invitrogen™

# StemPro® Alk Phos-expressing Rat Mesenchymal Stem Cells

**Catalog no. R7789-120**

**Version A**
14 November 2008
*A10855*

User Manual

# Table of Contents


Contents and Storage ..... v

Additional Products ..... vi

Introduction ..... 1

**Methods ..... 4**

General Information ..... 4

Thawing and Establishing Cells ..... 6

Subculturing Cells ..... 8

Freezing Cells ..... 10

Differentiation Media ..... 13

Differentiating StemPro® Alk Phos-expressing Rat MSCs ..... 15

**Appendix ..... 19**

Troubleshooting ..... 19

Technical Support ..... 21

Purchaser Notification ..... 22

References ..... 24

# Contents and Storage

## Shipping

StemPro® Alk Phos-expressing Rat Mesenchymal Stem Cells are shipped on dry ice.

## Contents and Storage

Contents and storage conditions for StemPro® Alk Phos-expressing Rat Mesenchymal Stem Cells are listed in the table below. For components of the freezing medium, see page 11.

| Product | Amount | Storage |
|---|---|---|
| StemPro® Alk Phos-expressing Rat Mesenchymal Stem Cells ($1 \times 10^6$ cells/ml in freezing medium) | 1 ml | Liquid nitrogen |


Handle cells as potentially biohazardous material under at least Biosafety Level 1 (BL-1) containment. This product contains Dimethyl Sulfoxide (DMSO), a hazardous material. Review the Material Safety Data Sheet (MSDS) before handling. Material Safety Data Sheets (MSDSs) are available on our website at www.invitrogen.com/msds.

## Information for European Customers

StemPro® Alk Phos-expressing Rat Mesenchymal Stem Cells are genetically modified and carry a chromosomal human Alkaline Phosphatase gene. As a condition of sale, this product must be in accordance with all applicable local legislation and guidelines including EC Directive 90/219/EEC on the contained use of genetically modified organisms.

# Additional Products

## Additional Products

The products listed in this section may be used with StemPro® Alk Phos-expressing Rat Mesenchymal Stem Cells. For more information, refer to our website (www.invitrogen.com) or contact Technical Support (see page 21).

| Item | Quantity | Cat. no. |
|---|---|---|
| Minimum Essential Medium (MEM) α Medium (1X) with GlutaMAX™-I, ribonucleosides and deoxyribonucleosides | 500 ml | 32571-036 |
| GlutaMAX™-I Supplement | 100 ml | 35050-061 |
| Fetal Bovine Serum (FBS), MSC-Qualified | 100 ml<br>500 ml | 12662-011<br>12662-029 |
| StemPro® Adipogenesis Differentiation Kit | 100 ml | A10070-01 |
| StemPro® Chondrogenesis Differentiation Kit | 100 ml | A10071-01 |
| StemPro® Osteogenesis Differentiation Kit | 100 ml | A10072-01 |
| 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 |
| TrypLE™ Express Dissociation Enzyme without Phenol Red | 100 ml<br>20 × 100 ml | 12604-013<br>12604-039 |
| Antibiotic-Antimycotic (100X), liquid | 100 ml | 15240-062 |
| Gentamycin Reagent Solution (10 mg/ml), liquid | 10 ml | 15710-064 |
| Gentamycin Reagent Solution (50 mg/ml), liquid | 10 ml | 15750-060 |
| Trypan Blue Stain | 100 ml | 15250-061 |
| LIVE/DEAD® Cell Vitality Assay Kit | 1000 assays | L34951 |
| Countess™ Automated Cell Counter (includes 50 Countess™ cell counting chamber slides and 2 ml of Trypan Blue Stain) | 1 unit | C10227 |
| ELF® 97 Endogenous Phosphatase Detection Kit | 1 kit | E6601 |
| CultureWell™ chambered coverglass (16 wells per coverglass, set of 8) | 1 set | C37000 |

# Introduction

## Introduction

StemPro® Alk Phos-expressing Rat Mesenchymal Stem Cells (MSCs) are produced from bone marrow isolated from transgenic Fischer 344 rats expressing the human placental alkaline phosphatase (hPAP) gene linked to the ubiquitously active ROSA26 (R26) gene promoter (Kisseberth *et al.*, 1999; Mujtaba *et al.*, 2002). The cells were isolated under sterile conditions and cryopreserved from primary cultures. Before cryopreservation, the MSCs are expanded for three passages in α-MEM medium supplemented with 10% MSC-Qualified FBS and antibiotic/antimycotic solution. The freezing medium consisted of 70% α-MEM, 20% MSC-Qualified FBS, and 10% DMSO.

