ARCTis™ Oncology

Product Manual

PM001, June 2023

Contents

Introduction

ARCTis™ – **A**lways **R**eady **C**ryo **T**issues

InSphero's ARCTis™ Oncology plates represent a novel concept that enables a 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™ Oncology 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™ Oncology

Zero development time & costs - ARCTis™ CryoTumors 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 , giving your team a head start generating valuable data for your project.

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.

Convenient scaffold-free formation of spheroids below 500 µm via cellular self-assembly in ultra-low attachment (ULA-treated) plates.

Zero spheroid loss during medium exchange - 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 includes all the components needed for a successful and comprehensive experience with the ARCTis™ technology platform. It is designed both for newcomers to 3D cell culture and experienced DIY-style spheroid generators alike. Our intent is to allow you a reproducible platform to test your hypotheses and to trust the data generated.

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 ARTCis™ Starter Pack elements are as follows:

- 6 x ARCTis™ plates – 2 plates for each of three different tumor cell lines: HCT116 (AT-NB00-0T0-001 HCT116), A549 (AT-NB00-0T0-001 A549) and T-47D (AT-NB00-0T0-001 T-47D)
- 6 x 30 ml Tumor Reaggregation Medium (CS-07-112-04)
- 4 x 125 ml Tumor Maintenance Medium (CS-07-112-03)
- 1 x Tilting stand (CS-AG11)
- Technical documentation as hard copy and as PDF file (USB drive)

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

ARCTis™ Oncology Plate

The ARCTis™ Oncology 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
- 96/96 wells contain enough cells to form a functional spheroid of ~ 200 µm diameter.
- Aggregation medium designed for thawing and post-cryopreservation recovery.

ARCTis™ oncology 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. The results can be found in the certificate of compliance for each production run.

ARCTis™ 3D Cell Models – Workflow & 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 is important. We have optimized this process to ensure high product quality and reliability. The total work effort is roughly 30 min. Preparing multiple plates can be done in parallel depending on available automation infrastructure and sufficient number of Tilting Stands (D). Each plate needs to thaw on its own Tilting Stand. Familiarize yourself with the shape of the wells in our Akura plate™ (Fig 1) and how to add or remove medium.

Additional Materials Required

- Box with dry ice.
- Inverted microscope with a 5x / 10x objective
- Cell counter, e.g., Neubauer chamber
- 8- or 12-channel pipette (e.g., Viaflo 10-300 µl, Integra Biosciences)
- Single channel 1000 µl pipette
- Medium reservoir for multichannel pipettes
- Microplate centrifuge
- Humidified 5 % CO2 incubator 37 °C

Preparation

- Prior to thawing any ARCTis™ plates, pre-warm the Re-aggregation medium to 37 °C that accompanies each plate and use this for all pipetting steps
- Prewarm the Tilting Stand (D) by placing it in the incubator and let it warm up to 37 °C (10-15 min) while preparing the cell culture hood.
- Prepare your workplace, e.g., a biosafety cabinet.
- Transport the frozen ARCTis™ plate within its transport bag to your lab in a box with dry ice to ensure it stays frozen during your preparations.

Thawing

1. Remove ARCTis™ plate with its packaging from -80 °C freezer and transfer it to the cell culture lab in a box with dry ice.
2. Wipe the ARCTis™ plate bag with 70% EtOH before, open it inside your biosafety cabinet on the triangular side carefully, and remove the plate from it.
3. Immediately place the ARCTis™ plate on the prewarmed Tilting Stand inside the incubator (37 °C, 5 % CO_2) and let the plate thaw for 7 min.

4. Spray the ARCTis™ plate with 70% EtOH before before you place it back into your safety cabinet and carefully remove the sealing foil.

5. Gently and stepwise dispense Reaggregation Medium (C) to each well according to the following procedure:
   i. Gently dispense20 µl of medium - incubate for 1 min
   ii. Gently dispense 20 µl of medium - incubate for 1 min
   iii. Gently dispense 60 µl of medium - incubate for 1 min
   iv. Gently dispense 80 µl of medium - incubate for 1 min
   → Total volume per well now 200 µl
6. Centrifuge plate at 250 rcf for 2 min
7. Slowly and carefully aspirate supernatant (10-20 µl/s if using an automated pipette) with the pipette tip in contact with the SureXchange™ ledge (see illustration), leaving a residual volume of 5 - 7 µl in each well
8. Dispense gently 70 µl medium (80-90 µl/s) in each well
9. Centrifuge the ARCTis™ plate at 250 rcf for 2 min
10. Transfer ARCTis™ plate to incubator and place on Tilting Stand for at least 48 - 72 h

Dosing of compounds should be done together with the regular medium exchange. Use Tumor Maintenance Medium (CS-07-112-3) for this.

