Subcutaneous Human Adipocytes Manual

Maintenance and Differentiation from Preadipocytes to Adipocytes

INSTRUCTION MANUAL ZBM0001.05

SHIPPING CONDITIONS

Human Adipocyte/Preadipocyte Cells

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. International orders are usually received in 2-4 days. 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. 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
- Cryopreserved cells: Vials of frozen preadipocytes are to be stored in vapor phase nitrogen (-150°C to -190°C).
- Live plated cells: Must be processed immediately upon receipt. Read this manual instructions 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

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U.S.A.

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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-F-1,-2,-3,-SL; SP-X	HUMAN SUBCUTANEOUS PREADIPOCYTES AND ADIPOCYTES
SPD-F	HUMAN SUBCUTANEOUS PREADIPOCYTES, TYPE 2 DIABETIC DONOR
BR-F	HUMAN SUBCUTANEOUS BREAST FAT TISSUE PREADIPOCYTES
SQE-F	CRYOPRESERVED SUBCUTANEOUS PREADIPOCYTES FROM THE FACE.

CONTENTS

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 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 storage conditions, media and handling protocols are followed. Cryopreserved primary human cells are assured to be viable when thawed according to Zen-Bio protocols and using the recommended cultureware. Cell viability depends greatly on the use of suitable media, reagents, and sterile plastic wear. If these parameters are not carefully observed, limited differentiation may occur and cell growth may be slow.

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 uses only. **It is not intended for human uses, including clinical, diagnostic, therapeutic, or veterinary uses.** 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.

By your acceptance and use of these products, you are acknowledging that these products will be:

1. Treated as potentially contaminated biological specimens even if accompanying serological reports are negative;
2. Handled by established users and following appropriate biological safety control procedures to ensure the safety of using these products.

All media, supplements, and tissue cultureware used in this protocol should be sterile.

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 Zen-Bio 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 Zen-Bio, Inc quality specifications. Preadipocytes are plated and differentiated as outlined in the Subcutaneous Adipocyte Care Manual (ZBM-0001). The resulting cultured mature adipocytes (2 weeks post-differentiation) accumulate lipid, respond to lipolytic agents, and secrete leptin and adiponectin.

MATERIALS PROVIDED FOR EACH CATALOG ITEM

- **Human Subcutaneous Preadipocytes , live plated cells**
  - Cat# SP-2096; SP-2048; SP-2024; SP-2012; SA-2006; SP-75; SP-25 ; SP-15)

  Approximately 100 %confluent upon arrival (ready for differentiation) unless otherwise noted
- **Human Subcutaneous Adipocytes, live plated cells**
  - Cat# SA-1096; SA-1048; SA-1024; SA-1012; SA-1006; SA-75; SA-25)- ***2-week lead time***
- **Cryopreserved Human Subcutaneous Preadipocyte from Type 2 Diabetic Donor** Cat# SPD-F
- **Cryopreserved Human Subcutaneous Preadipocytes isolated from Breast Fat** . Cat# BR-F
- **Cryopreserved Human subcutaneous preadipocytes from face area** Cat# SQE-F
- **Cryopreserved subcutaneous preadipocytes** (catalog # SP-F-1,-2,-3,-SL)
  - Frozen vial containing 2 million viable preadipocytes
  - SP-F-1 from donor with BMI <24.9
  - SP-F-2 from donor with BMI 25.0-29.9
  - SP-F-3 from donor with BMI >30.0
  - SP-F-SL from pooled donor lot (multiple donors mixed together)

All cryopreserved preadipocytes are to be stored in vapor phase liquid nitrogen immediately upon arrival

MEDIA COMPOSITIONS SUBCUTANEOUS (PRE) ADIPOCYTES

Preadipocyte Medium cat # PM-1	Adipocyte Differentiation Medium cat # DM-2	Adipocyte Maintenance Medium cat # AM-1
DMEM / Ham's F-12 (1:1, v/v)	DMEM / Ham's F-12 (1:1, v/v)	DMEM / Ham's F-12 (1:1, v/v)
HEPES pH 7.4	HEPES pH 7.4	HEPES pH 7.4
Fetal Bovine Serum (FBS)	Fetal Bovine Serum (FBS)	Fetal Bovine Serum (FBS)
Penicillin	Biotin	Biotin
Streptomycin	Pantothenate	Pantothenate
Amphotericin B	Human insulin	Human insulin
	Dexamethasone	Dexamethasone
	3-Isobutyl-1-methylxanthine (IBMX)	Penicillin
	PPARγ agonist	Streptomycin
	Penicillin	Amphotericin B
	Streptomycin	
	Amphotericin B	

