

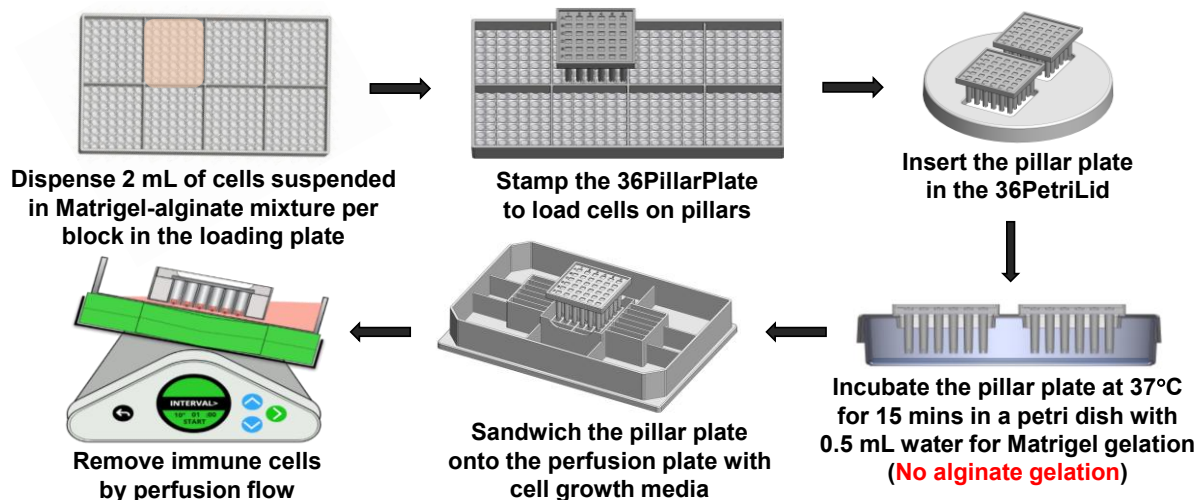
Immune Cell Removal for CTC Culture Using Pillar/Perfusion Plate

This standard operating procedure (SOP) provides step-by-step methods for efficiently removing immune cells for the culture of circulating tumor cells (CTCs) by using our pillar/perfusion plate system. CTCs are notoriously difficult to expand, partly due to the presence of inflammatory immune cells that are co-isolated during the CTC enrichment process. By leveraging the adherent nature of CTCs to extracellular matrices and the non-adherent behavior of immune cells, our system enables the effective separation of immune cells from the tumor cell population under dynamic conditions. Immune cells, encapsulated on the pillar plate, are rapidly removed through dynamic perfusion, thereby enhancing the purity and growth of the CTC culture. Please read this protocol carefully before performing experiments.

Materials:

- 36PillarPlate (Bioprinting Laboratories Inc., Cat. no. 36-01-00)
- 36PerfusionPlate (Bioprinting Laboratories Inc., Cat. no. 36-02-00)
- LoadingPlate (Bioprinting Laboratories Inc., Cat. no. 384-03-00)
- 36PetriLid (Bioprinting Laboratories Inc., Cat. no. 36-03-00)
- Petri dish, 90 mm x 15 mm (VWR, Cat. no. 75799-946)
- Growth factor reduced Matrigel (Corning, Cat. no. 354230)
- Alginate acid (Sigma Aldrich, Cat. no. A1112)
- OrganoFlow L Digital Rocker (MIMETAS)
- Vacusafe vacuum aspiration system (Integra Biosciences, Part no. 158320)

Methods:



The overall protocol of immune cell removal for CTC culture using the pillar/perfusion plate.

Cell encapsulation in Matrigel on 36PillarPlate and dynamic culture in 36PerfusionPlate

Preparation of 3% (w/v) alginate stock solution.

1. Add 300 mg of low viscosity alginate sodium salt in 10 mL of sterile distilled water in a 20 mL glass vial to prepare 3% (w/v) stock solution.
2. Dissolve the alginate sodium salt by continuously stirring for 3 days on a magnetic stirrer.
3. Store the alginate stock solution at 4°C until use.

