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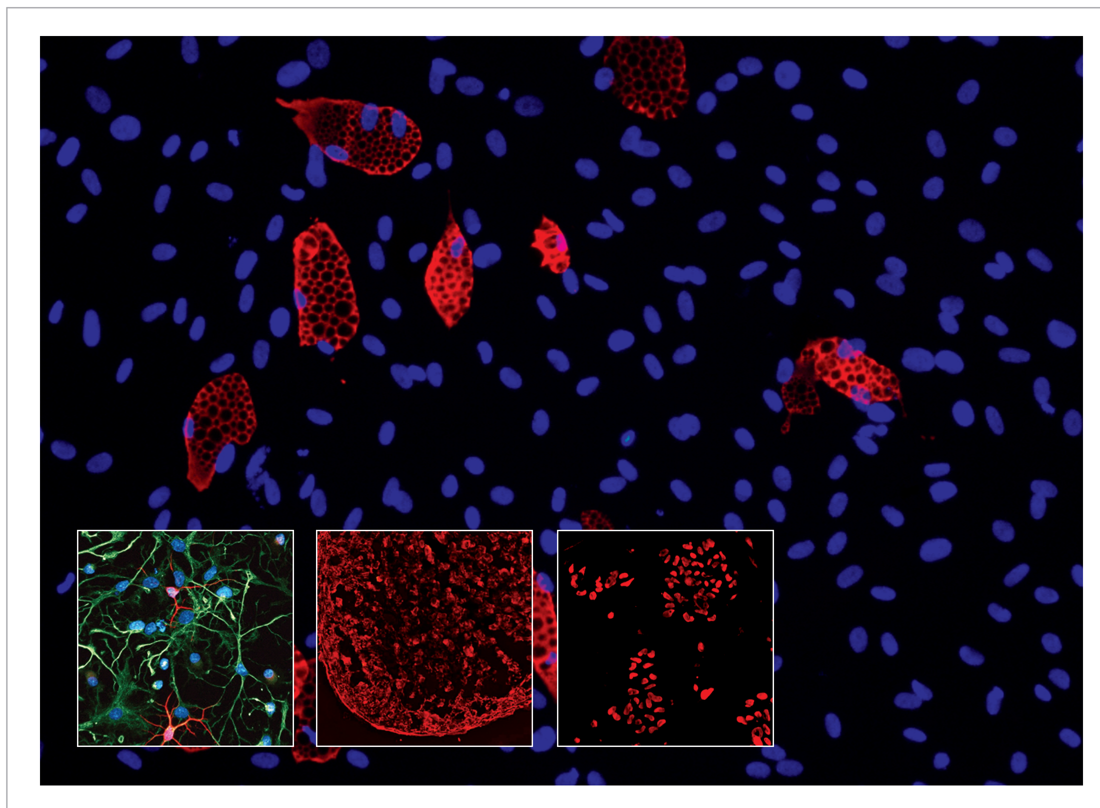
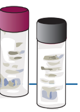
R&D Systems Stem Cell Culture Products & Protocol Guide

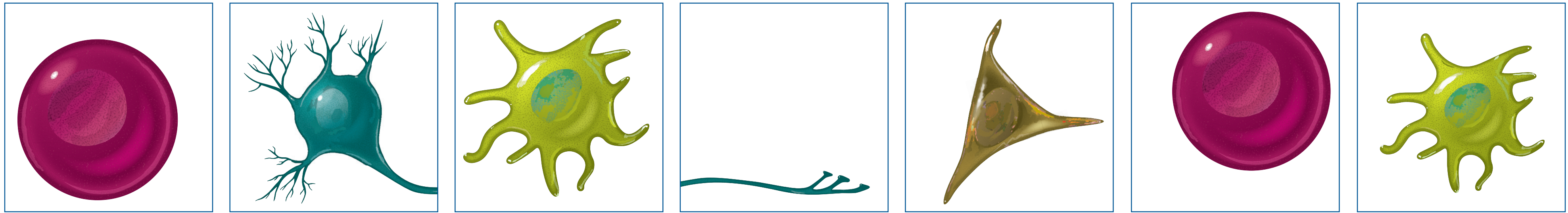
Stem Cell Culture Products & Protocol Guide



Tools for Cell Biology Research™

R&D SYSTEMS®





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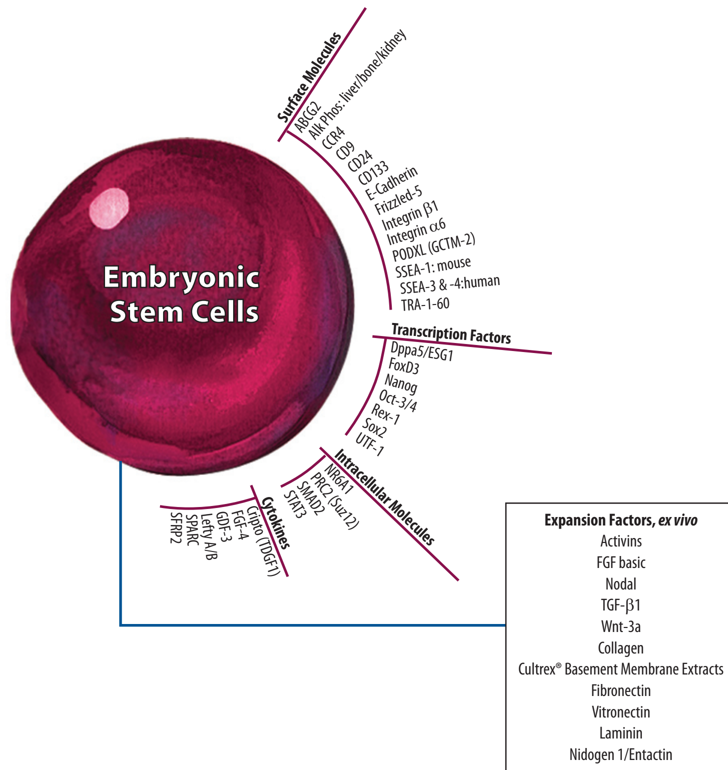


Embryonic stem (ES) cells, which are derived from the inner cell mass of preimplantation embryos, have been recognized as the most pluripotent stem cell population. These cells are capable of unlimited, undifferentiated proliferation *in vitro* and still maintain the capacity for differentiation into a wide variety of somatic tissues. In this capacity ES cells have widespread clinical potential in the treatments of heart disease, diabetes, spinal cord injury, and a variety of neurodegenerative disorders.

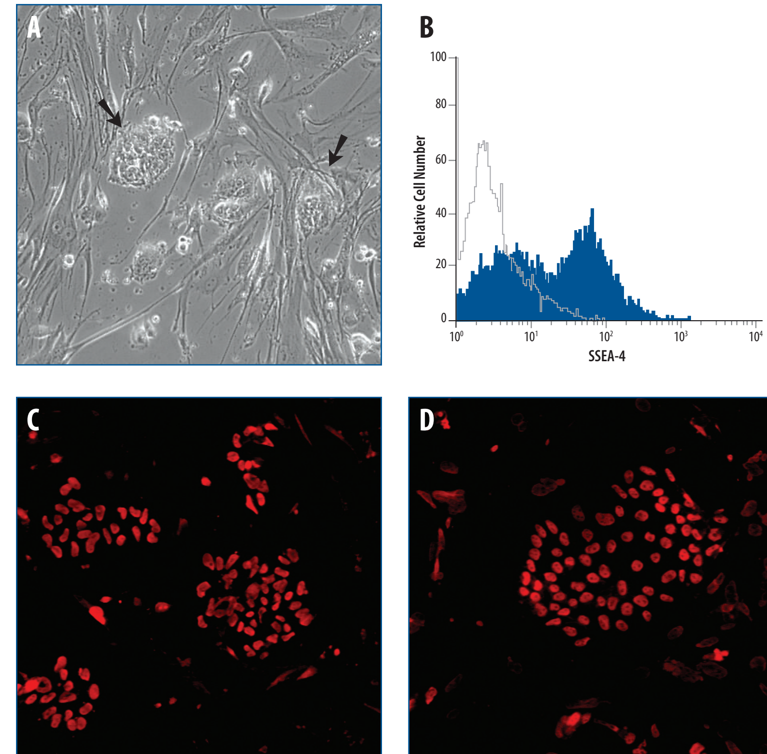
R&D Systems offers a range of products designed to maintain and expand ES cells in culture, as well as monitor their differentiation status.

Feeder Cells

R&D Systems offers cryopreserved irradiated mouse embryonic fibroblasts (iMEFs). These cells are isolated from pathogen-free E13.5 CF1 embryos and irradiated at passage 3 to arrest mitosis. Each order includes 5 vials, enough to prepare ten 10 cm tissue culture plates. Every lot of iMEF feeders is tested for its ability to maintain the expression levels of pluripotency markers as evaluated by immunocytochemistry or flow cytometry (see figure below).

Please see pages 12-13 for a complete protocol using iMEF.

Product	Description	Catalog #	Size
Irradiated Mouse Embryonic Fibroblasts	Tested for the ability to support the expansion of the BG01V human embryonic stem cell line in the undifferentiated state based on the expression of Oct-3/4, Nanog, and SSEA-4.	PSC001	5 Vials



Human Embryonic Stem Cells (Cell Line BG01V) Cultured on iMEFs are Assessed Using Markers of Pluripotency. BG01V colonies (highlighted by arrows) growing in culture on iMEFs **(A)** were analyzed for markers of pluripotency after 3 passages. Cells were evaluated for SSEA-4 expression by flow cytometry **(B)** using PE-conjugated anti-human SSEA-4 (Catalog # FAB1435P; filled histogram) or isotype control antibody (open histogram). BG01V colonies were incubated with anti-human Oct-3/4 (**C**; Catalog # AF1759) or anti-human Nanog (**D**; Catalog # AF1997) followed by staining with NorthernLights™ 557-conjugated anti-goat secondary antibody (Catalog # NL001).

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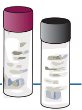
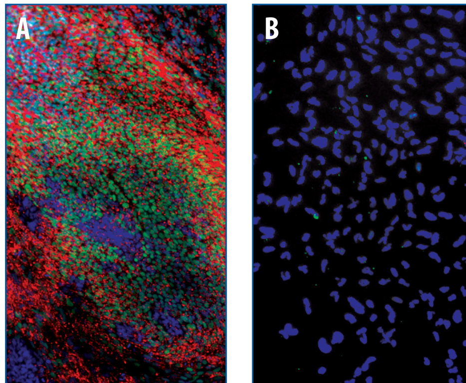


Feeder-Free Culture using Feeder Cell Conditioned Media

Human embryonic stem cells can be maintained and expanded using mouse or human feeder cell conditioned media. R&D Systems offers both Mouse Embryonic Fibroblast Conditioned Medium and Human Feeder Cell Conditioned Medium capable of supporting the growth of ES cells as assessed by the expression of pluripotency markers.

Please see page 14-15 for a complete protocol using conditioned media to grow the BG01V human ES cell line.

Product	Description	Catalog #	Size
Mouse Embryonic Fibroblast Conditioned Media	Tested for its ability to support the expansion of the BG01V human embryonic stem cell line in the undifferentiated state based on the expression of Oct-3/4, Nanog, and SSEA-4.	AR005	100 mL
Human Feeder Cell Conditioned Media	Tested for its ability to support the expansion of the BG01V human embryonic stem cell line in the undifferentiated state based on the expression of Oct-3/4, Nanog, and SSEA-4.	AR007	100 mL

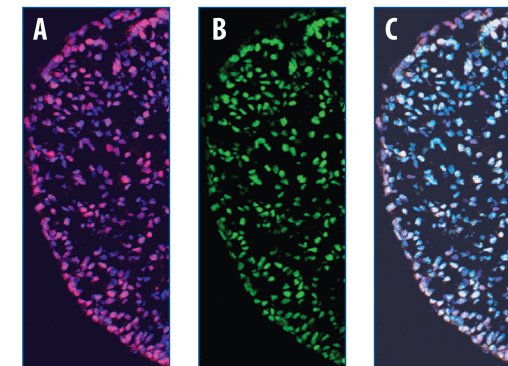


SSEA-4 Expression in Embryonic Stem Cells. Human embryonic stem cells were cultured with recombinant human FGF basic (Catalog # 233-FB) in the presence (**A**) or absence (**B**) of Mouse Embryonic Fibroblast (MEF)-conditioned medium (Catalog # AR005). SSEA-4 and Oct-3/4 were detected using anti-human SSEA-4 monoclonal antibody (Catalog # MAB1435) and anti-human Oct-3/4 polyclonal antibody (Catalog # AF1759). Cells were stained with Alexa Fluor® 568-conjugated anti-mouse secondary antibody (SSEA-4; red) and Alexa Fluor 488-conjugated anti-goat secondary antibody (Oct-3/4; green). Cells were counterstained with DAPI (blue). Image courtesy of Dr. Frank Soldner of the National Institutes of Health.

Embryonic Stem Cell Marker Panels

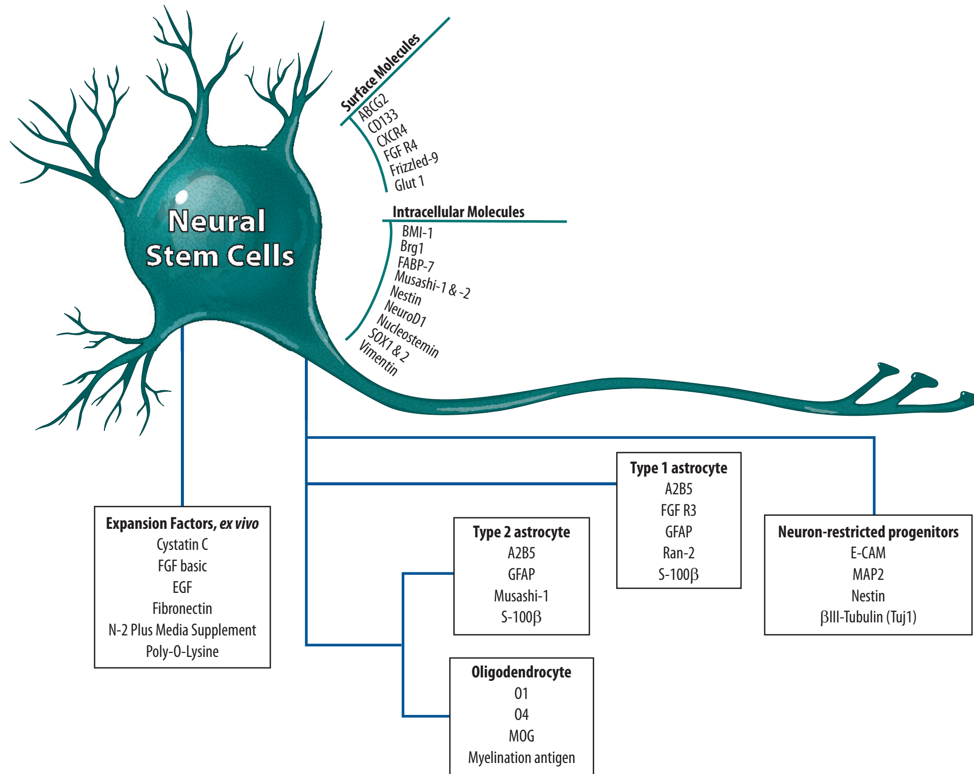
R&D Systems offers kits for assessing the differentiation status of human ES cells. Multiple kits are offered using either antibody or primer-based identification techniques. These kits offer an economical alternative to buying separate reagents.

Product	Kit Contents (1 Kit)	Catalog #
Human Embryonic Stem Cell Marker Antibody Panel	Anti-Alkaline phosphatase, Anti-Nanog, Anti-Oct-3/4, Anti-SSEA-1, Anti-SSEA-4.	SC008
Human Embryonic Stem Cell Marker Antibody Panel Plus	Anti-CD9, Anti-SOX2, Anti-Nanog, Anti-Oct-3/4, Anti-Podocalyxin, Anti-SSEA-4, Anti-E-Cadherin, Anti-SSEA-1.	SC009
Human Pluripotent Stem Cell Assessment Primer Pair Panel	Primer pairs for ESG1/DPPA5, Nanog, Oct-3/4, TP63/TP73L, Otx2, SOX2, AFP, GATA-4, Nestin, Brachyury, PDX-1, SOX17, HNF-3β, GAPDH, and Stella.	SC012
Mouse/Rat Pluripotent Stem Cell Assessment Primer Pair Panel	Primer pairs for ESG1/DPPA5, Nanog, Oct-3/4, TP63/TP73L, Otx2, SOX2, AFP, GATA-4, Nestin, Brachyury, PDX-1, SOX17, HNF-3β, GAPDH, and Stella.	SC015



An Embryoid Body Derived from Human Embryonic Stem Cells Stained for Pluripotency Markers. Cells were stained using antibodies from the Human Embryonic Stem Cell Marker Antibody Panel (Catalog # SC008) for Nanog (red; **A**) and the nuclei counterstained with DAPI (blue; **A**) or for Oct-3/4 (green; **B**). Merge of images in (**A**) and (**B**) shows the overlap of the 3 fluorochromes (white; **C**). Images courtesy of Dr. Ronald McKay, NINDS.

Alexa Fluor is a registered trademark of Invitrogen.



Neural stem cells (NSCs) are functionally defined by their capacity to self renew and their ability to differentiate into multiple specialized progenitor cells, which can commit to further maturation along specific lineages including astrocytes, neurons, and oligodendrocytes. *Ex vivo* expanded neural stem cells serve as excellent tools for researchers studying neural development and neurological disorders.

R&D Systems offers media and NSC expansion kits and functional identification kits to direct stem cells along neuronal lineages.

Recent Reference using Neural Stem Cell products from R&D Systems

Pierret, C. *et al.* (2007) Elements of a neural stem cell niche derived from embryonic stem cells. Stem Cells and Development **16**:1017.

