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Protocol for germ lineage differentiation of primed human pluripotent stem cells using chemically defined, nutrient-balanced media

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SUMMARY
Metabolism regulates cell fates during early mammalian cell differentiation. This protocol describes the steps for directed differentiation of primed human pluripotent stem cells (hPSCs) into the three primary germ lineages—ectoderm, endoderm, and mesoderm—using a chemically defined nutrient-balanced media formulation. Although the transient removal and addition of specific nutrients does not occur in vivo during embryonic development, manipulation of nutrients in vitro provides an accessible method for evaluating how extracellular and intracellular metabolites help determine hPSC fate.

For complete details on the use and execution of this protocol, please refer to Lu et al. (2019) and Lu et al. (2022).

BEFORE YOU BEGIN
We have had experience and success with commercially available ready-to-use human pluripotent stem cell (hPSC) differentiation kits, although these reagents may present a cost barrier for certain hPSC projects or applications. Additionally, the formulations of these kits are often proprietary and opaque to users. In designing experiments in which eliminating confounding variables is paramount, differing nutrient and/or metabolite concentrations within and between different differentiation media formulations may bias the interpretation of results unknowingly. Here, we provide a validated (Lu et al., 2019) and accessible methodology for differentiating primed hPSCs into early germ lineage-specific cells using chemically defined, nutrient-balanced media.

This protocol provides detailed instructions for differentiating primed hPSCs into endoderm, mesoderm, and ectoderm using readily available cytokines and equivalent media-supplied nutrient concentrations. We have used this protocol successfully for differentiating human embryonic stem cell (hESC) and induced pluripotent stem cell (iPSC) lines including H9 (WA09; RRID: CVCL_9773), H1 (WA01; RRID: CVCL_9771), HSF-1 (RRID: CVCL-D003), hiPS2 (RRID: CVCL_B508), and UCLA
We also include suggestions for biomarker analysis of primed-undifferentiated and differentiated hPSCs (Table 1), as well as a detailed proof-of-concept example with the manipulation of extracellular glutamine (Gln) availability.

The application of this protocol is only intended to be executed on primed (or often referred to as conventional) hPSCs. Although hPSCs have several states of pluripotency, naïve and primed, these states and the continuum between them differ in their biological properties (Weinberger et al., 2016). Naïve hPSCs in vitro cells correspond best to the inner cell mass of human embryonic pre-implantation blastocysts, whereas primed hPSCs resemble a later stage in post-implantation epiblast development (Takahashi et al., 2018).

The use of conventional hPSCs in downstream applications require effective maintenance media compositions that allow and ensure for the promotion of large-scale, quality-controlled expansion of hPSCs. It is well-established that culture conditions can affect several parameters in regulating the self-renewal, proliferation, survival, genetic stability, and functionality of hPSCs, which are important to evaluate for use in differentiation experiments (Dakhore et al., 2018). Currently, a number of different hPSC maintenance media options are commercially available, such as mTeSR(+), mTeSR1, mTeSR2, and mTeSR-E8 (Stemcell Technologies), StemPro, Essential 8, and StemFlex (Thermo Fisher Scientific), PluriSTEM (Millipore), and Nutristem (Corning). For our protocol, we choose to use mTeSR(+), an enhanced version of mTeSR1 (StemCell Technologies) based on experience. However, users can maintain hPSCs using their own preferred media and culturing methods.

Standard cell culture procedures and equipment are required for the maintenance and differentiation of primed hPSCs. In addition, all steps of this protocol utilize a humidified incubator at 37°C and 5% CO₂ without regulating the O₂ level, for cell culture and Matrigel® matrix gelation. It is also recommended that primed hPSCs in culture are tested bi-weekly for mycoplasma contamination, which can adversely affect mammalian cells.

### Institutional permissions

Depending on the organization where this protocol will be performed, prior approval for the use of hPSCs may be required, such as from an institutional Stem Cell Research Oversight Committee.

| Category                      | Biomarkers (full name) |
|-------------------------------|------------------------|
| pluripotent/ undifferentiated | SOX2 (SRY-box 2)       |
| pluripotent/ undifferentiated | NANOG (Nanog homeobox) |
| pluripotent/ undifferentiated | POU5F1 (POU class 5 homeobox 1) |
| endoderm                      | FOXA2 (Forkhead Box A2) |
| endoderm                      | SOX17 (SRY-box 17)     |
| endoderm                      | EOMES (Eomesodermin)   |
| endoderm                      | LEFTY1 (Left-right determination factor 1) |
| endoderm                      | AFP (Alpha fetoprotein) |
| mesoderm                      | MIXL1 (Mixed paired-like homeobox) |
| mesoderm                      | T (T brachyury transcription factor) |
| mesoderm                      | HAND1 (Heart and neural crest derivatives expressed 1) |
| mesoderm                      | DES (Desmin)           |
| mesoderm                      | CD34 (Transmembrane phosphoglycoprotein) |
| mesoderm                      | SNAI2 (Snail family transcriptional repressor 2) |
| ectoderm                      | NCAM1 (Neural cell adhesion molecule 1) |
| ectoderm                      | PAX6 (Paired box 6)    |
| ectoderm                      | OTX2 (Orthodenticle homeobox 2) |
| ectoderm                      | NES (Nestin)           |
| ectoderm                      | TFAP2A (Transcription factor AP-2 alpha) |
| ectoderm                      | SOX 1 (SRY-box 1)      |
Please ensure that all legal permissions from respective funding agencies and/or institutions are in place.

