STEMdiff™-ACF RPE Differentiation Kit
STEMdiff™-XF RPE Maturation Medium
STEMdiff™-ACF RPE Plating Supplement

Catalog #100-1367	1 Kit
Catalog #100-1365	500 mL
Catalog #100-1364	2.5 mL

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Product Description

STEMdiff™ retinal pigment epithelium (RPE) culture system comprises STEMdiff™-ACF RPE Differentiation Kit (Catalog #100-1367), STEMdiff™-XF RPE Maturation Medium (Catalog #100-1365), and STEMdiff™-ACF RPE Plating Supplement (Catalog #100-1364). This culture system is used to differentiate and mature RPE derived from human pluripotent stem cells (hPSCs).

STEMdiff™-ACF RPE Differentiation Kit includes four animal component-free (ACF) differentiation media for the rapid and robust generation of hPSC-derived immature RPE in 14 days. The resulting immature RPE cells are ≥ 50% positive for pre-melanosome protein PMEL17, and an average of 1 x 10^6 immature RPE cells/cm^2 can be harvested from cultureware. This kit is compatible with hPSCs maintained in mTeSR™1 (Catalog #85850) or mTeSR™ Plus (Catalog #100-0276) on Corning® Matrigel® or Gibco™ Vitronectin.

STEMdiff™-XF RPE Maturation Medium is a xeno-free (XF) medium for generating fully functional and mature RPE. Subculture of immature RPE cells in STEMdiff™-XF RPE Maturation Medium for five weeks is necessary for generating mature RPE. The resulting mature RPE cells are ≥ 90% PMEL17+, and ≥ 80% RPE65+, EZRIN+, and CRALBP+, and an average of 0.75 x 10^6 mature RPE cells/cm^2 can be harvested from cultureware. These cells display polygonal morphology, are pigmented, polarized, and are able to phagocytose photoreceptor outer segments by Day 49. hPSC-derived mature RPE can be used in various downstream applications and analyses.

STEMdiff™-ACF RPE Plating Supplement is required to enhance the survival and attachment of immature and mature RPE after harvesting. This supplement also increases the plating efficiency of cryopreserved immature or mature RPE.

Component Storage and Stability

The following components are sold as part of a complete kit (Catalog #100-1367) and are available for individual sale (Catalog #100-1364 and #100-1365).

COMPONENT NAME	COMPONENT #	SIZE	STORAGE	SHELF LIFE
STEMdiff™-ACF RPE Differentiation Kit (Catalog #100-1367)				
STEMdiff™ RPE Differentiation Medium A	100-1360	30 mL	Store at -20°C.	Stable for 12 months from date of manufacture (MFG) on label.
STEMdiff™ RPE Differentiation Medium B	100-1361	12 mL	Store at -20°C.	Stable for 12 months from date of manufacture (MFG) on label.
STEMdiff™ RPE Differentiation Medium C	100-1362	24 mL	Store at -20°C.	Stable for 12 months from date of manufacture (MFG) on label.
STEMdiff™ RPE Differentiation Medium D	100-1363	48 mL	Store at -20°C.	Stable for 12 months from date of manufacture (MFG) on label.
STEMdiff™-XF RPE Maturation Medium*	100-1365	500 mL	Store at 2 - 8°C.	Stable for 12 months from date of manufacture (MFG) on label.
STEMdiff™-ACF RPE Plating Supplement	100-1364	2.5 mL	Store at -20°C.	Stable for 2 years from date of manufacture (MFG) on label.

*This component 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.

