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# PROOF OF CONCEPT

# Protocol for CelXrocker® Test Flask with BioNOC II® and BioMESH® on CelXrocker®

## Contents

## Introduction

### Growing of Cells on *CelXrocker® Test Flask with BioNOC II® and BioMESH® in CelXrocker®*

The Tide Motion bioreactor achieves mixing, nutrient transport, and gas transport by pumping media in and out of a packed bed of the carriers (Figure 1).

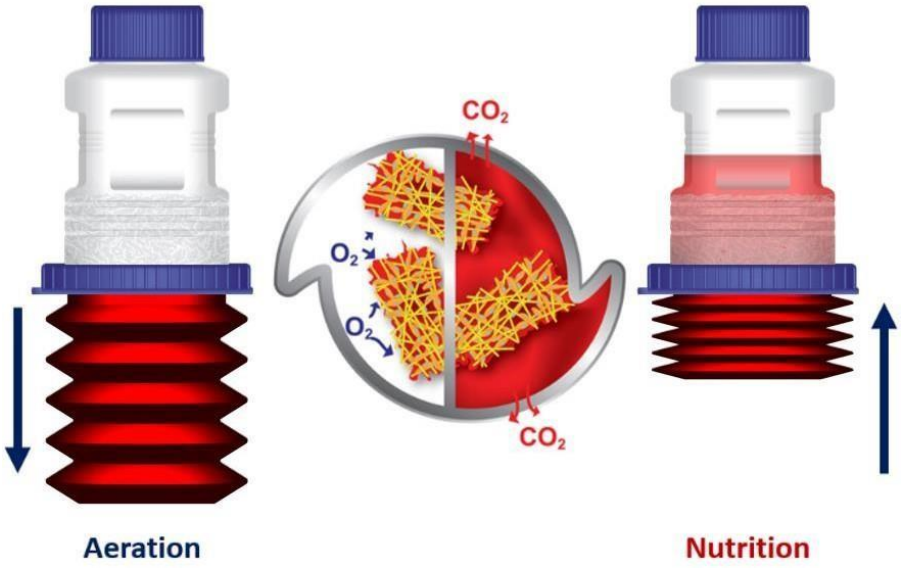



***Figure 1:*** *BelloCell® operates through the Tide Motion™ principle wherein adherent cells attached to BioNOC II® and BioMESH® carriers are alternately exposed to aeration and nutrition phases via the decompression and compression of the bellows holding the culture medium.*

The CelXrocker® serves as a small-scale system to conduct initial process optimization at lower costs. In this system, BioMESH® and BioNOC II® carriers are seeded with cells and transferred into tissue culture flasks before being placed on a 2-dimensional rocker for the culture period. The rocking motion of the CelXrocker® mimics Tide Motion™ systems by exposing the cells alternately to nutrient and aeration phases as well as providing for the gentle mixing of culture media (Figure 2).

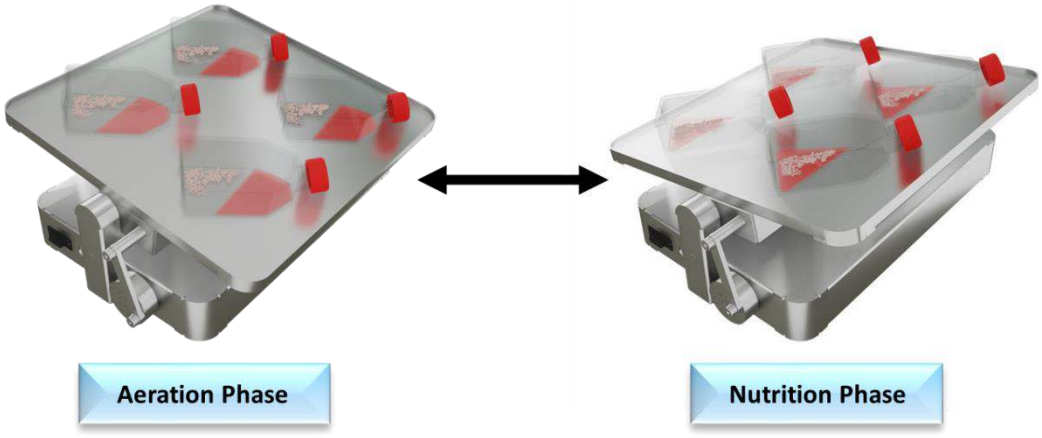


***Figure 2:*** *A schematic diagram showing how the rocking motion of CelXrocker® Test Flask mimics the Tide Motion™ principle observed in BelloCell® bioreactor by alternately exposing carriers to nutrition and oxygen supply on CelXrocker®.*

