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| CoA written by: | Mahfuz Chowdhury | CoA approved by: | Andrew Prowse |
| Signature: |  | Signature: | |
| Date: | 28/02/2023 | Date: | 24/1/2024 |

# Product Information and Certificate of Analysis

## Human Embryonic Stem Cell Line MEL-3

### Product Description

Originating from Australia, the MEL-3 cell line was derived by Stem Cell Sciences Ltd. in a collaboration with Melbourne IVF in an NHMRC-licensed project 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-3 |
| **Cell type** | Human embryonic stem cells |
| **Gender** | Female |
| **Bank designation** | Working Cell Bank (WCB) #2 |
| **Storage conditions** | ≤ -135°C |
| **Date of cryopreservation** | 15/08/2022 |
| **Passage number** | P40 |
| **Cell number** | $5 \times 10^6$ 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. No. |
|---|---|---|
| **ReLeSR™** | STEMCELL Technologies | 100-0484 |
| **mTeSR™ Plus Kit** | STEMCELL Technologies | 05825 |
| **Y-27632 hydrochloride** | Sapphire bioscience | 10005583-50MG |
| **Corning® Matrigel® hESC-Qualified Matrix** | Corning® | 354277 |
| **Accutase™ Cell Dissociation Reagent** | 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 > 80%. Cells reached confluence within 6 days. |
| **Mycoplasma** | Bioluminescence (Lonza MycoAlert detection kit) | Negative |
| **Expression of undifferentiated state markers** | Immunofluorescence | Positive for OCT4 and NANOG |
| | Flow cytometry | > 90% positive<br>(SSEA4, SOX2 and TRA-1-60) |
| | Gene expression by RT-qPCR | Similar expression levels of undifferentiated markers (NANOG, OCT4, DNMT3B and REX1) compared to H9 hES cells |
| **Genetic analysis** | Low resolution Copy number variation (CNV) test | No CNV detected |
| | Karyostat assay (high-resolution assay) | No CNV detected<br>Loss of heterozygosity (LOH) detected in Chromosome 4 and X |
| **Differentiation potential** | Immunofluorescence | <u>Positive for lineage specific markers:</u><br>Ectoderm (NESTIN, PAX6)<br>Mesoderm (NCAM1, BRACHYURY)<br>Endoderm (FOXA2, SOX17) |
| | Gene expression by RT-qPCR | <u>Increased expression of lineage-specific markers:</u><br>Ectoderm (PAX6, SOX1, NR2F2)<br>Mesoderm (HAND1, T, CDX2)<br>Endoderm (SOX17, FOXA2, EOMES, GATA6) |

# 1. Expression of Pluripotent Stem Cell Markers

## 1.1. Immunofluorescence

To assess pluripotency, MEL-3 cells were stained for nuclear markers OCT4 and NANOG.