Coating plates with Matrigel®

⊕ Timing: 30 min

hPSCs will be maintained in culture and differentiated in feeder-free conditions on tissue culture-treated polystyrene microplates coated with Corning Matrigel® basement membrane matrix. For our protocol, we describe hPSC culturing conditions using a 6-well plate format and differentiation with a 12-well plate setup.

Note: Corning Matrigel® matrix should be handled on ice as it will start to polymerize at temperatures above 10°C. Each lot of Matrigel® matrix varies in protein concentration and dilution, which is indicated on the Certificate of Analysis from the manufacturer. For additional information on the handling and use of Matrigel® matrix, visit the Corning® Matrigel® Matrix information page (here) for detailed instructions.

In general, we coat 6- and 12-well tissue culture-treated polystyrene microplates with 1 mL/well (6-well plate) or 0.5 mL/well (12-well plate) of cell feeder-free Matrigel® basement membrane matrix diluted in ice-cold DMEM/F-12 media (1:60 dilution) in a sterile hood, followed by incubation in a 5% CO₂ tissue culture incubator for gelation for at least 30 min at 37°C.

⚠ CRITICAL: Do not leave coated plates at 37°C incubation for more than 48 h. Coated plates left to incubate for extended periods of time are susceptible to evaporation and drying out. hPSCs will not adhere to dry plates.

Preparing media and stock reagents

Obtain media and reagents indicated in the key resources table. Prepare mTeSR(+) and working stocks of reagents as described in Tables 2 and 3, respectively.

Thawing hPSCs for culture

⊕ Timing: 30 min

| Reagent       | Solvent        | Stock concentration | Working concentration |
|---------------|----------------|---------------------|-----------------------|
| Y-27632       | water          | 10 mM               | 10 μM                 |
| Insulin       | water          | 10 mg/mL            | 0.7 μg/mL             |
| Transferrin   | n/a            | 30 mg/mL            | 15 μg/mL              |
| Activin A     | water          | 100 μg/mL           | 100 ng/mL             |
| Chir99021     | DMSO           | 10 mM               | 2 μM                  |
| Pt-103        | water          | 50 μM               | 50 nM                 |
| LDN 1931189   | DMSO           | 250 μM              | 250 nM                |
| VEGF-165      | water          | 100 μg/mL           | 100 ng/mL             |
| BMP4          | 5 mM HCl pH3, 0.1% BSA in DPBS | 100 μg/mL | 100 ng/mL |
| FGF2          | water          | 20 μg/mL            | 20 ng/mL              |
| N-2 supplement| n/a            | 100×                 | 1%                    |
| B-27 supplement| n/a           | 50×                  | 2%                    |
| SB431542      | DMSO           | 10 mM               | 10 μM                 |
| Dorosomorphin | DMSO           | 2 mM                | 1.2 μM                |

Store reagents as stated on the Certificate of Analysis from the manufacturer.
Here, we provide a general procedure for thawing and reviving hPSC lines onto a 6-well tissue culture-treated polystyrene microplate for culturing stock hPSCs for further differentiation.

1. Prepare a 6-well tissue culture-treated polystyrene microplate coated with Corning Matrigel®/C226 basement membrane matrix (as described above in coating plates with Matrigel®).
2. Obtain a frozen cryotube of hPSCs stored in liquid nitrogen and gently swirl the sealed vial in a 37 °C water bath until frozen liquid contents are almost completely melted.
3. In a sterile tissue culture hood, carefully transfer vial contents using a 5-mL serological pipette into a 15-mL conical tube containing 5 mL of 37 °C DMEM/F-12 media. Do not pipette up and down vigorously because cell colonies will dissociate.
4. Centrifuge cells at 300 × g at 20 °C for 5 min.
5. Aspirate the media off the coated 6-well tissue culture-treated polystyrene microplate.
6. Aspirate the supernatant and resuspend the cell pellet with 6 mL, 37 °C mTeSR(+) by gently pipetting up and down 2–3 times with a 10 mL serological pipette.
7. Pipette 1 mL/well of cell suspension into the 6-well tissue culture-treated polystyrene microplate. Make sure hPSC colonies are evenly distributed by shaking the plate in a “+” configuration.
   a. Then, incubate the microplate at 37 °C in a 5% CO2 tissue culture incubator without disturbing for 24 h.
   b. As an option to increase survival post-thawing, 10 μM Rho-associated protein kinase (ROCK) inhibitor, Y-27632 can be added.

