TECHNICAL MANUAL

In Vitro Hematopoietic Differentiation of Mouse ES & iPS Cells Using ES-Cult™

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1.0 Introduction

An exciting option in the study of hematopoiesis involves the use of mouse embryonic stem (ES) cells and mouse induced pluripotent stem (iPS) cells. ES cells are pluripotent cells derived from the inner cell mass (ICM) of a 3- to 4-day-old blastocyst. These cells possess properties of both the ICM and ectoderm-like cells.[1] Somatic cells can be reprogrammed to become iPS cells that closely resemble mouse ES cells using four defined factors.[2-5]

Under the appropriate culture conditions, ES and iPS cells retain the capacity to contribute to all cell lineages when implanted 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.[6-11]

In addition to their use for in vivo studies, mouse ES and iPS cells can differentiate in vitro into complex structures called embryoid bodies (EBs) that contain a number of different cell types. Assay systems have been devised for the detection of a variety of cell types including endothelial[12-14], neuronal[15-18], muscle[19-21], and hematopoietic progenitor cells[22-24]. The in vitro hematopoietic differentiation of mouse ES and iPS cells has been extensively examined at both the cellular and molecular levels.[25-27] Various techniques have been used to promote hematopoietic differentiation, including culture on stromal layers[28, 29], in chemically defined suspension media in the presence of hematopoiesis factors[30], or in methylcellulose-based semi-solid media containing cytokines[22, 25].

1.1 Two-Step In Vitro Differentiation of ES Cells in Methylcellulose

Differentiation of mouse ES and iPS cells in semi-solid methylcellulose-based media yields high numbers of hematopoietic progenitor cells, and the use of a two-step procedure has greatly enhanced the ability to quantitate hematopoietic development in this system.[25] In the first step, mouse ES and iPS cells 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 mouse ES and iPS cells 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 progenitor cells 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 progenitor cells 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 process.[25-27] These cellular and molecular studies have revealed that, in many ways, this in vitro model closely parallels in vivo developmental events.[22-24] The two-step in vitro differentiation procedure involving primary differentiation of mouse ES and iPS cells into EBs and secondary plating in methylcellulose cultures to form hematopoietic colonies is depicted in Figure 1.

Although differentiation in semi-solid media such as methylcellulose is the most quantitative method for the formation of EBs from mouse ES and iPS cells, and generally yields the highest numbers of hematopoietic progenitor cells per input mouse ES and iPS cells, other techniques exist that 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.

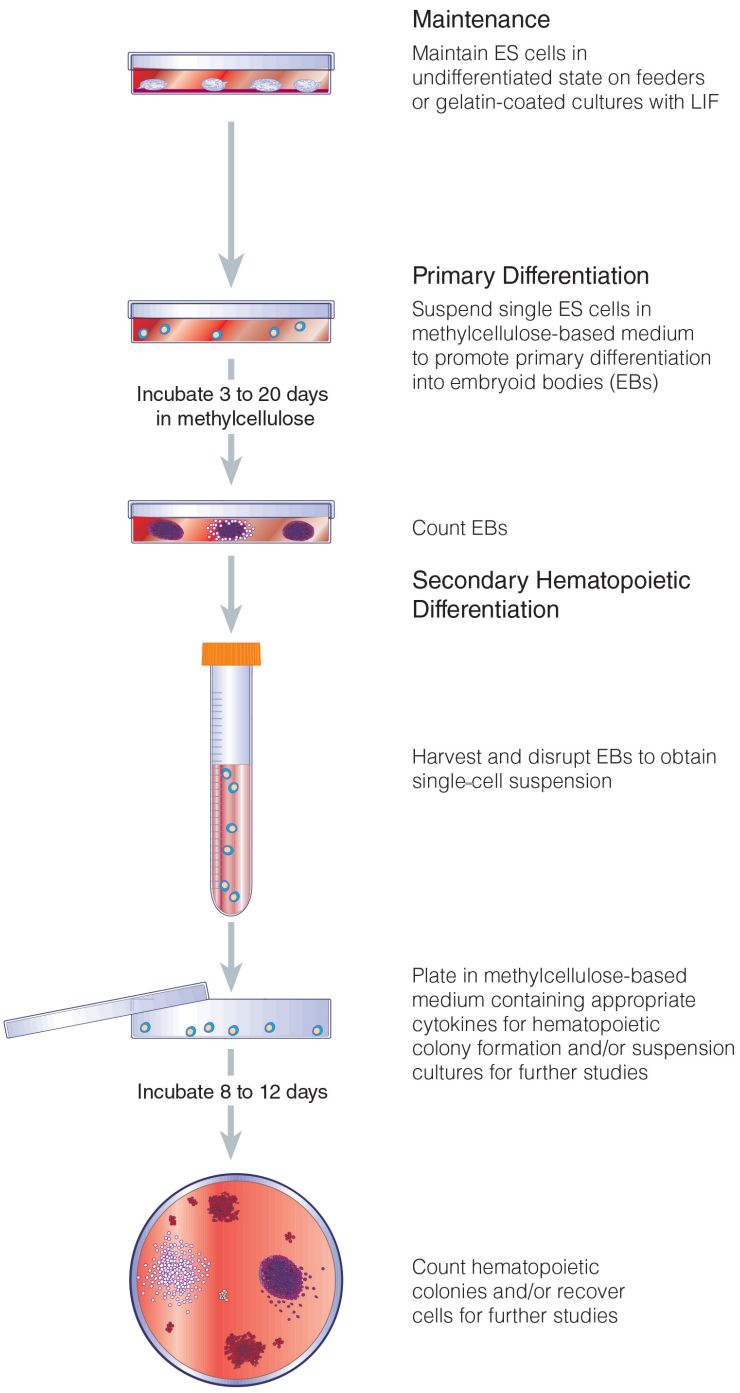


Figure 1. Hematopoietic Differentiation of Mouse ES & iPS Cells

A second suspension culture method involves differentiation in chemically defined media (CDM). These conditions allow mesodermal differentiation of EBs that give rise to hematopoietic progenitor cells.[30] 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.[30] 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 mouse ES/iPS cell model. Differentiation of mouse ES cells on selected stromal layers has also been shown to permit the generation of lymphoid cells[28, 29] as well as to enhance myeloid differentiation[31].

