

Protocols for Culturing Human Primary and Immortalized Muscle Cell Lines

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Nomenclature for Wellstone FSHD Human Muscle Cell Strains and Lines

- donors are designated by cohort (family) number (01, 03, 09, 12, etc.)
- the cohort number is immediately followed by a letter designation: FSHD donors with a confirmed 4qA deletion are assigned letters A, B, C... etc; control 1st degree relatives (who have been confirmed to lack a 4qA deletion) are assigned letters U, V, W...etc.
- the letter designation is followed by "bic" or "del" to describe the muscle of origin (biceps or deltoid)
- immortalized lines are denoted as "CT" (abbreviated for CDK4-hTERT) followed by the clone number

e.g. 03Udel: primary deltoid cells from the control subject of cohort 03

01Abic CT#6: immortalized biceps cells (clone #6) from the FSHD subject of cohort 01

Culture Conditions for Immortalized Human Muscle Cell Lines

Each vial contains between 5×10^4 to 1.5×10^5 cells; the approximate number of population doublings (PD) that each line has gone through is recorded on the vial. Thaw cells briefly in a 37°C water bath and resuspend in pre-warmed immortalized human muscle cell growth medium (LHCN). Centrifuge at 1,000xg for 5 minutes. Resuspend cell pellet in 4 mL culture medium and transfer to a gelatin-coated 6 cm dish. (Optional: when thawing frozen aliquots that are $>5 \times 10^4$ cells, you can seed onto a 10 cm dish instead). Culture at 37°C/5% CO₂.

LHCN medium should be refreshed between every one to three days; we exchange more frequently when culture density increases, and also initially post-thaw. When cells are ~70% confluent, rinse briefly with phosphate-buffered saline without calcium and magnesium (PBS) and dissociate with TrypLE (Invitrogen) for ~ five minutes at room temperature. Neutralize TrypLE with an equal volume of growth medium and count cells.

To expand, seed cells onto gelatin-coated dishes at ~2,000-4,000 cells/cm² and culture as described above.

To freeze, adjust volume as desired with growth medium; add an equal volume of ice-cold 2x freeze medium (50% FBS, 30% LHCN medium, 20% DMSO) and aliquot into cryovials. Place in liquid nitrogen vapour phase for 1 hour, then move to permanent storage.

To differentiate, culture cells until >90% confluent; aspirate growth medium, rinse with PBS and feed with Opti-MEM reduced serum media (Cat#31985070 from thermo fisher). Culture at 37°C/5% CO₂ untouched. Multinucleated myotubes are usually observed within 2-4 days.

Methods describing the derivation of the immortalized lines can be found in Stadler et al (2011): *Skeletal Muscle* 1:12.

Culture Conditions for Primary Human Muscle Cell Strains

On the side of the vial, the passage number (P) and population doubling number (PD) have been recorded. Please note that a passage number of "P2.3" indicates that CD56+ cells were sorted at P2, and subsequently expanded for three more passages (therefore, P2.3 = P5).

Thaw cells briefly in a 37°C water bath and resuspend in 10 mL pre-warmed primary human muscle cell growth medium (HMP). Centrifuge at 1,000xg for 5 minutes. Resuspend cell pellet in 4 mL HMP medium and transfer to a gelatin-coated 6 cm dish. Culture at 37°C/5% CO₂, **feeding daily**. When cells are ~50-70% confluent, rinse briefly with phosphate-buffered saline without calcium and magnesium (PBS) and dissociate with TrypLE (Invitrogen) for ~ five minutes at room temperature. Neutralize TrypLE with an equal volume of growth medium and count cells.

To expand, seed cells onto gelatin-coated dishes at ~2,000-4,000 cells/cm² and culture as described above.

To freeze, adjust volume as desired with growth medium; add an equal volume of ice-cold 2x freeze medium (50% FBS, 30% HMP medium, 20% DMSO) and aliquot into cryovials. Place in liquid nitrogen vapour phase for 1 hour, then move to permanent storage.

To differentiate, culture cells until >90% confluent; rinse with PBS, and feed differentiation medium. Cell fusion as well as maximal induction of myosin heavy chain gene expression is typically observed after 2 to 4 days. (Note that we have observed variable rates of fusion in both FSHD and control cells for different cohorts, *Homma et al (2011) Eur. J. Hum. Genet., in press*).

See Stadler et al (2011) Skeletal Muscle 1:12 for isolation methods.

Growth and Differentiation Media for Human Muscle Cell Strains and Lines

Immortalized Human Muscle Cell Growth Medium (LHCN)

LHCN	[final]	[stock]	volume
Media X			500 ml
FBS	15%	100%	91 ml
HEPES (add post-filtration)	0.02 M	1.0 M	12.1 ml
Zinc sulfate	0.03 ug/ml	60 ug/ml	0.302 ml
Vit. B12	1.4 ug/ml	14 mg/ml	0.060 ml
Dexamethasone	0.055 ug/ml	55 ug/ml	0.604 ml
HGF	2.5 ng/ml	5 ug/ml	0.302 ml
bFGF	10 ng/ml	20 ug/ml	0.302 ml
Total:			604.7 ml

Media X is 4:1 DMEM / medium 199; make up from powder according to manufacturer's directions; supplement with final concentrations of 0.8 mM sodium pyruvate (0.88 mg/L) and 3.4 g/L sodium bicarbonate (pH'd to 7.2-7.3).

