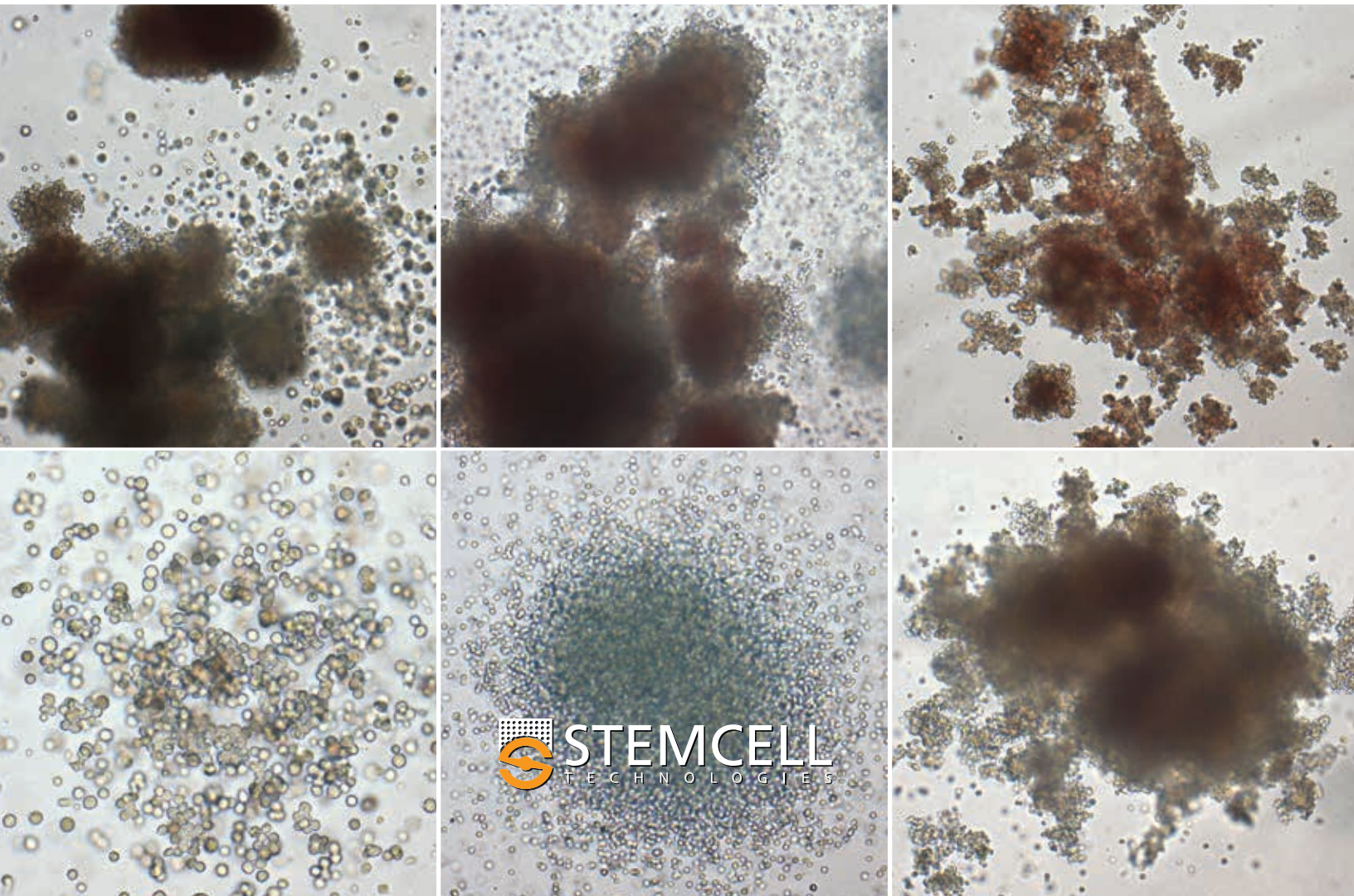
ATLAS OF HEMATOPOIETIC COLONIES FROM CORD BLOOD



STEMCELL TECHNOLOGIES

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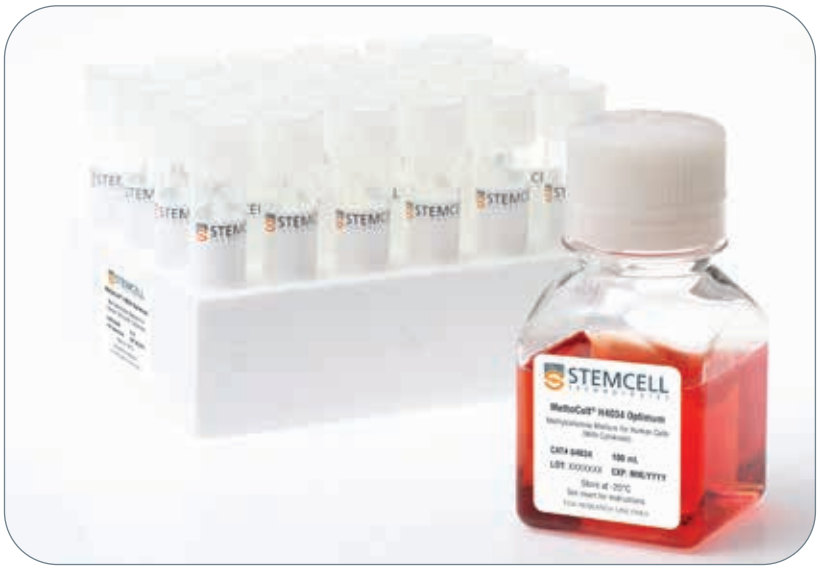
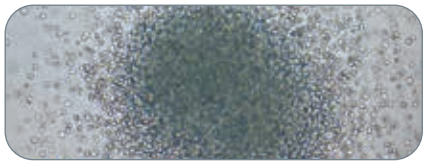
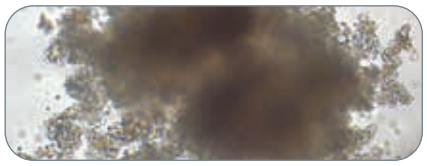
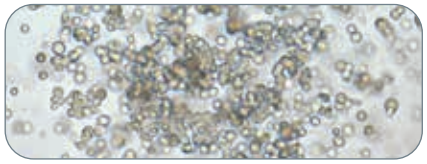








This atlas is a tool for the identification of colonies produced by hematopoietic progenitors in colony-forming cell (CFC) assays, specifically with human cord blood using semi-solid methylcellulose-based MethoCult® media from STEMCELL Technologies. The atlas is intended to be used as a tool for clinical and research staff performing CFC assays on cord blood samples. It can also be a useful reference for identification of hematopoietic CFCs in different human tissues, for example, bone marrow and mobilized blood, or purified hematopoietic stem and progenitor cell preparations. Shown are photographs of hematopoietic colonies from cord blood samples that have been cultured after removal of red blood cells. Also shown are colonies from cord blood samples that have been unprocessed or minimally processed before plating in MethoCult® media and that still contain red blood cells.

CHAPTER 1 - INTRODUCTION

1.1 HEMATOPOIESIS

Mature blood cells have a limited lifespan and are continuously replaced by the proliferation and differentiation of a very small population of pluripotent hematopoietic stem cells (HSCs). HSCs are found primarily in the bone marrow (BM) of healthy adults, in umbilical cord blood (CB) and in adult peripheral blood (PB) after mobilization from the bone marrow with cytokines such as G-CSF or other agents. These HSCs have the ability to replenish themselves by self-renewal, as well as to differentiate into all the mature hematopoietic cells found in the blood (Figure 1). A thorough review of the concepts and assays leading to this hierarchical model of hematopoiesis is available.[1]

1.2 ASSAYS FOR HEMATOPOIETIC CELLS

***In vivo* assays** to quantitate human HSCs are done by transplanting small numbers of HSCs into immuno-compromised NOD/SCID mice and determining the presence or absence of human myeloid and lymphoid cells in the circulation several weeks later. These limiting dilution *in vivo* assays are referred to as Competitive Repopulating Unit (CRU) assays or NOD/SCID Repopulating Cell (SRC) assays.[2-4]

***In vitro* assays** are used to gain insight into the frequencies and growth properties of hematopoietic progenitor cells at various stages of development. A subset of HSCs and closely related primitive progenitors are detected using *in vitro* culture systems such as the long-term culture-initiating cell (LTC-IC) assay.[5-7]

Other *in vitro* assays have been developed to detect hematopoietic progenitors, with more limited self-renewal capacity and reduced proliferative potential, by their ability to produce colonies of daughter cells in semi-solid media. The colony-forming cell (CFC) assay, also called the colony-forming unit (CFU) assay, initially detected only granulocyte-macrophage progenitors (CFU-GM).[8,9] This assay was subsequently adapted to detect erythroid progenitors (CFU-E and BFU-E),[10-12] megakaryocyte progenitors (CFU-Mk)[13] and progenitors of all three myeloid lineages (CFU-GEMM).[14] The CFC assay is the assay of choice to measure the number and viability of hematopoietic progenitors in blood and marrow, as well as to examine the effects of *ex vivo* manipulation such as red blood cell removal, T cell depletion, $CD34^+$ cell selection, volume reduction and cryopreservation.

Phenotypic assays depend on hematopoietic cells expressing certain cell surface phenotypic markers such as CD34 or CD133. Although the $CD34^+$ or $CD133^+$ content of a cell suspension indicates the presence of immature cells, it does not indicate whether these cells are stem cells or more mature progenitor cells. Unfortunately, phenotypic assays do not measure an individual cell's ability to display the critical functions of proliferation and differentiation. For example, only approximately 10 - 20% of purified $CD34^+$ cells are colony-forming cells.[15,16]

Importantly, clinical transplantation studies have demonstrated that the CFC content of a hematopoietic cell product used for a bone marrow transplant procedure correlates better with the time to platelet and neutrophil engraftment, and overall survival of the patient, than with other parameters such as the number of total cells, viable nucleated cells or $CD34^+$ cells in the graft.[17-21]

1.3 THE CFC ASSAY

The CFC assay has many applications in clinical and research laboratories and can be used to:

- Support the evaluation of donor samples (including CB) for stem cell transplants[17-21]
- Support patient diagnosis, prognosis and treatment[22-27]
- Quality control *ex vivo* manipulations, such as cryo-preservation, cell processing, T cell depletion and $CD34^+$ cell isolation[28-36]
- Study effects of cytokines, growth factors, hormones or mimetics on hematopoietic progenitors[37-41]
- Perform toxicity testing or drug screening assays[42-45]

CFC assays involve plating a single cell suspension at low cell density in media made semi-solid or viscous with either agar or methylcellulose. The media must also contain an appropriate combination of cytokines and other supplements that support the proliferation and differentiation of the hematopoietic progenitors for at least 14 days. Importantly, the number of cells initially plated must be kept low to ensure each colony can be seen to originate from a single progenitor. The semi-solid or viscous property of the media minimizes movement of cells in the culture. This ensures that the daughter cells derived from a single progenitor stay in close proximity to each other. Due to ease of handling, methylcellulose is most commonly used to provide the needed viscosity. It is chemically inert and its properties do not change with the pH. Most importantly, cells are not exposed to high temperatures, which can occur using agar-based media.

The **cellular composition** and the **size of a colony** are used to classify the cell of origin. Individual colonies containing all lineages (erythrocytes, granulocytes, monocytes/macrophages and megakaryocytes) are called CFU-GEMM, and considered to have arisen from a more primitive progenitor than those containing cells of a single lineage. Thus, colonies containing only one lineage are considered to have arisen from a more mature progenitor and are classified lower in the hematopoietic hierarchy as depicted in Figure 1 (CFU-GM, CFU-G, CFU-M, BFU-E, CFU-E, CFU-Mk). Similarly, larger colonies are considered to have originated from progenitors higher in the hematopoietic hierarchy than progenitors giving rise to smaller colonies. As discussed in this atlas, identifying the cellular composition and size of colonies requires some training and experience. Fortunately, in colonies that contain maturing erythroid cells, their erythroid nature can usually be identified by the red hemoglobin they contain. Optimal assessment of megakaryocyte progenitors (CFU-Mk) is best done in collagen-based cultures using MegaCult®-C medium where fixation and immunocytochemical staining can be carried out.

It should be noted that progenitors for human T and B lymphocytes and natural killer (NK) cells are usually not detectable in standard CFC assays, as lymphoid progenitors require special culture conditions and their proliferation and differentiation is not optimally supported by most methylcellulose-based media designed for CFC assays to detect human hematopoietic progenitor cells.

FIGURE 1. **The Hematopoietic Hierarchy.** This figure depicts the hierarchical nature of the hematopoietic system. Hematopoietic stem cells reside at the top of the pyramid and have the ability to differentiate into all mature hematopoietic cells (depicted at the bottom of the pyramid). Between these two cell types, there are several transient amplifying cells with increasing lineage-restriction and decreased proliferative capacity. The width of the pyramid reflects the relative number of cells present in the body during homeostasis.

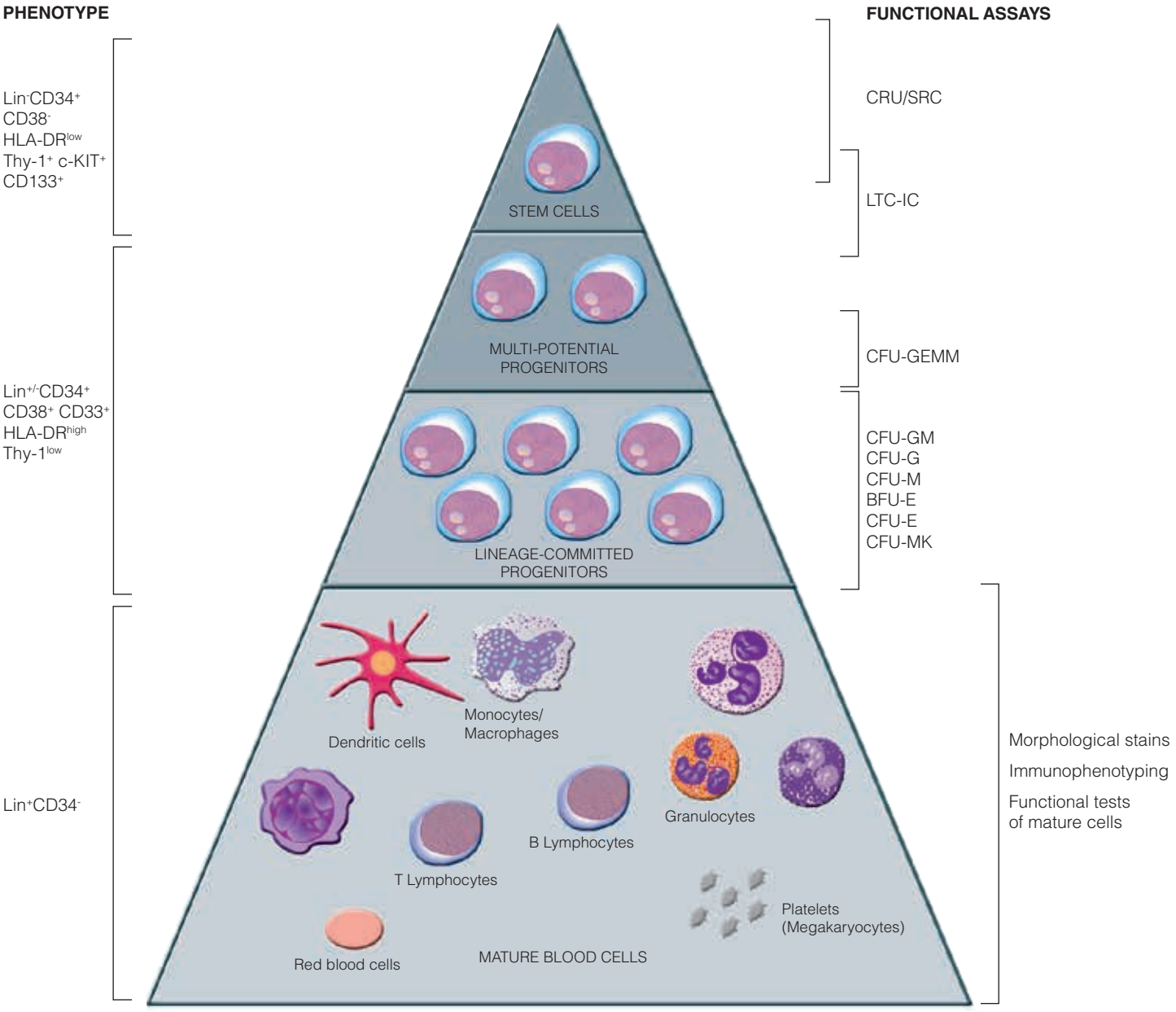


TABLE 1. **Applications for MethoCult® Products.** Note that all products come either in 100 mL or in 24 x 3 mL pre-aliquoted formats. For information about which cytokines are in each formulation, please refer to the appendix on page 41.

APPLICATION	PRODUCT NAME	CATALOG #	SIZE
Detection of BFU-E, CFU-GM, CFU-G, CFU-M, CFU-GEMM in routine colony assays*†	MethoCult® H4034 Optimum**	04034	100 mL
		04044	24 x 3 mL
	MethoCult® GF H84434**‡	84434	100 mL
		84444	24 x 3 mL
	MethoCult® H4434 Classic	04434	100 mL
		04444	24 x 3 mL
Detection of BFU-E, CFU-GM, CFU-G, CFU-M, CFU-GEMM in highly purified populations*†	MethoCult® H4435 Enriched	04435	100 mL
		04445	24 x 3 mL
	MethoCult® GF H84435‡	84435	100 mL
		84445	24 x 3 mL
Detection of CFU-GM, CFU-G, CFU-M in routine colony assays*†	MethoCult® H4035 Optimum without EPO**	04035	100 mL
		04045	24 x 3 mL
	MethoCult® GF H84534**‡	84534	100 mL
		84544	24 x 3 mL
	MethoCult® H4534 Classic without EPO	04534	100 mL
		04544	24 x 3 mL
Detection of CFU-GM, CFU-G, CFU-M in highly purified populations*†	MethoCult® H4535 Enriched without EPO	04535	100 mL
		04545	24 x 3 mL
	MethoCult® GF H84535‡	84535	100 mL
		84545	24 x 3 mL

* Also detects CFU-E but these progenitors are not typically found in cord blood samples

** This formulation differs from the MethoCult® H4434 Classic formulation by inclusion of G-CSF. This can assist in reducing subjectivity in identifying CFU-GM-derived colonies.

† Also suitable for BM, PB, MPB and purified samples

‡ CE marked for *in vitro* diagnostic use; available in the European Union

1.4 CLASSES OF HUMAN HEMATOPOIETIC PROGENITORS DETECTED USING METHOCULT® MEDIA

CFU-E: Colony-forming unit-erythroid. Produces one to two cell clusters containing a total of 8 - 200 erythroblasts. CFU-E are mature erythroid progenitors that require erythropoietin (EPO) for differentiation. They are present in BM and PB, but are usually not detected in CB samples.

