Mammalian Cell Culturing

Protocols:

AML-12, CHO-DG44, and NIH3T3

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This guide was developed for culturing and handling two commonly used mammalian cell lines, AML-12 and CHO-DG44, for use in synthetic biology and biotechnology. It is our intention that this guide may help future iGEM teams when working with mammalian cell lines.

-Team Cenozoic (2018)

Building upon the precedents set by team Cenozoic, we have updated and expanded the pre-existing protocols to include NIH3T3 cells. We hope our updates and clarifications will encourage future experimentation across mammalian cell lines within the iGEM community.

-Team Zorya (2019)

i. Table of Contents

I. Background

AML-12 cells are an immortal, epithelial cell line derived from mouse (*Mus musculus)* liver cells while NIH-3T3 cells are derived from mouse (*Mus musculus*) embryonic fibroblasts. CHO-DG44 is derived from chinese hamster ovarian cells (*Cricetulus griseus*), and is widely used in the pharmaceutical industry for production of therapeutic polypeptides. We chose to work with these strains in the context of iGEM because they are all BSL1 organisms, and accurately model most mammalian systems.

CHO cells are an immortal, epithelial cell line. CHO-DG44 is also commonly referred to as CHO-DHFR, and is deficient for dihydrofolate reductase, an enzyme required to synthesize Proline. Because of this, CHO-DG44 cells require glycine, hypoxanthine and thymidine (GHT) for growth. The two basic media formulations are IMDM and DMEM, each containing 30.00 mg/L of glycine.

NIH3T3 cells are an immortal, fibroblast cell line. Derived from NIH Swiss Mouse cells and named for the abbreviation of "**3** day **T**ransfer, inoculum of **3**x10^5 cells" in a 20cm^2 dish, the original experiments all followed this guideline. (Transfer or passage cells every 3 days with a starting dilution of 3x10^5 cells each time)

All three varieties (AML-12, CHO-DG44, and NIH-3T3) are adherent cell lines. The doubling time of CHO-DG44 is 20-24 hours, the doubling time of AML-12 is 37 hours, and the doubling time of NIH3T3 is 20-26 hours.

The following protocols for handling, growing, freezing, and quantifying cell viability/confluence are largely the same for all three cell lines, with the exception that they each require specialized media formulations.

II. Materials

Cell lines

- CHO-DG44 cells
- AML12 cells
- NIH3T3 cells

*Note: see appendix for further details on all media formulations

CHO-DG44 Complete Media

- Dulbecco's Modified Eagle Medium (DMEM)
- Fetal Bovine Serum (FBS)
- Penicillin/streptomycin
- Hypoxanthine
- Thymidine

AML12 Complete Media

- Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12, HEPES (DMEM/F12)
- Fetal Bovine Serum (FBS)
- Penicillin/streptomycin
- Insulin-Transferrin-Selenium (ITS-G)
- Dexamethasone (Dex)

NIH3T3 Complete Media

- Dulbecco's Modified Eagle Medium (DMEM)
- Fetal Bovine Serum (FBS)
- Penicillin/streptomycin

Phosphate-buffered Saline (PBS)

- NaCl
- KCl
- Na_2HPO_4
- KH_2PO_4

Dimethyl Sulfoxide (DMSO)

Trypsin (0.53 mM EDTA solution)

Trypan Blue

III. Safety

- Lab safety is the number one priority at all times.
- Before handling mammalian cells, be aware that there are additional safety regulations that must be met, compared to work with commonly used, exempt strains of bacteria such as DH5a. Many universities offer specialized safety training sessions and certifications. Ask your PI/advisors or your university's department of environmental health and safety about relevant safety certifications required for mammalian cell culturing and biotechnology. Likewise, obtain a biological use authorization (BUA) for any cell lines you will be using.
- Make sure to use appropriate PPE; i.e., full length pants, closed toed shoes, lab coat, nitrile gloves, and eye protection.
- Exercise caution when using liquid nitrogen. Use a full face mask and cryo-gloves when handling frozen vials. Vials may leak when submerged in liquid nitrogen and may slowly

fill with liquid nitrogen -- when thawed, conversion of liquid nitrogen to gas phase may result in vial exploding or blowing its cap off.

- Trypan blue is toxic and is a potential carcinogen; read Trypan Blue MSDS before use.
- DMSO is a combustible liquid, and is able to facilitate entry of organic molecules into tissues; read DMSO MSDS before use.
- **Biohazard Waste:** any items that have come into contact with cells must be disposed of in a biohazard waste receptacle (red bag in covered bin)
    - Sharps: should be disposed of in sharps biohazard containers
    - Decontaminate liquid waste using bleach. Add bleach to the liquid waste to a minimum final concentration of 20% volume. Allow the solution to sit for 30 minutes, and then dispose of down the drain.
- Ethanol solutions are flammable - keep away from ignition sources. Work with small volumes (less than 500 mL) of flammable liquids.

