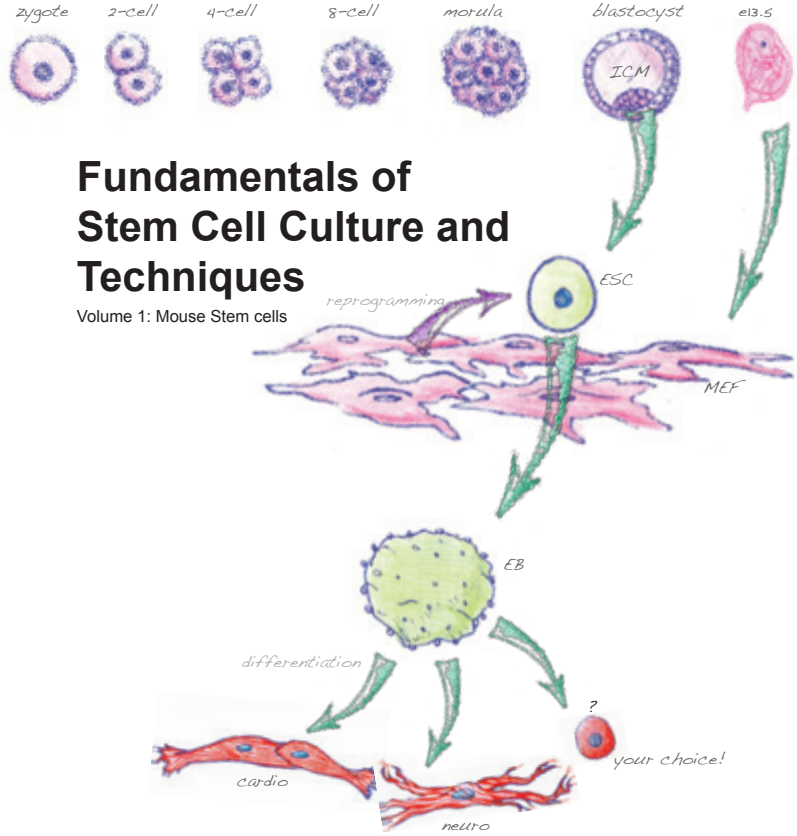

Fundamentals of Stem Cell Culture and Techniques

Volume 1: Mouse Stem cells

A laboratory manual for cell culture and molecular assays

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Acknowledgement

This technical guide is dedicated to Professor Tzvia Abramson at San Jose State University, CA. Tzvia hired us in the summer of 2015 to further develop her CIRM Bridges program funded by the California Institute of Regenerative Medicine. After developing and running the Stem Cell Laboratory course for two consecutive years, we synthesized this technical manual to guide students in stem cell biology molecular and cellular techniques.

Thank you Tzvia, for your passion in education and mentorship in biology.

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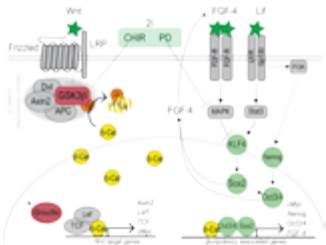
Stem cell laboratory

A stem cell is a cell that can self-renew and give rise to more differentiated daughter cells. There are many different types of stem cells, from different stages of embryonic stem cell development to adult stem cells. In this technical guide we synthesize established protocols utilizing mouse (murine) embryonic stem cells (mESC), mouse induced pluripotent stem cells (iPSC), and adult mouse marrow stromal cells (or mesenchymal stem cells, MSC) to introduce cell culture techniques and molecular biological assays to the novice stem cell biologist. Though there are differences between mouse and human ESC protocols, mouse stem cells are a great tool to introduce you to stem cell manipulations and maintenance.

Molecular pathway

While gaining experience with stem cell cultures throughout this laboratory, it is important to remember the molecular pathways that maintain stemness and to relate the type of cells you are working with to where it exist in the human body throughout development. Knowing these molecular pathways will help you understand why particular molecules and supplements are added to cultures to maintain stemness, or to direct differentiation. The more you know about the nature of these cell and how to control them, the better stem cell biologist you will be.



Fetal development

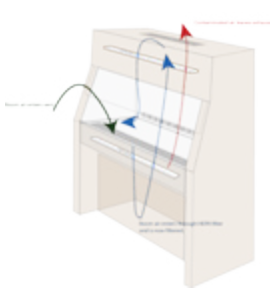
Embryonic stem cell cultures are derived from the Inner Cell Mass (ICM) of the developing embryo. The zygote through the morula contain totipotent cells that can give rise to any cell or tissue including the placenta; We are still trying to better understand the nature and conditions of totipotent cells. When the trophectoderm (pre-placenta) is established there is a condensed aggregate of cells within called the ICM that are pluripotent; These cells can give rise to any cell or tissue in the body except the placenta. We understand the conditions required to maintain the cells in a pluripotent state in vitro. To cultivate an immortal line of the stem cell cultures that were derived from the ICM, we need to provide them a chemical and physical environment that recapitulates the pluripotent state until we direct their differentiation as desired.

Aseptic techniques

Aseptic technique is critical for successful tissue culture, including the culture of mouse embryonic stem cells (mESC). Aseptic technique is an informed balance of procedures and skills that maintain sterility (free of all life) and asepsis (free of pathogens and biological contaminants). Biological contaminants include bacteria, fungi, viruses, mycoplasma, and cross-contamination with cells from another culture. It is good practice to take the appropriate measures to ensure sterility of surfaces and every inert object you work with. Being aware of potential sources of contamination and how to mitigate it will help you refine your skills to maintain aseptic cultures.

Every cell culture scientist will see contamination at some point. The trick to being a good cell culture scientist is to 1) prevent these contamination events with good laboratory practices and aseptic techniques, 2) always identifying potential sources of contamination and mitigating it, and 3) when you do see contamination, stop it quickly. The world is covered with microbial floras and the air is seeded with opportunistic bugs of all kinds, so preventing and fighting contamination is an ongoing battle. However if you develop good culture skills and awareness, this comes as second nature.

Sterilizing biosafety cabinet work area



Biosafety cabinets (hoods) maintain a sterile environment by creating a laminar flow of filtered air. The air flow from the room is pushed down through a HEPA filter and the resulting filtered air enters the biosafety cabinet through the ceiling and exits through vents in the back front of the cabinet. The succeeding current keeps a steady flow of sterile air circulating in the safety cabinet and maintains positive pressure so that outside air does not enter; The air current is affected by how you move inside the hood and where you place objects within the hood. Being aware of both your movements and object placement can help mitigate air eddies and reduce contamination.

Everyday, allow time to sterilize the biosafety cabinet before starting your cell culture.

1. Wipe down the workspace surface with 70% ethanol.
   Turn on the UV light in the Biosafety cabinets and expose the workspace for 15-20
2. minutes to sterilize. (IMPORTANT: Be sure no reagents are in the hood)
3. Turn on the blower to start the air circulation to create laminar flow of HEPA filtered air
4. Turn off the UV light and for good measure, re-spray the workspace with 70% ethanol.

Other items to keep in mind

- Minimize the items in the biosafety cabinets to reduce disruption of the laminar airflow and mitigate air eddies.
- Be aware of the airflow intake vents in the front and back of the workspace in the biosafety cabinet and avoid blocking these intake vents
- Spray 70% ethanol on all inert items that is brought into the biosafety cabinet packaging outside of the safety cabinet as soon as item is opened.

- Treat the outside surface of culture vessels, media bottles, and packaging of consumables as nonsterile. Spray the surfaces of the culture vessels and media bottle (especially the lips of the lid), but do not allow ethanol inside medias, culture vessels, or anything you want to retain biological activity. Discard all outer packaging outside of the safety cabinet as soon as item is opened.

Aseptic cell culture techniques

Being more aware of your work environment and movement in the biosafety cabinet will help reduce contaminants. Some guidelines to keep in mind when you are working.

- Before starting work in the biosafety cabinets, spray 70% ethanol onto your gloves as well as any inert materials brought into the biosafety cabinet prior to cell culture
- Only open vessels and bottles are inside the safety cabinet, and re-cap immediately when finished as to not leave reagents open and exposed to the moving air for too long.
  When opening and closing lids, do not touch the lip of the opening or the cap-
- threads with anything. If there is medium/or reagent inside lid, aspirate to reduce spread of contaminants
  Be aware of the tip of your pipette so that it does not contact other objects including the lips of flask and bottles.
- When working in the biosafety cabinet, arrange your material so that what you need first is easy to access. This will minimize reaching over open containers or vessels to access materials.
- When aspirating medium from cells, change the Pasteur pipettes between different cell lines to prevent contamination.

Other items to keep in mind

- All consumables (serological pipettes, plates, flasks, dishes, falcon tubes, and pipette tips) should be purchased sterile.
- Glasswares (including Pasteur pipettes) should be clean, rinse thoroughly to not leave residual detergent, and autoclaved.
- It is good practice to aliquot medium and reagents from the stock bottle when you first open it, and then to only use these aliquots during your culture. Compartmentalizing these smaller volumes of reagents will make troubleshooting contamination easier when it does happen.

Cleaning up

To reduce contamination in the hood, when work is complete clean your work area.

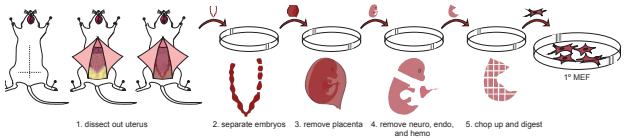
1. Wipe the work surface with 70% ethanol
2. Turn on the UV light in the Biosafety cabinets and expose the workspace for 15-20 minutes (or overnight) to sterilize. (Be sure reagents are put away and not in the hood)
3. Turn off the blower.

Derivation of Mouse Embryonic Fibroblast (MEF)

Purpose

It is often a requirement in biological research to derive your own primary (1º) cell lines. MEF are popular in research because they are easy to derive and have many applications (Garfield, 2010). Here, we will dissect e13.5 embryos from a timed-pregnant CF-1 strain mouse and isolate the 1º MEF for culture. MEF are adherent (stick to plastic) and will be expanded in a common cell culture container called T-flasks. The MEF derived in this protocol will have one of two fates: 1) to expand the MEF up to passage 3 or 4 and then inactivate them to be used as feeder layers for your mESC, and 2) to reprogram MEF into induced pluripotent stem cells (iPSC).

To maintain sterility during this procedure, it is important to use different sets of sterile tools in each step. This will help prevent cross-contamination or carryover of bacteria flora from the mouse into your cultures. In preparation, autoclave a variety of dissection tools. Large blunt tools are best used for the first few, more superficial cuts, to avoid puncturing of other tissues while breaking the skin. Fine tools are best for cutting deeper tissues, such as excising the uterus and removing the placenta. Switching to new sterile tools at every step of this dissection is highly recommended.



Materials

- 70% ethanol spray and a >200 mL in beaker
- Dissection pads and pins
- Minimum of 3 different packs of sterile dissection tools for external, internal, and embryo processing (small curved scissors, small pointed forceps, large straight scissors, large forceps)
- 3x to 5x sterile petri dishes (6mm)
- 1x to 3x sterile T75 flasks
- Standard sterile tissue culture supplies (gloves, falcon tubes, serological pipettes, ect.)

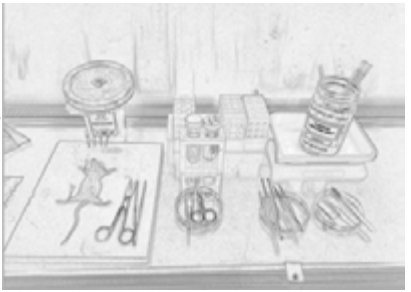
Reagents

- 1x PBS + 5% Penicillin Streptomycin (Pen/Strep)
- 0.25% trypsin (cold/on ice)
- MEF media (DMEM/F12, 2mM L-Glu, 10% FBS, 1x Pen/Strep)

Protocol

It is recommended that the hood be completely set up the laminar hood before the mice are sacrificed.

