. In the broadest sense, the method can comprise providing the gene delivery polynucleotide of any of the alternatives described herein, introducing the gene delivery polynucleotide into a T-cell, providing a vector encoding a Sleeping Beauty transposase, introducing the vector encoding the Sleeping Beauty transposase into the T-cell, selecting the cells comprising the gene delivery polynucleotide, wherein the selecting comprises a first round of selection and a second round of selection, wherein the first round of selection comprises adding a selection reagent at a first concentration range and the second round of selection comprises adding the same selection reagent at a second concentration range, wherein the second concentration range is greater than the first concentration range and, wherein the second concentration range is at least 1.5 fold higher than that of the first concentration range, and isolating the T-cells expressing a phenotype under this selective pressure. In some alternatives described herein, the selection reagent comprises an agent for selection. In some alternatives, the selection reagent is MTX.

[0061] An "inverted repeat" or IR is a sequence of nucleotides followed downstream by its reverse complement. Inverted repeats can have a number of important

biological functions. They can define the boundaries in transposons and indicate regions capable of self-complementary base pairing (regions within a single sequence which can base pair with each other). These properties play an important role in genome instability and contribute to cellular evolution, genetic diversity and also to mutation and disease. In some alternatives, a gene delivery polynucleotide is provided. In some alternatives, the gene delivery polynucleotide comprises a first inverted terminal repeat gene sequence and a second inverted terminal repeat gene sequence. In some alternatives, the gene delivery polynucleotide comprises a sleeping beauty transposon positioned between two inverted repeat sequences.

[0062] Sleeping beauty transposase binds specific binding sites that are located on the IR of the Sleeping beauty transposon. The sequence of IR (Inverted repeat) is as follows :

cagttgaagtcggaagtttacatacacttaagttggagtcattaaaactcgtttttcaactacTccacaaatttcttgttaacaaacaatagt
tttggcaagtcagttaggacatctactttgtgcatgacacaagtcatttttccaacaattgtttacagacagattatttcacttataattcactg
tatcacaattccagtgggtcagaagtttacatacactaagttgactgtgcctttaaacagcttggaaaattccagaaaatgatgtcatggct
ttagaagcttctgatagactaattgacatcatttgagtcaattggaggtgtacctgtggatgtatttcaagg (SEQ ID NO: 1)

[0063] A "polypeptide" is a polymer of amino acid residues joined by peptide bonds, whether produced naturally or synthetically. Polypeptides of less than about 10 amino acid residues are commonly referred to as "peptides."

[0064] A "protein" is a macromolecule comprising one or more polypeptide chains. A protein can also comprise non-peptide components, such as carbohydrate groups. Carbohydrates and other non-peptide substituents can be added to a protein by the cell in which the protein is produced, and will vary with the type of cell. Proteins are defined herein in terms of their amino acid backbone structures; substituents such as carbohydrate groups are generally not specified, but can be present nonetheless. In some alternatives, a gene delivery polynucleotide for stable insertion of a nucleic acid into a gene, wherein the nucleic acid for insertion is flanked by inverted terminal repeat gene sequences in the gene delivery polynucleotide and wherein the gene delivery polynucleotide is selectable, the gene delivery polynucleotide, is provided. In some alternatives, the gene delivery polynucleotide further comprises a sequence for at least one protein.

[0065] An "antibody" as described herein refers to a large Y-shape protein produced by plasma cells that is used by the immune system to identify and neutralize foreign objects such as bacteria and viruses. The antibody protein can comprise four polypeptide chains; two identical heavy chains and two identical light chains connected by disulfide bonds. Each chain is composed of structural domains called immunoglobulin domains. These domains can contain about 70–110 amino acids and are classified into different categories according to their size and function. In some alternatives, a gene delivery polynucleotide for stable insertion of a nucleic acid into a gene, wherein the nucleic acid for insertion is flanked by inverted terminal repeat gene sequences in the gene delivery polynucleotide and wherein the gene delivery polynucleotide is selectable, the gene delivery polynucleotide, is provided. In some alternatives, the gene delivery polynucleotide further comprises a sequence for at least one protein. In some alternatives, the gene delivery polynucleotide can comprise a sequence for an antibody or a portion thereof, which may be humanized.

[0066] A "chimeric antigen receptor" (CARs), also known as chimeric T-cell receptors, refers to artificial T-cell receptors that are engineered receptors, which graft an arbitrary specificity onto an immune effector cell. These receptors can be used to graft the specificity of a monoclonal antibody onto a T-cell, for example; with transfer of their coding sequence facilitated by retroviral vectors. The structure of the CAR can comprise single-chain variable fragments (scFv) derived from monoclonal antibodies, fused to CD3-zeta transmembrane and endodomain. Such molecules result in the transmission of a zeta signal in response to recognition by the scFv of its target. Some alternatives utilize a gene delivery polynucleotide for stable insertion of a nucleic acid into a gene, wherein the nucleic acid for insertion is flanked by inverted terminal repeat gene sequences in the gene delivery polynucleotide, and wherein the gene delivery polynucleotide is selectable. In some alternatives, the gene delivery polynucleotide further comprises a sequence for at least one protein. In some alternatives, the protein is a chimeric antigen receptor. Chimeric receptor can also be referred to as artificial T cell receptors, chimeric T cell receptors, chimeric immunoreceptors, and chimeric antigen receptors (CARs). These CARs are engineered receptors that can graft an arbitrary specificity onto an immune receptor cell. Chimeric antigen receptors or "CARs" are considered by some investigators in some contexts to

include the antibody or antibody fragment, spacer, signaling domain, and transmembrane region. However, due to the surprising effects of modifying the different components or domains of the CAR, such as the epitope binding region (for example, antibody fragment, scFv, or portion thereof), spacer, transmembrane domain, and/ or signaling domain), the components of the CAR are described herein in some contexts to include these features as independent elements. The variation of the different elements of the CAR can, for example, lead to stronger binding affinity for a specific epitope.

[0067] Artificial T-cell receptors, or CARs can be used as a therapy for cancer or viral infection using a technique called adoptive cell transfer. T-cells are removed from a patient and modified so that they express receptors specific for a molecule displayed on a cancer cell or virus, or virus-infected cell. The genetically engineered T-cells, which can then recognize and kill the cancer cells or the virus infected cells or promote clearance of the virus, are reintroduced into the patient. In some alternatives, the gene delivery polynucleotide can comprise a sequence for a chimeric antigen receptor. In some alternatives, a method of generating engineered multiplexed T-cells for adoptive T-cell immunotherapy is provided. In the broadest sense the method can comprise providing the gene delivery polynucleotide of any one of the alternatives described herein, introducing the gene delivery polynucleotide into a T-cell, providing a vector encoding a Sleeping Beauty transposase, introducing the vector encoding the Sleeping Beauty transposase into the T-cell, selecting the cells comprising the gene delivery polynucleotide, wherein selecting comprises a first round of selection and a second round of selection, wherein the first round of selection comprises adding a selection reagent at a first concentration range and the second round of selection comprises adding the selection reagent at a second concentration range, and wherein the second concentration range is at least 1.5 fold higher than that of the first concentration range and isolating the T-cells expressing a phenotype under selective pressure. In some alternatives, the selection reagent is MTX.

[0068] T-cell co-stimulation is desired for development of an effective immune response and this event occurs during the activation of lymphocytes. A co-stimulatory signal, is antigen non-specific and is provided by the interaction between co-stimulatory molecules expressed on the membrane of the antigen bearing cell and the T-cell. Co-stimulatory molecules can include but are not limited to CD28, CD80, and CD86. In some alternatives, a

method for generating engineered multiplexed T-cell for adoptive T-cell immunotherapy is provided. In some alternatives, the T-cell is a chimeric antigen receptor bearing T-cell. In some alternatives, the chimeric antigen receptor bearing T-cell is engineered to express co-stimulatory ligands. In some alternatives, methods are provided for treating, inhibiting, or ameliorating cancer or a viral infection in a subject. In the broadest sense the method can comprise administering to the subject a T-cell of any of the alternatives described herein. Preferably, genetically engineered T cells are used to treat, inhibit, or ameliorate a cancer or a viral disease, wherein the genetically engineered T cells are obtained by preferential amplification of T cells that are transformed to express multiple transgenes encoding receptors or chimeric receptors specific for a molecule presented by a virus or a cancer cell and selection pressure on the transformed T cells is applied in a two-stage MTX selection, utilizing increasing concentrations of MTX. In some of these alternatives, the subject is an animal, such as domestic livestock or a companion animal and on other alternatives, the subject is a human. In some of these alternatives, the chimeric antigen bearing T-cell is engineered to express a co-stimulatory molecule. In some alternatives, the gene delivery polynucleotide comprises a sequence for at least one co-stimulatory molecule. In some alternatives, the gene delivery polynucleotide is circular. In some alternatives, the gene delivery polynucleotide is at least 1kB to 6kB. In some alternatives, the gene delivery polynucleotide is a minicircle.

[0069] "T cell precursors" as described herein refers to lymphoid precursor cells that can migrate to the thymus and become T cell precursors, which do not express a T cell receptor. All T cells originate from hematopoietic stem cells in the bone marrow. Hematopoietic progenitors (lymphoid progenitor cells) from hematopoietic stem cells populate the thymus and expand by cell division to generate a large population of immature thymocytes. The earliest thymocytes express neither CD4 nor CD8, and are therefore classed as double-negative ($CD4^-CD8^-$) cells. As they progress through their development, they become double-positive thymocytes ($CD4^+CD8^+$), and finally mature to *single-positive* ($CD4^+CD8^-$ or $CD4^-CD8^+$) thymocytes that are then released from the thymus to peripheral tissues.

[0070] About 98% of thymocytes die during the development processes in the thymus by failing either positive selection or negative selection, whereas the other 2% survive and leave the thymus to become mature immunocompetent T cells.

[0071] The double negative (DN) stage of the precursor T cell is focused on producing a functional β-chain whereas the double positive (DP) stage is focused on producing a functional α-chain, ultimately producing a functional αβ T cell receptor. As the developing thymocyte progresses through the four DN stages (DN1, DN2, DN3, and DN4), the T cell expresses an invariant α-chain but rearranges the β-chain locus. If the rearranged β-chain successfully pairs with the invariant α-chain, signals are produced which cease rearrangement of the β-chain (and silence the alternate allele) and result in proliferation of the cell. Although these signals require this pre-TCR at the cell surface, they are dependent on ligand binding to the pre-TCR. These thymocytes will then express both CD4 and CD8 and progresses to the double positive (DP) stage where selection of the α-chain takes place. If a rearranged β-chain does not lead to any signaling (e.g. as a result of an inability to pair with the invariant α-chain), the cell may die by neglect (lack of signaling).

[0072] "Hematopoietic stem cells" or "HSC" as described herein, are precursor cells that can give rise to myeloid cells such as, for example, macrophages, monocytes, macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, dendritic cells and lymphoid lineages (such as, for example, T-cells, B-cells, NK-cells). HSCs have a heterogeneous population in which three classes of stem cells exist, which are distinguished by their ratio of lymphoid to myeloid progeny in the blood (L/M).

[0073] In some alternatives, a method of generating engineered multiplexed T-cells for adoptive T-cell immunotherapy is provided, wherein the method comprises providing a gene delivery polynucleotide, introducing the gene delivery polynucleotide into a T-cell, providing a vector encoding a Sleeping Beauty transposase, introducing the vector encoding the Sleeping Beauty transposase into the T-cell, selecting the cells comprising the gene delivery polynucleotide wherein selecting comprises a first round of selection and a second round of selection, wherein the first round of selection comprises adding a selection reagent at a first concentration range and the second round of selection comprises adding the selection reagent at a second concentration range, wherein the second concentration range is higher than the first concentration range and, wherein the second concentration range is at

least 1.5 fold higher than that of the first concentration range and isolating the T-cells expressing a phenotype under selective pressure. In some alternatives, the gene delivery polynucleotide comprises a first sequence, wherein the first sequence comprises a first inverted terminal repeat gene sequence, a second sequence, wherein the second sequence comprises a second inverted terminal repeat gene sequence, a third sequence, wherein the third sequence comprises a promoter region sequence, a fourth sequence, wherein the fourth sequence comprises at least one gene encoding a protein, and wherein the fourth sequence is optimized, a fifth sequence, wherein the fifth sequence comprises at least one selectable marker cassette encoding a double mutant of dihydrofolate reductase, wherein the double mutant of dihydrofolate reductase has a 15,000 fold or about 15,000 fold reduced affinity for methotrexate, wherein the methotrexate can be used as a selection mechanism to selectively amplify cells transduced with the gene delivery polynucleotide and wherein the fifth sequence is optimized, a sixth sequence, wherein the sixth sequence comprises a first attachment site (attP) and a seventh sequence, wherein the seventh sequence comprises a second attachment site (attB) wherein each of the first sequence, second sequence, third sequence, fourth sequence, fifth sequence, sixth sequence, and seventh sequence have a 5' terminus and a 3 ' terminus, and wherein the 3' terminus of the first sequence comprising the first inverted terminal repeat gene sequence is adjacent to the 5' terminus of the third sequence, the 3' terminus of the third sequence is adjacent to the 5' terminus of the fourth sequence, the 3' terminus of the fourth sequence is adjacent to the 5' terminus of the fifth sequence and the 3' terminus of the fifth sequence is adjacent to the 5' terminus of the second sequence comprising a second inverted terminal repeat. In some alternatives, the gene encoding the double mutant of human dihydrofolate reductase comprises the DNA sequence:

ATGGTTGGTTCGCTAAACTGCATCGTCGCTGTGTCCCAGAACATGGGCATCGGCA
AGAACGGGGACTTCCCCTGGCCACCGCTCAGGAATGAATCCAGATATTTCCAGA
GAATGACCACAACCTCTTCAGTAGAAGGTAAACAGAATCTGGTGATTATGGGTA
AGAAGACCTGGTTCTCCATTCCTGAGAAGAATCGACCTTTAAAGGGTAGAATTA
ATTTAGTTCTCAGCAGAGAACTCAAGGAACCTCCACAAGGAGCTCATTTTCTTTC
CAGAAGTCTAGATGATGCCTTAAAACTTACTGAACAACCAGAATTAGCAAATAA
AGTAGACATGGTCTGGATAGTTGGTGGCAGTTCTGTTTATAAGGAAGCCATGAAT
CACCCAGGCCATCTTAAACTATTTGTGACAAGGATCATGCAAGACTTTGAAAGTG

ACACGTTTTTTCCAGAAATTGATTTGGAGAAATATAAACTTCTGCCAGAATACCC AGGTGTTCTCTCTGATGTCCAGGAGGAGAAAGGCATTAAGTACAAATTTGAAGT ATATGAGAAGAATGATTAA (SEQ ID NO: 2). In some alternatives, the double mutant of human dihydrofolate reductase comprises the protein sequence: MVGSLNCIVA VSQNMGIGKN GDFPWPPLRN ESRYFQRMTT TSSVEGKQNL VIMGKKTWFS IPEKNRPLKG RINLVLSREL KEPPQGAHFL SRSLDDALKL TEQPELANKV DMVWIVGGSS VYKEAMNHPG HLKLFVTRIM QDFESDTFFP EIDLEKYKLL PEYPGVLSDV QEEKGIKYKF EVYEKND (SEQ ID NO: 3). In some alternatives, the gene delivery polynucleotide is circular. In some alternatives, the gene delivery polynucleotide is at least 1kB to 5kB. In some alternatives, the gene delivery polynucleotide is a minicircle. In some alternatives, the promoter region comprises an EF1 promoter sequence. In some alternatives, the fourth sequence comprises one, two, three, four, or five genes that encode proteins. In some alternatives, the fourth sequence is codon optimized to reduce the total GC/AT ratio of the fourth sequence. In some alternatives, the fourth sequence is optimized by codon optimization for expression in humans. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins, such as a plurality of antibody binding domains, which are specific for the same epitope. In some alternatives, the plurality of related proteins comprise a plurality of antibody binding domains, wherein the plurality of antibody binding domains are specific for the same epitope. In some alternatives, the fifth sequence is codon optimized to reduce the total GC/AT ratio of the fifth sequence. In some alternatives, the fifth sequence is optimized by codon optimization for expression in humans. In some alternatives, the protein is a protein for therapy. In some alternatives, the codon optimization and/or consensus sequence is generated by comparing the variability of sequence and/or nucleobases utilized in a plurality of related sequences. In some alternatives, the protein comprises an antibody or a portion thereof, which may be humanized. In some alternatives, the double mutant of dihydrofolate reductase comprises amino acid mutations of L22F and F31S. In some alternatives, the double mutant of dihydrofolate reductase comprises amino acid mutations of L22F and F31S. In some alternatives, the introducing is performed by electroporation. In some alternatives,

the selecting is performed by increasing selective pressure through the selective marker cassette. In some alternatives, the selection reagent comprises an agent for selection. In some alternatives, the agent for selection is methotrexate. In some alternatives, the first concentration range is at least 50nM – 100nM and the second concentration range is at least 75 to 150nM. In some alternatives, the first concentration is 50 nM, 60 nM, 70 nM, 80 nM, 90 nM, or 100nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 75 nM, 80 nM, 90 nM, 100 nM, 110 nM, 120 nM, 130 nM, 140 nM, or 150 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first concentration range is at least 75 nM – 150 nM and the second concentration range is at least 112.5 nM to 225 nM. In some alternatives, the first concentration is 75 nM, 85 nM, 95 nM, 105 nM, 115 nM, 125 nM, 135 nM, 145 nM, or 150 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 112 nM, 122 nM, 132 nM, 142 nM, 152 nM, 162 nM, 172 nM, 182 nM, 192 nM, 202 nM, 212 nM, or 225 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first concentration range is at least 300 nM – 675 nM and the first concentration range is at least 450 nM to 1012 nM. In some alternatives, the first concentration is 300 nM, 350 nM, 400 nM, 450 nM, 500 nM, 550 nM, 600 nM, 650 nM, or 675 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 450 nM, 500 nM, 550 nM, 600 nM, 650 nM, 700 nM, 750 nM, 800 nM, 850 nM, 900 nM, 1000 nM, or 1012 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first round of selection comprises exposing the T-cells to the selection agent for 2, 3, 4, 5, 6 or 7 days before the second round of selection. In some alternatives, the second round of selection comprises exposing the T-cells to the selection agent for at least 2, 3, 4, 5, 6,7, 8, 9, 10, 11, 12, 13, or 14 days or any time that is between a range of times defined by any two of the aforementioned time points before isolation. In some alternatives, the T cells comprise precursor T cells. In some alternatives, the precursor T cells are hematopoietic stem cells.

[0074] In some alternatives, a method of generating engineered cells for adoptive T-cell immunotherapy comprising, providing a gene delivery polynucleotide, introducing the gene delivery polynucleotide into a precursor T cell, providing a vector encoding a Sleeping Beauty transposase, introducing the vector encoding the Sleeping Beauty transposase into the precursor T cell, selecting the precursor T cells comprising the gene delivery polynucleotide; wherein selecting comprises a first round of selection and a second round of selection, wherein the first round of selection comprises adding a selection reagent at a first concentration range and the second round of selection comprises adding the selection reagent at a second concentration range, wherein the second concentration range is higher than the first concentration range and, wherein the second concentration range is at least 1.5 fold higher than that of the first concentration range and isolating the precursor T-cells expressing a phenotype under selective pressure. In some alternatives, the gene delivery polynucleotide is for stable insertion of a nucleic acid into an oligonucleotide wherein the nucleic acid for insertion is flanked by inverted terminal repeat gene sequences in the gene delivery polynucleotide and wherein the gene delivery polynucleotide is selectable, wherein the gene delivery polynucleotide comprises a first sequence, wherein the first sequence comprises a first inverted terminal repeat gene sequence, a second sequence, wherein the second sequence comprises a second inverted terminal repeat gene sequence, a third sequence, wherein the third sequence comprises a promoter region sequence, a fourth sequence, wherein the fourth sequence comprises at least one gene, wherein the at least one gene encodes a protein or encodes a sequence for mRNA transcription, and wherein the fourth sequence is optimized, a fifth sequence, wherein the fifth sequence comprises at least one selectable marker cassette encoding a double mutant of dihydrofolate reductase, wherein the double mutant of dihydrofolate reductase has a 15,000 fold or about 15,000 fold reduced affinity for methotrexate, wherein the methotrexate can be used to select for cells transduced with the gene delivery polynucleotide, to enhance the ratio of cells expressing the at least one gene and wherein the fifth sequence is optimized, a sixth sequence, wherein the sixth sequence comprises a first attachment site (attP) and a seventh sequence, wherein the seventh sequence comprises a second attachment site (attB); wherein each of the first sequence, second sequence, third sequence, fourth sequence, fifth sequence, sixth sequence, and seventh sequence have a 5' terminus and a 3 ' terminus, and wherein the 3' terminus of the

first sequence comprising the first inverted terminal repeat gene sequence is adjacent to the 5' terminus of the third sequence, the 3' terminus of the third sequence is adjacent to the 5' terminus of the fourth sequence, the 3' terminus of the fourth sequence is adjacent to the 5' terminus of the fifth sequence and the 3' terminus of the fifth sequence is adjacent to the 5' terminus of the second sequence comprising a second inverted terminal repeat. In some alternatives, the gene delivery polynucleotide is circular. In some alternatives, the gene delivery polynucleotide is at least 1kB to 5kB. In some alternatives, the promoter region comprises an EF1 promoter sequence. In some alternatives, the fourth sequence comprises one, two, three, four, or five genes that encode proteins. In some alternatives, the fourth sequence is codon optimized to reduce the total GC/AT ratio of the fourth sequence. In some alternatives, the fourth sequence is optimized by codon optimization for expression in humans. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins, such as a plurality of antibody binding domains, which are specific for the same epitope. In some alternatives, the plurality of related proteins comprise a plurality of antibody binding domains, wherein the plurality of antibody binding domains are specific for the same epitope. In some alternatives, the fifth sequence is codon optimized to reduce the total GC/AT ratio of the fifth sequence. In some alternatives, the fifth sequence is optimized by codon optimization for expression in humans. In some alternatives, the codon optimization and/or consensus sequence is generated by comparing the variability of sequence and/or nucleobases utilized in a plurality of related sequences. In some alternatives, the protein is a protein for therapy. In some alternatives, the protein comprises an antibody or a portion thereof, which may be humanized. In some alternatives, the double mutant of dihydrofolate reductase comprises amino acid mutations of L22F and F31S. In some alternatives, the gene delivery polynucleotide is a minicircle. In some alternatives, the introducing is performed by electroporation. In some alternatives, the selecting is performed by increasing selective pressure through the selective marker cassette. In some alternatives, the selection reagent comprises an agent for selection. In some alternatives, the agent for selection is methotrexate. In some alternatives, the first concentration range is at least 50nM – 100nM and the second concentration range is at least 75 to 150nM. In some alternatives,

the first concentration range is at least 75 nM – 150 nM and the second concentration range is at least 112.5 nM to 225 nM. In some alternatives, the first concentration range is at least 300 nM – 675 nM and the first concentration range is at least 450 nM to 1012 nM. In some alternatives, the first round of selection comprises exposing the T-cells to the selection agent for 2, 3, 4, 5, 6 or 7 days before the second round of selection. In some alternatives, the second round of selection comprises exposing the T-cells to the selection agent for at least 2, 3, 4, 5, 6,7, 8, 9, 10, 11, 12, 13, or 14 days or any time that is between a range of times defined by any two of the aforementioned time points before isolation. In some alternatives, the T cell precursor is a hematopoietic stem cell.

[0075] In some alternatives, a method of increasing protein production in a precursor T-cell is provided wherein the method comprises providing a polynucleotide, introducing the polynucleotide into a cell, providing a vector encoding a Sleeping Beauty transposase; introducing the vector encoding the Sleeping Beauty transposase into the precursor T-cell, selecting the precursor T cells comprising the gene delivery polynucleotide, wherein selecting comprises a first round of selection and a second round of selection, wherein the first round of selection comprises adding a selection reagent at a first concentration range and the second round of selection comprises adding the selection reagent at a second concentration range, wherein the second concentration range is higher than the first concentration range and, wherein the second concentration range is at least 1.5 fold higher than that of the first concentration range and isolating the precursor T cells expressing a phenotype under selective pressure. In some alternatives, the gene delivery polynucleotide is for stable insertion of a nucleic acid into an oligonucleotide wherein the nucleic acid for insertion is flanked by inverted terminal repeat gene sequences in the gene delivery polynucleotide and wherein the gene delivery polynucleotide is selectable, wherein the gene delivery polynucleotide comprises a first sequence, wherein the first sequence comprises a first inverted terminal repeat gene sequence, a second sequence, wherein the second sequence comprises a second inverted terminal repeat gene sequence, a third sequence, wherein the third sequence comprises a promoter region sequence, a fourth sequence, wherein the fourth sequence comprises at least one gene, wherein the at least one gene encodes a protein or encodes a sequence for mRNA transcription, and wherein the fourth sequence is optimized, a fifth sequence, wherein the fifth sequence comprises at least one

selectable marker cassette encoding a double mutant of dihydrofolate reductase, wherein the double mutant of dihydrofolate reductase has a 15,000 fold or about 15,000 fold reduced affinity for methotrexate, wherein the methotrexate can be used to select for cells transduced with the gene delivery polynucleotide, to enhance the ratio of cells expressing the at least one gene and wherein the fifth sequence is optimized, a sixth sequence, wherein the sixth sequence comprises a first attachment site (attP) and a seventh sequence, wherein the seventh sequence comprises a second attachment site (attB); wherein each of the first sequence, second sequence, third sequence, fourth sequence, fifth sequence, sixth sequence, and seventh sequence have a 5' terminus and a 3 ' terminus, and wherein the 3' terminus of the first sequence comprising the first inverted terminal repeat gene sequence is adjacent to the 5' terminus of the third sequence, the 3' terminus of the third sequence is adjacent to the 5' terminus of the fourth sequence, the 3' terminus of the fourth sequence is adjacent to the 5' terminus of the fifth sequence and the 3' terminus of the fifth sequence is adjacent to the 5' terminus of the second sequence comprising a second inverted terminal repeat. In some alternatives, the gene delivery polynucleotide is circular. In some alternatives, the gene delivery polynucleotide is at least 1kB to 5kB. In some alternatives, the promoter region comprises an EF1 promoter sequence. In some alternatives, the fourth sequence comprises one, two, three, four, or five genes that encode proteins. In some alternatives, the fourth sequence is codon optimized to reduce the total GC/AT ratio of the fourth sequence. In some alternatives, the fourth sequence is optimized by codon optimization for expression in humans. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins, such as a plurality of antibody binding domains, which are specific for the same epitope. In some alternatives, the plurality of related proteins comprise a plurality of antibody binding domains, wherein the plurality of antibody binding domains are specific for the same epitope. In some alternatives, the fifth sequence is codon optimized to reduce the total GC/AT ratio of the fifth sequence. In some alternatives, the fifth sequence is optimized by codon optimization for expression in humans. In some alternatives, the codon optimization and/or consensus sequence is generated by comparing the variability of sequence and/or nucleobases utilized in a plurality of related sequences. In some

alternatives, the protein is a protein for therapy. In some alternatives, the protein comprises an antibody or a portion thereof, which may be humanized. In some alternatives, the double mutant of dihydrofolate reductase comprises amino acid mutations of L22F and F31S. In some alternatives, the gene delivery polynucleotide is a minicircle. In some alternatives, the introducing is performed by electroporation. In some alternatives, the selecting is performed by increasing selective pressure through the selective marker cassette. In some alternatives, the selection reagent comprises an agent for selection. In some alternatives, the agent for selection is methotrexate.

[0076] In some alternatives, wherein the first concentration range is at least 50nM – 100 nM and the second concentration range is at least 75 to 150 nM. In some alternatives, the first concentration range is at least 75nM – 150nM and the second concentration range is at least 112.5 nM to 225 nM. In some alternatives, the first concentration range is at least 300 nM – 675 nM and the second concentration range is at least 450 nM to 1012 nM. In some alternatives, the first round of selection comprises exposing the cells to the selection agent for 2, 3, 4, 5, 6 or 7 days before the second round of selection. In some alternatives, the second round of selection comprises exposing the cells to the selection agent for at least 2, 3, 4, 5, 6, or 7 days before isolation. In some alternatives, the precursor T cells are hematopoietic stem cells.

[0077] A peptide or polypeptide encoded by a non-host DNA molecule is a "heterologous" peptide or polypeptide.

[0078] An "integrated genetic element" is a segment of DNA that has been incorporated into a chromosome of a host cell after that element is introduced into the cell through human manipulation. Within the present alternatives, integrated genetic elements can be derived from minicircles that are introduced into the cells by electroporation or other techniques. Integrated genetic elements are passed from the original host cell to its progeny. In some alternatives, an integrated genetic element is incorporated into a chromosome of a host cell by a gene delivery polynucleotide is circular. In some alternatives, the gene delivery polynucleotide is at least 1kB to 6kB. In some alternatives, the gene delivery polynucleotide is a minicircle.

[0079] A "cloning vector" or vector is a nucleic acid molecule, such as a minicircle, plasmid, cosmid, plastome, or bacteriophage that has the capability of replicating

autonomously in a host cell. Cloning vectors typically contain one or a small number of restriction endonuclease recognition sites that allow insertion of a nucleic acid molecule in a determinable fashion without loss of an essential biological function of the vector, as well as nucleotide sequences encoding a marker gene that is suitable for use in the identification and selection of cells transduced with the cloning vector. Marker genes typically include genes that provide tetracycline resistance or ampicillin resistance but in some alternatives can include a methotrexate resistance gene.

[0080] An "expression vector" is a nucleic acid molecule encoding a gene that is expressed in a host cell. Typically, an expression vector comprises a transcription promoter, a gene, and a transcription terminator. Gene expression is usually placed under the control of a promoter, and such a gene is said to be "operably linked to" the promoter. Similarly, a regulatory element and a core promoter are operably linked if the regulatory element modulates the activity of the core promoter. In some alternatives, an expression vector is provided. In some alternatives, the expression vector encodes a transposase. In some alternatives, the transposase is a Sleeping Beauty transposase. In some alternatives, expression vector is circular. In some alternatives, the expression vector is at least 1kB to 6kB. In some alternatives, the expression vector is a minicircle.

[0081] "Minicircles," as described herein, are small circular plasmid derivatives that have been freed from all prokaryotic vector parts. Minicircles can serve as an expression vector, where they have been applied as transgene carriers for the genetic modification of mammalian cells, with the advantage that, since they contain no bacterial DNA sequences, they are less likely to be perceived as foreign and destroyed. As such, typical transgene delivery methods involve plasmids, which contain foreign DNA. The smaller size of minicircles also extends their cloning capacity and facilitates their delivery into cells. Without being limiting, the preparation of minicircles can follow a two-step procedure, which can involve production of a parental plasmid (bacterial plasmid with eukaryotic inserts) in *E. coli* and induction of a site-specific recombinase at the end of this process but still in bacteria. These steps can be followed by the excision of prokaryotic vector parts via two recombinase-target sequences at both ends of the insert and recovery of the resulting minicircle (vehicle for the highly efficient modification of the recipient cell) and the miniplasmid by capillary gel electrophoresis (CGE).

[0082] The purified minicircle can be transferred into the recipient cell by transfection, by electroporation, or by other methods known to those skilled in the art. Conventional minicircles can lack an origin of replication, so they cannot replicate within the target cells and the encoded genes will disappear as the cell divides (which can be either an advantage or disadvantage depending on whether the application demands persistent or transient expression). Some alternatives utilize a gene delivery polynucleotide for stable insertion of a nucleic acid into a gene, wherein the nucleic acid for insertion is flanked by inverted terminal repeat gene sequences in the gene delivery polynucleotide, and wherein the gene delivery polynucleotide is selectable. In some alternatives, the gene delivery polynucleotide is a minicircle.

[0083] As used herein, "nucleofection", refers to a transfection method of exogenous nucleic acid(s) into a host cell and is performed by electroporation. In some alternatives, a method of generating engineered multiplexed T-cells for adoptive T-cell immunotherapy is provided. In the broadest sense the method can comprise providing the gene delivery polynucleotide of any of the alternatives described herein, introducing the gene delivery polynucleotide into a T-cell, selecting the cells comprising the gene delivery polynucleotide, wherein selecting comprises a first round of selection and a second round of selection, wherein the first round of selection comprises adding a selection reagent at a first concentration range and the second round of selection comprises adding the selection reagent at a second concentration range, wherein the second concentration range is higher than the first concentration range and, wherein the second concentration range is at least 1.5 fold higher than that of the first concentration range and isolating the T-cells expressing a phenotype under selective pressure. In some alternatives, the selection reagent is MTX. In some alternatives, introducing the gene delivery polynucleotide into a T-cell can be performed by electroporation.

[0084] "Host cell" as described herein, is a cell that contains one or more nucleases, for example endonucleases, end-processing enzymes, and/or endonuclease/end-processing enzyme fusion proteins encompassed by the present alternatives or a vector encoding the same that supports the replication, and/or transcription or transcription and translation (expression) of one or more nucleases, for example endonucleases, end-processing enzymes, and/or endonuclease/end-processing enzyme fusion proteins. In some

alternatives, host cells for use in the present alternatives can be eukaryotic cells. Host cells of the immune system can include T-cells. In some alternatives, a method of generating engineered multiplexed T-cells for adoptive T-cell immunotherapy is provided. In some alternatives, the method can comprise providing the gene delivery polynucleotide of any one of the alternatives described herein, introducing the gene delivery polynucleotide into a T-cell, providing a vector encoding a Sleeping Beauty transposase, introducing the vector encoding the Sleeping Beauty transposase into the T-cell, selecting the cells comprising the gene delivery polynucleotide, wherein the selecting comprises a first round of selection and a second round of selection, wherein the first round of selection comprises adding a selection reagent at a first concentration range and the second round of selection comprises adding the selection reagent at a second concentration range, wherein the second concentration range is higher than the first concentration range and, wherein the second concentration range is at least 1.5 fold higher than that of the first concentration range and isolating the T-cells expressing a phenotype under selective pressure. In some alternatives, the selection reagent is MTX.

[0085] As described herein, "transposable element" (TE), transposon or retrotransposon, can be referred to as a DNA sequence that can change its position within the genome, sometimes creating or reversing mutations and altering the cell's genome size. Transposition often results in duplication of the TE. TEs can make up a large fraction of the C-value of eukaryotic cells. "C-values," as described herein, refers to amount, in picograms, of DNA contained within a haploid nucleus of one half the amount in a diploid somatic cells of a eukaryotic organism. In some cases, the terms C-value and genome size are used interchangeably, however in polyploids the C-value can represent two or more genomes contained within the same nucleus. In *Oxytricha*, which has a unique genetic system, they play a critical role in development. They are also very useful to researchers as a means to alter DNA inside a living organism. In some alternatives, a gene delivery polynucleotide for stable insertion of a nucleic acid into a gene, wherein the nucleic acid for insertion is flanked by inverted terminal repeat gene sequences in the gene delivery polynucleotide and wherein the gene delivery polynucleotide is selectable, the gene delivery polynucleotide, is provided. In some alternatives, the gene delivery polynucleotide comprises a transposon.

[0086] The "Sleeping Beauty transposon system" as described herein, is composed of a Sleeping Beauty (SB) transposase and a transposon that was designed in 1997 to insert specific sequences of DNA into genomes of vertebrate animals. DNA transposons can translocate from one DNA site to another in a simple, cut-and-paste manner. Transposition is a precise process in which a defined DNA segment is excised from one DNA molecule and moved to another site in the same or different DNA molecule or genome.

[0087] An SB transposase can insert a transposon into a TA dinucleotide base pair in a recipient DNA sequence. The insertion site can be elsewhere in the same DNA molecule, or in another DNA molecule (or chromosome). In mammalian genomes, including humans, there are approximately 200 million TA sites. The TA insertion site is duplicated in the process of transposon integration. This duplication of the TA sequence is a hallmark of transposition and used to ascertain the mechanism in some experiments. The transposase can be encoded either within the transposon or the transposase can be supplied by another source, in which case the transposon becomes a non-autonomous element.

[0088] In some alternatives, a gene delivery polynucleotide for stable insertion of a nucleic acid into a gene, wherein the nucleic acid for insertion is flanked by inverted terminal repeat gene sequences in the gene delivery polynucleotide and wherein the gene delivery polynucleotide is selectable, the gene delivery polynucleotide, is provided. In some alternatives, the gene delivery polynucleotide comprises a transposon. In some alternatives, the transposon is a Sleeping Beauty transposon. In some alternatives, the nucleic acid to be inserted is a Sleeping Beauty transposon flanked by inverted terminal repeat gene sequences.

[0089] In some alternatives, the gene delivery polynucleotide for stable insertion of nucleic acid is a minicircle. In some alternatives, the gene delivery polynucleotide for stable insertion of nucleic acid comprises a Sleeping Beauty transposon. In some alternatives, methods of generating engineered multiplexed T-cells are provided. In some alternatives, the method comprises delivering a Sleeping Beauty transposase to a cell. In some alternatives, methods of increasing protein production in a T-cell are provided. In some alternatives, the method comprises providing a vector encoding a Sleeping Beauty transposase. In some alternatives, the method comprises delivering a vector encoding a Sleeping Beauty transposase to a cell.

[0090] "Codon optimization" as described herein, refers to the design process of altering codons to codons known to increase maximum protein expression efficiency in a desired cell. In some alternatives, codon optimization is described, wherein codon optimization can be performed by using algorithms that are known to those skilled in the art to create synthetic genetic transcripts optimized for high protein yield. Programs containing alogorithms for codon optimization are known to those skilled in the art. Programs can include, for example, OptimumGene™, GeneGPS® algorithms, etc. Additionally synthetic codon optimized sequences can be obtained commercially for example from Integrated DNA Technologies and other commercially available DNA sequencing services. In some alternatives, a gene delivery polynucleotide for stable insertion of a nucleic acid into a gene, wherein the nucleic acid for insertion is flanked by inverted terminal repeat gene sequences in the gene delivery polynucleotide and wherein the gene delivery polynucleotide is selectable, is provided. In some alternatives, the gene delivery polynucleotides are described, wherein the genes for the complete gene transcript are codon optimized for expression in humans. In some alternatives, the genes are optimized to have selected codons specifically for maximal protein expression in human cells, which can increase the concentration of proteins or CARs of a T-cell.

[0091] Codon optimization can be performed to reduce the occurrence of secondary structure in a polynucleotide, as well. In some alternatives, codon optimization can also be performed to reduce the total GC/AT ratio. Strict codon optimization can also lead to unwanted secondary structure or an undesirable GC content that leads to secondary structure. As such the secondary structures affect transcriptional efficiency. Programs such as GeneOptimizer can be used after codon usage optimization, for secondary structure avoidance and GC content optimization. These additional programs can be used for further optimization and troubleshooting after an initial codon optimization to limit secondary structures that may occur after the first round of optimization. Alternative programs for optimization are known to those skilled in the art. In some alternatives, a gene delivery polynucleotide for stable insertion of a nucleic acid into a gene, wherein the nucleic acid for insertion is flanked by inverted terminal repeat gene sequences in the gene delivery polynucleotide and wherein the gene delivery polynucleotide is selectable, provided. In some alternatives, the gene delivery polynucleotide comprises sequences that are codon optimized

for expression in humans and/or to remove secondary structure and/or to reduce the total GC/AT ratio. In some alternatives, the sequences are optimized for secondary structure avoidance. In some alternatives, the sequences are optimized to reduce the total GC/AT ratio.

[0092] In some alternatives, a method of generating engineered multiplexed T-cells for adoptive T-cell immunotherapy is provided. In the broadest sense, the method can comprise providing the gene delivery polynucleotide of any one of the alternatives described herein, introducing the gene delivery polynucleotide into a T-cell, providing a vector encoding a Sleeping Beauty transposase, introducing the vector encoding the Sleeping Beauty transposase into the T-cell, selecting the cells comprising the gene delivery polynucleotide, wherein selecting comprises a first round of selection and a second round of selection, wherein the first round of selection comprises adding a selection reagent at a first concentration range and the second round of selection comprises adding the selection reagent at a second concentration range, wherein the second concentration range is higher than the first concentration range and, wherein the second concentration range is at least 1.5 fold higher than that of the first concentration range, and isolating the T-cells expressing a phenotype under selective pressure. In some alternatives, the selection reagent is MTX.

Adoptive immunotherapy for cancer or a viral disease.

[0093] The premise of adoptive immunotherapy for cancer is transferring a patient's own tumor-specific T-cells into patients to facilitate the destruction of malignant cells. T-cells can be genetically-engineered to recognize tumor-specific antigens and exert cytotoxic activity against cancer cells. A method of adoptive immunotherapy for cancer is to isolate patient T-cells and introduce tumor recognition capability by expressing chimeric antigen receptors (CARs), membrane proteins that contain an extracellular tumor-binding domain linked to an intracellular signaling domain via a transmembrane segment. "Adoptive immunotherapy" or "T-cell adoptive transfer" refers to use of T-cell based cytotoxic response to attack cancer cells or specific cell targets. T-cells that have a natural or genetically engineered reactivity to a patient's cancer can be generated in vitro and then transferred back into the subject in need. Without being limiting, an example of adoptive transfer can be achieved by removing T-cells from a subject that has cancer or a viral disease and these T cells can be genetically engineered to express receptors specific for biomarkers

found on a cancer cell or virus such that the genetically engineered T cells attack the cancer cells or virus or virus infected cells once the genetically engineered T-cells are transferred back into the subject. In some alternatives, a method of generating engineered multiplexed T-cells for adoptive T-cell immunotherapy is provided. In some alternatives, methods of targeting malignant cells for destruction are provided. In some alternatives a method of treating, inhibiting, or ameliorating a cancer or a viral disease in a subject is provided. In some alternatives the method of treating, inhibiting, or ameliorating a cancer or a viral disease in a subject comprises administering to the subject an engineered multiplexed T-cells for adoptive T-cell immunotherapy. In some alternatives, the subject is human.

[0094] The co-integration of additional genes can further increase the anti-tumor or antiviral activity of CAR-expressing T-cells. Comprehensive T-cell activation requires, in addition to initial tumor or viral recognition and signal initiation by CAR, engagement of costimulatory and cytokine receptors, which may not be present within the immunosuppressive environment of the tumor or the viral infected subject. To address this immunosuppressive environment of the tumor, for example, expression of co-stimulatory ligands such as CD80 and 4-1BBL in engineered, CAR-expressing T-cells can result in greater T-cell expansion due to auto-co-stimulation compared to expression of co-stimulatory ligands on tumor cells. Another challenge in T-cell immunotherapy is cell survival after infusion into patients. Induced expression of anti-apoptotic proteins has been shown to improve *in vivo* survival of T-cells. Tumor homing and infiltration can be increased by introduction of chemokine receptors in engineered T-cells and this approach can be especially useful for tumors that express chemokines that are not normally recognized by T-cells. Finally, T-cells can be engineered to better resist the immunosuppressive tumor microenvironment or the immunocompromised virally infected subject through, for example, induced cytokine expression. Thus, methods to rapidly generate engineered T-cells expressing multiple transgenes are important and advantageous for clinical translation of T-cell immunotherapy. In some alternatives, methods of generating engineered multiplexed T-cells for adoptive T-cell immunotherapy are provided. In some alternatives, the T-cells express chimeric antigen receptors. In some alternatives, T-cells expressing chimeric antigen receptors are engineered to express co-stimulatory ligands. In some alternatives, the T-cells expressing chimeric antigen receptors express co-stimulatory ligands. In some alternatives

the co-stimulatory ligands are CD80. In some alternatives, the co-stimulatory ligands are 4-1BBL.

[0095] Adoptive cell transfer can refer to the transfer of cells, immune-derived cells, back into the same patient or into a different recipient host. For isolation of immune cells for adoptive transfer, blood can be drawn into tubes containing anticoagulant and the PBM (buffy coat) cells are isolated, typically by density barrier centrifugation. In T-cell based therapies, the cells can be expanded *in vitro* using cell culture methods relying heavily on the immunomodulatory action of interleukin-2 and returned to the patient in large numbers intravenously in an activated state. Anti-CD3 antibody can be used to promote the proliferation of T-cells in culture. Research into interleukin-21 indicates that it can also play an important role in enhancing the efficacy of T-cell based therapies prepared *in vitro*. Cells used in adoptive cell transfer can be used to deliver genetically modified lymphocytes, using recombinant DNA technology to achieve any number of goals. In alternatives described herein, adoptive cell transfer is used to transfer cells into a subject, wherein the cells are CAR expressing lymphocytes. In some alternatives, CAR expressing lymphocytes are host cells in methods for generating engineered multiplexed T-cells for adoptive T-cell immunotherapy. In some alternatives, the method comprises providing the gene delivery polynucleotide of the alternatives described herein, introducing the gene delivery polynucleotide into a T-cell, providing a vector encoding a Sleeping Beauty transposase, introducing the vector encoding the Sleeping Beauty transposase into the T-cell, selecting the cells comprising the gene delivery polynucleotide, wherein selecting comprises a first round of selection and a second round of selection, wherein the first round of selection comprises adding a selection reagent at a first concentration range and the second round of selection comprises adding the selection reagent at a second concentration range, wherein the second concentration range is higher than the first concentration range and, wherein the second concentration range is at least 1.5 fold higher than that of the first concentration range, and isolating the T-cells expressing a phenotype under selective pressure. In some alternatives, the gene delivery polynucleotide comprises a sequence for a co-stimulatory ligand. In some alternatives, the gene delivery polynucleotide comprises a sequence for a chimeric antigen receptor. In some alternatives, the T-cell expresses a CAR. In alternatives described herein, the CAR expressing lymphocytes are genetically modified by minicircles wherein the

minicircles comprise Sleeping Beauty transposons. In some alternatives, the selection reagent is MTX.

[0096] By way of example and not of limitation, genetically engineered T-cells can be created by infecting patient's cells with a transferring virus that contain a copy of a T-cell receptor (TCR) gene that is specialized to recognize, for example, tumor or viral antigens. It is important that the transferring virus is not able to reproduce within the cell however, but should integrate into the human genome. This is beneficial as new TCR gene remains stable in the T-cell. A patient's own T-cells are exposed to these transferring viruses and then are expanded non-specifically or stimulated using the genetically engineered TCR. The cells are then transferred back into the patient and are ready to mount an immune response against the tumor, virus, or viral infected cell. The use of adoptive cell transfer with genetically engineered T-cells is a promising new approach for the treatment of a variety of cancers or viral infections. In some alternatives, methods of adoptive immunotherapy for cancer are provided. In some alternatives, methods of adoptive immunotherapy for viral infections are provided.

[0097] The method of making genetically engineered T-cells by using a viral vector can have several drawbacks. Genetic modification of T-cells is typically accomplished using γ-retroviral or lentiviral vectors. While effective, drawbacks include cost of production, limited gene packaging capacity, and potential safety issues. Plasmids containing transposon systems such as *Sleeping Beauty* (SB) or *piggyBac* offer a non-viral approach for stably introducing genes into T-cells. Recently, the *piggyBac* system was used to produce stably-transfected mammalian cells expressing multiple transgenes of interest by delivery of multiple transposons. The SB system, first reactivated for mammalian cell use by Ivics and coworkers, has been used as the gene delivery modality in clinical trials of T-cell immunotherapy. Gene integration by SB has weaker preference for transcriptional units and their regulatory sequences compared to the γ-retroviral and lentiviral vectors and is therefore considered to be safer. In some alternatives described herein, genetic modification by minicircles comprising the Sleeping Beauty system are contemplated. In some alternatives described herein, genetic modification by minicircles comprising the *piggyBac* system are contemplated. In some alternatives described herein, genetic modification by minicircles comprising the Sleeping Beauty system are contemplated.

[0098] Minicircles are particularly attractive as transfection platforms for three reasons. First, the transfection efficiency of minicircles by electroporation is superior to that of their plasmid analogues. Second, transposition efficiency is higher in minicircles due to the shorter distance between the two transposon ends, which has been shown to affect transposase efficiency. Finally, as cell viability after nucleofection decreases with increasing construct size, minicircles are more advantageous given their smaller size compared to their analogous plasmids. To further improve transposition efficiency, the optimized SB100X hyperactive transposase developed by Izsvak et al. (*Nature Genet.* **2009**, *41*, 753-761; incorporated by reference in its entirety) can be used in combination with the T3 generation of SB previously by Yant et al (*Mol. Cell. Biol.* **2004**, *24*, 9239-9247; incorporated by reference in its entirety). In several alternatives described herein, methods for making a genetically modified T-cell for adoptive cell transfer are contemplated. In some alternatives, the methods comprise introducing a minicircle into a T-cell. In some alternatives, the introduction comprises electroporation delivery.

[0099] Another challenge in T-cell immunotherapy is cell survival after infusion into patients. Induced expression of anti-apoptotic proteins has been shown to improve *in vivo* survival of T-cells. Tumor homing and infiltration has been increased by introduction of chemokine receptors in engineered T-cells; this approach can be especially useful for tumors that express chemokines that are not normally recognized by T-cells. Finally, T-cells can be engineered to better resist the immunosuppressive tumor microenvironment through, for example, induced cytokine expression. Thus, methods to rapidly generate engineered T-cells expressing multiple transgenes are important and advantageous for clinical translation of T-cell immunotherapy. In some alternatives described herein, methods of introducing co-integration of additional genes for co-integration to further increase the anti-tumor activity of CAR-expressing T-cells are contemplated. In some alternatives, the additional genes encode co-stimulatory ligands. In some alternatives, the co-stimulatory ligand is CD80. In some alternatives, the co-stimulatory ligand is 4-1BBL. In some alternatives, the additional genes encode anti-apoptotic proteins. In some alternatives the additional genes encode chemokine receptors.

[0100] In some alternatives, methods of generating engineered multiplexed T-cells for adoptive T-cell immunotherapy are provided. In the broadest sense, the method can

comprise providing the gene delivery polynucleotide of any of the alternatives described herein, introducing the gene delivery polynucleotide into a T-cell, providing a vector encoding a Sleeping Beauty transposase, introducing the vector encoding the Sleeping Beauty transposase into the T-cell, selecting the cells comprising the gene delivery polynucleotide, wherein selecting comprises a first round of selection and a second round of selection, wherein the first round of selection comprises adding a selection reagent at a first concentration range and the second round of selection comprises adding the selection reagent at a second concentration range, wherein the second concentration range is higher than the first concentration range and, wherein the second concentration range is at least 1.5 fold higher than that of the first concentration range, and isolating the T-cells expressing a phenotype under selective pressure. In some alternatives, the T-cells are chimeric antigen receptor (CAR) expressing T-cells. In some alternatives, the selection reagent is MTX.

[0101] In some alternatives, methods of increasing protein production in a T-cell are provided. In the broadest sense, the method can comprise providing the gene delivery polynucleotide of any of the alternatives described herein, introducing the gene delivery polynucleotide into a T-cell, providing a vector encoding a Sleeping Beauty transposase, introducing the vector encoding the Sleeping Beauty transposase into the T-cell, selecting the cells comprising the gene delivery polynucleotide, wherein selecting comprises a first round of selection and a second round of selection, wherein the first round of selection comprises adding a selection reagent at a first concentration range and the second round of selection comprises adding the selection reagent at a second concentration range, wherein the second concentration range is higher than the first concentration range and, wherein the second concentration range is at least 1.5 fold higher than that of the first concentration range, and isolating the T-cells expressing a phenotype under selective pressure. In some alternatives, the selection reagent is MTX. In some alternatives, the T-cells are chimeric antigen receptor (CAR) expressing T-cells.