Trainings

*** Before you proceed, complete the following relevant safety trainings as well as any other certifications your university may require: ***

1. Safety fundamentals training
2. Laboratory safety training
3. Site specific safety training
4. Safe use of biological safety cabinets
5. Proper handling of materials at biosafety level 1
6. Biological use authorization for AML-12, CHO-DG44, and NIH3T3 cell lines

IV. Reagent Preparations

*Note: *Percentages are of the total volume, not the dilution of each solution**

AML-12 Complete Media Reagents (560 mL final volume)

- DMEM/F12 - 500 mL
- Penicillin/streptomycin (1%)- 5 mL
- FBS (10%)- 50 mL
- ITS (Insulin-Transferrin-Selenium) 100X (1X)- 5 mL
    - 5mL of 100X for final concentration of 1X
- Dexamethasone (40 ng/mL)- 10 uL

CHO-DG44 Complete Media Reagents (560 mL final volume)

- DMEM- 500 mL
- FBS (10%)- 50 mL
- Penicillin/streptomycin (1%)- 5 mL
- HT (hypoxanthine/thymidine) 100X (.1 mM/.016 mM)- 5 mL

NIH-3T3 Complete Media Reagents (555 mL final volume)

- DMEM- 500 mL
- FBS (10%)- 50 mL
- Penicillin/streptomycin (1%)- 5 mL

PBS Reagents (1 L final volume)

- NaCl (137 mM)- 8 g
- KCl (2.7 mM)- .2 g
- Na_2HPO_4 (10 mM)- 1.44 g
- KH_2PO_4 (1.8 mM)- .24 g

* FBS should be thawed overnight at 4°C. If not possible then thaw FBS at room temperature for 10 minutes, then finish thawing in 37°C water bath

* All filter sterilization was done using a 0.22 micron vacuum filter manifold

Prepare Stock Solution(s)

Dexamethasone (2mg/mL)

1. Dissolve 10 mg of Dex in 5 mL of ethanol(2mg/mL) to make Dex stock solution.
2. Filter sterilize using 0.22 micron filter and aliquot 50 uL samples
3. store in a -20C freezer

Hypoxanthine/Thymidine (HT 100X)

1. Dissolve 136 mg of hypoxanthine and 38 mg of thymidine in 100 mL of molecular biology grade water to make 100x stock solution of 10mM hypoxanthine and 1.6 mM thymidine.
2. Filter sterilize and dispense 5 mL aliquots into sterile tubes
3. store at -20C

FBS

1. Thaw FBS overnight at 4°C, if not possible then thaw FBS at room temperature for 10 minutes, then finish thawing in 37°C water bath
2. After initial overnight thaw, make 50 mL aliquots of FBS and refreeze unused aliquots. (Making aliquots keeps thawing/refreezing to a minimum and increases overall serum stability)

PBS

1. Fill 1 L media bottle with 800 mL of DI water
2. Weigh out then pour in all reagents. Mix thoroughly
3. Measure pH of solution and adjust pH to 7.4 with HCl
4. Add DI water to a final volume of 1 L.
5. Autoclave for 20 min at 15 psi or filter sterilize. Store at room temp

Prepare Complete Media

1. **If you are not planning to use the media right after, skip this step** ~20min before starting, put all the reagent bottles in the water bath at 37°C (If water bath is low, add DI water and wait for it to normalize at 37°C)
2. Turn on the UV light within the biosafety cabinet for ~5min then switch to fluorescent
3. Spray down all visible surfaces within the biosafety cabinet with 70% ethanol, including the glass window. Wipe down with a clean kimwipe.
4. Set up cabinet with all required tools, remember to spray everything that is going into the hood as it enters and again as it leaves with 70% ethanol
5. Make sure the sterile vacuum system (we used a 500ml Stericup-HV Sterile Vacuum Filtration System) is set up and ready to go. The two tubes must be attached to the white plastic connector under the biosafety cabinet. A clear pipe should be connected to a waste disposal bottle on one end.
6. Label the Millipore Filtration System with the type of media, what it contains, date of formation, and initials of the person that made it.
7. Transfer all the solutions in the correct volumes directly into a freshly opened DMEM bottle, gently swirl to homogenize.
8. Turn on the vacuum valve behind the hood. Connect the vacuum tube to the opening on the side of the 500 mL Millipore Filtration System and slowly pour the solution into the top compartment.
9. When all remaining solution has drained through the filter, disconnect the vacuum and discard the top portion of the container.
10. Securely screw on the provided lid. Be sure to spray bottle down with ethanol when removing from the biosafety cabinet. Store finished media at 2-8°C until use.

Freezing Media (10ml total volume)

Reagents: Complete Media (95%) - 9.5 mL + DMSO (5%) - 0.5 mL

1. Pipette complete media and DMSO* into a 15ml conical tube and filter sterilize the solution
2. Store at 2-8°C until use

**If DMSO is solid, it can be warmed to room temp to thaw it*

V. Thawing and Seeding

Notes before you begin:

- Thawing is stressful to cells, carefully follow protocol but work quickly to maximize cell survival
- Seeding densities: ratio of cells to media in a culture, different values for each
    - Vials that cells came in may have recommended dilution on them already
    - Thermo Fisher recommends general value is $2.1x10^6$ cells for T75 flask

Seeding Densities: Quick Check Reference Chart

Cell line	Preferred seeding concentration	For T75 flask ($75cm^2$)	For 24 well-plate ($1.9cm^2$ per well)
CHO-DG44	$2x10^4$ cells/cm^2	$1.5x10^6$ cells in 14ml media total	$0.5x10^5$ cells in 0.5-1ml media per well
AML12	$2x10^4$ cells/cm^2	$1.5x10^6$ cells in 14ml media total	$0.5x10^5$ cells in 0.5-1ml media per well
NIH3T3	$1.5x10^4$ cells/cm^2	$1x10^6$ cells in 14ml media total	$0.5x10^5$ cells in 0.5-1ml media per well

For more information and seeding densities in different plates/flasks, see Thermo Fisher

*24 well plate seeding densities were determined experimentally, and do not correspond with the normally recommended scaling proportions

Materials

- Cells
- Complete medium
- 15 ml conical tubes
- T75 flasks (culture vessels)
- Centrifuge

Prep Work

- Have water bath filled and set to 37°C
- Warm complete media in bath (for at least 15 min)
- Sterilize all surfaces and equipment with 70% ethanol, including the biosafety cabinet surfaces and any required tools before they go in

Protocol

1. Warm complete media in a 37°C water bath for 20 minutes prior to beginning of experiment. Spray with 70% ethanol before transferring to biosafety cabinet

