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Eliminating the Edge Effect with ReCO$_2$ver™

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ABSTRACT

The inability to use the outer ring of wells in a microtiter or multiwell cell culture plate, commonly known as the Edge Effect, has been plaguing researchers for too long. This phenomenon is due to uneven evaporation of the media from these outer wells. Using the tightly controlled relative humidity and quick recovery time after a door opening event of the ReCO$_2$ver™, we can virtually eliminate the Edge Effect seen in 96 and 384 well dishes when tested under extreme circumstances. This will help to protect sensitive cell cultures, save labs money and increase productivity.

INTRODUCTION

Science has been developing at a rapid pace, and with it, forcing matched technological developments to support it. The latest requirements have been in designing and performing experiments and testing larger, with more samples, faster. Medium to high throughput screening is quite commonplace. The instrumentation has come a long way, but there still seems to be a significant roadblock with the cell culture. The cells needed for each sample need to be grown in microtiter or multiwell plates. These plates come in a variety of sizes, 6-, 12-, 24-, 48-, 96-, 384- or 1536-wells per plate. The base of the plate remains a universal size to fit in any piece of lab equipment (automatic pipetting robots, imagers, etc.) so this means the volume of each well must decrease significantly. Due to the very small volumes of media present in each well and the loose fitting lid designed for air exchange, there is a significant amount of evaporation noticed in the outer ring of wells in the higher quantity microtiter plates (96-, 384- and 1536-well). This evaporation is widely known as the "Edge Effect" (**Figure 1**). The Edge Effect will lead to misleading or misconstruing of data in these outer wells, or can even be so severe as to dry out the cells and kill the samples. Evaporation of the media can cause treatments of unknown concentrations of media or drugs dissolved in the media. The osmolarity will be shifted. All of these conditions can be very stressful or even toxic to cell cultures.

To try to address this problem, the ingenious method of just-not-using-those-wells has been put into practice. The outer ring of wells is filled with water or extra media, but no cells added with a 96 well plate. For 384-well, the outer 2 rows are avoided. The "Inner 60" of a 96-well plate or "Inner 240" of a 384-well plate is used for experiments and the outer wells are used as an evaporation buffer (see **Figure 1**). This leaves 38% of the plate un-used. More plates need to be used to accommodate the same number of samples. With each plate used, all the appropriate controls need to be present on that plate as well, which leads to more conditions needing to be run on more plates. This will lead to laboratories having to incur additional expenses. For example, a screen of 5000 compounds run in a 384-well plate format would need 16 plates, assuming 2 columns each for a positive and negative control. If the outer 2 rows cannot be used, the same screen would need 27 plates. That is a difference of 11 plates, without running replicates of your samples. This near doubling of plates required means more cost (plates, media, reagents), space (double space needed to incubate/feed/store), and time (feeding, imaging, analyzing, etc.). These are three very important factors to labs of all sizes.

The $ReCO_2ver$™ is equipped with an ultrasonic nebulizer to accurately maintain a very high specific relative humidity (RH) within the incubator. It has been shown to return to ±3% of set point within 4 minutes after a 30 second door opening ($ReCO_2ver$™ brochure). This rapid recovery helps to maintain the ideal cellular environment. A traditional waterpan incubator has an unknown level of RH and a much slower recovery, averaging 20-30 minutes (Gallagher et al., 2014). Here we tested the evaporative loss within the two standard commonly used multiwell plates, 96- and 384-well.