[0102] As described herein, an alternative of the system comprises an engineered, non-viral gene delivery system comprising three key features: (1) *Sleeping Beauty* transposon system for stable gene expression, (2) minicircles for enhanced transfection, and (3) a double mutant of human dihydrofolate reductase (DHFRdm) as a selection mechanism (**Figure 1**).

[0103] Minicircles are particularly attractive as transfection platforms for three reasons. First, the transfection efficiency of minicircles by electroporation is superior to that of their plasmid analogues. Second, transposition efficiency is higher in minicircles due to the shorter distance between the two transposon ends, which has been shown to affect transposase efficiency. Finally, as cell viability after nucleofection decreases with increasing construct size, minicircles are more desirable given their smaller size compared to their analogous plasmids. To further improve transposition efficiency, the optimized SB100X hyperactive transposase developed by Izsvak *et al.* (*Nature Genet.* **2009**, *41*, 753-761; incorporated herein by reference in its entirety) was used in combination with the T3 generation of SB transposon previously by Yant *et al* (*Mol. Cell. Biol.* **2004**, *24*, 9239-9247; incorporated herein by reference in its entirety). In some alternatives described herein, genetic modification of T-cells is performed using minicircles. In some alternatives, the minicircles comprise transposons. In some alternatives, the transposons comprise Sleeping Beauty transposons. In some alternatives, an optimized SB100X hyperactive transposase is used in combination with a T3 generation of SB transposon.

[0104] A selection mechanism for rapid selection of engineered T-cells can also be employed. The double mutant of human dihydrofolate reductase (DHFRdm, with amino acid mutations L22F and F31S) exhibits a 15,000-fold reduced affinity for methotrexate, a potent inhibitor of DHFR that results in blockade of thymidylate and purine synthesis. Expression of DHFRdm in T-cells imparts MTX resistance without compromising proliferative ability, expression of T-cell markers, or cytolytic ability. Additional advantages of this selection system include availability of clinical grade MTX, the use of a non-genotoxic drug, and the small gene size of DHFRdm (561 bp). Therefore, MTX can be used as a selection mechanism to selectively amplify SB-transduced cells. In some alternatives, the minicircles comprise a genetic sequence encoding a double mutant of human dihydrofolate reductase. In some alternatives, a selection method for rapid selection of engineered T-cells is provided. In some alternatives, the selection method comprises contacting engineered T-cells with clinical grade methotrexate. In some alternatives, the T-cells comprise a minicircle wherein the minicircle comprises a sequence for a double mutant of human dihydrofolate reductase. In some alternatives, the double mutant of human dihydrofolate reductase exhibits a 15,000 fold or about 15,000 fold reduced specificity for

methotrexate. In some alternatives, methotrexate can be used to contact the T-cells for selectively amplifying cells transduced with minicircles, wherein the minicircles comprise a sequence for the double mutant of human dihydrofolate reductase. In some alternatives, the gene encoding the double mutant of human dihydrofolate reductase comprises the DNA sequence:

ATGGTTGGTTCGCTAAACTGCATCGTCGCTGTGTCCCAGAACATGGGCATCGGCA
AGAACGGGGACTTCCCCTGGCCACCGCTCAGGAATGAATCCAGATATTTCCAGA
GAATGACCACAACCTCTTCAGTAGAAGGTAAACAGAATCTGGTGATTATGGGTA
AGAAGACCTGGTTCTCCATTCCTGAGAAGAATCGACCTTTAAAGGGTAGAATTA
ATTTAGTTCTCAGCAGAGAACTCAAGGAACCTCCACAAGGAGCTCATTTTCTTTC
CAGAAGTCTAGATGATGCCTTAAAACTTACTGAACAACCAGAATTAGCAAATAA
AGTAGACATGGTCTGGATAGTTGGTGGCAGTTCTGTTTATAAGGAAGCCATGAAT
CACCCAGGCCATCTTAAACTATTTGTGACAAGGATCATGCAAGACTTTGAAAGTG
ACACGTTTTTTCCAGAAATTGATTTGGAGAAATATAAACTTCTGCCAGAATACCC
AGGTGTTCTCTCTGATGTCCAGGAGGAGAAAGGCATTAAGTACAAATTTGAAGT
ATATGAGAAGAATGATTAA (SEQ ID NO: 2). In some alternatives, the double mutant of human dihydrofolate reductase comprises the protein sequence: MVGSLNCIVA VSQNMGIGKN GDFPWPPLRN ESRYFQRMTT TSSVEGKQNL VIMGKKTWFS IPEKNRPLKG RINLVLSREL KEPPQGAHFL SRSLDDALKL TEQPELANKV DMVWIVGGSS VYKEAMNHPG HLKLFVTRIM QDFESDTFFP EIDLEKYKLL PEYPGVLSDV QEEKGIKYKF EVYEKND (SEQ ID NO: 3).

[0105] Stable transfer of up to three transgenes into the H9 T-cell line using multiplexed delivery of minicircles containing SB transposons followed by methotrexate (MTX) selection can be performed. Cells with higher number of gene integrations can be preferentially obtained by increasing selection pressure with MTX. Using a two-step selection method through two successive MTX selection rounds, 50% of cells expressing three transgene products can be obtained. In some alternatives, a method of stably transferring transgenes into a cell line is provided. In some alternatives, a method of introducing minicircles into a cell line is provided. In some alternatives, the minicircles comprise Sleeping Beauty transposons. In some alternatives, the method further comprises increasing selection pressure with methotrexate, wherein increasing the selection pressure

comprises contacting the cell line with increasing concentrations of methotrexate. In some alternatives, the two rounds of methotrexate selection are performed.

Additional Alternatives

[0106] In some alternatives, a gene delivery polynucleotide for stable insertion of a nucleic acid into a gene, wherein the nucleic acid for insertion is flanked by inverted terminal repeat gene sequences in the gene delivery polynucleotide and wherein the gene delivery polynucleotide is selectable, the gene delivery polynucleotide, is provided. In the broadest sense, the gene delivery polynucleotide comprises a first sequence, wherein the first sequence comprises a first inverted terminal repeat gene sequence, a second sequence, wherein the second sequence comprises a second inverted terminal repeat gene sequence, a third sequence, wherein the third sequence comprises a promoter region sequence, a fourth sequence, wherein the fourth sequence comprises at least one gene encoding a protein, and wherein the fourth sequence is optimized, a fifth sequence, wherein the fifth sequence comprises at least one selectable marker cassette encoding a double mutant of dihydrofolate reductase, wherein the double mutant of dihydrofolate reductase has a 15,000 fold or about 15,000 fold reduced affinity for methotrexate, wherein the methotrexate can be used as a selection mechanism to selectively amplify cells transduced with the gene delivery polynucleotide and wherein the fifth sequence is optimized, a sixth sequence, wherein the sixth sequence comprises a first attachment site (attP), and a seventh sequence, wherein the seventh sequence comprises a second attachment site (attB); wherein each of the first sequence, second sequence, third sequence, fourth sequence, fifth sequence, sixth sequence, and seventh sequence have a 5' terminus and a 3 ' terminus, and wherein the 3' terminus of the first sequence comprising the first inverted terminal repeat gene sequence is adjacent to the 5' terminus of the third sequence, the 3' terminus of the third sequence is adjacent to the 5' terminus of the fourth sequence, the 3' terminus of the fourth sequence is adjacent to the 5' terminus of the fifth sequence and the 3' terminus of the fifth sequence is adjacent to the 5' terminus of the second sequence comprising a second inverted terminal repeat. In some alternatives, the gene encoding the double mutant of human dihydrofolate reductase comprises the DNA sequence: ATGGTTGGTTCGCTAAACTGCATCGTCGCTGTGTCCCAGAACATGGGCATCGGCA

AGAACGGGGACTTCCCCTGGCCACCGCTCAGGAATGAATCCAGATATTTCCAGA
GAATGACCACAACCTCTTCAGTAGAAGGTAAACAGAATCTGGTGATTATGGGTA
AGAAGACCTGGTTCTCCATTCCTGAGAAGAATCGACCTTTAAAGGGTAGAATTA
ATTTAGTTCTCAGCAGAGAACTCAAGGAACCTCCACAAGGAGCTCATTTTCTTTC
CAGAAGTCTAGATGATGCCTTAAAACTTACTGAACAACCAGAATTAGCAAATAA
AGTAGACATGGTCTGGATAGTTGGTGGCAGTTCTGTTTATAAGGAAGCCATGAAT
CACCCAGGCCATCTTAAACTATTTGTGACAAGGATCATGCAAGACTTTGAAAGTG
ACACGTTTTTTCCAGAAATTGATTTGGAGAAATATAAACTTCTGCCAGAATACCC
AGGTGTTCTCTCTGATGTCCAGGAGGAGAAAGGCATTAAGTACAAATTTGAAGT
ATATGAGAAGAATGATTAA (SEQ ID NO: 2). In some alternatives, the double mutant of human dihydrofolate reductase comprises the protein sequence: MVGSLNCIVA VSQNMGIGKN GDFPWPPLRN ESRYFQRMTT TSSVEGKQNL VIMGKKTWFS IPEKNRPLKG RINLVLSREL KEPPQGAHFL SRSLDDALKL TEQPELANKV DMVWIVGGSS VYKEAMNHPG HLKLFVTRIM QDFESDTFFP EIDLEKYKLL PEYPGVLSDV QEEKGIKYKF EVYEKND (SEQ ID NO: 3). In some alternatives, the gene delivery polynucleotide is circular. In some alternatives, the gene delivery polynucleotide is at least 1kB to 6kB. In some alternatives, the gene delivery polynucleotide is a minicircle. In some alternatives, the promoter region comprises an EF1 promoter sequence. In some alternatives, the fourth sequence comprises one, two, three, four, or five genes that encode proteins. In some alternatives, the fourth sequence is codon optimized to reduce the total GC/AT ratio of the fourth sequence. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins, such as a plurality of antibody binding domains, which are specific for the same epitope. In some alternatives, the fifth sequence is codon optimized to reduce the total GC/AT ratio of the fourth sequence. In some alternatives, the codon optimization and/or consensus sequence is generated by comparing the variability of sequence and/or nucleobases utilized in a plurality of related sequences. In some alternatives, the protein is a protein for therapy. In some alternatives, the protein comprises an antibody or a portion thereof. In some alternatives, the double mutant of dihydrofolate reductase comprises amino acid mutations of L22F and F31S. In some alternatives, the minicircle comprises a sequence for the double mutant of dihydrofolate reductase, the sequence comprising the DNA

sequence :
ATGGTTGGTTCGCTAAACTGCATCGTCGCTGTGTCCCAGAACATGGGCATCGGCA
AGAACGGGGACTTCCCCTGGCCACCGCTCAGGAATGAATCCAGATATTTCCAGA
GAATGACCACAACCTCTTCAGTAGAAGGTAAACAGAATCTGGTGATTATGGGTA
AGAAGACCTGGTTCTCCATTCCTGAGAAGAATCGACCTTTAAAGGGTAGAATTA
ATTTAGTTCTCAGCAGAGAACTCAAGGAACCTCCACAAGGAGCTCATTTTCTTTC
CAGAAGTCTAGATGATGCCTTAAAACTTACTGAACAACCAGAATTAGCAAATAA
AGTAGACATGGTCTGGATAGTTGGTGGCAGTTCTGTTTATAAGGAAGCCATGAAT
CACCCAGGCCATCTTAAACTATTTGTGACAAGGATCATGCAAGACTTTGAAAGTG
ACACGTTTTTTCCAGAAATTGATTTGGAGAAATATAAACTTCTGCCAGAATACCC
AGGTGTTCTCTCTGATGTCCAGGAGGAGAAAGGCATTAAGTACAAATTTGAAGT
ATATGAGAAGAATGATTAA (SEQ ID NO: 2). In some alternatives, the double mutant of dihydrofolate reductase comprises the protein sequence: MVGSLNCIVA VSQNMGIGKN GDFPWPPLRN ESRYFQRMTT TSSVEGKQNL VIMGKKTWFS IPEKNRPLKG RINLVLSREL KEPPQGAHFL SRSLDDALKL TEQPELANKV DMVWIVGGSS VYKEAMNHPG HLKLFVTRIM QDFESDTFFP EIDLEKYKLL PEYPGVLSDV QEEKGIKYKF EVYEKND (SEQ ID NO: 3).

[0107] In some alternatives, a method of generating engineered multiplexed T-cells for adoptive T-cell immunotherapy is provided. In the broadest sense, the method can comprise providing the gene delivery polynucleotide of any of the alternatives described herein, introducing the gene delivery polynucleotide into a T-cell, providing a vector encoding a Sleeping Beauty transposase, introducing the vector encoding the Sleeping Beauty transposase into the T-cell, selecting the cells comprising the gene delivery polynucleotide, wherein selecting comprises a first round of selection and a second round of selection, wherein the first round of selection comprises adding a selection reagent at a first concentration range and the second round of selection comprises adding the selection reagent at a second concentration range, wherein the second concentration range is higher than the first concentration range and, wherein the second concentration range is at least 1.5 fold higher than that of the first concentration range, and isolating the T-cells expressing a phenotype under selective pressure. In some alternatives, introducing is performed by electroporation. In some alternatives, the selecting is performed by increasing selective

pressure through the selective marker cassette. In some alternatives, the selection reagent comprises an agent for selection. In some alternatives, the agent for selection is methotrexate. In some alternatives, the first concentration range is at least 50nM – 100nM and the second concentration range is at least 75 to 150nM. In some alternatives, the first concentration is 50 nM, 60 nM, 70 nM, 80 nM, 90 nM, or 100nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 75 nM, 80 nM, 90 nM, 100 nM, 110 nM, 120 nM, 130 nM, 140 nM, or 150 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first concentration range is at least 75 nM – 150 nM and the second concentration range is at least 112.5 nM to 225 nM. In some alternatives, the first concentration is 75 nM, 85 nM, 95 nM, 105 nM, 115 nM, 125 nM, 135 nM, 145 nM, or 150 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 112 nM, 122 nM, 132 nM, 142 nM, 152 nM, 162 nM, 172 nM, 182 nM, 192 nM, 202 nM, 212 nM, or 225 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first concentration range is at least 300 nM – 675 nM and the first concentration range is at least 450 nM to 1012 nM. In some alternatives, the first concentration is 300 nM, 350 nM, 400 nM, 450 nM, 500 nM, 550 nM, 600 nM, 650 nM, or 675 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 450 nM, 500 nM, 550 nM, 600 nM, 650 nM, 700 nM, 750 nM, 800 nM, 850 nM, 900 nM, 1000 nM, or 1012 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first round of selection comprises exposing the T-cells to the selection agent for 2, 3, 4, 5, 6 or 7 days before the second round of selection. In some alternatives, the second round of selection comprises exposing the T-cells to the selection agent for at least 2, 3, 4, 5, 6 or 7 days before isolation.

[0108] In some alternatives, a method of increasing protein production in a cell is provided. In the broadest sense, the method can comprise providing the gene delivery polynucleotide of any one of the alternatives described herein, introducing the gene delivery polynucleotide into a T-cell, providing a vector encoding a Sleeping Beauty transposase,

introducing the vector encoding the Sleeping Beauty transposase into the T-cell, selecting the cells comprising the gene delivery polynucleotide, wherein selecting comprises a first round of selection and a second round of selection, wherein the first round of selection comprises adding a selection reagent at a first concentration range and the second round of selection comprises adding the selection reagent at a second concentration range, wherein the second concentration range is higher than the first concentration range and, wherein the second concentration range is at least 1.5 fold higher than that of the first concentration range, and isolating the T-cells expressing a phenotype under selective pressure. In some alternatives, introducing is performed by electroporation. In some alternatives, selecting is performed by increasing selective pressure through the selective marker cassette. In some alternatives, the selection reagent comprises an agent for selection. In some alternatives, the agent for selection is methotrexate. In some alternatives, the low or first concentration range is at least 50 nM – 100 nM and the higher or second concentration range is at least 75 to 150 nM. In some alternatives, the low or first concentration range is at least 75 nM – 150 nM and the higher or second concentration range is at least 112.5 nM to 225 nM. In some alternatives, the low or first concentration range is at least 300 nM – 675 nM and the higher or second concentration range is at least 450 nM to 1012 nM. In some alternatives, the first round of selection comprises exposing the T-cells to the selection agent for 2, 3, 4, 5, 6 or 7 days before the second round of selection. In some alternatives, the second round of selection comprises exposing the T-cells to the selection agent for at least 2, 3, 4, 5, 6 or 7 days before isolation.

[0109] In some alternatives, an engineered multiplexed T-cell for adoptive T-cell immunotherapy generated by any one of the methods of is provided. In some alternatives, the engineered multiplexed T-cells for adoptive T-cell immunotherapy is generated by a method, wherein the method comprises providing a gene delivery polynucleotide, introducing the gene delivery polynucleotide into a T-cell, providing a vector encoding a Sleeping Beauty transposase, introducing the vector encoding the Sleeping Beauty transposase into the T-cell, selecting the cells comprising the gene delivery polynucleotide wherein selecting comprises a first round of selection and a second round of selection, wherein the first round of selection comprises adding a selection reagent at a first concentration range and the second round of selection comprises adding the selection reagent at a second concentration range, wherein the

second concentration range is higher than the first concentration range and, wherein the second concentration range is at least 1.5 fold higher than that of the first concentration range and isolating the T-cells expressing a phenotype under selective pressure. In some alternatives, the gene delivery polynucleotide comprises a first sequence, wherein the first sequence comprises a first inverted terminal repeat gene sequence, a second sequence, wherein the second sequence comprises a second inverted terminal repeat gene sequence, a third sequence, wherein the third sequence comprises a promoter region sequence, a fourth sequence, wherein the fourth sequence comprises at least one gene encoding a protein, and wherein the fourth sequence is optimized, a fifth sequence, wherein the fifth sequence comprises at least one selectable marker cassette encoding a double mutant of dihydrofolate reductase, wherein the double mutant of dihydrofolate reductase has a 15,000 fold or about 15,000 fold reduced affinity for methotrexate, wherein the methotrexate can be used as a selection mechanism to selectively amplify cells transduced with the gene delivery polynucleotide and wherein the fifth sequence is optimized, a sixth sequence, wherein the sixth sequence comprises a first attachment site (attP) and a seventh sequence, wherein the seventh sequence comprises a second attachment site (attB) wherein each of the first sequence, second sequence, third sequence, fourth sequence, fifth sequence, sixth sequence, and seventh sequence have a 5' terminus and a 3 ' terminus, and wherein the 3' terminus of the first sequence comprising the first inverted terminal repeat gene sequence is adjacent to the 5' terminus of the third sequence, the 3' terminus of the third sequence is adjacent to the 5' terminus of the fourth sequence, the 3' terminus of the fourth sequence is adjacent to the 5' terminus of the fifth sequence and the 3' terminus of the fifth sequence is adjacent to the 5' terminus of the second sequence comprising a second inverted terminal repeat. In some alternatives, the gene encoding the double mutant of human dihydrofolate reductase comprises the DNA sequence:

ATGGTTGGTTCGCTAAACTGCATCGTCGCTGTGTCCCAGAACATGGGCATCGGCA
AGAACGGGGACTTCCCCTGGCCACCGCTCAGGAATGAATCCAGATATTTCCAGA
GAATGACCACAACCTCTTCAGTAGAAGGTAAACAGAATCTGGTGATTATGGGTA
AGAAGACCTGGTTCTCCATTCCTGAGAAGAATCGACCTTTAAAGGGTAGAATTA
ATTTAGTTCTCAGCAGAGAACTCAAGGAACCTCCACAAGGAGCTCATTTTCTTTC
CAGAAGTCTAGATGATGCCTTAAAACTTACTGAACAACCAGAATTAGCAAATAA

AGTAGACATGGTCTGGATAGTTGGTGGCAGTTCTGTTTATAAGGAAGCCATGAAT CACCCAGGCCATCTTAAACTATTTGTGACAAGGATCATGCAAGACTTTGAAAGTG ACACGTTTTTTCCAGAAATTGATTTGGAGAAATATAAACTTCTGCCAGAATACCC AGGTGTTCTCTCTGATGTCCAGGAGGAGAAAGGCATTAAGTACAAATTTGAAGT ATATGAGAAGAATGATTAA (SEQ ID NO: 2). In some alternatives, the double mutant of human dihydrofolate reductase comprises the protein sequence: MVGSLNCIVA VSQNMGIGKN GDFPWPPLRN ESRYFQRMTT TSSVEGKQNL VIMGKKTWFS IPEKNRPLKG RINLVLSREL KEPPQGAHFL SRSLDDALKL TEQPELANKV DMVWIVGGSS VYKEAMNHPG HLKLFVTRIM QDFESDTFFP EIDLEKYKLL PEYPGVLSDV QEEKGIKYKF EVYEKND (SEQ ID NO: 3). In some alternatives, the gene delivery polynucleotide is circular. In some alternatives, the gene delivery polynucleotide is at least 1kB to 5kB. In some alternatives, the gene delivery polynucleotide is a minicircle. In some alternatives, the promoter region comprises an EF1 promoter sequence. In some alternatives, the fourth sequence comprises one, two, three, four, or five genes that encode proteins. In some alternatives, the fourth sequence is codon optimized to reduce the total GC/AT ratio of the fourth sequence. In some alternatives, the fourth sequence is optimized by codon optimization for expression in humans. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins, such as a plurality of antibody binding domains, which are specific for the same epitope. In some alternatives, the plurality of related proteins comprise a plurality of antibody binding domains, wherein the plurality of antibody binding domains are specific for the same epitope. In some alternatives, the fifth sequence is codon optimized to reduce the total GC/AT ratio of the fifth sequence. In some alternatives, the fifth sequence is optimized by codon optimization for expression in humans. In some alternatives, the protein is a protein for therapy. In some alternatives, the codon optimization and/or consensus sequence is generated by comparing the variability of sequence and/or nucleobases utilized in a plurality of related sequences. In some alternatives, the protein comprises an antibody or a portion thereof, which may be humanized. In some alternatives, the double mutant of dihydrofolate reductase comprises amino acid mutations of L22F and F31S. In some alternatives, the double mutant of

dihydrofolate reductase comprises amino acid mutations of L22F and F31S. In some alternatives, the introducing is performed by electroporation. In some alternatives, the selecting is performed by increasing selective pressure through the selective marker cassette. In some alternatives, the selection reagent comprises an agent for selection. In some alternatives, the agent for selection is methotrexate. In some alternatives, the first concentration range is at least 50nM – 100nM and the second concentration range is at least 75 to 150nM. In some alternatives, the first concentration is 50 nM, 60 nM, 70 nM, 80 nM, 90 nM, or 100nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 75 nM, 80 nM, 90 nM, 100 nM, 110 nM, 120 nM, 130 nM, 140 nM, or 150 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first concentration range is at least 75 nM – 150 nM and the second concentration range is at least 112.5 nM to 225 nM. In some alternatives, the first concentration is 75 nM, 85 nM, 95 nM, 105 nM, 115 nM, 125 nM, 135 nM, 145 nM, or 150 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 112 nM, 122 nM, 132 nM, 142 nM, 152 nM, 162 nM, 172 nM, 182 nM, 192 nM, 202 nM, 212 nM, or 225 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first concentration range is at least 300 nM – 675 nM and the first concentration range is at least 450 nM to 1012 nM. In some alternatives, the first concentration is 300 nM, 350 nM, 400 nM, 450 nM, 500 nM, 550 nM, 600 nM, 650 nM, or 675 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 450 nM, 500 nM, 550 nM, 600 nM, 650 nM, 700 nM, 750 nM, 800 nM, 850 nM, 900 nM, 1000 nM, or 1012 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first round of selection comprises exposing the T-cells to the selection agent for 2, 3, 4, 5, 6 or 7 days before the second round of selection. In some alternatives, the second round of selection comprises exposing the T-cells to the selection agent for at least 2, 3, 4, 5, 6,7, 8, 9, 10, 11, 12, 13, or 14 days or any time that is between a range of times defined by any two of the aforementioned time points before isolation. In some alternatives, the gene delivery

polynucleotide comprises a first sequence, wherein the first sequence comprises a first inverted terminal repeat gene sequence, a second sequence, wherein the second sequence comprises a second inverted terminal repeat gene sequence, a third sequence, wherein the third sequence comprises a promoter region sequence, a fourth sequence, wherein the fourth sequence comprises at least one gene encoding a protein, and wherein the fourth sequence is optimized, a fifth sequence, wherein the fifth sequence comprises at least one selectable marker cassette encoding a double mutant of dihydrofolate reductase, wherein the double mutant of dihydrofolate reductase has a 15,000 fold or about 15,000 fold reduced affinity for methotrexate, wherein the methotrexate can be used as a selection mechanism to selectively amplify cells transduced with the gene delivery polynucleotide and wherein the fifth sequence is optimized, a sixth sequence, wherein the sixth sequence comprises a first attachment site (attP) and a seventh sequence, wherein the seventh sequence comprises a second attachment site (attB) wherein each of the first sequence, second sequence, third sequence, fourth sequence, fifth sequence, sixth sequence, and seventh sequence have a 5' terminus and a 3 ' terminus, and wherein the 3' terminus of the first sequence comprising the first inverted terminal repeat gene sequence is adjacent to the 5' terminus of the third sequence, the 3' terminus of the third sequence is adjacent to the 5' terminus of the fourth sequence, the 3' terminus of the fourth sequence is adjacent to the 5' terminus of the fifth sequence and the 3' terminus of the fifth sequence is adjacent to the 5' terminus of the second sequence comprising a second inverted terminal repeat. In some alternatives, the gene encoding the double mutant of human dihydrofolate reductase comprises the DNA sequence:

ATGGTTGGTTCGCTAAACTGCATCGTCGCTGTGTCCCAGAACATGGGCATCGGCA
AGAACGGGGACTTCCCCTGGCCACCGCTCAGGAATGAATCCAGATATTTCCAGA
GAATGACCACAACCTCTTCAGTAGAAGGTAAACAGAATCTGGTGATTATGGGTA
AGAAGACCTGGTTCTCCATTCCTGAGAAGAATCGACCTTTAAAGGGTAGAATTA
ATTTAGTTCTCAGCAGAGAACTCAAGGAACCTCCACAAGGAGCTCATTTTCTTTC
CAGAAGTCTAGATGATGCCTTAAAACTTACTGAACAACCAGAATTAGCAAATAA
AGTAGACATGGTCTGGATAGTTGGTGGCAGTTCTGTTTATAAGGAAGCCATGAAT
CACCCAGGCCATCTTAAACTATTTGTGACAAGGATCATGCAAGACTTTGAAAGTG
ACACGTTTTTTCCAGAAATTGATTTGGAGAAATATAAACTTCTGCCAGAATACCC
AGGTGTTCTCTCTGATGTCCAGGAGGAGAAAGGCATTAAGTACAAATTTGAAGT

ATATGAGAAGAATGATTAA (SEQ ID NO: 2). In some alternatives, the double mutant of human dihydrofolate reductase comprises the protein sequence: MVGSLNCIVA VSQNMGIGKN GDFPWPPLRN ESRYFQRMTT TSSVEGKQNL VIMGKKTWFS IPEKNRPLKG RINLVLSREL KEPPQGAHFL SRSLDDALKL TEQPELANKV DMVWIVGGSS VYKEAMNHPG HLKLFVTRIM QDFESDTFFP EIDLEKYKLL PEYPGVLSDV QEEKGIKYKF EVYEKND (SEQ ID NO: 3). In some alternatives, the gene delivery polynucleotide is circular. In some alternatives, the gene delivery polynucleotide is at least 1kB to 5kB. In some alternatives, the gene delivery polynucleotide is a minicircle. In some alternatives, the promoter region comprises an EF1 promoter sequence. In some alternatives, the fourth sequence comprises one, two, three, four, or five genes that encode proteins. In some alternatives, the fourth sequence is codon optimized to reduce the total GC/AT ratio of the fourth sequence. In some alternatives, the fourth sequence is optimized by codon optimization for expression in humans. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins, such as a plurality of antibody binding domains, which are specific for the same epitope. In some alternatives, the plurality of related proteins comprise a plurality of antibody binding domains, wherein the plurality of antibody binding domains are specific for the same epitope. In some alternatives, the fifth sequence is codon optimized to reduce the total GC/AT ratio of the fifth sequence. In some alternatives, the fifth sequence is optimized by codon optimization for expression in humans. In some alternatives, the protein is a protein for therapy. In some alternatives, the codon optimization and/or consensus sequence is generated by comparing the variability of sequence and/or nucleobases utilized in a plurality of related sequences. In some alternatives, the protein comprises an antibody or a portion thereof, which may be humanized. In some alternatives, the double mutant of dihydrofolate reductase comprises amino acid mutations of L22F and F31S. In some alternatives, the introducing is performed by electroporation. In some alternatives, the selecting is performed by increasing selective pressure through the selective marker cassette. In some alternatives, the selection reagent comprises an agent for selection. In some alternatives, the agent for selection is methotrexate. In some alternatives, the first concentration range is at least 50nM – 100nM and the second

concentration range is at least 75 to 150nM. In some alternatives, the first concentration is 50 nM, 60 nM, 70 nM, 80 nM, 90 nM, or 100nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 75 nM, 80 nM, 90 nM, 100 nM, 110 nM, 120 nM, 130 nM, 140 nM, or 150 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first concentration range is at least 75 nM – 150 nM and the second concentration range is at least 112.5 nM to 225 nM. In some alternatives, the first concentration is 75 nM, 85 nM, 95 nM, 105 nM, 115 nM, 125 nM, 135 nM, 145 nM, or 150 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 112 nM, 122 nM, 132 nM, 142 nM, 152 nM, 162 nM, 172 nM, 182 nM, 192 nM, 202 nM, 212 nM, or 225 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first concentration range is at least 300 nM – 675 nM and the first concentration range is at least 450 nM to 1012 nM. In some alternatives, the first concentration is 300 nM, 350 nM, 400 nM, 450 nM, 500 nM, 550 nM, 600 nM, 650 nM, or 675 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 450 nM, 500 nM, 550 nM, 600 nM, 650 nM, 700 nM, 750 nM, 800 nM, 850 nM, 900 nM, 1000 nM, or 1012 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first round of selection comprises exposing the T-cells to the selection agent for 2, 3, 4, 5, 6 or 7 days before the second round of selection. In some alternatives, the second round of selection comprises exposing the T-cells to the selection agent for at least 2, 3, 4, 5, 6,7, 8, 9, 10, 11, 12, 13, or 14 days or any time that is between a range of times defined by any two of the aforementioned time points before isolation.

[0110] In some alternatives, a method of treating, inhibiting, or ameliorating cancer or a disease in a subject is provided, wherein the method comprises administering to the subject the modified or engineered multiplexed T-cell as described below. In some alternatives, the engineered multiplexed T-cells for adoptive T-cell immunotherapy is generated by a method, wherein the method comprises providing a gene delivery

polynucleotide, introducing the gene delivery polynucleotide into a T-cell, providing a vector encoding a Sleeping Beauty transposase, introducing the vector encoding the Sleeping Beauty transposase into the T-cell, selecting the cells comprising the gene delivery polynucleotide wherein selecting comprises a first round of selection and a second round of selection, wherein the first round of selection comprises adding a selection reagent at a first concentration range and the second round of selection comprises adding the selection reagent at a second concentration range, wherein the second concentration range is higher than the first concentration range and, wherein the second concentration range is at least 1.5 fold higher than that of the first concentration range and isolating the T-cells expressing a phenotype under selective pressure. In some alternatives, the gene delivery polynucleotide comprises a first sequence, wherein the first sequence comprises a first inverted terminal repeat gene sequence, a second sequence, wherein the second sequence comprises a second inverted terminal repeat gene sequence, a third sequence, wherein the third sequence comprises a promoter region sequence, a fourth sequence, wherein the fourth sequence comprises at least one gene encoding a protein, and wherein the fourth sequence is codon optimized for expression in humans, a fifth sequence, wherein the fifth sequence comprises at least one selectable marker cassette encoding a double mutant of dihydrofolate reductase, wherein the double mutant of dihydrofolate reductase has a 15,000 fold or about 15,000 fold reduced affinity for methotrexate, wherein the methotrexate can be used as a selection mechanism to selectively amplify cells transduced with the gene delivery polynucleotide and wherein the fifth sequence is codon optimized for expression in humans, a sixth sequence, wherein the sixth sequence comprises a first attachment site (attP) and a seventh sequence, wherein the seventh sequence comprises a second attachment site (attB) wherein each of the first sequence, second sequence, third sequence, fourth sequence, fifth sequence, sixth sequence, and seventh sequence have a 5' terminus and a 3 ' terminus, and wherein the 3' terminus of the first sequence comprising the first inverted terminal repeat gene sequence is adjacent to the 5' terminus of the third sequence, the 3' terminus of the third sequence is adjacent to the 5' terminus of the fourth sequence, the 3' terminus of the fourth sequence is adjacent to the 5' terminus of the fifth sequence and the 3' terminus of the fifth sequence is adjacent to the 5' terminus of the second sequence comprising a second inverted terminal repeat. In some alternatives, the gene encoding the double mutant of human dihydrofolate

reductase comprises the DNA sequence: ATGGTTGGTTCGCTAAACTGCATCGTCGCTGTGTCCCAGAACATGGGCATCGGCA AGAACGGGGACTTCCCCTGGCCACCGCTCAGGAATGAATCCAGATATTTCCAGA GAATGACCACAACCTCTTCAGTAGAAGGTAAACAGAATCTGGTGATTATGGGTA AGAAGACCTGGTTCTCCATTCCTGAGAAGAATCGACCTTTAAAGGGTAGAATTA ATTTAGTTCTCAGCAGAGAACTCAAGGAACCTCCACAAGGAGCTCATTTTCTTTC CAGAAGTCTAGATGATGCCTTAAAACTTACTGAACAACCAGAATTAGCAAATAA AGTAGACATGGTCTGGATAGTTGGTGGCAGTTCTGTTTATAAGGAAGCCATGAAT CACCCAGGCCATCTTAAACTATTTGTGACAAGGATCATGCAAGACTTTGAAAGTG ACACGTTTTTTCCAGAAATTGATTTGGAGAAATATAAACTTCTGCCAGAATACCC AGGTGTTCTCTCTGATGTCCAGGAGGAGAAAGGCATTAAGTACAAATTTGAAGT ATATGAGAAGAATGATTAA (SEQ ID NO: 2). In some alternatives, the double mutant of human dihydrofolate reductase comprises the protein sequence: MVGSLNCIVA VSQNMGIGKN GDFPWPPLRN ESRYFQRMTT TSSVEGKQNL VIMGKKTWFS IPEKNRPLKG RINLVLSREL KEPPQGAHFL SRSLDDALKL TEQPELANKV DMVWIVGGSS VYKEAMNHPG HLKLFVTRIM QDFESDTFFP EIDLEKYKLL PEYPGVLSDV QEEKGIKYKF EVYEKND (SEQ ID NO: 3). In some alternatives, the gene delivery polynucleotide is circular. In some alternatives, the gene delivery polynucleotide is a minicircle. In some alternatives, the gene delivery polynucleotide is at least 1kB to 5kB. In some alternatives, the promoter region comprises an EF1 promoter sequence. In some alternatives, the fourth sequence comprises one, two, three, four, or five genes that encode proteins. In some alternatives, the fourth sequence is codon optimized to reduce the total GC/AT ratio of the fourth sequence. In some alternatives, the fourth sequence is optimized by codon optimization for expression in humans. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins, such as a plurality of antibody binding domains, which are specific for the same epitope. In some alternatives, the plurality of related proteins comprise a plurality of antibody binding domains, wherein the plurality of antibody binding domains are specific for the same epitope. In some alternatives, the fifth sequence is codon optimized to reduce the total GC/AT ratio of the fifth

sequence. In some alternatives, the fifth sequence is optimized by codon optimization for expression in humans. In some alternatives, the protein is a protein for therapy. In some alternatives, the codon optimization and/or consensus sequence is generated by comparing the variability of sequence and/or nucleobases utilized in a plurality of related sequences. In some alternatives, the protein comprises an antibody or a portion thereof, which may be humanized. In some alternatives, the double mutant of dihydrofolate reductase comprises amino acid mutations of L22F and F31S. In some alternatives, the double mutant of dihydrofolate reductase comprises amino acid mutations of L22F and F31S. In some alternatives, the introducing is performed by electroporation. In some alternatives, the selecting is performed by increasing selective pressure through the selective marker cassette. In some alternatives, the selection reagent comprises an agent for selection. In some alternatives, the agent for selection is methotrexate. In some alternatives, the first concentration range is at least 50nM – 100nM and the second concentration range is at least 75 to 150nM. In some alternatives, the first concentration is 50 nM, 60 nM, 70 nM, 80 nM, 90 nM, or 100nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 75 nM, 80 nM, 90 nM, 100 nM, 110 nM, 120 nM, 130 nM, 140 nM, or 150 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first concentration range is at least 75 nM – 150 nM and the second concentration range is at least 112.5 nM to 225 nM. In some alternatives, the first concentration is 75 nM, 85 nM, 95 nM, 105 nM, 115 nM, 125 nM, 135 nM, 145 nM, or 150 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 112 nM, 122 nM, 132 nM, 142 nM, 152 nM, 162 nM, 172 nM, 182 nM, 192 nM, 202 nM, 212 nM, or 225 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first concentration range is at least 300 nM – 675 nM and the first concentration range is at least 450 nM to 1012 nM. In some alternatives, the first concentration is 300 nM, 350 nM, 400 nM, 450 nM, 500 nM, 550 nM, 600 nM, 650 nM, or 675 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 450 nM, 500 nM, 550 nM, 600 nM, 650 nM, 700 nM, 750 nM, 800 nM, 850 nM, 900 nM,

1000 nM, or 1012 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first round of selection comprises exposing the T-cells to the selection agent for 2, 3, 4, 5, 6 or 7 days before the second round of selection. In some alternatives, the second round of selection comprises exposing the T-cells to the selection agent for at least 2, 3, 4, 5, 6,7, 8, 9, 10, 11, 12, 13, or 14 days or any time that is between a range of times defined by any two of the aforementioned time points before isolation. In some alternatives, the subject is human.

[0111] Several of the material and methods are described in greater detail below.

Plasmids.

[0112] The pMC_T3/GFP-T2A-DHFRdm mini-circle (MC) plasmid that carries the T3 SB transposon cassette containing an EF1a promoter, maxGFP gene, *Thoseaasigna* virus 2A peptide (T2A) and a double mutant of dihydrofolate reductase (DHFRdm) insensitive to methotrexate (MTX) was constructed using pMC_T3/eGFP_IRES_FGFR (Nucleic Acids Research, 2012, 1–10 doi:10.1093/nar/gks213, incorporated in its entirety herein) as a backbone, implementing the cloning strategy described previously (Cold Spring Harbor Protoc; 2012; doi:10.1101/pdb.ip067876) to create the GFP-T2A-DHFRdm cassette. MaxGFP (Lonza) and pEGFRt-T2A-IMPDHdm-T2A-DHFRdm (generously provided by Michael Jensen) plasmids were used as templates for PCR. BmtI and BamHI sites were introduced for swapping genes for fluorescent proteins. Plasmid MC_SB100X was described previously (Nucleic Acids Research, 2012, 1–10 doi:10.1093/nar/gks213, incorporated in its entirety herein). Minicircles were produced and purified according to the System Biosciences user manual for minicircle DNA vector technology. All plasmids were amplified under endotoxin free conditions using an Endofree Plasmid Kit (Qiagen).

H9 culture and transfection.

[0113] H9 cells were cultured in DMEM with 10% FBS. The optimized nucleofection protocol forH9 cells (Lonza) was followed (program X-001, Nucleofector Kit V). Per nucleofection, $1x10^6$ cells were used with varying amounts of MC DNA. Cells were grown for a week after nucleofection to achieve stable transfection. For MTX selection, cells were cultured in DMEM with 10% FBS supplemented with different concentrations of MTX.

Flow cytometry analysis.

[0114] Live cells were selected based on propidium iodide exclusion by adding propidium iodide in the flow cytometry buffer to 2 μg/ml. Flow cytometry analysis was carried out on a MACSQuant Analyzer (Miltenyi Biotec) and LSRII (BD Biosciences). Collected data was analyzed with FlowJo software. Appropriate negative controls (untransfected H9 cells with and without propidium iodide staining, as well as cells transfected with single genes for GFP, BFP, and mCherry) were used for compensation and gating. A Becton Dickinson FACSAria II was used for cell sorting. Part of flow cytometry work was conducted at the UW Immunology Flow Cytometry Facility.

Determination of transposon copy number.

[0115] Genomic DNA was extracted with Puregene Kit A according to the manufacturer's instructions (Qiagen), and qPCR was performed using a 7300 Real-Time PCR System (Applied Biosystems) using Universal SYBR Green Supermix (BioRad). Primers for qPCR were designed using Primer3 software: maxGFP forward primer: 5'-ACAAGATCATCCGCAGCAAC-3' (SEQ ID NO: 4); reverse primer: 5'-TTGAAGTGCATGTGGCTGTC-3'(SEQ ID NO: 5); GAPDH forward primer: 5'-ACAACTTTGGTATCGTGGAAGG-3'(SEQ ID NO: 6); GAPDH reverse primer: 5'-GCCATCACGCCACAGTTTC-3' (SEQ ID NO: 7). MaxGFP primers are specific for the maxGFP gene in the transposon. Standard curves were generated using genomic DNA of a H9 clone with a single insertion of transposon ("gold standard") obtained by limiting dilution method. Copy number was calculated using the ΔΔCT method (Schmittgen,T.D. and Livak,K.J. (2008), incorporated in its entirety herein).

Characterization of SBTS integration distribution.

[0116] A population of T3/GFP-T2A-DHFRdm transfected-H9 cells selected with 200 nM MTX was plated in 96 well plates at a concentration of 0.5 cells/well in DMEM 10%FBS along with irradiated (5000 R) H9 feeder cells at 5,000 cells/well. Plates were incubated for 2-3 weeks, after which clonal populations were moved to larger plates and expanded. GFP expression was confirmed by flow cytometry. Relative RT-qPCR analysis was performed using DNA of 60 individual clones in order to determine transposon copy number.

Optimization of stable gene transfer to H9 cells.

[0117] Minicircle constructs, which have bacterial plasmid sequences removed, were used for all gene transfer studies. Minicircles can be generated as described previously by Kay *et al.* and colleagues (Chen, Z. Y.; He, C. Y.; Ehrhardt, A.; Kay, M. A. Molecular Therapy 2003, 8, 495-500 and Kay, M. A.; He, C. Y.; Chen, Z. Y. Nat. Biotechnol. 2010, 28, 1287-U96; incorporated herein by reference in their entirety). Three reporter minicircles containing transposons expressing different fluorescent proteins (maxGFP, mCherry, or BFP) under the EF1 alpha promoter were constructed. The selection gene, a double mutant of dihydrofolate reductase (DHFRdm) that confers metabolic resistance to MTX, was cloned in frame after the T2A sequence. The SB100X transposase gene was also prepared in a separate minicircle construct for co-delivery with transposon minicircles.

[0118] There are four transposase binding sites in a transposon (two per inverted terminal repeat). Bound transposase were proposed to interact with each other to promote juxtaposition of the two transposon ends. Overexpression of transposase has been hypothesized to lead to inhibition of transposition due to interaction of free transposase with bound transposase, thus preventing the juxtaposition step. Therefore, the optimal transposon/transposase ratio needed to be determined whether these genes are delivered on separate constructs. Reports of the inhibition phenomenon have been varied.

[0119] The efficiency of transient transfection were evaluated at 24 hours post-nucleofection and at stable transposition (7 days post-nucleofection) at various transposon/transposase ratios using the reporter minicircle expressing maxGFP by flow cytometry. Attention is drawn to **Figure 2,** which shows the optimization of transposon:transposase ratio. The H9 T-cell line was used as the transfection test-bed. Initial transfection efficiency ranged from 47.5% ± 2.2% to 66.9% ± 4.5%, increasing with increased amount of transposase minicircle. In the absence of transposase, minimal stable transfection (<1%) was detected 7 days post-nucleofection. The percentage of GFP^+ cells increased with the transposon/transposase ratio, reaching 39.2% ± 3.0% at 1:4 ratio, which reflects 58.6% integration efficiency of the initial transiently-integrated population. Higher ratios were not tested due to reduced cell viability. The overexpression inhibition effect was not observed in this tested range of transposon/transposase ratios. Therefore, from the results,

the transduction experiments were carried out using this optimized transposon/transposase ratio of 1:4.

Selection of engineered cells with methotrexate.

[0120] It was hypothesized that cells can be selected with multiple integration events using higher MTX concentrations due to increased selection pressure for DHFRdm expression. Cells stably transduced with the T3/maxGFP-T2A-DHRFdm transposon were therefore grown in the presence of increasing MTX concentrations (ranging from 50 to 200 nM) and GFP expression was evaluated by flow cytometry over 10 days. Attention is drawn to **Figure 3**, which shows the effect of methotrexate (MTX) concentration during selection. The initial selection efficiency, assessed with 3 days of MTX selection, was decreased with increasing MTX concentration (**Figure 3, panel A**). However, populations with >94% GFP+ cells were obtained by 7 days post-selection under all conditions. The mean GFP fluorescence in GFP^+ cells increased with selection pressure (**Figure 3, panel B**); the mean fluorescence in cells selected with 200 nM MTX was 6.4-fold higher than unselected cells and 3.3-fold higher than cells selected with 50 nM MTX. As shown, the positive correlation between mean GFP expression in GFP+ cells and MTX concentration suggests that increasing MTX concentration selects for cells with increased DHFRdm expression and therefore, multiple integration events.

[0121] The amplified cell populations selected with 2 weeks of MTX treatment maintained most of their transgene expression even upon MTX withdrawal up to 4 weeks. Attention is drawn to **Figure 4**, which shows the transgene persistence after methotrexate (MTX) withdrawal. Four weeks post-MTX withdrawal, the GFP^+ population remained >90% in all populations (**Figure 4, panel A**), although cells selected with 200 nM MTX had the highest GFP^+ population (97%), likely due to selection of cells with multiple integration events. The mean GFP expression in all populations decreased by 21%, 27%, and 28% for 200 nM, 100 nM, and 50 nM MTX selection, respectively by 4 weeks post-MTX withdrawal (**Figure 4, panel B**). As such, the decrease in mean GFP expression might be due to promoter silencing or preferential expansion of cells with lower GFP expression at the absence of selective pressure.

Analysis of distribution of integration.

[0122] To test the hypothesis that increased MTX selection pressure would select for cells with multiple integration events, the average number of transposon copy numbers in MTX-selected cell populations was determined using RT-qPCR with GFP primers. First, a "gold standard" clone with a single copy of integrated transposon was generated by limiting dilution method. The average number of integrations in the original stably-transduced population before MTX selection was determined by RT-qPCR analysis of the GFP^+ cells obtained by cell sorting. A trend of increasing average transposon copy number with increasing selection pressure was observed. Attention is drawn to **Figure 5A**, which shows the transposon copy number per human haploid genome. The average integration events in cells selected with 200 nM MTX was 2.1 ± 0.45 compared to an average of 1.1 ± 0.02 integration events in GFP+ cells before MTX selection. RT-qPCR was performed in triplicates and data represents a single biological replica for the sorted population and 3 biological replicas for MTX selection. Statistical difference was assessed by Student's t-test.

[0123] The distribution of integration events in cells selected with 200 nM MTX was then analyzed. Sixty clones were generated by limiting dilution method, GFP expression confirmed by flow cytometry, genomic DNA isolated, and the number of GFP genes per haploid genome analyzed by RT-PCR. The distribution of integration events is shown in **Figure 5B**. Most clones (~65%) contained multiple copies of GFP. The average number of integration events was 1.8 which correlates well with the average transposon copy number in the cell population selected with 200 nM MTX (**Figure 5A**).

Demonstration of multiplexed gene integration.

[0124] Since it was previously demonstrated that a majority of the population of transduced cells amplified under 200 nM MTX selection pressure contained multiple transposon copies, multiplexed gene integration was then assessed under these conditions. H9 cells were nucleofected with three minicircles containing three different reporter genes (maxGFP, mCherry, and BFP) in transposon cassettes and the SB100X transposase minicircle. Stably-transduced cells were then selected for 7 days with 200 nM and cell population assessed by flow cytometry analysis. Attention is drawn to **Figure 6**, which shows the flow cytometric analysis of H9 cell populations nucleofected with 3 minicircles

carrying transposons with different fluorescent proteins (MC_T3/GFP-T2A-DHFRdm, MC_T3/ BFP-T2A-DHFRdm, MC_T3/mCherry-T2A-DHFRdm), 2 μg each and 6 μg of MC_SB100X DNA at different time points after transfection. Initial transfection efficiency assessed 24 hours after nucleofection, was 68% (**Figure 6 panel A)**. The stably transduced population was 37 ± 1.4%, reflecting 54% integration efficiency. Of this population, 19 ± 0.6% expressed two or three different fluorescent proteins. Stably-transduced cells grown for 1 week in the presence of 200 nM MTX were then analyzed; 23 ± 1.0% of this selected population expressed all three reporter proteins (**Figure 6 panel A**). In order to further increase the population of cells expressing triple transgenes, cells selected by 200 nM MTX were subjected to a second selection step with increased MTX concentrations. Attention is drawn to **Figure 7**, which shows a bar graph demonstrating the results of an H9 cell population stably transfected with three transposons selected with 200 nM MTX for a week and then exposed to higher MTX concentrations of 500 and 1000 nM. As shown, cells that were cultured in 500 nM or 1000 nM MTX for an additional week resulting in an increased population (38.5 ± 1.0% and 53.1 ±0.3%, respectively) of cells expressing triple transgenes. Cell viability rebounded to ~70% during the second round of selection due to further selection for overexpression of the DHFRdm gene.

Stable expression of transposon DNA with Sleeping Beauty in T-cells with methotrexate selection.

[0125] Freshly thawed peripheral blood mononuclear cells (PBMCs) were electroporated using Amaxa™ Nucleofector™ Technology. The cells were transfected with 10μg of minicircle GFP (MC_T3/GFP-T2A-DHFRdm) and different amounts of SB100X hyperactive transposase (0, 5, or 10μg). Control cells were transfected with either the non-minicircle pMAXGFP vector (10 ug) or with no DNA. The cells were then stimulated with Miltenyi Transact beads 4 to 6 hours after transfection in the presence of IL-2 and IL-15. The cells were then aliquoted so that there were 400,000 cells per well of a 96-well U-bottomed plate. The cells were treated with methotrexate at 7 days after transfection with 0, 25, 50, or 100 nM of methotrexate. At days 2, 5, 7, 14, and 19, the cells were counted by trypan blue, stained, and analyzed.

[0126] Attention is drawn to **Figure 8** which shows an example of the flow analysis of the lymphocytes expressing GFP after minicircle transfection. Single cells (panel B) from the lymphocyte window (panel A) were analyzed for viability with the Invitrogen LIVE/DEAD red stain (panel C). Live lymphocytes were then analyzed for CD8 and GFP expression (panel D). As shown in **Figure 8** panel D, after selecting with 50 nM methotrexate, the majority of lymphocytes were CD8+ and expressed GFP.

Stable expression of transposon DNA with Sleeping Beauty in T-cells after one week.

[0127] In order to assess the expression of the minicircle DNA in the week before MTX selection, flow analysis was performed and then compared for cells transfected with pMAXGFP, 1:1 ratio of GFP transposon: SB100X, 1:2 ratio of transposon: SB100X, mcGFP alone, or no DNA control. Attention is drawn to **Figure 9**, which shows the results of a FACS assay on cells at two days (in the absence of Transact beads) and five days (in the presence of Transact beads) after electroporation. As shown, there is a loss of GFP expression over time without MTX. However, GFP expression persists in cells transfected with GFP transposon DNA only if there were co-transfected with SB100X transposase.

