

In Vitro Hematopoietic Differentiation of mESCs & miPSCs

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Toll-Free T. 1.800.667.0322
Toll-Free F. 1.800.567.2899
T. 1.604.877.0713
F. 1.604.877.0704
E. info@stemcell.com
E. orders@stemcell.com

In Europe

Toll-Free T. 00.800.7836.2355
Toll-Free F. 00.800.7836.2300
T. +33 (0)4.76.04.75.30
F. +33 (0)4.76.18.99.63
E. info.eu@stemcell.com

In Australia

Toll-Free T. 1.800.060.350
F. +61 (03)9338.4320
E. info.aus@stemcell.com

In Singapore

T. 65.6776.7754
F. 65.6776.7114
E. info.sg@stemcell.com

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1.0 Introduction

An exciting option in the study of hematopoiesis involves the use of mouse embryonic stem cells (mESCs) and mouse induced pluripotent stem cells (miPSCs). ESCs are totipotent cells derived from the inner cell mass (ICM) of a 3-4 day old blastocyst. These cells possess properties of both the ICM and ectoderm-like cells¹. Under the appropriate culture conditions, ESCs/iPSCs retain the capacity to contribute to all cell lineages when reimplanted back into a blastocyst. This potential, combined with their ease of genetic manipulation and selection, has revolutionized many fields by facilitating the ability to generate transgenic, chimeric, and knockout mice for gene function studies *in vivo*²⁻⁷.

In addition to their use for *in vivo* studies, ESCs & iPSCs can differentiate *in vitro* into complex structures called embryoid bodies (EBs) which contain a number of different cell types. Assay systems have been devised for the detection of a variety of cell types including endothelial⁸⁻¹⁰, neuronal¹¹⁻¹⁴, muscle¹⁵⁻¹⁷, and hematopoietic progenitors¹⁸⁻²⁰. The *in vitro* hematopoietic differentiation of mESCs/miPSCs has been extensively examined at both the cellular and molecular levels²¹⁻²³. Various techniques have been used to promote hematopoietic differentiation, including culture on stromal layers^{24, 25}, in chemically-defined suspension media in the presence of hematopoiesis factors²⁶, or in methylcellulose-based semisolid media containing cytokines^{18, 21}.

1.1 Two-Step In Vitro Differentiation of ESCs in Methylcellulose

Differentiation of mESCs/miPSCs in semisolid methylcellulose-based media yields high numbers of hematopoietic progenitors and the use of a two-step procedure has greatly enhanced the ability to quantitate hematopoietic development in this system²¹. In the first step, mESCs/miPSCs are suspended as single cells in methylcellulose-based medium which promotes their “primary differentiation” into EBs. This permits determination of the frequency with which differentiating mESCs/miPSCs form EBs and allows quantitation of EBs at various times throughout the “primary differentiation”. In the second step, EBs are disrupted into single cells and replated in methylcellulose-based medium containing a cocktail of hematopoietic cytokines. The various types of hematopoietic progenitors present in the EBs then grow out into discrete hematopoietic colonies that are easily identified in the methylcellulose cultures. Quantitation of these colonies allows a direct estimation of the number and type of hematopoietic progenitors present at various stages of the primary differentiation culture.

Numerous molecular analyses have been carried out to examine the expression of various developmental and hematopoietic genes (e.g. genes encoding cytokines, transcription factors, and cell surface antigens) during the primary differentiation process²¹⁻²³. These cellular and molecular studies have revealed that, in many ways, this *in vitro* model closely parallels *in vivo* developmental events¹⁸⁻²⁰. The two-step *in vitro* differentiation procedure involving primary differentiation of mESCs/miPSCs into EBs and secondary plating in methylcellulose cultures to form hematopoietic colonies is depicted in Figure 1.1.

Although differentiation in semi-solid media such as methylcellulose is the most quantitative method for the formation of EBs from mESCs/miPSCs and generally yields the highest numbers of hematopoietic progenitors per input mESCs/miPSCs, other techniques exist which might be better suited to particular situations. For example, when it is desirable to isolate EBs at early stages of the primary differentiation process (e.g. for isolation of RNA or early cells), differentiation in suspension culture facilitates the harvest of the small EBs.

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E. info.aus@stemcell.com

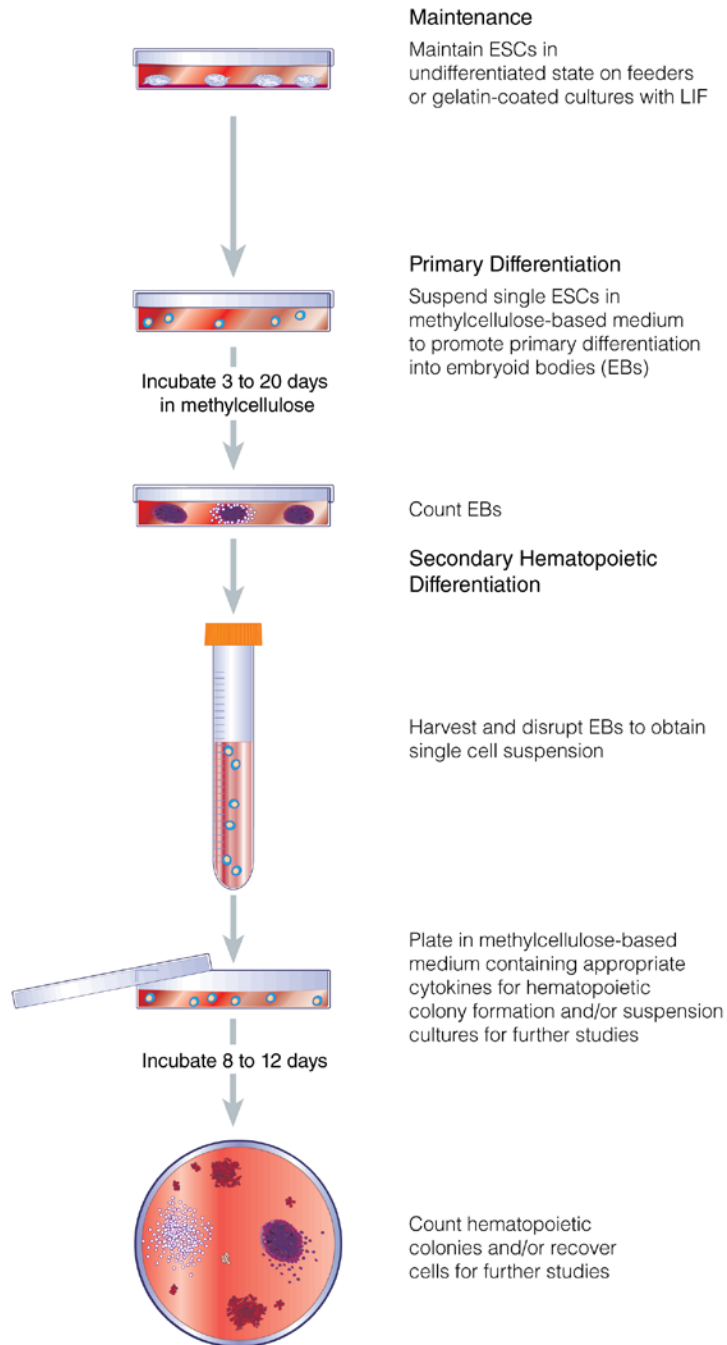
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Figure 1.1 Hematopoietic *In Vitro* Differentiation of mESCs & miPSCs



