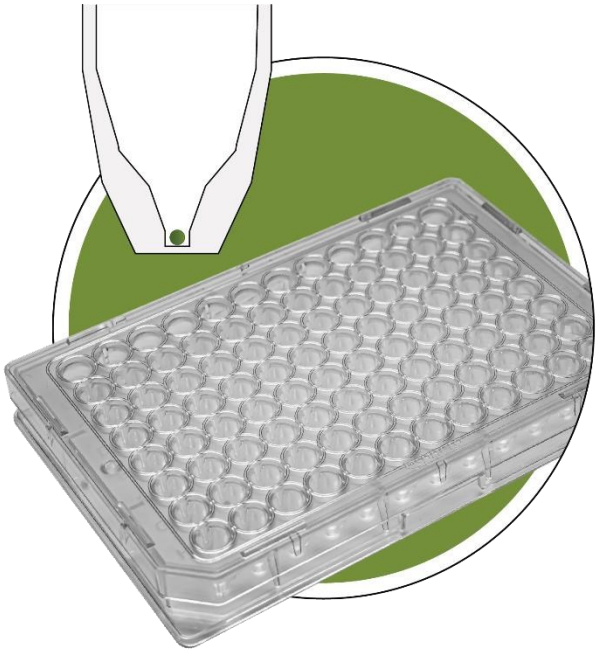
ARCTis™ Human Tumor
Product Manual



PM002, July 2024

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Introduction

ARCTis™ – Always Ready Cryo Tissues

InSphero's ARCTis™ Human Tumor plates represent a novel concept that enable fast and reliable use of 3D cultured microtissues for adoption in drug discovery programs. They are designed to support your testing strategy and help by being conveniently ready whenever you need them.
This convenience together with a continuously growing range of available cell lines gives you flexibility in study design. This also allows for re-testing therapeutic modalities to find out if they can be repurposed for novel indications. The Akura™ 96 Spheroid multi-well plate technology is the foundation on which our ARCTis™ products are designed. These microtiter plates are strictly designed with SLAS standards in mind and are completely automation compatible. Each plate is made of COP (Cyclo-olefin-polymer) and consists of a special ultra-low attachment (ULA) well and a low-evaporation lid. COP has exceptional optical properties that are comparable to glass, for example regarding its transparency, which makes these plates the ideal choice for imaging-based studies.
Like all other spheroids from InSphero the ARCTis™ Human Tumor 3D cell models are scaffold-free and allow for long-term cultivation, observation and testing in 96-well format. These 3D cell models are comprised of immortalized or modified cell lines as well as primary cells, either as a monoculture or co-culture. The comprehensive description for each 3D cell model together with their growth and culture characteristics (e.g., growth rate) may be found in their respective Specification Sheets. Please note that 3D cell model types are often referred to as spheroids or microtissues. For the remainder of the document, we term them as 'spheroids'.

Advantages of ARCTis™ Human Tumor

1. Zero development time & costs – ARCTis™ Human Tumor microplates are optimized for reliable formation, uniform size and cell composition, and growth window. From freezer to assay-ready 3D tumor spheroid in 3-5 days, depending on cell line, giving your team a head start generating valuable data for your project.
2. Broad range of cell lines - We are building the largest 3D CryoTumor bank in the world. (monoculture and co-culture. This ever-expanding cache of tumor models allows you to generate bigger, better, and more predictive data sets that capture the effects of tumor heterogeneity.
3. Convenient scaffold-free formation of spheroids below 300 µm via cellular self-assembly in ultra-low attachment (ULA-treated) plates.
4. SureXchange™ tapered ledge and culture chamber facilitates easy medium exchange and prevents spheroid loss during long-term spheroid growth and analysis. The 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.

ARCTis™ Starter Pack

The ARCTis™ Starter Pack contains all the necessary components for a successful and comprehensive experience with the ARCTis™ technology platform. It is designed for both newcomers to 3D cell culture and experienced DIY-style spheroid generators. Our goal is to provide a reproducible platform for testing hypotheses and generating trustworthy data.

All elements in the ARCTis™ Starter Pack were carefully selected to ensure the spheroids can be used reliably for testing. A wide range of endpoints can be chosen like size of a spheroids or its proliferation.

