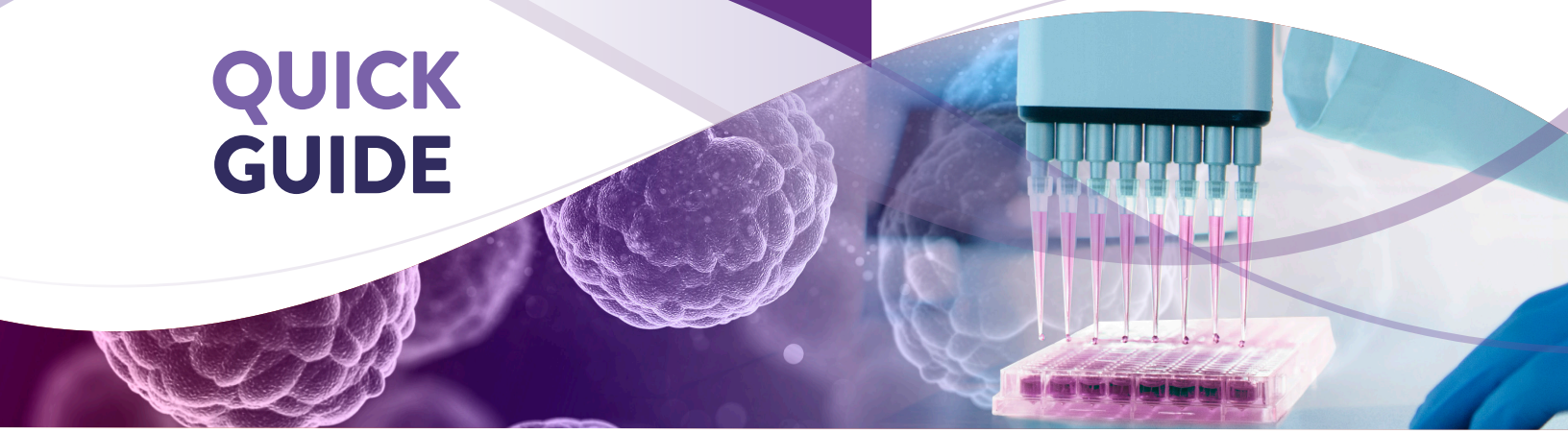
ATCC®

QUICK GUIDE



ThawReady™ by ATCC 3-D SPHEROID KITS
SPHEROID ASSAY PROTOCOL QUICK START GUIDE

Thank you for selecting ATCC's ThawReady™ 3-D Spheroid Kit for your 3D cell culture experiments. This Spheroid Assay Protocol Quick Start Guide is designed to kickstart your experiments promptly. For detailed instructions please refer to the ThawReady™ Spheroid Assay Product Manual and additional resources at www.atcc.org/thawready-spheroidkits.

CURRENTLY AVAILABLE THAWREADY™ 3-D SPHEROID KITS

ThawReady™ HCT 116 Spheroid Kit (ATCC® SCM-CCL-247-2PLT™)

ThawReady™ A549 Spheroid Kit (ATCC® SCM-CCL-185-2PLT™)

ThawReady™ T-47D Spheroid Kit (ATCC® SCM-HTB-133-2PLT™)

COMPONENTS OF EACH KIT:

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. 1 x 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™)

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

PREPARATIONS 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 being processed:
  - Remove packaging in biosafety cabinet.
  - Add 180 µL of sterile PBS to each of the well using a multichannel pipette.
  - Mark the plate as "Balance".
- Prepare a transport box with dry ice and move the frozen spheroid plate from the -80 °C storage to the biosafety cabinet to ensure it stays frozen during the following preparations.
- If using electric pipettes, please verify the set flow rates and adjust if needed.

THAWING SPHEROID PLATES

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 plastic pouch 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% CO_2) 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.: 10 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 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)**

