Visceral Adipocyte Care Manual

Maintenance and Differentiation from Preadipocytes to Adipocytes

INSTRUCTION MANUAL ZBM0022.03

SHIPPING CONDITIONS

Human Visceral Adipocyte/Preadipocyte 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:	4°C	6 months:	-20°C
Cells:	Frozen:	liquid nitrogen	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.

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 visceral preadipocytes are assured to be viable when thawed and maintained according to Zen-Bio protocols.

ORDERING INFORMATION AND TECHNICAL SERVICES

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U.S.A.

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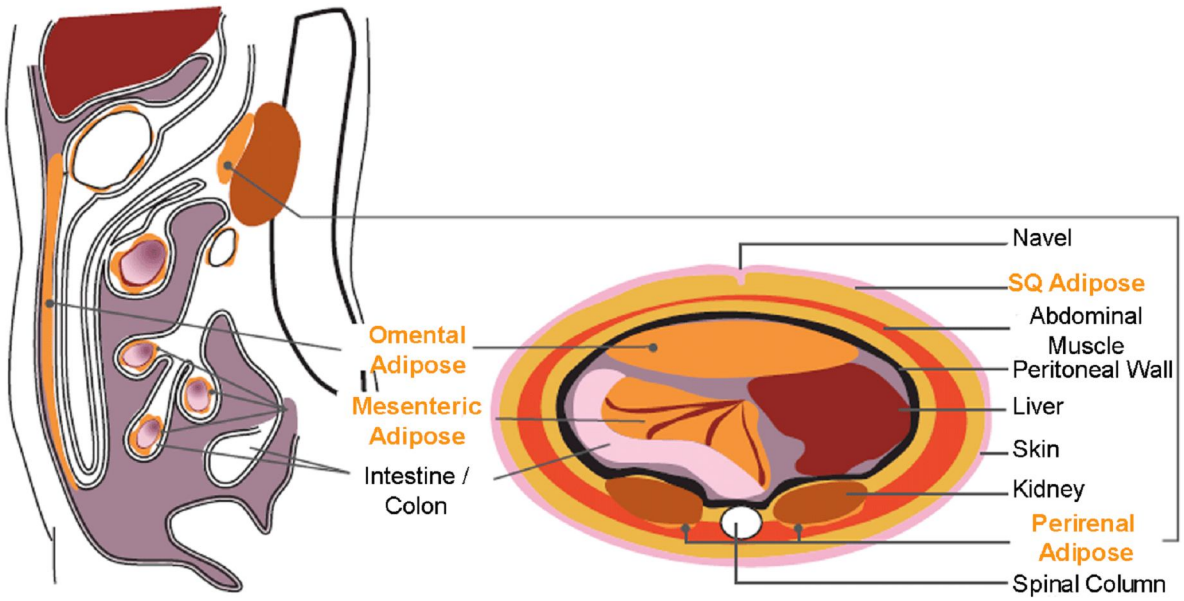
INTRODUCTION

Cultured human visceral adipocytes

Visceral 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 required 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.

A complication to commercial development of cultured visceral adipocytes is the varying definition of “visceral” within the scientific community. Some define omental and mesenteric fat as the only true visceral fat, whereas others include all intra-abdominal adipose tissue. Some researchers do not define it further than simply ‘not the subcutaneous layer’. The omentum (OM) is an immunologic organ composed of adipose, blood vessels and lymph nodes which overlays the abdominal organs within the peritoneal cavity. Mesenteric adipose tissue contained within the peritoneal cavity, is associated with the vasculature of the intestines and colon. Peri-renal adipose is attached to the kidneys.



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 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.

MATERIALS PROVIDED FOR EACH CATALOG ITEM

***Note**: Zen-Bio recommends that the Human Preadipocytes and Adipocytes be processed immediately upon receipt.*

- **Human Visceral Preadipocytes** (Includes Mesenteric, peri-renal, omental)
  - Cat# MP-2096,-2048,-2024,-2012,-2006,-75,-25 ; PR-2096,-2048,-2024,-2012,-2006,-75,-25 ; OP-2096,-2048,-2024,-2012,-2006,-75,-25)
  - Approximately 100 %confluent

- **Human Visceral Adipocytes** (Includes Mesenteric, peri-renal, omental)
  - Cat# MA-1096,-1048,-1024,-1012,-1006,-75,-25 ; PR-1096,-1048,-1024,-1012,-1006,-75,-25 ; OA-1096,-1048,-1024,-1012,-1006,-75,-25)