Product: Neural Stem Cell Expansion Kit – Neurosphere
Catalog # SC003

Sample type: Mouse primary neurospheres from ES Cells induced with retinoic acid

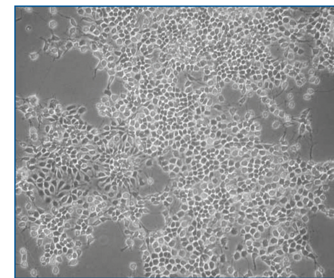
Application: Stem Cell Expansion

Serum-Free Medium & Neural Stem Cell Expansion Kits

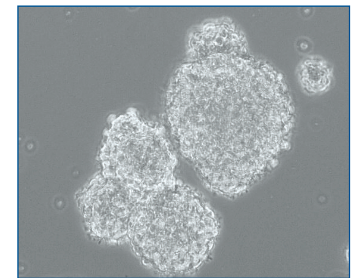
StemXVivo™ Serum-Free NSC Base Media is a serum-free formulation for the expansion and differentiation of neural stem and progenitor cells. All the components have been selected and optimized for culturing human, mouse, or rat cells. This media must be supplemented with cytokines/growth factors for the desired cell culture application. The cytokine/growth factor combinations used depends upon the experimental design of each researcher. The Neural Stem Cell (NSC) Expansion Kits contain specially formulated reagents to grow NSCs *in vitro* as monolayer cultures on an adhesive substrate or as free-floating "neurospheres." Cells grown using Neural Stem Cell Expansion Kits are Nestin-positive and proliferate in culture, while retaining the potential to differentiate into neurons, oligodendrocytes, and astrocytes.

Please see pages 16-19 for a protocol using StemXVivo Serum-Free NSC Base Media for the expansion of rat cortical stem cells.

Product	Description	Catalog #
Human/Mouse/Rat StemXVivo Serum-Free NSC Base Media*	StemXVivo Serum-free NSC Base Media is formulated for expansion and differentiation of human and rodent neural stem and progenitor cells. A volume of 250 mL is provided.	CCM002
Human/Mouse/Rat Neural Stem Cell Expansion Kit - Monolayer Plus*	Contains EGF, Fibronectin, FGF basic, N-2 Plus Media Supplement for neural stem cell expansion in monolayer form. Sufficient for 500 mL of medium.	SC005
Human/Mouse/Rat Neural Stem Cell Expansion Kit - Neurosphere*	Contains EGF, FGF basic, N-2 Plus Media Supplement for neural stem cell expansion in neurosphere form. Sufficient for 500 mL of medium.	SC003
N-2 Plus Media Supplement	A modification of Bottenstein's formulation, providing optimal conditions for neural stem cell expansion. Sufficient for 500 mL of medium.	AR003



Neural Stem Cell Monolayer. Expanded rat NSC in the monolayer system on day 3 after the second passage using the Neural Stem Cell Expansion Kit - Monolayer Plus (Catalog # SC005).



Neural Stem Cell Neurosphere. Expanded rat NSC in the neurosphere system on day 4 after the fifth passage using the Neural Stem Cell Expansion Kit -Neurosphere (Catalog # SC003).

* Sold under license from StemCells California, Inc. U.S. Patent Nos. 5,750,376; 5,851,832; 5,980,885; 5,968,829; 5,981,165; 6,071,889; 6,093,531; 6,103,530; 6,165,783; 6,238,922.

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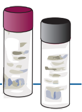


Neural Differentiation Kits

Kits are available for the serum-free differentiation of embryonic stem (ES) cells into dopaminergic neurons or oligodendrocytes. Each kit contains specially formulated media supplements and a growth factor panel designed to direct the differentiation to the specific neural lineage. These kits contain sufficient reagents for the induction of approximately 3 x 10^7 ES cells.

Product	Kit Contents (1 Kit)	Catalog #
Human/Mouse Dopaminergic Neuron Differentiation Kit	ITS Media Supplement, N-2 Plus Media Supplement, FGF basic, FGF-8b, Fibronectin, Shh-N.	SC001B
Mouse Oligodendrocyte Differentiation Kit	ITS Media Supplement, N-2 Plus Media Supplement, EGF, FGF basic, Fibronectin, PDGF-AA.	SC004

Neural Stem Cell Functional Identification Kits

Functional Identification kits are designed for *in vitro* identification of human or mouse/rat neural stem cells based on their ability to differentiate into multiple neural lineages. These kits contain specially formulated media supplements and a panel of antibodies for the differentiation and identification of neural precursors, astrocytes, neurons, and oligodendrocytes.

Product	Kit Contents (1 Kit)	Catalog #
Human Neural Stem Cell Functional Identification Kit*	Neural Stem Cell Maintenance Supplement, Neural Differentiation Supplement, Fibronectin, Anti-Neuron-Specific β-III Tubulin, Anti-GFAP, Anti-Nestin, Anti-04.	SC011
Mouse/Rat Neural Stem Cell Functional Identification Kit*	Neural Stem Cell Maintenance Supplement, Neural Differentiation Supplement, Fibronectin, Anti-Neuron-Specific β-III Tubulin, Anti-GFAP, Anti-Nestin, Anti-04.	SC013

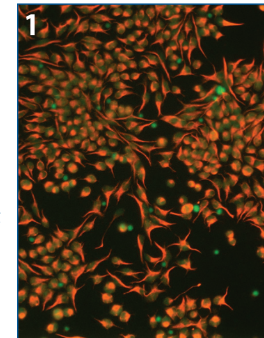
Neural Toxicity Assay

The Neural Toxicity Assay Kit is a 96 well plate kit designed as an *in vitro* screening tool to determine how bioactive agents, such as toxins, drugs, and growth factors, influence neural precursor differentiation and proliferation.

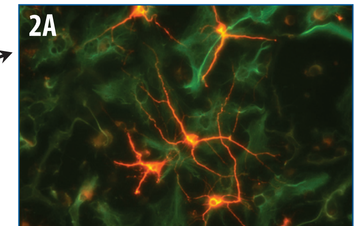
NEW Neural Toxicity Assay Kit	Neural Stem Cell Maintenance Supplement, Neural Differentiation Supplement, Fibronectin, HRP-conjugated Anti-Neuron-Specific β-III Tubulin, Resazurin, Buffers, Substrates, and Diluents.	SC014

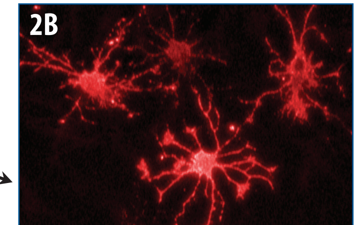
NEURAL STEM CELLS

PLATED ON POLY-L-ORNITHINE/ FIBRONECTIN-COATED PLATES FOR 48 HRS in medium containing maintenance supplement



CULTURED FOR 7-10 DAYS in medium containing differentiation supplement & stained with lineage-specific antibodies





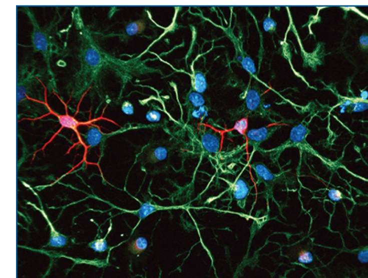
Expansion, Differentiation, and Identification of Neural Cell Populations using the Mouse/Rat NSC Functional Identification Kit. Nestin detected in rat cortical stem cells (Catalog # NSC001) with anti-rat Nestin (Figure 1; red). Three distinct cell types in differentiated neural stem cells were identified using the Mouse/Rat Neural Stem Cell Functional Identification Kit (Catalog # SC013). Neurons were detected using anti-neuron-specific β-III Tubulin (Figure **2A**; red); astrocytes using anti-human GFAP (Figure **2A**; green); and oligodendrocytes using anti-04 (Figure **2B**; red). Primary antibodies are included in the Neural Stem Cell Functional Identification Kit.

Primary Rat Cortical Stem Cells

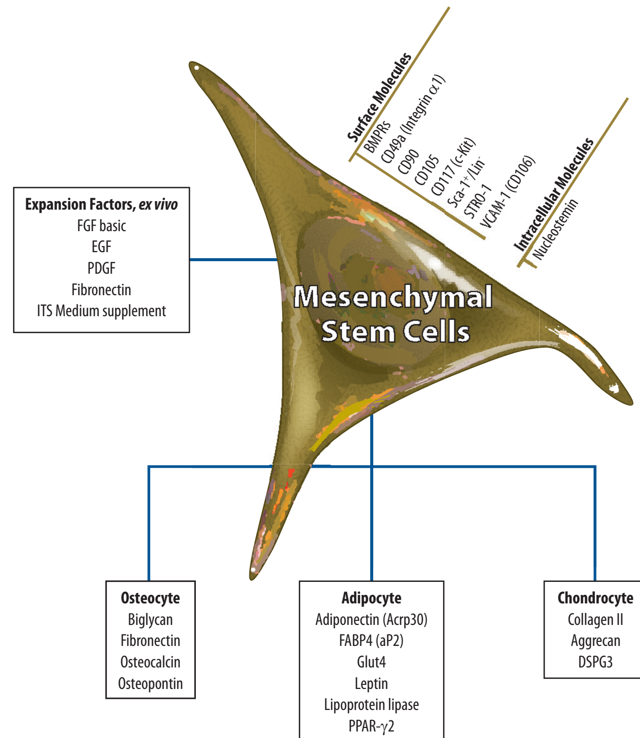
R&D Systems offers ready-to-use primary cortical stem cells isolated from E14.5 Sprague-Dawley rats. Every lot is validated for high levels of Nestin expression and the capacity for multi-lineage differentiation (astrocytes, neurons, and oligodendrocytes). Depending on your research needs, Rat Cortical Stem Cells can be optimally expanded as monolayers or neurospheres.

Please see pages 16-19 for a protocol using StemXVivo Serum-Free NSC Base Media for the expansion of rat cortical stem cells.

Product	Description	Catalog #	Size
Rat Cortical Stem Cells	Tested for its ability to proliferate using monolayer and neurosphere systems. Each vial contains 3 x 10^6 cells.	NSC001	1 vial



Differentiating Rat Cortical Stem Cells (Catalog # NSC001). Neural progenitors were labeled with anti-rat Nestin polyclonal antibody (Catalog # AF2736) and stained with anti-goat NorthernLights™-493 secondary antibody (Catalog # NL003; green). Differentiated neurons were labeled with neuron-specific anti-β-III Tubulin monoclonal antibody (TuJ-1; Catalog # MAB1195) and stained using anti-mouse NorthernLights-557 secondary antibody (Catalog # NL007; red). Nuclei were counterstained with DAPI (blue).



Mesenchymal stem cells (MSCs) are bone marrow-derived, self-renewing and multipotent progenitors. MSCs have been shown to be capable of differentiating into multiple cell types including adipocytes, chondrocytes, osteocytes, and cardiomyocytes.

R&D Systems offers kits for the maintenance and expansion of MSCs, as well as kits and reagents designed to promote and identify the progression of MSCs into osteogenic, adipogenic, and chondrogenic lineages.

Media & Supplements for Mesenchymal Stem Cell Expansion & Differentiation

StemXVivo MSC Expansion Media is a complete media for the expansion of mesenchymal stem cells (MSCs). All the components have been selected and optimized for culturing human and mouse MSCs. R&D Systems also offers base media and supplements specially formulated to direct MSC differentiation into adipogenic, chondrogenic, and osteogenic lineages.

Please see pages 20-21, 22-23, and 24-25 for protocols using StemXVivo Media and supplements for the differentiation of MSCs into osteogenic, adipogenic, and chondrogenic lineages, respectively. A protocol describing the expansion of MSCs can be found at **www.RnDSystems.com/go/MSCExpansionProtocol.**

Product	Description	Catalog #	Size
Human/Mouse StemXVivo Mesenchymal Stem Cell Expansion Media	StemXVivo MSC Expansion Media is a complete media for the expansion of MSCs.	CCM004	250 mL
Human/Mouse StemXVivo Chondrogenic Base Media	StemXVivo Chondrogenic Base Media is a base media for the differentiation of MSCs into chondrocytes. For use with StemXVivo Chondrogenic Supplement.	CCM005	50 mL
Human/Mouse StemXVivo Osteogenic/ Adipogenic Base Media	StemXVivo Osteogenic/Adipogenic Base Media can be used with the appropriate supplement for the differentiation of MSCs into osteocytes or adipocytes. For use with Osteogenic or Adipogenic Supplements.	CCM007	250 mL
Human/Mouse StemXVivo Chondrogenic Supplement	StemXVivo Chondrogenic Supplement is a media supplement for the differentiation of MSCs into chondrocytes. For use with Chondrogenic Base Media.	CCM006	0.5 mL
Human StemXVivo Osteogenic Supplement	StemXVivo Osteogenic Supplement is a media supplement for the differentiation of human MSCs into osteocytes. For use with Osteogenic/Adipogenic Base Media.	CCM008	12.5 mL
Mouse StemXVivo Osteogenic Supplement	StemXVivo Osteogenic Supplement is a media supplement for the differentiation of mouse MSCs into osteocytes. For use with Osteogenic/Adipogenic Base Media.	CCM009	12.5 mL
Human/Mouse StemXVivo Adipogenic Supplement	The StemXVivo Adipogenic Supplement is a media supplement for the differentiation of human or mouse MSCs into adipocytes. For use with Osteogenic/Adipogenic Base Media.	CCM011	3.5 mL

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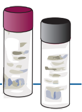
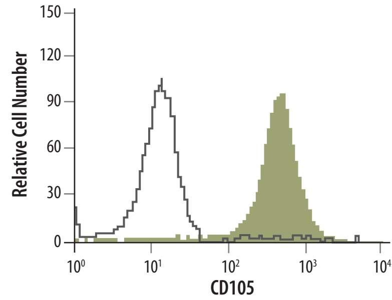
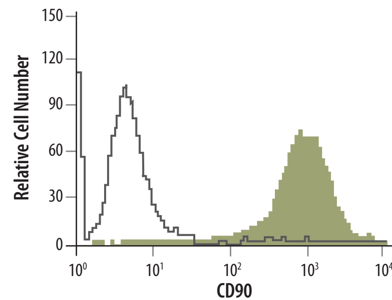
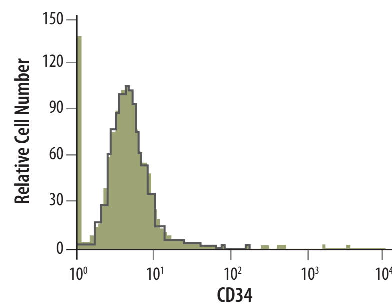








Phenotypic Analysis of Human MSCs by Flow Cytometry. MSCs were expanded using StemXVivo MSC Expansion Media (Catalog # CCM004). Green histograms indicate cells stained with markers of undifferentiated MSCs including, anti-CD105 (Catalog # FAB10971P) or CD90 (Catalog # FAB2067P). The open histograms show isotype-matched control staining. MSCs lack expression of CD34.

Mesenchymal Stem Cell Functional Identification Kits

Mesenchymal Stem Cell Functional Identification Kits contain specially formulated Adipogenesis, Chondrogenesis, and Osteogenesis Media Supplements that can be used to effectively differentiate MSCs or other candidate cell populations, into adipogenic, chondrogenic, or osteogenic lineages. A panel of antibodies are included for identification of the mature phenotypes of each lineage.

Product	Kit Contents (1 Kit)	Catalog #
Human Mesenchymal Stem Cell Functional Identification Kit	Adipogenic Supplement, Chondrogenic Supplement, ITS Supplement, Osteogenic Supplement, Anti-Aggrecan, Anti-Osteocalcin, Anti-FABP-4.	SC006
NEW Mouse Mesenchymal Stem Cell Functional Identification Kit	Adipogenic Supplement, Chondrogenic Supplement, ITS Supplement, Osteogenic Supplement, Anti-Collagen II, Anti-Osteopontin, Anti-FABP-4.	SC010

HUMAN MESENCHYMAL STEM CELL

CULTURE WITH CCM004

$CD34^-$, $CD90^+$, $CD105^+$

DIFFERENTIATION INDUCED WITH CCM007 & CCM008, OR SC006

DIFFERENTIATION INDUCED WITH CCM007 & CCM011, OR SC006

DIFFERENTIATION INDUCED WITH CCM005 & CCM006, OR SC006

Osteoblast

Pre-Adipocyte

Chondroblast

Cardiomyoblast, Myoblast

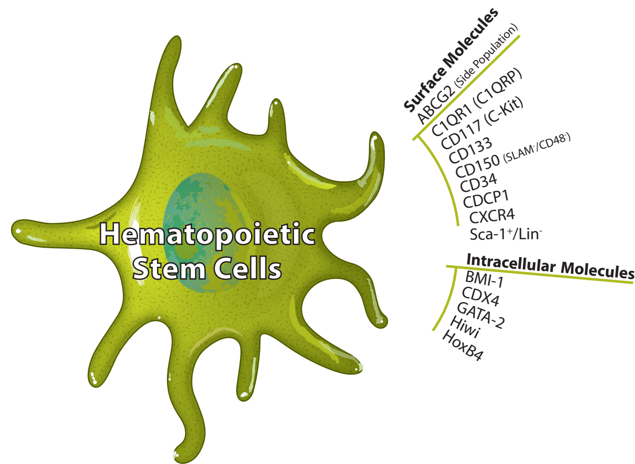
Osteocytes

Adipocytes

Chondrocytes

Cardiac Muscle
Skeletal Muscle
Smooth Muscle

Guide for the expansion and differentiation of human MSCs along the osteocyte, adipocyte, and chondrocyte lineages. Human MSCs are cultured and expanded in StemXVivo Human/ Mouse Mesenchymal Stem Cell Expansion Media, and osteogenesis, adipogenesis, and chondrogenesis can be induced with the indicated media and reagents.