1. Set up your hood well beforehand
    - *Left side will be for dissection and right side will be for the cell processing*
    - *Between the left and right sides, there should be 3-4 petri dishes containing 1x PBS + 5% Pen/Strep*
    - *The last petri dish on the cell culture side will be filled with cold trypsin while the 1st embryo is being eviscerated*



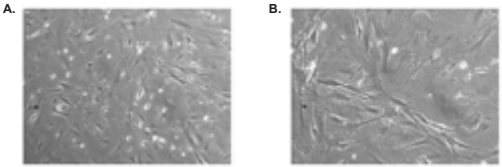
2. Euthanize the pregnant mouse according to your pre-approved Institution Animal Care and Use Committee (IACUC) protocol.
3. Submerge euthanized mouse in 70% ethanol in beaker and transfer beaker with submerged mouse into hood.
4. In the laminar flow hood, move the mouse from ethanol to dissection pad and pin limbs appropriately.
5. Use large 'external tools' to grasp the skin of the abdomen with forceps and make a large incision through the skin with scissors. Pin back the skin from the abdomen.
6. Select finer forceps from a second set of 'external tools' to grasp the peritoneum and make an incision with scissor. Pin back the peritoneum, exposing the abdominal organs.
7. Select forceps from the 'internal dissection tools' to remove uterus and place in first petri dish with 1x PBS + 5% Pen/Strep. Remove both sides of the ureteral "V" horn. *Embryos are fragile; gently remove them to ensure that they remain intact prior to removal of visceral tissues.*
8. Use new internal tools, release the embryo from the uterine wall by using fine tipped scissors to cut along the length of the uterus. Release each embryo into second petri dish with 1x PBS + 5% Pen/Strep.
   Each time that you transfer the content of the dish, make sure that your forceps do not touch the contents of the dish which you are transferring in order to not contaminate the new dish with the unsterile content.
9. Use new tools to remove embryos from its placenta and yolk sac from the uterus.
10. Place the embryo in third petri dish with 1x PBS + 5% Pen/Strep
11. Use new tools to remove the anterior half of the embryo (head) and dark red visceral tissues (internal organs).
12. Place eviscerated embryo into fourth petri dish with 1x PBS + 5% Pen/Strep.

13. Transfer all embryo into a 15 mL falcon tube containing 5 mL of cold 0.25% trypsin. Pipette up and down to break down the embryos smaller tissue. Place the falcon tube in the 37ºC water bath for 5 minutes. Remove from water bath, using aseptic techniques, continue to pipette up and down to break down the tissue into single cells.
    Alternatively, the embryos can be transferred to a 10 cm petri dish containing cold 0.25% trypsin and scissors and/or razor blade can be used to finely mince the embryos. Cover the dish and place minced embryo in 37ºC for 10 minutes. Pipette up and down to disperse cells and transfer content to 15 mL falcon tube.
14. Deactivate the trypsin by adding double volume of MEF media.
15. Centrifuge cells down and remove media supernatant with a serological pipet
    The 1º cell pellet is often fragile and is frequently accompanied by a mass of protein coagulate. This protein coagulate is often attached to the pellet so be careful when removing media. It may be safer to just pate the protein coagulate with the cells rather than trying to remove it.
16. Plate approximately 4-6 embryos per T75 flask. Place flasks in a 37°C, 5% CO_2 humidified incubator.
17. Check the flasks the following day after plating and passage when cultures are 80-95% confluent.

Mouse embryonic fibroblast feeder culture

MEF

- Mouse Embryonic Fibroblasts are a highly-proliferative primary (1°) adherent cell line derived from day e12.5-14.5 fetuses and do not proliferate indefinitely (they senesce).
- In this manual, we will mainly use MEF as feeder cells; using them in co-culture to support the growth of embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC). MEF contribute to the extracellular matrix (ECM) and secrete growth factors to support mESC and miPSC.
- For feeder-cell purpose, MEF are mitotically inactivated before being co-cultured with ES or iPS cells. The MEF remain metabolically viable, but do not proliferate .



Mouse embryonic fibroblast culture (A) 10x magnification and (B) 40x magnification.

Key points in MEF culture maintenance

- MEF should be fed every other day
- Passage MEF every 2-3 days when they reach 80-90% confluent.
- Recommended split ratio 1:3 to 1:5

MEF media preparation

Purpose: Media used to feed mouse embryonic fibroblast culture
Storage: Media is stored at 4°C. Discard unused media after one month

MEF culture media (500 mL)

Reagents	Concentration	Amount
DMEM/F12	--	440 mL
L-glutamine (200 mM)	2 mM	5 mL
MEM Non-essential amino acids (100x)	0.1 mM	5 mL
Fetal Bovine Serum (heat inactivated)	10%	50 mL
Penicillin Streptomycin (10,000 U/mL)	1x	5 mL
	Total volume	*500 mL*

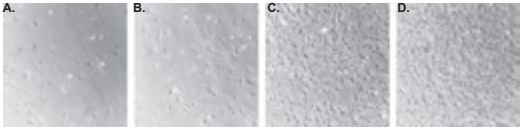
Protocol

1. Using aseptic techniques, combine the media reagents into a 0.2 µm filter system.
2. Attach the filter system to a vacuum and filter media through filter membrane to sterilize.

3. When the filtration is complete, cap the sterile bottle and clearly label:
   Name of media – "MEF media"
   Date prepared
   Your initials
4. Store the media at 2-8°C.

Estimating confluency

- Confluency = % area covered by the cells
- If you plate cells at less than 10% confluency, there is a high chance that they will senesce prematurely and stop expanding
- The optimal time to passage cells is when they are 80-90% confluent
- If you allow your cells to become 100% confluent they may die due to overgrowth (lack of surfaces spaces and nutrients to support cells)



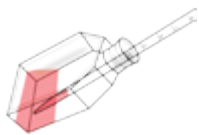
Mouse embryonic fibroblast culture at different confluencies (A) <10%, (B) 15-25%, (C) 55-65% and (D) 80-90%

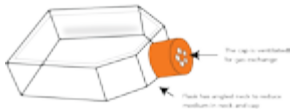
Passaging MEF feeder cells

When the MEF are 80-90% confluent, they should be split (passage) for expanding or frozen down. The recommended split ratio is 1:3 to 1:5.

Protocol

1. Aspirate the spent media from flasks and wash with 1x PBS to remove all residual trace of serum.
2. Add 1x volume 0.25% trypsin (dissociation enzyme) to cover the bottom of the flask. (example, ~7 mL for T75 flask)
3. Incubate flasks in a 37°C, 5% CO_2 humidified incubator for ~5 minutes.
   Check the cells under a microscope to make sure they are all detached.
4. Quench trypsin by adding equal volume (or more) of serum-containing media
5. Transfer the contents of the flask to a falcon tube.
6. Optional: At this point, you can remove a small aliquot of cell suspension (<100 µL) to count cells. While the cells are centrifuging, you can calculate the appropriate cell density for passaging.
7. Centrifuge at 500 xg for 5 minutes
8. Aspirate supernatant, resuspend pellet in MEF media and move to new flask.
9. Place flasks in a 37°C, 5% CO_2 humidified incubator.





MEF are typically expanded in T-flasks, but can be cultured in any adherent dish.

When placing the flask into the incubator, be sure that the neck of the flask is angled upwards to reduce medium from entering lid.

Cryopreservation (freezing) MEF feeder cells

Feeders can be cryopreserved in liquid nitrogen after slow freezing in isopropanol freezing containers such as 'Nalgene Mr. Frosty'. Pre-chill freezing media and the freezing container to 4ºC before usage.

MEF freezing media (10 mL)

Reagents	Concentration	Amount
DMEM	--	2-9 mL
Fetal Bovine Serum (heat inactivated)	FBS used can be between 20-90%*	2-9 mL
Dimethyl sulfoxide (DMSO)	10%	1 mL
	Total volume	*10 mL*

** In our experience we have found 50% DMEM, 40% serum, and 10% DMSO to be successful.*

Protocol

1. Harvest the inactivated MEF via trypsin chemical passaging. (see above protocol 'Passaging MEF feeder culture').
   PBS wash, trypsin, quench trypsin, centrifuge, aspirate supernatant
2. Resuspend in 1 mL cold freezing media per vial
3. Recommended freezing of 2-3 vials (approximately 3 to $4x10^6$ per vial) per T150 flasks at 1 mL freezing media per vial.
4. Place cryovials into COLD Mr. Frosty and incubate at -80°C for 48 hours before moving to liquid nitrogen for long term liquid nitrogen (LN_2) storage
5. When labeling the cryovial tubes, it is recommended to note if the MEF are active or inactivated, total cell number cryopreserved, passage number (if MEF are active), date and preparer's initials.

Thawing MEF feeder cells

Pre-thawing

*For **inactivated MEF only**: Before thawing inactivated MEF (xMEF), prepare 6-well plates coated with 1 mL of 0.1 % gelatin.*

Protocol

1. Remove one vial from the liquid nitrogen (LN_2) freezer. When handling contents of the LN2 freezer, wear eye protection and use cryogenic gloves.
   Due to temperature differences, vials stored in liquid nitrogen may accidentally explode when brought to room temperature.
2. Thaw cryovial in 37°C water bath taking care not to submerge the cap of the vial. Gently swirl the vial in the 37°C water bath until there is just a small bit of ice left in the vial.
   Thaw frozen cells rapidly (< 1 minute) in a 37°C water bath to reduce stress level

3. Spray the vial with 70% ethanol before placing it in the biosafety cabinet.
4. Add MEF media dropwise slowly to thaw cells. Do not add the whole volume of medium at once to the cells. This may result in decreased cell viability due to osmotic shock.
5. Transfer the resuspended cell suspension from vial gently into 15 mL falcon tube.
6. Centrifuge cells for 500xg for 5 minutes.
7. Resuspend cell pellet with MEF media and plate. For inactivated MEF, aspirate the gelatin from the wells and discard before adding cell suspension.
8. Incubate flask in 37°C, 5% CO_2 humidified incubator. When placing the flask in the incubator, be sure that the neck of the flask is higher than the bottom of the flask. Media should not be entering the cap area.

Counting cells via Hemocytometer

Purpose
Cell counting is used to calculate cell density in a cell suspension. Appropriate cell density is required when plating, passaging, and cryopreservation for optimal cell cultures.

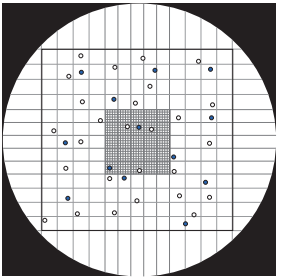
Protocol
There are multiple ways to achieve cell count. The method below is one of many.

1. Obtain a hemocytometer. Clean the chamber and coverslip with 70% ethanol. Dry with kimwipe and place the coverslip in position.
2. In 96-well plate, add 10 µL of the cells suspension and 10 µL of the trypan blue to a well. Mix the suspension with pipette.
3. Add 10 µL of cell suspension to the hemacytometer.
   Do not overfill the chamber. Allow the sample to be drawn out of the pipette by capillary action, the fluid should run to the edges of the grooves only.
4. Place the chamber in the inverted microscope using the 10x objective.
5. Count the unstained cells in the 4 corner squares from hemocytometer grid (1 mm2).
   Dead cells are stained by trypan blue
6. Calculate cell count.

$$\frac{S1+S2+S3+S4}{4} \times 2 \times 10^4 = X \times 10^4/mL$$

   Multiply by 2 to adjust for trypan blue dilution factor. Multiply by 10^4 to estimate number of cells per mL.
7. Calculate Viability Percentage. Count Live cells count and Total cell count (including trypan blue cells)

$$\frac{\text{Live cell count}}{\text{Total cell count}} = \text{percentage viability}$$



Inactivated MEF feeder cells

Introduction

Before use as feeder cells, MEF must be mitotically inactivated by γ-irradiation (irradiated to 3000-4000 rads) or Mitomycin-C treatment.

MEF do not proliferate indefinitely and when they begin to senesce they lose their capacity to support undifferentiated growth and proliferation of ESC. They are optimally used (inactivated for co-culture) between passage 3 and passage 6.

Mitomycin C is a cytotoxic antitumor agent that crosslinks the DNA to block cell division. The MEF feeder cells after mitomycin-C treatment are metabolically viable, but are no longer actively dividing.