△ CRITICAL: Avoid over-pipetting the resuspension to avoid single celling colonies and inducing apoptosis. Colonies in suspension should look no smaller than ~8–10-cell clusters under the microscope.

Maintaining hPSCs

© Timing: 30 min

This step describes daily maintenance of hPSCs in culture.

During primed hPSC maintenance and expansion, it is vital to assess and sustain the quality of cells and colonies until the time of seeding for differentiation experiments. Visible changes in hPSC colony morphology will be noticeable. Undifferentiated hPSC cells are small, tightly packed cells with a high nucleus/cytoplasm ratio. Differentiated cells must be removed to reduce unwanted genetic aberrations or loss of pluripotency due to poor cell quality and spontaneous differentiation (Garitaonnandia et al., 2015).

There are variations in the morphological appearance of hPSC colonies that a user may encounter, with the experience of the user helping to identify acceptable or unacceptable appearance variations. As an example, Figure 1 brightfield images illustrate different morphologies of high- and low-quality primed hPSC colonies. Desirable primed hPSC features include relatively uniform appearing colonies with defined borders, rounded shapes, and dense colony centers (Figure 1A). By contrast, undesirable primed hPSC colony features include non-uniform shapes and borders between colonies, especially with modest to large colony areas of apparent spontaneous differentiation showing increasingly ‘fibroblast-like’ shaped cells (Figure 1B). Note that culling may not be

| Table 3. Endoderm differentiation cytokines for Day 0 |
|------------------------------------------------------|
| Reagent     | Stock concentration | Final concentration | Per 1 mL EN media |
| Activin A   | 100 μg/mL            | 100 ng/mL           | 1 μL              |
| CHIR99021   | 10 mM                | 2 μM                | 0.2 μL            |
| PI-103      | 50 μM                | 50 nM               | 1 μL              |
necessary when differentiation involves <5% of cells and colonies within a well, but spontaneous differentiation regions should not exceed 20% of the well when the stock culture is high quality. It is best to revive a new cell stock when there are high levels of low-quality colonies.

It is recommended that a user visually inspects growing primed hPSC colonies every day to mark and remove low quality colonies or regions (Figure 1C). Use brightfield microscopy at low magnification (either 4× or 10×) to identify and mark unacceptable hPSC colonies using a microscope lens cell dotting marker, such as this, to stamp the bottom of each well. Remove selected regions by gently aspirating the marked colonies.

**Note:** Be sure to wear gloves and wipe down benchtop surfaces with 70% ethanol before beginning daily maintenance. Maintain culture sterility during visual inspection by keeping the microplate lid on at all times when working outside a sterile tissue culture hood. Do not leave the culture plate out of the incubator for more than 15 min at a time during inspection.

8. Aspirate spent media from each well of the 6-well plate and replace daily with 2 mL 37°C mTeSR(+).

9. Repeat daily maintenance until hPSCs are ready to seed for differentiation experiments.

**Passaging hPSCs**

© Timing: 30 min

Here, we provide Instructions for passaging hPSCs colonies, an essential step for hPSC maintenance and differentiation. Colonies are ready to passage or optimal for seeding differentiation experiments (as described in step-by-step method details) once they reach 70%–80% confluency. Below, we detail passaging of a single well from a 6-well hPSC culture plate onto a new 6-well tissue culture-treated polystyrene microplate for continued hPSC stock maintenance for subsequent differentiation seeding.

10. Prepare a 6-well tissue culture-treated polystyrene microplate coated with Corning Matrigel® basement membrane matrix (as described above in coating plates with Matrigel®).
11. Identify and remove regions of differentiation using aspiration (as described in Figure 1C) in the stock 6-well hPSC culture plate before harvesting.
12. Remove the media from one well of the 6-well hPSC culture plate by aspiration.
13. Wash hPSCs gently without disrupting colonies with 1 mL 1× Dulbecco’s phosphate-buffered saline (DPBS) without calcium or magnesium. Aspirate to remove the 1× DPBS.
14. Add 1 mL of Gentle Cell Dissociation Reagent to hPSC colonies and incubate at 37°C in a 5% CO₂ tissue culture incubator for 5–7 min. Aspirate to remove the Gentle Cell Dissociation Reagent.

**Note:** The Gentle Cell Dissociation Reagent incubation time depends on when the cells are dissociated from each other but are still attached to the plates. Use brightfield microscopy at low magnification (either 4× or 10×) to monitor cell dissociation.