The definitive hematopoietic system is made up of all adult blood cell types including megakaryocytes, erythrocytes and cells of the myeloid and lymphoid lineages. All of these cells are derived from multipotent hematopoietic stem cells (HSCs) through a succession of precursors with progressively limited potential. Hematopoietic stem cells (HSCs) are tissue-specific stem cells that exhibit remarkable self-renewal capacity and are responsible for the life-long maintenance of hematopoietic system. HSCs are rare cells that reside in adult bone marrow where hematopoiesis is continuously taking place, and they can also be found in cord blood, fetal liver, adult spleen and peripheral blood.

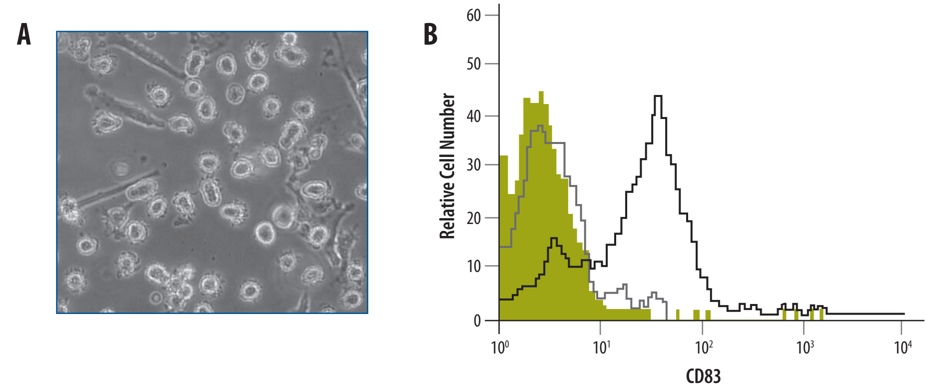
R&D Systems offers several products for assessing hematopoietic lineage cells including serum-free media, lineage depletion antibodies and kits, and reagents for performing colony forming cell (CFC) assays.

Serum-Free Media for Hematopoietic Cell Culture

The StemXVivo Serum-Free Dendritic Cell Base Media and T Cell Base Media are formulated and optimized for the culture of human dendritic cells and T cells, respectively. These media must be supplemented with cytokines/growth factors for the desired cell culture application. The cytokine/growth factor combinations used depends upon the experimental design of each researcher.

For a protocol highlighting the use of the StemXVivo Human Dendritic Cell Base Media, please visit our website at www.RnDSystems.com/go/DCProtocol. For a protocol highlighting the use of StemXVivo Serum-free T Cell Base Media, please visit our website at www.RnDSystems.com/go/ExVivoTCellProtocol

Product;	Description	Catalog #	Size
Human StemXVivo Serum-free Dendritic Cell Base Media	StemXVivo Serum-free Dendritic Cell Base Media is formulated and optimized for the culture and differentiation of human dendritic cells.	CCM003	250 mL
Human StemXVivo Serum-free T Cell Base Media	StemXVivo Serum-free T Cell Cell Base Media is formulated and optimized for the culture and differentiation of human T lymphocytes.	CCM010	250 mL



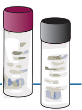
Dendritic Cell Culture and Maturation from CD14+ Monocytes. A: LPS-matured monocyte-derived dendritic cells were obtained after a 9-day culture of CD14-enriched monocytes in StemXVivo Serum-Free Dendritic Cell Base Media (Catalog # CCM003) supplemented with GM-CSF (Catalog # 215-GM) and IL-4 (Catalog # 204-IL). **B:** Dendritic cells cultured for 7 days were stained with the mature dendritic cell marker CD83 before (gray line) and after a two day treatment with LPS (black line). Isotype-matched control staining is also shown (filled histogram).

Supplements for Hematopoietic Stem Cell Expansion

R&D Systems offers the Mouse Hematopoietic Cytokine Panel containing supplements that support the expansion of hematopoietic stem cells. This panel provides an economical alternative to purchasing each protein separately.

Product;	Proteins Included	Catalog #	Size
Mouse Hematopoietic Stem Cell Expansion Cytokine Panel	Flt-3 Ligand, Tpo, SCF	SMPK9	100 µg of each

For research use only. Not for use in diagnostic procedures.



Hematopoietic Lineage Depletion

Mouse Hematopoietic Lineage Depletion Antibodies

Anti-mouse monoclonal antibodies can be used to efficiently bind lineage-committed bone marrow-derived cells. Antibodies included in the lineage depletion panel are optimized and contain sufficient amounts to process 1 x 10^9 bone marrow-derived cells. They can conveniently be used in conjunction with magnetic particle separation systems or flow cytometric cell sorting for the enrichment of uncommitted mesenchymal or hematopoietic stem cells.

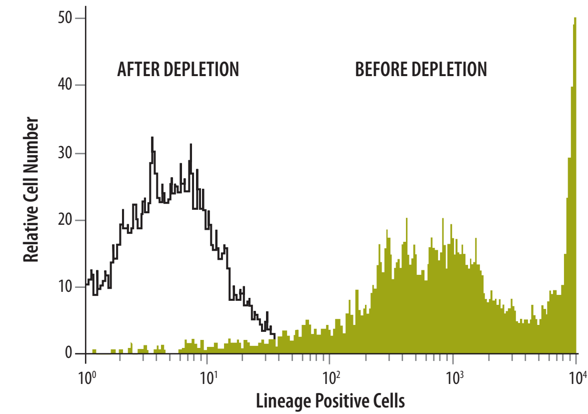
Analyte	Type	Clone #	Catalog #
Mouse B220/CD45R	Monoclonal Rat IgG_{2B}	RA3-6B2	MLDP7
Mouse CD3	Monoclonal Rat IgG_{2B}	172A2	MLDP1
Mouse CD4	Monoclonal Rat IgG_{2B}	GK1.5	MLDP2
Mouse CD5	Monoclonal Rat IgG_{2B}	53-7.3	MLDP3
Mouse CD8α	Monoclonal Rat IgG_{2B}	53-6.7	MLDP4
Mouse Integrin αM/CD Hb	Monoclonal Rat IgG_{2B}	M1/70	MLDP5
Mouse Gr-1/Ly-6G	Monoclonal Rat IgG_{2B}	RB6-8C5	MLDP6
Mouse TER-119	Monoclonal Rat IgG_{2B}	TER-119	MLDP8

Mouse Hematopoietic Cell Lineage Depletion Kit

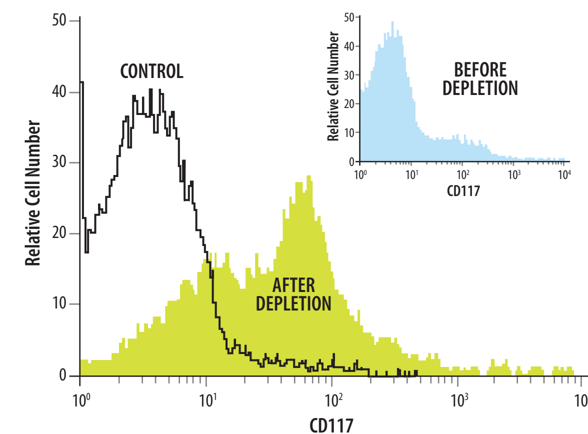
The Mouse Hematopoietic Cell Lineage Depletion Kit is designed to be used in conjunction with R&D Systems MagCellect™ cell enrichment system. Lineage committed cells (lineage positive) targeted for depletion include T cells, B cells, NK cells, monocytes/macrophages, granulocytes, and erythrocytes. The kit comes with reagents to process 1 x 10^9 cells.

A protocol for using the Mouse Hematopoietic Lineage Depletion Kit can be found on pages 26-27.

Product	Kit Contents (1 Kit)	Catalog #
Mouse Hematopoietic Cell Lineage Depletion Kit	Lineage depletion antibodies, MagCellect Ferrofluid, Blocking buffer, Wash buffer.	MAGM209



Hematopoietic Lineage Depletion. Lineage marker reactivity on BALB/c bone marrow (BM) cells processed with the MagCellect Mouse Hematopoietic Cell Lineage Depletion Kit (Catalog # MAGM209). Histograms show reactivity of BM cells labeled with the cocktail of biotinylated antibodies included in the kit both before (green histogram) and after (open histogram) magnetic depletion. Lineage marker reactivity was detected using Streptavidin-PE.



Enrichment of CD117⁺ Cells. CD117 (c-kit) staining of bone marrow lineage negative cells isolated from BALB/c mice using the MagCellect Mouse Hematopoietic Cell Lineage Depletion Kit (Catalog # MAGM209). Histograms reflect reactivity of all viable cells with anti-CD117-PE (Catalog # FAB1356P; light green) or a matched isotype control antibody (open histogram). Inset shows CD117 staining of cells before depletion.

Methylcellulose-based Reagents for Colony Forming Cell Assays

The colony forming cell (CFC) assay, also known as the clonal culture assay, is the standard *in vitro* assay for quantitation of clonogenic progenitors present in human, murine, and primate bone marrow, umbilical cord blood, peripheral blood, and G-CSF mobilized peripheral blood. This assay relies on the ability of hematopoietic progenitors to proliferate and differentiate into distinct colonies in a semi-solid media in response to growth factor stimulation. The unique morphology of the resulting colonies enables enumeration and characterization of the progenitors. Since methylcellulose has replaced agar as the immobilizing agent used in the CFC assay, this assay is also known as the methylcellulose assay. Hematopoietic colony-forming assays may be used to evaluate potential toxic effects of new compounds and to determine maximum tolerated doses (MTD) and inhibitory concentration values (IC_{50}).

Please see pages 28-31 for a protocol describing the use of methylcellulose-based reagents for colony forming cell assays.

Product	Description	Catalog #	Size
Methylcellulose Stock Solution*	Contains 2.8% methylcellulose in Iscove's MDM, and can be used in both human and murine hematopoietic stem cell research.	HSC001*	100 mL
Human Methylcellulose Base Media*	Contains all of the basic supplements with the exception of the cytokines required to perform CFC assays for hematopoietic stem cell research.	HSC002*	90 mL
Human Methylcellulose Complete Media	A specifically formulated media supplemented with recombinant human cytokines (GM-CSF, IL-3, SCF, and Epo) for routine assay of the human clonogenic hematopoietic progenitors from human bone marrow, peripheral blood, cord blood, and leukapheresis products. For the enumeration of CFU-E, BFU-E, CFU-G, CFU-M, CFU-GM, and CFU-GEMM.	HSC003	100 mL
Human Methylcellulose Complete Media *without Epo*	A specifically formulated media supplemented with recombinant human cytokines (GM-CSF, IL-3, and SCF) for routine assay of human clonogenic hematopoietic progenitors from human bone marrow, peripheral blood, cord blood, and leukapheresis products. For the enumeration of CFU-G, CFU-GM, and CFU-M.	HSC004	100 mL
Human Methylcellulose Enriched Media	A specifically formulated media supplemented with recombinant human cytokines (GM-CSF, G-CSF, IL-3, IL-6, Epo and SCF) for routine assay of purified human clonogenic hematopoietic progenitors enriched from human bone marrow, peripheral blood, cord blood, leukapheresis products, and purified $CD34^+$ cells. For the enumeration of BFU-E, CFU-E, CFU-GM, CFU-G, CFU-M, and CFU-GEMM.	HSC005	100 mL

*HSC001 and HSC002 do not contain any cytokines and will not support colony growth unless conditioned media, cytokines, or other culture supplements are added.

Recent references using Methylcellulose-based products from R&D Systems

Doepfner, K.T. *et al.* (2007) Autocrine insulin-like growth factor-I signaling promotes growth and survival of human acute myeloid leukemia cells via the phosphoinositide 3-kinase/Akt pathway. Leukemia **21**:1921.

Product: Human Methylcellulose Complete Media without Epo, Catalog # HSC004

Sample type: Human primary bone marrow $CD34^+$ cells

Application: CFC Assay

Wojciechowski, J.C. *et al.* (2008) Capture and enrichment of CD34-positive haematopoietic stem and progenitor cells from blood circulation using P-selectin in an implantable device. Brit. J. Haem. **140**:673.

Product: Human Methylcellulose Enriched Media, Catalog # HSC005

Sample type: Rat hematopoietic stem and progenitor cells extracted from lumen of implanted and P-Selectin-coated capture shunts

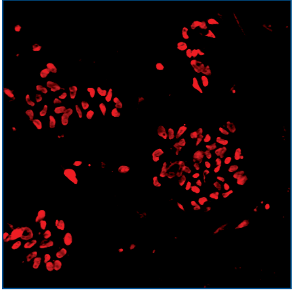
Application: CFC Assay

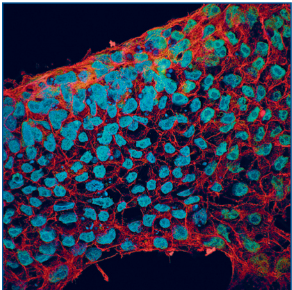
Additional Selected Stem Cell Culture-related Products

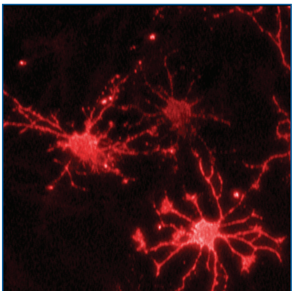
Please see our website at www.RnDSystems.com/go/StemCell for a complete product listing.

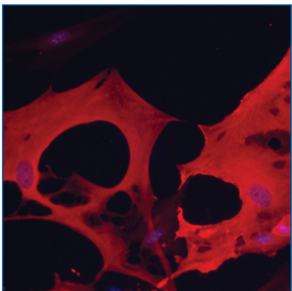
Product;	Description	Catalog #	Size
Recombinant Human FGF basic *Tissue Culture Grade*	FGF basic is required for the expansion of undifferentiated stem cells including embryonic stem cells, and neural stem cells. FGF basic tissue culture grade is tested for its ability to support the expansion of rat cortical neural stem cells and offers an excellent economical alternative for those using large quantities of FGF-basic.	4114-TC-01M	1 mg
Human Holo-transferrin	Holo-transferrin is a critical component frequently used to support hematopoietic progenitor expansion in serum-free culture.	2914-HT-001G 2914-HT-100MG	1 g 100 mg
Poly-L-Lysine	Poly-L-Lysine, a highly positively charged amino acid chain, is commonly used as a coating agent to promote cell adhesion in culture. This solution is provided ready to use at 0.01% and contains polymers in the 70,000-150,000 kDa range.	3438-100-01	100 mL
Cultrex® Basement Membrane Extract Reduced Growth Factor	A soluble basement membrane extract obtained from the murine Englebreth-Holm Swarm (EHS) tumor. Major components include Laminin, Collagen IV, Entactin, and Heparan Sulfate Proteoglycan. Other formulations of Basement Membrane Extract are also available. Please see our website for details.	3433-005-01 3433-010-01	5 mL 10 mL

Cultrex is a registered trademark of Trevigen Inc.

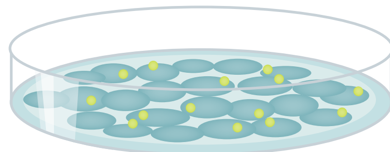


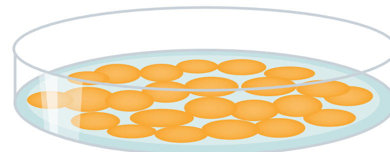


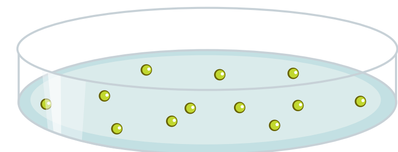


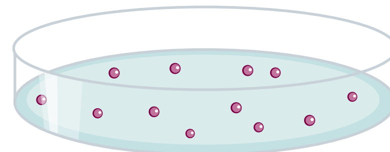


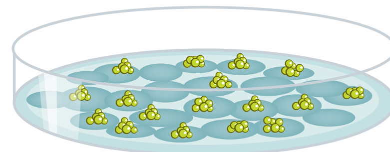
Stem Cell Protocols

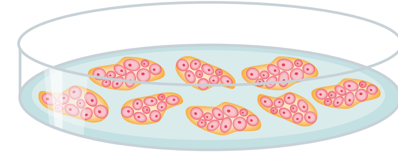












Protocol A: Culturing BG01V Human Embryonic Stem Cells on Irradiated Mouse Embryonic Fibroblasts (iMEF)

Human embryonic stem (hES) cells can be maintained on a layer of mitotically inactivated feeder cells such as irradiated mouse embryonic fibroblasts (iMEF, Catalog # PSC001). The protocol below has been used with the BG01V line of hES cells.

Please note that other hES cell lines may require modifications of this protocol. Optimal culture conditions must be determined by the investigator for each hES line. Please read the protocol in its entirety before starting.

SUPPLIES REQUIRED

REAGENTS

- Fetal bovine serum
- Knockout™ serum replacer (Invitrogen, Catalog # 10828)
- Non-Essential Amino Acids (100x)
- L-Glutamine (200 mM)
- Penicillin/Streptomycin (100x)
- β-mercaptoethanol
- DMEM/F12
- High glucose DMEM
- Recombinant human FGF basic (R&D Systems, Catalog # 233-FB; or Tissue Culture Grade, Catalog # 4114-TC)
- Accutase (Innovative Cell Technologies, Catalog # AT104 or equivalent)
- 0.1% w/v solution of gelatin in sterile deionized H_2O

MATERIALS

- BG01V human embryonic stem cells (ATCC # SCRC-2002)
- iMEF Feeder Cells (R&D Systems, Catalog # PSC001)
- Tissue culture plates (60 mm; the protocol can be adapted for other plate sizes)
- 15 mL centrifuge tubes
- 0.2 µm sterile filter unit
- Pipettes and pipette tips

EQUIPMENT

- 37° C, 5% CO_2 incubator
- Centrifuge (low speed clinical or equivalent)
- Hemocytometer
- Microscope

REAGENT & MEDIA PREPARATION

Note: Sterile technique is required when handling the reagents.

- **MEF Media -** MEF media consists of high glucose DMEM, 10% fetal bovine serum, 2 mM L-glutamine, and if desired, add a 1:100 dilution of penicillin/streptomycin (100x) stock. Filter sterilize the media using 0.2 µm sterile filter unit.

