21 Changi South Street 1 Singapore 486777
Tel: +65 65420833 | Fax: +65 65426920

PROTOCOL FOR TESTING BioNOC™ II CARRIERS

Contents

Introduction to Tide Motion

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

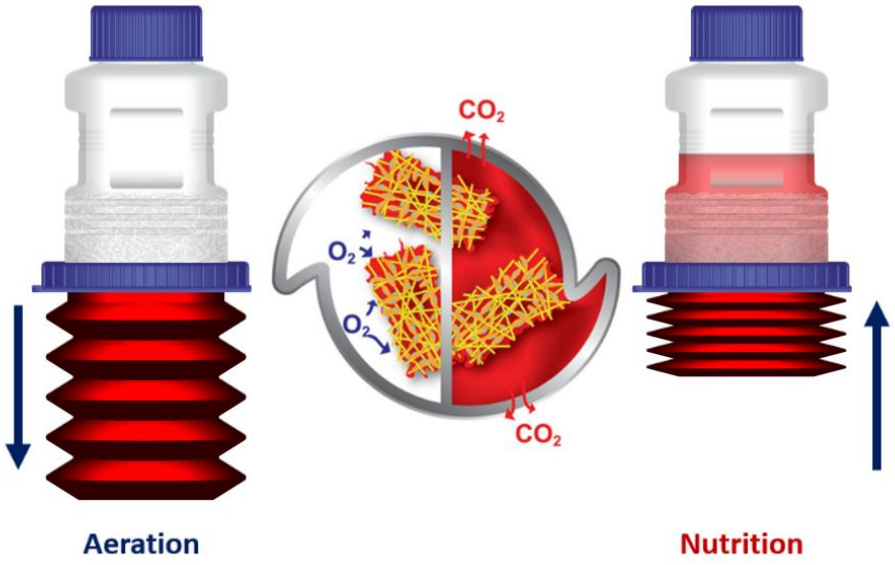


Figure 1: *CelCradle™ operates via Tide Motion with adherent cells attached to BioNOC™ II carriers. The packed bed is exposed to aeration and nutrition phases via decompression and compression of bellows.*

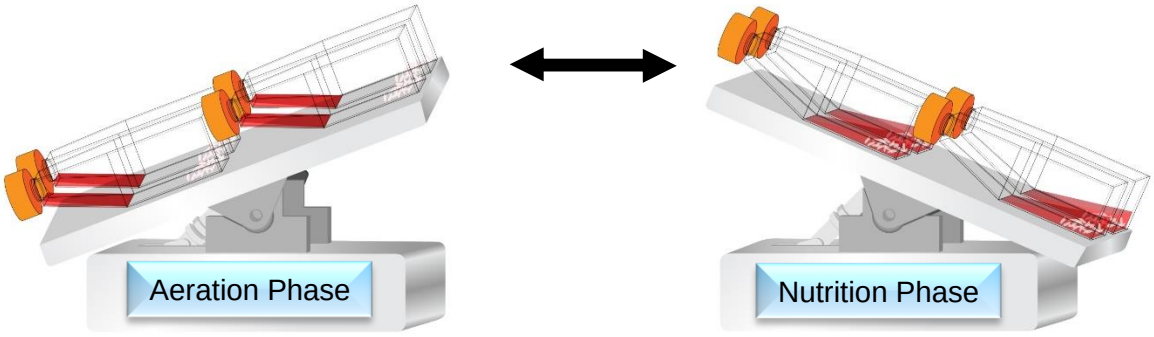


Figure 2: *The Mini Tide Motion System serves as a small-scale system to conduct initial process optimization. The carriers are seeded with cells and aseptically transferred into tissue culture flasks positioned on a rocker (Figure 2); this setup simulates the Tide Motion observed in CelCradle™.*

Mini Tide

Autoclaving Sterilisation

1. Place carriers in autoclavable container.
2. Submerge carriers in PBS; carriers should be sterilized in aqueous solution.
3. Autoclave at 121ºC for 20 min. Store the carriers in PBS until use.

