# Differentiation of cardiomyocytes and generation of human engineered heart tissue

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**Since the advent of the generation of human induced pluripotent stem cells (hiPSCs), numerous protocols have been developed to differentiate hiPSCs into cardiomyocytes and then subsequently assess their ability to recapitulate the properties of adult human cardiomyocytes. However, hiPSC-derived cardiomyocytes (hiPSC-CMs) are often assessed in single-cell assays. A shortcoming of these assays is the limited ability to characterize the physiological parameters of cardiomyocytes, such as contractile force, due to random orientations. This protocol describes the differentiation of cardiomyocytes from hiPSCs, which occurs within 14 d. After casting, cardiomyocytes undergo 3D assembly. This produces fibrin-based engineered heart tissues (EHTs)—in a strip format—that generate force under auxotonic stretch conditions. 10–15 d after casting, the EHTs can be used for contractility measurements. This protocol describes parallel expansion of hiPSCs; standardized generation of defined embryoid bodies, growth factor and small-molecule-based cardiac differentiation; and standardized generation of EHTs. To carry out the protocol, experience in advanced cell culture techniques is required.**

## INTRODUCTION

Human-pluripotent-stem-cell-derived cardiomyocytes are extraordinarily powerful tools within the field of cardiovascular research. Cardiomyocytes differentiated from hiPSCs via various protocols have been used to address questions related to cardiac toxicity and disease modeling[1,2]. The majority of these protocols are based on single-cell models. An important limitation of single-cell assays is their poor representation of the *in vivo* cardiovascular environment. Single cardiomyocytes lack important characteristics such as cellular networks, a 3D extracellular matrix and a defined auxotonic load. Furthermore, sarcomeric organization of myofilaments, a hallmark of mature functional cardiomyocytes, is primitively developed in single cardiomyocytes[3]. Evaluation of contractile force in single cells is therefore poorly established.

Engineered 3D heart tissues can improve these limitations. Several protocols for generating cardiac constructs from single cells have been established during the past two decades. These protocols mix single cells from neonatal animal hearts, or pluripotent-stem-cell-derived cardiomyocytes from mice or humans, with extracellular matrices such as fibrinogen, collagen or Matrigel. Depending on the specific protocol, different modes of tissue stretching (isometric/static and auxotonic) and contractile force analysis (force transducer, video–optical monitoring) are used. Progress in the field can be expected from improved protocols to differentiate cardiomyocytes with high efficiency at large scale and reasonable cost with suitable formats for generating EHTs at high levels of standardization. This protocol describes a step-by-step procedure for expanding undifferentiated hiPSCs, differentiating them into cardiomyocytes and generating fibrin-based EHTs in a 24-well plate format[4–6]. It includes detailed information regarding scalability and the optimal ratio of input cells to cell culture medium.

The scalability and efficiency of this differentiation protocol and the easy, reliable method of generating EHTs provide the opportunity to study physiological and pharmacological properties of human heart tissue *in vitro*. Using the protocol described here, it has been shown that EHTs with hiPSC-CMs have a high similarity to native human heart tissue, indicating that human EHTs are useful for predictive safety pharmacology and toxicology testing, as well as disease modeling[7]. Concerning the electrophysiological properties of hiPSC-CMs, it has been shown that they express L-type $Ca^{2+}$ current at the same density as human adult CMs and that the EHT culture format favors their maturation[8]. Furthermore, human EHTs formed large grafts after transplantation onto injured guinea pig hearts. They improved left ventricular and electrophysiological function and were able to electrically couple to host myocardium[9].

### Development of the protocol

Among the cell culture protocols currently in use for the expansion of undifferentiated hiPSCs, the largest differences are in the level of standardization and price[10–14]. We decided to optimize our protocol based on a cost-effective medium composition, in which pluripotency is driven by a combination of three growth factors (basic fibroblast growth factor (bFGF), transforming growth factor-β (TGFβ), activin A and the small-molecule bone morphogenetic protein (BMP) inhibitor dorsomorphin)[14]. bFGF, which is very important for maintaining hiPSC pluripotency[15–19], is both temperature and protease sensitive. To circumvent these problems, we replaced standard bFGF with an engineered bFGF variant with increased thermostability and higher resistance to proteases, while retaining the same biological properties as naturally occurring bFGF[20].

Several protocols for cardiac differentiation are available[21–25], but the inter- and intra-laboratory variability is high[26] (reviewed

in Moretti *et al.*[27]). We have optimized a previously published protocol based on embryoid body (EB) formation, as suspension-culture-based protocols are better suited to large-scale production. This is because, in contrast to 2D cultures, the number of cultivated cells is not limited to the growth surface, and the volume of culture medium can be adjusted accordingly. An important shortcoming of EB-based protocols is the difficulty involved in consistently producing homogeneous EBs. Spontaneous EB formation is difficult and sensitive to the confluency of the undifferentiated cells, and the EBs are often heterogeneous in size. EB formation by forced aggregation, on the other hand, does not, in our experience, depend on cell confluency, and it produces EBs of a defined size. However, microcavity plates are expensive, and scaling up this technique is difficult and very laborious. Conversely, spinner-flask-based protocols for EB formation require only dissociation of hiPSCs into single cells and subsequent culture of hiPSCs in stirred spinner flasks with glass bulb impellers. Such protocols have also been described for expansion of hiPSCs in suspension[28,29]. This method is, in our experience, very reliable and it provides EBs that are homogeneous in size, independent of cell line and confluency of the hiPSC culture. Furthermore, spinner-flask-based protocols have a high potential for scalability.

Mesoderm induction is achieved by the parallel addition of bFGF, BMP-4 and activin A in an RPMI-based medium composition. The small-molecule WNT-signaling activator CHIR99021 has also been shown to be an effective inducer of mesodermal progenitors[30–33]. As compared with protein growth factors, small molecules tend to be more consistent in quality and more cost-effective. Pilot experiments indicate that our protocol is compatible with CHIR99021 for mesoderm induction, leading to efficient cardiomyocyte differentiation from various stem cell lines. We chose to optimize our protocol for mesoderm induction with BMP4, activin A and bFGF, as this combination was previously shown to faithfully recapitulate mammalian embryonic development[23]. Given the complexity of embryonic cardiac development and the lack of an understanding of how to promote cardiomyocyte maturation, we hypothesized that a protocol that closely replicates the steps involved in mammalian mesoderm/cardiac specification represents the best possible starting point. Further cardiac specification is conducted with potent small-molecule WNT antagonists and subsequently with insulin[30,34]. Using this protocol, the onset of spontaneous beating is observed between days 8 and 10 (**Supplementary Videos 1–3**). WNT antagonists are omitted after day 11. hiPSC-CMs are dissociated into single cells and subjected to further analysis and EHT generation.

Several different techniques for the generation of EHTs are currently available[5,35–39]. They differ in the size of the construct, the mode of tissue anchoring, the choice of extracellular matrix and the techniques used to analyze the tissue. The fibrin-based, strip-format EHTs described in this protocol can be repeatedly generated with minimal manual handling in a 24-well format[4,5].

**Advantages and limitations of the method.** This protocol allows for the standardized production of hiPSC-CMs at both a scale and cost that are suitable for the generation of EHTs. In a successful differentiation run, $60 \times 10^6$ hiPSCs (four T80 flasks) on average give rise to $48 \times 10^6$ hiPSC–cardiomyocytes (70–97% Troponin T-positive; **Tables 1** and **2**, **Fig. 1** and **Supplementary Fig. 1**), which are enough cells to prepare two 24-well plates of EHTs (**Table 1**). The estimated cost associated with preparing one plate of EHTs is 180 , including costs incurred during hiPSC culture (for 4 d), cardiomyocyte differentiation, EHT generation and EHT cultivation (for 21 d).

The differentiation procedure described here is based on EB differentiation protocols, which were shown to recapitulate distinct stages of embryonic development and mesodermal germline commitment[23]. Compared with activating WNT signaling with the small-molecule CHIR99021, which has also been shown to be an effective inducer of mesodermal progenitors[30–33], there are disadvantages, including the limited stability of proteins, variability in their biologic activity and higher costs. However, although protein growth factors are more expensive than small molecules, the media compositions used in our protocol are cheaper overall as compared with those used in many of the published protocols using mTeSR, Stem Pro-34 or other media. For WNT signal inhibition during cardiac differentiation, an IWR-1 (4-(1,3,3a,4,7,7a-hexahydro-1,3-dioxo-4,7-methano-2H-isoindol-2-yl)-N-8-quinolinyl-benzamide) analog (DS-I-7) was used. This analog has been found to have a higher selectivity than IWP, as well as a higher potency and stability as compared with those of IWR-1 (ref. 34).

Contractile force is a very important parameter of cardiomyocyte function. Depolarization of a cardiomyocyte by an action potential leads to the entry of calcium into the cell through L-type

**TABLE 1** | Format, timing and concentrations for cardiomyocyte differentiation of hiPSCs.

| Step(s) | Culture format | Number of culture formats | Number of cells/EB volume | Volume of medium/format | Timing |
|---|---|---|---|---|---|
| 8: hiPSC expansion | T80 cell culture flask | 4 | $60 \times 10^6$ ($15 \times 10^6$ per T80) | 20 ml of FTDA | 4 d |
| 23–40: EB formation | Spinner flask | 1 | $60 \times 10^6$, suspension | 200 ml of EB formation medium | 1 d |
| 41–55: Mesoderm induction | T175 cell culture flask | 2 | 300-µl EB volume, suspension | 40 ml of mesoderm induction medium | 3 d |
| 56–66: Cardiac differentiation | T175 cell culture flask | 1 | 200-µl EB volume, suspension | 46 ml of cardiac differentiation medium I/II/III | 10 d |
| 67–76: Dissociation | T175 cell culture flask | 1 | $48 \times 10^6$ | 20 ml of collagenase II solution | 4 h |

**TABLE 2** | Differentiation efficiency and efficacy of different control and disease-specific cell lines.

| Cell line | Ctr. 1, *n* = 23 | Ctr. 2, *n* = 6 | Ctr. 3, *n* = 4 | CPVT, *n* = 6 | DCM-1, *n* = 3 | DCM-2, *n* = 11 | HCM, *n* = 5 | DM1, *n* = 6 | Average, *n* = 64 |
|---|---|---|---|---|---|---|---|---|---|
| cTNT-positive cells (%) | 87 ± 8 | 88 ± 5 | 86 ± 11 | 86 ± 4 | 89 ± 3 | 82 ± 11 | 86 ± 5 | 87 ± 7 | 85 ± 4 |
| CMs per hiPSC | 1.2 ± 0.8 | 0.3 ± 0.1 | 0.6 ± 0.5 | 1.5 ± 0.6 | 1.4 ± 0.6 | 0.6 ± 0.5 | 0.6 ± 0.2 | 0.5 ± 0.3 | 0.8 ± 0.5 |

Replicate numbers refer to the number of performed differentiations. CMs per hiPSC compare final numbers of CMs with the input of hiPSCs for embryoid body formation. Mean values ± s.d. are given. CPVT, catecholaminergic polymorphic ventricular tachycardia; cTNT, cardiac troponin T; Ctr., Control; DCM, dilated cardiomyopathy; DM, myotonic dystrophy; HCM, hypertrophic cardiomyopathy.

calcium channels and the subsequent release of calcium from the sarcoplasmic reticulum (SR) through ryanodine receptors. The free calcium binds to troponin-C at the regulatory complex of the actin filaments, which induces a conformational change of troponin-I. The resulting binding of actin to the myosin ATPase located on the myosin head results in ATP hydrolysis, which supplies energy for a conformational change to occur in the actin–myosin complex. The movement between the myosin heads and actin leads to shortening of the sarcomere length. At the end of the action potential, calcium is sequestered by the SR via an ATP-dependent calcium pump (SERCA, sarco-endoplasmic reticulum calcium-ATPase). In this way, the cytosolic calcium concentration and conformational changes are reversed, and the initial sarcomere length is restored. The majority of heart diseases have a direct (effect on sarcomeric proteins) or indirect (effect on ion channels) impact on cardiomyocyte force production. Therefore, the ability to measure and analyze force in a standardized and reproducible manner is highly beneficial. Furthermore, as the ultimate goal is the development of a surrogate for the human heart, human engineered tissues have inherent advantages over laboratory animal models because of their lack of species differences. This is partly balanced by engineering artifacts and a nonphysiological cellular composition. Analyzing cardiomyocyte contractile forces in single-cell assays is a very difficult task. Disorganized myofilaments in single cells make repeated and accurate force measurements difficult. Conversely, EHTs can be generated by standardized procedures, and are characterized by improved cellular alignment and sarcomeric organization of myofilaments. A key aspect of this technique is that the tissues are attached to flexible polydimethylsiloxane (PDMS) posts, which provide an auxotonic stretching environment. Auxotonic stretch is a combination of isometric and isotonic stretching, and is considered to be the physiological mode of stretching within the heart. This EHT protocol is complementary to other 3D[35,36,39–44] or muscular thin-film-based[45] technologies. Important characteristics are cell number per construct, depending on construct size ($1 \times 10^6$ cells per EHT, $1 \times 10^6$ cells per construct[36,39,42,44], 500 cells per construct[40], $6 \times 10^3$ to $10 \times 10^3$ cells per construct[45], $2.5 \times 10^5$ cells per construct[35] or $2.5 \times 10^6$ cells per construct[43]), and the requirement of additional fibroblasts or stromal cells for remodeling[36,40].

