NEXEL

Cardiosight®-S Application Protocol

for the Nanion Technologies CardioExcyte96 and FLEXcyte 96

Contents

1. Introduction

Contractility and electrophysiology recordings of NEXEL Cardiosight®-S iPSC-derived cardiomyocytes on CardioExcyte 96 and FLEXcyte 96 systems

NEXEL Co., Ltd. strives to provide high quality human cardiomyocytes derived from induced pluripotent stem (iPS) cells using optimized proprietary protocols. The Cardiosight® -S is a highly pure and electrophysiologically active population of cells, ensuring that researchers get a reliable product. These cardiomyocytes recover quickly upon thawing to form a synchronized monolayer of spontaneously beating cells

The CardioExcyte 96 is a fully automated hybrid system recording both contractility and electrophysiology of intact cardiomyocyte networks, in a 96 well format. Impedance and extracellular field potential measurements are performed at high resolution, are non-invasive and label-free. FLEXcyte 96, add-on to the CardioExcyte 96 system, uses 96 well plate with flexible silicone membranes. This system allows for contractility measurements of cells cultured in a more physiological environment, reflecting the mechanical conditions of the native human heart.

Cardiosight®-S cardiomyocytes can be successfully cultured, maintained and recorded on CardioExcyte 96 NSP-96 or FLEXcyte 96 FLX-96 plate for extended time spans, thereby allowing experimental flexibility and variety of acute, sub-acute and chronic drug-induced effects.

In this Application Protocol, we provide our users with guidance and instructions on how to plate, culture and acquire data from the Cardiosight®-S cardiomyocytes for cardiac *in vitro* screening of compound effects or safety screening applications. This document should be used in addition to the **Cardiosight®-S User Guide** and **CardioExcyte 96 User Guide**.

Cardiosight®-S Application Protocol
Nanion Technologies CE96 and FLEX96

Required Equipment, Consumables, and Software

Item	Provider	Catalog number
Equipment		
Multichannel pipettor: 8 or 12 channels	Multiple providers	
CardioExcyte 96 System	Nanion Technologies GmbH	
FLEXcyte 96	Nanion Technologies GmbH	
Consumables		
Cardiosight®-S, cryopreserved hiPSC-derived cardiomyocytes >5 million cells	NEXEL Co., Ltd.	C-002
Cardiosight®-S Media kit, large (200 ml)	NEXEL Co., Ltd.	CMS-002
Cardiosight®-S Advanced Media kit, large (200 ml)	NEXEL Co., Ltd.	CMS-002A
CardioExcyte 96 Sensor Plate (NSP-96 Type Standard: 2.0 mm)	Nanion Technologies GmbH	20 1001
CardioExcyte 96 Sensor Plate (NSP-96 Type Standard: 0.6 mm)	Nanion Technologies GmbH	20 1002
CardioExcyte 96 Sensor Plate (NSP-96 Type Standar Stim)	Nanion Technologies GmbH	20 1003
FLEXcyte 96 plates (FLX-96)	Nanion Technologies GmbH or Innovitro	20 1010
Fibronectin	Sigma-Aldrich	F0895
Sterile Reagent Reservoirs	Multiple providers	
Software		
CardioExcyte Control Software	Nanion Technologies GmbH	
DataControl 96 Software	Nanion Technologies GmbH	

2. CardioExcyte 96 Experimental Protocol

NSP-96 plate coating procedure

The NSP-96 plate is to be prepared and coated on the day of cardiomyocytes plating.

1. Fibronectin stock solution (eg. 1 mg/ml) should be diluted to a final concentration of 50 µg/ml in sterile D-PBS, immediately before use.
2. Each well of the NSP-96 plate needs to be coated with 80 µl of 50 µg/ml fibronectin solution. To coat one NSP-96 plate, prepare a total of 8 ml (excess of 200 µl to account for pipetting error).
3. Add 80 µl/well of the 50 µg/ml fibronectin solution to the center of the wells of an NSP-96 plate to evenly coat the bottom of the well.
4. Incubate the fibronectin coated NSP-96 plate at 37°C for at least 3 hours.

