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(54) Title: IMPROVED REPROGRAMMING METHODS AND CELL CULTURE PLATFORMS

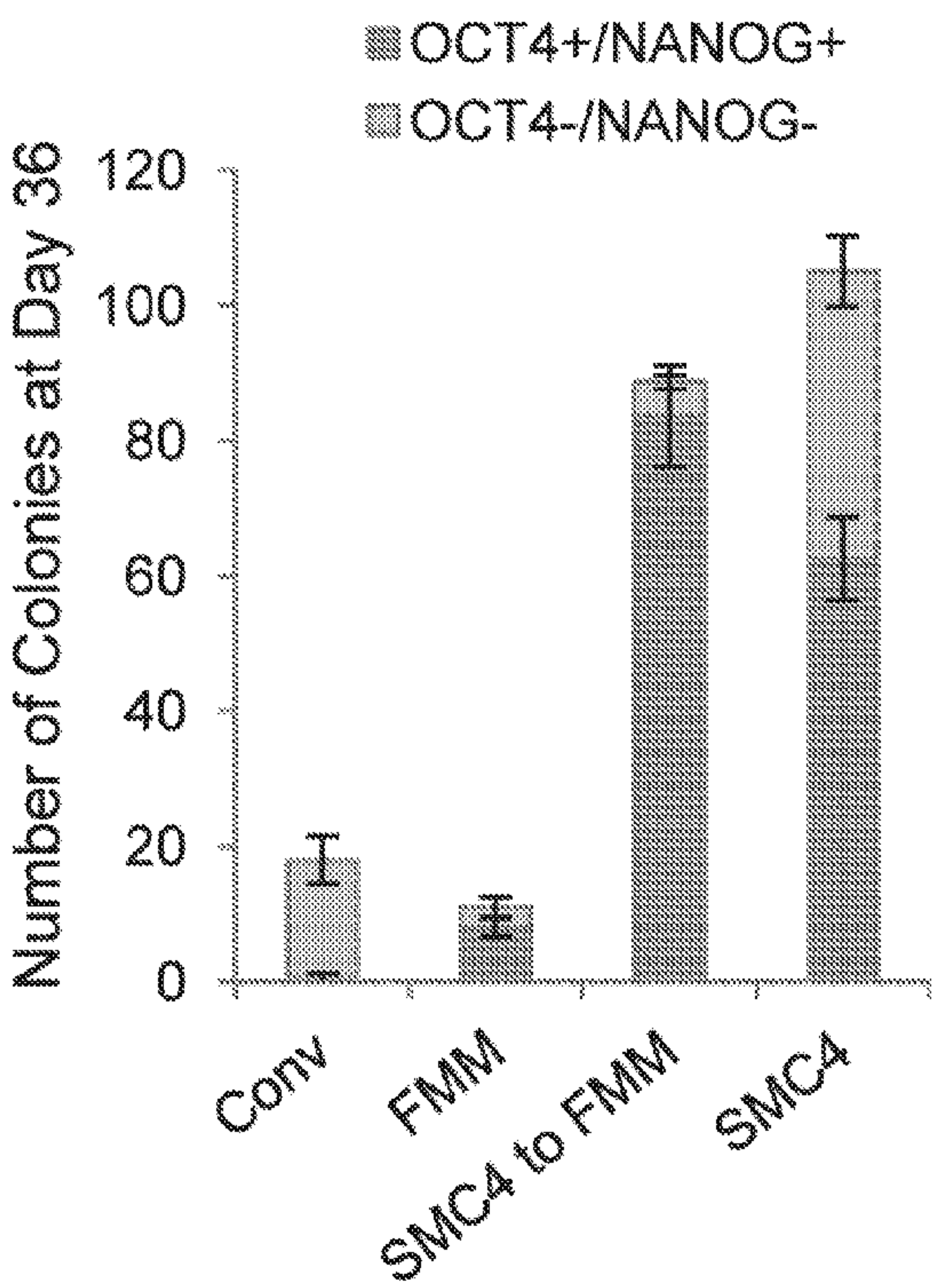

FIG. 1E

(57) Abrégé/Abstract:
The invention provides compositions and methods for manufacturing pluripotent cells. In particular, the invention provides improved culture platforms for manufacturing pluripotent cells with ground state pluripotency. In various embodiments, the invention contemplates, in part, a composition comprising: (a) a Wnt pathway agonist; (b) a MEK inhibitor; and (c) a ROCK inhibitor. In certain embodiments, the composition further comprises bFGF or LIF.

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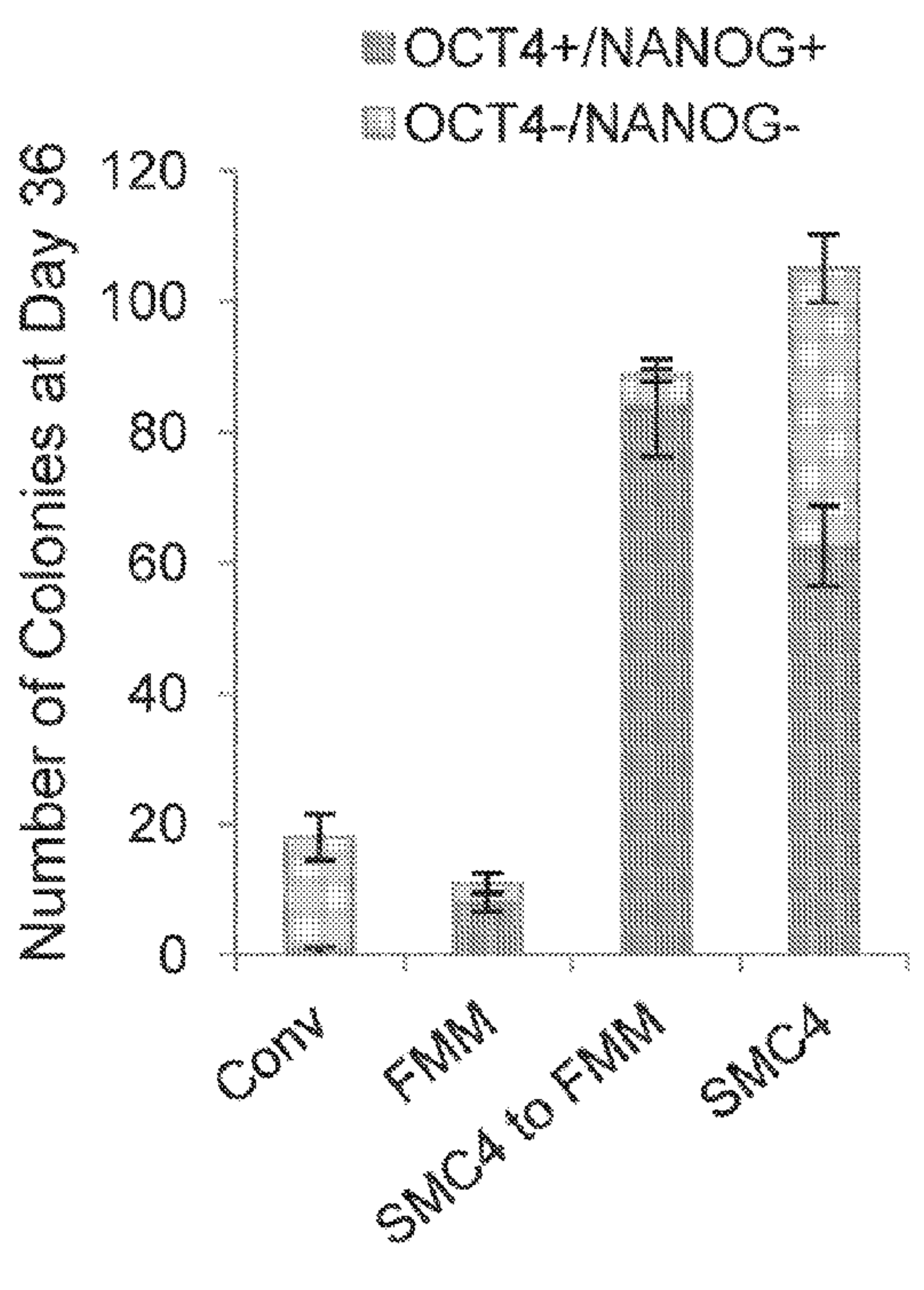

FIG. 1E

(57) Abstract: The invention provides compositions and methods for manufacturing pluripotent cells. In particular, the invention provides improved culture platforms for manufacturing pluripotent cells with ground state pluripotency. In various embodiments, the invention contemplates, in part, a composition comprising: (a) a Wnt pathway agonist; (b) a MEK inhibitor; and (c) a ROCK inhibitor. In certain embodiments, the composition further comprises bFGF or LIF.

WO 2015/134652 A1

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IMPROVED REPROGRAMMING METHODS AND CELL CULTURE PLATFORMS

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 61/947,979, filed March 4, 2014, which is incorporated by reference herein in its entirety.

STATEMENT REGARDING SEQUENCE LISTING

The Sequence Listing associated with this application is provided in text format in lieu of a paper copy, and is hereby incorporated by reference into the specification. The name of the text file containing the Sequence Listing is FATE_122_01WO_ST25.txt. The text file is 8 KB, was created on March 4, 2015, and is being submitted electronically via EFS-Web, concurrent with the filing of the specification.

BACKGROUND

Technical Field

The invention relates generally to compositions and methods for manufacturing pluripotent cells. In particular, the invention relates to improved culture platforms for manufacturing pluripotent cells with ground state pluripotency.

Description of the Related Art

Today's pluripotent stem cell-based disease and toxicology screening efforts and tomorrow's auto/allogeneic pluripotent stem cell therapies will require robust, reproducible methods of cell line generation and expansion of human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs). hiPSCs have been generated by the ectopic expression of pluripotency factors introduced through genome-integrating retro- and lentiviral expression systems. Efforts to eliminate as many integrating events as possible have included substituting small molecule inhibitors for a number of reprogramming factors. In addition, non-integrative methods have proven to be inefficient and labor intensive, requiring additional reprogramming factors or not being effective in reprogramming all somatic cells (Lee *et al.*, 2013).

Several challenges associated with the culture of pluripotent stem cells have yet to be addressed to allow the cells to become suitable for future industrial and clinical applications. In the most commonly used conventional culture system, hESCs and hiPSCs are maintained on feeder cells while passaged as clumps to prevent extensive cell death and genomic aberrations (Thomson *et al.*, 1998). The inability to single cell culture hiPSCs in a feeder-free (FF) environment severely limits potential industrial scale screening or cell therapy applications (Skottman *et al.*, 2007; Valamehr *et al.*, 2011). In addition, recent efforts on improving hiPSCs have focused on lentiviral derived hiPSC that were not transgene-free, limiting the therapeutic relevance of such efforts.

Another challenge yet to be successfully addressed, short of genome modification, is the propensity for spontaneous differentiation of human pluripotent stem cells in culture (Pera and Trounson, 2004; Sathananthan and Trounson, 2005; Valamehr *et al.*, 2011).

Studies in hESCs and hiPSCs have been described, but continuous ectopic expression of pluripotency genes were necessary to maintain the ground state resulting in genome modified human pluripotent stem cells (Hanna *et al.*, 2010a), which are unsuitable for industrial- and clinical-grade pluripotent cells.

Accordingly, the absence of compositions and methods for high-throughput, transgene or footprint free generation of human pluripotent cell products has thus far proven to be a substantial hurdle in the development and commercialization of future pluripotent stem cell therapies.

BRIEF SUMMARY

The invention generally provides improved cell culture platforms.

In various embodiments, the invention contemplates, in part, a composition comprising: (a) a Wnt pathway agonist; (b) a MEK inhibitor; and (c) a ROCK inhibitor, wherein the composition does not comprise a TGFβR inhibitor.

In particular embodiments, the Wnt pathway agonist is a GSK3 inhibitor.

In certain embodiments, the GSK3 inhibitor is CHIR99021 or BIO.

In additional embodiments, the MEK inhibitor is PD98059 or PD032901.

In further embodiments, the ROCK inhibitor is thiazovivin or Y27632.

In some embodiments, the GSK3 inhibitor is CHIR99021; the MEK inhibitor is PD032901; and the ROCK inhibitor is thiazovivin.

In certain embodiments, any of the foregoing compositions further comprises bFGF or LIF.

In further embodiments, any of the foregoing compositions further comprises bFGF and LIF.

In various embodiments, a culture medium is provided comprising any of the foregoing compositions, wherein the medium does not comprise a TGFβR inhibitor.

In some embodiments, a method of culturing one or more pluripotent cells comprising culturing the one or more pluripotent cells in a cell culture medium according to any of the foregoing culture media.

In additional embodiments, the one or more pluripotent cells are embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs).

In particular embodiments, the one or more pluripotent cells are iPSCs.

In certain embodiments, the composition comprises a population of pluripotent cells.

In further embodiments, the population of pluripotent cells is a homogenous population of pluripotent cells.

In particular embodiments, at least 95% of the population of pluripotent cells expresses SSEA4-FITC and TRA1-81 or TRA1-60.

In some embodiments, at most 5% of the population of pluripotent cells expresses α-smooth muscle actin (SMA), TUJ1, or FoxA2.

In particular embodiments, the pluripotent cells were previously cultured in a cell culture medium comprising a TGFβR inhibitor.

In additional embodiments, culturing the pluripotent cells in the cell culture medium reduces spontaneous differentiation of the cultured cells.

In one embodiment, expression of one or more differentiation marker genes in the cultured cells is decreased by at least about 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% compared to the expression of the one or more differentiation marker genes in a pluripotent cell cultured in a medium comprising a TGFβR inhibitor, wherein the differentiation marker genes are selected from the group consisting of: FOXA2, FGF5, SOX17, XIST, NODAL, COL3A1, OTX2, DUSP6, EOMES, NR2F2, NR0B1, CXCR4, CYP2B6, GATA3, GATA4, ERBB4, GATA6, HOXC6, INHA, SMAD6, RORA, NIPBL, TNFSF11, CDH11, ZIC4, GAL, SOX3, PITX2, APOA2, CXCL5, CER1, FOXQ1, MLL5, DPP10, GSC, PCDH10, CTCFL, PCDH20, TSHZ1, MEGF10, MYC, DKK1, BMP2, LEFTY2, HES1, CDX2, GNAS, EGR1, COL3A1, TCF4, HEPH, KDR, TOX, FOXA1, LCK, PCDH7, CD1D FOXG1, LEFTY1, TUJ1, T gene (Brachyury) and ZIC1.

In another embodiment, the one or more differentiation marker genes is selected from the group consisting of T gene, CXCR4, NODAL, GATA4, SOX17, FOXA2, OTX2, and TUJ1.

In yet another embodiment, expression of two or more differentiation marker genes is decreased in the cultured cells by at least about 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% compared to the expression of the two or more differentiation marker genes in a pluripotent cell cultured in a medium comprising a TGFβR inhibitor, wherein the differentiation marker genes are selected from the group consisting of: FOXA2, FGF5, SOX17, XIST, NODAL, COL3A1, OTX2, DUSP6, EOMES, NR2F2, NR0B1, CXCR4, CYP2B6, GATA3, GATA4, ERBB4, GATA6, HOXC6, INHA, SMAD6, RORA, NIPBL, TNFSF11, CDH11, ZIC4, GAL, SOX3, PITX2, APOA2, CXCL5, CER1, FOXQ1, MLL5, DPP10, GSC, PCDH10, CTCFL, PCDH20, TSHZ1, MEGF10, MYC, DKK1, BMP2, LEFTY2, HES1, CDX2, GNAS, EGR1, COL3A1, TCF4, HEPH, KDR, TOX, FOXA1, LCK, PCDH7, CD1D FOXG1, LEFTY1, TUJ1, T gene (Brachyury) and ZIC1.

In still yet another embodiment, the two or more differentiation marker genes are selected from the group consisting of T gene, CXCR4, NODAL, GATA4, SOX17, FOXA2, OTX2, and TUJ1.

In a particular embodiment, expression of three or more differentiation marker genes is decreased in the cultured cells by at least about 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% compared to the expression of the three or more differentiation marker genes in a pluripotent cell cultured in a medium comprising a TGFβR inhibitor, wherein the differentiation marker genes are selected from the group consisting of: FOXA2, FGF5, SOX17, XIST, NODAL, COL3A1, OTX2, DUSP6, EOMES, NR2F2, NR0B1, CXCR4, CYP2B6, GATA3, GATA4, ERBB4, GATA6, HOXC6, INHA, SMAD6, RORA, NIPBL, TNFSF11, CDH11, ZIC4, GAL, SOX3, PITX2, APOA2, CXCL5, CER1, FOXQ1, MLL5, DPP10, GSC, PCDH10, CTCFL, PCDH20, TSHZ1, MEGF10, MYC, DKK1, BMP2, LEFTY2, HES1, CDX2, GNAS, EGR1, COL3A1, TCF4, HEPH, KDR, TOX, FOXA1, LCK, PCDH7, CD1D FOXG1, LEFTY1, TUJ1, T gene (Brachyury) and ZIC1.

In a certain embodiment, the three or more differentiation marker genes are selected from the group consisting of T gene, CXCR4, NODAL, GATA4, SOX17, FOXA2, OTX2, and TUJ1.

In an additional embodiment, expression of five or more differentiation marker genes is decreased in the cultured cells by at least about 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% compared to the expression of the five or more differentiation marker genes in a pluripotent cell cultured in a medium comprising a TGFβR inhibitor, wherein the differentiation marker genes are selected from the group consisting of: FOXA2, FGF5, SOX17, XIST, NODAL, COL3A1, OTX2, DUSP6, EOMES, NR2F2, NR0B1, CXCR4, CYP2B6, GATA3, GATA4, ERBB4, GATA6, HOXC6, INHA, SMAD6, RORA, NIPBL, TNFSF11, CDH11, ZIC4, GAL, SOX3, PITX2, APOA2, CXCL5, CER1, FOXQ1, MLL5, DPP10, GSC, PCDH10, CTCFL, PCDH20, TSHZ1, MEGF10, MYC, DKK1, BMP2, LEFTY2, HES1, CDX2, GNAS, EGR1, COL3A1, TCF4, HEPH, KDR, TOX, FOXA1, LCK, PCDH7, CD1D FOXG1, LEFTY1, TUJ1, T gene (Brachyury) and ZIC1.

In a further embodiment, the five or more differentiation marker genes are selected from the group consisting of T gene, CXCR4, NODAL, GATA4, SOX17, FOXA2, OTX2, and TUJ1.

In a certain embodiment, culturing the pluripotent cells in the cell culture medium maintains or induces a ground state of pluripotency.

In particular embodiments, the ground state of pluripotency of the one or more pluripotent cells is maintained for at least 5 passages.

In certain particular embodiments, the ground state of pluripotency of the one or more pluripotent cells is maintained for at least 10 passages.

In further particular embodiments, the ground state of pluripotency of the one or more pluripotent cells is maintained for at least 50 passages.

In additional particular embodiments, the ground state of pluripotency of the one or more pluripotent cells is maintained for at least 100 passages.

In various embodiments, the foregoing methods further comprise dissociating the one or more pluripotent cells during passaging.

In certain embodiments, the viability of the one or more pluripotent cells is maintained during passaging.

In certain particular embodiments, the one or more pluripotent cells comprise a normal karyotype.

In certain additional embodiments, the one or more pluripotent cells are cultured in a feeder free environment.

In certain further embodiments, the genomic stability of the one or more pluripotent cells is maintained for at least 10 passages.

In certain related embodiments, the genomic stability of the one or more pluripotent cells is maintained for at least 50 passages.

In certain other embodiments, the genomic stability of the one or more pluripotent cells is maintained for at least 100 passages.

In various embodiments, the present invention contemplates, in part, a method of adapting pluripotent cells to a feeder-free culture comprising: (a) isolating one or more pluripotent cells that are cultured in the presence of feeder cells; (b) culturing the one or more pluripotent cell in a chemically defined cell culture medium comprising: a Wnt pathway agonist, optionally wherein the Wnt pathway agonist is a GSK3 inhibitor; a MEK inhibitor; and a ROCK inhibitor, wherein the medium does not comprise a TGFβR inhibitor.

In various particular embodiments, the present invention contemplates, in part, a method of culturing pluripotent cells enzymatically passaged as single cells comprising: (a) enzymatically treating one or more pluripotent cells to passage a single pluripotent cell; (b) culturing the single pluripotent cell in a feeder free environment;(c) culturing the single pluripotent cell in a chemically defined cell culture medium comprising: a Wnt pathway agonist, optionally wherein the Wnt pathway agonist is a GSK3 inhibitor; a MEK inhibitor; and a ROCK inhibitor, wherein the medium does not comprise a TGFβR inhibitor.

In various certain embodiments, the present invention contemplates, in part, a method of reducing spontaneous differentiation of one or more pluripotent cells comprising: (a) culturing the one or more pluripotent cells in a feeder free environment; (b) culturing the one or more pluripotent cells in a chemically defined cell culture medium comprising: a Wnt pathway agonist, optionally wherein the Wnt pathway agonist is a GSK3 inhibitor; a MEK inhibitor; and a ROCK inhibitor, wherein the medium does not comprise a TGFβR inhibitor.

In various additional embodiments, the present invention contemplates, in part, a method of manufacturing induced pluripotent stem cells (iPSCs) comprising: (a) obtaining one or more non-pluripotent cells; (b) reprogramming the one or more non-pluripotent cells to a pluripotent state; (c) culturing the pluripotent cells in a cell culture medium that does not comprise a TGFβR inhibitor thereby producing iPSCs.

In particular embodiments, the one or more non-pluripotent cells comprise a somatic cell.

In some embodiments, the one or more non-pluripotent cells comprise an adult stem cell.

In certain embodiments, the one or more non-pluripotent cells are reprogrammed to a pluripotent state by increasing the expression of endogenous OCT4 in the cell.

In further embodiments, reprogramming the one or more non-pluripotent cells to the pluripotent state comprises introducing one or more polynucleotides encoding one or more reprogramming factors selected from the group consisting of: OCT4, SOX2,

NANOG, KLF4, LIN28, C-MYC, ECAT1, UTF1, ESRRB, and SV40LT into the one or more non-pluripotent cells.

In additional embodiments, reprogramming the one or more non-pluripotent cells to the pluripotent state comprises introducing one or more polynucleotides encoding one or more copies of reprogramming factors selected from the group consisting of: OCT4, SOX2, NANOG, KLF4, LIN28, C-MYC, ECAT1, UTF1, ESRRB, and SV40LT into the one or more non-pluripotent cells.

In certain embodiments, reprogramming the one or more non-pluripotent cells to the pluripotent state comprises introducing one or more polynucleotides encoding one or more copies of reprogramming factors selected from the group consisting of: OCT4, SOX2, and NANOG into the one or more non-pluripotent cells.

In certain embodiments, reprogramming the one or more non-pluripotent cells to the pluripotent state comprises introducing one or more polynucleotides encoding one or more copies of reprogramming factors selected from the group consisting of: OCT4, NANOG, ECAT1, UTF1, and ESRRB into the one or more non-pluripotent cells.

In certain embodiments, reprogramming the one or more non-pluripotent cells to the pluripotent state comprises introducing one or more polynucleotides encoding one or more copies of reprogramming factors selected from the group consisting of: OCT4, ECAT1, and UTF1 into the one or more non-pluripotent cells.

In particular embodiments, the one or more polynucleotides are a lentiviral vector.

In some embodiments, the one or more polynucleotides are an episomal vector.

In related particular embodiments, the cell culture medium comprises a Wnt pathway agonist, optionally wherein the Wnt pathway agonist is a GSK3 inhibitor; a MEK inhibitor; and a ROCK inhibitor.

In further particular embodiments, reprogramming the one or more non-pluripotent cells comprises contacting the one or more non-pluripotent cells with a Wnt pathway agonist, optionally wherein the Wnt pathway agonist is a GSK3 inhibitor; a MEK inhibitor; and a TGFβR inhibitor, and optionally a ROCK inhibitor.

In additional embodiments, the iPSCs comprise a population of iPSCs.

In particular embodiments, the population of iPSCs is a homogenous population of iPSCs.

In particular embodiments, at least 95% of the population of iPSCs expresses SSEA4 and TRA1-81 or TRA1-60.

In certain embodiments, at most 5% of the population of pluripotent cells expresses α-smooth muscle actin (SMA), TUJ1, or FoxA2.

In particular embodiments, culturing the pluripotent cells in the cell culture medium reduce spontaneous differentiation, or maintain or induce a ground state of pluripotency.

In additional embodiments, expression of one or more, two or more, three or more, four or more, or five or more differentiation marker genes is decreased in the iPSCs by at least about 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% compared to the expression of the one or more differentiation marker genes in iPSCs cultured in a medium comprising a TGFβR inhibitor, wherein the differentiation marker genes are selected from the group consisting of: FOXA2, FGF5, SOX17, XIST, NODAL, COL3A1, OTX2, DUSP6, EOMES, NR2F2, NR0B1, CXCR4, CYP2B6, GATA3, GATA4, ERBB4, GATA6, HOXC6, INHA, SMAD6, RORA, NIPBL, TNFSF11, CDH11, ZIC4, GAL, SOX3, PITX2, APOA2, CXCL5, CER1, FOXQ1, MLL5, DPP10, GSC, PCDH10, CTCFL, PCDH20, TSHZ1, MEGF10, MYC, DKK1, BMP2, LEFTY2, HES1, CDX2, GNAS, EGR1, COL3A1, TCF4, HEPH, KDR, TOX, FOXA1, LCK, PCDH7, CD1D FOXG1, LEFTY1, TUJ1, T gene (Brachyury) and ZIC1.

In particular embodiments, the one or more differentiation marker genes is selected from the group consisting of T gene, CXCR4, NODAL, GATA4, SOX17, FOXA2, OTX2, and TUJ1.

In certain embodiments, the reduction in spontaneous differentiation is maintained for at least 5 passages.

In other embodiments, the reduction in spontaneous differentiation is maintained for at least 10 passages.

In particular embodiments, the reduction in spontaneous differentiation is maintained for at least 50 passages.

In additional embodiments, the reduction in spontaneous differentiation is maintained for at least 100 passages.

In further embodiments, the foregoing methods comprise dissociating the iPSCs during passaging.

In additional embodiments, the viability of the iPSCs is maintained during passaging.

In certain embodiments, the iPSCs comprise a normal karyotype.

In other embodiments, the iPSCs are cultured in a feeder free environment.

In particular embodiments, the genomic stability of the iPSCs is maintained for at least 10 passages.

In additional embodiments, the genomic stability of the iPSCs is maintained for at least 50 passages.

In particular additional embodiments, the genomic stability of the iPSCs is maintained for at least 100 passages.

In various embodiments, the invention provides, in part, an induced pluripotent stem cell (iPSC) comprising ground state pluripotency produced according to any one of foregoing embodiments.

In various certain embodiments, the invention provides, in part, an induced pluripotent stem cell (iPSC) comprising ground state pluripotency, wherein the iPSC does not comprise an exogenously introduced polynucleotide encoding a reprogramming factor polypeptide.

In various particular embodiments, the invention provides, in part, an method of manufacturing induced pluripotent stem cells (iPSCs) comprising: a) obtaining one or more pluripotent stem cells; (b) culturing the one or more pluripotent stem cells in a cell culture medium that does not comprise a TGFβR inhibitor thereby producing ground state iPSCs.

In certain embodiments, the one or more iPSCs comprises a reprogrammed somatic cell.

In additional embodiments, the one or more iPSCs comprises a reprogrammed adult stem cell.

In other embodiments, the one or more iPSCs were reprogrammed to a pluripotent state by increasing the expression of endogenous OCT4 in the one or more iPSCs.

In particular embodiments, the one or more iPSCs were reprogrammed by introducing one or more polynucleotides encoding one or more reprogramming factors selected from the group consisting of: OCT4, SOX2, NANOG, KLF4, LIN28, C-MYC, ECAT1, UTF1, ESRRB, and SV40LT into the one or more non-pluripotent cells.

In certain particular embodiments, the one or more iPSCs were reprogrammed by introducing one or more polynucleotides encoding one or more copies of reprogramming factors selected from the group consisting of: OCT4, SOX2, NANOG, KLF4, LIN28, C-MYC, ECAT1, UTF1, ESRRB, and SV40LT.

In additional embodiments, the one or more iPSCs were reprogrammed by introducing one or more polynucleotides encoding one or more copies of reprogramming factors selected from the group consisting of: OCT4, SOX2, and NANOG into the one or more non-pluripotent cells.

In additional embodiments, the one or more iPSCs were reprogrammed by introducing one or more polynucleotides encoding one or more copies of reprogramming factors selected from the group consisting of: OCT4, NANOG, ECAT1, UTF1, and ESRRB into the one or more non-pluripotent cells.

In another embodiment, the one or more iPSCs were reprogrammed by introducing one or more polynucleotides encoding one or more copies of reprogramming factors selected from the group consisting of: OCT4, ECAT1, and UTF1 into the one or more non-pluripotent cells.

In various embodiments, a lentiviral vector comprises the one or more polynucleotides.

In particular embodiments, an episomal vector comprises the one or more polynucleotides.

In other embodiments, the cell culture medium comprises a Wnt pathway agonist, optionally wherein the Wnt pathway agonist is a GSK3 inhibitor; a MEK inhibitor; and a ROCK inhibitor.

In certain embodiments, obtaining the one or more iPSCs comprises contacting the one or more non-pluripotent cells or partially pluripotent cells with a Wnt pathway agonist, optionally wherein the Wnt pathway agonist is a GSK3 inhibitor; a MEK inhibitor; and a TGFβR inhibitor, and optionally a ROCK inhibitor to produce the one or more iPSCs.

In additional embodiments, the iPSCs comprise a population of iPSCs.

In additional embodiments, the population of iPSCs is a homogenous population of iPSCs.

In particular embodiments, at least 95% of the population of iPSCs expresses SSEA4 and TRA1-81 or TRA1-60.

In particular embodiments, the one or more iPSCs are obtained by reprogramming a population of pluripotent cells, wherein at most 5% of the population of pluripotent cells expresses α-smooth muscle actin (SMA), TUJ1, or FoxA2.

In further embodiments, the foregoing methods comprise culturing the one or more iPSCs in the cell culture medium reduces spontaneous differentiation or maintains or induces a ground state of pluripotency.

In certain embodiments, the iPSCs with reduced spontaneous differentiation comprise a gene expression wherein expression of one or more, two or more, three or more, four or more, or five or more differentiation marker genes is decreased in the iPSCs by at least about 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% compared to the expression of the one or more differentiation marker genes in iPSCs cultured in a medium comprising a TGFβR inhibitor, wherein the differentiation marker genes are selected from the group consisting of: FOXA2, FGF5, SOX17, XIST, NODAL, COL3A1, OTX2, DUSP6, EOMES, NR2F2, NR0B1, CXCR4, CYP2B6, GATA3, GATA4, ERBB4, GATA6, HOXC6, INHA, SMAD6, RORA, NIPBL, TNFSF11, CDH11, ZIC4, GAL, SOX3, PITX2, APOA2, CXCL5, CER1, FOXQ1, MLL5, DPP10, GSC, PCDH10, CTCFL, PCDH20, TSHZ1, MEGF10, MYC, DKK1, BMP2, LEFTY2, HES1, CDX2, GNAS, EGR1, COL3A1, TCF4, HEPH, KDR, TOX, FOXA1, LCK, PCDH7, CD1D FOXG1, LEFTY1, TUJ1, T gene (Brachyury) and ZIC1.

In additional embodiments, the one or more differentiation marker genes is selected from the group consisting of T gene, CXCR4, NODAL, GATA4, SOX17, FOXA2, OTX2, and TUJ1.

In other embodiments, the reduced spontaneous differentiation is maintained for at least 5 passages.

In certain embodiments, the reduced spontaneous differentiation is maintained for at least 10 passages.

In additional embodiments, the reduced spontaneous differentiation is maintained for at least 50 passages.

In particular embodiments, the reduced spontaneous differentiation is maintained for at least 100 passages.

In further embodiments, the foregoing methods comprise dissociating the one or more iPSCs during passaging.

In particular embodiments, the viability of the one or more iPSCs are maintained during passaging.

In other embodiments, the one or more iPSCs comprise a normal karyotype.

In additional embodiments, the one or more iPSCs are cultured in a feeder free environment.

In certain embodiments, the genomic stability of the one or more iPSCs is maintained for at least 10 passages.

In additional embodiments, the genomic stability of the one or more iPSCs is maintained for at least 50 passages.

In particular embodiments, the genomic stability of the one or more iPSCs is maintained for at least 100 passages.

In various particular embodiments, the invention provides, in part, an induced pluripotent stem cell (iPSC) comprising ground state pluripotency produced according to any one of the foregoing embodiments.

In various embodiments, the invention provides, in part, an induced pluripotent stem cell (iPSC) comprising ground state pluripotency, wherein the iPSC does not comprise an exogenously introduced polynucleotide encoding a reprogramming factor polypeptide.

In some embodiments, a method for reprogramming a non-pluripotent cell to a pluripotent cell is provided comprising introducing into the nonpluripotent cell (i) one or more polynucleotides that encode at least one polypeptide selected from the group consisting of an OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, and an ESRRB polypeptide, or (ii) at least one polypeptide selected from the group consisting of an OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, and an ESRRB polypeptide, thereby reprogramming the non-pluripotent cell to a pluripotent cell.

In a particular embodiment, introducing comprises (i) introducing one or more polynucleotides that encode an OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, and an ESRRB polypeptide, or (ii) introducing a OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, and an ESRRB polypeptide.

In another embodiment, introducing comprises (i) introducing one or more polynucleotides that encode an OCT4 polypeptide, an ECAT1 polypeptide, and a UTF1 polypeptide, or (ii) introducing an OCT-4 polypeptide, an ECAT1 polypeptide, and a UTF1 polypeptide.

In some embodiments, the one or more polynucleotides are introduced by a retrovirus, Sendai virus, an adenovirus, an episome, mini-circle, vector system with expression cassette, or mRNA.

In a particular embodiment, the retrovirus is a lentivirus.

In another embodiment, the pluripotent cell is free of exogenous polynucleotides.

In another embodiment, the one or more polynucleotides is excised by CRE-mediated excision.

In some embodiments, the method further comprises introducing into the non-pluripotent cell (i) a polynucleotide that encodes a SV40LT polypeptide, or (ii) a SV40LT polypeptide.

In one embodiment, the method comprises contacting the nonpluripotent cell with at least one of a TGFβR inhibitor, a Wnt pathway agonist, a MEK inhibitor and a ROCK inhibitor, wherein the Wnt pathway agonist is optionally a GSK3 inhibitor.

In another embodiment, the nonpluripotent cell is contacted with a TGFβR inhibitor, a Wnt pathway agonist, a MEK inhibitor and a ROCK inhibitor, wherein the Wnt pathway agonist is optionally a GSK3 inhibitor.

In a particular embodiment, the method further comprises culturing the pluripotent cell in a culture medium comprising a Wnt pathway agonist, a MEK inhibitor and a ROCK inhibitor, wherein the Wnt pathway agonist is optionally a GSK3 inhibitor, and wherein the culture medium does not contain TGFβR inhibitor.

In some embodiments, the Rock inhibitor is thiazovivin or Y27632, the TGFβR inhibitor is A-83-01 or SB431542, the GSK3 inhibitor is CHIR99021 or BIO, or the MEK inhibitor is PD98059 or PD032901.

In another embodiment, pluripotency of the pluripotent cell is maintained for at least 5 cell divisions or at least 10 cell divisions.

In a particular embodiment, the one or more polynucleotides is introduced as a polycistronic vector comprising a plurality of polynucleotides that are separated by at least one 2A peptide.

In one embodiment, the polycistronic vector comprises a plurality of polynucleotides that encode an OCT4 polypeptide.

In some embodiments, the method comprises identifying the pluripotent cell by selecting for OCT4 expression in the pluripotent cell.

In one embodiment, selecting for OCT4 expression comprises selecting for ectopic Oct-4 expression.

In another embodiment, culturing produces a population of pluripotent stem cells.

In another embodiment, the population of pluripotent stem cells are at least 70% homogenous, at least 80% homogenous, or at least 90% homogenous.

In yet another embodiment, at least 70%, at least 80%, or at least 90% of the population of pluripotent cells expresses SSEA and Tra-181.

In an embodiment, the pluripotent cell or population of pluripotent cells are capable of single cell passaging.

In one embodiment, the cells produced by single cell passaging have a normal karyotype.

In one embodiment, the invention provides a pluripotent cell produced according to the method of any one of the methods above.

In another embodiment, the invention provides a composition comprising an isolated non-pluripotent cell that comprises (i) one or more exogenous polynucleotides that encode at least one polypeptide selected from the group consisting of an OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, and an ESRRB polypeptide, or (ii) at least one exogenous polypeptide selected from an OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, and an ESRRB polypeptide.

In one embodiment, the cell comprises (i) one or more exogenous polynucleotides that encode at least two of an OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, and an ESRRB polypeptide, or (ii) at least two exogenous polypeptides selected from an OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, and an ESRRB polypeptide.

In another embodiment, the cell comprises (i) one or more exogenous polynucleotides that encode at least three of an OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, and an ESRRB polypeptide, or (ii) at least three exogenous polypeptides selected from an OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, and an ESRRB polypeptide.

In one particular embodiment, the cell comprises (i) one or more exogenous polynucleotides that encode an OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, and an ESRRB polypeptide, or (ii) an exogenous OCT4 polypeptide, an exogenous ECAT1 polypeptide, an exogenous UTF1 polypeptide, an exogenous NANOG polypeptide, and an exogenous ESRRB polypeptide.

In another embodiment, the cell comprises one or more exogenous polynucleotides that encode an OCT4 polypeptide, an ECAT1 polypeptide, and a UTF1 polypeptide, or an exogenous OCT4 polypeptide, an exogenous ECAT1 polypeptide, and an exogenous UTF1 polypeptide.

In one embodiment, the cell has been contacted with at least one of a TGFβR inhibitor, a GSK3 inhibitor, a MEK inhibitor and a ROCK inhibitor.

In another embodiment, the cell has been contacted with a TGFβR inhibitor, a Wnt pathway activator, a MEK inhibitor and a ROCK inhibitor, wherein the Wnt pathway activator is optionally a GSK3 inhibitor.

In a particular embodiment, the Rock inhibitor is thiazovivin or Y27632, the TGFβR inhibitor is A-83-01 or SB431542, the GSK3 inhibitor is CHIR99021 or BIO, or the MEK inhibitor is PD98059 or PD032901.

In another embodiment, the one or more exogenous polynucleotides are introduced to the non-pluripotent cell by a retrovirus, Sendai virus, an adenovirus, an episome, mini-circle, vector system with expression cassette, or mRNA.

In another embodiment, the retrovirus is a lentivirus.

In a particular embodiment, the cell is free of exogenous polynucleotides.

In an embodiment, the one or more exogenous polynucleotides are removed by CRE-mediated excision.

In another embodiment, the cell comprises an exogenous polynucleotide that encodes a SV40LT antigen polypeptide, or an exogenous a SV40LT antigen polypeptide.

In one embodiment, the one or more exogenous polynucleotides is introduced as a polycistronic vector comprising a plurality of polynucleotides that are separated by at least one 2A peptide.

In another embodiment, the polycistronic vector comprises a plurality of polynucleotides that encode an OCT4 polypeptide.

In an embodiment, the exogenous polynucleotide encoding an OCT4 polypeptide is linked to a selectable marker.

In one particular embodiment, the invention provides a composition consisting of at least one of, at least two of, or at least three of (i) a cDNA encoding an OCT4 polypeptide, (ii) a cDNA encoding an ECAT1 polypeptide, (iii) a cDNA encoding a UTF1 polypeptide, (iv) a cDNA encoding a NANOG polypeptide, and (v) a cDNA encoding an ESRRB polypeptide.

In an embodiment, the composition consists of a cDNA encoding an OCT4 polypeptide, a cDNA encoding an ECAT1 polypeptide, a cDNA encoding a UTF1 polypeptide, a cDNA encoding a NANOG polypeptide, and a cDNA encoding an ESRRB polypeptide.

In another embodiment, the composition consists of a cDNA encoding an OCT4 polypeptide, a cDNA encoding an ECAT1 polypeptide, and a cDNA encoding a UTF1 polypeptide.

In a particular embodiment, each cDNA is encoded in a retrovirus, Sendai virus, an adenovirus, an episome, a mini-circle, a vector system with expression cassette, or mRNA.

In one embodiment, the invention provides a vector comprising of one or more polynucleotides that encode at least one reprogramming factor polypeptide selected from the group consisting of an OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, and an ESRRB polypeptide.

In an embodiment, the one or more polynucleotides encode an OCT polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, and an ESRRB polypeptide.

In another embodiment, the one or more polynucleotides encode an OCT polypeptide, an ECAT1 polypeptide, and a UTF1 polypeptide.

In another particular embodiment, the vector further comprises a polynucleotide that encodes an SV40LT antigen polypeptide.

In one embodiment, the vector is a retrovirus, Sendai virus, an adenovirus, an episome, mini-circle, vector system with expression cassette, or mRNA.

In one embodiment, the retrovirus is a lentivirus.

In one particular embodiment, the invention provides a kit for reprogramming a non-pluripotent cell to a pluripotent cell, the kit comprising: one or more polynucleotides that encode at least one polypeptide selected from the group consisting of an OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, or an ESRRB polypeptide; or at least one polypeptide selected from the group consisting of an OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, or an ESRRB polypeptide; and at least one of a TGFβR inhibitor, a Wnt pathway activator, a MEK inhibitor and a ROCK inhibitor, wherein the Wnt pathway activator is optionally a GSK3 inhibitor.

In an embodiment, the one or more polynucleotides encode an OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, and an ESRRB polypeptide, or the at least one polypeptide comprises an OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, and an ESRRB polypeptide.

In another embodiment, the one or more one or more polynucleotides encode an OCT4 polypeptide, an ECAT1 polypeptide, and a UTF1 polypeptide, or the at least one polypeptide comprises an OCT4 polypeptide, an ECAT1 polypeptide, and a UTF1 polypeptide.

In one embodiment, the kit comprises a polynucleotide that encodes a SV40LT antigen polypeptide, or a SV40LT antigen polypeptide.

In another embodiment, the kit comprises a TGFβR inhibitor, a Wnt pathway activator, a MEK inhibitor and a ROCK inhibitor, wherein the Wnt pathway activator is optionally a GSK3 inhibitor.

In one embodiment, the Rock inhibitor is thiazovivin or Y27632, the TGFβR inhibitor is A-83-01 or SB431542, the GSK3 inhibitor is CHIR99021 or BIO, or the MEK inhibitor is PD98059 or PD032901.

In another embodiment, the at least one polynucleotide is encoded in a retrovirus, Sendai virus, an adenovirus, an episome, a mini-circle, a vector system with expression cassette, or mRNA.

In another embodiment, the retrovirus is a lentivirus.

In one embodiment, the at least one polynucleotide is encoded in a polycistronic vector with each polynucleotide being separated by a 2A peptide.

In yet another embodiment, the polycistronic vector comprises two or more polynucleotides that encode an OCT4 polypeptide.

In a particular embodiment, the at least one polynucleotide that encodes an OCT4 polypeptide is linked to a selectable marker.

In another particular embodiment, the invention provides a method for producing a population of pluripotent stem cells comprising: providing a population of non-pluripotent cells; introducing into the population of non-pluripotent cells a polynucleotide that encodes an OCT4 polypeptide that is linked to a selectable marker; incubating the population of non-pluripotent cells with the polynucleotide under conditions sufficient to reprogram at least a portion of the population of non-pluripotent cells to pluripotent cells; selecting cells that express the selectable marker thereby providing a population of pluripotent stem cells.

In another embodiment, the polynucleotide is introduced as a polycistronic vector comprising a plurality of polynucleotides that encode an OCT4 polypeptide.

In one embodiment, the plurality of polynucleotides are separated by at least one 2A peptide.

In yet another embodiment, at least 10 % of the cells in the population of cells express SSEA and TRA-181.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Figures 1A-1E show results from a multistage culture platform for enhanced reprogramming and hiPSC maintenance. (1A) Lentiviral generated hiPSC clone FTi088 maintained a homogeneous population of undifferentiated cells in SMC4 while spontaneous differentiation was seen in the lentiviral generated hiPSC line FTi096 cultured in SMC4. Spontaneous differentiation was minimized when FTi096 was transitioned to FMM for 3 passages as shown by morphology (upper panels) and flow cytometry for SSEA4 and TRA1-81 (lower panels). (1B) qRT-PCR for transgene expression of viral element WPRE. Expression was normalized to GAPDH and relative to WPRE expression of parental fibroblast line four days post lentiviral infection (Day 4 P.I.). Uninfected fibroblast line (fibroblast) and human ESC line HUES9 were used as negative controls. Value of each set is indicated above the bar. (1C) Screen of various medium components' effect on SSEA4 and TRA1-81population of transgene free lentiviral-induced hiPSC after 10 passages; removal of SB431542 (-TGFβRi), increase from 10 to 100 ng/mL bFGF, addition of 10 ng/mL LIF. (1D) Fibroblast cell line was transfected with lentiviral construct containing gene set OCT4/KLF4/SOX2 and split into various media (conventional medium; Conv.), cultured for 17 days and sorted for SSEA4 and TRA1-81 double positive population at day 17. Sort gate is highlighted in blue. Each set was cultured for an additional 10 days in respective media except for the SMC4 set which was split into FMM and SMC4. At day 27, the cultures were resorted for SSEA4 and TRA1-81 double positive population, seeded at normalized density of sorted events and maintained in respective media for an additional 9 days. Conventional culture set gating was expanded to achieve normalized number of cells. At day 36, each culture was stained for OCT4 and NANOG expression. Representative immunocytochemistry images shown in the right panel for each set. (1E) Colony counts of day 36 staining as discussed in (1D). Error bars represent triplicates for FRM to FMM and FRM and duplicates for FMM and hESC.

Figures 2A-2E show that individual episomal reprogrammed hiPSCs are efficiently selected and seeded in 96-well plates for clonal expansion. (2A) Schematic timing illustration of episomal induction, multistage culture platform, flow cytometry sorting and clonal expansion. (2B) Flow cytometry profile of episomal induced reprogramming maintained in FRM to FMM transition in FF culture (outlined in 2A) at indicated days post transfection. Sort gating strategy used for each parental line (SSEA4+/TRA1-81+/CD30+ population) is illustrated in respective colors, corresponding to the bottom histogram panel representing the percent of wells of 96-well plate containing individual hiPSC clones. Wells containing multiple clones or differentiated clones were not scored. The solid line represents the average percentage amongst all derivations with dotted lines representing standard deviation. (2C) Flow profile of FTC007 induced to reprogram 19 days post transfection maintained in conventional medium in the presence of MEF cells. The induced population was taken from the same population of FTC007 in (2B), however treated in different culture thereafter. (2D) Immunocytochemistry analysis of various pluripotency markers of sorted colonies in 96-well plate. Right corner panels represent DAPI staining. (2E) qRT-PCR for NANOG expression for each well of a SSEA4/TRA1-81/CD30 direct sorted (FACS) 96-well plate at 3 cells per well. The expression range is between zero and four times expression relative to H1 human ESCs as described in the legend and normalized to GAPDH.

Figures 3A-3F show that episomal reprogrammed hiPSC clones maintain their undifferentiated state and are free of transgene sequence. (3A) Typical morphology of hiPSC clones 24 hours after single cell passage. (3B) Representative images of hiPSC clone during culture. (3C) PCR analysis for episomal DNA derived from various hiPSC clones. Lane 1, FTC007-c1 p4; Lane 2, FTC007-c21 p4; Lane 3, FTC016-c25 p5; Lane 4, FTC016-c36 p5; Lane 5, FTC017-c11 p7; Lane 6, FTC017-c14 p7; Lane 7, FTC017-c17 p6 (a line maintaining episomal constructs used a positive control); Lane 8, untransfected FTC007; Lane 9, hiPSC generated using lentiviral constructs (to serve as a control against cross contamination); Lane 10, episomal vector used as positive control. Input of 100ng genomic DNA and 35 PCR cycles were used for all sets. (3D) Pluripotency markers detected by immunofluorescence for the expression of OCT4, NANOG, TRA1-81 and TRA160. (3E) Flow cytometry profile for selected hiPSC clones from various parental lines. Upper row profiles SSEA4/TRA1-81 surface expression. Bottom row profiles OCT4/NANOG intracellular expression. (3F) qRT-PCR analysis for endogenous pluripotent gene expression. Data was normalized to GAPDH and relative to HUES9 hESCs. In the case of KLF4 expression two data points exceeded 15-times greater than HUES9 and were noted on the graph. Error bars represent standard deviation of replicates.

Figures 4A-4E show that genomic stability and pluripotency is maintained during continuous single cell and FF culture. (4A) Cytogenetic analysis on 20 to 40 G-banded metaphase cells from various hiPSC clones maintained in FF and single cell culture. (4B) Flow cytometry profile and cytogenetic analysis of long-term passaged (p25-30) hiPSC clones in FF and single cell culture. (4C) Three to four day directed differentiation of FTC017-c11. (4D) Embryoid body formation and differentiation of hiPSC clones demonstrating trilineage differentiation. Immunocytochemistry conducted 28 days post differentiation: Ectoderm, TUJ1; Mesoderm, alpha smooth muscle actin (aSMA); Endoderm, AFP. (4E) Histological sections of teratoma derived from FTC007-c21 and FTC016-c25 representing each somatic lineage. Black arrows, endoderm; white arrows, ectoderm; gray arrows, mesoderm.

Figures 5A-5J show derivation of hiPSC clones with minimal number of reprogramming factors. (5A) OCT4, SOX2 and NANOG were cloned into pCEP4 in various formats. Table represents vector systems and abbreviations. (5B) SSEA4 and TRA1-81 flow cytometry profile of reprogramming kinetics induced by various gene combinations at day 13 post induction. (5C) Efficiency histogram representing the presence of TRA1-81 positive hiPSC clones in wells of 96-well plate at 3 and 9 cells per well. (5D) PCR analysis for episomal DNA derived from various hiPSC clones. Lane 1 2xO+OS+ONS+T-c7 p6; Lane 2, 2xO+OS+ONS+T-c10 p6; Lane 3, 2xO+ONS+T-c5 p5; Lane 4, 2xO+ONS+T-c9 p5; Lane 5, 2xO+OS+T-c7 p7; Lane 6, 2xO+OS+T-c9 p6; Lane 7, untransfected FTC007; Lane 8, hiPSC generated using lentiviral constructs; Lane 9, episomal vector used as positive control. Input of 100ng genomic DNA and 35 PCR cycles were used for all sets. (5E) Morphology of clone 9 derived from 2xO+OS+T. (5F) Pluripotency markers detected by immunofluorescence for the expression of OCT4, NANOG, TRA1-81 and TRA160. Images taken at 10x magnification. (5G) Flow profile of hiPSC clones derived from selected gene sets. Upper row profiles SSEA4/TRA1-81 surface expression. Bottom row profiles OCT4/NANOG intracellular expression. (5H) Directed differentiation of selected hiPSC clones approximately 72 to 96 hrs post induction. (5I) Cytogenetic analysis of G-banded metaphase cells from various hiPSC clones maintained in FF and single cell culture. (5J) Histological sections of teratoma derived from hiPSC clone 2xO+OS+ONS+T-c10 representing each somatic lineage. Left panel, endoderm; middle panel, mesoderm; right panel, ectoderm.

Figures 6A-6B show the relative gene expression profile of minimal factor episomal-induced hiPSCs in FMM. Heatmap results derived from a Fluidigm dynamic array depicting relative gene expression levels (RQ) of pluripotency (6A) and differentiation (6B) genes of conventionally maintained hiPSC lines, conventionally maintained H1 hESCs, and episomal hiPSC lines derived using various gene combinations maintained in FMM. Relative gene expression for each line is noted within each box and color coded based on three expression levels summarized in the legend (lower right). All sets were conducted in duplicates, normalized to the average expression of two housekeeping genes (GAPDH and HPRT1) and referenced to the median expression level of six control conventional lines (OSK hiPSCs and H1 hESCs on MEF) representing 1x value.

Figures 7A-7G show that FMM maintained hiPSCs have reduced expression of differentiated genes and represent the ground state. (7A) A total of 339 probe sets were differentially expressed between conventional and FMM culture by greater or less than 2.5-fold. Hierarchical clustering on the 339 probe sets using a complete linkage method based on Euclidean distance measurements. (7B) Gene ontology biological process enrichment analysis (D.A.V.I.D.) of the 213 probe sets up-regulated 2.5-fold or greater with conventional culture (in comparison to FMM culture). (7C) Gene lists representative of ground or metastable pluripotency states. List derived from references noted in text. 7(D) Hierarchical clustering on the 231 probe sets corresponding to the genes in (7C) using a complete linkage method based on Euclidean distance measurements. (7E) RMA (log2) intensities for the probe sets corresponding to the genes in (7C). Left panel represents 39 probe sets for ground state, right panel represents 188 probe sets for metastable state. Average conventional culture intensity levels are plotted on the X-axis while the average FMM/SMC4 intensity is on the Y-axis, black line indicates equal expression. (7F) Gene expression comparison of X chromosome located genes between hiPSC clone derived and cultured in conventional medium culture and its counterpart adapted to SMC4 culture using Affymetrix probe sets. Probe sets associated with XIST gene expression are highlighted. (7G) Representative images of HEK27me3 on hiPSC clone maintained in FMM or adapted to conventional culture for 5 passages. Dotted arrow in the left panel points to a representative nucleus absent of H3K27me3 staining while solid arrow in the right panel points to a nucleus positive for H3K27me3 staining. Percentages of nucleus positive staining are indicated in the lower left side of each panel. FMM cultured cells have a larger nuclei. Scale bar = 50 µm.

Figures 8A-8D show episomal induced reprogramming with FRM and FMM. (8A) Day 10 SSEA4 and TRA1-81 flow profile of reprogramming pool. (8B) Representative morphology of typical colony seen during reprogramming. Image taken at day 13 post transfection. (8C) Episomal reprogrammed fibroblast cells maintained in FRM for the first 14 days were split and either maintained in FRM or switched to FMM. The reprogramming cultures were then sorted for SSEA4/TRA1-81/CD30 on day 21 post transfection and maintained in FRM or FMM for an additional 10 days prior to analysis. (8D) Morphology and flow profile of representative cultures in FRM or FMM. White arrow points to regions of differentiated cells in a culture that consists of a mixture of undifferentiated and differentiated population. Black arrow points to sharp edges of a mostly undifferentiated population. Lower panels are representative flow profiles. FSC; Forward Side Scatter.

Figures 9A-9B show reprogramming of various parental lines. (9A) Summary table of the starting cell lines used in this study. In addition to specific information related to each line, the percent positive SSEA4/TRA1-81/CD30 population at time of sort post episomal transfection is noted. (9B) Illustration depicting the sort and culture of CD34 enriched cord blood cells. A volume of 0.5 ml cord blood previously maintained in a bank was used to extract 65,000 CD34+CD45+Lin- cells which were cultured in suspension for 6 days prior to episomal transfection.

Figures 10A-10G show the characterization of hiPSCs during the reprogramming and maintenance process. (10A) Typical colony morphology three days post single cell 96-well plate sorting. Scale bar represents 400μm. (10B) Representative morphology of single cell derived hiPSC-like colony 7-9 days post sort from various starting cells. Scale bar represents 1000 μm. (10C) Immunocytochemistry for NANOG expression of hiPSC-like colonies in 96-well plates. (10D) Day 16 flow profile analysis of FTC007 induced to reprogram and maintained either on Matrigel or vitronectin coated culture plates. (10E) Bright-field image, (10F) immunofluorescence for OCT4 and NANOG or (10G) flow cytometry analysis for SSEA4 and TRA1-81 of FTC016-c28 maintained in FMM either continuously on Matrigel or for 5 passages Vitronectin.

Figures 11A-11C show examples of minimal gene reprogramming with the FMM culture platform. (11A) Morphology of cells treated with hygromycin from day 2 to 5 post transfection with episomal construct containing hygromycin selection cassette. (11B) Reprogramming pools were maintained for longer duration and profiled on day 16 post transfection. (11C) Appearance of culture maintained on either Matrigel or Vitronectin.

Figures 12A-12E show the characteristics of hiPSC cultured in multiple conditions. (12A) Lentiviral derived and SMC4 maintained FTi111 displayed the hallmarks of pluripotency and maintained genomic integrity. (12B) Depiction of thaw strategy of FTi111 p43. A single vial was thawed into four culture environments as noted. Surviving cultures were passaged in respective culture with the exception of conventional culture supplemented with thiazovivin on feeder cells, which was transitioned to conventional culture without Thiazovivin in the presence of feeder cells and passaged as clump. (12C) Morphology of recovering cells in various culture post thaw. No surviving cells were identified in the conventional culture without Thiazovivin in the presence of feeder cells. (12D) Morphology of culture sets at passage 3 post thaw. Larger colony morphology was associated with conventional culture. Scale bar 1000 μm. (12E) qRT-PCR analysis for endogenous pluripotent gene expression of each culture set. Data was normalized to GAPDH and relative to H1 hESCs.

Figures 13A-13E show the gene ontology of gene expression profiles of hiPSCs cultured in various conditions. (13A) Table describing the derivation and maintenance of each line described in global gene expression studies. (13B) A total of 300 probe sets were differentially expressed between the conventional and small molecule (FMM and SMC4) culture conditions by greater or less than 2.5-fold. Hierarchical clustering on the 300 probe sets using a complete linkage method based on Euclidean distance measurements. (13C) Gene ontology biological process enrichment analysis (D.A.V.I.D.) of the 133 probe sets up-regulated 2.5-fold or greater with conventional culture (in comparison to small molecule culture). (13D) Gene ontology biological process enrichment analysis (D.A.V.I.D.) of the 167 probe sets up-regulated 2.5-fold or greater with small molecule culture (in comparison to conventional culture). (13E) Gene ontology biological process enrichment analysis of the 126 probe sets up-regulated 2.5-fold or greater with FMM culture (in comparison to conventional culture).

Figures 14A-14F show cloning maps illustrating examples of the lentiviral constructs (14A-14B) and episomal constructs (14C-14F) used for reprogramming. Lentiviral constructs include an EF1α promoter and a LOXP site for CRE-mediated excision of transgenes. Episomal constructs also include an EF1α promoter.

Figures 15A-15C show representative flow analysis for various reprogramming factor combinations at days 8-15. Human fibroblast cells were induced with various combinations of lentiviral mediated reprogramming factors including OCT4, ECAT1, and UTF1.

Figures 16A-16D show representative flow analysis and iPSC morphology characteristics for various reprogramming factor combinations at days 21-27. Data shows unique reprogramming combinations can be used to derive SSEA4+/TRA181+ hiPSCs.

Figures 17A-17B show highly enhanced lentiviral reprogramming efficiency as illustrated by flow analysis (SSEA4+/TRA181+ and CD30+ populations) and iPSC morphology characteristics. Human fibroblast cells were reprogrammed with OCT4, ECAT1, UTF1, ESRRB, and NANOG. Cells were reprogrammed using FRM and maintained in FMM.

Figures 18A-18D show representative flow analysis and phase images of established four iPSC clones after 7-9 passages after 96-well sort. Clones were generated with lentiviral reprogramming factors (OCT4, ECAT1, UTF1, ESRRB, and NANOG) with FRM and maintained in FMM. Populations expressing high SSEA4+/TRA181+ indicate pluripotency.

Figures 19A-19B show representative flow analysis for expression of OCT4 and NANOG in human fibroblast cells reprogrammed with lentiviral reprogramming factors OCT4, ECAT1, UTF1, NANOG and ESRRB. Clones were reprogrammed using FRM and maintained in FMM. Populations expressing high OCT4+/NANOG+ indicate pluripotency.

Figures 20A-20C show the karyotype analysis of hIPSC clones derived from human fibroblast cells reprogrammed with lentiviral reprogramming factors OCT4, ECAT1, UTF1, NANOG and ESRRB. Clones were reprogrammed using FRM and maintained in FMM. Clones exhibit a normal, male karyotype.

Figure 21 shows 96 well plate sorting efficiency of reprogramming factor combination OCT4/ESRRB/NANOG/ECAT1/UTF1 compared to the reprogramming factor combination OCT4/NANOG/SOX2/LARGE T.

Figures 22A-22B show images of colonies during expansion out of 96 well for cells reprogrammed with (22A) OCT4-P2A-OCT4 / NANOG-P2A-ESRRB-T2A-LIN28 / ECAT1-T2A-UTF1 at 4, 6 and 11 days, and (22B) OCT4-P2A-ESRRB / OCT4-P2A-NANOG / ECAT1-T2A-UTF1 for two wells at 7 days and one well at 10 days.

Figures 23A-23C show (23A) a summary the results of flow analysis demonstrating the effect of reprogramming factor stoichiometry and use of a genetic marker for selection of cells with ectopic OCT4 expression, (23B) flow analysis for human fibroblasts reprogrammed with episomal OCT4-P2A-NANOG-T2A-SOX2 / SV40 Large T Antigen without selection of OCT4, and (23C) flow analysis for human fibroblasts reprogrammed with episomal OCT4-P2A-NANOG-T2A-SOX2 / SV40 Large T Antigen / OCT2-P2A-OCT4-Puromycin.

Figure 24 shows immunofluorescent analysis of derived iPSC clones stained for pluripotency markers OCT4 (green) and TRA181(red) with DAPI in blue.

Figure 25 shows images of SSEA4+/TRA181+/CD30+ 96-well plate sorted clones post CRE-mediated excision. Colonies were sorted from an iPSC clone originally derived from human fibroblast cells, reprogrammed with lentiviral factors OCT4, ECAT1, UTF1, NANOG, and ESRRB, and then excised for transgenes. Sorted colonies show an iPSC phenotype.

DETAILED DESCRIPTION

A. Overview

Existing methods for the production and maintenance of pluripotent cells have not yet realized homogenous cultures of footprint-free pluripotent cells free from spontaneous differentiation and amenable to high-resolution/high-clonality single cell passage and large scale expansion. Ground State pluripotent cells may confer qualities and characteristics that overcome these challenges. However, to date, no reliable or robust methods exist for the high-throughput generation of ground state pluripotent cells in feeder-free conditions. Thus, existing methods may not be suitable for the production of industrial- or clinical grade pluripotent cells. The invention contemplated herein addresses a need for the robust generation of stable pluripotent cells in or with characteristics of ground state pluripotency and solves problems in the manufacture of stable pluripotent cells suitable for industrial and clinical use.

In general, the invention relates to compositions and methods for the improved manufacture of pluripotent cells, particularly cells with reduced spontaneous differentiation, including ground state pluripotent cells. More particularly, the invention relates to a multistage culture platform that utilizes small molecule modulators of cellular signal transduction pathways, in a stage-specific manner and enables pluripotent cell derivation and maintenance to the point where culturing methods and methods of deriving pluripotent cells are no longer a source of variability and/or gating activity for downstream use. Moreover, the culture platform contemplated herein enables the derivation and maintenance of pluripotent cells in feeder-free conditions, with improved genomic stability, improved undifferentiated state, reduced spontaneous differentiation, improved culture homogeneity, improved survival in the culturing, dissociation, and passaging of single pluripotent cells, and improved methods of transgene or footprint free reprogramming cells to ground state pluripotency. Thus, the compositions and methods contemplated herein enable the manufacture of pluripotent cells that are appropriate for industrial and clinical use and/or ground state pluripotent cells.

To date no small molecule driven platform has demonstrated the ability to enhance reprogramming and support single cell and FF culture of footprint-free induced pluripotent stem cells (iPSCs) derived from human cells (Nichols and Smith, 2012). The culture platforms contemplated herein, provide for, in part, the application of specific combinations of small molecule inhibitors in a stage-specific manner to enable rapid and robust reprogramming and stable long term culture of pluripotent stem cells. In various embodiments, a culture platform for inducing or maintaining improved undifferentiated pluripotent state including ground state pluripotency is provided. The platform contemplated herein also provides a robust culture system for the production and maintenance of ground state pluripotency in human iPSCs (hiPSCs). In one embodiment, the culture platforms enable a transgene or footprint-free method of reprogramming. In particular embodiments, the platform contemplated herein represents an improved method for the manufacture of hiPSCs that overcomes key challenges in the multiplex derivation and maintenance of transgene-free hiPSC.

The practice of the present invention will employ, unless indicated specifically to the contrary, conventional methods of chemistry, biochemistry, organic chemistry, molecular biology, microbiology, recombinant DNA techniques, genetics, immunology, cell biology, stem cell protocols, cell culture and transgenic biology that are within the skill of the art, many of which are described below for the purpose of illustration. Such techniques are explained fully in the literature. *See*, *e.g.,* Sambrook, *et al.*, *Molecular Cloning: A Laboratory Manual* (3^{rd} Edition, 2001); Sambrook, *et al.*, *Molecular Cloning: A Laboratory Manual* (2^{nd} Edition, 1989); Maniatis *et al.*, *Molecular Cloning: A Laboratory Manual* (1982); Ausubel *et al.*, *Current Protocols in Molecular Biology* (John Wiley and Sons, updated July 2008); *Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology*, Greene Pub. Associates and Wiley-Interscience; Glover, *DNA Cloning: A Practical Approach*, vol. I & II (IRL Press, Oxford, 1985); Anand, *Techniques for the Analysis of Complex Genomes*, (Academic Press, New York, 1992); Guthrie and Fink, *Guide to Yeast Genetics and Molecular Biology* (Academic Press, New York, 1991); *Oligonucleotide Synthesis* (N. Gait, Ed., 1984); *Nucleic Acid Hybridization* (B. Hames & S. Higgins, Eds., 1985); *Transcription and Translation* (B. Hames & S. Higgins, Eds., 1984); *Animal Cell Culture* (R. Freshney, Ed., 1986); Perbal, *A Practical Guide to Molecular Cloning* (1984); Fire *et al.*, *RNA Interference Technology: From Basic Science to Drug Development* (Cambridge University Press, Cambridge, 2005); Schepers, *RNA Interference in Practice* (Wiley-VCH, 2005); Engelke, *RNA Interference (RNAi): The Nuts & Bolts of siRNA Technology* (DNA Press, 2003); Gott, *RNA Interference, Editing, and Modification: Methods and Protocols* (Methods in Molecular Biology; Human Press, Totowa, NJ, 2004); Sohail, *Gene Silencing by RNA Interference: Technology and Application* (CRC, 2004); Clarke and Sanseau, m*icroRNA: Biology, Function & Expression* (Nuts & Bolts series; DNA Press, 2006); *Immobilized Cells And Enzymes* (IRL Press, 1986); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Gene Transfer Vectors For Mammalian Cells* (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Harlow and Lane, *Antibodies*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1998); *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. Blackwell, eds., 1986); Riott, *Essential Immunology,* 6th Edition, (Blackwell Scientific Publications, Oxford, 1988); *Embryonic Stem Cells: Methods and Protocols* (Methods in Molecular Biology) (Kurstad Turksen, Ed., 2002); *Embryonic Stem Cell Protocols:*

Volume I: Isolation and Characterization (Methods in Molecular Biology) (Kurstad Turksen, Ed., 2006); *Embryonic Stem Cell Protocols: Volume II: Differentiation Models* (Methods in Molecular Biology) (Kurstad Turksen, Ed., 2006); *Human Embryonic Stem Cell Protocols* (Methods in Molecular Biology) (Kursad Turksen Ed., 2006); *Mesenchymal Stem Cells: Methods and Protocols* (Methods in Molecular Biology) (Darwin J. Prockop, Donald G. Phinney, and Bruce A. Bunnell Eds., 2008); *Hematopoietic Stem Cell Protocols* (Methods in Molecular Medicine) (Christopher A. Klug, and Craig T. Jordan Eds., 2001); *Hematopoietic Stem Cell Protocols* (Methods in Molecular Biology) (Kevin D. Bunting Ed., 2008) *Neural Stem Cells: Methods and Protocols* (Methods in Molecular Biology) (Leslie P. Weiner Ed., 2008); Hogan *et al.*, *Methods of Manipulating the Mouse Embyro* (2^{nd} Edition, 1994); Nagy *et al.*, *Methods of Manipulating the Mouse Embryo* (3^{rd} Edition, 2002), and *The Zebrafish book. A guide for the laboratory use of zebrafish* (Danio rerio), 4th Ed., (Univ. of Oregon Press, Eugene, OR, 2000).

All publications, patents and patent applications cited herein are hereby incorporated by reference in their entirety.

B. DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which the invention belongs. For the purposes of the present invention, the following terms are defined below.

The articles "a*,*" "an*,*" and "the" are used herein to refer to one or to more than one (*i.e.* to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

The use of the alternative (*e.g.,* "or") should be understood to mean either one, both, or any combination thereof of the alternatives.

The term "and/or" should be understood to mean either one, or both of the alternatives.

As used herein, the term "about" or "approximately" refers to a quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length that varies by as much as 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2% or 1% compared to a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length. In one embodiment, the term “about” or “approximately” refers a range of quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length ± 15%, ± 10%, ± 9%, ± 8%, ± 7%, ± 6%, ± 5%, ± 4%, ± 3%, ± 2%, or ± 1% about a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length.

As used herein, the term “substantially” or “essentially” refers to a quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length that is about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or higher compared to a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length. In one embodiment, the terms “essentially the same” or “substantially the same” refer a range of quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length that is about the same as a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length.

As used herein, the terms “substantially free of” and “essentially free of” are used interchangeably, and when used to describe a composition, such as a cell population or culture media, refer to a composition that is free of a specified substance, such as, 95% free, 96% free, 97% free, 98% free, 99% free of the specified substance, or is undetectable as measured by conventional means. Similar meaning can be applied to the term “absence of,” where referring to the absence of a particular substance or component of a composition.

As used herein, the term “appreciable” refers to a range of quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length or an event that is readily detectable by one or more standard methods. The terms “not-appreciable” and “not appreciable” and equivalents refer to a range of quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length or an event that is not readily detectable or undetectable by standard methods. In one embodiment, an event is not appreciable if it occurs less than 5%, 4%, 3%, 2%, 1%, 0.1%, 0.01%, 0.001% or less of the time.

Throughout this specification, unless the context requires otherwise, the words "comprise," "comprises" and "comprising" will be understood to imply the inclusion of a stated step or element or group of steps or elements but not the exclusion of any other step or element or group of steps or elements. In particular embodiments, the terms "include," "has," "contains," and "comprise" are used synonymously.

By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of." Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present.

By "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that no other elements are optional and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

Reference throughout this specification to "one embodiment," "an embodiment," "a particular embodiment," "a related embodiment," "a certain embodiment," "an additional embodiment," or "a further embodiment" or combinations thereof means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, the appearances of the foregoing phrases in various places throughout this specification are not necessarily all referring to the same embodiment. Furthermore, the particular features, structures, or characteristics may be combined in any suitable manner in one or more embodiments.

The term "*ex vivo*" refers generally to activities that take place outside an organism, such as experimentation or measurements done in or on living tissue in an artificial environment outside the organism, preferably with minimum alteration of the natural conditions. In particular embodiments, "*ex vivo*" procedures involve living cells or tissues taken from an organism and cultured in a laboratory apparatus, usually under sterile conditions, and typically for a few hours or up to about 24 hours, but including up to 48 or 72 hours, depending on the circumstances. In certain embodiments, such tissues or cells can be collected and frozen, and later thawed for *ex vivo* treatment.

Tissue culture experiments or procedures lasting longer than a few days using living cells or tissue are typically considered to be "*in vitro*," though in certain embodiments, this term can be used interchangeably with *ex vivo*.

The term "*in vivo*" refers generally to activities that take place inside an organism.

As used herein, the terms "reprogramming" or "dedifferentiation" or "increasing cell potency" or "increasing developmental potency" refers to a method of increasing the potency of a cell or dedifferentiating the cell to a less differentiated state. For example, a cell that has an increased cell potency has more developmental plasticity (*i.e.,* can differentiate into more cell types) compared to the same cell in the non-reprogrammed state. In other words, a reprogrammed cell is one that is in a less differentiated state than the same cell in a non-reprogrammed state.

As used herein, the term "potency" refers to the sum of all developmental options accessible to the cell (*i.e.,* the developmental potency). One having ordinary skill in the art would recognize that cell potency is a continuum, ranging from the most plastic cell, a totipotent stem cell, which has the most developmental potency to the least plastic cell, a terminally differentiated cell, which has the least developmental potency. The continuum of cell potency includes, but is not limited to, totipotent cells, pluripotent cells, multipotent cells, oligopotent cells, unipotent cells, and terminally differentiated cells.

As used herein, the term "pluripotent" refers to the ability of a cell to form all lineages of the body or soma (*i.e.,* the embryo proper). For example, an embryonic stem cell is a type of pluripotent stem cell that is able to form cells from each of the three germs layers: the ectoderm, the mesoderm, and the endoderm.

Pluripotency can be determined, in part, by assessing pluripotency characteristics of the cells. Pluripotency characteristics include, but are not limited to: (i) pluripotent stem cell morphology; (ii) the potential for unlimited self renewal (iii) expression of pluripotent stem cell markers including, but not limited to SSEA1 (mouse only), SSEA3/4; SSEA5, TRA1-60/81; TRA1-85, TRA2-54, GCTM-2, TG343, TG30, CD9, CD29, CD133/prominin, CD140a, CD56, CD73, CD90, CD105, OCT4, NANOG, SOX2, CD30 and/or CD50; (iv) ability to differentiate to all three somatic lineages (ectoderm, mesoderm and endoderm) (v) teratoma formation consisting of the three somatic lineages; and (vi) formation of embryoid bodies consisting of cells from the three somatic lineages;

Two types of pluripotency have previously been described: the "primed" or "metastable" state of pluripotency akin to the epiblast stem cells (EpiSC) of the late blastocyst and the "Naïve" or "Ground" state of pluripotency akin to the inner cell mass of the early/preimplantation blastocyst. While both pluripotent states exhibit the characteristics as described above, the naïve or ground state further exhibits; (i) preinactivation or reactivation of the X-chromosome in female cells (ii) improved clonality and survival during single-cell culturing (iii) global reduction in DNA methylation, (iv) reduction of H3K27me3 repressive chromatin mark deposition on developmental regulatory gene promoters, and (v) reduced expression of differentiation markers relative to primed state pluripotent cells. Standard methodologies of cellular reprogramming in which exogenous pluripotency genes are introduced to a somatic cell, expressed and then either silenced or removed from the resulting pluripotent cells are generally seen to have characteristics of the primed-state of pluripotency. Under standard pluripotent cell culture conditions such cells remain in the primed state unless the exogenous transgene expression is maintained, wherein characteristics of the ground-state are observed.

As used herein, the term "pluripotent stem cell morphology" refers to the classical morphological features of an embryonic stem cell. Normal embryonic stem cell morphology is characterized by being round and small in shape, with a high nucleus-to-cytoplasm ratio, the notable presence of nucleoli, and typical intercell spacing.

As used herein, the term "gene expression profile," "gene expression signature," "gene expression panel," "gene panel," or "gene signature" refers to the expression or levels of expression of a plurality of genes which serves to distinguish a cell or population of cells from another cell or population of cells. For example, a population of pluripotent cells maintained in a medium to prevent spontaneous differentiation may display a gene expression profile comprising decreased expression of differentiation genes relative to a control population of pluripotent cells of the same origin that are not maintained in the same medium.

As used herein, the term “differentiation marker gene,” or “differentiation gene,” refers to genes whose expression are indicative of cell differentiation occurring within a cell, such as a pluripotent cell. Differentiation marker genes include, but are not limited to, the following genes: FOXA2, FGF5, SOX17, XIST, NODAL, COL3A1, OTX2, DUSP6, EOMES, NR2F2, NR0B1, CXCR4, CYP2B6, GATA3, GATA4, ERBB4, GATA6, HOXC6, INHA, SMAD6, RORA, NIPBL, TNFSF11, CDH11, ZIC4, GAL, SOX3, PITX2, APOA2, CXCL5, CER1, FOXQ1, MLL5, DPP10, GSC, PCDH10, CTCFL, PCDH20, TSHZ1, MEGF10, MYC, DKK1, BMP2, LEFTY2, HES1, CDX2, GNAS, EGR1, COL3A1, TCF4, HEPH, KDR, TOX, FOXA1, LCK, PCDH7, CD1D FOXG1, LEFTY1, TUJ1, T gene (Brachyury) and ZIC1.

As used herein, the term “differentiation marker gene profile,” or “differentiation gene profile,” “differentiation gene expression profile,” “differentiation gene expression signature,” “differentiation gene expression panel,” “differentiation gene panel,” or “differentiation gene signature” refers to the expression or levels of expression of a plurality of differentiation marker genes.