Coating of Attachment Factors (if required)

1. Aseptically transfer autoclaved carriers into 50 ml tube.
2. Aspirate excess PBS and allow carriers to dry completely.
3. Submerge carriers in the coating agent. Coat carriers as per vendor's recommendation.
4. Remove coating solution.
5. Allow carriers dry completely; rinse with PBS if required for the coating.
6. Store as XX-coated carriers at appropriate coating temperature until use.

Inoculation

1. Aseptically transfer 30 carriers into 50 ml tube.
2. Inoculate carriers by pipetting required number of cells into 50 ml tube. Refer to Table 1 for optimal cell seeding number per carrier.
3. Add media into 50ml tube until carriers are completely submerged. pH value should be maintained between 7.0 and 7.4 (optimum pH value is 7.2)

Cell type	**Cell – Carrier Seeding Index**
Chick embryo fibroblasts (CEF)	300,000 – 500,000
Pulmonary epithelial cell line (A-549)	200,000 – 300,000
African green monkey kidney cell line (Vero) Chinese hamster ovary cells (CHO) Cellosaurus cell lines (HEK293T / PK-15 / IBRS-2) Hybridoma (OKT3)	100,000 – 300,000
Madin-Darby Canine Kidney (MDCK)	60,000 – 120,000
Subclone cell line (MARC-145)	50,000
Leghorn male hepatoma (LMH)	50,000 – 200,000
Human mesenchymal stem cells (hMSCs)	20,000 – 40,000
Diploid human cell lines (WI-38 / MRC-5)	15,000 – 20,000

Table 1: Optimal cell seeding density of various cell types.

Handling Tips: A wide variety of cell types can be used to seed onto carriers. Avoid seeding with cell concentrations of more than $1x10^6$ cells per ml to avoid cells clumping.

4. Tilt and rotate tube to provide gentle mixing (Figure 3).

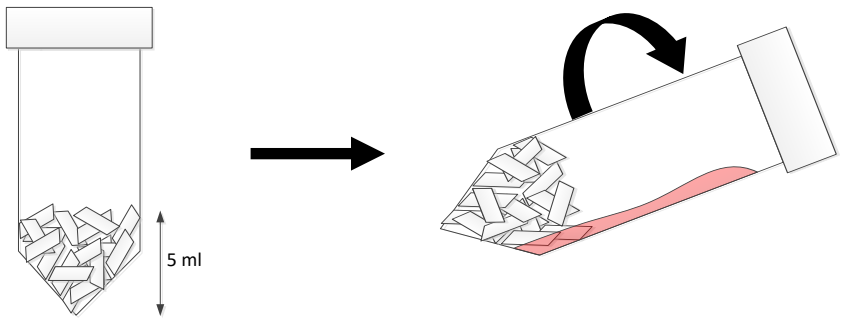


Figure 3: *Avoid tilting tube beyond 90° angle to minimize risk of cell loss.*

5. Incubate in upright position for 3 to 5 hours in an incubator (37°C, 5% CO_2) with cap loosened for CO_2 equilibration.
6. During first hour of incubation, tilt and rotate tube to re-suspend cells every 15 min. At the second hour of incubation, repeat this process at every 30 min interval.
7. After 3 or more hours of incubation, aseptically sample 50 µl media for cell counting. Perform cell count using a hemocytometer or automated cell counting device to determine % of attachment.
8. If attachment rate of 90% is achieved (i.e. less than 10% of cells remain in media) proceed to cell culture. Cell attachment is usually completed within 3 to 5 hours.

Cell Culture

1. Position rocker in CO2 incubator at 37°C and 5% CO_2. Adjust rocking speed to 4 cycles per min (each cycle is from left → right → left).
2. Use a pair of sterile forceps to transfer the carriers from the centrifuge tube to T-75 flask containing 18 ml (0.6 ml of media per carrier) of fresh medium.
3. Media used for inoculation can be spun down for counting of total unattached cells to determine final attachment efficiency.
4. Perform media change every 2 to 3 days or according to established protocols.