Basal Medium Cat# BM-1	Cryopreservation Medium Cat# FM-1-100
DMEM / Ham's F-12 (1:1, v/v)	DMEM / Ham's F-12 (1:1, v/v)
HEPES pH 7.4	HEPES pH 7.4
Biotin	Biotin
Pantothenate	Pantothenate
	Fetal Bovine Serum (FBS)

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°C upon arrival, the media is stable 30 days from the ship date
  - Use the +4°C expiration date listed on the bottle label.
- If stored at -20°C upon arrival, it is stable 6 months after the ship date
  - Use the -20°C expiration date listed on the bottle.
  - Upon thawing, add fresh antibiotics at 1% volume when you are ready to use.
  - The media will expire 30 days after the thawing date.
- Cryopreservation media expiration date will be 12 months from the ship date stored at -20°C.

MAINTENANCE OF PREADIPOCYTES

Your 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. 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 USE PROTECTIVE MEASURES WHEN HANDLING HUMAN PRIMARY 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 CO_2 incubator. Depending upon the plate configuration, please use the chart below to determine medium volumes 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
6 well plates	8.8 ml/well	5.8 ml
75cm^2 flask	260ml/flask	240 ml
25cm^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. Differentiation into adipocytes should be initiated immediately (see page 7). If cells are to be maintained as preadipocytes, they should be fed with Preadipocyte Medium (PM-1) every other day.

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). The majority of the preadipocytes will differentiate into adipocytes (see Figure 1-C) 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), diabetic status, smoking status and location of the sample removal (face, hips, abdomen, *etc*.) of the donor. Please read the Certificate of Analysis that came with your order for information specific to the cells you are using.

MAINTENANCE OF ADIPOCYTES

Your 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 for each plate. Call Zen-Bio if there is any problem with the 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 USE PROTECTIVE MEASURES WHEN HANDLING HUMAN PRIMARY 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 for incubation in a CO_2 incubator. When changing medium, do not remove all the liquid as the cells will detach and float. Depending upon the plate configuration, please use the chart below to determine medium volumes 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
6 well plates	8.8 ml/well	5.8 ml
75cm^2 flask	260ml/flask	240 ml
25cm^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) When feeding, we recommend you remove and replace approximately half of the volume of each well. The adipocytes should remain healthy and responsive for at least four 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 Zen-Bio to reserve a lot for any specific orders.

DIFFERENTIATION OF PREADIPOCYTES INTO 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 Zen-Bio, 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 37^0C and 5% CO_2.
3. After 7 days, cells should be fed by removing some of the media and replacing with fresh Adipocyte Medium (catalog # AM-1) (See Table 1. Feeding Volumes). Caution: Do not dry the wells. Add new medium gently. If using an automatic feeder, set the slowest flow rate possible.
4. Two (2) weeks after the initiation of differentiation, 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

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	150µl/ well	90 µl/ well	120 µl/ well	90 µl/ well	90 µl/ well
48 well plate	500 µl/ well	500µl/ well	500µl/ 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	5.6ml/flask	4.2 ml/flask	4.2 ml/flask

