CVCTD-General iPSC cellwork

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Category	Experimental Procedures
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Labels: iPSC | Thawing | Freezing | Coating Matrigel | Picking | Passaging

Introduction

This is an overview of all protocols used on a daily basis when working with iPSCs.

Volumes and amounts

	10cm dish	6-well plate	12-well plate	24-well plate
Matrigel	**5 ml**	**1 ml**	**750 µl**	**300 µl**
Medium	**9 ml**	**2 ml**	**1 ml**	**500 µl**
dPBS/EDTA$_{0,5mM}$	**1.5 ml**	**1 ml**	**750 µl**	**500 µl**
Number of cells	**+- 1x10^6**	**+- 200K**	**+- 70K**	**+- 40K**

Material and Reagents

Material

- Water bath
- 15mL conical tubes
- 50mL conical tubes
- 1,5mL Eppendorf tubes
- Centrifuge
- Cell culture plates: 24-well plates, 12-well plates, 6-well plates
- Cell culture 10cm dishes
- Cryovials
- Labels
- Leica cell counter
- Mr.Frosty
- Ice

- Parafilm
- Microscope

Reagents

- Matrigel *(Corning; cat. 354277)*
- E8 Flex medium *(ThermoFisher; cat. A2858501)*
  - Add 1mL E8 Flex supplement (1 aliquot) to 50mL E8 medium
  - Add 150µL P/S to complete the supplemented medium
- dPBS/EDTA$_{0,5mM}$
- dPBS *(ThermoFisher; cat. A14190144)*
- Acridin Orange *(Westburg; cat. LB F23002)*
- KSR + 10% DMSO *(ThermoFisher; cat. A3181501)*
- RevitaCell *(ThermoFisher; cat. A2644501)*
- DMEM/F12 *(ThermoFisher; Cat. 31330038)*
- Dettol
- 70% ethanol
- Umonium

Thawing iPSCs

Material and Reagents

- Water bath
- 15mL conical tube
- Centrifuge
- 6-well plate coated with Matrigel
- E8 Flex medium
- RevitaCell

Protocol

1. Pre-warm the water bath to 37ºC
2. Prepare a 15ml conical tube with 6ml of E8 medium at room temperature
3. Take the cells out of the liquid nitrogen stock and transfer them on ice to the cell lab
4. Immerse the cryo-vial with the cells in the water bath for up to 30s until the ice melts a bit, but not entirely
5. Spray the vial with 70% ethanol and transfer it under the flow

6. Add dropwise small amount of E8 medium from the prepared medium to the cells
7. Transfer the cells dropwise (to avoid osmotic shock) into the 15mL conical tube with E8 medium
8. Spin the tube down at 120g for 5 minutes at room temperature
9. Aspirate and discard supernatant
10. Very gently resuspend the cells with 1mL fresh medium
11. Remove the Matrigel suspension from the 6-well plate, and add 1mL fresh medium per well
12. Slowly add the cell suspension dropwise to the wells, spreading it evenly on a whole well
13. Add 20µl of RevitaCell supplement for each 2ml of medium (10% RevitaCell)
14. Gently mix the culture by shaking the plate in 8-like movements
15. Put the plate in the incubator (37°C, low oxygen if possible) and shake again the plate in 8-like movements
16. Change the medium after 24 hours

Coating with Matrigel

Material and Reagents

- Eppendorf tubes 1,5mL
- Labels
- Ice
- Plates/dishes for cell culture
- Parafilm
- 50mL conical tube
- Matrigel
- DMEM/F12

Protocol

Aliquot Matrigel

1. Thaw the bottle Matrigel overnight on ice in the fridge
2. Check at teh Corning website for the correct amounts per aliquot:

   https://www.corning.com/worldwide/en/products/life-sciences/resource-library.html
3. Put the Eppendorf tubes on ice

- Write MG on top and give each tube a label with expiration date and lot number

4. Aliquot the Matrigel solution
5. Store the aliquots at -20°C

Coating with Matrigel

1. Take the Matrigel aliquot out of the freezer and put it on ice to thaw
2. Fill a 50mL conical tube with 25 mL of cold DMEM/F12 medium
3. Add a bit of the DMEM/F12 medium to the Matrigel aliquot to speed up thawing, then add the mixture to the 50mL conical tube and pipette up and down to mix it properly
4. Immediately use the dilution to coat culture plates
5. Use the amount given above in the table
6. Spread the dilution evenly on a the wells by making 8-like movements
7. If the plates are not used immediately – seal the plate with Parafilm to prevent dehydration and store in the fridge for up to 7-14 days with date of preparation written on the parafilm
    1. It is also possible to keep the 50mL conical tube with Matrigel in the back of the fridge seeled with a Parafilm
8. Incubate in 37ºC for 1 hour before use
9. Before seeding the cells, aspirate the Matrigel dilution

Tips for picking

Colony picking

- Colonies will pop up 10 to 20 days after transduction
- Look for nice shaped (not spicky) colonies
- Pick from the original 6-well plate and transfer to a well in a 24-well plate (one colony per well)
- Name your colony eg: 1.1 if this is the first colony you picked from the first well
- Add 10% RevitaCell to each well to promote attachement of the cells
- Change medium 24h after picking a colony to remove the RevitaCell
- You should eliminate wells older then 9 days if no colonies can be picked
- Make 8-like movements before and after placing your cells in the incubator to spread the cells evenly in the well

iPSC passaging

Material and Reagents

- Plate or dish coated with Matrigel
- dPBS
- dPBS/$EDTA_{0,5mM}$
- E8 Flex medium
- RevitaCell

