# Subcutaneous Human Adipocyte Manual

Maintenance and Differentiation from Preadipocytes to Adipocytes

## INSTRUCTION MANUAL ZBM0001.08

## SHIPPING CONDITIONS

**Human Preadipocytes, Cryopreserved/ Plated Preadipocytes or Adipocytes**
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 primary human 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. |
| **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, North Carolina 27713 USA

| | |
|---|---|
| **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-F | CRYOPRESERVED SUBCUTANEOUS HUMAN PREADIPOCYTES |
| SPD-F | CRYOPRESERVED SUBCUTANEOUS HUMAN PREADIPOCYTES, DIABETIC DONOR |
| BR-F | CRYOPRESERVED SUBCUTANEOUS HUMAN BREAST FAT TISSUE PREADIPOCYTES |
| SQE-F | CRYOPRESERVED SUBCUTANEOUS HUMAN PREADIPOCYTES FROM THE FACE |
| SP/ SA | PLATED SUBCUTANEOUS HUMAN PREADIPOCYTES/ ADIPOCYTES |

## CONTENTS PAGE #


| | |
|---|---|
| Limited Product Warranty | 3 |
| Precautions | 3 |
| Introduction | 3 |
| Quality Control | 4 |
| Catalog Items | 4 |
| Media Compositions | 5 |
| Receiving and Maintaining Live Plated Cells | 6-7 |
| Differentiation Procedure | 7-8 |
| Plating Procedure | 9 |
| Expansion Procedure | 10 |
| Cryopreservation Procedure | 11 |
| Troubleshooting Guide | 12 |
| Frequently Asked Questions | 13-15 |
| Pathogen Testing | 16 |
| References | 16 |

# 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 of human origin, due to their potential biohazardous nature. **Always wear gloves and work behind a protective screen when handling primary human cells.** All media, supplements, and tissue cultureware used in this protocol should be sterile.

Human preadipocyte and adipocyte viability depends greatly on the use of suitable media, reagents, and sterile plastic wear. If these parameters are not carefully observed, cell growth may be slower than expected.

# INTRODUCTION

Subcutaneous human preadipocytes are isolated from human subcutaneous adipose tissue obtained from a competent volunteer adult donor undergoing elective surgery in the United States. Each donor has signed an Institutional Review Board (IRB) validated donor consent form that specifically lists both the intended uses for non-clinical research and confirms the procedures for processing the samples are Standard Operating Procedure (SOP) managed Good Laboratory Practices (GLP) protocols in compliance with ethical regulations. All samples are collected and processed in the United States.

The adipocyte precursor cells (preadipocytes) are isolated from subcutaneous adipose tissue of healthy non-diabetic and diabetic donors between undergoing elective surgery. The preadipocytes are isolated by centrifugal force after collagenase treatment. Preadipocytes can be cultured as growing precursor cells or differentiated into adipocytes using medium supplemented with adipogenic and lipogenic hormones. This instruction manual describes procedures to induce human preadipocytes to differentiate into mature adipocytes as well as culturing methods for human preadipocytes and adipocytes. The process of differentiating preadipocytes to adipocytes has been patent protected by ZenBio under US patent number 6153432.

# QUALITY CONTROL

Contaminating endothelial cells are undetectable (CD31 NEGATIVE) by flow cytometry in preadipocytes. This product has been tested and complies with ZenBio, Inc. quality specifications. Preadipocytes are plated and differentiated as outlined in the Subcutaneous Adipocyte Care Manual (ZBM0001). The resulting cultured mature adipocytes (2 weeks post-differentiation) accumulate lipid, respond to lipolytic agents, and secrete leptin and adiponectin.

