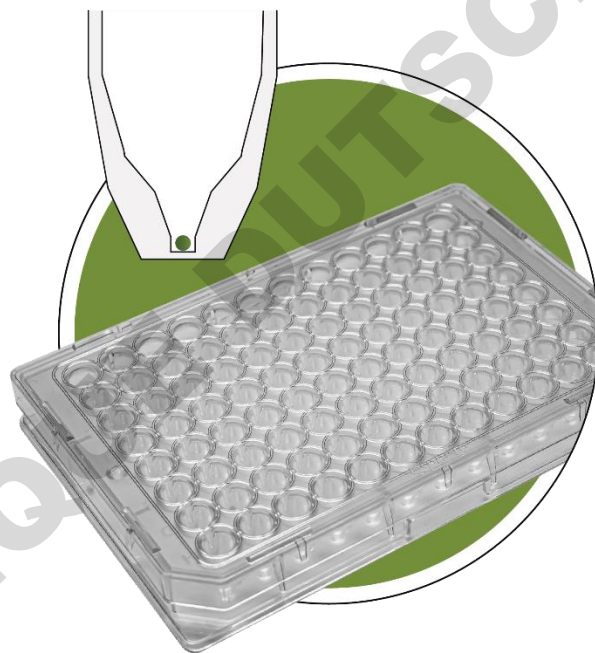




Akura™ 96 Spheroid Microplate

# Product Manual



PM001, July 2023

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## Introduction

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InSphero Akura™ 96 Spheroid Microplate represents a simple, flexible, and automation-compatible platform for the generation, long-term cultivation, observation, and testing of scaffold-free 3D cell models in 96-well format. Each plate is made of COP (Cyclo-olefin-polymer) and consists of a special ultra-low attachment (ULA) well and a low-evaporation lid.

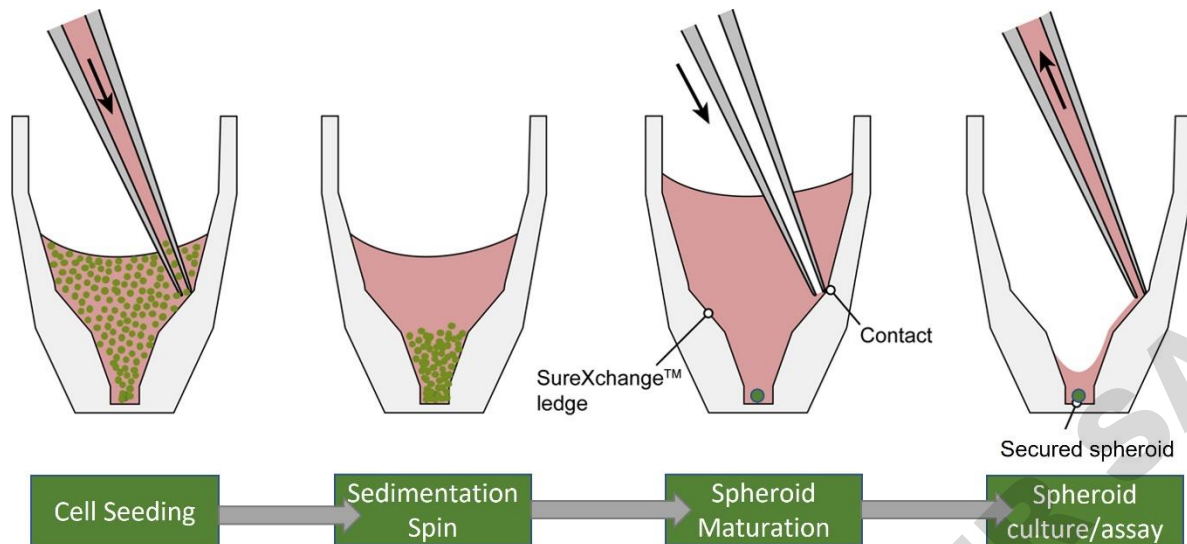
InSphero recommends Akura™ Plates for the generation of spheroids using immortalized or modified cell lines as well as primary or iPS-derived cells that are known to readily form 3D cell models, or as a starting point for investigating whether a cell type can form self-aggregating, scaffold-free spheroids.

The Akura™ 96 Spheroid Microplate is designed to generate several 3D cell model types, such as organoids, spheroids and microtissues. For the remainder of the document, we refer to these models as 'spheroids'.

## Advantages of Akura™ 96 Spheroid Microplate

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1. Convenient scaffold-free formation of spheroids below 750 µm via cellular self-assembly in ultra-low attachment (ULA-treated) plates.
2. SureXchange™ tapered ledge and culture chamber facilitates easy medium exchange and prevents spheroid loss during long-term spheroid growth and analysis.
3. 1 mm diameter flat bottom observation window enables simple spheroid observation, and greater distance between observation windows of adjacent wells reduces well-to-well imaging crosstalk compared to standard 96-well plates.



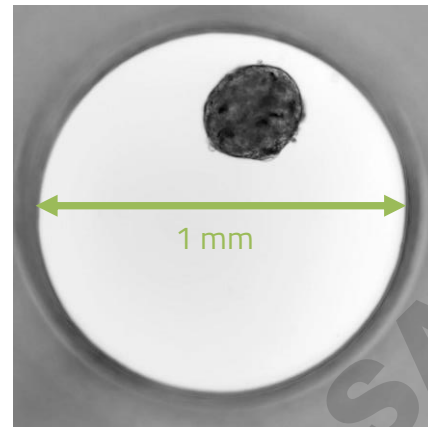
**Figure 1:** Spheroid formation in the Akura™ 96 Plate begins with initial seeding of cells in suspension, followed by a brief spin to concentrate cells. Following spheroid maturation, the SureXchange™ ledge of the tapered well facilitates medium exchange and compound dosing without disturbing or losing the spheroids.