In particular embodiments, a population of pluripotent cells showing decreased spontaneous differentiation may be characterized by a decrease in expression of a differentiation marker gene or a differentiation marker gene profile. For example, decreased spontaneous differentiation in a pluripotent cell or population of pluripotent cells may be indicated where a given set of culture conditions causes a decrease in expression of one or more a differentiation marker genes of at least 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more compared to the expression of the differentiation marker genes of a control pluripotent cell or population of pluripotent cells lacking the same culture conditions.

“Gene expression” as used herein refers to the relative levels of expression and/or pattern of expression of a gene in a biological sample, such as pluripotent cells, or a population of cells comprising pluripotent cells. In particular embodiments, the pluripotent cells are iPSCs.

Any methods available in the art for detecting expression of the genes characterizing the cells of the invention are encompassed herein. As used herein, the term "detecting expression" means determining the quantity or presence of an RNA transcript or its expression product of a gene. Methods for detecting expression of genes, that is, gene expression profiling, include methods based on hybridization analysis of polynucleotides, methods based on sequencing of polynucleotides, immunohistochemistry methods, and proteomics-based methods. The methods generally detect expression products (*e.g.,* mRNA) of the genes of interest. In some embodiments, PCR-based methods, such as reverse transcription PCR (RT-PCR) (Weis *et al.*, TIG 8:263-64, 1992), and array-based methods such as microarray (Schena *et al.*, Science 270:467-70, 1995) are used.

"Adhere" refers to cells attaching to a vessel, for example, a cell attaching to a sterile plastic (or coated plastic) cell culture dish or flask in the presence of an appropriate culture medium. Certain classes of cells are not sustained or do not grow in a culture unless they adhere to the cell culture vessel. Certain classes of cells ("non-adherent cells") are maintained and/or proliferate in culture without adhering.

"Culture" or "cell culture" refers to the maintenance, growth and/or differentiation of cells in an *in vitro* environment. "Cell culture media," "culture media" (singular "medium" in each case), "supplement" and "media supplement" refer to nutritive compositions that cultivate cell cultures.

"Cultivate" refers to the sustaining, propagating (growing) and/or differentiating of cells outside of tissue or the body, for example in a sterile plastic (or coated plastic) cell culture dish or flask. "Cultivation" may utilize a culture medium as a source of nutrients, hormones and/or other factors helpful to propagate and/or sustain the cells.

As used herein, a "dissociated" cell refers to a cell that has been substantially separated or purified away from other cells or from a surface (*e.g.,* a culture plate surface). For example, cells can be dissociated from an animal or tissue by mechanical or enzymatic methods. Alternatively, cells that aggregate *in vitro* can be dissociated from each other, such as by dissociation into a suspension of clusters, single cells or a mixture of single cells and clusters, enzymatically or mechanically. In yet another alternative embodiment, adherent cells are dissociated from a culture plate or other surface. Dissociation thus can involve breaking cell interactions with extracellular matrix (ECM) and substrates (*e.g.,* culture surfaces), or breaking the ECM between cells.

As used herein, the terms “enrich” and “enriching” refer to increasing the amount of a specified component in a composition, such as a composition of cells, and “enriched”, when used to describe a composition of cells such as a cell population, refers to a population of cells having an increased amount proportionally of a specified component as compared to the proportion of such component in the population of cells prior to being enriched. For example, a composition such as a population of cells may be enriched with respect to a target cell type (*i.e.*, cells having specified characteristics), thus having an increased proportion or percent of the target cell type as compared to the proportion of the target cells present in the population of cells before being enriched. A population of cells may be enriched for a target cell type by cell selection and sorting methods known in the art. In some embodiments, a population of cells is enriched by a sorting or selection process as described in the examples herein. In a particular embodiment, a method that enriches for a target cell population enriches the cell population with respect to the target cell population by at least about 20%, meaning that the enriched cell population comprises proportionately about 20% more of the target cell type than in the population before the population was enriched. In one embodiment, a method that enriches for a target cell population enriches the cell population with respect to the target cell population proportionately by at least about 30+%, 40+%, 50+%, 60+%, 70+%, 80%, 85%, 90%, 95%, 97%, 98% or 99%, or at least about 98%, or in particular embodiments, about 99%.

In certain embodiments, a population of cells is enriched with respect to the amount of pluripotent cells or cells exhibiting pluripotency characteristics. In particular embodiments of the invention, a population of cells undergoing reprogramming is enriched for target cells having characteristics of pluripotency, such as expression of pluripotency markers including, without limitation, SSEA3, SSEA4, TRA 1-60, TRA-1-81, CD30 or CD50.

In particular embodiments, a population of cells, such as a population of cells undergoing reprogramming, is depleted of nonpluripotent cells using surface markers specific to differentiated cell lineages or nonpluripotent cells, which may include, for example, CD13, CD26, CD34, CD45, CD31, CD46, or CD7. The resulting cell population can thus be described as a population of cells enriched for pluripotent cells.

In particular embodiments, the enriched cells comprises a distinct gene or protein expression profile, for example, cell surface expression of at least two pluripotency markers such as SSEA3, SSEA4, TRA 1-60, TRA-1-81, CD30 and CD50. In some embodiments, the enriched cells comprise two or more pluripotency markers. In particular embodiments, the enriched cells express SSEA4 in combination with either TRA-181 or TRA-160. In more particular embodiments, the enriched cells express SSEA4, TRA181, and CD30. In one embodiment, a population of cells comprises at least about 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 70%, 75%, 80%, 90%, 95%, 97%, 98%, or 99% of the enriched cells, such as pluripotent cells.

Thus, in some embodiments, methods of enriching a population of cells for pluripotent cells comprise sorting the cell population based on cell surface expression of pluripotency markers, such as SSEA3, SSEA4, TRA 1-60, TRA-1-81, CD30 and CD50, and collecting the fraction of cells expressing such markers to obtain a population of cells that is enriched for pluripotent cells. In other embodiments, a population of cells is enriched for pluripotent cells by sorting the cell population based on cell surface expression of markers of differentiating or differentiated cells, such as CD13, CD26, CD34, CD45, CD31, CD46, and CD7, and depleting the cell population of such cells to obtain a population of cells that is enriched for pluripotent cells. In particular embodiments, the cell population is sorted based on the expression of CD13, and $CD13^+$ cells are removed from the cell population to obtain a population of cells enriched for pluripotent cells.

As used herein, “feeder cells” or “feeders” are terms used to describe cells of one type that are co-cultured with cells of a second type to provide an environment in which the cells of the second type can grow, as the feeder cells provide growth factors and nutrients for the support of the second cell type. The feeder cells are optionally from a different species as the cells they are supporting. For example, certain types of human cells, including stem cells, can be supported by primary cultures of mouse embryonic fibroblasts, and immortalized mouse embryonic fibroblasts. The feeder cells may typically be inactivated when being co-cultured with other cells by irradiation or treatment with an anti-mitotic agent such as mitomycin c, to prevent them from outgrowing the cells they are supporting. Without limiting the foregoing, one specific feeder cell type may be a human feeder, such as a human skin fibroblast. Another feeder cell type may be mouse embryonic fibroblasts (MEF).

As used herein, a “feeder-free” (FF) environment refers to an environment such as a cell culture or culture media essentially free of feeder cells and/or which has not been pre-conditioned by the cultivation of feeder cells. “Pre-conditioned” medium refers to a medium harvested after feeder cells have been cultivated within the medium for a period of time, such as for at least one day. Pre-conditioned medium contains many mediator substances, including growth factors and cytokines secreted by the feeder cells cultivated in the medium.

Genomic stability refers to the ability of a cell to faithfully replicate DNA and maintain integrity of the DNA replication process. As used herein, the terms “genomically stable cells” and “cells having genomic stability” refer to cells that exhibit a frequency of mutations and chromosomal aberrations (such as translocations, aneuploidy, copy number variations and duplications) that is substantially similar to the frequency of mutations and chromosomal aberrations relative to normal somatic human cells.

“Ingredient” refers to any compound or other material, whether chemical or biological in origin that may be used in cell culture media to maintain and/or promote the growth and/or differentiation of cells. The terms “component” “nutrient” and “ingredient” may be used interchangeably. Conventional ingredients used for cell culture media may include but are not limited to amino acids, salts, metals, sugars, lipids, nucleic acids, hormones, vitamins, fatty acids, proteins and the like. Other ingredients that promote and/or maintain cultivation of cells *ex vivo* may be selected by those persons of ordinary skill in the art as required for a desired effect.

“Isolate” or “isolating” refers to separating and collecting a composition or material from its natural environment, such as the separating of individual cell or cell cultures from tissue or the body. In one aspect, a population or composition of cells is substantially free of cells and materials with which it can be associated in nature.

"Isolated" or "purified" or "substantially pure", with respect to a target population of cells, refers to a population of cells that is at least about 50%, at least about 75%, at least about 85%, at least about 90%, and in particular embodiments, at least about 95% pure, with respect to the target cells making up a total cell population. Purity of a population or composition of cells can be assessed by appropriate methods that are well known in the art. For example, a substantially pure population of pluripotent cells refers to a population of cells that is at least about 50%, at least about 75%, at least about 85%, at least about 90%, and in particular embodiments at least about 95%, and in certain embodiments about 98% pure, with respect to pluripotent cells making up the total cell population. The term "essentially pure" is used interchangeably herein with "substantially pure".

"Passage" or "passaging" refers to the act of subdividing and plating cells into multiple cell culture surfaces or vessels when the cells have proliferated to a desired extent. In some embodiments "passage" or "passaging" refers to subdividing, diluting and plating the cells. As cells are passaged from the primary culture surface or vessel into a subsequent set of surfaces or vessels, the subsequent cultures may be referred to herein as "secondary culture" or "first passage," *etc.* Each act of subdividing and plating into a new culture vessel is considered one passage.

"Plating" refers to placing a cell or cells into a culture vessel such that the cells adhere to and spread on a cell culture vessel.

A "pluripotency factor" refers to an agent capable of increasing the developmental potency of a cell, either alone or in combination with other agents. Pluripotency factors include, without limitation, polynucleotides, polypeptides, and small molecules capable of increasing the developmental potency of a cell. Exemplary pluripotency factors include, for example, transcription factors and small molecule reprogramming agents.

"Proliferate" refers to the property of one cell dividing into two essentially identical cells or a population of cells increasing in number (*e.g.,* to reproduce).

"Propagation" refers to growing (*e.g.,* reproducing via cell proliferation) cells outside of tissue or the body, for example, in a sterile container such as a plastic (or coated plastic) cell culture dish or flask.

"Primary culture" refers to cells, tissue and/or culture where the isolated cells are placed in a first culture vessel with culture medium. The cells, tissue and/or culture may be sustained and/or may proliferate, however, as long as the cells, tissue and/or culture remain in the first vessel the cells, tissue and/or culture are referred to as the primary culture.

The terms "small molecule reprogramming agent" or "small molecule reprogramming compound" are used interchangeably herein and refer to small molecules that can increase developmental potency of a cell, either alone or in combination with other pluripotency factors. A "small molecule" refers to an agent that has a molecular weight of less than about 5 kD, less than about 4 kD, less than about 3 kD, less than about 2 kD, less than about 1 kD, or less than about .5kD. Small molecules include, but are not limited to: nucleic acids, peptidomimetics, peptoids, carbohydrates, lipids or other organic or inorganic molecules. Libraries of chemical and/or biological mixtures, such as fungal, bacterial, or algal extracts, are known in the art and can be used as a source of small molecules in certain embodiments. In particular embodiments, the small molecule reprogramming agent used herein has a molecular weight of less than 10,000 daltons, for example, less than 8000, 6000, 4000, 2000 daltons, *e.g.,* between 50-1500, 500-1500, 200-2000, 500-5000 daltons.

C. *CELLS*

In a particular embodiment, one or more cells may be cultured, dissociated, and passaged using the compositions and methods contemplated herein. In one embodiment, single cells cultured, dissociated, and passaged using the compositions and methods contemplated herein. In another embodiment, a population of cells or a plurality of cells is cultured, dissociated, and passaged using the compositions and methods contemplated herein.

A starting population of cells suitable for use in particular embodiments may be derived from essentially any suitable source, and may be heterogeneous or homogeneous with respect to cell types or state of pluripotency. Suitable cells include fetal cells and adult cells. In addition, suitable cells may be mammalian in origin, *e.g.,* from a rodent, a cat, a dog, a pig, a goat, a sheep, a horse, a cow, or a primate. In one embodiment, the cells are human cells.

The cells may be somatic, non-pluripotent, incompletely or partially pluripotent stem cells, multipotent cells, oligopotent cells, unipotent cells, terminally differentiated cells, or a mixed population of cells comprising any combination of the foregoing. Pluripotent cells suitable for use in particular embodiments include, but are not limited to, naturally-occurring stem cells, embryonic stem cells, or iPSCs. A "mixed" population of cells is a population of cells of varying degrees of developmental potency. For example, a mixed population of cells may comprise cells undergoing reprogramming, so that the mixed population comprises pluripotent cells, partially pluripotent cells, and non-pluripotent cells, such as fully differentiated cells.

In one embodiment, the starting population of cells is selected from adult or neonatal stem/progenitor cells. In particular embodiments, the starting population of stem/progenitor cells is selected from the group consisting of: mesodermal stem/progenitor cells, endodermal stem/progenitor cells, and ectodermal stem/progenitor cells.

Illustrative examples of mesodermal stem/progenitor cells include, but are not limited to: mesodermal stem/progenitor cells, endothelial stem/progenitor cells, bone marrow stem/progenitor cells, umbilical cord stem/progenitor cells, adipose tissue derived stem/progenitor cells, hematopoietic stem/progenitor cells (HSCs), mesenchymal stem/progenitor cells, muscle stem/progenitor cells, kidney stem/progenitor cells, osteoblast stem/progenitor cells, chondrocyte stem/progenitor cells, and the like.

Illustrative examples of ectodermal stem/progenitor cells include, but are not limited to neural stem/progenitor cells, retinal stem/progenitor cells, skin stem/progenitor cells, and the like.

Illustrative examples of endodermal stem/progenitor cells include, but are not limited to liver stem/progenitor cells, pancreatic stem/progenitor cells, epithelial stem/progenitor cells, and the like.

In certain embodiments, the starting population of cells may be a heterogeneous or homogeneous population of cells selected from the group consisting of: pancreatic islet cells, CNS cells, PNS cells, cardiac muscle cells, skeletal muscle cells, smooth muscle cells, hematopoietic cells, bone cells, liver cells, an adipose cells, renal cells, lung cells, chondrocyte, skin cells, follicular cells, vascular cells, epithelial cells, immune cells, endothelial cells, and the like.

D. Culture Platforms for Reducing Spontaneous Differentiation and Inducing Ground State Pluripotency

Cell banking, disease modeling and cell therapy applications have placed increasing demands on manufacturing high quality pluripotent cells. For example, the high-throughput derivation of footprint-free iPSCs and their expansion in systems that allow scaled production remains technically elusive. In particular embodiments, culture platform are contemplated that allow for the rapid, parallel generation, selection and expansion of pluripotent cells using small molecule pathway inhibitors in stage-specific media compositions. The platforms contemplated herein support efficient and expedited reprogramming using minimal reprogramming factors in a completely feeder-free environment; enable single cell culture and expansion of pluripotent cells while maintaining a homogenous and genomically stable pluripotent population. Moreover, the culture platforms contemplated herein, provide culturing pluripotent cells, including hESCs and hiPSCs, to a reduced state of spontaneous differentiation and a common ground state of pluripotency, irrespective of genetic background and independent of transgene expression.

The culture platforms contemplated herein are useful, in part, for the production of industrial- or clinical-grade pluripotent cells having reduced spontaneous differentiation in culture. In one embodiment, non-pluripotent cells are induced to become pluripotent cells and cultured to maintain pluripotency. In another embodiment, non-pluripotent cells are induced to become pluripotent cells and cultured to achieve and/or maintain reduced spontaneous differentiation in culture. In another embodiment, non-pluripotent cells are induced to become pluripotent cells and cultured to achieve and/or maintain ground state pluripotency.

In various embodiments, the culture platforms contemplated herein maintain ground state pluripotency, normal karyotypes, and genomic stability of one or more pluripotency cells for at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100 or more passages, including any intervening number of passages.

In other embodiments, the culture platforms contemplated herein maintain reduced spontaneous differentiation in one or more pluripotency cells for at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100 or more passages, including any intervening number of passages.

In one embodiment, the culture platform comprises a cell culture medium comprising a cell culture medium and a GSK-3 inhibitor, a MEK inhibitor, and a Rho Kinase (ROCK) inhibitor. In various embodiments, the cell culture media contemplated herein do not comprise or lack an inhibitor of TGFβ/activin signaling pathways, including TGFβ receptor (TGFβR) inhibitors and ALK5 inhibitors. Without wishing to be bound to any particular theory, the inventors surprisingly discovered that while TGFβR/ALK5 inhibitors increase the efficiency of reprogramming, these inhibitors counteract the long-term maintenance, quality and homogeneity of a pluripotent cell population *i.e.* the inhibition of TGFβ pathway signaling improved the efficiency of cellular reprogramming but relief from this inhibition is required for subsequent maintenance of the pluripotent cell population in *in vitro* culture systems, particularly in systems using feeder-cell free and single cell, enzymatic passage where a homogeneous pluripotent population with reduced spontaneous differentiation is preferred and more particularly where transgene expression is absent. In addition, culturing metastable pluripotent cells in media comprising a GSK-3 inhibitor and a MEK inhibitor and optionally a ROCK inhibitor, but lacking TGFβR/ALK5 inhibitors, as disclosed herein, transition pluripotent cells to achieve reduced spontaneous differentiation and/or achieve ground state pluripotency. The culture media platform contemplated herein also enables efficient reprogramming and long-term culture of pluripotent cells in feeder free environments. Further, while “an ALK5 inhibitor” is not intended to encompass non-specific kinase inhibitors, an “ALK5 inhibitor” should be understood to encompass inhibitors that inhibit ALK4 and/or ALK7 in addition to

ALK5, such as, for example, SB- 431542 (see, *e.g.,* Inman, *et al., J Mol. Pharmacol.* 62(1): 65-74 (2002).

In a preferred embodiment, the culture platform comprises a cell culture medium comprising a GSK-3 inhibitor, a MEK inhibitor, a Rho Kinase (ROCK) inhibitor, and optionally, LIF and/or bFGF, and does not comprise a small molecule inhibitor of a TGFβ/activin signaling pathway including but not limited to TGFβR or ALK5 inhibitors.

In additional embodiments, the cell culture media is substantially free of cytokines and/or growth factors, and optionally is a feeder-free environment. In other embodiments, the cell culture media contains supplements such as serums, extracts, growth factors, hormones, cytokines and the like.

In one preferred embodiment, the culture platform comprises feeder-free cultures.

The culture platforms contemplated herein also offer numerous advantages such as manufacturing a homogenous population of industrial- or clinical-grade pluripotent cells having reduced spontaneous differentiation and/or achieving ground state pluripotency. As used herein, the term "homogenous" refers to a population of cells wherein each cell is the same or substantially the same as the other cells in the population. In one embodiment, a cell is the same as other cells in the population if each cell expresses one or more of the same pluripotency markers as contemplated herein, *e.g.,* SSEA4 and TRA1-81. In one embodiment, the population is homogenous if at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or more of the cells are the same or substantially the same as other cells in the population.

1. TGFβ Receptor/ALK5 Inhibitors

TGFβ receptor (*e.g.,* ALK5) inhibitors can include antibodies to, dominant negative variants of, and antisense nucleic acids that suppress expression of, TGFβ receptors (*e.g.,* ALK5). Exemplary TGFβ receptor/ALK5 inhibitors include, but are not limited to, SB431542 (see, *e.g.,* Inman, *et al.*, Molecular Pharmacology 62(1):65-74 (2002)), A-83-01, also known as 3-(6-Methyl-2-pyridinyl)-N-phenyl-4-(4-quinolinyl)-1H-p yrazole-1-carbothioamide (see, *e.g.,* Tojo, *et al.*, Cancer Science 96(11):791-800

(2005), and commercially available from, *e.g.,* Toicris Bioscience); 2-(3-(6-Methylpyridin-2-yl)-1H-pyrazol-4-yl)-1,5-naphthyridine, Wnt3a/BIO (see, *e.g.,* Dalton, *et al.*, WO2008/094597, herein incorporated by reference), BMP4 (see, Dalton, supra), GW788388 (-{4-[3-(pyridin-2-yl)-1H-pyrazol-4-yl]pyridin-2-yl}-N-(tetrahydro-2H-pyran-4-yl)benzamide) (see, *e.g.,* Gellibert, *et al.*, Journal of Medicinal Chemistry 49(7):2210-2221 (2006)), SM16 (see, *e.g.,* Suzuki, *et al.*, Cancer Research 67(5):2351-2359 (2007)), IN-1130 (3-((5-(6-methylpyridin-2-yl)-4-(quinoxalin-6-yl)-1H-imidazol-2-yl)methyl)benzamide) (see, *e.g.,* Kim, *et al.*, Xenobiotica 38(3):325-339 (2008)), GW6604 (2-phenyl-4-(3-pyridin-2-yl-1H-pyrazol-4-yl)pyridine) (see, *e.g.,* de Gouville, *et al.*, Drug News Perspective 19(2):85-90 (2006)), SB-505124 (2-(5-benzo[1,3]dioxol-5-yl-2-tert-butyl-3H-imidazol-4-yl)-6-methylpyridine hydrochloride) (see, *e.g.,* DaCosta, *et al.*, Molecular Pharmacology 65(3):744-752 (2004)) and pyrimidine derivatives (see, *e.g.,* those listed in Stiefl, *et al.*, WO2008/006583, herein incorporated by reference). Further, while "an ALK5 inhibitor" is not intended to encompass non-specific kinase inhibitors, an "ALK5 inhibitor" should be understood to encompass inhibitors that inhibit ALK4 and/or ALK7 in addition to ALK5, such as, for example, SB-431542 (see, *e.g.,* Inman, *et al.*, J, Mol. Pharmacol. 62(1): 65-74 (2002). Without intending to limit the scope of the invention, it is believed that ALK5 inhibitors affect the mesenchymal to epithelial conversion/transition (MET) process. TGFβ/activin pathway is a driver for epithelial to mesenchymal transition (EMT). Therefore, inhibiting the TGFβ/activin pathway can facilitate MET (*i.e.* reprogramming) process.

In view of the data herein showing the effect of inhibiting ALK5, it is believed that inhibition of the TGFβ/activin pathway will have similar effects of inhibiting ALK5. Thus, any inhibitor (*e.g.,* upstream or downstream) of the TGFβ/activin pathway can be used in combination with, or instead of, ALK5 inhibitors as described in each paragraph herein. Exemplary TGFβ/activin pathway inhibitors include but are not limited to: TGFβ receptor inhibitors, inhibitors of SMAD 2/3 phosphorylation, inhibitors of the interaction of SMAD 2/3 and SMAD 4, and activators/agonists of SMAD 6 and SMAD 7. Furthermore, the categorizations described below are merely for organizational purposes and one of skill in the art would know that compounds can affect one or more points within a pathway, and thus compounds may function in more than one of the defined categories.

TGFβ receptor (TGFβR) inhibitors can include antibodies to, dominant negative variants of and siRNA or antisense nucleic acids that target TGFβ receptors. Specific examples of TGFβ receptor inhibitors include but are not limited to SU5416; 2-(5-benzo[1,3]dioxol-5-yl-2-tert-butyl-3H-imidazol-4-yl)-6-methylpyridine hydrochloride (SB-505124); lerdelimumb (CAT-152); metelimumab (CAT-192); GC-1008; ID11; AP-12009; AP-11014; LY550410; LY580276; LY364947; LY2109761; SB-505124; SB-431542; SD-208; SM16; NPC-30345; Ki26894; SB-203580; SD-093; Gleevec; 3,5,7,2',4'-pentahydroxyflavone (Morin); activin-M108A; P144; soluble TBR2-Fc; and antisense transfected tumor cells that target TGFβ receptors. (See, *e.g.,* Wrzesinski, *et al.*, Clinical Cancer Research 13(18):5262-5270 (2007); Kaminska, *et al.*, Acta Biochimica Polonica 52(2):329-337 (2005); and Chang, *et al.*, Frontiers in Bioscience 12:4393-4401 (2007)).

Inhibitors of SMAD 2/3 phosphorylation can include antibodies to, dominant negative variants of and antisense nucleic acids that target SMAD2 or SMAD3. Specific examples of inhibitors include PD169316; SB203580; SB-431542; LY364947; A77-01; and 3,5,7,2',4'-pentahydroxyflavone (Morin). (See, *e.g.,* Wrzesinski, supra; Kaminska, supra; Shimanuki, *et al.*, Oncogene 26:3311-3320 (2007); and Kataoka, *et al.*, EP1992360, incorporated herein by reference).

Inhibitors of the interaction of SMAD 2/3 and smad4 can include antibodies to, dominant negative variants of and antisense nucleic acids that target SMAD2, SMAD3 and/or smad4. Specific examples of inhibitors of the interaction of SMAD 2/3 and SMAD4 include but are not limited to Trx-SARA, Trx-xFoxH1b and Trx-Lef1. (See, *e.g.,* Cui, *et al.*, Oncogene 24:3864-3874 (2005) and Zhao, *et al.*, Molecular Biology of the Cell, 17:3819-3831 (2006)).

Activators/agonists of SMAD 6 and SMAD 7 include but are not limited to antibodies to, dominant negative variants of and antisense nucleic acids that target SMAD 6 or SMAD 7. Specific examples of inhibitors include but are not limited to smad7-as PTO-oligonucleotides. (See, *e.g.,* Miyazono, *et al.*, US6534476, and Steinbrecher, *et al.*, US2005119203, both incorporated herein by reference).

2. *WNT PATHWAY AGONISTS*

As used herein, the terms "Wnt signal-promoting agent," “Wnt pathway activating agent,” or “Wnt pathway agonist,” refers to an agonist of the Wnt signaling pathway, including but not limited to an agonist of one or more of Wntl, Wnt2, Wnt2b/13, Wnt3, Wnt3a, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, Wnt7b, Wnt7c, Wnt8, Wnt8a, Wnt8b, Wnt8c, Wnt10a, Wntl0b, Wnt11, Wnt14, Wnt15, or Wnt16. Wnt pathway agonists further include, but are not limited to, one or more of the following polypeptides or a fragment thereof: a Dkk polypeptide, a crescent polypeptide, a cerberus polypeptide, an axin polypeptide, a Frzb polypeptide, a T-cell factor polypeptide, or a dominant negative disheveled polypeptide.

Non-limiting examples of Wnt pathway agonists further include one or more of the following: a nucleic acid comprising a nucleotide sequence that encodes a Wnt polypeptide, a polypeptide comprising an amino acid sequence of a Wnt polypeptide, a nucleic acid comprising a nucleotide sequence that encodes an activated Wnt receptor, a polypeptide comprising an amino acid sequence of an activated Wnt receptor, a small organic molecule that promotes Wnt/β-catenin signaling, , a small organic molecule that inhibits the expression or activity of a Wnt antagonist, an antisense oligonucleotide that inhibits expression of a Wnt antagonist, a ribozyme that inhibits expression of a Wnt antagonist, an RNAi construct, siRNA, or shRNA that inhibits expression of a Wnt antagonist, an antibody that binds to and inhibits the activity of a Wnt antagonist, a nucleic acid comprising a nucleotide sequence that encodes a β-catenin polypeptide, a polypeptide comprising an amino acid sequence of a β-catenin polypeptide, a nucleic acid comprising a nucleotide sequence that encodes a Lef-1 polypeptide, a polypeptide comprising an amino acid sequence of a Lef-1 polypeptide.

Wnt pathway agonists further include GSK3 inhibitors, such as, for example, a nucleic acid comprising a nucleotide sequence that encodes a dominant negative GSK-3, GSK3α, or GSK3β polypeptide, a polypeptide comprising an amino acid sequence of a dominant negative GSK-3, GSK3α, or GSK3β polypeptide, a small organic molecule that binds to and inhibits the expression or activity of GSK-3, GSK3α, or GSK3β, an RNAi construct, siRNA, or shRNA that binds to and inhibits the expression and/or activity of GSK-3, GSK3α, or GSK3β, an antisense oligonucleotide that binds to and inhibits the expression of GSK-3, GSK3α, or GSK3β, an antibody that binds to and inhibits the expression and/or activity of GSK-3, GSK3α, or GSK3β, a ribozyme that binds to and inhibits the expression of GSK-3, GSK3α, or GSK3β, and any GSK-3 -independent reagent that activates β-catenin target genes similar in effect to GSK-3 inhibition.

3. GSK-3B Inhibitors

GSK-3β inhibitors are specific exemplary Wnt pathway agonists suitable for use in compositions contemplated herein, and may include, but are not limited to, polynucleotides, polypeptides, and small molecules. GSK-3β inhibitors contemplated herein may decrease GSK-3β expression and/or GSK-3β activity. Illustrative examples of GSK-3β inhibitors contemplated herein include, but are not limited to, anti- GSK-3β antibodies, dominant negative GSK-3β variants, siRNA, shRNA, miRNA and antisense nucleic acids that target GSK-3β.

Other illustrative GSK-3β inhibitors include, but are not limited to: Kenpaullone, l-Azakenpaullone,CHIR99021, CHIR98014, AR-A014418, CT 99021, CT 20026, SB216763, AR-A014418, lithium, SB 415286, TDZD-8, BIO, BIO-Acetoxime, (5-Methyl- lH-pyrazol-3-yl)-(2-phenylquinazolin-4-yl)amine, Pyridocarbazole- cyclopenadienylruthenium complex, TDZD-8 4-Benzyl-2-methyl-l,2,4- thiadiazolidine-3,5-dione, 2-Thio(3-iodobenzyl)-5-(l-pyridyl)-[l,3,4]- oxadiazole, OTDZT, alpha-4-Dibromoacetophenone, AR-AO 144-18, 3- (l-(3-Hydroxypropyl)-lH-pyrrolo[2,3-b]pyridin-3-yl]-4-pyrazin-2-yl-pyrrole-2,5-dione; TWSl 19 pyrrolopyrimidine compound, L803 H-KEAPPAPPQSpP-NH2 or its myristoylated form; 2-Chloro-l- (4,5-dibromo-thiophen-2-yl)-ethanone; GF109203X; RO318220; TDZD-8; TIBPO; and OTDZT.

In particular illustrative embodiments, the GSK-3β inhibitor is CHIR99021, BIO, or Kenpaullone.

In a preferred embodiment, the GSK-3β inhibitor is CHIR99021.

4. *ERK/MEK INHIBITORS*

ERK/MEK inhibitors suitable for use in compositions contemplated herein include, but are not limited to, polynucleotides, polypeptides, and small molecules. ERK/MEK inhibitors contemplated herein may decrease MEK or ERK expression and/or MEK or ERK activity. Illustrative examples of MEK/ERK inhibitors contemplated herein include, but are not limited to, anti- MEK or anti-ERK antibodies, dominant negative MEK or ERK variants, siRNA, shRNA, miRNA and antisense nucleic acids that target MEK or ERK.

Other illustrative ERK/MEK inhibitors include, but are not limited to, PD0325901, PD98059, UO126, SL327, ARRY- 162, PD184161, PD184352, sunitinib, sorafenib, Vandetanib, pazopanib, Axitinib, GSKl 120212, ARRY-438162, RO5126766, XL518, AZD8330, RDEAl 19, AZD6244, FR180204 and PTK787.

Additional illustrative MEK/ERK inhibitors include those compounds disclosed in International Published Patent Applications WO 99/01426, WO 02/06213, WO 03/077914, WO 05/051301 and WO2007/044084.

Further illustrative examples of MEK/ERK inhibitors include the following compounds: 6-(4-Bromo-2-chloro-phenylamino)-7-fluoro-3-methyl-3H-benzoimidazol- e-5-carboxylic acid (2,3-dihydroxy-propoxy)-amide; 6-(4-Bromo-2-chloro-phenylamino)-7-fluoro-3-(tetrahydro-pyran-2-ylm- ethyl)-3H-benzoimidazole-5-carboxylic acid (2-hydroxy-ethoxy)-amide, 1-[6-(4-Bromo-2-chloro-phenylamino)-7-fluoro-3-methyl-3H-benzoimida- zol-5-yl]-2-hydroxy-ethanone, 6-(4-Bromo-2-chloro-phenylamino)-7-fluoro-3-methyl-3H-benzoimidazol- e-5-carboxylic acid (2-hydroxy-1,1-dimethyl-ethoxy)-amide, 6-(4-Bromo-2-chloro-phenylamino)-7-fluoro-3-(tetrahydro-furan-2-ylm- ethyl)-3H-benzoimidazole-5-carboxylic acid (2-hydroxy-ethoxy)-amide, 6-(4-Bromo-2-fluoro-phenylamino)-7-fluoro-3-methyl-3H-benzoimidazol- e-5-carboxylic acid (2-hydroxy-ethoxy)-amide, 6-(2,4-Dichloro-phenylamino)-7-fluoro-3-methyl-3H-benzoimidazole-5-- carboxylic acid (2-hydroxy-ethoxy)-amide, 6-(4-Bromo-2-chloro-phenylamino)-7-fluoro-3-methyl-3H-benzoimidazol- e-5-carboxylic acid (2-hydroxy-ethoxy)-amide, referred to hereinafter as MEK inhibitor 1; 2-[(2-fluoro-4-iodophenyl)amino]-N-(2-hydroxyethoxy)-1,5-dimethyl-6- -oxo-1,6-dihydropyridine-3-carboxamide; referred to hereinafter as MEK inhibitor 2; and 4-(4-bromo-2-fluorophenylamino)-N-(2-hydroxyethoxy)-1,5-dimethyl-6-- oxo-1,6-dihydropyridazine-3-carboxamide or a pharmaceutically acceptable salt thereof.

In a preferred embodiment, the MEK/ERK inhibitor is PD98059.

5. *ROCK Inhibitors*

Rho associated kinases (ROCK) are serine/threonine kinases that serve downstream effectors of Rho kinases (of which three isoforms exist--RhoA, RhoB and RhoC). ROCK inhibitors suitable for use in compositions contemplated herein include, but are not limited to, polynucleotides, polypeptides, and small molecules. ROCK inhibitors contemplated herein may decrease ROCK expression and/or ROCK activity. Illustrative examples of ROCK inhibitors contemplated herein include, but are not limited to, anti-ROCK antibodies, dominant negative ROCK variants, siRNA, shRNA, miRNA and antisense nucleic acids that target ROCK.

Illustrative ROCK inhibitors contemplated herein include, but are not limited to: thiazovivin, Y27632, Fasudil, AR122-86, Y27632 H-1152, Y-30141, Wf-536, HA-1077, hydroxyl-HA-1077, GSK269962A, SB-772077-B, N-(4-Pyridyl)-N'-(2,4,6-trichlorophenyl)urea, 3-(4-Pyridyl)-1H-indole, and (R)-(+)-trans-N-(4-Pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide and ROCK inhibitors disclosed in U.S. Patent No. 8,044,201, which is herein incorporated by reference in its entirety.

In one embodiment, the ROCK inhibitor is thiazovivin, Y27632, or pyrintegrin.

In a preferred embodiment, the ROCK inhibitor is thiazovivin.

The amount of the small molecules in the compositions and cell culture media contemplated herein can vary and may be optimized according to the specific culture conditions, including the specific molecules and combinations used, the type of cell being cultured in the media, and the specific application. In one embodiment, a small molecule is present in a composition at a concentration sufficient to induce pluripotency, improve the efficiency of reprogramming, increase or maintain the potency of a cell, or induce or maintain ground state pluripotency.

In particular embodiments, preferred concentrations and combinations of the small molecules in the cell culture media of the invention are shown in Table 1 as Fate

Maintenance Medium (FMM). The components of the medium may be present in the medium in amounts within an optimal range of about the optimal concentrations shown in Table 1. Fate Reprogramming Medium (FRM) is useful in culture platforms contemplated herein that includes the reprogramming of cells, but is not suitable for establishment and long-term maintenance of ground state pluripotent cells.

Table 1

Conventional hESC Medium (Conv.)	Fate Reprogramming Medium (FRM)	Fate Maintenance Medium (FMM)
DMEM/F12	DMEM/F12	DMEM/F12
Knockout Serum Replacement (20%)	Knockout Serum Replacement (20%)	Knockout Serum Replacement (20%)
	N2 (1x)	
	B27 (1x)	
Glutamine (1x)	Glutamine (1x)	Glutamine (1x)
Non-Essential Amino Acids (1x)	Non-Essential Amino Acids (1x)	Non-Essential Amino Acids (1x)
β-mercaptoethanol (100μM)	β-mercaptoethanol (100μM)	β-mercaptoethanol (100μM)
bFGF (10ng/mL)	bFGF (100ng/mL)	bFGF (100ng/mL)
	LIF (10ng/mL)	LIF (10ng/mL)
	Thiazovivin (5.0 μM)	Thiazovivin (5.0 μM)
	PD0325901 (0.4 μM)	PD0325901 (0.4 μM)
	CHIR99021 (1.0 μM)	CHIR99021 (1.0 μM)
	SB431542 (2.0 μM)	
In combination with MEF feeder cells	Feeder free, in combination with Matrigel or Vitronectin	

6. *Cytokines and Growth Factors*

In particular embodiments, the cell culture media of the invention is substantially free of cytokines and/or growth factors. In certain embodiments, the cell culture media contains one or more supplements including, but not limited to sera, extracts, growth factors, hormones, cytokines and the like.

In one illustrative embodiment, the culture media may comprise one or more of, ECM proteins, laminin 1, fibronectin, collagen IV isotypes, proteases, protease inhibitors, cell surface adhesion proteins, cell-signaling proteins, cadherins, chloride intracellular channel 1, transmembrane receptor PTK7, insulin-like growth factor, or Inhibin beta A, but does not comprise inducers of the TGFβ/Activin/nodal signaling pathway, and Activin A In other embodiments, the media may comprise inducers of the TGFβ/Activin/nodal signaling pathway.

In another illustrative embodiment, a culture medium comprises one or more of the following cytokines or growth factors: epidermal growth factor (EGF), acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), leukemia inhibitory factor (LIF), hepatocyte growth factor (HGF), insulin-like growth factor 1 (IGF-1), insulin-like growth factor 2 (IGF-2), keratinocyte growth factor (KGF), nerve growth factor (NGF), platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β), vascular endothelial cell growth factor (VEGF) transferrin, various interleukins (such as IL-1 through IL-18), various colony-stimulating factors (such as granulocyte/macrophage colony-stimulating factor (GM-CSF)), various interferons (such as IFN-γ) and other cytokines having effects upon stem cells such as stem cell factor (SCF) and erythropoietin (Epo). These cytokines may be obtained commercially, for example from R&D Systems, Minneapolis, Minn., and may be either natural or recombinant. In particular embodiments, growth factors and cytokines may be added at concentrations contemplated herein. In certain embodiments growth factors and cytokines may be added at concentrations that are determined empirically or as guided by the established cytokine art.

7. *CULTURE SUBSTRATES*

Any suitable vessel or cell culture container may be used as a support for cell cultures in the basal media and/or the cell culture supplements. No substrate coating on the support is necessary. Coating the surface of a culture vessel with adhesion-promoting substrata (for example, collagens, fibronectins, RGD-containing polypeptides, gelatins, and the like) however promotes attachment of the cells and in particular embodiments may enhance the effect of the cell culture media and supplements disclosed herein. Suitable substrates for culturing and passaging cells are known in the art and include, without limitation, vitronectin, gelatin, Laminin, Fibronectin, Collagen, Elastin, osteopontin, mixtures of naturally occurring cell line-produced matrices such as Matrigel™ and synthetic or man-made surfaces such as Polyamine monolayers and carboxy-terminated monolayers .

In one embodiment, a culture platform contemplated herein comprises a substrate comprising Matrigel™ or vitronectin.

8. FEEDER FREE ENVIRONMENTS

Existing methods for culturing pluripotent cells rely heavily on feeder cells or media pre-conditioned with feeder cells and containing fetal bovine serum; however, such environments may be unsuitable for producing cells for clinical and therapeutic use. For example, cells cultivated in such xeno-contaminated environments are generally considered unsuitable for human cell transplantation because the exposure to animal components may present a serious risk of immune rejection and transmitting unidentified pathogens to the treated patients, and could potentially reactivate animal retroviruses. Culture systems using animal-free culture media, such as the feeder free environments contemplated herein, facilitate the manufacture of clinical-grade cell lines, particularly hESC and hiPSC cell lines.

In particular embodiments, the feeder free environment is essentially free of human feeder cells, including without limitation mouse embryonic fibroblasts, human fibroblasts, keratinocytes, and embryonic stem cells, and is not pre-conditioned by feeder cells. The feeder free cell culture medium is suitable for use in culturing pluripotent cells, reprogramming cells, single-cell culture, dissociation, and passaging of pluripotent cells, cell sorting of pluripotent cells, generation of ground state pluripotent cells, and maintenance of ground state pluripotency. In particular embodiments, the feeder free environment is used to induce pluripotency, improve the efficiency of reprogramming, and/or increase or maintain the potency of a cell. In certain embodiments, the feeder free environment is substantially free of cytokines and growth factors, including bFGF.

9. Dissociation

One of the advantages offered by the culture platforms contemplated herein is the enhanced viability and survival of culturing, passaging, and dissociating single ground state pluripotent cells. Disassociation of cells into single cells, such as into a single cell suspension, can be accomplished by enzymatic or mechanical means. Any enzymatic agent known in the art to allow dissociation of cells into single cells may be used in the methods of the invention. In one embodiment, the dissociation agent is selected from Trypsin/EDTA, TrypLE-Select, Collagenase IV and Dispase.

A chelator, such as EDTA, Accutase, or AccuMax, may also be used, alone or in combination with an enzymatic agent, in dissociating cells in accordance with the methods contemplated herein. The dissociation agent may be dissolved in calcium and magnesium free PBS to facilitate dissociation to single cells.

To enhance the survival of the cells during and after dissociation, a survival promoting substance can be added (*e.g.,* growth factor, inhibitors of cellular pathways involved in cell death and apoptosis, or conditioned media), *e.g.,* a ROCK inhibitor such as thiazovivin.

Techniques in cell culture and media collection are outlined in Hu *et al.*, *Curr. Opin. Biotechnol.* 8:148, 1997; K. Kitano, *Biotechnology* 17:73, 1991; *Curr. Opin. Biotechnol.* 2:375, 1991; Birch *et al.*, *Bioprocess Technol.* 19:251, 1990; "Teratocarcinomas and embryonic stem cells: A practical approach" (E. J. Robertson, ed., IRL Press Ltd. 1987); "Guide to Techniques in Mouse Development" (P. M. Wasserman *et al.* eds., Academic Press 1993); "Embryonic Stem Cell Differentiation *in vitro*" (M. V. Wiles, *Meth. Enzymol.* 225:900, 1993); "Properties and uses of Embryonic Stem Cells: Prospects for Application to Human Biology and Gene Therapy" (P. D. Rathjen *et al.*, al., 1993).

Differentiation of stem cells is reviewed in Robertson, *Meth. Cell Biol.* 75:173, 1997; and Pedersen, *Reprod. Fertil. Dev.* 10:31,1998.

10. Enrichment and Depletion Strategies

In particular embodiments, strategies for enriching a population of cells for pluripotent cells, *e.g.,* iPSCs, are provided. In one embodiment, enrichment provides a method for deriving clonal iPSC colonies in a relatively short time, thereby improving the efficiency of iPSC generation. Enrichment may comprise sorting a population of cells, which have been induced to reprogram, to identify and obtain cells expressing markers of pluripotency, thereby obtaining a population of cells enriched for pluripotent cells. An additional enrichment methodology comprises the depletion of cells expressing markers of differentiation or non-pluripotent cells to obtain an enriched population of pluripotent cells. In some embodiments, the cells are cultured after reprogramming is induced for about 4 to 30 days, about 4 to 24 days, about 6 to 22 days, or about 8 to about 12 days.

In one embodiment, enriching a population of cells for pluripotent cells comprises making a single cell suspension by dissociating the cells in the population and resuspending the cells. The dissociated cells may be resuspended in any suitable solution or media for maintaining cells or performing cell sorting. In particular embodiments, the single cell suspension contains a GSK3 inhibitor, a MEK inhibitor, and a Rock inhibitor and lacks a TFGβ inhibitor. In certain embodiments, the GSK3 inhibitor is CHIR99021, the MEK inhibitor is PD0325901, and the Rock inhibitor is thiazovivin.

In a particular embodiment, a population of cells is sorted to positively select pluripotent cells, and/or the population is depleted of non-reprogrammed or non-pluripotent cells, thereby obtaining a population of cells enriched for pluripotent cells. In one embodiment, a single cell suspension is prepared, and then the single cells are prepared for sorting, such as by staining for markers of pluripotency using, *e.g.,* appropriate antibodies. Cells may be sorted by any suitable method of sorting cells, such as by magnetic bead or flow cytometry (FACS) sorting.

Cells may be sorted based on various markers of pluripotency, including expression of SSEA3/4, TRA1-60/81, TRA1-85, TRA2-54, GCTM-2, TG343, TG30, CD9, CD29, CD133/prominin, CD140a, CD56, CD73, CD105, , OCT4, NANOG, SOX2, KLF4, SSEA1 (Mouse), CD30, SSEA5, CD90 and CD50. In various embodiments, cells are sorted based on at least two, at least three, or at least four markers of pluripotency. In certain embodiments, cells are sorted based on expression of SSEA4, and in certain particular embodiments based on expression of SSEA4 in combination with TRA1-81 or TRA1-60. In certain embodiments, cells are sorted based on SSEA4, TRA1-81 or TRA1-60 and CD30 expression. In certain embodiments, cells are initially depleted for non-reprogrammed cells using surface markers of differentiating cells including, but not limited to, CD13, CD26, CD34, CD45, CD31, CD46, or CD7, and then enriched for pluripotent markers such as SSEA4, TRA1-81 and CD30.

A population enriched for pluripotent cells may be placed in a cell culture system, such as conventional hESC media or the cell culture media of the invention. The cell culture system may be supplemented with feeder cells, or optionally be a feeder free environment. In some embodiments, the sorted cells expressing markers of pluripotency are placed in a feeder cell supplemented culture system and then transferred to a feeder free environment. In one embodiment, the cell culture medium is a feeder free environment and comprises a GSK3 inhibitor, a MEK inhibitor, and a Rock inhibitor, and lacks a TGFβ inhibitor. In particular embodiments, the GSK3 inhibitor is CHIR99021, the MEK inhibitor is PD0325901, and the Rock inhibitor is thiazovivin. In other particular embodiments of the invention, the cell culture system is a feeder free environment comprising a Matrigel™ coated tissue plate. In one embodiment, the cell culture system comprises the FMM medium described in Table 1.

The enriched cell population may be cultured in the cell culture systems described herein to obtain ground state iPSC colonies, typically appearing about 3 to about 25 days post sort; about 5-9 days post sort, or about 5-7 days post sort. iPSC colonies can be picked or sorted for clonal expansion. Using the enrichment strategies contemplated herein, the cell population is enriched at least about 3-fold, 5-fold, or 10-fold or more for pluripotent cells.

In some embodiments, a population of cells undergoing reprogramming or a population of pluripotent cells is depleted of differentiated cells. In one embodiment, a population of pluripotent cells or cells induced to reprogram can be depleted of cells having cells surface markers of differentiated cells. Illustrative examples of cell surface markers of differentiating cells include but are not limited to, CD13, CD26, CD34, CD45, CD31, CD46, or CD7. In particular embodiments, CD13 is used as a surface marker of differentiating cells.

In other embodiments, a population of cells induced to differentiate into a desired lineage and is depleted of pluripotent cells to obtain an enriched population of differentiating or differentiated cells. In some embodiments, the population of differentiated cells comprises a population of cells, such as ESCs or iPSCs that has been induced to differentiate into a specific lineage. A population of cells may be depleted of pluripotent cells using the negative cell sorting techniques described above ("panning"), such as sorting cells in the population according to magnetic beads or FACs based on markers of pluripotency. In some embodiments, a population of cells comprising differentiated cells is sorted by FACs using pluripotency markers, and a fraction is obtained that is depleted of cells expressing pluripotency markers. In other embodiments, a population of cells is sorted by FACs based on markers of differentiation, such as lineage-specific markers like CD13, CD26, CD34, CD45, CD31, CD46, or CD7, to obtain a fraction depleted of markers of pluripotency. CD13 is used as a surface marker of differentiating cells in particular embodiments of the invention.

E. CULTURE PLATFORMS FOR REPROGRAMMING CELLS

Various strategies are being pursued to induce pluripotency, or increase potency, in cells (Takahashi, K., and Yamanaka, S., *Cell* 126, 663-676 (2006); Takahashi *et al.*, *Cell* 131, 861-872 (2007); Yu *et al.*, *Science* 318, 1917-1920 (2007); Zhou *et al.*, *Cell Stem Cell* 4, 381-384 (2009); Kim *et al.*, *Cell Stem Cell* 4, 472-476 (2009); Yamanaka *et al.*, 2009; Saha, K., Jaenisch, R., *Cell Stem Cell* 5, 584-595 (2009)), and improve the efficiency of reprogramming (Shi *et al.*, *Cell Stem Cell* 2, 525-528 (2008a); Shi *et al.*, *Cell Stem Cell* 3, 568-574 (2008b); Huangfu *et al.*, *Nat Biotechnol* 26, 795-797 (2008a); Huangfu *et al.*, *Nat Biotechnol* 26, 1269-1275 (2008b); Silva *et al.*, *Plos Bio* 6, e253. doi: 10.1371/journal. pbio. 0060253 (2008); Lyssiotis *et al.*, *PNAS* 106, 8912-8917 (2009); Ichida *et al.*, *Cell Stem Cell* 5, 491-503 (2009); Maherali, N., Hochedlinger, K., *Curr Biol* 19, 1718-1723 (2009b); Esteban *et al.*, *Cell Stem Cell* 6, 71-79 (2010); Feng *et al.*, *Cell Stem Cell* 4, 301-312 (2009)). However, existing methods have yet to realize a high-throughput solution for the manufacture of industrial- or clinical-grade pluripotent cells, *i.e.* clonal transgene- free pluripotent cell populations with homogeneous pluripotency, no significant spontaneous differentiation and an ability to culture and expand the cell population using single cell, enzymatic passage in defined, xeno-free, feeder-cell culture systems.

The culture platforms contemplated herein are useful, in part, for the production of high-grade induced pluripotent stem cells (iPSCs). In one embodiment, non-pluripotent cells are reprogrammed to pluripotency and cultured to maintain pluripotency. In another embodiment, iPSCs are cultured to ground state pluripotency.

In various embodiments, the culture platforms enable a transgene and/or footprint-free method of reprogramming. The culture platforms contemplated herein provide highly efficient episomal reprogramming with a significant reduction in the time and effort required for hiPSC generation. Without wishing to be bound to any particular theory, it is contemplated that by both blocking differentiation cues early in the reprogramming process and promoting mesenchyme-to-epithelial transition (MET) through small molecule inhibition of specific pathways (MEK, ERK, TGFβ and ROCK) the efficiency of hiPSC generation is significantly improved using episomal vectors, in FF and single cell culture systems.

In one embodiment, the culture platform comprises reprogramming one or more non-pluripotent cells to a pluripotent state comprising increasing the expression of endogenous OCT4 in the cell. Expression of endogenous OCT4 in the cell may be increased by introducing one or more polynucleotides, polypeptides, or small molecule inducers of OCT4 expression. In one embodiment, introduction of a polynucleotide encoding OCT4 or an OCT4 polypeptide into a cell is sufficient to induce endogenous expression of OCT4 in the cell.

In one embodiment, the culture platform comprises reprogramming one or more non-pluripotent cells comprising introducing one or more polynucleotides encoding one or more reprogramming factors selected from the group consisting of: OCT4, SOX2, NANOG, KLF4, LIN28, C-MYC, and SV40LT into the one or more non-pluripotent cells. In another embodiment, the culture platform comprises reprogramming one or more non-pluripotent cells comprising introducing one or more polypeptides selected from the group consisting of: OCT4, SOX2, NANOG, KLF4, LIN28, C-MYC, SV40LT, hTERT, SALL4, GLIS, ESRRB, DPPA2, ECAT1, SOX1, SOX3, KLF2, KLF5, L-MYC, N-MYC, LRH1 and UTF1 into the one or more non-pluripotent cells.

In one embodiment, the culture platform comprises reprogramming one or more non-pluripotent cells comprising introducing one or more polynucleotides encoding one or more reprogramming factors selected from the group consisting of: OCT4, NANOG, ESRRB, ECAT1 and UTF1 into the one or more non-pluripotent cells. In another embodiment, the culture platform comprises reprogramming one or more non-pluripotent cells comprising introducing one or more polypeptides selected from the group consisting of: OCT4, NANOG, ESRRB, ECAT1 and UTF1 into the one or more non-pluripotent cells.

As used herein, in particular embodiments, the term “introducing” refers to a process that comprises contacting a cell with a polynucleotide, polypeptide, or small molecule. An introducing step may also comprise microinjection of polynucleotides or polypeptides into the cell, use of liposomes to deliver polynucleotides or polypeptides into the cell, or fusion of polynucleotides or polypeptides to cell permeable moieties to introduce them into the cell.

In particular embodiments, one or more polynucleotides encoding 1, 2, 3, 4, 5 or more copies of one or more of the reprogramming factors selected from the group consisting of: OCT4, SOX2, NANOG, KLF4, LIN28, C-MYC,SV40LT, hTERT, SALL4, GLIS, ESRRB, DPPA2, ECAT1, SOX1, SOX3, KLF2, KLF5, L-MYC, N-MYC, LRH1 and UTF1 may be introduced into a non-pluripotent cell to reprogram the cell. The copy number of each reprogramming factor introduced into the cell may be the same or different in any combination suitable to achieve ground state pluripotency as contemplated herein.

In one embodiment, one or more polynucleotides encoding 1, 2, 3, 4, 5 or more copies of one or more of the reprogramming factors selected from the group consisting of: OCT4, SOX2, and NANOG are introduced into the non-pluripotent cell.

In one embodiment, one or more polynucleotides encoding 1, 2, 3, 4, 5 or more copies of each of OCT4, SOX2, and NANOG are introduced into the non-pluripotent cell.

In one embodiment, one or more polynucleotides encoding 1, 2, 3, 4, 5 or more copies of each of OCT4 and SOX2 are introduced into the non-pluripotent cell.

In one embodiment, one or more polynucleotides encoding 1, 2, 3, 4, 5 or more copies of OCT4 are introduced into the non-pluripotent cell.

In one embodiment, one or more polynucleotides encoding 2 copies of OCT4, 2 copies of SOX2, and 2 copies of NANOG are introduced into the non-pluripotent cell, with SV40LT optionally being introduced into the non-pluripotent cell.

In another embodiment, one or more polynucleotides encoding 3 copies of OCT4, 2 copies of SOX2, one copy of NANOG, and one copy of UTF1 are introduced into the non-pluripotent cell.

In various illustrative embodiments, a culture platform comprising reprogramming a non-pluripotent cell comprises introducing 1 to 5 copies of a polynucleotide encoding OCT4; 1 to 3 copies of a polynucleotide encoding SOX2, and optionally, 1 to 2 copies of a polynucleotide encoding NANOG. The polynucleotides may be introduced into the cell as any combination of one or more larger polynucleotides. In one non-limiting example, one or more polynucleotides encoding 1 to 4 copies of OCT4, 1 or 2 copies of SOX2, and 1 copy of NANOG are introduced into a non-pluripotent cell. In another non-limiting example reprogramming a non-pluripotent cells to the pluripotent state comprises introducing a first polynucleotide encoding 2 copies of OCT4, a second polynucleotide encoding 1 copy of OCT4 and 1 copy of SOX2; and a third polynucleotide encoding 1 copy of OCT4, 1 copy of SOX2, and 1 copy of NANOG, into the non-pluripotent cells. In a further non-limiting example, reprogramming one or more non-pluripotent cells comprises introducing a first polynucleotide encoding 2 copies of OCT4 and a second polynucleotide encoding 1 copy of OCT4, 1 copy of SOX2, and 1 copy of NANOG, into the one or more non-pluripotent cells. In yet a further non-limiting example, a first polynucleotide encoding 2 copies of OCT4 and a second polynucleotide encoding 1 copy of OCT4 and 1 copy of SOX2 are introduced into the one or more non-pluripotent cells to produce a pluripotent cell.

In one embodiment, a single vector comprising a polynucleotide comprising any number and combination of the reprogramming factors contemplated herein is introduced into a non-pluripotent cell and is sufficient to reprogram the cell to a pluripotent state.

In one embodiment, one or more vectors comprising 1, 2, 3, 4, 5 or more polynucleotides comprising any number and combination of the reprogramming factors contemplated herein is introduced into a non-pluripotent cell and is sufficient to reprogram the cell to a pluripotent state.

In a preferred embodiment, one or more vectors comprising the one or more polynucleotides contemplated herein for reprogramming a non-somatic cell are used to introduce the one or more polynucleotides into the cell and are sufficient to reprogram the cell.

In the most preferred embodiment, one or more episomal vectors comprising the one or more polynucleotides contemplated herein for reprogramming a non-somatic cell are used to introduce the one or more polynucleotides into the cell and are sufficient to reprogram the cell. Pluripotent cells displaying reduced spontaneous differentiation and/or the ground state may be manufactured with episomal vectors as contemplated herein, and then cultured until loss of the vector to obtain pluripotent cells displaying reduced spontaneous differentiation and/or the ground state which do not comprise exogenous nucleic acids encoding reprogramming factors.

It is further contemplated that when polynucleotide or vector comprising the same comprises a polynucleotide encoding at least two reprogramming factors or at least two copies of a reprogramming factor, the polynucleotide comprises an IRES sequence or a polynucleotide encoding a self-cleaving polypeptide sequence between each of the reprogramming factors as contemplated herein.

In some aspects, the efficiency of reprogramming non-pluripotent cells is increased by selecting for the ectopic expression of one or more reprogramming factor polynucleotides after the reprogramming factors polynucleotides are introduced into the non-pluripotent cells. Such selection may take place, for example, by linking one or more of the reprogramming factor polynucleotides to a selectable marker, introducing the reprogramming factor polynucleotides and selectable marker into the non-pluripotent cells, and selecting those cells that express the selectable marker, wherein the selection identifies cells having increased reprogramming efficiency relative to the cells that lack expression of the marker and its associated reprogramming factor polynucleotides. One skilled in the art will appreciate that in particular embodiments any selectable marker that identifies the expression of the introduced reprogramming polynucleotides by the non-pluripotent cell may be used.

One non-limiting example of such a selectable marker includes, but is not limited to, antibiotic resistance genes such as puromycin resistance. Selectable markers may be linked to one or more of the following reprogramming factor polynucleotides: OCT4, SOX2, NANOG, KLF4, LIN28, C-MYC, SV40LT, hTERT, SALL4, GLIS, ESRRB, DPPA2, ECAT1, SOX1, SOX3, KLF2, KLF5, L-MYC, N-MYC, LRH1 and UTF1. In some embodiments, a specific combination of reprogramming factor polynucleotides are introduced as a polycistronic vector, with the selectable marker being linked to the specific combination of reprogramming factor polynucleotides. The specific combination of reprogramming factor polynucleotides may encode two or more copies of the reprogramming factor polynucleotides disclosed herein.

In one non-limiting embodiment, the polycistronic vector encodes two or more copies of an OCT4 polynucleotides linked to a selectable marker, such as a gene encoding puromycin resistance.

In some aspects, a polycistronic vector encoding one or more reprogramming factor polynucleotides and a selectable marker are introduced to non-pluripotent cells in addition to one or more separate reprogramming factor polynucleotides, wherein selecting for cells that express the selectable marker produces a population of cells having greater reprogramming efficiency than cells that lack expression of the selectable marker.

In one non-limiting example of this selection process, OCT4, NANOG and SOX2 polynucleotides are introduced to non-pluripotent cells in addition to a polycistronic vector encoding two or more copies of OCT4 linked to a puromycin resistance gene. The subsequent selection of non-pluripotent cells expressing the selectable marker identifies non-pluripotent cells with greater reprogramming efficiency relative to the non-pluripotent cells that do not express the selectable marker. The selected cells may have a reprogramming efficiency of at least 5%, at least 10%, at least 15%, at least 20%, at least 30% or at least 40%.

Small molecules are often included in the reprogramming steps of particular preferred embodiments. Without wishing to be bound to any particular theory, it is contemplated that include of small molecule inhibitors of various differentiation pathways increases the efficiency and kinetics of reprogramming. Accordingly, in particular embodiments, culture platforms comprising reprogramming non-pluripotent cells comprise introducing one or more reprogramming factors into the cells as contemplated herein and contacting the cells with a GSK3 inhibitor; a MEK inhibitor; a TGFβR inhibitor, and a ROCK inhibitor.

Improvements in efficiency of reprogramming can be measured by (1) a decrease in the time required for reprogramming and generation of pluripotent cells (*e.g.,* by shortening the time to generate pluripotent cells by at least a day compared to a similar or same process without the small molecule), or alternatively, or in combination, (2) an increase in the number of pluripotent cells generated by a particular process (*e.g.,* increasing the number of cells reprogrammed in a given time period by at least 10%, 30%, 50%, 100%, 200%, 500%, *etc.* compared to a similar or same process without the small molecule). In some embodiments, a 2-fold to 20-fold improvement in reprogramming efficiency is observed. In some embodiments, reprogramming efficiency is improved by more than 20 fold. In some embodiments, a more than 100 fold improvement in efficiency is observed over the method without the small molecule reprogramming agent (*e.g.,* a more than 100 fold increase in the number of pluripotent cells generated).

In one embodiment, a culture platform contemplated herein comprises reprogramming non-pluripotent cells by introducing one or more reprogramming factors into the cells as contemplated herein and contacting the cells with a GSK3 inhibitor; a MEK inhibitor; and a TGFβR inhibitor, and optionally a ROCK inhibitor.

In one preferred embodiment, a culture platform contemplated herein comprises reprogramming non-pluripotent cells by introducing one or more reprogramming factors into the cells as contemplated herein and contacting the cells with a GSK3 inhibitor; a MEK inhibitor; a TGFβR inhibitor, and a ROCK inhibitor.

In a more preferred embodiment, a culture platform contemplated herein comprises reprogramming non-pluripotent cells by introducing one or more reprogramming factors into the cells as contemplated herein and contacting the cells with a GSK3 inhibitor; a MEK inhibitor; a TGFβR inhibitor, and a ROCK inhibitor, wherein the ROCK inhibitor is Thiazovivin.

However, to enable the long term culture of pluripotent cells in feeder-cell free and enzymatic passage culture systems with reduced or no significant spontaneous differentiation or to induce and/or maintain ground state pluripotency, iPSCs require subsequent culturing in a cell culture medium comprising a GSK-3 inhibitor, a MEK inhibitor, and optionally a Rho Kinase (ROCK) inhibitor, wherein the cell culture medium does not comprise, or lacks, an inhibitor of TGFβ/activin signaling pathways, including TGFβ receptor (TGFβR) inhibitors and ALK5 inhibitors, as contemplated herein. Without wishing to be bound to any particular theory, it is contemplated that long-term culture of pluripotent cells with a TGFβR/ALK5 inhibitor leads to spontaneous differentiation of the cultured transgene-free iPSCs and ultimately loss of ground state pluripotency.

In various embodiments, a two step culture platform is employed to stably reprogram somatic cells to achieve reduced spontaneous differentiation in culture, including ground state pluripotency. In certain embodiments, a non-pluripotent cell is reprogrammed by any suitable method disclosed in the art, and subsequently, the reprogrammed somatic cell is cultured to achieve reduced spontaneous differentiation in culture by culturing the cell in a medium comprising a GSK-3 inhibitor, a MEK inhibitor, and a Rho Kinase (ROCK) inhibitor, wherein the media lacks a TGFβR/ALK5 inhibitor. In some embodiments, the reprogrammed somatic cell is cultured to provide ground state pluripotent cells.

In particular embodiments, a non-pluripotent cell is reprogrammed by the methods disclosed herein and subsequently, the reprogrammed somatic cell is cultured to a stable ground state of pluripotency by culturing the cell in a medium comprising a GSK-3 inhibitor, a MEK inhibitor, and a Rho Kinase (ROCK) inhibitor, wherein the media lacks a TGFβR/ALK5 inhibitor.

In some embodiments, a non-pluripotent cell is reprogrammed by introducing one or more reprogramming factors and culturing the cell in a medium comprising a GSK-3 inhibitor, a MEK inhibitor, a Rho Kinase (ROCK) inhibitor, and a

TGFβR/ALK5 inhibitor, and subsequently, the reprogrammed somatic cell is cultured to a provide cells with reduced spontaneous differentiation by culturing the cell in a medium comprising a GSK-3 inhibitor, a MEK inhibitor, and a Rho Kinase (ROCK) inhibitor, wherein the media lacks a TGFβR/ALK5 inhibitor.

In some embodiments, a non-pluripotent cell is reprogrammed by introducing one or more reprogramming factors and culturing the cell in a medium comprising a GSK-3 inhibitor, a MEK inhibitor, a Rho Kinase (ROCK) inhibitor, and a TGFβR/ALK5 inhibitor, and subsequently, the reprogrammed somatic cell is cultured to a stable ground state of pluripotency by culturing the cell in a medium comprising a GSK-3 inhibitor, a MEK inhibitor, and a Rho Kinase (ROCK) inhibitor, wherein the media lacks a TGFβR/ALK5 inhibitor.

In preferred embodiments, a non-pluripotent cell is reprogrammed by introducing one or more reprogramming factors and culturing the cell in a medium comprising a GSK-3 inhibitor, a MEK inhibitor, a Rho Kinase (ROCK) inhibitor, and a TGFβR/ALK5 inhibitor, and subsequently, the reprogrammed somatic cell is cultured to a stable ground state of pluripotency by culturing the cell in a medium comprising a GSK-3 inhibitor, a MEK inhibitor, and a Rho Kinase (ROCK) inhibitor, wherein the media lacks a TGFβR/ALK5 inhibitor and wherein there is not significant residual expression of reprogramming transgene.

In one embodiment, a non-pluripotent cell is reprogrammed by introducing one or more reprogramming factors selected from the group consisting of: OCT4, SOX2, NANOG, KLF4, LIN28, C-MYC, SV40LT, hTERT, SALL4, GLIS, ESRRB, DPPA2, ECAT1, SOX1, SOX3, KLF2, KLF5, L-MYC, N-MYC, LRH1 and UTF1 as disclosed elsewhere herein and culturing the cell in a medium comprising a GSK-3 inhibitor, a MEK inhibitor, a Rho Kinase (ROCK) inhibitor, and a TGFβR/ALK5 inhibitor, and subsequently, the reprogrammed somatic cell is cultured in a medium comprising a GSK-3 inhibitor, a MEK inhibitor, and a Rho Kinase (ROCK) inhibitor, wherein the media lacks a TGFβR/ALK5 inhibitor.

In a preferred embodiment, a non-pluripotent cell is reprogrammed by introducing one or more reprogramming factors selected from the group consisting of: OCT4, SOX2, NANOG, KLF4, LIN28, C-MYC, SV40LT, hTERT, SALL4, GLIS,

ESRRB, DPPA2, ECAT1, SOX1, SOX3, KLF2, KLF5, L-MYC, N-MYC, LRH1 and UTF1 as disclosed elsewhere herein and culturing the cell in a medium comprising a GSK-3 inhibitor, a MEK inhibitor, a Rho Kinase (ROCK) inhibitor, and a TGFβR/ALK5 inhibitor, and subsequently, the reprogrammed somatic cell is cultured in a medium comprising a GSK-3 inhibitor, a MEK inhibitor, and a Rho Kinase (ROCK) inhibitor wherein the Rock Inhibitor is Thiazovivin, wherein the media lacks a TGFβR/ALK5 inhibitor.

In various embodiments, methods of manufacturing pluripotent cells with reduced spontaneous differentiation and/or ground state induced pluripotent stem cells (iPSCs) using the culture platforms contemplated herein are provided.

In particular embodiments, pluripotent cells with reduced spontaneous differentiation and/or ground state induced pluripotent stem cells (iPSCs) are manufactured using a starting material comprising one or more non-pluripotent or partially pluripotent stem cells and culturing the one or more pluripotent or partially-pluripotent stem cells in a culture medium that does not comprise a TGFβR inhibitor. The starting material may either be obtained or created. For example, non-pluripotent or partially pluripotent stem cells may be provided from a commercial supplier or other source or could be obtained *de novo*: non-pluripotent cells could also be isolated from a tissue or organ; and partially pluripotent cells could also be generated by reprogramming somatic cells or adult stem cells. In some embodiments, pluripotent embryonic stem cells, or pluripotent cells obtained by somatic nuclear transfer, may be induced to achieve ground state pluripotency using the culture media and platforms described herein.

In particular embodiments, a population of one or more IPSCs may comprise reprogrammed somatic cells or reprogrammed adult stem cells. In particular embodiments, the IPSCs may be generated by any known method either by performing the method or obtaining IPSCs generated by the method.

Exemplary methods of generating the IPSCs include, but are not limited to: increasing the expression of endogenous OCT4 in non-pluripotent cells; introducing one or more polynucleotides, optionally in one or more copies, encoding one or more reprogramming factors selected from the group consisting of: OCT4, SOX2, NANOG, KLF4, LIN28, C-MYC, SV40LT, hTERT, SALL4, GLIS, ESRRB, DPPA2, ECAT1, SOX1, SOX3, KLF2, KLF5, L-MYC, N-MYC, LRH1 and UTF1 into the one or more non-pluripotent cells; or introducing one or more polynucleotides, optionally in one or more copies, encoding one or more reprogramming factors selected from the group consisting of: OCT4, SOX2, and NANOG into the one or more non-pluripotent cells. Methods of generating IPSCs may further comprise contacting the one or more non-pluripotent cells or partially pluripotent cells with a GSK3 inhibitor; a MEK inhibitor; and a TGFβR inhibitor, and optionally a ROCK inhibitor to produce the one or more iPSCs.

In certain embodiments, the cell culture medium comprises a GSK3 inhibitor; a MEK inhibitor; and a ROCK inhibitor.

In preferred embodiments, the cell culture medium comprises a GSK3 inhibitor; a MEK inhibitor; and a ROCK inhibitor, wherein the ROCK inhibitor is Thiazovivin.

In particular embodiments, culturing the one or more pluripotent cells, *e.g.,* IPSCs, in the cell culture medium maintains or induces a ground state of pluripotency, viability, normal karyotype, genomic stability, and decreased rate of spontaneous differentiation that can be maintained for at least 5 passages, at least 10 passages, at least 50 passages, at least 100 passages, or more, including any intervening number of passages.

F. Characterizing Pluripotent Cells

Pluripotent cell manufactured using the culture platforms contemplated herein may further comprise selection or validation of the pluripotent cell product, including, for example, ground state pluripotent cells or pluripotent cells with reduced spontaneous differentiation. The pluripotent cells may be selected and/or validated after reprogramming and subsequent culture with the compositions and methods contemplated herein, or after pluripotent cells were transitioned to the culture methods contemplated herein, if the pluripotent cells were not reprogrammed. The pluripotency of the cells may be characterized and/or selected based on relevant and detectable morphological, molecular and/or biochemical changes associated with pluripotency.

Specific characteristics of cell pluripotency which may be monitored, separately or in combination, in assessing the potency of a cell include, but are not limited to, gene expression, methylation, and *in vivo* and *in vitro* characteristics such as: i) pluripotent stem cell morphology that is round; ii) expression of pluripotent stem cell markers including SSEA3/4 (human pluripotent stem cells); TRA1-60/81; TRA1-85, TRA2-54, GCTM-2, TG343, TG30, CD9, CD29, CD133/prominin, CD140a, CD56, CD73, CD105, OCT4, NANOG,SOX2, CD30, SSEA5, CD90 and/or CD50, and combinations of the foregoing; iii) teratoma formation of pluripotent stem cells; iv) formation of embryoid bodies and *in vitro* trilineage differentiation: and v) inactive X chromosome reactivation. In certain embodiments, a subset of any of the above characteristics is used for monitoring cell potency. In one embodiment, pluripotent cells are characterized by having a round colony morphology, expression of SSEA4, TRA1-81, and OCT4, and the ability to form embryoid bodies and teratomas.

In another embodiment, pluripotent cells having reduced spontaneous differentiation in *in vitro* culture may be identified by a gene expression signature that comprises at least about a 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90% decrease in the expression of one or more of the following differentiation marker genes compared to pluripotent cells cultured in the presence of a TGFβR inhibitor: FOXA2, FGF5, SOX17, XIST, NODAL, COL3A1, OTX2, DUSP6, EOMES, NR2F2, NR0B1, CXCR4, CYP2B6, GATA3, GATA4, ERBB4, GATA6, HOXC6, INHA, SMAD6, RORA, NIPBL, TNFSF11, CDH11, ZIC4, GAL, SOX3, PITX2, APOA2, CXCL5, CER1, FOXQ1, MLL5, DPP10, GSC, PCDH10, CTCFL, PCDH20, TSHZ1, MEGF10, MYC, DKK1, BMP2, LEFTY2, HES1, CDX2, GNAS, EGR1, COL3A1, TCF4, HEPH, KDR, TOX, FOXA1, LCK, PCDH7, CD1D FOXG1, LEFTY1, TUJ1, T gene (Brachyury) and ZIC1.

In one embodiment, pluripotent cells having reduced spontaneous differentiation are characterized by the decreased expression of one or more differentiation marker genes, including but not limited to: T gene, CXCR4, NODAL, GATA4, SOX17, FOXA2, OTX2, and TUJ1. In particular embodiments, pluripotent cells having reduced spontaneous differentiation may be identified by a gene expression signature that comprises at least about a 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%

decrease in the expression of one or more differentiation marker genes (*e.g.,* T gene, CXCR4, NODAL, GATA4, SOX17, FOXA2, OTX2, TUJ1) compared to pluripotent cells cultured in the presence of a TGFβR inhibitor. [In another particular embodiments, pluripotent cells having reduced spontaneous differentiation may be identified by a gene expression signature that comprises at least about a 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90% decrease in the expression of one or more differentiation marker genes (*e.g.,* T gene, CXCR4, NODAL, GATA4, SOX17, FOXA2, OTX2, TUJ1).

In particular embodiments, ground state pluripotent cells have significantly repressed Xist expression and expression of early markers of differentiated cells, *e.g.,* Foxa2, Sox17, and Brachyury, while conventional cultured pluripotent cells show only modest repression of Xist expression and significant expression of early differentiation markers.

In particular embodiments, ground state pluripotent cells retain characteristics of ground state pluripotency for multiple cell passages, such as for example, at least 1, 3, 5, 7, 10, 15, 20 or more passages.

G. Polynucleotides

In various illustrative embodiments, the present invention contemplates, in part, polynucleotides, polynucleotides encoding polypeptides and fusion polypeptides contemplated herein, and compositions comprising the same. In various other illustrative embodiments, the present invention contemplates, in part, reprogramming non-pluripotent cells with polynucleotides encoding one or more copies of at least one reprogramming factor. Reprogramming factors for use with the culture platforms described herein include, but are not limited to, : OCT4, SOX2, NANOG, KLF4, LIN28, C-MYC,SV40LT, hTERT, SALL4, GLIS, ESRRB, DPPA2, ECAT1, SOX1, SOX3, KLF2, KLF5, L-MYC, N-MYC, LRH1 and UTF1. In preferred embodiments, a polynucleotide comprises a sequence of a reprogramming factor as set forth herein.

As used herein, the term “gene” may refer to a polynucleotide sequence comprising enhancers, promoters, introns, exons, and the like. In particular embodiments, the term “gene” refers to a polynucleotide sequence encoding a polypeptide, regardless of whether the polynucleotide sequence is identical to the genomic sequence encoding the polypeptide.

An "isolated polynucleotide," as used herein, refers to a polynucleotide that has been purified from the sequences which flank it in a naturally-occurring state, *e.g.,* a DNA fragment that has been removed from the sequences that are normally adjacent to the fragment. In particular embodiments, an "isolated polynucleotide" refers to a complementary DNA (cDNA), a recombinant DNA, or other polynucleotide that does not exist in nature and that has been made by the hand of man.

In particular embodiments, one or more polynucleotides may be arranged in any suitable order within a larger polynucleotide, such as a vector. In preferred embodiments, the vector is an episomal vector.

