ReproCardio 2™

Cat. RCDC001N

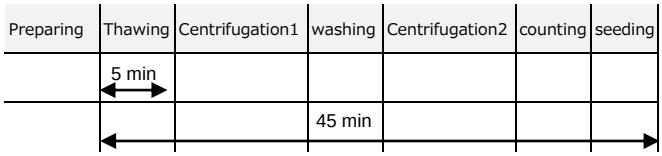
Version 1.0

Manuals

Thawing of ReproCardio2™

Critical Points for Cell Handling

- To maintain high viability, cell-vials should be stored in liquid nitrogen.
- Before thawing, briefly twist the cap a quarter of turn under the laminar flow hood. (Do not open it completely.)
- The time interval between thawing and cell transfer into the culture medium must be less than 5 minutes. The total procedures from thawing to seeding must not exceed 45 minutes.
- When transferring cell suspension from vial by using a pipette. Gentle suction of the cell suspension (with counting up to 3 seconds) is recommended to avoid damaging cells.
- Thaw only one vial at a time.



Items not included

Name	Catalog number	Note
ReproCardio2 Culture Medium™	ReproCELL, RCDC101	Store at 4°C
Fetal Bovine Serum	Life Technologies. (Gibco), 10437-028	Store at 4°C
Penicillin Streptomycin	Life Technologies. (Gibco), Cat. 15140	Store at 4°C
Prime surface (U-bottom 96-well plate)	Sumitomo baikuraito,MS-9096U	

Note 1. Add FBS and antibiotics to ReproCardio2 Culture Medium™ before use.

Note 2. Transfer frozen cell vial from package to liquid nitrogen as quickly as possible.

1. Preparation of the Culture Medium

1.1. Thaw ReproCardio2 Culture Medium™ at 4°C in a refrigerator overnight.

1.2. Add 25 mL of FBS and 1 mL of Penicillin-Streptomycin to the ReproCardio2 Culture Medium™. ReproCardio2 Culture Medium™ supplemented with FBS and Penicillin-Streptomycin is hence forth referred to as culture medium in this manual.

1.3. We recommend to aliquot the culture medium to 10-40 mL / tube and sealed by parafilm. This aliquot culture medium can be stored at 4°C up to 3 weeks. Please avoid repeated warming or cryopreserving, so that some components of the medium would not be degraded. The quality of cells would be affected negatively.

2. Thawing of Cells

2.1. Add 10 mL of the culture medium into two 15-mL plastic tubes (A and B) and add 14ml of the culture medium into a 15ml plastic tube (C). Warm the aliquots in a 37°C water bath for at least 15 minutes.

2.2. Bring tube A to the laminar flow hood and loose the cap.

2.3. Prepare reservoir for carrying the vials, and transfer the liquid nitrogen to the reservoir.

2.4. Take one vial of ReproCardio2™ cells out of the liquid nitrogen storage container and immediately place in the reservoir, and move it near the laminar flow hood.

2.5. Under the laminar flow hood, briefly twist the cap a quarter turn and close it again. Put the vial in the liquid nitrogen reservoir again as quickly as possible.

Note 3. Please ensure that this procedure is completed in no more than 30 seconds to prevent temperature increase.

Note 4. Do not open the cap completely to prevent contamination.

2.6. Bring the reservoir close to the water bath.

2.7. Dip the vial from the reservoir into the 37°C water bath as quickly as possible, and gently swirl it for 90 seconds.

Note 5. Please ensure that this procedure is completed within 90 seconds. The viability of the cells will decrease if the vial contents are thawed completely.

2.8. Bring the thawed vial to the laminar flow hood as quickly as possible, wipe water around the vial, and decant the content of the vial into tube A. Minimize the time of transferring the cell into tube A.

Note 6. From step 2.5. to step 2.8., ensure to finish within 2 minutes（90 seconds in water bath and 30 seconds in the transfer process）.

2.9. Transfer 1 mL of medium from tube A into the emptied vial, wash the vial, and transfer the content back into tube A.

2.10. Centrifuge tube A at 300 - 350 ×*g* for 5 minutes at room temperature.

2.11. Bring tube B to the laminar flow hood during centrifugation.

2.12. Gently bring tube A back to the laminar flow hood. Do not agitate tube A in order to avoid the cell pellet detaching from the bottom of tube.

2.13. Carefully aspirate supernatant from tube A. Be careful not to aspirate the cell pellet.

2.14. Transfer 10 mL of culture medium from tube B to tube A without pipetting

2.15. Centrifuge tube A at 300 - 350 ×*g* for 5 minutes at room temperature.

2.16. Carefully aspirate 9 mL of supernatant from tube A in the laminar flow hood.

2.17. Re-suspend the cell pellet with remaining 1 mL of culture medium by slowly and gently pipetting 6 times (6 seconds/pipetting). The cell suspension is 1.0×10^6 cells in 1 mL.

3. Seeding the cells

Note 7. Tube C is prepared for cell seeding.

Note 8. The time interval from thawing to seeding must be less than 45 minutes.

Please see cell seeding and culturing procedures in the following manuals.

- Measurement of the extracellular electric potential of cardiomyocytes grown in a thin layer using the QT-interval-prolongation assay (QTempo) ･･･page 5 (αMED)、page 9 (MCS)、and page 13 (Axion)
- Calcium-Imaging Assay ･･･page 17
- Culture Method for Patch Clamp Technique ･･･page 20
- Immunostaining･･･page 23

Q&A

Q1.Do you recommend a specific manufacturer for FBS?

A1. Any type of FBS is suitable for this protocol.

Q2.What happens when the cells thawing time in the water bath is shorter than 90 seconds?

A2. Since the content in the cryo-vial is not completely thawed, it will not be properly transferred into tube A.

Q3. What happens when the cells thawing in the water bath is longer than 90 seconds?

A3. The cells will be damaged and lower the viability, the cell could reform clump and cause poor adhesion.

Q4. How should I do to complete the thawing process in less than 5 minutes?

A4. We recommend rehearsing thawing process using a practice vial (no cells) beforehand.

Q5. How do I recognize whether or not the cells aggregate?

A5. Visual inspection is sufficient to identify aggregates.

Q6.How many times should I pipette to make the cell suspension?

A6.The upper limit of pipetting by using a micropipette (P-1000) is 10 times.

Q7. How do I seed cell suspension included in small aggregates even though pipetting 6 times was done?