2. In the biosafety cabinet, pipette 9 mL of complete media into a sterile 15 mL conical tube. Leave in biosafety cabinet while you warm cells
3. Remove frozen cells from dewar and thaw cell vial by gently swirling in a water bath at 37°C until there is almost no ice left, it should be fast and take less than 2 minutes. It is also possible to thaw in hand

    Note: Keep cap out water to prevent contamination and open carefully as pressure may have built while in the dewar and blow the cap off
4. Remove from water bath and decontaminate by dipping in or spraying the vial with 70% ethanol. Transfer to biosafety cabinet
5. Pipette total contents of vial slowly into the sterile 15 mL conical tube containing 9 mL of medium (recommended volume by ATCC)
6. Centrifuge tube for 10 minutes at 125 × g. Use Thermo Scientific Legend XTR or equivalent centrifuge
7. Carefully aspirate the supernatant, and resuspend the cells in 2 or 3 mL of complete growth medium. Ensure that you get a homogenous suspension by pipetting gently up and down

    **Media volume does not drastically influence results, but note volume selected for future counting and subculturing calculations*
8. If you need to count cells or measure viability then stop here and refer to the counting section, if not then continue
9. Aliquot cell suspension into T75 flasks to meet seeding density recommendations (total volume 15ml cells + media) and mix thoroughly by gently rocking. Make sure to add the media into the flask first, then aliquot the cell suspension in.

    * Use 15 mL total volume rather than 14 for seeding- more media helps dilute the remaining DMSO from freezing process, increasing initial stability
    * See table above for optimal seeding densities based on cell line
    * Check out our online density calculator to speed up the process

VI. Cell Maintenance

Notes before you begin:

- Cells should be maintained between 10%-90% confluency in a 37°C, 5% CO_2 incubator
- This typically will require passaging the culture around 3 times a week, approx every 3 days
    - Flasks that are around 80-90% confluency will typically have 2-3 million cells in a flask, CHO flasks tend to yield more cells than AML12 due to the AML12 cells covering more surface area per cell
- Media should be renewed every 2-3 days (recommended by ATCC)
    - You may need to wash cells with PBS prior to feeding cells (this would be to remove debris/dead cells, one might expect a large amount after thawing) -- described in protocol below
- Color pH indicator in media (phenol red) will provide information about the need to

change the media (metabolites released into media from cell growth will change pH and media will become more yellow over time)

Changing the Media

Materials

- Cells in culture vessel (ie. T75)
- Complete growth medium
- PBS

Prep Work

- Have water bath set to 37°C
- Warm media and PBS in bath to 37°C for approx 20 minutes prior to beginning
- Label flasks beforehand

Protocol

1. Check cell morphology and confluency using a microscope (see culture notes below for additional signs of contamination)
    a. **Morphology checks** include making sure there is no deterioration (i.e. granularity around the nucleus, detachment of cells, and cytoplasmic vacuolation)
    * *CHO and AML12 are epithelial and will flatten quite a bit when fully attached*
    b. **Media color-** we want the media to be pinkish and not yellowish. If the pinkish color is fading, it means that you need to change media ASAP, AML12 media starts off a little more yellow than the CHO media
2. Tilt flask so that liquid is at a corner where there are no adhered cells (towards lid of container rather than bottom) and aspirate media
3. If including a wash step, add 5ml of PBS followed by gently rocking back and forth, then aspirate using the same method as before
4. Pipette 14 mL of media into flask
5. Place the flask back in the incubator

Visually Inspecting Culture Health Using a Microscope

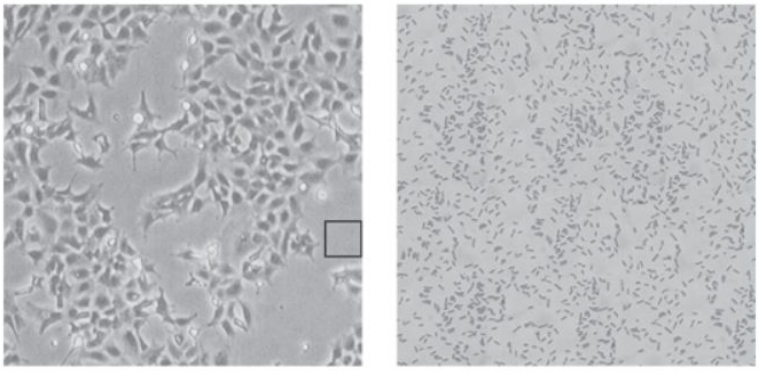
How often should I check on my culture? At least once a day, possibly more if it is getting close to reaching confluency

What is the goal of checking on my culture? Determine overall health, lack of contamination, track growth rate, and determine confluency

Check for Signs of Contamination

Media Indicator: Contamination will commonly change the pH of the media solution. For this reason, most media contains phenol red, a pH sensitive indicator dye. Presence of contamination can be inferred by a dramatic color change of the solution. (Pink to yellow)

1. **Bacterial Contamination:** small shimmering black dots within spaces between cells
   - Usually appear cloudy/turbid with a thin film on the surface
   - Drop in pH of medium, correlates with media color change (pink to yellow)



(A) Low power microscope image of bacterially infected adherent cell line, appear as small moving granules between cells
(B) Under high power microscope zoom, individual E. coli are visible

