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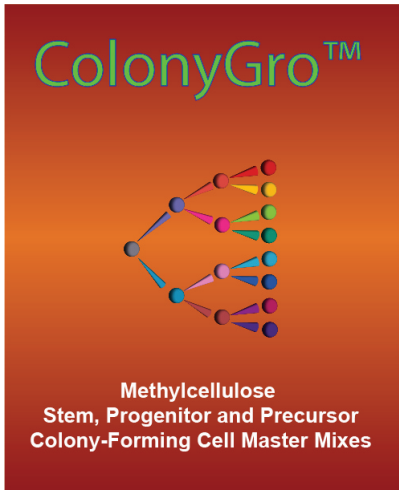

ColonyGro™

Methylcellulose Colony-Forming Cell Master Mixes for Lympho-Hematopoietic Stem, Progenitor and Precursor Cells

Assay Manual

(Version 3-2013)

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

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1. INTRODUCTION

ColonyGro™ is a methylcellulose-containing reagent for the clonal growth of stem, progenitor and precursor cells from the lympho-hematopoietic system. These are termed colony-forming cell (CFC) or colony-forming unit (CFU) assays. ColonyGro™ is available to detect up to 8 different stem cell populations, 7 progenitor cell populations and 4 precursor cell populations from up to 8 species (human, non-human primate, horse, sheep, pig, dog, rat and mouse). ColonyGro™ can be used for stem cell, basic and veterinary research applications as well as human cellular therapy applications.

HemoGenix® divides its cell-based assays into proliferation and differentiation assays. ColonyGro™ reagents and its equivalent complete CAMEO™-4 assay kits, detect the differentiation ability of lympho-hematopoietic cells, since the cells that produce colonies *in vitro* have to differentiate and mature in order for the investigator to identify the colonies. Although proliferation is required to produce the colonies, cell proliferation cannot be quantitatively measured using either ColonyGro™ or CAMEO™-4. CAMEO™-96 or HALO™-96 should be used to measure cell proliferation.

ColonyGro™ is a functional assay that was originally discovered in 1966 by Bradley and Metcalf in Melbourne, Australia and Pluznik and Sachs in Rehovot, Israel. In 1971, Stevenson and colleagues discovered a colony-forming population with limited proliferative capacity that produced small erythroid colonies under the influence of erythropoietin. These were called colony-forming units-erythroid (CFU-E). Shortly thereafter, Axelrad and coworkers discovered another erythropoietic population that required higher concentrations of erythropoietin and longer incubation times and was the progenitor of the CFU-E. This population was designated the burst-forming unit – erythroid (BFU-E). Colony-forming cells from multiple stem cell populations as well as every primary lympho-hematopoietic lineage can now be detected. In 1972, Iscove et al found that agar could be replaced with water-soluble methylcellulose, making the culture procedure easier to perform. In 1978, Bradley et al demonstrated that culturing GM-CFC under low oxygen tension was advantageous for growth. Similar results were found by Rich & Kubanek in 1982 for CFU-E and BFU-E using a miniaturized CFC/CFU assay that is now called CAMEO™-4.

The same reagents that are used for ColonyGro™ are also used for CAMEO™-4 and CAMEO™-96 assay kits. In addition to CAMEO™-4 being a miniaturized assay, the assay kit also includes the culture plates. CAMEO™-96 is a methylcellulose-based CFC/CFU assay performed in 96-well plates. This assay allows the differentiation ability of the cells to be detected by counting the number of colonies in a similar manner to that of ColonyGro™ and CAMEO™-4, but also include the most sensitive cell proliferation readout available. This readout is an ATP, luciferin/luciferase bioluminescence signal that is measured in a plate luminometer. Since this part of the assay readout is calibrated and fully standardized, it allows the "back standardization" of the CFC/CFU assay so that colony counts can be expressed as ATP concentration equivalents.

2. INTENDED USE and CELL POPULATIONS DETECTED

ColonyGro™ is a complete reagent that can be used for stem cell research, basic and veterinary research and stem cell therapy processing laboratories.

