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Technical Datasheet

HiDiff™ Osteogenesis Assay Kit

Product Code: CCK049

1. Introduction

HiDiff™ Osteogenesis Assay Kit has been developed for *in vitro* differentiation of MC3T3-E1 cells (pre-osteoblasts) into mature osteoblasts, determined by staining for mineralization and visualization.

2. Application

This kit facilitates studies on mechanisms of transcriptional regulation in bone formation, effects of various growth factors, drugs and toxic agents on the phenomenon of osteogenesis and screening of potential osteogenic molecules or drugs in pharmaceutical industry. This kit can also be used for studies based on stem cell differentiation into osteoblasts as well as tissue engineering.

3. About the Assay

MC3T3-E1 is an osteoblast precursor cell line derived from mouse calvaria. It expresses high levels of alkaline phosphatase, produces minerals like calcium and is capable of differentiation into osteocytes under optimal conditions and is thus, an appropriate model for osteogenesis studies. When cells are subjected to mitogenic and osteogenic signals provided by differentiation medium at optimum cell density, they acquire morphological and biochemical characteristics of mature osteoblasts. Osteogenesis can be visualized by Alizarin Red S staining which forms red color precipitates with calcium.

4. Kit contents

Reagents provided in the kit are sufficient for 100 tests (100 wells in a 96-well plate format).

Code	Contents	Quantity	Storage
CCK049(A)	Control Medium	30ml	2 - 8ºC
CCK049(B)	Osteogenesis Differentiation Medium 1	30ml	2 - 8ºC
CCK049(C)	Osteogenesis Differentiation Medium 2	50ml	2 - 8ºC
CCK049(D)	Osteogenesis Differentiation Supplement 1	1 vial (3.6ml)	-20ºC
CCK049(E)	Osteogenesis Differentiation Supplement 2	1 vial (6.05ml)	-20ºC
CCK049(F)	Washing Solution	20ml	RT
CCK049(G)	Fixing solution	20ml	RT
CCK049(H)	Alizarin red S Staining solution	20ml	RT

Note: Store the components of the kit at temperatures indicated in the table immediately upon arrival.

5. Materials required but not provided

- MC3T3-E1 cells (available from ATCC; Cat. No. CRL-2593)
- Dulbecco's Modified Eagle 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 (TCP177, TPP96)
- Consumables (Tips, Tubes, Pipettes)
- Hemocytometer
- Inverted microscope

6. Directions for Use

Users are advised to review entire procedure before starting the assay

6.1 Preparation of complete control medium

1. Aseptically remove 3ml of control medium from the bottle CCK049(A) and add 3ml of FBS to prepare complete control medium.
2. Swirl gently to mix the contents.

6.2 Preparation of complete osteogenesis differentiation medium 1 and 2

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

1. Thaw Osteogenesis Differentiation Supplement 1 CCK049 (D) and 2 CCK049 € overnight at 2-8 °C.
2. Aseptically add entire volume of Osteogenesis differentiation supplement 1 into Osteogenesis differentiation medium 1 to prepare complete osteogenesis differentiation medium 1.
3. Aseptically add entire volume of Osteogenesis differentiation supplement 2 into Osteogenesis differentiation medium 2 to prepare complete osteogenesis differentiation medium 2.

   Notes:
   - *Mix the contents by gentle swirling to avoid foaming.*
   - *Complete media should be stored at 2-8 ℃ until use and are stable for 4-6 weeks. Do not freeze.*
   - *Antibiotic-Antimycotic solution can be added to each medium at a final concentration of 1X, if desired.*

7. Assay procedure

7.1. Culturing and maintenance of MC3T3-E1 cells

1. Maintain MC3T3-E1 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 an amount sufficient to cover the monolayer.
8. Incubate at 37ºC 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 160g 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.

