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Mouse embryonic fibroblast isolation, culture, and maintenance

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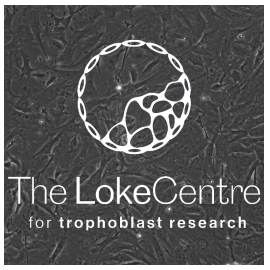

Busma Butt[1], Alice Truszczynska[1], Erin Slatery[1]

[1]Loke Centre for Trophoblast Research, Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge, CB2 3EG, UK

Busma W.Y. Butt
The Loke Centre for Trophoblast Research, University of Camb...

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Disclaimer

This protocol is provided for general informational and educational purposes only. It has not been peer reviewed. The procedures described involve handling of potentially hazardous materials, such as mitomycin C, and work with biological samples. It is the responsibility of users to comply with institutional policies, ethical standards, and applicable national regulations.

Individuals should rely on their professional expertise and ensure they are adequately trained and supervised where required.

Abstract

This protocol outlines the step-by-step isolation, culture, and maintenance of mouse embryonic fibroblasts (MEFs) derived from CD1 strain embryos. MEFs are widely used as feeder layers to support the growth and self-renewal of a variety of stem cells in vitro (Bryja et al. 2006, Jain et al. 2014) including mouse trophoblast stem cells (Hayakawa et al. 2015). The protocol provides detailed instructions for embryo dissection, MEF culture and passaging, cryopreservation, thawing, and mitotic inactivation using mitomycin C. It includes guidance on cell confluency and density to optimise viability and expansion, as well as medium recipes, reagent reference tables, and safety notes to support reproducibility and ease of use for both new and experienced researchers.

MEFs are isolated at embryonic day (E)13.5 by carefully removing extraembryonic tissues, followed by mechanical and enzymatic dissociation of the embryonic body. To function effectively as feeder cells, MEFs are rendered mitotically inactive to prevent overgrowth while retaining the metabolic activity necessary to support stem cell cultures. This is typically achieved through γ-irradiation or chemical treatment with mitomycin C (Nagy et al 2006). While γ-irradiation requires specialised equipment, mitomycin C offers a more accessible alternative, although it must be handled with care due to its cytotoxic nature. Thorough washing is essential after inactivation to avoid residual toxicity that could affect downstream applications.

By standardising each step of MEF preparation and maintenance, this protocol ensures consistent cell quality and optimised experimental performance. It serves as a comprehensive resource for researchers working in stem cell biology, developmental biology, and cell culture-based applications.

Image Attribution

MEF phase-contrast image taken by Busma W.Y. Butt, used with permission. Logo Loke Centre for Trophoblast Research, used with permission from the Centre.

Guidelines

Overview of the MEF protocol

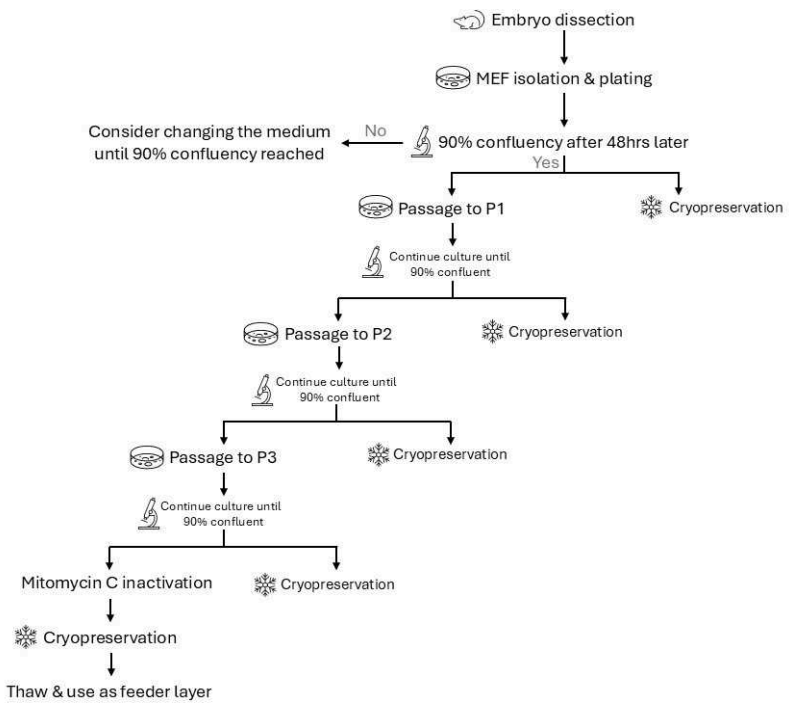

Figure 3: Flowchart outlining the MEF preparation protocol, from embryo dissection to cryopreservation.
MEFs are typically inactivated with mitomycin C at passage 3 (P3) in our centre, though this step is flexible and can be performed at earlier or later passages. However, we recommend inactivation no later than P5 to maintain optimal cell quality. At each passage, 8 out of 10 flasks are cryopreserved to ensure a consistent and reliable stock.

Time Consideration

- **Embryo collection:** MEF preparation requires embryos at embryonic day 13.5 (E13.5) of gestation.
- **Embryo dissection:** ~30 minutes per litter (about 10 embryos), depending on operator experience. Begin early in the day to accommodate subsequent processing steps.
- **Tissue processing and plating:** ~2 hours
- **Culture expansion:** MEFs typically reach 80–100% confluency within 1–2 days. Early-passage MEFs generally require passaging every 2 days.
- **Passaging:** ~2 hours
- **Cryopreservation:** ~2 hours
- **Thawing:** ~1 hour
- **Mitomycin C inactivation and cryopreservation:** ~4 hours, including at least 2 hours (maximum of 3 hours) of mitomycin C incubation.

Note: The above durations do not include media preparation and labelling of cryovials.

Strain Considerations and Embryo Quality

Embryo health, developmental stage, and genetic background are some of the factors that can influence the success of MEF isolation.

- **Litter size considerations by strain:** Different mouse strains produce varying litter sizes, which can impact how many breeding pairs are needed to obtain a sufficient number of embryos. For instance, C57BL/6 mice typically yield 5 to 8 embryos per litter, while CD1 mice may produce 11 to 15 embryos. These differences are strain-dependent and may be further influenced by genetic modifications, particularly if the alteration affects embryo viability or leads to embryonic lethality. When working with such lines, consider increasing the number of breeding females to compensate for potential losses.
- **Wild-type embryos:** Select embryos that are morphologically normal and consistent in size. Avoid embryos that are significantly smaller (≥25% smaller than littermates) or show developmental abnormalities, as these are less likely to yield healthy, proliferative MEFs. Embryonic age is also crucial. MEFs are commonly isolated from wild-type embryos between embryonic day (E) 12.5 and E14.5 (Bryja et al 2006, Jain et al 2014). During this period, fibroblasts are sufficiently abundant but have not yet undergone significant differentiation, ensuring a higher yield of proliferative MEFs. Harvesting embryos too early may result in low cell yields, while collecting them too late can lead to contamination with differentiated or non-proliferative cells.
- **Genetically modified embryos:** When working with transgenic or knockout mouse strains, the genetic alteration itself may lead to variation in embryo size, so discarding smaller embryos based on wild-type criteria may not be appropriate. Additionally, if the modification is associated with embryonic lethality, it is important to carefully select the timing of MEF harvest. Always dissect embryos individually using a clean set of sterilised tools to prevent cross-contamination between genotypes.

Sterility and Contamination Risk

Maintaining sterility is essential throughout the entire MEF preparation workflow, as with all tissue culture work. The highest risk of contamination typically arises during the dissection and initial plating stages. At our institute, uterine horns are provided by animal technicians, avoiding the need for whole animal dissection within the laboratory. This greatly reduces contamination risk. Whole animal dissection increases exposure to fur, faeces, and internal organs, all of which can introduce microbes into the workspace. It also involves more instruments and handling steps, each presenting an opportunity for contamination if not carefully controlled.

The following measures help minimise contamination:

- Washing uterine horns and embryos several times in sterile 1X DPBS.
- Using only tissue culture-approved, sterile consumables.
- Sterilising dissection instruments before use and between samples when required.
- Handling all media and reagents aseptically to prevent contamination.
- Aliquoting reagents and supplements into smaller sterile containers reduces the frequency of opening the main stock, thereby lowering the risk of contamination. If contamination does occur, only the affected aliquot is compromised, preserving the integrity of the remaining stock.
- Routinely screening all cultures for mycoplasma contamination.

Cell Growth and Expected Results

Proper seeding density and careful monitoring of MEF growth are essential for efficient expansion and maintaining cell quality.

- **Plating and expansion:** MEFs should be seeded at densities appropriate for the culture vessel. This protocol is based on the use of T175 flasks. Reference tools such as **Useful Numbers for Cell Culture | Thermo Fisher Scientific - UK** can guide estimates for optimal cell numbers and media volumes.
- **Growth rates:** Wild-type MEFs generally reach 80–100% confluency within 1–2 days post-isolation. *Figure 4* shows MEFs 1, 2, and 3 days after passaging, illustrating the increase in confluency and typical fibroblast-like morphology over time. Proliferation may vary depending on mouse strain or genetic background and should be monitored and adjusted accordingly.
- **Early culture expectations:** Some cell death and debris are typical during the first 24 hours. If confluency is below 80%, changing the medium can help remove unattached cells and debris that may hinder growth.
- **Morphology, senescence, and passage Limits:** MEFs exhibit typical mesenchymal, fibroblast-like morphology between passages 0 and 4–5, often forming compact, pavement-like arrangements *(see Figure 4)*. As they approach or exceed passage 5 or 6, signs of senescence become more evident, including slower proliferation (Manning and Kumar, 2010), larger and flatter cell shapes, some multinucleation, and irregular appearances *(see Figure 5A and 5B)*, all of which are hallmarks of declining culture quality. MEFs generally undergo ~20 divisions *in vitro* before entering senescence, so it is advisable not to expand cultures beyond passage 5.
- **Split ratios and passage guidance:** Early-passage MEFs (P0–P2) at 80–90% confluency and displaying uniform morphology can be split at a 1:4 to 1:6 ratio. Later-passage MEFs may require a more conservative 1:2 or 1:3 ratio due to slower proliferation. Always base split decisions on a combination of passage number, confluency, and cell morphology. Overconfluent cultures may cause cells to stop growing due to crowding and nutrient depletion, while sparsely seeded cultures may not establish enough cell-cell signaling for optimal proliferation.

