# 3T3-L1 Cell Care Manual

## Maintenance and Differentiation of 3T3-L1 Preadipocytes to Adipocytes

**INSTRUCTION MANUAL ZBM0009.08**

## SHIPPING CONDITIONS

Orders are delivered via Federal Express courier. All USA and Canada orders are shipped via Federal Express Priority service and are usually received the next day. Non North American International orders are usually received in 2-4 days. Cryopreserved cells can be sensitive to extended times at dry ice temperatures. If your transit time will exceed 3 days, please inquire about dry vapor shipper options for cryopreserved cells. Please inquire if alternate couriers are needed.

**All orders should be processed immediately upon shipment receipt.**

## STORAGE CONDITIONS

| | | |
|---|---|---|
| **Media:** | +4°C | Expires 30 days from ship date. |
| | -20°C | Expires 6 months from ship date. |
| **Cryopreservation Media:** | -20°C | Expires 12 months from ship date. |
| **Cryopreserved Cells:** | Store in vapor phase nitrogen (-150°C to -190°C) IMMEDIATELY UPON RECEIPT. **Any other use negates the warranty.** | |
| **Live Plated Cells:** | Must be processed IMMEDIATELY UPON RECEIPT. Read this manual for handling instructions. **Any other use negates the warranty.** | |

***All Zen-Bio Inc. products are for research uses only. Not approved for human or veterinary use or for use in diagnostic or clinical procedures or other uses in humans.***

## ORDERING INFORMATION AND TECHNICAL SERVICES


ZenBio, Inc.
3920 South Alston Avenue
Durham, NC 27713 U.S.A.

| | |
|---|---|
| **Telephone** | +1 (919) 547-0692 |
| **Toll free (continental US only)** | 1-866-ADIPOSE 1-(866)-234-7673 |
| **Electronic mail (e-mail)** | information@zen-bio.com |
| **World Wide Web** | http://www.zen-bio.com |


## THIS MANUAL IS SUITABLE FOR USE WITH THE FOLLOWING PRODUCTS:

| | |
|---|---|
| SP-L1-F | 3T3-L1 PREADIPOCYTES, CRYOPRESERVED (500,000 CELLS/VIAL) |
| SP-L1- | 3T3-L1 PREADIPOCYTES, PLATED |

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## LIMITED PRODUCT WARRANTY

This warranty limits our liability to replacement of this product. No other warranties of any kind, expressed or implied, including without limitation implied warranties of merchantability or fitness for a particular purpose, are provided by ZenBio, Inc. ZenBio, Inc. shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

ZenBio, Inc warrants the performance of cells only if ZenBio media are used and the recommended storage conditions and protocols are followed without amendment or substitution. ZenBio, Inc. cryopreserved cells are assured to be viable when stored as recommended and thawed according to ZenBio protocols and using the recommended protocol.

Contact ZenBio, Inc. within no more than 24 hours after receipt of products for all claims regarding shipment damage, incorrect ordering or other delivery issues. Delivery claims received after 7 days of receipt of products are not subject to replacement or refund.

## PRECAUTIONS

**This product is for research use only.** It is not intended for human, veterinary, or in vitro diagnostic use. Proper precautions and biological containment should be taken when handling cells to ensure their continued viability. All media, supplements, and tissue cultureware used in this protocol should be sterile.

## INTRODUCTION

3T3-L1 adipocytes have been fundamental in metabolic disease research for 30 years. Originally derived from Swiss mouse embryo tissue by Dr. Howard Green of Harvard Medical School, the 3T3-L1 system has been pivotal in advancing the understanding of basic cellular mechanisms associated with diabetes, obesity and related disorders.

## QUALITY CONTROL

3T3-L1 preadipocytes are assessed for viability, growth, morphology, lipolysis, and differentiation into adipocytes. 3T3-L1 preadipocytes are also assessed for pathogens (read page 12).

