NcEpic™ hPSC Medium

Product Manual

I. Product Introduction

NcEpic hPSC Medium is a completely defined, feeder-free, and animal protein-free medium for the culture of human pluripotent stem cells (hESC/hiPSC). hESC/hiPSC can rapidly proliferate in NcEpic hPSC Medium, while differentiated cells grow more slowly in this medium, thereby selectively expanding human pluripotent stem cells and achieving high purity.

II. Product Information

Table 1: NcEpic hPSC Medium Product Description

Product Information	Cat.NO.	Amount	Storage
NcEpic hPSC Medium Contains:	**RP01001**	**1 Kit**	**2℃～8℃***
NcEpic hPSC Basal Medium	RP01001-1	496 mL	2℃～8℃
NcEpic hPSC Supplement（125×）	RP01001-2	4mL	-20°C or -80°C

***Mix the basal medium and supplements to prepare the complete medium, which is stable at 2°C to 8°C for up to 2 weeks.**

III. Reagents and Materials

Table 2: Reagents & Materials

Reagents & Materials	Brand (e.g.)	Cat.NO. (e.g.)
NcEpic hPSC Medium	Shownin	RP01001
Vitronectin	Shownin	RP01002
hPSC Cryopreservation Medium	Shownin	RP01003
hPSC Dissociation Buffer	Shownin	RP01007
Blebbistatin (10 mM)	Shownin	RP01008
DMEM/F12 Medium	Thermo Sci.	11330
6-well Plate	Thermo Sci.	140685
1 mL/5 mL/10 mL/25 mL Pipettes	Thermo Sci.	N/A
15 mL/50 mL Centrifuge Tubes	Thermo Sci.	N/A
1.5/2 mL Cryovials	Thermo Sci.	N/A
Freezing Container	Thermo Sci.	5100-0001

IV. Reagent Preparation

(i) The complete NcEpic hPSC medium preparation (500 mL)

1. Thaw **NcEpic Supplement** at 4°C; **do not thaw at 37°C**.
2. In the biosafety cabinet, use a sterile pipette to mix the following two components to prepare 500 mL of complete culture medium.

   NcEpic Basal Medium：496 mL

   NcEpic Supplement：4 mL
3. The complete culture medium can be stored at 4°C and should be used within 2 weeks.

   TIPS: Aliquot and freeze NcEpic Supplement as needed. For 100 mL of complete medium, aliquot the Supplement into 0.8 mL × 5 vials. Thaw one vial and mix with 99.2 mL Basal Medium before use. Avoid more than 2 freeze-thaw cycles.

(ii) Vitronectin Coating of Culture Plates (Using VTN Coating Protein to Coat 6-Well Plates as an Example, the Procedure is Also Applicable to Other Culture Vessels)

1. Coat the culture dishes with Vitronectinunder sterile conditions.
2. Thaw Vitronectin at room temperature (15 - 25°C).

   TIPS: Store at 4°C after thawing for up to 2 weeks or aliquot and store at -20°C/-80°C. Avoid repeated freeze-thaw cycles.
3. Aliquot Vitronectin according to the coating protocol: For a 6-well plate (10 cm^2/well), use 60 μg (120 μL of 500 μg/mL). Aliquot into 120 μL (60 μg) vials and store at -20°C/-80°C. Use one vial per 6-well plate.
4. Dilute one vial (120 μL, 60 μg) in 9 mL DMEM/F12 and mix gently (no vortexing).
5. Add 1.5 mL/well to a 6-well plate and gently rock to ensure even coating.
6. Let stand at room temperature (15 - 25°C) for ≥1 hour. Aspirate the coating solution before use. Avoid scratching the coated bottom. No need for extra rinsing.

   TIPS: Seal coated plates to prevent evaporation for temperate storage at 4°C ≤ 1 week. Warm to room temperature (15 - 25°C) for 10-30 minutes before use. Drying of the coating solution will impair hESC/hiPSC adhesion.

(iii) Matrigel Coating of Culture Plates (Corning® Matrigel®-coated 6-well plates are used as an example**)**

A. Aliquoting Matrigel

Look up the concentration of Matrigel based on the batch number. Calculate the aliquot volume and quantity needed for the desired coating concentration and area.

