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FOR *IN VITRO* RESEARCH USE ONLY (RUO)
NOT FOR CLINICAL DIAGNOSTIC USE

CellExpand™

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

Manual

(Version 8-2013)

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

HemoGenix®, Inc

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7. 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, horse, pig, sheep, dog and rat bone marrow and peripheral blood. Murine peripheral blood should also be a MNC preparation. However, 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 37°C water bath or at room temperature.
- After use, CellExpand™ Master Mix is stable at 2-8°C for 1 month after thawing, but can be refrozen.

(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.

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1. INTRODUCTION

CellExpand™ is used to culture and/or expand lympho-hematopoietic stem and progenitor cells in suspension culture using different culture vessels 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 8 different stem cell populations and 7 progenitor cell populations from up to 8 species (human, non-human primate, horse, sheep, pig, dog, rat and mouse).

CellExpand™ is a complete Master Mix. It contains everything required to allow the cells to proliferate and expand. A CellExpand™ Base Master Mix is also available which contains no growth factor cocktails, thereby allowing the user to add any growth factors and/or cytokines at any concentration and for any species. Just add target cells at the required cell dose.

2. INTENDED USE: Applications and Advantages of Using CellExpand™

CellExpand™ is a complete reagent that can be used for stem cell research, basic and veterinary research and stem cell and regenerative cell therapy.

CellExpand™ can be used for lympho-hematopoietic stem and progenitor cells from:

- Embryonic tissue
- Fetal tissue
- Spleen
- Bone marrow
- Peripheral blood
- Cord blood

derived from the following species:

- Human
- Non-human primate
- Horse
- Pig
- Sheep
- Dog
- Rat
- Mouse

CellExpand™ is available for the following cell populations:

Cell Type	Cell Population	Growth Factors/Cytokines
	Not specified	No growth factors or cytokines included
Stem Cells	HPP-SP 1	IL-3, IL-6, SCF, TPO, Flt3-L
	HPP-SP 2	EPO, GM-CSF, IL2, IL-3, IL-6, IL-7, SCF, TPO, Flt3-L
	CFC-GEMM 1	EPO, GM-CSF, IL-3, IL-6, SCF, TPO, Flt3-L
	CFC-GEMM 2	EPO, GM-CSF, IL-3, IL-6, SCF, TPO
	CFC-GEMM 3	EPO, GM-CSF, G-CSF, IL-3, IL-6, SCF + TPO
	CFC-GEM 1	EPO, GM-CSF, IL-3, IL-6, SCF
	CFC-GEM 2	EPO, GM-CSF, IL-3, SCF
	CFC-GEM 3	EPO, GM-CSF, G-CSF, IL-3, SCF
Progenitor Cells	BFU-E 1	EPO, IL-3, SCF
	BFU-E 2	EPO
	GM-CFC 1	GM-CSF, IL-3, SCF
	GM-CFC 2	GM-CSF, G-CSF, IL-3, SCF
	GM-CFC 3	GM-CSF
	Mk-CFC 1	TPO, IL-3, SCF

treated with ACK lysis buffer.

- Removing excess density gradient reagent below the interface can result in granulocyte and platelet contamination of the MNCs.
- In some cases, a trade-off of higher cell recovery with increased platelet contamination may be necessary. In these cases, cells can be centrifuged for 10 minutes at 400 x g instead of 300 x g, with no detrimental effect on the cells.

STEP 2. CellExpand™ Cell Culture

Please refer to Section 7 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 bottle by inverting several times being careful not to cause bubbles.
3. Prepare the cell suspension as required and adjust to the preferred working cell concentration.
4. Prepare and label individual tubes or other culture vessels for each sample to be tested.
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. Dispense the cell suspension at the correct working concentration so that 10% is added to the CellExpand™ Master Mix . *This is now the Culture Master Mix.*
7. Mix the Culture Master Mix thoroughly by vortexing and leave for a few minutes to settle..
8. Dispense the Culture Master Mix into the vessels.
9. 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.
10. Lympho-hematopoietic stem cells initiate proliferation after about 24 hours and enter their exponential growth period after about 4-5 days. Progenitor cells usually demonstrate similar growth kinetics, but their total proliferation growth period will be shorter than for stem cells.

samples greater than 3ml, use a 50ml conical, plastic tube for separation.