Each vial of MSCs contains cells that can differentiate into multiple mature cell phenotypes *in vitro*, including adipocytes, osteocytes, and chondrocytes (De Ugarte *et al.*, 2003; Meirelles Lda & Nardi, 2003; Pittenger *et al.*, 1999; Wu *et al.*, 2002). *In vitro* differentiation into non-mesenchymal cell types, such as neuronal and myogenic cells have also been described (Anjos-Afonso *et al.*, 2004; Deng *et al.*, 2001; Han *et al.*, 2002; Han *et al.*, 2004; Moscoso *et al.*, 2005; Phinney *et al.*, 1999; Wakitani *et al.*, 1995). In addition, MSCs are shown to be involved in certain types of cancers (Houghton *et al.*, 2004; Singh *et al.*, 2004), and are known to secrete immunomodulatory, anti-angiogenic, anti-inflammatory, pro-cardiovasculogenic, and pro-arteriogenic factors (Djouad *et al.*, 2003; Gojo *et al.*, 2003; Houghton *et al.*, 2004; Kinnaird *et al.*, 2004; Krampera *et al.*, 2003; Oh *et al.*, 2008; Olivares *et al.*, 2004; Orlic *et al.*, 2001).

StemPro® Alk Phos-expressing Rat MSCs can be used for studies of adult stem cell differentiation, tissue engineering, cell and genetic therapy, and potential future clinical applications. These cells can also be used in transplant studies to track transplanted cells as they differentiate into mature phenotypes.

We recommend that you use α-MEM with GlutaMAX™-I and MSC-Qualified FBS (see page vi) for optimal growth and expansion.

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### Characteristics of StemPro® Alk Phos-Expressing MSCs

- Are prepared from low-passage (passage 3) adherent rat primary cell cultures
- Express a flow-cytometry cell-surface protein profile positive for CD29, CD73, and CD90 (> 70%), and negative for CD45 (< 10%)
- Stain positive for Alkaline Phosphatase (> 80%)
- Contain cells characteristic of at least tri-potential differentiation that can differentiate into osteogenic, adipogenic, and chondrogenic lineages.

### Isolation and Expansion

StemPro® Alk Phos-expressing Rat MSCs are extracted from the hind leg bones of alkaline phosphatase transgenic Fischer 344 rats through mechanical and enzymatic digestion. Cells are expanded using α-MEM medium supplemented with 10% MSC-Qualified FBS and antibiotic/antimycotic solution, which supports a cell doubling time of 30 ± 5 hours.

The *in vitro* growth capacity of MSCs has not been definitely established and can vary greatly depending on the culture conditions such as seeding density and growth factors used, but the cells can be expected to expand for at least 30 population doublings before their growth rate decreases significantly (Bruder *et al.*, 1997; Meirelles Lda & Nardi, 2003).

### Differentiation Potential

Multiple investigators have demonstrated that MSCs can be differentiated towards multiple mature cell phenotypes. In addition to traditional mesenchymal lineages, MSCs have been differentiated towards cardiomyocytic and neuronal phenotypes using specialized media. The *in vitro* differentiation potential of MSCs has not been definitely established, but long-term culture and high cell density are implicated in the loss of differentiation potential (Meirelles Lda & Nardi, 2003).