Example: For hPSC culture, the recommended coating concentration is 0.013 mg/cm^2. For a 6-well plate (9.6 cm^2/well), use 0.75 mg per plate. If Matrigel concentration is 11.3 mg/mL, aliquot 3 mg per tube (enough for 4 plates). Aliquot volume = 3 mg / 11.3 mg/mL = 0.265 mL. Number of aliquots = 10 mL / 0.265 mL = 37.74.

1. Prepare 38 sterile 1.5 mL EP tubes, pre-cool tubes, pipette tips, and tube racks at -20°C for 1 hour.

   TIPS: For hESC-Qualified Matrigel (Cat. No. 354277), follow the Dilution Factor provided (e.g., 238 μL for 4 plates). Number of Aliquots = 5 mL / 238 μL = 21.01.

2. Thaw Matrigel overnight at 4°C. Ensure it is fully liquid before aliquoting.

TIPS: Matrigel remains liquid only at 4°C. Temperature fluctuations may cause it to solidify.

3. Place thawed Matrigel, pre-cooled tubes, and pipette tips on ice.

4. Sterilely mix and aliquot Matrigel into pre-cooled 1.5 mL EP tubes. Keep tubes on ice. Replace pipette tips if clogged to ensure accurate volumes.

5. Store aliquots at -20°C.

B. Plating

1. Prepare 36 mL of chilled DMEM/F12 in a 50 mL centrifuge tube.
2. Pre-cool 1000 µL pipette tips at -20°C for 1 hour. Thaw one Matrigel aliquot (3 mg) at 4°C.
3. Place thawed Matrigel and pre-cooled pipette tips on ice.
4. Add 1 mL of cold DMEM/F12 to the thawed Matrigel (3 mg). Gently pipette to mix thoroughly.
5. Transfer the Matrigel mixture to the remaining DMEM/F12 in the centrifuge tube. Mix well using a 10 mL pipette.
6. Add 1.5 mL/well to 4 six-well plates. Gently rock to ensure even coating.
7. Let plates sit at room temperature for 1 hour or at 4°C overnight for immediate use. Or it can be stored at 4°C for use within 2 weeks.

V. Thawing and Recovery of hPSC (Using 6-well plate operation as an example, the procedure is also applicable to other culture vessels)

1. Preheat the water bath to 37°C.
2. Place a Matrigel-coated 6-well plate in the biosafety cabinet for 1 hour to warm to room temperature (15–30°C).
   Prepare 4 mL of NcEpic Complete Medium with 1 µL Blebbistatin (10 mM) at a 1:4000 ratio. Warm to room temperature (15–30°C).
   TIPS: Do not pre-warm the medium in a 37°C water bath.

3. Thaw a frozen cell vial in the 37°C water bath, gently shaking until ice crystals nearly disappear (within 1 minute).

4. Wipe the vial with 75% ethanol and transfer to the biosafety cabinet. Transfer the cell suspension to a 15 mL centrifuge tube. Slowly add 10 mL of DMEM/F12, mix gently, and centrifuge at 160 × g for 5 minutes.

5. Aspirate the supernatant. Resuspend the cell pellet in 4 mL of pre-warmed Blebbistatin + NcEpic Complete Medium. Avoid excessive pipetting.
6. Aspirate the Matrigel coating solution from two wells of the 6-well plate. Seed the cell suspension at 2 mL/well.

7. Gently rock the plate horizontally in a cross pattern three times. Place in a 37°C, 5% CO_2 incubator with saturated humidity. Rock the plate three more times and culture overnight.

8. Replace the medium with fresh NcEpic Complete Medium after 18–24 hours. Change the medium daily thereafter.

Table 3: Recommended Usage of Reagents for hPSC Passage and Culture Operations

Culture Vessel	Growth Area	DPBS (mL)	hPSC Dissociation Buffer	hPSC medium*
6-well Plate	9.6 cm^2 /well	2 mL/well	2 mL/well	2 mL/well
12-well Plate	4.5 cm^2 /well	1 mL/well	1 mL/well	1 mL/well
24-well Plate	2 cm^2 /well	0.5 mL/well	0.5 mL/well	0.5 mL/well
35mm Culture Dish	8 cm^2	2 mL	2 mL	2 mL

*** For routine hPSC culture, we recommend adding 50% more medium when confluence exceeds 50%. For example, add 3 mL/well to a 6-well plate during medium change.**

VI. Passaging of hPSC (Using 6-Well Plates and EDTA Dissociation as an Example; the Procedure is Also Applicable to Other Culture Vessels)

1. Determining the Timing for Passaging:

Passage iPSCs when either of the following conditions are met:

1.1 When cell confluence reaches approximately 85% (Figure 1), normally 4 days after seeding. Even if the clones are small and confluence is insufficient, continuous culture should not exceed 5 days.