15. Add 1 mL of 37°C mTeSR(+) to hPSC colonies.
16. Using a cell scraper, gently scrape colonies and cells off the bottom of the 6-well culture plate.
17. Aspirate the media off the coated 6-well tissue culture-treated polystyrene microplate.
18. Carefully transfer scraped colonies using a 10-mL serological pipette into a 15-mL conical tube containing 5 mL 37°C mTeSR(+).
   a. Gently pipette up and down 2–3× with a 10-mL serological pipette to mix the colonies in suspension.
19. Pipette 1 mL/well of cell suspension into the 6-well tissue culture-treated polystyrene microplate. Make sure that hPSC colonies are evenly distributed by shaking the plate in a “+” configuration. Then, incubate the microplate at 37°C in a 5% CO₂ tissue culture incubator without disturbing for 24 h.

**Note:** The steps provided for Passaging hPSCs are similar to those described in thawing hPSCs for culture with some modifications. Like Thawing hPSCs for culture, the user should avoid over-pipetting the cell suspension to avoid single celling colonies and inducing apoptosis. Colonies in suspension should look no smaller than ~8–10-cell clusters under the microscope.

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| 1-thioglycerol      | MilliporeSigma | Cat#M6145   |
| 2-mercaptoethanol   | Thermo Fisher Scientific | Cat#21985-023 |
| B27 supplement      | Gibco  | Cat#17504044 |
| BSA Fraction V, 7.5%| Gibco  | Cat#15260037 |
| Cell Culture Grade Water | Fisher Scientific | Cat# MT25055Cl |
| Chemically defined lipid concentrate (CDLC) | Gibco | Cat#11905031 |
| CHIR99021 (GSK3i)  | Stemcell Technologies | Cat#72052 |
| Dimethyl Sulfoxide (DMSO) | Fisher Scientific | Cat# MT-25950CQC |
| DMEM/F12            | Gibco  | Cat#11320033 |
| DMEM/F12, no Glc    | Gibco  | Cat#21331020 |
| Dorsomorphin        | Stemgent | Cat#04-0024 |
| DPBS, 1×, without calcium or magnesium | n/a | n/a |
| Gentle Cell Dissociation Reagent | Stemcell Technologies | Cat#07174 |
| GlutaMAX supplement | Thermo Fisher | Cat#35050-061 |
| Human recombinant Activin A | Stemcell Technologies | Cat#78001.1 |
| Human recombinant BMP-4 | PeproTech | Cat#120-05ET |
| Human recombinant FGF2 | Stemcell Technologies | Cat#78003 |
| Human recombinant insulin | MilliporeSigma | Cat#11376497001 |
| Human recombinant VEGF-165 | Stemcell Technologies | Cat#78073.1 |

(Continued on next page)
MATERIALS AND EQUIPMENT

All cell culture and material preparation must be performed under sterile conditions. When preparing media and reagents, we suggest the use of sterile cell culture materials, such as sterile centrifuge tubes, pipette tips, serological pipettes, and solvents. Sterilization using a 0.22-micron polyethersulfone (PES) membrane filter is also an option. Aliquot and pre-warm media to 37°C before usage. Excess warmed media should be discarded and not be re-stored at 4°C and re-warmed for usage due to reduced protein efficacy of media components essential for maintaining cell viability and proliferation.

Critical commercial assays
MycoAlert detection kit Lonza Cat#LT08-418

Experimental models: Cell lines
H9 (WA09); Human, Blastocyst stage, Female UCLA BSCRC hESC core bank RRID: CVCL_9773

Other
Cell scrapers Fisher Scientific Cat#08-100-241
Hemocytometer Fisher Scientific Cat#02-671-5
Polystyrene microplates Falcon Cat#087721
Q Exactive (MS) Thermo Scientific n/a
Sterile vacuum filtration system Fisher Scientific Cat#0974101
UltiMate 3000 RSLC (HPLC) Thermo Scientific n/a

mTeSR™ Plus (mTeSR(+)) media – commercially available kit

| Reagent                        | Final concentration | Amount  |
|--------------------------------|---------------------|---------|
| mTeSR™ Plus Basal Medium       | n/a                 | 400 mL  |
| mTeSR™ Plus 5X Supplement      | 1 x                 | 100 mL  |
| **Total**                      | n/a                 | **500 mL** |

Store at 4°C for up to 2 weeks.

**Note:** Visit Stemcell Technologies™ mTeSR™ Plus information page (here) for official guidelines on storage and handling of mTeSR™ Plus kit reagents and preparation and usage of complete mTeSR™ Plus.

**Note:** We store working aliquots at –20°C, except for transferrin, which we store at 4°C. We recommend making working aliquots of each reagent solution to avoid multiple freeze thaw cycles. Working aliquot amounts are based on the user’s experimental necessity.

**Base Media – mesoderm or endoderm (ME or EN) differentiation**

| Reagent        | Final concentration | Amount  |
|----------------|---------------------|---------|
| DMEM/F12       | n/a                 | 96.5 mL |
| 1-thioglycerol | 450 μM              | 3.9 μL  |

(Continued on next page)
STEP-BY-STEP METHOD DETAILS

The above section describes best practices for maintaining and expanding healthy primed hPSC colonies using visual inspection and culling, as needed, until the time of seeding for differentiation experiments. The following step-by-step method details the directed differentiation of primed hPSCs into each of the three primary germ lineages using chemically defined nutrient-balanced media formulations (Figure 2).

