# HiOsteoXL™ Osteocyte Differentiation Supplement

## Product Code: TCL168

### Product Description:

HiOsteoXL™ Osteocyte Differentiation Supplement is optimized for osteogenic differentiation of actively proliferating human mesenchymal stem cells *in vitro*.

Clonally expanded mesenchymal stem cells (MSCs) have ability to differentiate into three types of cells - adipocytes, chondrocytes and osteocytes. Differential potential of MSCs in these three cell types is considered as a reliable functional criterion to identify MSCs and distinguish them from preadipocytes, prechondrocytes and preosteocytes, each of which gives rise to only one cell type. Osteogenesis *in vitro* follows a highly ordered and well characterized temporal sequence.

TCL168 is a proprietary supplement formulated to contain induction factors that induce osteogenic differentiation of human mesenchymal stem cells.

### Materials required but not provided:

1. Expansion medium
   a. HiMesoXL™ Mesenchymal Stem Cell Expansion Medium (AL512) OR
      Dulbecco's Modified Eagle Medium, High glucose (AL007A)
2. Media supplements
   a. Fetal Bovine Serum (FBS) (RM1112/ RM10432)
   b. Antibiotic-Antimycotic Solution 100X (A002)
3. Reagents and medium for subculture
   a. Dulbecco's Phosphate Buffered Saline (DPBS) (TL1006)
   b. Trypsin/EDTA Solution 1X (TCL007)
   c. Soybean Trypsin Inhibitor Solution (TCL068)
   d. Trypan Blue 0.5% solution (TCL005)
4. Osteocyte staining
   a. Alizarin Red S (TC255)
5. Consumables

### General Guidelines:

Follow the below mentioned guidelines for optimal adipogenic differentiation.

**Passage number**
Use the cells with low passage number (less than 5 passages). Mesenchymal stem cells tend to lose their differentiation potential with increasing passage number. Use of cells with high passage number cells might lead to false-positive or false-negative results.

**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 loss of multipotency of the cells.

**Cell dissociation**
Avoid prolonged exposure of cells to trypsin during subculture. Prolonged exposure causes reduced viability and expansion capacity of cells. Monitor the trypsinization procedure carefully and neutralize it immediately upon dissociation.

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

### Directions:

*Users are advised to review entire procedure before starting the assay.*

**Preparation of mesenchymal stem cells for osteogenic differentiation**

1. Expand the mesenchymal cells in HiMesoXL™ Mesenchymal Stem Cell Expansion Medium (AL512) with 10% FBS.
2. Observe the cells every day 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.
   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 37°C in a 5% $CO_2$ humidified incubator.
9. Carefully monitor the cell dissociation.
10. As soon as the cells dissociate from the surface, neutralize the action of trypsin by adding complete medium or Soybean Trypsin Inhibitor solution (TCL068).
    Note: *Here, complete medium refers to AL007A with 10% FBS or AL512 with 10% FBS.*
11. Aseptically collect the cells in a sterile centrifuge tube and centrifuge at 500 - 600rpm for 5 - 7 minutes to remove the traces of trypsin.
12. Discard the supernatant and resuspend the pellet in complete medium.
13. Determine cell density and cell viability using trypan blue and hemocytometer.
14. Prepare the cell suspension in AL007A or AL512 with 10% FBS and seed with 5000 cells/$cm^2$ density in a desired culture vessel.
    Note: *Refer Table 1 for recommended culture volumes for different culture vessels.*

**Table 1: Suggested working volumes of media for different culture vessels**

| Culture vessel | Medium volume per well | No. of wells differentiated using 100ml AL522 |
|---|---|---|
| 48-well plate | 500µl | 200 |
| 24-well plate | 1ml | 100 |
| 12-well plate | 1ml | 100 |
| 6-well plate | 2ml | 50 |

15. Gently rock the plate back and forth and side to side to distribute the cells evenly before incubation. Do not swirl.
16. Incubate the plate at 37°C in a 5% $CO_2$ humidified incubator until the cells are 70% confluent (approximately 48-72 hours).
17. Once the cells reach 70% confluence, they are ready for osteogenic differentiation.

**Preparation of complete osteogenic differentiation medium**

1. Thaw HiOsteoXL™ osteogenic differentiation supplement (TCL168) at 2-8°C overnight.
   Note: *Precipitates in the supplement after thawing are normal. Precipitation will not affect performance of the medium.*
2. Disinfect the external surface of the bottles by spraying with isopropyl alcohol before placing in a bisafety hood.
3. Transfer the entire content of TCL168 to 100 ml of basal media under aseptic condition.
   Note: *If desired, 1ml antibiotic-antimycotic solution (A002) can be added to 100ml of complete medium.*
4. Tightly cap the bottle and swirl gently to ensure proper mixing.
   Note: *Do not mix vigorously. Doing so will cause formation of foam.*
5. Store the complete differentiation medium at 2 - 8°C until use.

