Product Information and Certificate of Analysis

Human Embryonic Stem (hES) Cell Line MEL-1

Product Description

Originating from Australia, the MEL-1 cell line was derived by Stem Cell Sciences Ltd. in a collaboration with Melbourne IVF in an NHMRC-licensed project (Licence No. 309709) from 'excess' human embryos donated by couples having IVF treatment. This line is registered on the USA's National Institute of Health's Human Embryonic Stem Cell Registry.

Properties

Cell line	MEL-1
Cell type	Human embryonic stem cells
Gender	Male
Bank designation	Working Cell Bank (WCB) #1
Storage conditions	≤ -135°C
Date of cryopreservation	24-Jul-2021
Passage number	P32
Cell number	5×10^6 cells per vial
Cryopreservation medium	CryoStor® CS10
Culture medium	mTeSR™ Plus
Matrix substrate	Matrigel® - hESC-Qualified Matrix
Recommended passage method and split ratio	ReLeSR™, cells can generally be split 1:4-1:6 every 5 days

Recommended Materials for Use

Reagent	Supplier	Cat. Number
ReLeSR™	STEMCELL Technologies	100-0484
mTeSR™ Plus Kit	STEMCELL Technologies	05825
RevitaCell Supplement (100X)	Thermo Fisher Scientific	A2644501
Corning® Matrigel® hESC-Qualified Matrix	Corning®	354277
Accutase™	Thermo Fisher Scientific	A1110501

CERTIFICATE OF ANALYSIS

The following testing specifications have been met for this product:

Test Description	Test Method	Test Results
Post-thaw cell viability and recovery	Cell count	Viable cell count at revival > 65%. Cells reached confluence within 5 days.
Mycoplasma	Bioluminescence (Lonza MycoAlert detection kit)	Negative
Expression of pluripotent stem cell markers	Immunofluorescence	Positive for Oct-4, Nanog and SSEA4
	Flow cytometry	> 90% positive (SSEA4 and Tra-1-60)
	Gene expression by qPCR	Similar expression levels of Nanog, Oct-4, DNMT3B and Rex1 compared to pluripotent control
Cytogenetics	G-banding (500 band level)	Normal - 46, XY
Genetic analysis	Copy number variation test qPCR	Normal
Differentiation potential	Immunofluorescence	Positive for lineage specific markers: Ectoderm (Nestin, Pax6) Mesoderm (NCAM1, Brachyury) Endoderm (FOXA2, Sox17)
	Gene expression by qPCR	Increased expression of lineage-specific markers: Ectoderm (PAX6, SOX1, NR2F2) Mesoderm (HAND1, Brachyury) Endoderm (FOXA2, SOX17, Eomes, Gata6)

Expression of Pluripotent Stem Cell Markers

1. Immunofluorescence

To assess pluripotency, MEL-1 cells are stained for nuclear markers Nanog and Oct-4, and surface marker SSEA4.

