SOP iPSC PT04-4v1	Title: **Culture and Maintenance of Pluripotent Stem Cells over feeder layer of iHFF-1**

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OBJECTIVE

This SOP describes the culture and maintenance of established Pluripotent Stem Cells (PSC) cell lines over feeder layer of irradiated human foreskin fibroblasts cells (iHFF).

DOCUMENTATION

SOP iPSC PT04-3v1 Cryopreservation and recovery upon thaw of iPSCs cells over a feeder layer of iHFF-1
SOP iPSC PT02-3v1 Preparation of culture media
SOP iPSC PT02-4v. Plate and culture of feeder layer cells of irradiated HFF-1

MATERIAL

Equipment

- Class II Microbiology Safety Cabinet
- Centrifuge
- Incubator (37ºC ± 0.5ºC/5% ± 0.5% CO_2)
- Micropipette Stripper (EMB/Midatlantic, Catalog #MID0001)

Consumables

- Matrigel (BD Biosciencies, Catalog #356231)
- DMEM (Life Technologies, Catalog #21969-035)
- Knock-Out DMEM (Life Technologies, Catalog #10829-018)
- Knock-Out Serum (Life Technologies, Catalog #10828-028)
- Fetal Bovine Serum (FBS) (Invitrogen # 10270-106) (-20ºC)
- Penicillin (10.000U/ml)/Streptomycin (10.000ug/ml) (100X)(Life Technologies, Catalog #15140-122)
- GlutaMAX 200mM (100X) (Life Technologies, Catalog #35050-038)
- MEM NEEA 100X (Cultek #B13-114E)
- 2-Mercaptoethanol 50mM (Life Technologies, Catalog #31350-010)
- *bFGF 1000µg (Millipore #GF003AF-MG)
- 6 well plates (Corning, Catalog #353-046)
- 50 mL centrifuge tubes
- 15 mL centrifuge tubes
- 5mL/10mL/25mL/50mL stripettes

PROCEDURE

NOTE: all following cell manipulations, tissue culture vessel preparations and media preparations must be performed under aseptic conditions within a microbiological safety cabinet.

NOTE: the microbiological safety cabinet must be cleaned thoroughly by wiping all base surfaces with 70/30 alcohol.

1. PSC in culture need daily media exchange with HES medium (see SOP iPSC PT02-3v1).
2. PSC lines must be observed daily for PSC-like morphology, the presence of differentiated cells and confluence. Using these observations, the operator must determine if the cells require further action (passage, cryopreservation or preparation for testing).
3. Plate and culture feeder layer of irradiated HFF-1cells (iHFF-1) in wells of a 6-well plate (see SOP iPSC PT02-4v1) 12-48hs before subculturing the colonies.

Split or disaggregation of colonies

NOTE: Cell lines should be passaged when the cells are approximately 80-85% confluent and in their log phase of growth.

NOTE: Split the colonies in the laminar flow hood.

4. Place a tube in a warm support with 1 mL of HES medium for each well to be collected; in this tube we will add the collected colonies. Mount a 175µm stripper in the micropipette stripper.

5. Take the plate and place it under the inverted microscope in the hood. Visualize the well at a magnification that allows seeing 3-4 colonies in a field with enough precision to collect them.

6. Split the colonies with the stripper without aspirating them, so that they remain in suspension. Try to scrape gently not breaking the feeder-layer monolayer.
   NOTE: Criteria to select which colonies to split: try to find undifferentiated colonies, which are flat, transparent, homogenous and with well-defined edges. Discard the more central part of a colony with differentiated morphology.
   The split ratio in new wells must be 1:2-1:3. The final density of the well will determine the subsequent growth speed of the colonies.

7. Once a high number of colonies (more or less 30) have been scraped, aspirate them and deposit them in the tube with medium prepared in step 8. The time spent doing the split of the colonies in one well cannot exceed 10 minutes. Save the split 24 h as back-up in case of passaging failure.

Plating the colonies over the feeder layer of iHFF

8. Take the feeder layer plate where the colonies will be plated and change the medium with 1 mL/well of HES medium.

9. Homogenize gently the colonies from the tube and place 1 mL of colonies in each well.

10. Write on the plate:

Name of the PSC line
Passage number
Date of the passage
Split ratio

11. To ensure even distribution of cell clusters, gently disperse the clusters by carefully moving the vessel side to side, back and forth several times before placing it in an incubator maintained at 37ºC ± 0.5ºC/5% ± 0.5% CO_2. Do not remove the plates from the incubator in the first 24h.

NOTE: routine testing for mycoplasma contamination must be carried out during this culture period.

12. After 48h of the passage, change the culture medium daily until the new colonies will be ready for a new passage (every 6-7 days).

PROTECTION MESSURES FOR USING THIS TECHNIQUE

- Take care when handling cutting material and needles during and after use, as well as throughout its clean-up and elimination procedures.

- Sharp objects (needles, syringes and other sharp instruments) must be deposited in appropriate containers with safety lids to prevent loss during transport. These containers are placed near the workplace and should not be overfilled. These objects must be disposed of as provided for specific medical waste or type III.

- Avoid wounds and scratches in handling of parts and accessories of instruments that can be sharp and in the access to difficult areas.

