

Assay Methods

Protocol: Human Embryonic Stem Cell Culture

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Basement membranes are continuous sheets of specialized extracellular matrix that are found at the dermal-epidermal junction, at the base of all lumen-lining epithelia throughout the digestive, respiratory, reproductive, and urinary tracts, and that underlie parenchyma of endocrine and exocrine glands.

Corning® Matrigel® hESC-qualified matrix is a soluble basement membrane extract of the Engelbreth-Holm-Swarm (EHS) mouse tumor that gels at room temperature to form a genuine reconstituted basement membrane.¹ The major components of Corning Matrigel matrix are laminin, collagen IV, entactin, and heparan sulfate proteoglycan.^{2,3} Growth factors, collagenases, plasminogen activators, and other undefined components have also been reported in Corning Matrigel matrix.^{4,5}

Corning Matrigel hESC-qualified matrix has been used extensively as a substrate for culturing human embryonic stem (hES) cells with various conditioned or defined media.¹⁻¹⁰ Historically, hES cell derivation and culturing techniques utilized serum and/or mouse embryonic fibroblast (MEF) feeder layers.⁶ An ideal environment for hES cell research consists of both a cell culture surface specifically qualified for hES cells and a serum-free defined medium. Matrigel hESC-qualified matrix and STEMCELL™ Technologies' mTeSR™1 (developed under the license from WiCell™ Research Institute⁷), a high-quality surface and medium combination, create the first complete environment to support feeder-independent expansion of hES cells.

Although Matrigel hESC-qualified matrix has been widely accepted as a feeder-free substrate for the culture of hES cells^{8,9,11}, Matrigel hESC-qualified matrix is an optimized surface that provides the additional reproducibility and consistency essential for hES cell research. Matrigel hESC-qualified matrix has been qualified as mTeSR1-compatible by STEMCELL Technologies and is also compatible with a variety of other culture media.

The following protocol is optimized for human embryonic stem (hES) cell culture using mTeSR1 media. To utilize an animal protein-free media, TeSR™2 can be used according to the manufacturer's recommendations.

Materials

- ▶ Corning Matrigel hESC-qualified matrix, 5 mL vial (Corning Cat. No. 354277)
- ▶ Falcon® standard tissue culture-treated plate or dish (6-well flat-bottom plate, Corning Cat. No. 353046; or 100 mm dish, Corning Cat. No. 353003)
- ▶ mTeSR1 Medium kit (STEMCELL Technologies, Cat. No. 05850; maintenance medium for human embryonic stem cells)
- ▶ Dulbecco's Phosphate Buffered Saline (DPBS) (e.g., Corning Cat. No. 21-031-CV)
- ▶ L-Glutamine 200 mM (e.g., Corning Cat. No. 25-005-CI or 25-005-CV)
- ▶ MEM non-essential amino acids (e.g., Corning Cat. No. 25-025)
- ▶ Fetal Bovine Serum (FBS) (e.g., Corning Cat. No. 35-011-CV) – Heat inactivated by heating to 56°C for 30 minutes
- ▶ DMEM/F12 medium (e.g., Corning Cat. No. 10-092)
- ▶ β-Mercaptoethanol

- ▶ Corning® Dispase (Corning Cat. No. 354235)
- ▶ 0.2 µm, low-protein binding filter (e.g., Corning Cat. No. 431227)
- ▶ Corning Delipidized BSA (Corning Cat. No. 354331)
- ▶ 10% Normal goat serum, or serum for blocking

Equipment Required for hESC Culture

- ▶ Vertical laminar flow hood certified for Level II handling of biological materials
- ▶ Humidified tissue culture incubator, 37°C, 5% CO₂ atmosphere
- ▶ Tabletop centrifuge
- ▶ Pipettor (e.g., Falcon®, Corning Lambda®, or Stripettor™)
- ▶ Hemacytometer (e.g., Neubauer, Reichert)
- ▶ Inverted microscope with 2X, 4X, and 10X phase objectives (e.g., Olympus CKX31)

Procedure

1.0 Preparing Aliquots of Corning Matrigel® hESC-qualified Matrix

Corning Matrigel hESC-qualified matrix should be reconstituted, aliquotted, and stored as recommended in the product Guidelines for Use. The dilution factor of Matrigel hESC-qualified matrix is lot specific and found on the Certificate of Analysis. The aliquot (dilution factor) volume is typically between 270 and 350 µL and is calculated for each lot based on the protein concentration. Matrigel hESC-qualified matrix and the coating medium must be kept ice-cold during the entire procedure.