There are several potential advantages to using the mouse ES/iPS cell system as a means to identify and analyze the molecules that 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 mouse ES and iPS cells can be genetically manipulated, clones isolated, and hematopoiesis accurately assessed makes this an exceptionally powerful screening technique for identifying and characterizing genes that 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 mouse ES/iPS cell experiments.

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 mouse ES and iPS cells. One is that they must preserve both the pluripotent, undifferentiated phenotype of the mouse ES and iPS cells 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 ES cell 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 ES cell functional assays.

The protocols in this manual have been designed to yield optimal levels of hematopoietic progenitor cells 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 progenitor cells. Results obtained will depend upon the actual mouse ES/iPS cell 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.

1.3 ES-Screened STEMCELL Products

CATALOG #	PRODUCT	DESCRIPTION
03120	ES-Cult™ M3120	Base methylcellulose medium tested for support of in vitro differentiation of mouse ES and iPS cells
03434	MethoCult™ GF M3434	Methylcellulose-based medium containing recombinant cytokines (including erythropoietin) for in vitro hematopoietic differentiation
36150	Iscove's MDM	Base culture medium for in vitro differentiation of mouse ES and iPS cells
09500	BIT 9500 Serum Substitute	Tested for efficient CFU formation during secondary plating
78042 78050 78017 78064	Recombinant Cytokines: Mouse Recombinant IL-3 Human Recombinant IL-6 Mouse Recombinant GM-CSF Mouse Recombinant SCF	Complete line of growth factors is available for the in vitro differentiation of mouse ES and iPS cells into hematopoietic colonies
02625	EPO, Human, Recombinant	For proliferation and differentiation of erythroid progenitor cells
37350	D-PBS (Without Ca++ and Mg++)	For mouse ES/iPS cell washing
07100	L-Glutamine	Medium supplements
07600	MEM Non-Essential Amino Acid Solution (100X)	
07901	Trypsin-EDTA (0.25%)	For disruption of mouse ES/iPS cell colonies and EBs
07902	Collagenase Type I (0.25%)	For disruption of EBs
07903	0.1% Gelatin in Water	For coating tissue culture surfaces
27100 27150	35 mm Culture Dishes 10 dishes 500 dishes/pack	Tested for efficient EB and CFU formation
28230	3 cc Syringes	For dispensing methylcellulose-based media
28110	Blunt-End Needles, 16 Gauge, 100/pack	For dispensing methylcellulose-based media

2.0 Coating Tissue Culture-Treated Vessels with Gelatin

In order for certain mouse ES cell lines (e.g. the CCE cell line), mouse iPS cell lines, or mitotically inactivated mouse embryonic fibroblast (iMEF) feeder layers to efficiently adhere to the surface of tissue culture-treated cultureware, it is necessary to coat the bottom of the cultureware with gelatin.

1. Dispense sufficient gelatin into a tissue culture-treated plate or flask so that it completely covers the bottom.

   Note: Suggested volumes are 3 mL per T-25 cm^2 flask or 60 mm tissue culture-treated dish; 7 - 8 mL per 100 mm tissue culture-treated dish or T-75 cm^2 flask. To coat other sizes of cultureware, scale the volume of gelatin by the surface area of the vessel to be coated.

2. Incubate at room temperature (15 - 25°C) for at least 20 minutes.

3. Aspirate the gelatin solution and allow any remainder to evaporate by leaving the container in the hood with the lid or cap open until no traces of the liquid remain. Replace the lid once the surface is dry.

   Note: If not used immediately, the gelatinized dishes or flasks can be stored at room temperature (15 - 25°C) for 1 day or at 2 - 8°C for up to 2 weeks.

3.0 Predifferentiation Culture of Mouse ES and iPS Cells

For in vitro differentiation to be successful, it is critical to use mouse ES and iPS cells of a low passage number that have been carefully maintained. One passage of mouse ES and iPS cells in IMDM (i.e. Predifferentiation Medium) 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 follows:
   i. Aspirate medium from culture vessel. Rinse cultures once with D-PBS (Without Ca++ and Mg++).
   ii. Add sufficient room temperature (15 - 25°C) Trypsin-EDTA (0.25%) to cover the cells. This is approximately 2 mL for a 60 mm dish or T-25 cm^2 flask, or 4 mL for a 100 mm dish or T-75 cm^2 flask.
   iii. For most mouse ES or iPS cell lines on gelatinized dishes, incubate at 37°C for 3 - 5 minutes or until the cells begin to lift off the plate. For mouse ES or iPS cells maintained on iMEF feeder layers, incubate at room temperature (15 - 25°C) for 2 - 3 minutes or until the cells begin to lift off the plate.

   Note: For plating and passaging MEF, or inactivation of MEF, refer to section 7.0 or 8.0 respectively.
   iv. Use a serological pipette to pipette up and down and dislodge any remaining cells on the plate. Transfer the cells to a 15 mL tube containing approximately 2 - 4 mL of a solution of DMEM with 4500 mg/L D-glucose containing 10% FBS. Immediately centrifuge the cells at 300 x *g* for approximately 8 minutes.

   Note: The serum is present in this wash step to inactivate residual trypsin activity.
2. Prepare gelatinized T-25 cm^2 flasks (section 2.0).
3. Prepare Predifferentiation Medium as follows:
   - Combine components in a 50 mL conical tube (e.g. Catalog #38010) as indicated in Table 1. Mix thoroughly.

