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Technical Datasheet

HiDiff™ Adipogenesis Assay Kit

Product Code: CCK011

1. Introduction

Abnormal increase in adipose tissue results in obesity, which in turn increases onset of Diabetes Type II and cardiovascular diseases. Hence understanding the mechanisms which contribute to increase in mass of adipose tissue mass are of clinical importance.

Since adipose tissue in humans is usually composed of loose connective tissue and adipocytes, changes in adipocyte size and/or number, results in changes in the mass of adipose tissue within the body.

Adipogenesis is the process of development of adipocytes from pre-adipocytes or re-engineered fibroblasts or mesenchymal stem cells and is achieved both through proliferation and differentiation of pre-adipocytes.

Pharmaceutical research therefore aims to identify cellular targets which influence proliferation and differentiation of pre-adipocytes with a hope to developing anti-obesity and/or anti-diabetic drugs.

Adipogenic differentiation is normally a highly controlled process, which requires stimulation of pre-adipocytes through extracellular matrix proteins, hormones such as insulin or insulin like growth factor culminating in activation of several transcription factors such as CCAAT/enhancer-binding protein (C/EBP) and PPAR-γ.

Immortalized pre-adipocytic cell lines like 3T3-L1 have been invaluable in determining the mechanisms involved in adipocyte proliferation, differentiation, adipokine secretion and gene/protein expression. Further they provide an easy, reproducible and affordable method to investigate adipogenesis as compared to primary cells or stem cells which have limited period of viability and differentiability.

2. About the Assay

3T3-L1 cells are pre-adipocytic in nature. Pre-adipocytes committed to adipogenesis have to withdraw from the cell cycle before undergoing adipose conversion. In this assay growth arrest required for adipocyte differentiation, is achieved through contact inhibition. For this purpose, initially the cells are cultured in control medium is to achieve an optimum density. Following growth arrest, preadipocytes must receive an appropriate combination of mitogenic and adipogenic signals to continue through the subsequent differentiation steps leading to the progressive acquisition of the morphological and biochemical characteristics of the mature adipocytes. This is achieved by culturing cells progressively in Differentiation medium 1 and Differentiation medium 2. Complete differentiation is usually achieved by day. Adipogenesis can be visualized or quantified by the ready–to-use Oil-red-O staining regents included in the kit.

3. Applications

- **Identification of novel cellular factors/ pathways involved in adipogenic differentiation.** Evaluation of effects of trophic factors, cytokines, and growth promoters, hormones, hormonal analogs and steroids on the differentiation process.
- **Proteomics:** Identification of protein-protein interactions which occur transiently during adipogenesis.
- **Drug discovery:** High-throughput screening of various anti-obesity and/or diabetic drugs.

4. Kit contents

Contents	Quantity	Storage
Control Medium(CCK011(A))	1 X 100 ml	2 - 8°C
3T3-L1 Differentiation Medium 1 (CCK011(B))	1 x 20ml	2 - 8°C
3T3-L1 Differentiation Medium 2 (CCK011(C))	1 x 20ml	2 - 8°C
3T3-L1 Differentiation Supplement 1 (CCK011(D))	1 vial x 2ml	-30°C to -10°C
3T3-L1 Differentiation Supplement 2 (CCK011(E))	1 vial x 2ml	-30°C to -10°C
Washing Solution (CCK011(F))	1 x 20 ml	15 to 30°C
Fixing solution (CCK011(G))	1 x 20 ml	15 to 30°C
Permeabilization Solution (CCK011(H))	1 x 20 ml	15 to 30°C
Oil-red-O Staining solution (CCK011(I))	1x 20 ml	15 to 30°C

* Sufficient for one 96-well plate (100 assays)

RT- Room temperature

Note: For other size multi-well plates, volume of media and other reagents to be added should be adjusted accordingly.

5. Materials required but not provided in the kit

- 3T3-L1 cells
- Dulbecco's Modified Essential Medium, high glucose (AL007A)
- Dulbecco's Phosphate Buffered Saline (TL1006)
- Fetal Bovine Serum (FBS) (RM1112/ RM10432)
- Trypsin-EDTA solution (TCL007)
- Antibiotic / Antimycotic solution (A002)
- 96-well plate for culturing the cells (TPC96, TPP96)
- Consumables

6. General Guidelines

Follow below mentioned guidelines for optimal adipogenic differentiation.

