invitrogen™

STEMPRO® Human Adipose-Derived Stem Cells

Catalog nos. R7788-110 and R7788-115

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User Manual

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Contents and Storage

Kit Configurations

Catalog no. R7788-110 includes cells plus media.

Catalog no. R7788-115 includes cells only.

Shipping

STEMPRO® Human Adipose-Derived Stem Cells and MesenPRO RS™ Growth Supplement are shipped on dry ice. MesenPRO RS™ Basal Medium is shipped at room temperature.

Kit Contents and Storage

Kit components and storage conditions for R7788-110 and R7788-115 are listed in the table below.

R7788-110	Amount	Storage
STEMPRO® Human Adipose-Derived Stem Cells (1×10^6 cells/ml in freezing medium)	1 ml	Liquid nitrogen
MesenPRO RS™ Basal Medium	500 ml	2 to 8°C **in the dark**
MesenPRO RS™ Growth Supplement	10 ml	–5 to –20°C **in the dark**

R7788-115	Amount	Storage
STEMPRO® Human Adipose-Derived Stem Cells (1×10^6 cells/ml in freezing medium)	1 ml	Liquid nitrogen

Handle cells as potentially biohazardous material under at least Biosafety Level 1 containment. This product contains Dimethyl Sulfoxide (DMSO), a hazardous material. Review the Material Safety Data Sheet (MSDS) before handling.

Product Qualification

The Certificate of Analysis provides detailed quality control information for each product. Certificates of Analysis are available on our website. Go to www.invitrogen.com/support and search for the Certificate of Analysis by product lot number, which is printed on the box.

Additional Products

Additional Products

The products listed in this section may be used with STEMPRO® Human Adipose-Derived Stem Cells. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Support (see page 21).

Item	Quantity	Catalog no.
MesenPRO RS™ Medium (includes Basal Medium and Growth Supplement)	1 kit	12746-012
L-glutamine (200 mM), liquid	100 ml	25030-081
STEMPRO® Osteogenesis Differentiation Kit	1 kit	A10072-01
STEMPRO® Adipogenesis Differentiation Kit	1 kit	A10070-01
Gentamicin (10 mg/ml)	10 ml	15710-064
Dulbecco's Phosphate Buffered Saline (DPBS), containing no calcium, magnesium, or phenol red	500 ml	14190-144
Fetal Bovine Serum, MSC-Qualified	100 ml	12662-011
TrypLE™ Express without phenol red	100 ml	12604-013
Antibiotic-Antimycotic (100X), liquid	100 ml	15240-062
Dulbecco's Modified Eagle Medium (DMEM) (1X) (low glucose) with 1,000 mg/l D-glucose and 110 mg/l sodium pyruvate—without L-glutamine and phenol red	500 ml	11054-020
L-glutamine (200 mM, liquid)	100 ml	25030-081
Dulbecco's Modified Eagle Medium (DMEM) (1X) (high glucose) with 4.5 g/l D-glucose and sodium pyruvate—without L-glutamine	500 ml	10312-021

Antibodies

A variety of antibodies for characterizing ADSCs are available from Invitrogen. The following table lists catalog numbers for purified antibodies only. For labeled antibodies or additional information, refer to our Web site (www.invitrogen.com) or contact Technical Support (see page 21).

Item	Quantity	Catalog no.
CD 31 Mouse Anti-Human, Purified	100 µg	MHCD3100
CD 90 Purified MS X HU (BioSource™)	100 µg	AHU0051
CD 29, Mouse Anti-Human, Purified	100 µg	CD2900
CD 14 Mouse Anti-Human, Purified	100 µg	MHCD1400
CD 105 Mouse Anti-Human, Purified	100 µg	MHCD10500
CD 44 Mouse Anti-Human, Purified	100 µg	MHCD4400
CD 45 Mouse Anti-Human, Purified	100 µg	MHCD4500
CD 73 (Host: Mouse, Clone: 7G2)	100 µg	41-0200

Introduction

Introduction

STEMPRO® Human Adipose-Derived Stem Cells (ADSCs) are isolated from human adipose tissue collected during liposuction procedures and cryopreserved from primary cultures. Before cryopreservation, the ADSCs are expanded for one passage in MesenPRO RS™ Medium. Each lot of ADSCs originates from a single donor of human lipoaspirate tissue.