Table 1. Predifferentiation Medium Components

COMPONENT	CATALOG #	AMOUNT TO ADD FOR 50 mL	FINAL CONCENTRATION
Fetal bovine serum (FBS; ES cell-qualified)	---	7.5 mL	15%
Sodium Pyruvate*	---	e.g. 0.5 mL of a 100 mM stock solution	1 mM
L-Glutamine	07100	0.5 mL	2 mM
MEM Non-Essential Amino Acid Solution (100X)	07600	0.5 mL	0.1 mM
Mouse Recombinant LIF†	78056	e.g. 5 µL of a 0.1 mg/mL stock solution	10 ng/mL
Monothiolglycerol (MTG); 1 in 100 dilution in IMDM	Sigma M6145	43 µL	100 µM
Iscove's Modified Dulbecco's Medium*	36150	To final volume of 50 mL	---

*It is not necessary to add sodium pyruvate if the ES-Cult™ IMDM (Catalog #36150) 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.
†Prepare stock solution as directed in the Product Information Sheet.

4. Aspirate medium from the cell suspension (prepared in step 1). Resuspend cells in Predifferentiation Medium and count live or dead cells using a viability stain.
5. Plate approximately 0.5 - 1 x 10^5 cells per gelatinized T-25 cm^2 flask (prepared in step 2).

4.0 Two-Step In Vitro Differentiation of Mouse ES and iPS Cells

This section provides protocols for the in vitro hematopoietic differentiation of mouse ES and iPS cells in methylcellulose-based cultures using a two-step method. The first step is the primary differentiation, in which the mouse ES and iPS cells form EBs containing a variety of hematopoietic progenitor cells. The second step involves the plating of cells originating from the EBs 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 progenitor cells that were present within the EBs. A great deal of variability exists amongst different mouse ES/iPS cell lines in their ability to differentiate in vitro. In addition, the ability of mouse ES and iPS cells to generate hematopoietic progenitor cells 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 mouse ES and iPS cells. As described in section 3.0, cells must be passaged once in Predifferentiation Medium prior to establishment of the primary differentiation culture.

4.1 Primary Differentiation of Mouse ES and iPS Cells into Embryoid Bodies (EBs)

4.1.1 Primary Plating

1. Prior to beginning the differentiation steps below, assess the status of your cultures. Mouse ES/iPS cell 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 in Predifferentiation Medium (section 3.0) at a lower density to improve morphology and differentiation potential. Alternatively, thaw a new vial of early passage mouse ES and iPS cells and begin again.

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

2. Harvest the mouse ES and iPS cells from the flask using Trypsin-EDTA as follows:
   i. Aspirate medium.
   ii. Rinse cultures once with D-PBS (Without Ca++ and Mg++).
   iii. Add 2 mL of room temperature (15 - 25°C) Trypsin-EDTA (0.25%) to cover the cells.
   iv. For most mouse ES or iPS cell lines, incubate at 37°C for 3 - 5 minutes or just until the cells begin to lift off the flask.
   v. Use a serological pipette to pipette up and down and dislodge any remaining cells on the flask. Transfer the cells to a 15 mL tube containing approximately 2 - 4 mL of IMDM with 10% FBS. Immediately centrifuge the cells at 300 x *g* for approximately 8 minutes.

   Note: The serum is present in this wash step to inactivate residual trypsin activity.

3. Aspirate medium and resuspend pellet in approximately 2 mL of IMDM, ensuring that a single-cell suspension is achieved.
4. Count live cells using a viability stain to ensure that cells are healthy.

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

5. Prepare 10 mL of a mouse ES/iPS cell suspension at a density of 2 - 5 x 10^3 cells/mL. The optimal cell density is cell line-dependent and will vary with the cells' differentiation capacity 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 mouse ES/iPS cell input.

   As an example, the appropriate range of mouse ES and iPS cells to plate is 200 - 500 cells per dish when using the CCE ES cell line that has been adapted for growth on gelatin. When using mouse ES and iPS

cells maintained on iMEFs, plating 1,000 - 5,000 cells per dish is generally required. These are approximate starting points, however, and each cell line should be evaluated empirically.

6. Prepare methylcellulose-based Primary Differentiation Medium as follows:
   i. Thaw 100 mL bottle of MethoCult™ GF M3434 at room temperature (15 - 25°C) or overnight at 2 - 8°C. Do not thaw MethoCult™ at 37°C.
   ii. Combine components in a 50 mL tube as indicated in Table 2.

Table 2. Primary Differentiation Medium Components

COMPONENT	CATALOG #	AMOUNT TO ADD FOR 90 mL	FINAL CONCENTRATION AFTER ADDITION OF CELLS
ES-Cult™ M3120	03120	40.0 mL (one bottle)	Approximately 1% methylcellulose
FBS, ES cell qualified	---	15.0 mL	15%
L-Glutamine	07100	1.0 mL	2 mM
Monothiolglycerol (MTG); 1 in 100 dilution in IMDM*	Sigma #M6145	0.124 mL	150 µM
Mouse Recombinant SCF**	78064	e.g. 40 µL of a 0.1 mg/mL stock solution	40 ng/mL
Iscove's Modified Dulbecco's Medium	36150	to final volume of 90 mL	---

*The MTG must be freshly prepared to achieve optimal levels of EB formation.

**Prepare stock solution as directed in the Product Information Sheet.

   iii. Mix thoroughly. Add this mixture to the 100 mL bottle of thawed MethoCult™ GF M3434.
   iv. Mix vigorously and then let stand for at least 5 minutes to allow bubbles to rise to the top before aliquoting.

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

7. Using a 12 mL syringe and a 16 gauge Blunt-End Needle, aliquot a maximum of 13.5 mL of Primary Differentiation Medium into a 50 mL conical tube. This volume is sufficient for 12 cultures of 1 mL each.

   Note: 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.

   Note: Do not use pipettes to dispense methylcellulose as the volume dispensed will not be accurate. Syringes and large bore blunt-end needles should be used for accurate dispensing of viscous methylcellulose medium and to prevent needle-stick injuries.

