Corning® CellCube® Culture System Cell Expansion Protocol

Guidelines for Use

CORNING

Introduction

The Corning CellCube system provides a simple, compact, and scalable method for mass culture of attachment-dependent cells. The CellCube modules provide a large, stable surface area for the immobilization and growth of attachment-dependent cells. Each CellCube module consists of a series of parallel, polystyrene plates joined to create thin, sealed laminar flow spaces between adjacent plates. CellCube modules are available in three basic sizes: 10- and 25-layer modules comprised of 10 and 25 culture plates, respectively, and a 100-layer module made up of four 25-layer modules in parallel. All three sizes are available with tissue culture treatment, and the 25- and 100-layer modules are also available with Corning CellBIND® surface treatment.

The basic closed system is configured with a peristaltic pump to drive continuous circulation through the CellCube module and is paired with a bioreactor and bioreactor controller for medium conditioning. As gas-conditioned culture medium is circulated through the CellCube system, the design of the modules allows for reliable distribution of nutrients and oxygen with low differential gradients across all cells within the modules.

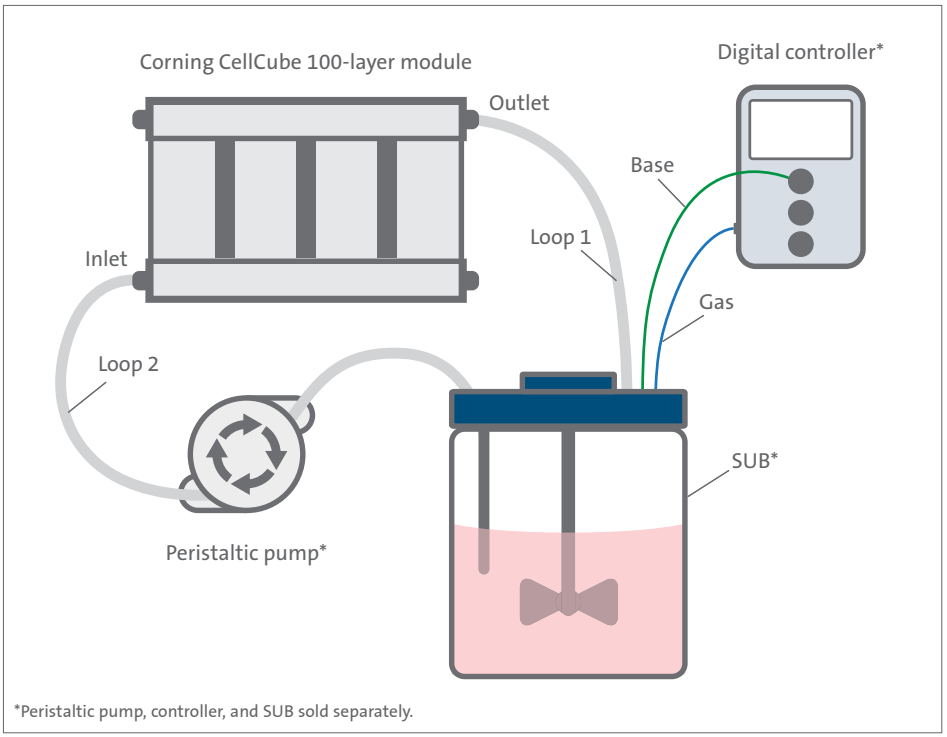

Figure 1. Schematic of the Corning CellCube Closed System. The culture medium (approximately 2L) within the system is removed from the single-use bioreactor (SUB) by a peristaltic pump and is then pumped into and distributed throughout the Corning CellCube 100-layer module. Medium flows from the outlet of the CellCube module, back to the SUB for conditioning. The controller automatically controls pH via $NaHCO_3$ and CO_2. Dissolved oxygen (DO) in the medium is also maintained by the controller, which continuously refreshes the gas mixture supplied to the headspace of the SUB and sparged directly into the medium. Fluid flow and gas exchange within the SUB are carefully controlled to help eliminate turbulence and foaming and to prevent protein degradation.