NOTE: If Matrigel hESC-qualified matrix is not thawed properly, clumps can be seen in the solution. Keep Matrigel vial on ice all the time while handling. All procedures should be performed under aseptic conditions except where indicated.

Precautions

- ▶ Matrigel hESC-qualified matrix will gel rapidly at 22°C to 35°C.
- ▶ Storage: Matrigel hESC-qualified matrix should be stored at -20°C.
DO NOT STORE IN FROST-FREE FREEZER.

2.0 Preparing a Working Solution of Corning Matrigel hESC-qualified Matrix

Add one aliquot (dilution factor) of Corning Matrigel hESC-qualified matrix to 25 mL of DMEM/F-12 to coat four 6-well plates, or three 100 mm dishes.

3.0 Coating Procedure

- 3.1 Add 1.0 mL of diluted Matrigel hESC-qualified matrix per well of a 6-well plate, or 8.0 mL per 100 mm dish.

NOTE: Volumes given in this section are for 6-well culture plates, or 100 mm culture dishes. Scale accordingly for different sized cultureware.

- 3.2 Swirl the plate or dish to distribute the Matrigel solution evenly across the surface.
- 3.3 Incubate cultureware at room temperature (15°C to 25°C) for at least 1 hour before use.
- 3.4 Aspirate the remaining liquid (coating solution) from cultureware just before use.
Ensure that the tip of the pipet does not scratch the coated surface.
- 3.5 Plates are now ready to use.

NOTE: Do not remove the coating solution until the plate(s) are ready to use. Coated plates can be stored at 4°C for up to a week. Make sure that the plates are sealed to prevent dehydration (e.g., Parafilm). Plate(s) are not optimal for hESC culture if the Corning® Matrigel® solution does not completely cover the surface. Therefore, plate(s) that have regions where the solution has evaporated are not recommended for use.

4.0 Human Embryonic Stem Cell Culture

NOTE: The following procedure is optimized for human ES cells cultured on Corning® Matrigel® hESC-qualified matrix-coated 6-well plate using mTeSR™1 media. Results may vary depending upon the cell line used, media, state of differentiation, and dissociation technique, etc. You should optimize conditions for your own system. Transitioning from conditioned media does not require any adaptation. Cells can be plated in mTeSR1 medium on Matrigel hESC-qualified matrix-coated 6-well plate at the time of passage. If using TeSR™2 medium, follow protocol according to the product specification sheet.

4.1 Preparation of mTeSR1

- 4.1.1 Thaw mTeSR1 5X Supplement (Component No. 05852 from mTeSR1 Medium kit) at room temperature (15°C to 25°C), or overnight at 2°C to 8°C.

NOTE: Please follow manufacturer's recommendations. For optimal results, ensure that lot numbers of both components of the mTeSR1 Medium kit (mTeSR1 Basal Medium and mTeSR1 5X Supplement) end with the same letter (e.g., D). If desired, 5X Supplement can be aseptically dispensed into working aliquots and stored at -20°C. Use frozen aliquots within 3 months. Thawed aliquots should be used within 1 day to prepare complete mTeSR1 medium. Do not refreeze aliquots after thawing.

- 4.1.2 Aseptically add the entire 100 mL of thawed 5X Supplement to 400 mL Basal Medium for a total volume of 500 mL. Mix well. Complete mTeSR1 is stable for up to 2 weeks when stored at 2°C to 8°C, or up to 6 months when frozen at -20°C. Thaw frozen medium at room temperature (15°C to 25°C), or overnight at 2°C to 8°C.

NOTE: If prepared aseptically complete mTeSR1 is ready for use, but the medium can also be filtered using a 0.2 µm, low-protein binding filter if desired.

