TECHNICAL MANUAL

Human Long-Term Culture-Initiating Cell (LTC-IC) Assays

STEMCELL™ TECHNOLOGIES

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1.0 Introduction

The long-term culture (LTC) system for primitive hematopoietic progenitors was initially developed for mouse marrow cells[1] and then successfully adapted for human cells.[2] In this sytem, primitive hematopoietic cells associate with an adherent stromal layer and are able to generate myeloid clonogenic progenitors and mature granulocytes and macrophages for many weeks if provided with appropriate medium, supplements and culture conditions. LTC systems have also been developed for generation and quantitation of lymphoid[3] and natural killer (NK)[4] cells.

The primary application of this culture system is the quantification of primitive hematopoietic progenitors capable of initiating and sustaining myelopoiesis for several weeks in vitro.[5] These cells share functional and phenotypic properties with in vivo repopulating stem cells[6,7] and are commonly referred to as long-term culture-initiating cells (LTC-IC).

The unique features of the long-term culture system have allowed the development of the LTC-IC assay[5] to detect and quantitate primitive hematopoietic cells which share phenotypic and functional properties with mouse and human in vivo repopulating stem cells.[6,7] In human long-term cultures, colony-forming units (CFUs) detected after > 5 weeks represent the progeny of LTC-IC since CFUs present in the input cell suspension have undergone terminal differentiation by this time.

Quantitation of LTC-IC in a test cell suspension requires culturing the cells on a supportive feeder layer of irradiated marrow cells or suitable human or mouse fibroblast cell lines. Limiting dilution analysis is used to determine the frequency of LTC-IC as well as the average number of CFUs produced per LTC-IC. Once the average number of CFUs per LTC-IC is established, the LTC-IC content of a sample can be determined by a bulk culture LTC-IC assay, provided that the same source of test cells is used and the assay conditions are identical. The LTC-IC content is then calculated by dividing the total CFU output by the average number of CFUs produced per LTC-IC.[5]

Variations of the assay have also been described in which genetically engineered feeder cell lines are used that express human growth factors, to enhance the sensitivity of the assay, or in which highly purified cell populations are deposited individually into culture wells using fluorescence-activated cell sorting.[8,9]

2.0 Glossary of Terms Used

ABBREVIATION	DESCRIPTION
LTC-IC	Long-term culture-initiating cell
HLTM	Human long-term culture medium
FBS	Fetal bovine serum
IMDM	Iscove's Modified Dulbecco's Medium
CFU	Colony-forming unit
D-PBS	Dulbecco's phosphate-buffered saline
BFU-E	Burst-forming unit - erythroid
CFU-GEMM	Colony-forming unit - granulocyte, erythrocyte, macrophage, megakaryocyte
CFU-GM	Colony-forming unit - granulocyte, macrophage

3.0 Equipment and Materials Required

3.1 Equipment

- Biohazard safety cabinet certified for Level II handling of biological materials

  Note: All procedures for cell processing and set-up of CFU assays should be performed using sterile technique and universal handling precautions.

- Incubator set at 37°C with 5% CO_2 in air and ≥ 95% humidity

  Note: Use of water-jacketed incubators with a water pan placed in the chamber is recommended. It is important to use medical-grade CO_2, as inhibition of CFU growth due to toxic substances present in the CO_2 gas source has been reported.

- Inverted microscope for colony counting
- Use of a quality inverted microscope equipped with a 10X or 12.5X eyepiece objective, 2X, 4X and 10X planar objectives and a blue filter is recommended
- Standard light microscope for cell counting
- Laboratory centrifuge
- Vortex
- Pipette-aid
- Micropipettors
- Multi-channel pipettor
- Automated cell counter or Neubauer hemocytometer

