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APPLICATION NOTE

ThawReady™ by ATCC

NEW EFFORTLESS 3-D TECHNOLOGY OVERCOMING THE CHALLENGE OF TRADITIONAL SPHEROIDS

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ABSTRACT

Three-dimensional (3-D) spheroid models have become essential tools in drug discovery and toxicology, offering superior physiological relevance as compared to conventional two-dimensional (2-D) cultures. However, their implementation is often hindered by technical challenges relating to media exchange, extensive handling, and endpoint compatibility. ThawReady™ 3-D Spheroid Kits introduce an effortless, integrated solution that overcomes these limitations by enabling uniform spheroid formation, near-complete media exchange, and compatibility with high-content imaging—all within a single plate. Comparative studies demonstrate improved reproducibility, reduced variability, and streamlined workflows, positioning ThawReady™ as a next-generation platform for 3-D culture applications.

INTRODUCTION

Conventional two-dimensional (2-D) cell cultures offer simplicity, cost-effectiveness, and ease of imaging and manipulation, making them a cornerstone for basic research and high-throughput screening. However, despite these advantages, they fail to replicate the complex three-dimensional (3-D) microenvironment of living tissues, which limits their predictive accuracy for pharmacological and toxicological studies. In recent years, 3-D models have become indispensable in drug discovery and toxicology due to their ability to mimic native tissue architecture. Unlike conventional 2-D culture systems, which rely on flat monolayers, 3-D culture encompasses a diverse array of techniques designed to recreate tissue-like architecture (e.g., organoids, transwells, 3-D cell printing, organ-on-a-chip, spheroids). These systems have been widely adopted and extensively reviewed in the literature.[1]

Spheroids—scaffold-free, 3-D aggregates of one or more cell types—offer a physiologically relevant alternative for tissue modeling by establishing gradients of oxygen, nutrients, and signaling molecules. Their size (50–500 µm) and architecture allow modeling of tumor biology, metabolic disorders, and organ-specific functions. However, despite the growing adoption of spheroid models, several technical hurdles limit their widespread implementation. First, media exchange is a critical challenge: incomplete removal of spent medium can lead to accumulation of toxic metabolites, dilution of nutrients, and interference with endpoint assays. Conventional plates often allow only partial exchange (≈50%), which compromises compound concentration control and IC_{50} calculations. Second, mechanical stress during spheroid handling—such as harsh aspiration and transfer steps—can cause shear-induced cell damage, apoptosis, or complete disintegration of loosely aggregated spheroids. These manipulations also increase variability and prolong workflows, particularly when manual transfers are required for downstream assays. Finally, endpoint incompatibility remains a bottleneck; many plates lack optically

clear bottoms for high-content imaging or geometries that support multiplexed biochemical and molecular analyses. Together, these limitations underscore the need for integrated solutions that minimize handling, enable efficient media exchange (>90%), and support diverse readouts within a single platform.

Addressing these limitations begins with an understanding of how spheroids are formed. The formation method not only influences morphology, compactness, and cellular behavior but also handling and workflow efficiency—even when using the same cell type. Common methods include (Figure 1):

- **Hanging drop:** This method produces uniform spheroids with minimal size variation; however, it is labor-intensive and prone to media evaporation.
- **Ultra-low attachment (ULA) plates:** These plates provide a simple and scalable solution, but they offer limited control over spheroid size and present challenges in media exchange.
- **U-bottom plates:** These plates improve spheroid uniformity as compared to ULA plates, but they require complex pretreatment steps that add time and variability to workflows.