In the EHT format, contractile force can be studied by conventional force transducers, as well as video–optical recording. Measurements with conventional force transducers[46–48] offer the advantage of absolute force determination under isometric conditions. On the other hand, they require transfer to organ baths and are therefore generally performed as end-point measurements under nonsterile conditions. The procedures are less standardized because more manual handling is required, provide a low level of automation and do not allow for long-term analyses. We therefore prefer video–optical recordings of auxotonically contracting 24-well EHTs under steady-state conditions.

A number of *in vitro* tools, such as adeno-associated virus transduction[49], electrophysiology[8], calcium-transient measurements[50] and afterload modification[7], can all be adapted to the human EHT format. Therefore, technologies for generating and analyzing EHTs will have a catalyzing impact on research areas such as disease modeling, regeneration of damaged heart tissue, predictive safety pharmacology/toxicology screening and basic research in cardiovascular medicine.

An important limitation of this technology, as compared with single-cell assays, is the high number of cells required ($1.0 \times 10^6$

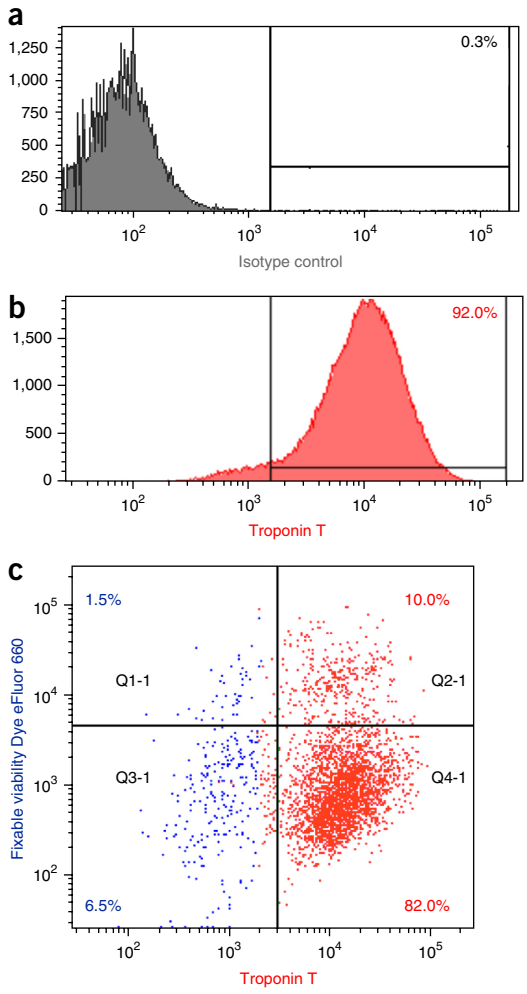


**Figure 1** | Quantitative analysis of cardiomyocytes differentiated from hiPSCs (day 17). (**a**) Isotype control. (**b**) Staining with an antibody directed against troponin T. (**c**) Staining of dead cells with Fixable Viability Dye eFluor 660 after thawing of cardiomyocytes, cryopreserved on day 17 of differentiation. Number of analyzed cells is $5 \times 10^4$ (**a**), $1 \times 10^5$ (**b**) and 5,000 (**c**). For details about antibodies and gating strategy, see **Supplementary Figure 1**.

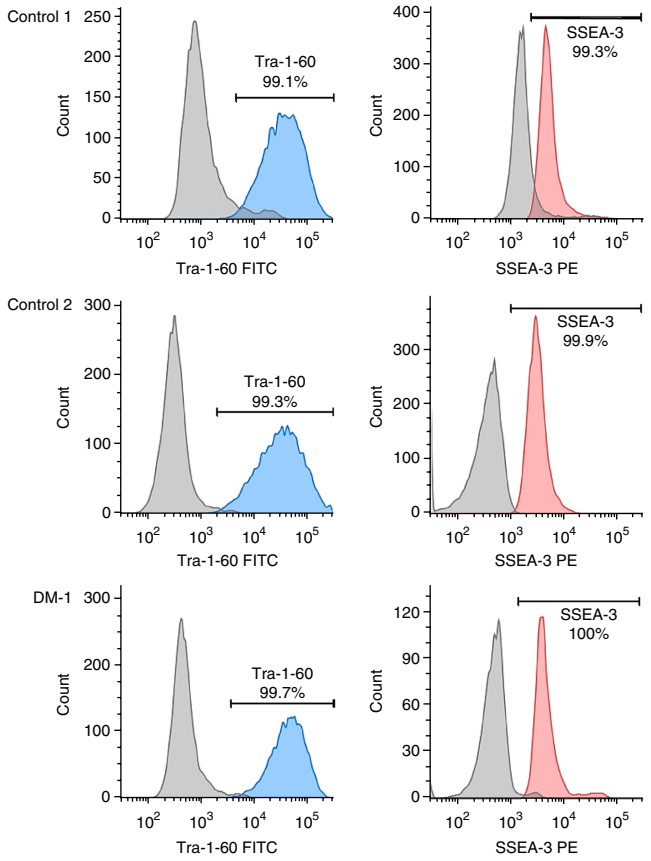


**Figure 2** | Expression of pluripotency markers by hiPSCs. Representative FACS analysis of two different control cell lines and a patient-derived myotonic dystrophy cell line (DM-1) after staining with antibodies directed against TRA-1-60 (blue) and SSEA-3 (pink) with respective isotype controls (gray). For details about antibodies, number of analyzed cells and gating strategy, see **Supplementary Figure 2**.

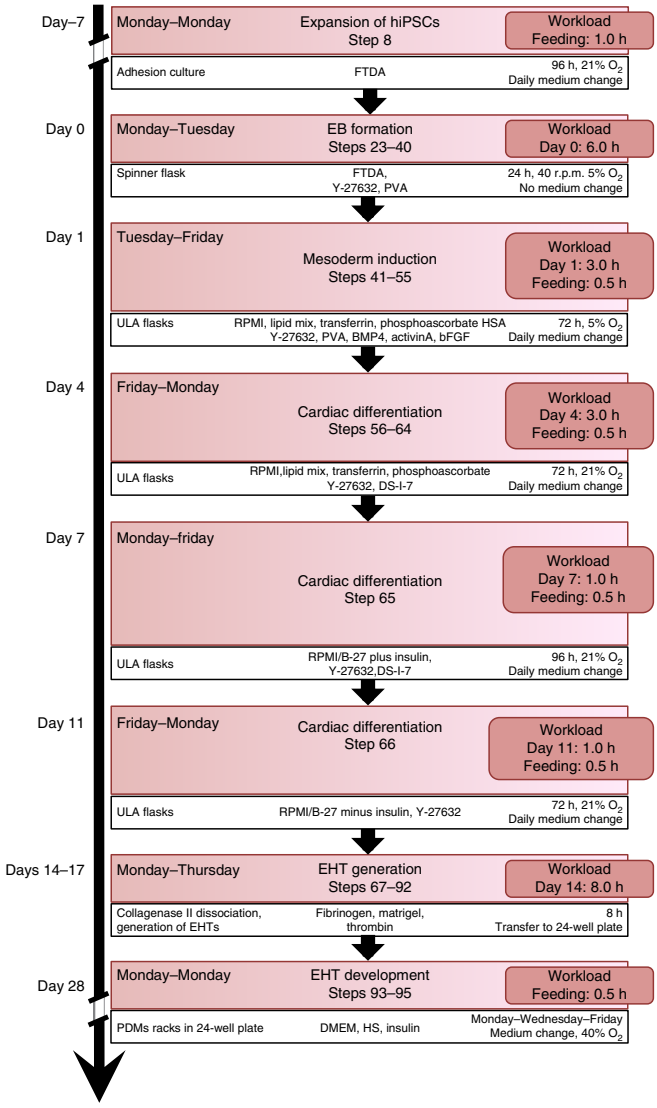


**Figure 3** | Schematic of the protocol for differentiation of cardiomyocytes from hiPSCs and EHT generation. BMP4, activin A and bFGF were used for mesoderm induction and DS-I-7 (IWR-1 analog) was used for WNT signal inhibition. FTDA, hiPSC medium with bFGF, TGFβ1, dorsomorphin and activin A; HSA, human serum albumin; HS, horse serum; PVA, polyvinyl alcohol; ULA, ultra-low-attachment.

cells per EHT). In addition, although our protocol is cost-effective, the differentiation efficiency and efficacy show variability between different cell lines and differentiation runs (see also the 'Anticipated Results' section and **Table 2**). In addition, smaller EHTs with fewer cardiomyocytes per tissue, in a 96-well format, are desirable, as they would result in a higher experimental throughput. However, achieving this would require advanced manufacturing technologies, and such small tissues would be difficult to handle manually.

### Experimental design

In this protocol, cardiomyocyte differentiation begins with the expansion of undifferentiated pluripotent stem cells. We passage undifferentiated hiPSCs twice per week (usually on Monday and Thursday) and seed $6.5 \times 10^4$ cells per $cm^2$, leading to a cell density of $1.9 \times 10^5$ to $2.5 \times 10^5$ cells per $cm^2$ ($15 \times 10^6$ to $20 \times 10^6$ cells per T80 flask) after 3 d in culture. EB formation is initiated 1 week after expanding hiPSC culture from a six-well maintenance culture to a T80 flask format. Important measures for quality control are the microscopic verification of typical morphology for undifferentiated hiPSC colonies, passaging intervals, FACS analysis of the pluripotency markers TRA-1-60 and SSEA-3 (ref. 32) (**Fig. 2**, **Supplementary Fig. 2**), PCR screening for mycoplasma contamination[51] and karyotyping.

After overnight EB formation, hiPSCs are prepared for the cardiac differentiation process and EHT generation as described in **Figures 3** and **4**. Corresponding bright-field images at different stages of the cardiac differentiation protocol are shown in **Figure 5**.

Mesoderm induction is initiated on day 1. The EBs are collected and pooled, in order to get an estimate of the total wet volume of the combined EB yield (**Fig. 6**). The EBs are transferred to T175 cell culture flasks coated with Pluronic F-127 (ref. 52), as a cost-effective alternative to commercially available ultra-low-attachment flasks. On this day, $30 \times 10^6$ undifferentiated input hiPSCs give rise to ~150 µl of EB wet volume. For mesoderm induction, this amount of EBs is cultivated in 40 ml of medium, of which 50% is exchanged on each of the two subsequent days (days 2 and 3). The volume of medium is proportionally adjusted to the measured wet volume of EBs (details are given in PROCEDURE Step 49).

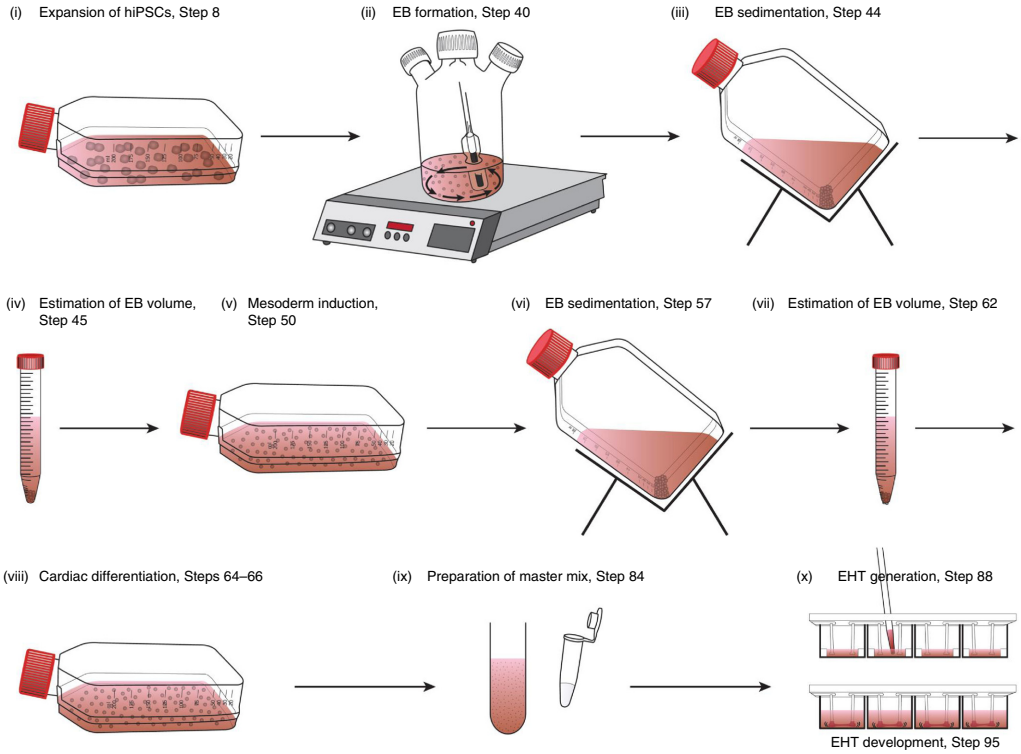


**Figure 4** | Graphical illustration of differentiation and EHT generation. (i) Undifferentiated colonies of hiPSCs are expanded in T80 flasks. (ii) EBs are formed in spinner flasks. (iii) EBs are sedimented on V-shaped sedimentation racks. (iv) EB volume is estimated in 15-ml Falcon tubes. (v) EBs are transferred to Pluronic-F-127-coated suspension flasks and mesoderm progenitors are induced. (vi) EBs are sedimented on V-shaped sedimentation racks. (vii) EB volume is estimated in 15-ml Falcon tubes. (viii) EBs are transferred to Pluronic-F-127-coated suspension flasks and cardiomyocytes are differentiated. (ix) Cardiomyocytes are dissociated and EHT master mix plus thrombin aliquot is prepared. (x) EHTs are cast and maintained under cell culture conditions.