3.2 Materials

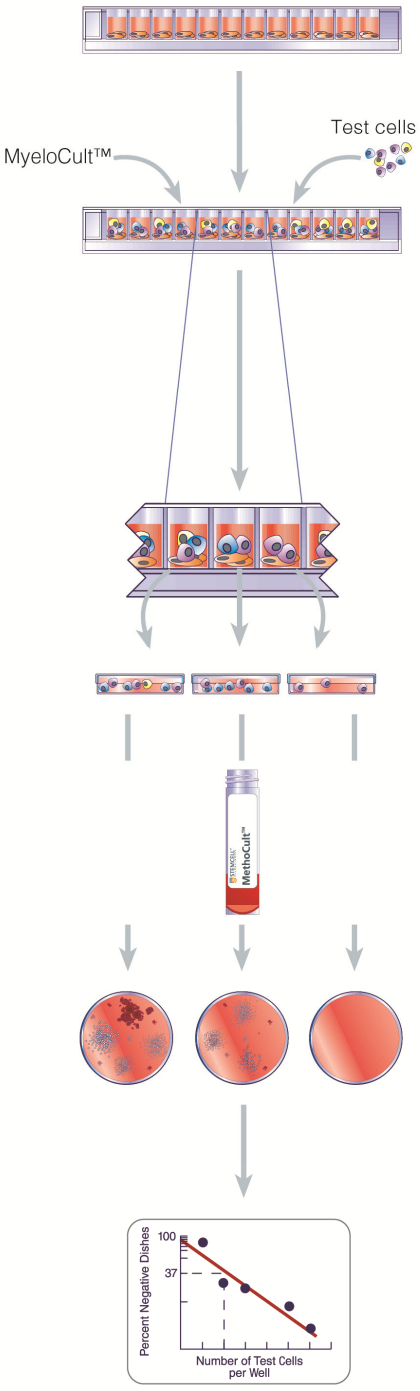
- Falcon® serological pipettes: 2 mL (Catalog #38002) and 5 mL (Catalog #38003)
- Sterile polystyrene tubes: 5 mL (Catalog #38007), 14 mL (Catalog #38008), 15 mL conical (Catalog #38009), 50 mL conical (Catalog #38010)
- Sterile pipette tips
- Syringes (luer lock): 3 mL (Catalog #28230), 6 mL, 12 mL
- 16 Gauge Blunt-End Needles (Catalog #28110)
- 35 mm tissue culture-treated dishes (Catalog #38050) or SmartDish™ 6-well culture plates (Catalog #27370)
- 100 mm tissue culture-treated dishes (Catalog #38046)
- 245 mm x 245 mm square culture dishes (e.g., 245 mm Square Dish, Tissue Culture-Treated, Catalog #38039) or 150 mm culture dishes
- 60 mm Gridded Scoring Dish (Catalog #27500) or STEMgrid™-6 counting grid (Catalog #27000)
- Permanent fine-tip marker
- 96-well plates

3.3 Additional Materials and Reagents

PRODUCT	CATALOG #
MyeloCult™ H5100	05150
MethoCult™ H4435 Enriched	04435
RPMI 1640 Medium	36750
HBSS, Modified (Without Ca++ and Mg++)	37250
Iscove's MDM with 2% FBS	07700
Hydrocortisone	07904
Trypsin in Citrate Saline (0.25%)	07400
Trypsin-EDTA (0.25%)	07901
Alpha MEM with Nucleosides	36450
Collagen Solution	04902
Lymphoprep™	07801
D-PBS (Without Ca++ and Mg++)	37350
L-Calc™ Limiting Dilution Software	28600

For a complete list of products available from STEMCELL Technologies Inc., visit www.stemcell.com.

4.0 LTC-IC Assay Procedure Diagram



1

Establish Feeders
(e.g. M2-10B4)

2

Add Serial Dilutions of Test Cells

3

Maintain Cultures
For ≥ 5 weeks with weekly 1/2 medium changes

4

Harvest and Plate
Harvest all cells from each well, including adherent and non-adherent cells, and transfer into individual methylcellulose cultures. Incubate for 14 - 16 days.

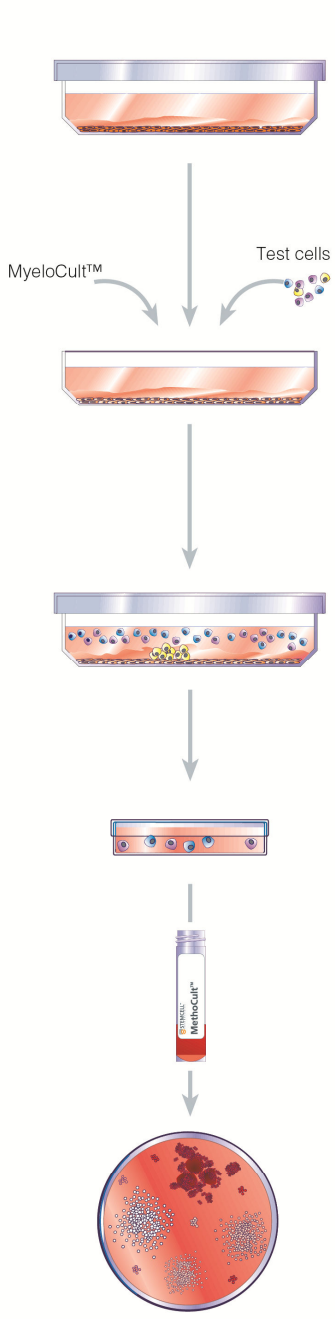
5

Score Dishes
Record growth (≥1 colony) or no growth (no colonies)