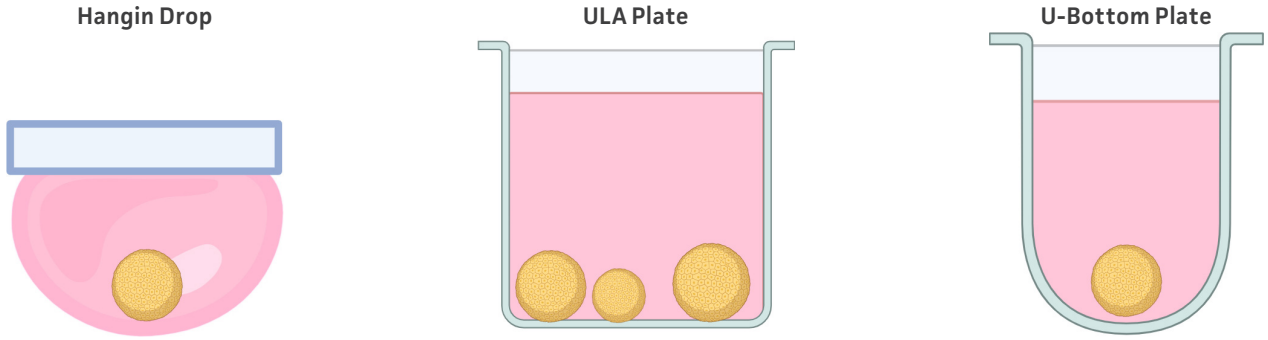


Figure 1: Representation of the most common spheroid formation methods. Image created with BioRender.com.

While these traditional methods allow spheroid formation, they often fall short when it comes to workflow integration. Researchers frequently struggle with incomplete media exchange, accidental spheroid aspiration, and incompatibility with high-content imaging. These issues not only increase variability but also introduce bias in drug screening and mechanistic studies.

To overcome these challenges, the ThawReady™ Spheroid Kit was developed as an integrated solution. This kit combines pre-plated tumor cells on 96-well microplates with optimized media and peripherals, enabling reliable spheroid formation and streamlined workflows. Its innovation lies in creating a system that supports the entire experimental lifecycle—from spheroid aggregation to endpoint analysis—within a single plate. By eliminating manual transfers, the kit reduces mechanical stress and improves data consistency.

Central to the kit is the ThawReady™ microplate, designed with a unique geometry and functionality to address common bottlenecks (Figure 2). The culture wells of the microplate feature a cell-repellent flat bottom and an angled ledge that allows for safe medium exchange (Figure 3). The angle ledge, a subtle but transformative design element, guides the pipette tip during aspiration and enables near-complete media removal (>90%) without disturbing the spheroid (Figure 3). This is critical for maintaining nutrient balance, preventing metabolite accumulation, and ensuring accurate compound dosing—factors that directly influence IC_{50} calculations and assay reproducibility.

Equally important is the protective cavity at the bottom of each well, which secures the spheroid during liquid handling and minimizes shear stress. Combined with an ultra-low attachment coating, this design ensures scaffold-free spheroid formation and long-term culture stability. The optically clear flat-bottom further enhances compatibility with high-content imaging systems, enabling researchers to capture high-resolution morphological and functional data without transferring samples.

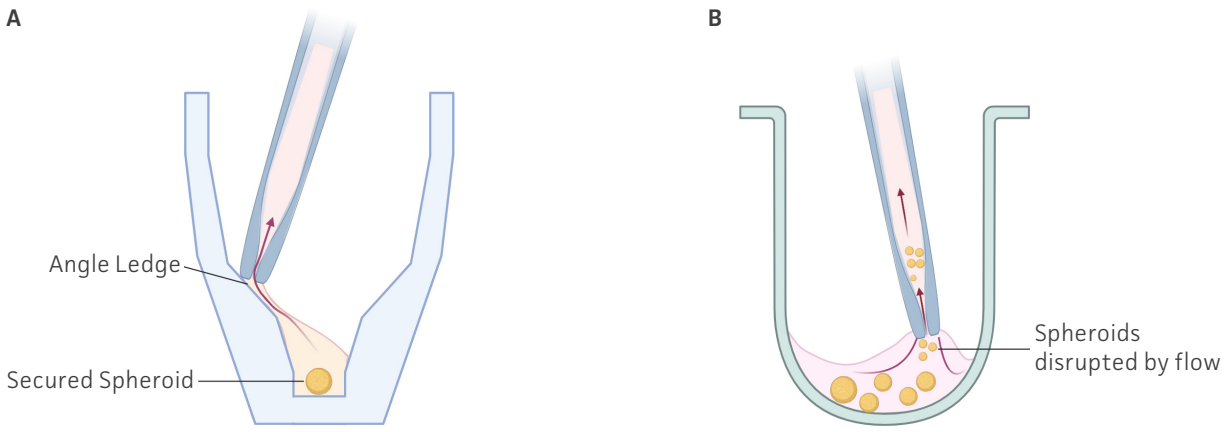


Figure 2: Comparison between a ThawReady™ well and U-bottom well. (A) The ThawReady™ well showing how more than 90% of the medium can be removed without disruption or aspiration of the spheroid. (B) The U-bottom well showing how spheroids can get disintegrated or disrupted with the flow, possibly causing accidental aspiration during medium exchange. Image created with Biorender.com.

