Improving assay consistency using Nunc Edge 2.0 96-Well Plates

Culturing and assaying cells for cancer research

Microplates are widely used in cell-based assays for cancer research. Researchers have long struggled with the “edge effect” in microplates caused by evaporation and temperature fluctuation in the perimeter wells, which becomes a major issue when assay complexity and incubation time increase. A built-in reservoir surrounding the 96 wells provides a barrier that helps to maintain a consistent microenvironment around all wells across the plate, even those at the perimeter.

Hypoxia in solid tumors *in vivo,* where cells rapidly outgrow the blood supply, can be an important factor affecting the clonal evolution of tumors, and is usually responsible for the failure of chemotherapies. The purpose of the present study is to establish a cell-based model that can consistently and reliably simulate this *in vivo* situation to provide improved relevancy to the benchtop research. Using this sample protocol, the HCT116 cell model of human colon cancer in 96-well microplates was evaluated using the Invitrogen™ Vybrant™ MTT Cell Proliferation Assay Kit. It was shown that the edge effect was greatly reduced in the microplates with the surrounding reservoir during prolonged incubation. Using Invitrogen™ Image-iT™ Hypoxia Reagent, uniform low oxygen tension was demonstrated across all 96 wells when exposed to a hypoxic environment. Applying this research cell model to the established colon cancer chemotherapy treatment by 5-fluorouracil, dose response curves were prepared to demonstrate assay consistency in the microplates under hypoxic conditions.

Part 1: Improving consistency using Thermo Scientific™ Nunc™ Edge 2.0 96-Well Plates in a cell proliferation assay

1. HCT116 cells were harvested and plated at 1.2 x 10^3 cells/well using 200 µL of cell suspension in Nunc Edge 2.0 96-Well Plates and in standard 96-well plates. The moat chambers of the Nunc Edge 2.0 plates were each filled with 1.7 mL of sterile water, and then all of the plates were incubated at 37°C in 5% CO_2 for 3 days.

2. After the 3-day incubation, the cells were labeled with Vybrant MTT cell proliferation assay reagent by replacing the growth medium in each well with 100 µL of assay medium and 10 µL of Vybrant MTT reagent, and incubating for 4 hours at 37°C in 5% CO_2.

3. After 4 hours, the assay medium with Vybrant MTT reagent was aspirated off the cells and replaced with 100 µL of isopropanol to dissolve the formazan crystals. The plates were incubated at room temperature for 2 hours and then mixed well. The absorbance was then read at 570 nm using a Thermo Scientific™ Varioskan™ LUX Multimode Microplate Reader.

Conclusion

The Nunc Edge 2.0 96-Well Plate significantly minimizes variation in cell viability between perimeter wells and center wells, allowing full use of the 96 wells in cell-based assays (Figure 1).

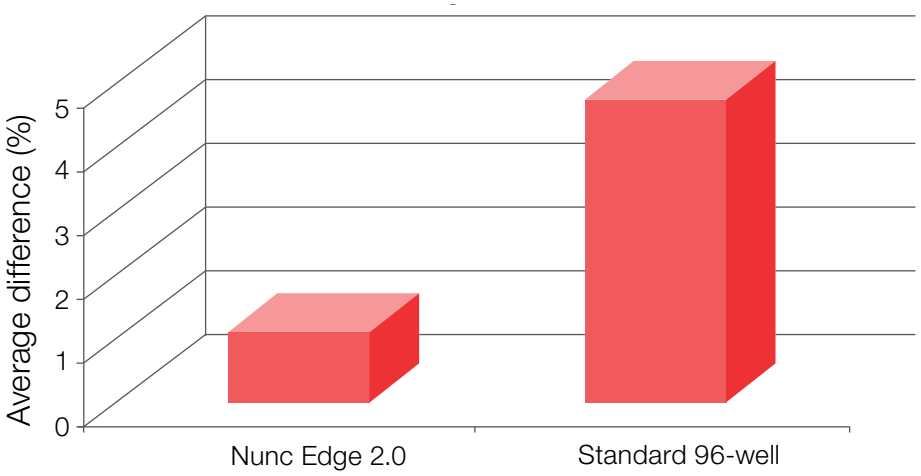

Figure 1. Difference in cell viability between perimeter wells and center wells. The average difference between optical density readings of the perimeter wells and the center wells of the Nunc Edge 2.0 96-Well Plate was greatly reduced, compared to that of a standard 96-well microplate.

