Melo-DM
Technical Manual

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1.0 Introduction

Human pluripotent stem cells (hPSCs) have developmental capacity identical to that demonstrated by human embryonic inner cell mass cells. This means an entire adult organism can theoretically be produced using these cells, and any cell type by extension. Using hPSCs to produce cell types of the nervous system and related neural crest lineage is especially attractive, as these cell types are difficult to acquire without damaging the organism.

The nervous system is exquisitely complex. An estimated 10,000 different neuronal subtypes originate within highly specific anatomical locations during development, and may or may not migrate to other anatomical locations later in development. Manufacture of specific neuron subtypes from hPSCs has proven to be a substantial technical challenge given this eventual complexity, but even the primary fate decision required to enter the ectodermal germ layer is a substantial barrier.

From the beginnings of hPSC culture and ectodermal differentiation, formation of ectoderm has required at least one week in vitro. This is only the first step in development with many more to occur, resulting in protracted 30 to 60-day protocols for producing neurons and glia. To put the difficulties working with ectoderm into context, terminally differentiated cardiomyocytes can be produced in seven days. An entire cardiomyocyte differentiation protocol can be executed in the same amount of time that is required for the first step in producing any particular neuron. This places neuroscience at a substantial disadvantage when attempting to utilize hPSC platforms, which are essential for understanding human neuroscience.

For the first time, ectoderm can be produced from hPSCs efficiently within 24 hours, which grants immediate access to understanding the complexity of the nervous system. Melo-DM builds upon these findings to produce a highly pure population of immature melanocytes neurons within only 7 days. Melo-DM is useful for generating melanocytes useful for cosmetic testing, studying vitiligo, and melanoma.

2.0 Related Products

Anatomic provides an integrated ecosystem of products meant to streamline your research and production experiences. We provide a portfolio of melanocyte products that encompass a broad range of use-cases. Whether you want to study early or intermediate developmental events, or are interested in the terminal cell type produced, Anatomic can provide a product to fit your specific needs.

Product	Cat #	Use
RealMELO™ Melanocytes	2020	Cryopreserved melanocytes for downstream assays

+Check our website frequently for an updated list of new and exciting products: www.anatomic.com

3.0 Materials Required for Rapid Production of Melanocytes from hPSCs

Melo-DM is very simple to use and requires few materials and standard equipment to ensure success.

3.1 Product Contents and Storage

Component	Temperature	Packaging	Total Volume	Stability
Matrix 1	-20°C	3 x 1 mL Tube	75 µL	Until labeled expiry
Basecamp	-20°C	120 mL PET Bottle	130 mL	Until labeled expiry
Melo DM1	-20°C	2 mL Tube	1.5 mL	Until labeled expiry
Melo DM2	-20°C	2 mL Tube	1.5 mL	Until labeled expiry
Melo DM3	-20°C	2 mL Tube	1.5 mL	Until labeled expiry
Melo DM4	-20°C	2 mL Tube	1.5 mL	Until labeled expiry
Melo DM5	-20°C	2 mL Tube	1.5 mL	Until labeled expiry
Melo DM6	-20°C	2 mL Tube	1.5 mL	Until labeled expiry
Melo DM7	-20°C	2 mL Tube	1.5 mL	Until labeled expiry

3.2 Materials Required but not Included

Product Name	Supplier	Cat #	Use
dPBS with Calcium and Magnesium	Corning™	21-030-CM	Coating Buffer
Chroman 1	Medchem Express	HY-15392	Day -1 Plating
Versene Solution	ThermoFisher	15040066	Day -1 Dissociation
DMEM F-12	ThermoFisher	11330107	Day -1 Dissociation
Trypsin/EDTA Solution	Lonza	CC-5012	Day 7 Dissociation
Trypsin Neutralizing Solution	Lonza	CC-5002	Day 7 Dissociation
dPBS without Calcium and Magnesium	Corning™	21031CV	Day 7 Dissociation
15 & 50 mL conicals	Your preferred supplier	-	Day 7 Dissociation
CryoStor© CS10	BioLife Solutions	210102	Cryopreservation
Cryovials	Your preferred supplier	-	Cryopreservation
Hemocytometer	Your preferred supplier	-	Cryopreservation
Trypan Blue	Your preferred supplier	-	Cryopreservation
Medium 254	Gibco	M254500	Maturation
Human Melanocyte Growth Supplement (HMGS)	Gibco	S0025	Maturation