For medium changes, the flasks are transferred to V-shaped sedimentation racks and placed in the incubator (for a maximum of 20 min) to enable EB sedimentation in the bottom corner of the flask (**Fig. 6b**). Approximately half of the medium is then exchanged for new medium to reduce sedimentation time and the time in which EBs are condensed into a pellet, which could

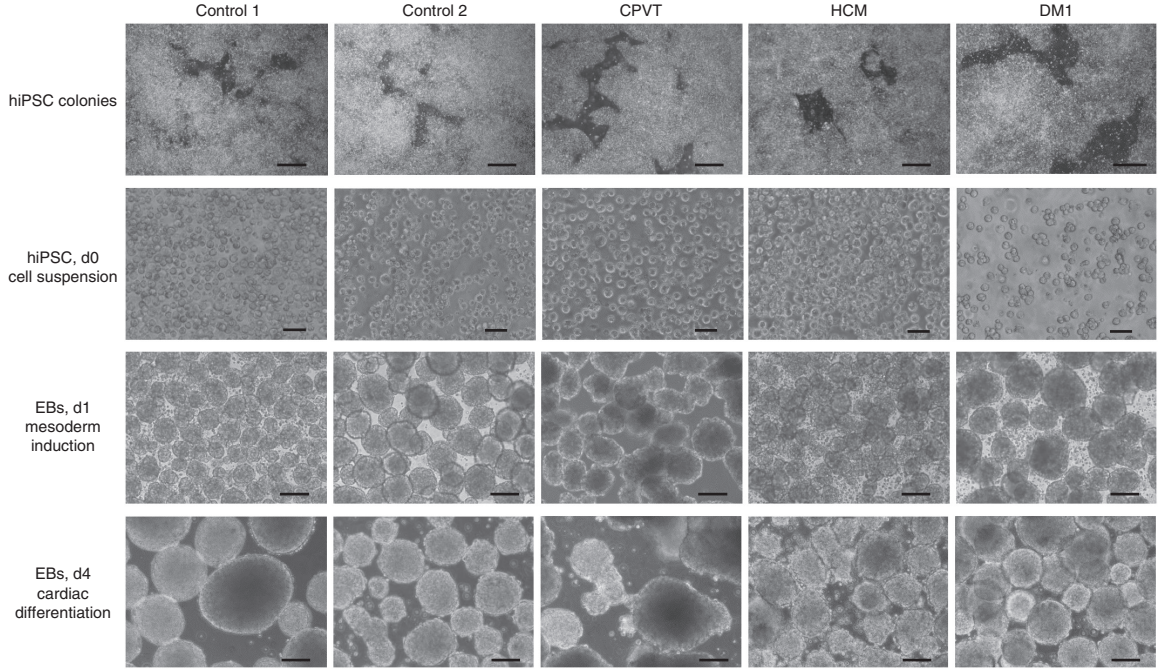


**Figure 5** | Bright-field images at different time points of cardiac differentiation with five different cell lines, showing the typical morphology. Scale bars, 1,000 µm (first row); 100 µm (second row); 250 µm (third and fourth rows).

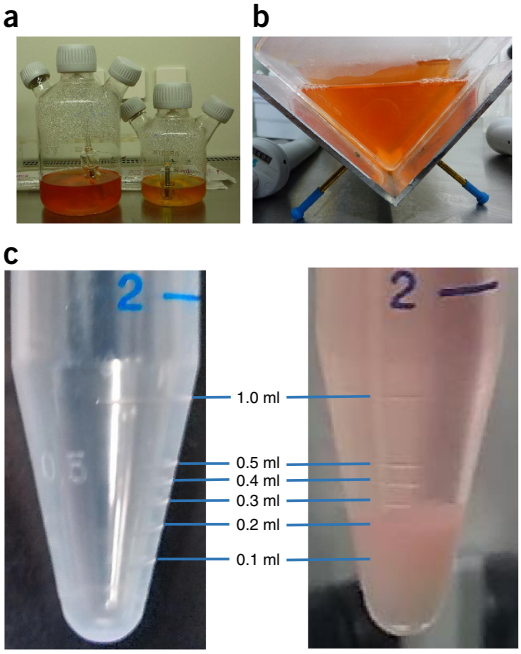


**Figure 6** | Estimation of EB volume after EB formation. (**a**) Spinner flasks with EBs in EB formation medium. (**b**) Sedimentation of transferred EBs in T175 flasks on a V-shaped sedimentation rack. (**c**) 15-ml tube with scale in the lower volume range (left) and example of EB volume (0.2 ml; right).

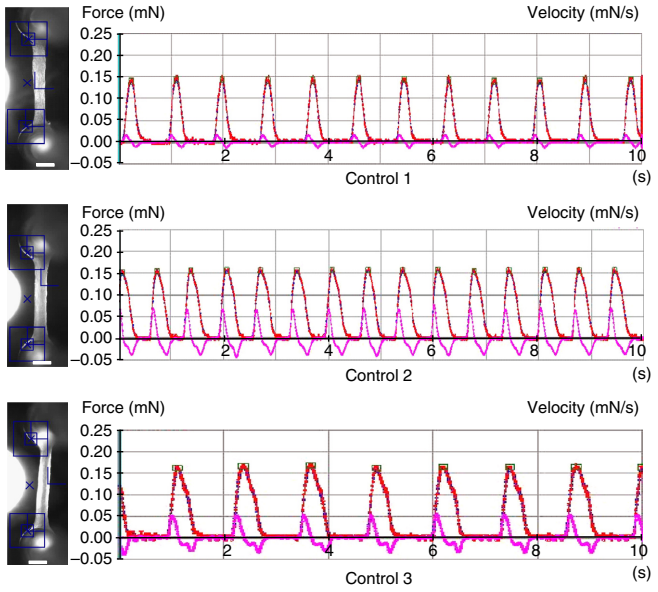


**Figure 7** | Pictures of EHTs from hiPSC-CMs of three different control cell lines taken during a contractility measurement (left) and representative force recording (right). The blue crosses define the top and bottom reference positions for each EHT (left). The red line indicates force, and the purple line indicates velocity (right). Measurements were done in medium (control 1 and control 2) or Tyrode's solution (1.8 mM $Ca^{2+}$, control 3). Scale bars, 1 mm.

lead to disintegration of EBs because of cell death. In addition, by leaving more medium, potential (not yet fully defined) paracrine signaling effects are taken advantage of[53]. Only when initiating the next differentiation step is the medium is changed completely and the medium retention reduced to 10–20% to remove the previous medium composition. **Table 1** provides details on the suggested ratio for EB volume, cell culture medium and cell culture flask size. In our experience, BMP-4 (10 ng/ml), activin A (3 ng/ml) and bFGF (5 ng/ml) in the absence of insulin are able to effectively differentiate stem cells from various hiPSC lines into cardiac mesoderm progenitors. Insulin, a key regulator of cardiac differentiation, has previously been shown to inhibit cardiac mesoderm formation and promote cardiac differentiation of human pluripotent stem cells[54–56].

Cardiac differentiation is initiated on day 4 (Friday) by sedimenting the EBs, removing most of the cell culture medium and pooling the EBs in a graduated 15-ml Falcon tube to estimate EB volume (**Fig. 6c**). Cardiac differentiation is performed with 80–150 µl of EB volume per T75 flask or 200–250 µl of EB volume per T175 flask. WNT signal inhibition and insulin are the two most important components of this differentiation step. WNT signal inhibition is started on day 4 and is omitted on day 11. Insulin is added on day 7. In comparative studies using FACS analysis, we found these timings for treatment to be the most efficient in inducing differentiation to cardiomyocytes. The first spontaneous beating is generally observed by days 8–10. **Supplementary Videos 1–3** show beating EBs. Between days 14 and 17, the cardiomyocytes can be dissociated from EBs into single cells and cast as EHTs. A sample of these dissociated cells was FACS-analyzed to determine its cardiomyocyte purity (**Fig. 1,Supplementary Fig. 1**). A more detailed FACS analysis of hiPSC-CMs directly after differentiation and after cultivation in EHT format can be found elsewhere[7,9].

The EHT generation procedure relies on the generation of agarose casting molds and formation of fibrin gels with single-cell suspension around flexible PDMS posts[5]. To produce EHTs, a mixture of isolated cardiomyocytes, medium, fibrinogen and thrombin is cast around two PDMS posts, within an agarose mold. Within 2 h, the mixture polymerizes and forms a fibrin block around the posts. **Supplementary Video 4** illustrates the process of EHT generation. Fibrin was chosen as the extracellular matrix for this protocol because it is readily available from different species, it is biodegradable, it has short gelation times and it readily adheres to PDMS. In the beginning of EHT construction, when the fibrin gel is formed, the flexible PDMS posts are oriented parallel to one another. Subsequent remodeling of the matrix by the resident cells leads to shortening of the muscle strip and, thus, bending of the flexible PDMS posts. This configuration of the PDMS posts is a key asset of this EHT system. The tension in the posts applies an auxotonic load to the tissues, and thereby facilitates the longitudinal alignment of the embedded cardiomyocytes. Static stretch, in contrast, is not a physiological environment and promotes a pathological form of hypertrophy in neonatal rat cardiomyocyte EHTs[6]. Approximately 10–15 days after the production of the tissues, EHTs begin to spontaneously beat. As the tissues beat, they further deflect the PDMS posts to which they are attached. This deflection can then be recorded video–optically and measured to assess the force-producing capability of the tissues (**Fig. 7**; **Supplementary Videos 5–8**). The analysis of EHTs by video–optical recording has proven very useful, as it is performed with high levels of standardization and reproducibility under sterile conditions[5,7]. This last point cannot be overemphasized, as it enables repeated and long-term analysis.

