CellExpand™

Suspension Expansion Culture™ (SEC™) Master Mixes for Lympho-Hematopoietic Stem and Progenitor Cells

Technical Manual

(Version 8-19)

This manual should be read in its entirety prior to using this product

For *In Vitro* Research Use Only.
Not for clinical diagnostic use.

Preferred Cell Systems™

KM014.001

TABLE OF CONTENTS

1. LIMITATIONS OF THE ASSAY AND PRECAUTIONS

1. *CellExpand™ is not approved by either the U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMA)*
2. *CellExpand™ is for research use only and has not been approved for clinical GMP use.*
3. *Reagents and supplies in this kit are STERILE. Perform all procedures under sterile conditions, except where indicated.*
4. *This reagent should not be used beyond the expiration date on the kit label.*
5. *Do not mix or add other reagents to CellExpand™. The final concentrations will otherwise be diluted.*
6. *Always use professionally calibrated and, preferably, electronic pipettes for all dispensing procedures. Small discrepancies in pipetting can lead to large pipetting errors. Although electronic pipettes self-calibrate themselves, they still need to be professionally calibrated on a regular basis.*
7. *Good laboratory practices and universal protective precautions should be undertaken at all times when handling the kit components as well as human cells and tissues. Material safety data sheets (MSDS) are included in each literature packet.*

2. Introduction

CellExpand™ are complete Master Mixes contain either individual or cocktails of ready-mixed and ready-to-use growth factors and/or cytokines to culture and/or expand lympho-hematopoietic stem and progenitor cells in suspension culture. Cells can be cultured in different culture vessels ranging from microwell culture plates to large flasks, reactors and 3-dimensional culture systems. CellExpand™ Suspension Expansion Culture™ (SEC™) Master Mixes are available to culture up to different stem cell and progenitor cell populations from up to 5 species.

To quantitatively measure cell proliferation after CellExpand™ use, Preferred Cell Systems recommends HALO®, HemoFLUOR™, MultiCellGro™ or HemoLIGHT™ Assays kits. To determine differentiation ability and/or capacity, AllColonies™, ColonyGro™, CAMEO™-4 or CAMEO™-96 methylcellulose assays are highly recommended.

3. Use and Availability

Applications of CellExpand™

- Expansion of stem and progenitor cells in suspension culture.
- Cell growth in virtually any cell culture vessel from microwell plates to cells reactors and 3-dimensional cultures.
- Allows easy and rapid high-content screening for both proliferation and differentiation processes.
- Flow cytometry.
- Cell signaling studies.
- Genetic analysis.
- Mechanism of Action (MOA) studies.
- Immune cell studies.

CellExpand™ can be used for the following tissues:

- Bone marrow
- Peripheral blood
- Umbilical cord blood
- Spleen
- Fetal liver
- Embryonic tissue (e.g. yolk sac)

CellExpand™ incorporating low serum is available for the following species:

- Human
- Non-human primate
- Dog
- Rat
- Mouse

CellExpand™ Serum-Free reagents are available for:

- Human
- Primate
- Mouse.

CellExpand™ should be used with cells that have the following degrees of purity:

- Mononuclear cell (MNC) fractions
- Purified stem or progenitor cells obtained by flow cytometry or magnetic bead separation.

CellExpand™ reagents:

- Allows cell-cell interaction.
- Produces greater assay sensitivity.
- Shorter cell incubation times; cell proliferation is measured on the exponential part of the growth curve.

- Coefficients of variation ≤15%.

Cell Populations Expanded using CellExpand™

Cell Type	Expanded Cell Population	Equivalent Colony-Forming Cell Population	Growth Factors/Cytokines
Stem Cells	SC-HPP 1	HPP-SP 1	IL-3, IL-6, SCF, TPO, Flt3-L
	SC-HPP 2	HPP-SP 2	EPO, GM-CSF, IL2, IL-3, IL-6, IL-7, SCF, TPO, Flt3-L
	SC-GEMM 1	CFC-GEMM 1	EPO, GM-CSF, IL-3, IL-6, SCF, TPO, Flt3-L
	SC-GEMM 2	CFC-GEMM 2	EPO, GM-CSF, IL-3, IL-6, SCF, TPO
	SC-GEMM 3	CFC-GEMM 3	EPO, GM-CSF, G-CSF, IL-3, IL-6, SCF + TPO
	SC-GEM 1	CFC-GEM 1	EPO, GM-CSF, IL-3, IL-6, SCF
	SC-GEM 2	CFC-GEM 2	EPO, GM-CSF, IL-3, SCF
Progenitor Cells	P-BFU 1	BFU-E 1	EPO, IL-3, SCF
	P-BFU 2	BFU-E 2	EPO
	P-GM 1	GM-CFC 1	GM-CSF, IL-3, SCF
	P-GM 3	GM-CFC 3	GM-CSF
	P-Mk 1	Mk-CFC 1	TPO, IL-3, SCF
	P-Tcell	T-CFC	IL-2
	P-Tcell	T-CFC	CD3 + CD28 (serum-free only)
	P-Tcell	T-CFC	IL-2 + CD3 + CD28 (serum-free only)
	P-Bcell	B-CFC	IL-7

