



InSphero Akura™ PLUS Hanging Drop System **Product Manual**



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Introduction

The Akura™ PLUS Hanging Drop System¹ represents the most reliable, versatile, and complete platform for the generation, long-term cultivation, observation and testing of 3D cell culture models in 96 well format.

Each two-plate system consists of one Akura™ PLUS Hanging Drop Plate ("PLUS Plate") and one Akura™ 96 Spheroid Microplate. InSphero uses this system for routine large-scale production of assay-ready microtissues.

Advantages of the Akura™ PLUS Hanging Drop System:

1. Robust hanging-drop spheroid formation using the PLUS Plate
2. Straightforward spheroid transfer to the Akura™ 96 Plate
3. Easy long-term growth, assay and observation in the Akura™ 96 Plate
4. Protocols available for assays and analysis in the Akura™ 96 Plate

¹ The Akura™ PLUS Hanging Drop System, including Akura™ PLUS and Akura™ 96 Spheroid Microplates and related technology, is protected by several granted and pending patents worldwide.

The Akura™ PLUS Hanging Drop System 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'.

Akura™ PLUS Hanging Drop System Components

Akura™ PLUS Plate

The complete Akura™ PLUS Plate assembly consists of the following components (Fig. 1):

1. Bottom plate with reservoir
2. Akura™ PLUS Plate (Frame with 12x8-well strips)
3. Lid
4. Humidifier pad (provided in bags with tweezer)

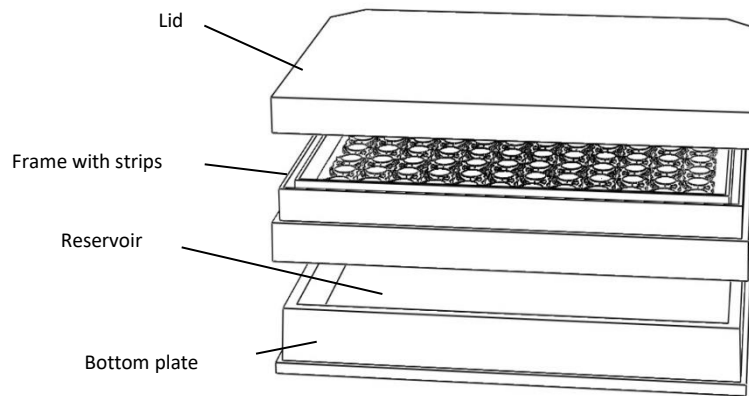


Figure 1: Components of the Akura™ PLUS Plate.

Spheroid production with the Akura™ PLUS Plate is very simple. A cell suspension is delivered from the top through the SureDrop™ inlet funnels of the individual wells of the Akura™ PLUS Plate using a pipette or robotic liquid handler. At the outlets under the plate, hanging drops will form and the cells will form spheroids by gravity-assisted assembly within 2-4 days (Fig. 2).

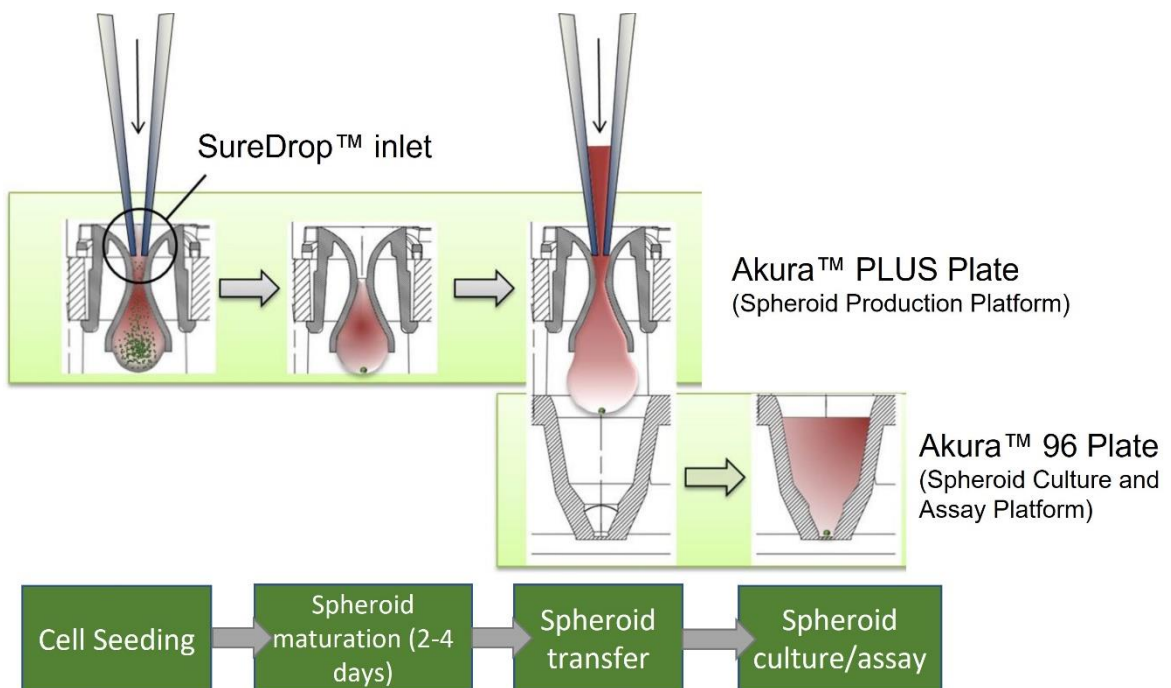


Figure 2: 3D cell culture model formation in the Akura™ PLUS Plate and subsequent transfer to the corresponding non-adhesively coated wells of the Akura™ 96 Plate for further cultivation and downstream applications.

After formation, spheroids are transferred into the Akura™ 96 Plate. This format facilitates long-term maintenance, optical visualization, compound dosing and biochemical assays. If required, dosing and medium exchange can also be performed directly in the Akura™ PLUS Plate - see Annex C.

Akura™ 96 Spheroid Microplate

The Akura™ 96 Plate is a special non-adhesively coated 96-well microtiter plate. It is designed to accommodate 3D cell culture models for convenient long-term cultivation and analysis. Akura™ tapered wells feature a SureXchange™ ledge to prevent inadvertent spheroid aspiration and disruption during medium exchange and compound dosing (Fig. 3).

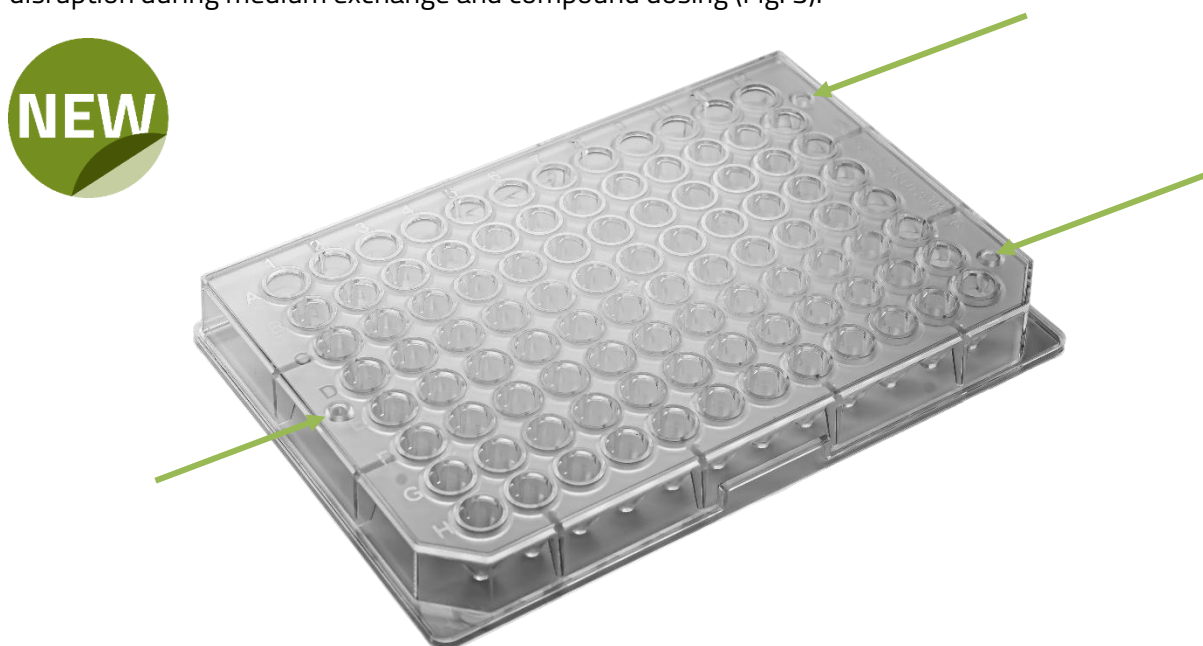
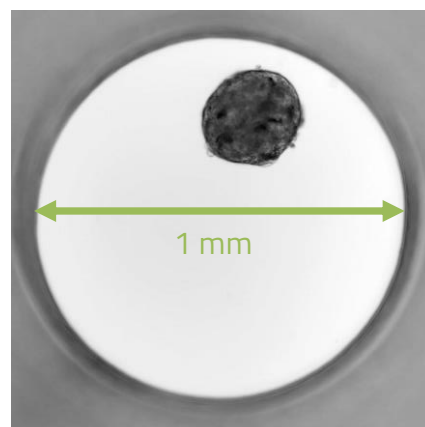