Each vial of ADSCs contains cells that can differentiate into multiple mature cell phenotypes *in vitro*, including adipocytes, osteoblasts, and chondrocytes (Fraser & Schreiber *et al.*, 2006; Fraser & Wulur *et al.*, 2006; Schäffler & Büchler, 2007; Strem *et al.*, 2005). *In vitro* differentiation into non-mesenchymal cell types, such as neuronal and glial progenitors, hepatocytes and vascular endothelial progenitors have also been described (Rehman *et al.*, 2004; Safford & Rice, 2005; Strem *et al.*, 2005). In addition, ADSCs are known to secrete pro-angiogenic, immunomodulatory and anti-apoptotic factors (Puissant *et al.*, 2005; Rehman *et al.*, 2004; Yañez *et al.*, 2006). ADSCs can be used for studies of adult stem cell differentiation, tissue engineering, and potential future clinical applications. They may also be used for the delivery of recombinant DNA constructs.

MesenPRO RS™ Medium is recommended for use with these cells for optimal growth and expansion.

Characteristics of STEMPRO® ADSCs

- Are prepared from low-passage (passage 1) adherent human adipose-derived primary cell cultures
- Express a flow-cytometry cell-surface protein profile positive for CD29, CD44, CD73, CD90, CD105, and CD166 (> 95%), and negative for CD14, CD31, CD45, and Lin1 (< 2%).
- Contain cells characteristic of at least bi-potential differentiation

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Introduction, continued

Isolation and Expansion

ADSCs are extracted from human adipose tissue through mechanical and enzymatic digestion. Cells are expanded using MesenPRO RS™ Medium, which supports a much shorter cell doubling time (36 +/- 4 hours) than traditional medium (DMEM + 10% FBS), resulting in a cell doubling time of 54 +/– 4 hours.

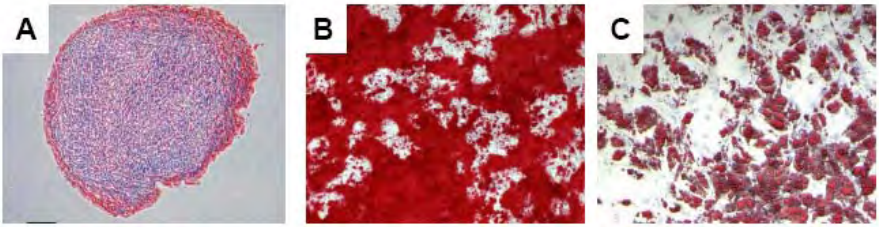
ADSCs can be expanded to 4–5 passages before they lose their ability to grow or differentiate into all potential phenotypes.

Differentiation Potential

Multiple investigators have demonstrated that ADSCs can be differentiated towards multiple mature cell phenotypes. In addition to traditional mesenchymal lineages, ADSCs have been differentiated towards cardiomyocytic, pancreatic, epithelial, and other phenotypes using specialized media.

Differentiation into Mesenchymal Cell Types

The images below show the differentiation of ADSCs into mesenchymal cell types.



A. ADSCs induced to differentiate towards chondrocytes for 29 days and then stained with safranin orange dye (pellet cross-sectional staining) for proteoglycan content; image captured using 4x objective lens.

B. ADSCs induced to differentiate towards osteoblasts for 29 days and then stained with alizarin red dye (which stains mineralized extracellular matrix); image captured using 4x objective lens.

C. ADSCs induced to differentiate towards adipocytes for 14 days and then stained with oil-red-O (which stains lipid vacuoles) and counterstained with hematoxylin; image captured using 10x objective lens.

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MesenPRO RS™ Medium

MesenPRO RS™ Basal Medium and Growth Supplement have been developed for the growth and expansion of human mesenchymal stem cell-like cells, including ADSCs, in tissue-culture vessels. Complete MesenPRO RS™ Medium is a reduced-serum medium (2% FBS) for reduced MSC doubling times, improved MSC expansion, and improved multilineage differentiation capability.

Complete MesenPRO RS™ Medium provides the following advantages for culturing human ADSCs:

- Consistently improves expansion compared to traditional medium (DMEM + 10% FBS).
- Maintains multilineage differentiation capabilities
- Eliminates time and money spent pre-qualifying FBS lots

MesenPRO RS™ Basal Medium and Growth Supplement are included with catalog no. R7788-110 and are available separately for catalog no. R7788-115 (see page vi for ordering information).