BFU-E: Burst-forming unit-erythroid. Produces a colony containing >200 erythroblasts in a single cluster or multiple clusters and can be sub-classified based on the number of cells or cell clusters per colony, if desired. BFU-E are more immature progenitors than CFU-E and require EPO and other cytokines, such as Interleukin-3 (IL-3) and Stem Cell Factor (SCF), for optimal colony growth.

CFU-GM: Colony-forming unit-granulocyte, macrophage. Produces a colony containing at least 20 granulocytes (CFU-G), macrophages (CFU-M) or cells of both lineages (CFU-GM). Colonies arising from primitive CFU-GM may contain thousands of cells in single or multiple clusters.

CFU-GEMM: Colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte. A multi-potential progenitor that produces a colony containing erythroblasts and cells of at least two other recognizable lineages. Due to their primitive nature, CFU-GEMM tend to produce large colonies containing >500 cells.

CFU-Mk: Colony-forming unit-megakaryocyte. Produces a colony containing three or more megakaryocytic cells.

Although megakaryocytic progenitors can be cultured in methylcellulose-based medium containing the appropriate growth factors, it is difficult to distinguish CFU-Mk based on cellular and colony morphology. Therefore, we recommend that CFU-Mk are enumerated by culturing in collagen-based, serum-free, MegaCult®-C media, followed by immunocytochemical staining of the dehydrated gels. For more details please contact us or visit our website at www.stemcell.com.

1.5 METHOCULT® MEDIA

Methylcellulose-based CFC assay medium commonly contains components including fetal bovine serum (FBS), bovine serum albumin (BSA), a reducing agent such as 2-mercaptoethanol, basal medium such as Iscove's MDM, supplements such as insulin and transferrin, and cytokines. Insulin and transferrin are usually present in serum-free formulations (i.e. formulations that do not contain FBS). FBS and BSA batches show lot-to-lot variability in their ability to support the growth and differentiation of hematopoietic CFCs. Therefore batches of FBS, BSA and other components used in MethoCult® are rigorously pre-screened to select optimal batches and ensure batch-to-batch reproducibility.

Detection of the different classes of human hematopoietic progenitors and their optimal proliferation and differentiation is largely dependent on the combination of recombinant cytokines present in the methylcellulose-based medium. Many cytokines that exert biological effects on hematopoietic progenitors *in vitro*, including supporting cell survival, promoting proliferation and inducing differentiation, have been identified and characterized. Although individual cytokines demonstrate one or more of these properties, most show additive or synergistic effects when used in combination with other cytokines.

The proliferation and differentiation of hematopoietic progenitors in CFC assays is also affected by other components in the medium, including, metals, steroid hormones and other factors present in FBS, BSA, insulin, transferrin and basal medium components. Differences in biological properties of hematopoietic cells isolated at different stages of development (i.e. fetal and adult) and from different tissue sources (i.e. bone marrow, peripheral blood and cord blood) have also been documented. For these reasons, various MethoCult® formulations have been developed for performing hematopoietic CFC assays on different cell preparations. An overview of the various MethoCult® media available for colony assays on human cells is presented in Table 1.

MethoCult® H4034 Optimum (MethoCult® GF H4034; Catalog #04034/04044) is the recommended medium for standard colony assays on human cord blood samples and the examples of colonies presented in this atlas were obtained using this medium. This medium supports the proliferation and differentiation of progenitors in unfractionated as well as processed CB samples. It is also recommended for other cell samples such as BM, PB and mobilized PB (MPB).

MethoCult® H4434 Classic (MethoCult® GF H4434; Catalog #04434/04444) is STEMCELL's original formulation and used in standard colony assays on CB, BM, PB and MPB.

MethoCult® H4435 Enriched (MethoCult® GF+ H4435; Catalog #04435/04445) is recommended for colony assays with purified progenitors, for example, CD34+ cells or lineage-depleted cells prepared with RosetteSep®, EasySep® or StemSep® cell separation procedures, or prepared by cell sorting.

MethoCult® H4034 Optimum, MethoCult® H4434 Classic and MethoCult® H4435 Enriched are complete formulations; no additional components are required prior to inoculating cells for the CFC assay. Depending on the cells used, colony numbers and size may vary between different media, but colony morphology is usually very similar and the examples shown in this atlas are relevant for results obtained with MethoCult® H4034 Optimum, MethoCult® H4435 Enriched and MethoCult® H4434 Classic.

MethoCult® media are available without EPO and are recommended for applications in which only granulocyte/macrophage colonies need to be identified, as erythroid colonies will not develop in the absence of EPO. Formulations without EPO include MethoCult® H4035 Optimum without EPO (MethoCult® GF H4035; Catalog #04035/04045), MethoCult® H4535 Enriched without EPO (MethoCult® GF+ H4535; Catalog #04535/04545) and MethoCult® H4434 Classic without EPO (MethoCult® GF H4534; Catalog #04534/04544).

1.6 METHOCULT® EXPRESS

The MethoCult® media listed in Table 1 have been optimized for detection of the different types of progenitors present in CB, specifically BFU-E, CFU-GM and CFU-GEMM, which require a culture period of 14 days to develop into morphologically distinguishable colonies.

For some time-sensitive procedures, particularly in cord blood banks and transplant centers, it may be important to obtain accurate information about the total progenitor content of a CB sample, irrespective of progenitor lineage, after a much shorter period than the 14 days required for standard assays.

STEMCELL Technologies provides a methylcellulose-based medium, **MethoCult® Express** (Catalog #04437/04447), which has been designed for accurate enumeration of total progenitors in CB after a 7 day culture period instead of the 14 days of standard CFC assays.

MethoCult® Express has been formulated for accelerated progenitor proliferation and produces colonies that are still morphologically immature and not yet identifiable as BFU-E, CFU-GM or CFU-GEMM, but that can be counted as early as after 7 days of culture (Figure 2). Colonies counted after 7 days in MethoCult® Express show excellent correlation with total colony numbers in standard 14-day assays in MethoCult® H4034 Optimum (Figure 3). For more information about MethoCult® Express and examples of the morphology of colonies in 7-day CFC assays of CB please refer to Chapter 5, as well as to our website at www.stemcell.com.

1.7 CORD BLOOD

Umbilical cord blood has become an important source of hematopoietic stem cells for clinical transplantation. However, the numbers of stem and progenitor cells in individual CB units is usually limited and can vary significantly between individual CBs, while the *ex vivo* manipulations to which most CB units are subjected, specifically red blood cell removal, volume reduction, cryopreservation and thawing, have variable effects on cell yield and viability. For these reasons, assessing the quality of CB units is important and several studies have demonstrated a significant correlation between the numbers of CFCs post-thaw and the success of transplantation, as measured by the time to neutrophil and platelet engraftment, and overall survival after transplantation. [22-24]

FIGURE 2. **Colonies derived from cord blood progenitors in MethoCult® Express** at (A) day 7 and (B) day 14 with a 2X objective lens; at (C) day 7 and (D) day 14 with a 4X objective lens.

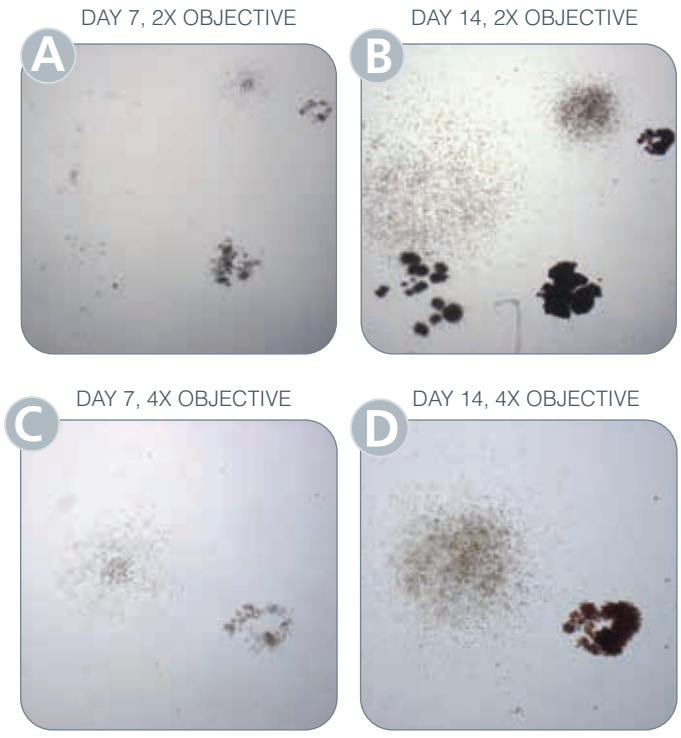
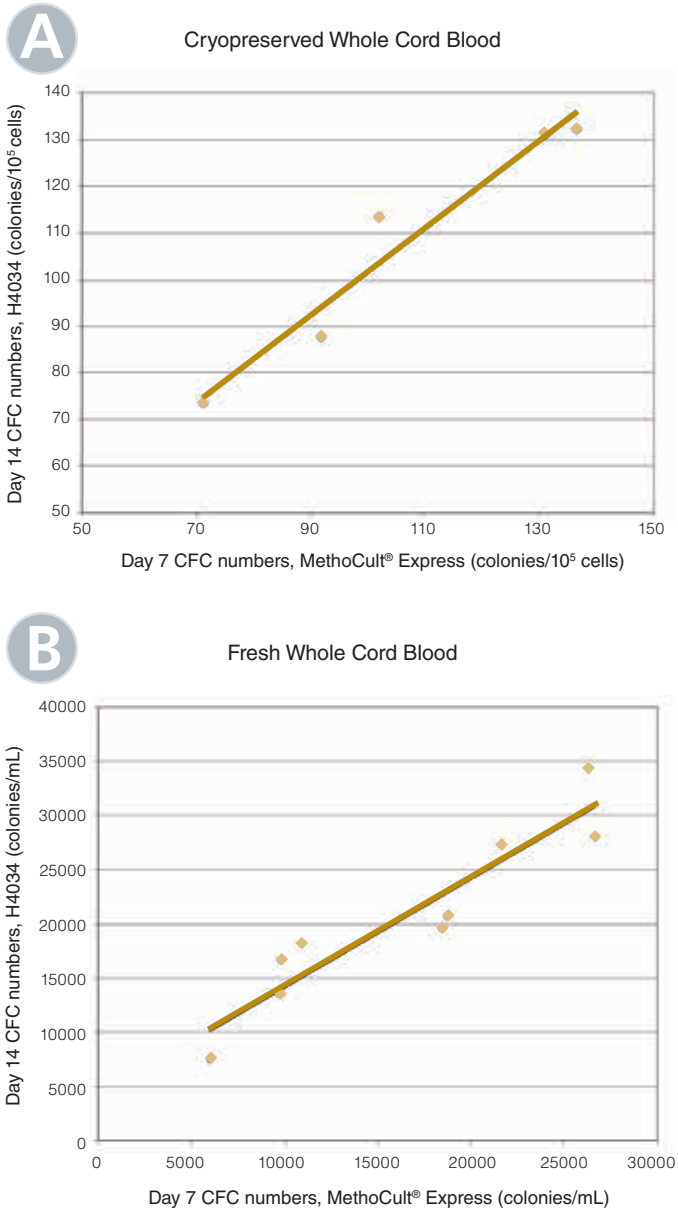


FIGURE 3. **Correlation between 7-day colony assays in MethoCult® Express and standard 14-day colony assays in MethoCult® H4034 Optimum.** (A) Thawed cells from previously cryopreserved whole cord blood samples were plated into MethoCult® Express (x axis) or MethoCult® H4034 Optimum (y axis) and counted after 7 and 14 days, respectively. Samples were highly correlated with a Pearson product moment correlation coefficient of 0.973 ($p<0.05$, $n=5$). (B) Cells from fresh whole cord blood samples were either plated without any red blood cell depletion into MethoCult® H4034 Optimum (y-axis) or subjected to red blood cell depletion by ammonium chloride lysis before plating in MethoCult® Express (x-axis). Cultures were counted at day 14 and day 7, respectively. Samples were well correlated with a Pearson product moment correlation coefficient of 0.941 ($p<0.05$, $n=9$).



1.8 CORD BLOOD CELL PROCESSING METHODS

CFC assays can be performed on unprocessed CB as well as on CB samples that have undergone cell processing and/or cryopreservation. Unfractionated fresh CB and minimally processed CB samples, for example, units that have had the plasma removed or that have been processed using automated systems (e.g. Sepax™), usually contain large numbers of red blood cells (RBCs). The presence of red blood cells does not usually inhibit colony growth, but it can cause a background in CFC assays that can make colony detection and counting more difficult. For this reason, the atlas shows examples of CFC assays on CB samples from which red blood cells have been removed, as well as of assays on samples that contain large numbers of RBCs. Samples processed using the following methods will contain few RBCs and are not likely to produce a RBC background in the dish:

- Density-based separation methods, for example, using Ficoll-Paque™ PLUS*
- Red blood cell lysis, for example, using ammonium chloride lysis
- Cryopreservation and thawing, in particular when using cryopreservation media containing dimethyl sulfoxide (DMSO)
- Progenitor enrichment methods, e.g. $CD34^+$ cell isolation or $lineage^+$ cell depletion methods

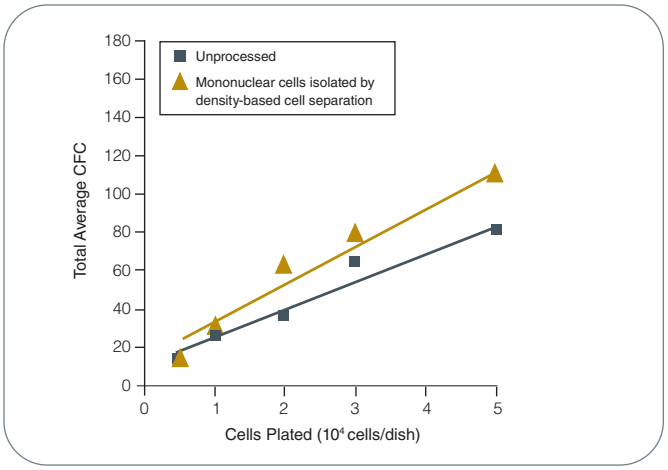
*Ficoll-Paque™ PLUS is a trademark of GE Healthcare Ltd.

1.9 ASSAY SET-UP

In order to obtain optimal colony numbers it is important to select an appropriate cell plating concentration in the MethoCult® medium. Under optimal plating concentrations and culture conditions there is a linear relationship between the number of cells plated and the number of colonies counted (Figure 4).

Too few colonies (underplating) or too many colonies per dish (overplating) can present problems. When the culture is underplated colonies are easy to count, but the variation between replicates may be relatively large and the information obtained on progenitor frequencies in the sample can be inaccurate. Overplating will also result in inaccurate results and may underestimate the progenitor frequency in a sample, as adjacent colonies may be difficult to distinguish and colony growth can be inhibited due to depletion of medium components and accumulation of toxic waste products.

FIGURE 4. **The importance of cell plating concentration.** Under optimal conditions, the number of cells plated and the number of resulting colonies should have a linear relationship. In order to achieve this, optimal plating numbers need to be determined. Recommendations are given in Table 2.



Fifty to sixty colonies per standard 35 mm dish are considered to be optimal for CFC assays on CB. The optimal cell plating density depends on the progenitor frequency in the cell sample. Recommended plating concentrations for unprocessed CB and CB preparations processed using various methods are presented in Table 2. Because of the potential large variability in the progenitor content between different CB units and in progenitor recovery after cell processing and cryopreservation, testing each sample at two or more cell plating concentrations to help ensure that at least one of the plating concentrations produces a number of colonies in the optimal range is recommended.

Detailed instructions are included in the Technical Manual for Human Colony-Forming Cell Assays Using MethoCult® (Manual Catalog #28404) and MethoCult® Product Information Sheets, available upon request and on our website at www.stemcell.com. An overview of supplies needed for the CFC assay is provided in Table 3.

TABLE 2. **Recommended plating concentrations for cord blood cells.**

CELL SOURCE	CELLS PLATED PER 35 mm DISH
Unprocessed or minimally processed CB	2×10^4 (1×10^4 - 4×10^4)
CB, ammonium chloride treated	2×10^4 (1×10^4 - 4×10^4)
CB mononuclear cells**	1×10^4 (5×10^3 - 2×10^4)
Lineage-depleted CB	1000 (500 - 2×10^3)
$CD34^+$ CB cells	500 (250 - 2×10^3)

** Mononuclear cells (MNCs) are isolated by density-based cell separation (e.g. sedimentation over Ficoll-Paque™ PLUS). Ficoll-Paque™ PLUS is a trademark of GE Healthcare Ltd.

TABLE 3. **Other equipment required to perform colony assays.** All the consumable supplies are available from STEMCELL Technologies.

PRODUCT DESCRIPTION	CATALOG #	QUANTITY	APPLICATIONS
Iscove's Modified Dulbecco's Medium (IMDM) with 2% Fetal Bovine Serum	07700	100 mL	Washing hematopoietic cells
Blunt-end needles	28110	100	Aliquoting MethoCult® and plating cultures. Recommended for prevention of needle-stick injuries.
	28120	2000	
3 cc syringe	28230	30	For aliquoting MethoCult® and plating cultures. Syringes and blunt-end needles are recommended when dispensing MethoCult®. The semi-solid medium will stick to the inside of a standard pipette, resulting in a less accurate volume.
	28240	100	
35 mm dishes for culture in MethoCult®	27100	10	Optimal colony growth without supporting adherent cells.
	27150	50	
60 mm gridded scoring dishes	100-0082	20	A 35 mm culture dish fits inside the 60 mm gridded scoring dish, which is a standard size for most microscope stages. Allows for reproducible and accurate scoring of colonies by ensuring areas of the dish are not counted more than once or not missed.
High-quality inverted microscope with 2X, 4X and 10X planar objectives, stage holder for a 60 mm gridded dish and a blue filter	N/A	N/A	Use a 2X objective to scan the whole dish prior to counting. With a 2X objective, large colonies typical of cord blood samples can be viewed as a whole. This allows the individual scoring to gain a perspective of the dish, including general spacing between colonies. The colonies can be counted using a 2X objective if they are very large. A 4X objective can be used to count colonies if they are not too large. If the colonies generally take up the whole field of view when a 4X objective is used, a 2X objective for counting is recommended. A 10X objective allows colonies to be viewed more closely to confirm cellular morphology and lineage. A stage holder allows the gridded dish to be held in place for accurate and reproducible colony counts. A blue filter enhances the red color of hemoglobinized erythroblasts, facilitating easier determination of lineage.
Differential counter	N/A	N/A	Multiple buttons allow colonies of different lineage to be counted at the same time. Both electronic and manual differential counters are available.