### Continued

| Reagent                      | Final concentration | Amount    |
|------------------------------|---------------------|-----------|
| BSA                          | 1 mg/mL             | 1.33 mL   |
| 2-mercaptoethanol            | 0.11 μM             | 182 μL    |
| GlutaMAX supplement          | 1%                  | 1 mL      |
| Insulin                      | 0.7 μg/mL           | 7 μL      |
| Transferrin                  | 15 μg/mL            | 50 μL     |
| Chemically defined lipid concentrate | 1 mL/100 mL | 1 mL     |
| **Total**                    | n/a                 | 100 mL    |

Store at 4°C for up to 2 weeks.

### Base Media – ectoderm (EC) differentiation

| Reagent                      | Final concentration | Amount    |
|------------------------------|---------------------|-----------|
| DMEM/F12                     | n/a                 | 97.5 mL   |
| 1-thioglycerol               | 450 μM              | 3.9 μL    |
| BSA                          | 1 mg/mL             | 1.33 mL   |
| 2-mercaptoethanol            | 0.11 μM             | 182 μL    |
| GlutaMAX supplement          | 1%                  | 1 mL      |
| **Total**                    | n/a                 | 100 mL    |

Store at 4°C for up to 2 weeks.

### Gln-free Base Media – mesoderm or endoderm (ME or EN) differentiation

| Reagent                      | Final concentration | Amount    |
|------------------------------|---------------------|-----------|
| DMEM/F12, no Gln             | n/a                 | 97.5 mL   |
| 1-thioglycerol               | 450 μM              | 3.9 μL    |
| BSA                          | 1 mg/mL             | 1.33 mL   |
| 2-mercaptoethanol            | 0.11 μM             | 182 μL    |
| Insulin                      | 0.7 μg/mL           | 7 μL      |
| Transferrin                  | 15 μg/mL            | 50 μL     |
| Chemically defined lipid concentrate | 1 mL/100 mL | 1 mL     |
| **Total**                    | n/a                 | 100 mL    |

Store at 4°C for up to 2 weeks.

### Gln-free Base Media – ectoderm (EC) differentiation

| Reagent                      | Final concentration | Amount    |
|------------------------------|---------------------|-----------|
| DMEM/F12, no Gln             | n/a                 | 98.5 mL   |
| 1-thioglycerol               | 450 μM              | 3.9 μL    |
| BSA                          | 1 mg/mL             | 1.33 mL   |
| 2-mercaptoethanol            | 0.11 μM             | 182 μL    |
| **Total**                    | n/a                 | 100 mL    |

Store at 4°C for up to 2 weeks.
CRITICAL: No antibiotics are present or used in hPSC culture medium. All steps must be performed under sterile conditions. Take extra caution in all steps by using sterile reagents and filtration. If any cloudiness or opaqueness appear in culture media, or when colonies are dying in suspension, throw the stock away and start with new cells, as contamination is likely to have occurred.

Seeding hPSCs for differentiation

Timing: 1–1.5 h, two days prior to differentiation

This section covers harvesting and single cell seeding of primed hPSCs for directed differentiation experiments. Typically, we discard hPSCs that have exceeded 10 passages post-thawing and start from another cryovial from a well-characterized working bank (Gu et al., 2016b; Hong et al., 2019; Kuilman et al., 2010). We encourage users to test cells for mycoplasma contamination using standard kits of choice, such as the Lonza MycoAlert Kit, prior to starting the differentiation procedure. Additionally, take care to visually inspect the spent culture media for any cloudiness or opaqueness, as this may indicate bacterial or fungal contamination. Users should routinely check stock hPSC cells for the presence of pluripotency and absence of differentiation biomarkers using flow cytometry or RT-qPCR to ensure starting cell populations are of high quality (Table 1). Growth and expansion of hPSC colonies to 70%–80% confluency, which yields \( \sim 2.0–3.0 \times 10^6 \) cells in the 6-well microplate format, is optimal for harvest and seeding of growing colonies at the start of the differentiation procedure. The protocol below provides detailed procedures for harvesting hPSCs from a 6-well tissue culture-treated polystyrene microplate and seeding into 12-well plates. These steps can be adapted to other plating formats by adjusting the numbers of cells or colonies and media volumes accordingly.

