PEPROTECH®
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Instruction Manual

PeproGrow hMSC Medium

Maintenance Media for
Human Mesenchymal Stem Cells

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XENO-FREE

Product Use Limitations: Not for human use.

Made in the United States.

Expansion of Human Mesenchymal Stem Cells (MSCs) Using PeproGrow hMSC Medium

A. Introduction

PeproGrow hMSC (Mesenchymal Stem Cell) Medium is a xeno-free, human serum-containing, phenol red-free complete media formulation originally designed for the *in vitro* expansion of adipose-derived human mesenchymal stem cells (ADMSCs) while maintaining full multipotency. Since its design, this media formulation has been shown to be suitable for the sustained growth of adipose tissue-derived, bone marrow-derived, umbilical cord-derived, placental-derived, and urine-derived MSCs in both adherent and suspension culture. For optimal results, culturing should be conducted on a surface coated with PeproTech's Animal-Free Human Vitronectin Matrix as a surface-coating reagent; however, other suitable extracellular matrix (ECM) proteins, such as fibronectin or vitronectin, can be used. PeproGrow hMSC Medium was designed and developed in collaboration with American CryoStem Corporation, and is supplied as a 500mL bottle of PeproGrow hMSC Basal Medium (Catalog# BM-XF-HMSC-500) containing a human serum component, and a separate, lyophilized vial of animal-free PeproGrow hMSC Growth Factor Supplement (Catalog# GF-XF-HMSC-500). The addition of the separate, lyophilized growth factor supplement to the basal medium results in a complete medium containing all growth factors and supplements necessary for optimal expansion of human mesenchymal stem cells in culture. Additional companion products, including PeproTech's Animal-Free Vitronectin Matrix and Buffer Kit, are available separately.

B. Materials and Reagents.

1. PeproGrow hMSC Medium:

Kit/Components	Catalog Number:	Size:
PeproGrow hMSC Medium Kit	**XF-HMSC-500**	**500mL**
PeproGrow hMSC Basal Medium	BM-XF-HMSC-500	500mL
PeproGrow hMSC Growth Factor Supplement	GF-XF-HMSC-500	Vial for 500mL Basal Medium

2. Refer to Appendix I for additional materials and reagents, including the product listing of PeproTech's media and companion products.

C. Preparation of Medium and Growth Factor Supplement

1. To reconstitute the lyophilized PeproGrow hMSC Growth Factor Supplement, first warm the 500mL Bottle of PeproGrow hMSC Basal Medium in a 37˚C incubator and aseptically add 1mL of the warmed Basal Medium to the vial. Gently pipette the solution up and down several times to encourage resuspension of the lyophilized Growth Factor Supplement, and let the solution stand at room temperature for at least 5 minutes (Note: Do not vortex). After letting solution stand at room temperature, gently pipette again several times to ensure full solubilization.
2. Aseptically add the entire reconstituted PeproGrow hMSC Growth Factor Supplement to the bottle of remaining PeproGrow hMSC Basal Medium, and mix well by swirling. Filtration is not necessary when prepared aseptically.
3. **OPTIONAL**: Antibiotic/antimycotic agents may be added to the PeproGrow hMSC Basal Medium at the user's discretion.
4. **OPTIONAL**: If the full 500mL volume of medium is not needed, the amount of complete medium prepared can be scaled down, taking care to keep the ratio of basal medium volume to reconstituted growth factors consistent. Under these circumstances, a portion or aliquoted portions of the reconstituted growth factors may be frozen for future use.
5. Label the bottle with both the date of mixture and the newly calculated expiration date (2 weeks from the date of mixture). Store in the dark at 2˚C to 8˚C.

Storage/Stability: Keep medium in the dark.

Product Form	Temperature	Storage Time
Liquid Basal Medium	2°C to 8°C; Keep in the dark	6 months
Lyophilized Growth Factor Supplement	-20°C to -80°C	2 years from date of receipt
	2°C to 8°C	6 Months
Liquid Basal Medium + Reconstituted Growth Factor Supplement	2°C to 8°C; Keep in the dark	2 weeks from date of preparation

Avoid repeated freeze-thaw cycles.

6. Prior to cell culture, determine the volume of complete medium required for feeding by using the general recommendations listed below in **Table 1.**

Table 1: Recommended Volumes of Complete PeproGrow hMSC Medium

Flask Size	Surface Area (cm^2)	Recommended Volumes (mL)
T-12.5	12.5	2-3
T-25	25	5-6
T-75	75	14-16
T-225	225	40-60

7. Remove only the calculated amount of chilled complete medium and warm to room temperature (RT) prior to feeding cells. While the chilled medium can be warmed using a 37˚C water bath where necessary, please note that water baths can be a source of microbial contamination. Where time permits, it is preferable to warm the medium using a 37˚C tissue culture incubator, or by covering and leaving on a clean bench top for at least 20 to 30 minutes.

