Human Adult Stem Cell Manual

Differentiation protocols for human adipose-derived adult stem cells

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INTRODUCTION

The adipose derived adult stem cells are isolated from subcutaneous adipose tissue of healthy non-diabetic donors 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 specific media formulations and protocols . This instruction manual describes procedures to induce human adipose derived adult stem cells (ASC) to differentiate into 1) mature adipocytes, 2) osteoblasts, and 3) chondrocytes. The process of differentiating human adipose-tissue derived adult stem cells to adipocytes has been patent protected by under US patent number 6153432.

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.

MATERIALS PROVIDED FOR EACH CATALOG ITEM

- **Cryopreserved Human Adipose-Derived Adult Stem Cells (catalog # ASC-F)**

Frozen vial containing either 1.0 or 2.0 x10^6 viable adult stem cells (store in liquid nitrogen upon receipt)
50 ml Preadipocyte Medium *[NOTE: this medium is suitable as a plating medium for the adipose derived adult stem cells]*

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	Fetal bovine serum	Fetal bovine serum
Penicillin	Biotin	Biotin
Streptomycin	Pantothenate	Pantothenate
Amphotericin B	Human insulin	Human insulin
	Dexamethasone	Dexamethasone
	Isobutylmethylxanthine	Penicillin
	PPARγ agonist	Streptomycin
	Penicillin	Amphotericin B
	Streptomycin	
	Amphotericin B	

Adipocyte Basal Medium 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-high glucose
HEPES pH 7.4	Fetal Bovine serum	Fetal bovine serum
Biotin	β-glycerophosphate	Transforming growth factor β_1(TGF-β1)
Pantothenate	Ascorbate-2-phosphate	Ascorbate-2-phosphate
	Dexamethasone	Dexamethasone
	1,25 $(OH)_2$ Vitamin D_3	Insulin-transferrin-selenium plus (ITS+)
	Penicillin	Penicillin
	Streptomycin	Streptomycin

NOTE:

All media except Chondrocyte Medium contains 3.15g/L (17.5mmol/L) D-glucose. Chondrocyte Differentiation Medium contains 4.5g/L (25.0mmol/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 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.

PLATING AND EXPANSION PROCEDURES

Cryopreserved Adult Stem Cells

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 Growth Medium (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 hemacytometer.
4. Place approximately 6.7 X 10^5 cells in T-75 culture flasks using PM-1.
5. Incubate cells until they are 85-90% confluent (in about 4-5 days). Do not let the cells become 100% confluent (see Figure 1-A, page 8 for picture of 100% confluent cells). Cells will need to be fed every other day with PM-1.
6. Aspirate medium and wash adult stem cells 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 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. 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. *OPTIONAL* – Cryopreserve stem cells after counting. Centrifuge at 280 x g, 20°C, 5 minutes. Suspend in cold cryopreservation medium (Cat# FM-1-100) at a concentration of 1X10^6 cells/ml. Do not exceed a 6:1 ratio of cells (per million): volume cryopreservation medium (per ml). Remember to account for the volume of the cell pellet before adding the volume of cryopreservation medium necessary for cell suspension. 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. 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

Please note: Primary cells can be very sensitive to brands of cultureware. ***AMSBIO 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 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 1.0 or 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 hemacytometer.
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 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 AMSBIO immediately.

7. 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.
8. 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.**
9. 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. Adipogenesis: 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	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

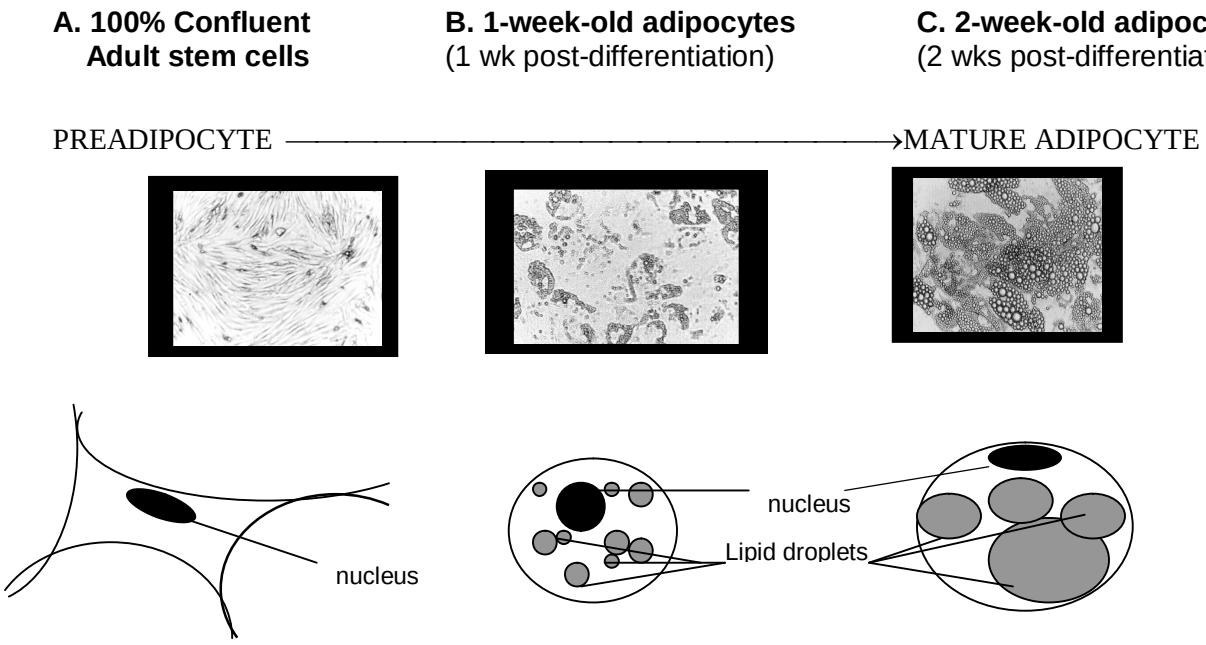


Figure 1: Photographs of 100% confluent Adult stem cells (ASC) (A), 1-week-old (post-differentiation) cultured adipocytes (B) and mature (2 weeks post-differentiation) cultured Adipocytes (C). These are unstained photographs of human adult stem cell morphology (20X). The cells should appear comparable in appearance to these pictures.

