

Cardiomyocyte differentiation from PSC suspension culture

This protocol is for culture of human pluripotent stem cells (hPSCs) and differentiation of hPSCs to cardiomyocytes in 6-well shaking plates. Use of other vessels requires optimization of the stirring speed to maintain discrete spheroids of favorable size. It will also be necessary to determine an appropriate stirring speed when using equipment other than what is listed here. Please see the Gibco™ StemScale™ PSC Suspension Medium user guide (thermofisher.com/stemscale) for help with speed conversion.

In this protocol, cardiomyocyte differentiation is initiated by the GSK-3 α/β inhibitor CHIR-99021. The results are very sensitive to changes in concentration of this

small molecule, and the optimal concentration may be dependent on cell line. Before attempting differentiation of a new cell line, we recommend following the support protocol to test a range of CHIR-99021 concentrations in small cultures.

Before beginning, the hPSC line should be cultured in suspension for at least one passage in StemScale PSC Suspension Medium (Cat. No. A4965001). One well of a 6-well plate of suspension culture should yield enough hPSCs to inoculate at least 12 mL of StemScale medium at 0.15×10^6 cells/mL.

Materials

Product	Source	Quantity	Cat. No.
Gibco™ RPMI 1640 Medium (ATCC modification)	Thermo Fisher Scientific	3 x 500 mL	A1049101
Gibco™ B-27™ Supplement, XenoFree, minus insulin	Thermo Fisher Scientific	10 mL	A3695201
Gibco™ B-27™ Supplement (50X), serum free	Thermo Fisher Scientific	10 mL	17504044
Gibco™ Antibiotic-Antimycotic (100X)	Thermo Fisher Scientific	20 mL	15240096
Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent	Thermo Fisher Scientific	100 mL	A1110501
CHIR-99021 HCl	Selleck Chemicals	5 mg	S2924
IWP-2	Selleck Chemicals	10 mg	S7085
ROCK inhibitor Y-27632 2HCl	Selleck Chemicals	10 mg	S1049

Equipment and cultureware

Product	Source	Cat. No.
Thermo Scientific™ CO ₂ Resistant Shaker, 100–240 V, US	Thermo Fisher Scientific	88881101
Thermo Scientific™ Nunc™ Non-treated Multidishes, 6-well plates	Thermo Fisher Scientific	150239

Antibodies

Product	Source	Cat. No.
Invitrogen™ Sarcomeric Alpha Actinin Monoclonal Antibody	Thermo Fisher Scientific	MA1-22863
Anti-Cardiac Troponin T Rabbit Polyclonal Antibody	Abcam	AB45932
Invitrogen™ Donkey Anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488	Thermo Fisher Scientific	A21202
Invitrogen™ Donkey Anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 594	Thermo Fisher Scientific	A21207

Chemical stocks

10 mM CHIR-99021 stock: To 5 mg of CHIR-99021, add 996 µL of DMSO aseptically. Mix well. Aliquot and freeze at –20°C. Thaw each aliquot only once and discard any remnant.

5 mM IWP-2 stock: Warm 4.29 mL of DMSO to 50°C in a water bath. Add warm DMSO to 10 mg of IWP-2 aseptically, and mix by pipetting until clear. Aliquot and freeze at –20°C. Thaw each aliquot only once and discard any remnant.

10 mM Y-27632 stock: To 10 mg of Y-27632 2HCl, add 3.1225 mL of sterile distilled water aseptically. Mix until clear. Aliquot and freeze at –20°C. Thaw each aliquot only once and discard any remnant.

Media

RPMI/B-27 Supplement, minus (–) insulin: Thaw a 10 mL bottle of B-27 Supplement, XenoFree, minus insulin (Cat. No. A3695201), either overnight at 4°C or briefly in a 37°C water bath. Mix the supplement well with a sterile pipette and transfer it into 500 mL of RPMI 1640 Medium (Cat. No. A1049101). Store in the dark at 4°C. Each day, take the needed volume of medium and warm it to 37°C.