General recommendations:

- **Recommended Coating for Cell Cultureware:** Culturing on uncoated plastic is not recommended. It is recommended that MSCs be cultured on culture surfaces coated using PeproTech's Animal-Free Recombinant Human Vitronectin Matrix as a surface-coating reagent. However, other suitable ECM proteins can also be used. Fibronectin has been noted to encourage much stronger adherence of MSCs to culture surfaces than vitronectin, which can mean quicker cellular dissociation and passaging with cells grown on a vitronectin coating. PeproTech's Animal-Free Recombinant Human Vitronectin Matrix and Buffer Kit (catalog number AF-VMB-220) is recommended to be used at 5µg/mL, and performs optimally with standard cell dishes. Refer to "D. Preparation of Animal-Free Human Vitronectin Matrix-Coated Cell Plates/Dishes."
- **Please Note:** Where possible, hMSCs should be expanded in a low-oxygen environment of 5% O_2, as culturing at ambient O_2 concentration (roughly 21%) presents an unnatural environment for hMSCs and may result in altered growth kinetics and phenotypes.
- Refer to the Appendix for additional reagents and protocols.

D. Preparation of Animal-Free Human Vitronectin Matrix-Coated Cell Cultureware

1. Reconstitute the Animal-Free Human Vitronectin Matrix according to the instructions provided on the product's Certificate of Analysis (CoA) using the osmotically-compatible Animal-Free Human Vitronectin Matrix Buffer (PBS + Kolliphor P 188) from PeproTech's Animal-Free Recombinant Human Vitronectin Matrix and Buffer Kit (catalog number AF-VMB-220).

2. Coat cell cultureware with 5µg/mL of the reconstituted Animal-Free Human Vitronectin Matrix according to **Table 2** below. For other cultureware, adjust the volume in relation to the surface area, and ensure surface area is completely covered.

Table 2: Recommended Volumes of Animal-Free Human Vitronectin Matrix

Cultureware Type	Cultureware Size	Surface Area (cm^2)	Volumes (mL)
Flask	T12.5	12.5	1-2
Flask	T25	25	2-3
Flask	T75	75	8-9
Flask	T225	225	20-30
Dish	3.5 cm	8	1-2
Dish	6 cm	21	2-3
Dish	10 cm	55	4-6
Dish	15 cm	148	10-15
Plate	48 wells	0.95	0.1-0.2
Plate	24 wells	1.9	0.25-0.35
Plate	12 wells	3.8	0.50-0.75
Plate	6 wells	9.5	1.0-1.5

NOTE: It is critical that the entire culture surface is covered during coating and/or storage. Do not allow the dish surface to dry out as this may result in suboptimal performance.

3. After removal from 4˚C storage, coated cultureware should be warmed at 37°C for approximately 30 minutes prior to use, using either in a humidified cell culture incubator with gentle, periodic swirling, or a rocking, heated plate shaker.
4. After incubation of coated cultureware, aspirate the Animal-Free Human Vitronectin Matrix solution and immediately add complete medium. Do not allow the dish surface to dry as this can lead to the matrix not functioning as expected.
5. Allow medium to warm to room temperature by returning cultureware to a cell culture incubator or leaving in the laminar flow hood. Use the dish the same day.

E. Thawing Cryopreserved Human Mesenchymal Stem Cells

NOTE: Ideally, thawing of cryogenically frozen cell samples should be a rapid process wherein the entire frozen mass is more-or-less warmed at the same rate throughout. If warming is conducted too slowly or occurs at different rates in different parts of the frozen cell pellet, temperature gradients may form which can compromise the health of cells, especially those present at gradient interfaces. If done correctly, thawing a single vial of cells should take approximately 1 to 1.5 minutes. It is critical that cell suspensions, once thawed, be transferred into fresh, warmed cell culture medium as quickly as possible to immediately dilute the cryopreservative, especially if it is DMSO-inclusive as DMSO is known to exert cytotoxic effects during prolonged exposure.