Thawing and plating Cardiosight®-S cardiomyocytes onto the NSP-96 plate

1. Thaw the cardiomyocytes according to the Cardiosight®-S User Guide to a final volume of 10 ml Plating Media (or Advanced Plating Media). Dilute the 1 ml cell suspension from the cryovial in 1 ml of Plating Media and rinse with 8 ml of additional Plating Media.
2. To confirm cell viability and count, remove a sample of cells to be examined in a hemocytometer with trypan blue or an automated cell counter.
3. Calculate the final volume of Plating Media needed to obtain a final cell plating density of 5 x 10^5 viable cardiomyocytes/ml.
4. Aspirate the fibronectin solution from the NSP-96 plate and immediately add 100 µl/well of the cell suspension (50,000 cells/well) to the center of the wells using a multichannel pipettor.
5. Culture the cardiomyocytes in Plating Media (or Advanced Plating Media) in a cell culture incubator at 37°C, 5% CO_2 for one day.

   The freshly plated NSP-96 plate should be placed in a low traffic cell culture incubator and away from the door to minimize fluctuations in temperature and air movement in a low traffic incubator.

Cell culture maintenance of the NSP-96 plate

1. Prepare the Cardiosight®-S Maintenance Media (or Advanced Maintenance Media) as described in Cardiosight®-S User Guide.
2. Immediately before use, equilibrate an aliquot of Maintenance Media (or Advanced Maintenance Media) at room temperature for 30 minutes.
3. One day post-plating, replace the Plating Media with Maintenance Media. To remove the spent media, slightly tilt the NSP-96 plate and aspirate the media using a multichannel pipettor. Then, gently add 200 µl/well of pre-warm Manteinance Media to the side of the well to avoid disturbing the cardiomyocyte monolayer.

   Be careful not to touch the bottom of the well during media removal or addition. Also, be aware that media exchange may cause transient alterations to beating rhythm. Prior to the experiment, always allow normal beating patterns to recover after media exchange.
4. Maintain the cardiomyocyte culture on the NSP-96 plate by replacing 100% of the spent media with 200 µl/well of fresh pre-warm Maintenance Media every 48 hours.
5. Continue to culture the cardiomyocytes in a cell culture incubator at 37°C, 5% CO_2.
6. We recommend to perform experiments from day 5 post plating when the cardiomyocytes have reached a stable baseline (Figure 1).

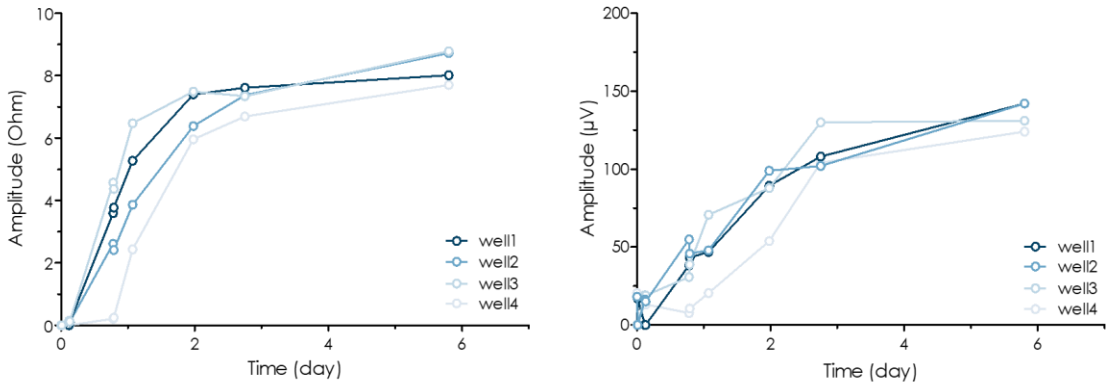


Figure 1. Cardiosight®-S cardiomyocytes form functional monolayers as recorded on CardioExcyte 96.

