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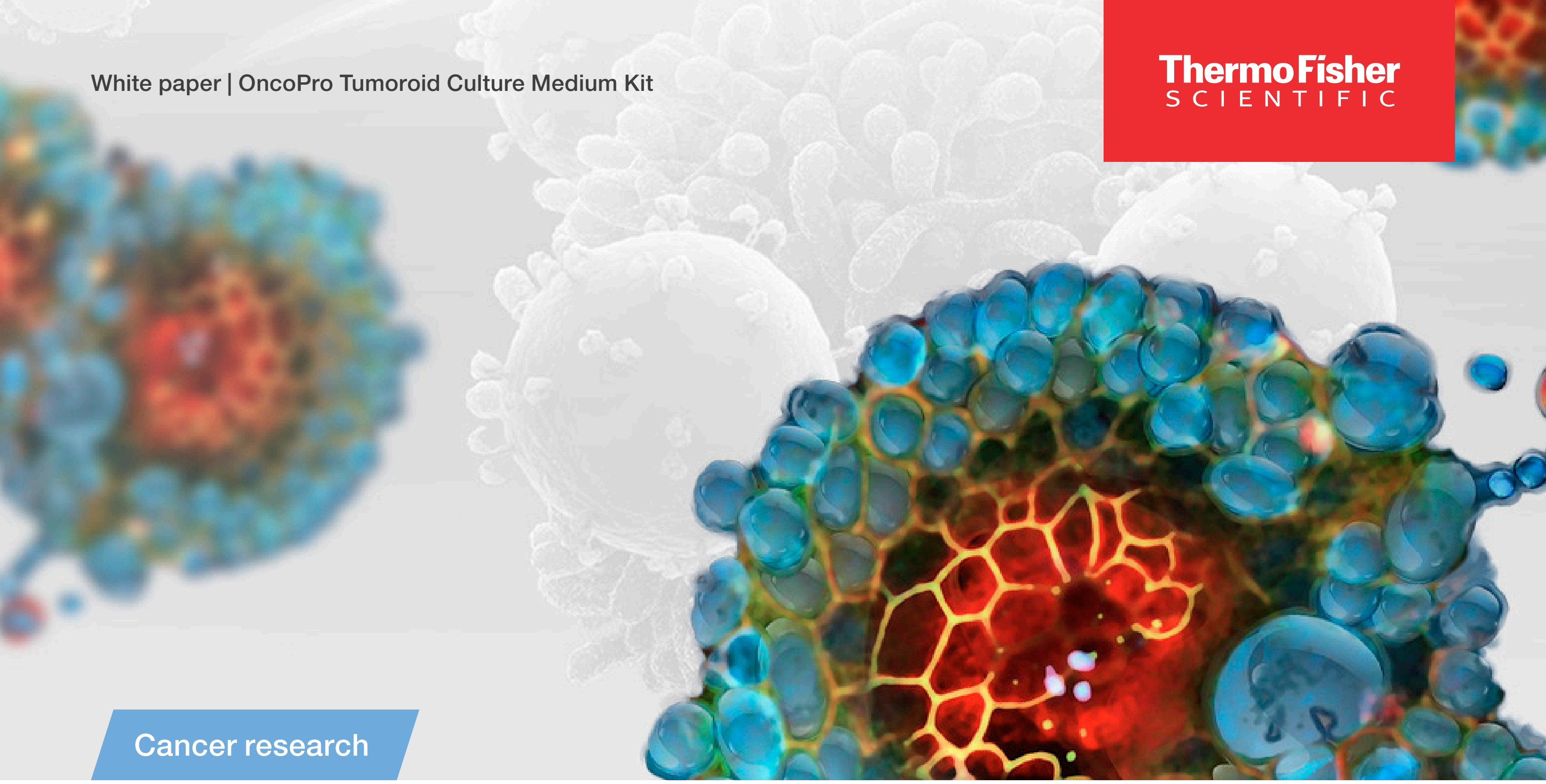

Cancer research

Using assay-ready, cryopreserved tumoroids for drug screening in cancer research

Keywords
Assay-ready, cancer research, cryopreservation, drug screening, tumoroids

The power of tumoroids in cancer research

Patient-derived tumoroids, also known as cancer organoids, are three-dimensional (3D) cell culture models that largely recapitulate the genetic profiles, gene expression levels, and architectures of *in vivo* tumors. Studies have shown that the drug responses of these models often mirror the clinical responses of patients, making the models invaluable in drug development, immunotherapy evaluation, and personalized medicine.

