Application Note #470

Advanced Cell Culture Surfaces: BD PureCoat surfaces provide improved cell attachment and growth of many cell types compared to standard tissue culture vessels

Elizabeth J. Abraham, Ph.D., and Kerry Thompson
BD Biosciences – Discovery Labware, Bedford, MA

Introduction

Cell attachment, growth, and cell-to-cell interactions on a surface or extracellular matrix substrate are highly complex events involving cell adhesion molecules such as selectins, integrins or cadherins. Another factor governing the growth of cells is the composition of the culture medium, especially serum which not only provides the necessary nutrients for cells but also influences cell attachment. Serum is known to contain many extracellular matrix proteins well known for enhancing cell attachment. However, there are known limitations to serum in culture medium. Apart from being expensive, it can interfere with specific assays and introduce variability due to lot-to-lot inconsistencies and the presence of undefined components. Therefore, reducing or eliminating serum in growth media is highly desirable and helps to reduce costs involved in cell culture. However, many cell types do not fare well in the absence of serum because of loss of cell attachment. BD PureCoat™ surfaces allow robust cell attachment and therefore are amenable tools to culturing cells in reduced serum and/or serum-free medium. Enhanced cell attachment on BD PureCoat surfaces is also beneficial for recovery of cells from a freeze-thaw cycle. More importantly, cells cultured on BD PureCoat surfaces remain functional for a variety of assays including *in vitro* cytoxicity and transfection-based assays.

Materials and Methods

Cell Attachment Assay

The CellTiter 96® Aqueous One Solution Cell Proliferation MTS Assay (Promega™) was utilized for attachment and growth studies. MTS is a tetrazolium compound that is reduced by metabolically active living cells into a soluble product, formazan that gives a purple hue. The amount of formazan product is directly proportional to the number of living cells. Therefore, cell proliferation or attachment can be quantified by reading the absorbance of formazan. In most growth assays, MTS was added directly into culture medium whereas for cell attachment protocols, exhausted medium was refreshed with medium containing MTS. Plates were then read on a Tecan Safire microplate reader at 490 nm. A row of wells containing media+MTS served as experimental blank and data are expressed as background-subtracted values. In most instances, cell enumeration was performed using a hemacytometer, except for BHK-21 experiments where cells were counted on a Coulter Counter. Cells were originally purchased from ATCC, except BHK-21 Cl.13 which was obtained from Sigma.

LnCAP cell attachment

Cryopreserved LnCAP cells (passage 4 -7) were thawed in a 37ºC water bath for 1-2 minutes and transferred into 10 mls of growth medium (RPMI-1640 + L-Glutamine + 10% fetal bovine serum). An aliquot was removed to obtain a cell count using a hemacytometer. Cells were seeded at 16,000 cells/well onto 96-well plates in 100 µl of growth medium and incubated at 37ºC in a humidified 5% CO_2 atmosphere. The next day, plates were visualized under a microscope and images captured. Cell attachment was quantified using a MTS-based assay. Briefly, exhausted culture media was gently removed, replaced with media containing MTS reagent (Promega) and incubated at 37ºC for 2h and absorbance read on a Tecan Safire2 microplate reader.

HT-1080 attachment assay

HT-1080 cells were grown in DMEM (4.5 mM glucose) containing 10% FBS in T175 flasks. To initiate this study, cells were trypsinized and washed with serum-free growth medium. Cells were seeded at 25,000 cells/cm^2 onto 6-well plates in 2 mls serum-free medium and incubated at 37ºC in a humidified 5% CO_2 atmosphere. The next day, cells were fixed with methanol and stained with 1% Toluidine Blue/ Borax. Plates were visually examined under a microscope and images were captured.

BHK-21 cell growth

BHK-21 Cl.13 cell line was grown in growth medium (GMEM containing 2 mM L-Glutamine, 5% tryptose-phosphate broth) containing 10% FBS in T175 flasks. For this study, cells (passage 4-8) were trypsinized (0.25% Trypsin-EDTA) for a few minutes and immediately neutralized with growth medium. Cell suspensions were centrifuged and pellet was reuspended in growth medium now containing 1% serum. Cells were enumerated using a Coulter Counter. Then, cells were seeded onto 96- or 24-well plates at 10-20,000 cells/cm^2 in 100 µl or 400 µl, respectively in growth medium supplemented with 1% FBS and were incubated at 37°C in a humidified 5% CO_2 atmosphere. After a 3d period, plates were visually examined, and MTS added to wells directly. The plates were then read after a 2h incubation on a Tecan Safire2 microplate reader.

