

Proliferation and cell death analyses of 3D cultures using Revvity CellCarrier Spheroid ULA microplates and ATPlite 3D products.

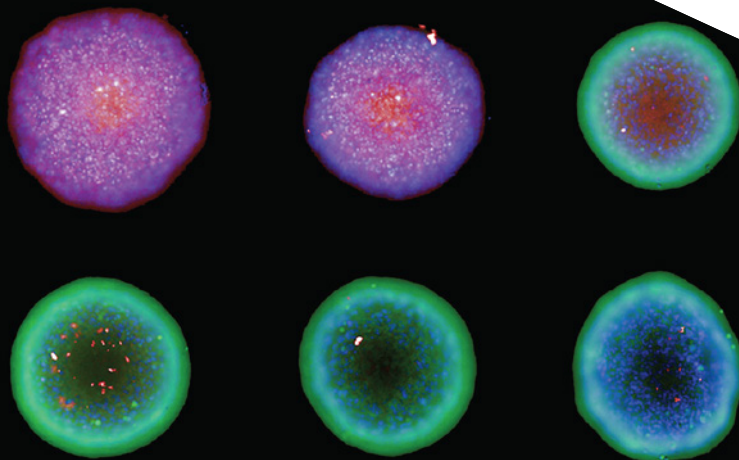
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Introduction

In the process of developing new therapeutic molecules, toxicity - whether it is to be avoided or whether it is desired - is an important feature to consider as early as possible in the development flow. This should be analyzed in a cellular model as predictive as possible of the native physiology in order to avoid the high development cost of compounds failing later in clinical testing phases. Over the past 15 years, 3D cell culture has become an increasingly popular technique that's been implemented into a variety of workflows pertaining to many applications by scientists across the globe. Growing cell cultures in 3D promotes native cellular interactions, mimicking *in vivo* microarchitecture for more biologically-relevant information. This is achieved by allowing cells *in vitro* to grow in all directions as opposed to only two dimensions in traditional adherent or monolayer (2D) cultures. By providing more physiologically-relevant data, the incorporation of 3D culture techniques into drug discovery workflows has demonstrated the ability to reduce downstream costs caused by the failure of 2D-assays to accurately predict toxicity and efficacy of drugs in development. Revvity's CellCarrier™ Spheroid ULA 96-well microplates have a unique Ultra-Low Attachment (ULA)-coated surface that enables the formation of consistently round spheroids from numerous different cellular models. It's important to note that this ULA coating also helps to eliminate satellite spheroid growth, allowing for easier imaging data acquisition and analysis and better well-to-well consistency in the cellular material analyzed.



An easy, sensitive and widely-accepted way to assay cell viability, metabolism, and proliferation is to look at the Adenosine triphosphate (ATP) content of cells in cultures. ATP is a vital energy transfer molecule used by all cells. The concentration of ATP in cell cultures drops rapidly when cell viability is negatively impacted. In particular, ATP levels decline rapidly when cells undergo necrosis or apoptosis, making the monitoring of ATP concentration a good indicator of a compound's cytotoxic and cytostatic effects. In addition, as the global ATP levels of a cell population will increase when cells are replicating, ATP levels can also be used as a good marker for analyzing proliferation effects.

Revvity's ATPlite™ and ATPlite 1step Luminescence Assay System are established kits that have been successfully used to quantify ATP content in traditional 2D culture models to measure cellular viability and proliferation. The assays are based on the production of light caused by the reaction of ATP generated by cells in culture with added firefly (*Photinus pyralis*) luciferase and D-luciferin. Revvity's ATPlite™ 3D and ATPlite 1step 3D Luminescence Assay Systems are also available for the specific analysis of the viability and growth of cells in 3D spheroid cultures. As in 2D applications, cells are first lysed in order to make ATP accessible to the reporter luciferase enzyme. Subsequently, light is generated when both the enzyme and its cofactors are present, each of which are provided in excess. ATP is the limiting factor in this reaction, thus the intensity of light emitted will be proportional to the ATP content of the sample.

In both the 1-step and 2-steps assays, the rate of the enzyme is carefully controlled by the use of a specific assay buffer, so that the naturally fast rise ("flash") and drop of light emission by firefly luciferase is transformed into a stable "glow" signal, allowing ample time between the cell lysis process and measuring the signal. In the ATPlite 1step 3D assay, the addition of a single reagent provides the detergents necessary for spheroid cell lysis and the enzyme and cofactors necessary for the generation of light. In the 2-step ATPlite 3D assay, the spheroids are first lysed using a sodium hydroxide-based lysis solution. In these conditions, ATP is very stable and the basic pH permanently inactivates the cellular ATPases, preventing later competition against firefly luciferase. In a second addition step, the reagents necessary for light generation, in a pH neutralizing solution, are added to the sample. As a result of these two addition steps, cell lysis with ATPlite 3D is highly efficient, and the signal generated is substantially more stable over time, with a half-life of more than four hours.

We demonstrate here that ATPlite 3D and ATPlite 1step 3D can be reliably used to assess ATP levels of various cell types cultured in 3D spheroids in Revvity's CellCarrier™ Spheroid ULA 96-well microplates.

Materials and methods

Cell culture methods

HeLa (human cervical cancer), DU 145 (human prostate cancer), and HEK293 (human embryonic kidney) cells were thawed from frozen stocks (ATCC® catalog numbers CCL-2, HTB-81, and CRL-1573), re-suspended and maintained in culture in Eagle's Minimum Essential Medium (EMEM; ATCC Cat. No. 30-2003) in 75 mm² flasks. HEK293 cells were grown up on PDL-coated flasks (VWR Cat. No. 89020-034). All growth media contained 10% Fetal Bovine Serum (FBS; ThermoFisher Cat. No. 26140079), and Penicillin/Streptomycin (ThermoFisher Cat. No. 15140122). Culture media used for 3D spheroid formation was the same used for growing 2D cell cultures, including the same concentration of serum.