Streamlining functional assays with cryopreserved tumoroid biobanks

In many functional assays, cells are kept in culture for 1–2 passages post-thaw prior to experiments to stabilize growth rates and ameliorate issues that could arise due to recovery from cryopreservation. However, keeping cells in culture requires lengthy lead times prior to experiments and extensive cell culture work, especially when testing multiple cell or tumoroid lines. Here, we present an approach in which cryopreserved tumoroid biobanks established and expanded using the Gibco™ OncoPro™ Tumoroid Culture Medium Kit can be directly used for small molecule and immunotherapy cytotoxicity assays ("assay-ready" tumoroids). These advanced models offer a robust platform for high-throughput drug screening while significantly reducing the cell culture burden on researchers.

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Advantages of assay-ready tumoroids

Cryopreservation involves freezing tumoroids at ultralow temperatures to preserve their viability and functionality for future use. Assay-ready, cryopreserved tumoroids offer several advantages for drug screening applications:

- **Reduced cell culture burden**—Traditionally, maintaining active tumoroid cultures requires continuous and labor-intensive cell culture work. The use of cryopreserved, assay-ready tumoroids allows scientists to bypass this step, significantly reducing the cell culture burden. Tumoroids can be thawed and directly used in assays without the need for extensive preculture steps.
- **Consistent biological inputs**—Cryopreserved tumoroids help ensure a consistent supply of biologically relevant cancer models, reducing variability in experimental results. Researchers can use the same batch of tumoroids across multiple experiments.
- **Assay flexibility**—Drug screening can require a large number of cells. When combining the OncoPro Tumoroid Culture Medium Kit with a suspension culture approach, tumoroids can be produced in bulk, cryopreserved in large quantities, and thawed as needed. This helps ensure an ample supply for extensive screening campaigns without the need for continuous cell culture maintenance. Additionally, and as discussed in more detail in subsequent sections, assay-ready tumoroids can be used for multiple assays, including compound cytotoxicity and immune cytotoxicity testing.

Implementing cryopreserved tumoroids in drug screening

The workflow for using cryopreserved tumoroids in drug screening is straightforward and efficient. A typical process involves:

1. **Cryopreservation:** Tumoroids are expanded in culture, dissociated, and cryopreserved at desired cell densities.
2. **Thawing and seeding:** When needed, the cryopreserved tumoroids are thawed and seeded (based on viable cell number) into appropriate assay plates.
3. **Compound and/or effector cell addition:** After a culture period to allow for tumoroid formation, compounds or immune cells are added to the tumoroids.
4. **Assay readout:** Following a period of incubation with compounds or immune cells, parameters of interest are measured to gather assay results.
5. **Data analysis:** Results are collected and analyzed, providing insights into the effectiveness of the tested compounds.

High-content imaging, well-based viability assays, and other analytical techniques can be used to quantify the effects of perturbations on tumoroid number, proliferation, and phenotypic state, providing insights into drug efficacy, toxicity, and potential mechanisms of action. For instance, fluorescent indicators such as Invitrogen™ CellEvent™ Caspase-3/7 Detection Reagents can reveal apoptotic activity in response to treatment. Next we present two case studies using cryopreserved, assay-ready tumoroids.

Case study: assay-ready tumoroids for compound screening

We first examined how cryopreservation density could affect growth and viability in an assay-ready workflow by cryopreserving different numbers of cells per vial (Figure 1). Two human colorectal cancer tumoroid models (HuCo090123 and HuCo111622) were expanded in OncoPro Tumoroid Culture Medium, dissociated, and cryopreserved at densities of 2 x 10^6, 10 x 10^6, or 20 x 10^6 cells/mL in Gibco™ Recovery™ Cell Culture Freezing Medium. Tumoroids were thawed, and cells were collected for RNA sequencing and targeted mutational profiling using the Ion AmpliSeq™ Transcriptome Human Gene Expression Kit and the Ion Torrent™ Oncomine™ Comprehensive Assay v3C, respectively (Figure 1A). As a control, tumoroids in active culture (passaged at least twice after thaw and prior to assaying) were dissociated and sequenced. Thawed cells following cryopreservation at various cell densities were plated at equivalent numbers of viable cells per culture vessel, and cell counts and viability were monitored over four passages.

Cell expansion (as measured by cumulative population doublings (PDs)) and cell viabilities were nearly identical over the range of cryopreservation densities tested for both tumoroid lines (Figure 1B). Additionally, gene expression levels for a panel of 1,423 cancer-related genes were highly correlated within a given tumoroid line across freezing densities and similar to those from dissociated tumoroid samples from active culture (Figure 1C). The variant allelic frequency (VAF) of single-nucleotide variants (SNVs) was also donor-specific and nearly identical across conditions tested for a given tumoroid line (Figure 1D).