# CATALOG ITEMS

❖ **Cryopreservation Medium for Subcutaneous Human Preadipocytes**
- Cat # FM-1-100
- Store -20°C

❖ **Preadipocyte Medium**
- Cat # PM-1 (500mL), PM-1-250 (250mL)

❖ **Adipocyte Maintenance Medium**
- Cat # AM-1 (500mL), AM-1-250 (250mL)

❖ **Adipocyte Differentiation Medium**
- Cat # DM-2 (100mL), DM-2-500 (500mL)

❖ **Basal Medium**
- Cat # BM-1

❖ **Live, Plated Subcutaneous Human Preadipocytes** **SP-20##-X**

- Cat # SP - 2006 (6-Well Plate) - 1 (BMI < 24.9)
  2012 (12-Well Plate) 2 (BMI 25.0-29.9)
  2024 (24-Well Plate) 3 (BMI ≥ 30.0)
  2048 (48-Well Plate) SL (Multiple Donors)
  2096 (96-Well Plate)
- Approximately 100% confluent upon arrival (ready for differentiation) unless otherwise noted.

❖ **Live, Plated Subcutaneous Human Adipocytes** **SA-1###-X / SA-##-X**

- Cat # SA - 1006 (6-Well Plate) - 1 (BMI < 24.9)
  1012 (12-Well Plate) 2 (BMI 25.0-29.9)
  1024 (24-Well Plate) 3 (BMI ≥ 30.0)
  1048 (48-Well Plate) SL (Multiple Donors)
  1096 (96-Well Plate)
  1384 (384 Well Plate)
  25 (T-25 Flask)
  75 (T-75 Flask)
- **Ensure a 2 week lead time**, adipocytes need time to differentiate from preadipocytes.

❖ **Cryopreserved Subcutaneous Human Preadipocytes** **(2 million cells/vial)**
- Cat # SP-F-1 (BMI <24.9), SP-F-2 (BMI 25.0-29.9), SP-F-3 (BMI ≥30.0), SP-F-SL (Multiple Donors)

❖ **Cryopreserved Subcutaneous Human Preadipocytes from a Diabetic Donor** **(1 million cells/vial)**
- Cat # SPD-F-1 (LEAN, BMI <24.9), SPF-F-2 (BMI 25.0-29.9), SPD-F-3 (BMI ≥30.0)

❖ **Cryopreserved Subcutaneous Human Breast Fat Tissue Preadipocytes** **(1 million cells/vial)**
- Cat # BR-F

❖ **Cryopreserved Subcutaneous Human Preadipocytes from the Face** **(1 million cells/vial)**
- Cat # SQE-F

**All cryopreserved cells MUST be stored in vapor phase liquid nitrogen IMMEDIATELY upon receipt.** ***Any other storage negates the warranty.***

# MEDIA COMPOSITIONS

| **Preadipocyte Medium** Cat # PM-1 500 mL |
|---|
| DMEM / Ham's F-12 (1:1, v/v) |
| HEPES pH 7.4 |
| Fetal Bovine Serum (FBS; USA Origin) |
| Penicillin |
| Streptomycin |
| Amphotericin B |

| **Adipocyte Differentiation Medium** Cat # DM-2 100 mL |
|---|
| DMEM / Ham's F-12 (1:1, v/v) |
| 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 |

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

| **Basal Medium** Cat # BM-1 500 mL |
|---|
| DMEM / Ham's F-12 (1:1, v/v) |
| HEPES pH 7.4 |
| Biotin |
| D-Pantothenic Acid |

| **Cryopreservation Medium** Cat # FM-1-100 100 mL |
|---|
| DMEM / Ham's F-12 (1:1, v/v) |
| HEPES pH 7.4 |
| Biotin |
| D-Pantothenic Acid |
| Fetal Bovine Serum (FBS; USA Origin) |
| Dimethyl Sulfoxide (DMSO) |

All media contain 3.15g/L (17.5 mmol/L) D-glucose.
All media are also available as phenol red free and/or without serum. 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, it is stable 6 months. Add fresh antibiotics 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