For the generation of 3D spheroids, cell lines were harvested to single cell suspensions by first rinsing with PBS (without Ca²⁺ and Mg²⁺, ThermoFisher Cat. No. 14190144) and applying approximately 1 mL of 0.25% Trypsin-EDTA (ThermoFisher Cat. No. 25200056) to separate cells. Cells were counted, spun down, and re-suspended in fresh medium to the cell density needed to obtain the final number of cells desired per well for each experiment (cell density was varied to obtain various spheroid sizes: cell numbers are specified in the Results section and figure legends for each experiment). 100 µL per well of cell suspension (or of media for negative control wells) were seeded in CellCarrier Spheroid ULA microplates, with a manual multichannel pipettor (8- or 12-wells at a time). The round well shape and ULA-coating prevents cells from adhering to the polystyrene plate and promotes cellular interaction and the scaffold-free self-assembly of small spherical microtissues. Figure 1 illustrates the workflow for seeding and growing 3D spheroid cultures. Cultures were fed with fresh media by halves (removing 50 µL of "old" medium and adding 50 µL of fresh medium, in order to avoid losing the spheroid by aspiration when removing the "old" medium) within two days after seeding and every two to three days afterwards. In some cases, cultures were treated with a toxic compound, added with 50 µL of culture medium during a feeding step containing 2X the final concentration.

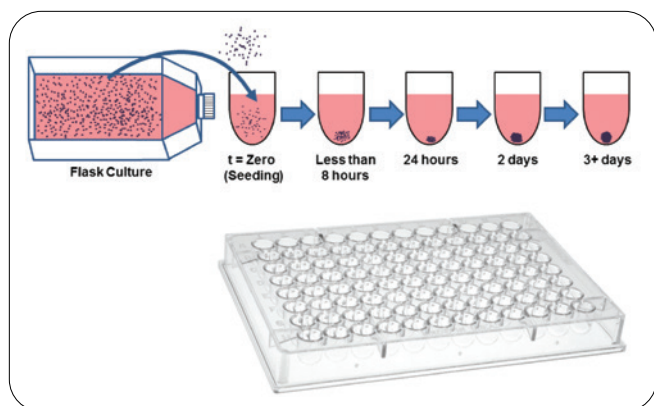


Figure 1: Above - Workflow for generating and growing cells in 3D spheroid cultures in Revvity CellCarrier Spheroid microplates; Below - Image of a 96-well CellCarrier Spheroid ULA microplate.

CellCarrier Spheroid ULA 96-well microplates are available as a 10-pack of individually pouched plates with lids (Cat. No. 6055330) or as a case containing 40 lidded plates packed as two bags with 20 plates each (Cat. No. 6055334). For more detailed information on growing, staining and imaging 3D spheroids in CellCarrier Spheroid microplates, please refer to the “User’s Guide to Cell Carrier Spheroid ULA Microplates” available on the Revvity website.

Live cell staining and spheroid imaging

In order to assess spheroid size and cell health in some experiments, cultures were stained with Hoechst 33342 (5 µg/mL; Life Technologies, Cat. No. H3570), Calcein AM (0.5 µM; Life Technologies, Cat. No. C3100MP), and SYTOX® Red (1:1000 dilution; Life Technologies, Cat. No. S34859) by direct addition of each fluorescent marker to the culture medium. Images were acquired in all microplates using the Operetta® High Content Imaging system equipped with Harmony Data Acquisition and Analysis Software. Brightfield and fluorescence imaging was performed using the 10X long WD objective in either non-confocal (for rapid visualization of all wells) or confocal mode (for better visualization of individual, fluorescently-labeled cells inside spheres). All plate wells for each experiment were imaged in brightfield mode to generate whole-plate images as a back-up measure to assess the presence of debris in the culture or absence of a spheroid in case of outliers observed in luminescence data.

ATP-based luminescence assays

ATPlite is the assay of choice when a long-lived luminescent signal with a half-life greater than four hours is required. The kits are shipped at ambient temperature and stored at 4 °C so there is no thawing required before use. The standard ATPlite protocol for “2D” adherent and suspension cell cultures is as follows: Grow cells in opaque microplates (white CulturPlates are recommended, all Revvity catalog numbers are presented in Table 3 on the last page). Equilibrate buffers and lyophilized reagent to room temperature, prepare [ATP] standard curve in media (if wanting to convert luminescent counts from the samples into absolute ATP concentrations), and add 50 µL of mammalian cell lysis solution to 100 µL of cells in culture medium (for 96-well plate format). Shake the plate vigorously for five minutes (700 RPM, with a plate-shaker with 3.0 mm orbit diameter). Reconstitute the lyophilized substrate solution with substrate buffer solution and add 50 µL per well. Shake again for another five minutes, dark adapt the plates (if necessary) and measure luminescence. ATPlite 1step is a rapid, single addition assay that does not require a separate lysis step but presents a shorter half-life. It also has a higher light-output and a reduced Phenol Red dependency compared to the 2-steps assay. The standard ATPlite 1step protocol for adherent and suspension cell cultures is as follows: Grow cells in white opaque cell culture plates as noted above. Equilibrate buffers and lyophilized substrate to room temperature, prepare [ATP] standard curve in media (if conversion to [ATP] values is desired), reconstitute the lyophilized substrate with buffer, and add 100 µL to 100 µL of cells in culture medium (for 96-well plate format). Shake plate vigorously for two minutes, dark adapt plates (if necessary) and measure luminescence.

ATPlite™ 3D and ATPlite 1step 3D Kits perform like their counterparts used in traditional 2D cultures but with some additional steps added to the protocols that allow for maximal extraction of ATP from cells in spheroid microtissues. In addition to the ATPlite assay reagents, each 3D kit also comes with a 96-well CellCarrier Spheroid ULA Microplate for spheroid culture development, and with an OptiPlate-96 HS (Gray) microplate for the luminescence reading. The protocol and procedure adaptations are discussed in the next section. All luminescence reads were performed using the Revvity EnSight™ Multimode Plate Reader. All cellular imaging and quantification of 2D cultures was performed using the cellular imaging mode of the EnSight plate reader and Kaleido™ Analysis software.

Results and discussion

Optimizing protocols for using ATPlite 3D kits with spheroids

Standard ATPlite and ATPlite 1step assays, developed for use with monolayer and suspension cultures, have not proven to be sufficient for lysing spheroid microtissues effectively, especially in microtissues seeded with larger cell numbers (producing larger spheres). This is due mostly to the tight cell-to-cell adhesions in a spheroid that act to protect cells in the central region from the detergents and other lytic components of the ATPlite buffers. In a 2D or suspension culture, each cell's membrane is at least in part exposed to the liquid environment in the well.

Another concern of using the standard ATPlite procedures is that it calls for reading luminescence directly in the assay plate, which is not recommended with the translucent plates we (and many others) use for culturing spheroid tissue as they suffer from considerable cross-talk. One option would be to transfer spheroids into an opaque plate and running the assay in that plate. However, that procedure is not straightforward to perform manually or simple to automate, can affect spheroid morphology, and can produce significantly more variability in your assay - in particular if the spheroid is lost in the transfer process for some wells.

