Ncardia
Stem cell experts

Xpress.4U™ LightPace Cor.4U® Kit

Manual

Version 1.0

Contents

1. Getting Started

Please make sure to read this entire Xpress.4U™ LightPace Cor.4U® Manual carefully before you start using the Xpress.4U™ LightPace Cor.4U® Kit. A material Safety Data Sheet (MSDS) for Xpress.4U™ LightPace is available at https://ncardia.com/resources/#certificate-of-analysis
Application notes demonstrating compound effects as analyzed by LUMOS™ (Axion Biosystems) can be found with our application browser at http://ncardia.com/application/#applications

2. Technical Support and Training

Our scientists are ready to help you with any questions you may have regarding this manual or the Xpress.4U™ LightPace Cor.4U® Kit. In addition, in-lab training is available upon request. For further information please visit our website, or contact us directly by e-mail (support@ncardia.com).

3. Kit Contents

This kit contains sufficient reagents to transfect two Lumos OptiClear 48-well plates or one Nanion CardioExcyte 96 Sensor plate.

Xpress.4U LightPace Cor.4U Kit	Nc-S-X-LP
LightPace mRNA	30 µL
Neutralization Buffer	20 µL
Liposomes	25 µL
Dilution Buffer	10 mL
Cor.4U® cardiomyocytes	4 M
Cor.4U® Complete Culture Medium	250 mL

4. Storage Conditions

Store the complete box containing the mRNA, Neutralization Buffer, Liposomes and Dilution Buffer at -80°C. Transfer the cryopreserved Cor.4U® vials directly to the vapor phase of liquid nitrogen. Store Cor.4U® Complete Culture Medium at -20°C. When stored at the conditions described in this section, the Xpress.4U™ LightPace Cor.4U® Kit can be used for 6 months after purchase date.

5. Important Notes

- Protect the reagents and specifically the liposomes from direct light
- Prevent RNAse contamination of the Xpress4U™ LightPace Cor.4U® Kit
    - Wear protective gloves and change them frequently
    - Keep tubes closed as much as possible
    - Use RNAse-free sterile disposable polypropylene tubes (e.g. Eppendorf tubes)
- Thaw the Neutralization Buffer, Dilution Buffer and Liposomes to room temperature (18 - 25°C) shortly before use
- Let the Dilution Buffer reach room temperature
- Thaw the mRNA on ice shortly before use
- Add chunks of crushed ice sparingly during each sonication process to the sonication bath to prevent overheating of the sample(s)

6. Quality Control

Xpress.4U™ LightPace Cor.4U® Kit is manufactured at Ncardia's ISO 9001:2015 certified site. Each lot is tested to certify consistent product quality. More information about this can be found in the Certificate of Analysis, which can be downloaded from our website at http://ncardia.com/resources/#certificate-of-analysis

7. Intended Use

Xpress.4U™ LightPace has been validated on Cor.4U® Cardiomyocytes (Ncardia).
Xpress.4U™ LightPace is not intended for direct administration into humans or animals.
Xpress.4U™ LightPace enables optical pacing of Cor.4U® Cardiomyocytes to investigate acute effects. Chronic pacing will cause light toxicity and is not recommended.

8. Introduction

Human induced pluripotent stem cell (hiPSC)-derived cardiomyocytes are spontaneously beating by nature and form a suitable model to study cardiac safety and toxicity, as well as drug efficacy in early drug discovery and development. To study the effect of compounds on the behavior of hiPSC-derived cardiomyocytes, electrophysiology-based platforms including microelectrode arrays (MEA) are very relevant.

Most cardioactive drugs affect the spontaneous beat rate of the hiPSC-derived cardiomyocytes. Since the repolarization of hiPSC-derived cardiomyocytes is dependent on the beat rate, it is necessary to either introduce a frequency correction to the repolarization duration or to standardize the beat rate by external pacing to assess the pure drug effect on repolarization correctly.

Certain compounds are known to exhibit different effects at different beat rates. Compounds in which a higher beat rate increases their effect are known as use-dependent compounds. Reverse use-dependence compounds however, show an increase in their effect at lower beat rates. Only by controlling the beat rate of the hiPSC-derived cardiomyocytes can these effects be detected.