In the following study, we compare the performance of the ThawReady™ Spheroid Kit microplate with a conventional U-bottom plate during spheroid formation and within a drug sensitivity assay.

MATERIAL AND METHODS

CELL LINE PREPARATION

HCT-116 cells (ATCC® CCL-247™) were used as the model in this study and were seeded in a U-Bottom plate. Prior to seeding, cells were expanded in T75 flasks according to the ATCC product sheet, using complete culture medium comprising McCoy's 5A (ATCC® 30-2007™) supplemented with 10% Fetal Bovine Serum (ATCC® 30-2020™). Once the cells reached confluency, they were collected and total viable cell count was assessed using a Vi-CELL BLU Cell viability analyzer (Beckman Coulter). We then redistributed 9.6 x 10^4 total viable cells across 48 wells of a U-bottom plate using complete culture medium (described above) and 9.6 x 10^4 total viable cells across the remaining 48 wells using ThawReady™ Spheroids Aggregation Medium (Figure 5); an average of 2000 cells were added per well. A regular cell-treated flat-bottom plate was seeded as well to evaluate a comparison against 2-D culture conditions.

On the same day, a ThawReady™ HCT-116 Spheroid Kit was thawed following ATCC protocol. In brief, the plate was positioned on the spheroids assay stand (provided in the kit) for 7 minutes at 37°C in a 5% CO_2 incubator, followed by slow dispensing of 180 µL of medium per well inside a biosafety cabinet, holding the pipette in a vertical position against the well ledge (Figure 3A). Subsequently, the plate was centrifuged at 250 x g for 2 min, followed by the gentle removal of 175 µL of medium using the pipette in an angled position against the angled ledge (Figure 3B).

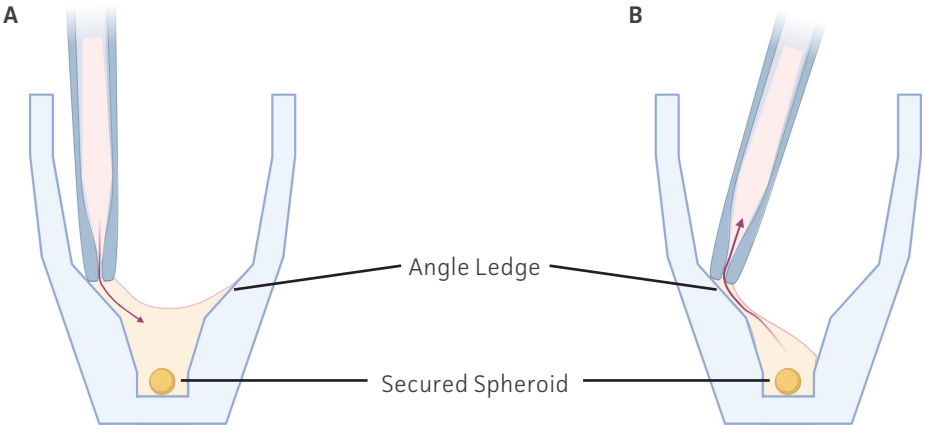


Figure 3: Pipette tip against angled ledge. (A) Using the pipette tip in vertical position is ideal for adding medium. (B) Using the pipette tip in tilted position is recommended during medium exchange to minimize disturbance of the spheroids. Image created with Biorender.com.

Spheroids were then washed by adding 70 µL of ThawReady™ Spheroids Aggregation Medium or complete culture medium to each well (Figure 5), followed by a centrifugation step at 250 X g for 2 min. The plate was then transferred to a 37°C incubator with 5% CO_2 using the spheroid assay stand.

The spheroids' size was evaluated using BioTek Cytation 5 Cell Imaging Multimode Reader (Agilent) 5 days after thaw (Figure 4).