HEK-293 cell growth

HEK-293 cells were grown on T175 flasks in growth medium (DMEM containing 4.5 mM glucose, 2mM L-Glutamine, 1 mM sodium pyruvate) containing 10% fetal bovine serum. To initiate this study, cells (passage 5-12) were trypsinized with 0.25% Trypsin-EDTA for 5 minutes and neutralized with growth medium. Cell suspensions were centrifuged and pellet was resuspended in growth medium. Post cell enumeration, an aliquot of cells required for experimentation was removed, mixed with serum-free growth medium (1:3 ratio) and centrifuged. The cell pellet that was obtained was then resuspended in growth medium now containing 1% FBS. Cells were seeded onto 24-well plates at 60,000 cells/cm^2 in growth medium containing 1% FBS at a volume of 400 µl/well and incubated at 37°C in a humidified 5% CO_2 atmosphere. After a 4d period, plates were visually examined and images captured. Exhausted medium was removed and fresh growth medium (+1% FBS) containing MTS Reagent was added. After a 2h incubation at 37°C, plates were read on a Tecan Safire2 microplate reader.

HepG2 cell growth

HepG2 cell line was grown in growth medium (MEM containing 2 mM L-Glutamine) containing 10% FBS in T-175 flasks. For this study, cells were trypsinized (0.25% Trypsin-EDTA), centrifuged, washed and seeded onto 96- or 24-well plates at 10,000-20,000 cells/cm^2 in 100 µl or 400 µl, respectively in growth medium now containing 5% or 10% FBS. Plates were incubated at 37°C in a humidified 5% CO_2 atmosphere. After a 4d period, cells were visually examined and MTS added to wells directly. The plates were then read after a 2h incubation on a Tecan Safire2 microplate reader.

Transfection

Passaged HepG2 cells were seeded at 25,000 cells/cm^2 onto 24-well plates and cultured in HepG2 growth medium containing 10% FBS. The next day, cells were transfected with pZsGreen1-C1 plasmid DNA (Clontech Cat# 632447) using Lipofectamine 2000 (according to manufacturer's recommendations). Cells were transfected with 0.2 ug DNA/2 µl Lipofectamine™ (Gibco Cat# 11668-027) per well in OPTI-MEM I for a period of 4.5h after which transfection-mixture was aspirated and wells were refreshed with fresh growth medium. The next day, plates were visualized and fluorescent images captured on a BD Pathway™ 800 Imager using a 10X objective. Montage capture settings were utilized to provide a larger microscopic field of view.

Cytotoxicity assay

Passaged HepG2 cells were seeded at 25,000-50,000 cells/cm^2 onto 96-well plates and cultured in HepG2 growth medium containing 10% FBS. The next day, cells were either untreated (received an equal volume of DMSO) or challenged with 10 µM, 30 µM and 60 µM of Tamoxifen for 3h at 37°C. Then, each well received 20 µl of MTS reagent and plates were incubated for 2h and read on a Tecan Safire2 microplate reader.

Results and Discussion

While freeze-thaw cryopreservation of cells is a routine technique, damage from cryopreservation can lead to cell losses. There is considerable interest in improving freeze-thaw recovery of cells. Here, we demonstrate that BD PureCoat™ carboxyl enhances cell attachment of LnCAP cells freeze-thaw. LnCAP (a prostate cancer cell line) cells frozen in growth medium containing 5% DMSO were thawed and seeded onto the surfaces shown in **Figure 1A**. Images of cells grown on BD PureCoat carboxyl show even attachment and appear more dense and less clumpy than TC-treated plates (**Figure 1A**). Cell attachment was further challenged by removal of exhausted medium resulting in removal of cells that were loosely attached. Results from a representative experiment indicate that more cells attached on multiple-lots of BD PureCoat carboxyl freeze-thaw than TC or Enhanced TC Competitor C plates (**Figure 1B, panel 1**). This experiment was repeated and the increase in cell growth was quantified. We find a >25% increase in cell attachment of LnCAP cells freeze-thaw on BD PureCoat carboxyl vs. TC or Enhanced TC Competitor C plates (**Figure 1B, panel 2**).