Pacing of hiPSC-derived cardiomyocytes can be achieved by blue light pulses after introducing a light-sensitive ion channel into the hiPSC-derived cardiomyocytes. The Xpress.4U™ LightPace Cor.4U® Kit enables pacing with easy and non-toxic introduction of channelrhodopsin-2 into Cor.4U® Cardiomyocytes using Xpress.4U™ Technology:

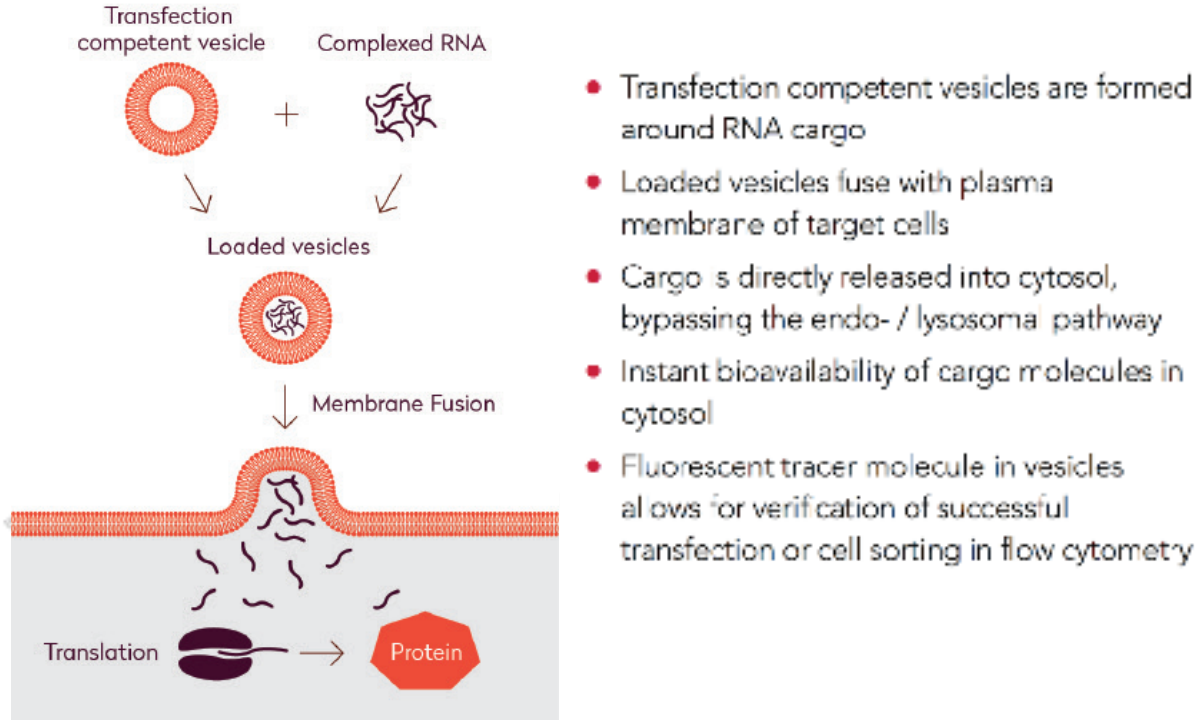

Figure 1: Xpress.4U™ technology based on fusion avoids induction of cytotoxic pathways

Cor.4U® Cardiomyocytes transfected with Xpress.4U™ LightPace adapt to optical stimulation applied through the Axion Maestro Lumos™.
Note: The successful adaptation of Cor.4U® Cardiomyocytes to the applied pacing frequency (especially high frequencies) mainly depends on the condition of the cells.