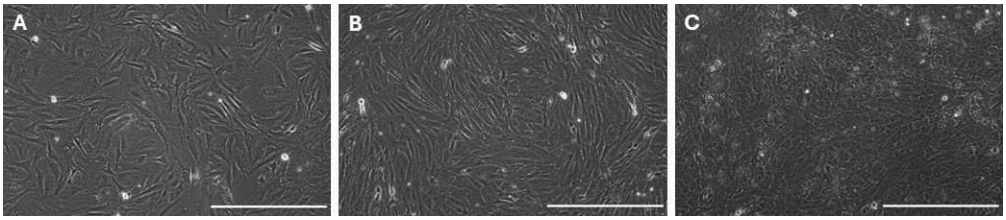

Figure 4. MEF Confluency Progression Post-Passaging
A. Phase-contrast image of P1 MEFs 1 day after passaging.
B. Phase-contrast image of P1 MEFs 2 days after passaging. Cultures show increased cell density and coverage of the growth surface. Cells maintain uniform spindle-shaped morphology and are actively proliferating.
C. Phase-contrast image of P1 MEFs 3 days after passaging, approaching 100% confluency. Cells form a nearly continuous monolayer with close cell-cell contact. At this stage, cultures are ready for the next passage. The images collectively demonstrate the typical growth kinetics and morphological progression of early-passage MEFs.

The scale bars indicate 400um.

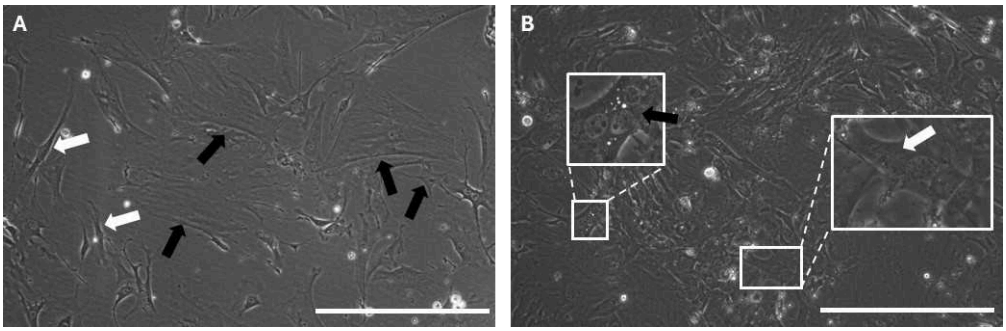

Figure 5. Senescent Morphology and Culture Decline in MEFs
A. Phase-contrast image showing a mixed population of MEFs, including normal proliferative fibroblasts and senescent cells. Actively dividing MEFs exhibit the characteristic elongated, spindle-shaped morphology with uniform size and bipolar alignment (white arrow). In contrast, several cells in the field display a markedly enlarged, flattened appearance with broad cytoplasmic spread (black arrows) - a morphology typical of senescent fibroblasts. These irregularly shaped cells often have indistinct borders. They are less suitable for use as feeder layers as they no longer provide effective support for cell growth and potential secretion of stress-associated factors. Identifying and avoiding cultures dominated by these senescent morphologies is important for maintaining MEF quality in stem cell co-culture systems.
B. Phase-contrast image showing MEFs displaying hallmark features of culture decline. Many cells in the field exhibit granular cytoplasm (white arrow) and some also show multinucleation (black arrow). The fibroblasts also display irregular morphologies, in contrast to the typical spindle-shaped appearance of healthy, proliferative MEFs. A tendency toward clustering or clumping is observed, where cells lose their uniform distribution and instead form dense, overlapping aggregates. These morphological changes are indicative of cellular stress and senescence. To maintain the high quality of MEFs produced in our laboratory, we do not use cultures containing these cell types.

The scale bars indicate 400um.

Freezing and Thawing MEFs

Effective freezing and thawing protocols are essential for preserving MEF viability and function:

- **Freeze gradually:** Use a controlled-rate freezing method (e.g., Mr. Frosty) to ensure slow, uniform cooling and minimise cryo-injury.
- **Thaw rapidly:** Only place the vial in the water bath when you are fully ready to continue. Once thawed, cells are vulnerable and exposed to DMSO, which is toxic at room temperature.
- **Remove DMSO promptly:** Proceed immediately to dilute the thawed suspension in culture medium and remove DMSO after thawing to protect cell viability.

Materials

This content is divided into two parts: **Section 1 - Media Recipes**, detailing the formulations used throughout the procedures, and **Section 2 - Reagents, Materials and Equipment**, listing the tools, consumables, and reagents required for each step.

Section 1 - Media Recipes:

Table 1 - MEF medium (with P/S) recipe (total volume 500mL, filtered):

	Component	Stock concentration	Final concentration	Volume
	DMEM, high glucose (Gibco 21969-035)	-	-	500mL
	L-glutamine (Gibco 25030-024)	200mM	1%	5mL
	Penicillin/streptomycin (P/S) (Gibco 15140-122)	100X	1%	5mL
	Foetal Calf Serum (Gibco 10270-106)	-	10%	50mL
	β-mercaptoethanol (Gibco – 31350-010)	50mM		250uL

Table 1: Recipe for MEF culture medium (total volume: 500 mL), supplemented with penicillin/streptomycin. Filter-sterilise the final solution using a 0.22 µm filter within a biological safety cabinet. Store at 4 °C and use within a reasonable timeframe, ensuring sterility is maintained.

Table 2 - MEF culture medium (without P/S) recipe for mycoplasma testing (total volume 1mL):

	Component	Stock concentration	Final concentration	Volume
	DMEM, high glucose (Gibco 21969-035)	-	-	1000µL
	L-glutamine (Gibco 25030-024)	200mM	1%	10µL
	Foetal Calf Serum (Gibco 10270-106)	-	10%	100µL
	β-mercaptoethanol (Gibco – 31350-010)	50mM		0.5µL

Table 2: Recipe for MEF culture medium without penicillin/streptomycin (total volume: 1000µL), intended for use in mycoplasma testing if using the TransDetect PCR Mycoplasma Detection Kit. It is not filter-sterilized due to the small volume and should be prepared under sterile conditions. Store at 4 °C and use promptly, as the absence of antibiotics increases the risk of contamination.

Table 3 - Freezing medium recipe (total volume 1mL):

	Component	Final concentration	Volume
	Foetal Calf Serum (Gibco 10270-106)	50%	500µL
	DMSO (Santa Cruz sc-358801)	10%	100µL
	MEF medium	40%	400µL

Table 3: Recipe for freezing medium used for cryopreservation of MEFs (total volume: 1000µL). The medium consists of fetal bovine serum (FBS) supplemented with 10% dimethyl sulfoxide (DMSO) to protect cells during freezing. Prepare fresh on the day of use, under sterile conditions.

Table 4 - 0.4mg/ml Mitomycin C solution recipe (total volume 25mL):

	Component	Stock concentration	Final concentration	Volume
	Mitomycin C (StemCell Technologies 73274)	10mg	-	-
	DPBS (no MgCl2, no CaCl2) (Merck D8537-500ML)	-	-	25mL

Table 4: Recipe for preparing a 0.4 mg/mL mitomycin C stock solution. Prepare under sterile conditions and protect from light. The solution can be stored at 4 °C for up to 1 week in the dark, or frozen at −20 °C for up to 1 month. (Storage guidance adapted from **Journal of Glaucoma**.) The stock solution is now ready to be diluted in MEF culture medium to achieve the desired working concentration.