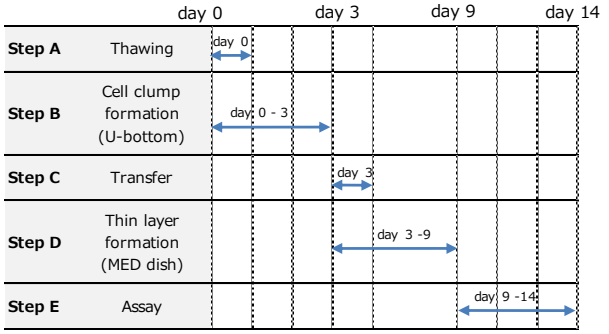
A7.It is OK to seed the cell suspension with occasional small aggregates. As long as the cell aggregates reform in U bottom low binding plate, re-beating will occur.

ReproCardio2™: Measurement of the extracellular electric potential of cardiomyocytes grown in thin layer using QT-interval-prolongation assay (QTempo)
Ver. MED64 SYSTEM (ALPHA MED SCIENTIFIC, α MED)

Critical Points for Cell Handling

1. 15,000 cells are used to make loose cell clumps. If the cell number is less than 15,000, thin layer may not be formed on the MED dish.
2. Transfer loose cell clumps from the U-bottom plate to a MED dish on day 3 when clumps have been formed in almost all wells.
3. If the cell clumps remain in the U-bottom plate past day 4, they become rigid and may not form a thin layer.
4. If cell clumps are still loose by day 3, the cell clumps should be transferred very gently to the MED dish by using a pipette.
5. After day 5, culture medium (1,000 µL/dish) should be changed every other day by using a micropipette. Be very gentle to avoid detachment of the thin layer from the MED dish.
6. Assay should be performed under 37℃, 5% CO_2 environment. The control of CO_2 can contribute to sustainable beating cycle.
7. Recommended size of electrode is 20 µm×20 µm.

Schedule for thin layer formation on the MED dish



Volume of required culture medium

	Cultivation Time				
Cell clump number per MED dish	by day 7	by day 8-9	by day 10-11	by day 12-13	by day 14
1	171.9 mL	234.9 mL	297.9 mL	360.9 mL	423.9 mL
2	134.7 mL	166.7 mL	198.7 mL	230.7 mL	262.7 mL
3	121.5 mL	142.5 mL	163.5 mL	184.5 mL	205.5 mL
4	115.5 mL	131.5 mL	147.5 mL	163.5 mL	179.5 mL

* Please purchase ReproCardio2 Culture Medium ™ (RCDC101) as appropriate..

Items not included

Name	Catalog number	Note
ReproCardio2 Assay Medium ™	ReproCELL, RCDC301	Store at 4°C
ReproCardio2 Culture Medium ™	ReproCELL, RCDC101	Store at 4°C
Fibronectin Human, Plasma	Life Technologies, 33016-015	Store at 4°C
5% CO_2 mixed with air gas	–	Room temperature

Note 1. Read carefully the protocol for cell thawing (page 2-4).

Step A: Preparation of the cell suspension (day 0)

Add12.3 mL medium (tube C) per tube of cell suspension in tube A to make final concentration of 0.75×10^5 cells/mL. **Note 2.** The cell suspension should be kept at 37°C in a water bath to maintain high viability.

Step B: Formation of loose cell clumps in a U-bottom 96-well plate (day 0–3)

1. Add 200 µL of the cell suspension into each well of a 96-well U-bottom plate.

Note 3. 1.5×10^4 cells should be used to form thin

layer

2. Place the 96-well plate in an incubator maintained at 37°C with 5% CO_2. Remove 100 µL/well medium on the next day (day 1) (total volume of medium: 200 µL/well).
3. Remove 100 µL of the medium and add 100 µL/well of fresh medium on day 2.
4. Single cells start to aggregate spontaneously to form loose clumps.
   Clump formation: day 1: 30–50%, day 2: 80%, day 3: 100% in 96-well plate

Step C: Transfer of loose cell clumps to the MED dish (day 3)

Note 4. Once cell clumps have formed in the U-bottom plate, transfer them to the MED dish on day 3. If the cell clumps remain in the U-bottom plate past day 4, they become rigid and may not form a thin layer.

Note 5. The chance of thin layer formation is lower on a glass surface (e.g., MEA dish such as Multi Channel Systems, 50%) than on a plastic surface (over 90%). A few additional MED dishes are advised to be prepared for the first experiment.

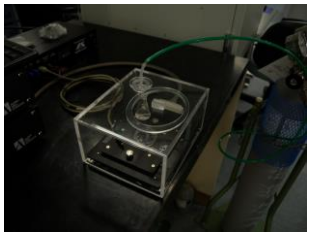
1. Place a MED dish (MED-P210A) into a Φ 100-mm dish.
2. Dilute 5 mg of human plasma fibronectin with phosphate-buffered saline (-) to a final volume of 50 µg/mL.
3. To coat the MED dish, add **20 µL** of diluted human plasma fibronectin solution (50 µg/mL) onto the electrode nodes of the MED dish. In order to prevent evaporation of the buffer, add 3 mL phosphate-buffered saline in Φ 100-mm dish (outside the MED dish). Incubate the MED dish for 1 hour at 37°C with 5% CO_2.
4. Add 1 mL/well of PBS (-), and remove the coating solution from the MED dish.
5. Set a micropipette (P-200) to **150** µL.
6. Transfer loose cell clumps very gently from the U-bottom plate to the MED dish by using a micropipette.
   Note 6. For 4-well dish, set a micropipette (P-200) to 75 µL.
7. Distribute the medium in entire culture area of dish or well.
8. Move the clumps gently to the electrodes in the MED dish by using a pipette tip or a glass rod under a phase contrast microscope.
9. Incubate the MED dish at 37°C with 5% CO_2.

Step D: Thin Layer Formation on the MED Dish (day 3–9)

1. Add 200 µL/dish of medium on day 4.
   Note 7. For the 4-well dish, add 125 µL/well of medium.
2. The cell clumps start to contract and flatten spontaneously to form a thin layer on the MED dish.
   Note 8. The outline of the cell clump will gradually become less distinct (day 3–7). Please refer to the presentive image of a thin layer of Cardiomyocytes on the MED dish.
3. Change the culture medium every other day. When changing the medium, remove the old medium completely and add 1,000 µL/dish of fresh and warm medium with a micropipette.
   Note 9. The culture medium is changed every other day with a micropipette not with an aspirator. Be very gentle to avoid detachment of the thin layer from the MED dish.
   Note 10. The culture medium MUST NOT be added directly onto the cells, because cells may detach easily upon mechanical disturbance.
   Note 11. For the 4-well dish, add 200 µL/well of medium per well.

Step E: Assay (day 9–14)

1. Prepare the test compound solution (×10) and maintain it at 37°C.
2. Set the temperature inside the MED dish to 37°C using a thermal controller. Cover the MED dish with the lid of a 35-mm dish to prevent evaporation of the medium. Moreover, shield the MED dish with lip by using an airtight chamber.