1.10 IDENTIFYING AND ENUMERATING COLONIES

Colonies are enumerated after 14 - 16 days of culture at 37°C, 5% CO_2 in a humidified incubator. Please refer to the Technical Manual for Human Colony-Forming Cell Assays Using MethoCult® (Manual Catalog #28404) for detailed instructions on culture conditions.

Before counting the colonies, scanning the whole dish at low magnification (2X objective) is recommended. This is to review the colony density (e.g. too few or too many colonies, general distance between colonies) and the size and morphology of the colonies, which will vary with each sample. A 60 mm gridded scoring dish is recommended as a guide. Colonies are then counted, using a 2X or 4X objective depending on the size of the colonies, by gradually moving the dish through the field of view.

Each colony is identified based on the morphology of cells within the colony. Erythroid colonies can also usually be identified by their high contrast and red color. A 10X objective can be used to confirm the cellular morphology within the colony but counting colonies at this magnification is not recommended.

The following chapters show photographs of colonies obtained in CFC assays on human CB with detailed descriptions of hematopoietic progenitors detected.

1.11 STANDARDIZATION OF CFC ASSAYS

High quality methylcellulose medium provides a solid foundation for reproducibility and standardization of the CFC assay. MethoCult® methylcellulose-based medium from STEMCELL Technologies offers the following advantages in order to promote standardization:

- Each lot is performance tested for low lot-to-lot variability
- Components are extensively prescreened for optimal growth and differentiation of hematopoietic progenitors
- It utilizes STEMCELL's extensive expertise in manufacturing methylcellulose-based media
- It is available in different formats, including 100 mL bottles and racks of 3 mL tubes, for flexibility
- Custom formulations and sizes are available upon request

Other aspects of the CFC assay can potentially contribute to variability in results. Sources of variability include equipment and culture conditions, cell processing, and colony recognition and enumeration.

STEMCELL Technologies offers a variety of tools to assist with standardization of the CFC assay with respect to cell processing, and colony recognition and enumeration. These tools include training courses, proficiency testing programs and quality control kits.

The Hematopoietic Progenitor Assay Training Course (Catalog #00215) is designed to increase knowledge in performing hematopoietic colony assays. The course is held over two days and includes lectures and practical laboratory sessions. The following topics are covered in the practical laboratory sessions: cell processing, assay set-up, identification of hematopoietic progenitor-derived colonies and comparative counting exercises. Each course is tailored to the interests of the participants.

STEMCELL Technologies' **Contract Assay Services** offers customized educational courses and on-site training. These courses are designed to suit your needs and are taught by scientists with expertise in hematopoietic progenitor assays, as well as neural, mesenchymal and mammary stem cell assays and culture. Contact contractassay@STEMCELL.com for more information.

Proficiency Testing Programs promote standardization in cell thawing, counting, dilution and plating; colony enumeration; and colony identification. Participants receive detailed personalized reports for each of these parameters, allowing for anonymous comparison with overall program results.

Benefits of Proficiency Testing Programs include:

- Comparison of progenitor counts with participants from centers around the world
- Reduction in variability of cell processing and colony scoring techniques
- Identification of specific areas that may benefit from continued education and training

Proficiency Testing Programs are offered for fresh and frozen cord blood, as well as bone marrow. Contact us at proficiency@STEMCELL.com for program dates and catalog numbers.

Quality Control Kits provide a convenient mechanism for monitoring reproducibility in setting up and counting hematopoietic colony assays over a one-year period. Variations in the numbers of colonies counted when cells from the same sample are plated each month can reveal inconsistencies in assay set-up and colony identification, as well as equipment malfunction.

The Quality Control Kits contain all the supplies necessary for 12 monthly tests, including cells, media and software to facilitate cell dilution calculations and results. They are available with Human Cord Blood (Catalog #00651) and Bone Marrow (Catalog #00650) cells.

CHAPTER 2 - COLONY DESCRIPTIONS

2.1 COLONY DESCRIPTIONS

This chapter provides a general description of the different colonies and tips for determining the number of colonies when multiple clusters of cells are close together.

CFU-E (colony-forming unit-erythroid) are not typically found in cord blood samples. The CFU-E is a more mature erythroid progenitor than the BFU-E, and would typically give rise to a colony containing 8 - 200 erythroblasts in one to two clusters.

Note that the fields of view are presented based on the objective lens. When viewing colonies directly using an inverted microscope with a 10X or 12.5X occular lens, total magnification is 20 - 25X with a 2X objective, 40 - 50X with a 4X objective and 100 - 125X with a 10X objective lens. Total magnification is not presented in this atlas as it may be slightly altered when a photograph is taken with a digital camera, presented on a computer monitor and then printed.

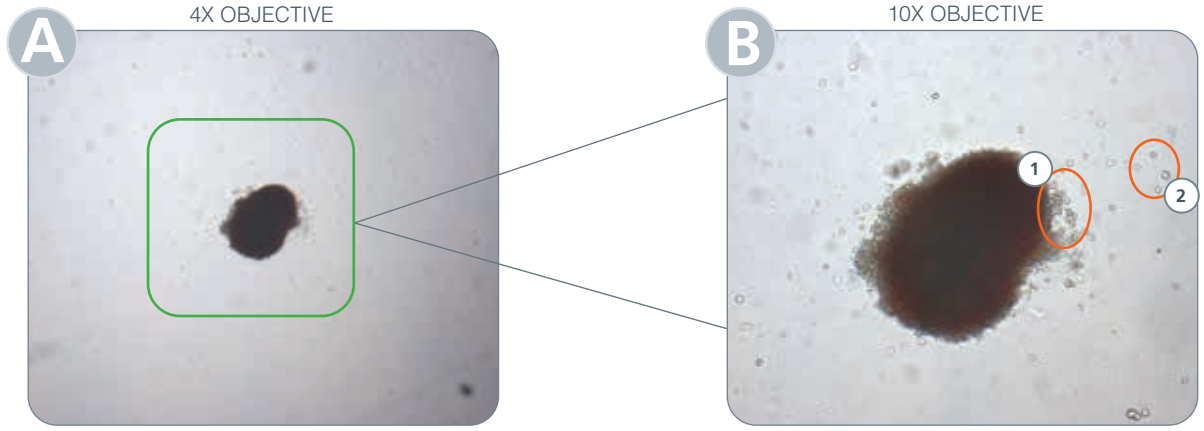
2.1.1 BFU-E

A BFU-E (burst-forming unit-erythroid) is a progenitor cell which produces a colony containing more than 200 erythroblasts, usually in more than one cluster. Each colony arises from a single erythroid progenitor.

KEY FEATURES:

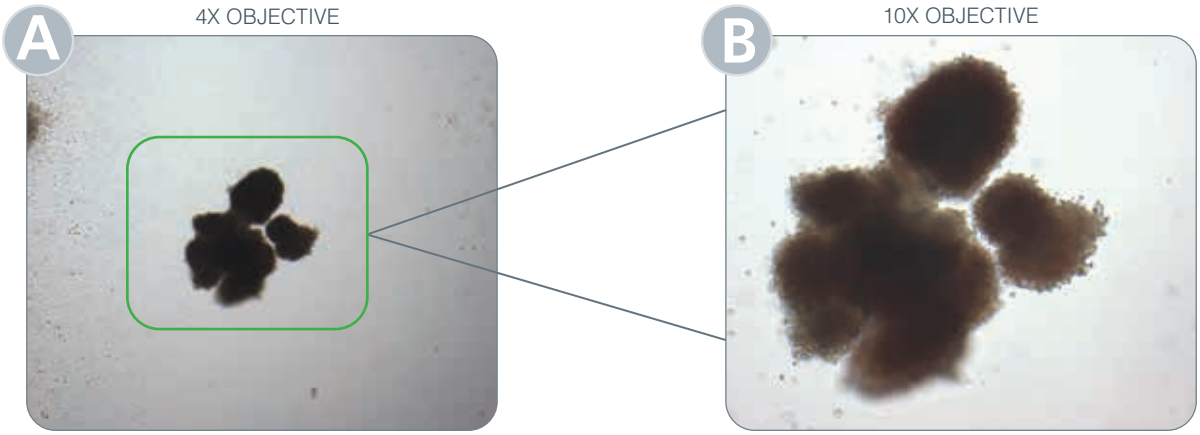
- Colonies appear red or brown as the cells are hemoglobinized.
- It is difficult to distinguish individual cells in the center or at the edge of the colony.
- Detection of erythroid progenitors requires erythropoietin (EPO) in the medium.
- Colonies can appear as one distinct compact cluster or with multiple clusters (several bursts).

FIGURE 5. BFU-E



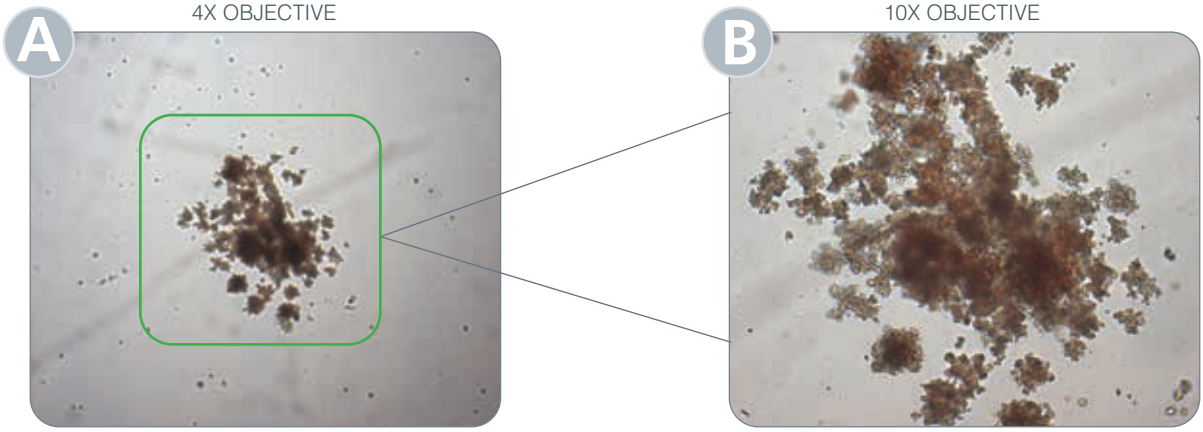
This is an example of a compact colony derived from a BFU-E. Note the red/brown color and the fact that the majority of individual cells cannot be easily distinguished. You may notice small groups of cells (erythroblasts) at the edge of the colony (Circle 1). The individual cells cannot be made out and resemble a bag of marbles or a bunch of cauliflower. This is in contrast to the cells in Circle 2 that are clear and morphologically different than those in Circle 1.

FIGURE 6. BFU-E



This is another example of a compact colony derived from a BFU-E.

FIGURE 7. BFU-E



This is an example of a multi-cluster colony derived from a BFU-E. Although there are multiple clusters, the colony was formed by a single progenitor cell. This is because the morphology of the clusters is similar, there are no other colonies in the area surrounding this colony and the clusters are all in the same plane of focus. Tips for determining whether multiple clusters make up one or two colonies are discussed in more detail in section 2.2.

2.1.2 CFU-GM

A CFU-GM (colony-forming unit-granulocyte, macrophage) is a progenitor cell which produces a colony containing more than 40 granulocyte and macrophage cells.

KEY FEATURES:

- The cells in this type of colony are not hemoglobinized and therefore do not appear red or brown.
- Individual cells in the colony can be distinguished.
- CFU-GM (including CFU-G and CFU-M) do not require erythropoietin for growth and differentiation. The morphology of colonies derived from these progenitors is similar when grown in medium formulations that do or do not contain EPO.

MORPHOLOGY:

Cells within a CFU-GM-derived colony can be spread out without a distinct center (Figure 8) or grouped together with dense centers, forming a "starburst pattern." (Figures 9 and 10).

- If the cells in the colony are spread out, it is possible to distinguish individual cells throughout the colony.
- If the cells in the colony are grouped together, individual cells in the center of the colony cannot be easily distinguished and may appear dark in color. This should not be confused with hemoglobinization. If unsure, look at the cells at the edge of the colony at a higher magnification.

CFU-GM, CFU-G AND CFU-M:

Some colonies contain only granulocytes or only macrophages and are derived from CFU-G (colony-forming unit-granulocyte) or CFU-M (colony-forming unit-macrophage) progenitors, respectively.

No Distinctions

Distinctions are not usually made between CFU-GM-, CFU-G- and CFU-M-derived colonies. If distinctions are not made, CFU-G- and CFU-M-derived colonies are both scored as CFU-GM-derived colonies.

Distinctions

If distinctions are made between the different colony types, CFU-GM-derived colonies are identified based on the fact that they contain cells of different sizes. This is because mature granulocytes and macrophages are different sizes.

CFU-G- and CFU-M-derived colonies are described in sections 2.1.3 and 2.1.4.

FIGURE 8. CFU-GM

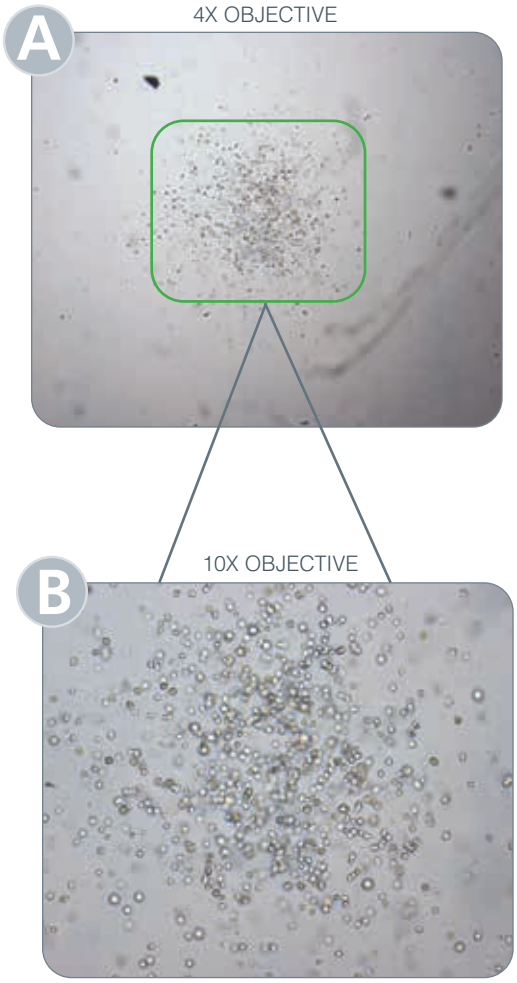
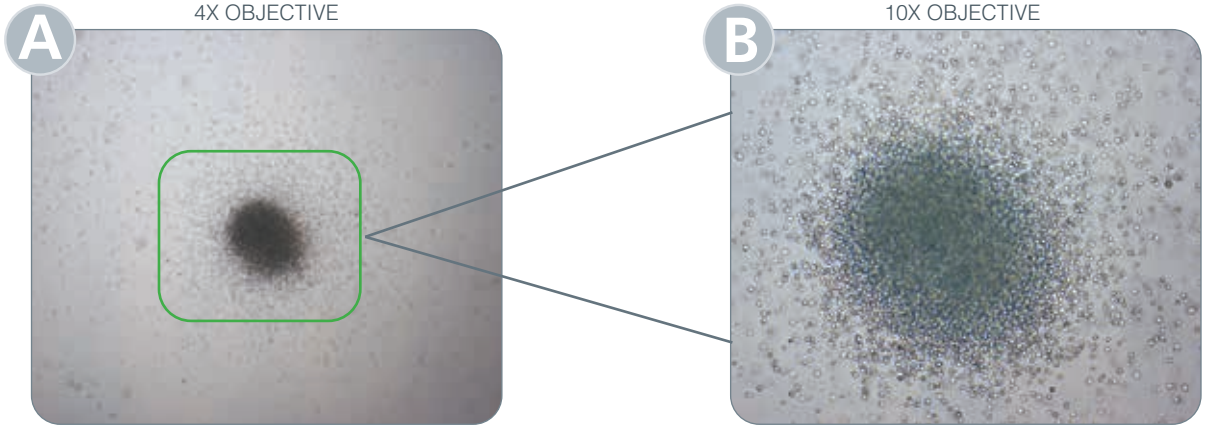


FIGURE 9. CFU-GM

FIGURE 10. CFU-GM



NOTES

2.1.3 CFU-G

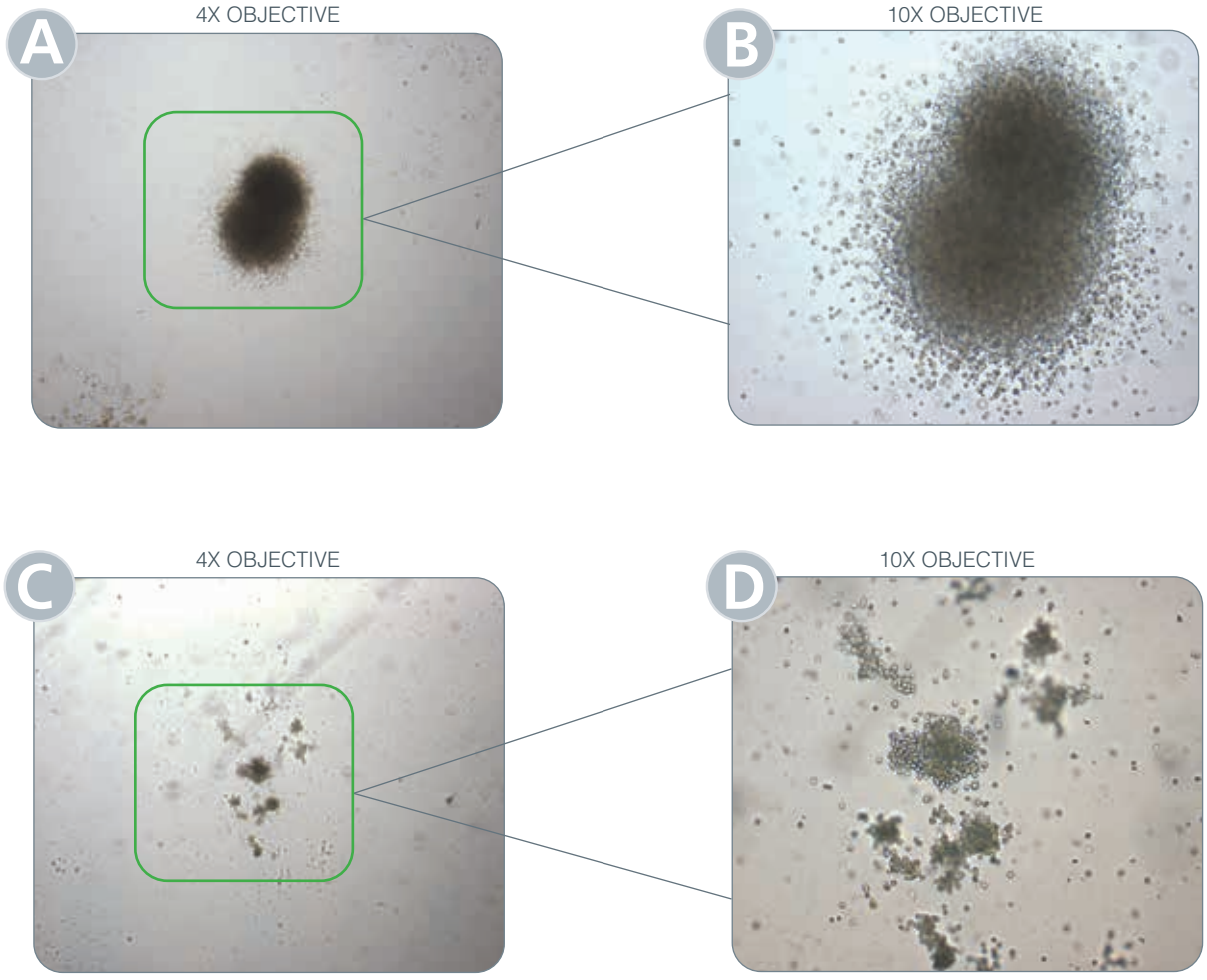
A CFU-G is a progenitor cell which produces a colony containing more than 40 granulocytes.