## The Akura™ 96 Spheroid Microplate

The Akura™ 96 Plate is a special non-adhesively coated 96-well microtiter plate. It is designed to accommodate production of 3D cell models for convenient long-term cultivation and analysis. Akura™ 96 tapered wells feature a SureXchange™ ledge to prevent inadvertent spheroid aspiration and disruption during medium exchange and compound dosing (Fig. 1). Spheroids are positioned in a 1.0 mm observation chamber at the bottom of each well, which enables automated imaging processes (Fig. 2). Biochemical assays as well as optical analytical methods such as inverted bright field and fluorescence microscopy can be performed.

Spheroid production with Akura™ 96 Plates is very simple and recommended for cell types that are known to readily form spheroids in ULA conditions, or as a first step in characterizing the spheroid-forming capabilities of a particular cell type of interest. A cell suspension is delivered to the plate using a multi-channel pipette or a robotic liquid handler. Following brief centrifugation to concentrate cells near the bottom of the tapered chamber, spheroids begin forming by gravity-assisted self-assembly. Spheroid maturation typically occurs within 2-5 days of seeding depending on the cell type and culture conditions (Fig. 1).

**Figure 2:** Brightfield image of 3D InSight™ Human Liver Microtissue. Picture acquired with a Zeiss Axiovert 25 inverted microscope, 5x objective, Canon® PowerShot digital camera, zoom 8.9x.



## Generating 3D Cell Models

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Generating 3D cell models in the Akura™ 96 Plate is a straightforward process, but one that must be optimized for each cell type. Cell type, growth medium, and intended downstream applications will impact the starting density and desired culture volume. In addition to the process overview in this chapter, optimization is recommended for each cell type and application.

## Additional Materials Required

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1. Mammalian cells (primary or cell line) of interest
2. Your specific maintenance medium
3. Inverted microscope with a 5x/10x objective
4. Cell counter, e.g., Neubauer chamber
5. 8- or 12-channel pipette (e.g., Viaflo 10-300 µl, Integra Biosciences)
6. Single channel 1000 µl pipette
7. Medium reservoir for multichannel pipettes
8. Microplate centrifuge
9. Humidified 5% CO<sub>2</sub> incubator 37°C

## Preparation

1. Prior to seeding, pre-warm the cell maintenance medium to 37°C.
2. Wipe the Akura™ 96 Plate bag with 70% EtOH before opening.
3. Carefully open the bag under sterile working conditions e.g., inside a biosafety cabinet and take out the Akura™ 96 Plate assembly.

## Pre-wetting

### IMPORTANT

- Pre-wetting the wells of the Akura™ 96 Plate according to the procedure below is recommended prior to seeding cells to prevent inclusion of air bubbles.
  - Perform all following steps under sterile conditions.
1. Add 40 µl of cell line medium containing FCS or BSA to each well by placing the tip near to, but not touching the bottom of the well. It is recommended to use a multichannel pipette (8- or 12-channel).
  2. Gently pipette the pre-wetting solution up and down and remove pre-wetting solution by placing the tip at the ledge of the upper cavity of the well (Fig. 3). Aspirate until medium is completely removed from each well. A negligible amount of medium (<5-7 µl) may remain in the bottom of the chamber.

## Cell seeding

1. Prepare a single-cell suspension of your preferred cell type or cell mixture, according to your standard protocol.
2. Count the cells using a Neubauer chamber or cell counting instrument to determine the starting cell concentration.
3. Prepare the final cell suspension for seeding, using a final volume per well of 70 µl.  
Recommended cell concentrations: For long-term growth profiling start with low cell numbers (250–500 cells per well). If non-proliferating cells or rapid production of larger spheroids are required, start with 2500–25,000 cells per 70 µl.

IMPORTANT – To generate spheroids with uniform size and cell composition, it is essential to assure a homogeneous distribution of the cell suspension by gently pipetting up and down prior to seeding into the Akura™ 96 Plate.

4. Gently ( $\leq 10$  µ/sec) add 70 µl of the cell suspension to the Akura™ 96 Plate by placing the pipette tips far into the wells (avoid touching the well bottom).

## Cell sedimentation and spheroid maturation

Following seeding, it is recommended (but optional) to briefly centrifuge the plate to remove any air bubbles, and to force cells to the bottom of the well to promote aggregation and spheroid formation.