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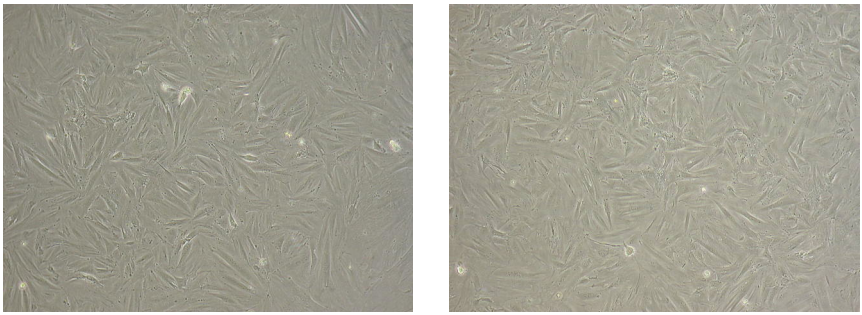
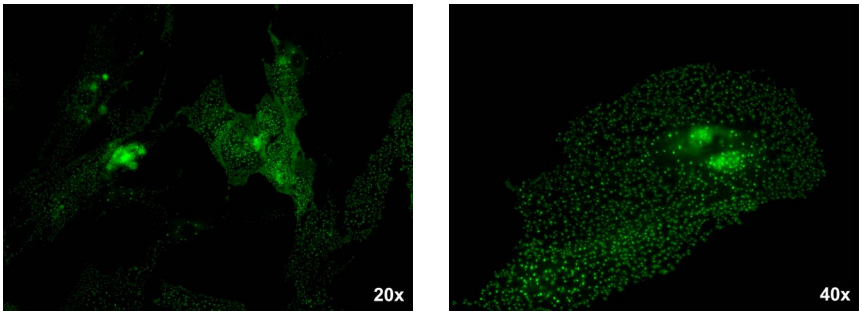
### Alkaline Phosphatase Expression

*In vivo* tracking of implanted MSCs in cell and gene therapy protocols is very important as the success of these therapies depends on MSCs' engraftment abilities, especially after systemic infusion (Meirelles Lda & Nardi, 2003). Further, it has been shown that MSCs can fuse with other cells and acquire their characteristics (Spees *et al.*, 2003). The StemPro® Alk Phos-expressing Rat MSCs allow the user to track the implanted cells (Han *et al.*, 2002; Han *et al.*, 2004; Mujtaba *et al.*, 2002) using a simple, fluorescence-based enzymatic assay, where the removal of the phosphate from the substrate provided in the assay kit causes an intense yellow-green fluorescence (ELF® 97 Endogenous Phosphatase Detection Kit, see page vi for ordering information).



Bright field images (10X) of StemPro® Alk Phos-expressing Rat MSCs at P4 that have been in culture for 14 days.




Fluorescence images (20X and 40X) of StemPro® Alk Phos-expressing Rat MSCs at P4 that have been in culture for 5 days. Alkaline phosphatase expression is detected using the ELF® 97 Endogenous Phosphatase Detection Kit

# Methods

## General Information

### General Cell Handling

Follow the general guidelines below to grow and maintain StemPro® Alk Phos-expressing Rat Mesenchymal Stem Cells.

- **All solutions and equipment that come in contact with the cells must be sterile.** Always use proper aseptic 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).


As with other mammalian cell lines, when working with MSCs, handle as potentially biohazardous material under at least Biosafety Level 1 (BL-1) containment. For more information on BL-1 guidelines, refer to *Biosafety in Microbiological and Biomedical Laboratories,* 4th ed., published by the Centers for Disease Control, or see the following website: www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm

**Important**

It is very important to strictly follow the guidelines for culturing StemPro® Alk Phos-expressing Rat Mesenchymal Stem Cells in this manual to keep them undifferentiated.

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### Media Requirements

We recommend using Minimum Essential Medium (MEM) α medium (α-MEM medium) with GlutaMAX™-I and supplemented with 10% MSC-Qualified Fetal Bovine Serum (FBS) for optimal growth and expansion of StemPro® Alk Phos-expressing Rat MSCs, and to keep them undifferentiated (see page vi for ordering information).

- Prepare your growth medium prior to use.
- When thawing or subculturing cells, transfer cells into pre-warmed medium at 37°C.
- You may store the complete growth medium **in the dark** at 4°C for up to four weeks.
- Avoid repeated freeze-thaw cycles of MSC-Qualified FBS.

**Important**

We have observed that StemPro® Alk Phos-expressing Rat MSCs adhere poorly when plated on media other than α-MEM medium supplemented with 10% **MSC-Qualified** FBS after their initial thaw. Although they recover and adhere well after their first passage, we suggest that you use the recommended media. If you prefer to culture your MSCs on growth media other than the recommended, we advise you to optimize your growth conditions and treat the your cells gently (*i.e.*, do not vortex, bang the flasks to dislodge the cells, or centrifuge the cells at high speeds).

# Thawing and Establishing Cells

## Introduction

To thaw StemPro® Alk Phos-expressing Rat MSCs and to initiate cell culture, follow the protocol below.

