Supplementary Figure Legends

Figure 1: Single Cell qPCR analysis.

Heatmap analysis of 232 single cells analysed across 45 genes using a Fluidigm BioMark system, hierarchical clustering was performed for the genes assessed. The heatmap visualises the individual gene expression after normalisation across genes and samples. White coloured genes indicates undetected levels. Black arrows indicate cells which appear to express pluripotency associated genes (particularly *SOX2, NANOG* and *POU5F1*) at a high level whilst also expressing early differentiation markers. Cells co-expressing pluripotency and differentiation associated genes were readily detected in the *MIXL1*(**+**)/SSEA-3(**+**) fraction but not the other fractions.

Figure 2: Assessing the stem cell potential of *MIXL1*(+)/SSEA-3(+) substate.

a) Live TRA-1-81 staining fluorescent images of colonies derived after the first passage into a 48 well plate at 4x, TRA-1-81(RED) and *MIXL1*-GFP (GREEN). Wells marked with white stars indicates clones that survived the passage and stained positive for TRA-1-81. Of the 44 colonies passaged, 27 survived and stained positive for TRA-1-81. **b)** Immunofluorescent analysis of NANOG expression in HES3 *MIXL1*-GFP clones 2-D2 and 3-C6 growing in E8V conditions. Merged images display Hoechst (Nuclei) in blue and NANOG positive cells in red. Secondary only staining control is also shown. **c)** Bar chart showing the percentage positive cells for the stem cell associated antigens BF4, CD9, SSEA-3, SSEA4, TRA-1-60s, TRA-1-81 and TRA-2-49 for six clonal lines established (Mean of all lines +/- SD). All lines displayed high expression of these surface markers.

Figure 3: Averaged qPCR Signature Comparison

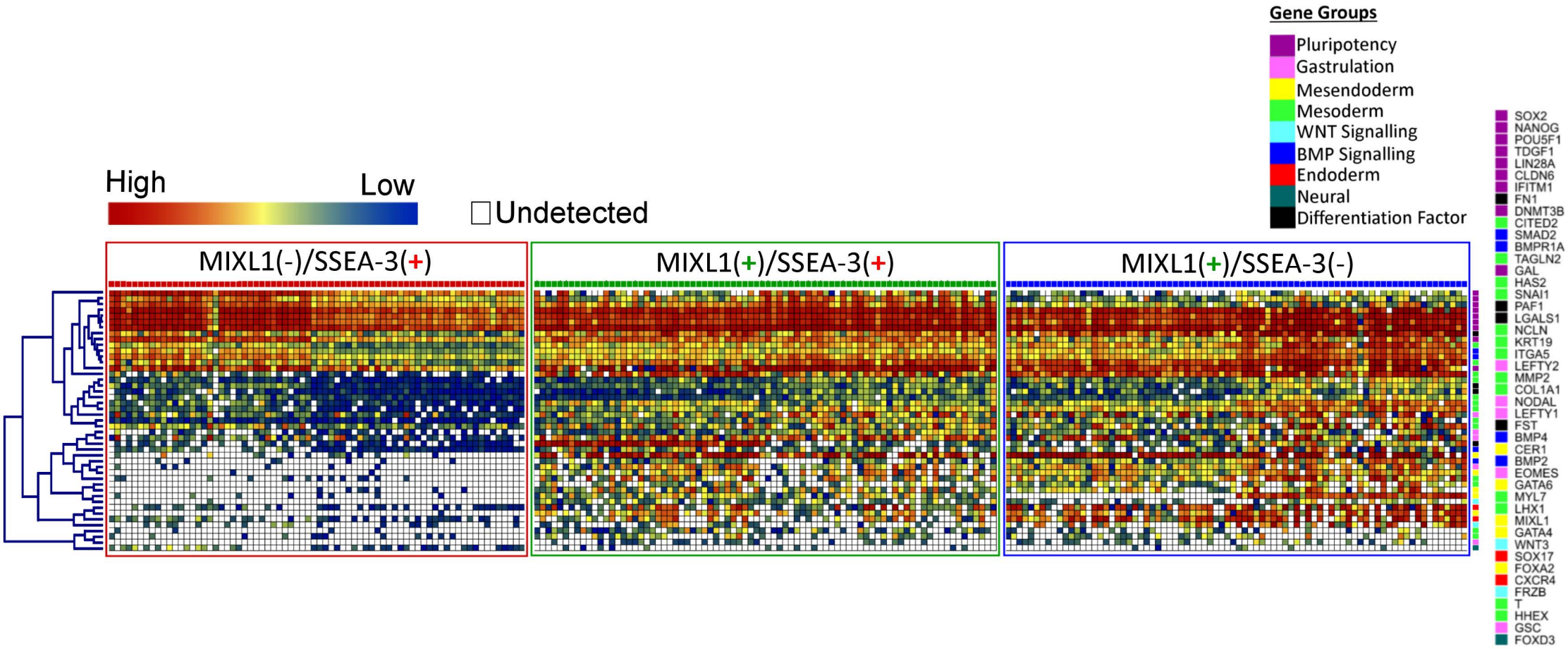
The average 1/Ct values for 47 genes from single cell qPCR analysis. Genes were ordered from highest to lowest expression based on the *MIXL1*(-)/SSEA-3(+) fraction. A solid line connects the mean expression points to give a state “signature” with surrounding shaded

796 area represents the 95% confidence interval of the data. **a)** Displays the state signatures of
797 *MIXL1*(-)/SSEA-3(+)(red), *MIXL1*(+)/SSEA-3(+) (green) and *MIXL1*(+)/SSEA-3(-) (blue)
798 grown in MEF/KOSR conditions. **b)** Displays the state signatures of *MIXL1*(+)/SSEA-3(+)
799 cells grown in MEF/KOSR (green) and Primo (purple) conditions. The state signature of the
800 both *MIXL1*(+)/SSEA-3(+) were very similar.
801
802 **Figure 4: Single Cell Gene Expression Plots.**
803 The single cell gene expression distribution was similar between the two *MIXL1*(+)/SSEA-
804 3(+) fractions from MEF/KOSR (green) and Primo (purple) conditions. 1/Ct values for each
805 single cell for a given gene. Mean and standard deviation are displayed on top of data sets
806 as black bars. Cells are split into their respective sorted fractions MEF/KOSR conditions
807 *MIXL1*(-)/SSEA-3(+) cells in red, *MIXL1*(+)/SSEA-3(+) cells in green, *MIXL1*(-)/SSEA-3(+)
808 cells in blue and Primo conditions *MIXL1*(+)/SSEA-3(+) cells in purple. **a)** Contains a
809 collection of plots from genes associated with pluripotency. **b)** Contains a collection of plots
810 from key genes associated with mesendoderm differentiation. **c-f)** Contains plots from the
811 remaining genes assessed by single cell qPCR.
812
813 **Figure 5: Differentiation Time Course**
814 Flow cytometry density plots of HES3 *MIXL1*-GFP cells stained for SSEA-3 at indicated time
815 points after induction of differentiation in E8 containing 3μM CHIRON. Red boxes indicate
816 the sorting gates for each timepoint. The expression of *MIXL1*-GFP increases first, before
817 the eventual loss of SSEA-3.
818
819 **Figure 6: Clones generated from the *MIXL1*(+)/SSEA-3(+) from Primo medium exhibit**
820 **normal stem cell growth and characteristics.**
821 **a)** Live TRA-1-81 staining fluorescent images of colonies derived from single cell deposition
822 of *MIXL1*(+)/SSEA-3(+) from PRIMO Plus conditions after the first passage into a 48 well
823 plate at 4x, TRA-1-81(RED) and *MIXL1*-GFP (GREEN). Wells marked with white stars

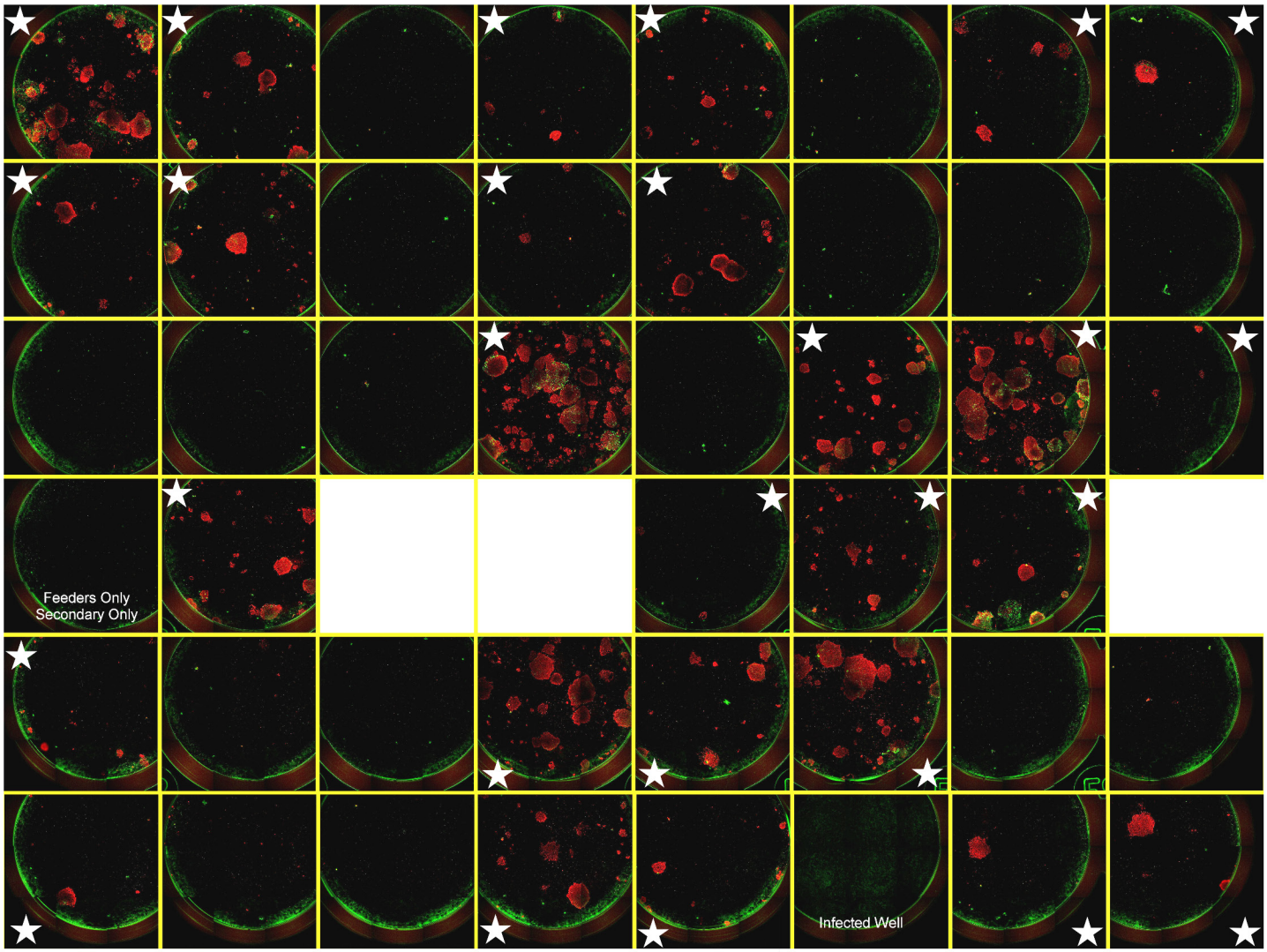
824 indicates clones that survived the passage and stained positive for TRA-1-81. **b)** Flow
825 cytometry density plot of *MIXL1*-GFP versus SSEA-3 from clone 12-F11 grown in
826 MEF/KOSR conditions. **c)** Bar chart of percentage positive stem cell associated antigen
827 SSEA-3 and *MIXL1*-GFP expression for five clonal lines during initial expansion in
828 MEF/KOSR conditions. **d)** Flow cytometry density plot of *MIXL1*-GFP versus SSEA-3 from
829 clone 12-F11 after being transitioned into E8V conditions. **e)** Bar chart of percentage positive
830 stem cell associated antigens BF4, CD9, SSEA-3, SSEA4, THY-1 and TRA-1-81 for six
831 clonal lines. All lines displayed high expression of these surface markers.
832
833 **Figure 7: Components of Primo can be substituted for others that target the same**
834 **pathway.**
835 **a-e)** Flow cytometry density plots of T-venus and SSEA-3 expression in different conditions.
836 **a)** In standard E8V conditions. **b)** Using PRIMO Plus formulation, IWP2 was replaced for
837 DKK1 at 100ng/mL, density plot reveals high double expression 4 days after the first
838 passage. **c)** CHIR99021 was replaced with SB216763 at 10μM, density plot reveals high
839 double expression after 3 days of induction. **d)** LPA was replaced with S1P, density plots
840 demonstrate the ability of S1P to block differentiation, 1.92μM S1P maintained a high
841 proportion of double positive cells after 3 days of induction (optimal concentrated highlighted
842 with a red box). **e)** LPA was replaced with GRI977143, density plots demonstrate the ability
843 of GRI to block differentiation, 4μM GRI maintained a high proportion of double positive cells
844 after 3 days of induction (optimal concentrated highlighted with a red box).
845
846 **Figure 8: Passage 10 NANOG and SOX2 expression Analysis**
847 Cells in all conditions at the tenth passage show high expression of NANOG and SOX2. **a-b)**
848 Immunofluorescence analysis of Hoechst, *MIXL1*-GFP, and **a)** NANOG or **b)** SOX2
849 expression of HES3 *MIXL1*-GFP cells in PRIMO Plus, E8+LPA (0.96μM), E8 alone, E8 with
850 1μM IWP-2 added and E8+LPA (0.96μM) (Secondary antibody only staining) for 3 days post
851 to 9 passages in PRIMO Plus. A merged image of all three channels is present below