- **hES Media -** hES media consists of DMEM/F12, 15% fetal bovine serum, 5% Knockout serum replacer, 1:100 dilution of non-essential amino acids stock (100x), 1:100 dilution of penicillin/streptomycin (100x) stock, and 0.1 µM β-mercaptoethanol. Filter sterilize the media using 0.2 µm sterile filter unit. Just prior to use, the media should be supplemented with recombinant human FGF basic at 4 ng/mL.

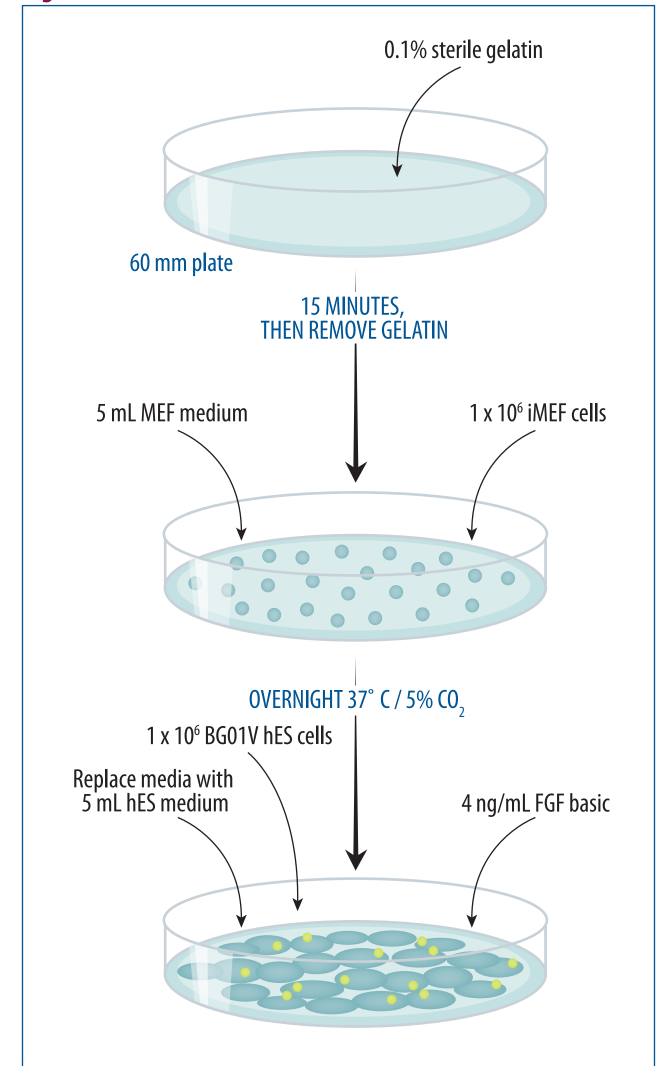
PROCEDURES

Note: When handling biohazard materials such as human cells, safe laboratory procedures should be followed and protective clothing should be worn.

I. Thawing and Plating of iMEF Feeder Cells (plate feeder cells one day prior to stem cell seeding) (Figure 1)

1. Coat the appropriate sized plate(s) for the desired number of cells by covering the surface of the dish with 0.1% sterile gelatin for 15 minutes. For example, one vial of 6 x 10^6 iMEF can be plated on two 100 mm dishes, six 60 mm dishes, or two 6 well plates.
2. Warm MEF media to 37° C.
3. Thaw the desired number of vials of iMEF cells by quickly warming the cryotube(s) in a 37° C water bath until the cells are just thawed and then immediately transferring the contents of one vial to a 15 mL conical tube containing at least 5 mL of pre-warmed MEF media. Rinse the cryotube with an additional 1 mL of media to ensure the removal of all the cells.
4. Spin at 200 x g in a clinical centrifuge for 5 minutes.
5. Remove the supernatant and gently flick the pellet.
6. Aspirate the 0.1% gelatin from the plate(s).

Figure 1



7. Resuspend the iMEF cells in MEF media and transfer to the gelatin-coated plates at a density of approximately 1 x 10^6 cells/60 mm plate.
8. Incubate overnight in a 37° C, 5% CO_2 incubator before seeding with stem cells.

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II. Thawing the BG01V hES cells

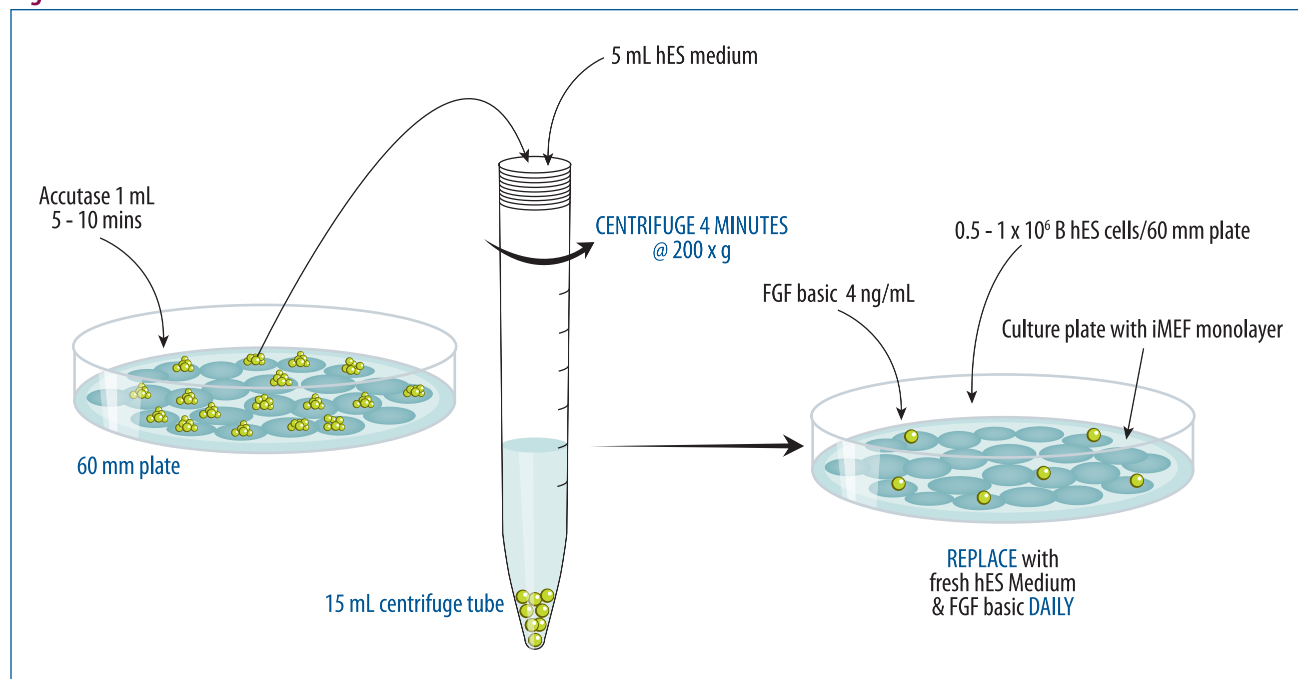
1. Warm the hES media to 37° C.
2. Place the cryovial of hES cells in a 37° C water bath until just thawed and then transfer the cells immediately to a 15 mL centrifuge tube containing at least 5 mL of pre-warmed hES media. Rinse the cryovial with an additional 1 mL of media to ensure the removal of all the cells.
3. Spin at 200 x g in a clinical centrifuge for 4 minutes.
4. Remove the supernatant and gently flick the pellet.
5. Resuspend the pellet in an appropriate amount of hES media freshly supplemented with 4 ng/mL of recombinant human FGF basic (typical volume is 5 mL per 60 mm plate).
6. Remove the MEF media from a plate containing iMEF cells and add the hES cell suspension. Typically 1 x 10^6 hES cells are thawed for a 60 mm plate.
7. Place the cells in a 37° C, 5% CO_2 incubator. Cells should be fed daily with hES media freshly supplemented with recombinant human FGF basic. Passage the cells before the hES colonies touch at their edges.

III. Passaging of the BG01V hES cells (Figure 2)

Note: Plate iMEF feeder cells on the desired number of plates 1 day prior to passaging.

1. Warm the hES media to 37° C.
2. Remove the hES media from BG01V cells. Add 1 mL of Accutase solution to each 60 mm plate. Incubate at room temperaturefor 5 - 10 minutes or until cells begin to slough off the plate.
3. Pipette Accutase gently over the plate until all the cells have been detached.
4. Gently pipette cell suspension up and down to break up large cell clumps.
5. Transfer the cell suspension to a 15 mL centrifuge tube containing 5 mL of hES media and spin at 200 x g for 4 minutes.
6. Remove the supernatant and gently flick the pellet.
7. Resuspend the pellet in pre-warmed hES media and count the cells using a hemocytometer.
8. Plate the desired number of cells (approximately 0.5 - 1.0 x 10^6 cells/60 mm plate) on the iMEF monolayer in hES media supplemented with 4 ng/mL of recombinant human FGF basic.
9. Feed the cells daily with hES media freshly supplemented with recombinant human FGF basic.

Figure 2



Knockout is a trademark of Invitrogen Corporation. BG01V Cells are licensed from BresaGen, Inc.

Protocol B: Culturing BG01V Human Embryonic Stem Cells with Mouse Embryonic Fibroblast (MEF)-Conditioned Media or Human Feeder Cell-Conditioned Media

If culturing in the absence of a feeder cell layer is desired, human embryonic stem (hES) cells can be maintained using Mouse or Human-Conditioned Media (Catalog # AR005, AR007). The protocol below has been used with the BG01V line of hES cells.

Please note that other hES cell lines may require modifications of this protocol. Optimal culture conditions must be determined by the investigator for each hES line. Please read the protocol in its entirety before starting.

SUPPLIES REQUIRED

REAGENTS

- MEF-Conditioned Media (R&D Systems, Catalog # AR005) or Human Feeder Cell-Conditioned Media (R&D Systems, Catalog # AR007)
- Recombinant human FGF basic (R&D Systems, Catalog # 233-FB) or tissue culture grade FGF basic (R&D Systems, Catalog # 4114-TC)
- Accutase (Innovative Cell Technologies, Catalog # AT104 or equivalent)
- Cultrex® Reduced Growth Factor Basement Membrane Extract (BME) (R&D Systems, Catalog # 3433-005-01)
- DMEM/F12

MATERIALS

- BG01V human embryonic stem cells (ATCC # SCRC-2002)
- 60 or 100 mm tissue culture plates
- 15 mL centrifuge tubes
- Pipettes and pipette tips

EQUIPMENT

- 37° C, 5% CO_2 incubator
- Centrifuge (low speed clinical or equivalent)
- Hemocytometer
- Microscope

PROCEDURES

Note: When handling biohazard materials such as human cells, safe laboratory procedures should be followed and protective clothing should be worn.

Note: Sterile technique is required when handling the reagents.

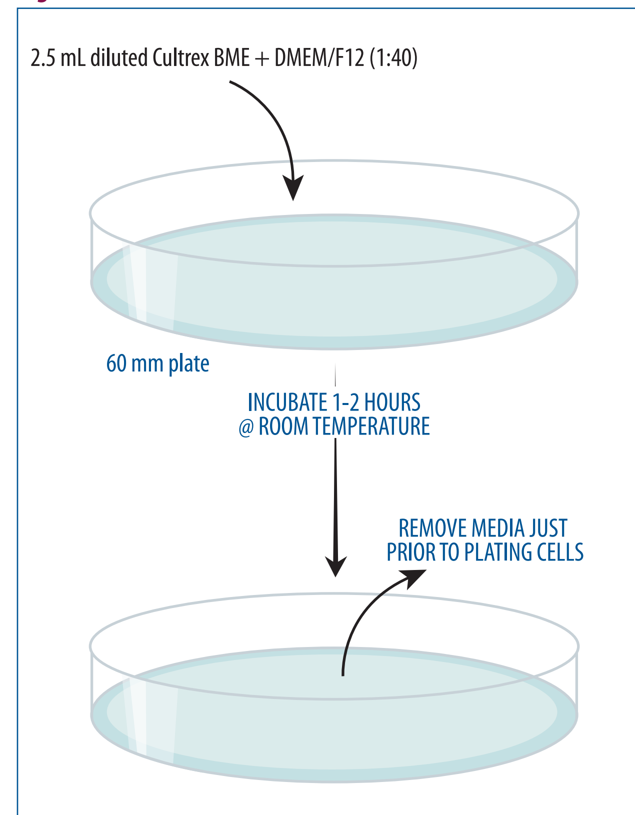
I. Prepare the Cultrex BME-coated plate. (Figure 1)

1. Thaw Cultrex BME on ice at 2 - 8° C overnight.
2. Aliquot thawed Cultrex BME into pre-cooled tubes and store at -20° C.
3. Thaw the aliquot on ice or at 2 - 8° C overnight.
4. Dilute Cultrex BME 1:40 in DMEM/F12. This can be stored at 4° C for up to 2 weeks.
5. Coat the desired number of plates with diluted Cultrex BME (approximately 2.5 mL/60 mm plate) and incubate for 1-2 hours at room temperature.
6. Remove the Cultrex BME solution immediately prior to plating the cells.

II. Preparation and plating of BG01V Cells. (Figure 2)

1. Warm the MEF or Human Feeder Cell-Conditioned Media to 37° C.
2. Warm the vial of BG01V hES cells until just thawed and then immediately transfer to a 15 mL centrifuge tube containing at least 5 mL of pre-warmed Conditioned Media. Rinse the cryovial with an additional 1 mL of media to ensure the removal of all the cells.
3. Spin in a clinical centrifuge at 200 x g for 4 minutes.
4. Remove the supernatant and gently flick the pellet. Resuspend the pellet in an appropriate amount of Conditioned Media supplemented with 4 ng/mL of recombinant human FGF basic.

Figure 1



5. Add the BG01V hES cell suspension to the Cultrex BME-coated plate.
6. Grow in a 37° C, 5% CO_2 incubator. Change the media daily and monitor the cells. Passage the cells at the desired confluency.

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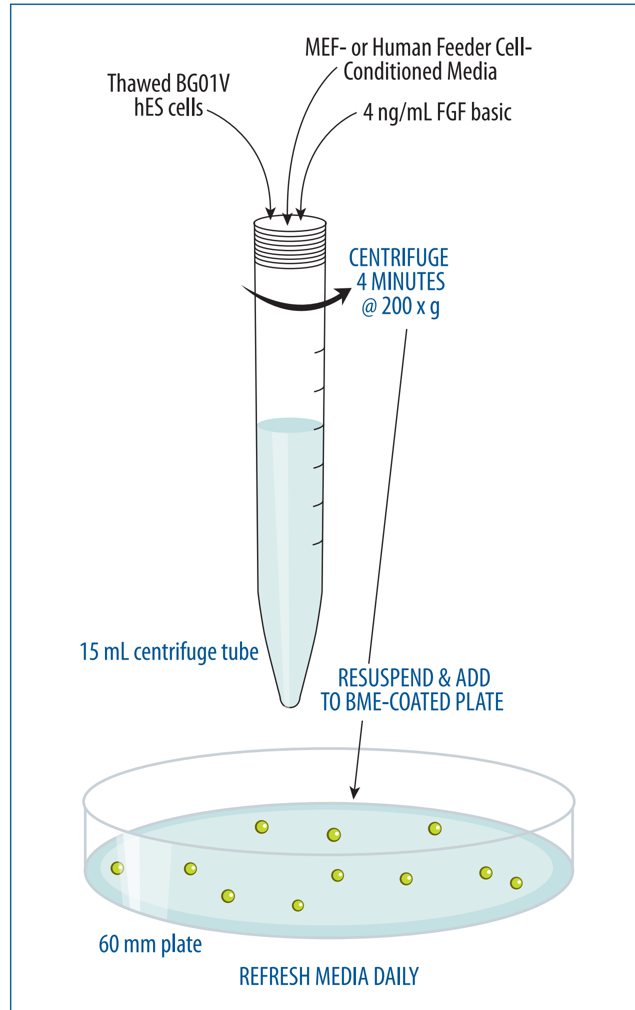
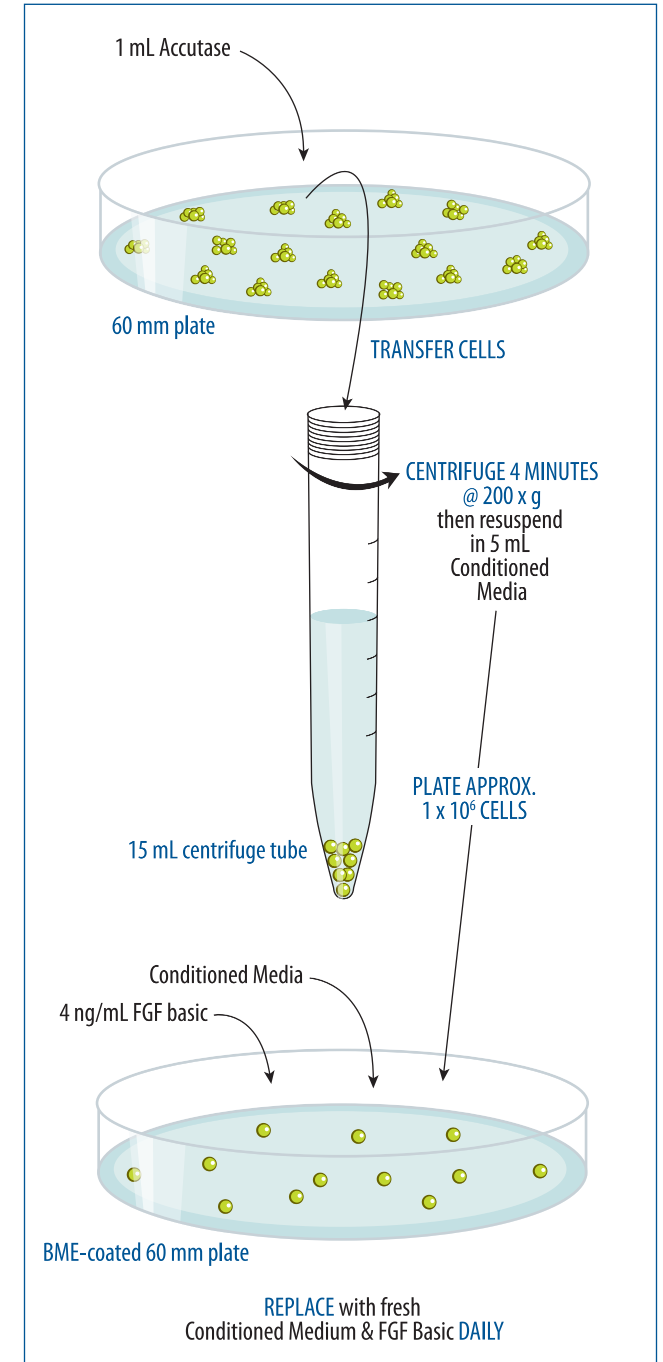
Figure 2



III. Passaging BG01V Cells (Figure 3)

1. Prepare the desired number of plates by coating with Cultrex BME, as described above, 1 - 2 hours prior to passaging the cells.
2. Warm the MEF- or Human Feeder Cell-Conditioned Media to 37° C.
3. Remove the Conditioned Media from cells. Add 1 mL of Accutase solution to each 60 mm plate. Incubate at room temperature for 5 - 10 minutes or until cells begin to slough off the plate.
4. Pipette gently over the plate until all the cells have been detached.
5. Pipette the cell suspension up and down to break up large cell clumps.
6. Remove the cell suspension to a 15 mL centrifuge tube containing 5 mL of Conditioned Media and spin at 200 x g for 4 minutes.
7. Resuspend the pellet in Conditioned Media and count the viable cells using a hemocytometer.
8. Plate the desired number of cells (approximately 1.0 x 10^6 cells/60 mm plate) on the Cultrex BME coated plate in Conditioned Media containing 4 ng/mL of recombinant human FGF basic.
9. Change the media daily. Monitor the cells for the desired confluency.