In order to overcome this, we developed the ATPlite 3D and ATPlite 1step 3D kits and optimized the protocols to provide for the most effective lysis of spheroid microtissues and the least assay variability. To do this, the assay is started directly in the CellCarrier spheroid microplate with the final step requiring transfer of a quarter of the volume to an opaque plate for reading luminescence. The assay procedure for each kit is illustrated by a flowchart in Figure 2. Please note that the substrate solutions listed in the protocol must first be suspended with the appropriate buffer provided in the kit with the volume indicated in the manual and solutions should all be equilibrated to room temperature beforehand (as with the original 2D assay kits).

The protocol for both assays calls for the addition of an initial reagent solution that contains components which lyse individual cells in spheroids. In the original 2D kit protocols, the subsequent step of shaking the plate is sufficient for cell lysis of adherent or suspension cells. However, since this procedure calls for eventual transfer of the reaction to another plate in the "3D" assay, it is not advised to seal the plates. Therefore, violent shaking of plates is not recommended here as it could lead to well-to-well

cross-contamination. The 3D protocol requires a gentler shaking step (in our case, we used 700 RPM on a DELFIA Plateshake, a Revvity plate shaker with a 1.5 mm orbital diameter). Even with lids on the plates, gentler shaking is recommended to prevent splashing between wells. For other plate shakers, we recommend that you slowly increase the speed until you're comfortable that there is no overflow of reagents in a well.

Even with more vigorous shaking of the plate, we determined that it's not possible to shake the plate violently enough to get the reagents to penetrate the spheroid in the round-bottomed plates - partly due to plate geometry and due to tissue thickness. To compensate for the need for aggressive shaking, a longer incubation time and the addition of pipette mixing steps during reagent additions was included.

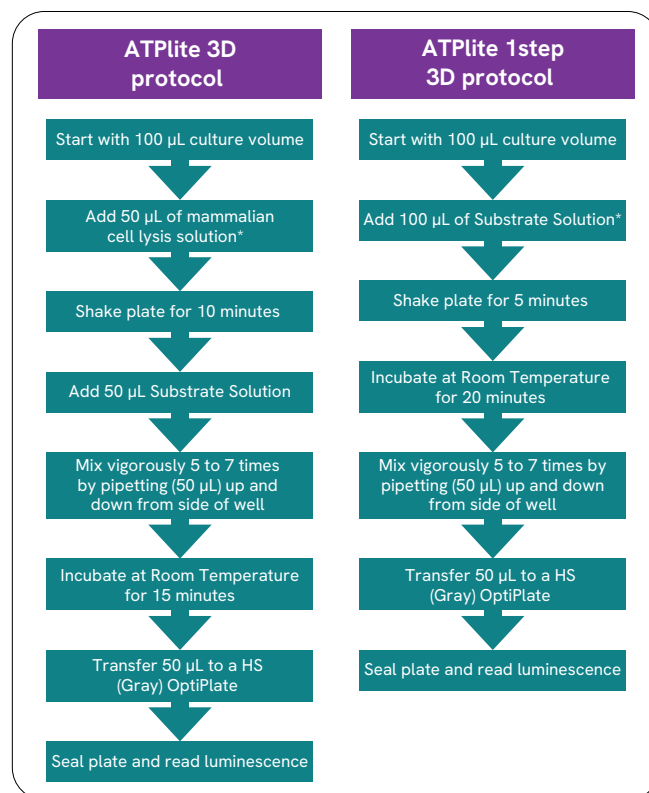


Figure 2: Overview of ATPlite 3D and ATPlite 1step 3D Assay protocols. The workflow for both assay formats is represented here in two flow-charts. * For larger, more tightly-packed microtissues (over 5,000 cells seeded per well), additional pipette mixing steps can be added upon the initial addition of mammalian cell lysis solution for ATPlite 3D or Substrate Solution for ATPlite 1step 3D to further enhance reagent penetration.

The additional incubation time and pipette mixing steps are important to allow for more penetration of the lytic components of the buffers into larger microtissues. To save on the number of pipette tips used and to simplify the protocol, we combined the pipette-mixing steps with the addition of substrate solution in the ATPlite 3D protocol or with the transfer step in the ATPlite 1step 3D protocol, and we have validated that these protocols were suitable to generate good assay reproducibility with the spheroid sizes and cell types used here. However, if the spheroids are more densely packed or larger in size, you may choose to include additional pipette mixing steps to improve the ATP extraction (in case you would find this necessary for your cellular system). We found that the protocol described here worked well with spheroids seeded with up to 5,000 cells per well. One way to do this is to increase mixing to more than seven times or to include a few pipette mixing steps in the initial addition step for ATPlite 1step 3D substrate solution and for addition of mammalian cell lysis solution in ATPlite 3D (see * in Figure 2).

The last two steps indicated in the protocol for both assays is to transfer 50 μ L (of the 200 μ L) assay volume to a gray OptiPlate (catalog #'s in Table 3). Please note that we used 384-well plates to generate the data included in this application note, but a gray Optiplate-96 HS works as well, and is the one included in the ATPlite 3D 300 assay kit and ATPlite 1step 3D 100 assay kit as manual pipetting in 96-well is considered to be more convenient. It is necessary to transfer the assay to an opaque plate before reading to prevent cross-talk between wells that would occur if reading the signal directly from the CellCarrier Spheroid ULA 96-well Microplates (a 5% (diagonal) or 12-15% (direct neighbors) cross-talk is expected if reading directly from the ULA plate). However, we determined that it was not necessary to transfer the entire assay volume. Therefore, we chose for this Application note to transfer a smaller amount (1/4 of the volume) into an OptiPlate-384 HS, in order to expand time and cost-saving options downstream by allowing for assays in up to 4 x 96-well spheroid plates to be transferred to a single Optiplate-384 for reading. We recommend the use of the gray microplates rather than using white as gray (HS) OptiPlates are a good compromise between getting a high luminescence signal and minimizing the amount of well-to-well cross-talk. Additionally, we determined that it is not necessary to dark adapt the Gray OptiPlates before reading them in Revvity Multimode Plate Readers such as the EnSight, EnVision™, or EnSpire®.