1.2 When cell confluence is low, but the colonies are overly large, leading to poor growth in the central region of colonies.

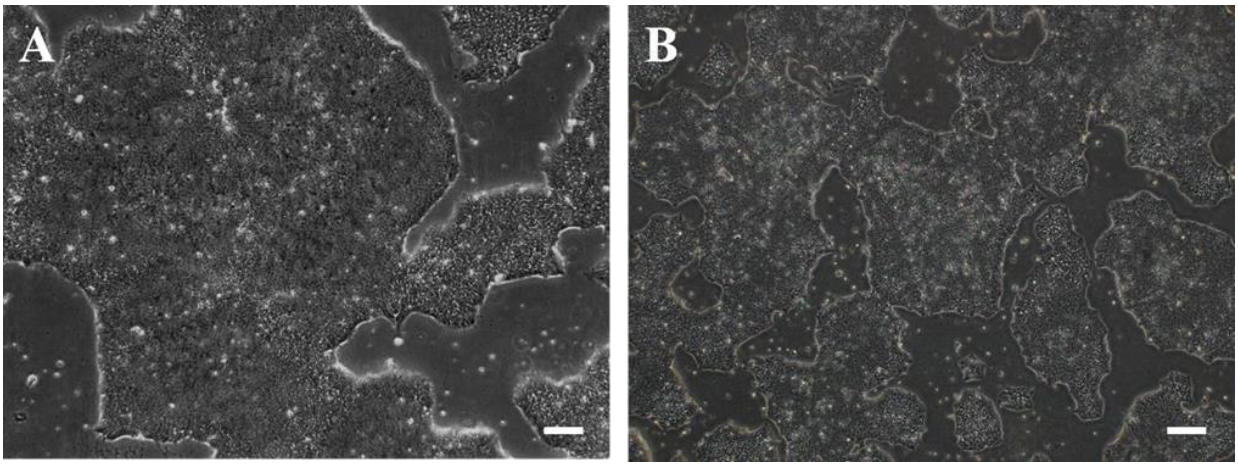

Figure 1: hiPSC clones at approximately 85% confluence.

(A) on a matrigel coated plate; (B) on a vitronectin coated plate.

Scale bar: 200 μm.

2. Passaging ratio:
   The passaging ratio can range from 1:5 to 1:20 depending on cell growth status and experimental requirements. If cells are healthy, with 85% confluence and uniform clone size (Figure 1), a 1:10 ratio is recommended. Reduce the ratio if cell density is low; increase the ratio if density is high.

3. Preparation of Culture Vessels:
   Place Vitronectin-coated 6-well plates in the biosafety cabinet for approximately 1 hour to equilibrate to room temperature (~25°C).

4. Preparation of Medium:
   Prepare 2 mL/well of NcEpic complete medium for the number of wells to be passaged. Add Blebbistatin (10 mM) at a 1:4000 ratio and equilibrate to room temperature (~25°C).
   TIP: Add 0.5 µL of Blebbistatin (10 mM) to 2 mL of NcEpic complete medium.

5. Aspirate the medium from the iPSC wells and add 2 mL/well of DPBS (without calcium and magnesium). Gently swirl and aspirate.

6. Add 2 mL/well of **hPSC Dissociation Buffer** to fully cover the well surface.

7. Incubate in a 37°C incubator for 7-8 minutes.
   TIPS: (1) Observe cell morphology under a microscope after 7-8 minutes. Stop dissociation when most cells appear bright and round but have not yet detached or floated (Figure 2C). If most cells remain unchanged, extend the dissociation time (Figures 2A & 2B). (2) Place the 6-well plate directly on the metal shelf in the incubator for even heating. Do not stack plates.