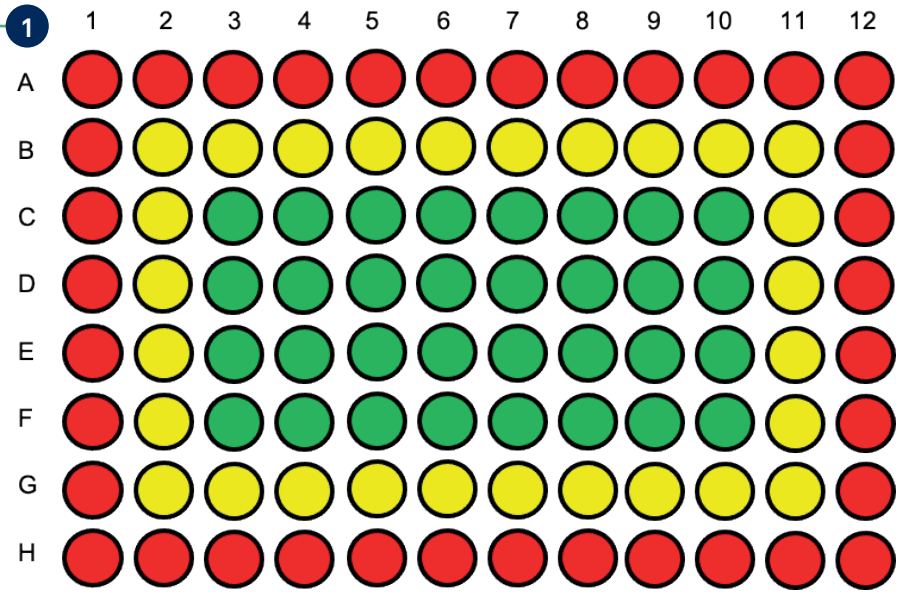


FIGURE 1.

Diagram of the Edge Effect.
In this example, a 96-well plate is being shown with the outer ring of wells unusable (red) and the inside 60 wells ("Inner 60") shown in yellow and green. Yellow may still have some evaporative effects, while green would be considered safe.

MATERIALS AND EQUIPMENT

MATERIALS	CATALOG #	SUPPLIER
1. ReCO$_2$ver™ Plus Incubator	REC602 Plus	The Baker Company, Sanford, ME
2. Basic Waterpan Incubator		Brand X
3. White 96-well cell culture dishes, tissue culture treated	165306	Thermo Scientific
4. Clear 384-well tissue culture treated flat bottom dish with lid	353961	Falcon
5. WFI Quality Water	25-055-CM	Corning
6. Trypan Blue Solution	T8154	Sigma-Aldrich
7. FinnTip Flex 300 disposable pipette tips	94060513	Fisher Scientific
8. Pipettors, 10 and 100uL		Gilson
9. BenchMate Multi-8, 40-200uL		Oxford

METHODS

The effectiveness of the RH regulation of ReCO$_2$ver™ was tested in 3 ways. First, the overall evaporation in 96 well plates was challenged. Secondly, the location of the plates within a ReCO$_2$ver™ was tested, to see if a "Sweet Spot" or prime location existed within the incubator to have less evaporation. Thirdly, the effects on 384-well plates were tested.

Overall Evaporation of 96-well plates

Standard flat bottomed, tissue culture treated 96 well plates were plated with 50 and 100µL of 0.2% Trypan Blue in water using a multichannel pipette for uniformity in duplicate. The ReCO$_2$ver™ and the Brand X water pan incubator were given identical samples to test. The plates were placed in the center of the incubators. ReCO$_2$ver™ was set at 90% RH, 37˚C, and 5% CO$_2$. Brand X was set at 37˚C, 5% CO$_2$ and a full water pan for the duration of the experiment.

The outer and inner doors were opened to 90˚ for 30 seconds 7 times per day for 4 days. This is to simulate a multi-use lab checking on their cells frequently. We also tested small volumes of liquid to show extreme examples.

The volume of liquid in each well was tested by reverse pipetting. Percent loss was calculated based on the initial volume plated.

"Sweet Spot" testing

Four 96 well plates containing 100µL of 0.2% Trypan Blue were placed throughout each incubator to test the different locations (see **Figure 4A**). It was hypothesized that the outer walls would have the most evaporation due to the direct heat of the incubator wall.

The plates were subjected to the same extreme testing as above. The outer and inner doors were opened to 90˚ for 30 seconds 7 times per day for 4 days. The volume of liquid in each well was tested by reverse pipetting. Percent loss was calculated based on the initial volume plated.

384-well evaporation

Flat bottomed, tissue culture treated 384-well plates were distributed with 20 and 40µL of 0.2% Trypan Blue solution and placed in the center of each incubator. The plates underwent the above test. The outer and inner doors were opened to 90˚ for 30 seconds 7 times per day for 4 days. The volume of liquid in each well was tested by reverse pipetting. Percent loss was calculated based on the initial volume plated.