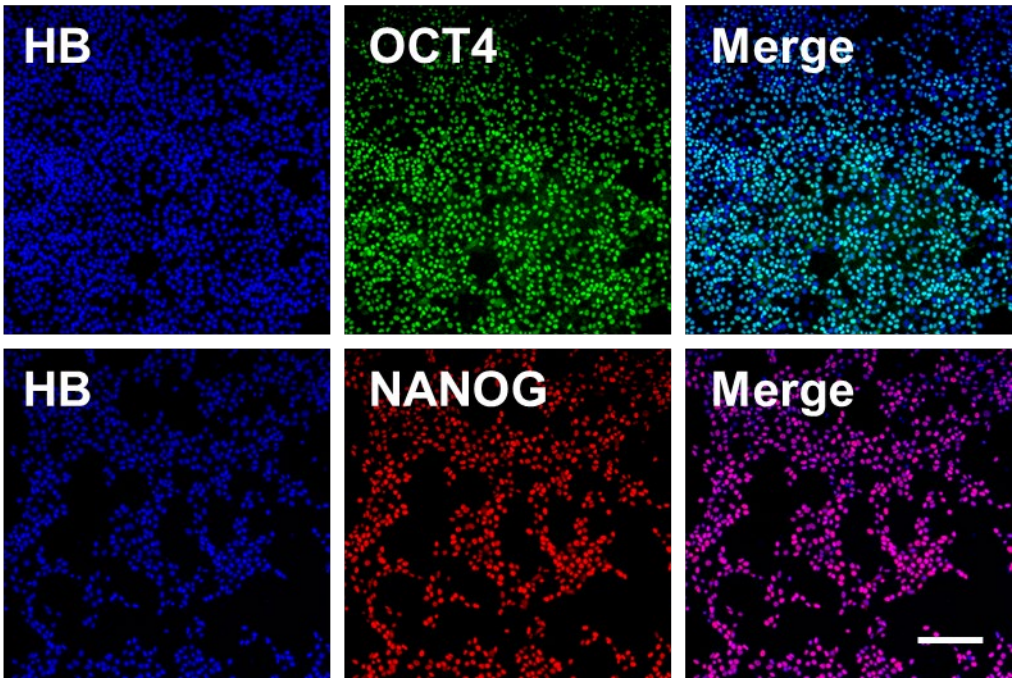



**Figure 1**. Expression of marker for the undifferentiated state, OCT4 (green) and NANOG (red) in MEL-3 hES cells detected by immunostaining. HB: Hoechst Blue; Scale bar represents 200 µm.

## 1.2. Flow Cytometry

In addition to immunofluorescence, flow cytometry analysis confirmed that > 90% of cells expressed undifferentiated state markers SSEA4, SOX2 and TRA-1-60.

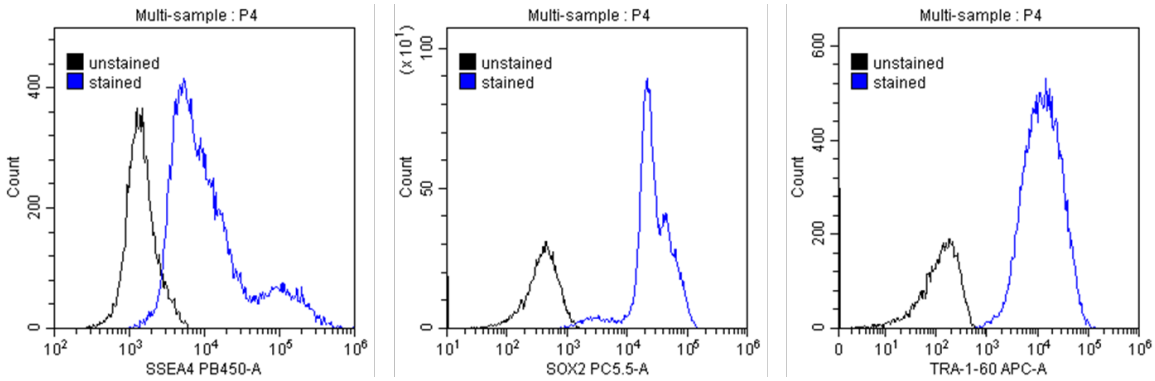



**Figure 2**. Expression of undifferentiated cell markers SSEA4 (left box), SOX2 (middle box) and TRA-1-60 (right box) in the cell line detected by flow cytometry. Black and blue histograms represent unstained and stained cells, respectively.

## 1.3. Gene Expression Analysis

Relative gene expression was measured by Reverse Transcription Quantitative PCR (RT-qPCR) using mRNA extracted from the cells. GAPDH was used as the endogenous housekeeping gene. Expression of the undifferentiated markers in MEL-3 cells was comparable to H9 hESC control.