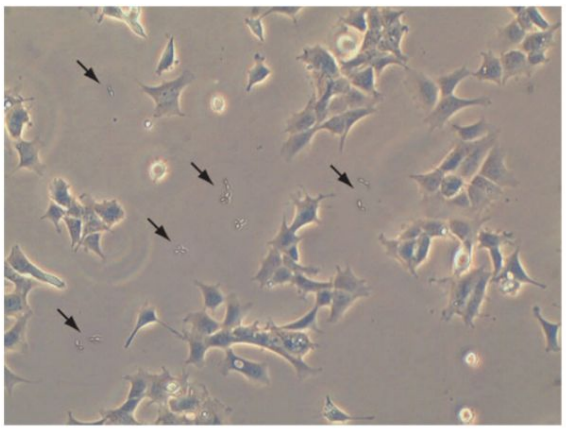
Thermo Fisher scientific Guide

2. **Yeast Contamination:** Yeast appear as small (approx 3-4 micrometer, but occasionally reaching 40 micrometers at largest) ovoid or circular units, spouting off smaller progeny as they divide

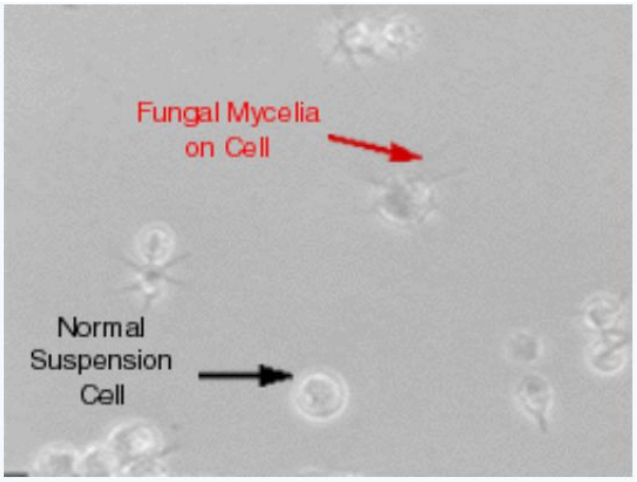
- pH of media will normally remain unchanged until contamination becomes severe

Thermo Fisher Scientific Guide

Image of adherent cell line 24hrs after yeast contamination >



3. **Fungal Contamination:** Usually in the form of thin, wisp-like, filamentous mycelia growing out from the culture



- pH will remain relatively stable, similar to yeast contamination, followed by rapid increase when contamination becomes severe

- Spores are durable and can survive harsh environments in a dormant stage until the conditions become more favorable

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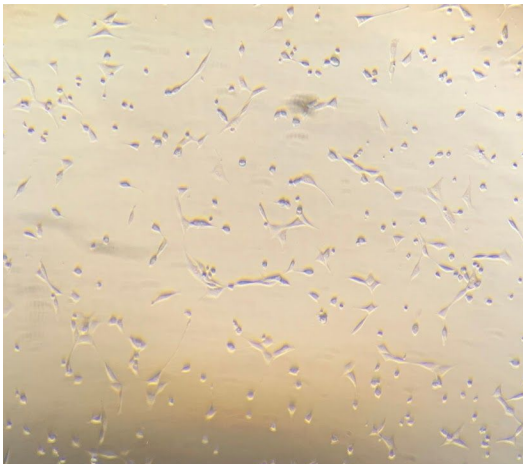
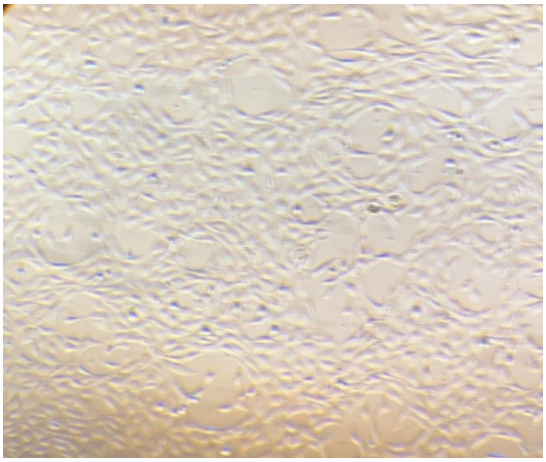
Check for Confluency

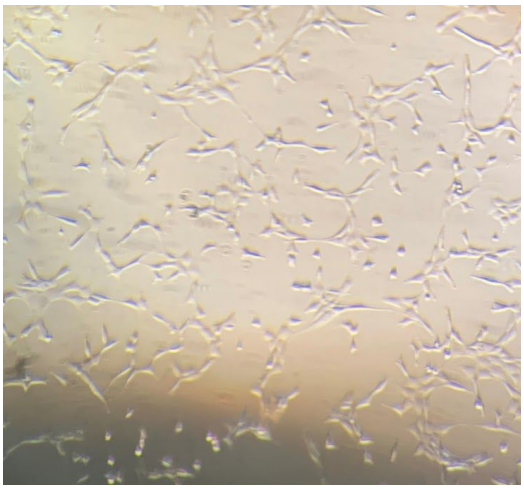
Confluency: a measure of cell density, in the form of a % (surface area cells/total SA)



