Framingham Heart Study

Manual of Procedures

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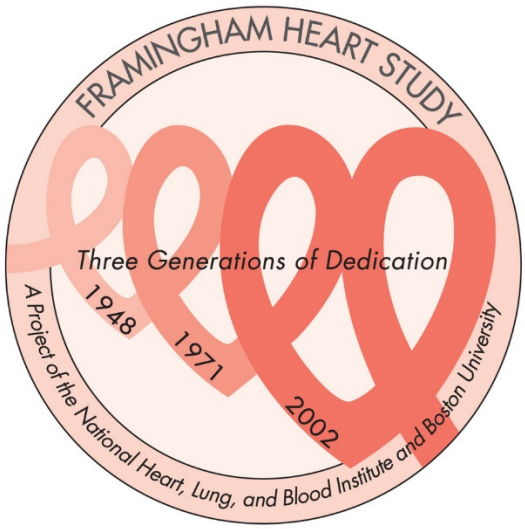
Cell Culture



Tracking of Revisions to this FHS Protocol MOP

Revised Section	Revision Author	Date (s) of Revisions; source	Approved by, Date	Revisions	Previous Pages #s section changed	Distribution Date

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Cell Culture Procedures

1.0 Culturing Lymphoblast Cell Lines

Background

Once established, lymphoblast cell lines (LCLs) are robust, fast growing cell lines. They grow in suspension and healthy cultures form cell clumps as they multiply. LCLs require a 37^{o} C incubator with 5% CO_2. There are two different commercially available mediums that will support lymphoblast cell line growth. One is RPMI 1640 and the other is Iscove's Modified Dulbecco's Medium (IMDM). Using either medium, one can quickly gauge culture status through unaided visual inspection by noting the color change of the medium. Freshly prepared IMDM contains a phenol red indicator making the medium cherry red. As a cell culture metabolizes media components, the pH changes and the media will turn orange which is a positive indicator of healthy cell growth. As media components continue to be depleted, the color will change to yellow. Standard complete media preparation to support lymphoblast cell growth is IMDM supplemented with 10% Fetal Bovine Serum (50mL), 1%, 10,000 units each penicillin/streptomycin/L-Glutamine solution (5mL). Once a culture has been successfully transitioned from a t-25 into a t-75 the culture can be maintained in a 10% Horse Serum medium. Complete media preparations are quality control tested by incubation at 37^{o}C for 24 hours.

Healthy lymphoblast cultures are fed 2-3 times a week by adding media. Generally, the volume of the culture should be doubled with each feeding until the maximum capacity of the growth vessel is reached. Healthy LCLs grow in clumps, these cultures grow better when maintained at higher cell densities. When judging how much to feed a culture, always err on the side of caution and under feed rather than overfeed. Cultures with cell suspensions that become too dilute tend to die out. Visual indicators as described above allow a trained eye to accurately assess the health of a culture and approximate cell density. Probably 90% of lymphoblast cell lines grow at similar rates and will quickly pass into larger capacity growth vessels until the desired cell count is attained. The other 10% of these cell cultures will grow at a slower rate and will require small feedings less frequently to keep them healthy.

It is assumed that all culture manipulations take place in a Biological Safety Cabinet (BSC). Aseptic technique is employed and all consumables used are sterile.

1.1. Initiating a LCL culture

Equipment and Consumables

- Table-top swing bucket centrifuge with 750 mL capacity.
- Rotor and adapters to accommodate 15 and 50 mL conical centrifuge tubes.
- 37^{0} C Water bath
- 50 ml polypropylene conical centrifuge tubes

- 15 ml polypropylene conical centrifuge tubes
- 10 ml pipets
- 5 ml pipets
- 1 ml pipets
- 20% FBS RPMI 1640 or IMDM Complete Media
- Cryovial rack
- Waste container
- 70% ethanol spray bottle
- Labels & tracking sheet
- Rubber bands

1. Label all labware with barcode labels containing the Cell Culture number (CC#). Set up labeled labware in order of CC#. Warm media in incubator or 37º water bath ahead of time.

2. Pipet 9 mL of warmed media into a 15 ml tube for each cell line to be cultured.

3. Vials of cryogenically preserved cell lines retrieved from storage are immediately immersed in a 37^0 C water bath and are kept in constant motion to hasten the thawing process. Thawing six vials at a time is manageable.

4. As soon as the vials have thawed, spray the vials down with 70% ETOH and wipe dry. Match the cellid on the cryovial to the cellid on the 15 mL tube and promptly transfer the contents of each vial using a 1 ml pipette to a prepared 15 mL centrifuge tube (see #2).

5. Using a tabletop swing bucket centrifuge, pellet the cells @1000 rpm for 10 minutes. Discard the supernatant into a waste bottle.

6. Re-suspend the cell pellet in 2-3 ml (depending on pellet size) in 20% FBS RPMI 1640 medium and inoculate into a t-25 cell culture flask.