Figure 3: Akura™ 96 Plate – arrows indicating positioning pins for precise transfer of spheroids from the Akura™ PLUS Plate.

Spheroids are centered in a 1.0 mm observation chamber at the bottom of each well, which enables automated imaging processes (Fig. 4). Biochemical assays as well as optical analytical methods such as inverted bright field and fluorescence microscopy can be performed.

Figure 4: Figure 4. 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 Culture Models

Generating 3D cell cultures models is a straightforward process that works with the vast majority of cell types capable of forming spheroids in vivo.

Additional materials required

1. Mammalian cells, either cell lines, stem cells, or primary cells
2. Your cell culture medium of choice
3. Inverted microscope with a 5× objective or a 10× long working distance objective (see also Annex A)
4. Cell counter, e.g., Neubauer chamber
5. 8- or 12-channel pipette (e.g., Viaflo 10.0–300.0 µl, Integra Biosciences. All aspiration and dispensing speeds in this document are based on Integra Viaflo electronic pipettes)
6. Medium reservoir (sterile) for multichannel pipettes
7. For microscopic observation of the full Akura™ PLUS Plate, an additional Akura™ PLUS bottom plate is recommended
8. Microplate centrifuge
9. Humidified 5% CO₂ incubator, 37°C
10. PBS

Preparation

1. Wipe the Akura™ PLUS Plate bag with 70% EtOH before opening.
2. Carefully open the bag under sterile working conditions and take out the Akura™ PLUS Plate assembly.
3. Prepare a reservoir (e.g., a 15 cm diameter petri dish) with 20 ml 0.5x PBS.

4. Open the bag containing humidifier pads. Using the tweezers, remove one humidifier pad and place it in the dedicated reservoir containing the 0.5x PBS.
5. Wait until the humidifier pad is completely soaked with PBS (approx. 5 min).
6. While pad is soaking, open the Akura™ PLUS Plate package and remove the frame (Fig. 1).
7. Place the soaked humidifier pad in the bottom plate (Fig. 1) of the Akura™ PLUS Plate.
8. Prepare a single cell suspension of your preferred cell type or cell mixture according to your standard protocol.
9. Count the cells using the Neubauer chamber or cell counting instrument

Recommended cell concentration: For long-term growth profiling start with low cell numbers (250–500 cells per 40 μ l drop). If non-proliferating cells or rapid production of larger spheroids are required, then start with 2500–25,000 cells/40 μ l.

Hanging-drop formation

IMPORTANT: To generate 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 the seeding into the Akura™ PLUS Plate

1. Gently deliver 40 μ l of cell suspension into each well of the Akura™ PLUS Plate (Fig. 5, left). Ensure a tight contact between the pipette tip and the well inlet by applying a slight pressure to form the SureDrop™ seal (Fig. 5, right).

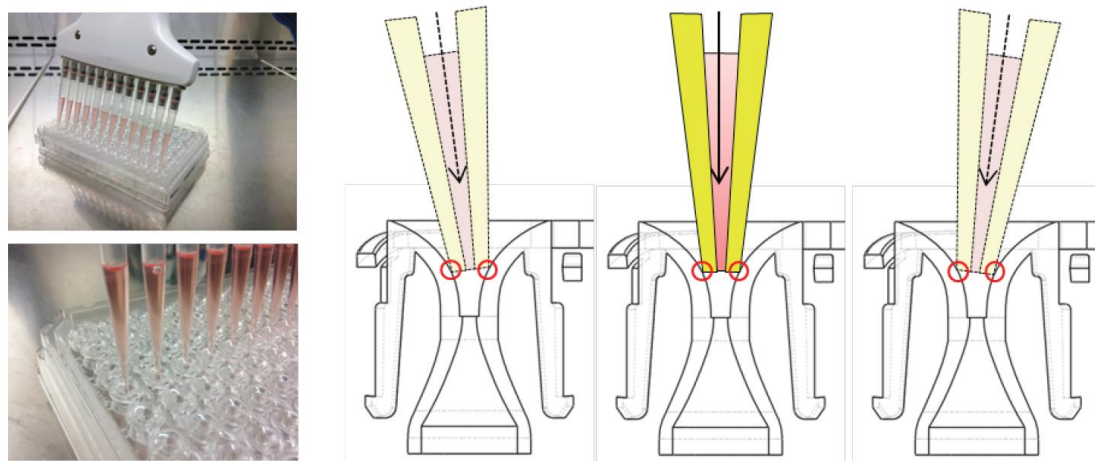


Figure 5: Filling Akura™ PLUS wells. The pipette (8- or 12-channel) is positioned into the inlet of the well in an upright or slightly tilted orientation. It is important that the pipette tips make sufficient contact with the well surface to assure complete liquid transfer and uniform drop formation. The weight of the pipette alone is usually sufficient to provide adequate contact pressure.

2. Place the lid (Fig. 1) on the Akura™ PLUS Plate.
3. Place the Akura™ PLUS Plate assembly in a humidified CO₂ incubator at 37°C.
4. Important – Please handle the PLUS Plate carefully to avoid any loss of drops.
5. Assess spheroid formation regularly. After 4 days in culture most cell types aggregate and form a compact spheroid.

Spheroid formation can be observed by inverted microscopy through the cut-out section of the humidifier pad in the bottom tray (Fig. 6). For detailed instructions about microscopy with the Akura™ PLUS Plate please refer to Annex A.

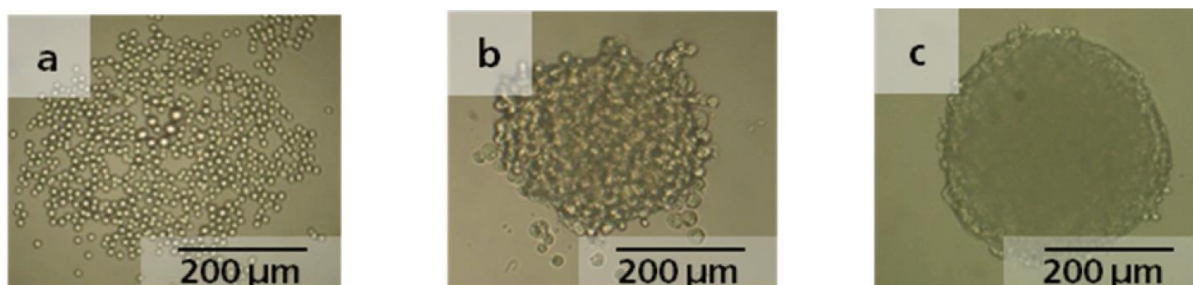


Figure 6: Microscopic images of spheroid formation of 500 HCT-116 cells visualized directly in the drop. (a) cells are located at the meniscus of the drop 30 min after seeding, (b) first cell-to-cell contacts 1 day after seeding, (c) compactly formed spheroid at day 3 after seeding.

Transferring Spheroids

For long-term cultivation and assays, transfer of 3D cell culture models from the Akura™ PLUS Plate to the Akura™ 96 Plate is required.