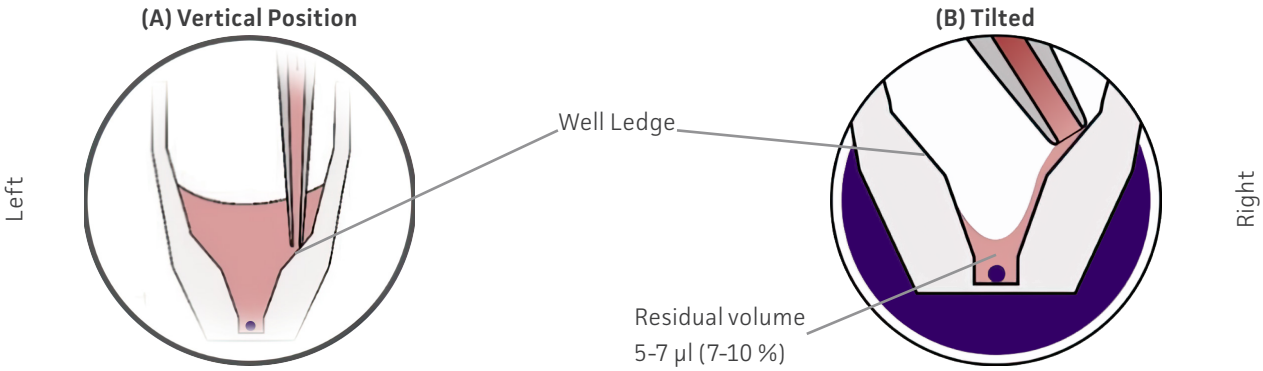


Figure 1: **(A).** Pipette tip in Vertical Position is ideal for adding medium. **(B)** Pipette tip in Tilted Position is suggested for use when exchanging medium, to avoid the disturbance of the spheroids.

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
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):

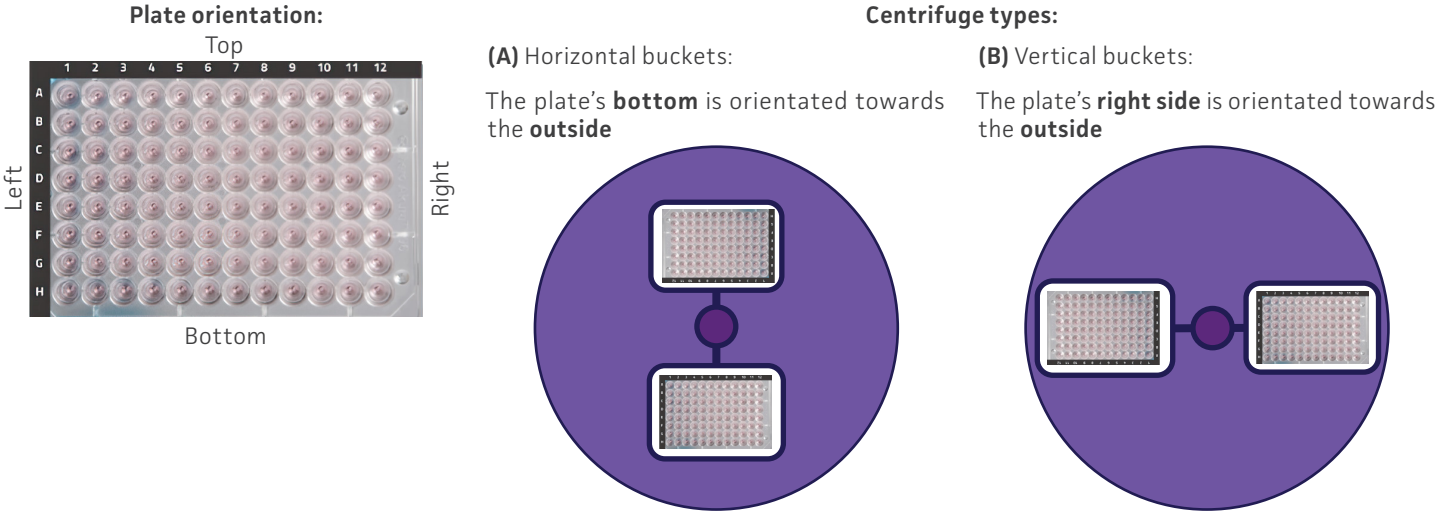


Figure 2: **Figure 2 (A).** The plate's bottom is orientated towards the outside for horizontal centrifuge. **(B)** The plate's right side is orientated towards the outside for vertical centrifuge.

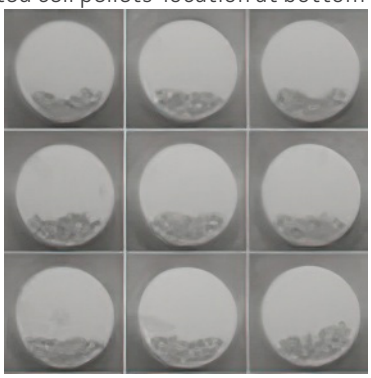
NOTE: It is key to remember the orientation of the plate, as that will allow for a successful aggregation.

