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Primary Human Fibroblast Cell Culture

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1 Works for me dx.doi.org/10.17504/protocols.io.427gyhn

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ABSTRACT

Growing and maintaining primary human skin fibroblast cell cultures.

Additional links for protocols can be found:

- https://www.coriell.org/0/PDF/Fibroblast_Culture_FAQ.pdf
- https://www.ncbi.nlm.nih.gov/pubmed/23852182

GUIDELINES

Follow biosafety level 2 guidelines handling human tissue.

MATERIALS

NAME	CATALOG #	VENDOR
1.5ML Microcentrifuge Tube	BT620-NS	Bio Basic Inc.
EZ-LINE Serological Pipettes, 5ml stripette, Sterile, indiv wrapped, 250 per case	CR4113.SIZE.1CS	Bio Basic Inc.
DMSO	D8418	Sigma
Falcon® Serological Pipettes, 10 mL 200 Pipettes	38004	Stemcell Technologies
0.1% Gelatin in Water 500 mL	7903	Stemcell Technologies
Corning® Cryogenic Vials with Orange Caps, 2 mL 500 Vials	38053	Stemcell Technologies
FBS		Invitrogen - Thermo Fisher
Trypsin		Thermo Fisher Scientific
PBS		Invitrogen - Thermo Fisher
Trypan Blue Solution 0.4% (w/v) in PBS pH 7.5 ± 0.5	25-900-CI	Corning
AmnioMAX™ C-100 Basal Medium	17001074	Thermo Fisher Scientific
Remel™ 70% Ethanol	R2470110	Thermo Fisher Scientific
Nunc™ EasYFlask™ Cell Culture Flasks T25 Solid	156340	Thermo Fisher Scientific
Falcon™ 15mL Conical Centrifuge Tubes	14-959-53A	Fisher Scientific
Thermo Scientific™ Molecular BioProducts™ SoftFit-L Reload™ System: Sterile Tips	21-402-556	Fisher Scientific
Fisherbrand™ Aspirating Pipets	14-955-135	Fisher Scientific
Corning LX CoolCell Freezing System for Cryogenic Vials green 12 exposed vials; 1/ea	UX-04392-02	Cole Parmer
Fungizone (Amphotericin B)	15290018	Thermo Fisher Scientific
CHANG Medium C Frozen Supplement-14mL	C106	Fujifilm Wako Pure Chemical

MATERIALS TEXT

Reagents

Medium, AmnioMax C100, kit
0.1% Gelatin in Water, autoclaved
Trypan Blue
Ethanol 70%
PBS
FBS
20% DMSO
.25% Trypsin
Fungizone (Amphotericin B)
CHANG Medium C Frozen Supplement-14mL

Equipment

Labconco Biosafety Cabinet
Benchtop Centrifuge
Nanotek EVE Automated Cell Counter
37 °C Water bath
P1000 Rainin Pipet-Lite™
P20 Rainin Pipet-Lite™
-80 °C freezer
Liquid Nitrogen tank

Materials

EVE Cell Counting Slide
Falcon™ 15mL Conical Centrifuge Tubes
50 mL Fisherbrand™ Sterile Polystyrene Disposable Serological Pipet
25 mL Fisherbrand™ Sterile Polystyrene Disposable Serological Pipet
10 mL Fisherbrand™ Sterile Polystyrene Disposable Serological Pipet
5 mL Fisherbrand™ Sterile Polystyrene Disposable Serological Pipet
Fisherbrand™ T25 flask
Fisherbrand™ Premium Microcentrifuge Tubes: 1.5mL
Thermo Scientific™ Molecular BioProducts™ SoftFit-L Reload™ System: Sterile Tips
2 mL Fisherbrand™ Sterile Polystyrene Disposable Aspiration Pipet
2ml Cryogenic Vials with Orange caps
CoolCell Freezing System for Cryogenic Vials

SAFETY WARNINGS

Use appropriate safety meassures when working with nitrogen tank and **-80 °C** freezer such as cryogenic gloves, full face visor and lab coats. Abide by biosafety level 2 standard safety procedures as human materials are used.

BEFORE STARTING

Turn on biosafety cabinet 10-15 min before start and clean all working surfaces with 70% ethanol. Make sure all materials in the cabinet are sterile and utilize standard aseptic technique.

Note: For plating density of human fibroblasts use 1.0-1.4 x 10^4 cells/cm^2.