Materials Required but Not Included

PRODUCT NAME	CATALOG #
70 µm Reversible Strainer, Large	27260
Cell lifter	e.g. 200-0596
Conical tubes, 15 and 50 mL	e.g. 38009 and 38010

PRODUCT NAME	CATALOG #
Corning® Matrigel® hESC-Qualified Matrix OR Gibco™ Vitronectin (VTN-N) Recombinant Human Protein, Truncated	Corning 354277 OR Thermo Fisher Scientific A14700
CryoStor® CS10	07930
D-PBS (Without Ca++ and Mg++)	37350
Gentle Cell Dissociation Reagent	100-0485
Tissue culture-treated cultureware	e.g. 38016
Trypan Blue	07050
TrypLE™ Express Enzyme	Thermo Fisher Scientific 12604013

Preparation of Reagents and Materials

A. STEMDIFF™-ACF RPE DIFFERENTIATION MEDIA

Thaw STEMdiff™ RPE Differentiation Medium A, B, C, or D at room temperature (15 - 25°C) or overnight at 2 - 8°C. Mix thoroughly.

NOTE: If not used immediately, store at 2 - 8°C for up to 1 month. Alternatively, aliquot and store at -20°C. After thawing aliquots, do not re-freeze. Do not exceed the shelf life of the media.

B. RPE PLATING MEDIUM

Use sterile technique to prepare RPE Plating Medium (STEMdiff™-XF RPE Maturation Medium + STEMdiff™-ACF RPE Plating Supplement). The following example is for preparing 25 mL of complete medium. If preparing other volumes, adjust accordingly.

1. Thaw STEMdiff™-ACF RPE Plating Supplement at room temperature (15 - 25°C). Mix thoroughly.

   NOTE: Once thawed, use immediately or 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 250 µL of STEMdiff™-ACF RPE Plating Supplement to 24.75 mL of STEMdiff™-XF RPE Maturation Medium. Mix thoroughly. Warm to room temperature before use.

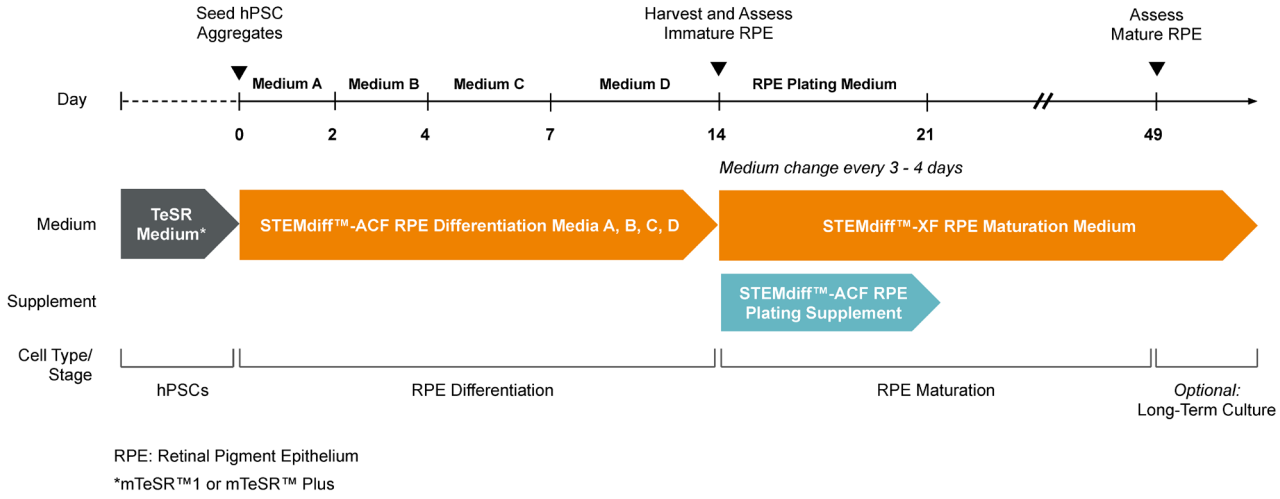
   NOTE: Use on day of preparation; do not store.

C. COATING CULTUREWARE WITH CORNING® MATRIGEL® OR GIBCO™ VITRONECTIN

For complete instructions on coating cultureware with Corning® Matrigel®, 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.

