



3T3-L1 Cell Care Manual

Maintenance and Differentiation of 3T3-L1 Preadipocytes to Adipocytes

INSTRUCTION MANUAL ZBM0009.07

SHIPPING CONDITIONS

Cells are shipped using dry ice or dry vapor shipper. Orders are delivered via Federal Express or DHL courier. All US and Canada orders are shipped via Federal Express Priority service and are usually received the within 1-2 days. International orders are usually received in 2-4 days. Alternate couriers and dry vapor shippers are available if needed. Please inquire.

STORAGE CONDITIONS

Media: Short Term (30 days from ship date) 4°C 6 months -20°C

Cryopreserved cells: Store in vapor phase nitrogen (-150°C to -190°C) IMMEDIATELY UPON RECEIPT.

Live plated 3T3-L1 preadipocytes: Must be processed immediately upon receipt. See manual for handling instructions.

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

ORDERING INFORMATION AND TECHNICAL SERVICES

ZenBio, Inc.

3920 South Alston Avenue

PO Box 13888

Durham, NC 27713

U.S.A.

Telephone

(919) 547-0692

Facsimile (FAX)

(919) 547-0693

Toll free (continental US only)

1-866-ADIPOSE 1-(866)-234-7673

Electronic mail (e-mail)

information@zenbio.com

World Wide Web

<http://www.zenbio.com>

THIS MANUAL IS SUITABLE FOR USE WITH THE FOLLOWING PRODUCTS:

SP-L1-F	3T3-L1 PREADIPOCYTES, CRYOPRESERVED
SP-2096; SP-2048; SP-2024; SP-2012	3T3-L1 PREADIPOCYTES, PLATED

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LIMITED PRODUCT WARRANTY

This warranty limits our liability for 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 Zen-Bio, Inc. Zen-Bio, 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.

Zen-Bio, Inc warrants the performance of cells only if Zen-Bio media are used and the recommended protocols and storage conditions are followed. Cryopreserved cells are assured to be viable when thawed according to Zen-Bio protocols and using the recommended cultureware.

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.

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.

MATERIALS PROVIDED FOR EACH CATALOG ITEM

❖ **Plated 3T3-L1 Preadipocytes**

- Cat# SP-2096; SP-2048; SP-2024; SP-2012
- Sub-confluent cells
- Follow the MAINTENANCE OF PLATED 3T3-L1 PREADIPOCYTES Instructions below

❖ **Cryopreserved 3T3-L1 preadipocytes**

- Catalog # SP-L1-F
- Frozen vial containing at least 500,000 3T3-L1 preadipocytes- Passage 8 (store in liquid nitrogen upon receipt)

3T3-L1 MEDIA COMPOSITIONS

3T3-L1 Adipocyte Medium (cat # AM-1-L1) DMEM / Ham's F-12 medium (1:1, v/v) HEPES pH 7.4 Fetal Bovine Serum (FBS) Biotin Pantothenate Human insulin Dexamethasone Penicillin Streptomycin Amphotericin B	3T3-L1 Preadipocyte Medium (cat # PM-1-L1) DMEM, 4.5 g/L D glucose HEPES pH 7.4 Bovine Calf Serum (BCS) Penicillin Streptomycin Amphotericin B
3T3-L1 Differentiation Medium (cat # DM-2-L1) DMEM / Ham's F-12 medium (1:1, v/v) HEPES pH 7.4 Fetal Bovine Serum (FBS) Biotin Pantothenate Human insulin Dexamethasone Penicillin Streptomycin Amphotericin B 3-Isobutyl-1-methylxanthine (IBMX) PPAR γ agonist	3T3-L1 Basal Medium (cat # BM-1-L1) DMEM/Ham's F-12 medium (1:1, v/v) HEPES pH 7.4 Biotin Pantothenate 3T3-L1 Cryopreservation Medium (cat# FM-1-L1) DMEM, high glucose Dimethyl sulfoxide (DMSO) Bovine Calf Serum (BCS)

NOTE:

All media except cat# PM-1-L1 contain 3.15g/L D-glucose.

PM-1-L1 contains 4.5g/L D-glucose.

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°C upon arrival, the media is stable until the +4°C expiration date on the bottle label.

If stored at -20°C upon arrival, the media is stable until the -20°C 6 months post ship date listed on the bottle label. Add fresh antibiotics at 1% when you are ready to use. The media will expire 30 days after the thaw date.

MAINTENANCE OF PLATED 3T3-L1 PREADIPOCYTES

Your 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. 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. 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 CO₂ 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
12 well plates	5.8 ml/well	3.8 ml

5. Keep the plates at 37°C with 5% CO₂ 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. See page 6 for differentiation protocol.