3. For samples of 3ml diluted to 6ml with dPBS, dispense 5ml of the density gradient reagent into the tube. For samples greater than 3ml, dispense 15ml of the density gradient reagent into a 50ml tube.
4. Using a sterile, serological pipette, dispense the diluted sample gently on top of the density gradient reagent by holding the tube at approx. 45° and using a Pipette Aid on slow delivery. The cleaner the interface between the density gradient reagent and cell suspension, the better the separation will be. Do not allow the cells to mix with the reagent.
5. Centrifuge for 10 min at 1,000 x g or 20 min at 600 x g at room temperature with NO brake.
6. After centrifugation, remove the tube(s) gently and carefully aspirate the top layer above the MNC interface leaving approx. ¼" above the interface. Discard the supernatant.
7. Harvest the MNCs from the interface and transfer the cells to another sterile tube. It is best to harvest the cells using a manual 1ml pipette. Do not remove cells below the interface.
8. Add approx. 10-20 ml dPBS, mix gently and centrifuge the cells for 10 min at 300 x g at room temperature.
9. Aspirate the supernatant after centrifugation taking care not to aspirate the cell pellet.
10. Add 1-2ml of IMDM and resuspend the cells, breaking up any clumps using a 1ml manual pipette.
11. Perform a nucleated cell count and viability. The cell viability must be greater than 85%. Using cells with a viability lower than 85% will produce results with low proliferation ability.

Fractionating the MNCs by density gradient centrifugation will usually dramatically improve viability.

Density Gradient Centrifugation Troubleshooting

- Specimens that are not fresh or greater than 24 hours old, may not separate cleanly. These specimens may need to have the procedure performed twice.
- Fibrin strands can appear after separation. They are attached to the mononuclear cell layer. Care should be taken in this situation.
- Aspirate the supernatant using a serological pipette and save to a sterile tube. Do not discard the supernatant until a clean transfer has been made. Try to avoid the fibrin strands.
- If fibrin strands get caught up with the cells, transfer entire supernatant to a sterile tube and centrifuge for 10 minutes @ 300 x g. The resulting cells will have some residual platelets; however, most of the platelets will be removed with the supernatant after centrifugation.
- Bone marrow often has fat globules and bone spicules; these can get caught in the cell interface. The fat will rise to the top during the second spin and the spicules can be "picked out" using a manual 1ml pipette.
- If the cell separation is not clean and distinct, transfer the supernatant to a sterile tube using a serological pipette and reserve until the cell count is performed. If the cell count is low, centrifuge the supernatant to recover additional cells.
- Specimens that have a high red cell concentrations can be separated again or

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Cell Type	Cell Population	Growth Factors/Cytokines
	T-CFC	IL-2 (will usually require the addition of co-stimulator(s)
	B-CFC	IL-7

Abbrevitions used. 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.

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.

Advantages of Using CellExpand™

- Pre-made Master Mixes require no further additions. Just add cells.
- Available to detect up to 8 different stem cell populations and 8 progenitor cell populations from up to 8 different species.
- Setup as many replicates as necessary.
- Allows easy and accurate dispensing using normal pipettes and liquid handlers.
- CellExpand™ allows for cell interactions providing (a) shorter lag times to initiate cell proliferation; (b) more rapid culture completion; (c) greater sensitivity.
- Available in 10ml, 40ml and bulk quantities.

IMPORTANT:
CellExpand™ is for research use only and has not been approved for clinical diagnostic use or for cGMP conditions.

3. 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 by using 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. The reagent is usually provided in 10ml or 40ml bottles. 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.

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E. Cell Thawing after Cryopreservation

Thawing of 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. Thawing of the vial contents is initiated 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 culture medium. If necessary, add 6µg/ml DNase.

F. Mononuclear Cell Preparation

Density Gradient Centrifugation
Red blood cells and other contaminating cells can be removed by density gradient centrifugation. It should be emphasized that when the red blood cell content is high, even density gradient centrifugation may not provide optimal depletion. In most cases, however, density gradient centrifugation will produce a high quality MNC fraction and will also increase the viability of the cell sample.

