3T3-L1 Differentiation Protocol

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- MATERIALS

✓ Dulbecco's Modified Eagles Medium (D-MEM) (High Glucose) with L-Glutamine and Phenol Red High glucose (Wako Chem 044-29765, 500 mL)
✓ 10× D-PBS(-) (Phosphate Buffered Saline, Wako Chem 048-29805)
✓ Fetal Bovine Serum
✓ Isobutylmethylxanthine (IBMX; Sigma Aldrich, I5879)
✓ Dexamethasone (Sigma Aldrich, D4902)
✓ Insulin, human, recombinant (Wako Chem, 090-03446, 50 mg)
✓ Penicillin G potassium salt (Aldrich P7794-10MU)
✓ Streptomycin sulfate (Wako Chem. 196-08511, 5 g)
✓ Gentamicin sulfate (Wako Chem. 073-04914, 1 g)
✓

- SOLUTIONS

✧ Antibiotic stock solution (×1000 times)

1. Weigh 100 mg gentamicin sulfate, 200 mg streptomycin sulfate, 200,000 unit penicillin G potassium salt and dissolve in 2 mL of milliQ water (final concentration 50 mg/mL gentamicin sulfate, 100 mg/mL streptomycin sulfate, 100,000 unit/mL penicillin G potassium salt)
2. Filter sterilized through 0.2 μm filter in clean bench (use 1.5 mL microtube)
3. Store in freezer (-20ºC)

✧ 10% FBS/DMEM

1. Add 55 mL Fetal Bovine Serum (heat inactivated) in 500mL D-MEM (high glucose) bottle
2. Add 0.5 mL of antibiotic stock solution to the bottle
3. Store in refrigerator

✧ PBS

1. Prepare 450 mL of sterilized milliQ water in 500 mL bottle (use autoclave)
2. Add 50 mL of 10× D-PBS(-) in the bottle

✧ Trypsin solution

1. Take 40 mL of PBS in 50 mL centrifuge tube
2. Weigh 40 mg of trypsin and 4 mg of EDTA 2Na
3. Add 2 to 1 and dissolve
4. Keep the solution in refrigerator for 1 day
5. Filter sterilized through 0.2 μm filter in clean bench (use 15 mL centrifuge tube, 10 mL each)
6. Leave one for use (store in refrigerator) and store others in freezer

For differentiation of 3T3-L1

✧ Dexamethazone stock solution

1. Prepare 0.5 mM solution in DMSO
2. Filter sterilized through 0.2 μm filter (for DMSO!) in clean bench (use 1.5 mL microtube)
3. Store in freezer (-20ºC)

✧ Insulin Stock Solution

1. Prepare 10 mg/mL solution in PBS
2. Add 1 M HCl aq. drop by drop and dissolve insulin
3. Filter sterilized through 0.2 μm filter in clean bench (use 1.5 mL microtube)
4. Store in freezer (-20ºC)

✧ Differentiation medium (make fresh, only usable for 1-2 weeks)

1. Dissolve 5.6 mg IBMX in a 10 mL 10%FBS/DMEM (sonication required)*
2. Filter sterilize through 0.2 μm filter in clean bench (use 50 mL tube)
3. Add 40 mL 10% FBS/DMEM
4. Add 25 uL dexamethasone stock and 50 uL Insulin stock
5. Store in refrigerator

* IBMX may be prepared as stock solution in DMSO. However it precipitates upon addition and still needs sonication except the required time will be shorter. Also, be carefull of DMSO concentration.

✧ Insulin Medium (Differentiation enhancer)

Add insulin (final concentration 5 μg/mL) to 10% FBS/DMEM

METHOD for culturing 3T3-L1 cells

- Preadipocyte maintenance and passage:

Plate the cells in 10% FBS/DMEM on culture dishes (10 or 6 cm) and incubate them at 37°C in 10% CO_2.

Important!! Feed the preadipocytes every couple of days and never let them get too confluent (>70%).

- Seeding from stock (1×10^6 cells/mL stock)

1. Add 10 mL of 10% FBS/DMEM to 10 cm dishes
2. Move the cell out of the deep freezer and thaw in water bath
3. Seed the cells in 1 by Pasteur pipette
4. Disperse the cells
5. Place in CO2 incubator
6. Check the cells every day and split and re-seed them before confluent.

