Cell Growth Protocol and Differentiation treatment for the LHCN-M2 Cell Line
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LHCN-M2 cell culture, differentiation treatment, and cross-linking protocol.

LHCN-M2 are a line of human skeletal myoblasts derived from satellite cells from the pectoralis major muscle of a 41 year old Caucasian male heart transplant donor. They are immortalized with lox-hygro-hTERT ("LH"), and Cdk4-neo ("CN"). M2 is an extremely well-differentiating subclone of LHCN (thus "LHCN-M2"). The cells are adherent in culture, and are grown on gelatinized plastic culture dishes. They grow as undifferentiated myoblasts when in growth medium (15% fetal bovine serum). Myogenic differentiation is initiated by switching the cells to serum-free medium. See: Zhu et al. (2007) in *Aging Cell*, vol. 6, pp 515-523.

Cell culture protocol for cycling (exponentially growing) cells:
Cells are grown at 37°C in a humidified incubator with 5% CO_2.

Myoblast growth medium:

	final	stock	example
DMEM / medium 199		4:1	500 mL
FBS (fetal bovine serum)	15%	100%	90.6 mL
HEPES	0.02M	1M	12.09 mL
Zinc Sulfate	0.03 ug/mL	60 ug/mL	302 uL
Vitamin B12	1.4 ug/mL	14 mg/mL	60 uL
Dexamethasone	0.055 ug/mL	55 ug/mL	604 uL
Hepatocyte Growth Factor, recombinant human	2.5ng/mL	5 ug/mL	302 uL
Basic FGF	10 ng/mL	20 ug/mL	302 uL
Final			604.6 mL

Materials:

Medium 199 (with bicarbonate)	GIBCO # 11150-059
DMEM (high glucose + glutamine + bicarbonate)	GIBCO #11965-084
HEPES (1M)	Gibco #15630
Dexamethasone	Sigma-Aldrich # D-2915
Zinc Sulfate	Sigma-Aldrich #Z4750
Vitamin B12 (cyanocobalamin)	Sigma-Aldrich #V2876
HGF (Hepatocyte Growth Factor, recombinant human)	Chemicon Int'l #: GF116
porcine skin gelatin	Sigma-Aldrich # G1890-500G
basic FGF-2, human recombinant	BioPioneer # HRP-0011

Antibiotics: If you choose to use antibiotics in the culture medium, we recommend 0.6X Penicillin/Streptomycin (100X stock = Gibco # 15140). This comes out to final concentrations of 60 units/mL penicillin and 60 ug/mL streptomycin.

Although the culture medium is buffered with HEPES, we use M199 and DMEM formulations that also contain sodium bicarbonate as buffer. We find that this keeps the pH more tightly controlled and is especially important for the serum-free differentiation medium (below), which will turn acidic and kill your cells if not buffered with sodium bicarbonate.

Gelatinizing dishes:

Autoclave a 1% solution of Sigma Pigskin Gelatin made in molecular biology grade water. Dilute this 1% stock 1:10 with sterile water, add enough to cover the bottom of each dish, and let stand in the culture hood for >4 hours. Do not turn the UV light on. Aspirate off liquid and store dry in a sterile bag or container at 4°C. It is possible to add medium and cells immediately after drying, if needed. Make sure to dry the gelatinized plates well before putting them in the fridge for storage, we usually let them sit overnight in the TC hood. We use 15cm Nunc delta surface plates, although other types of standard tissue culture plates are acceptable.

Liquid Nitrogen Storage:

Freeze cells in growth medium supplemented with 10% (v/v) DMSO in 1 ml aliquots of approximately 0.5-1 x 10^6 cells. When grown on 15 cm dishes, the cells reach confluence at ~4.0 X 10^6 cells per dish.

Cell culture and passage

1. Thaw a 1 ml aliquot of cells as quickly as possible in a water bath at 37°C. Transfer cells to 10 mL warm media in a 15 mL conical tube. Spin down at 115 x g at 4°C for 5 minutes. Remove the medium from the tube by aspiration, being careful not to aspirate the cell pellet. Add 10 mL of fresh medium, resuspend the cells, and plate on a new 10 cm gelatinized dish. Place in incubator. Expand from 10 cm plate to 15 cm plate at 50% confluence.