Hoechst (Blue), *MIXL1*-GFP (Green) and NANOG or SOX2 (Red). **c-d)** Stacked percentage bar charts displaying cell profiler analysis of 3 wells for each condition (Bars are mean ± SD, n= 3 technical repeats) for *MIXL1*-GFP, and **c)** NANOG or **d)** SOX2 expression grown in PRIMO Plus, E8+LPA (0.96μM), E8 alone and E8 with 1μM IWP-2 added, for 3 days post to 9 passages in PRIMO Plus.

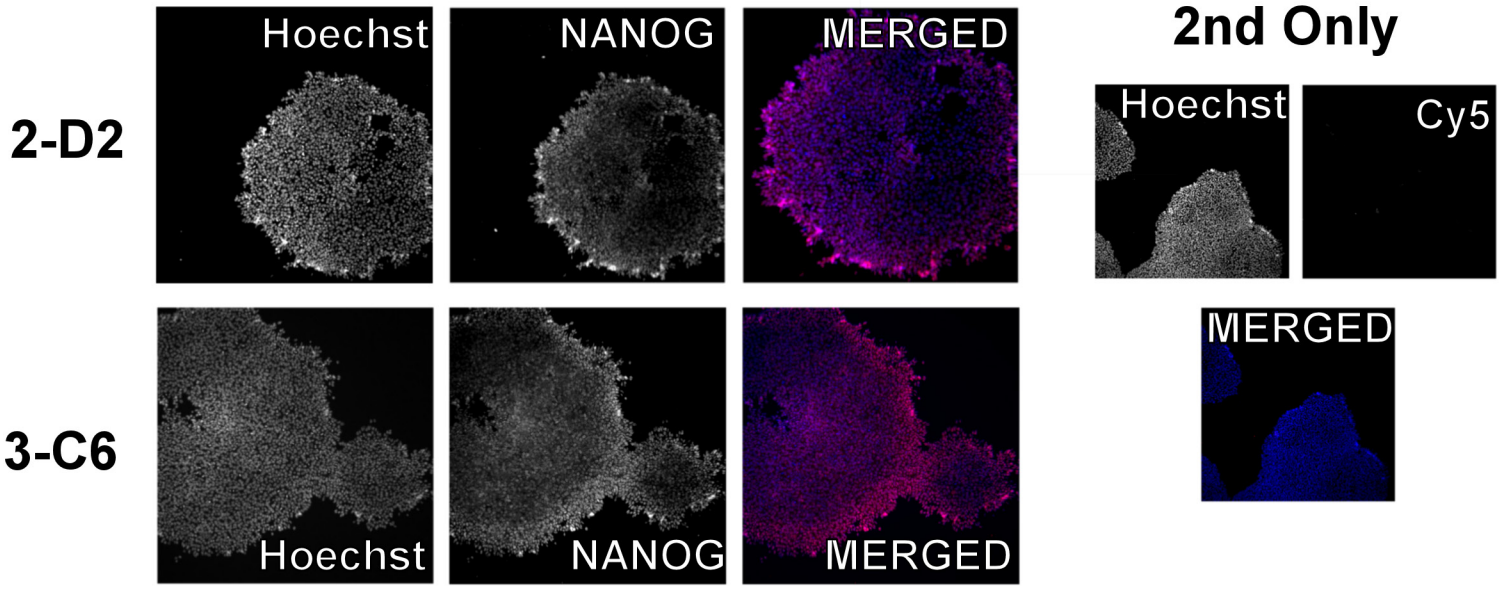
S1


Gene Groups
Pluripotency
Gastrulation
Mesendoderm
Mesoderm
WNT Signalling
BMP Signalling
Endoderm
Neural
Differentiation Factor
High
Low
Undetected
MIXL1(-)/SSEA-3(+)
MIXL1(+)/SSEA-3(+)
MIXL1(+)/SSEA-3(-)
SOX2
NANOG
POU5F1
TDGF1
LIN28A
CLDN6
IFITM1
FN1
DNMT3B
CITED2
SMAD2
BMPR1A
TAGLN2
GAL
HAS2
SNAI1
PAF1
LGALS1
NCLN
KRT19
ITGA5
LEFTY2
MMP2
COL1A1
NODAL
LEFTY1
FST
BMP4
CER1
BMP2
EOMES
GATA6
MYL7
LHX1
MIXL1
GATA4
WNT3
SOX17
FOXA2
CXCR4
FRZB
T
HHEX
GSC
FOXD3

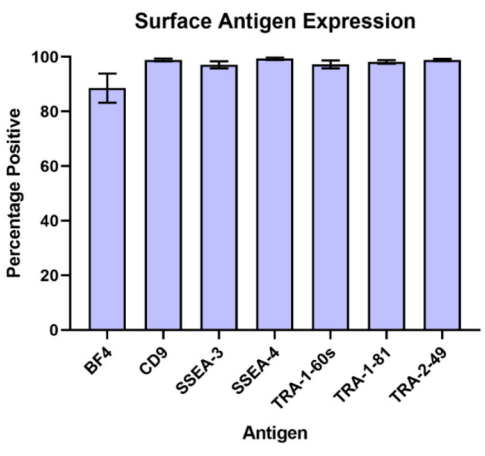
a


Feeders Only
Secondary Only
Infected Well

b


2-D2
Hoechst
NANOG
MERGED
2nd Only
Hoechst
Cy5
3-C6
MERGED
Hoechst
NANOG
MERGED

c


Surface Antigen Expression
Percentage Positive
100
80
60
40
20
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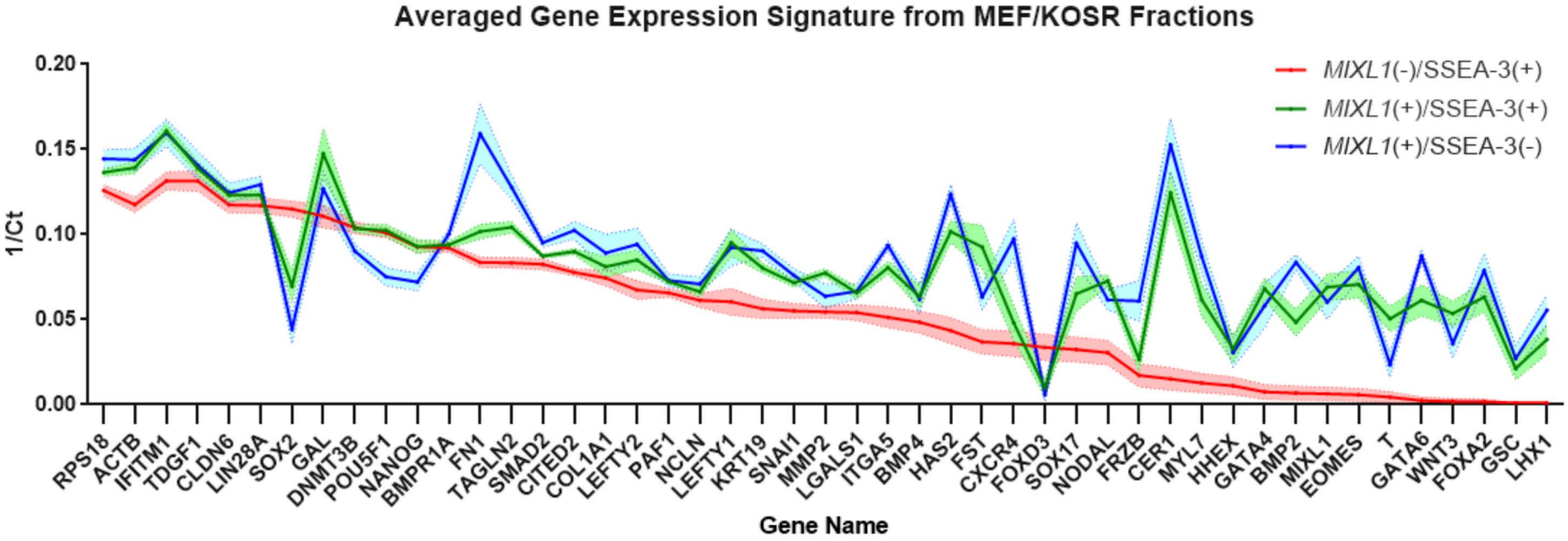
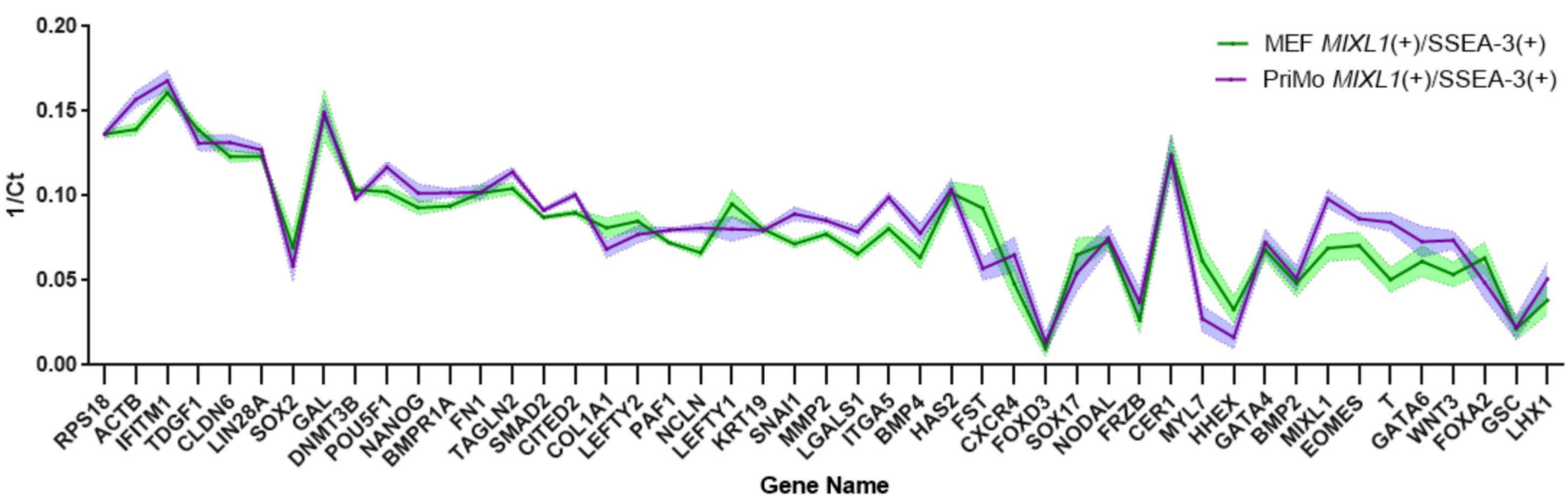
BF4
CD9
SSEA-3
SSEA-4
TRA-1-60s
TRA-1-81
TRA-2-49
Antigen