Your live, plated preadipocytes or adipocytes 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 on each plate. Discard any plate where the vacuum seal has been compromised during shipment. Please be aware that these cells are of human origin. Please treat them as potentially infectious since we cannot test for all pathogens. ALWAYS WEAR GLOVES AND WORK BEHIND A PROTECTIVE SCREEN WHEN HANDLING PRIMARY HUMAN CELLS.
2. Place the package into a sterile environment using sterile technique. **THIS IS VERY IMPORTANT AS BREAKING THE VACUUM SEAL MAY POTENTIALLY INTRODUCE CONTAMINATION INTO THE PLATE.** Use scissors to cut 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 the 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 what volume of media should be 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 |
| 6 well plates | 8.8 mL/well | 5.8 mL |
| 75 $cm^2$ flask | 260 mL/flask | 240 mL |
| 25 $cm^2$ flask | 72 mL/flask | 65 mL |

5. Keep the plates at 37°C with 5% $CO_2$ in a humidified incubator until ready for use.
6. Once all above steps have been completed, proceed to one of the following sections:

## ❖ PLATED PREADIPOCYTES

7. If the cells are to be differentiated into adipocytes, differentiation should be initiated immediately. [see page 7, Differentiation Procedures]
8. If the cells are to be maintained as preadipocytes and not differentiated, they should be fed with Preadipocyte Medium (PM-1) every other day until they are confluent.

Preadipocytes are flat, phase-dark spindle-shaped cells. The cells have a similar appearance in culture to fibroblasts or smooth muscle cells [see Figure 1-A, page 8]. The majority of the preadipocytes will differentiate into adipocytes [see Figure 1-C, page 8] using Adipocyte Differentiation Medium (cat# DM-2) and Adipocyte Maintenance Medium (cat# AM-1) as described in this manual. The differentiation

efficiency varies depending on the donor. The donor information provided on the certificate of analysis is limited to age, gender, race, Body Mass Index (BMI), smoking status, medications, collection date, and location that the sample was taken from (face, hips, abdomen, *etc*.). Please read the Certificate of Analysis that came with your order for information specific to the cells you are using.

## ❖ PLATED ADIPOCYTES

7. When feeding adipocytes, we recommend you remove and replace approximately half of the volume of each well with Adipocyte Maintenance Media (cat# AM-1). The adipocytes should remain healthy and responsive for at least 4 weeks after induction of differentiation. Unless otherwise stated on the plate, cultured adipocytes will be 2-3 weeks old upon receipt. Different lots will vary due to donor variation. We recommend doing one whole set of experiments using cells from the same lot number. When large numbers of plates are needed, please contact ZenBio to reserve a lot for any specific orders or long-term experiments.

# DIFFERENTIATION PROCEDURE

## PREADIPOCYTES TO ADIPOCYTES

1. Preadipocytes are plated confluent in Preadipocyte Medium (cat# PM-1) and shipped the same day via overnight delivery. Differentiation should be initiated within 24 hours after receiving the cells. Please contact ZenBio, Inc. to coordinate the shipping date with your schedule.

2. To start the process, aspirate the entire volume of Preadipocyte Medium from all wells. Add the appropriate volume of Adipocyte Differentiation Medium (catalog # DM-2) to the wells [see Table 1: Feeding Volumes]. Incubate plate for 7 days at $37^0$C and 5% $CO_2$.

3. After 7 days, cells should be fed by removing a portion of the media [See Table 1: Feeding Volumes] and replacing with fresh Adipocyte Medium (catalog # AM-1). Add new medium gently. If using an automatic feeder, set the slowest flow rate possible.

4. Two (2) weeks after differentiation has been initiated, cells should appear rounded with large lipid droplets apparent in the cytoplasm [see Figure 1-C]. Cells are now considered mature adipocytes and are suitable for most assays.