ADD HEPES AFTER FILTRATION OF LHCN MEDIUM (the medium becomes more alkaline following filtration).

Medium 199	Invitrogen Cat# 31100-035
DMEM	Invitrogen Cat# 12100-061
HEPES	Sigma-Aldrich Cat# H3375
Dex	Sigma-Aldrich Cat# D2915
Zinc Sulfate	Fisher Scientific Corp. Cat# Z68-500
B12	Sigma-Aldrich Cat# V2876
HGF	Chemicon International Cat#: GF116
bFGF	BioPioneer HRP-0011

The addition of bFGF has been reported by the Wright lab to be optional in culture of immortalized cell strains; however, these lines have been cultured in bFGF-containing medium, only.

Growth and Differentiation Media for Human Muscle Cell Strains and Lines, Cont'd

Primary Human Muscle Cell Growth Medium (HMP)

Ham's F-10 (Cellgro 10-070-CV) supplemented with:
 20% FBS, characterized (Hyclone SH30071.03)
 0.5% chick embryo extract (see protocol below)

1.2 mM CaCl₂ (EMD OmniPur 3000)
1% antibiotic/antimycotic (Cellgro 30-004-CI)

Human Muscle Cell Differentiation Medium (Primary and Immortal Cells):

Media X (DMEM and Medium 199 in a ratio of 4:1), supplemented with *EITHER*:

2% horse serum (Invitrogen 16050-122)
2 mM L-glutamine (Invitrogen 25030081)
1% antibiotics/antimycotics (Cellgro 30-004-CI)
1 mM sodium pyruvate (Invitrogen 11360070)
20 mM HEPES buffer (Invitrogen 15630080)

OR:

10 mg/l insulin (Sigma-Aldrich)
100 mg/l apo-transferrin (Sigma-Aldrich or Invitrogen)
20 mM HEPES buffer (Invitrogen 15630080)

N.B. Ham's F10 medium can be substituted for Media X for differentiation of primary cells.

Opti-MEM Reduced Serum Medium (Gibco 31985-070) can be used in place of Differentiation Medium, either directly from the bottle or supplemented with 1X antibiotics/antimycotics (Corning/Cellgro 30-004-CI)

Chick Embryo Extract

Protocol adapted from Frank Stockdale's Lab, Stanford

You will need:

- day 12 SPF Premium Fertilized White Leghorn Chicken Eggs (Charles River, North Franklin, CT): 5 dozen eggs makes ~200-250 mL final CEE; we have processed up to 10 dozen at one time.
- surgical scissors (2 pairs: one for cutting through eggshells, and one for decapitating embryos) and long forceps, sterilized
- ice-cold Hanks Balanced Saline Solution, sterile (HBSS; Invitrogen)
- 15 cm petri dishes, sterile
- two 500 mL or 1 L beakers, one 500 mL graduated cylinder, and one magnetic stirbar, **sterilized**
- 60 mL syringe (no needle), sterile
- sterile 50 mL centrifuge tubes (or appropriate size for your centrifuge)

Protocol:

1. Spread out benchcoat and/or diapers to contain the mess. Fill three 15 cm petri dishes with ice-cold HBSS.
2. Using the pointed end of a pair of scissors, stab a hole into each egg. Cut a window out of each eggshell. Remove the embryo and place in large petri dish with ice-cold HBSS. Using sterile scissors, decapitate embryo, leaving as much of the neck as possible.
3. Rinse embryos two times in ice-cold HBSS to remove blood. (Refresh HBSS as necessary.) Store embryos in a sterile beaker with HBSS on ice until all embryos have been collected.
4. Macerate embryos by pushing through a 60 ml syringe (no needle) into an ice-cold, sterile graduated cylinder.
5. Add an equal volume of ice-cold HBSS, transfer to a sterile beaker, cover with aluminum foil and stir gently at 4°C for 1 hour.
6. Transfer to sterile centrifuge tubes and centrifuge at 4°C for 1 hour at 10,000 rpm*.
7. Collect supernatant and store in aliquots at –80°C.

*Alternatively, extract can be centrifuged at 3600 rpm for 1 hour at 4°C in sterile 50 mL centrifuge tubes. Extract prepared in this way tends to be fairly thick and difficult to filter; therefore, prior to use, thaw an aliquot of chick embryo extract in a room temperature waterbath and re-centrifuge for 10 minutes at 3600 rpm at 4°C in 15 mL tubes. Add the supernatant to medium and filter-sterilize. Leftover extract can be stored at –20°C.

Gelatin Coating Tissue Culture Dishes

Make 0.1% gelatin solution by combining 1 g gelatin (e.g. Sigma G9391) and 1 L tissue culture grade water; autoclave for 30 minutes to dissolve and sterilize. (Alternatively, gelatin can be dissolved by heating; filter-sterilize solution when cool.) We usually treat one sleeve of dishes at a time: in a biosafety cabinet, add sufficient 0.1% gelatin to coat the first dish; using a sterile pipette, aspirate the solution and use it to coat the second dish, etc until all of the dishes have been coated. Airdry and store in original sleeve at room temperature.