3.3 Required Equipment

- Biosafety cabinet certified for handling of biological materials
- 37°C, 5% CO2, 95% humidity incubator
- Pipette-aid with appropriate serological pipettes
- Inverted microscope
- -20°C freezer
- Refrigerator (2 - 8°C)
- Controlled rate freezer

4.0 Important Parameters for Successful Use of Melo-DM

Excellence, Defined. Melo-DM is currently the most modern system available for production of melanocytes. Melo-DM utilizes serum-free, chemically defined media, and has been validated for use downstream from multiple defined systems for the undifferentiated culture of hPSCs. Melo-DM has not been validated for use on hPSCs grown in serum replacement-containing medium on human or murine feeder layers, or feeder-free using serum-replacement medium conditioned by feeders. The benefits of Melo-DM are best enjoyed looking toward the future, rather than living in the past.

Density is Critical. While use of Melo-DM is a robust process, every technology has its limits. Under-seeding of hPSCs before exposure to Melo-DM can result in substantial loss of viability, while over-seeding can reduce control of downstream differentiation processes. Best results are usually enjoyed using a seeding density of 4,000 cells per cm^2 (300,000 cells per T75 flask). Once 4,000 cells per cm^2 has been attempted, densities between 2,000 and 10,000 cells per cm^2 can be attempted to maximize yield and efficiency based on your hPSC line's intrinsic growth rate. To ensure success, view Section 6.0: Production of Melanocytes Using Melo-DM for a guide to appropriate day-one density before addition of Melo-DM.

Volume Is Critical. While this will be clearly stated in the instructions, it's worth highlighting in its own section. Melo DM1 should be applied to hPSC cultures on day-one using 0.2 mL/cm^2. Exceeding this volume on day-one could result in a substantial loss in cell viability.

Apart from the previously stated caveats, Melo-DM is actually very easy to use. You should be ready to go!

5.0 Preparation of Reagents and Media

5.1 Coating with Matrix 1

5.1.1. Thaw Matrix 1 at room temperature
5.1.2. Dilute 75 uL of Matrix 1 into 7.5 mL dPBS with Calcium and Magnesium (1:100 dilution)
5.1.3. Add 7.5 mL of diluted Matrix to a T75 tissue culture-treated flask
5.1.4. Swirl the vessel to evenly spread the solution across the surface
5.1.5. Incubate the vessel overnight at 4°C or at least three hours at 37°C

!!!CRITICAL!!!: Do not let vessels dry out during storage and when aspirating matrix prior to cell seeding.

NOTE: Vessels can also be wrapped with parafilm and stored at 4°C overnight and up to two weeks before use

5.2 Preparation of Melo-DM

Compounding differentiation medium is a basal + supplement affair. For each day of differentiation, 1.5 mL of Melo DM(x) supplement will be added to 13.5 mL of Basecamp to create 15 mL of complete differentiation medium that can then be fed immediately to cultures or stored short-term in the refrigerator prior to feeding.

5.2.1. Thaw Melo-DM for the appropriate day at room temperature
5.2.2. Aliquot 13.5 mL Basecamp into a 50 mL conical
5.2.3. Combine the 1.5 mL Melo DM(x) supplement with the 13.5 mL Basecamp aliquot

Note: It is important that all volumes are combined to ensure the appropriate final concentrations are produced. The supplement can be briefly centrifuged to ensure no liquid remains in the cap, and the supplement tube can be rinsed with the combined supplement/basal to collect any residual-high concentration factors.

6.0 Production of Melanocytes from hPSCs using Melo-DM

Melo-DM will promote the conversion of hPSCs into melanocytes within 7 days. Resulting melanocytes can be characterized by immunolabeling using a standard antibody panel, which includes primary antibodies that are immunoreactive toward melanocytes specific markers MITF+, SOX10+, and TYRP1+. Follow standard immunocytochemical protocols for verification of appropriate immunolabeling.