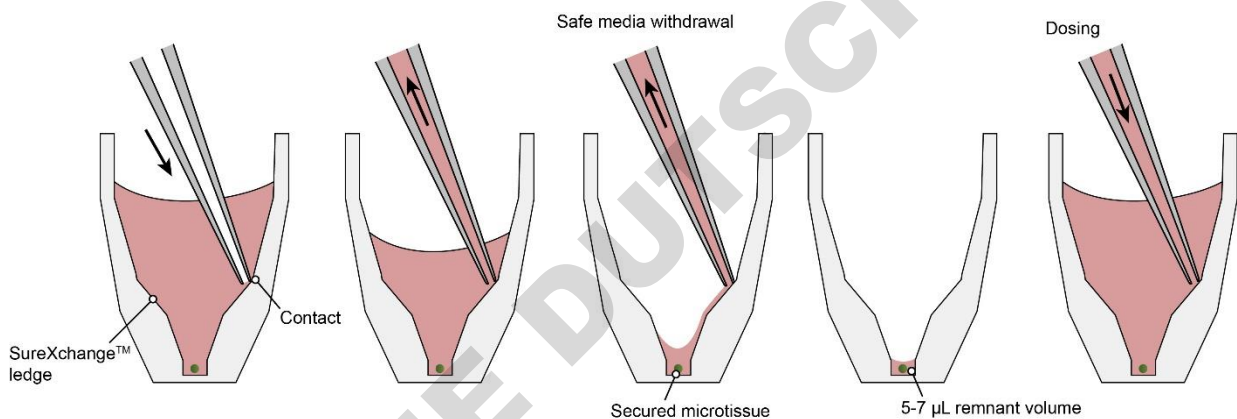
1. Place the lid on the Akura™ 96 Plate and spin in a microtiter-plate centrifuge for 2 minutes at 250 RCF.
2. Following centrifugation, remove the plate and incubate the plate in a humidified CO<sub>2</sub> incubator at 37°C for 2-5 days, checking daily to observe spheroid maturation and exchanging medium as necessary.
3. Tilt the plate in the incubator to approximately 30° or use Akura™ Tilting Stand (InSphero, CS-AG11) to improve the maturation process.

NOTES - To achieve optimal spheroid formation it may be necessary to modify the aggregation conditions such as modifying the cell concentration or media composition, or by the addition of supporting cell types (e.g., matrix secreting cells) or supplements (e.g., ECM). For new cell types we recommend trying a variety of aggregation conditions.

## Medium Exchange in the Akura™ 96 Spheroid Microplate

The special Akura™ 96 Plate design allows routine medium exchange for longer-term cultivation without the risk of spheroid loss. The SureXchange™ ledge at the inside wall of the well serves as an anchoring point for the pipette tip.

1. Place the pipette tip at the ledge of the well (Fig. 3, left).
2. Remove the medium at low pipetting speed (<30  $\mu\text{l}/\text{sec}$ ) by aspirating an excess of volume. A minimal volume of ~5-7  $\mu\text{l}$  medium will remain in the well.
3. Add 70  $\mu\text{l}$  of fresh medium by placing the pipette tip at the ledge (Fig. 3, right). Use a dispensing rate <50  $\mu\text{l}/\text{sec}$ .



**Figure 3:** Medium exchange in the Akura™ 96 Plate. Left: Medium removal with the pipette tip placed at the ledge of the well. Right: Medium addition.

### Medium exchange with multi-channel electronic pipettes

Cultivating spheroids typically requires 2-3 medium exchanges per week, but recommended frequency may vary by spheroid type. To exchange medium, please follow these steps and review our recommendations (Table 1).

1. Place pipette tip at the ledge by slowly sliding down along the inside wall of the well until a subtle resistance can be felt (Fig. 3, left)
2. Carefully and slowly remove the medium by aspirating an excess of volume. This will lead to an almost complete removal of the medium.



3. Add 70 µl of pre-warmed medium by placing the pipette tip at the ledge of the plate well (Fig. 3, right) and gently dispense at low pipetting speed (speed dependent on spheroid type, ~10 – 30 µl/sec if using an automated multi-channel pipette).
4. Optional: For a more thorough medium exchange, repeat steps 2-3.
5. Place the lid on the Akura™ 96 Plate and incubate the spheroids in a humidified 37°C CO<sub>2</sub> incubator.

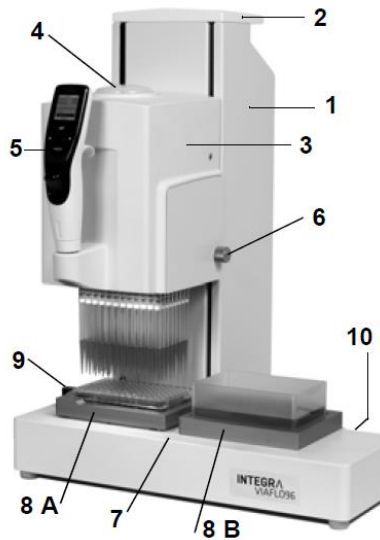
**Table 1**

*Recommendations for culturing spheroids in Akura™ 96 Plates*

Material/Process	Recommendation
Culture medium	See 3D InSight™ platform guides for media recommendations for spheroid models and applications
Culture medium volume	70 µl/well
Medium exchanges	2-3 times per week or frequency recommended for specific spheroids
Pipettes	INTEGRA VIAFLO multichannel pipette with Integra 300 µl pipette tips with filters
Aspiration speed	Slow (set automated pipette to < 20 µl/second)
Dispense speed	Slow to moderate (set automated pipette to < 50 µl/second)

## Semi-automated medium exchange with INTEGRA VIAFLO 96

The unique design of the Akura™ 96 Plate enables the use of multi-channel pipetting systems for parallel liquid handling without the risk of spheroid loss. We recommend the INTEGRA VIAFLO system as it is a compact, easy-to-use semi-automated pipette with 96 channels (Figure 4) for increased productivity.



- 1 Base unit, to move left/right (X-axis)
- 2 Carrying handle of base unit
- 3 Pipetting unit, to move up/down (Z-axis)
- 4 Tip load button
- 5 Control unit
- 6 Knob of side cover
- 7 Instrument deck
- 8 Plate holders on position A and B
- 9 Plate slider
- 10 Main switch

*Figure 4. INTEGRA VIAFLO 96/384 device*

The following guidelines are for using the INTEGRA VIAFLO system with Akura™ 96 Plates. Some parameters may vary due to different hardware and software versions and/or different accessories of the system. Please refer to the *INTEGRA VIAFLO 96/384 Operating Instructions* for additional details.