4.2 Culture of hESC Using mTeSR1

NOTE: Generally, hESCs from 1 well of a 6-well plate cryopreserved in STEMCELL Technologies' defined, serum-free cryopreservation medium, mFreSR™ (Cat. No. 05854 or 05855) can be successfully thawed and cultured into 1 well of a 6-well plate format. If the cells have been cryopreserved using other methods, this may vary. Human ESCs cultured using other maintenance protocols (e.g., with mouse embryonic feeders or conditioned medium) can be thawed into mTeSR1 or TeSR2 using this protocol. Have all tubes, warmed medium, and plates ready before starting the protocol to ensure that the thawing procedure is done as quickly as possible.

- 4.2.1 Quickly thaw the hESCs in a 37°C waterbath by gently shaking the cryovial continuously until only a small frozen pellet remains. Remove the cryovial from the waterbath and wipe with 70% ethanol.
- 4.2.2 Use a 2 mL pipet to transfer the contents of the cryovial to a 15 mL conical tube.

NOTE: Use of a 2 mL pipet will minimize breakage of cell clumps.
- 4.2.3 Add 5 to 7 mL of warm mTeSR1 dropwise to the tube, gently mixing as the medium is added.
- 4.2.4 Centrifuge cells at 300 x g for 5 minutes at room temperature (15°C to 25°C).
- 4.2.5 Aspirate the medium, leaving the cell pellet intact. Using a 2 mL pipet, gently resuspend the cell pellet in 1 to 2 mL of mTeSR1, taking care to maintain the cells as aggregates.
- 4.2.6 Remove excess medium (coating solution) from the Matrigel hESC-qualified matrix-coated 6-well plate by gently tilting the plate onto one corner and allowing the excess Corning Matrigel solution to collect in that corner. Remove the solution using a serological pipet or by aspiration. Ensure that the tip of the pipet does not scratch the coating surface.

NOTE: If the plate(s) have been stored at 2°C to 8°C, allow the plate(s) to come to room temperature (15°C to 25°C) for 30 minutes before removing the Matrigel hESC-qualified matrix coating solution.

4.2.7 Transfer 2 mL cell aggregates per well to a Corning® Matrigel® hESC-qualified matrix-coated 6-well plate. Ensure that clumps are evenly distributed between wells. Move the plate in quick side to side, forward to back motions to evenly distribute the clumps within the wells.

4.2.8 Culture the cells in a humidified incubator at 37°C, with 5% CO₂.

4.2.9 Perform daily medium changes. Check for undifferentiated colonies that are ready to passage (dense centered) approximately 5 to 7 days after thawing.

NOTE: If only few colonies are observed after thawing, it may be necessary to passage and replate them in the same size well on a new Matrigel hESC-qualified matrix-coated 6-well plate.

5.0 Passage of Human ES Cells on Corning Matrigel hESC-qualified Matrix-coated 6-well Plate

5.1 Warm Corning Dispase, aliquotted mTeSR™1 and DMEM/F-12 media at 37°C.

5.2 Use a microscope to visually identify regions of differentiation, and remove regions of differentiation by scraping with a pipet tip or by aspiration.

NOTE: This selection should not exceed 20% of the well if the culture is of high quality.

5.3 Aspirate medium from the hESC culture and rinse with DMEM/F-12 (2 mL/well).

5.4 Add 1 mL per well of Dispase (1 mg/mL) to the hES cells, incubate for 6 to 8 minutes at 37°C, or until the edges of the colony start to curl up when observed under the microscope.

5.5 Aspirate Dispase and gently rinse each well 3 times with 2 mL of DMEM/F-12 per well to remove any remaining Dispase. After wash, add 2 mL/well of mTeSR1 and using a sterile 5 mL pipet, gently break-up and scrape the colonies using small circular motions. Start at the outside edge of the colony and work your way towards the middle. Try to cut as many small pieces as possible. This is easiest to do while viewing the process using a phase contrast microscope (2X or 4X objective).

5.6 Transfer the detached cell aggregates to a 15 mL conical tube and rinse the well with an additional 2 mL of DMEM/F-12 or mTeSR1 to collect any remaining aggregates. Add the rinsate to the 15 mL tube.