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A second suspension culture method involves differentiation in chemically-defined media (CDM). These conditions allow mesodermal differentiation of EBs which give rise to hematopoietic progenitors²⁶. This protocol permits the researcher to examine the ability of certain conditions, chemicals or gene products to induce hematopoietic differentiation. For example, Johansson *et al*²⁶ developed and used this technique to demonstrate the role of bone morphogenetic protein- 4 (BMP-4) in the induction of hematopoietic development in the *in vitro* mESC/miPSC model. Differentiation of mESCs on selected stromal layers has also been shown to permit the generation of lymphoid cells^{24,25}, as well as to enhance myeloid differentiation²⁷.

There are several potential advantages to using the mESC/miPSC system as a means to identify and analyze the molecules which regulate early hematopoietic development. First, at all stages of the developmental process there is accessibility to sufficient numbers of cells for analysis. Second, one can examine the effects of genetic manipulations on the cell types of interest without concern for embryonic lethality. In addition, the relative ease with which mESCs/miPSCs can be genetically manipulated, clones isolated, and hematopoiesis accurately assessed makes this an exceptionally powerful screening technique for identifying and characterizing genes which may be involved in the process of hematopoiesis.

1.2 The ES-Cult[®] Family of Products

We strongly recommend that you read this entire Technical Manual before commencing your mESC/miPSC experiments. For maintenance, we offer the ES-Cult[®] Maintenance Kit (Catalog #03150). Please refer to the Technical Manual “Maintenance of mESCs & miPSCs Using ES-Cult[®]” (Catalog #29141) for more information.

Two main criteria must be considered regarding the media, fetal bovine serum (FBS), supplements and accessory reagents utilized in experiments involving *in vitro* differentiation of mESCs/miPSCs. One is that they must preserve both the totipotent, undifferentiated phenotype of the mESCs/miPSCs and their subsequent ability to differentiate efficiently *in vitro*. The second is that the reagents used in the differentiation procedure effectively support this process. All reagents used must be of the highest quality and must be carefully screened to ensure they support the desired ESC characteristics. This can be a very tedious and time-consuming process for individual laboratories to contend with. All ES-Cult[®] products from STEMCELL Technologies have been pre-tested or screened in the appropriate ESC functional assays. ES-Cult[®] ES-tested FBS (Catalog #06902 and #06952) has been selected for its ability to maintain mESCs/miPSCs in the undifferentiated state for the generation of knockout transgenic mice and/or the generation of embryoid bodies and hematopoietic progenitors by two-stage *in vitro* differentiation. Therefore, ES-Cult[®] products allow you to confidently perform your mESC/miPSC experiments without delay.

The protocols in this manual have been designed to yield optimal levels of hematopoietic progenitors when the quality-controlled ES-Cult[®] reagents suggested at each step are used. They were devised and tested with the CCE cell line specifically selected for growth on gelatin and for the ability to differentiate into hematopoietic progenitors. Results obtained will depend upon the actual mESC/miPSC line, the maintenance conditions, and the supplements and growth factors used. Results may also vary if non-ES-Cult[®] reagents are substituted within the protocols.

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E. info.eu@stemcell.com

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F. +61 (03)9338.4320
E. info.aus@stemcell.com

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F. 65.6776.7114
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1.3 ES-Cult® Products from STEMCELL Technologies Inc.

1.3.1 Products for Growth of Undifferentiated mESCs and miPSCs

Catalog #	Product Description	Comments
03150	ES-Cult® Maintenance Kit	Contains reagents for the maintenance of undifferentiated mESCs & miPSCs

1.3.2 Kits for *In Vitro* Hematopoietic Differentiation of mESCs and miPSCs

ES-Cult® Hematopoietic Starter Kit with Cytokines			Catalog #03161
Catalog #	Description	Quantity	Unit Size
36150	Iscove's MDM	1	500 mL
06900	ES-Cult® Fetal Bovine Serum	1	100 mL
03120	ES-Cult® M3120 Medium	2	40mL
02731	rm Stem Cell Factor	3	10 µg
02733	rm Interleukin-3	1	10 µg
02506	rh Interleukin-6	1	10 µg
02625	rh EPO	1	500 U
09500	BIT 9500	1	100 mL
07902	Collagenase	2	5 mL
28110	Blunt- end needles	1	100 needles
28230	3cc syringes	1	30 syringes
27100	35 mm Culture dishes	3	10 dishes
07901	Trypsin-EDTA	1	500 mL