### Table 1: Feeding Volumes

| Cultureware Format | Plating | Change PM-1 to DM-2 | | Change DM-2 to AM-1 | | Change AM-1 to AM-1 | |
|---|---|---|---|---|---|---|---|
| | IN | OUT | IN | OUT | IN | OUT | IN |
| 96 well plate | 150 µL/well | 150 µL/well | 150 µL/well | 90 µL/well | 120 µL/well | 90 µL/well | 90 µL/well |
| 48 well plate | 500 µL/well | 500 µL/well | 500 µL/well | 300µL/well | 400 µL/well | 300 µL/well | 300 µL/well |
| 24 well plate | 1.0 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 | 2.0 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 | 3.0 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 | 20 mL/flask | 20 mL/flask | 20 mL/flask | 12 mL/flask | 16 mL/flask | 12 mL/flask | 12 mL/flask |
| T-25 flask | 7 mL/flask | 7 mL/flask | 7 mL/flask | 4.2mL/flask | 5.6 mL/flask | 4.2 mL/flask | 4.2 mL/flask |

**CAUTION:** Do not allow live plated cells to dry out.

## Figure 1: Preadipocytes to Adipocytes

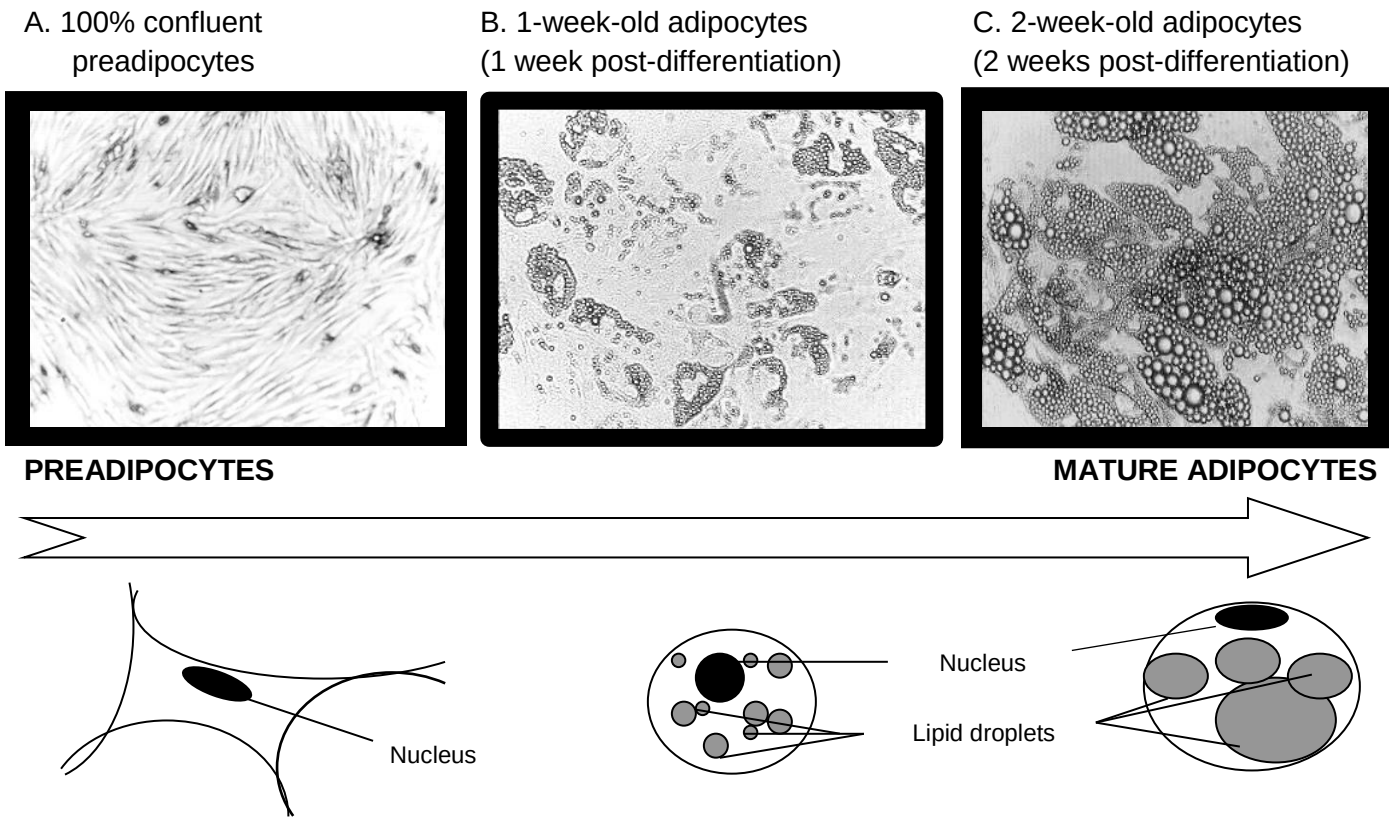


Figure 1: Photographs of 100% confluent Preadipocytes (A), 1-week-old (post-differentiation) cultured adipocytes (B) and mature (2 weeks post-differentiation) cultured Adipocytes (C).

These are unstained photographs of human preadipocyte morphology (20X). The cells should appear comparable in appearance to these pictures. The preadipocytes should be confluent 24-48 hours after plating for differentiation. If they are not 100% confluent, the cells will not differentiate well. Please see the Troubleshooting Guide [page 12] for any problems.

# PLATING PROCEDURE

## Cryopreserved Subcutaneous Preadipocytes (Catalog # SP-F)

Please note: Primary human preadipocyte 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 thawing, transfer the cells to a sterile conical bottom centrifuge tube containing 10 mL of Preadipocyte Medium (cat # PM-1).

3. Centrifuge at 1,200 rpm (282 X *g*) / 20°C for 5 minutes. Aspirate the supernatant. DO NOT ASPIRATE ANY OF THE CELL PELLET.