ColonyGro™ can be used to test hematopoietic stem, progenitor and precursor 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

ColonyGro™ 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
	B-CFC	IL-7

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Cell Type	Cell Population	Growth Factors/Cytokines
Precursor Cells	CFU-E	EPO
	G-CFC	G-CSF
	M-CFC	M-CSF
	Mk-CFC	TPO

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. CFU-E: Colony-Forming Unit Erythroid. G-CFC: Granulocyte Colony-Forming Cell. M-CFC: Macrophage Colony-Forming Cell. B-CFC: B-lymphocyte Colony-Frming Cell. EPO: Erythropoietin. SCF: Stem Cell Factor. TPO: Thrombopoietin. CSF: Colony Stimulating Factor. IL: Interleukin.

ColonyGro™ for stem cell processing laboratories

Five ColonyGro™ reagents and CAMEO™-4 assay kits are available for stem cell processing laboratories that are equivalent to corresponding MethoCult® reagents.

TABLE 1
CAMEO™-4 Equivalent Assay Kits for Stem Cell processing Laboratories

ColonyGro™ Catalog Number	CAMEO™-4 Catalog Number	Equivalent MethoCult® Reagent	Growth Factor/ Cytokine Cocktail
CFC-GEMM3-100H	KC-GEMM3-50H	H4435	EPO, GM-CSF, G-CSF, IL-3, IL-6, SCF, TPO*
CFC-GEM2-100H	KC-GEM2-50H	H4434	EPO, GM-CSF, IL-3, SCF
CFC-GEM3-100H	KC-GEM3-50H	H4034	EPO, GM-CSF, G-CSF, IL-3, SCF
CFC-GM1-100H	KC-GM1-50H	H4534	GM-CSF, IL-3, SCF
CFC-GEM2-100H	KC-GM2-50H	H4035	GM-CSF, G-CSF, IL-3, SCF

* Please note that thrombopoietin (TPO) is not included in H4435 formulation.

These assay kits are for human normal and mobilized peripheral blood, umbilical cord blood and bone marrow and purified cell populations (e.g. $CD34^+$ cells) from these tissues.

IMPORTANT:
ColonyGro™ is for research use only and has not been approved for clinical diagnostic use.

3. OVERVIEW of the ColonyGro™ PROCEDURE

Using a ColonyGro™ CFC/CFU assay is a 3 step process.

Step 1 – Cell Preparation
Cells are not provided with ColonyGro™. 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
ColonyGro™ is a complete, ready-to-use methylcellulose Master Mix for detection of a specific hematopoietic cell populations. The reagent is usually provided in 2 bottles containing 50ml. For each sample and total of 2.25ml of the ColonyGro™ Master Mix is dispensed into a sterile 5ml plastic tube followed by 0.25ml (10% of the total culture volume) of the cell suspension adjusted to the correct cell concentration. The contents of the tubes are mixed thoroughly on a vortex mixer and 1ml is dispensed into each of two 35mm Petri dishes. The plates are transferred to a 37°C, humidified incubator gassed with 5% CO_2 and preferably 5% O_2.

Step 3 - Colony Counting
The cells are incubated usually for the same length of time as a traditional CFC assay. However, it is recommended to view the plates after 9-10 days for human cells and 2-7 days for animal cells, since ColonyGro™ reagents usually allow the cells to grow more rapidly into colonies. After incubation, the total colony number and/or the colony number of differentiated colonies are recorded.

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8. Troubleshooting

A. Colonies grow together so that they cannot be counted
The source of the cells used, the cell density and the incubation time will define the growth of the cells and colony formation. To count and differentiate colonies with any degree of accuracy, it is important that each colony is distinct and separated from its neighbor. If colonies grow into each other so that they cannot be properly counted, the cultures have been left in the incubator too long. Either reduce the incubation time or the cell density so that the number of colonies can be counted correctly.