Table 5 - 10ug/ml Mitomycin C inactivation medium recipe (total volume 200mL):

	Component	Stock concentration	Final concentration	Volume
	Mitomycin C solution	0.4mg/mL	-	5mL
	MEF medium	-	-	195mL

Table 5: Recipe for preparing mitomycin C inactivation medium at a final concentration of 10 µg/mL. Prepare under sterile conditions by diluting the 0.4 mg/mL mitomycin C stock solution in MEF culture medium. Inactivation medium can be frozen and stored for up to 6 months. (Reference: https://cdn.cytivalifesciences.com/api/public/content/digi-17788-pdf)

Table 6 - 5 x TBE stock solution recipe (total volume 1000mL)

	Component	Stock concentration	Final concentration	Amount
	Tris base (Roche 10708976001)	-	0.45M	54g
	Boric acid (Honeywell Fluka 31146-500G)	-	0.45M	27.5g

	Component	Stock concentration	Final concentration	Amount
	0.5M EDTA pH 8.0 (invitrogen by Thermo Fisher Scientific AM9261)	0.5M	0.01M	20mL
	Type 2 water	-	-	up to 1000mL

Table 6: Recipe for preparing 5x TBE stock solution with a total volume of 1000mL. Initially add all reagents to 900mL of Type 2 water and dissolve them using a magnetic stirrer then bring the volume to 1000mL. (Reference: https://cshprotocols.cshlp.org/content/2006/1/pdb.rec8458.short)

Section 2 - Reagents, Materials and Equipment

Supplement note 1 - Derivation and culture of MEFs

Reagents:

1. 0.1% gelatin, Cambridge Stem Cell Institute Tissue Culture Facility, https://www.stemcells.cam.ac.uk/facilities/tissue-culture
2. Dulbecco's Phosphate Buffered Saline (without MgCl2 and CaCl2) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D8537-500ML
3. Trypsin-EDTA (0.25%), phenol red **Thermo Fisher Catalog #**25200072
4. MEF culture medium (with P/S), In-house preparation - See Table 1

Materials and Equipment:

	Item	Description / Notes	Sterility / Preparation	Vendor (Catalogue or Model #)
	T175 culture flasks	For growing MEFs	Sterile	Greiner Bio-One (660175)
	Dissection tools	Scissors, forceps	Autoclaved or immersed in 70% ethanol	No specific vendor
	100mm plastic dishes	Used for dissection steps; at least 4 per experiment	Sterile	Greiner (664160)
	Dissection microscope	Optional, for improved visibility during embryo isolation	Wipe with 70% ethanol	No specific vendor
	Class II biological safety cabinet	For aseptic handling of cells and reagents; provides personnel, product, and environmental protection	Sterile	Haier (HR1200-11A2)

	Item	Description / Notes	Sterility / Preparation	Vendor (Catalogue or Model #)
	Scalpel	Disposable or sterilized reusable scalpel	Sterile	No specific vendor
	5mL sterile serological pipette	For transferring cell suspensions, adding and removing media and reagents	Sterile	Corning (4487)
	10mL sterile serological pipette	For transferring cell suspensions, adding and removing media and reagents	Sterile	Corning (4488)
	Pipette controller	For accurate aspiration and dispensing of media and reagents	Sterile	No specific vendor
	15mL centrifuge tubes	For cell collection	Sterile	Greiner (188271)
	Benchtop centrifuge	For pelleting cells during processing steps; compatible with 15mL and 50mL centrifuge tubes	Sterility not required	Eppendorf (5702)
	Aspiration pipette	For removing media and reagents	Sterile	Greiner (710183)
	Aspiration vacuum system	For safe waste collection	Sterility not required	Integra (Vacusafe)
	Tissue culture incubator	37 °C, humidified, with 5% CO_2	Sterile	ESCO (CCL-170B-8)

Supplement table 1. Materials and Equipment Required for MEF Derivation and Culture. This table lists the essential tools, consumables, and equipment used during the isolation and culture of MEFs, presented in approximate order of use. Specific sizes and brands listed are those used in this protocol; equivalent alternatives may be used as appropriate.

Supplement note 2 - Passaging and expanding MEFs

Reagents:

1. 0.1% gelatin, Cambridge Stem Cell Institute Tissue Culture Facility, https://www.stemcells.cam.ac.uk/facilities/tissue-culture
2. Dulbecco's Phosphate Buffered Saline (without MgCl2 and CaCl2) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D8537-500ML
3. Trypsin-EDTA (0.25%), phenol red **Thermo Fisher Catalog #**25200072
4. MEF culture medium (with P/S), In-house preparation - See Table 1
5. MEF culture medium (without P/S), In-house preparation - See Table 2

Materials and Equipment:

	Item	Description / Notes	Sterility / Preparation	Vendor (Catalogue or Model #)
	Inverted microscope	For assessing cell morphology and confluency	Wipe with 70% ethanol	Motic (AE31E)
	T175 culture flasks	For growing MEFs	Sterile	Greiner Bio-One (660175)
	Aspiration pipette	For removing media and reagents	Sterile	Greiner (710183)
	Aspiration vacuum system	For safe waste collection	Sterility not required	Integra (Vacusafe)
	10mL sterile serological pipette	For transferring cell suspensions, adding and removing media and reagents	Sterile	Corning (4488)
	25mL sterile serological pipette	For transferring cell suspensions, adding and removing media and reagents	Sterile	Corning (4489)
	Pipette controller	For accurate aspiration and dispensing of media and reagents	Sterile	No specific vendor
	Benchtop centrifuge	For pelleting cells during processing steps; compatible with 15mL and 50mL centrifuge tubes	Sterility not required	Eppendorf (5702)
	Tissue culture incubator	37 °C, humidified, with 5% CO_2	Sterile	ESCO (CCL-170B-8)
	50mL centrifuge tubes	For cell collection	Sterile	Greiner (227261)
	12 well culture plate	Used for mycoplasma testing; conserves medium. Other formats suitable.	Sterile	Corning (3513)

Supplement table 2. Materials and Equipment Required for MEF Passaging. This table lists the essential tools, consumables, and equipment used during the passaging of MEFs, presented in approximate order of use. Specific sizes and brands listed are those used in this protocol; equivalent alternatives may be used as appropriate.

Supplement note 3 - Freezing MEFs

Reagents:

1.

Dulbecco's Phosphate Buffered Saline (without MgCl2 and CaCl2) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D8537-500ML

2. Trypsin-EDTA (0.25%), phenol red **Thermo Fisher Catalog #**25200072
3. MEF culture medium (with P/S), In-house preparation - See Table 1
4. Freezing medium, In-house preparation - See Table 3

Materials and Equipment:

	Item	Description / Notes	Sterility / Preparation	Vendor (Catalogue or Model #)
	Inverted microscope	For assessing cell morphology and confluency.	Wipe with 70% ethanol	Motic (AE31E)
	Aspiration pipette	For removing media and reagents	Sterile	Greiner (710183)
	Aspiration vacuum system	For safe waste collection	Sterility not required	Integra (Vacusafe)
	5mL sterile serological pipette	For transferring cell suspensions, adding and removing media and reagents	Sterile	Corning (4487)
	10mL sterile serological pipette	For transferring cell suspensions, adding and removing media and reagents	Sterile	Corning (4488)
	25mL sterile serological pipette	For transferring cell suspensions, adding and removing media and reagents	Sterile	Corning (4489)
	Pipette controller	For accurate aspiration and dispensing of media and reagent	Sterile	No specific vendor
	Tissue culture incubator	37 °C, humidified, with 5% CO_2	Sterile	ESCO (CCL-170B-8)
	50mL centrifuge tubes	For cell collection	Sterile	Greiner (227261)
	0.5mL Microcentrifuge tubes	For cell counting	Sterility not required	Eppendorf (30121023)
	Automated cell counter	Used for rapid and accurate cell counting; manual hemocytometer counting can also be used	Sterility not required	Invitrogen (Countess 3)
	Benchtop centrifuge	Used to pellet cells during processing steps; compatible with 15mL and 50mL centrifuge tubes	Sterility not required	Eppendorf (5702)

	Item	Description / Notes	Sterility / Preparation	Vendor (Catalogue or Model #)
	Cryovials	Fro freezing MEFs. (Important to choose internal thread and self-standing)	Sterile	Grenier (122263)
	Freezing container (e.g. Mr. Frosty)	Enables controlled-rate freezing (~−1 °C/min) when placed in a −80 °C freezer	Sterility not required	No specific vendor

Supplement table 3. Materials and Equipment Required for MEF Freezing. This table lists the essential tools, consumables, and equipment used during the freezing of MEFs, presented in approximate order of use. Specific sizes and brands listed are those used in this protocol; equivalent alternatives may be used as appropriate.

Supplement note 4 - Thawing MEFs

Reagents:

1. 0.1% gelatin, Cambridge Stem Cell Institute Tissue Culture Facility, https://www.stemcells.cam.ac.uk/facilities/tissue-culture
2. MEF culture medium (with P/S), In-house preparation - See Table 1

Materials and Equipment:

	Item	Description / Notes	Sterility / Preparation	Vendor (Catalogue or Model #)
	Aspiration pipette	For removing media and reagents	Sterile	Greiner (710183)
	Aspiration vacuum system	For safe waste collection	Sterility not required	Integra (Vacusafe)
	5mL sterile serological pipette	For transferring cell suspensions, adding and removing media and reagents	Sterile	Corning (4487)
	10mL sterile serological pipette	For transferring cell suspensions, adding and removing media and reagents	Sterile	Corning (4488)
	25mL sterile serological pipette	For transferring cell suspensions, adding and removing media and reagents	Sterile	Corning (4489)
	Pipette controller	For accurate aspiration and dispensing media and reagents	Sterile	No specific vendor

	Item	Description / Notes	Sterility / Preparation	Vendor (Catalogue or Model #)
	Tissue culture incubator	37 °C, humidified, with 5% CO_2	Sterile	ESCO (CCL-170B-8)
	Water bath	For thawing the frozen MEF vials	Sterile	Grant (JB Aqua 5)
	Inverted microscope	For assessing cell morphology and confluency.	Wipe with 70% ethanol	Motic (AE31E)
	50mL centrifuge tubes	For cell collection	Sterile	Greiner (227261)
	Benchtop centrifuge	Used to pellet cells during harvesting and washing steps; compatible with 15mL and 50mL centrifuge tubes	Sterility not required	Eppendorf (5702)

Supplement table 4. Materials and Equipment Required for MEF Thawing. This table lists the essential tools, consumables, and equipment used during the thawing of MEFs, presented in approximate order of use. Specific sizes and brands listed are those used in this protocol; equivalent alternatives may be used as appropriate.

