Limiting Dilution Assays to Determine Frequencies of Lymphohematopoietic Progenitors

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[Abstract] This protocol is useful to determine the frequencies of lymphohematopoietic progenitors in tested samples. To effectively support the growth and differentiation of primitive lymphohematopoietic progenitors, complex signals from stromal cells are important. Several stromal cell lines are known to support both lymphoid and myeloid cells simultaneously in mouse. In this protocol, we introduce two stromal co-culture systems for murine lymphohematopoietic progenitors and their application for limiting dilution assays.

Materials and Reagents

1. Stromal cells (MS5 cells or OP9 cells)
2. Growth factors
   a. rm SCF (10 ng/ml) (*e.g.* R&D systems, catalog number: 455-MC-010)
   b. rm Flt3-ligand (20 ng/ml) (*e.g.* R&D systems, catalog number: 427-FL-025)
   c. rm IL-7 (1 ng/ml) (*e.g.* R&D systems, catalog number: 407-ML-005)
3. Antibodies
   a. Anti-mouse Mac1 (BD Biosciences, catalog number: 557396)
   b. Gr1 (BD Biosciences, catalog number: 553128)
   c. CD19 (BD Biosciences, catalog number: 557399)
   d. CD45R/B200 (BD Biosciences, catalog number: 553092)
   e. CD45 (BD Biosciences, catalog number: 550994)
4. Trypsin/EDTA solution (Nacalai Tesque, catalog number: 35554-64)
5. EDTA solution (Nacalai Tesque, catalog number: 14367-74)
6. Alpha Modification of Eagle's Medium (Corning, cellgro®, catalog number: 50-012)
7. Minimum Essential Medium Alpha Medium (Gibco®, catalog number: 41061-029)
8. FCS (MP Biomedicals, catalog number: 2916754)
9. L-glutamine (Corning, cellgro®, catalog number: 61-030)
10. Penicillin-Streptomycin (Nacalai Tesque, catalog number: 26253-84)
11. MS-5 growth media (see Recipes)
12. OP-9 growth media (see Recipes)

Equipment

1. Cell sorting machines (BD Biosciences, FACS Aria™) with the automated cell deposition system
2. 96-well flat bottom plates
3. 10 cm plastic dishes
4. CO_2 incubators
5. Flow cytometry system

Procedure

1. Grow stromal cells to a sub-confluent state in 10 cm-dishes. Use MS-5 growth media to grow MS-5 stromal cells. Use OP-9 growth media to grow OP-9 stromal cells.
2. Wash the stromal cells twice with PBS and trypsinize with trypsin/EDTA solution at 37 °C for 5 min. Dissociate the cells by pipetting. After centrifugation at 300 *x g* for 5 min, re-suspend the cells in the growth medium. Seed half of the cells from a 10 cm dish into one 96-well plate on a day before the start of co-culture. The density and volume of stromal cells are 40~50 cells/l and 50 l/well when they are seeded into 96-well plates.
3. Prepare testing lymphohematopoietic progenitors (the testing progenitors should differ according to the aim of each experiment). Put the progenitors with the different densities (for example 1, 2, 4, 8, 16, 32, 64 cells per well) onto the prepared stromal layers by using a cell-sorting machine. Culture the progenitors on stromal cells in alpha Modification of Eagle's Medium supplemented with 10% FCS, rmSCF (10 ng/ml), rmFlt3-ligand (20 ng/ml), and rmIL7 (1 ng/ml).
4. Feed the cultures twice a week by removing half of the medium and replacing it with fresh medium. Cytokines should be freshly added with each feeding.

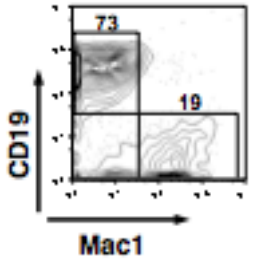

Figure 1. Shown above is a representative profile of flow cytometry showing the expression of Mac1 and CD19 on recovered CD45$^+$ cells (from Yokota *et al.*, 2009)

5. At 10 days of culture, inspect the wells for the presence of hematopoietic clones. Positive wells are harvested and analyzed by flow cytometry for the presence of $CD45^+$ hematopoietic cells, $CD45R/B220^+$ $CD19^+$ $Mac1^-$ (and/or $Gr1^-$) B-lineage cells, and $Mac1^+$ (and/or $Gr1^+$) myeloid-lineage cells (Figure 1).
6. Determine the frequencies of progenitors by calculating with linear regression analysis on the basis of Poisson distribution. The frequencies are given as the reciprocal of the concentration of test cells that result in 37% negative cultures. See Figure 2 for an example.

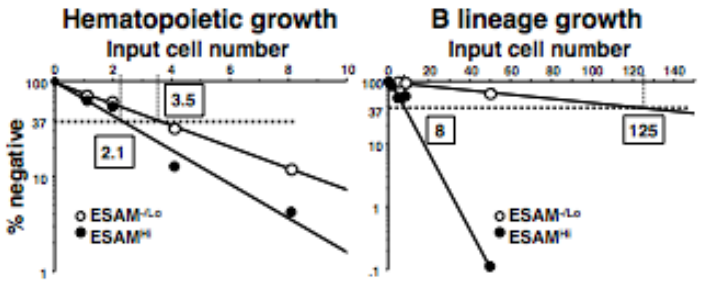

Figure 2. Semilogarithmic plots for hematopoietic cells and B lineage cells are made of % of negative cultures as a function of the dose of input $ESAM^{-/Lo}$ or $ESAM^+$ progenitors in each well. One in 2.1 $ESAM^{Hi}$ cells and 1 in 3.5 $ESAM^{-/Lo}$ cells gave rise to $CD45^+$ blood cells, indicating that both populations are potent sources of hematopoietic progenitors (left). However, while 1 in 8 $ESAM^{Hi}$ cells gave rise to $CD19^+$ B lineage cells, only 1 in 125 $ESAM^{-/Lo}$ cells produced B lineage cells under these conditions (right) (from Yokota *et al.*, 2009).

Recipes

1. MS-5 growth media
   Alpha Modification of Eagle's Medium
   10% FCS
   2 mM L-glutamine
   Penicillin-Streptomycin
2. OP-9 growth media
   Minimum Essential Medium Alpha Medium
   20% FCS
   Penicillin-Streptomycin

Acknowledgments

This protocol was adapted from our previously published papers Satoh *et al.* (2013) and Yokota *et al.* (2009). The representative data shown in the protocol was adapted from Yokota *et al.* (2009).

References

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2. Yokota, T., Oritani, K., Butz, S., Kokame, K., Kincade, P. W., Miyata, T., Vestweber, D. and Kanakura, Y. (2009). The endothelial antigen ESAM marks primitive hematopoietic progenitors throughout life in mice. *Blood* 113(13): 2914-2923.