2. When cells are 50-60% confluent, split 1:3 (at most). They are density-sensitive, so don't over-split. It is important to avoid letting the cells become fully confluent; the index of myogenin positive cells increases as you get closer to confluence. To passage, remove and discard culture medium. Rinse twice with 12 mL PBS (calcium and magnesium free), being sure to drain the dish thoroughly. This allows the trypsin to work more effectively. For a 15 cm dish, add 2.5 mL of 0.25% (w/v) trypsin + 0.53 mM EDTA solution (Gibco #25300) pre-warmed to 37°C, for 2 minutes. (If using 10cm dishes, the volume of trypsin is reduced to 1 mL, and the time is reduced to 1 minute in trypsin). Do not aspirate the trypsin from the dish. Trypsinization is enhanced by swirling the dishes regularly during the interval to keep the trypsin solution dispersed over the cells. Add 10mL of myoblast growth medium to the dish, and dislodge the cells by vigorously pipetting. Collect the cells into a 15 mL centrifuge tube, then check the plate under the microscope to ensure that the cells have been removed. Spin down at 115 x g at 4°C for 5 minutes. Remove the medium/trypsin from the tube by aspiration, being careful not to aspirate the cell pellet. Add fresh medium, resuspend the cells, and then dilute in a larger flask to an appropriate volume (25 mL per 15 cm plate, 10 mL per 10 cm plate) with growth medium and plate on new gelatinized dishes. It is important when resuspending the cells after spin down that you triturate vigorously. Seeding density becomes non-uniform if the cells are not well-dispersed. The cells will begin to differentiate if they become locally dense, which causes problems when collecting early time points in a differentiation time course. Feed again 2 days after plating. This is a fairly slow-growing cell line; you will have several days before the cells reach a suitable density for passage again.

The Nunc plates that we use have the following measurements:

nominal diameter (cm)	actual diameter (cm)	radius (cm)	area (cm^2)	cell plating volume (mL)
15.0	13.5	6.8	143.1	25.0
10.0	8.5	4.3	56.7	9.9
6.0	5.0	2.5	19.6	3.4
3.5	3.4	1.7	9.1	1.6

Be sure to account for these measurements when plating 6 cm immunocytochemistry plates, etc.

Differentiation treatment

Differentiate for 7 days by rinsing fully confluent cells once with PBS and adding 25mL of serum free differentiation medium. Feed with fresh differentiation medium every other day.

Differentiation medium:

	final	stock	example
DMEM/M199		4:1	479.7 mL
HEPES	0.02M	1M	10 mL
Zinc Sulfate	0.03 ug/mL	60 ug/mL	250 uL
Vitamin B12	1.4 ug/mL	14 mg/mL	50 uL
Insulin	10 ug/mL	1 mg/mL	5 mL
apo-transferrin	100 ug/mL	10 mg/mL	5 mL
Final			500 mL

Materials:

Medium 199 (with bicarbonate)	GIBCO # 11150-059
DMEM (high glucose + glutamine + bicarbonate)	GIBCO #11965-084
HEPES (1M)	Gibco #15630
Zinc Sulfate	Sigma-Aldrich #Z4750
Vitamin B12 (cyanocobalamin)	Sigma-Aldrich #V2876
apo-transferrin	Sigma-Aldrich #T-1147
insulin	Sigma-Aldrich #I-6634

Insulin: 100X stock is 1mg/mL in 4:1 DMEM/M199 with 10-20 µl of 10M NaOH added to alkalinize the medium so it dissolves (use minimum possible). Filter sterilize with 0.2 um filter.

Transferrin: 100X stock is 10 mg/ml of apo-transferrin in 4:1 DMEM/M199.

Antibiotics: If you choose to use antibiotics in the culture medium, we recommend 0.6X Penicillin/Streptomycin (100X stock = Gibco # 15140). This comes out to final concentrations of 60 units/mL penicillin and 60 ug/mL streptomycin.

Cell cross-linking and harvest – protocol from Katherine Fisher

1. Remove the medium from the culture plates and add a pre-warmed (37°C) solution of PBS with 1% formaldehyde (Sigma-Aldrich # F87750). Swirl gently, and incubate at room temperature for 10 minutes.

2. Stop the cross-linking reaction by adding glycine to a final concentration of 0.125 M and swirl gently to mix. Incubate for 5 minutes.

3. Remove PBS/FA/glycine from plates and gently wash cells twice with 15 mL room temperature PBS.

4. To permeabilize the cells, add dilute trypsin (2mL PBS + 0.4mL of Gibco trypsin+EDTA (Gibco #25300)) for 10 min at 37°C, then quench with 100uL horse serum or FBS.

5. Add 2 mL of PBS and scrape into a 15mL falcon tube, rinse plate once with 5mL of PBS and combine.

6. Pellet cells at 360 X g for 5 minutes at 4°C.

7. Aspirate PBS/trypsin solution and resuspend cells in 5 ml cold (4°C) PBS + 1 uM PMSF.

8. Pellet cells at 360 X g for 5 minutes at 4°C.

9. Carefully aspirate PBS and add 6 ml cold (4°C) Farnham lysis buffer (5 mM PIPES pH 8.0 / 85 mM KCl / 0.5% NP-40) + Roche Protease Inhibitor Cocktail Tablet (Complete 11836145001). This step lyses the cell membrane, leaving the nuclear envelope intact.

10. Pellet nuclei at 360 X g for 5 minutes at 4°C.

11. Place the nuclear pellet on ice. Carefully remove supernatant and either proceed to sonication step or snap freeze in liquid nitrogen and store at -80°C or in liquid nitrogen.

RNA yields

A 15 cm dish of undifferentiated cells yields about 45 ugs of total RNA collected with Qiagen RNEasy reagents. A 15 cm dish of differentiated cells yields about 61 ugs of total RNA.