# CATALOG ITEMS

- **3T3-L1 Cryopreservation Medium**
  - Cat # FM-1-L1
  - Store -20°C
- **3T3-L1 Preadipocyte Medium**
  - Cat # PM-1-L1
  - Store according to label
- **3T3-L1 Adipocyte Differentiation Medium**
  - Cat # DM-2-L1
  - Store according to label
- **3T3-L1 Adipocyte Maintenance Medium**
  - Cat # AM-1-L1
  - Store according to label
- **3T3-L1 Basal Medium**
  - Cat # BM-1-L1
  - Store according to label
- **Live, Plated 3T3-L1 Preadipocytes** (sub-confluent) **(SP-L1-X##)**
  - Cat# SP-L1 - 24 (24-well plate)
    48 (48-well plate)
    96 (96-well plate)
    T25 (T-25cm$^2$ flask)
  - Follow protocol on page 6 **IMMEDIATELY UPON RECEIPT**
- **Cryopreserved 3T3-L1 Preadipocytes**
  - Cat # SP-L1-F
  - Cryopreserved vial containing at least 500,000 viable 3T3-L1 cells per vial **(store in vapor phase liquid nitrogen IMMEDIATELY upon receipt)** *any other storage negates the warranty*

# MEDIA COMPOSTIONS

| **3T3-L1 Preadipocyte Medium** |
|---|
| Cat # PM-1-L1 500 mL |
| DMEM, 4.5 g/L (25 mmol/L) D-glucose |
| HEPES pH 7.4 |
| Bovine Calf Serum (BCS; USA Origin) |
| Penicillin |
| Streptomycin |
| Amphotericin B |

| **3T3-L1 Adipocyte Differentiation Medium** |
|---|
| Cat # DM-2-L1 100 mL |
| DMEM / Ham's F-12 (1:1, v/v) 3.15 g/L (17.5 mmol/L) D-glucose |
| HEPES pH 7.4 |
| Fetal Bovine Serum (FBS; USA Origin) |
| Biotin |
| D-Pantothenic Acid |
| Human Insulin, recombinant |
| Dexamethasone |
| 3-Isobutyl-1-methylxanthine (IBMX) |
| PPAR γ agonist |
| Penicillin |
| Streptomycin |
| Amphotericin B |

| **3T3-L1 Adipocyte Maintenance Medium** |
|---|
| Cat # AM-1-L1 500 mL |
| DMEM / Ham's F-12 (1:1, v/v) 3.15 g/L (17.5 mmol/L) D-glucose |
| HEPES pH 7.4 |
| Fetal Bovine Serum (FBS; USA Origin) |
| Biotin |
| D-Pantothenic Acid |
| Human Insulin, recombinant |
| Dexamethasone |
| Penicillin |
| Streptomycin |
| Amphotericin B |

| **3T3-L1 Basal Medium** |
|---|
| Cat # BM-1-L1 500 mL |
| DMEM / Ham's F-12 (1:1, v/v) 3.15 g/L (17.5 mmol/L) D-glucose |
| HEPES pH 7.4 |
| Biotin |
| D-Pantothenic Acid |

| **3T3-L1 Cryopreservation Medium** |
|---|
| Cat # FM-1-L1 100 mL |
| DMEM, 4.5 g/L (25 mmol/L) D-glucose |
| Bovine Calf Serum (BCS; USA Origin) |
| Dimethyl Sulfoxide (DMSO) |

All media are also available without serum and/or phenol red free.
Please inquire for custom media requests.

## MEDIA EXPIRATION DATES:

*If placed at +4℃ upon arrival, the media is stable until the expiration date on the bottle label.*

*If stored at -20℃ upon arrival, the media is stable for 6 months. Add fresh antibiotics at 1% when you are ready to use. The media will expire 30 days after the thaw date.*

*Cryopreservation media expiration date will be 12 months from the ship date if stored at -20°C.*

# RECEIVING AND MAINTAINING LIVE PLATED CELLS

## 3T3-L1 PREADIPOCYTES

Your live, plated 3T3-L1 preadipocytes have arrived in our patented CellPorter™ packaging system. Upon receiving the plates, please follow the instructions carefully to ensure your safety and the optimal performance of these cells.

1. Check the seal for each plate. Discard any plate where the vacuum seal has been compromised during shipment. ALWAYS WEAR GLOVES AND USE PROTECTIVE MEASURES WHEN HANDLING CULTURED CELLS.

2. Place the package into a sterile environment using sterile technique. **THIS IS VERY IMPORTANT SINCE BREAKING THE VACUUM SEAL MAY POTENTIALLY INTRODUCE CONTAMINATION INTO THE PLATE.** Use scissors to snip open the bag at any end. The vacuum seal should be released at this time. You may notice some bubbling of the medium in the plate at this time. This is normal and will not affect cell performance.

3. Still in a sterile environment, remove the plate from the bag, taking care to not disturb the cover top from the plate. Open the lid and remove the white liner using sterile forceps or a hemostat and discard. Carefully remove the clear adhesive seal by grabbing the edge with sterile forceps or hemostat and lifting the film slowly towards the other end. Discard adhesive film in appropriate biohazard waste container. Replace lid on plate.