RPMI/B-27 Supplement, plus (+) insulin: Thaw a 10 mL bottle of B-27 Supplement (50X), serum free (Cat. No. 17504044), either overnight at 4°C or briefly in a 37°C water bath. Mix the supplement well with a sterile pipette and transfer it into 500 mL of RPMI 1640 Medium (Cat. No. A1049101). Add 5 mL of Antibiotic-Antimycotic (100X) (Cat. No. 15240096) and mix. Store in the dark at 4°C. Each day, take the needed volume of medium and warm it to 37°C.

Differentiation of cardiomyocytes

1. If the PSC line is growing in attached culture, expand cells in StemScale PSC Suspension Medium for at least one passage.
 - a. See the StemScale PSC Suspension Medium user guide for help with establishing suspension cultures.
2. Passage PSCs with StemPro Accutase Cell Dissociation Reagent to single cells. Seed each well of a non-treated 6-well plate (Cat. No. 150239) with 0.15×10^6 cells/mL in 2 mL of StemScale medium with 10 μ M Y-27632.
 - a. Incubate at 37°C/5% CO₂ with rotation.
 - b. For help with selecting the correct rotation speed, refer to the StemScale PSC Suspension Medium user guide.
3. After 24 hours, tilt plate at a 45° angle for 5 minutes and pipet off 1 mL/well; replace each well with 1 mL of StemScale medium without Y-27632.
4. Grow PSCs in suspension for 3–5 days, following suggested feeding schedules in the StemScale PSC Suspension Medium user guide.
5. When PSC spheroids reach an average diameter of 280–360 μ m, they are ready for cardiac induction.

6. Cardiac induction step 1 (day 0):

- a. For each 6-well plate, prewarm 15 mL of RPMI medium without supplements, and 15 mL of RPMI/B-27 Supplement (– insulin) to 37°C.
- b. Add CHIR-99021 (4.5 μ L of 10 mM stock) to the RPMI/B-27 Supplement (– insulin) to a final concentration of 3 μ M.

The concentration of CHIR-99021 is critical, and requirements may vary by cell line. The optimal concentration should be empirically determined in a small-scale experiment prior to starting (see support protocol).
- c. Collect the entire volume of spheroids and medium by pipetting into sterile centrifuge tubes.
- d. Centrifuge at 250 x g for 1 minute to gently pellet the spheroids. Aspirate supernatant without disturbing the pellet.

- e. Rinse each well with 2 mL of RPMI medium without supplements and add this to the centrifuge tube. Agitate gently to resuspend the pelleted spheroids.
- f. Centrifuge at 250 x g for 1 minute. Aspirate supernatant without disturbing the pellet.
- g. For each well, resuspend the pelleted spheroids in 2 mL of RPMI/B-27 Supplement (– insulin) with 3 μ M CHIR-99021.
- h. Return the spheroids and medium to the original multiwell plate.
- i. Incubate at 37°C/5% CO₂ with agitation: we recommend 70 rpm as an initial setting for 6-well plates.*
- j. Note the time at which cells were returned to the incubator. Proceed with cardiac induction step 2 precisely 48 hours later.

7. Cardiac induction step 2 (day 2):

- a. For each 6-well plate, prewarm 15 mL of RPMI medium without supplements, and 15 mL of RPMI/B-27 Supplement (– insulin) to 37°C.
- b. Add IWP-2 (22.5 μ L of 5 mM stock) to the RPMI/B-27 Supplement (– insulin) to a final concentration of 7.5 μ M.
- c. Collect the entire volume of spheroids and medium by pipetting into sterile centrifuge tubes.
- d. Centrifuge at 250 x g for 1 minute to gently pellet spheroids. Aspirate supernatant without disturbing the pellet.
- e. Rinse each well with 2 mL of RPMI medium without supplements and add this to the centrifuge bottle. Agitate gently to resuspend the pelleted spheroids.
- f. Centrifuge at 250 x g for 1 minute. Aspirate supernatant without disturbing the pellet.
- g. Resuspend the pelleted spheroids in 2 mL of RPMI/B-27 Supplement (– insulin) with 7.5 μ M of IWP-2 per well.
- h. Return the spheroids and medium to the original multiwell plate.
- i. Incubate at 37°C/5% CO₂ with agitation: we recommend 70 rpm.

