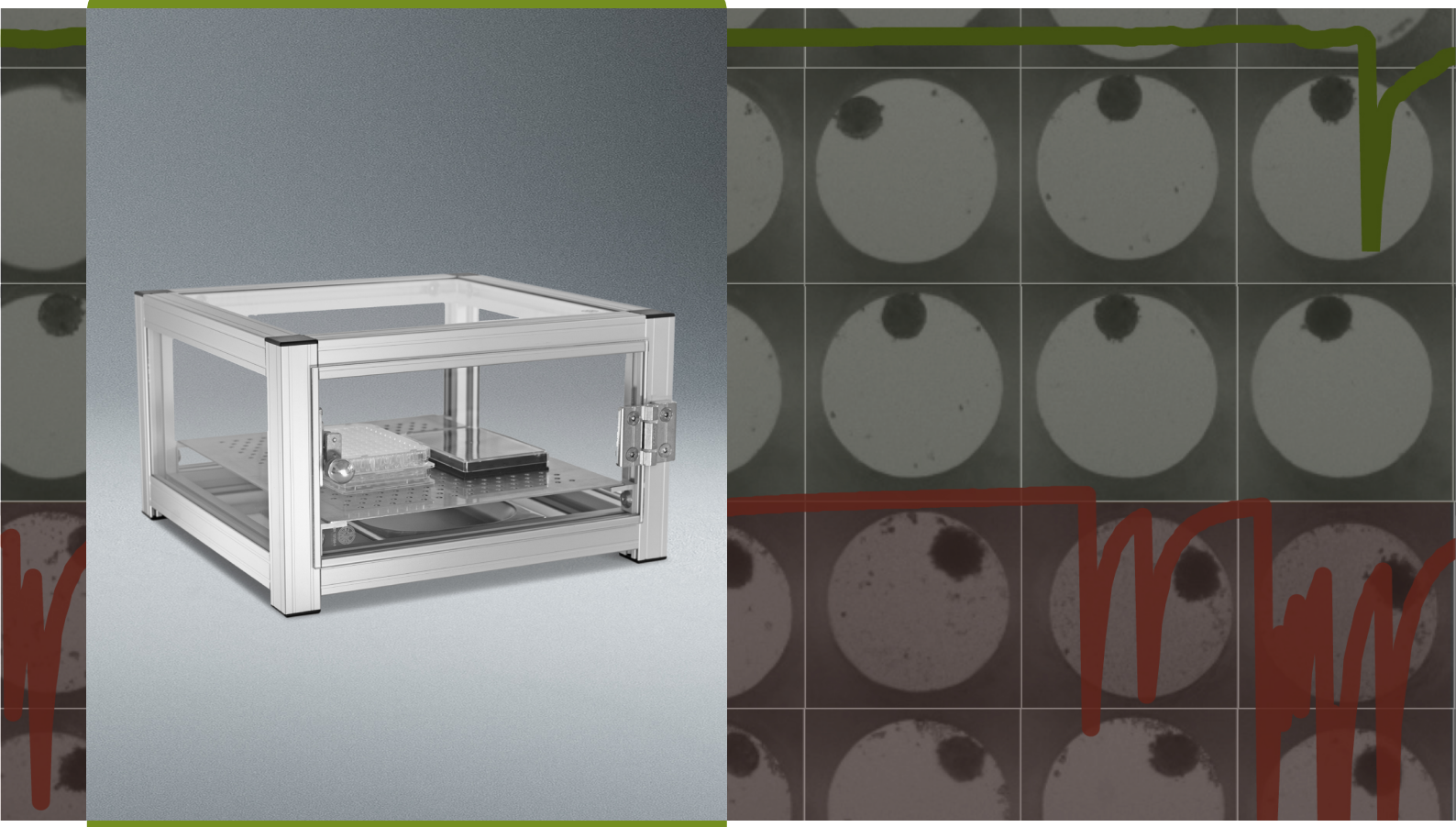

APPLICATION NOTE

Utility of the Incubox™ to reduce edge-effects and minimize well-to-well variability

The application of *in vitro* 3D cell culture models in drug discovery programs continues to expand. One important characteristic supporting their higher physiological relevance is the longevity of the tissue structures enabling assay times of several weeks with repeated compound dosing. Maintaining a controlled and stable culturing climate over time poses challenges to the equipment. Medium evaporation in culture dishes, for example, is significantly reduced by maximizing the humidity inside an incubator. In busy labs with many projects, the doors of incubators are frequently opened, leading to large humidity drops that are slow to recover, especially in incubators with large chamber volumes.

