Considerations When Using Frozen Cells for High Throughput Cell-Based Assays

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SnAPPShots

A brief report from the Corning Applications Group

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Introduction and Purpose

The use of cell-based assays in High Throughput Screens (HTS) is growing in acceptance and popularity. Advantages of cell-based assays include: (1) distinguishing between agonist and antagonist, (2) identification of allosteric modulators, (3) characterization of compound attributes, such as permeability and stability inside of cells, and, (4) assessment of cytotoxicity.[1] Ideally, the goal is to generate data that are more physiologically predictive of the clinical outcome. The rise in the use of HTS is predominantly due to wider choices in technology and the availability of large batches of cryopreserved cells. Using cryopreserved cells directly in HTS assays allows for:[2]

- Improved assay performance and uniformity due to the availability of large batches of cells at the same cell line passage.
- Increased flexibility for running screens due to readily available stocks of frozen cells.
- Reduced costs for screens, due to the decreased time and reagent requirements for maintaining parallel cell lines in culture.

Although beneficial, the use of cells (and cryopreserved cells) for *in vitro* assays should be approached with caution, as cells can demonstrate dramatically differing growth and functional characteristics depending on environmental and experimental conditions.

The following study investigated the impact on performance of batch-frozen vs. continuously cultured cells in multiple assay formats. HEK-293 and CHO-K1-M1WT2 adherent cell lines were used in a luciferase reporter gene and calcium flux assay, respectively. C166-GFP cells were used to assess the impact on cellular retention on the microplate surfaces after the various treatments. For all assay formats, the impact of cell treatment on assay performance was assessed. The study also examined the impact of surface chemistry on assay performance using cryopreserved cells, comparing the standard tissue culture surface (TCT), Corning® CellBIND® surface and poly-D-lysine coating.

Methods and Results

All cells were frozen at a concentration of $1x10^6$ to $2x10^6$ cells per mL in 90% FBS (Mediatech Inc.) with 10% DMSO (Invitrogen Corp.). To study the impact of using frozen HEK293-Cre-Luc cells (Panomics Cat. No. RC0007) on assay performance, the luciferase reporter gene assay was utilized (Figure 1). Cells were seeded into Corning 384 well white tissue culture-treated (TCT) microplates (Corning Catalog No. 3570) at a density of 5,000 cells per well. Cells were cultured in IMDM (Mediatech Inc.) without phenol red with 10% FBS and 0.1 mg/mL hygromycin (Mediatech Inc.). Three cell treatments were used for this study:

1. Continuously cultured (CC) cells were passed through several passages.
2. Cells, one passage from freezer (1P), were thawed into flasks and passed into the microplates.
3. Cells thawed directly into a microplate approximately 20 hours prior to Forskolin addition (o/n).

The HEK293-Cre-Luc cell line is stably transfected with a luciferin reporter gene driven by a promoter responsive to the cyclic AMP response element-binding (CREB) protein. A subset of the cells were induced with 20 µM Forskolin and incubated overnight in a humidity controlled incubator at 37°C with 5% CO_2. Prior to reading, the microplates were equilibrated to room temperature for 30 minutes. The SteadyLite HTS Luciferase reagent (Perkin Elmer™) was

then added to the appropriate wells and the microplates incubated for five minutes and then read on a Aquest® plate reader (Molecular Devices). We observed that cells thawed directly into the microplates can be successfully used in the luciferase reporter gene assay (Figure 1), although we observed reduced signal strength compared to the other treatments.

To study the physiologic impact of using cryopreserved cells in a cell based signal transduction assay, CHO-K1 M1WT2 cells (ATCC®, CRL-1984™) stably transfected with the rat M1 muscarinic acetylcholine receptor were used in a calcium flux assay (Figure 2). The following 96 well microplates were seeded at 20,000 cells per well in Ham's F-12 (Mediatech Inc.) with 10% FBS and 50 µg/mL G418 (Invitrogen Corp).