The polynucleotides contemplated herein, regardless of the length of the coding sequence itself, may be combined with other DNA sequences, such as expression control sequences, promoters and/or enhancers, untranslated regions (UTRs), Kozak sequences, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, internal ribosomal entry sites (IRES), recombinase recognition sites (*e.g.,* LoxP, FRT, and Att sites), termination codons, transcriptional termination signals, and polynucleotides encoding self-cleaving polypeptides, epitope tags, as disclosed elsewhere herein or as known in the art, such that their overall length may vary considerably. It is therefore contemplated that a polynucleotide fragment of almost any length may be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol.

Polynucleotides can be prepared, manipulated and/or expressed using any of a variety of well established techniques known and available in the art. In order to express a desired polypeptide, a nucleotide sequence encoding the polypeptide, can be inserted into appropriate vector. Examples of vectors are plasmid, autonomously replicating sequences, and transposable elements. Additional exemplary vectors include, without limitation, plasmids, phagemids, cosmids, artificial chromosomes such as yeast artificial chromosome (YAC), bacterial artificial chromosome (BAC), or P1-derived artificial chromosome (PAC), bacteriophages such as lambda phage or M13 phage, and animal viruses. Examples of categories of animal viruses useful as vectors include, without limitation, retrovirus (including lentivirus), adenovirus, adeno-associated virus, herpesvirus (*e.g.,* herpes simplex virus), poxvirus, baculovirus, papillomavirus, and papovavirus (*e.g.,* SV40). Examples of expression vectors are pClneo vectors (Promega) for expression in mammalian cells; pLenti4/V5-DEST™, pLenti6/V5-DEST™, and pLenti6.2/V5-GW/lacZ (Invitrogen) for lentivirus-mediated gene transfer and expression in mammalian cells. In particular embodiments, coding sequences of polypeptides disclosed herein can be ligated into such expression vectors for the expression of the polypeptides in mammalian cells.

In particular embodiments, the vector is an episomal vector or a vector that is maintained extrachromosomally. As used herein, the term “episomal” refers to a vector that is able to replicate without integration into host’s chromosomal DNA and without gradual loss from a dividing host cell also meaning that said vector replicates extrachromosomally or episomally. The vector is engineered to harbor the sequence coding for the origin of DNA replication or “ori” from a lymphotrophic herpes virus or a gamma herpesvirus, an adenovirus, SV40, a bovine papilloma virus, or a yeast, specifically a replication origin of a lymphotrophic herpes virus or a gamma herpesvirus corresponding to oriP of EBV. In a particular aspect, the lymphotrophic herpes virus may be Epstein Barr virus (EBV), Kaposi's sarcoma herpes virus (KSHV), Herpes virus saimiri (HS), or Marek's disease virus (MDV). Epstein Barr virus (EBV) and Kaposi's sarcoma herpes virus (KSHV) are also examples of a gamma herpesvirus. Typically, the host cell comprises the viral replication transactivator protein that activates the replication.

“Expression control sequences,” “control elements,” or “regulatory sequences” present in an expression vector are those non-translated regions of the vector—origin of replication, selection cassettes, promoters, enhancers, translation initiation signals (Shine Dalgarno sequence or Kozak sequence) introns, a polyadenylation sequence, 5' and 3' untranslated regions—which interact with host cellular proteins to carry out transcription and translation. Such elements may vary in their strength and specificity. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including ubiquitous promoters and inducible promoters may be used.

The term “operably linked”, refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. In one embodiment, the term refers to a functional linkage between an expression control sequence (such as a promoter, and/or enhancer) and a second polynucleotide sequence, wherein the expression control sequence directs transcription of the nucleic acid corresponding to the second sequence.

Illustrative ubiquitous expression control sequences suitable for use in particular embodiments of the invention include, but are not limited to, a cytomegalovirus (CMV) immediate early promoter, a viral simian virus 40 (SV40) (*e.g.,* early or late), a Moloney murine leukemia virus (MoMLV) LTR promoter, a Rous sarcoma virus (RSV) LTR, a herpes simplex virus (HSV) (thymidine kinase) promoter, H5, P7.5, and P11 promoters from vaccinia virus, an elongation factor 1-alpha (EF1a) promoter, early growth response 1 (EGR1), ferritin H (FerH), ferritin L (FerL), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), eukaryotic translation initiation factor 4A1 (EIF4A1), heat shock 70kDa protein 5 (HSPA5), heat shock protein 90kDa beta, member 1 (HSP90B1), heat shock protein 70kDa (HSP70), β-kinesin (β-KIN), the human ROSA 26 locus (Irions *et al.*, *Nature Biotechnology* 25, 1477 - 1482 (2007)), a Ubiquitin C promoter (UBC), a phosphoglycerate kinase-1 (PGK) promoter, a cytomegalovirus enhancer/chicken β-actin (CAG) promoter, and a β-actin promoter.

Illustrative examples of inducible promoters/systems include, but are not limited to, steroid-inducible promoters such as promoters for genes encoding glucocorticoid or estrogen receptors (inducible by treatment with the corresponding hormone), metallothionine promoter (inducible by treatment with various heavy metals), MX-1 promoter (inducible by interferon), the “GeneSwitch” mifepristone-regulatable system (Sirin *et al.*, 2003, *Gene*, 323:67), the cumate inducible gene switch (WO 2002/088346), tetracycline-dependent regulatory systems, *etc.*

Conditional expression can also be achieved by using a site specific DNA recombinase. According to certain embodiments of the invention, polynucleotides comprise at least one (typically two) site(s) for recombination mediated by a site specific recombinase. As used herein, the terms “recombinase” or “site specific recombinase” include excisive or integrative proteins, enzymes, co-factors or associated proteins that are involved in recombination reactions involving one or more recombination sites (*e.g.,* two, three, four, five, six, seven, eight, nine, ten or more.), which may be wild-type proteins (see Landy, Current Opinion in Biotechnology 3:699-707 (1993)), or mutants, derivatives (*e.g.,* fusion proteins containing the recombination protein sequences or fragments thereof), fragments, and variants thereof. Illustrative examples of recombinases suitable for use in particular embodiments of the present invention include, but are not limited to: Cre, Int, IHF, Xis, Flp, Fis, Hin, Gin, ΦC31, Cin, Tn3 resolvase, TndX, XerC, XerD, TnpX, Hjc, Gin, SpCCE1, and ParA.

In particular embodiments, polynucleotides contemplated herein, include one or more polynucleotides that encode one or more polypeptides. In particular embodiments, to achieve efficient translation of each of the plurality of polypeptides, the polynucleotide sequences can be separated by one or more IRES sequences or polynucleotide sequences encoding self-cleaving polypeptides. As used herein, an "internal ribosome entry site" or "IRES" refers to an element that promotes direct internal ribosome entry to the initiation codon, such as ATG, of a cistron (a protein encoding region), thereby leading to the cap-independent translation of the gene. *See, e.g.,* Jackson *et al.*, 1990. *Trends Biochem Sci* 15(12):477-83) and Jackson and Kaminski. 1995. *RNA* 1(10):985-1000. Examples of IRES generally employed by those of skill in the art include those described in U.S. Pat. No. 6,692,736. Further examples of "IRES" known in the art include, but are not limited to IRES obtainable from picornavirus (Jackson *et al.*, 1990).

H. POLYPEPTIDES

The present invention contemplates, in part, compositions comprising polypeptides, fusion polypeptides, and vectors that express polypeptides. In preferred embodiments, a polypeptide comprises the amino acid sequence set forth herein. "Polypeptide," "polypeptide fragment," "peptide" and "protein" are used interchangeably, unless specified to the contrary, and according to conventional meaning, *i.e.,* as a sequence of amino acids. In one embodiment, a "polypeptide" includes fusion polypeptides and other variants. Polypeptides can be prepared using any of a variety of well known recombinant and/or synthetic techniques. Polypeptides are not limited to a specific length, *e.g.,* they may comprise a full length protein sequence, a fragment of a full length protein, or a fusion protein, and may include post-translational modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like, as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

An "isolated peptide" or an "isolated polypeptide" and the like, as used herein, refer to *in vitro* isolation and/or purification of a peptide or polypeptide molecule from a cellular environment, and from association with other components of the cell, *i.e.*, it is not significantly associated with *in vivo* substances.

In one embodiment, where expression of two or more polypeptides is desired, the polynucleotide sequences encoding them can be separated by and IRES sequence as discussed elsewhere herein. In another embodiment, two or more polypeptides can be expressed as a fusion protein that comprises a polypeptide cleavage signal between each of the polypeptide domains described herein. In addition, polypeptide site can be put into any linker peptide sequence. Exemplary polypeptide cleavage signals include polypeptide cleavage recognition sites such as protease cleavage sites, nuclease cleavage sites (*e.g.,* rare restriction enzyme recognition sites, self-cleaving ribozyme recognition sites), and self-cleaving viral oligopeptides (see deFelipe and Ryan, 2004. *Traffic*, 5(8); 616-26).

Suitable protease cleavages sites and self-cleaving peptides are known to the skilled person (*see, e.g.,* in Ryan *et al.*, 1997. *J. Gener. Virol.* 78, 699-722; Scymczak *et al.* (2004) Nature Biotech. 5, 589-594). Exemplary protease cleavage sites include, but are not limited to the cleavage sites of potyvirus NIa proteases (*e.g.,* tobacco etch virus protease), potyvirus HC proteases, potyvirus P1 (P35) proteases, byovirus NIa proteases, byovirus RNA-2-encoded proteases, aphthovirus L proteases, enterovirus 2A proteases, rhinovirus 2A proteases, picorna 3C proteases, comovirus 24K proteases, nepovirus 24K proteases, RTSV (rice tungro spherical virus) 3C-like protease, PYVF (parsnip yellow fleck virus) 3C-like protease, heparin, thrombin, factor Xa and enterokinase. Due to its high cleavage stringency, TEV (tobacco etch virus) protease cleavage sites are preferred in one embodiment, *e.g.,* EXXYXQ(G/S) (SEQ ID NO:29), for example, ENLYFQG (SEQ ID NO:30) and ENLYFQS (SEQ ID NO:31), wherein X represents any amino acid (cleavage by TEV occurs between Q and G or Q and S).

In certain embodiments, the self-cleaving polypeptide site comprises a 2A or 2A-like site, sequence or domain (Donnelly *et al.*, 2001. *J. Gen. Virol.* 82:1027-1041). In a particular embodiment, the viral 2A peptide is an aphthovirus 2A peptide, a potyvirus 2A peptide, or a cardiovirus 2A peptide.

In one embodiment, the viral 2A peptide is selected from the group consisting of: a foot-and-mouth disease virus (FMDV) 2A peptide, an equine rhinitis A virus (ERAV) 2A peptide, a Thosea asigna virus (TaV) 2A peptide, a porcine teschovirus-1 (PTV-1) 2A peptide, a Theilovirus 2A peptide, and an encephalomyocarditis virus 2A peptide.

TABLE 2: Exemplary 2A sites include the following sequences:

SEQ ID NO: 1	LLNFDLLKLAGDVESNPGP
SEQ ID NO: 2	TLNFDLLKLAGDVESNPGP
SEQ ID NO: 3	LLKLAGDVESNPGP
SEQ ID NO: 4	NFDLLKLAGDVESNPGP
SEQ ID NO: 5	QLLNFDLLKLAGDVESNPGP
SEQ ID NO: 6	APVKQTLNFDLLKLAGDVESNPGP
SEQ ID NO: 7	VTELLYRMKRAETYCPRPLLAIHPTEARHKQKIVAPVKQT

SEQ ID NO: 8	LNFDLLKLAGDVESNPGP
SEQ ID NO: 9	LLAIHPTEARHKQKIVAPVKQTLNFDLLKLAGDVESNPGP
SEQ ID NO: 10	EARHKQKIVAPVKQTLNFDLLKLAGDVESNPGP

In preferred embodiments, a vector encoding one or more reprogramming factor polypeptides comprises one or more of the same or different protease cleavage sites between each of the reprogramming factors.

All publications, patent applications, and issued patents cited in this specification are herein incorporated by reference as if each individual publication, patent application, or issued patent were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims. The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of noncritical parameters that could be changed or modified to yield essentially similar results.

EXAMPLES

The Examples disclosed herein identified a platform for the rapid, parallel generation, selection and expansion of hiPSCs using small molecule pathway inhibitors in stage-specific media compositions. The platform supported efficient and expedited episomal reprogramming using minimal reprogramming factors in a completely feeder-free environment. The resulting hiPSCs were free of transgene, readily cultured and expanded as single cells while maintaining a homogenous and genomically stable pluripotent population. hiPSCs generated or maintained in the media compositions contemplated in the Examples exhibit properties associated with the ground state of pluripotency and represent a robust high-throughput system for the manufacture of uniform industrial- or clinical-grade hiPSCs.

EXAMPLE 1

IDENTIFICATION OF A MEDIUM PLATFORM FOR LONG-TERM MAINTENANCE AND EXPANSION OF IPSCS

OVERVIEW

The majority of lentiviral-derived hiPSC lines in SMC4-supplemented cultures maintain a homogeneous population of undifferentiated cells; however; silencing of the transgenic reprogramming factor in a subset of lines displayed various degrees of spontaneous differentiation in extended culture (Figure 1A and B). Therefore, various cell culture components were assessed in order to identify conditions for the maintenance of pluripotency during continuous FF culture and single-cell enzymatic passage irrespective of residual transgene expression. A multi-stage culture system that targets unique pathways at different stages of the reprogramming and maintenance process was identified as an efficient and robust approach to hiPSC generation.

RESULTS

Inhibition of TGFβ pathway during long-term maintenance was identified as a significant factor in the spontaneous differentiation of hiPSC lines with silenced transgene expression (Figure 1C). One of the iPSC cell lines found to undergo spontaneous differentiation was transitioned to culture in a new medium formulation, Fate Maintenance Medium (FMM) (Table 1). Spontaneous differentiation was eliminated and a homogenous population of SSEA4/TRA1-81 positive cells was established within 2-3 passages (Figure 1A).

OCT4/KLF4/SOX2 (OKS) lentiviral reprogramming in conventional culture (hESC medium on MEF feeder cells), SMC4-supplemented medium in FF culture or the newly formulated FMM in FF culture (Figure 1D) was also compared. Seventeen days after the induction of lentiviral reprogramming, SSEA4/TRA1-81 positive cells were selected by FACs and re-plated in either SMC4 or FMM for comparison (Figure 1D). SMC4 improved the kinetics of reprogramming and resulted in significantly more SSEA4/TRA1-81 positive cells at day 17 post induction (2.72% versus 0.76% for FMM and 0.10% for conventional culture; Figure 1D) than reprogramming with FMM.

After the initial sort, cells were maintained in their respective conditions for 10 days, followed by a second SSEA4/TRA1-81 positive flow cytometry selection (Figure 1D). The cultures were maintained for an additional 9 days (total of 36 days post infection) and scored for undifferentiated colonies based on OCT4 and NANOG co-expression (Figure 1D and 1E). The combination of initial reprogramming in SMC4 followed by a transition to FMM ultimately resulted in more OCT4/NANOG positive colonies and a significantly reduced number of OCT4/NANOG negative colonies relative to continuous maintenance in SMC4 (Figure 1D and 1E). Although OCT4/NANOG positive colonies were detected in cultures maintained exclusively in FMM, the number and size of the colonies appeared inferior to the stage-specific media approach.

These results show that a novel multi-stage culture system that targets unique pathways at different stages of the reprogramming and maintenance process resulted in the efficient manufacture of high quality hiPSCs.

EXAMPLE 2

PLATFORM FOR MANUFACTURING TRANSGENE-FREE HIPSCS IN A SINGLE CELL PASSAGE AND FF FORMAT

OVERVIEW

The efficiency of non-integrative reprogramming methods using episomal vector systems is extremely low (<0.001%), especially in FF environments (Narsinh *et al.*, 2011; O'Doherty *et al.*, 2013). Episomal-induction was tested in a multi-stage culture system including two media: Fate Reprogramming Medium (FRM) containing SMC4 and medium additives shown to improve reprogramming and FMM (Figure 2A and Table 1).

RESULTS

An episomal expression system consisting of gene combination OCT4/SOX2/NANOG/KLF4/LIN28/MYC/SV40LT (OSNKLMT) was used to transfect various fibroblast cells. Twenty-four hours after induction of episomal expression, the reprogramming culture is transitioned to FRM to enhance reprogramming kinetics. Early colony formation was observed within the first week and by day 10, a large population of SSEA4/TRA1-81 positive cells was detected (>1%) (Figure 8A and 8B). On Day 14, the reprogramming culture supported by FRM was split into either FRM or FMM media. On day 21, FACS was used to identify SSEA4/TRA1-81/CD30 positive cells in the cultures (Figure 8C). FRM maintained cultures contained both differentiated and undifferentiated cells, whereas FMM cultures contained mostly undifferentiated cells (Figure 8D).

The throughput and robustness of this approach was tested with fibroblasts and CD34+ cells expanded from minimal volumes of umbilical cord blood from donors of different ages, genders and ethnicity (Figure 9A and 9B). Somatic cell reprogramming was induced as outlined in Figure 2A with the episomal gene combination set OSNKLMT and 96-well plate flow cytometry sorted for individual hiPSCs between days 16 and 21 (Figure 2B). A large population of SSEA4/TRA1-81/CD30 positive cells was observed for the majority of lines tested. Compared to a parallel reprogramming experiment using conventional medium and feeder cells, the FRM and

FMM media system was resulted in a significant increase in the number of hiPSC clones (8.55% in FRM/FMM versus 0.02% in conventional culture for the FTC007 fibroblast line; Figure 2B and 2C). On average 22 clonal hiPSCs per 96-well plate were seen for each somatic line (Figures 2B, 10A and 10B) including the fibroblast FTC008 which had been previously observed to be refractory to lentiviral reprogramming with SMC4 medium. Colonies were subsequently confirmed as bona fide hiPSC clones by analysis of intracellular and surface marker expression and direct qRTPCR for NANOG (Figure 2D, 2E and 10C). The efficiency of reprogramming using the 96-well sorting and selection process was also increased (Figure 2E). A similar reprogramming efficiency was also observed with the defined surface coating vitronectin (Figure 10D).

These data showed that the platform was robust and reproducible when applied to episomal reprogramming and allowed for multiple reprogramming experiments to be performed in parallel in a high-throughput fashion with minimal effort and without compromising quality of the iPSC end product.

EXAMPLE 3

LONG-TERM PASSAGE AND EXPANSION OF TRANSGENE-FREE HIPSC LINES IN FMM

OVERVIEW

The long-term passage and expansion of hiPSC using the multi-stage media platform of FRM and FMM was studied using hiPSC clones from Example 2, expanded as single cells in FF culture (Figure 3A and 3B).

RESULTS

hiPSC lines reprogrammed according to Example 2 lost episomal DNA by passage 4-7 and thus, pluripotent independent of the transgene-based reprogramming factors (Figure 3C). The hiPSC lines maintained a homogeneous population of undifferentiated cells positive for SSEA4, TRA1-81, OCT4 and NANOG. Moreover, these lines maintained pluripotent characteristics (Figure 3F) in the absence of any cleanup strategies that are commonly utilized in pluripotent culture (Figure 3D and 3E). Similar expansion of uniform hiPSC cultures were observed when Matrigel was replaced with the defined surface coating Vitronectin during routine single cell passaged culture (Figure S10E-10G).

Genomic abnormalities are often detected in hESC and hiPSC lines cultured as single cells in a FF environment (Laurent *et al.*, 2011; Taapken *et al.*, 2011). Karyotype analysis of all analyzed hiPSC lines demonstrated genomic stability in FMM culture (Figure 4A). In addition, single cell and FF cultured hiPSC clones maintained in FMM for an extended period (25-30 passages) continued to maintain their undifferentiated profile and genomic stability without the need for culture cleaning or selection (Figure 4B).

Episomal-derived hiPSC clones maintained in FMM also readily gave rise to all three somatic lineages, *in vitro* differentiation via embryoid bodies, and *in vivo* differentiation by teratoma formation (Figure 4C-E).

These data demonstrated that the FRM and FMM multi-stage media platform enables transgene-free hiPSC clones to be readily generated and expanded in FF and single cell enzymatic passage format while maintaining homogeneous population of pluripotent and genomically stable cells.

EXAMPLE 4

MULTI-STAGE MEDIA PLATFORM ENABLES EPISOMAL REPROGRAMMING WITH MINIMAL GENES

OVERVIEW

An efficient footprint-free expression system with the reduced dependency for oncogenes such as KLF4, MYC and LIN28 or the need to knockdown P53 in the reprogramming process would be of great value for pluripotent stem cell therapies (Lee *et al.*, 2013; Okita *et al.*, 2011; Yu *et al.*, 2009). Because the multi-stage media platform demonstrated an extremely efficient and robust platform for the generation and expansion of transgene-free hiPSC cells using OSNKLMT reprogramming factors, the robustness of the platform was measured against the requirement for reprogramming factor.

RESULTS

Several episomal-expression cassettes containing minimal gene sets were constructed, including OCT4/NANOG/SOX2 (ONS), OCT4/SOX2 (OS) or OCT4/OCT4 (2xO) in an attempt to vary gene expression combination and dosage (Figure 5A). A fibroblast cell line was transfected with combinations of OCT4, NANOG, SOX2, and SV40LT and cultured using the FRM/FMM platform. SV40LT alone did not produce any true SSEA4/TRA1-81 positive cells at day 13 of reprogramming, but improved cell survival post transfection (Figures 5B and 11A). The various reprogramming factor combinations, resulted in efficient reprogramming as demonstrated by emergence of SSEA4/TRA1-81 positive populations early in the reprogramming process (>0.5% for OCT4/SOX2/SV40LT and >3.5% for OCT4/NANOG/SOX2/SV40LT by day 13; Figure 5B). Cultures reprogrammed for additional days significantly increased the SSEA4/TRA1-81 positive population (>4.0% for OCT4/SOX2/SV40LT by day 16; Figure 11B). Surprisingly, the percentage of reprogrammed cells observed was comparable to lentiviral and episomal-induced systems containing the oncogenes KLF4 and MYC (Figures 2B, 5B and 11B).

Several reprogramming factor combinations were carried forward and transitioned to FMM medium prior to flow cytometry sorting and selection of individual hiPSC clones. Similar to OSNKLMT episomal reprogramming, clonal hiPSC lines were readily derived from combinations containing minimal genes OCT4, SOX2 and SV40LT (2xO + OS + T) as well as other combinations (Figure 5C). hiPSC clones that had lost transgenes and episomal vector markers by passages 5-7 were carried forward for further analysis (Figure 5D). Selected clones were continuously passaged as single cells in a FF environment and maintained a homogeneous population of genomically stable undifferentiated cells and displayed the ability to efficiently differentiate into the three somatic lineages (Figures 5E-J).

Collectively, these data indicated that hiPSC were readily generated by transient expression of minimal reprogramming genes in the FRM/FMM, flow cytometry-based reprogramming platform.

EXAMPLE 5

THE FMM PLATFORM SUPPORTS GROUND STATE PLURIPOTENCY

OVERVIEW

In order to further evaluate the FMM platform, the gene expressions characteristics of somatic cells reprogrammed by existing methods was compared with the gene expressions characteristics of somatic cells reprogrammed using the FMM platform contemplated herein. The gene expression differences between small molecule and conventionally maintained hiPSC cultures (Hanna *et al.*, 2010b; Saha and Jaenisch, 2009) were evaluated.

RESULTS

In one set of experiments, the gene expression patterns between small molecule mediated and conventional culture were evaluated. A lentiviral-induced hiPSC clone FTi111 was generated and maintained in small molecule culture and shown to be pluripotent. The clone was thawed directly into various culture environments including: i) conventional medium with feeder cells and ii) small molecule inhibitor-containing medium with feeder cells or on FF surfaces (Figure 12A and 12B). hiPSC colonies in conventional culture were only recovered in the presence of Thiazovivin, a ROCK inhibitor and subsequently converting the recovered cells to clump culture (Figure 12B and 12C). Each set of culture conditions demonstrated a unique colony morphology (Figure 12D) and distinct pattern of gene expression for pluripotent markers (Figure 12E). The conventionally maintained culture on feeder cells more closely resembled hESC controls H1 and HUES9 maintained in conventional culture than its counterpart cultures maintained in small molecules (Figure 12E). These data showed that distinct gene expression patterns exist between small molecule mediated and conventional culture.

Differences in gene expression were also assessed between hiPSCs derived using lentiviral induction and conventional ESC/feeder culture, and episomal derived lines and further between episomal lines derived with different combinations of reprogramming factors maintained in the FRM/FMM platform. High-content qRT-PCR analysis was used to quantify gene expression associated with pluripotency and differentiation. The majority of the pluripotency genes surveyed displayed comparable expression patterns between hiPSC maintained in FMM culture or conventional cultures containing feeder cells (Figure 6A). However, differences between cell lines were observed on assessment of genes associated with differentiation (Figure 6B). FMM maintained hiPSCs displayed lower expression of most genes associated with the three somatic lineages when compared to both hiPSCs and H1 hESCs maintained in conventional medium and on feeder cells. A subset of lines induced by episomal gene set OSNKLMT appeared to show expression of ectoderm lineage, OTX2 and TUJ1, whereas this expression was negligible in the hiPSC episomally-derived without the use of Lin28, KLF4 and c-MYC (Figure 6B). Surprisingly, the expression of all differentiation genes tested were fully suppressed in all hiPSCs derived from episomal minimal genes sets and maintained in FMM (Figure 6B). Together, these data indicated that the FRM/FMM platform can robustly reprogram cells with few episomal-based reprogramming factors and can maintain the hiPSCs in a stable ground pluripotent state.

The global gene expression patterns were determined for hiPSCs derived from the following methods: i) episomal induction maintained in FMM, ii) episomal induction maintained in FMM but switched to conventional medium for three passages, iii) lentiviral induction maintained in SMC4; and iv) lentiviral induction maintained in conventional culture (Figure 13A, 13B). Prior to evaluating the gene expression profiles, all lines were determined to be pluripotent, genomically stable; and able to differentiate to all three somatic lineages. Cluster analysis of differentially genes expressed between small molecule culture and conventional culture revealed that the hiPSC lines grouped based on current culture conditions and not by original derivation method and culture (Figure 13B). Gene ontology classification of 300 genes displaying 2.5 fold expression differences identified differentiation and development as the main categories highly enriched in the conventional culture group while genes upregulated in small molecule culture group were mostly associated with regulation of cell proliferation and sex development (Figure 13C and 13D).

Gene expression analyses were repeated and FMM, conventional, or transition culture systems were directly compared (FMM, Conv, FMM→Conv, Conv→FMM; Figure 13A). Cluster analysis produced two groups separated based on the current culture system regardless of method of generation or the prior culture system (Figure 7A). For example, hiPSC cloneFTC016-c28 was generated and maintained under the FRM/FMM platform, prior to transition to conventional culture. This clone grouped with cultures maintained exclusively in conventional culture and not with its parental line maintained in FMM; comparable results were seen with lines such as an OKS lentiviral-induced hiPSC clone which was generated in conventional culture and grouped within the conventional set until transition to FMM, upon which it grouped within the FMM cluster (Figure 7A). Gene ontology categorized conventional culture to be enriched with genes associated with differentiation and development (*i.e.*, p-value=2.2E-10, pattern specification process; Figures 7B and 13E). Collectively, these data showed that genes associated with differentiation propensity are significantly reduced in FMM culture and hiPSCs can be adapted to the FMM culture platform to reduce spontaneous differentiation potential.

Gene lists were compiled to represent ground state and metastable states of human pluripotent stem cells (De Los Angeles *et al.*, 2012; Han *et al.*, 2011; Hanna *et al.*, 2010a; Hirata *et al.*, 2012; Nichols and Smith, 2012; Valamehr *et al.*, 2012; Zhou *et al.*, 2010) (Figure 7C). Gene clustering based on these gene lists was performed for hiPSC lines in FMM or conventional culture (Figure 7D). Similar to global gene expression comparison, the focused gene clustering showed a separation of the cell lines based on their current culture conditions with profiles appearing to be interconvertible. For example, hiPSC clone FTC016-c28 transitioned from FMM to conventional culture grouped with H1 hESC and not with its parental hiPSC line maintained in FMM (Figure 7D). Similarly, a lentiviral hiPSC clone derived from a fibroblast line maintained in conventional culture grouped with HUES9 hESC and other hiPSC clones in conventional culture; however, when switched to FMM, it grouped with an episomal hiPSC derived from umbilical cord blood as well as other FMM cultured lines (Figure 7D). The distribution of genes representative of the ground and metastable states within the two clusters was determined by plotting the average intensities for each probe set with respect to small molecule (SMC4/FMM) versus conventional culture (Figure 7E). Surprisingly, the majority of genes associated with the ground state showed elevated expression in small molecule culture cluster, and increased expression of genes associated with metastable state was detected in the conventional culture cluster (Figure 7E).

The X-inactivation state of hiPSCs cultured and maintained in conventional culture was compared to its counterpart adapted to small molecule culture and maintained for 10 passages (Figure 7F). The hiPSC maintained in small molecule culture showed an increase in X chromosome gene expression when compared to conventional culture, which suggested reactivation of the silenced X chromosome (Figure 7F). The noticeable exception was the X-inactive specific transcript (XIST) which was down-regulated in the switch to small molecule culture (Figure 7F). Further evidence of X activation was provided by the differential staining of H3K27me3 in hiPSCs cultured in FMM relative to their counterpart culture adapted to conventional medium (Figure 7G). The majority of hiPSCs in FMM lacked H3K27me3 staining; whereas, the majority of hiPSCs in conventional culture displayed H3K27me3 nuclear foci with the appearance of reduced nuclear size, which suggested X inactivation (<10% H3K27me3 staining in FMM compared to >90% H3K27me3 staining in conventional culture; Figure 7G).

EXAMPLE 6

HIPSC MAINTENANCE IN SMALL MOLECULE CULTURE

Derived hiPSCs (fibroblasts or blood-cell induced with various combinations of reprogramming factors including OCT4/NANOG/SOX2, OCT4/ECAT1/UTF1, or OCT4/ECAT1/UTF1/ESRRB/NANOG) were routinely passaged as single cells once confluency of the culture reached 75-90%. For single cell dissociation, hiPSCs were washed once with phosphate buffered saline (PBS) (Mediatech) and treated with Accutase (Millipore) for 3 to 5 min at 37°C followed with pipetting to ensure single cell dissociation. The single cell suspension was then mixed in equal volume with conventional medium, centrifuged at 225 g for 4 min, resuspended in Fate Maintenance Media (FMM) and plated on hESC-qualified Matrigel (Corning) coated surfaces. Matrigel was prepared and used to coat surfaces per manufacturer's instructions. Passages were typically 1:3-1:6, tissue culture plates were previously coated with Matrigel for 1-4hrs at 37°C, and fed every two to three days with FMM. Cell cultures were maintained in a humidified incubator set at 37°C and 5% CO_2. Conventional medium consists of DMEM/F12 (Mediatech), 20% Knock-Out Serum Replacement (Life Technologies), 1x Glutagro (Mediatech), 1x Non-Essential Amino Acids (NEAA) (Mediatech), 1x Pen/Strep (Mediatech), and 100μM β-Mercaptoethanol. FMM consists of conventional medium supplemented with 5μM Thiazovivin (synthesized in-house), 0.4μM PD0325901 (Biovision), 1μM CHIR99021 (Biovision), 100ng/mL bFGF (Life Technologies), and 10ng/mL hLIF (Millipore). Flow analysis and morphology for cultures expanded in FMM is presented in Figs. 18A-D and 19A-B. Cells expanded in FMM also demonstrated a normal karyotype over multiple passages as shown in Figures 20A-D.

EXAMPLE 7

REPROGRAMMING WITH MINIMAL GENES IN SMALL MOLECULE CULTURE

To initiate reprogramming, ectopic expression of reprogramming factors was induced by lentiviral transduction using NIL, traditional integrating lentivirus, or electroporation with episomal vectors. As illustrated in Figures 14A-B, the lentiviral expression system consisted of several features including an EF1α promoter, specific gene combinations (Table 3) and a LOXP site at the 3' end to allow for CRE-mediated excision of the integrated transgenes. Upon CRE-excision, the derived hiPSCs genome no longer contained transgenes and were essentially footprint-free. As illustrated in Figures 14C-F, the episomal constructs had unique features including an EF1α promoter and unique reprogramming factors. Upon transfection, the episomal constructs resided in the nucleus and acted in a trans-mediated fashion that did not integrate into the genome.

For lentivirus infection, the starting human fibroblast cells were seeded at $7x10^4$-$1x10^5$ cells per well of a 6-well plate coated with Matrigel (Corning) per manufacturer's instructions. Fresh lentiviral supernatant from 293T cells was added to the starting cells at a dilution of 1:2 (one part lentiviral supernatant: one part fibroblast medium). NIL viral supernatant was used at a 1x concentration and not diluted. If previously frozen virus was used, it was not diluted and used at a 1x concentration. Viral supernatants of various factors were combined (Table 3) up to a total of 2mL of media per 6-well. This was supplemented with 5μg/mL polybrene (Millipore) and 10mM Hepes (Meditech) followed by spin infection. Six well plates were sealed with parafilm and centrifuged at 600 g for 90min at 32°C. Plates were then transferred to 37°C and 5% CO_2 incubators for 12-16hrs. After incubation with lentivirus, the cells were washed with PBS and the culture medium was switched to 50/50 medium containing one part Fate Reprogramming Medium (FRM) and one part fibroblast medium. The medium was completely switched to FRM between 4 to 6 days post infection. FRM consists of conventional medium (described above) supplemented with 5μM Thiazovivin (synthesized in-house), 0.4μM PD0325901 (Biovision), 1μM CHIR99021 (Biovision), 2μM SB431542 (Biovision), 100ng/mL bFGF (Life Technologies), 10ng/mL hLIF (Millipore), 1x N2 Supplement (Life Technologies), and 1x B27 Supplement (Life Technologies). Once wells became confluent, cells were passaged onto 10cm dishes previously coated with Matrigel. Passaging consisted of dissociation with Accutase (Millipore) onto Matrigel coated surface (as described above). Between days 14 and 18 or when iPSC colonies became present, the culture media was switched from FRM to FMM. The single cell dissociated cells were expanded onto Matrigel coated plates with FMM and maintained until flow cytometry sorting. Results for expression of hiPSC phenotype are presented in Table 3, Figures 15A-C (at 8-15 days), and Figures 16A-D, Figures 17A-B (Oct-4/Ecat1/UTF1/Esrrb/Nanog at week 4).

TABLE 3: Reprogramming Factor Combinations and Expression of Pluripotent Phenotype

Vector System	SSEA4/Tra181 Expression by Flow Cytometry		iPSC Morphology
	Day 13-18 Flow (%)	Day 21-27 Flow (%)	
OCT4-P2A-OCT4 ECAT1-P2A-UTF1	0.00	0.06	+
OCT4-P2A-OCT4 NANOG-P2A-ESRRB-T2A-LIN28 ECAT1-P2A-UTF1	0.02	0.19	++
OCT4-P2A-ESRRB OCT4-P2A-NANOG	0.10	1.29	++

ECAT1-P2A-UTF1			
OCT4-P2A-NANOG ECAT1-P2A-UTF1	0.05	0.14	++
OCT4 –P2A-NANOG-T2A-SOX2 SV40LT	0.14	0.90	++
OCT4-P2A-OCT4 OCT4-P2A-NANOG-T2A-SOX2 SV40LT	0.00	1.46	++
OCT4-P2A-OCT4 ECAT1-P2A-UTF1 SV40LT	0.03	0.90	+
OCT4-P2A-DPPA2 OCT4-P2A-ESRRB ECAT1-P2A-UTF1	0.03	0.11	+
OCT4-P2A-OCT4 OCT4-P2A-ESRRB ECAT1-P2A-UTF1	0.02	0.12	+

For episomal vector reprogramming, transfection of fibroblast or cord blood cells using the plasmids illustrated in Figure 13 was conducted using the NEON Transfection System (Life Technologies). Approximately, a total of 3μg of episomal plasmids containing reprogramming factors was co-transfected with EBNA (either in the form of mRNA or as a cassette in cloning plasmid pCDNA) into $5x10^5$ fibroblast cells or $2.5x10^5$ cord blood cells using settings 1650v/10ms/3pulses in appropriate buffers as described by product manual. The transfected cells were seeded directly onto a well of a 6-well plate coated with Matrigel containing either fibroblast medium or cord blood culture medium (depending on the cell type) supplemented with 4 ng/mL bFGF and 5 μg/mL fibronectin (BD Biosciences) without antibiotics. Cord blood culture medium consists of SFMII + CC110 (Stem Cell Technologies). Twenty-four hours post transfection, FRM was added to the culture in equal volume. For fibroblast cultures, forty-eight hours post transfection 50 μg/mL hygromycin (Corning) was added to the culture. The culture medium was switched entirely to FRM on day 5 with hygromycin removed 7 days post transfection. All reprogramming cultures were switched to FMM 14 days post transfection. For cord blood cultures, twenty-four hours post transfection, FRM was added in equal volume and continuously added every few days until day 14 post transfection where the culture was aspirated and replaced with entirely FMM. In both cases, clusters of adherent rounded cells were seen around 5 to 7 days post transfection. Once in FMM, all reprogramming cultures were maintained and single cell passaged using Accutase on Matrigel coated surfaces (described above). The single cell dissociated cells were expanded onto Matrigel coated plates with FMM and maintained until flow cytometry sorting.

EXAMPLE 8

INFLUENCE OF REPROGRAMMING FACTORS AND THEIR STOICHIOMETRY

Human fibroblast cells were spin infected with lentivirus containing several reprogramming factors. All samples were infected with OCT4, SOX2, NANOG, and SV40LT using a lentiviral plasmid not containing an antibiotic selection factor. Cells were co-infected with a single lentiviral plasmid containing a puromycin selection cassette as well as various reprogramming factors. These factors included either OCT4-P2A-SOX2, OCT4-P2A-NANOG-T2A-SOX2, or OCT4-P2A-OCT4. Two days post infection, 500ng/mL of Puromycin (Life Technologies) in 50/50 media was added to each to well. On Day 5, after three days of Puromycin selection, media was changed to FRM without Puromycin. On Day 14, media was switched to FMM. Between Days 24 and 27, flow analysis was conducted for SSEA4+/TRA181+ populations. It was observed that increased OCT4 expression significantly improves reprogramming efficiency (Figure 23A).

EXAMPLE 9

EXPERIMENTAL PROCEDURES

HIPSC MAINTENANCE IN CONVENTIONAL CULTURE SYSTEM

Conventionally cultured hiPSCs were maintained on mitomycin C treated MEF (Millipore) feeder cells and cultured with conventional medium (referred to as conventional medium in the text) containing DMEM/F12 (Mediatech), 20% v/v knockout serum replacement (Life Technologies), 1% v/v non-essential amino acids (Mediatech), 2 mM L-glutamine (Mediatech), 100 μM β-mercaptoethanol (Life Technologies) and 10 ng/mL bFGF (Life Technologies). Upon confluency, conventionally cultured hiPSCs were enzymatically dissociated using 1 mg/mL collagenase IV (Life Technologies) for 7 min at 37°C followed by mechanical dissociation into small pieces (termed as clump passaging), collected and dilute passaged 1:3–1:4 onto freshly seeded feeder cells every 5–7 days with daily addition of conventional medium. In case of excessive spontaneous differentiation, colonies were manually picked and cut into small pieces using the tip of Insulin Syringe (Becton Dickinson) and transferred to freshly seeded feeder cells. Cell cultures were maintained in a humidified incubator set at 37°C and 5% CO2.

REPROGRAMMING OF SOMATIC CELLS

To initiate reprogramming, ectopic expression of reprogramming factors were induced by lentiviral transduction or episomal vector transfection. Lentiviral transfection was followed as previously described (Valamehr *et al.*, 2012). Briefly, the starting cells were plated at $1x10^5$ cells per well of a 6-well plate on Matrigel (BD Biosciences) coated surface. Unless specified, all Matrigel coatings consists of adding Matrigel solution (1 aliquot of Matrigel resuspended in 25 mL DMEM/F12) to tissue culture surfaces and allowing for 2-4 hrs incubation at 37°C. Supernatant from 293T cells generating lentivirus expressing transgene OCT4/SOX2/KLF4 was added to the starting cells at a dilution of 1:2 (one part lentiviral supernatant : one part fibroblast medium), supplemented with 4 μg/mL polybrene (Millipore), and transferred to 37°C and 5% CO2 for 12-16 hrs. Fibroblast medium: DMEM (Mediatech), 10% FBS (Life Technologies), 1x glutamax (Life Technologies), 1x non-essential amino acids (Mediatech). After incubation with lentivirus, the cells were washed three times with PBS and fed with fibroblast medium. 48hrs post transfection, the culture medium was switched to 50/50 medium containing one part FRM (or SMC4) and one part fibroblast medium. The medium was completely switched to FRM (or SMC4) once the culture was passaged into a larger vessel, usually between days 4 to 6 post infection. Passaging consists of dissociation with Accutase onto Matrigel coated surface (as described below). Cultures were maintained in FRM (or SMC4) until the next application.

For episomal vector reprogramming, transfection of fibroblast or cord blood cells using gene set OCT4/SOX2/NANOG/KLF4/LIN28/MYC/SV40LT (A14703, Life Technologies) was conducted using NEON Transfection System (Life Technologies). Approximately, 4μg of vector set was transfected into $5x10^5$ fibroblast cells or $2.5x10^5$ cord blood cells using settings 1650v/10ms/3pulses in appropriate buffers as described by product manual. The transfected cells were plated directly into a 10 cm dish (fibroblast) or a well of 6-well plate (cord blood) coated with Matrigel and containing either fibroblast culture medium or cord blood culture medium (depending on the cell type) supplemented with 10 ng/mL bFGF and 5 μg/mL fibronectin (BD Biosciences). Cord blood culture medium: SFMII + CC110 (Stem Cell Technologies). Twenty-four hours post transfection, FRM was added to the culture in equal volume. For fibroblast cultures, forty-eight hours post transfection 50 μg/mL hygromycin (Mediatech) was added to the culture. The culture medium was switched to entirely FRM on day 5 with hygromycin removed on day 7 post transfection. All reprogramming cultures were switched to FMM on day 14 post transfection. For cord blood cultures, twenty-four hours post transfection, FRM was added in equal volume and continuously added every few days until day 14 post transfection where the culture was aspirated and replaced with entirely FMM. In both cases, cluster of adherent rounded cells were seen around days 5 to 7 post transfection. Once in FMM all reprogramming cultures were maintained and single cell passaged using Accutase. The single cell dissociated cells were expanded onto Matrigel coated plates with FMM and maintained until flow cytometry sorting. *In vitro*nectin (Life Technologies) surface coating studies, all aspects were kept the same except for the substitution of Matrigel for Vitronectin. For reduced factor episomal reprogramming, pCEP4 (Life Technologies) vector backbone was constructed to contain OCT4-P2A-OCT4, OCT4-P2A-SOX2 or OCT4-P2A-NANOG-T2A-SOX2 under the regulation of EF1α promoter. The transfection of reduced factor episomal vectors followed the same protocol as described above with the exception of few modifications. EBNA was co-transfected as either EBNA mRNA (20 μg) or vector cassette (2 μg) (Howden *et al.*, 2006). Hygromycin selection was maintained for 10 days and FMM was introduced on day 16.

GENERATION OF LENTIVIRUS

293Ts (ATCC) were maintained in fibroblast media without antibiotics and were not allowed to reach over 80% confluency. Fibroblast medium consisted of DMEM (Mediatech), 10% Fetal Bovine Serum (FBS) (Life Technologies), 1x Glutagro (Mediatech), and 1x NEAA (Mediatech). Cells were passaged by first washing with PBS followed by a 4min incubation at 37°C with 0.05% Trypsin (Mediatech). Dissociated cells were resuspended in fibroblast media, centrifuged at 225 g for 4min and seeded onto desired plates. To generate integrating lentivirus, 293Ts were passaged on Day 1 at $3.5x10^6$ cells per 10cm dish for each viral prep. On Day 2, the media was changed to 10mL of fresh fibroblast media 1 hour prior to transfection. DNA was transfected using CalPhos Kit (Clontech). The following were combined for the transfection: 5μg of lentiviral cloning plasmid containing the gene(s) of interest, 3.2μg of packaging plasmid pPAX, 630ng of packaging plasmid pMDG, 87μL Calcium Solution, and water up to 700μL. 700μL of HBS Solution was added while creating bubbles using a 1mL serological pipette. This was incubated at room temperature for 15min and then added drop-wise to a 10cm plate of 293Ts. On Day 3, the viral supernatant was removed, discarded, and 15mL of fresh fibroblast media added to the plate. On Day 4, 48hrs post transfection, the viral supernatant was collected and stored at 4°C. 15mL of fibroblast media was added to the plate. On Day 5, 72hrs post transfection, the viral supernatant was collected and added to the Day 4 supernatant. This viral pool was filtered using a 0.45μm filter and checked for titer using a Lenti-X GoStix (Clontech). Virus was either used for an infection or frozen in aliquots at -80°C. To generate Non-Integrating Lentivirus (NIL) (Invivogen), the protocol was followed per manufactures instructions using a T75 flask for each viral prep. Viral supernatants were collected 48, 72, and 96 hours post-transfection, pooled, filtered and titered as described above. NIL virus was either used for an infection or frozen in aliquots at -80°C.

HIPSC MAINTENANCE IN SMALL MOLECULE CULTURE

Derived hiPSCs were routinely passaged as single cells once confluency of the culture reached 75-90%. Note that over-confluency may result in differentiation. For single cell dissociation, hiPSCs were washed once with phosphate buffered saline

(PBS) (Mediatech) and treated with Accutase for 3 to 5 min at 37°C followed with pipetting to ensure single cell dissociation. The single cell suspension was then mixed in equal volume with conventional medium, centrifuged at 225 g for 4 min, resuspended in FMM and plated on Matrigel coated surface. Passages were typically 1:4–1:8, transferred tissue culture plates previously coated with Matrigel for 2-4 hrs in 37°C and fed every other day with FMM. Cell cultures were maintained in a humidified incubator set at 37°C and 5% CO2. Medium formulations for FMM and FRM are described in Table 1. SMC4 culture is discussed previously (Valamehr *et al.*, 2012). Briefly, small molecules 0.4 mM PD0325901 (Biovision), 1 mM CHIR99021 (Biovision), 5 mM Thiazovivin and 2 mM SB431542 (Biovision) are added to conventional culture medium and passaged according to protocol.

FLOW CYTOMETRY ANALYSIS AND SORTING

Single cell dissociated (described above) reprogramming pools were resuspended in chilled staining buffer containing Hanks' Balanced Salt Solution (MediaTech), 4% fetal bovine serum (Invitrogen), 1x penicillin/streptomycin (Mediatech) and 10 mM Hepes (Mediatech). Conjugated primary antibodies, including SSEA4-FITC, TRA1-81-Alexa Fluor-647 and CD30-PE (BD Biosciences), were added to the cell solution and incubated on ice for 15 min. All antibodies were used at 7-10 µL in 100 µL staining buffer per million cells. The solution was washed once in staining buffer, spun down at 225 g for 4min and resuspended in staining buffer containing 10 µM Thiazovivin and maintained on ice for flow cytometry sorting. Flow cytometry sorting was performed on FACS Aria II (BD Biosciences) using gating strategy described *supra*. The sorted cells were directly ejected into 96-well plates using the 100 µM nozzle, at concentrations of 3 and 9 events per well. Sorting 3 cells per well was our preferred concentration as we noticed that events sorted did not necessarily correlate to actual number of cells seen in each well post sort and that 3 cells per well gave us a preferred number of wells containing individual colonies. Each well was prefilled with 200 µL FMM supplemented with 5 µg/mL fibronectin and 1x penicillin/streptomycin (Mediatech) and previously coated overnight with 5x Matrigel. 5x Matrigel precoating includes adding one aliquot of Matrigel into 5 mL of DMEM/F12, then incubated overnight at 4°C to allow for proper resuspension and finally added to 96-well plates at 50 μL per well followed by overnight incubation at 37°C. The 5x Matrigel is aspirated immediately before the addition of media to each well. Upon completion of the sort, 96-well plates were centrifuged for 1-2 min at 225 g prior to incubation. The plates were left undisturbed for seven day. On the seventh day, 150 μL of medium was removed from each well and replaced with 100 μL FMM. Wells were refed with an additional 100 μL FMM on day 10 post sort. Colony formation was detected as early as day 2 and most colonies were expanded between days 7-10 post sort. In the first passage, wells were washed with PBS and dissociated with 30 μL Accutase for approximately 10 min at 37°C. The need for extended Accutase treatment reflects the compactness of colonies that have sat idle in culture for prolonged duration. After cells are seen to be dissociating, 200 μL of FMM is added to each well and pipetted several times to break up the colony. The dissociated colony is transferred to another well of a 96-well plate previously coated with 5x Matrigel and then centrifuged for 2 min at 225 g prior to incubation. This 1:1 passage is conducted to spread out the early colony. Subsequent passages are done routinely with Accutase treatment for 3-5 min and expansion of 1:4 into larger wells previously coated with 1x Matrigel in FMM. Flow cytometry analysis was performed on Guava EasyCyte 8 HT (Millipore) and analyzed using FCS Express 4 (De Novo Software).

Real-Time RT-PCR and Fluidigm Analysis

Total RNA was isolated using Pico Pure RNA Isolation Kit (Life Technologies). Complimentary DNA (cDNA) was reverse transcribed from 100ng of isolated total RNA using the iScript cDNA Synthesis Kit (Bio-Rad). The cDNA was then used for pre-amplification of 22 specific target genes and two reference control genes using the TaqMan PreAmp Master Mix Kit (Life Technologies) and a 0.2x concentration of pooled TaqMan assays. Specific target amplification (STA) from cDNA was performed using 14 cycles of amplification with the standard cycling conditions stated in the manufacturer's protocol. The pre-amplified cDNA reactions (n=48) were diluted 1:5 (in sterile water) and used as template for the real-time quantitative PCR reactions. 48.48 Dynamic arrays (Fluidigm) were loaded using a NanoFlex IFC Controller MX (Fluidigm) with TaqMan assays loaded in duplicate and real-time reactions were performed using a BioMark Real-Time PCR System

(Fluidigm). Results were analyzed using BioMark Real-Time PCR Analysis software (Fluidigm). Samples with cycle thresholds (Cts) above 32 were excluded from the calculations. Average Cts were calculated from the assay duplicates and delta-delta Cts (ΔΔCt) were calculated using the mean of two reference genes (GAPDH and HPRT1) against the median of six control MEF cell lines (OSK hiPSCs on MEF and H1 ESCs). Relative gene expression (RQ) results are displayed in Excel (Microsoft) in heat map format.

Table 4. FAM-labeled TaqMan probes

Assay ID	Catalog Number (Life Tech.)	Gene Symbol	RefSeq
Hs00232764_m1	4331182	FOXA2	NM_021784.4;NM_153675.2
Hs00173490_m1	4331182	AFP	NM_001134.1
Hs00171403_m1	4331182	GATA4	NM_002052.3
Hs00751752_s1	4331182	SOX17	NM_022454.3
Hs00610080_m1	4331182	T	NM_003181.2
Hs00607978_s1	4331182	CXCR4	NM_003467.2;NM_001008540.1
Hs00415443_m1	4331182	NODAL	NM_018055.4
Hs02330075_g1	4331182	MYOD1	NM_002478.4
Hs00240871_m1	4331182	PAX6	NM_001127612.1
Hs00801390_s1	4331182	TUBB3	NM_001197181.1;NM_006086.3
Hs00374280_m1	4331182	STAT3	NM_139276.2;NM_213662.1;NM_003150.3
Hs04260366_g1	4331182	NANOG	NM_024865.2
Hs00602736_s1	4331182	SOX2	NM_003106.3
Hs00399279_m1	4331182	ZFP42	NM_174900.3
Hs01003405_m1	4331182	DNMT3B	NM_001207055.1;NM_001207056.1;NM_006892.3;NM_175848.1;NM_175850.2;NM_175849.1
Hs00702808_s1	4331182	LIN28A	NM_024674.4
Hs99999003_m1	4331182	MYC	NM_002467.4

Hs01081364_m1	4331182	DNMT3L	NM_013369.2;NM_175867.1
Hs00360439_g1	4331182	KLF2	NM_016270.2
Hs00222238_m1	4331182	OTX2	NM_172337.1;NM_021728.2
Hs00242962_m1	4331182	PAX7	NM_001135254.1;NM_002584.2;NM_013945.2
Hs00414521_g1	4331182	DPPA2	NM_138815.3
Hs00216968_m1	4331182	DPPA4	NM_018189.3
Hs99999905_m1	4331182	GAPDH	NM_002046.4
Hs01003267_m1	4331182	HPRT1	NM_000194.2

Custom-made TaqMan Gene Expression Assays		
Gene	Forward Primer	Reverse Primer
OCT4	GGGTTTTTGGGATTAAGTTCTTCA (SEQ ID NO:27)	GCCCCCACCCTTTGTGTT (SEQ ID NO:11)
KLF4	AGCCTAAATGATGGTGCTTGGT (SEQ ID NO:28)	TTGAAAACTTTGGCTTCCTTGTT (SEQ ID NO:12)

TESTING PRESENCE OF TRANSGENES

Genomic DNA was isolated using QIAamp® DNA Mini Kit and Proteinase K digestion (Qiagen). 100 ng of the genomic DNA was amplified using transgene-specific primer sets (Table 5 below) (Yu *et al.*, 2007) using Taq PCR Master Mix Kit (Qiagen). The PCR reactions were run for 35 cycles as follows: 94°C for 30 sec (denaturation), 60-64°C for 30 sec (annealing) and 72°C for 1 min (extension). Genomic DNA from fibroblasts and hiPSCs generated using lentiviral methods were used as negative controls. DNA of the episomal constructs was used as positive control.

Table 5. Transgene specific primer sets

Amplified region	Forward	Reverse
Oct4-Oct4 region of episomal transgene	CAGGCCCGAAAGAGAAAGCG (SEQ ID NO: 13)	GGAGGGCCTTGGAAGCTTAG(SEQ ID NO: 14)
Oct4-NANOG region of episomal transgene	TATACACAGGCCGATGTGGG (SEQ ID NO: 15)	TTGACCGGGACCTTGTCTTC (SEQ ID NO: 16)
OCT4-SOX2 region of episomal transgene	GTGGTCCGAGTGTGGTTCTG (SEQ ID NO: 17)	GTTCTCCTGGGCCATCTTGC (SEQ ID NO: 18)
Lin28-SV40pA episomal transgene	AAGCGCAGATCAAAAGGAGA (SEQ ID NO: 19)	CCCCCTGAACCTGAAACATA (SEQ ID NO: 20)
WPRE lentiviral element	TGCTTCCCGTATGGCTTTC (SEQ ID NO: 21)	AAAGGGAGATCCGACTCGTCTG (SEQ ID NO: 22)

EBNA1	ATCGTCAAAGCTGCACACAG (SEQ ID NO: 23)	CCCAGGAGTCCCAGTAGTCA (SEQ ID NO: 24)
Human GAPDH	GTGGACCTGACCTGCCGTCT (SEQ ID NO: 25)	GGAGGAGTGGGTGTCGCTGT (SEQ ID NO: 26)

IMMUNOCYTOCHEMISTRY ANALYSIS

Cells were fixed using 4% v/v paraformaldehyde (Alfa Aesar), washed three times with PBS containing 0.2% v/v Tween (PBST) (Fisher Scientific) and permeablized using 0.15% v/v TritonX-100 (Sigma-Aldrich) in PBS for 1 hr at 25°C. After permeabilization, cells were blocked with 1% v/v BSA (Invitrogen) in PBST (PBSTB) (Fisher Scientific) for 30 min at 25°C. After gentle removal of PBSTB, cells were incubated with primary antibody in PBSTB overnight at 4°C. Primary antibodies used in this study include OCT4 (Santa Cruz), NANOG (Santa Cruz), TRA160 (Millipore), TRA1-81 (Millipore), SSEA4 (Millipore), β-III Tubulin (TUJ1, R&D Systems), α-Smooth Muscle Actin (Sigma), FoxA2 (R&D Systems), Sox17 (R&D Systems), NESTIN (Abcam) and Alpha-1-Fetoprotein (Dako). After the overnight incubation, cells were washed three times with PBST and stained with secondary antibody (Alexa Fluor 488 or 555; Invitrogen) diluted 1:250 in PBSTB for 1 hr at 37°C. The cells were washed three times in PBST and stained with Hoechst dye (Invitrogen). For H3K27me3 staining analysis, hiPSCs were grown 72 to 96 hrs on cover slips and fixed with 4% paraformaldehyde (Electron Microscopy Science, EMS) in PBS for 15 min at 25°C. Cell permeabilization was performed with 0.1% Triton X-100 in PBS for 1 hour at 25°C, and then cells were incubated with blocking solution (1% BSA in PBS) for 30 min at 25°C. After blocking, cover slips were incubated with 1:1600 dilution of anti-trimethyl-histone H3 (Lys27) antibody (Millipore 07-449, H3K27me3) in blocking solution, overnight at 4°C. Secondary antibodies were Alexa Fluor 555 Goat-anti-Rabbit IgG (Life Technologies, A21429). The nuclei were counterstained with DAPI and viewed with an Axio Observer Inverted Microscope (Carl Zeiss). Images were captured with the AxioVS40 v4.8.1.0 (Carl Zeiss Imaging Solutions Gmbh).

Cells reprogrammed according to Example 7 were fixed using 4% v/v paraformaldehyde (Alfa Aesar), washed with PBS (Mediatech) and permeabilized using 0.15% v/v TritonX-100 (Sigma-Aldrich) in PBS for 1hr at 25°C. After permeabilization, the cells were blocked with 1% v/v BSA (Sigma) in PBS (PBSB) for

30 min at 25°C. After gentle removal of PBSB, cells were incubated with primary antibody in PBSB overnight at 4°C. Primary antibodies used in this study include OCT4 (Santa Cruz) and TRA181 (Millipore). After the overnight incubation, cells were washed three times with PBS and stained with secondary antibody (Alexa Fluor 488 or 555; Life Technologies) diluted 1:250 in PBSB for 1hr at 37°C. The cells were washed three times in PBS and stained with Hoechst dye (Invitrogen). Stained cells were viewed with an Axio Observer Inverted Microscope (Carl Zeiss). Images were captured with the AxioVS40 v4.8.1.0 (Carl Zeiss Imaging Solutions Gmbh).

Differentiation Analysis (EB and Directed)

hiPSC were differentiated as EBs in differentiation medium containing DMEM/F12 (Mediatech), 20% fetal bovine serum (Invitrogen), 1% non-essential amino acids (Mediatech), 2 mM L-glutamine (Mediatech) and 100 µM β-mercaptoethanol. Briefly, for EB formation hiPSCs were seeded in FMM and switched to conventional the following day to prime the cells. After 3 to 4 days in conventional medium, cultures were single cell dissociated with Accutase (Millipore) and resuspended in differentiation medium including 10 µM Y27632 to a final concentration of 100,000 cells/mL. Note that ROCK inhibitor Y27632 instead of Thiazovivin is used for EB formation. Cells were seeded at 100 µL/well in V-bottom 96-well non-tissue culture plate (Nunc) and centrifuged at 950 g for 5 min. The following day compact "ball-like clumps" were transfer to ultra-low binding 6-well plate (Corning) using P1000 at approximately 30–40 EBs/well in differentiation medium. After 7 days, EBs were transferred at 1:1 to Matrigel coated 6-well plate and fed with differentiation medium every three days. After 3 weeks in culture, cells were fixed and stained. For directed monolayer differentiation, hiPSCs were seeded on Matrigel coated wells in FMM to deliver 50% and 90% confluency the following day. Both densities were induced to differentiate. For neural induction, FMM media was replaced with hESC media supplemented with 10 µM SB431542 and 100 nM LDN-193189 (both SMAD inhibitors, Biovision). Following 2 days, differentiation media with supplemented with 3 µM CHIR99021 (Biovision) in addition to the dual SMAD inhibitors. Cells were fixed two days later and stained for Nestin (Abcam). For mesoderm differentiation, media was replaced with RPMI (Mediatech) supplemented with 1x B27 media additive

(Life Technologies), 3 μM CHIR99021, 4 ng/ml bFGF and 10 ng/ml BMP4. Media was changed every other day and cells were fixed on the 4th day and stained for αSMA (Sigma). Endoderm differentiation was performed using the Human Pluripotent Stem Cell Functional Identification Kit (R&D Systems). hiPSCs were incubated with endoderm differentiation media for 3 days, fixed and stained for SOX17 (R&D Systems).

GENE EXPRESSION ANALYSIS

RNA was extracted using the PicoPure RNA Isolation kit (Life Technologies) using the manufacturers recommended protocol. Total RNA was quantified using the Nanodrop 2000 Spectrophotometer (Thermo Scientific). In brief, biotinylated aRNA was prepared from roughly 100 ng of total RNA using the standard protocol for MessageAmp II aRNA Amplification Kit (Applied Biosystems/Ambion, Austin, TX) utilizing the optional Second Round Amplification and then transcribed into biotin labeled aRNA using MessageAmp II Biotin Enhanced Kit (Applied Biosystems/Ambion, Austin, TX) using the standard protocol. Biotin labeled aRNA was purified and fragmented according to Affymetrix recommendations. 20 μg of fragmented aRNA were used to hybridize to the Human Genome U133-plus-2.0 chips (Affymetrix Inc. Santa Clara, CA) for 16 hours at 45°C. The arrays were washed and stained in the Affymetrix Fluidics Station 450 and scanned using the Affymetrix GeneChip Scanner 3000 7G. Raw expression data files are available on Gene Expression Omnibus (GSE50868). The image data were analyzed using Affymetrix Expression Console software using default analysis settings. Arrays were normalized by log scale robust multi-array analysis (RMA, Affymetrix) and visualized in Spotfire for Genomics 4.5 (Tibco Spotfire, Palo Alto, CA). Biological pathway enrichment analysis of the differentially expressed probes was performed against the Gene Ontology (GO) database (Singular Enrichment to GO Biological Process and p-value < 0.01) using Database for Annotation, Visualization and Integrated Discovery (DAVID v6.7). Hierarchical clustering was performed to compare the gene expression profiles between samples based on Log2 expression levels using a complete linkage clustering method with Euclidean distance measurements (Spotfire for Genomics 4.5). Probe sets for clustering were selected by either an overall differential in expression levels (><

2.5-fold) or presence on targeted gene lists defining a ground or metastable state. For X Chromosome gene expression comparison, RMA normalized Affymetrix gene chip intensities were converted to linear expression values by taking the 2^[RMA log2 intensity]. Linear expression ratios were calculated as the naïve expression set divided by the primed expression set. The expression ratios for all probe sets mapped to the X chromosome were visualized in Spotfire 4.5 with the probe sets greater or less than 2 fold enrichment ratio highlighted.

KARYOTYPE ANALYSIS

Cytogenetic analysis was performed on twenty to forty G-banded metaphase cells by WiCell Research Institute (Madison, WI).

TERATOMA FORMATION

Single cell dissociated hiPSCs, at concentrations of 0.5 and 3 million cells per 200 μL solution (100 μL FMM and 100 μL Matrigel) were injected subcutaneously into NOD/SCID/γ null mice. After 5-6 weeks (3 million cells injection) and 7-8 weeks (0.5 million cells injection), teratomas were harvested in PBS, fixed overnight at room temperature in 4% paraformaldehyde and maintained thereafter in 70% ethanol at room temperature for processing. Samples were submitted to UCSD Histology Core Facility for sectioning and hematoxylin and eosin staining. Sections were examined, interpreted and photographed using a Nikon Eclipse TS100 microscope equipped with a Nikon DS-Fi1 camera.

STATISTICAL ANALYSIS

Student's t test was used for statistical evaluations pertaining to standard deviation. StepOne Software v2.2 (Life Technologies) was used to determine RQ minimum and maximum values (error bars) pertaining to qRTPCR data.

In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims, but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

CLAIMS

1. A composition comprising:

(a) a Wnt pathway agonist;

(b) a MEK inhibitor; and

(c) a ROCK inhibitor,

wherein the composition does not comprise a TGFβR inhibitor.

2. The composition of claim 1, wherein the Wnt pathway agonist is a GSK3 inhibitor.

3. The composition of claim 2, wherein the GSK3 inhibitor is CHIR99021 or BIO.

4. The composition of claim 1, wherein the MEK inhibitor is PD98059 or PD032901.

5. The composition of claim 1, wherein the ROCK inhibitor is thiazovivin or Y27632.

6. The composition of claim 1, wherein the GSK3 inhibitor is CHIR99021; the MEK inhibitor is PD032901; and the ROCK inhibitor is thiazovivin.

7. The composition according to any one of claims 1 to 6, further comprising bFGF or LIF.

8. The composition according to any one of claims 1 to 6, further comprising bFGF and LIF.

9. A culture medium comprising the composition of claim 1,
wherein the medium does not comprise a TGFβR inhibitor.

10. A method of culturing one or more pluripotent cells comprising culturing the one or more pluripotent cells in a cell culture medium according to claim 9.

11. The method of claim 10, wherein the one or more pluripotent cells are embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs).

12. The method of claim 10, wherein the one or more pluripotent cells are iPSCs.

13. The method of claim 10, wherein the composition comprises a population of pluripotent cells.

14. The method of claim 13, wherein the population of pluripotent cells is a homogenous population of pluripotent cells.

15. The method of claim 13, wherein at least 95% of the population of pluripotent cells expresses SSEA4-FITC and TRA1-81 or TRA1-60.

16. The method of claim 13, wherein at most 5% of the population of pluripotent cells expresses α-smooth muscle actin (SMA), TUJ1, or FoxA2.

17. The method of claim 10, wherein the pluripotent cells were previously cultured in a cell culture medium comprising a TGFβR inhibitor.

18. The method of claim 13, wherein culturing the pluripotent cells in the cell culture medium reduces spontaneous differentiation of the cultured cells.

19. The method of claim 18, wherein expression of one or more differentiation marker genes in the cultured cells is decreased by at least about 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% compared to the expression of the one or more differentiation marker genes in a pluripotent cell cultured in a medium comprising a TGFβR inhibitor, wherein the differentiation marker genes are selected from the group consisting of: FOXA2, FGF5, SOX17, XIST, NODAL, COL3A1, OTX2, DUSP6, EOMES, NR2F2, NR0B1, CXCR4, CYP2B6, GATA3, GATA4, ERBB4, GATA6, HOXC6, INHA, SMAD6, RORA, NIPBL, TNFSF11, CDH11, ZIC4, GAL, SOX3, PITX2, APOA2, CXCL5, CER1, FOXQ1, MLL5, DPP10, GSC, PCDH10, CTCFL, PCDH20, TSHZ1, MEGF10, MYC, DKK1, BMP2,

LEFTY2, HES1, CDX2, GNAS, EGR1, COL3A1, TCF4, HEPH, KDR, TOX, FOXA1, LCK, PCDH7, CD1D FOXG1, LEFTY1, TUJ1, T gene (Brachyury) and ZIC1.

20. The method of claim 19, wherein the one or more differentiation marker genes is selected from the group consisting of T gene, CXCR4, NODAL, GATA4, SOX17, FOXA2, OTX2, and TUJ1.

21. The method of claim 18, wherein expression of two or more differentiation marker genes is decreased in the cultured cells by at least about 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% compared to the expression of the two or more differentiation marker genes in a pluripotent cell cultured in a medium comprising a TGFβR inhibitor, wherein the differentiation marker genes are selected from the group consisting of: FOXA2, FGF5, SOX17, XIST, NODAL, COL3A1, OTX2, DUSP6, EOMES, NR2F2, NR0B1, CXCR4, CYP2B6, GATA3, GATA4, ERBB4, GATA6, HOXC6, INHA, SMAD6, RORA, NIPBL, TNFSF11, CDH11, ZIC4, GAL, SOX3, PITX2, APOA2, CXCL5, CER1, FOXQ1, MLL5, DPP10, GSC, PCDH10, CTCFL, PCDH20, TSHZ1, MEGF10, MYC, DKK1, BMP2, LEFTY2, HES1, CDX2, GNAS, EGR1, COL3A1, TCF4, HEPH, KDR, TOX, FOXA1, LCK, PCDH7, CD1D FOXG1, LEFTY1, TUJ1, T gene (Brachyury) and ZIC1.

22. The method of claim 21, wherein the two or more differentiation marker genes are selected from the group consisting of T gene, CXCR4, NODAL, GATA4, SOX17, FOXA2, OTX2, and TUJ1.

23. The method of claim 18, wherein expression of three or more differentiation marker genes is decreased in the cultured cells by at least about 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% compared to the expression of the three or more differentiation marker genes in a pluripotent cell cultured in a medium comprising a TGFβR inhibitor, wherein the differentiation marker genes are selected from the group consisting of: FOXA2, FGF5, SOX17, XIST, NODAL, COL3A1, OTX2, DUSP6, EOMES, NR2F2, NR0B1, CXCR4, CYP2B6, GATA3, GATA4, ERBB4, GATA6, HOXC6, INHA, SMAD6, RORA, NIPBL, TNFSF11, CDH11, ZIC4, GAL, SOX3, PITX2, APOA2, CXCL5, CER1, FOXQ1, MLL5, DPP10, GSC, PCDH10, CTCFL, PCDH20, TSHZ1, MEGF10, MYC, DKK1, BMP2, LEFTY2, HES1, CDX2, GNAS, EGR1, COL3A1, TCF4, HEPH, KDR,

TOX, FOXA1, LCK, PCDH7, CD1D FOXG1, LEFTY1, TUJ1, T gene (Brachyury) and ZIC1.

24. The method of claim 23, wherein the three or more differentiation marker genes are selected from the group consisting of T gene, CXCR4, NODAL, GATA4, SOX17, FOXA2, OTX2, and TUJ1.

25. The method of claim 18, wherein expression of five or more differentiation marker genes is decreased in the cultured cells by at least about 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% compared to the expression of the five or more differentiation marker genes in a pluripotent cell cultured in a medium comprising a TGFβR inhibitor, wherein the differentiation marker genes are selected from the group consisting of: FOXA2, FGF5, SOX17, XIST, NODAL, COL3A1, OTX2, DUSP6, EOMES, NR2F2, NR0B1, CXCR4, CYP2B6, GATA3, GATA4, ERBB4, GATA6, HOXC6, INHA, SMAD6, RORA, NIPBL, TNFSF11, CDH11, ZIC4, GAL, SOX3, PITX2, APOA2, CXCL5, CER1, FOXQ1, MLL5, DPP10, GSC, PCDH10, CTCFL, PCDH20, TSHZ1, MEGF10, MYC, DKK1, BMP2, LEFTY2, HES1, CDX2, GNAS, EGR1, COL3A1, TCF4, HEPH, KDR, TOX, FOXA1, LCK, PCDH7, CD1D FOXG1, LEFTY1, TUJ1, T gene (Brachyury) and ZIC1.

26. The method of claim 25, wherein the five or more differentiation marker genes are selected from the group consisting of T gene, CXCR4, NODAL, GATA4, SOX17, FOXA2, OTX2, and TUJ1.

27. The method of claim 18, wherein culturing the pluripotent cells in the cell culture medium maintains or induces a ground state of pluripotency.

28. The method of claim 27, wherein the ground state of pluripotency of the one or more pluripotent cells is maintained for at least 5 passages.

29. The method of claim 27, wherein the ground state of pluripotency of the one or more pluripotent cells is maintained for at least 10 passages.

30. The method of claim 27, wherein the ground state of pluripotency of the one or more pluripotent cells is maintained for at least 50 passages.

31. The method of claim 27, wherein the ground state of pluripotency of the one or more pluripotent cells is maintained for at least 100 passages.

32. The method of any one of claims 10 to 31, further comprising dissociating the one or more pluripotent cells during passaging.

33. The method of claim 32, wherein the viability of the one or more pluripotent cells is maintained during passaging.

34. The method of any one of claims 10 to 33, wherein the one or more pluripotent cells comprise a normal karyotype.

35. The method of any one of claims 10 to 34, wherein the one or more pluripotent cells are cultured in a feeder free environment.

36. The method of any one of claims 10 to 35, wherein the genomic stability of the one or more pluripotent cells is maintained for at least 10 passages.

37. The method of any one of claims 10 to 35, wherein the genomic stability of the one or more pluripotent cells is maintained for at least 50 passages.

38. The method of any one of claims 10 to 35, wherein the genomic stability of the one or more pluripotent cells is maintained for at least 100 passages.

39. A method of adapting pluripotent cells to a feeder-free culture comprising:

(a) isolating one or more pluripotent cells that are cultured in the presence of feeder cells;

(b) culturing the one or more pluripotent cell in a chemically defined cell culture medium comprising: a Wnt pathway agonist, optionally wherein the Wnt pathway agonist is a GSK3 inhibitor; a MEK inhibitor; and a ROCK inhibitor, wherein the medium does not comprise a TGFβR inhibitor.

40. A method of culturing pluripotent cells enzymatically passaged as single cells comprising:

(a) enzymatically treating one or more pluripotent cells to passage a single pluripotent cell;

(b) culturing the single pluripotent cell in a feeder free environment;

(c) culturing the single pluripotent cell in a chemically defined cell culture medium comprising: a Wnt pathway agonist, optionally wherein the Wnt pathway agonist is a GSK3 inhibitor; a MEK inhibitor; and a ROCK inhibitor, wherein the medium does not comprise a TGFβR inhibitor.

41. A method of reducing spontaneous differentiation of one or more pluripotent cells comprising:

(a) culturing the one or more pluripotent cells in a feeder free environment;

(b) culturing the one or more pluripotent cells in a chemically defined cell culture medium comprising: a Wnt pathway agonist, optionally wherein the Wnt pathway agonist is a GSK3 inhibitor; a MEK inhibitor; and a ROCK inhibitor, wherein the medium does not comprise a TGFβR inhibitor.

42. A method of manufacturing induced pluripotent stem cells (iPSCs) comprising:

(a) obtaining one or more non-pluripotent cells;

(b) reprogramming the one or more non-pluripotent cells to a pluripotent state;

(c) culturing the pluripotent cells in a cell culture medium that does not comprise a TGFβR inhibitor thereby producing iPSCs.

43. The method of claim 42, wherein the one or more non-pluripotent cells comprises a somatic cell.

44. The method of claim 42, wherein the one or more non-pluripotent cells comprises an adult stem cell.

45. The method of claim 42, wherein the one or more non-pluripotent cells is reprogrammed to a pluripotent state by increasing the expression of endogenous OCT4 in the cell.

46. The method of claim 42, wherein reprogramming the one or more non-pluripotent cells to the pluripotent state comprises introducing one or more polynucleotides encoding one or more reprogramming factors selected from the group consisting of: OCT4, SOX2, NANOG, KLF4, LIN28, C-MYC, ECAT1, UTF1, ESRRB, and SV40LT into the one or more non-pluripotent cells.

47. The method of claim 42, wherein reprogramming the one or more non-pluripotent cells to the pluripotent state comprises introducing one or more polynucleotides encoding one or more copies of reprogramming factors selected from the group consisting of: OCT4, SOX2, NANOG, KLF4, LIN28, C-MYC, ECAT1, UTF1, ESRRB, and SV40LT into the one or more non-pluripotent cells.

48. The method of claim 42, wherein reprogramming the one or more non-pluripotent cells to the pluripotent state comprises introducing one or more polynucleotides encoding one or more copies of reprogramming factors selected from the group consisting of: OCT4, SOX2, and NANOG into the one or more non-pluripotent cells.

49. The method of claim 42, wherein reprogramming the one or more non-pluripotent cells to the pluripotent state comprises introducing one or more polynucleotides encoding one or more copies of reprogramming factors selected from the group consisting of: OCT4, NANOG, ECAT1, UTF1, and ESRRB into the one or more non-pluripotent cells.

50. The method of claim 42, wherein reprogramming the one or more non-pluripotent cells to the pluripotent state comprises introducing one or more polynucleotides encoding one or more copies of reprogramming factors selected from the group consisting of: OCT4, ECAT1, and UTF1 into the one or more non-pluripotent cells.

51. The method of any one of claims 46 to 50, wherein the one or more polynucleotides is a lentiviral vector.

52. The method of any one of claims 46 to 50, wherein the one or more polynucleotides is an episomal vector.

53. The method of any one of claims 42-52, wherein the cell culture medium comprises a Wnt pathway agonist, optionally wherein the Wnt pathway agonist is a GSK3 inhibitor; a MEK inhibitor; and a ROCK inhibitor.