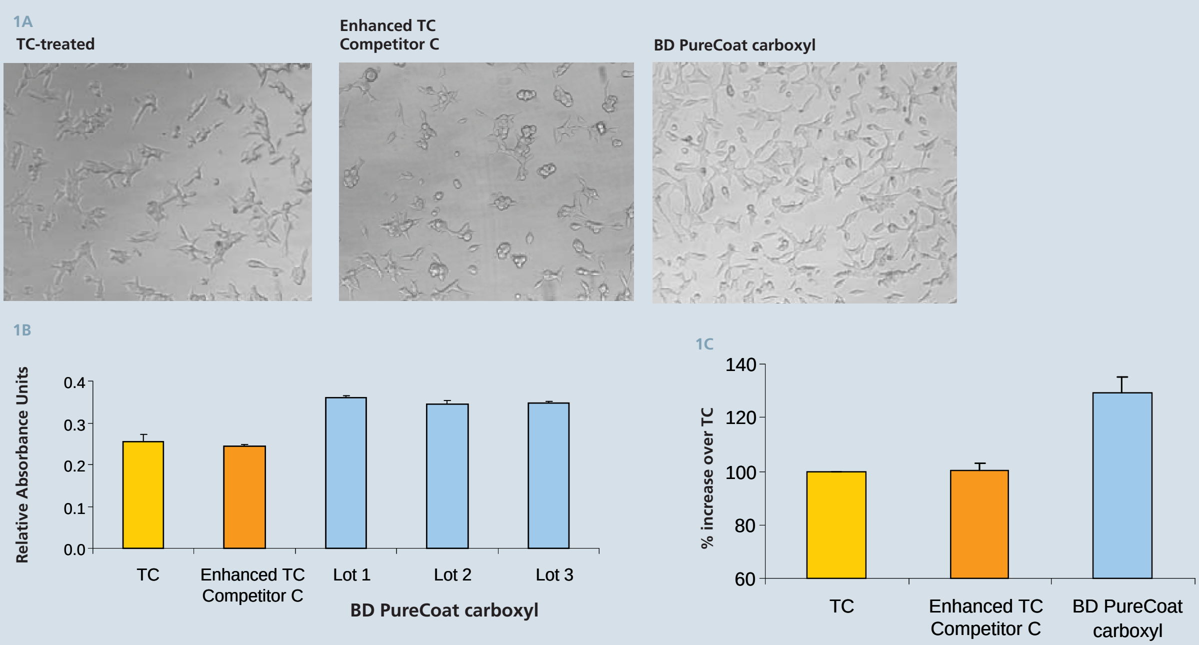
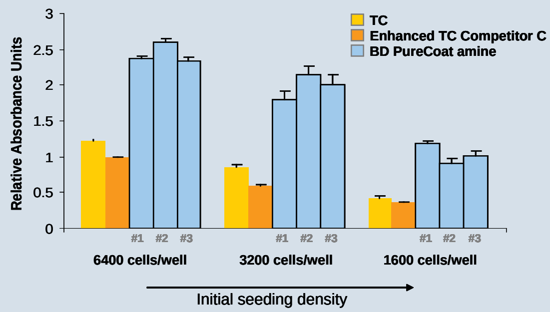