B. Colony growth is rapid
Unlike other methylcellulose formulations, ColonyGro™ has been designed for rapid colony growth. It will be noticed that the methylcellulose is more fluid than that of other formulations. In other words, the methylcellulose is less viscous. The more viscous the methylcellulose, the more difficult it is for the cells comprising the colony to expand. This can lead to reduced colony size or prevent colony formation all together. Monitor colony growth until and optimum incubation time is reached. However, the first 24 - 48 hours days are the most critical for colony growth. For hematopoietic stem and progenitor cells, do not remove the cultures from the incubator for at least 3 - 4 days.

A. Cultures dry out
Small volume cultures tend to dry out very quickly despite a "fully humidified" incubator. If this occurs, place the culture plates in 100mm Petri dishes containing a 35mm Petri dish without a lid with 2-3ml of sterile water. For large numbers of culture dishes, use a large container and place a beaker full of sterile water in the center of the container. This container can be glass or a plastic sandwich box. Place aluminium foil loosely over container or drill holes in the sandwich box lid to allow for gas exchange.

B. High replicate or experimental variation
This is usually caused by several factors. The first is variations due to dispensing methylcellulose. Methylcellulose is notoriously difficult to dispense accurately. Do not use syringes and needles. This method is inaccurate and can lead to extremely high variations. Use a positive displacement repeater pipette to dispense all methylcellulose reagents. Also ensure that all pipettes are properly calibrated. Small errors in dispensing can lead to large variations.

Ensure that cell concentration calculations have been performed properly and that volumes are correctly dispensed. Similarly, check all growth factor/cytokine doses if these are added separate and that the volumes are correctly dispensed.

Colony counting is a subjective procedure regardless of whether it is performed manually or automatically. Unfortunately, there are no standards and controls for the CFC/CFU assay and therefore the assay cannot be calibrated, standardized or validated.

7. RECOMMENDATIONS AND TIPS PRIOR TO USING ColonyGro™.

(i) Cell Suspension

a. 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.

b. High concentrations of red blood cells can make it extremely difficult to view and identify colonies. Colony formation producing a false negative result. It is therefore recommended to use an MNC preparation.

c. If cells have been treated (e.g. with cytotoxic drugs etc.) prior to cell culture, higher cell concentrations than those shown in Table 1 may be required.

(ii) Thawing and Storage of the ColonyGro™ Master Mix

a. Prior to using the ColonyGro™, remove the bottle from the freezer and thaw either at 37ºC or at room temperature.

b. After use, ColonyGro™ Master Mix is stable at 2-8°C for 1 month after thawing.

(iii) Dispensing ColonyGro™ Master Mix and Culture Master Mix

Methylcellulose is notoriously difficult to dispense accurately. DO NOT use a syringe and needle to dispense any methylcellulose reagent, since it will result is serious dispensing errors and high coefficients of variation (CVs). It is recommended to use positive displacement (preferably electronic) repeater, syringe pipettes to dispense both the ColonyGro™ Master Mix and Culture Master Mix (which contains cells).

(iv) Number of Replicates Performed

ColonyGro™ has been designed for samples to be tested in duplicate (2 replicates). The total volume, including cell suspension, for each sample prepared is 2.5ml. Sufficient ColonyGro™ Master Mix is provided for up to 44 samples.

(v) Humidity Chamber

Duplicate cultures should be placed in a 100mm sterile Petri dish humidity chamber containing a 35mm Petri dish without a lid and filled with 2-3 ml of sterile water. Even fully humidified incubators do not provide sufficient humidity to prevent evaporation of the culture reagents.

(vi) Incubation Times

The culture time depends on a number of different factors, including cell source, species and cell concentration. The plates can be removed at any time from the incubator to monitor colony growth. Do not allow the colonies to grow into each other. It will be difficult to count the colonies. The cells should be cultured until the colony type can be identified and the colonies have grown so that they can be discreetly counted individually.

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4. ColonyGro™ Reagents and Storage

ColonyGro™ is usually supplied as two 50ml bottles of complete, ready-to-use Master Mix containing all the components necessary to grow the required cell population(s). The reagent is shipped complete and no other components should be added.