RESULTS

Overall 96-well evaporation

The 96-well plates placed in the center of the incubator with initially 100µL of 0.2% Trypan Blue solution were analyzed for the amount of remaining liquid after the 4 day incubation. Each well was measured using reverse pipetting. The percent loss was then calculated and graphed for each location of the well in the 96-well plate as seen in **Figure 2**. The traditional "Inner 60" phenomenon can be seen in **Fig. 2A**, where the Basic Waterpan Incubator X has created an outer ring of wells with greater than 30% volume loss, and the most loss occurring in the four corners. The inside 60 wells have experienced less than 20% loss, excepting of course the corners (Wells B2, B11, G2 and G11).

This level of evaporation has become acceptable. When comparing to the ReCO$_2$ver™ (**Fig. 2B**), a vast increase in volume retention can be seen. All wells have less than 20% initial volume loss, excepting the corners, which are less than 30%. The whole plate mirrors the acceptable Inner 60 profile seen in the Basic Waterpan Incubator. If a very small amount of initial media is added (50µL of 0.2% Trypan Blue solution), the amount of evaporation is exaggerated. The entire plate from the Basic Waterpan Incubator is deemed unusable due to excessive evaporation (**Figure 3A**), while the ReCO$_2$ver™ has only lost the outer ring of wells (**Fig. 3B**). This shows the extensive level of protection the ultrasonic nebulizer of the ReCO$_2$ver™.

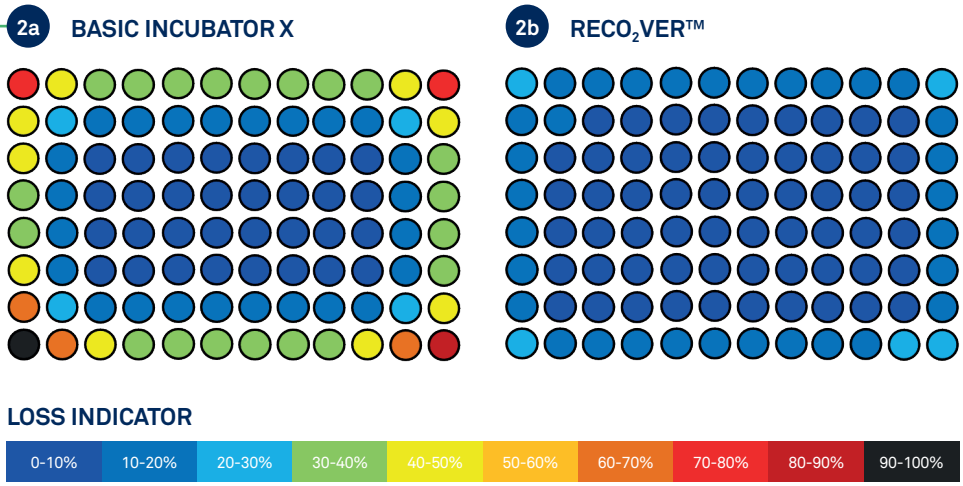


FIGURE 2.

96-well 100µL media calculated percent loss.
The percent loss is color coded as shown in the loss indicator below. (**A**) shows the Edge Effect in a Basic Waterpan Incubators and (**B**) shows no evaporation effects in a ReCO$_2$ver™ incubator.