6

Calculate LTC-IC Frequency
Use a statistical software program for limiting dilution analysis, e.g. L-Calc™

5.0 Bulk Culture LTC-IC Assay Procedure Diagram



1
Establish Feeders
(e.g. M2-10B4)

2
Add Known Number of Test Cells

3
Maintain Cultures
For ≥ 5 weeks with weekly 1/2 medium changes

4
Harvest and Plate
Harvest entire dish, including adherent and non-adherent cells, and plate appropriate aliquot in methylcellulose cultures. Incubate for 14 -16 days.

5
Count Total Number of Colonies
Calculate number of LTC-IC in test cell suspension based on the average output of CFU per LTC-IC as determined by a limiting dilution assay

6.0 Maintenance of Feeder Cells

M2-10B4 mouse fibroblast cell line can be obtained from American Type Culture Collection (ATCC® CRL-1972™). Alternatively, STEMCELL Technologies can provide SI/SI and M2-10B4 fibroblast cell lines producing human growth factors. Contact us at techsupport@stemcell.com for further details.

Fresh M2-10B4 cells should be maintained in RPMI 1640 Medium containing 10% FBS in tissue culture flasks (T-25 cm^2 or T-75 cm^2).

Passaging Fresh M2-10B4 cells

1. Remove culture medium by careful decanting or suctioning.
2. Add 2 mL of HBSS, Modified (Without Ca++ and Mg++). Rotate flask gently and discard medium.
3. Add 2 mL of Trypsin in Citrate Saline (0.25%) per T-25 cm^2 flask.
4. Incubate at 37°C in 5% CO_2 with ≥ 95% humidity for 2 - 10 minutes or until adherent cells start to detach from the surface of the tissue culture flask.
5. Add 0.2 mL FBS to neutralize trypsin and mix with pipette to disperse cells.
6. Transfer cells to a 15 mL sterile tube and fill with 10% FBS in RPMI 1640 Medium. Centrifuge at 300 x *g* for 7 - 10 minutes.
7. Wash cells once with 10% FBS in RPMI 1640 Medium. Transfer 1/50 to 1/100 of the volume to a new T-25 cm^2 flask containing 8 mL of 10% FBS in RPMI 1640 Medium.
8. Incubate cells at 37°C in 5% CO_2 with ≥ 95% humidity. The cells should be passaged once they have reached 80% confluency (i.e. every 7 - 10 days).

Note: Irradiated cells cannot be passaged, as irradiation causes mitotic arrest.

7.0 Preparation of Human Long-term Culture Medium (HLTM) With Hydrocortisone

1. Thaw human long-term culture medium (HLTM; MyeloCult™ H5100) in a 37°C waterbath, at room temperature (15 - 25°C), or overnight at 2 - 8°C. Mix well.

   Note: If not used immediately, store at 2 - 8°C for up to 1 month.
2. Prepare stock solution of Hydrocortisone by dissolving Hydrocortisone powder in Alpha MEM with Nucleosides to yield a concentration of 1 x 10^{-3} M. Filter sterilize using a 0.2 µm low protein binding syringe filter.

   Note: Prepare fresh Hydrocortisone each week.
3. Prepare a 1 in 10 dilution of the 1 x 10^{-3} M Hydrocortisone solution to yield 1 x 10^{-4} M. Dilute this 1 x 10^{-4} M working stock solution 1 in 100 in HLTM to yield a final Hydrocortisone concentration of 1 x 10^{-6} M (e.g. 1 mL of 1 x 10^{-4} M Hydrocortisone in 99 mL HLTM).

   Note: Store HLTM with Hydrocortisone at 2 - 8°C for up to 1 week.

8.0 Collagen-Coating Tissue Culture Dishes for LTC-IC Assay

Note: Collagen-coating the tissue culture dish promotes adherence of feeder cells.