## Materials Needed

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

- StemPro® Alk Phos-expressing Rat MSCs, stored in liquid nitrogen
- Ethanol or 70% isopropanol
- α-MEM medium with GlutaMAX™-I containing 10% **MSC-Qualified** FBS plus antibiotic/antimycotic or gentamycin; pre-warmed to 37°C
- Disposable, sterile 15-ml tubes
- 37°C water bath
- 37°C incubator with a humidified atmosphere of 5% $CO_2$
- Microcentrifuge
- Tissue-culture treated flasks, plates or dishes
- Hemacytometer, cell counter and Trypan Blue, LIVE/DEAD® Cell Vitality Assay Kit, or the Countess™ Automated Cell Counter

**Note**

Invitrogen's Countess™ Automated Cell Counter is a benchtop counter designed to measure cell count and viability (live, dead, and total cells) accurately and precisely in less than a minute per sample, using the standard Trypan Blue technique (see page vi for ordering information).

Using the same amount of sample that you currently use with the hemocytometer, the Countess™ Automated Cell Counter takes less than a minute per sample for a typical cell count and is compatible with a wide variety of eukaryotic cells and provides information on cell size.

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### Thawing Procedure, continued

To thaw and establish StemPro® Alk Phos-expressing Rat MSCs:

1. Pre-warm the prepared α-MEM medium with GlutaMAX™-I containing 10% MSC-Qualified FBS and antibiotic/antimycotic or gentamycin to 37°C.
2. Remove the cells from liquid nitrogen storage, and wipe the cryovial with ethanol or 70% 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 15-ml sterile tube and add pre-warmed complete α-MEM medium **dropwise** up to 10 ml.
5. Centrifuge cells for 5 minutes at 300 × *g*.
6. Aspirate supernatant and resuspend cells in 2 ml of complete α-MEM medium
7. Determine the viable cell count using your method of choice, and plate the resuspended cells at a seeding density of 5,000 cells per $cm^2$. If necessary, add complete α-MEM medium to the cells to achieve the desired cell concentration and recount the cells.
8. Incubate at 37°C, 5% $CO_2$ and 90% humidity and allow cells to adhere for at least 24 hours.
9. The next day, replace the medium with an equal volume of fresh, pre-warmed complete α-MEM medium.
10. Change the medium every 3–4 days.

# Subculturing Cells

## Introduction

Follow the protocol below to culture StemPro® Alk Phos-expressing Rat MSCs. Subculture cells when needed (before colonies start contacting each other), typically every 7–10 days.

## Materials Needed

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

- Culture vessels containing StemPro® Alk Phos-expressing Rat MSCs
- Tissue-culture treated flasks, plates or dishes
- α-MEM medium with GlutaMAX™-I supplemented with 10% **MSC-Qualified** FBS and containing antibiotic/antimycotic or gentamycin, pre-warmed to 37°C
- Disposable, sterile 50-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, pre-warmed to 37°C
- Hemacytometer, cell counter and Trypan Blue, LIVE/DEAD® Cell Vitality Assay Kit, or the Countess™ Automated Cell Counter

## Passaging Cells

1. Aspirate the complete α-MEM medium from the cells.
2. Rinse the surface of the cell layer with DPBS without $Ca^{2+}$ and $Mg^{2+}$ (approximately 2 ml DPBS per 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. Aspirate the DPBS and discard.
4. To detach the cells, add a sufficient volume of pre-warmed TrypLE™ Express to cover the cell layer (approximately 0.5 ml/10 $cm^2$).
5. Incubate at 37°C for approximately 5–8 minutes.

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### Passaging Cells, continued

*Procedure continued from previous page*

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 **gently** to expedite cell detachment.
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 TrypLE™ Express) of pre-warmed complete α-MEM medium. Disperse the medium by pipetting over the cell layer surface several times.
8. Transfer the cells to a 50-ml conical tube and centrifuge at 300 × *g* for 5 minutes at room temperature. Aspirate and discard the medium
9. Resuspend the cell pellet in a minimal volume of pre-warmed complete α-MEM medium and remove a sample for counting.
10. Determine the total number of cells and percent viability using your method of choice. If necessary, add complete α-MEM medium to the cells to achieve the desired cell concentration and recount the cells.
11. 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)
12. Add complete α-MEM medium to each vessel so that the final culture volume is 0.2–0.5 ml per $cm^2$.
13. Add the appropriate volume of cells to each vessel and incubate at 37°C, 5% $CO_2$ and 90% humidity.
14. 3–4 days after seeding, completely remove the medium. Replace with an equal volume of complete α-MEM 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 StemPro® Alk Phos-expressing Rat MSCs
- α-MEM 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
- Hemacytometer, cell counter and Trypan Blue, LIVE/DEAD® Cell Vitality Assay Kit, or the Countess™ Automated Cell Counter
- Sterile freezing vials

