RSeT™ Feeder-Free Medium

Serum-free medium for naïve-like human pluripotent stem cells

Catalog #05975 500 mL Kit

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Product Description

RSeT™ Feeder-Free Medium is a serum-free cell culture medium used for the reversion of primed human embryonic stem (ES) and induced pluripotent stem (iPS) cells to a naïve-like state and maintains cells in a naïve-like state under hypoxic conditions without bFGF or feeder cells. RSeT™ Feeder-Free Medium was developed under license from the Weizmann Institute of Science. With pre-screened quality components that ensure batch-to-batch consistency, this medium produces robust cultures with features of a naïve-like state such as tightly packed, domed colonies with refractive edges. Key transcripts associated with naïve-like human ES/iPS cells, such as KLF17, KLF2, KLF4, and TFCP2L1, show increased expression in human ES/iPS cells cultured in RSeT™ Feeder-Free Medium. RSeT™ Feeder-Free hPSCs can either be differentiated, or converted back to a primed state by culture in mTeSR™1 and then differentiated. Products that can be used for differentiation include STEMdiff™ Definitive Endoderm Kit (Catalog #05110), STEMdiff™ SMADi Neural Induction Kit (Catalog #08581), and STEMdiff™ Mesoderm Induction Medium (Catalog #05220).

Product Information

The following components are sold as a complete kit (Catalog #05975) and are not available for individual sale.

COMPONENT NAME	COMPONENT #	SIZE	STORAGE	SHELF LIFE
RSeT™ Feeder-Free Basal Medium	05976	450 mL	Store at 2 - 8°C.	Stable until expiry date (EXP) on label.
RSeT™ Feeder-Free 10X Supplement*	05977	50 mL	Store at -20°C.	Stable until expiry date (EXP) on label.

*This product contains material derived from human plasma. Donors have been tested and found negative for HIV-1 and -2, hepatitis B, and hepatitis C prior to donation. However, this product should be considered potentially infectious and treated in accordance with universal handling precautions. Please refer to the Safety Data Sheet (SDS) for hazard information.

Materials Required But Not Included

PRODUCT NAME	CATALOG #
Corning® Matrigel® hESC-Qualified Matrix	07181
D-PBS (Without Ca++ and Mg++)	37350
12-Well Flat-Bottom Plate, Tissue Culture-Treated	38052
DMEM/F-12 with 15 mM HEPES	36254
mTeSR™1	85850
Gentle Cell Dissociation Reagent	07174
TrypLE™ Express Enzyme (1X), phenol red	Thermo Fisher 12605028
Y-27632	72304
Conical tubes, 15 mL and 50 mL	e.g. 38009 and 38010
Trypan Blue	07050

Preparation of Reagents and Materials

Complete RSeT™ Feeder-Free Medium

Use sterile techniques when preparing complete RSeT™ Feeder-Free Medium (Basal Medium + 10X Supplement + Corning® Matrigel®). The following example is for preparing 100 mL of complete medium. If preparing other volumes, adjust accordingly.

1. Thaw the supplement at room temperature (15 - 25°C) or at 2 - 8°C overnight. Ensure supplement is evenly resuspended after thawing.

   NOTE: Precipitate may be observed in the supplement; this will not affect performance of the medium. If desired, filter the supplement using a 0.2 µm low protein binding polyethersulfone (PES) filter unit (e.g. Fisher 09-741-04).

   NOTE: If not used immediately, aliquot and store at -20°C. Do not exceed the shelf life of the supplement. After thawing the aliquots, use immediately. Do not re-freeze.
2. Add 10 mL of 10X Supplement to 90 mL of Basal Medium. Mix thoroughly.
3. Store medium on ice or at 2 - 8°C.
4. Thaw 200 µL of Matrigel® on ice for 2 hours, or overnight on ice at 2 - 8°C. Ensure to keep Matrigel® on ice when thawing and handling to prevent gelling.
5. Add 200 µL of Matrigel® to cold medium. Mix thoroughly.