[0128] Attention is drawn to **Figure 10,** which shows graphs of the levels of GFP expression and cell growth from days 2 to 7. As shown in panel A of **Figure 10,** the amounts of percent GFP expression decreases over time (pMAXGFP (10 ug), mcGFP: MC_SB100X 1:1, and mcGFP: MC_SB100X 2:1). There was a slow increase of live cells in the presence of Transact beads (panel B), but not without the beads (panel C), indicating the importance of the beads for cell growth.

Cell selection with MTX for 7 days and 12 days.

[0129] After 1 week, samples of the transfected cells were exposed to different levels of MTX (25, 50, or 100 nM) to enrich for cells expressing the minicircle transposon. Cells that stably express the DHFRdm MTX-resistance gene as well as GFP due to transposase integration should survive higher MTX concentrations. Attention is drawn to **Figure 11,** which shows the results of a FACS assay of the transfected cells after treatment with 100 nM methotrexate for 7 days. In cells treated with 100 nM MTX, only cells transfected with both transposon and transposase DNA express GFP. As shown, 100 nM

MTX selection was effective with GFP expression at both ratios of mcGFP to SB at a 2:1 mcGFP: MC_SB100X ratio and at a 1:1 mcGFP: MC_SB100X ratio after cell selection with MTX for seven days.

[0130] Attention is drawn to **Figure 12** and **Figure 13** which show the results of a FACS assay of the transfected cells after treatment with methotrexate for 7 and 12 days, respectively. Scatter plots and CD8+/GFP expression for live lymphocytes are shown for each condition. Percent GFP expression in lymphocytes is given in boxes in **Figure 12**.

[0131] As shown in **Figure 12** at 7 days, cells treated with 0 or 25 nM MTX show about 25% or 75% of the cells expressing GFP, respectively. In contrast, at least 90% of the cells express GFP at 50 and 100 nM MTX. As shown, MTX selection was equally effective for enrichment of GFP expression at 50 nM and 100 nM, and at both ratios of mcGFP to SB at a 2:1 mcGFP: MC_SB100X ratio and a 1:1 mcGFP: MC_SB100X ratio. As expected, in the absence of SB transposase and in the no DNA controls there is no appreciable GFP expression. Note that the expression of GFP is similar in CD8+ and CD8- lymphocytes.

Stable expression of transposon DNA with Sleeping Beauty in T-cells with methotrexate selection–cell counts.

[0132] The cell growth of PBMC that stably expressing the transposon DNA under MTX selection, was later assessed. Note that due to stimulation with Transact beads and growth in the presence of IL2 and IL-15, the majority of the surviving cells are T cells by 1 week. As shown in **Figure 14**, the amounts of live cells following treatment with MTX at 0 nM, 25 nM, 50 nM, and 100 nM methotrexate was determined with trypan blue cell counts at days 7, 14, and 19 days (days 0, 7, and 12 of MTX). In the control (0 nM MTX), the number of live cells increased over time for all DNA conditions. However, in the presence of MTX, only the cells that were transfected with both the SB transposase and the minicircle transposon that coexpresses GFP and the DHFRdm resistance gene were able to divide, indicating that SB is required for stable expression of the transposon.

Stable expression of transposon DNA with Sleeping Beauty in T-cells with methotrexate selection- GFP expression.

[0133] The stable expression of transposon DNA with Sleeping Beauty in T-cells during MTX selection was assessed by determining the GFP expression of the transfected

cells over 19 days. Attention is drawn to **Figure 14** which shows increasing GFP expression over time in cells transfected with transposon DNA and Sleeping Beauty in T-cells following methotrexate selection starting at day 7 (0, 25, 50, and 100 nM). As shown in the control with no methotrexate selection from days 2, 5, 7, 14, and 19, the expression of GFP in the cells transfected with mcGFP and SB is maintained at ~20%, while the expression steadily decreases in the mcGFP alone and pMAXGFP controls. In the presence of MTX selection, GFP expression increases over time, with highest levels seen with 50 and 100 nM MTX. As shown, the ratios of mcGFP: MC_SB100X had no difference between a ratio of 1:1 and 2:1. Additionally there was minimal difference in the mean fluorescence intensity in cells that were exposed to either 50 nM or 100nM MTX. The low levels of GFP expression (~20%) with mcGFP alone in the presence of MTX is likely due to transposon-independent stable integration, and the absolute number of cells in these conditions is very low as shown in **Figure 14**.

[0134] In one alternative, a gene delivery polynucleotide for stable insertion of a nucleic acid into a gene, wherein the nucleic acid for insertion is flanked by inverted terminal repeat gene sequences in the gene delivery polynucleotide and wherein the gene delivery polynucleotide is selectable is provided, wherein the gene delivery polynucleotide comprises a first sequence, wherein the first sequence comprises a first inverted terminal repeat gene sequence, a second sequence, wherein the second sequence comprises a second inverted terminal repeat gene sequence, a third sequence, wherein the third sequence comprises a promoter region sequence, a fourth sequence, wherein the fourth sequence comprises at least one gene encoding a protein, and wherein the fourth sequence is optimized, a fifth sequence, wherein the fifth sequence comprises at least one selectable marker cassette encoding a double mutant of dihydrofolate reductase, wherein the double mutant of dihydrofolate reductase has a 15,000 fold or about 15,000 fold reduced affinity for methotrexate, wherein the methotrexate can be used as a selection mechanism to selectively amplify cells transduced with the gene delivery polynucleotide and wherein the fifth sequence is optimized, a sixth sequence, wherein the sixth sequence comprises a first attachment site (attP) and a seventh sequence, wherein the seventh sequence comprises a second attachment site (attB) wherein each of the first sequence, second sequence, third sequence, fourth sequence, fifth sequence, sixth sequence, and seventh sequence have a 5' terminus and a 3 '

terminus, and wherein the 3' terminus of the first sequence comprising the first inverted terminal repeat gene sequence is adjacent to the 5' terminus of the third sequence, the 3' terminus of the third sequence is adjacent to the 5' terminus of the fourth sequence, the 3' terminus of the fourth sequence is adjacent to the 5' terminus of the fifth sequence and the 3' terminus of the fifth sequence is adjacent to the 5' terminus of the second sequence comprising a second inverted terminal repeat. In some alternatives, the gene encoding the double mutant of human dihydrofolate reductase comprises the DNA sequence: ATGGTTGGTTCGCTAAACTGCATCGTCGCTGTGTCCCAGAACATGGGCATCGGCA AGAACGGGGACTTCCCCTGGCCACCGCTCAGGAATGAATCCAGATATTTCCAGA GAATGACCACAACCTCTTCAGTAGAAGGTAAACAGAATCTGGTGATTATGGGTA AGAAGACCTGGTTCTCCATTCCTGAGAAGAATCGACCTTTAAAGGGTAGAATTA ATTTAGTTCTCAGCAGAGAACTCAAGGAACCTCCACAAGGAGCTCATTTTCTTTC CAGAAGTCTAGATGATGCCTTAAAACTTACTGAACAACCAGAATTAGCAAATAA AGTAGACATGGTCTGGATAGTTGGTGGCAGTTCTGTTTATAAGGAAGCCATGAAT CACCCAGGCCATCTTAAACTATTTGTGACAAGGATCATGCAAGACTTTGAAAGTG ACACGTTTTTTCCAGAAATTGATTTGGAGAAATATAAACTTCTGCCAGAATACCC AGGTGTTCTCTCTGATGTCCAGGAGGAGAAAGGCATTAAGTACAAATTTGAAGT ATATGAGAAGAATGATTAA (SEQ ID NO: 2). In some alternatives, the double mutant of human dihydrofolate reductase comprises the protein sequence: MVGSLNCIVA VSQNMGIGKN GDFPWPPLRN ESRYFQRMTT TSSVEGKQNL VIMGKKTWFS IPEKNRPLKG RINLVLSREL KEPPQGAHFL SRSLDDALKL TEQPELANKV DMVWIVGGSS VYKEAMNHPG HLKLFVTRIM QDFESDTFFP EIDLEKYKLL PEYPGVLSDV QEEKGIKYKF EVYEKND (SEQ ID NO: 3). In some alternatives, the gene delivery polynucleotide is circular. In some alternatives, the gene delivery polynucleotide is at least 1kB to 5kB. In some alternatives, the gene delivery polynucleotide is a minicircle. In some alternatives, the gene delivery polynucleotide is a minicircle. In some alternatives, the promoter region comprises an EF1 promoter sequence. In some alternatives, the fourth sequence comprises one, two, three, four, or five genes that encode proteins. In some alternatives, the fourth sequence is codon optimized to reduce the total GC/AT ratio of the fourth sequence. In some alternatives, the fourth sequence is optimized by codon optimization for expression in humans. In some alternatives, the fourth sequence is a

consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins, such as a plurality of antibody binding domains, which are specific for the same epitope. In some alternatives, the plurality of related proteins comprise a plurality of antibody binding domains, wherein the plurality of antibody binding domains are specific for the same epitope. In some alternatives, the fifth sequence is codon optimized to reduce the total GC/AT ratio of the fifth sequence. In some alternatives, the fifth sequence is optimized by codon optimization for expression in humans. In some alternatives, the protein is a protein for therapy. In some alternatives, the codon optimization and/or consensus sequence is generated by comparing the variability of sequence and/or nucleobases utilized in a plurality of related sequences. In some alternatives, the protein comprises an antibody or a portion thereof, which may be humanized. In some alternatives, the double mutant of dihydrofolate reductase comprises amino acid mutations of L22F and F31S.

[0135] In some alternatives, a method of generating engineered multiplexed T-cells for adoptive T-cell immunotherapy is provided, wherein the method comprises providing a gene delivery polynucleotide as described herein, introducing the gene delivery polynucleotide into a T-cell, providing a vector encoding a Sleeping Beauty transposase, introducing the vector encoding the Sleeping Beauty transposase into the T-cell, selecting the cells comprising the gene delivery polynucleotide wherein selecting comprises a first round of selection and a second round of selection, wherein the first round of selection comprises adding a selection reagent at a first concentration range and the second round of selection comprises adding the selection reagent at a second concentration range, wherein the second concentration range is higher than the first concentration range and, wherein the second concentration range is at least 1.5 fold higher than that of the first concentration range and isolating the T-cells expressing a phenotype under selective pressure. In some alternatives, the gene delivery polynucleotide comprises a first sequence, wherein the first sequence comprises a first inverted terminal repeat gene sequence, a second sequence, wherein the second sequence comprises a second inverted terminal repeat gene sequence, a third sequence, wherein the third sequence comprises a promoter region sequence, a fourth sequence, wherein the fourth sequence comprises at least one gene encoding a protein, and

wherein the fourth sequence is optimized, a fifth sequence, wherein the fifth sequence comprises at least one selectable marker cassette encoding a double mutant of dihydrofolate reductase, wherein the double mutant of dihydrofolate reductase has a 15,000 fold or about 15,000 fold reduced affinity for methotrexate, wherein the methotrexate can be used as a selection mechanism to selectively amplify cells transduced with the gene delivery polynucleotide and wherein the fifth sequence is optimized, a sixth sequence, wherein the sixth sequence comprises a first attachment site (attP) and a seventh sequence, wherein the seventh sequence comprises a second attachment site (attB) wherein each of the first sequence, second sequence, third sequence, fourth sequence, fifth sequence, sixth sequence, and seventh sequence have a 5' terminus and a 3 ' terminus, and wherein the 3' terminus of the first sequence comprising the first inverted terminal repeat gene sequence is adjacent to the 5' terminus of the third sequence, the 3' terminus of the third sequence is adjacent to the 5' terminus of the fourth sequence, the 3' terminus of the fourth sequence is adjacent to the 5' terminus of the fifth sequence and the 3' terminus of the fifth sequence is adjacent to the 5' terminus of the second sequence comprising a second inverted terminal repeat. In some alternatives, the gene encoding the double mutant of human dihydrofolate reductase comprises the DNA sequence: ATGGTTGGTTCGCTAAACTGCATCGTCGCTGTGTCCCAGAACATGGGCATCGGCA AGAACGGGGACTTCCCCTGGCCACCGCTCAGGAATGAATCCAGATATTTCCAGA GAATGACCACAACCTCTTCAGTAGAAGGTAAACAGAATCTGGTGATTATGGGTA AGAAGACCTGGTTCTCCATTCCTGAGAAGAATCGACCTTTAAAGGGTAGAATTA ATTTAGTTCTCAGCAGAGAACTCAAGGAACCTCCACAAGGAGCTCATTTTCTTTC CAGAAGTCTAGATGATGCCTTAAAACTTACTGAACAACCAGAATTAGCAAATAA AGTAGACATGGTCTGGATAGTTGGTGGCAGTTCTGTTTATAAGGAAGCCATGAAT CACCCAGGCCATCTTAAACTATTTGTGACAAGGATCATGCAAGACTTTGAAAGTG ACACGTTTTTTCCAGAAATTGATTTGGAGAAATATAAACTTCTGCCAGAATACCC AGGTGTTCTCTCTGATGTCCAGGAGGAGAAAGGCATTAAGTACAAATTTGAAGT ATATGAGAAGAATGATTAA (SEQ ID NO: 2). In some alternatives, the double mutant of human dihydrofolate reductase comprises the protein sequence: MVGSLNCIVA VSQNMGIGKN GDFPWPPLRN ESRYFQRMTT TSSVEGKQNL VIMGKKTWFS IPEKNRPLKG RINLVLSREL KEPPQGAHFL SRSLDDALKL TEQPELANKV

DMVWIVGGSS VYKEAMNHPG HLKLFVTRIM QDFESDTFFP EIDLEKYKLL PEYPGVLSDV QEEKGIKYKF EVYEKND (SEQ ID NO: 3). In some alternatives, the gene delivery polynucleotide is circular. In some alternatives, the gene delivery polynucleotide is at least 1kB to 5kB. In some alternatives, the gene delivery polynucleotide is a minicircle. In some alternatives, the promoter region comprises an EF1 promoter sequence. In some alternatives, the fourth sequence comprises one, two, three, four, or five genes that encode proteins. In some alternatives, the fourth sequence is codon optimized to reduce the total GC/AT ratio of the fourth sequence. In some alternatives, the fourth sequence is optimized by codon optimization for expression in humans. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins, such as a plurality of antibody binding domains, which are specific for the same epitope. In some alternatives, the plurality of related proteins comprise a plurality of antibody binding domains, wherein the plurality of antibody binding domains are specific for the same epitope. In some alternatives, the fifth sequence is codon optimized to reduce the total GC/AT ratio of the fifth sequence. In some alternatives, the fifth sequence is optimized by codon optimization for expression in humans. In some alternatives, the protein is a protein for therapy. In some alternatives, the codon optimization and/or consensus sequence is generated by comparing the variability of sequence and/or nucleobases utilized in a plurality of related sequences. In some alternatives, the protein comprises an antibody or a portion thereof, which may be humanized. In some alternatives, the double mutant of dihydrofolate reductase comprises amino acid mutations of L22F and F31S. In some alternatives, the introducing is performed by electroporation. In some alternatives, the selecting is performed by increasing selective pressure through the selective marker cassette. In some alternatives, the selection reagent comprises an agent for selection. In some alternatives, the agent for selection is methotrexate. In some alternatives, the first concentration range is at least 50nM – 100nM and the second concentration range is at least 75 to 150nM. In some alternatives, the first concentration is 50 nM, 60 nM, 70 nM, 80 nM, 90 nM, or 100nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 75 nM, 80 nM, 90 nM, 100 nM, 110 nM, 120 nM, 130 nM, 140 nM,

or 150 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first concentration range is at least 75 nM – 150 nM and the second concentration range is at least 112.5 nM to 225 nM. In some alternatives, the first concentration is 75 nM, 85 nM, 95 nM, 105 nM, 115 nM, 125 nM, 135 nM, 145 nM, or 150 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 112 nM, 122 nM, 132 nM, 142 nM, 152 nM, 162 nM, 172 nM, 182 nM, 192 nM, 202 nM, 212 nM, or 225 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first concentration range is at least 300 nM – 675 nM and the first concentration range is at least 450 nM to 1012 nM. In some alternatives, the first concentration is 300 nM, 350 nM, 400 nM, 450 nM, 500 nM, 550 nM, 600 nM, 650 nM, or 675 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 450 nM, 500 nM, 550 nM, 600 nM, 650 nM, 700 nM, 750 nM, 800 nM, 850 nM, 900 nM, 1000 nM, or 1012 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first round of selection comprises exposing the T-cells to the selection agent for 2, 3, 4, 5, 6 or 7 days before the second round of selection. In some alternatives, the second round of selection comprises exposing the T-cells to the selection agent for at least 2, 3, 4, 5, 6,7, 8, 9, 10, 11, 12, 13, or 14 days or any time that is between a range of times defined by any two of the aforementioned time points before isolation.

[0136] In some alternatives, a method of increasing protein production in a T-cell is provided, wherein the method comprises providing a polynucleotide described herein, introducing the polynucleotide into a cell, providing a vector encoding a Sleeping Beauty transposase, introducing the vector encoding the Sleeping Beauty transposase into the T-cell, selecting the cells comprising the gene delivery polynucleotide wherein selecting comprises a first round of selection and a second round of selection, wherein the first round of selection comprises adding a selection reagent at a first concentration range and the second round of selection comprises adding the selection reagent at a second concentration range, wherein the second concentration range is higher than the first concentration range and, wherein the

second concentration range is at least 1.5 fold higher than that of the first concentration range and isolating the cells expressing a phenotype under selective pressure. In some alternatives, the introducing is performed by electroporation. In some alternatives, the selecting is performed by increasing selective pressure through the selective marker cassette. In some alternatives, the selection reagent comprises an agent for selection. In some alternatives, the agent for selection is methotrexate. In some alternatives, the first concentration range is at least 50nM – 100nM and the second concentration range is at least 75 to 150nM. In some alternatives, the first concentration is 50 nM, 60 nM, 70 nM, 80 nM, 90 nM, or 100nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 75 nM, 80 nM, 90 nM, 100 nM, 110 nM, 120 nM, 130 nM, 140 nM, or 150 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first concentration range is at least 75 nM – 150 nM and the second concentration range is at least 112.5 nM to 225 nM. In some alternatives, the first concentration is 75 nM, 85 nM, 95 nM, 105 nM, 115 nM, 125 nM, 135 nM, 145 nM, or 150 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 112 nM, 122 nM, 132 nM, 142 nM, 152 nM, 162 nM, 172 nM, 182 nM, 192 nM, 202 nM, 212 nM, or 225 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first concentration range is at least 300 nM – 675 nM and the first concentration range is at least 450 nM to 1012 nM. In some alternatives, the first concentration is 300 nM, 350 nM, 400 nM, 450 nM, 500 nM, 550 nM, 600 nM, 650 nM, or 675 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 450 nM, 500 nM, 550 nM, 600 nM, 650 nM, 700 nM, 750 nM, 800 nM, 850 nM, 900 nM, 1000 nM, or 1012 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first round of selection comprises exposing the T-cells to the selection agent for 2, 3, 4, 5, 6 or 7 days before the second round of selection. In some alternatives, the second round of selection comprises exposing the T-cells to the selection agent for at least 2, 3, 4, 5, 6,7, 8, 9, 10, 11,

12, 13, or 14 days or any time that is between a range of times defined by any two of the aforementioned time points before isolation.

[0137] In some alternatives, the gene delivery polynucleotide comprises a first sequence, wherein the first sequence comprises a first inverted terminal repeat gene sequence, a second sequence, wherein the second sequence comprises a second inverted terminal repeat gene sequence, a third sequence, wherein the third sequence comprises a promoter region sequence, a fourth sequence, wherein the fourth sequence comprises at least one gene encoding a protein, and wherein the fourth sequence is optimized, a fifth sequence, wherein the fifth sequence comprises at least one selectable marker cassette encoding a double mutant of dihydrofolate reductase, wherein the double mutant of dihydrofolate reductase has a 15,000 fold or about 15,000 fold reduced affinity for methotrexate, wherein the methotrexate can be used as a selection mechanism to selectively amplify cells transduced with the gene delivery polynucleotide and wherein the fifth sequence is optimized, a sixth sequence, wherein the sixth sequence comprises a first attachment site (attP) and a seventh sequence, wherein the seventh sequence comprises a second attachment site (attB) wherein each of the first sequence, second sequence, third sequence, fourth sequence, fifth sequence, sixth sequence, and seventh sequence have a 5' terminus and a 3 ' terminus, and wherein the 3' terminus of the first sequence comprising the first inverted terminal repeat gene sequence is adjacent to the 5' terminus of the third sequence, the 3' terminus of the third sequence is adjacent to the 5' terminus of the fourth sequence, the 3' terminus of the fourth sequence is adjacent to the 5' terminus of the fifth sequence and the 3' terminus of the fifth sequence is adjacent to the 5' terminus of the second sequence comprising a second inverted terminal repeat. In some alternatives, the gene encoding the double mutant of human dihydrofolate reductase comprises the DNA sequence:
ATGGTTGGTTCGCTAAACTGCATCGTCGCTGTGTCCCAGAACATGGGCATCGGCA
AGAACGGGGACTTCCCCTGGCCACCGCTCAGGAATGAATCCAGATATTTCCAGA
GAATGACCACAACCTCTTCAGTAGAAGGTAAACAGAATCTGGTGATTATGGGTA
AGAAGACCTGGTTCTCCATTCCTGAGAAGAATCGACCTTTAAAGGGTAGAATTA
ATTTAGTTCTCAGCAGAGAACTCAAGGAACCTCCACAAGGAGCTCATTTTCTTTC
CAGAAGTCTAGATGATGCCTTAAAACTTACTGAACAACCAGAATTAGCAAATAA
AGTAGACATGGTCTGGATAGTTGGTGGCAGTTCTGTTTATAAGGAAGCCATGAAT

CACCCAGGCCATCTTAAACTATTTGTGACAAGGATCATGCAAGACTTTGAAAGTG ACACGTTTTTTCCAGAAATTGATTTGGAGAAATATAAACTTCTGCCAGAATACCC AGGTGTTCTCTCTGATGTCCAGGAGGAGAAAGGCATTAAGTACAAATTTGAAGT ATATGAGAAGAATGATTAA (SEQ ID NO: 2). In some alternatives, the double mutant of human dihydrofolate reductase comprises the protein sequence: MVGSLNCIVA VSQNMGIGKN GDFPWPPLRN ESRYFQRMTT TSSVEGKQNL VIMGKKTWFS IPEKNRPLKG RINLVLSREL KEPPQGAHFL SRSLDDALKL TEQPELANKV DMVWIVGGSS VYKEAMNHPG HLKLFVTRIM QDFESDTFFP EIDLEKYKLL PEYPGVLSDV QEEKGIKYKF EVYEKND (SEQ ID NO: 3). In some alternatives, the gene delivery polynucleotide is circular. In some alternatives, the gene delivery polynucleotide is at least 1kB to 5kB. In some alternatives, the gene delivery polynucleotide is a minicircle. In some alternatives, the gene delivery polynucleotide is a minicircle. In some alternatives, the promoter region comprises an EF1 promoter sequence. In some alternatives, the fourth sequence comprises one, two, three, four, or five genes that encode proteins. In some alternatives, the fourth sequence is codon optimized to reduce the total GC/AT ratio of the fourth sequence. In some alternatives, the fourth sequence is optimized by codon optimization for expression in humans. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins. In some alternatives, the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins, such as a plurality of antibody binding domains, which are specific for the same epitope. In some alternatives, the plurality of related proteins comprise a plurality of antibody binding domains, wherein the plurality of antibody binding domains are specific for the same epitope. In some alternatives, the fifth sequence is codon optimized to reduce the total GC/AT ratio of the fifth sequence. In some alternatives, the fifth sequence is optimized by codon optimization for expression in humans. In some alternatives, the codon optimization and/or consensus sequence is generated by comparing the variability of sequence and/or nucleobases utilized in a plurality of related sequences. In some alternatives, the protein comprises an antibody or a portion thereof, which may be humanized. In some alternatives, the double mutant of dihydrofolate reductase comprises amino acid mutations of L22F and F31S. In some alternatives, the introducing is performed by electroporation. In some alternatives, the

selecting is performed by increasing selective pressure through the selective marker cassette. In some alternatives, the selection reagent comprises an agent for selection. In some alternatives, the agent for selection is methotrexate. In some alternatives, the first concentration range is at least 50nM – 100nM and the second concentration range is at least 75 to 150nM. In some alternatives, the first concentration is 50 nM, 60 nM, 70 nM, 80 nM, 90 nM, or 100nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 75 nM, 80 nM, 90 nM, 100 nM, 110 nM, 120 nM, 130 nM, 140 nM, or 150 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first concentration range is at least 75 nM – 150 nM and the second concentration range is at least 112.5 nM to 225 nM. In some alternatives, the first concentration is 75 nM, 85 nM, 95 nM, 105 nM, 115 nM, 125 nM, 135 nM, 145 nM, or 150 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 112 nM, 122 nM, 132 nM, 142 nM, 152 nM, 162 nM, 172 nM, 182 nM, 192 nM, 202 nM, 212 nM, or 225 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first concentration range is at least 300 nM – 675 nM and the first concentration range is at least 450 nM to 1012 nM. In some alternatives, the first concentration is 300 nM, 350 nM, 400 nM, 450 nM, 500 nM, 550 nM, 600 nM, 650 nM, or 675 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations, and the second concentration range is 450 nM, 500 nM, 550 nM, 600 nM, 650 nM, 700 nM, 750 nM, 800 nM, 850 nM, 900 nM, 1000 nM, or 1012 nM or any concentration that is between a range of concentrations defined by any two of the aforementioned concentrations. In some alternatives, the first round of selection comprises exposing the T-cells to the selection agent for 2, 3, 4, 5, 6 or 7 days before the second round of selection. In some alternatives, the second round of selection comprises exposing the T-cells to the selection agent for at least 2, 3, 4, 5, 6,7, 8, 9, 10, 11, 12, 13, or 14 days or any time that is between a range of times defined by any two of the aforementioned time points before isolation.

[0138] In some alternatives, a method of treating, inhibiting, or ameliorating cancer or a disease in a subject, the method comprising administering to the subject a modified T-cell as described herein. In some alternatives, the subject is human.

[0139] With respect to the use of substantially any plural and/or singular terms herein, those having skill in the art can translate from the plural to the singular and/or from the singular to the plural as is appropriate to the context and/or application. The various singular/plural permutations may be expressly set forth herein for sake of clarity.

[0140] It will be understood by those within the art that, in general, terms used herein, and especially in the appended claims (*e.g.*, bodies of the appended claims) are generally intended as "open" terms (*e.g.*, the term "including" should be interpreted as "including but not limited to," the term "having" should be interpreted as "having at least," the term "includes" should be interpreted as "includes but is not limited to," etc.). It will be further understood by those within the art that if a specific number of an introduced claim recitation is intended, such an intent will be explicitly recited in the claim, and in the absence of such recitation no such intent is present. For example, as an aid to understanding, the following appended claims may contain usage of the introductory phrases "at least one" and "one or more" to introduce claim recitations. However, the use of such phrases should not be construed to imply that the introduction of a claim recitation by the indefinite articles "a" or "an" limits any particular claim containing such introduced claim recitation to embodiments containing only one such recitation, even when the same claim includes the introductory phrases "one or more" or "at least one" and indefinite articles such as "a" or "an" (*e.g.*, "a" and/or "an" should be interpreted to mean "at least one" or "one or more"); the same holds true for the use of definite articles used to introduce claim recitations. In addition, even if a specific number of an introduced claim recitation is explicitly recited, those skilled in the art will recognize that such recitation should be interpreted to mean at least the recited number (*e.g.*, the bare recitation of "two recitations," without other modifiers, means at least two recitations, or two or more recitations). Furthermore, in those instances where a convention analogous to "at least one of A, B, and C, etc." is used, in general such a construction is intended in the sense one having skill in the art would understand the convention (*e.g.*, " a system having at least one of A, B, and C" would include but not be limited to systems that have A alone, B alone, C alone, A and B together, A and C together, B and C together,

and/or A, B, and C together, etc.). In those instances where a convention analogous to "at least one of A, B, or C, etc." is used, in general such a construction is intended in the sense one having skill in the art would understand the convention (*e.g.*, " a system having at least one of A, B, or C" would include but not be limited to systems that have A alone, B alone, C alone, A and B together, A and C together, B and C together, and/or A, B, and C together, etc.). It will be further understood by those within the art that virtually any disjunctive word and/or phrase presenting two or more alternative terms, whether in the description, claims, or drawings, should be understood to contemplate the possibilities of including one of the terms, either of the terms, or both terms. For example, the phrase "A or B" will be understood to include the possibilities of "A" or "B" or "A and B."

[0141] In addition, where features or aspects of the disclosure are described in terms of Markush groups, those skilled in the art will recognize that the disclosure is also thereby described in terms of any individual member or subgroup of members of the Markush group

WHAT IS CLAIMED IS:

1. A gene delivery polynucleotide for stable insertion of a nucleic acid into an oligonucleotide, wherein the nucleic acid for insertion is flanked by inverted terminal repeat gene sequences in the gene delivery polynucleotide and, wherein the gene delivery polynucleotide is selectable, the gene delivery polynucleotide comprising:

a first sequence, wherein the first sequence comprises a first inverted terminal repeat gene sequence,

a second sequence, wherein the second sequence comprises a second inverted terminal repeat gene sequence;

a third sequence, wherein the third sequence comprises a promoter region sequence;

a fourth sequence, wherein the fourth sequence comprises at least one gene, wherein the at least one gene encodes a protein or encodes a sequence for mRNA transcription, and wherein the fourth sequence is optimized;

a fifth sequence, wherein the fifth sequence comprises at least one selectable marker cassette encoding a double mutant of dihydrofolate reductase, wherein the double mutant of dihydrofolate reductase has a 15,000 fold or about 15,000 fold reduced affinity for methotrexate, wherein the methotrexate can be used - to select for cells transduced with the gene delivery polynucleotide to enhance the ratio of cells expressing the at least one gene and wherein the fifth sequence is optimized;

a sixth sequence, wherein the sixth sequence comprises a first attachment site (attP); and

a seventh sequence, wherein the seventh sequence comprises a second attachment site (attB); wherein each of the first sequence, second sequence, third sequence, fourth sequence, fifth sequence, sixth sequence, and seventh sequence have a 5' terminus and a 3 ' terminus, and wherein the 3' terminus of the first sequence comprising the first inverted terminal repeat gene sequence is adjacent to the 5' terminus of the third sequence, the 3' terminus of the third sequence is adjacent to the 5' terminus of the fourth sequence, the 3' terminus of the fourth sequence is adjacent to the 5' terminus of the fifth sequence and the 3' terminus of the fifth sequence is adjacent to the 5' terminus of the second sequence comprising a second inverted terminal repeat.

2. The gene delivery polynucleotide of claim 1, wherein the gene delivery polynucleotide is circular.

3. The gene delivery polynucleotide of claim 1 or 2, wherein the gene delivery polynucleotide is at least 1kB to 5kB.

4. The gene delivery polynucleotide of any one of claims 1–3, wherein the promoter region comprises an EF1 promoter sequence.

5. The gene delivery polynucleotide of any one of claims 1–4, wherein the fourth sequence comprises one, two, three, four, or five genes that encode proteins.

6. The gene delivery polynucleotide of any one of claims 1–5, wherein the fourth sequence is codon optimized to reduce the total GC/AT ratio of the fourth sequence.

7. The gene delivery polynucleotide of any one of claims 1–5, wherein the fourth sequence is optimized by codon optimization for expression in humans.

8. The gene delivery polynucleotide of any one of claims 1–7, wherein the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins.

9. The gene delivery polynucleotide of claims 1-8, wherein the fourth sequence is a consensus sequence generated from a plurality of nucleic acids that encode a plurality of related proteins, such as a plurality of antibody binding domains, which are specific for the same epitope.

10. The gene delivery polynucleotide of any one of claims 8-9, wherein the plurality of related proteins comprise a plurality of antibody binding domains, wherein the plurality of antibody binding domains are specific for the same epitope.

11. The gene delivery polynucleotide of any one of claims 1–10, wherein the fifth sequence is codon optimized to reduce the total GC/AT ratio of the fifth sequence.

12. The gene delivery polynucleotide of any one of claims 1–10, wherein the fifth sequence is optimized by codon optimization for expression in humans.

13. The gene delivery polynucleotide of any one of claims 6-12, wherein the codon optimization and/or consensus sequence is generated by comparing the variability of sequence and/or nucleobases utilized in a plurality of related sequences.

14. The gene delivery polynucleotide of any one of claims 1-13, wherein the protein is a protein for therapy.

15. The gene delivery polynucleotide of any one of claims 1–14, wherein the protein comprises an antibody or a portion thereof, which may be humanized.

16. The gene delivery polynucleotide any one of claims 1–15, wherein the double mutant of dihydrofolate reductase comprises amino acid mutations of L22F and F31S.

17. A method of generating engineered multiplexed T-cells for adoptive T-cell immunotherapy comprising,

providing the gene delivery polynucleotide of any one of claims 1-16 or 40;

introducing the gene delivery polynucleotide into a T-cell;

providing a vector encoding a Sleeping Beauty transposase;

introducing the vector encoding the Sleeping Beauty transposase into the T-cell;

selecting the cells comprising the gene delivery polynucleotide; wherein selecting comprises a first round of selection and a second round of selection, wherein the first round of selection comprises adding a selection reagent at a first concentration range and the second round of selection comprises adding the selection reagent at a second concentration range, wherein the second concentration range is higher than the first concentration range and, wherein the second concentration range is at least 1.5 fold higher than that of the first concentration range; and

isolating the T-cells expressing a phenotype under selective pressure.

18. The method of claim 17, wherein the introducing is performed by electroporation.

19. The method of claim 17 or 18, wherein the selecting is performed by increasing selective pressure through the selective marker cassette.

20. The method of any one of claims 17-19, wherein the selection reagent comprises an agent for selection.

21. The method of claim 20, wherein the agent for selection is methotrexate.

22. The method of any one of claims 17-21, wherein the first concentration range is at least 50nM – 100nM and the second concentration range is at least 75 to 150nM.

23. The method of any one of claims 17-21, wherein the first concentration range is at least 75 nM – 150 nM and the second concentration range is at least 112.5 nM to 225 nM.

24. The method of any one of claims 17-21, wherein the first concentration range is at least 300 nM – 675 nM and the first concentration range is at least 450 nM to 1012 nM.

25. The method of any one of claims 17-24, wherein the first round of selection comprises exposing the T-cells to the selection agent for 2, 3, 4, 5, 6 or 7 days before the second round of selection.

26. The method of any one of claims 17-25, wherein the second round of selection comprises exposing the T-cells to the selection agent for at least 2, 3, 4, 5, 6,7, 8, 9, 10, 11, 12, 13, or 14 days or any time that is between a range of times defined by any two of the aforementioned time points before isolation.

27. A method of increasing protein production in a T-cell comprising:

providing the polynucleotide of any one of claims 1-16 or 40;

introducing the polynucleotide into a cell;

providing a vector encoding a Sleeping Beauty transposase;

introducing the vector encoding the Sleeping Beauty transposase into the T-cell;

selecting the cells comprising the gene delivery polynucleotide; wherein selecting comprises a first round of selection and a second round of selection, wherein the first round of selection comprises adding a selection reagent at a first concentration range and the second round of selection comprises adding the selection reagent at a second concentration range, wherein the second concentration range is higher than the first concentration range and, wherein the second concentration range is at least 1.5 fold higher than that of the first concentration range; and

isolating the cells expressing a phenotype under selective pressure.

28. The method of claim 27, wherein the introducing is performed by electroporation.

29. The method of claim 27 or 28, wherein the selecting is performed by increasing selective pressure through the selective marker cassette.

30. The method of any one of claims 27-29, wherein the selection reagent comprises an agent for selection.

31. The method of claim 30, wherein the agent for selection is methotrexate.

32. The method of any one of claims 27-31, wherein the first concentration range is at least 50nM – 100 nM and the second concentration range is at least 75 to 150 nM.

33. The method of any one of claims 27-31, wherein the first concentration range is at least 75nM – 150nM and the second concentration range is at least 112.5 nM to 225 nM.

34. The method of any one of claims 27-31, wherein the first concentration range is at least 300 nM – 675 nM and the second concentration range is at least 450 nM to 1012 nM.

35. The method of any one of claims 27-34, wherein the first round of selection comprises exposing the cells to the selection agent for 2, 3, 4, 5, 6 or 7 days before the second round of selection.

36. The method of any one of claims 27-35, wherein the second round of selection comprises exposing the cells to the selection agent for at least 2, 3, 4, 5, 6, or 7 days before isolation.

37. An engineered multiplexed T-cells for adoptive T-cell immunotherapy generated by any one of the methods of Claims 16-36 or 63-64.

38. A method of treating, inhibiting, or ameliorating cancer or a disease in a subject, the method comprising:

administering to the subject the modified T-cell of claims 37.

39. The method of claim 38, wherein the subject is human.

40. The gene delivery polynucleotide of any one of claims 1–16 wherein the gene delivery polynucleotide is a minicircle.

41. A method of generating engineered cells for adoptive T-cell immunotherapy comprising:

providing the gene delivery polynucleotide of any one of claims 1-16 or 40;

introducing the gene delivery polynucleotide into a precursor T cell;

providing a vector encoding a Sleeping Beauty transposase;

introducing the vector encoding the Sleeping Beauty transposase into the precursor T cell;

selecting the precursor T cells comprising the gene delivery polynucleotide; wherein selecting comprises a first round of selection and a second round of selection, wherein the first round of selection comprises adding a selection reagent at a first concentration range and

the second round of selection comprises adding the selection reagent at a second concentration range, wherein the second concentration range is higher than the first concentration range and, wherein the second concentration range is at least 1.5 fold higher than that of the first concentration range; and

isolating the precursor T-cells expressing a phenotype under selective pressure.

42. The method of claim 41, wherein the introducing is performed by electroporation.

43. The method of claim 41 or 42, wherein the selecting is performed by increasing selective pressure through the selective marker cassette.

44. The method of any one of claims 41-43, wherein the selection reagent comprises an agent for selection.

45. The method of claim 44, wherein the agent for selection is methotrexate.

46. The method of any one of claims 41-45, wherein the first concentration range is at least 50nM – 100nM and the second concentration range is at least 75 to 150nM.

47. The method of any one of claims 41-45, wherein the first concentration range is at least 75 nM – 150 nM and the second concentration range is at least 112.5 nM to 225 nM.

48. The method of any one of claims 41-45, wherein the first concentration range is at least 300 nM – 675 nM and the first concentration range is at least 450 nM to 1012 nM.

49. The method of any one of claims 41-48, wherein the first round of selection comprises exposing the T-cells to the selection agent for 2, 3, 4, 5, 6 or 7 days before the second round of selection.

50. The method of any one of claims 41-49, wherein the second round of selection comprises exposing the precursor T-cells to the selection agent for at least 2, 3, 4, 5, 6,7, 8, 9, 10, 11, 12, 13, or 14 days or any time that is between a range of times defined by any two of the aforementioned time points before isolation.

51. The method of any one of claims 41-50, wherein the T cell precursor is a hematopoietic stem cell.

52. A method of increasing protein production in a precursor T-cell comprising:

providing the polynucleotide of any one of claims 1-16 or 40;

introducing the polynucleotide into a precursor T cell;

providing a vector encoding a Sleeping Beauty transposase;

introducing the vector encoding the Sleeping Beauty transposase into the precursor T-cell;

selecting the precursor T cells comprising the gene delivery polynucleotide; wherein selecting comprises a first round of selection and a second round of selection, wherein the first round of selection comprises adding a selection reagent at a first concentration range and the second round of selection comprises adding the selection reagent at a second concentration range, wherein the second concentration range is higher than the first concentration range and, wherein the second concentration range is at least 1.5 fold higher than that of the first concentration range; and

isolating the precursor T cells expressing a phenotype under selective pressure.

53. The method of claim 52, wherein the introducing is performed by electroporation.

54. The method of claim 53 or 54, wherein the selecting is performed by increasing selective pressure through the selective marker cassette.

55. The method of any one of claims 52-24, wherein the selection reagent comprises an agent for selection.

56. The method of claim 55, wherein the agent for selection is methotrexate.

57. The method of any one of claims 52-56, wherein the first concentration range is at least 50nM – 100 nM and the second concentration range is at least 75 to 150 nM.

58. The method of any one of claims 52-56, wherein the first concentration range is at least 75nM – 150nM and the second concentration range is at least 112.5 nM to 225 nM.

59. The method of any one of claims 52-56, wherein the first concentration range is at least 300 nM – 675 nM and the second concentration range is at least 450 nM to 1012 nM.

60. The method of any one of claims 52-59, wherein the first round of selection comprises exposing the cells to the selection agent for 2, 3, 4, 5, 6 or 7 days before the second round of selection.

61. The method of any one of claims 52-60, wherein the second round of selection comprises exposing the cells to the selection agent for at least 2, 3, 4, 5, 6, or 7 days before isolation.

62. The method of any one of claims 52-61, wherein the precursor T cells are hematopoietic stem cells.

63. The method of any one of claims 17-36, wherein the T cells are precursor T cells.

64. The method of claim 63, wherein the precursor T cells are hematopoietic stem cells.

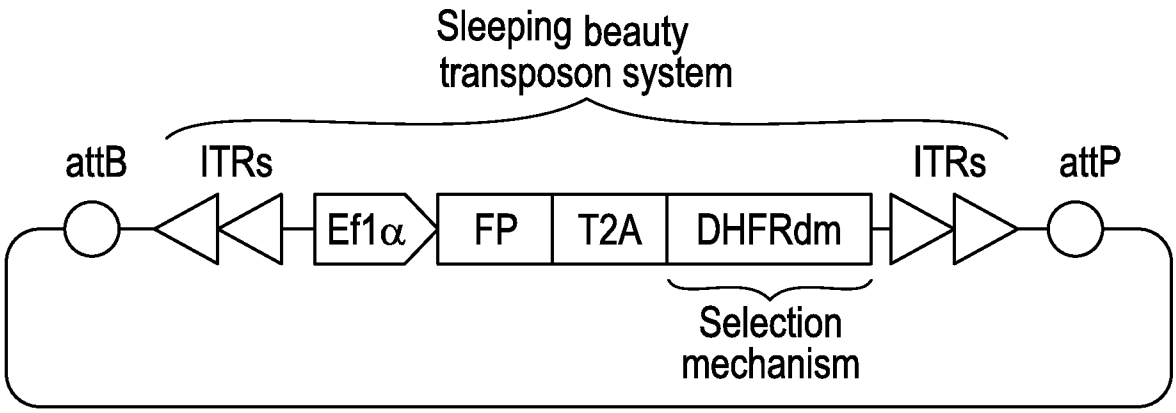

Sleeping beauty
transposon system
attB
ITRs
Ef1α
FP
T2A
DHFRdm
ITRs
attP
Selection
mechanism

FIG. 1

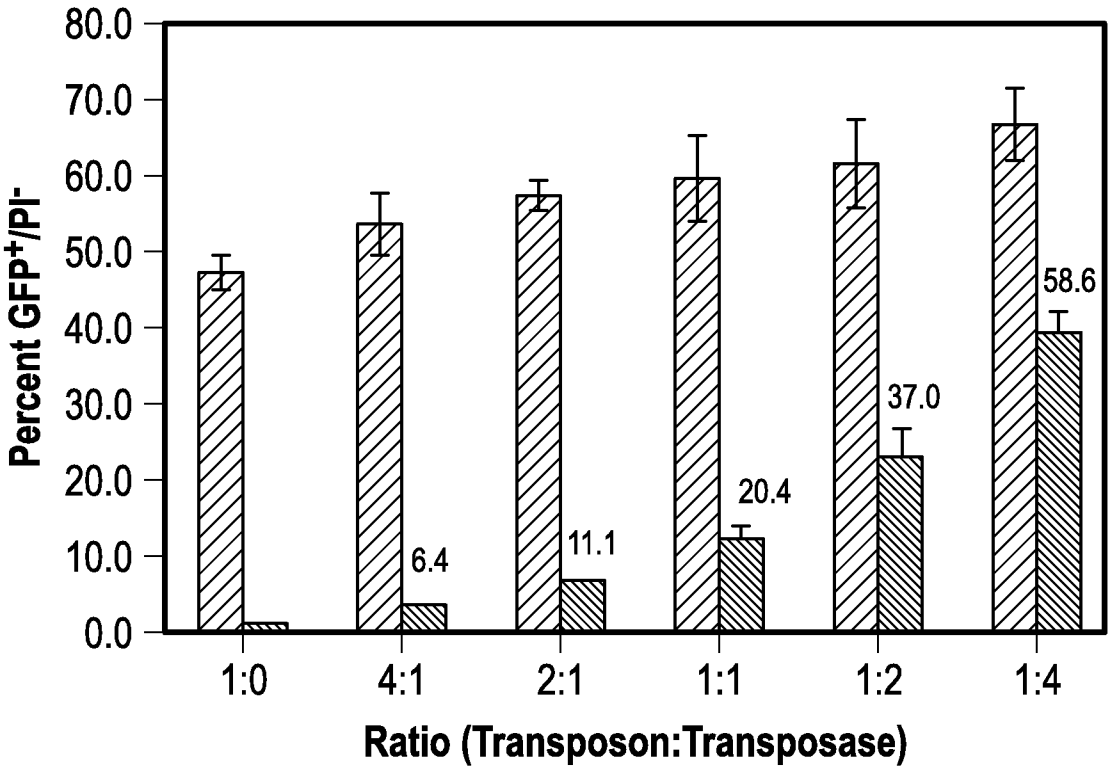

80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Percent GFP+/PI-
6.4
11.1
20.4
37.0
58.6
1:0
4:1
2:1
1:1
1:2
1:4
Ratio (Transposon:Transposase)

FIG. 2

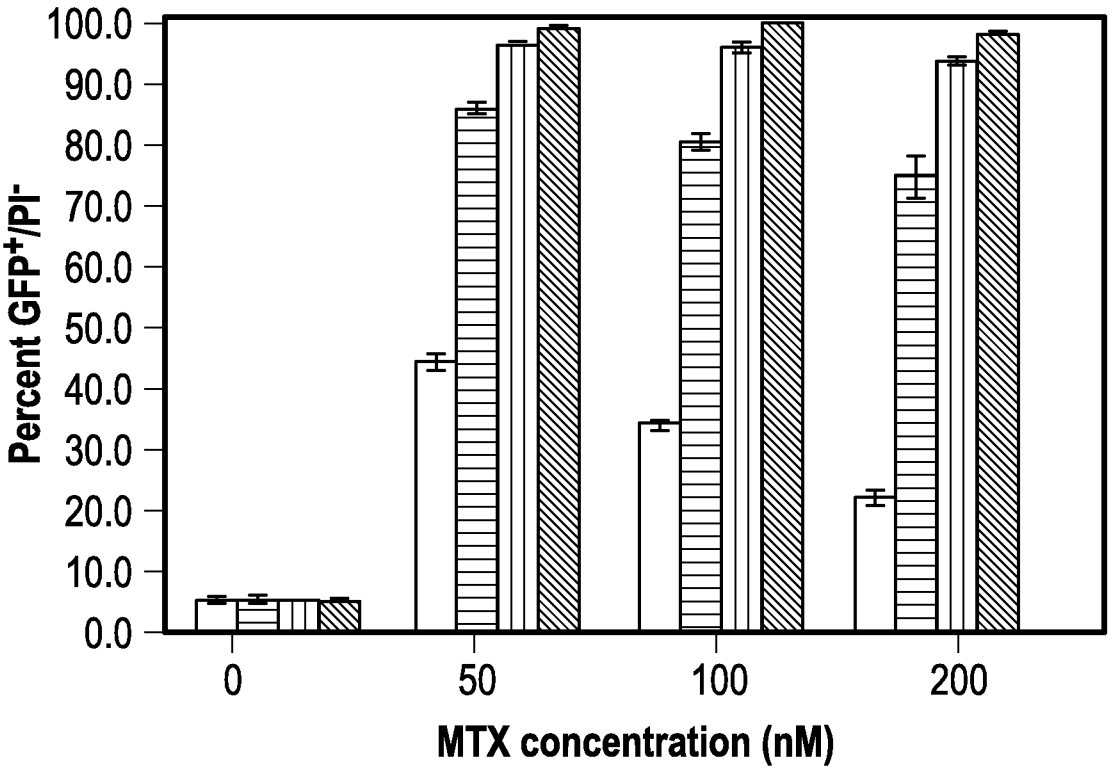

100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Percent GFP+/PI-
0
50
100
200
MTX concentration (nM)

FIG. 3A

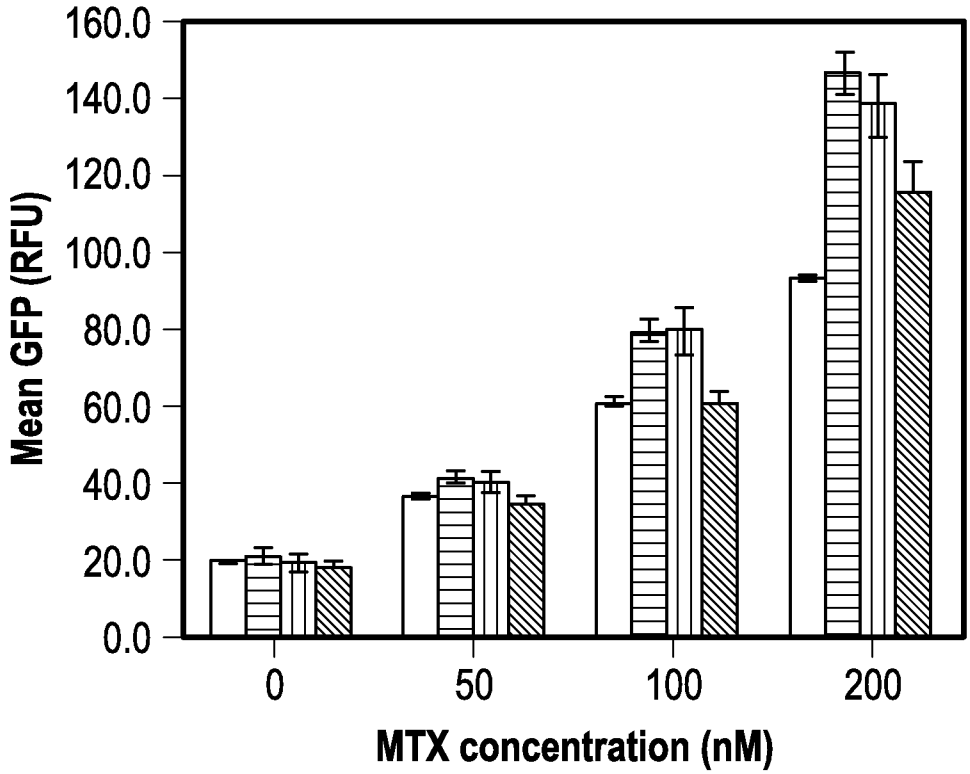

160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Mean GFP (RFU)
0
50
100
200
MTX concentration (nM)

