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ThawReady™ 3-D Spheroid Kits Spheroid Assay Product Manual

Contents

Introduction

ThawReady™ 3-D Spheroid Kits – Assay Ready Spheroids

ATCC's ThawReady™ 3-D Spheroid Kits represent a novel concept that enables fast and reliable use of 3-D cultured assay ready spheroids 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. The ThawReady™ Spheroid Plates are strictly designed with SLAS standards in mind and are completely automation compatible. Each plate is made of Cyclo-olefin-polymer (COP) and consists of innovatively designed ultra-low attachment (ULA) wells 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.

ThawReady™ spheroid 3-D cell models are scaffold-free in a 96-well format that provides an efficient end-to-end workflow, allowing users to simply thaw, observe, and test the spheroids all in one plate. The comprehensive description for each 3-D cell model together with their growth and culture characteristics (e.g., growth rate) may be found in their respective Technical Specification Sheets. Please note that 3-D cell model types are often referred to as spheroids or microtissues (MT). For the remainder of the document, we term them as 'spheroids'.

Advantages of ThawReady™ 3-D Spheroid Kits

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

	Standard ULA Round Bottom Plate	ThawReady Spheroid Plate
Formation of single spheroid/well	✖	✔
Easy medium exchange without spheroids loss	✖	✔
Allows for confocal microscopy	✖	✔
Reduces well- to-well imaging crosstalk	✖	✔
Reproducibility guaranteed within plate and across multiple plates	✖	✔

ThawReady™ 3-D Spheroid Kit

The ThawReady™ 3-D Spheroid Kit contains all the necessary components for a successful experiment and is designed for both newcomers to 3-D cell culture and experienced DIY-style spheroid generators. Our goal is to provide a reproducible platform for testing hypotheses and generating trustworthy data.

The ThawReady™ 3-D Spheroid Kit components are as follows:

- 2 x ThawReady™ Spheroid Plates: (ATCC® SCM-CCL-247™); (ATCC® SCM-CCL-185™); or (ATCC® SCM-HTB-133™) depending on kit
- 2 x 30 mL ThawReady™ Spheroid Aggregation Medium (ATCC® SCM-1000™)
- 1 x Spheroid Assay Stand (ATCC® SCM-STAND™)
- 1 x 125 mL ThawReady™ Spheroid Maintenance Medium (ATCC® SCM-2000™)
- 1 ThawReady™ Spheroid Assay Balance Plate (ATCC® SCM-PLATE™)
- Technical documentation as hard copy and as a PDF file

ThawReady™ Spheroid Plates

Characteristics of each ThawReady™ spheroid plate:

- Uses ATCC authenticated cell lines
- 96 wells containing 20 µL cell suspension with 500-2000 cells cryopreserved in cryopreservation agent
- Optimized for formation of ~200 mm diameter spheroids with the cells' doubling time dictating the number of cells needed for forming spheroids
- Optimized for post-cryopreservation recovery by thawing the cells directly in their wells with use of ThawReady™ Spheroid Aggregation Medium

Simply thaw, observe, and test the spheroids all in one plate. Phenotypic observation of the spheroids can be observed over a period of several days with the length of the observation window depending on the doubling time of the cells. This makes it unique for each cell line but typically ranges from 9 to 11 days. Everything from thawing to measuring optical endpoints is performed in the same ThawReady™ spheroid plate unless one wants to extract the spheroids for histological analysis outside the wells.

ThawReady™ 3-D Cell Models – Handling Protocol

Revitalizing the cryopreserved 3-D cell models in the ThawReady™ 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 performance, product quality and reliability.

ThawReady™ 3-D Spheroid Kit Components

A. 2 x ThawReady™ Spheroid Plates: (ATCC® SCM-CCL-247™); (ATCC® SCM-CCL-185™); or (ATCC® SCM-HTB-133™) depending on kit
B. 2 x 30 mL ThawReady™ Spheroid Aggregation Medium (ATCC® SCM-1000™)
C. Spheroid Assay Stand (ATCC® SCM-STAND™)
D. 1 x 125 mL ThawReady™ Spheroid Maintenance Medium (ATCC® SCM-2000™)
E. 1 ThawReady™ Spheroid Assay Balance Plate (ATCC® SCM-PLATE™)