### INTEGRA VIAFLO 384 System Configuration

VIAFLO 96 (2<sup>nd</sup> Generation, Part No. 6001)

96-channel pipette head, 10-300 µl (Part No. 6103)

Spring loaded plate holder (8A) with slide function (384 offset) (Part No. 6215)

Standard plate holder for 96 well plate in position 8B (Part No. 6205)

Grip tips, 300 µl, sterile, with filter (Part No. 6435)

Reservoirs, 300 mL in tray (Part No. 6327)

Firmware Base unit: 3.27

Firmware Control unit: 3.11

Medium exchange is executed in two steps: First, medium is **aspirated** from one or more plates and discarded or sampled, then fresh medium is **dispensed** to wells from reservoirs.

#### Medium aspiration

1. Place waste reservoir or plate for samples in position 8B (plate holder on right).
2. Place Akura™ 96 Plate containing spheroids in position 8A (plate holder on left).  
**Note:** Place the Akura™ 96 Plate onto an Akura™ 96 lid. This allows the plate to be positioned with a slight horizontal offset (1-2 mm) with respect to the tips.
3. Program the pipette for medium aspiration (Table 2, Specifications for: 96-channel pipette head, 300 µl volume pipette tips and Akura™ 96 Plate lid as base for Akura™ 96 Plate).

**Table 2**

*Medium aspiration pipetting program (for 4 plates in a row)*

Step	Instruction	Notes
1	Tip Align A3	Move pipette head to position 8A above Akura™ 96 Plate.
2	Z-Height pos. A, 38.5 mm	Gently immerse pipette tips into Akura™ 96 Plate wells, until reaching Z-Height.  Displace Akura™ 96 Plate by 1-2 mm, repositioning pipette tips along well wall. Hold plate in this position.
3	Aspirate 75 µl, speed 1	Aspirate 75 µl with speed 1, repeat as necessary, depending on plate quantity.
4	Tip Align B3	Move pipette head to position 8B (right) into reservoir.
5	PURGE, speed 4	Set purge speed.

\*See Figure 4 for reference

#### Medium dispensing

1. Place medium reservoir position 8B (plate holder on right).  
**Note:** Calculate up to 10 mL of extra medium to prevent aspiration of air.
2. Place Akura™ 96 Plate containing spheroids in position 8A (plate holder on left).  
**Note:** Place the Akura™ 96 Plate onto an Akura™ 96 lid. This allows the plate to be positioned with a slight horizontal offset (1-2 mm) with respect to the tips.

3. Program the pipette for medium dispensing (Table 3, Specifications for: 96-channel pipette head, 300 µl volume pipette tips and Akura™ 96 Plate lid as base for Akura™ 96 Plate).

**Table 3**

*Medium dispensing pipetting program (for 4 plates in a row)*

Step	Instruction	Notes
1	Tip Align B3	Move pipette head to position 8B (right) into reservoir.
2	Aspirate 290 µl, speed 3	Aspirate 290 µl <sup>1</sup> with speed 3. (aspiration volume dependent on plate quantity; 70 µl plus excess per plate).
3	Tip Align A3	Move pipette head to position 8A above Akura™ 96 Plate.
4	Z-Height position 8B, 38.5 mm	Gently immerse the pipette tips into the wells of the Akura™ 96 Plate, until reaching Z-Height. Displace Akura™ 96 Plate by 1-2 mm, repositioning pipette tips along well wall. Hold plate in this position.
5	Dispense 70 µl, speed 1	Dispense 70 µl in to well with speed 1, repeat as necessary depending on plate quantity.
6	PURGE, speed 4	

<sup>1</sup> 4x 70 µl plus 10 µl excess volume remaining in the tip.

## Analysis and Assays in Akura™ 96 Spheroid Microplate

The Akura™ 96 Plate format is compatible with a broad variety of biochemical methods and allows for spectrometric measurements with a multiwell plate reader or for visual inspection of spheroids by an inverted microscope (similar to analysis of standard 2D cultures):

### Fluorescent/luminescent multiwell plate reader compatibility

Growth changes and profiles in tumor spheroids expressing GFP/RFP can easily be analyzed using fluorescent plate readers, as the signal intensity is stronger than with monolayer cultured cells.

### Automated imaging

The Akura™ 96 Plate is ideal for use in automated imaging equipment, such as the SCREEN Cell3iMager, automated microscopes and high content imaging systems (e.g., Yokogawa CQ1 or

PerkinElmer Operetta), as the 1 mm diameter optically clear base of each well will be positioned exactly in the center of the field of view.

NOTES - The flat Cyclo-olefin-polymer (COP) bottom of the Akura™ 96 Plate provides superior imaging quality relative to round-bottom spheroid plates. However, modifications to the plate settings and/or autofocus settings on your imaging instrument may be required to achieve optimal results. In general, these are relatively simple changes that can be made by a knowledgeable instrument operator. Please review the following points, in advance of your study.

- Due to the tapered well bottom and the 0.8 mm bottom thickness, the creation of a new 96 well plate definition (a.k.a. form factor) may be required for optimal imaging performance (use the specifications provided in Annex A as the starting point for the new plate definition).
- The non-continuous well bottoms and 0.8 mm bottom thickness may necessitate the use of an extended autofocus range to ensure accurate focus across the entire plate.
- If image acquisition through the entire Z height of the spheroids is required, the working distance of the selected objective must be equal to the bottom thickness (0.8 mm) plus the Z height of your specimen.
- Objectives with correction collars should be set for a 0.8 mm bottom thickness.

By adhering to the suggestions above, the Akura™ 96 Plate can be used successfully with nearly all high content imaging platforms. One exception is the Sartorius Incucyte platform which is currently incompatible with the Akura™ 96 Plate due to the firmware configuration.