Impedance (left) and EFP (right) amplitudes depict the formation of a confluent monolayer of beating cells. As shown, stable signal values are reached within 5 days post plating.

Data acquisition, compound application, and analysis

1. At least 4 hours prior to the experiment, exchange the complete media inside the wells with 180 µl pre-warmed Cardiosight®-S Manteinance Media (or Advanced Maintenance Media). Place the cardiomyocytes in a cell culture incubator at 37°C, 5% CO_2.

   Evaporation rates can vary across the NSP-96 plate. Changing the Maintenance Media before compound treatment is required to ensure uniform media volumes across the plate.

2. Prepare test compounds in Maintenance Media (or Advanced Maintenance Media) at 10X the final concentration in a regular 96-well cell culture plate.

   Final DMSO concentrations above 0.1% should be used with caution. Therefore, if test compounds are dissolved in DMSO, the 10X compound solutions should not exceed 1% DMSO.

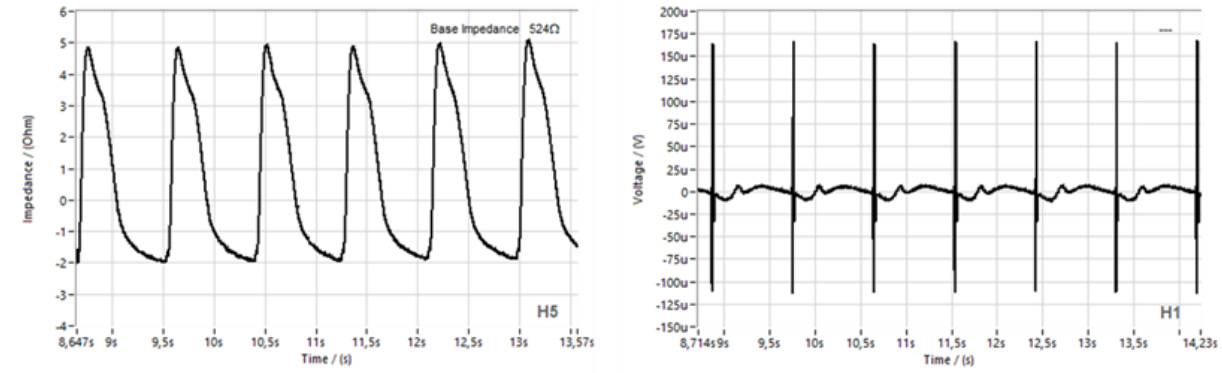
3. Equilibrate the 96-well cell culture plate containing the 10X compound solutions in a cell culture incubator at 37°C, 5% CO_2.

4. Quickly transfer 20 µl/well of the 10X compound solutions from the 96-well cell culture plate to the CE96 plate. Gently mix by pipetting 3-5 times.

   Beating rate and amplitude are temperature-dependent. For this reason it is recommended to add the compounds while the CE96 plate is placed on the CardioExcyte system. If this is not possible the CE96 plate should not be kept outside the incubator for more than 5 minutes while compounds are added.

5. The representative traces of CardioExcyte 96 for control and compound conditions using Cardiosight®-S are shown in Figure 2.