The ARCTis™ Starter Pack elements are as follows:

- 6 x ARCTis™ plates – 2 plates for each of three different tumor cell lines: HCT116, A549 and T-47D
- 6 x 30ml Aggregation Medium
- 4 x 125 ml Tumor Maintenance Medium
- 1 x extra Akura™ 96 Spheroid Microplate
- 1 x Tilting Stand
- Technical documentation as hard copy and as PDF file

It can be ordered in our web shop or through our sales representatives with the catalog number: AK-NB00-0T0-001.

ARCTis™ Human Tumor Plate

The ARCTis™ Human Tumor multiwell plates are based on the successful Akura™ 96 Spheroid Microplate (from here on in this document coined Akura™ plate). The ARCTis™ plates contain cryopreserved tumor models that allows for phenotypic observation over a period of several days. The length of the observation window depends on the doubling time of the cells and thus is unique for each cell line. Typically, it ranges from 9 to 11 days.

All 96 wells contain 20 µl cell suspension with 500-2000 cells cryopreserved in cryopreservation agent. The cells' doubling time dictates the number of cells needed for forming spheroids of approximately 200 µm in diameter. We have optimized this for your convenience and use elaborate production protocols to ensure they give you comparable start points.

We deliver each ARCTis™ plate with 30 ml Aggregation Medium that is used for thawing the cells in their wells. Please note that you do not need any other microplates. Everything from thawing to measuring

optical endpoints is performed in the same ARCTis™ plate unless one wants to extract the spheroids for histological analysis outside the wells. The ARCTis™ plate characteristics are:

- Based on Akura™ 96 Spheroid Microplate
- All 96 wells contain enough cells to form a functional spheroid of ~ 200 µm diameter.
- Aggregation Medium is designed for thawing and post-cryopreservation recovery.

ARCTis™ Human Tumor plates can be ordered in our web shop or through our sales representatives with the catalog number: AT-NB00-0T0-001 + Cell line name (e.g., AT-NB00-0T0-001 HCT116 for the cell line HCT116). They are shipped on dry ice with the non-frozen Aggregation medium in a separate compartment.

To ensure the cell lines are as identified, we use vials directly delivered to us from ATCC. Our production is tested for viruses and mycoplasma. Furthermore, we use certified laboratories for verifying their identity using STR. The results can be found in the certificate of compliance for each production run.

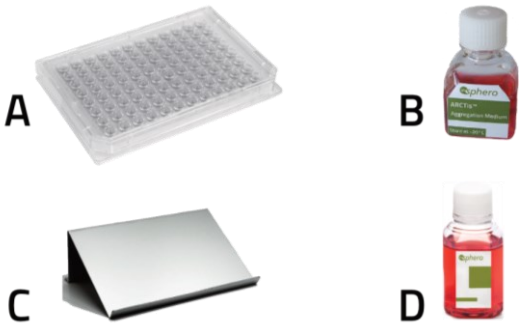
ARCTis™ 3D Cell Models – Handling Protocol

Revitalizing the cryopreserved 3D cell models in the ARCTis™ plate is a straightforward process. In general, cryopreservation is stressful for any cell type or tissue. Therefore, the thawing procedure as well as the recovery time directly after it are important. We have optimized this process to ensure high product quality and reliability.

ARCTis™ Human Tuman Platform

A – ARCTis™ Plate (AT-NB00-0T0-001)
B – Aggregation Medium (CM-0T-99-00-T)
C – Tilting Stand (CS-AG11)
D – Tumor Maintenance Medium (CM-TT-00-00-C) &

1 extra Akura™ 96 Spheroid Microplate (CS-PB11)



The total work effort required is 30 minutes. It is possible to prepare multiple plates in parallel, depending on the available infrastructure. To ensure consistency, use InSphero's Aggregation Medium (B) at 37°C for all steps. Use the extra Akura™ 96 Microplate for training and as counterbalance in the centrifuge.