Note 12. To ensure that the temperature of the electrodes of the MED dish is adequate, set the thermal controller to 37°C.

3. Add 5% CO_2 mixed with air gas by bubbling through MilliQ water (30 mL) in the bottle.
4. Remove the old culture medium completely from the MED dish with a P-1000 micropipette and add 1,000 µL of warmed ReproCardio Assay Medium™ to the MED dish. Incubate the cells for 10 minutes at 37°C with 5% CO_2.

   Note 13. For the 4-well dish, add 200 µL of ReproCardio Assay Medium™ per well.
5. Connect the MED dish to the MED64 system and incubate for 10 minutes.

   Note 14. Aspirate gentry by using Pipetman in changing the medium.

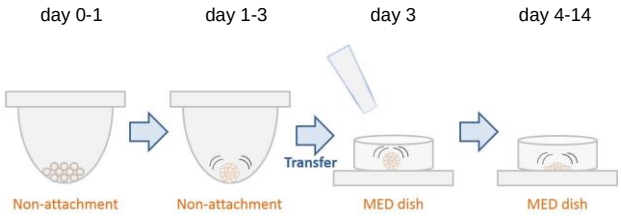
   Note 15. The assay should be performed during day 9–14, because beating of the thin layer is usually stabilized by day 9. On the other hand, there is the possibility of detachment of cells after day 15.
6. Measure the electrical signals (beating rate, Na^+ amplitude, K^+ amplitude, and Na^+–K^+ interval, etc.).
7. For the addition of testing compound, remove 1/10 of the medium gently from the assay medium and add an equal volume of the testing compound solution.
8. Mix the medium by gentle pipetting.
9. Expose the cells to each dose of the compound for 4 or 10 minutes and record/analyze the last wave at 2–4 minutes or 8–10 minutes.

   Note 16. Repeat steps 7–9 until the examinations are completed.

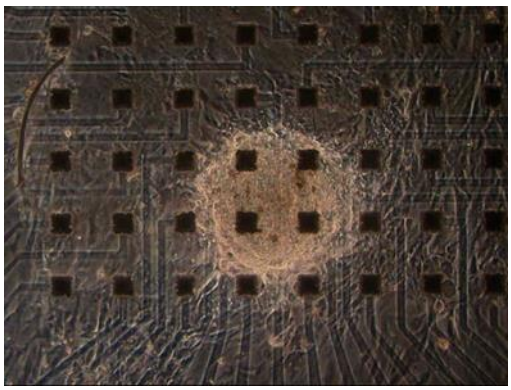
   Note 17. Please determine the appropriate exposure time for each compound.

Example Images

1. Review of the steps.



2. A thin layer of cardiomyocytes on the MED dish.



Q&A

Q1. Cell clumps do not contract in the U-bottom well plate

A1. Cell clumps usually start to contract on day 2–3. However, even if the loose cell clumps do not contract, please transfer them to the MED dish on day 3. They will start to contract on the MED dish.

Q2. How often should the medium be changed?

A2. The medium should be changed every other day. We suggest to aliquot the medium before use.

Q3. No thin layer forms on the MED dish after transfer

A3. We propose to prepare additional MED dishes for the first experiment.

Q4. Detachment of the thin layer from the MED dish

A4. We think that there is a high possibility that the thin layer detaches when the cells are cultured beyond day 16 or during medium change. We suggest using the thin layer for the assay between day 9 and day 14.

Q5. Extracellular action potential cannot be detected

A5. Check whether the electrode nodes are covered by the beating area of the thin layer.

Q6. Extracellular action potential does not stabilize

A6. Beating of the thin layer is usually stabilized on day 9. Please continue to culture the cells and observe them.

Q7. Is 5% CO_2 mixed with air gas required?

A7. We recommend using 5% CO_2 mixed with air gas because it contributes to stable beating in the periodic cycle.

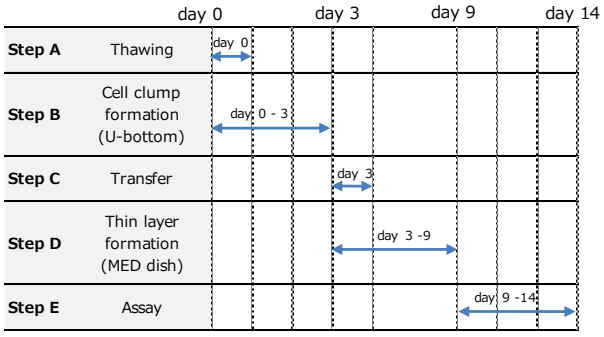
ReproCardio2™: Measurement of the extracellular electric potential of cardiomyocytes grown in thin layer using QT-interval-prolongation assay (QTempo)

Ver. MEA system (Multi Channel Systems, MCS)

Critical Points for Cell Handling

1. 15,000 cells are used to make loose cell clumps. If the cell number is less than 15,000, thin layer may not be formed on the MED dish.
2. Transfer loose cell clumps from the U-bottom plate to a MED dish on day 3 when clumps have been formed in almost all wells.
3. If the cell clumps remain in the U-bottom plate past day 4, they become rigid and may not form a thin layer.
4. If cell clumps are still loose by day 3, the cell clumps should be transferred very gently to the MED dish by using a pipette.
5. After day 5, culture medium (1,000 µL/dish) should be changed every other day by using a micropipette. Be very gentle to avoid detachment of the thin layer from the MED dish.
6. Assay should be performed under 37℃, 5% CO_2 environment. The control of CO_2 can contribute to sustainable beating cycle.
7. Recommended size of electrode is 20 µm×20 µm.

Schedule for thin layer formation on the MEA dish



Volume of required culture medium

Cell clump number per MED dish	Cultivation Time: by day 7	by day 8-9	by day 10-11	by day 12-13	by day 14
1	171.9 mL	234.9 mL	297.9 mL	360.9 mL	423.9 mL
2	134.7 mL	166.7 mL	198.7 mL	230.7 mL	262.7 mL
3	121.5 mL	142.5 mL	163.5 mL	184.5 mL	205.5 mL
4	115.5 mL	131.5 mL	147.5 mL	163.5 mL	179.5 mL

* Please purchase ReproCardio2 Culture Medium™ (RCDC101) as appropriate.

Items not included

Name	Catalog number	Note
ReproCardio2 Assay Medium ™	ReproCELL, RCDC301	Store at 4°C
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Fibronectin Human, Plasma	Life Technologies, 33016-015	Store at 4°C
5% CO_2 mixed with air gas	–	Room tenpaerature

Note 1. Read carefully the protocol for cell thawing (page 2-4).