Control conditions



Compound addition

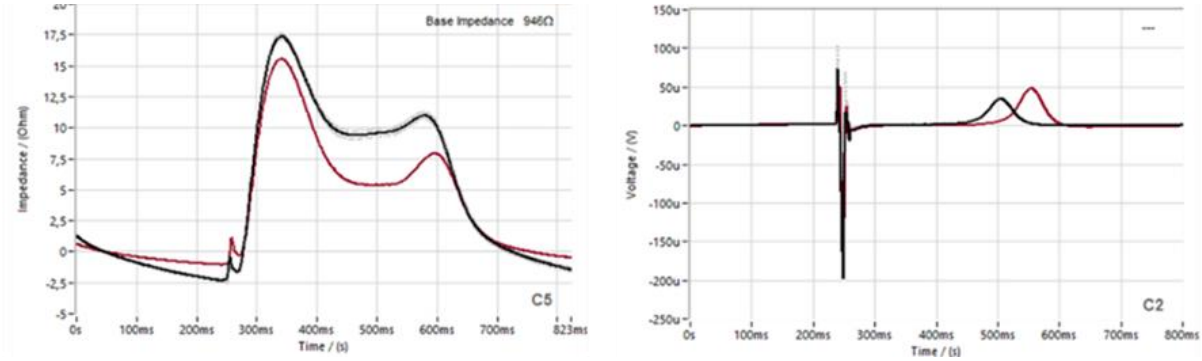


Figure 2. Impedance and EFP signals of Cardiosight®-S cardiomyocytes as recorded on CardioExcyte 96.

Upper traces show impedance (left) and EFP (right) original recording traces at day 5 of Cardiosight®-S cardiomyocytes cultured on the NSP-96 plate.

Lower traces represent impedance (left) and EFP (right) signals before (black trace) and after a compound was added (red trace).

3. FLEXcyte 96 Experimental Protocol

FLX-96 plate coating procedure

The FLX-96 plate is to be prepared and coated on the day of cardiomyocytes plating.

1. Fibronectin stock solution (eg. 1 mg/ml) should be diluted to a final concentration of 50 µg/ml in sterile D-PBS, immediately before use.
2. Each well of the FLX-96 plate needs to be coated with 80 µl of 50 µg/ml fibronectin solution. To coat a one FLX-96 plate, prepare a total of 8 ml (excess of 200 µl to account for pipetting error).
3. Add 80 µl/well of the 50 µg/ml fibronectin solution to the center of the wells of a FLX-96 plate to evenly coat the bottom of the well.
4. Incubate the fibronectin coated FLX-96 plate at 37°C for at least 3 hours.

Thawing and plating Cardiosight®-S cardiomyocytes onto the FLX-96 plate

1. Thaw the cardiomyocytes according to the Cardiosight®-S User Guide to a final volume of 10 ml Plating Media (or Advanced Plating Media). Dilute the 1 ml cell suspension from the cryovial in 1 ml of Plating Media and rinse with 8 ml of additional Plating Media.
2. To confirm cell viability and count, remove a sample of cells to be examined in a hemocytometer with trypan blue or an automated cell counter.
3. Calculate the final volume of Plating Media needed to obtain a final cell plating density of 5 x 10^5 viable cardiomyocytes/ml.
4. Aspirate the fibronectin solution from the FLX-96 plate. Immediately add 100 µl/well of cell suspension (50,000 cells/well) to the center of the wells using a multichannel pipettor.
   - Do not touch the membrane with the pipette tip to prevent punctures.
5. Culture the cardiomyocytes in Plating Media (or Advanced Plating Media) in a cell culture incubator at 37°C, 5% CO_2 for one day.
   - The freshly plated FLX-96 plate should be placed in a low traffic cell culture incubator and away from the door to minimize fluctuations in temperature and air movement in a low traffic incubator.

Cell culture maintenance of the FLX-96 plate

1. Prepare the Cardiosight®-S Maintenance Media (or Advanced Maintenance Media) as described in Cardiosight®-S User Guide.
2. Immediately before use, equilibrate an aliquot of Maintenance Media (or Advanced Maintenance Media) at room temperature for 30 minutes.
3. One day post-plating, replace the Plating Media with Maintenance Media. To remove the spent media, slightly tilt the FLX-96 plate and aspirate the media using a multichannel pipettor. Then, gently add 200 µl/well of pre-warm Manteinance Media to the from top of the well to avoid disturbing the cardiomyocyte monolayer and make sure not to touch the membrane.