Additional Materials Required

- Box with dry ice or cooling elements at -80°C
- Inverted microscope with a 5x/10x objective
- Automated multichannel pipette* (e.g. 8- or 12-channel pipette, Viaflo, IntegraBiosciences)
- Medium reservoir for multichannel pipettes
- Microplate compatible centrifuge with swing bucket (horizontal or vertical)

- Humidified Incubator at 37°C with 5% CO_2
- Cell culture hood
- Timer

  * the use of manual pipettes is possible too

General preparation

- Check the availability and integrity of the components in the Starter Kit
- Thaw Aggregation & Tumor Maintenance Medium at 4°C over night, the day before the experiment
- Check on availability of components listed in additional required materials

Preparation prior to thawing ARCTis™ plates

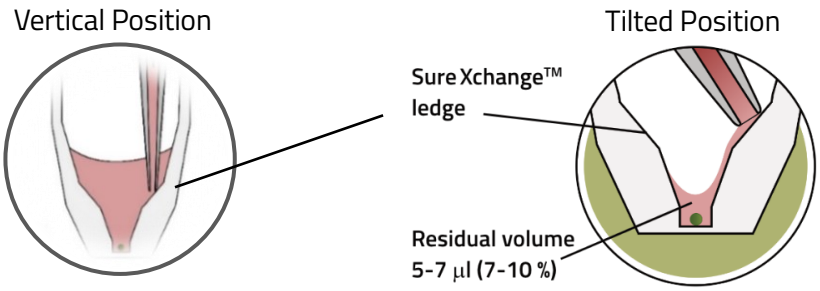
Note! It is recommended to thaw medium bottles 1 day prior to their use by transferring them from their -20 °C storage to a 2° – 8 ° C refrigerator and let them thaw slowly over night.

- Prior to thawing ARCTis™ plates, pre-warm the Aggregation medium to 37 °C that accompanies each plate
- Prewarm the Tilting Stand (C) in the incubator (37°C during the cell culture hood preparation
- Prepare a transport box with dry ice and move the frozen ARCTis™ plate from the -80°C storage to the lab in a box to ensure it stays frozen during the following preparations.
- If using electric pipettes (such as INTEGRA Viaflow), please check the set flow rates and adjust if needed.
- Prepare centrifugation counterbalance plate in case a single ARCTis™ plate is processed
    - Remove bag in biosafety cabinet
    - Add 100 µl of sterile PBS to each of the well using a multichannel pipette

Thawing (adhere to time sensitive process)

1. Wipe the ARCTis™ plate bag with 70% EtOH before and open it inside your biosafety cabinet.
2. Unpack ARCTis™ plate (A), keep the lid on and immediately place it in the incubator (37 °C, 5% CO_2) on the Tilting Stand (C) and let it thaw in there for 7 min.
3. Transfer only the ARCTis™ plate to the cell culture hood.
4. Remove lid and dispense medium volume stepwise to all wells at 1min intervals according to the steps below, start the timer (1min) and:
    4.1 Dispense 20 µl of medium at normal speed in the vertical position, wait until the 1 min incubation interval is completed
    4.2 Add 20 µl of medium at normal speed in the vertical position, complete the 1 min incubation interval
    4.3 Add 60 µl of medium at normal speed in the vertical position, complete the 1 min incubation interval
    4.4 Add 80 µl of medium at normal speed in the vertical position, complete the 1 min incubation interval

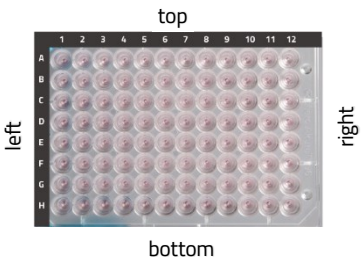
NOTE! For adding medium hold the pipette in vertical position for optimal wash (see illustration)



NOTE! Total volume per well reaches 200 µl which is towards maximal well capacity. Ensure the lid does not touch the inner plate wells.

