Low Adherent Microwell Plate Setup for ClinoReactor

Protocol

To achieve the most uniform and size-controlled organoids or spheroids, CelVivo recommend using a low adherent microwell plate e.g., Spherical 5D plate from Kugelmeiers® or AggreWell™ from STEMCELL™ Technologies for initial cell aggregation prior to ClinoReactor® setup. The plates are available in different formats depending on the desired results. This protocol describes the procedure for preparing HepG2/C3A (ATCC HB-8065) hepatoma cell line.

Reagents and Materials

- Low adherent micro pattern plate e.g., AggreWell™ 400 or Kugelmeiers® SPD5
- Cell culture medium with supplements
- Sterile water

Additional information

Recommended protocols to consult for additional information:
Explanation of parts and preparation of the ClinoReactor® is found in protocol **003_Protocol_Preparation_of_ClinoReactor**. Cell culture media change **004_Protocol_Cell_Culture_Media_Change_ClinoReactor.** The protocol should be individually optimized according to the cell type.

Protocol

Preparation of low adherent microwell plates

1. Prepare the plate according to manufacturer's protocol in a sterile environment.
2. After addition of fresh medium, we recommend to centrifuge to remove air bubbles trapped in the wells.
3. Centrifuge 2 min at 3.000xG (or maximum allowed speed depending on rotor type, increase centrifugation time if needed) to remove air bubbles.
4. Verify that air bubbles have been removed using an optical microscope. Repeat step 3 if necessary.
5. Place the plate in the incubator at 37°C.

Generation of initial spheroids

1. Prepare a single-cell suspension in desired cell culture medium.
2. Perform a cell count and evaluate cell viability via a haemocytometer or an automated cell counter. We recommend seeding density of approximately 800 cells pr microwell.
3. Transfer the plate from the incubator to the sterile hood and add the desired number of cells to each well.
4. Centrifuge the plate at 100xG for 3 minutes.
5. Verify equal cell distribution in the microwells across the plate by optical microscopy.
6. Place the plate in the incubator overnight (37°C, 5 % CO_2).
7. Prepare the ClinoReactor® according to **Protocol 003_Protocol_Preparation_Of_ClinoReactor**.

Transferring of spheroids from plates to ClinoReactor®

1. Collect the ClinoReactor®, remove the cell culture media used for equilibration.
2. Fill with 5-6 mL prewarmed media using the top port (**Figure 1 A**).

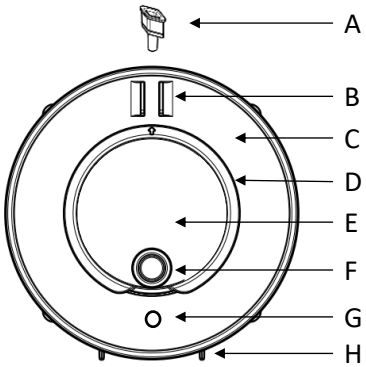


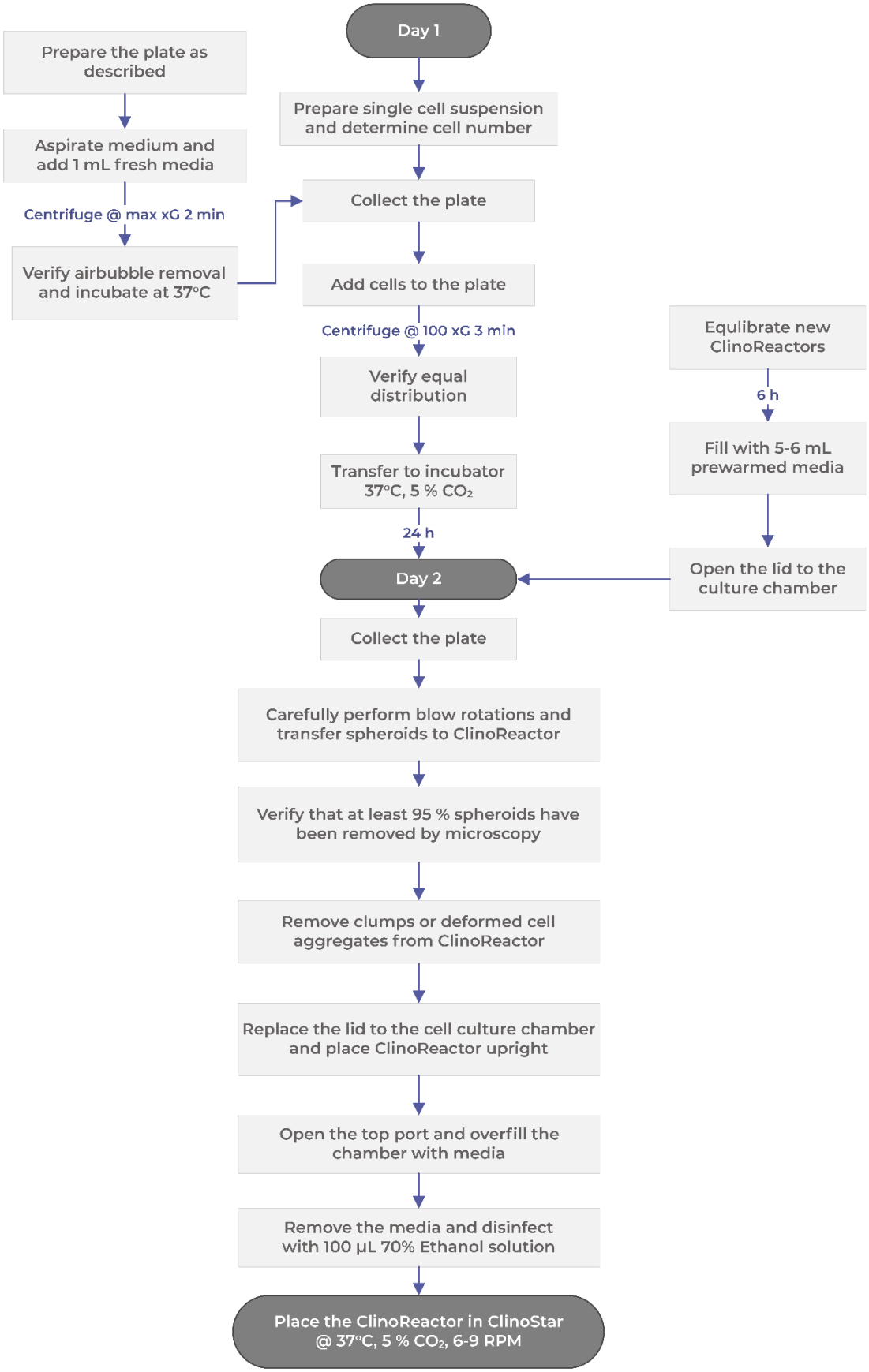
Figure 1 ClinoReactor® for single use (A) Top plug enables media dispensing and removal. **(B) Vents** to ensure correct gas exchange and humidification in the culture chamber. **(C)** Humidification chamber containing the unhydrated humidification beads. **(D) Petri dish lid** for opening the entire culture chamber in a petri dish fashion. **(E)** Cell culture chamber. **(F) Front port** giving access to the culture chamber. **(G) Hydration port** for hydration of the humidification beads with sterile water. **(H)** Feet allowing the ClinoReactor® to stand upright.