   Be careful when you replace the media in order to avoid touching the bottom of the wells and rupture the silicone membranes.
4. Maintain the cardiomyocyte culture on the FLX-96 plate by replacing 100% of the spent media with 200 µl/well of fresh pre-warm Maintenance Media (or Advanced Maintenance Media) every 48 hours.
5. Continue to culture the cardiomyocytes in a cell culture incubator at 37°C, 5% CO_2.
6. We recommend to perform experiments from day 6 post plating when the cardiomyocytes have reached a stable baseline (Figure 3).

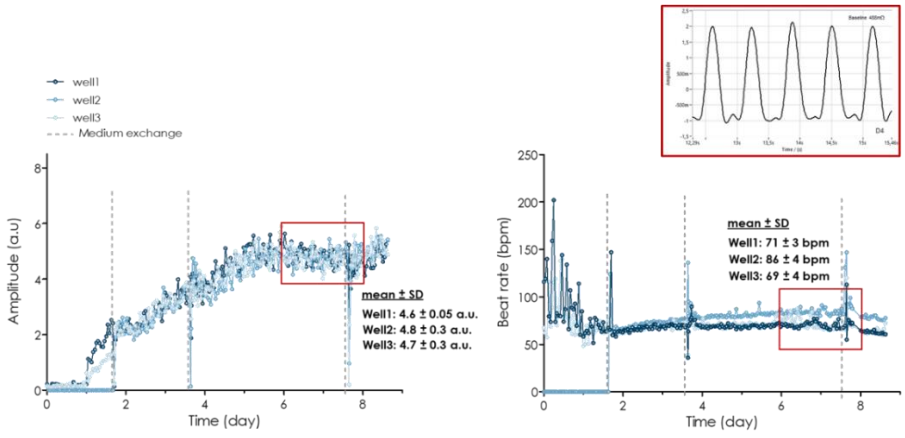


Figure 3. Cardiosight®-S cardiomyocytes form functional monolayers as recorded on FLEXcyte 96.

Amplitude (left) and beat rate (right) plots depict the formation of a confluent monolayer of beating cells in FLX-96 plate. As shown, stable signal values are reached approximately 6 days post plating. Insert depicts the morphology of a typical signal of Cardiosight®-S cardiomyocytes contracting on FLEXcyte 96 flexible membrane.

Data acquisition, compound application, and analysis

1. The day before the experiment, change the media completely with 180 µl pre-warmed Cardiosight®-S Manteinance Media (or Advanced Maintenance Media) for each well about 12 hours prior to the main experiment. Place the cardiomyocytes in a cell culture incubator at 37°C, 5% CO_2.

   - Changing the Maintenance Media 12 hours before compound treatment is required to ensure that the cardiomyocytes have completely recovered after media replacement.

2. Prepare test compounds in Maintenance Media (or Advanced Maintenance Media) at 10X the final concentration in a regular 96-well cell culture plate.

   - Final DMSO concentrations above 0.1% should be used with caution. Therefore, if test compounds are dissolved in DMSO, the 10X compound solutions should not exceed 1% DMSO.

3. Equilibrate the 96-well cell culture plate containing the 10X compound solutions in a cell culture incubator at 37°C, 5% CO_2.

4. Quickly transfer 20 µl/well of the 10X compound solutions from the 96-well cell culture plate to the FLX-96 plate. Gently mix by pipetting 3-5 times.

   - Beating rate and amplitude are temperature-dependent. It is recommended to add the compounds while the FLX-96 plate is placed on the FLEXcyte 96 system, because Cardiosight®-S cardiomyocytes plated on FLEXycte are really sensitive to temperature change. If this is not possible, the FLEXcyte plate should not be kept outside the incubator for more than 5 minutes while compounds are added.

5. The representative traces of FLEXcyte 96 for control and compound conditions using Cardiosight®-S are shown in Figure 4.