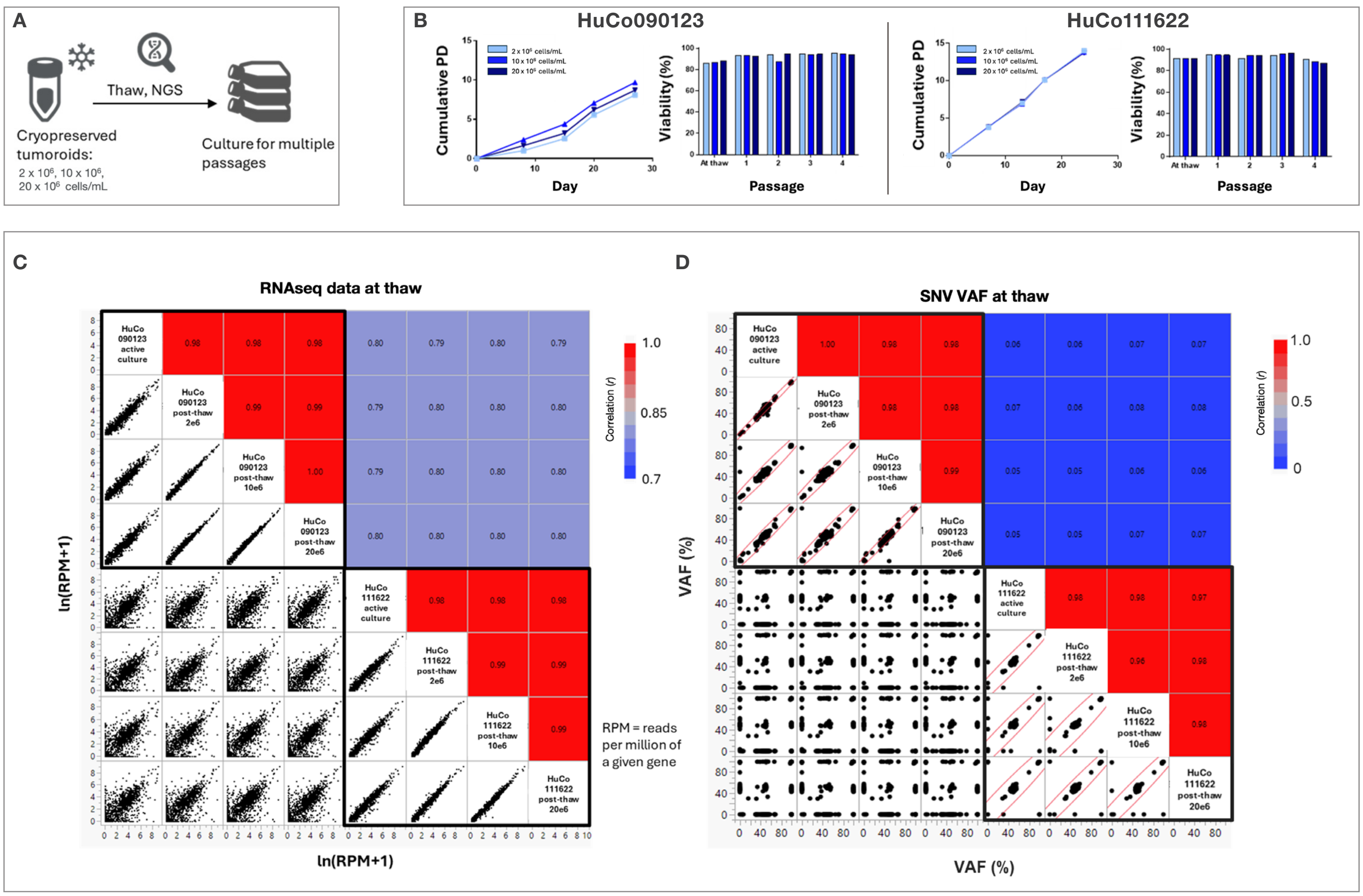
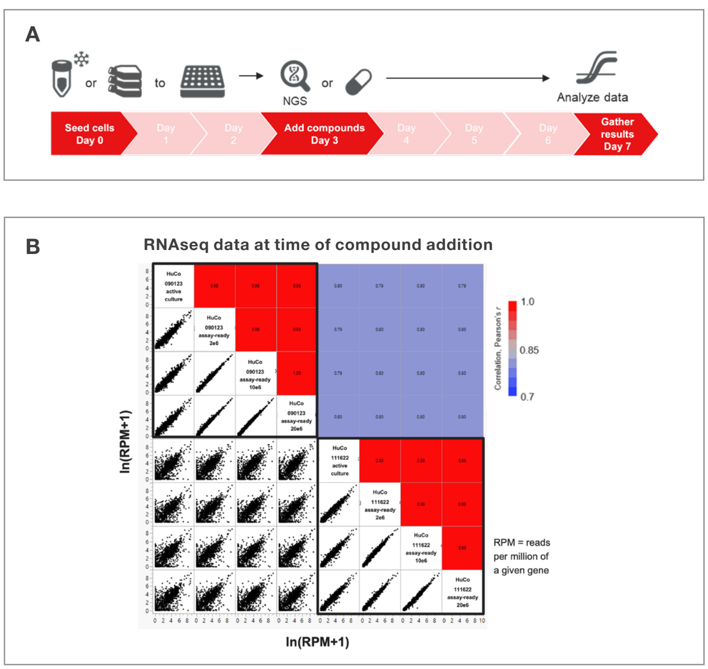