   NOTE: If not used immediately, store complete RSeT™ Feeder-Free Medium at 2 - 8°C for up to 2 weeks. Storage in a PETG (polyethylene terephthalate, glycol modified) bottle is recommended. Storage in polypropylene tubes may result in formation of Matrigel® aggregates, but performance of the medium will not be affected. Do not aliquot complete medium into additional containers. Warm complete medium to room temperature (15 - 25°C) before use. Protect from exposure to direct light.

Coating Cultureware

Coat cultureware at least 1 hour prior to use. Corning® Matrigel® should be aliquoted and frozen. Consult the Matrigel® Certificate of Analysis for the recommended aliquot size ("Dilution Factor") to prepare 25 mL of diluted matrix. Keep Matrigel® on ice when thawing and handling to prevent it from gelling.

NOTE: Use tissue culture-treated cultureware.

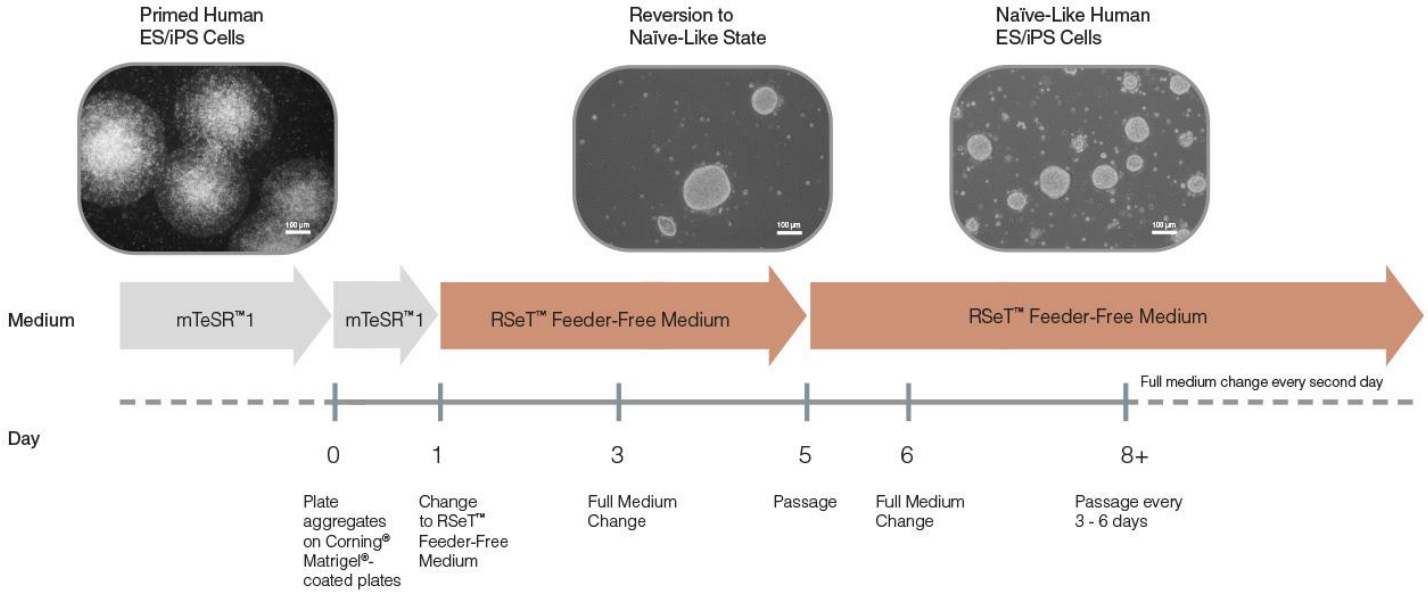
The following protocol is for coating a 12-well tissue culture-treated plate. If using other cultureware, adjust volumes accordingly.