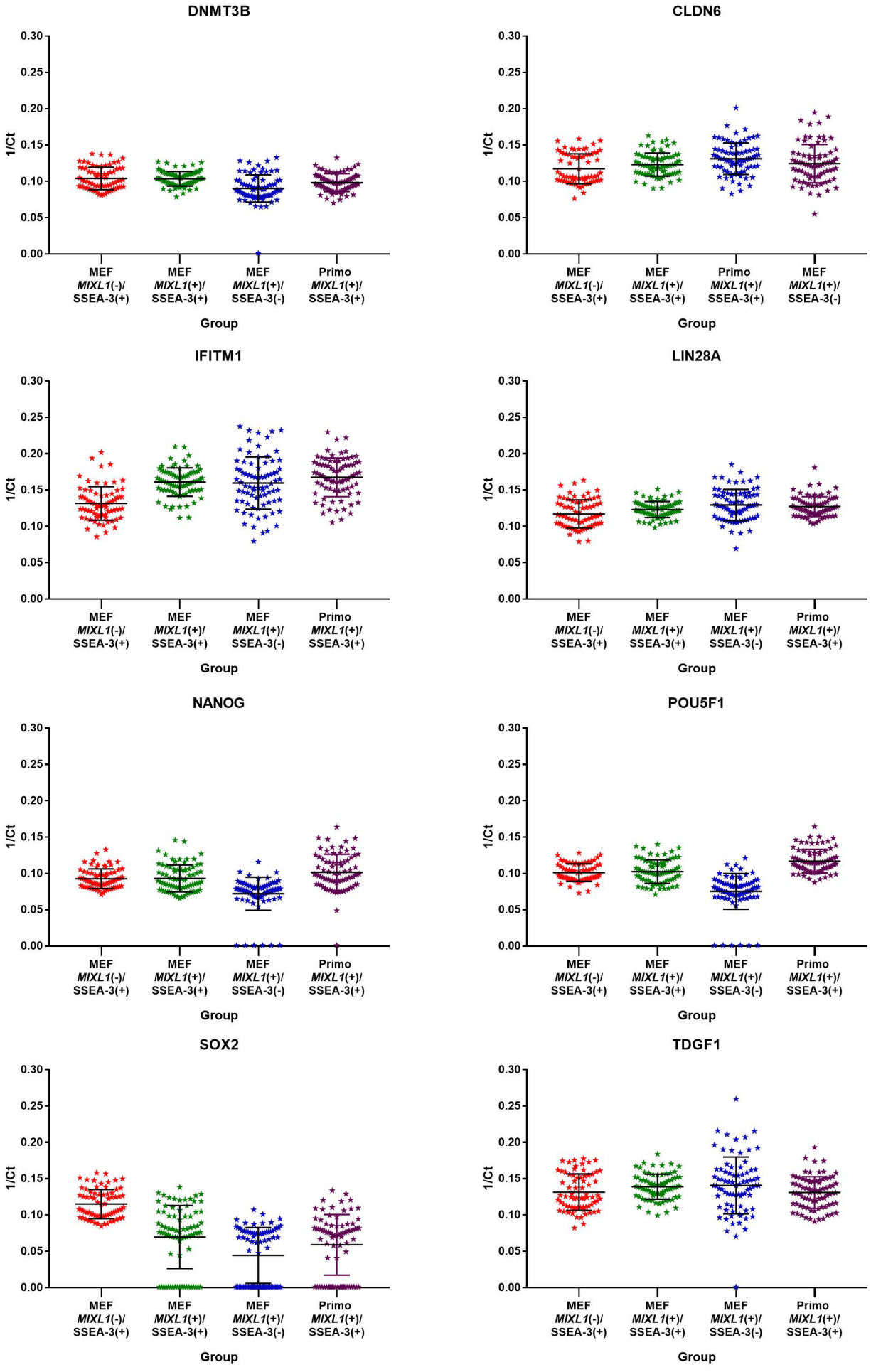
a


Averaged Gene Expression Signature from MEF/KOSR Fractions
MIXL1(-)/SSEA-3(+)
MIXL1(+)/SSEA-3(+)
MIXL1(+)/SSEA-3(-)
1/Ct
0.20
0.15
0.10
0.05
0.00
RPS18 ACTB IFITM1 TDGF1 CLDN6 LIN28A SOX2 GAL DNMT3B POU5F1 NANOG BMPR1A FN1 TAGLN2 SMAD2 CITED2 COL1A1 LEFTY2 PAF1 NCLN LEFTY1 KRT19 SNAI1 MMP2 LGALS1 ITGA5 BMP4 HAS2 FST CXCR4 FOXD3 SOX17 NODAL FRZB CER1 MYL7 HHEX GATA4 BMP2 MIXL1 EOMES T GATA6 WNT3 FOXA2 GSC LHX1
Gene Name

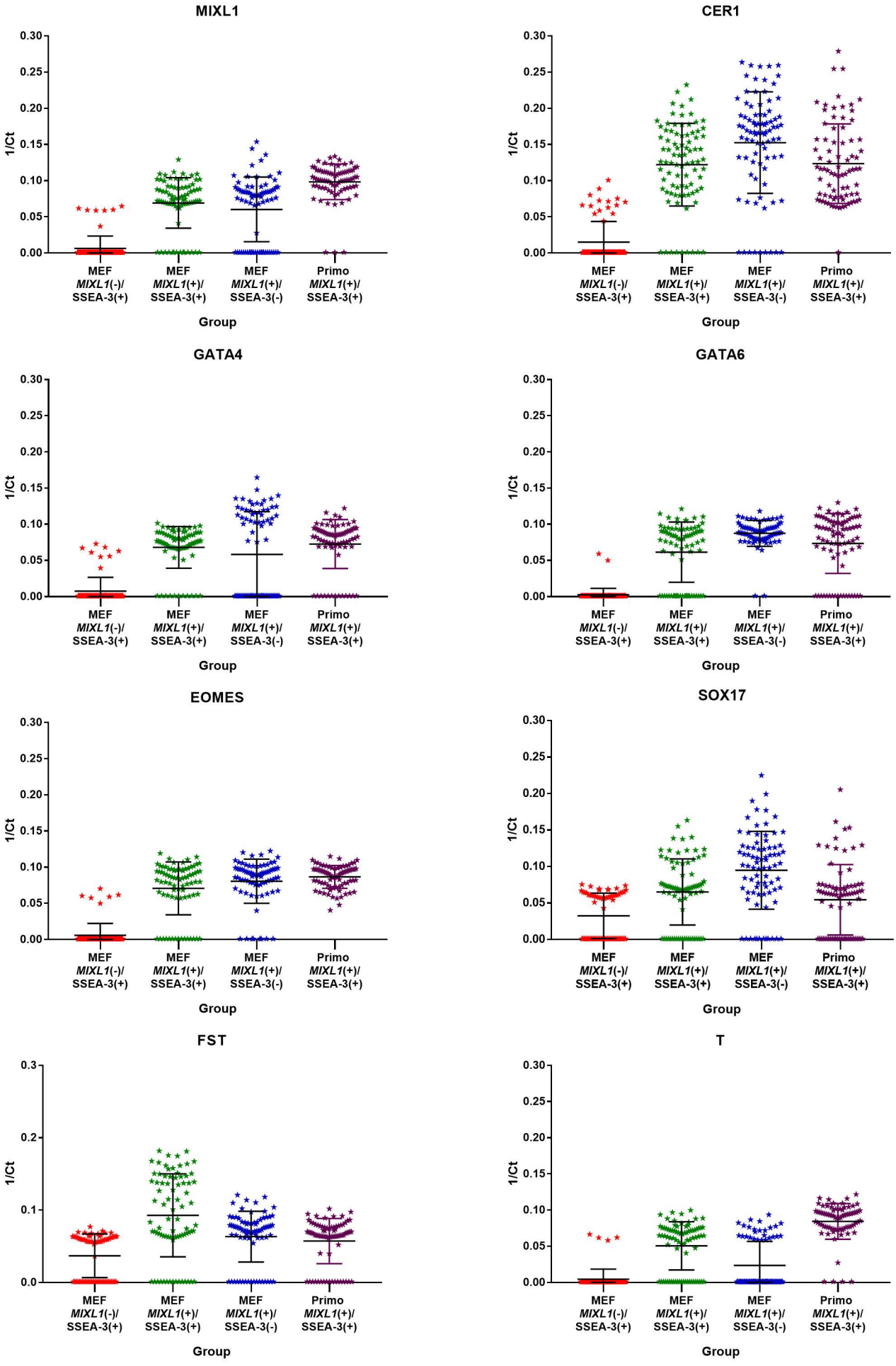
b


Averaged Gene Expression Signature from MIXL1(+)/SSEA-3(+) Fractions
MEF MIXL1(+)/SSEA-3(+)
PriMo MIXL1(+)/SSEA-3(+)
1/Ct
0.20
0.15
0.10
0.05
0.00
RPS18 ACTB IFITM1 TDGF1 CLDN6 LIN28A SOX2 GAL DNMT3B POU5F1 NANOG BMPR1A FN1 TAGLN2 SMAD2 CITED2 COL1A1 LEFTY2 PAF1 NCLN LEFTY1 KRT19 SNAI1 MMP2 LGALS1 ITGA5 BMP4 HAS2 FST CXCR4 FOXD3 SOX17 NODAL FRZB CER1 MYL7 HHEX GATA4 BMP2 MIXL1 EOMES T GATA6 WNT3 FOXA2 GSC LHX1
Gene Name