4. The cell vial contains a minimum of 2 million viable cells; however, we recommend performing a cell count to determine a more exact number of cells. Resuspend the cell pellet in 2 mL PM-1, dilute an aliquot in 0.4% trypan blue solution. We suggest withdrawing an aliquot of 50 µL of the cells and mixing with 100µL of the trypan blue solution, resulting in a dilution factor of 3. Count live (unstained) cells on a hemocytometer.

5. Plate approximately 40,625 cells/$cm^2$ using the media volumes from the table below. Refer to the manufacturer's specifications for the plating area ($cm^2$) of the cultureware brand you are using.

| FORMAT | VOLUME PER WELL | TOTAL VOLUME PER FORMAT* |
|---|---|---|
| 96 well plate | 150 µL | 14.4 mL |
| 48 well plate | 500 µL | 24.0 mL |
| 24 well plate | 1 mL | 24.0 mL |
| 12 well plate | 2 mL | 24.0 mL |
| 6 well plate | 3 mL | 18.0 mL |
| 10 cm dish | 15 mL | 15.0 mL |
| T-75 flask | 20 mL | 20.0 mL |
| T25 flask | 7 mL | 7.0 mL |

**We recommend preparing slightly larger volumes to allow for loss due to foam and pipet error.*

6. Plate cells in desired format and place in a humidified 37ºC incubator with 5% $CO_2$. Do not agitate the plate, as cells will not plate evenly.

7. Twenty-four (24) hours after plating, check the plates for confluence. If they are not completely confluent, leave for an additional 24 hours maximum before inducing differentiation. If the cells are not confluent after 48 hours, DO NOT INDUCE DIFFERENTIATION (differentiation will be poor). Contact ZenBio immediately.

8. To differentiate the cells, see the section Differentiation Procedure [page 7], starting at step 2.

# EXPANSION PROCEDURE

## Cryopreserved Subcutaneous Preadipocytes

Please note: Primary human preadipocyte 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 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, 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 Preadipocyte Medium (cat # PM-1).

3. Centrifuge at 1,200 rpm (282 X *g*) / 20°C for 5 minutes. Aspirate the supernatant. DO NOT ASPIRATE ANY OF THE CELL PELLET. Count using a hemocytometer.

4. Place approximately 670,000 cells in T-75 culture flasks using PM-1.

5. Incubate cells until they are 85-90% confluent (in about 4-5 days). Do not let the cells become 100% confluent [see Figure 1-A, page 8]. Cells will need to be fed every other day with PM-1.