7. Visually inspect the cultures the next day for growth. Unaided visual inspection should observe the formation of clumps and mild color changes in the media. Microscopic inspection should find spherically formed cell clumps with individual cells around the periphery of the clump to be round and clear and the center of the clump looking dense and dark.

***Some cultures may show signs of little or no growth. Upon visual inspection a poorly performing culture may show no color change of the media, no cell clumping, cloudy or hazy appearance which usually indicates contamination (mold, bacteria or fungus). Under microscopic inspection there will be an abundance of dead or bursting cells. At the slightest hint of any of these, bring to the attention of the Lab Supervisor to discuss options.

1.2. Proliferation of Cell Lines

What you'll need:

T-25 & T-75 flasks
Warmed enriched & regular RPMI media
Filters
Rubber bands
10 ml pipettes
25 ml pipettes
Labels & tracking sheet
70% Ethanol spray bottle

1. When feeding your cultures, the media ALWAYS has to be warmed to 37^0 C. Filter media before feeding to avoid contamination. When new bottles are made up, they must be kept in the incubator for AT LEAST 48 hours to make sure there is no contamination present so plan accordingly.

2. Cell lines are maintained in a t-25 up to a maximum volume of ~15 ml. When a 15 ml saturated cell suspension has been achieved, ~13 ml of the culture is transferred to a t-75 cell culture flask and 10 ml of fresh media is added. The original t-25 is then fed 2 ml, labeled and maintained as a backup for the cell line. Backups are kept in a separate incubator chamber from the now larger primary cultures and are maintained in case of future failure of larger culture. When feeding T-25 samples, always use a pipette.

3. Healthy, saturated cultures are fed with fresh media when the color nears a vibrant yellow (the feeding times vary from culture to culture but a healthy and robust cell line should take approximately 1 month from thaw to extraction). When healthy growth is apparent in the t-25 the switch can be made to Regular Growth Media** that is supplemented with 10% Horse Serum rather than FBS. Horse Serum supports growth of lymphoblast cell lines and is, in orders of magnitude, less expensive to buy.

4. Cultures in a t-75 can be fed up to a maximum volume of ~125 ml and the flask is laid down on its back at ~50 ml to maximize the surface area for CO_2 exchange through the depth of medium in the flask. When a ~75-100 ml saturated cell suspension has been achieved, the entire culture is ready for extraction. When feeding T-75 samples, always use a pipette.

*Enriched RPMI 1640 Growth Medium:
20% Heat Inactivated FBS (100 ml)
1% Pen/Strep/L-Glutamine Solution (5 ml)

**Regular Growth Media:
10% Horse or Bovine Serum (50 ml)
1% Pen/Strep/L-Glutamine Solution (5 ml)

Heat inactivation of serum: heat at 56º C for 30 minutes.

2.0 Freezing Lymphoblast Cell Lines

Cryopreserving Lymphoblast Cell Lines (LCLs)

2.1. Introduction

In order to maintain the inventory of cryopreserved LCLs, new aliquots are cryopreserved as need when a line is cultured. These are referred to as refreezes because they are not the original aliquots sent to FHS from the contracting lab.

The volume of cell suspension required will vary depending upon the number of refreezes that need be done. If 3 vials need to be refrozen, the cell culture volume to be harvested is 35-40 mL. The minimum volume of saturated cell suspension required to cryopreserve a 1 mL aliquot is 10 mL. To ensure an adequate cell density per refreeze vial the harvesting volume is slightly over the minimum.

The freeze medium MUST BE COLD before you start this process. The DMSO in the freeze medium allows the cells to survive in liquid nitrogen storage, but kills the cells at room temperature. DMSO can be dangerous and absorbs through the skin. Wear appropriate PPE while working with this solution. Once the samples are aliquoted into cryovials, immediately place them in a chilled storage box.

2.2. Supplies and Equipment

DMSO
50 ml conical centrifuge tubes
Pipets
Waste container
Ice and ice bucket
Beaker
10% FBS RPMI 1640 complete media
Cryogenic freezer vials
Storage box with 9x9 divider
Labels
Cell culture worksheet
Tabletop centrifuge

Protocol

1. Prepare a 10% DMSO freeze media solution using previously prepared 10% FBS RPMI 1640 complete media. Make enough to use 1 mL of freeze media per vial to be frozen. Store in a 50 mL centrifuge tube. KEEP ON ICE IN A BEAKER AS YOU WORK.

2. Place prepared barcoded labels on the cryovials and 50 mL tubes in order of LN2 freezer position and loosen the cap of the cryovials.

3. Line the cell culture flasks to be harvested and labeled 50 mL tubes in order of freezer position. Gently shake the flask and transfer the needed volume of cell suspension (10 mL per vial to freeze) into the 50 mL tube and centrifuge at 1000 rpms for 10 minutes to pellet the cells.

4. A large cell pellet should be visible. Promptly pour off supernatant into waster container.

5. Re-suspend the pellet in the appropriate volume of cold freeze media (i.e. 3 refreezes require 3 mL of freeze media) by pipetting up and down to break up the cell pellet, avoiding air bubbles. Aliquot 1 mL cell suspension per cryovial. Immediately put on ice to be transferred to LN2 storage.