Supplement note 5 - Inactivating MEFs

Reagents:

1. Dulbecco's Phosphate Buffered Saline (without MgCl2 and CaCl2) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D8537-500ML
2. Trypsin-EDTA (0.25%), phenol red **Thermo Fisher Catalog #**25200072
3. Mitomycin C inactivation medium, In-house preparation - See Table 4
4. MEF culture medium (with P/S), In-house preparation - See Table 1
5. Freezing medium, In-house preparation - See Table 3

Materials and Equipment:

	Item	Description / Notes	Sterility / Preparation	Vendor (Catalogue or Model #)
	2mL Syringe	For preparing mitomycin C	Sterile	No specific vendor
	16G Needle	For preparing mitomycin C	Sterile	No specific vendor
	50mL centrifuge tubes	For preparing mitomycin C and cell collection	Sterile	Greiner (227261)
	Waste collection container	For collecting mitomycin C-contaminated PBS washes and small items (e.g. syringes, needles).	Sterile	No specific vendor

	Item	Description / Notes	Sterility / Preparation	Vendor (Catalogue or Model #)
		Dispose of as hazardous waste according to local regulations.		
	Heavy duty waste collection bags	Double-bagged for mitomycin C-contaminated large items (e.g. serological pipettes). Dispose of as hazardous waste according to local regulations.	Sterility is not required, as the bags are placed outside the biological safety cabinet.	No specific vendor
	Inverted microscope	For assessing cell morphology and confluency.	Wipe with 70% ethanol	Motic (AE31E)
	Aspiration pipette	For removing media and reagents	Sterile	Greiner (710183)
	Aspiration vacuum system	For safe waste collection	Sterility not required	Integra (Vacusafe)
	5mL sterile serological pipette	For transferring cell suspensions, adding and removing media and reagents	Sterile	Corning (4487)
	10mL sterile serological pipette	For transferring cell suspensions, adding and removing media and reagents	Sterile	Corning (4488)
	25mL sterile serological pipette	For transferring cell suspensions, adding and removing media and reagents	Sterile	Corning (4489)
	Pipette controller	For accurate aspiration and dispensing media and reagents	Sterile	No specific vendor
	Tissue culture incubator	37 °C, humidified, with 5% CO_2	Sterile	ESCO (CCL-170B-8)
	0.5mL Microcentrifuge tubes	For cell counting	Sterility not required	Eppendorf (30121023)
	Automated cell counter	Used for rapid and accurate cell counting; manual hemocytometer counting can also be used	Sterility not required	Invitrogen (Countess 3)
	Benchtop centrifuge	Used to pellet cells during harvesting and washing steps; compatible with 15mL and 50mL centrifuge tubes	Sterility not required	Eppendorf 5702

	Item	Description / Notes	Sterility / Preparation	Vendor (Catalogue or Model #)
	Cryovials	Fro freezing MEFs. (Important to choose internal thread and self-standing)	Sterile	Grenier (122263)
	Freezing container (e.g. Mr. Frosty)	Enables controlled-rate freezing (~−1 °C/min) when placed in a −80 °C freezer	Sterility not required	No specific vendor

Supplement Table 5. Materials and Equipment Required for MEF Inactivation. This table lists the essential tools, consumables, and equipment used during the mitomycin C-based inactivation of MEFs, presented in approximate order of use. Specific sizes and brands listed are those used in this protocol; equivalent alternatives may be used as appropriate.

Supplement note 6 - Preparing Mitomycin C Stock Solution

Safety information

CAUTION: Mitomycin C is a cytotoxic agent. Handle with care in a biological safety cabinet. Wear appropriate PPE, including lab coat, nitrile gloves (double gloving if needed), and eye protection (if required). Change gloves frequently to avoid contamination of surfaces. Refer to the manufacturer's Safety Data Sheet (SDS) for detailed handling instructions.

All mitomycin C waste must be disposed of in accordance with the local safety guidelines for hazardous chemical waste. Never discard contaminated items in general waste bins.

Reagents:

1. Mitomycin C 10 mg **STEMCELL Technologies Inc. Catalog #**73274
2. Dulbecco's Phosphate Buffered Saline (without MgCl2 and CaCl2) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D8537-500ML

Materials and Equipment:

	Item	Description / Notes	Sterility / Preparation	Vendor (Catalogue or Model #)
	2mL Syringe	For preparing mitomycin C	Sterile	No specific vendor
	16G Needle	For preparing mitomycin C	Sterile	No specific vendor
	50mL centrifuge tubes	For preparing mitomycin C	Sterile	Greiner (227261)

	Item	Description / Notes	Sterility / Preparation	Vendor (Catalogue or Model #)
	Heavy duty waste collection bags	Double-bagged for mitomycin C-contaminated large items (e.g. serological pipettes). Dispose of as hazardous waste according to local regulations.	Sterility is not required, as the bags are placed outside the biological safety cabinet.	No specific vendor
	5mL sterile serological pipette	For transferring media and reagents	Sterile	Corning (4487)
	10mL sterile serological pipette	For transferring media and reagents	Sterile	Corning (4488)
	25mL sterile serological pipette	For transferring media and reagents	Sterile	Corning (4489)
	Pipette controller	For accurate aspiration and dispensing media and reagents	Sterile	No specific vendor

Supplement Table 6. Materials and Equipment Required for Mitomycin C Stock Solution Preparation. This table lists the essential tools, consumables, and equipment used during the preparation of mitomycin C stock solution, presented in approximate order of use. Specific sizes and brands listed are those used in this protocol; equivalent alternatives may be used as appropriate.

Supplement note 7 - Preparing Mitomycin C Inactivation Medium

Reagents:

1. Mitomycin C (0.4mg/ml) stock solution, In-house preparation - See Table 4
2. MEF culture medium (with P/S), In-house preparation - See Table 1

Materials and Equipment:

	Item	Description / Notes	Sterility / Preparation	Vendor (Catalogue or Model #)
	Sterile bottle or container	For preparing mitomycin C	Sterile	No specific vendor
	Heavy duty waste collection bags	Double-bagged for mitomycin C-contaminated large items (e.g. serological pipettes). Dispose of as hazardous waste according to local regulations.	Sterility is not required, as the bags are placed outside the biological safety cabinet.	No specific vendor
	25mL sterile serological pipette	For transferring media and reagents	Sterile	Corning (4489)
	10mL sterile serological pipette	For transferring media and reagents	Sterile	Corning (4488)

	Item	Description / Notes	Sterility / Preparation	Vendor (Catalogue or Model #)
	5mL sterile serological pipette	For transferring media and reagents	Sterile	Corning (4487)
	Pipette controller	For accurate aspiration and dispensing media and reagents	Sterile	No specific vendor

Supplement Table 7. Materials and Equipment Required for Mitomycin C Inactivation Medium Preparation. This table lists the essential tools, consumables, and equipment used during the preparation of mitomycin C inactivation medium, presented in approximate order of use. Specific sizes and brands listed are those used in this protocol; equivalent alternatives may be used as appropriate.

Supplement note 8 - Mycoplasma testing

Reagents:

1. TransDetect PCR Mycoplasma Detection Kit **Clinisciences Limited Catalog #**FM311-01
2. Agarose, ultrapure, 500g **Thermofisher Catalog #**16500500
3. SYBR SAFE DNA Gel Stain **Invitrogen - Thermo Fisher Catalog #**S33102
4. GeneRuler 100bp DNA Ladder **Thermofisher Catalog #**SM0241
5. TBE buffer, In-house preparation - See Table 6

Materials and Equipment:

	Item	Description / Notes	Sterility / Preparation	Vendor (Catalogue or Model #)
	Benchtop centrifuge	To prepare culture media supernatant	Sterility not required	Eppendorf (5702)
	Eppendorf tubes	To collect media from samples	Sterile	Eppendorf (0030120086)
	PCR tubes	To test culture media supernatant	Sterility not required	No specific vendor
	Thermocycler	For denaturation and PCR	Sterility not required	No specific vendor
	Microwave	To prepare agarose gel	Sterility not required	No specific vendor
	Conical flask	To prepare agarose gel	Sterility not required	No specific vendor
	Gel electrophoresis tank and power supply	To run agarose gel	Sterility not required	No specific vendor

	Item	Description / Notes	Sterility / Preparation	Vendor (Catalogue or Model #)
	Gel imaging system (e.g. Bio-Rad GelDoc Go)	To image agarose gel	Sterility not required	No specific vendor

Supplement Table 8. Materials and Equipment Required for Mycoplasma Testing. This table lists the essential tools, consumables, and equipment used for Mycoplasma testing, presented in approximate order of use. Specific sizes and brands listed are those used in this protocol; equivalent alternatives may be used as appropriate.

Safety warnings

Safety information

CAUTION: Mitomycin C is a cytotoxic agent. Please refer to the manufacturer's safety datasheet for proper handling procedures.

Ethics statement

All animal research at the University of Cambridge is conducted in accordance with internationally accepted standards for the care and use of laboratory animals, including the principles of the 3Rs (Replacement, Reduction, and Refinement). All procedures are reviewed and approved by the University's Animal Welfare and Ethical Review Body (AWERB) to ensure compliance with relevant legislation and the highest standards of animal welfare.

This protocol uses only natural mating and Schedule 1 methods of euthanasia, which fall outside the scope of regulated procedures under the UK Animals (Scientific Procedures) Act 1986 (ASPA). Therefore, a Home Office licence is not required. All work is documented under non-regulated procedure reference NR2025/20.

Schedule 1 euthanasia and dissection of pregnant mice are carried out by trained and competent animal care staff from the University's Biomedical Services (UBS), in accordance with institutional protocols and ethical standards.

Before start

All work must be performed under sterile conditions in a biological safety cabinet unless stated otherwise.