STEMPRO® Differentiation Kits

The STEMPRO® Osteogenesis Differentiation Kit and Adipogenesis Differentiation Kit provide specialized media and reagents to promote pathway-specific differentiation of human MSC-like cells, including ADSCs, in tissue-culture vessels. Each kit contains media and reagents for inducing MSCs to be committed to the osteogenic or adipogenic pathway.

Using STEMPRO® Differentiation Kits in combination with MesenPRO RS™ Medium or STEMPRO® MSC SFM provides a standardized culture workflow solution for MSC isolation, expansion, and differentiation into matrix-forming osteoblasts or lipid vesicle-forming adipocytes.

Methods

General Information

General Cell Handling

Follow the general guidelines below to grow and maintain STEMPRO® Human Adipose-Derived Stem Cells.

- **All solutions and equipment that come in contact with the cells must be sterile.** Always use proper sterile technique and work in a laminar flow hood.
- Before starting experiments, ensure cells have been established (at least 1 passage), and also have some frozen stocks on hand.
- For differentiation studies and other experiments, we recommend using cells below passage 5.
- For general maintenance of cells, cell confluency should be 60–80%, cell viability should be at least 90%, and the growth rate should be in mid-logarithmic phase prior to subculturing.
- When thawing or subculturing cells, transfer cells into pre-warmed medium.
- Antibiotic-antimycotic containing penicillin, streptomycin, and amphotericin B may be used if required (see page vi for ordering information).

Important

It is very important to strictly follow the guidelines for culturing ADSCs in this manual to keep them undifferentiated.

As with other human cell lines, when working with ADSCs, handle as potentially biohazardous material under at least Biosafety Level 1 containment.

Preparing Complete MesenPRO RS™ Medium

Introduction

Follow the instruction in this section for preparing Complete MesenPRO RS™ Medium.

Materials Needed

The following materials are required:

- MesenPRO RS™ Basal Medium and MesenPRO RS™ Growth Supplement (included with catalog no. R7788-110 and available separately for catalog no. R7788-115; see page vi for ordering information)
- L-glutamine, 200 mM, liquid (see page vi for ordering information)

Note

- Store all media components in the dark.
- Thaw MesenPRO RS™ Growth Supplement at 2 to 8°C prior to use. Avoid repeated freeze-thaw cycles of the supplement.
- Do not store the prepared complete MesenPRO RS™ Medium longer than 15 days.

Preparing Complete MesenPRO RS™ Medium

Prepare Complete MesenPRO RS™ Medium with MesenPRO RS™ Growth Supplement and L-glutamine prior to use, as follows. Store the complete medium **in the dark** at 2 to 8°C and use within 15 days.

1. Aseptically add 10 ml of MesenPRO RS™ Growth Supplement to 500 ml of MesenPRO RS™ Basal Medium
2. Aseptically add L-glutamine to the medium to a final concentration of 2 mM (*e.g.*, add 5 ml of 200 mM L-glutamine stock to 500 ml of medium).

Thawing and Establishing Cells

Introduction

Follow the protocol below to thaw STEMPRO® ADSCs to initiate cell culture.

Materials Needed

The following materials are required (see page vi for ordering information).

- STEMPRO® Human Adipose-Derived Stem Cells, stored in liquid nitrogen
- Ethanol or isopropanol
- Prepared Complete MesenPRO RS™ Medium (see previous page), prewarmed to 37°C
- Disposable, sterile 50-ml tubes
- 37°C water bath
- 37°C incubator with a humidified atmosphere of 5% CO_2
- Microcentrifuge
- Tissue-culture treated 35-mm dish

Thawing Procedure

To thaw and establish STEMPRO® ADSCs:

1. Prewarm prepared Complete MesenPRO RS™ Medium to 37°C.
2. Remove the cells from liquid nitrogen storage, and wipe the cryovial with ethanol or isopropanol before opening. In an aseptic field, briefly twist the cap a quarter turn to relieve pressure and then retighten. Do not expose cells to air before thawing.
3. Quickly thaw the vial of cells by swirling it in a 37°C water bath and removing it when the last bit of ice has melted, typically < 2 minutes. Do not submerge the vial completely. Do not thaw the cells for longer than 2 minutes.
4. When thawed, immediately transfer cells into a 50-ml sterile tube and add prewarmed Complete MesenPRO RS™ Medium dropwise up to 10 ml.
5. Centrifuge cells for 5 minutes at 210 × *g*.
6. Aspirate supernatant.