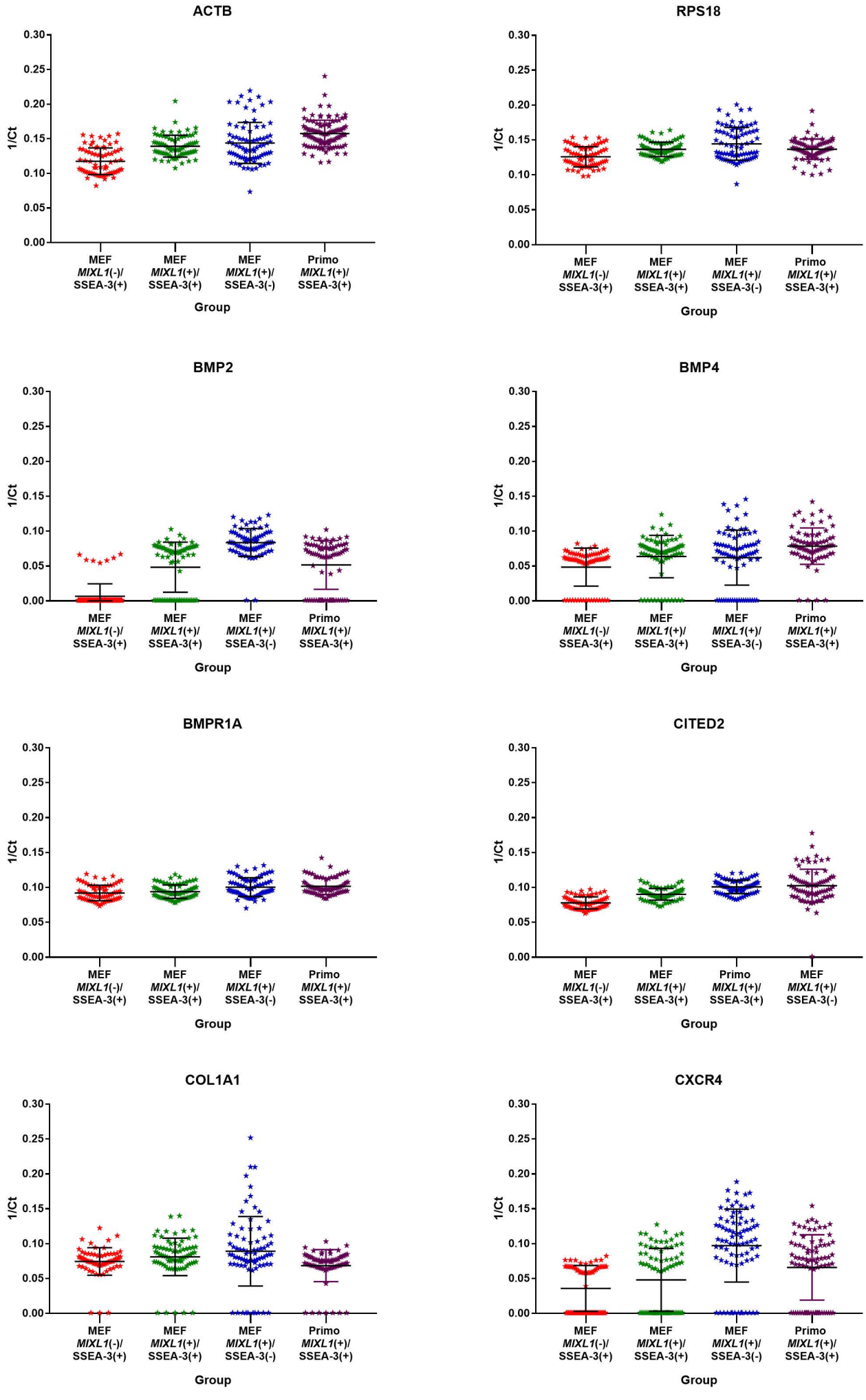
S4. a


DNMT3B
CLDN6
IFITM1
LIN28A
NANOG
POU5F1
SOX2
TDGF1
1/Ct
0.30
0.25
0.20
0.15
0.10
0.05
0.00
MEF MIXL1(-)/ SSEA-3(+)
MEF MIXL1(+)/ SSEA-3(+)
MEF MIXL1(+)/ SSEA-3(-)
Primo MIXL1(+)/ SSEA-3(+)
Group

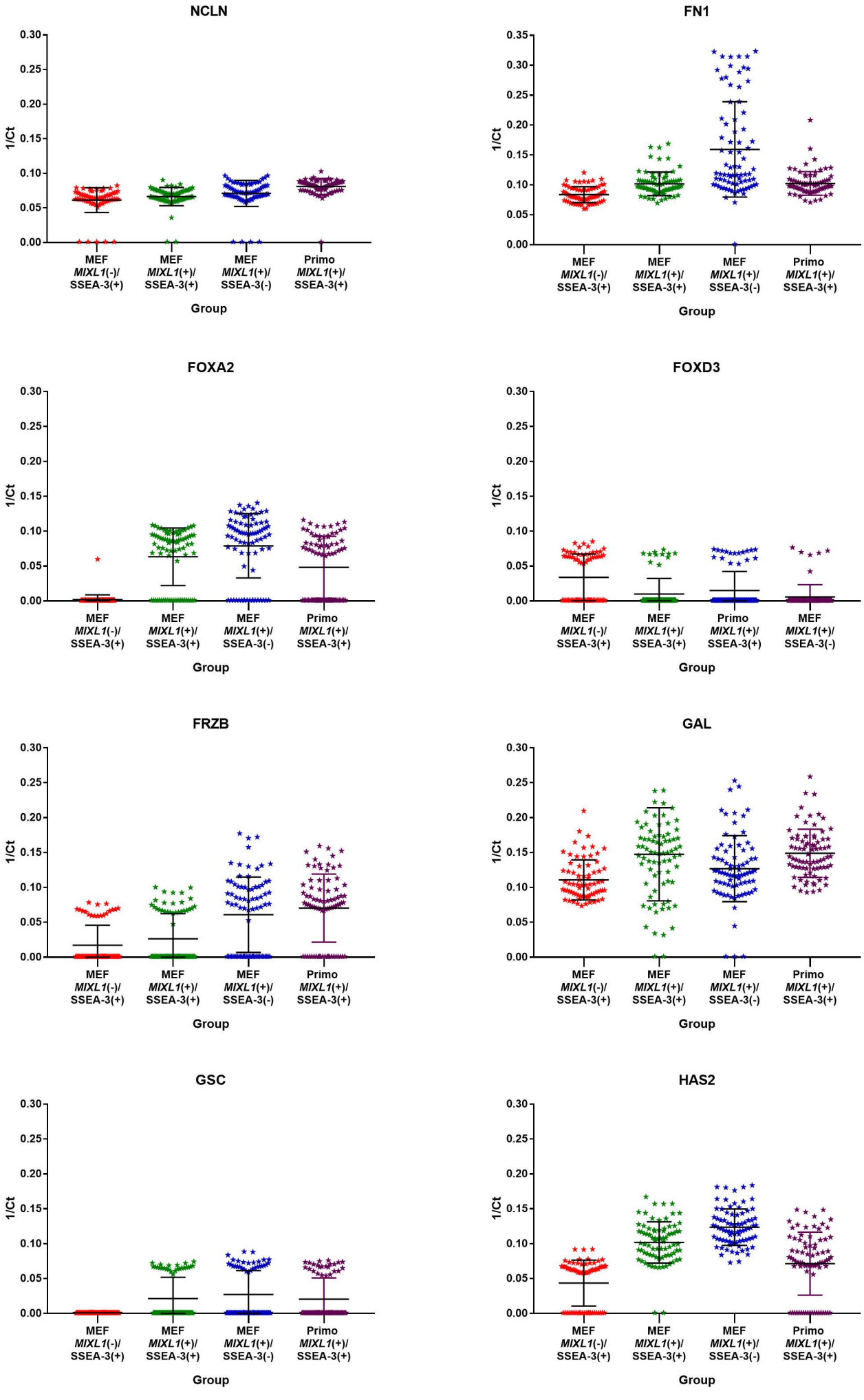
S4. b


MIXL1
CER1
GATA4
GATA6
EOMES
SOX17
FST
T
1/Ct
Group
MEF MIXL1(-)/ SSEA-3(+)
MEF MIXL1(+)/ SSEA-3(+)
MEF MIXL1(+)/ SSEA-3(-)
Primo MIXL1(+)/ SSEA-3(+)

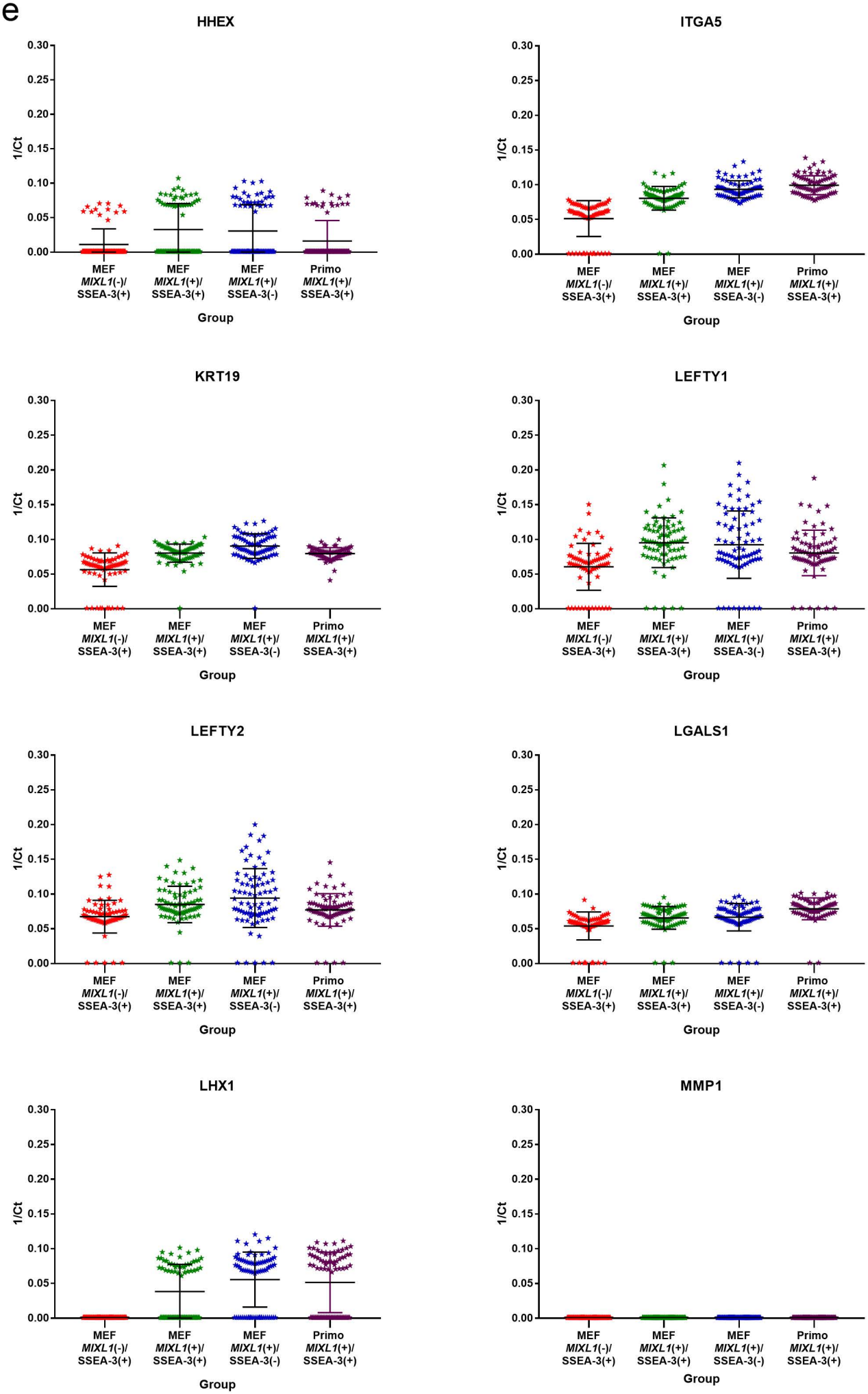
S4. c


ACTB
RPS18
BMP2
BMP4
BMPR1A
CITED2
COL1A1
CXCR4
1/Ct
0.30
0.25
0.20
0.15
0.10
0.05
0.00
MEF MIXL1(-)/ SSEA-3(+)
MEF MIXL1(+)/ SSEA-3(+)
MEF MIXL1(+)/ SSEA-3(-)
Primo MIXL1(+)/ SSEA-3(+)
Group