- **Cryopreserved visceral preadipocytes** (Includes Mesenteric, peri-renal, omental)
  - catalog # MSN-F, PR-F,OP-F
  - Frozen vial containing 1 x10^6 viable visceral preadipocytes (store in liquid nitrogen upon receipt)
  - 50 ml Omental Preadipocyte Medium (cat# OM-PM)

VISCERAL MEDIA COMPOSTIONS

Omental Adipocyte Medium

(cat # OM-AM)

DMEM / Ham's F-12 medium (1:1, v/v)
HEPES pH 7.4
Fetal bovine serum
Biotin
Pantothenate
Human insulin
Dexamethasone
Penicillin
Streptomycin
Amphotericin B

Omental Preadipocyte Medium

(cat # OM-PM)

DMEM/Ham's F-12 medium (1:1, v/v)
HEPES pH 7.4
Fetal bovine serum
Penicillin
Streptomycin
Amphotericin B

Omental Differentiation Medium

(cat # OM-DM)

Omental Adipocyte medium (OM-AM)
Isobutylmethylxanthine (IBMX)
PPARγ agonist

Omental Basal Medium

(cat # OM-BM)

DMEM/Ham's F-12 medium (1:1, v/v)
HEPES pH 7.4
Biotin
Pantothenate

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 added.
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 expire 30 days after the thaw date.**

MAINTENANCE OF VISCERAL PREADIPOCYTES

Your human visceral 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 as a guide to determine medium volumes to remove from each well. Please note some settling may occur during shipping. Please leave sufficient volume of medium to cover the cell monolayer.

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^{o}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 Omental Preadipocyte Medium (OM-PM) every other day.

6. Preadipocytes are flat, phase-dark spindle-shaped cells. The cells have a similar appearance in culture to fibroblasts or smooth muscle cells. The majority of the preadipocytes will differentiate into adipocytes using Omental Differentiation Medium (cat# OM-DM) and Omental Adipocyte Medium (cat# OM-AM) as described in this manual. The differentiation efficiency varies depending on the donor. Please see the Certificate of Analysis that came with your order for information specific to the cells you have received.

MAINTENANCE OF VISCERAL 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 as a guide to determine medium volumes to remove from each well. Please note some settling may occur during shipping. Please leave sufficient volume of medium to cover the cell monolayer.

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^{o}C with 5% CO_2 in a humidified incubator until ready for use.

6. The adipocytes should be fed with Omental Adipocyte Medium (OM-AM) if not used within 5-7 days of arrival**.** When feeding, we recommend you remove and replace approximately half of the volume of each well.

7. The adipocytes should remain healthy and responsive for at least three weeks after induction of differentiation. Unless otherwise stated on the plate, cultured adipocytes will be 2-3 weeks old upon receipt. Human differentiated adipocytes do not replicate and cannot be trypsinized without cell lysis. Different lots will vary due to patient variation. When large numbers of a particular lot are needed, please contact Zen-Bio to reserve a lot for any specific orders.

DIFFERENTIATION OF PREADIPOCYTES INTO ADIPOCYTES

1. Visceral preadipocytes are plated to be confluent in Omental Preadipocyte Medium (cat# OM-PM) 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 Omental Preadipocyte Medium from all wells.
3. Add the appropriate volume of Omental Differentiation Medium (catalog # OM-DM) to the wells (see Table 1. Feeding Volumes). Incubate plate for 7 days at 37°C and 5% CO_2.
4. After 7 days, cells should be fed by removing some of the medium and replacing with fresh Omental Adipocyte Medium (catalog # OM-AM; 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.**
5. Two (2) weeks after the initiation of differentiation, cells should appear rounded with large lipid droplets apparent in the cytoplasm. Cells are now considered mature adipocytes and are suitable for most assays.

Table 1. Feeding Volumes

Format	Plating	Change OM-PM to OM-DM		Change OM-DM to OM-AM		Change OM-AM to OM-AM	
	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	300 µl /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	4.2 ml/flask	5.6 ml/flask	4.2 ml/flask	4.2ml/flask

Table 2. Summary Culture area, Zen-Bio Recommended Cultureware

Multi-well Plate Format	6	12	24	48	96
Greiner Bio-One Cellstar, cm²/well	**9.62**	**3.87**	**1.94**	**1.02**	**0.35**
Cat#	657160	665180	662160	677180	655180
Costar/Corning, cm²/well	**9.50**	**3.80**	**1.90**	**0.95**	**0.32**
Cat#	3516	3513	3526	3548	3595
Nunc, cm²/well	**9.60**	**3.50**	**1.90**	**1.10**	**0.33**
Cat#	152795	150628	143982	150687	167008

