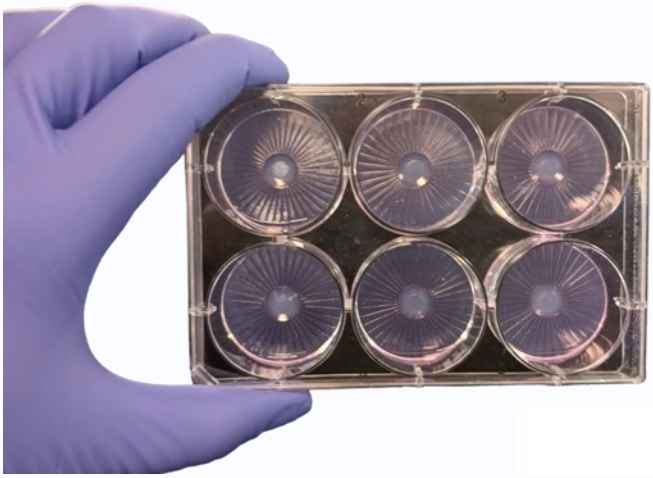
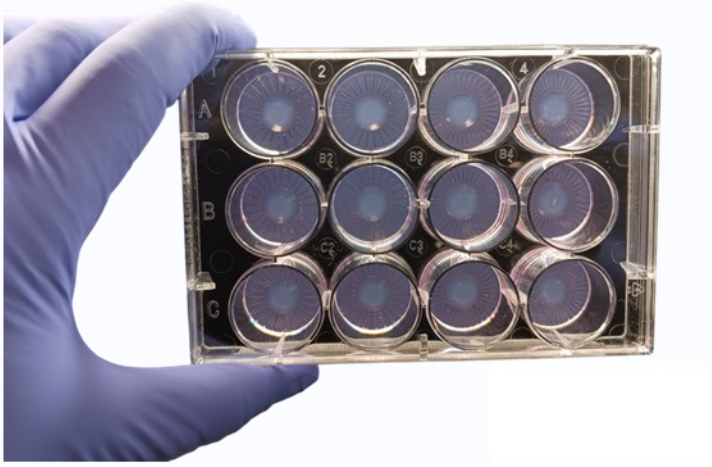
APRICELL BIOTECHNOLOGY

TruSphere Plate™

Next Gen 3D Tissue Culture





User Protocol

TruSphere Plate™ User Guide

Materials Included

- Twelve transparent hydrogel inserts.
- One standard EPS 6,12 and 24-well plates

Shipping & storage

The inserts are shipped pre-inserted within each well of a standard multi-well plate in sealed sterile bags. The plates can be stored at 4° C temperature for up to 12 months from the date of production.

Starting Note

The plates are shipped in a sterilized condition. **However, we recommend putting the plates under the UV light of the biosafety cabinet for 15 min before usage.**

To avoid contamination, only manipulate the plates and inserts using aseptic techniques in a laminar flow hood.

If condensation forms on the plates, gently wipe with an aseptic wiper.

To preserve the integrity of the insert structures, avoid touching the gels with any hard or sharp objects.

This plate was optimized for making tumor spheroids from various types of mesenchymal and epithelial cancer cells including but not limited to ovarian, glioblastoma, breast and pancreatic cancers. The protocol may need adjustments to satisfy your specific needs. Each insert contains one (1) seeding zone and forty (40) microwells suitable for making spheroids of uniform size. The number of cells per seeding can be adjusted from 100K to 400K cells according to your desired spheroid size, microwell diameter and well plate format[1].

[1] Using the higher range of cell numbers when moving from 24 well plates to 6 well plates is recommended.

Protocol

1- Cell Seeding & Spheroid Formation

The number of cells seeded onto each insert can range from 100K to 400K cells according to your desired spheroid size and microwell diameter. Each seeding produces 40 spheroids. This protocol is optimized for cancer cell spheroids

1-1- Using aseptic technique under a laminar flow hood, open the sealed bag and aspirate the PBS solution intended to keep the inserts hydrated. Add 300-800 µL of culture media in the Seeding Zone of the insert. Ensure that the Seeding Zone hydrogel is still slightly above the culture media level[2].

1-2- Prepare a cell suspension with a density of 1×10^5 to 4×10^5 cell/mL and pipette 100-200 µL of the cell suspension gently, dropwise, and close to the gel in the Seeding Zone of the insert[3].

1-3- Leave the well plate for 5-10 min under the BSC. Then, gently transfer it to the incubator[4]. Spheroids will typically be formed between 2-4 days, depending on the cell type and number of cells seeded. A formed spheroid is a cell aggregate that is visibly separated from the outer walls of the microwell and has a dark center under a brightfield microscope.