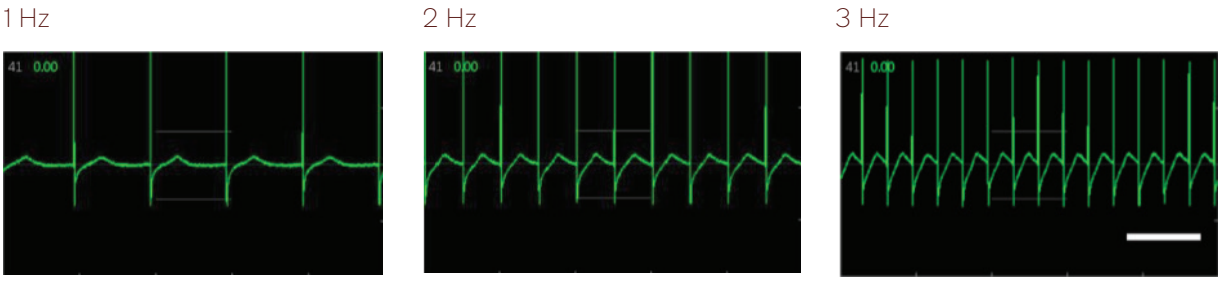

Figure 2: Cor.4U® Cardiomyocytes were paced with the Axion LUMOS™ up to 3 Hz (scale bar: 1 sec).

A demonstration of a series of compound effects at different beat rates can be found in the two corresponding application notes available through our application browser http://ncardia.com/application/#applications

9. User-supplied Equipment, Materials and Reagents

Table 1: Required equipment to be supplied by the user

Equipment	Supplier	Catalog number
Laminar flow cabinet (class II or higher)		
Cell culture incubator (37°C, 5% CO_2, humidified)		
Vacuum pump		
Ultrasonic bath Power: 30-800 W Frequency: 35-50 kHz	VWR or similar	USC200T

Table 2: Required materials to be supplied by the user

Materials	Supplier	Catalog number
1-10 µL pipette	e.g. Thermo Scientific	Various
1-10 µL pipette	e.g. Thermo Scientific	Various
20-200 µL pipette	e.g. Thermo Scientific	Various
100-1000 µL pipette	e.g. Thermo Scientific	Various
300 µL multichannel pipette	e.g. Thermo Scientific	Various
2 mL polypropylene reaction tubes (RNAse free)		
Multichannel aspiration adaptor		
50 mL reagent reservoir		
Pipette Controller		
25 mL serological pipette		
Optional: electronic stepper	e.g. Thermo Scientific	

Table 3: Required reagents to be supplied by the user

Reagents	Supplier	Catalog number
Crushed ice		

10. Methods

10.1 Recommendations for working with Cor.4U® Cardiomyocytes

A successful optical pacing experiment using the Xpress.4U™ LightPace Cor.4U® Kit depends on the health and quality of the Cor.4U® Cardiomyocytes at the time of transfection. For optimal results, it is recommended to use the Cor.4U® Cardiomyocytes batch provided in this Xpress.4U™ LightPace Cor.4U® Kit.

To enable a healthy culture of Cor.4U® cardiomyocytes we recommend to:

- Refresh the Cor.4U® Complete Culture Medium on a daily basis, when cells are cultured in assay-specific plates
- Pre-warm the culture medium to 37°C prior to refreshing the culture medium in the plate
- Keep pipette tips above the bottom of the wells to avoid disturbing the cardiomyocyte monolayer

For detailed instructions on seeding and maintenance of Cor.4U® Cardiomyocytes in multi- electrode array (MEA) plates, please refer to the MEA User Guide (Axion Maestro/ LUMOS™). Please note that in order to prevent working during the weekends, in combination with Xpress.4U™ LightPace a slight adjustment in the workflow of the preculture of Cor.4U® Cardiomyocytes is recommended. The recommended workflow can be found in section 10.2.

10.2 Procedure overview

The protocol described in this document consist of a number of sequential steps. In order to prevent activities during the weekend, the following planning is recommended:

Starting on a Thursday (day -6), cells are thawed for pre-cultivation preferably in the afternoon. The next day (day -5) a medium refreshment is performed. On Monday (day -2) the Cor.4U® Cardiomyocytes are ready to be reseeded on the Axion Lumos™ OptiClear 48 plates. The following days are used to refresh the medium and perform the transfection. On Friday (day 2), the cells are ready to be optically stimulated.

*Pacing can be done 1-3 days after transfection. Most optimal results with Cor.4U® cardiomyocytes are obtained at 2 days after transfection.

Cor4U® Cardiomyocytes can be precultured 1 to 4 days, according to the experimental planning. For detailed instructions on seeding and maintenance of Cor.4U® Cardiomyocytes in multi- electrode array (MEA) plates please refer to the MEA User Guide.