## Culturing of Cells in CelXrocker®

### Coating of Attachment Factors on Carriers (if required)

1. Aseptically transfer carriers from the CelXrocker® Test Flask into a 50 ml tube.
2. Coat the carriers as per the vendor's recommendation. The carriers should be submerged in the coating agent for the recommended amount of time and temperature.
3. Remove coating solution.
4. Rinse with DPBS if needed (coating dependent).
5. Aseptically transfer carriers a 50 ml tube into the CelXrocker® Test Flask.
6. Store as XX-coated BioMESH® or BioNOC II® carriers (store at appropriate coating temperature until use).

### Inoculation

1. Prepare cells with suggested total numbers and culture medium on the table below the recommended medium in a 50 ml tube. The pH value of the medium should be maintained between 7.0 and 7.4 (optimum pH value is 7.2)

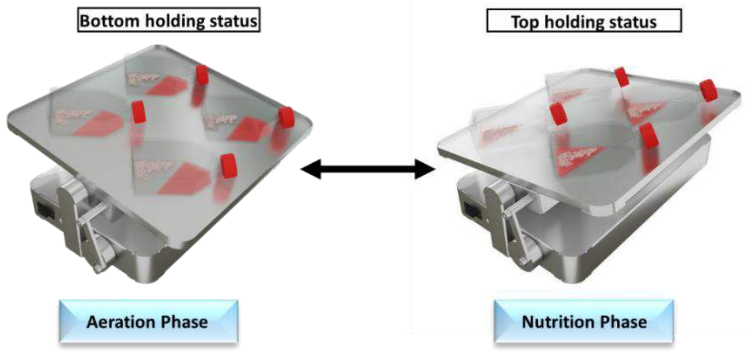
| **The recommended inoculating volume** | |
|---|---|
| **Product type** | **Inoculating volume (ml)** |
| **Flask - T75** | |
| CXR-T75 BioNOC II_5 pcs | 3 |
| CXR-T75 BioNOC II_10 pcs | 5 |
| CXR-T75 BioNOC II_20 pcs | 9 |
| CXR-T75 BioNOC II_30 pcs | 10 |
| CXR-T75 BioMESH_5 pcs | 3 |
| CXR-T75 BioMESH _10 pcs | 5 |
| CXR-T75 BioMESH _20 pcs | 9 |
| CXR-T75 BioMESH _30 pcs | 12 |
| **Flask – T175** | |
| CXR-T175 BioNOC II_10 pcs | 5 |
| CXR-T175 BioNOC II_20 pcs | 9 |
| CXR-T175 BioNOC II_30 pcs | 12 |
| CXR-T175 BioNOC II_50 pcs | 20 |
| CXR-T175 BioMESH_10 pcs | 5 |
| CXR-T175 BioMESH _20 pcs | 9 |
| CXR-T175 BioMESH _30 pcs | 12 |
| CXR-T175 BioMESH _50 pcs | 22 |

**Note:** The flask size and carrier amounts depend on the user's experimental design. For example, if the users want to take samples from a flask once during the culture period, the users can select the products of CXR-T75_5 pcs, 10 pcs or CXR-T175_10 pcs, 20 pcs. However, if customers want to take samples from a flask lots of times or daily, the users can select the products of CXR-T75_20 pcs, 30 pcs or CXR-T175_30 pcs, 50 pcs.

| **Product type** | CelXrocker® Test Flask-BioNOC II® |
|---|---|
| **Cell type** | **Seeding density (Cells/carrier)** |
| Chick embryo fibroblasts (CEF) | 300,000 – 500,000 |
| A-549 | 200,000 – 300,000 |
| Chinese hamster ovary cells (CHO) | 100,000 – 300,000 |
| HEK293T / PK-15 / IBRS-2 | 100,000 – 300,000 |
| Vero | 100,000 – 300,000 |
| Hybridoma (OKT3) | 100,000 – 300,000 |
| MDCK | 60,000 – 120,000 |
| Leghorn male hepatoma (LMH) | 50,000 – 200,000 |
| MARC-145 | 50,000 |
| Human mesenchymal stem cells (hMSCs) | 60,000 |
| Human diploid cells (WI-38 / MRC-5) | 15,000 – 20,000 |

| **Product type** | CelXrocker® Test Flask-BioMESH® |
|---|---|
| **Cell type** | **Seeding density (Cells/carrier)** |
| Chick embryo fibroblasts (CEF) | 60,000 |
| Human mesenchymal stem cells (hMSCs) | 60,000 – 90,000 |

