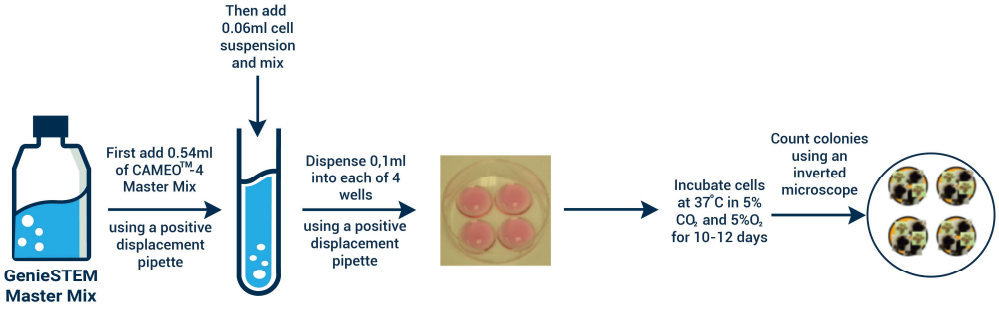
4 CFC/CFU Differentiation Assay Kit for Human Cells VIII, EPO free

(Catalog #BN00577; 50 assays; Store at -20°C)

I. Introduction:

4 CFC/CFU is a miniaturized, *in vitro* colony-forming cell (CFC) or unit (CFU) differentiation assay that detects the differentiation ability and/or potential of lympho-hematopoietic stem, progenitor or precursor cells to form colonies in semi-solid methylcellulose. Unlike the traditional assay, 4 CFC/CFU assay utilizes specially designed 35 mm petri dishes containing 4 mini-wells (0.1 ml each) which allow colonies to be easily viewed, differentiated and counted up to the edge of the well. The assay kit includes a superior **Master Mix** to support optimal growth and enumeration of your lympho-hematopoietic cell population of interest, including erythroid progenitors (CFU-E and BFU-E), granulocyte/macrophage progenitors (CFC-GM, CFU-M, CFU-G), multi-potential granulocyte, erythroid, macrophage and megakaryocyte progenitors (CFU-GEMM), etc., in just 10-14 days.



II. Applications:

- Basic, applied, and veterinary research
- Hematopoietic stem cell transplantation and cord blood bank processing laboratories

III. Sample Type:

- Bone marrow, peripheral blood (normal and mobilized), umbilical cord blood, spleen, fetal liver, yolk sac, purified cell populations (e.g. $CD34^+$ cells) from these tissues.

IV. Species

- Human

V. Kit Contents:

Components	BN00577	Part Number
4-wel, 35 mm Petri Dishes	50 dishes	BN00577-1
Master Mix VIII	38 ml	BN00577-2

VI. User Supplied Reagents and Equipment:

- Inverted microscope with 10X ocular and 4X and 10X objectives
- Tissue culture hood
- Single channel pipettes for volumes between 1-1000 µl and sterile tips
- Repeater pipette with positive displacement and sterile syringes
- Hemocytometer for cell counting and/or viability
- PBS
- Iscoves' Modified Dulbecco's Medium (IMDM)
- Density gradient medium (e.g. NycoPrep 1.077, Axis-Shield)
- Trypan blue for viability assay. (7-AAD or Propidium iodide if using FACS for viability determination)

VII. Storage Conditions and Reagent Preparation:

Store kit at -20°C, protected from light. Components can be stored up to 12 months, if unopened. Briefly centrifuge small vials prior to opening. Read entire protocol before performing the assay.

- **4 CFC/CFU Master Mix:** Thaw the reagent in a 37°C incubator or in a water bath at room temperature. Once opened the reagent can be stored at 4°C till the date of expiry.

VIII. 4 CFC/CFU Differentiation Assay Protocol:

1. Sample (Cell) Preparation:

a. Cells: Use NycoPrep 1.077 (Axis-Shield) density gradient centrifugation media to separate the mononuclear cells (MNC) from the red blood cells (RBC), platelets and neutrophils as per the manufacturer's protocol. (A high concentration of RBCs can inhibit cell growth as well as make it extremely difficult to count colonies. Therefore a hematocrit of 10% or less is required to avoid interference by RBCs). Resuspend the cells in IMDM or PBS.

b. **Human Umbilical Cord blood, peripheral blood (normal/mobilized) or bone marrow cells**: Deplete the cord blood of erythrocyte using a current Hetastarch® protocol or a density gradient separation per the manufacturer's instructions.

c. **Isolation of Hematopoietic Subpopulations:** Use magnetic cell isolation procedures (e.g. Miltenyi Biotech) for rapid isolation of stem and progenitor cell populations with substantial purity, viability and yield. Please see Table 1 to determine the optimal, final cell concentration to use for the assay.

d. **Cell Viability, Cell Counting and Cell Culture Suspension Preparation:** For dye exclusion viability methods, use trypan blue and a hemocytometer or automated method. A viability of 85% or greater should be obtained when using dye exclusion method. Determine the cell concentration and then adjust to the working cell concentration (refer to Table 1) using IMDM or PBS.

