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# Generating Blood-Generating Heart-Forming Organoids from human pluripotent stem cells

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Protocol for generating Bl…

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**Protocol status:** Working
**We use this protocol and it's working**

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**Keywords:** Organoids, Cardiogenesis, Embryo development, Hematopoiesis, Hematopoietic progenitor cells, Human pluripotent stem cells

## Abstract

Our established human pluripotent stem cell (hPSC)-derived heart-forming organoids (HFOs) recapitulate aspects of heart, vasculature, and foregut co-development. Modulating HFO differentiation, we here report the protocol for generating blood-generating (BG)-HFOs; while maintaining a functional ventricular-like heart anlagen, BG-HFOs comprise a mesenchyme embedded hemogenic endothelial layer encompassing multiple hematopoietic derivatives and hematopoietic progenitor cells with erythro-myeloid and lymphoid potential, reflecting aspects of primitive and definitive hematopoiesis. The model enables the morphologically structured co-development of cardiac, endothelial, and multipotent-hematopoietic tissues equivalent to the intra-embryonic hematopoietic region in vivo, promoting research on hematopoiesis in vitro.

## Guidelines

Our research complies with all relevant ethical regulations; experiments using hESCs lines were performed under allowance ‘108 Genehmigung nach dem Stammzellgesetz’; granted by the Robert Koch Institute.

## Materials

**Materials:**

*synthesized by the Institute for Organic Chemistry (Leibniz University Hannover)

| MATERIAL | COMPANY |
|---|---|
| Accutase | Thermo Fisher Scientific, cat. no. A11105-01 |
| Ascorbic acid 2-phosphate | Sigma-Aldrich, cat. no. A8960 |
| B-27 supplement (minus insulin) | Thermo Fisher Scientific, cat. no. A1895601 |
| B-27 supplement (plus insulin) | Thermo Fisher Scientific, cat. no. 17504-044 |
| Basic fibroblast growth factor (bFGF) | PeproTech, cat. no. AF-100-18B |
| Bone morphogenetic protein 4 (BMP4) | PeproTech, cat. no. 120-05 HeLa |
| CHIR99021 (CHIR) | * |
| DMEM/F-12 medium | Thermo Fisher Scientific, cat. no. 11330-057 |
| Erythropoietin (EPO) | PeproTech, cat. no. 100-64 |
| Fms related receptor tyrosine kinase-3 (FLT-3) | PeproTech, cat. no. 300-19 |
| Geltrex LDEV-Free hESC-qualified Reduced Growth Factor Basement Membrane Matrix | Thermo Fisher Scientific, cat. no. A1413202 |
| Insulin | Sigma-Aldrich, cat. no. I9278 |
| Insulin-like growth factor 1 (IGF-1) | PeproTech, cat. no. 100-11 |
| (Interleukin-3) IL-3 | PeproTech, cat. no. 200-03 |
| (Interleukin-6) IL-6 | PeproTech, cat. no. 200-06 |
| (Interleukin-11) IL-11 | PeproTech, cat. no. 200-11 |
| (Inhibitor of WNT production 2) IWP2 | Tocris, cat. no. 3533 |
| Matrigel | Corning, cat. no. 354234; tested lots: no. 100007, 1335001, 1336002, 1013001, 1335001, 1336002, 2033002, 2237001 |
| Phosphate-buffered saline (PBS) (Ca2+/Mg2+-free; 10x) | Thermo Fisher Scientific, cat. no. 70011051 |
| Penicillin/ Streptomycin (P/S) | Sigma-Aldrich, cat. no. P0781 |
| Rho kinase inhibitor Y-27632 | Tocris Bioscience, cat. no. 1254, or * |
| RPMI/1640 medium | Thermo Fisher Scientific, cat. no. 21875-091 |
| Sonic Hedgehog (SHH) | PeproTech, cat. no. 100-45 |
| Sodium bicarbonate ($NaHCO_3$) | Sigma-Aldrich, cat. no. S5761 |
| Sodium selenite ($Na_2SeO_3$) | Sigma-Aldrich, cat. no. S5261 |
| Stem Cell Factor (SCF) | PeproTech, cat. no. 300-07 |
| STEMdiff Cardiomyocyte Dissociation Kit | Stemcell Technologies, cat. no. 05025 |
| Tissue-Tek | Sakura Finetek, cat. no. 4583 |
| (Transforming growth factor β) TGFβ | PeproTech, cat. no.100-21C |
| Thrombopoietin (TPO) | PeproTech, cat. no. 300-18 |
| Transferrin | Sigma-Aldrich, cat. no. T3705 |
| Vascular endothelial growth factor (VEGF) | PeproTech, cat. no. 100-20 |
| Trypan blue solution | Sigma-Aldrich, cat. no. T8154 |