ColonyGro™ reagents are shipped frozen. Upon arrival, transfer the reagents to a -20°C freezer until used.

Important for Users of ColonyGro™ with No Growth Factors

If using ColonyGro™ with No Growth Factors as a background control, Iscove's Modified Dulbecco's Medium (IMDM) must be added to the Master Mix to achieve the correct volume and viscosity.

ColonyGro™ with No Growth Factors can also be used to add growth factors and/or cytokines specified by the investigator. In this case, the volume of IMDM is replaced by the growth factor/cytotkine cocktail.

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

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 ColonyGro™ 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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irregular in shape and contain many dark centers. Each dark center represents a single "proliferation unit" (PU) in which the cells are actually proliferating. With time, these PUs grow together to form an irregular-shaped colony. Counting the individual PUs within a colony provides an indication of colony proliferation. However, this should not be misunderstood as a means to quantify cell proliferation, which must be measured using a different assay (e.g. HALO®-96, HemoFluor™-96 or HemoLIGHT™-96).

2. Counting all PUs within a colony as 1 colony. This is the normal manner in which colonies are counted. No consideration is made regarding whether the colony is spherical or irregular in shape. If it is separate entity from another colony, it is counted as a single colony.
3. Use a camera fitted to a microscope or an electronic colony counter to image the colonies and save the image for later enumeration either to count the colonies manually or using image analysis software. This type of colony enumeration can be problematic since the walls of the well cause a shadow around the outer rim of the well. In addition, the software may have to be "taught" how to count colonies. If this type of colony enumeration is to be performed, it is suggested to compare manual counting with electronic counting to ensure that the correct number of colonies are counted in both instances.

Important Considerations When Evaluating CFC/CFU Results

1. The number of colonies counted provides no quantitative information on cell proliferation. Cell proliferation is inferred, since without it no colony growth can occur.
2. The CFC/CFU assay detects cell differentiation since the colonies can only be identified by the ability of the cells to differentiate and mature.
3. Manual colony counting is a subjective process and semi-quantitative at best. Colonies grow in a multitude of forms and sizes. When counting a colony, it should be a distinct entity and separate from its neighbors.
4. In general, the size of the colony is indicative of the primitiveness of the cell that produced the colony; that is, the larger the colony, the more primitive the cell.
5. Colony evaluation requires considerable time to learn.
6. Due to the notorious inaccuracy of dispensing methylcellulose reagents coupled with colony evaluation, the CFC/CFU assay usually produces very high coefficients of variation (CV) which are usually unacceptable for clinical use or *in vitro* toxicology evaluations.
7. As an extension of (6) and coupled with the lack of standards and controls, the CFC/CFU assay cannot be validated according to regulatory requirements.
8. For clinical or toxicological applications that need to comply with regulatory requirements, it is recommended to use CAMEO™-96 or HALO® Assays that have been specifically developed for these applications.

10. Accurately dispense 1.0 ml of the Culture Master Mix into the middle of each of the two 35mm Petri dishes.
11. Leave the Culture Master Mix to spread over the surface of the Petri dish. If the Culture Master Mix has not covered the surface of the Petri dish completely, gently rock the Petri dish so that the Culture Master Mix covers the surface completely.
12. To prevent the Petri dishes from drying out, transfer both dishes to a sterile 100mm Petri dish containing an open 35 mm Petri dish filled with about 2-3ml of sterile water, or transfer all plates to a large container containing a beaker of water and cover the container with aluminum foil.
13. Incubate 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. Table 3 shows the suggested incubation times.