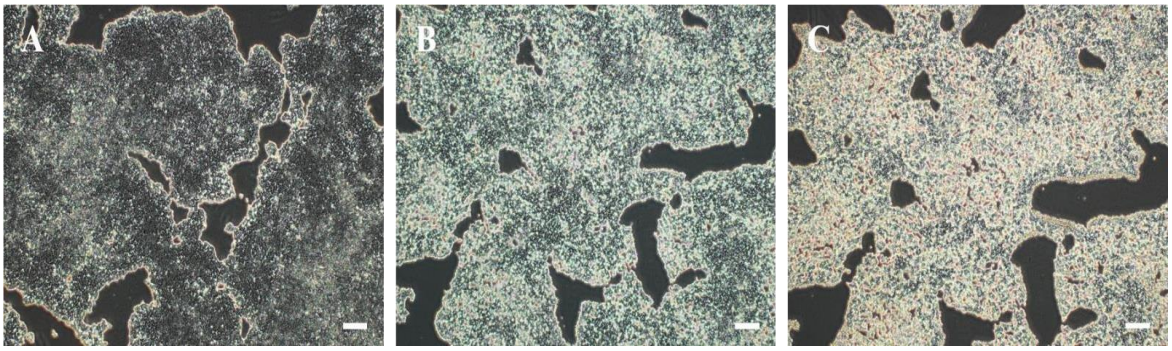

Figure 2: (A) Digestion for 4 min; (B) Digestion for 6 min; (C) Digestion for 8 min. Scale bar: 200 µm.

8. Bring the plate to the biosafety cabinet without shaking. Tilt the plate and aspirate the EDTA dissociation buffer.

9. Immediately add 2 mL/well of pre-warmed Blebbistatin-supplemented NcEpic complete medium. Gently rock the plate in a horizontal cross pattern to detach cells.
   TIPS: (1) Gently pipette 1-2 times after adding the medium. Avoid excessive pipetting (>2 times).
   (2) Avoid scraping cells. It is normal for 10-15% of cells to remain attached. If a large number of cells remain attached, extend the dissociation time.
   (3) Process no more than one 6-well plate at a time. Quickly aspirate the medium after adding NcEpic medium, as EDTA dissociation is rapidly neutralized and the cells re-attach quickly in medium. hPSCs should not remain in EDTA dissociation buffer for >15 minutes.

10. Seeding:
    10.1 Aspirate the Vitronectin solution from the 6-well plate and add 2 mL/well of pre-warmed Blebbistatin + NcEpic Complete Medium.
    10.2 Gently mix the cell suspension obtained in step 9 and evenly distribute it into the wells according to the predetermined passage ratio.
    TIPS: Alternatively, calculate the total cell number required per plate, transfer the suspension accordingly to a 15 mL centrifuge tube, and bring up the volume to 12 mL with pre-warmed Blebbistatin-supplemented NcEpic complete medium. Distribute evenly into Matrigel-coated 6-well plates after aspirating the coating solution.

11. Gently rock the 6-well plate horizontally in a cross pattern three times. Place it in a 37°C, 5% CO_2, humidified

incubator incubator. Rock the plate again three times and culture overnight.

12. After 18-24 hours, replace with fresh NcEpic Complete Medium. Change the medium daily thereafter, followed with passaging or cryopreservation 4-5 days later (Figure 3-4).