Protocol 1 - Derivation and culture of MEFs

1

Note

Reference Tables for Reagents, Materials and Equipment:
Refer to the Materials section *(Supplement Note 1)* for all reagents, materials, and equipment used in the *Derivation and culture of MEFs* procedure.

2 ***T175 flask preparation:***

Add 10 mL of 0.1% gelatin to each T175 flask and leave them in the biological safety cabinet for 00:10:00 at Room temperature . Gelatin can also be left to coat for longer than 10 minutes if required.

Note

Guidance for estimating flask coating needs based on embryo yield:
It is not absolutely essential to pre-prepare flasks for deriving MEFs, but it is a routine practice for our institute to save time at critical downstream steps when performing large batch preparations of MEFs.

At our institute, a typical CD1 litter yields ~15-17 embryos. On average, 3 embryos are sufficient to seed one T175 flask. In this protocol, embryos are pooled and seeded without prior cell counting. To make the most of the time and effort involved, we typically use two pregnant mice per experiment, allowing us to make a large batch of MEFs (~2 x 10^9) for laboratory use and backup storage. In such cases, we usually prepare and coat 10 T175 flasks. The actual number of flasks required will depend on factors such as mouse strain, expected embryo yield, culture dish sizes, and desired cell seeding density. Please refer to published literature for recommended seeding densities for different culture flasks and dishes (e.g. **Useful Numbers for Cell Culture | Thermo Fisher Scientific - UK**).

Steps #2–5 (flask preparation) may alternatively be performed after the total number of embryos is determined in Step #7. This allows adjustment of the number of flasks prepared based on the actual yield.

3 After 10 minutes, **aspirate** the gelatin from each flask.

4 **Add** 25 mL of MEF culture medium *(prepared as described in Materials section, Table 1)* to each flask.

5 **Transfer** the flasks to a humidified incubator set at 37 °C with 5% CO_2. The flasks are now ready for seeding with MEFs.

6 ***Pre-dissection setup:***
Wipe down the workspace with 70% ethanol or an appropriate disinfectant. Immerse dissection tools in 70% ethanol for ~10 minutes, or spray with ethanol and wipe clean before use.
Optional: Autoclave instruments in advance as an added sterility measure.

Note

The dissection of uterine horns can be performed outside the biological safety cabinet up to Step 11, after which the procedure should continue under sterile conditions in the cabinet. We have not experienced contamination when following this approach.

7 **Prepare** four (or more) sterile 100 mm culture dishes, each containing 1X DPBS, for handling embryos.

Note

The exact number of dishes is flexible; four is the amount we usually use. Multiple dishes are useful to wash away blood during dissection and to separate tissues as needed. For example, transferring embryos to a fresh dish during dissection helps keep the workspace and dishes clean by removing non-essential tissues such as heads or other discarded parts.

Filling each dish about halfway with DPBS is usually sufficient for washing and handling embryos.

8 **Transfer** the uterine horns from the 50 ml Falcon tube (provided by the animal technician) into one of the prepared DPBS dishes.

Optional: Record the number of embryos to track cell yield.

Note

At our institute, the pregnant female mouse is dissected by the animal technician; therefore, only the uterine horns are handled during this protocol. If dissection from the pregnant demale is required, please refer to the *Supplementary Protocol: Dissection of Pregnant Mouse for Uterine Horn Collection* at the end of the protocol.

9 ***Embryo dissection:***

Using fine scissors, **open** the uterine wall to release embryos, along with placental tissue and membranes, into the 1X DPBS. Discard the empty uterus according to local safety guidelines for animal waste.

Note

Select only morphologically normal embryos for further processing. Discard any that appear unhealthy or show signs of resorption (see Guidelines for more details).

10 **Decapitate** the embryo and remove the non-mesenchymal red organs (such as liver and lungs) using fine-point dissection forceps.

Optional: Use a dissection microscope to assist with this step.

Note

Complete removal of visceral tissue is not essential, as these cells will not survive the subsequent culture and will be lost during passaging.

11 **Transfer** the dissected embryo carcasses into fresh 1X DPBS to wash off any residual blood, then **move** the dish into a biological safety cabinet to continue the procedure under sterile conditions.

12 **Add** a small volume (1mL) of 1X DPBS to a new sterile 100 mm culture dish.

Note

A minimal volume helps keep the tissue in a confined area, making it easier to mince. If too much liquid is added, the tissue fragments can float or spread out, making them difficult to mince efficiently.

13 **Transfer** the washed embryo carcasses to this dish.

14 **Mince** the embryo tissue thoroughly at Room temperature using a sterile blade until the tissue pieces becomes smooth and viscous. When cutting no longer visibly reduces the size of individual pieces, proceed to the next step.

15 **Add** 5 mL of 1X DPBS to the minced tissue to aid in transfer.

16 **Collect** all tissue pieces using a 10mL serological pipette and evenly distribute them into two 15 mL
centrifuge tubes.

Note

Embryos from one pregnant female are typically divided between two 15 mL tubes.

17 Allow the tissues to **settle** by gravity for 1–2 minutes, or until they have visibly sunk to the bottom.

18 **Aspirate** the supernatant, then rinse the dish with additional 5 mL of 1X DPBS to collect any remaining tissue pieces and transfer them into the tubes.

19 Allow the tissues to **settle** again.

20 **Aspirate** the supernatant carefully, leaving the tissues undisturbed.

Note

Two settling steps help with faster sedimentation in smaller volumes, but combining everything at once is also acceptable.

21 ***Trypsinisation:***
Add 3 mL of 0.25% trypsin-EDTA to each of the centrifuge tube.

Note

The volume of trypsin can be adjusted depending on the number of embryos in each tube. In our routine procedure, 3 mL is typically used for digesting tissues from one litter of 15 x CD1 mice.

22 **Incubate** the tubes at 37 °C in a humidified 5% CO_2 incubator for 00:15:00 , gently shaking every 5 mins to ensure even exposure to trypsin.

23 **Retrieve** the tubes from incubator.

24 **Pipette** the tissue suspension up and down approximately 20 times using a 5mL sterile serological pipette to dissociate the cells into a single-cell suspension.

Note

Adjust the number of pipetting based on how well the tissue has been dissociated.

25 **Evenly distribute** the cell suspension from each tube into separate pre-warmed T175 flasks, each containing 25 ml of MEF culture medium, as prepared in Step #5. Gently tilt the flask back and forth and side to side to ensure even distribution of cells across the entire growth surface.

This is passage 0.

Note

- In this protocol, the cell suspension is evenly distributed into pre-warmed T175 flasks without prior cell counting. This approach is based on standard practice in our laboratory, where ~3 CD1 embryos are enough to seed one T175 flask. From experience, this results in healthy, steadily proliferating MEFs that typically reach 80–90% confluency within 48 hours post-isolation. Pooling embryos and dividing the suspension evenly across flasks simplifies workflow during large batch preparations, while still maintaining reproducibility and cell quality. If greater accuracy is required for downstream applications, cells can be counted and seeded at a defined density. As a general guide, please refer to published resources such as **Useful Numbers for Cell Culture | Thermo Fisher Scientific - UK** for recommended seeding densities across different vessel types.
- The serum in the MEF culture medium inactivates the trypsin, so there is no need to wash the cells.
- Avoid circular motions, as this can cause cells to concentrate in the centre of the flask.

26 **Incubate** the T175 flasks Overnight at 37 °C in a humidified 5% CO_2 incubator.

27 **Examine** the cultures under an inverted microscope the following day to assess cell confluency.

Note

Guidance for assessing confluency and deciding next steps:
It is normal to observe some cell debris and undigested tissue 24 hours after plating (see *Figure 1A, black arrows*). At this stage, MEFs typically exhibit partial confluency, generally below 80%, and need further incubation to support continued growth.

By 48 hours post-plating, MEFs often reach 80–100% confluency (see *Figure 1B*). They can be either:

- Passaged into T175 flasks for further expansion (see Protocol 2 – Passaging and Expanding MEFs), or
- Prepared for cryopreservation (see Protocol 3 – Freezing MEFs), depending on experimental needs. For optimal recovery, MEFs should ideally be frozen during the exponential growth phase and at a low passage number; either P0 (directly post-harvest) or between P1 and P4 following expansion.

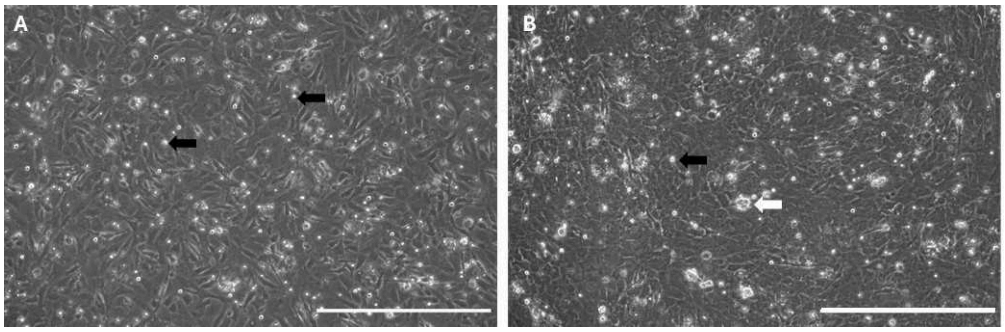

Figure 1. Confluency Assessment of MEFs Post-Plating
A. Phase-contrast image of MEFs 24 hours after plating. Cultures display partial confluency with visible cell debris (white arrows) and undigested tissue (black arrows).
B. Phase-contrast image of MEFs 48 hours after plating, showing cultures that have progressed to complete confluency.

If confluency remains below 80% at 48 hours, consider changing the medium: carefully aspirate the old medium to remove debris and undigested material, add fresh MEF culture medium, and return the flasks to the incubator to support further growth.