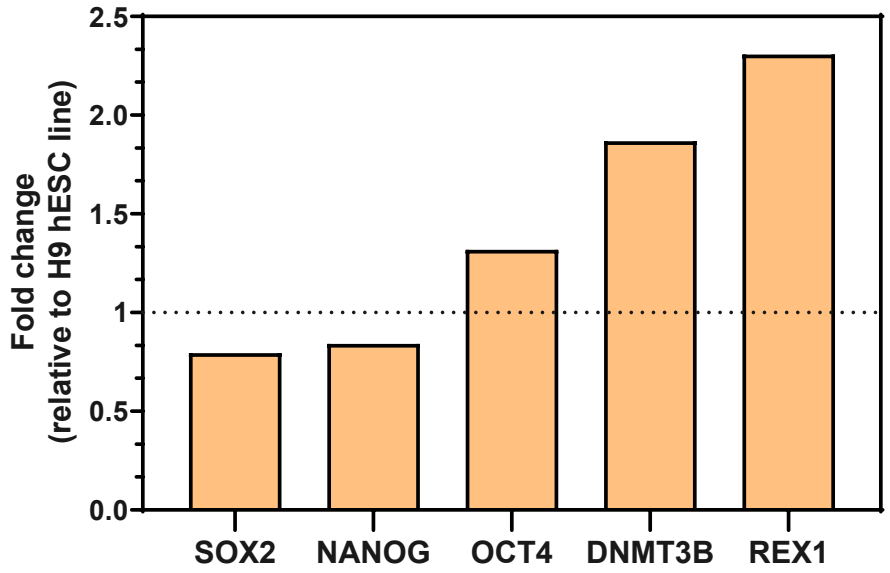



**Figure 3**. Expression of undifferentiated state markers (SOX2, NANOG, OCT4, DNMT3B and REX1) in MEL-3 hES cells. H9 hES cell line (WiCell) is used as the undifferentiated cell control (dotted line). Data represents mean for three technical replicates from one biological replicate.

## 2. Genetic Analysis

### 2.1. Copy Number Variation Test

MEL-3 hES cells were screened for recurrent CNVs commonly reported in hES cells using the hPSC Genetic Analysis Kit (STEMCELL Technologies). Genomic DNA was extracted, and qPCR was performed using this kit. qPCR data was analysed using the application available at STEMCELL Technologies Genetic Analysis Tool. Analysis did not detect any CNVs.