3D Spheroid and 2D culture data from cell titrations

To demonstrate the difference made by using our new 3D kits and protocols, we compared the ATPlite 1step and ATPlite 1step 3D assays run in both spheroid cultures and adherent, 2D cultures using a prostate cell line (DU 145 cells). For this assay, we seeded DU 145 cells at seven concentrations from 5,000 cells down to 250 cells per well, plus negative control wells containing media only into CellCarrier Spheroid microplates and CellCarrier 96-well microplates. The cultures were grown for four days and assays were run following each assay protocol in both plate types. Data (Figure 3) showed that the ATPlite 1step assay is not sufficient for extracting ATP from spheroids produced from greater than 1,000 cells and that the variability of the assay is high in spheroids made from less than 1,000 cells. The ATPlite 1step 3D assay was efficient at lysing all tested sizes of spheroid microtissues and showed significant improvement over ATPlite 1step when testing spheroids, especially at higher seeding densities (Figure 3A, blue squares).

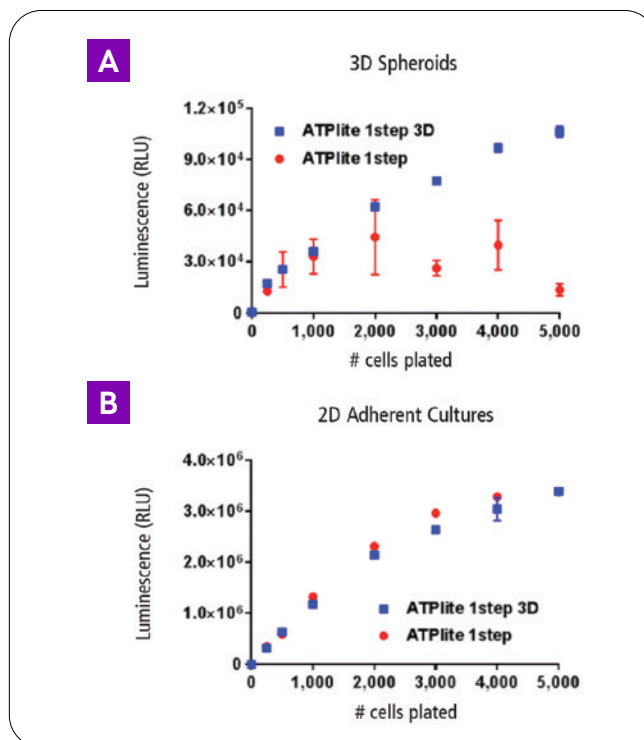


Figure 3: Comparison of ATPlite 1step and ATPlite 1step 3D in adherent and Spheroid cultures. DU 145 cells were seeded at the numbers indicated (x-axes) into CellCarrier Spheroid microplates (A) and standard CellCarrier 96-well microplates (B) and grown for 4 days. ATPlite 1step and 1step 3D assays were run on both plate types with the final step being to transfer 1/4 of the volume (50 μ L) of the reaction to a Gray OptiPlate-384 for luminescence reading on the EnSight. Each data point reflects the average of triplicate wells; Error bars = SEM.

When using the ATPlite 1step 3D protocol for testing 2D adherent cultures, the data is comparable to the original ATPlite 1step data generated. Therefore, the increases in exposure time and mixing steps necessary in the ATPlite 1step 3D protocol does not greatly affect overall signal or variability in adherent cultures, which could be a concern considering the significantly longer assay time (25 versus two minutes).

To examine assay linearity, ATPlite 1step 3D signals were examined for their correlation to the number of initial DU 145 cells that were seeded and were found to correlate well (Figure 4A). Additionally, the spheroid size was measured by labeling cell nuclei with Hoechst and using the Operetta high content imager to capture fluorescent images (at a single plane through the approximate middle of each sphere) using UV filters and Harmony software to select for an intensity cutoff and automatically measure area. Spheroid area also correlates well with both the number of cells initially seeded (Figure 4B) and with the amount of ATP measured as represented by luminescence in the raw ATPlite 1step 3D assay (Figure 4D). Similarly, we observe that the ATPlite 1step signal correlates linearly with the number of cells in adherent 2D cultures. Cell number in each well was measured using the Revvity EnSight Multimode Plate Reader cellular imaging module by imaging Hoechst-stained cells in the UV channel. Cell number was quantified automatically using the "Count Nuclei" algorithm in the EnSight's Kaleido software. As expected and previously reported, the original ATPlite 1step assay correlates well with the number of cells per well. Also, note the magnitude of difference in luminescence between overall signals in 2D and 3D cultures (in the y-axes in Figure 3A vs 3B and 4B vs 4C). This is due mostly to the proliferative growth observed in 2D cultures, whereas the cell contact that occurs in 3D spheroids inhibits proliferation and this is reflected in the data indicating the difference in overall cell numbers between each culture type. This proliferation can be seen in the numbers on the x-axis of Figure 4C where distinct groupings of cell data can be observed: when correlating these numbers with initial cell numbers seeded, the number of cells counted exceeds the number plated by as much as 12 times (compare seeded numbers from figure legend to counted numbers after four days on the x axis).