The total work effort required is approximately 30 minutes. It is possible to prepare multiple spheroid plates simultaneously, depending on the available infrastructure. In order to ensure consistency, use ThawReady™ Spheroid Aggregation Medium (B) at 37°C for all steps in day 1. Use the ThawReady™ Spheroid Assay Balance Plate 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
- Manual or automated multichannel pipette (e.g. 8- or 12-channel pipette)
- Medium reservoir for multichannel pipettes
- Microplate compatible centrifuge with swing bucket (horizontal or vertical)
- Humidified Incubator at 37°C with 5% CO2
- Biosafety cabinet class II for cell culture handling (BSC)
- Timer

General Preparation

- Check the availability and integrity of the components in the ThawReady™ 3-D Spheroid Kit
- Defrost Spheroid Aggregation Medium (B) at 4°C overnight, the day before the experiment
- Check on availability of components listed in additional materials

Preparation Prior to Thawing Spheroid Plates

Note: It is recommended to thaw Spheroid Aggregation 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 overnight.

- Prior to thawing the spheroid plates, pre-warm the Spheroid Aggregation Medium (B) to 37 °C that accompanies each plate
- Pre-warm the Spheroid Assay Stand (C) in the incubator at 37 °C.

- Prepare centrifugation counterbalance plate (E) in case a single spheroid plate is processed:
  - Remove packaging in biosafety cabinet.
  - Add 180 µL of sterile PBS (ATCC® 30-2200™) to each of the wells using a multichannel pipette.
  - Mark the plate as "Balance"
- If using electric pipettes, please verify the set flow rates and adjust if needed.

Thawing Spheroid Plates (adhere to time sensitive process)

The total work effort required is approximately 30 minutes. It is possible to prepare multiple spheroid plates simultaneously, depending on the available infrastructure. In order to ensure consistency, use ThawReady™ Spheroid Aggregation Medium (B) at 37°C for all steps in day 1.

NOTE: Thawing spheroid plates takes ~30 minutes with about 15 mins of total hands-on time

1. Move the spheroid plate from the -80 °C storage to the biosafety cabinet using a box with dry ice to ensure it stays frozen.
2. Wipe the spheroid plate packaging with 70% EtOH beforehand and open it inside the biosafety cabinet.
3. Unpack Spheroid Plate (A), keep the lid on and immediately place it in the incubator (37 °C, 5% CO2) on the Spheroid Assay Stand (C) and let it thaw in there for 7 minutes.
4. During the incubation period, transfer 25 mL of the Spheroid Aggregation Medium (B) per plate processed in the medium reservoir (ex.: 50 mL are needed if 2 plates are processed at the same time)
5. After 7 minutes, transfer the ThawReady™ Spheroid Plate (not the Spheroid Assay Stand) to the biosafety cabinet.
6. Remove lid and, using the multichannel pipette, dispense medium volume stepwise to all wells at 1 min intervals according to the steps below, start the timer (1 min) and:
   - 6.1 Slowly dispense 20 µL of medium in the vertical position, wait until the 1 min incubation interval is completed
   - 6.2 Slowly add 20 µL of medium in the vertical position, complete the 1 min incubation interval
   - 6.3 Slowly add 60 µL of medium in the vertical position, complete the 1 min incubation interval
   - 6.4 Slowly add 80 µL of medium in the vertical position, complete the 1 min incubation interval

NOTE: For adding medium, hold the pipette in vertical position for optimal wash (see Figure 1)