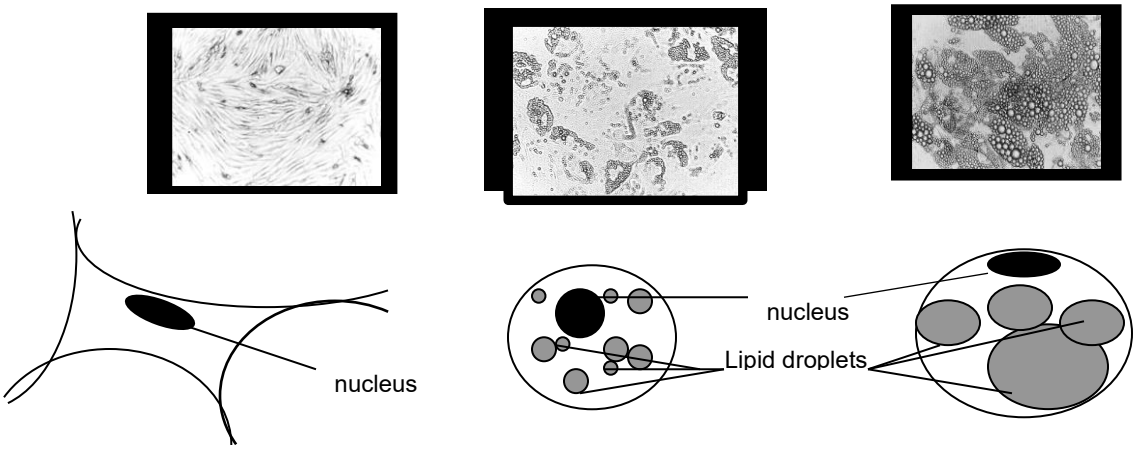

Figure 1: Photographs of 100% confluent Preadipocytes (A), 1-week-old (post-differentiation) cultured adipocytes (B) and mature (2 weeks post-differentiation) cultured Adipocytes (C). These are unstained photographs of human preadipocyte morphology (20X). The cells should appear comparable in appearance 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 for any problems.

PLATING PROCEDURE

Cryopreserved Subcutaneous Preadipocytes (Catalog # SP-F)

Please note: Primary cells can be very sensitive to brands of cultureware. Our scientists are currently using Nunc, Corning Costar, or Greiner Bio-One Cellstar 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 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. 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). Centrifuge: 1,200 rpm (282 X g) / 20°C / 5 minutes. Aspirate the supernatant. TAKE CARE TO NOT ASPIRATE ANY OF THE CELL PELLET.
3. The cell vial contains a minimum of 2.0 x 10^6 viable cells; however, we recommend performing a cell count to determine a more exact number of cells. Resuspend the cell pellet in 2 ml Preadipocyte Medium, dilute an aliquot in 0.4% trypan blue solution. We suggest withdrawing an aliquot of 50 µl of cells and mixing with 100 µl of the trypan blue solution, resulting in a dilution factor of 3. Count live (unstained) cells on a hemocytometer.
4. Plate approximately 40,625 cells / cm^2 using the media volumes from the table below. Refer to the manufacturer's specifications for the specific 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.

5. Plate cells in desired format and place in a humidified 37°C incubator with 5% CO_2. Do not agitate the plate, as cells will not plate evenly.
6. Twenty-four 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 Zen-Bio immediately.
7. To differentiate the cells please see the protocol on page 7 starting at step 2.

EXPANSION PROCEDURE

Cryopreserved Subcutaneous Preadipocytes

Please note: Primary cells can be very sensitive to brands of cultureware. Our scientists are using ThermoFisher Nunc, Corning Costar, Greiner Bio-One, CytoOne, Sarstedt 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 Preadipocyte Medium (PM-1).
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 hemocytometer.
4. Place approximately 670,000 cells in T-75 culture flasks using Preadipocyte Medium (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 for picture of 100% confluent cells). 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 (PBS) to remove all traces of serum (until there is no foaming of the medium). Remove the PBS and release the cells from the flask bottom by adding 2 mL/T-75 flask (or 6 ml/T-225 flask) of 0.25% trypsin/ 2.21mM EDTA solution. Allow cells to detach for 5 minutes at 37°C. Tap the outside of the flask gently with your hand to loosen the cells.
7. Neutralize the trypsin using 7 ml Preadipocyte Medium (cat# PM-1) per T-75 flask (or 21 ml per T-225 flask). Check the flask under a microscope to ensure all cells are free of the flask bottom.
8. Count the cells and plate in desired format (see page 10 for plating protocol). 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.
9. Follow the differentiation protocol as outlined on page 8.
10. 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 Phosphate Buffered Saline without calcium or magnesium (PBS) 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 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 (cat# TRP-100).
4. Incubate cells with trypsin solution 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 removed.
6. Add the trypsin-medium-cells to a sterile conical centrifuge tube then centrifuge at 280 x g, 20°C, 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 280 x g, 20°C, 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): 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 2. Differentiation conditions not optimal 3. Cells were plated at a low density 4. Cultureware used not optimal for human primary adipocytes 5. Differences in cultureware brand surface area may affect plating density if unknown	1. Use cells of a lower passage number 2. Use our defined differentiation media. Make sure that wells are confluent BEFORE initiating differentiation. 3. Use the cell density recommended in our manual 4. Zen-Bio scientists are using tissue culture treated culture-ware from Thermo Fisher Nunc, Corning Costar, Greiner Bio-One, CytoOne, Sarstedt 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 2. Cells expanded too high 3. Cultureware used not optimal for human primary adipocytes	1. Use cells of a lower passage number 2. Do not exceed 1:6 expansion ratio 3. Use tissue culture treated culture-ware from Thermo Fisher Nunc, Corning Costar, Greiner Bio-One, CytoOne, Sarstedt 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 2. Fresh medium was added too quickly	1. Make sure to follow instructions listed in Table 1 Feeding Volumes 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.
	3. Cells placed on uneven surface in the incubator	3. Place cultureware are on a level surface in the incubator to ensure cells attach evenly.