54. The method of any one of claims 42 to 53, wherein reprogramming the one or more non-pluripotent cells comprises contacting the one or more non-pluripotent cells with a Wnt pathway agonist, optionally wherein the Wnt pathway agonist is a GSK3 inhibitor; a MEK inhibitor; and a TGFβR inhibitor, and optionally a ROCK inhibitor.

55. The method of claim 42, wherein the iPSCs comprise a population of iPSCs.

56. The method of claim 55, wherein the population of iPSCs is a homogenous population of iPSCs.

57. The method of claim 55, wherein at least 95% of the population of iPSCs expresses SSEA4 and TRA1-81 or TRA1-60.

58. The method of claim 55, wherein at most 5% of the population of pluripotent cells expresses α-smooth muscle actin (SMA), TUJ1, or FoxA2.

59. The method of any one of claims 42-58, wherein culturing the pluripotent cells in the cell culture medium reduces spontaneous differentiation, or maintains or induces a ground state of pluripotency.

60. The method of claim 59, wherein expression of one or more, two or more, three or more, four or more, or five or more differentiation marker genes is decreased in the iPSCs by at least about 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% compared to the expression of the one or more differentiation marker genes in iPSCs cultured in a medium comprising a TGFβR inhibitor, wherein the differentiation marker genes are selected from the group consisting of: FOXA2, FGF5, SOX17, XIST, NODAL, COL3A1, OTX2,

DUSP6, EOMES, NR2F2, NR0B1, CXCR4, CYP2B6, GATA3, GATA4, ERBB4, GATA6, HOXC6, INHA, SMAD6, RORA, NIPBL, TNFSF11, CDH11, ZIC4, GAL, SOX3, PITX2, APOA2, CXCL5, CER1, FOXQ1, MLL5, DPP10, GSC, PCDH10, CTCFL, PCDH20, TSHZ1, MEGF10, MYC, DKK1, BMP2, LEFTY2, HES1, CDX2, GNAS, EGR1, COL3A1, TCF4, HEPH, KDR, TOX, FOXA1, LCK, PCDH7, CD1D FOXG1, LEFTY1, TUJ1, T gene (Brachyury) and ZIC1.

61. The method of claim 60, wherein the one or more differentiation marker genes is selected from the group consisting of T gene, CXCR4, NODAL, GATA4, SOX17, FOXA2, OTX2, and TUJ1.

62. The method of claim 60, wherein the reduction in spontaneous differentiation is maintained for at least 5 passages.

63. The method of claim 60, wherein the reduction in spontaneous differentiation is maintained for at least 10 passages.

64. The method of claim 60, wherein the reduction in spontaneous differentiation is maintained for at least 50 passages.

65. The method of claim 60, wherein the reduction in spontaneous differentiation is maintained for at least 100 passages.

66. The method of any one of claims 42 to 65, comprising dissociating the iPSCs during passaging.

67. The method of claim 66, wherein the viability of the iPSCs is maintained during passaging.

68. The method of any one of claims 42 to 67, wherein the iPSCs comprise a normal karyotype.

69. The method of any one of claims 42 to 68, wherein the iPSCs are cultured in a feeder free environment.

70. The method of any one of claims 42 to 69, wherein the genomic stability of the iPSCs is maintained for at least 10 passages.

71. The method of any one of claims 42 to 69, wherein the genomic stability of the iPSCs is maintained for at least 50 passages.

72. The method of any one of claims 42 to 69, wherein the genomic stability of the iPSCs is maintained for at least 100 passages.

73. An induced pluripotent stem cell (iPSC) comprising ground state pluripotency produced according to any one of claims 42 to 72.

74. An induced pluripotent stem cell (iPSC) comprising ground state pluripotency, wherein the iPSC does not comprise an exogenously introduced polynucleotide encoding a reprogramming factor polypeptide.

75. A method of manufacturing induced pluripotent stem cells (iPSCs) comprising:

a) obtaining one or more pluripotent stem cells;

(b) culturing the one or more pluripotent stem cells in a cell culture medium that does not comprise a TGFβR inhibitor thereby producing ground state iPSCs.

76. The method of claim 75, wherein the one or more iPSCs comprises a reprogrammed somatic cell.

77. The method of claim 75, wherein the one or more iPSCs comprises a reprogrammed adult stem cell.

78. The method of claim 75, wherein the one or more iPSCs were reprogrammed to a pluripotent state by increasing the expression of endogenous OCT4 in the one or more iPSCs.

79. The method of claim 75, wherein the one or more iPSCs were reprogrammed by introducing one or more polynucleotides encoding one or more reprogramming factors

selected from the group consisting of: OCT4, SOX2, NANOG, KLF4, LIN28, C-MYC, ECAT1, UTF1, ESRRB, and SV40LT into the one or more non-pluripotent cells.

80. The method of claim 75, wherein the one or more iPSCs were reprogrammed by introducing one or more polynucleotides encoding one or more copies of reprogramming factors selected from the group consisting of: OCT4, SOX2, NANOG, KLF4, LIN28, C-MYC, ECAT1, UTF1, ESRRB, and SV40LT.

81. The method of claim 75, wherein the one or more iPSCs were reprogrammed by introducing one or more polynucleotides encoding one or more copies of reprogramming factors selected from the group consisting of: OCT4, SOX2, and NANOG into the one or more non-pluripotent cells.

82. The method of claim 75, wherein wherein the one or more iPSCs were reprogrammed by introducing one or more polynucleotides encoding one or more copies of reprogramming factors selected from the group consisting of: OCT4, NANOG, ECAT1, UTF1, and ESRRB into the one or more non-pluripotent cells.

83. The method of claim 75, wherein the one or more iPSCs were reprogrammed by introducing one or more polynucleotides encoding one or more copies of reprogramming factors selected from the group consisting of: OCT4, ECAT1, and UTF1 into the one or more non-pluripotent cells.

84. The method of any one of claims 79 to 83, wherein a lentiviral vector comprises the one or more polynucleotides.

85. The method of any one of claims 79 to 83, wherein an episomal vector comprises the one or more polynucleotides.

86. The method of claim 75, wherein the cell culture medium comprises a Wnt pathway agonist, optionally wherein the Wnt pathway agonist is a GSK3 inhibitor; a MEK inhibitor; and a ROCK inhibitor.

87. The method of any one of claims 75 to 86, wherein obtaining the one or more iPSCs comprises contacting the one or more non-pluripotent cells or partially pluripotent cells with a Wnt pathway agonist, optionally wherein the Wnt pathway agonist is a GSK3 inhibitor; a MEK inhibitor; and a TGFβR inhibitor, and optionally a ROCK inhibitor to produce the one or more iPSCs.

88. The method of claim 75, wherein the iPSCs comprise a population of iPSCs.

89. The method of claim 88, wherein the population of iPSCs is a homogenous population of iPSCs.

90. The method of claim 88, wherein at least 95% of the population of iPSCs expresses SSEA4 and TRA1-81 or TRA1-60.

91. The method of claim 88, wherein the one or more iPSCs are obtained by reprogramming a population of pluripotent cells, wherein at most 5% of the population of pluripotent cells expresses α-smooth muscle actin (SMA), TUJ1, or FoxA2.

92. The method of claim 75, wherein culturing the one or more iPSCs in the cell culture medium reduces spontaneous differentiation or maintains or induces a ground state of pluripotency.

93. The method of claim 92, wherein the iPSCs with reduced spontaneous differentiation comprise a gene expression wherein expression of one or more, two or more, three or more, four or more, or five or more differentiation marker genes is decreased in the iPSCs by at least about 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% compared to the expression of the one or more differentiation marker genes in iPSCs cultured in a medium comprising a TGFβR inhibitor, wherein the differentiation marker genes are selected from the group consisting of: FOXA2, FGF5, SOX17, XIST, NODAL, COL3A1, OTX2, DUSP6, EOMES, NR2F2, NR0B1, CXCR4, CYP2B6, GATA3, GATA4, ERBB4, GATA6, HOXC6, INHA, SMAD6, RORA, NIPBL, TNFSF11, CDH11, ZIC4, GAL, SOX3, PITX2, APOA2, CXCL5, CER1, FOXQ1, MLL5, DPP10, GSC, PCDH10, CTCFL, PCDH20, TSHZ1, MEGF10, MYC, DKK1, BMP2, LEFTY2, HES1, CDX2, GNAS, EGR1, COL3A1,

TCF4, HEPH, KDR, TOX, FOXA1, LCK, PCDH7, CD1D FOXG1, LEFTY1, TUJ1, T gene (Brachyury) and ZIC1.

94. The method of claim 93, wherein the one or more differentiation marker genes is selected from the group consisting of T gene, CXCR4, NODAL, GATA4, SOX17, FOXA2, OTX2, and TUJ1.

95. The method of claim 93, wherein the reduced spontaneous differentiation is maintained for at least 5 passages.

96. The method of claim 93, wherein the reduced spontaneous differentiation is maintained for at least 10 passages.

97. The method of claim 93, wherein the reduced spontaneous differentiation is maintained for at least 50 passages.

98. The method of claim 93, wherein the reduced spontaneous differentiation is maintained for at least 100 passages.

99. The method of any one of claims 75 to 98, comprising dissociating the one or more iPSCs during passaging.

100. The method of claim 90, wherein the viability of the one or more iPSCs are maintained during passaging.

101. The method of any one of claims 75 to 100, wherein the one or more iPSCs comprise a normal karyotype.

102. The method of any one of claims 75 to 101, wherein the one or more iPSCs are cultured in a feeder free environment.

103. The method of any one of claims 75 to 102, wherein the genomic stability of the one or more iPSCs is maintained for at least 10 passages.

104. The method of any one of claims 75 to 102, wherein the genomic stability of the one or more iPSCs is maintained for at least 50 passages.

105. The method of any one of claims 75 to 102, wherein the genomic stability of the one or more iPSCs is maintained for at least 100 passages.

106. An induced pluripotent stem cell (iPSC) comprising ground state pluripotency produced according to any one of claims 75 to 105.

107. An induced pluripotent stem cell (iPSC) comprising ground state pluripotency, wherein the iPSC does not comprise an exogenously introduced polynucleotide encoding a reprogramming factor polypeptide.

108. A method for reprogramming a non-pluripotent cell to a pluripotent cell comprising introducing into the nonpluripotent cell (i) one or more polynucleotides that encode at least one polypeptide selected from the group consisting of an OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, and an ESRRB polypeptide, or (ii) at least one polypeptide selected from the group consisting of an OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, and an ESRRB polypeptide, thereby reprogramming the non-pluripotent cell to a pluripotent cell.

109. The method of claim 108, wherein introducing comprises (i) introducing one or more polynucleotides that encode an OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, and an ESRRB polypeptide, or (ii) introducing a OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, and an ESRRB polypeptide.

110. The method of claim 108, wherein introducing comprises (i) introducing one or more polynucleotides that encode an OCT4 polypeptide, an ECAT1 polypeptide, and a UTF1 polypeptide, or (ii) introducing an OCT-4 polypeptide, an ECAT1 polypeptide, and a UTF1 polypeptide.

111. The method of any one of claims 108-110, wherein the one or more polynucleotides are introduced by a retrovirus, Sendai virus, an adenovirus, an episome, mini-circle, vector system with expression cassette, or mRNA.

112. The method of claim 111, wherein the retrovirus is a lentivirus.

113. The method of claim 111, wherein the pluripotent cell is free of exogenous polynucleotides.

114. The method of claim 113, wherein the one or more polynucleotides is excised by CRE-mediated excision.

115. The method of any one of claims 108-114, further comprising introducing into the non-pluripotent cell (i) a polynucleotide that encodes a SV40LT polypeptide, or (ii) a SV40LT polypeptide.

116. The method of any one of claims 108-115, further comprising contacting the nonpluripotent cell with at least one of a TGFβR inhibitor, a Wnt pathway agonist, a MEK inhibitor and a ROCK inhibitor, wherein the Wnt pathway agonist is optionally a GSK3 inhibitor.

117. The method of claim 116, wherein the nonpluripotent cell is contacted with a TGFβR inhibitor, a Wnt pathway agonist, a MEK inhibitor and a ROCK inhibitor, wherein the Wnt pathway agonist is optionally a GSK3 inhibitor.

118. The method of any one of claims 108-117, further comprising culturing the pluripotent cell in a culture medium comprising a Wnt pathway agonist, a MEK inhibitor and a ROCK inhibitor, , wherein the Wnt pathway agonist is optionally a GSK3 inhibitor, and wherein the culture medium does not contain TGFβR inhibitor.

119. The method of any one of claims 116-118, wherein (a) the Rock inhibitor is thiazovivin or Y27632, (b) the TGFβR inhibitor is A-83-01 or SB431542, (c) the GSK3 inhibitor is CHIR99021 or BIO, or (d) the MEK inhibitor is PD98059 or PD032901.

120. The method of claim 118 or 119, wherein pluripotency of the pluripotent cell is maintained for at least 5 cell divisions or at least 10 cell divisions.

121. The method of any one of claims 108-120, wherein the one or more polynucleotides is introduced as a polycistronic vector comprising a plurality of polynucleotides that are separated by at least one 2A peptide.

122. The method of claim 121, wherein the polycistronic vector comprises a plurality of polynucleotides that encode an Oct4 polypeptide.

123. The method of any one of claims 108-122, further comprising identifying the pluripotent cell by selecting for Oct34 expression in the pluripotent cell.

124. The method of claim 123, wherein selecting for Oct4 expression comprises selecting for ectopic Oct4 expression.

125. The method of any one of claims 118-124, wherein culturing produces a population of pluripotent stem cells.

126. The method of claim 125, wherein the population of pluripotent stem cells are at least 70% homogenous, at least 80% homogenous, or at least 90% homogenous.

127. The method of claim 125 or 126, wherein at least 70%, at least 80%, or at least 90% of the population of pluripotent cells expresses SSEA and Tra-181.

128. The method of any one of claims 108-127, wherein the pluripotent cell or population of pluripotent cells are capable of single cell passaging.

129. The method of claim 128, wherein the cells produced by single cell passaging have a normal karyotype.

130. A pluripotent cell produced according to the method of any one of claims 108-129.

131. A composition comprising an isolated non-pluripotent cell that comprises (i) one or more exogenous polynucleotides that encode at least one polypeptide selected from the group consisting of an OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, and an ESRRB polypeptide, or (ii) at least one exogenous polypeptide selected from an OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, and an ESRRB polypeptide.

132. The composition of claim 131, wherein the cell comprises (i) one or more exogenous polynucleotides that encode at least two of an OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, and an ESRRB polypeptide, or (ii) at least two exogenous polypeptides selected from an OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, and an ESRRB polypeptide.

133. The composition of claim 131, wherein the cell comprises (i) one or more exogenous polynucleotides that encode at least three of an OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, and an ESRRB polypeptide, or (ii) at least three exogenous polypeptides selected from an OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, and an ESRRB polypeptide.

134. The composition of claim 131, wherein the cell comprises (i) one or more exogenous polynucleotides that encode an OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, and an ESRRB polypeptide, or (ii) an exogenous OCT4 polypeptide, an exogenous ECAT1 polypeptide, an exogenous UTF1 polypeptide, an exogenous NANOG polypeptide, and an exogenous ESRRB polypeptide.

135. The composition of claim 131, wherein the cell comprises (i) one or more exogenous polynucleotides that encode an OCT4 polypeptide, an ECAT1 polypeptide, and a UTF1 polypeptide, or (ii) an exogenous OCT4 polypeptide, an exogenous ECAT1 polypeptide, and an exogenous UTF1 polypeptide.

136. The composition of any one of claims 121-135, wherein the cell has been contacted with at least one of a TGFβR inhibitor, a GSK3 inhibitor, a MEK inhibitor and a ROCK inhibitor.

137. The composition of claim 136, wherein the cell has been contacted with a TGFβR inhibitor, a Wnt pathway activator, a MEK inhibitor and a ROCK inhibitor, wherein the Wnt pathway activator is optionally a GSK3 inhibitor.

138. The composition of claim 136 or 137, wherein (a) the Rock inhibitor is thiazovivin or Y27632, (b) the TGFβR inhibitor is A-83-01 or SB431542, (c) the GSK3 inhibitor is CHIR99021 or BIO, or (d) the MEK inhibitor is PD98059 or PD032901.

139. The composition of any one of claims 131-138, wherein the one or more exogenous polynucleotides are introduced to the non-pluripotent cell by a retrovirus, Sendai virus, an adenovirus, an episome, mini-circle, vector system with expression cassette, or mRNA.

140. The composition of claim 139, wherein the retrovirus is a lentivirus.

141. The composition of claim 139, wherein the cell is free of exogenous polynucleotides.

142. The method of claim 141, wherein the one or more exogenous polynucleotides are removed by CRE-mediated excision.

143. The composition of any one of claims 131-142, wherein the cell further comprises (i) an exogenous polynucleotide that encodes a SV40LT antigen polypeptide, or (ii) an exogenous a SV40LT antigen polypeptide.

144. The composition of any one of claims 131-143, wherein the one or more exogenous polynucleotides is introduced as a polycistronic vector comprising a plurality of polynucleotides that are separated by at least one 2A peptide.

145. The composition of claim 144, wherein the polycistronic vector comprises a plurality of polynucleotides that encode an OCT4 polypeptide.

146. The composition of any one of claims 131-145, wherein the exogenous polynucleotide encoding an OCT4 polypeptide is linked to a selectable marker.

147. A composition consisting of at least one of, at least two of, or at least three of (i) a cDNA encoding an OCT4 polypeptide, (ii) a cDNA encoding an ECAT1 polypeptide, (iii) a cDNA encoding a UTF1 polypeptide, (iv) a cDNA encoding a NANOG polypeptide, and (v) a cDNA encoding an ESRRB polypeptide.

148. The composition of claim 147, wherein the composition consists of a cDNA encoding an OCT4 polypeptide, a cDNA encoding an ECAT1 polypeptide, a cDNA encoding a UTF1 polypeptide, a cDNA encoding a NANOG polypeptide, and a cDNA encoding an ESRRB polypeptide.

149. The composition of claim 148, wherein the composition consists of a cDNA encoding an OCT4 polypeptide, a cDNA encoding an ECAT1 polypeptide, and a cDNA encoding a UTF1 polypeptide.

150. The composition of any one of claims 147-149, wherein each cDNA is encoded in a retrovirus, Sendai virus, an adenovirus, an episome, a mini-circle, a vector system with expression cassette, or mRNA.

151. A vector comprising of one or more polynucleotides that encode at least one reprogramming factor polypeptide selected from the group consisting of an OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, and an ESRRB polypeptide.

152. The vector of claim 151, wherein the one or more polynucleotides encode an OCT polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, and an ESRRB polypeptide.

153. The vector of claim 152, wherein the one or more polynucleotides encode an OCT polypeptide, an ECAT1 polypeptide, and a UTF1 polypeptide.

154. The vector of any one of claims 151-153, wherein the vector further comprises a polynucleotide that encodes an SV40LT antigen polypeptide.

155. The vector of any one of claims 151-154, wherein the vector is a retrovirus, Sendai virus, an adenovirus, an episome, mini-circle, vector system with expression cassette, or mRNA.

156. The vector of claim 155, wherein the retrovirus is a lentivirus.

157. A kit for reprogramming a non-pluripotent cell to a pluripotent cell, the kit comprising:

(a)one or more polynucleotides that encode at least one polypeptide selected from the group consisting of an OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, or an ESRRB polypeptide; or

(b) at least one polypeptide selected from the group consisting of an OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, or an ESRRB polypeptide; and

(c)at least one of a TGFβR inhibitor, a Wnt pathway activator, a MEK inhibitor and a ROCK inhibitor, wherein the Wnt pathway activator is optionally a GSK3 inhibitor.

158. The kit of claim 157, wherein (i) the one or more polynucleotides encode an OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, and an ESRRB polypeptide, or (ii) the at least one polypeptide comprises an OCT4 polypeptide, an ECAT1 polypeptide, a UTF1 polypeptide, a NANOG polypeptide, and an ESRRB polypeptide.

159. The kit of claim 157, wherein (i) the one or more one or more polynucleotides encode an OCT4 polypeptide, an ECAT1 polypeptide, and a UTF1 polypeptide, or (ii) the at least one polypeptide comprises an OCT4 polypeptide, an ECAT1 polypeptide, and a UTF1 polypeptide.

160. The kit of any one of claims 157-159, wherein the kit further comprises (i) a polynucleotide that encodes a SV40LT antigen polypeptide, or (ii) a SV40LT antigen polypeptide.

161. The kit of any one of claims 157-160, wherein the kit comprises a TGFβR inhibitor, a Wnt pathway activator, a MEK inhibitor and a ROCK inhibitor, wherein the Wnt pathway activator is optionally a GSK3 inhibitor.

162. The kit of any one of claims 157-161, wherein (a) the Rock inhibitor is thiazovivin or Y27632, (b) the TGFβR inhibitor is A-83-01 or SB431542, (c) the GSK3 inhibitor is CHIR99021 or BIO, or (d) the MEK inhibitor is PD98059 or PD032901.

163. The kit of any one of claims 157-162, wherein the at least one polynucleotide is encoded in a retrovirus, Sendai virus, an adenovirus, an episome, a mini-circle, a vector system with expression cassette, or mRNA.

164. The kit of claim 163, wherein the retrovirus is a lentivirus.

165. The kit of any one of claims 157-164, wherein the at least one polynucleotide is encoded in a polycistronic vector with each polynucleotide being separated by a 2A peptide.

166. The kit of claim 165, wherein the polycistronic vector comprises two or more polynucleotides that encode an OCT4 polypeptide.

167. The kit of any one of claims 157-166, wherein the at least one polynucleotide that encodes an OCT4 polypeptide is linked to a selectable marker.

168. A method for producing a population of pluripotent stem cells comprising:

a) providing a population of non-pluripotent cells;

b) introducing into the population of non-pluripotent cells a polynucleotide that encodes an OCT4 polypeptide that is linked to a selectable marker;

c) incubating the population of non-pluripotent cells with the polynucleotide under conditions sufficient to reprogram at least a portion of the population of non-pluripotent cells to pluripotent cells; and

d) selecting cells that express the selectable marker thereby providing a population of pluripotent stem cells.

169. The method of claim 168, wherein the polynucleotide is introduced as a polycistronic vector comprising a plurality of polynucleotides that encode an OCT4 polypeptide.

170. The method of claim 169, wherein the plurality of polynucleotides are separated by a 2A peptide.

171. The method of claim 169, wherein at least 10 % of the cells in the population of cells express SSEA and TRA-181.

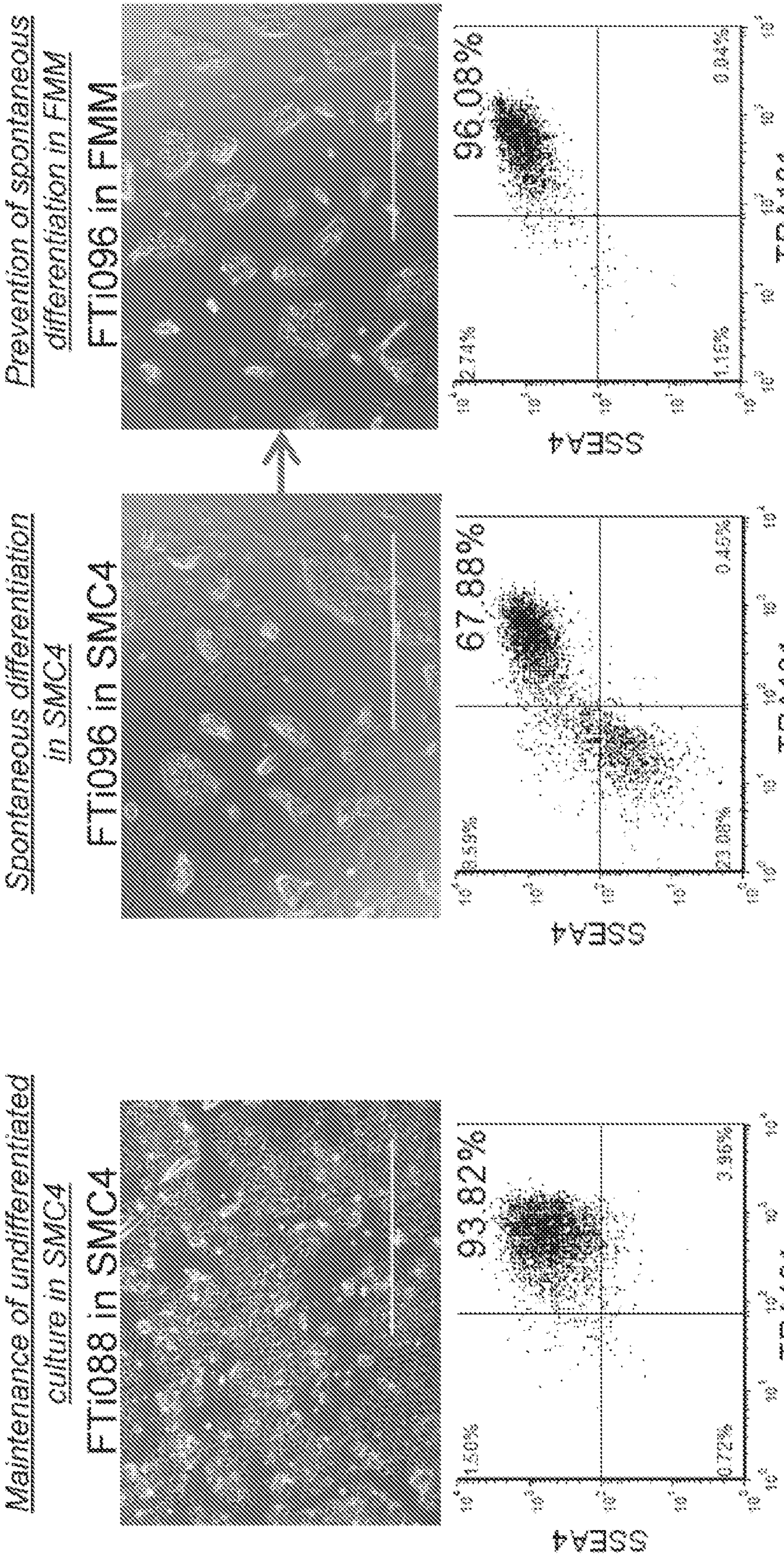

Maintenance of undifferentiated culture in SMC4
FTi088 in SMC4
93.82%
1.50%
0.72%
3.96%
SSEA4
TRA181
Spontaneous differentiation in SMC4
FTi096 in SMC4
67.88%
3.55%
23.08%
0.45%
SSEA4
TRA181
Prevention of spontaneous differentiation in FMM
FTi096 in FMM
96.08%
2.74%
1.16%
0.04%
SSEA4
TRA181

FIG. 1A

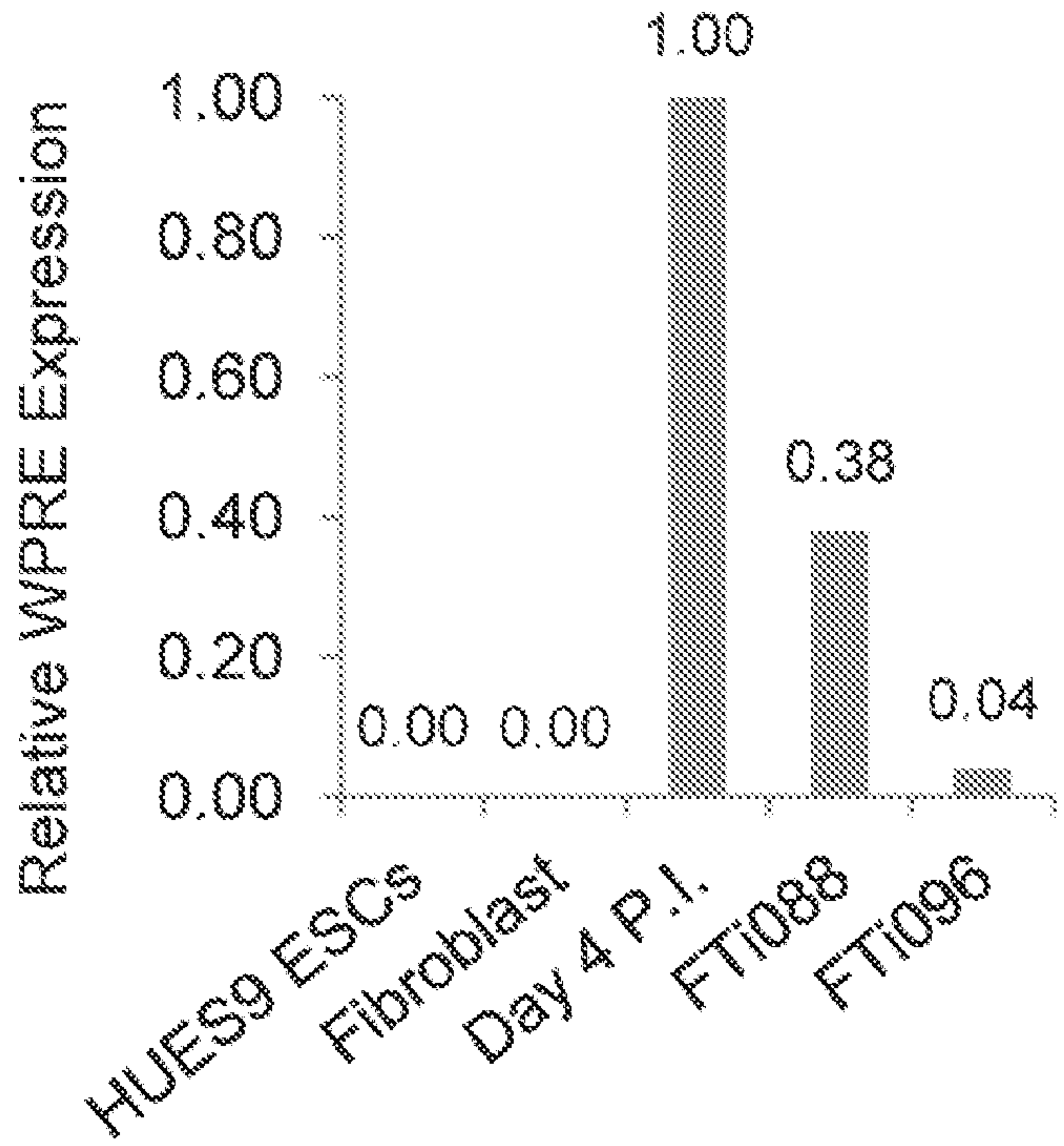

Relative WPRE Expression
1.00
0.80
0.60
0.40
0.20
0.00
1.00
0.38
0.04
0.00
0.00
HUES9 ESCs
Fibroblast
Day 4 P.I.
FTi088
FTi096

FIG. 1B

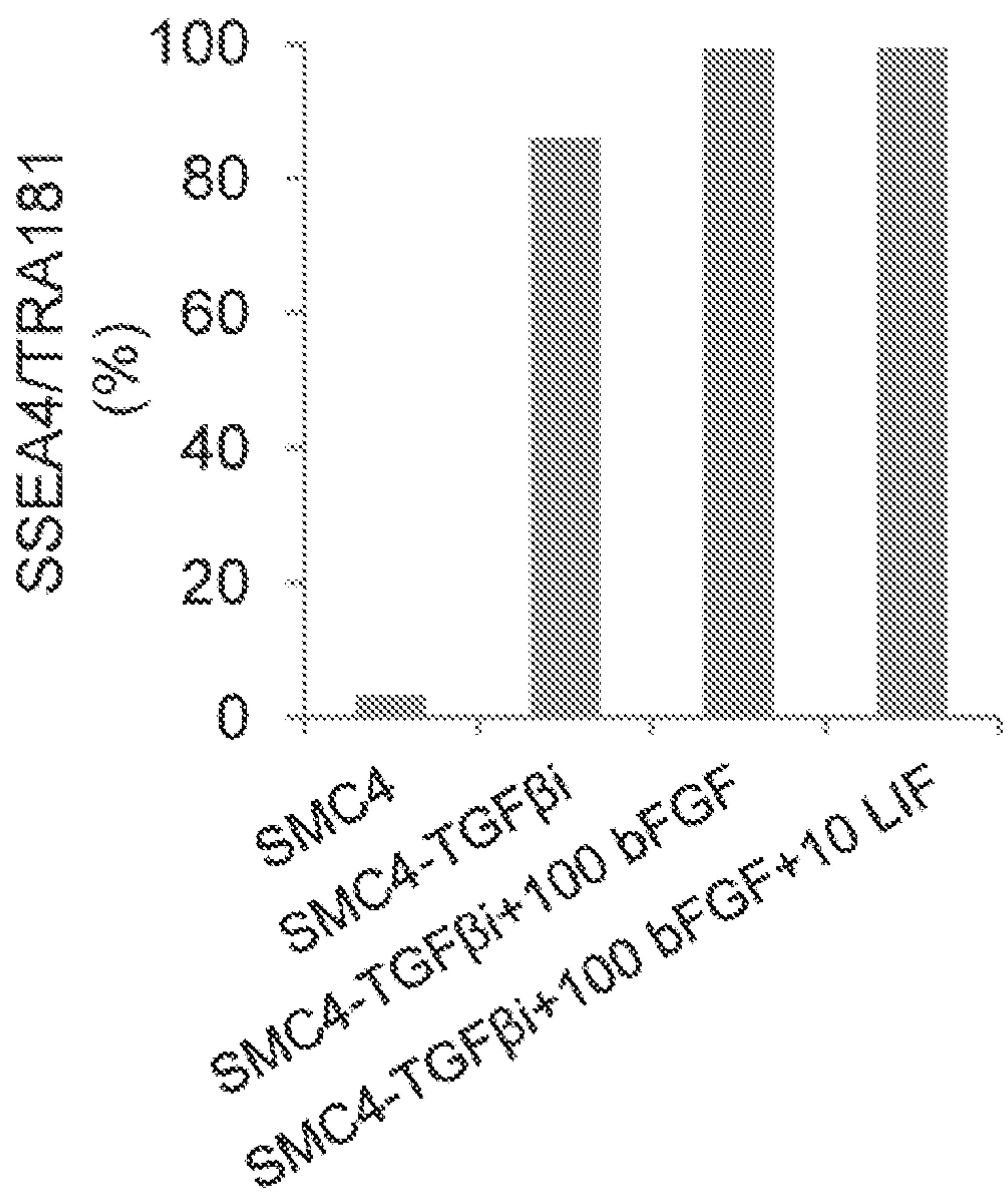

SSEA4/TRA181
(%)
100
80
60
40
20
0
SMC4
SMC4-TGFβi
SMC4-TGFβi+100 bFGF
SMC4-TGFβi+100 bFGF+10 LIF

FIG. 1C

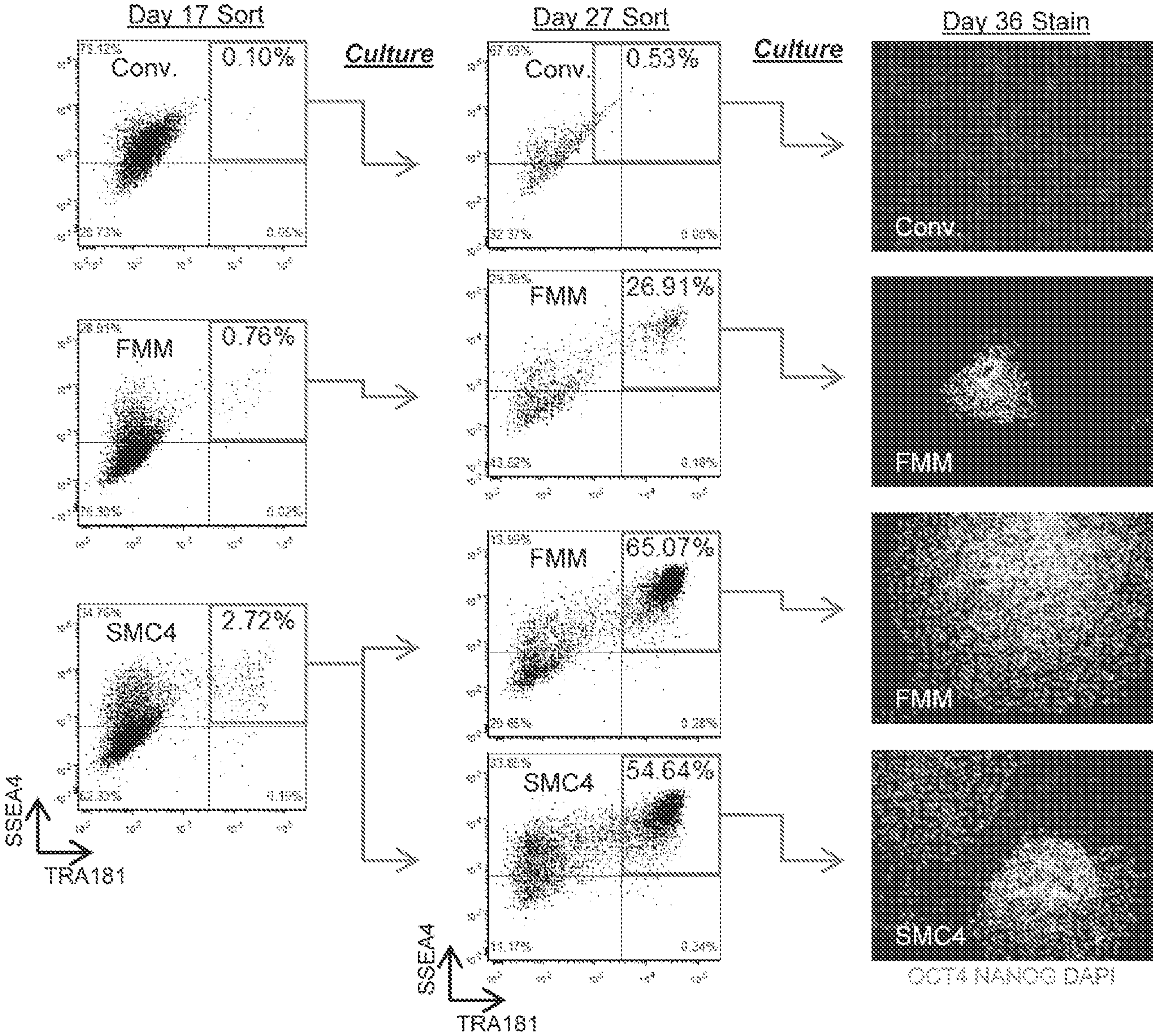

Day 17 Sort
Culture
Day 27 Sort
Culture
Day 36 Stain
Conv.
0.10%
FMM
0.76%
SMC4
2.72%
Conv.
0.53%
FMM
26.91%
FMM
65.07%
SMC4
54.64%
Conv.
FMM
FMM
SMC4
OCT4 NANOG DAPI
SSEA4
TRA181
SSEA4
TRA181

FIG. 1D

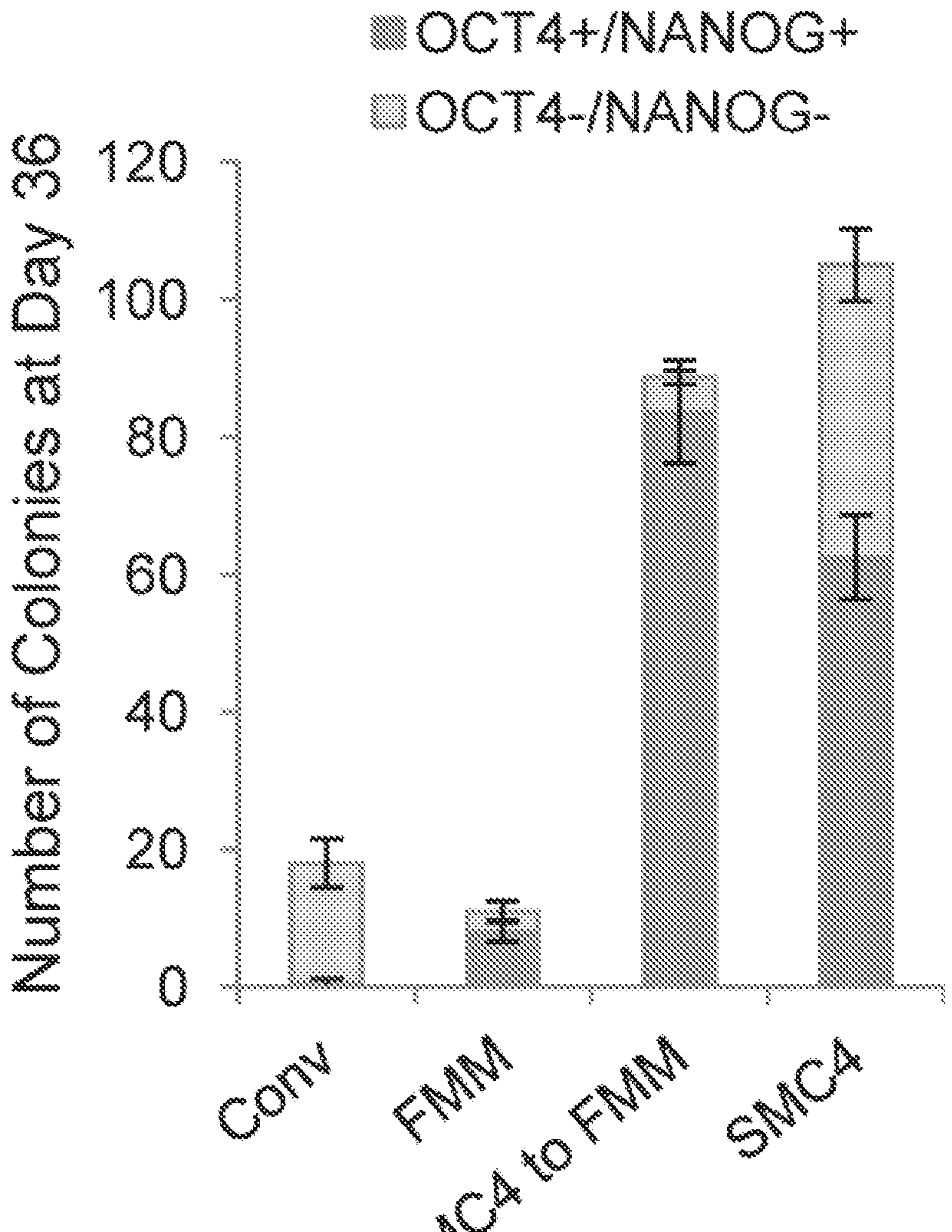

OCT4+/NANOG+
OCT4-/NANOG-
Number of Colonies at Day 36
120
100
80
60
40
20
0
Conv
FMM
SMC4 to FMM
SMC4

FIG. 1E

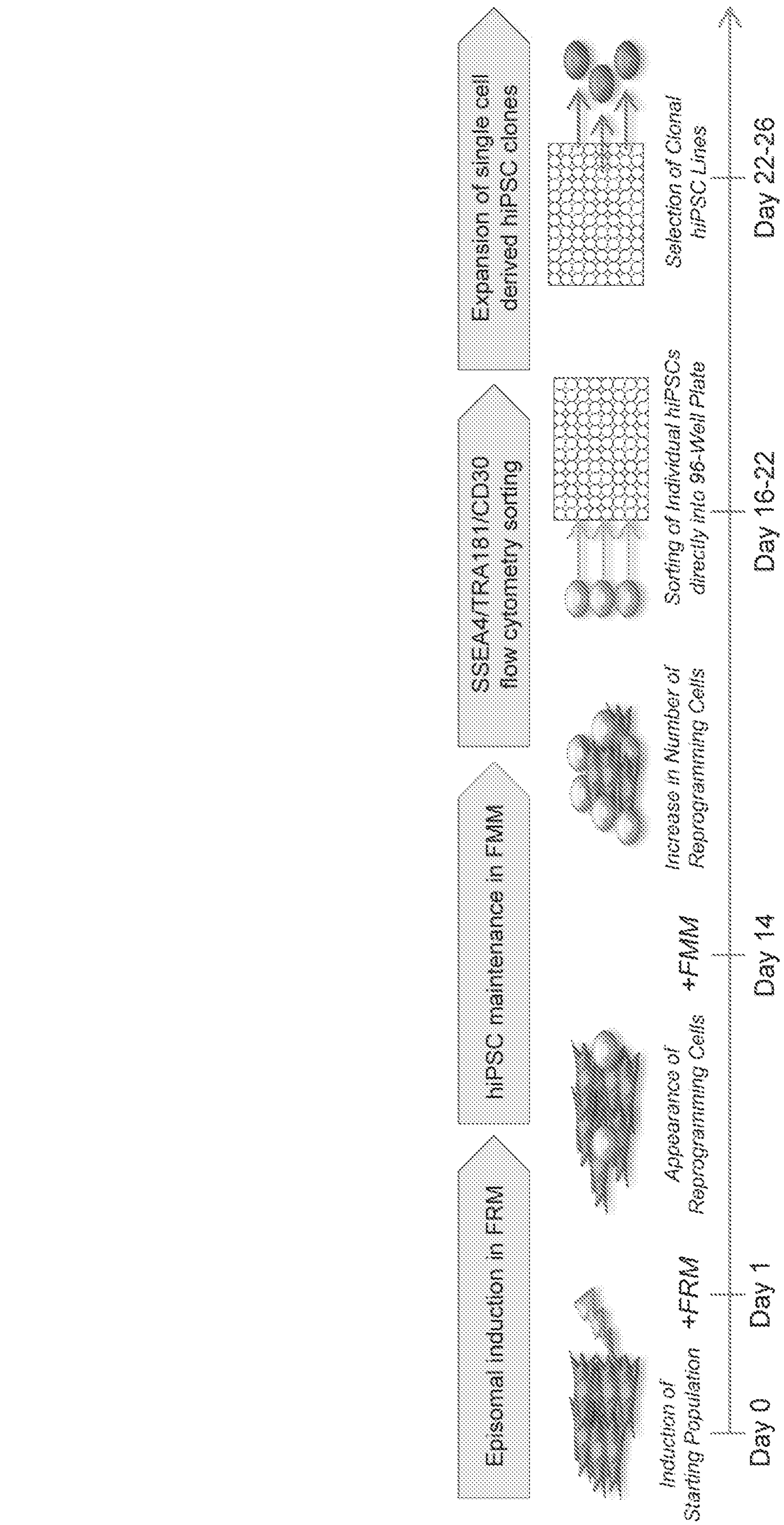

Episomal induction in FRM
hiPSC maintenance in FMM
SSEA4/TRA181/CD30 flow cytometry sorting
Expansion of single cell derived hiPSC clones
Induction of Starting Population
+FRM
Appearance of Reprogramming Cells
+FMM
Increase in Number of Reprogramming Cells
Sorting of Individual hiPSCs directly into 96-Well Plate
Selection of Clonal hiPSC Lines
Day 0
Day 1
Day 14
Day 16-22
Day 22-26

FIG. 2A

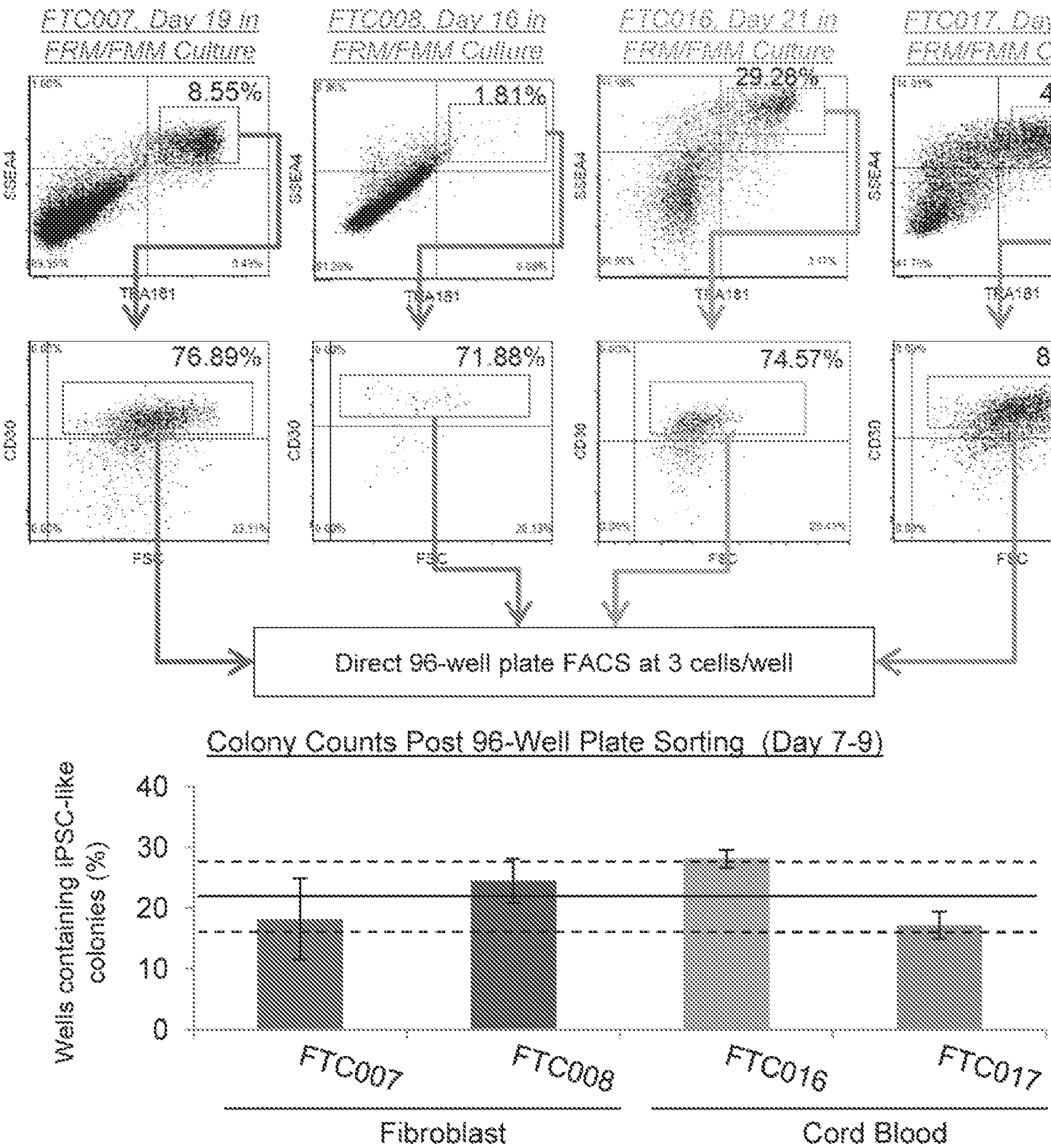

FTC007, Day 19 in FRM/FMM Culture
FTC008, Day 16 in FRM/FMM Culture
FTC016, Day 21 in FRM/FMM Culture
FTC017, Day 21 in FRM/FMM Culture
8.55%
1.81%
29.28%
41.22%
SSEA4
TRA181
76.89%
71.88%
74.57%
85.61%
CD30
FSC
Direct 96-well plate FACS at 3 cells/well
Colony Counts Post 96-Well Plate Sorting (Day 7-9)
Wells containing iPSC-like colonies (%)
40
30
20
10
0
FTC007
FTC008
FTC016
FTC017
Fibroblast
Cord Blood

FIG. 2B

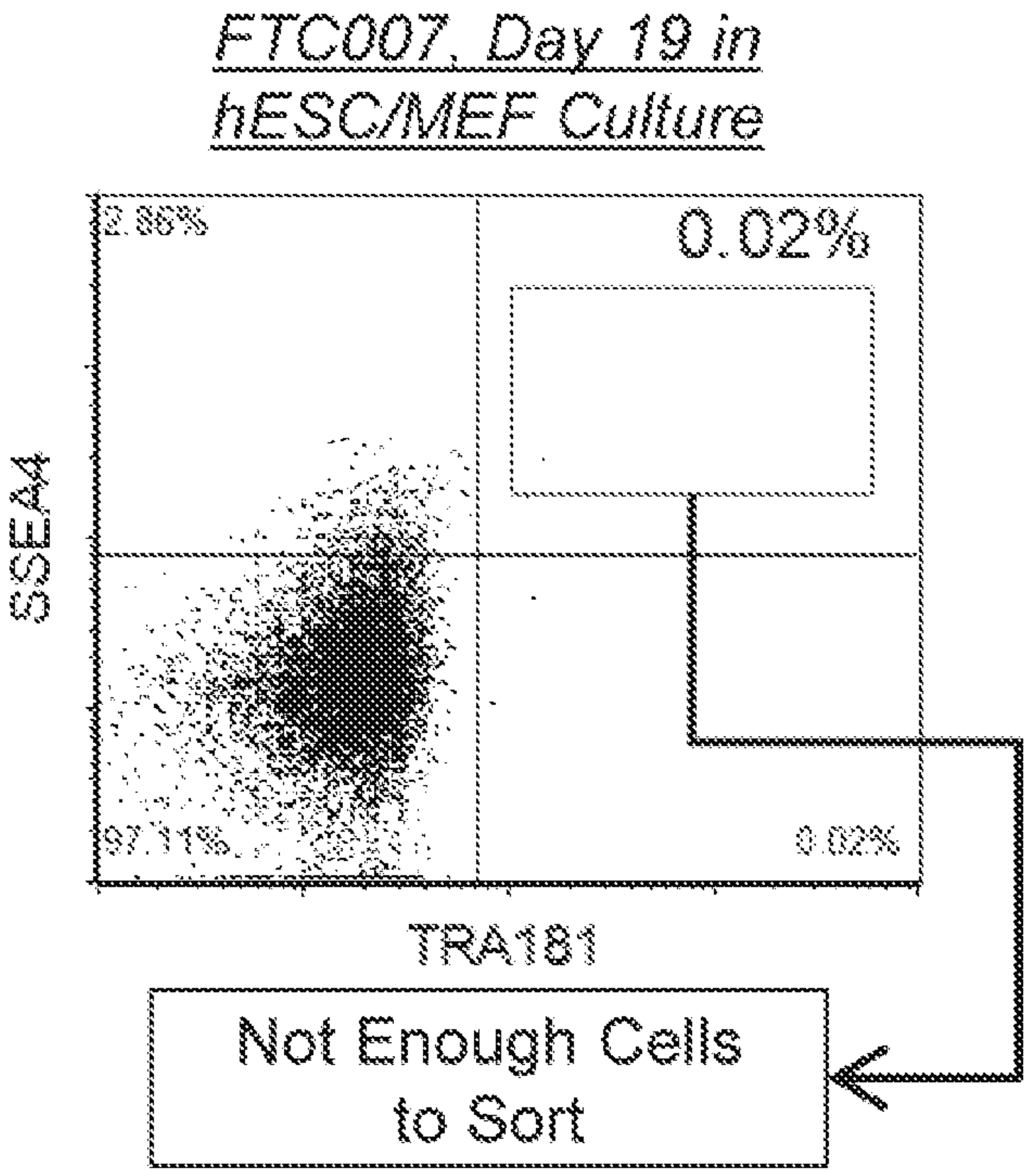

FTC007, Day 19 in
hESC/MEF Culture
2.86%
0.02%
97.11%
0.02%
SSEA4
TRA181
Not Enough Cells
to Sort

FIG. 2C

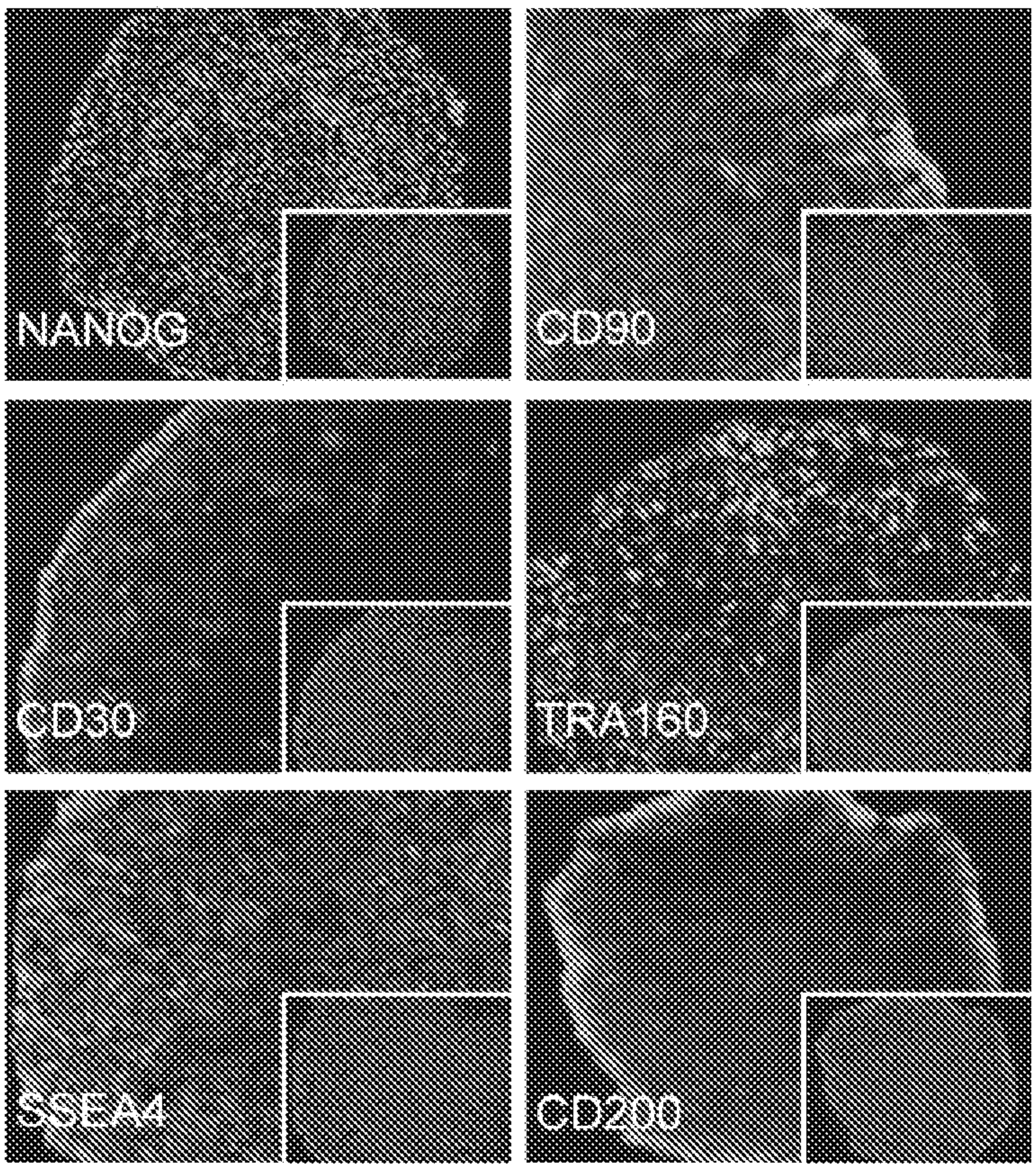

NANOG
CD90
CD30
TRA160
SSEA4
CD200

FIG. 2D

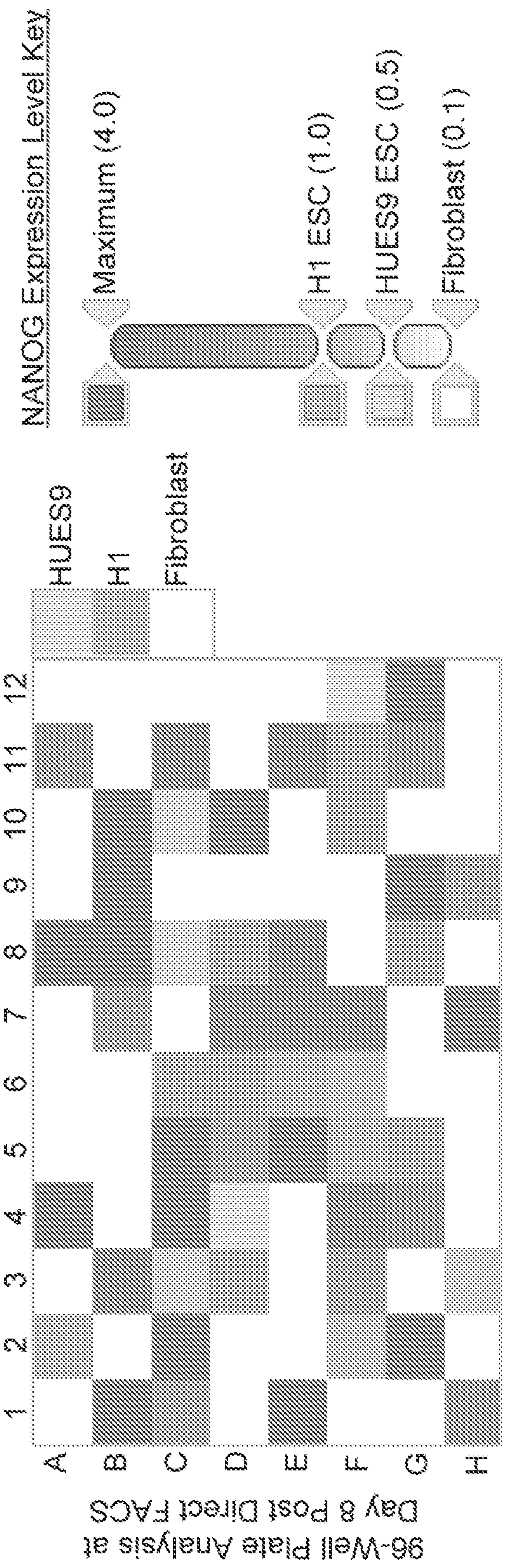

NANOG Expression Level Key
Maximum (4.0)
H1 ESC (1.0)
HUES9 ESC (0.5)
Fibroblast (0.1)
HUES9
H1
Fibroblast
1
2
3
4
5
6
7
8
9
10
11
12
A
B
C
D
E
F
G
H
96-Well Plate Analysis at
Day 8 Post Direct FACS

FIG. 2E

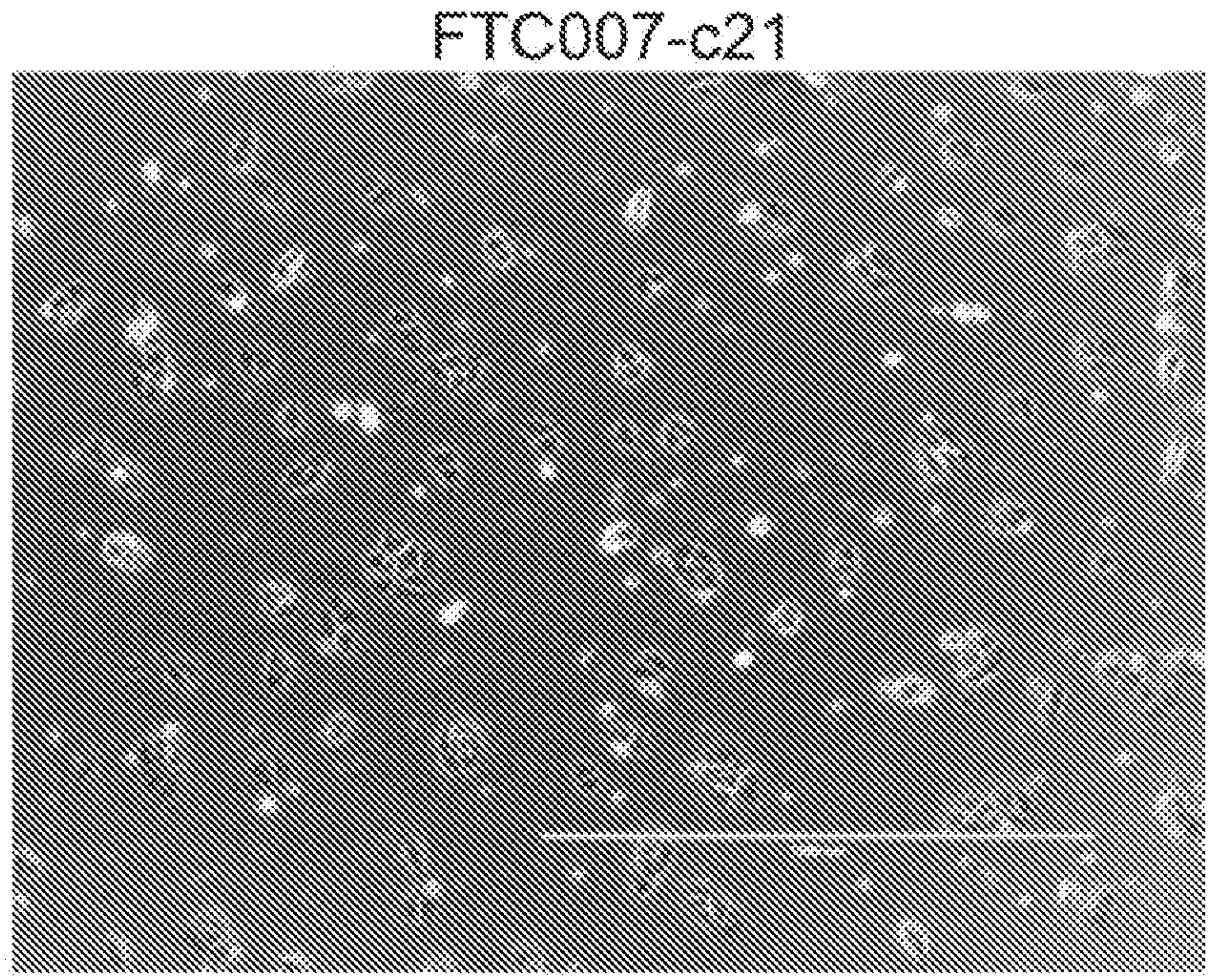


FIG. 3A

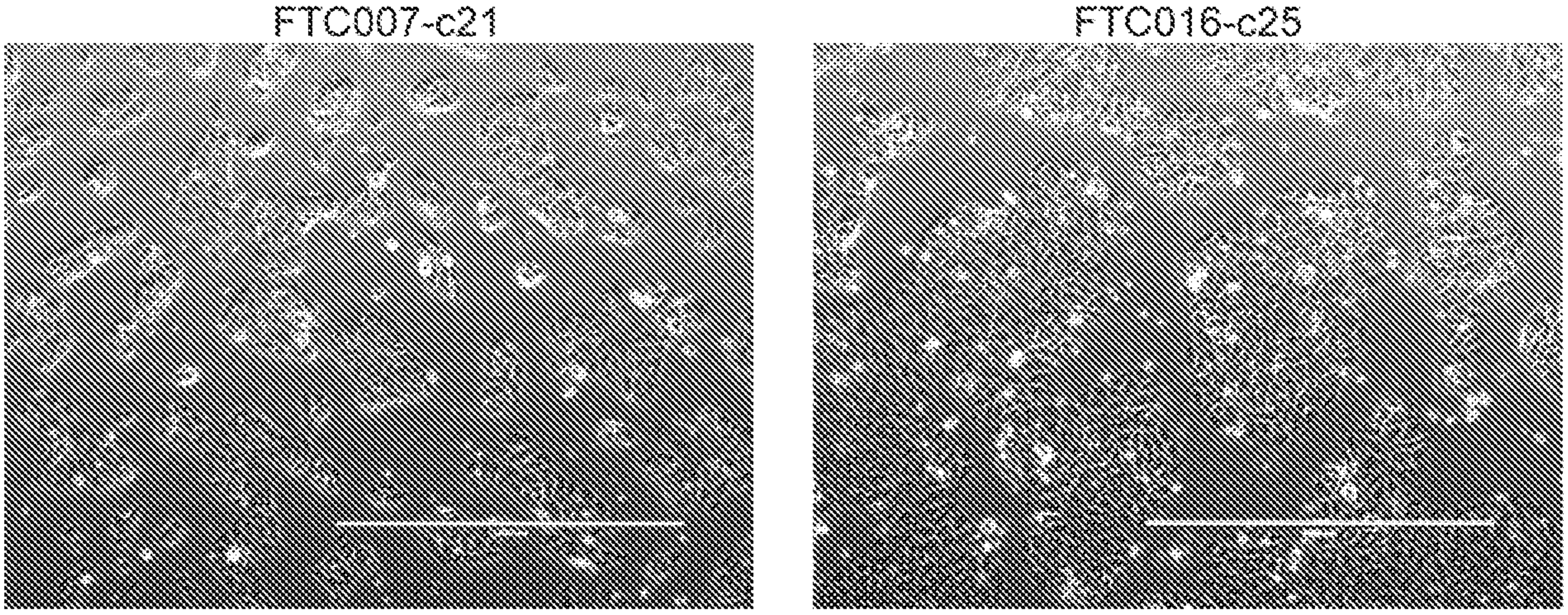


FIG. 3B

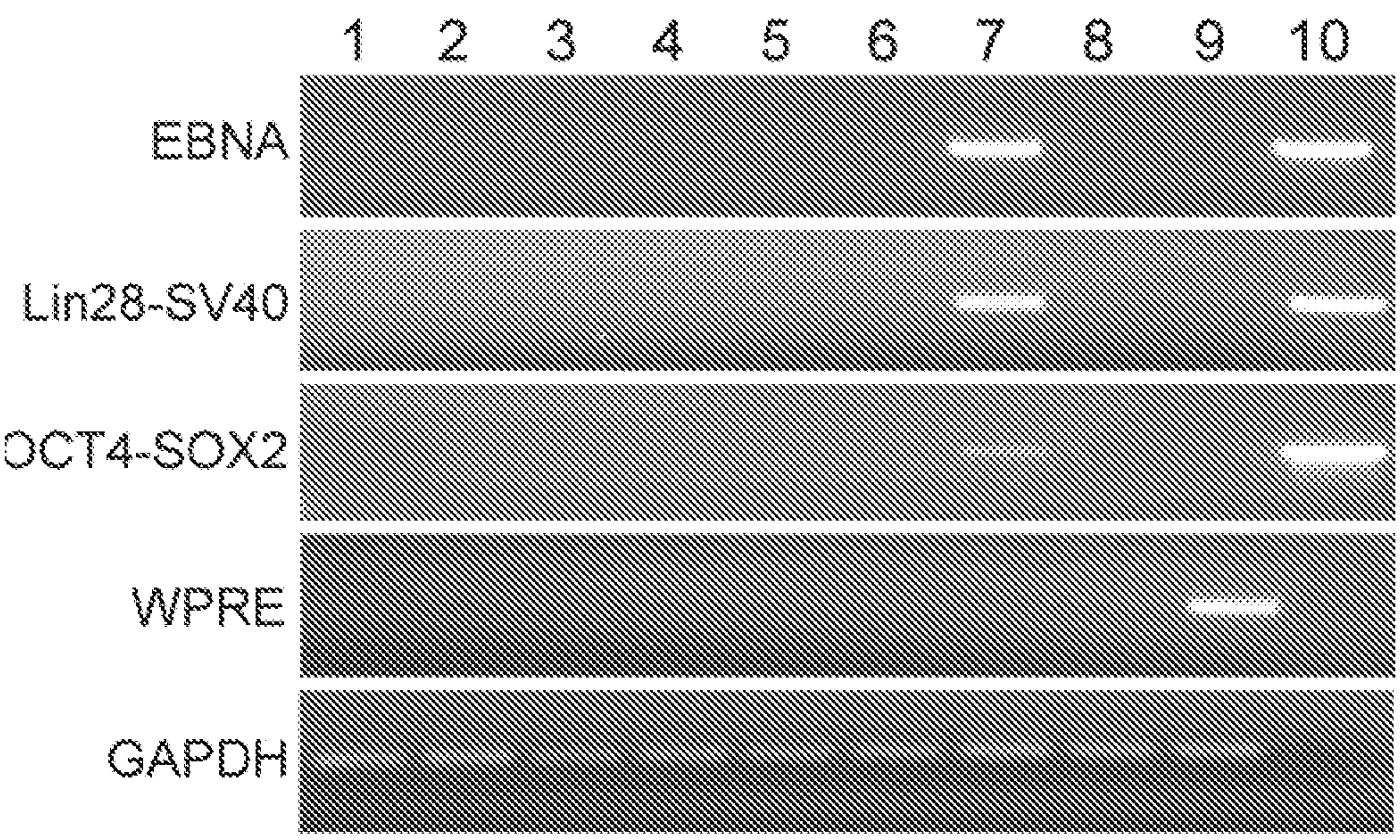


FIG. 3C

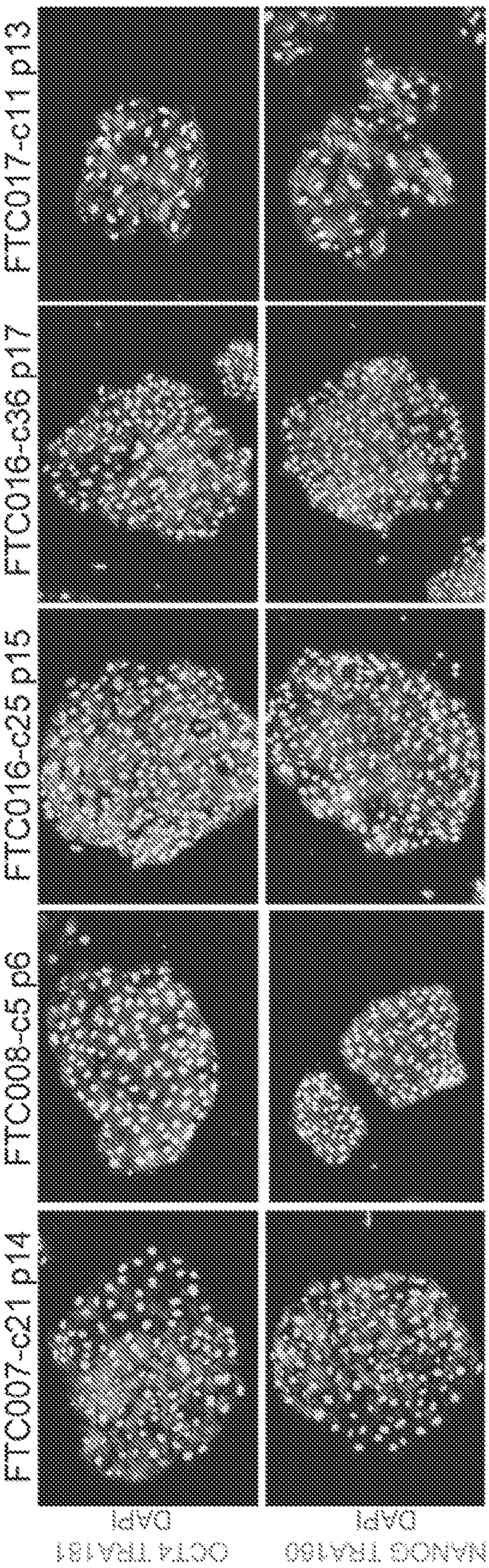

FTC017-c11 p13
FTC016-c36 p17
FTC016-c25 p15
FTC008-c5 p6
FTC007-c21 p14
OCT4 TRA181 DAPI
NANOG TRA160 DAPI

FIG. 3D

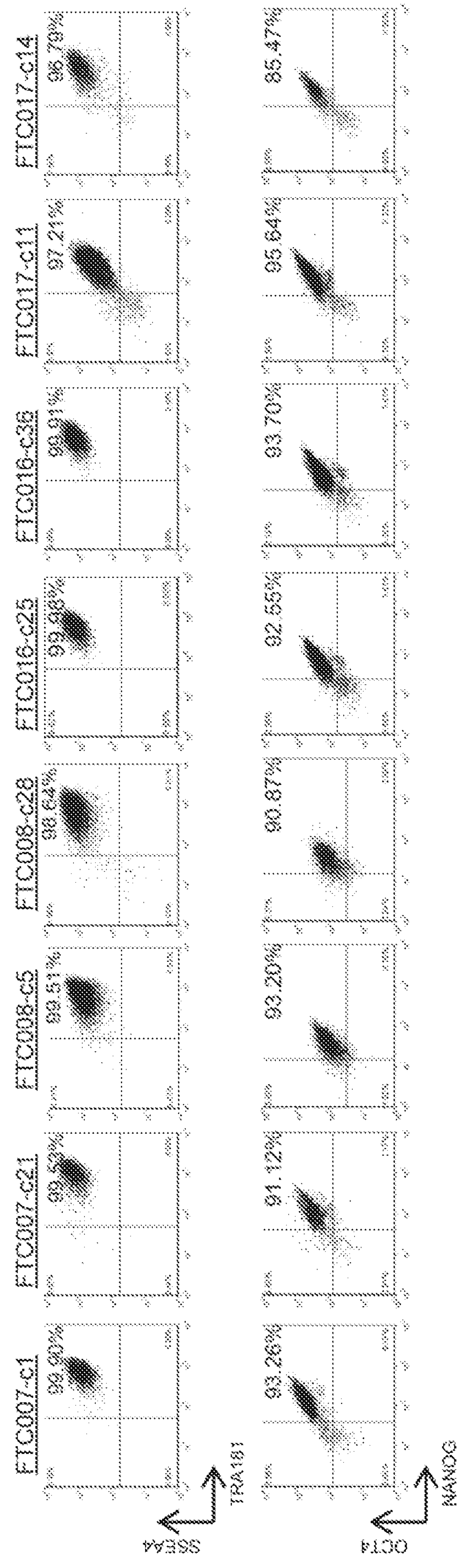

FTC007-c1
FTC007-c21
FTC008-c5
FTC008-c28
FTC016-c25
FTC016-c36
FTC017-c11
FTC017-c14
99.90%
93.26%
91.12%
99.51%
93.20%
98.64%
90.87%
92.55%
99.91%
93.70%
97.21%
95.64%
85.47%
SSEA4
TRA181
OCT4
NANOG