Concepts and terms in Culturing Aging Fibroblasts
These include cumulative population doublings (CPD), senescence and senesced. CPD refers to the number of times that the cell number has been doubled. It has been used to measure the total number of cell divisions and it can be affected by several biological factors including the maximum lifespan of the species, age of the donor, the site of the biopsy, and the culture conditions. If cultures fail to reach confluency in 1 week, the culture is termed as “senescence”. Cultures are considered to be “senescent” (at the end of their replicative lifespan) when they are unable to complete one population doubling during a 4-week period that includes 3 consecutive weeks of re-feeding with fresh medium containing 10% FBS. Reference: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3873382/

Preparing the Media

1 Thaw 15 ml Chang C supplement and wipe bottle with 70% alcohol before placing in a biosafety cabinet.

2 Take the AmnioMax Basal Media and the Fungizone and wipe both bottles with 70% alcohol before placing in a biosafety cabinet.

3 Using a 50 ml pipette, aliquot 90 ml and using a 25 ml pipette, add the 15 ml Chang C supplement to the media.

4 Add 100 µl of Fungizone to the media.

5 Wrap the media container in foil to protect in the light and place in 4 °C until ready for use. Complete media can be stored at 4 °C for one month.

Preparing for Culturing

6 Store cryovials in liquid nitrogen storage tank upon arrival. Place vials in dry ice when ready to work with cells (do not thaw immediately).

7 Record the label of the vial (ID, date frozen, any other documentation) and document in inventory.

8 Prepare culture dish to thaw fibroblasts by adding 2 ml of 0.1% gelatin (autoclaved) to a culture dish and incubate at room temperature for 00:20:00 .

Note: For plating density of human fibroblasts use 1.0-1.4 x 10^4 cells/cm^2, e.g. for T25 flask plate 2.5 x 10^5 – 3.5 x 10^5. If cells are plated too sparse the grow slows down and cells go into senescence.

9 Warm up AmnioMax media in water bath, wipe bottle with 70% alcohol before placing in a biosafety cabinet. Aliquot 9 ml of the AmnioMax media into a 15 ml conical tube.

10 Aspirate gelatin and add 3 ml of AmnioMax media to the culture dish.

Culturing Cells

11 Thaw the cells by holding the lower half of the vial in 37 °C water bath and carefully swirling the vial around until only a small amount of ice is left in the vial. Do not let cells thaw completely. Wipe vial with 70% alcohol, before placing in biosafety cabinet.

12 Carefully remove the cap of vial and gently mix by triturating using a P1000 micropipette to resuspend the cells without causing foam.

13 Using a p20 micropipette, remove 10 µl from the vial for cell counting and add to a 1.5 ml microcentrifuge tube or onto a piece of

parafilm.

14 Using a p1000 micropipette, take **400 µl** of media from the 15 ml conical tube and add to the vial with cells drop by drop. Note: depending on the size of the croyvial adjust the volume. Standard cryovials hold 1.5-2.0 ml.

15 Using a p1000 micropipette, aspirate solution from cryovial and slowly add to prepared conical tube with **9 ml** Amniomax (Step 4) in a drop by drop fashion.

16 Centrifuge the 15 ml conical tube containing the cells and the medium at **1000 rpm** for **00:05:00** . Note: always balance centrifuge.

Cell counting

17 Take the 1.5 ml microcentrifuge tube or drop on parafilm from step 6 and add **10 µl** of Trypan Blue and gently mix by pipetting up and down.

18 Take **10 µl** of the solution and fill cell counting slide.

19 Insert the cell counting slide into the cell counter and count the cells. Record the number of cells and viability and then dispose of the cell counting slide.

Based on cell counts and viability adjust the culture vessel size to plate 1.0-1.4x10^4 of viable cells per cm^2.

Culturing Cells

20 Take the 15 ml conical tube from the centrifuge and aspirate Amniomax media until there is only a little left above the cell pellet.

21 Add **1 ml** of Amniomax media and gently triturate to resuspend the cell pellet.

22 Add 1ml cell solution from step 16 to the culture dish and gentle rock the culture dish back and forth to evenly distribute the cell over entire surface. Place flask in **37 °C**, 5% CO2 humidified incubator.