5.7 Gently triturate cell clumps a few times by pipetting up and down (do not make a single cell suspension). Add 0.5 mL of cell suspension to each well on Matrigel hESC-qualified matrix-coated 6-well plates containing mTeSR1 and place plates gently at 37°C incubator with 5% CO₂ and humidified air. As a rough guideline, split cells at a 1:3 to 1:6 ratio. Ensure that clumps are evenly distributed between wells. Move the plate in quick side to side, forward to back motions to evenly distribute the clumps within the wells.

NOTE: If the colonies are at an optimal density, the cells can be split every 4 to 7 days using 1:6 to 1:10 splits (i.e., the aggregates from 1 well of a 6-well plate can be plated in 6 to 10 wells of a 6-well plate). If the colonies are too dense or too sparse, adjust the split ratio accordingly. It is important not to swirl contents of plate as this will result in colonies crowding in the center of the plate. Gently rock the plate side to side and back and forth just prior to placing into the incubator to evenly distribute the cell clumps on the plate surface.

6.0 Maintenance of Human ES Cells

NOTE: Following dissociation of human ES cells, the cultures should be left undisturbed for the next day (Day 2). Start changing media from next day after plating cells.

- 6.1 Aspirate exhausted media from Corning® matrigel® hESC-qualified matrix-coated 6-well plates and add 2.5 to 3.0 mL of pre-warmed mTeSR™1 media.
- 6.2 Change media on human ES cell cultures every day from Day 3 to Day 7. Monitor colonies to ensure that they are mostly undifferentiated.
- 6.3 Cells will typically require passaging on Day 5 to 7.

NOTE: hESCs cultured in mTeSR1™ are ready to passage when the colonies are large, beginning to merge, and have centers that are dense and phase-bright compared to their edges. Depending on the size and density of seeded aggregates, cultures are usually passaged 5 to 7 days after seeding in mTeSR1.

7.0 Characterization of Undifferentiated hESCs

7.1 Morphology

Monitor for undifferentiated hESCs. Undifferentiated hESCs grow as compact, multicellular colonies. They should also exhibit a high nuclear-to-cytoplasm ratio and prominent nucleoli. These colonies are characterized by a distinct border.¹² Healthy hESC colonies will be multi-layered in the center, resulting in clusters of phase-bright cells when viewed under phase contrast. Differentiation is characterized by loss of border integrity, gross non-uniformity of cell morphology within a colony, and the emergence of obvious alternate cell types.¹³

7.2 Immunohistochemical Detection of Cell Surface Markers

- 7.2.1 Remove culture media from the cultureware. Wash the hES cells twice with 2 mL of PBS.
- 7.2.2 Fix the cells with 1 mL of 4% paraformaldehyde for 20 minutes at room temperature.
- 7.2.3 Wash the cells twice with 2 mL of PBS for 5 minutes.
- 7.2.4 Block the cells with 1 mL of 0.1% BSA, 10% normal goat serum* in PBS at room temperature for 45 minutes to 1 hour.

NOTE: For Oct-3/4 staining, permeabilize in 0.1% Triton X-100, and block with 1% BSA, 10% normal rabbit serum in PBS at room temperature for 45 minutes.
- 7.2.5 During the blocking step, prepare the primary antibody working solution with PBS containing 1% BSA and 10% normal goat serum* to a final desired concentration.
- 7.2.6 After blocking, incubate the cells with 1 mL/well of diluted primary antibody working solution overnight at 2°C to 8°C, or 1 hour at room temperature.
- 7.2.7 Wash the cells three times with 2 mL of PBS containing 1% BSA for 5 minutes each.
- 7.2.8 Dilute the secondary antibody (fluorescence-conjugated) in PBS containing 1% BSA. Incubate the cells with secondary antibody at 1 mL per well for 60 minutes at room temperature in the dark.

NOTE: If using pre-conjugated antibody, secondary antibody will not be required.
- 7.2.9 Wash the cells three times with 2 mL of PBS containing 1% BSA for 5 minutes each.
- 7.2.10 Cover the cells with 4 mL of PBS and visualize using a fluorescence microscope.

*Substitute normal serum from appropriate species depending on the host species of the secondary antibody. Further characterization using flow cytometry analysis and quantitative real-time RT-PCR may also be performed.

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