- Accutase **Gibco - Thermo Fischer Catalog #** A1110501
- L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A8960
- B-27™ Supplement, minus insulin **Gibco - Thermo Fisher Catalog #**A1895601
- B-27 Supplement **Gibco - Thermo Fischer Catalog #**17504044
- Animal-Free Recombinant Human FGF-basic **peprotech Catalog #**AF-100-18B
- Recombinant Human BMP-4 **peprotech Catalog #**120-05
- DMEM/F-12, HEPES **Thermo Fisher Catalog #**11330057
- Recombinant Human EPO **peprotech Catalog #**100-64
- Recombinant Human Flt3-Ligand **peprotech Catalog #**300-19
- Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix **Thermo Fisher Catalog #**A1413202
- Insulin solution human **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**I9278
- Recombinant Human IGF-I **peprotech Catalog #**100-11
- Recombinant Human IL-3 **peprotech Catalog #**200-03
- Recombinant Human IL-6 **peprotech Catalog #**200-06
- Recombinant Human IL-11 **peprotech Catalog #**200-11
- IWP 2 **Tocris Catalog #**3533
- Corning® Matrigel® Basement Membrane Matrix, LDEV-free, 10 mL **Corning Catalog #**354234
- PBS (10X), pH 7.4 **Thermo Fisher Scientific Catalog #**70011051
- Penicillin-Streptomycin **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P0781
- Y-27632 dihydrochloride **Tocris Catalog #**1254
- RPMI 1640 Medium **Thermo Fisher Catalog #**21875091
- Recombinant Human Sonic Hedgehog **peprotech Catalog #**100-45
- Sodium bicarbonate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S5761
- Sodium selenite **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S5261
- Recombinant Human SCF **peprotech Catalog #**300-07
- STEMdiff™ Cardiomyocyte Dissociation Kit **STEMCELL Technologies Inc. Catalog #**05025
- SDS Tissue-Tek® **Sakura Finetek Catalog #**4583
- Recombinant Human TGF-β1 (CHO derived) **peprotech Catalog #**100-21C
- Recombinant Human TPO **peprotech Catalog #**300-18

Transferrin human **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T3705

Recombinant Human VEGF **peprotech Catalog #**100-20

Trypan Blue solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T8154

**Culture Media:**

| Medium/buffer | Composition |
|---|---|
| Essential 8 (E8) medium | DMEM/F-12, HEPES-supplemented with<br>64 mg/L ascorbic acid 2-phosphate<br>100 µg/L bFGF<br>20 mg/L insulin<br>543 mg/L NaHCO3<br>14 µg/L Na2SeO3<br>10.7 mg/L transferrin<br>2 µg/L TGFβ<br>P/S 1:100 |
| RB- | RPMI/1640<br>2% B-27 supplement (minus insulin)<br>P/S 1:100 |
| RB+ | RPMI/1640<br>2% B-27 supplement (plus insulin)<br>P/S 1:100 |

**Equipment:**

| EQUIPMENT | COMPANY, CATALOG NUMBER |
|---|---|
| Conical tubes, 15 mL | Greiner Bio-One, cat. no. 188271 |
| T25 flasks (TPP, cat. no. 90026) | TPP, cat. no. 90026 |
| U-bottom ultra-low attachment 96-well plates | Nunclon Sphera, cat. no. 174925<br>FaCellitate cat. no. 202003 |
| Hemocytometer, Neubauer improved | Brand, cat. no. 717805 |
| Pipets (2–20 µL, 20–200 µL, 100–1,000 µL) | Eppendorf |
| Sterile scissors (heated to at least 180 °C for at least 3 h) | |
| Centrifuge, Heraeus Multifuge X3R | Thermo Fisher Scientific |
| Humidified incubator (37 °C, 5% $CO_2$) | SANYO |
| Fluorescence microscope AXIO Observer A1 | Zeiss |
| Inverted microscope Olympus CKX41 | Olympus |