The current protocol was developed for HEK293T and Vero cell expansion using a Corning® CellCube® 100-layer module (Corning Cat. No. 3233) with the Eppendorf BioFlo® 120 (controller) (Eppendorf Cat. No. B120ACS000) and BioBLU® 3c single-use bioreactor (SUB) (Eppendorf Cat. No. 1386000300) to adequately control medium conditioning[1]. However, CellCube modules are to be handled in the same manner regardless of the bioreactor or controller used for medium conditioning. Moreover, the general process is applicable to most cell types. Medium formulation and conditioning parameters, process timing, and culture volume should be determined empirically for each cell type and application.

Protocol

Prior to system setup, calibrate the external circulation pump as well as the built-in controller pumps. Sterilize tubing and connections for base control. If using any in-vessel probes, calibrate (if required), and sterilize according to manufacturer's instructions.

NOTE: The following protocol utilizes several custom products for connection to the closed system outside of the biosafety cabinet. Custom AseptiQuik® S Connector-to-MPC adapters and AseptiQuik S connector cross adapters are utilized for multiple connections into the circulation loops. Custom CellCube modules are configured with AseptiQuik G connectors at both the inlet and outlet. As such, the circulation tubing sets are configured with AseptiQuik G connectors at the terminal ends to facilitate connections after the CellCube modules are positioned in the warm room. In addition, the origins of the circulation tubing are fit with AseptiQuik S connectors to connect via AseptiQuik S Connector-to-MPC adapters into the SUB outlet and SUB return. For assistance with custom closed systems orders, contact Corning Scientific Support[2].

Equilibration (Day -1)

1. Prepare a collection bag (Corning Cat. No. 91-200-43, 91-200-45, or 91-200-47) with the required volume of warm medium (Table 1) for system equilibration. Connect an AseptiQuik® S Connector-to-MPC adapter (Corning Cat. No. 3237 or 3238) to the bag.

Table 1. Corning CellCube Module Medium Volume

Module	Area (cm^2)	Module Volume	Oxygenator and Circulation Loop	Total Volume
10-layer	8,500	0.6L	2.0L*	2.6L
25-layer	21,250	1.5L	2.0L*	3.5L
100-layer	85,000	6L	2.0L*	8.0L

*Starting volume. Circulation volumes are up to the discretion of the user.

2. In a biosafety cabinet, connect a disposable 60 mL Luer lock syringe filled with antifoam solution to the SUB via Luer connection to the accessory port.

   NOTE: To avoid filter wet-out, antifoam is added to the SUB manually on an as-needed basis to reduce foaming. Addition of antifoam may not be necessary depending upon the headspace of the bioreactor, the gassing strategy, and percentage of FBS in the medium.

3. For base control, fill a 1L bottle with 7.5% $NaHCO_3$ solution (Corning Cat. No. 25-035-CI) and fit with an aseptic transfer cap connected to a length of pump-grade silicone tubing that terminates in a Luer connection. Connect the bottle to the SUB via the Luer connection to the accessory port.

   NOTE: Before moving the closed system to the warm room, insert any in-vessel probes through open ports in the SUB in the biosafety cabinet.

4. Connect AseptiQuik S Connector-to-MPC adapters to the bioreactor return and outlet (with dip tube), respectively, of the SUB.

5. Place the SUB adjacent to the controller in a warm room (37°C).

   NOTE: Once the closed system is set up in the warm room, any connections into the SUB or circulation loops must be made in an aseptic manner, via tube welding or other aseptic connection.

6. Remove the CellCube module (Corning Cat. No. 3231, 3232, or 3233) from its packaging and connect the inlet (Loop 2; Corning Cat. No. 3235) and outlet (Loop 1; Corning Cat. No. 3234) circulation loops via the AseptiQuik G connectors.

7. Connect Loops 1 and 2 to the bioreactor return and outlet (with dip tube) of the SUB, respectively, via the AseptiQuik S connectors.

8. Thread Loop 2 through the external peristaltic pump. Route the bicarbonate bottle tubing through Pump 1 of the controller.

