Cell Growth Protocol and Differentiation treatment for the C2C12 Cell Line
From: Wold mouse ENCODE
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C2C12 cell culture, differentiation treatment, and cross-linking protocol.

The cell line C2C12 is an immortal line of mouse skeletal myoblasts originally derived from satellite cells from the thigh muscle of a two month old female C3H mouse donor 70h after a crush injury (Yaffe and Saxel, 1977; karyotyping available in Casas-Delucchi, 2011). From the C2s the immortal subline C2C12 was selected (Blau et al., 1985). These cells differentiate well into myocytes under appropriate culture conditions given below. The cells are adherent in culture and are grown on Nunc delta surface plastic culture dishes. They grow as undifferentiated myoblasts in growth medium (15-20% fetal bovine serum, with 20% used here). Myogenic differentiation is initiated upon reaching confluence by switching the cells to medium containing 2% horse serum supplemented with insulin. C2C12's are commercially available but because variable handling of this line can select for cells with different kinetics or poor differentiation performance, the Wold lab will provide plugs of these C2C12's upon request. See: (1) Yaffe and Saxel, 1977; Nature Vol. 270, 725-727; (2) Casas-Delucchi et al., 2011; Nature Communications Vol. 2, 222. (3) Blau et al., 1985; Science Vol. 230, 758-766.

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			395 mL
FBS (fetal bovine serum)	20%	100%	100 mL
Final			500 mL

Materials:

DMEM (high glucose + glutamine, no Sodium Pyruvate)	GIBCO #11965
FBS	HyClone #30071.03

Antibiotics: We use 1X Penicillin/Streptomycin (100X stock = Gibco # 15140). This comes out to final concentrations of 100 units/mL penicillin and 100 ug/mL streptomycin.

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 ~2.6 X 10^6 cells per dish.

Cell culture and passage

1. Thaw a 1-ml aliquot of cells as quickly as possible in water bath at 37°C. Transfer cells to 24 mL warm media in a 50 mL conical tube. Mix gently. Plate the cells in a 15cm Nunc delta surface plates. Place in incubator. After one day, remove the medium and add fresh media.

2. When cells are 50-60% confluent (meaning that very few of them are physically touching each other), split 1:4 or 1:5 (at most). It is important to not let the cells become fully confluent because they can begin to fuse and partially differentiate upon cell-cell contact. To passage, remove and discard culture medium. Rinse twice with PBS (Calcium and Magnesium free). For a 15 cm dish, add 2.5mL of 0.25% (w/v) trypsin + 0.53 mM EDTA solution (Gibco #25300) pre-warmed to 37°C, and observe cells under an inverted microscope until cell aspect changes to round (usually within 60-90 seconds). Aspirate the majority of the trypsin and let stand for an additional 1-2 minutes, then tap the plate to dislodge cells. Add 10mL of myoblast growth medium to the dish, and collect cells by gently pipetting. (If using 10cm dishes, the volume of trypsin is reduced to 1 mL, and the time is reduced to 1 minute in trypsin). Dilute cells in a larger flask to the appropriate volume using growth media and aliquot to new Nunc dishes. There is no need to feed the cells in between passages. This is a fairly quickly growing cell line (doubling time is approximately 12h); you will need to passage them every 1-2 days.

Differentiation treatment

Differentiate for 24 hours to 7 days by rinsing fully confluent cells once with PBS and adding 25mL of low-serum differentiation medium. Feed with fresh differentiation medium every 24 hours up to the 72h timepoint and after that, every 12 hours (as these cells differentiate, they begin to deplete and acidify the medium more quickly). The timepoints we typically use are 24h, 60h, 5D, and 7D. Feed the cells no closer than 6h before fixation to avoid seeing serum-response effects in the cell prep.

Differentiation medium:

	final	stock	example
DMEM			489.5mL
Donor equine serum	2%	100%	10 mL
Insulin (add no more than 24h before use)	1uM	1mM	0.5 mL
Final			500 mL

Materials:

DMEM (high glucose + glutamine, no Sodium Pyruvate)	GIBCO #11965
Donor equine serum	HyClone #SH30074.02
insulin	Sigma-Aldrich #I-6634

Insulin: 1,000X stock is 1mg/mL in water with 10-20 µl of acetic acid added to acidify the water so it dissolves (use minimum possible). Filter sterilize with 0.2 um filter. Store at -20°C in small aliquots until use.

Antibiotics: We use 1X Penicillin/Streptomycin (100X stock = Gibco # 15140). This comes out to final concentrations of 100 units/mL penicillin and 100 ug/mL streptomycin.

Cell cross-linking and harvest for ChIP

1. Remove the medium from the culture plates and add a 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. Use a stock solution of 2.5M glycine dissolved in H20. Incubate for 10 minutes.

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

4. To detach the cells from the dishes, 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. Transfer to ice or 4°C.

5. Add 2 mL of cold PBS and scrape into a 15mL falcon tube; rinse plate once with 5mL of cold 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 20 ugs of total RNA collected with Qiagen RNEasy reagents. A 15 cm dish of differentiated cells yields about 60 ugs of total RNA.