Human Adult Stem Cell Care Manual

Differentiation Protocols for Human Adipose-Derived Adult Stem Cells

INSTRUCTION MANUAL ZBM0015.05

SHIPPING CONDITIONS

Human Adult Stem Cells (ASC), Cryopreserved

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. 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.

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

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

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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:

ASC-F	**CRYOPRESERVED ADULT STEM CELLS, 1 MILLION CELLS/VIAL**
ASC-F-SL	**CRYOPRESERVED ADULT STEM CELLS, POOLED DONOR (MULTIPLE DONORS IN LOT), 1 MILLION CELLS/VIAL**

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LIMITED PRODUCT WARRANTY

This warranty limits our liability to replacement of this product. No other warranties of any kind, expressed or implied, including without limitation implied warranties of merchantability or fitness for a particular purpose, are provided by 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 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 Zen-Bio 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 adult stem 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.

INTRODUCTION

ZenBio, Inc. adipose derived adult stem cells are collected from subcutaneous adipose tissue from a consented, healthy non-diabetic donor between 18 and 60 years old undergoing elective surgery. The cells are isolated by centrifugal force after collagenase treatment. Adult stem cells can be differentiated into various lineages using Zen-Bio media formulations and protocols.

This instruction manual describes procedures to induce human adipose derived adult stem cells (ASC) to differentiate into three different cell types:

1) **Mature Adipocytes** Adipogenesis Procedure (Pages 8-10)
2) **Osteoblasts** Osteogenesis Procedure (Pages 11-12)
3) **Chondrocytes** Chondrogenesis Procedure (Page 13)

The process of differentiating human adipose-tissue derived adult stem cells to adipocytes has been patent protected by Zen-Bio under US patent number 6153432.

QUALITY CONTROL

Adipose-derived adult stem cells (ASC) are evaluated for surface markers indicative of stem cells via flow cytometry. The adult stem cells stain >99% positive for CD105 and CD44, and negative for CD31 and CD45.

The adult stem cells are also assessed for differentiation and morphology. Adipogenesis is confirmed by assessing lipid formation by lipid staining and total trglyceride content through lipolysis assays. Osteogenesis is confirmed by Alizarin red staining to determine the degree of mineralization of the cells. Chondrogenesis is confirmed by Alcian blue staining to determine whether or not collagen is being produced.

CATALOG ITEMS

- **Preadipocyte Medium**
  - Cat # PM-1 (500mL), PM-1-250 (250mL)
  - Store according to label
- **Adipocyte Differentiation Medium**
  - Cat # DM-2 (100mL), DM-2-500 (500mL)
  - Store according to label
- **Adipocyte Maintenance Medium**
  - Cat # AM-1 (500mL), AM-1-250 (250mL)
  - Store according to label
- **Basal Medium** (for adipocytes)
  - Cat # BM-1 (500mL)
  - Store according to label
- **Osteoblast Differentiation Medium**
  - Cat # OB-1 (500mL)
  - Store according to label
- **Chondrocyte Differentiation Medium**
  - Cat # CM-1 (500mL), CM-1-100 (100mL)
  - Store according to label
- **Cryopreserved Human Adipose-Derived Adult Stem Cells (ASC)**
  - Cat # ASC-F (single donor), ASC-F-SL (multiple donors)
  - Cryopreserved vial containing 1 million viable adult stem cells per vial **(store in vapor phase liquid nitrogen IMMEDIATELY upon receipt)** *any other storage negates the warranty*

MEDIA COMPOSTIONS

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; USA Origin)	Fetal Bovine Serum (FBS; USA Origin)	Fetal Bovine Serum (FBS; USA Origin)
Penicillin	Biotin	Biotin
Streptomycin	D-Pantothenic Acid	D-Pantothenic Acid
Amphotericin B	Human Insulin, recombinant	Human Insulin, recombinant
	Dexamethasone	Dexamethasone
	3-isobutyl-1-methylxanthine (IBMX)	Penicillin
	PPAR γ agonist	Streptomycin
	Penicillin	Amphotericin B
	Streptomycin	
	Amphotericin B	