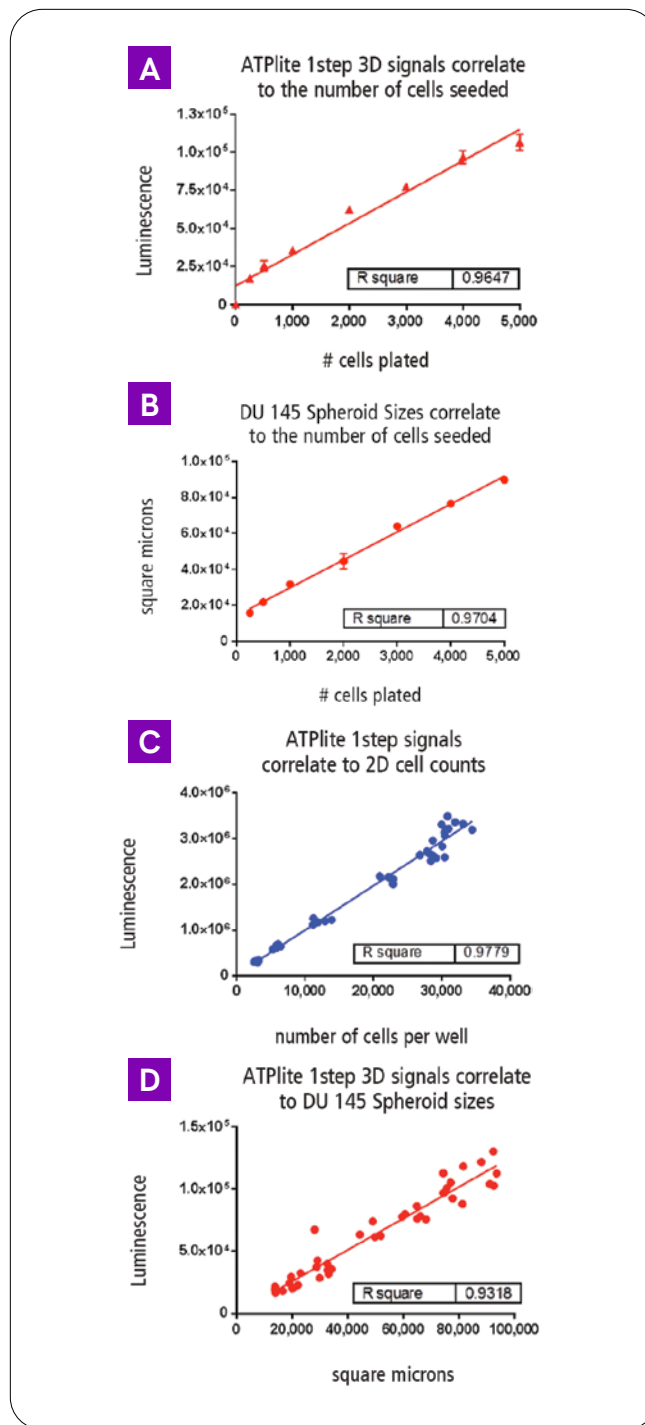


Figure 4: Data from ATPlite 1step and ATPlite 1 step 3D assays correlate with culture size. DU 145 cells were titrated and seeded into CellCarrier Spheroid (3D) microplates (4A, B, D) and standard 96-well black CellCarrier plates (4C) at the cell numbers specified (on the X-axis of 4A and B) and allowed to grow for four days. Cultures were labeled with Hoechst and spheroids were imaged in the UV channel on the Operetta at the same plane through each sphere. Cross-sectional spheroid area was measured using an intensity cutoff set for the whole plate. 2D culture wells were imaged and cells counted using the EnSight plate reader and Kaleido software. (Graph A data = average of triplicate wells; Graph B data = average of six spheres per data point, Error bars = SEM; Graphs C and D present data from individual wells).

Toxicity assays with Spheroids generated from multiple cell types

To illustrate the effectiveness of ATPlite 3D and ATPlite 1step 3D in toxicity assays, spheroid cultures generated from three different cell lines were seeded at 4,000 cells per well, grown for three days and then treated for one day with the compound Staurosporine (ThermoFisher Cat. No. 569397, 10 mM stock made in DMSO, then directly diluted in culture medium to make 2X concentrations that were added to the ULA plates) to induce a toxic response. Data from these assays are shown in Figure 5. Some cell lines grow and divide slower in 3D spheroids than others and some cell lines produce more ATP overall, therefore overall signal differences between cell types may not be reflective of different cell numbers or proliferation. Either way, in our experiments, we found HEK293 spheroids to produce the most ATP in culture as illustrated by the highest luminescence values seen in Figure 5A and B. In order to visually compare the IC_{50} curves between the different cell types, luminescence signals were normalized independently for each line to the average of wells containing the lowest concentration of compound.

ATP concentration and extraction from 3D spheroids

In order to determine the total amount of ATP quantified per well, standard curves with an ATP standard (provided in each kit) may be titrated in growth media and assayed concurrently following the exact same protocol as for the spheroid cultures. To convert samples' luminescence signal values into extracted ATP concentrations, the best-fit regression curve is calculated from raw luminescence values generated by the standard curve and the sample luminescent signal values are interpolated on this curve to determine the corresponding amount of ATP. These values for the HeLa and DU 145 spheroids are presented in Figure 6. The data was interpolated from the raw data shown in Figure 5. From this experiment (and from others, data not shown), we observe that ATPlite 3D appears to extract more ATP from spheroid tissues than ATPlite 1step 3D, which is consistent with the more aggressive nature of the chemistry used to lyse the cells in the ATPlite 3D kit (compared to ATPlite 1step 3D).

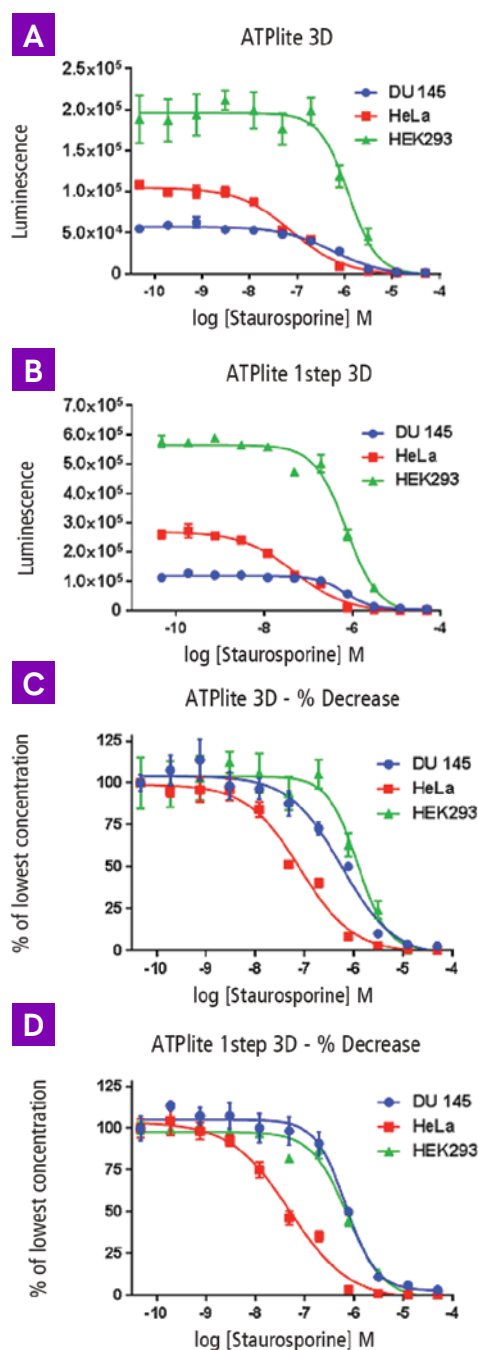


Figure 5: Toxic compound IC_{50} curves for 3 cell types. DU 145, HeLa, and HEK293 cells were plated at 4,000 cells per well and grown for 3 days before treatment with varying concentrations of the compound Staurosporine (12-point titration) to induce a toxic response. The next day, ATPlite 3D (A, C) and ATPlite 1step 3D (B, D) assays were run to detect toxicity and the resulting IC_{50} curves for each cell type were graphed (A and B). To compare IC_{50} values between cell types, data were normalized to the average of wells with the lowest concentration of compound and graphed in C and D. IC_{50} values for ATPlite 3D are: 571 nM for DU 145, 77 nM for HeLa, and 1.2 μ M for HEK293. For ATPlite 1step 3D, IC_{50} values are: 683 nM for DU 145, 50 nM for HeLa, & 738 nM for HEK293. Each data point is an average of four wells, bars = SEM.