Abbrevitions used. SC: Stem Cell. P: Progenitor cell. HPP-SP: High Proliferative Potential - Stem and Progenitor. CFC-GEMM: Colony-Forming Unit - Granulocyte, Erythroid, Macrophage, Megakaryocyte. CFC-GEM: Colony-Forming Unit - Granulocyte, Erythroid, Macrophage. BFU-E: Burst-Forming Unit - Erythroid. GM-CFC: Granulocyte-Macrophage Colony-Forming Cell. Mk-CFC: Megakaryocyte - Colony-Forming Cell. T-CFC: T-lymphocyte Colony-Forming Cell. B-CFC: B-lymphocyte Colony-Frorming Cell. EPO: Erythropoietin. SCF: Stem Cell Factor. TPO: Thrombopoietin. CSF: Colony Stimulating Factor. IL: Interleukin.

4. OVERVIEW of the CellExpand™ PROCEDURE

Using a CellExpand™ is usually just a 2 step process.

Step 1 – Cell Preparation
Cells are not provided with CellExpand™. Cells are prepared either with a user-defined, pre-validated protocol to obtain a single cell suspension or procedures that are suggested in this manual. A dye exclusion viability and/or metabolic viability and nucleated cell count should be performed on all samples.

Step 2 – Cell Culture
CellExpand™ is a complete, ready-to-use Master Mix for growing and/or expanding specific lympho-hematopoietic cell populations. Depending on the volume of CellExpand™ to be used, cells are added at 10% of the final volume. Culture vessels containing lympho-hematopoietic cells should be transferred to a 37°C, humidified incubator gassed with 5% CO_2 and preferably 5% O_2.

5. CellExpand™ Reagents and Storage Conditions

CellExpand™ reagents are supplied in 40mL volumes. For serum-free P-Tcell reagents, these are also supplied as 10 x concentrated in 2mL volumes. All CellExpand™ reagents are shipped complete and no other components should be added.

CellExpand™ reagents are shipped frozen. Upon arrival, transfer the reagents to a -20°C freezer until used.

Once thawed, CellExpand™ can be stored at 4°-8°C for 1 month, but can also be refrozen and thawed at a later time.

Growth factors and/or cytokines should only be added if the No Growth Factor CellExpand™ Base Master Mix was ordered.

6. Equipment, Supplies and Other Reagents Required, but not Provided

Equipment and Supplies

1. Laminar Flow Biohood
2. Plate luminometer (e.g. Berthold LB962 CentroLIA/pc; Molecular Devices, SpectraMaxL)
3. Sterile plastic tubes (5ml, 10ml, 50ml)
4. Single channel pipettes, preferably electronic (e.g. Rainin EDP pipettes for variable volumes between 1µl and 1000µl).
5. 8 or 12-channel pipette, preferably electronic (e.g. Rainin EDP pipettes for fixed or variable volumes between 10µl and 100µl).
6. Reservoir for 8- or 12 channel pipette
7. Sterile pipette tips.
8. Vortex mixer.
9. Tissue culture incubator, humidified at 37°C with 5% CO_2 (minimum requirement) and 5% O_2 (preferable).
10. 1.5ml plastic vials (5 for each ATP dose response).
11. Hemocytometer or electronic cell counter to determine cell concentration.
12. Flow cytometer or hemocytometer for determining viability.

Reagents

1. HemoGro™ Hematopoietic Growth Medium for cell suspensions and dilutions (Preferred Cell Systems™)
2. Iscove's Modified Dulbecco's Medium (IMDM)
3. Density-gradient medium (e.g. Ficoll).
4. 7-AAD, propidium iodide or trypan blue for viability assay.
5. LIVEGlo™ metabolic viability assay (Preferred Cell Systems™)

7. The CellExpand™ PROTOCOL

PLEASE READ THE FOLLOWING PROTOCOL CAREFULLY.
SEE SECTION 8 BEFORE PERFORMING THE ASSAY

Good laboratory practices and universal protective precautions should be undertaken at all times

Using CellExpand™ is usually a 2 step process.

