pluriomics

MANUAL

PLURICYTE® CARDIOMYOCYTE DIFFERENTIATION KIT

Version 1.1

MANUAL

Pluricyte® Cardiomyocyte Differentiation Kit

Cat# PM-CDK-805

Table of Contents

1. Introduction

Pluricyte® Cardiomyocyte Differentiation Kit consists of 3 ready-to-use defined serum-free media for the generation of a monolayer of cardiomyocytes (CMs) from human pluripotent stem cell (hPSC) lines maintained in different culture methods. Within 14 days, functional spontaneously contracting CMs that express relevant cardiac markers and exhibit relevant pharmacological responses to cardioactive compounds are generated. Typically, contracting areas appear between day 7-11 of differentiation, which is dependent on the hPSC line that is used.

hPSC-derived CMs (hPSC-CMs) provide an attractive model for development studies, disease modelling and drug discovery/safety screenings, as well a potential source for cell therapies [1]. Applications of hPSC-CMs generated with the Pluricyte® Cardiomyocyte Differentiation Kit include, but are not limited to, biochemical/molecular studies (e.g. gene expression or protein profiling), mechanical/contraction studies, electrophysiological characterization and calcium assays.

2. Product Description

Pluricyte® Cardiomyocyte Differentiation Kit is a ready-to-use defined serum-free media kit suitable for the generation of contracting cardiomyocytes in an adherent monolayer format. Our kit is compatible with hPSC lines maintained in different culture methods.

3. Product Use Statement

FOR RESEARCH USE ONLY. Not intended for human or animal diagnostic or therapeutic use.

4. Safety information

Read the Material Safety Data Sheet on our website (MSDS) and follow the handling instructions. Safe laboratory procedures should be followed and appropriate protective eyewear, clothing, and gloves should be worn when handling kit reagents.

5. Kit components and storage conditions

The following components are sold as a complete kit. For enquiries regarding additional maintenance medium C please contact our product support team (support@pluriomics.com).

Component name	Component number	Size	Storage	Retest interval
Differentiation Medium A*	PM-CDK-805A	0.07l	2-8 °C	3 months upon receipt
Differentiation Medium B*	PM-CDK-805B	0.07l	2-8 °C	3 months upon receipt
Differentiation Medium C*	PM-CDK-805C	0.5l	2-8 °C	3 months upon receipt

*Protect from light.

6. Additional required reagents, materials and equipment

Reagent
hPSC medium containing 20% KOSR
DMEM/F12
D-PBS without Ca^{2+}/Mg^{2+} (PBS-)
Accutase® solution (Sigma, cat no A6964)
Matrigel™ hESC-qualified Matrix (Corning, cat no 354277)
Trypan blue 0.4% solution
mTeSR™1 medium (STEMCELL Technologies, cat no 05850)
E8 medium (or custom-specific hPSC maintenance medium)
Recombinant Trypsin (rTrypsin)
1x TrypLE™ Select (Thermo Scientific, cat no 12563)

Material
Human pluripotent stem cells
12-well culture plates
15ml and 50ml centrifuge tubes
Pipettes and pipette tips
Serological pipettes

Equipment
Biological safety cabinet class II
Incubator at 37°C, 5% CO_2 and humidified air
37°C water bath
Centrifuge
Cell counting chamber or automated cell counter
Inverted microscope

7. Quick guideline

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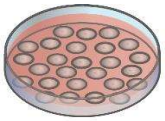

Day -4 (or day -3): hPSC seeding on a 12-well plate

Coat a 12-well plate with recommended substrate and seed hPSCs in their parental maintenance medium

Single cell hPSCs grown on **feeder cultures**

- Routine passaging interval: **3-4 days**
- Dissociation method: **Enzymatic (Accutase/rTrypsin)**
- Typical seeding cell conditions: **12.5 - 15x10³ cells/cm²**
- Confluency at day 0: **60-80%**
- No refreshment needed until day 0

hPSCs grown in **E8 medium or other feeder-free media**

- Routine passaging interval: **3-4 days**
- Dissociation method: **EDTA (aggregates) or Enzymatic (rTrypsin or equivalent)**
  Typical seeding cell conditions: **1:8 to 1:20 or 2.5 - 15x10³ cells/cm²**
- Confluency at day 0: **30-80%**
- Refresh the medium daily from the 2nd day after passaging