6. Aspirate medium and wash preadipocytes 4-5 times using sterile 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 flask by adding 2 mL/ T-75 flask (or 6 mL/T-225 flask) of 0.25% trypsin/2.21mM EDTA solution.

8. Allow cells to detach for 5 minutes at 37°C. Tap the outside of the flask gently with your hand to loosen the cells. Check the flask under a microscope to ensure all cells are detached from the bottom of the flask.

9. Neutralize the trypsin using 7 mL PM-1 per T-75 flask (or 21 mL per T-225 flask).

10. Count the cells and plate in desired format [see page 9, Plating Procedure, step 5]. Ensure cells are evenly suspended when plating large numbers of plates or flasks. Do not agitate plates and flasks after plating. Place in a humidified incubator at 37°C and 5% $CO_2$, making sure the surface is level for even cell distribution.

11. To differentiate the cells, see the section Differentiation Procedure [page 7], starting at step 2. We DO NOT recommend differentiating preadipocytes that are older than Passage 5. Cells will arrive at Passage 2 or 3.

# CRYOPRESERVATION PROCEDURE

1. Aspirate medium and wash cells 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).
2. Warm the Trypsin-EDTA (0.25% trypsin/2.21 mM EDTA) solution (cat# TRP-100) in a 37°C water-bath.
3. Remove the DPBS-1000 and release the cells from the cultureware bottom by adding 2 mL/T-75 flask of TRP-100.
4. Incubate cells with trypsin for 5 minutes at 37°C.
5. Neutralize the trypsin using 0.1 mL Preadipocyte Medium (cat# PM-1) per $cm^2$ cultureware surface area (7.5 mL for T-75 flask). Check under a microscope to ensure all cells are detached.
6. Add the trypsin-medium-cell mixture to a sterile conical centrifuge tube then centrifuge at 1,200 rpm (282 X *g*) / 20°C for 5 minutes. Aspirate the medium and suspend cells in a volume of PM-1 appropriate for counting the cells. Count using a hemocytometer.
7. Centrifuge again at 1,200 rpm (282 X *g*) / 20°C for 5 minutes.
8. Suspend in cold cryopreservation medium at a concentration of 1-2 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.
9. 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.
10. 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.** | 1. Cells have been passaged too many times.<br>2. Differentiation conditions not optimal.<br>3. Cells were plated at a low density.<br>4. Cultureware used was not optimal for human primary adipocytes.<br>5. Differences in cultureware brand surface area may affect plating density if unknown. | 1. Use cells of a lower passage number.<br>2. Use our defined differentiation media. **Make sure that wells are confluent BEFORE initiating differentiation.**<br>3. Use the cell density recommended in our manual.<br>4. Use sterile tissue culture treated cultureware. ZenBio scientists are using cultureware from Thermo Fisher Nunc, Corning Costar, Greiner Bio-One, CytoOne, and Sarstedt.<br>5. Verify the surface area for the cultureware brand you are using. |
| **Preadipocytes do not attach well or do not grow.** | 1. Cells have been passaged too many times.<br>2. Cells expanded too high.<br>3. Cultureware used not optimal for human primary preadipocytes/adipocytes.<br>4. Cells not viable upon thawing. | 1. Use cells of a lower passage number.<br>2. Do not exceed 1:6 expansion ratio.<br>3. Use sterile tissue culture treated cultureware. ZenBio scientists are using cultureware from Thermo Fisher Nunc, Corning Costar, Greiner Bio-One, CytoOne, and Sarstedt.<br>4. Primary human cells are to be stored in vapor phase liquid nitrogen immediately upon arrival. **Any other temperature storage will result in poor quality and negate the warranty.** |
| **Edge effects.** | 1. Medium in outside wells evaporated. | 1. Ensure a saturated humidity in the incubator. Make sure multiple plates are stacked no more than 3 plates high. |
| **Adipocytes appear uneven in each well.** | 1. Medium was completely removed during feeding.<br>2. Fresh medium was added too quickly.<br>3. Cells placed on uneven surface in the incubator. | 1. Make sure to follow instructions listed in Table 1: Feeding Volumes.<br>2. Add media slowly to each well. Position the pipet tips halfway down, pressing on the side of the wells and slowly release the medium.<br>3. Place cultureware are on a level surface in the incubator to ensure cells attach evenly. |