8. Transfer both the spheroid plate and 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.

NOTE: It is the key to success to maintain the plate always in the same orientation when centrifuge.

12. Using a microscope, check correct cell pellet allocation in edge of the well (see **Figure 3**). 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

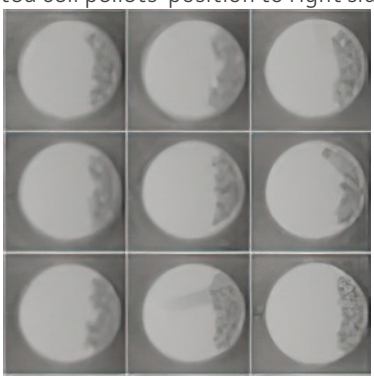
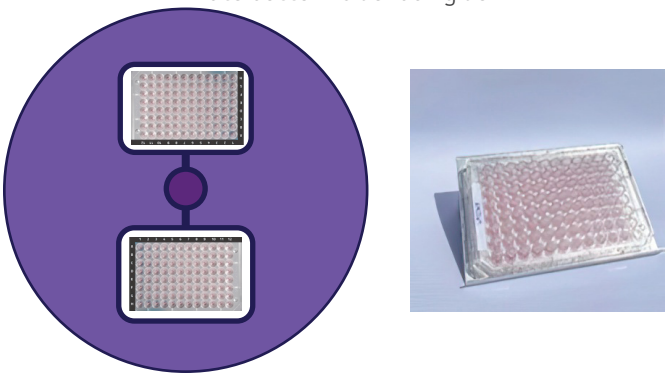


Figure 3: **(A).** Correct cell pellet allocation is at the bottom of the wells for horizontal centrifuge. **(B)** Correct cell pellet allocation is to the right side of the wells for vertical centrifuge.

NOTE: In case the cells appear dispersed in the well and do not pellet, apply **Troubleshooting Step 1**.

13. Transfer spheroid plate back to incubator and place on Spheroid Assay Stand as shown below (see **Figure 4**).

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



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

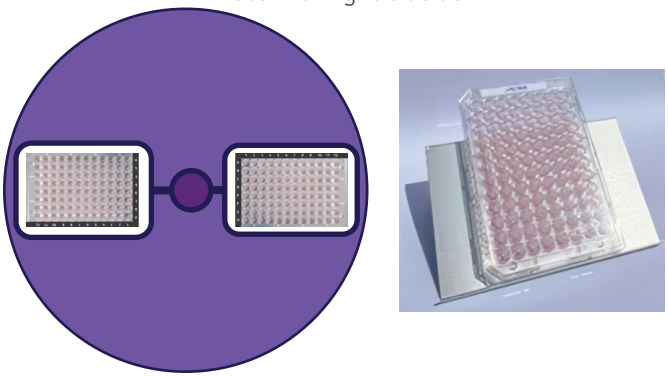


Figure 4: **(A).** Place spheroid plate with bottom-side facing down for horizontal centrifuge. **(B)** Place spheroid plate with right-side facing down for vertical centrifuge.

Note: Spheroid formation will take place over a time course from 72 to 96 h (dependent on cell model). You can stack up to three plates on the Spheroid Assay Stand if required.

14. 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.

ASSAY INITIATION/ MEDIUM EXCHANGE/ DOSING

1. Have Spheroid Maintenance Medium warmed at 37 °C.
2. Once Spheroid Maintenance Medium is warmed, transfer the appropriate amount in reservoir in the biosafety cabinet.
3. Remove the spheroid plate from the incubator.

4. Set the multichannel pipette to 75 µL, place the tip on tilted position, touching the well ledge and slowly remove all medium above the ledge.
5. Replace with 70 µL fresh Spheroid Maintenance Medium per well.
6. If a compound needs to be added, have it diluted in the Spheroid Maintenance Medium according to your experiment scheme.

TECHNICAL NOTE

Each ThawReady™ spheroid plate contains 20 µL cryopreserved cell suspension of 500 to 2000 cells per well. The number of cells has been optimized depending on the observed doubling time of the respected cell line. As a result, the formed spheroid has the correct size to allow for a testing period of 10 days after aggregation. Throughout this 10-days test-window, a variety of different endpoints can be measured such as growth rate, ATP content, and viability.

It is possible that it takes 1-2 days longer for the cells to aggregate. In such case, simply let them continue with their spheroid formation in the incubator while the ThawReady™ spheroid plate is placed on the Spheroid Assay Stand.