Notes:

- Proliferation rates between hPSCs can vary, therefore we recommend testing different seeding densities for each hPSC line
- More detailed information can be found in our Manual Sheet
- For further guidance on seeding cell densities, contact us at support@pluriomics.com

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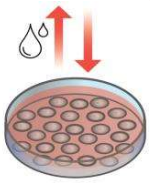

Day 0 - 2: Differentiation Medium A

Day 0: Replace the medium with 1ml/well pre-warmed (37°C) Differentiation Medium A

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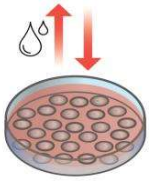
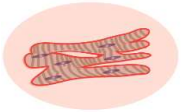


Day 2 - 4: Differentiation Medium B

Day 2: Replace the medium with 1ml/well pre-warmed (37°C) Differentiation Medium B (at day 0 + 48h)

3

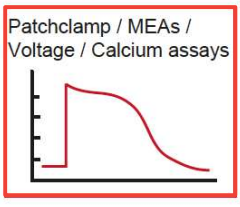


Day 4 - 14: Differentiation Medium C

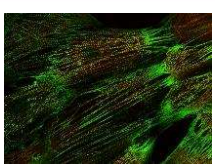
Day 4: Replace the medium with 1ml/well pre-warmed (37°C) Differentiation Medium C (at day 0 + 96h)
Day 6-14: Refresh with Differentiation Medium C every 2 days

Characterization assays using hPSC-derived cardiomyocytes:

Electrophysiology

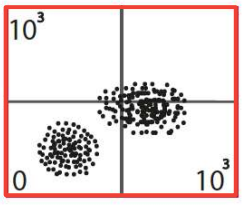


Contraction



Gene Expression

Flow Cytometry



8. Before starting: important recommendations

- The protocol is designed on the assumption that the operator using the kit is experienced in the routine maintenance of the specific hPSC line.
- For successful differentiations, use karyotypically normal hPSCs of high quality with no signs (or minimal signs) of differentiation and that express pluripotency markers.
- After thawing cells, passage hPSCs for at least 2 weeks or perform 3 passages before initiating differentiations.
- Optimal seeding density is crucial to the efficiency of the differentiation and is directly linked to the proliferation rate of the distinct hPSC line. To achieve best results, the optimal confluency at day 0 of differentiation needs to be established. This optimum may vary per line between 30-80% (See **Table 1** for details). We recommend initially testing of three different seeding densities.
- For hPSC lines passaged as small clumps or aggregates, establishment of the splitting ratio (based on routine passaging) is needed to decide optimal seeding densities (See **Table 1** for details).

Table 1: Recommended seeding parameters of different hPSC culture systems

Culture System	Routine Passaging interval	Dissociation method	Seeding densities	Pre-plating time	Confluency at day 0
Feeder-based	3-4 days	Enzymatic/Single cells Accutase®/ rTrypsin (or equivalent)	12.5 to 15x10^{3}cells/cm^2	4 days	50-80%
E8 medium	3-4 days	Aggregates/EDTA	1:8- 1:12	4 days	50-80%
(and other feeder-free cultures)			1:12-1:20 (faster growing cell lines)	3 days	30-60%
	3-4 days	Enzymatic/Single cells* rTrypsin (or equivalent)	2.5 to 15x10^3 cells/cm^2	3-4 days	30-80%

*ROCK inhibitor treatment is recommended.

The recommendations listed in Table 1 are general guidelines. Please contact us at support@pluriomics.com for additional guidance and trouble shooting.

- The differentiation protocol (see section 9) gives instructions for 4 days of pre-plating, however the differentiation can also be initiated 3 days after pre-plating. This is especially recommended when fast growing hPSC lines are used.
- It depends on the maintenance medium whether daily refreshment of the pre-seeded cells is required prior to starting differentiation at day 0 (see section 9.1. and 9.2. for details).
- When plating hPSCs for differentiation, ROCK inhibitor treatment is optional and can be used to improve cell survival and plating consistency. In this case, refresh medium the day after seeding to remove the ROCK inhibitor.
- Although this differentiation protocol has been optimized for hPSCs grown on feeder-based cultures and E8 medium, it may also be suitable for cells grown on other cultures systems such as L7™ hPSC Culture System (Lonza), NutriStem™ XF/FF Culture Medium (Stemgent), StemMACS IPS-Brew XF (Miltenyi) and

mTeSR™1 medium (STEMCELL Technologies). Follow manufacturer's instructions for substrate coating and medium refreshments during the pre-differentiation step and use this manual for optimal seeding density and differentiation procedures.