Step A: Preparation of the cell suspension (day 0)

Add12.3 mL medium (tube C) per tube of cell suspension in tube A to make final concentration of 0.75×10^5 cells/mL. **Note 2.** The cell suspension should be kept at 37°C in a water bath to maintain high viability.

Step B: Formation of loose cell clumps in a U-bottom 96-well plate (day 0–3)

1. Add 200 µL of the cell suspension into each well of a 96-well U-bottom plate.

Note 3. 1.5×10^4 cells should be used to form thin layer

2. Place the 96-well plate in an incubator maintained at 37°C with 5% CO_2. Remove 100 µL/well medium on the next day (day 1) (total volume of medium: 200 µL/well).
3. Remove 100 µL of the medium and add 100 µL/well of fresh medium on day 2.
4. Single cells start to aggregate spontaneously to form loose clumps.
   Clump formation: day 1: 30–50%, day 2: 80%, day 3: 100% in 96-well plate

Step C: Transfer of loose cell clumps to the MEA dish (day 3)

Note 4. Once cell clumps have formed in the U-bottom plate, transfer them to the MEA dish on day 3. If the cell clumps remain in the U-bottom plate past day 4, they become rigid and may not form a thin layer.

Note 5. The chance of thin layer formation is lower on a glass surface (e.g., MEA dish such as Multi Channel Systems, 50%) than on a plastic surface (over 90%). A few additional MEA dishs should be prepared for the first experiment.

1. Place a MEA dish (200/30) (MCS60MEA200/30iR-Ti-gr) into a Φ 100-mm dish.
2. Dilute 5 mg of human plasma fibronectin with phosphate-buffered saline (-) to a final volume of 50 µg/mL.
3. To coat the MEA dish, add **20 µL** of the human plasma fibronectin solution (50 µg/mL) to the electrode nodes of the MEA dish. In order to prevent evaporation of the medium, add 3 mL phosphate-buffered saline in Φ 100-mm dish (outside the MEA dish). Incubate the MEA dish for 1 hour at 37°C with 5% CO_2.
4. Add 1 mL/dish of PBS (-), and remove the coating solution from the MEA dish.
5. Set a micropipette (P-200) to **150** µL.
6. Transfer loose cell clumps very gently from the U-bottom plate to the MEA dish by using a micropipette.

   Note 6. For the 6-well dish, set a micropipette (P-200) to 33 µL.
7. Distribute the medium in the entire culture area of dish or well.
8. Move the clumps gently to the electrodes in the MEA dish by using a pipette tip or a glass rod under a phase contrast microscope.
9. Incubate the MEA dish at 37°C with 5% CO_2.

Step D: Thin Layer Formation on the MEA Dish (day 3–9)

1. Add 200 µL/dish of medium on day 4.

   Note 7. For the 6-well dish, add 100 µL/well of medium.
2. The cell clumps start to contract and flatten spontaneously to form a thin layer on the MEA dish.

   Note 8. The outline of the cell clump will gradually become less distinct (day 3–7). Please refer to the presentive image of a thin layer of Cardiomyocytes on the MEA dish.
3. Change the culture medium every other day. When changing the medium, remove the old medium completely and add 1,000 µL/dish of fresh and warm medium with a micropipette.

   Note 9. The culture medium is changed every other day with a micropipette not with an aspirator. Be very gentle to avoid detachment of the thin layer from the MEA dish.

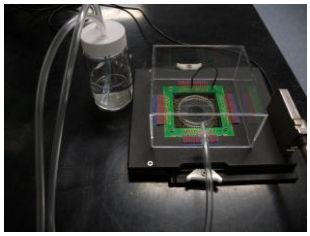
   Note 10. The culture medium MUST NOT be added directly onto the cells, because cells may detach easily upon mechanical disturbance.

   Note 11. For the 6-well dish, add 150 µL/well of medium per well.

Step E: Assay (day 9–14)

1. Prepare the test compound solution (×10) and maintain it at 37°C.

2. Set the temperature inside the MEA dish to 37°C using a thermal controller. Cover the MEA dish with the lid of a 35-mm dish to prevent evaporation of the medium. Moreover, shield the MEA dish by using an airtight chamber.



Note 12. To ensure that the temperature of the electrodes of the MEA dish is adequate, set the thermal controller at 37°C.

3. Add 5% CO_2 mixed with air gas by bubbling through MilliQ water (30 mL) in the bottle.
4. Remove the old culture medium completely from the MEA dish with a P-1000 micropipette and add 800 µL of warmed ReproCardio Assay Medium™ to the MEA dish. Incubate the cells for 10 min at 37°C with 5% CO_2.

   Note 13. For the 6-well dish, add 150 µL of ReproCardio Assay Medium™ / well.
5. Connect the MEA dish to the MEA amplifier and incubate it for 10 minutes.

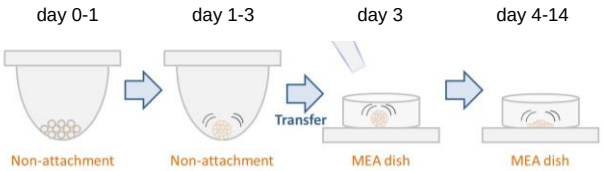
   Note 14. The MEA assay should be performed during day 9–14, because beating of the thin layer is usually stabilized by day 9. On the other hand, there is the possibility of detachment of cells after day 14.
6. Measure the electrical signals (beating rate, Na^+ amplitude, K^+ amplitude, and Na^+–K^+ interval, etc.).
7. For the addition of testing compound, remove 1/10 of the medium gently from the assay medium and add an equal volume of the testing compound solution.
8. Mix the medium by gentle pipetting.
9. Expose the cells to each dose of the compound for 4 or 10 minutes and record/analyze the last wave at 2–4 minutes or 8–10 minutes.

   Note 15. Repeat steps 7–9 until the examinations are completed.

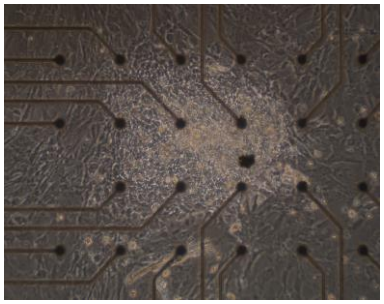
   Note 16. Please determine the appropriate exposure time for each compound.

Example Images

1. Review of the steps.



A thin layer of cardiomyocytes on the MEA dish.



Q&A

Q1. Cell clumps do not contract in the U-bottom well plate

A1. Cell clumps usually start to contract on day 2–3. However, even if the loose cell clumps do not contract, please transfer them to the MEA dish on day 3. They will start to contract on the MEA dish.

Q2. How often should the medium be changed?

A2. The medium should be changed every other day. We suggest to aliquot the medium before use.