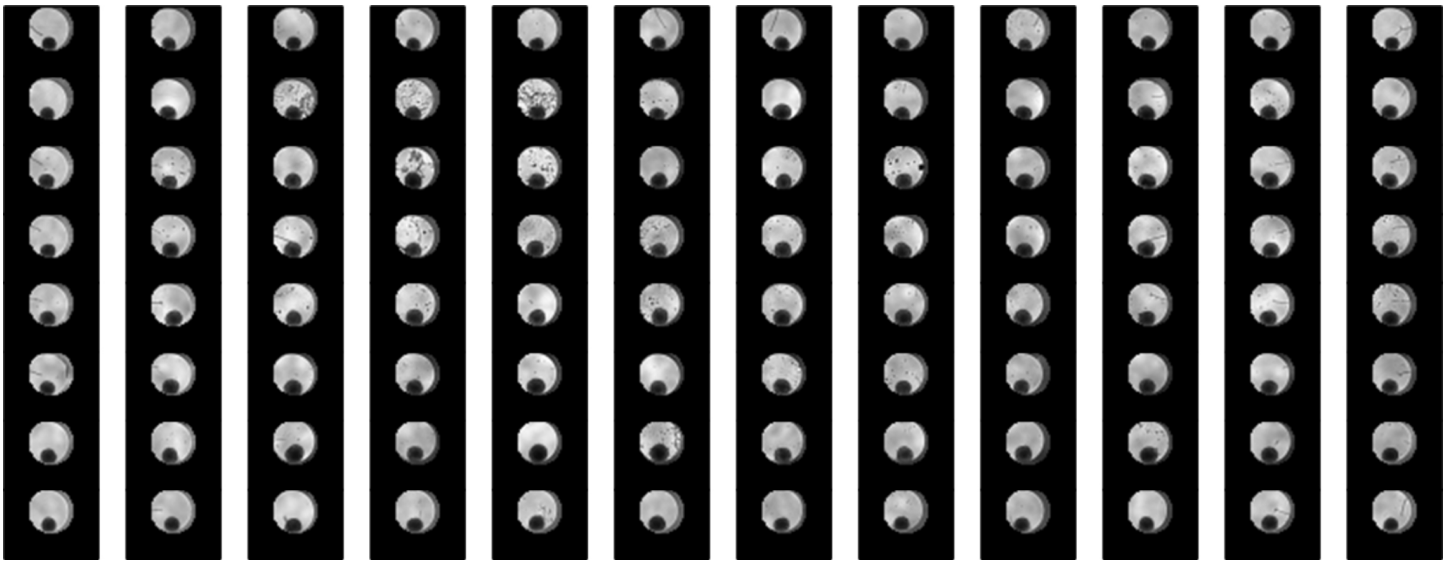


Figure 4: Well-by-well imaging showing consistency across the plate. Using the Cytation 5 Cell Imaging Multimode Reader (Agilent), each well was quickly and accurately imaged. An automatic program would report the spheroids' size

DRUG SENSITIVITY ASSAY

For each plate—ThawReady™ HCT-116 Spheroid, U-Bottom, and cell-treated flat bottom (2-D)—Staurosporine (Sigma) was used as a reference compound to assess the feasibility and reproducibility of the drug sensitivity assay (Figure 5). In brief, once the spheroids formed, Staurosporine was resuspended in DMSO (ATCC® 4-X™) and added to the cells at 5 different concentrations: 2 µM, 0.4 µM, 0.08 µM, 0.016 µM, and 0.0032 µM. Each concentration had 4 replicates for each independent media formulation. Medium + DMSO was used as a negative control or 0 value.

Cells were in contact with medium + Staurosporine for 72 hours. After incubation, the CellTiter-Glo 3D Cell Viability Assay (Promega) was carried out according to the manufacturer's instructions. ATP readout levels were measured using a BioTek Cytation 5 Cell Imaging Multimode Reader (Agilent).