2. Make the carriers fall to bottom of test flask.
3. Gently and slowly drop the cell suspension onto the carriers until the suspension was dropped completely and confirmed that submerged the carriers by the culture medium.
4. Position CelXrocker® in $CO_2$ incubator at 37°C and 5% $CO_2$. Adjusting the parameters in inoculated mode of CelXrocker®, **Setpoint speed: 1 rpm, Top angle: 8°, Bottom angle: 15°, Setpoint Tide Delay Top (Bottom holding status): 10 sec, Setpoint Tide Delay Bottom (Top holding status): 60 sec**.
5. Place the test flask on the platform of CelXrocker®. The placement and direction of test flask follows the schematic diagram below.



6. Make sure the tilt angle could expose the carriers entirely from the culture medium after starting rocking motion.
7. After inoculating 3 ~ 4 hours, transfer the test flask to the biosafety cabinet, gently tilt and sample ~50 µl medium for cell counting. Count suspended cells remaining in the culture medium and determine % of attachment.
8. If a satisfactory attachment rate of 90% or more is achieved (i.e. less than 10% of cells remain in media), the additional culture medium can be added to the cultured volume recommended in the table below under the background of the biosafety cabinet. Adjusting the parameters in cultured mode of CelXrocker® which **Setpoint speed: 1 to 3 rpm, Setpoint Tide Delay Top&Bottom: 10 sec.** If the attachment rate is unsatisfactory, the cells were incubated for longer inoculating time and sample ~50 µl medium for cell counting again. Typically, cell attachment is completed within 4 hours.

| **The recommended cultured volume** | |
|---|---|
| **Product type** | **Cultured volume (ml)** |
| **Flask - T75** | |
| CXR-T75 BioNOC II_5 pcs | 7-12 ml |
| CXR-T75 BioNOC II_10 pcs | |
| CXR-T75 BioNOC II_20 pcs | |
| CXR-T75 BioNOC II_30 pcs | |
| CXR-T75 BioMESH_5 pcs | |
| CXR-T75 BioMESH _10 pcs | |
| CXR-T75 BioMESH _20 pcs | |
| CXR-T75 BioMESH _30 pcs | |
| **Flask – T175** | |
| CXR-T175 BioNOC II_10 pcs | 20-25 ml |
| CXR-T175 BioNOC II_20 pcs | |
| CXR-T175 BioNOC II_30 pcs | |
| CXR-T175 BioNOC II_50 pcs | |
| CXR-T175 BioMESH_10 pcs | |
| CXR-T175 BioMESH _20 pcs | |
| CXR-T175 BioMESH _30 pcs | |
| CXR-T175 BioMESH _50 pcs | |

9. Check the cell growth on day 3, 5 and 7 or after during culture. Usually, cells will grow to plateau by day 5 to 7 if the sufficient cells are added initially. Cell count could be done by detachment solution or crystal violet nuclei count method. Users can fix and stain the cells to observe the cell morphology under microscope.

**Note:** After inoculation, the cells are mildly attached to the carriers. Be gentle while handling the carriers to prevent cells from dislodging.

**Note:** The parameters of top and bottom holding time can be adjusted by the growth situation of cells after culturing. If the cells need more nutrients from the medium, the top holding time can be prolonged. If lots of cells were cultured in a carrier, the bottom holding time can be prolonged to increase aeration time.

**Note:** Use Esco CelXrocker® or a 2D rocker that has a slow and gentle rocking speed of 1 rpm. If the rocking speed is too fast, cells may detach or have reduced growth.