5. Centrifuge the plate at 250 rcf for 2 min (plate positioning below) plate positioning in centrifuge:

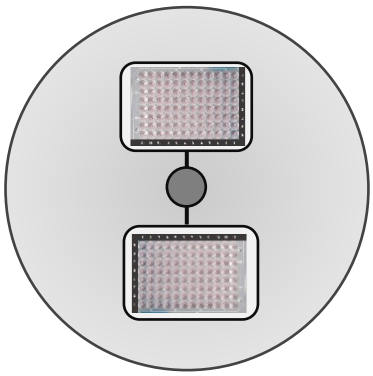
Plate orientation:



Centrifuge types:

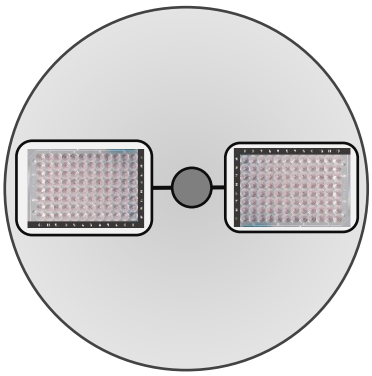
Horizontal buckets:

- The plate's bottom is orientated towards the outside



Vertical buckets:

- The plate's right side is orientated towards the outside

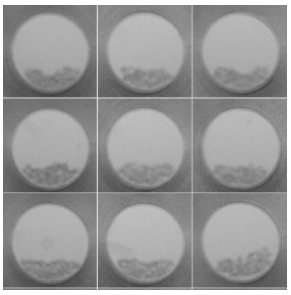


6. Transfer the plate back to the cell culture hood
7. Gently aspirate supernatant with slow speed with the pipette tip in contact with the SureXchange™ ledge, tilted position (see illustration), 5 µl medium remains in each well
8. Dispense 70 µl medium in each well at normal speed using vertical tip positioning (pellet wash)
9. Re- Centrifuge the plate according to plate positing above (horizontal and vertical centrifuge) at 250 rcf for 2 min
10. Check correct cell pellet allocation in edge of the well (see illustration). In case of deviation move to trouble shouting section.

In Process Control: Check correct cell pellet allocation in edge of the well (see illustration)

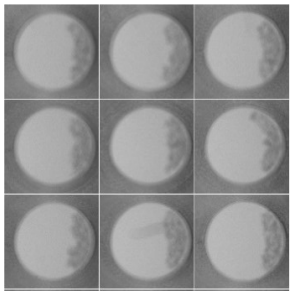
Horizontal buckets:

Expected cell pellets location at bottom of well



Vertical buckets:

Expected cell pellets' position to right side of well

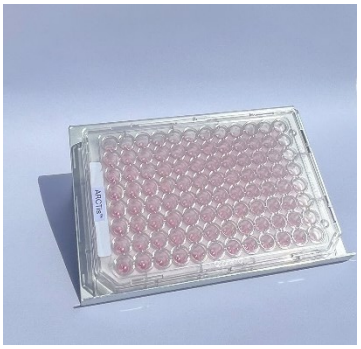


Note! In case cells allocate disperse throughout the well apply trouble shoot step 1

11. Transfer ARCTis™ plate back to incubator and place on Tilting Stand as shown below.

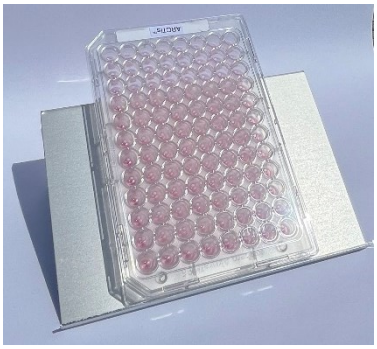
Horizontal buckets:

Plate bottom-side facing down



Vertical buckets:

Plate with right-side facing down



NOTE! spheroids formation will take place over a time course from 72 to 96 h (dependent on cell model). You can stack up to three plates on the Tilting Stand if required.

12. Depending on the cell line the tissue formation is completed within 3-5 days and assays can be initiated. Please refer to the exact time given in the specification sheet for each cell line.

Pipetting Speeds:

Slow speed = 10-20 µl/s (e.g. Speed 1 for INTEGRA Viaflow with 300 µl pipette tip)
Normal speed = 80- 90 µl/s (e.g. Speed 5 for INTEGRA Viaflow with 300 µl pipette tip)

Trouble Shooting Step 1

In case the cells appear disperse in the well and do not pellet, move on with the following trouble shoot step.