## Spheroid Collection

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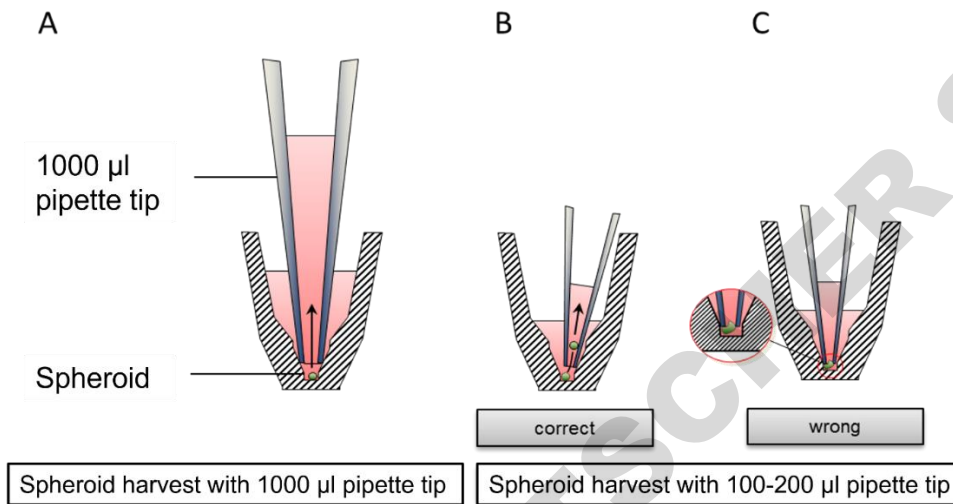
The special coating of the Akura™ 96 Plate minimizes the adherence of the spheroids to the bottom of the well. This facilitates collection of spheroids for transfer into another plate format or for further processing, such as embedding for histological analysis. To harvest the spheroids, we recommend two different options:

### **Spheroid transfer using manual or automated, single- or multi-channel pipettes**

1. Before beginning the spheroid collection steps below, prewet the pipette tip with at least 60  $\mu$ l 100% FCS. Pre-wetting the tip will discourage spheroids from sticking to the inside of the tip.
2. Gently immerse a pipette, holding a 1000  $\mu$ l tip, along the inside wall of the well, until feeling a slight resistance. The pipette tip is now positioned slightly above the spheroid on the well bottom (Figure 5A). Use of 1000  $\mu$ l tips prevents the spheroid from being squeezed inadvertently because the tip diameter exceeds the size of the well bottom.
3. Alternatively, use a 100–200  $\mu$ l tip and carefully lower the tip at a slightly angled position along the wall until it touches the well bottom. Aspirate by placing the head of the tip close to the

edge of the well bottom (Figure 5B). Note that incorrect positioning of the 100–200 µl pipette may damage the spheroids (Figure 5C).

4. Collect the spheroid by aspirating 50 µl of the medium. Avoid aspiration of air bubbles to prevent spheroid loss in the pipette tip.
5. Transfer the spheroid in medium into another vessel or plate.



**Figure 5:** Pipette positioning when collecting spheroids using A. a 1000 µl pipette tip or B. a 200 µl pipette tip. C. The incorrect way to position a 200 µl pipette tip during transfer, causing spheroid damage.

## Annex A: Akura™ 96 Spheroid Microplate Specifications

The Akura™ 96 Spheroid Microplate format is compliant with standard microtiter-plate definitions as specified by the SLAS Microplate Standards Advisory Committee ANSI SLAS 1-2004 (R2012). The 96 wells are arranged in 8 rows and 12 columns, identified by alphanumeric labels (Figure 1A). Individual wells show a regular wide opening at the top narrowing down into a small cavity at the well bottom, with a flat optically clear base (Figure 6B), designed to accommodate spheroids of up to 750 µm in diameter. The Akura™ 96 Spheroid Microplate technical specifications are provided as a reference for automation system programming (Figure 7, 8 and 9).

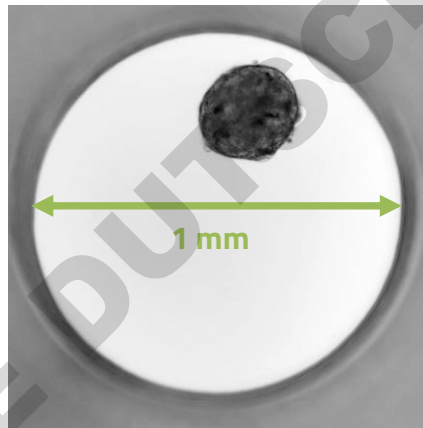
### Plate Dimensions:

Plate length:	127.76 mm
Plate width:	85.48 mm
Height of plate:	14.35 mm
Height of plate with lid:	15.35 mm
Height of well:	12.95 mm
Skirt height:	0.4 mm
Diameter well opening:	6.70 mm
Diameter well bottom:	1 mm
Thickness well bottom:	0.8 mm
Working volume:	70-80 µl
Well-to-well distance:	9 mm
SureXchange™ tip position:	1.71 mm horizontal offset; 9.86 mm in z-height (see Fig. 9)
Plate and lid material:	COP (Cyclo-olefin-polymer), Polystyrene

**A**



**B**



**Figure 6:** **A.** Angled view of Akura™ 96 Plate. **B.** Human liver microtissue in Akura™ 96 Plate. The well diameter is exactly 1 mm.