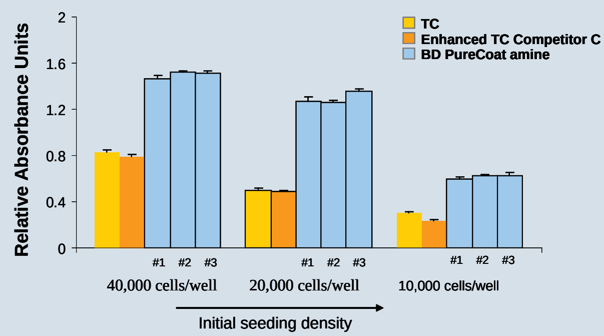
Figure 1. Improved freeze-thaw recovery of LnCAP, a prostate cancer cell line on BD PureCoat carboxyl. Early passage LnCAP cells cryopreserved in growth medium (RPMI-1640, 10% fetal bovine serum) and 5% DMSO were thawed in a 37°C water bath and immediately seeded onto 96-well black/clear tissue culture (TC), Enhanced TC Competitor C, or BD PureCoat carboxyl plates at 16,000 cells/well. (A) After an overnight incubation, cultures were observed and images captured at 100X magnification. Results from a representative experiment show greater number of LnCAP cells are evenly attached on BD PureCoat carboxyl vs. TC or Enhanced TC Competitor C plates. (B) Spent media was gently removed, replaced with growth medium containing MTS reagent (Promega™) and incubated at 37°C for 2h. Relative absorbance units (mean ± SEM) are higher (n=16 wells) on multiple lots of BD PureCoat carboxyl compared to those on tissue culture or Enhanced TC Competitor C plates (C). This increase in cell attachment and proliferation rates was significantly ($p<0.05$) greater than other surfaces tested (N=3 experiments).

BD PureCoat™ surface treatment supports robust attachment and growth of fastidious cell lines even under demanding assay conditions such as low or no serum. Although, serum is the choice supplement in cell culture media, it lends itself to certain limitations. Apart from being expensive, it can interfere with specific assays and introduce variability due to lot-to-lot inconsistencies and contain undefined components. Therefore, reducing or eliminating serum in growth media can be highly desirable, both to better define the system and to reduce the cost. However, many cell systems do not fare well in the absence or at lower serum levels. This is because serum contains attachment factors that enable cells to adhere to the substrate. BD PureCoat surface treatment enhances cell attachment and proliferation in reduced serum and serum-free medium conditions. BHK-21 cells grown in reduced serum are able to attach and proliferate well on BD PureCoat amine. As shown in **Figure 2A and 2B**, BHK-21 cells grown on multiple lots of BD PureCoat amine in 1% serum-supplemented medium show enhanced cell growth over TC or Enhanced TC Competitor C surfaces (>60% vs. TC) in all three seeding densities tested across multiple well-formats.

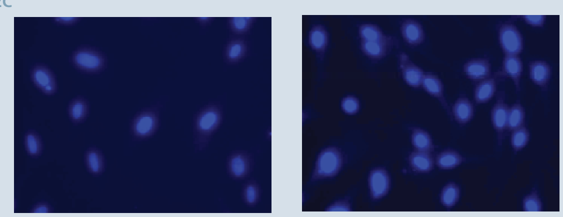
2A



2B



2C



Tissue Culture

BD PureCoat amine

Figure 2. Enhanced cell growth of Baby Hamster Kidney (BHK-21) cells in reduced serum on BD PureCoat amine. BHK-21 cells were seeded on 96-well black/clear tissue culture, Enhanced TC Competitor C or BD PureCoat amine plates at densities between 1,600-6,400 cells/well and grown in culture medium containing 5% tryptose and 1% fetal bovine serum for 72h. MTS reagent (Promega) was added directly into culture wells (n=10 wells/condition) and incubated for 2h. Representative relative absorbance units (mean ± SEM) from one of three experiments is shown in panel (A). A similar experiment was also done on 24-well formats of the above-mentioned surfaces and cells were seeded between 10,000-40,000 cells/well as shown in panel (B). Increased cell proliferation was observed on multiple lots of BD PureCoat amine in all three seeding densities across both formats tested compared to TC or Enhanced TC Competitor plates. Shown is a representative image of BHK-21 cells grown on 24-well tissue culture or BD PureCoat amine plates and fixed with 4% paraformaldehyde. A nuclear stain, DAPI was added to cultures and fluorescence images were captured at 200X magnification as shown in Panel (C). An increase in DAPI-staining was observed on BD PureCoat amine indicating more cells attach and proliferate on the BD PureCoat amine surface vs. TC.

We also report a similar outcome when using HEK-293 cells, a popular cell line choice for many cell-based assays. HEK-293 cells grown in 1% serum-supplemented medium on BD PureCoat surfaces show enhanced cell growth over TC or Enhanced TC Competitor surfaces (>25% vs. TC; **Figure 3**). BHK-21 and HEK-293 do not appear to require special adaptation steps when the serum was decreased and the cells were cultured on BD PureCoat surfaces.