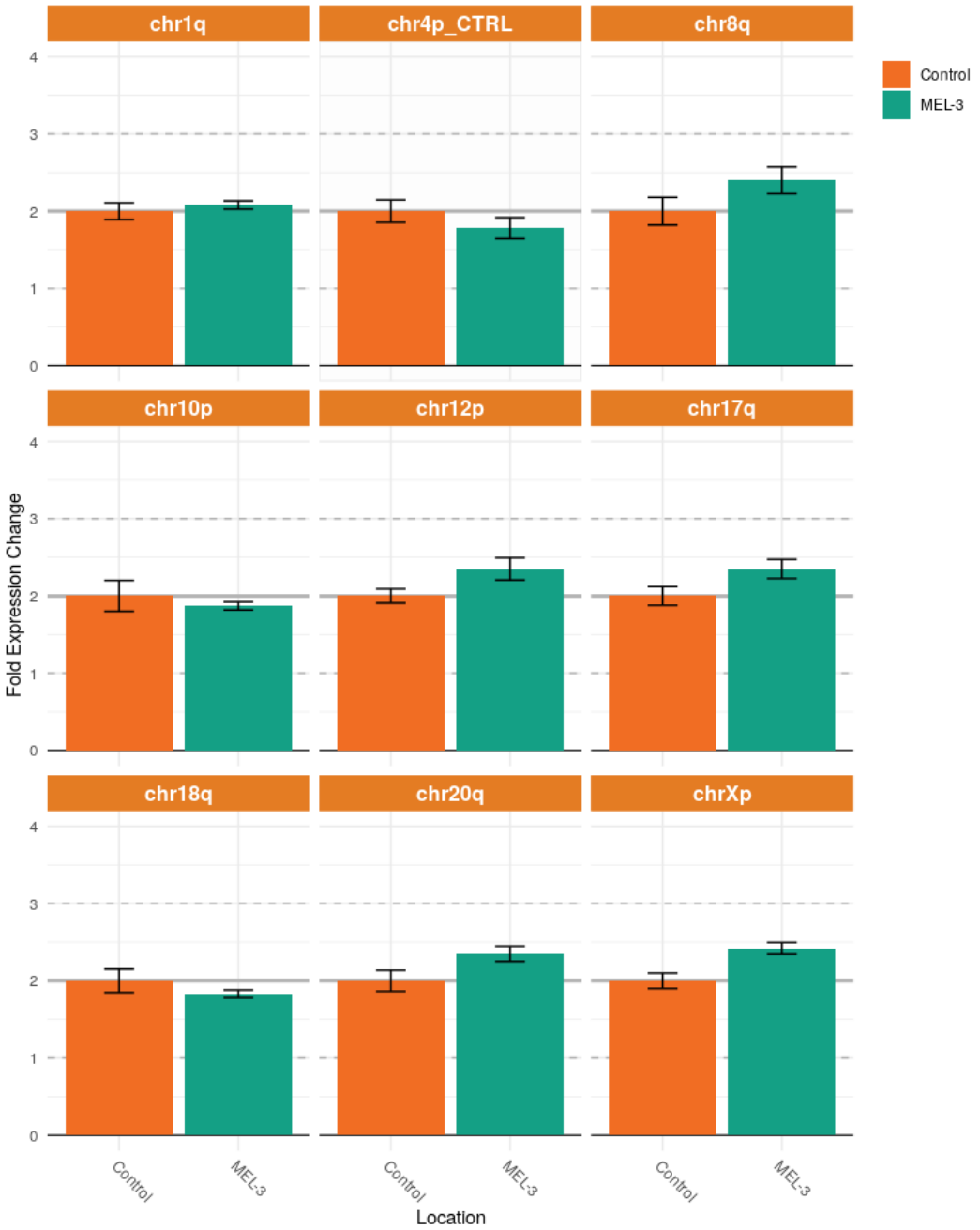
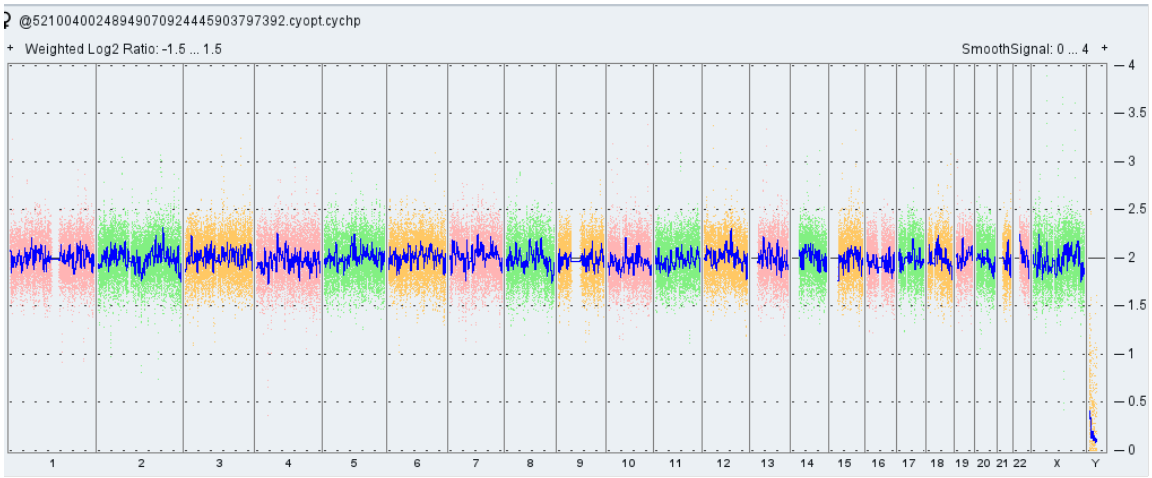