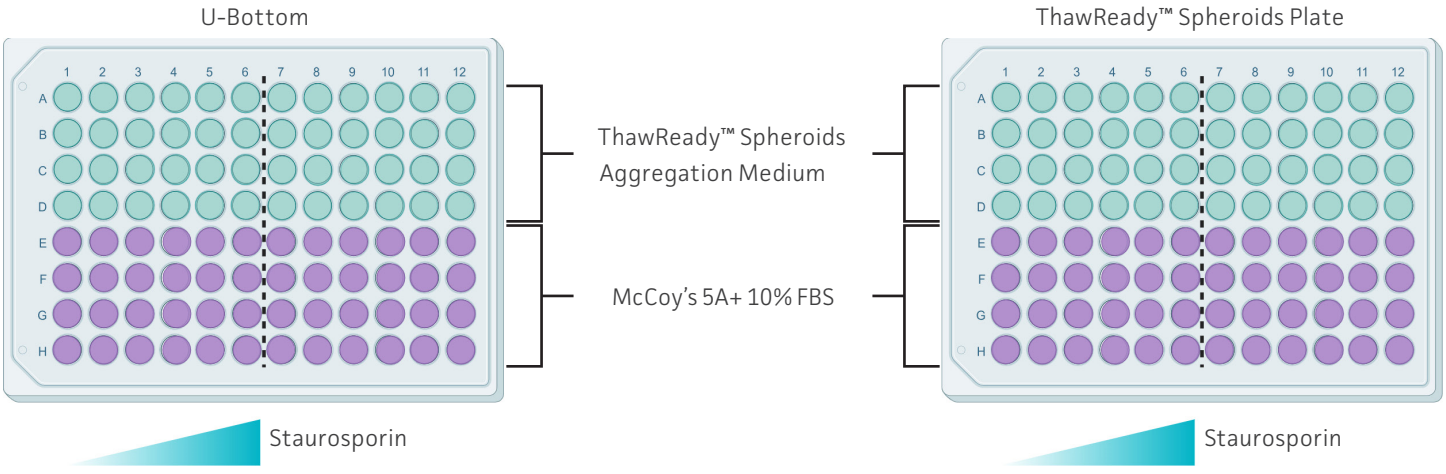


Figure 5: Schematic of spheroids preparation and Staurosporine treatment. Figure created with BioRender.com.

FORMALIN-FIXED, PARAFFIN-EMBEDDED SPHEROIDS

On day 11 after initial seeding, spheroids were gently harvested using a P1000 micropipette and fixed in 10% neutral buffered formalin (NBF) at a ratio of 20:1 (NBF to cell pellet). Samples were incubated overnight at room temperature to ensure complete fixation, followed by washing with Dulbecco's phosphate-buffered saline (D-PBS; ATCC® 30-2200™). Spheroids were then embedded in HistoGel (Fisher Scientific), previously liquefied at 55°C, using a 1:1 ratio of spheroids to HistoGel. After solidification, the gel disk was subjected to a graded ethanol dehydration series (80%, 95%, and 100%), followed by clearing in xylene. All the following steps are performed at room temperature. The HistoGel disk was immersed in 100% xylene for 45 minutes, transferred to a histology cassette, and incubated twice for 30 minutes each in molten paraffin. Finally, the cassette was placed into a mold, residual paraffin was added using a transfer pipette, and the sample was mounted for downstream histological processing.

HEMATOXYLIN AND EOSIN STAINING

Hematoxylin and Eosin (H&E) staining was performed on the formalin-fixed, paraffin-embedded spheroids to visualize their structure. Slides were stained following several steps (Table 1): First, the wax was removed using xylene, followed by hydration through graded alcohols to eliminate residual xylene and finally rinsing in water. Once hydrated, hematoxylin nuclear stain was applied, followed by a water rinse and application of eosin as the counterstain.