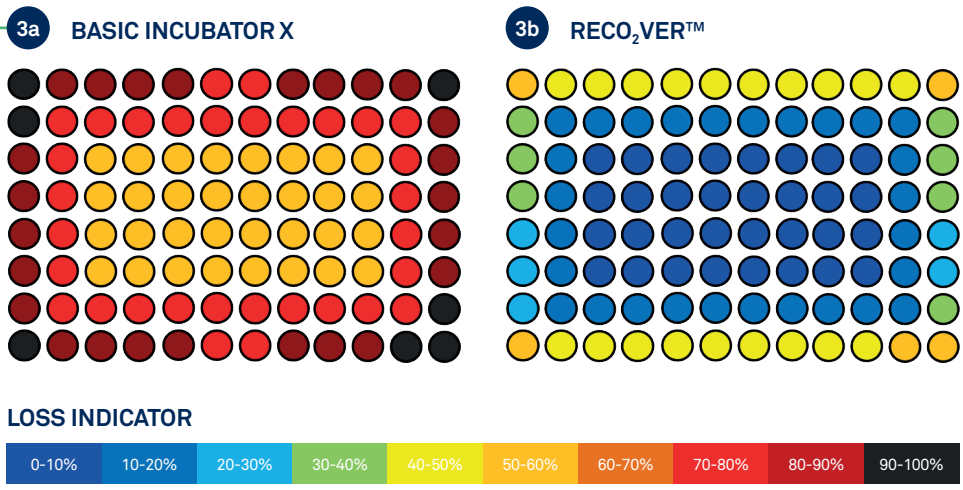


FIGURE 3.

Media loss in a 96-well plate using 50µL initial volume.
Percent loss color coded according to the Loss indicator below. Basic Waterpan Incubator X on left (**A**) and ReCO$_2$ver™ on right (**B**).

RESULTS AND CONCLUSION

The internal "Sweet Spot" detection

Four 96 well plates were placed in various positions throughout the incubator to test for the prime location for the most volume retention, also known as the "sweet spot" (**Figure 4A**). When all four locations were measured, the greatest defect was seen in the corner wells. The average percent retention for the 4 corners of each plate location was calculated. As seen in **Figure 4B**, the Basic Waterpan Incubator had a lot of variability depending on where the plate was placed, whereas $ReCO_2ver$™ upheld its high level of retention independent of where the plate was located. It was expected that the most evaporation would be on the edges, as the incubator is a direct heat incubator, and the walls should be warmer than the interior, but that is not seen here.

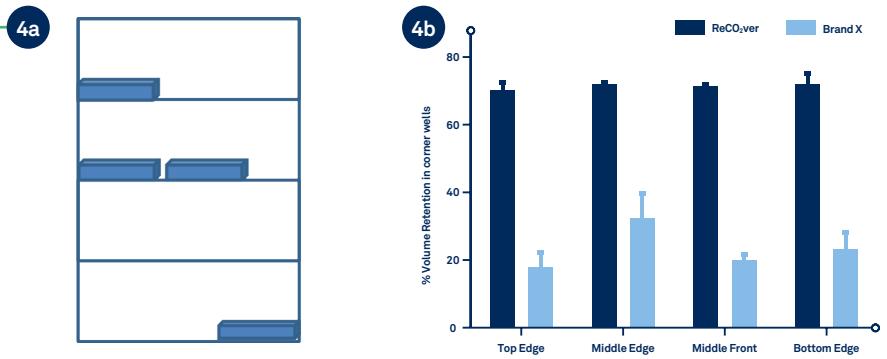


FIGURE 4.

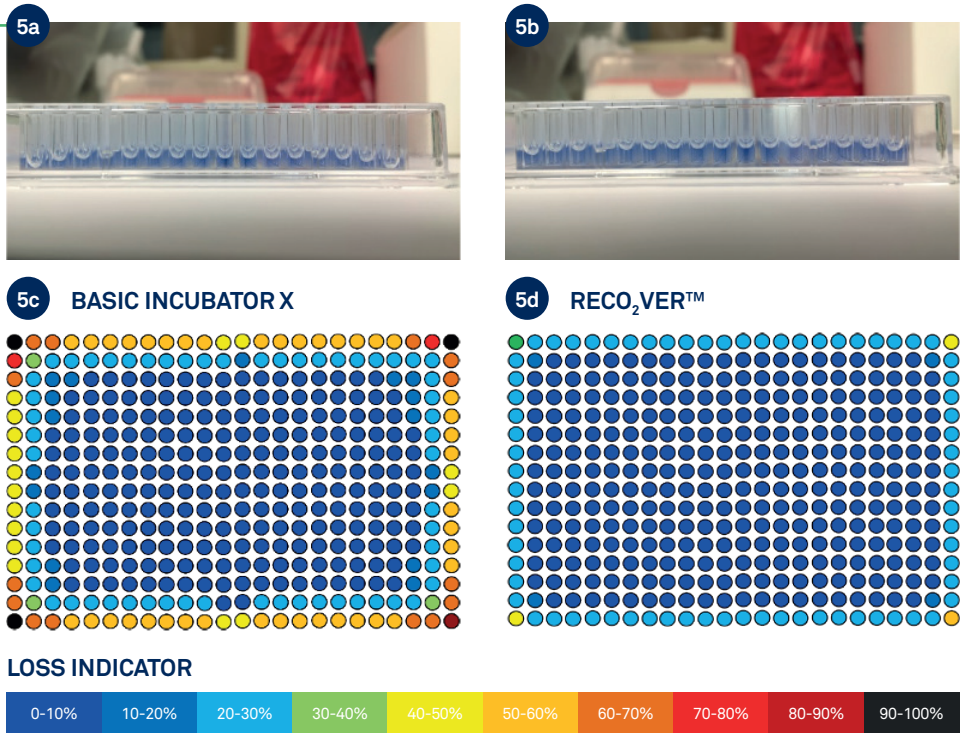
"Sweet Spot" Location test.
Four locations within the incubator were tested as diagramed (**A**). (**B**) The percent of media remaining is shown with $ReCO_2ver$™ in dark blue and Basic Waterpan X in light blue.