Mitomycin C treatment of MEF feeder cells

Protocol

1. Make a 10 µg/mL Mitomycin-C media
   Note: Dilute 2 mg of Mitomycin-C into 200 mL MEF culture medium.
2. Observe MEF cells under a microscope. Start with a confluent flask to inactivate with Mitomycin-C.
3. Aspirate medium from T flasks containing growing MEF cells.
4. Add 10 µg/mL Mitomycin-C medium to the T flasks. Incubate in 37°C, 5% CO_2 humidified incubator.for approximately 5 hours.
5. After incubation time, aspirate Mitomycin-C media from the flasks.
6. Wash flasks 3x with 5 mL of 1x DPBS.
7. Harvest the MEF via trypsin.
8. Inactivated MEF cells (xMEF) can now be plated or cryopreserved for long-term storage.
   Recommended that before usage in co-culture, the xMEF are plated and observed for 2-3 days to ensure the xMEF were properly inactivated.

Cryopreservation (freezing) MEF feeder cells

In this protocol, the optimal seeding density we will use for one 6-well plate of xMEF to support mESC is $3x10^6$ cells/plate, which is equivalent to $5x10^5$ cells/well. Since the viability is not 100% when thawing cells, we recommend freezing at a higher cell density of $4x10^6$ cells/mL/vial.

Protocol

1. Harvest the inactivated MEF via trypsin chemical passaging.
   DPBS wash, trypsin, quench trypsin, COUNT CELLS and centrifuge, aspirate supernatant
2. Resuspend pellet in freezing media (90% FBS + 10% DMSO). Freeze at $4x10^6$ cells/mL/vial.
3. Place cryovials into COLD isopropanol freezing container and incubate at -80°C for 48 hours before moving to liquid nitrogen for long term liquid nitrogen (LN2) storage
4. When labeling the cryovial tubes, it is recommended to note that MEF are inactivated, total cell number cryopreserved, date and preparer's initials.

Plating inactivated MEF (xMEF)

In this manual, the optimal seeding density for one 6-well plate of xMEF to support mESC is $3x10^6$ cells/plate, which is equivalent to $5x10^5$ cells/well.

For inactivated MEF, the culture dishes are prepared with gelatin prior to plating. Plating the MEF in gelatin creates physically better co-culture conditions for subsequent plating of mESC as gelatin matrices support ESC/iPSC.

Protocol

1. In each well of a 6-well plate, add 1 mL of 0.1% gelatin. Let the gelatin sit in the wells for at least 30 minutes at room temperature.
   Swirl plate for even coating on the bottom of the well.
2. Obtain $3x10^6$ cells of inactivated MEF cells
3. Resuspend cells in 12 mL of MEF media.
   $3x10^6$ cell/6 well is equivalent to $5x10^5$ cells/well
4. Aspirate gelatin from wells in the 6-well plate.
5. Pipet the MEF cell suspension up and down to resuspend cells that have settled. Add 2 mL of cell suspension to each well.
6. Label the 6-well plate as follows:
   "xMEF, date, your initials"
7. Place the lid back on the plate and shift the plate back and forth to evenly distribute the cells.
8. Place plates in the 37°C, 5% CO_2 humidified incubator

   NOTE: If you are not plating mESC on the xMEF soon, feed the xMEF ~every two days to maintain their metabolic activity until you do plate on them. Plated inactivated MEF cells can be maintained for up to 2 weeks before they should be discarded.

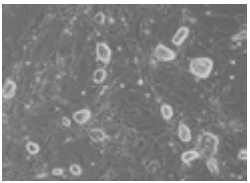
Mouse embryonic stem cell (mESC) culture

Introduction

Mouse embryonic stem cells (mESCs) are derived from blastocysts obtained from post-coitum day 3.5 in vitro fertilized embryos. mESC are pluripotent cells derived from the inner cell mass (ICM) of preimplantation mammalian embryos. The early mouse embryonic stem cell lines were derived in 1993 by plating the embryos individually onto feeder layer of mitomycin C-treated fibroblasts. After plating, the cells attach and the inner cell masses (ICMs) expands (Nagy et al., 1993).

mESC

- mESC are capable of unlimited, undifferentiated proliferation in vitro
- Undifferentiated mouse ES cells can be maintained in vitro in media containing the cytokine leukemia-inhibitory factor (LIF)
- mESC morphology includes formation of tight colonies with defined borders and high nucleus-to-cytoplasm ratio
- If colonies are close to or touching each other they will grow beyond a certain size; the overgrowth will results in differentiation in the center or its periphery



mESC culture. Note refractory edges (“halo”)

Key points in mESC culture maintenance

- mESCs should be fed every day
- Passage mESCS every 1-2 days when colonies are still distinct and not touching edges with one another. The recommended split ratio 1:6, 1:10, or 1:12
- mESC are typically cultured in a 6-well plates with or without feeder depending on the matrix and media used

Complete mESC media preparation

Purpose: Media used to feed mESC
Storage: Media is stored at 2-8°C.
Note: Ready-to-use Complete ES medium with 15% FBS and LIF can be purchase and can support mESC both with feeder and feeder-free. The key component in the media that supports pluripotent state is Leukemia inhibitory factor (LIF).

Complete ES cell media (500 mL)

Reagents	Concentration	Amount
Complete ES media w/ 15% FBS and LIF	--	495 mL
Penicillin Streptomycin (10,000 U/mL)	1%	5 mL
	Total volume	*500 mL*

Protocol

1. Using aseptic techniques, combine the media reagents into a 0.2 µm filter system.
2. Attach the filter system to a vacuum and filter media through filter membrane to sterilize.
3. When the filtration is complete, tightly cap the bottle, and clearly label:
   Name of media – "Complete mESC media"
   Date prepared
   Your initials
4. Store the media at 2-8°C.

Complete ES cell media, version 2 (500 mL)
An alternate recipe for the ESC medium if not purchasing the ready-to-use Complete ES medium

Reagents	Concentration	Amount
DMEM high glucose	--	413.95 mL
Fetal Bovine Serum (heat inactivated)	15%	65 mL
L-glutamine	2 mM	5 mL
Non-essential amino acids	0.1 mM	5 mL
Sodium pyruvate	1 mM	5 mL
Beta-mercaptoethanol	0.1 mM	5 µL
LIF	1000 u/Ml	50 µL
Penicillin Streptomycin (10,000 U/mL)	1x	1 mL
	Total volume	*500 mL*

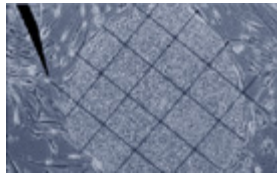
Passaging mESC culture

Passaging mESC is based on colony size, colony morphology, and confluency. Colonies should be passaged before they are contacting each other and while their edges are well defined and retain refractile propertied (halos). When the colonies come in contact with each other or break their halos they begin to differentiate. mESC media should be changed well before it becomes yelllowish, as spent media will cause the cells to differentiate. Healthy mESC cultures should be tightly monitored and passaged every 1-2 days. The recommended split ratio is between 1:6 to 1:20.

Mechanical dissociation
Used when you want to select for specific colonies. A tool, such as a needle, is used to dissect the colonies into smaller cell clumps containing a few hundred cells each and they are transferred to a new plate of feeders.

Protocol

1. Change the media on the well of interest
2. Aseptically under a dissection microscope, mechanically break up the colonies.
   Recommend a sterile 23G needle is used to break up the colonies. A 20 µL pipettor with sterile filter tip attached can be used to gently scrape the colony to lift from plate.



3. Transfer the cell suspension to a new xMEF feeder well.
4. Place plate in a 37°C, 5% CO_2 humidified incubator.

Enzymatic dissociation
Used for single cell dissociation.

Protocol

1. Aspirate the spent media from well and wash with PBS to remove all traces of serum.
2. Add ~1 mL of Accutase to cover the bottom of the well.
3. Incubate 6-well plates in a 37°C, 5% CO_2 humidified incubator for ~5 minutes. *Check the cells under a microscope to make sure they are all detached.*
4. Dilute the accutase by adding equal volume of serum containing medium.
5. Transfer the contents of the flask to a 15 mL falcon tube.
6. Centrifuge at 500xg for 5 minutes.
7. Aspirate supernatant, resuspend pellet in Complete mESC medium and transfer to new xMEF feeder well.
8. Place plate in a 37°C, 5% CO_2 humidified incubator.

Cryopreservation (freezing) of mESC cultures

One confluent 6-well plate of mESC has approximately 1x106 - 2x106 cells and can be cryopreserved in one vial per confluent well.

Protocol

1. Harvest the mESC via Accutase (enzymatic dissociation medium) PBS wash, accutase, dilute medium, centrifuge, aspirate supernatant
2. Resuspend in 1 mL of freezing medium (recommend Bambanker)
3. Place cryovials into COLD Mr. Frosty and incubate at -80°C for 48 hours before moving to liquid nitrogen (LN_2) for long term storage.

Thawing mESC cultures

Pre-thawing
A plate of inactivated MEF should be prepared at least one day prior.

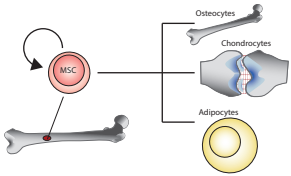
Protocol

1. Remove one vial from the liquid nitrogen freezer. Carefully thaw cryovial in 37°C water bath until there is just a small bit of ice left in the vial.
2. Spray the vial with 70% ethanol before placing it in the biosafety cabinet.
3. Add media (containing serum) dropwise to thaw cells.
4. Transfer the resuspended cell suspension from vial gently into the prepared 15 mL falcon tube.
5. Centrifuge cells for 500xg for 5 minutes.
6. Resuspend cell pellet with Complete mESC medium and set aside.
7. Aspirate the MEF media from the xMEF wells and plate your mESC cell suspension.
8. Incubate plate in 37°C, 5% CO_2 humidified incubator.

Isolation of bone marrow mesenchymal stem cells (MSC)

Introduction

Marrow stromal cells (or mesenchymal stem cells, MSC) are adult stem cells that have multipotential properties and can be differentiated into adipocytes, chondrocytes, and osteocytes. MSC are classically derived from bone marrow, but different types of MSC can be isolated from different tissues.



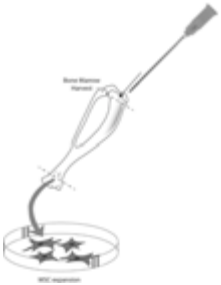
Materials

- 70% ethanol
- Minimum of 2 different packs of sterile dissection tools for external and internal (small curved scissors, small pointed forceps, large straight scissors, large forceps)
- 4x petri dishes
- 1x T25 flasks
- Standard tissue culture supplies (falcon tubes, serological pipettes, …)

Reagents

- 1x PBS + 5% Penicillin Streptomycin (Pen-Strep)
- α-Minimum essential medium (α-MEM)
- Complete MSC medium (see below for recipe)

Protocol



1. Set up your hood well before hand
2. Submerge euthanized mouse in 70% ethanol in beaker and transfer beaker with submerged mouse into hood.
3. In the laminar flow hood, move the mouse from ethanol to dissection pad and pin limbs appropriately.
4. On only the hind limbs, cut the skin all the way around the limb at the hip. (Higher is better)
5. With forceps, pull the skin down and off the limb (inverting the skin) like a sleeve.
6. The skin will hang-up at the foot. With scissors, cut through the foot slightly below the ankle to separate the foot and skin from the intact femur and tibia.
7. Cut through the hip right above the femur hear to remove the intact femur and tibia.
8. Place the femurs and tibias in a sterile petri dish with PBS + 5% Pen/Strep. Carefully (as sterile as possible) remove muscles, ligaments, and tendons using scissors, scalpel, and or forceps. Soft issues are removed to avoid cellular contamination.
9. Rinse the bones in a petri dish containing α-MEM medium + 5% Pen/Strep.
10. Move the bones to a new petri dish with α-MEM medium + 5% Pen/Strep.