Procedure continued on next page

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Thawing Procedure, continued

Procedure continued from previous page

7. Resuspend cells in Complete MesenPRO RS™ Medium (2 ml for a 35-mm dish) and plate the resuspended cells. The recommended seeding density for Adipose-Derived Stem Cells is 5,000 cells per cm^2.
8. Incubate at 37°C, 5% $C0_2$ and 90% humidity and allow cells to adhere for several hours (or overnight).
9. When the cells have attached to the growth surface, replace the medium with an equal volume of fresh, prewarmed Complete MesenPRO RS™ Medium.
10. Change the medium every 3-4 days.

Subculturing Cells

Introduction

Follow the protocol below to culture ADSCs. Subculture cells when needed (before colonies start contacting each other), typically every 10-14 days.

Materials Needed

The following materials are required (see page vi for ordering information).

- Culture vessels containing ADSCs
- Tissue-culture treated flasks, plates or dishes
- Complete MesenPRO RS™ Medium, prewarmed to 37°C
- Disposable, sterile 15-ml tubes
- 37°C incubator with humidified atmosphere of 5% CO_2
- Dulbecco's Phosphate Buffered Saline (DPBS), containing no calcium, magnesium, or phenol red
- TrypLE™ Express, without phenol red
- Hemacytometer or cell counter
- Trypan blue

Passaging Cells

1. Aspirate the Complete MesenPRO RS™ Medium from the cells.
2. Rinse the surface of the cell layer with DPBS (approximately 2 ml DPBS/10 cm^2 culture surface area), by adding the DPBS to the side of the vessel opposite the attached cell layer and rocking back and forth several times.
3. Remove the DPBS by aspiration and discard.
4. To detach the cells, add a sufficient volume of prewarmed TrypLE™ Express without phenol red to cover the cell layer (approx. 0.5 ml/10 cm^2).
5. Incubate at 37°C for approximately 7 minutes.
6. Observe the cells under a microscope. If the cells are less than 90% detached, continue incubating and observe within 2 minutes for complete detachment of the cells. Tap the vessel to expedite cell detachment.

Procedure continued on next page

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Passaging Cells, continued

Procedure continued from previous page

7. When ≥ 90% of the cells have detached, tilt the vessel for a minimal length of time to allow the cells to drain. Add the equivalent of 2 volumes (twice the volume used for the TrypLE™ Express) of temperature-equilibrated Complete MesenPRO RS™ Medium.
8. Disperse the medium by pipetting over the cell layer surface several times.
9. Transfer the cells to a 15-ml conical tube and centrifuge at 210 × *g* for 5 minutes at room temperature.
10. Resuspend the cell pellet in a minimal volume of temperature-equilibrated Complete MesenPRO RS™ Medium and remove a sample for counting.
11. Determine the total number of cells and percent viability using a hemacytometer or cell counter and Trypan Blue exclusion. If necessary, add Complete MesenPRO RS™ Medium to the cells to achieve the desired cell concentration and recount the cells.
12. Determine the total number of vessels to inoculate by using the following equation:

    Number of vessels = Number of viable cells ÷ (growth area of vessel in cm^2 × 5,000 cells per cm^2 recommended seeding density)
13. Add Complete MesenPRO RS™ Medium to each vessel so that the final culture volume is 0.2–0.5 ml per cm^2.
14. Add the appropriate volume of cells to each vessel and incubate at 37°C, 5% $C0_2$ and 90% humidity.
15. Three to four days after seeding, completely remove the medium. Replace with an equal volume of Complete MesenPRO RS™ Medium.

Freezing Cells

Introduction

Guidelines and procedures for preparing freezing medium and freezing cells are provided in this section.

Materials Needed

The following materials are required (see page vi for ordering information).

- Culture vessels containing ADSCs
- Complete MesenPRO RS™ Medium
- Fetal Bovine Serum, MSC-Qualified
- DMSO (use a bottle set aside for cell culture; open only in a laminar flow hood)
- Disposable, sterile 15-ml conical tubes.
- DPBS, containing no calcium, magnesium, or phenol red
- TrypLE™ Express, without phenol red
- Hemacytometer or cell counter
- Sterile freezing vials

Guidelines

When freezing ADSCs, we recommend the following:

- Freeze cells at a density of 1–2 × 10^6 viable cells/ml.
- Use a freezing medium composed of final concentrations of 20% Fetal Bovine Serum (MSC Cell-qualified) and 10% DMSO.
- Bring the cells into freezing medium in two steps, as described in this section.