1. Prepare 12-well tissue culture-treated polystyrene microplates coated with Corning Matrigel® basement membrane matrix (as described in before you begin).
2. Identify and remove regions of differentiation using aspiration (as described in Figure 1C) in the stock 6-well hPSC culture plate before harvesting.
3. Remove the media from the 6-well hPSC culture plate by aspiration.
4. Wash hPSCs gently to not disrupt colonies with 1 mL 1 x DPBS. Aspirate to remove the 1 x DPBS.
5. Add 1 mL/well of Gentle Cell Dissociation Reagent to hPSC colonies and incubate at 37°C in a 5% CO₂ tissue culture incubator for 10 min.
6. Add 1 mL/well of 37°C DMEM/F-12 media to dilute out Gentle Cell Dissociation Reagent.
7. Use a cell scraper to gently scrape colonies and cells off the bottom of the 6-well culture plate and collect combined contents of the plate into a 50 mL conical tube.

Note: Ensure they are dissociated into single cells by pipetting against the surface of each well with a P1000 3 x before collecting into a 50 mL conical tube.

8. Count the number of hPSCs harvested for use with a hemocytometer.

Optional: Automated cell counters are acceptable, but we recommend validating automated counting machines using a hemocytometer.

9. Centrifuge the harvested hPSCs at 450 x g for 5 min.
10. Aspirate media gently to avoid disrupting the cell pellet.
11. Resuspend the hPSCs in 1 mL of 37°C mTeSR(+) containing 10 μM ROCK inhibitor, Y-27632.

△ CRITICAL: It is critical to add ROCK inhibitor, Y-27632, into the media because it prevents apoptosis induced by single cell dissociation of hPSC colonies. Otherwise, the survival of hPSCs post-seeding will be markedly reduced (Kurosawa, 2012).

12. Calculate the volume required to plate primed hPSCs in mTeSR(+) media with 10 μM Y-27632 at 6.5 x 10⁴ cells/cm² final concentration.
13. Aspirate the media off the coated 6-well tissue culture-treated polystyrene microplate.
14. Place 1 mL/well of hPSCs in mTeSR(+) media with 10 μM Y-27632 into Matrigel®-coated 12-well tissue culture-treated polystyrene microplates.

△ CRITICAL: Make sure that hPSCs are evenly distributed.

15. Incubate the seeded microplate at 37°C in a 5% CO₂ tissue culture incubator for 24 h.
16. 24 h post-plating (Figure 3A), gently wash the primed hPSCs with 1 mL 1 x DPBS without calcium or magnesium. Aspirate the 1 x DPBS gently.
17. Add 1 mL/well of 37°C mTeSR(+) media (without ROCK inhibitor).
18. Return the microplate to the 37°C, 5% CO₂ tissue culture incubator.
19. Allow the primed hPSCs to grow in mTeSR(+) media for 24 h before beginning the directed differentiation procedure.

Note: Expected morphology of single cells plated with Y-27632 will appear “spiky” and relatively flatter and larger than non-dissociated hPSCs in colonies.

△ CRITICAL: In the event that the primed hPSCs do not appear ~70%–80% confluent in their seeded wells (Figure 3B) on Day 0 (Figure 2), allow the hPSCs to continue growing in replaced fresh 1 mL mTeSR(+) media for an additional 24 h prior to initiating the directed differentiation procedure. In case of over-confluence (>85%), repeat the seeding as efficacy of differentiation will be decreased.

Making chemically defined, nutrient-balanced media for tri-lineage differentiation

△ Timing: 1 h

This section details the preparation of media for chemically defined, nutrient-balanced directed differentiation of primed hPSCs into ectoderm, mesoderm, and endoderm lineages. Of note, the
protocol endpoint for endoderm is three days (72 h) post-start of differentiation, whereas the endpoint for mesoderm and ectoderm differentiation is five days (120 h) post-start of differentiation (Figure 2). Additionally, be sure to exclude insulin, transferrin, and chemically defined lipid concentrate from the base media for ectoderm differentiated cells because B-27 and N2 supplements already contain these factors. The Base Media Preparation Tables provided below note this difference. These cytokine combinations have been optimized and adapted from previous studies (Chambers et al., 2009; Loh et al., 2014, 2016). Here, we also include formulation instructions for Gln-free medium as an example; however, this protocol for chemically defined, nutrient-balanced media is non-limiting, meaning that it can be adjusted for any nutrient deprivation study.

20. Make mesoderm/endoderm or ectoderm base media according to the provided recipes for lineage differentiation of choice in materials and equipment.
   a. A range of experimental manipulations can be made by modifying DMEM/F12 nutrient composition. Additionally, we also provide an example for Gln-free base media in materials and equipment. In this instance, DMEM/F12 no Gln is used and GlutaMAX was excluded.
Note: We recommend first sterile filtering base media and then adding sterile cytokines to generate differentiation media because lineage-inducing cytokines could stick to the filter membrane, affecting their final concentrations, and causing undesirable reduction or variability in hPSC differentiation within or between experiments.

Optional: A substitute for GlutaMAX is L-Gln. However, L-Gln is not stable at 4°C and is therefore not recommended.

21. Sterilize each final media preparation using a 0.22-micron polyethersulfone (PES) membrane filter.

Pause point: At this step, base media preparations can be stored at 4°C for use within two weeks.