The following scheme provides a visual representation of the steps required to promote formation of melanocytes from hPSCs using Melo-DM. As can be seen, conversion using Melo-DM is relatively simple to execute when compared to legacy methods.

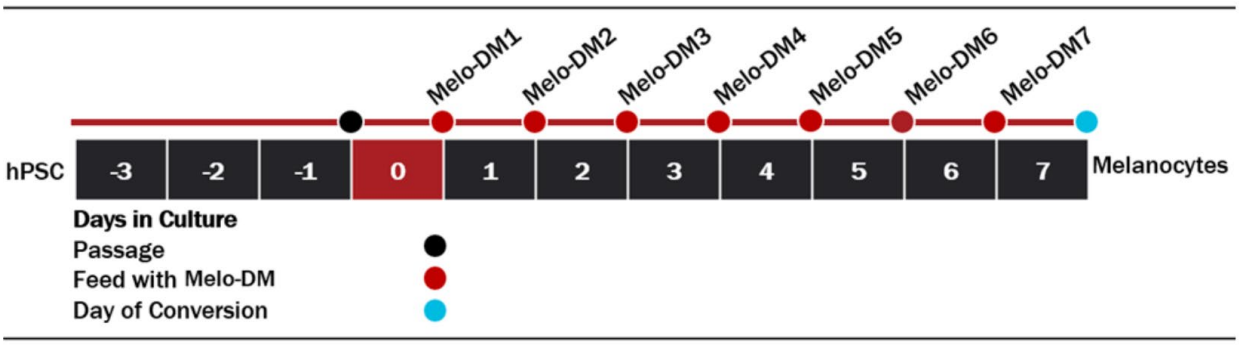
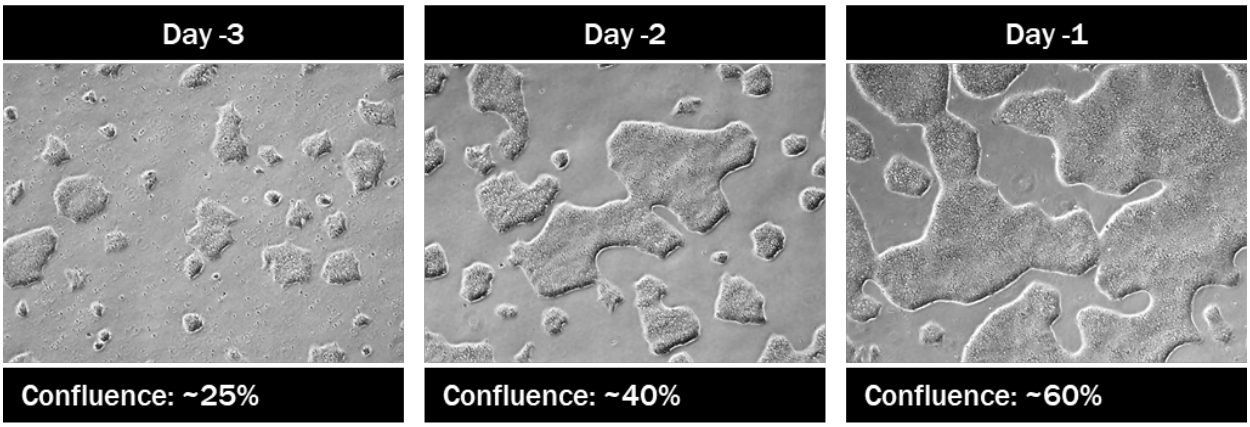


Figure: Schematic for production of melanocytes using Melo-DM.

Days -4 to -1: Maintaining undifferentiated cultures in defined media.

Melo-DM is best used for hPSC cultures maintained under defined conditions. Day -3 represents the first day following passage when cultures are at their lowest confluence. Cultures should be fed as recommended, usually every day to maintain cultures in good condition. Confluence on Day -3 should be roughly 20%, and confluence should increase to roughly 60% by day -1 when cultures will be passaged. The following figure represents the usual confluence and growth rate from days -3 to -1.



Day -1: Seeding undifferentiated hPSC colonies for conversion with Melo-DM

By day -1, your hPSC cultures should have achieved roughly 60% confluence and are ready to passage for the purposes of differentiation. You will have already Matrix 1 coated T-75 flask as described in Section 5.1: Coating with Matrix 1. Now you will single-cell dissociate the hiPSC culture (T25 flask assumed below) using the non-enzymatic passaging solution Versene, a solution containing 0.55 mM EDTA.