1-4- Monitor spheroid formation and growth daily and change with 1mL of media every two days until spheroids reach your desired size.

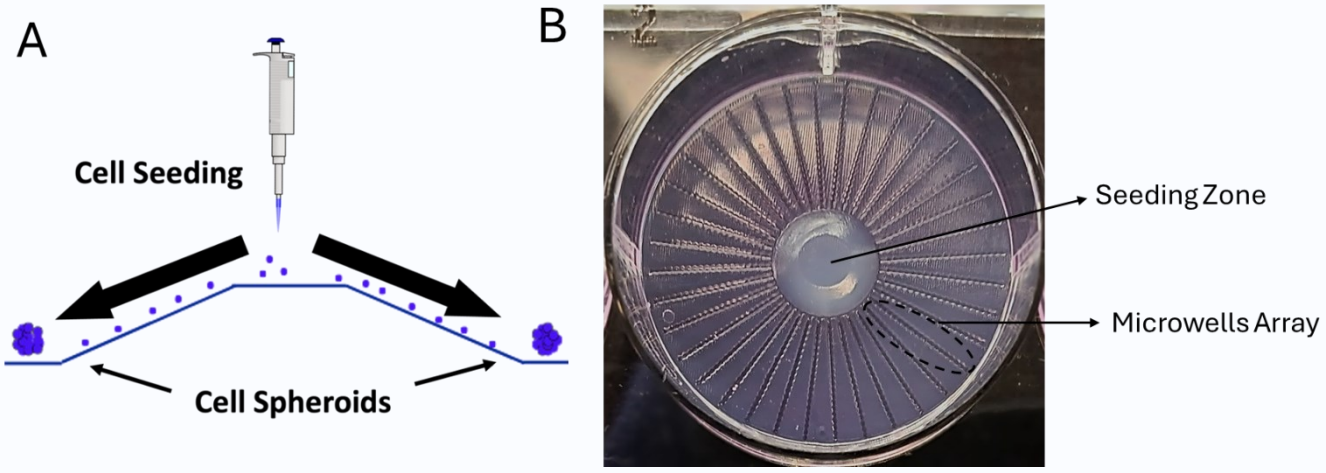


Fig. 1. A) Labeled side view of the TruSphere Plate™, B) Labeled birds-eye view of the TruSphere Plate™

[2] The recommend amount of primary media on seeding zone is 300 µL, 500 µL and 800 µL for 24, 12 and 6 well plates respectively.
[3] The recommend amount of cell suspension for seeding is 100 µL, 150 µL and 200 µL for 24, 12 and 6 well plates respectively.
[4] Let the remaining cells roll into the wells through the guiding channels.

2- Live/Dead Staining of Spheroids in the TruSphere Plate

Live/Dead reagent is used through labeling of live cells with Calcein AM and of dead cells with EthD-1.[5] For preparing 10 mL reagent of an approximately 2 µM Calcein AM and 4 µM EthD-1 solution:

2-1- Add 20 µL of 2 mM EthD-1 stock solution (Component B) to 10 mL of sterile, tissue culture–grade D-PBS, vertexing to ensure thorough mixing. This gives an approximately 4 µM EthD-1 solution. Cover solution from light exposure.

2-2- Combine the reagents by transferring 5 µL of 4 mM Calcein AM stock solution (Component A) to the 10 mL EthD-1 solution. Vortex the resulting solution to ensure thorough mixing [6].

2-3- Carefully aspirate the culture media from the outer diameter of the well plate, making sure that the spheroids are not aspirated or disturbed. Then, add 500 µL of the prepared and warmed Live/Dead solution to the Seeding Zone.

2-4- Leave the well plate for 2 hours in the incubator and then remove the Live/Dead solution by aspirating from the edge of the well plate. Then, wash the inserts gently with 1× PBS two times.

2-5- Perform immunofluorescent IF imaging by adjusting Ex/Em of Calcein on 494/517 nm and EthD-1 on 528/617 nm.

3- Presto Blue Analysis of Tumor Spheroids in the TruSphere Plate

Presto Blue (PB) assays are used for metabolic activity, viability and proliferation analysis of tumor spheroids after exposure to different interventions including various drug treatments.

3-1- Add 1 mL of PB stock solution to 9 mL of sterile DMEM culture medium. Vortex to ensure thorough mixing while avoiding light exposure to the solution. The final concentration of the PB solution is 10% (v/v) for measuring the metabolic activity of the spheroids.

3-2- Carefully aspirate all the media from side of the insert. Then, add 500 µL of the 10% PB solution to the well plate.