Table 1: H&E staining steps

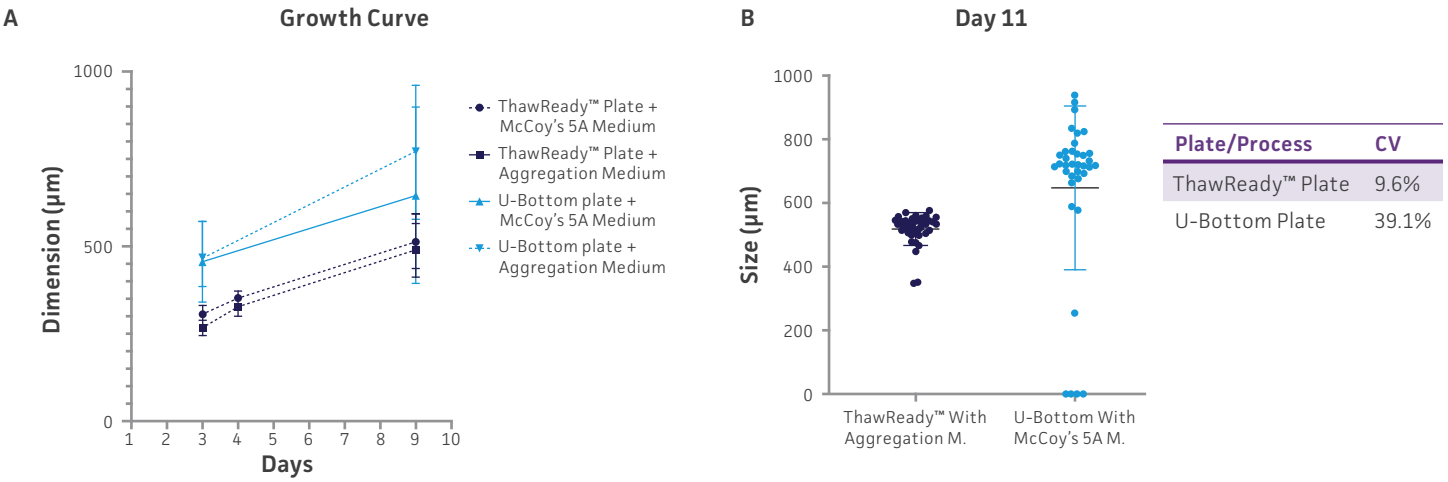
Reagent	Time
Xylene	5 minutes
Xylene	5 minutes
100% ethanol	30 seconds
95% ethanol	30 seconds
70% ethanol	30 seconds
Water wash	30 seconds
Hematoxylin	15-30 seconds
Water wash	30 seconds
Scott's Tap water	20 seconds
70% ethanol	20 seconds
Eosin	3-10 seconds
95% ethanol	30 seconds
100% ethanol	30 seconds
100% ethanol	30 seconds
Xylene	1 minutes
Xylene	1 minutes
Coverslip	

RESULTS

COMPARISON BETWEEN DIFFERENT PLATES AND MEDIA

Cells were seeded in the ThawReady™ plate with both Aggregation and McCoy's 5A complete medium, and in a more traditional U-Bottom plate with both Aggregation and McCoy's 5A complete medium. Size was calculated using an automatic protocol on the imager Cytation 5. The objective was to compare the functional impact of plate architecture and the culture medium on spheroid formation efficiency and long-term viability, emphasizing their combined importance in optimizing 3-D cell culture systems. We found that the U-bottom plate with the Aggregation medium or McCoy's 5A complete medium had larger spheroids that showed a necrotic core already by day 9 (Figure 6A). It is important to note that during the medium change, 7 spheroids were lost in the U-bottom plate, while 0 spheroids were lost in the ThawReady™ plate.

We then took a closer look at the difference between spheroids grown in the ThawReady™ plate with Aggregation medium and spheroids grown in the U-bottom plate with McCoy's 5A complete medium. The comparison provided insight into how the two established methods align in terms of performance and applicability. In addition to substantial size disparities (average of 700 µm in the U-bottom plate versus an average of 400 µm in the ThawReady™ plate), the analysis revealed significant variability among spheroids generated in different plate configurations. In fact, for the ThawReady™ plate, the CV is <10%, while for the U-bottom plate, the variability within the plate was above 39%.



Plate/Process	CV
ThawReady™ Plate	9.6%
U-Bottom Plate	39.1%

Figure 6: Comparison between different plates and media. (A) The growth curve for all four conditions is shown over time. (B) Spheroid size was evaluated for the two established methods: ThawReady™ plate with Aggregation medium and U-Bottom Plate with McCoy's 5A complete medium. The CV was evaluated.

DRUG SENSITIVITY

Drug sensitivity for Staurosporine was evaluated in the ThawReady™ plate, the U-bottom plate, and in traditional 2-D conditions using a flat-bottom cell-treated plate. For each plate, spheroids and cells were established in 48 wells using ThawReady™ Aggregation medium, and in another 48 wells, spheroids and cells were established and treated in McCoy's 5A complete culture medium, as per ATCC recommendations. We found that the cells behaved very differently based on the media or plate used (Figure 7).