IMPORTANT

- Pre-wetting the wells of the Akura™ 96 Plate according to the procedure below is recommended prior to transferring spheroids to prevent inclusion of air bubbles and to ensure precise positioning of the spheroid in the center of the well.
- Perform all the following steps under sterile conditions.

Pre-wetting

1. Prior to transfer, pre-warm the medium required for spheroid culture.
2. Wipe the Akura™ 96 Plate bag with 70% EtOH before unwrapping the plate.
3. Open the bag under sterile working conditions and take out the Akura™ 96 Plate.

4. Add 40 μl of cell line media containing FCS or to each well by placing the tip near, but not touching the bottom of the wells. It is recommended to use a multichannel pipette (8- or 12-channel).
5. Gently pipette the medium up and down and remove medium by placing the tip at the ledge of the upper cavity of the well (Fig. 7). Aspirate until medium is completely removed from each well.

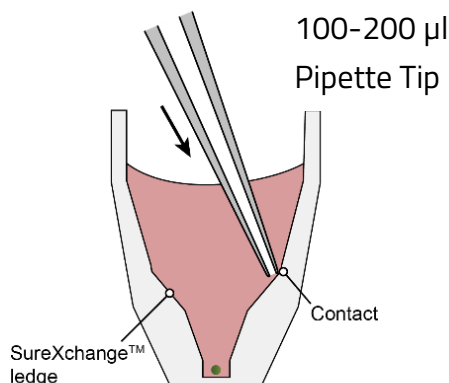


Figure 7: Placing the pipette tip at the SureXchange™ ledge to remove medium from an Akura™ 96 Plate well.

Spheroid transfer

1. Place the frame with stripes (Fig. 1) of the Akura™ PLUS Plate onto the Akura™ 96 Plate by positioning the three pins into the corresponding holes on the top surface of the Akura™ 96 Plate (Fig. 3). The drops under the Akura™ PLUS Plate will then be perfectly aligned with the wells of the Akura™ 96 Plate underneath.
2. Slowly (i.e., $\leq 10 \mu\text{l}/\text{sec}$ when using Integra Viaflo pipettes) add 70 μl of medium through the inlet of the Akura™ PLUS Plate wells. The pipette tips should be in direct contact with the well inlets by simultaneously applying a subtle pressure with the pipette. The drops will fall into the Akura™ 96 Plate (Fig. 8).
3. Verify the transfer by a microscopic inspection of the wells (use an inverted microscope). After transfer of the spheroids, place the Akura™ 96 Plate in a microplate centrifuge and spin for 2 min at 250 RCF. The centrifugation step will force the tissues to the bottom of the well and remove trapped air bubbles.
4. To assure defined medium volumes in the wells, the solution in the wells may be replaced by aspiration and addition of 70 μl of fresh medium. See next section for details on medium exchange.

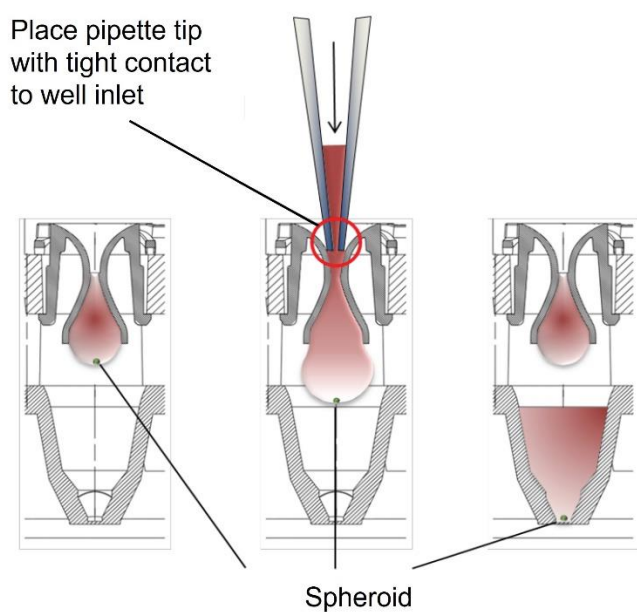


Figure 8: 3D cell culture model transfer from the Akura™ PLUS Plate into the Akura™ 96 Plate.

Medium Exchange in the Akura™ 96 Plate

The unique Akura™ 96 Plate design allows routine medium exchange for long-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. 9, 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 μl medium will remain in the well.
3. Add 70 μl of fresh medium by placing the pipette tip at the ledge (Fig. 9, right). Use a dispensing rate <50 $\mu\text{l}/\text{sec}$ when using Integra Viaflo pipettes.

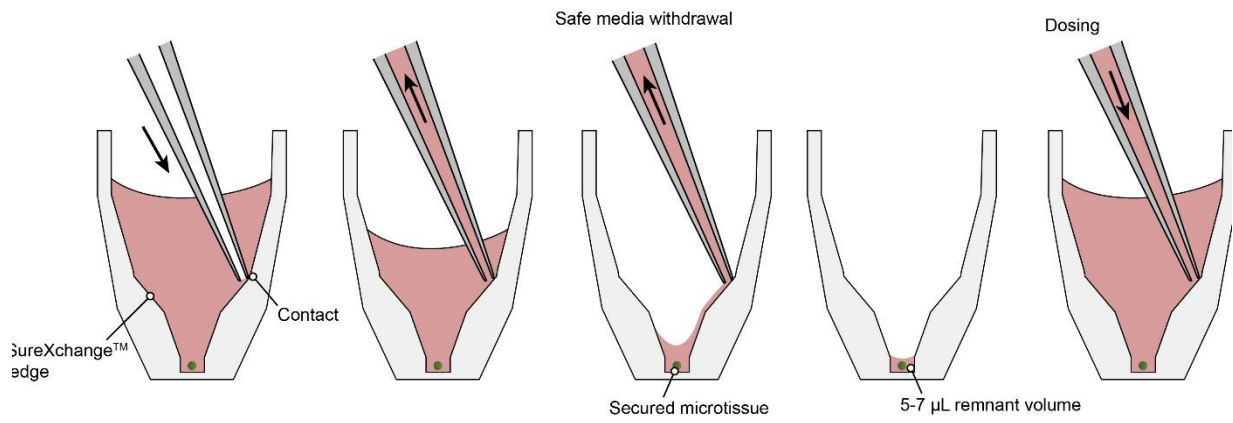


Figure 9: Medium exchange in the Akura™ 96 Plate. Left: Medium removal with the pipette tip placed at the ledge of the well. Right: Medium addition.

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. 9, 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. 9, 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 CO₂ 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 10) for increased productivity.

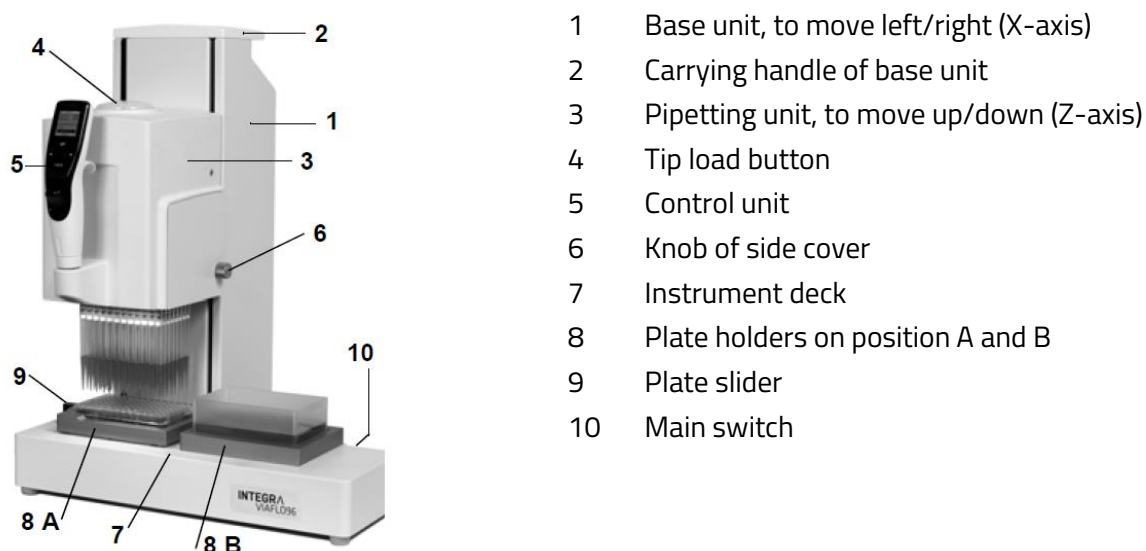


Figure 10. INTEGRA VIAFLO 96/384 device

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 (2nd 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 10 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 ¹ 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	

¹ 4x 70 µl plus 10 µl excess volume remaining in the tip.