11. Excise the two ends of the bones just below the end of the marrow cavity using new microdissecting scissors.
12. Using a 27-gauge needle attached to a 10 mL syringe filled with α-MEM medium, insert the needle into the bone cavity.
13. Slowly flush the marrow out of the bones into a 15 mL falcon tube.
14. Repeat 2-3x times flushing the marrow out of the bone cavities with α-MEM medium until the bones become pale.
15. Transfer the suspension collected and strain the cells through a 70 µm cell strainer placed on a 50 mL falcon tube.
16. Centrifuge cell suspension at 400xg for 10 minutes.
17. Aspirate supernatant and resuspend the pellet in 10 mL of Complete MSC medium.
18. Transfer cell suspension to T25 flask and place flask in 37°C, 5% CO_2 humidified incubator

Complete MSC medium

Purpose: Media used to feed MSC. Alternatively, to enriched for MSC, MesenCult™ MSC Basal Medium from StemCell Technologies can be used.
Storage: Media is stored at 4°C. Discard unused media after one mon**th.**

Complete MSC medium (500 mL)

Reagents	Concentration	Amount
α-MEM	--	430 mL
Fetal Bovine Serum (heat inactivated)	15%	65 mL
Penicillin Streptomycin (10,000 U/mL)	1x	5 mL
	Total volume	*500 mL*

MSC purification via magnetic column negative selection

Introduction

There are 2 popular ways to enrich cell populations: 1. Fluorescent Activated Cell Sorting (FACS), and 2. Magnetic Activated Cell Sorting (MACS). Here we will use MACS to negatively select MSC form our primary bone marrow cultures.

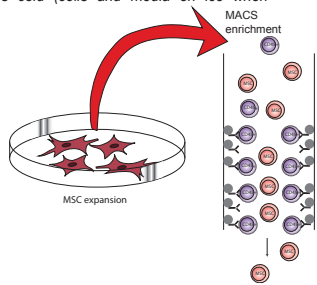
The bone marrow is largely composed of hematopoietic cells and has a smaller population of MSC (which are not hematopoietic). We already enriched for these cells using culture techniques 1) because many hematopoietic cells are not adherent, we rinsed them away before we enzymatically lifted the adherent cells (which contain our MSC), 2) we added a media which promotes the growth of MSC specifically, so the MSC should outgrow and overcome the other cells. However, we would like to further enrich this population before expanding our cultures.

The CD45 protein is expressed on the surface of hematopoietic cells, so we can use this marker to remove these cells. We will stain our cultures with an anti-CD45 antibody that has a magnetic conjugate. We will then run our cells through a magnetic column, so that all the CD45+ cells stick inside the column to the magnetic beads. All the cells that flow through the column will be CD45- and the resulting culture will be greatly enriched for MSC. We will use flow cytometry later to verify the purity.

CD45- selection with an LD column

Protocol

IMPORTANT: Work fast and keep the cells cold (cells and media on ice when not being used)



1. Centrifuge your lifted cells at 500xg for 5 minutes
2. Aspirate media
3. Resuspend pellet in 500 µL cold MACS buffer (PBS, 0.5% FCS, 1 mM EDTA).
4. Add 10 µL of anti-CD45 Microbeads (per 10^7 cells)
5. Mix well and incubate for 20 minutes in the refrigerator at 4°C. (not on ice)
6. Wash the cells by adding 2 mL cold MACS buffer
7. Centrifuge cell suspension at 200xg for 10 minutes and aspirate supernatant.
8. Resuspend the cells in 500 µL of cold MACS buffer
9. Equilibrate the LD column by rinsing with 3 mL cold MACS buffer (can discard flow through here)
10. Transfer 500 µL of cell suspension through 70 µm cell strainers onto the column and collect flow through containing unlabeled cells in 15 mL falcon tube placed below the column.
11. Wash column with 3 mL of MACS buffer three times.
12. Remove 15 mL tube with cells and centrifuge 500xg for 5 minutes.
13. Resuspend cells in MSC media and plate in T25 flask for further culture.
14. Place flask in 37°C, 5% CO_2 humidified incubator.

Passaging MSC cultures

The MSC cultures are confluent within 2 days. When the MSC are 70-90% confluent, they should be split for expanding or frozen down. Passaging should be every 4 to 6 days at a recommended split ratio of 1:3. At passage 3, there are fewer macrophages and blood cells and less fat compared to passage 1 and 2 (Huang et al., 2015)

Protocol

1. Aspirate the spent media from flasks and wash with PBS to remove all traces of FBS and loosen the adhesive force of MSCs to the dish.
2. Add 0.25% trypsin (~5 mL for T25 flask) to cover the bottom of the flask.
3. Incubate flasks in a 37°C, 5% CO2 humidified incubator for ~2 minutes. Digestion should be limited to 2 minutes as longer digestion could lift non-MSCs
4. Quench the trypsin by adding equal volume of serum-free media
5. Transfer the contents of the flask to a 15 mL falcon tube.
6. Centrifuge the cell suspension at 500xg for 5 minutes.
7. Aspirate supernatant, resuspend pellet in MSC media and move to new flask.
8. Incubate flasks in a 37°C, 5% CO2 humidified incubator overnight.

Induced pluripotent stem cells (iPSC) reprogramming

Introduction

Direct reprogramming of somatic cells to induced pluripotent stem cells (iPSC) is an attractive alternative to overcome some of the concerns that are associated with embryonic stem cells (ESC). iPSC have been derived from a number of different species, including human (Takahashi et al. 2007), by expression of the four Yamanaka factor; Oct, Sox2, Klf4, and c-Myc. A number of different approaches have been devised to reprogram somatic cells and their efficiency and quality of resultant iPSCs vary (Stadtfeld et al 2015; Mahendra et al 2013). In this protocol, we will be reprogramming utilizing Sendai vectors.

iPSC reprogramming methods

Methods	Pro	Con	Efficiency
Retrovirus	Good efficiency, easy to implement, validated for multiple cell types	Genome integration, even with excisable vector, there is a small footprint retained in reprogrammed cells	0.001-0.01%
Inducible lentiviral vectors	Expression can be controlled by inert drug doxycycline allows for selection of fully reprogrammed iPSCs. More efficient than retroviral vector.	Genome integration and less efficient silencing than retroviral vectors.	0.1-2.0%
mRNA	Zero footprint, high efficiency, and mRNAs for reprogramming factors available commercially	Cost if purchased commercially, technically challenging if mRNA generated by researcher, labor intensive, and published work only on fibroblasts	>1%
Sendai virus	Zero footprint, Good efficiency, validated for multiple cell types, and reprogramming factor viral extracts available commercially	Cost if purchased commercially, if virus is generated by researcher the method is technically challenging, and licensing/patent issues may exist	0.01-1%

Reprogramming MEF to iPSC via Sendai virus

Introduction

The CytoTune™-iPS 2.0 reprogramming kit utilizes Sendai vectors that include the four Yamanaka factors, Oct, Sox2, Klf4, and c-Myc to reprogram ES cells into iPS cells (Takahashi et al., 2007). The advantages of this kit is that the Sendai vectors do not integrate into the chromosomes of the target cells, there is zero footprint, the virus are non-pathogenic to humans, and the expression of the transgenes are high level and easy to detect after transduction.

To transduce the MEF to iPSC, use the following MOIs to transduce 2.0x105 cells: KOS MOI=5, hc-Myc MOI = 5, and hKlf4 MOI = 3

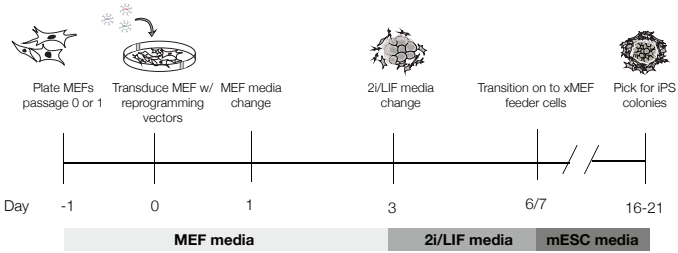
Titer of each CytoTune 2.0 Sendai reprogramming vector is lot-dependent. For the specific titer of the vectors refer to Certificate of Analysis (CoA). The CoA can be found on the vendor's website (http://www.thermofisher.com/order/catalog/product/A16519?CID=fl-we112444 if purchased through Thermo Fisher)

$$\frac{\text{MOI (CIU/cell)} \times \text{number of cells}}{\text{Titer of virus (CIU/mL)} \times 10^3\ (\mu\text{L/mL})} = \text{Volume of virus } (\mu\text{L})$$

Calculate what volume is needed per Sendai vector to transduce 2.0x105 MEF cells in a 12-well plate.

Component	Titer (CIU/mL)	Volume
CytoTune 2.0 hKOS	5	_____
CytoTune 2.0 hc-Myc	5	_____
CytoTune 2.0 hKlf4	3	_____

Reprogramming timeline



Day 3 post-transduction cells are cultured in 2i medium. 2i medium contains CT99021 and PD325901. CT99021 is a GSK-3α and GSK-3β inhibitor. PD325901 is a mitogen-activated protein kinase (MEK) inhibitor.

Materials

- Plated 5x105 MEF cells passage 0 or 1 in 12-well plate
- Inactivated MEF feeder cells plated in 6-well plate

Reagents

- MEF medium (DMEM/F12, 10%FBS, 0.1 mM NEAA, 2 mM L-glu, 1x Pen/Strep)
- 2i medium (ESGROW-2i medium, Millipore catalog # SF016-200)
- Cytotune-iPS 2.0 kit (Invitrogen catalog # A16517)
- 0.25% trypsin-EDTA solution
- Accutase
- PBS

Protocol

[Day -1]

1. Plate two wells of a 12-well plate with MEF at the concentration of 5x105 cells/ well of a 12-well plate.
   Note: It is recommended to use MEF of as early passage as possible.

[Day 0]

1. Thaw each of Sendai vectors in 37°C water bath, spin down and place on ice.
2. Prepare the transducing media; In 1 mL of MEF media and add appropriate volume of each vector so that the MOI of KOS and hc-Myc is 5 and hKlf4 is 3 (against 2.0x105 cells).
3. Mix by pipetting and proceed to next step within 5 minutes
4. Remove culture medium and add the transducing medium prepared.

[Day 1]

Incubate cells for 24 hr and change medium with fresh MEF media.

Note: You should expect to see some cytotoxicity 24-48 hours post-transduction which can affect >50% of your cells because of the high uptake of the virus. Continue culturing cells.

[Day 2-3]

1. Incubate cells for additional 2 days, change MEF medium every day.

[Day 3]

1. Replace MEF medium to **2i medium**.

[Day 6/7]

1. Incubate cells for 3-4 days. Every day, change medium with 2i medium.
2. Prepare an inactivated MEF feeder 100 mm tissue culture dish for transduced cells.
   Note: To prepare an inactivated MEF feeder 100 mm tissue culture dish, incubate tissue culture dish with 0.1% gelatin for 30 minutes, aspirate gelatin, and plate $1x10^6$ - $1.5x10^6$ inactivated feeder cells cultured in MEF media.

[Day 7]: Transition to feeders

1. Six to seven days after the transduction, separate transduced cells by trypsinization 0.05% trypsin (use 5 times dilution of 0.25% trypsin-EDTA solution with PBS). Count cells and seed $0.1x10^6$ to $1x10^6$ transduced cells on plated feeder. Cells are cultured in 2i medium.

[Day 16-21]

1. Continue to culture and maintain cells while change the spent medium every day with fresh 2i medium until colonies grow at appropriate size for analysis or cloning.

Note: After iPSC colonies are selected, you can analyze that your cells are free of CytoTune™ 2.0 Sendai reprogramming vectors. You can do an immunochemistry staining with an anti-SeV antibody or you can reverse transcribe cells' RNA to cDNA, use primer targeting the reprogram transgene via PCR, and run an agarose gel to detect bands of transgene product.