Conical tube, 15 ml **greiner bio-one Catalog #**188271

Tissue Culture Flasks **Techno Plastic Products (tpp) Catalog #**90026

Nunclon™ Sphera™ Microplates, 96U-Well Plate **Thermo Fisher Catalog #**174925

BRAND® counting chamber BLAUBRAND® Neubauer improved New without clips, double ruled **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**BR717805

## Pre-culture

1 Grow the human pluripotent stem cell (hPSC) lines of interest (in our case hES3 NKX2.5-eGFP[1, 2] and HSC_ADCF_SeV-iPS2[3]) on irradiated embryonic mouse fibroblasts in the incubator at 37 °C, 5%$CO_2$.

- At 80% colony confluence, passage the cells and seed either onto fresh irradiated fibroblasts or, to start organoid differentiation, transfer to Geltrex-coated T25 flasks in Essential 8 (E8) medium supplemented with [M] 10 micromolar (µM) Y-27632.

2 Passage the cells every 3-4 days and change the medium daily except the day immediately after the passaging.

## Generation of BG-HFOs

2d 0h 56m

3

**Note**

- The protocol for generation of BG-HFOs relies on the published protocol for generating Heart-Forming Organoids (HFOs)[4, 5]; to have specifics regarding the key protocol passages please refer to the cited manuscript[4].
- The HFO protocol adaptation in order to generate BG-HFOs consists of the addition of growth factors and cytokines at specific time-points during the differentiation, described as follows:

4 On the starting day (day minus 4; d-4), detach hPSCs from Geltrex-coated flasks using Accutase.

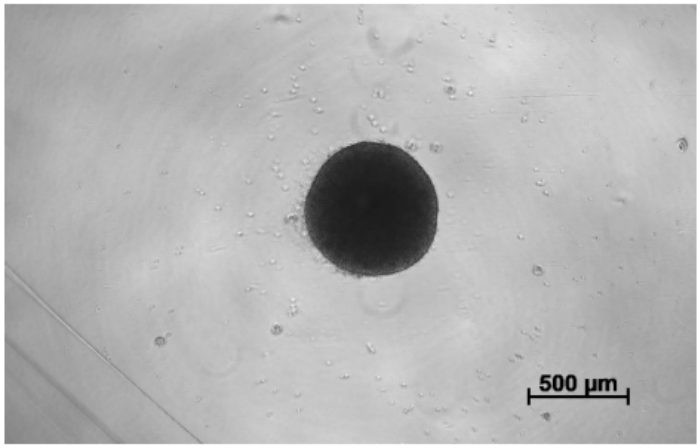
4.1 Count the cells using a hemocytometer and trypan blue solution; seed 5000 cells per well in a U-shaped ultralow-attachment 96-well plate in E8 medium supplemented with 10 µM Y-27632.

4.2 Centrifuge the plate is at 300 x g, 4°C, 00:06:00 and placed in the incubator to let one hPSC aggregate per well form Overnight.

6m

5 After 52 h (d−2), only the hPSC aggregates looking round and with a sharp edge will be further differentiated.

- Embedd the selected aggregates in a 20 µL Matrigel droplet. Embedding is performed seeding Matrigel droplets using a p200 pipet set to 20 µl, and in each droplet seed one single aggregate using a p20 pipet set to 3 µl.
- In order to maintain the integrity of the aggregates, the edge of the 20 µl plastic tip is enlarged by cutting it with a pair of sterilized scissors; for specifics please refer to the HFO protocol[4].




hES3 NKX2.5-eGFP-derived aggregate at d-2 of the protocol with round and sharp edge; such organoids are selected for subsequent Matrigel embedding.