FREQUENTLY ASKED QUESTIONS

1	What media to I need to differentiate the cells?	In order to complete the differentiation process, you will need Preadipocyte Medium (cat# PM-1, PM-1-250), Adipocyte Differentiation Medium (cat# DM-2) and Adipocyte Maintenance Medium (cat# AM-1, AM-1-250).
2	When do I use the Basal Medium?	– 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]
4	Can I pass the cells?	– Adipocytes cannot be passed since they are terminally differentiated and will NOT reattach to cultureware nor remain intact. – Preadipocytes can be trypsinized and re-plated several times. – Preadipocytes grow slower with each passage and differentiate poorly after passage 4. – Cells are shipped at Passage 2-3. – Do not exceed a 1:6 expansion ration 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 ~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 AM-1 medium

		every 7 days.
8	Should antibiotics be included in the medium?	– 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 (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 cultured in multiple-well plates are sealed using our patented CellPorter™ package method and shipped to customers via Federal Express overnight delivery.
11	How long do I have to wait before receiving the cells?	– US orders are shipped Mondays –Thursdays. – Canada orders are shipped Mondays –Wednesdays. – International orders are shipped on Mondays and on Fridays. – We do not ship orders to domestic locations on Fridays unless you are available for Saturday delivery (small fee applies). – 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 2 weeks.
12	Is the β3 receptor present on these cells?	– Yes, to a small extent. The subcutaneous adipocytes express β1 adrenoceptor (~35%) and β2 adrenoceptor (~65%).
13	Do the 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 venders
14	Can I get brown fat cells?	– No. We do not have brown fat preadipocytes or brown fat tissues
15	Do the cells respond to insulin treatment?	– Yes. Insulin does stimulate both glucose uptake and lipid accumulation.
16	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 this manual.
17	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. – 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 at Biosafety Level 1 (BSL-1) or higher
18	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 PPARγ agonist.
19	Can I order visceral (omental) adipose?	– Yes. Please see our online pricelist for visceral (omental) adipose tissue derived cells and related reagents. – Contact us for our current inventory
20	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 20 μg total RNA from a 10 cm dish of preadipocytes and 40-60 μg of RNA from a 10 cm dish of adipocytes.
21	What donor information do I receive?	– The donor's gender, age, race (if known), type 2 diabetes status, smoking status, BMI and a current medications list will be provided.
22	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 and mixed donor (called a superlot) lots at time order is placed.
23	What if I want to test my own compounds in differentiation?	– We have Adiposight Differentiation kit to test your potential PPAR gamma agonists (cat#-DIF-AG, DIF-AG-NC). – Contact ZenBio at **information@zenbio.com** or **contracts@zenbio.com** for custom requests.
24	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.
25	What is the formulation of	– Zen-Bio's serum-free media are not enhanced to

	Zen-Bio's serum-free media?	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 medium.
26	What quality control is performed on the cells?	– Subcutaneous preadipocytes are tested for viability, correct vial counts, sterility; NEGATIVE for mycoplasma, HIV-1, HIV-2, hepatitis B, hepatitis C, CD31 (cell surface marker for endothelial cells). – Preadipocytes differentiated into adipocytes are tested for triglyceride accumulation, isoproterenol induced lipolysis, and glucose uptake
	I have had my media in the freezer for 8 months. Is it sill 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

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 using US Food and Drug Administration (FDA) tests. 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 at Biosafety Level 1 (BSL-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 at our website (http://www.zenbio.com).