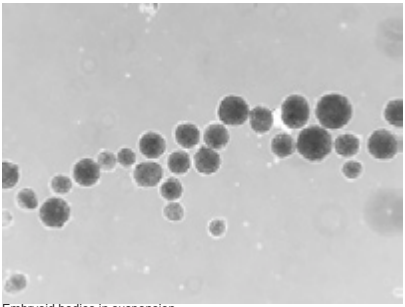
The CytoTune™ 2.0 Sendai hc-Myc reprogramming vector may persist longer in the cells compared to the other vector. The vector has been designed to contain a temperature sensitive mutation. To enhance the removal of this vector, you can incubate your cells at 38–39°C for ~5 days. If the RT-PCR to show that hKlf4

Embryoid bodies (EBs) formation

Introduction

Differentiation can be achieved by growing cells in suspension as embryoid bodies (EB). EB formation recapitulates the early stages of postimplantation development when gastrulation occurs with formation of the three germ layers (ectoderm, endoderm, and mesoderm). EB can be cultured in media that contains specific cytokines and growth factors to promote proliferation and push cells toward one of the three germ layers.

To initiate EB, the removal of the feeder cells and LIF will encourage the cells to begin early differentiation into the three germ layers. EB differentiation protocols vary widely. Depending on what cell type you are going for, some say size matters (Ohnuki and Kurosawa, 2012) and some say growth factors matter (Puceat 2007). One way to control EB size is to use the hanging drop method whereby you control the cell density and volume to dictate the number of cells per EB and use gravity to generate uniform spherical EBs.



Embryoid bodies in suspension

NOTE

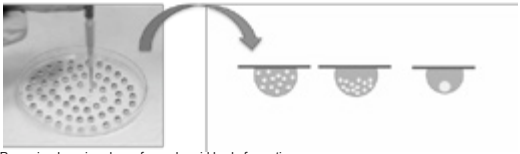
mES cells should be in pristine condition before attempting to form embryoid bodies or attempting any differentiation protocol. The mES cells should be undifferentiated and characterized by distinct round colonies, a high nucleus to cytoplasm ratio, and distinct phase bright borders (halos). Beginning differentiation protocols with cells that have started to differentiate may affect the cells in the dish.

Hanging drop EB culture

Protocol

(Adapted from Spelke 2011)

1. Harvest cells via accutase.
2. Resuspend cell pellet in media - LIF
   Concentration will be dependent on differentiation protocol and are typically in the range of 10^4 to 10^5. Concentration of ESC and size of drops will affect the size of EB
3. Plate 30 mL drops of cell suspension onto lid of 100-mm petri dish
4. Add PBS + 1% Pen/Strep to the bottom of the dish to avoid evaporation.
5. Reverse the lid and place it on the bottom of the dish
6. Place culture dish in 37°C, 5% CO_2 humidified incubator.
   ESC drops will promote ESC aggregation and EB formation.
7. After 24 hours to 3 days, add 5 mL of media -LIF over lid of dish to collect EBs.



Preparing hanging drops for embryoid body formation

Rocking EB culture

Another way to generate EB is to rock or shake your cells in non-adherent dishes. This method is much less labor intensive, but results in random sized and shaped EB as the cells will aggregate on their own.

1. Harvest mES cells via accutase.
2. Resuspend cell suspension in media - LIF
3. Transfer the cell suspension to a 60-mm petri dish (Be sure it's not a tissue culture dish)
4. Place the petri dish on the rocker inside the 37°C, 5% CO_2 humidified incubator.
5. Incubate cells. Be sure and check that media is not depleted.

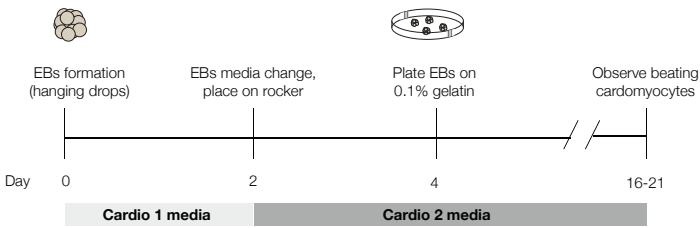
Cardiomyocyte differentiation

Introduction

One way to evaluate pluripotency is to differentiate your cells into different lineages. Cardiomyocytes are the contractile muscle cells of the heart. There are many different reasons to try to differentiate cardiac tissue in regenerative medicine: to recapitulate cardiac diseases in a dish, to help repair myocardial infarctions and heart malformations, or to test the effects of drugs on heart tissue in vitro before prescribing it to a patient.

Cardiomyocytes are of the mesodermal lineage, so it is important to begin differentiation by pushing them towards mesoderm. One way to promote mesodermal differentiation in murine cells is by adding TGFβs and BMPs (Puceat, 2007) to the early EB formation to block ectoderm differentiation. Compared to rodent and human serums, bovine serum has elevated BMP2 (Hu et al., 2010). Therefore, one way to improve murine mesodermal differentiation is to increase the concentration of fetal bovine serum (FBS) (Fuegemann et al., 2010). Another way to promote cardiomyocytes differentiation is by adjusting EB size (Ohnuki, 2013).

Here we evaluate the pluripotency of our mESC by differentiating them into beating cardiomyocytes using the hanging drop method of embryoid body (EB) formation. The cells are cultured in the presence of TGFβ and then an increased bovine serum concentration.



Cardiomyocyte media

Two distinct medias will be used to differentiate mESC into cardiomyocytes. The first media contains hTGFβ-1 to block the ectoderm lineage and to push the cells toward the mesoderm lineage during the first few days of differentiation. The later media contains higher level of bovine serum (contains elevated BMP2) to maintain mesodermal lineage momentum during the later days of differentiation.
Storage: Media is stored at 4°C

Cardio D0-2 medium (10 mL)

Reagents	Concentration	Amount
DMEM/F12	--	QS to 10 mL
TGFβ-1*	2.5 ng/mL	_____*
Fetal Bovine Serum (heat inactivated)	10%	1.0 mL
L-glutamine	2 mM	100 µL
Penicillin Streptomycin (10,000 U/mL)	1x	100 µL
	Total volume	*10 mL*

**Small molecule concentration is dependent on how lab prepared the stock.*

Cardio D2+ medium (50 mL)

Reagents	Concentration	Amount
DMEM/F12	--	35.0 mL
Fetal Bovine Serum (heat inactivated)	28%	14.0 mL
L-glutamine	2 mM	500 µL
Penicillin Streptomycin (10,000 U/mL)	1x	500 µL
	Total volume	*50 mL*

Protocol

mES cells should be in pristine condition before attempting to form embryoid bodies. The mES cells should be undifferentiated characterized by distinct round colonies, a high nucleus to cytoplasm ratio, and distinct phase bright borders.

[Day 0]: Remove the MEFs from mESC culture
Follow protocol under EB formation (hanging drops)

1. Count cells to determine cell concentration and cell total
2. Resuspend cell suspension for a concentration of $6.67x10^4$ cells/mL in Cardio D0-2 media
3. In the hood, pipet 30 µL drops onto the lid of a sterile 10-cm petri dish/low attachment plate until the lid is well-populated with singular uniform drops
   Do not put drops too close to edge as they may contact the edge of the bottom, creating a mess and encouraging contamination.
4. Add 3 mL of PBS + 1% Antibiotic/Antimycotic to the bottom of the plate.
   The PBS increases available moisture to prevent EBs from desiccating.
5. Gently and confidently, invert the lid with drops onto bottom of dish so drops are hanging in sterile environment
6. Gently, label your dish of hanging drops and transfer to 37ºC, 5% CO_2 humidified incubator for 2-3 days

[Day 2-3]: Collect EBs

1. The hanging drop EB have been incubated for 2 to 3 days.
2. Rinse drops into new petri dish with Cardio D2+ media.
3. Optional: Remove a portion for EB in suspension for RNA analysis to study gene expression
4. Transfer the cell suspension to a new 60 mm and add ~3 mL Cardio D2+ medium.
   Do not overfill the plate to prevent leakage and reduce contamination when plates are placed on rockers
5. Place plate on rocker in 37ºC, 5% CO_2 humidified incubator for two more days.

[Day 4]

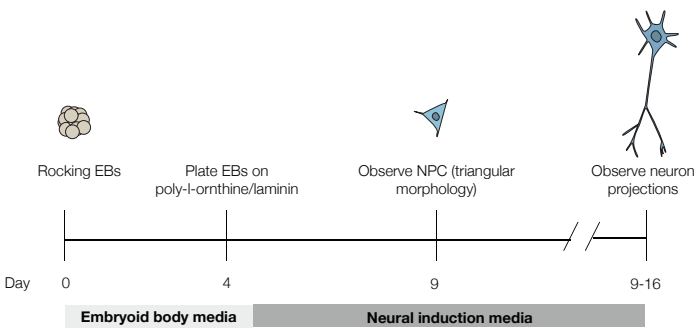
1. Coat a 6-well plate with 0.1% gelatin. Set aside and let incubate for 30 minutes at room temperature.
2. With a serological pipet, gently move your EB into a 15 mL falcon tube.
3. Allow EB to settle (5-15 minutes dependent on EB size)
4. Gently aspirate majority of the media and resuspend in Cardio D2+ media.
   Be gentle and slow during aspiration to not lose too many cells
5. Aliquot EB suspension into wells so that there is approximately 10 EBs per well
6. *Optional: Save some EBs for RNA analysis*
7. Maintain cells for 16 to 21 days until beating cardiomyocytes are observed. The culture should be fed less frequently, approximately every ~5 days.

Neuronal differentiation

Introduction

To further evaluate the pluripotency of our stem cells, we will differentiate our cells into different lineages. In this lab we will be differentiating mESC to neurons, which are of the ectodermal lineage and are highly research in regenerative medicine in hopes of treatment for many diseases including Parkinson's disease, Multiple sclerosis, Alzheimer.

The generation of embryonic stem cell derived neural cells have been established by treating embryoid bodies with retinoic acid, then plated onto laminin coated tissue culture plastic (Frichard et al., 1995; Wu et al., 2012; Stavridis et al., 2003). Neuronal cells were found to express neurofilament flight chain, microtubule-associated proteins 2 and 5, and respond to a range of neurotransmitter and depolarizing currents. Cells found in culture include glial cell types, astrocytes, and oligodendrocytes (Stavridis 2003).



Neuronal differentiation media

Two distinct media will be used to differentiate the mES cells toward neurogenesis. The first media removes LIF to push the cells toward spontaneous differentiation. The second media contains retinoic acids to induce the cells toward neuronal differentiation.
Storage: Media is stored at 4°C Storage: Media is stored at 4°C

Embryoid body media (50 mL)

Reagents	Concentration	Amount
DMEM/F12	--	QS to 50 mL
Knock-out serum	20%	10 mL
L-glutamine	2 mM	500 µL
Non-essentital amino acids	0.1 mM	500 µL
β-mercaptoethanol	0.1 mM	500 µL
Penicillin Streptomycin (10,000 U/mL)	1x	500 µL
	Total volume	*50 mL*

Neural induction media (NIM) (50 mL)

Reagents	Concentration	Amount
Neurobasal medium	--	QS up to 50 mL
N2 supplement	1x	500 µL
All-trans retinoic acid	1µM	_____ *
β-mercaptoethanol	55 µM	50 µL
L-glutamine	2 mM	500 µL
Heat inactivated Fetal Bovine Serum	25%	2.5 mL
Penicillin Streptomycin (10,000 U/mL)	1x	500 µL
B27 supplement	***1x***	***1 mL***
	Total volume	*50 mL*

**Small molecule concentration is dependent on how lab prepared the stock.*

1. Combine all ingredients **except for B27 supplement**. Filter sterilize with 2 µM filter.
2. Add B27 to filtered media.
3. Store at 4°C.

Protocol

[Day 0]: Rocking EBs formation

1. Harvest mES cells via accutase.
2. Resuspend cell suspension for a concentration of 50,000 cells/1mL for a volume of 3 mL in embryoid body media.
3. Transfer the cell suspension to a 60-mm petri dish (Be sure it's not a tissue culture dish)
4. Place the petri dish on the rocker inside the 37°C, 5% CO_2 humidified incubator.
5. Incubate cells. Be sure and check that media is not depleted.

[Day 2-4]: Media change EBs

1. On day 2, collect the cell suspension culture containing EBs into 15 mL conical tube.
2. Wash the 60-mm petri dish 2x with 3 mL MEF media to collect any remaining EBs. Transfer the media into same 15 mL conical tube.
3. Leave conical tube at RT for 10 minutes to allow EBs to settle to the bottom of the tube.
4. Carefully remove the supernatant and discard.
5. Resuspend the EBs in fresh embryoid body media.
6. Plate the EBs suspension to a new 10-cm petri dish
7. Place plate on a rocker and incubate at 37ºC, 5% CO_2 humidified incubator.