ES-Cult® Hematopoietic Starter Kit without Cytokines			Catalog #03160
Catalog #	Description	Quantity	Unit Size
36150	Iscove's MDM	1	500 mL
06900	ES-Cult® Fetal Bovine Serum for Hematopoietic Differentiation	1	100 mL
03120	ES-Cult® M3120 Medium	2	40mL
09500	BIT 9500 Serum Substitute	1	100 mL
07902	Collagenase	2	5 mL
28110	Blunt end needles, 16 gauge	1	100 needles
28230	Syringes, 3 mL	1	30
27100	35 mm Culture dishes	3	10 dishes
07901	Trypsin-EDTA	1	500 mL

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E. orders@stemcell.com

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T. +33 (0)4.76.04.75.30
F. +33 (0)4.76.18.99.63
E. info.eu@stemcell.com

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E. info.aus@stemcell.com

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T. 65.6776.7754
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1.3.3 ES Screened Supplements and Accessories

Catalog #	Product Description	Comments
06900 06950	ES-Cult [®] Fetal Bovine Serum 100 mL 500 mL	Tested for efficient <i>in vitro</i> hematopoietic differentiation of mESCs/miPSCs
03120	ES-Cult [®] M3120 Medium	Tested for support of <i>in vitro</i> differentiation of mESCs/miPSCs
03434	MethoCult [®] GF M3434 Medium	Contains recombinant cytokines and recombinant erythropoietin for <i>in vitro</i> hematopoietic differentiation
36150	Iscove's MDM	Base culture medium for <i>in vitro</i> differentiation of mESCs/miPSCs
09500	BIT 9500 Serum Substitute	Tested for efficient CFC formation during secondary plating
02733 02506 02732 02731	Recombinant Cytokines: mIL3 hIL6 mGM-CSF mSCF	Complete line of growth factors is available for the <i>in vitro</i> differentiation of mESCs/miPSCs into hematopoietic colonies
37350	Dulbecco's PBS (without Ca ⁺⁺ or Mg ⁺⁺)	For mESC/miPSC cell washing
07100	L-glutamine, 200 mM	Media supplements
07000	Pyruvate, 100 mM	
07600	MEM Non Essential Amino Acids, 10 mM	
07500	Penicillin G + Streptomycin	Antibiotics
07901	Trypsin-EDTA	For disruption of mESC/miPSC colonies and EBs
07902	Collagenase	For disruption of EBs
07903	Gelatin	For coating tissue culture surfaces
27100 27150	35 mm culture dishes 10 dishes 500dishes/case	Tested for efficient EB and CFC formation
28110	Blunt-end needles, 100/pack	For dispensing methylcellulose-based media

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2.0 The Maintenance of mESCs and miPSCs

2.1 Maintenance of mESCs and miPSCs

For the maintenance of mESCs and miPSCs, please refer to the Technical Manual “Maintenance of mESCs & miPSCs Using ES-Cult[®]” (Catalog #29141) where there is a full procedure and list of items required.

If non-ES-Cult[®] products are substituted, it is essential that they are pre-tested to ensure their ability to maintain mESCs/miPSCs in the undifferentiated state.

2.2 Predifferentiation Culture of mESCs/miPSCs

For the *in vitro* differentiation to be successful, it is critical to use mESCs/miPSCs of a low passage number that have been carefully maintained. A good rule of thumb is to use cells which have been in culture for 10 days or less, although this is not always possible. Cells cultured for longer periods may be used, but the efficiency of *in vitro* differentiation will be reduced. In addition, one passage of the mESCs/miPSCs in IMDM instead of DMEM greatly enhances the efficiency of *in vitro* differentiation.

1. 48 hours prior to the start of the *in vitro* hematopoietic differentiation procedure, harvest and centrifuge cells as described the plating and passaging section of Technical Manual “Maintenance of mESCs & miPSCs Using ES-Cult[®]” (Catalog #29141).
2. Prepare gelatinized T-25 cm² flasks. Refer to ‘Gelatin Coating of Tissue Culture-Treated Vessels’ section of the Technical Manual “Maintenance of mESCs & miPSCs Using ES-Cult[®]” (Catalog #29141) for instructions.
3. Prepare Predifferentiation Medium as follows:

Predifferentiation Medium		
Component	Volume Added for 50 mL	Final Concentration
ES-Cult [®] FBS (Catalog #06902/06952)	7.5 mL	15%
Sodium Pyruvate 100 mM* (Catalog #07000)	0.5 mL	1 mM
Penicillin and Streptomycin Solution (Catalog #07500)	0.5 mL	100 U/mL Penicillin 100 µg/mL Streptomycin
Glutamine 200 mM (Catalog #07100)	0.5 mL	2 mM
Non-Essential Amino Acids 10mM (Catalog #07600)	0.5 mL	0.1 mM
LIF (Leukemia Inhibitory Factor) 10 µg/mL (Catalog #02740)	50 µL	10 ng/mL
Monothiolglycerol (MTG) [†] ; (1:100 dilution)	43 µL	100 µM
IMDM* (Catalog #36150)	to final volume of 50 mL	

* It is not absolutely necessary to add sodium pyruvate if the ES-Cult[®] IMDM is used, as it already contains this supplement. If other sources of IMDM are used, check the formulation to determine if addition of sodium pyruvate is required.

[†] MTG working solution is prepared by diluting MTG (Sigma #M6145) 1/100 in IMDM (Catalog #36150)

4. Resuspend cells in Predifferentiation Medium and count live or dead cells using a viability stain.
5. Plate approximately 0.5 - 1 x 10⁵ cells per gelatinized T-25 cm² flask (refer to ‘Gelatin Coating of Tissue Culture-Treated Vessels’ section of the Technical Manual “Maintenance of mESCs & miPSCs Using ES-Cult[®]” (Catalog #29141) for instructions).