1. Transfer hPSC culture vessel to biosafety cabinet
2. Aspirate culture medium from hPSC culture vessel
3. Dispense 2.5 mL Versene Solution into the T25 flask
4. Aspirate Versene Solution from culture vessel
5. Dispense 2.5 mL Versene Solution into the T25 flask
6. Aspirate Versene Solution from culture vessel
7. Dispense 2.5 mL Versene Solution into the T25 flask
8. Return culture vessel to incubator and treat for 20 minutes

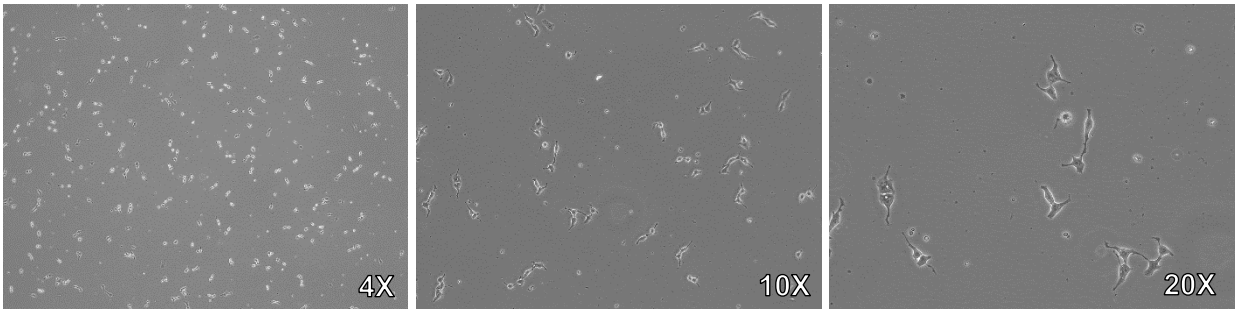
NOTE: Following 20 minutes incubation, cells will have completely dislodged from culture surface, but have not yet singularized.

9. Transfer treated culture to biosafety cabinet
10. Tilt flask vertically to collect dislodged culture into the bottom of the flask
11. With a 10 mL pipet, triturate culture 5 to 10 times to dissociate the cells
12. Collect hPSC culture into sterile 50 mL conical tube

13. Rinse previous culture vessel with 7.5 mL DMEM F-12 medium to collect remaining hPSCs
14. Add this rinse to the hPSC culture in the 50 mL conical tube.
15. Evenly mix the culture to reduce sampling error
16. Sample roughly 45 uL of culture for hemacytometer counting (or appropriate amount necessary given your counting method.)
17. Centrifuge the culture 5 minutes at 300 x G.
18. Observe pellet has formed and carefully aspirate supernatant
19. Resuspend culture to 1 million cells per mL in your hPSC medium of choice
20. Inoculate 15 mL hPSC medium containing 50 nM Chroman 1 with 300 uL of cell suspension (adjust appropriately for targeted seeding density).
21. Aspirate the Matrix 1 coating from its culture vessel
22. Dispense the roughly 15.4 mL of hPSC culture into the coated flask
23. Return flask to incubator and distribute cells evenly by motioning flask front-to-back and side-to-side

Day 0: Addition of Melo-DM1 for production of ectoderm from hPSCs.