- Splitting

1. Remove the medium and wash the cell with PBS
2. Remove PBS and add Trypsin solution by Pasteur pipette (12-15 drops for 10 cm dish, 7-8 drop for 6 cm dish)
3. Tilt the dish and disperse the trypsin
4. Place in CO2 incubator for 5 min
5. Prepare new dishes, plates, medium during this time
6. Take the dish out of incubator and see the cells by microscope
7. Add 10% FBS/DMEM to the dish to stop trypsin (4-5 mL for 10 cm, 2-3 mL for 6 cm)
8. Remove the cells from the dish by using Pipette
9. Seed the cells depending on your use

- Making stock

1. Follow the procedure for splitting to 7
2. Remove the cells from dish and move them to centrifuge tube
3. Count the cell density
4. Centrifuge for 5 min at 1000rpm
5. Remove the supernatant (leave about 0.5 mL)
6. Disperse the cells
7. Add cellbanker (prepare 1×10^6 cell/mL solution)
8. Dispense the cell solution in stock tube (0.5 mL each)

9. Label the name of the cell and the date
10. Store in deep freezer

- Adipocyte Differentiation Protocol:

1. Grow preadipocytes to confluency in 10% FBS/DMEM
2. Two days post confluency (DAY 0) change the medium to differentiation medium. You will notice a distinct change in the morphology of the cells (become more spindly) in the next 2 days.
3. On DAY 2 and 4, change the media to Insulin Medium.
4. On DAY 6 and 8 change media to 10% FBS/DMEM.
5. Ready for use on DAY 10

*After changing the medium to differentiation medium, cells get quite sensitive to physical shock and easily detach from the plate. Change the medium slowly and neat to avoid detaching.

- **PPAR activity assay**

✓ KRPH buffer

1. ×10 KRPH buffer (-Mg, -Ca)
   200 mM HEPES, 50 mM KH2PO4, 136 mM NaCl, 4.7 mM KCl, pH 7.4 (dissolve each chemical in 400 mL of milliQ water, adjust pH by 1 M NaOH aq. and add water to 500 mL), Sterilize by autoclave, store in refrigerator
2. 1 mM MgSO4, 1 mM CaCl2 solution: prepare 900 mL, sterilize by autoclave
3. Add 100 mL of ×10 KRPH buffer (-Mg, -Ca) to 1 mM MgSO4, 1 mM CaCl2 solution (do not mix when hot after autoclave, always cool to room temperature and then mix)

✓ 0.1 M HCl aq.

✓ 0.1 M NaOH aq.

✓ 150 mM triethanolamine buffer (pH8.1)

Procedure

1. Change the medium to the sample medium (appropriate concentration of sample dissolved in 10% FBS/DMEM)
2. Incubate for 2 days and change the medium to the same sample containing medium
3. On the 4th day, wash the cells with DMEM (without FBS), add DMEM and incubate for 2 hours (serum starvation)
4. Wash the cells with KRPH buffer twice, and add 100 nM insulin/KRPH buffer (Ins+) or KRPH buffer (Ins-)
5. Incubate for 30 min in CO2 incubator
6. Remove the buffer and add 1 mM 2-deoxyglucose/KRPH buffer
7. Incubate for 30 min in CO2 incubator
8. Wash cells three times by cold saline (0.9% NaCl w/v)
9. Add 0.1 M NaOH aq. and lyse the cells
10. Freeze in deep freezer
11. Thaw and heat at 85°C for 40 min
12. Add 0.1 M HCl aq and neutralize
13. Add the same amount of 150 mM triethanolamine buffer (pH 8.1)
14. Measure the amount of protein by Bradford method
15. Measure the amount of 2-DG

- **Bradford method: follow the manual, run in triplicate**

- **2-DG measure**

✓ TEA buffer : 50 mM Triethanolamine hydrochloride, 50 mM KCl, 0.5 mM MgCl2, pH8.1

✓ Assay Cocktail : 1.3 mM ATP, 20 μM NADP+, 50 μM resazurin sodium salt, 12 units/mL hexokinase, 32 units/mL G6PDH, 4 unit/mL diaphorase in TEA buffer

Prepare followings and mix A/B/C/D = 1/1/1/2, prepare fresh every time

A: 6.5 mM ATP, 100 μM NADP+, in TEA buffer (store in freezer)

B: 250 μM resazurin sodium salt in TEA buffer (store in freezer, avoid light)

C: 12 units/mL hexokinase, 32 units/mL G6PDH, 4 unit/mL diaphorase in TEA buffer (store in refrigerator, use within 10 days)

D: TEA buffer

✓ 2-DG standard

Prepare 0, 2.5, 5, 10, 20, 40, 80, 160 μM solution in TEA buffer

Procedure

1. Mix 50 μL of sample or standard and 50 μL of assay cocktail in 96 well black plate.
2. Mix thoroughly and incubate for an hour at 37°C (avoid light)
3. Measure fluorescence (Ex 530 nm, Em 590 nm)