Passage number

Use 3T3-L1 cells with low passage number. 3T3-L1 monolayers are fragile and tend to curl up and form bundles that are difficult to analyze for adipocyte differentiation. This property is increases with increase in passage number. Hence it is recommended to use 3T3-L1 cells at low passage number to get optimum differentiation.

Passage timing

During regular maintenance, subculture the cells when they are 70 - 80% confluent. Do not allow them to reach 100% confluency as it results in gradual loss of differentiation capacity of the cells.

Assay controls

Use appropriate assay controls for comparing differentiated cells with undifferentiated cells.

- Medium control - Medium without cells
- Cell control - Cells in control medium throughout the protocol
- Positive control - Factors that are known to increase adipogenic differentiation (Indomethacin, biotin, pantothenate, etc.)
- Experimental drug
- Vehicle control - Solvent in which experimental drug is dissolved

Staining

- During entire staining procedure, do not leave the cell monolayer dry for more than 30 seconds.
- Oil-Red-O stains skin and clothing. Wear (Personal Protective Equipment) while handling the solution.

Technical aspects

- During differentiation, cells tend to attach loosely to the vessel surface. Gently add and remove the medium from wells to avoid detachment of cells from plate surface. Add the medium along the side of culture wells.
- Do not tilt the plate during aspiration or addition of medium. Exposure of cells to air may result in bursting of lipid vacuoles.

7. Directions for use

Users are advised to review entire procedure before starting the assay

7.1 Media Preparation

7.1.1 Preparation of Control Medium

1. Disinfect the external surface of the bottle of control medium by spraying with isopropyl alcohol before placing in a biosafety hood.
2. Aseptically add 10ml of FBS to make the Complete Medium.
3. If necessary, add Antibiotic / Antimycotic solution to a final concentration of 1X.
4. Store at 2-8 °C until use.

7.1.2 Preparation of Complete 3T3-L1 Differentiation Medium 1 and 2

Note: Complete 3T3-L1 Differentiation Medium 1 is required on Day 2 of differentiation and complete 3T3-L1 Differentiation Medium 2 is required on Day 4 of differentiation. Prepare both the media just prior to use.

1. Thaw 3T3-L1 Differentiation Supplement 1 and 3T3-L1 Differentiation Supplement 2 at 2-8˚C overnight.

2. Disinfect the external surface of supplement vials and bottles of 3T3-L1 Differentiation medium 1 and 2 by spraying with isopropyl alcohol before placing them in a biosafety hood.
3. Aseptically add entire quantity of 3T3-L1 Differentiation Supplement 1 in 3T3-L1 Differentiation Medium 1. (This is complete 3T3-L1 Differentiation Medium 1).
4. Aseptically add entire quantity of 3T3-L1 Differentiation Supplement 2 in 3T3-L1 Differentiation Medium 2. (This is complete 3T3-L1 Differentiation Medium 2).
5. Add 0.2ml Antibiotic-Antimycotic solution to each medium if desired.
6. Swirl gently to mix the contents.
   Note: *Do not mix vigorously as it will result in foaming in the medium.*
7. Store at 2-8 °C until use.
   Note: *Both the complete media are stable for 4 – 6 weeks at 2 – 8°C. Do not freeze the complete media.*

7.2 3T3-L1 Differentiation Procedure:

Day 0

1. Maintain 3T3-L1 cells in DMEM, high glucose supplemented 10% FBS (RM1112 / RM10432).
2. Observe the cells under the microscope for morphology and confluency.
3. Replace the medium if required.
4. When the cells are 70% confluent, they are ready for subculturing.
5. Aseptically remove spent medium and wash the monolayer gently using appropriate volume of DPBS (TL1006).
   Note: Rock the flask gently. Take care not to disturb the monolayer.
6. Remove and discard DPBS.
7. Add trypsin in amount sufficient to cover the monolayer.
8. Incubate at 37°C for 3 – 5 minutes in a 5% CO_2 humidified incubator.
9. Carefully monitor the cell dissociation.
10. As soon as the cells dissociate from vessel surface, neutralize the action of trypsin by adding equal amount of complete control medium or Soybean Trypsin Inhibitor (TCL068).
11. Transfer the cell suspension to a sterile centrifuge tube and centrifuge at 800rpm for 8 – 10 minutes.
12. Discard the supernatant and resuspend the pellet in 1 – 2ml of complete medium.
13. Determine the cell density and cell viability using trypan blue and hemocytometer.
14. Adjust the cell density to 1.5 X 10^5 to 2.0 X 10^5 cells/ml using complete control medium.
15. Seed 200µl of the cell suspension in each well of 96-well multi-well plate.
    Note: Each well contains 3 X 10^4 to 4 X 10^4 cells.
16. Incubate the plate at 37°C in a 5% CO_2 humidified incubator for 48 hours.
17. Observe them microscopically every day for morphology and confluency. Cells are ready for differentiation once they are 70 -80% confluent.

Day 2

18. After 48 hours, prepare complete 3T3-L1 differentiation medium 1 as described in section 7.1.2.
19. Aseptically aspirate off the spent complete medium from each well and add 200µl of 3T3-L1 differentiation medium 1.
    Note: Do not add 3T3-L1 differentiation medium 1 in control wells. Add complete control medium to control wells.
20. Incubate the plate at 37°C in a 5% CO_2 humidified incubator for 48 hours.
21. Observe them microscopically every day for morphology.
    Note: Cells tend to become spherical during this period and attachment of the cells with plate becomes loose.

Day 4

22. After 48 hours, prepare 3T3-L1 differentiation medium 2 as described in section 7.1.2
23. Aseptically aspirate off the spent differentiation medium 1 from each well and add 200µl of 3T3-L1 differentiation medium 2.
    Note: Do not add 3T3-L1 differentiation medium 2 in control wells. Add complete control medium to control wells.
24. Incubate the plate at 37°C in a 5% CO_2 humidified incubator for 48 hours.
25. Observe them microscopically every day for morphology.
    Note: Population of spherical cells increases after incubation with 3T3-L1 Differentiation Medium 2. Cells look round and show presence of intracellular lipid vacuoles.

Day 6

26. After 48 hours, aspirate off the spent differentiation medium 2 and add 200µl of complete control medium to all the wells including control wells.
27. Incubate the plate at 37°C in a 5% CO_2 humidified incubator for 48 hours.
28. Observe them microscopically every day for morphology.
    Note: Lipid vacuoles tend to increase in number as well size as differentiation progresses.
29. After every 48 hours, replace the spent medium with complete control medium.
30. Complete differentiation is achieved between Day 7 to Day 15.
31. Stain the lipid droplets using Oil-Red-O staining method.
    Note: Refer section 7.3.

7.3 Oil-Red-O Staining Procedure:

7.3.1 Washing the cells

1. Aspirate off the spent medium from control wells and differentiated wells.
2. Add 100µl of washing solution along the side of each well.
3. Swirl gently to wash the cell monolayer.

7.3.2 Fixation and permeabilization of cells

1. Aspirate off the washing solution and add 100µl of fixing solution to each well.
2. Incubate the plate at room temperature for 30 -60 minutes in fume hood.
3. After incubation aspirate off the fixing solution and add 100µl of distilled water along the side of each well.
4. Swirl gently to remove any traces of fixing solution.
5. Aspirate off the water and add 100µl of permeabilization solution.
6. Incubate the plate at room temperature for 5 minutes.

7.3.3 Staining the cells

Preparation of working stain solution

1. Mix 3 parts of Oil-Red-O staining solution with 2 parts of distilled water.
2. Incubate for 10 minutes at room temperature.
3. Filter the working Oil-Red-O solution through Whatmann filter paper
   Note: Working stain solution is stable for only 2 hours. Prepare only the required quantity just prior to use.