Medium Exchange

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 (Fig. 1, left).
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.

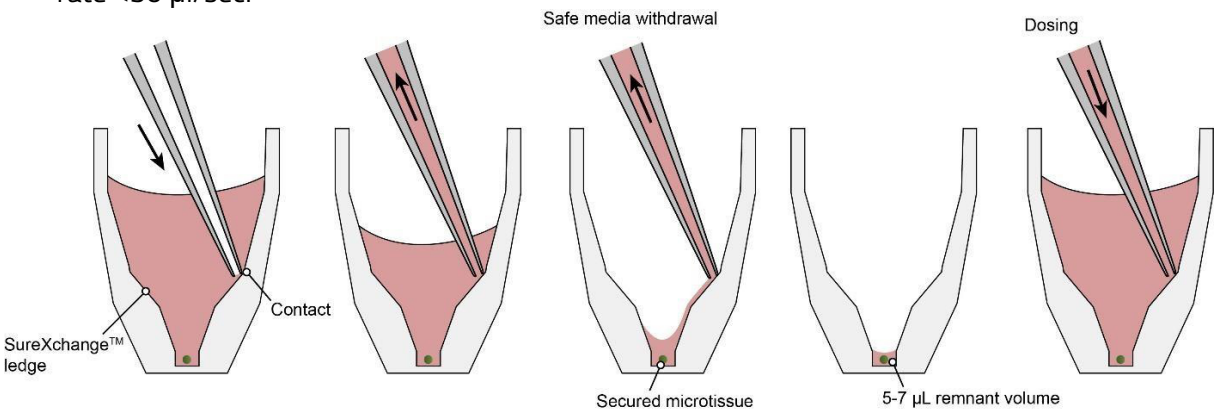


Figure 1: Medium exchange in the Akura™ 96 Plate. Left: Medium removal with the pipette tip placed at the ledge of the well. Right: Medium addition.

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. 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 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 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 2A). 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 2B). Note that incorrect positioning of the 100–200 µl pipette may damage the spheroids (Figure 2C).
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.

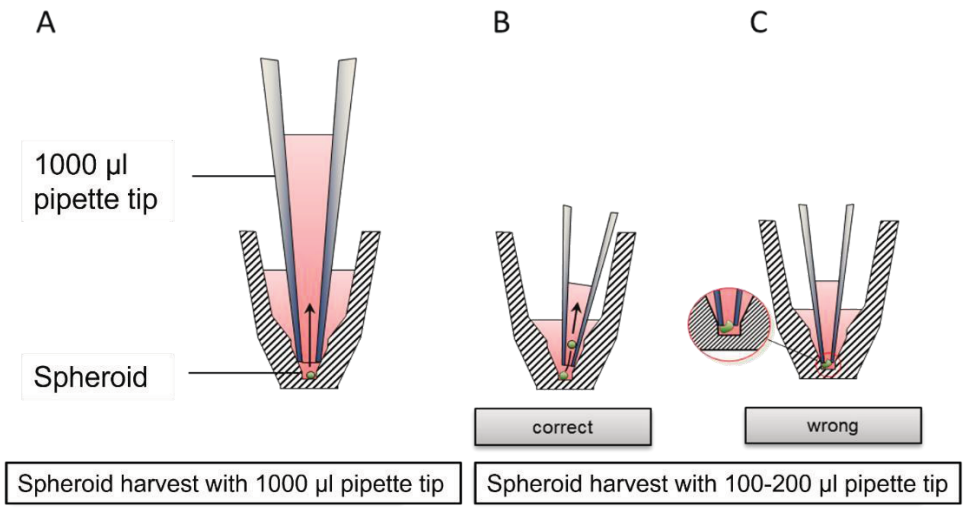


Figure 2: Pipette positioning when collecting spheroids using A. a 1000 µl pipette tip or B. a 200 µl pipette tip. C. The incorrect way to position a 200 µl pipette tip during transfer, causing spheroid damage.