Preparation of Matrigel and cell culture medium in 36PerfusionPlate

4. Thaw Matrigel[®] stock overnight by submerging the unopened bottle in an ice bucket filled with ice in a 4°C refrigerator. Prepare 500 µL aliquots of Matrigel and store at - 20°C for future use.
5. Thaw Matrigel[®] aliquots overnight in a 4°C refrigerator.
Note: *It is important to thaw Matrigel aliquots in advance in a 4°C refrigerator and maintain Matrigel chilled on ice during use since Matrigel starts to solidify above 10°C. Do not freeze and thaw Matrigel aliquots.*
6. For cell culture in a 36PerfusionPlate, dispense 400 µL of a cell growth medium in each reservoir of the 36PerfusionPlate (800 µL cell culture medium per fluidic channel) and place it on a flat surface for 1 - 2 minutes to fill all perfusion wells with the cell culture medium (Fig. 1).
Note: Adding an excessive volume of cell culture medium to the perfusion plate can cause overflow after the pillar plate is sandwiched and the assembly is placed on the digital rocker. Avoid wetting the bottom of the pillars with cell culture medium, as it can lead to cross-talk or contamination during dynamic cell culture. If any perfusion wells are not filled with cell growth medium, manually dispense 60 µL of the medium in the empty perfusion wells.



Figure 1. Manual dispensing of a cell growth medium in reservoirs of the 36PerfusionPlate.

7. Cover the 36PerfusionPlate with a lid and place it on the digital rocker in a 5% CO₂ incubator at 37°C for 30 minutes.
Note: *Ensure uniform flow of the cell growth medium in the perfusion plate by verifying the levels in the upper and lower reservoirs and the perfusion wells. Pre-warming the cell growth medium ensures minimal bubble formation on the 36PillarPlate after loading cells/spheroids in hydrogel.*
8. Take the perfusion plate out of the incubator and check the level of the cell growth medium in upper and lower reservoirs as well as perfusion wells (**Fig. 2**).

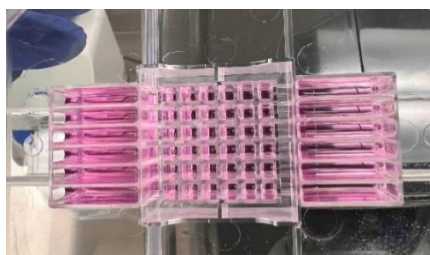


Figure 2. Uniform level of the cell growth medium in the 36PerfusionPlate.

Preparation of pillar plate and cell suspension

9. Hydrate the surface of the pillar plate by inserting two 36PillarPlates in the 36PetriLid on a 90 x 15 mm petri dish containing 500 µL of sterile, distilled water and placing it in a 5% CO₂ incubator at 37°C for 20 - 30 minutes (**Fig. 3**).
Note: *Hydrating the surface of the pillar plate in a humid environment is necessary to make it hydrophilic and minimize air bubble entrapment on the pillars after cell loading in Matrigel.*

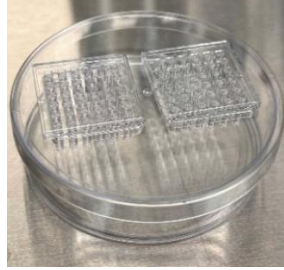


Figure 3. Hydration of the pillar plate surface in a 90 x 15 mm petri dish with 500 μL of sterile, distilled water to minimize air bubble entrapment.

10. Obtain approximately 1.0 mL of cell suspension in a 15 mL centrifuge tube, which contains CTCs and immune cells from the CTC enrichment process.
11. Gently mix 833 μL of the cell suspension with 1.0 mL of **cold Matrigel** and 167 μL of 3% alginate solution to generate a homogenous mixture of cells and Matrigel-alginate without air bubbles entrapped.

Note: *The resulting cell suspension contains a final concentration of 50% (v/v) Matrigel and 0.25% (w/v) alginate. Alginate is supplemented to facilitate immune cell removal under dynamic conditions as alginate can be gradually leached out of Matrigel. Ensure to use the cell-Matrigel-alginate mixture immediately as cells in the hydrogel could settle down in 5 – 10 minutes, which could lead to non-uniform cell loading on the pillar plate. Keep resuspending the cell-Matrigel-alginate mixture before cell loading.*

Loading cell suspension in Matrigel-alginate on single pillar plate using a 1 mL pipette tip

12. Aspirate 250 μL of the cell-Matrigel-alginate mixture using a 1 mL pipette tip for single 36PillarPlate.
13. Separate the 1 mL pipette tip from the pipette gently to prevent cell-Matrigel-alginate spillage.
14. Using the index finger, block the back of the pipette tip (i.e., the large open area of the tip) to prevent overflow of the cell-Matrigel-alginate mixture while tapping the pillar surface.
15. Gently tap the 1 mL pipette tip with the cell-Matrigel-alginate mixture in the center of the pillar to load the cell-Matrigel-alginate mixture while blocking the large open area of the tip using the index finger (**Fig. 4**).