1. Keep vials of cells frozen until ready to use. If possible, frozen cell stocks should be stored in the presence of vapor-phase nitrogen as immersion of cells in liquid can permit cross-sample contamination; although, storage in liquid nitrogen may suffice, if that is the only option available. Cells should never be stored long-term in -80ºC or -20ºC freezers as these temperatures are not low enough to maintain cellular stasis. Immersion in liquid or vapor phase nitrogen allows temperatures to reach -180ºC, which is necessary for maintaining cellular stasis.
2. Thaw cells in a 37ºC water bath by gently swirling vials back-and-forth through warm water. After 45 seconds, invert vials 2-3 times to facilitate mixing of warming cell suspension and break up any temperature gradients which may have formed. Continue swirling in warm water until cell pellet has melted (generally a total of 60-70 seconds).
3. Decontaminate the exterior of the vial by spraying with 70% alcohol, and transfer to a sterile tissue culture hood.
4. Remove the vial's cap, and pipette the contents up and down several times to resuspend.
5. Add cell suspension drop-by-drop to fresh, warm cell culture medium. Take care to do so slowly as cells may experience osmotic shock, resulting in lower post-thaw viability, if this process is rushed. It is recommended that 1mL of cryopreserved cell suspension be resuspended in at least 9mL of culture medium to sufficiently dilute the cryopreservative.
6. Once the cells have been added to the medium, pellet them by centrifugation in a swinging bucket centrifuge at 500-800g for 10 minutes. Aspirate the supernatant, taking care not to disturb the cell pellet.
7. Resuspend cells in 3mL of fresh cell culture medium, and determine cell count by the technique common to your laboratory.

8. Aspirate ECM solution from coated flask or dish, and add the appropriate volume of cell culture medium and cell suspension to make the total volume listed in **Table 2.** It is recommended that cells be seeded at ≥ 4,000 viable cells/cm^2; although, a slightly higher cell density (5,000-6,000 viable cells/cm^2) may be preferable for culture initiation as well as older cultures exhibiting slower growth rates.

NOTE: Cells in culture secrete a variety of their own endogenously-produced cytokines and growth factors. These secreted factors can be helpful in initiating cultures as well as supporting the growth of older ones, hence the above recommendation.

F. Passaging Mesenchymal Stem Cells

NOTE: Enzymatic passaging of cells being grown in culture has become standard procedure in all cell-based laboratories. Due to individual differences and preferences, we will not attempt to supplant existing methodologies. However, a few points should be raised.

1. First, enzymatic digestion processes will not only digest the ECM proteins to which the cells adhere, they will also digest the proteins present on the cell surface, including growth factor and cytokine receptors and antigenic marker proteins. The end user should always be mindful that overexposure to digestive enzymes can have the potential to damage cells or alter cellular phenotypes in the case of pluripotent or multipotent stem cells. Care should be taken to prevent overexposure by careful monitoring of cells during passage. Once cells have begun to "lift off" from the culture surface, gentle agitation of the flask by tapping may be employed to dislodge any weakly adherent cells.
2. Once cells have completely lifted off, no further enzymatic treatment is necessary. It is recommended that passaging be done with the TrypLE Cell Dissociation reagents (GIBCO) as experience has shown that they allow for gentler passaging, greater viability, and less disruption of cellular phenotypes. It is further recommended that a commercial Trypsin Inhibitor product, such as Soybean Trypsin Inhibitor, be used following cellular collection to neutralize enzymes and terminate digestive activity. While some may choose not to use this, we strongly recommend its use as experience has shown that residual digestive enzymes may alter cellular physiology.

NOTE: The steps below detail a suggested procedure for harvesting cells from a vitronectin-coated T75 flask and seeding another vitronectin-coated T-75. These recommendations are not intended to replace existing protocols in laboratory groups; they are merely suggestions. For all other cultureware, make reference to **Table 2** and adjust the volumes in relation to the surface area.

NOTE: Cells should not be allowed to reach absolute confluency. Passaging at 80-85% confluency is generally recommended as cells in that state will still be in an active growth phase.