Materials	Cat. No.
Nunc Edge 2.0 96-Well Plates	167542
Nunc MicroWell 96-Well Microplates	161093
HRE-*bla* HCT116 Cells	KV1183
McCoy's 5A Medium	16600082
Opti-MEM I Reduced Serum Medium	11058021
Dialyzed FBS	26400044
Penicillin-Streptomycin	15140122
Vybrant MTT Cell Proliferation Assay Kit	V13154
Image-iT Hypoxia Reagent	H10498
Heracell VIOS 160i Tri-Gas CO_2 Incubator	51030410
E1-ClipTip Pipette	4672080
Varioskan LUX Multimode Microplate Reader	VLBLATD2
EVOS FL Auto Imaging System	AMAFD1000
5-Fluorouracil	Acros Organics 228440010

Part 2: Inducing hypoxia in tumor cells in the Thermo Scientific™ Heracell™ VIOS 160i Tri-Gas CO_2 Incubator

1. HCT116 cells were plated using 2 x 10^4 cells/well in 100 µL and allowed to grow overnight at 37°C in a Heracell VIOS 160i Tri-Gas CO_2 Incubator set for normoxic conditions (~20% O_2).
2. A 1 mM stock solution of Image-iT Hypoxia Reagent was prepared by adding 1.4 mL of DMSO to 1 mg of the lyophilized powder.
3. The Image-iT Hypoxia Reagent was added (1 µL per well) to the Nunc Edge 2.0 96-Well Plates containing the medium, to a final concentration of 10 µM.
4. The cells were incubated for 12–18 hours in the Heracell VIOS 160i incubator set for hypoxic conditions (~1% O_2).
5. Cells were placed on the Invitrogen™ EVOS™ FL Auto Imaging System equipped with the Invitrogen™ EVOS™ Onstage Incubator. When the EVOS Onstage Incubator reached the required temperature (37°C), O_2 level (1%), and CO_2 level (5%), images were captured at 20x magnification using a custom Invitrogen™ EVOS™ light cube containing a GFP excitation source (488 nm) and an RFP emission filter (610 nm).

Conclusion

The Image-iT reagent revealed growth of the HCT116 cells under the hypoxic (1% O_2) conditions in the Heracell VIOS 160i incubator (Figure 2).

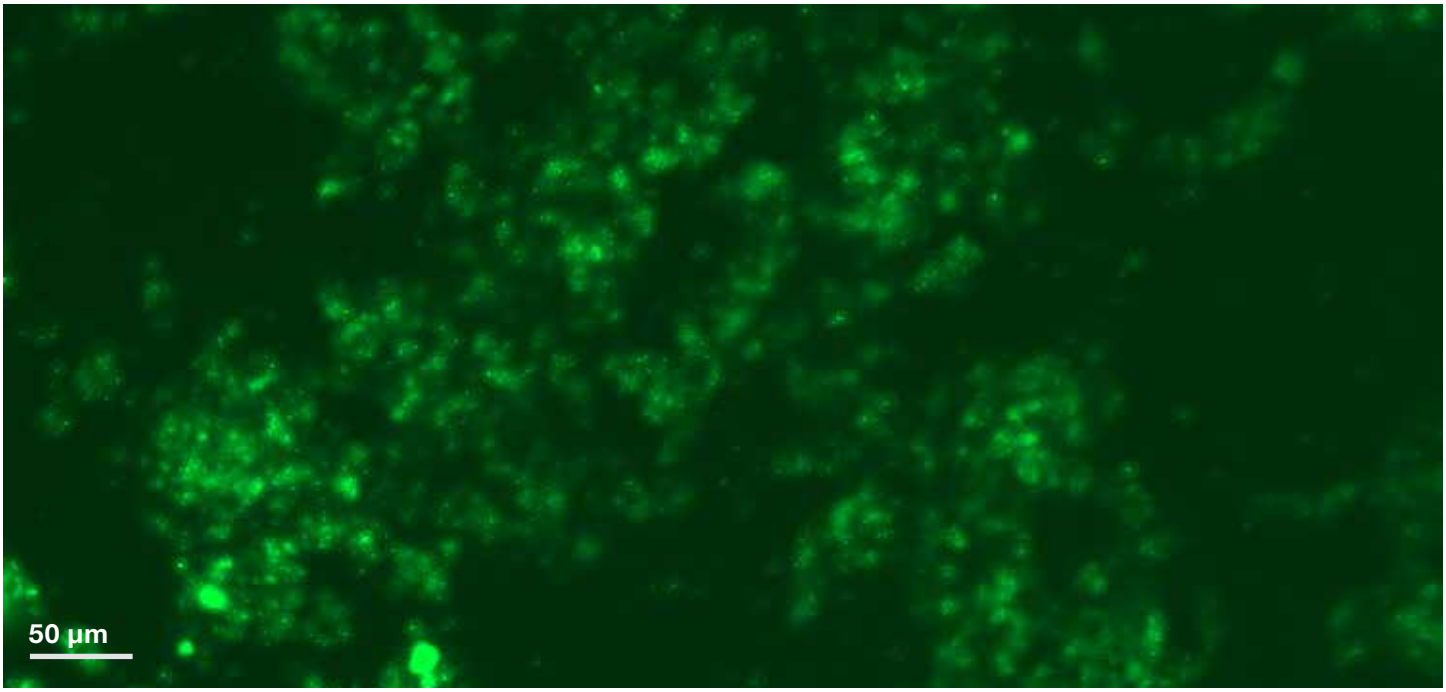

Figure 2. Fluorescence image of HCT116 cells under hypoxic conditions. HCT116 cells were viewed using a 20x objective on the EVOS FL Auto Imaging System after being stained with Image-iT Hypoxia Reagent and exposed to 1% O_2. The Image-iT fluorogenic reagent, taken up by the live cells, begins to fluoresce when atmospheric oxygen drops below 5%, confirming hypoxia in the cells.