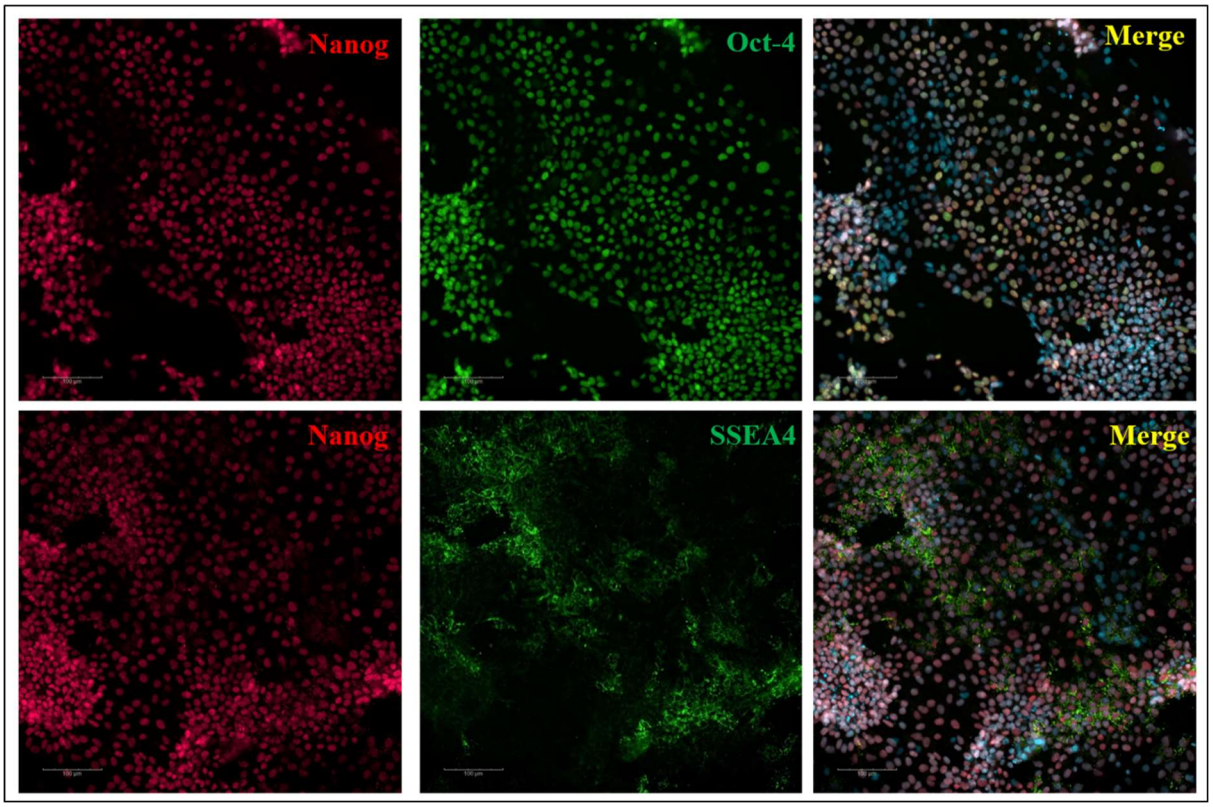

Figure 1. Pluripotency marker expression in MEL-1 WCB #1 cells demonstrating the presence of Nanog, Oct-4 and SSEA4. HB: Hoechst blue; Scale bar 100 µm.

2. Flow Cytometry

In addition to immunofluorescence, flow cytometry analysis confirms the presence of surface markers characteristic of pluripotent cells - SSEA4 and Tra-1-60.

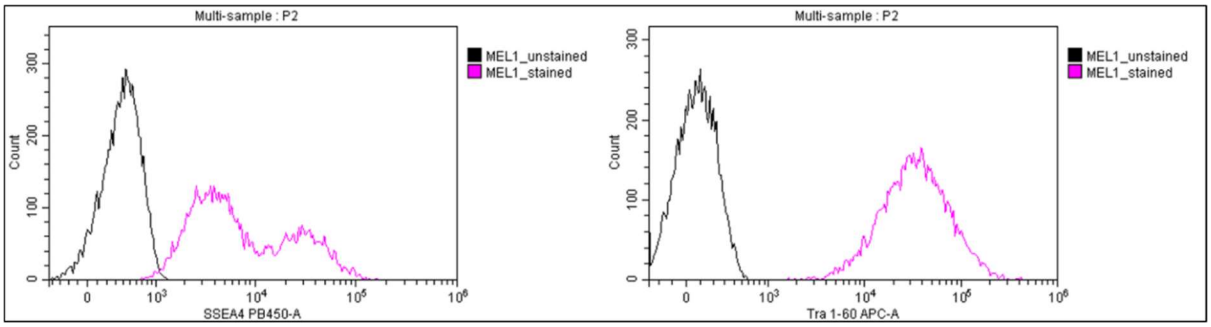

Figure 2. Pluripotency marker expression as assessed by flow cytometry confirming the presence of SSEA4 and Tra-1-60. Black histograms are unstained samples overlayed with stained samples for MEL-1 WCB #1.