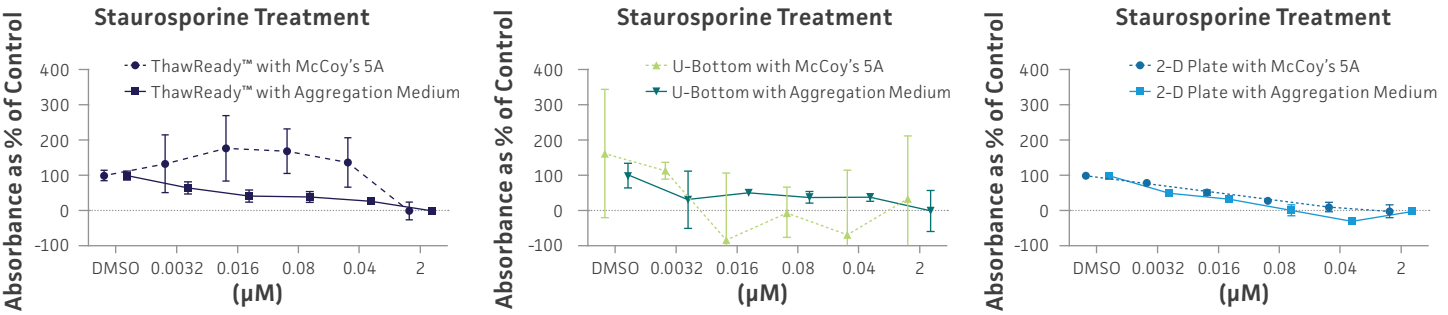


Figure 7: Drug sensitivity assay using different platforms and media. Sensitivity to Staurosporine was evaluated using McCoy's 5A complete culture medium and it was compared against sensitivity to Staurosporine in Aggregation Medium in ThawReady™ plate (A). The same comparison was performed in the (B) U-bottom plate and (C) 2-D flat bottom cell culture treated plate.

Under 2-D culture conditions (Figure 7C), the IC_{50} value for Staurosporine remained relatively consistent across media types (IC_{50} = 0.024 µM in Aggregation medium versus IC_{50} = 0.015 µM in McCoy's 5A complete medium), indicating that medium composition had a negligible impact on drug sensitivity in monolayer cultures. In contrast, spheroids cultured in U-bottom and ThawReady™ plates exhibited a markedly different response, underscoring the influence of 3-D architecture and plate format on compound efficacy.

For the U-bottom plate, the Aggregation medium helped reduce variability within the same drug concentration (Figure 7B); although, variability remains evident, with two out of six data points displaying relatively large error margins. The combination of U-bottom plate and McCoy's 5A complete culture medium showed a pronounced variation in response from spheroids treated with the same drug concentration. This inconsistency is likely attributable to the substantial initial heterogeneity in spheroid size, which can influence drug penetration, diffusion gradients, and overall sensitivity.

We also saw that combining the ThawReady™ plate with Aggregation medium yielded the most favorable outcome (Figure 7A), characterized by minimal variability and highly consistent results across replicates, indicating superior reproducibility and reliability of the experimental conditions.

FORMALIN-FIXED, PARAFFIN-EMBEDDED SPHEROIDS

At day 11, spheroids from both U-bottom and ThawReady™ plate were fixed in formalin and embedded in paraffin. Paraffin blocks were then sliced using a Leica Microtome (Leica) in 6 µm slices, and slides were stained with hematoxylin and eosin to evaluate the morphology. Spheroids generated in U-bottom plates with McCoy's 5A complete medium (Figure 8A) exhibited a pronounced necrotic core, indicative of compromised viability and diffusion limitations. In contrast, spheroids formed in the ThawReady™ plate using Aggregation medium were smaller yet structurally compact and morphologically healthy, underscoring the superior performance and functional reliability of the optimized plate-medium combination.