Step 1 – Cell preparation.
Step 2 – CellExpand™ culture setup.

All steps must be performed in a laminar flow biohazard hood

STEP 1 – Cell Preparation

A. Human, Non-Human Primate, Canine and Rat Cells

1. For best results, CellExpand™ requires that target cells be separated from red blood cells since these can interfere with cell growth and expansion. Neutrophils and platelets should also be removed. Therefore, the starting cell suspension should be a mononuclear cell (MNC) suspension or purified population. Preferred Cell Systems™ recommends preparing MNCs using density gradient centrifugation media. This separation procedure should be used for human, non-human primate, canine and rat cells. Ficoll-Paque can also be used, but cells should be washed after this separation procedure since Ficoll can be toxic to cells. Follow the manufacturer's protocol to prepare the MNCs.
2. Resuspend the cells in HemoGro™ low serum or serum-free medium.

Human Umbilical Cord Blood and Bone Marrow Donor/Patient Samples.
Human umbilical cord blood and bone marrow samples are routinely left as a so-called total nucleated cell (TNC) fraction. This is produced by plasma or red blood cell depletion. The TNC fraction usually contains high concentrations of red blood cells, neutrophils and platelets that can interfer with the cell expansion process. It is recommended perform a density gradient separation per the manufacture's protocol so that the majority of cell impurities are removed from the cell suspension.

B. Preparation of Rat or Murine Bone Marrow

1. Remove organs (femora and tibia (optional)) under aseptic conditions.
2. Remove as much muscle from the bones as possible.
3. Using a sterile blade, first cut off the proximal (hip joint) end below the ball joint at right angles to the longitudinal length of the bone. Then cut off the distal end (above the patella or knee).
4. Transfer sufficient sterile medium to a tube so that it will cover the whole bone, approximately 1-2ml. (Some of the medium provided with the kit can be used for this purpose).
5. Half fill a syringe (1-3ml) with sterile medium and, using a needle gauge that will enter the bone cavity without cracking the bone, insert the needle into the proximal end and immerse the whole bone in the medium contained in the tube.
6. Flush out the marrow through the bone cavity and withdraw part of the cell suspension through the bone and into the syringe.
7. Flush the cell suspension through the bone and repeat steps 6 and 7 two to three times. When finished, the bone should appear translucent, indicating that most of the cells have been flushed out of the cavity.
8. Remove the empty bone and replace it with the next bone until the marrow from all bones has been flushed out of the cavities.
9. Let the cell suspension settle for 1-2 minutes to allow large debris to fall to the bottom of the tube.
10. Using a small gauge (22-25) needle and syringe, slowly withdraw the cell suspension leaving the large debris in the tube and transfer it to a new tube, noting the volume.
11. If necessary, add medium to achieve the required volume.

It is recommended to purify rat cells using density gradient centrifugation. This procedure is not necessary for mouse cells.

C. Cell Thawing after Cryopreservation

Thawing of Cells

CellExpand™ can be used with cryopreserved cells. If cells are cryopreserved as a total nucleated cell product, they will contain red blood cells, granulocytes and other cell populations. When the cells are thawed, granulocytes and other cell components will rupture and release DNA. Large amounts of released DNA will clump together encasing cells. If the cell preparation originally cryopreserved was a MNC or similar fraction, the chances of clumping will be low. However to reduce or alleviate the possibility of clumping during cell thawing, it is recommended that DNase be added to the cell suspension. The following procedure is used for small aliquots of cells only (1-1.5ml).

1. Thaw the vial contents in a 37°C water bath, by swirling the vial for approx. 1 min.
2. When a small ball of ice still remains in the vial (1-2 min), remove the vial from the water bath, sterilized the outside of the vial by spraying with 70% ethanol and carefully unscrew the vial lid.
3. It is possible that clumping can occur at this stage, in which case, add DNase to the total volume in the vial to achieve a concentration of 6µg/mL before proceeding to the next step.
4. Using a 1mL pipette, gently mix the contents of the vial and transfer to a 50mL tube containing 20mL of thaw medium. Up to 3 vials of the same cells can be added to this 20mL of thaw medium. However, clumping can also occur at this stage. In this case, DNase at a final concentration of 6µg/mL should be added before proceeding to the next step.
5. Gently mix the cells by swirling the contents of the tube. Do not use repeat pipetting to mix the cells. This could cause further rupture of cells and the release of DNA resulting in increased clumping.
6. Centrifuge the cells at 300 x g for 10 min at room temperature and discard the supernatant after centrifugation.
7. Resuspend the cells in 1mL of HemoGro™ medium. If necessary, add 6µg/mL DNase.