* Other shaker platforms may have a different orbital radius and thus a different optimal speed. For help with speed conversion, see the StemScale PSC Suspension Medium user guide. Choose a rotation speed fast enough to prevent aggregation while keeping spheroids intact.

8. Cardiac induction step 3 (day 4):

- a. Prewarm 2 mL of RPMI/B-27 Supplement (– insulin) per well to 37°C.
- b. Collect the entire volume of spheroids and medium by pipetting into a sterile centrifuge tube. Allow spheroids to sediment by gravity; centrifugation can be performed as above (250 x g, 1 minute) if necessary.
- c. Aspirate as much medium as possible without disturbing the sedimented spheroids.
- d. Add 2 mL of RPMI/B-27 Supplement (– insulin) to each well to resuspend the spheroids. Return spheroids and medium to the same multiwell plate.
- e. Incubate at 37°C/5% CO₂ with agitation.

9. Cardiac induction step 4 (day 6):

- a. Prewarm 2 mL of RPMI/B-27 Supplement (+ insulin) per well to 37°C.
- b. Collect the entire volume of spheroids and medium by pipetting into a sterile centrifuge tube. Pellet spheroids by sedimentation or centrifugation (250 x g, 1 minute).
- c. Aspirate as much medium as possible without disturbing the sedimented spheroids.
- d. Add 2 mL of RPMI/B-27 Supplement (+ insulin) to each well to resuspend the spheroids. Return spheroids to the same multiwell plate.
- e. Incubate at 37°C/5% CO₂ with agitation.

10. Maintenance of cardiac spheroids:

- a. Every 2nd day, exchange half of the medium with fresh RPMI/B-27 Supplement (+ insulin) as follows:
 - i. Prewarm 1.2 mL of RPMI/B-27 Supplement (+ insulin) to 37°C for each well.
 - ii. Move the multiwell plate into the sterile cabinet and rest it at a 45° angle. Allow the spheroids to settle by gravity for 1–5 minutes.
 - iii. Aspirate or pipet off half the medium (1 mL/well) without disturbing the sedimented spheroids.
 - iv. Replace with 1 mL of prewarmed RPMI/B-27 Supplement (+ insulin) per well.
 - v. Incubate at 37°C/5% CO₂ with agitation.
- b. From day 7 onward, examine the spheroids for spontaneous beating under a microscope.

Expected results

Around day 7 of differentiation, one should observe morphological changes, including formation of internal fluid-filled spaces. Although elongated or irregularly shaped spheroids may beat, higher proportions of cardiomyocytes are found in smooth, rounded spheroids. Spontaneous beating can be observed under high magnification starting anywhere from day 7 to day 14.

If the spheroids slough off large numbers of dead cells or shrink in size, the agitation speed may be too high. Formation of large aggregates (>3 mm diameter) of spheroids is a sign that agitation is too slow or interrupted. We have not observed spontaneous beating in such large aggregates.

Support protocol: small-scale differentiation to optimize concentration of CHIR-99021 for induction.