6 After embedding, place the plate at 37 °C in the incubator for 00:50:00 to let the Matrigel solidify. 50m

- Afterwards, supplement E8 medium with 10 ng $ml^{-1}$ BMP4 is added on top of the Matrigel-embedded aggregates.
- Add 100 µL for each well. Further details are described in our previous publication[4].

7 After 2 days, on d0, replace E8 with 200 µL per well of RB− supplemented with 7.5 µM CHIR 10 ng $ml^{-1}$ BMP4, and bFGF 5 ng $ml^{-1}$.

- Remove the medium by using first a P1000 pipet to remove the majority of the medium and then a p200 pipet to remove the remaining medium.
- It is important to do not disturb the Matrigel droplet containing the aggregate during the media change, but gently moving it with the edge of the tip.
- Perform all the subsequent media changes following this criteria.

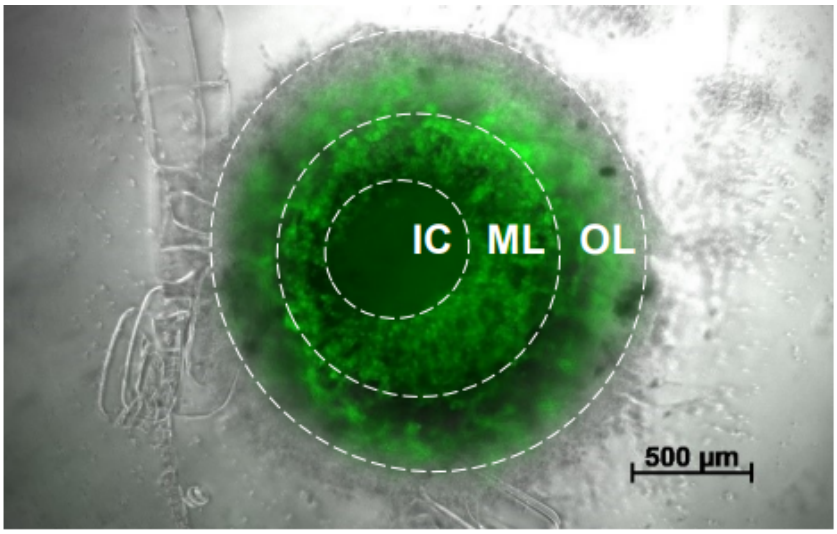
8 After 24 h, on d1, exchange the medium by RB− supplemented with 50 ng $ml^{-1}$ VEGF and 10 ng $ml^{-1}$ bFGF.

9 2 days afterwards, on d3, add RB− supplemented with [M] 5 micromolar (μM) IWP2, 50 ng $ml^{-1}$ VEGF, 10 ng $ml^{-1}$ bFGF, 100 ng $ml^{-1}$ SCF, 17 ng $ml^{-1}$ EPO, 10 ng $ml^{-1}$ IL-6, 10 ng $ml^{-1}$ IL-11 and 25 ng $ml^{-1}$ IGF-1, for 48:00:00 . 2d

10 After 2 days, on d5, exchange the medium by RB- supplemented with the same molecules of d3 plus 30 ng $ml^{-1}$ TPO, 10 ng $ml^{-1}$ FLT3, 30 ng $ml^{-1}$ IL-3, 10 ng $ml^{-1}$ BMP4, and 20 ng $ml^{-1}$ SHH.

11 2 days after, from d7 onwards, cultivate BG-HFOs in RB+ supplemented with the same cytokines added on d5.

12 After 3 days, on d10, complete BG-HFO differentiation; keep the organoids in culture for downstream analysis until d14.




hES3 NKX2.5-eGFP-derived BG-HFO at d10 of the differentiation. NKX2.5-eGFP, expressed in early cardiomyocytes, allows for the visualization of the organoid´s structure; an inner core (IC) NKX2.5-eGFP$^{neg}$, a myocardial layer (ML) NKX2.5-eGFP$^{pos}$, and an outer layer (OL) only partially positive for NKX2.5eGFP. Such organoids are used for downstream analyses.

## Protocol references

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