3. Gene Expression Analysis

RNA of pluripotent cells is extracted and gene expression is measured by quantitative RT-PCR. GAPDH was used as the endogenous housekeeping gene. Relative gene expression is shown as fold difference in expression as compared to a pluripotent control (WA09, WiCell line H9). Pluripotency markers in MEL-1 WCB #1 are not more than 3-fold different to H9 control.

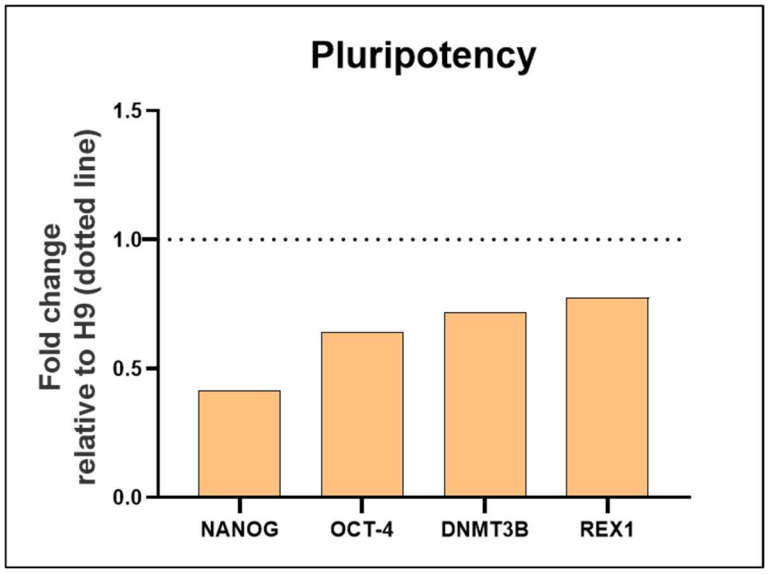

Figure 3. Gene expression analysis of pluripotency markers in MEL-1 cells demonstrating the expression of Nanog, Oct-4, DNMT3B and Rex1. The hES line H9 (WiCell) is used as positive pluripotent control (dotted line).

Genetic Analysis

1. Copy Number Variation Test

Genomic DNA from MEL-1 is screened for the 8 most common karyotypic abnormalities reported in human pluripotent stem cells using the hPSC Genetic Analysis Kit (STEMCELL Technologies).