Figure 3



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Protocol C: Culturing Rat Cortical Stem Cells: Expansion using the Monolayer System

Ex vivo expanded neural stem cells serve as excellent tools for researchers studying neural development and neurological disorders. Ready-to-use primary cortical stem cells, isolated from E14.5 Sprague-Dawley rats (Catalog # NSC001), can be grown in monolayer, as described here, or as neurospheres. These cells retain capacity for multi-lineage differentiation into astrocytes, neurons, and oligodendrocytes.

Please read the protocol in its entirety before starting.

SUPPLIES REQUIRED

REAGENTS

- StemXVivo™ Serum-Free NSC Base Media (R&D Systems, Catalog # CCM002)
- Recombinant FGF basic (R&D Systems, Catalog # 233-FB or Tissue Culture Grade FGF basic, Catalog # 4114-TC)
- Bovine Fibronectin (R&D Systems, Catalog # 1030-FN)
- PBS
- Penicillin-Streptomycin (100x)
- Poly-L-Ornithine
- Ca^{2+}/Mg^{2+}-free Hank's Balanced Salt Solution (HBSS) (10x)
- HEPES
- BSA, very low endotoxin
- Trypan blue, 0.4%
- Deionized water

MATERIALS

- 10 cm tissue culture plates
- 50 mL centrifuge tubes
- 0.2 µm, sterile filter unit
- Plastic cell scraper
- Pipettes and pipette tips

EQUIPMENT

- 37° C, 5% CO_2 incubator
- Centrifuge
- Hemocytometer
- Microscope

REAGENT & MEDIA PREPARATION

Note: Sterile technique is required when handling the reagents.

- **NSC Base Media** - Thaw the StemXVivo Serum-Free NSC Base Media at 2 - 8° C or room temperature. Aliquot any remaining thawed media and store at -20° C. Use within 10 days when stored in the dark at 2 - 8° C.
- **Completed NSC Base Media** - Add Penicillin-Streptomycin (100x) to the NSC Base Media at a 1:100 dilution.
- **Buffered HBSS (1x)** - Add 100 mL of HBSS (10x) and 3.9 g HEPES to 900 mL of deionized water to make 1000 mL of buffered HBSS 1x. Adjust the pH to 7.2 ± 0.2. Sterile filter the solution using a 0.2 µm filter unit and store at room temperature for up to 6 months.
- **FGF basic Stock (1000x)** - Add sterile 0.1% BSA in PBS to the human FGF basic vial to make a 20 µg/mL stock. Aliquot and store at -20° C in a manual defrost freezer for up to 6 months. Avoid repeated freeze-thaw cycles.

PROCEDURES

I. Culture Dish Preparation

1. Dissolve Poly-L-Ornithine in sterile PBS to make a 15 mg/mL stock (1000x). Aliquot and store at -20° C in a manual defrost freezer for up to 6 months. Avoid repeated freeze-thaw cycles.
2. Dilute the 1000x Poly-L-Ornithine Stock 1000-fold in sterile PBS to make a 15 µg/mL (1x) solution. Prepare fresh as needed.
3. Add 10 mL of the (1x) Poly-L-Ornithine solution to each 10 cm tissue culture dish. Incubate overnight at 37° C.
4. Discard the Poly-L-Ornithine solution. Wash each dish 3 times with 10 mL of PBS.
5. Add 10 mL of PBS to each dish. Incubate overnight at 37° C.
6. Allow the vial of bovine Fibronectin to warm to room temperature without agitation. Make a 1 µg/mL solution by pipetting the bovine Fibronectin into sterile PBS and gently inverting the tubes. Prepare fresh as needed.
7. Discard the PBS from each Poly-L-Ornithine-coated dish. Wash each dish once with 10 mL of PBS.
8. Add 10 mL of 1 µg/mL bovine Fibronectin solution to each dish. Incubate at 37° C for 3 - 30 hours.
9. Discard the bovine Fibronectin solution. Wash each dish once with 10 mL of PBS before use.

II. Thawing Cryopreserved Rat Cortical Stem Cells (Review the following section in detail before thawing the cells)

1. Warm 30 mL of Completed NSC Base Media supplemented with 20 ng/mL FGF basic in a 37° C water bath.
2. Add 20 mL of pre-warmed Completed Base NSC Media and FGF basic to a 50 mL tube. Reserve the remaining 10 mL pre-warmed Completed Base NSC Media for step # 5.
3. Remove the cryovial containing frozen rat cortical stem cells from the liquid nitrogen. Using a 2 mL pipette, immediately add 1 mL of fresh pre-warmed media to the vial by gently pipetting up and down. As cells begin to thaw, transfer the thawed portion into the pre-warmed media in the 50 mL tube. Repeat this process with the warmed media until all of the cells have thawed.

   Note: Most of the frozen cells will be at the bottom of the cryovial.
4. Centrifuge the cells at 200 x g for 5 minutes.
5. Aspirate off 95% of the supernatant carefully and resuspend by gently pipetting the cell pellet up and down with 10 mL of Completed Base Media supplemented with FGF basic.

   Note: Rapid resuspension of frozen cells in warmed media during thawing is critical. Allowing cells to thaw slowly in the DMSO will dramatically reduce viability. Around 90% cell viability is expected from the freshly thawed cells when the appropriate thawing procedure is followed.
6. Seed cells at a density according to the expansion protocol described below.

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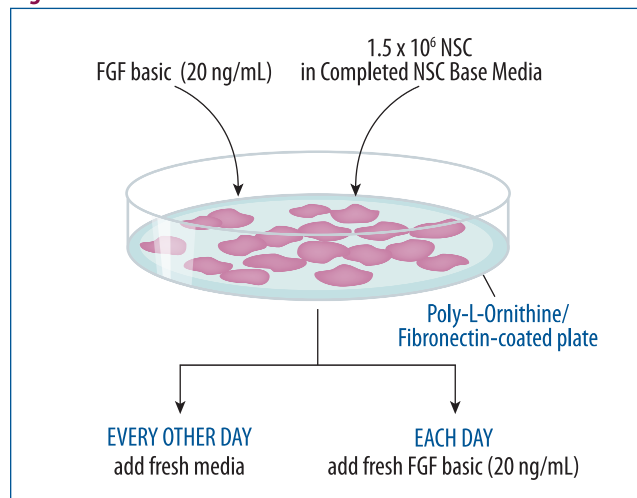
III. Cell Expansion (Figure 1)

1. Seed 1 - 1.5 x 10^6 NSCs in 10 mL of Completed NSC Base Media supplemented with 20 ng/mL of FGF basic on a Poly-L-Ornithine/Fibronectin-coated 10 cm plate.
2. Incubate the cells at 37° C, 5% CO_2. Cells should become adherent after 24 hours.
3. After 24 hours, add 10 µL of FGF basic stock (1000x) to the culture.
4. Every second day, replace the media with fresh Completed NSC Base Media.
5. Supplement the media with FGF basic (to 20 ng/mL) each day.
6. Passage the cells according to the procedure described below when they reach 60 - 70% confluency (approximately 4 days after initial plating).

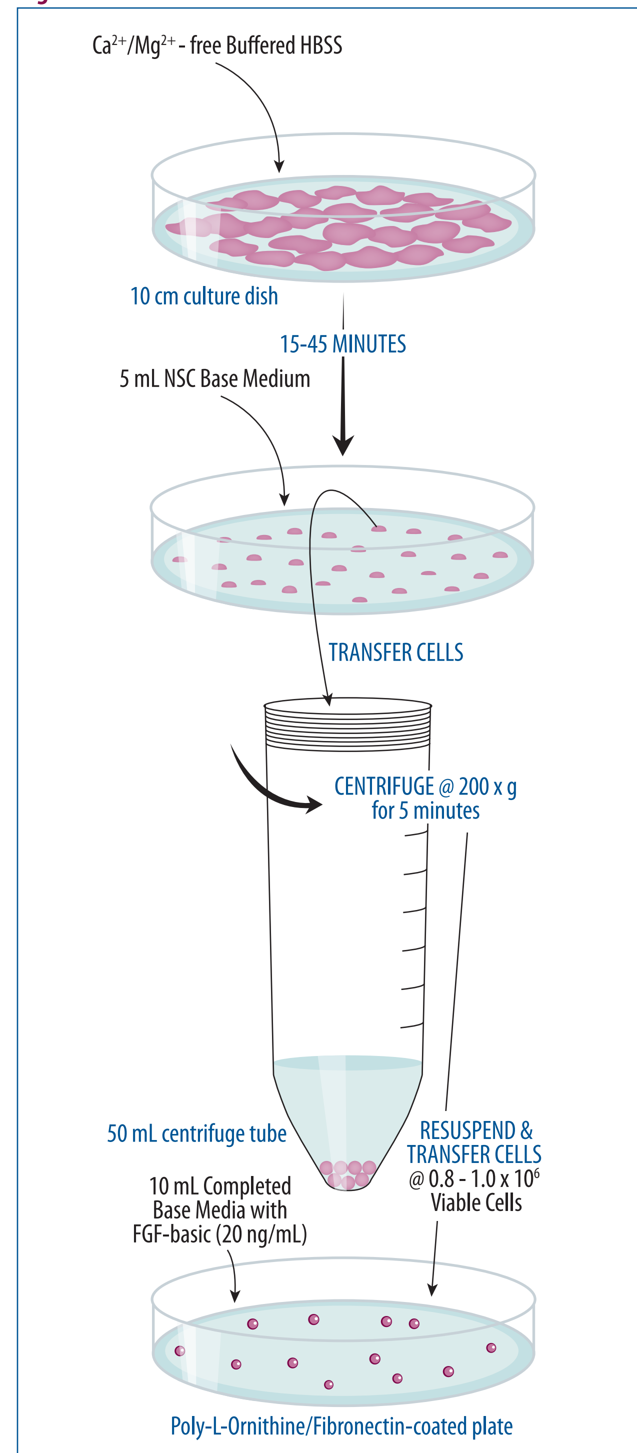
Figure 1



IV. Passaging Cells (Figure 2)

1. Warm the buffered HBSS (1x) and Completed NSC Base Media supplemented with FGF basic (20 ng/mL) to 37° C.
2. Remove the media from the cells. Wash once in 10 mL of buffered HBSS (1x).
3. Add 5 mL of buffered HBSS (1x). Incubate at room temperature for 15 - 45 minutes until the cells round up (check frequently).
4. Scrape the cells from the plate with a hard plastic cell scraper. Transfer the cells to a 50 mL centrifuge tube.
5. Centrifuge for 5 minutes at 200 x g and remove the supernatant.
6. Resuspend the cells with 5 mL of Completed Base Media containing FGF basic by slowly pipetting up and down approximately 5 times with a 5 mL pipette.
7. Mix 10 µL of the cell suspension with 10 µL of 0.4% Trypan blue and count the live cells on a hemocytometer.
8. Seed 0.8 - 1.0 x 10^6 viable cells in 10 mL of Completed NSC Base Media containing FGF basic on a Poly-L-Ornithine/Fibronectin-coated plate.
9. Incubate the cells at 37° C, 5% CO_2. Repeat steps 4 and 5 in the Expansion section (see above). Passage the cells after 3 days or when cells reach 70% confluence.

Figure 2



Ex vivo expanded neural stem cells serve as excellent tools for researchers studying neural development and neurological disorders. Ready-to-use primary cortical stem cells, isolated from E14.5 Sprague-Dawley rats (Catalog # NSC001), can be grown in monolayer or as neurospheres as described here. These cells retain capacity for multi-lineage differentiation into astrocytes, neurons, and oligodendrocytes.

Please read the protocol in its entirety before starting.

SUPPLIES REQUIRED

REAGENTS

- StemXVivo Serum-Free NSC Base Media (R&D Systems, Catalog # CCM002)
- Recombinant human FGF basic (R&D Systems, Catalog # 233-FB/CF; Tissue Culture Grade FGF basic, Catalog # 4114-TC)
- Recombinant human EGF (R&D Systems, Catalog # 236-EG)
- PBS
- Penicillin-Streptomycin (100x)
- BSA, very low endotoxin
- Acetic acid
- Trypan blue
- Deionized water

MATERIALS

- 6-well plates
- 15 mL centrifuge tubes
- Pasteur pipettes
- Pipettes and pipette tips

EQUIPMENT

- 37° C and 5% CO_2 incubator
- Centrifuge
- Hemocytometer
- Microscope

REAGENT & MEDIA PREPARATION

Note: Sterile technique is required when handling the reagents.

- **NSC Base Media** - Thaw the StemXVivo Serum-Free NSC Base Media at 2 - 8° C or room temperature. Aliquot any remaining thawed media and store at -20° C. Use within 10 days when stored in the dark at 2 - 8° C.
- **Completed NSC Base Media** - Add Penicillin-Streptomycin (100x)to the NSC Base Media at a 1:100 dilution.
- **FGF basic Stock** (1000x) - Add sterile 0.1% BSA in PBS to the Human FGF basic vial to make a 20 µg/mL stock. Aliquot and store at -20° C in a manual defrost freezer for up to 6 months. Avoid repeated freeze-thaw cycles.
- **EGF Stock** (1000x) - Add sterile 0.1% BSA in 10 mM acetic acid to the Human EGF vial to make a 20 µg/mL stock. Aliquot and store at -20° C in a manual defrost freezer for up to 6 months. Avoid repeated freeze-thaw cycles.

PROCEDURES

I. Thawing Cryopreserved Rat Cortical Stem Cells (Review the following section in detail before thawing the cells)

1. Warm 30 mL of Completed NSC Base Media in a 37° C water bath.
2. Add 20 mL of pre-warmed Completed Base NSC Media supplemented with FGF basic (20 ng/mL) and EGF (20 ng/mL) to a 50 mL tube. Reserve the remaining 10 mL pre-warmed Completed Base NSC Media for step # 5.
3. Remove the cryovial containing frozen rat cortical stem cells from the liquid nitrogen. Using a 2 mL pipette, immediately add 1 mL of fresh pre-warmed media to the vial by gently pipetting up and down. As cells begin to thaw, transfer the thawed portion into the pre-warmed media in the 50 mL tube. Repeat this process with the warmed media until all of the cells have thawed.

   Note: Most of the frozen cells will be at the bottom of the cryovial.
4. Centrifuge the cells at 200 x g for 5 minutes.
5. Aspirate off 95% of the supernatant carefully and resuspend by gently pipetting the cell pellet up and down with 10 mL of Completed Base Media with growth factors.

   Note: Rapid resuspension of frozen cells in warmed media during thawing is critical. Allowing cells to thaw slowly in the DMSO will dramatically reduce viability. Around 90% cell viability is expected from the freshly thawed cells when the appropriate thawing procedure is followed.
6. Seed cells at a density according to the appropriate expansion protocol described below.

II. Neurosphere Expansion (Figure 1)

1. Seed approximately 1 x 10^5 NSCs in 5 mL of Completed NSC Base Media supplemented with 20 ng/mL of EGF and 20 ng/mL of FGF basic per well in a 6-well plate.
2. Incubate the cells at 37° C and 5% CO_2.
3. Add fresh EGF (20 ng/mL) and FGF basic (20 ng/mL) each day to the media. Every fourth day, based on the number of neurospheres, replace the media according to the steps described below.

   a. **Less than 50 neurospheres** - Transfer the neurospheres, using a Pasteur pipette, directly into 2.5 mL of Completed NSC Base Media containing EGF (20 ng/mL) and FGF basic (20 ng/mL) in one well of a 6-well plate. DO NOT DISCARD THE CONDITIONED Media. Add 2.5 mL of this conditioned media to the well. When there are fewer neurospheres, conditioned media is required. Only half of the media is replaced with fresh Completed NSC Base Media containing EGF (20 ng/mL) and FGF basic (20 ng/mL).

   b. **More than 50 neurospheres** - Transfer the media containing the neurospheres to a 15 mL tube. Centrifuge for 5 minutes at 100 x g and remove the media. Gently resuspend the pellet using a small quantity of fresh Completed NSC Base Media containing EGF (20 ng/mL) and FGF basic (20 ng/mL). Add the neurosphere suspension to 5 mL of fresh Completed Base Media containing EGF (20 ng/mL) and FGF basic (20 ng/mL) in one well of a 6-well plate.