Protocol 2 - Passaging and Expanding MEFs

28

Note

Reference Tables for Reagents, Materials and Equipment:
Refer to the Materials section *(Supplement Note 2)* for all reagents, materials, and equipment used in the *Passaging and Expanding of MEFs* procedure.

29 - Prepare MEF medium (without penicillin/streptomycin) for mycoplasma testing as described in the Materials section - Table 2, for use in Step 43. For details on the mycoplasma testing, see Supplementary Protocol: Mycoplasma Testing.

Note

The volume required will depend on the number and size of culture flasks. For guidance, refer to published recommendations such as the **Useful Numbers for Cell Culture | Thermo Fisher Scientific - UK**.

30 ***T175 flask preparation:***
Depending on cell confluency, passage the cells at a 1:5 ratio if they have reached approximately 90% confluency. **Follow** steps #2–5 to coat the T175 flasks with gelatin, but use 20 mL of MEF culture medium instead of 25 ml.

Note

Guidance on MEF split ratios based on passage number and morphology:
The appropriate split ratio depends on culture confluency, cell morphology, and passage number. Early-passage MEFs (P0–P2) that are healthy and tightly packed can often be split at a ratio of 1:4 to 1:6. In contrast, MEFs at later passages may exhibit slower proliferation rates and may require a more conservative split ratio of 1:2 or 1:3 to maintain optimal growth. It's crucial to avoid seeding densities that are too low, as this may not establish sufficient cell-cell signaling for optimal proliferation. Likewise, allowing cultures to become overly confluent may cause cells to stop growing due to crowding and nutrient depletion.

31 **Retrieve** T175 flasks containing cultured MEFs from the incubator.

32 **Aspirate** the medium from each flask.

33 **Add** 15 mL of sterile 1X DPBS to each T175 flask. Gently **rinse** the cell surface by drawing up and dispensing the PBS with a sterile serological pipette 3 times within the same flask.

34 **Aspirate** the DPBS from each flask.

35 (Optional) **Repeat** steps #33–34 for a second DPBS wash.

Note

Residual serum components in MEF medium inhibit trypsin activity; thorough washing is essential for effective cell detachment.

36 ***Trypsinisation:***
Add 7 mL of 0.25% trypsin-EDTA (or just enough to fully cover the surface) to each T175 flask. Gently tilt the flask back and forth and side to side to ensure the cell monolayer is fully covered.

37 **Incubate** the flasks at 37 °C in a humidified 5% CO_2 incubator for 00:02:00 .

38 **Retrieve** the flasks from the incubator and **examine** under an inverted microscope to assess cell detachment. Most cells should appear rounded and detached. Firmly tap the sides of each flask to dislodge any remaining attached cells.

39 **Add** 15 mL of MEF culture medium to each flask to inactivate the trypsin. Gently rinse the flasks by pipetting the medium up and down a few times using a sterile serological pipette.

40 **Transfer** the cell suspension to a 50 mL sterile centrifuge tube.

41 **Add** an additional 10 mL of MEF culture medium to the empty flask and rinse again to collect any remaining cells.

42 Combine the second rinse with the initial cell suspension in the same 50 mL tube.

43 ***Reserve cells for mycoplasma testing:***

Plate 100 µL of the pooled cell suspension into a well of a 12-well plate containing 500 µL MEF culture medium (without penicillin/streptomycin) prepared in Step #29, for routine mycoplasma testing. A 12-well plate is typically used to minimise the volume of medium and number of cells required, but other sterile plates or dishes are also suitable.

Note

Mycoplasma testing is performed routinely on every batch of MEFs prepared in our laboratory as part of our standard quality control practices. A supplementary protocol outlining the testing procedure is provided at the end of this document (See *Supplementary Protocol: Mycoplasma Testing*). After testing, MEFs are cultured exclusively in a designated mycoplasma-free tissue culture room, where the risk of contamination is minimal and regular monthly testing is carried out to maintain a clean cell culture environment.

44 **Centrifuge** the remaining cell suspension at 1200 rpm, Room temperature, 00:05:00 .

45 **Aspirate** the supernatant carefully, leaving the cell pellet undisturbed.

46 **Resuspend** each cell pellet in 25 mL of fresh pre-warmed MEF culture medium.

Note

The resuspension volume is calculated based on a 1:5 split ratio, allowing for even seeding density across flasks and maintaining optimal cell-to-medium ratio that supports efficient attachment and proliferation in T175 flasks.

47 **Pipette** the suspension up and down 5 times using a 10mL sterile serological pipette to break up cell clumps and achieve a single-cell suspension.

Note

Adjust the number of pipetting based on how well the tissue has dissociated.

48 **Seed** 5 mL of the cell suspension into each pre-warmed T175 flask containing 20 mL MEF culture medium. Gently tilt the flask back and forth and side to side to ensure even distribution of cells across the entire growth surface.

This is passage 1.

Note

Avoid circular motions, as this can cause cells to concentrate in the centre of the flask.

49 **Incubate** the T175 flasks Overnight at 37 °C in a humidified 5% CO_2 incubator.

50 **Examine** the cultures under an inverted microscope the following day to assess cell confluency. If the cultures have reached 80-90% confluency, proceed with either passaging (see steps #30-49) or cryopreservation (see next section) depending on your experimental needs.

Protocol 3 - Freezing MEFs

51

Note

Reference Tables for Reagents, Materials and Equipment:
Refer to the Materials section *(Supplement Note 3)* for all reagents, materials, and equipment used in the *Freezing of MEFs* procedure.

Note

This section describes two freezing approaches:
1. For downstream experiments, freeze MEFs at a standard concentration of 5×10^6 cells/mL (see Steps 52–60).
2. For our internal backup storage (to allow future regrowth of MEFs), freeze without prior cell counting (see Steps 52, 53, 55-60). We typically freeze one T175 flask into 1 cryovial.

52 **Label** cryovials with the name of the cell line, passage number, number of cells, and date.

53 **Follow** steps #31-42 to detach the cells from the flask.

54 **Take** two 10 µL aliquots of the cell suspension and transfer them to microcentrifuge tubes for cell counting. Based on the total cell count, calculate the volume of freezing medium required to resuspend the cells at the desired final concentration (5×10^6 cells/mL).

55 **Prepare** freezing medium *(as described in Materials section, Table 3)*.

- For standard downstream experiments, prepare the freezing medium based on the total number of cells with a final concentration of 5×10^6 cells/mL.
- For internal backup storage, prepare the freezing medium based on the number of flasks. For example, if freezing cells from 8 flasks, prepare 8 mL of freezing medium (1 mL per T175 flask).

56 **Centrifuge** the remaining cell suspension at
1200 rpm, Room temperature, 00:05:00 .

57 **Aspirate** the supernatant carefully, leaving the cell pellet undisturbed.

58 **Resuspend** the pellet in freezing medium. Pipette up and down gently to dissociate any cell aggregates.

59 **Transfer** 1 mL cell suspension in freezing medium into each labelled cryovial using a 5mL sterile serological pipette. This method is used both for standard downstream applications (5×10^6 cells/ml) and for internal backup storage, where cells are frozen without prior counting.

Note

- Mix the cell suspension by pipetting up and down frequently during aliquoting to maintain an even distribution of cells across all vials - this is especially important when working with a large volume of suspension.
- Work swiftly, as DMSO in the freezing medium is toxic to cells at room temperature.

60 Immediately **place** cryovials into an isopropanol freezing container (e.g. Mr. Frosty) and **transfer** to a −80 °C freezer for controlled-rate cooling (~−1 °C/minute). **Transfer** vials from the −80 °C freezer to a liquid nitrogen storage system the following day.

Note

To minimise DMSO exposure time:
- Load cryovials into the freezing container in small batches and place them in the −80 °C freezer promptly.
- Or keep the cell suspension on ice during preparation.

Protocol 4 - Thawing MEFs

61

Note

Reference Tables for Reagents, Materials and Equipment:
Refer to the Materials section *(Supplement Note 4)* for all reagents, materials, and equipment used in the *Thawing of MEFs* procedure.

62 ***T175 flask preparation:***
Follow steps #2–5 to coat the T175 flasks with gelatin, but use 20 mL of MEF culture medium instead of 25 ml.

63 **Retrieve** the frozen cryovial of MEFs from liquid nitrogen storage and immediately place into a 37 °C water bath. Thaw as quickly as possible with gently agitation until no ice crystals remain.

Note

If not proceeding directly to thawing, place the cells on dry ice or in a liquid nitrogen container. Only place the vial in the water bath when you are fully ready to continue. Once thawed, cells are vulnerable and exposed to DMSO, which is toxic at room temperature. Proceed immediately to dilute the thawed suspension in culture medium to protect cell viability.

64 **Sterilise** the outside of the cryovial extensively with 70% ethanol to minimise the risk of contamination before opening in the biological safety cabinet.

65 **Transfer** the contents of the cryovial into a 15mL centrifuge tube containing 10 mL MEF culture medium using a P1000 pipette.

66 **Add** 1 mL of MEF culture medium to the cryovial, gently rinse to collect remaining cells, and **transfer** to the centrifuge tube. Repeat with another wash.

67 **Centrifuge** the cell suspension at 1000 rpm, Room temperature, 00:05:00 .

Note

This is to remove the DMSO in the freezing medium.

68 **Aspirate** the supernatant carefully, leaving the cell pellet undisturbed.

