

## **A. Preparing Primary Cultures from Muscle Biopsy Specimens II**

This is the present protocol, it has been used since 6/05

1. Aspirate transport media and wash specimen with 10 ml PBS.
2. Aspirate PBS and replace with 5ml PBS.
3. Pour specimen into sterile Petri dish and cut into small pieces using two sterile #10 scalpels.
4. Pipette up as much of the specimen as possible and transfer to a 15ml sterile tube. Wash the plate with an additional 5ml of PBS.
5. Let the specimen settle to the bottom of the tube. Aspirate off PBS, sparing as much of the specimen as possible.
6. Pipette 10 ml of 1x PBS onto the specimen. Cap tube and shake well. Let the specimen fall to the bottom of the tube, then aspirate off the PBS
7. Repeat step 5 twice (for a total of three times).
8. Aspirate the PBS, add 1.6ml of Dispase II solution, 2ml of Collagenase 4 and 40ul of 0.25M CaCl<sub>2</sub>.
9. Incubate in 37°C waterbath for 15min.
10. Triturate (pipette up and down) 10 times, try to avoid producing foam, return to waterbath for an additional 15 mins.
11. Repeat trituration.
12. If tissue does not appear disassociated (by eye), repeat incubation and trituration.
13. Place a 100um nylon cell strainer on top of a 50ml tube, prewet filter with PBS.
14. Pass sample through cell strainer and rinse strainer with an additional 5ml PBS.
15. Pellet cells, aspirate supernatant and resuspend in 10ml of Primary Culture Media (see below).
16. Preplate for 60-90 mins on a standard Petri dish (non TC coated). This will allow contaminating fibroblasts to adhere, but myoblasts will not.
17. Transfer medium to a T75 flask, discard Petri dish.
18. On the 3<sup>rd</sup> or 4<sup>th</sup> day after initiating the culture, supplement the media with 4ul of bFGF in 1 ml of F10 media, myoblasts may not have adhered at this stage and will not have a normal appearance.
19. For the first day or so, cells will appear rounded and may not be all adhered. It can take up to a week for cells to adhere and flatten out. If original biopsy sample was small or from a severely affected FSHD patient or other disease controls you may not easily see any cells for a few days.
20. Supplement fresh bFGF every 3-4 days. Change media for 1<sup>st</sup> time after one week. If it is necessary to perform first media change earlier than 1 week, pellet old media to recover any non- adherent cells and resuspend in fresh media.
21. Subsequently, change media approx 2x per week until growth foci are seen. Do not allow these foci to overgrow.
22. Redistribute cells as foci develop. Aspirate medium and rinse with 10ml of PBS, rock flask gently to cover and aspirate. Add 1ml 0.1% trypsin, rock flask to cover surface and either place in incubator or leave at room temp. Cells will begin to detach within a couple of minutes. Gently tap side of flask and check under

- microscope that cells are detached. Add 10ml of media and return to incubator in same flask.
23. Grow until culture is approx. 60-80% confluent. Trypsinize as before, split to 3-4 flasks (10ml media per flask). P1
  24. Grow cultures to 60-80% confluence. Trypsinize using 1ml of 0.1% trypsin per flask. When cells are detached inactivate trypsin with 10ml of F10 media (no conditioned media) per flask. Pool media containing cells and pellet at speed 7 for 5mins (use old centrifuge in cell culture lab).
  25. Aspirate supernatant and resuspend cell pellet carefully in freezing medium. Use 2-3ml per flask depending on degree of confluency, (aim for 8-10 vials per line). This is passage 2. Aliquot into cryogenic vials 1ml/vial and freeze slowly (Nalgene Cryo freezing container, 5100-0001) at least overnight and up to a few days. Transfer to liquid nitrogen for long term storage.

Grow cells in F10/conditioned media until just after 1<sup>st</sup> passage if growing well, or until step 18 (freeze down) if growing slowly. After thawing, Maintenance media may be used.

Aliquot bFGF and store at -20C, store thawed aliquot at 4C for up to one month. Add bFGF at 4ul/10ml media immediately prior to use.

Do not allow cultures to overgrow as they may start to differentiate.

For human lines, label cryovials with subject code, date of freeze and passage #. Do not label vials with subject name, vials shipped to other labs must have names removed, this is difficult to do on frozen vials with alcohol resistant ink.