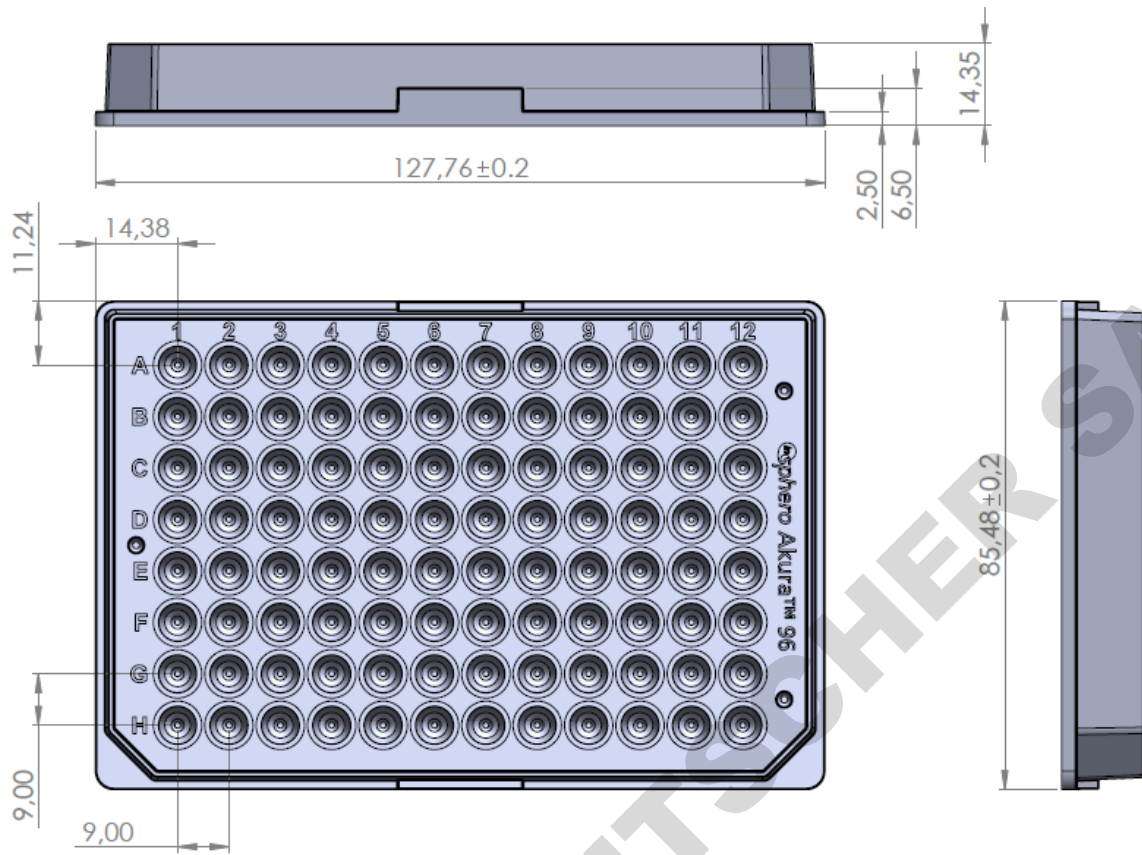


Figure 7: Technical specifications of Akura™ 96 Plate in mm.

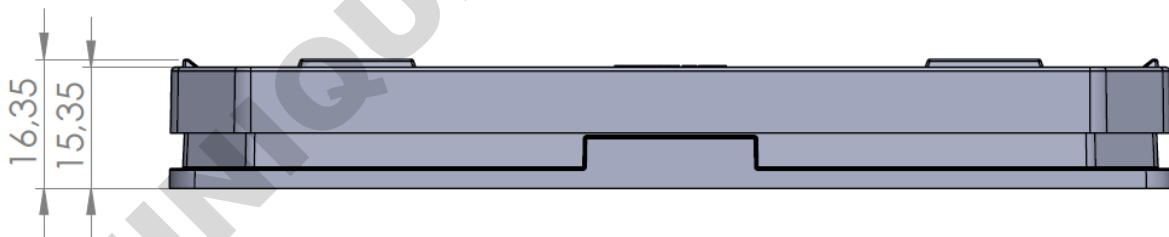


Figure 8: Height of well with lid in mm.