Figure 1. Dissociated colorectal tumoroids can be cryopreserved at various densities while retaining donor-specific growth rates, gene expression patterns, and mutations post-thaw. (A) Schematic of experimental setup. **(B)** Post-thaw cumulative PD and viabilities during culture of tumoroids following cryopreservation at 2 x 10^6, 10 x 10^6, and 20 x 10^6 cells/mL. **(C)** Pearson's correlation (*r*) in gene expression levels (1,423 cancer-related genes) between tumoroid cells dissociated from active culture and cells analyzed immediately post-thaw following cryopreservation at 2 x 10^6, 10 x 10^6, and 20 x 10^6 cells/mL. **(D)** Pearson's *r* for VAF of SNVs, indicated by dots, comparing cells from active culture and cells collected immediately post-thaw following cryopreservation at densities of 2 x 10^6, 10 x 10^6, and 20 x 10^6 cells/mL. The plot includes regression lines, confidence intervals, and density ellipses encompassing 90% of the data. Each dot represents the VAF for a genetic locus covered by the Oncomine Comprehensive Assay v3C. Data from two colorectal tumoroid lines (HuCo090123 and HuCo111622) are shown in panels (C) and (D).

Having established that tumoroids could be cryopreserved at various cell densities, we tested whether assay-ready tumoroids could be used for compound screening experiments. Assay-ready tumoroid cells were thawed, counted, and plated in 96-well plates at a density of 12,100 viable cells per well (Figure 2A). In parallel, tumoroids from active cultures were dissociated and plated at identical viable cell density. We first compared the mutational profile and transcriptomic state of tumoroids from active culture to the mutational profile and transcriptomic state of assay-ready tumoroids by isolating and analyzing RNA and DNA from untreated samples at three days post-plating, which is the timepoint at which we typically add compounds in screening experiments (Figure 2A). When HuCo111622 and HuCo090123 colorectal tumoroid cells were characterized by next-generation sequencing (NGS) at the point at which they “see” drug compounds, the transcriptomes of cells seeded directly post-thaw were donor-specific, highly correlated with those of cells seeded from active culture, and independent of cryopreservation density (Figure 2B). Additionally, colorectal tumoroids (HuCo111622) for which we had material available for sequencing also showed nearly identical VAF of SNVs) at the time of compound addition regardless of the condition from which they were plated (Figure 2C).

We next tested the functional response of assay-ready HuCo111622 colorectal tumoroids. Compounds were added after three days of culture in post-thaw or post-passage 9-point dilution series. Tumoroid survival was assessed 7 days after plating using ATP quantification, and normalized relative luminescence units (RLU) were plotted for each condition (Figure 2A). The dose responses of HuCo111622 colorectal tumoroids to azaserine and staurosporine were nearly identical when comparing cells seeded from active culture to cells seeded from cryopreservation following freezing at densities of 2 x 10^6, 10 x 10^6, and 20 x 10^6 cells/mL (Figure 2D). To test the generalizability of the assay-ready approach to tumoroids from other cancer indications, a human lung tumoroid line, HuLu051421, was plated in the assay-ready condition after cryopreservation at 2 x 10^6 cells/mL and showed functional response similar to cells from active culture (Figure 2E). These results suggest that HuCo111622 and HuLu051421 tumoroid cells can be plated directly at thaw and assayed according to this protocol, with results expected to be comparable to those seen when using cells in active culture. Discussion of the results using HuCo090123 tumoroids is included below.