69 **Resuspend** the pellet in an appropriate volume of MEF culture medium, depending on your intended use. For downstream experiments, adjust the volume based on the desired seeding density. For internal expansion, resuspending in 5 mL is typically sufficient for direct seeding into one T175 flask containing 20 mL MEF culture medium (as prepared in Step #62). From experience, this method yields healthy, proliferative cultures that typically reach 80–90% confluency within 48 hours.

70 **Pipette** the suspension up and down 5 times using a 10mL sterile serological pipette to break up cell clumps and achieve a single-cell suspension.

Note

Adjust the number of pipetting based on how well the tissue has dissociated.

71 **Seed** an appropriate amount of the cell suspension into each pre-warmed T175 flask containing the required MEF culture medium. Gently tilt the flask back and forth and side to side to ensure even distribution of cells across the entire growth surface.

Note

Avoid circular motions, as this can cause cells to concentrate in the centre of the flask.

72 **Incubate** the T175 flasks Overnight at 37 °C in a humidified 5% CO_2 incubator for further growth.

Protocol 5 - Inactivating MEFs using Mitomycin C

73

Note

Reference Tables for Reagents, Materials and Equipment:
Refer to the Materials section *(Supplement Note 5)* for all reagents, materials, and equipment used in the *Inactivating of MEFs* procedure.

74 **Prepare** Mitomycin C inactivation medium as described in the *Materials section (Table 4-5)* and follow the detailed procedure in *Supplementary Protocol Mitomycin C Stock and Working Solution Preparation.*

Safety information

CAUTION: Mitomycin C is a cytotoxic agent. Handle with care in a biological safety cabinet. Wear appropriate PPE, including lab coat, nitrile gloves (double gloving if needed), and eye protection (if required). Change gloves frequently to avoid contamination of surfaces. Refer to the manufacturer's Safety Data Sheet (SDS) for detailed handling instructions.

Note

Precautions When Working with Mitomycin C:
The following waste-handling practices are those implemented in our laboratory. Please consult your institution's safety office for local guidance.

- **Liquid waste:** Set up a clearly labelled, sturdy chemical-resistant waste collection bottle inside the biological safety cabinet for collecting all mitomycin C-contaminated liquid waste
- **Solid waste:** Prepare double-bagged, sturdy plastic waste bags for disposing of larger contaminated items (e.g. gloves, serological pipettes).
- **Sharps:** Dispose of any contaminated sharps (e.g., needles) in a dedicated, puncture-resistant sharps container.
- **Disposal:** All mitomycin C waste must be disposed of in accordance with the local safety guidelines for hazardous chemical waste. Never discard contaminated items in general waste bins.

75 **Retrieve** T175 flasks containing cultured MEFs from the incubator.

76 **Aspirate** the medium from each flask.

77 **Add** 10 mL of mitomycin C inactivation medium (containing 10 µg/mL mitomycin C) to each T175 flask.

78 **Incubate** the flasks at 37 °C in a humidified 5% CO_2 incubator for 02:00:00 .

Note

Incubation can be extended up to 3 hours if necessary.

79 **Remove** the inactivation medium into designated collection bottle using a serological pipette.

80 **Follow** steps #33-42 to wash and remove the cells from the flask.

Note

Three separate PBS washes are recommended after Mitomycin C treatment to ensure thorough removal of residual medium. All liquid waste must be disposed of in the designated collection bottle.

81 **Follow** steps #52-60 to count and freeze the cells.

Supplementary Protocol: Dissection of Pregnant Mouse for Uterine Horn Collection

82

Note

If dissection of a pregnant mouse is required to obtain embryos, carry out the following steps before Step #7.

83 **Kill** the pregnant female mouse using cervical dislocation or another approved method in accordance with institutional guidelines.

84 **Sterlise** the entire mouse by spraying 70% ethanol to reduce surface contamination.

85 Using forceps (sterility not essential at this stage), gently **lift** the abdominal skin and make a small horizontal incision with scissors.

86 **Pull** the skin apart above and below the incision using the forceps to expose the underlying abdominal muscles.

87 Switch to sterile dissection instruments. Carefully **incise** the abdominal wall to open the peritoneal cavity. Gently move aside any overlying intestines using sterile forceps to reveal the uterus.

88 Locate the base of the uterus near the cervix. Use sterile forceps to **grasp** this region and **cut** just below the utero-vaginal junction with sterile scissors.

89 Gently **separate** the uterine horns from the surrounding mesometrium and **trim** any excess fat or connective tissue.

90 Proceed to Step #7 of the main protocol to continue with embryo isolation.

Supplementary Protocol: Mitomycin C Stock Solution Preparation

91

Note

Reference Tables for Reagents, Materials and Equipment:
Refer to the Materials section (Supplement Note 6) for all reagents, materials, and equipment used in the *mitomycin C stock solution preparation* procedure.

For the composition of the mitomycin C stock solution, refer to the recipe provided in Table 4.

92 **Retrieve** vial of mitomycin C powder (2mg) from −20°C storage (some suppliers provide it at 4°C).

Safety information

CAUTION: Mitomycin C is a cytotoxic agent. Handle with care in a biological safety cabinet. Wear appropriate PPE, including lab coat, nitrile gloves (double gloving if needed), and eye protection (if required). Change gloves frequently to avoid contamination of surfaces. Refer to the manufacturer's Safety Data Sheet (SDS) for detailed handling instructions.

Why use the needle method for reconstitution?
Mitomycin C is supplied as a powder and is cytotoxic even in small quantities. Removing the rubber stopper can expose your gloves and surrounding surfaces to contamination from the dry powder, even when working inside a biological safety cabinet. Instead, puncturing the rubber stopper with a sterile needle allows for safe, controlled reconstitution by keeping the powder enclosed and minimising direct contact. This method reduces the risk of contaminating gloves, surfaces, and equipment with hazardous material.

Note

Precautions When Working with Mitomycin C:
The following waste-handling practices are those implemented in our laboratory. Please consult your institution's safety office for local guidance.

- **Liquid waste:** Set up a clearly labelled, sturdy chemical-resistant waste collection bottle inside the biological safety cabinet for collecting all mitomycin C-contaminated liquid waste
- **Solid waste:** Prepare double-bagged, sturdy plastic waste bags for disposing of larger contaminated items (e.g. gloves, serological pipettes).
- **Sharps:** Dispose of any contaminated sharps (e.g., needles) in a dedicated, puncture-resistant sharps container.
- **Disposal:** All mitomycin C waste must be disposed of in accordance with the local safety guidelines for hazardous chemical waste. Never discard contaminated items in general waste bins.

93 **Prepare** a labelled 50mL centrifuge tube with 21 mL 1X DPBS.

94 ***Insert vent needle:***
Insert a sterile needle (without syringe attached) into the rubber stopper of the mitomycin C vial to act as a vent.

Note

Use of vent needle:
When injecting liquid into a vial, the internal pressure increases, especially if the vial is sealed (as in the case of our mitomycin C bottle with a rubber lid). This pressure may cause resistance during injection or even force the solution to spray back. This poses a safety risk, especially when working with hazardous chemicals. Inserting a second needle (without a syringe) as a vent allows displaced air to escape as liquid enters, preventing pressure buildup and enabling safer, more controlled reconstitution.

95 Using a 2ml syringe with a needle, **draw up** 2 mL of fresh 1X DPBS.

96 While the vent needle is in place, **carefully inject** the 2 mL 1X DPBS into the mitomycin C vial to rinse.

97 **Gently swirl** the vial to dissolve powder.

98 **Slowly transfer** the reconstituted mitomycin C down the **inner wall** of the 50mL tube to avoid splashing or aerosol formation.

99 **Draw** another 2 mL of fresh 1X DPBS using the same syringe and repeat the rinse:

- Inject into the vial, swirl gently, and withdraw the solution.
- Transfer the second rinse into the same 50mL tube.

Note

This ensures maximum retrieval of remaining mitomycin C.

100 Discard all items that have come into contact with mitomycin C as hazardous waste, following local safety guidelines.

101 The reconstituted mitomycin C (0.4 mg/mL) is now ready to be diluted in MEF medium. The total volume in the 50mL tube should now be approximately 25 mL . The prepared solution may be stored at 4^{o}C for up to 1 week, or at -20^{o}C for up to 1 month (Kinast et al, 2016). In both cases, protect the solution from light by storing it in a dark container or wrapping the tube in foil.

Supplementary Protocol: Mitomycin C Inactivation Medium Preparation

102 55

Note

Reference Tables for Reagents, Materials and Equipment:
Refer to the Materials section (Supplement Note 7) for all reagents, materials, and equipment used in the *mitomycin C stock solution preparation* procedure.

For the composition of the mitomycin C inactivation medium, refer to the recipe provided in Table 5.

Refer to Step 92 for safety precautions when handling Mitomycin C.

103 Retrieve the 0.4 mg/mL mitomycin C stock solution from 4°C or −20°C storage and thaw if frozen. Protect the tube from light (e.g. wrap in foil).

104 In a sterile, labelled bottle or container, add 195 mL of MEF culture medium.

105 Add 5 mL of the 0.4 mg/mL mitomycin C stock solution to the medium. Gently pipette up and down to mix well. This dilution yields a final mitomycin C concentration of 10 µg/mL, suitable for MEF inactivation.

Note

When adding the 5 mL mitomycin C stock solution, handle carefully to avoid any spills or droplets on gloves or surrounding surfaces. Clean any splashes immediately following your laboratory's hazardous waste protocol.

106 The reconstituted mitomycin C is now ready to be used for MEF inactivation. Store at -20^{o}C for up to 6 months. Protect from light. (Reference: https://cdn.cytivalifesciences.com/api/public/content/digi-17788-pdf)

Supplementary Protocol: Mycoplasma Testing

107

Note

As mentioned in Step 43, every batch of MEF must be rigorously tested for mycoplasma to ensure they are uncontaminated before being made available for use in experiments. We test for mycoplasma at the first passage and immediately before inactivation.