**Procedure for induction of osteogenic differentiation**

1. Take out the plate from incubator and observe microscopically for confluence and morphology of cells. If the cells exhibit healthy morphology and are 70% confluent, use the plate for osteogenic differentiation.
2. Aseptically remove half the volume of spent medium from each well and add appropriate volume of fresh medium.
   Note: *For e.g., in case of a 6-well plate, total culture volume is 2ml. Remove 1ml spent medium from each well and then add 2ml of fresh medium.*
3. Refer table 2 for recommended volumes of medium to be added depending on culture vessel.
4. Incubate the plate at 37°C in a 5% $CO_2$ humidified incubator.

**Table 2: Suggested volumes of media for medium change for different culture vessels**

| Culture vessel | Volume of medium per well at the time of seeding | Volume of fresh medium to be added per well |
|---|---|---|
| 48-well plate | 500µl | 500µl |
| 24-well plate | 1ml | 1ml |
| 12-well plate | 1ml | 1ml |
| 6-well plate | 2ml | 2ml |

Note: *Calcium deposits formed by osteocytes are extracellular. Removal of entire spent medium may lead to loss of calcium deposits. Do not tilt the plate while changing the medium. Tilting may also lead to exposure of cell monolayer to air and loss of calcium deposits.*

*During differentiation procedure, cells tend to peel off from the vessel surface. Gently add and remove the medium from culture vessel to avoid detachment of cells from vessel surface. Add the medium along the side of culture wells.*

*This medium change is considered as Day 1 of differentiation.*

5. Observe the cells microscopically and replace the medium with fresh complete differentiation medium after every 48 - 72 hours. Osteoblasts appear tightly packed and linear in shape.

   Note: *At this step, volume of the fresh medium to be removed and added will remain the same.*

   *For. e.g. in case of a 6-well plate, remove 2ml of spent medium and add 2ml of fresh medium.*
6. Incubate the plate at 37°C in a 5% $CO_2$ humidified incubator.
7. Continue steps 5 and 6 for next 18 to 21 days.
8. Differentiation of mesenchymal stem cells to osteocytes can be detected by several methods such as surface marker analysis by immunostaining, gene expression analysis, protein detection, Alizarin Red S staining for calcium deposits and alkaline phosphatase detection.

## Suggested staining methods for detection of osteogenic differentiation:

**Alizarin Red S Staining:**

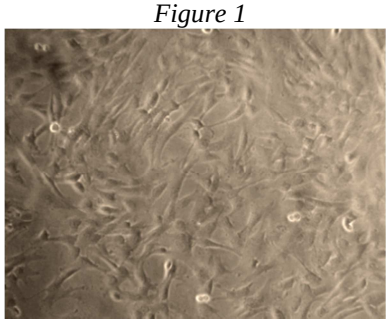
Undifferentiated mesenchymal stem cells have no extracellular calcium deposits whereas differentiated osteoblasts exhibit large amount of extracellular calcium deposits *in vitro*. Calcium deposits are therefore an indication of successful differentiation of MSCs into osteoblasts. Calcium deposits can be specifically stained with Alizarin Red S. Osteoblasts appear bright orange-red in colour. Undifferentiated MSCs do not exhibit the colour.

**Alkaline Phosphatase (AP) Staining:**

Undifferentiated mesenchymal stem cells exhibit weak alkaline phosphatase activity whereas differentiated osteoblasts show very high alkaline phosphatase activity. AP activity is therefore an indication of successful differentiation of MSCs into osteoblasts.

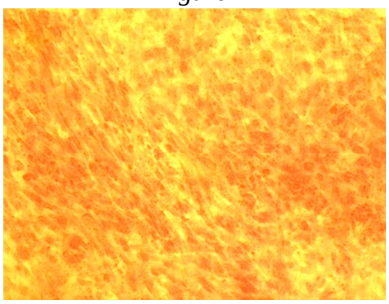
## Observation:

*Figure 1*



*Undifferentiated Human Adult Mesenchymal Stem cells (40X)*

*Figure 2*



*Calcium deposits in mesenchymal stem cells differentiated into osteocytes stained red in colour by Alizarin Red S (40X)*

## Quality control:

**Appearance**

Pale yellow coloured clear solution

**Sterility**

No bacterial or fungal growth is observed after 14 days of incubation, as per USP specification.

**Cultural Response**

Osteogenic differentiation potential of the medium is assessed by differentiating mesenchymal stem cells for 18 - 21 days in the medium and analyzing them qualitatively for presence of calcium deposits by Alizarin red S and alkaline phosphatase staining method.

## Storage and Shelf Life:

Shelf life is 12 months at -20°C

Shelf life of complete medium after reconstitution with TCL168 is 6 weeks at 2-8°C

Note: *Freezing of the basal medium and complete medium is not recommended. Avoid repeated freezing and thawing of the growth supplement.*

| | |
|---|---|
| IVD | In vitro diagnostic medical device |
| CE | CE Marking |
|  | Consult instructions for use |
|  | Do not use if package is damaged |
|  | HiMedia Laboratories Pvt. Limited, B /4-6 , MIDC, Dindori, Nashik MH<br>www.himedialabs.com |
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