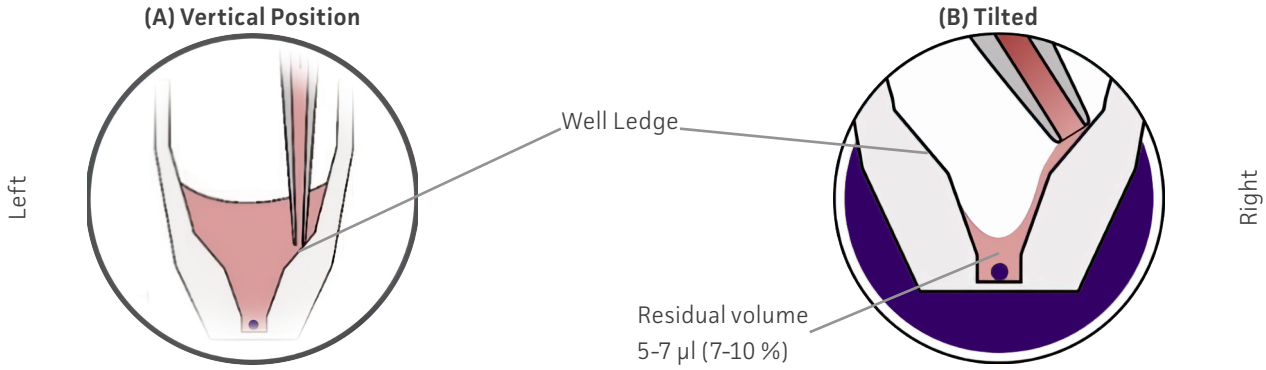


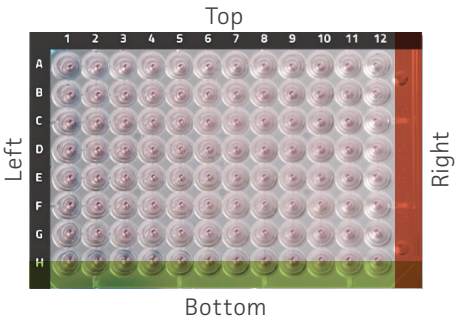
Figure 1: **(A)** Pipette tip in Vertical Position is ideal for adding medium. **(B)** Pipette tip in Tilted Position is suggested for use when exchanging medium, to avoid the disturbance of the spheroids.

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

If automatic multichannel pipette is used, refer to the following range of speeds: Slow = 10-20 µL/s and Normal = 80-90 µL/s

7. Use the previously prepared balance plate and centrifuge the plate at 250 X g for 2 min (**see plate positioning in Figure 2 below**):

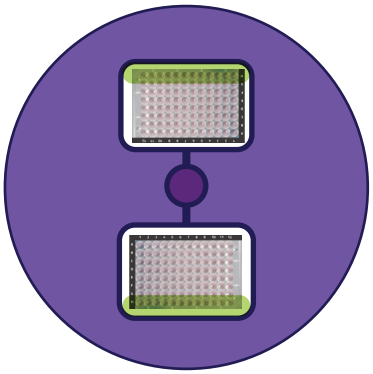
Plate orientation:



Centrifuge types:

(A) Horizontal buckets:

The plate's **bottom** is orientated towards the **outside**



(B) Vertical buckets:

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

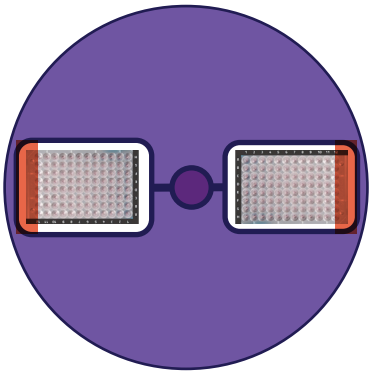


Figure 2: **(A)** The plate's bottom is orientated towards the outside for horizontal centrifuge. **(B)** The plate's right side is orientated towards the outside for vertical centrifuge.

NOTE: It is key to remember the orientation of the plate, as that will allow for a successful aggregation.

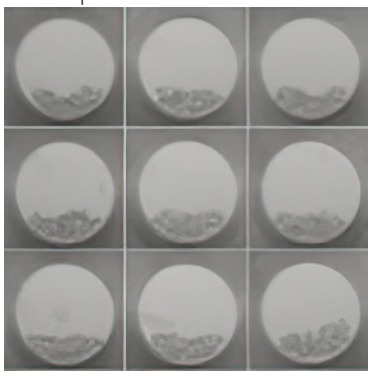
8. Transfer both the spheroid plate and the balance plate back to the biosafety cabinet.
9. Prepare the spheroid plate for the pellet wash step as follow:
   9.1 Using a multichannel pipette in a **tilted position (see Figure 1B)**, gently remove 175 µL of supernatant (use slow speed with an automatic pipette). The pipette tip will be in contact with the well ledge. 5-7 µL medium should remain in each well to minimize cell loss.

   Microscope Checkpoint: Using a microscope, verify that the cells are present. This will help in further possible troubleshooting.