- Pre-coat cell culture treated plates with appropriate substrate, following manufacturer's recommended instructions, before seeding cells.
- Once differentiation is initiated, medium should be replaced every 2 days. After day 4, refresh the medium of the cells every 2 days. Refreshment could be performed on Friday and Monday to prevent weekend work.
- For extended maintenance of cardiomyocytes, Differentiation Medium C can be used. To enhance maturation, cardiomyocytes can be dissociated and replated in Pluricyte® Cardiomyocyte Medium (PM-2100-100ml).
- From day 14 of differentiation, cardiomyocytes can be dissociated for further characterization and/or can be used in a variety of applications/assays.

9. Cardiomyocyte Differentiation Protocol

This cardiomyocyte differentiation protocol has been optimized for hPSCs grown on feeder-based cultures and E8 medium. However, it may also be suitable for cells grown in other cultures systems such as L7™ hPSC Culture System (Lonza), NutriStem™ XF/FF Culture Medium (Stemgent), StemMACS IPS-Brew XF (Miltenyi) and mTeSR™1 medium (STEMCELL Technologies).

For feeder-based cultures, we recommend to plate cells on Matrigel™ in mTeSR™1 medium. There is no requirement to refresh medium during the pre-differentiation step from Day -4 to Day 0. For E8 cultures, cells can be plated on Matrigel™, Vitronectin or Geltrex® and E8 medium should be refreshed daily from the second day after passaging until start of differentiation.

For hPSCs grown on feeder-based cultures go to page 8

For hPSCs grown in E8 medium go to page 9

Transferring hPSCs for differentiation as small aggregates page 9

Transferring hPSCs for differentiation as single cells page 10

For hPSCs grown in other non-feeder based cultures, please refer to **Table 1** (page 5) for recommendations on culture conditions and/or contact us for further support via support@pluriomics.com.

9.1. Starting differentiation from hPSCs grown on feeder-based cultures

Before starting the procedure of dissociating hPSCs, prepare culture plates coated with Matrigel™ according to the manufacturer's instruction. Coating should be performed at least 1 hour before use.

Day-4, Transferring hPSCs from a 6-well feeder-based culture to a 12-well plate coated with Matrigel™

This protocol uses Accutase® solution to singularize cells. Other methods, such as recombinant Trypsin (rTrypsin) or TrypLE™ Select, can also be used.

1. Pre-warm Accutase® solution and mTeSR™1 medium to room temperature (RT) and the hPSC medium containing 20% KOSR to 37°C.
2. Aspirate medium from the hPSC culture and rinse each well with 2ml PBS- (when using a 6-well plate format).
3. Add 0.7ml Accutase® solution at RT per well. Swirl the dish to cover the entire cell surface.
4. Incubate cells for 5 minutes at 37°C, 5% CO_2.
   Note: when monitoring dissociation under a microscope, cells should start to separate and round up.
5. Pipette up and down to dissociate the cells and collect the cell suspension in a 15ml conical tube containing 4.3ml hPSC medium with 20% KOSR. Centrifuge the cell suspension for 3 min at 250xg at RT.
6. Aspirate supernatant and resuspend pelleted cells in 5ml of mTeSR™1 medium.
7. Take a sample of the cell suspension to count the cells and add 1:1 Trypan blue 0.4% solution to determine cell viability.
8. Calculate the number of viable cells and prepare a cell suspension of 50-60k cells per ml in mTeSR™1 medium.
   Note: when plating hPSCs for differentiation, ROCK inhibitor *treatment is* optional and can be used *to improve cell survival and plating consistency.*
9. Aspirate the Matrigel™ solution from the pre-coated 12 well plates.
10. Add 1ml cell suspension per 12-well plate well. This should be suitable to reach 60-80% confluency at day 0 of differentiation.
11. Move the plate to disperse cells over the well surface.
12. Place the plates in a 37°C incubator with 5% CO_2 until the start of the differentiation at day 0.
13. No refreshment is required until day 0 of differentiation.