Preparations

dPBS/$EDTA_{0,5mM}$ ► Add 53µL $EDTA_{0,5M;\ pH8}$ to 50mL dPBS

Protocol

1. Prepare Matrigel coated dish an hour beforehand
2. Discard the old medium
3. Wash with dPBS and discard it
4. Add dPBS/EDTA$_{0,5mM}$ to each well

- See above for the amount of dPBS/EDTA$_{0,5mM}$ needed

5. Incubate for 4 minutes or look at large colonies under the microscope until holes appear in the middle

- Incubation with dPBS/EDTA$_{0,5mM}$ up to 7 minutes if colonies don't loosen easily

6. Aspirate and discard the dPBS/EDTA$_{0,5mM}$
7. Add 1 or 2 mL of fresh E8 Flex medium directly onto the cells and wash the cells out gently
8. Remove Matrigel from the wells/dish
9. Collect the cells
10. Wash the plates again with fresh E8 Flex medium to collect all the remaining cells
11. Add extra E8 Flex medium to the total volume needed for this well
12. Add 10% RevitaCell
13. Make 8-like movements with the plate to divide the cells evenly in the wells
14. Put back in the incubator and gently shake plate again in 8-like movemnets before closing the incubator
15. Change medium after 24h to remove RevitaCell

iPSC validation

Set 3 good clones for validation by expanding to 4x 10 cm dishes

These dishes should be used for:

1. **DNA extraction** ► SNP array: CNV analysis and identity check

- +/- 2,5-3x10^6cells or 1/2 10cm dish
- Spin the cells down at 120g for 5 minutes at RT, remove medium and store the pellet at **-20°C**

2. **RNA extraction** ► SeV test, qPCRs
    1. +/- 2,5-3x10^6cells or 1/2 10cm dish
    2. Spin the cells down at 120g for 5 minutes at RT, remove medium, add **RNA lysis buffer** and store at **-80°C**
        1. 300µL RNA lysis buffer for < 5x10^6 cells
        2. 600µL RNA lysis buffer for > 5x10^6 cells

- Follow protocol **Quick-RNA™ Miniprep Kit ZYMO** for RNA extraction
- Follow protocol **First strand cDNA Synthesis** for cDNA synthesis

3. **Staining** for Tra1-60, Tra1-81, Oct3/4, Nanog, Sox2 ► Validation pluripotency marker expression
    1. 100.000 cells/well in a 24-well plate with cover slips and Matrigel
    2. Follow protocol **Immunocytochemistry staining iPSC's**

4. Trilineage **differentiation** ► differentiation potential

5. **Freeze** 2 vials and seed the rest for expansion to freeze down in a second step

Note:

- Choose clones to validate from different reprogramming events (= different starting numbers)
- Add all clones to the iPSC-database

Freezing iPSCs

Material and Reagents

- Cryovials
- Labels
- Leica cell counter
- Centrifuge
- 15mL conical tube
- Mr.Frosty freezing container
- dPBS
- dPBS/EDTA$_{0,5mM}$
- E8 Flex medium
- Acridin Orange
- KSR + 10% DMSO

Protocol

1. Label the cryo-vials with cell line, passage number (same as the one on a plate), freeze date, your initials and a vial number (V1/2/3/...)
    1. ***iPSC_disease_gene_patientID_PBMC_CxPx***

    2. Use the 'serial' bottom on the label printer to add a vial number
    3. Give a vial number to each vial frozen down from the same cell line (same passage, date...) so it is easier to find samples back

2. Put the prepared vials on ice under the hood
3. (Remove differentiated cells from the cell culture you want to freeze if necessary using the picking tool or a 100µL pipette)
4. Discard culture medium from the plate, wash with PBS
5. Add PBS/$EDTA_{0,5mM}$ for 4 minutes (1,5mL for 10cm dish)
6. Aspirate the PBS/$EDTA_{0,5mM}$ and discard it
7. Dispense 2ml of medium over the plate, collect cells by washing them out
8. Collect cells in a 15ml tube
9. Wash the plates with fresh medium to collect all the cells (10cm dish: up to 4/5ml of medium in total)
10. Take 18µl of cell suspension into an Eppendorf tube to count the cells, +2µl of Acridine Orange
    1. Turn on the Luna Dual Fluorescence cell counter – Select Fluorescent measurement
    2. Load the correct protocol (iPSC = default)
    3. Put 12µl in the counter slide
    4. Focus on 2x magnification
11. Count cells, write down Total amount /ml + Life amount /ml. Proceed with life count
12. Freeze down 1,5-2 million cells/vial
13. Spin cells down at 120g for 5 minutes at room temperature
14. Re-suspend the cells in freezing medium (90% Knock-out SeRum + 10% DMSO)
15. Distribute 1ml of cell suspension to each vial
16. Quickly place vials on ice and transfer them into the freezing container with isopropanol
17. Place the freezing container in -80ºC freezer for minimum 4 hours
18. Transfer the vials to liquid nitrogen storage

Attachments

No file attachments

*This procedure was originally created by **Gerarda van de Beek***