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3.0 Two-Step *In Vitro* Differentiation of mESCs and miPSCs

This section provides protocols for the *in vitro* hematopoietic differentiation of mESCs and miPSCs in methylcellulose-based cultures using a two-step method. The first step is the primary differentiation in which the mESCs/miPSCs form embryoid bodies containing a variety of hematopoietic progenitors. The second step involves the plating of cells originating from the embryoid bodies into methylcellulose cultures containing a variety of cytokines for hematopoietic colony formation. This allows the detection and quantitation of the specific types and numbers of hematopoietic progenitors that were present within the embryoid bodies. A great deal of variability exists amongst different mESC/miPSC lines in their ability to differentiate *in vitro*. In addition, the ability of mESCs/miPSCs to generate hematopoietic progenitors *in vitro* is also highly dependent upon the maintenance of the cells prior to setting up the differentiation cultures. In general, it is best to use low-passage mESCs/miPSCs which have been maintained *in vitro* for less than 10 days. As described in the previous section, cells must be passaged once in Predifferentiation Medium (refer to Section 2.2) prior to establishment of the primary differentiation culture.

3.1 Primary Differentiation of mESCs/miPSCs into EBs

3.1.1 Primary Plating

1. Prior to beginning the differentiation steps below, assess the status of your cultures. mESC/miPSC colonies should cover no more than 30 to 50% of the surface area of the tissue culture vessel and should show little or no evidence of differentiation. If cultures differ dramatically from this, the efficiency of EB formation will be significantly decreased. In this case, passage cells once again in Predifferentiation Medium (refer to Section 2.2) at a lower density to improve morphology and differentiation potential. Alternatively, thaw a new vial of early passage mESCs/miPSCs and begin again.

Passaging again reduces the number of differentiated cells since they do not replate well.

2. Harvest the mESCs/miPSCs from the flask using Trypsin-EDTA (Catalog #07901) as described in the plating and passaging section of Technical Manual "Maintenance of mESCs & miPSCs Using ESC-Cult[®]" (Catalog #29141).
3. Wash cells once in IMDM with 10% FBS (Catalog #06900/06950) and resuspend pellet in approximately 2 mL of IMDM, ensuring that a single cell suspension is achieved.

Note that this and subsequent steps employ pre-screened differentiation FBS (Catalog #06900/06950).

4. Count live cells using viability stain to ensure that cells are healthy.

Viability should be greater than 90%, otherwise differentiation will be suboptimal.

5. Prepare 10 mL of a mESC/miPSC suspension at a density of $2 - 5 \times 10^3$ cells/mL. The cell density of the suspension will be cell line dependent and will vary on their ability to differentiate in methylcellulose. Optimally, there will be 50-100 EBs per dish in 1 mL of methylcellulose. As a first step, it may be necessary to perform a dose curve to determine the number of cells required to yield the optimal number of EBs. The number of EBs obtained should be linear with mESC/miPSC input.

As an example, we have found the appropriate range of mESCs/miPSCs plated to be between 200 to 500 cells per dish when using the CCE ESC line that has been adapted for growth on gelatin. When using mESCs/miPSCs maintained on MEFs, we find that plating 1,000 to 5,000 cells per dish is

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generally required. These are approximate starting points, however, and each cell line should be evaluated empirically

- Prepare the methylcellulose-based Primary Differentiation Medium as indicated in the table below. Combine all components except for the methylcellulose in a 50 mL tube, mix thoroughly and then add this mixture directly into the bottle of methylcellulose.

Prepare all methylcellulose-based media in this way to ensure proper mixing of all reagents. The ES-Cult® FBS (Catalog #06900/06950) has been tested and selected to yield high levels of EB formation and generation of hematopoietic progenitors.

Substitution of this FBS for other manufacturers may yield fewer progenitors than expected.

Primary Differentiation Medium		
Component	Volume Added	Final Concentration Upon Addition of Cells
Basic methylcellulose (MC) (Catalog #03120)	40.0 mL (one bottle)	approximately 1% MC
ES-Cult® FBS (Catalog #06900/06950)	15.0 mL	15%
L-Glutamine 200 mM (Catalog #07100)	1.0 mL	2 mM
MTG* (1:100)	0.124 mL	150 µM
murine Stem Cell Factor, 10 µg (mSCF) (Catalog #02731)	0.4 mL	40 ng/mL
IMDM (Catalog #36150)	to final volume of 90 mL	

* It is very important that the MTG be freshly prepared to achieve optimal levels of EB formation. MTG working solution is prepared by diluting MTG (Sigma #M6145) 1/100 in IMDM (Catalog #36150).

- Once the reagent mixture is added to the bottle of methylcellulose, mix vigorously and then allow the air bubbles to dissipate prior to dispensing.

Thorough mixing is critical since the methylcellulose solution is quite viscous.

- Using a 12 mL syringe and a 16g blunt-end needle (Catalog #28110), aliquot a maximum of 13.5 mL of the Primary Differentiation Medium into a 50 mL conical tube. This volume is sufficient for 12 cultures of 1 mL each.

Do not put a greater volume than this into one 50 ml tube or mixing will not be adequate and the number of EBs obtained per dish will be highly variable.

Syringes with blunt-end needles should be used to aliquot methylcellulose solutions, as the viscosity makes pipetting impossible.

- Add the single-cell suspension of mESCs/miPSCs to the tube of Primary Differentiation Medium to yield a 1/10 dilution (e.g. 1.5 mL cells to 13.5 mL medium for a final volume of 15 mL).
- Vortex vigorously and allow air bubbles to dissipate.
- Aliquot 1.0 mL of the mESC/miPSC suspension using a 3 mL syringe (Catalog #28230) and a 16g blunt-end needle (Catalog #28110) into each 35 mm culture dish (Catalog #27100 or 27150) and swirl

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gently to ensure that the suspension is evenly distributed on the bottom of the dish without touching the lid.

Do not coat these dishes with gelatin, as adherence is not desirable at this stage. The 35 mm culture dishes are non-coated and have been pre-tested to ensure they do not allow significant attachment of adherent cells.

- Place dishes into a larger covered culture dish along with an open 35 mm culture dish containing 3 mL of sterile water and incubate at 37°C, 5% CO₂, >95% humidity until further analysis is performed.

EBs will be visible within two to three days and will be large enough to quantitate using an inverted microscope by day 5 or 6 of culture.

If counted too early, EB estimates may be high since some EBs fail to thrive.

- Store all unused Primary Differentiation Medium at -20°C in 15 mL aliquots. It can be used to “feed” the differentiation cultures (refer to Section 3.1.2).