Staining procedure

1. After incubation, aspirate off the permeabilization solution and add 100µl of staining solution.
2. Incubate the plate at room temperature for 5 minutes.
3. After incubation period aspirate off the staining solution and add 100µl of distilled water along the side of each well.
4. Swirl gently to remove any traces of staining solution.
5. Aspirate off the water and repeat washing with water till clear solution is obtained.
6. Again add distilled water to each well and observe under phase contrast microscope at 40X magnification.

7.3.4 Quantification of staining

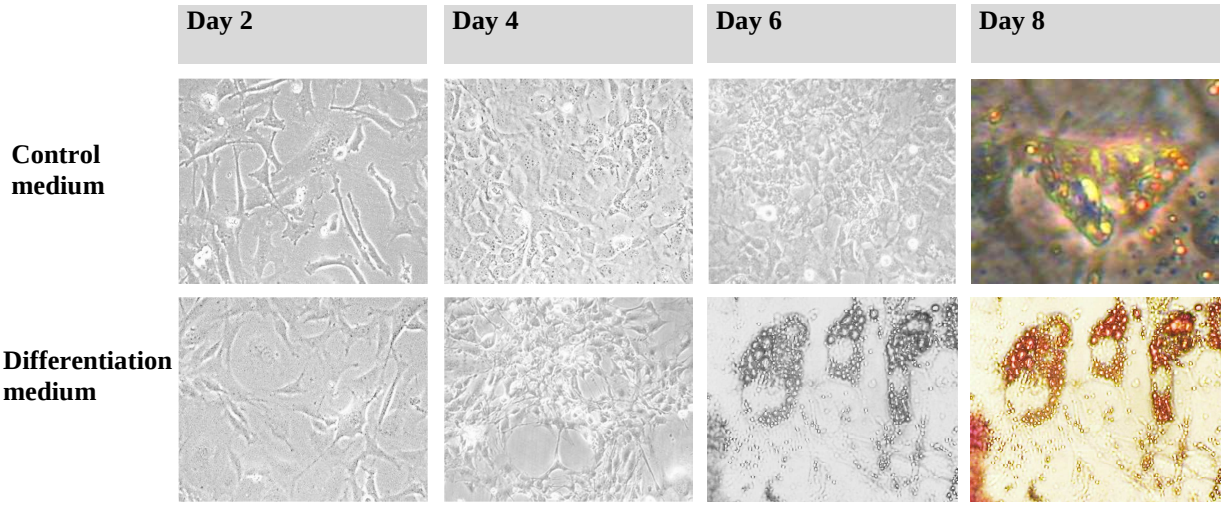
1. Aspirate off the water from wells.
2. Add 100% isopropyl alcohol.
3. Incubate at room temperature for 1 minute.
4. Measure the absorbance at 500nm using isopropyl alcohol as blank on spectrophotometer or 96-well plate reader.

8. Storage and Shelf Life

- The un-reconstituted media, if stored at 2-8 °C remains stable for a period of 12 months.
- The components of the frozen vial are stable for 12 months, if stored at -30°C to -10°C undisturbed.
- Media once reconstituted with factors is stable at 2-8°C for a period of 4-6 weeks.

9. Observations

Results of a typical 3T3-L1 pre-adipocyte differentiation to adipocytes



10. Trouble Shooting

Problem	Cause	Solution
Pre-adipocytes do not grow well/ differentiate well.	Cells have reached a higher passage number.	• Ensure that the cells used for differentiation are of low passage number. • Ensure that media and supplements have been stored at correct temperatures. • Ensure that cells have reached confluence prior to beginning the differentiation protocol.
High background of staining in untreated cells.	Inadequate washes after staining	Wash the cell layer with distilled water until it is no longer red/pink in color.
	Precipitation in Oil-Red O staining solution.	Filter the staining solution through. Whatmann filter paper before use.
	Improper selection of filter in spectrophotometer or 96-well plate reader.	• Choose appropriate filters. • Stain blank wells (containing media but no cells) to assess background staining.
Non-uniform staining	Monolayer disturbed during addition or removal of media and reagents.	Perform addition and removal gently along the side walls of the well.
	Cells growing in patches	Use uniformly spread confluent cells for staining.

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