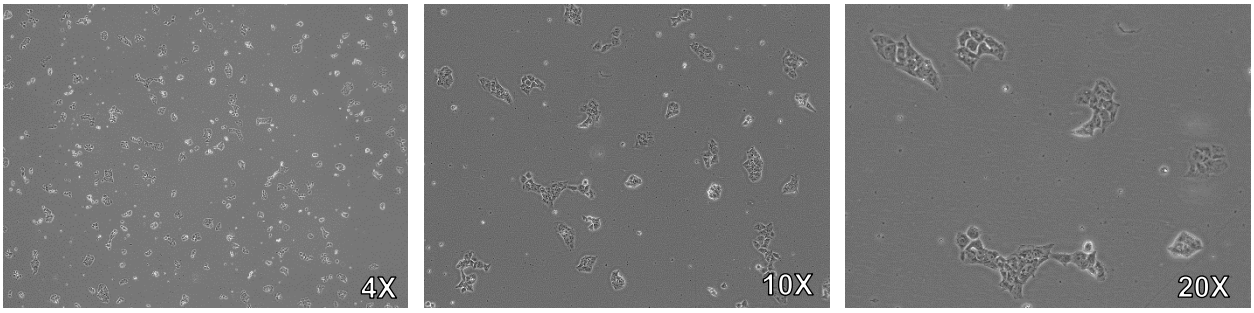
Your culture should be scattered with small spiky hPSC colonies typical of single-cell dissociation with ROCK inhibitors. The figure below depicts a potential culture you may witness.



Prepare Melo-DM1 as indicated in Section 5.2: Preparation of Melo-DM.

1. Transfer hPSC culture vessel to biosafety cabinet
2. Aspirate culture medium from hPSC culture vessel
3. Dispense 15 mL Melo-DM1 into the culture vessel
4. Return culture to incubator
5. Incubate approximately 24 hours

Day 1: Observing ectoderm following conversion



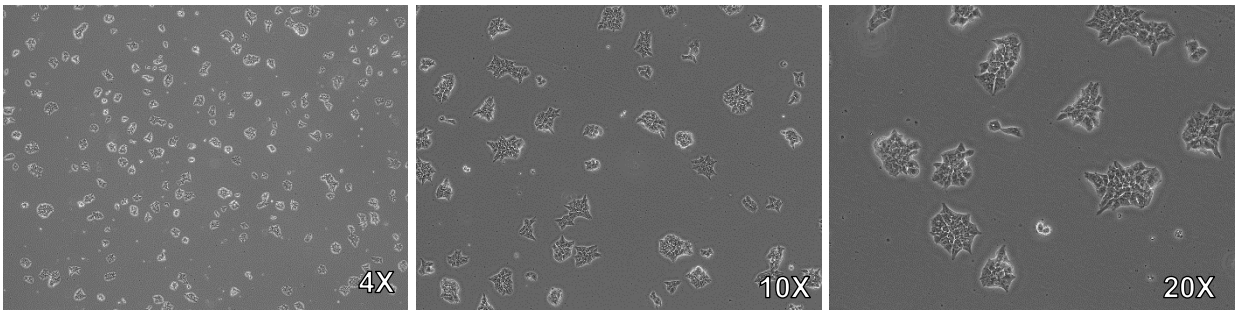
The culture of hPSCs exposed to Melo-DM1 the previous day should have converted to homogenous ectoderm. The culture may appear less dense due some minor cell death, as well as due to the merging of smaller colonies into larger colonies that occupy less surface area in aggregate.

Prepare Melo-DM2 as indicated in Section 5.2: Preparation of Melo-DM.

1. Transfer hPSC culture vessel to biosafety cabinet
2. Aspirate culture medium from culture vessel
3. Dispense 15 mL Melo-DM2 into the culture vessel
4. Return culture to incubator
5. Incubate approximately 24 hours

Day 2: Observe spinal neural culture

Cultures exposed to Melo-DM2 produce spinal neural populations indicated by immuncytochemical staining with antibodies specific to HOXB4 and SOX2. Colonies become more bright and edges are smoother. The center of colonies become increasingly more dense over the next couple of days.