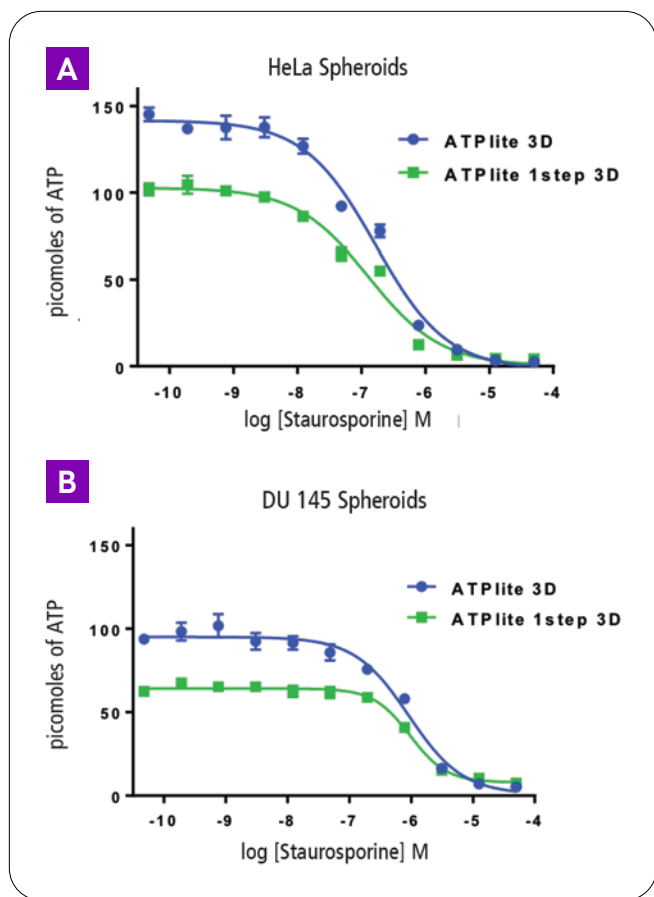


Figure 6: ATPlite 3D and ATPlite 1step 3D Assays produce comparable IC_{50} values though the lysis step in ATPlite 3D assay appears to extract ATP more efficiently. To quantify the amount of ATP from 3D culture in Figure 5, ATP standard curves were generated for each assay, raw luminescence values were interpolated, and data plotted in picomoles of ATP for HeLa (A) and DU 145 spheroids (B). The IC_{50} values HeLa spheroids: 166 nM for ATPlite 3D, 129 nM for 1step 3D; For DU 145 spheroids: 912 nM for ATPlite 3D, 954 nM for 1step 3D). n = 4; Error bars = SEM.

Toxicity assays in spheroids compared to cells grown in adherent cultures

Of interest to many in the field is whether cells grown in 3D spheroids respond differently than 2D cultures to toxic compounds. Some evidence supports the idea that cells may be more protected in 3D cultures and less susceptible to some toxic compounds due to their increased cell-cell surface contacts and cellular interactions. To illustrate how we can use the ATPlites 3D kits to look at this, we examined the responses of 2D and 3D cultures generated from HeLa and HEK293 cells to Staurosporine with ATPlite 1step and ATPlite 1step 3D assays (see Figure 7A and 7B). The data indicate that the IC_{50} values for HeLa cells are similar between 3D and adherent cultures,

whereas there is an almost 10-fold difference between 3D and 2D cultures generated from HEK293 cells suggesting that these cells grown in spheroids may be more sensitive to this compound than in 2D cultures.

One concern when generating spheroid microtissues is the health of cells in the center of the sphere. To examine cell health and viability, live cell fluorescent stains for cell viability were added to spheroid cultures treated with a titration of Staurosporine and spheroids were imaged in the appropriate channels using the Operetta (see Figure 7C and D). Our first observation is that spheroid tissues from both cell types remain intact with even the highest concentration of Staurosporine. Spheres with the highest concentrations of toxic compound were entirely labeled with SYTOX Red, indicating they were almost entirely composed of dead cells whereas the spheres with little to no Staurosporine stained brightest for the green, Calcein AM label. These stains appear to correlate well with the IC_{50} curves in Figure 7A. The lack of red staining in the center of spheres without treatment also suggests that those cells are still living and healthy. Additionally, the toxic effects of Staurosporine on the spheroids appears to be working more on the interior cell layers first, which does not support the idea that these cells are more protected, but suggest that they are more vulnerable. One more observation to note from this experiment (and the one in Figure 4) is that fluorescent, live cell stains do not appear to interfere with ATPlite 3D luminescence signals or affect the assay read-out (since wells that are stained were included in the averages and did not appreciably influence assay variability). This enables users to use the same plates for high-content imaging experiments as well as for quantifying ATP in luminescence mode.