   9.2 Dispense 70 µL Spheroid Aggregation Medium in each well using the micropipette tip in **vertical position (see Figure 1A).**
10. Prepare the balance plate, removing 100 µL of PBS, leaving a final volume of 75 µL/well
11. Re-centrifuge the plates according to plate positioning (**Figure 2A or Figure 2B**) at 250 X g for 2 min
12. Using a microscope, check correct cell pellet allocation in edge of the well (**see Figure 3A and B**). In case of deviation move to trouble shooting section.

(A) Horizontal buckets:

Expected cell pellets' location at bottom of well



(B) Vertical buckets:

Expected cell pellets' position to right side well

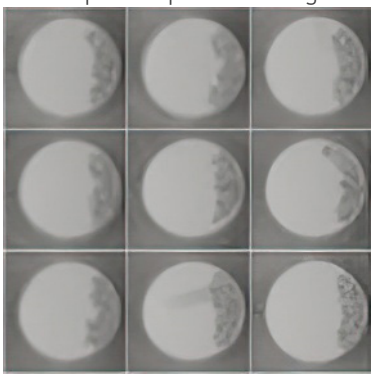
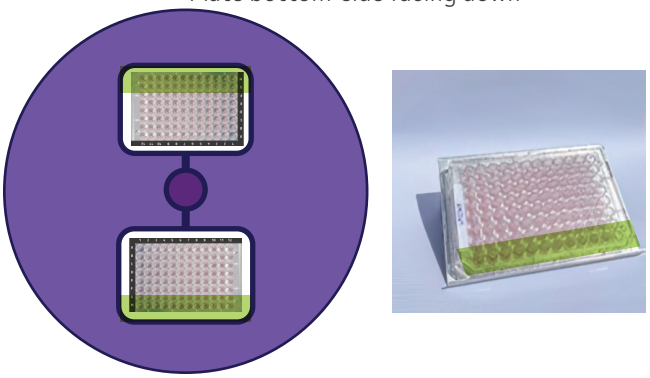


Figure 3: **(A)** Correct cell pellet allocation is at the bottom of the wells for horizontal centrifuge. **(B)** Correct cell pellet allocation is to the right side of the wells for vertical centrifuge.

Note: In case the cells appear dispersed in the well and do not pellet, apply **Trouble Shooting Step 1** before proceeding. Do NOT proceed if the cells appear dispersed.

13. Transfer spheroid plate back to incubator and place on Spheroid Assay Stand as shown below (**see Figure 4**).
14. To obtain optimal results, it is important to position the plate on the Spheroid Assay Stand based on the centrifuge bucket used. Proper formation of the spheroids cannot be achieved if this step is not executed properly.

(A) Horizontal buckets:
Plate bottom-side facing down



(B) Vertical buckets:
Plate with right-side down

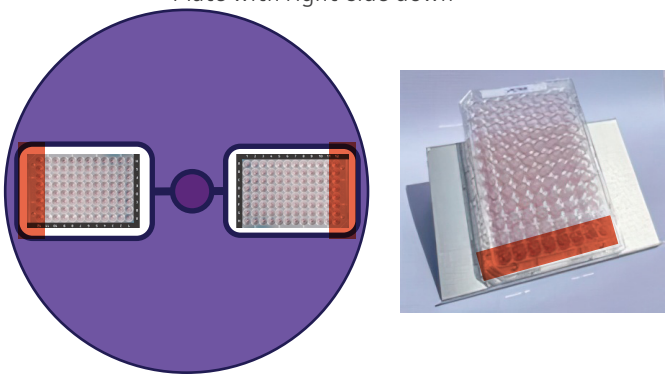


Figure 4: **(A)** Place spheroid plate with bottom-side facing down for horizontal centrifuge. **(B)** Place spheroid plate with right-side facing down for vertical centrifuge.

NOTE: Spheroid formation will take place over a time course from 3-4 days (dependent on cell model). You can stack up to three plates on the Spheroid Assay Stand if required.

15. Depending on the cell line, spheroid formation is completed within 3-5 days and assays can be initiated. Please refer to the exact time given in the Technical Specification Sheet for each cell line.