9. Connect an AseptiQuik S connector cross adapter (Corning Cat. No. 3236) to the port in Loop 2 upstream of the pump.

10. Connect the prepared collection bag to the port in Loop 2 via the AseptiQuik S connector. Fill the CellCube module and SUB with complete medium via gravity fill.

    NOTE: For the initial gravity fill of the CellCube module and SUB, the air vent on the SUB can be used for venting the system. Subsequent empty and fill steps rely on the vent filter engineered into Loop 1 of the circulation tubing to prevent pressurization of the system.

11. Ready the SUB and accompanying controller for operation by first setting the motor atop the magnetic coupling. Wrap the exhaust filter and SUB with the filter heater and heating jacket, respectively. Set the temperature, pH, and dissolved oxygen probes (DO) into the designated ports. Connect the gas line(s) from the controller to the sparge and overlay filters.

    NOTE: For detailed instructions on the use of Eppendorf bioreactor systems, contact Eppendorf Customer Support[3].

12. Set the controller for temperature control only with a set point of 37°C.

13. Start circulation to run at 0.5L/minute and allow the system to reach working temperature before proceeding with any calibrations. Once the temperature stabilizes, turn off circulation.

14. Calibrate the pH probe according to the manufacturer's operation manual.

    NOTE: If using an in-vessel pH probe calibrated prior to autoclaving and insertion into the SUB, the controller pH values should be re-standardized to the offline pH reading at this point.

15. Calibrate the dissolved oxygen (DO) probe according to the manufacturer's operation manual.

16. Following calibration, set the controller for the desired set points (for example see Table 2), and resume circulation. Allow the entire system to equilibrate overnight.

    NOTE: Set points for agitation, pH, and DO, as well as the control cascades should be determined empirically for each controller system, cell type, and application.

Table 2. Example Medium Conditioning Parameters

	HEK293T	Vero
Agitation	100 rpm	100 rpm
Temperature	37°C	37°C
pH level	7.40 ± 0.04	7.40 ± 0.05
pH Control	$NaHCO_3$, CO_2	$NaHCO_3$, CO_2
DO Level	30% ± 10%	60% ± 10%
DO Control	Air, O_2, N_2 overlay and sparge	Air, O_2 overlay and sparge

Seeding (Day 0)

Follow standard culturing protocols to prepare cells for introduction into the CellCube system. The recommended seed train starts from thaw, with scale-up through 2 to 3 passages into Corning CellSTACK 5- or CellSTACK 10-layer culture chambers, depending upon the size of the CellCube module and the desired seeding density.

1. Draw a medium sample from the SUB to check pH. If offline analysis indicates a difference in pH greater than the controller deadband value, re-standardize the controller to the offline pH value. Allow pH to stabilize before proceeding with seeding process.

2. Harvest cells from the source vessel to generate a single-cell suspension.

    NOTE: Addition of 0.1% Poloxamer 188 (Corning Cat. No. 13-901-CI) to the cell dissociation reagent can help to reduce cell aggregation and clumping.

3. Stop circulation in the CellCube system and drain the medium from the CellCube module into a collection bag via gravity.

    WARNING: During fluid exchange between the closed CellCube system and an external collection container be sure to vent the system appropriately to avoid pressurization.

4. Inoculate the collection bag with the correct volume of single-cell suspension to seed both sides of the CellCube module (total module surface area; Table 1) at the desired seeding density, and mix by gentle massage.

Alternative Double Seeding

Inoculate the collection bag with cell suspension to seed the first side (half module surface area) of the CellCube module. Fill the CellCube module with cell suspension via gravity-fill, clamp the inlet and outlet, and rotate the CellCube module vertically with the outlet facing up. Incubate in this position for 2 hours. Near the end of the first seeding period, harvest the second set of source vessels for the second seeding. Fill a second seeding bag with fresh media to fill the CellCube module and inoculate with cell suspension to seed the second side of the CellCube module. Return the CellCube module to resting position and empty into the original collection bag. Fill the CellCube module with the second seeding cell suspension, clamp the inlet and outlet, and rotate the CellCube module vertically with the outlet facing down to seed the second side of the vessel. Incubate for 2 hours, then return to resting position for 30 minutes before resuming circulation.