DIFFERENTIATION OF 3T3-L1 PREADIPOCYTES INTO ADIPOCYTES

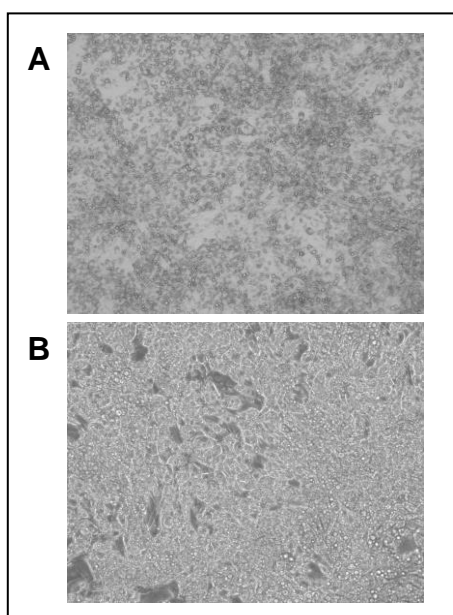
Cryopreserved 3T3-L1 Preadipocytes (Catalog # SP-L1-F)

Please note: Some cells can be very sensitive to brands of cultureware. Zen-Bio does not currently recommend the use of Corning Falcon or Sarstedt brand plates or flasks. Our scientists are using Nunc, Corning Costar, or Greiner Bio-One tissue culture treated plates and flasks. Please contact us if you have any questions.

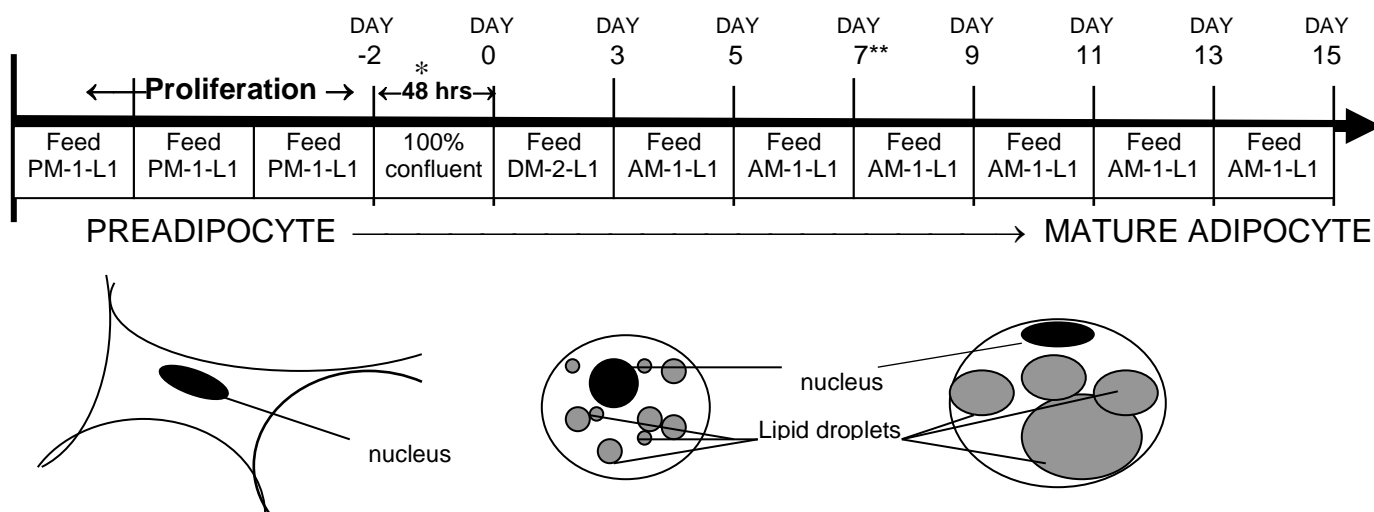
1. Remove cells from liquid nitrogen and place immediately into a 37°C water bath with agitation. Be careful not to submerge the cap of the vial into water. Do not leave the vials in water bath after most of the content has thawed. Rinse the vials with 70% ethanol before taking them to the culture hood.
2. Upon the thawing, add the cells to a sterile conical bottom centrifuge tube, containing 10 ml of 3T3-L1 Preadipocyte Medium (PM-1-L1).
3. Centrifuge at 280 x g, 20°C, 5 minutes. Aspirate the medium and resuspend cells in a volume of PM-1-L1 appropriate for counting the cells. Count using a hemacytometer.
4. Place approximately 5,000 cells/cm² in tissue culture treated cultureware using 3T3-L1 Preadipocyte Medium (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% CO₂. Cells will need to be fed every other day with PM-1-L1 during this time. See 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 3T3-L1 Preadipocyte Medium (cat# PM-1-L1) and replace with an appropriate volume 3T3-L1 Differentiation Medium (cat# DM-2-L1; see table 1 below for recommended volumes). Incubate for 3 days.
8. Remove the 3T3-L1 Differentiation Medium and replace with 3T3-L1 Adipocyte Maintenance Medium. Incubate for 2-3 days.
9. Feed cells every 2-3 days using 3T3-L1 Adipocyte Maintenance Medium 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	90µl/well	90µl/well	150µl/well	150 µl / well	90 µl /well	120µl /well	90 µl /well	90µl /well
48 well plate	300 µl /well	300 µl /well	500µl/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.