Q3. No thin layer forms on the MEA dish after transfer

A3. Although the loose cell clumps are transferred to the MEA dish such as Multi Channel Systems on day 3, the likelihood of successful thin layer formation is 50%. We propose to prepare additional MEA dishs for the first experiment.

Q4. Detachment of the thin layer from the MEA dish

A4. We think that there is a high possibility that the thin layer detaches when the cells are cultured beyond day 16 or during medium change. We propose to use the thin layer for the assay between day 9 and day 14.

Q5. Extracellular action potential cannot be detected

A5. Check whether the electrode nodes are covered by the beating area of the thin layer.

Q6. Extracellular action potential does not stabilize

A6. Beating of the thin layer is usually stabilized by day 9. Please continue to culture the cells and observe them.

Q7. Is 5% CO_2 mixed with air gas required?

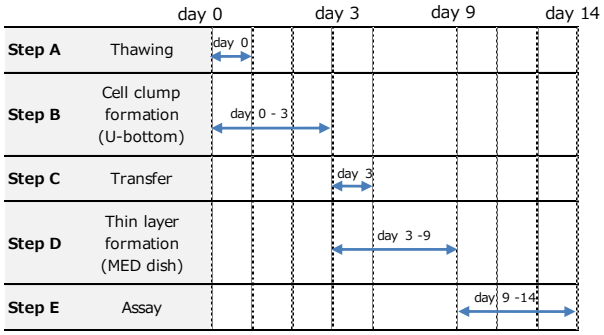
A7. We recommend using 5% CO_2 mixed with air gas because it contributes to stable beating in the periodic cycle.

ReproCardio2™: Measurement of the extracellular electric potential of cardiomyocytes grown in thin layer using QT-interval-prolongation assay (QTempo)
Ver. Maestro (Axion)

Critical Points for Cell Handling

1. 15,000 cells are used to make loose cell clumps. If the cell number is less than 15,000, thin layer may not be formed on the MED dish.
2. Transfer loose cell clumps from the U-bottom plate to a MED dish on day 3 when clumps have been formed in almost all wells.
3. If the cell clumps remain in the U-bottom plate past day 4, they become rigid and may not form a thin layer.
4. If cell clumps are still loose by day 3, the cell clumps should be transferred very gently to the MED dish by using a pipette.
5. After day 5, culture medium (1,000 µL/dish) should be changed every other day by using a micropipette. Be very gentle to avoid detachment of the thin layer from the MED dish.
6. Assay should be performed under 37℃, 5% CO_2 environment. The control of CO_2 can contribute to sustainable beating cycle.
7. Recommended size of electrode is 20 µm×20 µm.

Schedule for thin layer formation on the MEA probe



Volume of required culture medium

	Cultivation Time				
Cell clump number per MED dish	by day 7	by day 8-9	by day 10-11	by day 12-13	by day 14
1	171.9 mL	234.9 mL	297.9 mL	360.9 mL	423.9 mL
2	134.7 mL	166.7 mL	198.7 mL	230.7 mL	262.7 mL
3	121.5 mL	142.5 mL	163.5 mL	184.5 mL	205.5 mL
4	115.5 mL	131.5 mL	147.5 mL	163.5 mL	179.5 mL

* Please purchase ReproCardio2 Culture Medium™ (RCDC101) as appropriate.

Items not included

Name	Catalog number	Note
ReproCardio2 Assay Medium ™	ReproCELL, RCDC301	Store at 4°C
ReproCardio2 Culture Medium ™	ReproCELL, RCDC101	Store at 4°C
Fibronectin Human, Plasma	Life Technologies, 33016-015	Store at 4°C
5% CO_2 mixed with air gas	–	Room tenpaerature

Note 1. Read carefully the protocol for cell thawing (page 2-4).

Step A: Preparation of the cell suspension (day 0)

Add12.3 mL medium (tube C) per tube of cell suspension in tube A to make final concentration of 0.75×10^5 cells/mL. **Note 2.** The cell suspension should be kept at 37°C in a water bath to maintain high viability.

Step B: Formation of loose cell clumps in a U-bottom 96-well plate (day 0–3)

1. Add 200 µL of the cell suspension into each well of a 96-well U-bottom plate.

Note 3. 1.5×10^4 cells should be used to form thin layer

2. Place the 96-well plate in an incubator maintained at 37°C with 5% CO_2. Remove 100 µL/well medium on the next day (day 1) (total volume of medium: 200 µL/well).
3. Remove 100 µL of the medium and add 100 µL/well of fresh medium on day 2.
4. Single cells start to aggregate spontaneously to form loose clumps.
   Clump formation: day 1: 30–50%, day 2: 80%, day 3: 100% in 96-well plate

Step C: Transfer of loose cell clumps to the MEA probe (day 3)

Note 4. Once cell clumps have formed in the U-bottom plate, transfer them to the MEA probe on day 3. If the cell clumps remain in the U-bottom plate past day 4, they become rigid and may not form a thin layer.

Note 5. The chance of thin layer formation is lower on a glass surface (e.g., MEA probe such as Multi Channel Systems, 50%) than on a plastic surface (over 90%). A few additional MEA probe should be prepared for the first experiment.

1. Prepare a MEA probe.
2. Dilute 5 mg of human plasma fibronectin with phosphate-buffered saline (-) to a final volume of 50 µg/mL.
3. To coat the MEA probe, add **20 µL** of the human plasma fibronectin solution (50 µg/mL) to the electrode nodes of the MEA probe. In order to prevent evaporation of the buffer, add 2 mL phosphate-buffered saline outside the MEA probe. Incubate the MEA probe for 1 hour at 37°C with 5% CO_2.
4. Add 1 mL/well of PBS (-) and remove the coating solution from the MEA probe.
5. Set a micropipette (P-200) to **50 µL**.
6. Transfer loose cell clumps very gently from the U-bottom plate to the MEA probe by using a micropipette.
7. Distribute the medium in entire culture area of probe or well.
8. Move the clumps gently to the electrodes in the MEA probe by using a pipette tip or a glass rod under a phase contrast microscope.
9. Incubate the MEA probe at 37°C with 5% CO_2.

Step D: Thin Layer Formation on the MEA probe (day 3-9)

1. Add 200 µL/well of medium on day 4.
2. The cell clumps start to contract and flatten spontaneously to form a thin layer on the MEA probe.

   Note 6. The outline of the cell clump will gradually become less distinct (day 3–7).Please refer to the presentive image of a thin layer of Cardiomyocytes on the MED probe.
3. Change the culture medium every other day. When changing the medium, remove the old medium completely and add 1,000 µL/well of fresh and warm medium with a micropipette.

   Note 7. The culture medium is changed every other day with a micropipette not with an aspirator. Be very gentle to avoid detachment of the thin layer from the MEA probe.