KEY FEATURES:

- Cells within a CFU-G-derived colony are small, uniform in size and usually tightly packed.
- Individual cells can be distinguished, particularly at the edge of the colony.
- Individual cells in the center of the colony are difficult to distinguish and may appear dark in color. If unsure about the lineage, look at the cells at the edge of the colony at higher magnification.

In comparison to CFU-M-derived colonies (described in section 2.1.4), the cells in a CFU-G-derived colony are smaller and more densely packed within the colony.

FIGURE 11. CFU-G



Both of these colonies highlight the importance of confirming colony lineage at a higher magnification. Using a 4X objective, these colonies could be mistakenly identified as being derived from a BFU-E or a CFU-GEMM.
When viewed at 10X objective, it is apparent that they are derived from a CFU-G (see Key Features listed above).

2.1.4 CFU-M

A CFU-M is a progenitor cell which produces a colony containing more than 40 macrophages.

KEY FEATURES:

- Individual cells in the colony can be distinguished.
- Cells are large, appear "glossy," are uniform in size, and are usually spread out.
- Cells are typically spread out but may have macrophages clustered together in the center of the colony, making it appear dark.

In contrast to a CFU-G-derived colony, cells in a CFU-M-derived colony are larger and more spread out.

FIGURE 12. CFU-M

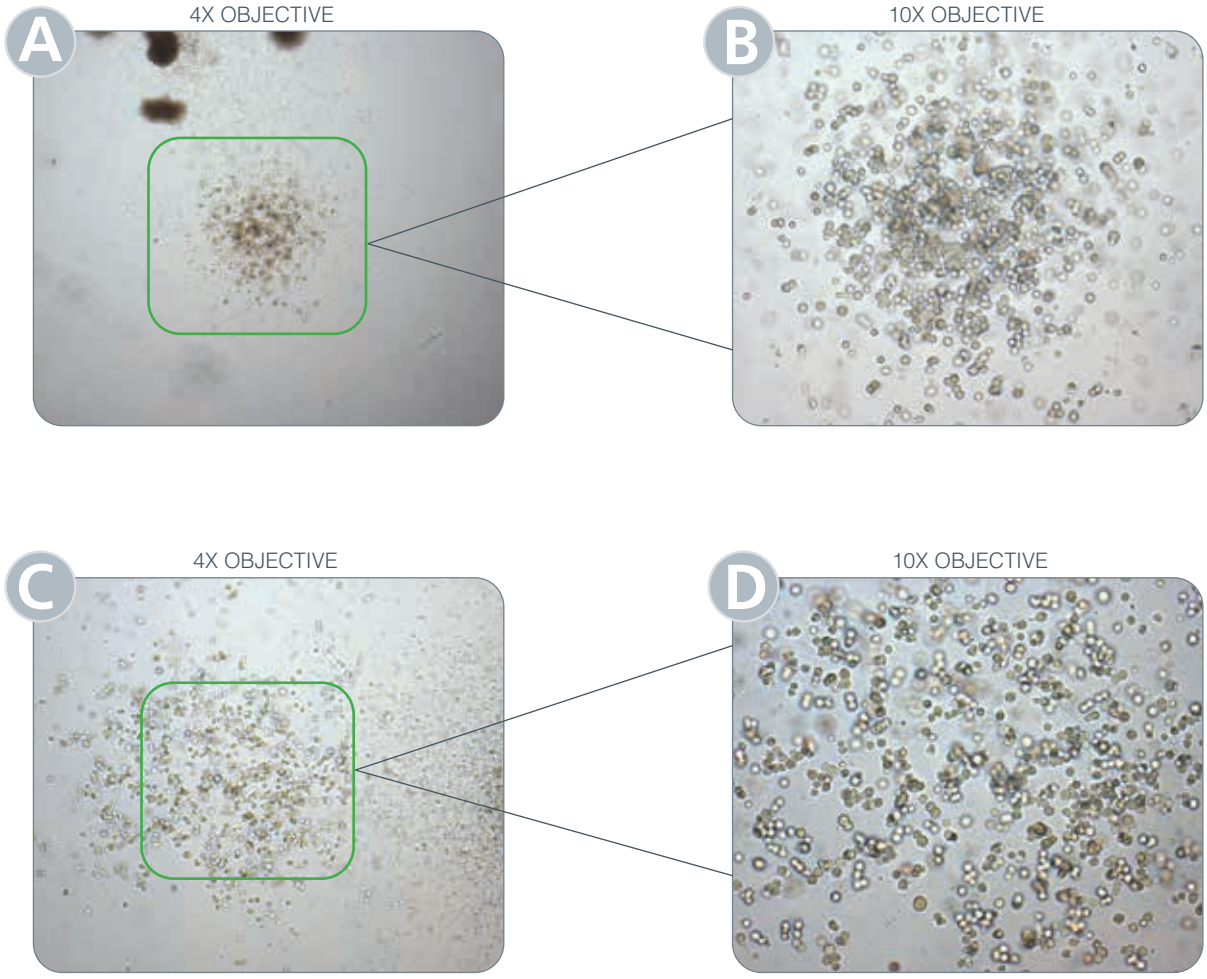
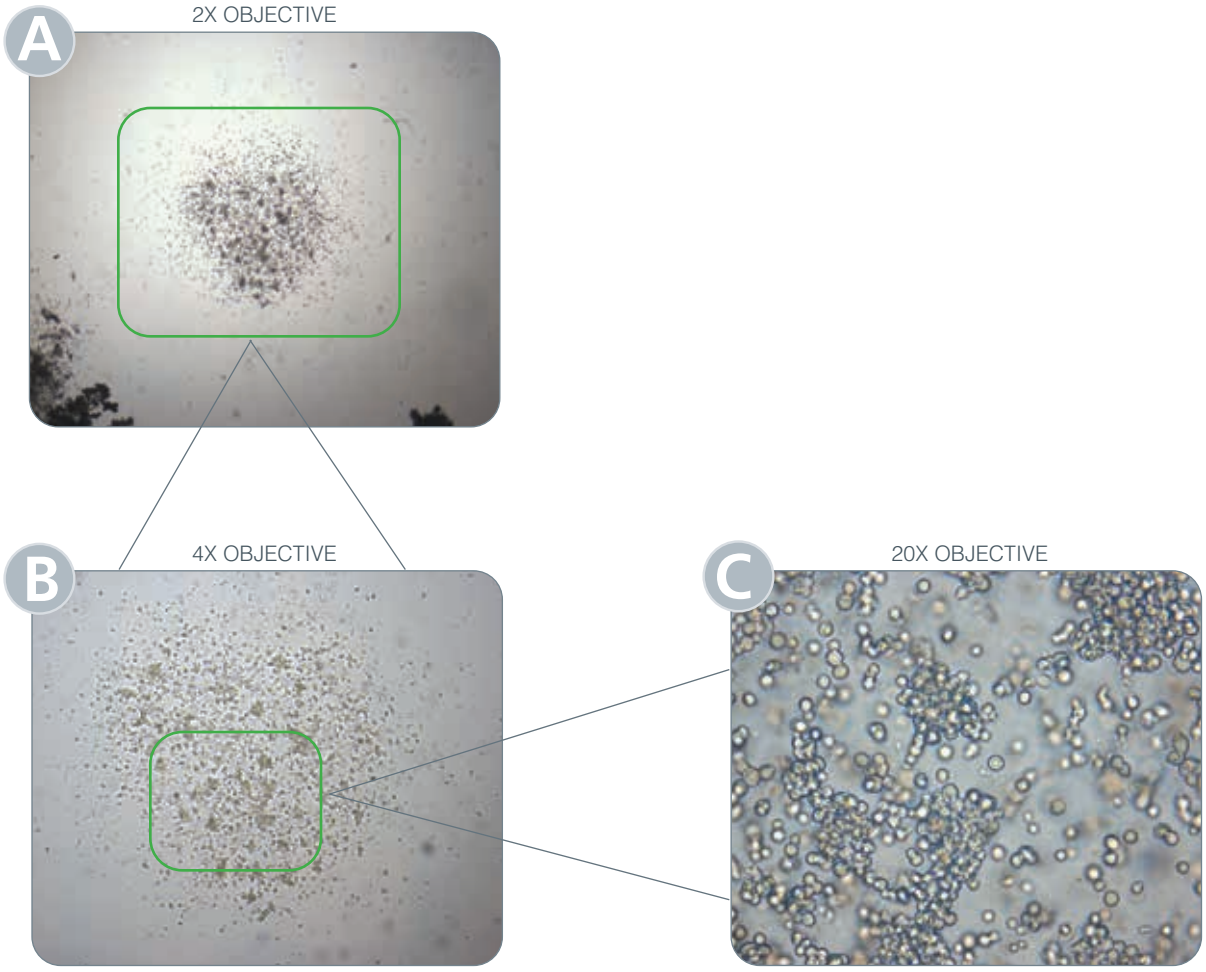


FIGURE 13. CFU-M



NOTES

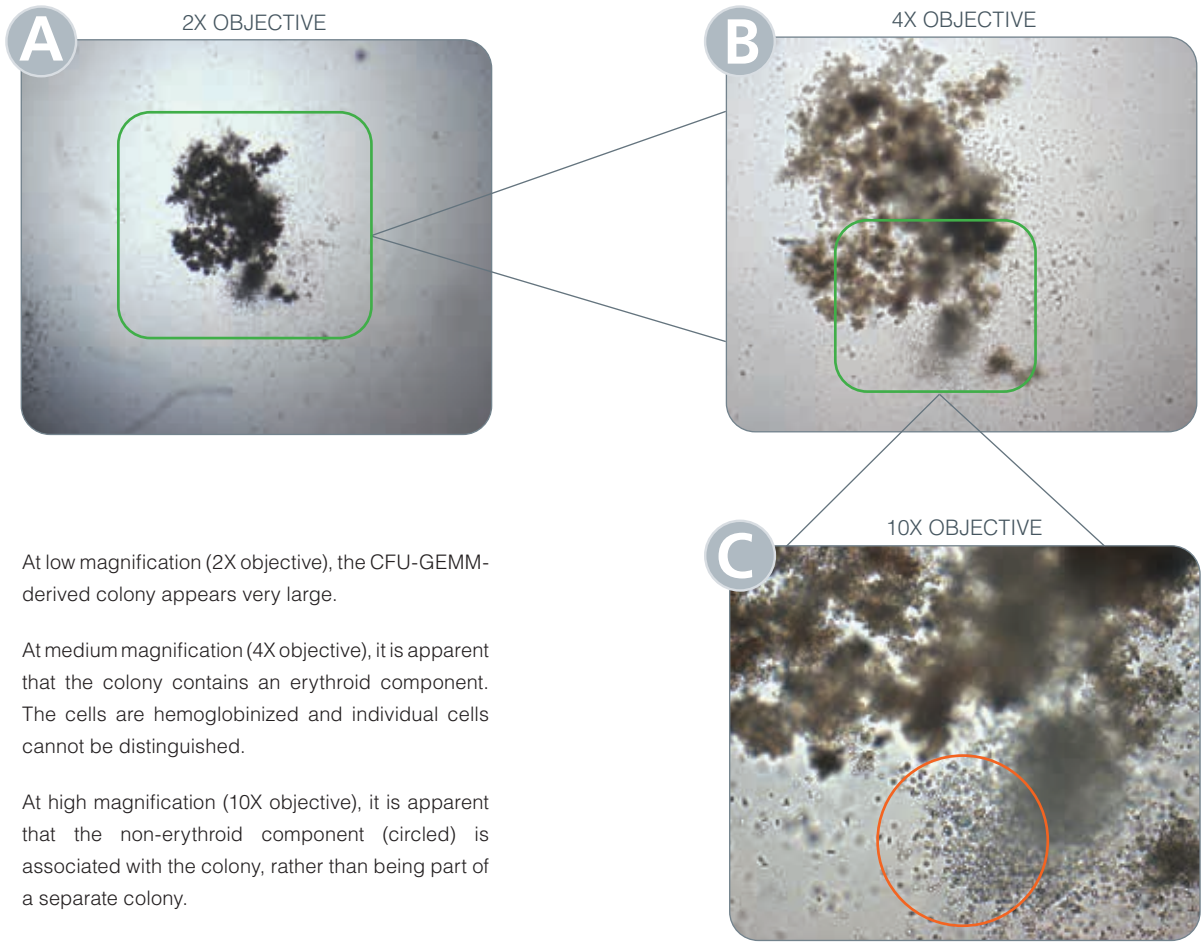
2.1.5 CFU-GEMM

A CFU-GEMM (colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte) is a progenitor cell which produces a colony containing erythroid cells as well as 20 or more non-erythroid cells, including granulocyte, macrophage or megakaryocyte cells. These colonies arise from multi-potential progenitors, which are more immature than the progenitors that form CFU-GM- and BFU-E-derived colonies. Cord blood samples often contain a higher frequency of multi-potential progenitors than other cell sources (e.g. bone marrow).

CFU-GEMM-derived colonies are often large as they arise from immature progenitors and therefore have a larger capacity to proliferate.

Often, a CFU-GEMM-derived colony will have erythroid cells in the center and non-erythroid cells surrounding them. However, the non-erythroid cells can be concentrated on one side of the erythroid cells.

FIGURE 14. CFU-GEMM

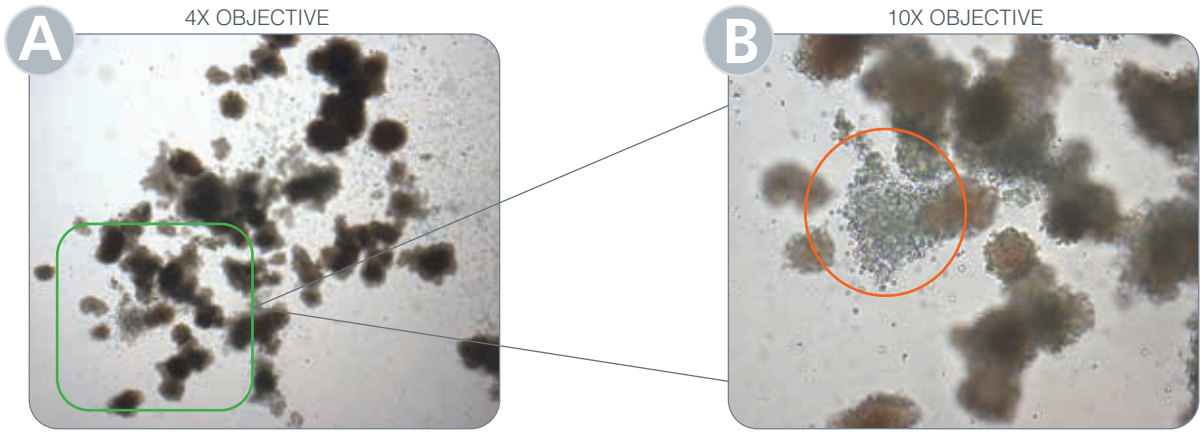


At low magnification (2X objective), the CFU-GEMM-derived colony appears very large.

At medium magnification (4X objective), it is apparent that the colony contains an erythroid component. The cells are hemoglobinized and individual cells cannot be distinguished.

At high magnification (10X objective), it is apparent that the non-erythroid component (circled) is associated with the colony, rather than being part of a separate colony.

FIGURE 15. CFU-GEMM



This is another example of a colony derived from a CFU-GEMM. It contains both an erythroid component and a non-erythroid component (indicated by the circle in B).

NOTES

2.2 ONE OR MULTIPLE COLONIES?

It can sometimes be difficult to determine whether a cluster of cells is actually one or multiple colonies. There are three factors to consider when making this decision: distribution of colonies throughout the dish, planes of focus and morphology of cells within the colony.

2.2.1 DISTRIBUTION OF COLONIES

Before beginning to count colonies, scan the entire dish at low power. This will give you a general idea of how close together the colonies are. If the dish is not overplated and the progenitor cells were evenly distributed throughout the MethoCult®, colonies should be distinguishable. In this case it is unlikely that multiple progenitors would be located in close proximity to each other. Therefore if multiple clusters are found together, they are likely derived from a single progenitor and should be counted as one colony. In contrast, if the dish is overplated, the likelihood of multiple colonies that are overlapping increases.