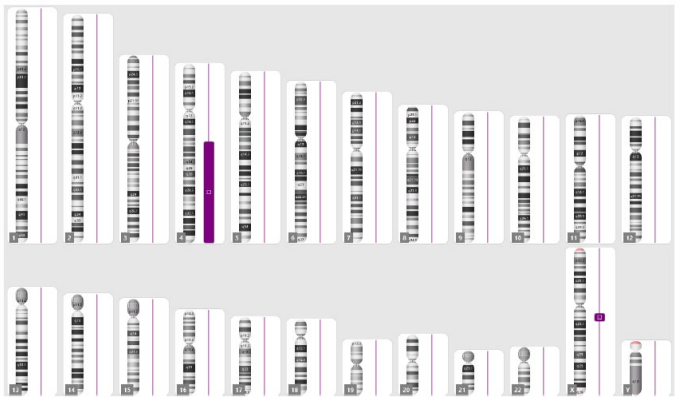

**Figure 4.** Genetic abnormality assessment showing that all tested loci are normal in MEL-3 WCB #2. Data represents mean ± SEM for three technical replicates of each sample.

## 2.2. KaryoStat Assay

The KaryoStat Assay provides much better resolution than G-banding karyotyping or CNV test offering detection of multiple types of chromosomal abnormalities in the whole-genome. The Karyostat assay on MEL-3 WCB#2 sample was performed by using CytoScan Optima Suite that can detect copy-number variations (CNVs) in 18,018 regions, single-nucleotide polymorphism (SNP) in 148,450 regions as well as allelic imbalance, loss of heterozygosity (LOH).

The analysis of the assay data demonstrated that there was no detectable CNV (Figure 5 – top panel) in any chromosomes. SNP analysis-facilitated assessment of the allelic composition indicated LOH in chromosome 4 and X (Figure 5 – bottom panel). Note that, copy neutral LOH in chromosomes can be observed in research/clinical grade hESC [1].



| Chr | Cytoband(s) | Marker boundaries | Size (kbp) |
|---|---|---|---|
| chr4 | q21.22-q35.2 | 82,357,640-190,000,554 | 107,643 |
| chrX | q13.1-q21.1 | 70,112,580-78,280,499 | 8,168 |

**Figure 5.** Summary of the KaryoStat assay data analysed with ChAS 4.4. Top panel shows fold change (right y-axis, blue line indicates mean of the signal) of the regions on chromosomes (x-axis) analysed for CNVs. Mean value hovering around value of 2 indicates no CNVs in MEL-3 WCB#2 cells. Panel at bottom-left shows presence of any LOH as indicated by bars adjacent to chromosome cytobands. Table at bottom-right shows some details on LOH regions.

## 3 Differentiation Potential Analysis

Pluripotent state of MEL-3 cells was confirmed by differentiating them in three germ layers (Ecto, Meso and Endoderm) by using the STEMdiff™ Trilineage Differentiation Kit (STEMCELL Technologies) and characterising the differentiated cells with immunofluorescence and qPCR.

### 3.1 Immunofluorescence

Differentiated cells were stained for two markers of each germ layer.