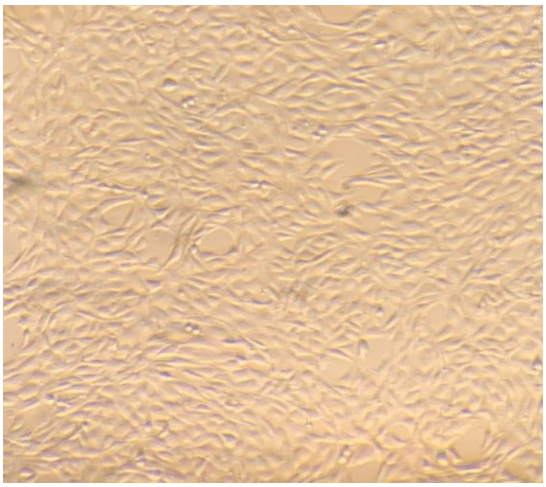
~10% confluent NIH3T3 sample



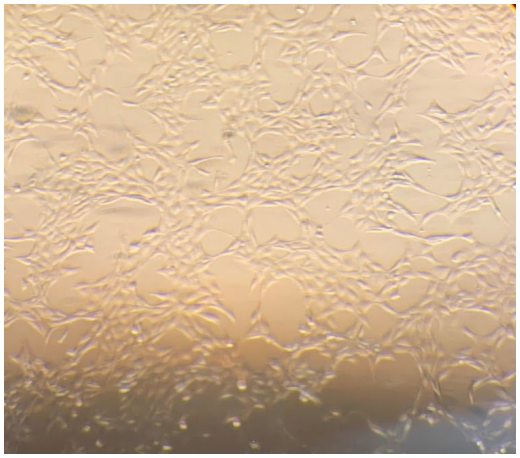
~90% confluent NIH3T3 sample



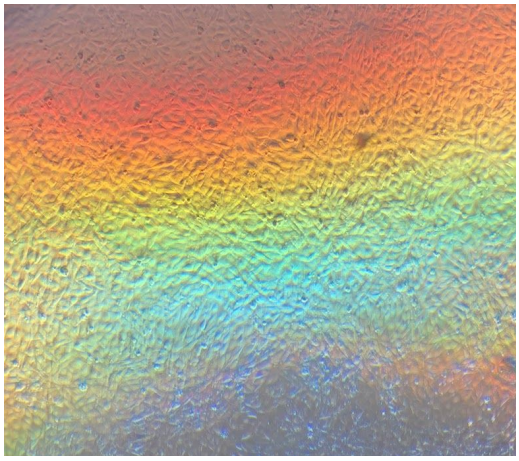
~50% confluent NIH3T3 sample



Over confluent

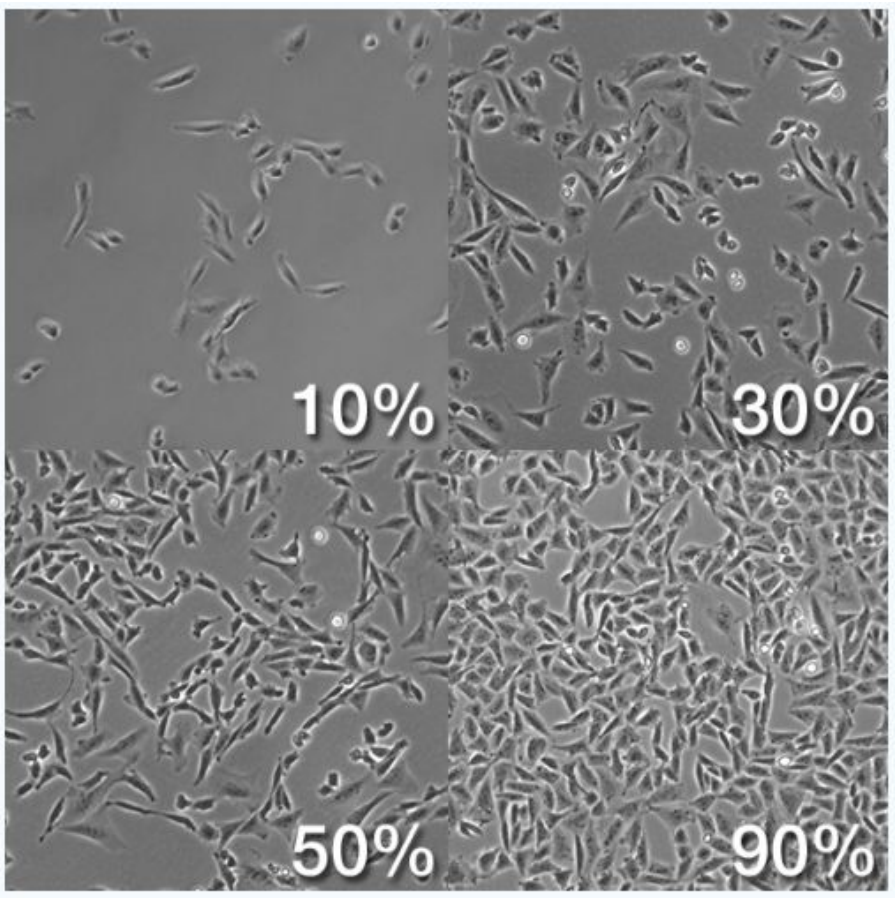


~80% confluent NIH3T3 sample



severely over confluent NIH3T3 sample

*all above images were taken in our own lab of the same flask of NIH3T3 cells over the course of experimentation



Creative Bioarray primary cell culture guide - fibroblast confluency reference and further info

VII. Passaging/Subculturing

Notes before you begin:

- Passage number: # times cells have been subcultured (i.e., harvested/reseeded into daughter flasks)
    - When cells are trypsinized and then thawed/reseeded, this represents one passage
- Population doubling (PD) = approximate number of doublings the cell population has undergone since isolation

Subcultivation ratio for AML12	Subcultivation ratio for CHO-DG44	Subcultivation ratio for NIH-3T3
1:4 to 1:6 (from ATCC)	1:4 to 1:8 (from ATCC)	3 to $5x10^3$ cells/cm^2

*Subculture cells around 70-80% confluency, aim for 80%

Materials

- Cells in culture vessel
- Trypsin
- New culture vessel
- Cell counting materials

Prep Work

- Have water bath set to 37°C
- Warm media and Trypsin in bath to 37°C
- Label flasks beforehand