Continued on next page

Freezing Cells, continued

Preparing Freezing Media

Prepare Freezing Medium A and B immediately before use. You will need enough of each freezing medium to resuspend cells at a density of 1–2 × 10^6 cells/ml (see the freezing procedure below).

1. In a sterile 15-ml tube, mix together the following reagents for every 1 ml of **Freezing Medium A** needed:

Complete MesenPRO RS™ Medium	0.6 ml
Fetal Bovine Serum, MSC-Qualified	0.4 ml

2. In another sterile 15-ml tube, mix together the following reagents for every 1 ml of **Freezing Medium B** needed:

Complete MesenPRO RS™ Medium	0.8 ml
DMSO	0.2 ml

3. Place tube with Freezing Medium B on ice until use (leave Freezing Medium A at Room Temperature).

Note: Discard any remaining freezing medium after use.

Freezing Cells Procedure

1. Aspirate Complete MesenPRO RS™ Medium from the flask, well, or dish.
2. Rinse the surface with DPBS (approximately 2 ml DPBS/10 cm^2 culture surface area) by adding the DPBS to the side of the vessel opposite the attached cell layer and rocking back and forth several times.
3. Remove the DPBS by aspiration and discard.
4. To detach the cells, add a sufficient volume of prewarmed TrypLE™ Express without phenol red to cover the cell layer (approx. 0.5 ml/10 cm^2).
5. Incubate at 37°C for approximately 7 minutes.
6. Observe the cells under a microscope. If the cells are less than 90% detached, continue incubating and observe within 2 minutes for complete detachment of the cells. Tap the vessel to expedite cell detachment.

Procedure continued on next page

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Freezing Cells Procedure, continued

Procedure continued from previous page

7. When ≥90% of the cells have detached, tilt the vessels on end for a minimal length of time to allow the cells to drain. Add the equivalent of 2 volumes (twice the volume used for the TrypLE™ Express) of temperature-equilibrated Complete MesenPRO RS™ Medium to each vessel.
8. Disperse the medium by pipetting over the cell layer surface several times.
9. Transfer the cells to a 15-ml conical tube and centrifuge at 210 × *g* for 5 minutes at room temperature.
10. Resuspend the cell pellet in a minimal volume of temperature-equilibrated Complete MesenPRO RS™ Medium and remove a sample for counting.
11. Determine the total number of cells using a hemacytometer or cell counter.
12. Gently aspirate media from the vessel and resuspend the cells to a concentration of 4×10^6 cells/ml in Freezing Medium A.
13. Add the same volume of Freezing Medium B to cells in a dropwise manner.
14. Aliquot 1 ml to each freezing vial and store at –80°C overnight in an isopropanol chamber.
15. The next day, transfer the frozen vials to a liquid nitrogen tank for long-term storage.

Note: You may check the viability and recovery of frozen cells 24 hours after storing cryovials in liquid nitrogen by following the procedure outlined in **Thawing and Establishing Cells**, page 6.

Osteogenic Differentiation Media and Methods

Introduction

This section provides media-preparation guidelines and a protocol for inducing STEMPRO® ADSCs to differentiate towards osteoblasts using the STEMPRO® Osteogenesis Differentiation Kit.

Materials Needed

The following materials are required (see page vi for ordering information).

- STEMPRO® Osteogenesis Differentiation Kit
- Gentamicin (10 mg/ml)
- Culture vessels containing ADSCs
- DPBS without Ca^{++} and Mg^{++}
- TrypLE™ Express, without phenol red
- Tissue-culture treated vessels
- Disposable, sterile 15-ml tubes
- 37°C incubator with humidified atmosphere of 5% CO_2
- Hemacytometer or cell counter

StemPro® Osteogenesis Differentiation Kit

The STEMPRO® Osteogenesis Differentiation Kit provides specialized media and reagents for osteogenic differentiation of ADSCs in tissue-culture vessels. See the insert provided with the kit for detailed information and protocols.

Preparing Complete Differentiation Medium

To prepare Complete STEMPRO® Osteogenesis Differentiation Medium, thaw the STEMPRO® Osteogenesis Supplement at 4°C, room temperature, or in a 37°C water bath, and prepare as below.