Very small volume retention in a 384-well format

384-well plates initially with 40µL of 0.2% Trypan Blue solution after 4 days of incubation are shown in **Figure U**. While substantial evaporation was seen in the outer wells of the Basic Waterpan Incubator X (**Fig. 5A**), very little was seen in the $ReCO_2ver$™ plate (**Fig. 5B**). When quantified and the percent loss was calculated, it can be seen that the outer 2 rows of the Basic Waterpan Incubator must be sacrificed due to excessive evaporation (**Fig. 5C**), while the whole $ReCO_2ver$™ plate can be used, excepting the four corners (wells A1, A24, P1 and P24). This is a significant improvement on the quantity of usable wells per plate, from 62.5% used (excluding the outer 2 rows) to 99% usable (380/384 wells). When this experiment was repeated using a smaller initial volume (20µL) of 0.2% Trypan Blue dye, the results were expected to be more evident, due to a very high rate of evaporation. This hypothesis held true, especially for the Basic Waterpan Incubator X (**Figure 6A, C**). Dramatic volume loss can be seen in the outer wells (**Fig. 6A**), and were measured to be 90% to complete loss of liquid from all the shorter edges of the plate (**Fig. 6C**). $ReCO_2ver$™ was not completely immune to the harsh conditions of this test (4 days, 7 door openings, 20µL volume), but the results were much improved as compared to the Waterpan Incubator. More volume retention was observed (**Fig. 6B**), and measured (**Fig. 6D**) leading to only the outer edge of the plate being effected. This increases the usable plate area from 61.5% to 79.7%, Waterpan versus $ReCO_2ver$™, respectively.

FIGURE 5.

Evaporative media loss in a 384-well plate, 40µL initial volume.
Side profile of the 384-well plate shown (**A**, Basic Waterpan and **B**, ReCO$_2$ver™). Dramatic loss indicated by red arrows. Calculated percent loss color coded according to Loss Indicator (below) for Basic Waterpan X (**C**) and ReCO$_2$ver™ (**D**).