   Note 8. The culture medium MUST NOT be added directly onto the cells, because cells may detach easily upon mechanical disturbance.

Step E: Assay (day 9-14)

1. Prepare the test compound solution (×10) and maintain it at 37°C.
2. Set temperature inside the MEA probe to 37℃ using a thermal controller.

   Note 9. To ensure that the temperature of electrodes of the MEA probe is adequate, set the thermal controller to at **37℃**.
3. Remove the old culture medium completely from the MEA probe with a P-1000 micropipette and add 1,000 µL/well of warmed ReproCardio Assay Medium™ to the MEA probe. Incubate the cells for 10 minutes at 37℃ with 5% CO_2.
4. Connect the MEA probe to the AXION MEA system and incubate it for 10 minutes at 37℃ with 5% CO_2.

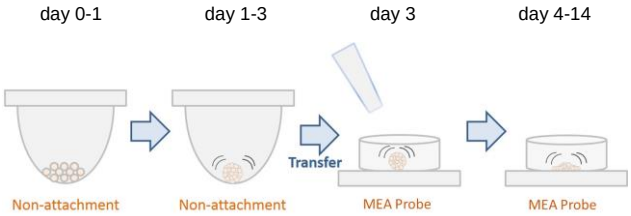
   Note 10. The assay should be done during day 9-14, because beating of the thin layer usually stabilizes from day 9. On the other hand, there is the possibility of detachment of cells after day 16.
5. Measure the electrical signals (beating rate, Na^+ amplitude, K^+ amplitude, and Na^+–K^+ interval, etc.).
6. For the addition of testing compound, remove 1/10 of the medium from the assay medium and add an equal volume of the testing compound solution.
7. Mix the medium by gentle pipetting.
8. Expose the cells to each dose of compound for 4 or 10 minutes and also recording /analyze the last wave at 2-4 minutes or 8-10 minutes.

   Note 11. Repeat steps 7–9 until examinations are completed.

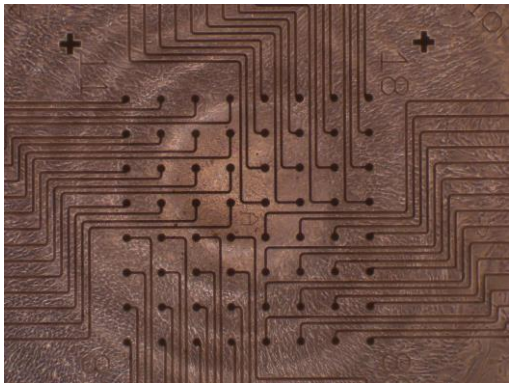
   Note 12. Please determine the exposure time for each compound.

Example Images

1. Review of the steps.



2. Thin layer of cardiomyocytes on the MEA probe.



Q&A

Q1. Cell clumps do not contract in the U-bottom well plate

A1. Cell clumps usually start to contract on day 2–3. However, even if the loose cell clumps do not contract, please transfer them to the MEA probe on day 3. They will start to contract on the MEA probe.

Q2. How often should the medium be changed?

A2. The medium should be changed every other day. We suggest to aliquot the medium before use.

Q3. No thin layer forms on the MEA probe after transfer

A3. Although the loose cell clumps are transferred to the MEA probe such as Multi Channel Systems on day 3, the likelihood of successful thin layer formation is 50%. We propose to prepare additional MEA probes for the first experiment.

Q4. Detachment of the thin layer from the MEA probe

A4. There is a high possibility that the thin layer detaches when the cells are cultured beyond day 16 or during medium change. We propose to use the thin layer for the assay between day 9 and day 14.

Q5. Extracellular action potential cannot be detected

A5. Check whether the electrode nodes are covered by the beating area of the thin layer.

Q6. Extracellular action potential does not stabilize

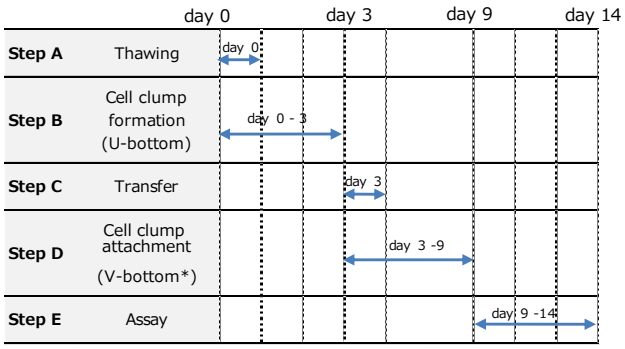
A6. Beating of the thin layer is usually stabilized on day 9. Please continue to culture the cells and observe them.

ReproCardio2™ : Calcium-Imaging Assay

Critical Points for Cell Handling

1. Place the cell clump in the center of the assay plate during transfer.
2. Cells have to be cultured until day 7 to acquire stable beating thin-layer for assay.
3. Cal-520 mixture should not be thawed repeatedly.

Schedule for Thin Layer Formation



Items not included

Name	Catalog Number	Note
ReproCardio2 Assay Medium™	ReproCELL RCESD301	Store at 4°C
ReproCardio2 Culture Medium™	ReproCELL RCESD101	Store at 4°C
ReproCoat™	ReproCELL RCHEOT001	R.T
Cal-520™AM	ABD Bioquest 21130	Store at -20°C
Cremophor EL	Sigma C5135-500G	R.T.
96-well plate (V-bottom)	Sumitomo bakelite MS-9096V	R.T.
DMSO	WAKO 049-07213	R.T.

Note 1. Read carefully the protocol for cell thawing (page 2-4).

Step A: Preparation of the cell suspension (day 0)

Add 9 mL medium (from tube C) per tube of cell suspension in tube A to make final concentration of 1×10^5 cells/mL. **Note 2** .The cell suspension should be kept at 37°C in a water bath to maintain high viability.

Step B: Cell Clump Formation (Non-attachment U-bottom 96-well Plate) (day 0-3)

1. Add 100 µL of the cell suspension into each well of a 96 well U-bottom plate.
2. Add 100µL of the Culture medium into each well of a 96 well U-bottom plate.

   Note 3. To form a thin layer, we recommend to adhere cell clumps (1.0×10^4 cells in 200 µL/well of the cell suspension).
3. Place the 96-well plate in an incubator maintained at 37°C with 5% CO_2.
4. Remove 100 µL/well of medium and add 100 µL/well fresh medium at day 2
5. Single cells start to aggregate spontaneously to form loose clumps.

   Clump formation: day 1: 30–50%, day 2: 80%, day 3: 100% in 96-well plate.