9.2. Starting from hPSCs grown in E8 medium

Before starting procedure of dissociation, coat cell culture plates with Matrigel™, Vitronectin or Geltrex® according to the manufacturer's instructions. Coating should be performed at least one hour before use.

9.2. A. Day-4, Transferring hPSCs from a 6-well E8 medium culture to a 12-well plate coated with Matrigel™ as small aggregates

This protocol uses EDTA to dissociate cells into small aggregates.

1. Pre-warm E8 Medium at RT.
   Note: do not warm medium in a 37°C water bath.
2. Aspirate medium from the hPSC culture and rinse each well with 2ml PBS- (when using a 6-well plate format).
3. Add 1ml 0.5mM EDTA solution (0.5M EDTA diluted in PBS-) at RT per well. Swirl the dish to cover the entire cell surface.
4. Incubate for 4-5 minutes at 37°C, 5% CO_2.
   Note: when monitoring dissociation under a microscope, cells should start to separate and round up. For certain cell lines, dissociation may take longer than 5 min.
5. Aspirate the EDTA solution and add 2ml E8 Medium per well.
6. Gently pipet up and down to dissociate the cells and collect the cell suspension in a 15ml conical tube.
7. Aspirate the Matrigel™ solution from the pre-coated 12-well plates.
8. Add the cell suspension to the 12-well plate at a split ratio suitable to reach 30-80% confluency at day 0 of differentiation (for split ratio recommendations, see **Table 1**).
9. Move the plate to disperse the cells over the well surface.
10. Place the plates in a 37°C incubator with 5% CO_2.
11. Refresh the medium daily from the second day after passaging.

9.2. B. Day-4, Transferring hPSCs from a 6-well E8 medium culture to a 12-well plate coated with Matrigel™ as single cells

This protocol uses rTrypsin or TrypLE™ Select to dissociate hPSCs into single cells. In addition, the use of ROCK inhibitor treatment upon cell seeding is recommended.

1. Pre-warm E8 Medium at RT.
   Note: do not warm medium in a 37°C water bath.
2. Aspirate medium from the hPSC culture and rinse each well with 2ml PBS- (when using a 6-well plate format).
3. Add 0.7ml rTrypsin or TrypLE™ Select at RT per well. Swirl the dish to cover the entire cell surface.
4. Incubate cells for 5 minutes at 37°C, 5% CO_2.
   Note: when monitoring dissociation under a microscope, cells should start to separate and round up.
5. Pipette up and down to dissociate the cells and collect the cell suspension in a 15ml conical tube containing 4.3ml E8 medium. Centrifuge the cell suspension for 3 min at 250xg at RT.
6. Aspirate supernatant and resuspend pelleted cells in 5ml of E8 medium.
7. Take a sample of the cell suspension to count the cells and add 1:1 Trypan blue 0.4% solution to determine cell viability.
8. Calculate the number of viable cells and prepare a cell suspension of 10-60k cells per ml in E8 medium.
   Note: when plating hPSCs for differentiation, ROCK inhibitor *treatment is* optional and can be used *to improve cell survival and plating consistency.*
9. Aspirate the Matrigel™solution from the pre-coated 12 well plates.
10. Add 1ml cell suspension per 12-well plate well. This should be suitable to reach 30-80% confluency at day 0 of differentiation.
11. Move the plate to disperse cells over the well surface.
12. Place the plates in a 37°C incubator with 5% CO_2 until the start of the differentiation at day 0.
13. Refresh the medium daily from the second day after passaging.

10. Differentiation Procedure

After allowing hPSCs to expand and reach optimal confluency (30-80%, please refer to **Table 1**) in the different maintenance culture systems, the differentiation procedure is generally applicable to all of them.
Note: the differentiation procedure is described for a 12-well plate format.

Day 0, Start differentiation using Differentiation Medium A

1. Pre-warm 12ml Differentiation Medium A by incubating 15 minutes in a water bath of 37°C.
2. Aspirate the medium from all wells, and replace with 1ml pre-warmed Differentiation Medium A per well.
3. Culture the cells in a 37°C, 5% CO_2 incubator for 2 days.

