Preparing Primary Cultures from Muscle Biopsy Specimens II

This is the present protocol, it has been used since 6/05, with the exception of the F/M culture (point 17).

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 50ul of 0.2M $CaCl_2$.
9. Incubate in 37^{o}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 90 mins in a T75 flask. This will allow contaminating fibroblasts to adhere, but myoblasts will not.
17. Transfer medium to a fresh T75 flask, label this **M**, add 10ml fibroblast media to the first flask, label this **F/M** (fibroblasts from muscle) to differentiate it from fibroblasts isolated from skin.
18. On the 3rd or 4th 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 10 days 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 1st time after one week. (if small foci have not yet developed leave up to 10 days before changing media, but continue to supplement with 4ul bFGF every 3-4 days). 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 detatch within a couple of minutes. Gently tap side of flask and check under microscope that cells are detatched. 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 detatched 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 1st 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 **10**ml Maintenance Media in a T75 flask (do not pellet cells at this point, viabiltity will be reduced). Change media the following day to remove DMSO.

Media Recipies and Culture Supplies

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 15070-063

Collagenase 4
Worthington Chemical (800-445-9603), CLSS-4, 100mg filtered and lyophilized, resuspended in 25ml sterile PBS, no need to filter as it is already sterile and aliquoted. Store –20°C

Dispase II
Dispase II / Roche 04 942 078 001 5 x 1g use at 2.4units/ml
Provided at 0.99u/mg i.e. 990u/1 gram vial
Dilute 1g to 100ml PBS = 10mg/ml (9.9u/ml), dilute again 10ml to 41.25ml = (2.4u/ml),
Store -20°C in small aliquots, store 4°C once thawed for 1-2 weeks

Calcium Chloride
Prepare 0.2M solution and filter through 0.22um filter, Store -20°C in small aliquots, store 4°C once thawed.

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 of a 25ug/ml sterile stock per 10ml of media)
1uM Dexamethazone (10ul of our 1mM stock per 10ml media).

MRC-5 conditioned media
This 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.

MRC-5 culture media
DMEM low glucose Gibco 11885-084
10% FBS Gibco 26140-079
1% Pen/Strep Gibco 15070-063

Maintenance media
F-10 Nutrient Media Gibco 11550-043
20% FBS Gibco 26140-079
1% Pen/Strep Gibco 15070-063
10ng/ml bFGF Promega G5071
1uM Dexamethazone

Mix F10, FBS and Pen/Strep, filter through 0.22um filter (Corning 430767). Store 4^0C.
Add bFGF; 4ul of a 25ug/ml sterile stock per 10ml media just before use.
Add Dexamethazone; 10ul of a 1mM sterile stock per 10ml media just before use

bFGF stock (25ng/ul)
add 1ml sterile PBS to 25ug vial, dispense into 50ul aliquots, store frozen -20C. Once thawed store in refrigerator at 4^0C.

Dexamethazone stock (1mM)

Dexamethazone , sodium phosphate for injection, supplied as 1ml vials 10mg/ml of dexamethazone phosphate (8.31mg/ml dexamethazone base), preservative free, 500601, NDC 63323-506-01. American Pharmaceutical Industries

Vial is 10mg/ml of which 8.31mg is Dexamethazone base.
Take 472ul from vial and dilute into sterile PBS to give a total of 10ml = 0.392ug/ul
Store as 500ul aliquots, -20C. Once thawed store in refrigerator 4^0C
Add 10ul of this to each 10ml of media = 3.92ug/10ml = 1uM final conc in media

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 37^0C 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 T75 flask)

Freezing Medium

90% FBS
10% DMSO

Plastics

T75 flasks, Corning 430725
Cell strainer 100um nylon, BD Falcon 352360.

JES 4/14 University of Rochester, Dept Neurology
janet_sowden@urmc.rochester.edu