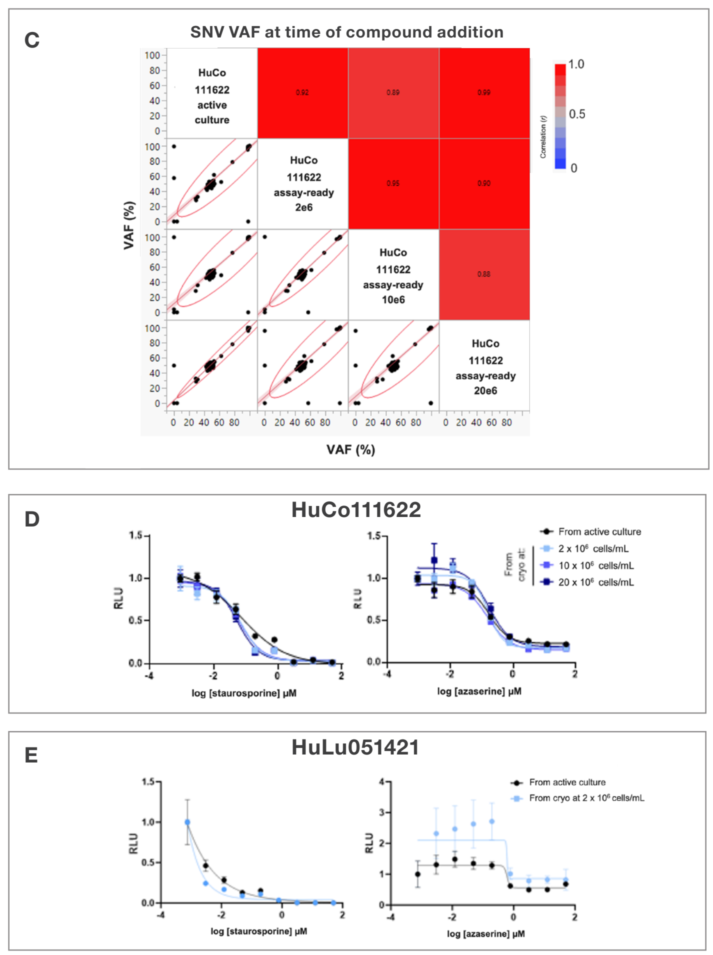


Figure 2. Use of cryopreserved tumoroid banks as assay-ready cells in compound screening experiments. (A) Schematic of experimental setup. **(B)** Pearson’s *r* in gene expression levels (1,423 cancer-related genes) at three days post-seeding (i.e., at time of compound addition) between tumoroid cells dissociated from active culture and cells seeded immediately post-thaw following cryopreservation at 2 x 10^6, 10 x 10^6, or 20 x 10^6 cells/mL. Data from two colorectal tumoroid lines (HuCo090123 and HuCo111622) are shown. **(C)** Pearson’s *r* for VAF of SNVs comparing HuCo111622 tumoroid cells from active culture and cultured for three days to cells plated post-thaw following cryopreservation at densities of 2 x 10^6, 10 x 10^6, and 20 x 10^6 cells/mL and cultured for three days. The plot includes regression lines, confidence intervals, and density ellipses encompassing 90% of the data. Each dot represents the VAF for a genetic locus covered by the Oncomine Comprehensive Assay v3C. **(D)** Dose-response curves for colorectal (HuCo111622) tumoroids seeded for treatment with staurosporine or azaserine from active culture or directly post-thaw following cryopreservation at the indicated density. Plots show mean ± standard error of the mean (SEM) from triplicate wells. **(E)** Dose-response curves for lung (HuLu051421) tumoroids seeded for treatment with staurosporine or azaserine from active culture or directly post-thaw following cryopreservation at the indicated density. Plots show mean ± SEM from triplicate wells.

Case study: assay-ready tumoroids for immune cell cytotoxicity testing

To test whether cryopreserved banks of dissociated tumoroid cells could be thawed, plated, and used directly for immunotherapy cytotoxicity assays, HuCo1044-GFP colorectal tumoroids were dissociated and seeded from active cultures ("fresh") or thawed from cryovials (following cryopreservation at 2 x 10^6 cells/mL; "frozen"), counted, and plated (Figure 3A). After a three-day period to allow for tumoroid formation, natural killer (NK) cells differentiated from induced pluripotent stem cells (iNK cells) were added to the tumoroids at various effector (iNK cell) to target tumoroid cell (E:T) ratios.

Cells were stained with Invitrogen™ CellEvent™ Caspase-3/7 Red Detection Reagent and imaged over time on a Sartorius™ Incucyte™ Live-Cell Analysis System. Green fluorescent protein (GFP) intensity and caspase signal over time were quantified, and results were nearly identical when comparing tumoroid cells seeded from active culture versus those seeded directly from cryopreserved biobanks (Figure 3B, C).