## Monitoring of Cell Growth on BioNOC II® and BioMESH® Carriers

### Cell Harvesting and Counting

**1. By Dissociation Reagent**

**Enzymatic reagents for dissociation: Collagense, TrypLE Express**

1. Aseptically transfer 1 carrier from the flask into a 15 mL microcentrifuge tube.
2. Gently wash the carriers with 5 mL DPBS thrice. Discard DPBS.
3. Add 1 mL of 300U Collagenase (ThermoFisher, Cat. No. 17101015, Collagenase, Type II, powder) into the tube and incubate at 37°C for 30 min.
4. Collect the collagenase enzyme in a 15 mL collection tube.
   (Collagenase is specific to the cells which produced lots of ECM, If the cells would not produce ECM, the step 3 and 4 can be neglected.)
5. Add 1 mL of TrypLE Select in the tube and incubate at 37°C for 15 min.
6. Collect the enzyme in the 15 mL collection tube.
7. Add 1 mL of neutralization media into the carrier filled tube and flick the tube against a pen/forceps for 40 seconds.
8. Transfer the solution to the 15 mL collection tube.
9. Add 2 mL DPBS and pipette up and down to wash out the cells from the carriers and flick the tube against a 15 mL tube/forceps for 40 seconds.
10. Transfer the solution to the 15 mL collection tube.
11. Repeat step 9 and 10 at least 2 more times.
12. Centrifuge the collection tube, discard the supernatant and re-suspend cells in media for counting cells on a hemocytometer. Calculate the average cell number for one carrier and use this value to estimate the total number of cells in the T flask.

**Note:** To harvest an entire flask of carriers, harvesting could be done directly in the flask following the above protocol with slight protocol modifications: Add sufficient volume of enzyme to completely submerge the carriers. The flask may have to be tilted to a corner during the incubation period to completely submerge the carriers.

**2. By CVD (Crystal Violet Dye) Reagent**

**Note**: CVD reagent as supplied by ESCO. Not suitable for stem cells due to the large secretion of ECM.

1. Aseptically transfer 1 or 2 carriers from the test flask into a 1.5 ml micro-centrifuge tube.
2. Add 0.5 ml or 1 ml CVD reagent in each micro-centrifuge tube.
3. Vortex each micro-centrifuge tube for 60 seconds.
4. Place micro-centrifuge tube into a 37ºC incubator for 1 hour.
5. Vortex several times during incubation.
6. Count the nuclei and calculate the average cell number in one carrier.

## Cell Staining and Observation

### 1. Staining with Dyes

1. Aseptically transfer 1 BioMESH® or BioNOC II® carrier from the test flask into a 6 well plate.
2. Dehydrate and fix the cells using ethanol 70% - 100% for 10 minutes.
3. Wash off the ethanol once, using either DI water or DPBS.
4. Stain the cells with hematoxylin, or H&E dye for 5-10 mins.
5. Wash off the excess dye with DI water.
6. Observe the carriers with cells under light microscope with bright field.

**Note:** Other types of dyes may be used, e.g. Trypan blue, Liu's A&B staining. Use fluorescence dye for staining to obtain better visualization of cells left on carriers post harvesting. Refrain from using colored dyes for post-harvest stains.

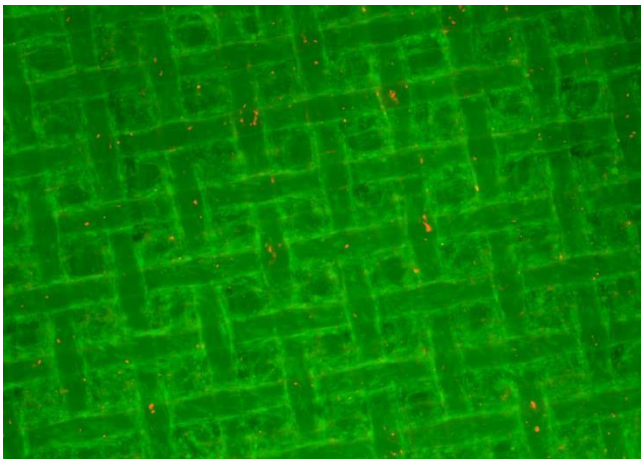
### 2. Live Cell Staining with Fluorescence Dyes