1. gently resuspend the cells at slow speed holding the pipette in vertical position.
2. Re-centrifuge the plate according to the process described above horizontal and vertical centrifuge) at 250 rcf for 2 min.
3. Check correct cell pellet allocation in edge of the well as above
4. Repeat in case cells stay disperse in the wells OR move to step 10 in the process

Medium Exchange Day 3 (and subsequent)

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. Spheroids are positioned in a 1.0 mm observation chamber at the bottom of each well, which enables automated imaging processes. Biochemical assays as well as optical analytical methods such as inverted bright field and fluorescence microscopy can be performed.
The medium exchange is performed like this:

1. Place the pipette tip at the ledge of the well (tilted position, Figure 1).
2. Remove the medium at low pipetting speed (<30 µl/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. 1, right). Use a dispensing rate <50 µl/sec.

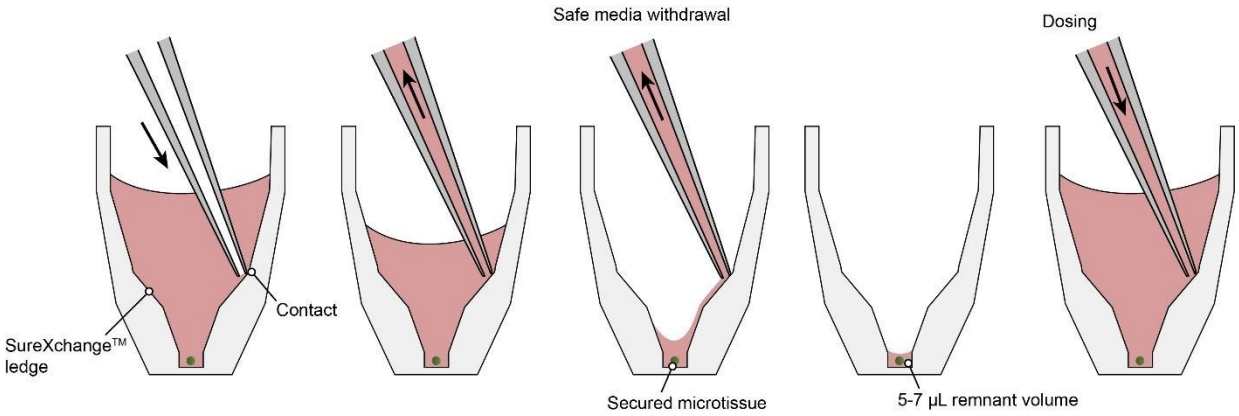


Figure 1: Medium exchange in the Akura™ 96 Plate. Left: Medium removal with the pipette tip placed at the ledge of the well. Right: Medium addition.

Analysis and Assays

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

Changes in spheroids' sizes as well as levels of expressions of 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. 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 not yet configured for Akura™ 96 Plates. This could be resolved by future firmware updates.

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 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 µl 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 spheroid on the well bottom (Figure 5A). Use of 1000 µl 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 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 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.