For complete instructions on coating plates with Gibco™ Vitronectin (VTN-N) Recombinant Human Protein, Truncated, refer to the manufacturer's instructions.

Protocol Diagram



RPE: Retinal Pigment Epithelium

*mTeSR™1 or mTeSR™ Plus

Directions for Use

Please read the entire protocol before proceeding. Use sterile technique when performing the following protocols:

A. Seeding hPSCs as Aggregates (Day 0)
B. Differentiation to Immature RPE Cells (Day 1 - 13)
C. RPE Cell Maturation (Day 14 - 49+)
D. Cryopreservation & Thawing

A. SEEDING hPSCs AS AGGREGATES (DAY 0)

This protocol is for harvesting a 6-well plate of hPSCs maintained in mTeSR™1 or mTeSR™ Plus. If using other cultureware, adjust volumes accordingly. It is critical to start with high-quality hPSC cultures for efficient RPE differentiation. hPSC cultures are ready for aggregate passaging when the majority of colonies are large and compact and the culture is 50 - 80% confluent. One well of hPSCs at 50 - 80% confluence can typically seed 5 - 10 wells of a 12-well plate.

For complete instructions on maintaining hPSCs in TeSR™ media, refer to the Technical Manual for mTeSR™1 or mTeSR™ Plus, available at www.stemcell.com, or contact us to request a copy.

Day 0:

1. Coat cultureware with Corning® Matrigel® or Gibco™ Vitronectin and bring to room temperature (15 - 25°C) for at least 1 hour prior to use.
2. Thaw STEMdiff™ RPE Differentiation Medium A (see Preparation of Media section A) and warm to room temperature.
3. Aliquot a sufficient volume of Gentle Cell Dissociation Reagent (GCDR) and warm to 37°C.
4. Use a microscope to visually identify regions of differentiation in the hPSC culture and mark them using a felt tip or lens marker on the bottom of the plate. Assess confluence of hPSC cultures to determine the number of wells required for seeding.

   NOTE: The seeding density may need to be adjusted depending on the cell line and hPSC maintenance medium used. Observe the confluency of the resulting culture on Day 4, 7, and 14 to determine which adjustments are needed.
5. 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. Removal of differentiated cells will result in improved differentiation efficiency.
6. Harvest the hPSC culture as follows:
   a. Aspirate the medium and rinse each well with 1 mL of pre-warmed GCDR.

      NOTE: Do not rinse with phosphate-buffered saline or culture medium. Washing the hPSC culture with GCDR results in reduced incubation time and improved aggregate formation.
   b. Add 1 mL of warm GCDR per well and incubate the plate at room temperature for 3 - 5 minutes.
   c. Aspirate the GCDR and add 1 mL/well of STEMdiff™ RPE Differentiation Medium A.
   d. Gently detach the colonies by scraping with a cell lifter. Take care to minimize the breakup of colonies.
   e. Transfer the hPSC aggregate suspension to a conical tube.
   f. Aspirate the coating from cultureware prepared in step 1 and add STEMdiff™ RPE Differentiation Medium A according to the recommended volumes in Table 1.

Table 1. Recommended Volumes and hPSC Seeding Densities for Various Cultureware

CULTUREWARE	VOLUME OF DIFFERENTIATION MEDIUM	SEEDING VOLUME OF hPSC AGGREGATE SUSPENSION
12-well plate	1 mL per well	100 - 200 µL per well
6-well plate	2 mL per well	300 - 600 µL per well
T-25 cm^2 flask	5 mL per flask	750 - 1500 µL per flask
100 mm dish	12 mL per dish	1650 - 3300 µL per dish
T-75 cm^2 flask*	15 mL per flask	2250 - 4500 µL per dish

* A total of two STEMdiff™-ACF RPE Differentiation Kits are required to prepare enough medium to differentiate RPE in one T-75 cm^2 flask.

   g. Carefully pipette the hPSC aggregate suspension up and down to break up the aggregates as needed.
   h. Seed the prepared cultureware containing STEMdiff™ RPE Differentiation Medium A with the hPSC aggregate suspension according to the recommended seeding volumes in Table 1.