4. The excess medium added to each well for shipping should be removed before incubation in a humidified atmosphere $CO_2$ incubator. Depending upon the plate configuration, please use the chart below to determine medium volume to remove from each well.

| Cultureware | Total shipping volume per well | Removal volume per well |
|---|---|---|
| 96 well plates | 300 µL/well | 150 µL |
| 48 well plates | 1.3 mL/well | 0.8 mL |
| 24 well plates | 3.0 mL/well | 2.0 mL |
| 25 $cm^2$ flask | 72 mL/flask | 65 mL |

5. Keep the plates at 37ºC with 5% $CO_2$ in a humidified incubator until ready for use. The cells should be fed with 3T3-L1 Preadipocyte Medium (PM-1-L1) every 2-3 days until confluent. Read the next section (Differentiation Procedure) for differentiation protocol.

# DIFFERENTIATION PROCEDURE

## 3T3-L1 PREADIPOCYTES TO 3T3-L1 ADIPOCYTES

Note: Primary cells require use of sterile tissue culture treated cultureware. No extracellular matrix coatings are required.

1. Remove cells from liquid nitrogen and place immediately into a 37°C water bath and agitate while in bath. Be careful not to submerge the cap of the vial into water. Do not leave the vials in water bath after most of the content has thawed, no longer than 1 minute. Rinse the vials with 70% ethanol before taking them to the culture hood.

2. Upon the thawing, add the cells to a sterile conical bottom centrifuge tube, containing 10 mL of 3T3-L1 Preadipocyte Medium (cat# PM-1-L1).

3. Centrifuge at 280 x *g* / 20°C for 5 minutes. Aspirate the supernatant being careful not to disturb the cell pellet and resuspend cells in a volume of PM-1-L1 appropriate for counting the cells. Count using a hemocytometer.

4. With a seeding density of 5,000 cells/cm$^2$, plate in tissue culture treated cultureware using PM-1-L1.

5. Maintain cells until they are 100% confluent (in about 6-7 days) in a humidified incubator, 37°C, with 5-10% $CO_2$. Cells will need to be fed every other day with PM-1-L1 during this time. Read Table 1 for feeding volumes.

6. **Once the cells are confluent, incubate an additional 48 hours before initiating differentiation.**

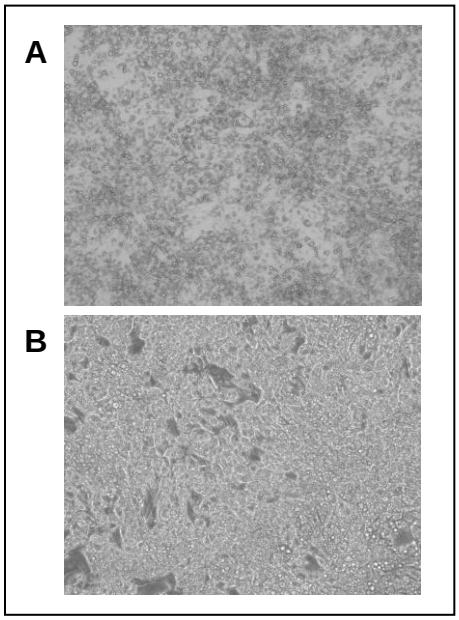
7. Two days after the cells have been confluent, remove the PM-1-L1 and replace with an appropriate volume 3T3-L1 Differentiation Medium (cat# DM-2-L1) using Table 1 below for recommended volumes. Incubate for 3 days after initiating differentiation.

8. Remove the DM-2-L1 and replace with 3T3-L1 Adipocyte Maintenance Medium (cat# AM-1-L1). Incubate for 2-3 days.

9. Feed cells every 2-3 days using AM-1-L1 until ready for assay. 3T3-L1 adipocytes are suitable for most assays 7-14 days post differentiation (see Figure 1 and Figure 2).