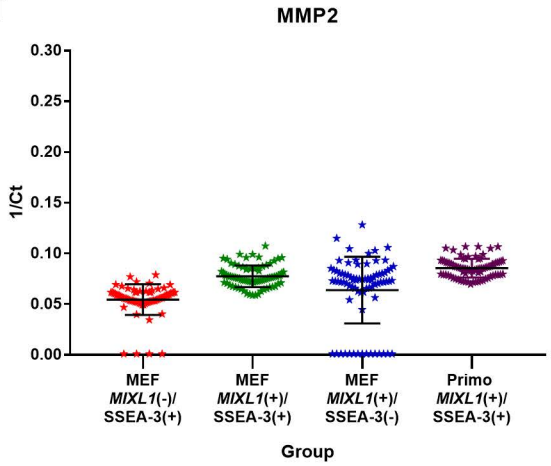
S4. d

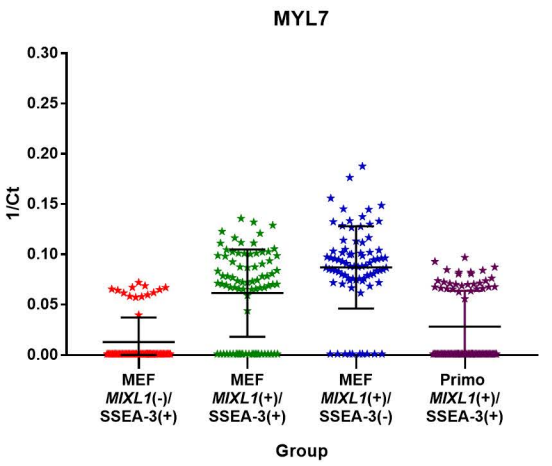

NCLN
FN1
FOXA2
FOXD3
FRZB
GAL
GSC
HAS2
1/Ct
0.30
0.25
0.20
0.15
0.10
0.05
0.00
MEF MIXL1(-)/ SSEA-3(+)
MEF MIXL1(+)/ SSEA-3(+)
MEF MIXL1(+)/ SSEA-3(-)
Primo MIXL1(+)/ SSEA-3(+)
Group

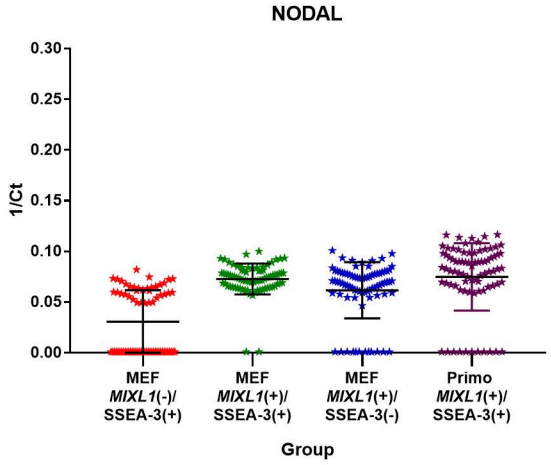
S4. e

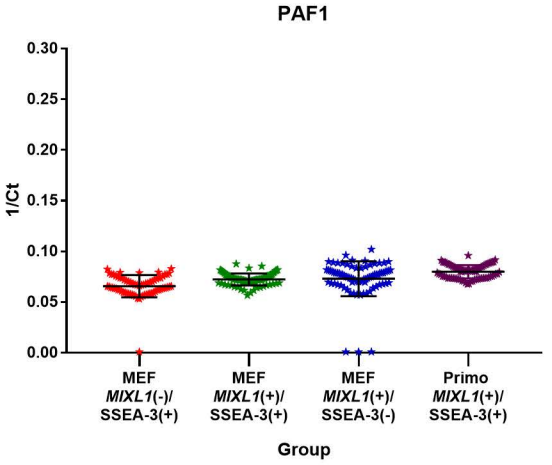

HHEX
ITGA5
KRT19
LEFTY1
LEFTY2
LGALS1
LHX1
MMP1
1/Ct
0.30
0.25
0.20
0.15
0.10
0.05
0.00
MEF MIXL1(-)/ SSEA-3(+)
MEF MIXL1(+)/ SSEA-3(+)
MEF MIXL1(+)/ SSEA-3(-)
Primo MIXL1(+)/ SSEA-3(+)
Group

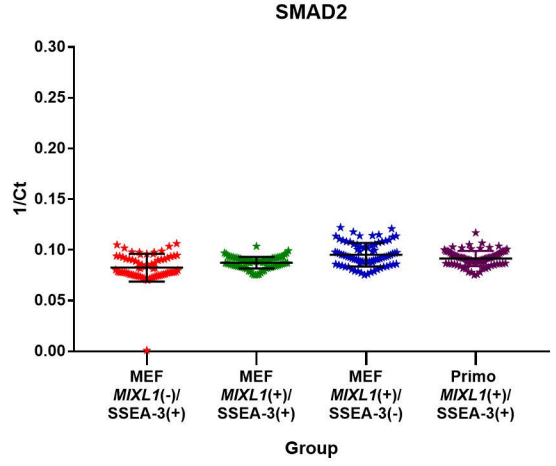
S4. f

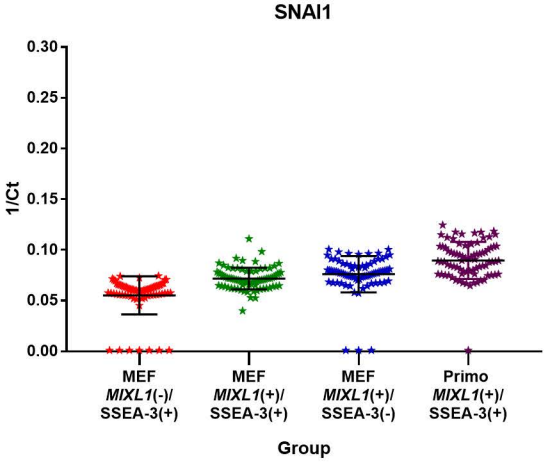

MMP2
1/Ct
0.30
0.25
0.20
0.15
0.10
0.05
0.00
MEF MIXL1(-)/ SSEA-3(+)
MEF MIXL1(+)/ SSEA-3(+)
MEF MIXL1(+)/ SSEA-3(-)
Primo MIXL1(+)/ SSEA-3(+)
Group

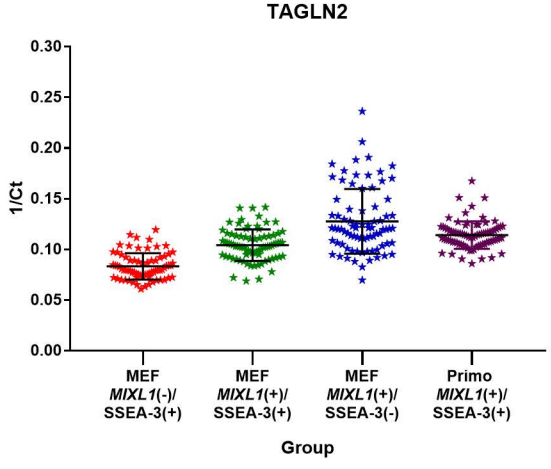

MYL7
1/Ct
0.30
0.25
0.20
0.15
0.10
0.05
0.00
MEF MIXL1(-)/ SSEA-3(+)
MEF MIXL1(+)/ SSEA-3(+)
MEF MIXL1(+)/ SSEA-3(-)
Primo MIXL1(+)/ SSEA-3(+)
Group

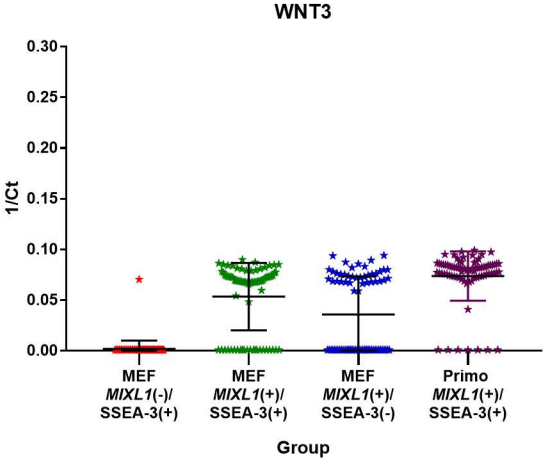

NODAL
1/Ct
0.30
0.25
0.20
0.15
0.10
0.05
0.00
MEF MIXL1(-)/ SSEA-3(+)
MEF MIXL1(+)/ SSEA-3(+)
MEF MIXL1(+)/ SSEA-3(-)
Primo MIXL1(+)/ SSEA-3(+)
Group


PAF1
1/Ct
0.30
0.25
0.20
0.15
0.10
0.05
0.00
MEF MIXL1(-)/ SSEA-3(+)
MEF MIXL1(+)/ SSEA-3(+)
MEF MIXL1(+)/ SSEA-3(-)
Primo MIXL1(+)/ SSEA-3(+)
Group


SMAD2
1/Ct
0.30
0.25
0.20
0.15
0.10
0.05
0.00
MEF MIXL1(-)/ SSEA-3(+)
MEF MIXL1(+)/ SSEA-3(+)
MEF MIXL1(+)/ SSEA-3(-)
Primo MIXL1(+)/ SSEA-3(+)
Group

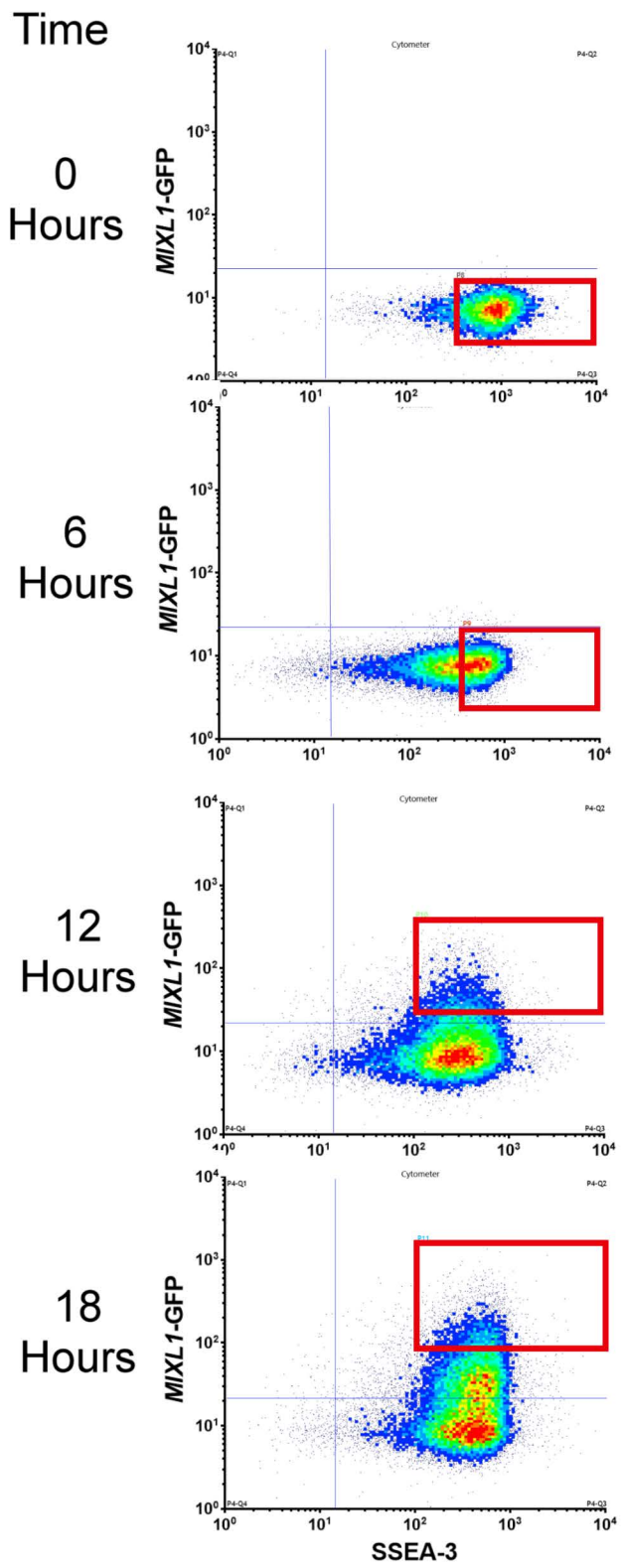

SNAI1
1/Ct
0.30
0.25
0.20
0.15
0.10
0.05
0.00
MEF MIXL1(-)/ SSEA-3(+)
MEF MIXL1(+)/ SSEA-3(+)
MEF MIXL1(+)/ SSEA-3(-)
Primo MIXL1(+)/ SSEA-3(+)
Group