1. Add 1 mL of Collagen Solution per 35 mm tissue culture dish or 2 mL per 60 mm tissue culture dish for 1 - 2 minutes. Ensure solution is spread uniformly over the surface of the dish.
2. Remove excess collagen using sterile technique. This collagen solution can then be used to coat remaining dishes.
3. Allow the dishes to air dry at room temperature (15 - 25°C) for a minimum of 1 hour within a biosafety cabinet with the tissue culture lids partially or completely removed. Dishes can be used immediately or tightly wrapped and stored at 4°C for up to 2 weeks.
4. Rinse coated tissue culture dishes once with sterile D-PBS (Without Ca++ and Mg++) or culture medium to neutralize the acidity prior to use.

9.0 Preparation of M2-10B4 Feeder Layers

9.1 Preparation of Fresh M2-10B4 Feeder Cells

1. Trypsinize M2-10B4 cells from flasks as described in section 6.0 and wash twice in RPMI 1640 culture medium containing 2% FBS.
2. Following the final wash, resuspend cells in 1 - 2 mL of HLTM with Hydrocortisone and perform a nucleated cell count.
3. Irradiate cells with 8000 cGy using X-ray or ^{137}Cesium source.

   Note: It may be necessary to confirm that this dose of irradiation allows M2-10B4 to support LTC-IC but is sufficient to inhibit cell proliferation.
4. Plate the appropriate number of M2-10B4 cells in HLTM with Hydrocortisone in collagen-coated tissue culture dishes or in 96-well flat-bottom culture plates for limiting dilution analyses (Table 1).

Table 1. Recommended Plating Volumes/Densities for Preparation of M2-10B4 Feeder Layers

VOLUME	CULTUREWARE	CELL DENSITY
8.0 mL	60 mm tissue culture dish	1 x 10^6 cells/dish
2.5 mL	35 mm tissue culture dish	3 x 10^5 cells/dish
0.10 - 0.15 mL/well	96-well flat-bottom plate	1.25 x 10^4 cells/well

5. Incubate at 37°C in 5% CO_2 with ≥ 95% humidity for up to 10 days.

   Note: Although test cells can be added immediately, it is recommended that the cultures be incubated for a minimum of 24 hours prior to the addition of test cells.

10.0 Test Cell Suspensions

The numbers of LTC-IC can be measured in populations of bone marrow, cord blood, peripheral blood and mobilized peripheral blood. The LTC-IC content of normal peripheral blood is usually too low for accurate quantitation unless they are first enriched by density gradient centrifugation (e.g. Lymphoprep™) or other enrichment strategies. In addition, T and B cell depletion of peripheral blood samples is recommended to avoid the possible outgrowth of Epstein-Barr virus-transformed B cells.[10] The LTC-IC content of bone marrow can be assayed in samples following lysis of red blood cells by ammonium chloride treatment or density gradient centrifugation.[10] Methods to obtain cell suspensions enriched for LTC-IC content have been described in the literature.[5,10-14]

11.0 LTC-IC Limiting Dilution Assay

Refer to section 4.0 for the LTC-IC assay procedure diagram.

1. Establish feeder cells in 96-well flat-bottom culture plates as described in section 9.0.
2. On day of assay, carefully remove medium using multi-channel pipettor with sterile tips and discard.

   Note: Do not disturb the adherent feeder cells; the ends of pipette tips should not contact the adherent layer. To avoid contamination, care must be taken not to touch tips on the exterior of the wells. New tips must be used each time cells and/or medium are removed from wells. Do not allow the cells to dry (leave approximately 10 µL of medium in well).
3. Add test cells in 0.1 mL of HLTM with Hydrocortisone.

   Note: The number of cells seeded per well is dependent on the source of the cells and the expected enrichment of LTC-IC in the test cell suspension. The use of 3 or 4 different initial test cell concentrations with 12 - 16 wells per concentration is recommended.
4. Incubate LTC-IC cultures at 37°C in 5% CO_2 with ≥ 95% humidity for ≥ 5 weeks. Place cultures in suitable containers (allowing gas exchange) with open 35 or 60 mm dishes containing sterile water to reduce evaporation and potential contamination.
5. For weekly half-medium exchanges, remove one half of the medium and non-adherent cells and replace with one half of the original volume of fresh HLTM with Hydrocortisone.

   Note: Add fresh HLTM dropwise to avoid disrupting the adherent layer.

   Example: 100 µL cultures in 96-well flat-bottom plate: Remove 40 µL of medium using a multi-channel pipettor and sterile tips. Add 50 µL of fresh HLTM with Hydrocortisone.

12.0 Harvest of Cells From Limiting Dilution Cultures

LTC-IC are cultured for ≥ 5 weeks with weekly half-medium exchanges.