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 4). 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 3B), 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 4, 5 and 6).

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:	0.40 mm
Diameter of well opening:	6.70 mm
Diameter of well bottom:	1.00 mm
Thickness well bottom:	0.80 mm
Working volume:	70-80 µl
Well-to-well distance:	9.00 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

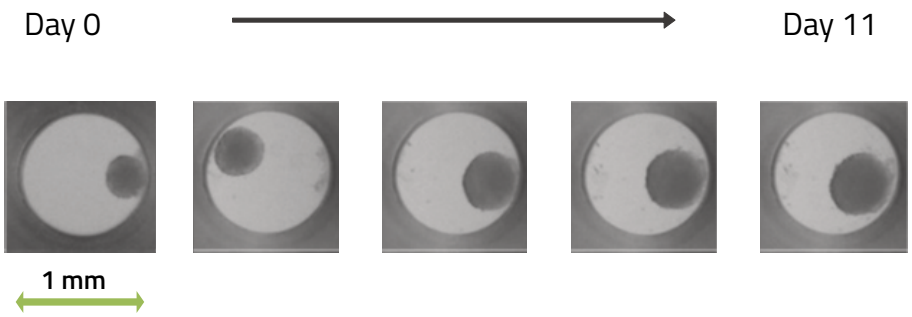


Figure 3: A. Angled view of Akura™ 96 Plate. B. Brightfield images of the same microtissue of HCT 116 colorectal tumor cells growing over a period of 11 days in a well of an Akura™ 96 Plate. The well diameter is exactly 1 mm.