D. Isolation of Hematopoietic Subpopulations

CellExpand™ can also be used with purified cell populations prepared by cell sorting or magnetic bead separation.

E. Cell Viability, Cell Counting and Cell Culture Suspension Preparation

1. For dye exclusion viability methods, use trypan blue and a hemocytometer or automated method, flow cytometry using 7-AAD or another vital stain.
   Note *that dye exclusion viability methods detect membrane integrity. They do not detect cellular and mitochondrial integrity and therefore metabolic viability.*
   A viability of 85% or greater should be obtained when using dye exclusion viability methods only. It is recommended not to use cell suspensions with a viability of less than 85% since these cells will not be able to sustain proliferation ability. Use LIVEGlo™ (HemoGenix®, Inc) as a metabolic viability assay.
2. Determine the cell concentration using either a hemocytometer or electronic cell/particle counter.
3. Adjust the cell suspension concentration to that recommended in Table 1.
4. Prepare the total volume of cell suspension required in HemoGro™ medium. The volume of the adjusted cell suspension required will be 10% of the total volume of the CellExpand™ Culture Master Mix.

STEP 2. CellExpand™ Cell Culture

Please refer to Section 8 for recommendations and tips prior to beginning this stage of the procedure.

Perform all cell culture procedures under sterile conditions in a biosafety cabinet.

With the exception of CellExpand™ that contains no growth factor cocktails, all CellExpand™ Master Mixes are complete and ready-to-use. No additional components should be added since these will dilute the components and result in non-optimized culture conditions.

The CellExpand™ Method

1. Transfer one or more bottles of frozen CellExpand™ Master Mix to a 37ºC water bath or thaw at room temperature.
2. When thawed, mix the contents of the bottle by gently inverting several times being careful not to cause bubbles.
3. Prepare the cell suspension as required and adjust to the working cell concentration that should be 10 - 100 times higher than the required final concentration.
4. Prepare and label the culture vessels that are going to be used for cell expansion.
5. When the CellExpand™ Master Mix has thawed dispense the required amount of reagent into the culture vessel(s) being used minus 10%.
   NOTE*: If using a CellExpand™ Assay Kit with no growth factors (Base Master Mix), first dispense the reagent followed by the specific growth factor(s) and or cytokine(s).*
6. Add the cell suspension to the CellExpand™ Master Mix.
7. Mix thoroughly.
8. Culture the cells at 37˚C in a fully humidified incubator containing an atmosphere of 5% CO_2. If possible, use a 3-gas incubator to displace the atmospheric oxygen concentration (21%) to 5% O_2 with nitrogen. This increases the plating efficiency by reducing oxygen toxicity to the cells.
9. Lympho-hematopoietic stem cells initiate proliferation after about 24 hours and enter their exponential growth period after about 2-4 days depending on the species. Progenitor cells usually demonstrate similar growth kinetics, but their total proliferation growth period will be shorter than for stem cells.

8. RECOMMENDATIONS AND TIPS PRIOR TO USING CellExpand™

(i) Cell Suspension

- The preferred cell suspension is a mononuclear cell suspension (MNC) of human umbilical cord blood, human, primate, dog and rat bone marrow or peripheral blood. Murine bone marrow can usually be used without any fractionation.
- High concentrations of red blood cells can inhibit cell growth and expansion. It is therefore recommended to use an MNC preparation.
- If cells have been treated (e.g. with cytotoxic drugs etc.) prior to cell culture, or are to be treated in cell culture, higher cell concentrations may be required.

(ii) Thawing and Storage of the CellExpand™ Master Mix

- Prior to using the CellExpand™, remove the bottle from the freezer and thaw either in a 37ºC water bath or at room temperature.
- After use, CellExpand™ Master Mix is stable at 2-8°C for 1 month after thawing. Do not subject CellExpand™ to repeated freeze/thaw cycles.

(iii) Humidity Chamber

If microwell cultures or similar small volume cultures are performed, it is recommended to use an additional humidity chamber to prevent evaporation and drying out of the cultures. Even fully humidified incubators do not provide sufficient humidity to prevent evaporation.

Ordering Information
Toll free: 1-888-436-6869
Tel: (719) 264-6251
Fax: (719) 264-6253
Email: info@preferred-cell-systems.com
Order online at preferred-cell-systems.com

Technical Support
Tel: (719) 264-6251
Email: info@preferred-cell-systems.com

Preferred Cell Systems™
1485 Garden of the Gods Road
Suite 152
Colorado Springs, CO 80907
U.S.A.
Website: www.preferred-cell-systems.com

Preferred Cell Systems™