TABLE 3
Suggested Culture Incubation Times

Species	Cell Type	Cell Populations	Incubation Period (days)
Human	Bone marrow, normal and mobilized peripheral blood, umbilical cord blood	Stem cells Progenitor cells Precursor cells	9 - 12 9 - 12 5 - 7
Non-human primate	Bone marrow, peripheral blood	Stem cells Progenitor cells Precursor cells	9 - 12 9 - 12 5 - 7
Horse, Pig, Sheep, Dog	Bone marrow, peripheral blood	Stem cells Progenitor cells Precursor cells	5 - 9 5 - 9 2 - 5
Rat, Mouse	Bone marrow	Stem cells Progenitor cells Precursor cells	5 - 7 5 - 7 2-4

STEP 3 - COUNTING COLONIES

Table 3 shows the incubation times for different hematopoietic tissues derived from different species. It is important to emphasize that the number of colonies is determined by the number of colony-forming cells present in the cell suspension. Therefore, the number of colonies that can be produced is "set in stone" very early in the culture. Increasing the time period does not increase the number of colonies; it only increases the size of the colonies and the state of differentiation and maturation of the cells that identify the colonies.

There are three ways of counting colonies grown in methylcellulose.

1. Count each individual center within a colony. Colonies are rarely spherical with a single dark center indicating the center of proliferation. Most colonies are

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6. The ColonyGro™ 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

Performing a ColonyGro™ CFC/CFU assay is a 3 step process.

Step 1 – Cell preparation.
Step 2 – ColonyGro™ cell culture master mix preparation, plating and incubation in 35mm Petri dishes.
Step 3 - Colony counting to detect differentiation ability/potential.

Step 1 and Step 2 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, ColonyGro™ requires that target cells be separated from red blood cells. Red blood cells should be removed because these can interfere with colony growth as well as make it extremely difficult to count colonies. Neutrophils and platelets should also be removed. Therefore, a mononuclear cell (MNC) suspension is the cell suspension of choice. 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 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 in the ColonyGro™ assay. 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 ColonyGro™ 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 ColonyGro™ Culture Master Mix.

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STEP 2. ColonyGro™ 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 ColonyGro™ that contains no growth factor cocktails, all ColonyGro™ 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 ColonyGro™ Method

1. Transfer one or more bottles of frozen ColonyGro™ Master Mix to a 37ºC water bath or thaw at room temperature.
2. When thawed, mix the contents of bottle thoroughly on a vortex mixer.
3. Prepare the cell suspension as required and adjust to the preferred working cell concentration. Table 2 shows the working cell concentrations. Ideally, the final cell concentration should produce sufficient colonies to allow statistical significance between samples. This requires that a minimum of between 20-25 colonies should be obtained. However, this can vary depending on the cell source and species.
4. Prepare and label individual tubes for each sample to be tested.
5. Prepare and label duplicate, 35mm Petri dishes for each sample to be tested.
6. When the ColonyGro™ Master Mix has thawed and mixed, dispense 2.25 ml accurately into each tube. See Section 7 for important information on dispensing methylcellulose reagents.
   NOTE*: If using ColonyGro™ with No Growth Factors as a background control, first dispense only 2.0 ml of the ColonyGro™ Master Mix into the tube(s) followed by 0.25ml of IMDM. Vortex mix the contents prior to adding cells.*
   If adding a specific growth factor, cytokine or cocktail, the concentrations and volumes of working solution must be calculated so that a total volume of 0.25 ml can be added mixed prior to adding the cell suspension.

   VERY IMPORTANT:
   DO NOT *use a syringe and needle to dispense the methylcellulose reagents as this is extremely inaccurate and results in high coefficients of variation (%CV). HemoGenix® strongly recommends using a positive displacement repeater pipette for this purpose (See Section 7).*

7. Dispense 0.25 ml of the working cell concentration into each tube using a calibrated pipette (see Section 8). 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. HemoGenix® strongly suggests using electronic pipettes .*
8. Mix the Culture Master Mix thoroughly by vortexing and leave for a few minutes to settle..
9. Using a positive displacement repeater pipette (see Section 7), withdraw approximately 2.25-2.3 ml the Culture Master Mix, taking care not to withdraw bubbles.