Reduced humidity inside incubators leads to increased evaporation in wells, especially wells at the edge of the microtiter plates. This so-called edge effect is particularly an issue for long-term studies, in which medium is only exchanged every 3-5 days. As a result, the concentration of medium ingredients and test compounds increases, which can lead to precipitation in the wells and increased variability between inner and outer wells.

These edge effects cause many researchers to avoid using edge or outer wells, thereby wasting up to 38% of the wells of a 96-well plate. As a result, the treatment groups have to be distributed over more plates, increasing workload and cost of goods and at the same time decreasing flexibility for designing experimental plate layouts.

In this application note, we use InSphero's Incubox™ in a 14-day toxicity assay, which is frequently used to determine drug-induced liver injury[1]. A key feature of the assay is a repeated dosing with 5 days of intermediate incubation without medium exchange. We demonstrate that using the Incubox™, edge effects and related data variation can be eliminated, thereby enabling the use of all 96 wells of a plate without compromise.

HANDLING

Imaging

Aggregation

The Incubox™

InSphero's Incubox™ is placed directly onto one of the shelves inside a conventional cell culture incubator. Up to 20 microplates can be loaded through the magnetically secured front door. The Incubox™ is compatible with a wide range of solvents and decontamination procedures commonly used for incubators.

Figure 1: *Photo of the Incubox™ including Akura™ Spheroid Microplates*

Small holes ensure stable O_2 and CO_2 conditions controlled by the incubator itself. The Incubox™ includes its own water bath, which creates a high-humidity microclimate and protects the microplates from large humidity drops and fluctuations when the door of the incubator is opened.

Figure 2 compares relative humidity measurements inside the incubator and inside the Incubox™ over the course of a few hours. Opening the door of the incubator results in a significant drop of humidity inside the incubator chamber, a humidity above 90% is re-established only after approximately 30 min. The humidity level maintained inside the Incubox™ is by default higher (>95%) than in the incubator chamber. Opening the incubator door only minimally affects humidity inside the Incubox™. Opening both the incubator door and Incubox™ door (e.g. to perform medium exchanges) causes a much less pronounced humidity drop in the Incubox™ than in the Incubator chamber (down to 80%, versus 30%-65%), which then recovers to >90% relative humidity within just a few minutes. As a result, evaporation in the wells, especially outer and edge wells is substantially reduced.

Figure 3 shows the remaining medium volume after five days inside a standard incubator and inside the Incubox™. The Incubox™ was equilibrated for at least 1 hour before placing the plates inside.

Discover more about Incubox™ at shop.insphero.com

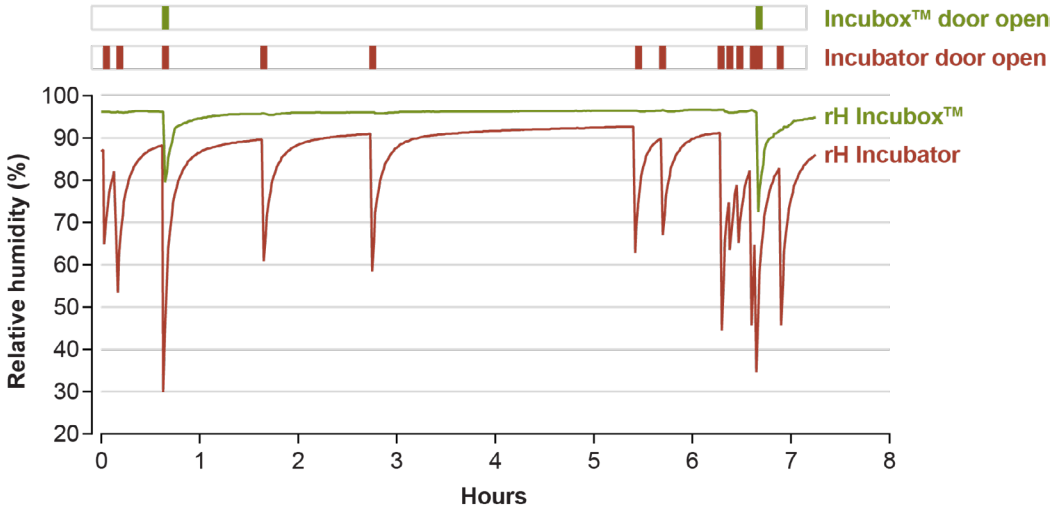

Figure 2: *Relative humidity measured inside the incubator chamber and inside the Incubox™ placed on the central shelf of the same incubator. The humidity inside the Incubox™ is by default higher and is not affected when the incubator door is opened and re-establishes within a few minutes when both doors are opened to remove or load microplates into the Incubox™.*