7.2. Osteogenic Differentiation Procedure:

PROCEDURE AT A GLANCE

Day	Activity
0	Harvest and plating of MC-3T3-E1 cells
2	Addition of complete differentiation medium 1 to positive control and complete control medium to negative control
4	Addition of complete differentiation medium 2 to positive control and complete control medium to negative control
6	Addition of complete control medium to all the wells
6 - 18	Addition of complete control medium to all the wells every alternate day
18 - 21	Staining and visualization of osteocytes

Day 0

1. Harvest MC3T3-E1 cells from the culture vessel as mentioned in section 7.1.
2. Adjust the cell density to 0.15 – 0.2x 10^6 cells per ml using complete control medium.
3. Label required number of wells of a 96 well plate as "Negative Control" and "Positive control" wells.
4. Add 100µl of the adjusted cell suspension to each well so that each well contains 15000 to 20000 cells.
5. Incubate at 37°C and 5% CO_2 incubator until the cells reach 70-80% confluence (typically 48 hours).

Day 2

6. After 48 hours, prepare complete Osteogenesis Differentiation Medium 1 as mentioned in section 6.2
7. Aspirate off the complete control medium from each well. Add 100 µl of Complete Osteogenesis Differentiation medium 1 to the wells labelled as "Positive control"
8. Add 100 µl of complete control medium 1 to the wells labelled as "Negative control".
9. Incubate at 37°C and 5% CO_2 incubator for next 48 hours
   Note: Addition of media should be performed carefully along the walls of the plate. Cells tend to detach if additions are done forcefully.

Day 4

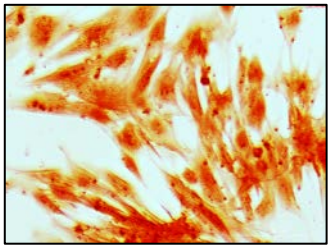
10. After 48 hours, prepare complete Osteogenesis Differentiation Medium 2 as mentioned in section 6.2.
11. Aspirate off differentiation medium 1 from "Positive control" wells and gently add 100 µl of complete differentiation medium 2 along the sides of the walls.
12. Aspirate off control medium from "Negative control" wells and gently add 100µl of complete control medium along the sides of the walls.
13. Incubate at 37°C and 5% CO_2 incubator for next 48 hours.

Day 6

14. Aspirate off media from all the wells and add 100µl of complete control medium to all the wells (positive control as well as negative control wells).
15. Incubate at 37°C and 5% CO_2 incubator for next 48 hours.
16. Replace the spent medium with complete control medium after every 48 hours.
17. Observe the plate microscopically on each day for cell morphology and health.
18. Complete differentiation is typically achieved between Day 18 to Day 21. Stain the calcium deposits in osteocytes using Alizarin Red S staining method.

7.3. Alizarin Red S Staining Procedure:

1. Aspirate off spent medium from all the wells and wash the cells gently with 100µl of washing solution CCK049(F).
2. Aspirate off the washing solution and add 100µl of fixing solution CCK049(G) to each well and incubate for 30-60 minutes at room temperature (preferably in a fume hood).
3. 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.
   Note: pH of Alizarin Red S is very critical for optimum staining of calcium deposits. Before use, ensure that the pH of staining solution is between 4.1 to 4.3. If pH is found to be deviated, adjust it to 4.1 to 4.3 before use.
5. Aspirate off water and add 75µl of staining solution CCK049(H) and incubate for 45 minutes in dark at room temperature.
6. Wash the cells with 100µl of distilled water repeatedly until solution becomes clear.
7. Observe under phase contrast microscope at 20X or 40X objective.



MC3T3-E1 cells differentiated into osteocytes. Calcium deposits in differentiated cells stained with Alizarin Red S (20X).

Key points

- Use appropriate assay controls for comparing differentiated cells with undifferentiated cells. Negative control – Cells in control medium throughout the protocol; Positive control – Cells treated with Differentiation Medium 1 and 2.
- 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.
- During entire staining procedure, do not leave the cell monolayer dry for more than 30 seconds.
- Alizarin Red S stains skin and clothing. Wear (Personal Protective Equipment) while handling the solution.

8. Trouble Shooting

Problem	Cause	Solution
Pre-osteocytes 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 70 – 80% 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 colour
	Precipitation in Alizarin Red S staining solution	Filter the staining solution through a filter paper before use
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

HiMedia Laboratories Pvt. Ltd.
Plot No. C40, Road No. 21Y, MIDC, Wagle Industrial Area, Thane (West) 400604, Maharashtra, India.Tel No.022-69034800
Email: atc@himedialabs.com Website: www.himedialabs.com.