Trouble Shooting Step 1

In case the cells appear dispersed in the well and do not pellet, use the below trouble shooting step:

1. Gently resuspend the cells at slow speed holding the pipette in vertical position (**Figure 1A**).
2. Re-centrifuge the spheroid plate according to the process described for horizontal (**Figure 2A**) or vertical (**Figure 2B**) centrifuge at 250 X g for 2 min. **NOTE: The orientation of the plate is key!**
3. Check correct cell pellet allocation in edge of the well (**Figure 3**).
4. Repeat in case cells stay dispersed in the wells OR move to Step 12 in the process.

Medium Exchange Day 3 (and Subsequent Spheroid Maintenance Medium Exchange)

The ThawReady™ spheroid plate is a special non-adhesively coated 96-well microtiter plate. It is optimized to produce 3-D cell models for convenient thaw-and-go cultivation and analysis. ThawReady™ spheroid plate wells feature a tapered well 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 as follow:

1. Thaw Spheroid Maintenance Medium overnight at 4-8 °C.
2. Warm up Spheroid Maintenance Medium for 30-40 minutes at 37 °C.
3. Place the pipette tip at the ledge of the well (**tilted position, Figure 5A**).
4. Slowly, remove the medium by aspirating an excess of volume. A minimal volume of ~5-7 µL medium will remain in the well.
5. Slowly, add 70 µL of fresh Spheroid Maintenance Medium by placing the pipette tip at the ledge (**Figure 5B**).

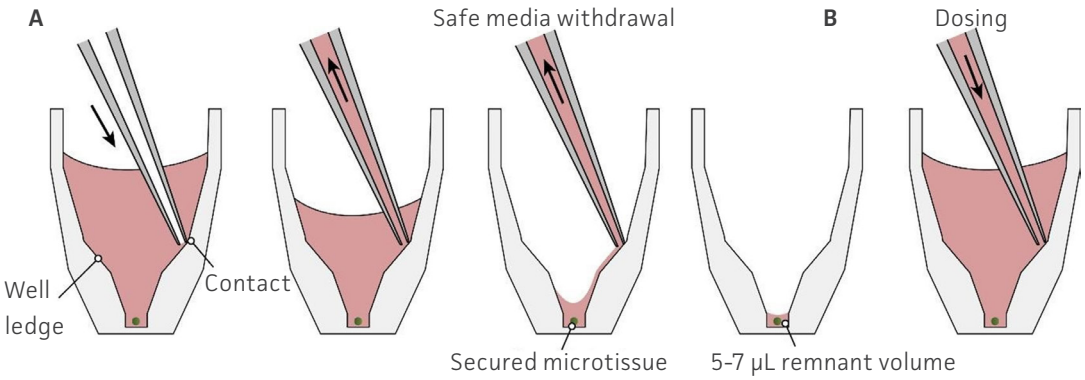


Figure 5: Medium exchange in the ThawReady™ spheroid plate. (A) Medium removal with the pipette tip placed at the ledge of the well. **(B)** Medium addition.

Spheroid Collection

The special coating of the ThawReady™ spheroid 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 PBS. 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 6A**). 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.
4. Aspirate by placing the head of the tip close to the edge of the well bottom **(Figure 6B**). Note that incorrect positioning of the 100–200 µL pipette may damage the spheroids (**Figure 6C**).
5. Collect the spheroid by aspirating 50 µL of the medium. Avoid aspiration of air bubbles to prevent spheroid loss in the pipette tip.
6. Transfer the spheroid in medium into another vessel or plate.