## Guidelines

When freezing MSCs, 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% **MSC-Qualified** FBS and 10% DMSO.
- Bring the cells into freezing medium in two steps, as described in this section.

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# 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:

| | |
|---|---|
| α-MEM medium | 0.6 ml |
| FBS, 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:

| | |
|---|---|
| α-MEM 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 α-MEM medium from the flask, well, or dish.
2. Rinse the surface with DPBS without $Ca^{2+}$ and $Mg^{2+}$ (approximately 2 ml DPBS per 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. Aspirate the DPBS and discard.
4. To detach the cells, add a sufficient volume of pre-warmed TrypLE™ Express to cover the cell layer (approximately 0.5 ml/10 $cm^2$).
5. Incubate at 37°C for approximately 5–8 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. Gently tap the vessel to expedite cell detachment.

*Procedure continued on next page*

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### Freezing Cells Procedure, continued

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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 pre-warmed complete α-MEM medium to each vessel. Disperse the medium by pipetting over the cell layer surface several times.
8. Transfer the cells to a 15-ml conical tube and centrifuge at 300 × *g* for 5 minutes at room temperature. Aspirate the medium used for washing the cells (step 7).
9. Resuspend the cell pellet in a minimal volume of pre-warmed complete α-MEM medium and remove a sample for counting.
10. Determine the total number of cells using your method of choice.
11. Gently aspirate media from the vessel and resuspend the cells to a concentration of $4 \times 10^6$ cells/ml in Freezing Medium A.
12. Add the same volume of Freezing Medium B to cells in a **dropwise** manner.
13. Aliquot 1 ml to each freezing vial and store at –80°C overnight in an isopropanol chamber.
14. 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.

# Differentiation Media

## Introduction

One critical hallmark of MSCs is their ability to differentiate into three or more mature cell types. Traditional and modern bioassays are used to demonstrate the multipotency of MSCs to differentiate along the osteogenic, adipogenic, and chondrogenic lineages. This section provides guidelines for preparing media that are used for inducing StemPro® Alk Phos-expressing Rat MSCs to differentiate into osteogenic, adipogenic and chondrogenic cell types.

## Mesenchymal Stem Cell Basal Medium

MSC basal medium is used a cell attachment medium and as a negative control during differentiation experiments. It consists of α-MEM medium with GlutaMAX™-I containing 10% **MSC-Qualified** FBS and 5 µl/ml gentamicin (see page vi).

| Component | Final Conc. | For 500 ml |
|---|---|---|
| α-MEM medium with GlutaMAX™-I | 1X | 450 ml |
| FBS, MSC-Qualified | 10% | 50 ml |
| Gentamicin (10 mg/ml) | 5 µg/ml | 250 µl |

## Osteogenic Differentiation Medium

To prepare osteogenic differentiation (OD) medium, combine the following in a sterile flask. Although you may use the StemPro® Osteocyte/Chondrocyte Differentiation Basal Media, differentiation appears to be more efficient with α-MEM as the basal media. Store the OD medium at 4°C **in the dark** up to four weeks.

| Component | Final Conc. | For 100 ml |
|---|---|---|
| α-MEM medium with GlutaMAX™-I | 1X | 90 ml |
| StemPro® Osteogenesis Supplement | 1X | 10 ml |
| Gentamicin (10 mg/ml) | 5 µg/ml | 50 µl |

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### Adipogenic Differentiation Medium

To prepare adipogenic differentiation (AD) medium, combine the following in a sterile flask. Although you may use the StemPro® Adipocyte Differentiation Basal Media, differentiation appears to be more efficient with α-MEM as the basal media. Store the AD medium at 4°C **in the dark** up to four weeks.