Step C: Transfer of the Clumps (day 3)

1. Prepare the V-bottom 96-well plate, which is not included in kit.
2. For coating the V-bottom 96-well plate, add 50 µL/well of ReproCoat™ on the V-bottom 96-well plate and incubate it at least 12 hours at 37°C with 5% CO_2 .
3. Remove the coating solution by aspirator.
4. Adjust a micropipette (P-200) to 200 µL.
5. Transfer all of the content of the non-attachment U-bottom 96-well plate to the wells of V-bottom plate* by using the P-200 micropipette. Please make sure that the

clump is taken out slowly and gently by pipetting while counting to 3 seconds. This is because the clumps at day 3 are still loose and fragile. Add 1 clump to each well.

Note 4. The cell clump should be placed in the center of the assay plate during transfer by tilting the plate.

6. Culture the clumps for more than day 9. The cells can then be assayed between day 9 and day 14.

Step D: Attachment and Maintenance (Steps to be followed after the Transfer)

1. Warm the necessary amount of ReproCardio Culture Medium 2 ™ (+FBS) at 37°C for at least 15 minutes.
2. Change the culture medium every other day from day 4. For the medium change, please remove 100 µL/well of the culture medium and add 100 µL/well of fresh and warm ReproCardio Culture Medium 2 ™ (FBS+). (Medium is necessary to change half volume of supernatant.)

   Note 5. Ensure that the medium is added along the wall of well, not directly on to the cells.
3. Culture the cells by placing the plate in the incubator at 37°C with 5% CO_2.

Step E: Procedure for Calcium Imaging Assay (day 9-14)

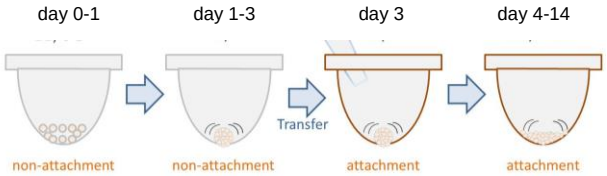
1. Aliquot necessary amount of the ReproCardio Assay Medium™ (For example, 20 mL/96 well), and subsequently add final concentration of 5 µM Cal-520™AM/DMSO and 0.05% Cremophor EL (Ca indicator solution).
2. Remove all of the culture medium from the plate.
3. Voltex Ca indicator solution for 10 seconds and add 100 µL/well of Ca indicator solution.
4. Incubate the thin layers for 120 minutes at 37°C with 5% CO_2.
5. Prepare stock solution of testing compound (×10), which various concentrations of testing compound can be prepared from. Warm the test solutions at 37°C.
6. Remove the 180 µL/well of medium from the plate.
7. Add 80 µL/well of warm ReproCardio Assay Medium™ in the absence of Cal-520™AM, or Cremophor EL.
8. Incubate the thin layers for 10 minutes in the instrument under heat control for Ca imaging assay.
9. Before the treatment of testing compound, obtain control fluorescence images at Ex488 nm/Em 514 nm for 30 seconds by using inverted microscope equipped with an appropriate filter set.

   Note 6. Representative result image were shown by using ECLIPSE Ti; Nikon, Japan (page 19).
10. Remove 10 µL/well of the medium, and add 10 µL/well of the test compound stock solution (x10). Mix the medium by pipetting once.
11. After 10 minutes, obtain fluorescence images at Ex488 nm/Em 514 nm for 30 seconds.
12. Repeat steps 9 and 10 until the examinations are complete.

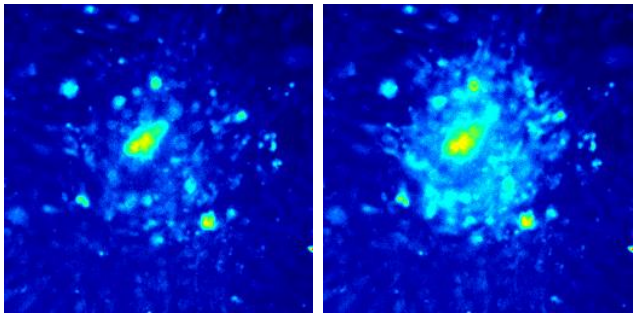
    Note 7. All the images should be obtained within 180 minutes after stained. The fluorescence is high possible to fade off after 180 minutes.
13. Quantify the acquired images by using analysis software such as AQUACOSMOS or Wave analysis (HAMAMATSU PHOTONICS).

Example Images

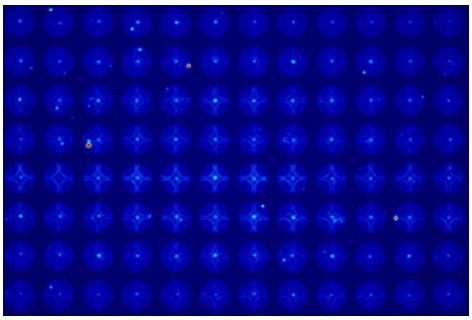
1. Review of the steps.



2. Representative images of Fluo-8-AM™ fluorescence at Ex488 nm/ Em 514 nm during calcium imaging. Left: Thin layer in its resting state. Right: Thin layer in its activated state



3. 96 well plat form by FDSS/μCELL (HAMAMATSU PHOTONICS)



Q&A

Q1. The beating was stopped after transfer

A1. Cells will start to beat again after incubation over 1 hour at 37°C with 5% CO_2.

Q2. What if the cell does not emit fluorescence

A2. First, you should observe whether or not cells were beating. If the cell are not beating, continue to culture for another 2 or 3 days. On the other hands, if the cells are beating, you should check whether or not the cell has fluorescence emission under the medium including Ca indicator solution. If not, we recommend using new or fresh Ca indicator.

Q3. Can the cells be used for assay that are not central position in assay plate?

A3. If the cells are beating, it is not a problem to measure the intensity of fluorescence with microscope. However, we recommend to place the cells on the central position when using HTS instruments (For example, FDSS, HAMAMATSU PHOTONICS)

Q4. Can the cells measure fluorescence (Ca imaging) over 1 hour

A4. Add the Cal-520 (2 μM) directly. In the case of the high background, please add quencher.

Movies of beating thin layers available:
https://www.reprocell.com/en/cardio-layer/

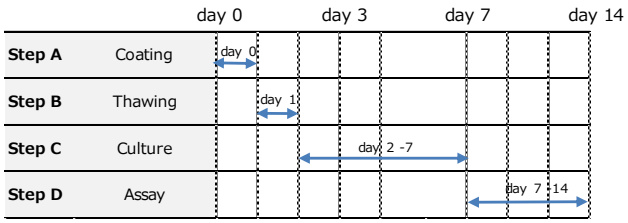
ReproCardio2™ : Culture Method for Patch Clamp Technique

Coverslip	MATSUNAMI C013001I	R.T.