Day 2, Replace medium with Differentiation Medium B

4. Pre-warm 12ml Differentiation Medium B by incubating 15 minutes in a water bath of 37°C.
5. Aspirate the medium from all wells, and replace with 1ml pre-warmed Differentiation Medium B per well at the same time as day 0 (= day 0 + 48h).
6. Culture the cells in a 37°C, 5% CO_2 incubator for 2 days.

Day 4, Replace medium with Differentiation Medium C

7. Pre-warm 12ml Differentiation Medium C by incubating 15 minutes in a water bath of 37°C.
8. Aspirate the medium from all wells, and replace it with 1ml pre-warmed Differentiation Medium C per well at the same time as day 0 (= day 0 + 96h).
9. Culture the cells in a 37°C, 5% CO_2 incubator for 2 days.

Day 6-14, Replace medium with Differentiation Medium C

10. Refresh with Differentiation Medium C every 2 days. Cells should start beating between day 7 and day 11, dependent on the hPSC line (see note below).
    Note: depending on hPSC line, it is normal to observe some (substantial) cell death between day 5 and day 8.

Typically, contracting areas appear between day 7-11 of differentiation, dependent on the hPSC line. Around day 14, cardiomyocytes can be dissociated (see **Appendix I** for Dissociation of Cardiomyocytes) for further characterization and/or for use in a variety of applications/assays [2].
For more information on cell culture and maintenance, please visit our website (www.pluriomics.com) or contact us via support@pluriomics.com.
For performing safety pharmacology studies we recommend using our quality controlled Pluricyte® Cardiomyocytes (see related products below).

11. Data examples

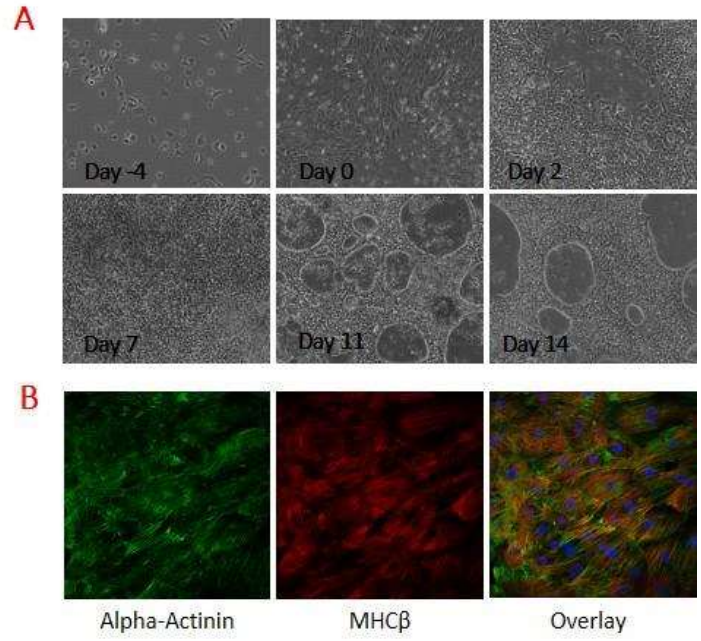


Fig.1. Characterization of hPSC-CMs generated with the Pluricyte® Cardiomyocyte Differentiation Kit. (A) Representative bright field images of hPSCs during cardiac differentiation. Contracting areas were observed at day 11. (B) Immunofluorescence staining of hPSC-CMs (Green: alpha actinin, red: β-MHC, blue: nuclei). At day 14 of differentiation, hPSC-CMs were replated in Pluricyte® Cardiomyocyte Medium for 7 days. Organized sarcomeric structures are observed.