Transfection of Cor.4U® cardiomyocytes using Xpress.4U™ LightPace should be performed 2 days after seeding the Cor.4U® Cardiomyocytes in the MEA plates, when a stably beating monolayer has formed. Pacing of transfected Cor.4U® Cardiomyocytes can be performed 1-3 days after transfection, although pacing responsiveness is optimal 2 days after transfection.

For each Xpress.4U™ LightPace experiment, we recommend including a number of controls to assure data quality and validity:

- Transfection control: Do not add transfection mixture to a selected number of wells
- Baseline measurement: Record field potentials of cardiomyocytes before optical stimulation
- Compound control (optional): Treat cells with compound dissolvent only (e.g. 0.1% DMSO)

10.3 Protocol

i. Transfection using Xpress.4U™ LightPace Cor.4U® Kit

Important notes before starting:

- Indicated volumes are sufficient to transfect 1 LUMOS™ OptiClear 48 plate (48 wells).

Xpress.4U™ LightPace Cor.4U® Kit contains enough reagents to transfect 2 Lumos™ OptiClear 48 plates. However, it is recommended to execute transfection of both plates consecutively.

- Important! Add crushed ice to the ultrasonic bath before starting each sonication step.

To prevent the buildup of frictional heat between the tube(s) and the floater.

- Incorporate transfection controls.

Do not add transfection mixture to a selected number of wells.

1. Disperse the Liposomes (L) by pipetting thoroughly up and down three times. Transfer 12 µL into a 2 mL tube. Label this tube as Tube 1.
2. Sonicate the Liposomes (Tube 1) in the ultrasonic bath for 5 minutes.
   Note: keep the temperature in the ultrasonic bath below room temperature by adding crushed ice.
3. Mix 8.8 µL Neutralization Buffer (NB) and 13.2 µL mRNA in a separate 2 mL tube (label as Tube 2) and incubate 5 minutes at room temperature.
4. Transfer 10 µL of the mRNA - Neutralization Buffer mix (Tube 2) to a 2 mL tube (label as Tube 3). Transfer the remaining 10 µL to another 2 mL tube (label as Tube 4).

5. Add 5 µL of the sonicated Liposomes (Tube 1) to each of the 2 mL tubes containing the mRNA - Neutralization Buffer mixtures (Tubes 3 and 4).
   Note: avoid repeated pipetting. Mixing will occur during sonication.
6. Sonicate the mixtures (Tubes 3 and 4) in the ultrasonic bath for 5 minutes.
   Note: keep the temperature in the ultrasonic bath below room temperature by adding crushed ice.
7. Add 500 µL of Dilution Buffer to each of the sonicated complexes (Tubes 3 and 4) and mix by vortexing briefly (i.e. 2 seconds).
8. Sonicate the mixtures (Tubes 3 and 4) in the ultrasonic bath for 5 minutes.
   Note: keep the temperature in the ultrasonic bath below room temperature by adding crushed ice.
9. Vortex the transfection mixtures (Tubes 3 and 4) briefly (i.e. 2 seconds).
10. Carefully remove all culture medium from the cells, one column or row at a time, to prevent the cells from drying out.
    Note: all medium has to be removed to prevent dilution of the transfection mixture.
11. Add 20 µL of the transfection mixture (Tubes 3 and 4) carefully to the center of each well. Incubate the cells at 37°C and 5% CO2 for **30 minutes**.
    Note: briefly vortex tube 4 again before adding the mixture to the wells.
12. Carefully add 200 µL of culture medium using a multichannel pipette. Incubate the cells overnight at 37°C and 5% CO2.
13. Refresh the medium using a multichannel pipette the next day, **but no later than 24 hours after transfection.**

ii. Studying drug effects

To study acute drug effects, we recommend to:

- Perform an endpoint assay (culture of cells will not be continued after finishing the compound assay)
- Dilute test compounds in Cor.4U® Complete Culture Medium at ≥10x the desired final concentration
- Add the compound in a volume of maximum 10% of the total medium volume of the well

  For example - 20 µL in a total volume of 200 µL for the OptiClear 48 plate
- Do not exceed a final concentration of DMSO above 0.1%

1. Replace the medium in the OptiClear 48 plate at least 2 hours before the compound assay and return the plate back to the incubator (37°C and 5% CO_2).
2. Turn on the Maestro MEA system and warm the instrument to 37°C.
   Note: if desired, the cells can be optically paced before the addition of compounds to verify responsiveness and to act as a baseline for compound effect quantification.