Handling Tips:

- Amount of media and size of T-flask should be adjusted in accordance to quantity of carriers.
- An initial test volume of 0.6 ml (adjustable) of fresh culture media per carrier is recommended; this amount equates to the media volume (500 ml) in Celcradle (850 carriers).
- No more than 18 ml (30 carriers in T-75 flask) or 30 ml (50 carriers in T-175 flask) of media should be used to avoid wetting of filter vent during rocker; precaution steps should be taken to avoid contamination.
- It is recommended to monitor cell growth (via harvesting) during culture. 1 to 2 carriers can be stained to observe cell growth directly on the carriers (refer to below procedures).
- If a rocker is not used, add sufficient media to just submerge carriers and leave at static conditions.

Monitoring of Cell Growth on Carriers

Cell Harvesting and Counting

1. Aseptically transfer 3 carriers from the T-flask into 1.5 ml micro-centrifuge tubes.
2. Rinse carriers with 1 ml calcium and magnesium free PBS thrice (gently invert tube for each wash).
3. Perform enzyme dissociation:

i. Trypsin or TrypLE Express: (most cell types)
   - Add 1 ml 0.25% Trypsin – EDTA, incubate at 37ºC for 10 to 15 min.

ii. Accumax or Accutase: (hMSCs/ primary cells)
   - Add 1 ml Accumax or Accutase, incubate at room temperature for 15 to 30 min. An empirical testing of different incubation periods of 15 min, 20 min and 30 min is highly recommended.

iii. Collagenase: (hMSCs/ primary cells)
   - Dilute collagenase to achieve a final working solution of collagenase containing 100 units per ml in HBSS.
   - Add 1 ml of collagenase and incubate for 15 to 30 min; increase incubation time as required.

4. After the enzyme incubation period, transfer the enzyme solution into a 15 ml collection tube.
5. Add suitable neutralizing solution or PBS into the carrier filled 1.5 ml tube.
6. Flick 1.5 ml tube continuously using back of a 15 ml tube or pen 40 to 60 times.
7. Transfer solution to 15 ml collection tube.
8. Repeat steps 5 and 7 three additional times with PBS instead of neutralizing solution.
9. Centrifuge 15 ml collection tube, discard supernatant and re-suspend cells in media. Perform cell count using a hemocytometer or automated cell counting device and calculate average number of cells per carrier.
10. Re-plate cells. Observe cell morphology and viability the following day.

Handling Tips:

- For mass harvesting, harvesting can be performed directly in T-flask with above protocol. Add sufficient volume of enzyme to completely submerge carriers (1.5 to 2 ml of enzyme for every 10 carriers). Tilt flask during enzyme incubation to completely submerge carriers.

Cell Staining

With Dyes

1. Aseptically transfer carriers into 24 well plate.
2. Dehydrate and fix cells using ethanol (70% to 100%) for 15 min.
3. Wash off ethanol once, using either DI water or PBS.
4. Stain cells with hematoxylin, H&E or Trypan Blue for 5 to 10 min.
5. Wash off excess dye with DI water.
6. Observe carriers with cells under bright-field microscopy.

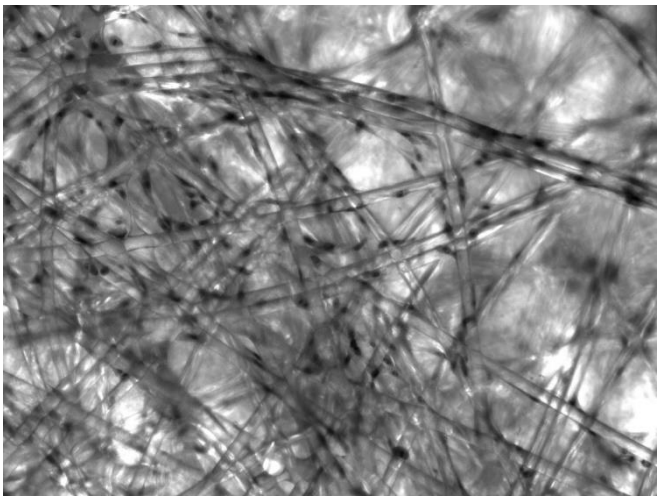
With Fluorescence Dyes

1. Aseptically transfer carriers into 24 well plate.
2. Add 500 µl of culture media to well.
3. Add dyes at following final concentrations: 1 µg/ml of Hoescht 33342 (Thermo Fisher, H3570), 1 uM Calcein green (Thermo Fisher, C34852) and 1 µg/ml Propidium iodide (Sigma Aldrich P4170) in culture media. Fluorescein diacetate, cell tracker, DAPI are alternatives.
4. Incubate carriers for 30 min at 37ºC, 5% CO_2. Capture images using appropriate filters.