T75 passaging Protocol

1. Check cell morphology and confluency using a microscope (80% confluency is desired), see above microscopy guide
2. Aspirate old media from a corner of flask not in contact with the cells adhered to the bottom surface
3. Briefly rinse the cell layer with 5 mL of PBS solution. Gently rock back and forth to remove all traces of serum that contains trypsin inhibitor
4. Aspirate PBS using same technique as step 2
5. Add 5 mL of Trypsin-EDTA solution and place in incubator for 4-6 minutes to allow all cells to separate from the bottom of the flask.
6. Observe cells under an inverted microscope until cell layer is dispersed (usually within a few minutes)
    a. **For CHO-DG44 and AML-12:** To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal. If stubborn, a gentle isolated tapping with your finger can be used.
    b. **For NIH3T3:** Cells tend to form large clumps of cells when Trypsinized. Hold flask firmly in one hand and hit the side of the T75 4-5 times with the palm of your opposite hand to separate large clumps

Image 1: (left) NIH3T3 cells 4 minutes post Trypsin

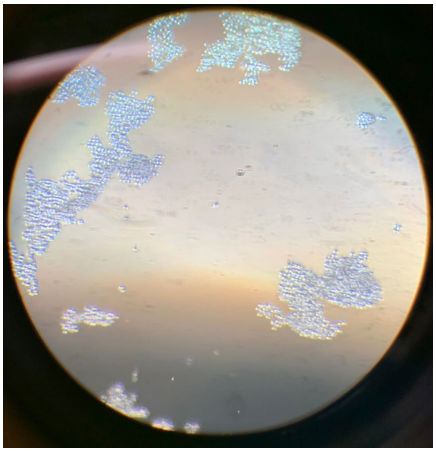
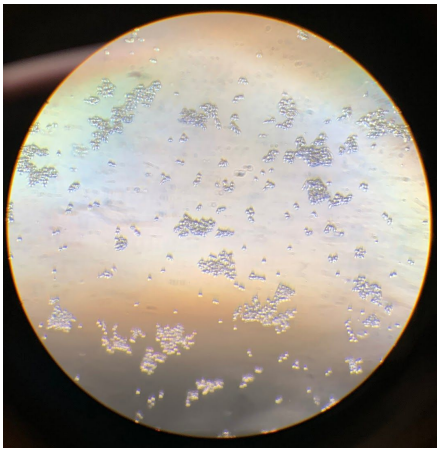


Image 2: (right) NIH3T3 cells post tapping



7. Add 5 mL of complete growth medium (add 0.5mL media per well for 24 well plate)
8. Pipette cells to a 15 mL conical tube and spin down at 125 x g for 5 minutes accel 7
9. Aspirate media, being careful not to disturb the pellet. It is better to leave a couple microliters of media than to accidentally aspirate the cells.
10. Resuspend pellet in 3ml of media via slow and gentle pipetting to create homogenous solution, then add the rest of the media- volume dependent on if counting step is desired (in which case transfer 10uL of cells to microcentrifuge tube and see “counting cells” protocol below) and desired split ratio, see our dilution calculator.
11. Add appropriate aliquots of fresh media and the cell suspension to new labeled culture vessels
12. Incubate cultures at 37°C

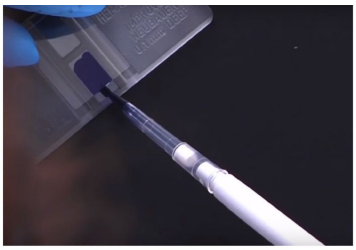
VIII. Counting Cells

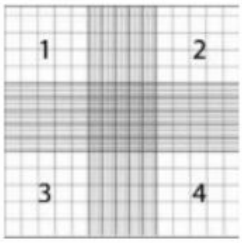
Materials

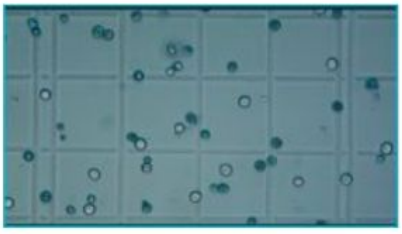
- Hemocytometer
- Trypan Blue

Protocol

1. Transfer 10uL of evenly homogenized cells into a 1.5 mL microcentrifuge tube
2. Add 10 uL of 1X Trypan blue, mix by pipetting up and down gently.
3. Incubate 2-5 min before measuring in hemocytometer to give cells time to digest the Trypan blue.
4. Prepare and assemble hemocytometer
    a. Clean hemocytometer and glass coverslip with ethanol and thoroughly dry with Kimwipes. A trick for assembling the coverslip is to dampen the ridges of the coverslip mounting structures with ethanol and then put the coverslip on, making sure there is resistance when trying to slide it across the ridges
5. Transfer 10 uL of cell suspension + trypan blue mixture to the edge of the counting chamber. Allow the cell suspension to be drawn into the counting chamber by capillary action.
    a. Some people load the sample directly in the middle of the slit -- alternatively, you can aim the pipette at the edge of where the coverslip meets the surface just to the right of the slit (even though it looks like the sample won’t really go inside of the chamber, capillary action will indeed pull that liquid inward)







6. Place the hemocytometer under an inverted microscope (be sure to clean the microscope stage with an ethanol dampened kimwipe before you begin) and view cells at 100x magnification
7. Using a hand counter/clicker tool to keep track, record the number of cells in each of the four sections (non-stained = alive, blue-stained = dead; count both separately)
    a. You should see the above grid on the hemocytometer consisting of four isolated squares (1,2,3,and 4). Choose two edges of the square to count -- if cells are on the line (e.g., if there are a few cells on the borders of the square, count the cells on the bottom/right edge but not any that are on the line of the top/left edge)
    b. If one of the four squares had drastically different numbers than the other three, you can count the center square as well to increase overall accuracy
8. Record the number of cells in each section and calculate total cell count (see our concentration calculator) or if by hand:
    a. Total # of cells in resuspended volume = Average # of cells/square * Volume Resuspended * Trypan Blue Dilution Factor * Hemocytometer Factor
        i. Hemocytometer factor = 10,000 (*based on each square having an area of 0.1mm^2 and depth being 0.1mm*)
        ii. Dilution factor = 2 (If using 10 uL cell suspension and 10 uL trypan blue (1:1) ratio)
9. Cell viability is calculated as the number of unstained or viable cells divided by the total number of cells, expressed as a percentage
    a. For best results, adjust the concentration of the suspension so that 50-100 cells are in each section
10. Clean hemocytometer with alcohol after use. Dry using Kimwipes. Paper towels should not be used, as they will scratch the glass. Disassemble, put away parts.