[Day 3] Prepare poly-L-ornithine and laminin 8-well chamber slide
See protocol under Matrices, pg.

[Day 4-16]: Expansion of Neuronal Cells

1. On day 2, determine approximately how many EBs you have. This can be done by gently swirling the 10-cm Petri dish containing the neural induced EBs counterclockwise for several circular rotations to localize all the EBs to the center of the 10-cm Petri dish.
2. Based on the number of EBs you have, transfer your cell suspension to a new 15 mL conical tube with approximately 100-200 EBs. collect the cell suspension culture containing EBs into 15 mL conical tube.

[Day 4-16]: Expansion of Neuronal Cells (continue)

3. Incubate the conical tube at room temperature for 10 minutes to allow the EBs to settle to the bottom of the tube.
4. Carefully remove the supernatant and discard.
5. Resuspend the EBs in 2 mL Neural Induction Medium (NIM).
6. Plate approximately 10 to 20 EBs to each well of a poly-L-ornithine and laminin coated 8-well chamber slide.
7. Exchange the media in each well with 0.5 mL fresh NIM Medium every 2 days for a total of 10-15 days.

Day 9+ triangular cells with small projection will begin to emerge; these are neural progenitor cells. The neural cells will continue to mature and projections will elongate. When changing media, gently aspirate on the side of the wells to not disturb projections.

Matrices

Different matrices are important in cell culture to mimic the cells' natural physical micro-environment and promote appropriate behavior/differentiation. There are labs that focus solely on the effects that different matrices have on cell cultures and cells transplants. It is important to research the appropriate matrices for your cells of interest before planning your culture experiments.

Gelatin

Gelatin is a heterogeneous mixture of water-soluble proteins that is derived from porcine. A solution of 0.1% is used to coat cell culture plates that are used for growth of mouse embryonic stem cells.

Protocol

1. Using aseptic technique, add 1-2 mL of 0.1% gelatin solution to cover the whole surface of a 6-well plate.
2. Incubate plate at room temperature for 30-60 minutes to allow the gelatin to coat the culture plate.
3. Just before use, aspirate the gelatin solution just prior to adding culture medium. *Do not allow the gelatin solution to evaporate or dry prior to addition of cell culture Media. If gelatin plate not used immediately, parafilm sealed plates can be stored for up to one week at 2-8°C.*

Matrigel

A solubilized basement membrane preparation that is extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumor that is rich in extracellular matrix proteins, such as laminin, collagen IV, heparan sulfate proteoglycans, entactin/nidogen, and growth factors (heparan sulfate proteoglycan (perlecan), TGF-β, epidermal growth factor, insulin-like growth factor, fibroblast growth factor, tissue plasminogen activator) (Corning, 2016). An alternative coating of tissue culture plates for neuronal and neural cells is matrigel. Usage of this matrix include 2D and 3D cell culture in vitro, in vivo propagation of human tumors in immunosuppressed mice, maintenance of human ES and human iPS cells, and neuronal matrix and differentiation.

Protocol

1. 10 mg/mL matrigel is aliquoted and stored at -80°C *(Check with the manufacturer for concentration as it may varies).*
2. Thaw the matrigel **on ice** until liquid. *Matrigel solidifies at 8°C and it recommended working with cold tubes and serological pipettes when aliquoting to reduce premature solidification.*
3. Dilute matrigel aliquot with COLD media dependent on usage.
   - Thin gel: 50 µL/cm^2
   - Thick gel: 150 - 200 µL/cm^2
4. Immediately add the diluted matrigel solution to coat tissue culture treated plates. Incubate overnight at 4°C. *Alternatively, plates can be incubated at 37°C for 30 minutes or at room temperature for 45 minutes*
5. The next day, remove plates from 4°C and incubate the plates at 37°C for at least 20 minutes before removing matrigel solution.

Neuronal culture matrices; poly-L-ornithine and laminin

Poly-L-Ornithine is a highly positively charged amino acid chain that is generally used to coat surface to promote cell adhesion. The combination with laminin is used to enhance attachment of neuronal and neural stem cells

Reagent

- 50 µg/mL (in tissue culture grade water) Poly-L-Ornithine
- 5 µg/mL (in PBS) Laminin

Protocol

Coating of 8 well chamber slides

1. Using aseptic technique, add 0.05 mL of 50 µg/mL poly-L-ornithine solution to cover the whole surface of the each 8-well chamber slide. Incubate overnight at room temperature.
2. Alternatively, plates can be incubated at 37°C for 2 hours
3. The next day, rinse each well with sterile water.
4. Add 0.5 mL of 5 µg/mL laminin solution to each 8-well. Incubate for 2 hours at 37°C. Coated slides can be stored in the laminin solution at -20°C for 6-8 months. The tray of slides should be wrapped in plastic saran wrap before storage at -20°C.
5. Just before use, aspirate the laminin solution in the coated wells and wash the wells once with 1x sterile water.

Molecular techniques

Immunostaining

Immunostains are qualitative assays that involve staining for protein expression with antibodies. Antibodies can be made to bind to a protein of interest and tagged with a reporter (usually enzymatic or fluorescent). A well-designed stain can localize expression of specific proteins within a cell, which can indicate cellular qualities. We will use immunofluorescence (IF) to evaluate the pluripotency of your stem cells, then to evaluate the efficacy of our neuronal differentiation. Afterwards, you will design your own IF assay to evaluate your own differentiation protocol.

Immunofluorescent staining for pluripotency markers

Introduction

The 'gold standard' of pluripotency is the ability to incorporate into morulas/ICM of early embryos and produce viable beings in which the cells are components of all tissues. The 'silver standard' of pluripotency is to form teratomas (all 3 germ layers) when injected into immunocompromised mice. The 'bronze standard' is to express all 3 of the pluripotency markers Sox2, Oct3/4, and Nanog. These are the main standards, but additional criteria can bolster your claim, such as: the ability to be differentiate into multiple lineages, gross morphology (colonies with refractory hailos), expression of surface markers including SSEA and TRA antigens and transcription factors Oct3/4, Sox2 and Nanog, and a high level of expression of Alkaline Phosphatase (AP) activity.

Purpose

To evaluate the expression of markers Sox2 and Oct4 we will use Immunofluorescence (IF). The IF staining should reveal nuclear specific localization of the markers in each pluripotent cell.

Example immunofluorescence for pluripotency

Host/clone/class	1º antigen	Cell localization (Surface or Intracellular)	2º antibody (α-1º antibody)
Rat IgG α-mouse	SSEA-1	Surface	FITC Donkey α-Rat IgG
Rabbit IgM α-mouse	Sox2	Intracellular	PE Hamster α-Rabbit IgM
~~IgM α-mouse~~		Nucleus	DAPI Nuclear counterstain

Immunofluorescence for your project

Host/clone/class	1º antigen	Cell localization (Surface or Intracellular)	2º antibody (α-1º antibody)
Ig α-mouse			α- Ig
Ig α-mouse			α- Ig
Ig α-mouse			

Protocol

[Day 0]

1. Decant media from wells of 8-well chamber slides. Wash well with 500 µL DPBS.
2. Do not let cells dry out.
3. Add 250 µL of 4% Paraformaldehyde in PBS to fix cells. Let incubate for 5 minutes. Do not overfix.
4. Decant fixative and permeabilized with 0.1% Triton-X100 (a detergent) in PBS 1-5 minutes.
   Permeabilizing step is only required for detection of nuclear proteins (eg., Oct4, Sox2, Nanog). Surface markers do not require permeabilization. Do not allow wells to dry
5. Decant permeabilize buffer and rinse with PBS for 3 minutes. Repeat wash 3x.
6. Decant PBS and block with 5% serum (or FBS) in PBS for 30 minutes
7. Prepare primary (1º) antibodies (1:100 dilution in 5% serum in PBS) in a final volume of 250 µL/well.
8. Decant blocking buffer and add 1º antibody working dilutions. Incubate overnight at 4ºC.
9. Alternatively, the primary antibody can be incubated for 1 hour at room temperature.

[Day 1]

1. Prepare secondary (2º) antibodies (1:100 dilution in 5% serum in PBS) in a final volume of 250 µL/well.
2. Decant 1º antibodies and wash with PBS for 5 minutes. Repeat wash 3x.
3. Aspirate PBS and add 2º antibodies. Incubate 30 minutes - 1 hr at RT.
4. Wash with PBS for 5 minutes. Repeat wash 3x.
5. Add 1 mg/mL DAPI in PBS if desired for nuclear counterstain. Wash to remove counterstain.
6. Add antifade or aqueous mounting media and coverslip if wanting to store samples or mount slides before microscopy.

Alkaline phosphatase (ALP) staining to verify pluripotency

Purpose

Alkaline phosphatase is an enzyme that is expressed by certain types of cells including osteocytes, dermal papilla, dental pulp, and embryonic stem cells. The enzyme is also used as an enzymatic conjugate on antibodies for immunohistochemistry and western blots. ALP is a colorimetric assay (like Beta-galactosidase and Xgal), whereby introducing its specific substrate results in a color change where the enzyme is active. ALP staining ranges from blue-purple to red-pink, depending on the buffers you add to the substrates. Although its expression in multiple different cellular compartments examples its lack of specificity, ALP staining is a quick and easy assay, therefore it remains very common as a preliminary readout for pluripotent state. However, since enzyme activity is not exclusive to mESC, the assay should be used in conjunction with other assays for pluripotency.

***Note**: When using ALP-conjugated antibodies in tissue or heterogenous cells, you must chemically attenuate the endogenous background ALP activity before you introduce your conjugate.*

Protocol

There are various kits and reagents that can be purchased that includes the alkaline phosphatase enzyme and buffers. In our experience, we have had success with the Alkaline Phosphatase Detection Kit. You can also use a precipitating substrate for detection of alkaline phosphatase activity.

Pre-staining protocol

Staining is best if ES cells are cultured for five days at low to medium density prior to analyzing AP activity. Passage mESC at split ratio indicated below.

ALP and DAPI nuclei counter-staining protocol

1. Aspirate media from wells. Wash well with 1x PBS.
2. Fix cells with 4% Paraformaldehyde in PBS. Let incubate for 1-2 minutes. DO NOT OVERFIX. Longer fixation result in the irreversible inactivation of alkaline phosphatase.
3. Permeabilize cells with 0.1% Triton-x100 in PBS
   Leave in permeabilized buffer while preparing AP substrate stain. Do not allow wells to dry
4. Depending on the kit/reagent you use, prepare your substrate staining solution. Reagents are light sensitive and it is recommended you prepare reagents in the dark.
   Millipore Alkaline Phosphatase Detection Kit. Prepare reagents 2 Fast Red Violet (FRV) : 1 Naphthol AS-BI phosphate : 1 distilled water with a final volume of 0.5 ml per well. Leave reagents on ice
5. Decant buffer and add ALP stain solution to cover each well
   Be careful to not disrupt the cell monolayer

6. Incubate plate in dark at room temperature for 15 minutes or until stain is visible. Higher AP activity, higher intensity of stain. Over incubation becomes unspecific.
7. Decant staining solution. Add 250 µL of 1x DAPI and incubate for 5 minutes
8. Decant DAPI and wash twice with 1x PBS.
9. Evaluate the colonies.
    - **Brightfield microscope,** evaluate the number of colonies expressing AP (red stem cell colonies) versus the number of differentiated colonies (colorless).
    - **Fluorescent microscope,** evaluate the DAPI stained nuclei in colony formation.