PLATING PROCEDURE

Cryopreserved Visceral Preadipocytes (Catalog # MSN-F, PR-F, OP-F)

Please note: Primary cells can be very sensitive to brands of cultureware. Zen-Bio does not currently recommend the use of Falcon or Sarstedt brand plates or flasks. Our scientists are using Nunc, Costar/Corning, 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 Omental Preadipocyte Medium (cat # OM-PM).
3. Centrifuge: 1,200 rpm (282 X g) / 20°C / 5 minutes. Aspirate the supernatant. TAKE CARE TO NOT ASPIRATE ANY OF THE CELL PELLET.
4. The cell vial contains a minimum of 1.0 or 2.0 x 10^6 viable cells (see vial label); however, we recommend performing a cell count to determine a more exact number of cells. Resuspend the cell pellet in 2 ml Omental Preadipocyte Medium and 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 hemacytometer.
5. 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 (see Table 2).

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 37°C incubator with 5% CO_2. Do not agitate the plate, as cells will not plate evenly.
7. Twenty-four hours after plating, check the plates for confluence. If they are not completely confluent, leave for and 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.
8. To differentiate the cells please see the protocol on page 7 starting at step 2.

TROUBLESHOOTING GUIDE

Observation	Possible causes	Suggestions
Preadipocytes do not differentiate	1. Cells have been passaged too many times	1. Visceral cells will arrive at passage 2, 3 or 4. We do not recommend expanding the visceral cells.
	2. Differentiation conditions not optimal	2. Use our defined Differentiation Medium (OM-DM). Make sure that wells are confluent BEFORE initiating differentiation.
	3. Cells were plated at a low density	3. Use the cell density recommended in our manual
	4. Cultureware used not optimal for human primary adipocytes	4. Zen-Bio does not recommend the use of Falcon or Sarstedt cultureware for all cell culture applications
	5. Differences in cultureware brand surface area may affect plating density if unknown	5. Verify the surface area for the cultureware brand you are using.
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	1. Make sure to follow instructions listed in Table 1. Feeding Volumes
	2. Fresh medium was added too quickly	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

- **When do the cells differentiate?**

Oil droplets should appear within 7-8 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 locules. Please note that omental preadipocytes and adipocytes are distinct from subcutaneous preadipocytes and adipocytes. The level of lipid accumulation and morphology in culture may appear different from that which you have normally observed in the subcutaneous human adipocytes.

- **Can I pass the cells?**

Adipocytes cannot be passed since they float after trypsinization. We do not recommend expanding the visceral preadipocytes. Cells are shipped at Passage 2, 3 or 4; please see vial or plate label to determine passage number of the lot of cells you have received. Please contact Zen-Bio for your studies in which large numbers of visceral cells are required.

- **How long do the cells last in culture?**

Adipocytes retain similar morphology and express adipocyte specific genes for at least 3-4 weeks. [NOTE: Cultured adipocytes are usually shipped at 2 weeks old.]

- **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 Omental Basal Medium (OM-BM).

- **From where are the cells sourced?**

The preadipocytes are isolated from human visceral adipose tissue obtained under informed donor consent: mesenteric or peri-renal or omental adipose tissue.

- **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.

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

We do not ship to domestic locations on Fridays. In general, preadipocytes (in culture or cryopreserved) can be shipped the second day after the purchase order is confirmed. Please inquire as to the availability of the adipocytes when ordering. All visceral cultured adipocyte orders require 2 weeks to prepare.

- **Can I differentiate the cells myself?**

Yes. You can order preadipocytes and pre-made culture media for adipocyte differentiation. Simple instructions for differentiating the cells are found in this manual.

- **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, HTLV I, HTLV II, 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 gender, age, and BMI will be provided. Additional clinical information such as fasting blood glucose or medications taken may be available. Please contact Zen-Bio for more specific details.

- **Are the cells from one donor?**

Yes. We can also provide lot numbers containing cells mixed from 5 to 8 donors to get average responses. Please inquire about availability of single donor and mixed donor (called a superlot) lots at time order is placed.

- **What if I want to test my own compounds in differentiation?**

Please call or fax special media requests.

- **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. Do not differentiate preadipocytes in serum-free medium.

PATHOGEN TESTING

Samples from each donor are tested via PCR to confirm non-reactivity for HIV-1, HIV-2, HTLV I, HTLV II, hepatitis B and hepatitis C. However, no known test can offer complete assurance that the cells are pathogen free. 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.

REFERENCES

Lists of articles using Zen-Bio, Inc cultured human cultured preadipocytes and adipocytes may be found at our website (http://www.zenbio.com) under the COMPANY button.

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