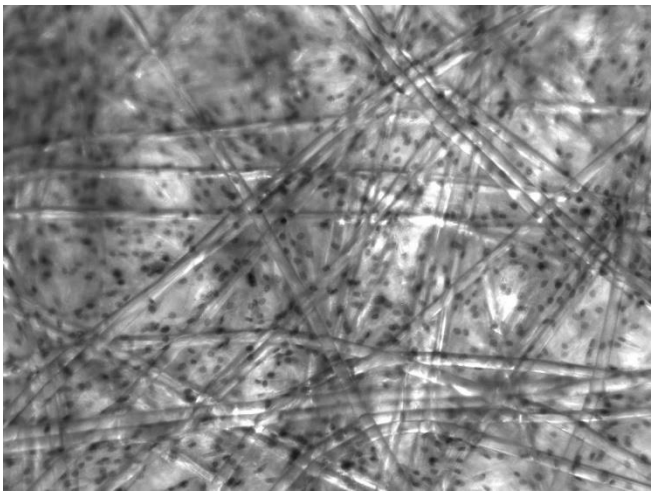
Appendix

1. Hematoxylin staining of fixed hMSCs

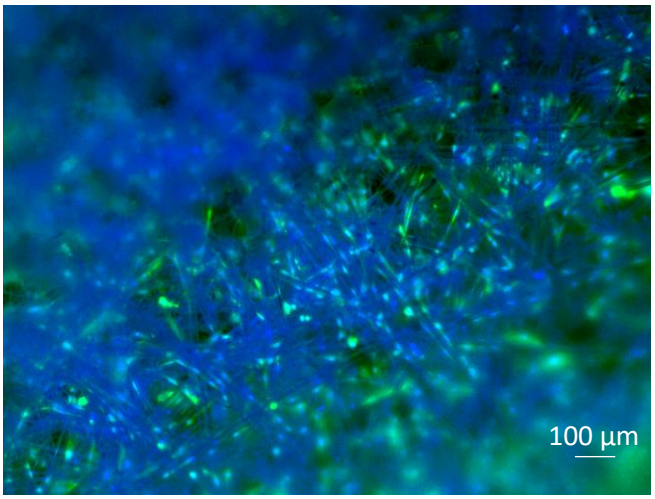
Day 1

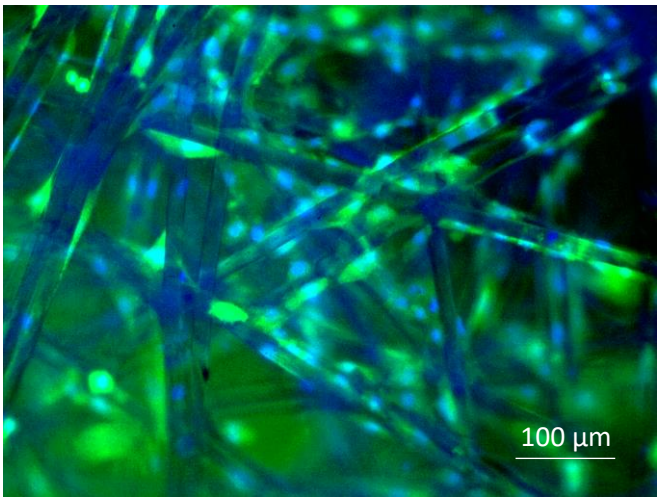


Day 3



2. Fluorescence staining of viable hMSCs





Calcein green: cytoplasm

Hoechst 33342: nucleus

Propidium iodide: non-viable cells (none or little observed)