Always record the date of a refreeze on the cell culture worksheet. This worksheet must be accurately maintained as it is the source of the permanent electronic file.

3.0 Liquid Nitrogen Freezer Storage Formats

LN2 Freezer LCL Inventory Formats

FHS participant lymphoblast cell lines (LCLs) were created by 2 different labs; one at Mass General Hospital (MGH) and one at the University Minnesota (aka: Fairview). Each lab labels the vials differently and cryopreserves a different number of vials for storage. Thus, the storage format used the cell lines differently and are therefore inventoried in FHS LN2 freezers in different formats depending on the source lab.

Fairview returned 6-1 mL vials. Each vial is labeled, in text and barcode, with the cellid, specid, and freeze date. MGH returned a total of 8-1 mL vials, usually 4 “A” vials and 4 “B” vials. Each vial from MGH is labeled with a handwritten “MIN” number (the MGH ID system) and freeze date. Half of the vials from each source are stored in LN2 freezers located on BUMC in L402 (primary location). The other half are stored in LN2 freezers at our offsite biorepository, Fisher BioServices, in Franklin, MA (backup location). Primary and back up inventory formats are the same. The lab uses 9x9 or 81 position boxes and an alpha-numeric position identification.

3.1. Fairview cell line inventory format

Maximum number of vials per cell line is 6. Three primary vials and 3 back-up vials.
Three positions per cell line are assigned by row.
The starting positions are always A1, A4, and A7, etc. for each row.
When pulling a vial to thaw, always take from the last position available. For example, if a cell line occupies positions B1, B2, and B3, take the vial from the B3 position.

If there are 2 vials left in inventory (primary or back up), no need to refreeze stock. If one vial is left after a sample is pulled, 2 vials will need to be refrozen to replenish stock (R2); if no vials are left, 3 vials will need to be frozen down its an R3.
If none are there at all, back up inventory will need to be accessed. Note the culture as an R3 and write “Franklin” in the comments section of the work list.

A1 * *	A4 * *	A7 * *
B1 * *	B4 * *	B7 * *
C1 * *	C4 * *	C7 * *
D1 * *	D4 * *	D7 * *
E1 * *	E4 * *	E7 * *
F1 * *	F4 * *	F7 * *
G1 * *	G4 * *	G7 * *
H1 * *	H4 * *	H7 * *
I1 * *	I4 * *	I7 * *

The freezer racks that hold inventory from Fairview are:
2, 6, 7, 8, 10, 12, 19-current rack

3.2. MGH cell line inventory format

Maximum number of vials per cell line is 8. Four primary vials and 4 back-up vials.
Two different cell lines are inventoried per ROW and 2 different cell lines are placed down the center of the box.
The starting positions are always A1 and A6, etc. for each row.
The starting positions are always M1 & M2 in the middle column at top and bottom.

When pulling a vial to thaw, always take from the last space available.
If there are 3 vials left after pulling, it's an R0, if there are 2 it's an R2, if there is 1 it's an R3 and if there are none, it's an R6 and it must be noted as "Franklin" in the comments section of the work list.

A1 * * *	M1	A6 * * *
B1 * * *	*	B6 * * *
C1 * * *	*	C6 * * *
D1 * * *	*	D6 * * *
E1 * * *		E6 * * *
F1 * * *	M2	F6 * * *
G1 * * *	*	G6 * * *
H1 * * *	*	H6 * * *
I1 * * *	*	I6 * * *

The freezer racks that hold inventory from MGH are:
1, 3, 4, 5, 9, 11, 13, 14, 15, 16, 17, 18

4.0 Routine Cleaning in the Tissue Culture Room

4.1. Daily

- ALWAYS spray down the BioSafety Cabinets (BSC) with 70% ethanol inside and out before starting to work and the work has been completed. Wipe up spills immediately as they occur while executing protocols, the spray the area with 70% ethanol.
- Keep aisles clear and supplies well organized. This will make ordering much easier.
- Always discard liquid waste down the sink with copious amount of water after adding bleach to the waste and letting sit overnight.
- Wipe down all benches with 70% ethanol at the end of the day.
- Turn on the BSC UV lights at the end of the day.

4.2. Weekly

- Clean and change the water in the pans in the incubators. Use purified water and add water conditioner. (2ml/L)
- Clean and change the water in the water baths. Use DI water and add water conditioner.
- Scrape any ice build-up in the LN2 freezers.

4.3. Monthly

- Clean the incubator chambers.
  - -Remove all cultures and place in another chamber.
  - -Turn the power off to the chamber to be cleaned.
  - -Remove the shelves, sides, top.
  - -Place these removable parts in an autoclave bag and autoclave.
  - -Wipe down the entire interior surface with 70% Ethanol. Be sure to wipe the rubber gasket around the door and the rubber gasket in the top left corner.
  - -Leave door open to air out ~10 minutes.
  - -Replace autoclaved parts.
  - -Turn power back on and allow it to equilibrate.
  - -Return cultures and document cleaning.