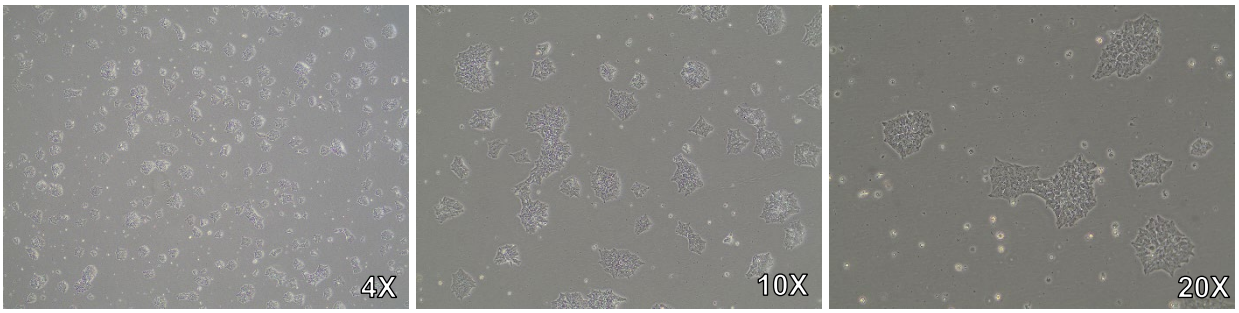


Prepare Melo-DM3 as indicated in Section 5.2: Preparation of Melo-DM.

1. Transfer hPSC culture vessel to biosafety cabinet
2. Aspirate culture medium from culture vessel
3. Dispense 15 mL Melo-DM3 into the culture vessel
4. Return culture to incubator
5. Incubate approximately 24 hours

Day 3: Observe neural plate border culture

Cultures exposed to Melo-DM3 produce a neural plate border population evident with immuncytochemical staining with antibodies specific to PAX3. The edges of colonies should become spikier. This culture is now competent to produce neural crest by applying Melo-DM4.

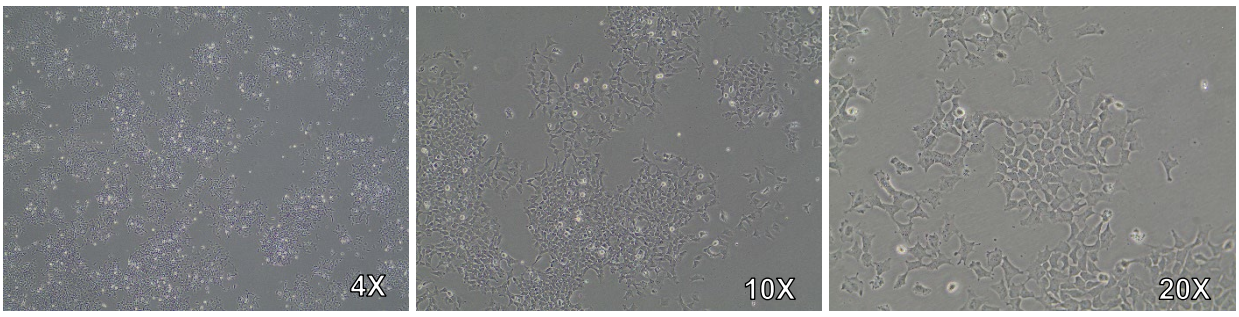


Prepare Melo-DM4 as indicated in Section 5.2: Preparation of Melo-DM.

1. Transfer hPSC culture vessel to biosafety cabinet
2. Aspirate culture medium from culture vessel
3. Dispense 15 mL Melo-DM4 into the culture vessel
4. Return culture to incubator
5. Incubate approximately 24 hours

Day 4: Observe neural crest culture

Cultures exposed to Melo-DM4 initiate commitment into neural crest. This is evident with immunocytochemical staining with antibodies specific to PAX3 and SOX10. Neural crest progenitors begin to extensively migrate in a sheet-like mass from the edges of colonies. Centers of colonies may become more three-dimensional and yellow in coloration. This culture now requires users to expand the neural crest by applying Melo-DM5.

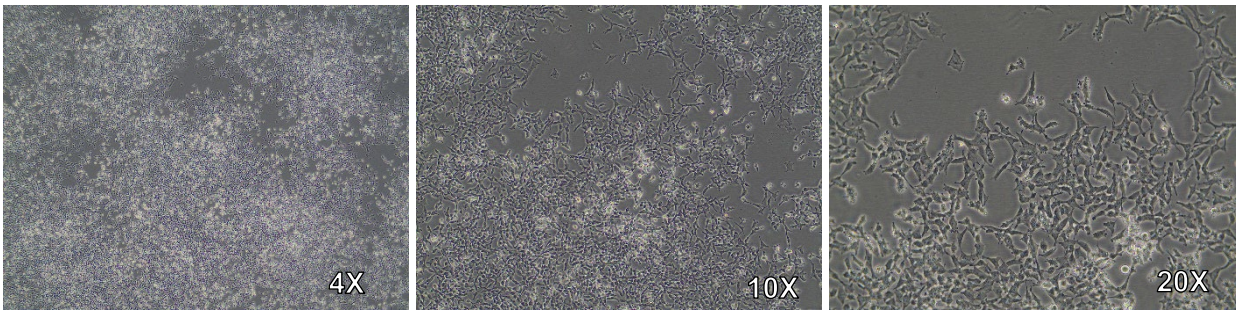


Prepare Melo-DM5 as indicated in Section 5.2: Preparation of Melo-DM.