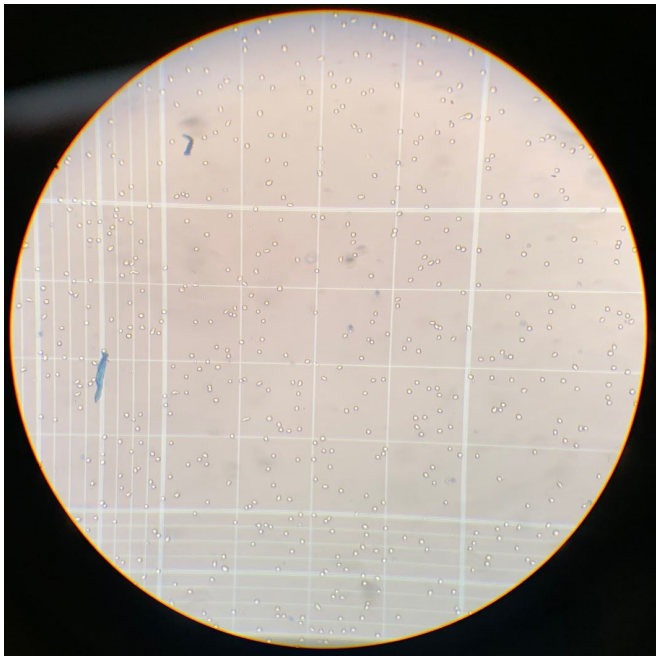


Image of counting high confluency NIH3T3 cells in quadrant 2

IX. Freezing Cells

Notes before you begin

- As soon as a small surplus of cells become available, they should be frozen as seed stock
- Ideally, you will freeze cells at a high concentration but low passage number
- Cells should be 75% viable but aim for 90% viable prior to freezing
    - Able to see this through cell counting
- Cells should be frozen slowly -- ~1°C per minute using a controlled-rate cryo-freezer or a cryo-freezing container (Styrofoam) -- this is to minimize formation of intracellular ice, which can damage or kill cells (water can escape by osmosis instead)
- DMSO is a cryoprotective agent -- cells won't shrink too much/lose viability
    - Store DMSO in aliquots, protected from light
- Cells should be frozen at approximately $1x10^6$ cells/vial

Materials

- Cells
- Cryovials
- Dewar
- Complete growth media with 5% DMSO
- Controlled-rate freezing apparatus
- Cell counting material

Prep Work

- Warm media, PBS and 10% FBS to 37°C in water bath
- Prepare Freezing Medium (see stock solutions), store at 2-8°C until use:
    - Complete growth medium + 5% (v/v) DMSO
- Pre-cool insulated container that will be used to put in the -80

Protocol

1. Prepare freezing media (stock solutions) and pre-cool styrofoam tube container in -80C
2. Follow Passaging protocol steps 1-10
3. Pipette desired amount of cells into new conical tube
    a. Depending on how many cells are left, they can be put into a new culture vessel or bleached
4. Spin down at 125 x g for 5-10 minutes
5. Aspirate media, careful not to disturb pellet
6. Resuspend pellet in 1ml freezing media (via slow and gentle pipetting to create homogenous solution) then add the rest of the freezing media to desired ratio
    a. Cells should be at a density of $1x10^6$ cells/mL
7. Aliquot into labeled cryovials (1 mL per vial)
8. Allow cells to equilibrate in the freezing medium at room temp for a minimum of 15 minutes but no longer than 40 minutes
9. Place vials in a pre-cooled insulated container for slow cooling, and store overnight at –80°C for at least 24 hours
10. Transfer to dewar
11. After 24 hours at –130°C, remove one vial, restore the cells in culture medium and determine their viability/sterility through counting

X. Appendix

Notes on Media formulation

CHO DG-44 Media (also known as CHO-DHFR)

Reagent	Final Concentration	Purpose	Further Reading
DMEM (with and without phenol red)	--	Food, nutrients, pH indicator	Animal cell culture media: History, characteristics, and current issues
FBS	10%	Growth factors, nutrients	Culture conditions and types of growth media for mammalian cells
Penicillin/ streptomycin	1% (100 units/mL pen., 100ug/mL strep)	Prevent bacterial contamination	Together, Penicillin and Streptomycin function against both gram-positive and gram-negative bacterial contaminants. Penicillin does so by targeting the cell wall, and Streptomycin by targeting the 30S ribosomal subunit and inhibiting translation and protein synthesis. (More at Thermo Fisher)
hypoxanthine	0.1mM	Cell line is deficient for this nutrient	Hypoxanthine-ATCC
thymidine	0.016mM	Cell line is deficient for this nutrient	Thymidine-ATCC

*** Media temp and pH: 37°C, 5% CO_2**

- IMDM should be used at 5% CO2
- DMEM should ideally be used at 10% CO2 but almost everybody in the field uses 5% CO2. It means that the pH will be 7.6 instead of 7.2-7.4, and does not appear to have a significant effect on cell growth