FIGURE 16. Appropriate plating concentrations

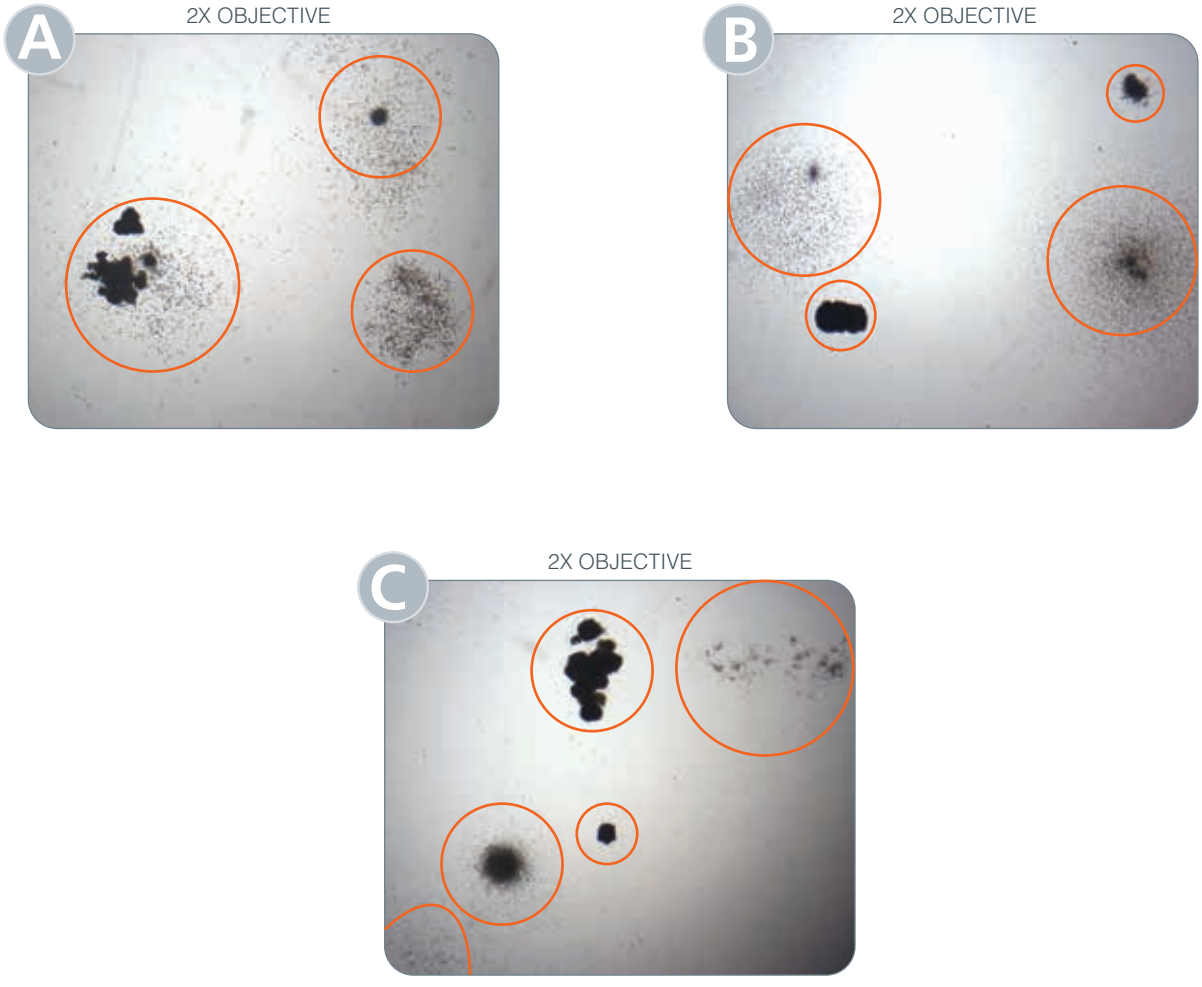


Figure 16 shows fields of view in a dish where the progenitor cells were plated at an appropriate concentration. The colonies are relatively far apart so they can be distinguished. Individual colonies are identified by circles.

FIGURE 17. Overplated dishes

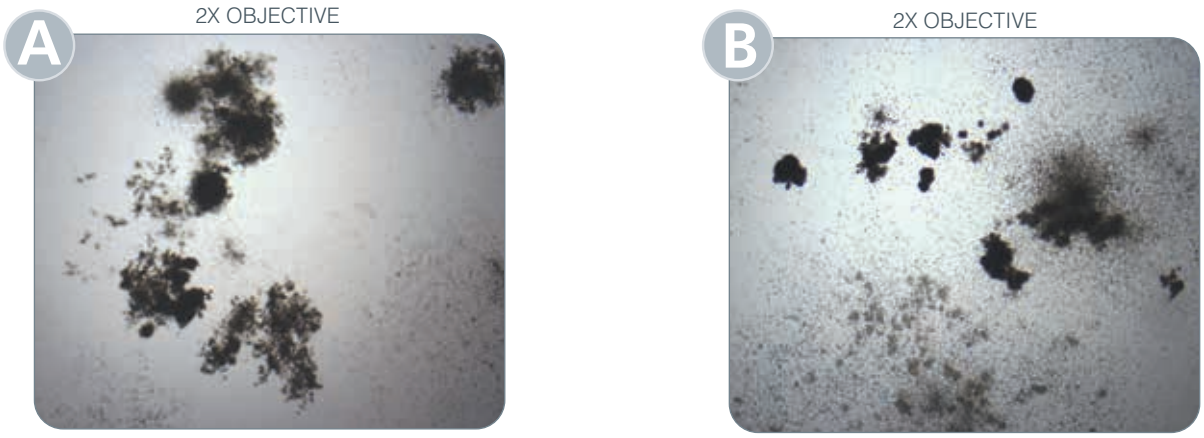


Figure 17 shows two fields of view from a dish that has been overplated. As all of the colonies are very close together, it is difficult to determine which clusters of cells actually make up an individual colony. With this number of colonies, it is also likely that colony growth is inhibited by waste products and metabolites. The medium will turn yellow when dishes are overplated.

2.2.2 PLANE OF FOCUS

If you come across several clusters of cells in close proximity, you should first determine whether they are in the same plane of focus by focusing up and down. Generally, multiple clusters that make up one colony will be in the same plane of focus. If multiple clusters are in a different plane of focus, it is more likely that they are separate colonies (Figures 18 and 19). However, this is not always the case, particularly if the colony is large (Figure 20).

FIGURE 18. Two colonies determined by different planes of focus

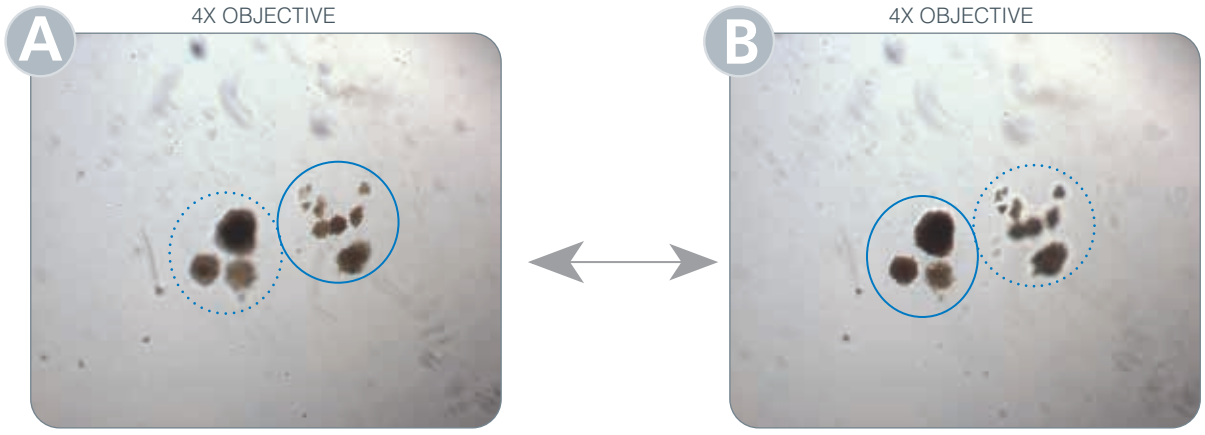


Figure 18 shows two colonies derived from BFU-E progenitors. Although they are close together and there are no other colonies in the field of view, they are in different planes of focus.

FIGURE 19. Two colonies determined by different planes of focus

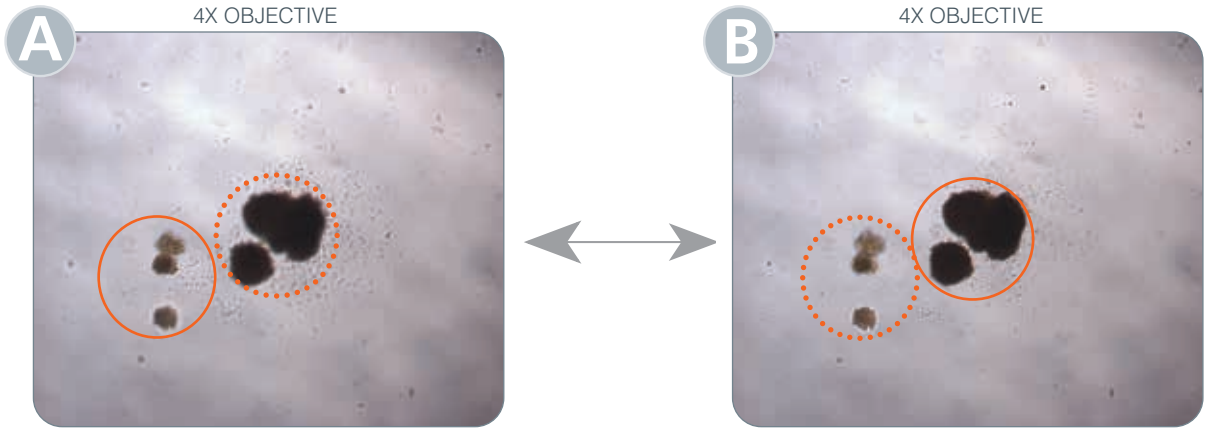


Figure 19 shows a BFU-E-derived colony on the left-hand side of the image and a CFU-GEMM-derived colony on the right-hand side of the image. Although the colonies are close together and there are no other colonies in the field of view, they are in different planes of focus.

FIGURE 20. One colony in different planes of focus

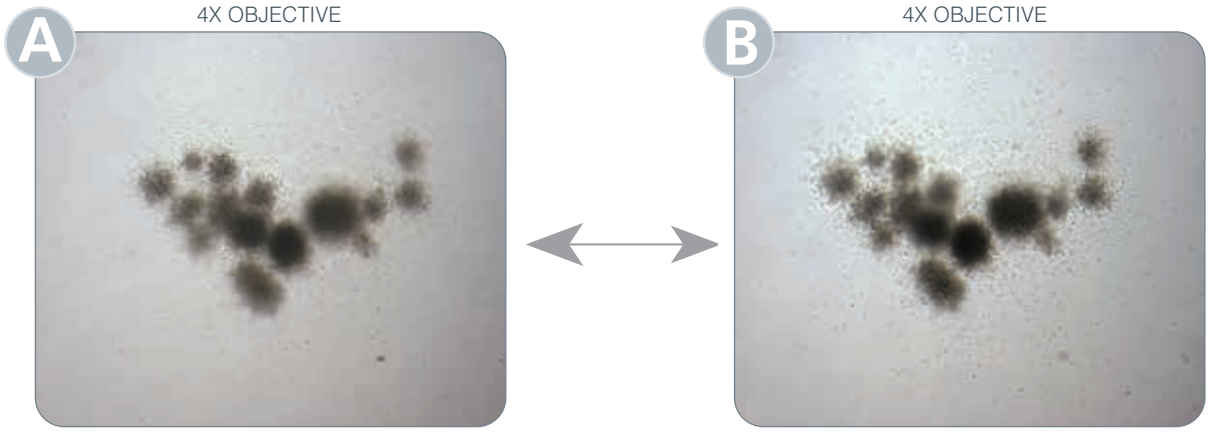


Figure 20 shows a large multi-cluster CFU-GM-derived colony. Although parts of the colony are in different planes of focus, it should be scored as one colony. All of the clusters of cells are very close together and the cellular morphology is very similar throughout the colony.

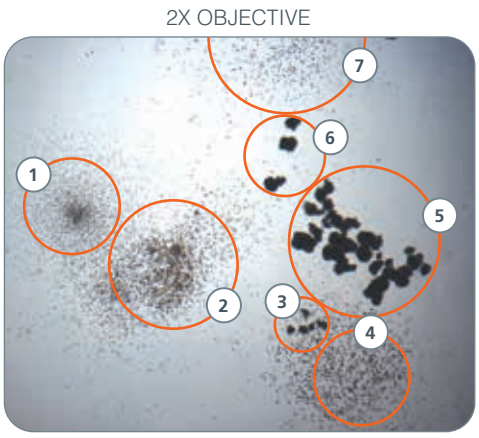
2.2.3 MORPHOLOGY

Morphology should be considered when deciding whether adjacent clusters are to be counted as one or multiple colonies. Multiple clusters that make up one colony generally contain cells that show a similar morphology. The exception is a colony derived from a CFU-GEMM. These progenitors are multi-potential and will produce a colony containing different mature cells.

Morphological characteristics to consider include:

- whether the cells that make up the colony are spread out or clustered together
- the degree to which cells in a BFU-E-derived colony are hemoglobinized
- the distribution of mature cell types in a CFU-GM-derived colony

FIGURE 21. Multiple colonies



Scanning using a 2X objective gives an estimate of seven colonies. Colony lineage should be confirmed using a 4X or a 10X objective.

Using a 2X objective, it is possible to determine that Circle 1 and Circle 2 indicate two different colonies based on cellular morphology within the colony.

Circle 1 shows a colony made up of small cells in a "starburst" pattern. Circle 2 shows a colony made up of larger cells in a more irregular pattern.

FIGURE 22. Two colonies

This is an example of two colonies in close proximity to one another that can be identified as separate colonies based on morphology. The morphology of cells within each colony (identified by a circle) is very different.

BFU-E-DERIVED COLONY ON THE LEFT-HAND SIDE

- The cells are hemoglobinized and individual cells cannot be distinguished.

CFU-GM-DERIVED COLONY ON THE RIGHT-HAND SIDE

- The cells are not hemoglobinized and individual cells at the periphery can be distinguished.

THIS SHOULD NOT BE MISTAKEN FOR ONE COLONY DERIVED FROM A CFU-GEMM

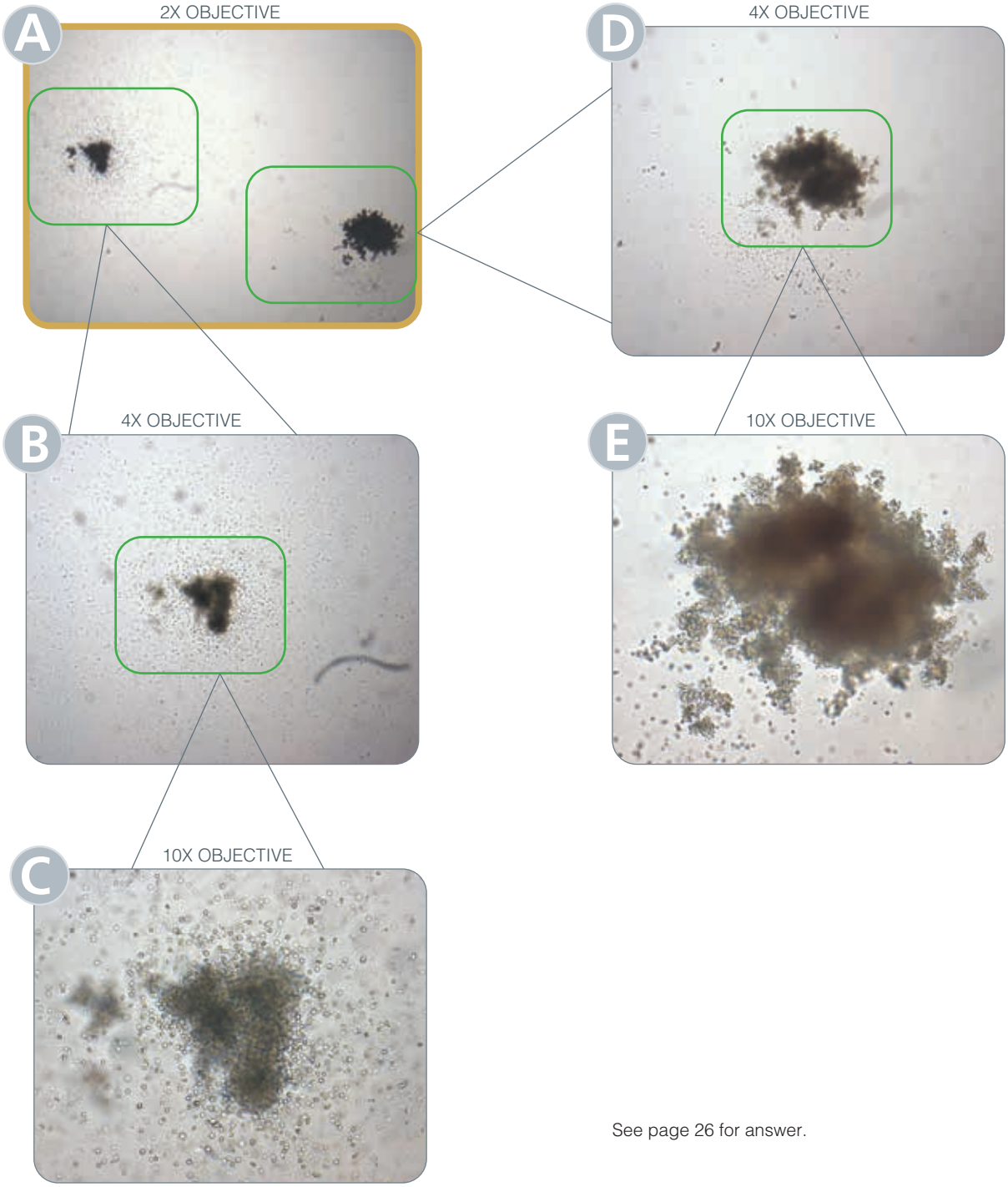
- The CFU-GM-derived colony shows a "starburst" pattern, the center of which is not close to the BFU-E-derived colony.

CHAPTER 3 - COLONY IDENTIFICATION CHALLENGES (NO RED CELL BACKGROUND)

Colonies presented in this chapter are derived from cord blood samples that have been processed to remove the red blood cells, or derived from samples that have not been processed but have been cryopreserved. Refer to Chapter 1 for a description of processing methods that will eliminate a red blood cell background in the CFC assay.

CHALLENGE 1 - Identify the Colonies

FIGURE 23.



See page 26 for answer.

CHALLENGE 1 - Answer

LEFT-HAND SIDE OF FIGURE 23

- The cells in the colony are not hemoglobinized and individual cells can be distinguished at the periphery.
- Lineage should be determined at 4X and 10X objective (B and C). It is not possible to definitively determine the lineage using a 2X objective (A).
- **Colony derived from a CFU-GM**

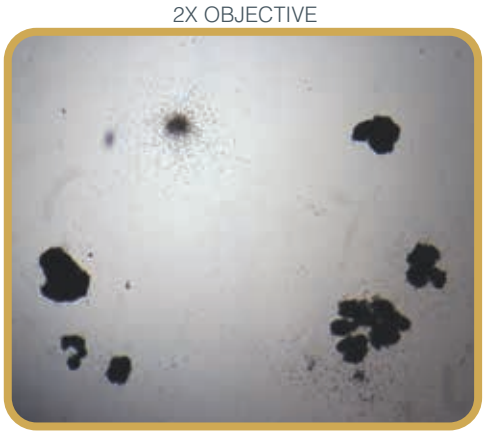
RIGHT-HAND SIDE OF FIGURE 23

- The cells are hemoglobinized and individual cells cannot be distinguished.
- Determine lineage using a 4X and 10X objective (D and E).
- **Colony derived from a BFU-E**
- This is not a colony derived from a CFU-GEMM, even though there is a cluster of non-erythroid cells near the bottom left-hand side of the colony. There is no significant overlap between the erythroid colony and the non-erythroid cells, and both components are in a slightly different plane of focus.
- The non-erythoid cells could be scored as a colony derived from a CFU-GM, depending on the institution's criteria for minimum number of cells in a colony.