FIG. 3B

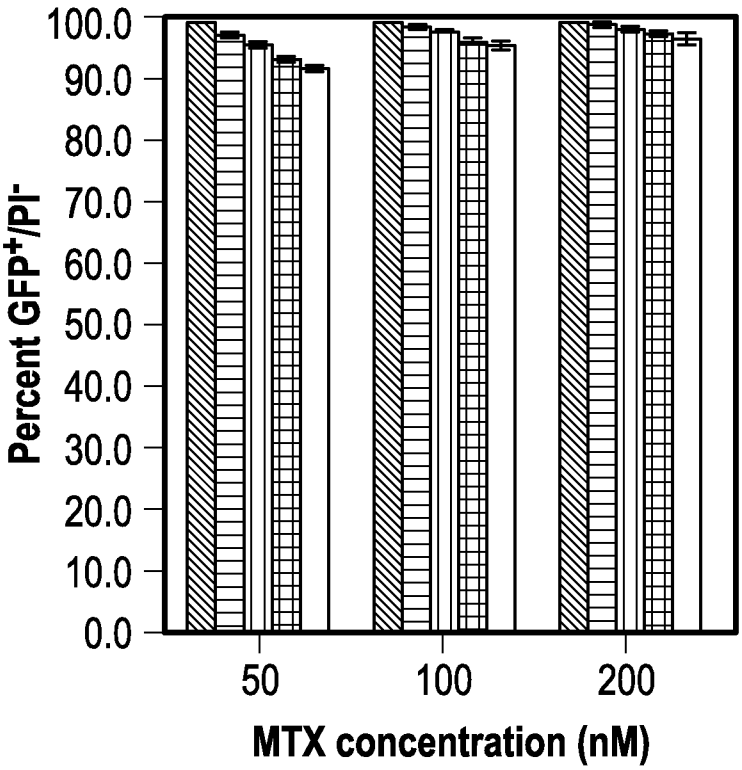

100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Percent GFP+/PI-
50
100
200
MTX concentration (nM)

FIG. 4A

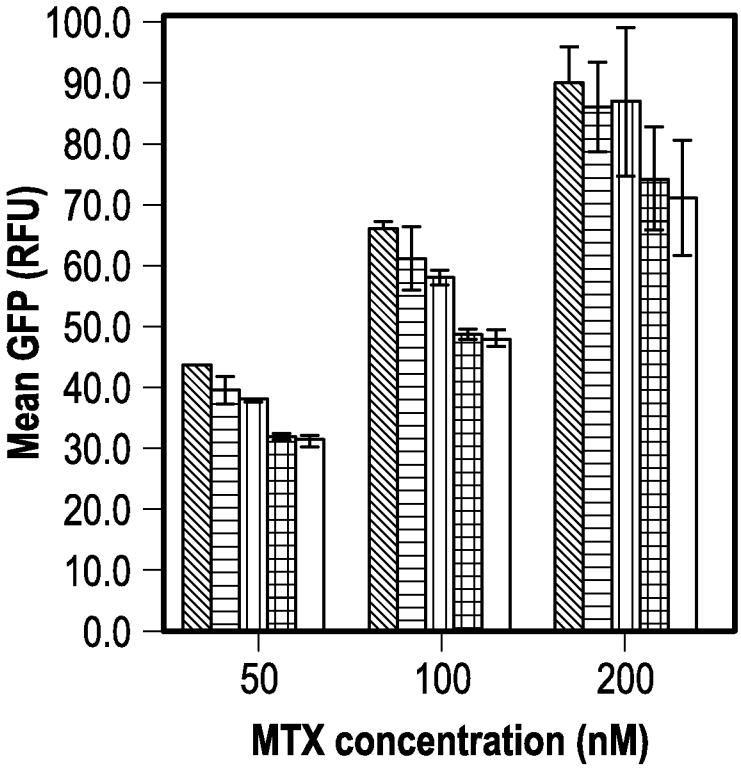

100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Mean GFP (RFU)
50
100
200
MTX concentration (nM)

FIG. 4B

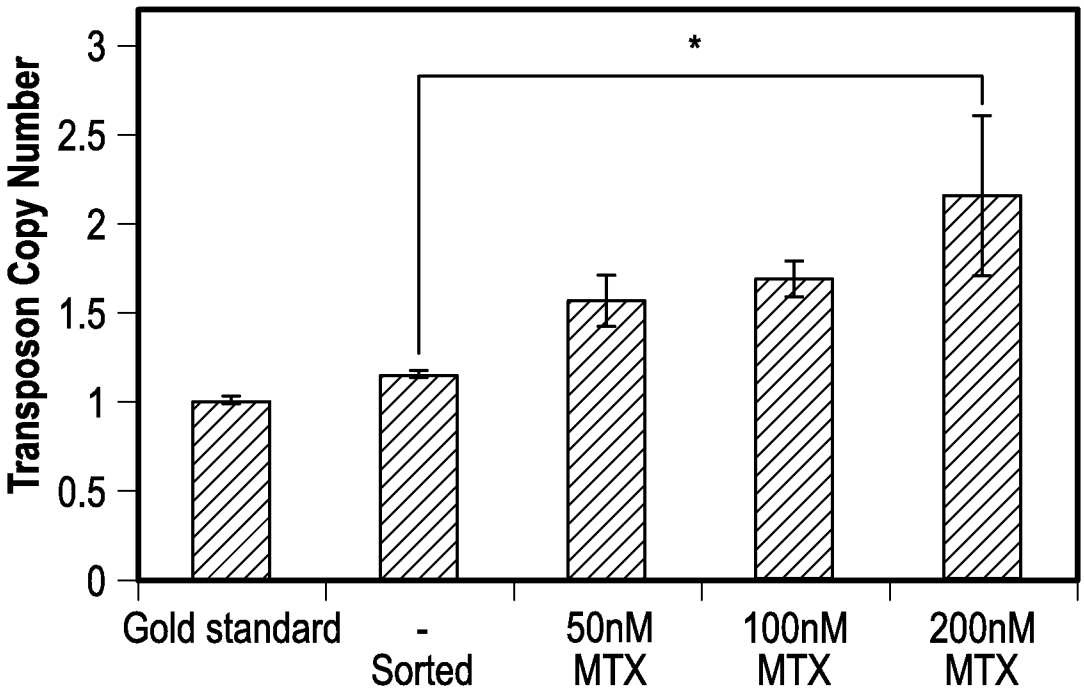

Transposon Copy Number
3
2.5
2
1.5
1
0.5
0
*
Gold standard
-
Sorted
50nM
MTX
100nM
MTX
200nM
MTX

FIG. 5A

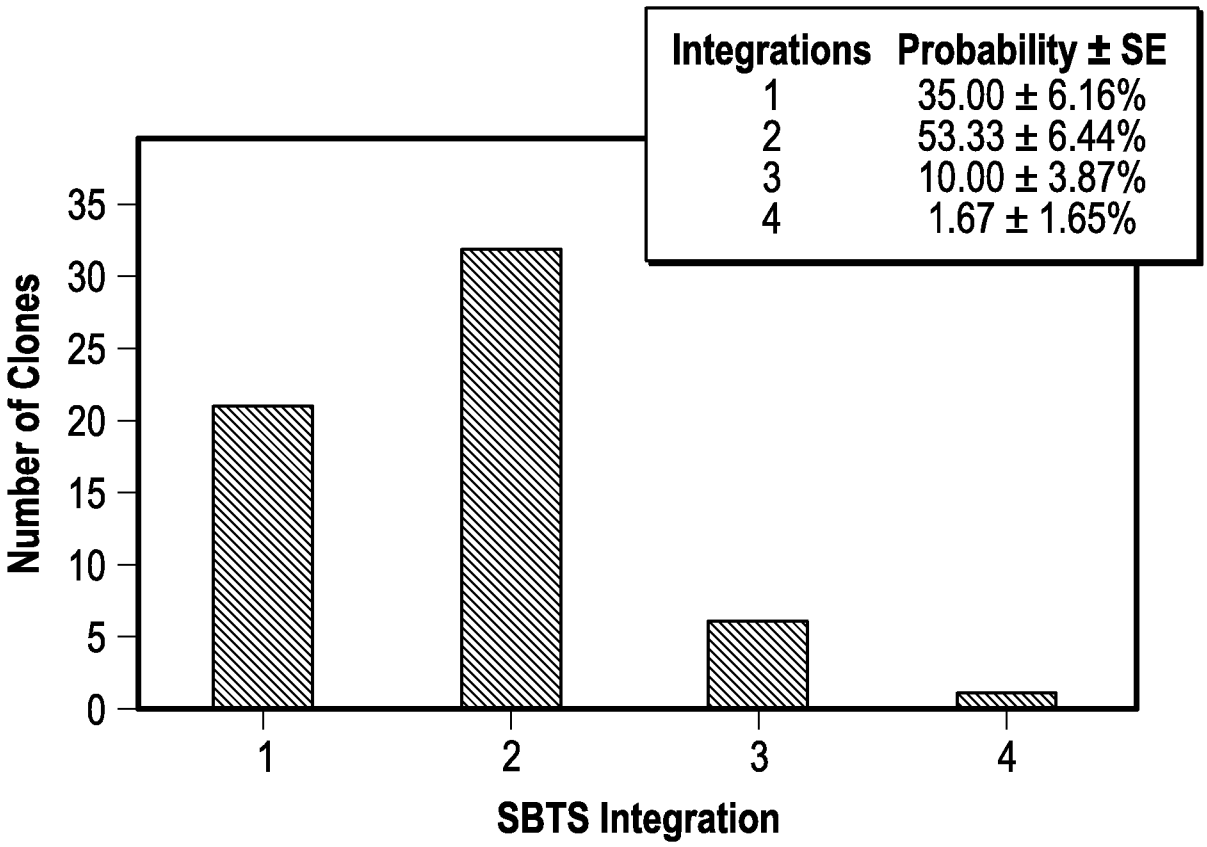

Integrations Probability ± SE
1 35.00 ± 6.16%
2 53.33 ± 6.44%
3 10.00 ± 3.87%
4 1.67 ± 1.65%
Number of Clones
35
30
25
20
15
10
5
0
1
2
3
4
SBTS Integration

FIG. 5B

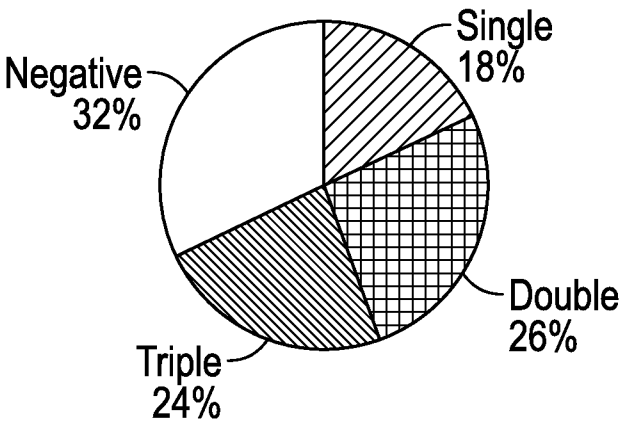


FIG. 6A

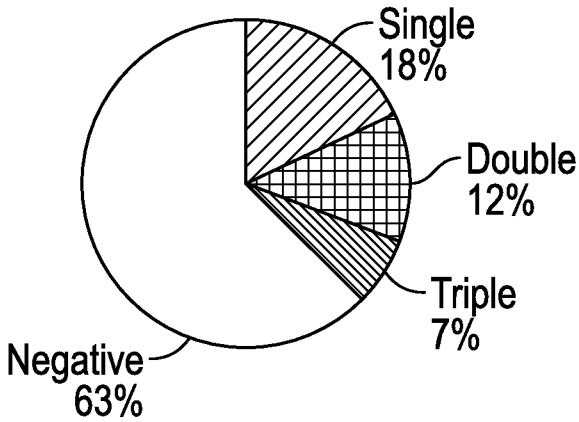


FIG. 6B

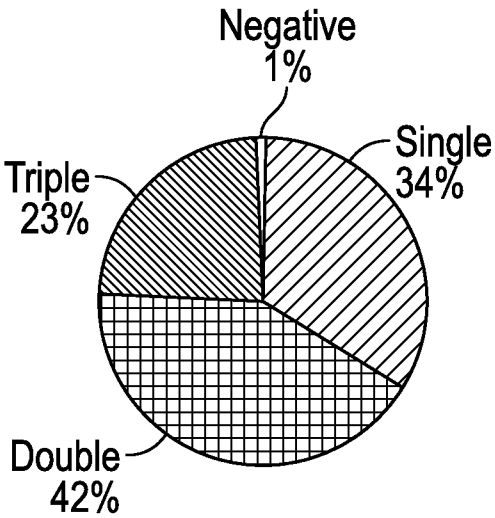


FIG. 6C

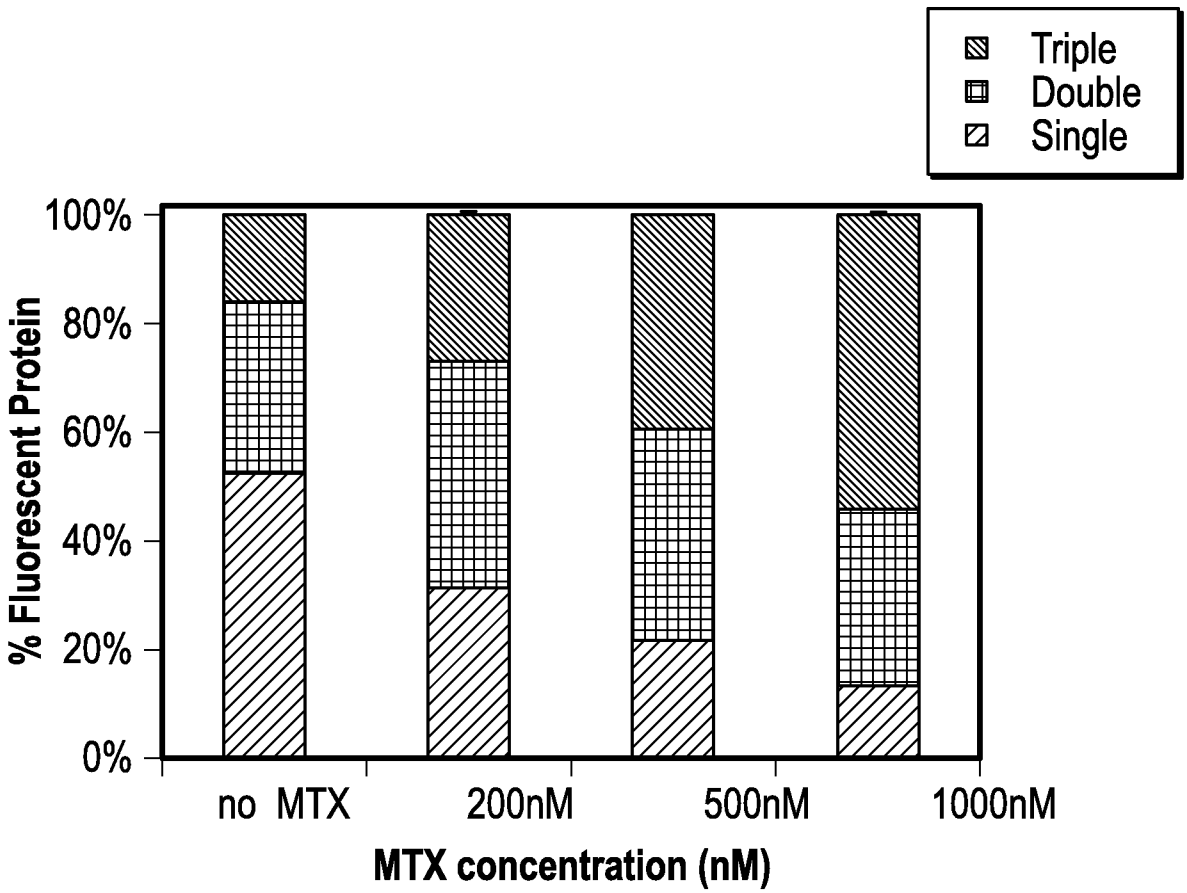

Triple
Double
Single
100%
80%
60%
40%
20%
0%
% Fluorescent Protein
no MTX
200nM
500nM
1000nM
MTX concentration (nM)

FIG. 7

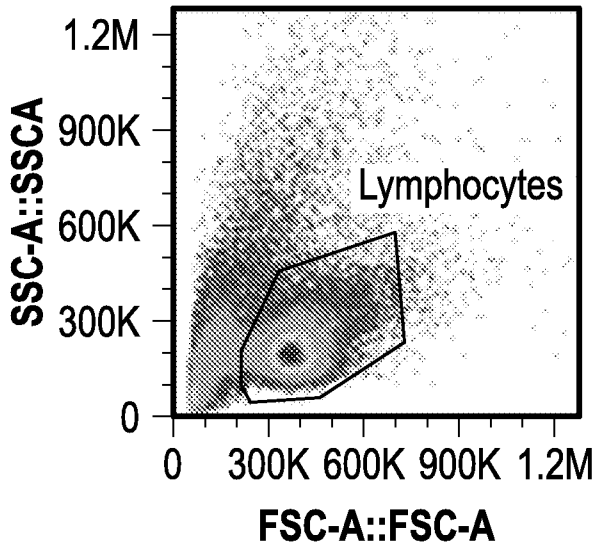


FIG. 8A

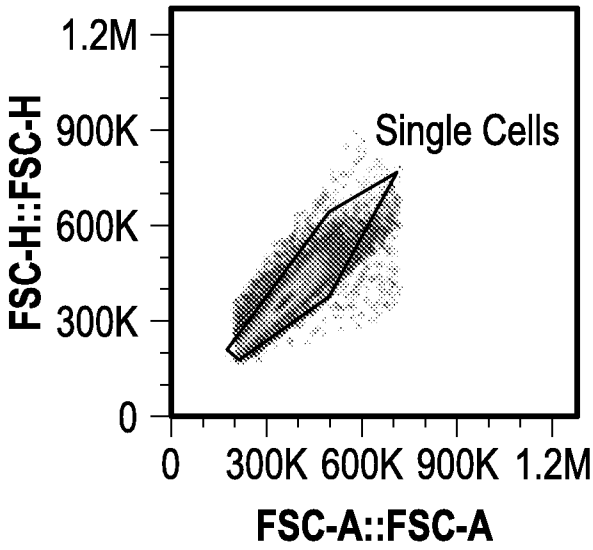


FIG. 8B

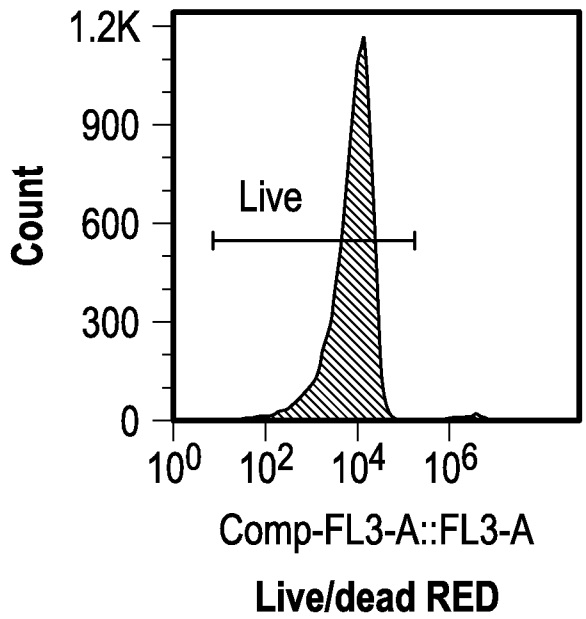


FIG. 8C

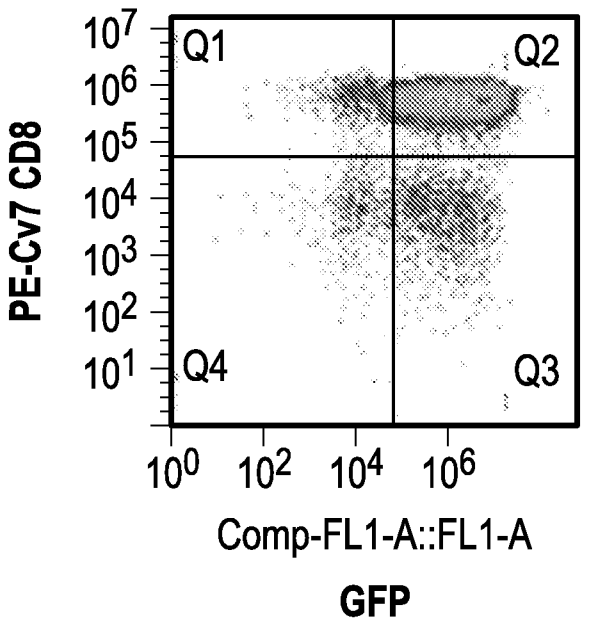
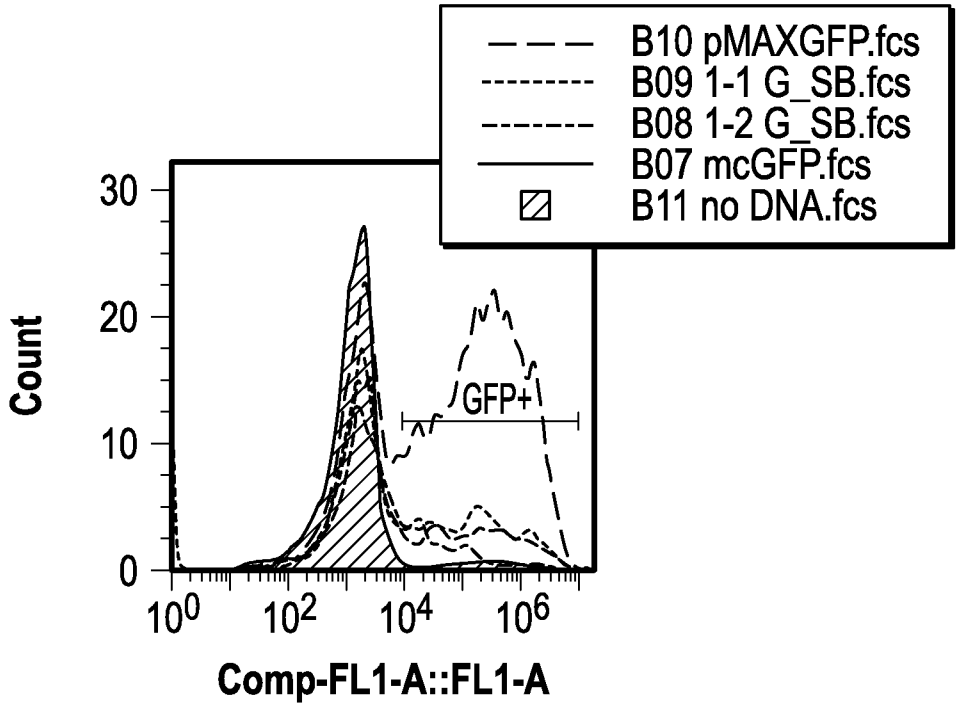
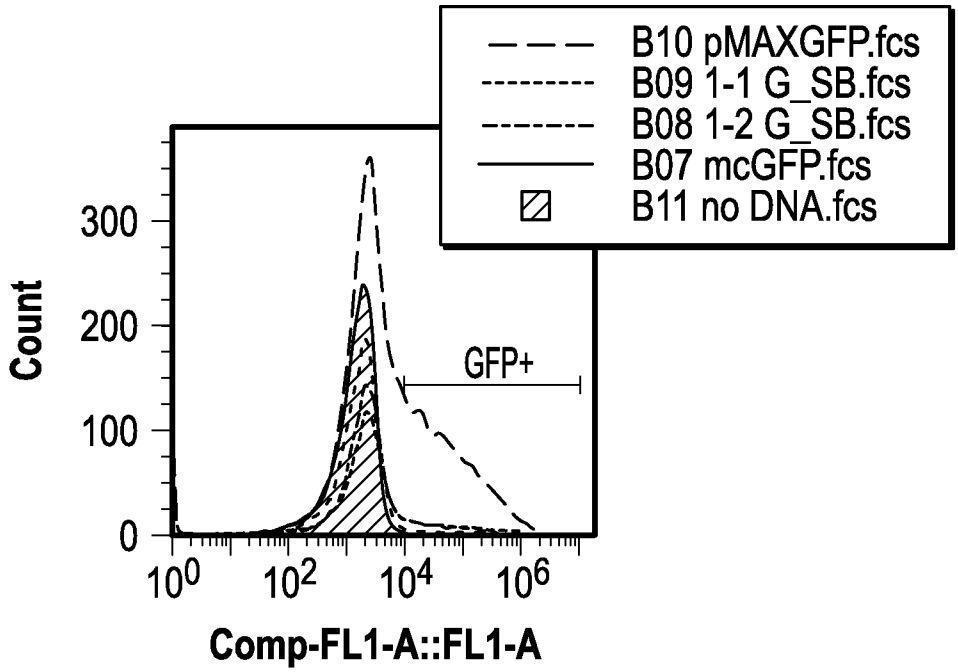


FIG. 8D



day 2

9A



day 5

9B

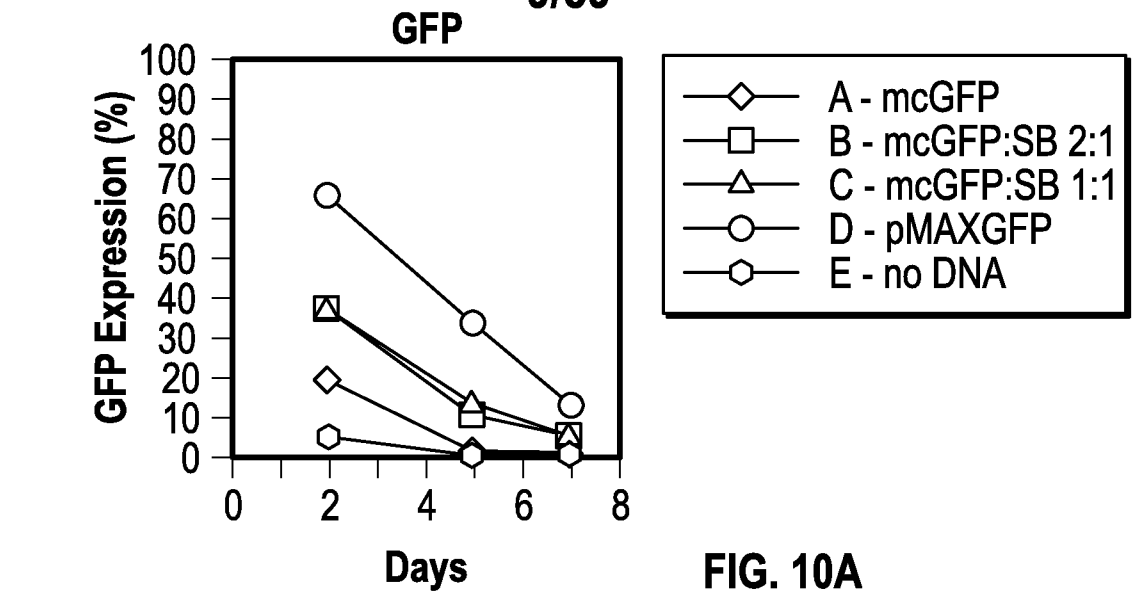


FIG. 10A

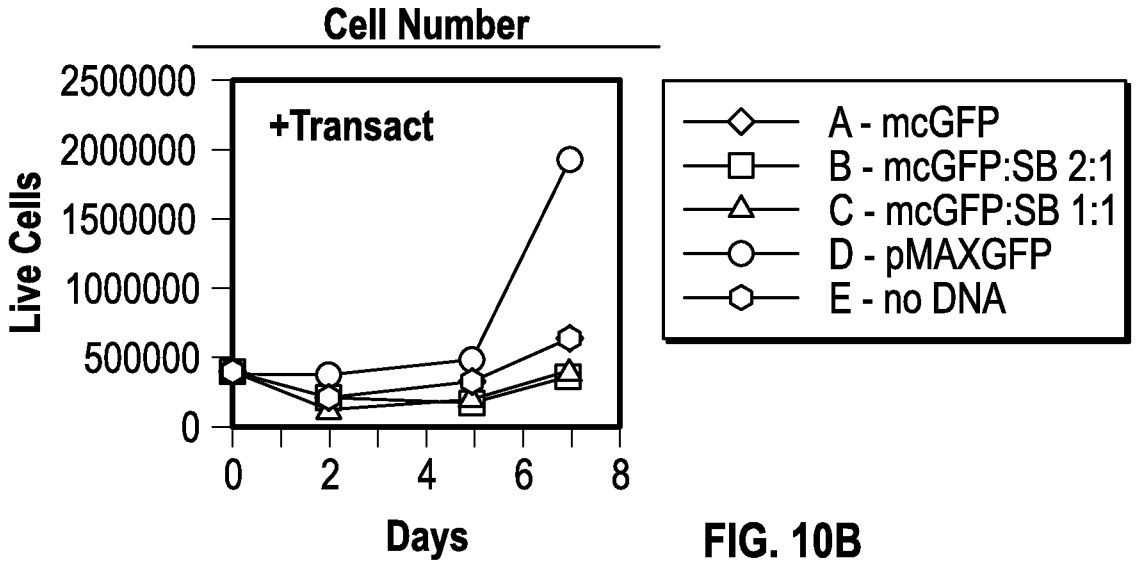


FIG. 10B

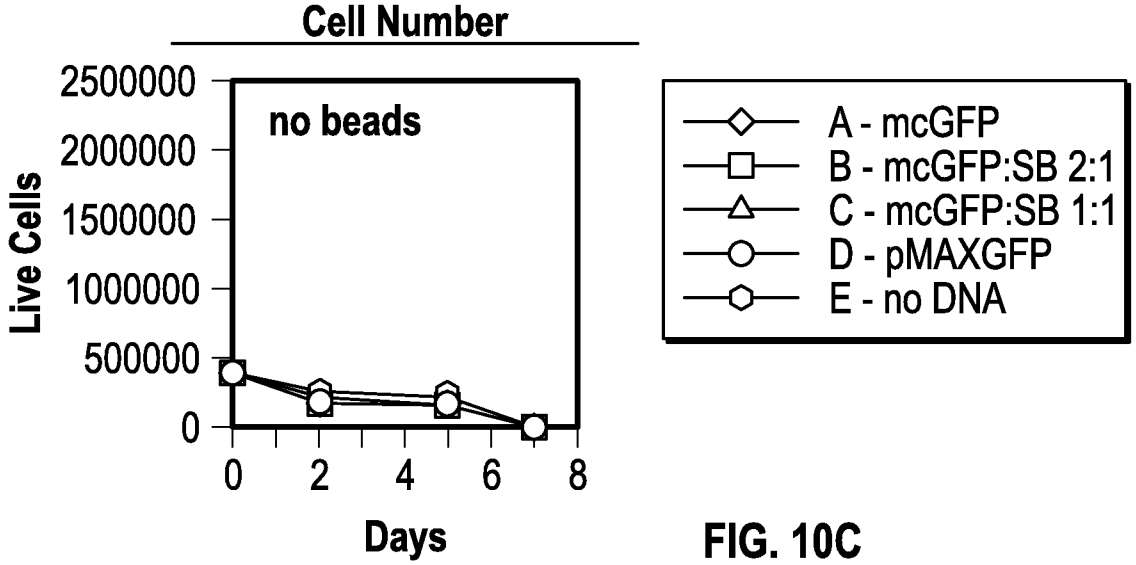
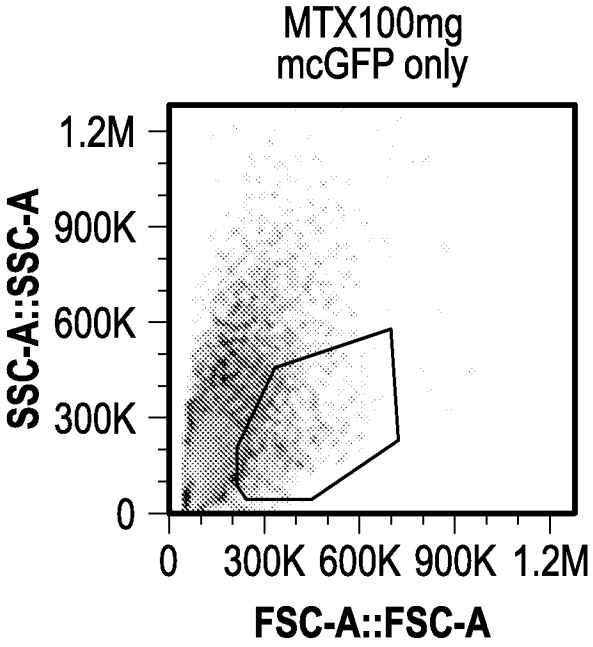
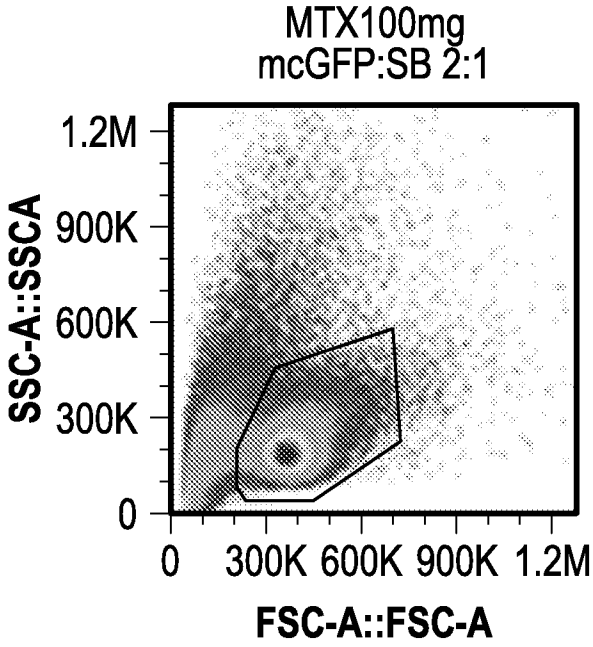


FIG. 10C



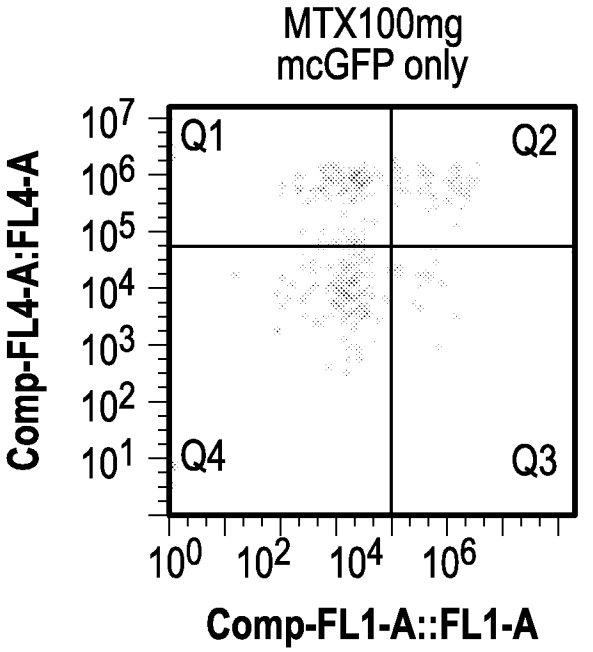
B07 A MTX 100.fcs
Ungated
15919

FIG. 11A



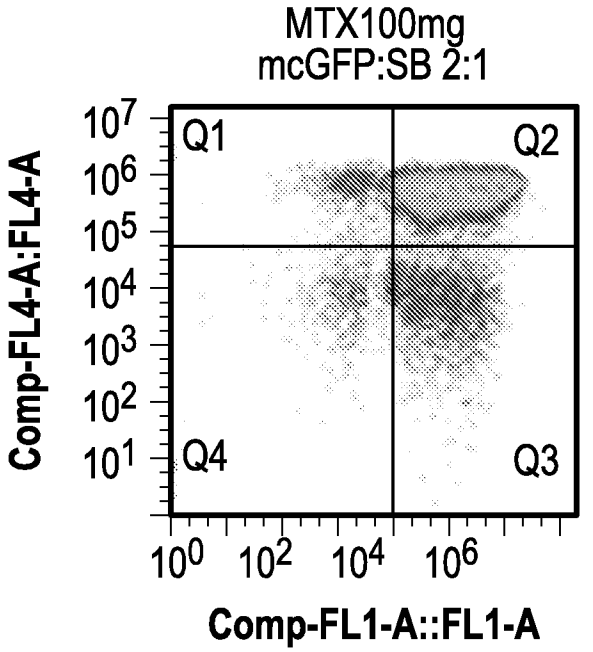
B08 B MTX 100.fcs
Ungated
320953

FIG. 11B



B07 A MTX 100.fcs
live
409

FIG. 11C



B08 B MTX 100.fcs
live
54047

FIG. 11D

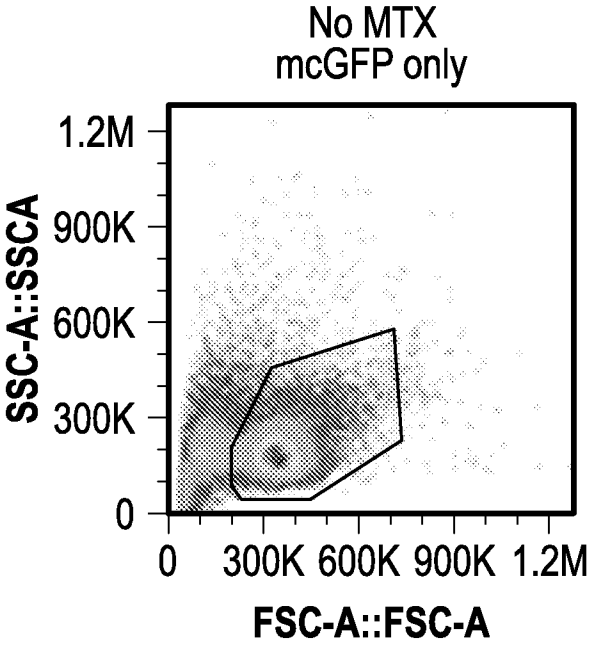


FIG. 11E

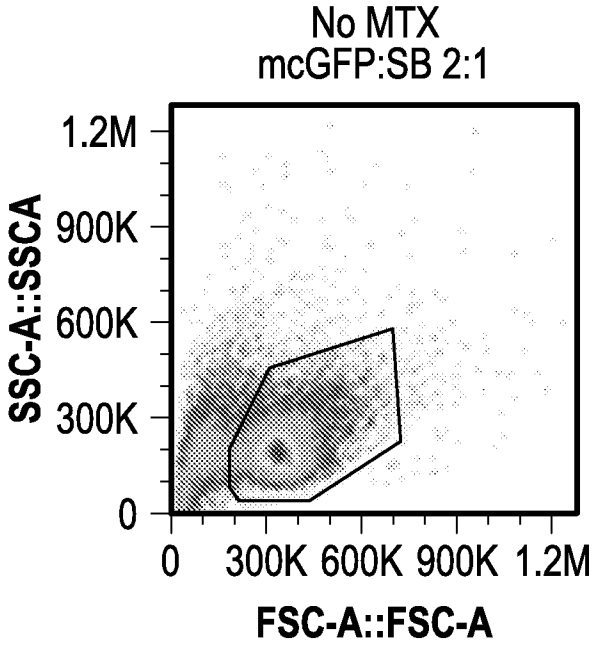


FIG. 11F

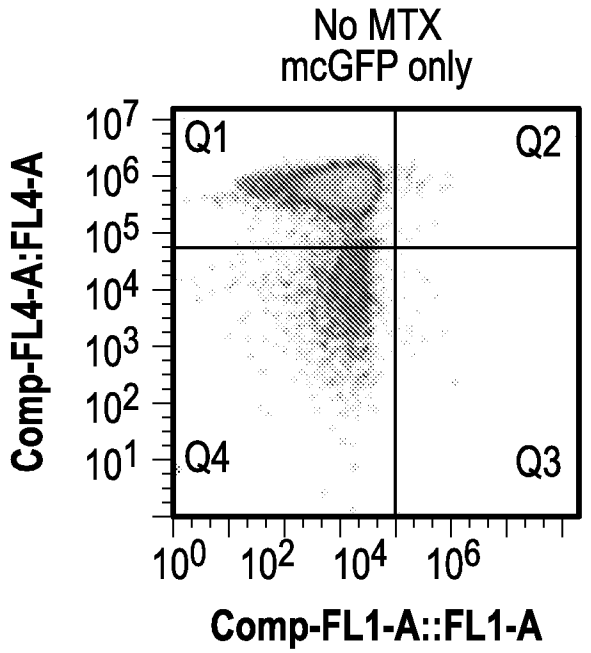


FIG. 11G

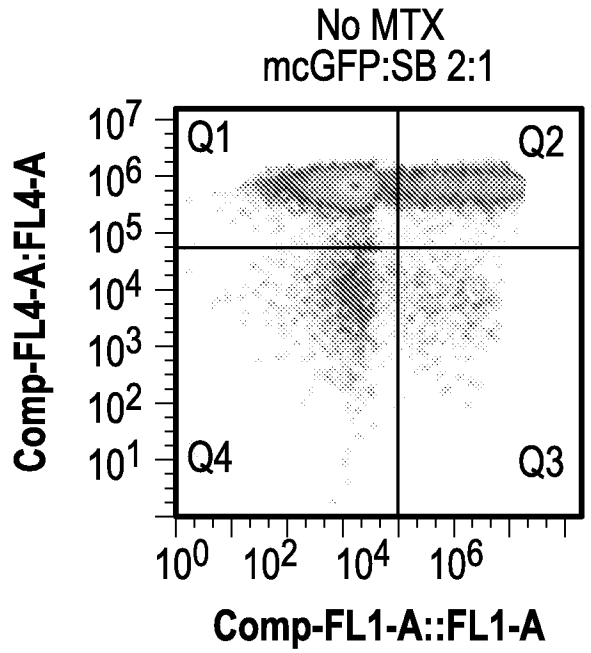


FIG. 11H

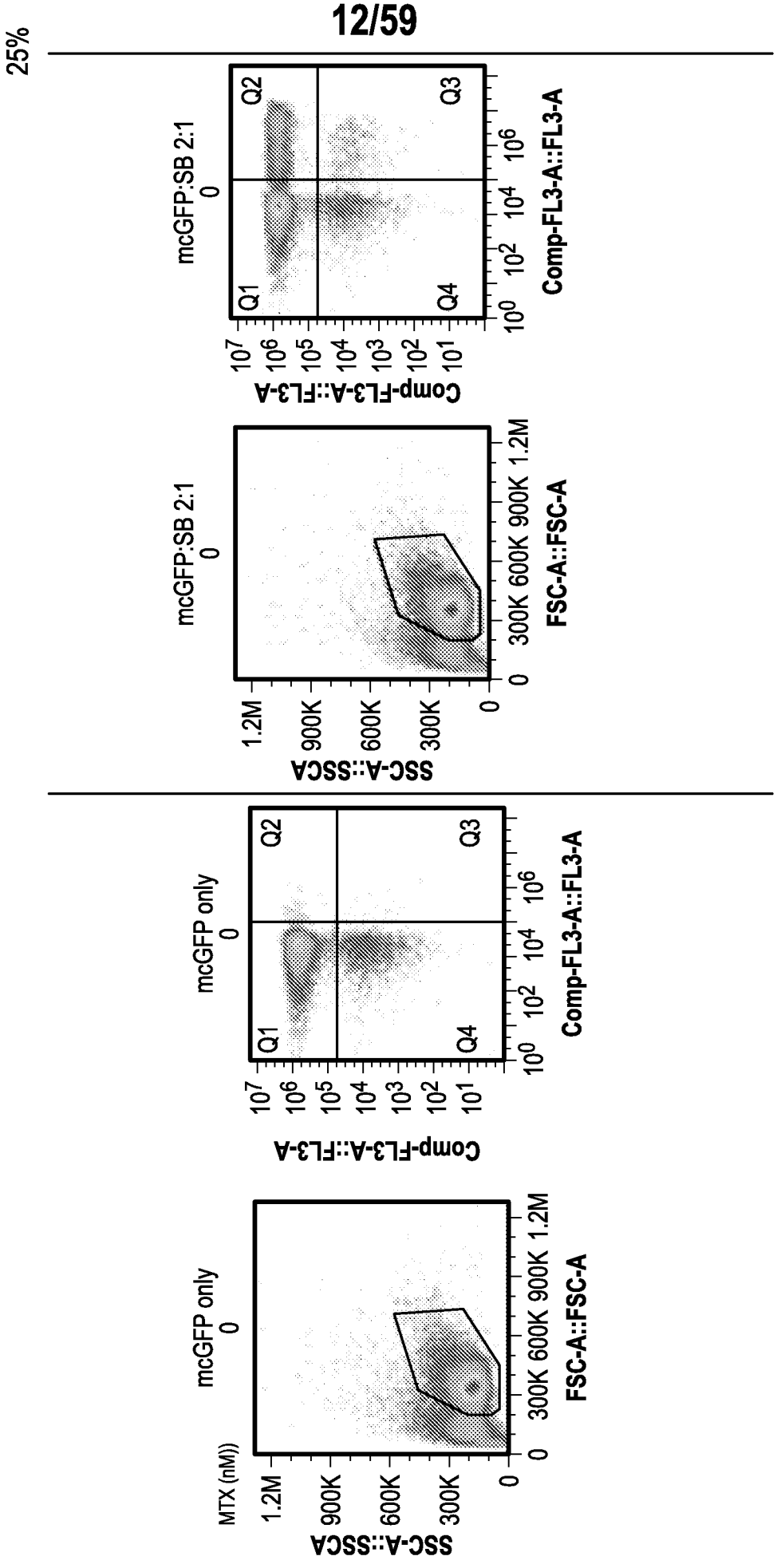

25%
mcGFP:SB 2:1
0
Q1
Q2
Q3
Q4
Comp-FL3-A::FL3-A
Comp-FL3-A::FL3-A
FSC-A::FSC-A
SSC-A::SSCA
mcGFP only
0
MTX (nM))
1.2M
900K
600K
300K
0
300K 600K 900K 1.2M

FIG. 12A-1

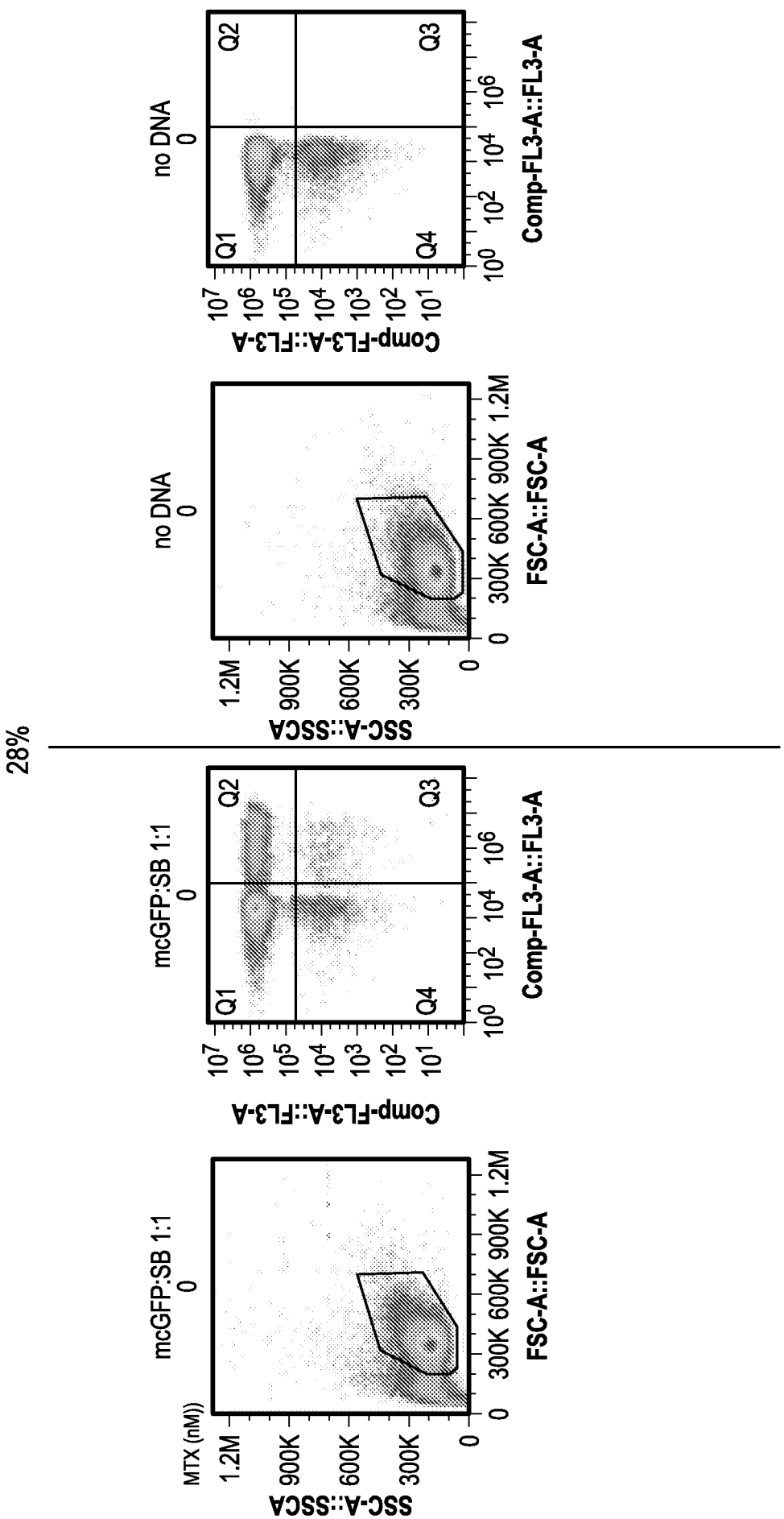

28%
no DNA
0
mcGFP:SB 1:1
0
MTX (nM))
SSC-A::SSCA
FSC-A::FSC-A
Comp-FL3-A::FL3-A
Q1
Q2
Q3
Q4
0
300K
600K
900K
1.2M
10^0
10^2
10^4
10^6
10^1
10^3
10^5
10^7