Basal Medium (for adipocytes) cat# BM-1	**Osteoblast Differentiation Medium** cat# OB-1	**Chondrocyte Differentiation Medium** cat# CM-1
DMEM/ Ham's F-12 (1:1, v/v)	DMEM/ Ham's F-12 (1:1, v/v)	DMEM, 4.5 g/L (25 mmol/L) D-glucose
HEPES pH 7.4	Fetal Bovine Serum (FBS; USA Origin)	Fetal Bovine Serum (FBS; USA Origin)
Biotin	β-glycerophosphate	Transforming growth factor β_1 (TGF-β1)
D-Pantothenic Acid	L-Ascorbic acid 2-phosphate	L-Ascorbic acid 2-phosphate
	Dexamethasone	Dexamethasone
	1,25 $(OH)_2$ Vitamin D_3	Insulin-transferrin-selenium (ITS)
	Penicillin	Linoleic Acid
	Streptomycin	Bovine Serum Albumin (BSA), Low Endotoxin
	Amphotericin B	Penicillin
		Streptomycin
		Amphotericin B

All media (except CM-1) contains 3.15g/L (17.5mmol/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 (BM-1 should remain antibiotic/antimycotic-free). The media will expire 30 days after the thaw date.

PLATING AND EXPANSION PROCEDURES

THAWING AND CULTURING OR CRYOPRESERVING ADULT STEM CELLS

Note: Primary human cell viability is greatly dependent on the use of appropriate sterile tissue culture treated cultureware. No extracellular matrix coatings required.

1. Pre-warm Preadipocyte Medium (cat# PM-1) at 37°C, and prepare all pipets and vessels.
2. Transfer 9.5 mL of warm PM-1 to a sterile 15 mL conical centrifuge tube.
3. Remove cryovial of human adult stem cells (ASCs) from liquid nitrogen and place **immediately** into a 37°C water bath with mild agitation. Be careful not to submerge the cap of the vial into water. For best results, the thawing step should not take more than 1 minute, and should be stopped when there is still visible ice within the vial.
4. Rinse cryovial with 70% ethanol, and wipe cryovial with lint-free lab wiper. Open cryovial under laminar flow hood and resuspend cells in previously prepared 9.5 mL of warmed PM-1.
5. Centrifuge cell suspension at 282 x *g* (1200 rpm) for 5 minutes at 20ºC.
6. Carefully aspirate the supernatant, being careful not to disturb the cell pellet, and resuspend in a volume of PM-1 appropriate for counting the cells. Count cells using a hemocytometer or automated cell counter.
7. The plating density of ASCs is 8,900-9,000 cells per cm^2 for standard proliferation. Calculate the necessary culture surface area according to the plating density (being sure to reference the manufacturer specifications for cell culture area).
8. Place vessel in an incubator (37°C, 5% CO_2) for cell attachment. Ensure cells are evenly suspended when plating large numbers of plates or flasks. Do not agitate the plates or flasks after plating, making sure the vessel surface is level for even cell distribution.
9. Replace medium after 16-24 hours. Medium should be changed every other day until cells reach 85-90% confluency (in about 4-5 days). **Do not let the cells become 100% confluent!** (For a guide to visual identification of 100% confluence, see Figure 1-A, page 10)
10. If expanding further, proceed to the subsection titled “OPTIONAL – ADULT STEM CELL SUBCULTURE” directly below. If cryopreserving, proceed to the subsection titled “OPTIONAL – ADULT STEM CELL CRYOPRESERVATION” in this section.

OPTIONAL – ADULT STEM CELL SUBCULTURE

Note: Adult stem cells should not be expanded after passage 4.