## MATERIALS

### REAGENTS

**General reagents**

- 10 × DMEM (Gibco, cat. no. 52100-021)
- 1-Thioglycerol (Sigma-Aldrich, cat. no. M6145)
- 2-propanol (Sigma-Aldrich, cat. no. 278475)
- Agarose (Invitrogen, cat. no. 15510-027)
- Aprotinin (Sigma-Aldrich, cat. no. A1153)
- Sterile water for injection (Baxter, cat. no. 001428)
- B27 plus insulin (Gibco, cat. no. 17504-044)
- BIOMYC-1 (PromoCell, cat. no. PK-CC03-036-1B)
- BIOMYC-2 (PromoCell, cat. no. PK-CC03-037-1B)
- *N*-benzyl-*p*-toluenesulfonamide (BTS; TCI, cat. no. B3082-25G)
- Cytoseal 60 mounting medium (Science Services, cat. no. 18006)
- DMEM (Biochrom, cat. no. F0415)
- DMEM/F-12 without glutamine (Gibco, cat. no. 21331-046)
- DMSO (Sigma-Aldrich, cat. no. D4540)
- EDTA (Roth, cat. no. 8043.2)
- FBS superior (Biochrom, cat. no. S0615)
- Fibrinogen (Sigma-Aldrich, cat. no. F8630)
- Fixable Viability Dye eFluor 660 (eBioscience, cat. no. 65-0864)
- Gelatin (Sigma-Aldrich, cat. no. G1890)
- Geltrex (Gibco, cat. no. A1413302)
- Giemsa stain (Sigma-Aldrich, cat. no. G9641)
- Glacial acetic acid (Roth, cat. no. 3738.1)
- HBSS minus $Ca^{2+}/Mg^{2+}$ (Gibco, cat. no. 14175-053)
- HBSS plus $CaCl_2/MgCl_2$ (Gibco, cat. no. 14025-092)
- HEPES (Roth, cat. no. 9105.4)
- Horse serum (Life Technologies, cat. no. 26050088)
- Human pluripotent stem cell lines: We have used C25 (control 1)[57], and all other cell lines used were reprogrammed from skin fibroblasts[58] of healthy donors (controls 2 and 3) or from patients suffering from catecholaminergic polymorphic ventricular tachycardia (CPVT), hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM; DCM-1 and DCM-2) and myotonic dystrophy (DM; DM1) **! CAUTION** The cell lines used in your research should be regularly checked to ensure that they are authentic and that they are not infected with mycoplasma.
- KaryoMax Colcemid in HBSS (10 µg/ml; Life Technologies, cat. no. 15210-040)
- KCl (75 mM; Sigma-Aldrich, cat. no. P9541)
- L-Glutamine (Gibco, cat. no. 25030-081)
- Lipid mix (Sigma-Aldrich, cat. no. L5146)
- Matrigel Basement Membrane Matrix (BD, cat. no. 354234)
- Methanol (J.T. Baker, cat. no. 8045)
- Mucasol (Sigma-Aldrich, cat. no. Z637181)
- Ham's F10 Nutrient Mix (Life Technologies, cat. no. 31550-015)
- PBS (Gibco, cat. no. 10010-049)
- Penicillin/streptomycin (Gibco, cat. no. 15140)
- Phosphoascorbate (2-Phospho-L-ascorbic acid trisodium salt; Sigma-Aldrich, cat. no. 49752)
- Pluronic F-127 (Sigma-Aldrich, cat. no. P2443)
- Polyvinyl alcohol (Sigma-Aldrich, cat. no. P8136)
- Potassium chloride (KCl; Merck, cat. no. 1.04936)
- RPMI 1640 (Gibco, cat. no. 21875)
- Saponin from Quillaja bark (Sigma-Aldrich, cat. no. S7900)
- Selenium (Sigma-Aldrich, cat. no. S5261)
- Sodium selenite (Sigma-Aldrich, cat. no. S5261)
- Thrombin (Biopur, cat. no. BP11-10-1104)
- Titrisol (Sigma-Aldrich, cat. no. 31103)
- Trypsin/EDTA (Gibco, cat. no. 25300)

**Proteins and small molecules**

- Activin-A, stock concentration, 50 µg/ml (R&D Systems, cat. no. 338-AC)
- Basic FGF, improved sequence, stock concentration, 100 µg/ml (Miltenyi Biotec, cat. no. 130-104-921)
- BMP-4, stock concentration, 50 µg/ml (R&D Systems, cat. no. 314-BP)
- Collagenase II (Worthington, cat. no. LS004176)
- DNase II, type V (from bovine spleen; Sigma, cat. no. D8764)
- DS-I-7(IWR-1 analog, 4-(*cis*-endo-1,3-dioxooctahydro-*2H*-4,7-methanoisoindol-2-yl)-*N*-(quinolin-8-yl)-*trans*-cyclohexylcarboxamide), stock concentration, 100 mM in DMSO
- Dorsomorphin (Abcam, cat. no. ab120843)
- Human serum albumin (Biological Industries, cat. no. 05-720-1B)
- Insulin, stock concentration, 10 mg/ml (Sigma-Aldrich, cat. no. I9278)
- IWR-1, stock concentration, 20 mg/ml (Sigma-Aldrich, cat. no. I0161)
- TGFβ1 (Peprotech, cat. no. 100-21)
- Thrombin, stock concentration, 100 U/ml (Biopur, cat. no. BP11-10-1104)
- Transferrin (Sigma-Aldrich, cat. no. T8158)
- Y-27632, stock concentration, 10 mM (Biorbyt, cat. no. orb60104)

**Primary antibodies**

- Anti-α-actinin, clone EA-53, monoclonal, 1:800 (Sigma-Aldrich, cat. no. A7811)
- Anti-cardiac troponin T-FITC, clone REA400, 1:10 (Miltenyi Biotec, cat. no. 130-106-687)
- Anti-myosin light chain 2 (MLC-2V; Proteintech, cat. no. 10906-1-AP, 1:100)
- Anti-myosin light chain A, clone 56F5, monoclonal, 1:100 (MLC-2A, Synaptic Systems, cat. no. 311011)
- Anti-human TRA-1-60-FITC, clone TRA-1-60, 1: (BD Biosciences, cat. no. 5608765)
- Anti-SSEA-3-PE, clone MC-631, 1:5 (BD Biosciences, cat. no. 560879)
- Mouse IgG1 isotype control, clone MOPC-21, 1:250 (BD Biosciences, cat. no. 554121)
- FITC mouse IgM, κ isotype control, clone G155-228, 1:200 (BD Biosciences, cat. no. 553474)
- PE rat IgM, κ isotype control, clone R4-22, 1:80 (BD Biosciences, cat. no. 553943)
- REA Control (I)-FITC (IgG1 isotype control, clone REA400, 1:10; Miltenyi Biotec, cat. no. 130-104-611)

**Secondary antibodies**

- Anti-mouse Alexa Fluor 488 (Life Technologies, cat. no. A-11001, 1:800)
- Anti-mouse Alexa Fluor 546 (Life Technologies, cat. no. A-11060, 1:800)
- Anti-rabbit Alexa Fluor 488 (Life Technologies, cat. no. A-11034, 1:800)

### EQUIPMENT

- 6-Well cell culture plates (Nunc, cat. no. 140675)
- 24-Well plates (Nunc, cat. no. 144530) **▲ CRITICAL** Use Nunc plates (cat. no. 144530) for EHT generation, as 24-well plates do not have standardized dimensions and plates from other suppliers might not be compatible with the PDMS racks or Teflon spacers.
- 250-ml 'rapid' Filtermax vacuum filtration unit (TPP, cat. no. 99250)
- 500-ml 'rapid' Filtermax vacuum filtration unit (TPP, cat. no. 99500)
- Cell scraper (Sarstedt, cat. no. 83.1830)
- Centrifuge tubes, 15 ml (Sarstedt, cat. no. 62.554.502 and Greiner, cat. no. 188280)
- Coplin jar (Sigma-Aldrich, cat. no. S6016-6EA)
- Coverslips (Sigma-Aldrich, cat. no. Z692271)
- CytoVision image analysis system (Leica Biosystems)
- Flow Cytometer (FACSCanto II; BD Bioscience)
- Flow cytometry tubes (Sarstedt, cat. no. 55.1579)
- EHT contractility analysis instrument (EHT Technologies, cat. no. A0001)
- PCR cycler (e.g., Mastercycler Pro; Eppendorf, cat. no. 6321000515)
- PDMS racks (dimensions: length/width of rack, 79 × 18.5 mm; length of posts, 12 mm; diameter,1 mm; plate diameter, 2 mm; distance (center–center),8.5 mm; Young's modulus,1.7 MPa; EHT Technologies, cat. no. C0001)
- Slides (76 × 26 × 1 mm; Marienfeld, cat. no. REF 1000200)
- Spinner flask (1,000 ml; Integra, cat. no. 182 101)
- Spinner flask (500 ml; Integra, cat. no. 182 051)
- Stirrer, Variomag/Cimarec Biosystem 4 Direct (Thermo Fisher Scientific, cat. no. 50088060)
- Stirrer, Variomag/Cimarec Biosystem Direct (Thermo Fisher Scientific, cat. no. 70101)
- T175 cell culture flask (Sarstedt, cat. no. 83.1812.002)
- T75 cell culture flask (Sarstedt, cat. no. 83.1813.002)
- T80 cell culture flask (Nunc, cat. no. 178905)
- T175 suspension cell culture flask (Sarstedt, cat. no. 83.3912.502)
- Teflon spacers (dimensions: length,12 mm; width,3 mm; height, 13.5 mm; EHT Technologies, cat. no. C0002)
- Video–optical force analysis system (EHT Technologies, cat. no. A0001)
- V-shaped sedimentation rack (custom-made; dimensions of the two side panels of the metal bracket: 30 cm × 10 cm; angle: 90°)

## REAGENT SETUP

**10× DMEM** Dissolve 134 mg/ml DMEM powder in 5 ml of sterile water for injection. Sterilize the solution by passing it through a 0.2-µm filter, and store the solution at 4 °C for up to 2 months. Store the DMEM powder at 4 °C for up to 36 months, and make sure that the box is properly closed, as DMEM is hygroscopic.

**Agarose** Dissolve 2% agarose (wt/vol) in 300 ml of PBS and autoclave the solution. Store it at 60 °C immediately in a heated cabinet for up to 2 months. ▲ **CRITICAL** Do not use water to dissolve the agarose because this will substantially compromise EHT development. Keep agarose at room temperature (18–22 °C) only for up to 10 min; otherwise, it will solidify.

**Aprotinin** Dissolve 33 mg/ml aprotinin in sterile water for injection and make aliquots of 250 µl. Store the aliquots at −20 °C for up to 1 year.

**BTS solution** Dissolve BTS in DMSO to a concentration of 30 mM and make aliquots of 250 µl. Store the aliquots at −20 °C for up to 1 year.

**DNase solution** Dissolve 100 mg of DNase II, type V in 50 ml of PBS, and make aliquots of 2 ml. Store the aliquots at −20 °C for up to 1 year.

**EDTA solution** Dissolve EDTA in PBS to a concentration of 0.5 mM and sterilize the solution by passing it through a 0.2-µm filter; then make aliquots of 50 ml. Store the aliquots at 4 °C for up to 6 months.

**Fibrinogen** Prewarm 0.9% (wt/vol) NaCl solution (37 °C). Grind the lumps of fibrinogen to a fine powder. Dissolve the fibrinogen at a concentration of 200 mg/ml in warm 0.9% (wt/vol) NaCl solution. Incubate the solution in a water bath (37 °C) until the fibrinogen is completely dissolved. Add aprotinin stock (33 mg/ml) to a final concentration of 100 µg/ml. Make aliquots of 500 µl and store them at −20 °C for up to 2 months, or at −80 °C for up to 1 year.

**Gelatin solution** Dissolve gelatin in water to make a 0.1% (wt/vol) solution and prepare 500-ml aliquots. Store the aliquots at 4 °C for up to 6 months.

**HEPES stock solution** Dissolve HEPES in PBS to a concentration of 1 M, and adjust the pH to 7.4 with potassium hydroxide. Sterilize the solution by passing it through a 0.2-µm filter, and make 500-ml aliquots. Store the aliquots at 4 °C for up to 1 year.

**Sodium selenite** Dissolve 33 mg of sodium selenite (382 µM) in 500 ml of PBS and prepare 500-ml aliquots. Store the aliquots at 4 °C for up to 1 year.

**Pluronic F-127 solution** Dissolve Pluronic F-127 in PBS to a concentration of 1% (wt/vol). Sterilize the solution by passing it through a 0.2-µm filter, and make 500-ml aliquots. Store the aliquots at 4 °C for up to 1 year.

**Polyvinyl alcohol stock solution (50×)** Dissolve 20 g of polyvinyl alcohol in 100 ml of $dH_2O$ by adding the polyvinyl alcohol slowly and stirring at ~20 °C. After dispersal, the temperature should be increased to 80 °C, while stirring constantly, and then should be held until the polyvinyl alcohol is fully dissolved. Afterward, while stirring, the temperature should be decreased to below 35 °C. Make aliquots of 50 ml and store them at 4 °C for up to 1 year. After the addition of polyvinyl alcohol to the medium, filter-sterilize the medium using a 0.2-µm filter.

**Thrombin** Dissolve thrombin at 100 U/ml in 60% (vol/vol) PBS and 40% (vol/vol) sterile water for injection. Mix the solution thoroughly. Make 450-µl aliquots (stock) or 3-µl aliquots for EHT generation, and store them at −20 °C for up to 1 year.

**Transferrin–selenium** Dissolve 100 mg of transferrin in 2 ml of sodium selenite and make aliquots of 55 µl. Store them at −20 °C for up to 1 year.

**hiPSC medium with bFGF, TGFß1, dorsomorphin and activin A (FTDA)** hiPSC medium contains DMEM/F-12 without glutamine, L-Glutamine (2 mM), lipid mix (1/1,000), penicillin/streptomycin (0.5% (vol/vol)), transferrin–selenium stock solution (1/10,000; 5 mg/liter transferrin; 5 µg/liter selenium), human serum albumin (0.1% (vol/vol)), insulin (5 µg/ml), activin-A (2.5 ng/ml), basic FGF (30 ng/ml), dorsomorphin (50 nM) and TGFß1 (0.5 ng/ml). Filter-sterilize the medium by passing it through a 0.2-µm filter, and store it at 4 °C for up to 1 week.

We recommend aliquotting cold FTDA for daily medium changes and only prewarming the aliquot. ▲ **CRITICAL** We recommend using basic FGF with improved sequence, because of its increased thermostability and higher resistance to proteases. As the manufacturer does not further specify bFGF kinetics, we recommend considering use of the same precautions as those for normal bFGF, to ensure the highest bFGF stability. Basic FGF is added immediately before using the medium, as diminished concentrations of bFGF can lead to spontaneous differentiation of hiPSCs.