Incubator

	1	2	3	4	5	6	7	8	9	10	11	12
A	50	55	55	55	55	55	55	55	55	55	50	40
B	55	65	70	70	70	70	70	70	70	70	65	50
C	55	70	70	70	70	70	70	70	70	70	70	50
D	55	70	70	70	70	70	70	70	70	70	70	50
E	55	70	70	70	70	70	70	70	70	70	70	50
F	55	70	70	70	70	70	70	70	70	70	70	50
G	55	65	65	65	68	68	68	68	68	68	65	50
H	40	55	55	55	55	55	55	55	55	55	50	35

Incubox™

	1	2	3	4	5	6	7	8	9	10	11	12
A	50	60	60	60	60	60	60	60	60	60	60	55
B	60	65	70	70	70	70	70	70	70	70	65	60
C	60	70	70	70	70	70	70	70	70	70	70	65
D	60	70	70	70	70	70	70	70	70	70	70	65
E	60	70	70	70	70	70	70	70	70	70	70	65
F	60	70	70	70	70	70	70	70	70	70	70	65
G	55	65	70	70	70	70	70	70	70	70	65	60
H	50	60	60	60	60	60	60	60	60	60	60	50

Figure 3: *Measured remaining liquid volumes in each well of microtiter plates after 5 days cultured in a standard incubator and inside the Incubox™. The starting volume was 70 µl. Values in µl are means of two microplates.*

Case Study: 14-day toxicity test

The utility of the Incubox™ has been investigated in InSphero's 14-day toxicity test, which is routinely used for assessing drug-induced liver injury (DILI) of pharmaceutical compounds. In this test, primary human liver microtissues (hepatocyte/non-parenchymal cell co-culture) are exposed to different concentrations of compounds over 14 days. The medium is exchanged, and a re-dosing is performed only every 4 to 5 days enabling the investigation of metabolite effects. For such long incubation times, minimizing evaporation is critical.

1. Experimental layout

Microtissues are commercially available and have been produced in Akura™ 96 Spheroid Microplates using InSphero's validated production protocol[2]. The experimental protocol of the assay is outlined in ***Figure 4***: Medium is exchanged at day 0, 5 and 9 including a redosing of the compound at respective concentrations. In this experiment, Chlorpromazine, a known liver toxic substance <10 µM, has been used. The plate layout is shown in ***Figure 4***, on the lower image. Single microtissues cultured in each well were exposed column-wise to increasing concentrations of Chlorpromazine. For this experiment, only the first 4 rows A-D were considered. Viability was determined at day 14 through measurement of the cellular ATP levels of each microtissue using Promega's CellTiter-Glo 3D assay[3].

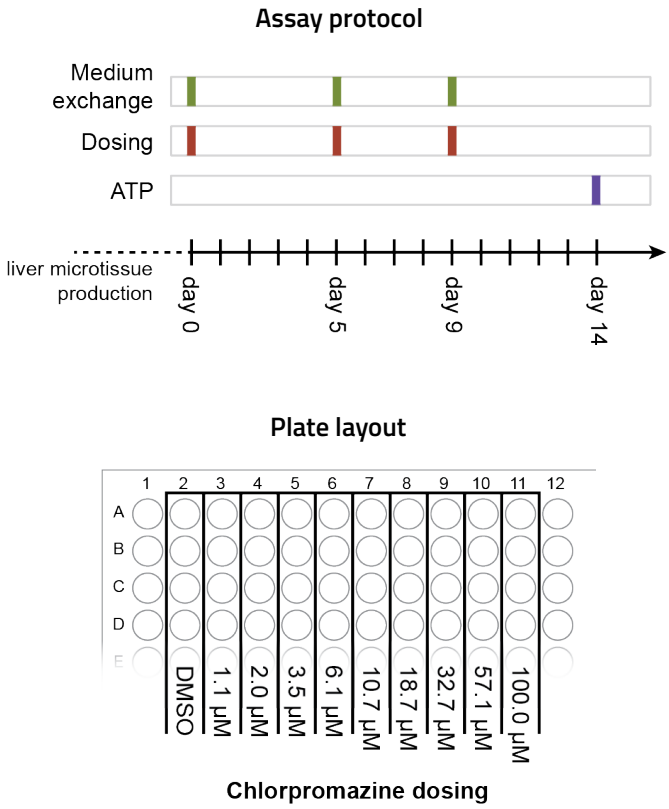

Figure 4: *Timeline and plate layout of the 14-day tox testing protocol. Liver microtissues were aggregated in Akura™ 96 Spheroid Microplates. After quality control, rows of the plates were exposed to different concentration of Chlorpromazine as indicated in the plate layout with a re-dosing at day 5 and 9. Cellular ATP was determined at day 14.*

2. Results

Plate scans were taken at day 5 (see ***Figure 5***) following incubation without medium exchange since day 0. The plate subjected to standard incubator conditions shows evidence of compromised liver microtissues, likely due to increased evaporation in outer wells. Precipitations and less compact microtissues can be clearly identified in row A of the plate cultured in the standard incubator. Whereas good homogeneity can be observed throughout the plate cultured in the Incubox™ (lower plate in ***Figure 5***).