Gene expression by qRT-PCR

Introduction

In each cell, gene expression can be examined by level of proteins or RNAs to reflect gene transcription. Quantitative reverse transcription PCR (or quantitative real time PCR) is commonly used to evaluate gene expression differences at the RNA level. To determine RNA expression differences using a qPCR assay, you must first decide which genes you would like to evaluate and what cells/tissues you would like to compare. Microarrays or RNAseq assays can be used to evaluate global expression changes, but they are very expensive and are commonly outsourced. To take a closer look at the expression levels of specific genes, qPCR is commonly employed. qPCR is so sensitive that it may be employed to quantify the exact number of RNA transcripts there are in sample –this is called absolute qPCR. Absolute qPCR requires many controls including a ladder for each gene assessed, which includes a serial dilution of known concentrations of each gene to be analysed. Thus, qPCR results are commonly expressed in a relative fashion, whereby gene expression of experimentals are compared to that of controls and the resulting data is presented in fold-expression using the 2-ΔΔCt method (Livak, 2001). There are 3 main steps to obtaining this sensitive gene expression data: 1) RNA extraction, 2) reverse transcription, and 3) qPCR.

Purpose

For pluripotency, we can evaluate the expression of Sox2, Oct4, and Nanog, using either gene and protein expression assays as readouts. Using qRT-PCR to evaluate expression of these markers at both the RNA and protein levels, the output should reveal higher relative expression of these 3 markers in pluripotent state compared to all other cell states.

Working with RNA

IMPORTANT! RNA is very unstable, so precaution must be observed to prevent degradation. In addition, RNases (ribonucleases) are ubiquitous enzymes that are difficult to denature and will significantly degrade your RNA sample in minutes. RNAses are on your hands and in your breath, so work smartly.

- RNaseZapTM or 10% bleach to reduce RNase contamination on all surfaces, inert equipments/materials
- Have an ice bucket ready to place samples. Keep samples on ice, work quickly, and handle the tubes as little as possible
- Always wear gloves when handling reagents and samples
- Don't breath on open samples
- Use certified RNAses-free consumables including polypropylene tubes, pipette tips. Diethyl pyrocarbonate (DEPC) can be utilize to treat water and equipment (plastic consumables, glassware) to inhibit RNase

Cell harvest and storage for RNA extraction

Protocol

1. Pellet up to $1x10^7$ cells by centrifugation at 250xg for 5 minutes.
2. Aspirate supernatant.
3. Wash cell pellet with 1x DPBS and centrifuge to re-pellet
4. Resuspend cells in 100 µL RNAlater®
5. Move to -20ºC for up to 4 weeks or -80ºC for archival storage
   If you are preparing multiple samples, the cells can be store cells on ice for 10 to 30 minutes while you work.

RNA extraction

RNA can be purified using phenol and chloroform, however this procedure use hazardous material, requires technical skills and is time consuming. Commercially available RNA purification kits provide an easier and less hazardous method. There are many kits available and they typically include buffers, other reagents, and silica-based nucleic acid affinity columns. The protocol below has been adapted from the GeneJET™ RNA purification kit and may require modification depending on the kit you choose

See note above regarding working with RNA. ***IMPORTANT*** *before you start.*

Protocol

1. Thaw cell pellet (stored in RNAlater) on ice
2. To each sample, add 600 µL Lysis Buffer supplemented with β-mercaptoethanol or DTT to lyse cells. Vortex for 10 seconds or pipet to mix.
   Incomplete loosening of the cell pellet may lead to inefficient lysis and reduced RNA yields.
3. Homogenized the sample by passing the lysate at through a blunt 20-gauge needle (0.9 mm diameter) fitted to a certified RNase-free syringe.
4. Add 600 µL of 70% ethanol to the homogenized lysate, and mix well by pipetting. Do not centrifuge.
   Precipitates may be observed after addition of ethanol to your sample. This is not a concern and should not affect the procedure.
5. Load the sample, including any precipitate that may have formed, to the RNA spin column placed in a 2-mL collection tube. Centrifuge the column for 1 minute at ≥12000xg. Discard the flow-through and place the spin column back into the collection tube
6. Add the remainder of the sample to the column. Centrifuge the column for 1 minute at ≥12000xg and discard the flow-through.
7. Add 700 µL Wash Buffer 1 to the RNA spin column. Close the lid and centrifuge for 1 minute at ≥12000xg to wash the spin column membrane. Discard the flow-through and place the spin column back into the 2-mL collection tube
8. Add 500 µL Wash Buffer 2 (supplemented with ethanol) to the RNA spin column. Close the lid and centrifuge for 1 minute at ≥12000xg to wash the spin column membrane. Discard the flow-through and place the spin column back into the 2-mL collection tube.
9. Add an additional 500 µL Wash Buffer 2 to the RNA spin column. Close the lid and centrifuge for 1 minute at ≥12000xg to wash the spin column membrane. Discard the flow-through and place the spin column back into the 2-mL collection tube.
10. **Optional**: Centrifuge the spin column for an additional 1 minute at ≥12000xg to remove any residual solution and ethanol in the column.
11. Place the RNA spin column over a new 2-mL collection tube.
12. Add 50 µL of RNase-free water directly to the center of the spin column membrane. Close the lid gently and centrifuge for 1 min at 8000xg to elute RNA into eluate. The flow through is your eluate sample, do not discard.
13. To increase total yield (but reduce concentration), add an additional 50 µL of RNase-free water directly to the center of the spin column membrane. Close the lid gently and centrifuge again for 1 minute at 8000xg. Discard the purification column.
14. Pipette 5 µL of eluate into another centrifuge tube for quantifying via nanodrop.
15. Proceed immediately to cDNA synthesis or store RNA at -80°C until use.

Quantifying RNA via nanodrop

Before proceeding with cDNA synthesis, it is necessary to determine the RNA concentrations of your extractions. A NanoDropTM spectrophotometer can be utilized to quantify your sample. This instrument allows measurement of the optical density (OD) of the RNA sample at wavelength of 260 nm and 280 nm to determine the concentration of the sample and presence of DNA, protein, salts, and other contaminants. A "pure" DNA sample has a 260/280 ratio of 1.8 and a "pure" RNA sample has a 260/280 ratio of 2.0. (Maniatis 1982). A 260/230 ratio of 1.8–2.1 at pH 7.5 is widely accepted as indicative of highly pure RNA. A low ratio is indicative of possible contaminants from salt, carbohydrates, and phenol (Luebbehusen 2004).

cDNA synthesis using reverse transcriptase

In order to complete a PCR reaction, the DNA polymerase enzyme must have a 5' to 3' DNA template of its target amplicon to copy, thus we need to use reverse transcriptase to make a complementary DNA (cDNA) library of your biologic's RNA transcriptome. DNA is also more stable than RNA, so it's best to complete the reverse transcriptase (RT) reaction immediately after purifying your RNA samples. The two main methods of RT reactions are to use poly-T primers or to use random decamers. There are benefits to both, but once you pick a method, you should stick with it till the end of your project.

Note: it is very important to always use the same RT method, because comparing expression between samples which were RT'd with two different methods has caveats. Different RT methods may have preferences for certain transcripts over other others, thus respect the theory of relativity and make all samples equal by being consistent.

The RT and PCR can be broken into in two separate processes or completed together in one process. In one-step RT-PCR, the enzymes required for both the RT and PCR steps are included in one reaction. In two step RT-PCR, the RT reaction is carried out prior to the PCR reaction. The benefit of the one-step method is that the reaction is faster, but you have to thaw and work with your RNA everytime you want to run a plate. We have found (especially in a class setting) it can be easier to make cDNA libraries from RNA samples one day, and then on a separate day, load and run the qPCR plates. The benefit of this, is that the scientists/students now have a stable cDNA library that they can turn back to to run additional plates or to re-run plates that didn't work. Also, it is less stressful working with cDNA on the benchtop rather than RNA. Thus, in this guide, we provide the protocols for the RT-PCR in two steps adapted from RETROscript® Reverse Transcription Kit.

Protocol

See note above regarding working with RNA. ***IMPORTANT*** *before you start.*

1. Thaw RNA sample on ice
2. For each RNA sample, use 1 µg of RNA per reaction. All reactions must have the sample amount of RNA. Less RNA can be used if the target is abundant
   Prepare the Primer Master Mix in sterile 0.2-mL PCR tube(s) and pipette mix.

   Creating a master mix of the common regents assist in controlling variations and reducing pipetting errors.

   Primer master mix (per reaction)

Primers(oligo dT or decamers)	2 µL
Diluted total RNA (1 µg)	8 µL
RNase-free water	up to 12 µL
Total	*12 µL*

4. Briefly centrifuge tube(s).
5. Close the tube lid(s) and place the tube(s) in a 70–85ºC cycler for 3 minutes.
   The exact temperature is generally not critical. If a target is GC-rich or has high degree of secondary structure, 85ºC might be more appropriate.
6. Remove tube and briefly centrifuge. Place the tube(s) on ice for at least 1 minute.
7. Add the remaining RT components

RT master mix (1 reaction)

Primer master mix	*12 µL*
10x RT buffer	2 µL
dNTP mix	4 µL
RNase inhibitor	1 µL
Reverse transcriptase (MMLV-RT)	1 µL
Total	*20 µL*

8. Pipette mix gently up and down. Briefly centrifuge tube(s)
9. Close the tube lids and placed the tube(s) in a 44ºC cycler for 60 minutes
   For templates that do not reverse transcribe well at lower 44ºC temperature, elevate the incubation temperature to a maximum of 55ºC.
10. Inactivate the reverse transcriptase at 92ºC for 10 minutes. Place sample(s) on ice.
11. Store cDNA sample(s) at –20ºC.

qPCR

Now that you have cDNA from the transcriptomes of your biologic samples, we can setup and run the quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Its fundamentally the same as PCR in that the technique is used to amplify, but it also utilizes a laser that detects fluorescent and reads the relative fluorescence of each reaction on each cycle to quantify the gene of interest. The detection is real time (live) and based on when the PCR product is amplified above the cycle threshold number (Ct).

Here we will be using SybrGreen as our fluorescent reporter. SybrGreen emits a strong fluorescent signal when it intercalates into double-stranded DNA… take a minute and think about what the resulting amplification plots will look and how this will allow you quantify expression levels of specific genes. There are other available detection chemistry currently available for qRT-PCR that relies on probe based qPCR to generate fluorescence signal.

You will be using a using a 96-well plate to run the qPCR reaction. Each samples should be run in triplicate, which means we will have 4 biological samples and 8 primer sets (or visa versa). To efficiently load the plate, we will divide the reaction into 2 cocktails; The first cocktail contains the primer set and the other cocktail containing our biologic samples.

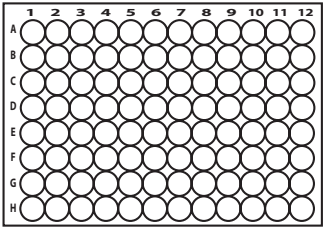
Before you start, it is important to have an organizational plan

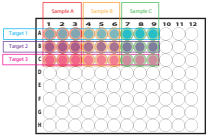
- What are your variables?
- How many conditions are you running?
- How many replicates?
- Did you include a housekeeping gene for normalizing copy number?
- Can they all fit on the plate?
- What will your pattern for loading the plate be?

Common mRNA markers for pluripotency are OCT4, SOX2 and Nanog. Common housekeeping genes (used for normalizing copy numbers) are Beta-actin and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

IMPORTANT. If you cannot fit all the reactions on the same plate then you will need to run your control biologic with the same primers the exact same way on each consecutive plate. Because this assay is relative and conditions may change, you'll need to use this standard to pool your data in the end.

Make a brief sketch of the microplate and label what will be in the rows and columns.





Samples = cell samples
Targets = gene target eg., GADPH as housekeeping gene

Amplification general reaction

(we will be dividing this reaction into two cocktails to efficiently load plate)

General reaction

2x SybrGreen master mix	10 µL
10 µM Forward primer	1 µL
10 µM Reverse primer	1 µL
cDNA [consistent amount]	1 µL
molecular grade H20	7 µL
Total	*20 µL*

Protocol

1. Design your plates so that you can calculate the total volume needed for each cocktail for assay. Add 5 reaction volumes extra for each reagent
2. Label 8 tubes with primer set targeting your genes of interest (Cocktail 1)
3. Label 4 eppendorf tubes for your biologic cocktails (Cocktail 2)
   Prepare the cocktails and **store on ice**.