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Human Dermal Fibroblast Manual

INSTRUCTIONAL MANUAL ZBM0023.05

SHIPPING CONDITIONS

Human Adult or Neonatal Dermal Fibroblast Cells

Orders are delivered via Federal Express courier. All US and Canada orders are shipped via Federal Express Priority service and are usually received the next day. International orders are usually received in 3-4 days.

Must be processed upon shipment receipt.

STORAGE CONDITIONS

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

Cells: Frozen: Vapor phase liquid nitrogen

Live Plated: 37°C incubator

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

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Research Triangle Park, NC 27709
U.S.A.

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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:

DF-F	HUMAN DERMAL FIBROBLASTS
DFN-F	HUMAN NEONATAL DERMAL FIBROBLASTS
DF-F-PS	HUMAN DERMAL FIBROBLASTS FROM PSORIASIS DONOR
DF-D-F	HUMAN DERMAL FIBROBLASTS FROM TYPE 2 DIABETIC DONOR
DF-1, DF-1-PRF	DERMAL FIBROBLAST MEDIUM, - PHENOL RED FREE
DFM-100	DERMAL FIBROBLAST CRYOPRESERVATION MEDIUM

CONTENTS PAGE

INTRODUCTION

Adult dermal fibroblasts are isolated from the dermis of healthy non-diabetic adult donors undergoing elective surgery. Neonatal dermal fibroblasts are isolated from the foreskins of healthy male newborns. The cells are isolated by centrifugal force following enzymatic treatment or from an explant culture. This instruction manual describes procedures to passage and culture the human dermal fibroblast cells.

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 dermal fibroblast cell viability depends greatly on the use of suitable media, reagents, and sterile plastic wear. If these parameters are not carefully observed, limited differentiation may occur and cell growth may be slow.

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 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 its cells only if Zen-Bio media are used and the recommended protocols are followed. Cryopreserved cells are assured to be viable when thawed and maintained according to Zen-Bio protocols.

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.

CATALOG ITEMS

NOTE: Effective February 1, 2016, we will no longer provide 50ml support medium with each cryopreserved vial ordered. Please order media according to your needs.

- **Human Adult Dermal Fibroblasts** (96-,48-,24-,12-,6-well plates; 75-,25-cm^2 flasks)
  - Cat # DF-4096, -4048, -4024, -4012, -4006, -4075, -4025

- **Dermal Fibroblast Cryopreservation Medium**
  - Cat# DFM-100

- **Cryopreserved Human Adult Dermal Fibroblasts**
  - Cat # DF-F
  - Vial containing 1 x10^6 viable adult dermal fibroblasts (store in vapor phase liquid nitrogen)

- **Cryopreserved Human Neonatal Dermal Fibroblasts**
  - Cat # DFN-F
  - Vial containing 500,000 viable neonatal dermal fibroblasts (store in vapor phase liquid nitrogen)

MEDIA COMPOSTIONS

Dermal Fibroblast Medium cat # DF-1 500ml	**Dermal Fibroblast Basal Medium cat # DF-2 500ml**	**Dermal Fibroblast Cryopreservation Medium cat # DFM-100 100ml**
DMEM	DMEM	DMEM
Fetal bovine serum	Penicillin	Fetal bovine serum
Penicillin	Streptomycin	DMSO
Streptomycin	Amphotericin B	
Amphotericin B		

All media contain 4.5 g/L (25 mmol/L) D-glucose.
All media are also available as without serum and/or phenol red.
Please inquire for custom media requests.