1. Remove HLTM and non-adherent cells from wells and place into individual 5 mL sterile tubes using a pipettor and sterile tips. Harvest 12 - 16 wells at a time.

   Note: A multi-channel pipettor can be used to harvest 3 wells at a time, providing test tube racks allowing uncapped 12 x 75 mm tubes to be close together are being used. To avoid contamination, care must be taken not to touch tips on the exterior of the tubes. New tips must be used each time cells and/or medium are removed from wells.

2. Rinse each well once with 0.1 mL HBSS, Modified (Without Ca++ and Mg++) and add to tube.
3. Add 0.1 mL Trypsin in Citrate Saline (0.25%) or Trypsin-EDTA (0.25%) to each well and incubate at 37°C in 5% CO_2 with ≥ 95% humidity. After 3 - 5 minutes of incubation, scan plate using inverted microscope to determine if adherent layer has started to detach. Continue incubation until adherent cells loosen. Trypsin should not be left in wells for more than approximately 10 minutes. If desired, add 10 µL of FBS to neutralize trypsin.
4. Resuspend cells with pipettor and sterile tips or multichannel pipettor with sterile tips to obtain a single-cell suspension. Add all cells and medium to the appropriate tube.
5. Rinse each well once with Iscove's MDM with 2% FBS and add to tube.
6. Fill tube with Iscove's MDM with 2% FBS and centrifuge at 300 x *g* for 7 - 10 minutes. Remove supernatant without disturbing cell pellet and leave approximately 0.1 mL of medium.
7. Vortex tube and add 1 mL of Methocult™ H4435 Enriched. Vortex and leave for 5 minutes to allow bubbles to rise to the top.
8. Plate each tube (contents of one well) individually into a 35 mm culture dish or individual wells of a SmartDish™ using a 1 mL syringe (without needle attached). Place 2 dishes within a 100 mm Petri dish containing a third 35 mm dish (without lid) with 3 mL sterile water.
9. Incubate at 37°C in 5% CO_2 with ≥ 95% humidity for 14 - 16 days.
10. Count colonies. A well is scored as positive if one or more BFU-E, CFU-GM or CFU-GEMM are detected or scored as negative if no colonies are present. For further information, refer to the Technical Manual: Human Colony-Forming Unit Assays using MethoCult (Document #28404), available at www.stemcell.com or contact us to request a copy.
11. The LTC-IC frequency in the test cell population is calculated from the proportion of negative wells (no CFU present) and the method of maximum likelihood.[16] Statistical analysis can be performed using L-Calc™ Limiting Dilution Software.
12. Appropriate statistical analysis can be used to determine the number of CFUs per LTC-IC using standard culture conditions.[5]

13.0 Quantitation of LTC-IC Using Bulk Cultures

Refer to section 5.0 for the bulk culture LTC-IC procedure diagram.

1. Resuspend test cells in the required volume of HLTM with Hydrocortisone (dependent on the type of cultureware used; see Table 1). Refer to Table 2 for the recommended concentration of cells for various cell sources. Gently remove the medium from tissue culture dishes containing irradiated feeder cells (section 9.0) and replace with HLTM with Hydrocortisone containing test cells.

Table 2. Recommended Concentration of Cells for Initiation of LTC-IC on M2-10B4 Feeder Layers

CELL SOURCE	CELLS PER CULTURE (per 35 mm dish)
Bone marrow - nucleated cells*	2×10^6
Bone marrow - mononuclear cells	1×10^6
Bone marrow - $CD34^+$ - Enriched	3000 - 5000
Peripheral blood - mononuclear cells	8×10^6
Cord blood - mononuclear cells	5×10^5

*Red blood cells removed using ammonium chloride red blood cell lysis buffer.

2. Place the 35 mm culture dishes within a 100 mm Petri dish. Include an additional uncovered 35 mm dish containing 3 mL sterile distilled water. This will allow easier handling of the cultures and help to maintain sterility and humidity.
3. Incubate at 37°C in 5% CO_2 with ≥ 95% humidity.
4. Every week for 4 - 5 weeks, remove one half of the medium containing cells and replace with freshly prepared HLTM with Hydrocortisone, as follows:
   a. Rotate dish gently to mix contents.
   b. Using a sterile 2 or 5 mL pipette draw up the contents of well and add back a volume equal to half the original volume of HLTM and cells (e.g. for 35 mm tissue culture dishes, add back 1.25 mL of HLTM and cells). Discard remaining medium and cells.
   c. Add a volume of fresh HLTM with Hydrocortisone equal to the amount of HLTM and cells added back in step 4b.