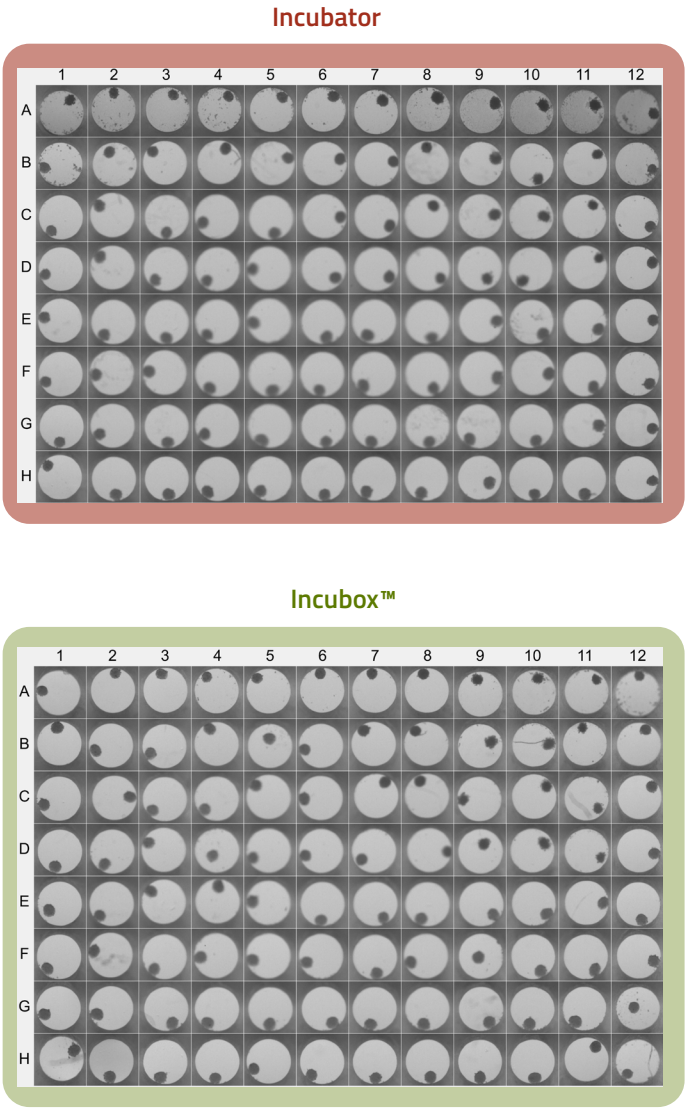

Figure 5: *Plate scans showing brightfield images of individual wells at day 5. Increased evaporation in standard incubators (upper scan) causes morphological changes of microtissue and precipitations in outer wells (row A). No morphological changes are visible in plates cultured in the Incubox™ (lower scan).*

Figure 6 presents microtissue viability by measured cellular ATP content of all microtissues after 14 days of compound exposure as well as corresponding dose curves. The heat maps representing absolute ATP values in pmol show a significant viability loss in row A of the plate cultured in the incubator also at low Chlorpromazine concentration (left side). These results indicate that viability is compromised by evaporation. In contrary, the plate cultured in the Incubox™ shows similar values along the columns respectively same concentrations from row A to D.