△ CRITICAL: Sterile, non-expired cytokines that have not undergone multiple freeze-thaw cycles should be added immediately prior to the start of each experiment to ensure efficacy.

22. At the start of differentiation (D0) (Figure 2), add sterile lineage-inducing cytokines to each respective base media for each desired indication (Tables 3, 4, 5, 6, and 7).
   a. Below, we provide tables to guide the addition of cytokines to the different types of base media for lineage-specific differentiations.
   b. Dilute stock cytokine solutions using sterile techniques and store according to the manufacturers’ instructions (varies).

△ CRITICAL: Prepare aliquots of stock cytokine solutions to extend storage life and avoid repeated freeze-thaw cycles that can reduce potency.

Note: Be careful because each day of differentiation may require the addition of different cytokine combinations to enhance the success of this protocol. See specific schedules in the tables, below.

Directed differentiation of hPSCs to primary germ lineages

© Timing: 30 min–1 h per day

This section provides instructions for the execution of hPSC directed differentiation into each of the three germ lineages, starting with the transition of mTeSR(+) hPSC maintenance cultures to lineage-specific differentiation media.

| Table 4. Endoderm differentiation cytokines for Days 1–3 |
|-----------------------------------------------|
| Reagent | Stock concentration | Final concentration | Per 1 mL EN media |
|----------|----------------------|----------------------|------------------|
| Activin A | 100 µg/mL | 100 ng/mL | 1 µL |
| LDN 1931189 | 250 µM | 250 nM | 1 µL |

Table 5. Mesoderm differentiation cytokines for Day 0

| Reagent | Stock concentration | Final concentration | Per 1 mL ME media |
|----------|----------------------|----------------------|------------------|
| VEGF-165 | 100 µg/mL | 100 ng/mL | 1 µL |
| BMP4 | 100 µg/mL | 100 ng/mL | 1 µL |
| FGF2 | 20 µg/mL | 20 ng/mL | 1 µL |
| Activin A | 100 µg/mL | 100 ng/mL | 1 µL |
23. Remove spent mTeSR(+) maintenance media from wells and wash the hPSC colonies gently with 20°C 1× DPBS w/o calcium & magnesium.

24. Add 1 mL/well (~263 µL/cm²) of 37°C appropriate differentiation media.

25. Incubate cells at 37°C in a 5% CO₂ incubator for 24 h.

26. Every 24 h, repeat gentle washing and media changes according to the cytokine schedule above (Tables 3, 4, 5, 6, and 7) until hPSCs have committed to a directed lineage (Table 1, Figures 2 and 4).

27. Harvest cells according to planned downstream applications and analyses.

**EXPECTED OUTCOMES**

There is growing, multidisciplinary appreciation for the role of nutrients in governing developmental cell fate decisions (Lu et al., 2021). An increased understanding for how specific nutrients that are present in certain niches in vivo, and our ability to alter these nutrients in hPSC culture media, provides a valuable experimental tool to yield new insights into the regulation of early cell fate transitions, potentially within a developing human embryo. This protocol provides an accessible and customizable way to uncover the influence of nutrients in in vitro culture, with the following comments providing details of the expected outcomes of implementing this protocol. To confirm equivalent amounts of nutrients in each lineage differentiation media, we used ultra high-performance liquid chromatography mass spectrometry (UHPLC-MS) to quantify carbon sources and amino acids present in freshly prepared, unspent media. Results indicate that unspent media for induction of each lineage consisted of equivalent levels of metabolites (Figure 5). This is the expected outcome when directed differentiation media is prepared properly since the base media composition and cytokine cocktails net out to the same concentration of nutrients. Successful, directed hPSC differentiation into each specific germ lineage was also verified using RNA-Seq transcriptome profiling, qRT-PCR analysis (Figure 6), and lineage-specifying biomarker protein expression by flow cytometry and immunoblot analyses (Table 1) (Lu et al., 2019, 2022).

**Proof-of-concept example**

Using our chemically defined, nutrient-balanced differentiation media, our group recently showed that there is an essential need for Gln during germ lineage differentiation, with each lineage having its own unique Gln dependency (Lu et al., 2019). Specifically, we uncovered that initial ectoderm is independent of extracellular Gln (Lu et al., 2022). Directed tri-lineage differentiation with and without Gln in the media revealed varying reliance for this nutrient in the formation and viability of hPSCs undergoing cell fate transitions (Figure 7). For the full execution and proof-of-concept for this protocol that examined Gln availability during hPSC differentiation, please refer to Lu et al. (2022).