3.1.2 Feeding of Differentiation Cultures

In order to ensure the viability of the primary differentiation cultures over an extended period of time, the cultures are “fed” on day 7 with a dilute methylcellulose medium containing hematopoietic growth factors.

- Prepare the methylcellulose-based Feed Medium as follows:

Feed Medium		
Component	Volume Added for 30 mL	Final Concentration
Primary Differentiation Medium (from above or freshly prepared)	15.0 mL	approximately 0.5% MC
ES-Cult [®] FBS (Catalog #06900/06950)	2.25 mL	15%
MTG (1:100 dilution)*	38 µL	150 µM
murine Stem Cell Factor 10 µg/mL (m SCF; Catalog #02731)	0.48 mL	160 ng/mL
murine Interleukin-3 10 µg/mL (mIL-3; Catalog #02733)	90 µL	30 ng/mL
human Interleukin-6 10 µg/mL (hIL-6; Catalog #02506)	60 µL	20 ng/mL
Human Erythropoietin (rhEPO) (Catalog #02625)		3 U/mL
IMDM (Catalog #36150)	to final volume of 30 mL	

* The MTG must be freshly prepared to achieve optimal levels of EB formation. MTG working solution is prepared by diluting MTG (Sigma #M6145) 1/100 in IMDM (Catalog #36150).

- Layer 0.5 mL of Feed Medium onto the surface of each differentiation culture drop-wise using a 3cc syringe (Catalog #28230) and a 16g blunt-end needle (Catalog #28110).

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3.1.3 Harvest of Embryoid Bodies from Methylcellulose Cultures

Regardless of the age of the EBs in the primary differentiation cultures, the initial stages of the harvest are the same.

1. Flood each culture dish with 1 mL of medium (IMDM plus 2% ES-Cult[®] FBS (Catalog #06900/06950) and mix the liquid medium with the methylcellulose. Transfer contents of dish into a 14 mL round-bottom tube (polystyrene preferred). Transfer no more than two or three dishes into this size tube or 10 to 12 dishes into a 50 mL tube.

A pipette with a 1 mL tip works best for mixing and transferring the methylcellulose cultures to the tubes.

Polystyrene tubes are preferable because the EBs are easier to see and they do not stick to the sides.

2. Wash dish with 1 mL of medium and add this to the tube to ensure all EBs are collected.
3. Mix and centrifuge at 300 x g for 10 minutes. Remove supernatant carefully so as not to disturb the loose pellet.
4. Disrupt EB with Trypsin-EDTA or Collagenase depending upon the age of the EBs, as outlined below.
 - a) For EBs that are up to eight days old:

Add 2 to 3 mL Trypsin-EDTA (Catalog #07901) to pellet and incubate for 2-3 minutes at room temperature. Disrupt EBs by passing through a 21g 1 1/2" needle on a 3cc syringe three times (up and down). Make sure you obtain a single cell suspension.

Do not keep the EBs in the Trypsin-EDTA solution for longer than the indicated time, or cell viability will decrease.

- b) For EBs that are nine or more days old:

Add 2 to 3 mL of Collagenase (Catalog #07902) and incubate at 37°C for one hour, swirling gently following 30 minutes of incubation. Ensure the EBs stay in solution and are not on walls of tube. Disrupt EBs by passing through a 21g 1 1/2" needle on a 3 mL syringe three times (up and down). Make sure you obtain a single cell suspension.

If more than two to three dishes were harvested, increase the amount of Collagenase used to about 5mL.

5. Add IMDM containing 5% FBS (Catalog #06900/06950) to neutralize the Trypsin or Collagenase and pellet cells by centrifugation at 300 x g for 5-8 minutes.
6. Remove supernatant carefully so as not to disturb the loose pellet and and resuspend the cells in a minimum volume (1 to 3 mL) of IMDM plus 2% ES-Cult[®] FBS (Catalog #06900/06950).

The volume required depends on the number of cells present, which relates to both the age of the cultures and the number of dishes harvested.

7. Count cells and adjust their concentration to a density of 1 - 5 x 10⁵ cells/mL (refer to Section 3.2.1, step 4).

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3.1.4 Formation of Simple Embryoid Bodies in Suspension Culture

In general, the use of suspension culture for the formation of embryoid bodies is not very useful to study hematopoiesis since the number of hematopoietic progenitors is greatly reduced in comparison to formation in methylcellulose. Secondly, quantitation is not possible as it is in methylcellulose (i.e. cannot calculate the efficiency of EB formation or the number of hematopoietic progenitors per EB).

However, some cell lines are inefficient in the formation of embryoid bodies in methylcellulose. In this case, it may help to start cultures in suspension and then transfer to methylcellulose 24 - 48hrs later. For the analysis of gene expression at days 3 to 4 of EB formation, the suspension culture system allows for access to a greater number of cells at earlier time points than in methylcellulose²⁹.

1. Prior to beginning the differentiation steps below, assess the status of your cultures. mESC/miPSC colonies should cover no more than 30-50% of the surface area of the tissue culture vessel and should show little or no evidence of differentiation. If cultures differ dramatically from this, the efficiency of EB formation will be significantly decreased. In this case, passage cells once again at a lower density to improve morphology and differentiation potential. Alternatively, thaw a new vial of early passage mESCs/miPSCs and begin again.

Passaging again reduces the number of differentiated cells since they do not replate well.