AML-12 Media

Reagent	Final Concentration	Purpose
DMEM-F12 media (with and without Phenol red)	--	DMEM/F-12 is a 1:1 mix of Dulbecco's modified Eagle medium and Ham's F-12 medium. Major feedstock source in the culture contains amino acids, salts, glucose, and vitamins. DMEM is a modified version of Eagle's Minimal Essential Media, with a higher concentration of vitamins, amino acids, and glucose, as well as phenol red and iron. The phenol red is used to indicate pH- it should normally be pink when culturing mammalian cells, and if it turns yellow, the solution has become acidic, usually due to bacterial contamination. We can also order DMEM without phenol red, as it will interfere when measuring green fluorescence.
Fetal bovine serum	10%	DMEM/F12 contains no proteins, lipids, or growth factors. Therefore, it needs to be supplemented with 10% FBS. Fetal bovine serum is commonly used in culturing eukaryotic cells. It contains growth factors and a large amount of Bovine Serum Albumin (BSA) which is a small and stable, mainly unreactive protein.
insulin	10 µg/ml	Used to stimulate growth and proliferation of cultured cells. IT is also a component of serum-free media formulations used for most primary-cells and cell lines (for long term growth). In addition to stimulation of cell growth, classical insulin responses such as increased fatty acid and glycogen synthesis are seen in serum-free medium. Insulin initiates it activity by binding to glycoprotein receptors on the surface of the cell. Binding to the receptor eventually results in insulin's action on glucose, lipid, and protein metabolism. In hepatocytes, insulin limits autophagy that occurs after isolation.

transferrin	5.5 µg/ml	Transferrins facilitate extracellular iron storage and transport, and play the role of important extracellular antioxidants. They bind iron so tightly under physiological conditions that virtually no free iron exists to catalyze the production of free radicals. The delivery of iron to cells by transferrins is a receptor mediated and controlled process. Cells regulate the amount of iron they receive from the extracellular environment by varying transferrin receptor expression.
selenium	5 ng/ml	Selenium is an essential trace element for normal cell growth and development in vivo and in vitro. It is incorporated into enzymes that protect cells by reducing peroxides, organic hydroperoxides, and peroxynitrites to non-harmful species. Seleno-enzymes with various antioxidant functions, and different substrate specificities are localized inside, on and outside the cell, and together these enzymes provide a comprehensive range of defenses against oxidative damage.
dexamethasone	40 ng/ml	Promotes the differentiation of mature hepatocytes from mouse and human embryonic stem (ES) cells. Promotes the maturation of fetal mouse hepatocytes.

*** Media temp and pH: 37°C, 5% CO_2**

- DMEM/F12 uses a sodium bicarbonate buffer system and therefore requires a 5%-10% CO2 environment to maintain physiological pH.

NIH3T3 Media

Reagent	Final Concentration	Purpose	Further Reading
DMEM (with and without phenol red)	--	Food, nutrients, pH indicator	Animal cell culture media: History, characteristics, and current issues
FBS	10%	Growth factors, nutrients	Culture conditions and types of growth media for mammalian cells
Penicillin/ streptomycin	1% (100 units/mL pen., 100ug/mL strep)	Prevent bacterial contamination	Together, Penicillin and Streptomycin function against both gram-positive and gram-negative bacterial contaminants. Penicillin does so by targeting the cell wall, and Streptomycin by targeting the 30S ribosomal subunit and inhibiting translation and protein synthesis. (More at Thermo Fisher)

*** Media temp and pH: 37°C, 5% CO_2**

- DMEM should ideally be used at 10% CO2 but almost everybody in the field uses 5% CO2. It means that the pH will be 7.6 instead of 7.2-7.4, and does not appear to have a significant effect on cell growth

Notes on Storage of media

- CHO-DG44 Complete Media may be stored for 3 to 4 weeks at 2º to 8ºC before use
  - Dulbecco's Modified Eagle Medium (DMEM)-may be stored at 2º to 8ºC
  - Fetal Bovine Serum (FBS)- 10% store at -20ºC in aliquots
  - Penicillin/streptomycin- 1% (100U/mL)(may be stored at -5°C to -20°C)
  - Hypoxanthine(0.1 mM) - stock solutions may be stored at -20°C for up to a year
  - Thymidine(0.016 mM)- stock solutions may be stored at -20°C for up to a year. See protocol here

- AML-12 Complete Media may be stored for 3 to 4 weeks at 2º to 8ºC before use
  - Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12, HEPES (DMEM/F12)-may be stored at 2º to 8ºC
  - Fetal Bovine Serum (FBS)- 10% store at -20ºC in aliquots
  - Penicillin/streptomycin- 1% (100U/mL)(may be stored at -5°C to -20°C)
  - Insulin-Transferrin-Selenium (ITS-G)(10 µg/mL- 5.5 µg/mL5 - 5 ng/mL (may be stored at 2º to 8ºC)

    - Dexamethasone (40 ng/mL)
        - "Dexamethasone solutions are stable for at least 30 days when stored at 4°C and protected from light. The solution may be aliquoted and stored at -20°C." (Sigma).
        - powder should be stored at 2-8 °C; stock should be frozen in working aliquots, avoid repeated freeze/thaw cycles
    - Dimethyl Sulfoxide (DMSO)-may be stored at 2° to 8°C
    - Trypsin- 0.53 mM EDTA solution
    - Trypan Blue

- NIH-3T3 Complete Media may be stored for 3 to 4 weeks at 2º to 8ºC before use
    - Dulbecco's Modified Eagle Medium (DMEM)-may be stored at 2º to 8ºC
    - Fetal Bovine Serum (FBS)- 10% store at -20ºC in aliquots
    - Penicillin/streptomycin- 1% (100U/mL)-may be stored at -5°C to -20°C

XI. References

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