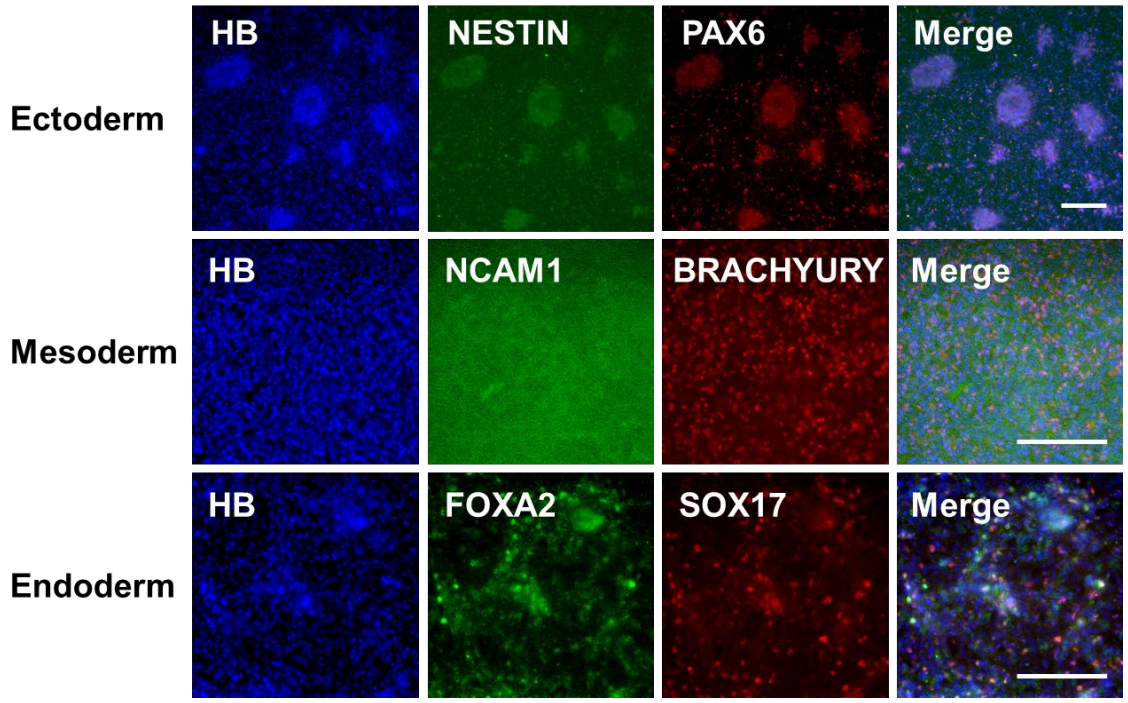



**Figure 6.** Trilineage differentiation of MEL-3 cells demonstrating the presence of markers from the three germ layers – Ectoderm (NESTIN and PAX6), Endoderm (FOXA2 and SOX17) and Mesoderm (NCAM1 and BRACHYURY). HB: Hoechst blue; Scale bar represents 100 µm.

### 3.2 Gene Expression Analysis

Relative expression of genes specific for each germ layers was measured from extracted mRNA by using RT-qPCR. GAPDH was used as the endogenous housekeeping gene.