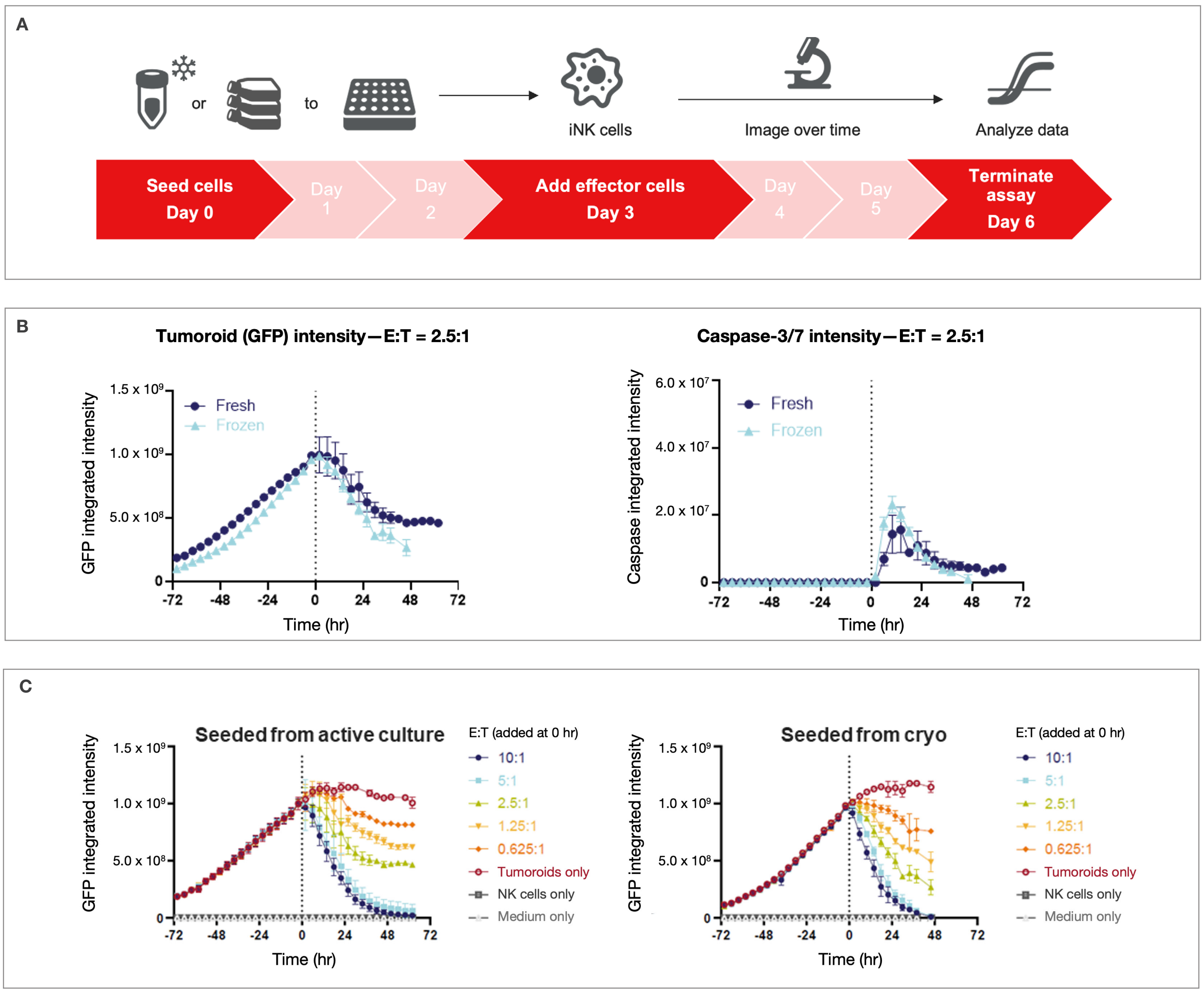


Figure 3. Assay-ready tumoroids can be used to evaluate iNK cell cytotoxicity. (A) Schematic of experimental setup. **(B)** Plots of tumoroid (GFP) intensity and caspase-3/7 intensity over time (as total intensity per well, GCU x µm²/well) when using tumoroids seeded from active culture or directly post-thaw for an E:T ratio of 2.5:1. **(C)** Tumoroid (GFP) intensity over time (as total intensity per well, GCU x µm²/well) at various E:T ratios when using tumoroids seeded from active culture or directly post-thaw. In frames B and C, the dotted lines represent time of iNK cell addition, and plots show mean ± standard deviation (SD) from 3–4 wells.

Challenges with assay-ready tumoroids

Direct use of cryopreserved tumoroids offers numerous advantages and has generated results concordant with those obtained using cells in active culture for the majority of tumoroid lines we have tested. However, there are instances in which certain tumoroid lines from cryopreserved cells may generate different results if used in the assay-ready protocol compared to tumoroid lines from active cultures. Tumoroids may not be compatible with the assay-ready approach if they exhibit:

- **Slow recovery from cryopreservation**—Some tumoroid lines may significantly lag in growth upon post-thaw culture following cryopreservation and require multiple passages for the growth rate to stabilize. For these cultures, tumoroid number may decrease over the first passage post-thaw, remain stable, or increase at a lower rate than that seen in later passages (see, for example, post-thaw growth curve of colorectal tumoroids HuCo083123 in Figure 4A compared to growth curves for HuCo111622 and HuCo090123 in Figure 1B). This extended recovery time can affect the response of tumoroids to compounds, particularly those that target metabolic activity or cell division, leading to differential response when compared to cells assayed from active culture (Figure 4B). This result contrasts with that seen with HuCo111622, where cells expand immediately upon thaw and generate results similar to cells in culture (see Figures 1 and 2).