NOTE: The seeding volume is determined by the confluence of the hPSC culture on the day of passaging. An hPSC culture harvested at ~80% confluence may require a lower seeding volume (e.g. 100 µL/well of a 12-well plate), while a culture harvested closer to ~50% confluence may require a higher seeding volume (e.g. 200 µL/well of a 12-well plate).

7. Place the plate in a 37°C incubator. Move the plate in several quick, short, back-and-forth and side-to-side motions to distribute the cell aggregates. Do not disturb the plate for 24 hours.

Proceed to section B for RPE differentiation.

B. DIFFERENTIATION TO IMMATURE RPE CELLS (DAY 1 - 13)

The following instructions are for differentiating the seeded hPSC aggregates from section A to immature RPE cells. Perform full-medium changes by removing the medium from each well and adding fresh STEMdiff™ RPE Differentiation Medium as directed in Table 2. If using different cultureware, adjust volumes accordingly (see Table 1). Incubate at 37°C and 5% CO_2 between medium changes. Assess culture morphology throughout the protocol.

Table 2. Differentiation Media for Full-Medium Changes on Days 1 - 13

DAY	DIFFERENTIATION MEDIUM
1	STEMdiff™ RPE Differentiation Medium A
2	STEMdiff™ RPE Differentiation Medium B
4	STEMdiff™ RPE Differentiation Medium C
6	STEMdiff™ RPE Differentiation Medium C
7	STEMdiff™ RPE Differentiation Medium D
9	STEMdiff™ RPE Differentiation Medium D
11	STEMdiff™ RPE Differentiation Medium D
13	STEMdiff™ RPE Differentiation Medium D

Day 2: Cultures should display rosette-like morphology (Figure 1A; indicated by the white arrow).

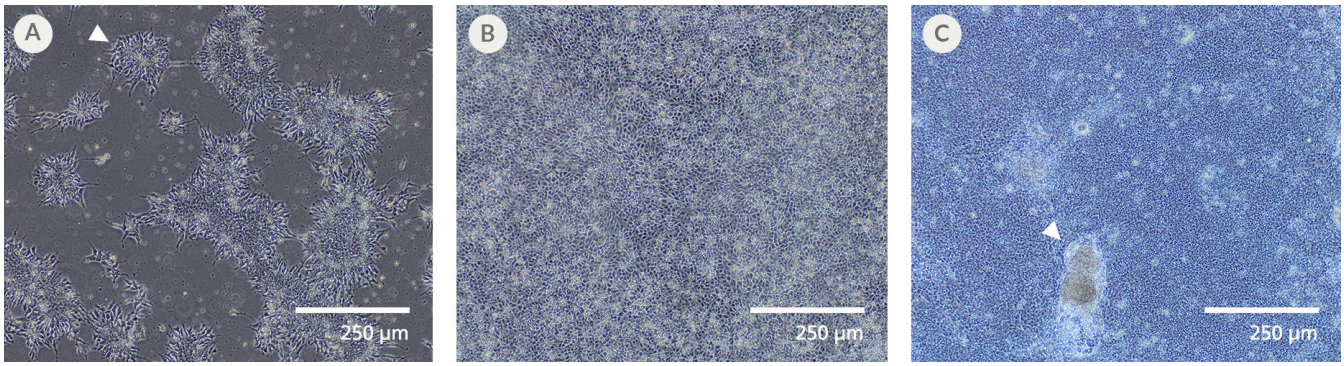


Figure 1. Representative RPE Cultures on (A) Day 2, (B) Day 7, and (C) Day 14

Day 4: Assess cells for confluency. Ideally, cultures should be ~100% confluent for optimal differentiation.