1. Human adult stem cells (ASCs) should be sub-confluent (less than 90% confluent) upon harvest for expansion.
2. Carefully aspirate medium from cell culture vessel and wash cells using sterile Dulbecco’s phosphate buffered saline without calcium or magnesium (cat# DPBS-1000) to remove all traces of serum, or until there is no foaming of the medium.
3. Remove the DPBS-1000 and release the cells from the cultureware bottom by adding Trypsin/EDTA solution (cat# TRP-100) at 0.1-0.2 mL per cm^2 cultureware surface area.
4. Incubate cells for 5-10 minutes at 37°C in Trypsin/EDTA.
5. Examine cells under microscope, and once cells begin detaching, gently tap the side of the vessel to loosen the remaining cells.

6. Neutralize Trypsin/EDTA solution using PM-1 at 0.1-0.2 mL per cm^2 cultureware surface area. Carefully transfer the cell suspension to an appropriate centrifuge tube.
7. If expanding further, repeat steps 5-9. If differentiating or cryopreserving, proceed to the appropriate section.

OPTIONAL – ADULT STEM CELL CRYOPRESERVATION

Note: Only cryopreserve undifferentiated adult stem cells.

1. Human adult stem cells (ASCs) should be sub-confluent (less than 90% confluent) upon harvest for cryopreservation.
2. Aspirate medium and wash cells using sterile Dulbecco's phosphate buffered saline without calcium or magnesium (cat# DPBS-1000) to remove all traces of serum, or until there is no foaming of the medium.
3. Remove the DPBS-1000 and release the cells from the cultureware bottom by adding Trypsin/EDTA solution (cat# TRP-100) at 0.1-0.2 mL per cm^2 cultureware surface area.
4. Incubate cells for 5-10 minutes at 37°C if using Trypsin/EDTA.
5. Examine cells under microscope, and once cells begin detaching, gently tap the side of the vessel to loosen the remaining cells.
6. Neutralize Trypsin/EDTA solution using Preadipocyte Medium (cat# PM-1) at 0.1-0.2 mL per cm^2 cultureware surface area. Carefully transfer the cell suspension to an appropriate centrifuge tube.
7. Centrifuge cell suspension at 282 x *g* (1200 rpm) for 5 minutes at 20ºC.
8. Carefully aspirate supernatant, being careful not to disturb the cell pellet, and resuspend the cells in a volume of PM-1 appropriate for counting the cells. Count cells using a hemocytometer or automated cell counter.
9. Centrifuge cell suspension at 282 x *g* (1200 rpm) for 5 minutes at 20ºC.
10. Carefully aspirate supernatant, being careful not to disturb the cell pellet, and suspend in cold Cryopreservation Medium (cat# FM-1-100) at a concentration of 1 million cells per 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.
11. 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.
12. 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.

ADIPOGENESIS PROCEDURE

DIFFERENTIATION OF ADULT STEM CELLS INTO ADIPOCYTES

Note: Primary human cell viability is greatly dependent on the use of appropriate sterile tissue culture treated cultureware. No extracellular matrix coatings required.

1. Pre-warm Preadipocyte Medium (cat# PM-1) at 37°C, and prepare all pipets and vessels.
2. Transfer 9.5 mL of warm PM-1 to a sterile 15 mL conical centrifuge tube.
3. Remove cryovial of human adult stem cells (ASCs) from liquid nitrogen and place **immediately** into a 37°C water bath with mild agitation. Be careful not to submerge the cap of the vial into water. For best results, the thawing step should not take more than 1 minute, and should be stopped when there is still visible ice within the vial.
4. Rinse cryovial with 70% ethanol, and wipe cryovial with lint-free lab wiper. Open cryovial under laminar flow hood and resuspend cells in previously prepared 9.5 mL of warmed PM-1.
5. Centrifuge cell suspension at 282 x *g* (1200 rpm) for 5 minutes at 20ºC.
6. Carefully aspirate the supernatant, being careful not to disturb the cell pellet, and resuspend in a volume of PM-1 appropriate for counting the cells. Count cells using a hemocytometer or automated cell counter.
7. The plating density of ASCs for Adipogenesis is approximately 40,625 cells per cm^2. Calculate the necessary culture surface area according to the plating density (being sure to reference the manufacturer specifications for cell culture area) using the media volumes from Table 1 below.

