PROTOCOL **Nunc Edge 2.0 96-well plates**

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 micro environment around all wells across the plate, even those at the perimeter.

Hypoxia in solid tumours ***in vivo***, where cells rapidly outgrow the blood supply, can be an important factor affecting the clonal evolution of tumours, 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 bench top 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.

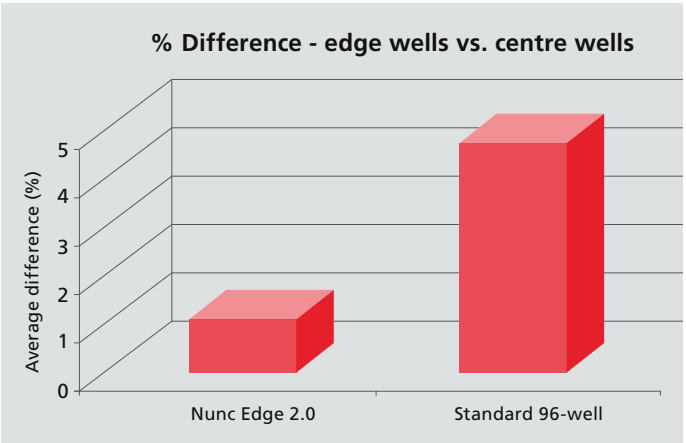

Figure 1. Difference in cell viability between perimeter wells and centre wells. The average difference between optical density readings of the perimeter wells and the centre wells of the Nunc Edge 2.0 96-well plate was greatly reduced, compared to that of a standard 96-well microplate.

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 1,2x10^3 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 labelled 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 minimises variation in cell viability between perimeter wells and centre wells, allowing full use of the 96 wells in cell-based assays (Figure 1).

thermo scientific

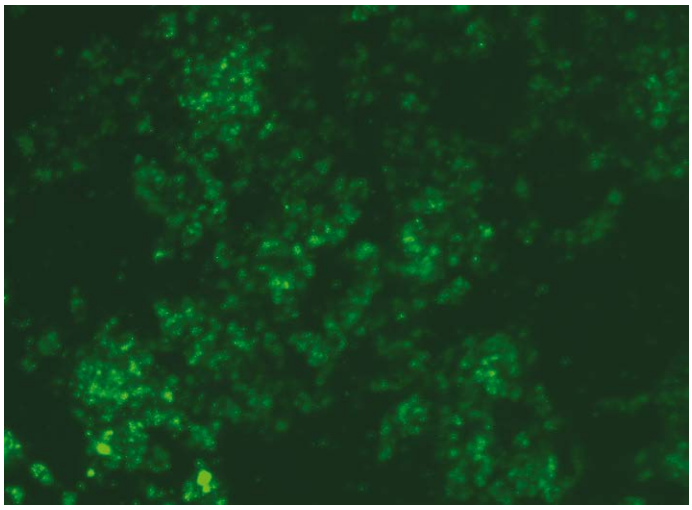

Figure 2. Fluorescence image of HCT116 cells under hypoxic conditions. HCT116 cells were viewed using a 20x objective on the EVOS FL auto imaging system after being stained with Image-iT hypoxia reagent and exposed to 1% O_2. The Image-iT fluorogenic reagent, taken up by the live cells, begins to fluoresce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

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

1. HCT116 cells were plated using 2x10^4 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), 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).

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

1. HCT116 cells were plated into two sets (three plates each) of Nunc Edge 2.0 96-well plates at 3x10^3 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 anti-cancer 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% 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 in the Part 1 protocol.

Conclusion

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

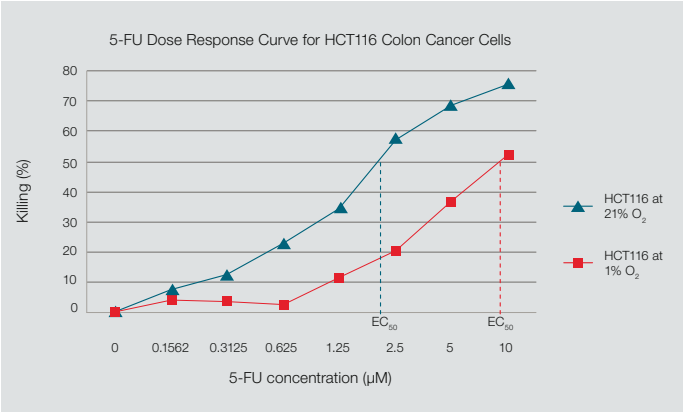

Figure 3. A 5-fluorouracil (5-FU) dose response curve for HCT116 colon cancer cells. A 4-fold increase in EC_{50} was seen under the hypoxic condition (1% O_2) compared to the normoxic condition (21% O_2).

Materials	Cat. No.
Nunc Edge 2.0 96-well plates	735-0327
Nunc MicroWell 96-well microplates	734-2073
HRE-bla HCT116 cells	-
McCoy's 5A medium	733-1705
Opti-MEM I reduced serum medium	LONZ12-743F
Dialyzed FBS	HYCLSH30079.01
Penicillin-Streptomycin	LONZ17-602E

Materials	Cat. No.
Vybrant MTT cell proliferation assay kit	30006.
Image-iT Hypoxia reagent	-
Heracell VIOS 160i Tri-Gas C incubator	-
E1-ClipTip pipette	613-6017
Varioskan LUX multimode microplate reader	735-0324
EVOS FL auto imaging system	-
5-Fluorouracil	228440010.