- **Inconsistent morphology upon thaw**—For colorectal tumoroid line HuCo090123, we have observed that, although cell growth, viability, and gene expression levels post-thaw are similar to those from tumoroids in culture (Figures 1 and 2), differences in cell adhesion can affect relative sensitivity to compounds (Figure 5). These differences are likely due to accessibility of cells within the tumoroids to compounds, which underscores the importance of structural components of culture vessels during assays. Other tested tumoroids showed minimal adhesion to non-treated culture vessels and retained consistent 3D morphology. To address the issue of sticky tumoroid lines, a practical workaround involves coating all vessels with 7.5% bovine serum albumin (BSA) for at least 30 minutes prior to seeding. This coating helps reduce adhesion.

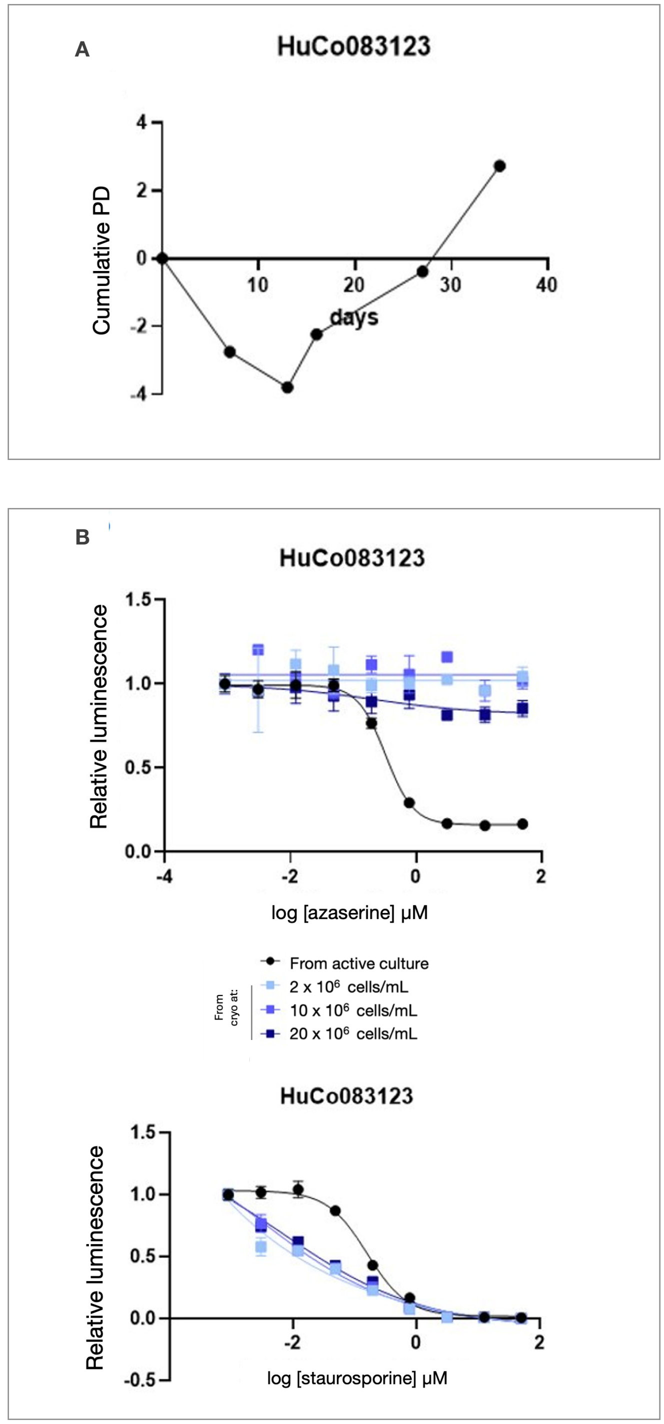


Figure 4. Tumoroids that would not be considered assay-ready due to slow recovery from cryopreservation. **(A)** Post-thaw cumulative population doublings during culture of colorectal (HuCo083123) tumoroids following cryopreservation at 2 x 10^6 cells/mL. **(B)** Dose-response curves for colorectal (HuCo083123) tumoroids seeded for treatment with azaserine or staurosporine from active culture or directly post-thaw following cryopreservation at indicated density. Plots show mean ± SEM from triplicate wells.