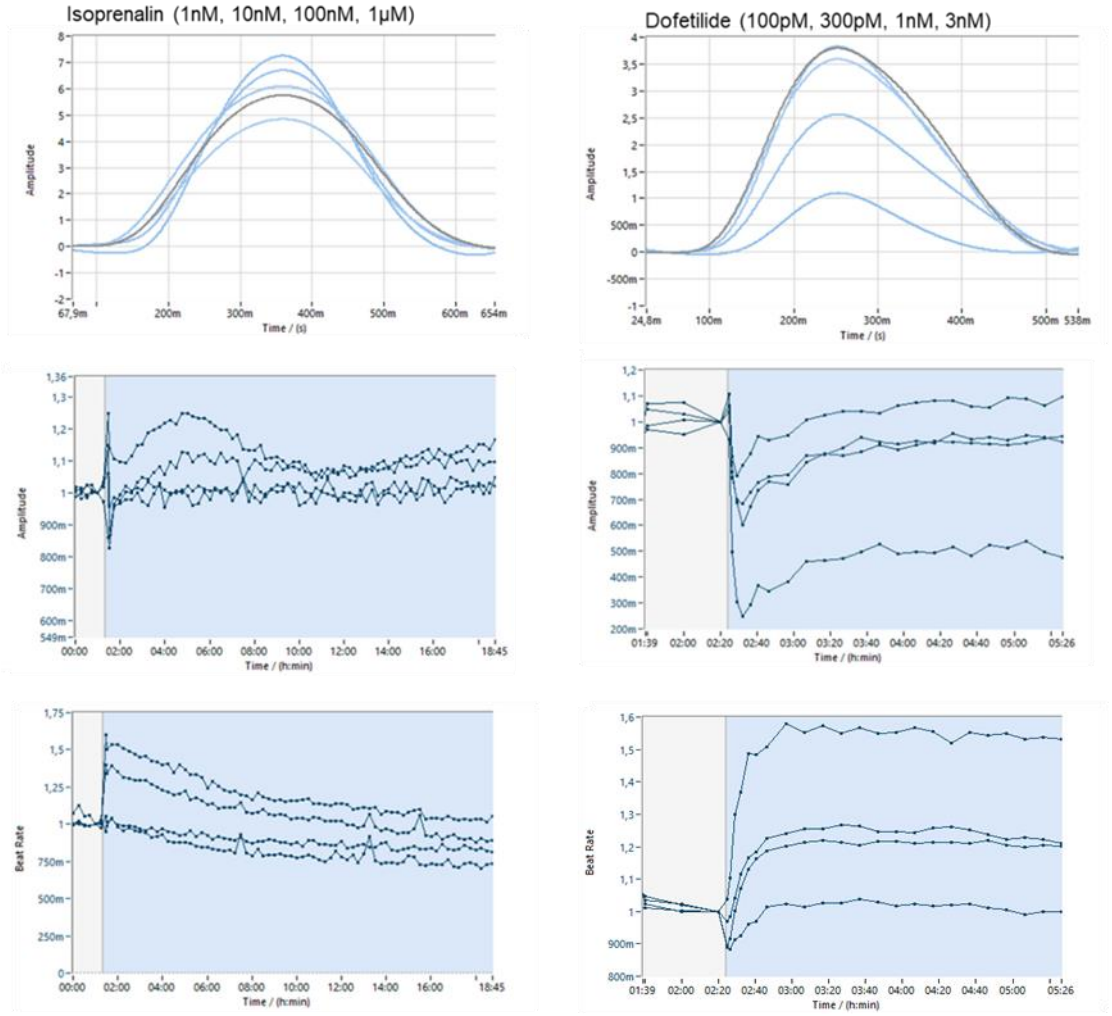


Figure 4. Responses of Cardiosight®-S cardiomyocytes to compound application recorded on FLEXcyte 96.

Effects of isoprenalin (left) and dofetilide (right) analyzed in DataControl96 software with raw traces (top), amplitude (middle) and beat rate (bottom) change over time are shown. Gray areas represent control conditions, while light blue areas indicate that the compound was added.