3

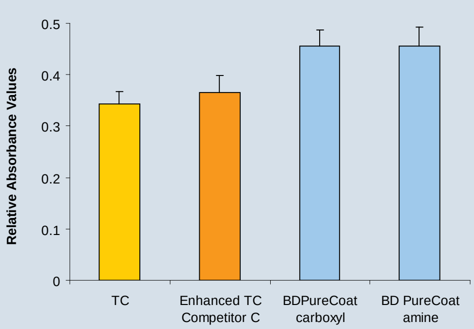


Figure 3. Enhanced cell growth of Human Embryonic Kidney (HEK-293) cells in reduced serum on BD PureCoat amine. HEK-293 cells were seeded on 24-well black/clear tissue culture, Enhanced TC Competitor C, or BD PureCoat carboxyl and BD PureCoat amine plates at 120,000 cells/well and grown in culture medium (DMEM-high glucose, L-Glutamine and sodium pyruvate) containing 1% fetal bovine serum. On the 4th day, exhausted medium was aspirated and refreshed with fresh growth medium (+1% FBS) containing MTS reagent (Promega; n=4 wells/condition) and incubated for 2h. Representative relative absorbance units (mean ± SEM) is shown. Increased cell proliferation was observed on BD PureCoat surfaces compared to TC or Enhanced TC Competitor C plates.

Serum-free attachment assays are also frequently used in drug discovery, however this feature is generally more difficult to achieve. More so, standard TC vessels are unable to support cell attachment of some cell types in the absence of serum. Here, we show that BD PureCoat™ carboxyl is able to support HT-1080, a fibrosarcoma cell line attachment in the absence of serum. As shown in **Figure 4**, HT-1080 cells attach and spread uniformly on BD PureCoat carboxyl. Cells plated on TC or Enhanced TC Competitor C surfaces are sparse and show uneven cell attachment, indicating poor cell attachment in these vessels in serum-free medium conditions.

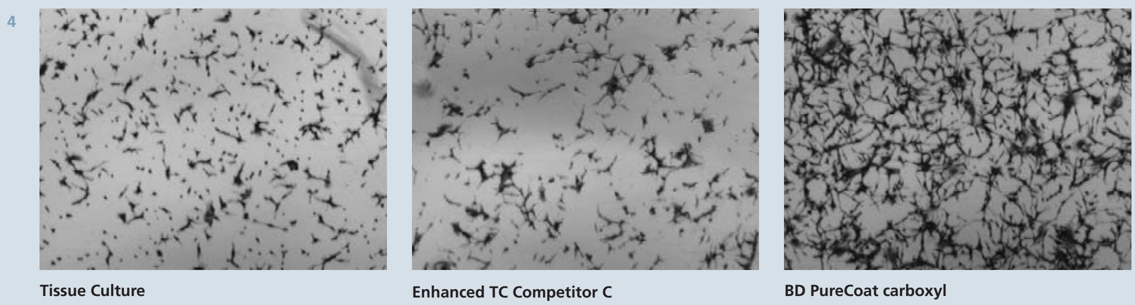


Figure 4. Enhanced attachment of HT-1080 cells on BD PureCoat carboxyl surface in serum-free media. HT-1080 cells were seeded onto 24-well clear tissue culture, Enhanced TC Competitor C, or BD PureCoat carboxyl plates at 25,000 cells/cm² in serum-free medium. Care was taken to ensure complete removal of serum prior to initial seeding. After an overnight incubation, cultures were fixed with methanol and stained with 1% Toluidine Blue/Borax. Shown are representative images captured at 40X magnification. More HT-1080 cells evenly attach and spread on BD PureCoat carboxyl whereas less dense uneven cell attachment was observed on tissue culture or Enhanced TC Competitor C plates.