2. Harvest the mESCs/miPSCs from the flask as described in the plating and passaging section of Technical Manual "Maintenance of mESCs & miPSCs Using ES-Cult[®]" (Catalog #29141) using Trypsin-EDTA (Catalog #07901). If you have trouble with EB formations, it may help to use the Trypsin-EDTA for an even shorter length of time (1 - 2 min), so that small clumps of cells are still present. The clumps will encourage EB formation
3. Neutralize the trypsin using DMEM – 10% FBS (Catalog #06900/06950). Centrifuge to pellet the cell suspension at 300 x g for ~ 5-7 minutes.
4. Aspirate the medium and resuspend in DMEM – 10% FBS (Catalog #06900/06950), ensuring a single cell suspension. If you are using difficult cultures, only pipet up and down against the bottom once or twice to disrupt the cell pellet into small clumps.
5. Plate into 35 mm culture dishes (Catalog #27100/27150) at 4×10^5 cells per dish. Small aggregates (simple embryoid bodies) will be visible in 24hrs. These simple EBs can be transferred into methylcellulose between 24 and 48hrs.
6. If you are continuing in the liquid culture system, the media must be changed every 3-4 days. The EB's will tend to aggregate into clumps with regions of necrosis. To avoid this, break clumps apart by using a large mouth pipet (25mL) such that you do not disrupt the EBs themselves. Transfer the EBs to a tube and allow them to sink to the bottom. Carefully aspirate off the old media, replace with fresh and replate into the culture dish.
7. To disrupt EBs into single cells, refer to 3.1.3, steps 4-7.

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3.2 Plating for Detection of Hematopoietic Progenitors

3.2.1 Secondary Plating

1. Prepare methylcellulose-based Hematopoietic Differentiation Medium as follows:

As before, mix all supplements in a 50 mL tube before adding to the bottle of methylcellulose. Rinse the tube with the last bit of IMDM.

Hematopoietic Differentiation Medium		
Component	Volume Added for 100 mL	Final Concentration
Basic methylcellulose (Catalog #03120)	40 mL	approximately 1% MC
ES-Cult [®] FBS (Catalog # 06900/06950)	15.0 mL	15%
L-glutamine 200 mM (Catalog #07100)	1.0 mL	2 mM
MTG* (1:100)	0.124 mL	150 µM
BIT 9500 Serum Substitute (Catalog #09500)	20 mL	1% BSA 10 µg/mL Insulin 200 µg/mL Transferrin
Murine Stem Cell Factor (Catalog #02731)	1.6 mL	150 ng/mL
Murine Interleukin-3 (Catalog #02733)	0.3 mL	30 ng/mL
Human Interleukin-6 (Catalog #02506)	0.3 mL	30 ng/mL
Human Erythropoietin (Catalog #02625)		3 U/mL
IMDM (Catalog #36150)	to final volume of 100 mL	

* The MTG must be freshly prepared to achieve optimal levels of EB formation. MTG working solution is prepared by diluting MTG (Sigma #M6145) 1/100 in IMDM (Catalog #36150).

2. Aliquot 3.0 mL per 14 mL polypropylene tube using a syringe with a 16g blunt-end needle (Catalog #28110). Store excess tubes at -20°C until needed.
3. Add 0.3 mL of cells at a concentration of $1-5 \times 10^5$ cells per mL to each tube containing the 3 mL Hematopoietic Differentiation Medium and vortex thoroughly. Let stand three to five minutes to allow bubbles to dissipate.
4. Plate 1.1 mL of the cell suspension per 35 mm culture dish (Catalogue #27100/27150).

*This yields a final number of $1 - 5 \times 10^4$ cells per dish. The actual number plated will vary depending on the cell line and conditions used, as well the age of the EBs, but this density should provide a useful starting range. **When first establishing optimal plating densities, it is advisable to try two different cell concentrations which differ by two- to three-fold.***

5. Place dishes into a larger covered dish along with an open 35 mm culture dish containing 3 mL of sterile water and incubate at 37°C, 5% CO₂, >95% humidity.
6. Score the numbers and types of hematopoietic colonies after approximately 10 days of culture.

Colony morphology is best viewed at this time. If the cells are left in culture for longer, they become difficult to identify.

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4.0 Identification and Scoring of Embryoid Bodies and Hematopoietic Colonies

4.1 Example of Quantitation of Hematopoietic EBs and Hematopoietic Progenitors from Two Step *In Vitro* Differentiation of mESCs and miPSCs

Primary Differentiation

Number of mESCs/miPSCs plated per 35 mm dish	300
Number of Dishes plated	5

Day 10

Total EBs scored per dish	mean = 120
Hematopoietic EBs scored per dish	mean = 75

Efficiency of EBs formation	$120/300 = 40\%$
Proportion of Hematopoietic EBs	$75/120 = 65\%$

Harvest at Day 10

Number of Dishes harvested	5
Total Cells harvested	7.5×10^6
Average cells per culture	$7.5 \times 10^6 / 5 \text{ dishes} = 1.5 \times 10^6 \text{ cells per culture}$
Average cells per EB	$7.5 \times 10^6 / 600 = 1.2 \times 10^4 \text{ cells per EB}$

Secondary Plating

- 1.) Plated 2×10^4 cells per mL in methylcellulose-based media.
- 2.) On day 10, an average of 50 colonies per methylcellulose culture were detected.

Average number of colonies per culture:

$50 \text{ CFC per } 2 \times 10^4 \text{ cells plated} = 3,750 \text{ CFC per } 1.5 \times 10^6 \text{ (average cells harvested per culture)}$

Average number of colonies per EBs:

3,750 CFC per culture

3,750 CFC per 120 EBs (average EBs per culture)

31 CFC per EB

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4.2 The Morphology of Undifferentiated mESCs and miPSCs

Photographs 1 and 2

Undifferentiated mESCs/miPSCs have a large nucleus, minimal cytoplasm, and one or more prominent dark nucleoli. It should be difficult to identify individual cells within the mESC/miPSC colony, as there are non-distinct cytoplasmic membranes between the cells. Colonies appear amorphous without a distinct or common shape.

Signs of differentiation include the ability to distinguish individual cells within the mESC/miPSC colony by the defined cytoplasmic membrane for the cells. The colony may appear to spread and cells appear flattened. Cells may lift off of the dish.

4.3 Identification of Embryoid Bodies

Photographs 3-6

Individual mESCs/miPSCs plated in primary differentiation methylcellulose-based medium will proliferate and differentiate into multi-cellular structures called embryoid bodies (EBs) within days. Morphologically, the EBs appear as a dense mass of cells surrounded by a cellular envelope. Clumps of disorganized or non-viable cells should not be scored as EBs. By day 10 to 12 or later, under the appropriate conditions of culture, hematopoietic EBs (Photo 5 and 6 in Section 4.5) can be detected. Morphologically, these can be identified by the presence of macrophages, erythroid cells and occasionally granulocytic cells at the edges of the EB. Hemoglobinization of erythroid cells is often visible.