Maintaining Cell Culture

23 20 - 30% confluency should be reached 24 hours after the cells have been plated (the following day) 24hrs after plating. Figure Legend: Example of a human fibroblast cell culture

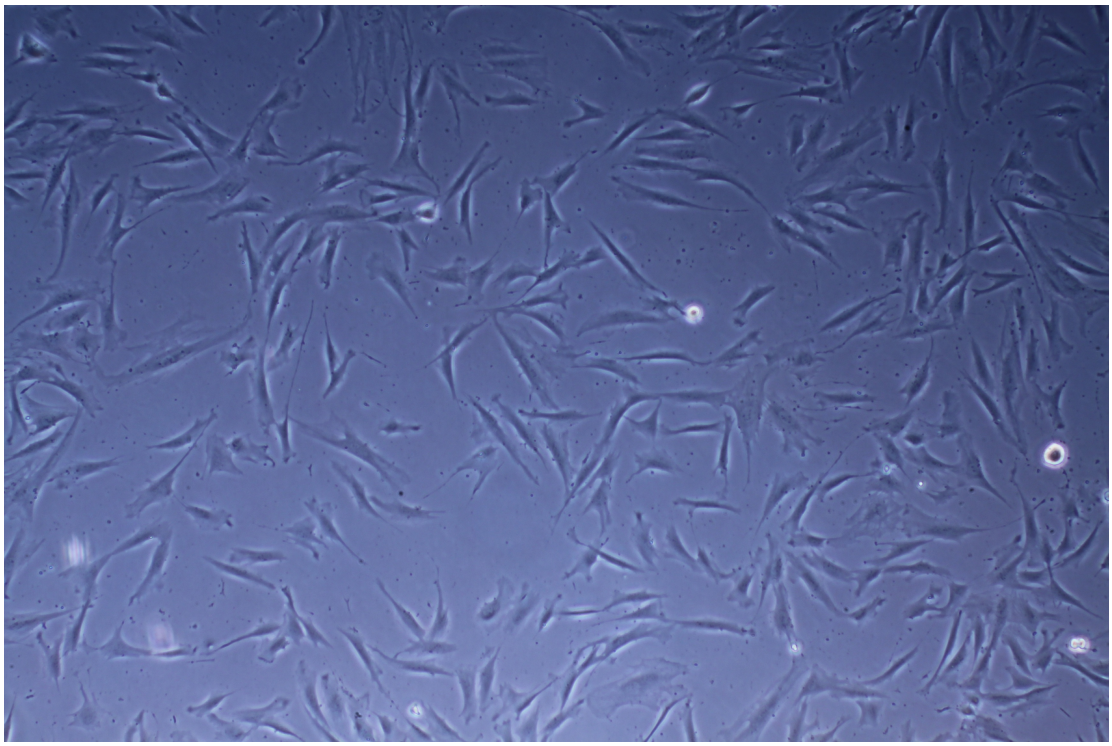

Figure Legend: Example of a human fibroblast cell culture 24hrs after plating 1.2x10^4 cells per cm^2.

24 Aspirate the media the cells are in and then add new media (the media should be warmed up and the bottles should be wiped down with 70% alcohol before use).

25 The whole media should be changed every 2 days until 90-100% confluency is reached, at which point the cells should be passaged.

Preparation for Passaging Cells

26 Add **4 ml** of 0.1% gelatin (autoclaved) to culture dishes (x2 T25) and incubate at room temperature for **00:20:00** . Note: For plating density of human fibroblasts use 1.0-1.4 x 10^4 cells/cm^2, e.g. for T25 flask plate 2.5 x 10^5 – 3.5 x 10^5. If cells are plated too sparse the grow slows down and cells go into senescence.

27 Warm up AmnioMax media in water bath, wipe bottle with 70% alcohol before placing in a biosafety cabinet.

28 Aspirate gelatin and add **3 ml** of AmnioMax media to the culture dish.

Passaging the Cells

29 Remove Fibroblasts from incubator, place culture vessel in biosafety cabinet and aspirate media.

30 Add **2 ml** of PBS using 5ml serological pipette, swirl culture vessel, aspirate and discard PBS.

31 Add **2 ml** of 0.25% Trypsin and incubate for **00:05:00** at **37 °C** and 5% CO2. Firmly adherent cells can be detached quickly at 37°C. Observe the cells under microscope. The detached cells appear rounded under microscope. Tap the culture vessel gently to detach all cells.

Note: Prevent cell exposure to trypsin solution for longer periods (>=10 min)

32 Add **4 ml** of media to neutralize the trypsin. Note: add 2 volumes of pre-warmed complete growth media (containing serum) to inactivate trypsin.

33 Using a pipette, completely mix cells with no clumps, transfer the cell suspension to a 15 ml conical centrifuge tube.

34 For cell counting, remove **10 µl** of solution from the tube using a P20 micropipette and add to a 1.5 ml microcentrifuge tube, see steps under "Cell Counting".