FIG. 3E

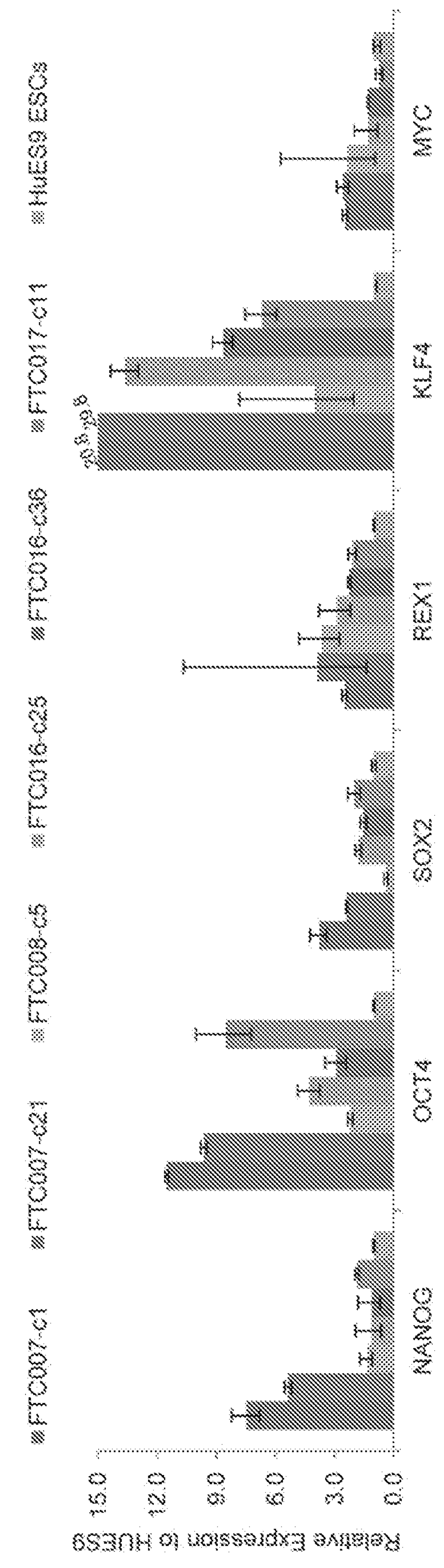

FTC007-c1
FTC007-c21
FTC008-c5
FTC016-c25
FTC016-c36
FTC017-c11
HuES9 ESCs
Relative Expression to HUES9
15.0
12.0
9.0
6.0
3.0
0.0
NANOG
OCT4
SOX2
REX1
KLF4
MYC
20.8
20.8

FIG. 3F

FTC007-c1 p10
46,XY

FTC007-c21 p10
46,XY

FTC016-c25 p11
46,XX

FTC016-c36 p14
45,XX

FTC016-c28 p13
46,XX

FTC017-c11 p13
46,XY

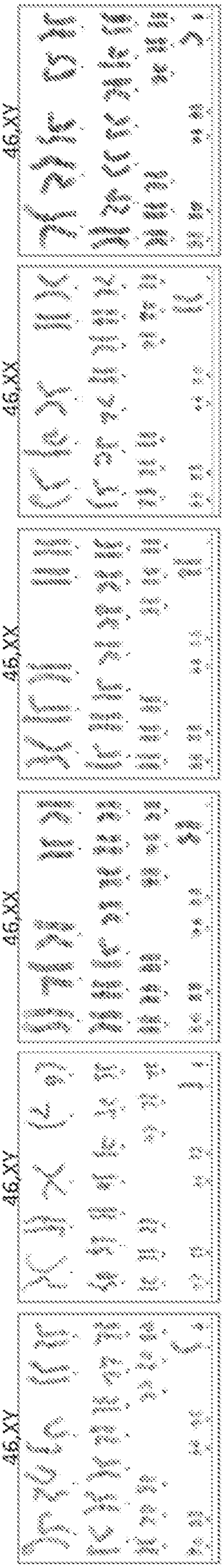


FIG. 4A

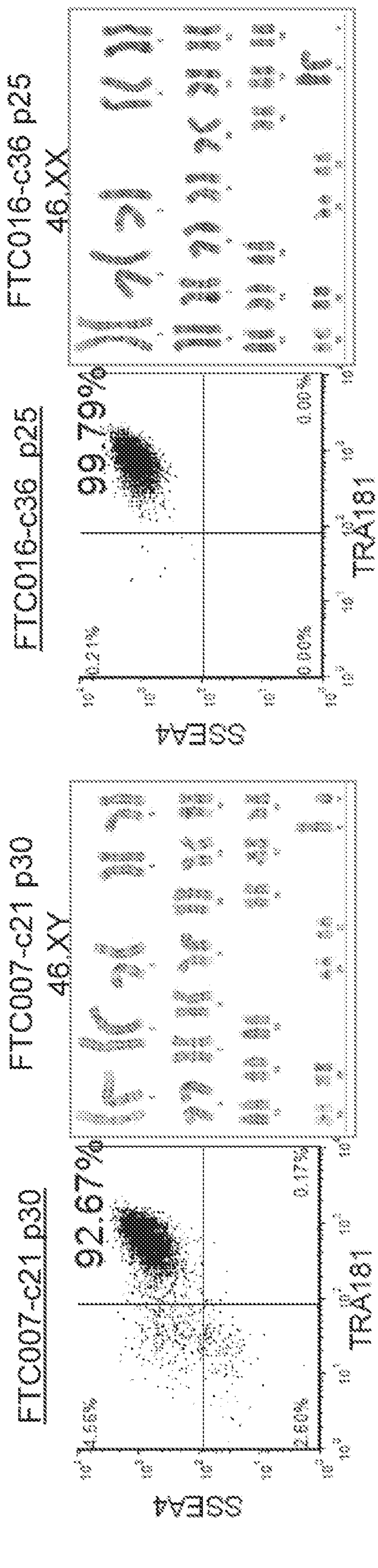

FTC007-c21 p30
92.67%
4.56%
2.60%
0.17%
SSEA4
TRA181
FTC007-c21 p30
46,XY
FTC016-c36 p25
99.79%
0.21%
0.00%
0.00%
SSEA4
TRA181
FTC016-c36 p25
46,XX

FIG. 4B

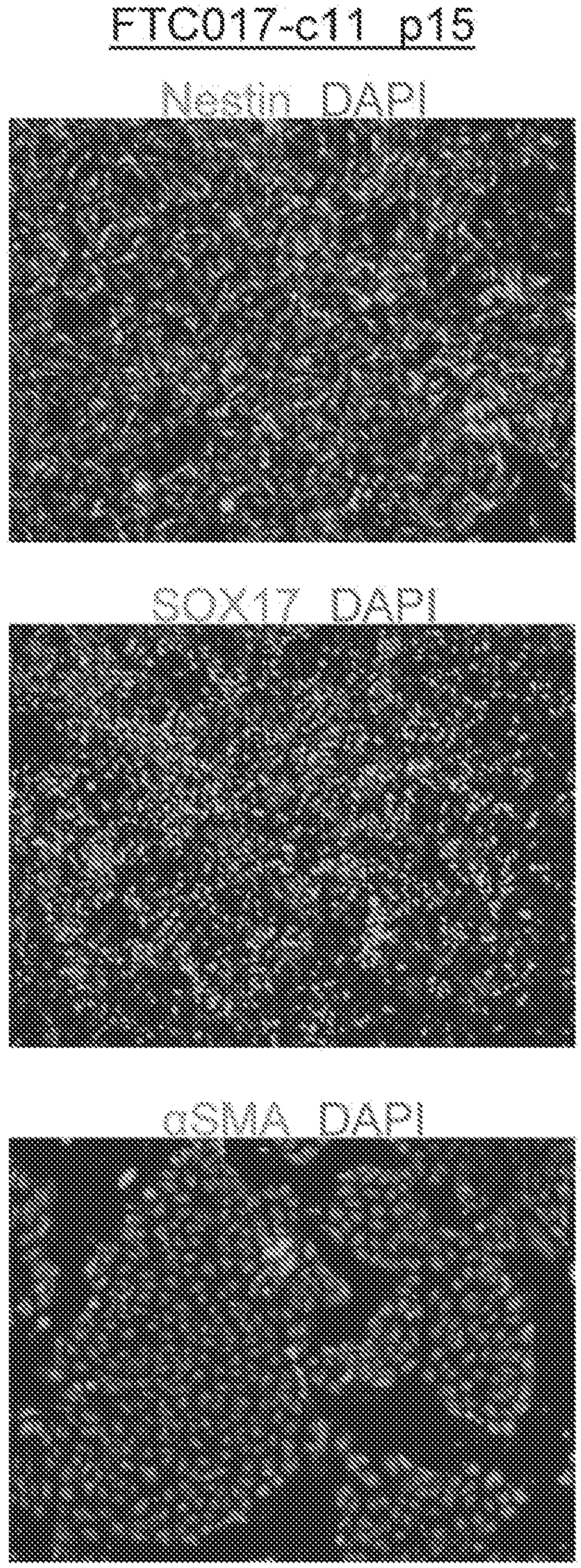


FIG. 4C

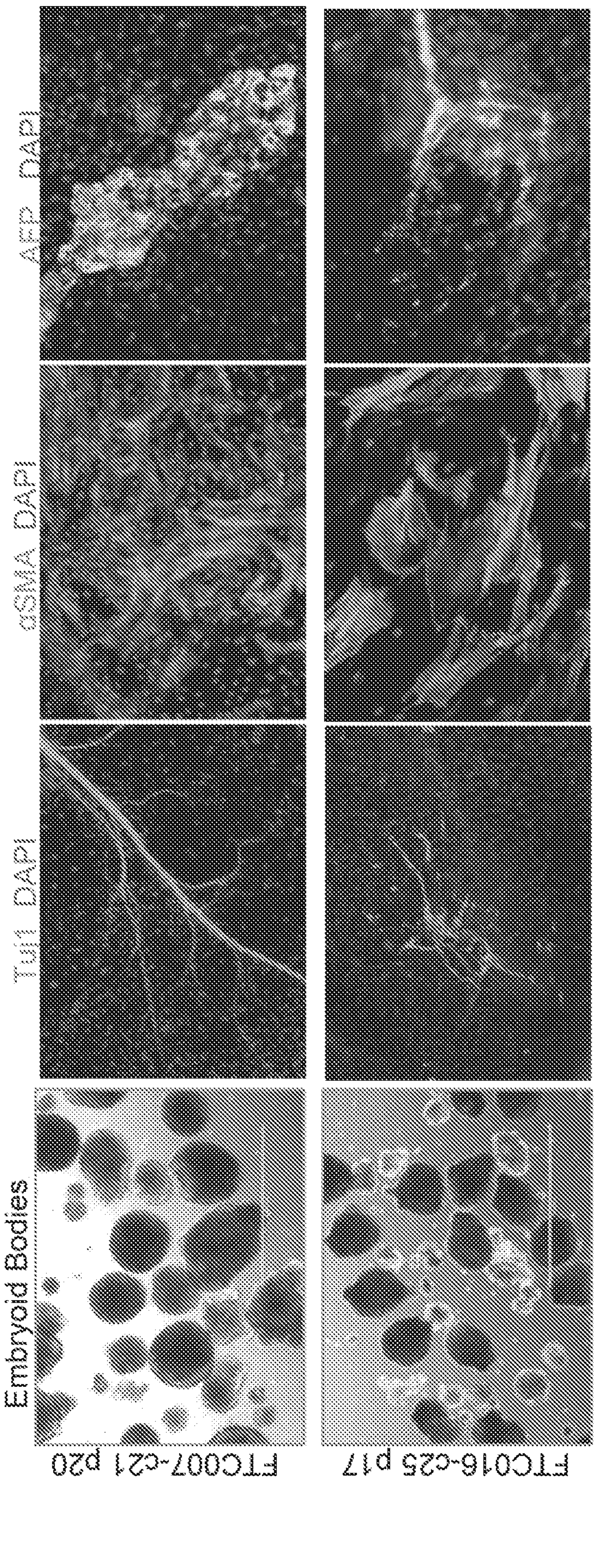
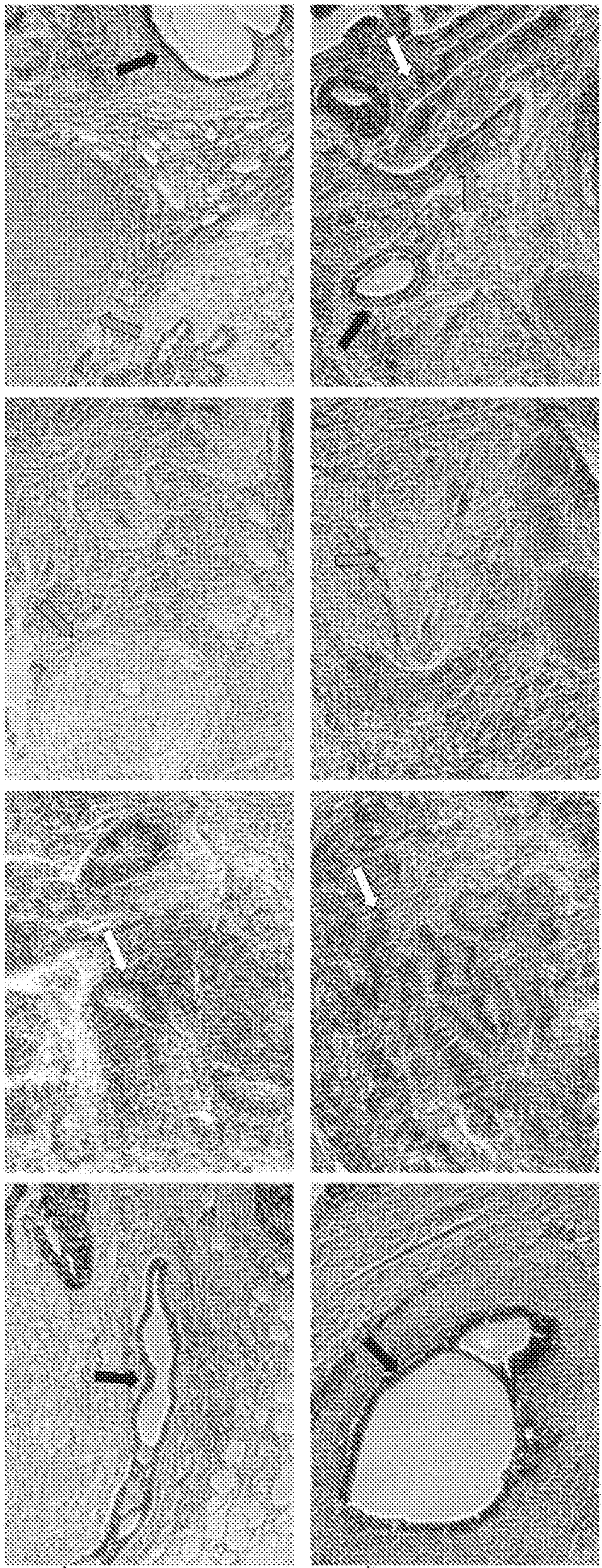

Embryoid Bodies
Tuj1 DAPI
αSMA DAPI
AFP DAPI
FTC007-c21 p20
FTC016-c25 p17

FIG. 4D



FTC007-c21 p20

FTC016-c25 p17

FIG. 4E

Vector System	Abbreviation
EF1a-OCT4-2A-NANOG-2A-SOX2	ONS
EF1a-OCT4-2A-SOX2	OS
EF1a-OCT4-2A-OCT4	2xO
EF1a-SV40LT	T

FIG. 5A

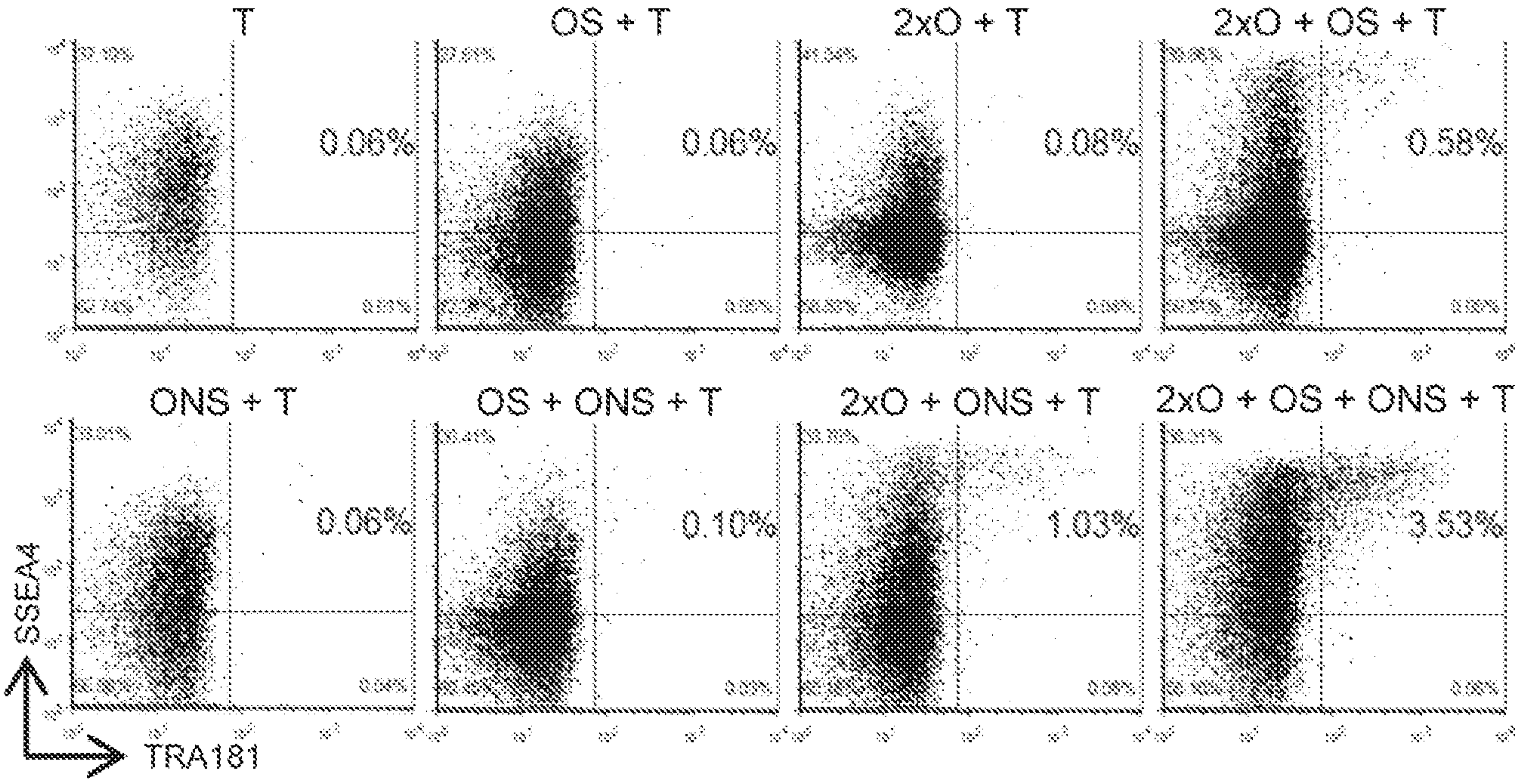


FIG. 5B

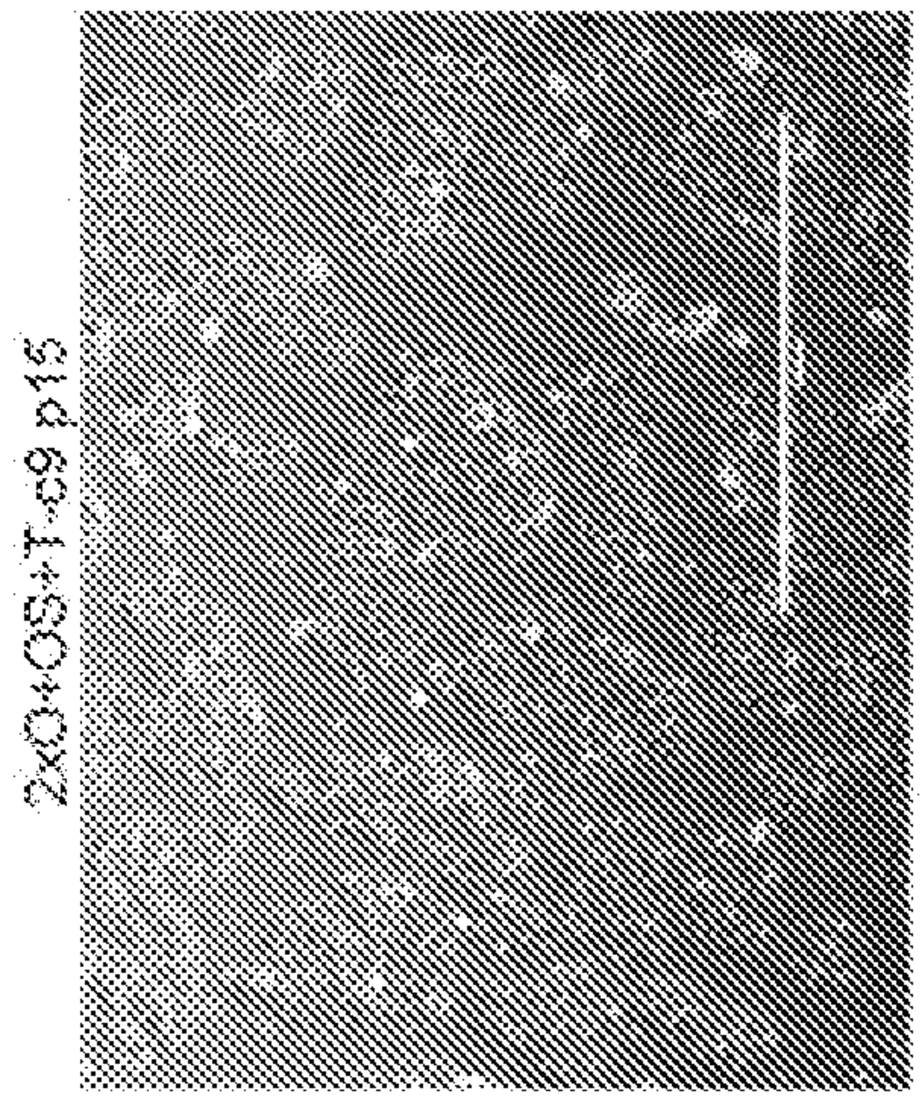


FIG. 5E

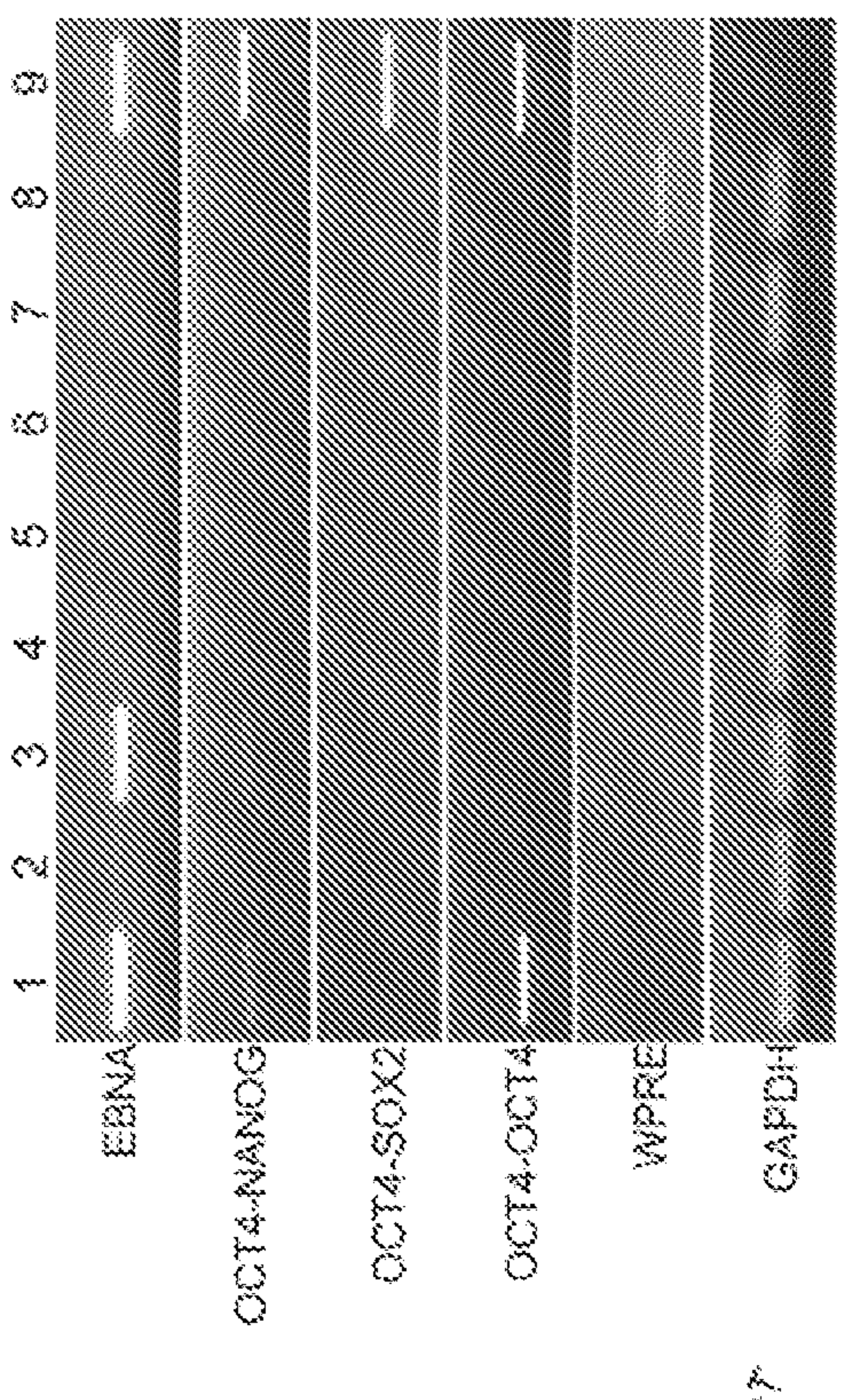


FIG. 5D

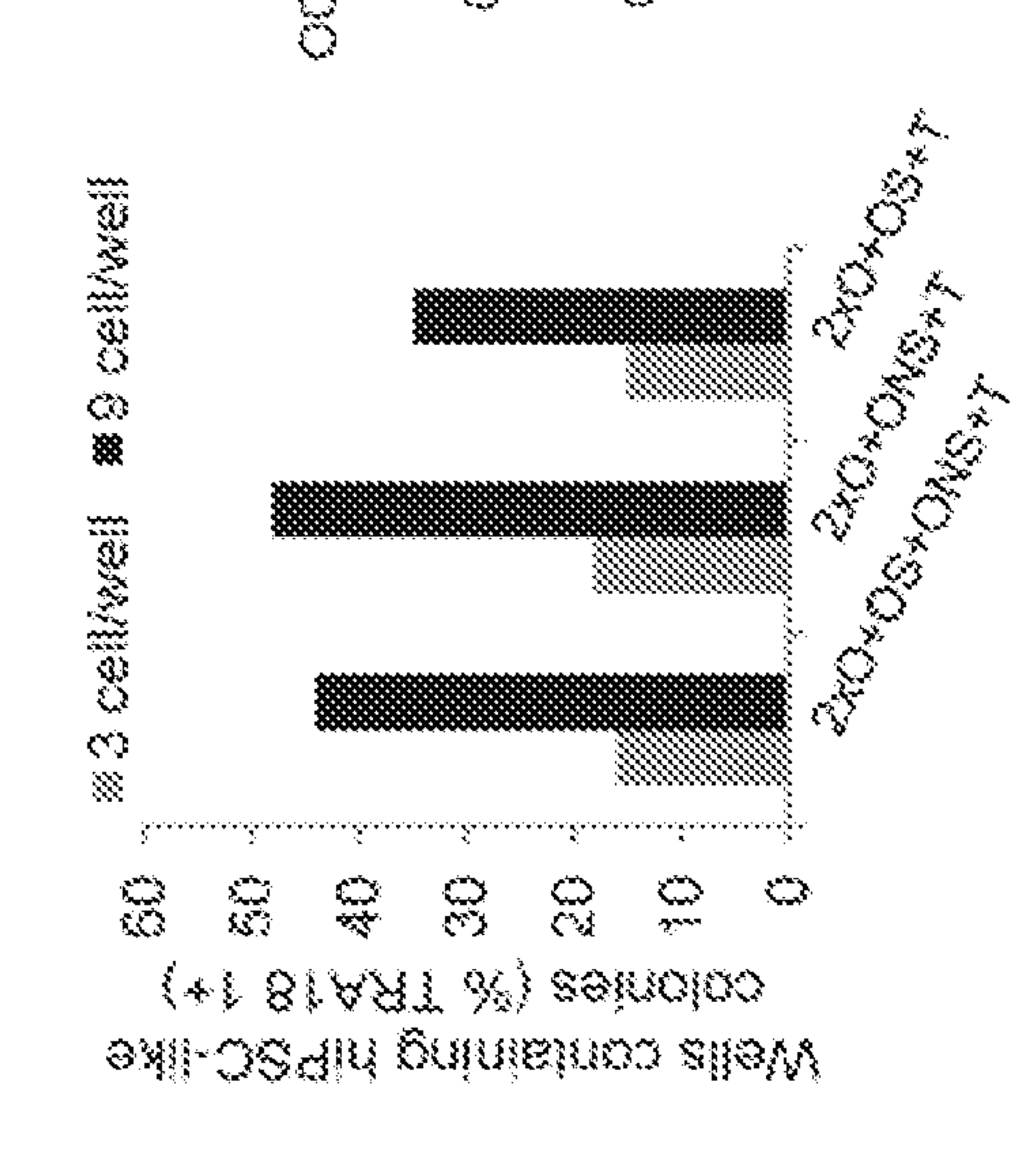


FIG. 5C

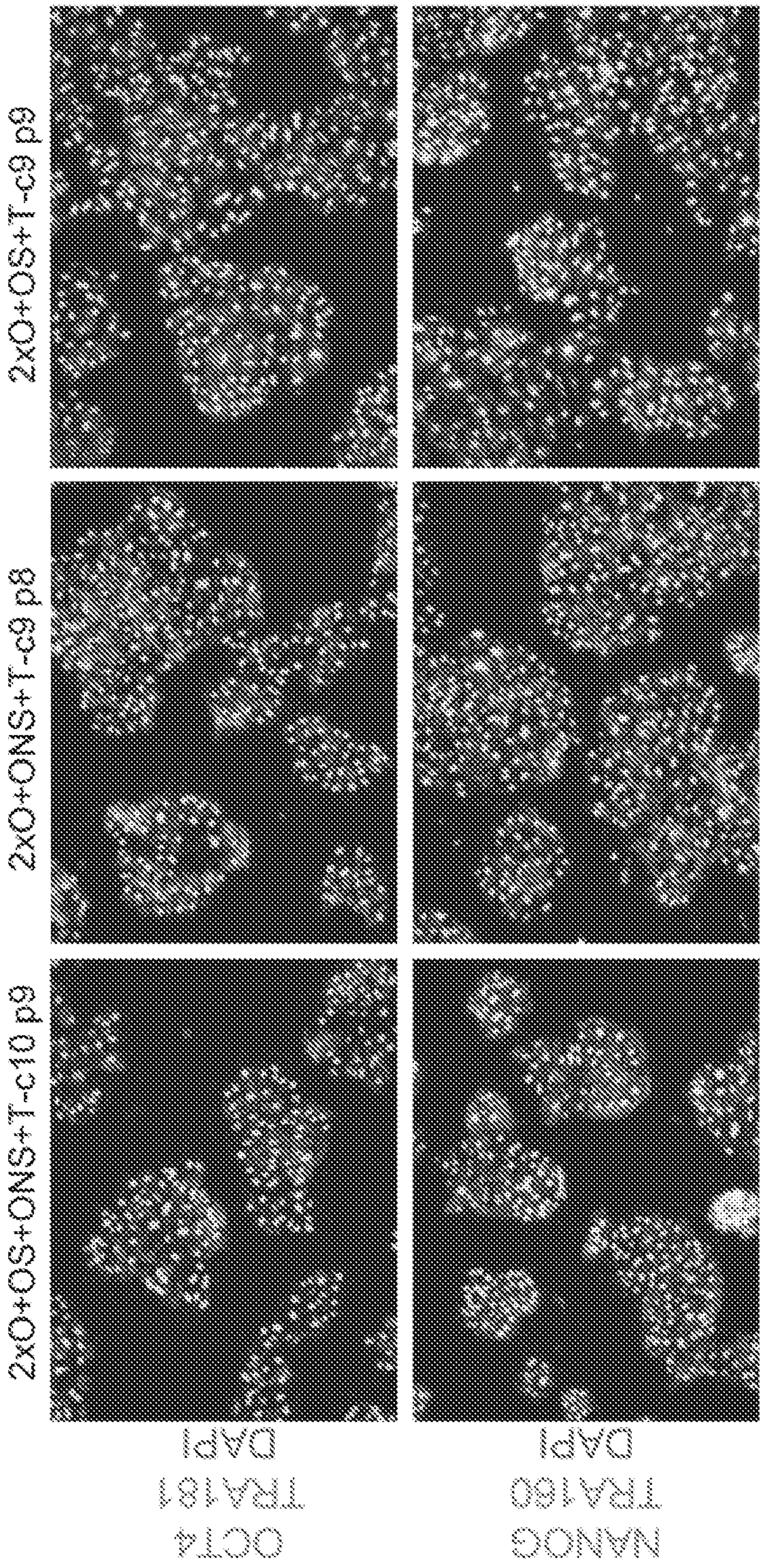

2xO+OS+T-c9 p9
2xO+ONS+T-c9 p8
2xO+OS+ONS+T-c10 p9
OCT4
TRA181
DAPI
NANOG
TRA160
DAPI

FIG. 5F

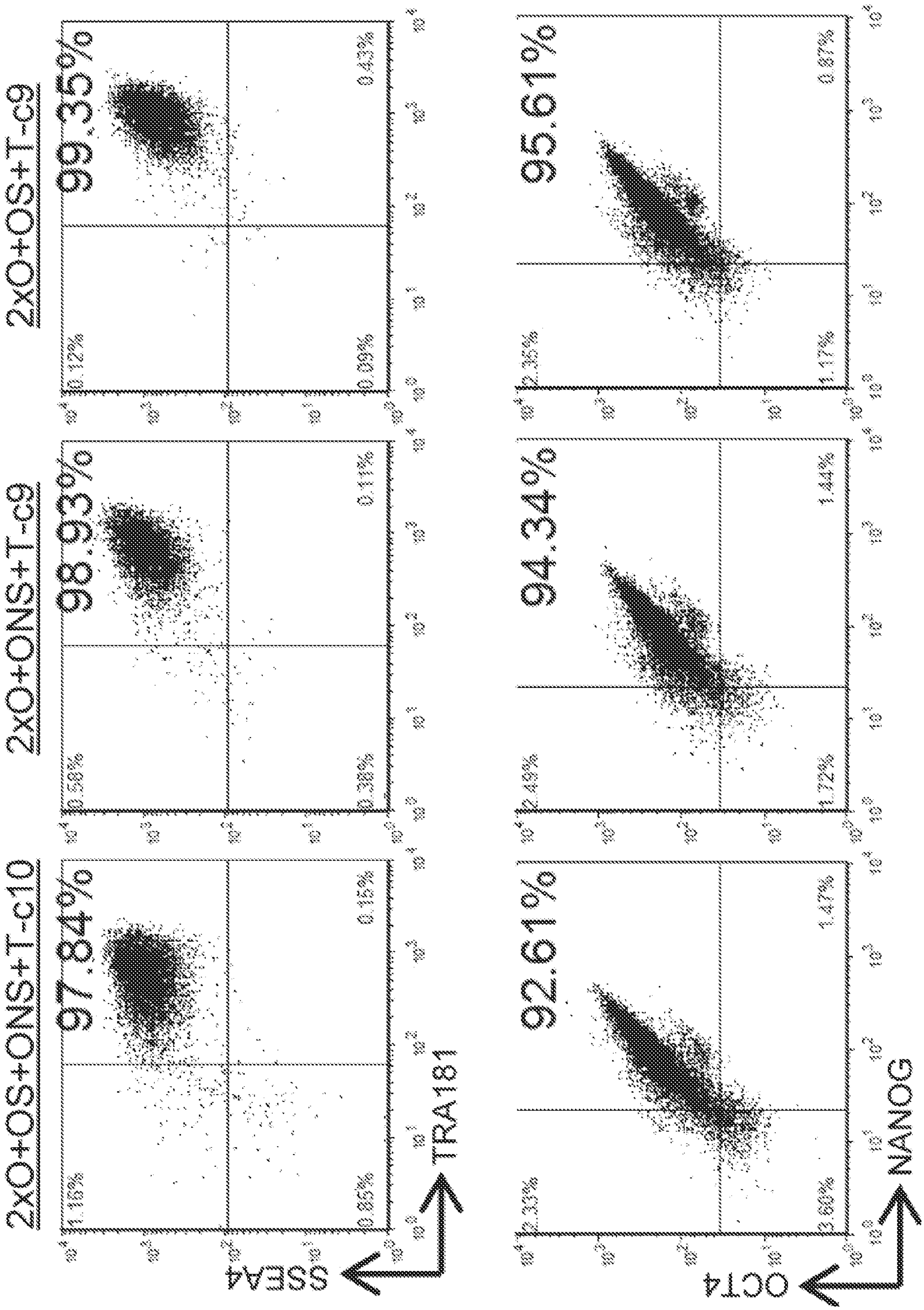

2xO+OS+ONS+T-c10
97.84%
2xO+ONS+T-c9
98.93%
2xO+OS+T-c9
99.35%
92.61%
94.34%
95.61%
SSEA4
TRA181
OCT4
NANOG

FIG. 5G

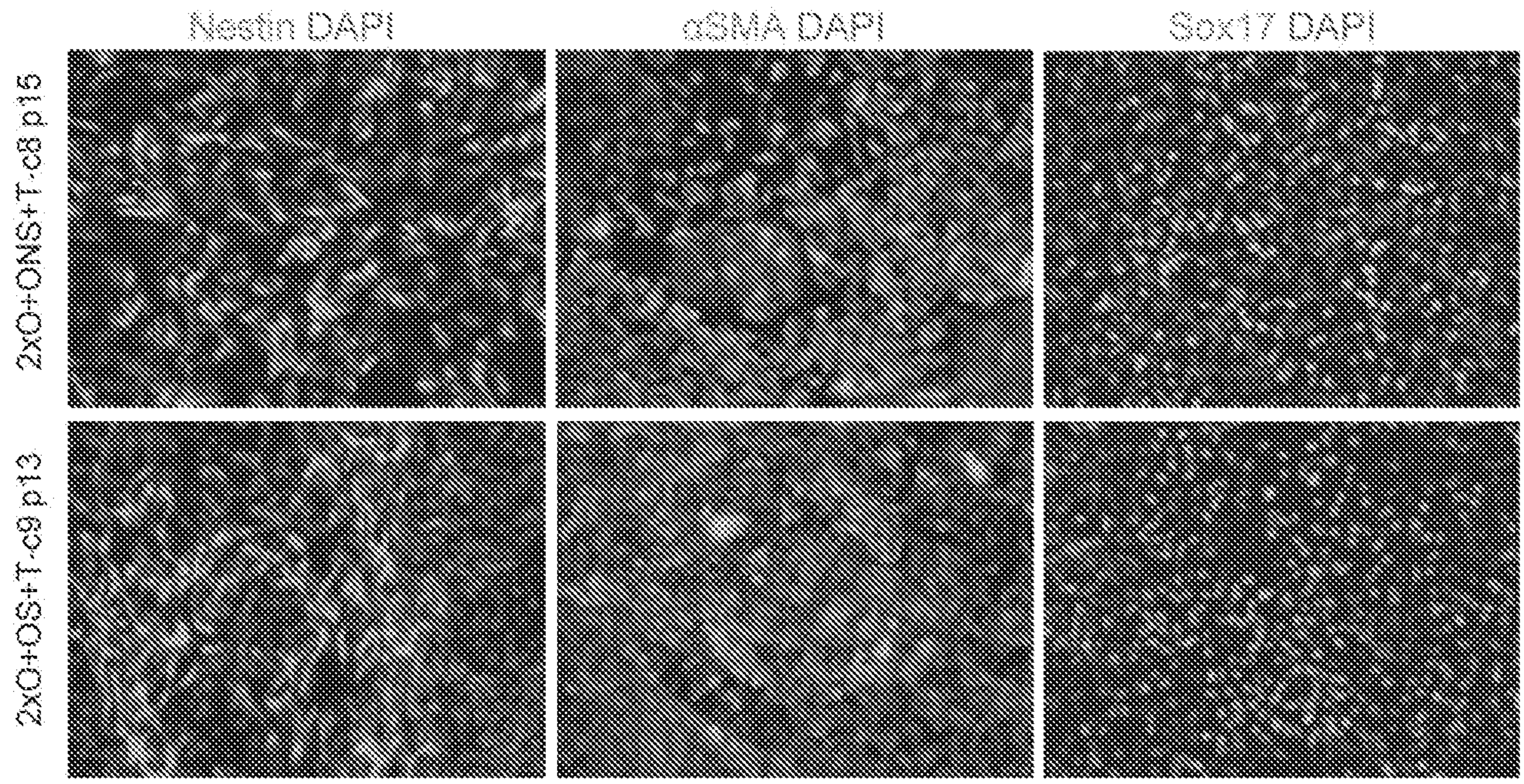

Nestin DAPI
αSMA DAPI
Sox17 DAPI
2xO+ONS+T-c8 p15
2xO+OS+T-c9 p13

FIG. 5H

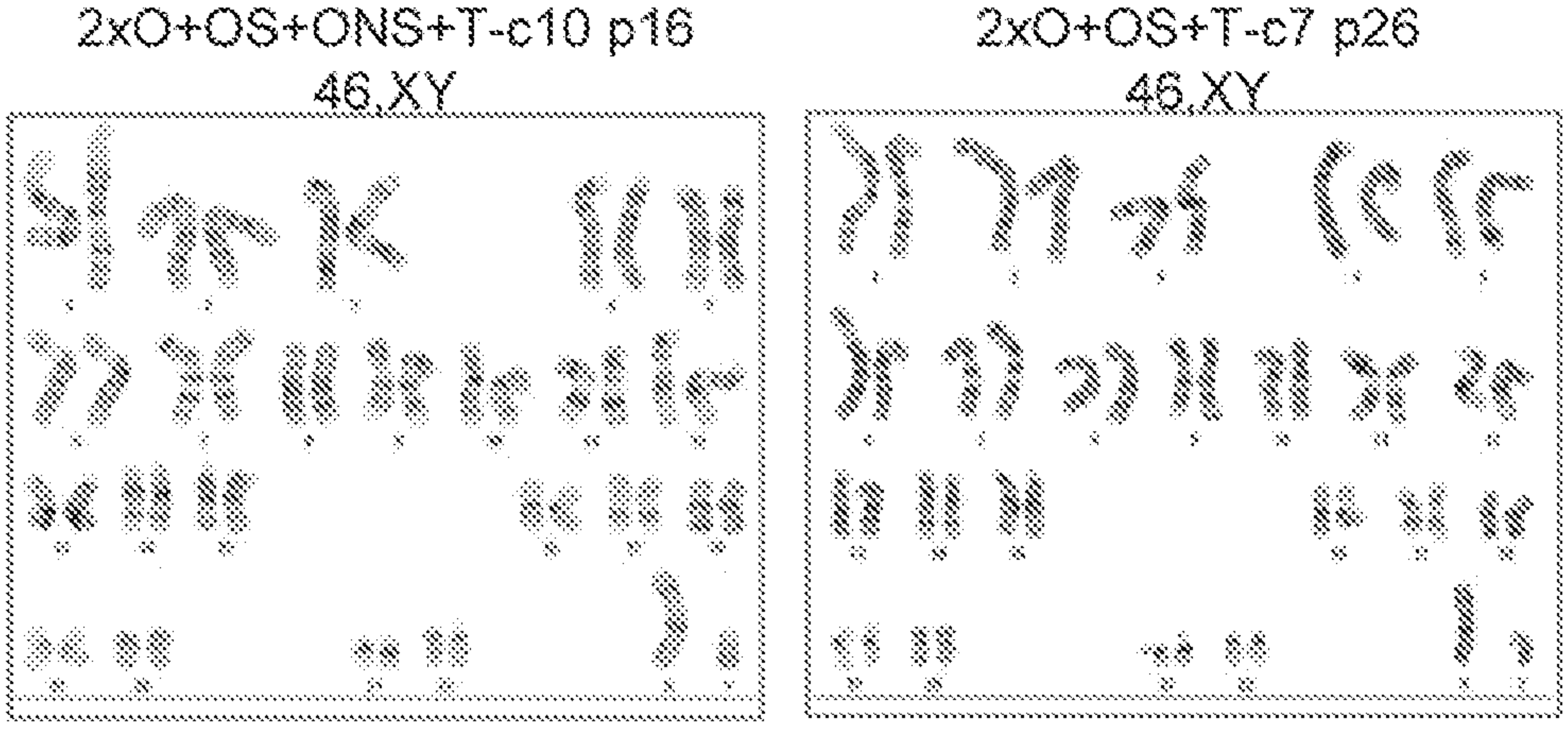
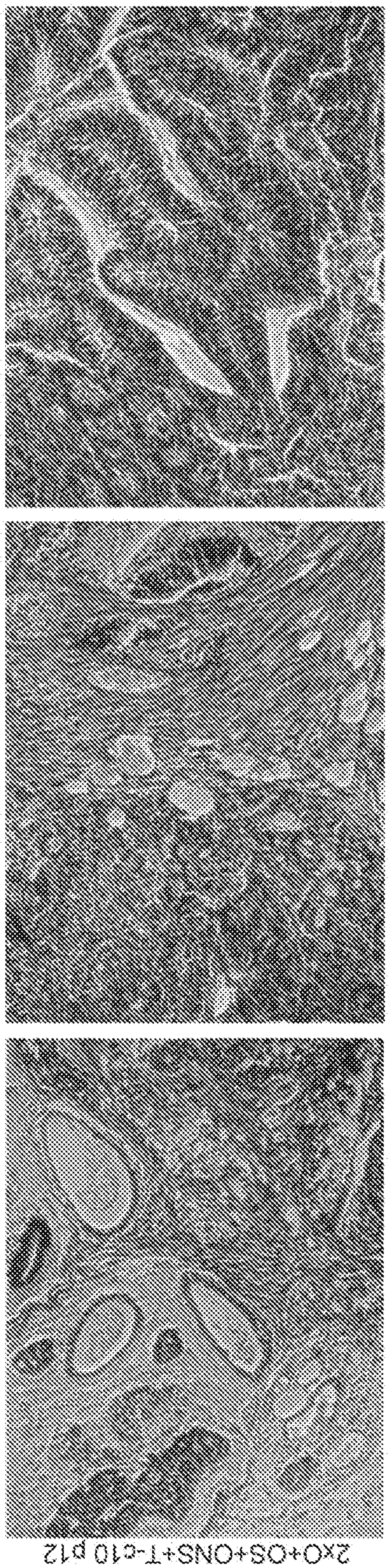

2xO+OS+ONS+T-c10 p16
46,XY
2xO+OS+T-c7 p26
46,XY

FIG. 5I



2xO+OS+ONS+T-c10 p12

FIG. 5J

Cell Type	Cell Line	NANOG	OCT4	SOX2	KLF4	REX1	DNMT3B	LIN28	MYC
Conventionally maintained hiPSCs on MEF	OKS hiPSC	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	OKS hiPSC	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	OKS hiPSC	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	OKS hiPSC	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	OKS hiPSC	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
hESC on MEF	H1 ESCs	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
OSNKLMT Episomal hiPSCs in FMM	FTC016-c5	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	FTC007-c1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	FTC016-c25	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	FTC016-c36	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	FTC016-c28	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	FTC017-c11	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	FTC016-c31	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	FTC017-c14	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	FTC007-c21	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Minimal Factor Episomal hiPSCs in FMM	ONS+OS+2xO-c7	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	ONS+OS+2xO-c10	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	ONS+2xO-c5	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	ONS+2xO-c8	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	ONS+2xO-c9	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	OS+2xO-c7	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	OS+2xO-c9	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

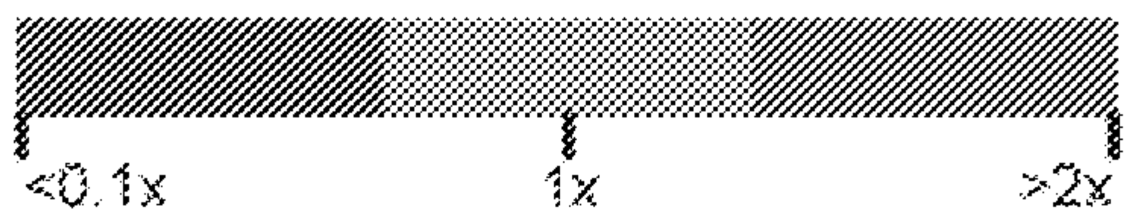


FIG. 6A

Cell Type	Cell Line	T	CXCR4	NODAL	GATA4	SOX17	FOXA2	OTX2	TUJ1
Conventionally maintained hiPSCs on MEF	OKS hiPSC	0.351	0.032	0.000	1.997	2.358	0.001	0.050	0.050
	OKS hiPSC	1.431	1.488	2.013	0.963	0.593	1.036	0.648	1.863
	OKS hiPSC	1.209	2.280	0.673	2.128	1.209	1.234	1.770	1.041
	OKS hiPSC	0.102	1.185	0.484	0.029	0.581	0.830	1.543	1.282
	OKS hiPSC	1.417	3.804	3.068	2.412	3.322	4.476	5.126	0.854
hESC on MEF	H1 ESCs	1.333	0.001	0.000	0.836	1.000	0.000	0.000	0.000
OSNKLMT Episomal hiPSCs in FMM	FTC016-c5	0.011	0.046	0.171	0.001	0.002	0.001	0.862	2.023
	FTC007-c1	0.006	0.128	1.108	0.020	0.006	0.046	2.090	2.031
	FTC016-c25	0.025	0.001	0.000	0.015	0.001	0.000	0.002	0.061
	FTC016-c36	0.026	0.001	0.000	0.010	0.005	0.000	0.000	0.001
	FTC016-c28	0.004	0.075	0.000	0.000	0.001	0.011	1.337	2.297
	FTC017-c11	0.046	0.001	0.000	0.004	0.001	0.000	0.000	0.061
	FTC016-c31	0.015	0.095	0.403	0.002	0.002	0.009	0.231	2.860
	FTC017-c14	0.469	0.377	0.003	0.146	0.455	0.141	0.007	0.016
	FTC007-c21	0.064	0.003	0.000	0.099	0.012	0.001	0.000	0.031
Minimal Factor Episomal hiPSCs in FMM	ONS+OS+2xO-c7	0.081	0.003	0.000	0.020	0.003	0.001	0.001	0.004
	ONS+OS+2xO-c10	0.322	0.007	0.000	0.069	0.006	0.001	0.000	0.083
	ONS+2xO-c5	0.044	0.001	0.000	0.023	0.004	0.000	0.000	0.001
	ONS+2xO-c8	0.011	0.001	0.000	0.027	0.002	0.000	0.000	0.001
	ONS+2xO-c9	0.074	0.001	0.000	0.035	0.003	0.000	0.000	0.000
	OS+2xO-c7	0.005	0.001	0.000	0.019	0.000	0.000	0.000	0.082
	OS+2xO-c9	0.145	0.001	0.000	0.026	0.009	0.000	0.000	0.001

<0.1x 1x >2x

FIG. 6B

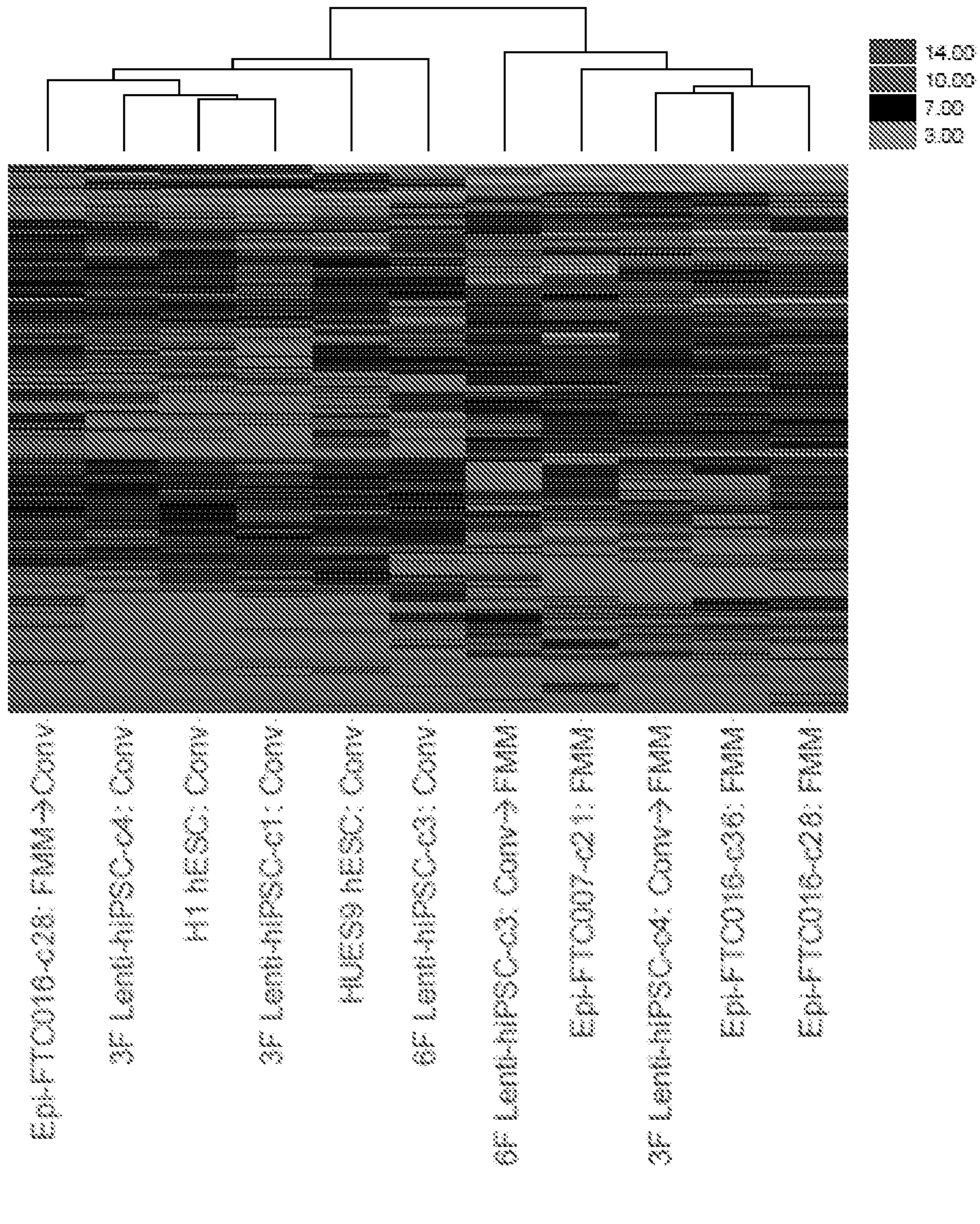

14.00
10.00
7.00
3.00
Epi-FTC016-c28: FMM→Conv
3F Lenti-hiPSC-c4: Conv
H1 hESC: Conv
3F Lenti-hiPSC-c1: Conv
HUES9 hESC: Conv
6F Lenti-hiPSC-c3: Conv
6F Lenti-hiPSC-c3: Conv→FMM
Epi-FTC007-c21: FMM
3F Lenti-hiPSC-c4: Conv→FMM
Epi-FTC016-c36: FMM
Epi-FTC016-c28: FMM

FIG. 7A

213 Probe sets upregulated in conventional hESC culture group vs. FMM culture group			
GO ID	**GO Term**	**%**	**p-value**
GO:0007389	pattern specification process	11.2	2.20E-10
GO:0003002	regionalization	8.1	1.90E-07
GO:0048598	embryonic morphogenesis	8.1	1.90E-05
GO:0009952	anterior/posterior pattern formation	5.6	3.60E-05
GO:0043009	chordate embryonic development	6.2	2.90E-03
GO:0009792	embryonic development ending in birth or egg hatching	6.2	3.10E-03
GO:0001756	somitogenesis	2.5	3.40E-03
GO:0001775	cell activation	5.6	4.20E-03
GO:0001667	ameboidal cell migration	2.5	4.30E-03
GO:0048568	embryonic organ development	4.4	4.50E-03
GO:0040029	regulation of gene expression, epigenetic	3.1	5.00E-03
GO:0010557	positive regulation of macromolecule biosynthetic process	8.8	5.70E-03
GO:0010628	positive regulation of gene expression	8.1	5.80E-03
GO:0045321	leukocyte activation	5	6.00E-03
GO:0048562	embryonic organ morphogenesis	3.8	6.80E-03
GO:0030111	regulation of Wnt receptor signaling pathway	2.5	8.00E-03
GO:0031328	positive regulation of cellular biosynthetic process	8.8	8.20E-03
GO:0046649	lymphocyte activation	4.4	8.90E-03
GO:0035282	segmentation	2.5	9.00E-03
GO:0009891	positive regulation of biosynthetic process	8.8	9.20E-03
GO:0010604	positive regulation of macromolecule metabolic process	10	9.40E-03

FIG. 7B

Ground State Associated Gene Set
KLF4
KLF2
POU5F1
STAT3
UTF1
FGF4
ID4
TBX3
GDF3
DNMT3L
ESRRB
DPPA2
DPPA3
DPPA5
ZFP42
ZSCAN4
TRIM43
TFCP2L1

Metastable State Associated Gene Set		
MYC	FOXA2	T
DKK1	FGF5	SOX17
BMP2	XIST	NODAL
LEFTY2	COL13A1	OTX2
HES1	DUSP6	EOMES
CDX2	NR2F2	NR0B1
GNAS	CXCR4	CYP2B6
EGR1	GATA3	ERBB4
COL3A1	GATA6	HOXC6
TCF4	INHA	SMAD6
HEPH	RORA	NIPBL
KDR	TNFSF11	CDH11
TOX	ZIC4	GAL
FOXA1	SOX3	PITX2
LCK	APOA2	CXCL5
PCDH7	CER1	FOXQ1
CD1D	MLL5	DPP10
FOXG1	GSC	PCDH10
LEFTY1	CTCFL	PCDH20
ZIC1	TSHZ1	MEGF10

FIG. 7C

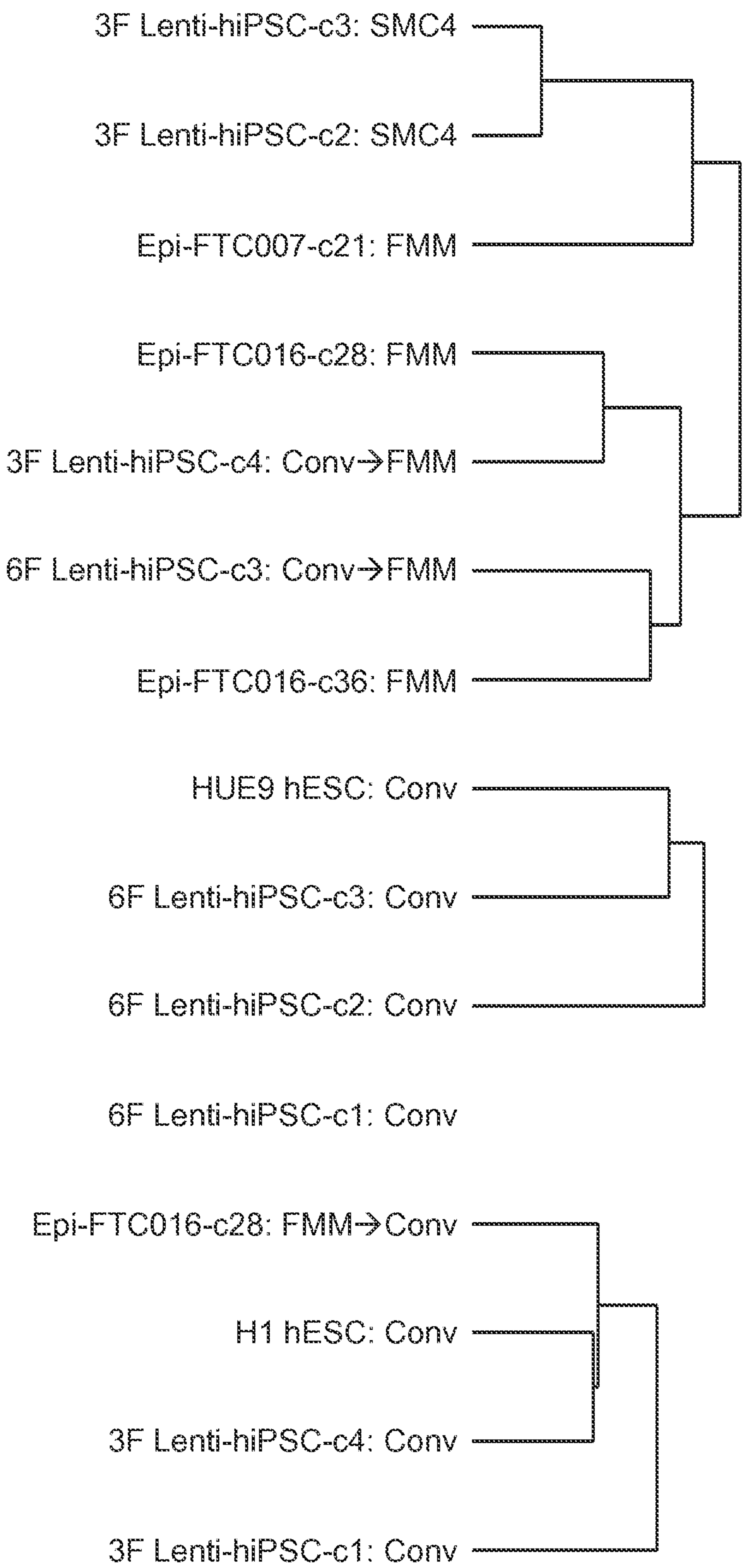

3F Lenti-hiPSC-c3: SMC4
3F Lenti-hiPSC-c2: SMC4
Epi-FTC007-c21: FMM
Epi-FTC016-c28: FMM
3F Lenti-hiPSC-c4: Conv→FMM
6F Lenti-hiPSC-c3: Conv→FMM
Epi-FTC016-c36: FMM
HUE9 hESC: Conv
6F Lenti-hiPSC-c3: Conv
6F Lenti-hiPSC-c2: Conv
6F Lenti-hiPSC-c1: Conv
Epi-FTC016-c28: FMM→Conv
H1 hESC: Conv
3F Lenti-hiPSC-c4: Conv
3F Lenti-hiPSC-c1: Conv

FIG. 7D

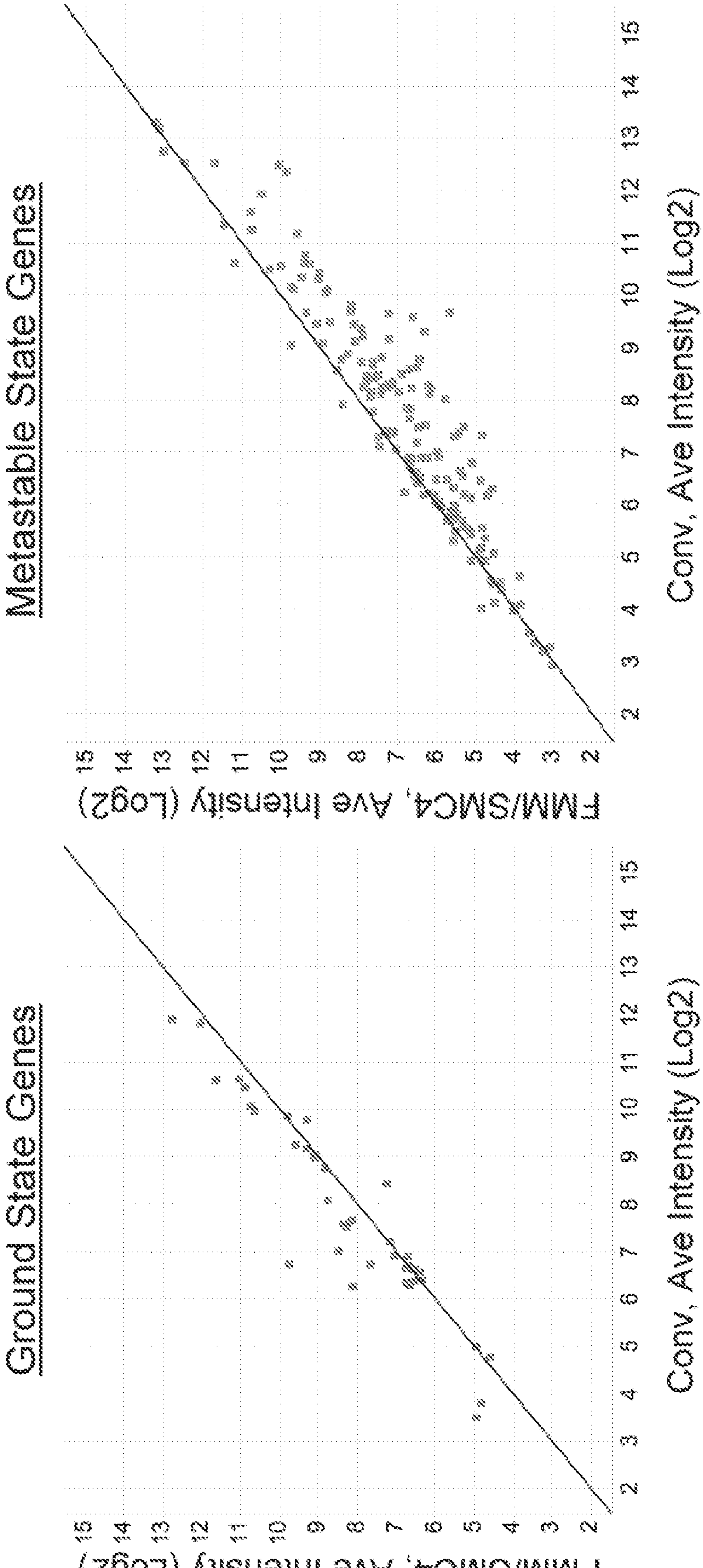

Metastable State Genes
Ground State Genes
FMM/SMC4, Ave Intensity (Log2)
Conv, Ave Intensity (Log2)
2
3
4
5
6
7
8
9
10
11
12
13
14
15

FIG. 7E

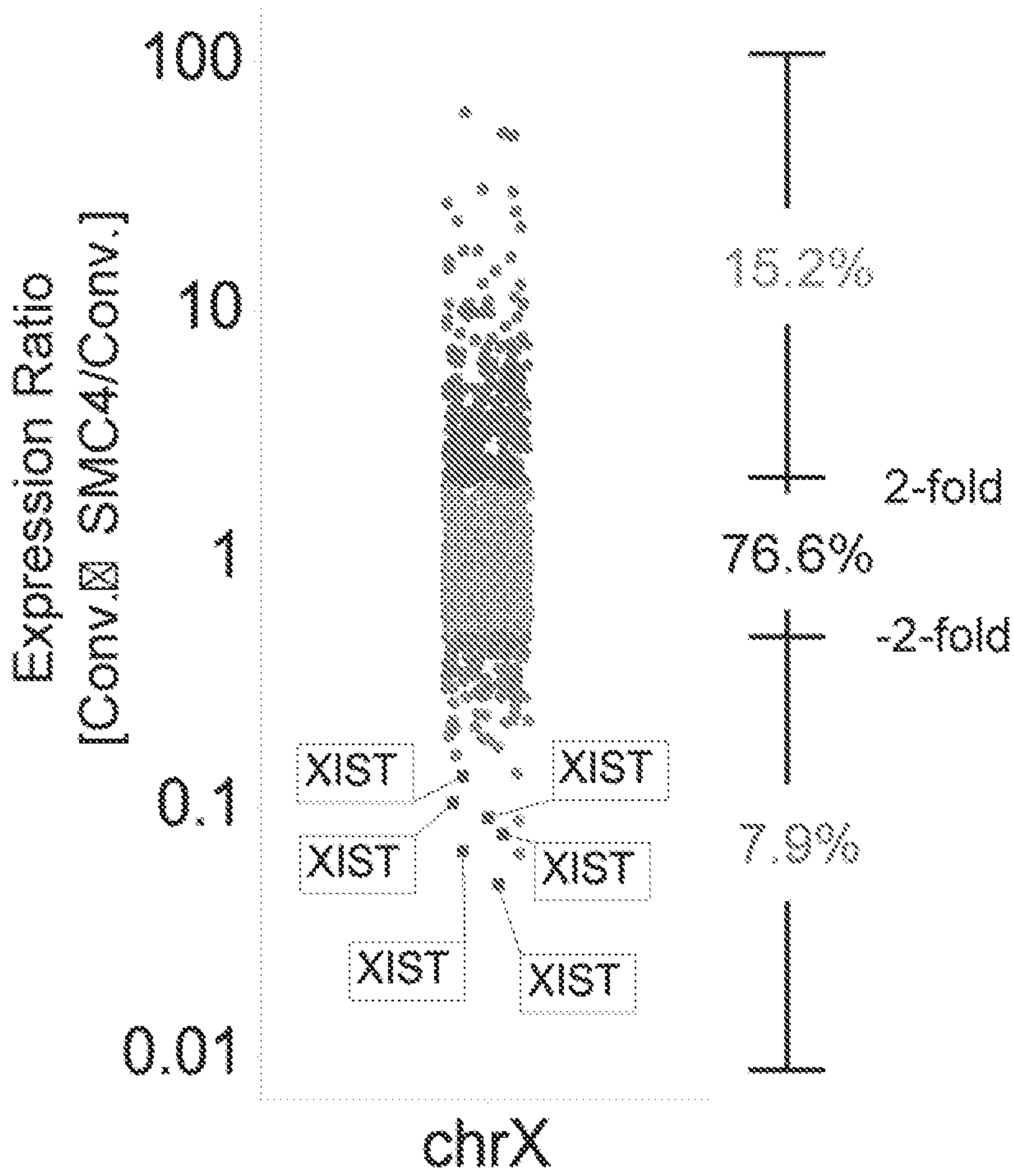

100
10
1
0.1
0.01
Expression Ratio
[Conv.⊠ SMC4/Conv.]
XIST
XIST
XIST
XIST
XIST
XIST
chrX
15.2%
2-fold
76.6%
-2-fold
7.9%