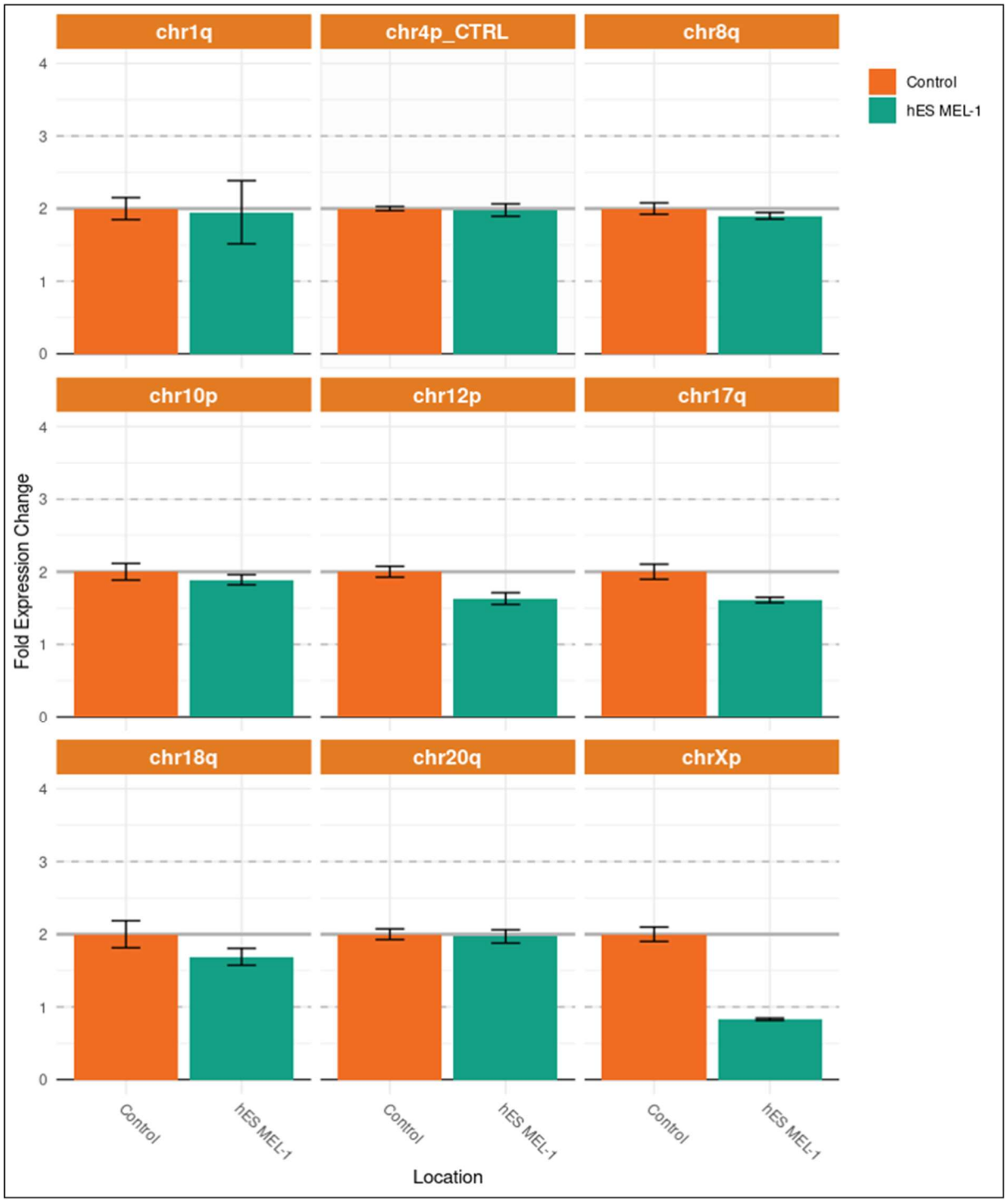

Figure 4. Genetic abnormality assessment showing that all tested loci are normal in MEL-1 WCB #1. NOTE: only one copy of chrXp detected as expected for sex difference between male sample and female control.

2. Cytogenetics

Test Method	G-Banding
G-band level	500
Passage at analysis	P33
Metaphase cells counted	10
Metaphase cells analysed	5
Short ISCN	46, XY