1. Seed two non-treated 6-well plates (Cat. No. 150239) with 0.15×10^6 cells/mL in 2 mL/well of StemScale medium with 10 μ M Y-27632.
2. Incubate on an orbital shaker at 37°C/5% CO₂ with 70 rpm agitation.
3. After 24 hours, tilt plate at a 45° angle for 5 minutes and pipet off 1 mL/well; replace with 1 mL/well of StemScale medium without Y-27632.
4. Feed cells every day by changing half the medium as above, until spheroids reach an average diameter of 280–360 μ m (~3–4 days).
5. When spheroids have grown to the required size, prewarm 30 mL of RPMI medium and 50 mL of RPMI/B-27 Supplement (– insulin) to 37°C.
6. Label 4 tubes A–D, and fill each with 10 mL of RPMI/B-27 Supplement (–insulin) at 37°C.
 - a. To tube A, add 3.0 μ L of 10 mM CHIR-99021 and mix.
 - b. To tube B, add 4.5 μ L of 10 mM CHIR-99021 and mix.
 - c. To tube C, add 6.0 μ L of 10 mM CHIR-99021 and mix.
 - d. To tube D, add 7.5 μ L of 10 mM CHIR-99021 and mix.

7. Cardiac induction step 1 (day 0):

- a. Tilt plates at a 45° angle in a laminar flow cabinet and allow spheroids to sediment.
- b. Pipet off (do not aspirate) as much medium as possible without damaging spheroids.
- c. Rinse each well with 2 mL of RPMI medium and allow spheroids to sediment again.
- d. Pipet off (do not aspirate) as much medium as possible.
- e. Add 2 mL from tubes A–D to three wells each.

- f. Incubate on an orbital shaker at 37°C/5% CO₂ with 70 rpm agitation.
 - i. Note the time at which cells were returned to the incubator. Proceed with cardiac induction step 2 precisely 48 hours later.

8. Cardiac induction step 2 (day 2):

- a. Prewarm 30 mL of RPMI medium to 37°C.
- b. Add 45 μ L of 5 mM IWP-2 stock to 30 mL of RPMI/B-27 Supplement (– insulin) and prewarm to 37°C.
- c. Tilt plates at a 45° angle in a laminar flow cabinet and allow spheroids to sediment.
- d. Pipet off (do not aspirate) as much medium as possible.
- e. Rinse each well with 2 mL of RPMI and allow spheroids to sediment again.
- f. Pipet off (do not aspirate) as much medium as possible.
- g. Add 2 mL of RPMI/B-27 Supplement (– insulin) and 7.5 μ M of IWP-2 to each well.
- h. Incubate on an orbital shaker at 37°C/5% CO₂ with 70 rpm agitation.

9. Cardiac induction step 3 (day 4):

- a. Prewarm 30 mL of RPMI/B-27 Supplement (– insulin) to 37°C.
- b. Tilt plates at a 45° angle in a laminar flow cabinet and allow spheroids to sediment.
- c. Pipet off (do not aspirate) as much medium as possible.
- d. Add 2 mL of RPMI/B-27 Supplement (– insulin) to each well.
- e. Incubate on an orbital shaker at 37°C/5% CO₂ with 70 rpm agitation.

10. Cardiac induction step 4 (day 6):

- a. Prewarm 30 mL of RPMI/B-27 Supplement (+ insulin) to 37°C.
- b. Tilt plates at a 45° angle in a laminar flow cabinet and allow spheroids to sediment.
- c. Pipet off (do not aspirate) as much medium as possible.
- d. Add 2 mL RPMI/B-27 Supplement (+ insulin) to each well.
- e. Incubate on an orbital shaker at 37°C/5% CO₂ with 70 rpm agitation.

11. Maintenance of cardiac spheroids:

- a. Every second day, exchange half of the medium with fresh RPMI/B-27 Supplement (+ insulin) as follows:
 - i. Prewarm 20 mL of RPMI/B-27 Supplement (+ insulin) to 37°C.
 - ii. Tilt plates at a 45° angle in a laminar flow cabinet and allow spheroids to sediment.
 - iii. Pipet off half the medium (1 mL) from each well without disturbing the sedimented spheroids.
 - iv. Replace with 1 mL of prewarmed RPMI/B-27 Supplement (+ insulin).
 - v. Incubate on an orbital shaker at 37°C/5% CO₂ with 70 rpm agitation.
12. From day 7 onward, examine the spheroids for spontaneous beating under a microscope. The optimal concentration of CHIR-99021 for a cell line is the one that produces the highest frequency of beating organoids.