| Component | Final Conc. | For 100 ml |
|---|---|---|
| α-MEM medium with GlutaMAX™-I | 1X | 90 ml |
| StemPro® Adipogenesis Supplement | 1X | 10 ml |
| Gentamicin (10 mg/ml) | 5 µg/ml | 50 µl |

### Chondrogenic Differentiation Medium

To prepare chondrogenic differentiation (CD) medium, combine the following in a sterile flask. Although you may use the StemPro® Osteocyte/Chondrocyte Differentiation Basal Media, differentiation appears to be more efficient with α-MEM as the basal media. Store the CD medium at 4°C **in the dark** up to four weeks.

| Component | Final Conc. | For 100 ml |
|---|---|---|
| α-MEM medium with GlutaMAX™-I | 1X | 90 ml |
| StemPro® Chondrogenesis Supplement | 1X | 10 ml |
| Gentamicin (10 mg/ml) | 5 µg/ml | 50 µl |

# Differentiating StemPro® Alk Phos-expressing Rat MSCs

## Introduction

This section provides guidelines and instructions for inducing StemPro® Alk Phos-expressing Rat MSCs to differentiate into osteogenic, adipogenic, and chondrogenic cell types.

## Materials Needed

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

- Culture vessels containing your MSCs
- Tissue-culture treated flasks, plates, or dishes
- MSC Basal Medium, prewarmed to 37°C (see page 13)
- Appropriate Differentiation Medium, pre-warmed to 37°C (see pages 13–14)
- Dulbecco's Phosphate Buffered Saline (DPBS), containing no calcium, magnesium, or phenol red
- Disposable, sterile 50-ml tubes
- 37°C incubator with humidified atmosphere of 5% $CO_2$
- TrypLE™ Express, pre-warmed to 37°C
- Hemacytometer, cell counter and Trypan Blue, LIVE/DEAD® Cell Vitality Assay Kit, or the Countess™ Automated Cell Counter

## Harvesting MSCs

Follow the protocol below to harvest your StemPro® Alk Phos-expressing Rat MSCs for differentiation experiments. We recommend that you expand your cells to ≤ 70% confluency in a tissue-culture treated T-225 flask, and prepare the appropriate differentiation medium ahead of time.

1. Aspirate complete α-MEM medium from the flask and rinse the surface with DPBS without $Ca^{2+}$ and $Mg^{2+}$ (approximately 2 ml DPBS per 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.
2. Aspirate the DPBS and discard.
3. To detach the cells, add a sufficient volume of pre-warmed TrypLE™ Express to cover the cell layer (approx. 0.5 ml/10 $cm^2$).
4. Incubate at 37°C for approximately 5–8 minutes.

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# Differentiating StemPro® Alk Phos-expressing Rat MSCs, continued

## Harvesting MSCs, continued

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5. 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. Gently tap the vessel to expedite cell detachment.
6. Spin for 5 minutes at 300 × *g* at room temperature. While the cells are spinning, perform a viable cell count using your method of choice; note total cell number. Calculate required amount of MSC basal medium to obtain the appropriate seeding concentration (see below).
7. Resuspend cells in the appropriate amount of MSC basal medium.
8. Dispense cell solution according to differentiation condition being tested (see protocols below).

## Osteogenic Differentiation Protocol

Follow the protocol below to differentiate your StemPro® Alk Phos-expressing Rat MSCs into an osteogenic phenotype.

1. Seed the MSCs into culture vessels at $1.9 \times 10^4$ 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.
2. To six wells of a 12-well plate, add 1 ml of cell solution per well and let attach in the 37°C, 5% $CO_2$ incubator for a minimum of two hours.
3. Replace three wells with MSC basal medium as negative controls, and other three wells with fresh OD medium. Let culture at 37°C with 5% $CO_2$.
4. Refeed cultures every 2–3 days with media prepared at initiation of differentiation. MSCs will continue to expand as they differentiate under osteogenic conditions.
5. 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. For long term culture (>21 days), we recommend that you reduce the seeding density by half ($9.5 \times 10^3$ cells/cm$^2$) to prevent overgrowth and cell detachment.

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# Differentiating StemPro® Alk Phos-expressing Rat MSCs, continued

## Adipogenic Differentiation Protocol

Follow the protocol below to differentiate your StemPro® Alk Phos-expressing Rat MSCs into an adipogenic phenotype.