   Note: Place the tip of the pipette against the side of the culture dish and add or remove medium very slowly in order not to disturb the adherent layer. Examine the culture periodically using an inverted microscope to assess hematopoiesis and to detect any contamination.

14.0 Harvest of Cells from Bulk Cultures

Following 5 - 6 weeks of incubation, harvest LTC-IC cultures (both adherent and non-adherent cells) and assay for clonogenic progenitors in methylcellulose-based media.

The following example is for harvesting cells from 35 mm culture dishes.

1. Pipette non-adherent cells and medium from the tissue culture dish into a sterile 15 mL centrifuge tube (the "harvest tube").
2. Rinse the adherent layer twice with 1 mL HBSS, Modified (Without Ca++ and Mg++) (to remove any remaining medium containing serum and loosely attached cells). Add all medium to the harvest tube.
3. Add 1.0 mL of Trypsin in Citrate Saline (0.25%) or Trypsin-EDTA (0.25%) and incubate at 37°C in 5% CO_2 with ≥ 95% humidity for approximately 1 - 2 minutes (maximum 10 minutes). At intervals, swirl culture gently and examine using an inverted microscope for evidence of detachment of the adherent layer. Once adherent layer has started to detach, add 0.2 mL FBS to neutralize the trypsin.
4. Using a sterile pipette, repeatedly pipette the trypsin solution over the surface of the dish to ensure all adherent cells are detached and to make a single-cell suspension. Add all cells and medium to the harvest tube.
5. Rinse the culture dish twice with Iscove's MDM with 2% FBS. Add all cells and medium to the harvest tube.
6. Fill harvest tube with Iscove's MDM with 2% FBS and centrifuge at 300 x *g* for 7 - 10 minutes.
7. Remove supernatant and discard. Resuspend cells in approximately 400 µL of Iscove's MDM with 2% FBS. Record volume and perform a nucleated cell count.
8. Adjust to give the appropriate cell concentration in Iscove's MDM with 2% FBS and assay the number of colony forming units (CFU) using Methocult™ H4435 Enriched. Proportions of the culture can be assayed, or plate 2 - 4 dishes at a concentration of 2 - 5 x 10^4 cells per dish.

   Example: *For 5 x 10^4 cells per dish, adjust cells to 5 x 10^5 cells per mL in Iscove's MDM with 2% FBS. Add 0.3 mL of cells to 3 mL of MethoCult™ H4435 Enriched (for duplicate assays) or 0.5 mL of cells to 5 mL of MethoCult™ H4435 Enriched (for quadruplicate assays) and vortex. Let stand for 5 minutes to allow bubbles to rise to the top. Plate 1.1 mL per 35 mm Petri dish or individual well of a SmartDish™ using a 3 mL syringe attached to a 16 Gauge Blunt-End Needle. Rotate gently to spread MethoCult™ medium over the surface of the dish and place 2 dishes within a 100 mm Petri dish containing a third uncovered 35 mm dish with 3 mL sterile water.*
9. Incubate cultures at 37°C in 5% CO_2 with ≥ 95% humidity for 14 - 16 days.
10. Score and record the total number of colonies per dish.
11. Calculate the number of LTC-IC present in the initial test cell suspension by dividing the total number of CFUs detected in the culture by the average number of clonogenic progenitors per LTC-IC as determined by a limiting dilution assay performed on the same source of test cells and using the same assay conditions (section 11.0). Alternatively, values can be expressed as LTC-IC derived CFUs per number of test cells.

    Example: *A 35 mm culture dish containing irradiated M2-10B4 feeder cells was initiated with 2 x 10^6 ammonium chloride-treated normal bone marrow cells and incubated for 5 weeks with weekly half-medium exchanges.*

Number of test cells per 35 mm dish:	*2 x 10^6 bone marrow cells*
Total cells harvested at week 5:	*1 x 10^6*
Number of cells plated per dish of MethoCult™ H4435 Enriched:	*5 x 10^4*
Average number of CFUs obtained per methylcellulose culture:	*50*
Average CFU output per LTC-IC[8]:	*8*

Calculation:

Total CFU:

50 CFUs per 5 x 10^4 or 1000 per 1 x 10^6 total cells (harvested from LTC-IC culture)

Total LTC-IC:

1000 LTC-IC derived CFUs per 2 x 10^6 bone marrow cells

or

125 LTC-IC (divide 1000 by 8) per 2 x 10^6 bone marrow cells

15.0 References

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