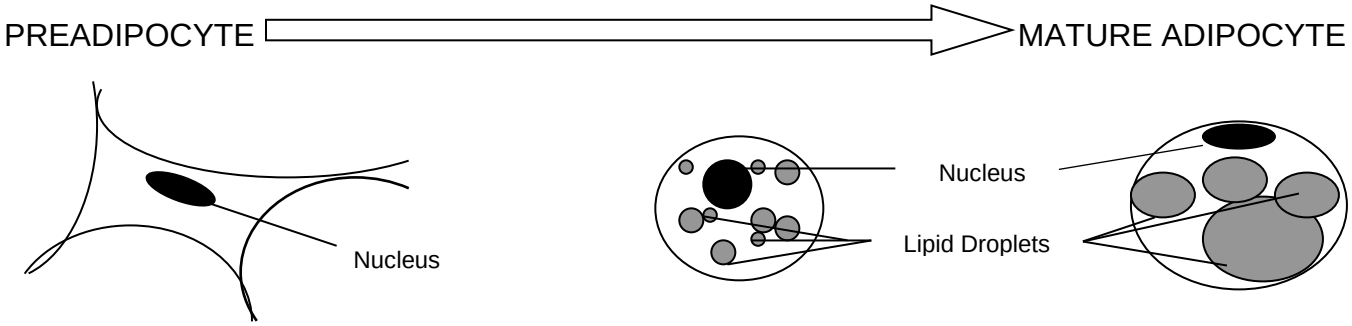
**Table 1** **Feeding Volumes**

| Format | Change PM-1-L1 to PM-1-L1 | | Change PM-1-L1 to DM-2-L1 | | Change DM-2-L1 to AM-1-L1 | | Change AM-1-L1 to AM-1-L1 | |
|---|---|---|---|---|---|---|---|---|
| | OUT | IN | OUT | IN | OUT | IN | OUT | IN |
| 96 well plate | 90 µL/well | 90 µL/well | 150 µL/well | 150 µL/well | 90 µL/well | 120 µL/well | 90 µL/well | 90 µL/well |
| 48 well plate | 300 µL/well | 300 µL/well | 500 µL/well | 500 µL/well | 300 µL/well | 400 µL/well | 300 µL/well | 300 µL/well |
| 24 well plate | 0.6 mL/well | 0.6 mL/well | 1.0 mL/well | 1.0 mL/well | 0.6 mL/well | 0.8 mL/well | 0.6 mL/well | 0.6 mL/well |
| 12 well plate | 1.2 mL/well | 1.2 mL/well | 2.0 mL/well | 2.0 mL/well | 1.2 mL/well | 1.6 mL/well | 1.2 mL/well | 1.2 mL/well |
| 6 well plate | 1.8 mL/well | 1.8 mL/well | 3.0 mL/well | 3.0 mL/well | 1.8 mL/well | 2.4 mL/well | 1.8 mL/well | 1.8 mL/well |
| T-75 flask | 12 mL/flask | 12 mL/flask | 20 mL/flask | 20 mL/flask | 12 mL/flask | 16 mL/flask | 12 mL/flask | 12 mL/flask |
| T-25 flask | 4.2 mL/flask | 4.2 mL/flask | 7 mL/flask | 7 mL/flask | 4.2 mL/flask | 5.6 mL/flask | 4.2 mL/flask | 4.2 mL/flask |

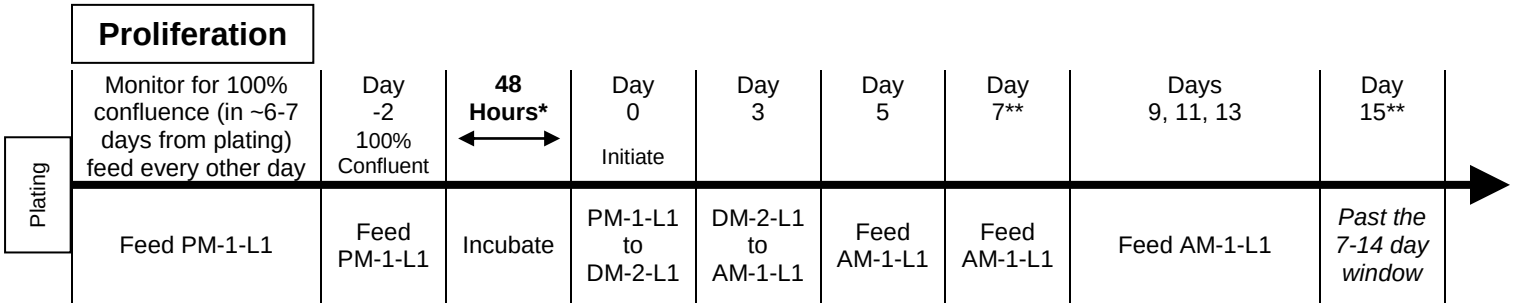



## Figure 1
## Lipid accumulation in 3T3-L1 cells cultured in Zen Bio media.

3T3-L1 preadipocytes were seeded in 24 well plates and induced to differentiate 2 days post confluent using Zen Bio's DM-2-L1 for 3 days. Cells were then fed Zen Bio's AM-1-L1, with fresh media being added every other day. Phase contrast images were taken on day 7 (Panel A) and day 14 (Panel B) of differentiation using an Olympus IX60 microscope equipped with a STOP digital camera (20X magnification)




## Figure 2
## 3T3-L1 Growth and Differentiation Feeding Schedule




* Once the cells are 100% confluent, incubate an additional 48 hours before initiating differentiation. The cells require this time to initiate growth arrest.