Toxicity assays are indispensable for clinical research and enable rapid evaluation of potential toxicity of large numbers of compounds/drugs. The value of *in vitro* cytotoxicity tests is based on the premise that chemical compounds affect cell functions and that the toxicity of compounds can be measured by assessing cell damage. This assay system is especially desirable because it can limit animal experimentation when possible. HepG2, a cell line derived from human hepatocellular carcinoma is commonly employed in toxicity studies. Here we demonstrate that BD PureCoat surfaces are able to support HepG2 cell growth. As shown in **Figure 5A and 5B**, HepG2 cells grown on BD PureCoat carboxyl show enhanced growth over TC surfaces both in standard growth medium as well as in reduced serum conditions.

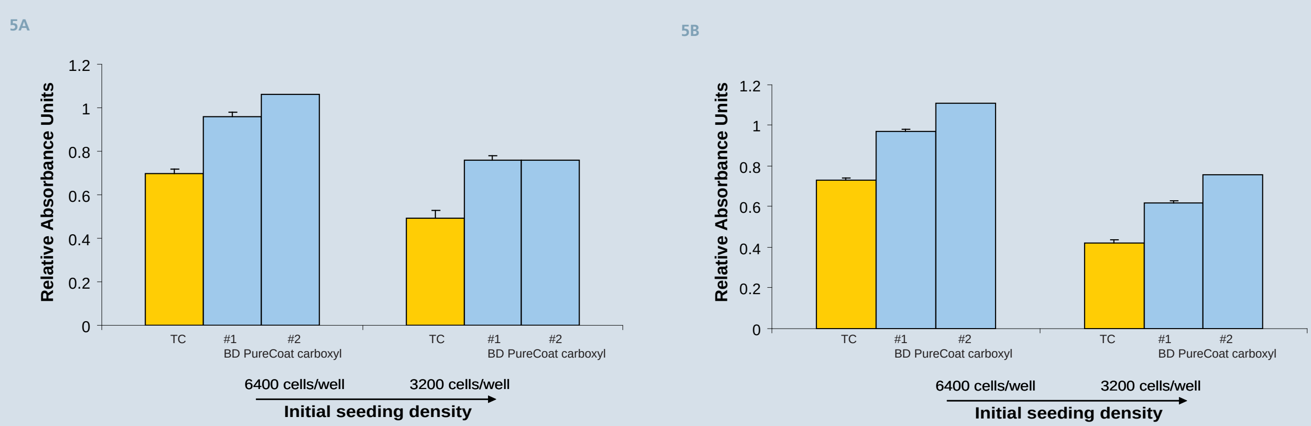


Figure 5. Improved cell growth of HepG2 cells in standard growth medium as well as in reduced serum on BD PureCoat carboxyl. HepG2 cells were seeded onto 96-well black/clear tissue culture or BD PureCoat carboxyl plates at densities between 3200-6400 cells/well and grown in culture medium containing either 5% (5A) or 10% fetal bovine serum (5B). After a 4d period, MTS reagent (Promega) was added directly into culture wells (n=10 wells/condition) and incubated for 2h. Representative relative absorbance units (mean ± SEM) from one of three experiments are shown in 5A and B. Increased cell growth was observed on multiple lots of BD PureCoat carboxyl compared to TC plates in both standard growth medium (supplemented with 10% serum) as well as in reduced serum-supplemented medium (+5% serum).

Furthermore, HepG2 cells grown on BD PureCoat™ surfaces remain responsive in toxicity assays. HepG2 cell viability decreases in response to increasing concentrations of tamoxifen (**Figure 6A and B**) on all surfaces tested. The potency (EC_{50}) of tamoxifen in HepG2 cells grown on BD PureCoat surfaces is within two fold of that observed when cells were grown on TC surfaces.