Note: *Do not touch the bottom of the pillar with the pipette tip so as not to damage the surface coating. Use hydrated pillar plates in the petri dish within 5 minutes before complete water drying.*

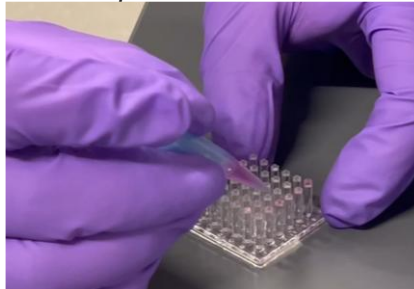


Figure 4. Loading the cell-Matrigel-alginate mixture on the pillar plate using a 1 mL pipette tip.

16. Repeat **Step 15** for all pillars.
17. After loading the cell-Matrigel-alginate mixture on all pillars, scrape excess the cell-Matrigel-alginate mixture off the pillars by sliding the 1 mL pipette tip in a horizontal position on all pillars (**Fig. 5**).

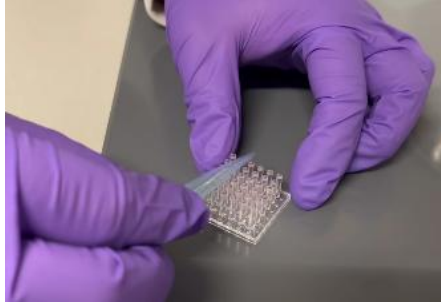


Figure 5. Scraping excess cell-Matrigel-alginate mixture off the pillars using the 1 mL pipette tip.

Loading cell suspension in Matrigel-alginate on multiple pillar plates using a LoadingPlate

18. Place the LoadingPlate on a flat surface, dispense 1.5 - 2 mL of the cell-Matrigel-alginate mixture per small block without big bubbles, and spread it properly with the pipette tip (**Fig. 6**).

Note: Do not leave the cell-Matrigel-alginate mixture on the LoadingPlate for longer than 5 minutes to avoid premature gelation during stamping. Since the stamping process is quick, we don't need to place the LoadingPlate with the cell-Matrigel-alginate mixture on ice.

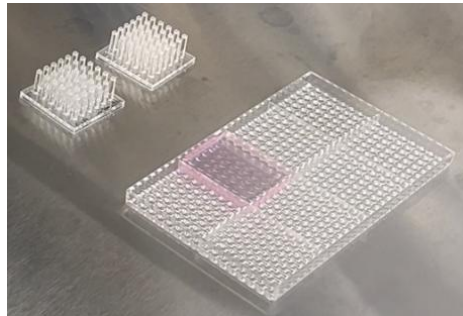


Figure 6. Dispensing 1.5 - 2 mL of the cell-Matrigel-alginate mixture per block in the LoadingPlate for rapid loading of the cells on the pillar plate.

19. Stamp the 36PillarPlate on the LoadingPlate and press gently to load the cell-Matrigel-alginate mixture evenly on the entire pillar plate (**Fig. 7**). Repeat this cell loading step for other pillar plates as needed.

Note: With 1.5 - 2 mL of the cell-Matrigel-alginate mixture, we can prepare at least three 36PillarPlates (5 μ L cell-Matrigel-alginate mixture per pillar or 180 μ L the cell-Matrigel-alginate mixture per 36PillarPlate) without introducing macro-bubbles on the pillars. For uniform wetting of the pillars and robust cell loading, gently wiggle the pillar plate during stamping.

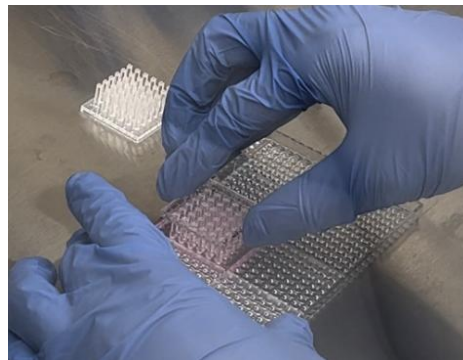


Figure 7. Stamping of the 36PillarPlate on the LoadingPlate to load cells suspended in Matrigel-alginate on pillars.

