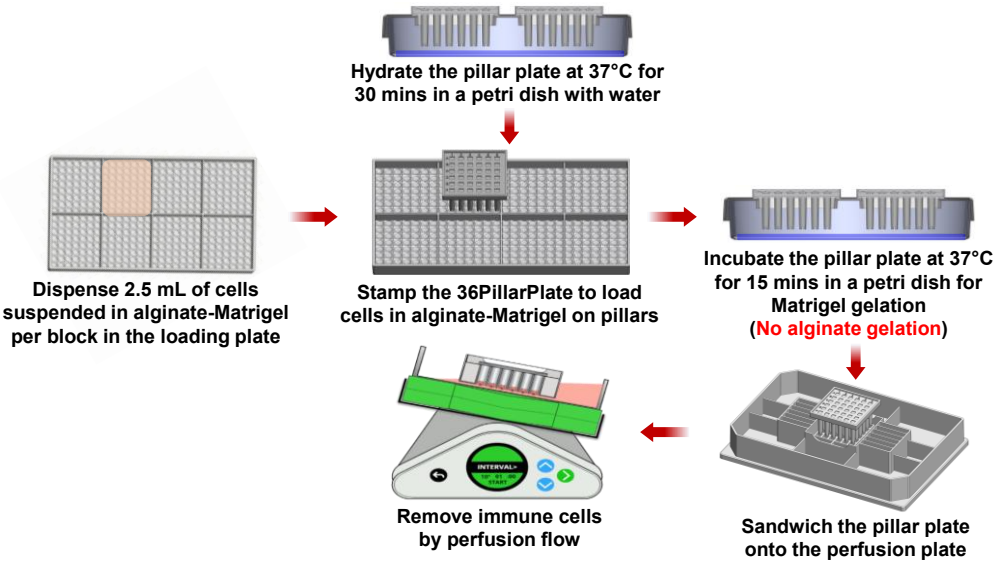
Immune Cell Removal for CTC Culture Using Pillar/Perfusion Plate

This standard operating procedure (SOP) provides step-by-step methods for the efficient removal of immune cells during the culture of circulating tumor cells (CTCs) using the pillar/perfusion plate system. CTCs are notoriously difficult to expand *in vitro*, partly due to inflammatory immune cells that are co-isolated during the CTC enrichment process. By leveraging the differential adhesion properties of cells, this system enables selective retention of CTCs while facilitating the removal of non-adherent immune cells under dynamic perfusion conditions. CTCs attach to extracellular matrix hydrogels on the pillar plate, whereas immune cells are gradually washed away through continuous bidirectional perfusion. This process improves CTC purity and supports enhanced long-term growth and expansion. 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)
- 36PetriLid (Bioprinting Laboratories Inc., Cat. no. 36-03-00)
- LoadingPlate (Bioprinting Laboratories Inc., Cat. no. 384-03-00)
- Deep petri dish, 100 mm x 20 mm (Corning, Cat. no. 70165-102)
- Growth factor reduced Matrigel (Corning, Cat. no. 354230)
- Alginic acid, low viscosity (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 alginate stock, Matrigel stock, and cell culture medium in 36PerfusionPlate

1. To prepare 3% (w/v) alginate stock solution, add 300 mg of low-viscosity alginic acid sodium salt

in 10 mL of sterile distilled water in a 20 mL glass vial.

2. Dissolve the low-viscosity alginate by continuously stirring for 3 days on a magnetic stirrer.
   Note: *Use a large magnetic bar for stirring to ensure proper dissolution of alginate since the use of a small magnetic bar for stirring will lead to improper dissolution of alginate due to high viscosity.*
3. Sterile the 3% alginate solution by passing it through a Millex™ PVDF syringe filter (0.45 µm pore size) using a sterile syringe inside a biosafety cabinet.
   Note: *Because the 3% alginate solution is highly viscous, apply slow and steady pressure during filtration to avoid filter clogging*.
4. Store the sterile alginate stock solution at 4°C until use.
5. 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.
6. Thaw Matrigel® aliquots overnight in a 4°C refrigerator prior to mixing with cell suspension.
   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.*
7. For cell culture, dispense 800 µL/fluidic channel of cell growth medium into a 36PerfusionPlate. Cover the plate with an appropriate well plate lid and incubate it in a humidified 5% CO_2 incubator at 37°C for at least 1 hour prior to use.
   Note: *Prewarming the medium helps minimize temperature shock and reduces air bubble formation during plate assembly and culture*. *Adding an excessive volume of cell culture medium to the 36PerfusionPlate may cause overflow after the pillar plate is sandwiched with the plate. Avoid wetting the bottom of the pillars with culture medium during this process, as it may result in cross-talk or contamination between wells.*
8. Hydrate the surface of the pillar plate by inserting two 36PillarPlates into a 36PetriLid placed on a 100 x 20 mm petri dish containing 5 mL of sterile distilled water. Incubate the assembly in a humidified 5% CO_2 incubator at 37°C for 30 minutes prior to hydrogel loading (**Fig. 1**).
   Note: *Hydrating the surface of the pillar plate in a humid environment is necessary to increase surface hydrophilicity and minimize air bubble entrapment on the pillars after cell loading. Ensure that the pillars are not immersed in water when transferring the assembly to the CO_2 incubator, as excess water on the pillars may interfere with uniform hydration.*