Day 7: Cultures must be 100% confluent (Figure 1B). Do not proceed with under-confluent cultures as this may impact RPE purity and yield.

Day 14: A uniform monolayer of immature RPE cells should be observed (Figure 1C). Neural retina differentiation (indicated by the white arrow) may also be present, and will appear spherical or cup-shaped.

NOTE: The presence of excessive neural retina differentiation in the culture indicates that the initial seeding density was too high. If the culture is under-confluent and/or displays fibroblast-like cell morphology, the initial seeding density was too low.

Day 14 immature RPE cells can be harvested and assessed for expression of PMEL17 via flow cytometry to determine culture purity:

- **< 50% PMEL17+**: Proceed with caution. Cultures may still enrich to > 90%. It is recommended to repeat hPSC seeding and perform optimizations as needed.
- **50 - 100% PMEL17+:** Proceed to section C for RPE maturation.

Optionally, Day 14 immature RPE cells can be cryopreserved to generate an 'intermediate' cell bank of immature RPE cells. Refer to section D for instructions on how to cryopreserve and thaw RPE cells.

C. RPE MATURATION (DAY 14 - 49+)

The following instructions are for harvesting and seeding Day 14 immature RPE for maturation. Immature RPE must be cultured in STEMdiff™-XF RPE Maturation Medium for at least 5 weeks to generate fully functional and mature RPE by Day 49.

Day 14 (continued):

1. Prepare RPE Plating Medium (Preparation of Medium section B). Warm RPE Plating Medium and a sufficient volume of STEMdiff™-XF RPE Maturation Medium to room temperature (15 - 25°C).
2. Prepare Corning® Matrigel®- or Gibco™ Vitronectin-coated cultureware according to the manufacturer's instructions.
3. Examine the culture for neural retina differentiation under a microscope. If excessive differentiation is observed, mark the regions using a felt tip or lens marker on the bottom of the plate and remove by scraping with a pipette tip or by aspiration. Otherwise, proceed directly to step 4.
4. Harvest the RPE culture as follows:
   a. Remove the medium and rinse the culture with D-PBS (Without Ca++ and Mg++).
   b. Add TrypLE™ Express Enzyme and incubate at 37°C for 20 - 30 minutes. Refer to Table 3 for recommended volumes.

Table 3. Recommended Volumes for Harvesting RPE Cells from Various Cultureware

CULTUREWARE	VOLUME OF TRYPLE™ EXPRESS ENZYME	VOLUME OF STEMDIFF™-XF RPE MATURATION MEDIUM
12-well plate	0.5 mL/well	2 mL/well
6-well plate	1 mL/well	4 mL/well
T-25 cm^2 flask	2.5 mL/flask	10 mL/flask
100 mm dish	5 mL/dish	20 mL/dish
T-75 cm^2 flask	7.5 mL/flask	30 mL/flask

   c. Gently tap the side of the culture vessel to detach the monolayer. If the monolayer remains attached, use a cell lifter to remove the remaining cells.
   d. Using a 10 mL serological pipette, pipette the cells up and down several times to generate a uniform single-cell suspension and transfer to a 50 mL conical tube.
   e. Rinse the cultureware with warm STEMdiff™-XF RPE Maturation Medium (as directed in Table 3) to collect the remaining cells and quench the TrypLE™. Transfer the rinse to the same conical tube.
   f. Pass the cell suspension through a 70 µm Reversible Strainer attached to a new 50 mL conical tube and collect the flow-through.
   g. Centrifuge the tube at 300 x g for 10 minutes at room temperature.
   h. Discard the supernatant and resuspend the cells in a small volume of RPE Plating Medium (prepared in step 1).
   i. Perform a viable cell count using Trypan Blue and a hemocytometer (e.g. Catalog #100-1181).
      OPTIONAL: Assess PMEL17 expression by flow cytometry. Cells should be ≥ 50% PMEL17+ at this stage.
      OPTIONAL: Extra cells may be cryopreserved using CryoStor® CS10 (see section D).
   j. Aspirate the coating from the cultureware prepared in step 2. Add RPE Plating Medium and seed the cells as directed in Table 4.