**LIMITATIONS**

Using this protocol, chemically defined, nutrient-equivalent lineage directed differentiation of primed hPSCs is limited to feeder-free systems that use Matrigel® matrix, gelatin, or other solid...
substrates. Studies comparing feeder-free and feeder-dependent systems reported differences in hPSC metabolic characteristics and differentiation potential (Gu et al., 2016a; Zhang et al., 2016), which guided our initial decision to optimize this protocol in feeder-free hPSC culture. Other factors that limit the translational impact of this protocol include: (1) shifting nutrient concentrations that exclude one nutrient or another almost certainly do not occur in vivo during development and (2) nutrient concentration levels used in this in vitro culture system may be supra-physiologic and likely do not resemble in vivo levels.

**TROUBLESHOOTING**

**Problem 1**
Stock or seeded hPSCs exhibit a high amount of cell death and/or release from culture plate surface as flakes or whole colonies (before you begin steps 8 and 9; step-by-step method details step 16).

**Potential solution**
This could be from several issues related to the sterility of the cell culture. Note that this protocol does not utilize antibiotics, so extra precaution is required. In addition, it is advisable to routinely examine the stock hPSC culture for mycoplasma infection by testing spent media removed from plate wells with 70%–80% confluent hPSC growth, followed by mycoplasma testing using commercially available kits, such as the Lonza MycoAlert Kit. Additionally, visual checks for clarity or cloudiness of spent media could suggest or exclude bacterial, fungal or yeast contamination as a potential source for this problem. Another reason for high death could be exclusion of ROCK inhibitor to single cell suspensions during the hPSC seeding step, which will induce apoptosis due to single cell dissociation.

**Problem 2**
Stock, seeded, or differentiating cells are flaking off into suspension during media changes and/or during DPBS washes (before you begin steps 8 and 9; step-by-step method details steps 16, 17, 24, 25, and 27).

**Potential solution**
Primed hPSC colonies are sensitive to physical dislodging from a plate surface. When performing daily culture media changes, gently pipette the media to the wall of the well to avoid direct interaction of the media stream with the colonies. Additionally, when aspirating media from wells, take care

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### Figure 4. Morphologies of primed hPSCs differentiate into specific embryonic germ lineages
Representative brightfield images of directed germ lineage differentiation. Expected morphologies of differentiated hPSCs at the end of this protocol: ectoderm and mesoderm after five days and endoderm after three days. Scale bar: 400 μM.
not to directly touch the bottom of the well with the aspirator tip, as doing so may disrupt the Matrigel® coating and/or aspirate off colonies.

Problem 3
Cells are not differentiating properly (step-by-step method details step 27), as verified by morphological (Figure 4) or biomarker assessments (Table 1).

Potential solution
A problem could be the potency of small molecules or cytokines used to induce differentiation. Ensure that cytokines are not used beyond their expiration date after aliquoting and fresh dilution before each addition to base media. For example, failed mesoderm differentiation could be from multiple freeze-thaw cycles, or old and expired, low potency BMP4.
Problem 4
There is high variability of differentiation efficiencies between biological replicates (step-by-step method details step 27).

Potential solution
It is typical for different hPSC lines to vary in functional differentiation capacities (Cahan and Daley, 2013). However, when an experimentalist is experiencing high differentiation variability between replicate studies of the same hPSC line, several issues may be at fault. First, ensure primed hPSC seeding is consistent and confluence at the start of differentiation (Day 0) is 70%–80% in each plate well. When the starting hPSC confluence is too low, differentiating cells may experience high cell death rates. When the starting hPSC confluence is too high, differentiation may be impaired because amount of available cytokine of interest is insufficient. Second, it is crucial to ensure all cytokines, reagents, and base media components are presumably active (non-expired, not continuously freeze-thawed, correct storage conditions, non-contaminated). In our experience, it may be helpful to contact technical support to inquire whether other users are having similar issues with a particular lot. Third, check the genomic integrity and stability of the hPSC stock cells to ensure the starting cells are high quality. Genetic abnormalities can be detected using qPCR analysis with products such as this hPSC Genetic Analysis Kit.

Problem 5
Low yield of germ lineage cells at the end of differentiation (step-by-step method details step 27).

Potential solution
The seeding volume provided in seeding hPSCs for differentiation is a recommended, optimized plating density of $6.5 \times 10^4$ cells/cm$^2$ that will provide committed germ lineage cells at the end.
of this protocol. However, an experimentalist may run into the issue of low yield of a specific germ lineage, especially with mesoderm where hPSCs have a slightly higher percentage of dead cells when inducing differentiation. A user can either increase the initial seeding density from a working range of $6.5 \times 10^4$ cells/cm$^2$ up to $7.8 \times 10^4$ cells/cm$^2$ or starting the differentiation (Day 0) when hPSCs are at 75%–80%.

**RESOURCE AVAILABILITY**

**Lead contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Michael A. Teitell (mteitell@mednet.ucla.edu).

**Materials availability**

This study did not generate new unique reagents.

**Data and code availability**

This protocol did not generate new datasets or code.
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AUTHOR CONTRIBUTIONS

V.L., M.T.D., and M.A.T. conceptualized and wrote the manuscript. I.J.R. and A.T. assisted in developing the protocol.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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