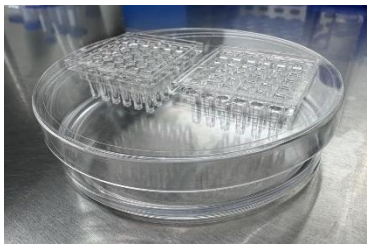


Figure 1. Hydration of the pillar plate surface in a 100 x 20 mm petri dish with 5 mL of sterile, distilled water to minimize air bubble entrapment.

Preparation of single-cell suspension in Matrigel-alginate

9. 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.
10. Gently mix 833 µL of the warm 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 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

11. Aspirate 250 µL of the cell-Matrigel-alginate mixture using a 1 mL pipette tip for single 36PillarPlate.
    Note: *It will require 5 µL of the cell-Matrigel-alginate mixture per pillar (at least 180 µL per 36PillarPlate).*
12. Separate the 1 mL pipette tip from the pipette gently to prevent cell-Matrigel-alginate spillage.
13. Using the index finger, block the back opening of the pipette tip to prevent cell-Matrigel-alginate overflow while tapping the pillar surface.
14. Gently tap the 1 mL pipette tip containing the cell-Matrigel-alginate mixture onto the center of the pillar to load single-cell suspension in Matrigel-alginate while blocking the large back opening of the tip with the index finger (**Fig. 2**).
    Note: *Do not touch the bottom surface of the pillar with the pipette tip to avoid damaging the surface coating. Use the hydrated pillar plates within 5 minutes after removal from the humidified petri dish to prevent complete dying of the surface.*

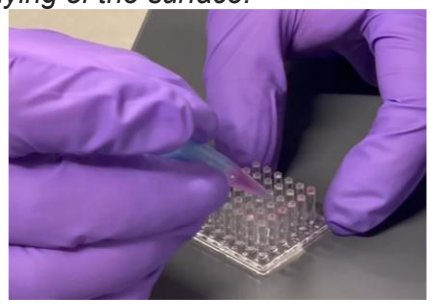


Figure 2. Loading the cell-Matrigel-alginate mixture on the pillar plate using a 1 mL pipette tip.

15. Repeat **Step 14** for all pillars.
16. After loading the cell-Matrigel-alginate mixture on all pillars, remove excess cell-Matrigel-alginate by horizontally sliding a 1 mL pipette tip across the pillar surfaces (**Fig. 3**).

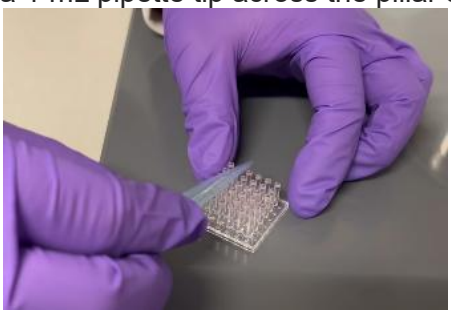


Figure 3. Scraping excess cell-Matrigel-alginate mixture from the pillars using a 1 mL pipette tip.

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

17. Place a LoadingPlate on a flat surface. Dispense 2 - 2.5 mL of the cell-Matrigel-alginate mixture into each small block without introducing big bubbles, and spread the solution evenly using the pipette tip (**Fig. 4**).
    Note: *Ensure to use the cell-Matrigel-alginate mixture immediately as cells in Matrigel-alginate could settle down in 5 minutes, which could lead to non-uniform cell loading on the pillar plate. Keep resuspending the cell-Matrigel-alginate mixture before dispensing on the LoadingPlate.*

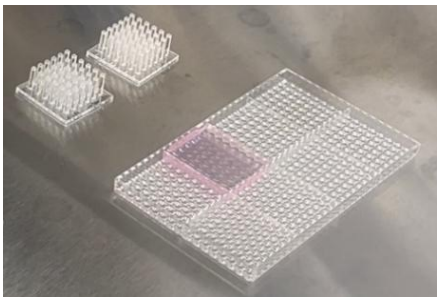
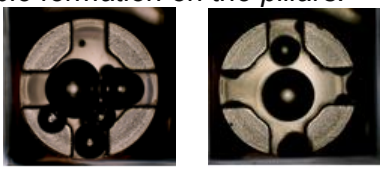


Figure 4. Dispensing 2 - 2.5 mL of the cell-Matrigel-alginate mixture per block in the LoadingPlate for rapid loading of the cells on the pillar plate.