3. Remove the lid (**Figure 1 D**) on the ClinoReactor® to access the cell culture chamber.
4. Keep the plate in a slightly tilted position while performing the spheroid detachment procedure. Remember to change the position of the tip; close to the surface when aspirating and close to the bottom when blowing.
5. Carefully detach the spheroids by gently blowing medium over the microwells. Perform only 2-3 blow/aspirate cycle before collecting and transferring spheroids into the ClinoReactor® vessel.
6. Aspirate the spheroids from plate with a p200 wide bore or cut tip and transfer them to the ClinoReactor®. It is recommended to keep the plate in slight tilted position when collecting the detached spheroid seeds. Perform the collection every 2-3 aspiration/blow cycles, keep the tip close to the bottom of the well when aspirating the spheroid seeds.
7. Observe the wells by optical microscopy (x4 objective) to determine how many spheroids have been collected and if any spheroids are remaining inside the microwells.
8. Repeat step 5 and 6 if needed. Avoid emptying the wells completely, it may be necessary to add some extra medium to the wells during the transfer procedure.
9. Verify by optical microscopy that 95% -100% of spheroids have been transferred.
10. Observe the spheroids inside the ClinoReactor® and remove clumps or deformed cell aggregates.
11. Replace the lid (**Figure 1 D**) to the culture chamber, place the ClinoReactor® in a vertical position.
12. Slowly refill the ClinoReactor® with media without disturbing the spheroids. Cell culture media should be clearly visible in the collar NOTE: placing the needle end directly against the front or back ClinoReactor® wall disperse the medium flow and create more gentle re-filling).
13. Overfill the chamber with media. The media should be visible in the top port collar.
14. Remove air bubbles trapped in the cell culture chamber by gently tapping the ClinoReactor®.
15. Replace the top plug (**Figure 1 A**) and remove the excess media with a pipette.
16. Check for any remaining bubbles by careful rotating the vessel. If no bubbles could be observed proceed directly to step 17.
17. If the air bubbles are present in the cell culture chamber place the ClinoReactor® in a vertical position, open the top port (**Figure 1 A**) then add a small amount of fresh medium to overfill the cell culture chamber to the point where medium filles the collar around the port. Remove air bubbles trapped in the cell culture chamber by gently tapping the ClinoReactor®.

18. Replace the top plug and remove the media in the top collar.
19. Disinfect the collar with 100 µL 70 % Ethanol Solution.
20. Place the ClinoReactor® in the ClinoStar® and adjust the speed. The speed is correctly set when spheroids or organoids are in a statically orbit. For spheroid seeds created out of 800 cells per seed the starting speed should be set depending on the compactness of the constructs between 6 and 9 RPM. Routinely adjust the speed accordingly.

Warranty/disclaimer: This equipment is for research use only. Materials produced by the use of this equipment must not be used for diagnosis or treatment in any type or form.

For additional product or technical information visit www.celvivo.com or consult CelVivo Aps at info@celvivo.com or +45 70 228 228.


Day 1
Prepare the plate as described
Aspirate medium and add 1 mL fresh media
Centrifuge @ max xG 2 min
Verify airbubble removal and incubate at 37°C
Prepare single cell suspension and determine cell number
Collect the plate
Add cells to the plate
Centrifuge @ 100 xG 3 min
Verify equal distribution
Transfer to incubator 37°C, 5 % CO2
24 h
Day 2
Collect the plate
Carefully perform blow rotations and transfer spheroids to ClinoReactor
Verify that at least 95 % spheroids have been removed by microscopy
Remove clumps or deformed cell aggregates from ClinoReactor
Replace the lid to the cell culture chamber and place ClinoReactor upright
Open the top port and overfill the chamber with media
Remove the media and disinfect with 100 µL 70% Ethanol solution
Place the ClinoReactor in ClinoStar @ 37°C, 5 % CO2, 6-9 RPM
Equlibrate new ClinoReactors
6 h
Fill with 5-6 mL prewarmed media
Open the lid to the culture chamber