4. Pass the cells according to the procedure described below at 5-7 days, or when the neurospheres have a dark clump inside or ruffling on the outside of the neurosphere.

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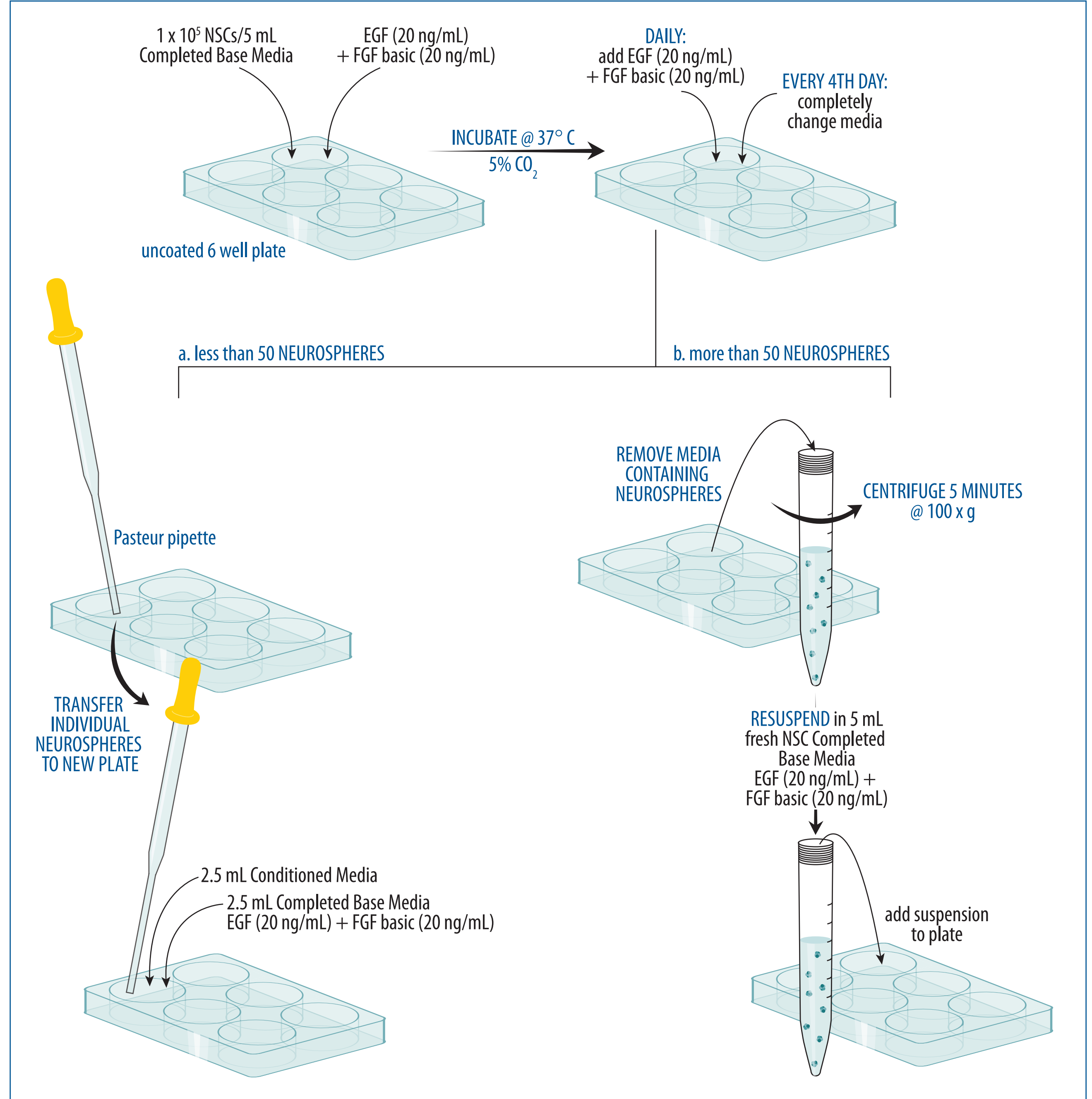
III. Passaging Neurospheres

1. Transfer the media containing the floating neurospheres to a 15 mL tube. DO NOT DISLODGE ATTACHED NEUROSPHERES FOR PASSAGE.
2. Centrifuge for 5 minutes at 100 x g.
3. Partially dissociate the neurospheres by pipetting up and down 20 times with a P200 pipette, being careful not to create bubbles in the suspension.
4. At passages 1 and 2 the cells should be split 1:1. After passage 2 the cells can be split 1:2.

Figure 1



Protocol E: Osteogenic Differentiation of Human/Mouse Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are functionally defined by their capacity to self renew and their ability to differentiate into multiple cell types including adipocytes, chondrocytes and osteocytes. This protocol describes a technique to promote the osteogenic differentiation of human and mouse MSCs.

Please read the protocol in its entirety before starting.

SUPPLIES REQUIRED

REAGENTS

- Bone marrow derived MSCs
- StemXVivo Human/Mouse Osteogenic/Adipogenic Base Media (R&D Systems, Catalog # CCM007)
- StemXVivo Human Osteogenic Supplement (R&D Systems, Catalog # CCM008) or StemXVivo Mouse Osteogenic Supplement (R&D Systems, Catalog # CCM009)
- Penicillin-Streptomycin (100x)

MATERIALS

- 10 cm tissue culture plates
- 15 mL centrifuge tubes
- Serological pipettes
- Pipettes and pipette tips

EQUIPMENT

- 37º C, 5% CO_2 humidified incubator
- Centrifuge
- Hemocytometer
- Microscope
- Water bath

REAGENT & MEDIA PREPARATION

Note: Sterile technique is required when handling the reagents.

- **StemXVivo Osteogenic/Adipogenic Base Media** - Thaw the StemXVivo Osteogenic/Adipogenic Base Media at 2 - 8° C or room temperature. Aliquot any unused thawed media and store at -20° C in a manual defrost freezer. Thawed media may be stored in the dark at 2 - 8° C for up to 1 month.
- **Completed StemXVivo Osteogenic/Adipogenic Base Media** - Add Penicillin-Streptomycin to the StemXVivo Osteogenic/Adipogenic Base Media at a 1:100 dilution.

  Note: If Penicillin-Streptomycin is not needed for the experiment, it can be omitted.
- **Completed StemXVivo Osteogenic Differentiation Media** - Add StemXVivo Osteogenic Supplement to the completed StemXVivo Osteogenic/Adipogenic Base Media at a 1:20 dilution.

  Note: With the exception of Step 5, the reagent and material preparation for mouse and human completed StemXVivo Osteogenic/Adipogenic Base Media and completed StemXVivo Osteogenic Differentiation Media are the same using either the mouse or the human Supplement.

PROCEDURE (Figure 1)

Note: When handling biohazard materials such as human cells, safe laboratory procedures should be followed and protective clothing should be worn.

1. Pre-warm the Completed StemXVivo Osteogenic/Adipogenic Base Media in a 37° C water bath. This procedure uses 10 mL for each 10 cm tissue culture plate used.
2. Resuspend 2.3 - 2.5 x 10^5 MSCs in 10 mL of the pre-warmed Completed StemXVivo Osteogenic/Adipogenic Base Media.

   Note: If using another size tissue culture vessel, seed cells at approximately 4.2 x 10^3 cells/cm^2/0.2 - 0.3 mL media.
3. Add this cell suspension to a 10 cm tissue culture plate. The cells should be 50 - 70% confluent in 1 - 2 days.
4. At 50 - 70% confluency, replace the media with 10 mL of pre-warmed completed StemXVivo Osteogenic Differentiation Media to induce osteogenesis.
5. Every 3 - 4 days (for mouse every 2 - 3 days) remove and discard spent media and replace with 10 mL of pre-warmed completed StemXVivo Ostegenic Differentiation Media.

   Note: Dispense media down the side of the plate so as not to disrupt cells.
6. After 2 - 3 weeks induced cells will exhibit phenotypic changes consistent with osteogenic differentiation (Figures 2 and 3).

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Figure 1

2.3 - 2.5 x 10^5 MSCs

Completed StemXVivo Osteogenic/Adipogenic Base Media

1:100 Penicillin/ Streptomycin (optional)

ADD CELL PREPARATION TO 10 cm CULTURE PLATE

15 mL centrifuge tube

10 cm plate

CELLS SHOULD BE 50-70% CONFLUENT IN 1 - 2 DAYS

AT 50-70% CONFLUENCY ADD StemXVivo Osteogenic Differentiation Media

Osteogenic cells

REPLACE BASE MEDIA EVERY 3 - 4 DAYS (2 - 3 DAYS FOR MOUSE)

OSTEOGENIC DIFFERENTIATION IS COMPLETE AFTER 2 - 3 WEEKS

Figure 2. **Osteocyte Differentiation of Human MSCs.** Human mesenchymal stem cells differentiated *in vitro* for 21 days using Human/Mouse StemXVivo Osteogenic/Adipogenic Base Media (Catalog # CCM007) and Human StemXVivo Osteogenic Supplement (Catalog # CCM008) were stained with an antibody to the osteogenic lineage marker Osteocalcin (Catalog # MAB1419) and NorthernLights 557-conjugated anti-mouse secondary antibody (Catalog # NL007; red). Cells were counterstained with DAPI (blue).

Figure 3. **Osteocyte Differentiation of Mouse MSCs.** The Mouse Mesenchymal Stem Cell Functional Identification Kit (Catalog # SC010) was used to differentiate MSCs into the osteogenic lineage. Mature differentiated cells were labeled using an antibody to the osteogenic lineage marker Osteopontin supplied with the kit (red). The nuclei were counterstained with DAPI (blue). Similar results may be obtained using the Human/Mouse StemXVivo Osteogenic/Adipogenic Base Media (Catalog # CCM007) and Mouse StemXVivo Osteogenic Supplement (Catalog # CCM009).

Protocol F: Adipogenic Differentiation of Human/Mouse Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are functionally defined by their capacity to self renew and their ability to differentiate into multiple cell types including adipocytes, chondrocytes and osteocytes. This protocol describes the adipogenic differentiation of human and mouse MSCs using the StemXVivo Human/Mouse Osteogenic/Adipogenic Base Media (Catalog # CCM007) and StemXVivo Adipogenic Supplement (Catalog # CCM011).

Please read the protocol in its entirety before starting.

SUPPLIES REQUIRED

REAGENTS

- Bone marrow derived MSCs
- StemXVivo Human/Mouse Osteogenic/Adipogenic Base Media (R&D Systems, Catalog # CCM007)
- StemXVivo Adipogenic Supplement (R&D Systems, Catalog # CCM011)
- Penicillin-Streptomycin (100x)

MATERIALS

- 10 cm tissue culture plates
- 15 mL centrifuge tubes
- Serological pipettes
- Pipettes and pipette tips

EQUIPMENT

- 37° C, 5% CO_2 humidified incubator
- Centrifuge
- Hemocytometer
- Microscope
- Water bath

REAGENT & MEDIA PREPARATION

Note: Sterile technique is required when handling the reagents.

- **StemXVivo Osteogenic/Adipogenic Base Media** - Thaw the StemXVivo Osteogenic/Adipogenic Base Media at 2 - 8° C or room temperature. Aliquot any unused thawed media and store at ≤ 20° C in a manual defrost freezer. Thawed media may be stored in the dark at 2 - 8° C for up to 1 month.
- **Completed StemXVivo Osteogenic/Adipogenic Base Media** - Add Penicillin-Streptomycin to the StemXVivo Osteogenic/Adipogenic Base Media at a 1:100 dilution.

  Note: If Penicillin-Streptomycin is not needed for the experiment, it can be omitted.
- **Completed StemXVivo Adipogenic Differentiation Media** - If a precipitate forms, warm the Adipogenic Supplement vial in a 37° C water bath for 5 minutes. Vortex until the precipitate dissolves. Add StemXVivo Adipogenic Supplement to the completed StemXVivo Osteogenic/Adipogenic Base Media at a 1:100 dilution.

PROCEDURE (Figure 1)

Note: When handling biohazard materials such as human cells, safe laboratory procedures should be followed and protective clothing should be worn.

1. Pre-warm the Completed StemXVivo Osteogenic/Adipogenic Base Media in a 37° C water bath. This procedure uses 10 mL for each 10 cm tissue culture plate used.
2. Resuspend 1 x 10^6 MSCs into 10 mL of the pre-warmed Completed StemXVivo Osteogenic Base Media.

   Note: If using another size tissue culture vessel, seed cells at approximately 2.1 x 10^4 cells/cm^2/0.2 - 0.3 mL.
3. Add this cell suspension to a 10 cm tissue culture plate and incubate overnight in a 37° C, 5% CO_2 incubator. Cells should be 100% confluent after overnight incubation. If they are not confluent, replace media every 2 - 3 days with Osteogenic/Adipogenic Base Media until 100% confluency is reached.
4. At 100% confluency, replace the media with 10 mL of pre-warmed completed StemXVivo Adipogenic Differentiation Media to induce adipogenesis.
5. Every 3 - 4 days remove and discard the spent media and replace with 10 mL of freshly prepared, pre-warmed Completed StemXVivo Adipogenic Differentiation Media.

   Note: Differentiation is complete after 7 - 21 days, at which time adipogenic induced cells will have morphological changes and lipid vacuoles (Figure 2) and express markers of the adipocyte lineage (Figure 3).

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Figure 1

1 x 10^6 MSC

10 mL Stem XVivo Osteogenic/Adipogenic Base Media

TRANSFER CELLS & MEDIA TO 10 cm CULTURE DISH

15 mL centrifuge tube

10 cm plate

REPLACE BASE MEDIA EVERY 3 - 4 DAYS UNTIL CONFLUENT

INCUBATE @ 37° C 5% CO_2

Stem XVivo Osteogenic/Adipogenic Differentiation Media

Adipocytes

ADIPOGENIC DIFFERENTIATION IS COMPLETE AFTER 7 - 21 DAYS

Figure 2. Adipogenic Differentiation of Human MSCs. Human mesenchymal stem cells differentiated *in vitro* for 21 days using the Human/Mouse StemXVivo Osteogenic/Adipogenic Base Media (Catalog # CCM007) and Human Mouse StemXVivo Adipogenic Supplement (Catalog # CCM011) were stained with the adipogenic lineage-specific antibody to FABP-4 (Catalog #AF3150) and NorthernLights 557-conjugated anti-goat secondary antibody (Catalog # NL001; red) and counterstained with DAPI (blue).

Figure 3. **Adipogenic Differentiation of Mouse MSCs.** The Mouse Mesenchymal Stem Cell Functional Identification Kit (Catalog # SC010) was used to differentiate mouse MSCs into the adipogenic lineage. Mature differentiated cells were labeled using the adipogenic lineage-specific antibody to FABP4 supplied with the kit (red). The nuclei were counterstained with DAPI (blue). Similar results may be obtained using the Human/Mouse StemXVivo Osteogenic/Adipogenic Base Media (Catalog # CCM007) and Human/Mouse StemXVivo Adipogenic Supplement (Catalog # CCM011).

Protocol G: Chondrogenic Differentiation of Human/Mouse Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are functionally defined by their capacity to self renew and their ability to differentiate into multiple cell types including adipocytes, chondrocytes and osteocytes. This protocol describes the chondrogenic differentiation of MSCs using the StemXVivo Human/Mouse Chondrogenic Base Media (Catalog # CCM005) and StemXVivo Human/Mouse Chondrogenic Supplement (Catalog # CCM006).

Please read the protocol in its entirety before starting.

SUPPLIES REQUIRED

REAGENTS

- Bone marrow derived MSCs
- StemXVivo Human/Mouse Chondrogenic Base Media (R&D Systems, Catalog # CCM005)
- StemXVivo Human/Mouse Chondrogenic Supplement (R&D Systems, Catalog # CCM006)
- Penicillin-Streptomycin (100x)

MATERIALS

- 15 mL centrifuge tubes
- Serological pipettes
- Pipettes and pipette tips

EQUIPMENT

- 37° C, 5% CO_2 humidified incubator
- Centrifuge
- Hemocytometer
- Water bath

REAGENT & MEDIA PREPARATION

Note: Sterile technique is required when handling the reagents.

- **StemXVivo Chondrogenic Base Media** - Thaw the StemXVivo Chondrogenic Base Media at 2 - 8° C or room temperature. Aliquot any unused thawed media and store at ≤ 20° C in a manual defrost freezer. Thawed media may be stored in the dark at 2 - 8° C for up to 1 month.
- **Completed StemXVivo Chondrogenic Base Media** - Add Penicillin-Streptomycin to the StemXVivo Chondrogenic Base Media at a 1:100 dilution.

  Note: If Penicillin-Streptomycin is not needed for the experiment, it can be omitted.
- **Completed StemXVivo Chondrogenic Differentiation Media** - Add StemXVivo Chondrogenic Supplement to the completed StemXVivo Chondrogenic Base Media at a 1:100 dilution.

  Note: This procedure will use 0.5 mL of completed StemXVivo Chondrogenic Differentiation Media for each 15 mL centrifuge tube.

PROCEDURE (Figure 1)

Note: When handling biohazard materials such as human cells, safe laboratory procedures should be followed and protective clothing should be worn.