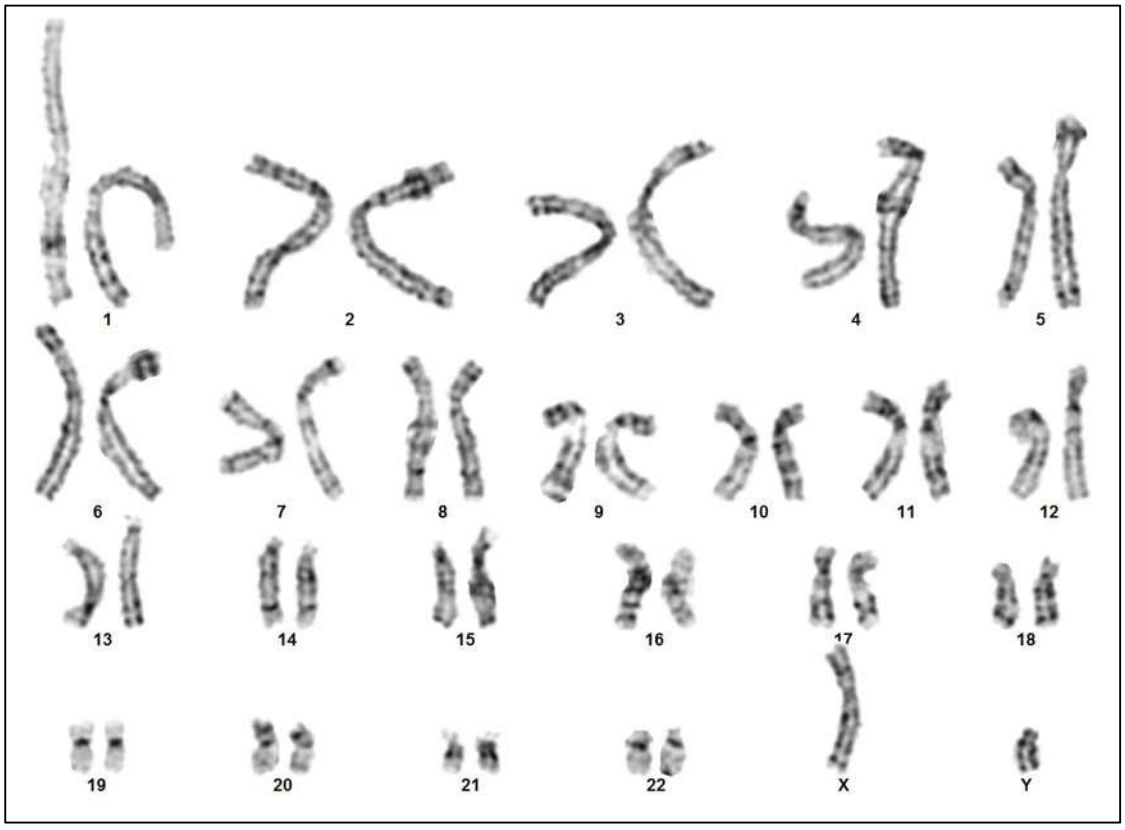

Figure 5. G-banding karyogram of MEL-1 WCB #1 cells showing a normal male karyotype – 46, XY (testing performed by Virtus Diagnostics).

Differentiation Potential Analysis

1. Immunofluorescence

Cells are differentiated using the STEMdiff™ Trilineage Differentiation Kit (STEMCELL Technologies) and stained for two markers of each germ layer.

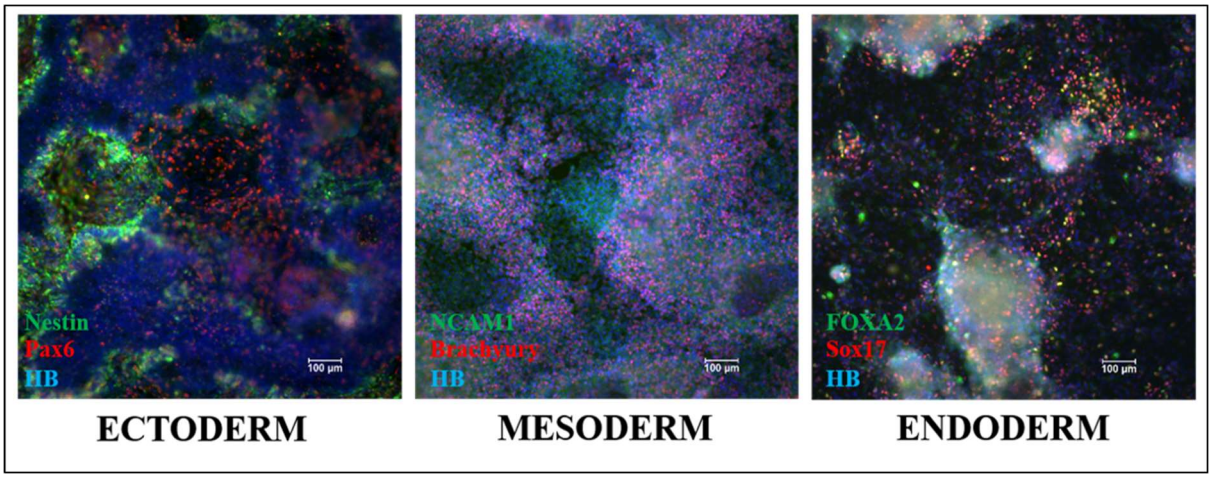

Figure 6. Trilineage differentiation of MEL-1 WCB #1 cells demonstrating the presence of markers from the three germ layers – Ectoderm (Nestin and Pax6), Mesoderm (NCAM1 and Brachyury) and Endoderm (FOXA2 and Sox17). HB: Hoechst blue; Scale bar 100 µm.