Store complete medium at 2–8°C in the dark.

Component	Final Conc.	For 100 ml
STEMPRO® Osteocyte/Chondrocyte Differentiation Basal Medium	1X	90 ml
STEMPRO® Osteogenesis Supplement	1X	10 ml
Gentamicin (10 mg/ml)	5 µg/ml	50 µl

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Preparing an Osteogenic Cell Culture

1. Observe the ADSC monolayer to ensure mid-log growth phase confluence (60–80%). Aspirate the medium and floating cells from the culture flask and discard.
2. Add 5–10 ml DPBS to the flask. Gently rinse the cell monolayer.
3. Remove DPBS and add 5–7 ml of pre-warmed TrypLE™ Express to the flask and completely coat the culture surface. Incubate for 5–8 minutes at 36–38°C or until cells have fully detached.
4. Gently pipet detached cells into a single-cell solution and verify on inverted microscope.
5. Remove the cell suspension from the flask, transfer into a centrifuge tube, and pellet cells at 100 × g for 5–10 minutes.
6. Determine cell viability and total cell density using Trypan Blue Stain and electronic (*i.e.,* Coulter Counter) or manual (*i.e.,* hemocytometer) cell counting method.
7. Resuspend the pellet in an appropriate volume of pre-warmed Complete MesenPRO RS™ Medium.
8. Seed the ADSCs into culture vessels at 5×10^3 cells/cm^2. For classical stain differentiation assays, seed into a 12-well plate. For gene-expression profile studies, seed into a T-75 flask. For immunocytochemistry studies, seed into a 16-well CultureWell™ chambered coverglass or 96-well plate.
9. Incubate in Complete MesenPRO RS™ Medium at 36–38°C in a humidified atmosphere of 4–6% CO_2 for a minimum of 2 hours up to 4 days.
10. Replace media with pre-warmed Complete STEMPRO® Osteogenesis Differentiation Medium and continue incubation. ADSCs will continue to expand as they differentiate under osteogenic conditions. Refeed cultures every 3–4 days.
11. After specific periods of cultivation, osteogenic cultures can be processed for alkaline phosphatase staining (7–14 days) or Alizarin Red S staining (>21 days), gene expression analysis, or protein detection.

Adipogenic Differentiation Media and Methods

Introduction

This section provides media-preparation guidelines and a protocol for inducing STEMPRO® ADSCs to differentiate towards adipocytes using the STEMPRO® Adipogenesis Differentiation Kit.

Materials Needed

The following materials are required (see page vi for ordering information).

- STEMPRO® Adipogenesis Differentiation Kit
- Gentamicin (10 mg/ml)
- Culture vessels containing ADSCs
- DPBS without Ca^{++} and Mg^{++}
- TrypLE™ Express, without phenol red
- Tissue-culture treated vessels
- Disposable, sterile 15-ml tubes
- 37°C incubator with humidified atmosphere of 5% CO_2
- Hemacytometer or cell counter

StemPro® Adipogenesis Differentiation Kit

The STEMPRO® Adipogenesis Differentiation Kit provides specialized media and reagents for adipogenic differentiation of ADSCs in tissue-culture vessels. See the insert provided with the kit for detailed information and protocols.

Complete Adipogenic Differentiation Medium

To prepare the complete medium, thaw the supplement in a 37±2°C water bath, swirl and warm the supplement to promote dissolution of the precipitate (see **Note** on the following page), and prepare the medium as described in the table below. Store complete medium at 2–8°C in the dark.

Component	Final Conc.	For 100 ml
STEMPRO® Adipocyte Differentiation Basal Medium	1X	90 ml
STEMPRO® Adipogenesis Supplement	1X	10 ml
Gentamicin (10 mg/ml)	5 µg/ml	50 µl

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Note

It is normal to see a precipitate formed in the supplement after thawing. To promote dissolution of the precipitate, warm the supplement with swirling for no more than 30 minutes prior to preparing complete media. Any remaining precipitate should be suspended in solution before it is added to STEMPRO® Adipocyte Differentiation Basal Medium, and will dissolve completely when mixed with the Basal Medium and warmed.