Note: *It is critical to maintain a sufficient volume of the cell-Matrigel-alginate mixture in each block of the LoadingPlate; a minimum volume of 2 mL per block is recommended. Single-cell suspension in Matrigel-alginate should be distributed uniformly to ensure complete wetting of all pillars. Improper loading of the cell-Matrigel-alginate mixture onto the pillars during the stamping process may result in macro-bubble formation on the pillars.*



Note: *Do not leave the cell-Matrigel-alginate mixture on the LoadingPlate for longer than 5 minutes to avoid premature Matrigel gelation during the stamping process. Because pillar stamping is performed rapidly, it is generally not necessary to place the LoadingPlate containing the cell-Matrigel-alginate mixture on ice during this step.*

18. Stamp the 36PillarPlate on the LoadingPlate and press gently to load the cell-Matrigel-alginate mixture evenly on the entire pillar plate. Repeat this cell loading step for another pillar plate (**Fig. 5**).
    Note: *With 2 - 2.5 mL of the cell-Matrigel-alginate mixture, it is possible to prepare at least four 36PillarPlates (5 µL cell-Matrigel-alginate mixture per pillar or 180 µL per 36PillarPlate) without introducing macro-bubbles on the pillars. For uniform pillar wetting and robust cell loading, gently wiggle the pillar plate during stamping. Add additional cell-Matrigel-alginate solution to the LoadingPlate as needed.*

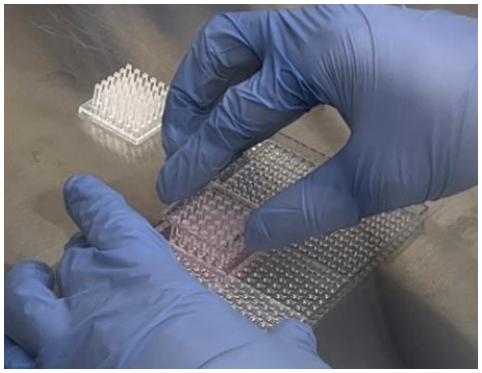


Figure 5. Stamping of the 36PillarPlate onto the LoadingPlate to load cells suspended in Matrigel-alginate on pillars.

Matrigel gelation and cell culture on the pillar plate

19. For minimizing water evaporation during Matrigel gelation, insert two 36PillarPlates loaded with

cells in Matrigel-alginate into a 36PetriLid placed on a 100 x 20 mm petri dish containing 5 mL of sterile, distilled water (**Fig. 1**).

20. Incubate the assembly in a humidified 5% CO_2 incubator at 37°C for 15 minutes to allow complete gelation of Matrigel.
    Note: *Matrigel forms a gel, whereas alginate does not. It is critical to minimize water evaporation during Matrigel gelation to maintain high cell viability. Ensure that the pillars are not immersed in water when transferring the assembly to the CO_2 incubator, as excess water on the pillars may interfere with proper gelation.*

Perfusion culture in 36PerfusuionPlate

21. Separate the 36PillarPlate with CTC cells in Matrigel-alginate and sandwich it onto the 36PerfusionPlate with 800 µL cell culture medium per fluidic channel (**Fig. 6**).

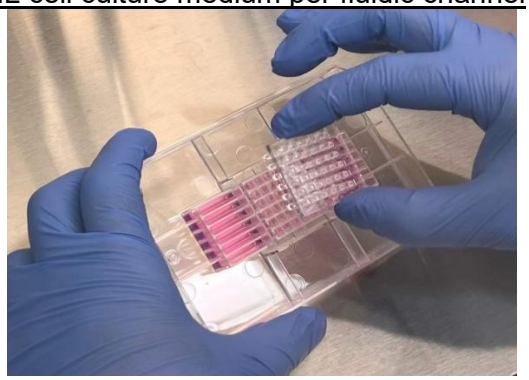
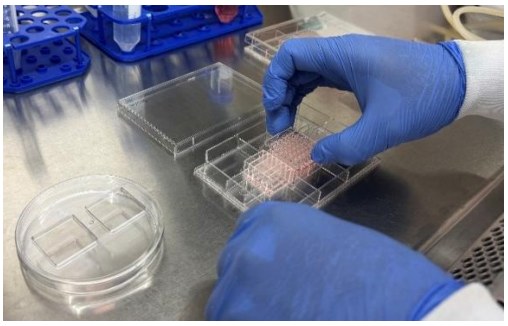


Figure 6. Sandwiching the 36PillarPlate with CTC cells in Matrigel-alginate onto the perfusion wells of the 36PerfusionPlate for dynamic immune cell removal and CTC culture.