2. Gene Expression Analysis

Cells are differentiated using the STEMdiff™ Trilineage Differentiation Kit (STEMCELL Technologies). RNA is extracted and gene expression is measured by quantitative RT-PCR. GAPDH is used as the endogenous housekeeping gene. Relative gene expression is shown as the fold difference in expression as compared to undifferentiated pluripotent MEL-1 cells. Calculations are performed using the $2^{-\Delta\Delta CT}$ method. (Livak KJ, Schmittgen TD. Methods. 2001 Dec;25(4):402-8.PMID:11846609).

Germ Layer Gene Expression During Lineage Differentiation

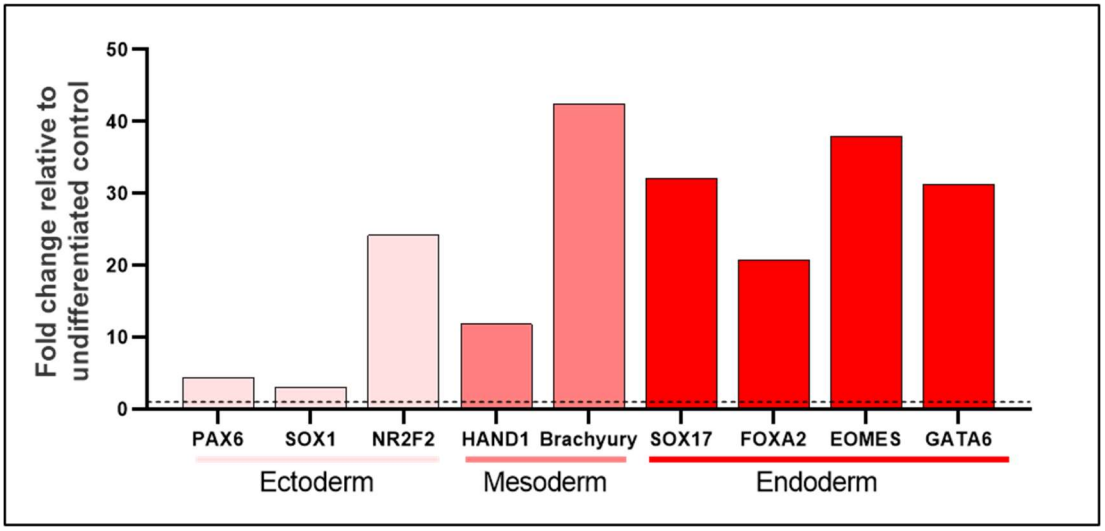

Figure 7. Gene expression analysis of differentiated MEL-1 WCB #1 cells demonstrating the expression of genes from the three germ layers - Ectoderm (PAX6, SOX1 and NR2F2), Mesoderm (HAND1 and Brachyury) and Endoderm (SOX17, FOXA2, EOMES and GATA6) relative to undifferentiated control (dotted line).