8. Add the single-cell suspension of mouse ES and iPS cells (prepared in step 5) to the tube of Primary Differentiation Medium at a 1 in 10 dilution (e.g. 1.5 mL cells to 13.5 mL medium for a final volume of 15 mL).
9. Vortex vigorously and allow air bubbles to dissipate.
10. Aliquot 1.0 mL of the mouse ES/iPS cell suspension using a 3 cc Syringe and a 16 gauge Blunt-End Needle into each 35 mm culture dish and swirl gently to ensure that the suspension is evenly distributed on the bottom of the dish without touching the lid.

    Note: 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.

11. 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 and 5% CO_2 with > 95% humidity until further analysis is performed.

    Note: EBs will be visible within 2 - 3 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.

12. Store all unused Primary Differentiation Medium at -20°C in 15 mL aliquots. It can be used to "feed" the differentiation cultures (section 4.1.2).

4.1.2 Feeding Differentiation Cultures

In order to ensure the viability of the primary differentiation cultures over an extended period of time, "feed" the cultures on day 7 with a dilute methylcellulose medium containing hematopoietic growth factors (Feed Medium) as described below.

1. On day 7, prepare methylcellulose-based Feed Medium as follows:
   - Combine components as indicated in Table 3 in a 50 mL tube. Mix thoroughly.

Table 3. Feed Medium Components

COMPONENT	CATALOG #	AMOUNT TO ADD FOR 30 mL	FINAL CONCENTRATION
Primary Differentiation Medium (section 4.1.1)	N/A	15.0 mL	Approximately 0.5% methylcellulose
FBS, ES cell qualified	---	2.25 mL	15%
Monothiolglycerol (MTG); 1 in 100 dilution in IMDM*	Sigma #M6145	38 µL	150 µM
Mouse Recombinant SCF†	78064	e.g. 48 µL of a 0.1 mg/mL stock solution	160 ng/mL
Mouse Recombinant IL-3†	78042	e.g. 9 µL of a 0.1 mg/mL stock solution	30 ng/mL
Human Recombinant IL-6†	78050	e.g. 6 µL of a 0.1 mg/mL stock solution	20 ng/mL
EPO, Human, Recombinant†	02625	e.g. 180 µL of a 500 U/mL stock solution	3 U/mL
Iscove's Modified Dulbecco's Medium	36150	to final volume of 30 mL	---

*The MTG must be freshly prepared to achieve optimal levels of EB formation.

†Prepare stock solution as directed in the Product Information Sheet.

2. Layer 0.5 mL of Feed Medium onto the surface of each differentiation culture dropwise using a 3 cc Syringe and a 16 gauge Blunt-End Needle.
3. Incubate at 37°C and 5% CO_2 with > 95% humidity. EBs are typically harvested between day 7 and 15; refer to section 5.3 and 5.4 for representative images. Proceed to section 4.1.3 for harvesting EBs.

4.1.3 Harvesting EBs from Methylcellulose Cultures

Regardless of the age of the EBs in the primary differentiation cultures, the initial stages of the harvest (steps 1 - 3) are the same.

1. Fill each culture dish (from section 4.1.2) with 1 mL of IMDM with 2% FBS and mix the liquid medium with the methylcellulose. Transfer contents of the dish into a 14 mL round-bottom polystyrene tube (e.g. Catalog #38008). Transfer no more than 2 - 3 dishes into this size tube or 10 - 12 dishes into a 50 mL tube.

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

   Note: 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 EBs with Trypsin-EDTA (0.25%) or Collagenase Type I (0.25%) depending on the age of the EBs, as outlined below.

   For EBs ≤ 8 days old:

   Add 2 - 3 mL Trypsin-EDTA (0.25%) and incubate for 2 - 3 minutes at room temperature (15 - 25°C). Disrupt EBs by passing through a 21 gauge needle on a 3 cc Syringe 3 times (up and down). Ensure a single-cell suspension is obtained.

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

   For EBs ≥ 9 days old:

   Add 2 - 3 mL of Collagenase Type I (0.25%) and incubate at 37°C for 1 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 21 gauge needle on a 3 cc Syringe 3 times (up and down). Ensure a single-cell suspension is obtained.

   Note: If more than 2 - 3 dishes were harvested, increase the amount of Collagenase Type I used to approximately 5 mL.
5. Add an equal volume of IMDM with 5% FBS to neutralize the Trypsin-EDTA (or Collagenase Type I). Centrifuge at 300 x *g* for 5 - 8 minutes.
6. Remove supernatant carefully so as not to disturb the loose pellet. Resuspend the cells in a minimum volume (1 - 3 mL) of IMDM with 2% FBS.

   Note: 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. Proceed to section 4.2 for secondary plating.

4.1.4 Forming Simple EBs in Suspension Culture

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

However, some cell lines are inefficient in the formation of EBs in methylcellulose. In this case, it may help to start cultures in suspension and then transfer to methylcellulose 24 - 48 hours later. For the analysis of gene expression at day 3 - 4 of EB formation, the suspension culture system allows access to a greater number of cells at earlier time points than in methylcellulose.[29]

1. Prior to beginning the differentiation steps below, assess the status of your cultures. Mouse ES/iPS cell 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 mouse ES/iPS cells and begin again.

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

2. Harvest the mouse ES/iPS cells from the flask as described in section 4.1.1 step 2.

   Note: If you have difficulty with EB formation, it may help to use Trypsin-EDTA for an even shorter length of time (1 - 2 minutes), so that small clumps of cells are still present. The clumps will encourage EB formation.

3. Aspirate medium and resuspend the pellet in DMEM with 10% FBS, ensuring a single-cell suspension is obtained. If you are using cell lines that are sensitive to Trypsin-EDTA, only pipette up and down against the bottom once or twice to disrupt the cell pellet into small clumps.

4. Plate into 35 mm culture dishes at 4 x 10^5 cells per dish. Incubate at 37°C. Small aggregates (simple EBs) will be visible after 24 hours of incubation. These simple EBs can be transferred into methylcellulose after 24 - 48 hours of incubation (see section 4.1.1).