TABLE 2
Recommended Cell Concentrations (See Step 2)

Species	Cell Type	Cell Preparation	Cell State	Working Cell Concentration Required (10 x Final Cells/Well)	Final Cell Dose / Well
Human	Bone marrow	MNC	Fresh/ Frozen	0.1-2 x 10^6	0.1-2 x 10^5
	Peripheral blood	MNC	Fresh/ Frozen	0.5-2 x 10^6	0.5-2 x 10^5
	Mobilized peripheral blood	MNC	Fresh/ Frozen	0.1-2 x 10^6	0.1-2 x 10^5
	Umbilical cord blood	MNC	Fresh/ Frozen	0.1-2 x 10^6	0.1-2 x 10^5
	Bone marrow	$CD34^+$	Fresh	0.1-1 x 10^5	0.1-1 x 10^4
	Mobilized peripheral blood*	$CD34^+$	Fresh/ Frozen	0.1-1 x 10^5	0.1-1 x 10^4
	Umbilical cord blood	$CD34^+$	Fresh/ Frozen	0.1-1 x 10^5	0.1-1 x 10^4
Non-human primate	Bone marrow	MNC	Fresh/ frozen	0.1-2 x 10^6	0.1-2 x 10^5
	Peripheral blood	MNC	Fresh/ Frozen	1-5 x 10^6	1-5 x 10^5
Horse, Pig, Sheep	Bone marrow	MNC	Fresh/ Frozen	0.1-2 x 10^6	0.1-2 x 10^5
	Peripheral blood	MNC	Fresh/ frozen	1-5 x 10^6	1-5 x 10^5
Dog	Bone marrow	MNC	Fresh/ Frozen	0.5-2 x 10^6	0.5-2 x 10^5
Rat	Bone Marrow	MNC	Fresh	0.1-2 x 10^6	0.1-2 x 10^5
	Peripheral blood	MNC	Fresh	1-5 x 10^6	1-5 x 10^5
Mouse	Bone Marrow	MNC	Fresh	0.1-2 x 10^6	0.1-2 x 10^5
	Spleen	MNC	Fresh	1-10 x 10^6	1-10 x 10^5
	Fetal liver	MNC	Fresh	0.1-2 x 10^6	0.1-2 x 10^5

* Lot dependent

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E. Thawing of Cells and Procedures to Reduce Red Blood Cell Content and Produce a Mononuclear Cell Suspension

(i) 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.

(ii) Reduction or Removal of Red Blood Cells

The red blood cell content must be reduced to 10% or less for assay cell suspensions used in CAMEO™-96. This is because red blood cells contain high levels of ATP that will cause false positive results. If the sample to be assayed is fresh, the red blood cells can be subjected to a cold lysis procedure. Lysis should not be used to remove red blood cells from a cryopreserved sample. Density gradient centrifugation is recommended for cryopreserved samples.

Red Blood Cell Cold Lysis

To perform a cold lysis, ACK Lysis Buffer (Cat. No. K-Lysis-100, HemoGenix®) is recommended. The procedure is performed as follows:

1. Refrigerate the ACK Lysis Buffer.
2. Prepare an ice water bath in a 200ml beaker.
3. Transfer the sample to a centrifuge tube.
4. Add 10 parts of the cold ACK Lysis Buffer to 1 part cell suspension. For 1ml of cell suspension, add 10ml ACK Lysis Buffer.
5. Place the tube in the ice water bath and set a lab timer for 5 min.

6. At 2.5 min, invert the tube once and replace in the ice water bath.
7. At 5 min, remove the tube. There should be a cherry red clear solution. If lysis has not taken place, a cloudy cell suspension will still be observed. Replace the tube in the ice water bath for another 2.5 min. Continue this for a maximum of 10 min.
8. Centrifuge the tube at 400 x g for 5 min and discard the supernatant after centrifugation.
9. Resuspend the cells in IMDM to the original cell volume.
10. Perform a cell and differential count and viability assay.
11. The white blood cell recovery should be at least 95% and the viability must be greater than 85%.

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 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.

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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 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.