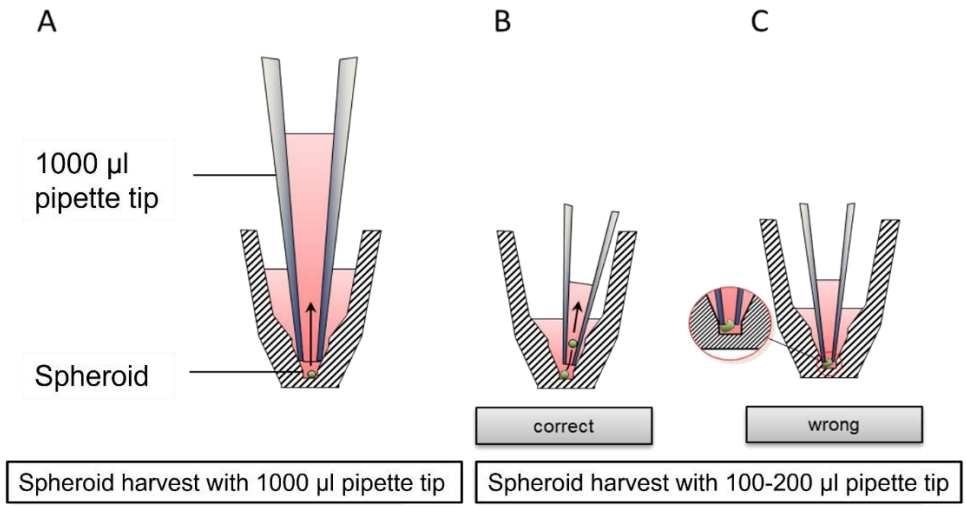


Figure 5: Pipette positioning when collecting spheroids using A. a 1000 µl pipette tip or B. a 200 µl pipette tip. C. The incorrect way to position a 200 µl pipette tip during transfer, causing spheroid damage.

Annex A: ARCTis™ Portfolio of Human Tumor Models

February 2024

The list of available cell lines is continuously growing. For the latest update please check our website.

Cell Line	Tissue
HMC3	Brain
U-87 MG	Brain
T47D	Breast
DLD-1	Colon
HCT116	Colon
HCT15	Colon
HT-1080	Connective tissue
NCI-N87	Stomach
A498	Kidney
A549	Lung
Panc-1	Pancreas
A375	Skin
HepG2	Liver
HeLa	Cervix

Annex B: 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:	12.75 mm
Skirt height:	0.4 mm
Diameter well opening:	6.70 mm
Diamter 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

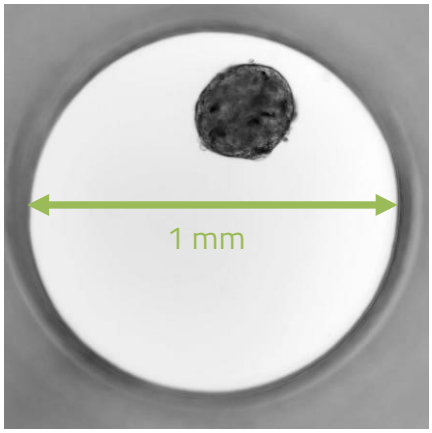


Figure 6: A. Angled view of Akura™ 96 Plate. B. Human liver microtissue in Akura™ 96 Plate. The well diameter is exactly 1 mm.