Table 1: PLATING FOR ADIPOGENESIS

FORMAT	VOLUME PER WELL	*TOTAL VOLUME PER FORMAT
96 well plate	150 µL	14.4 mL/plate
48 well plate	500 µL	24.0 mL/plate
24 well plate	1.0 mL	24.0 mL/plate
12 well plate	2.0 mL	24.0 mL/plate
6 well plate	3.0 mL	18.0 mL/plate
10 cm dish	15.0 mL	15.0 mL/dish
T-75 flask	20.0 mL	20.0 mL/flask
T25 flask	7.0 mL	7.0 mL/ flask

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

8. Place vessel in an incubator (37°C, 5% CO_2) for cell attachment. Ensure cells are evenly suspended when plating large numbers of plates or flasks. Do not agitate the plates or flasks after plating, making sure the vessel surface is level for even cell distribution.
9. 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 Zen-Bio immediately.

10. To start the process of adipogenesis, aspirate the entire volume of PM-1 from all wells. Add the appropriate volume of Adipocyte Differentiation Medium (catalog # DM-2) to the wells (volumes found in Table 2). Incubate plate for 7 days at 37^0C and 5% CO_2.

11. After 7 days, cells should be fed by removing some of the media and replacing with fresh Adipocyte Maintenance Medium (cat# AM-1) (volumes found in Table 2).

Caution: **Do not dry the wells. Add new medium gently. If using an automatic feeder, set the slowest flow rate possible.**

12. 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 2: ADIPOGENESIS FEEDING VOLUMES

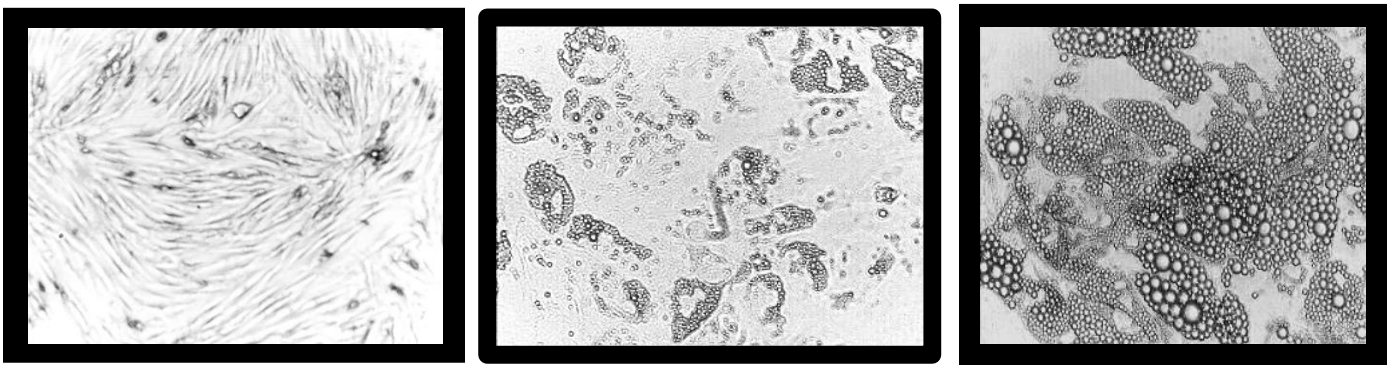
Format	Plating	Change PM-1 to DM-2		Change DM-2 to AM-1		Change AM-1 to AM-1	
		Day 0 (24-48 hours after plating)		Day 7		Day 14 (Ready for most assays)	
	IN	OUT	IN	OUT	IN	OUT	IN
96 well plate	150 µL/well	150 µL/well	150 µL/ well	90 µL/well	120 µL/well	90 µL/well	90 µL/well
48 well plate	500 µL/well	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	4.2 mL/flask