- Use of biosafety hoods in combination with additional personal protective equipment (Biological Safety Cabinets Class II will be used).

- Washing hands after handling biological material and before leaving the laboratory.

PROTECTION EYES / FACE

Use of biosafety hoods. If it is not possible, safety goggles should be used in those cases where, by the nature of the procedure performed, splashes affecting the mucous membranes of eyes are expected.

Face shields should be used in situations of risk where eye protection should be extended to the face.

The use of surgical masks could be considered sufficient against biological risks coming from splashes. However, these masks are not considered personal protective equipment for the respiratory system.

SKIN PROTECTION

The continuous use of gloves is mandatory in all operations.

Hands and arms are normally the parts of the body that more frequently come into contact with sharp objects and splashing. Gloves and sleeves garments are ideal for protecting hands and arms.

Gloves to protect against biological agents must be waterproof, flexible and with great sensitiveness to enable use in all types of work. When it is required, they should be sterile.

USE CHEMICAL PRODUCTS

ALWAYS CONSULT THE SAFETY DATA SHEETS BEFORE YOU BEGIN WORKING WITH A CHEMICAL PRODUCT

- Use lab coat
- Work in a gas extractor hoods. If you cannot do so, use a respiratory mask
- Use safety glasses with side protection (EN 166)
- Use nitrile chemical protective gloves (EN 374)
- Remove the gloves without touching the surface of the glove to avoid skin contact with the product
- Throw the gloves in the correct container
- Wash and dry your hands immediately after using the substance

WHAT TO DO IN CASE OF EMERGENCY: BIOLOGICAL AGENT (leak, spillage, etc.)

In case of a leak, spillage and accident, such as inoculation, cut and pricks to the skin, inform immediately the person in charge of Emergencies, your direct head (Head of Department, Platform or Laboratory), and the person in charge for the Safety at the workplace.

FIRST AID INSTRUCTIONS

After inoculations, cuts or pricks to the skin: A small hemorrhage has to be provoked and the wound has to be washed with water and neutral soap, do not rub, and add some iodized Povidona.

After sprinklings on the skin: Wash the affected area with abundant cold water and neutral soap, without rubbing, for 10 minutes.

After sprinklings in the eyes: Wash the eyes with water in the special basin for the eyes and keep the eyelids open, for 20 minutes.

In all cases and after the first cure, the biological agent involved in the accident and the origin of the leak has to be identified, inform the person in charge for the Safety at the workplace and go to see the doctor of the insurance company for work-related accidents (Mutua).

Biological spillage

Disinfect the area contaminated with a 10 % dilution of lye.

If it is necessary, disinfect the area with antifog fluid.

If the paper forms of the laboratory or other manuscript or printed paper are contaminated, the information shall be copied in another document and the original has to be thrown in the container for contaminated waste.

Emission of potentially infectious aerosols (out of a camera of biological safety)

Everyone should evacuate the affected area immediately; those exposed to the emission should be sent immediately to receive medical attention. Nobody will be able to enter the area during a specified time, so that the aerosols could go out and the heaviest particles settle. If the laboratory is not fitted with a central air evacuation system, the access will be delayed.

Signs will be placed indicating that entry is forbidden. After the appropriate time, the decontamination has to be done under the supervision of the person in charge of the Laboratory. For this it will be necessary to use protective clothes and suitable breathing equipment.

WHAT TO DO IN CASE OF EMERGENCY: SPILLAGE OR EMISSION OF CHEMICAL SUBSTANCES

Spillage of a Chemical Substance

- Notify the situation to the person in charge of Emergencies, your direct head (Head of Department or Platform), or the Waste Supervisor. Alert the employees who work nearby.

- Protect yourself against the possible risks that could be caused by the chemical substance. Do not take action if you do not have facial, respiratory and skin protection (gloves and suit).

- In the case of small quantities, control the spillage the nearest to the possible origin. Use the adequate absorbent method for the product spilled. (KIT SEKUROKA)

- Ensure that the product doesn't enter the drainage system or closed rooms.

- Remove the product, following the instructions given in the Safety File.

- If the spillage is great, notify the person in charge of Emergencies, your direct head (Head of Department or Platform), the Waste Supervisor or the Safety Control Centre of the PRBB and get away from there.

Emission of substances

- If, when entering a zone where chemical products are stored you detect an intense smell or your eyes, nasal mucous or respiratory tract start itching while you are in the room:

- Leave the zone.

- Close the entrance.

- Notify the incident to the person in charge of Emergencies, your direct head (Head of Department or Platform), the Waste Supervisor or the Center of Control of the PRBB, indicating the characteristics of the accident and the location of the same.

FIRST AID

In case of inhalation: quickly breathe in fresh air.

In case of contact with skin: remove contaminated clothing and wash the affected area with plenty of water, without rubbing, for 15 minutes.

In case of contact with eyes: wash eyes with eye bath using plenty of water with the eyelids open for 15 minutes.

In all cases, once immediate action has been taken, inform the work hazards prevention service and visit Mútua d'Accidents de Treball i Malalties Professionals (Work Accidents and Occupational Diseases Mutual Benefit Society) doctor.