**2× Freezing medium for pluripotent stem cells** 2× Freezing medium contains FTDA (80% (vol/vol)), and DMSO (20% (vol/vol)). Store the medium at 4 °C for up to 2 months.

**EB formation medium** EB formation medium contains FTDA, polyvinyl alcohol (4 mg/ml) and Y-27632 (10 µM). Filter-sterilize the medium by passing it through a 0.2-µm filter, and store it at 4 °C for up to 1 week.

**Mesoderm induction medium** Mesoderm induction medium contains RPMI 1640, polyvinyl alcohol (4 mg/ml), HEPES (10 mM), penicillin/streptomycin (0.5% (vol/vol)), human serum albumin (0.05% (vol/vol)), phosphoascorbate (250 µM), transferrin–selenium stock solution (1/10,000; 5 mg/l transferrin; 5 µg/l selenium), lipid mix (1/1,000), Y-27632 (10 µM), activin-A (3 ng/ml), BMP-4 (10 ng/ml), and basic FGF (5 ng/ml). Filter-sterilize the medium by passing it through a 0.2-µm filter, and store it at 4 °C for up to 1 week without growth factors. We do not recommend storing the medium supplemented with BMP4, activin A and bFGF because growth factor activity may decline during storage. ▲ **CRITICAL** Cardiomyocyte differentiation is substantially hampered if insulin is present during mesoderm induction. We recommend using basic FGF with improved sequence because of its increased thermostability and higher resistance to proteases. As the manufacturer does not further specify bFGF kinetics, we recommend considering use of the same precautions as those for normal bFGF to ensure the highest bFGF stability. Basic FGF is added immediately before using the medium, as diminished concentrations of bFGF can lead to spontaneous differentiation of hiPSCs.

**Cardiac differentiation medium I** Cardiac differentiation medium I contains RPMI 1640, HEPES (10 mM), penicillin/streptomycin (0.5% (vol/vol)), human serum albumin (0.05% (vol/vol)), phosphoascorbate (250 µM), transferrin–selenium stock solution (1/10,000; 5 mg/l transferrin; 5 µg/l selenium), lipid mix (1/1,000), Y-27632 (1 µM), and DS-I-7 (IWR-1 analog (100 nM)). Filter-sterilize the medium by passing it through a 0.2-µm filter, and store it at 4 °C for up to 1 week.

**Cardiac differentiation medium II** Cardiac differentiation medium II contains RPMI 1640, B27 plus insulin (2% (vol/vol)), HEPES (10 mM), penicillin/streptomycin (0.5% (vol/vol)), 1-Thioglycerol (500 µM), Y-27632 (1 µM), and DS-I-7 (IWR-1 analog (100 nM)). Filter-sterilize the medium by passing it through a 0.2-µm filter, and store it at 4 °C for up to 1 week.

**Cardiac differentiation medium III** Cardiac differentiation medium III contains RPMI 1640, B27 minus insulin (2% (vol/vol)), HEPES (10 mM), penicillin/streptomycin (0.5% (vol/vol)), 1-Thioglycerol (500 µM), and Y-27632 (1 µM). Filter-sterilize the medium by passing it through a 0.2-µm filter, and store it at 4 °C for up to 1 week.

**Collagenase II solution** Collagenase II solution contains HBSS minus $Ca^{2+}$/$Mg^{2+}$, Collagenase II (200 units per ml), HEPES (10 mM), Y-27632 (10 µM), and BTS (30 µM). Filter-sterilize the solution by passing it through a 0.2-µm filter, and store it at 4 °C for up to 1 week or at −20 °C for up to 1 year. Add Y-27632 and BTS directly before use.

**Dissociation medium** Dissociation medium contains DMEM and DNase (6 µl/ml). We do not recommend storing this solution.

**Freezing medium for hiPSC-derived cardiomyocytes** Freezing medium contains heat inactivated FBS (90% (vol/vol) and DMSO (10% (vol/vol)). We do not recommend storing this solution.

**2× DMEM** 2× DMEM contains 10× DMEM (20% (vol/vol)), heat inactivated horse serum (20% (vol/vol)), penicillin/streptomycin (2% (vol/vol)) and sterile water for injection (58% (vol/vol)). Filter-sterilize the solution by passing it through a 0.2-µm filter, and store it at 4 °C for up to 2 months. ▲ **CRITICAL** Use heat-inactivated horse serum to prevent resolving of the EHT.

**EHT medium** EHT medium contains DMEM, heat-inactivated horse serum (10% (vol/vol)), penicillin/streptomycin (1% (vol/vol)), aprotinin (0.1% (wt/vol); 33 µg/ml), and insulin (0.1% (wt/vol); 10 µg/ml). Filter-sterilize the medium by passing it through a 0.2-µm filter, and store it at 4 °C for up to 1 week. ▲ **CRITICAL** Use heat-inactivated horse serum to prevent resolving of the EHT.

**Normal cardiomyocyte medium** Normal cardiomyocyte medium (NCM) contains DMEM, heat-inactivated FBS (10% (vol/vol)), glutamine (1% (vol/vol)), and penicillin/streptomycin (1% (vol/vol)). Store the medium at 4 °C for up to 2 weeks. ▲ **CRITICAL** Use heat-inactivated FBS to prevent resolving of the EHT. For heat inactivation, incubate FBS for 1 h in a 60 °C water bath.

**Washing medium** Washing medium contains RPMI 1640, HEPES (10 mM), and penicillin/streptomycin (0.5% (vol/vol)). Store the medium at 4 °C for up to 2 months.

**FACS buffer for staining of intracellular antigens** This buffer contains 500 ml of PBS, FBS (5% (vol/vol); 25 ml), Saponin (0.5% (wt/vol); 2.5 g), and sodium azide (0.05% (wt/vol); 0.25 g). Store it at 4 °C for up to 2 months. ! **CAUTION** Saponin is very hazardous in case of eye contact or inhalation, and must be weighed in a fume hood.

**FACS buffer for staining of extracellular antigens** This buffer contains 500 ml of PBS, FBS (5% (vol/vol); 25 ml), and sodium azide (0.05% (wt/vol); 0.25 g).

Store the buffer at 4 °C for up to 2 months.

**F10 complete medium** F10 complete medium contains 10 ml of FBS and 100 ml of Ham's F10 Nutrient Mix . Store the medium at 4 °C for up to 1 week.

**Fixative solution for karyotyping** Add 10 ml of glacial acetic acid to 30 ml of methanol, and keep the solution at −20 °C until use.

We do not recommend storing this solution.

**EQUIPMENT SETUP**

**Geltrex coating of cell culture plates and flasks** Thaw Geltrex on ice and dilute it 1:100 in ice-cold DMEM. Coat the growth surface of the desired culture plates with 1 ml per 10 $cm^2$. Incubate the plates at 37 °C for 30 min. Coated plates can be stored at 2–8 °C for up to 2 weeks.

**Pluronic F-127 coating of cell culture flasks** Add 1 ml of 1% (wt/vol) Pluronic F-127 solution to the suspension cell culture flasks per 10-$cm^2$ growth area.

Incubate the flasks at 37 °C overnight. Wash the flasks twice with 1.5 ml of PBS per 10-$cm^2$ culture surface. Remove the PBS and add 2 ml of mesoderm induction medium per 10 $cm^2$. The cell culture flasks are now ready to use. Coated flasks can be stored at 37 °C for up to 2 weeks. ▲ **CRITICAL** Do not let the surfaces dry out. Longer coating times (for several days) are not detrimental.

**Cleaning protocol for spinner flasks** Clean the spinner flasks and the impellers with $dH_2O$ and a bottle brush.

Reassemble the spinner flasks and autoclave them. ▲ **CRITICAL** Do not use soap/detergent to clean the spinner flasks, and ensure that the spinner flask lids are not fully closed during autoclaving.

**Preparation of Teflon spacers and PDMS racks** Clean the Teflon spacers and PDMS racks with $dH_2O$. Boil the Teflon spacers and PDMS racks in $dH_2O$ twice. Autoclave the Teflon spacers and PDMS racks. ▲ **CRITICAL** Do not use soap/detergent to clean the Teflon spacers and PDMS racks. Make sure to place the PDMS racks upside down in the autoclave containers, so that the flexible posts are not bent or distorted during the autoclaving process.

## PROCEDURE

### Thawing and expansion of undifferentiated pluripotent stem cells ● TIMING 30 min plus 4 d for expansion before EB formation, including continuous maintenance

**1|** Remove the frozen tube of hiPSCs from liquid nitrogen or –150 °C and thaw the cells at 37 °C.

**2|** Transfer the cells to a 15-ml tube.

**3|** Add 10 ml of FTDA, drop by drop for the first 2 ml.

**4|** Centrifuge the mixture at 200*g* for 5 min at room temperature.

**5|** Remove the supernatant and resuspend the hiPSCs in the desired volume of FTDA including 10 μM Y-27632.

**6|** Aspirate the liquid from the Geltrex-coated plate, and seed the hiPSCs into the Geltrex-coated wells.

**7|** Put the plate into the incubator and distribute the cells in the well by moving the plate in short side-to-side and back-and-forth motions. Incubator conditions should be set to 37 °C, 5% $CO_2$, 21% $O_2$ and 90% humidity.

**8|** Feed the hiPSCs daily with 2 ml of warm FTDA per 10-$cm^2$ growth surface until they are 80% confluent. When feeding cells, leave 10–20% of the old medium in the well.

**? TROUBLESHOOTING**

### Passaging of undifferentiated hiPSCs ● TIMING 10 min

**9|** Wash the hiPSCs twice with warm (37 °C) PBS.

**10|** Incubate the hiPSCs with 0.5 mM EDTA for 4–5 min at room temperature (1 ml of EDTA per 10-$cm^2$ growth surface).

**11|** Remove the EDTA solution.

## Box 1 | Freezing of undifferentiated hiPSCs ● TIMING 15 min

1. Mix 500 μl of freezing medium with 500 μl of the resuspended cells.
2. Add 10 μM Y-27632.
3. Transfer the hiPSCs to a freezing tube.
4. Freeze the tube at –80 °C overnight in a 2-propanol-filled freezing container.
5. Transfer the tubes to liquid nitrogen or –150 °C for long-term storage.





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## Box 2 | Mycoplasma screening and treatment ● TIMING 3.5 h or up to 2 weeks

**Additional reagents**
Taq DNA Polymerase Kit (Qiagen, cat. no. 201205) containing:
10× buffer
Q-Solution
$MgCl_2$ (25 mM)
Deoxynucleoside triphosphate (DNTP)
Polymerase
BIOMYC-1 (PromoCell, cat. no. PK-CC03-036-1B)
BIOMYC-2 (PromoCell, cat. no. PK-CC03-037-1B)

1. Remove 250 µl of the cell culture medium after overnight incubation and transfer it to a sterile reaction tube.
2. Add 750 µl of sterile water and mix.
3. Incubate the diluted sample for 10 min in a thermoblock at 100 °C.
4. Centrifuge at 250*g* for 5 min at room temperature.
5. Prepare the PCR master mix by combining the following reagents.

| Component | Per reaction |
|---|---|
| $H_2O$ | 26.75 µl |
| 10× buffer | 5.00 µl |
| Q-Solution | 10.00 µl |
| $MgCl_2$ (25 mM) | 4.00 µl |
| Primer pool (10 pM) Myco-dw[51]: 5′-TGC ACC ATC TGT CAC TCT GTT AAC CTC-3′ | 1.00 µl |
| Primer pool (10 pM) Myco-up[51]: 5′-ACT CCT ACG GGA GGC AGC AGT A-3′ | 1.00 µl |
| DNTPs | 1.00 µl |
| Polymerase | 0.25 µl |
| Sum | 48.00 µl |

6. Take 2 µl of the sample supernatant and add it to 48 µl of the master mix solution. Use water (negative control) and a contaminated supernatant (positive sample) as references.
7. Immediately start the PCR Cycler set to the following program:

| Cycler | time (*t*) | °C | Cycles |
|---|---|---|---|
| Initiation | 15 min | 95 | 1 |
| Denaturation | 30 s | 94 | |
| Annealing | 30 s | 56 | 40 |
| Extension | 1 min | 72 | |
| Final extension | 10 min | 72 | 1 |

8. After completing the PCR, load the samples on a 1% (wt/vol) agarose gel and run the reaction for 25 min. If cells test positive for mycoplasma, please follow the next steps; if cells are not infected, please continue to follow the main procedure from Step 23.
9. For the treatment of mycoplasma infections, sequentially treat the contaminated cell culture with BIOMYC-1 for 4 d and then with BIOMYC-2 for 3 d, according to the manufacturer's instructions.
10. Repeat two to three treatment cycles to avoid antibiotic resistance.

**12|** Vigorously flush the surface of the wells with 3 ml of FTDA per 10-$cm^2$ growth surface to detach cell clusters. At this step, the cells can be frozen for storage; see **Box 1** for details.