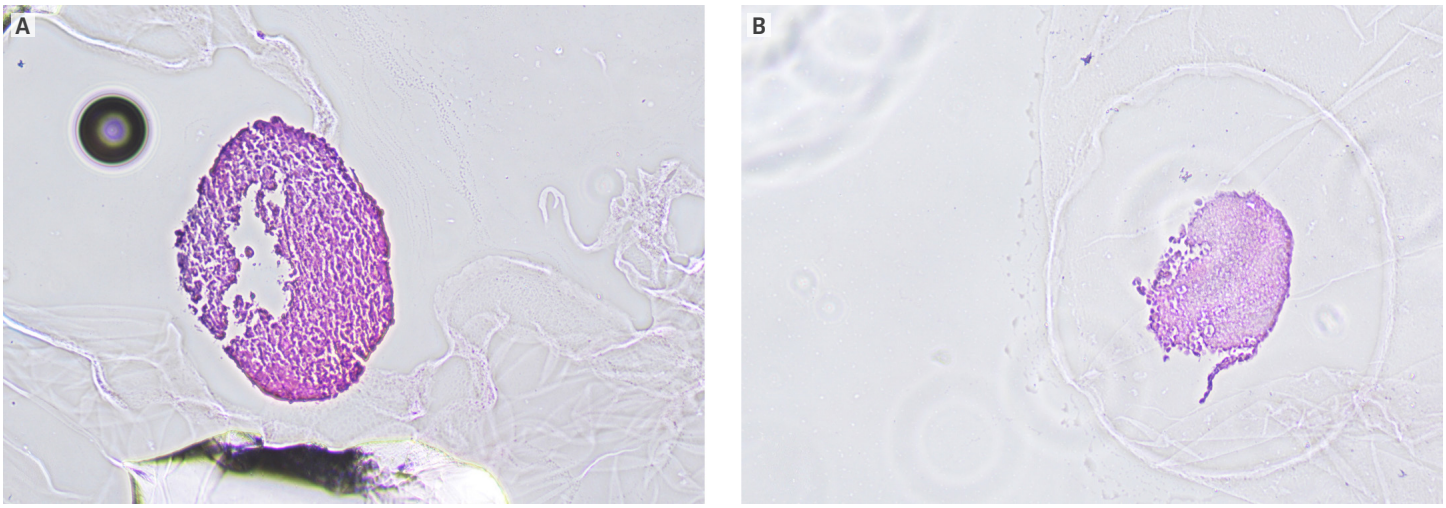


Figure 8: Formalin-Fixed, Paraffin-Embedded Spheroids. (A) The U-bottom spheroid with McCoy's 5A complete medium condition produced a large-sized spheroid with a necrotic core, while the (B) ThawReady™ plate with Aggregation medium produced a smaller-sized and structurally healthy spheroid.

CONCLUSION

Across formation, handling, and assay readouts, the ThawReady™ platform—particularly when paired with the Aggregation medium—consistently delivered spheroids that were small yet uniform (≈400 µm; CV < 10%) and structurally intact. In contrast, traditional U-bottom plates produced large spheroids (≈700 µm) with pronounced necrotic cores by day 9 and higher intra-plate variability (>39%). Notably, seven spheroids were lost during medium changes in U-bottom plates versus none with ThawReady™, underscoring the impact of well geometry and liquid-handling guidance on culture integrity.

Drug-sensitivity testing with staurosporine further emphasized these differences: 2-D monolayers showed comparable IC_{50} values across media, but 3-D responses depended strongly on plate format and medium. In U-bottom plates, McCoy's 5A complete culture medium supported spheroid formation but yielded inconsistent responses within the same concentration, likely due to initial size heterogeneity, while the Aggregation medium reduced (but did not eliminate) variability. In contrast, ThawReady™ combined with Aggregation medium produced minimal variability and highly reproducible dose-response behavior across replicates, indicating superior assay fidelity for compound testing in 3-D.

From a workflow perspective, the angled ledge and protective cavity of ThawReady™ enabled nearly complete medium exchange (>90%) without disturbing spheroids, reduced shear-induced damage, and eliminated the need for manual transfers between formation and endpoints. The optically clear flat bottom supported high-content imaging, providing a unified platform from aggregation through analysis.

Collectively, these results demonstrate that ThawReady™'s integrated design addresses major bottlenecks of traditional spheroid systems—media exchange, mechanical stress, and endpoint compatibility—thereby improving reproducibility, reducing bias in drug screening, and enhancing biological relevance. For studies requiring reliable IC_{50} estimation, longitudinal phenotyping, and histopathological evaluation, adopting ThawReady™ with the Aggregation medium offers a practical and robust path to high-quality 3-D data.

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TR-012626-v01