FIG. 7F

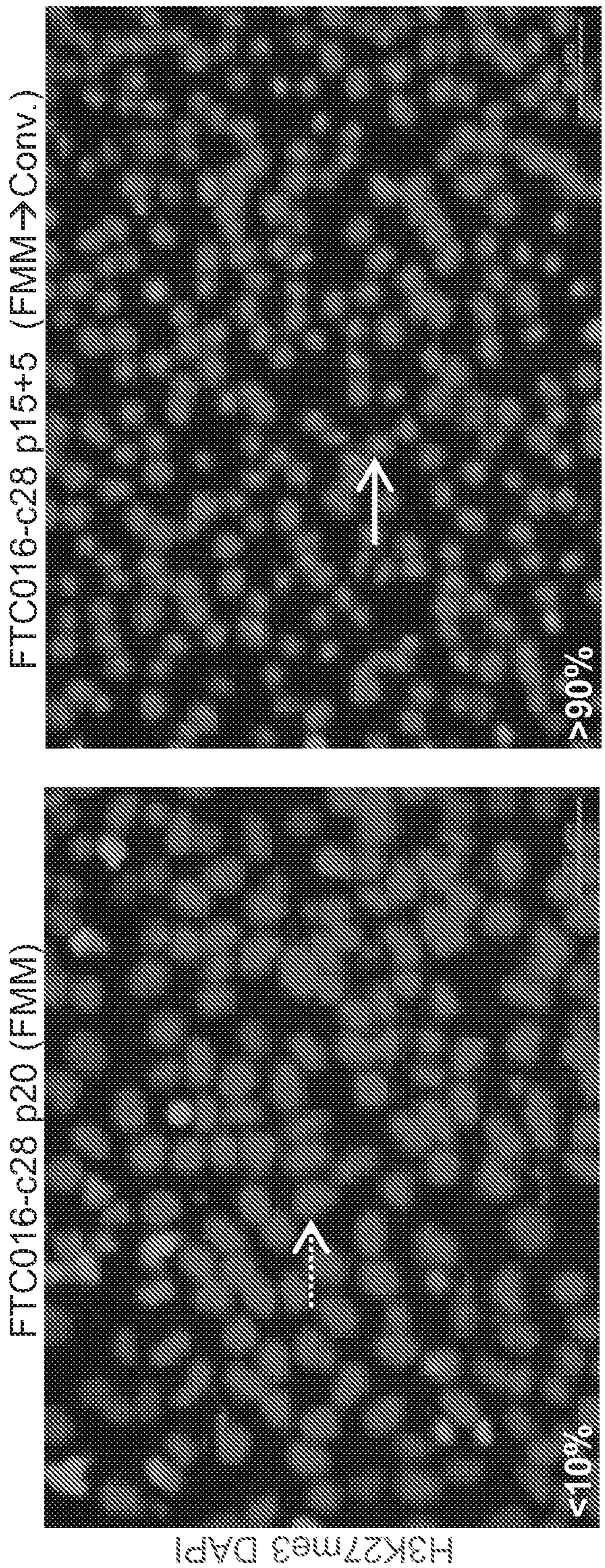

FTC016-c28 p15+5 (FMM→Conv.)
>90%
FTC016-c28 p20 (FMM)
<10%
H3K27me3 DAPI

FIG. 7G

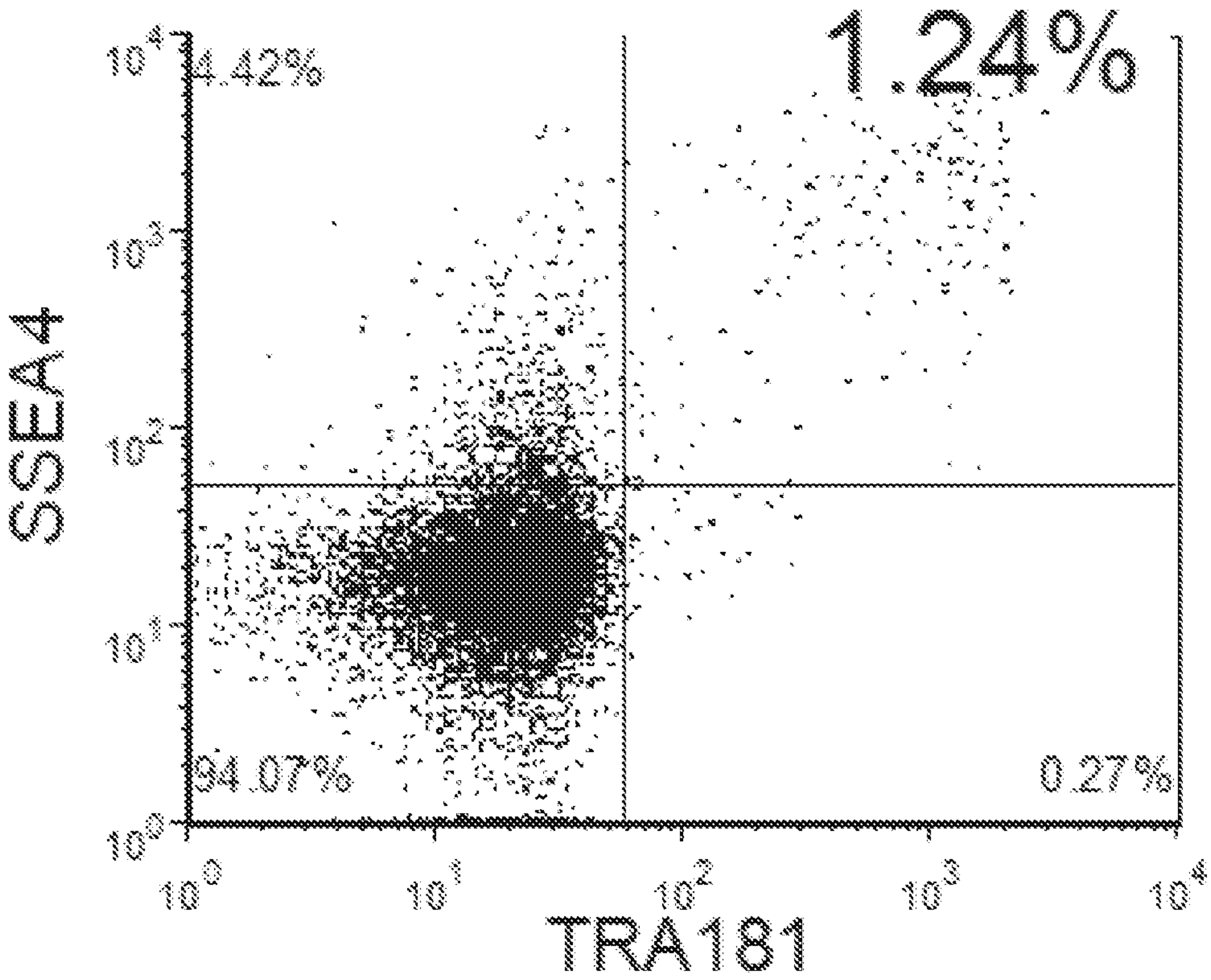


FIG. 8A

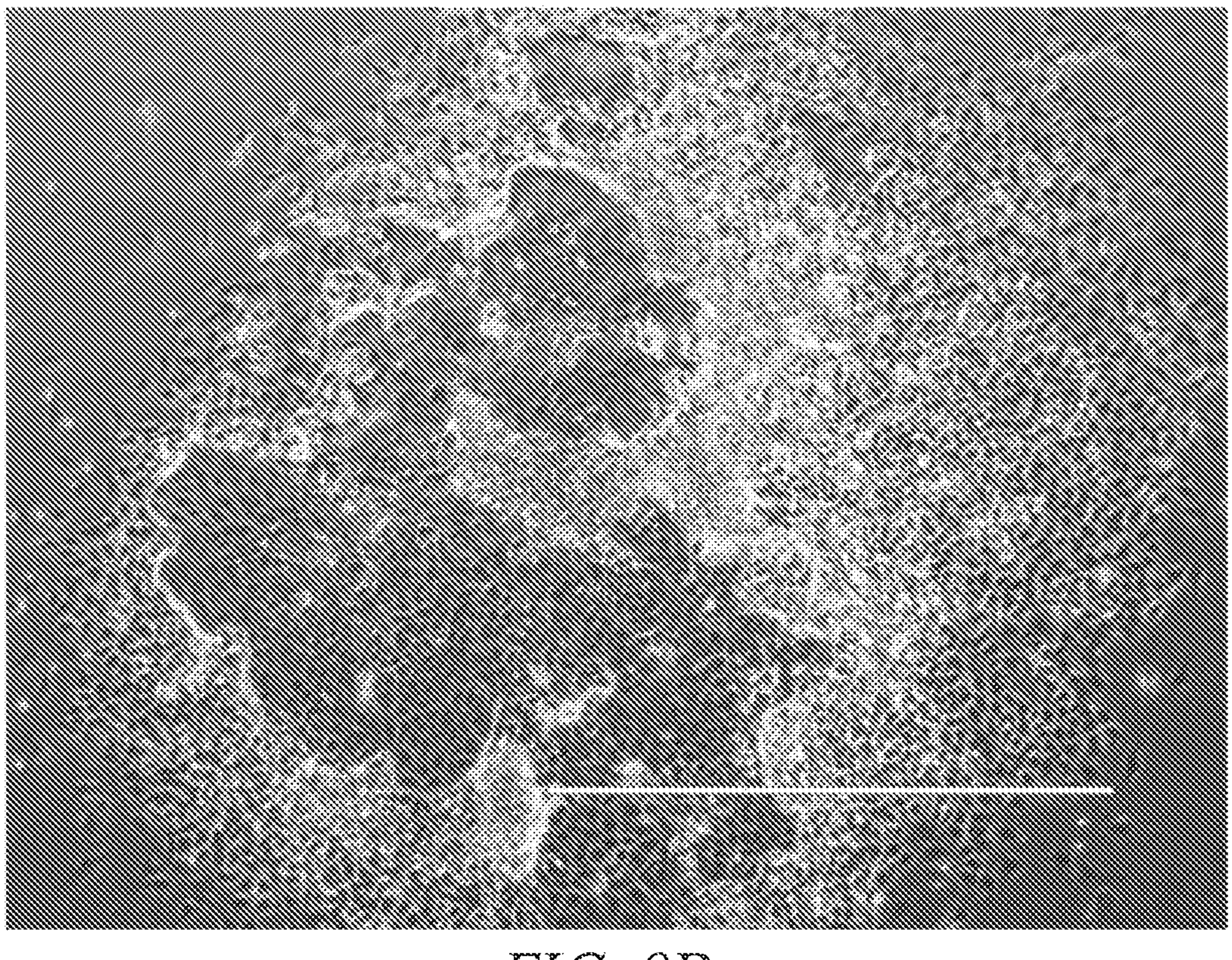


FIG. 8B

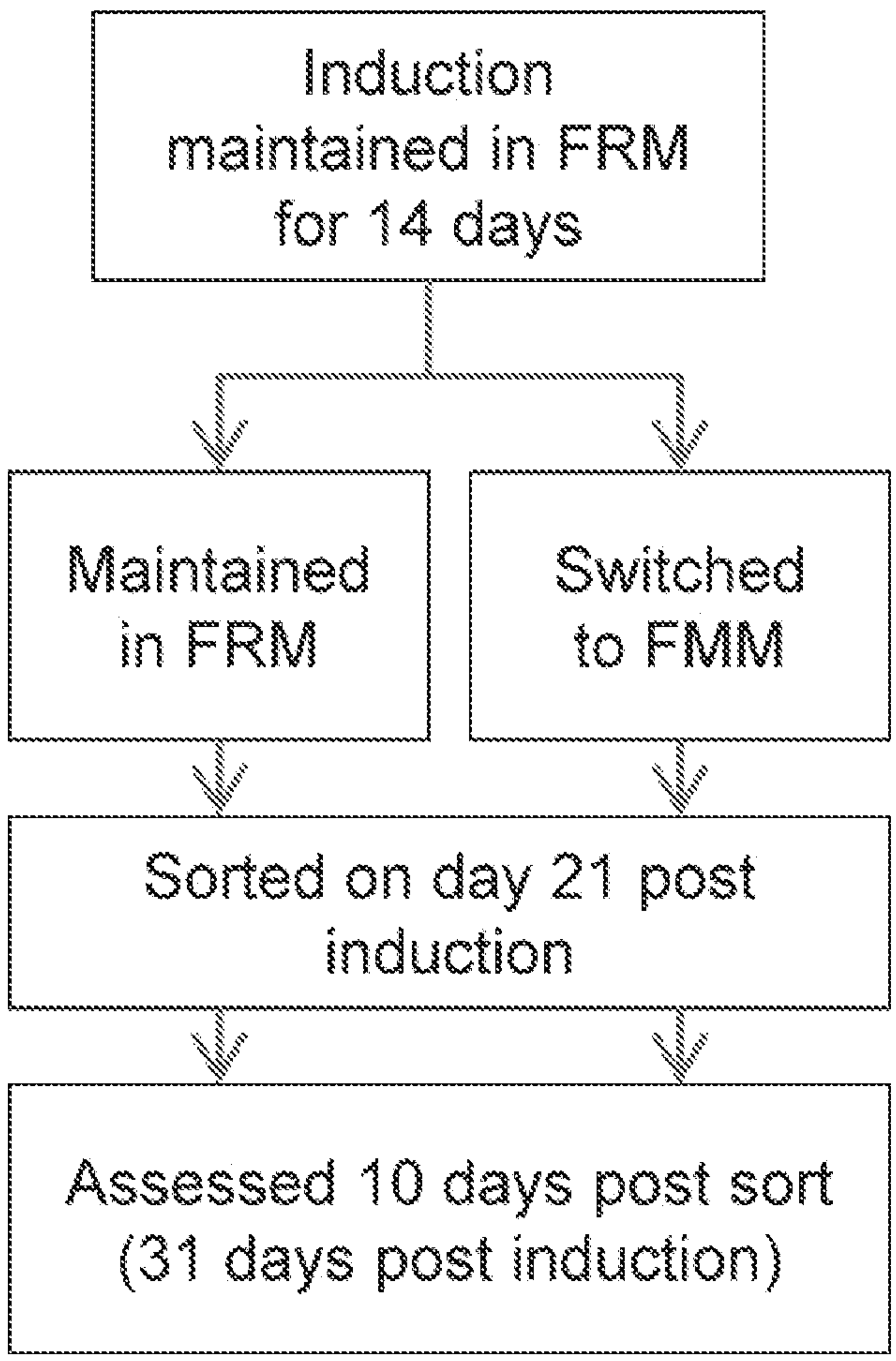

Induction maintained in FRM for 14 days
Maintained in FRM
Switched to FMM
Sorted on day 21 post induction
Assessed 10 days post sort (31 days post induction)

FIG. 8C

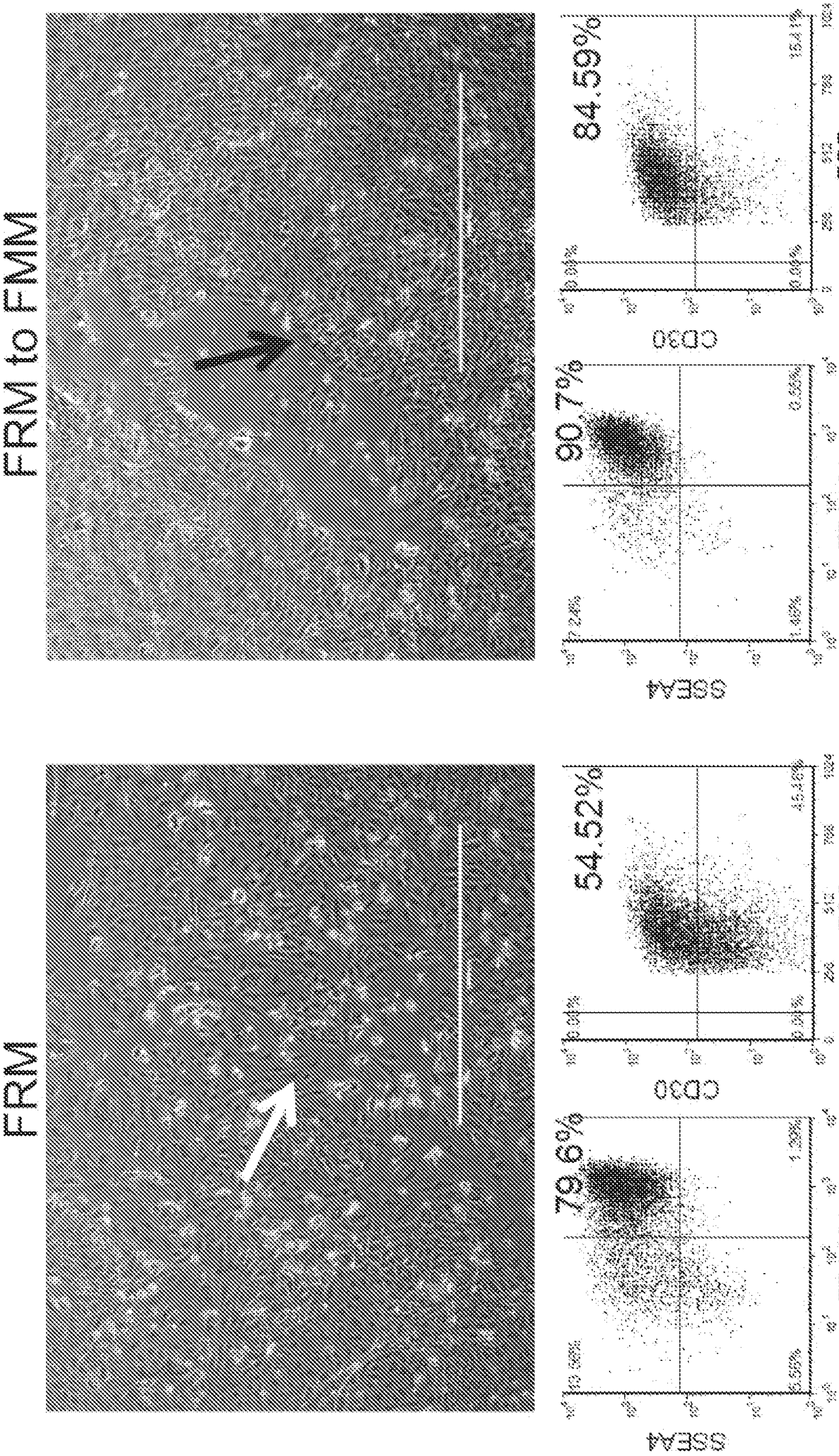


FIG. 8D

Nomenclature	Cell Type	Source	Ethnicity	Gender	Age	Culture Medium	Percent SSEA4/TRA181 Positive Population At Time of 96-Well Plate FACS
FTC007	Fibroblast	Neonatal Foreskin	Caucasian	Male	Neonatal	DMEM + 10%FBS	8.55% (Day 19 post induction)
FTC008	Fibroblast	Dermal Skin	Asian	Female	Adult	DMEM + 10%FBS	1.81% (Day 16 post induction)
FTC016	CD34+ Hematopoietic Cells	Cord Blood, AllCells	Pool	Pool	Neonatal	Stempro + CC110	29.3% (Day 19 post induction)
FTC017	CD34+ Hematopoietic Cells	Cord Blood, Fate derived	African American	Male	Neonatal	Stempro + CC110	41.2% (Day 21 post induction)

FIG. 9A

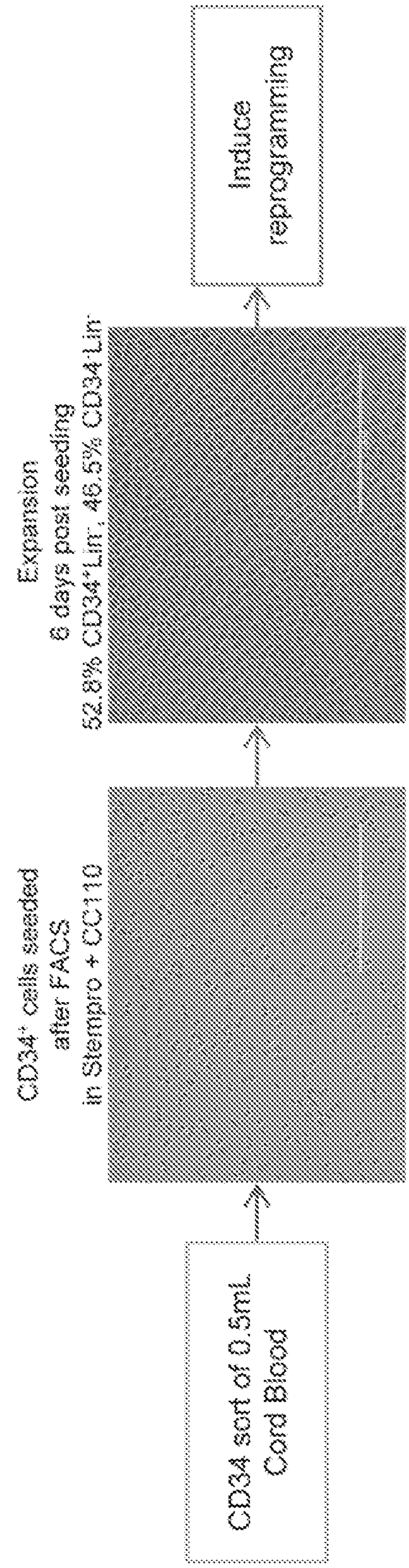

CD34 sort of 0.5mL Cord Blood
CD34+ cells seeded after FACS in Stempro + CC110
Expansion 6 days post seeding 52.8% CD34+Lin-, 46.5% CD34-Lin-
Induce reprogramming

FIG. 9B

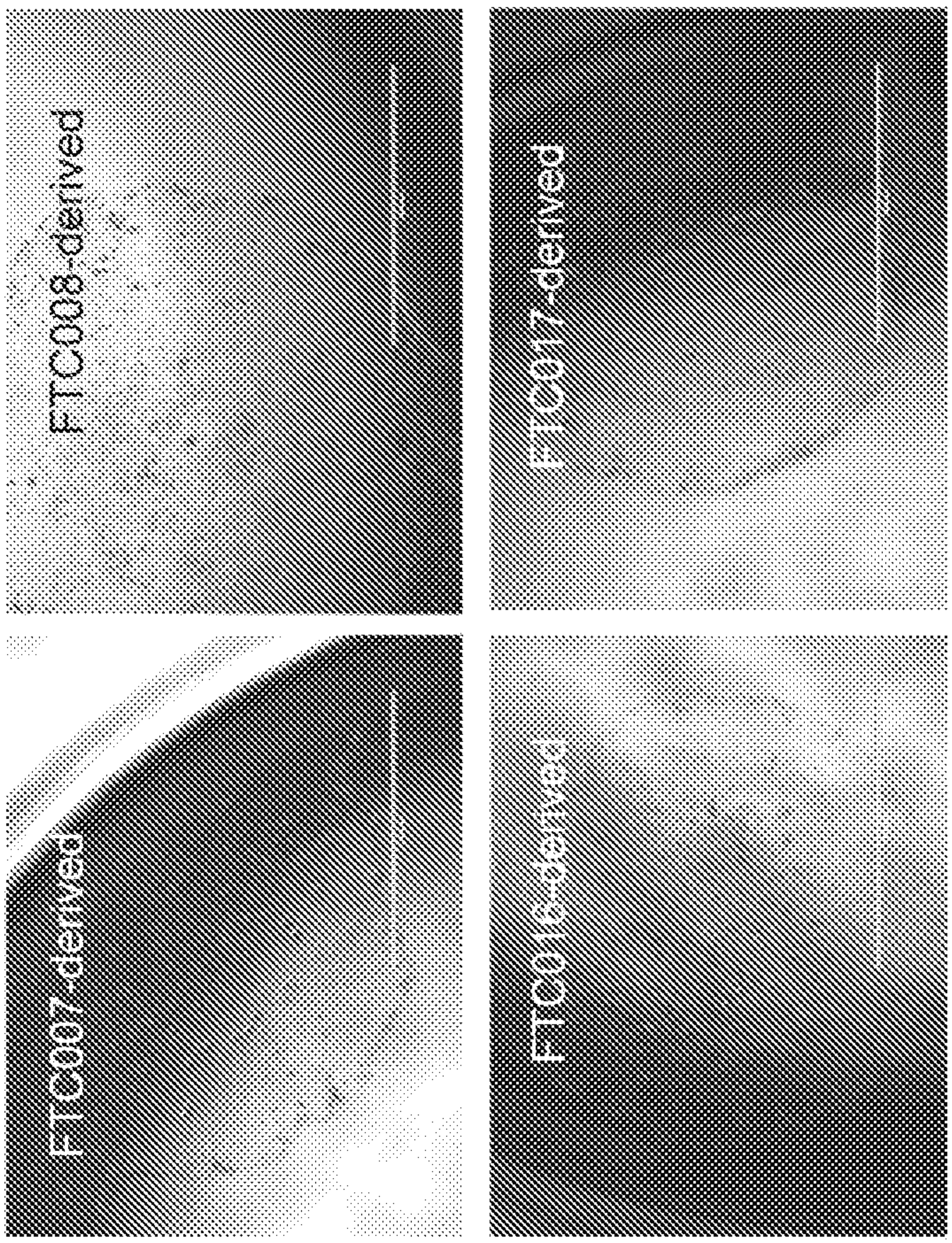


FIG. 10B

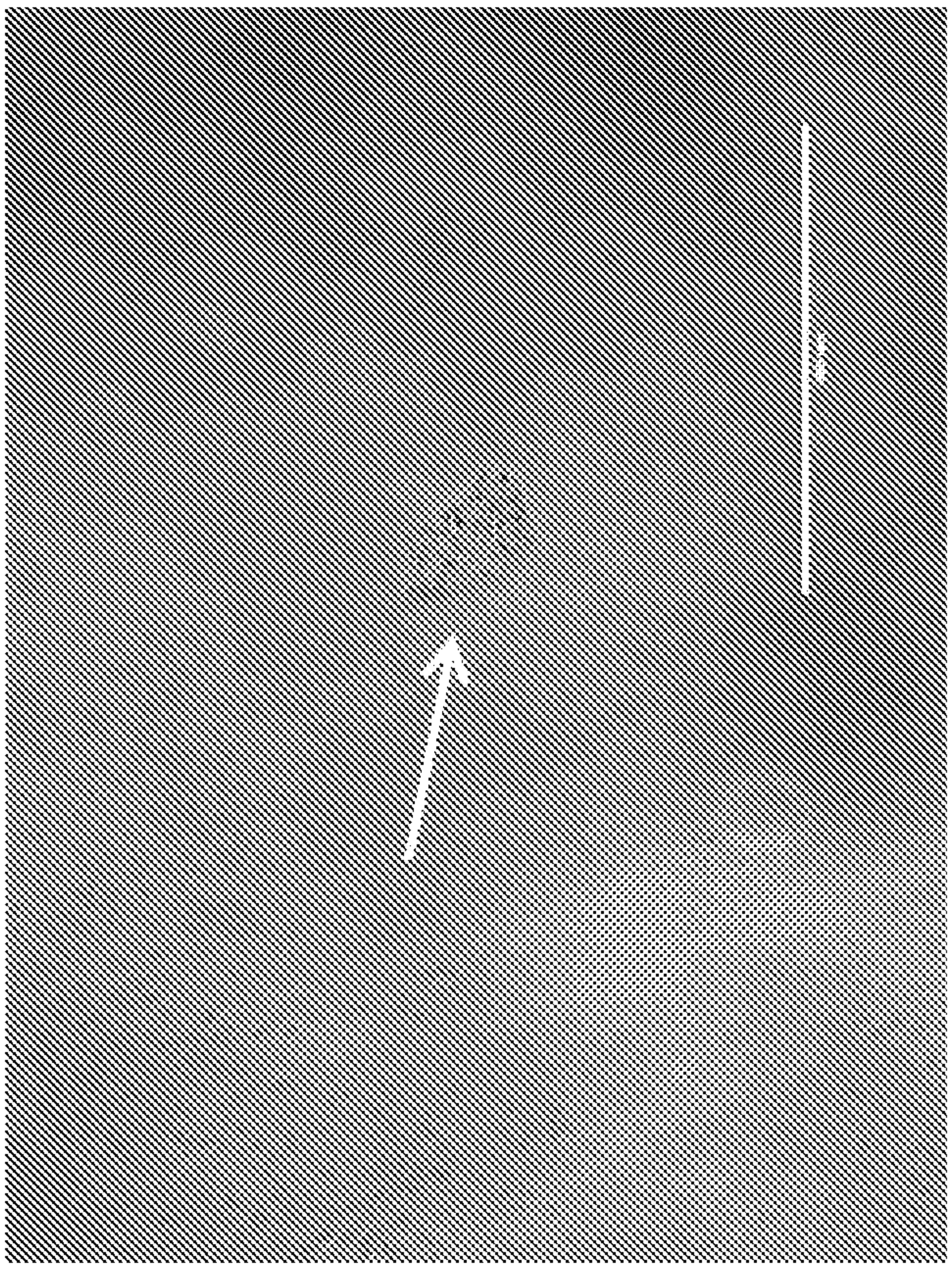


FIG. 10A

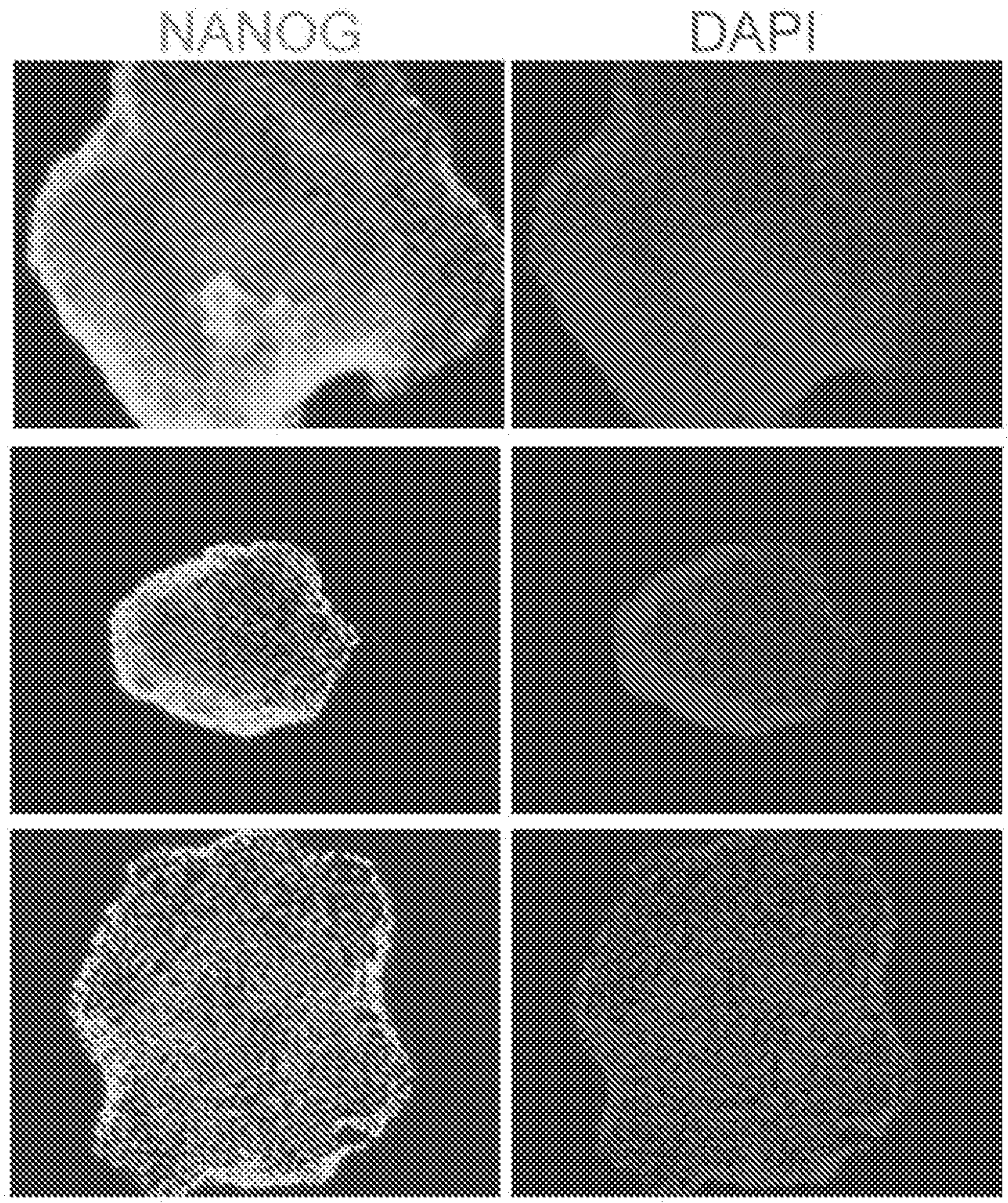


FIG. 10C

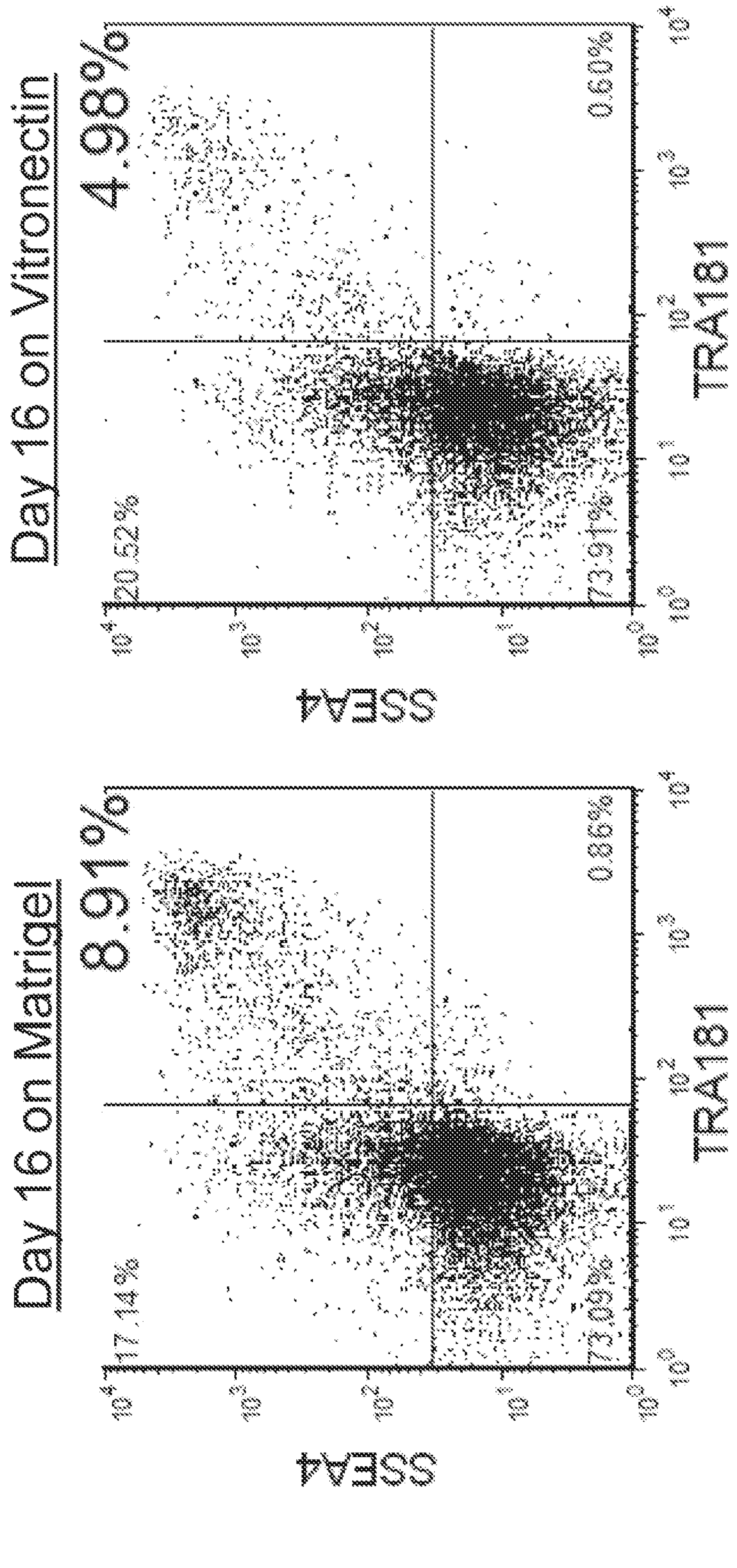

Day 16 on Vitronectin
4.98%
20.52%
0.60%
73.91%
SSEA4
TRA181
Day 16 on Matrigel
8.91%
17.14%
0.86%
73.09%
SSEA4
TRA181

FIG. 10D

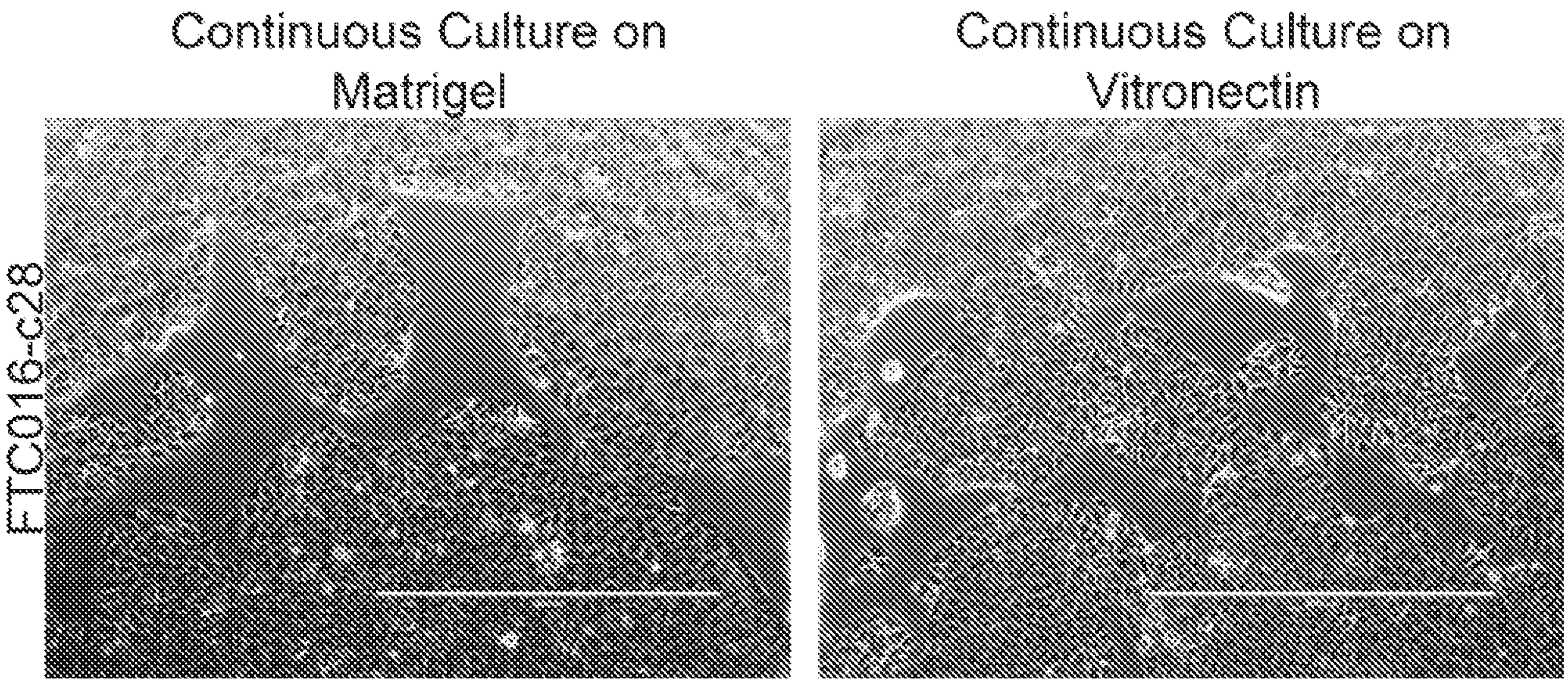


FIG. 10E

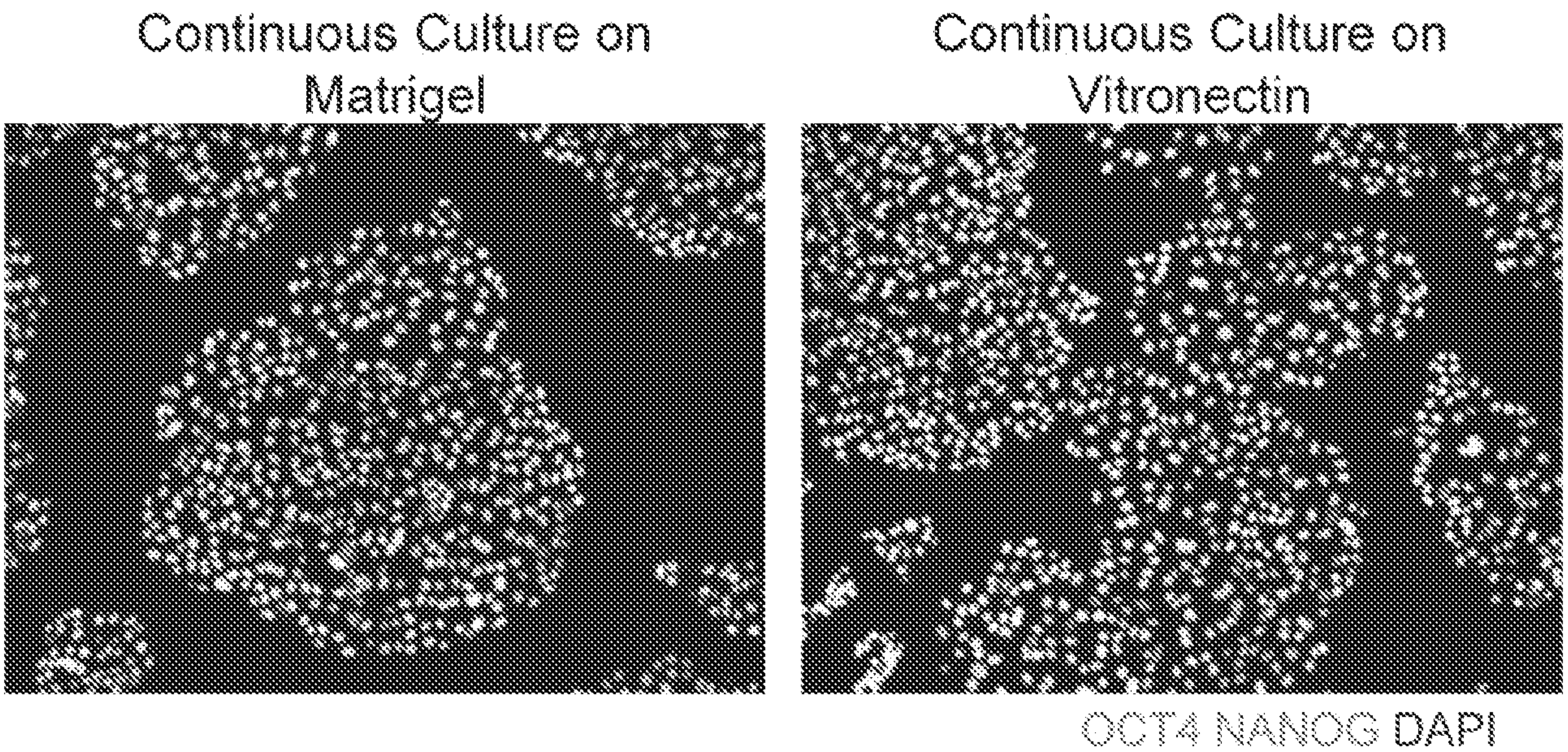


FIG. 10F

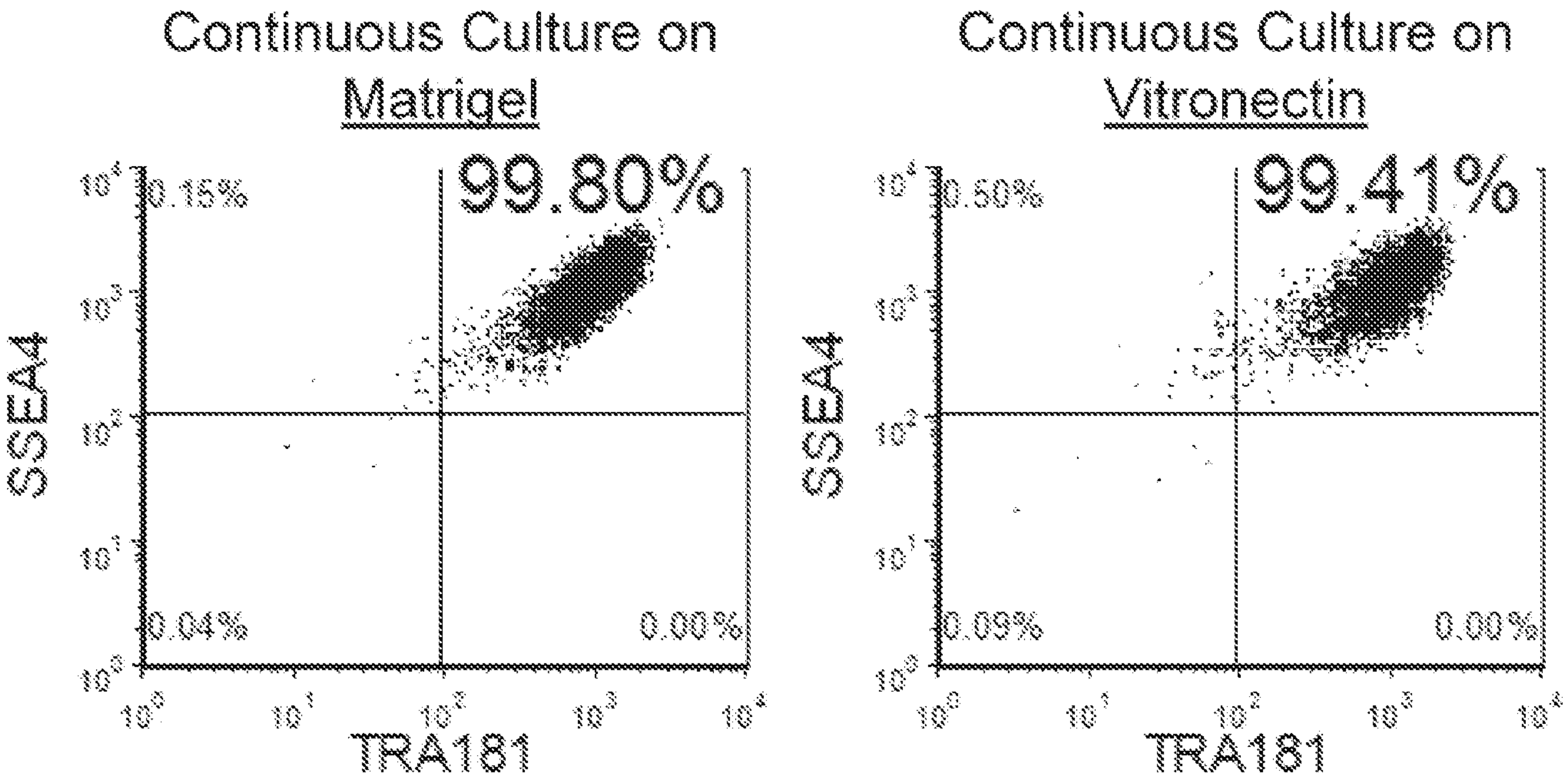

Continuous Culture on Matrigel
99.80%
0.15%
0.04%
0.00%
SSEA4
TRA181
Continuous Culture on Vitronectin
99.41%
0.50%
0.09%
0.00%
SSEA4
TRA181

FIG. 10G

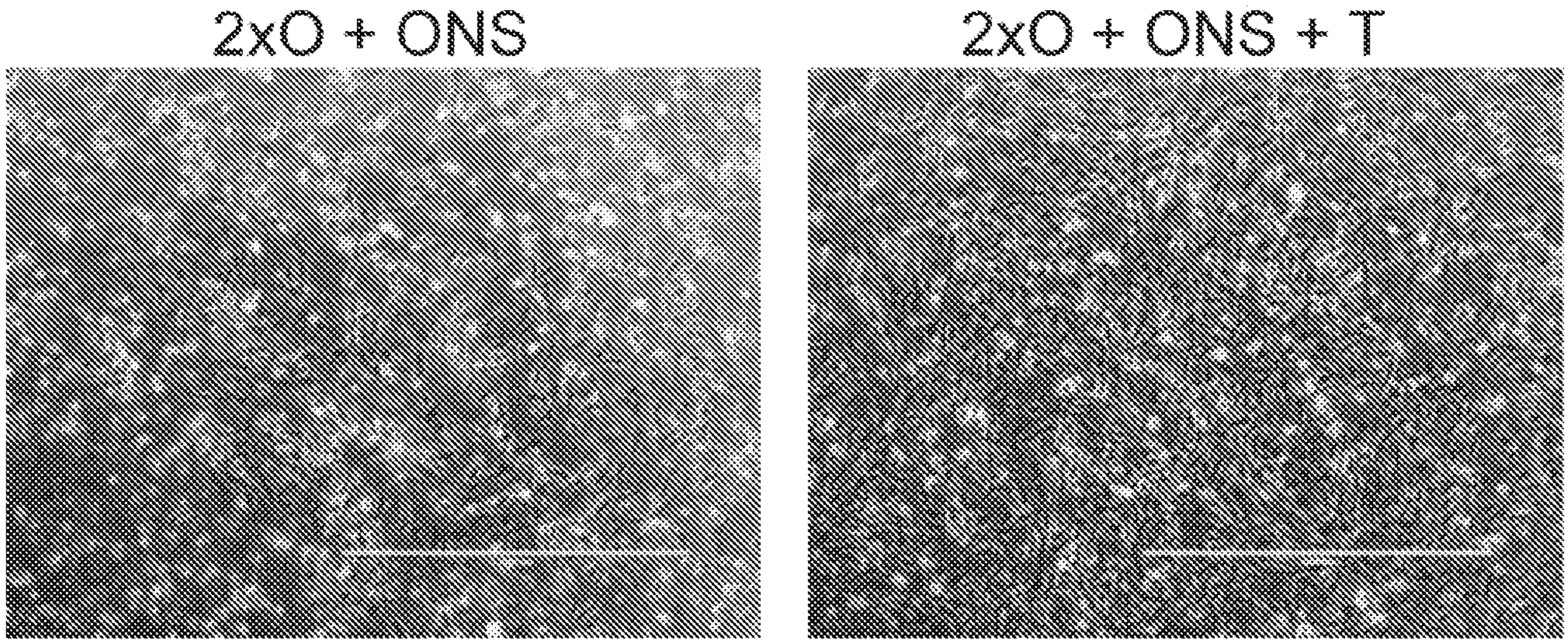


FIG. 11A

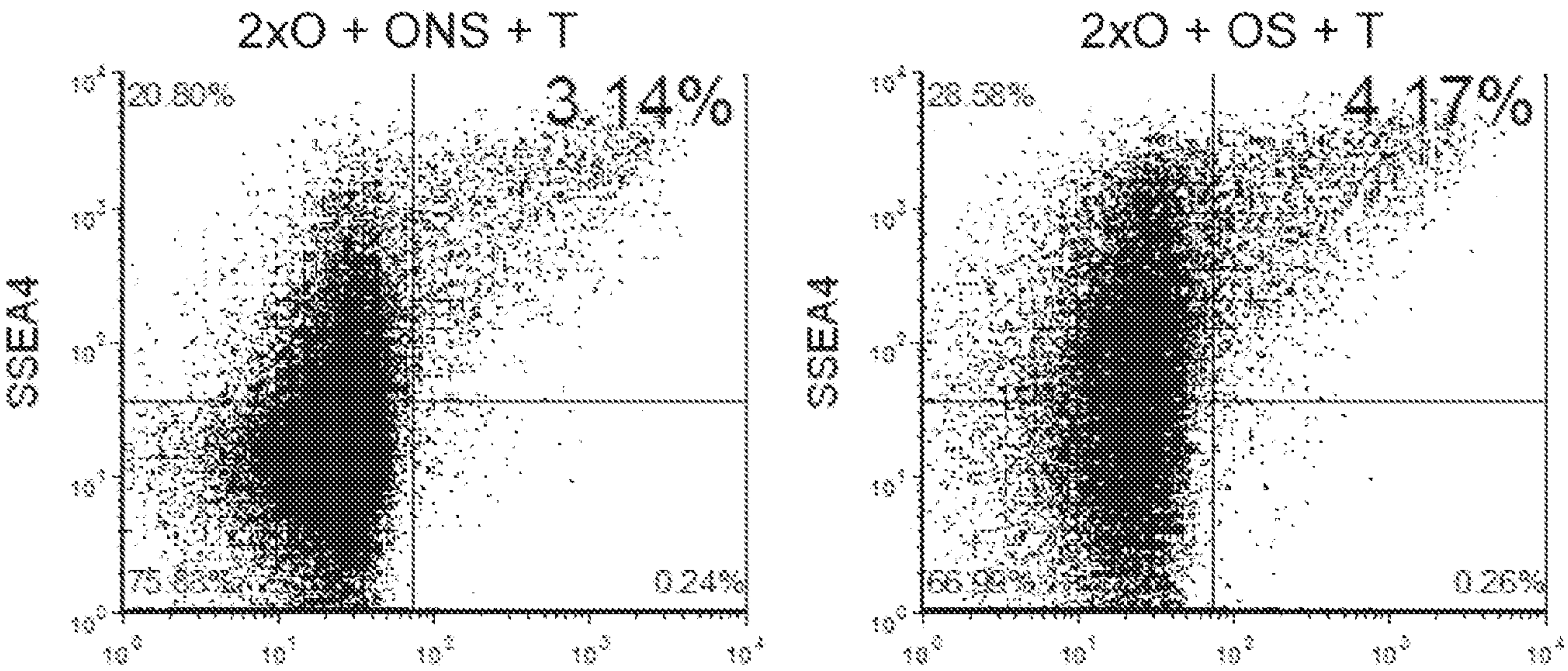


FIG. 11B

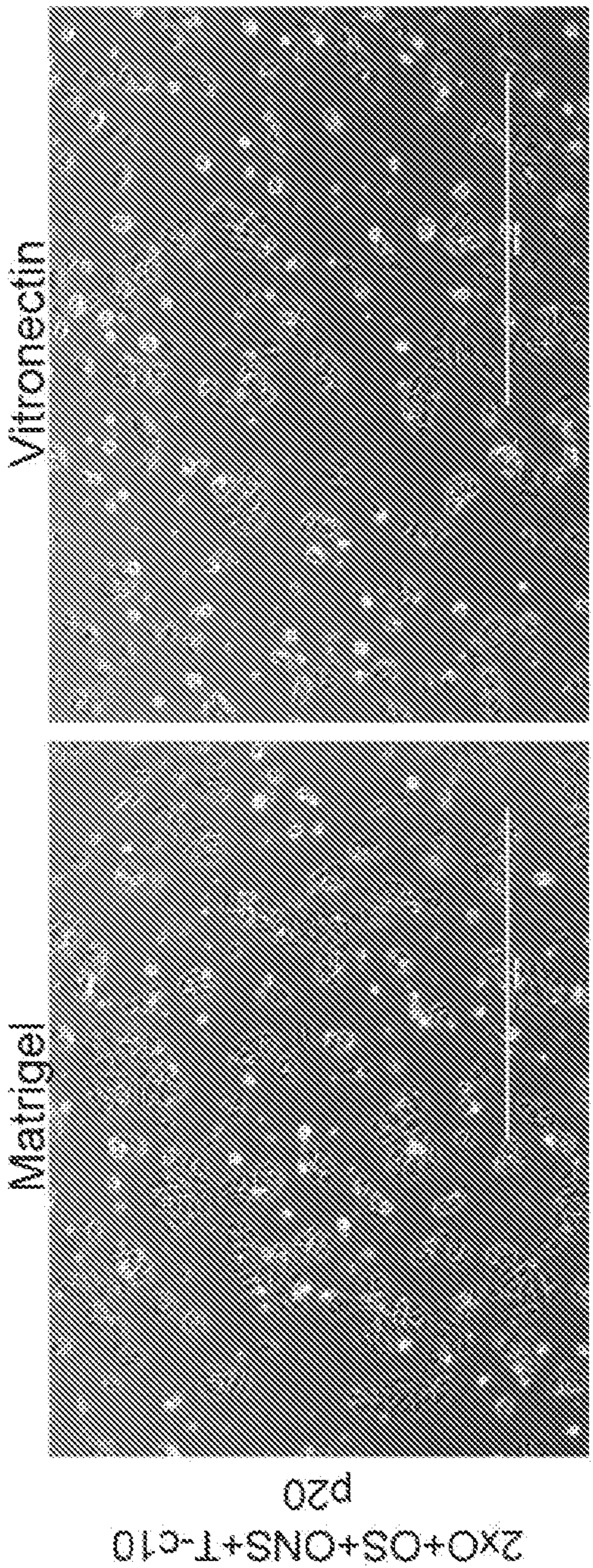


FIG. 11C

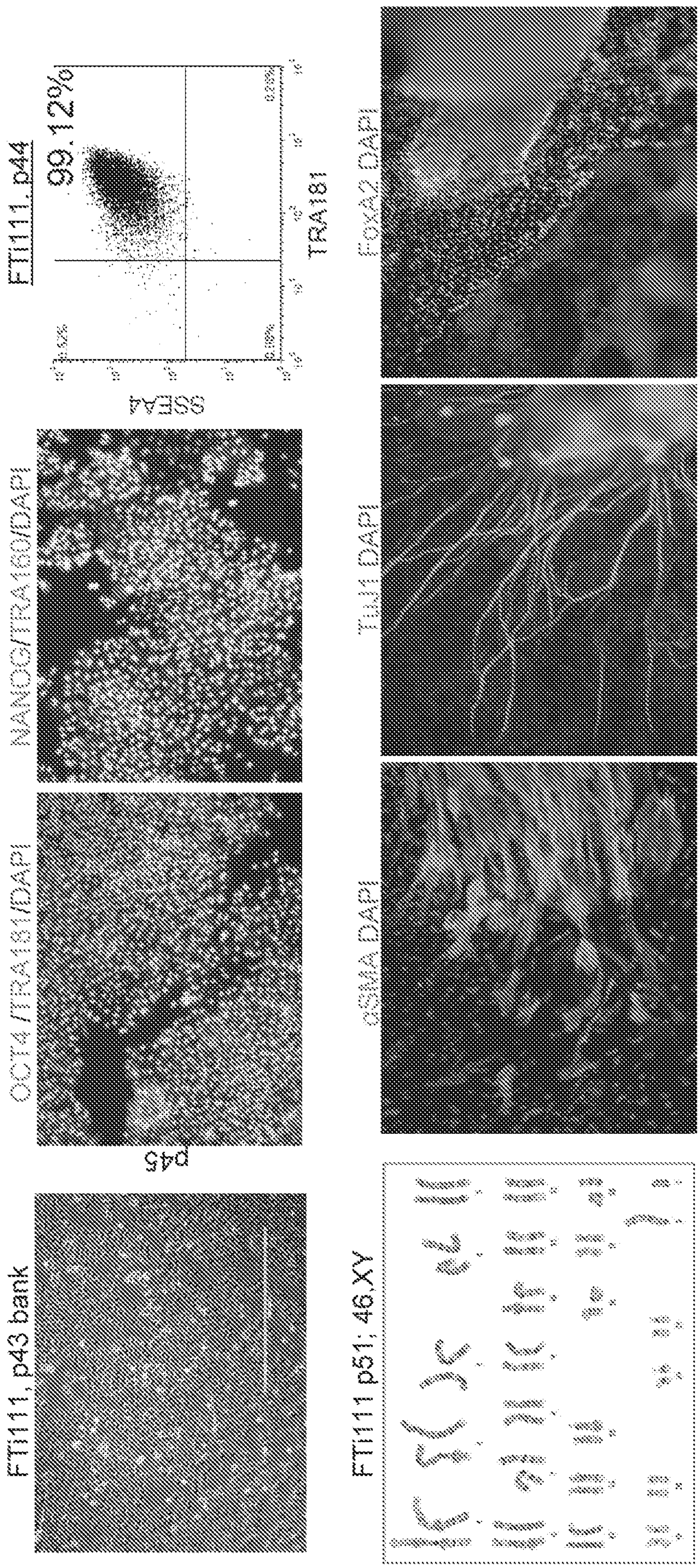

FTi111, p43 bank
p45
OCT4 /TRA181/DAPI
NANOG/TRA160/DAPI
FTi111, p44
99.12%
SSEA4
TRA181
FTi111 p51; 46,XY
αSMA DAPI
TuJ1 DAPI
FoxA2 DAPI

FIG. 12A

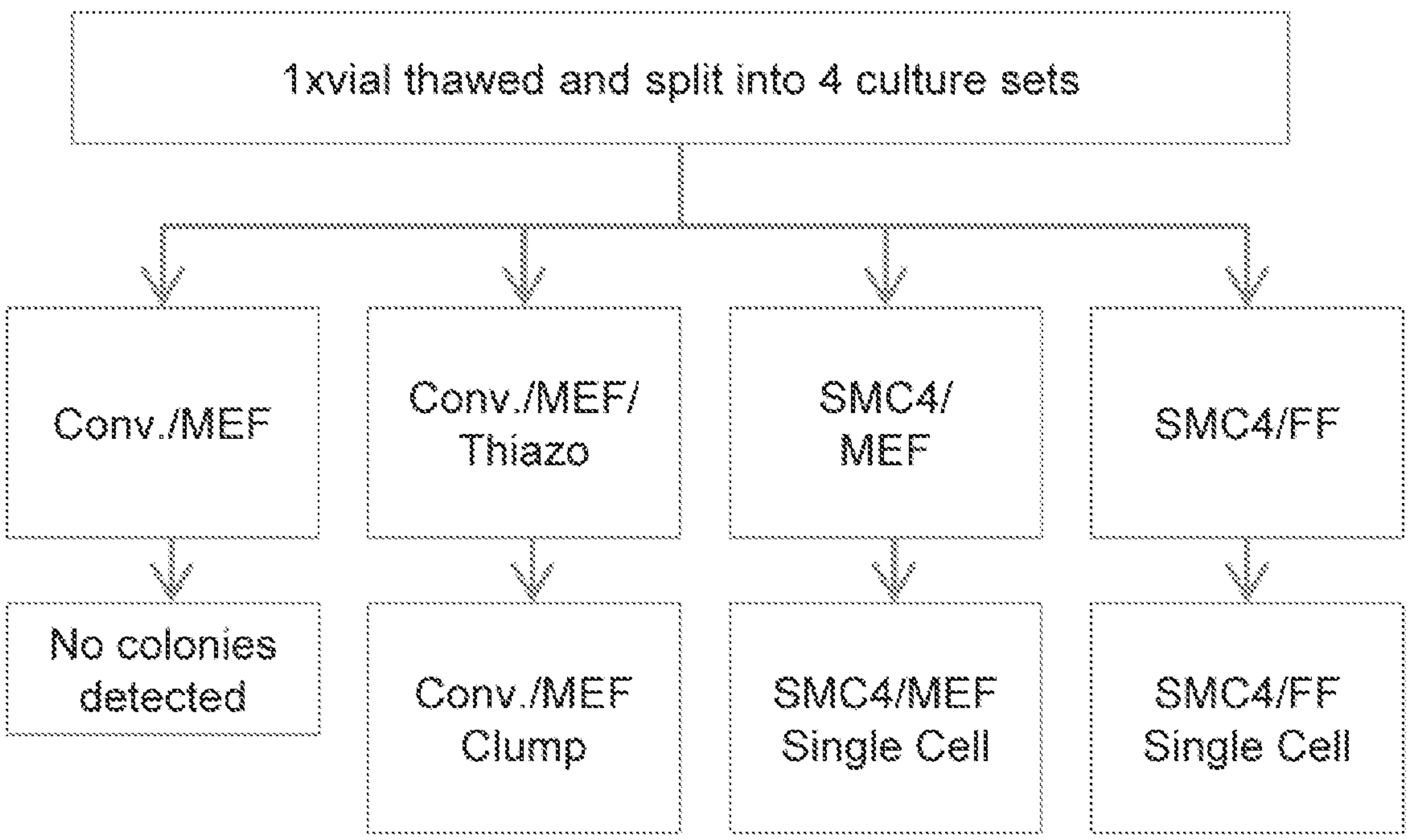

1xvial thawed and split into 4 culture sets
Conv./MEF
Conv./MEF/ Thiazo
SMC4/ MEF
SMC4/FF
No colonies detected
Conv./MEF Clump
SMC4/MEF Single Cell
SMC4/FF Single Cell

FIG. 12B

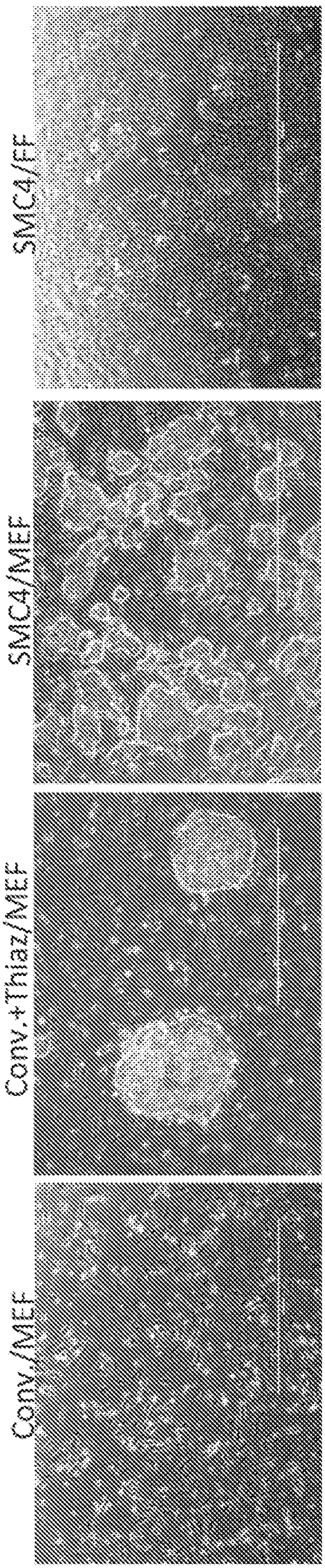


FIG. 12C

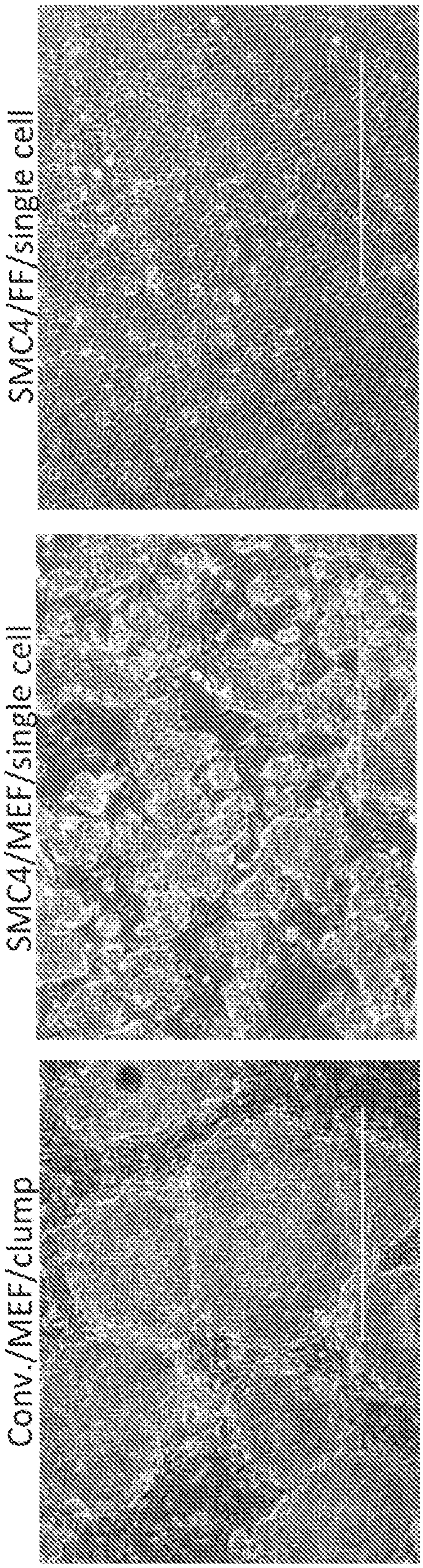


FIG. 12D

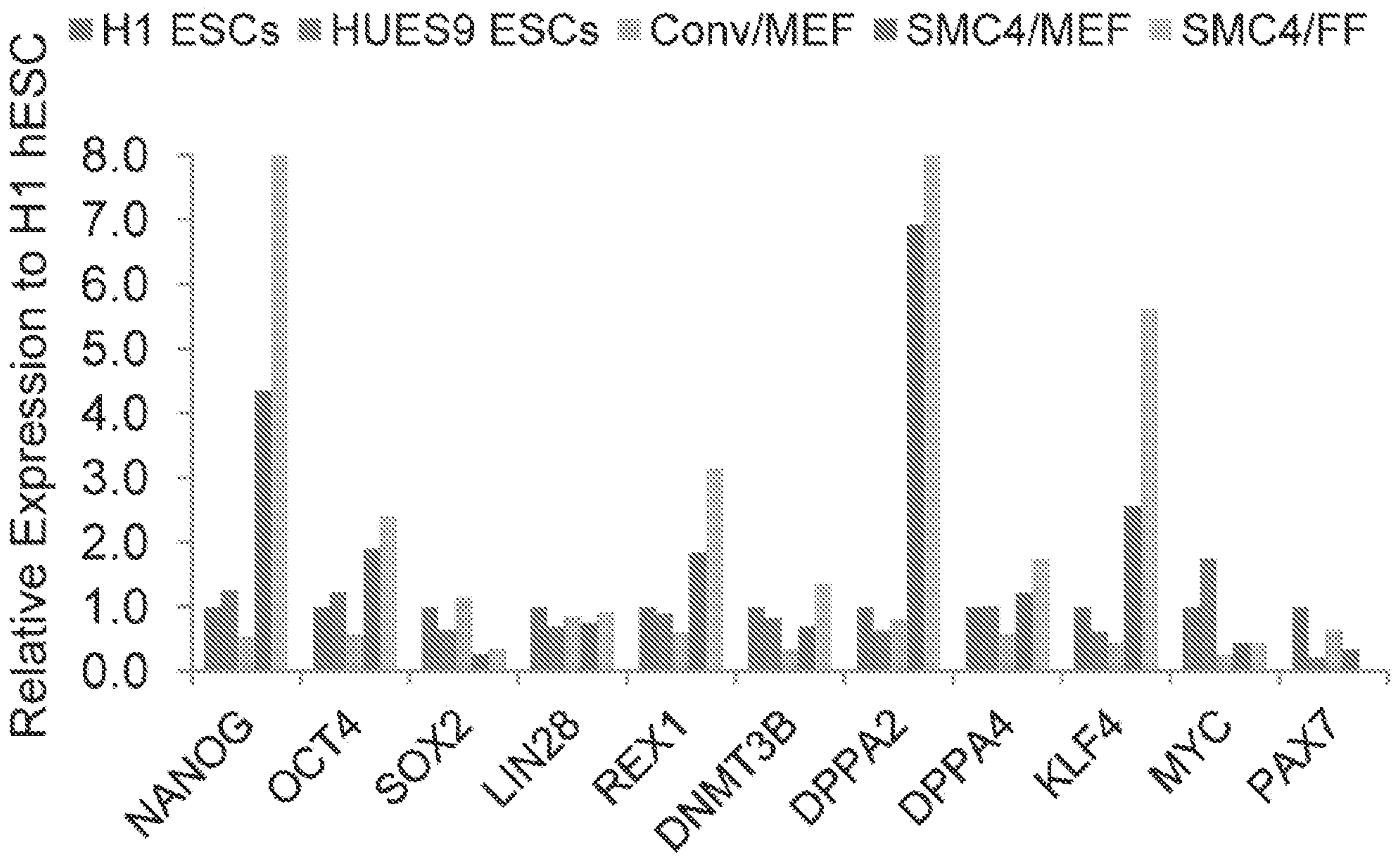

H1 ESCs
HUES9 ESCs
Conv/MEF
SMC4/MEF
SMC4/FF
Relative Expression to H1 hESC
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
NANOG
OCT4
SOX2
LIN28
REX1
DNMT3B
DPPA2
DPPA4
KLF4
MYC
PAX7

FIG. 12E

Cell Line	Description		Cell Line	Description
FTC016-c28 & -c36	Cord blood parental, episomal-generated and maintained in FRM/FMM		6F Lenti-hiPSC-c1, -c2 & -c3	Fibroblast parental, OSKMNL lentiviral hiPSC clone generated and maintained in conventional hESC culture
FTC007-C21	Fibroblast parental, episomal-generated and maintained in FRM/FMM		3F Lenti-hiPSC-c1 & -c4	Fibroblast parental, OKS lentiviral hiPSC clone generated and maintained in conventional hESC culture
3F Lenti-hiPSC-c2 & -c3	Fibroblast parental, OKS lentiviral hiPSC clone generated and maintained in SMC4			

FIG. 13A

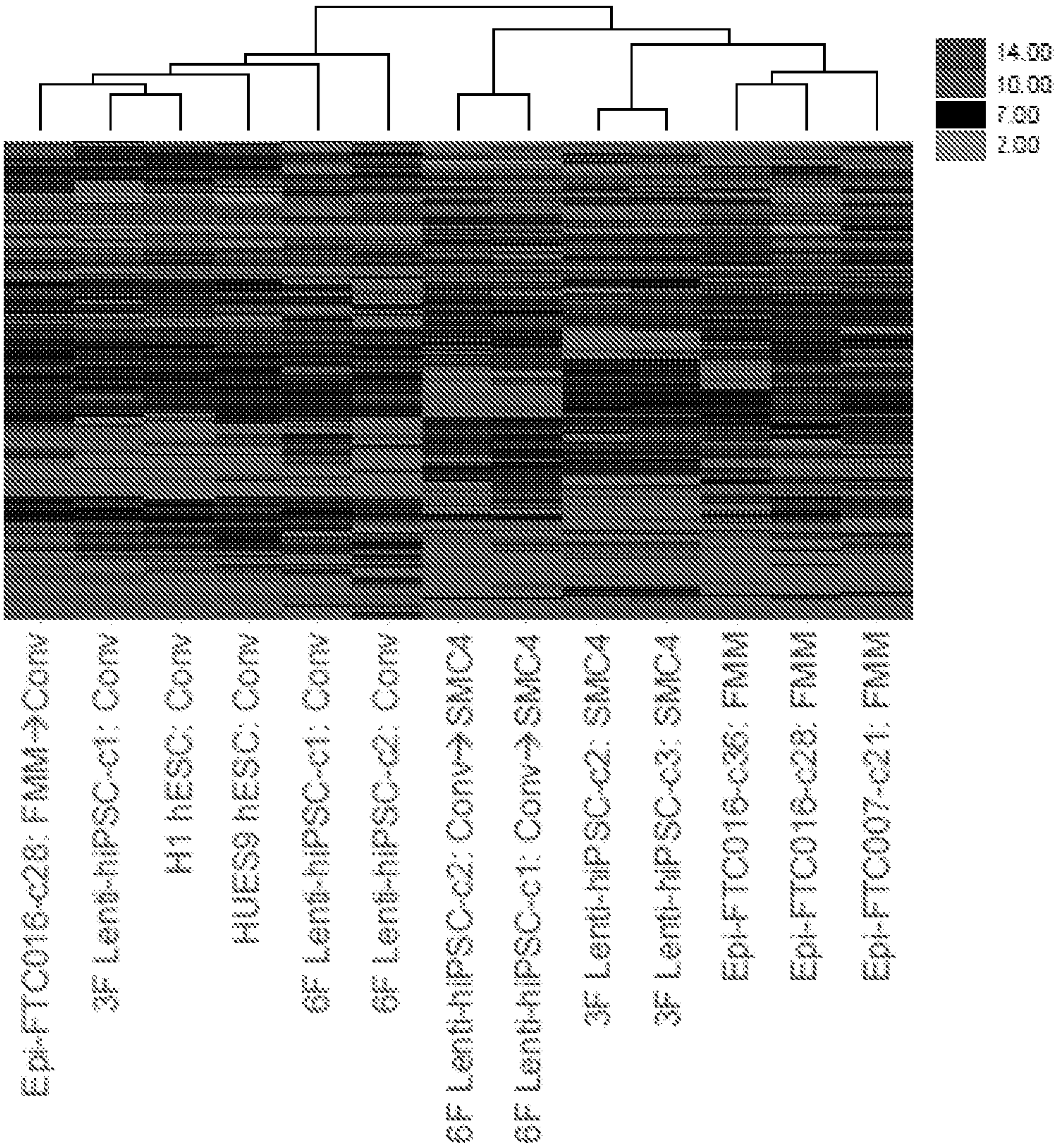

14.00
10.00
7.00
2.00
Epi-FTC016-c28: FMM→Conv
3F Lenti-hiPSC-c1: Conv
H1 hESC: Conv
HUES9 hESC: Conv
6F Lenti-hiPSC-c1: Conv
6F Lenti-hiPSC-c2: Conv
6F Lenti-hiPSC-c2: Conv→SMC4
6F Lenti-hiPSC-c1: Conv→SMC4
3F Lenti-hiPSC-c2: SMC4
3F Lenti-hiPSC-c3: SMC4
Epi-FTC016-c36: FMM
Epi-FTC016-c28: FMM
Epi-FTC007-c21: FMM

FIG. 13B

133 Probe sets upregulated in conventional hESC culture group vs. FMM/SMC4 culture group			
GO ID	**GO Term**	**%**	**p-value**
GO:0007389	pattern specification process	9.7	3.50E-05
GO:0048598	embryonic morphogenesis	8.6	5.90E-04
GO:0001775	cell activation	7.5	2.30E-03
GO:0046649	lymphocyte activation	6.5	2.50E-03
GO:0042110	T cell activation	5.4	3.00E-03
GO:0002521	leukocyte differentiation	5.4	3.40E-03
GO:0030217	T cell differentiation	4.3	3.60E-03
GO:0030097	hemopoiesis	6.5	5.10E-03
GO:0045321	leukocyte activation	6.5	5.70E-03
GO:0007610	behavior	8.6	6.50E-03
GO:0048534	hemopoietic or lymphoid organ development	6.5	7.60E-03
GO:0048568	embryonic organ development	5.4	8.90E-03
GO:0002520	immune system development	6.5	9.80E-03

FIG. 13C

167 Probe sets upregulated in small molecule culture group vs. conventional culture group			
GO ID	**GO Term**	**%**	**p-value**
GO:0042127	regulation of cell proliferation	12.4	5.20E-04
GO:0051205	protein insertion into membrane	2.5	1.50E-03
GO:0009725	response to hormone stimulus	7.4	2.60E-03
GO:0051668	localization within membrane	2.5	4.20E-03
GO:0009719	response to endogenous stimulus	7.4	4.80E-03
GO:0008284	positive regulation of cell proliferation	7.4	5.50E-03
GO:0008406	gonad development	4.1	6.10E-03
GO:0009636	response to toxin	3.3	7.30E-03
GO:0014070	response to organic cyclic substance	4.1	8.00E-03
GO:0043066	negative regulation of apoptosis	6.6	8.20E-03
GO:0048545	response to steroid hormone stimulus	5	8.30E-03
GO:0008585	female gonad development	3.3	8.30E-03
GO:0043069	negative regulation of programmed cell death	6.6	8.80E-03
GO:0060548	negative regulation of cell death	6.6	9.00E-03
GO:0048608	reproductive structure development	4.1	9.20E-03
GO:0045137	development of primary sexual characteristics	4.1	9.40E-03
GO:0046660	female sex differentiation	3.3	1.00E-02
GO:0046545	development of primary female sexual characteristics	3.3	1.00E-02