1. Transfer hPSC culture vessel to biosafety cabinet
2. Aspirate culture medium from culture vessel
3. Dispense 15 mL Melo-DM5 into the culture vessel
4. Return culture to incubator
5. Incubate approximately 24 hours

Day 5: Observe expanded neural crest culture

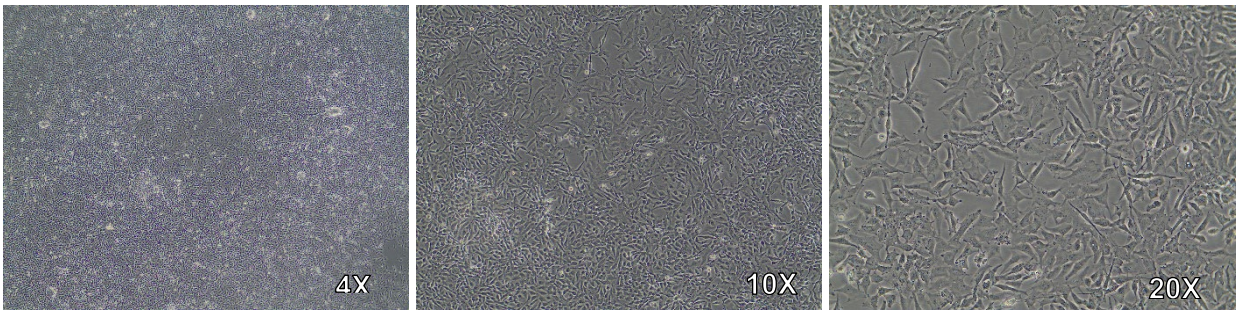
Cultures exposed to Melo-DM5 increase the number of PAX3 and SOX10 expressing neural crest. Day 5 cultures is now competent to produce a population of melanoblasts by applying Melo-DM6.



Prepare Melo-DM6 as indicated in Section 5.2: Preparation of Melo-DM.

1. Transfer hPSC culture vessel to biosafety cabinet
2. Aspirate culture medium from culture vessel
3. Dispense 15 mL Melo-DM6 into the culture vessel
4. Return culture to incubator
5. Incubate approximately 24 hours

Day 6: Observe initial dendrites

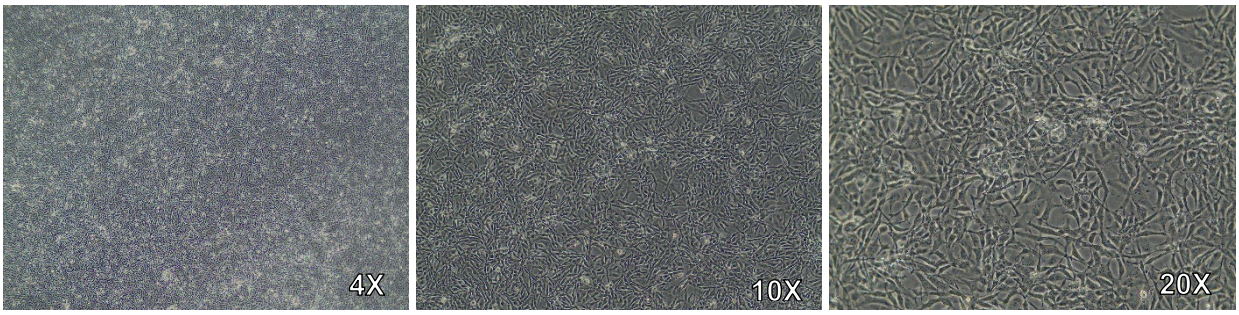


Day 6 cultures exposed to Melo-DM6 initiated commitment into a mix of melanoblasts evident with immuncytochemical staining with antibodies specific to MITF, SOX10, and PAX3. The culture should begin extending dendrites. The MITF/SOX10/PAX3 positive melanocytes will further enrich with application of Melo-DM7.