To thaw cells, thaw rapidly by holding in a 37C water bath and wash outside of vial with ethanol. Transfer contents of vial to 10ml Maintenance Media in a T75 flask (do not pellet cells at this point, viability will be reduced). Change media the following day to remove DMSO.

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## **B. MYOBLAST RECIPES**

### **Transport Media**

Muscle tissue should be placed in this at biopsy for transport to laboratory. If necessary tissue may be sent to laboratory by FedEx priority overnight with cold packs (blue ice not dry ice).

F-10 Nutrient Media    Gibco 11550-043  
10% FBS                Gibco 26140-079  
2% Pen/Strep        Gibco 1514-122

#### **Collagenase 4**

Worthington Chemical (800-445-9603), CLS-4, 100mg lyophilized, resuspended in 25ml PBS, filtered through 0.2um filter and aliquoted. Store -20°C.

#### **Dispase II**

Boehringer Mannheim/Roche #295 825, aliquot and store at -20°C.

#### **Primary culture Media (to establish line)**

50% Maintenance media without bFGF, (below)

50% MRC-5 conditioned F10 media.

Then just before use, add,

10ng/ml bFGF (4ul per 10ml)

Dexamethazone (10ul per 10ml)

MRC-5 conditioned media is prepared by overlaying a 70% confluent MRC-5 culture with 10ml Maintenance media (without bFGF or Dexamethazone) and incubating overnight. The media is pooled, filtered through a 0.45um filter and aliquoted into 5ml batches. It is stored at -20°C until use.

#### **Maintenance media**

F-10 Nutrient Media Gibco 11550-043

20% FBS Gibco 26140-079

1% Pen/Strep Gibco 1514-122

10ng/ml bFGF Promega G5071 or Atlanta Biologicals 15140-122.

Mix F10, FBS and Pen/Strep, filter through 0.22um filter (Corning 430767). Store 4°C.

Add bFGF at 4ul per 10ml as used.

Add Dexamethazone 10ul per 10ml as used.

#### **Trypsin**

Use at 0.1% in PBS.

Stock is 0.25%, Gibco 25200-056.

-wash with PBS

-add 1 ml of 0.1% trypsin, incubate 37°C 1-5 mins (usually closer to 1 min)

-gently tap to remove cell sheet

-add 10 ml of media to flask

-split this 10 ml between required # of flasks, (should be 10ml media total in each flask)

#### **Freezing Medium**

90% FBS

10% DMSO

#### **Plastics**

T75 flasks, Corning 430725

Cell strainer 100um nylon, BD Falcon 352360.

### **C. DESMIN STAINING**

Set up culture in mini dish (35 x 10 mm) corning 430165, cell culture grade.  
Allow to grow to 50-80% confluency. Note 1 T75 flask is equivalent to 5 of these plates.

Rinse 1x in PBS

Fix in 2% PFA for 1 min.

Wash 5x with PBS, 2 mins each wash.

Fix with 2% chilled acetone/PBS for 5 mins RT (pre-chill 15 mins at -20C)

Rinse 2x in PBS. Store under PBS at 4C for up to 2 days, (sodium azide can be used to store longer).

Incubate in primary antibody 1-2 hours at RT. Desmin antibody (sigma D1033, raised in mouse) is used at 1:400 in 0.5% BSA/PBS. Overlay 200-400ul into centre of dish and cover with a coverslip (cut plastic 60mm coverslips into squares). Do not press coverslip down.

Wash 3x in PBS.

Incubate in secondary antibody, 30 mins RT. Add DAPI at 1:20,000 (1:20 of Matt's working stock) to the secondary. Secondary will be anti mouse, with a fluorescent tag. After addition of secondary antibody, all subsequent steps should be carried out under dimmed lights. Cover with foil for incubations.

Rinse 3x in PBS

Aspirate PBS, add 2 drops of gel mount, cover with coverslip and aspirate around the outside of the coverslip to dry the remainder of the plate. Allow to dry in a dark place at RT overnight.

Cut side wall off plate and tape onto a microscope slide.

Store at -20C in the dark.

**REFERENCE:** Pavlath G.K. Isolation, purification and growth of human skeletal muscle cells. In: Methods in Molecular Medicine : Human Cell Culture Protocols. G. E. Jones, Editor, Humana Press, Totowa, NJ, p 307-317.

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