Figure 1: ADULT STEM CELLS TO MATURE ADIPOCYTES

A. 100% confluent adult stem cells

B. 1-week-old adipocytes (1 week post-differentiation)

C. 2-week-old adipocytes (2 weeks post-differentiation)



ADULT STEM CELLS **MATURE ADIPOCYTES**

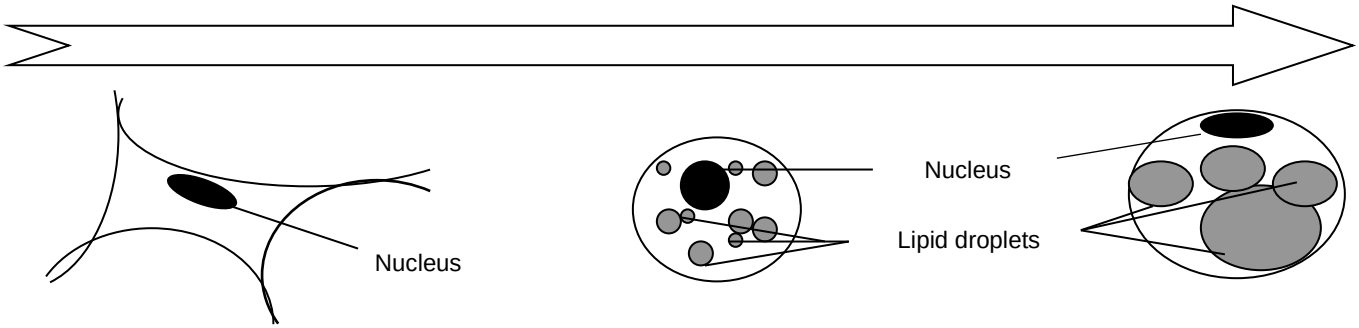


Figure 1 Description: These are unstained photographs of human adult stem cell morphology (20X). The cells should appear comparable in appearance to these pictures.

(1-A) Photographs of 100% confluent adult stem cells (ASC).

(1-B) 1-week-old (post-differentiation) cultured adipocytes.

(1-C) Mature (2 weeks post-differentiation) cultured Adipocytes.

OSTEOGENESIS PROCEDURE

DIFFERENTIATION OF ADULT STEM CELLS INTO OSTEOBLASTS

Note: Primary human cell viability is greatly dependent on the use of appropriate sterile tissue culture treated cultureware. No extracellular matrix coatings required.

1. Pre-warm Preadipocyte Medium (cat# PM-1) at 37°C, and prepare all pipets and vessels.
2. Transfer 9.5 mL of warm PM-1 to a sterile 15 mL conical centrifuge tube.
3. Remove cryovial of human adult stem cells (ASCs) from liquid nitrogen and place **immediately** into a 37°C water bath with mild agitation. Be careful not to submerge the cap of the vial into water. For best results, the thawing step should not take more than 1 minute, and should be stopped when there is still visible ice within the vial.
4. Rinse cryovial with 70% ethanol, and wipe cryovial with lint-free lab wiper. Open cryovial under laminar flow hood and resuspend cells in previously prepared 9.5 mL of warmed PM-1.
5. Centrifuge cell suspension at 282 x *g* (1200 rpm) for 5 minutes at 20ºC.
6. Carefully aspirate the supernatant, being careful not to disturb the cell pellet, and resuspend in a volume of PM-1 appropriate for counting the cells. Count cells using a hemocytometer or automated cell counter.
7. The plating density of ASCs for Osteogenesis is approximately 30,000 cells per cm^2. Calculate the necessary culture surface area according to the plating density (being sure to reference the manufacturer specifications for cell culture area) using the media volumes from Table 3 below

Table 3: PLATING FOR OSTEOGENESIS

FORMAT	VOLUME PER WELL	*TOTAL VOLUME PER FORMAT
96 well plate	150 µL	14.4 mL /plate
48 well plate	500 µL	24.0 mL/plate
24 well plate	1.0 mL	24.0 mL/plate
12 well plate	2.0 mL	24.0 mL/plate
6 well plate	3.0 mL	18.0 mL/plate
10 cm dish	15.0 mL	15.0 mL/dish
T-75 flask	20.0 mL	20.0 mL/flask
T25 flask	7.0 mL	7.0 mL/ flask