Pluripotent Gene Expression During Lineage Differentiation

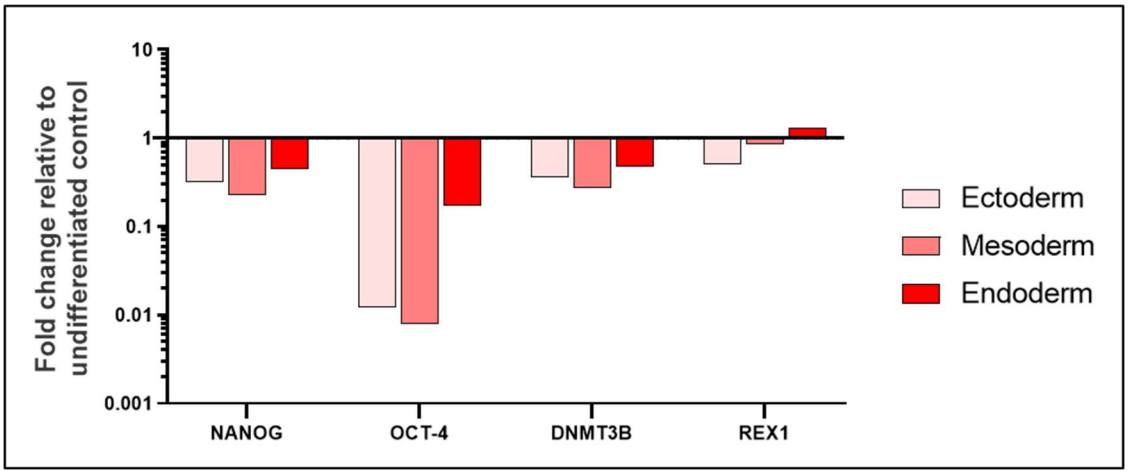

Figure 8. Gene expression analysis of differentiated MEL-1 WCB #1 cells demonstrating downregulation of pluripotency genes in the three germ layers.

Directions for Use

The following instructions are for preparing one cryovial of MEL-1 for plating in 1 × 75cm^2 flask. Use aseptic technique throughout the protocol.

Thawing and plating MEL-1

1) Coat culture-ware flask with Matrigel® (made to manufacturer's specifications) and incubate for at least 30 minutes at 37°C or overnight at 4°C. Prior to cell seeding, the flask should be pre-warmed to 37°C and the mTeSR™ Plus medium warmed to room temperature.
2) Gently warm the cryovial in a water bath until a small ice pellet remains and transfer into a biosafety cabinet.
3) Add 1 ml of mTeSR™ Plus media to cryovial and transfer content into a 15 ml centrifuge tube containing 2 ml of mTeSR™ Plus media.
4) Centrifuge at 200 × g for 3 minutes.
5) Add RevitaCell to mTeSR™ Plus medium according to manufacturer's specifications.
6) Aspirate the media avoiding disruption to the cell pellet and add 1 ml of mTeSR™ Plus with RevitaCell.
7) Remove the substrate coating prior to transferring the cells and add mTeSR™ Plus medium with RevitaCell to flask (11 ml/T75 flask).
8) Gently re-suspend the cell pellet and transfer to the Matrigel-coated flask containing mTeSR™ Plus and RevitaCell.
9) Transfer flask into an incubator at 37°C and do not disturb for 24 hours.
10) Perform a full media exchange 24 hours later (without RevitaCell) and then replace daily or every second day (depending on cell confluence).

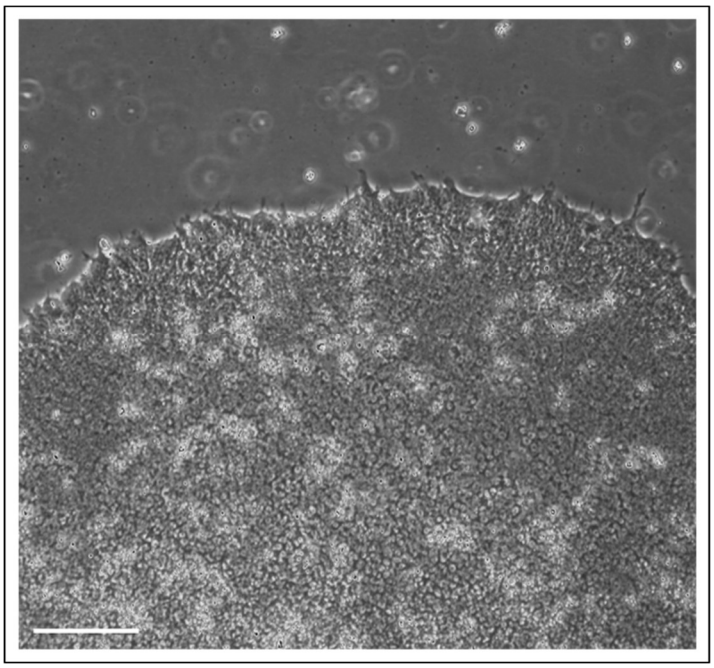

Figure 9. MEL-1 colony at Day 4 post-recovery from cryostorage. Scale bar 200 µm.