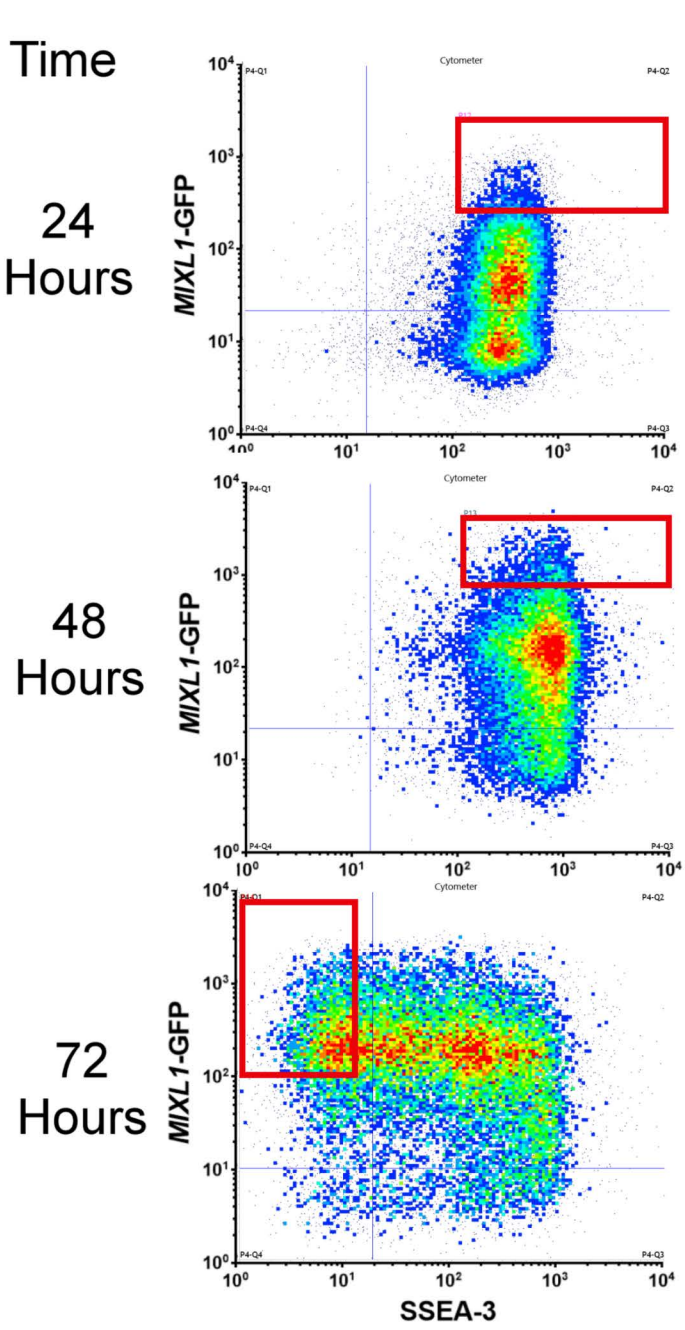

TAGLN2
1/Ct
0.30
0.25
0.20
0.15
0.10
0.05
0.00
MEF MIXL1(-)/ SSEA-3(+)
MEF MIXL1(+)/ SSEA-3(+)
MEF MIXL1(+)/ SSEA-3(-)
Primo MIXL1(+)/ SSEA-3(+)
Group


WNT3
1/Ct
0.30
0.25
0.20
0.15
0.10
0.05
0.00
MEF MIXL1(-)/ SSEA-3(+)
MEF MIXL1(+)/ SSEA-3(+)
MEF MIXL1(+)/ SSEA-3(-)
Primo MIXL1(+)/ SSEA-3(+)
Group