1. Pre-warm 5 mL of the completed StemXVivo Chondrogenic Base Media and 0.5 mL of completed StemXVivo Chondrogenic Differentiation Media in a 37° C water bath.
2. Resuspend 2.5 x 10^5 MSCs (for mouse, 1.25 x 10^5 MSCs) in 5 mL of the pre-warmed completed StemXVivo Chondrogenic Base Media.
3. Centrifuge the cells at 200 x g for 5 minutes at room temperature. Remove the media and resuspend the cells with 0.5 mL of pre-warmed completed StemXVivo Chondrogenic Differentiation Media.
4. Centrifuge the cells at 200 x g for 5 minutes at room temperature. Do not remove the media. Loosen the cap of the tube to allow gas exchange and incubate upright at 37° C and 5% CO_2.
5. After 1 - 2 days the cell pellet will form a round ball approximately 1 - 2 mm in diameter. This pellet will remain about the same size for the entire culturing time.
6. Every 2 - 3 days remove and discard the spent media and replace with 0.5 mL of pre-warmed completed StemXVivo Chondrogenic Differentiation Media.

   Note: Use caution when removing the media to avoid aspirating the pellet.
7. Chondrogenic pellets can be harvested after 14 - 21 days in culture and used for the desired analysis.

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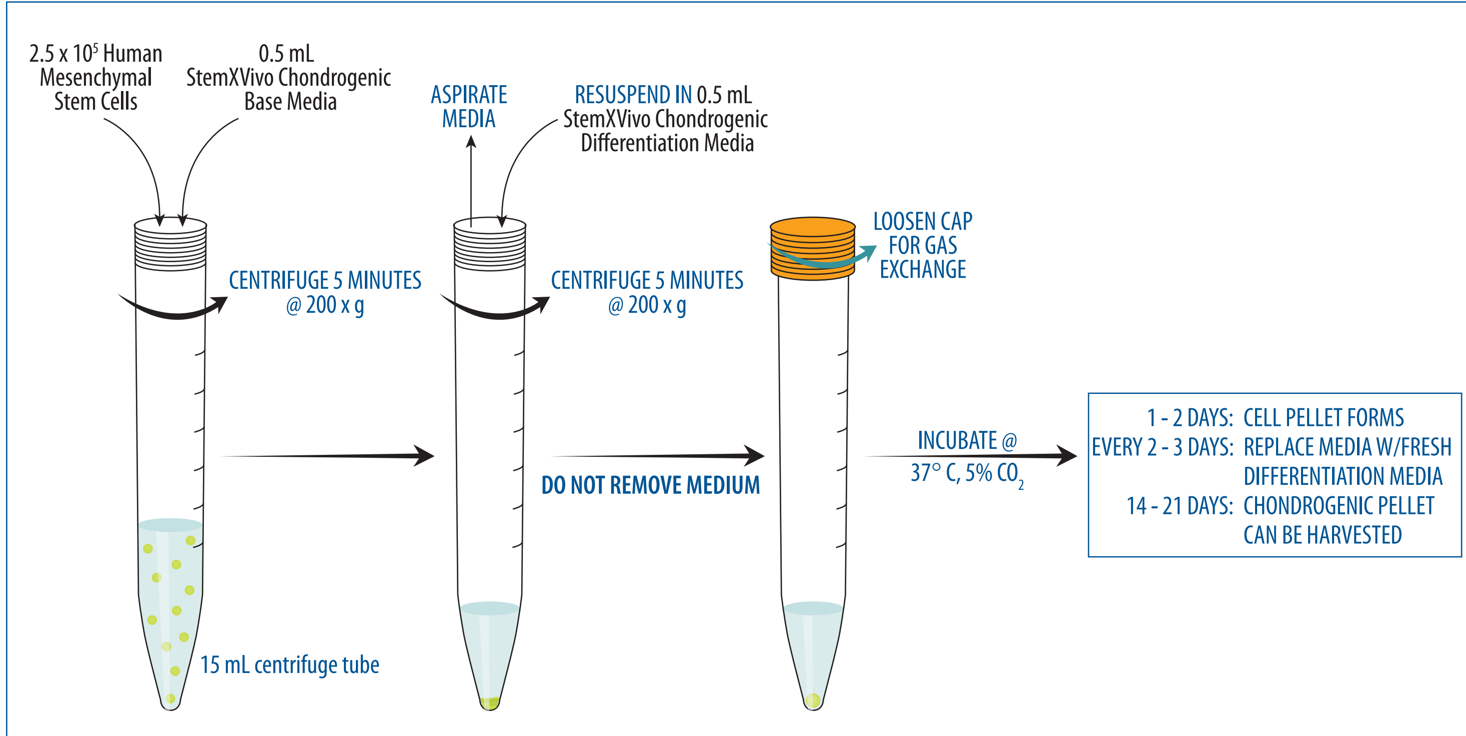
Figure 1



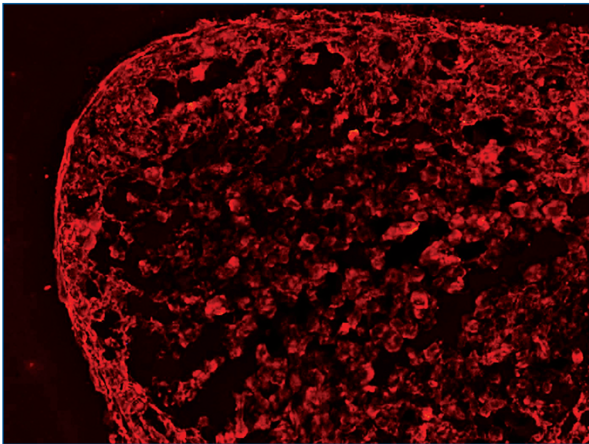


Figure 2. **Chondrogenic Differentiation of MSCs.** Mouse mesenchymal stem cell pellet differentiated *in vitro* for 21 days using the Human/Mouse StemXVivo Chondrogenic Base Media (Catalog # CCM005) and Human/Mouse StemXVivo Chondrogenic Supplement (Catalog # CCM006) was sectioned and stained with an antibody to the chondrogenic lineage marker, Collagen II (Catalog # AF3615) and NorthernLights 557-conjugated anti-sheep secondary antibody (Catalog # NL010).

Antibodies including anti-mouse CD5, CD11b, CD45R (B220), Ly-6G (Gr-1), and TER-119 can be used to efficiently tag bone marrow-derived cells committed to major hematopoietic lineages, including T lymphocytes, B lymphocytes, monocytes/macrophages, granulocytes, and erythrocytes. These antibodies can be used, in conjunction with magnetic particle separation systems or flow cytometric cell sorting, to deplete lineage-committed cells and enrich for uncommitted hematopoietic progenitors. This procedure describes the depletion of lineage committed cells using the MagCellect™ Mouse Hematopoietic Lineage Depletion Kit (Catalog # MAGM209). The resulting cell population is enriched for $CD117^{+}$ cells (from 40 to 70% depending on mouse strain) with less than 5% residual lineage positive cells.

Please read the protocol in its entirety before starting.

SUPPLIES REQUIRED

REAGENTS

- MagCellect Mouse Hematopoietic Lineage Depletion Kit (R&D Systems, Catalog # MAGM209)
- PBS
- Fetal bovine serum (FBS)
- Sterile deionized water

MATERIALS

- 50 mL centrifuge tubes
- 15 mL centrifuge tubes
- Round-bottom Polypropylene tubes: 12 x 75 mm (5 mL) or 17 x 100 mm (15 mL)
- 0.2 µm, 500 mL filter units
- 70 µm cell strainers
- Sterile Pasteur pipettes
- Pipettes and pipette tips

EQUIPMENT

- Centrifuge
- Hemocytometer
- Microscope
- MagCellect Magnet (R&D Systems, Catalog # MAG997)

REAGENT & MEDIA PREPARATION

- **PBS with 2% FBS** - Add 10 mL of FBS to 490 mL of PBS. Sterile filter the solution using a 0.2 µm, 500 mL filter unit. Store at 2 - 8° C for up to 1 month.
- **Preparation of MagCellect Buffer** - Prepare 25 mL of 1x MagCellect Buffer for each 1.0×10^{8} cells to be processed by mixing 2.5 mL of MagCellect 10x Buffer with 22.5 mL sterile deionized or distilled water. The 1x MagCellect Buffer should be kept on ice (or refrigerated) and used within 24 hours.
- **Preparation of Bone Marrow Cells** - Prepare a suspension of mononuclear cells from mouse bone marrow using traditional methods. Both femurs and tibiae from one mouse typically yield $2.0 - 6.0 \times 10^{7}$ hematopoietic cells, $0.3 - 1.0 \times 10^{6}$ of which are lineage negative. A detailed protocol can be found in Current Protocols in Immunology, Isolation of Murine Macrophages (1994), Coligan, J.E. *et al*. eds. John Wiley & Sons, Inc., Volume 3, Supplement 11, 14.1.4. To remove cell clumps and debris after harvesting the bone marrow cells, pass the cell suspension through a 70 µm nylon cell strainer.

  Wash the cells in 50 mL centrifuge tubes with cold PBS with 2% FBS by centrifuging at 300 x g for 8 minutes at 2 - 8° C. Remove the supernatant completely and resuspend the cells in 2 mL cold 1X MagCellect Buffer. Count the cells and bring the cell suspension to 2×10^{7} cells/mL in 1x MagCellect Buffer. Lysing of red blood cells is not required.

  Note: Reaction incubations must be carried out at 2 - 8° C in a refrigerator and not in ice baths to avoid excessively low temperatures that can slow optimized kinetic reactions.

  Note: If sterile cells are required following lineage depletion, the entire procedure should be carried out in a laminar flow hood to maintain sterile conditions. Use sterile equipment when pipetting reagents that will be reused at a later date.

  Note: Avoid antibody capping on cell surfaces and non-specific cell tagging by working fast, keeping cells and solutions cold through the use of pre-cooled solutions, and by adhering to the incubation times and temperatures specified in the protocol. Increased temperature and prolonged incubation times may lead to non-specific cell labeling, thus lowering cell purity and yield.

PROCEDURE (Figure 1)

I. Lineage Depletion of Bone Marrow Cells using the MagCellect Mouse Hematopoietic Lineage Depletion Kit

1. Place 1×10^{8} cells (5.0 mL volume) into a sterile 15 mL centrifuge tube. Add 50 µL of MagCellect Blocking Reagent-1. Mix gently and incubate at 2 - 8° C in a refrigerator for 15 minutes.

   Note: See below for processing different cell numbers.

2. Add 100 µL of Lineage Depletion Biotinylated Antibody Cocktail. Mix gently and incubate at 2 - 8° C in a refrigerator for 15 minutes.
3. Wash away excess antibody by filling the tube to the 15 mL mark with cold 1x MagCellect Buffer and centrifuge at 300 x g for 8 minutes.
4. Remove the supernatant completely and resuspend the cell pellet with 1 mL of cold 1x MagCellect Buffer by gently pipetting up and down.
5. Transfer the cells to a clean 5 mL tube (12 x 75 mm). Retain 0.5×10^{6} cells to assess the proportion of lineage positive cells by staining with a suitable streptavidin-fluorochrome conjugate.
6. Add 150 µL of MagCellect Streptavidin Ferrofluid. Mix gently and incubate at 2 - 8° C in a refrigerator for 15 minutes.
7. At the end of the incubation period bring the volume of the reaction in the tube to 2 mL by adding 0.85 mL of 1x MagCellect Buffer. Mix gently to ensure that all reactants in the tube are in suspension.

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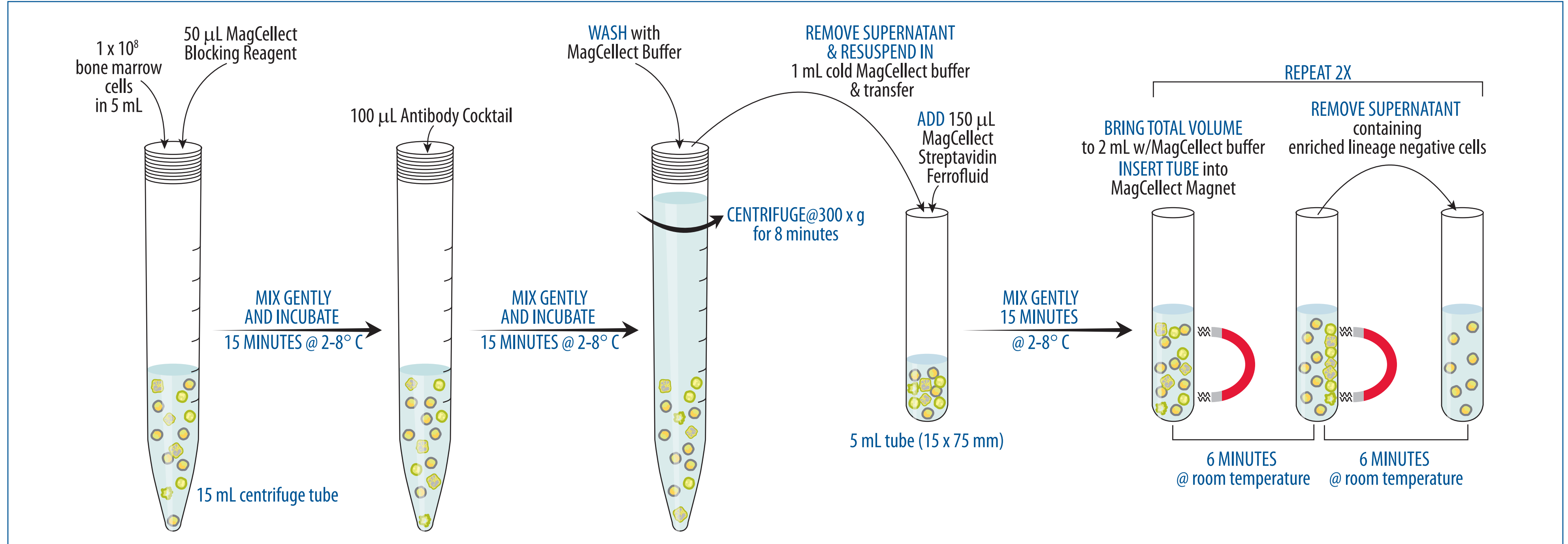
Figure 1



8. Place the reaction tube in the MagCellect Magnet that has been positioned horizontally to accommodate 5 mL tubes, and incubate for 6 minutes at room temperature. Magnetically tagged cells will migrate toward the magnet (these are the unwanted cells), leaving the desired cells suspended in the supernatant.

9. Recovery of desired cells is achieved as follows: while the tube is in the magnet use a sterile Pasteur pipette or transfer pipette to carefully aspirate all of the reaction supernatant. Place the supernatant in a new 5 mL tube and discard the tube containing the magnetically trapped cells.

10a. To ensure that all of the magnetic nanoparticles have been removed, repeat the magnetic depletion (step # 8 and # 9) with the new tube containing the recovered cells. The supernatant obtained at the end of these steps is the final depleted cell fraction containing the lineage negative cells. The cells are now ready for counting, staining or other downstream applications.

10b. **When processing different numbers of cells, observe the following guidelines:**

Add 5 µL of Blocking Reagent-1, 10 µL of Antibody Cocktail, and 15 µL of Streptavidin Ferrofluid per each 1 x 10^7 cells processed.

10c. **When processing 2 x 10^8 cells or fewer:**

Use the 12 x 75 mm (5 mL) tubes with the MagCellect Magnet horizontally positioned to accommodate up to six 5 mL tubes. *Do not process more than 2 x 10^8 cells in each 5 mL tube and do not exceed a total reaction volume of 3 mL in each tube.* A reaction volume of 2 mL is recommended for processing 1 x 10^8 cells. A reaction volume of 1 mL is recommended when processing 5 x 10^7 or fewer cells. *Reaction volume adjustments must be made using 1x MagCellect Buffer just prior to the magnetic separation step.*

10d. **When processing greater than 2 x 10^8 cells:**

Use 17 x 100 mm (15 mL) tubes with the MagCellect Magnet positioned vertically to accommodate up to two tubes. *Do not process more than 6 x 10^8 cells in each 15 mL tube and do not exceed a total reaction volume of 9 mL in each tube.* When using this larger tube, increase the reaction volume before the magnetic separation step according to the following formula: 3 mL for each 2 x 10^8 cells processed. Also increase the magnetic incubation time described in step # 8 to 8 minutes. *Reaction volume adjustments must be made using 1x MagCellect Buffer just prior to the magnetic separation step.*

Protocol I: The Human Colony Forming Cell (CFC) Assay using Methylcellulose-based Media

The colony forming cell (CFC) assay, also referred to as the methylcellulose assay, is an *in vitro* assay used in the study of hematopoietic stem cells. The assay is based on the ability of hematopoietic progenitors to proliferate and differentiate into colonies in a semi-solid media in response to cytokine stimulation. The colonies formed can be enumerated and characterized according to their unique morphology.

Please read the protocol in its entirety before starting.

SUPPLIES REQUIRED

REAGENTS

- Cells derived from bone marrow, blood, or enriched $CD34^+$ cells
- Methylcellulose-based Media (R&D Systems, Catalog # HSC001, HSC002, HSC003, HSC004 or HSC005)
- Iscove's Modified Dulbecco's Media (IMDM)
- Ca^{2+}/Mg^{2+} -free Hank's Balanced Salt Solution (HBSS)
- Ficoll-Paque™ PLUS (GE Healthcare, Catalog # 17-1440-03)

MATERIALS

- 100 mm culture plates
- 35 mm culture plates
- 15 mL centrifuge tubes
- 50 mL centrifuge tubes
- 10 mL syringes
- 3 mL syringes
- 5 mL vials
- 16 gauge 1½ inch needle
- 14 gauge laboratory pipetting needle (Popper & Sons, Catalog # 7941 or Fisher Scientific (Catalog # 14-825-16M)
- Heparinized syringes or Vacutainers®
- Serological pipettes
- Pipettes and pipette tips

Vacutainer is a registered trademark of Becton Dickinson & Co. Ficoll-Paque PLUS is a trademark of GE Heathcare, Ltd.

EQUIPMENT

- 37° C, CO_2 humidified incubator
- Centrifuge
- Vortex mixer
- Hemocytometer
- Inverted Microscope

REAGENT & MEDIA PREPARATION

Note: Sterile technique is required when handling the reagents.