The efficiency of EB formation and the proportion of hematopoietic embryoid bodies obtained will be dependent upon the ESC line, and the conditions of culture.

4.4 Identification of Hematopoietic Progenitors

It is important to look at all colonies under both low and high power to identify cell types.

The numbers and types of hematopoietic colonies detected in methylcellulose cultures derived from disaggregated EBs is dependent on the mESC/miPSC line, the day of harvest of EBs, and hematopoietic cytokines used in the secondary differentiation.

Primitive Erythroid:

- predominant class of progenitor derived from day 3 to day 8 EBs
- macrophage colonies are also seen
- small clusters containing 8-200 erythroblasts
- score at seven to 10 days of culture

Erythroid cells are larger than erythroblasts present in burst-forming unit-erythroid (BFU-E) from murine adult bone marrow.

Definitive Erythroid: Photographs 7, 9, 11

- detectable from EBs cultured for seven days or longer
- similar in appearance to BFU-E derived from murine bone marrow (smaller cells than primitive erythroid cells)
- multiple clusters each containing 8 to 200 erythroblasts
- score at day 10 to 12 of culture

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Colony-forming unit-granulocyte/macrophage (CFU-GM): Photographs 8, 10

- colonies contain ≥ 30 cells
- usually detectable from EB's cultured for seven to 14 days
- mast cell colonies are predominant after day 12 of culture
- similar in appearance to colony forming unit-granulocyte/macrophage (CFU-GM) derived from murine bone marrow
- colonies contain monocyte-macrophages and/or granulocytes

macrophages: large, round cells with uniform cytoplasmic membrane (can be irregular in shape)

granulocytes: smaller than monocyte-macrophages. Maturing granulocytes have a short half-life and non-viable cells will vary in size.

mast cells: small, uniform refractile cells which can form clusters within the colony.

Mixed Colonies (CFU-Mixed): Photograph 12

- detectable from EBs cultured for seven days or longer.
- contain granulocytes/macrophages and erythroid cells. Megakaryocytes (large cells, slightly irregular in shape, present singularly or in clusters of two to 10 cells) are often present
- similar in appearance to colony forming unit-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM) derived from murine bone marrow.

Cautionary note: It is important to distinguish mixed colonies from partially disrupted EBs or large definitive erythroid colonies which often contain macrophages.

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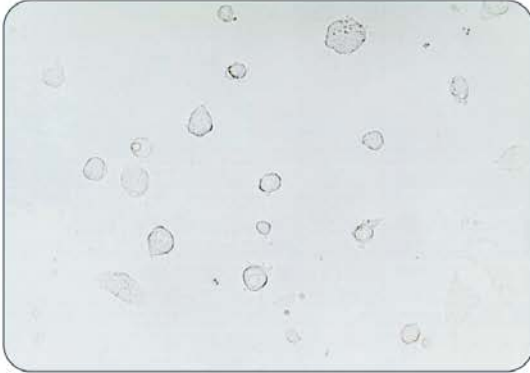
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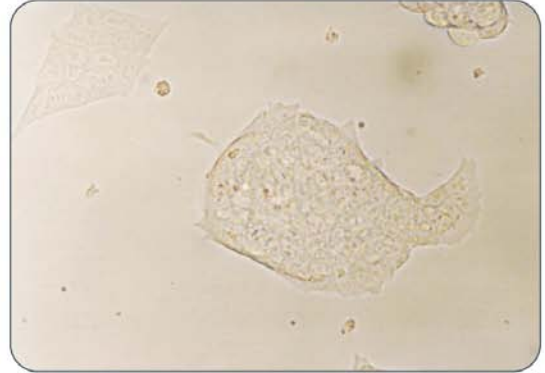
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4.5 Photographs of ESCs, mESC- and miPSC-Derived Embryoid Bodies (EBs) and Hematopoietic Colonies