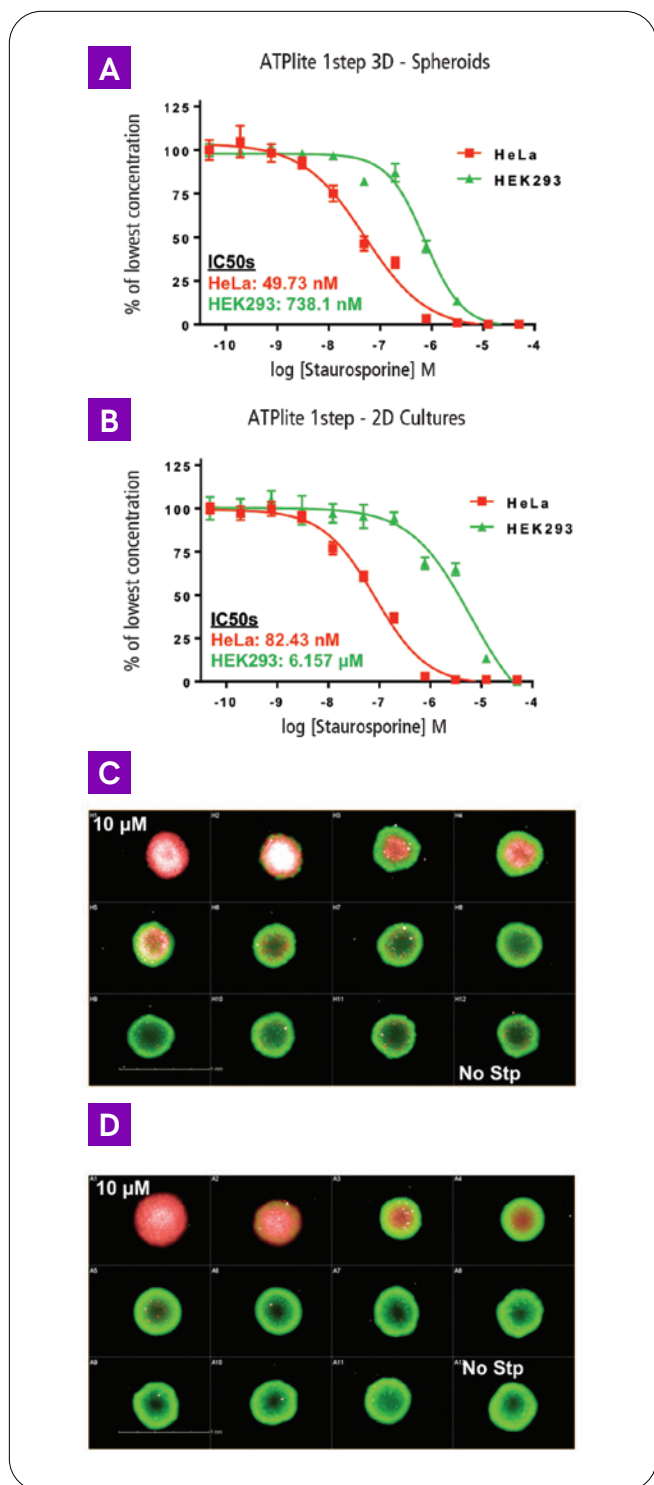


Figure 7: Inhibition curves compared between 3D and 2D cultures. HeLa and HEK293 cells were plated at 4,000 cells per well, grown for 3 days and treated overnight with Staurosporine. ATPlite 1step 3D and ATPlite 1step data presented in A and B as luminescence data normalized to wells treated with the lowest concentration. In panels C and D, spheroids treated with 12 concentrations of Staurosporine were live-stained with Calcein AM to label live cells and Sytox Red for dead cells and imaged on the Operetta in confocal mode using the 10x objective. Each data point reflects the average of four wells (including 1 well per condition containing fluorescence stains); Error bars = SEM.

Comparison to a competitor assay

We compared the effectiveness of ATPlite 3D and ATPlite 1 step 3D against a competitor kit that was a luminescence-based ATP assay specialized for 3D cultures. We followed strictly the protocol of the competitor kit, with one adaptation to the final two steps where 50μL of the assay was transferred to an Optiplate-384 HS for luminescence reads, congruent to the ATPlite 3D protocol.

In the first assay comparison, HeLa spheroids were seeded at 1,000 cells per well in two plates and treated the next day with a DMSO titration for one more day before running ATPlite 3D (two-step assay) and the competitor assay (“Competitor”) in parallel (data presented in Figure 8A). The raw luminescence data generated from Competitor (RLUs in graph) were of considerably higher magnitude (~16 times higher signals at top values). However, when the scale for ATPlite 3D is adjusted (left axis) to raise the top luminescence to that of Competitor (right axis), the IC₅₀ curves for both assays are identical, indicating that ATPlite 3D is measuring the same effect of DMSO. Additionally, the Error bars were larger in the Competitor assay with the higher luminescence values, suggesting that greater signals go along with greater variability.

A comparison with larger spheroid cultures is illustrated in Figure 8 in which 4,000 HeLa cells were seeded and then treated the next day with a Staurosporine titration (for three days) and all three assays (ATPlite 3D, ATPlite 1step 3D, and Competitor) were run concurrently on different plates following the specified protocols. The data from this experiment is presented in Figure 8B with the left y-axis adjusted for ATPlite 1step 3D to be equivalent at the top range with Competitor. In this experiment, it’s clear that the maximal signals represent a little less than 10-fold higher for Competitor than for ATPlite 1step 3D, but it still appears that variability is lower in the ATPlite 3D assays.

To test this observation more closely, we ran another assay to determine variability and calculate z-prime values. For this assay, three plates were prepared where 32 wells each were seeded with 1,000 cells from HeLa, HEK293, or DU145 cell lines. The spheres were grown for two days before half the wells were treated overnight with Staurosporine (5 μM) and the other half with vehicle control (0.5% DMSO). All three assays were run as in the previous experiment and data are presented in Table 1.