1. Thaw one aliquot of Matrigel® on ice.
2. Dispense 25 mL of cold DMEM/F-12 with 15 mM HEPES into a 50 mL conical tube and keep on ice.
3. Add thawed Matrigel® to the cold DMEM/F-12 and mix well. If desired, the vial may be washed with cold medium.
4. Immediately add 0.5 mL of the diluted (approximately 1:100) Matrigel® solution per well of a 12-well plate.
5. Swirl cultureware to spread the Matrigel® solution evenly across the surface of the wells. Place the lid on the plate.

   NOTE: If the cultureware surface is not fully coated by the Matrigel® solution, it should not be used for human ES/iPS cell culture.
6. Incubate at room temperature (15 - 25°C) for at least 1 hour before use. Do not let the Matrigel® solution evaporate.

   NOTE: If not used immediately, seal cultureware (e.g. with Parafilm®) to prevent evaporation of the Matrigel® solution. Store at 2 - 8°C for up to 1 week after coating. Allow stored coated cultureware to warm to room temperature (15 - 25°C) for 30 minutes before use.
7. Aliquot sufficient mTeSR™1 and D-PBS. Warm to room temperature (15 - 25°C) before use.
8. Gently tilt the coated cultureware onto one side and allow the excess Matrigel® solution to collect at the edge. Remove the excess solution using a serological pipette or by aspiration. Ensure that the coated surface is not scratched.
9. Add 1 mL of mTeSR™1 per well and incubate at 37°C under **normoxic** conditions (20% O_2, 5% CO_2).

Procedure Diagram



Directions for Use

Use sterile techniques when performing the following protocols:

A. Passaging Aggregates of mTeSR™1-Cultured ES/iPS Cells

B. Plating and Reversion of Human ES/iPS Cells to a Naïve-Like State

C. Passaging Naïve-Like Human ES/iPS Cells

The following protocols are for the reversion of primed human ES/iPS cells cultured in mTeSR™1 under feeder-free conditions. For complete instructions on maintaining human ES/iPS cells in mTeSR™1, refer to the Technical Manual: Maintenance of Human Pluripotent Stem Cells in mTeSR™1, available at www.stemcell.com or contact us to request a copy.

A. Passaging Aggregates of mTeSR™1-Cultured Human ES/iPS Cells

The following protocol is for passaging human ES/iPS cells cultured in mTeSR™1 in a 6-well tissue culture-treated plate. If using other cultureware, adjust volumes accordingly.

1. Aliquot sufficient volumes of mTeSR™1 and Gentle Cell Dissociation Reagent. Warm to room temperature (15 - 25°C) before use.
2. Use a microscope to visually identify regions of differentiation in the ES/iPS cell culture. Mark these with a felt tip or lens marker on the bottom of the plate.
3. Remove regions of differentiation by scraping with a pipette tip or by aspiration. Avoid having the culture plate out of the incubator for more than 15 minutes at a time.

   NOTE: Selection may not be required if differentiation is < 5%. Selection should not exceed 20% of the well if the culture is of high quality. Primed human ES/iPS cell cultures containing a high proportion of differentiated cells may not revert to a naïve-like state and could further increase rates of differentiation during reversion in RSeT™ Feeder-Free Medium. For suggestions on measuring pluripotency of primed cultures, refer to the Technical Manual: Maintenance of Human Pluripotent Stem Cells in mTeSR™1, available at www.stemcell.com or contact us to request a copy.
4. Aspirate medium from the well and add 1 mL of Gentle Cell Dissociation Reagent per well. Incubate at room temperature (15 - 25°C) for 5 - 7 minutes.
5. Aspirate the Gentle Cell Dissociation Reagent and add 1 mL of mTeSR™1.
6. Gently detach the colonies by scraping with a serological glass pipette or a cell scraper (e.g. Catalog #38065).
7. Transfer the detached cell aggregates to a 15 mL conical tube.

   Optional: Rinse the well with an additional 1 mL of mTeSR™1 to collect remaining cell aggregates. Add the rinse to the 15 mL tube.

   NOTE: Centrifugation of cell aggregates is not required.
8. Carefully pipette the cell aggregate mixture up and down to break up the aggregates as needed. A uniform suspension of large aggregates (approximately 100 - 200 µm in diameter) is optimal; do not create a single-cell suspension.