# FREQUENTLY ASKED QUESTIONS

## 1. What media do I need to differentiate the cells?

- In order to complete the differentiation process, you will need the following:
  - Preadipocyte Medium (cat # PM-1, or PM-1-250)
  - Adipocyte Differentiation Medium (cat # DM-2, or DM-2-500)
  - Adipocyte Maintenance Medium (cat # AM-1, or AM-1-250).

## 2. When do I use the Basal Medium (cat # BM-1)?

- Basal Medium (cat # BM-1) is only needed if you need to rest your cells from antibiotics, serum, or hormones such as insulin prior to assay.
- The differentiated adipocytes are stable in BM-1 medium for up to 3 days.

## 3. When do the cells differentiate?

- Oil droplets should appear within 4-7 days after differentiation is induced. They look extremely small initially. Lipid accumulation continues throughout the first two weeks. The oil droplets gradually fuse to several big fat droplets. [See Figure 1, page 8]

## 4. Can I passage the cells?

- Adipocytes cannot be passaged because they are terminally differentiated and will NOT reattach to cultureware nor remain intact after being trypsinized.
- Preadipocytes can be trypsinized and re-plated several times, however preadipocytes grow slower with each passage and differentiate poorly after passage 4.
- Cells are shipped at passage 2 or 3.
- Do not exceed a 1:6 expansion ratio as listed above in the Troubleshooting section.

## 5. Do I have to use your Cryopreservation Medium?

- It is highly recommended to use our Cryopreservation Medium (cat # FM-1-100) to preserve the cells.

## 6. How fast do the cells replicate?

- The average doubling time is approximately 48 hours. However, please note that the replication rate for human preadipocytes varies from donor to donor.

## 7. How long do the cells last in culture?

- Adipocytes retain similar morphology and express adipocyte-specific genes for at least 4 weeks after induction of differentiation.
- Cultured adipocytes are usually shipped at 2 weeks old.
- Adipocytes should only be fed fresh Adipocyte Maintenance Medium (cat # AM-1) every 7 days.

## 8. Should antibiotics be included in the medium?

- Yes. Antibiotics and anti-fungal agents are always recommended for primary cells.
- All Zen-Bio media contain antibiotics and anti-fungal agents except Basal Medium (cat # BM-1).

## 9. Where do you obtain your cells?

- The preadipocytes are isolated from human subcutaneous adipose tissue obtained from competent volunteer consented adult donors undergoing elective surgeries in the United States.

**10. How are the cells shipped?**

- Cells cultures in multiple-well plates are sealed using our patented CellPorter™ package method and are shipped to customers via Federal Express overnight delivery.

**11. How long do I have to wait before receiving the cells?**

- US orders are shipped Monday through Thursday.
- Canadian orders are shipped Monday through Wednesday.
- International orders are shipped only on Mondays or Fridays.
- We do not ship orders to domestic locations on Fridays unless you are confirmed to be available for Saturday delivery (small fee applies). This means a person must be present who can process the cells and store them properly on the arrival day.
- Cryopreserved preadipocytes are usually shipped no later than the second day after the purchase order is confirmed unless otherwise specified on the order.
- In general, preadipocytes in culture are shipped the second day after the purchase order is confirmed. The cells are plated early in the morning and shipped the same day via FedEx Priority Overnight courier.
- Preadipocytes are plated to be 100% confluent and ready for differentiation upon arrival unless otherwise noted on your order.
- Lead time for plated adipocytes is always at least 2 weeks.

**12. Is the β3receptor present on these cells?**

- Yes, to a small extent. The subcutaneous adipocytes express β1 adrenoceptor (~35%) and β2 adrenoceptor (~65%).