** 3T3-L1 adipocytes are suitable for most assays 7-14 days post differentiation.

# EXPANSION PROCEDURE

## 3T3-L1 PREADIPOCYTES

1. Remove cells from liquid nitrogen and place immediately into a 37°C water bath and agitate while in bath. Be careful not to submerge the cap of the vial into water. Do not leave the vials in water bath after most of the content has thawed, no longer than 1 minute. Rinse the vials with 70% ethanol before taking them to the culture hood.

2. Upon the thawing, add the cells to a sterile conical bottom centrifuge tube, containing 10 mL of 3T3-L1 Preadipocyte Medium (cat# PM-1-L1).

3. Centrifuge at 280 x *g* / 20°C for 5 minutes. Aspirate the supernatant being careful not to disturb the cell pellet and resuspend cells in a volume of PM-1-L1 appropriate for counting the cells. Count using a hemocytometer.

4. With a seeding density of 3,000 - 5,000 cells/$cm^2$, plate in tissue culture treated cultureware using PM-1-L1.

5. Incubate cells until they are 80-85% confluent (in about 5-6 days). Do not let the cells become 100% confluent. Cells will need to be fed every other day with PM-1-L1 while in incubation.

6. Aspirate medium and wash preadipocytes 4-5 times using sterile Dulbecco's Phosphate Buffered Saline without calcium or magnesium (cat# DPBS-1000) to remove all traces of serum (until there is no foaming of the medium).

7. Remove the DPBS-1000 and release the cells from the bottom of the cultureware vessel by adding 30 µL/$cm^2$ of 0.25% trypsin/ 2.21mM EDTA solution (cat# TRP-100). Allow cells to detach for 5 minutes at 37°C. Tap the flask gently to loosen the cells.

8. Neutralize the trypsin using at least 100 µL/$cm^2$ PM-1-L1. Check under a microscope to ensure all cells are detached.

9. Count the cells and plate in desired format (refer back to step 4 for plating density for further expansion, or refer to page 7, step 4 for plating density for differentiation). Ensure cells are evenly suspended when plating large numbers of plates or flasks. Place in a humidified incubator at 37°C and 5-10% $CO_2$, making sure the surface is level for even cell distribution.

10. If expanding the cells for differentiation, follow the differentiation protocol as outlined on pages 7-8. If expanding to cryopreserve, follow the cryopreservation protocol as outlined on page 10.

11. We DO NOT recommend expanding 3T3-L1 preadipocytes that are older than Passage 12-13. 3T3-L1 is NOT an immortalized cell line and the cells will not perform well. Cells will arrive at Passage 8.

# CRYOPRESERVATION PROCEDURE

## 3T3-L1 PREADIPOCYTES

1. 3T3-L1 preadipocytes should be sub confluent (less than 85% confluent) upon harvest for expansion or cryopreservation.

2. Aspirate medium and wash preadipocytes 4-5 times using sterile Dulbecco's Phosphate Buffered Saline without calcium or magnesium (cat# DPBS-1000) to remove all traces of serum (until there is no foaming of the medium).

3. Remove the DPBS-1000 and release the cells from the bottom of the cultureware vessel by adding 30 µL/cm$^2$ of 0.25% trypsin/ 2.21mM EDTA solution (cat# TRP-100). Allow cells to detach for 5 minutes at 37°C. Tap the flask gently to loosen the cells.

4. Neutralize the trypsin using 0.1 mL 3T3-L1 Preadipocyte Medium (cat# PM-1-L1) per cm$^2$ cultureware surface area (7.5 mL for T-75 flask). Check under a microscope to ensure all cells are detached.

5. Centrifuge at 280 x *g* / 20°C for 5 minutes. Aspirate the medium and suspend cells in a volume of PM-1-L1 appropriate for counting the cells. Count using a hemocytometer.