- **Methylcellulose-based Media** - Thaw the bottle of media at 2 - 8° C overnight. After the media is completely thawed, shake the bottle vigorously to thoroughly mix the contents. Allow air bubbles to escape by placing the bottle either at room temperature or at 2 - 8° C for 0.5 - 1 hour.

  Aliquot the exact amount of media required for a single experiment (Table 1) into sterile 5 mL vials using a sterile 14 gauge laboratory pipetting needle and a 10 mL syringe.

  Note: Due to the high viscosity of methylcellulose media, the use of a syringe is necessary to accurately measure volume. The 14 gauge laboratory pipetting needle referred to in the Supplies Required section is recommended due to its larger diameter. The needle is autoclavable and reusable.

  Store the aliquots at -20° C in a manual defrost freezer until use.
  Do not use past the kit expiration date.

Table 1. Due to the different requirements for each product in the CFC assay, the recommended volume for each is listed.

REAGENT *	CATALOG #	VOLUME x 2 (mL) ◆	VOLUME x 3 (mL) ✦
Methylcellulose Stock Solution	HSC001	1.4	2.1
Human Methylcellulose Base Media	HSC002	2.7	3.6
Human Methylcellulose Complete Media	HSC003	3.0	4.0
Human Methylcellulose Complete Media without EPO	HSC004	3.0	4.0
Human Methylcellulose Enriched Media	HSC005	3.0	4.0

* Please see page 10 for a more detailed description of each media

◆ Volume for Duplicate Experiments (mL)

✦ Volume for Triplicate Experiments (mL)

- **Cell Resuspension Solution:** Thaw the bottle at 2 - 8° C. Mix the solution thoroughly using a serological pipette. Aliquot and store at -20° C in a manual defrost freezer. **Do not use past the expiration date.**

PROCEDURE

Preparation of Mononuclear Cells:

Note: When handling biohazardous materials such as human blood, safe laboratory procedures should be followed and protective clothing should be worn.

1. Collect peripheral blood in heparinized syringes or Vacutainers. Immediately mix the samples gently to prevent clotting. Cord blood and leukapheresis product from mobilized peripheral blood should already be heparinized.
2. Dilute the sample with HBSS before proceeding to Ficoll-Paque gradient centrifugation. For whole blood, dilute with an equal volume of HBSS. For leukapheresis product, dilute with three volumes of PBS.
3. Add the diluted sample to 50 mL sterile centrifuge tubes. Underlay the diluted sample with 15 mL of sterile Ficoll-Paque PLUS. Centrifuge at 400 x g for 20 minutes with the brake off.
4. Carefully harvest the mononuclear cells from the interface between the Ficoll-Paque PLUS and sample buffer using a sterile Pasteur pipette. Transfer the cells to sterile centrifuge tubes.
5. Wash with an equal volume of HBSS and centrifuge for 10 minutes at 400 x g to remove the Ficoll-Paque PLUS residue.
6. If multiple tubes are used, pool the cells together and wash a second time in a large volume of HBSS.

Note: At this point, it is optional to enrich the $CD34^+$ population using standard enrichment procedures.

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Methylcellulose Assay (Figures 1 & 2)

1. Thaw aliquots of Methylcellulose-based Media and Cell Resuspension Solution at room temperature for approximately 30 minutes without disturbance. Cell Resuspension Solution is included in Catalog # HSC002, HSC003, HSC004, and HSC005 only.
2. While the aliquots are thawing, resuspend the mononuclear cells in 10 mL (or other appropriate volume) of IMDM and count.
3. Calculate the total number of cells using **Table 2** to determine the recommended final cell number per 35 mm culture plate. Transfer the appropriate volume of cells (plus a slight excess) into a new 15 mL centrifuge tube and centrifuge for 10 minutes at 300 x g.
4. Remove the supernatant and resuspend the cells in Cell Resuspension Solution (or an appropriate media) to the desired stock cell number found in **Table 2.** The stock cell number is approximately 10x the final number needed for the experiment.

   Notes: For Catalog # HSC002, HSC003, HSC004 and HSC005, resuspend the cells in the Cell Resuspension Solution provided. When using Catalog # HSC001, resuspend the cells in the appropriate media determined by each laboratory for the specific assay.

   Due to the limited volume of Cell Resuspension Solution provided, it is important that only the required number of cells be resuspended.

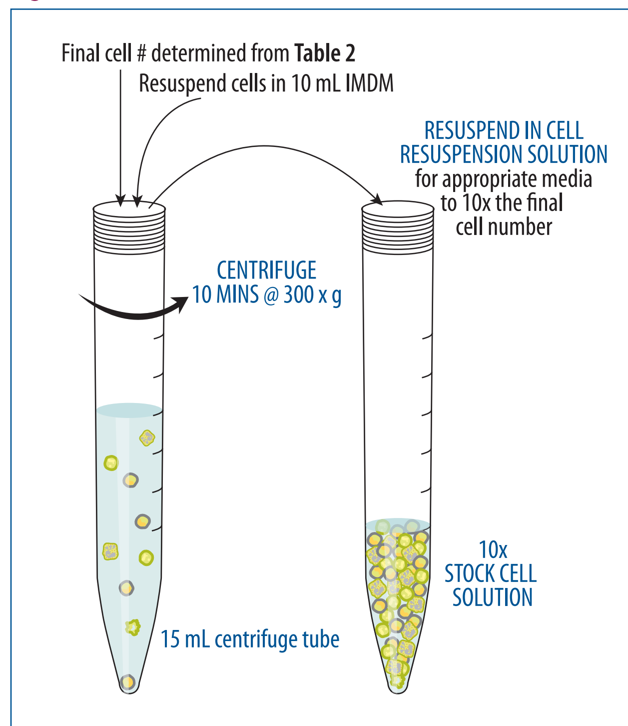
Table 2. Determining the approximate cell number needed for each 35 mm culture plate.

SAMPLE SOURCE	FINAL CELL NUMBER [1]	STOCK CELL NUMBER (10x final)
Enriched $CD34^+$ Cells	5.0×10^2 - 2.0×10^3	5.0×10^3 - 2.0×10^4
Cord Blood	5.0×10^3 - 2.5×10^4	5.0×10^4 - 2.5×10^5
Peripheral Blood	1.0×10^5 - 2.0×10^5	1.0×10^6 - 2.0×10^6
Mobilized Peripheral Blood	1.0×10^4 - 5.0×10^4	1.0×10^5 - 5.0×10^5
Bone Marrow (low-density)	1.0×10^4 - 5.0×10^4	1.0×10^5 - 5.0×10^5

[1] Final Cell Number per 35 mm Culture Plate (or 1.1 mL media)

Note: The cell plating numbers listed above serve as a reference only. Optimal cell plating concentration should be determined by each laboratory for each cell type.

Figure 1



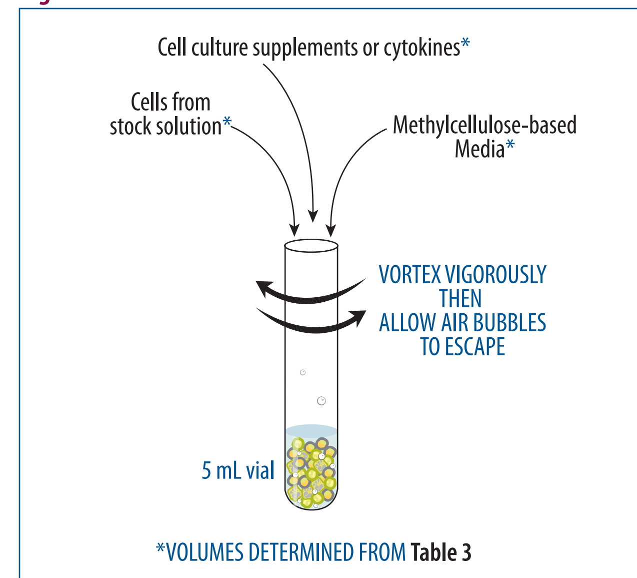
5. **Table 3** provides the recommended volume of cells from the 10x cell stock and additional culture supplements added to the indicated volume of methylcellulose media. The methylcellulose concentration in the final cell mixture should be approximately 1.17%.

Table 3. Volumes necessary for experiments using 35 mm culture platees in duplicate or triplicate.

	Catalog # HSC001		Catalog # HSC002		Catalog # HSC003, Catalog # HSC004, Catalog # HSC005	
FOR EXPERIMENTS USING CELL SAMPLES IN →	DUPLICATE	TRIPLICATE	DUPLICATE	TRIPLICATE	DUPLICATE	TRIPLICATE
Methylcellulose-based media	1.4 mL	2.1 mL	2.7 mL	3.6 mL	3.0 mL	4.0 mL
Culture supplements or cytokines	1.6 mL	2.4 mL	0.3 mL	0.4 mL	None*	None*
Cells	0.3 mL	0.45 mL	0.3 mL	0.4 mL	0.3 mL	0.4 mL

*No additional culture supplements or cytokines are needed.

Figure 2



6. Vigorously vortex the vial to thoroughly mix cells with the media.
7. Wait for approximately 20 minutes before continuing to allow air bubbles to escape.

8. Add 1.1 mL of the final cell mixture to a 35 mm culture plate using a 3 mL syringe fitted with a 16 gauge needle. Spread the media evenly by gently rotating the plate. (Figure 3)
9. Place two sample plates and an uncovered plate containing 3 - 4 mL sterile water in a 100 mm culture plate and cover. The sterile water plate serves to maintain the humidity necessary for colony development.
10. Incubate the cells for 14 - 16 days at 37° C and 5% CO_2. Avoid disturbing the plate during the incubation period to prevent shifting of the colonies.

Colony Scoring (Figure 4)

Score the colonies at the end of the incubation period. Identify and count the individual colonies using an inverted microscope and a scoring grid.

- Prepare the scoring grid as described in the Scoring Grid section. The diagram provided below can be used as a template to reproduce the scoring grid on a 100 mm culture plate. Mark the grid on a new 100 mm culture plate by placing the culture plate on the template and tracing the grid with a marker.
- Refer to the Counting Criteria section for guidance on how to identify and count colonies.

Figure 3

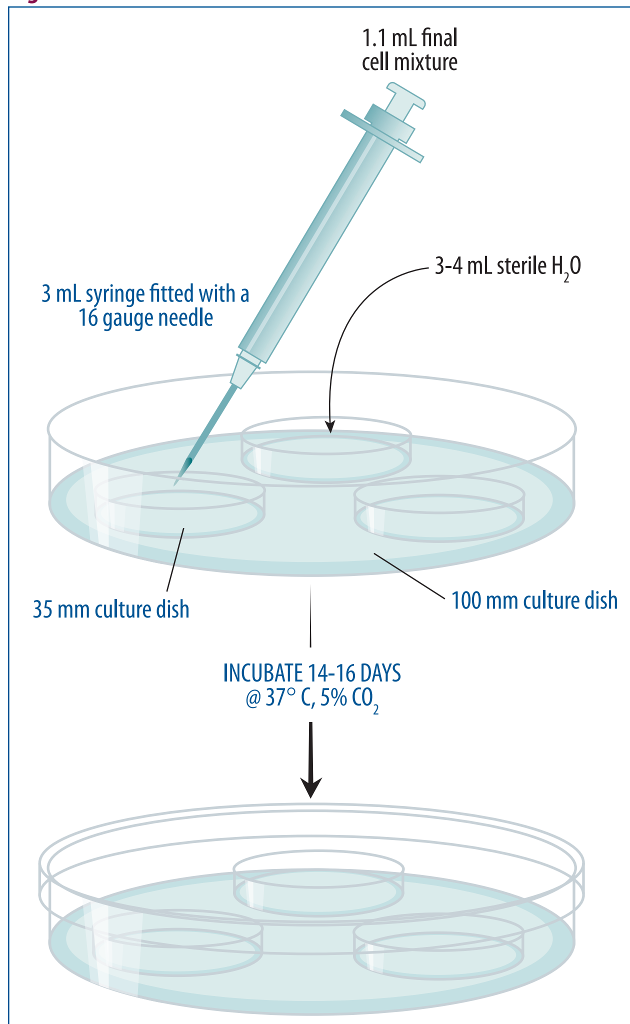
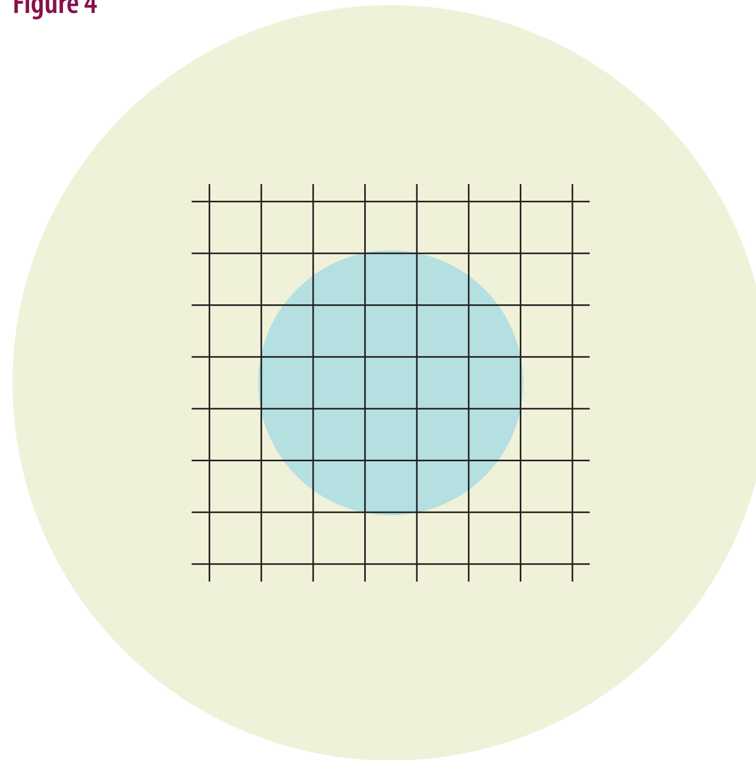


Figure 4



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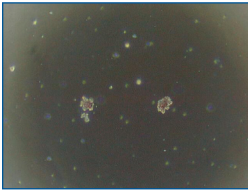
Counting Criteria

Colonies consisting of at least 40 cells are counted (or the minimum cell count set by each laboratory).

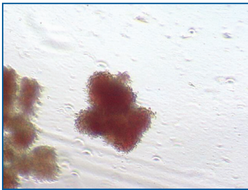
COLONY TYPES

- **CFU-E (Colony forming unit-erythroid):**



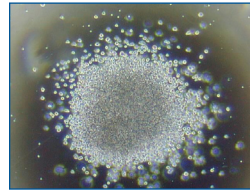
Clonogenic progenitors that produce only one or two clusters with each cluster containing from 8 to approximately 100 hemoglobinized erythroblasts. It represents the more mature erythroid progenitors that have less proliferative capacity. Erythroblasts reach maturity by 10 to 12 days and can be distinguished by the red color displayed. After day 14, the colony may appear brownish due to lysed erythroblasts.

- **BFU-E (Burst forming unit-erythroid):**



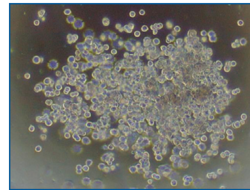
The size of the colony can be described as small (3 to 8 clusters), intermediate (9 to 16 clusters), or large (more than 16 clusters) according to the number of clusters present. A single large cluster is occasionally observed. These are primitive erythroid progenitors that have high proliferative capacity. Due to the variation in the differentiation stage, some colonies may not be completely hemoglobinized and mature until days 18 to 20.

- **CFU-G (Colony forming unit-granulocyte):**



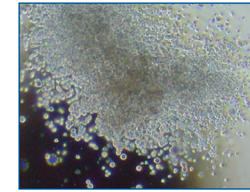
Clonogenic progenitors of granulocytes that give rise to a homogeneous population of eosinophils, basophils or neutrophils. The developed colony is colorless, unlike the red color displayed by CFU-E and BFU-E colonies. Individual cells can be discriminated from macrophages by their smaller size. Both compact and diffuse colony morphology is observed.

- **CFU-M (Colony forming unit-macrophage):**



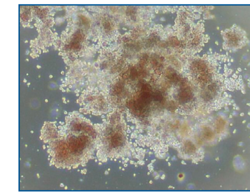
Clonogenic progenitors of macrophages that give rise to a homogenous population of macrophages. The developed colony is colorless. Macrophages are large cells in comparison to granulocytes and erythrocytes and they continue to grow in size after day 14. The colony can drastically expand its size by days 16 to 18.

- **CFU-GM (Colony forming unit-granulocyte, macrophage):**



Progenitors that give rise to colonies containing a heterogeneous population of macrophages and granulocytes. The morphology is similar to the CFU-M and CFU-G descriptions.

- **CFU-GEMM (Colony forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte):**



Multi-lineage progenitors that give rise to erythroid, granulocyte, macrophage and megakaryocyte lineages, as the name indicates. It can be identified by red cells (erythroid) mixed with colorless cells (granulocytes, macrophages, and megakaryocytes) in a single colony.

PRODUCT GUIDE (FOR USE IN HUMAN STUDIES)

Table 4. Colonies Supported by Methylcellulose Products.

Product Name	Catalog #	Colonies Supported					
		CFU-E	BFU-E	CFU-G	CFU-M	CFU-GM	CFU-GEMM
Methylcellulose Stock Solution	*HSC001	NA*	NA*	NA*	NA*	NA*	NA*
Human Methylcellulose Base Media	*HSC002	NA*	NA*	NA*	NA*	NA*	NA*
Human Methylcellulose Complete Media	HSC003	Yes	Yes	Yes	Yes	Yes	Yes
Human Methylcellulose Complete Media without Epo	HSC004	No	No	Yes	Yes	Yes	No
Human Methylcellulose Enriched Media	HSC005	Yes	Yes	Yes	Yes	Yes	Yes

*HSC001 and HSC002 do not contain any cytokines and will not support colony growth unless conditioned media, cytokines, or other culture supplements are added.

Notes

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