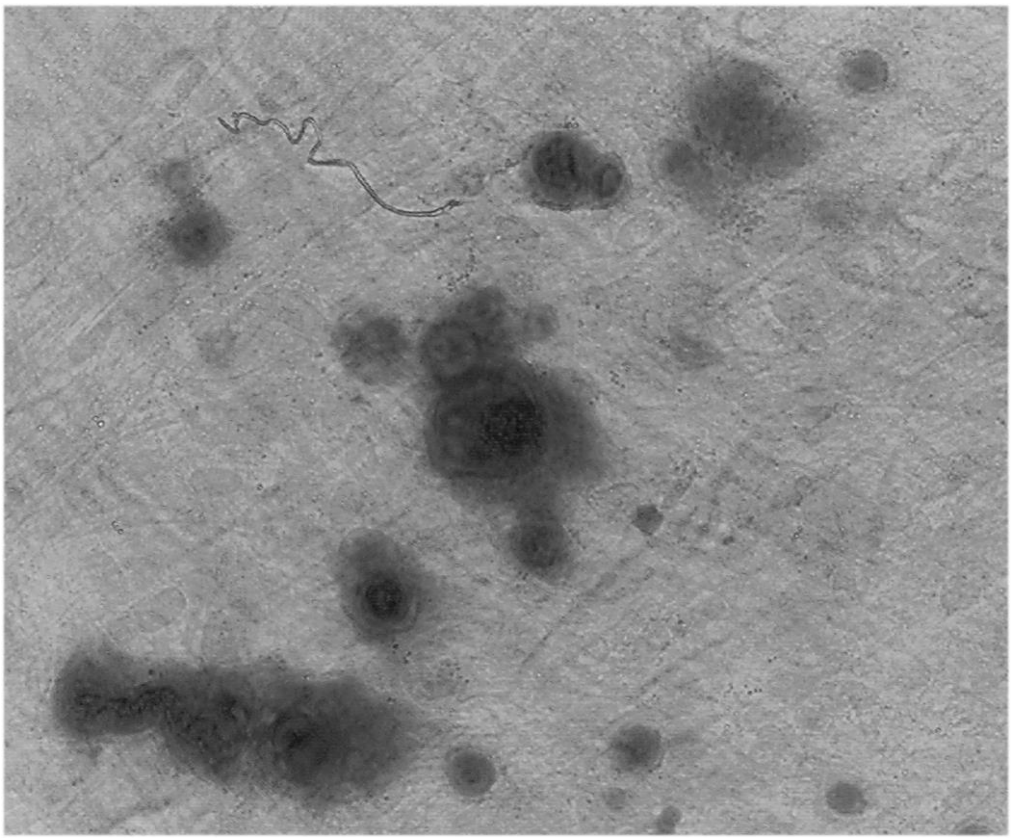
***Note: We recommend preparing slightly larger volumes to allow for loss due to foam and pipet error.**

8. Place vessel in an incubator (37°C, 5% CO_2) for cell attachment. Ensure cells are evenly suspended when plating large numbers of plates or flasks. Do not agitate the plates or flasks after plating, making sure the vessel surface is level for even cell distribution.
9. Twenty-four (24) hours after plating, aspirate the entire volume of PM-1 from all wells, ensuring you do not disturb the attached cells. Gently add the appropriate volume of Osteoblast Differentiation Medium (cat# OB-1) to the wells (volumes found in Table 4).
10. Incubate cells at 37^0C and 5% CO_2 feeding cells every 3 days with OB-1. Osteoblasts can remain in culture for up to 2 weeks.

Table 4: OSTEOGENESIS FEEDING VOLUMES

Format	Volume per well
96 well plate	150 µL/well
48 well plate	300 µL/well
24 well plate	1.0 mL/well
12 well plate	2.0 mL/well
6 well plate	3.0 mL/well
T-75 flask	12 mL/flask
T-25 flask	7 mL/flask

Figure 2: STAINED OSTEOBLASTS (20X)



Alizarin red staining of 14 day old cells shows significant mineralization.