6. Centrifuge at 280 x *g* / 20°C for 5 minutes. Suspend in cold 3T3-L1 Cryopreservation Medium (cat# FM-1-L1) at a concentration of 1 million cells/mL. Do not exceed a 6:1 ratio of cells (per million) to volume cryopreservation medium (per mL). Remember to account for the volume of the cell pellet before adding the volume of cryopreservation medium necessary for cell suspension.

7. If using a controlled-rate freezer: Freeze by reducing the temperature 1°C per minute until the temperature reaches -80°C. If using a cell cryopreservation container, prepare according to the manufacturer's instructions.

8. For best results we recommend transferring the vials to the vapor phase of a liquid nitrogen storage facility as soon as possible after the cells have reached -80°C.

# TROUBLESHOOTING GUIDE

| Observation | Possible Cause(s) | Suggestion(s) |
|---|---|---|
| **3T3-L1 Preadipocytes do not grow.** | • Cells have been passaged too many times. | • Use cells of a lower passage number. The 3T3-L1 cell line is NOT immortalized. It is recommended not to use them past passage 12-13. The cells arrive at passage 8.<br>• 3T3-L1 cells grow faster in an incubator set to 10% CO2. |
| **Edge effects.** | • Medium in outside wells evaporated. | • Ensure a saturated humidity in the incubator and feed the cells no less than every 3 days. Make sure multiple plates are stacked no more than 3 plates high. |
| **3T3-L1 Preadipocytes do not differentiate well.** | • Cells have been passaged too many times. | • Use cells of a lower passage number. The 3T3-L1 cell line is NOT immortalized. It is recommended not to use them past passage 12-13. The cells arrive at passage 8.<br>• Ensure cells are 100% confluent for 48 hours prior to initiating differentiation.<br>• Do not use fetal bovine serum during the proliferation process. It will affect later differentiation potential. We recommend using Zen-Bio's 3T3-L1 Preadipocyte Medium (cat # PM-1-L1). |
| **3T3-L1 Preadipocytes don't differentiate after thawing.** | **1.** Incorrect media used.<br><br>**2.** Cells too confluent upon harvest. | **1.** Use ZenBio 3T3-L1 Preadipocyte Medium (cat# PM-1-L1) for growth and Cryopreservation Medium (cat# FM-1-L1) for storage to ensure optimal 3T3-L1 performance. 3T3-L1 cells exposed to FBS during expansion do not differentiate consistently.<br>**2.** Collect cells when no more than 85% confluent. |

# FREQUENTLY ASKED QUESTIONS

**What media do I need to differentiate the cells?**

- In order to complete the differentiation process, you will need 3T3-L1 Preadipocyte Medium (cat# PM-1-L1), 3T3-L1 Differentiation Medium (cat# DM-2-L1) and 3T3-L1 Adipocyte Maintenance Medium (cat# AM-1-L1). At a minimum, PM-1-L1 is needed to maintain the cells.
- Please order media according to your needs.

**What is the formulation of Zen-Bio's serum-free media?**

- Zen-Bio's serum-free media are not enhanced to supplement the absence of serum. These media are available for assay procedures where cells are rested from serum.

**Should antibiotics be included in the medium?**

- Yes. Antibiotics and anti-fungal agents are always recommended since the cells are primary cells. All listed Zen-Bio media in this manual contain antibiotics and anti-fungal agents except 3T3-L1 Basal Medium (cat# BM-1-L1) and 3T3-L1 Cryopreservation Medium (cat# FM-1-L1).

**When do the cells differentiate?**

- Lipid droplets should appear within 4-7 days after differentiation is induced. They will look extremely small initially. Lipid accumulation continues throughout the first two weeks. The lipid droplets gradually fuse to several big locules. (See Figures 1 & 2, page 8)

**Do you provide ready-to-use plated 3T3-L1 adipocytes?**

- No. At this time they are too sensitive to the stresses of shipping during differentiation. Only cryopreserved and sub-confluent preadipocytes are provided as live plated cells.

**What plated formats do you provide for 3T3-L1 preadipocytes?**

- We provide plated 3T3-L1 preadipocytes in the formats listed in the section Catalog Items on page 4.

**What is the concentration of ingredients in your media?**

- We do not disclose the concentrations of the components of our media. We are happy to prepare custom media to your specifications. Please inquire for custom formulations.

# PATHOGEN TESTING

Our 3T3-L1 cells are tested for sterility and for mycoplasma contamination via direct plating and DNA fluorochrome staining; mycoplasma contamination is not detected.