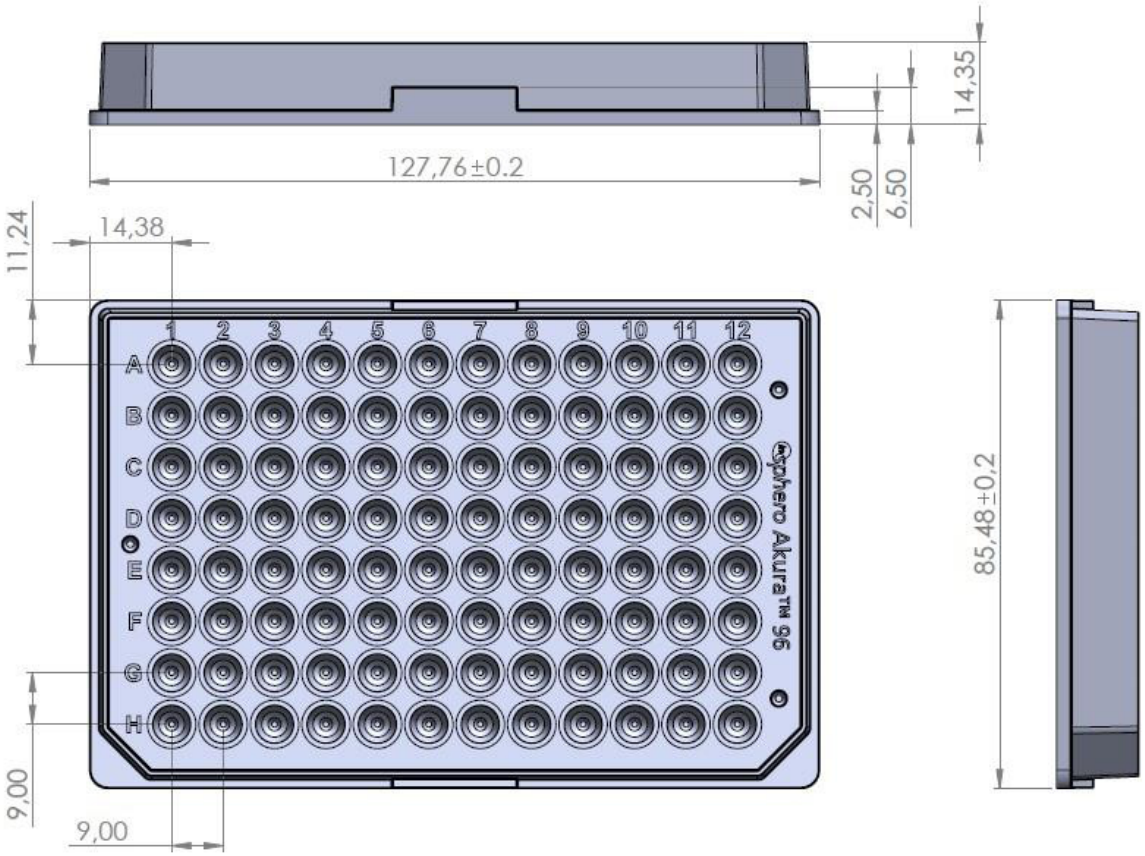


Figure 4: Technical specifications of Akura™ 96 Plate in mm.

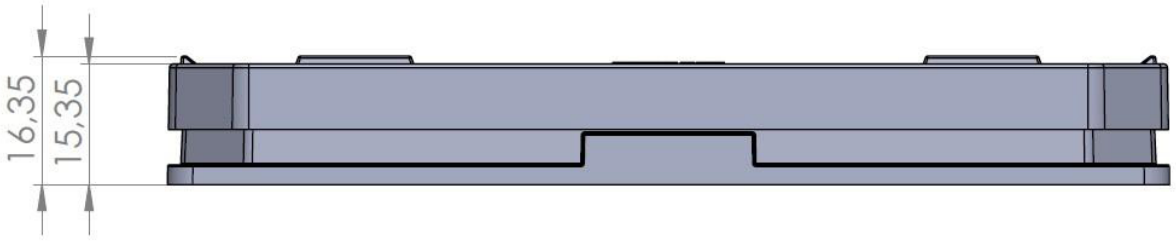


Figure 5: Height of well with lid in mm.