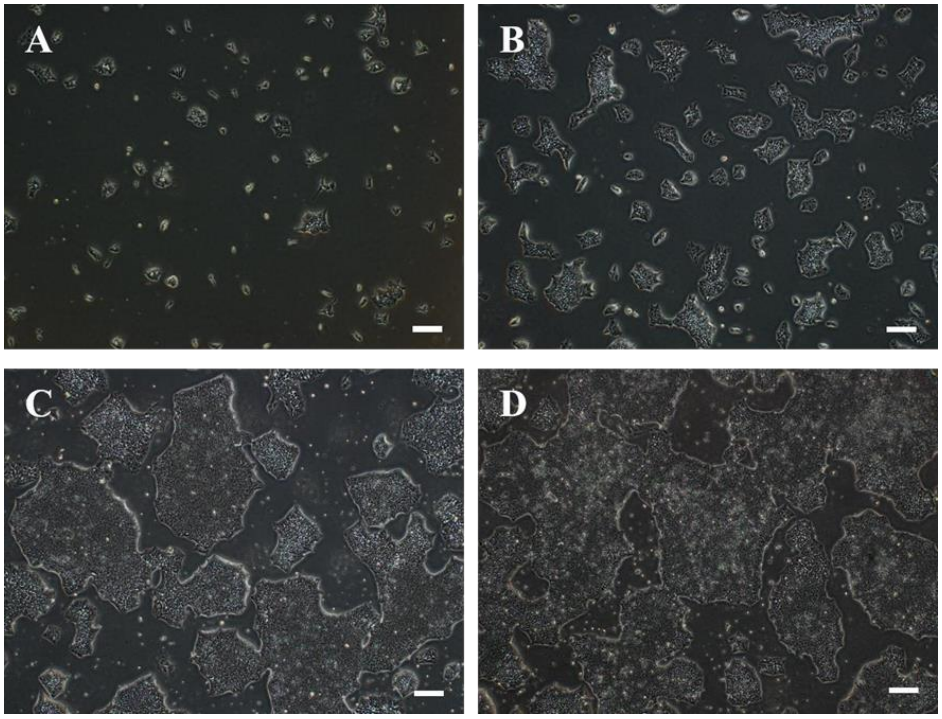

Figure 3: Morphology of hiPSCs cultured in NcEpic human pluripotent stem cell medium on a vitronectin Plate.

(A, B, C, D) Morphology on days 1, 2, 3, and 4, respectively. Scale bar: 200 μm.

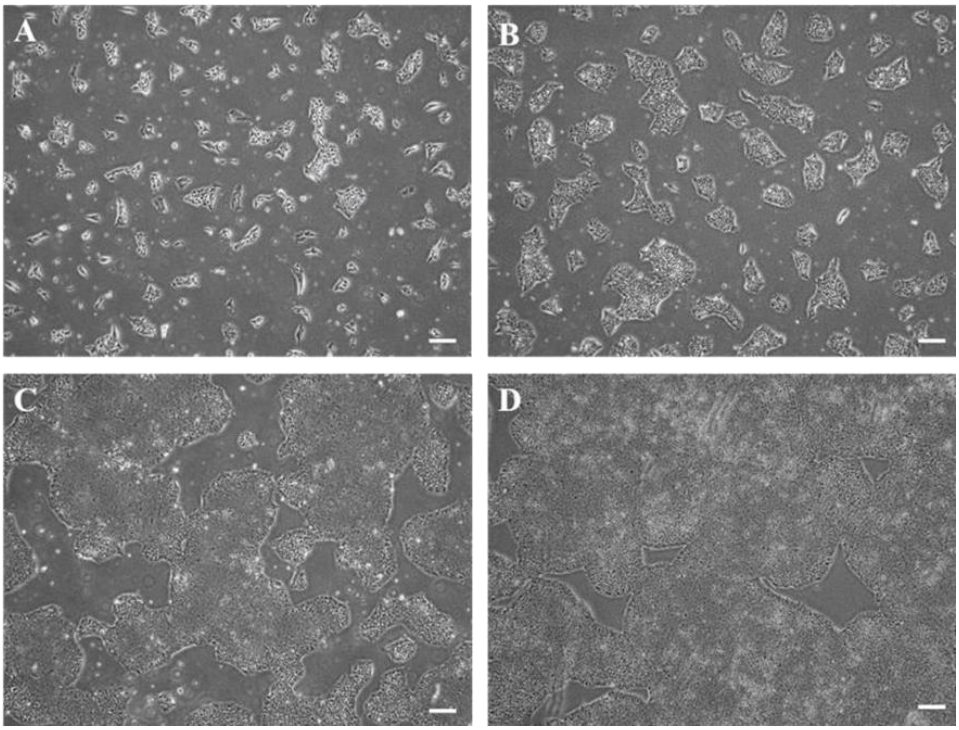

Figure 4: Morphology of hiPSCs cultured in NcEpic human pluripotent stem cell medium on matrigel Plate.

(A, B, C, D) Morphology on days 1, 2, 3, and 4, respectively. Scale bar: 200 μm.

VII. Cryopreservation of hPSC

1. When the cell confluence reaches about 85% (Figures 1), cells can be harvest for cryopreservation. Typically, 2×10^6-4×10^6 viable cells per well of a 6-well plate can be collected and frozen in one cryovial.