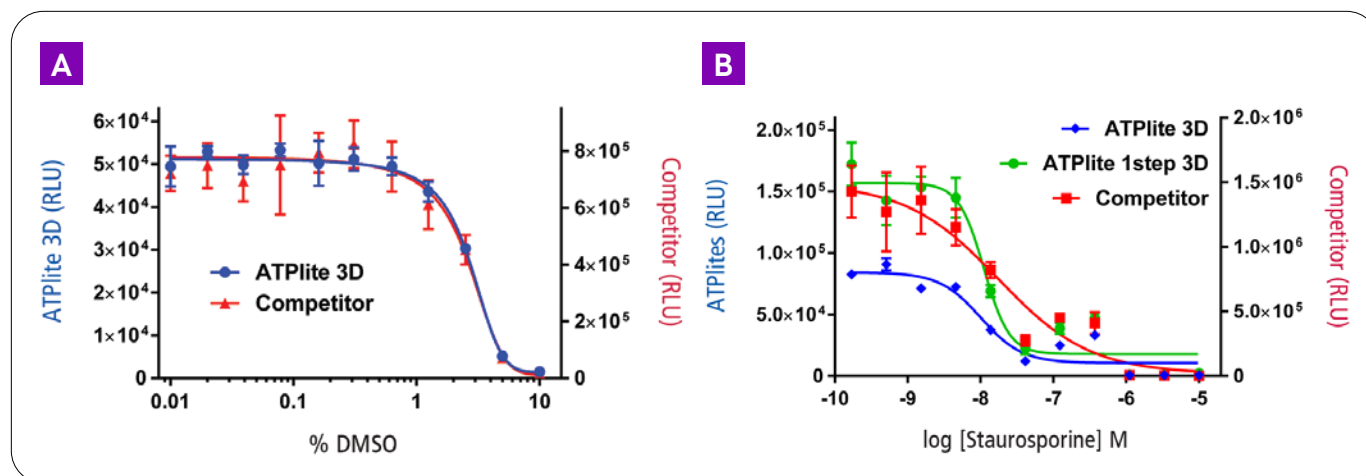


Figure 8: ATPlites demonstrate Lower signal but less variability and comparable IC_{50} s compared to a Competitor Assay. (A) HeLa spheroid cultures were seeded at 1,000 cells per well in two plates and treated the next day with a DMSO titration for one more day before running ATPlite 3D and Competitor assays in parallel. (B) For the comparison in larger spheres, HeLa cells were seeded into three plates at 4,000 per well and treated the next day with a titration of Staurosporine. Spheres were then assayed on day four. Each data point reflects the average of four wells; Error bars = StDev.

Table 1: Variability data for ATPlite 3D, ATPlite 1step 3D, and a Competitor assay. Z-primes were calculated from raw luminescence signals from spheroids seeded at 1,000 cells per well from three cell types, grown for three days and treated overnight (~22 hours) with Staurosporine vehicle control. Note – All wells were imaged using the Operetta in Brightfield mode before the assay was performed to ascertain the presence of a healthy, spheroid culture (thus only 15 out of 16 wells were included for a few conditions).

Cell type	Assay	mean 1	mean 2	S/B	n 1	n2	%CV1	%CV2	z'
HeLa	ATPlite 3D	296	37,129	125	16	16	20.1	7.0	0.78
HeLa	ATPlite 1step 3D	799	75,257	94	16	16	37.2	8.8	0.72
HeLa	Competitor	3,222	588,976	183	16	16	40.5	13.6	0.58
HEK293	ATPlite 3D	23,660	79,832	3	15	15	22.7	8.1	0.37
HEK293	ATPlite 1step 3D	37,818	181,486	5	16	16	15.3	4.3	0.72
HEK293	Competitor	493,647	1,361,293	3	16	16	53.5	9.4	-0.36
DU145	ATPlite 3D	840	21,516	26	16	15	16	9.7	0.68
DU145	ATPlite 1step 3D	3,818	53,908	14	16	16	14.2	5.8	0.78
DU145	Competitor	10,908	330,624	30	16	16	27.7	10.9	0.63

The data in Table 1 indicate that, although the competitor assay produces higher raw signals and greater signal-to-background, ATPlite 3D and ATPlite 1step 3D assays out-performed in measures of variability by producing lower well-to-well variability.

Other benefits of using ATPlite 3D assays are that: (1) They are stored at 4 °C, and therefore are rapidly available for use. In contrast, the competitor assay requires thawing of the frozen reagent at 4 °C the day before the assay is to be run, followed by equilibration of the reagent vials to room temperature. (2) The shelf life of the ATPlite 3D kits are considerably longer (at least one year) and they

can be stored at 4 °C, thus saving on your freezer space. (3) Another great benefit of the ATPlite 3D protocols is that spheroid microtissues do NOT need to be transferred before running the assay (hence avoiding the risk of losing the spheroid in the transfer step) and the majority of the assay is done directly in the spheroid producing plates. (4) The ATPlite kits do not contain DTT, avoiding an unpleasant odor and the need to work under a chemical hood, while being environment friendly. (5) The stability of the ATPlite 3D signal is unsurpassed, with a half-life of more than four hours, providing you comfort in the organization of your work.

Table 2: Features and benefits of each reagent kit.

ATPlite 3D	ATPlite 1step 3D
Shipped at room temperature: no need to get rid of dry ice, friendly shipping cost, environment-friendly	
Stored at 4 °C: does not consume freezer space, reagent rapidly available to work with	
No DTT formulation: no smell, no work under the hood, environment-friendly	
Shelf life at least one year from manufacturing date	
Best efficiency of spheroid lysis, but two additions steps	1 step: a single reagent to add
Less light intensity, but similar Z' values	About 3x higher light output than ATPlite 3D
Glow luminescence, Half-life > four hours	Glow luminescence, Measure within 30 min

The protocols for using Revvity CellCarrier Spheroid ULA microplates and ATPlite 3D protocols may be easily adapted for use with automation. Things to consider when developing these protocols would be: placement of pipette tips to the sides of wells when performing the pipette-mixing steps, using a faster pipetting speed when mixing in the wells, and combining pipette-mixing steps with reagent additions or transfer steps. The mixing steps have been found to be most important to the consistency of the assay and, if an orbital plate shaker is not available, the shaking steps can be eliminated from the protocol and substituted by adding in additional pipette-mixing. This should be optimized for use with your specific cultures. Additionally, the volume transferred and opaque plate type in the last two steps of the protocol can be easily adjusted to accommodate different plate types (can adjust from 384-well to 96-well or 96-well half-area plates if desired).

Table 3: List of all Revvity products listed in this Application Note and their associated Catalog numbers.

Revvity product description	Catalog numbers
ATPlite 3D	6066943 (300 assay points in 96 well-plates)
ATPlite 1step 3D	6066736 (100 assay points in 96 well-plates)
ATPlite	6016943 (300 assay points in 96 well-plates)
	6016941 (1,000 assay points in 96 well-plates)
	6016947 (5,000 assay points in 96 well-plates)
	6016949 (10,000 assay points in 96 well-plates)
ATPlite 1step	6016736 (100 assay points in 96 well-plates)
	6016731 (1,000 assay points in 96 well-plates)
	6016739 (10,000 assay points in 96 well-plates)
CellCarrier Spheroid ULA 96-well Microplates	6055330 (10 lidded, individually-wrapped plates) 6055334 (40 lidded plates - two bags with 20/bag)
OptiPlates, 96-well HS (Gray)	6005330 (case of 50)
	6005339 (case of 200)
OptiPlates, 384-well HS (Gray)	6005310 (case of 50)
	6005300 (case of 200)
CulturPlates, 96-well, white, opaque, sterile, TC	6005680 (case of 50)
	6005688 (case of 160)
	6005689 (case of 200)
CulturPlates, 384-well, white, opaque, sterile, TC	6007680 (case of 50)
	6007688 (case of 160)
	6007689 (case of 200)