Preparing an Adipogenic Cell Culture

1. Observe the ADSC monolayer to ensure mid-log growth phase confluence (60–80%). Aspirate the medium and floating cells from culture flask and discard.
2. Add 5–10 ml DPBS. Gently rinse the cell monolayer.
3. Remove the DPBS and add 5–7 ml of pre-warmed TrypLE™ Express to the flask and completely coat the culture surface. Incubate for 5–8 minutes at 36–38°C or until cells have fully detached.
4. Gently pipet the detached cells into a single-cell solution and verify on inverted microscope.
5. Remove the cell suspension from the flask, transfer into a centrifuge tube, and pellet cells at 100 × g for 5–10 minutes.
6. Determine cell viability and total cell density using Trypan Blue Stain and electronic (*i.e.,* Coulter Counter) or manual (*i.e.,* hemocytometer) cell counting method.
7. Resuspend the pellet in an appropriate volume of pre-warmed Complete MesenPRO RS™ Medium.
8. Seed the ADSCs into culture vessels at 1×10^4 cells/cm^2. For classical stain differentiation assay, seed into a 12 well plate. For gene expression profile studies, seed into a T-75 flask. For immunocytochemistry studies, seed into a 16-well CultureWell™ chambered coverglass or 96-well plate.
9. Incubate in Complete MesenPRO RS™ Medium at 36–38°C in a humidified atmosphere of 4–6% CO_2 for a minimum of 2 hours up to 4 days.

Procedure continued on the next page

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Preparing an Adipogenic Cell Culture, continued

Procedure continued from the previous page

10. Replace media with pre-warmed Complete Adipogenesis Differentiation Medium and continue incubation. ADSCs will continue to undergo limited expansion as they differentiate under adipogenic conditions. Refeed cultures every 3–4 days.
11. After specific periods of cultivation, adipogenic cultures can be processed for Oil Red O or LipidTOX™ staining (beginning at 7–14 days), gene expression analysis or protein detection.

Chondrogenic Differentiation Media and Methods

Introduction

This section provides media-preparation guidelines and a protocol for inducing STEMPRO® ADSCs to differentiate towards chondrocytes using published recipes (Mackay *et al.*, 1998; Pittenger *et al.*, 1999).

Materials Needed

The following materials are required (see page vi for ordering information).

- Chondrogenic differentiation medium (see below)
- Culture vessels containing ADSCs
- DPBS without Ca^{++} and Mg^{++}
- TrypLE™ Express, without phenol red
- Tissue-culture treated vessels
- Disposable, sterile 15-ml tubes
- 37°C incubator with humidified atmosphere of 5% CO_2
- Hemacytometer or cell counter

Chondrogenic Differentiation (CD) Medium

Chondrogenic Differentiation Medium is prepared as follows. Store the prepared medium in the dark at 2 to 8°C.

Component	Source	Cat. no.	Volume	Conc.
DMEM low glucose, without L-glutamine and phenol red	Invitrogen	11054-020	100 ml	—
200 mM L-glutamine	Invitrogen	25030-081	1 ml	2 mM
Insulin-Transferrin-Selenium-Plus	BD Biosciences	354352	1 ml	1X
50 mM L-ascorbic acid	Sigma	A8960-5G	100 µl	50 µM
40 mg/ml L-proline	Sigma	P5607	100 µl	40 µg/ml
100 µM dexamethasone	Sigma	D8893	100 µl	0.1 µM
10 µg/ml recombinant human TGF-beta 3 (BioSource)	Invitrogen	PHG9304	100 µl	10 ng/ml
10 mg/ml gentamicin	Invitrogen	15710-064	50 µl	5 µg/ml

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Chondrogenic Differentiation Media and Methods, continued

Preparing a Chondrogenic Cell Culture

1. Detach cells using TrypLE™ Express and perform a cell count as described in **Passaging Cells**, pages 8–9 (through Step 10).
2. Resuspend the cells in DMEM (low glucose) with 10% MSC-qualified FBS to a concentration of 8×10^6 cells/ml.
3. To six wells in a 12-well tissue-culture dish, spot 10 µl of cells per well.
4. Incubate for two hours at 37°C, 5% $C0_2$ and 90% humidity.
5. To three of the spotted wells, add 1 ml of DMEM (low glucose) with 10% MSC-qualified FBS. To the other three wells, add 1 ml of Chondrogenic Differentiation Medium (prepared as described above).
6. Incubate at 37°C, 5% $C0_2$ and 90% humidity. Refeed cultures every three to four days with same media, prepared at the initiation of differentiation.
7. Check for chondrogenesis after a set period of cultivation. You can perform alcian blue staining to detect glycosaminoglycans or collagen 2a immunostaining after ~28 days.