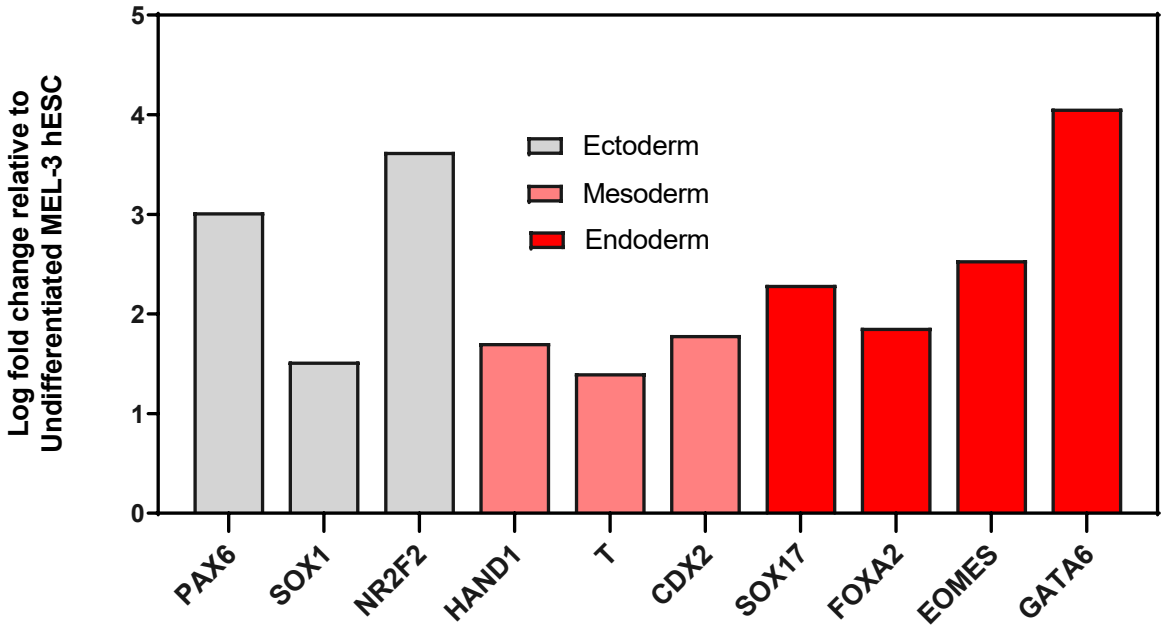



**Figure 7.** Gene expression analysis of differentiated MEL-3 cells demonstrating the expression of genes from the three germ layers - Ectoderm (PAX6, SOX1 and NR2F2), Mesoderm (HAND1, T and CDX2) and Endoderm (SOX17, FOXA2, EOMES and GATA6) relative to undifferentiated control (expression level = 0). Data represents mean for three technical replicates of each sample.

# 4. Directions for Use

The following instructions are for preparing one cryovial of MEL-3 cells for plating in 1 × 75cm$^2$ flask. Use aseptic technique throughout the protocol.

## 4.1 Thawing and plating MEL-3 hES cells

1) Coat culture-ware flask with Matrigel® (made to manufacturer's specifications) and incubate for at least 30 min at 37°C or overnight at 4°C. Prior to cell seeding, the flask should be brought to room temperature.
2) Aliquot required volume of mTeSR plus media (~ 15 mL – 20 mL for seeding in one T75) in a 50 ml tube, add Y-27632 (i.e., rock inhibitor) for 10 µM final concentration and pre-warm to 37°C. Use this media in steps 3 to 9.
3) Gently warm the cryovial in a water bath until a small ice pellet remains and transfer into a biosafety cabinet.
4) Add 1 mL of mTeSR media to cryovial and transfer content into a 15 mL centrifuge tube containing 2 mL of mTeSR media.
5) Centrifuge at 300 × g for 3 min.
6) Aspirate the media avoiding disruption to the cell pellet and add 1 mL of mTeSR media.
7) Remove the substrate coating prior to transferring the cells and add mTeSR to flask (11 mL/T75 flask).
8) Gently re-suspend the cell pellet and transfer to the flask.
9) Transfer flask into an incubator at 37°C and do not disturb it for 24 hrs.
10) Perform a full media exchange after 24 hrs (without Y-27632) and then every other day.