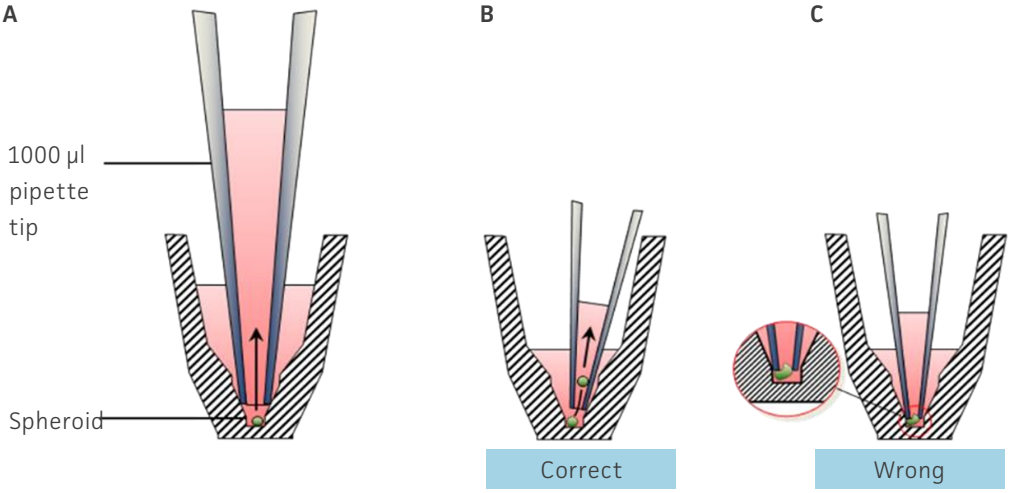


Figure 6: Pipette positioning when collecting spheroids using (A) a 1000 µL pipette tip or **(B)** a 200 µL pipette tip. **(C)** The incorrect way to position a 200 µL pipette tip during transfer, causing spheroid damage.

Analysis and Assays

The ThawReady™ spheroid 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 2-D cultures):

Automated imaging

The ThawReady™ spheroid plate is ideal for use in automated imaging equipment and automated microscopes and high content imaging systems, as the 1 mm diameter optically clear base of each well will be positioned exactly in the center of the field of view.

NOTE: The flat Cyclo-olefin-polymer (COP) bottom of the ThawReady™ spheroid 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 Appendix 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 ThawReady™ spheroid 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 ThawReady™ spheroid plates. This could be resolved by future firmware updates.

Appendix A: ThawReady™ Portfolio of Human Tumor Models

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

Cell Line	Tissue	Item #	Description
T47D	Breast	SCM-CCL-185	ThawReady™ Spheroids - Breast
HCT116	Colon	SCM-CCL-247	ThawReady™ Spheroids - Colon
A549	Lung	SCM-HTB-133	ThawReady™ Spheroids - Lung

Appendix B: ThawReady™ Spheroid Plate Specifications

The ThawReady™ spheroid plate 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 7A**). 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 7B**), designed to accommodate spheroids of up to 750 µm in diameter. The ThawReady™ spheroid plate technical specifications are provided as a reference for automation system programming (**Figure 8, Figure 9 and Figure 10**).

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
Well ledge tip position:	1.71 mm horizontal offset; 9.86 mm in z-height (**see Figure 10**)
Plate and lid material:	COP (Cyclo-olefin-polymer), Polystyrene

Figure 7: **(A**) Angled view of ThawReady™ spheroid plate. **(B)** Spheroid in ThawReady™ spheroid plate. The well diameter is exactly 1 mm.