5. If you are continuing in the liquid culture system, the medium must be changed every 3 - 4 days. The EBs will tend to aggregate into clumps with regions of necrosis. To avoid this, break clumps apart by using a large pipette (e.g. 25 mL) such that the EBs are not disrupted. Transfer the EBs to a tube and allow them to sink to the bottom. Carefully aspirate medium, replace with fresh medium, and replate into the culture dish.

6. To disrupt EBs into single cells, refer to section 4.1.3, steps 4 - 6.

4.2 Secondary Plating for Detection of Hematopoietic Progenitor Cells

1. Prepare methylcellulose-based Hematopoietic Differentiation Medium as follows:
   i. Combine components in a 50 mL tube as indicated in Table 4.

Table 4. Hematopoietic Differentiation Medium Components

COMPONENT	CATALOG #	AMOUNT TO ADD FOR 100 mL	FINAL CONCENTRATION
FBS, ES cell qualified	---	15.0 mL	15%
L-Glutamine	07100	1.0 mL	2 mM
Monothiolglycerol (MTG); 1 in 100 dilution in IMDM*	Sigma #M6145	0.124 mL	150 µM
BIT 9500 Serum Substitute	09500	20 mL	1% BSA 10 µg/mL insulin 200 µg/mL transferrin
Mouse Recombinant SCF†	78064	e.g. 150 µL of a 0.1 mg/mL stock solution	150 ng/mL
Mouse Recombinant IL-3†	78042	e.g. 30 µL of a 0.1 mg/mL stock solution	30 ng/mL
Human Recombinant IL-6†	78050	e.g. 30 µL of a 0.1 mg/mL stock solution	30 ng/mL
EPO, Human, Recombinant†	02625	e.g. 600 µL of a 500 U/mL stock solution	3 U/mL

*The MTG must be freshly prepared to achieve optimal levels of EB formation.
†Prepare stock solution as directed in the Product Information Sheet.

   ii. Add component mixture to bottle of ES-Cult™ M3120 (Catalog #03120; 40 mL).
   iii. Rinse the tube with Iscove's Modified Dulbecco's Medium (Catalog #36150) and add to the bottle to a final volume of 100 mL.
2. Aliquot 3.0 mL per 14 mL polypropylene tube using a 3 cc Syringe with a 16 gauge Blunt-End Needle. Store excess tubes at -20°C until needed.
3. Add 0.3 mL of cells at a concentration of 1 - 5 x 10^5 cells per mL to each tube containing 3 mL of Hematopoietic Differentiation Medium and vortex thoroughly. Let stand 3 - 5 minutes to allow bubbles to dissipate.
4. Plate 1.1 mL of the cell suspension per 35 mm culture dish.

   Note: This yields 1 - 5 x 10^4 cells per dish. This is a good starting range; the optimal number of cells to plate will vary depending on the cell line and culture conditions, as well the age of the EBs. ***When determining optimal plating densities, it is advisable to try two cell concentrations that differ by 2- to 3-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 and 5% CO_2 with > 95% humidity.
6. Count the numbers and types of hematopoietic colonies after approximately 10 days of culture. Refer to section 5.0 for identification and counting of EBs and hematopoietic colonies.

   Note: Colony morphology is best viewed at this time. If the cells are left in culture for longer than 10 days, they become difficult to identify.

5.0 Identification and Counting of Embryoid Bodies (EBs) and Hematopoietic Colonies

5.1 Example of Quantitation of Hematopoietic EBs and Hematopoietic Progenitor Cells from Two-Step In Vitro Differentiation of Mouse ES and iPS Cells

PRIMARY DIFFERENTIATION

Number of mouse ES and iPS cells plated per 35 mm dish	300
Number of dishes plated	5
Day 10	
Total EBs counted per dish	mean = 120
Hematopoietic EBs counted per dish	mean = 75
Efficiency of EB 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	7.5×10^6 / 5 dishes = 1.5×10^6 cells/culture
Average cells per EB	7.5×10^6 / 600 = 1.2×10^4 cells/EB

SECONDARY PLATING

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

Average number of colonies per culture:
50 CFUs per 2×10^4 cells plated = 3,750 CFUs per 1.5×10^6 (average cells harvested per culture)

Average number of colonies per EB:
3,750 CFUs per culture
3,750 CFUs per 120 EBs (average EBs per culture)
31 CFUs per EB

5.2 Morphology of Undifferentiated Mouse ES and iPS Cells

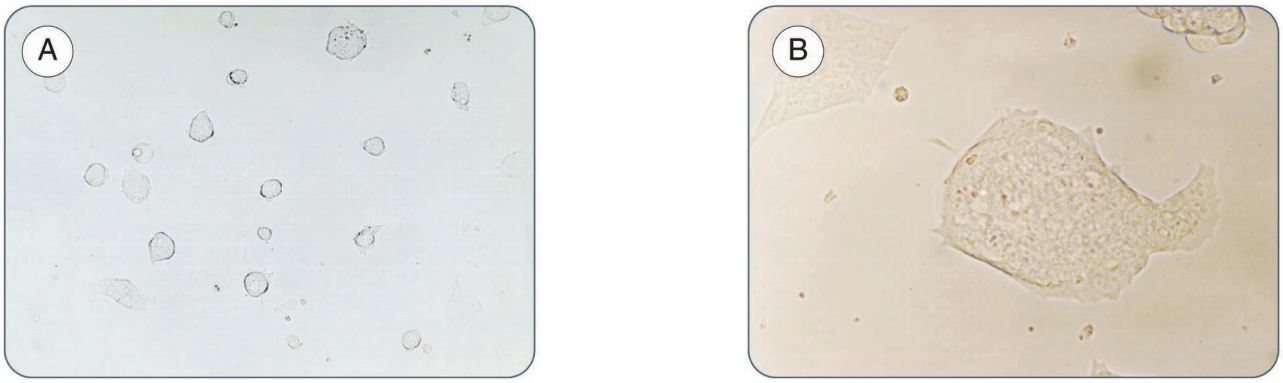


Figure 2. Undifferentiated ES cells: (A) Low power and (B) High power
Undifferentiated mouse ES and iPS cells have a large nucleus, minimal cytoplasm, and one or more prominent dark nucleoli. It should be difficult to identify individual cells within the mouse ES/iPS cell colony, as there are non-distinct 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 mouse ES/iPS cell 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.