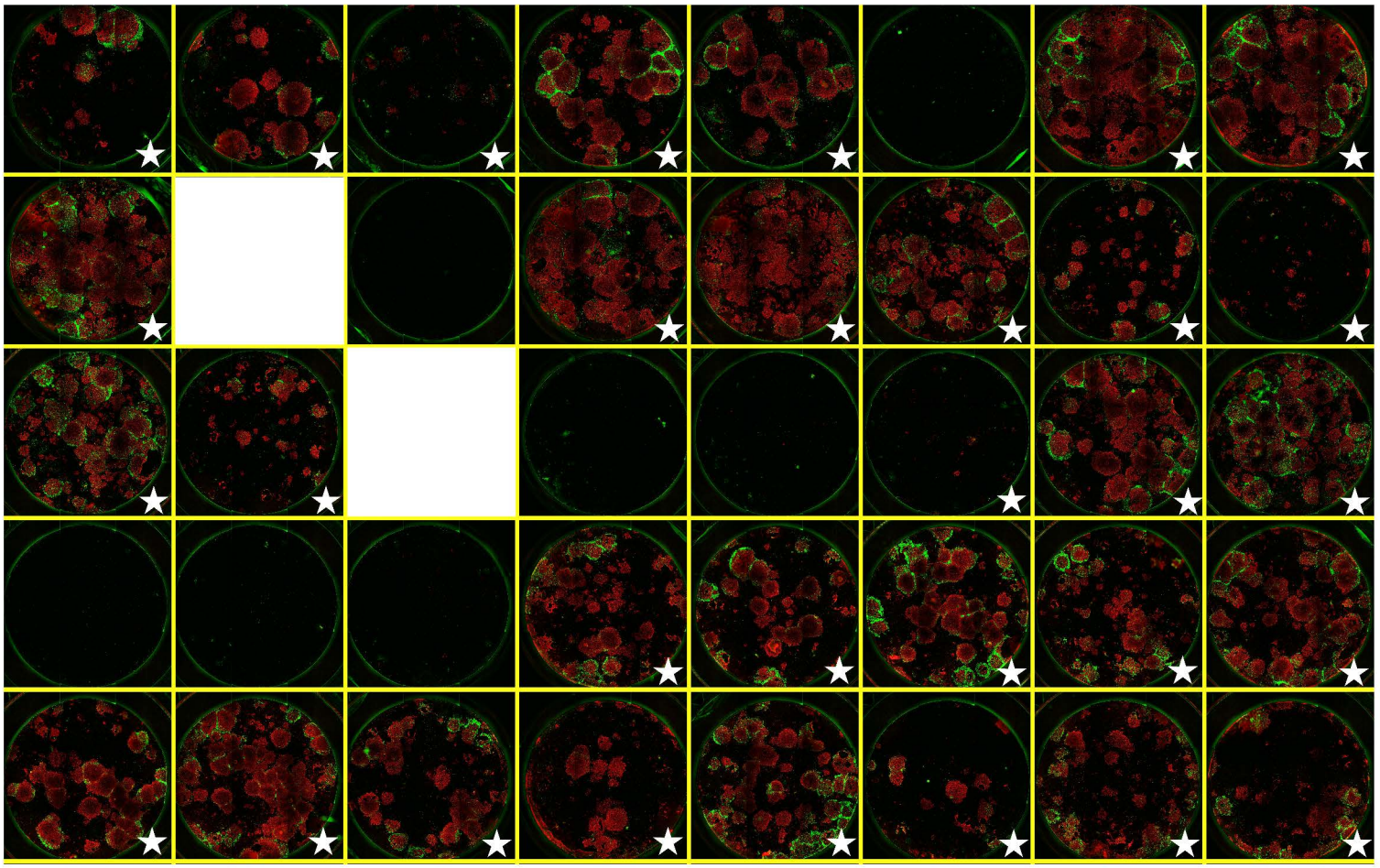
a

S5


Time
0 Hours
6 Hours
12 Hours
18 Hours
MIXL1-GFP
SSEA-3

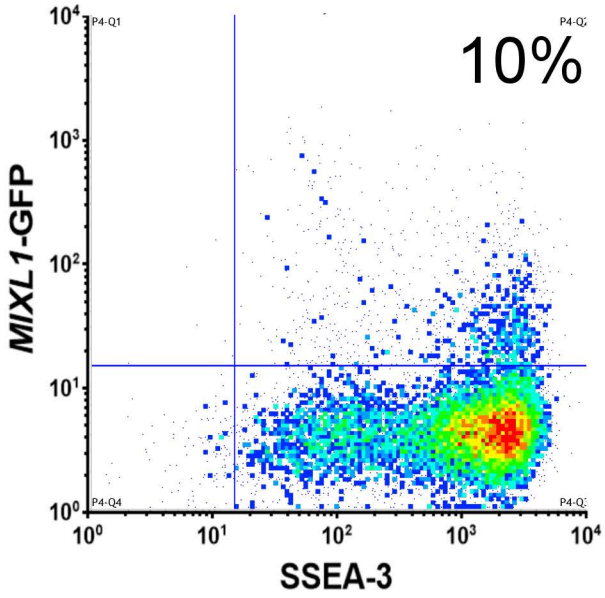

Time
24 Hours
48 Hours
72 Hours
MIXL1-GFP
SSEA-3

a S6

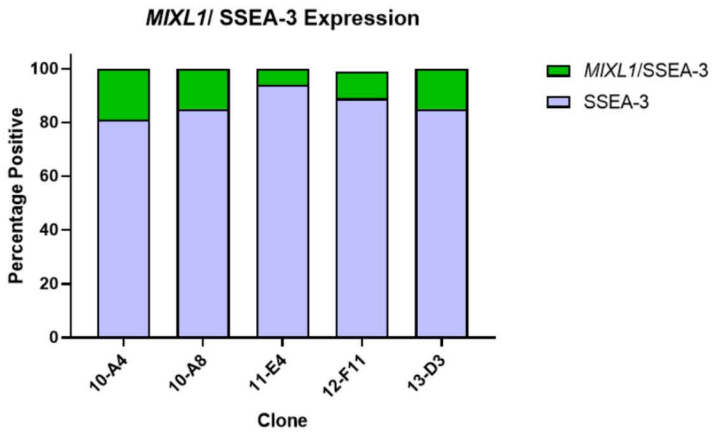


b

MEF/ KOSR

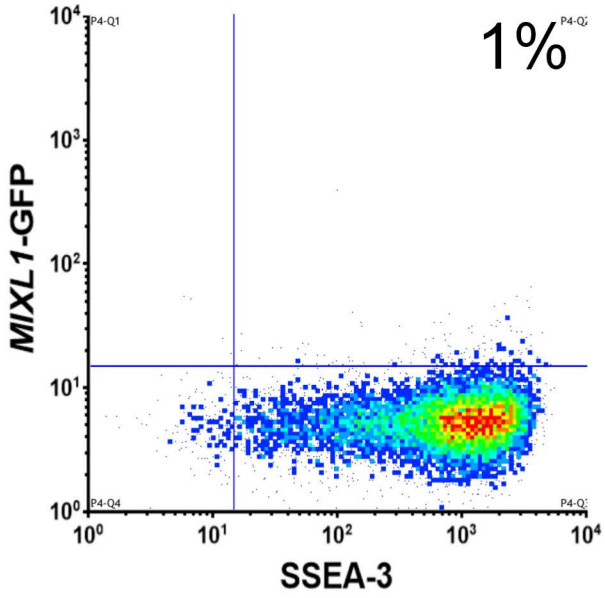


c

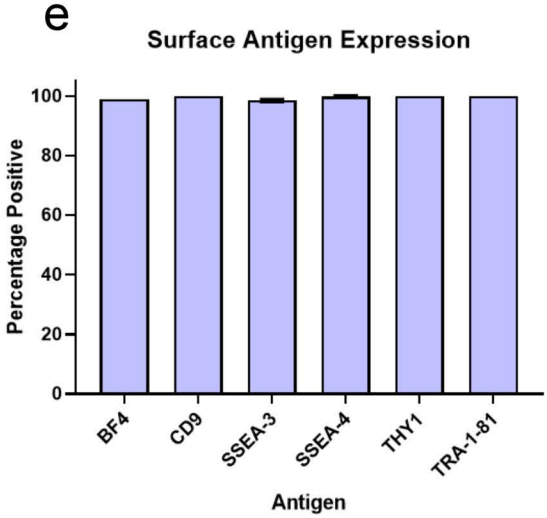


d

E8V

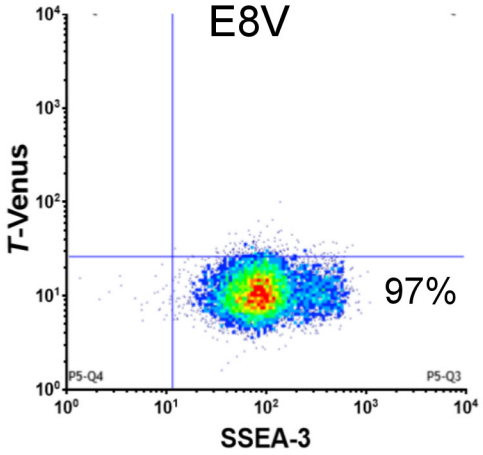


e



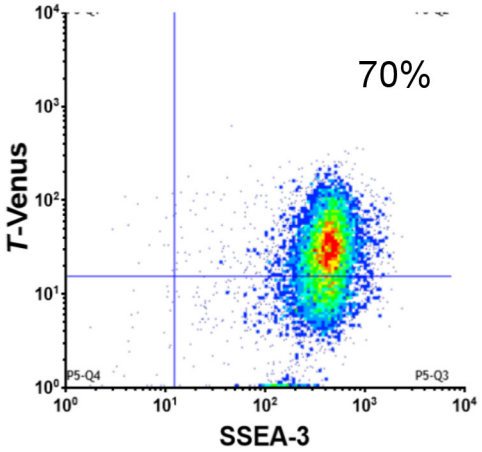
S7

a



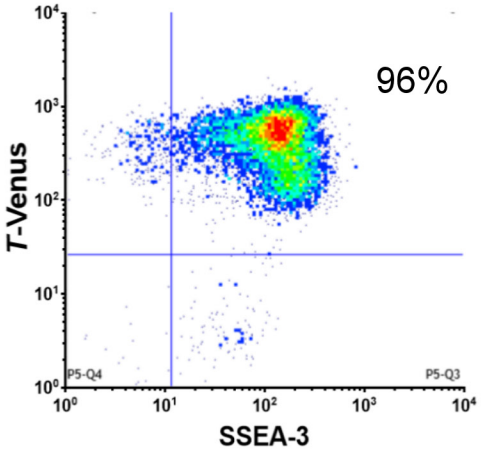
b

IWP2 replaced with DKK1 100ng/mL



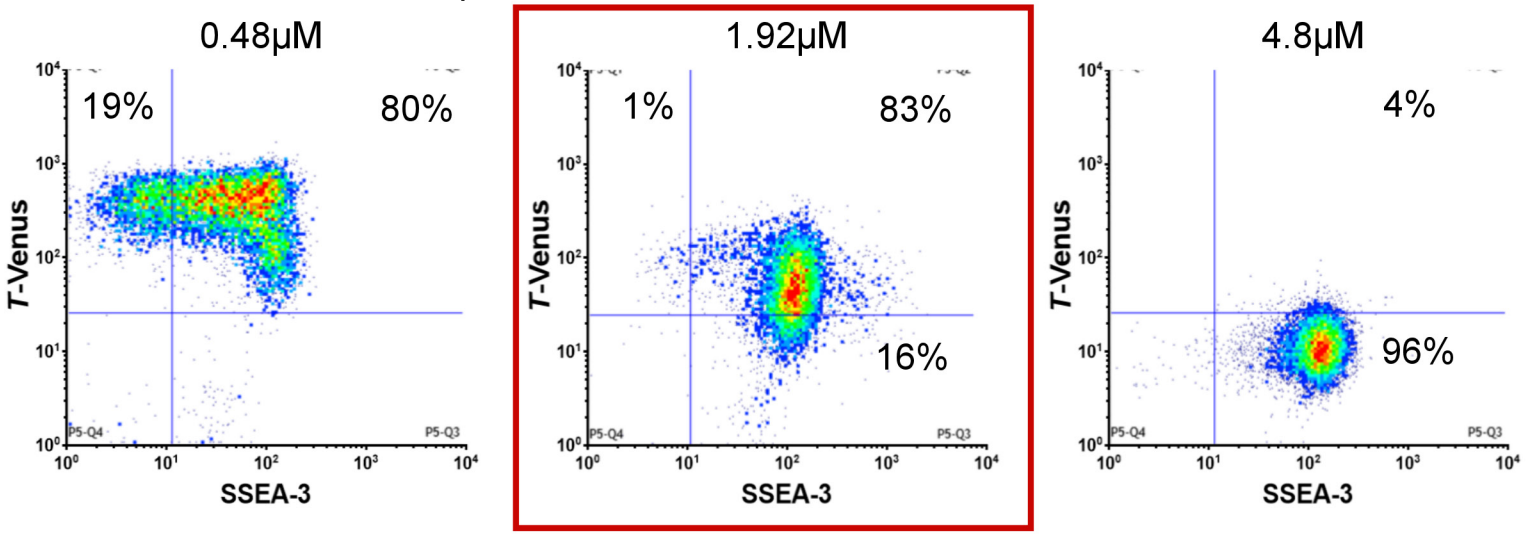
c

CHIR99021 replaced with SB216763 at 10μM



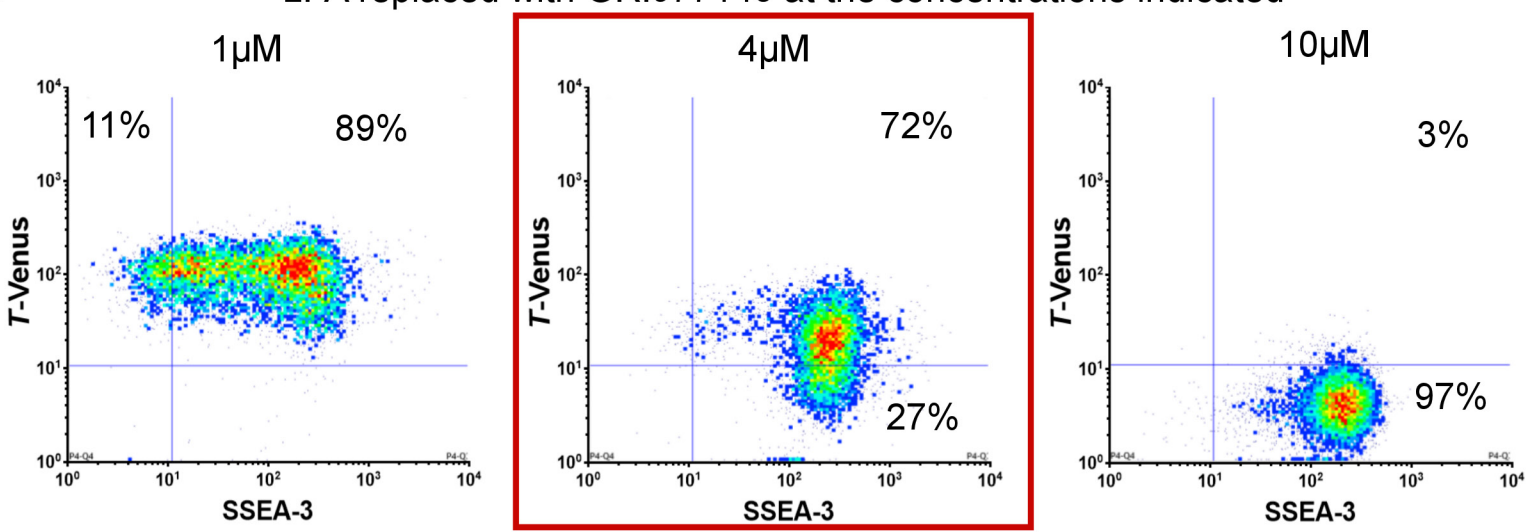
d

LPA replaced with S1P at the concentrations indicated

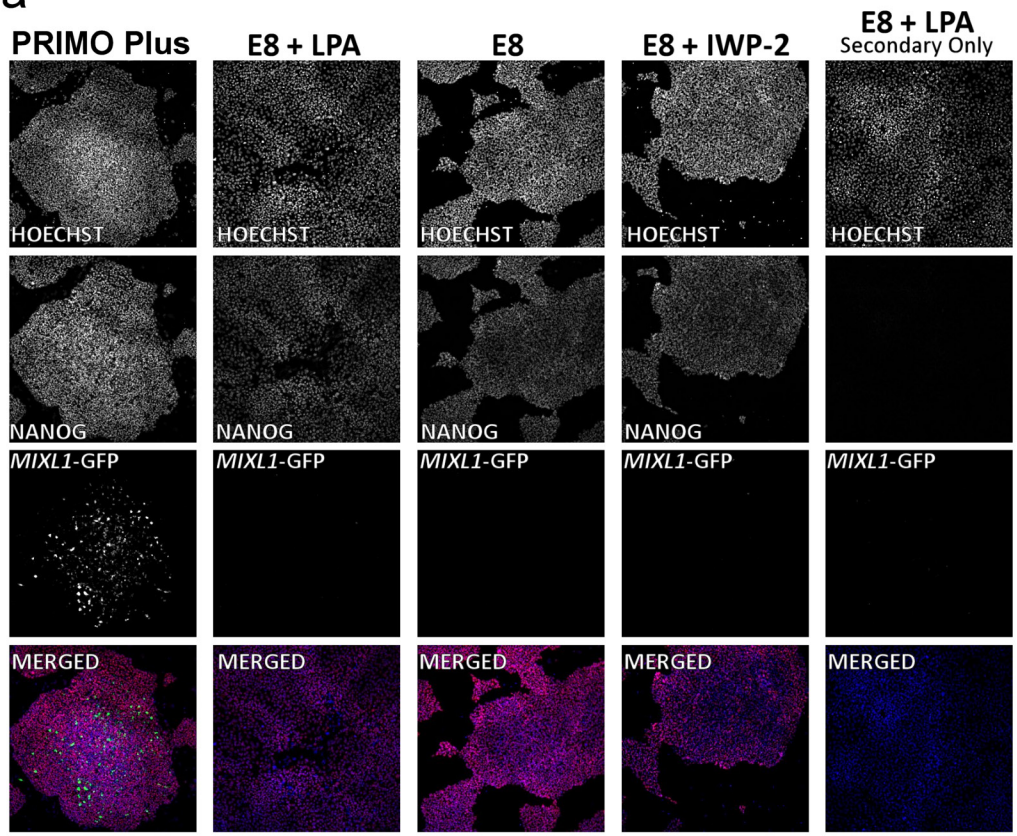


e

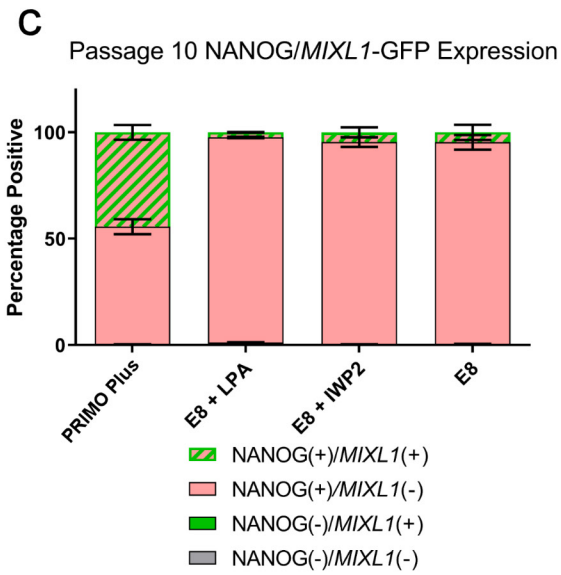
LPA replaced with GRI977143 at the concentrations indicated



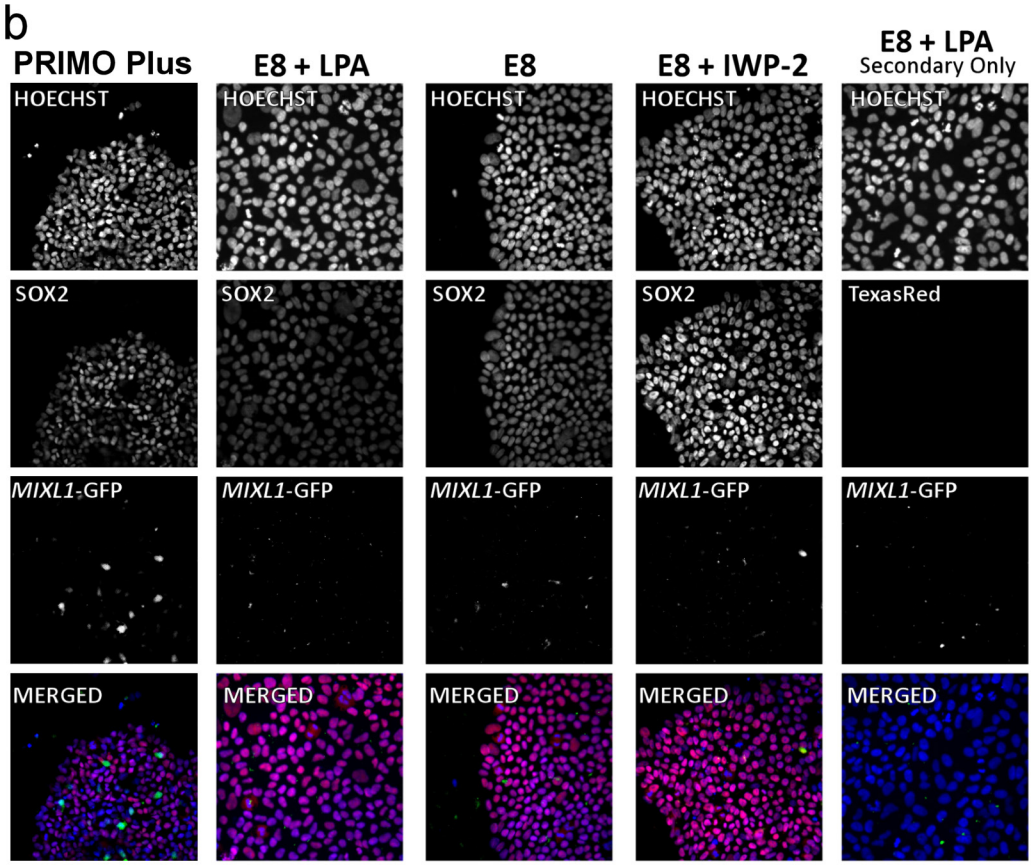
a


PRIMO Plus
E8 + LPA
E8
E8 + IWP-2
E8 + LPA
Secondary Only
HOECHST
NANOG
MIXL1-GFP
MERGED

c


Passage 10 NANOG/MIXL1-GFP Expression
Percentage Positive
100
50
0
PRIMO Plus
E8 + LPA
E8 + IWP2
E8
NANOG(+)/MIXL1(+)
NANOG(+)/MIXL1(-)
NANOG(-)/MIXL1(+)
NANOG(-)/MIXL1(-)

b


PRIMO Plus
E8 + LPA
E8
E8 + IWP-2
E8 + LPA
Secondary Only
HOECHST
SOX2
TexasRed
MIXL1-GFP
MERGED

d

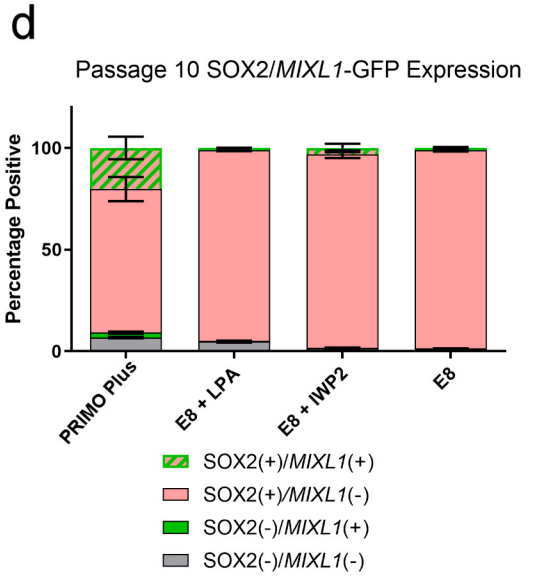

Passage 10 SOX2/MIXL1-GFP Expression
Percentage Positive
100
50
0
PRIMO Plus
E8 + LPA
E8 + IWP2
E8
SOX2(+)/MIXL1(+)
SOX2(+)/MIXL1(-)
SOX2(-)/MIXL1(+)
SOX2(-)/MIXL1(-)

Table 1 Single Cell qPCR assay list

Assay ID	Gene Symbol	RefSeq	Amplicon Length	Detects gDNA	Best Coverage
Hs01060665_g1	*ACTB*	NM_001101.3	63	Yes	Yes
Hs00154192_m1	*BMP2*	NM_001200.2	60	No	Yes
Hs03676628_s1	*BMP4*	NM_130850.2;NM_001202.3; NM_130851.2	116	Yes	Yes
Hs01034913_g1	*BMPR1A*	NM_004329.2	94	Yes	Yes
Hs00193796_m1	*CER1*	NM_005454.2	92	No	Yes
Hs01897804_s1	*CITED2*	NM_001168388.2;NM_001168389.2;NM_006079.4	106	Yes	Yes
Hs00607528_s1	*CLDN6*	NM_021195.4	154	Yes	Yes
Hs00164004_m1	*COL1A1*	NM_000088.3	66	No	Yes
Hs00976734_m1	*CXCR4*	NM_003467.2;NM_001008540.1	153	No	No