**13|** Pool the detached cell clusters.

**14|** Remove the Geltrex solution from new six-well dishes/T80 flasks and immediately add the cell solution to the prepared culture flasks/plates at ratios of 1:2 to 1:6 ($6.5 \times 10^4$ cells per $cm^2$). Before preparing hiPSCs for cardiomyocyte differentiation, we recommend performing mycoplasma testing as described in **Box 2** and regularly performing karyotyping as described in **Box 3**.
**? TROUBLESHOOTING**

**FACS analyses of hiPSCs for pluripotency markers: staining of extracellular antigens ● TIMING 90 min**
**15|** Harvest hiPSCs as described in Steps 9–13.

**16|** Resuspend the cells in 500 µl of FACS buffer and incubate for 15 min at 4 °C.





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## Box 3 | Karyotyping of hiPSCs ● TIMING 2.5 h, plus analysis of arresting cells in metaphase

1. Use at least 4 × $10^6$ hiPSCs (20-$cm^2$ growth area) for karyotyping to ensure that you have a sufficient amount of metaphases for analysis.
2. Add 40 µl of colcemid to each well and incubate for 5 h at 37 °C and 5% $CO_2$.

**Harvesting of hiPSCs**

3. Wash the hiPSCs twice with PBS. Incubate the hiPSCs with 0.5 mM EDTA for 5 min at room temperature (1 ml of EDTA per 10-$cm^2$ growth surface). Remove the EDTA solution. Vigorously flush the growth surface with 2 ml of F10 complete medium per 10 $cm^2$ to detach the hiPSCs.
4. Triturate the cells four times with a 10-ml pipette.
5. Transfer the hiPSCs to a centrifuge tube.
6. Centrifuge at 250*g* for 10 min at room temperature.
7. Remove the supernatant, and while swirling the cell suspension on a vortex mixer keep the centrifuge tube in a slanted position and pour the 7 ml of warm 75 mM KCl solution dropwise gently along the inner wall of the tube onto the cell suspension.

▲ **CRITICAL STEP** This step is critical because the hiPSCs swell in the hypotonic solution and the chromosomes can spread out, which provides better results for chromosome analysis.

8. Invert the tube gently to mix, and incubate for 15 min at 37 °C.
9. With a glass Pasteur pipette, add 13 drops of fixative solution to the suspension, invert gently and centrifuge at 250*g* for 10 min at room temperature.
10. Remove up to 1 ml of the supernatant.
11. Resuspend the cell pellet on a vortex mixer and add 6 ml of ice-cold fixative, drop by drop, while swirling to prevent cell clumping.
12. Centrifuge at 250*g* for 10 min at room temperature, and repeat the fixative addition step two more times. At this point the fixative can be added more quickly to the suspension.
13. After the last fixative addition step, remove enough fixative so that the resuspended cell solution appears slightly cloudy.
14. The cell suspension is now ready for slide making or may be stored.

■ **PAUSE POINT** The cell suspension can be stored at –20 °C for several years. If cells were stored for more than 4 weeks, repeat steps 12 and 13.

**Slide preparation**

15. Wipe the slides with a tissue soaked in 70% (vol/vol) ethanol on both sides. Arrange the slides alternately straight across and diagonally in a 150-ml slide staining dish. Rinse the slides with the detergent solution Mucasol in hot water and cover. Let the slides sit overnight at room temperature. Remove the washing solution and rinse the slides with water for 15 min. Rinse two more times with purified water and fill the dish with sterile water for injection. The slides are now ready to use and should be kept in water at 4 °C until use.

■ **PAUSE POINT** The slides can be kept at 4 °C for 4 weeks.

**Slide making**

16. Preheat a laboratory oven to 80 °C and place a beaker inside with 50 ml of $dH_2O$ to increase humidity.

▲ **CRITICAL STEP** Optimal environmental conditions to ensure good slide quality are 45–50% humidity and a temperature of 20–22 °C.

17. Place the centrifuge tube with the hiPSC suspension on ice.
18. Cut off the end of a pipette tip from a 100-µl pipette and use it to resuspend the cells by gently pipetting.
19. Take a slide from step 15 out of the Coplin jar and place 80–100 µl of hiPSCs onto the wet slide from a height of ~20 cm.
20. Wipe off excess fluid from the back of the slide, label the slide and place it in the oven on top of the beaker until all the fixative has evaporated.
21. Check the cell density and metaphase spreading using a phase-contrast microscope.

**? TROUBLESHOOTING**

22. Incubate the slides before staining in a laboratory oven at 95 °C for 15 min or keep them at room temperature for several days.

**Trypsin G-banding**

23. For Trypsin G-banding (GTG-banding), prepare three Coplin jars, one with 0.25% (vol/vol) trypsin in HBSS (jar 1), another with 1× PBS (jar 2) and a final jar containing 10% (vol/vol) Giemsa Stain in Titrisol, pH 7.2 (jar 3).
24. Place the slide into jar 1 for 5 s. Then dab the slide onto a wiper to remove excess trypsin.
25. Dip the slide into jar 2 twice and dab the slide onto a wiper to remove excess fluid.
26. Place the slides into jar 3 for 6 min.
27. Rinse the slide with cold water.
28. Use a wiper to clean the backs of the slides, and then let the slides air-dry for a few minutes.

**Analysis of chromosomes**

29. Cover the slides with a coverslip and Cytoseal 60. For each cell line, analyze 15 metaphases under a light microscope at a 1,000× magnification.

▲ **CRITICAL** For analysis of the chromosomes, a banding resolution of at least 400 b.p.h. (bands per haploid) is generally required, and the chromosomes should be well separated to permit their clear visualization.

**? TROUBLESHOOTING**

30. Choose the best mitotic spreads for karyotypes. Long chromosomes, few overlaps and clear bands are important criteria for judging good spreads. At least four metaphases of each cell line should be karyotyped using an appropriate cytogenetic image analysis system.

**? TROUBLESHOOTING**

**17|** Centrifuge the cell suspension at 4 °C for 5 min at 200*g*, and discard the supernatant.

**18|** In one FACS tube, resuspend 2 × $10^5$ cells in 100 µl of FACS buffer containing the directly labeled antibody (e.g., FITC-anti-human TRA-1-60 (1:5 dilution) or PE-anti-SSEA-3 (1:5 dilution)) and in another FACS tube resuspend 2 × $10^5$ cells in 100 µl of FACS buffer with the respective isotype control (e.g., FITC mouse IgM, κ isotype (1:200 dilution) or PE rat IgM, κ isotype (1:80 dilution)).

**19|** Incubate the cells for 30 min at 4 °C.

**20|** Wash the cells with 2.5 ml of FACS buffer and centrifuge at 200*g* for 5 min at 4 °C; discard the supernatant and repeat the wash.

**21|** Resuspend the cells in 150 µl of PBS.

**22|** Analyze the cells with a flow cytometer, adjusting the gates according to the isotype control (**Supplementary Fig. 2**). If the cells express pluripotency markers, continue with EB formation (Step 23).

**Embryoid body formation ● TIMING 2 h per cell line and 10 flasks, plus 1 d for incubation**

**23|** Expand the hiPSCs from Step 22 in T80 flasks to 80% confluency.

**24|** Add 10 µM Y-27632 to the T80 flasks containing 80% confluent hiPSCs in FTDA.

**25|** Incubate the hiPSCs for 1 h at 37 °C.

**26|** Remove the FTDA.

**27|** Wash the hiPSCs twice with 10 ml of PBS.

**28|** Incubate the hiPSCs with 0.5 mM EDTA for 5–10 min at room temperature (1 ml of EDTA per 10-$cm^2$ growth surface). **▲ CRITICAL STEP** Monitor the hiPSCs during EDTA incubation because detachment varies between cell lines. If the hiPSCs detach very easily, shortening of the incubation time might be necessary to avoid cell loss. At the same time, make sure that you obtain a single-cell suspension.

**29|** Remove the EDTA solution.

**30|** Add 1 ml of warm PBS per 10-$cm^2$ growth surface.

**31|** Tap the flasks on the bench to detach the hiPSCs.

**32|** Triturate the cells three times with a 10-ml pipette.

**33|** Transfer the hiPSCs to a 50-ml Falcon tube containing 10 ml of EB formation medium.

**34|** Rinse the cell culture flasks with 5 ml of $Ca^{2+}$-containing RPMI medium and add it to the cell suspension from Step 33.

**35|** Centrifuge the hiPSCs at 250*g* for 5 min at room temperature.

**36|** Resuspend the hiPSCs in 10 ml of EB formation medium by triturating five times with a 10-ml pipette.

**37|** Count the hiPSCs.

**38|** Resuspend the hiPSCs in the EB formation medium at a concentration of 30 × $10^6$ cells per 100 ml.

**39|** Add the hiPSC suspension to the spinner flask.

**40|** Incubate the flask overnight at 37 °C, 5% $CO_2$, 5% $O_2$, 90% humidity, rotating at 40 r.p.m.





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**Induction of mesodermal progenitor cells ● TIMING 1 h per spinner flask, plus 3 d for cultivation**

**41|** Transfer 50 ml of the EB suspension from the spinner flask to a Falcon tube.

**42|** Transfer the rest of the EB suspension to T175 flasks (a maximum of 200 ml per flask).

**43|** Place the T175 cell culture flasks on a prewarmed V-shaped sedimentation rack.

**44|** Put the rack back in the incubator, and let the EBs sediment in the lower corner of the flask for 5–20 min (maximum), depending on EB (~100–300 µm).
▲ **CRITICAL STEP** Once sedimented, process the EBs as soon as possible.

**45|** Remove the supernatant from the 50-ml Falcon tube, add 5 ml of washing medium, transfer the EBs to a 15-ml Falcon tube with a marked scale to estimate the EB volume (**Fig. 6**), and calculate the total EB volume by extrapolating to the total volume of EB formation medium.
▲ **CRITICAL STEP** EBs should not be left in the Falcon tube for more than 5 min after sedimentation.

**46|** Remove 90% of EB formation medium in the T175 flask from Step 44, and add the EBs from the Falcon tube. If there is more than one flask, you can pool all the EBs together.

**47|** Wash the EBs with prewarmed washing medium.

**48|** Remove at least 90% of the washing medium, and resuspend the EBs carefully in mesoderm induction medium.

**49|** Transfer the EBs to Pluronic-F-127-coated suspension cell culture flasks. If you are using a T75 cell culture flask, transfer 50–100 µl of EBs in 20 ml of mesoderm induction medium; if you are using a T175 cell culture flask transfer 150–250 µl of EBs in 40 ml of mesoderm induction medium.
▲ **CRITICAL STEP** If you have more than one T175 flask per cell line, check the volume of medium in which the EBs are kept, divide it by the number of flasks you need and then remove the volume for one flask at a time to make sure that the EBs are distributed equally.

**50|** Incubate the flasks for 3 d at 37 °C, 5% $CO_2$, 5% $O_2$ and 90% humidity. Change the medium daily.
**? TROUBLESHOOTING**

**51|** For medium change, place the T175 cell culture flask on a V-shaped sedimentation rack.

**52|** Let the EBs sediment in the lower corner of the flask (~5 min).

**53|** Remove 50% of the medium (20 ml).

**54|** Add 20 ml of medium/200 µl of EB volume.

**55|** Place the flasks back into the incubator.

**Cardiac differentiation ● TIMING 1 h per cell line and 10 flasks, plus 10 d for cultivation**

**56|** Place the T175 cell culture flask on a V-shaped sedimentation rack.

**57|** Let the EBs sediment in the lower corner of the flask (5 min).
▲ **CRITICAL STEP** Once sedimented, process the EBs as soon as possible.

**58|** Remove 90% of mesoderm induction medium and add 20 ml of washing medium. Let the EBs sediment.

**59|** Remove 90% of washing medium and add 5 ml of cardiac differentiation medium I.

**60|** Transfer the EBs to a 15-ml Falcon tube.

**61|** Let the EBs sediment.

**62|** Estimate the EB volume with the aid of the scale on the 15-ml Falcon tube (**Fig. 6**).
▲ **CRITICAL STEP** Use 15-ml tubes with a marked scale to facilitate estimation of EB volume (**Fig. 6**). Once sedimented, process the EBs as soon as possible. EBs should not be left in sediment in the Falcon tube for >5 min.

**63|** Transfer the EBs to Pluronic-F-127-coated cell culture flasks. If you are using a T75 flask, transfer 80–150 µl of EBs in 20 ml of medium; if you are using a T175 flask, transfer 200–250 µl of EBs in 46 ml of medium.