MEDIA EXPIRATION DATES:

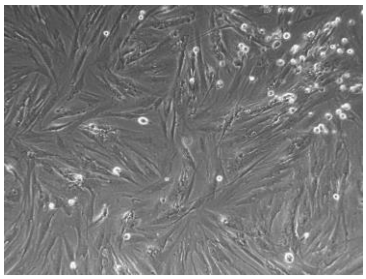
- If placed at 4°C upon arrival, the media is stable until the expiration date on the bottle label.
- If stored at -20°C upon arrival, the media is stable for 6 months. Add fresh antibiotics when you are ready to use. The media will now expire 30 days after the thaw date

PLATING AND EXPANSION PROCEDURES

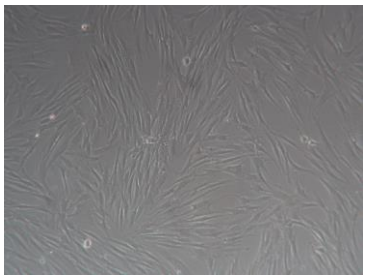
Cryopreserved Adult and Neonatal Dermal Fibroblasts

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 9 ml of Dermal Fibroblast Growth Medium (DF-1).
3. Centrifuge at 400 x g, 20°C, 10 minutes. Aspirate the medium and resuspend cells in a volume of DF-1 appropriate for counting the cells. Count using a hemacytometer.
4. Place approximately 10,000 cells/cm^2 (i.e. 0.75 X 10^6 cells in T-75 culture flask) using DF-1.
5. Incubate cells until they are 85-90% confluent (in about 3-5 days). Cells will need to be fed every 3 days with DF-1.
6. Aspirate medium and wash adult fibroblasts 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 flask bottom by adding 2 mL/T-75 flask (or 6 ml/T-225 flask) of 0.25% trypsin/ 2.21mM EDTA solution. Allow cells to trypsinize for 5 minutes at 37°C. Tap the flask gently to loosen the cells.
7. Neutralize the trypsin using 7 ml DF-1 per T-75 flask (or 21 ml per T-225 flask). Check the flask under a microscope to ensure all cells are free of the flask bottom.
8. Count the cells and plate in desired format at 10,000 cells/cm^2. 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.
9. Cells will need to be fed with fresh DF-1 every 3 days until the desired confluence is reached. The cells may be confluent within 3-8 days when plated at the recommended seeding density.

Figure 1. Neonatal and adult dermal fibroblasts, 3 days post-plating



A. Neonatal dermal fibroblasts



B. Adult dermal fibroblasts

CRYOPRESERVATION

10. ***OPTIONAL* – Cryopreserve dermal fibroblasts after counting.**
    a. Centrifuge at 280 x g, 20°C, 5 minutes.
    b. Suspend in cold Dermal Fibroblast Cryopreservation medium (Cat# DFM-100) at a concentration of $1X10^6$ cells/ml. Do not exceed a 6:1 ratio of cells (per million): volume freeze medium (per ml).
    c. Remember to account for the volume of the cell pellet before adding the volume of freeze medium necessary for cell suspension.
    d. 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
    e. . 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.

FREQUENTLY ASKED QUESTIONS

- **Can I passage the cells?**
  Dermal fibroblast cells can be trypsinized and replated several times. All cells are shipped after establishing a primary culture. We do not have any data on the limit of expansion of the cells.
- **How fast do the cells replicate?**
  The average doubling time ranges from 18-24 hours. However, keep in mind that the replication rate for human dermal fibroblasts varies from donor to donor.

- **Should antibiotics be included in the medium?**
  Yes. Antibiotics and anti-fungal agents are always recommended since the cells are primary cells.
- **Where are the cells obtained?**
  The adult dermal fibroblast cells are isolated from the dermal layer of human skin tissue. The neonatal cells are isolated from the dermal layer of newborn human foreskin tissue.
- **Do you test for pathogens? Which ones?**
  Yes. Samples from each donor are tested via PCR to confirm non-reactivity for HIV-1, HIV-2, hepatitis B and hepatitis C. However, since we cannot test all pathogens, please treat the culture as a potentially infectious agent.
- **What donor information do I receive?**
  The donor's age, gender, and BMI are provided in the certificate of analysis that accompanies each lot of cells.
- **Are there recommendations for cultureware to use with the cells?**
  Yes. Primary 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 Cellstar tissue culture treated plates and flasks.
- **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.

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-2 (Biosafety Level 2) or higher. Always wear gloves and work behind a protective screen when handling primary human cells.

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