9. Count aggregates > 100 µm in diameter. For a cell aggregate counting method, refer to the Technical Manual: Maintenance of Human Pluripotent Stem Cells in mTeSR™1, available at www.stemcell.com or contact us to request a copy.
10. Proceed to section B for plating.

B. Plating and Reversion of Human ES/iPS Cells to a Naïve-Like State

The following protocol is for plating and reversion of human ES/iPS cells in a 12-well Matrigel®-coated tissue culture-treated plate. If using other cultureware, adjust volumes accordingly.

1. **Day 0**: Coat cultureware with Corning® Matrigel® (see Preparation of Reagents and Materials).
2. Plate 500 - 1000 aggregates into each well of a coated 12-well plate containing mTeSR™1 (prepared in step 1).

   NOTE: Human embryonic stem (ES) cell lines may require 1000 - 2000 aggregates to be plated, whereas 500 - 1000 aggregates are sufficient for human induced pluripotent stem (iPS) cell lines.
3. Incubate at 37°C for 24 - 36 hours under **normoxic** conditions (20% O_2, 5% CO_2).
4. **Day 1**: Aspirate mTeSR™1 and replace with 1 mL of RSeT™ Feeder-Free Medium per well.
5. Incubate at 37°C under **hypoxic** conditions (5% O_2, 5% CO_2). Perform a full medium change with RSeT™ Feeder-Free Medium every second day.
6. **Day 4 or 5**: Colonies are typically ready to be passaged. Colonies will begin to adopt a tightly-packed, highly domed morphology with the edges of the colony becoming more refractive and smooth.
7. Proceed to section C for passaging.

C. Passaging Naïve-Like Human ES/iPS Cells

The following protocol is for passaging naïve-like human ES/iPS cells from a 12-well plate to a 12-well plate. If using other cultureware, adjust volumes accordingly.

1. **Day 4 or 5**: Aliquot D-PBS and warm to room temperature (15 - 25°C). Warm the bottle of RSeT™ Feeder-Free Medium to room temperature.
2. Add Y-27632 to RSeT™ Feeder-Free Medium to a final concentration of 5 µM. Immediately proceed to step 3.
3. Add 1 mL of RSeT™ Feeder-Free Medium + 5 µM Y-27632 per well of a 12-well tissue culture-treated (non-coated) plate.

   NOTE: If desired, Matrigel®-coated plates may be used at this stage. However, it is not required, as Matrigel® is already in the medium.
4. Aliquot D-PBS and TrypLE™ and warm to room temperature (15 - 25°C).
5. Aspirate medium from wells containing naïve-like cells.
6. Wash with 1 mL of D-PBS. Aspirate D-PBS.
7. Add 250 µL of TrypLE™ to each well.
8. Incubate cells at 37°C for approximately 8 minutes under **hypoxic** conditions (5% O_2, 5% CO_2).
9. Without aspirating TrypLE™, add 750 µL of RSeT™ Feeder-Free Medium to the well.
10. Using a 1 mL pipette tip, gently pipette up and down 2 - 3 times to dislodge cells from well. The cell suspension should contain a mixture of single cells.
11. Transfer to a 15 mL conical tube. Centrifuge at 300 x *g* for 5 minutes.
12. Remove supernatant and gently resuspend the cell pellet in 100 µL of RSeT™ Feeder-Free Medium.
13. Count cells using Trypan Blue and a hemocytometer.
14. Add 8 x 10^4 cells to the plate prepared in step 3.

    NOTE: During the early stages of reversion, low-density cultures may arise; as a result, a 1:1 split ratio may be necessary for several passages. Avoid high-density cultures, as this may lead to a flattened colony morphology and passaging every 2 days.
15. Incubate at 37°C under **hypoxic** conditions (5% O_2, 5% CO_2) for 24 hours.
16. **Day 5 or 6**: Perform a full medium change with RSeT™ Feeder-Free Medium. Incubate at 37°C under **hypoxic** conditions (5% O_2, 5% CO_2).
17. **Day 7+ or 8+**: Perform a full medium change every second day. Visually assess cultures to monitor growth; they are ready for passaging when colonies are 50 - 100 µm diameter. See Figure 1 for an image of ES cells reverted after one passage.