**64|** Incubate the cells for 3 d. Leave the cells for 1 d and on days 2 and 3 change the medium (50% medium exchange). Incubate the cells at 37 °C, 5% $CO_2$, 21% $O_2$ and 90% humidity.
▲ **CRITICAL STEP** When changing the medium, ensure that the EBs are not left in sediment and are processed as soon as possible.
**? TROUBLESHOOTING**

**65|** Switch to cardiac differentiation medium II, and incubate the cells for an additional 4 d with daily medium changes (50% medium exchange). Incubate the cells at 37 °C, 5% $CO_2$, 21% $O_2$ and 90% humidity.
▲ **CRITICAL STEP** When changing the medium, ensure that the EBs are not left in sediment and are processed as soon as possible.
**? TROUBLESHOOTING**

**66|** Switch to cardiac differentiation medium III and incubate the cells for an additional 3 d with daily medium changes (50% medium exchange). Incubate the cells at 37 °C, 5% $CO_2$, 21% $O_2$ and 90% humidity.
▲ **CRITICAL STEP** When changing the medium, ensure that the EBs are not left in sediment and are processed as soon as possible.
**? TROUBLESHOOTING**

**Generation of human EHT ● TIMING 1 d, plus 2–3 weeks for maintenance**
**67|** For dissociation of hiPSC-CMs, remove cardiac differentiation medium III.

**68|** Wash the hiPSC-CMs carefully with HBSS twice.

**69|** Add 1 ml of collagenase II solution per 10-$cm^2$ growth surface.

**70|** Incubate the mixture for 3–4 h at 37 °C, 5% $CO_2$, 21% $O_2$ and 90% humidity. During this time, monitor the progress of cell dissociation microscopically.

**71|** In the meantime, prewarm DMEM to 37 °C, prepare dissociation medium and have sterile Teflon spacers, sterile PDMS racks, fibrinogen stock, thrombin aliquots, 24-well plates, liquid sterile agarose (keep in the heated cabinet at 60 °C), 2× DMEM, EHT medium and a cell counting instrument ready to use for the generation of EHTs.

**72|** Transfer the dissociated hiPSC-CMs to a 50-ml Falcon tube.

**73|** Flush the cell culture flask with dissociation medium and place the medium in the same Falcon tube.

**74|** Centrifuge the hiPSC-CMs for 10 min at 100*g* at room temperature.
▲ **CRITICAL STEP** Cardiomyocytes are sensitive to centrifugation at high speed. Do not centrifuge faster than 100*g*.

**75|** Discard the supernatant and resuspend the hiPSC-CMs in 20 ml of DMEM, or in 40 ml of medium (for a larger pellet to aid cell counting).

**76|** Count the cells with an automated cell counter.
▲ **CRITICAL STEP** Cell counting, especially with a Neubauer chamber, is difficult to standardize. We suggest using an automated procedure for cell counting.
**? TROUBLESHOOTING**

**77|** Centrifuge the hiPSC-CMs at 100*g* for 10 min at room temperature. After this step, continue to the next step to prepare the fibrinogen containing the master mix solution and the casting molds for EHT generation. Alternatively, follow **Box 4** for cryopreservation and thawing of cardiomyocytes or **Box 5** for FACS analyses of dissociated hiPSC–cardiomyocytes.
▲ **CRITICAL STEP** Cardiomyocytes are sensitive to centrifugation at high speed. Do not centrifuge faster than 100*g*.





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## Box 4 | Freezing and thawing of cardiomyocytes ● TIMING 30 min, plus 4 h for dissociation of cardiomyocytes

▲ CRITICAL Volumes are given for one cryovial. When thawing two or three vials of the same cells, pool the cell suspension before adding the rinse and final volumes of medium. Adjust the media volumes accordingly.

1. Dissociate the cardiomyocytes as described in the 'Generation of human EHT' section (Steps 67–76).
2. Resuspend the cardiomyocyte pellet in cold freezing medium for hiPSC-CMs. Use 1 ml of freezing medium per cryovial. Freeze at least 2 million hiPSC-CMs per vial.
3. Distribute the cells into cryovials. Avoid making bubbles or touching the cryovial cap.
4. Place the cryovials into a cryobox.
5. Place the cryobox at –80 °C for 12–24 h.
6. Transfer the cryovials to a –150 °C freezer or liquid nitrogen.

■ PAUSE POINT The cells can be stored at –150 °C for several years.

7. For thawing of cardiomyocytes, remove the cryovial from the storage tank (–150 °C).
8. Warm one vial at a time in your hand until the freezing medium is almost completely thawed.

▲ CRITICAL STEP Do not thaw more than three vials of cardiomyocytes at one time.

9. If necessary, cryovials can be placed on dry ice for up to 10 min before thawing.
10. Gently transfer the cryovial cell suspension to a sterile 50-ml Falcon tube using a 1-ml pipette.
11. Rinse the empty cryovial with 1 ml of room-temperature RPMI to recover any residual cells from the vial.
12. Transfer the 1 ml of RPMI from the cryovial dropwise over 90 s to the 50-ml Falcon tube containing the cell suspension.

▲ CRITICAL STEP Dropwise addition of medium to the cell suspension is critical in order to minimize osmotic shock.

13. Gently swirl the tube while adding the medium.
14. Slowly add 8 ml of room-temperature RPMI to the 50-ml Falcon tube. Add the first 1 ml dropwise over 30–60 s. Then add the remaining 7 ml over 30 s. Gently swirl the tube while adding the medium.
15. Gently mix the Falcon tube by inverting two to three times.

▲ CRITICAL STEP Avoid pipetting or vortexing.

16. Count the cells within the cell suspension.

? TROUBLESHOOTING

17. Centrifuge the cell suspension to pellet the cardiomyocytes at 100*g* for 5 min at room temperature before EHT generation (Steps 78–92).

**78|** Prepare the master mix based on the cell count. Calculate the number of EHTs that will be generated ($1.0 \times 10^6$ per EHT) (see table below for guidance).

| | Per EHT | Per 24-well plate |
|---|---|---|
| NCM medium | 81.9 µl | 1,965.6 µl |
| Fibrinogen stock | 2.5 µl | 60.0 µl |
| Matrigel Basement Membrane Matrix | 10.0 µl | 240.0 µl |
| 2× DMEM | 5.5 µl | 132.0 µl |
| Y-27632 | 0.1 µl | 2.4 µl |
| Dissociated cells | $1.0 \times 10^6$ | $24 \times 10^6$ |
| Total | 100.0 µl | 2,400.0 µl |

**79|** Resuspend the corresponding volume of fibrinogen stock in cold NCM.

▲ CRITICAL STEP Fibrinogen must be room temperature for pipetting. Dissolve the fibrinogen thoroughly.

**80|** Add Y-27632 (final concentration 10 µM).

**81|** Add 2× DMEM.

**82|** Add Matrigel Basement Membrane Matrix.





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## Box 5 | FACS analyses of dissociated hiPSC–cardiomyocytes ● TIMING 3 h, plus 4 h for dissociation of cardiomyocytes

1. Dissociate the cardiomyocytes as described in the 'Generation of human EHT' section (Steps 67–76).
2. Prepare two FACS tubes, each with 200,000 freshly dissociated hiPSC-CMs.
3. Wash with 3 ml of PBS, centrifuge for 5 min at 200*g* at room temperature and discard the supernatant. Repeat this wash step.
4. Resuspend the cells in 500 µl of cold methanol (–20 °C) and fix the cells for 20 min on ice.
5. Wash twice with 500 µl of FACS buffer.
6. For further permeabilization, resuspend the fixed cells in 500 µl of FACS buffer and incubate for at least 45 min at 4 °C.
7. Staining of intracellular antigens: resuspend permeabilized cells from one FACS tube in 100 µl of FACS buffer containing the primary or directly labeled antibody (e.g., anti-cardiac Troponin T-FITC, 1:10 dilution) and the resuspend the cells from the other tube with the respective isotype control (e.g., REA Control (I)-FITC). Incubate for 30 min at 4 °C.
8. Wash twice with 2.5 ml of FACS buffer.
9. If you are using a secondary antibody, repeat steps 7 and 8 with the secondary antibody.
10. Resuspend the cells in 150 µl of PBS.
11. Analyze the cells with a flow cytometer, adjusting the gates according to the isotype control (**Supplementary Fig. 2**).

**83|** Pipette up and own thoroughly until the fibrinogen is dissolved.

**84|** Resuspend the cell pellet in the fibrinogen containing the master mix and keep it on ice.
▲ **CRITICAL STEP** Cardiomyocytes are sensitive to shear stress and repeated resuspension. Prepare the fibrinogen containing the master mix and resuspend the hiPSC-CMs directly in the master mix.
▲ **CRITICAL STEP** Avoid air bubble formation during pipetting.
▲ **CRITICAL STEP** Calculate 10% extra volume to compensate for loss during pipetting.

**85|** *Generation of casting molds*. Pipette 1.6 ml of liquid agarose into 8 wells of a 24-well plate. Place the Teflon spacers onto these wells directly after pipetting, as agarose solidifies very quickly. Repeat with the following wells. Let the agarose solidify at room temperature (10–15 min).
▲ **CRITICAL STEP** Prolonged solidification results in fine cracks in the surface of the agarose. This makes the casting molds leaky and decreases the efficiency of EHT generation.

**86|** Remove the Teflon spacers.

**87|** Position the PDMS racks on the 24-well plate such that each pair of PDMS posts is positioned within one agarose casting mold.

**88|** *Pipetting of EHTs*. Mix 100 µl of master mix with one thrombin aliquot (3 µl) briefly. Pipette the mixture quickly into an agarose casting mold with the PDMS racks placed on top. Repeat this step for each EHT separately.
▲ **CRITICAL STEP** Use a new filter pipette tip for pipetting each EHT. Resuspend the master mix carefully after 4–8 EHTs. Avoid air bubble formation during pipetting, by pressing the pipette only until the first pressure point.

**89|** Place the 24-well plate in the incubator at 37 °C, 7% $CO_2$, 40% $O_2$ and 90% humidity for 120 min.

**90|** Supplement the fibrin gels in the casting molds with 200–300 µl of DMEM per well.
▲ **CRITICAL STEP** Adding the medium on top of the EHT before transfer will ease the removal from the casting molds and improve the efficiency of EHT generation.

**91|** Incubate the gels for an additional 10 min.

**92|** Prepare a 24-well plate with prewarmed EHT medium (1.5 ml per well), and transfer the PDMS racks with fibrin gels carefully from the casting mold plate to the new cell culture plate.

**93|** Incubate the EHTs at 37 °C, 40% $O_2$, 7% $CO_2$ and 90% humidity for at least 2 weeks.
**? TROUBLESHOOTING**





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**94|** *EHT medium change*. Perform medium changes on Mondays, Wednesdays and Fridays. To do this, prepare a second 24-well plate with new EHT medium, and transfer the PDMS rack/EHTs carefully.

Make sure that the EHTs do not touch the edges of the wells while doing this.

▲ **CRITICAL STEP** Use two 24-well plates for the entire batch of EHTs and refill with new medium to transfer the EHTs back and forth between the two plates.

**95|** Monitor the EHTs during development by observing single-cell contraction and coherent contraction of small areas of cells microscopically and coherent contraction of entire EHTs by video–optical analysis in the following steps.

**? TROUBLESHOOTING**

**Video–optical analysis of contractile force ● TIMING 20 min for a baseline measurement of one 24-well plate—20 s per EHT**

**96|** 1 h before the experiment, turn on the hardware, heating, gas supply ($O_2$, $N_2$ and $CO_2$), axis system, LEDs and computer.

**97|** Make sure that the temperature, humidity and $CO_2$ in the internal incubator box have reached target values. We recommend using 37 °C, 90% humidity and 7% $CO_2$.

**98|** Start the software.

**99|** Place the 24-well cell culture plate with the EHTs into the internal incubator box on top of the LED panel on the small metal pins.

**100|** Use the manual 'EHT contractility analysis instrument' to set up the experiment.

**101|** Define the parameters for contractility analysis. Standard parameters for human EHT are as follows: peak force, 4; filter level, 10; baseline level, 0.95; force threshold, 0.02–0.05 mN; minimum factor, 0.2; maximum peak dist., 60 s; contraction velocity/relaxation velocity (CV/RV) threshold factor, 0.2.

**102|** Define the top and bottom reference positions (blue crosses) for each EHT per well by optimizing the *xyz* coordinates.

**103|** Start the measurement. Details in regard to the analysis of data are provided in the manual of the EHT contractility analysis instrument.

**? TROUBLESHOOTING**

Critical steps throughout this protocol are emphasized for each differentiation step. Low differentiation efficiencies can be the result of diverse problems that must be excluded systematically. As mentioned above, the quality of hiPSC culture is the most important prerequisite for efficient differentiation. Undesired spontaneous differentiation of hiPSCs may occur in cultures that are either too sparsely or too densely populated. 80% confluency is the optimal cell density for starting a differentiation. In addition, the passage number may have an impact on hiPSC quality. Usually it takes some passages after thawing before the hiPSCs show their normal growth behavior. On the other hand, mutations may accumulate over time. We regularly perform karyotyping and dispose of cultures with a passage number >100. Therefore, it is important to maintain a large cell bank of hiPSCs with a low passage number. Media composition is also critical to maintaining the stem cells' undifferentiated state. FTDA contains bFGF, TGFβ1 and activin A, which cooperatively support proliferation, whereas dorsomorphin inhibits spontaneous differentiation[14]. bFGF is not stable at 37 °C, which leads to large fluctuations in bFGF levels in the culture medium[59]. To improve the efficiency of this essential growth factor, we use bFGF which has an increased thermostability and higher resistance to proteases, and which retains the same biological properties as naturally occurring bFGF[20]. This growth factor variant is also advantageous for the induction of mesodermal progenitor cells. We observed that the absence of bFGF during mesoderm induction is detrimental to the differentiation efficiency.