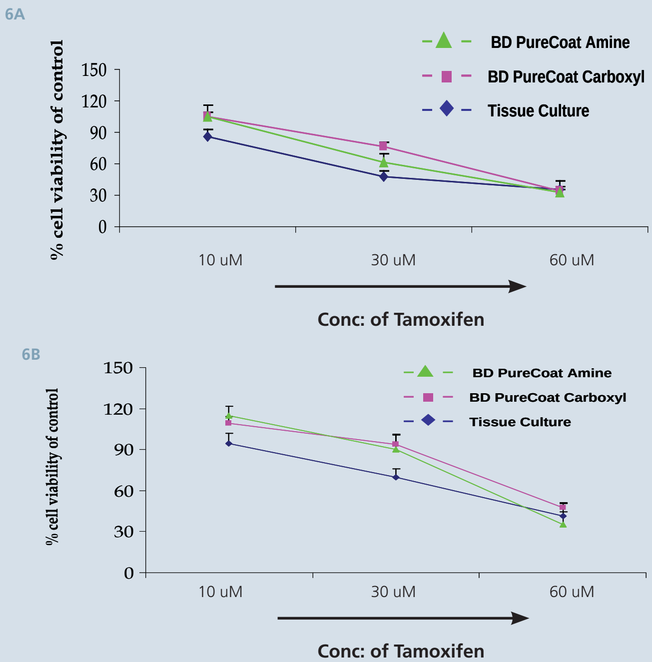
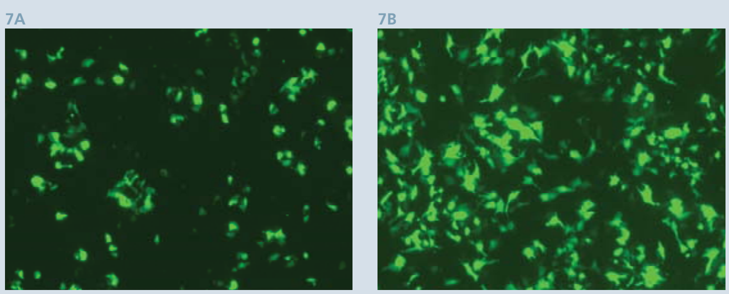


Figure 6. HepG2 cells grown on BD PureCoat surfaces remain functional in *in vitro* toxicity assays. HepG2 cells were seeded onto 96-well black/clear tissue culture or BD PureCoat surfaces at 8,000 and 16,000 cells/well in culture medium containing 10% fetal bovine serum (panel A and B, respectively). After 24h, cells were exposed to increasing concentrations of Tamoxifen for 3h. Then, MTS reagent (Promega) was added directly into culture wells (n=10 wells/condition) and absorbance read 1.5h later. As shown, HepG2 cells respond to increasing concentrations of Tamoxifen with decreasing cell viability. Toxicity was more pronounced at the lower cell seeding density than the higher density (panel A vs. B)

Mammalian expression systems have an undisputed legacy for the generation of recombinant proteins and transfection of DNA is a method by which this target may be achieved. We evaluated whether cells grown on BD PureCoat surfaces can be transfected with plasmid DNA and find that both HepG2 and HT-1080 cells seeded on BD PureCoat amine or carboxyl, respectively, can be readily transfected with pZsGreen1-C1. As shown in **Figure 7A and B**, many fluorescent cells were visible post transfection on PureCoat surfaces. We report that although HepG2 cells show an altered morphology on BD PureCoat surfaces, they still remain functional both in transfection and cytoxicity assays.



Tissue Culture **BD PureCoat amine**

Figure 7. Transient transfection of DNA in HepG2 cells grown on BD PureCoat amine. HepG2 cells were seeded onto 24-well tissue culture or BD PureCoat amine at 50,000 cells/well in culture medium containing 10% fetal bovine serum. After 24h, cells were exposed to transiently transfected with pZsGreen1-C1 plasmid DNA and Lipofectamine™ 2000 (0.8 ug DNA and 2 ul Lipofectamine/well) according to manufacturer's recommendation. The transfection mixture was removed after 4h and cells received fresh growth medium. The next day, cells were visualized and fluorescent images captured on a BD Pathway™ 800 Imager. Representative images show many ZsGreen positive cells on BD PureCoat amine (B).

Conclusions

- BD PureCoat surfaces provide enhanced cell attachment and growth in serum reduced or serum-free culture media
- Improved freeze-thaw recovery of cryopreserved cells when cultured on BD PureCoat surfaces
- Cells grown on BD PureCoat™ surfaces remain functional in cytoxicity and transfection assays

Acknowledgments

We thank Susan Qian, William Galbraith, and Paula Flaherty for assistance with manuscript preparation and Robert Barton for providing application samples.

BD Biosciences
Two Oak Park
Bedford, MA 01730 USA
tel: 877.232.8995
fax: 800.325.9637