As an example, see the image series of an untreated HCT116 (ATCC® CCL-247™) cell line spheroid illustrating a typical growth over 13 days from thawing (d0) to last day of measurement (Day 10). The image from d0 was taken a few hours after the 2nd centrifugation step. It can be seen that the cells migrate to a common center point when the plate is on the Spheroid Assay Stand.

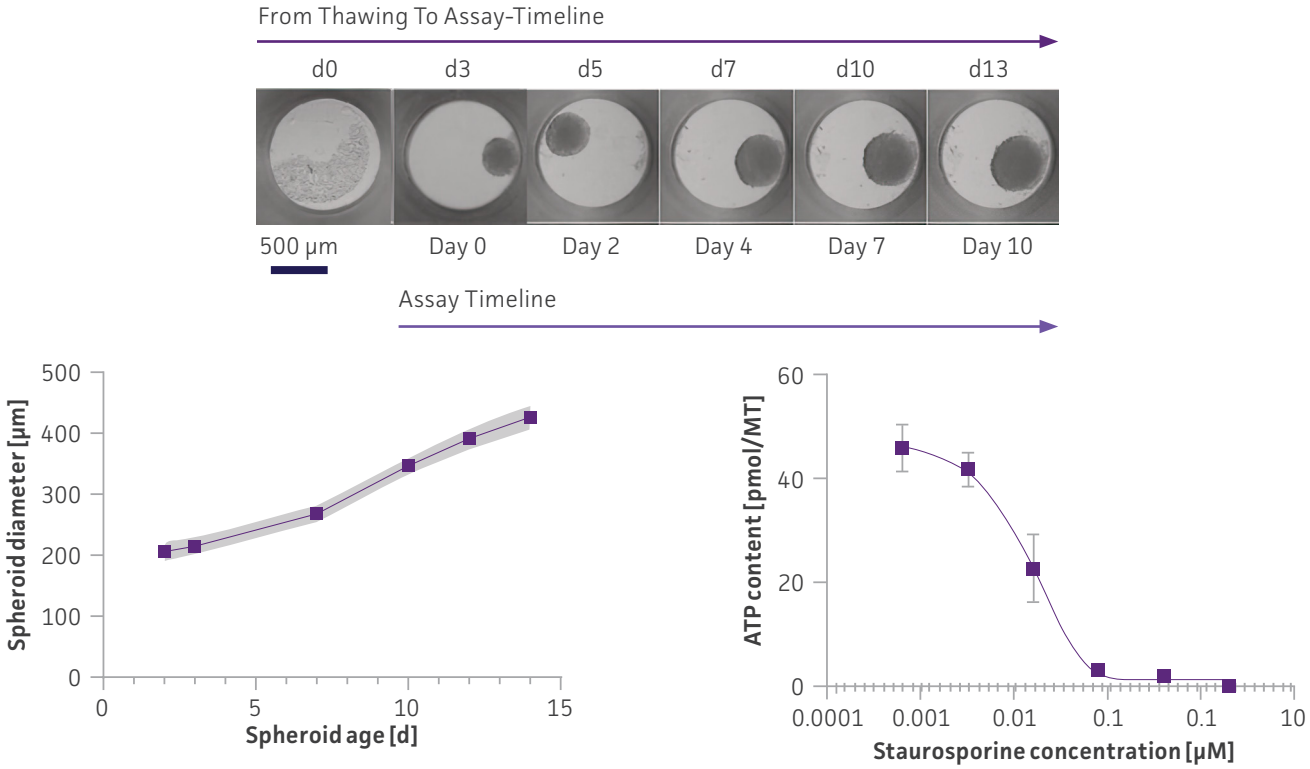


Figure 5: Typical growth over 13 days. Top. Micrograph showing growth of HCT 116 spheroids over 13 days; Left) Typical growth curve for HCT 116 spheroids over 13 days; Right) Dose response for HCT 116 spheroid using the apoptotic agent staurosporin (as an example for compound testing).

FREQUENTLY ASKED QUESTIONS:

Q: How many spheroids should form in each well?

A: ThawReady™ spheroid plates are designed to hold a single spheroid per well. Multiple satellite spheroids can appear during the aggregation process which over time consolidate into a single one.

Q: I don't see any spheroids in any well. What could have gone wrong?

A: All cells were removed during the aspiration step. Check the plate after the centrifugation. The cells should be at the very bottom of the well. Check that you used the correct angled position was used for the pipette.

10801 University Boulevard
Manassas, Virginia 20110-2209

703.365.2700

703.365.2701

sales@atcc.org

www.atcc.org

TRQG-012025-v01