35 Centrifuge the cells in the 15 ml conical tube for **00:05:00** at **1000 rpm**.

36 Aspirate the supernatant until there is only a little left above the cell pellet.

37 Resuspend the cell pellet in media by gently triturating.

38 Remove 2.5 x 10^5 – 3.5 x 10^5 cells using a pipette (the amount of solution to pipette out is based on the number of cells which was determined by counting the cells) and add to each of the gelatin coated T25 flasks. Note: For plating density of human fibroblasts use 1.0-1.4 x 10^4 cells/cm^2, e.g. for T25 flask plate 2.5 x 10^5 – 3.5 x 10^5. If cells are plated too sparse the grow slows down and cells go into senescence.

39 Place cells in incubator at **37 °C** and 5% CO2 and follow protocol for "Maintaining Cell Culture."

Cryofreezing Cells

40 If there are cells left in the 15 ml conical centrifuge tube that need to be cryofrozen, place the tube in the centrifuge and centrifuge for **00:05:00** at **1000 rpm**.

41 Aspirate any supernatant until there is only a little left above the cell pellet and resuspend the pellet by tapping the side of the tube.

42 Add **500 µl** of pre-warmed complete growth media (containing serum) to the conical tube with the cells using a P1000 micropipette and gently triturate.

43 Add **500 µl** of 20% DMSO (diluted in complete growth media) to the conical tube with the cells and gently triturate with a P1000 micropipette.

44 Using the pipette, transfer the cells to a Cryogenic Vial and label the vial. Note: If the time it will take between filling the vials and placing them in **-80 °C** will be quite long, keep the vials in ice as they are being filled and transfer them to the freezing container once you are at the **-80 °C** freezer.

45 Place the Cryogenic Vial in a Cryogenic Vial freezing system container and place in -80 °C. Note: Make sure all of the holes in the freezing conatiner are filled so that the temperature spreads evenly.

46 Transfer the vials from the -80 °C to a Liquid Nitrogen tank no earlier than 24:00:00 after putting them in -80 °C.

Results

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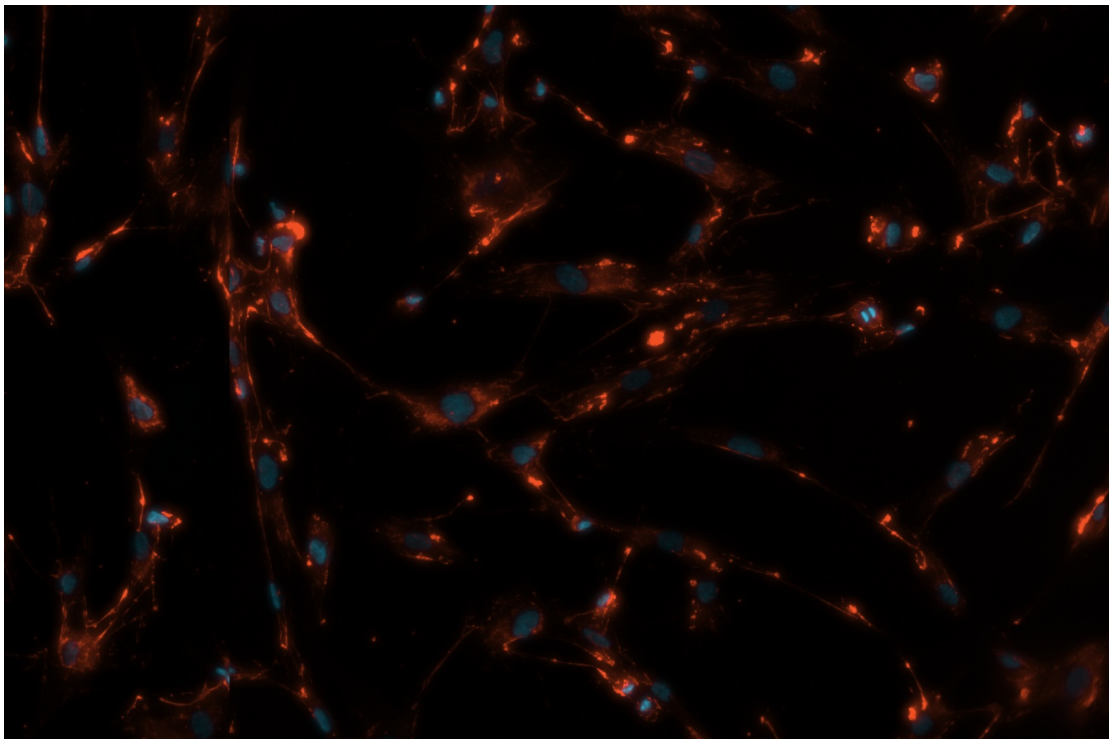

Figure Legend: Fibroblasts stained with Fibronectin EP5 (555) by Santa Cruz Biotechnology (sc-8422) and Hoechst.