1. Seed the MSCs into culture vessels at $7.6 \times 10^4$ 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.
2. To six wells of a 12-well plate, add 1 ml of cell solution per well, and let attach in the 37°C, 5% $CO_2$ incubator for a minimum of two hours.
3. Replace three wells with MSC basal medium as negative controls, and other three wells with fresh AD medium. Let culture at 37°C and 5% $CO_2$.
4. Refeed cultures every 3–4 days with media prepared at initiation of differentiation. MSCs will continue to undergo limited expansion as they differentiate under adipogenic conditions.
5. 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.

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# Differentiating StemPro® Alk Phos-expressing Rat MSCs, continued

## Chondrogenic Differentiation Protocol

Follow the protocol below to differentiate your StemPro® Alk Phos-expressing Rat MSCs into a chondrogenic phenotype.

1. Detach cells using TrypLE™ Express and perform a cell count as described in **Harvesting MSCs**, pages 15–16 (through Step 6).
2. Resuspend the cells in MSC basal medium to a concentration of $8 \times 10^6$ 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% $CO_2$ and 90% humidity.

   **Note**: If this step is not performed under high humidity conditions, the spots may dehydrate and the formation of chondrogenic pellets inhibited.
5. To three of the spotted wells, add 1 ml of MSC basal medium as a negative control. To the other three wells, add 1 ml of CD medium.
6. Incubate at 37°C, 5% $CO_2$, and 90% humidity. Refeed cultures every 2–3 days with same media, prepared at the initiation of differentiation.
7. Check for chondrogenesis after a set period of cultivation. You may perform alcian blue staining on the pellets (to detect glycosaminoglycans) after 14 days, or paraffin section of pellets for collagen 2a immunohistological staining after ~21 days.

**Important**

# 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® Alk Phos-expressing Rat MSCs. |
| | Thawing medium not correct | Use pre-warmed complete α-MEM medium, prepared as described on page 5. Be sure to use **MSC-Qualified** FBS. |
| | Cells too diluted | Generally we recommend thawing one vial at a density of 5,000 cells per $cm^2$. |
| | Cell not handled gently. | StemPro® Alk Phos-expressing Rat MSCs are fragile; treat your cells gently, do not vortex, bang the flasks to dislodge the cells, or centrifuge the cells at high speeds. |
| Cells grow slowly | Growth medium not correct | Use prewarmed complete α-MEM medium. |
| | Cells too old | Use healthy MSCs, under passage 5; do not overgrow. |
| Cells differentiated | Culture conditions not correct | Thaw and culture fresh vial of new StemPro® Alk Phos-expressing Rat MSCs. Follow thawing instructions (page 6) and subculture procedures (page 8) exactly. |
| | Cells too old | MSCs above passage 5 may become differentiated. |

*Continued on next page*

### Culturing Cells, continued

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

| Problem | Cause | Solution |
|---|---|---|
| Cells not adherent after initial thaw | Used serum other than MSC-Qualified FBS | Be sure to prepare your culture medium using **MSC-Qualified** FBS (see page vi for ordering information). |
| Cannot detect expression of alkaline phosphatase | Assay system not sensitive enough | Use ELF® 97 Endogenous Phosphatase Detection Kit (see page vi for ordering information). |

### Differentiating Cells

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

| Problem | Cause | Solution |
|---|---|---|
| Cells fail to differentiate | Used StemPro® Osteocyte/Chondrocyte or Adipocyte Differentiation Basal Media | Although you may use the StemPro® Osteocyte/Chondrocyte or Adipocyte Differentiation Basal Media for your differentiation studies, we have observed that differentiation is more efficient with α-MEM as the basal media. Repeat your differentiation studies using α-MEM as the basal media |
| | Initial spotting step not performed under high humidity (if differentiating into chondrocytes) | If this step is not performed under high humidity conditions, the spots may dehydrate and the formation of chondrogenic plates inhibited. Repeat the initial spotting step at 37°C, 5% $CO_2$, and 90% humidity. |
| Cells have overgrown the culture plates and have detached | Initial seeding density too high | For long term culture (>21 days), we recommend that you seed at a lower cell density of $3 \times 10^3$ cells/$cm^2$ to prevent overgrowth and cell detachment. |

# Technical Support

## Web Resources

Visit the Invitrogen website at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
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# Notes

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