Prepare Melo-DM7 as indicated in Section 5.2: Preparation of Melo-DM.

1. Transfer hPSC culture vessel to biosafety cabinet
2. Aspirate culture medium from culture vessel
3. Dispense 15 mL Melo-DM7 into the culture vessel
4. Return culture to incubator
5. Incubate approximately 24 hours

Day 7: Differentiation is complete



Day 6 cultures exposed to Melo-DM7 should have fully differentiated into a monolayer of cells beginning to extend dendrites. The percentage of melanocytes is variable depending on optimized seeding density, but should be no worse than 70%, and can be used for downstream uses.

This is the optimal timing for cryopreservation, as these melanocytes are immature and dissociate and survive cryopreservation well.

7.0 Dissociation of melanocytes produced with Melo-DM.

Melanocytes produced with Melo-DM are best cryopreserved on Day 7 when melanocytes are immature. The following procedure can be followed to dissociate these melanocytes.

1. Carefully aspirate culture medium.
2. Add 7.5 mL cold Trypsin/EDTA solution to culture
3. Return to incubator for 3 minutes
4. Confirm dissociation progress under microscope
5. Add 15 mL Trypsin Neutralizing Solution directly to the flask, bringing total volume in the flask up to 22.5 mL
6. Triturate up and down to ensure all cells have dissociated from the flask and clumps are broken up
7. Transfer entire volume of cells into a 50 mL conical

NOTE: Single-cells can be observed in solution when viewing conical over objective lens.

8. Sample well-mixed culture for a cell count
9. Centrifuge cells 5 minutes at 300 x g
10. Perform a cell count during centrifugation
11. Observe compacted cell pellet
12. Carefully aspirate supernatant by tipping supernatant toward aspirator
13. Smear pellet by flicking conical vigorously, which aids in resuspension
14. Re-suspend pellet into an appropriate volume of cryopreservative according to application

8.0 Cryopreservation of melanocytes produced using Melo-DM

Melanocytes produced using Melo-DM can be cryopreserved when differentiation has been completed, 24 hours following application of Melo-DM7. Following step 7.13, after the centrifuged cell pellet has been smeared, ice cold CryoStor® CS10 can be used for resuspension following manufacturer instructions. This suspension can be vialed (>1 million cells per vial) and placed into an isopropanol bath at -80°C for controlled rate freezing before long-term storage in liquid nitrogen. The expected recovery rate (total viable cells post-thaw divided by number of viable cells vialed) ranges from 50 to 60% when using isopropanol containers.

9.0 Maturation of melanocytes using Melo-MM

Melanocytes produced using Melo-DM can be cultured on Matrix 1 and rapidly matured using Medium 254 (Gibco, Cat#M254500) supplemented with Human Melanocyte Growth Supplement (HMGS) (Gibco, Cat# S0025) to express key melanogenesis enzymes and begin pigment production within one week.

Please feel free to reach out to us for more information and to discuss your research goals with the Melo Products.

10.0 Troubleshooting

Things go wrong. It's understandable. The following are potential issues that may occur when using Melo-DM. This is not an exhaustive list, so please contact us by email: support@anatomic.com to report any issues.

PROBLEM	SOLUTION
Everything immediately died 24 hours after using Melo-DM1	• Ensure no more than 0.2 mL/cm² of Melo-DM1 was used on Day 0
I don't notice a morphology change on day 1	• Morphologically, the change is very minute. Look under higher magnification (20x to 40X magnification), although it is normal to still not notice a difference.
Cells died mid-differentiation	• Manually extract culture media with serological pipet rather than vacuum aspirate to prevent cultures from drying out
My cultures didn't characterize to be highly positive for melanocyte markers	• Ensure use of Melo-DM in proper sequence Melo-DM1→Melo-DM2→Melo-DM3→Melo-DM4→Melo-DM5→Melo-DM6→Melo-DM7 • Cultures continue to mature in Melo-MM, during which melanocytic marker expression increases.