Insulin, in contrast, inhibits differentiation of hiPSCs to mesodermal progenitor cells[54]. Therefore, it is added only during the progression of mesodermal cells toward cardiomyocytes (day 7 until day 11). During this stage, potent small-molecule inhibitors of canonical WNT signaling promote cardiac differentiation.

With regard to handling of the EBs, it is important to keep all processing steps as short as possible and to avoid long resting times in the sedimented state. In addition, EBs must be pipetted carefully at early steps and should not be centrifuged, because this will lead to disaggregation of the cell clusters.

Handling of cardiomyocytes must be performed carefully as well, as they are sensitive to shear stress and more susceptible to high-speed centrifugation than other cells. Therefore, dissociation of differentiated cardiomyocytes and generation of EHTs must be carried out in a conscientious manner. EHTs can also be generated from cryopreserved cardiomyocytes. Viability after thawing is usually 80–90% (**Fig. 1c**). With regard to EHT generation, it is important to prepare the master

mix on ice, to avoid bubbles during pipetting and to use heat-inactivated serum to avoid dissolving of the fibrin matrix. Overall, EHT generation is a very robust process if differentiation efficiency is sufficient and cardiomyocyte viability is high.

Further troubleshooting advice can be found in **Table 3**.

**TABLE 3** | Troubleshooting table.

| Step | Problem | Possible reasons | Solution |
|---|---|---|---|
| 8: Thawing of hiPSCs | Poor attachment of hiPSCs to Geltrex-coated plates | No ROCK inhibitor Y-27632 was included in FTDA medium | Add Y-27632 to the FTDA medium |
| 8: Cultivation of hiPSCs | Spontaneous differentiation of hiPSCs | Cultures were too sparsely or too densely populated cultures | Adjust passage ratio |
| | | Growth factors with low activity | Always prepare fresh FTDA |
| | hiPSCs are proliferating very slowly | After thawing, hiPSCs must be passaged several times before they show their normal growth behavior | Wait for some passages to occur |
| | | Mycoplasma infection | Perform mycoplasma test |
| | | Karyotype abnormalities | Perform karyotyping |
| | | Imperfect coating | Passage hiPSCs to newly coated plates/flasks |
| 14: Passaging of hiPSCs | Many dead cells | EDTA incubation lasted too long | Monitor the cells under a microscope and stop dissociation at the right time |
| | | An enzyme was used for dissociation | Enzyme-free dissociation is more gentle and increases the likelihood of cell survival |
| **Box 3**, step 21: Karyotyping | Cell density was too high; (ii) | Volume was not sufficient for the number of cells | Dilute the suspension with a few drops of ice-cold fixative |
| | Cell density was too low | The number of cells was too low for the volume used | Centrifuge at 250*g* for 10 min at 4 °C and resolve the pellet in a smaller volume of fixative |
| | Chromosomes do not show satisfactory spreading | Humidity and/or temperature was unfavorable for spreading | Try to adjust the humidity to 45–50% and keep the slide at 20 °C–30 °C while placing the drops on the slide |
| **Box 3**, step 30: Karyotyping | Staining does not give a clear distinction between pale staining and dark staining of different chromosomal regions | Trypsin was incubation too long/short | Adjust trypsin treatment time |
| | | Giemsa staining was not sufficient | Optimize Giemsa staining |
| 50 and 64–66: Differentiation of cardiomyocytes | EBs are dissolving | EBs were kept sedimented too long | Keep transfer steps as short as possible—e.g., 50% medium exchange within a differentiation step |
| | | EBs were processed carelessly | Pipette EBs carefully and do not centrifuge |
| 66: Differentiation of cardiomyocytes | No spontaneously contracting cells on day 14 | Poor-quality hiPSC culture | See solutions concerning problems during cultivation of hiPSCs |
| | | Low bFGF activity during EB formation and/or mesoderm induction | Use stable bFGF and add it directly before use of medium |
| 76: Dissociation of cardiomyocytes | Many dead cardiomyocytes | Incubation in collagenase solution lasted too long | Monitor dissociation microscopically |
| | | Cardiomyocytes were processed carelessly | Pipette cardiomyocytes carefully and do not centrifuge faster than 100*g* |
| 93: Generation of human EHT | EHTs are dissolving | Active serum was used for one of the components or the medium | Only use heat-inactivated serum |
| | | Reduced activity/degradation of aprotinin | Replace aprotinin |

(continued)





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**TABLE 3** | Troubleshooting table (continued).

| Step | Problem | Possible reasons | Solution |
|---|---|---|---|
| 95: Generation of human EHT | EHTs are not contracting on day 15 | Differentiation efficiency was too low | Only use differentiated cell populations with ≥60% troponin-T-positive cells |
| **Box 4**, step 16: Thawing of cardiomyocytes | Many dead cardiomyocytes after thawing | Freezing medium was unsuitable<br>Freezing process damaged cardiomyocytes<br>Thawing process damaged cardiomyocytes | Use 90% (vol/vol) FBS plus 10% DMSO (vol/vol) for freezing cardiomyocytes<br>Always use cryoboxes, freeze at –80 °C and store at –150 °C<br>Thaw cardiomyocytes as quickly as possible, but add medium slowly to minimize osmotic shock |



## ● TIMING

Steps 1–7, thawing of hiPSCs: 30 min
Step 8, daily maintenance of hiPSCs: 4 d
Steps 9–14, passaging of hiPSCs: 10 min
Steps 15–22, FACS analysis of hiPSCs: 90 min
Steps 23–66, differentiation of cardiomyocytes: 14 d
Steps 23–40, EB formation: 2 h per cell line and 10 flasks, plus 24-h for incubation
Steps 41–49, induction of mesodermal progenitor cells: 1 h per spinner flask
Steps 50–55, induction of mesodermal progenitor cells: 3 d
Steps 56–63, cardiac differentiation: 1 h per cell line and 10 flasks
Steps 64–66, cardiac differentiation: 10 d
Steps 67–92, generation of human EHT: 1 d
Steps 93–95, maintenance of human EHT: 14 d
Steps 96–103, video–optical analysis of contractile force: 20 min for baseline measurement
**Box 1**, freezing of undifferentiated hiPSCs: 15 min
**Box 2**, mycoplasma screening and treatment: 3.5 h or up to 2 weeks
**Box 3**, karyotyping of hiPSCs: 2.5 h, plus analysis of arresting cells in metaphase
**Box 4**, freezing and thawing of cardiomyocytes: 30 min, plus 4 h for dissociation of cardiomyocytes
**Box 5**, FACS analyses of dissociated hiPSC-cardiomyocytes: 3 h, plus 4 h for dissociation of cardiomyocytes

## ANTICIPATED RESULTS

This protocol describes how to perform efficient differentiation of cardiomyocytes and subsequent EHT generation from hiPSC lines. Suspension culture facilitates upscaling because, in contrast to 2D cultures, the number of cultivated cells is not limited to the growth surface, and this allows differentiation of high cell numbers. Differentiation efficiencies and efficacies for several control and disease-specific cell lines are given in **Table 2**. High-quality hiPSC culture, free of differentiated cells, is critical to successful cardiac differentiation. One T80 cell culture flask of hiPSCs grown to 80% confluency contains ~15 × $10^6$ hiPSCs. A few hours after growth of the hiPSC suspension in the spinner flasks, EB formation can be observed. 60 × $10^6$ hiPSCs give rise to an average EB volume of 300 µl (**Table 1**). During induction of mesodermal progenitor cells, there is a decline in EB volume because of apoptotic cells (**Table 1**). However, we have observed that this has no adverse effects on the outcome of the differentiation. The first spontaneously contracting EBs can generally be observed between days 8 and 10, depending on the cell line and quality of the starting hiPSC culture. **Supplementary Videos 1–3** show hiPSC-CMs in EB format. The time point of first beating activity is a good indicator of the differentiation efficiency, with earlier onset (day 8) in cell populations with higher cardiomyocyte content. A successful differentiation experiment results in 18–174 × $10^6$ cardiomyocytes (70–97% Troponin T-positive; **Fig. 1** and **Supplementary Fig. 1**) from 60 × $10^6$ hiPSCs, depending on cell line and quality of undifferentiated hiPSCs (**Table 2**). Directly after differentiation, hiPSC-CMs express MLC2a (refs. 7,9). Bright-field images at different time points of cardiac differentiation are shown in **Figure 5**.

For the successful development of EHTs, the differentiation efficiency should be at least 60%, as we observed a correlation between cardiomyocyte purity and force development. With an increasing number of noncardiomyocytes such as fibroblasts in the input cell population, EHTs shrink rapidly after casting and do not start contracting. For some disease-specific cell lines with mutations leading to weaker contraction of the cardiomyocytes, a higher differentiation efficiency than that needed for healthy cardiomyocytes can be necessary for sufficient EHT performance. In contrast to cardiomyocyte purity, the differentiation method does not influence EHT performance. We recently used commercial hiPSC-CMs (CDI iCell; Axiogenesis Cor.4U)

**TABLE 4** | Details of contraction parameters.

| Cell line | Frequency (b.p.m.) | Force (mN) | T1 contraction time (s) | T2 relaxation time (s) |
|---|---|---|---|---|
| Control 1, *n* = 75/77 | 61 ± 17 | 0.152 ± 0.052 | 0.120 ± 0.017 | 0.163 ± 0.026 |
| Control 2, *n* = 46/3 | 92 ± 31 | 0.138 ± 0.019 | 0.174 ± 0.083 | 0.215 ± 0.049 |
| Control 3, *n* = 23/3 | 61 ± 15 | 0.115 ± 0.044 | 0.151 ± 0.024 | 0.238 ± 0.085 |

Replicate number is the number of EHTs/number of batches. Intra-batch variability is negligible. Values are given as ± s.d. Age: control 1: 20–25 days; control 2: 15–20 days, control 3: 20–30 days. Measurements were done in culture medium (control 1 and 2) or Tyrode's solution (1.8 mM $Ca^{2+}$, Ctr. 3). Data for control 1 were taken from Mannhardt *et al*[7]. T1 is time of 20% to peak force; T2 is time of peak force to 80% relaxation (=20% above baseline).

for comparison with our in-house cell line and observed no differences in contractile function of the EHTs[7]. Normally, 2–5 d after EHT generation, single-cell contractions can be observed. Between 5 and 10 d, small clusters of hiPSC-CMs begin contracting, and coherent contraction of the entire EHT generally occurs between days 10 and 15. We usually perform experiments and video–optical analysis between days 14 and 28, although EHTs show a high robustness in long-term culture (up to day 100; **Supplementary Video 8**). Details of contraction parameters are given in **Table 4**. On average, 95% of cast EHTs can be analyzed. Sometimes single EHTs detach from the post while the PDMS rack is being transferred from the casting molds to the culture plate or cannot be analyzed because of enclosed air bubbles. **Supplementary Videos 5–8** show EHTs from control cell lines. After casting, EHTs are progressively remodeled, and after 3 weeks in culture they have a diameter of 223.4 ± 10.4 µm (*n* = 10), a length of 6.09 ± 0.23 mm (*n* = 10) and an estimated volume of 0.24 ± 0.03 $mm^3$ (*n* = 10). Over time of EHT cultivation, an increase in cell length, longitudinal orientation and network formation, paralleled by a reduction of extracellular space, can be observed histologically[60]. Myosin light-chain expression changes during cultivation from MLC2a to MLC2v (refs. 7,9), indicating maturation. hiPSC-CMs possess L-type $Ca^{2+}$ currents ($I_{Ca,L}$) as high as those in human adult cardiomyocytes, but in contrast to adult cardiomyocytes T-type $Ca^{2+}$ currents ($I_{Ca,T}$) coexist. Furthermore, serotonin does not increase $I_{Ca,L}$ values in hiPSC-CMs, indicating an immature ventricular phenotype[8].

*Note: Any Supplementary Information and Source Data files are available in the online version of the paper.*

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**AUTHOR CONTRIBUTIONS** K.B. designed and performed the experiments, analyzed the data and wrote the paper. T.S., I.M., B.U., M.C.R., T.W., A.B., D.L.-B., B.K., M.P., G.M., S.L., A.S., D.S., S.F., C.N., E.K., U.S., M.L.S. and M.L.R. contributed to the development or validation of this protocol. A.H. supervised the project. A.H. and T.E. wrote and approved the final paper.

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