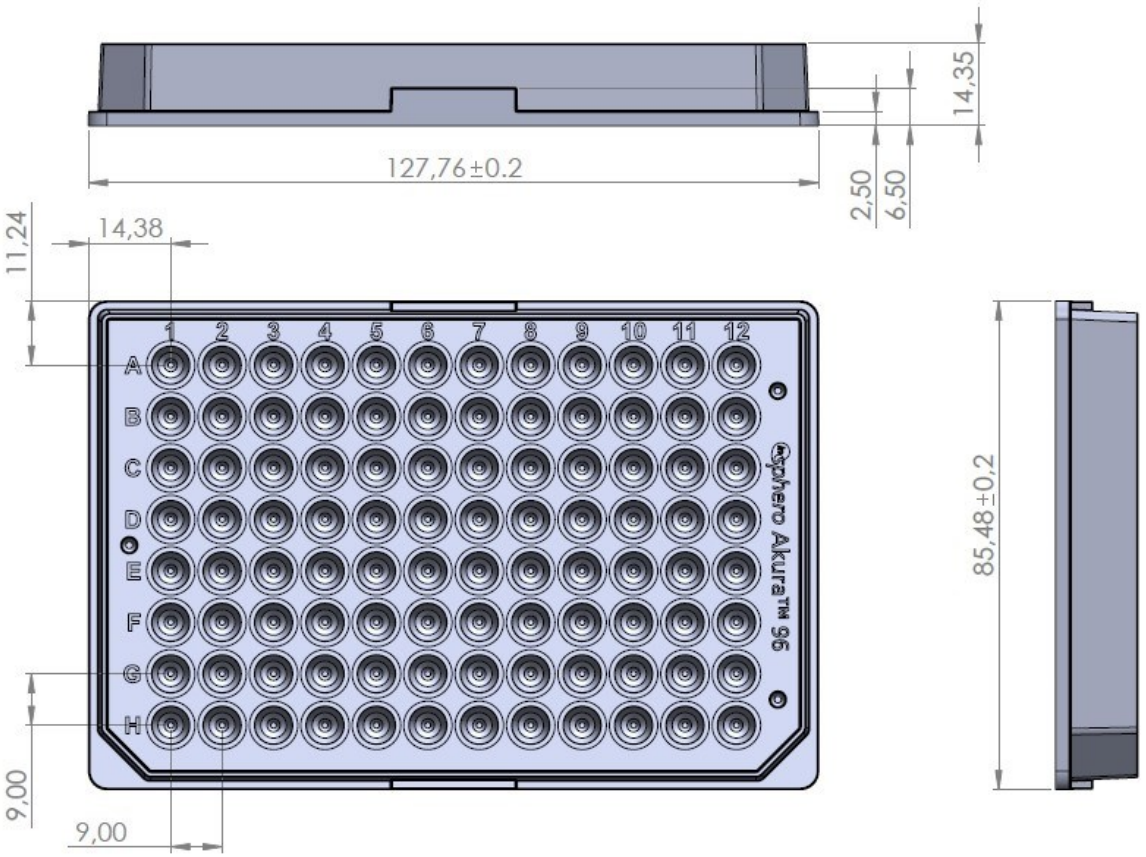


Figure 7: Technical specifications of Akura™ 96 Plate in mm.

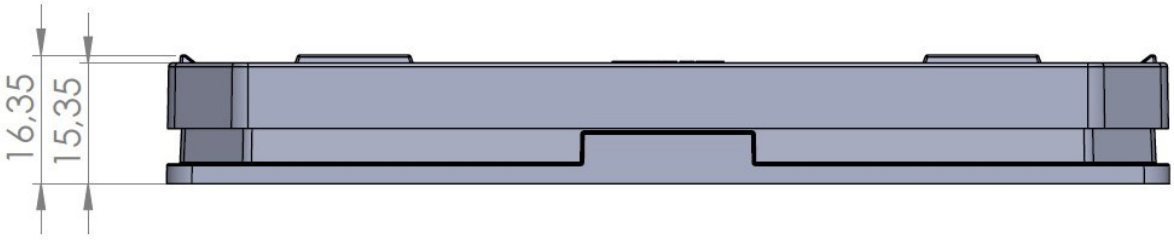
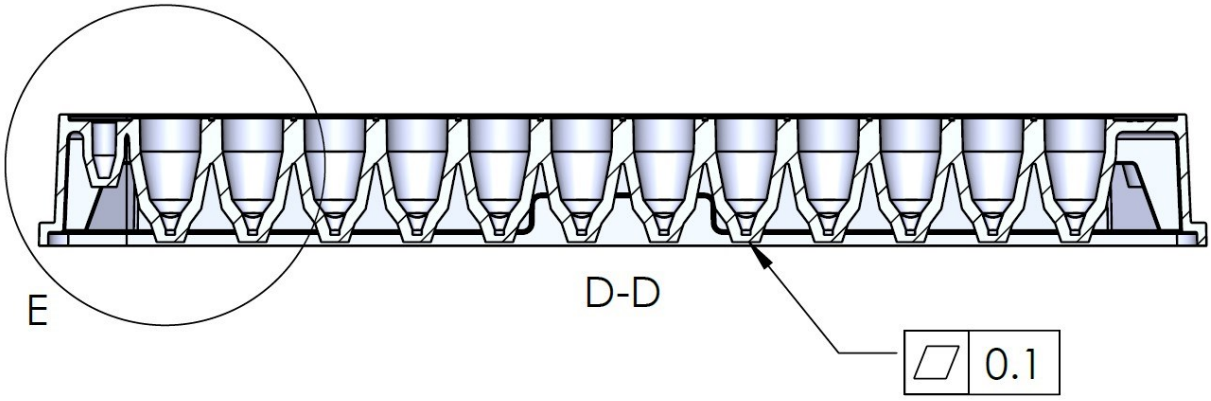


Figure 8: Height of well with lid in mm.