Hs00171876_m1	*DNMT3B*	NM_001207055.1;NM_001207056.1;NM_175848.1;NM_175849.1;NM_175850.2;NM_006892.3	55	No	Yes
Hs00172872_m1	*EOMES*	NM_001278183.1;NM_001278182.1;NM_005442.3	81	No	Yes
Hs01549976_m1	*FN1*	NM_212482.1;NM_054034.2; NM_002026.2;NM_212478.1; NM_212474.1;NM_212476.1	81	No	Yes
Hs00232764_m1	*FOXA2*	NM_021784.4;NM_153675.2	66	No	Yes
Hs00255287_s1	*FOXD3*	NM_012183.2	78	Yes	Yes
Hs00173503_m1	*FRZB*	NM_001463.3	108	No	Yes
Hs00246256_m1	*FST*	NM_006350.3;NM_013409.2	108	No	No
Hs00544355_m1	*GAL*	NM_015973.3	125	No	Yes
Hs00171403_m1	*GATA4*	NM_002052.3	68	No	Yes
Hs00232018_m1	*GATA6*	NM_005257.4	91	No	Yes
Hs00906630_g1	*GSC*	NM_173849.2	100	No	No
Hs00193435_m1	*HAS2*	NM_005328.2	63	No	Yes
Hs00242160_m1	*HHEX*	NM_002729.4	110	Yes	Yes
Hs00705137_s1	*IFITM1*	NM_003641.3	93	Yes	Yes
Hs01547673_m1	*ITGA5*	NM_002205.2	54	No	Yes
Hs00761767_s1	*KRT19*	NM_002276.4	116	Yes	Yes
Hs00764128_s1	*LEFTY1*	NM_020997.3	136	Yes	Yes
Hs00745761_s1	*LEFTY2*	NM_001172425.1;NM_003240.3	102	Yes	Yes
Hs00355202_m1	*LGALS1*	NM_002305.3	63	No	Yes
Hs00232144_m1	*LHX1*	NM_005568.3	60	No	Yes
Hs00702808_s1	*LIN28A*	NM_024674.4	143	Yes	Yes
Hs00430824_g1	*MIXL1*	NM_031944.1	152	No	No
Hs00899658_m1	*MMP1*	NM_001145938.1;NM_002421.3	64	No	Yes

Assay ID	Gene Symbol	RefSeq	Amplicon Length	Detects gDNA	Best Coverage
Hs01548727_m1	*MMP2*	NM_004530.4;NM_001127891.1	65	No	Yes
Hs01085598_g1	*MYL7*	NM_021223.2	74	No	Yes
Hs04399610_g1	*NANOG*	NM_024865.2	101	Yes	No
Hs00378379_m1	*NCLN*	NM_020170.3	65	No	Yes
Hs00415443_m1	*NODAL*	NM_018055.4	68	No	Yes
Hs00219496_m1	*PAF1*	NM_019088.3;NM_001256826.1	100	No	Yes
Hs04260367_gH	*POU5F1*	NM_001173531.1;NM_002701.4;NM_203289.4	77	Yes	Yes
Hs01375212_g1	*RPS18*	NM_022551.2	93	Yes	Yes
Hs00183425_m1	*SMAD2*	NM_001135937.2;NM_001003652.3;NM_005901.5	129	No	No
Hs00195591_m1	*SNAI1*	NM_005985.3	66	Yes	Yes
Hs00751752_s1	*SOX17*	NM_022454.3	149	Yes	Yes
Hs01053049_s1	*SOX2*	NM_003106.3	91	Yes	Yes
Hs00610080_m1	*T*	NM_001270484.1;NM_003181.3	132	No	Yes
Hs00761239_s1	*TAGLN2*	NM_001277224.1;NM_001277223.1;NM_003564.2	163	No	Yes
Hs02339499_g1	*TDGF1*	NM_003212.3;NM_001174136.1	170	Yes	No
Hs00902257_m1	*WNT3*	NM_030753.4	76	No	Yes