NOTES

CHALLENGE 2 - How many colonies?

FIGURE 24.



Figures 25 - 27 show some of the colonies from Figure 24 at higher magnification.

See page 28 for answer.

FIGURE 25. CFU-GEMM (Circle 3 from Figure 28)

FIGURE 26. CFU-GM (Circle 1 from Figure 28)

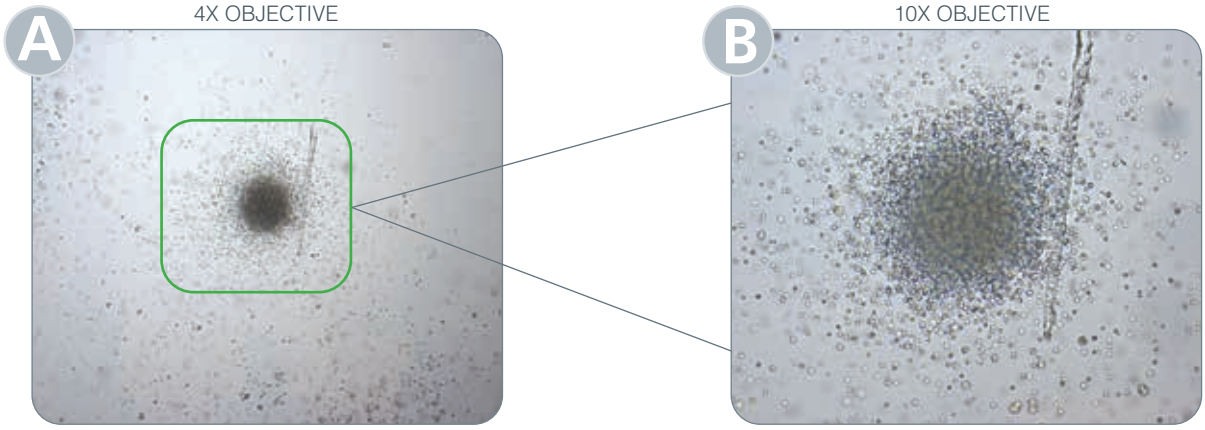
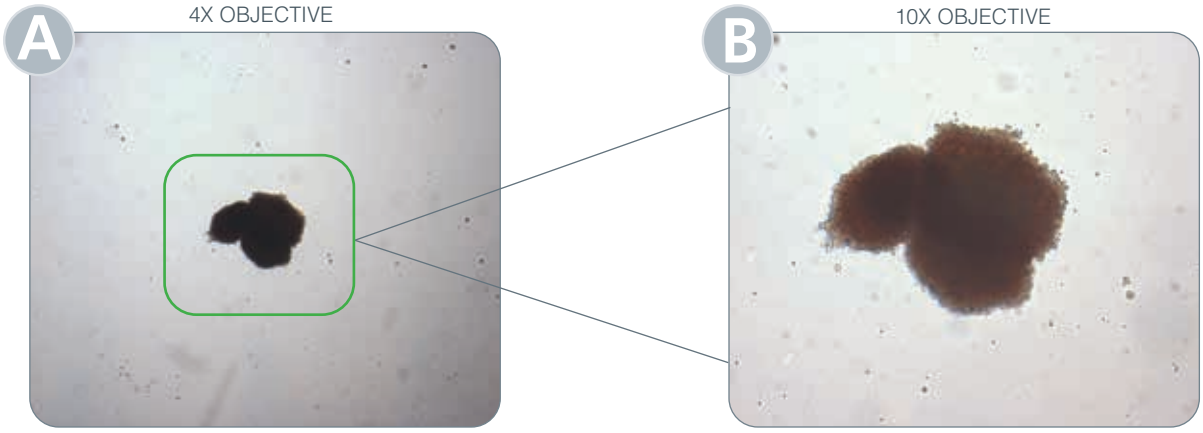


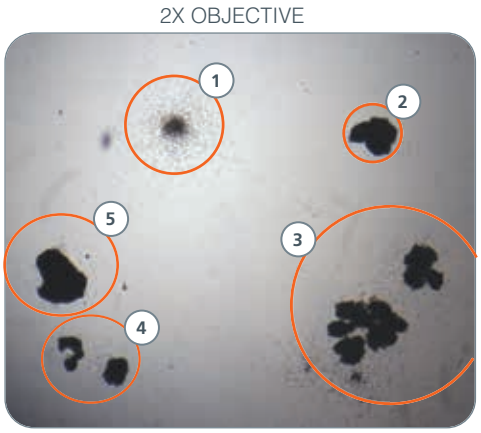
Figure 26 shows a colony derived from a CFU-GM. Note the piece of debris on the right-hand side of the colony.

FIGURE 27. BFU-E (Circle 2 from Figure 28)



CHALLENGE 2 - Answer

FIGURE 28.



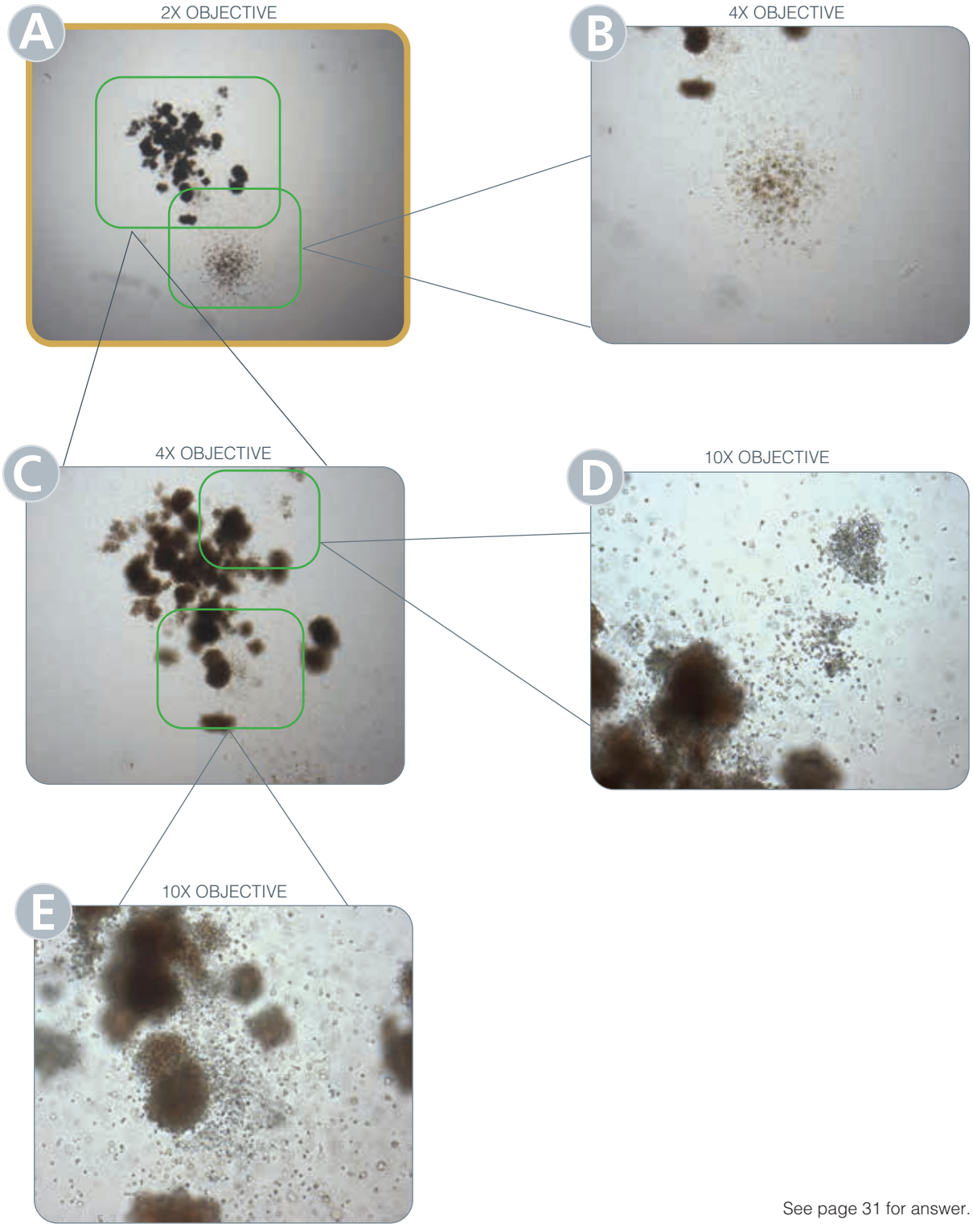
There are five colonies in the field of view (each one is indicated with a circle). The clusters indicated by Circle 4 may be two separate colonies, although they are more likely to be one colony. There is another colony in close proximity (Circle 5) so it is unlikely that there were three separate progenitors in this area at the time of plating, particularly as the dish appears to have been plated at an appropriate concentration. The two clusters shown by Circle 4 also appear very similar and are approximately the same size.

Figure 25 shows a colony derived from a CFU-GEMM (Circle 3 in Figure 28). This colony shows the importance of using a 2X objective when counting. If this field of view was only observed using a 4X objective, this colony would most likely be counted as two separate colonies. However, at 2X objective (Figure 24), it is apparent that the clusters are relatively close together and should be counted as one colony.

NOTES

CHALLENGE 3 - One colony or multiple colonies?

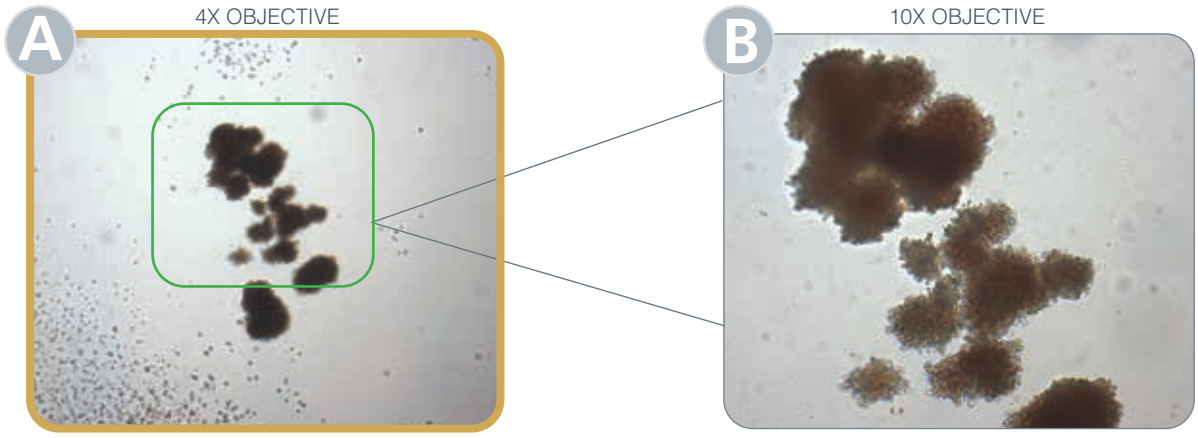
FIGURE 29.



See page 31 for answer.

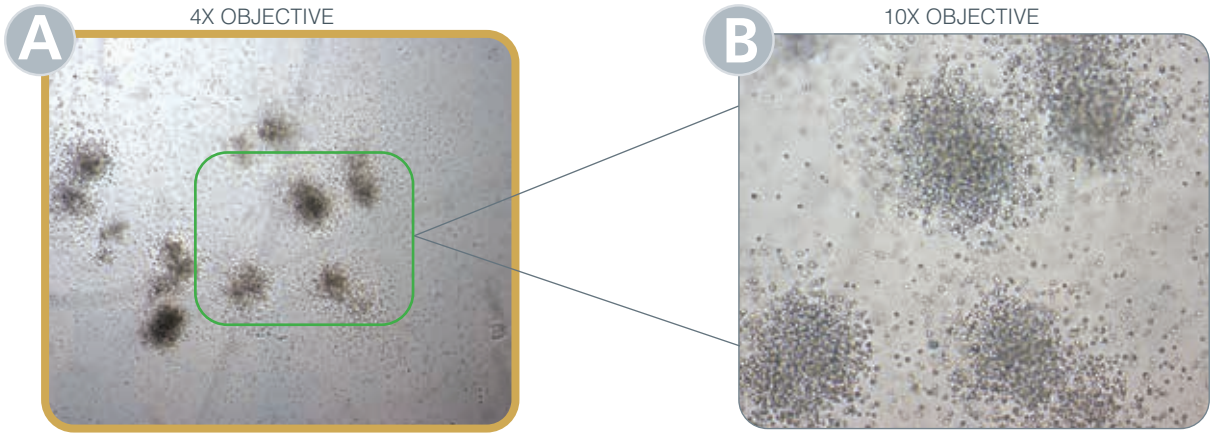
CHALLENGE 4 - One Colony or Multiple Colonies?

FIGURE 30.



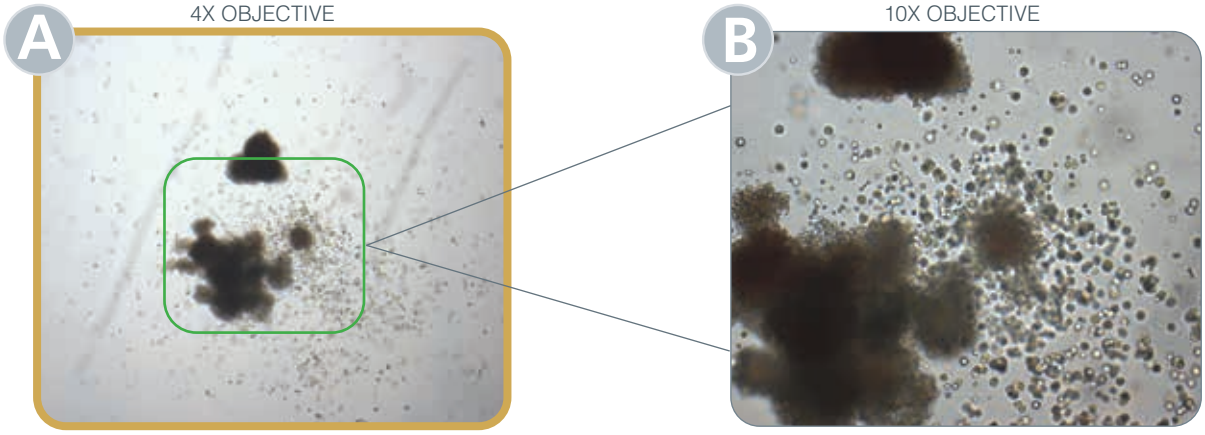
CHALLENGE 5 - One Colony or Multiple Colonies?

FIGURE 31.



CHALLENGE 6 - One Colony or Multiple Colonies?

FIGURE 32.



See page 31 for answers.

CHALLENGE 3 - Answer

There are two colonies: one derived from a CFU-GEMM (top) and one derived from a CFU-GM (bottom).

- The colony derived from the CFU-GM has a distinct center (i.e. the cells are not spread out) and it is far from the erythroid clusters (B). This means it should be scored as a separate colony.
- There are multiple erythroid clusters in the colony derived from a CFU-GEMM (C, D and E). View the colony at low magnification (A) and it is apparent that the erythroid clusters are part of the same colony as they are all relatively close together and they are of similar morphology (density and degree of hemoglobinization).
- The erythroid clusters in the CFU-GEMM-derived colony are not completely surrounded by non-erythroid cells. However, there are sufficient non-erythroid cells for it to be classified as a CFU-GEMM-derived colony (D and E).

CHALLENGE 4 - Answer

ONE COLONY DERIVED FROM A BFU-E

- This is a multi-cluster colony derived from a BFU-E (the cells are hemoglobinized and individual cells cannot be distinguished).
- This should be scored as one colony even though there are multiple clusters in slightly different planes of focus. The clusters have similar morphologies (color or degree of hemoglobinization and density of cells) and are very close together.
- There are granulocyte and macrophage cells at the top and bottom of the photograph but they are not part of the colony highlighted in this figure.

CHALLENGE 5 - Answer

ONE COLONY DERIVED FROM A CFU-G

- The cells are not hemoglobinized, individual cells can be distinguished at the periphery of each cluster and the cells are small and uniform in size.
- Each cluster of cells is in a slightly different plane of focus but each cluster is of a similar size and with similar morphology.
- There are approximately ten clusters in this colony. If the dish was plated at a high enough concentration for this many progenitor cells to be in such close proximity, additional colonies would be visible at the edges of the photograph. If there were this many progenitors in a small area, it is highly unlikely that all of them would be of the same lineage.

CHALLENGE 6 - Answer

ONE COLONY DERIVED FROM A CFU-GEMM

- The colony contains both an erythroid component and a non-erythroid component.
- Although there are two clusters of erythroid cells, they are part of the same colony. They are relatively close together and have similar morphologies (degree of hemoglobinization and density of cells).

CHAPTER 4 - COLONY IDENTIFICATION WITH RED BLOOD CELL BACKGROUND

Colonies presented in this section are derived from fresh minimally processed or unprocessed samples.

Fresh, minimally processed or unprocessed samples plated in a CFC assay will produce a red blood cell background. The same minimally processed or unprocessed sample, after freezing and thawing, will produce a significantly reduced red blood cell background. This is because red blood cells are sensitive to cryopreservation and will lyse, as will other mature cells.

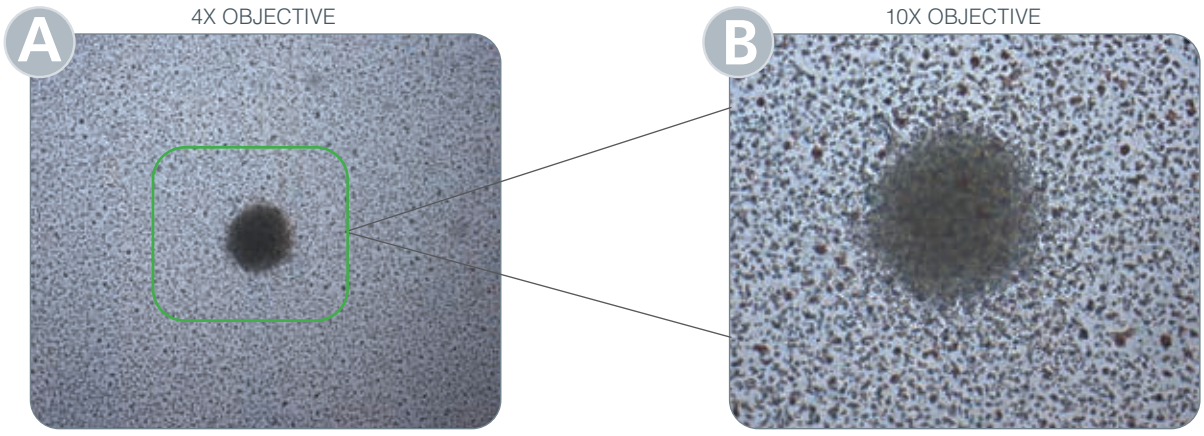
A red blood cell background can vary in appearance, depending on the length of time between taking the sample and plating in the CFC assay, and the concentration of red blood cells in the sample. The red cell background can appear either as small black dots or large red cells that are about the same size as a single macrophage.