FIG. 13D

126 Probe sets upregulated in FMM samples group vs. conventional culture group			
GO ID	GO Term	%	p-value
GO:0007267	cell-cell signaling	16.5	8.20E-07
GO:0040008	regulation of growth	10.3	1.10E-04
GO:0042981	regulation of apoptosis	15.5	1.20E-04
GO:0043067	regulation of programmed cell death	15.5	1.30E-04
GO:0010941	regulation of cell death	15.5	1.30E-04
GO:0010817	regulation of hormone levels	7.2	1.90E-04
GO:0045926	negative regulation of growth	6.2	3.60E-04
GO:0031100	organ regeneration	4.1	3.90E-04
GO:0001558	regulation of cell growth	7.2	7.10E-04
GO:0043066	negative regulation of apoptosis	9.3	7.40E-04
GO:0043069	negative regulation of programmed cell death	9.3	8.20E-04
GO:0060548	negative regulation of cell death	9.3	8.30E-04
GO:0016358	dendrite development	4.1	9.40E-04
GO:0006916	anti-apoptosis	7.2	9.70E-04
GO:0045596	negative regulation of cell differentiation	7.2	1.20E-03
GO:0030308	negative regulation of cell growth	5.2	1.70E-03
GO:0050768	negative regulation of neurogenesis	4.1	2.00E-03
GO:0045792	negative regulation of cell size	5.2	2.20E-03
GO:0010721	negative regulation of cell development	4.1	2.40E-03
GO:0042445	hormone metabolic process	5.2	2.80E-03
GO:0032535	regulation of cellular component size	7.2	3.90E-03
GO:0034754	cellular hormone metabolic process	4.1	4.30E-03
GO:0051270	regulation of cell motion	6.2	4.30E-03
GO:0048754	branching morphogenesis of a tube	4.1	5.60E-03
GO:0008361	regulation of cell size	6.2	5.70E-03
GO:0001569	patterning of blood vessels	3.1	5.90E-03
GO:0031099	regeneration	4.1	6.60E-03
GO:0035295	tube development	6.2	7.50E-03
GO:0009991	response to extracellular stimulus	6.2	7.50E-03
GO:0001763	morphogenesis of a branching structure	4.1	8.00E-03
GO:0006917	induction of apoptosis	7.2	8.60E-03
GO:0012502	induction of programmed cell death	7.2	8.70E-03

FIG. 13E

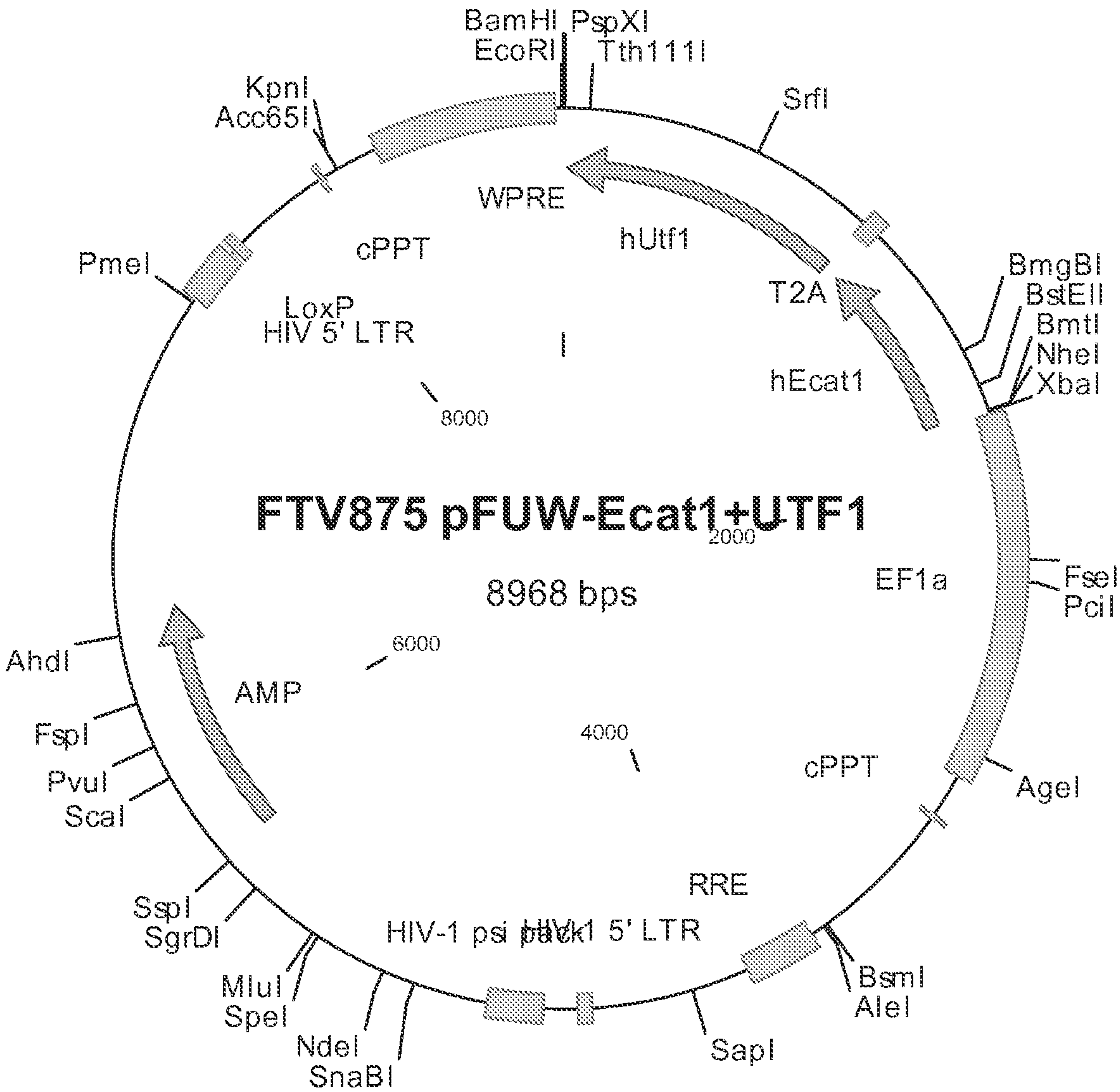

BamHI
PspXI
EcoRI
Tth111I
SrfI
KpnI
Acc65I
WPRE
hUtf1
cPPT
PmeI
LoxP
HIV 5' LTR
T2A
BmgBI
BstEII
BmtI
NheI
XbaI
hEcat1
8000
FTV875 pFUW-Ecat1+UTF1
2000
8968 bps
EF1a
FseI
PciI
AhdI
6000
AMP
FspI
PvuI
ScaI
4000
cPPT
AgeI
RRE
SspI
SgrDI
HIV-1 psi pack
HIV-1 5' LTR
MluI
SpeI
BsmI
AleI
NdeI
SnaBI
SapI

FIG. 14A

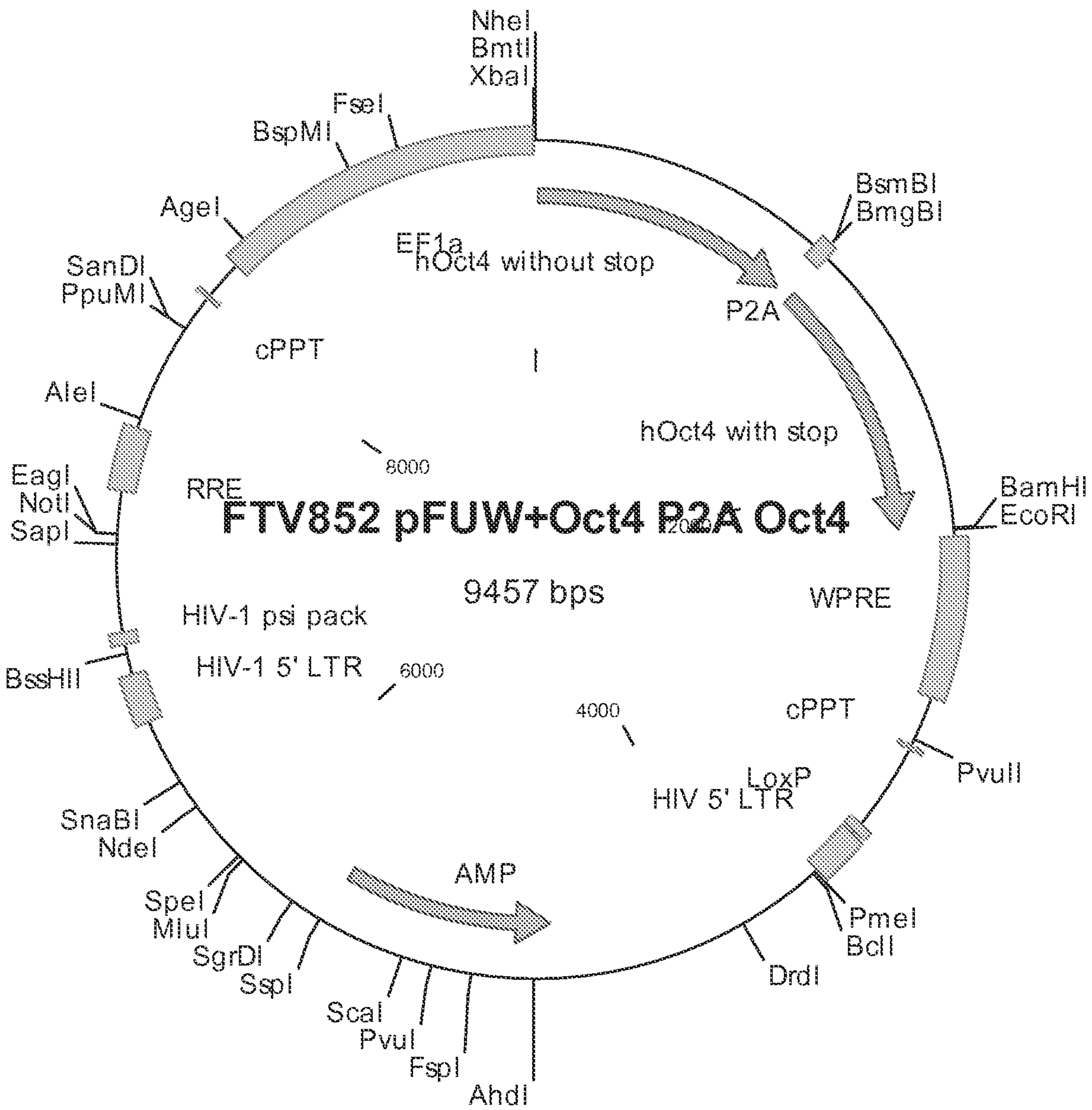

FTV852 pFUW+Oct4 P2A Oct4
9457 bps
NheI
BmtI
XbaI
FseI
BspMI
AgeI
SanDI
PpuMI
AleI
EagI
NotI
SapI
BssHII
SnaBI
NdeI
SpeI
MluI
SgrDI
SspI
ScaI
PvuI
FspI
AhdI
DrdI
PmeI
BclI
PvuII
BamHI
EcoRI
BsmBI
BmgBI
EF1a
hOct4 without stop
P2A
hOct4 with stop
cPPT
RRE
HIV-1 psi pack
HIV-1 5' LTR
WPRE
cPPT
LoxP
HIV 5' LTR
AMP
8000
6000
4000
2000

FIG. 14B

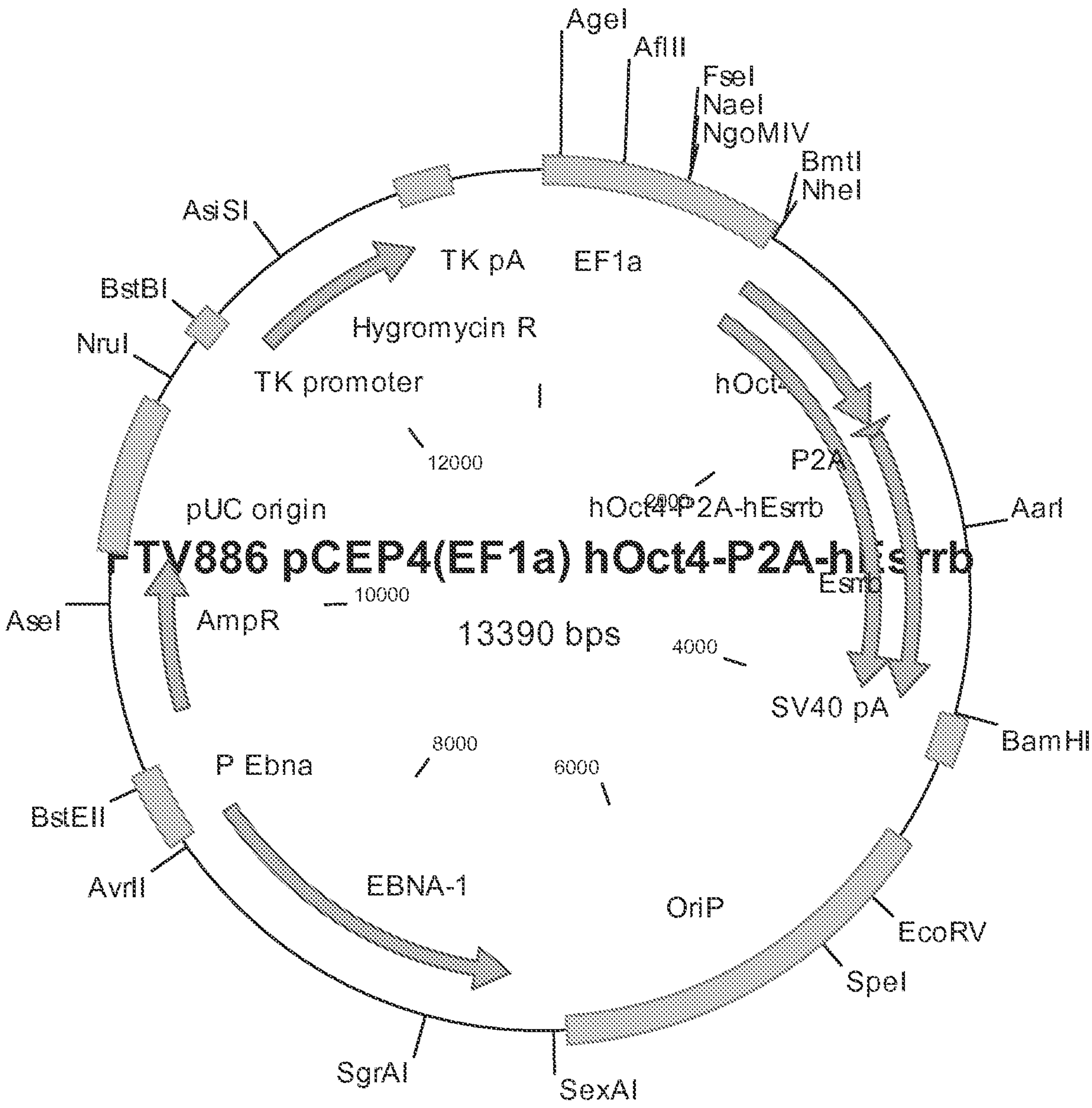

AgeI
AflII
FseI
NaeI
NgoMIV
BmtI
NheI
AsiSI
BstBI
NruI
TK pA
EF1a
Hygromycin R
TK promoter
hOct4
12000
P2A
pUC origin
2000
hOct4-P2A-hEsrrb
AarI
pTV886 pCEP4(EF1a) hOct4-P2A-hEsrrb
Esrrb
AseI
10000
AmpR
13390 bps
4000
SV40 pA
8000
BamHI
P Ebna
6000
BstEII
AvrII
EBNA-1
OriP
EcoRV
SpeI
SgrAI
SexAI

FIG. 14C

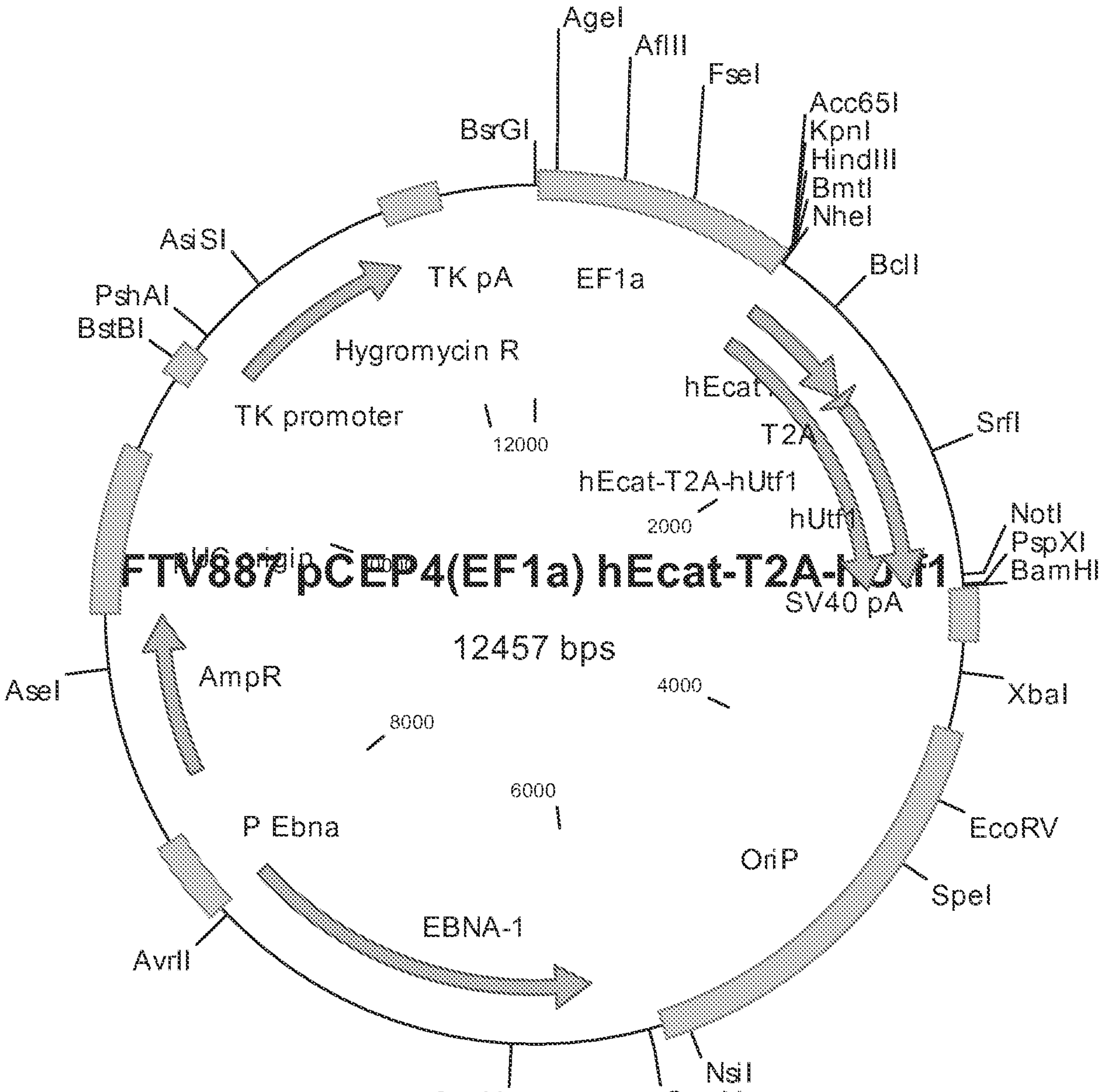

AgeI
AflII
FseI
Acc65I
KpnI
HindIII
BmtI
NheI
BsrGI
AsiSI
PshAI
BstBI
BclI
SrfI
NotI
PspXI
BamHI
XbaI
EcoRV
SpeI
NsiI
SexAI
SgrAI
AvrII
AseI
TK pA
EF1a
Hygromycin R
TK promoter
12000
T2A
hEcat-T2A-hUtf1
2000
hUtf1
FTV887 pCEP4(EF1a) hEcat-T2A-hUtf1
SV40 pA
12457 bps
AmpR
4000
8000
6000
P Ebna
OriP
EBNA-1

FIG. 14D

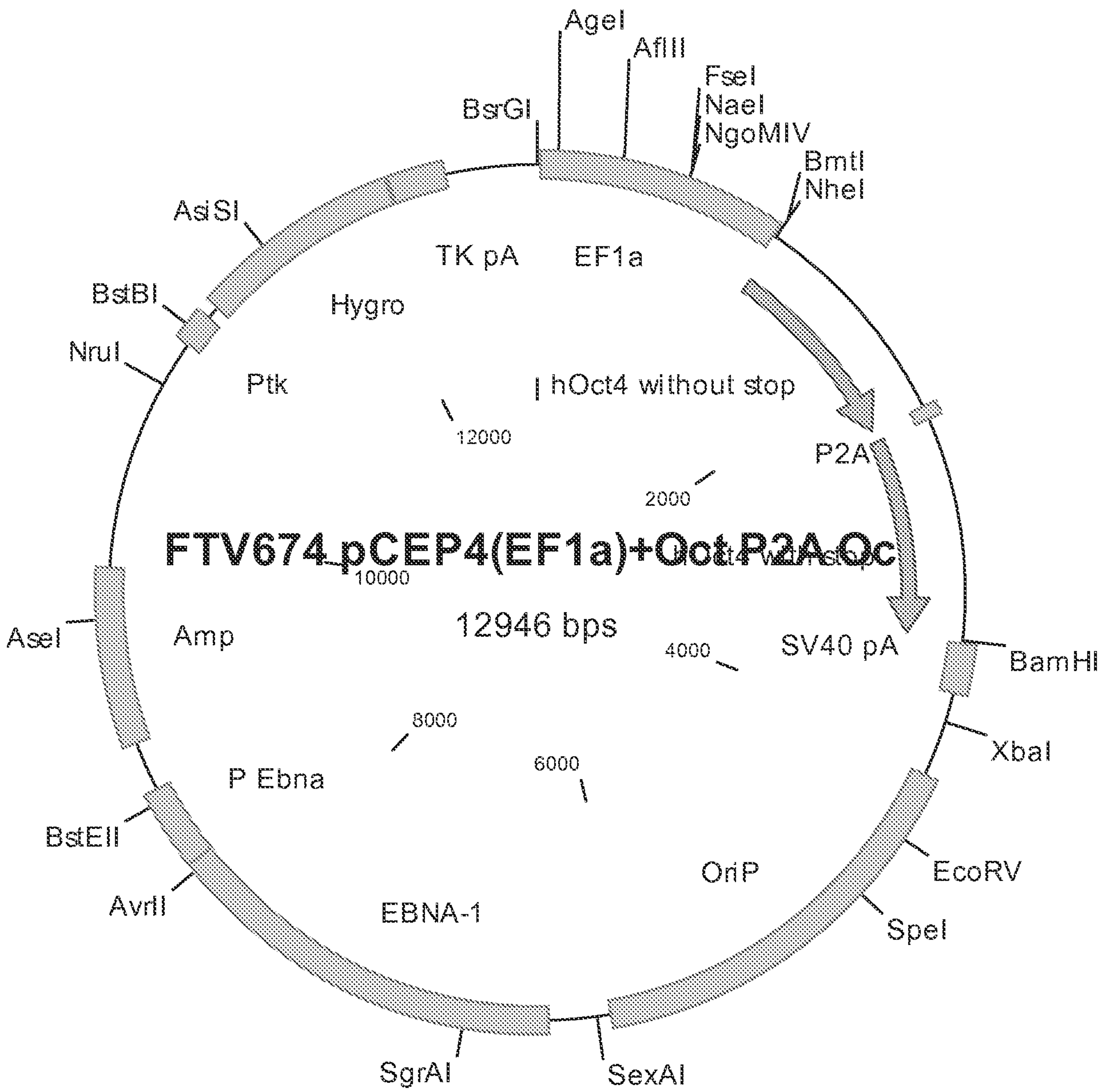

AgeI
AflII
FseI
NaeI
NgoMIV
BmtI
NheI
BsrGI
AsiSI
BstBI
NruI
TK pA
EF1a
Hygro
Ptk
hOct4 without stop
12000
P2A
2000
FTV674 pCEP4(EF1a)+Oct P2A Oc
10000
12946 bps
AseI
Amp
4000
SV40 pA
BamHI
8000
XbaI
6000
P Ebna
BstEII
OriP
EcoRV
AvrII
EBNA-1
SpeI
SgrAI
SexAI

FIG. 14E

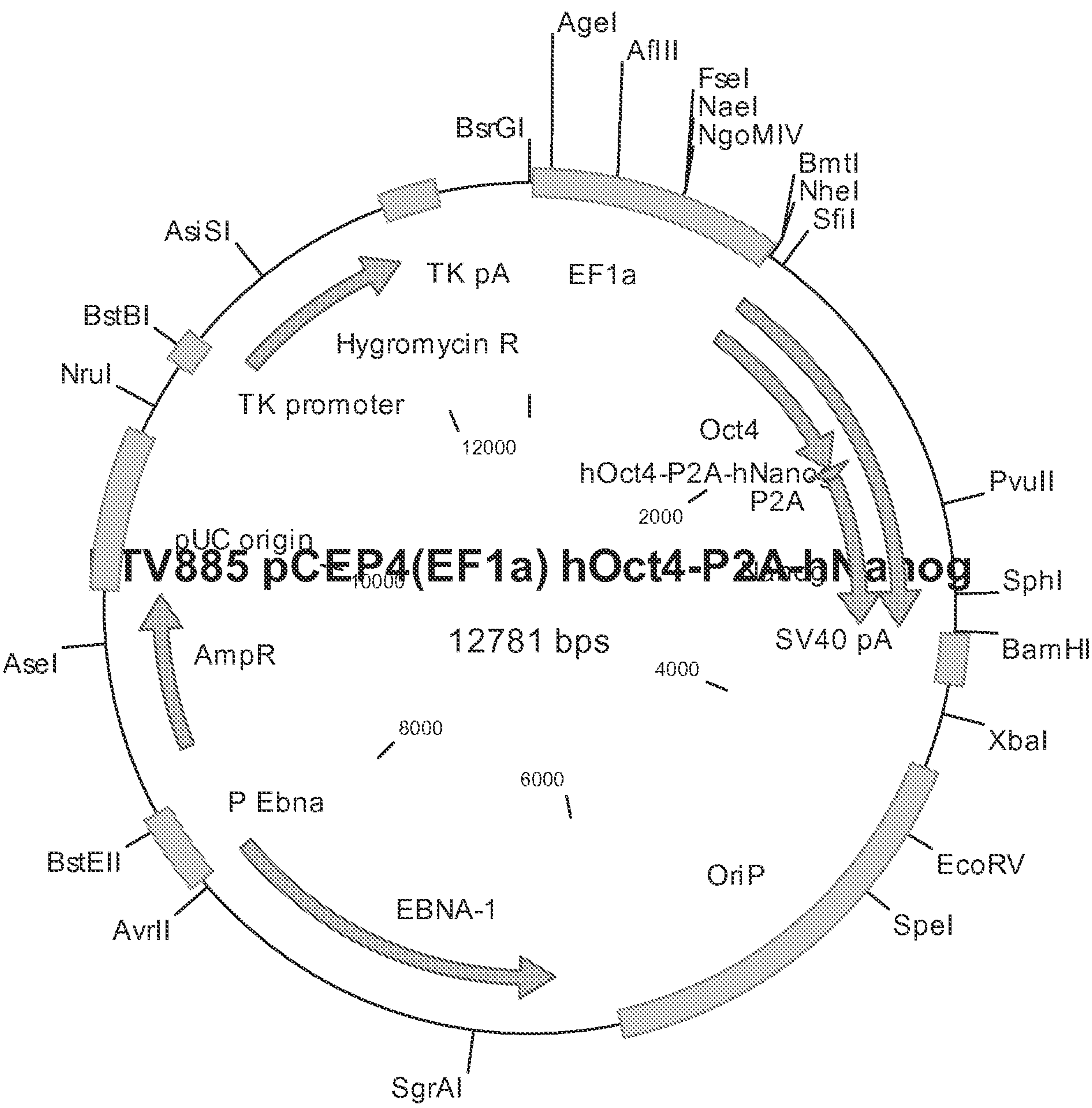

AgeI
AflII
FseI
NaeI
NgoMIV
BsrGI
BmtI
NheI
SfiI
AsiSI
TK pA
EF1a
BstBI
Hygromycin R
NruI
TK promoter
12000
Oct4
hOct4-P2A-hNanog
P2A
2000
PvuII
pUC origin
TV885 pCEP4(EF1a) hOct4-P2A-hNanog
10000
SphI
12781 bps
SV40 pA
BamHI
AseI
AmpR
4000
8000
XbaI
6000
P Ebna
BstEII
OriP
EcoRV
EBNA-1
AvrII
SpeI
SgrAI

FIG. 14F

OCT4/NANOG/ECAT1/UTF1

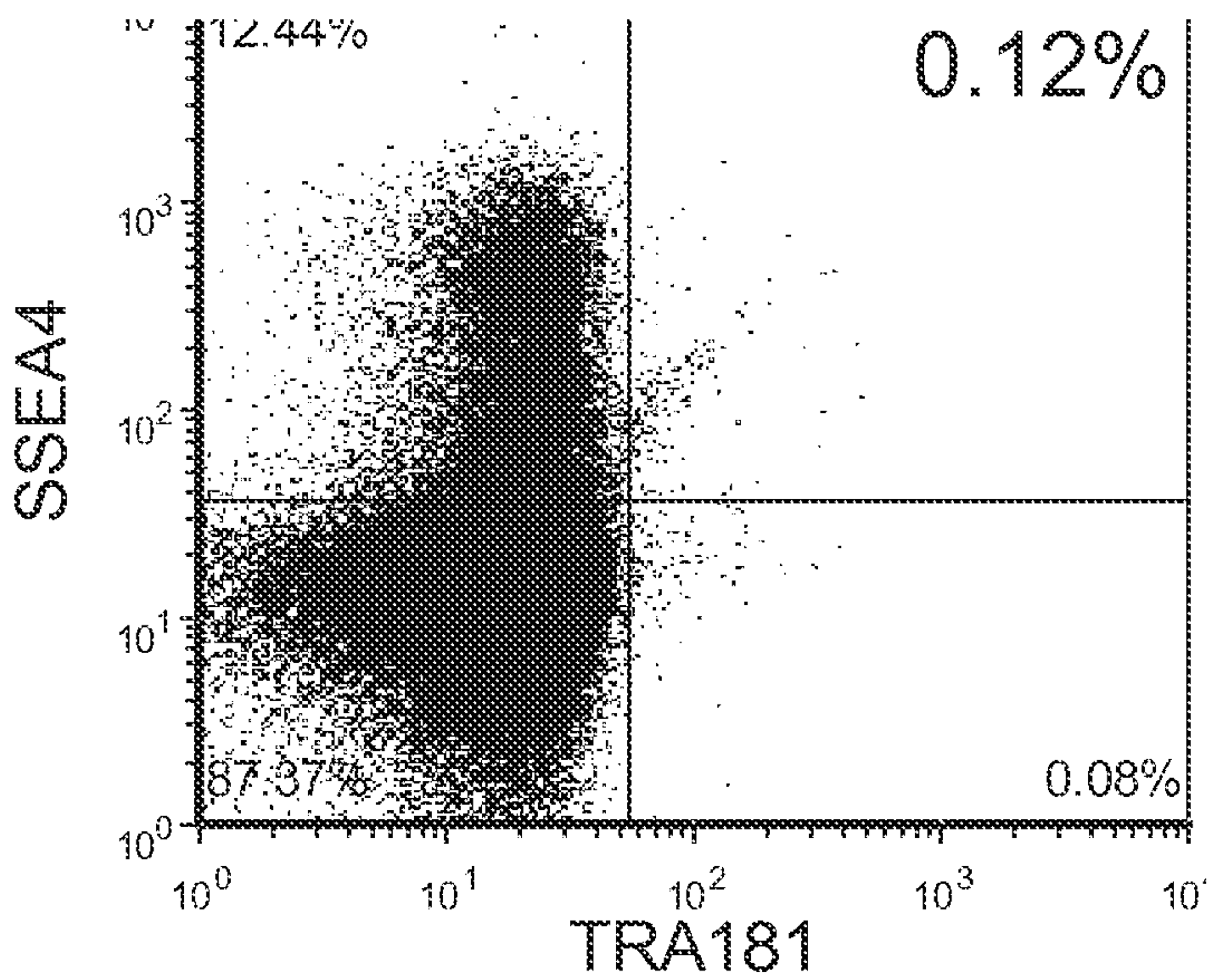


FIG. 15A

OCT4/ESRRB/NANOG/ECAT1/UTF1

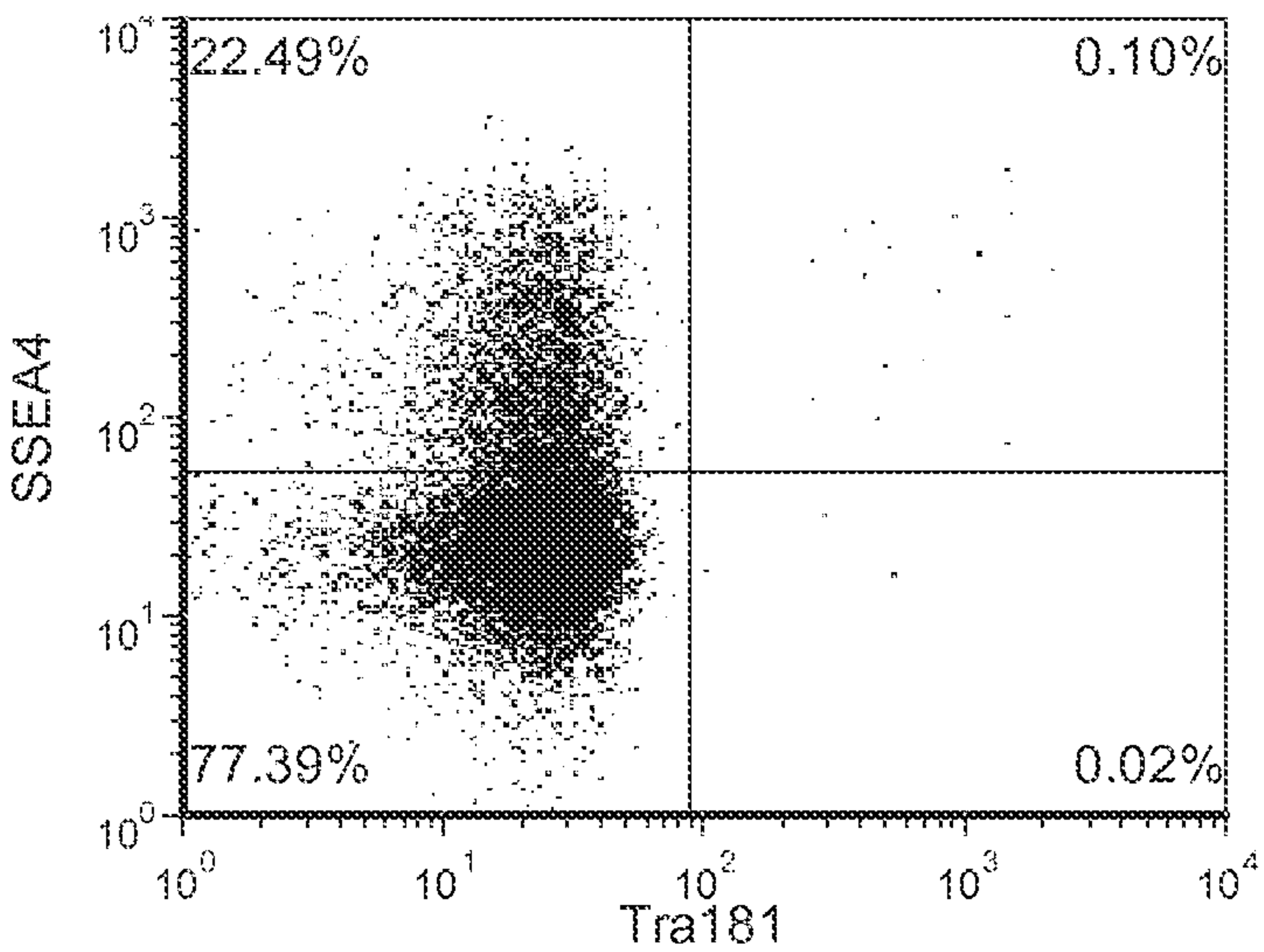


FIG. 15B

OCT4/ESRRB/LIN28/ECAT1/UTF-1

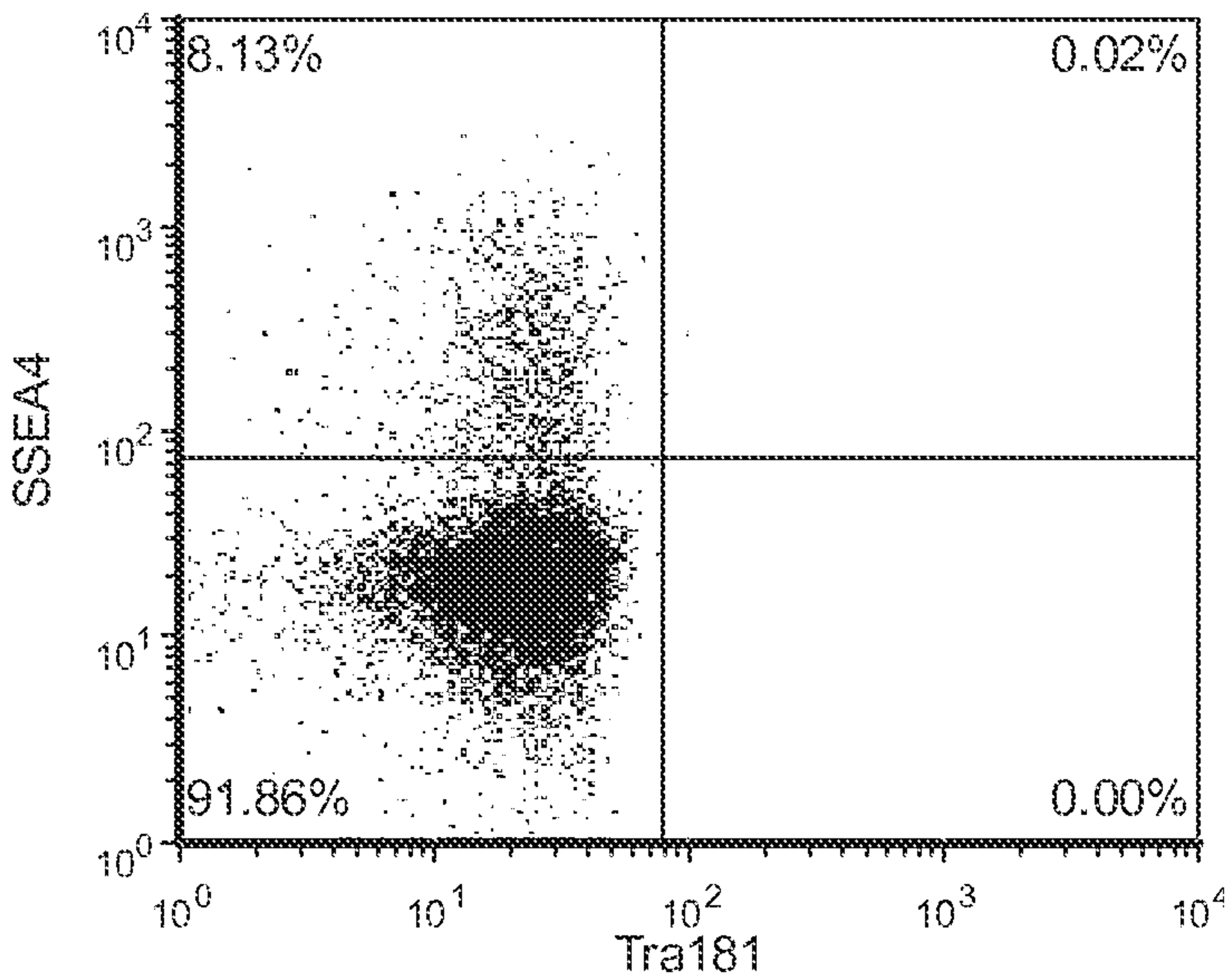


FIG. 15C

OCT4-P2A-OCT4 / NANOG-P2A-ESRRB-P2A-LIN28 / ECAT1-T2A-UTF1

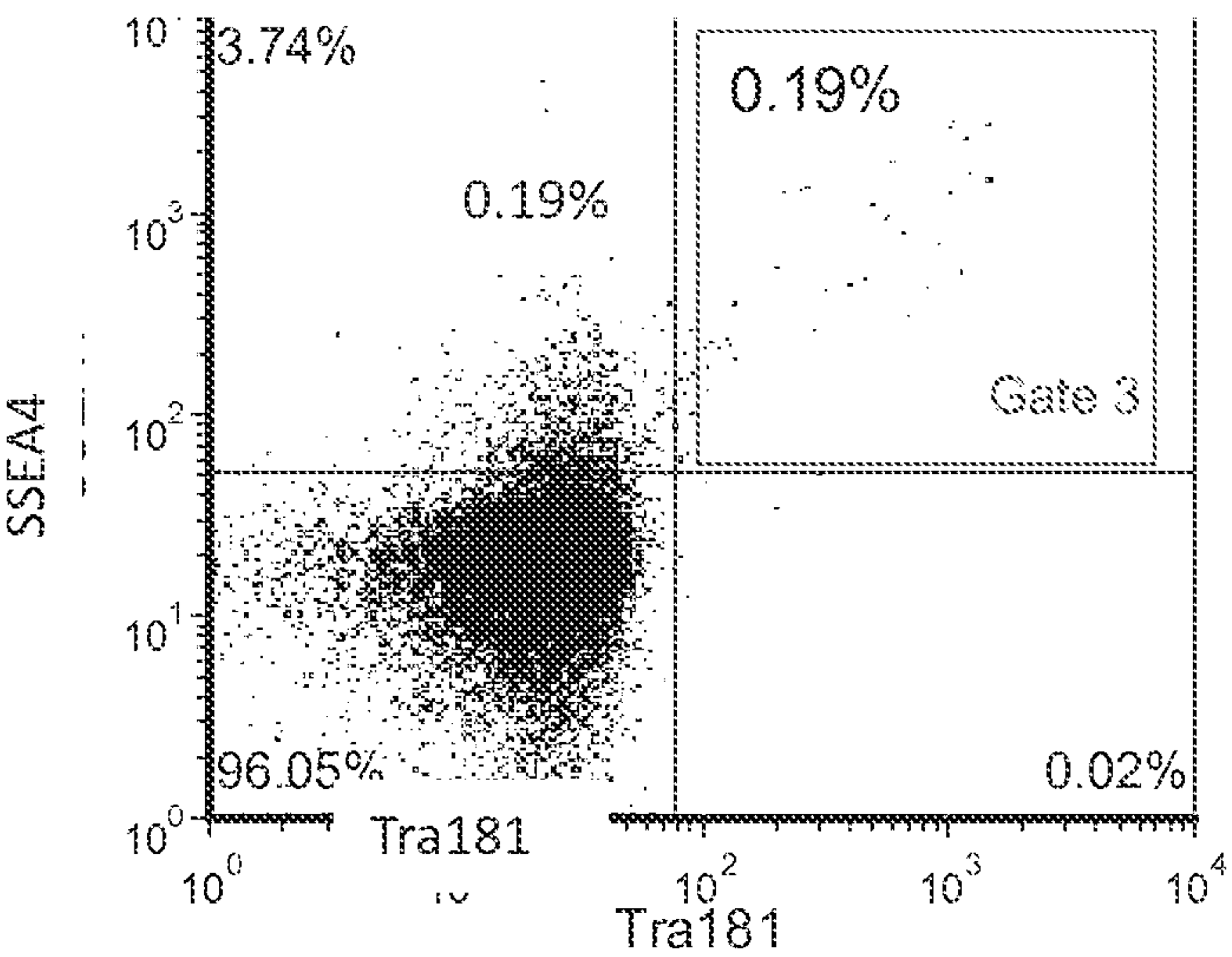
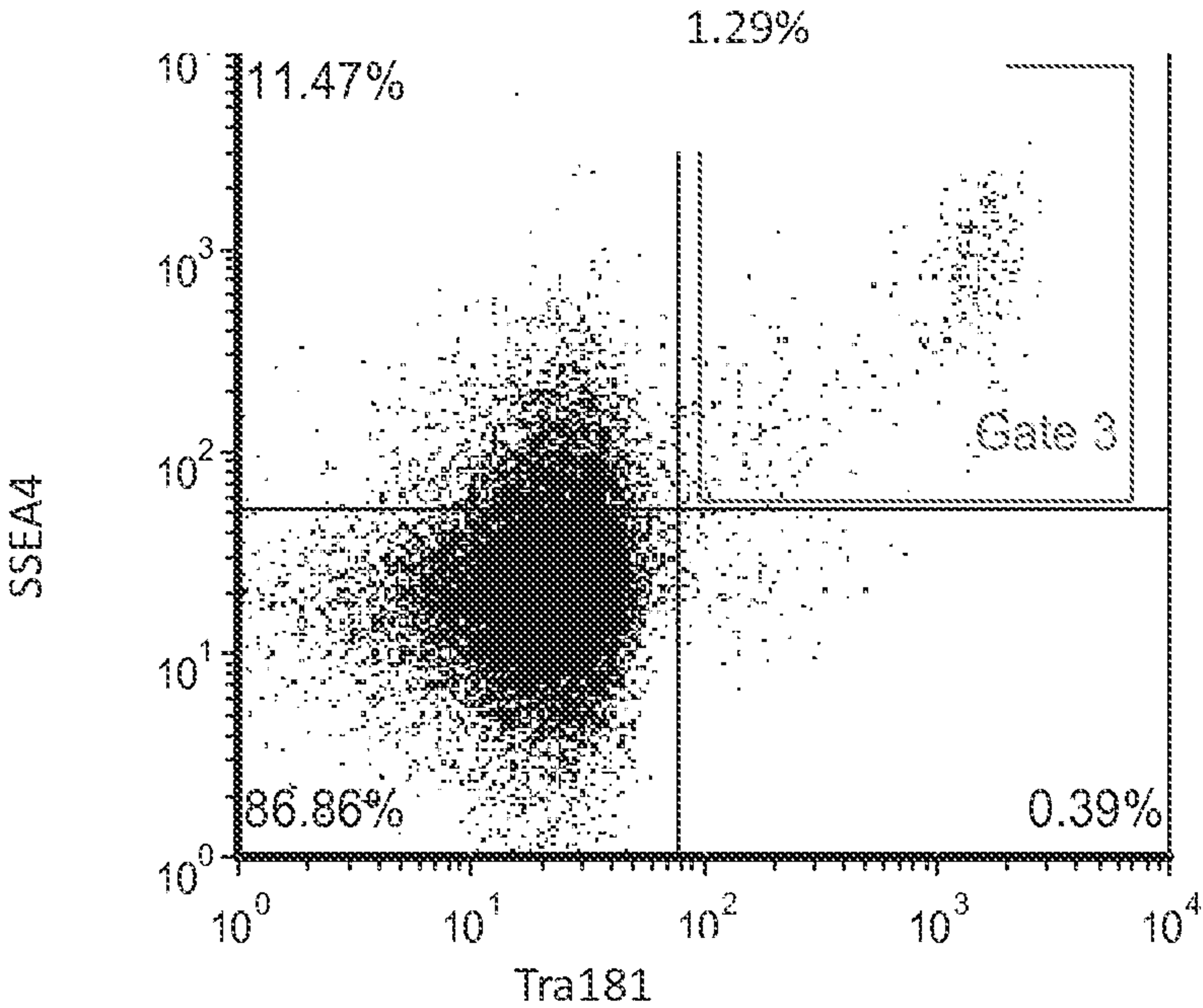


FIG. 16A

FIG. 16B

OCT4-P2A-ESRRB / OCT4-P2A-NANOG / ECAT1-T2A-UTF1



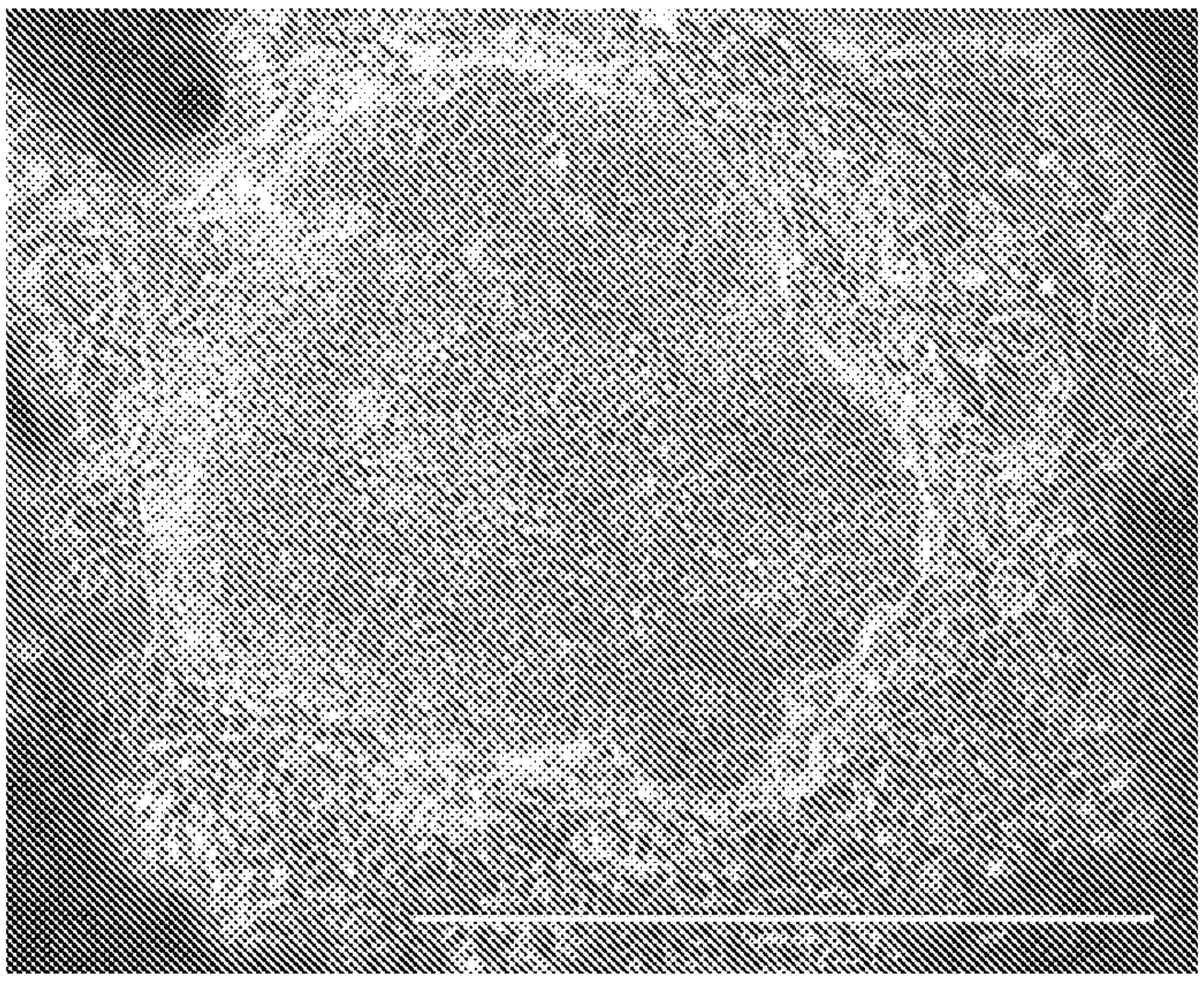
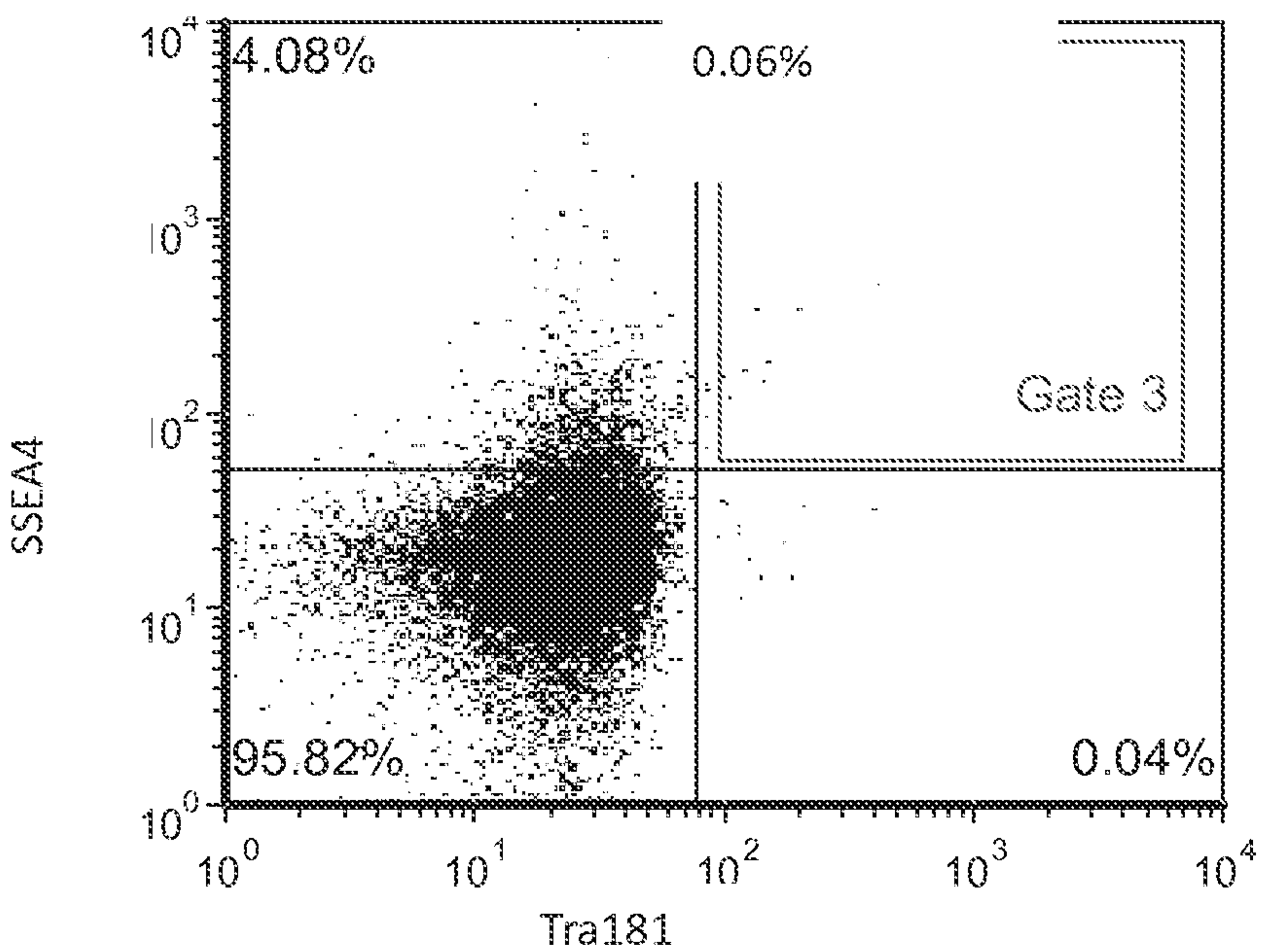


FIG. 16C

OCT4-P2A-OCT4 / ECAT1-T2A-UTF1



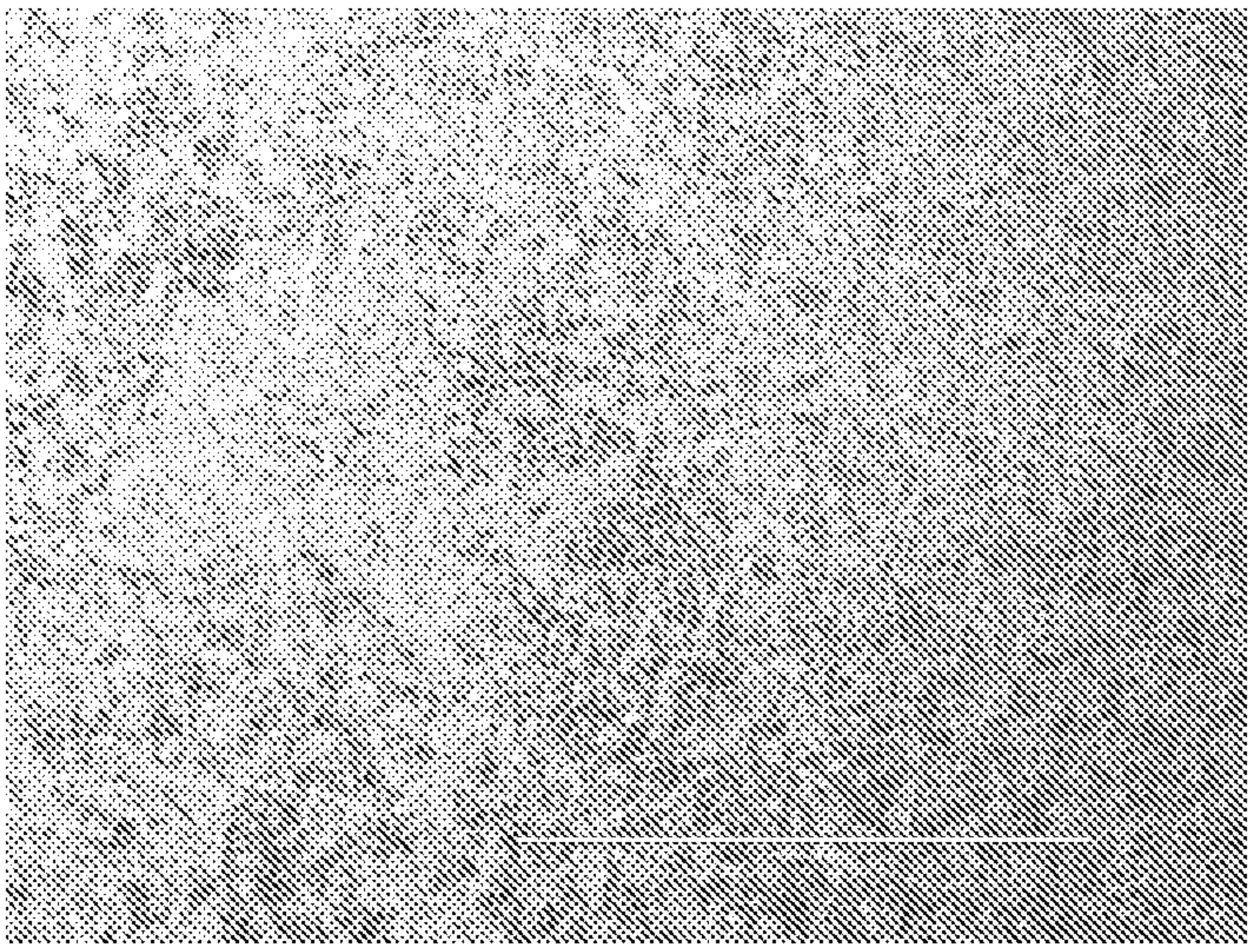
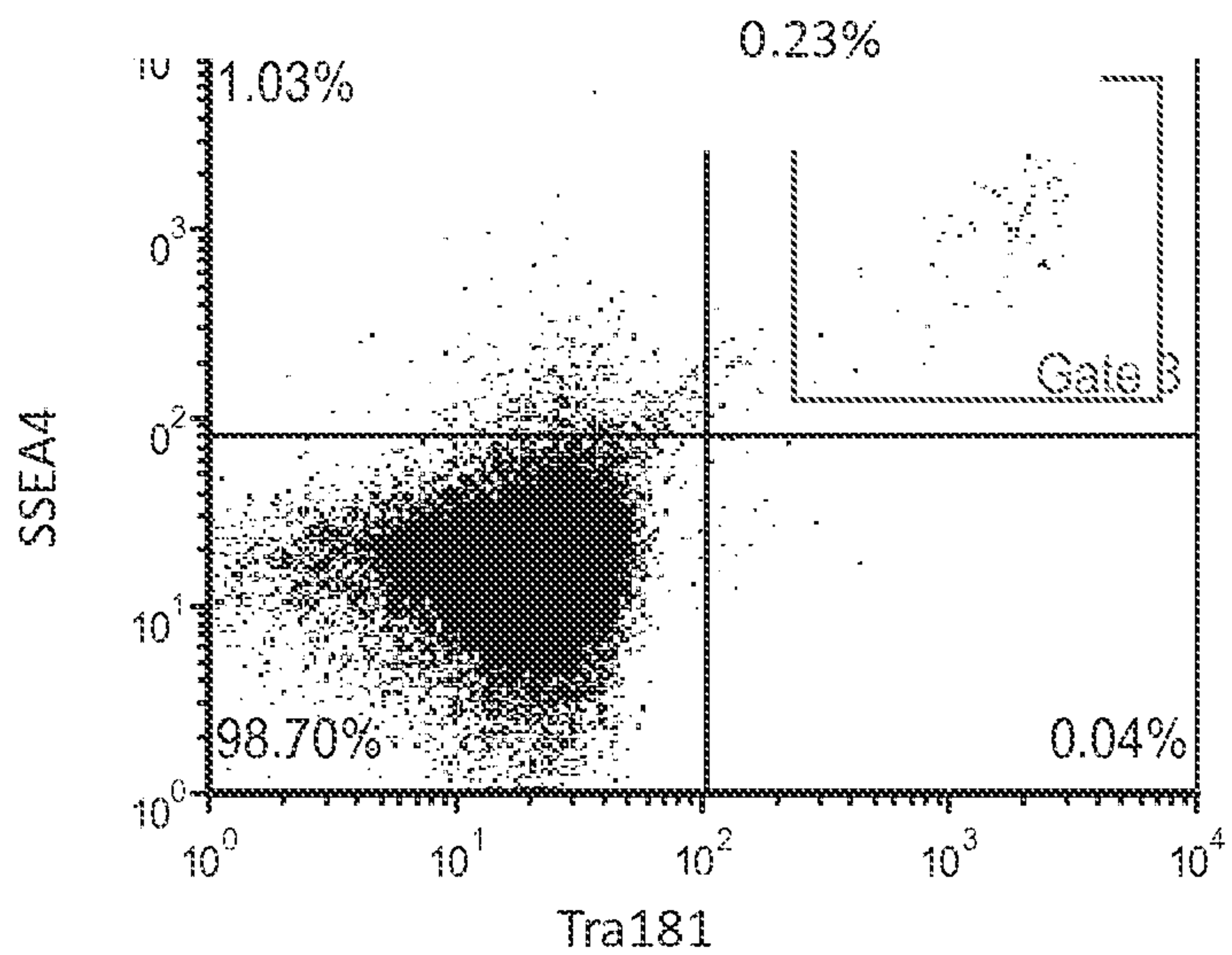


FIG. 16D

OCT4-P2A-NANOG / ECAT1-T2A-UTF1



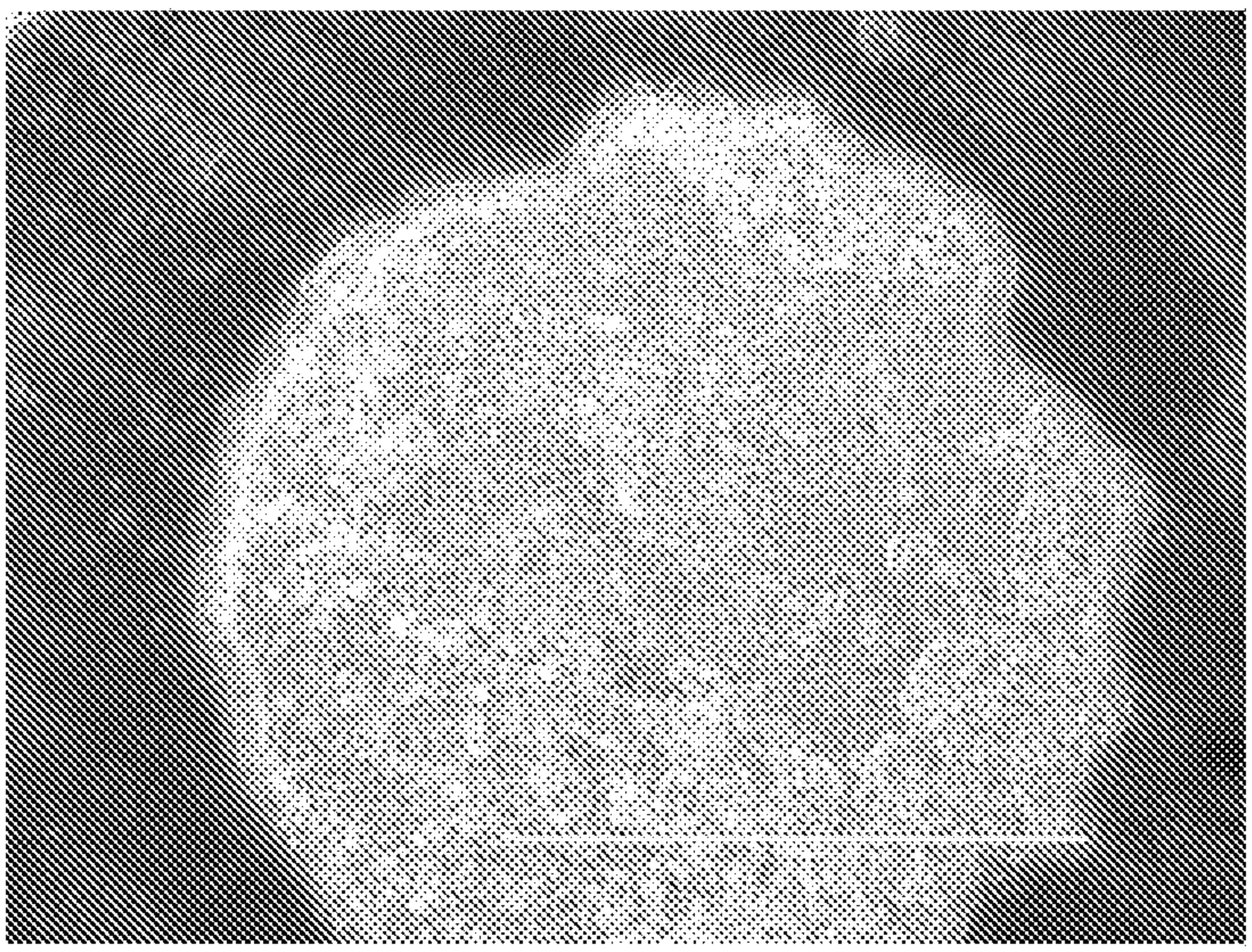
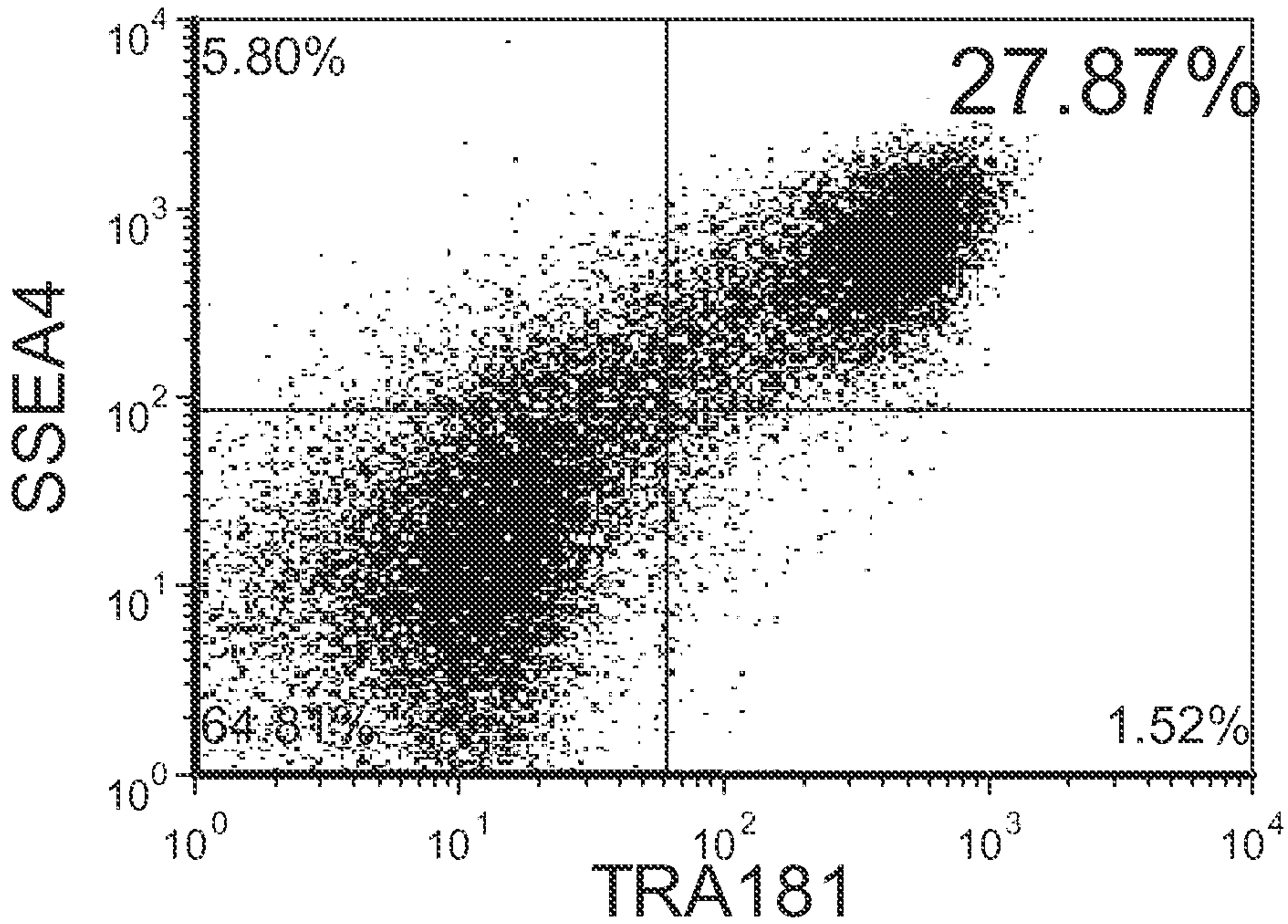


FIG. 17A



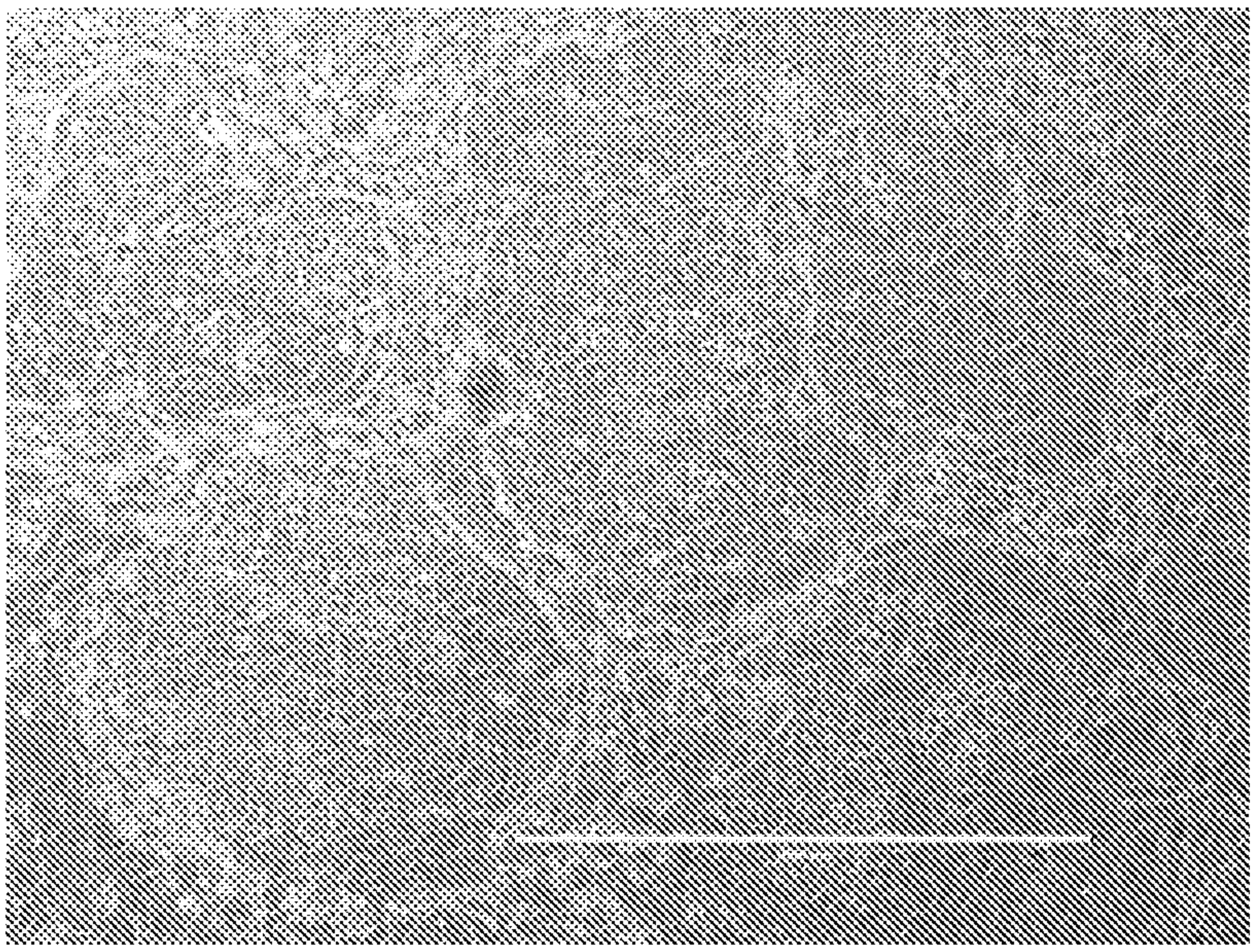
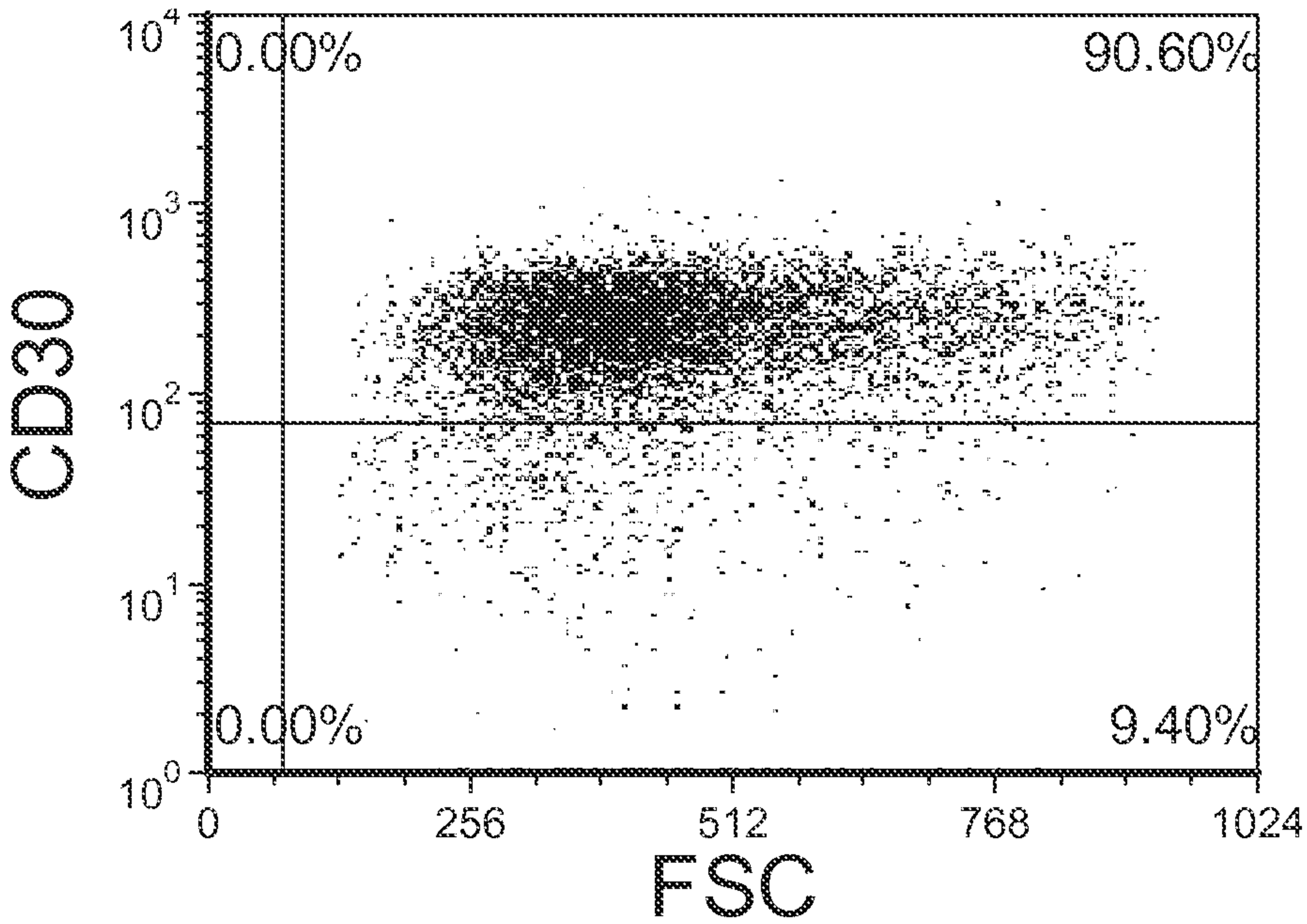


