Introduction

Primary human bone marrow hematopoietic progenitors are multipotent cells that can differentiate into several lineages (e.g. myeloid, erythroid and megakaryocytic cells). The in vitro differentiation of primary human hematopoietic progenitors can be accomplished by treatment with specific combinations of growth factors and assayed by the traditional colony assay (1). Colonies of differentiating blood cells appear over a time course of 12 to 14 days and are counted manually. While the parameters of the colony assay have been optimized to obtain quantitative data, the extremely low throughput and subjective nature of this assay preclude its use in library screening, early drug discovery and high-throughput toxicity screening.

The Myeloid, Erythroid and Megakaryocytic EasyDiff™ Supplements allow differentiation of CD34+ cells into three different hematopoietic lineages in 96-well plates. Each Supplement provides enough reagent for 96-wells. These EasyDiff™ Supplements can be used for both drug discovery and toxicology. Bone marrow progenitors are extremely sensitive to the toxic side effects of many drugs. Bone marrow toxicity is often a limiting factor in the dosing and duration of chemotherapy. Early discovery and elimination of toxic compounds is an important component of the drug discovery process. Studies have shown that the inhibitory activity of several drugs, as measured in in vitro assays of hematopoietic progenitor differentiation, correlate with their in vivo activities in various animal models (2, 3). Toxic effects of drugs can also be lineage-specific (4, 5).

NOTE: READ AND UNDERSTAND THE ENTIRE INSTRUCTION MANUAL BEFORE INITIATING AN ASSAY. Lonza’s warranty of the EasyDiff™ Supplement is dependent upon strict adherence to the detailed instructions provided in this instruction manual. Note: EasyDiff™ is Quality Control tested on bone marrow derived cells only; there may be variation in results using other sourced material or from donor-to-donor variation.

Components

PT-4510 Myeloid-Specific Differentiation Supplement including: SCF, GM-CSF, G-CSF and Fetal Bovine Serum.

Materials Required But Not Supplied with the EasyDiff™ Myeloid Supplement

- Human Bone Marrow CD34+ progenitor cells, Lonza No. 2M-101, 2M-101A, or 2M-101B
- LGM-3™ Medium, Lonza No. CC-3211
- L-Glutamine, Lonza No. 17-605C
- FBS, Lonza No. 14-501E
- Humidity chamber (e.g. Nunc Bio-Assay Dishes, No. 240835)

Thawing of Cells

The thawing procedure described below has been developed to provide optimal recovery and viability for Poietics™ CD34+ Progenitor Cells. Failure to follow this protocol will result in lower yields of viable progenitor cells.

1. Mix 5 ml FBS with 45 ml LGM-3™.
2. Warm LGM-3™ medium containing 10% FBS to 37°C.
3. Quickly thaw the vial of frozen cells in a 37°C water bath. Wipe the outside of the vial with 70% ethanol.
4. Aseptically transfer the cell suspension to a 50 ml conical tube.
5. Rinse the cryovial with 1 ml of LGM-3™ with FBS. Add the rinse dropwise to the cells while gently swirling the tube (~ one minute).
6. Slowly add enough medium dropwise to the cells until the total volume is 5 ml, while gently swirling after each addition of several drops of medium (~ three minutes).
7. Slowly bring the volume up to fill the tube by adding 1 ml to 2 ml volumes of LGM-3™ with FBS dropwise, while gently swirling after each addition of medium (~ five to ten minutes).
8. Centrifuge the cell suspension at 200 X g at room temperature for 15 minutes.
9. Carefully remove by pipet (and save in a second tube) most of the wash, leaving a few milliliters behind so the cell pellet is not disturbed. Gently resuspend the cell pellet in the remaining medium.

10. Dilute 20 ul of the cell suspension in 20 ul of 0.4% Trypan Blue and do a cell count and determine % viability. Recovery should be greater than 90%. If the cell count is lower than expected, centrifuge the wash saved in step #8 at a higher speed, count the pelleted cells and combine if necessary.

**Initiation of culture process**

1. Thaw contents of EasyDiff™ Supplement at room temperature.

2. Add the following components to the contents of the EasyDiff™ Supplement: 5.1 ml of LGM-3™ (without FBS), and 200 µl of L-Glutamine to make 8 ml of 2.5X Myeloid EasyDiff™ Medium. Mix gently. Unused 2.5X Myeloid EasyDiff™ Medium may be stored at 4°C for up to 10 days and still be used in another experiment.

3. For each well needed, mix 2000 CD34+ cells in a total of 20 µl of LGM-3 with 80 µl of 2.5X Myeloid EasyDiff™ Medium. It is suggested to combine cells and media for multiple wells together in a pool.

4. Transfer the diluted cell suspension to a sterile plastic reservoir and use a multichannel pipette to seed the primary human progenitors in the wells of the 96-well plate at a density of 2000 cells/well (100 µl/well). DO NOT SEED MORE THAN 2000 CELLS PER WELL. Please note that unused wells should be filled with 200 µl of sterile water. In addition, please note that the final Myeloid EasyDiff™ Medium Concentration should be 1x in all wells, which is achieved by adding either LGM-3™ and/or test samples (see section below) so a final volume of 200 µl /well is achieved.

**Treatment of the culture with test samples**

1. Test samples are added to the cell culture as 2X solutions in LGM-3™. Serial dilutions of the test samples can be made in 24-well tissue culture plates (1 ml/well). Test samples are diluted to concentrations twice the desired final concentration - they will be diluted 2-fold when 100 µl of the test sample is added to 100 µl of pre-seeded cells.

2. Starting with the lowest sample concentration, use a multichannel pipette to add 100 µl of each test sample dilution to 4 wells of primary human progenitors. Alternatively, assays can be done with triplicate samples. When treating cells with the test samples, a different set of pipette tips should be used for each test compound.

3. Add 100 µl of LGM-3™ to several wells as needed for controls lacking test samples.

4. A negative (inhibitory) compound (not supplied) can also be included in the assay as a positive control for cytotoxicity assays.

5. If the test sample stock solutions are in solvents such as DMSO or ethanol, solvent controls must be included in the assay. Solvent controls can be prepared by making serial dilutions to produce a series of 2X solutions of the solvent(s) at concentrations equal to those found in the test sample solutions. Add 100 µl of each solvent control to 4 adjacent wells of hematopoietic progenitors.

   It is important to use minimal concentrations of solvents in long-term *in vitro* assays; excess solvent concentrations can be toxic to cultured cells. Interestingly, DMSO is known to stimulate *in vitro* erythropoiesis (6) — this can complicate interpretation of assay results. Solvent concentrations should be kept at 0.1% or lower if possible.

6. Cover the 96-well plate and incubate for 1 hour at room temperature in the hood. This has been shown to minimize edge effect, thus improving assay performance (7).

7. Place the plate in a clean humidity chamber, following the instructions provided with the chamber. Culture the cells in the chamber at 37°C in 5% CO₂ for up to 10 days.

   **Note:** Use of both an appropriately humidified incubator and the humidity chamber is critical. The incubator must be >98% saturation. Do not culture the cells beyond 10 days.

---

**Example Differentiation Data:** Effect of drug compounds on bone marrow CD34+ cell differentiation using Myeloid EasyDiff™ Medium. CD11b positive cells were assessed using the Molecular Devices IsoCyte cytometer. (Data courtesy of Molecular Devices, Inc)
Safety Statement
THESE PRODUCTS ARE FOR RESEARCH USE ONLY. Not approved for human or veterinary use, for application to humans or animals, or for in vitro diagnostic or clinical procedures.

References