Colonies derived from CFU-M can be difficult to see in a dish with a red blood cell background. This is due to the fact that the colonies generally do not contain a dense core.

Colonies derived from CFU-GEMM can be difficult to distinguish from colonies derived from BFU-E in a dish with a red blood cell background. This is due to the fact that the non-erythroid component of the colony may be obscured by the background, particularly if it is small relative to the colony as a whole.

When counting a dish with a red cell background, it is important to observe several planes of focus in each field of view (i.e. focus "up and down"). Look for cells that appear "shiny" in comparison to red blood cells, which appear "dull." This will help to ensure all the colonies are scored and none are missed.

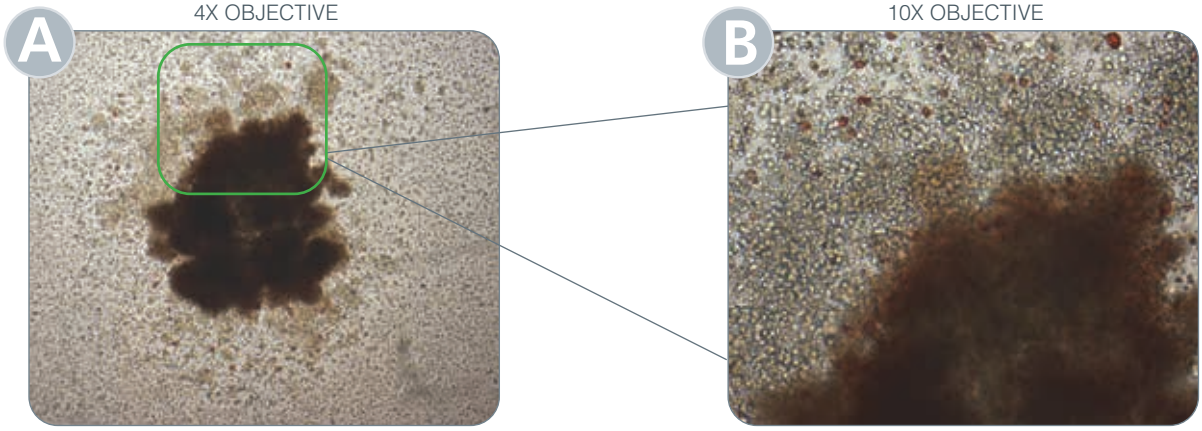
FIGURE 33. CFU-GM



CFU-GM-DERIVED COLONY

- The cells in the colony are not hemoglobinized.
- There are red cells (especially when viewed at high magnification in Figure 33B) but these are part of the red cell background. Note that they are evenly distributed throughout the photograph.
- The cells at the edge of the colony can be distinguished. It can be difficult to identify the edge of the colony with a red cell background in the dish, but compare the edge of this colony to the edge of other colonies derived from BFU-E in this section.

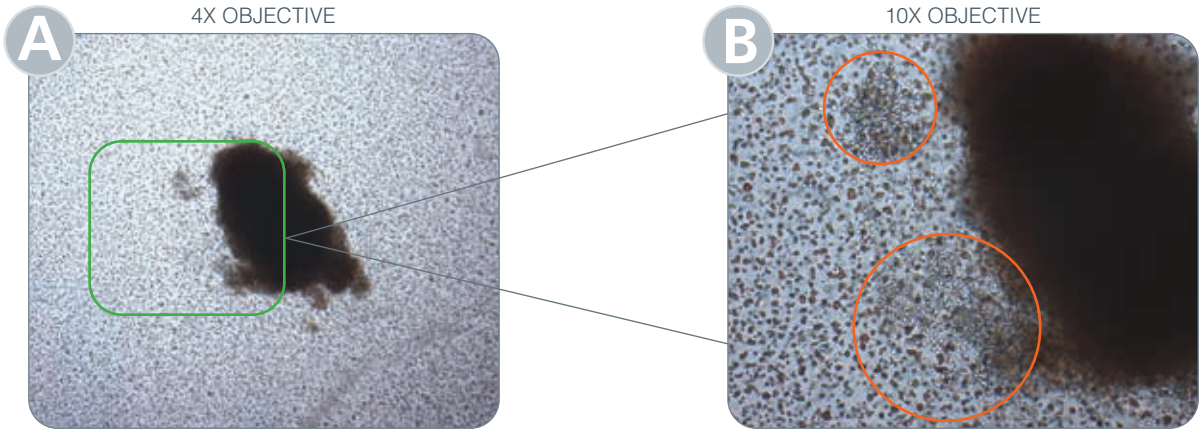
FIGURE 34. CFU-GEMM



CFU-GEMM-DERIVED COLONY

- Erythroid component: These cells are hemoglobinized and cannot be easily distinguished.
- Non-erythroid component: The individual cells can be distinguished and they are not hemoglobinzed (they do not appear red or brown).
- These two components do not appear distinct and separate from each other, indicating that they are derived from a single CFU-GEMM

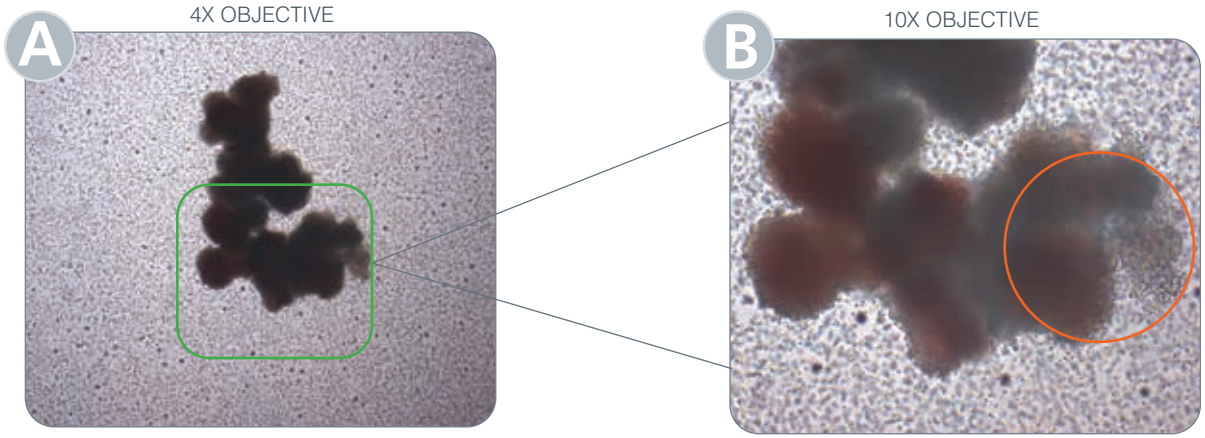
FIGURE 35. CFU-GEMM



CFU-GEMM-DERIVED COLONY

- This is an example of a CFU-GEMM-derived colony that is difficult to distinguish from a BFU-E-derived colony with the red blood cell background in the dish.
- The colony contains both an erythroid component (cells are hemoglobinized and cannot be distinguished) and a non-erythroid component (cells are not hemoglobinized and can be distinguished). The non-erythorid component is indicated by circles in Figure 35B.
- Both components are part of the same colony as they are closely associated with one another and are in the same plane of focus.

FIGURE 36. BFU-E

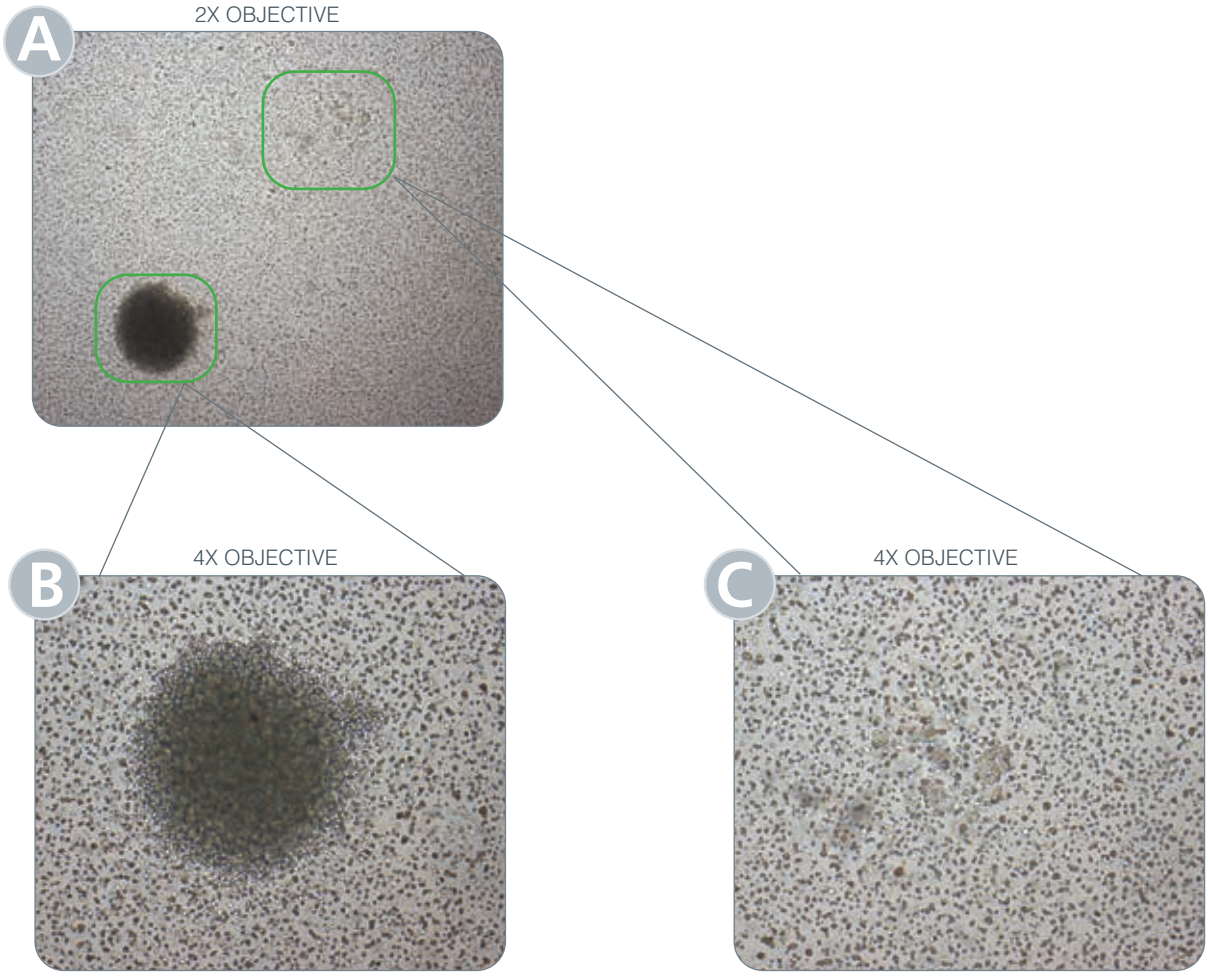


BFU-E-DERIVED COLONY

- The cells that make up the colony are hemoglobinized.
- There are erythroblasts at the edge of the colony (shown by the circle in Figure 36B). These should not be confused with cells that make up a non-erythroid component. The individual cells cannot be made out and they resemble a bag of marbles or a bunch of cauliflower. Cells making up a non-erythroid component would be larger and distinguishable.
- The colony is surrounded by a red blood cell background that is evenly spread throughout the dish, as opposed to a non-erythroid component.

NOTES

FIGURE 37. 2 CFU-G

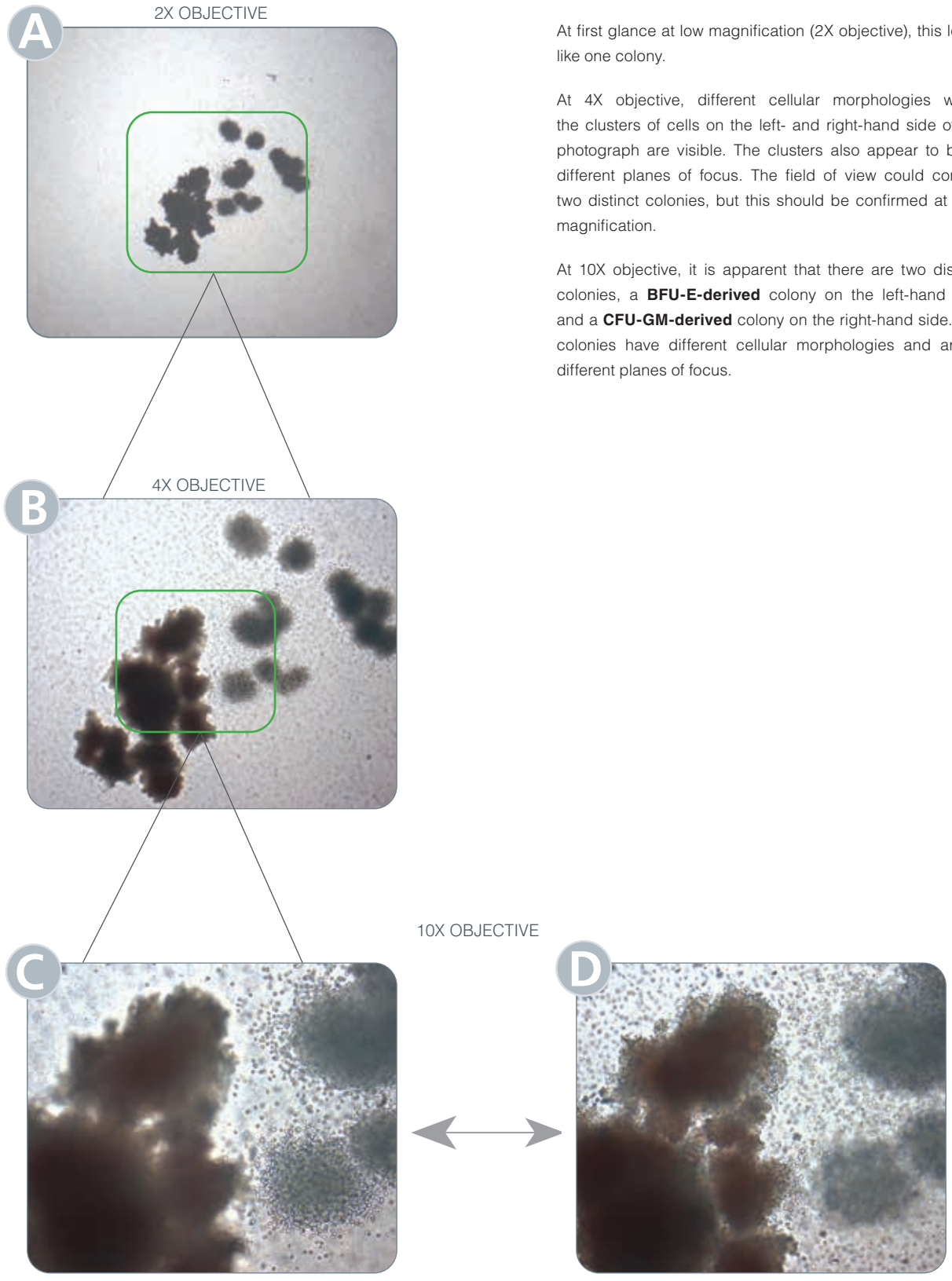


This is an example of a more challenging field of view in a dish with a red blood cell background.

2 CFU-G-DERIVED COLONIES

- Each colony is derived from a unique CFU-G.
- Note that the cells are small and homogeneous in morphology but each colony has a different density.
- Each colony shows a different morphology. The colony in Figure 37B is larger and more compact than the colony in Figure 37C.
- The colony in Figure 37B is derived from a more primitive progenitor as there are more cells. Colony size is indicative of the maturity of the progenitor cell from which it originated.

FIGURE 38. BFU-E and CFU-GM

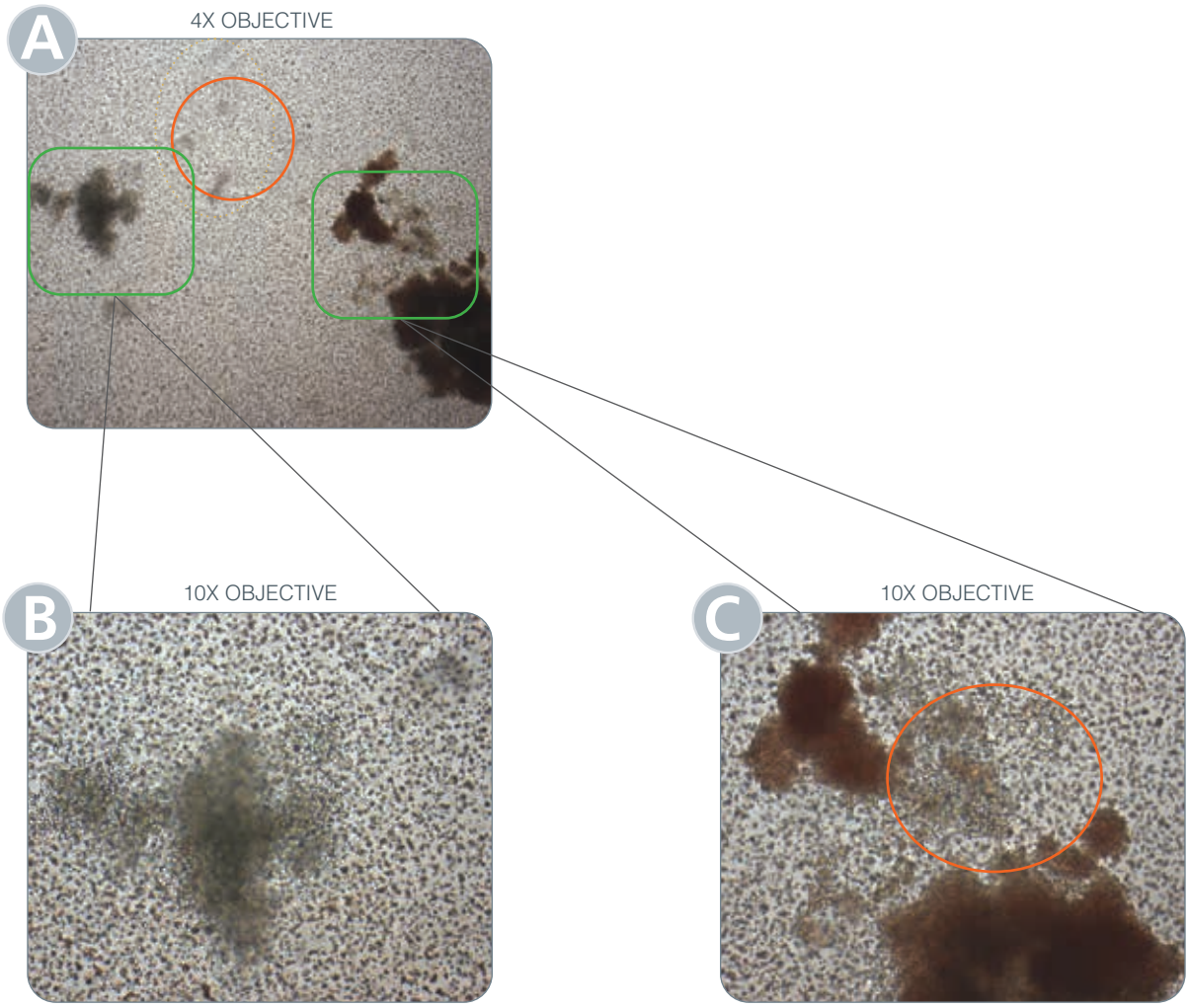


At first glance at low magnification (2X objective), this looks like one colony.