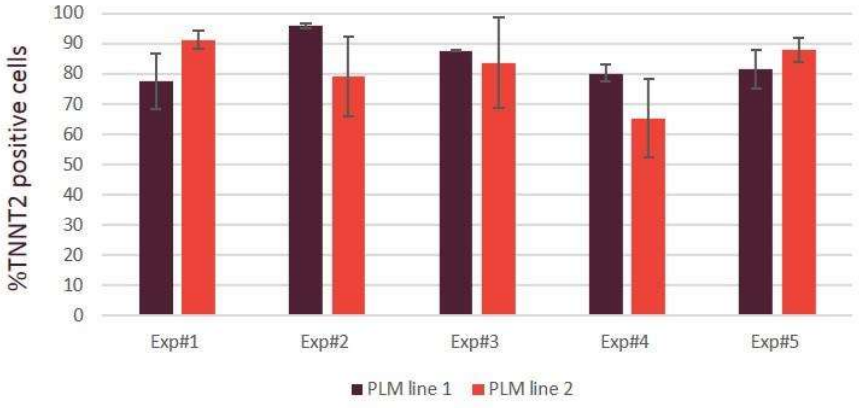


Fig.2. Flow cytometry analysis upon cardiac differentiation based on TNNT2 expression. Reproducibility over 5 independent experiments in 2 different hPSC lines.

12. References

[1] Braam SR et al. Cardiomyocytes from human pluripotent stem cells in regenerative medicine and drug discovery. Trends in Pharmacological Sciences (2009) Vol.30 No.10.

[2] Ribeiro MC et al. Functional maturation of human pluripotent stem cell derived cardiomyocytes in vitro-correlation between contraction force and electrophysiology. Biomaterials (2015) 51:138-50.

Appendix I. Dissociation of cardiomyocytes for characterization and further applications / assays

Before starting the procedure below, prepare surfaces for replating as desired. We recommend using Matrigel™ or Fibronectin. For coating procedure protocols, we refer to our Pluricyte® Cardiomyocyte Manual on our website.

Dissociation procedure for one 12-well plate

1. Pre-warm 6ml 1x TrypLE™Select to 37°C.
2. Prepare a 50ml conical tube with 20ml Differentiation Medium C at RT to neutralize after dissociation.
3. Prepare a 50ml conical tube with 6ml Differentiation Medium C at RT to rinse all wells after dissociation (0.5ml/well).
4. Aspirate Differentiation Medium C from all wells.
5. Rinse each well 2x with 1ml PBS- per well.
6. Add 0.5ml of pre-warmed 1x TrypLE™ Select per well and incubate at 37°C, 5% CO_2 for 10 min.
7. At 10 min, pipet about 6x up and down using a P1000 pipette, set at 0.8ml, to detach cells from the well.
8. Tilt the plate and pipet up and down until clumps are dissociated.
9. Transfer the cell suspension to the tube containing 20ml Differentiation Medium C at RT.
10. Rinse the plate with 0.5ml Differentiation Medium C per well and also add this to the neutralization tube.
11. Take 20µl of the cell suspension, add 1:1 Trypan blue 0.4% solution to count cells and to determine cell viability.
12. Use the amount of viable cells to calculate the medium volume that is needed to obtain the appropriate cell density for replating.
13. Centrifuge the cell suspension for 3 minutes at 250xg at RT.
14. Aspirate the supernatant and gently resuspend the cell pellet in an appropriate amount of Differentiation Medium C. See Table below for cell density recommendations:

Plating density in cells / well	Format	Volume Differentiation Medium C:
40k	96-well	100µl
150k	24-well	500µl
300k	12-well	1000µl

Maintenance of culture

15. Refresh cultures with Differentiation Medium C 24 hours after dissociation and replating.
16. Refresh cultures with Differentiation Medium C every 2 days.

Note: Cardiomyocytes can be replated in Pluricyte® Cardiomyocyte Medium for enhanced maturation, more information on this product can be found on our website (www.pluriomics.com).

General Terms & Conditions of Sale

Certificate of analysis (CoA) and Material safety data sheets (MSDS) for any Pluriomics product can be downloaded from www.pluriomics.com.

For further information and technical assistance, contact support@pluriomics.com.

Related products

- Pluricyte® Cardiomyocyte Kit (Cat# PCK-1.5)

  1 Pluricyte® Cardiomyocyte Kit includes:
  - 1 vial of **Pluricyte® Cardiomyocytes** (1.5 million cells): Fully functional human iPSC-derived ventricular cardiomyocytes, differentiated without any genetically modification or purification/selection procedures.
  - 1 bottle of **Pluricyte® Cardiomyocyte Medium** (100ml): Well defined serum free culture medium designed to promote cell maturation and function.