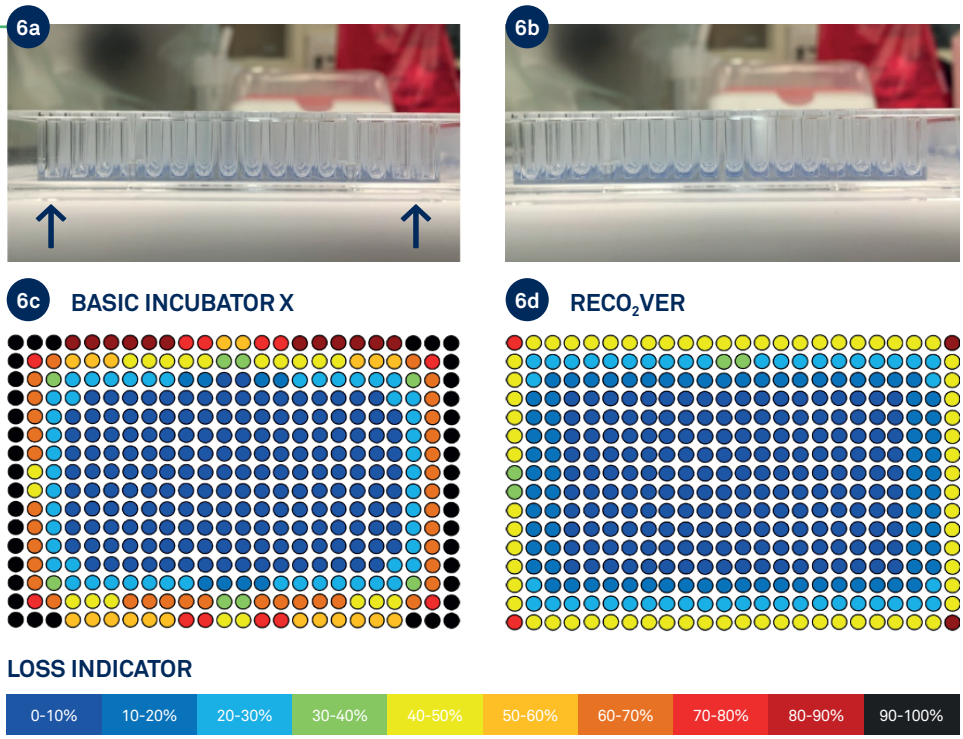


RH monitoring

The incredible difference in the levels of evaporation between the Basic Waterpan Incubator X and the ReCO$_2$ver™ is most likely due to the method of humidification each incubator employs. The Basic Incubator had a full waterpan on the basin floor. Its RH fluctuated drastically, averaging at 51% RH. It also has a slow recovery time (20-30 minutes) back to setpoint. The ReCO$_2$ver™ has a built in RH sensor that maintains setpoint at 90%, with a recovery time of less than 4 minutes back to setpoint (Gallagher et al., 2014, ReCO$_2$ver™ brochure). The level of continuous high RH will undoubtedly make a significant difference in the level of media evaporation of a multiwell plate.

FIGURE 6.

Media Evaporation of 20µL initial volume in a 384-well plate.
Side profile of the 384-well plate shown for Basic Waterpan Incubator X and ReCO$_2$ver™ (**A** and **B**, respectively). Calculated percent loss color coded according to the Loss Indicator (below) for Basic Waterpan X and ReCO$_2$ver™ (**C** and **D**, respectively).



CONCLUSIONS

When working with multiwell plates, volume retention is a key determining factor of the experiment's viability. With too much volume loss, the results can be skewed, or at worst, lost completely due to cellular death. Maintaining an appropriate amount of the initial volume desired in each well, uniformly across the plate is a hallmark of good experimental design. The Edge Effect destroys this. To work around the Edge Effect by using less wells for experiments per plate ("Inner 60") would require more plates and more control wells to be needed, which will increase the space required to store the plates, time required to feed and check them and cost to purchase more plates and media. To simplify this problem, here we show that the fast RH recovery of the ultrasonic nebulizer unique to the ReCO$_2$ver™ CO$_2$ incubator creates a buffer to protect multiwell plates from dramatic media evaporation. Using a standard amount per well (greater than 100µL in a 96-well plate and greater than 40µL in a 384-well plate) will eliminate the Edge Effect (**Figures 2 and 5**). This is true in all locations within the incubator (**Figure 4**).

If very small volumes must be used in the multiwell plates, some evaporation will still occur, but much less occurred in the ReCO$_2$ver™ incubator than in the Basic Waterpan Incubator (**Figures 3 and 6**). The amount of usable plate area was significantly increased in both the 96- and 384-well formats.

The quality of the assays conducted will be dramatically improved when the cells are maintained in the ideal environment initially designed for them for the entire extent of the experiment. The amount of time, reagents, storage space and cost saved for each experiment can also snowball very quickly, just by having less evaporation of media. We can conclusively say that control of one variable, RH, can significantly alter the entire outcome of a cell culture experiment, as well as the overall functioning of any cell culture laboratory.

REFERENCES

1. Gallagher, M., Eagleson, D., Held, K. Controlling Relative Humidity and Condensation in a CO$_2$ incubator. The Baker Company, 2021.