5.3 Identification of EBs

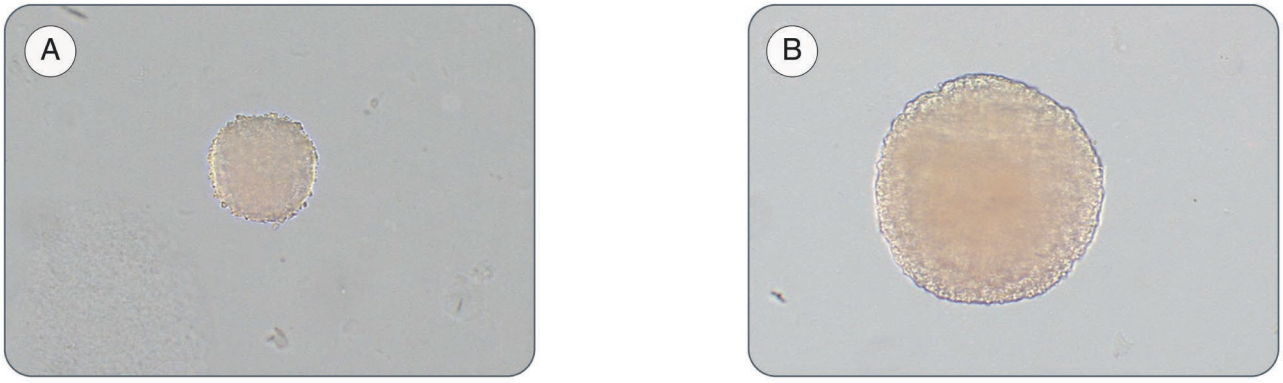


Figure 3. EBs: (A) Day 6, low power and (B) Day 9, low power
Individual mouse ES and iPS cells plated in primary differentiation methylcellulose-based medium will proliferate and differentiate into multi-cellular structures called embryoid bodies (EBs) within days. Morphologically, the EBs appear as a dense mass of cells surrounded by a cellular envelope. Clumps of disorganized or non-viable cells should not be counted as EBs.

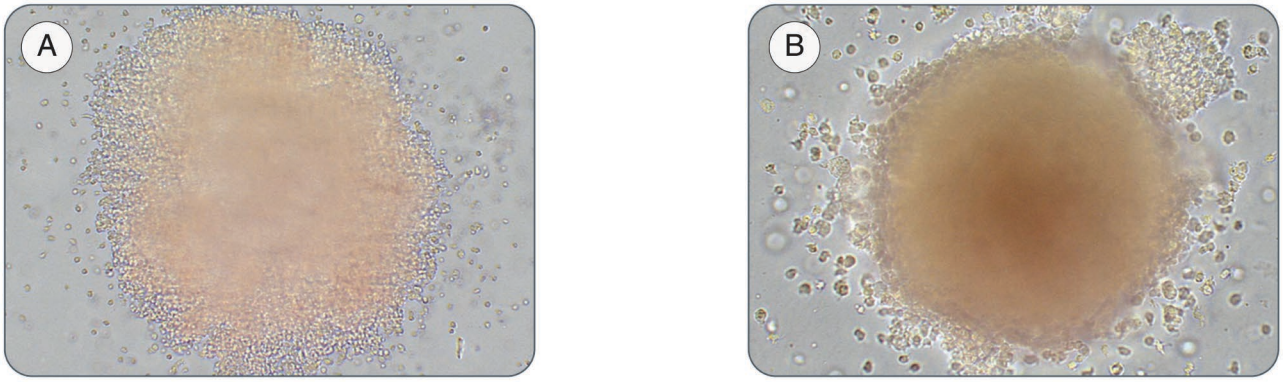


Figure 4. Hematopoietic EBs: (A) Day 13, low power and (B) Day 15, low power
By day 10 to 12 or later, under the appropriate conditions of culture, hematopoietic EBs can be detected. Morphologically, these can be identified by the presence of macrophages, erythroid cells, and occasionally granulocytic cells at the edges of the EB. Hemoglobinization of erythroid cells is often visible. The efficiency of EB formation and the proportion of hematopoietic EBs obtained will be dependent upon the ES cell line and the conditions of culture.

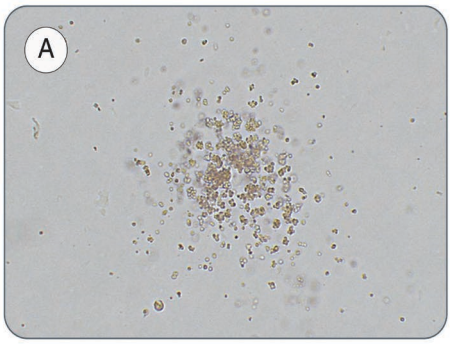
5.4 Identification of Hematopoietic Progenitor Cells

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

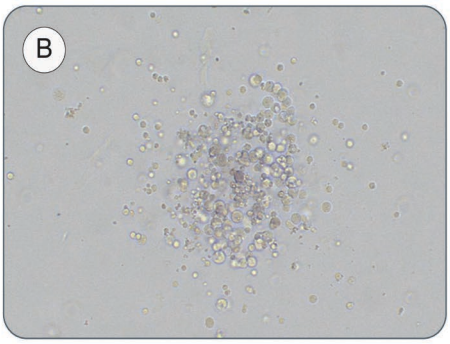
The numbers and types of hematopoietic colonies detected in methylcellulose cultures derived from disaggregated EBs is dependent on the mouse ES/iPS cell line, the day of harvest of EBs, and hematopoietic cytokines used in the secondary differentiation.