Analysis and Assays in Akura™ 96 Plate

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 F 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

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 spheroids, we recommend two different options:

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

1. Before beginning the spheroid collections steps below, prewet the pipette tip with at least 60 μl of 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 μl tip, along the inside wall of the well, until feeling a slight resistance. The pipette tip is now positioned slightly above the spheroids on the well bottom (Figure 11A). Use of 1000 μl tips prevents the spheroids from being squeezed inadvertently because the tip diameter exceeds the size of the well bottom.
3. Alternatively, use a 100–200 μl 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 11B). Note that incorrect positioning of the 100–200 μl pipette may damage the spheroids (Figure 11C).
4. Collect the spheroids by aspirating 50 μl of the medium. Avoid aspiration of air bubbles to prevent spheroid loss in the pipette tip.
5. Transfer the spheroids in medium into another vessel or plate.

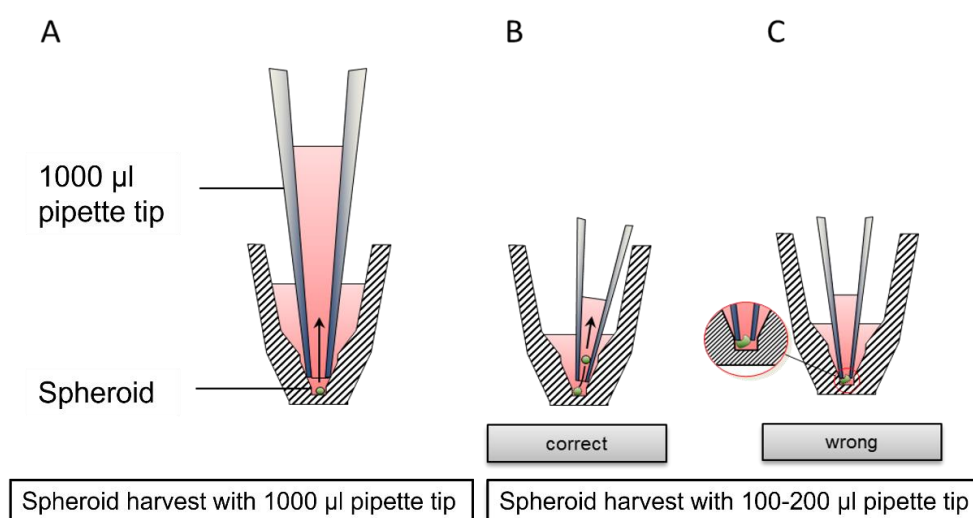


Figure 11: Pipette positioning when collecting spheroids using A. a 1000 μl pipette tip or B. a 200 μl pipette tip. C. The incorrect way to position a 200 μl pipette tip during transfer, causing spheroid damage.

Annex A: Microscopy of Spheroids in Akura™ PLUS Plates

3D cell culture model formation, appearance and growth profiles can be assessed using an inverted brightfield microscope (Fig. 12). A long-working-distance objective (LWDO), preferentially of 10× magnification, is required for proper imaging.

Depending on the minimal gap (D1) between the objective plane and the microscope stage, the specifications of the objective should include a working distance of minimally 11.5 mm+D1. As long-working-distance objectives are commonly shorter in height than regular objectives.

If microscopic assessment of all 96 wells is desired an additional empty bottom plate is required.

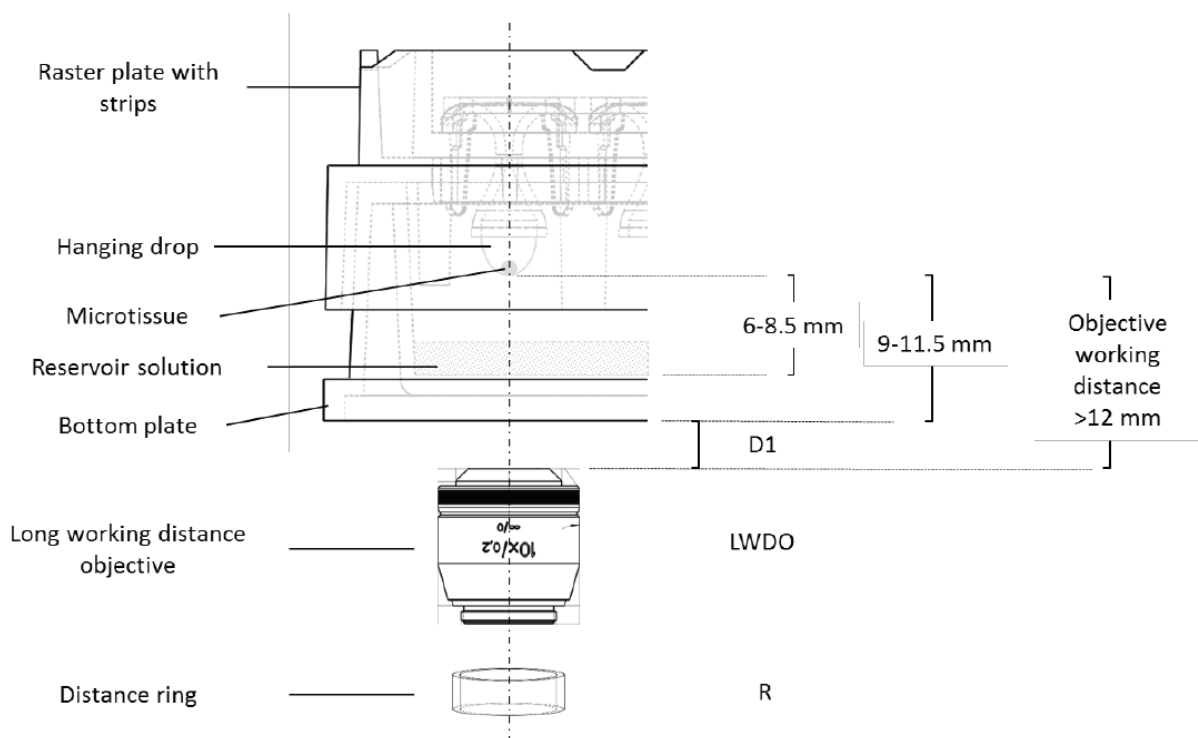


Figure 12: Inspection of spheroids in Akura™ PLUS Plate using an inverted brightfield

Annex B: Preventing Evaporation in Akura™ PLUS Plates

As previously described, a buffer-soaked fleece (humidifier pad) provided with the Akura™ PLUS Plate is placed in the bottom tray to ensure optimal humidity for minimal evaporation from the hanging drops.

For incubators with poor humidity control, hypotonic buffer solutions (e.g., 0.2× PBS) may be applied to the humidifier pad.

For incubators with good humidity control (>95% of rel. humidity), buffers of higher ionic strength may be used (e.g., 0.5–0.75× PBS).

Due to the nature of hanging drops with a high exposed surface/volume ratio, evaporation is a critical factor for long-term cultures in the Akura™ PLUS Plate, with edge wells typically experiencing greater evaporation rates than central wells. InSphero recommends transfer of spheroids to the Akura™ 96 Plate for long-term cultivation, observation, and assays.

Annex C: Dosing and Medium Exchange in Hanging Drops

Compound addition

This following section describes the procedure for compound treatment in the Akura™ PLUS Hanging Drop Plate without the recommended transfer into the Akura™ 96 Plate. A two-step dilution of the compound (e.g., 1000× working concentration in DMSO) first in medium (1:250) and then in the drop (1:4) is exemplified:

1. Ideally use an electronic 8- or 12- channel pipette to aspirate medium to obtain a drop volume of 30 µl. Medium removal should be done at low pipetting speed.
2. Add 10 µl of a 4× concentrated solution of your compound in medium.