108 The cells reserved for mycoplasma testing in Step 43 must be cultured for a minimum of 4 days in the MEF media without penicillin/streptomycin and without a media change. This allows any potential mycoplasma to reach detectable levels. Shorter incubation times may lead to false negative results.

109 After 4-7 days, **collect** approximately 1 mL of the culture media into a sterile 1.5 mL Eppendorf tube.

110 **Centrifuge** the culture media for 00:03:00 at 1100 rpm to pellet any cells as mycoplasma accumulates in the culture supernatant.

111 **Transfer** the supernatant into a new sterile 1.5 mL Eppendorf tube; **discard** any pellet.

112

Note

The following PCR protocol for mycoplasma testing is based on the manufacturer's instructions; however, reaction volumes have been reduced by 50% for economy without compromising sensitivity. The kit used is the *TransDetect* PCR Mycoplasma Detection Kit.

113 **Remove** the kit's reagents: *TransDetect* PCR Myco SuperMix (2×), Myco Primer Mix, Myco Positive Control Template, and MycoFree Water from -20°C storage and **thaw** on ice.

Note

- *TransDetect* PCR Myco SuperMix (2×) contains loading dye so no additional loading dye is needed for gel electrophoresis.
- Myco Primer Mix targets the 16S gene fragment that is conserved across common Mycoplasma species.
- Myco Positive Control Template confirms the functionality of the PCR.
- Nuclease-free water serves as the negative control.

114 **Transfer** 40 µL of the culture supernatant you are testing to a clean PCR tube of the size appropriate to your thermocycler.

Note

Ensure the lid of the tube is secure.

115 **Heat** the PCR tube to 95 °C for 00:10:00 in a thermocycler to denature the sample. This breaks open the mycoplasma cells and denatures the proteins which releases the target DNA allowing it to be amplified.

116

Note

The PCR master mix can be prepared whilst the thermocycler is running.

Prepare sufficient master mix for the number of samples + the positive control and negative control, in a 1.5 mL Eppendorf tube, using the volumes of each reagent as shown in the table below:

	Component	Volume for 1 tube (uL)
	TransDetect PCR Myco SuperMix (2×)	5
	Myco Primer Mix	0.2

	Component	Volume for 1 tube (uL)
	MycoFree Water	3.8

Note

It is advised that additional volume equivalent for the amount for one sample is also prepared to account for pipetting error e.g. if you have 5 samples + a positive and negative control, prepare a master mix for 8 reactions instead of the 7 that you actually have.

117 **Mix** the PCR master mix by gently pipetting up and down then store on ice until needed.

118 Once sample denaturation program has finished on the thermocycler, **spin** the tube(s) briefly using a mini centrifuge.

119 **Take** 1 µL of the denatured sample into a clean PCR tube.

120 **Add** 9 µL of the reaction mix to the sample, giving a total volume of 10 µL.

121 Make sure the lid of the PCR tube is secure and **spin** briefly using a mini centrifuge.

122 **Transfer** the tube to a PCR thermocycler and run the following programme:

122.1 *Initial denaturation* for 00:04:00 at 94 °C : This denatures the double-stranded DNA as the high temperature breaks the hydrogen bonds between the nucleotide bases, resulting in single-stranded DNA the primers are able to bind to later.

122.2 *Denaturation* for 00:00:30 at 94 °C : This step separates the DNA strands at the start of each
PCR cycle, ensuring that primers can anneal to the DNA templates.

122.3 *Annealing* for 00:00:30 at 60 °C : This temperature is optimal for the *TransDetect* kit mycoplasma-specific primers, which are targeting conserved regions of the 16S rRNA gene, to anneal to their complimentary sequences on the single-stranded DNA templates.

122.4 *Extension* for 00:00:30 at 72 °C : Taq DNA polymerase extends the primers, synthesising new DNA strands that are complementary to the target region. This is the optimal temperature for enzyme activity, enabling rapid and accurate DNA strand extension.

122.5 **Repeat** steps #122.2-122.4 for 35 cycles which amplifies the target DNA exponentially, making billions of copies of the mycoplasma DNA if present.

122.6 *Final extension* for 00:05:00 at 72 °C : This step makes sure that any partially synthesised DNA strands are fully extended, resulting in complete amplicons and improving their stability and visibility on the gel later.

122.7 **Hold** at 4 °C until the next step. This preserves the PCR products until the gel is ready to
load, as Taq DNA polymerase activity is stopped and degradation is minimised.

Note

Alternatively, the PCR tubes can be stored in a 4 °C fridge until the next step.

123 **Set up** a gel electrophoresis system in order to run the samples. Make sure the gel tray with comb insert is taped and ready for liquid agarose gel to be poured into it.

124

Note

A standard sized (~14 wells) 1% agarose/Tris-Borate-EDTA (TBE) gel can be prepared whilst the PCR thermocycler program is running. Adjust the volumes below to your standard gel preparation procedure and according to how many wells you need for the number of samples.

124.1 **Weigh** out 1 g of agarose powder in a weigh boat and **transfer** to a **250 mL** conical flask.

124.2 **Measure** out 100 mL of **1x TBE** using a measuring cylinder and **add** it to the agarose in the conical flask.

124.3 **Microwave** gently (e.g. for 900w, an initial burst of 30 seconds, then 10-second intervals thereafter) to dissolve the agarose powder in 1× TBE, avoiding the solution bubbling over.

125 Allow the liquid gel to cool slightly by swirling and running the base of the conical flask under cold water until it can be held in the palm of a gloved hand for more than 10 seconds without discomfort.

Safety information

Wear a heatproof glove and safety goggles when handling the hot conical flask to protect against burns or splashes. Please also follow any additional safety precautions established by your institution for handling hot objects.

126 **Add** 10 µL of **SYBR Safe** DNA gel stain to the liquid gel (1:10,000) and swirl the conical flask to mix thoroughly.

Safety information

Ethidium Bromide (EtBr) or another alternative DNA gel stain can be used in place of SYBR Safe, but it is vital to follow your institution's risk assessment as EtBr is carcinogenic.

127 **Pour** the gel into the gel tray with comb and leave it to cool and solidify for approximately 00:20:00 .

128 **Load** 5 µL of 100 bp DNA ladder into the first well, then 10 µL of the PCR reaction products into subsequent wells.

Note

The addition of loading buffer is not necessary as it is already present in the *TransDetect* PCR Myco SuperMix (2 ×) used for the samples.

129 **Run** the gel at 100 V for 00:45:00 or until the ladder bands are distinct.

130 **Visualise** the gel using a standard gel imaging system (e.g., Bio-Rad GelDoc Go, as used in our laboratory).

130.1 Example of how the visualised gel could look:

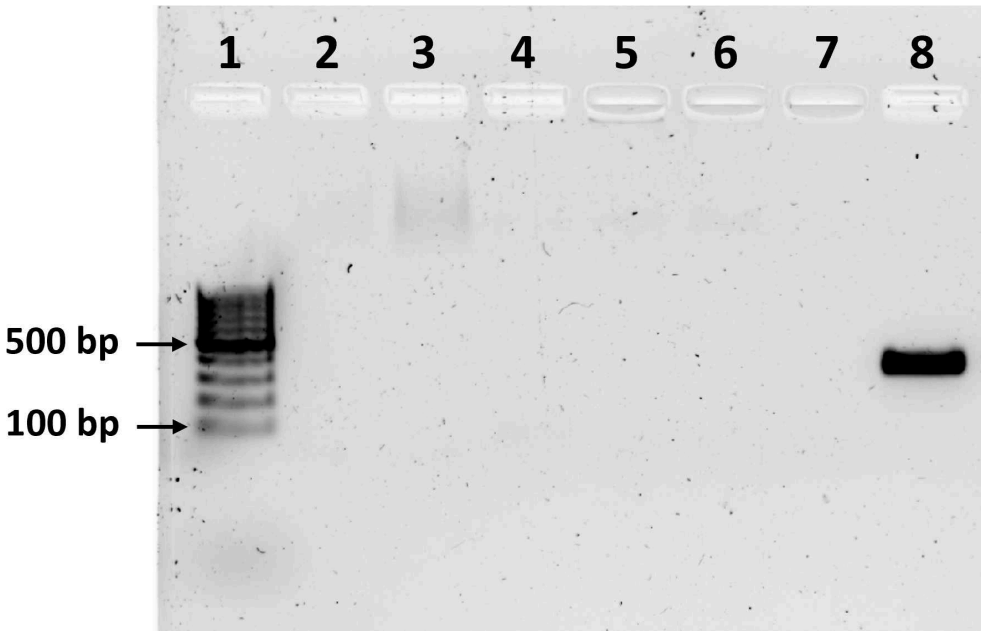

Figure 2: PCR gel electrophoresis for the detection of mycoplasma contamination.
Lane 1 - DNA ladder (GeneRuler Ready-to-Use DNA Ladder, ThermoFisher, SM0313), Lane 2-6 - Test samples (cell culture media supernatant), Lane 7 - Negative control (MycoFree water + Master Mix), Lane 8 - Positive control (Myco Positive Control Template + Master Mix).

131

Note

Repeat the test if a band appears in the negative control, or if the positive control fails to amplify and no band is present.

The presence of a band should be considered a potential indication of mycoplasma contamination, while the absence of a band indicates that the culture is free of mycoplasma.

If the test indicates mycoplasma contamination and a repeat test confirms this, follow your laboratory's established protocol for handling contaminated cultures.

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