Matrigel gelation and cell culture on the pillar plate

20. Insert the pillar plate with cells in Matrigel-alginate in the 36PetriLid on a 90 x 15 mm petri dish containing 500 μL of sterile, distilled water to prevent water evaporation during Matrigel gelation (**Fig. 8**).

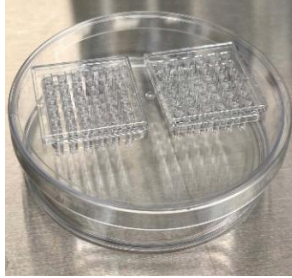


Figure 8. Gelation of Matrigel on the pillar plate inserted in the 36PetriLid on a 90 x 15 mm petri dish with 500 μL of sterile, distilled water to prevent evaporation during gelation.

21. For complete Matrigel gelation on the pillar plate, incubate the petri dish with the pillar plate in a 5% CO_2 incubator for 15 minutes at 37°C.
Note: Matrigel forms a gel, whereas alginate does not.
22. Separate the 36PillarPlate and sandwich it onto the 36PerfusionPlate with 800 μL cell culture medium per fluidic channel (**Fig. 9**).

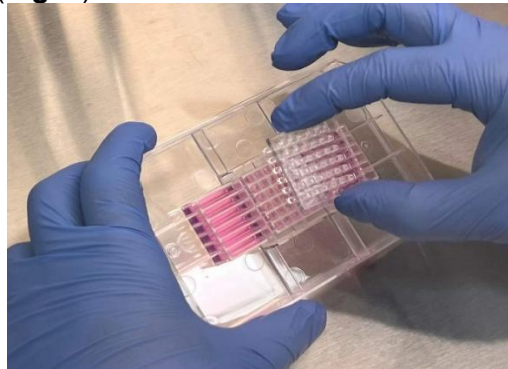


Figure 9. Sandwiching the 36PillarPlate with cells in Matrigel-alginate onto the perfusion wells of the 36PerfusionPlate for dynamic immune cell removal and CTC culture.

23. Inspect the pillar plate under the microscope to ensure uniform cell loading throughout the entire pillar plate.
24. Place the sandwiched plates on the digital rocker (at 10° tilting angle with a 30-second interval between tilting angle changes) in the 5% CO_2 incubator at 37°C and change the medium every 6 hours until most immune cells are removed.
Note: Frequent medium changes will be necessary initially to remove immune cells. For medium change in the perfusion plate, refer to the protocol titled “Dynamic Cell Culture in Perfusion Plate”.
25. To replace the old cell culture medium, carefully separate the pillar plate containing cells from the perfusion plate, and immediately insert the pillar plate into the 36PetriLid (or 144PetriLid) positioned on a 90 x 15 mm petri dish (**Fig. 10**).

Note: *Do not place the wet pillar plate directly on a flat surface. This causes cell culture medium to drip from the pillar tip to the bottom of the pillar. If the bottom of the pillar becomes wet, it can lead to “overflow” when the pillar plate is reinserted into the perfusion plate. Therefore, always keep the pillar plate inserted in the PetriLid during medium replacement to prevent pillar bottom wetting and minimize evaporation of residual moisture.*

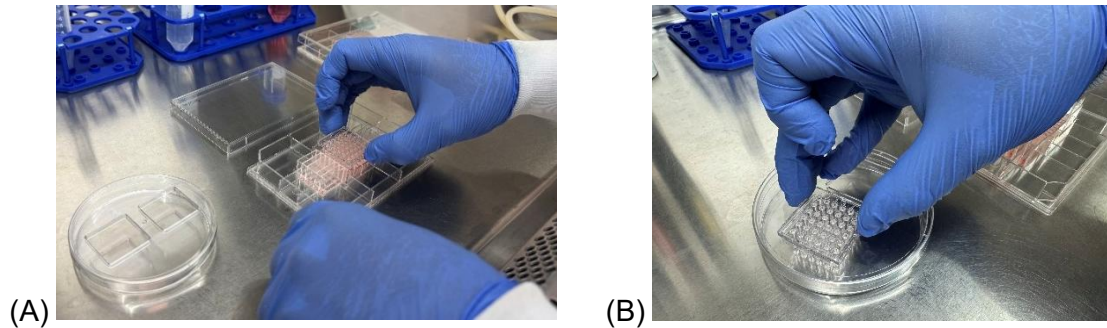


Figure 10. (A) Separation of the 36PillarPlate from the 36PerfusionPlate. **(B)** Insertion of the 36PillarPlate into the 36PetriLid placed on a 90 x 15 mm petri dish to maintain the pillars in a downward orientation and prevent wetting of the pillar bottoms during medium replacement.