Table 4. Recommended Seeding Density and Volume of Medium for Various Cultureware

CULTUREWARE	SEEDING DENSITY	VOLUME OF MEDIUM
12 mm cell culture inserts*	0.4 x 10^6 cells/cm^2	0.5 mL apical and 1.5 mL basal
12-well plate	0.1 x 10^6 cells/cm^2	2 mL/well
6-well plate		4 mL/well
T-25 cm^2 flask		8 mL/flask
T-75 cm^2 flask		30 mL/flask
T-175 cm^2 flask		70 mL/flask

*RPE cultures should optimally be ≥ 85% PMEL17+ before seeding on 12 mm cell culture inserts. If cultures are 50 - 84% PMEL17+ on Day 14, seed on alternative cultureware and proceed with maturation until Day 49 before reassessing PMEL17 purity and seeding RPE on 12 mm cell culture inserts.

5. Incubate at 37°C and 5% CO_2 for 3 - 4 days.

Day 18:

6. Perform a full-medium change with RPE Plating Medium. Incubate at 37°C and 5% CO_2 for 3 - 4 days.

Day 21 - 48:

7. Perform full-medium changes with STEMdiff™-XF RPE Maturation Medium every 3 - 4 days. Incubate at 5% CO_2 and 37°C.

Day 49:

8. Harvest mature RPE (repeat steps 4 - 5).
9. Resuspend mature RPE in RPE Plating Medium and seed at the appropriate density according to Table 4.
10. Incubate at 5% CO_2 and 37°C. Perform a full-medium change every 3 - 4 days with STEMdiff™-XF RPE Maturation Medium.

    OPTIONAL: Perform flow cytometry on the harvested cells to assess expression of PMEL17, RPE65, EZRIN, and CRALBP.

Day 49+:

Mature RPE can be maintained long-term (~120 days) while maintaining functionality and polarity. Long-term mature RPE cultures may be harvested every 28 days (steps 4 - 5) or maintained as a monolayer. Perform full-medium changes every 3 - 4 days.

Mature RPE cells seeded on Day 49 can be harvested at ~50% confluence and cryopreserved for cell banking. Refer to section D for instructions on how to cryopreserve and thaw RPE cells.

D. CRYOPRESERVATION & THAWING

RPE may be cryopreserved for cell banking. Immature RPE can be harvested on Day 14, while mature RPE can be harvested on Day 50 - 52 (i.e. 1 - 3 days after seeding on Day 49).

Cryopreserving Immature RPE:

1. Harvest Day 14 immature RPE as directed in section C, steps 4a - 4i.
2. Centrifuge the tube at 300 x g for 5 minutes at room temperature.
3. Discard the supernatant and resuspend the cell pellet by gently flicking the tube.
4. Add 1 mL of cold (2 - 8°C) CryoStor® CS10 per 4 x 10^6 cells. Mix thoroughly and transfer the suspension to a cryovial.
5. Freeze cells using a standard slow rate-controlled cooling protocol (approximately -1°C/minute) or an isopropanol freezing container and store at liquid nitrogen temperature (-135°C).

   NOTE: Long-term storage at -80°C is not recommended.

Cryopreserving Mature RPE:

1. Monitor the mature RPE seeded on Day 49 (section C) until they are approximately 50% confluent and have not regained pigmentation, typically 1 - 3 days after seeding.
2. Harvest cells on Day 50 - 52 as directed in section C, steps 4a - 4i.
3. Centrifuge the tube at 300 x g for 5 minutes at room temperature.
4. Discard supernatant and resuspend the cell pellet by gently flicking the tube.
5. Add 1 mL of cold (2 - 8°C) CryoStor® CS10 per 4 x 10^6 cells. Mix thoroughly and transfer the suspension to a cryovial.
6. Freeze cells using a standard slow rate-controlled cooling protocol (approximately -1°C/minute) or an isopropanol freezing container and store at liquid nitrogen temperature (-135°C).