3-3 Return the well plate to the incubator. The incubation time can vary depending on the size of the spheroids[7].

3-4- After incubation is complete, transfer 100 µL of PB media from each well into a fresh 96-well plate. Make sure to take note of where you transferred each well to in the new 96-well plate. Duplicate or triplicate 100 µL samples are recommended for experimental accuracy.

3-5- Wrap the 96-well plate with aluminum foil to protect the plate from light before transferring the plate to a plate reader. The plate can be in the fridge for up to 48 hours before reading with a plate reader.

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[5] The optimal concentrations of live–dead solution might vary depending on the cell type. In general, it is best to use the lowest dye concentration that generates a more signal-to-noise ratio and less background in the fluorescent images.

[6] The concentration of the Calcein and EthD-1 can be adjusted based on the final desired volume.

[7] 20 to 30 min is usually sufficient for a PB assay. For 3D culture, the incubation time is affected by the diffusion of the fluorescent component of PB in the hydrogel or spheroids. It is better to incubate for too long and have to dilute your samples rather than to risk having poor signal separation for different conditions.

3-6- Read the plate at 560 nm excitation and 590 nm emission. If any of the wells read outside of the plate reader's working range, you will have to either reduce the volume of the samples or dilute the wells for all the wells. This will not affect your final results as you are just comparing the conditions to your control condition. Repeat this step until all your samples read within the working range of the instrument.

3-7- When processing the data, subtract the blank well value from all other wells and set the control condition to be 100% viability.

4- Imaging & Analysis

- The TruSphere Plate is compatible with different imaging modalities including inverted, upright, fluorescent and confocal microscopes.
- For fluorescent imaging, the spheroids can be stained within their microwells without needing to remove them from the insert.

4-1- **Immunostaining of spheroids in TruSphere Plate**

This protocol is for general immunostaining of the spheroids.

4-1-1 Prepare 4% formaldehyde by diluting the stock 37% formaldehyde in PBS (for 10 mL, add 1.08 mL stock formaldehyde solution to 8.92 mL PBS). Aspirate the media from the side of the insert and add 300–500 µL of 4% solution dropwise on top of the Seeding Zone. Incubate at room temperature for 30 min[8].

4-1-2- Remove the formaldehyde solution and gently wash the spheroids three times with PBS. Washing can be done by adding 300–500 µL of PBS to the side of the well and incubating it for 2 min. This will allow the formaldehyde solution to diffuse out of the gel and into the washing solution.

4-1-3- Block the sample using BSA or normal serum related to the primary/secondary antibodies. Follow the following procedure to make the blocking buffer.

(a) Warm up PBS to 45–50 °C.
(b) Dissolve 0.3% wt/vol Triton X-100 in the warm PBS until it is clear.
(c) Weigh enough BSA to make a 5% (w/v) solution in a falcon tube. Wait until the Triton solution cools down to room temperature. Then add the 0.3% Triton solution to make the blocking buffer.

4-1-4- Aspirate the last wash solution from the side of the insert. Then, add 300-500 µL of the blocking buffer. Keep in the room temperature overnight.

4-1-5- Make the primary antibody solution. Similar to the procedure for blocking buffer, make a solution of 1% (w/v) BSA and 0.3% (w/v) Triton in PBS. According to the protocol (for 3D culture use a decreased dilution factor), dilute the primary antibody in the 1% (w/v) BSA and 0.3% (w/v) Triton solution.

4-1-6- Aspirate the blocking buffer from the side of the insert and add the primary antibody solution. 200 µL volume is enough and no washing step is required at this stage. Incubate overnight[9].

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[8] Fixing reagent can be chosen by the user.
[9] Steps 7 and 8 are for non-conjugated primary antibodies. If the primary antibody is already conjugated go directly to step 9.

4-1-7- Remove the primary antibody solution from the insert and wash it three times according to Step 2, Subheading 3-1-2.

4-1-8- Add secondary antibody solution. To make the secondary antibody solution, make 1% BSA and 0.3% Triton solution similar to step 5 and dilute the secondary antibodies according to the supplier's protocol (for 3D culture use a decreased dilution factor). Incubate for 4–6 hours in the fridge.

4-1-9- Remove the solution of secondary antibodies and add the DAPI solution. To make the DAPI solution, dilute stock solution of DAPI in PBS according to supplier's protocol (for 3D culture use a decreased dilution factor). Incubate for 2–3 hours at room temperature.

4-1-10- Remove the DAPI solution and wash the spheroids three times with PBS. Then, add 500 µL of PBS from the side of the insert. The spheroids will then be ready for immunofluorescence microscopy.