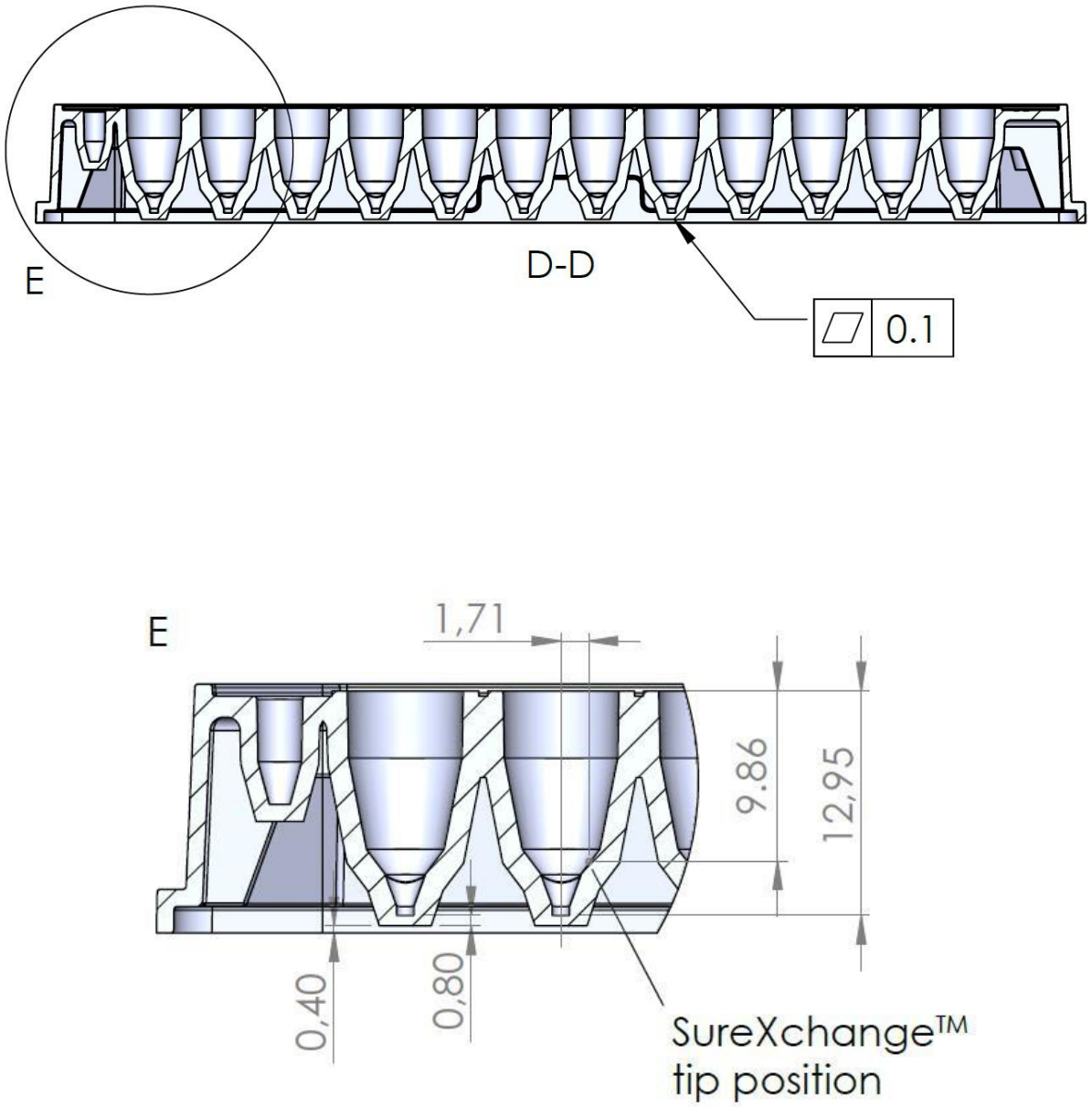


Figure 6: Height of well, skirt height, well bottom thickness and SureXchange™ tip position in mm.

Annex B: 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).

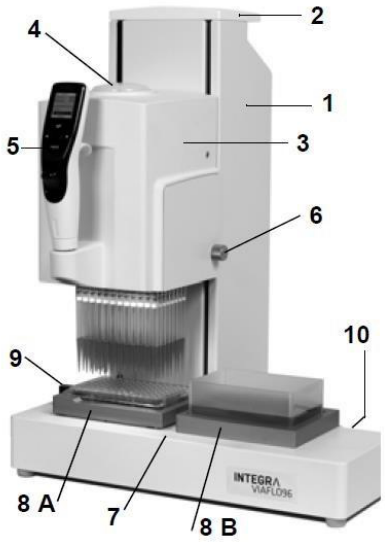
1. Place pipette tip at the ledge by slowly sliding down along the inside wall of the well until a subtle resistance can be felt (Fig. 1, left)
2. Carefully and slowly remove the medium by aspirating an excess of volume. This will lead to an almost complete removal of the medium.
3. Add 70 µl of pre-warmed 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 CO2 incubator.

Table 1
Recommendations for culturing ARCTis™ Oncology Plates

Material/Process:	Recommendation
Culture Medium:	Tumor Maintenance (CS-07-112-03)
Culture Medium volume:	70 µl/ well
Medium exchanges:	2-3 times per week or frequency recommended of specific spheroids
Pipettes:	Integra VIAFLO multichannel pipette with Integra 300 µl pipette tips with filters
Aspiration speed:	Slow (set automated pipette to < 20 µl/s)
Dispense speed:	Slow to moderate (set automated pipette to < 50 µl/s

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 7) 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

Figure7. 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).

Table2

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.

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).

Table3

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 µl1 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	

Annex C: License Agreement

License Agreement ARCTis™, 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 ARCTis™ Oncology, 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 ARCTis™ 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 ARCTis™ Plates to any third party or entity. End User will not sell, or transfer cellbased products generated using the ARCTis™ Plates to any third party or entity.

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

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:

i. COP (Cyclo-Olefin Polymer, 92% transparency 400-800 nm) as plate material instead of Polystyrene.
ii. Thinner well bottom of 0.8 mm, before 1.3 mm.
iii. 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:
iv. Excellent planarity across plate (below 80 µm) for reliable spheroid transfer and precise medium exchange

Less evaporation:

i. Optimized distance (200 µm) between customized low-evaporation lid and plate reduces evaporation in outer and edge wells
ii. Standard SLAS plate height:
iii. 14.35 mm plate height instead of 11.48 mm
iv. 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 airbubbles. 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 CO2 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 lowvolume 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:

- 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).
- 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.
- We recommend the use of the InSphero Incubox™ (CS-10-001-00) (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.

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.

Figure 10: InSphero Incubox™

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