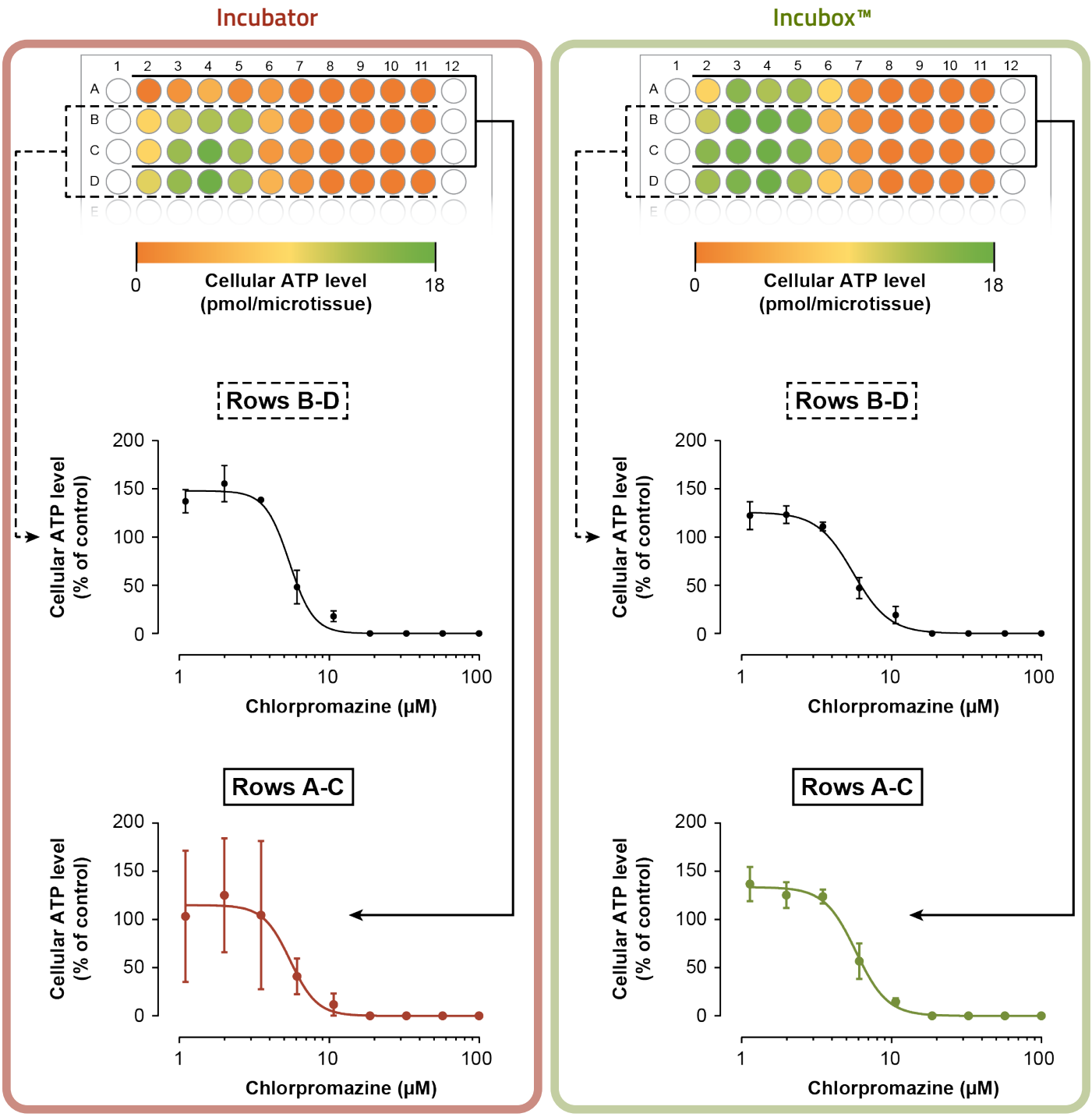

Figure 6: *Results of the 14-day toxicity test displayed in effective ATP values of each liver microtissue as plate heatmaps and deduced dose response curves from either rows B-D or A-C. Results on the left side are from a plate cultured in the incubator, results on the right side are from a plate cultured in the Incubox™. Data show means and standard deviations (n=3).*

In order to illustrate the impact of evaporation effects, dose response curves were plotted for either rows B-D or rows A-C (n=3) in both cases. The B-D scenario replicates the use of only the inner 60 wells of a 96-well plate, an approach often chosen by researchers to prevent edge effects. In both cases – incubator and Incubox™ – dose response curves with small standard deviations can be extracted. When outer wells are included (i.e. rows A-C are selected), the plates cultured in the incubator show high variations at low compound concentrations. For the plate cultured in the Incubox™, no difference between rows B-D and A-C can be observed due to the significant reduction of edge effects.

3. Conclusion

The Incubox™ creates a high humidity environment inside standard cell culture incubators. Opening the door of the incubator only minimally affects humidity fluctuations inside the Incubox™. As a result, evaporation in well plates, especially the outer wells, is significantly reduced. In the example of a long-term toxicity experiment with relatively few medium exchanges, edge effects could be significantly reduced which allows the use of all wells without evaporation-induced variability. With Incubox™, all the wells in a 96 well plate can be used with high confidence, enabling more experimental conditions per plate with higher flexibility, ultimately reducing effort and cost.

References

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