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Part 3: Using Nunc Edge 2.0 plates to study drug resistance by tumor cells under hypoxia

1. HCT116 cells were plated into two sets (3 plates each) of Nunc Edge 2.0 96-Well Plates at 3 x 10^3 cells/well using 100 µL of cell suspension. The moat chambers of the plates were each filled with 1.7 mL of sterile water, and the plates were incubated at 37°C in 5% CO_2 overnight.
2. The growth medium was replaced with 200 µL of fresh growth medium containing the anticancer agent 5-fluorouracil. Each row of the plates was treated with a different concentration of the drug.
3. One set of plates was returned to the normoxic conditions in the 37°C, 5% CO_2 incubator, while the other set was incubated at 37°C in a Heracell VIOS 160i Tri-Gas CO_2 Incubator with 1% O_2 and 5% CO_2 to induce hypoxia in the cells. On day 3 of incubation, the moat chambers of the plates were refilled to a volume of 1.7 mL with sterile water.
4. After 5 days of incubation, the dose response curve was determined using the Vybrant MTT cell proliferation assay as described in steps 2 and 3 of the Part 1 protocol.

Conclusion

With improved assay consistency in the Nunc Edge 2.0 96-Well Plates, HCT116 cells demonstrated resistance to the anticancer agent 5-fluorouracil under hypoxic conditions (Figure 3).

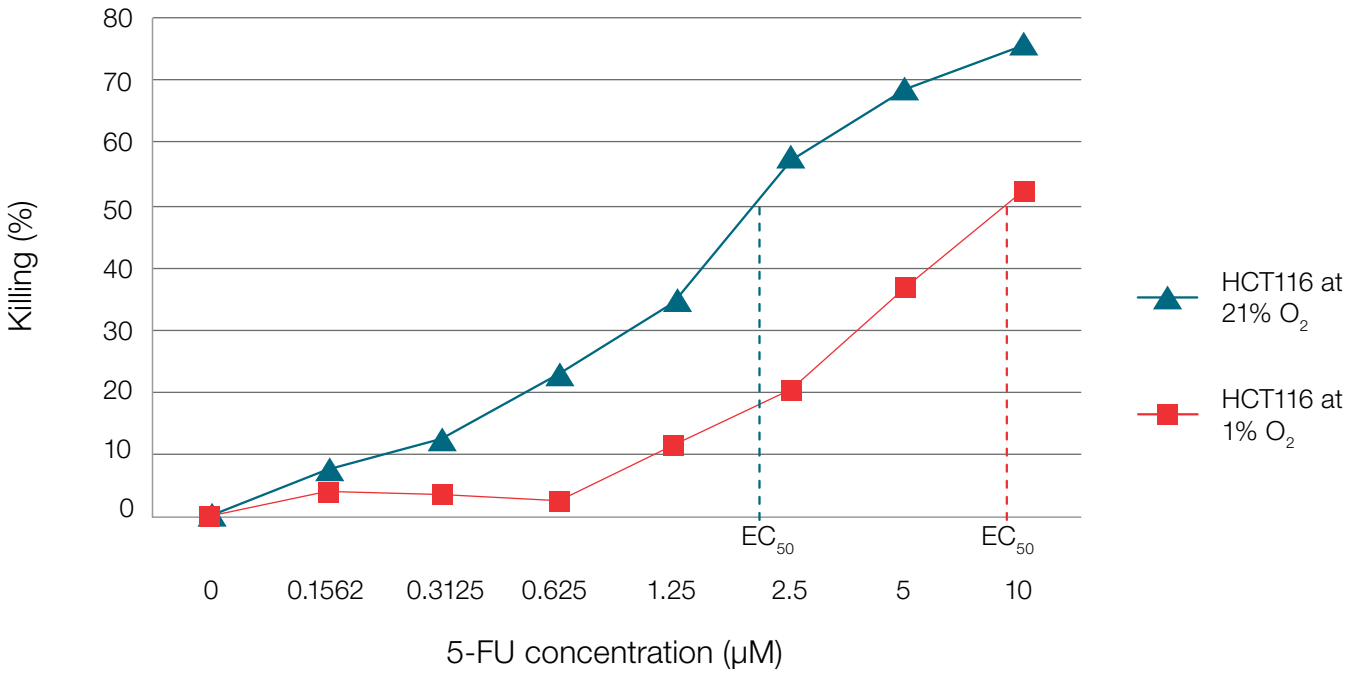

Figure 3. A 5-fluorouracil (5-FU) dose response curve for HCT116 colon cancer cells. A 4-fold increase in EC_{50} was seen under the hypoxic condition (1% O_2) compared to the normoxic condition (21% O_2).

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