5. Fill the CellCube module with the cell suspension via gravity fill, adjusting the height of the collection bag to fill the module evenly.
6. Clamp the inlet and outlet tubing and rotate the CellCube module vertically with the outlet facing up. Incubate in this position for 20 minutes.

   NOTE: Incubation time for seeding each side of the CellCube module is dependent upon cell type. Conduct preliminary studies in flat stock culture vessels to determine time for cell attachment and spreading at the same seeding density prior to working with the CellCube module.
7. Rotate CellCube in the opposite direction (outlet facing down) to seed the second side of the vessel for the next 30 minutes.
8. Repeat rotation at 20 minutes and then at 30 minutes to continue seeding first and second sides, respectively, of the CellCube module.
9. After seeding, return the CellCube module to the resting position for 30 minutes to ensure cells have begun to spread on the culture surface.
10. Open CellCube module inlet and outlet clamps and resume system circulation.

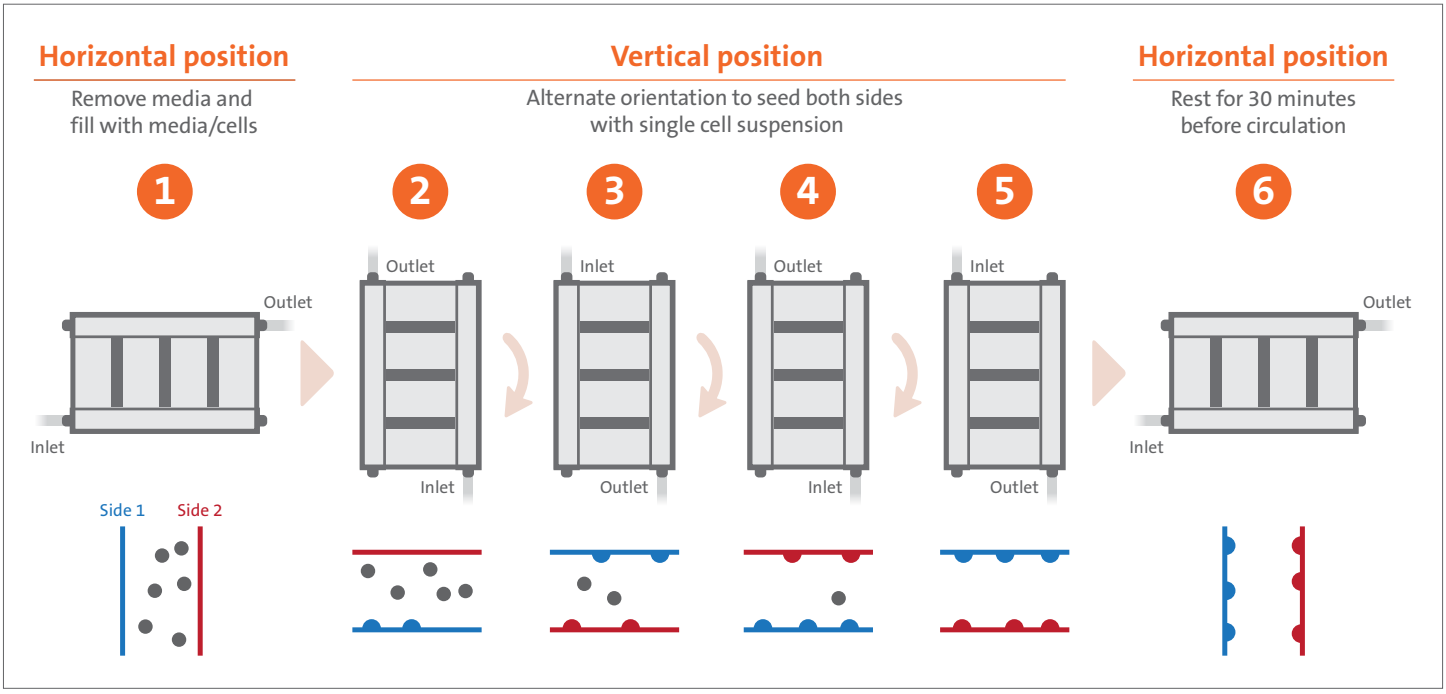

Figure 2. Corning CellCube module single seeding process. (1) Equilibrated medium is emptied from the Corning CellCube module, inoculated with cell suspension to seed both sides of the CellCube module, and the CellCube module is refilled with cell suspension. Next (2 to 5), the tubing is clamped at both the inlet and outlet, and the Corning CellCube module is rotated vertically with the outlet facing up. Three more rotational seedings are performed to complete the seeding. (6) After seeding, the CellCube module is returned to the resting position for 30 minutes before resuming circulation.

Expansion (Day 1 through Day N)

1. Draw daily samples from the SUB for offline gas, electrolyte, and metabolite analysis. Re-standardize the controller to the offline pH value if offline analysis indicates a difference in pH greater than the controller deadband value.
2. Check for condensation on the SUB vent, gas overlay, and gas sparge filters.

   NOTE: It may be necessary to add antifoam (approximately 1 mL) through the accessory port to reduce foaming in the SUB.

Harvest (Day N)

1. Prepare a collection bag with the harvest solution: warm Trypsin EDTA (Corning Cat. No. 25-052-CV) plus 0.1% Poloxamer 188.

   NOTE: Addition of 0.1% Poloxamer 188 to the cell dissociation solution can help to reduce cell aggregation and clumping and protects cells during harvest.