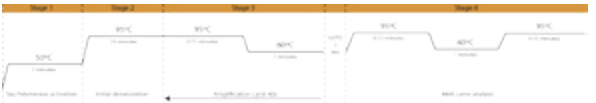
Cocktail 1 (per reaction)		**Cocktail 2 (per reaction)**	
2x SybrGreen master mix	10 µL	cDNA	1 µL
10 µM Forward primer	1 µL	RNase-free water	7 µL
10 µM Reverse primer	1 µL		

These are the volumes per 1 rxn (per well). You will run in triplicate (x3) and the cocktails are designed to be used for multiple samples. Calculate the total volumes you will need in each cocktail to complete this assay and add at least 5 reaction volumes extra for each reagent (this is always a good idea, because you lose volume due to evaporation, the thin coating of liquids on the surfaces of the tube and pipet tip, and to supernatural forces).

The reaction is light and temp sensitive, so you should load your plate with this in mind. You can run the loaded plate right away or store it in the dark at 4ºC overnight.

4. Load the reaction into the wells of the microplate. Ensure that each well has equal volume and that you do not introduce bubble.
   If there are bubbles, you can also use the swinging centrifuge (centrifuge at 4ºC)
5. Place a clear adhesive seal across the top of your microplate. Touch only the edges to not introduce fingerprints, dust or other contaminant that can interfere with sample reading. Be sure that the adhesive is tightly across over all the wells to prevent evaporation of your samples.
6. Before loading the plate into the qPCR thermocycler, make sure your temperature protocol matches your DNA polymerase and the appropriate annealing temp for your primers.
7. Run the thermocycler

Thermocycler program



Thermocycler program includes Taq polymerase activation, Initial denaturation, Amplification cycle, and Melt curve analysis

8. Once the run is complete, review the amplification plot and adjust the threshold so that it lies within the exponential growth region of the amplification curve. Remove the microplate carefully from the cycler.

Amplification plot illustrate the phases of amplification. Amplification efficiency between dilutions of samples is indicated by the interval of CT values. Place threshold line within the exponential phase.

Flow cytometry

Introduction

Gene expression in cells can be examine by the level of proteins expressed by the genes. The proteins can be stained using protein-specific antibodies and then assay by flow cytometry. Flow cytometry can be used to evaluate cell phenotypes to the nth dimension at a single cell level (n being the number of available channels you have on your cytometer). Here, we use flow cytometry to evaluate the heterogeneity of our ESC and iPSC cultures. The multiple characteristics of individual cells can be measure as it flows in a single file in a stream of fluid. The light scattering at different angles allows us to distinguish the cells based on size and complexity. In addition, the use of fluorescently labeled antibodies can be use to identify an array of cell surface and cytoplasmic antigens. In this protocol, we will be using a multi-color antibody panel with both extracellular and intracellular H/M Pluripotent Stem Cell markers. Take care to design your panel so that your fluorophores work with your flow cytometer and don't overlap and that your antibodies are anti mouse proteins since we are working with murine cells

Cell surface and intracellular staining

Protocol

(Adapted from Albu D 2010, Menon V 2014)
Saponin-mediated cell permeabilization is a reversible process, it is important to keep the cells in the presence of saponin during intracellular staining.

1. Harvest mESC cells via accutase.
2. Wash mESC 2x in DPBS.
3. Centrifuge at 500xg for 5 minutes. Decant buffer from pelleted cells.
4. Prepare two FACS tubes, each with a resuspension approximately 5 x 10^5 washed cells in 0.5 mL of Fixation/Permeabilization Buffer (1 - 4% paraformaldehyde in PBS). Vortex cells to maintain single cell suspension.
5. Incubate at 2-8°C for 30 minutes.
6. Centrifuge the cells at 500xg for 5 minutes. Discard supernatant and resuspend the pellet in 100-200 µL of the Permeabilization/Wash Buffer (3% formaldehyde, 0.1% Saponin, 0.5% Tween 20 in PBS).
7. In tube 1, add an appropriate dilution of fluorophore conjugated antibody to the cells. In tube 2, add corresponding isotype control antibody.
   The working dilution for each antibody is determine prior to the experiment based on antibody titer or manufacturer recommendation.
8. Incubate the mixture for 30-45 minutes at room temperature in the dark.
9. Following the incubation, remove any excess antibody by washing the cells in 2 mL of Permeabilization/Wash Buffer (0.5% Saponin, 0.5% Tween 20, 1% bovine serum albumin, 0.01% sodium azide in PBS).
10. Centrifuge and resuspend cell pellet is resuspended in 200-400 µL of DPBS for flow cytometric analysis.

Characterize mESC using flow cytometry

Embryonic stem cells are characterized by pluripotency markers. Using markers for intracellular transcription factors such as Sox2, Oct 3/4 or surface markers such as SSEA-1, we can analyze the pluripotency status of the cell cultures. Other markers that would be useful for characterization include Tra-1, Tra-60, Nanog, and SSEA-4. Note that some markers are used to character pluripotency in mouse but differentiation in human and vice versa.

mESC staining panel

	Marker	Description	Fluorophore/Channel
+	SOX2-Mouse	Pluripotency transcription factor	
+	Oct 3/4-Rat	Pluripotency transcription factor	
+	SSEA-1 Mouse	Surface marker for pluripotency (-) in human	
-	SSEA-4 Mouse	Surface marker for pluripotency (-) in human	

Characterize MSC using flow cytometry

Bone marrow is largely composed of hematopoietic cells (CD45+) and a smaller population of MSC. After enrichment of MSC using the CD45+ depletion column, we can use flow cytometry to characterize our enriched cell population to evaluate the purity of our 1o MSC line.

MSC staining panel

	Marker	Description	Fluorophore/Channel
+	Sca-1	Mesodermal	
-	CD45	Hematopoietic	
+	CD106	Vascular and mesodermal adhesion	
-	CD31	Vascular Adhesion (Endothelial cells)	
-	CD34	Hematopoietic stem cells	
+	CD90.1	T cells and MSC	

Flow cytometry tips

Flow panel design

- Design your panel so that your fluorophores work with your flow cytometer and don't overlap.
- Use antibodies are anti mouse proteins since we are working with murine cells
- Multiple fluorochromes requires proper flow cytometric compensation to remove the spillover fluorescence from a particular probe to a certain channel.

General steps in flow cytometry

1. Stain cells with intracellular and surface staining. Include an unstained control.
2. Instrument set-up: Set up scatter voltages, forward scatter (size) and side scatter (complexity), to place cells in acquisition window.
3. Use single-color stainings to define voltages for fluorochrome channels for proper compensation
4. Run unstained cells and set-up primary gates to exclude dead cells and debris. A threshold is recommended to further exclude debris.
5. Ensure that your voltages were set properly and run samples.

Appendix A: Recommended surface area and volume

Each cell line has different characteristics and the same cell lines may behave different in different people's hands, so seeding requirements do vary. However, this chart provides useful guidance for general plating and harvesting of adherent cells.

	surface area (cm^2)	seeding density (~$2x10^4/cm^2$)	approx confluent cell density	feeding volume (mL)
8-well chamber slide	0.8	2.0×10^4	8.0×10^4	0.2 - 0.3
48-well plate	0.95	2.5×10^4	1.0×10^5	0.2 - 0.3
12-well plate	3.8	1.0×10^5	4.0×10^5	0.7 - 1.25
6-well plate	9.5	2.0×10^5	1.0×10^6	1.5 - 3.0
60-mm dish	20	4.0×10^5	2.5×10^6	3.0 - 5.0
10-cm dish	55	1.5×10^6	6.0×10^6	10.0 - 20.0
T25 flask	25	7.0×10^5	3.0×10^6	5.0 - 7.5
T75 flask	75	2.0×10^6	8.0×10^6	15.0 - 30.0
T150 flask	150	4.0×10^6	1.5×10^7	30.0 - 45.0

Appendix B: Reagent list

Partial list of commonly used reagents in mammalian cell culture

Reagent	Manufacturer	Catalog #	Units
MEF			
DMEM/F12	Millipore	DF-042-B	500 mL
Fetal Bovine Serum (FBS)			
Penicillin/Streptomycin (10,000 U/mL)	Gibco	15140-122	100 mL
L-glutamine	Gibco		100 mL
Non-essential amino acids (NEAA) (100x)	Gibco	11140050	100 mL
0.25% Trypsin-EDTA	Gibco	25200-072	
0.1% Gelatin solution	Millipore	ES-006-B	500 mL
DMSO			
Timed pregnant CF-1 strain mouse	Charles River	023	
Mitomycin-C	Fisher Scientific	BP2531-2	2 mg
Embryonic stem cells and induced pluripotent stem cellls			
Complete ES Cell Media w/ 15% FBS and LIF	Millipore	ES-101-B	500 mL
Accutase cell dissociation solution	Millipore	SCR005	100 mL
Alkaline Phosphatase Detection Kit	Millipore	SCR004	1 kit
CytoTuneTM-IPSC 2.0 reprogramming kit	Invitrogen		1 kit
EsGrow2i medium	Millipore	SF016-200	100 mL
Recombinant Murine LIF	Peprotech	250-02	25 µg
Mesenchymal stem cells			
α-MEM medium			
70 µm cell strainer			
27-gauge needle			
MesenCult MSC basal media	Stem Cell Technologies	05501	

Supplies and consumable	Manufacturer	Catalog #	Units
MEF			
T flask			
6-well plates			
Petridishes			
Serological pipettes			

Reagent	Manufacturer	Catalog #	Units
Immunofluorescence staining			
4% Paraformaldehyde in 1x PBS (fixative solution)			
4'-6-Diamidino-2-phenylindole (DAPI) / PBS counterstain			
Phallodin			
Triton X-100			
Anti-fade mounting solution (DABCO/PVA)			
Millicell EZ Slide 8-well glass			
Hoechst mounting medium	Millipore	EZGS0816	
qPCR			
RNase Zap			
RNA later	Ambion	AM7020	
SyberGreen marker mix	Applied biosystem		
MicroAmp Optical 96-Well Reaction Plate	ThermoFisher	N80 0560	
ABsolute qPCR Plate Seals	ThermoFisher	AB 170	
Flow cytometry			
Fixation/Permeabilization buffer			
Oct 3/4			
SSEA-1			
SSEA-4			
Sox2			
Saponin			
Tween 20			
DPBS			
Fetal calf serum			
Sodium Azide			

Appendix C: Terminology worksheet

Fill in the definition for key terms used in mammalian cell culture.

adult (somatic) stem cells _____

animal cloning _____

asymmetric division _____

blastocyst _____

chimera _____

clonal _____

dedifferentiation _____

directed differentiation _____

ectoderm _____

endoderm _____

embryoid bodies (EBs) _____

embryonic stem cells (ESC) _____

enucleated _____

enzymatic cell dissociation _____

feeder cells _____

feeder-free culture system _____

fertilization _____

gastrulation _____

germ layers _____

GLP (good laboratory practices) _____

GMP (good manufacturing practices) _____

implantation _____

induced pluripotent stem cells (iPSC) _____

inner cell mass _____

intraperitoneal (IP) _____

mesenchymal stem cells (MSC) _____

mesoderm_____

mouse embryonic fibroblast (MEF)_____

multipotent_____

niche_____

pluripotent_____

primary cell culture_____

primitive streak_____

reporter gene_____

reporter mice_____

reprogramming_____

subcloning_____

symmetrical division_____

teratoma_____

terminally differentiated cells_____

totipotent_____

transdifferentiation_____

transfection_____

trophectoderm_____

twinning (monozygotic vs dizygotic)_____

undifferentiated_____

unipotent_____

Yamanaka factors_____

xeno free_____

zygote_____

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Recommended reading materials

1. Stem Cells: From Biology to Therapy Robert A. Meyers [ISBN: 978-0-470-61074-9]
2. Essential of Stem Cell Biology; Edition 3. 2013 Robert Lanza [ISBN:9780124104273]
3. Stem Cells: A Short Course. 2016 Rob Burgess [ISBN: 978-1-118-43919-7]
4. Human Stem Cell Manual. 2007 Jeanne F Loring [ISBN: 978-0-12-370465-8]
5. Culture of Animal Cells. 2005, R. Ian Freshney
6. STEM BOOK http://www.stembook.org