OSTEOGENESIS PROCEDURE

Differentiation of Adult Stem Cells into Osteoblasts

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 1.0 or 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 hemacytometer.
4. Plate approximately 30,000 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	NUMBER CELLS PER FORMAT
96 well plate	150 µl	15 ml /plate	0.93 X 10^6 cells/15 ml
48 well plate	500 µl	24ml/plate	1.25 X 10^6 cells/25 ml
24 well plate	1 ml	25ml/plate	1.44X 10^6 cells/26ml
12 well plate	2 ml	25ml/plate	1.5 X 10^6 cells/26ml
6 well plate	3 ml	18 ml/plate	1.8 X 10^6 cells/20ml
10 cm dish	15 ml	15ml/dish	2.25 X 10^6 cells/15 ml
T-75 flask	20 ml	20 ml/flask	2.25 X 10^6 cells/20ml
T25 flask	7 ml	7 ml/ flask	0.75 X 10^6 cells/7ml

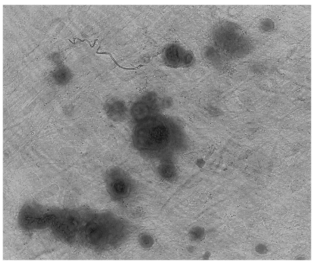
***We recommend preparing slightly larger volumes to allow for loss due to foam and pipet error.**

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, aspirate the entire volume of Preadipocyte Medium from all wells. Add the appropriate volume of Osteoblast Differentiation Medium (catalog # OB-1) to the wells (see Table 3. Osteogenesis Feeding Volumes).
7. Incubate cells at 37°C and 5% CO_2.
8. Feed cells every 3 days with Osteoblast Differentiation Medium (catalog # OB-1).

Table 3. 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. Osteoblasts, (20X)
Alizarin red staining of 14 day old cells shows significant mineralization.

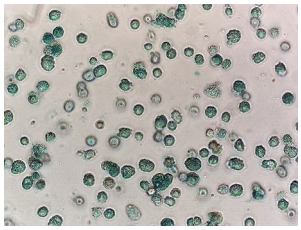


CHONDROGENESIS PROCEDURE

Differentiation of Adult Stem Cells into Chondrocytes

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 1.0 or 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 (PM-1), 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.
4. Remove the supernatant and resuspend the cell pellet in 1.2 % alginate solution (made in 150mM NaCl) at 4 x 10^6 cells/ml. Pipette up and down without creating bubbles to mix thoroughly.
5. Draw the cell-seeded alginate suspension into a sterile 10 cc syringe using a 22 gauge needle.
6. Add 3 ml of $CaCl_2$ (102 mM) into one well of a 6 well culture plate.
7. 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.
8. Using a glass pipette, aspirate the $CaCl_2$ solution off the beads. Be careful not to aspirate the beads with the solution.
9. Wash the cell-seeded alginate beads three times with NaCl (150 mM) and then one more time with DMEM-HG.
10. Add 3 ml of the Chondrogenic Differentiation Medium (cat# CM-1).
11. Incubate at 37°C, 5.0 % CO_2, and 95% relative humidity for the duration of the experiment.
12. Change media every three days.

Figure 3. Differentiated cells stained for collagen production.



TROUBLESHOOTING GUIDE

Observation	Possible causes	Suggestions
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 in 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. AMSBIO does not recommend the use of Falcon or Sarstedt cultureware for all Adipogenesis cell culture applications 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 adult stem cells	1. Use cells of a lower passage number 2. Do not exceed 1:6 expansion ratio 3. Use only Costar, Nunc or Greiner cultureware
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.
Adipogenesis: Adipocytes appear uneven in each well	1. Medium was completely removed during feeding 2. Fresh medium was added too quickly 3. Cells placed on uneven surface in the incubator	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. Place cultureware are on a level surface in the incubator to ensure cells attach evenly.

FREQUENTLY ASKED QUESTIONS

- **Can I pass the cells?**
  Adult Stem Cells can be trypsinized and replated several times. They grow slower with each passage and undergo adipogenesis poorly after passage 4. All cells are shipped at Passage 2-3.

- **How fast do the cells replicate?**
  The average doubling time is 48-84 hours. However, keep in mind that the replication rate for human adult stem cells varies slightly 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. All media contain antibiotics and anti-fungal agents except Adipocyte Basal Medium (cat# BM-1).

- **Where are the cells obtained?**
  The adult stem cells are isolated from human subcutaneous adipose 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, 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.

- **How do I obtain RNA from the cells? How much RNA can I expect?**
  Use RNA Tri-reagent (Molecular Products), RNeasy kit (Qiagen), or a guanidine thiocyanate solution (Chomzynski protocol). You can expect approximately 20 μg total RNA from a 10 cm dish of adult stem cells.

- **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.

- **What is the formulation of the serum-free media?**

  The 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 quality control testing is conducted on the cells?**

  We do 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; negative for CD31 and CD45. Our quality control for adipocytes is lipid staining, total triglyceride content, and functional lipolysis; for osteoblasts it is measurement of degree of mineralization as assessed by Alizarin Red staining; for chondrocytes it is collagen 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. See selected references below.

- 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 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.