1. Aspirate the medium from the flask, or alternatively appropriate cultureware.

2. **OPTIONAL**: Rinse with 5mL of PBS-EDTA. This will remove any dead cells and residual proteins, and will allow for quicker enzymatic digestion.
3. Add 5-8mL TrypLE and incubate at 37˚C for 8-10 minutes to allow sufficient time for detachment to occur.
4. If cells have not yet detached, gentle agitation of the flask by tapping may be employed to dislodge any weakly adherent cells. Likewise, drawing up the digestive enzyme and gently pipetting it over the culture surface may aid in collection.
5. Add cell suspension to an equal volume of Trypsin Inhibitor in a sterile collection tube (15mL or 50mL). Gentle pipetting of the entire volume of the cell suspension is generally sufficient to break up clumps.
6. **OPTIONAL:** Following cell collection, rinse the flask with 5-10mL of sterile HBSS or PBS to capture any cells that might remain in the flask, then add flask contents to the cell suspension.
7. Pellet cells by centrifugation. We recommend a range of 500-800g for 10 minutes. Centrifugation may be done at ambient room temperature or at 4°C. Both methods have been shown to result in equivalent cell qualities.
8. Return culture tubes to the biosafety cabinet, and sterilize the tube exteriors with 70% ethanol. Carefully aspirate supernatant (growth medium/TrypLE) so as not to disturb the pellet, and resuspend in 3-5mL of growth medium.
9. Determine the concentration of your cell suspension using the cell counting method typically used by your laboratory group.
10. Determine the desired cell volume necessary to achieve optimal cell density and add this volume to the cell culture flask. Best results have been seen when cells are seeded at a density of 4,000 viable cells/cm^2, which is 300,000 viable cells for a T-75 flask. For later passages that have begun to grow more slowly, increase the cell density to 5,000-7000 viable cells/cm^2. Allow cells to settle for at least 1 hour before observing.
11. Return flask to cell culture incubator and feed every second to third day.

G. Freezing Mesenchymal Stem Cells

NOTE: Ideally, cryogenic freezing of any cell type should be conducted in a gradual, step-wise fashion that allows the solution to uniformly cool throughout its volume. The use of a suitable cryoprotectant will ensure retention of cellular viability during storage. In general, DMSO-inclusive cryoprotectants are preferred for cellular storage. It has been experimentally determined that a DMSO concentration of 5% is adequate to preserve MSCs.

1. Passage and count cells as described above in **Section F**.

2. Pellet the cells by centrifugation as previously described and aspirate the supernatant, taking care not to disturb the cell pellet.
3. Resuspend the cells in sufficient cryopreservative to give a final concentration of 500,000-3,000,000 viable cells/mL. Aliquot cells into cryovials.
4. Where possible, it is recommended that cells be frozen using a controlled-rate freezing unit with an optimized cooling protocol to gradually bring the final temperature to -80°C. Alternatively, where this is not possible, a CoolCell (Biocision) or "Mr. Frosty" cooling unit (Nalgene) can be placed in a -80°C freezer.
5. Once the vials have been frozen to -80˚C, they should be transferred to a liquid nitrogen (LN_2) vapor phase storage tank, or an equivalent liquid or vapor phase nitrogen storage system. Do not keep vials stored at -80˚C for more than 3 days' time.

Appendix I – Reagents and Materials

PeproTech Human Mesenchymal Cell Culture Media Products

PeproGrow hMSC Medium

Kit/Components	Catalog Number:	Size:
PeproGrow hMSC Medium Kit	**XF-HMSC-500**	**500mL**
PeproGrow hMSC Basal Medium	BM-XF-HMSC-500	500mL
PeproGrow hMSC Growth Factor Supplement	GF-XF-HMSC-500	Vial for 500mL Basal Medium

Companion Products

Companion Product/Components	Catalog Number:	Size:
Animal-Free Human Vitronectin Matrix and Buffer Kit	**AF-VMB-220**	**Kit**
Animal-Free Human Vitronectin Matrix		500µg
PBS + Kolliphor P 188		220mL

Appendix II – Tables, Figures and Descriptions

Table 3: Sources of MSCs tested PeproGrow hMSC Medium

Source	Acronym	Growth
Adipose Tissue Aspirate	AD-MSC	+
Bone Marrow Aspirate	BM-MSC	+
Umbilical Cord Blood Buffy Coat	UCB-MSC	+
Placental Stroma	PL-MSC	+
Urine-derived	U-MSC	+

Table 4: Immunophenotyping of MSC populations via immunofluorescence microscopy and flow cytometry

Marker	BM-MSCs	UCB-MSCs	PL-MSCs	AD-MSCs
CD13	+	+	+	+
CD14	-	-	-	-
CD19	-	-	-	-
CD29	+	+	+	+
CD31	-	-	-	-
CD34	-	-	-	-
CD44	+	+	+	+
CD45	-	-	-	-
CD73	+	+	+	+
CD90	+	+	+	+
CD105	+	+	+	+
CD146	+	+	+	+

Appendix III – References

1. Salasznyk, RM; et al. 2004. Adhesion to Vitronectin and Collagen I Promotes Osteogenic Differentiation of Human Mesenchymal Stem Cells. Journal of Biomedicine & Biotechnology 2004(1): 24-34.