Appendix

Troubleshooting

Culturing Cells

The table below lists some potential problems and solutions that help you troubleshoot your cell culture problems.

Problem	Cause	Solution
No viable cells after thawing stock	Stock not stored correctly	Order new stock and store in liquid nitrogen. Keep in liquid nitrogen until thawing.
	Home-made stock not viable	Freeze cells at a density of 1–2 × 10^6 viable cells/ml.
		Use low-passage cells to make your own stocks.
		Follow procedures in **Freezing Cells** (page 10) exactly. Slow freezing and fast thawing is the key. Add Freezing Medium B drop wise manner (slowly). At time of thawing, thaw quickly and do not expose vial to the air but quickly change from nitrogen tank to 37°C water bath.
		Obtain new STEMPRO® ADSCs.
	Thawing medium not correct	Use prewarmed Complete MesenPRO RS™ Medium, prepared as described on page 5.
	Cells too diluted	Generally we recommend thawing one vial in a 35-mm dish at a density of 5,000 cells per cm^2.
Cells grow slowly	Growth medium not correct	Use prewarmed Complete MesenPRO RS™ Medium.
	Cells too old	Use healthy ADSCs, under passage 5; do not overgrow.
Cells differentiated	Culture conditions not correct	Thaw and culture fresh vial of STEMPRO® ADSCs. Follow thawing instructions (page 6) and subculture procedures (page 8) exactly.
	Cells too old	ADSCs above passage 5 may become differentiated.

Technical Support

Web Resources

Visit the Invitrogen Web site at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical service contact information
- Access to the Invitrogen Online Catalog
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References

Fraser, J. K., Schreiber, R., Strem, B., Zhu, M., Alfonso, Z., Wulur, I., and Hedrick, M. H. (2006) Plasticity of human adipose stem cells toward endothelial cells and cardiomyocytes. *Nat Clin Pract Cardiovasc Med, 3*, S33-37

Fraser, J. K., Wulur, I., Alfonso, Z., and Hedrick, M. H. (2006) Fat tissue: an underappreciated source of stem cells for biotechnology. *Trends Biotechnol., 24*, 150-154

Mackay, A. M., Beck, S. C., Murphy, J. M., Barry, F. P., Chichester, C. O., and Pittenger, M. F. (1998) Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. *Tissue Eng, 4*, 415-428

Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M. A., Simonetti, D. W., Craig, S., and Marshak, D. R. (1999) Multilineage potential of adult human mesenchymal stem cells. *Science, 284*, 143-147

Puissant, B., Barreau, C., Bourin, P., Clavel, C., Corre, J., Bousquet, C., Taureau, C., Cousin, B., Abbal, M., Laharrague, P., Penicaud, L., Casteilla, L., and Blancher, A. (2005) Immunomodulatory effect of human adipose tissue-derived adult stem cells: Comparison with bone marrow mesenchymal stem cells. *Br J Haematol, 129*, 118-129

Rehman, J., Traktuev, D., Li, J., Merfeld-Clauss, S., Temm-Grove, C. J., Bovenkerk, J. E., Pell, C. L., Johnstone, B. H., Considine, R. V., and March, K. L. (2004) Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. *Circulation, 109*, 1292-1298

Safford, K. M., and Rice, H. E. (2005) Stem cell therapy for neurologic disorders: Therapeutic potential of adipose-derived stem cells. *Current Drug Targets, 6*, 57-62(56)

Schäffler, A., and Büchler, C. (2007) Concise review: adipose tissue-derived stromal cells--basic and clinical implications for novel cell-based therapies. *Stem Cells, 25*, 818 – 827

Strem, B. M., Hicok, K. C., Zhu, M., Wulur, I., Alfonso, Z., Schreiber, R. E., Fraser, J. K., and Hedrick, M. H. (2005) Multipotential differentiation of adipose tissue-derived stem cells. *Keio J Med, 54*, 132-141

Yañez, R., Lamana, M. L., García-Castro, J., Colmenero, I., Ramírez, M., and Bueren, J. A. (2006) Adipose tissue-derived mesenchymal stem cells have in vivo immunosuppressive properties applicable for the control of the graft-versus-host disease. *Stem Cells, 24*, 2582-2591

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