Passaging:

1) When cells reach 80-90% confluence prepare the following reagents and volumes per T75 flask (adjust volumes as required for specific culture flask):
   a) 5 ml ReLeSR™ (pre-warm to 37°C).
   b) 10 ml of PBS (calcium and magnesium free (CMF), pre-warm to 37°C).
   c) T75 flasks pre-coated with extracellular matrix (ECM) of choice according to manufacturer's instructions.
   d) 15 ml mTeSR™ Plus (pre-warm to 37°C) containing 1x RevitaCell at recommended dilution (or 10 µM ROCK inhibitor).
   e) 5 ml of DMEM (pre-warm to 37°C, specific formulation not strictly important).
2) Remove flask from incubator and transfer to biosafety cabinet.
3) Aspirate media and wash cells with 10 ml PBS CMF. Aspirate PBS.
4) Add 5 ml of ReLeSR™. Lay down flask and ensure entire surface is covered by ReLeSR™.
5) Incubate in biosafety cabinet for no more than 30 seconds.
6) Aspirate ReLeSR™ and immediately transfer flask to 37°C incubator.
7) Incubate for 2 min. Check cells under microscope for rounding of cells. Incubate for 1 minute longer if required.
8) In the biosafety cabinet, add 5 ml of DMEM down the side of the flask.
9) Tilt flask so surface is covered with DMEM and tap the side of the flask 2 or 3 times. If incubation time in ReLeSR™ is sufficient, cells will lift off with tapping.
10) Wash the surface with the 5 ml DMEM/cell suspension and transfer to a centrifuge tube.
11) Pellet the cells at 300 × g for 3 minutes.
12) Aspirate supernatant and add 1 ml of mTeSR™ Plus with RevitaCell. Mix well to resuspend pellet.
13) Aspirate ECM from T75 and immediately add 12 ml of mTeSR™ Plus with RevitaCell down the side of the flask to avoid removing ECM from surface.
14) Take 1:6 to 1:8 of the cell suspension and transfer to one T75 with media.
15) Transfer to CO_2 incubator. Gently agitate flask back and forth to ensure even spread of cells in the flask.
16) Change media 24 hrs later to remove RevitaCell. 12-20 ml per T75 flask is recommended based on confluence at the time.
17) Cells will reach confluence within 4-5 days.

Cryopreservation:

1) Follow the passaging protocol up to step 10.
2) Take a small aliquot of the cell suspension (volume of your choice) and transfer to a second centrifuge tube to perform a cell count.
3) Centrifuge both tubes at 300 × g for 3 minutes.
4) Aspirate media and resuspend cell suspension for cryopreservation in 5 ml of mTeSR™ Plus.
5) Resuspend cell count aliquot in 1 ml of pre-warmed Accutase™ (include a PBS CMF wash before this if desired. Cells are currently in small clusters of 5 - 10 cells and don't require much effort for single cell dissociation).
6) Incubate at room temperature for 3 minutes with regular gentle agitation using a 200 µl pipette.
7) Perform cell count using desired method.
8) Back calculate to determine cell number of original cell suspension.
9) Centrifuge cells at 300 × g for 3 minutes.
10) Aspirate mTeSR™ Plus.
11) Gently flick tube to dislodge cell pellet.
12) Add cold, CryoStor® CS10 to a final cell density of 1 - 5 × 10^6 cells/ml. We recommend not cryopreserving densities lower than 1 × 10^6 cells/ml . Higher densities improve cell recovery at thaw.
13) Aliquot ≥ 500 µl of cells in CryoStor® CS10 per cryovial.
14) Transfer to isopropanol tanks and then to -80°C for 2 hours before transfer to cryostorage.
15) Depending on confluence, each T75 flask contains 18 - 25 × 10^6 cells.