Notes:

- If using flow cytometry use 7-AAD or other vital stain.
- 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.

Table 1: Recommended Cell Plating Concentrations for Cell Types, Cell Populations and Cell States for 4 CFC/CFU

Species	Cell Type	Cell Preparation	Cell State	Working Cell Concentration Required (100 x Final Cells/Well)	Final Cell Dose / Well
Human	Bone marrow, Peripheral blood (normal & mobilized), Umbilical cord blood	MNC	Fresh/ Frozen	0.5-0.75 x 10^6	5,000-7,500
	Bone marrow	$CD34^+$	Fresh	0.1-1 x 10^5	100-1,000
	Mobilized peripheral blood*, Umbilical cord blood	$CD34^+$	Fresh/ Frozen	0.1-5 x 10^5	100-5,000

*Lot Dependent

2. 4 CFC/CFU Cell Culture

a. Mix the contents of the bottle of Master Mix thoroughly, avoid the formation of bubbles or foam.

b. Prepare and label tubes and the culture plates.

c. Dispense 0.54 ml of 4 CFC/CFU Master Mix accurately into each tube.

Notes:

- *If using a 4 CFC/CFU Assay Kit with no growth factors, first dispense only 0.48ml of the Master Mix into the tube(s). If adding a specific growth factor/cytokine cocktail, ensure that the working and final concentrations are prepared so that 0.06ml can be added to produce a final volume of 0.54ml prior to adding the cell suspension. Use IMDM to ensure that the correct volumes are added.*
- ***Important:*** DO NOT use a syringe and needle to dispense the methylcellulose-containing Master Mix as this is extremely inaccurate and results in high coefficients of variation (%CV). Use a positive displacement repeater syringe pipette for this purpose.

d. Dispense 0.06 ml of the working concentration cell suspension into the Master Mix using a calibrated pipette. This now produces the Culture Master Mix.

IMPORTANT: If using manual pipettes, ensure that the mechanism is working correctly and that the pipette is properly calibrated. Electronic pipettes are strongly recommended.

e. Mix the Culture Master Mix thoroughly by vortexing and leave for a few minutes for the mix to settle.

f. Using a positive displacement repeater syringe pipette, dispense 0.1 ml of the Culture Master Mix into each of the 4 replicate wells of each plate.

g. Using the same syringe tip, disperse the Culture Master Mix in the well so that the Mix completely covers the growth surface of the well.

h. To prevent the culture plates from drying out, either transfer 2 plates to a sterile 100 mm Petri dish containing an open 35 mm Petri dish filled with about 2-3 ml of sterile water, or transfer all plates to a large container containing a beaker of water and cover the container with aluminum foil.

i. Incubate 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 helps to increase the plating efficiency by reducing oxygen toxicity to the cells. Table 2 shows the suggested incubation times.

Table 1: Recommended Cell Plating Concentrations for Different Species, Cell Types, Cell Preparations and Cell States for 4 CFC/CFU

Species	Cell Type	Cell Populations	Incubation Period (days)
Human	Bone marrow, normal and mobilized peripheral blood, umbilical cord blood	Stem and progenitor cells Precursor cells	10-14 7

3. **Colony Enumeration:** After incubation, enumerate the colonies under an inverted microscope as per your institution's standard operating procedure for the CFC assay. The colonies should be enumerated using an inverted microscope equipped with 10x oculars and 4x pan objective to give an overview of the culture and a 10x objective for better colony identification. Higher objective can also be used (e.g. 20x).

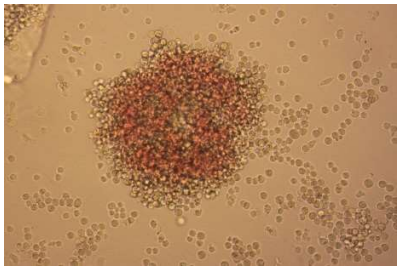


Figure: An example of a BFU-E (Burst forming unit-erythroid) colony: These are early committed erythroid progenitor cells.

IX. Troubleshooting Guide:

Problem	Causes	Solution
Cultures dry out	• Small culture volume • Insufficient humidity	• Put the culture dishes in a glass or plastic sandwich box. Place a beaker in the middle of the box full of sterile water
High Replicate Variation	• Problem in dispensing Methylcellulose-containing medium • Mistakes in cell concentration calculations	• Use a positive displacement repeater pipette to dispense all methylcellulose reagent • Check calculations and dispense cell volumes using calibrated pipettes
Colonies looking distorted or streaky	• Methylcellulose dishes were moved/disturbed during incubation	• Do not disturb the dishes during the incubation period.

FOR RESEARCH USE ONLY! Not to be used on humans