22. Inspect the pillar plate under a brightfield microscope to ensure uniform cell loading throughout the entire pillar plate.
23. Place the sandwiched plates on the digital rocker (at 10° tilt angle with a 30-second rocking interval) in a humidified 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".*
24. 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 positioned on a 100 x 20 mm petri dish containing 5 mL of sterile distilled water (**Fig. 7**).
    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.*

(A) 

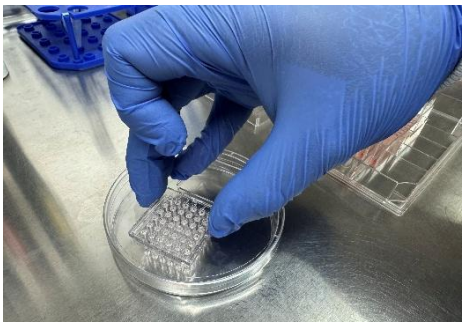
(B) 

Figure 7. (A) Separation of the 36PillarPlate from the 36PerfusionPlate. **(B)** Insertion of the 36PillarPlate into the 36PetriLid placed on a 100 x 20 mm petri dish to maintain the pillars in a downward orientation and prevent wetting of the pillar bottoms during medium replacement.

25. 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. 8**).
    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.*

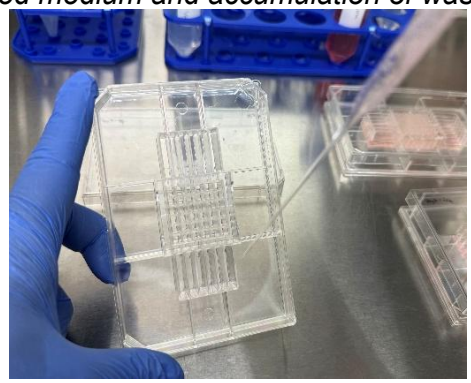


Figure 8. Vacuum aspiration of the old medium from the far end of the lower reservoirs.

26. Carefully remove the pillar plate from the 36PetriLid and reinsert it onto the corresponding perfusion plate (**Fig. 9**).
    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.*

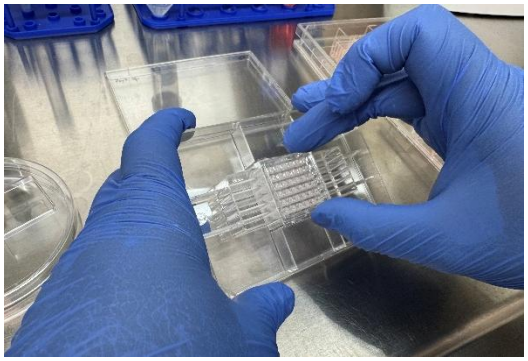


Figure 9. Reinserting the pillar plate onto the perfusion plate after old medium removal.

27. Slowly dispense 400 µL of fresh, warm cell growth medium into each reservoir of the 36PerfusionPlate (for a total of 800 µL per fluidic channel) (**Fig. 10**).
    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.*

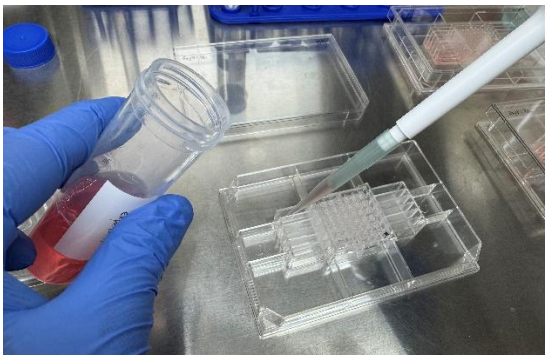


Figure 10. Dispensing fresh, pre-warmed cell growth medium from the far end of each reservoir in the 36PerfusionPlate to prevent capillary-driven overflow into the perfusion wells.

28. 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.
29. Place the sandwiched plates containing fresh cell growth medium onto the digital rocker, and resume dynamic cell culture inside the CO_2 incubator.