NOTE: Depending on the humidity of the incubator some medium may evaporate from the drops. In case of low incubator humidity, it is recommended to study the evaporation rate for your specific incubator (see Annex B).

Addition of assay reagents

For reagent addition it is recommended to add 20 µl of the 2× concentrated reagent to a 20 µl drop. This assures drop stability even when working with detergents. Here an example for a lytic step prior to a biochemical assay:

1. Use an electronic 8- or 12-channel pipette to aspirate medium to obtain a drop volume of 20 µl. Medium removal should be done at low pipetting speed.

2. Add 20 µl of your 2× concentrated reagent and mix with moderate pipetting speed (~20 µl/sec) for reagents that require lysis of the spheroid.
3. Transfer the lysed tissue into the analysis plate by complete aspiration. For non-lytic assays, tissue transfer for further analysis is recommended as described below.

Medium exchange/sampling

Depending on the cell types and initial size of the spheroids the first medium exchange should be performed at 2-6 days after cell seeding. Spheroids are located at the bottom of the drop due to their higher density. This allows for the aspiration of culture medium through the inlet without removing the spheroid, facilitating medium exchange and medium sampling.

1. Ideally an electronic 8- or 12-channel pipette is used to aspirate medium at controlled, low pipetting speed (<10 µl/sec).
2. Up to 50% of the medium volume can be exchanged (e.g., if 40 µl starting volume was used up to 20 µl can be removed).
3. Gently re-apply fresh medium to each well of the Akura™ PLUS Plate by ensuring a tight contact of the pipette tip with the well inlet by applying subtle pressure.

Medium removal and replenishing can be repeated if a more thorough medium exchange is required.

Annex D: Trouble-shooting Guide

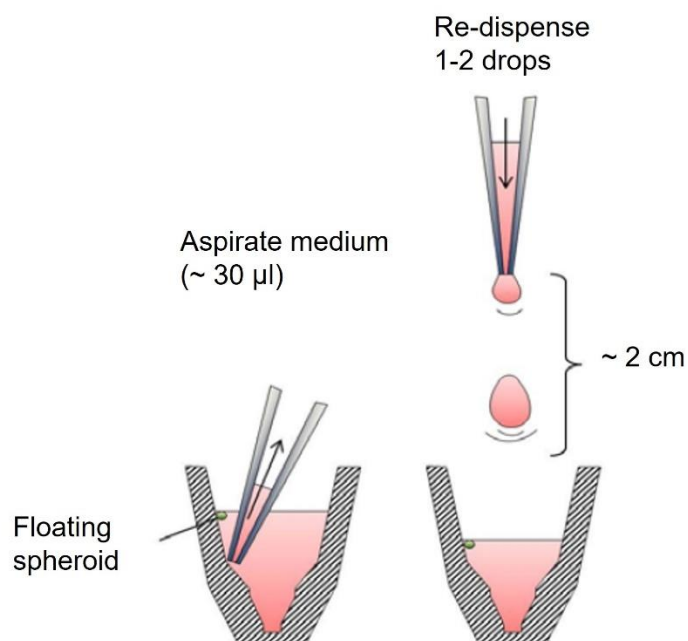
Issue:	Background:	Solution:
Uneven drop volume due to evaporation	Low humidity in incubator needs to be compensated.	Use humidifier pad, add 12 ml of 0.75× PBS. At high evaporation rates, reduce ionic strength of humidifier pad as low as 0.2× PBS.
Transfer rate of spheroids is below 100%	Spheroids not detectable due to air bubbles. Pre-wetting of the wells will prevent inclusion of air bubbles.	Pre-wet Akura™ 96 Plate as described. Transfer spheroids by applying medium from the top at low pipetting speed (<10ul/ sec). (InSphero recommends Integra Viaflo electronic pipettes).
	Spheroid(s) did not settle at the bottom of the well(s).	Centrifuge Akura™ 96 Plate at 250 RCF for 2' to deposit MT at the bottom of the cavity. Alternatively transfer spheroid by aspiration of entire drop volume from the top (MT size should be less than 500 μm in diameter). For recovery of floating spheroids, please consult trouble-shooting guide Appendix E.
	Occasionally certain types of spheroids tend to stay afloat after the transfer into the Akura™ 96 Plate due to hydrophobic interactions with the medium surface.	
Drops fall off	Too low ionic strength of humidifier solution in the bottom tray may result in growing drops, which eventually fall off.	Increase ionic strength of the humidifier pad solution

Annex E: Recovery of Floating Spheroids

Missing Spheroids after the transfer into Akura™ 96 Plates might be related to the fact that spheroids may adhere to the air/liquid boundary due to surface tension interactions. This is a rare observation and only related to certain types of cells. In this case, we recommend the following procedure for recovery of spheroids:

1. In wells where spheroids are missing, remove 30 μ l of medium from the well (Fig. 13, left). Take care not to remove the spheroid floating on top of the medium at the air/liquid boundary.
2. Re-dispense the same medium from above a distance of approx. 2 cm from the well and release 1-2 drops into the well. By this procedure the floating spheroid will be submerged again and will sink to the bottom of the well (Fig. 13, right).
3. Verify the presence of spheroids at the bottom of the wells by an inverted bright-field microscope.

Figure 3: Recovery procedure for floating

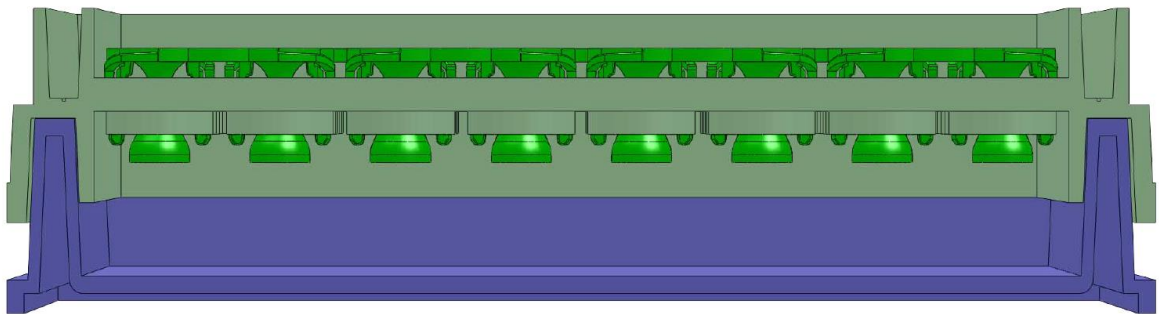


Annex F: Akura™ PLUS Hanging Drop System Specifications

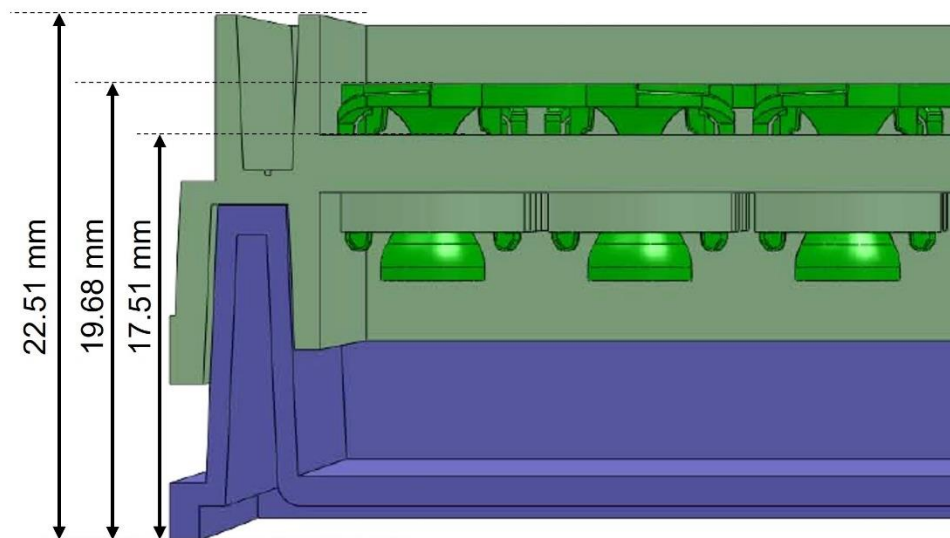
Akura™ PLUS Plate:

The Akura™ PLUS Plate consists of hourglass shaped, clear 96-well. The plates feature a SureDrop™ microtechnology, which allows for precise dispensing into the individual wells of the Akura™ PLUS Plate. At the outlet on the underside of the plate, hanging drops facilitate gravity-assisted formation of spheroids following incubation of seeded cells. The Akura™ PLUS Plate assembly consists of a bottom plate with reservoir, frame with 12x8 well strips, lid and a humidifier pad (Fig. 14).