FIG. 17B



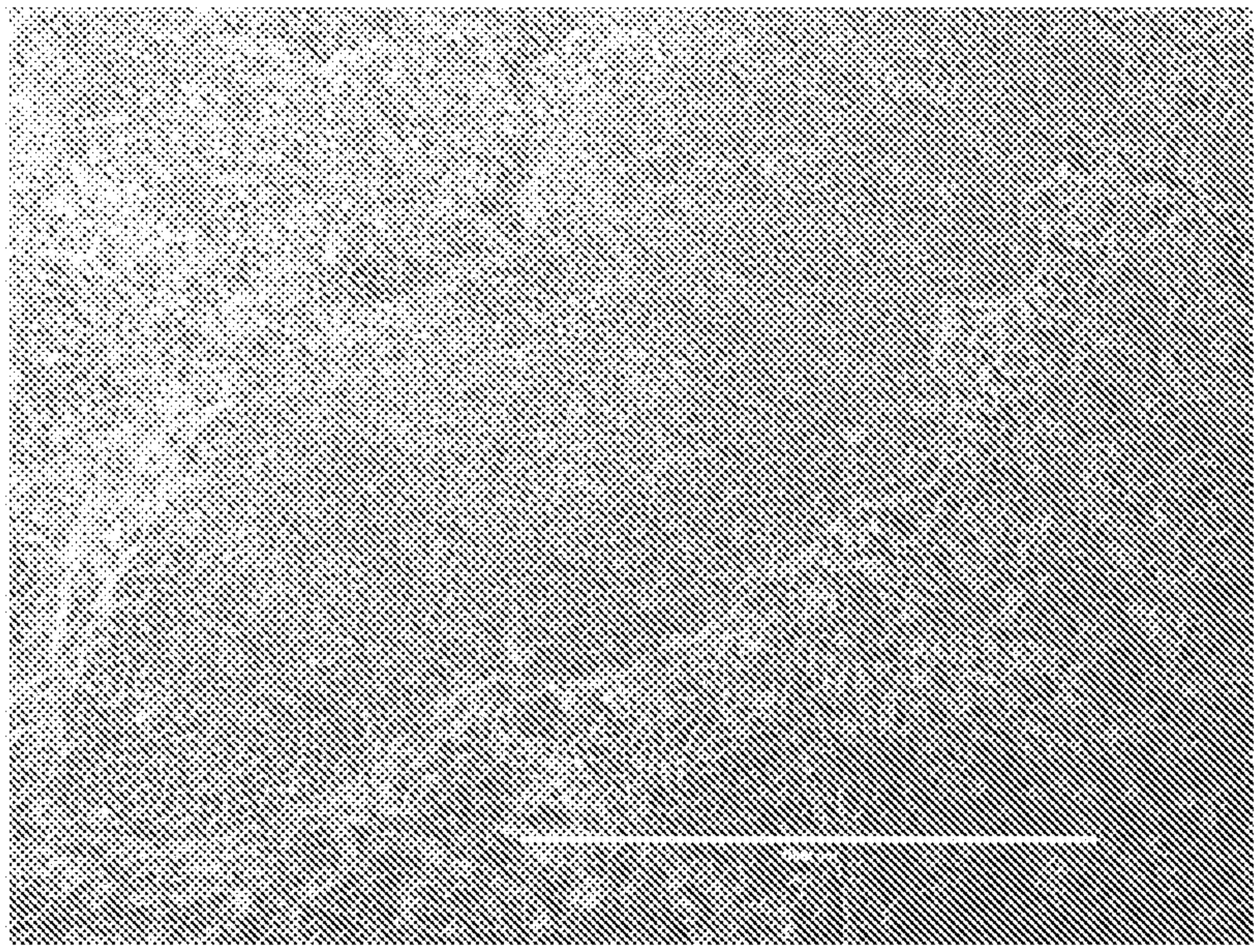
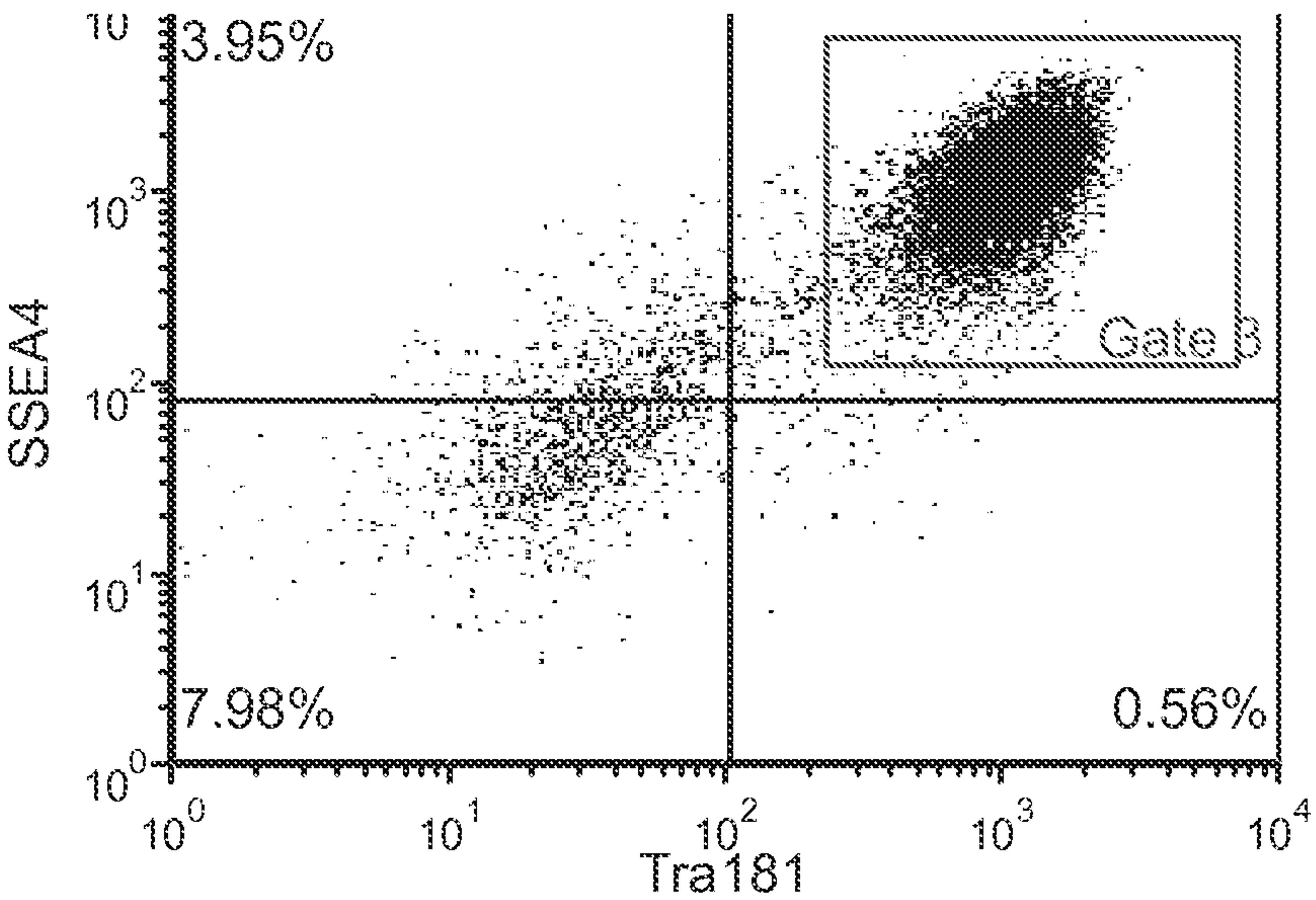


FIG. 18A



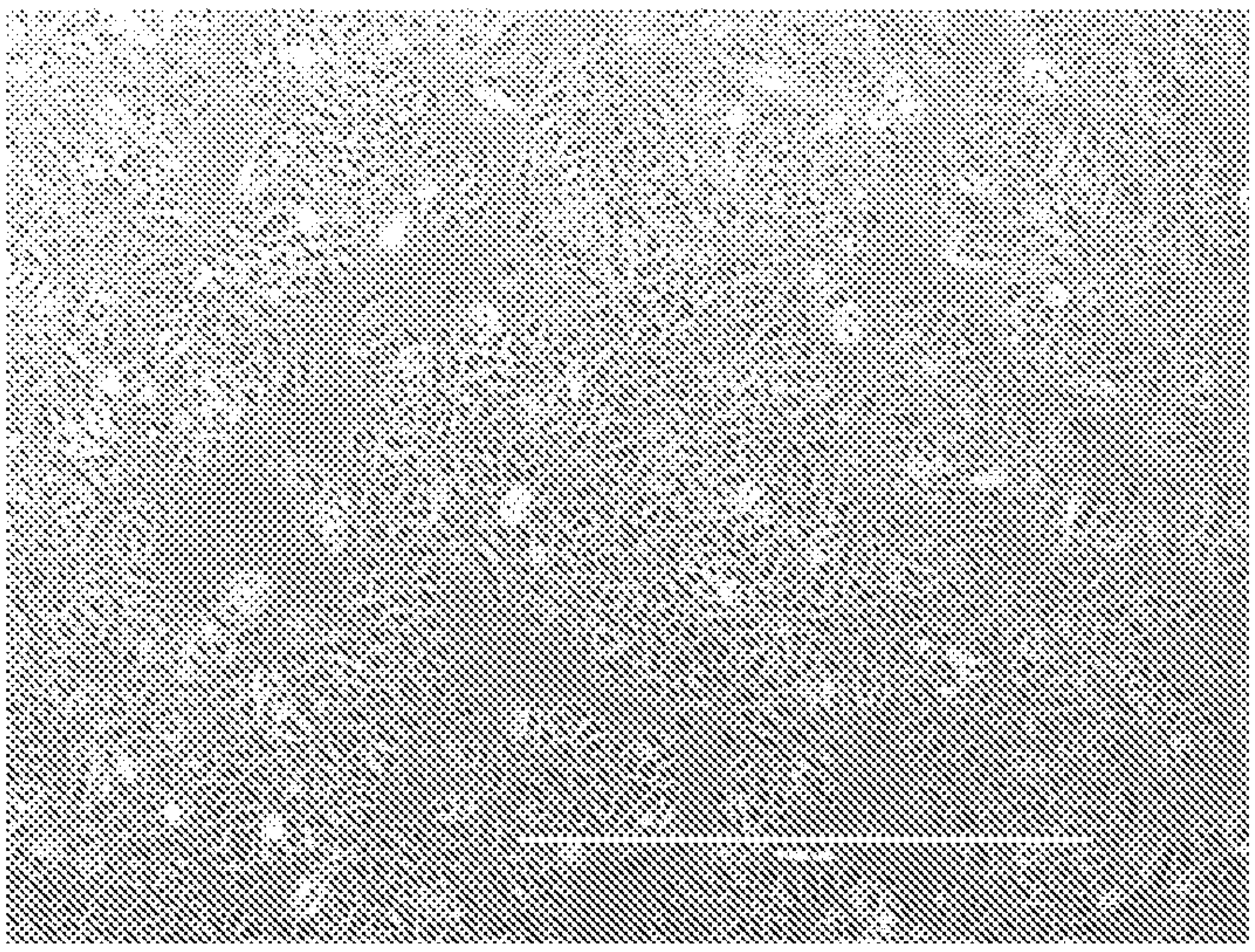
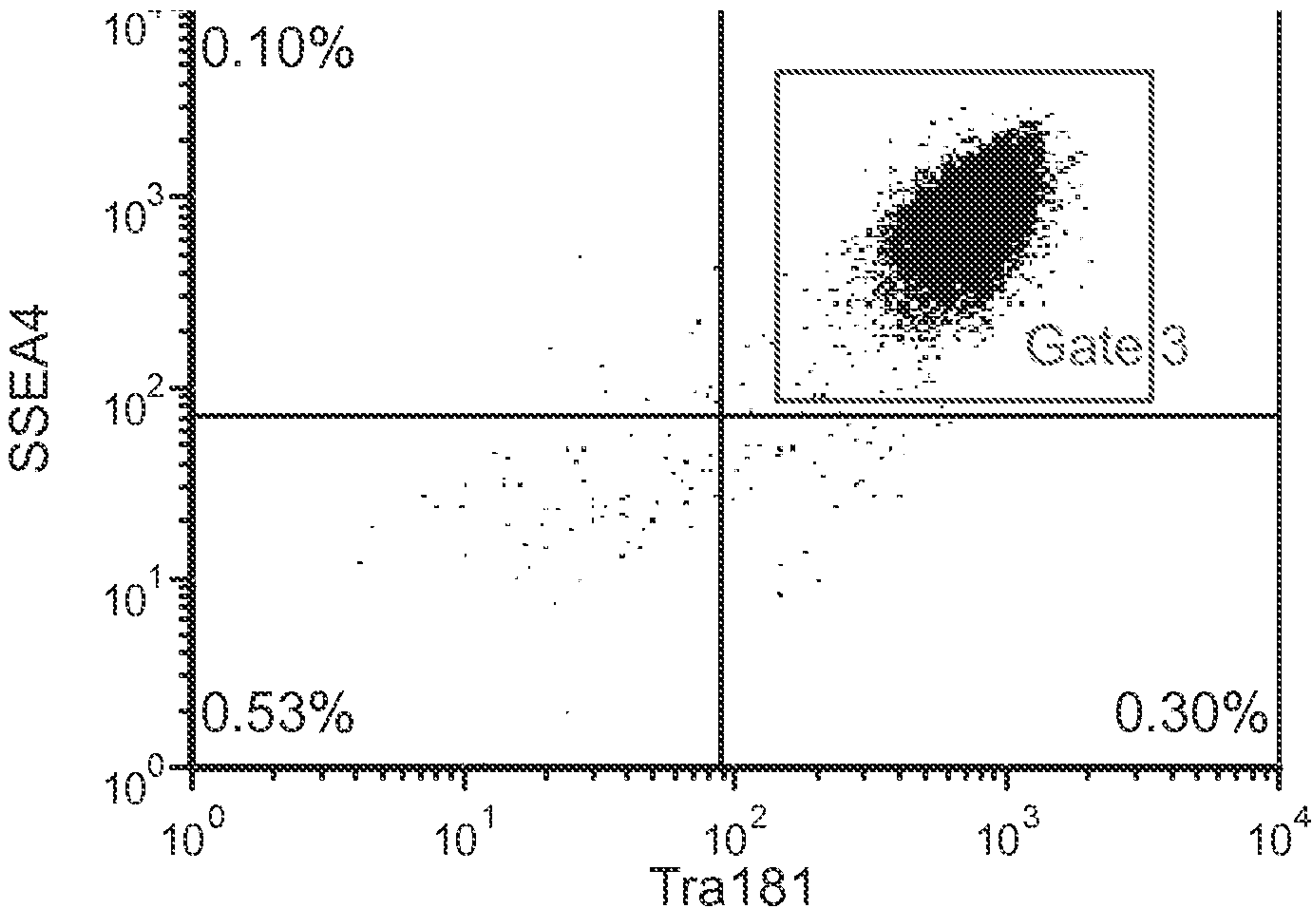


FIG. 18B



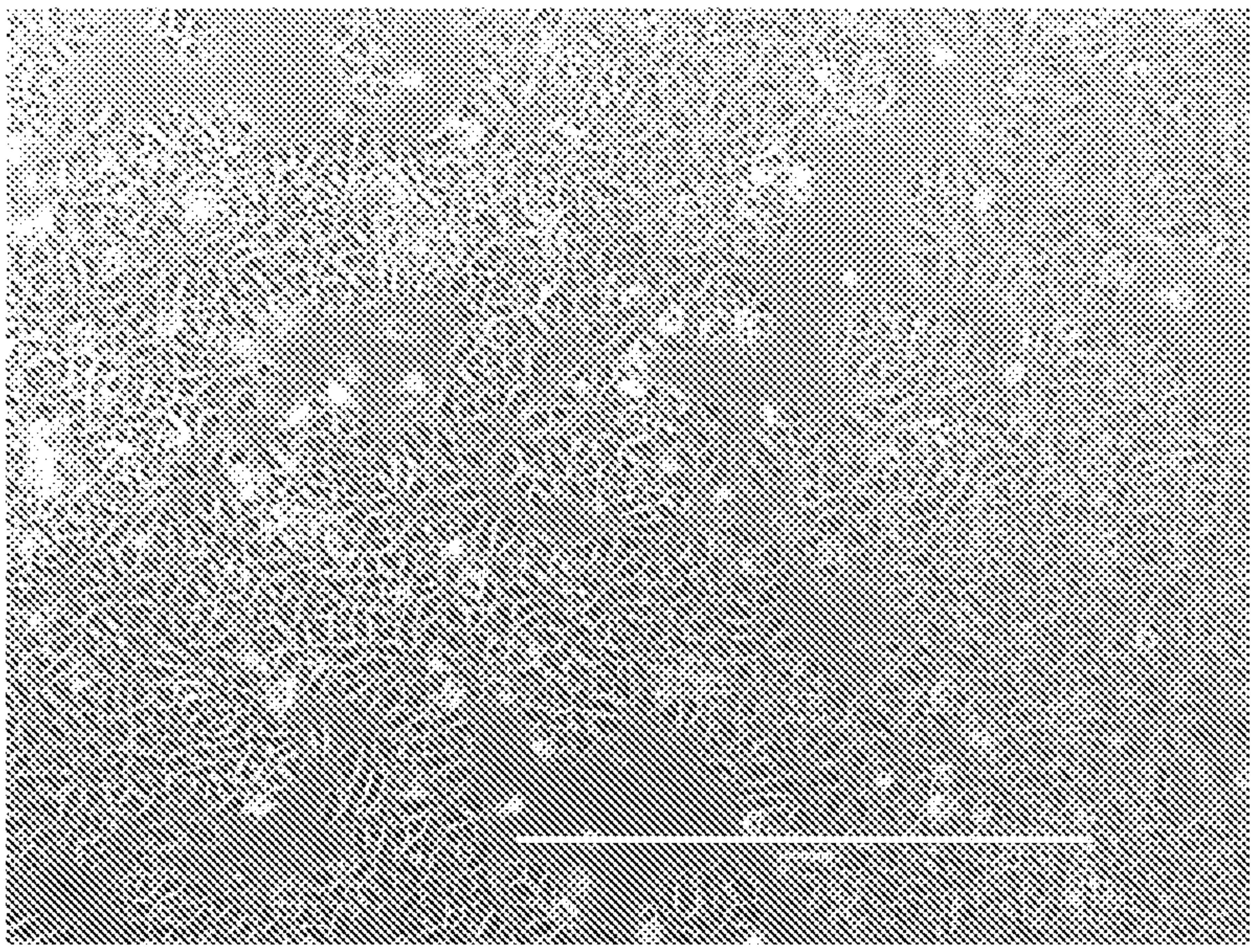
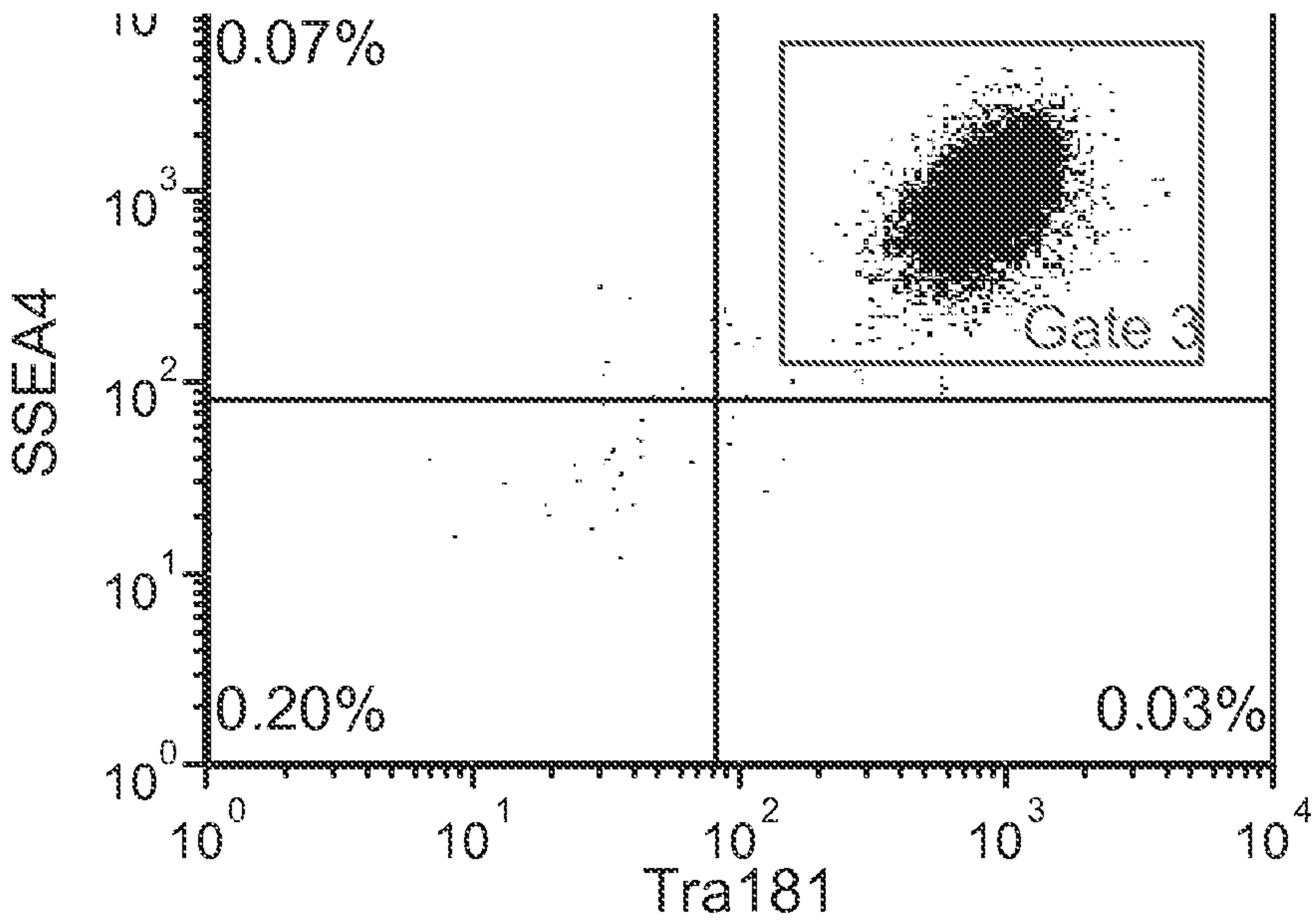


FIG. 18C



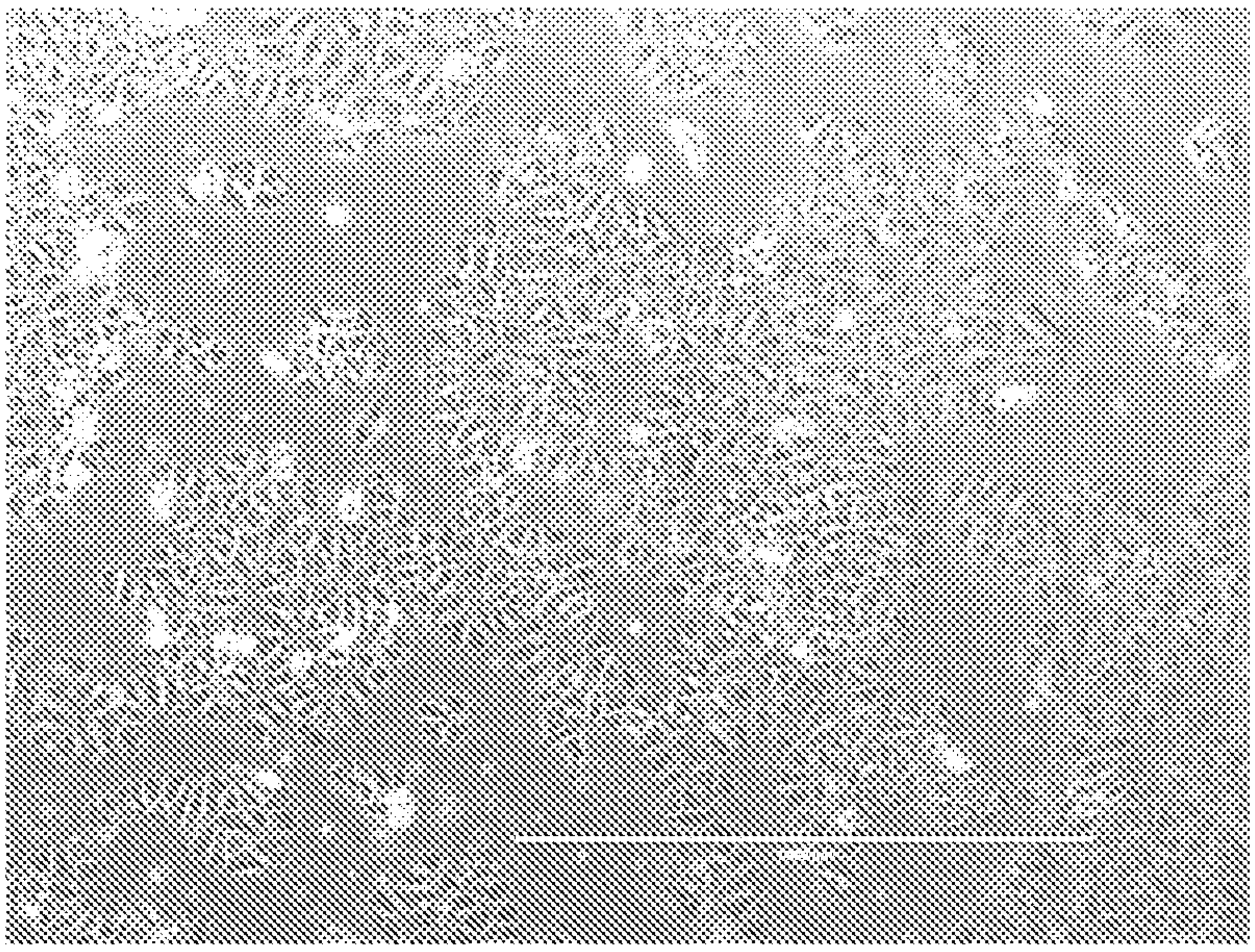
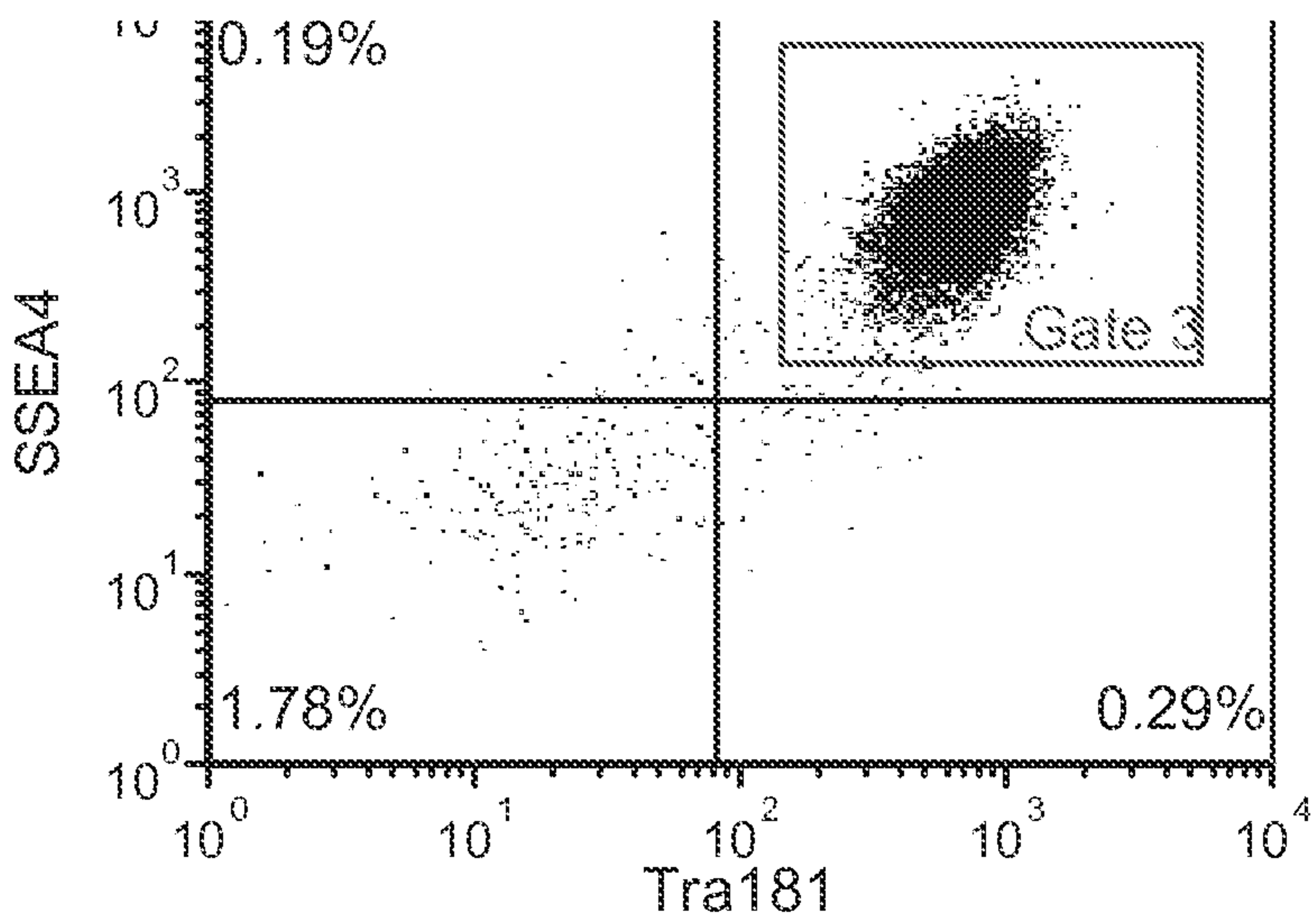


FIG. 18D



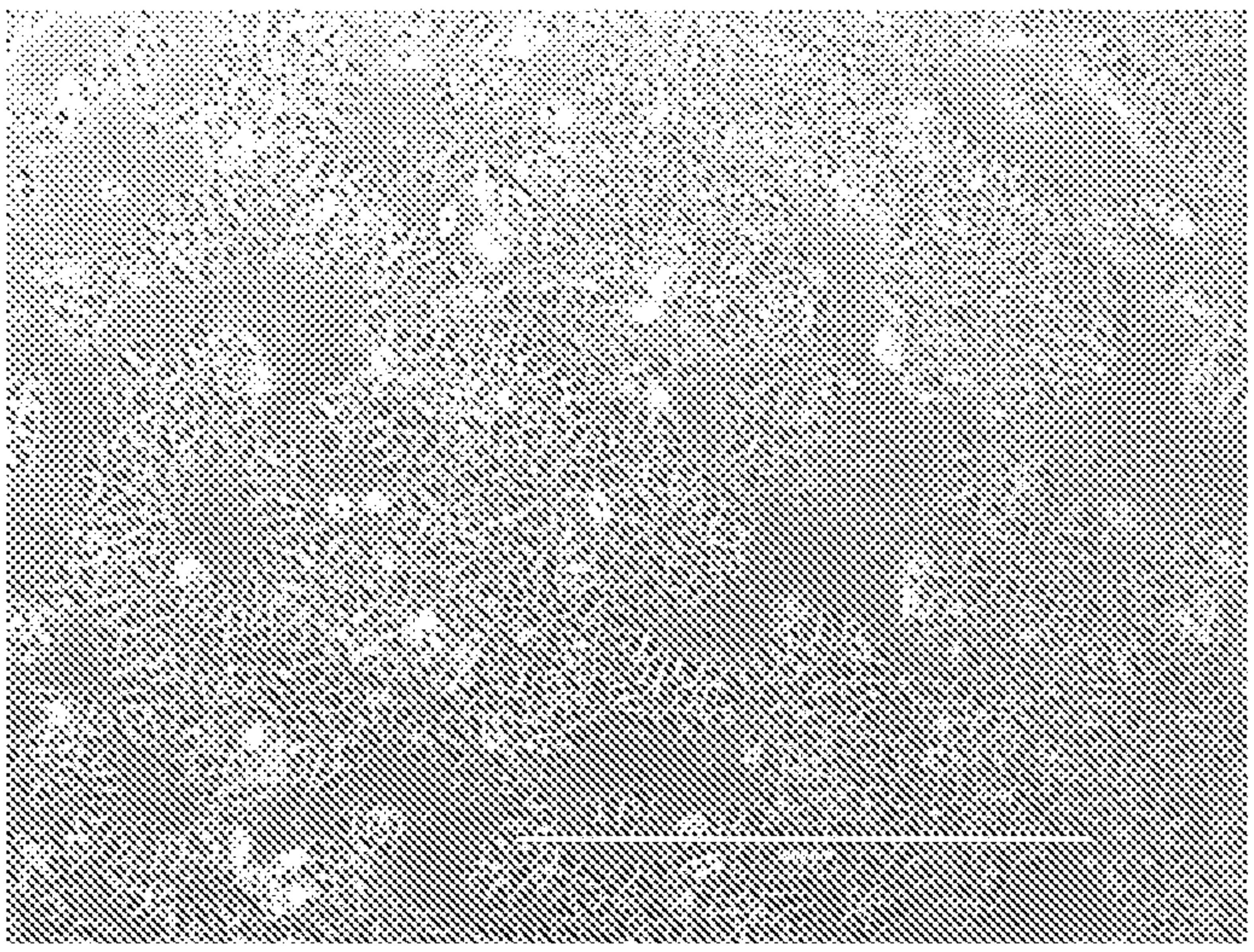
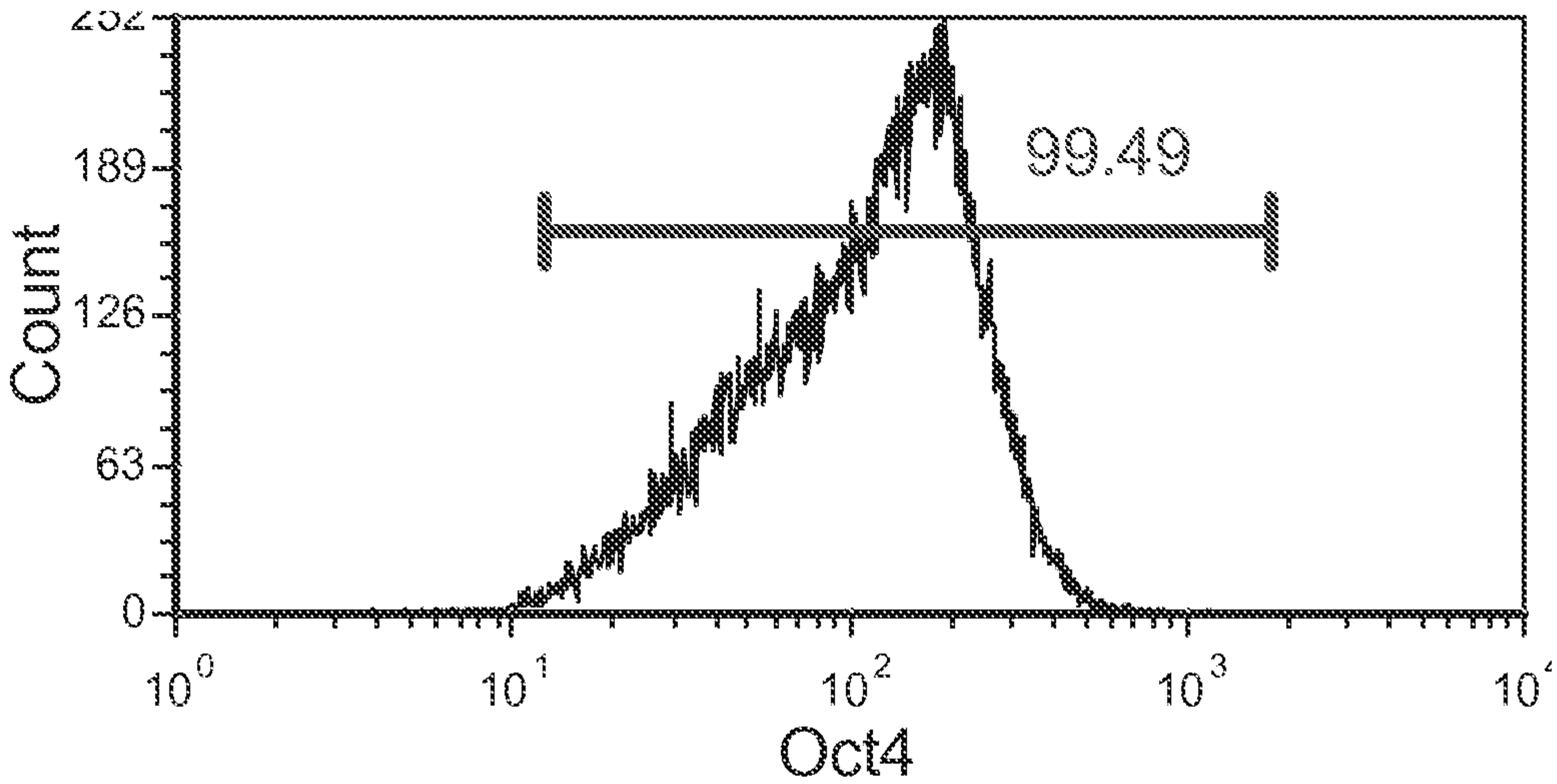


FIG. 19A



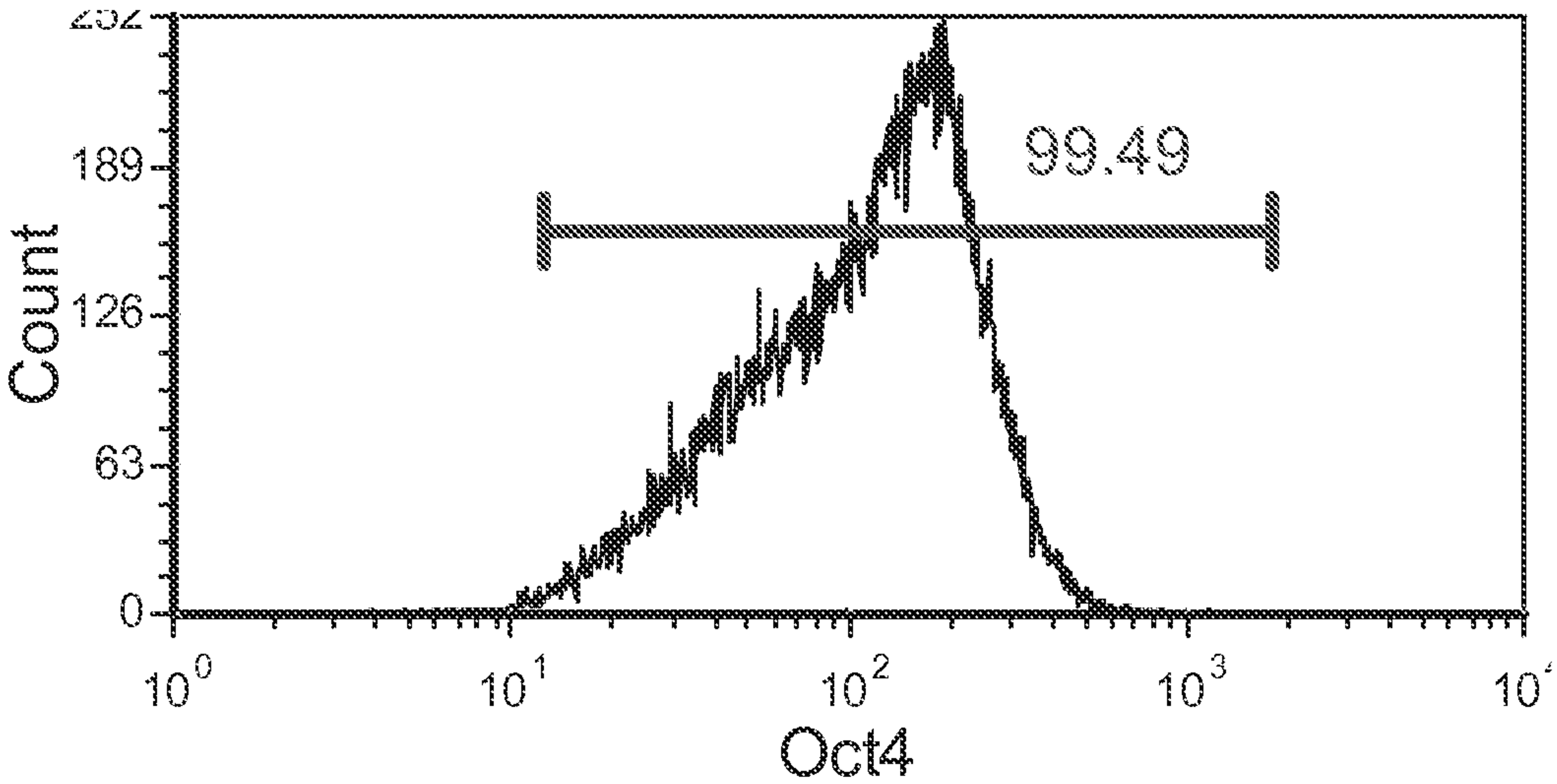


FIG. 19B

Clone 5, Passage 9

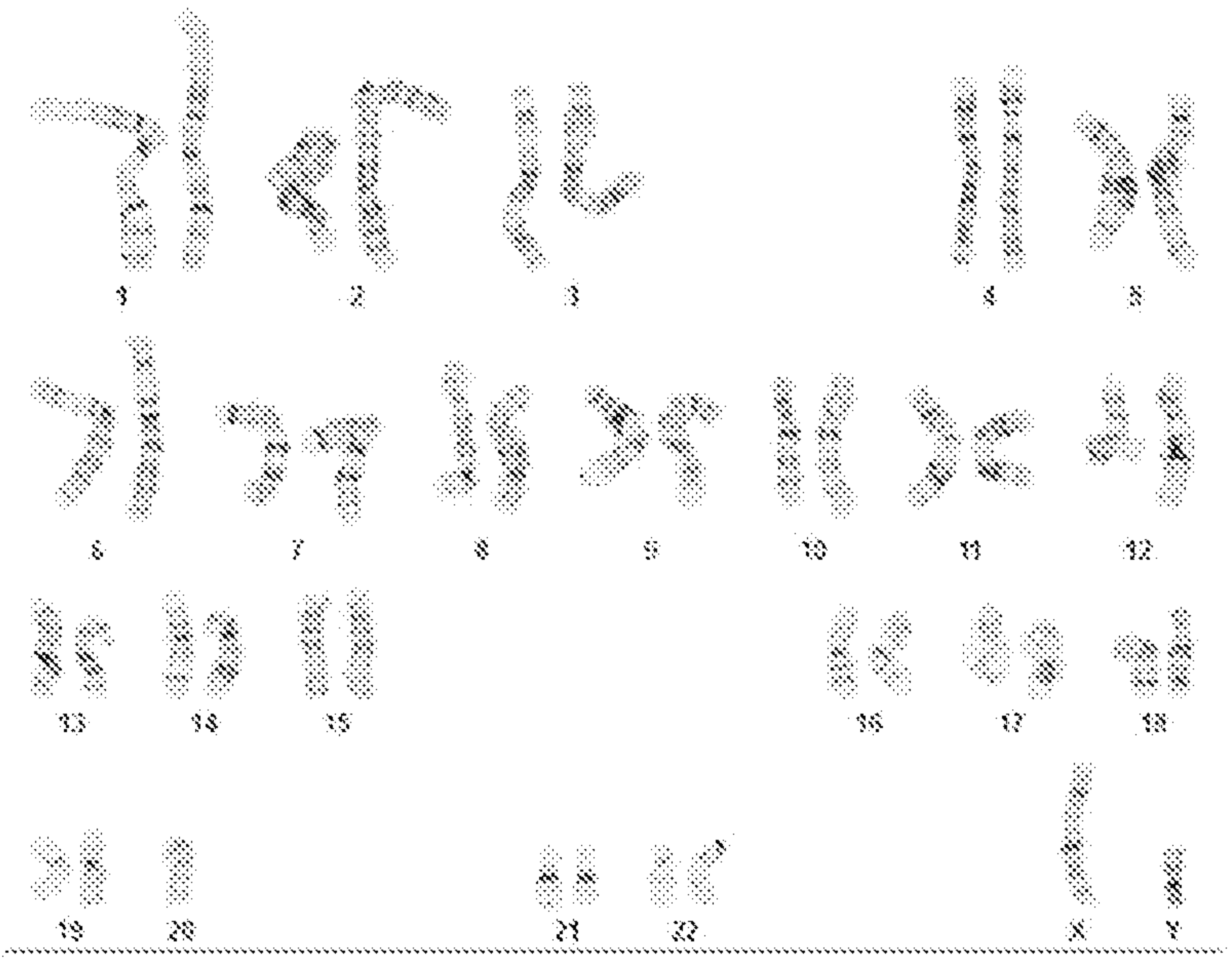


FIG. 20A

Clone 10, Passage 8

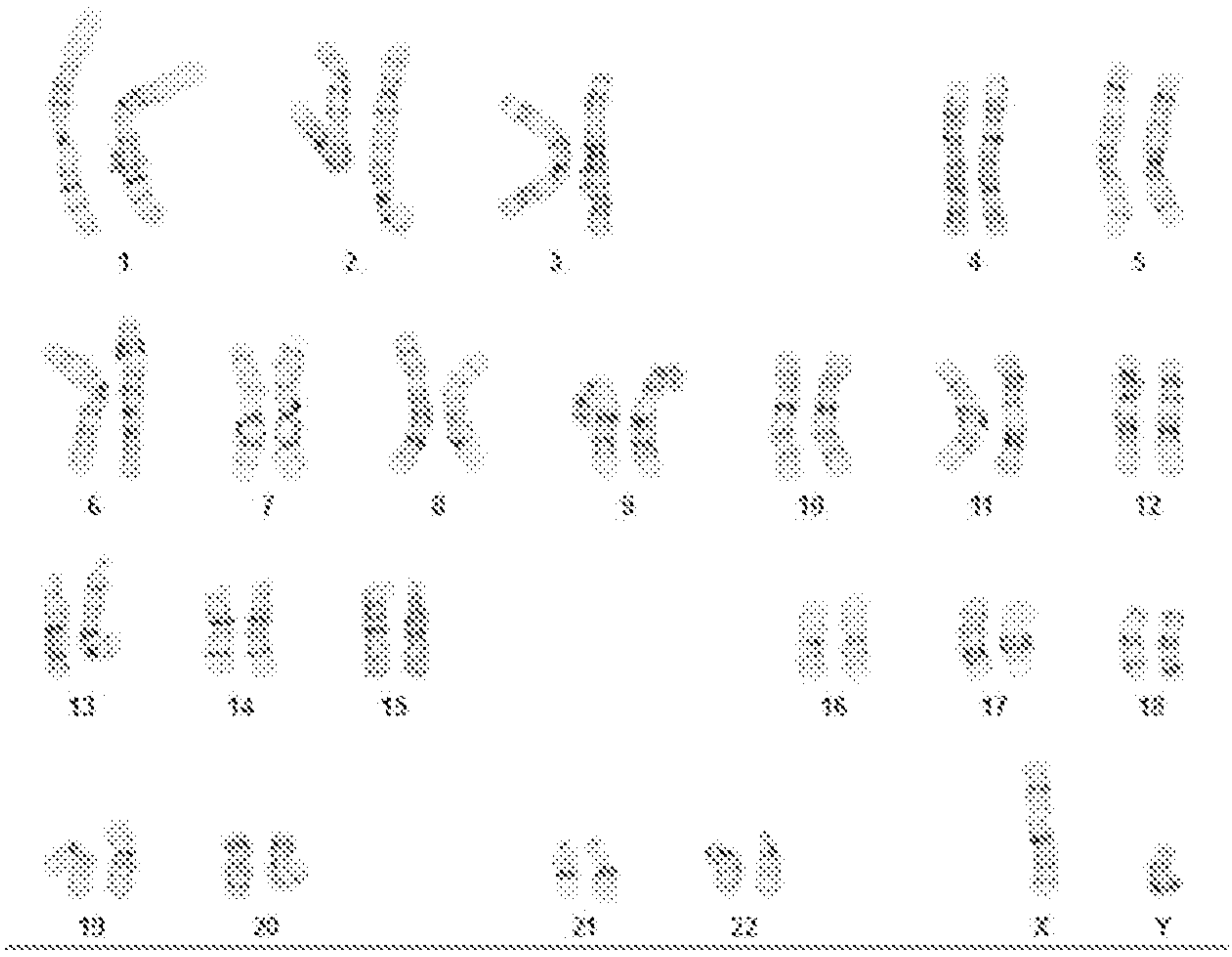


FIG. 20B

Clone 3, Passage 9

FIG. 20C

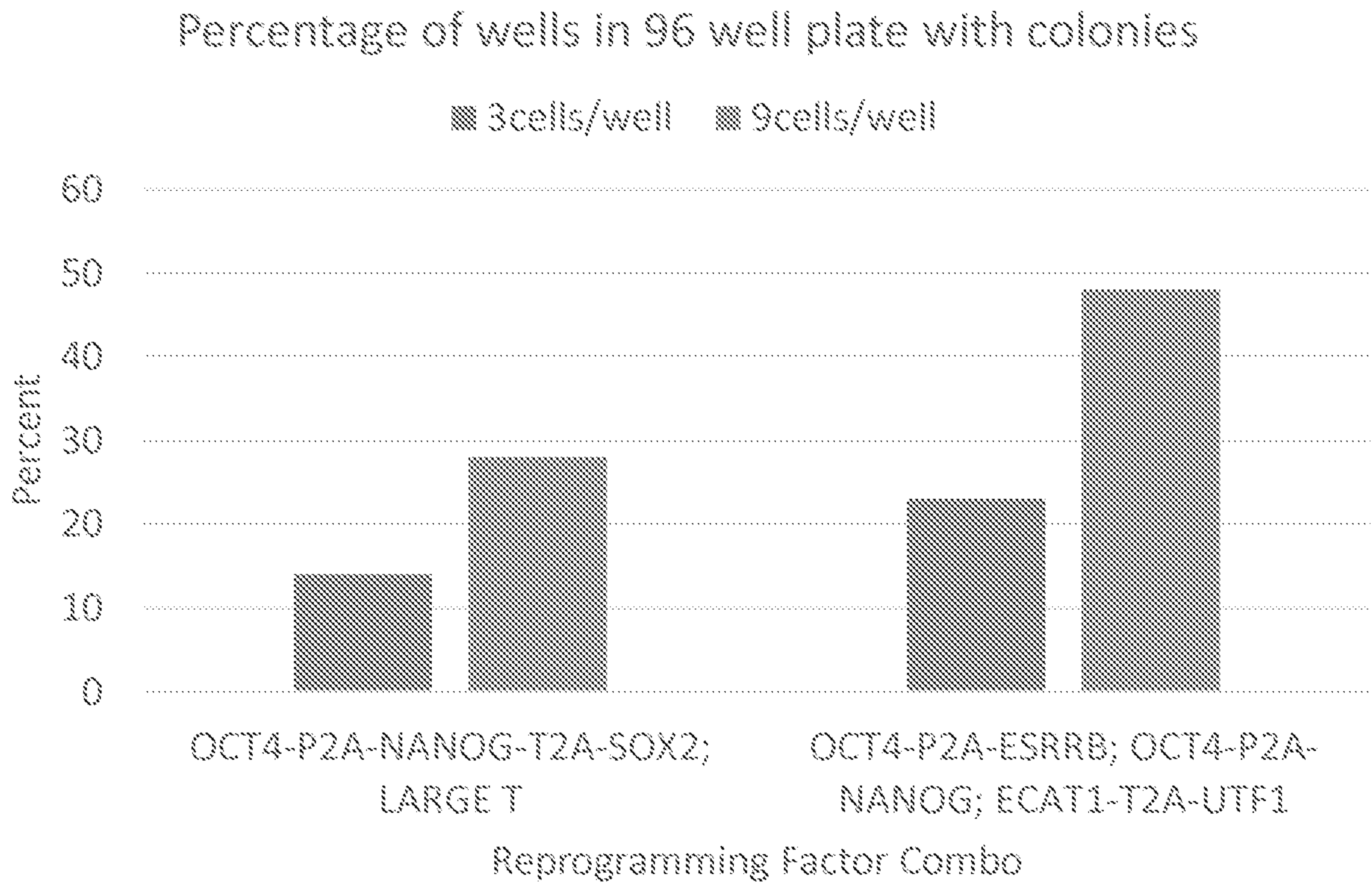


FIG. 21

OCT4-P2A-OCT4 / NANOG-P2A-ESRRB-T2A-LIN28 / ECAT1-T2A-UTF1

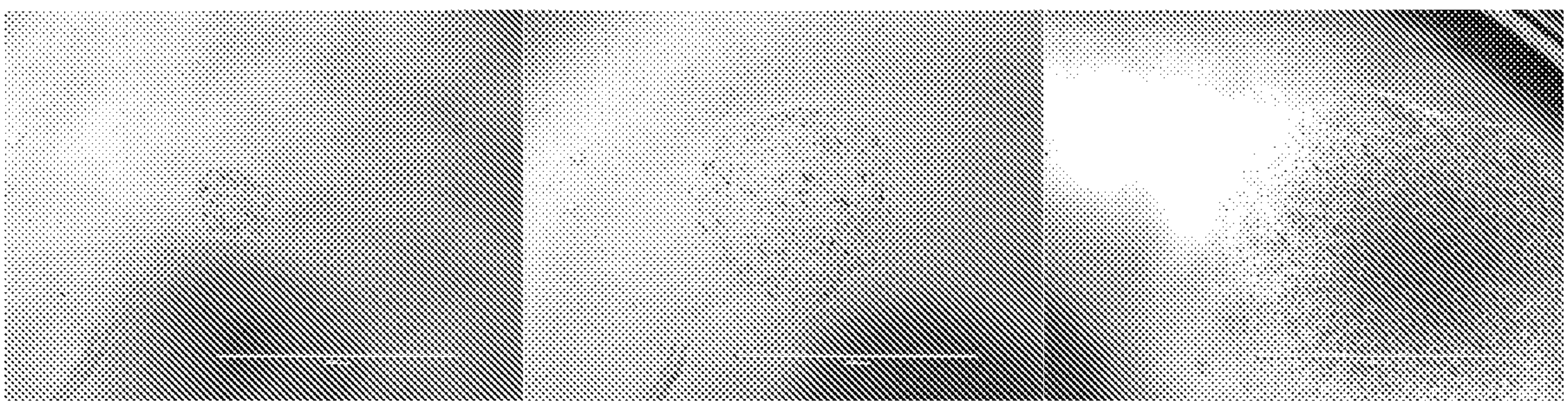


FIG. 22A

OCT4-P2A-ESRRB / OCT4-P2A-NANOG / ECAT1-T2A-UTF1

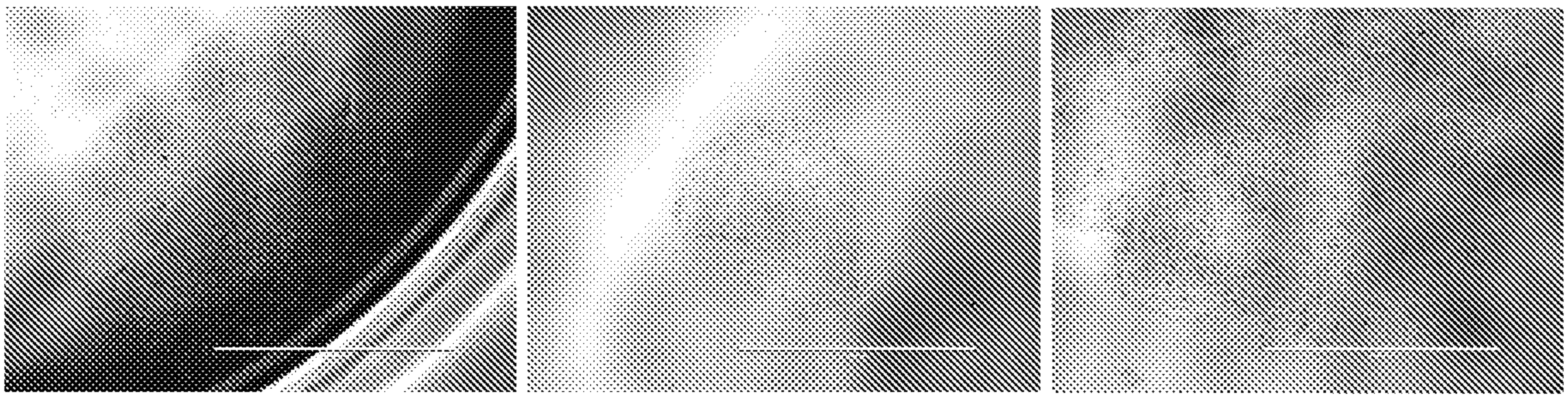


FIG. 22B

Constant Expression	Selection for	SSEA4/Tra181 Positive by flow analysis
OCT4-P2A-NANOG-T2A-SOX2 SV40 Large T Antigen	No Selection	0.9%
OCT4-P2A-NANOG-T2A-SOX2 SV40 Large T Antigen OCT4-P2A-SOX2-Puro	OCT4/SOX2	0.24%
OCT4-P2A-NANOG-T2A-SOX2 SV40 Large T Antigen OCT4-P2A-NANOG-T2A-SOX2-Puro	OCT4/NANOG/SOX2	0.86%
OCT4-P2A-NANOG-T2A-SOX2 SV40 Large T Antigen OCT2-P2A-OCT4-Puro	OCT4	13.93%

FIG. 23A

OCT4-P2A-NANOG-T2A-SOX2 /SV40 Large T Antigen

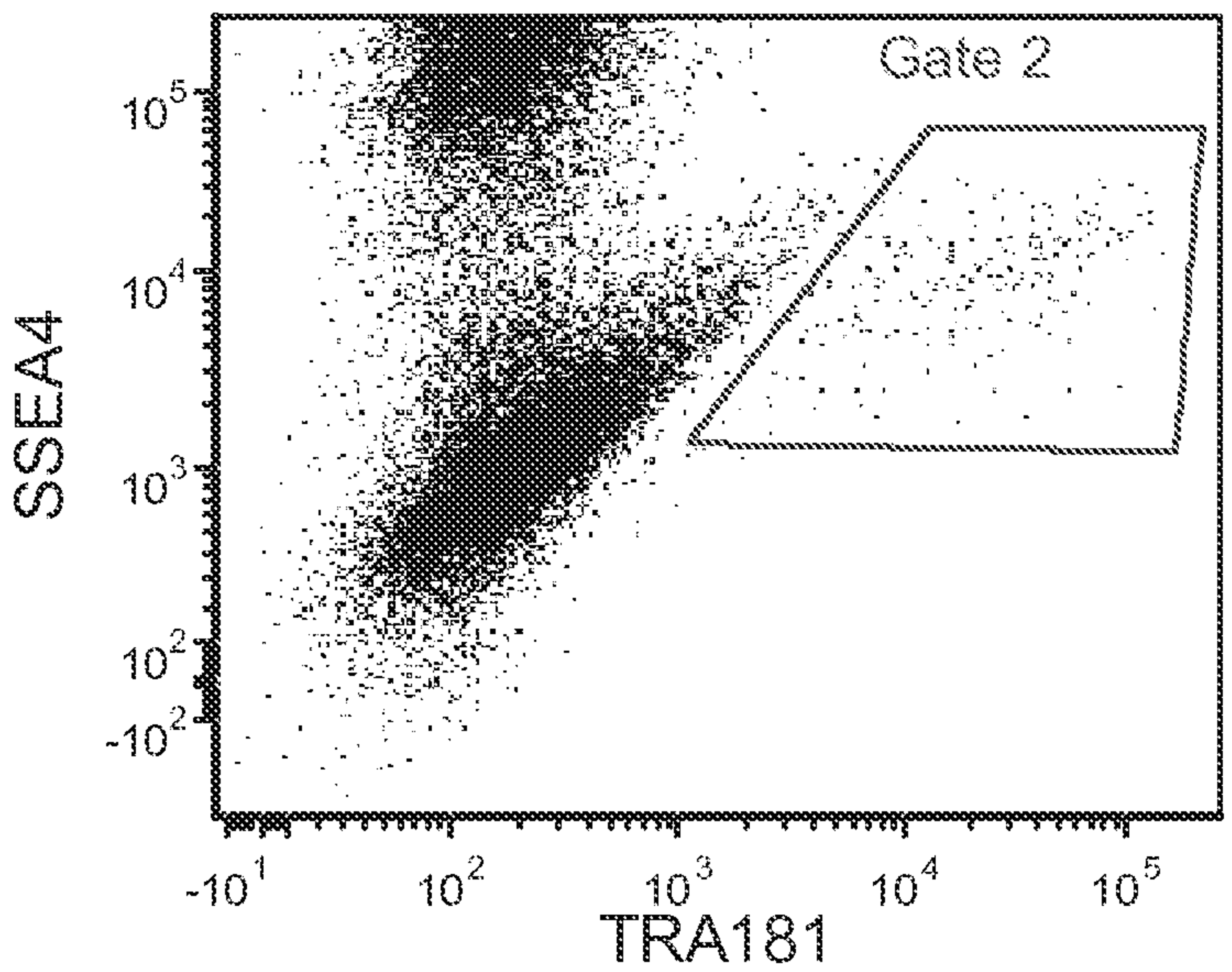


FIG. 23B

OCT4-P2A-NANOG-T2A-SOX2 / SV40 Large T Antigen / OCT2-P2A-OCT4-Puro

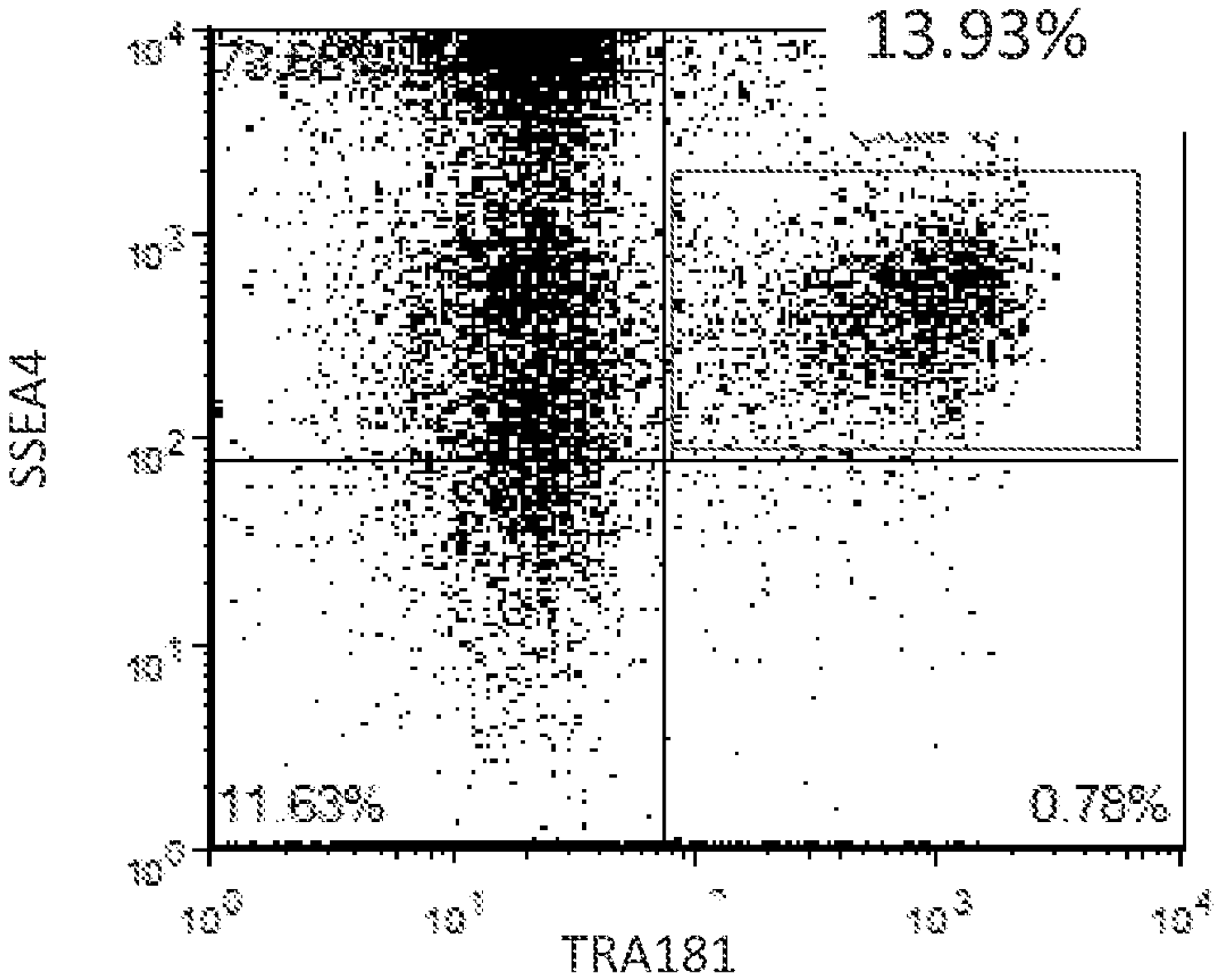


FIG. 23C

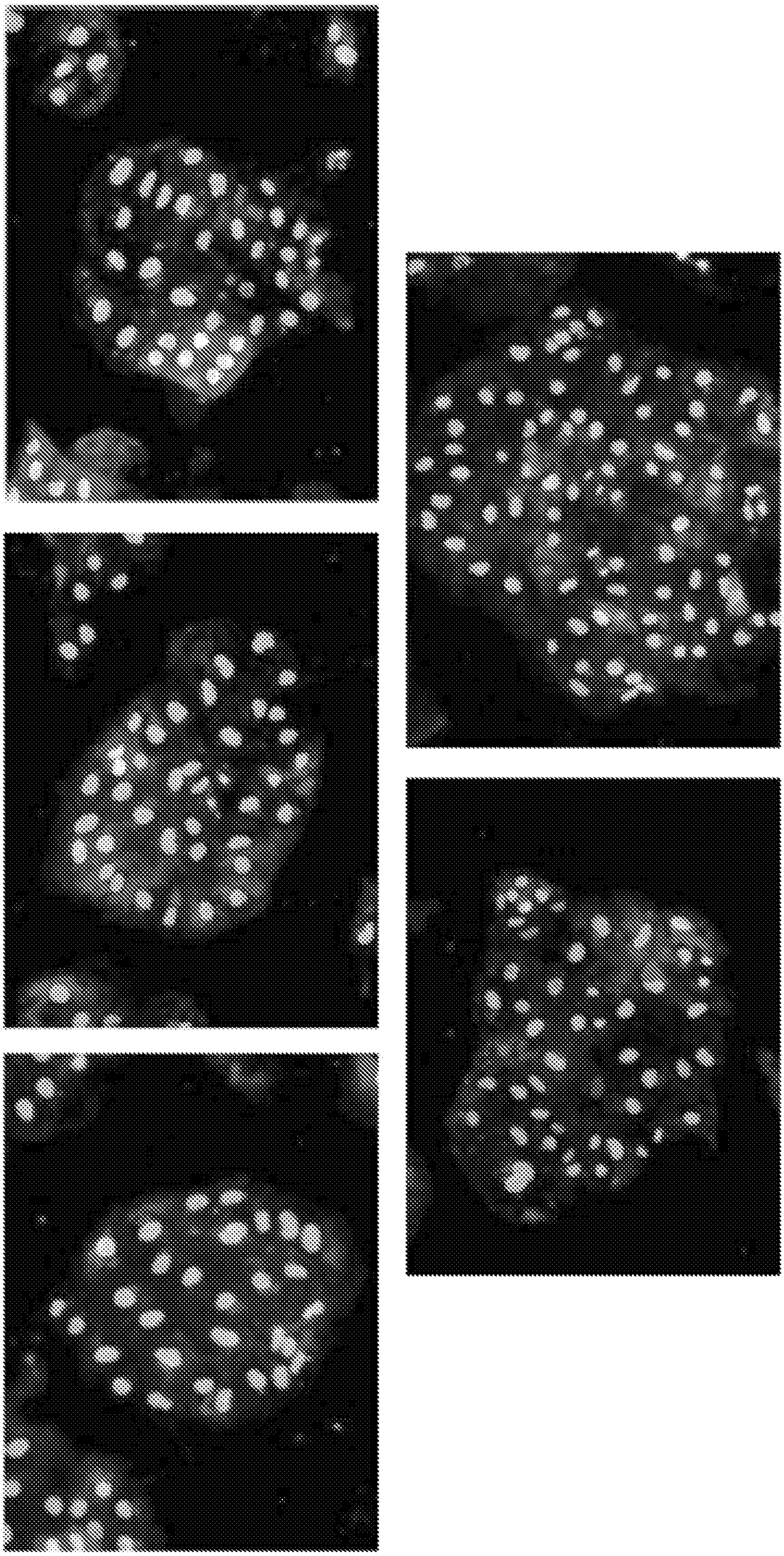


FIG. 24

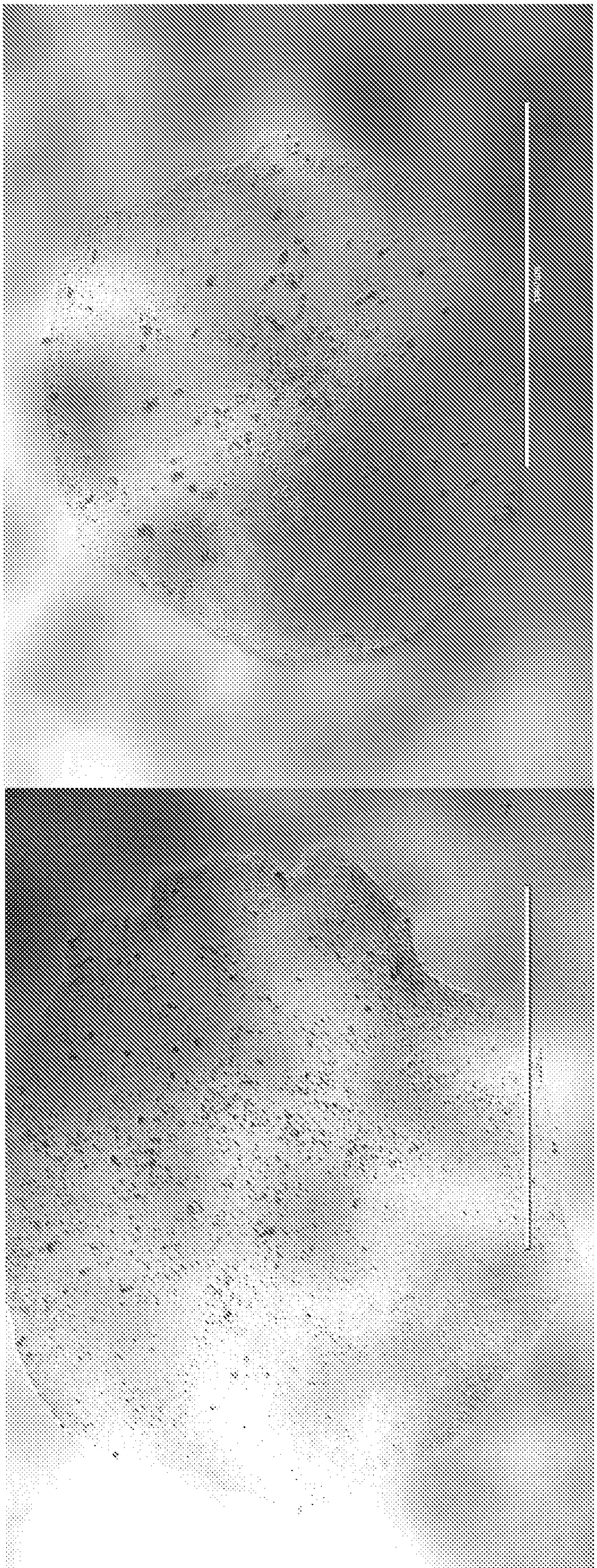


FIG. 25

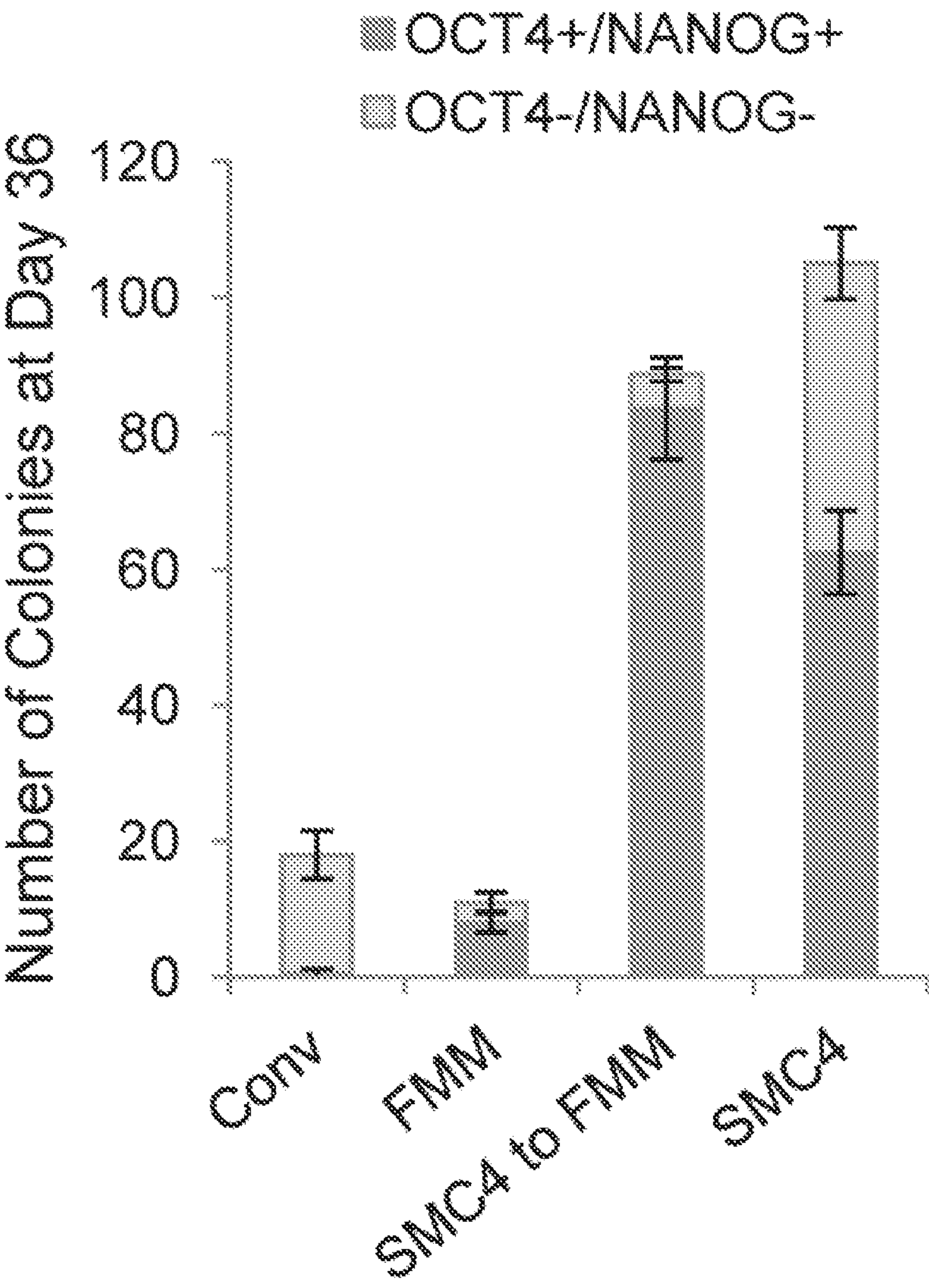

OCT4+/NANOG+
OCT4-/NANOG-
Number of Colonies at Day 36
120
100
80
60
40
20
0
Conv
FMM
SMC4 to FMM
SMC4

FIG. 1E