CHONDROGENESIS PROCEDURE

DIFFERENTIATION OF ADULT STEM CELLS INTO CHONDROCYTES

Note: You will need to prepare a solution of 1.2% alginate in 150mM NaCl and a solution of 102mM $CaCl_2$ before thawing. Set aside some of the 150mM NaCl solution without alginate.

Note: Primary human cell viability is greatly dependent on the use of appropriate sterile cultureware. No extracellular matrix coatings required. Adult stem cells may perform poorly past passage 4.

1. Pre-warm Preadipocyte Medium (cat# PM-1) at 37°C, and prepare all pipets and vessels.
2. Transfer 9.5 mL of warm PM-1 to a sterile 15 mL conical centrifuge tube.
3. Remove cryovial of human adult stem cells (ASCs) from liquid nitrogen and place **immediately** into a 37°C water bath with mild agitation. Be careful not to submerge the cap of the vial into water. For best results, the thawing step should not take more than 1 minute, and should be stopped when there is still visible ice within the vial.
4. Rinse cryovial with 70% ethanol, and wipe cryovial with lint-free lab wiper. Open cryovial under laminar flow hood and resuspend cells in previously prepared 9.5 mL of warmed PM-1.
5. Centrifuge cell suspension at 282 x *g* (1200 rpm) for 5 minutes at 20ºC.
6. Carefully aspirate the supernatant, being careful not to disturb the cell pellet, and resuspend in a volume of PM-1 appropriate for counting the cells. Count cells using a hemocytometer or automated cell counter.
7. Centrifuge cell suspension at 282 x *g* (1200 rpm) for 5 minutes at 20ºC.
1. Remove the supernatant and resuspend the cell pellet in a solution of 1.2% alginate in 150mM NaCl at 4 million cells/mL. Pipette up and down without creating bubbles to mix thoroughly.
2. Draw the cell-seeded alginate suspension into a sterile 10 cc syringe using a 22 gauge needle.
3. Add 3 mL of a 102mM $CaCl_2$ solution into one well of a 6 well culture plate.
4. Carefully and slowly dispense equal-sized droplets of the cell-seeded alginate solution into the $CaCl_2$ solution (dispense10-30 beads per well taking care to avoid clumping the beads). Cure the beads in the $CaCl_2$ solution for 10 minutes at room temperature.
5. Using a glass pipette, aspirate the $CaCl_2$ solution off the beads. Be careful not to aspirate the beads with the solution.
6. Wash the cell-seeded alginate beads three times with the 150mM NaCl solution ***without*** alginate and then one more time with DMEM high glucose, 4.5g/L (cat# DMEMHG).
7. Add 3 mL of the Chondrocyte Differentiation Medium (cat# CM-1).
8. Incubate at 37°C, 5.0 % CO_2, and 95% relative humidity for the duration of the experiment.
9. Change media every three days. Chondrocytes can remain in culture for up to 4 weeks.

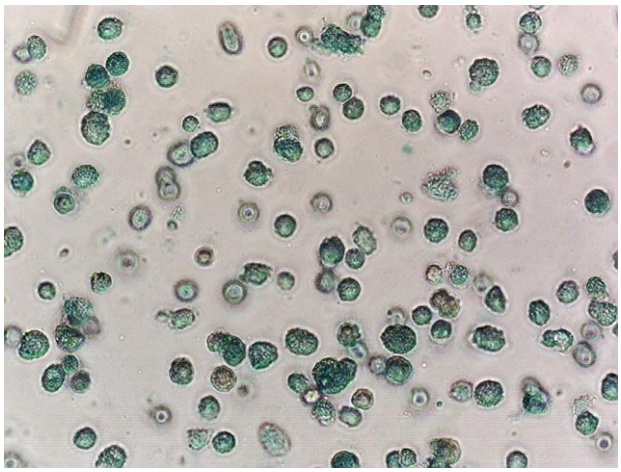


Figure 3: STAINED CHONDROCYTES
(Right)

Alcian blue-stained chondrocytes to show collagen production.