FIG. 12A-2

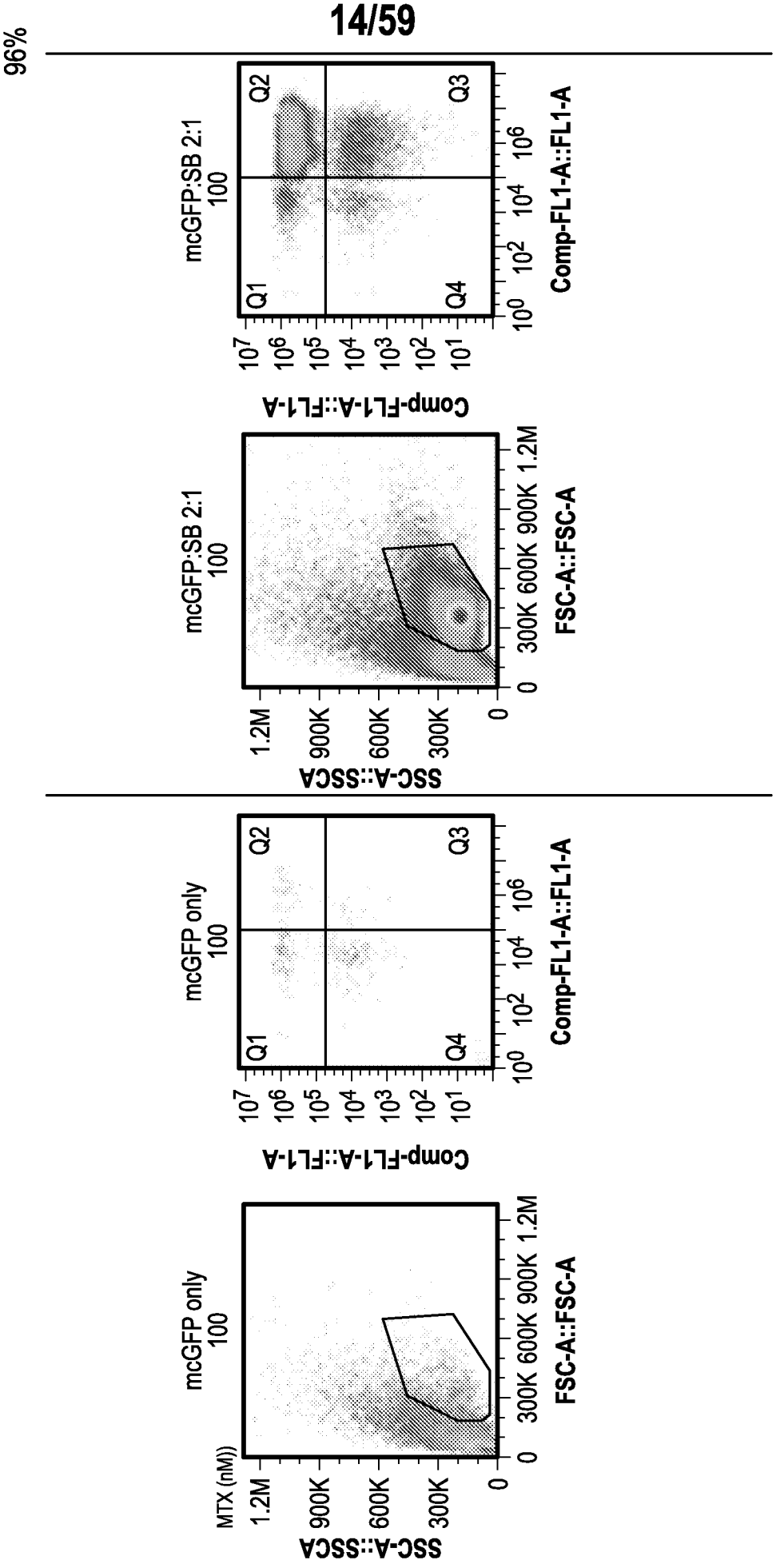

96%
mcGFP:SB 2:1
100
Q1
Q2
Q3
Q4
Comp-FL1-A::FL1-A
FSC-A::FSC-A
SSC-A::SSCA
mcGFP only
100
MTX (nM))
1.2M
900K
600K
300K
0
300K 600K 900K 1.2M

FIG. 12B-1

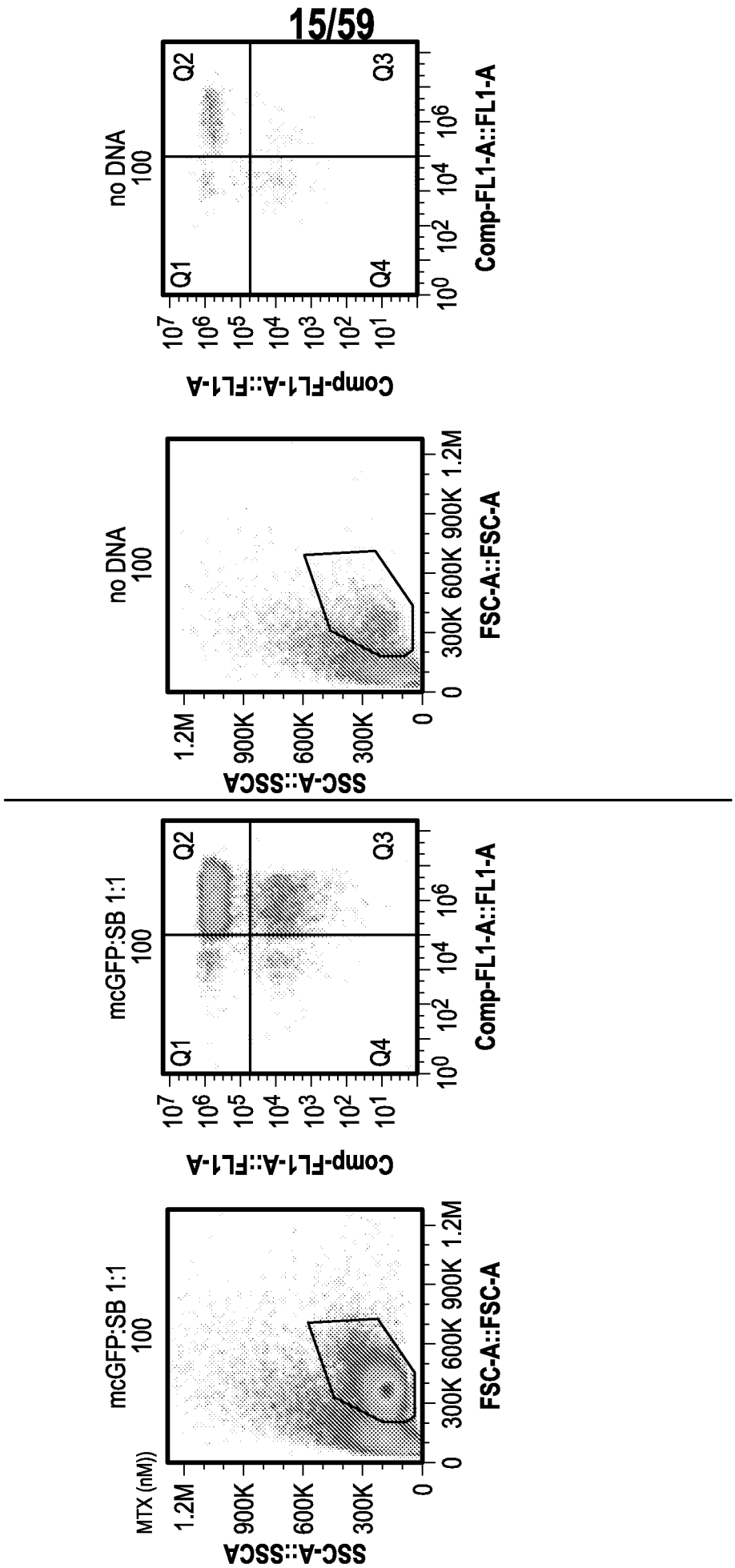

no DNA
100
mcGFP:SB 1:1
100
MTX (nM))
SSC-A::SSCA
FSC-A::FSC-A
Comp-FL1-A::FL1-A
Q1
Q2
Q3
Q4

FIG. 12B-2

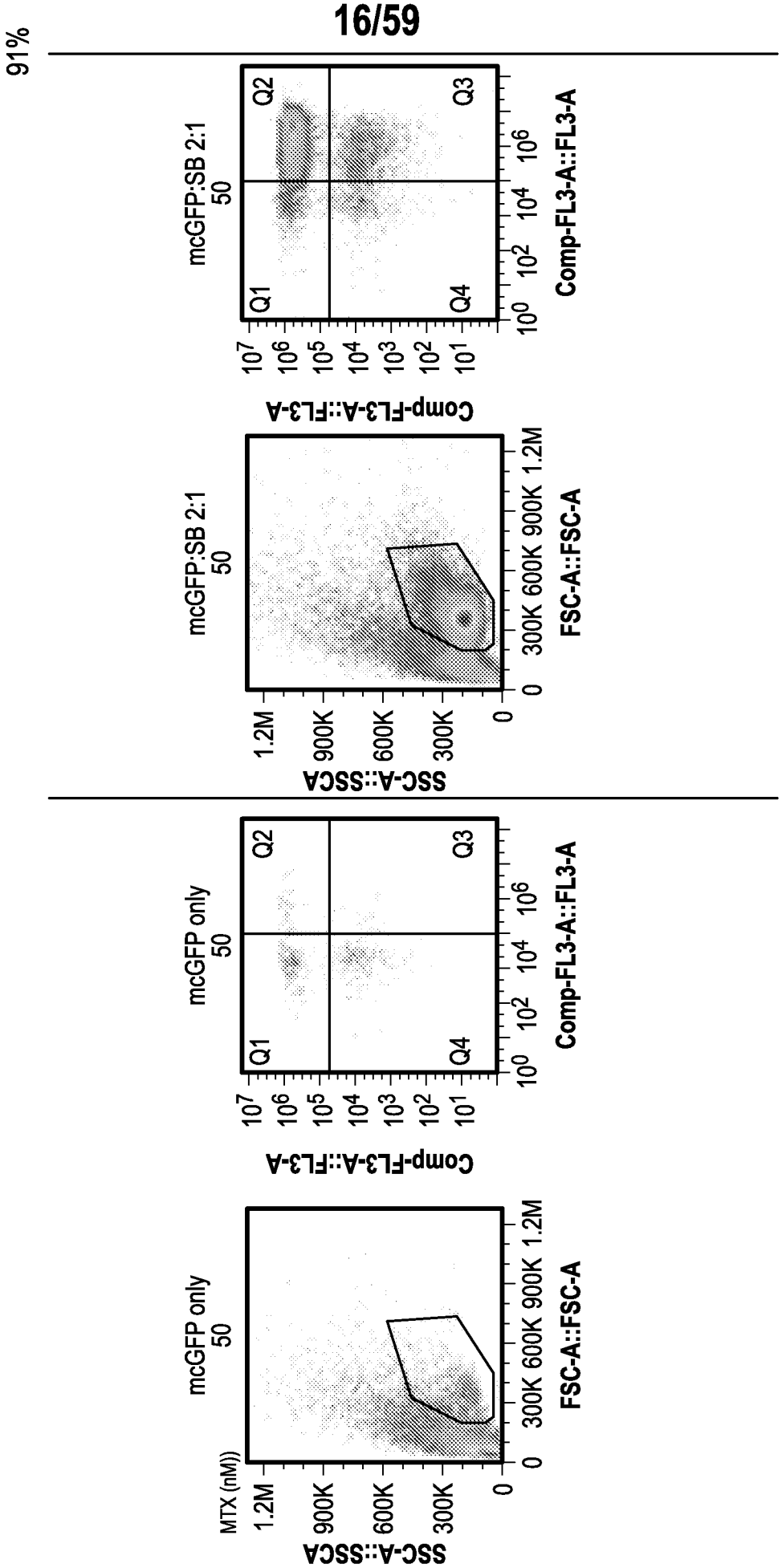

91%
mcGFP:SB 2:1
50
mcGFP only
50
MTX (nM))
SSC-A::SSCA
FSC-A::FSC-A
Comp-FL3-A::FL3-A
Q1
Q2
Q3
Q4
0
300K
600K
900K
1.2M
10^0
10^2
10^4
10^6
10^1
10^3
10^5
10^7

FIG. 12C-1

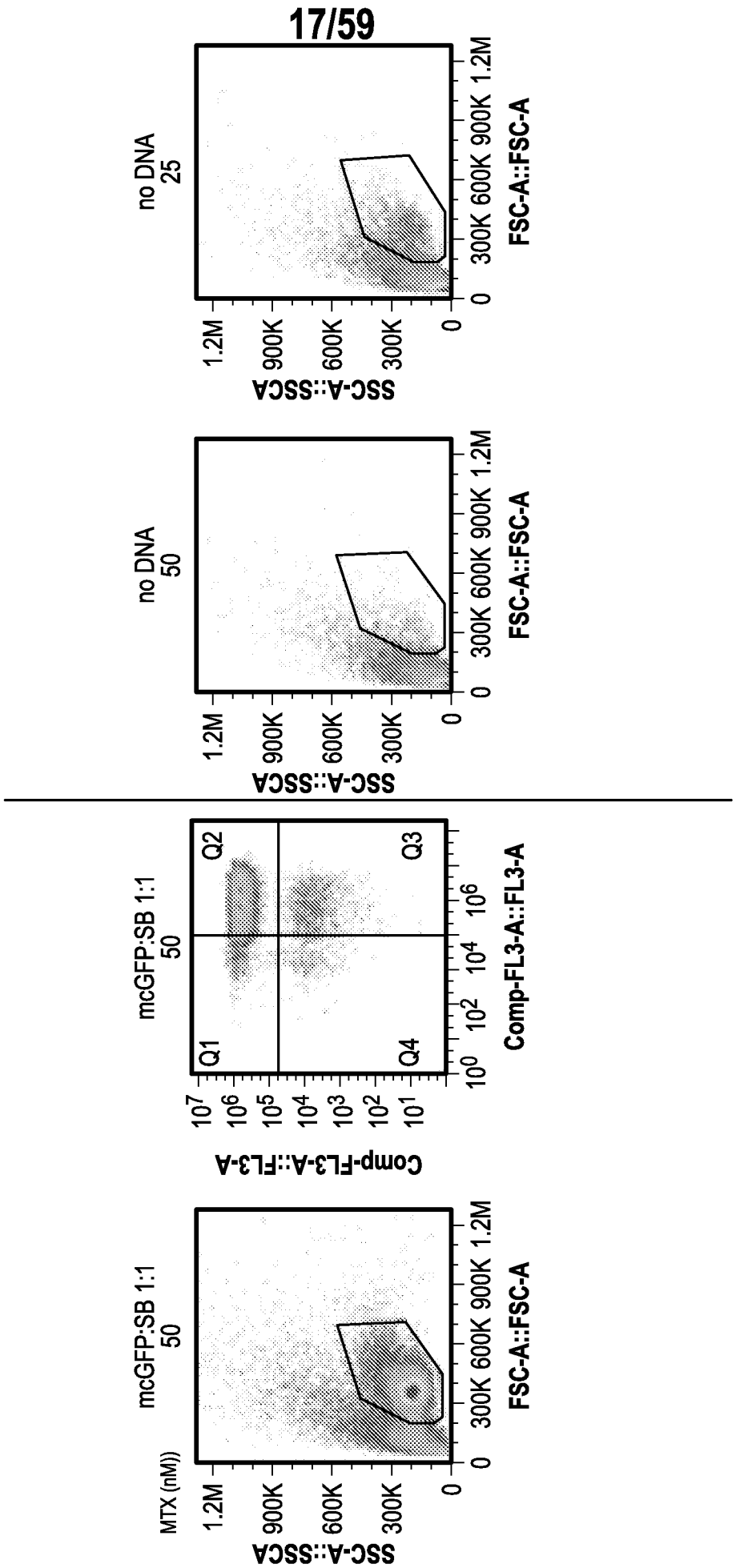

no DNA
25
no DNA
50
mcGFP:SB 1:1
50
mcGFP:SB 1:1
50
MTX (nM))
SSC-A::SSCA
FSC-A::FSC-A
Comp-FL3-A::FL3-A
Q1
Q2
Q3
Q4
0
300K
600K
900K
1.2M

FIG. 12C-2

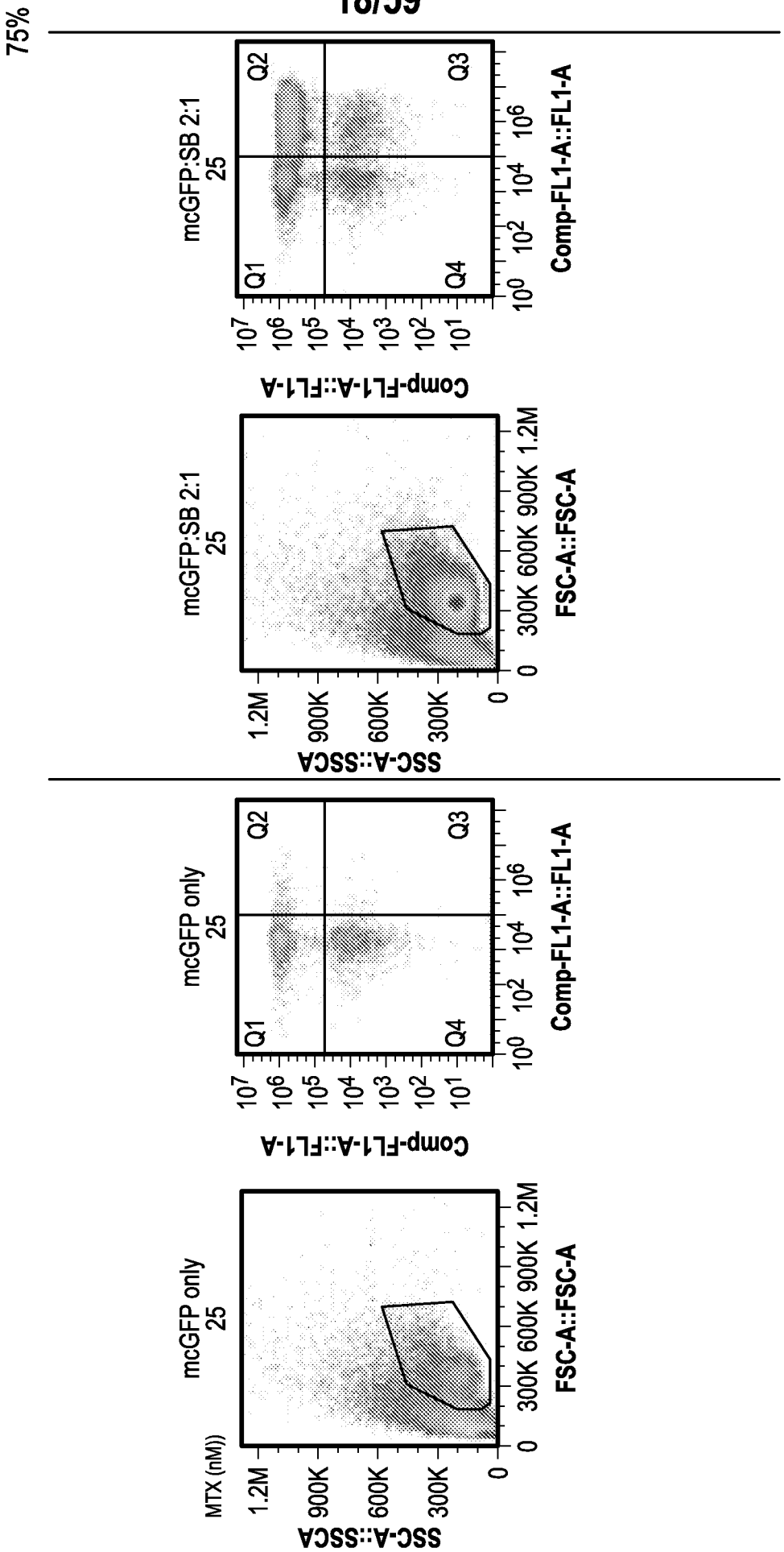

75%
mcGFP:SB 2:1
25
mcGFP only
25
MTX (nM))
SSC-A::SSCA
FSC-A::FSC-A
Comp-FL1-A::FL1-A
Q1
Q2
Q3
Q4
0
300K
600K
900K
1.2M
10^0
10^2
10^4
10^6
10^1
10^3
10^5
10^7

FIG. 12D-1

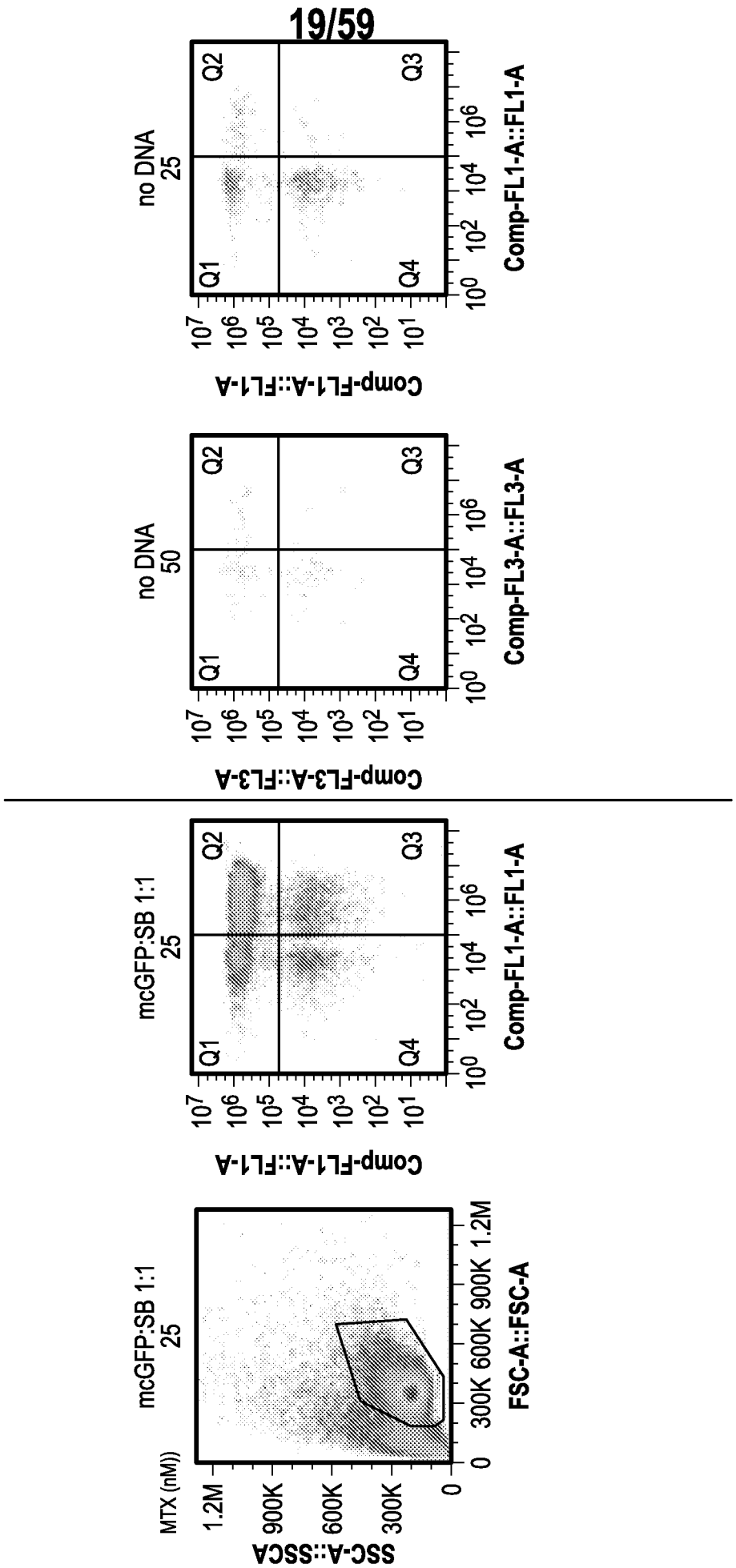

mcGFP:SB 1:1
25
MTX (nM))
1.2M
900K
600K
300K
0
SSC-A::SSCA
0
300K
600K
900K
1.2M
FSC-A::FSC-A
mcGFP:SB 1:1
25
Q1
Q2
Q3
Q4
Comp-FL1-A::FL1-A
Comp-FL1-A::FL1-A
no DNA
50
Comp-FL3-A::FL3-A
Comp-FL3-A::FL3-A
no DNA
25
Comp-FL1-A::FL1-A
Comp-FL1-A::FL1-A

FIG. 12D-2

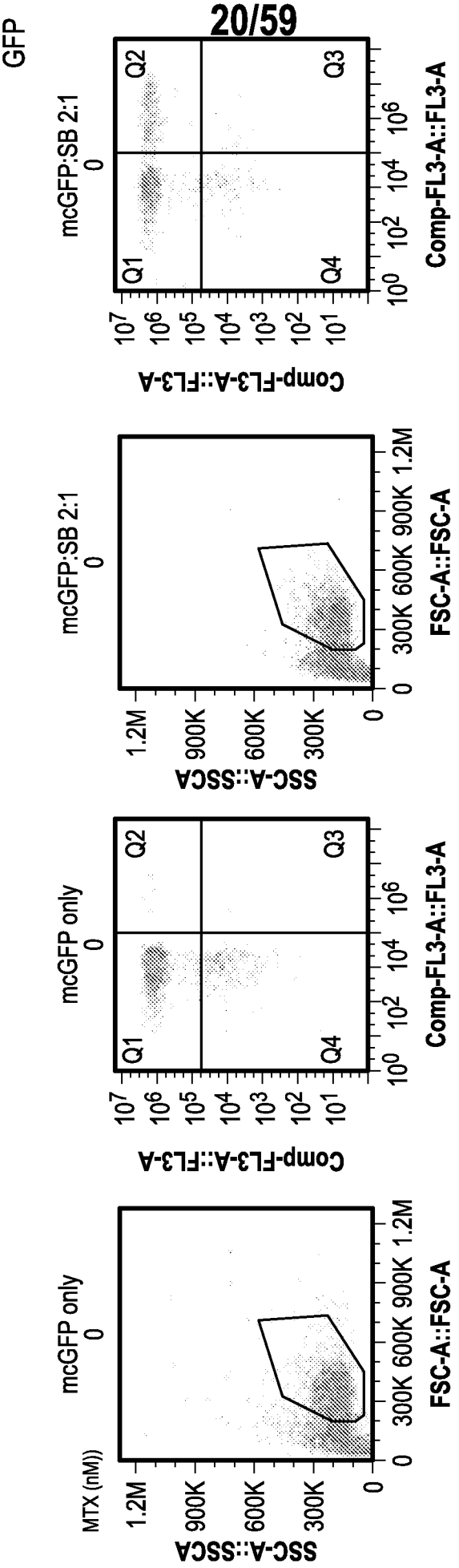

GFP
mcGFP:SB 2:1
mcGFP only
MTX (nM))
0
300K
600K
900K
1.2M
SSC-A::SSCA
FSC-A::FSC-A
Comp-FL3-A::FL3-A
Q1
Q2
Q3
Q4

FIG. 13A-1

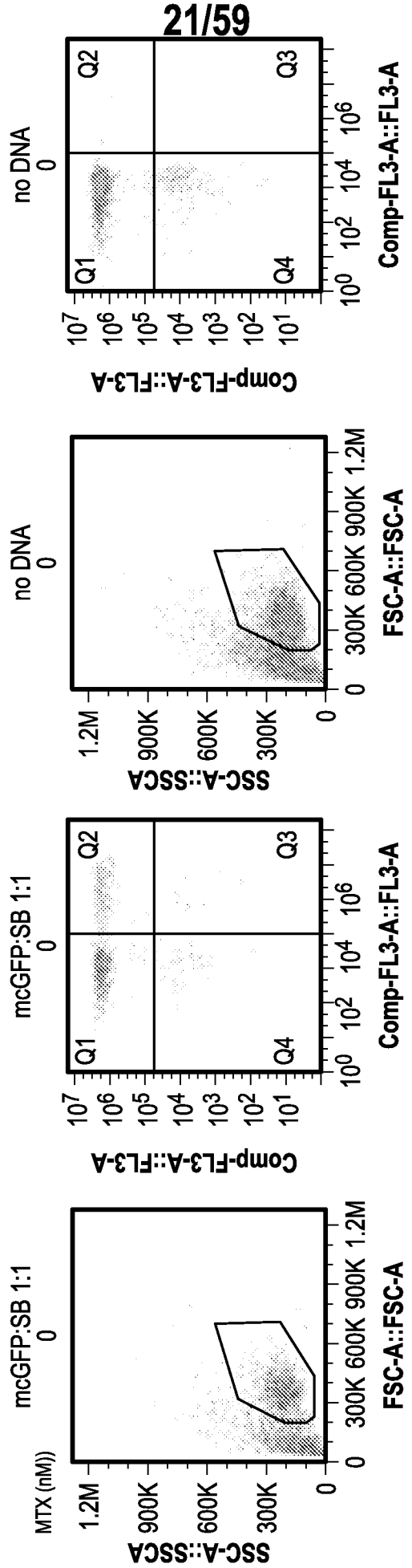

MTX (nM))
mcGFP:SB 1:1
no DNA
SSC-A::SSCA
FSC-A::FSC-A
Comp-FL3-A::FL3-A
Q1
Q2
Q3
Q4
0
300K
600K
900K
1.2M
10^0
10^2
10^4
10^6
10^1
10^3
10^5
10^7

FIG. 13A-2

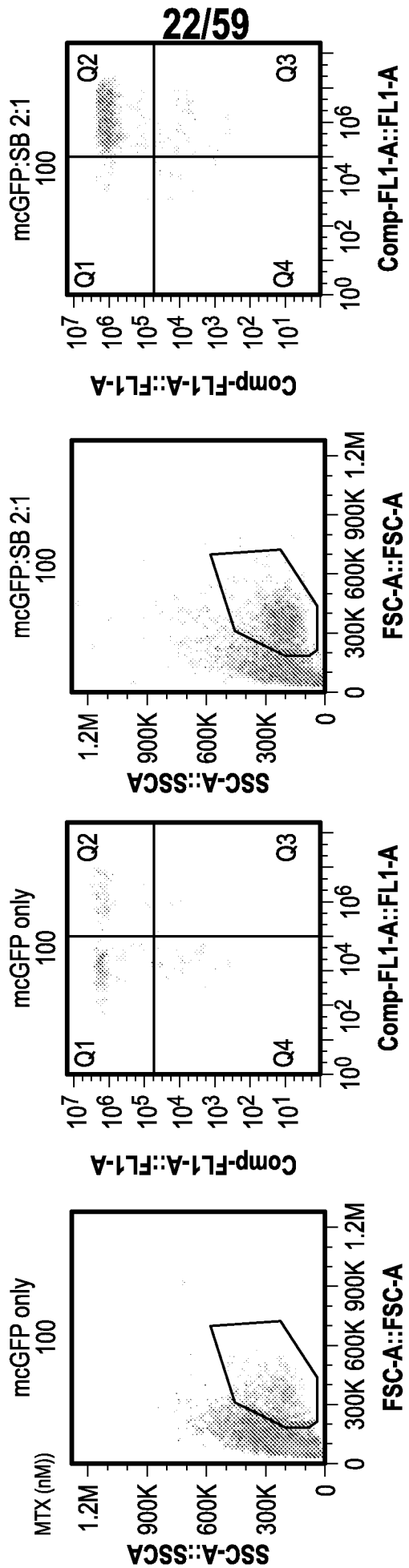

mcGFP only
100
MTX (nM))
SSC-A::SSCA
FSC-A::FSC-A
mcGFP only
100
Comp-FL1-A::FL1-A
Comp-FL1-A::FL1-A
mcGFP:SB 2:1
100
SSC-A::SSCA
FSC-A::FSC-A
mcGFP:SB 2:1
100
Comp-FL1-A::FL1-A
Comp-FL1-A::FL1-A
Q1
Q2
Q3
Q4

FIG. 13B-1

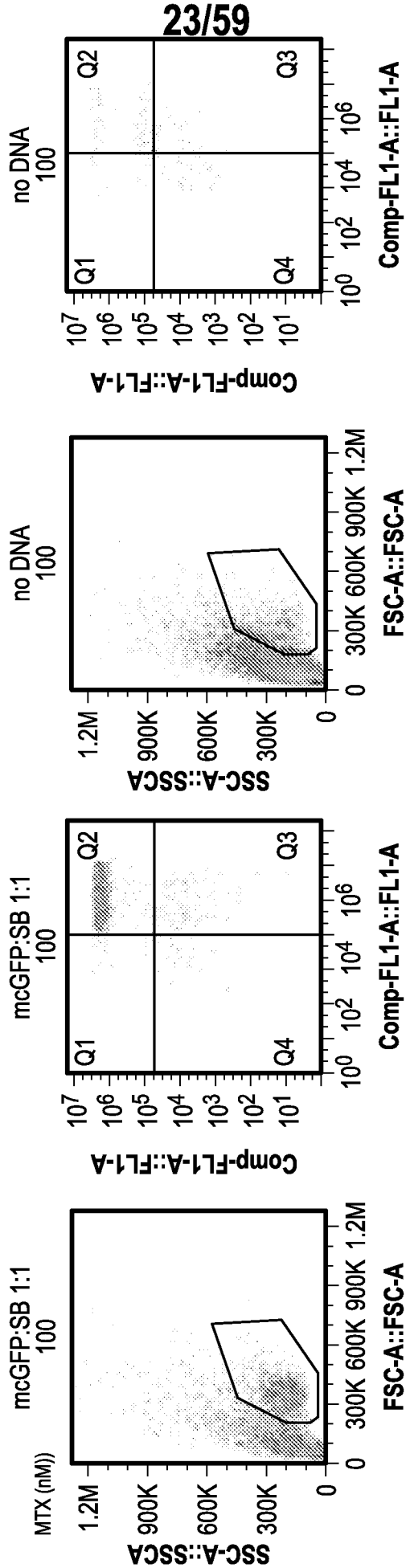

MTX (nM))
mcGFP:SB 1:1
100
no DNA
100
SSC-A::SSCA
FSC-A::FSC-A
Comp-FL1-A::FL1-A
Comp-FL1-A::FL1-A
Q1
Q2
Q3
Q4
0
300K
600K
900K
1.2M
10^0
10^2
10^4
10^6
10^1
10^2
10^3
10^4
10^5
10^6
10^7

FIG. 13B-2

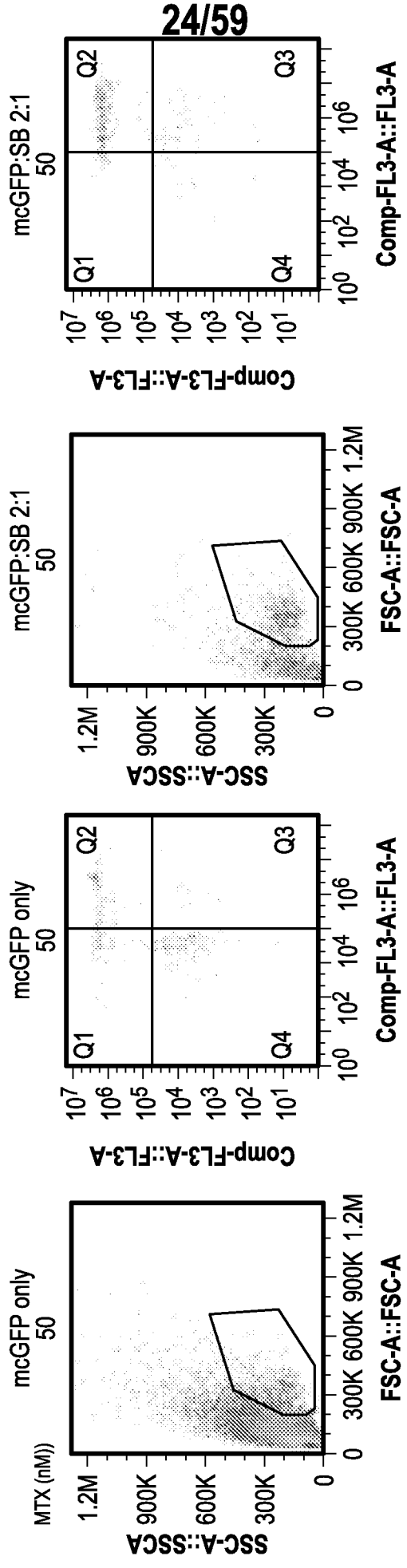

mcGFP only
50
MTX (nM))
SSC-A::SSCA
FSC-A::FSC-A
mcGFP only
50
Comp-FL3-A::FL3-A
Comp-FL3-A::FL3-A
mcGFP:SB 2:1
50
SSC-A::SSCA
FSC-A::FSC-A
mcGFP:SB 2:1
50
Comp-FL3-A::FL3-A
Comp-FL3-A::FL3-A
Q1
Q2
Q3
Q4

FIG. 13C-1

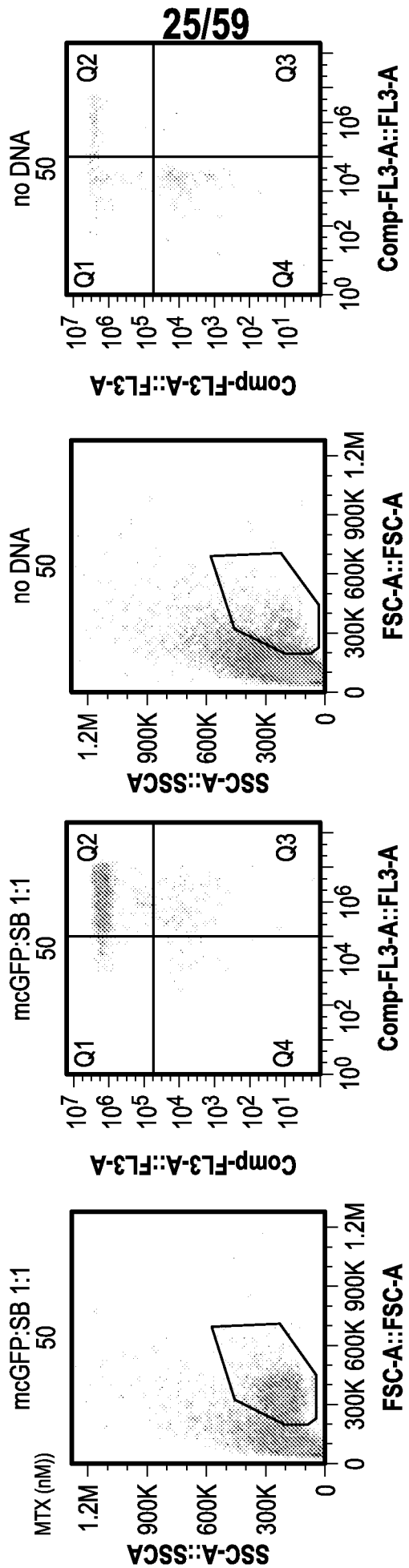

MTX (nM))
1.2M
900K
600K
300K
0
SSC-A::SSCA
0
300K
600K
900K
1.2M
FSC-A::FSC-A
mcGFP:SB 1:1
50
Comp-FL3-A::FL3-A
Q1
Q2
Q3
Q4
10^0
10^2
10^4
10^6
Comp-FL3-A::FL3-A
10^1
10^2
10^3
10^4
10^5
10^6
10^7
mcGFP:SB 1:1
50
SSC-A::SSCA
1.2M
900K
600K
300K
0
FSC-A::FSC-A
0
300K
600K
900K
1.2M
Comp-FL3-A::FL3-A
Q1
Q2
Q3
Q4
10^0
10^2
10^4
10^6
Comp-FL3-A::FL3-A
10^1
10^2
10^3
10^4
10^5
10^6
10^7
no DNA
50
SSC-A::SSCA
1.2M
900K
600K
300K
0
FSC-A::FSC-A
0
300K
600K
900K
1.2M
Comp-FL3-A::FL3-A
Q1
Q2
Q3
Q4
10^0
10^2
10^4
10^6
Comp-FL3-A::FL3-A
10^1
10^2
10^3
10^4
10^5
10^6
10^7
no DNA
50

FIG. 13C-2

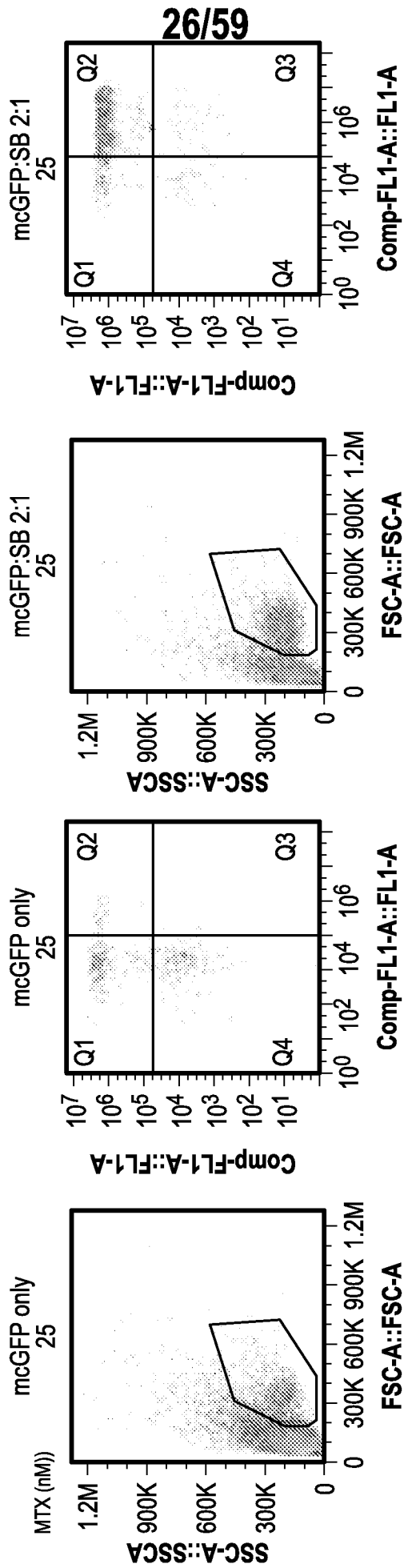

mcGFP only
25
MTX (nM))
SSC-A::SSCA
FSC-A::FSC-A
mcGFP only
25
Comp-FL1-A::FL1-A
Comp-FL1-A::FL1-A
mcGFP:SB 2:1
25
SSC-A::SSCA
FSC-A::FSC-A
mcGFP:SB 2:1
25
Comp-FL1-A::FL1-A
Comp-FL1-A::FL1-A
Q1
Q2
Q3
Q4

FIG. 13D-1

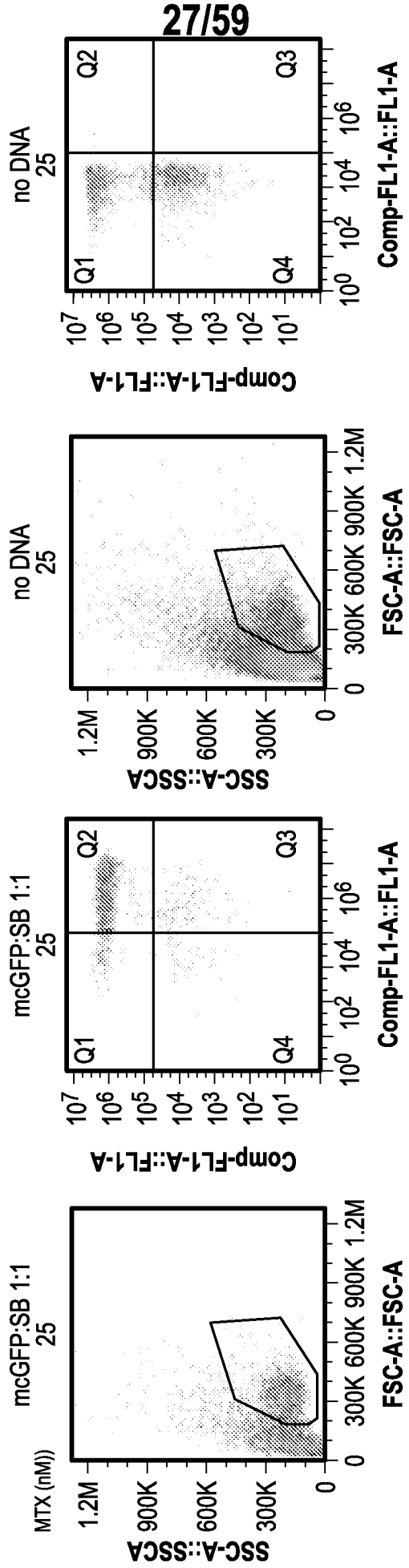

MTX (nM))
mcGFP:SB 1:1
25
SSC-A::SSCA
1.2M
900K
600K
300K
0
0
300K
600K
900K
1.2M
FSC-A::FSC-A
mcGFP:SB 1:1
25
Comp-FL1-A::FL1-A
Q1
Q2
Q3
Q4
10^0
10^2
10^4
10^6
Comp-FL1-A::FL1-A
10^1
10^2
10^3
10^4
10^5
10^6
10^7
no DNA
25
SSC-A::SSCA
1.2M
900K
600K
300K
0
0
300K
600K
900K
1.2M
FSC-A::FSC-A
no DNA
25
Comp-FL1-A::FL1-A
Q1
Q2
Q3
Q4
10^0
10^2
10^4
10^6
Comp-FL1-A::FL1-A
10^1
10^2
10^3
10^4
10^5
10^6
10^7