**13. Do these cells express leptin? How do you measure it?**

- Yes. Mature adipocytes (greater than 2 weeks post-differentiation) do secrete leptin.
- Commercial leptin ELISA kits are available from many vendors.

**14. Do the cells respond to insulin treatment?**

- Yes. Insulin does stimulate both glucose uptake and lipid accumulation.

**15. Can I differentiate the cells myself?**

- Yes. You can order preadipocytes and pre-made culture media for adipocyte differentiation. Instructions for differentiating the cells are found in the section titled Differentiation Procedure.

**16. Do you test for pathogens? Which ones?**

- Yes. Each lot of primary cells is tested via PCR and found non-reactive to viral DNA from HIV and hepatitis B and viral RNA from hepatitis C. However, no known test can offer complete assurance that these viruses are not present.
- Because we cannot test all pathogens, always treat the culture as a potentially infectious reagent. We recommend using the US Centers for Disease Control (CDC) Universal Precautions for prevention of blood-borne pathogens as a minimum guideline for standards of practice at Biosafety Level 1 (BLS-1) or higher.

**17. Do the cells express the uncoupling proteins? Which ones?**

- The UCP1 and UCP2 mRNA can be detected by PCR only after stimulating the cells with a PPAR γ agonist.

### 18. Can I order visceral (omental) adipose?

- Yes. Please visit our website for visceral (omental) adipose tissue derived cells and related reagents. Contact us for our current inventory.

### 19. How do I obtain RNA from the cells? How much RNA can I expect?

- Use RNeasy kit (Qiagen), or similar column based isolation kit.
- You can expect approximately 10µg total RNA from a 10cm dish of preadipocytes and 40-60µg of RNA from a 10cm dish of adipocytes.

### 20. What donor information do I receive?

- The donor's gender, age, race (if known), diabetes status, smoking status, BMI, and current medications list will be provided on the certificate of analysis.

### 21. Are the cells from one donor?

- We have both single donor lots from different body mass indices (BMI) and mixed donor lots available.
- Please inquire about availability of single donor lots and mixed donor lots (also called a superlot or a pooled donor lot) at the time the order is placed.

### 22. What if I want to test my own compounds in differentiation?

- Contact Zen-Bio at information@zen-bio.com or contracts@zen-bio.com for custom requests.

### 23. 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.

### 24. 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. They are simply prepared in the absence of fetal bovine or calf serum. These media are available for assay procedures where cells are rested from serum. Do not differentiate preadipocytes in serum-free media.

### 25. What quality control is performed on the cells?

- Subcutaneous preadipocytes are tested for viability, correct vial counts, and sterility. They have also tested NEGATIVE for CD31 (cell surface marker for endothelial cells). Samples have also undergone PCR testing, and have been found non-reactive to viral DNA from HIV and Hepatitis B, and viral RNA from Hepatitis C.
- Preadipocytes differentiated into adipocytes are tested for triglyceride accumulation, isoproterenol induced lipolysis, and glucose uptake.

### 26. I have had my media in the freezer for 8 months. Is it still suitable to use?

- Our only data are for suitability after 6 months stored at -20°C. We cannot make any guarantees on performance beyond that time.

## PATHOGEN TESTING

Samples from each donor are tested via PCR and found non-reactive to viral DNA from HIV and Hepatitis B and viral RNA from Hepatitis C. However, no known test can offer complete assurance that these viruses are not present. Since we cannot test all pathogens, always treat the culture as a potentially infectious reagent. We recommend using the US Centers for Disease Control (CDC) Universal Precautions for prevention of blood-borne pathogens as a minimum guideline for standards of practice. Our products are tested and are free from mycoplasma contamination. Proper precautions and biological containment should be taken when handling cells of human origin, due to their potential biohazardous nature. All human based products should be handled at a BSL-1 (Biosafety Level 1) or higher. Always wear gloves and work behind a protective screen when handling primary human cells.

## REFERENCES

Lists of articles using ZenBio, Inc. cultured human cultured preadipocytes and adipocytes may be found on our website (http://www.zenbio.com).