HemoGenix® does not recommend using a Ficoll density gradient centrifugation procedure. This is because all Ficoll-derived reagents are toxic to cells. It is recommended to use NycoPrep 1.077, which is non-toxic and can also be added to cultures without any deleterious effects. It is essential that the density gradient centrifugation reagent is brought to room temperature prior to use. The procedure for density gradient centrifugation using NycoPrep is as follows:

1. Dilute the sample to be fractionated with an equal volume of Dulbecco's phosphate buffered saline (dPBS) and mix gently by inversion.
2. For samples 3ml or less use a 15ml conical plastic tube for separation. For

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.

To further purify a cell suspension, it is recommended to underlay 1-2ml of NycoPrep 1.077 (Axis-Shield) under the cell suspension in a sterile, 15ml conical tube with screw cap and centrifuge the cells according to the manufacturer's protocol.

C. Isolation of Hematopoietic Subpopulations

Providing sufficient cells are available, subpopulations of stem and progenitor cells can be isolated and purified for use with CellExpand™. HemoGenix® recommends using magnetic cell isolation procedures (e.g. Miltenyi Biotech), since these allow rapid isolation of different cell populations with substantial purity, viability and yield. Please see table of recommended cell concentrations to determine the optimal, final cell concentration to use with CellExpand™ reagents.

D. 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.
   Note *the working cell concentration per ml is 100 x the final cell concentration per well.* If cells have been treated prior to cell culture, higher cell concentrations may be required.
4. Prepare the total volume of cell suspension required using IMDM or PBS. The volume of the adjusted cell suspension required will be 10% of the total volume of the CellExpand™ Culture Master Mix.

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4. CellExpand™ Reagents and Storage

CellExpand™ is usually supplied as 10ml or 40ml volumes of complete, ready-to-use Master Mix containing all the components necessary to grow and/or expand the required cell population(s). The reagent is 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.

Good laboratory practices and universal protective precautions should be undertaken at all times when handling the kit components as well as cells and tissues. Material safety data sheets (MSDS) are included in each literature packet.

5. EQUIPMENT, SUPPLIES AND REAGENTS REQUIRED, BUT NOT PROVIDED

The following is a selection of equipment, supplies and reagents that might be required for use with CellExpand™.

Equipment and Supplies

1. 35mm Petri dishes that are not tissue culture treated.
2. Inverted microscope fitted with 10x oculars and a 4x and 10x objectives
3. Laminar Flow Biohood.
4. Sterile plastic tubes (5ml, 10ml).
5. Single channel pipettes, preferably electronic (e.g. ViaFlow or Rainin EDP pipettes for variable volumes between 1µl and 1000µl).
6. Sterile pipette tips.
7. Repeater pipette with positive displacement (e.g. Eppendorf Repeater Strean, Oxford, Gilson Distriman or Rainin AutoRep E) for dispensing CellExpand™ reagent.
8. Sterile syringes for repeater pipette.
9. Vortex mixer.
10. Tissue culture incubator, humidified at 37°C with 5% CO_2 (minimum requirement) and 5% O_2 (preferable).
11. Hemocytometer or electronic cell counter to determine cell concentration.
12. Flow cytometer or hemocytometer for determining viability.

Reagents

1. Sterile Phosphate Buffered Saline (PBS)
2. Iscove's Modified Dulbecco's Medium (IMDM)
3. Density-gradient medium (e.g. NycoPrep 1.077, Axis-Shield).
4. 7-AAD, propidium iodide or trypan blue for viability assay.

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6. The CellExpand™ PROTOCOL

**PLEASE READ THE FOLLOWING PROTOCOL CAREFULLY.
SEE SECTION 7 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, Horse, Pig, Sheep or Canine 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. HemoGenix® recommends separating the MNCs using NycoPrep 1.077 (Axis-Shield) density gradient centrifugation media. This separation procedure should be used for human, non-human primate, horse, pig, sheep and canine cells. Ficoll-Paque can also be used, although this is toxic to cells. Follow the manufacturer's protocol to prepare the MNCs.
2. Resuspend the cells in IMDM or PBS

Human umbilical cord blood.
It is recommended to deplete human umbilical cord blood of erythrocytes using a current Hetastarch® protocol or a density gradient separation per the manufacture's protocol so that the majority of the erythrocytes are removed from the cell suspension. It is not recommended to perform the CFC assay with a high concentration of erythrocytes present for the same reasons as above.

B. 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