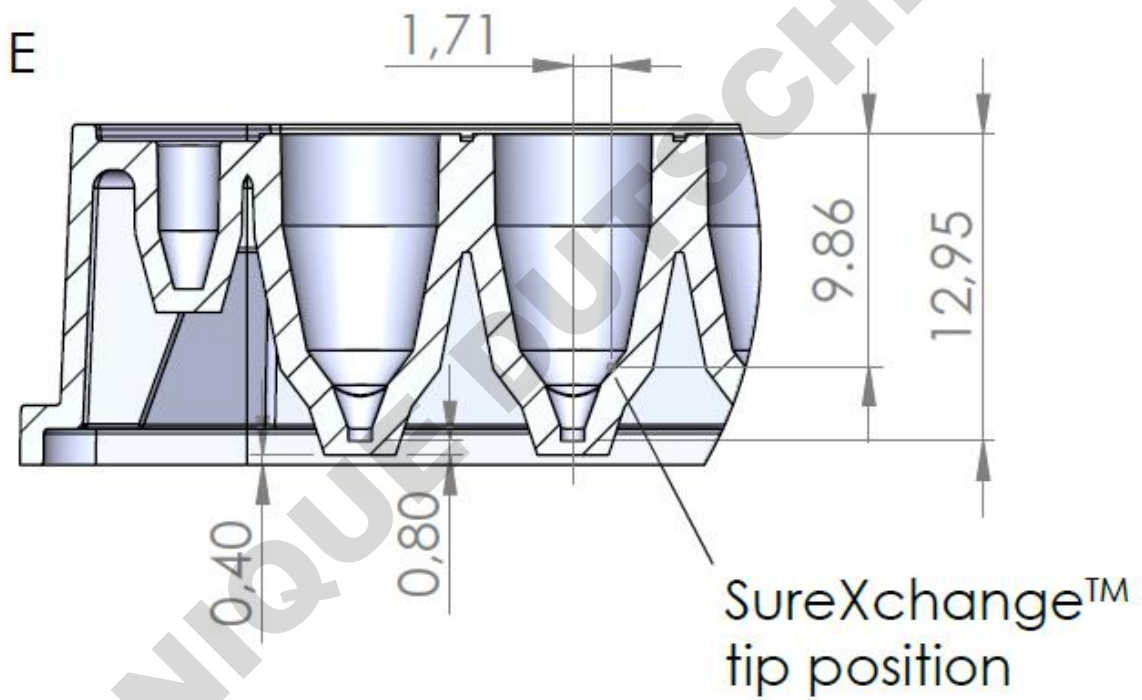
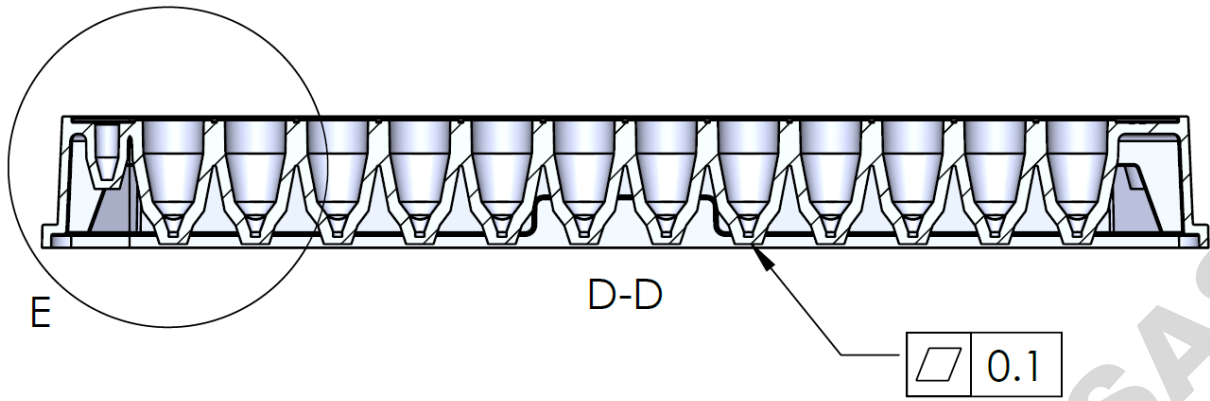


Figure 9: Height of well, skirt height, well bottom thickness and SureXchange™ tip position in mm.

## Annex B: License Agreement

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### License Agreement Akura™ 96 Spheroid Microplate, Akura™ 384 Spheroid Microplate and Akura™ PLUS Hanging Drop System

This License Agreement (the „License Agreement“) is a legal agreement between the end user („End User“) and InSphero AG or its subsidiaries („InSphero“) to use the Akura™ 96 Spheroid Microplate, Akura™ 384 Spheroid Microplate and Akura™ PLUS Hanging Drop System („Akura Plates“) covered by patents owned or controlled by InSphero which are provided to you.

1. Warranties: The End User hereby irrevocably warrants to keep and use the Akura Plates in accordance with the restrictions and limitations contained in this License Agreement.
2. Proprietary rights of the Akura Plates may be covered by one or more of the following patents: US 9126199 B2, CA 2737627 C, EP 2342317 B, DK 2342317 T3, ES 2401640 T3, CN 102257123 B, JP 5490803 B2, and other pending patent applications. By entering into this License Agreement, End User acknowledges that the Akura Plates are so covered.
3. Excluded Fields: No permission is granted hereunder for the use of the Akura Plates:
  - a. for selling cell-based products generated using the Akura Plates to third parties;
  - b. for using with human or animal primary pancreatic islets, or islet like cells (e.g., stem cell derived islet like cells);
  - c. for screening or testing of more than 10,000 distinct compounds (high throughput screening);
  - d. in veterinary applications, in diagnostics, *in vivo* use in humans and/or uses related to food products.
4. Use by the End User Subject to Clause 3 above End User will use the Akura Plates solely for *in vitro* research in-house for the discovery and development of compounds outside the Excluded Fields by End User. End User will not sell, transfer, disclose or otherwise provide access to the Akura Plates to any third party or entity. End User will not sell, or transfer cell-based products generated using the Akura Plates to any third party or entity.

## Annex C: Frequently Asked Questions Regarding the Akura™ 96 Spheroid Microplate

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A detailed protocol for production of spheroids in the Akura™ 96 Spheroid Microplate is provided in the product manual. Below are answers to some frequently asked questions to help get you started.