TROUBLESHOOTING GUIDE

Observation	Possible Cause(s)	Suggestion(s)
Adult stem cells 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 medium. 3. Use the cell density recommended in our manual. 4. Use sterile tissue culture treated cultureware with a flat culture surface area. 5. Verify the surface area for the cultureware brand you are using.
Adult stem cells 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 sterile tissue culture treated cultureware with a flat culture surface area.
Edge effects.	Medium in outside wells evaporated.	• Ensure a saturated humidity in the incubator. • Make sure multiple plates are stacked no more than 3 plates high.
(Adipogenesis) **Adipocytes appear uneven in each well.**	1. Medium was completely removed during feeding. 2. Fresh medium was added too quickly. 3. Cell placed on uneven surface in the incubator.	1. Make sure to follow instructions listed in Table 2: Adipogenesis 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. Place cultureware on a level surface in the incubator to ensure cells attach evenly.

FREQUENTLY ASKED QUESTIONS

Can I passage these cells and what is the maximum passage if so?

Yes, adult stem cells can be trypsinized and re-plated. Adult stem cells grow slower with each passage and undergo adipogenesis poorly after passage 4. All cells are shipped at passage 2-3 after establishing a primary culture.

How fast do the cells replicate?

Average doubling time ranges from 48 to 84 hours. Please keep in mind that doubling time varies from donor to donor.

My cells were not confluent after 24-48 hours. Can I trypsinize (detach) cells and start again?

No. The cells will not differentiate. Use a new vial of cells.

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

Use RNA Tri-reagent (vendor: Molecular Products), RNeasy kit (vendor: Qiagen) or other column based kits. You can expect approximately 20 µg total RNA from a 10 cm dish of undifferentiated adult stem cells.

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 adult stem cells into adipocytes using medium without serum.

What is the concentration of ingredients in your media?

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

Are antibiotics included in the medium?

Yes, an antibiotics/antimycotic solution (penicillin, streptomycin, and amphotericin B) is recommended since the cells are primary cells. Most Zen-Bio media contain antibiotics and/or anti-fungal agents. However, there are no antibiotics in Basal Medium (cat# BM-1).

Where are the cells obtained?

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

What donor information do I receive?

The donor's age, gender, and body mass index (BMI) are provided in the certificate of analysis that accompanies each lot of cells.

Do you test for pathogens? Which ones?

Yes. Refer to the section titled Pathogen Testing on the next page.

What quality control testing is performed on the cells?

We confirm the presence of several cell surface markers indicative of stem cells via flow cytometry. The adult stem cells stain >99% positive for CD105 and CD44, and negative for CD31 and CD45.

Further, quality control is performed after differentiation:

- Adipocytes: Lipid staining, total triglyceride content, and functional lipolysis.
- Osteoblasts: Measurement of degree of mineralization as assessed by Alizarin Red staining.
- Chondrocytes: Measurement of collagen production by Alcian Blue staining.

Can the adult stem cells be differentiated into any additional cell types?

Other researchers have differentiated adult stem cells into smooth muscle, neuronal, and hepatocyte lineages. However, the procedures are not currently offered by ZenBio, Inc. See selected references below for further information:

- Mizuno, *et al.* Myogenic differentiation by human processed lipoaspirate cells. Plastic and Reconstructive Surgery (2002) 109:199-209.
- Seo, *et al*. Differentiation of human adipose stromal cells into hepatic lineage in vitro and in vivo. Biochem Biophys. Res. Comm. (2005) 328: 258-264.
- Rodríguez, RV, *et al.* Clonogenic multipotent stem cells in human adipose tissue differentiate into functional smooth muscle cells. Proc Natl Acad Sci USA. (2006), 103(32): 12167–12172.
- Wouter, JFM *et al.* Effect of tissue-harvesting site on yield of stem cells derived from adipose tissue: implications for cell-based therapies. Cell Tissue Res. (2008) 332(3): 415–426.

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 human adult stem cells may be found at our website (http://www.zenbio.com).