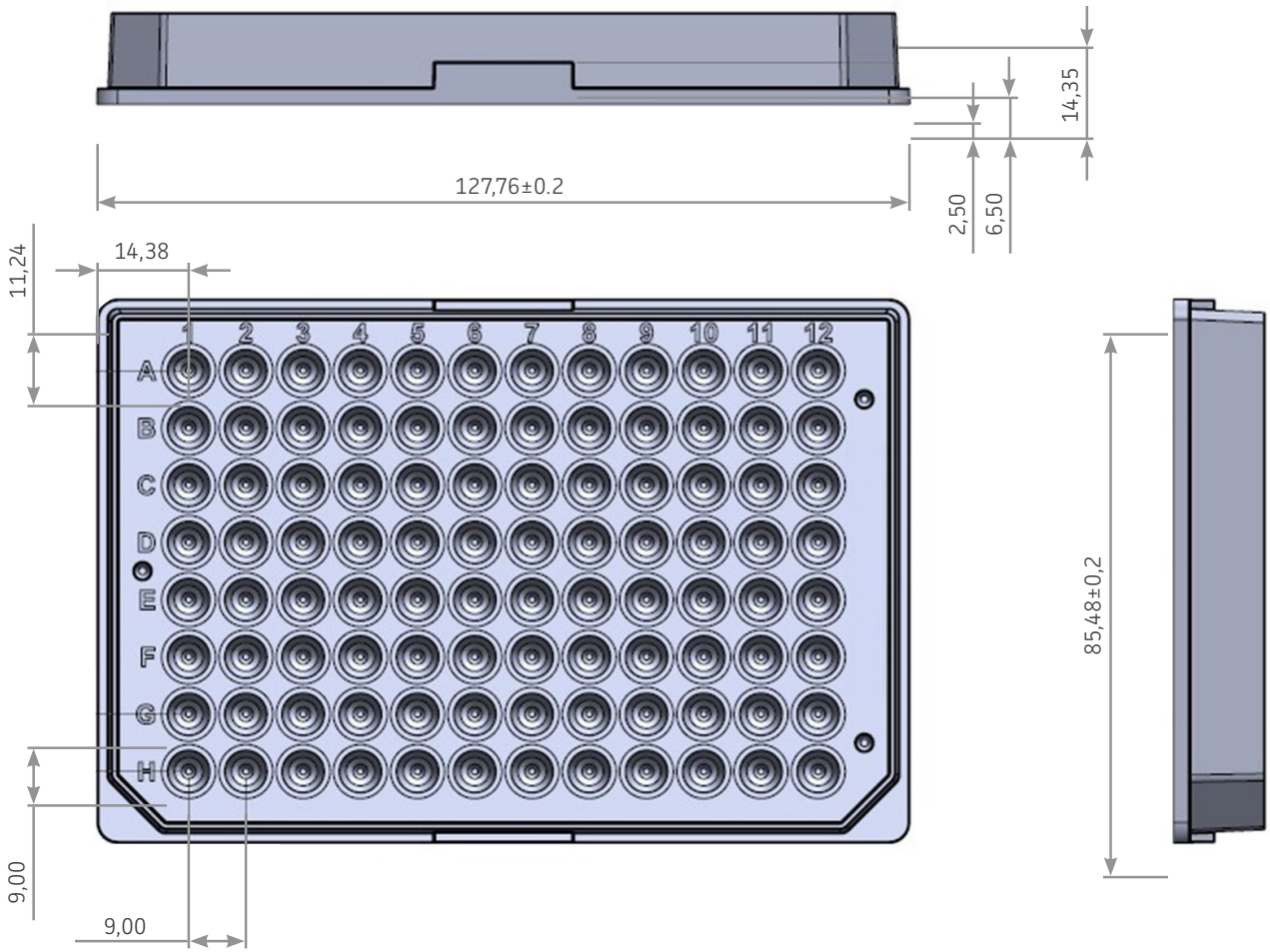


Figure 8: **Technical specifications of ThawReady™ spheroid plate in mm.**

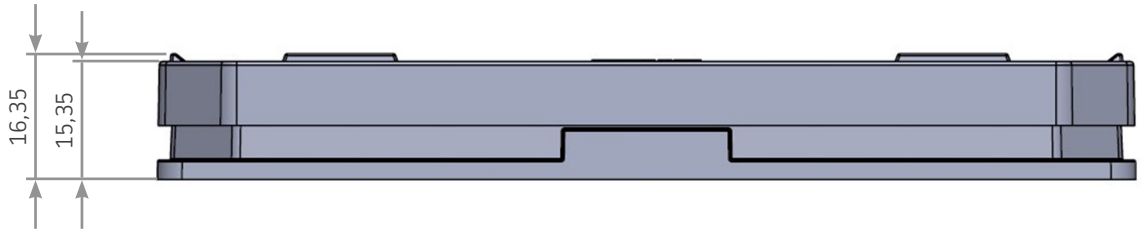


Figure 9: **Height of well with lid in mm.**

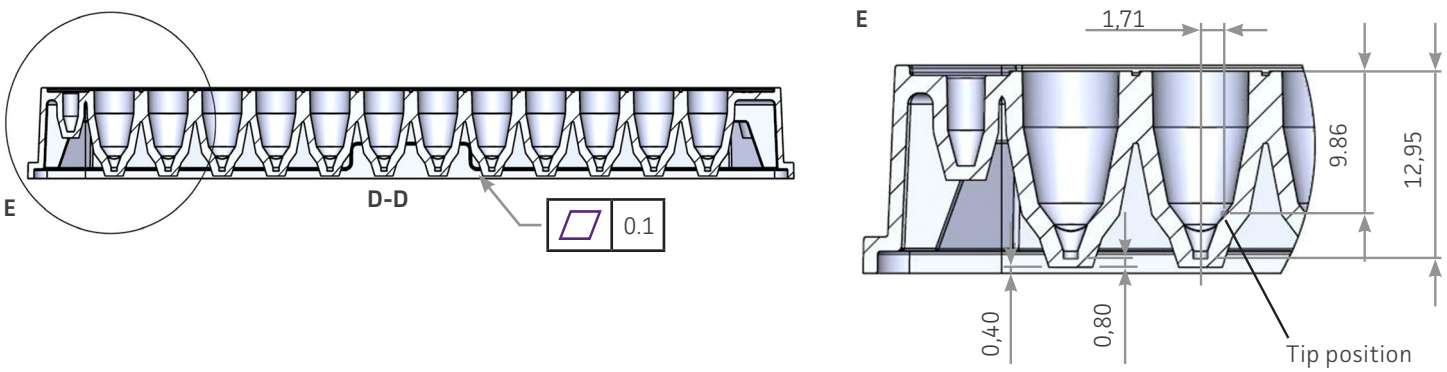


Figure 10: **Height of well, skirt height, well bottom thickness and well ledge tip position in mm.**

Appendix C: Frequently Asked Questions Regarding the ThawReady™ Spheroid Plate

Q: What makes the ThawReady™ spheroid plate unique?

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
- 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
- Maximum volume 280 µL

Q: What is the optimal volume per well in the ThawReady™ spheroid plate?

A: To achieve optimal conditions, gently deliver 70 µL (pipetting speed < 10 µL/sec) of medium into each well of the ThawReady™ spheroid plate by placing the pipette tips on the well ledge while not touching the bottom of the wells.

Q: How do I exchange the medium in the ThawReady™ spheroid plate without disturbing or losing the spheroids?

A: To prevent spheroid/organoid loss during the exchange of media, the well 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 5**) 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.

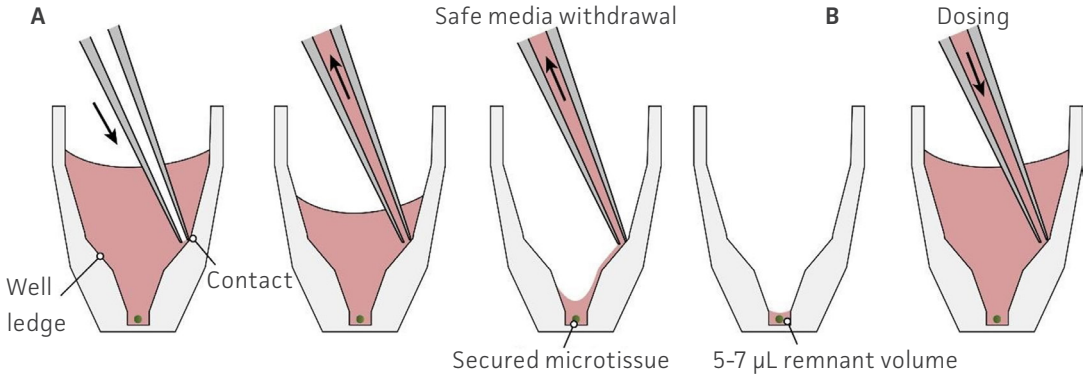


Figure 5: Medium exchange in the ThawReady™ spheroid plate. (A) Medium removal with the pipette tip placed at the ledge of the well. **(B)** Medium addition.

NOTE: 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 ThawReady™ spheroid 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 ThawReady™ spheroid 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.

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 ThawReady™ spheroid 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 on our website 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.

Appendix D: 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 (**Figure 5A**)
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 (**Figure 5B**) 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 ThawReady™ spheroid plate and incubate the spheroids in a humidified 37°C CO_2 incubator.

Table 1: Recommendations for culturing ThawReady™ spheroid plates

Material/Process	Recommendation
Culture medium	Spheroid Maintenance medium (SCM-2000)
Culture medium volume	70 µL/well
Medium exchanges	2-3 times per week or frequency recommended for specific spheroids
Aspiration speed	Slow (set automated pipette to < 20 µL/second)

Table 2: 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* 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 ThawReady™ spheroid plate.
4	Z-Height position 8B, 38.5mm	Gently immerse the pipette tips into the wells of the ThawReady™ spheroid plate, until reaching Z-Height. Displace ThawReady™ spheroid 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	

*4x 70 µL plus 10 µL excess volume remaining in the tip.

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