26. While holding the perfusion plate at approximately a 45° angle, allow the old cell culture medium to drain toward the lower reservoirs. Aspirate and remove the medium completely from the far end of the lower reservoirs using a Vacusafe vacuum aspiration system (Integra Biosciences) (**Fig. 11**).

Note: Ensure complete removal of the old medium from all perfusion wells and reservoirs to prevent dilution of freshly added medium and accumulation of waste metabolites.

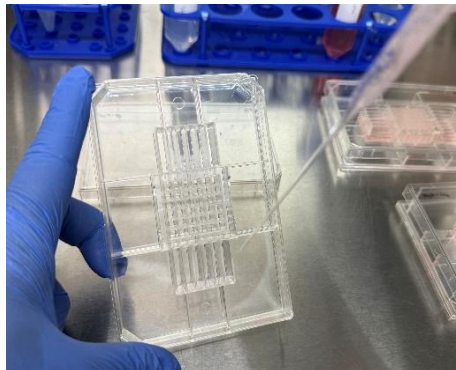


Figure 11. Vacuum aspiration of the old medium from the far end of the lower reservoirs.

27. Carefully remove the pillar plate from the 36PetriLid (or 144PetriLid) and reinsert it onto the corresponding perfusion plate (**Fig. 12**).

Note: Before insertion, ensure that all pillars are properly aligned with the perfusion wells. Misalignment may cause mechanical disturbance of the cultured cells on the pillars.

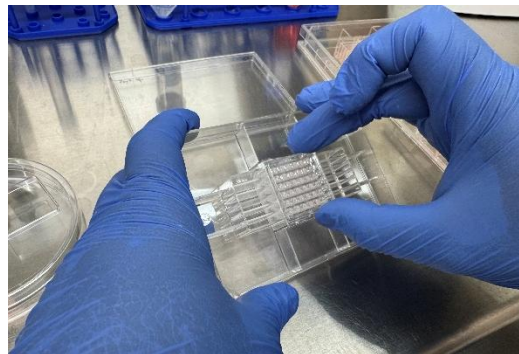


Figure 12. Reinserting the pillar plate onto the perfusion plate after old medium removal.

28. Slowly dispense 400 μ L of fresh, warm cell growth medium into each reservoir of the 36PerfusionPlate (for a total of 800 μ L per fluidic channel) (**Fig. 13**). For the 144PerfusionPlate, dispense 800 μ L into each reservoir (1,600 μ L per fluidic channel).

Note: Dispense the fresh, pre-warmed cell growth medium from the far end of the reservoirs

(away from the microchannels) to prevent overflow in the perfusion wells caused by capillary action.

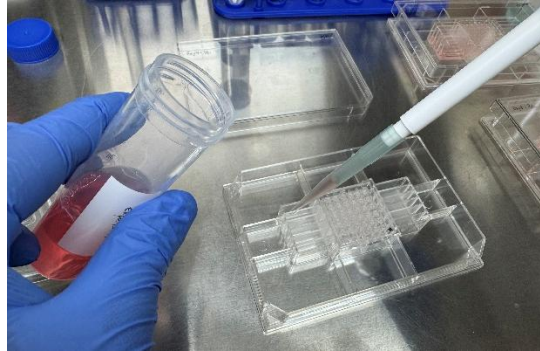


Figure 13. Dispensing fresh, pre-warmed cell growth medium from the far end of each reservoir in the 36PerfusionPlate to prevent capillary-driven overflow into the perfusion wells.

29. Leave the sandwiched plates on a flat surface for approximately 1 minute to allow the medium to equilibrate and distribute evenly across all perfusion wells and channels.
30. Place the sandwiched plates containing fresh cell growth medium onto the digital rocker, and resume dynamic cell culture inside the CO₂ incubator.