A.



B.



C.

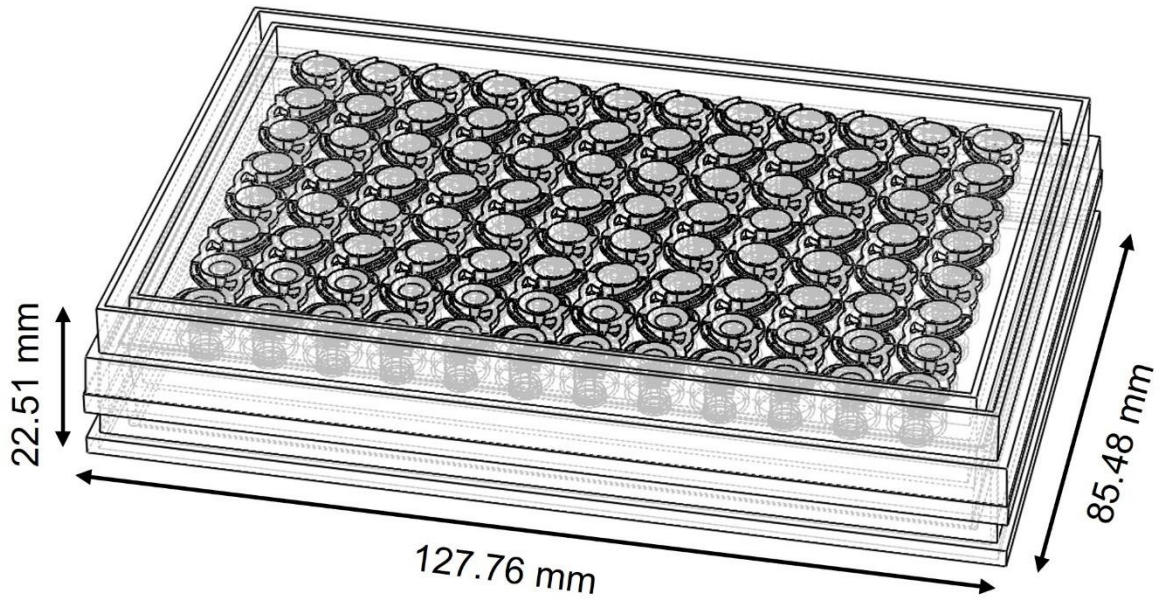


Figure 14: A. Side view of Akura™ PLUS Plate (Frame with 12x8-well strips) attached to bottom plate. B. Dimensions of Akura™ PLUS Plate. C. Angled view to see SureDrop™ technology.

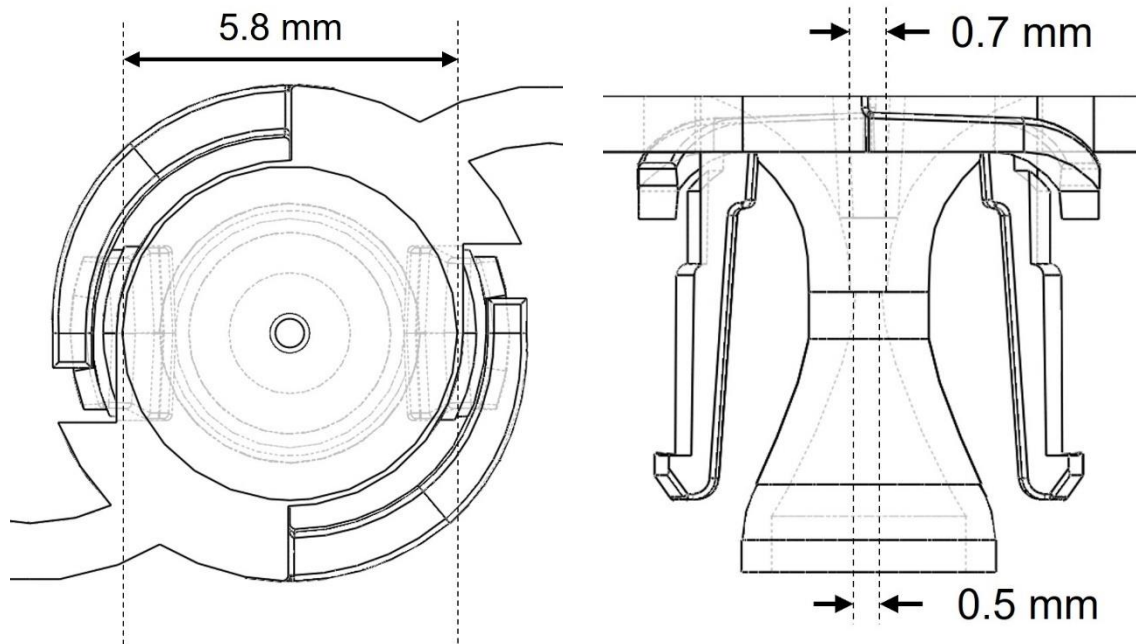


Figure 15: A. Top view well opening. B. Side view of hourglass shaped well.



Akura™ 96 Spheroid Microplate:

The Akura™ 96 plate format is compliant with standard microtiter-plate definitions as specified by the Society for Biomolecular Screening (SBS). The 96 wells are arranged in 8 rows and 12 columns, identified by alphanumeric labels (Figure 16A). 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 16B), designed to accommodate microtissues of up to 1 mm in diameter. Plate technical specifications are provided as a reference for automation system programming (Figure 17,18 and 19).

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:	12.75 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. 19)
Plate and lid material:	COP (Cyclo-olefin-polymer), Polystyrene

A



B

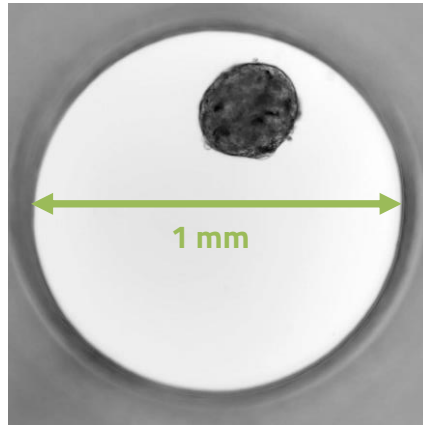


Figure 16: A. Angled view of Akura™ 96 Plate. B. Human liver microtissue in Akura™ 96 Plate. The well diameter is exactly 1 mm.