Please let the cells recover for at least 1 hour after baseline pacing and before drug application.

3. Prepare the test compounds in Cor.4U® Complete Culture Medium at ≥10x the desired final concentration in a normal tissue culture plate. Place this "compound-plate" in an incubator at 37°C, 5% CO_2 for at least 30 minutes.
4. Transfer the OptiClear 48 plate to the Maestro device and equilibrate for at least 10 minutes.
   Note: keep the OptiClear 48 plate in the Maestro device until the end of the experiment. Any additional transport will disturb the cells and may require new equilibration.
5. Remove the chosen volume from each well of the OptiClear 48 plate.
   Example: 20 µL in a total volume of 200 µL for the OptiClear 48 plate.
6. Mix the Cor.4U® Complete Culture Medium containing the compounds gently in the compound-plate by pipetting up and down 3 times to ensure a homogeneous solution.
7. Transfer the required volume of compound solution to the dedicated wells of the OptiClear 48 plate to reach final compound concentration.
   Note: the plate will be potentially unsterile after compound addition. Culture should not be continued after finishing the assay.
8. Incubate for 30 minutes.
9. Start measurements with optical stimulation with the Axion LUMOS™.

iii. Optical stimulation using the Axion LUMOS™

1. Switch on Lumos™ device.
2. Wait until status LED on top of the lid turns cyan.
3. Place Lumos™ LED lid on top of OptiClear 48 plate in Maestro device.
   Note: the status LED will turn from cyan to green.
4. Open "Stimulation Studio" in the Axion Integrated Studio (AxIS) software by clicking the lightbulb button in the bottom panel.
5. Open the "Stimulation Type" dropdown menu in the top left of the window and select "Optical".
6. Drag-and-drop a yellow "Repeat" box from the flow control panel into the blue lane in the center of the window. Double-click the top right corner to change number of loop cycles.
7. Drag-and-drop "Optical pulse stimulation (ON block)" from the optical pulses panel into the yellow box.
   Note: ON time and light intensity can be adjusted by double-clicking.
8. Drag-and-drop "Optical pulse stimulation (OFF block)" from the optical pulses panel into the yellow box.
   Note: OFF time can be adjusted by double-clicking.
9. Repeat steps 4 – 6 to build the pulse protocol (see example in table on the next page):

Table 4: Pulse protocol example

	Frequency	Pulse duration (ON time)	OFF time	Intensity	Cycles	Duration
1	Spontaneous	5 ms	995 ms	0 %	60	1 minute
2	1 Hz	5 ms	995 ms	100 %	60	1 minute
3	1.5 Hz	5 ms	661.66 ms	100 %	90	1 minute
4	2 Hz	5 ms	495 ms	100 %	120	1 minute
5	2.5 Hz	5 ms	395 ms	100 %	150	1 minute
6	3 Hz	5 ms	328.33 ms	100 %	180	1 minute

10. Once the protocol is established, assign it to the plate (see screenshot below).

11. Start recording and immediately press the “Trigger” button in the bottom right of the AxIS software to start the stimulation protocol.
    Note: Status LED on the LUMOS™ lid will start flashing green.
    Optical stimulation will end, when all cycles of the last “Repeat” box have been executed, indicated by status LED on the LUMOS™ lid turning to permanent green again.
12. Stop the recording.

11. Troubleshooting

Problem	Comment and suggestion
Aggregates of lipids visible after thawing	• Ensure to resuspend them by thorough pipetting.
Melting of reaction tubes during sonication	• The reaction tubes are sitting too tightly in the floater causing frictional heat. • Make sure to add a sufficient amount of ice to the sonicator. • Immerse the floater every once in a while, in order to cool down the walls of the tube.
Macroscopic precipitates visible after addition of lipids to the mRNA/NB complexes.	• Lipids should only be dispended into the reaction tube containing mRNA/NB complexes. Mixing by repeated pipetting should be avoided. The actual mixing occurs during sonication.
Not all wells follow optical stimulation two days after transfection.	• Check correct positioning of light delivery device on assay plate. • Check lid of assay plate for condensation that might reduce light intensity. • Reduce media volume per well to 200 µL /well (OptiClear 48). • Increase pulse duration.

12. Limited Use Label

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