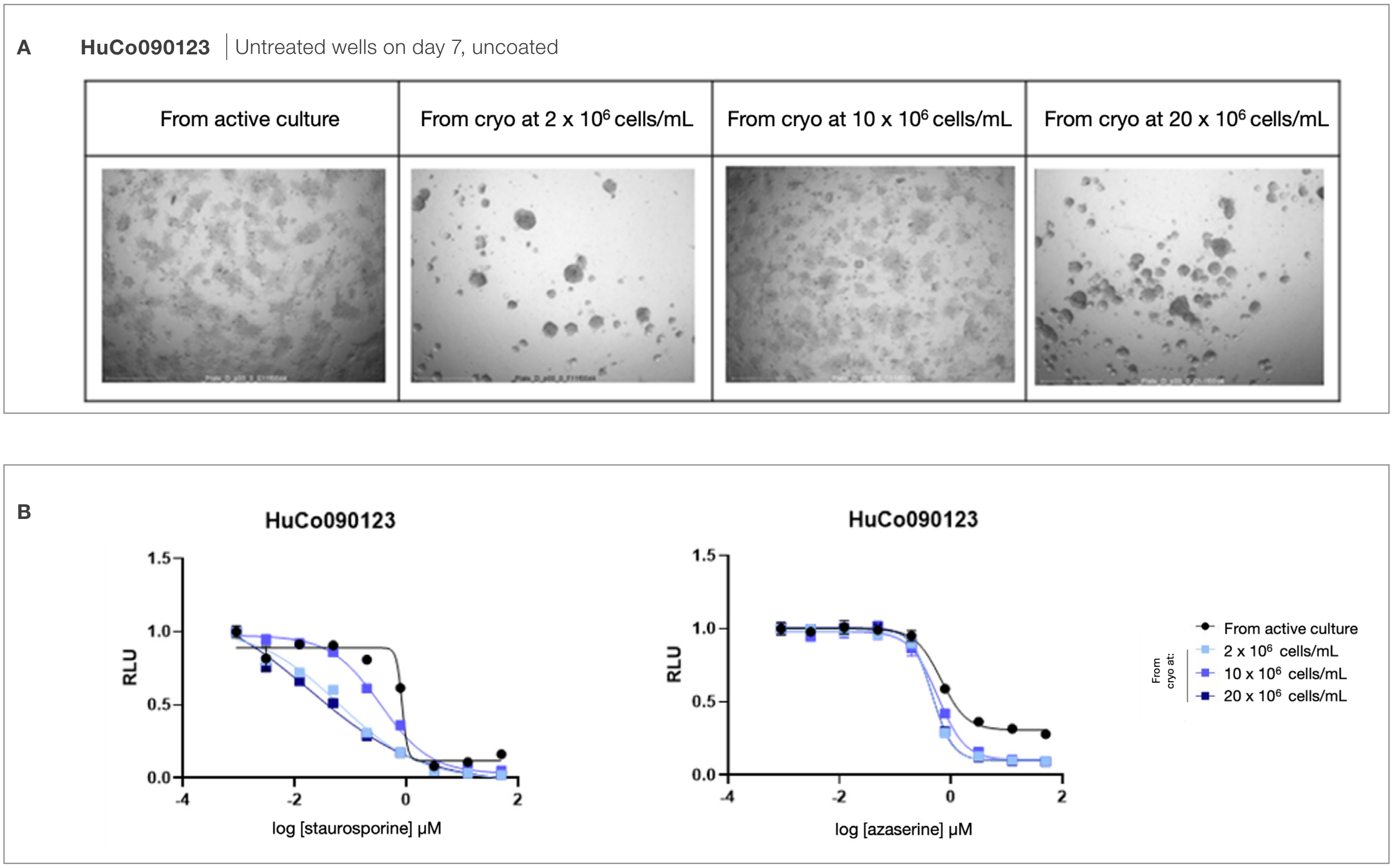


Figure 5. Tumoroids with inconsistent morphology. (A) Images of untreated colorectal (HuCo090123) tumoroids after 7 days of growth from active culture or indicated cryopreserved densities. Vessels were not coated prior to seeding. **(B)** Dose-response curves for colorectal (HuCo090123) tumoroids seeded for treatment with azaserine or staurosporine from active culture or directly post-thaw following cryopreservation at indicated density. Plots show mean ± SEM from triplicate wells.

These results suggest that tumoroid lines should be functionally qualified prior to use in assay-ready formats on a line-by-line basis to help ensure that results are consistent with those seen when tumoroids in active culture are used.

Conclusion

Cryopreserved, assay-ready tumoroids offer a reliable and efficient platform for high-throughput drug screening. By combining the physiological relevance of 3D tumoroid models with the convenience and consistency of cryopreservation, researchers can streamline their workflows and reduce cell culture burden, ultimately accelerating the discovery of effective cancer therapies. As the field continues to evolve, the integration of cryopreserved tumoroids into drug screening workflows promises to enhance our understanding of cancer biology and improve treatment outcomes for patients.

Ordering information

Product	Cat. No.
OncoPro Tumoroid Culture Medium Kit	A5701201
CellEvent Caspase-3/7 Detection Reagents	C10423
OncoPro Tumoroid Cell Lines	Varies
Recovery Cell Culture Freezing Medium	12648010
Ion AmpliSeq Transcriptome Human Gene Expression Kit	A26326
Oncomine Comprehensive Assay v3C	A35806

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