FIG. 13D-2

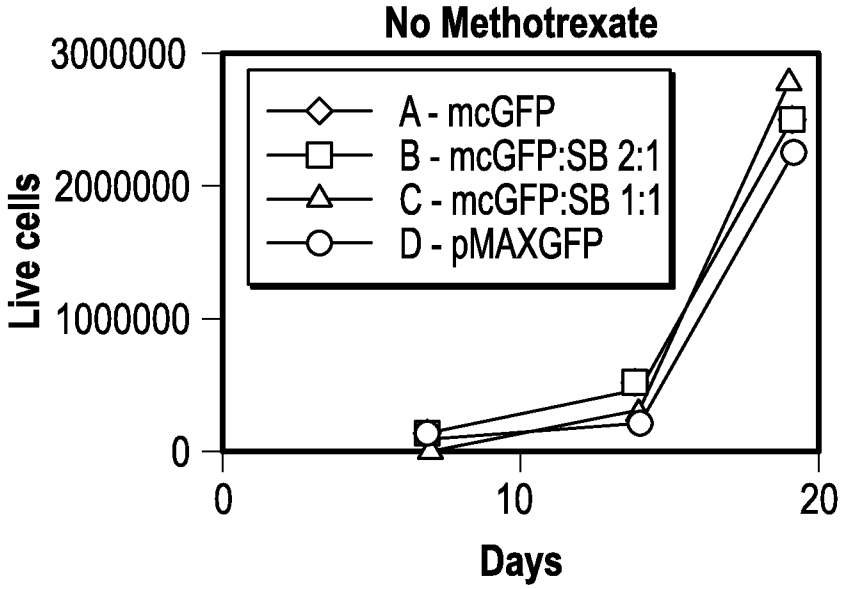


FIG. 14A

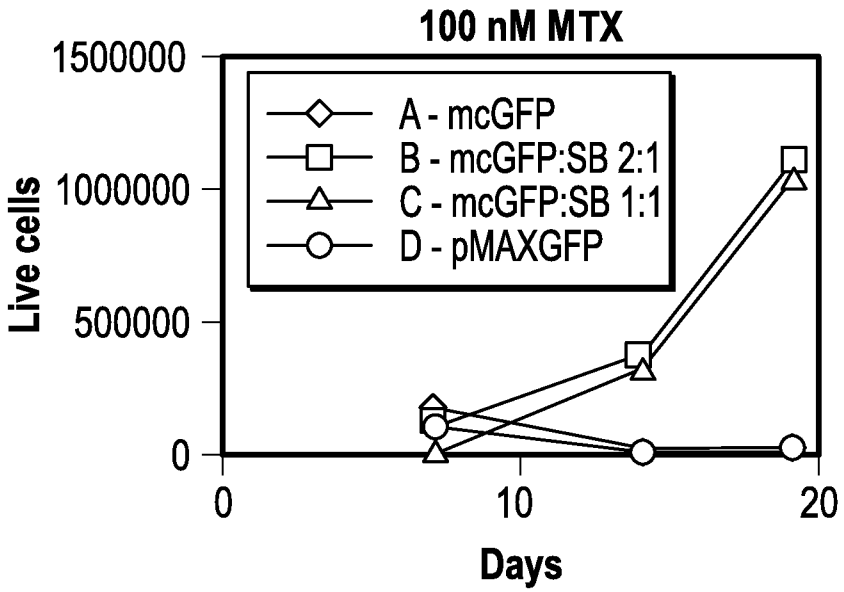


FIG. 14B

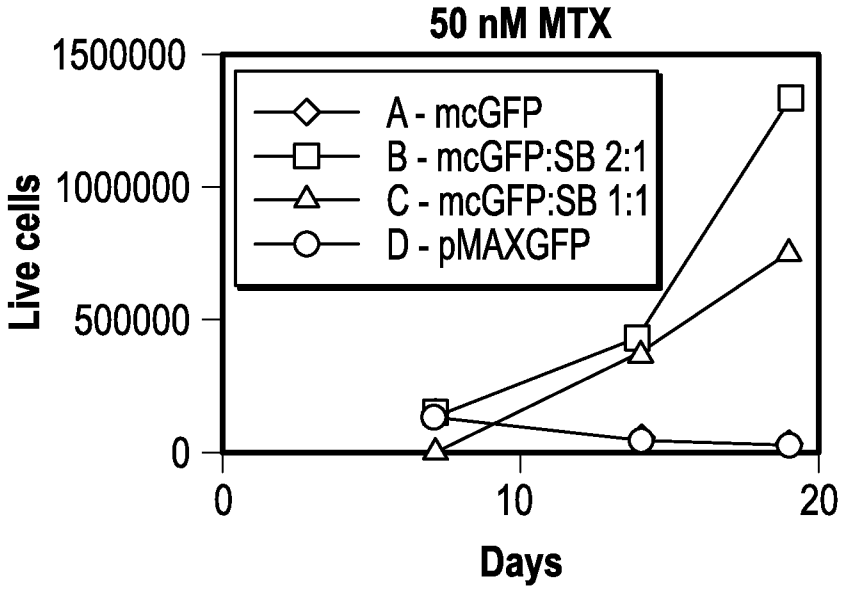


FIG. 14C

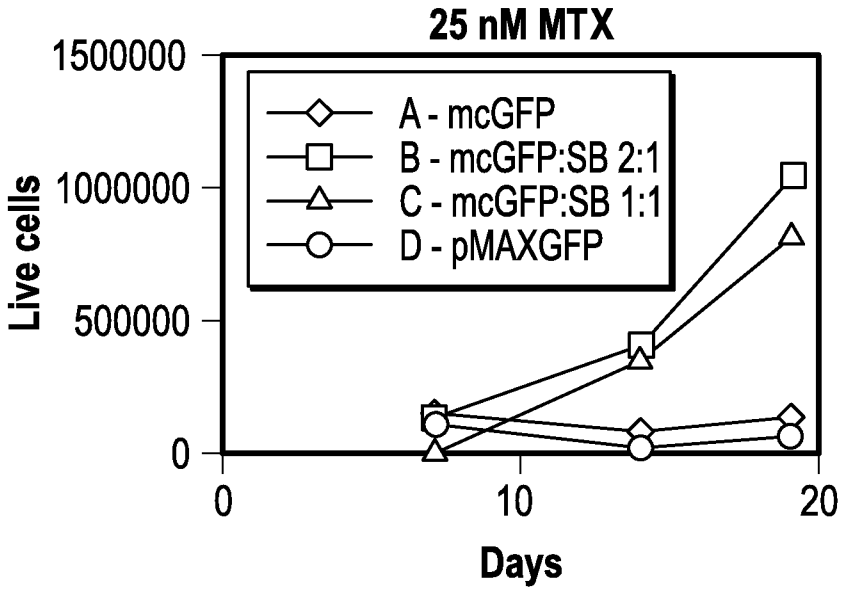


FIG. 14D

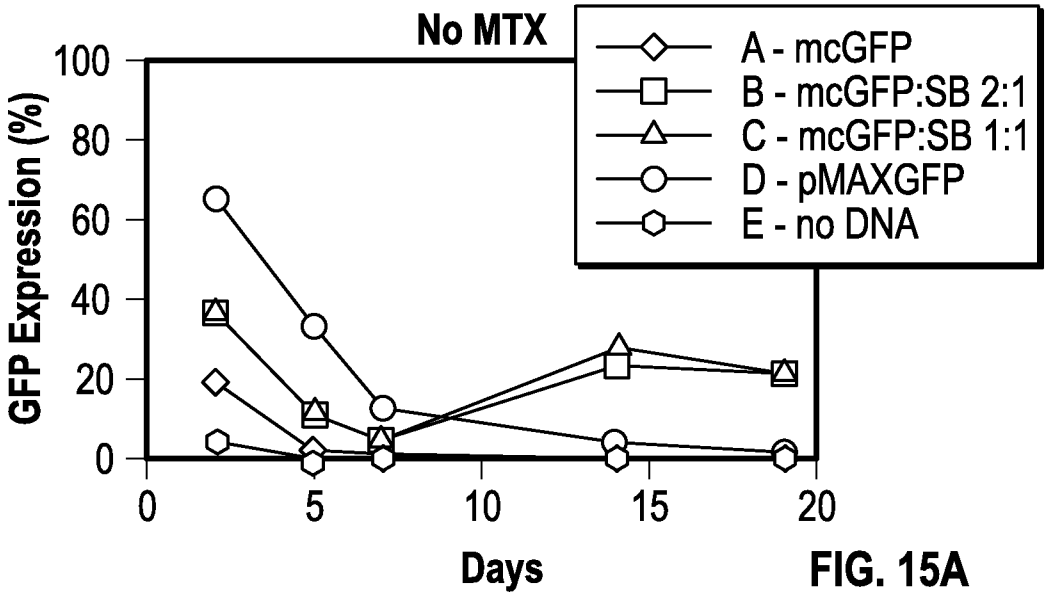


FIG. 15A

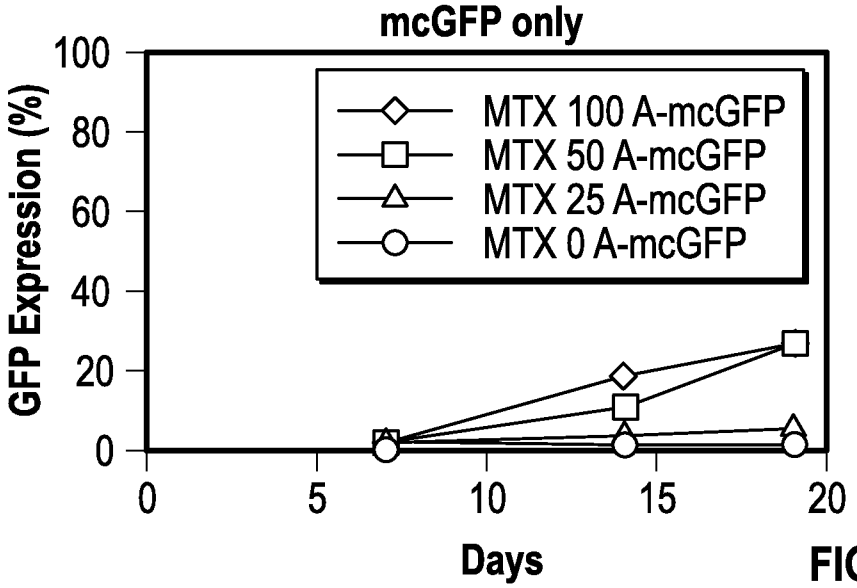


FIG. 15B

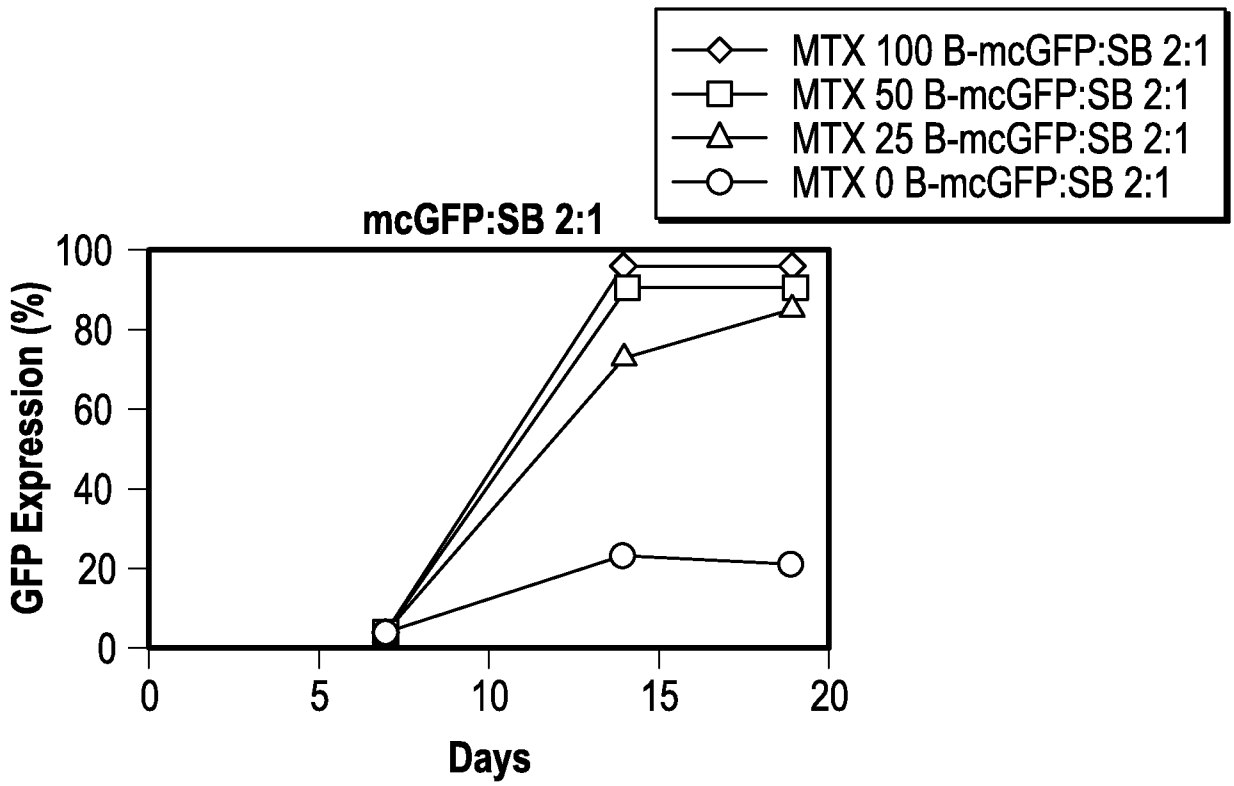


FIG. 15C

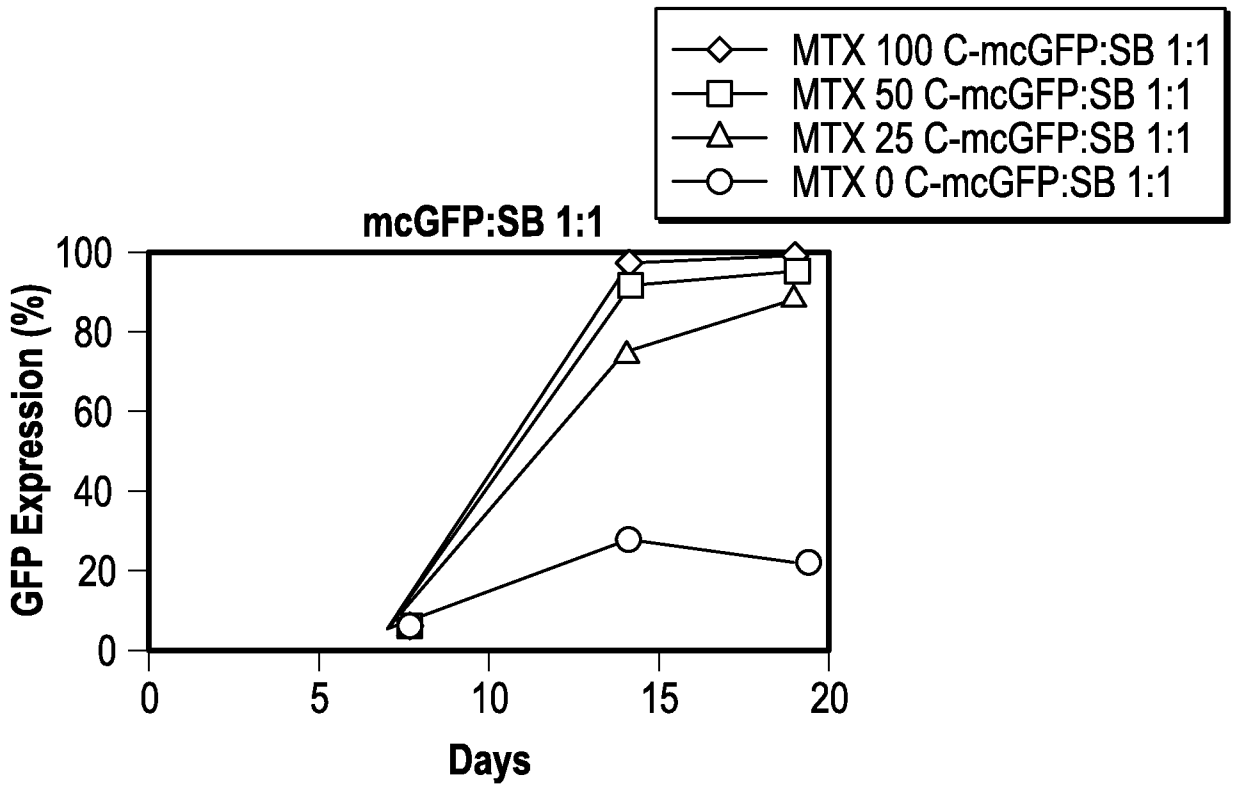


FIG. 15D

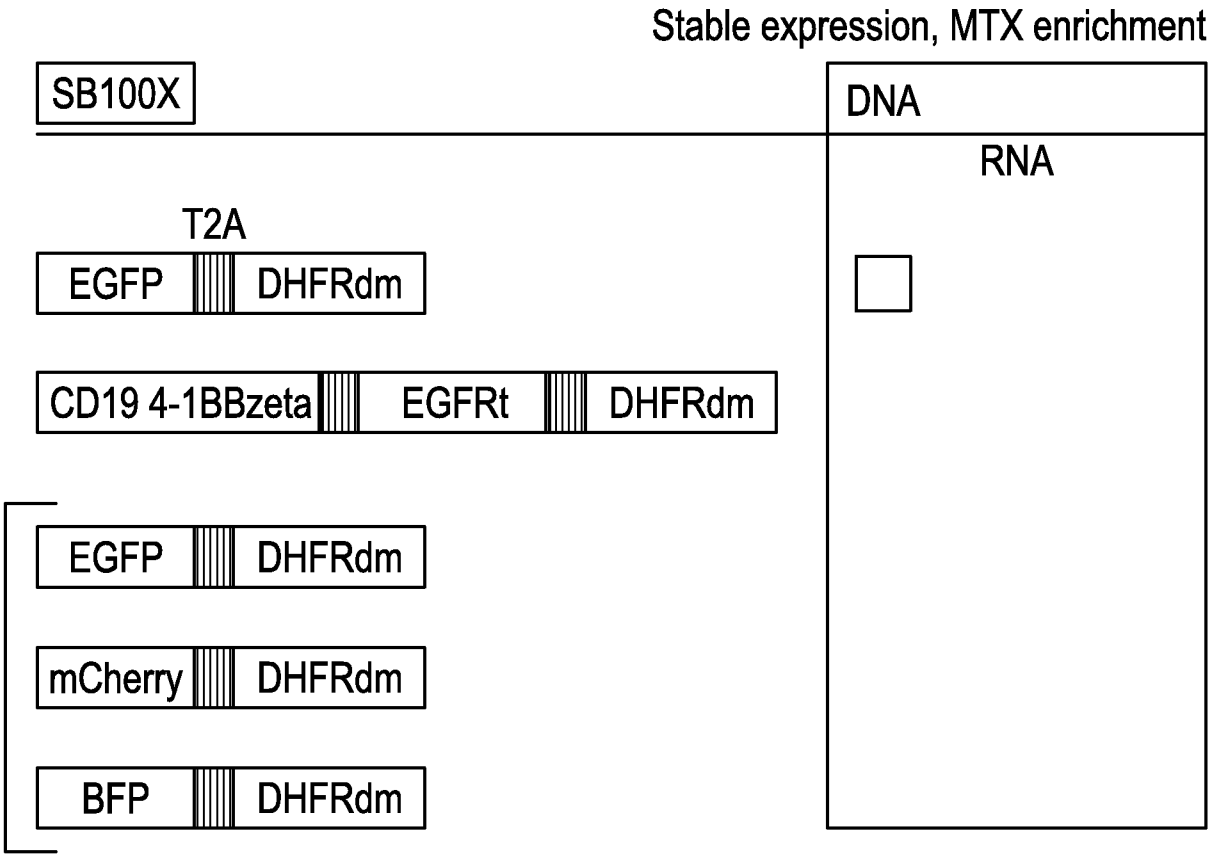


FIG. 16

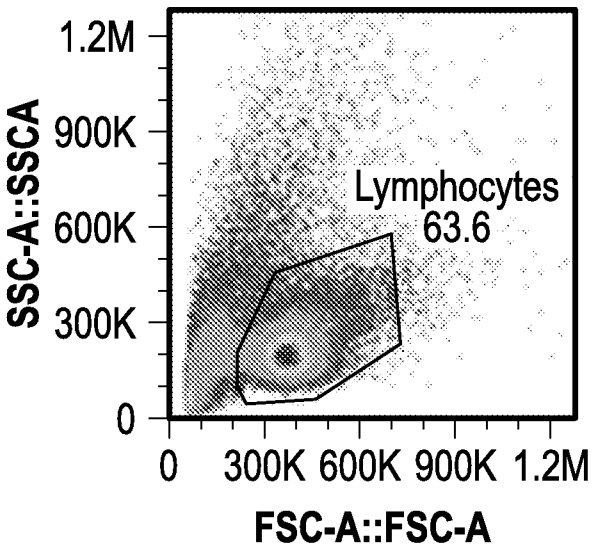


FIG. 17A

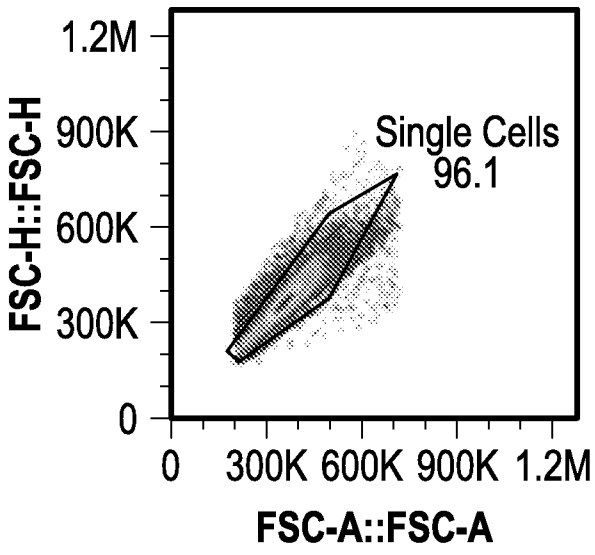


FIG. 17B

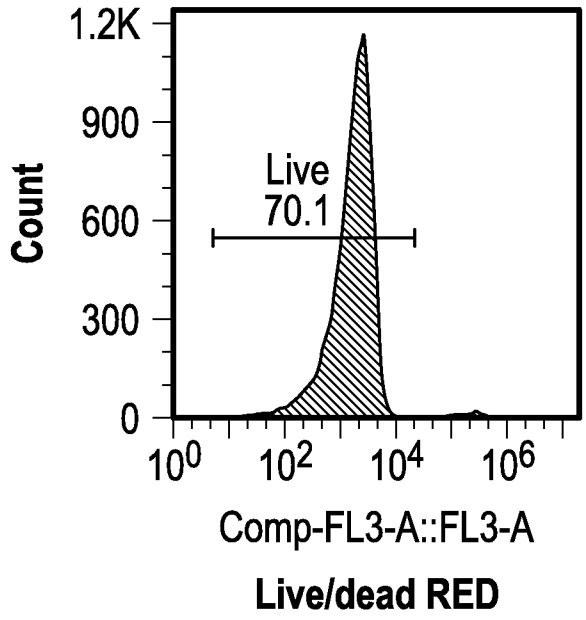


FIG. 17C

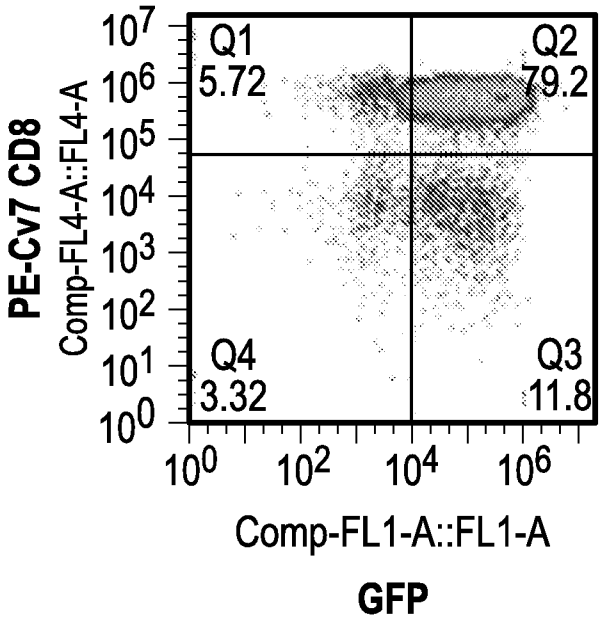


FIG. 17D

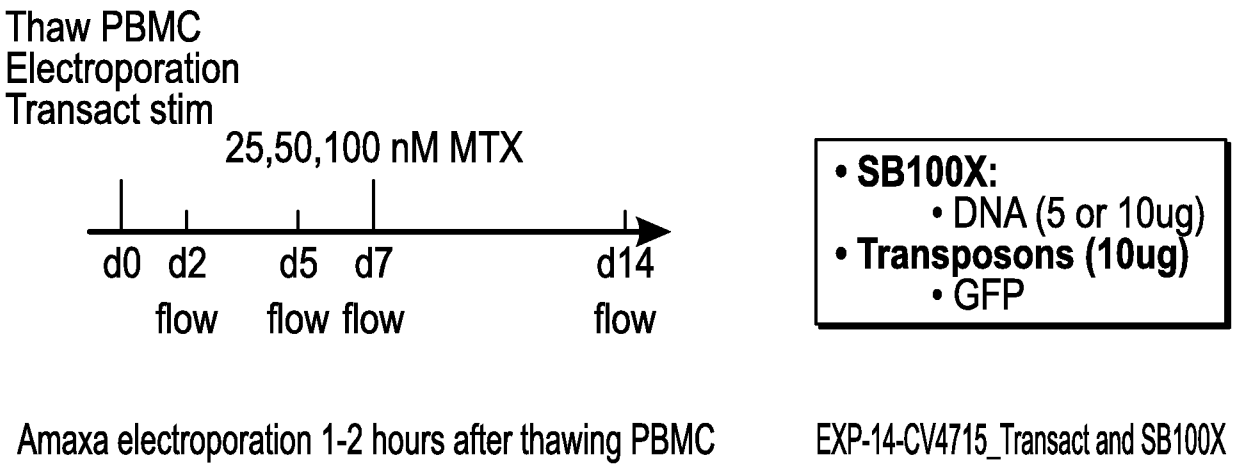


FIG. 17E

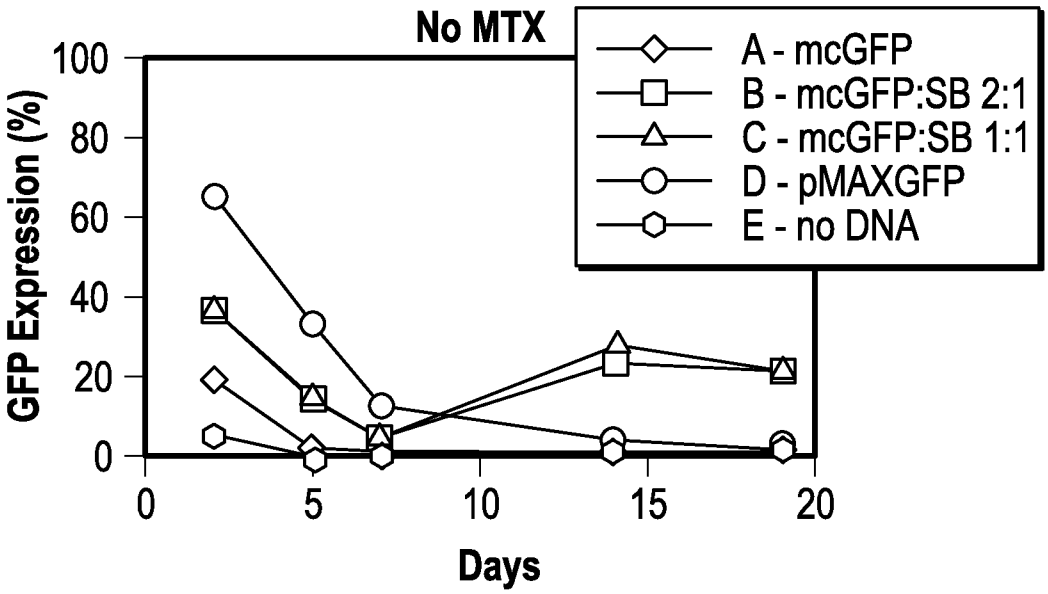


FIG. 18A

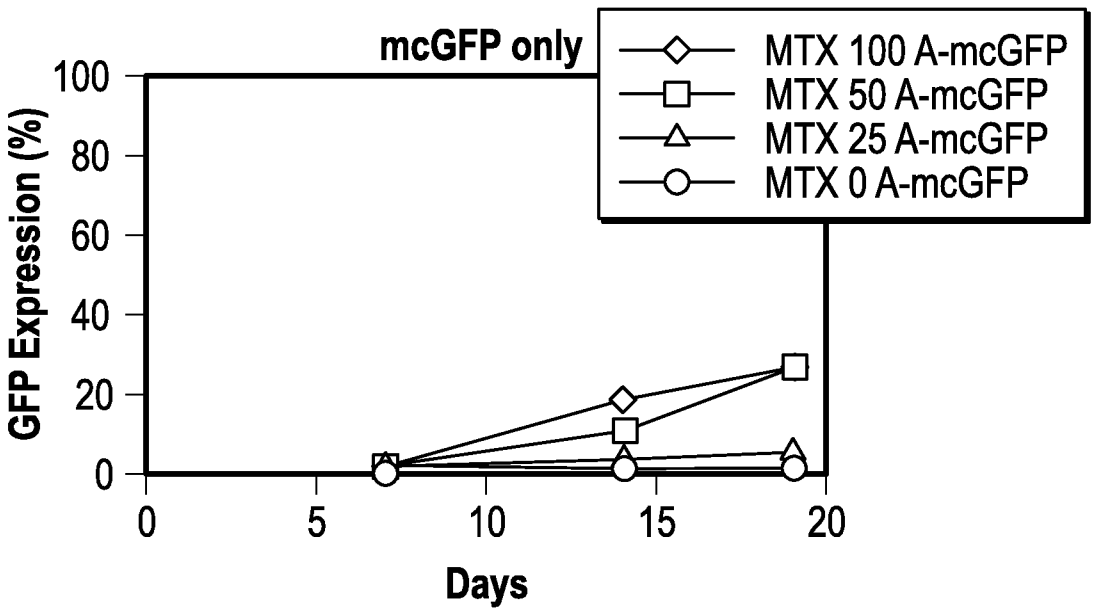


FIG. 18B

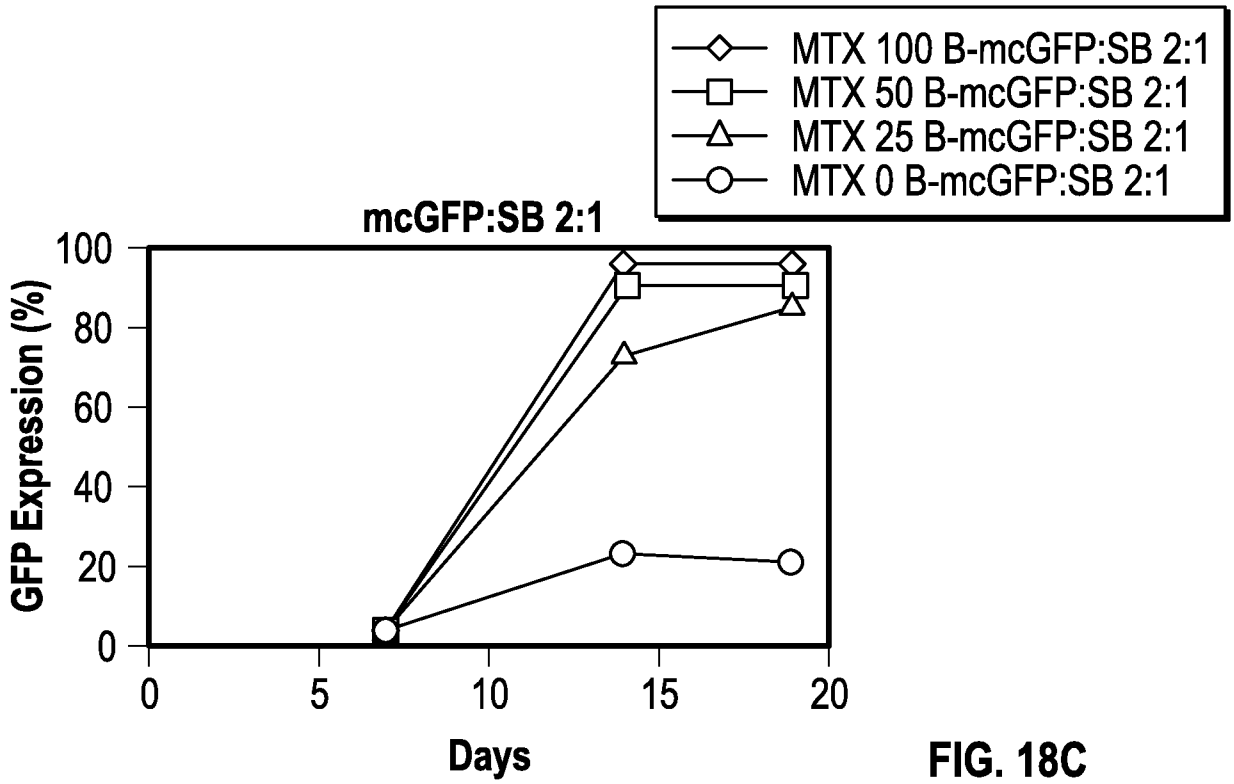
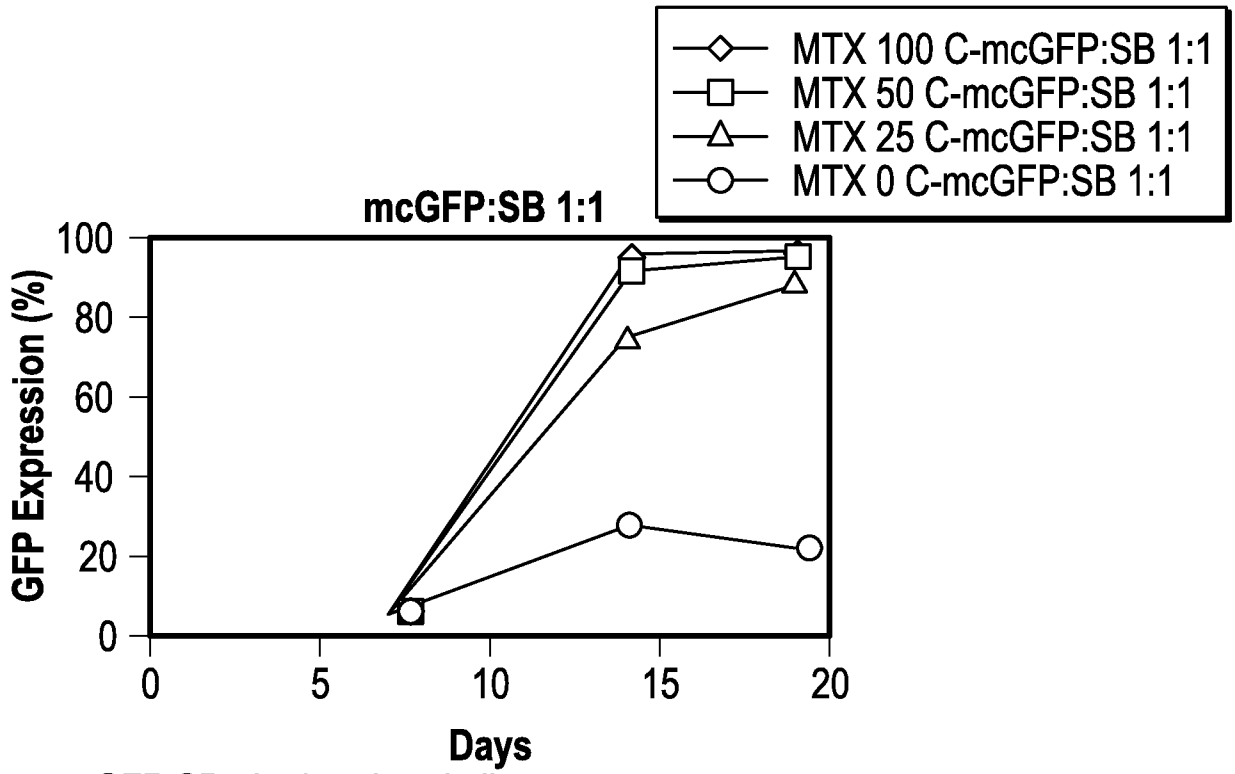


FIG. 18C



- mcGFP:SB - both ratios similar
- >90% GFP positive after 1 week
- Minimal difference in MFI for 50 versus 100 nM MTX

EXP-14-CV4715_Transact and SB100X

FIG. 18D

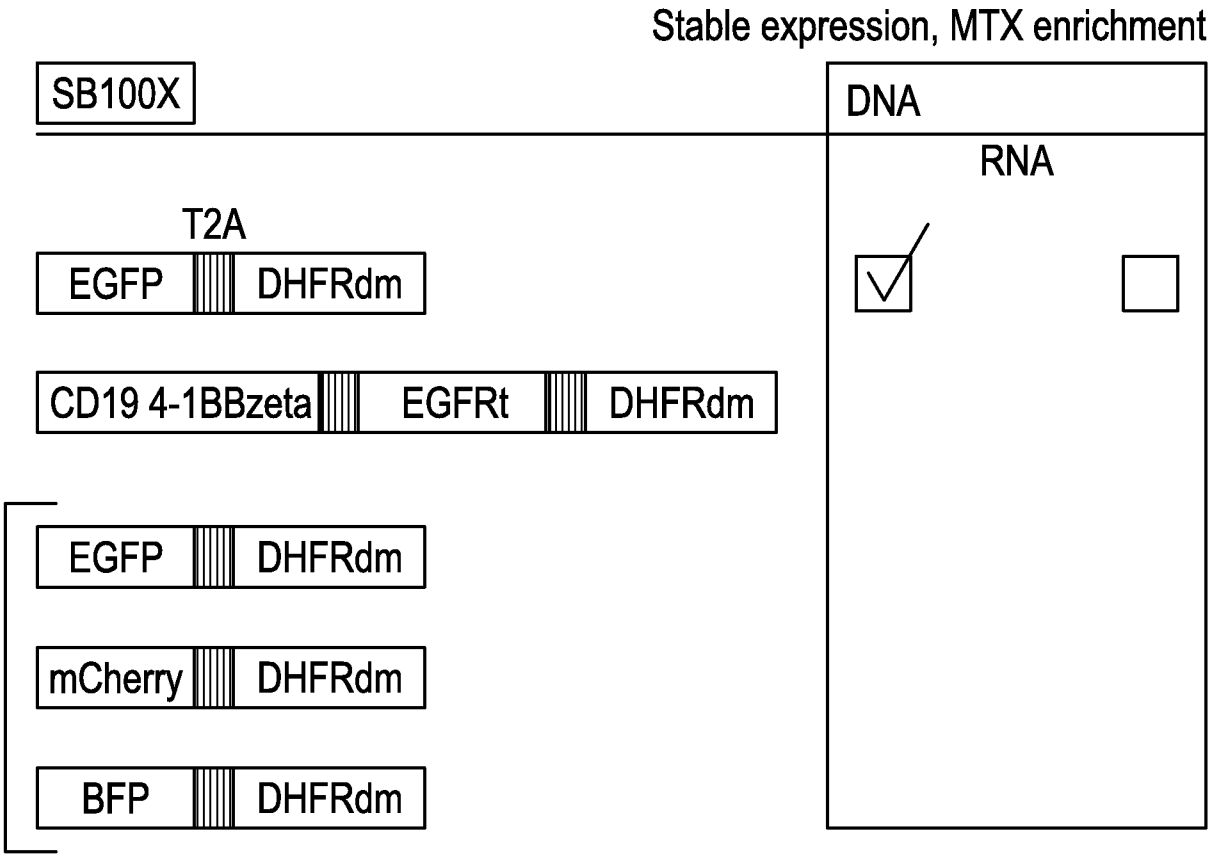


FIG. 19

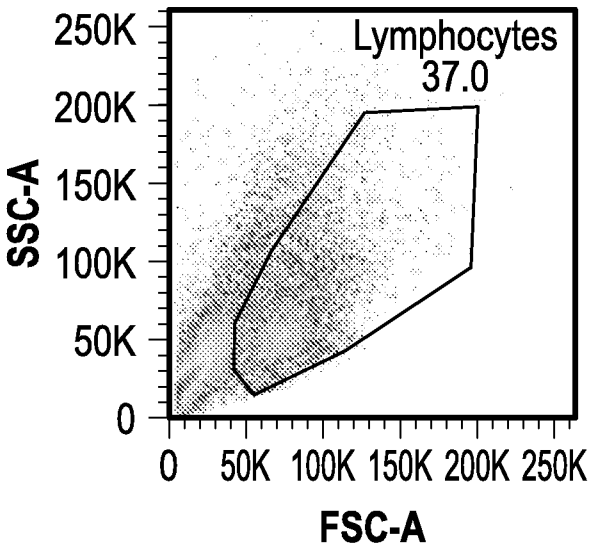


FIG. 20A

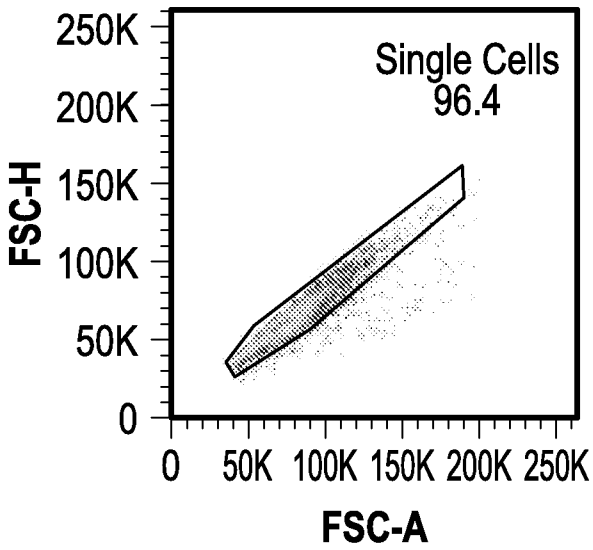


FIG. 20B

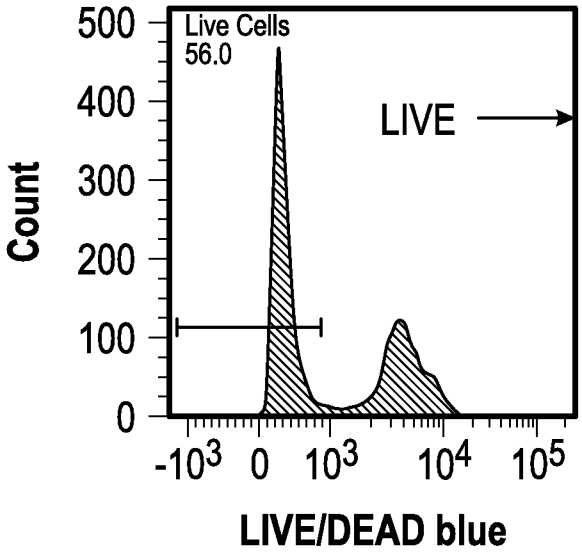


FIG. 20C

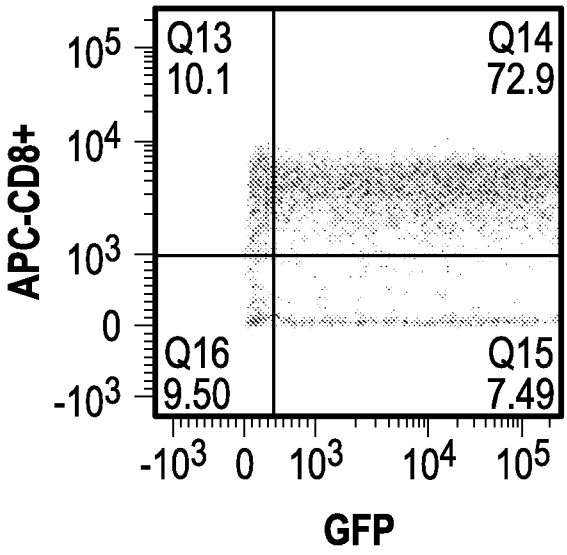
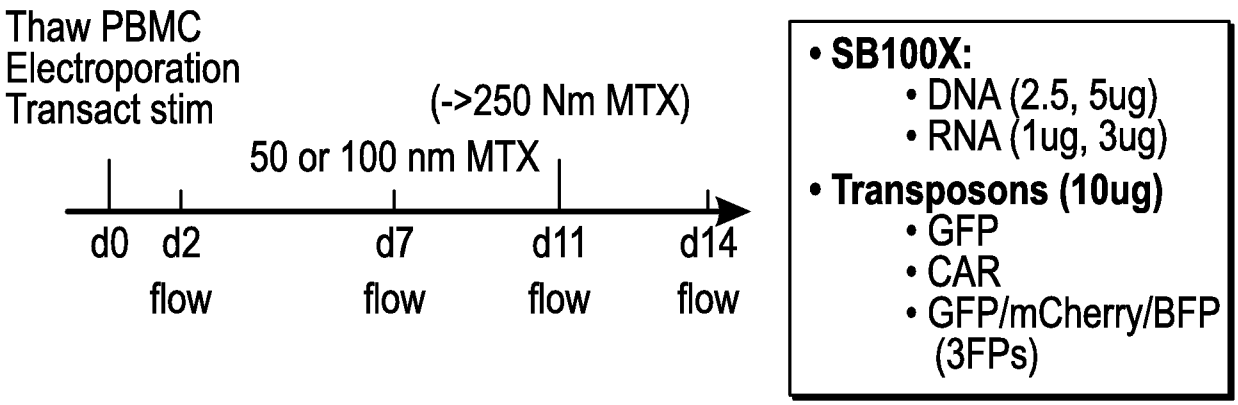


FIG. 20D



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LHOROW-EXP-14-CV4717_248481-SB100X RNA and multiplex electroporation