Critical Points for Cell Handling

1. It is necessary to make the mixture of coating solutions (ReproCardio2 Coat for Patch clamp solution B™ and C™) in accordance with the steps mentioned in this manual. Be sure that you should add it onto coverslips after mix within 15 minutes.
2. Ensure that the cardiomyocytes do not detach Do not use an aspirator; instead use a micropipette while removing the entire culture medium, otherwise the cells might detach easily from coverslips. Please add the medium along the wall of well.
3. Cells have to be cultured between day 1-7 and they can be assayed by day 14.

Schedule for Patch Clamp



Items not included

Name	Catalog number	Note
ReproCardio2 Coat for Patch Clamp Solution A™ (12 mL)	ReproCELL RCDC201	4℃
ReproCardio2 Coat for Patch Clamp Solution B™ (0.4 mL)	ReproCELL RCDC201	−20℃
ReproCardio2 Coat for Patch Clamp Solution C™ (1.4 mL)	ReproCELL RCDC201	−20℃
ReproCardio2 Culture Medium™	ReproCELL RCDC101	4℃
24-well plate; flat bottom	TPP Z707791-126EA	R.T.

Step A: Coating (day 0)

Note 1. Ensure that the mixture of coating solutions was quickly added into coverslips within 15 minutes after mix the ReproCardio2 Coat for Patch clamp solution B™ and C™.

1. Sterilize the coverslips by autoclaving.
2. Place coverslips on the 24-well plate.
3. Thaw ReproCardio2 Coat for Patch Clamp solution B™ (Coat B) and C™ (Coat C) on ice at least 1 hour.
4. Prepare ReproCardio2 Coat for Patch Clamp A™ after thawing Coat B and C.
5. Mix Coat B and C in 15 mL tube at room temperature by following the guidelines mentioned below.

   Note 2. Residues of Coat B and C can be stored in -20℃ again.
6. Add requisite amount of Coat A into the 15 mL tube and mix it with the Coat B and C.

Number of wells	Volume (µL)	ReproCardio Coat (µL)		
		A	B	C
1	500	437.5	12.5	50
5	3,000	2,625	75	300
10	5,000	4,375	125	500
15	7,500	6,562	187	750
24	12,000	10,500	300	1,200

7. Add 500 µL of the solution prepared in step 6 onto the coverslip placed on each well of the 24-well plate.
8. Incubate the 24-well plate at room temperature for 6 hours.
9. After incubation, remove the supernatant from the well by using a pipette. Then, add 500 µ L/well of ReproCardio2 Culture Medium™ and incubate it at 37°C with 5% CO_2.

Note 3 Read carefully the protocol for cell thawing (page 2-4).

Step B: Thawing (day 1)

1. Add 9 mL medium (tube C) per tube of cell suspension in tube A to make final concentration of 1.0×10^5 cells/mL.

   Note 4. The cell suspension should be kept at 37°C in a water bath to maintain high viability.
2. Remove all the culture medium in coated coverslip well (Prepared in Step A) and add 750 µL/well of ReproCardio2 Culture Medium™ (FBS+).
3. Add 250 µL of cell suspension (2.5×10^4 cells) slowly onto each coverslip.
4. Incubate the cells at 37℃ with 5% CO_2.

   Note 5. Please aspirate and add gentry on changing the medium

Step C: Culturing (day 2-7)

1. Change ReproCardio2 Culture Medium™ (FBS+) on day 3, day 5, day 7 (every other day).
2. Warm the necessary amount of medium at 37°C for at least 15 min.
3. By using P-1000 pipet, remove 500 µL/well of the culture medium from the supernatant, and subsequently add 500 µL/well of ReproCardio2 Culture Medium™ (FBS+) along the wall of well (Medium is necessary to change half volume of supernatant.)
4. Incubate the culture medium at 37°C with 5% CO_2.

   Note 6. Ensure that medium is added along the wall of well until day 7 because adherent ability of cardiomyocytes is very weak.

Step D: Assay (day 7-14)

Cells can be assayed between day 7 and day 14. It is difficult to patch the cells cultured until day 6.

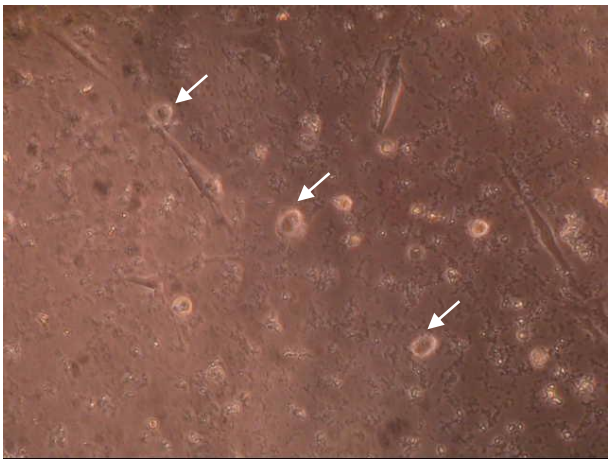


Figure: Cardiomyocytes (white arrows) attached onto the coverslip (Magnification: 200×).These cells are used for patch crump.

Q&A

Q1. How did you acquire the patch clamp data on your web page?

A1. They were acquired by whole cell patch clamp.

Q2. Would you show the inside and outside solutions on the whole cell patch clamp in detail?

A2. Please look at the table as below. However, they are not specialized for ReproCardio2™.

【Inside solution】

140mM KCl
1mM MgCl2
5mM EGTA
10mM HEPES
5mM MgATP
(Finally, adjust to pH7.2 by KOH)

【Outside solution】

150mM NaCl
4mM KCl
1mM MgCl2
1.2mM CaCl2

10mM HEPES

10mM Glucose

(Finally, adjust to pH7.4 by KOH)

Q3. Cell cannot be adhered onto coverslip

A3. We recommend to keep as still as possible.

Q4. Cardiomyocyte is not beating after seeding

A4. Please observe the cardiomyocyte under the magnification of × 200 because it is difficult to judge whether or not single cardiomyocyte is beating.

The Cardiomyocyte should start beating by day 3. However, please perform the patch clump procedure after day 7.

Q5. Can the non-beating cells patch?

A5. We recommend that you should patch the beating cells, not non-beating cells because beating cells means to have ability of induction of action potential.

Q6. What happen if the mixture of coating solutions is prepared over 15 minutes?

A6. It has a high possibility not to coat the coverslip. So ensure that you should calculate requisite amount of Coat A, B and C before thawing.

Conditions for Use

This product is for research use only and not for therapeutic or diagnostic purposes. The sale of this product to a third party or its use for commercial purposes is not allowed without prior permission from ReproCELL.

ReproCELL Inc.
Website: https://www.reprocell.com/en
E-mail: info_en@reprocell.com