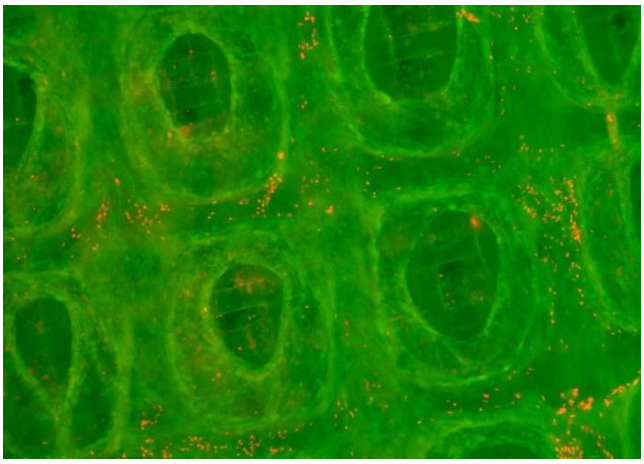
1. Aseptically transfer 1 BioMESH® or BioNOC II® carriers from the test flask into a 6 well plate.
2. Add 500 µl of culture media to the well. Add dyes at the following final concentrations: 1 µg/ml of Hoescht 33342 (Thermo Fisher, H3570), 1 uM calcein green (Thermo Fisher, C34852 and 1 µg/ml PI (propidium iodide, Sigma Aldrich P4170) in culture media.
3. Incubate the carriers for 30 mins at 37°C, 5% $CO_2$ before capturing images at their respective filters (Blue for Hoechst 33342, green for calcein green and red for PI).

**Note:** Other fluorescence dyes can be used to visualize the cells. Eg. fluorescein diacetate, Cell tracker etc.

## Carrier Staining Examples

1. Fluorescence staining of live human Mesenchymal Stem Cells





Green: Calcein green for cytoplasm, red: propidium iodide for dead cells (none or little observed)

## Related Products

| Product Name | Model | Item Code | Description |
|---|---|---|---|
| **CelXrocker®** | CXR-1-280X300-C | 2231015 | Tide 2D rocker, corded power supply |
| **Motor** | EQR/EL-DC MOTOR, 12VDC | 1620675 | Brushless DC planetary gear motor |

| Item Code | Product Name | Package |
|---|---|---|
| **BioNOC II®** | | |
| **1400376** | CXR-T75 BioNOC II_5 pcs, pack of 6 | 6 × T75 flask, prefilled with 5 pcs of BioNOC II® |
| **1400377** | CXR-T75 BioNOC II_10 pcs, pack of 6 | 6 × T75 flask, prefilled with 10 pcs of BioNOC II® |
| **1400378** | CXR-T75 BioNOC II_20 pcs, pack of 6 | 6 × T75 flask, prefilled with 20 pcs of BioNOC II® |
| **1400379** | CXR-T75 BioNOC II_30 pcs, pack of 6 | 6 × T75 flask, prefilled with 30 pcs of BioNOC II® |
| **1400380** | CXR-T175 BioNOC II_10 pcs, pack of 6 | 6 × T175 flask, prefilled with 10 pcs of BioNOC II® |
| **1400381** | CXR-T175 BioNOC II_20 pcs, pack of 20 | 6 × T175 flask, prefilled with 20 pcs of BioNOC II® |
| **1400382** | CXR-T175 BioNOC II_30 pcs, pack of 30 | 6 × T175 flask, prefilled with 30 pcs of BioNOC II® |
| **1400383** | CXR-T175 BioNOC II_50 pcs, pack of 50 | 6 × T175 flask, prefilled with 50 pcs of BioNOC II® |
| **BioMESH®** | | |
| **1400388** | CXR-T75 BioMESH_5 pcs, pack of 6 | 6 × T75 flask, prefilled with 5 pcs of BioMESH® |
| **1400389** | CXR-T75 BioMESH_10 pcs, pack of 6 | 6 × T75 flask, prefilled with 10 pcs of BioMESH® |
| **1400390** | CXR-T75 BioMESH_20 pcs, pack of 6 | 6 × T75 flask, prefilled with 20 pcs of BioMESH® |
| **1400391** | CXR-T75 BioMESH_30 pcs, pack of 6 | 6 × T75 flask, prefilled with 30 pcs of BioMESH® |
| **1400392** | CXR-T175 BioMESH_10 pcs, pack of 6 | 6 × T175 flask, prefilled with 10 pcs of BioMESH® |

| | | |
|---|---|---|
| **1400393** | CXR-T175 BioMESH_20 pcs, pack of 6 | 6 × T175 flask, prefilled with 20 pcs of BioMESH® |
| **1400394** | CXR-T175 BioMESH_30 pcs, pack of 6 | 6 × T175 flask, prefilled with 30 pcs of BioMESH® |
| **1400395** | CXR-T175 BioMESH_50 pcs, pack of 6 | 6 × T175 flask, prefilled with 50 pcs of BioMESH® |