FIG. 20E

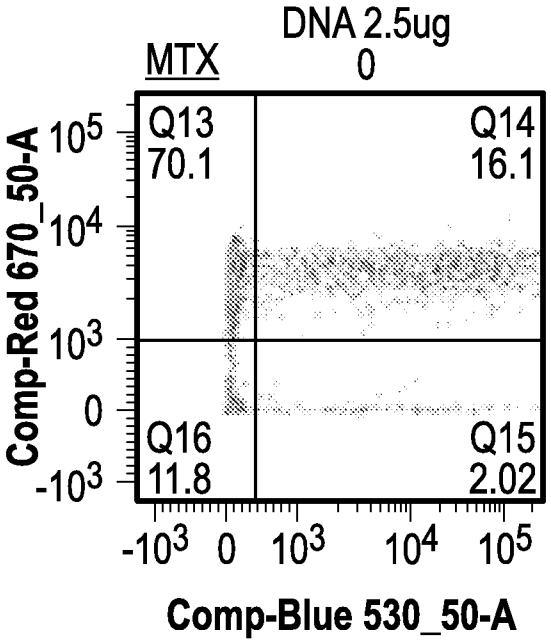


FIG. 21A

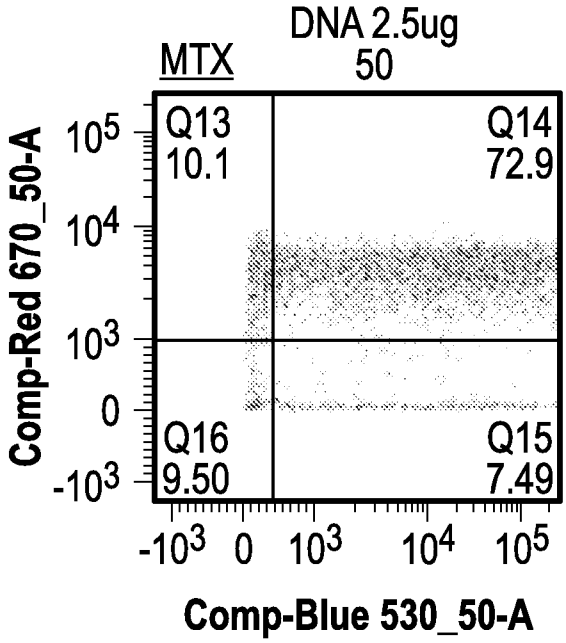


FIG. 21B

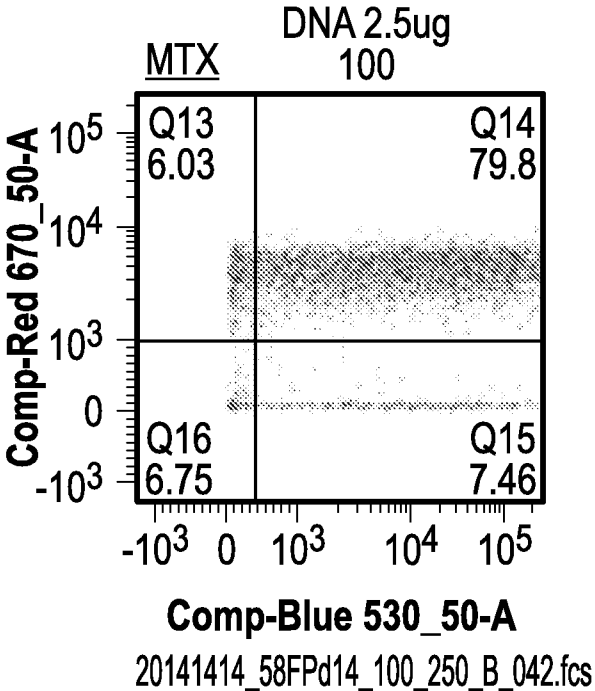


FIG. 21C

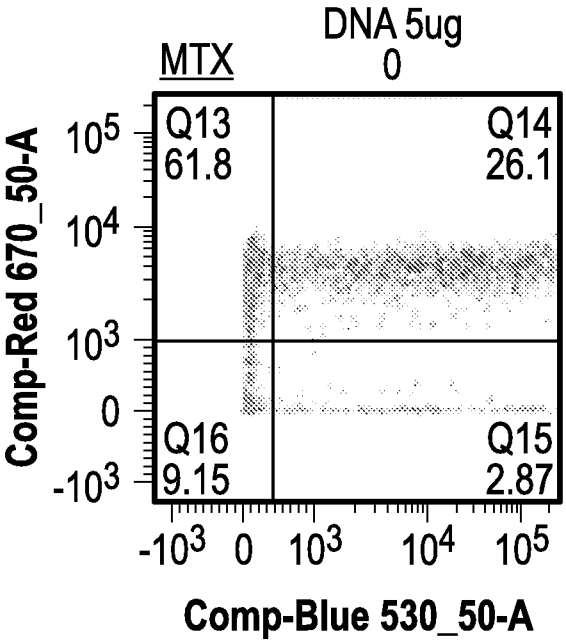


FIG. 21D

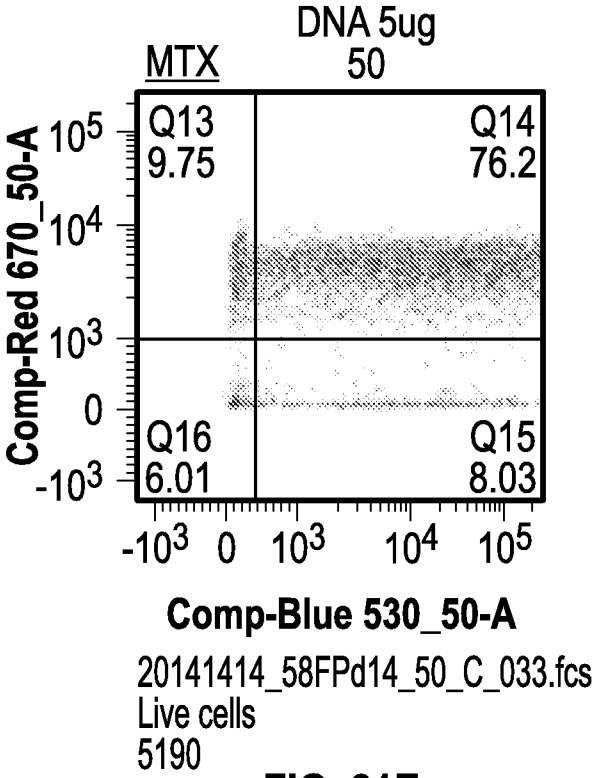


FIG. 21E

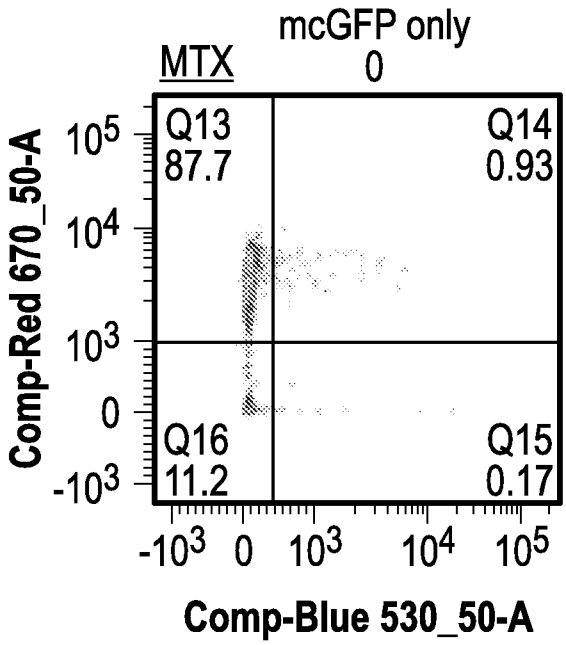


FIG. 21F

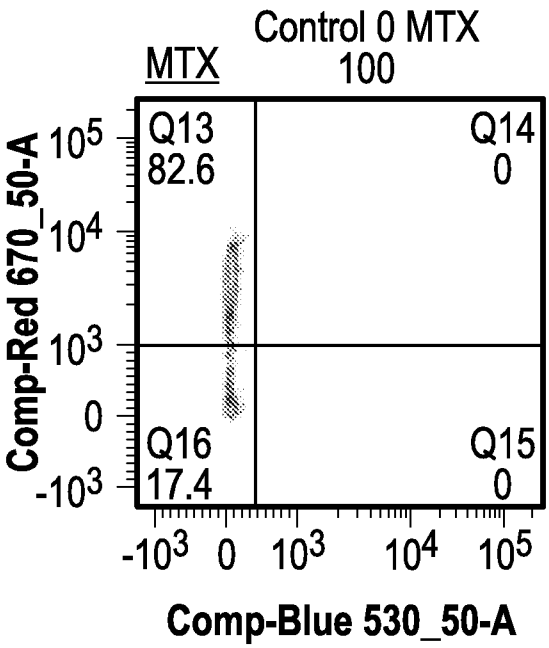


FIG. 21G

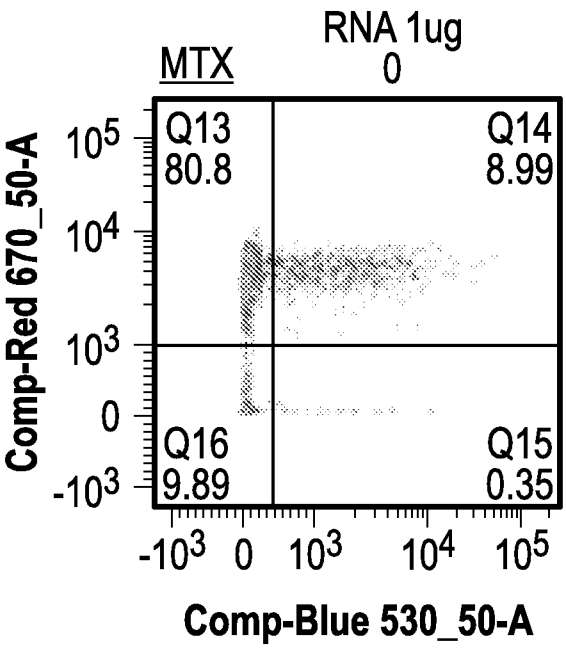


FIG. 21H

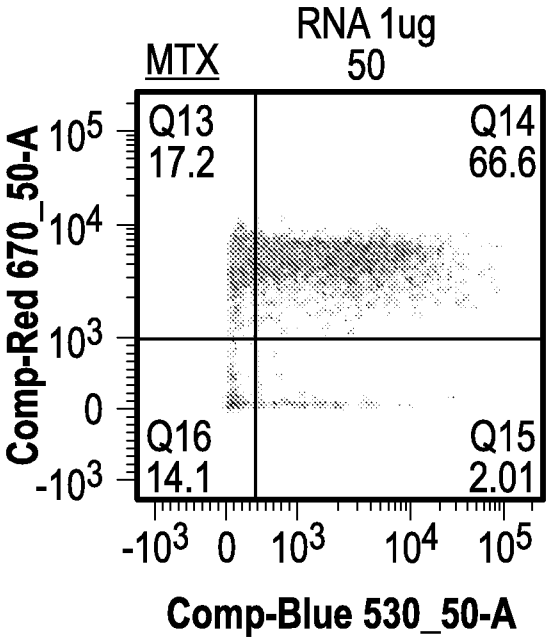


FIG. 21I

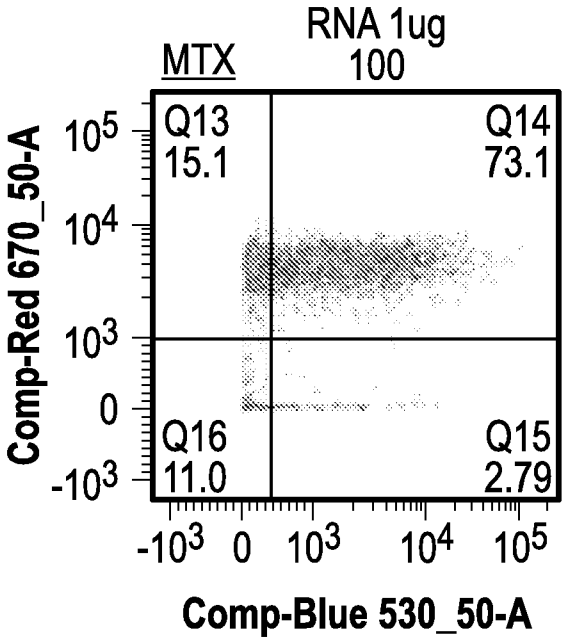


FIG. 21J

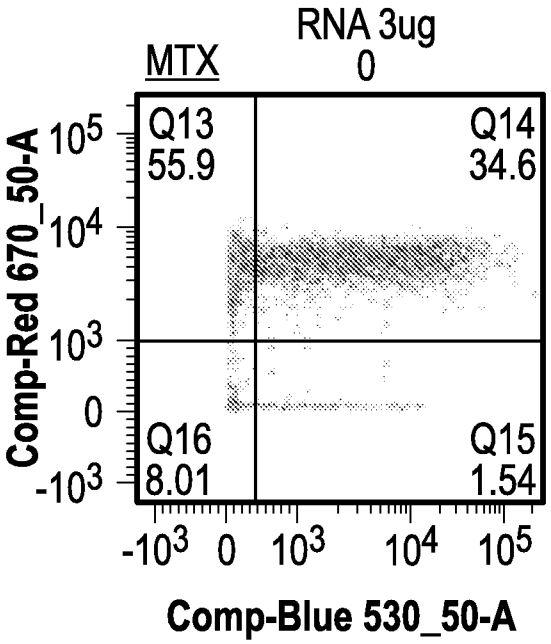


FIG. 21K

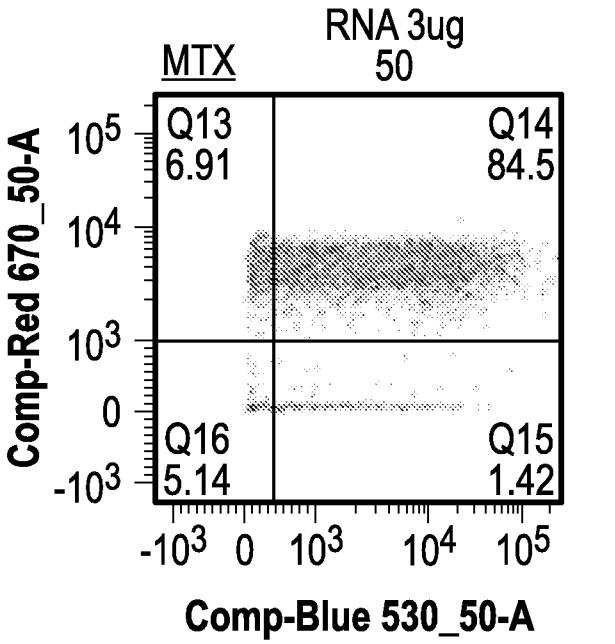


FIG. 21L

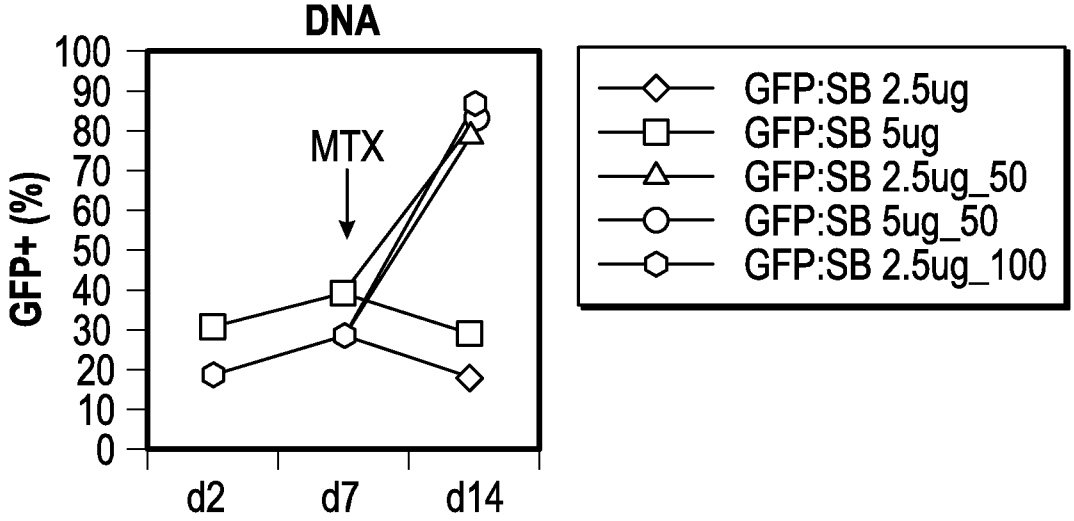


FIG. 22A

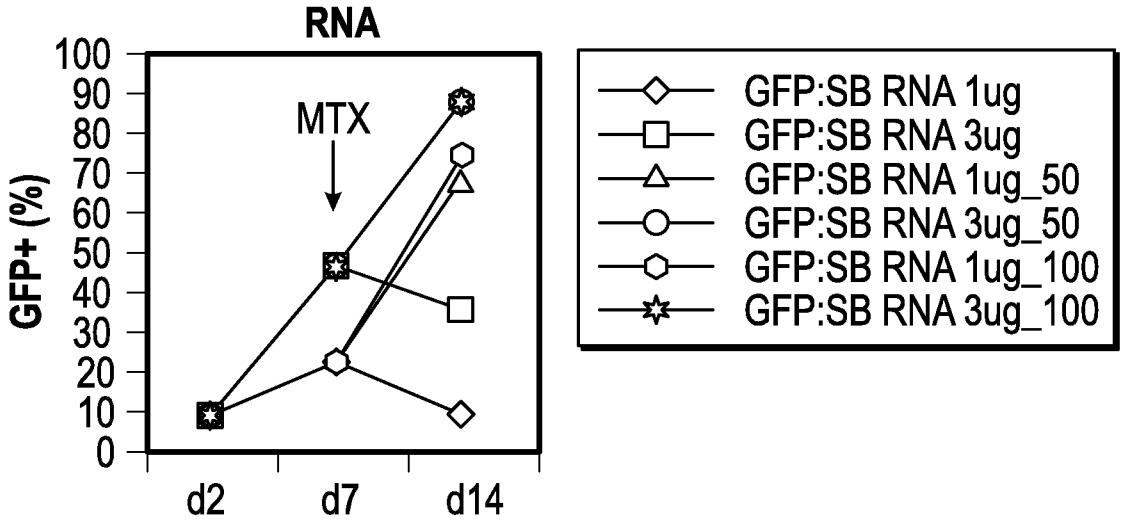


FIG. 22B

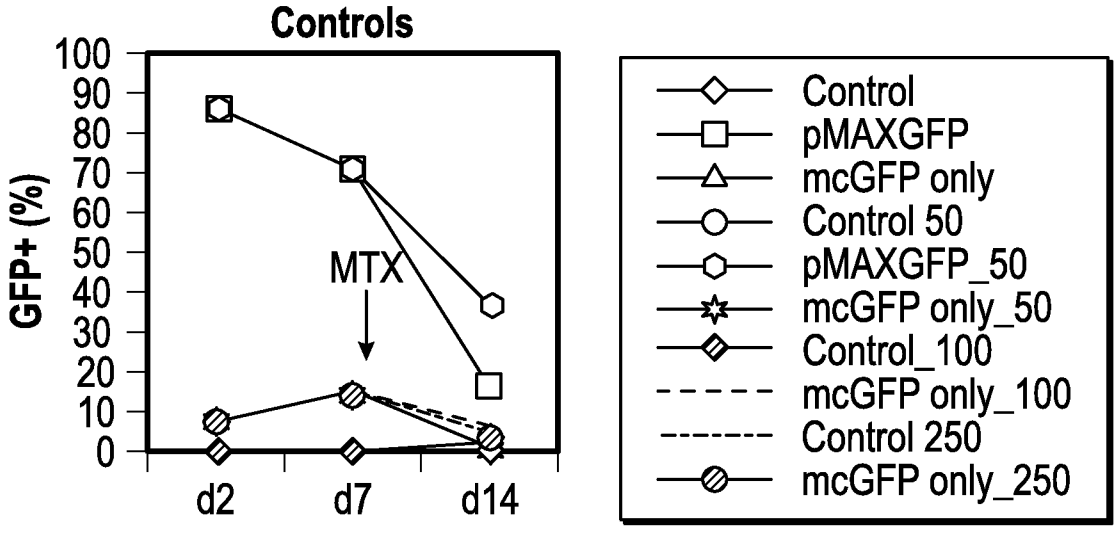


FIG. 22C

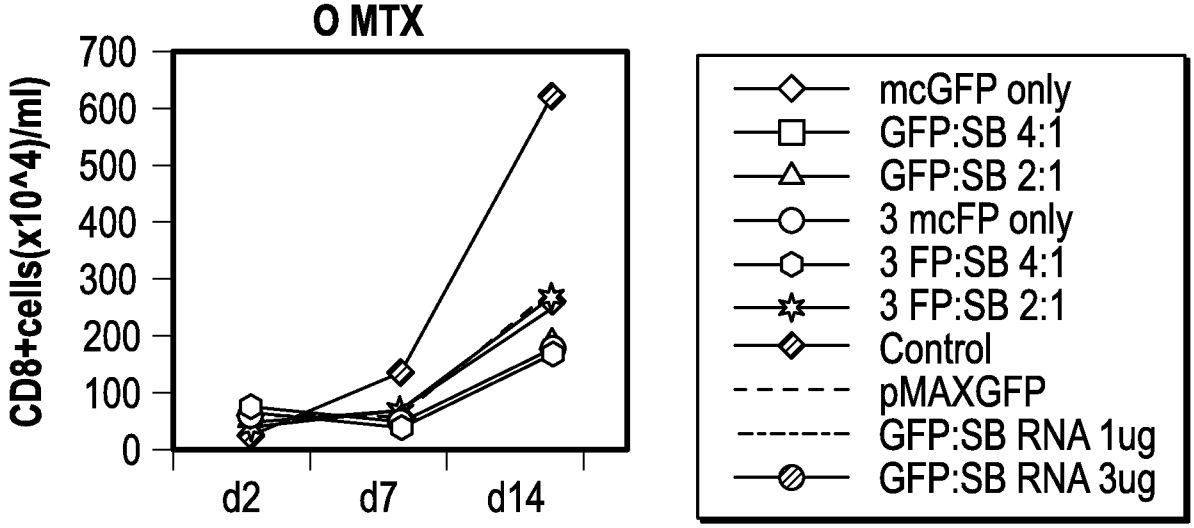


FIG. 23A

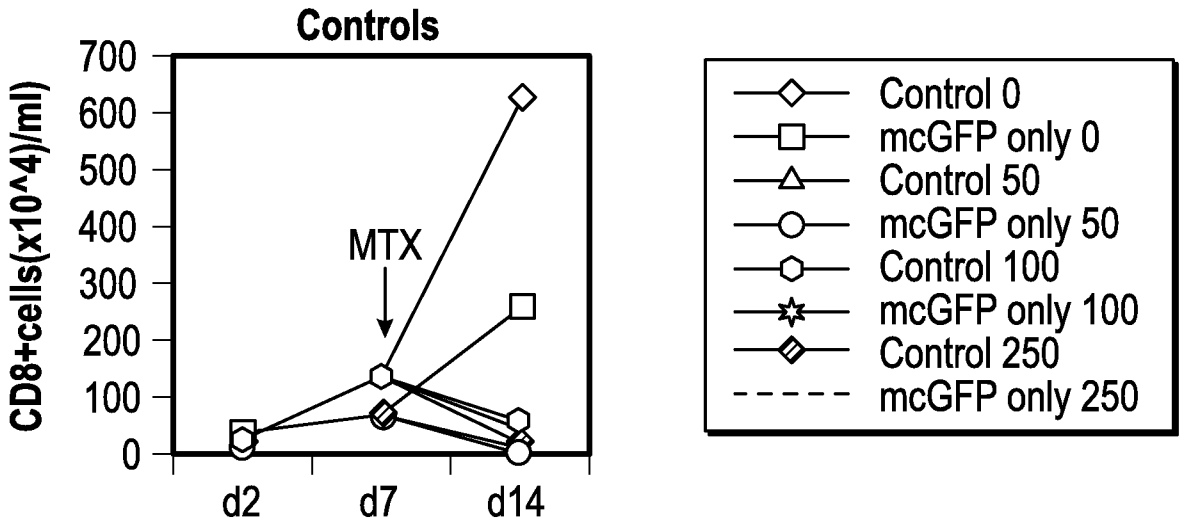


FIG. 23B

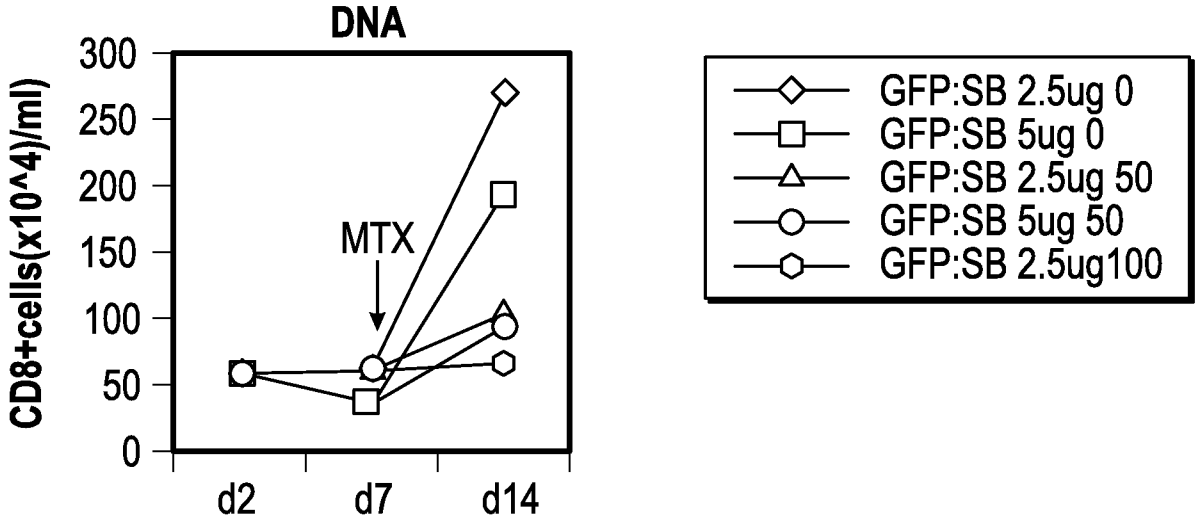


FIG. 23C

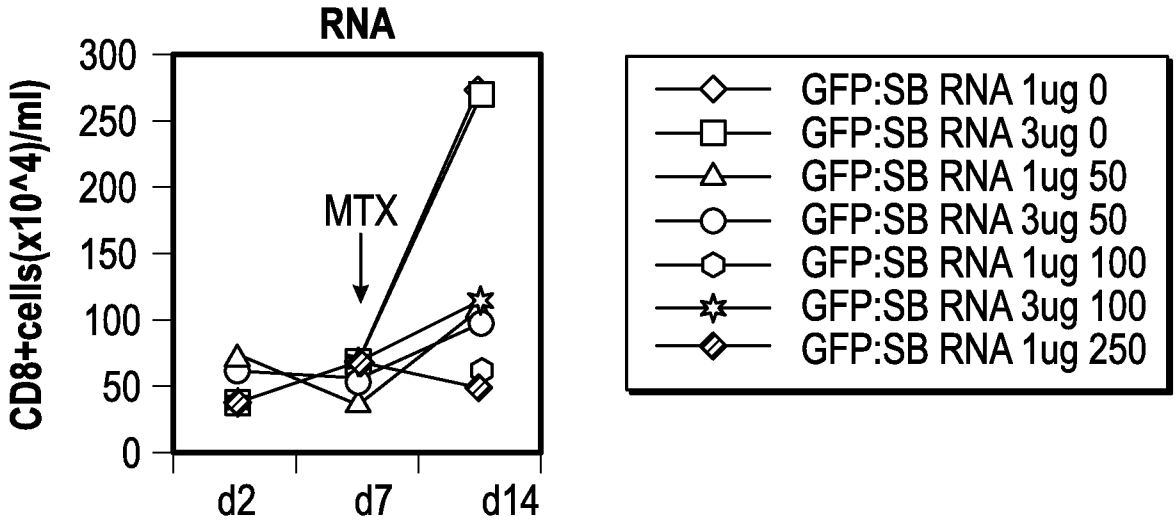
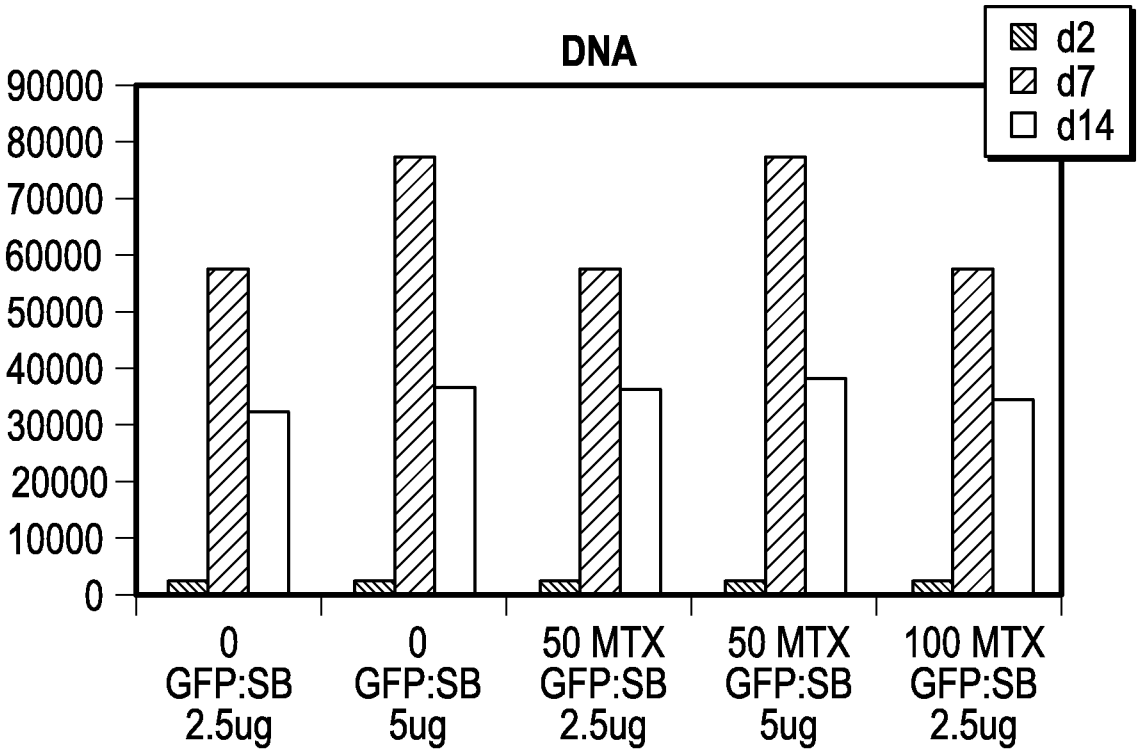
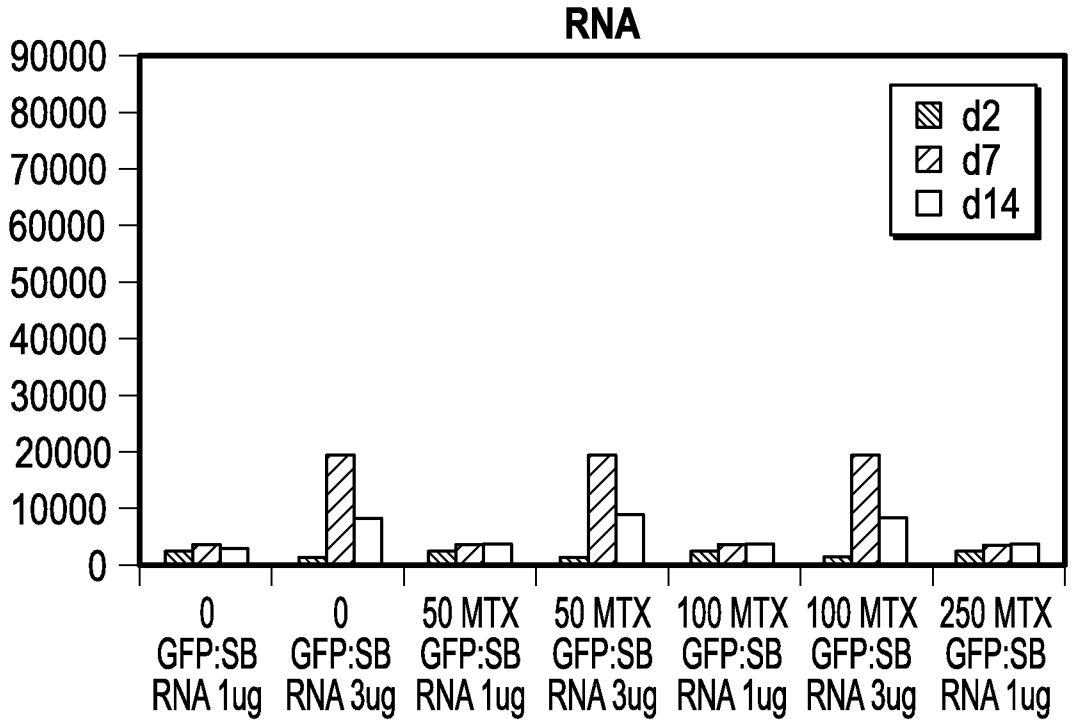


FIG. 23D



- DNA-**lower** concentration lower efficiency but better growth.
- RNA-**higher** concentration higher efficiency and MFI, similar growth.
- **DNA** higher initial efficiency than RNA, higher MFI.
- → try higher RNA concentrations? SB100X toxicity?

FIG. 24A



- DNA-**lower** concentration lower efficiency but better growth.
- RNA-**higher** concentration higher efficiency and MFI, similar growth.
- **DNA** higher initial efficiency than RNA, higher MFI.
- → try higher RNA concentrations? SB100X toxicity?

FIG. 24B

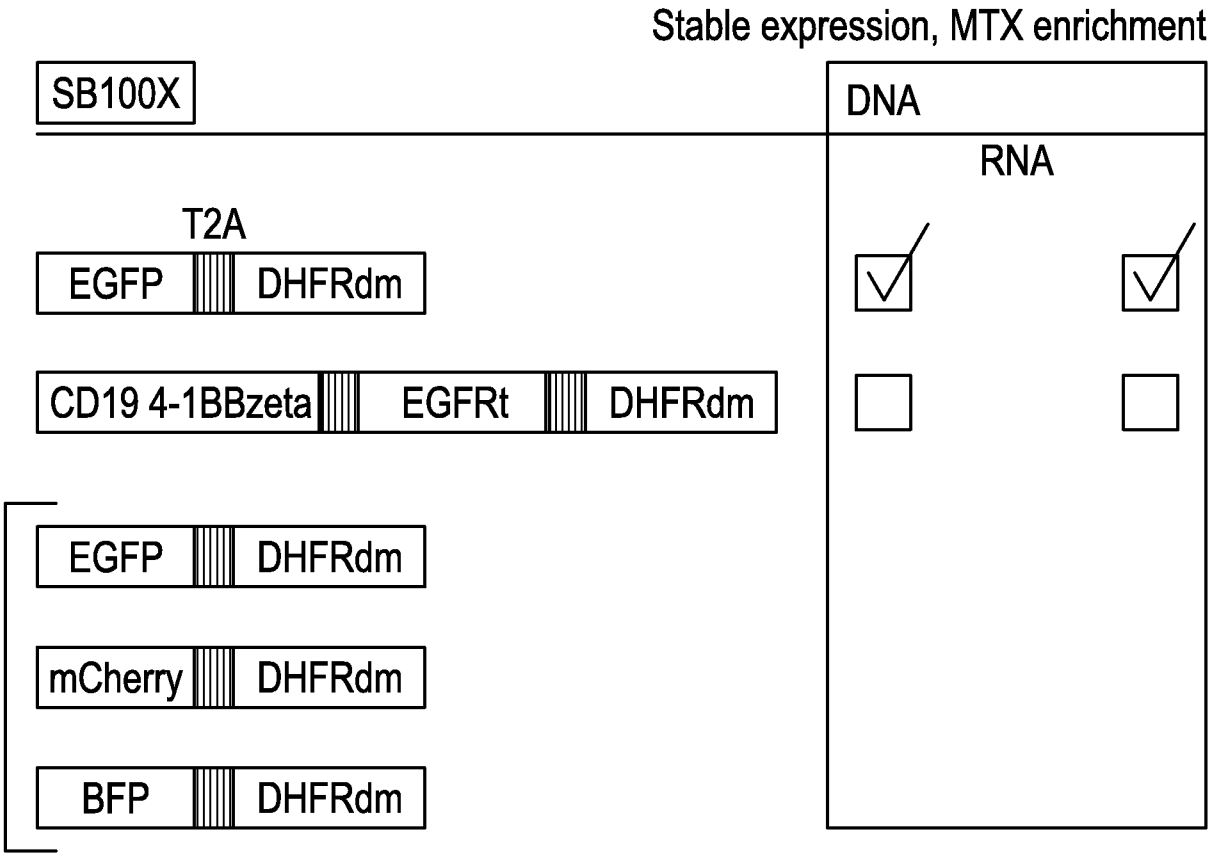


FIG. 25

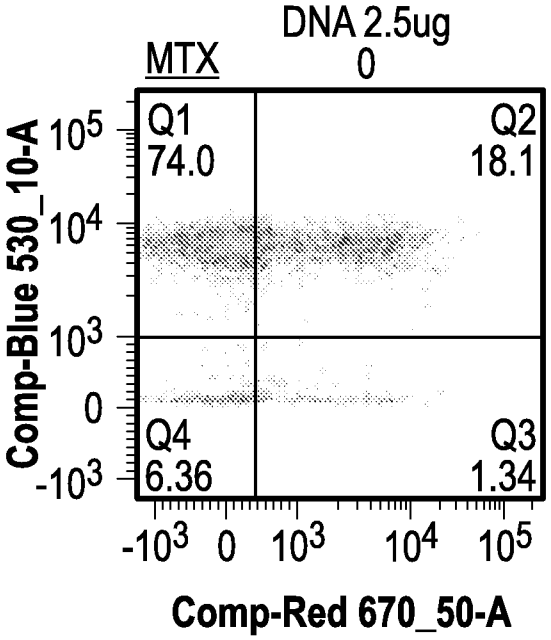


FIG. 26A

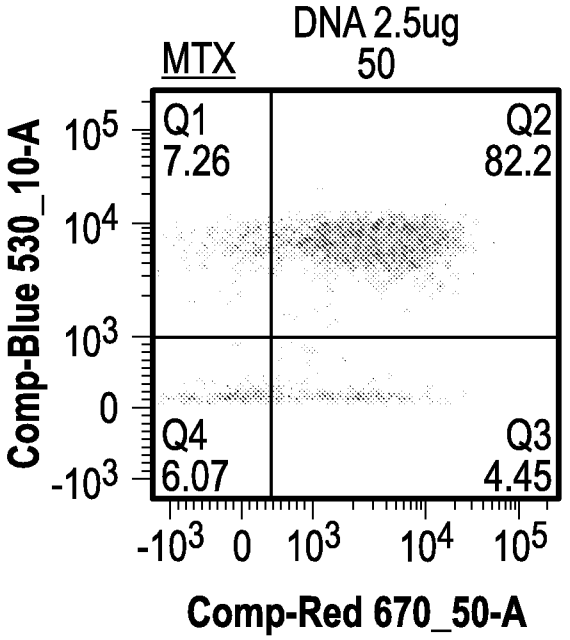


FIG. 26B

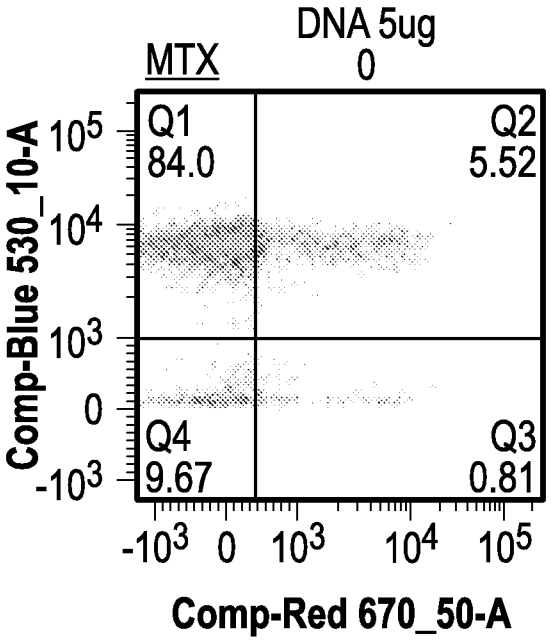
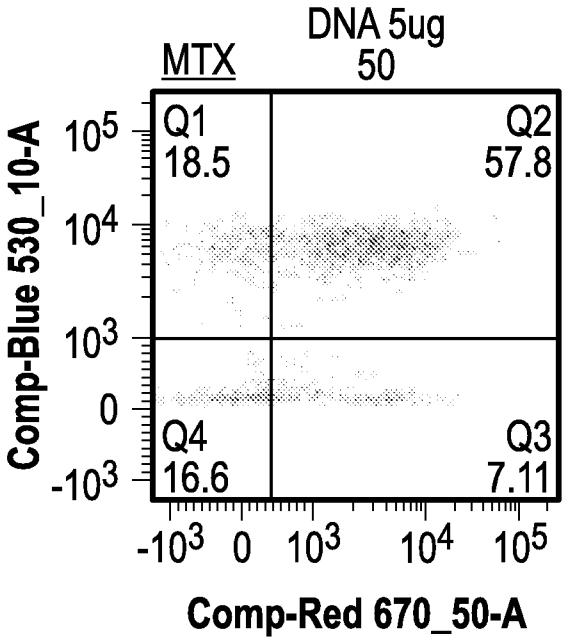
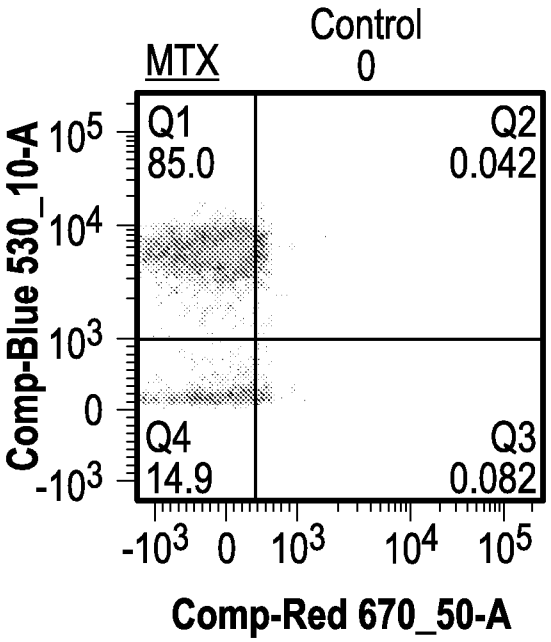


FIG. 26C



20141414_SBCARd14_50_H_027.fcs
Live cells
1921

FIG. 26D



20141414_SBCARd14_0_I_025.fcs
Live cells
8455

FIG. 26E

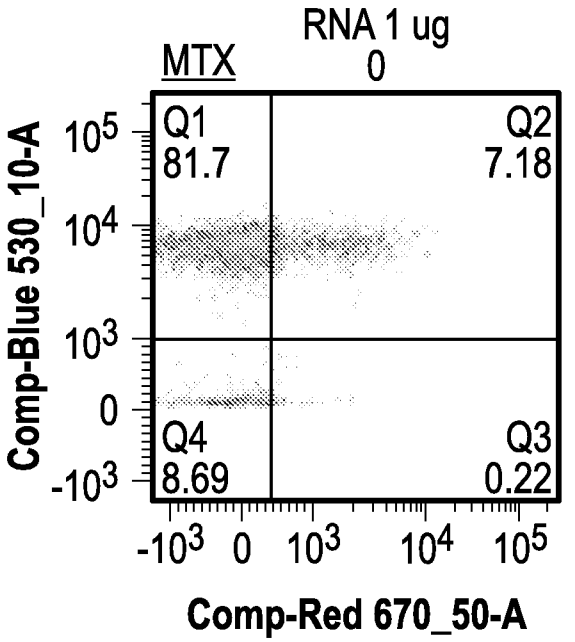


FIG. 26F

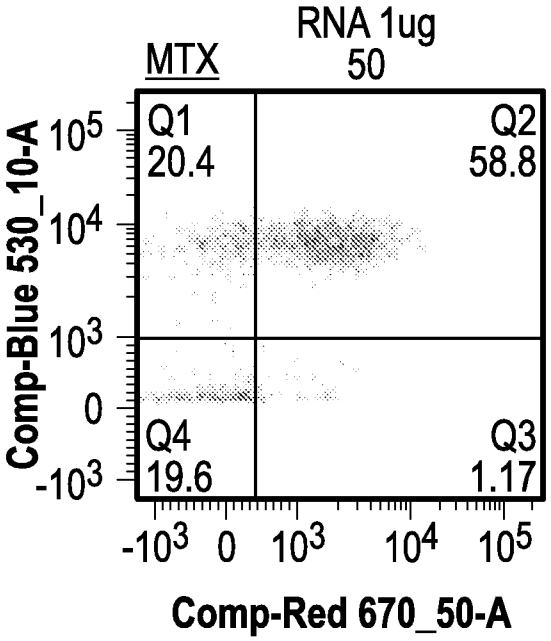


FIG. 26G

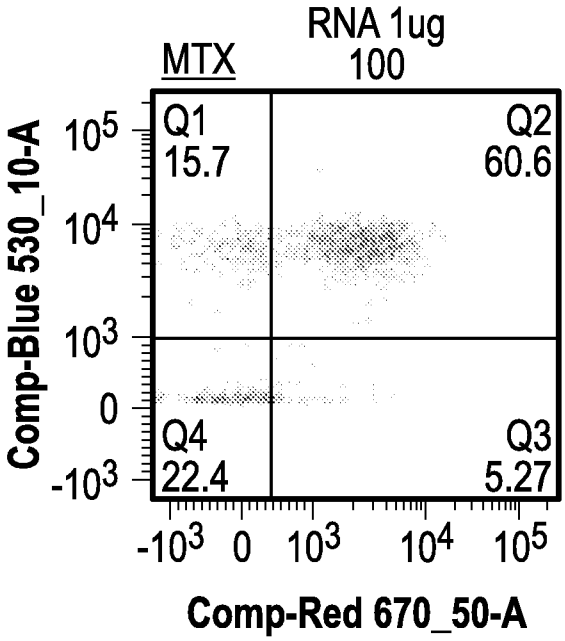


FIG. 26H

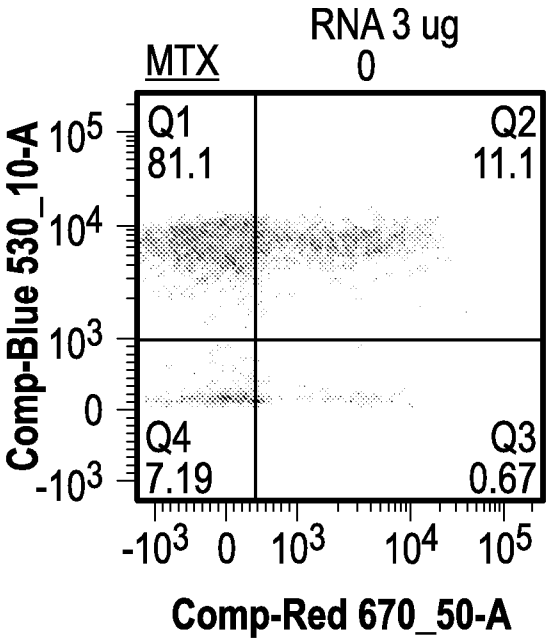


FIG. 26I

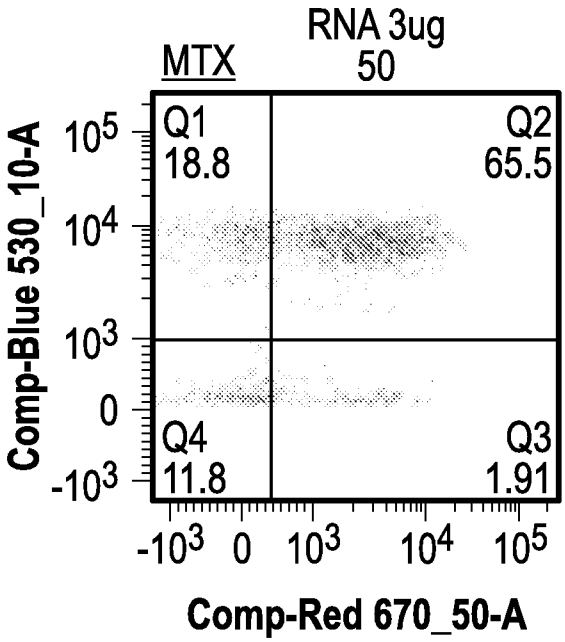


FIG. 26J

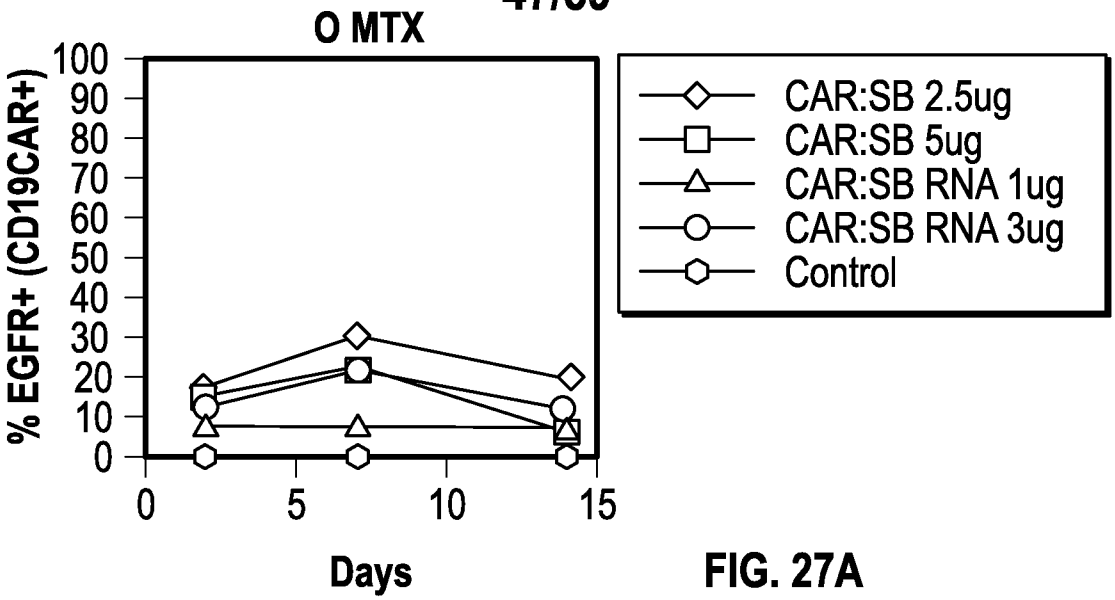


FIG. 27A

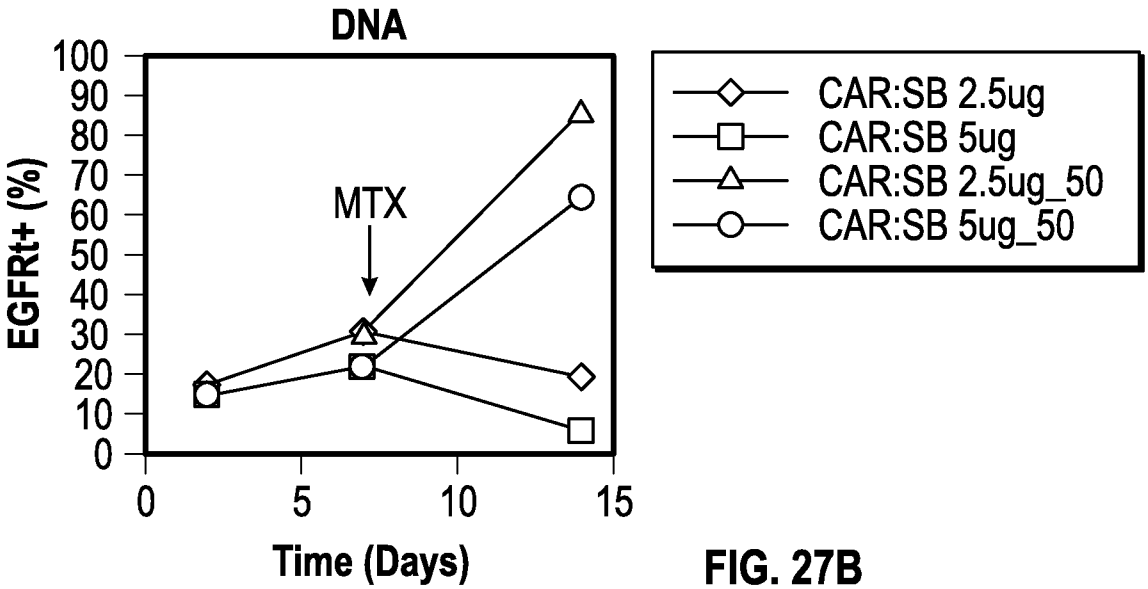


FIG. 27B

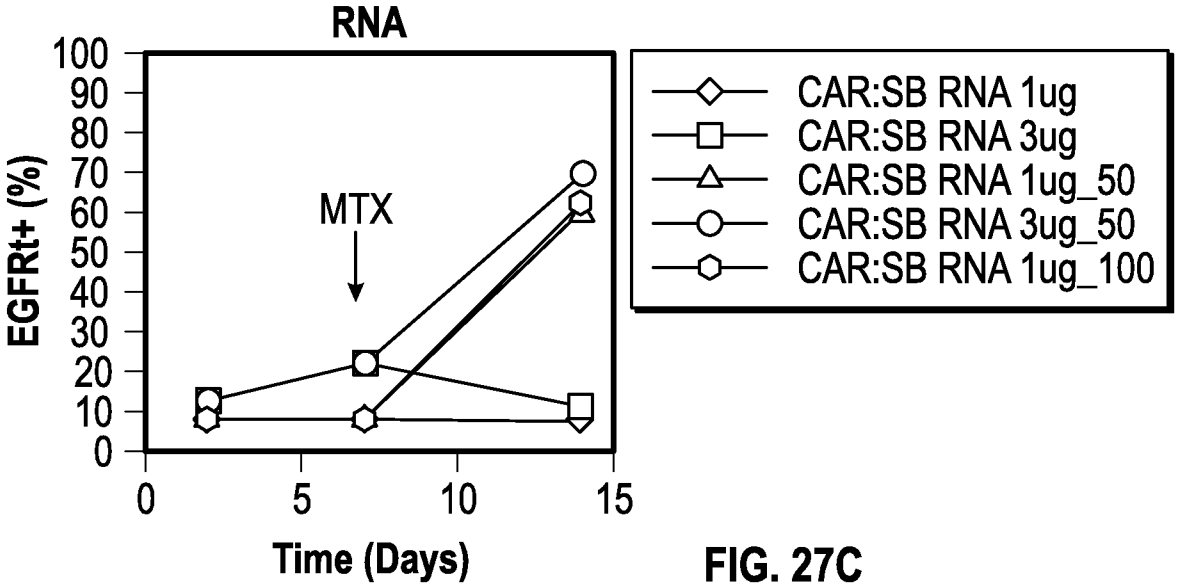


FIG. 27C

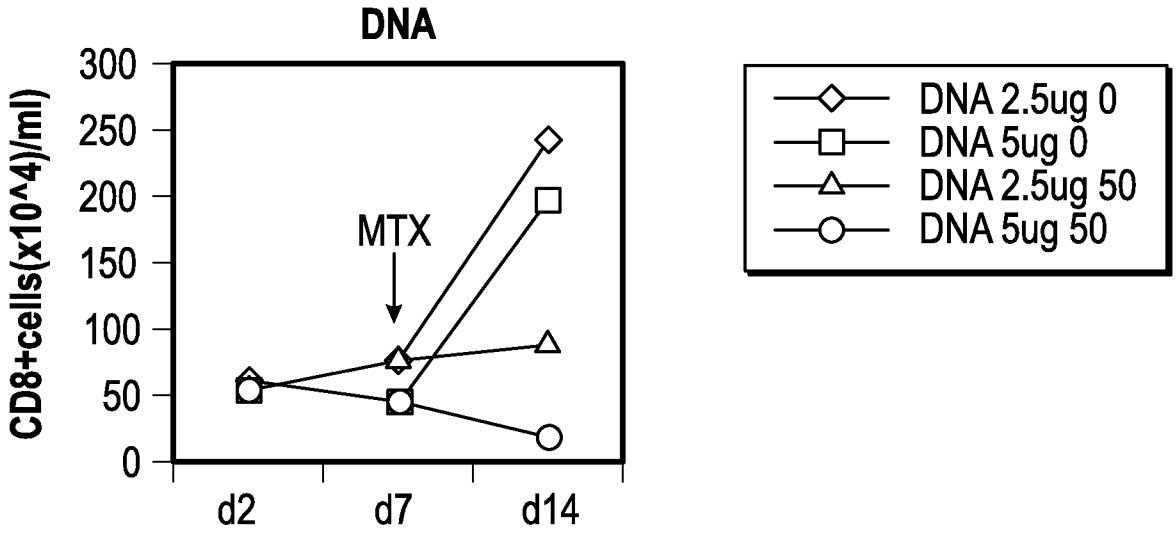


FIG. 28A

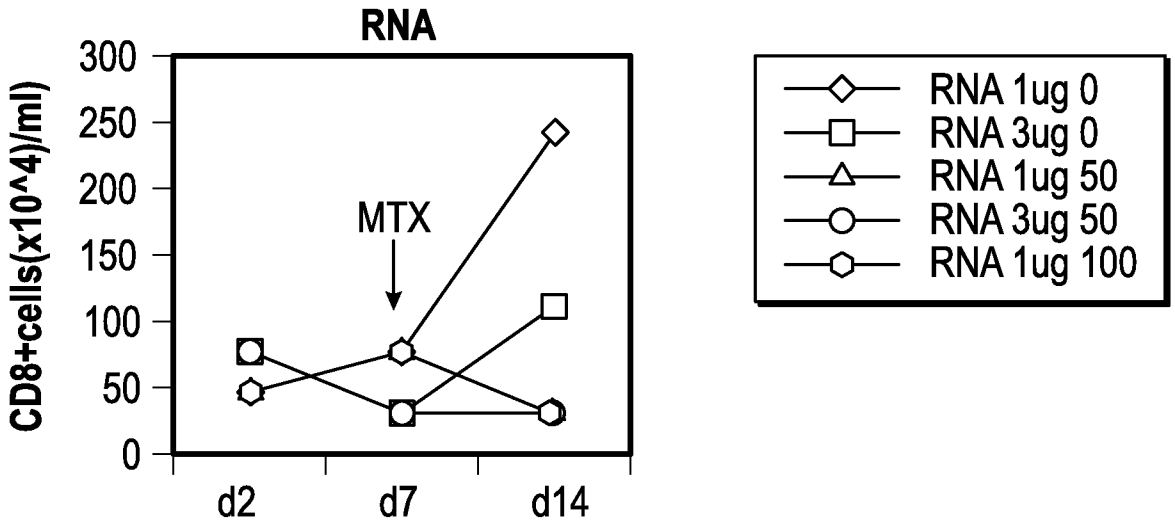
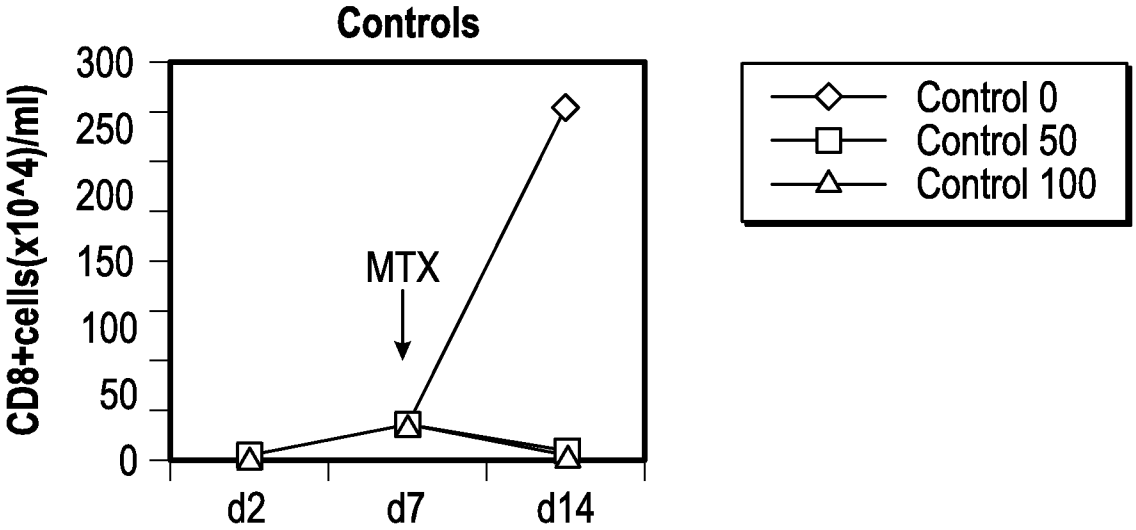


FIG. 28B



LHOROW-EXP-14-CV4717_248481-SB100X RNA and multiplex electroporation

- DNA-**lower** concentration better growth.
- RNA-**higher** concentration better expression, **lower** concentration better growth initially--- too few + cells/well
- **DNA** better than RNA
- → try higher RNA concentrations? SB100X toxicity?