1. Undifferentiated ESCs, low power



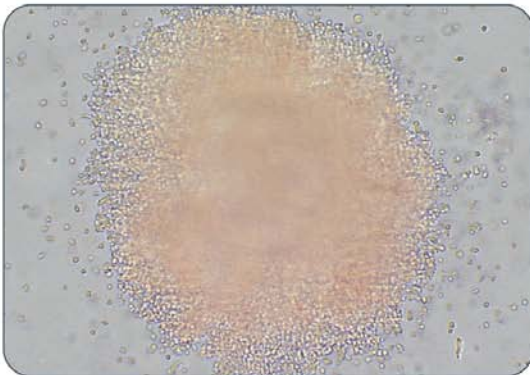
2. Undifferentiated ESCs, high power



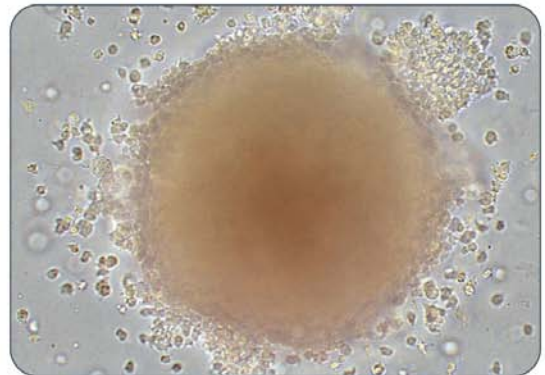
3. EB, day 6, low power



4. EB, day 9, low power



5. EB, day 13 (hematopoietic), low power



6. EB, day 15 (hematopoietic), low power

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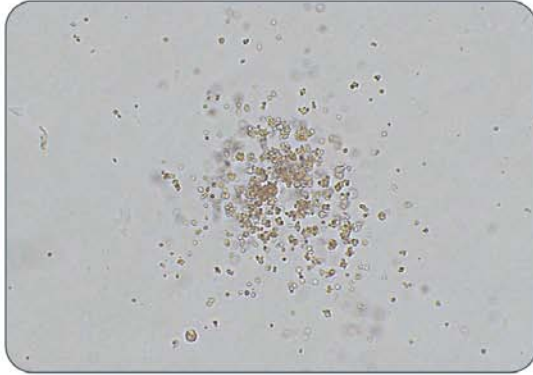
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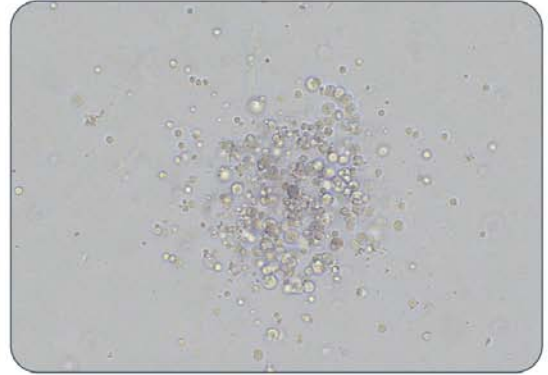
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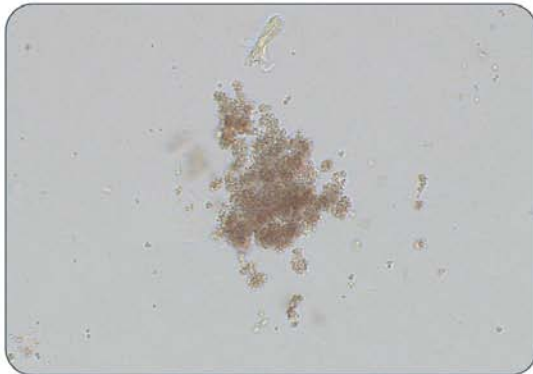
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7. BFU-E from a day 11 EB, low power



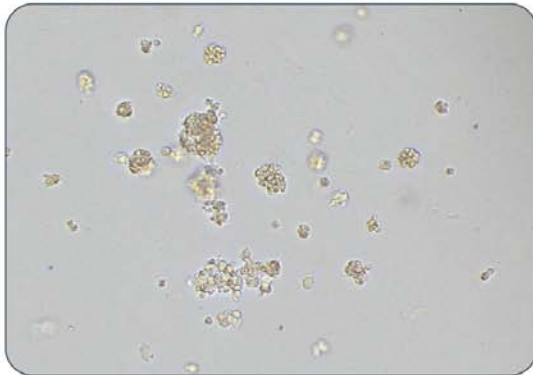
8. CFU-GM from a day 11 EB, low power



9. BFU-E from a day 11 EB, low power



10. CFU-GM from a day 14 EB, low power



11. BFU-E from a day 11 EB, high power



12. CFU-mixed from a day 11 EB

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F. +61 (03)9338.4320
E. info.aus@stemcell.com

In Singapore

T. 65.6776.7754
F. 65.6776.7114
E. info.sg@stemcell.com

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5.0 Helpful Hints

Listed below are the most common problems associated with the maintenance and *in vitro* hematopoietic differentiation of mESCs/miPSCs and the possible causes for these difficulties:

5.1 mESC/miPSC Maintenance

1. Cell death:

- Lack of MTG in the maintenance media
- Passaging the cells at too low of a density
- Toxicity of one of the reagents

All ES-Cult® products have been pre-screened and found to exhibit no observable toxicity to a commonly used ESC line.

2. mESC/miPSC colonies lift off the culture plate during maintenance:

- Petri dishes rather than tissue culture ware used
- Gelatin solution prepared incorrectly
- Excessive differentiation

To avoid improperly made gelatin solutions, we recommend using ES-Cult® gelatin.

3. Excessive differentiation:

- Failure to obtain a single cell suspension when passaging ESCs/miPSCs
- Insufficient or inactive LIF
- Plating too many cells in the culture vessel

Excessive differentiation is characterized by the presence of large numbers of flattened colonies in which the individual cells are visible; mESC/miPSC colonies lifting off the culture dish; the presence of many round floating cells; or the presence of round mESC/miPSC colonies with a clearly defined external membrane surrounding the colony.

5.2 Primary Differentiation

1. Low numbers of EBs generated:

- Differentiation occurred during maintenance steps
- MTG not freshly prepared
- Maintenance FBS used instead of differentiation FBS

2. Variable and inconsistent numbers of EBs per dish:

- Inadequate mixing of growth factors or cells with methylcellulose-based media

Due to its viscosity, care must be taken to completely mix all components of media containing methylcellulose.

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Toll-Free F. 1.800.567.2899
T. 1.604.877.0713
F. 1.604.877.0704
E. info@stemcell.com
E. orders@stemcell.com

In Europe

Toll-Free T. 00.800.7836.2355
Toll-Free F. 00.800.7836.2300
T. +33 (0)4.76.04.75.30
F. +33 (0)4.76.18.99.63
E. info.eu@stemcell.com

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5.3 Secondary Differentiation

1. Few hematopoietic progenitors detected:
 - Insufficient or inactive growth factors used
 - EBs exposed to Trypsin-EDTA for too long during harvesting
 - Previous differentiation of ESCs during maintenance
 - Too few cells plated

5.4 Use of Alternative Products

1. We are often asked if β -mercaptoethanol can be used in place of monothioglycerol. The procedures in this Technical Manual have been developed and optimized for the CCE cell line. If you are using another cell line, use the appropriate reducing agent for your cells.
2. Some of STEMCELL Technologies' other murine methylcellulose products can be used for the differentiation of ESCs. These products have not been extensively pre-tested or screened in the appropriate ESC functional assays.

Product	Catalog Number	Application
MethoCult [®] M3134	03134	alternate to 03120, base methylcellulose
MethoCult [®] M3234	03234	may be used as a base for secondary plating, adding only the appropriate cytokines
MethoCult [®] M3334	03334	substitute base for hematopoietic differentiation medium that contains Erythropoietin but no other cytokines
MethoCult [®] M3434	03434	alternative to preparing hematopoietic differentiation medium (contains the same cytokines but at lower concentrations)

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