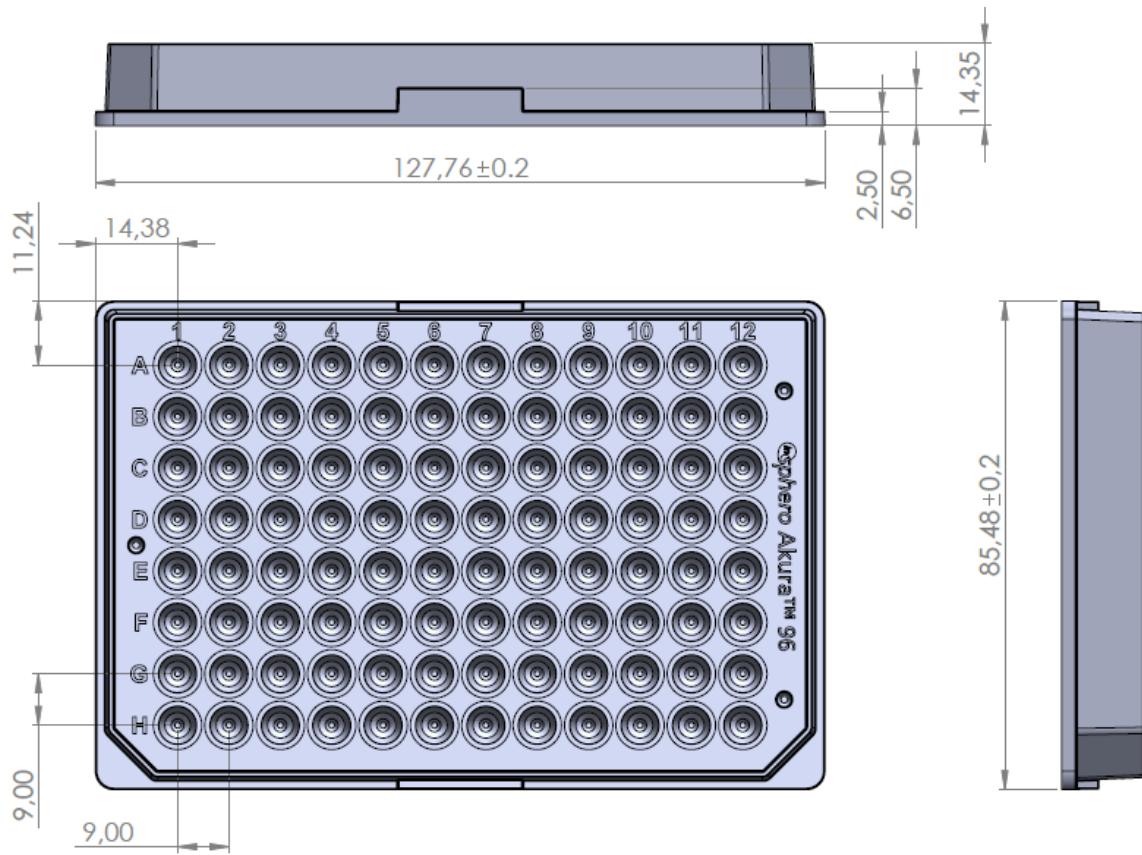


Figure 17: Technical specifications of Akura™ 96 Plate in mm.

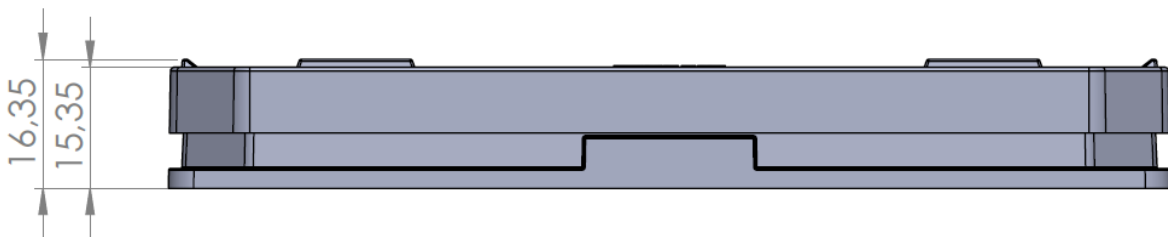


Figure 18: Height of well with lid in mm.

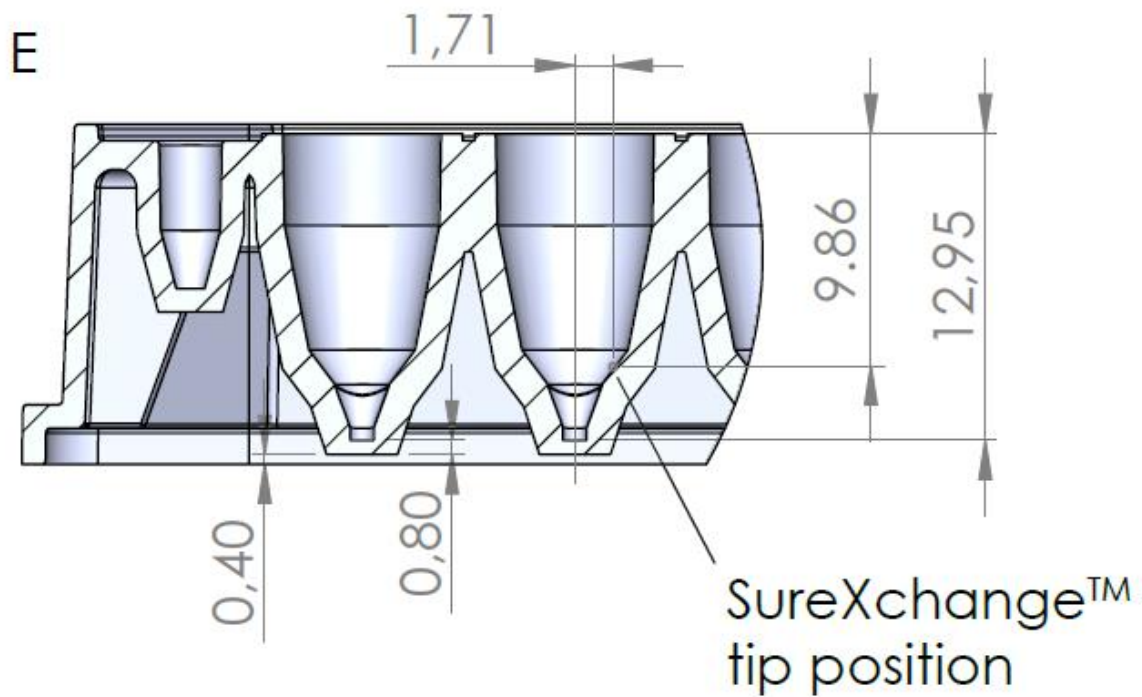
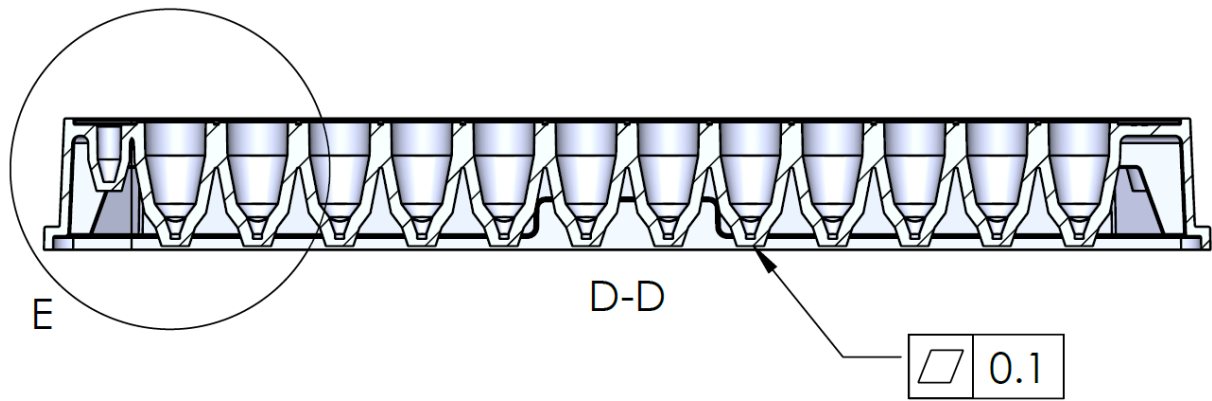


Figure 19: Height of well, skirt height, well bottom thickness and SureXchange™ tip position in mm.