FIG. 28C

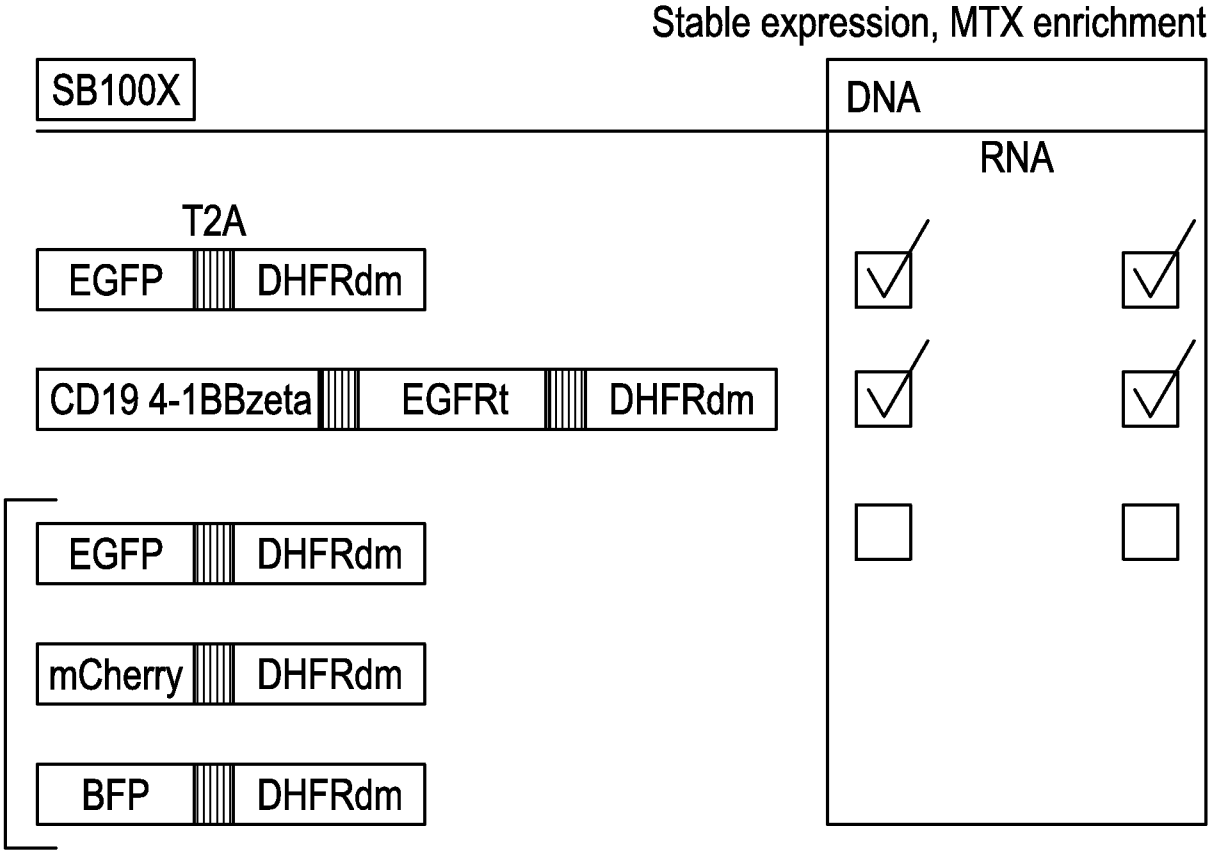


FIG. 29

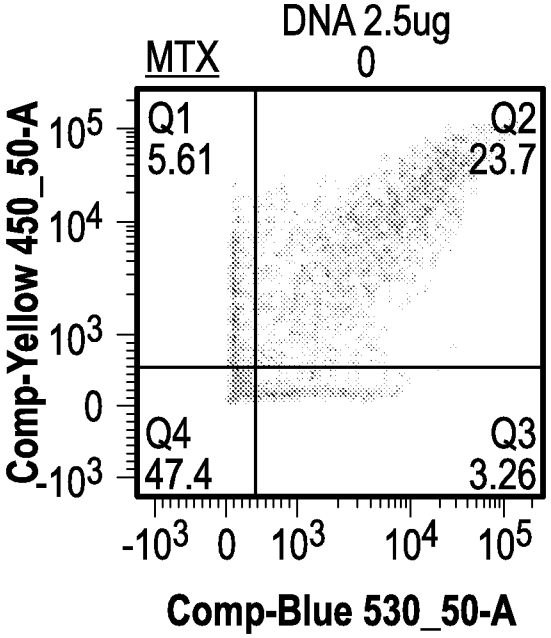


FIG. 30A

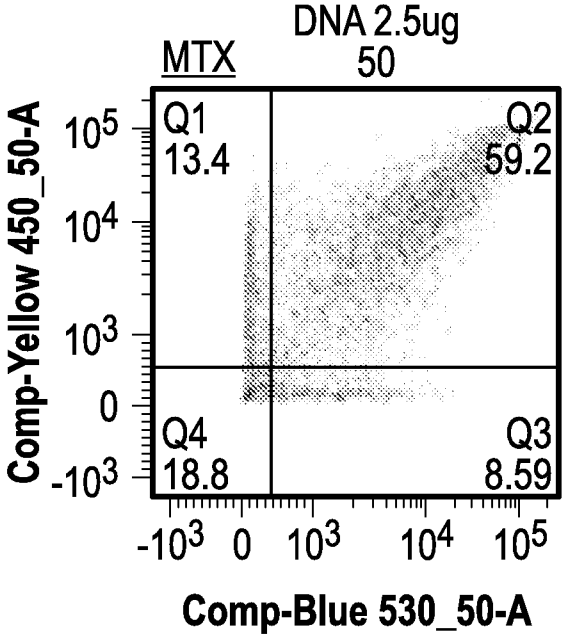


FIG. 30B

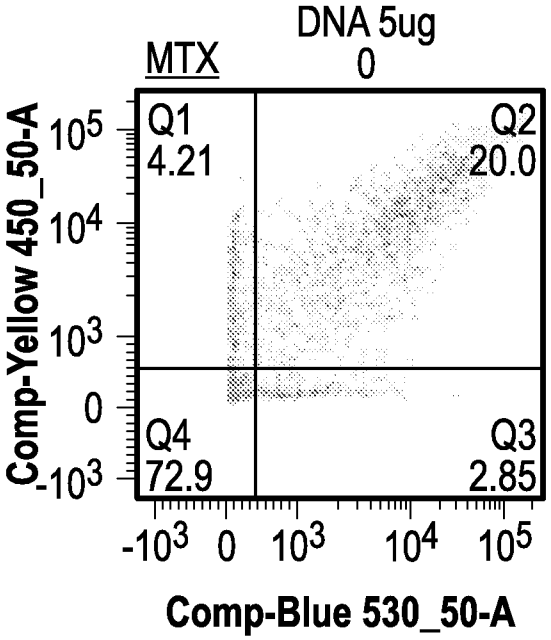


FIG. 30C

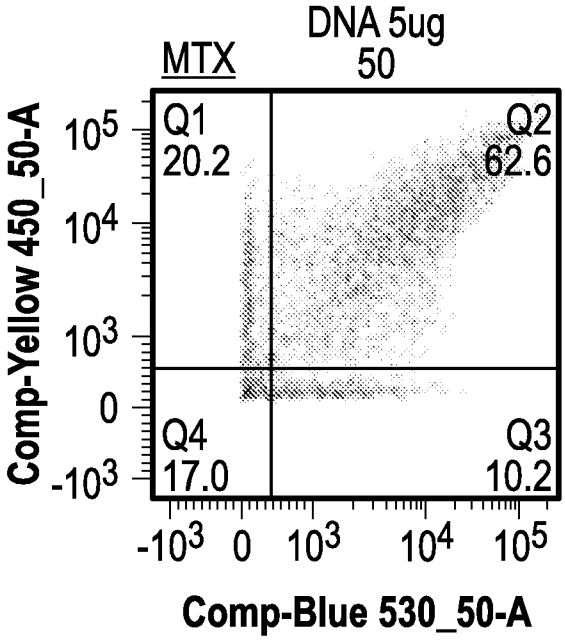


FIG. 30D

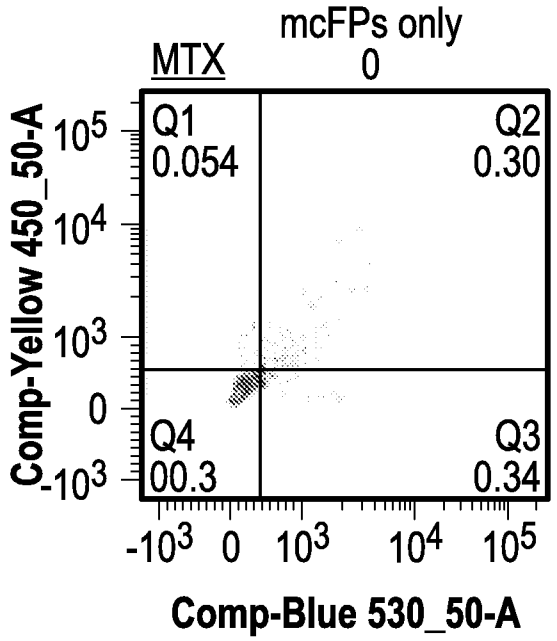


FIG. 30E

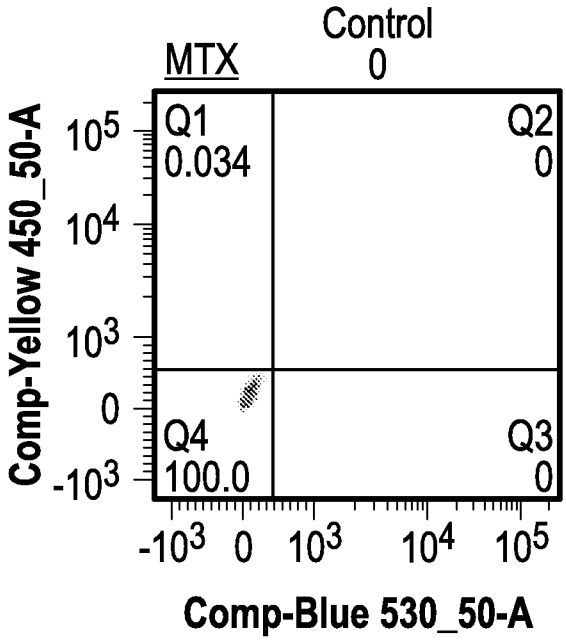


FIG. 30F

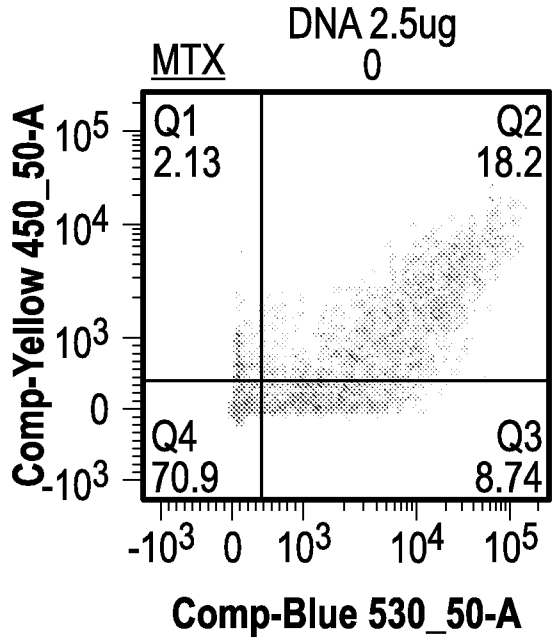


FIG. 30G

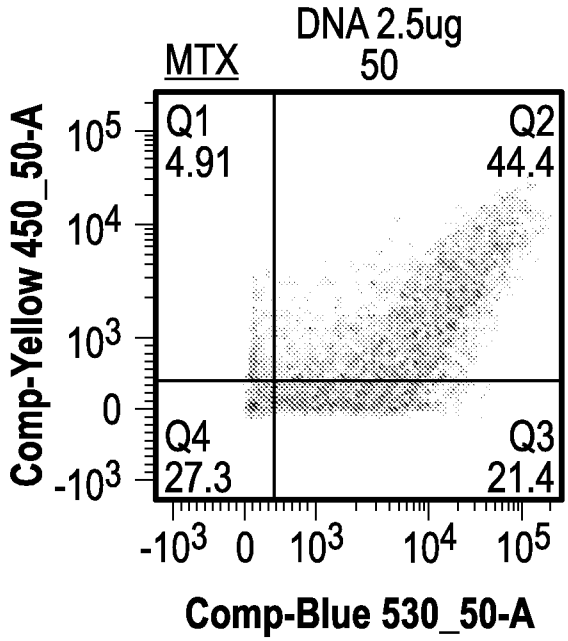


FIG. 30H

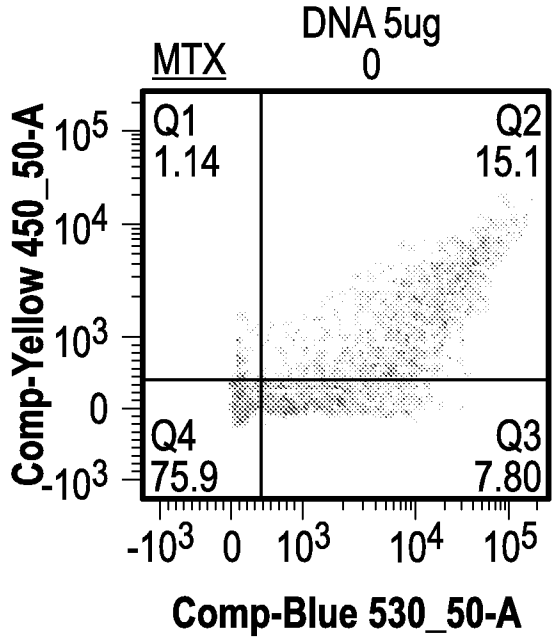


FIG. 30I

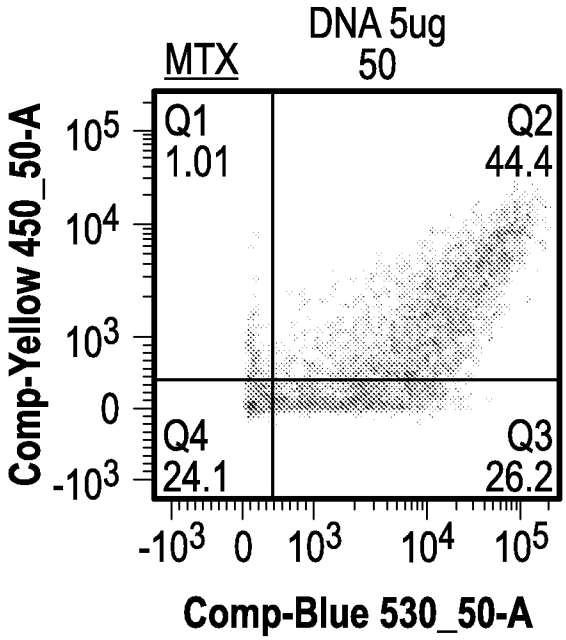


FIG. 30J

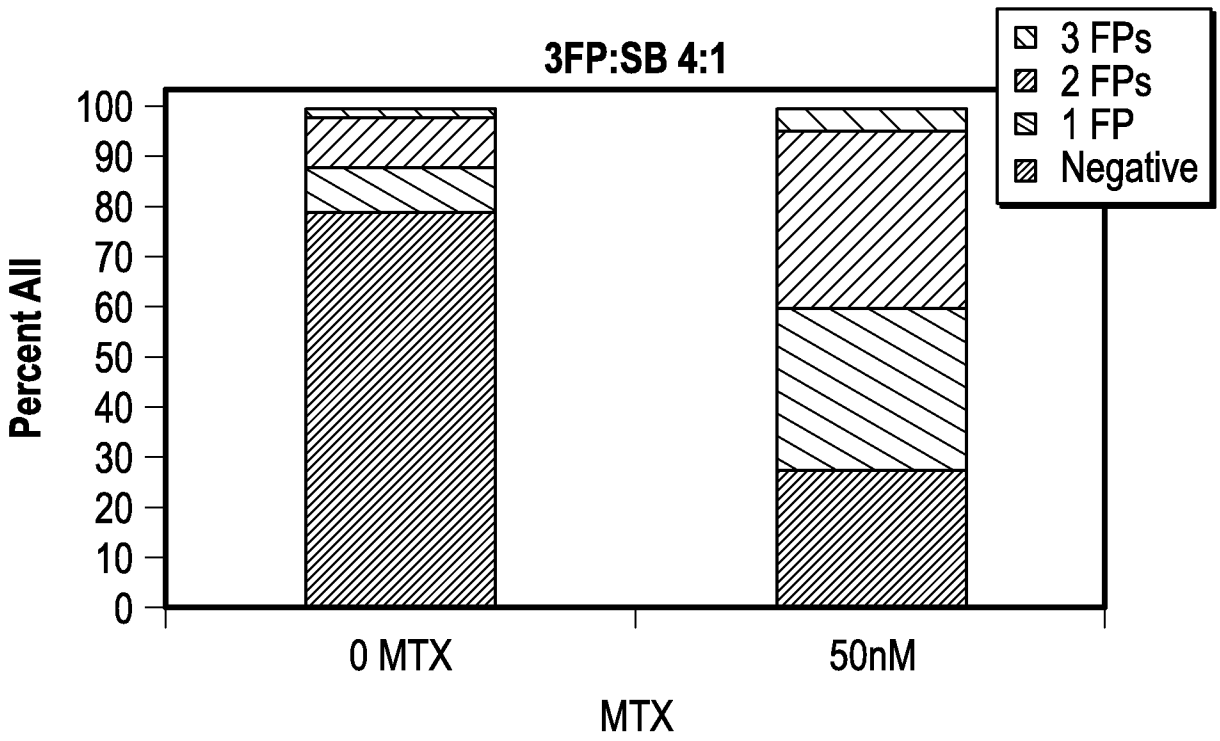


FIG. 31A

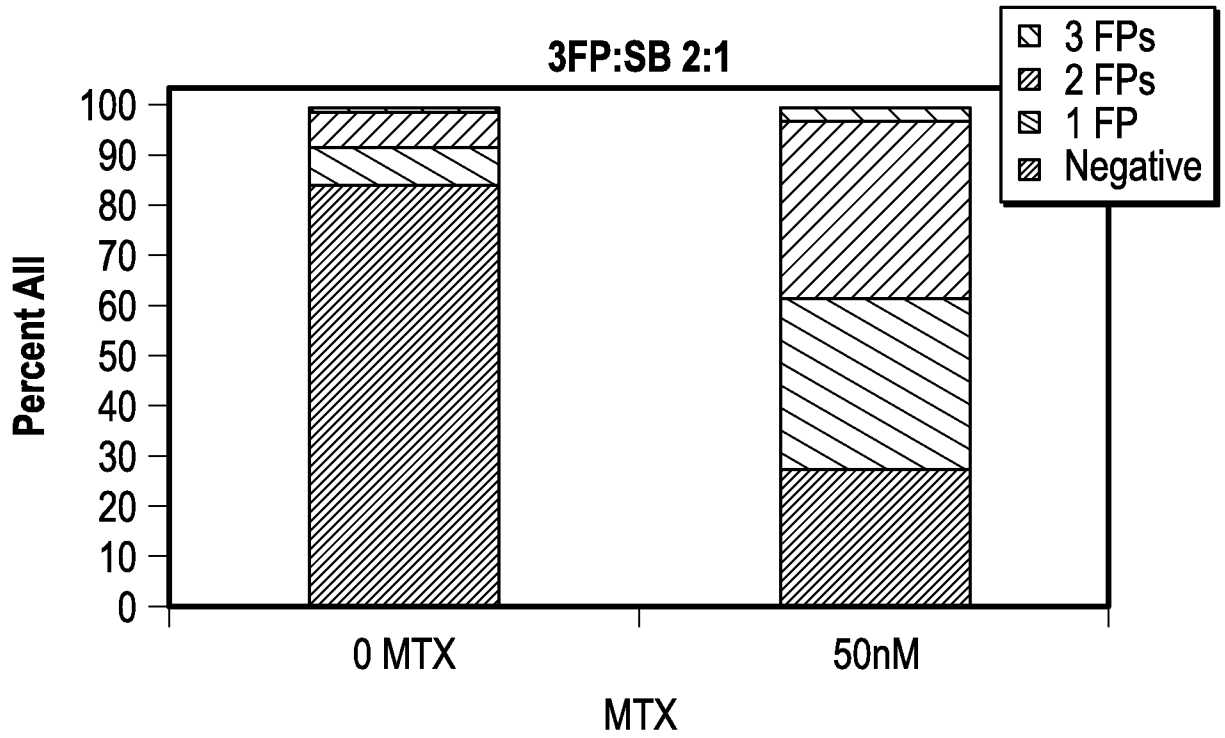


FIG. 31B

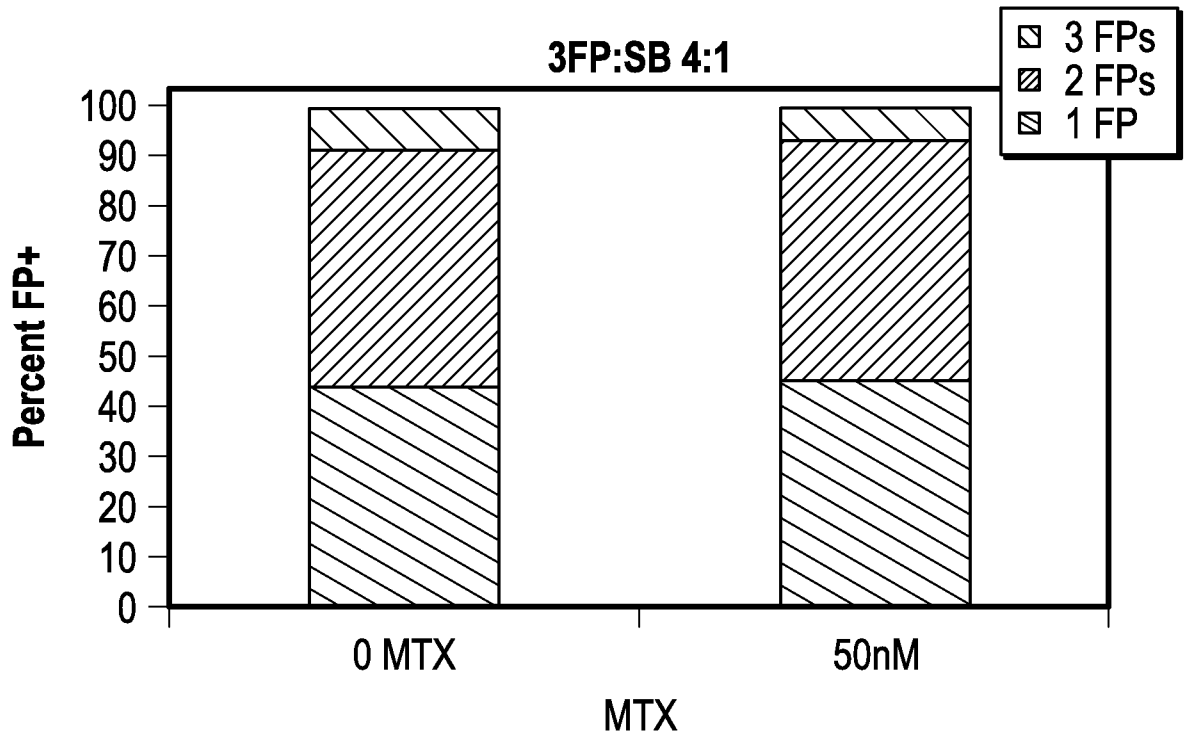
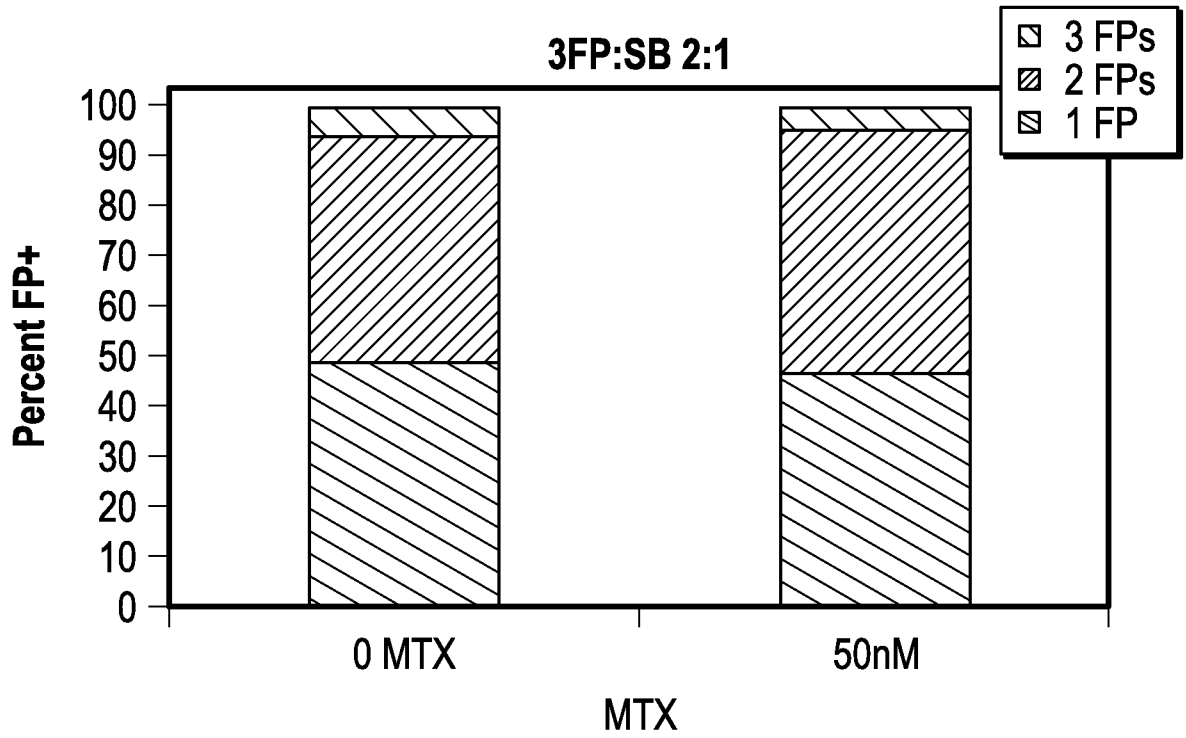


FIG. 31C



MTX enrichment for positive cells. For multiple copies? NO-higher MTX?

FIG. 31D

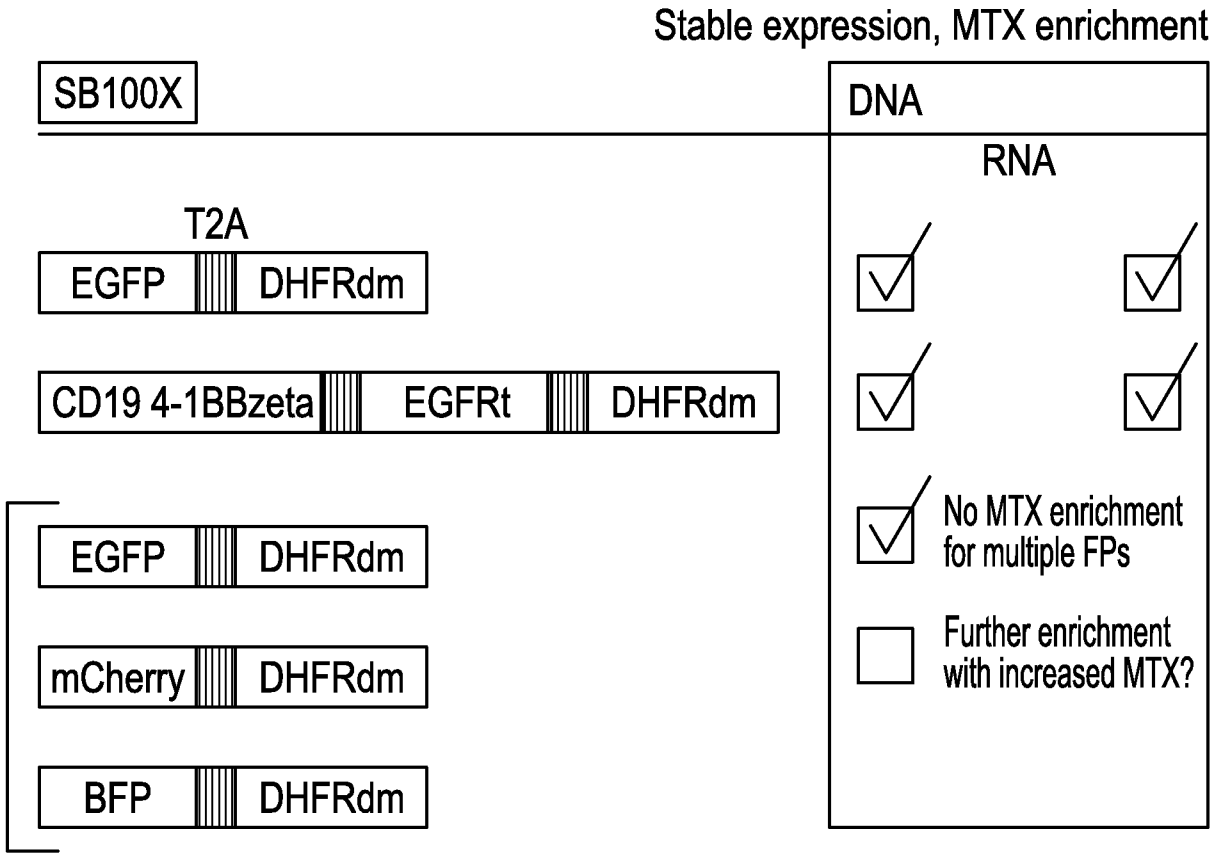
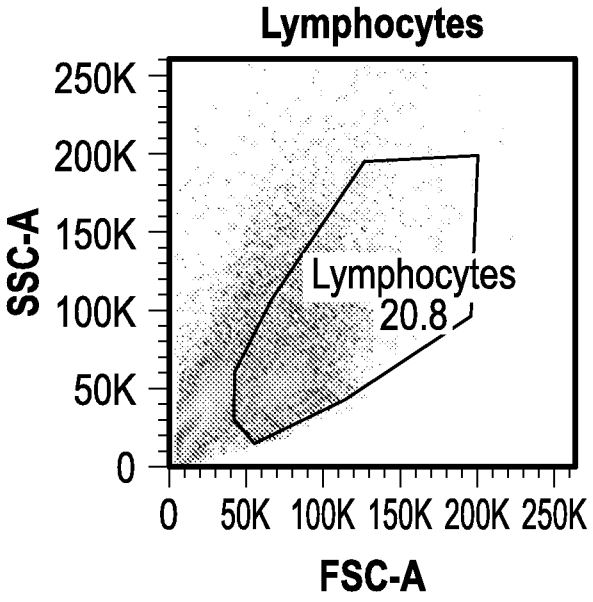


FIG. 32

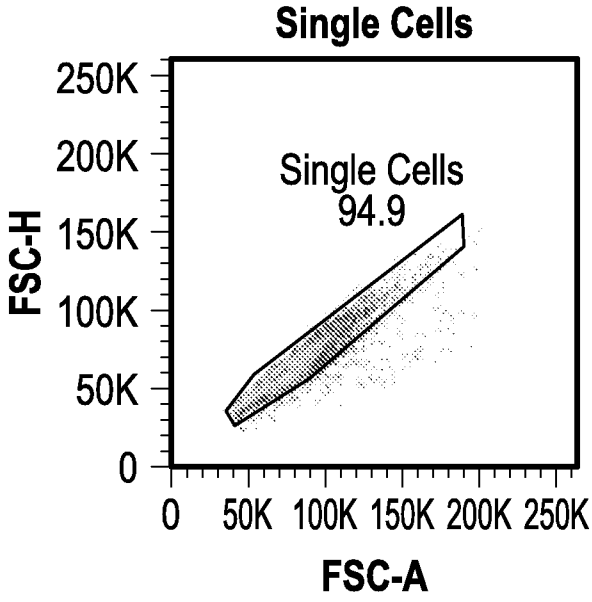
Module #3
Sleeping Beauty
transposon system

Sleeping Beauty Transposons:
Multiplexing with increased MTX selection



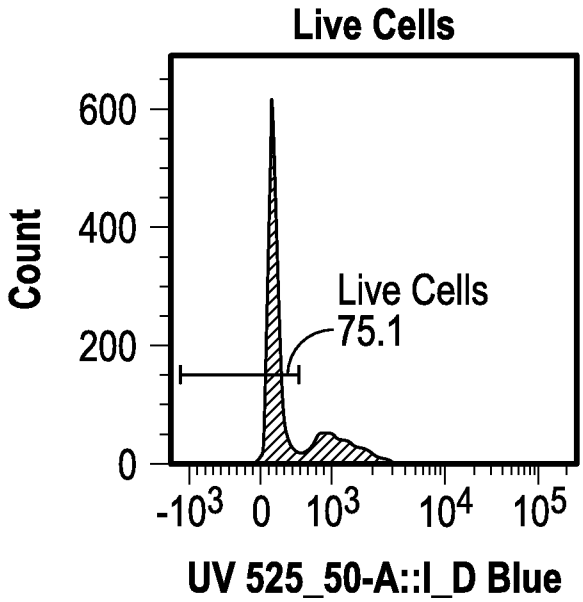
20141121_SBFPd30_250_8A_080.fcs
Ungated
33252

FIG. 33A



20141121_SBFPd30_250_8A_080.fcs
Lymphocytes
6933

FIG. 33B



20141121_SBFPd30_250_8A_080.fcs
Single Cells
6578

FIG. 33C

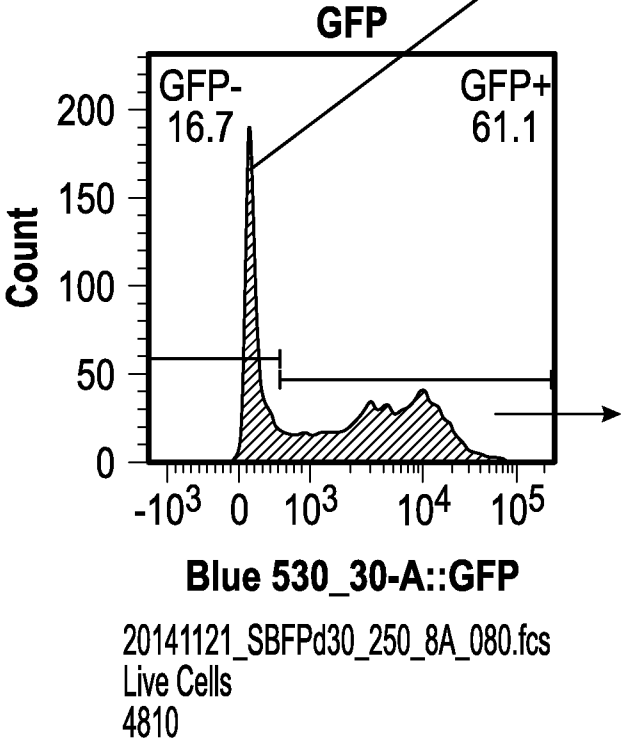


FIG. 33D

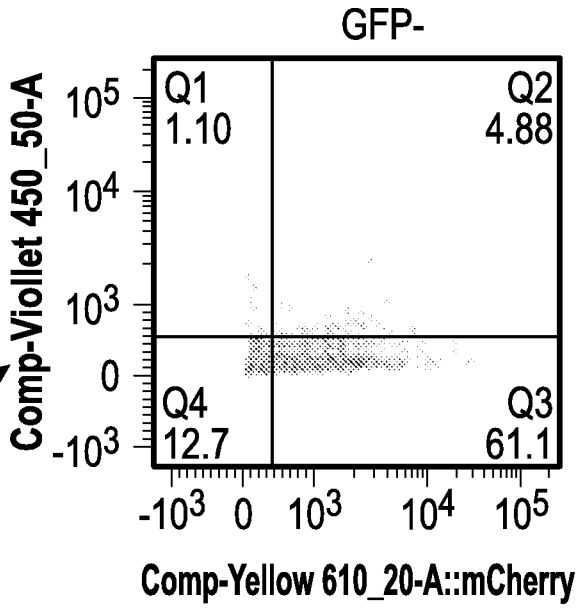


FIG. 33E

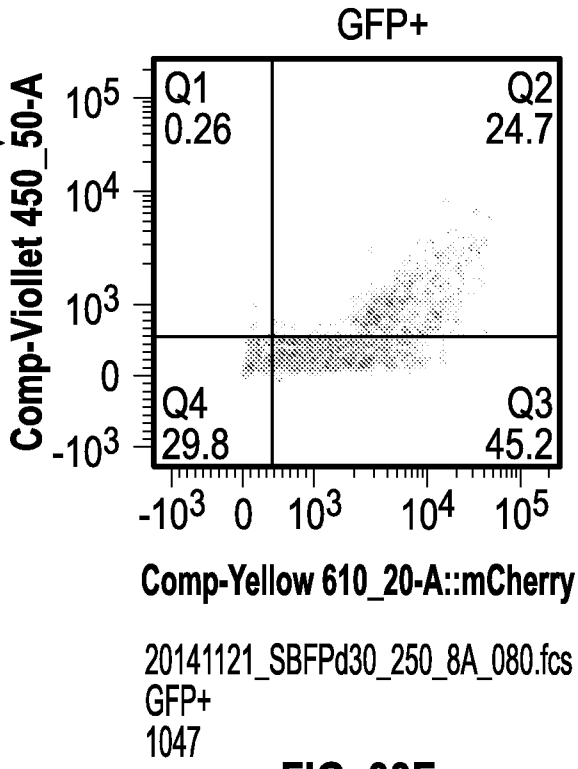
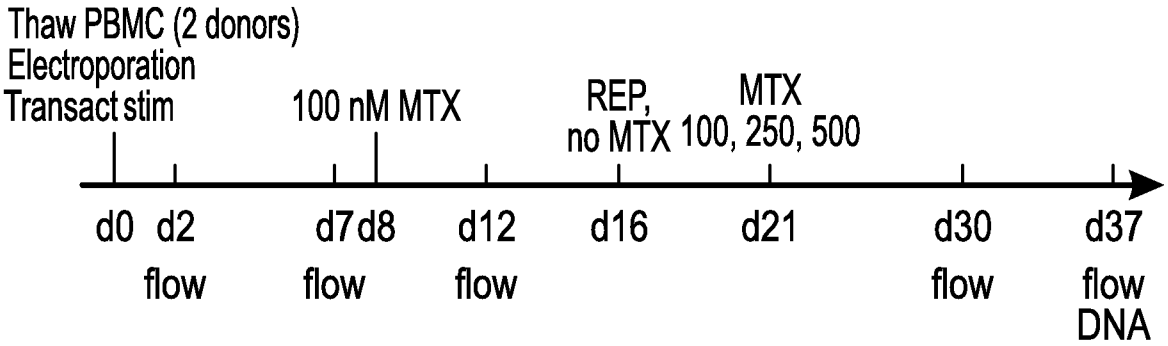


FIG. 33F



- Transposons with DHFRdm (10ug total, minicircle):
  - GFP, mCherry, BFP
  - 1, 2, or 3 colors
- +/- SB100X transposase (5ug, minicircle)

2014-11-21 LHOROW-EXP-14-CV4716_248480-Tricolor SB100X

FIG. 33G

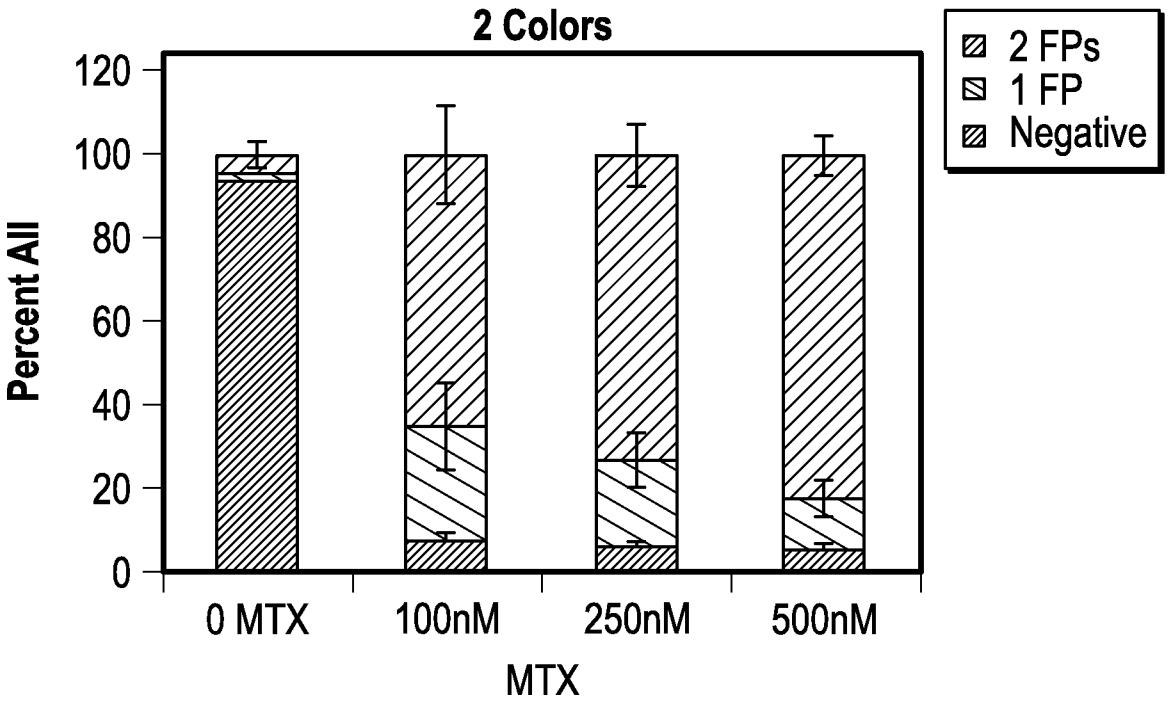


FIG. 34A

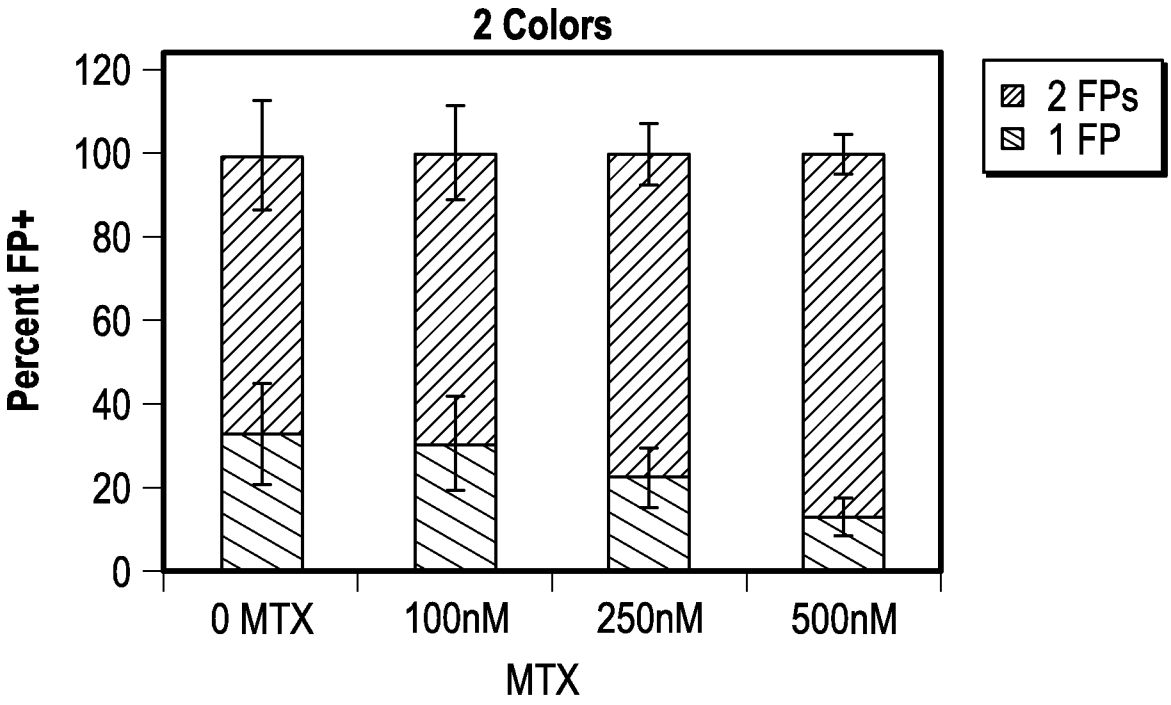


FIG. 34B

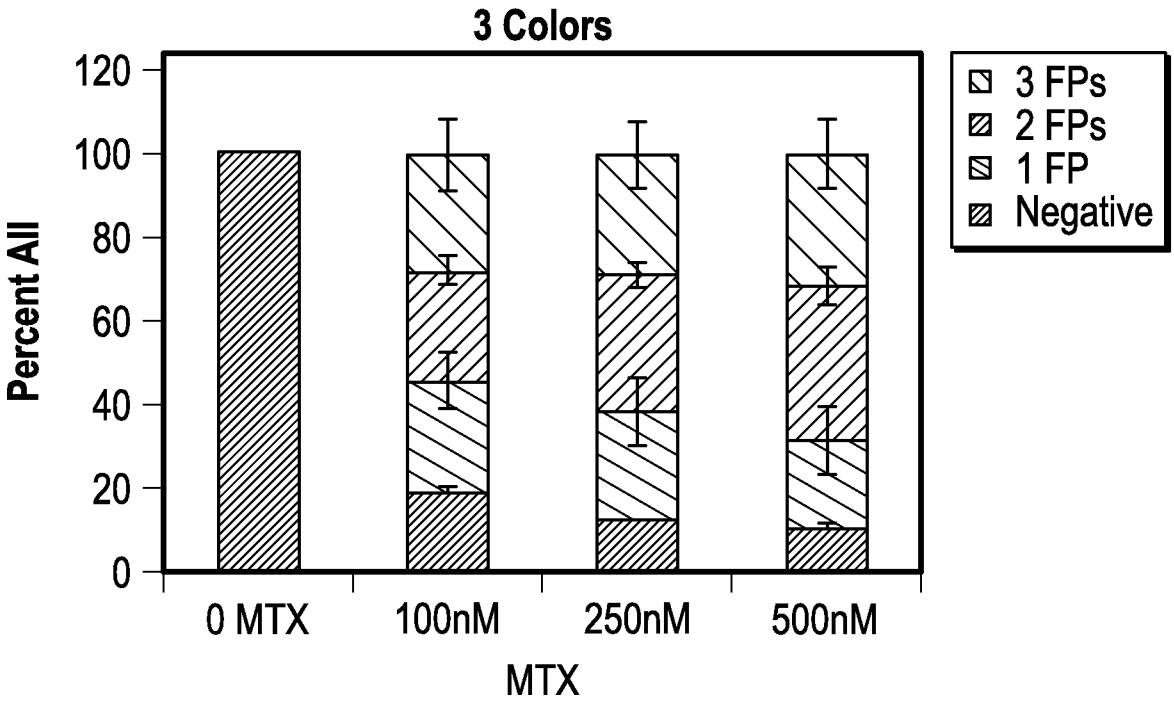


FIG. 34C

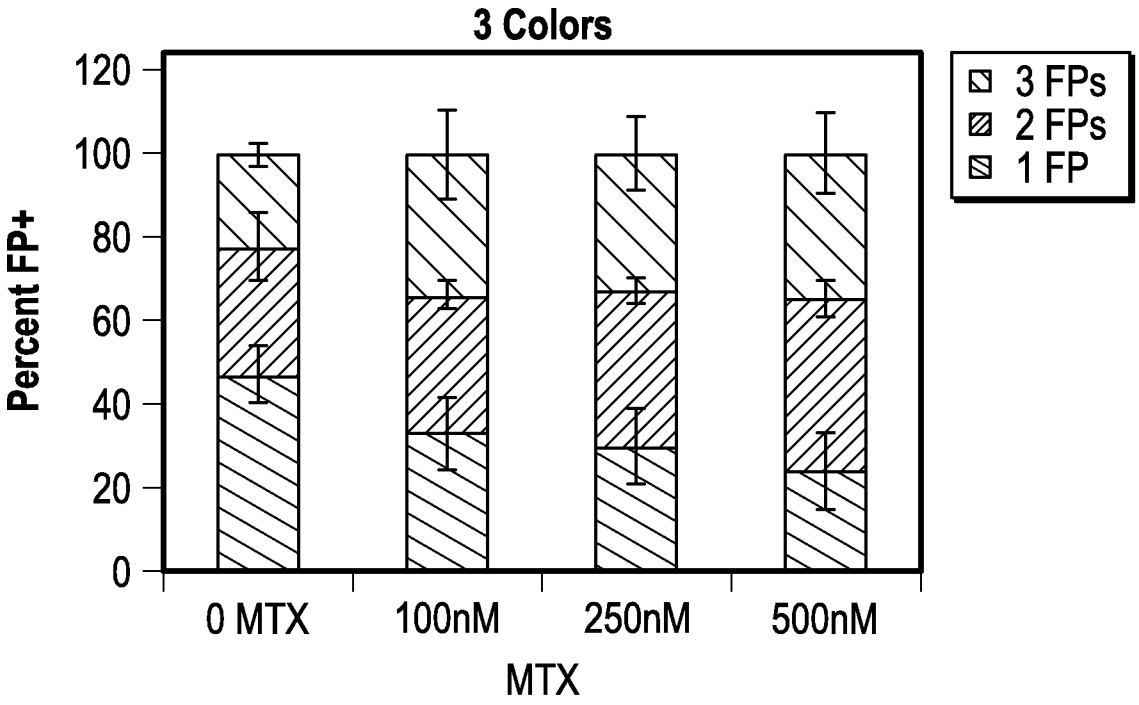


FIG. 34D

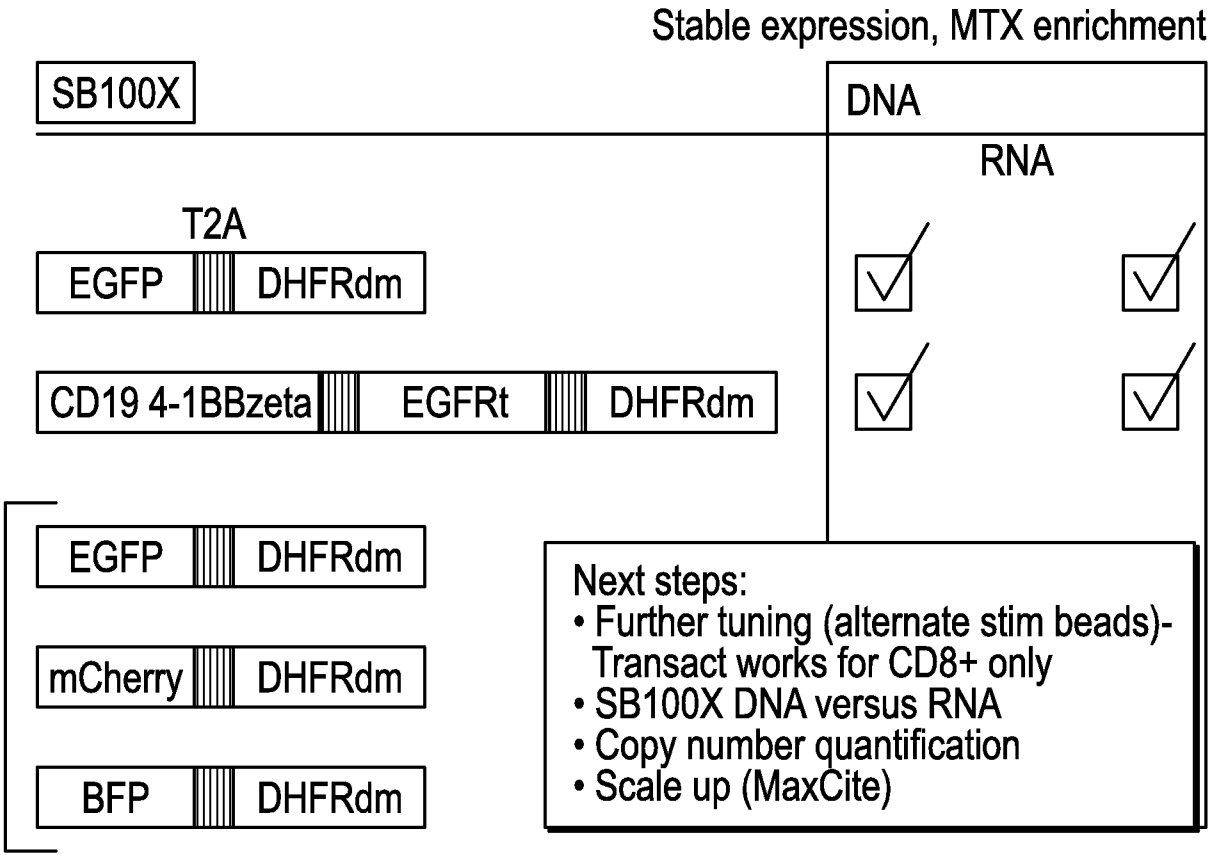


FIG. 35

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US15/24868

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 15/09, 5/10 (2015.01)
CPC - C12N 2830/85, 2740/13043, 2830/15
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): C12N 1/15, 15/09, 1/21, 1/19, 5/10; C12P 21/02, 21/08; C07K 16/00, 16/12; A61K 48/00 (2015.01)
CPC: C12N 2830/85, 2830/00, 2740/13043, 2830/15; C07K 16/1228, 16/3007, 16/1282; A61K 48/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, INPADOC Data); Google; Google Scholar; NCBI/PubMed; Dialog ProQuest; vector, gene, delivery, selectable, 'nucleic acid,' nucleotide, sequence, 'T cell,' immunotherapy, promoter

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2005/0060762 A1 (BLECK, GT); March 17, 2005; figure 10d; paragraphs [0043], [0044], [0061], [0088]-[0089], [0093], [0099], [0137]-[0140], [0163], [0180], [0248]	1-3
Y	ERCIKAN-ABALI, EA et al. Active Site-Directed Double Mutants Of Dihydrofolate Reductase. Cancer Res. 15 September 1996, Vol. 56, No. 18; pages 4142-5; page 4143, column 2, paragraph 3; Table 2	1-3
Y	US 2012/0297493 A1 (COOPER, R et al.); November 22, 2012; paragraphs [0012], [0087], [0208]	2, 3/2

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 June 2015 (24.06.2015)	10 JUL 2015
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US15/24868

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

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

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

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

3. ☒ Claims Nos.: 4-64
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.