   NOTE: Long-term storage at -80°C is not recommended.

Thawing Cryopreserved RPE:

1. Warm RPE Plating Medium and STEMdiff™-XF RPE Maturation Medium to room temperature (15 - 25°C). Prepare Corning® Matrigel®- or Gibco™ Vitronectin-coated cultureware before starting the protocol to ensure that the thawing procedure is done as quickly as possible.
2. Add 8 mL of warm STEMdiff-XF™ RPE Maturation Medium to a 15 mL conical tube.
3. Wipe the outside of the vial of cells with 70% ethanol or isopropanol.
4. In a biosafety cabinet, twist the cap a quarter-turn to relieve internal pressure, then retighten.
5. Quickly thaw cells in a 37°C water bath by gently shaking the vial. Remove the vial when a small frozen cell pellet remains. Do not vortex cells.
6. Wipe the outside of the vial with 70% ethanol or isopropanol.
7. Use a 1 mL pipette to transfer the contents of the cryovial to the 15 mL conical tube prepared in step 2.
8. Rinse the cryovial with 1 mL of STEMdiff™-XF RPE Maturation Medium and transfer to the same conical tube.
9. Centrifuge cells at 300 x g for 5 minutes at room temperature.
10. Carefully remove the supernatant with a pipette, leaving a small amount of medium to ensure the cell pellet is not disturbed.
11. Gently resuspend the cell pellet in 4 mL of warm RPE Plating Medium.
12. Perform a viable cell count using Trypan Blue and a hemocytometer.

13. Aspirate the coating from the cultureware prepared in step 1 and add warm RPE Plating Medium as directed in Table 4.
14. Seed RPE at a density of 0.15 x 10^6 cells/cm^2.
15. Place the cultureware in a 37°C and 5% CO_2 incubator.
16. Perform a full-medium change with RPE Plating Medium 3 - 4 days after seeding the thawed cells. Incubate at 37°C and 5% CO_2 for an additional 3 - 4 days.
17. Perform a full-medium change every 3 - 4 days with STEMdiff™-XF RPE Maturation Medium for at least 4 weeks before harvesting RPE or performing downstream assays.

Assessment of hPSC-Derived RPE

Marker expression of RPE may be assessed by immunocytochemistry or flow cytometry after labelling with the following antibodies:

- Anti-human PMEL17 antibody, clone HMB-45 (BioLegend #911505)
- Anti-human RPE65 antibody, clone 401.8B11.3D9 (Thermo Fisher Scientific #MA1-16578)
- Anti-human EZRIN antibody, clone 3C12 (Santa Cruz Biotechnology #sc-59487)
- Anti-human CRALBP antibody, clone B2 (Santa Cruz Biotechnology #sc-59487)
- Anti-human BEST1 antibody, clone E6-6 (Millipore Sigma #ZMS1043)

Results may vary depending on the maturity of the RPE culture and the cell line used. Refer to Table 5 for expected expression levels. RPE may require longer than Day 49 to display the expected RPE marker expression.

Antibodies against RPE65, EZRIN, CRALBP and BEST1 require staining with a secondary antibody. The differentiation efficiency of mature RPE may also be assessed by functional assays, such as melanin production, polarized secretion of VEGF and PEDF (as determined by ELISA), transepithelial resistance, and ability to phagocytose bovine or porcine photoreceptor outer segments.

Table 5. Expected Expression Levels for Assessment of Immature and Mature RPE

ANTIBODY TARGET	EXPECTED EXPRESSION
PMEL17	Day 14: ≥ 50% Day 49: ≥ 90%
RPE65	Day 49: ≥ 80%
EZRIN	Day 49: ≥ 80%
CRALBP	Day 49: ≥ 80%
BEST1	Positive

Related Products

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