Primitive erythroid colonies are the predominant class of progenitor derived from day 3 to day 8 EBs. The colonies will be small clusters containing 8 - 200 erythroblasts and can be counted after 7 - 10 days of culture. Macrophage colonies are also visible at this stage. Erythroid cells are larger than erythroblasts present in burst-forming unit-erythroid (BFU-E) from adult mouse bone marrow. Representative images of hematopoietic progenitor cells are shown in Figure 5.

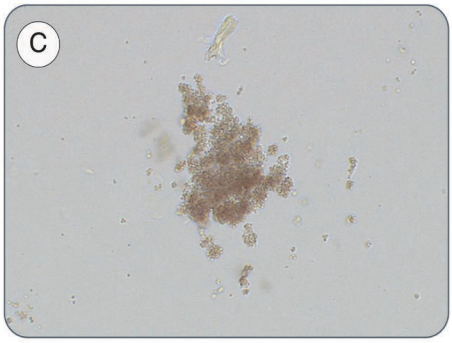
Note: It is important to distinguish mixed colonies from partially disrupted EBs or large definitive erythroid colonies that often contain macrophages.



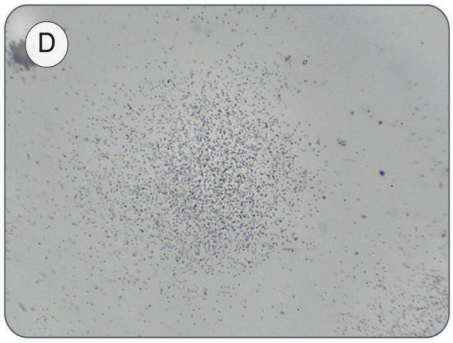
BFU-E from a day 11 EB, low power



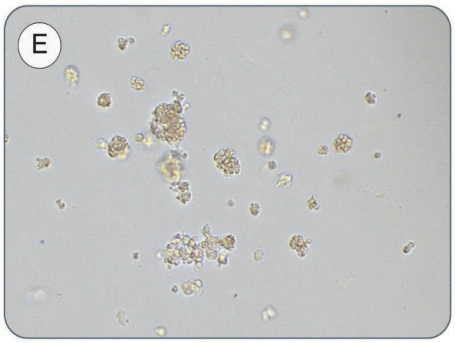
CFU-GM from a day 11 EB, low power



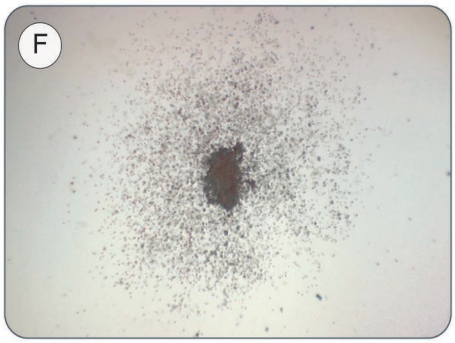
BFU-E from a day 11 EB, low power



CFU-GM from a day 14 EB, low power



BFU-E from a day 11 EB, high power



CFU-mixed from a day 11 EB

Figure 5. Hematopoietic Progenitor Cells

A, C, E: **Definitive Erythroid Colonies (BFU-E).** These are detectable from EBs cultured for 7 days or longer and are similar in appearance to BFU-E colonies derived from mouse bone marrow (smaller cells than primitive erythroid cells). They consist of multiple clusters, each containing 8 - 200 erythroblasts. Count at day 10 - 12 of culture.

B, D: **Colony-forming unit-granulocyte/macrophage (CFU-GM).** These contain $\geq$ 30 cells and are usually detectable from EBs cultured for 7 - 14 days. They contain monocyte-macrophages and/or granulocytes. Mast cell colonies are predominant after day 12 of culture. These are similar in appearance to colony-forming unit-granulocyte/macrophage (CFU-GM) derived from mouse bone marrow.

F: Mixed colonies (CFU-Mixed). These are detectable from EBs cultured for 7 days or longer, and contain granulocytes/macrophages and erythroid cells. Megakaryocytes (large cells, slightly irregular in shape, present singularly or in clusters of 2 - 10 cells) are often present. These are similar in appearance to colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM) derived from mouse bone marrow.

6.0 Troubleshooting

The most common problems associated with the maintenance and in vitro hematopoietic differentiation of mouse ES and iPS cells are described below, along with the possible causes.

6.1 Mouse ES/iPS Cell Maintenance

1. Cell death:
   - Lack of monothiolglycerol in the maintenance medium
   - Passaging the cells at a low density
   - Toxicity of one of the reagents

   Note: All ES-Cult™ products have been pre-screened and found to exhibit no observable toxicity to a commonly used ES cell line.
2. Mouse ES/iPS cell colonies lift off the culture plate during maintenance:
   - Petri dishes rather than tissue cultureware used
   - Gelatin solution prepared incorrectly
   - Excessive differentiation
3. Excessive differentiation:
   - Failure to obtain a single-cell suspension when passaging mouse ES/iPS cells
   - Insufficient or inactive LIF
   - Plating too many cells in the culture vessel

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

6.2 Primary Differentiation

1. Low numbers of EBs generated:
   - Differentiation occurred during maintenance steps
   - Monothiolglycerol not freshly prepared

2. Variable and inconsistent numbers of EBs per dish:
   - Inadequate mixing of growth factors or cells with methylcellulose-based medium

     Note: Due to its viscosity, media containing methylcellulose must be thoroughly mixed.

6.3 Secondary Differentiation

Few hematopoietic progenitor cells detected:

- Insufficient or inactive growth factors used
- EBs exposed to Trypsin-EDTA for too long during harvesting
- Previous differentiation of ES cells during maintenance
- Too few cells plated

6.4 Use of Alternative Products

- 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.
- Some of STEMCELL Technologies' other mouse methylcellulose products can be used for the differentiation of ES cells (Table 5). These products have not been extensively pre-tested or screened in the appropriate ES cell functional assays.