EXPANSION OF 3T3-L1 PREADIPOCYTES _____

Cryopreserved 3T3-L1 Preadipocytes (Catalog # SP-L1-F)

1. Remove cells from liquid nitrogen and place immediately into a 37°C water bath with agitation. 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. 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 (PM-1-L1).
3. Centrifuge at 280 x g, 20°C, 5 minutes. Aspirate the medium and resuspend cells in a volume of PM-1 appropriate for counting the cells. Count using a hemacytometer.
4. Place approximately 3,000- 5,000 cells/cm² in tissue culture treated cultureware using 3T3-L1 Preadipocyte Medium (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.
6. Aspirate medium and wash preadipocytes 4-5 times using sterile Phosphate Buffered Saline (PBS) to remove all traces of serum (until there is no foaming of the medium). Remove the PBS and release the cells from the bottom of the cultureware vessel by adding 30µl/cm² 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.
7. Neutralize the trypsin using at least 100µl/cm² 3T3-L1 Preadipocyte Medium (cat# PM-1-L1). Check the vessel under a microscope to ensure all cells are free of the flask bottom.
8. Count the cells and plate in desired format (see page 6). Ensure cells are evenly suspended when plating large numbers of plates or flasks. Place in a humidified incubator at 37°C and 5-10% CO₂, making sure the surface is level for even cell distribution.
9. Follow the differentiation protocol as outlined on pages 6- 7.
10. We DO NOT recommend expanding the 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.

3T3-L1 CRYOPRESERVATION PROCEDURE

1. 3T3-L1 preadipocytes should be sub confluent (less than 85% confluent) upon harvest for expansion or cryopreservation.
2. Aspirate medium and wash cells 4-5 times using sterile Phosphate Buffered Saline without magnesium or calcium (PBS) to remove all traces of serum (until there is no foaming of the medium).
3. Remove the PBS and release the cells from the cultureware bottom by adding 2 ml/T-75 flask of 0.25% trypsin/ 2.21 mM EDTA solution.
4. Incubate cells with trypsin solution for 5 minutes at 37°C.
5. Neutralize the trypsin using 0.1 ml 3T3-L1 Preadipocyte Medium (cat# PM-1-L1) per cm² cultureware surface area (7.5 ml for T-75 flask). Check under a microscope to ensure all cells are removed.
6. Centrifuge at 280 x g, 20°C, 5 minutes. Aspirate the medium and suspend cells in a volume of PM-1-L1 appropriate for counting the cells. Count using a hemocytometer.
7. Centrifuge at 280 x g, 20°C, 5 minutes. Suspend in cold 3T3-L1 Cryopreservation Medium (cat# FM-1-L1) at a concentration of 1×10^6 cells/ml. Do not exceed a 6:1 ratio of cells (per million): 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.
8. 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.
9. 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 causes	Suggestions
Preadipocytes do not differentiate well	Cells have been passaged too many times	<ul style="list-style-type: none"> - Use cells of a lower passage number. The 3T3-L1 cell line is NOT immortalized and is suitable until passage 12-13 - Ensure cells are 100% confluent for 48 hours prior to initiating differentiation - 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)
Preadipocytes do not grow	Cells have been passaged too many times	<ul style="list-style-type: none"> -Use cells of a lower passage number. The 3T3-L1 cell line is NOT immortalized and is suitable only until passage 12-13. Cells will arrive at Passage 8. -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 don't differentiate after thawing	<ul style="list-style-type: none"> -Incorrect media used. -Cells too confluent upon harvest 	<ul style="list-style-type: none"> -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. -Collect cells when no more than 85% confluent.

FREQUENTLY ASKED QUESTIONS

QUESTION	ANSWER
What media do I need to differentiate the cells?	<ul style="list-style-type: none"> - 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). - Please order media according to your needs.
What is the formulation of Zen-Bio's serum-free media?	<ul style="list-style-type: none"> - 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?	<ul style="list-style-type: none"> - Yes. Antibiotics and anti-fungal agents are always recommended since the cells are primary cells. All Zen-Bio media contain antibiotics and anti-fungal agents except Basal Medium (BM-1-L1).
When do the cells differentiate?	<ul style="list-style-type: none"> - 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]
Do you provide ready to use plated 3T3-L1 adipocytes?	<ul style="list-style-type: none"> - 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?	<ul style="list-style-type: none"> - We provide 3T3-L1 preadipocytes in the following formats: - 96-well, 48-well, 24-well, 8-chamber slides
What are the concentrations of components in your media?	<ul style="list-style-type: none"> - We do not disclose the concentrations of the components of our media. - We are happy to prepare custom media to your specifications. Please inquire

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.