At 4X objective, different cellular morphologies within the clusters of cells on the left- and right-hand side of the photograph are visible. The clusters also appear to be in different planes of focus. The field of view could contain two distinct colonies, but this should be confirmed at high magnification.

At 10X objective, it is apparent that there are two distinct colonies, a **BFU-E-derived** colony on the left-hand side and a **CFU-GM-derived** colony on the right-hand side. The colonies have different cellular morphologies and are in different planes of focus.

FIGURE 39. CFU-GM and BFU-E



CFU-GM-DERIVED COLONY ON LEFT-HAND SIDE

- The colony is dark but the cells are not hemoglobinized (compare to the color of the BFU-E-derived colony, on the right-hand side).
- The cells at the periphery can be distinguished. Although they are difficult to distinguish with the red blood cell background, compare to the periphery of the BFU-E-derived colony.

BFU-E-DERIVED COLONY ON RIGHT-HAND SIDE

- The cells are hemoglobinized and individual cells cannot be distinguished, even at the periphery of the colony.
- There is an area of immature erythroid cells (indicated by the circle in Figure 39C) that should not be confused as the non-erythroid component of a CFU-GEMM-derived colony. These cells are beginning to hemoglobinize. They are not “glossy” and clear (compare to the cells in the CFU-GM-derived colony in Figure 39B).

The orange circle in Figure 39A shows a cluster of cells that might be part of the colony derived from the CFU-GM or part of a separate colony. It would be necessary to scan the dish to make this decision.

CHAPTER 5 - COLONY IDENTIFICATION WITH METHOCULT® EXPRESS

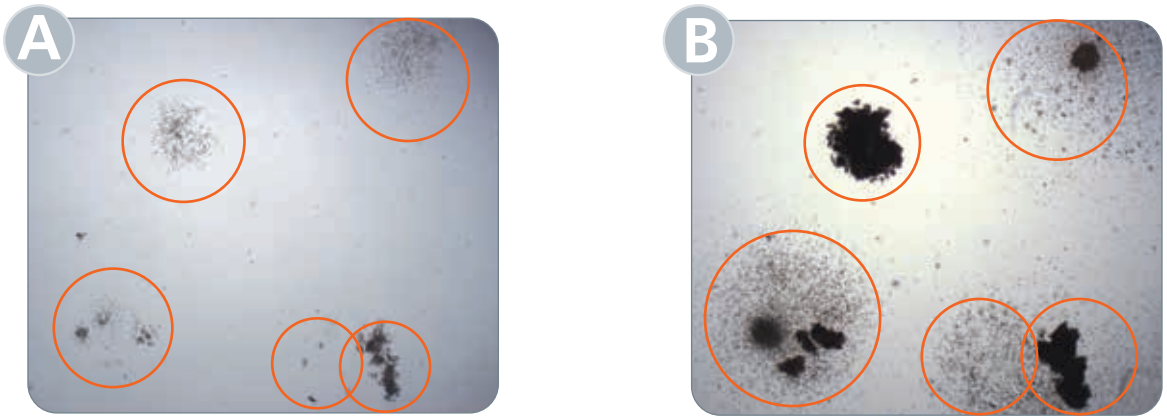
MethoCult® Express (Catalog #04437/04447) was developed by STEMCELL Technologies to allow for accurate enumeration of total progenitors in CB after a 7 day culture period. MethoCult® Express is formulated for accelerated progenitor proliferation, which ensures that colonies are large enough to be counted after only 7 days and that results correlate with results from a 14-day assay in standard MethoCult® medium. Additional background information, including data correlating the 7-day colony assay and the 14-day colony assay is provided in Section 1.6. Detailed instructions on the use of MethoCult® Express are included in the Technical Manual for Human Colony-Forming Cell Assay Using MethoCult® Express (Manual Catalog #29926), available upon request and on our website at www.stemcell.com.

Counting colonies after 7 days in MethoCult® Express is different than counting colonies after 14 days in standard MethoCult® medium. After 7 days of culture, cells within the colonies are still immature and it is not yet possible to distinguish the different lineages on the basis of colony morphology. Therefore only total colonies are counted in 7-day CFC assays in MethoCult® Express.

The spacing between cells within one colony after 7 days can appear to be larger than what is typically seen after 14 days. Progenitors and their immediate progeny are mobile during the first days of culture and may not remain in exactly the same position within the semi-solid medium. This may result in the formation of separate cell clusters that are relatively far apart from each other after 7 days of culture. As a result of further proliferation and reduced mobility of the daughter cells, the spaces between these cell clusters are usually filled between days 7 and 14.

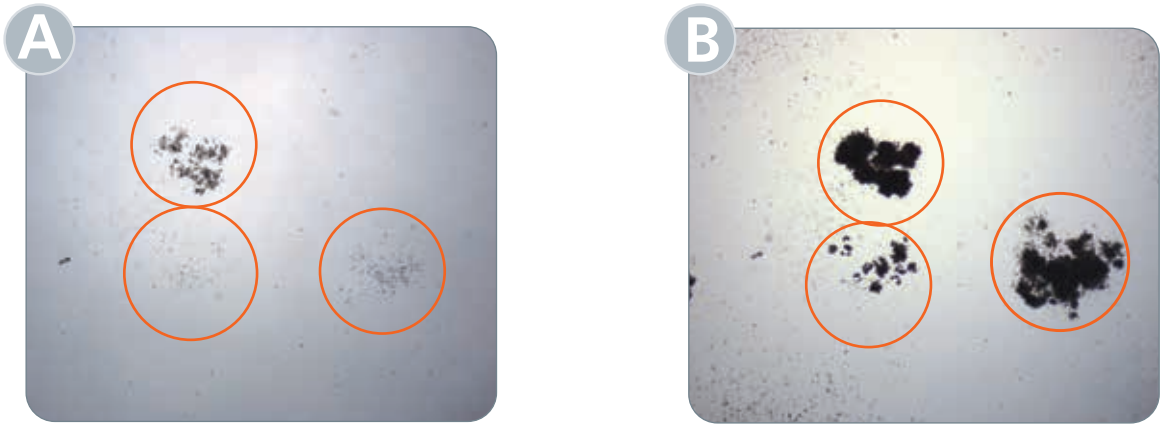
It is important to consider the total number of colonies in the dish as well as the relative size, distance and appearance of separate cell clusters when enumerating colonies in MethoCult® Express after 7 days. When two adjacent clusters have a similar size and appearance as compared to other nearby cell clusters, these two clusters are likely to be derived from the same progenitors and should be counted as one colony. If two adjacent clusters, are different in size and/or appearance, it is more likely that they belong to two separate colonies.

FIGURE 40.



Figures 40 and 41 show examples of colonies derived from cord blood progenitors in MethoCult® Express at days 7 (A) and 14 (B). Colonies are indicated by circles. Note that the different colony lineages can be distinguished after 14 days in MethoCult® Express. However, a standard medium such as MethoCult® H4034 Optimum is recommended for the 14-day assay. If MethoCult® Express is used for 14-day assays, a lower plating concentration is recommended. Colonies derived from cord blood progenitors can become very large and may be difficult to distinguish from one another if colony numbers per dish are high.

FIGURE 41.



Note that in Figure 41B (14 days), cells can be seen at the periphery of the photograph that were not visible in Figure 41A (7 days). This is because the cells are part of colonies that were much smaller at day 7 and were not visible in the field of view.

FIGURE 42.

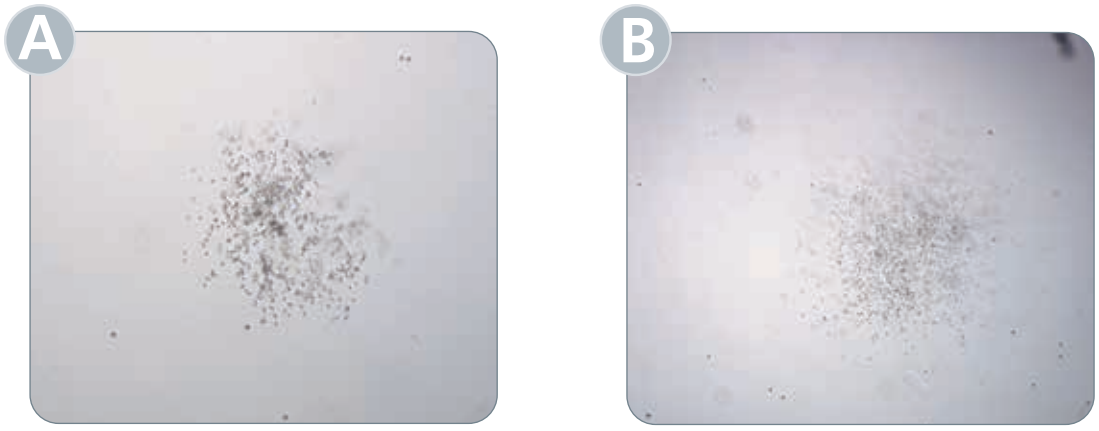


Figure 42 shows photographs of two separate colonies after 7 days in MethoCult® Express. Each photograph shows one colony and the lineage cannot be distinguished. These are examples of colonies with relatively small spaces between cell clusters.

FIGURE 43.

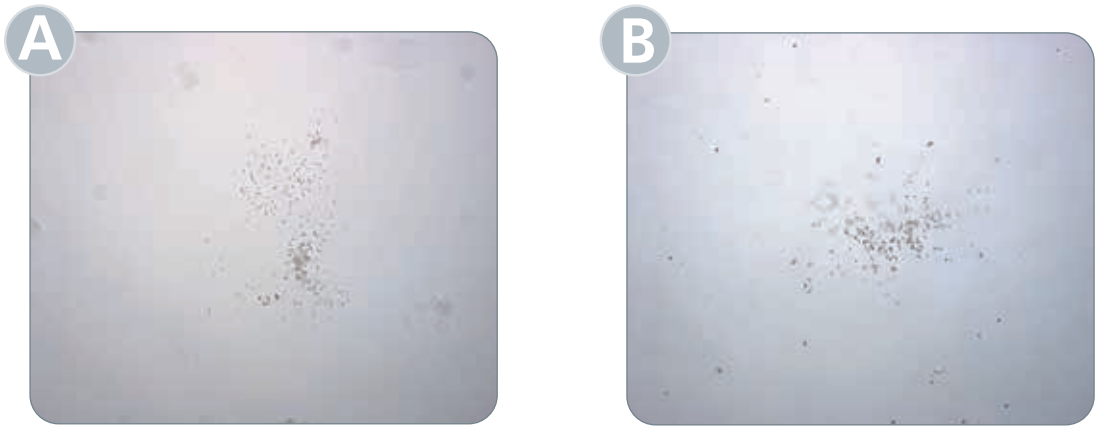


Figure 43 shows photographs of two separate colonies after 7 days in MethoCult® Express. Each photograph shows one colony and the lineage cannot be distinguished. These are examples of colonies with large spaces between cell clusters. Note that they are each counted as one colony due to the fact that there are no other clusters of cells in the surrounding area. If cultured for another 7 days, the spaces between cell clusters would be filled by further proliferation.

APPENDIX

PRODUCT NAME	CATALOG #	CYTOKINES					
		SCF	IL-3	GM-CSF	G-CSF	IL-6	EPO
MethoCult® H4034 Optimum	04034	•	•	•	•		•
	04044						
MethoCult® GF H84434‡	84434						
	84444						
MethoCult® H4434 Classic	04434	•	•	•			•
	04444						
MethoCult® H4435 Enriched	04435	•	•	•	•	•	•
	04445						
MethoCult® GF H84435‡	84435						
	84445						
MethoCult® H4035 Optimum without EPO	04035	•	•	•	•		
	04045						
MethoCult® GF H84534‡	84534						
	84544						
MethoCult® H4534 Classic without EPO	04534	•	•	•			
	04544						
MethoCult® H4535 Enriched without EPO	04535	•	•	•	•	•	
	04545						
MethoCult® GF H84535‡	84535						
	84545						

‡ CE marked for *in vitro* diagnostic use; available in the European Union

GLOSSARY OF TERMS USED

ABBREVIATION	DESCRIPTION
BM	Bone Marrow
BFU-E	Burst-Forming Unit-Erythroid
BSA	Bovine Serum Albumin
CB	Cord Blood
CFC	Colony-Forming Cell
CFU	Colony-Forming Unit
CFU-E	Colony-Forming Unit-Erythroid
CFU-G	Colony-Forming Unit-Granulocyte
CFU-GEMM	Colony-Forming Unit-Granulocyte, Erythroid, Macrophage, Megakaryocyte
CFU-GM	Colony-Forming Unit-Granulocyte, Macrophage
CFU-M	Colony-Forming Unit-Macrophage
CFU-Mk	Colony-Forming Unit-Megakaryocyte
CRU	Competitive Repopulating Unit
FBS	Fetal Bovine Serum
HSC	Hematopoietic Stem Cell
LTC-IC	Long-Term Culture-Initiating Cell
rh EPO	Recombinant Human Erythropoietin
rh G-CSF	Recombinant Human Granulocyte Colony-Stimulating Factor
rh GM-CSF	Recombinant Human Granulocyte Macrophage Colony-Stimulating Factor
rh IL-3	Recombinant Human Interleukin-3
rh IL-6	Recombinant Human Interleukin-6
rh SCF	Recombinant Human Stem Cell Factor
MPB	Mobilized Peripheral Blood
PB	Peripheral Blood
SRC	SCID Repopulating Cells

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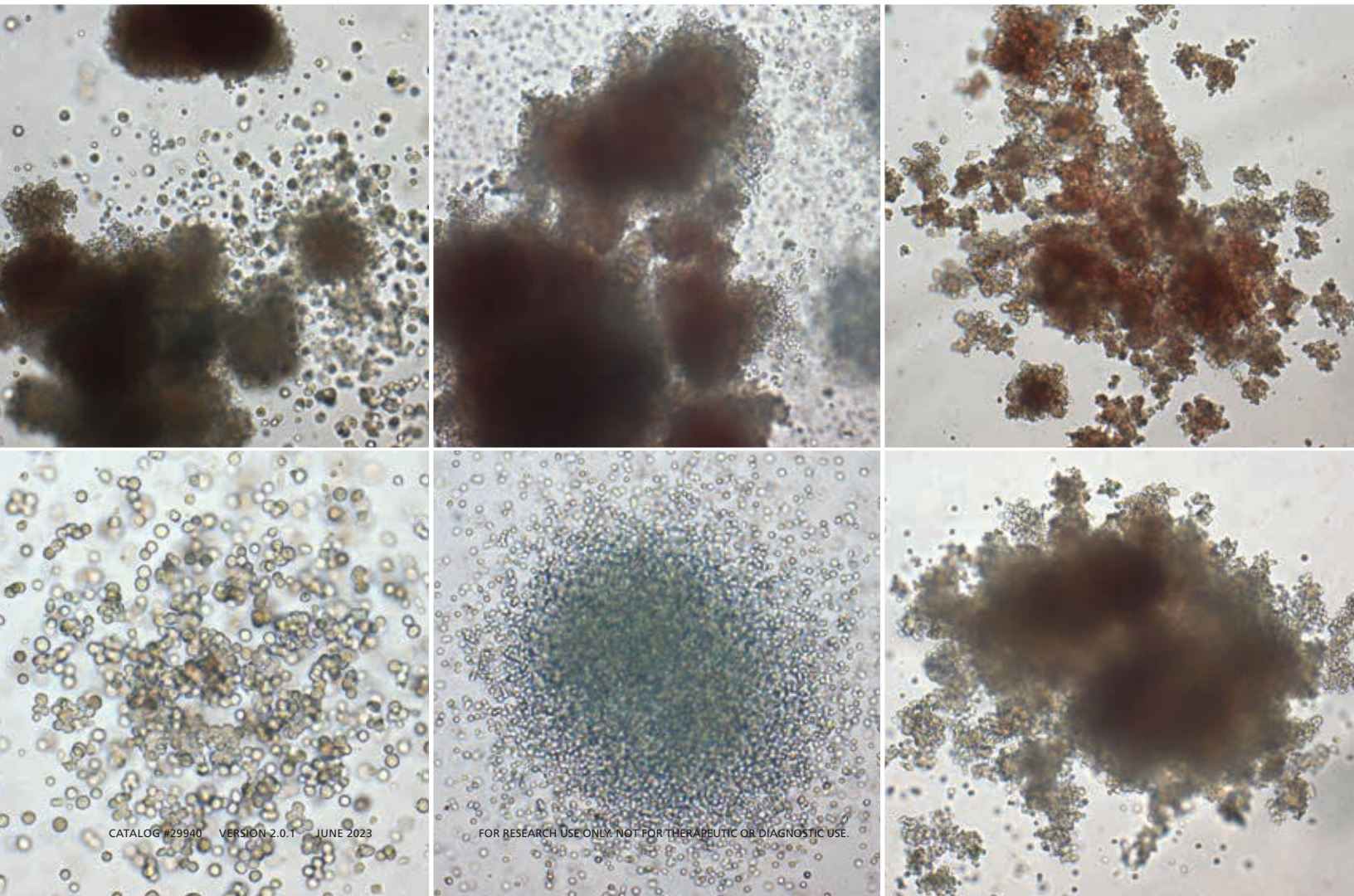
T. +33 (0)4 76 04 75 30 F. +33 (0)4 76 18 99 63 E. INFO.EU@STEMCELL.COM

IN AUSTRALIA TOLL-FREE T. 1 800 060 350 F. +61 (03) 9338 4320 E. INFO.AUS@STEMCELL.COM

IN SINGAPORE T. +(65) 6776 7754 F. +(65) 6776 7114 E. INFO.SG@STEMCELL.COM



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