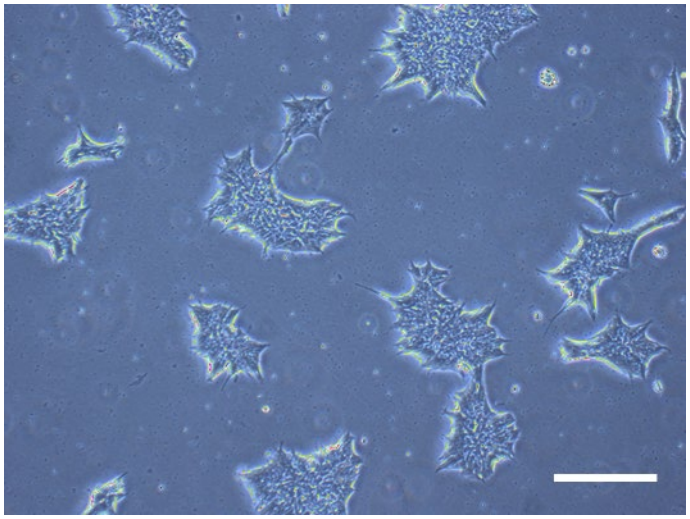


**Figure 8**. MEL-3 hESC colonies at Day 3 post-recovery from cryostorage. Scale bar 200 µm.

## 4.2 Passaging MEL-3 hES cells

1) When cells reach 80-90% confluence prepare the following reagents and volumes per T75 flask (adjust volumes as required for specific culture flask):
2) 5 mL ReLeSR™ (pre-warm to 37°C).
3) 10 mL of PBS (calcium and magnesium free (CMF), pre-warm to 37°C).
4) T75 flasks pre-coated with extracellular matrix (ECM) of choice according to manufacturer's instructions.
5) 18 mL mTeSR™ Plus (pre-warm to 37°C) containing 10 µM Y-27632.
6) 10 mL of DMEM (pre-warm to 37°C, specific formulation not strictly important).
7) Remove flask from incubator and transfer to biosafety cabinet.
8) Aspirate media and wash cells with 10 mL PBS CMF. Aspirate PBS.
9) Add 5 mL of ReLeSR™. Lay down flask and ensure entire surface is covered by ReLeSR™.
10) Incubate in biosafety cabinet for no more than 30 sec.
11) Aspirate ReLeSR™ and immediately transfer flask to 37°C incubator.
12) Incubate for 2 min. Check cells under microscope for rounding of cells. Incubate for 1 min longer if required.
13) In the biosafety cabinet, add 5 mL of DMEM down the side of the flask.
14) 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. Collect the cell suspension in a 15 mL centrifuge tube.
15) Wash the surface with another 5 mL DMEM and transfer to the centrifuge tube.
16) Pellet the cells at 300 × g for 3 min.
17) Aspirate supernatant and add 5 mL of mTeSR™ Plus with Y-27632. Mix well to resuspend pellet.
18) Aspirate ECM from T75 and immediately add 12 mL of mTeSR™ Plus with Y-27632 down the side of the flask to avoid removing ECM from surface.
19) Take 1:8 to 1:10 (or $0.75 \times 10^6$ to $1 \times 10^6$ cells) of the cell suspension and transfer to one T75 with media.
20) Transfer to $CO_2$ incubator. Gently agitate flask back and forth to ensure even spread of cells in the flask.
21) Exchange media after 24 hrs with 20 mL mTeSR plus without Y-27632 and then every other day.
22) Cells will reach 80-90% confluence within 4 days.

### 4.3 Cryopreservation MEL-3 hES cells

1) Follow the passaging protocol up to step 17 and take 0.5 or 1 mL of the cell suspension into a 15 mL tube for cell counting.
2) Centrifuge the 15 mL tube at 300 × g for 3 min.
3) Resuspend cell count aliquot in 0.5 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).
4) Incubate at 37°C for 3 min with regular gentle agitation. After incubation, add 0.5 mL mTeSR+ media and pipette 5-10 times using a 200 µL pipette.
5) Perform cell count using desired method.
6) Back calculate to determine cell number of original cell suspension. Depending on confluence, each T75 flask may contain 15 - 25 × $10^6$ cells.
7) Centrifuge the original cell suspension at 300 × g for 3 min and aspirate media.
8) Gently flick tube to dislodge cell pellet.
9) Add cold, CryoStor® CS10 to a final cell density of 2 - 5 × $10^6$ cells/mL. We recommend not cryopreserving densities lower than 2 × $10^6$ cells/mL. Higher densities improve cell recovery at thaw.
10) Aliquot ≥ 500 µL of cells in CryoStor® CS10 per cryovial.
11) Transfer to isopropanol tanks and then to -80°C for 2 hrs before transferring to LN2 cryostorage.

## References

[1] Jacquet, L., Wood, V., Kadeva, N., Cornwell, G., Codognotto, S., Stephenson, E., & Ilic, D. (2016). Generation of KCL040 clinical grade human embryonic stem cell line. *Stem Cell Research*, *16*(1), 173-176.