2. Prepare a second collection bag with a volume of warm FBS (Table 4) as a quench for the Trypsin EDTA solution.

   NOTE: Alternatively, the Trypsin can be quenched 1:1 with complete medium containing 10% FBS. If using other cell dissociation reagents, dilute or quench the reagent according to established protocols.

Table 3. Trypsin Quench Volumes

Module	Module Volume	FBS Volume*	Medium Volume*
10-layer	0.6L	32 mL	0.6L
25-layer	1.5L	79 mL	1.5L
100-layer	6L	315 mL	6L

* Volume based on a final concentration of 5% FBS to quench protease activity.

3. Turn off control of the SUB and stop circulation.
4. Disconnect Loop 1 and Loop 2 from the SUB and connect to each other to create a circulation loop through only the CellCube module.
5. Empty spent medium from the CellCube module into an empty collection bag.
6. Fill the CellCube module with the harvest solution via gravity fill, adjusting the height of the collection bag to fill the module evenly.
7. Clamp the inlet and outlet tubing, and rotate the CellCube module vertically with the outlet facing up. Incubate in this position for 5 to 10 minutes.

   NOTE: Harvest timing is dependent upon the cell type and confluence of the culture as well as the cell dissociation reagent. Determine harvest timing in preliminary studies in flat stock culture vessels prior to working in the CellCube module.

   NOTE: Monitor cell dissociation in the CellCube module during harvest using a handheld USB microscope (e.g., Bysameyee Microscope 1000X).
8. Empty half of the volume of the CellCube module into the quench solution, mix well, and drain back into the CellCube module.
9. Resume circulation for an additional 5 minutes.

   NOTE: Air bubbles in the circulation path help to push cells off the surface of the CellCube module. In the horizontal position, rock the CellCube module side to side during the circulation to allow air bubbles to reach to the outer edges of the culture plates, ensuring all cells are dissociated from the vessel.
10. Empty the full harvest volume into the quench bag.
11. Stop circulation. Mix the total harvest thoroughly, and draw off samples for enumeration.

References

1. Maximizing Yield for Attachment-dependent Cells with the Corning CellCube System (Corning App. Note CLS-AN-568).
2. Corning Life Sciences Scientific Support at https://www.corning.com/worldwide/en/products/life-sciences/support-main/technical-support.html
3. Eppendorf Customer Support at https://www.eppendorf.com/US-en/about-us/eppendorf-north-america/contact-us/

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