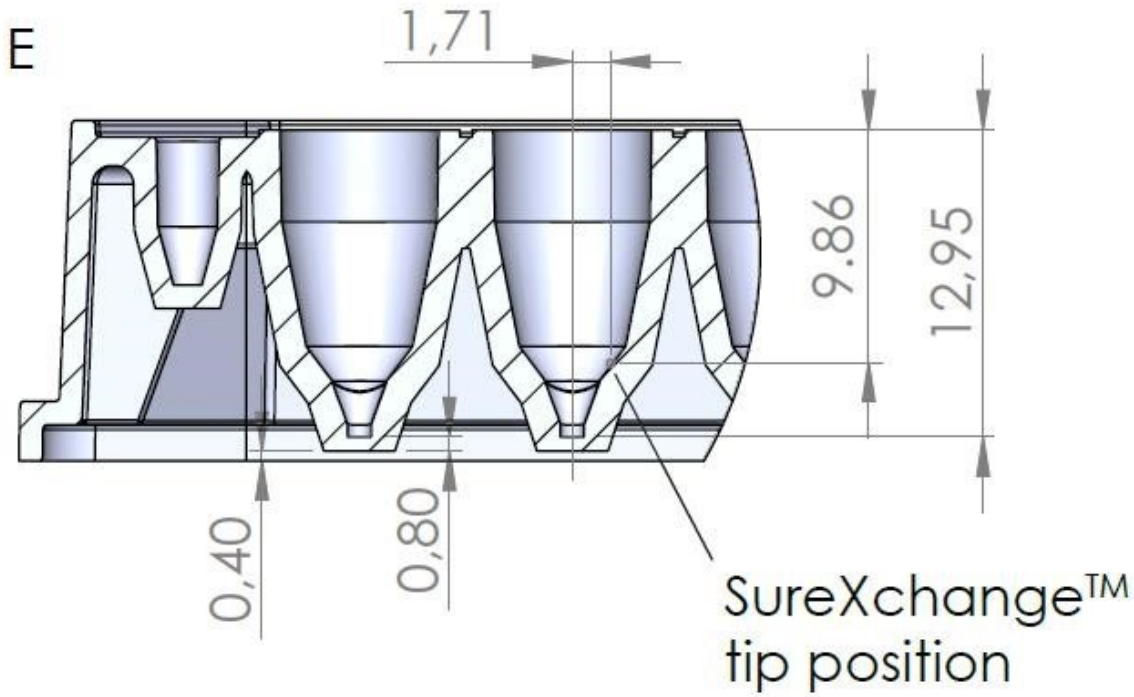


Figure 9: Height of well, skirt height, well bottom thickness and SureXchange™ tip position in mm.

Annex C: 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. 1, 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. 1, 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_2 incubator.

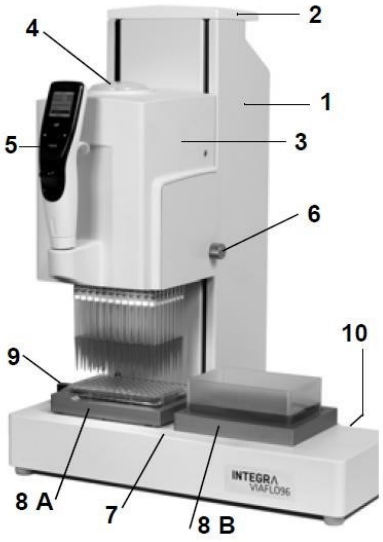
Table 1

Recommendations for culturing ARCTis™ Human Tumor Plates

Material/Process	Recommendation
Culture medium	Tumor Maintenance medium (CS-07-112-03)
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 (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 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[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.

Annex D: 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.

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 E: Frequently Asked Questions Regarding the Akura™ 96 Spheroid Microplate

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: 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 medium into each well of the Akura™ 96 Plate by placing the pipette tips on the SureXchange™ ledge not touching, the bottom of the wells.

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-10-001-00) (Figure 2) to reduce edge effects when performing long-term culture with low-frequency medium exchange. The InSphero Incubox™ is available on shop.insphero.com.

Figure 2: 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 set for a 0.8 mm bottom thickness.

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