    NOTE: Passage every 3 - 6 days; do not exceed 7 days between passages.

    NOTE: For cultures at 10+ passages, we recommend monitoring for common karyotypic abnormalities using the hPSC Genetic Analysis Kit (Catalog #07550).

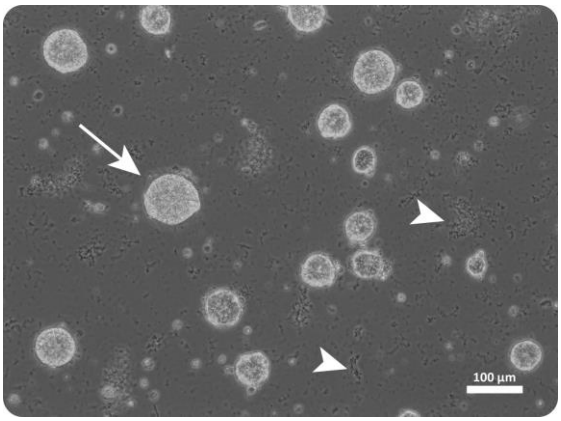


Figure 1. Human ES Cells Reverted to a Naïve-Like State

Human ES cells (H9) reverted in RSeT™ Feeder-Free Medium after one passage. During reversion, colonies change from a flat morphology to a domed morphology characteristic of naïve-state hPSCs (arrow). Naïve-like cultures are established within one passage in RSeT™ Feeder-Free Medium and > 90% of colonies have dome-shaped morphology throughout long-term culture. Corning® Matrigel® aggregates are commonly observed attached to the plate or floating in medium after adding fresh RSeT™ Feeder-Free Medium (arrowheads).

Troubleshooting

When culturing naïve-like cells in RSeT™ Feeder-Free Medium, most problems can be addressed by assessing a small number of key parameters, as described in the table below. When passaging cells, the split ratio being used will have an effect on the quality of the cultures. If there is a decrease in cell growth or in naïve-like qualities of cells cultured in RSeT™ Feeder-Free Medium, cultures can be rescued by subsequent careful passaging with the appropriate split ratios.

PROBLEM	RECOMMENDED ACTION
Few aggregates initially attach when transferred from primed conditions to RSeT™ Feeder-Free Medium in initial plating stage (section B step 4)	Seed larger aggregates (> 100 µm diameter) and a higher number of aggregates (> 1000) per well (section B step 2)
Colonies begin to detach on Day 5 in the initial reversion stage (section B step 6)	Passage cells on Day 4 of the initial reversion stage
High heterogeneity of colonies (mixture of flat and domed)	• Continue passaging for at least 3 passages to see improvement of culture morphology • Re-examine quality of starting primed human ES/iPS cell cultures
Poor expansion and growth of cultures despite ideal domed morphology	Passage cells at a low split ratio (1:1) for several passages and gradually increase split ratio over time when possible. An increase in cell proliferation has been observed as cells revert in RSeT™ Feeder-Free Medium. If cells do not expand after 5 passages in RSeT™ Feeder-Free Medium, restart the culture and seed a higher number of primed aggregates per well (section B step 2).

Assessment of Human ES/iPS Cells

The following antibodies can be used to characterize human ES/iPS cells by flow cytometry or immunocytochemistry:

- Anti-Human SSEA-4 Antibody, Clone MC-813-70 (Catalog #60062)
- Anti-Human TRA-1-60 Antibody, Clone TRA-1-60R (Catalog #60064)
- Anti-Human OCT4 (OCT3) Antibody, Clone 3A2A20 (Catalog #60093)

Related Products

For related products, including specialized cell culture and storage media, matrices, antibodies, cytokines, and small molecules, visit www.stemcell.com/hPSCworkflow or contact us at techsupport@stemcell.com.

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