Table 5. Mouse Methylcellulose Products

PRODUCT	CATALOG #	APPLICATION
MethoCult™ M3134	03134	Alternative to ES-Cult™ M3120 base methylcellulose medium
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 (section 4.2) (contains the same cytokines but at lower concentrations)

7.0 Appendix I: Plating and Passaging Mouse Embryonic Fibroblasts (MEF)

MEF (Catalog #00321/00322) and drug-resistant MEF (Catalog #00323/00324/00325) are available from STEMCELL Technologies; these can be expanded and used up to passage 5. Drug-resistant MEF can be used for experiments where drug selection of undifferentiated cells is required.

Note: High serum concentrations and gelatin increase the plating efficiency of MEF from the frozen state. Gelatinized tissue culture dishes or flasks should be used for the first passage of MEF after thawing.

1. Prepare a 50 mL tube with 20 mL of medium consisting of DMEM with 4500 mg/L D-glucose containing 30% FBS and 10^{-4} M ß-mercaptoethanol.
2. Quickly thaw one vial of frozen MEF by gently shaking in a 37°C water bath. Remove the vial from the water bath and wipe with 70% ethanol to sterilize the outside of the vial.
3. Add cells *dropwise* to the 50 mL tube of medium containing 30% FBS (prepared in step 1) with gentle agitation. Using a sterile 5 mL or 10 mL pipette, mix by gently pipetting up and down 2 - 3 times.
4. Transfer the appropriate amount of medium containing the cells to a gelatinized, tissue culture-treated plate or flask.
5. Incubate the plate(s) or flask(s) at 37°C and 5% CO_2 with 95% humidity overnight.
6. The next day, prepare 20 mL of medium consisting of DMEM with 4500 mg/L D-glucose containing 10% FBS and 10^{-4} M ß-mercaptoethanol. Replace the culture medium with this fresh medium and allow the cells to grow to confluence, usually 2 - 4 days.

   Note: Once MEF have become confluent, they can be passaged up to 5 times.
7. To passage cells, aspirate medium and rinse cultures once with D-PBS (Without Ca++ and Mg++).
8. Add sufficient Trypsin-EDTA (0.25%) at room temperature (15 - 25°C) to cover the cells.

   Note: Use approximately 2 mL for a 60 mm dish or T-25 cm^2 flask; 4 mL for a 100 mm dish or T-75 cm^2 flask.
9. Incubate for 3 - 5 minutes at 37°C or just until the cells begin to lift off the plate.
10. After the incubation period, use a serological pipette to transfer the cells to a 15 mL tube containing approximately 2 - 4 mL of a solution of DMEM with 4500 mg/L D-glucose containing 10% FBS. Then immediately centrifuge the cells at 300 x *g* for approximately 8 minutes to pellet the cell suspension.

    Note: The serum is present in this wash step to inactivate residual trypsin activity.
11. Aspirate the medium and resuspend the cell pellet in approximately 2 mL of DMEM with 4500 mg/L D-glucose containing 10% FBS. Pipette up and down against the bottom of the tube 4 - 6 times to ensure that the cell pellet is disrupted to a single-cell suspension.
12. Re-plate MEF in an appropriate amount of DMEM with 4500 mg/L D-glucose containing 10% FBS. It is recommended to re-plate at a ratio of 1:5. However, this ratio may vary depending on the preparation and passage number of the MEF.

8.0 Appendix II: Inactivation of MEF

MEF used for feeder layers must be mitotically inactivated before use. This is often conveniently done while the cells are still attached to the tissue culture plate. Alternatively, if cells are to be maintained and/or expanded further, harvest the fibroblasts with Trypsin-EDTA (0.25%) and re-plate a portion at a 1:5 dilution. The remainder of the cells can then be irradiated in a tube.

1. Mitotically inactivate the MEF using either irradiation or treatment with mitomycin C.

   Inactivation by irradiation:

   Expose the MEF either on plate(s) or in tube(s) to 6,000 - 10,000 rads (60 - 100 Gy) from a gamma or x-ray source. Large batches of MEF may be inactivated and frozen at recommended densities (see step 2 below) so that they may be thawed and used directly at a later date.

   Inactivation by mitomycin C treatment:

   Mitomycin C is light-sensitive and cytotoxic. Please refer to the SDS for hazard information. Stock solutions should be divided into 10 mL aliquots and stored at -20°C in the dark.

   i. Once the MEF are confluent, replace the growth medium with a solution of DMEM with 4500 mg/L D-glucose containing 5% FBS and 10 µg/mL mitomycin C and return the MEF to the 37°C incubator with 5% CO_2 and 95% humidity for 3 hours.

      Note: Use 7 - 10 mL of solution per 100 mm plate or 20 - 25 mL of solution per T-75 cm^2 tissue culture-treated flask.

   ii. Remove the solution and wash the MEF at least 2 times with DMEM with 4500 mg/L D-glucose if the plate is to be used directly, or with D-PBS (Without Ca++ and Mg++) if the cells are to be trypsinized and re-plated.

2. If dividing a large plate of inactivated MEF between several smaller plates, or if MEF were inactivated in tubes, re-plate inactivated MEF in maintenance medium such that a uniform feeder monolayer of cells is formed.

   Recommended cell densities for plating inactivated MEF feeder layers are as follows:

   - 100 mm dish: 1.7 - 2 x 10^6 cells
   - 60 mm dish: 1 x 10^6 cells
   - 24-well plate: 1 x 10^5 cells per well
   - 96-well plate: 3 - 5 x 10^4 cells per well

   Note: Once MEF have been inactivated, gelatinized dishes are no longer required – instead, use high-quality tissue culture-treated plates. Inactivated feeder layers should be used for mouse ES or iPS cell culture within 10 days.

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