Annex G: Frequently Asked Questions Regarding the Akura™ PLUS Hanging Drop System

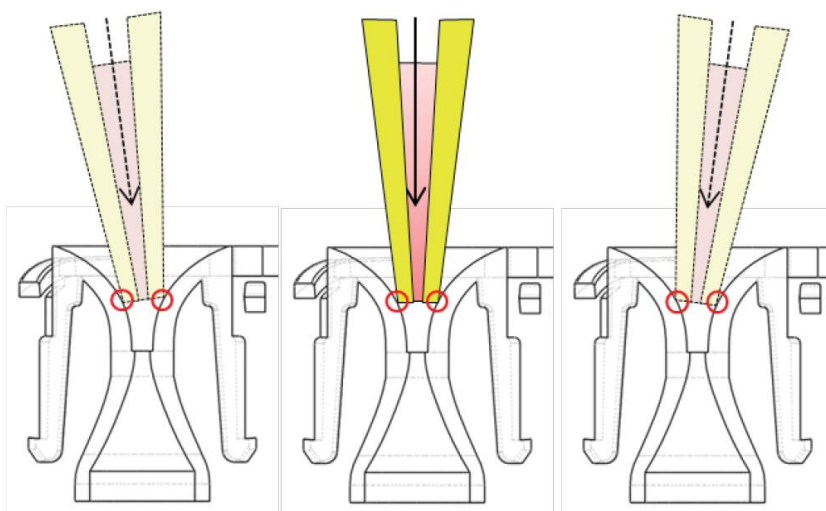
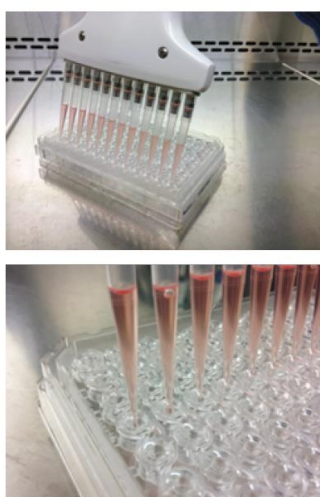
A detailed protocol for production and transfer of spheroids in the Akura™ PLUS Hanging Drop System is provided in the product manual. Below are answers to some frequently asked questions to help get you started.

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

A: For long-term growth profiling, we recommend starting with low cell numbers (250 – 500 cells per drop of 40 µl). If use of non-proliferating cells or rapid production of larger spheroids are required, start with higher numbers (from 2500+ cells per 40 µl). Generally, we recommend trying different concentrations for defining your optimal range when using new cell types.

Q: What is the SureDrop™ inlet?

A: InSphero's Akura™ PLUS Hanging Drop Plate features the patent pending SureDrop™ microtechnology, which allows for precise dispensing into and aspirating from hanging drops. As the drop volume corresponds to the seeding cell number, spheroids produced in Akura™ PLUS Hanging Drop Plates display outstanding size consistency, with variation in diameter of 5% and less across an entire 96-well plate. To assure the SureDrop™ seal, it is important that the pipette tips make sufficient contact with the well surface to assure complete liquid transfer and uniform drop formation. The spring-loaded wells elasticity adjusts to ensure contact is maintained when downward pressure is applied during pipetting.



Q: What is the optimal volume per drop in the Akura™ PLUS Hanging Drop Plate?

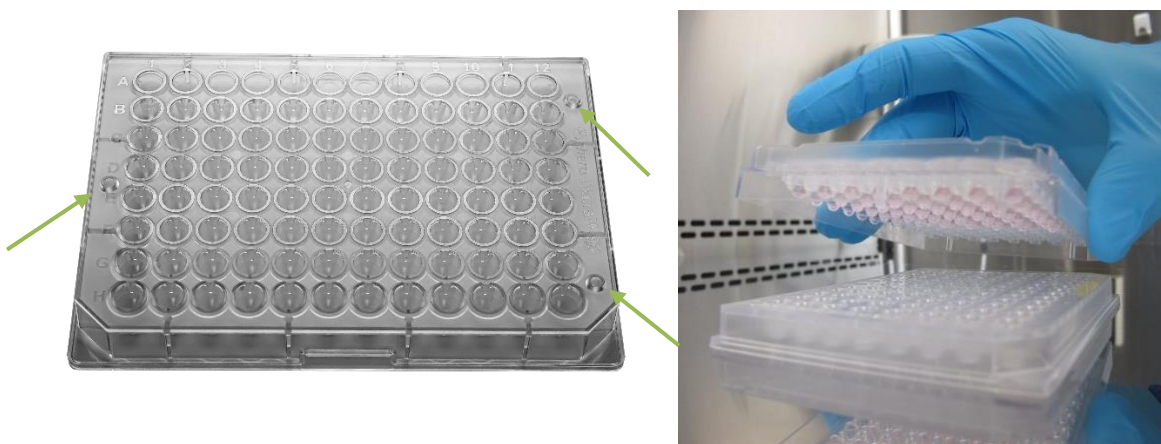
A: To achieve an optimal and stable hanging drop, gently deliver 40 μ l (pipetting speed < 50 μ l/sec) of cell suspension into each well of the Akura™ PLUS Hanging Drop Plate. Important: To generate spheroids that are uniform in size and cell composition it is essential to assure a homogeneous distribution of the cells by gently pipetting up and down prior to seeding into the Akura™ PLUS Hanging Drop Plate.

Q: Why do you recommend pre-wetting Akura™ 96 Plate wells prior to spheroids transfer?

A: Pre-wetting the wells of the Akura™ 96 Plate is required prior to transfer to prevent inclusion of air-bubbles. For that, apply 40 μ l of aggregation 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 μ l) may remain in the bottom of the chamber.

Q: How do you transfer spheroids into the receiver (Akura™ 96) Plate?

A: Place the frame with stripes of the Akura™ PLUS Plate onto the Akura™ 96 Plate by positioning the three pins into the corresponding holes on the top surface of the Akura™ 96 Plate. The drops under the Akura™ PLUS Plate will then be perfectly aligned with the wells of the Akura™ 96 Plate underneath. Using a slow pipetting speed (\leq 10 μ l /sec), add 70 μ l of medium or buffer through the inlet of the Akura™ PLUS Hanging Drop Plate wells. Make sure that the pipette tip forms a tight contact with the well inlets.



1. Merge the Akura™ 96 Plate with the Akura™ PLUS Hanging Drop Plate.
2. Specific pins and pinholes allow for easy connection of the 2 plates.
3. Additional 'pins' provide proper distance of the plates.

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 maintain proper humidity control. Evaporation in the outer rows of hanging drops (Akura™ PLUS Plate) or wells of the Akura™ 96 Plate although infrequent when following the recommendations below, is a possibility. If not controlled, pronounced evaporation can result in concentration or precipitation of media components (e.g., 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™ PLUS Hanging Drop System, we recommend the following:

- 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).
- During microtissue formation in the Akura™ PLUS Hanging Drop Plate, always use the supplied humidifier pad in the base plate, thoroughly soaking it with sterile 0.5x diluted PBS. This is key to maintaining proper humidity in the Akura™ PLUS Plate during microtissue maturation in the hanging drop.
- For incubators with poor humidity control, hypotonic buffer solutions (e.g., 0.2x PBS) may be applied to the humidifier pad.
- For culture in the Akura™ 96 Plate, 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.
- We recommend the use of the InSphero Incubox™ (InSphero CS-00-030-00) (Figure 20) to reduce edge effects when performing long-term studies with low-frequency medium exchanges. The InSphero Incubox™ is available on shop.insphero.com



Figure 20: InSphero Incubox™

Q: Why do my cells not form spheroids?

A: There are several reasons why a particular cell line, cell type, or co-culture may not re-aggregate and form a spheroid. Pre-treatment/harvesting conditions, cell seeding density (or ratio in co-culture models), media formulation, incubation time, media evaporation, and many other factors can impact the success or failure of microtissue production and culture.

Many tumor cell lines are known to readily form microtissues in ultra-low attachment (ULA) treated plates, and we recommend our Akura™ 96 Plates CS-09-001-03 as a starting point if the literature suggests this is the case for your cell line of interest, or if you are unsure.

For primary cells, more complex co-cultures, or cell lines that fail to form spheroids in ULA plates, we recommend the Akura™ PLUS Hanging Drop System (CS-06-001-02). Some cells may require co-culture with fibroblasts or supplemental growth factors in order to form dense, spheroids, or to establish and maintain an organotypic phenotype.

Q: What is the best way to image spheroids in the hanging drop?

A: Spheroid formation, appearance and growth profiles can be assessed using an inverted brightfield microscope. A long-working-distance objective (LWDO), preferentially of 10x magnification, is required for proper imaging. Depending on the minimal gap (D1) between the objective plane and the microscope stage, the specifications of the objective should include a working distance of minimally $11.5 \text{ mm} + D1$. The included humidifier pad has a rectangular opening in the center to allow easy visualization of hanging drops in the most central wells. If microscopic assessment of all 96 wells is desired, simply transfer the Hanging Drop Plate to an empty, sterile base plate for imaging, then return to the humidified base plate prior to returning to the incubator.

Annex H: License Agreement

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.

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.



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