### **Q: What improvements did you make to the new Akura™ 96 Plate?**

A:

#### **Improved optical properties:**

- COP (Cyclo-Olefin Polymer, 92% transparency 400-800 nm) as plate material instead of Polystyrene.
- Thinner well bottom of 0.8 mm, before 1.3 mm.
- Reduced skirt height of 0.4 mm. High NA objectives (e.g., 20X and 40X) may be used to image the outer wells of the plate

#### **Automation friendly:**

- Excellent planarity across plate (below 80 µm) for reliable spheroid transfer and precise medium exchange

#### **Less evaporation:**

- Optimized distance (200 µm) between customized low-evaporation lid and plate reduces evaporation in outer and edge wells

#### **Standard SLAS plate height:**

- 14.35 mm plate height instead of 11.48 mm
- Maximum volume 280 µl instead of 170 µl

### **Q: Why do you recommend pre-wetting of the wells prior to cell seeding?**

A: Pre-wetting the wells of the Akura™ 96 Plate is required prior seeding to prevent inclusion of air-bubbles. For that, apply 40 µl of your cell medium to each well by placing the tips far into the wells. Remove the pre-wetting solution by placing the tip at the ledge of the upper cavity of the well. Aspirate medium until is completely removed from each well. A negligible amount (< 5-7 µl) may remain in the bottom of the chamber.

**Q: Can I create spheroids from any cell type?**

A: Not all cells aggregate to spheroids. First, it highly depends on the architecture, function, and morphology of the tissue/organ of which they are isolated from and how the isolation affected the cells. Further, we see variations in successful aggregation between donors for the same cell type. Plateable cells, in general, have a good chance to form spheroids. To achieve optimal spheroid formation, it may be necessary to modify the aggregation conditions such as modifying the cell concentration or media composition, or by the addition of supporting cell types (e.g., matrix secreting cells) or supplements (e.g., ECM). For new cell types we recommend trying a variety of aggregation conditions.

**Q: Could you recommend a cell concentration for my cell suspension for the generating of spheroids/organoids?**

A: For long-term growth profiling, we recommend starting with low cell numbers (250 – 500 cells per well of 70 µl). If use of non-proliferating cells or rapid production of larger spheroids are required, start with higher numbers (from 2500+ cells per 70 µl). Generally, we recommend trying different concentrations for defining your optimal range when using new cell types.

**Q: What is the optimal volume per well in the Akura™ 96 Spheroid Microplate?**

A: To achieve optimal conditions, gently deliver 70 µl (pipetting speed < 10 µl/sec) of cell suspension into each well of the Akura™ 96 Plate by placing the pipette tips near, but not touching, the bottom of the wells.

Important - For spheroids with uniform size and cell composition it is essential to assure a homogeneous distribution of the cells by gently pipetting up and down prior to seeding into the Akura™ 96 Plate.

**Q: Why do you recommend centrifuging the Akura™ 96 Spheroid Microplate after cell seeding?**

A: We recommend to briefly centrifuge the plate after cell seeding to remove any air bubbles and to force the cells to the bottom of the well to promote cell-aggregation and spheroid formation.

For that, place the lid on the plate and spin in a microtiter-plate centrifuge for 2 minutes at 250 RCF. Afterwards, incubate the plate in a humidified CO<sub>2</sub> incubator at 37 °C for 2-5 days.

**Q: How do I exchange the medium in the Akura™ 96 Spheroid Microplate without disturbing or losing the spheroids?**

A: To prevent spheroid/organoid loss during the exchange of media, the SureXchange™ ledge at the inside wall of each well serves as an anchoring point for the pipette tip. Just place the tip at the ledge of the well, see figure below, and remove the medium at low pipetting speed (>30 µl/sec). A minimal volume of ~5-7 µl will remain in the well.

Then, add 70 µl of fresh medium by placing the pipette tip at the ledge, use dispensing rate <50 µl/sec.

Important - when using automated liquid handling devices, an off-center alignment of the vertical pipette tip will achieve the same effect.

**Q: What is the best way to prevent evaporation in the outer wells of my plates?**

A: Evaporation in the outer (perimeter) rows of wells is a phenomenon common to most low-volume culture platforms, and thus requires careful attention to maintaining proper humidity control. If not controlled, pronounced evaporation can result in concentration or precipitation of media components (serum, salt) that can impact spheroid formation or health, and can alter the effective concentration of a compound/additive in the medium over the course of a long-term experiment. To provide maximum humidity control when using the Akura™ Plates, we recommend the following:

1. Use an incubator with good humidity control (>95% of rel. humidity), and exercise best practice in maintaining and minimizing loss of humidity (e.g., minimize incubator door opening and closing).
2. For culture in the Akura™ 96 Spheroid Microplate, at least 50-70 µl of medium in each well is recommended and can be increased to a maximum of 80 µl if incubator humidity control is a persistent issue. Medium exchange frequency can also be increased to every other day or daily if conditions dictate.
3. We recommend the use of the InSphero Incubox™ (CS-AH11) (Figure 10) to reduce edge effects when performing long-term culture with low-frequency medium exchange. The InSphero Incubox™ is available on [shop.insphero.com](http://shop.insphero.com).



*Figure 10: InSphero Incubox™*

**Q: What do I need to consider when using the plates for imaging?**

A: In order to achieve optimal results, a few relatively simple changes need to be made by a knowledgeable instrument operator. By adhering to the suggestions below, the Akura™ 96 Plate can be used successfully with nearly all high content imaging platforms:

- Due to the tapered well bottom and the 0.8 mm bottom thickness, the creation of a new 96 well plate definition (a.k.a. form factor) may be required for optimal imaging performance (use the specifications provided in our online store as the starting point for the new plate definition).
- The non-continuous well bottoms and 0.8 mm bottom thickness may necessitate the use of an extended autofocus range to ensure accurate focus across the entire plate.
- If image acquisition through the entire Z height of the spheroids is required, the working distance of the selected objective must be equal to the bottom thickness (0.8 mm) plus the Z height of your specimen.
- Objectives with correction collars should be set for a 0.8 mm bottom thickness.

DOMINIQUE DUTSCHER SAS



InSphero AG  
Schlieren, Switzerland

InSphero Inc.  
Brunswick, ME, USA

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