2. Prepare the appropriate number of 1.5/2 mL cryovials and label properly.

3. Take out hPSC High-Efficiency Cryopreservation Medium from the 4°C refrigerator and equilibrate to room temperature. Mix thoroughly before use.

   TIPS: DMSO in the cryopreservation medium tends to settle at the bottom. Inadequate mixing may result in insufficient DMSO concentration initially and excessive concentration later, leading to instability in cryopreserved cells.

4. Aspirate the culture medium from the plate and add 2 mL/well of DPBS (without calcium and magnesium). Gently rock swirl several times and aspirate.

5. Add 2 mL/well of hPSC dissociation working solution. Place the plate in a 37°C incubator for 7-8 minutes (refer to Section VI, Step 7).

6. After digestion, gently take out the culture plate and aspirate the EDTA buffer.

7. Mix the pre-warmed hPSC cryopreservation medium thoroughly. Add 1 mL of cryopreservation medium to each well. Gently pipette to resuspend the cells, then rock the plate in a horizontal cross pattern three times. Transfer the cell suspension into the labeled 1.5/2 mL cryovials..

8. Place the vials in a cell freezing container and store at -80°C overnight. Transfer the vials to a liquid nitrogen tank for long-term storage the next day. Alternatively, use a programmable controlled rate freezer to cool the cells below -80°C before transferring to liquid nitrogen storage.

VIII. Adaptation of hPSC from Other Culture Systems to NcEpic Culture Conditions

Other feeder-free cultured hPSCs can be transitioned to NcEpic complete medium when the cells are in good condition. Initially, replace the medium with a 1:1 mixture of the original medium and NcEpic complete medium. After two medium changes, culture the cells in NcEpic complete medium for 2–3 passages to allow adaptation to the NcEpic pluripotent stem cell medium.

IX. Troubleshooting

➢ **Differentiation Observed in hiPSC Culture**
• Ensure NcEpic complete medium is stored at 4°C and used within 2 weeks. Only pre-warm the amount of medium required for the current experiment to minimize temperature fluctuations and prevent degradation of ingredients in the medium. • If hPSC colonies exhibit good overall morphology with sporadic differentiated cells (<1%) at the edges, these cells can be removed during EDTA passaging. • Ensure that the size of the hPSC cell clusters during passaging is uniform, with the size of about 20 cells being ideal. For larger clusters, gently pipette no more than 3 times using a 5 mL pipette, applying light and even pressure to avoid cell damage or differentiation. • Avoid removing cells from the incubator for more than 15 minutes during observation. • If hPSC clones appear loose internally with irregular edges and differentiation exceeds 20%, discard the culture.
➢ **Can Dispase or Collagenase Be Used for hPSC Passaging?**
• Dispase or collagenase can be used, but digestion efficiency may be suboptimal, affecting post-passaging cell viability and potentially accumulating differentiated cells. • For hPSCs cultured in the NcEpic system, non-enzymatic, gentle dissociation methods are recommended for passaging. • If single-cell dissociation is required for experiments, use Accutase for 5-10 minutes.
➢ **hPSCs Fail to Adhere or Exhibit Low Adhesion After Passaging**
• Avoid excessive passaging ratios (>1:20). • EDTA dissociation time should not be too long. While some cell lines may require extended dissociation beyond 8 minutes, do not exceed 15 minutes. • Avoid excessive pipetting (>3 times) to prevent clusters disruption or cell damage. • Ensure culture plates are coated with Vitronectin, Matrigel, or other matrices suitable for pluripotent stem cell growth. • ROCKi is necessary in the medium after passaging.
➢ **Cells detach after medium change**
• Perform the first medium change 18-24 hours after seeding to ensure proper cell adhesion. • Handle medium changes gently to avoid dislodging cell clusters from the matrix. If cell seeding density is very low (e.g., for cell cloning experiments), avoid medium changes for 2-3 days, ensuring the medium contains ROCKi.
➢ **Uneven distribution of hiPSC clusters in wells**
• Ensure the coating matrix is evenly distributed across the bottom of the culture vessel. • During passaging, ensure cells are evenly dispersed. After rocking the plate in a horizontal cross pattern, avoid moving the plate to prevent cell aggregation in the center. After placing the plate in the incubator, rock it again in a horizontal cross pattern to ensure even distribution.