- Corning® black 96 well flat clear bottom TC-treated surface (Corning Cat. No. 3904)
- Corning CellBIND® surface black 96 well flat clear bottom (Cat. No. 3340)
- Corning black 96 well flat clear bottom poly-D-lysine coated (Corning Cat. No. 3667)

All cultures were grown in a humidity controlled incubator with 5% CO_2 at 37°C. The calcium mobilization assay was then performed using a Calcium 3 kit (Molecular Devices, Inc.). The same cell preparations were used as previously explained with the addition of thawing the cells two hours prior to the study (2h). After removal of old medium and the addition of fresh medium, 10 µL of Calcium 3 dye solution was added to appropriate wells and the microplates were incubated for one hour at 37°C. Microplates were then equilibrated to room temperature for 30 minutes before being placed into a FlexStation® reader (Molecular Devices, Inc.). The fluidic module on the FlexStation was programmed to transfer 5 µL of carbachol (Sigma) to induce the calcium response or 5 µL of vehicle for negative controls. Calcium signal was monitored over a period of 45 seconds at excitation and emission wavelengths of 485 and 525 nm, respectively.

Flux kinetic curves demonstrated that cells plated approximately 20 hours prior to the assay (o/n) can perform similar to continuously cultured cells (CC) cells (Figure 2a). Cells thawed and plated two hours prior to the assays (2h) had a significantly reduced assay window. Surprisingly, cells thawed and passed one time in flasks (1P) also demonstrated a shorter assay window. However, these cells were passed for less than 24 hours from a previous experiment's (o/n) vials, and were likely affected by the presence of residual cryopreservative. The use of poly-D lysine or Corning CellBIND surface does not appear to add any benefit to the performance of frozen cells on the assay. Figure 2b reveals that the EC_{50}s are equivalent between the different samples, with the cells seeded from the single passage (1P) showing lower EC_{50} values due to poor activation curves (refer to the activation curves from figure 2A).

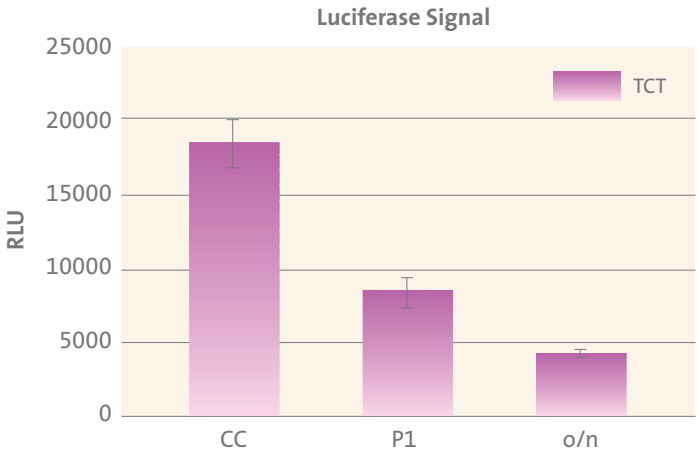

Figure 1. Graph showing the impact of using frozen HEK293-Cre-Luc cells (Panomics Cat. No. RC0007) on assay performance in the luciferase reporter gene assay. The following culturing methods were used: (CC) continuously cultured cells which were passed through several passages; (1P) cells passed once from the freezer and (o/n) cells thawed directly into a microplate approximately 20 hours prior to Forskolin addition.

To examine whether the different microplate surfaces impact the retention of cryopreserved cells on the microplate, C166-GFP cells (ATCC CRL-2583™) were assayed using the Cellomics ArrayScan II (ThermoFisher Scientific). The C166-GFP endothelial cell line, derived from F1 embryos obtained by mating a female NMRI/GSF mouse with a male CD-1 mouse, were transfected with the pEGFP-N1 reporter vector, which encodes an enhanced green fluorescent protein (GFP). Cells cryopreserved as previously described were thawed and plated into Corning 96 well black clear bottom plates with the following surface treatments: poly-D lysine (Corning Cat. No. 3372), tissue culture treated (TCT) (Corning Cat. No. 3904) and Corning CellBIND treated surface (Cat. No. 3340). Once seeded, the cells were allowed to adhere to the various microplate surfaces as described in Figure 2. Microplates were then washed three times with PBS (Mediatech, Inc.) using a ELx405cw plate washer (Bio-Tek Inc.). Cells were then fixed with 4% paraformaldehyde in conjunction with 0.1 µg/ml Hoechst nuclear dye (Invitrogen Corp.) and incubated for 15 minutes prior to scanning on the ArrayScan II. Figure 3 reveals that on both the continuously cultured (CC) and the one pass (1P) samples, there were greater cell numbers retained than those thawed directly into the various microplates 20 hours previously (o/n) or thawed the morning of the screen (2h). In both the (CC) and (1P) samples those cells thawed on to plates with the Corning CellBIND surface had greater retention compared to those cultured on TCT and poly-D lysine microplates.

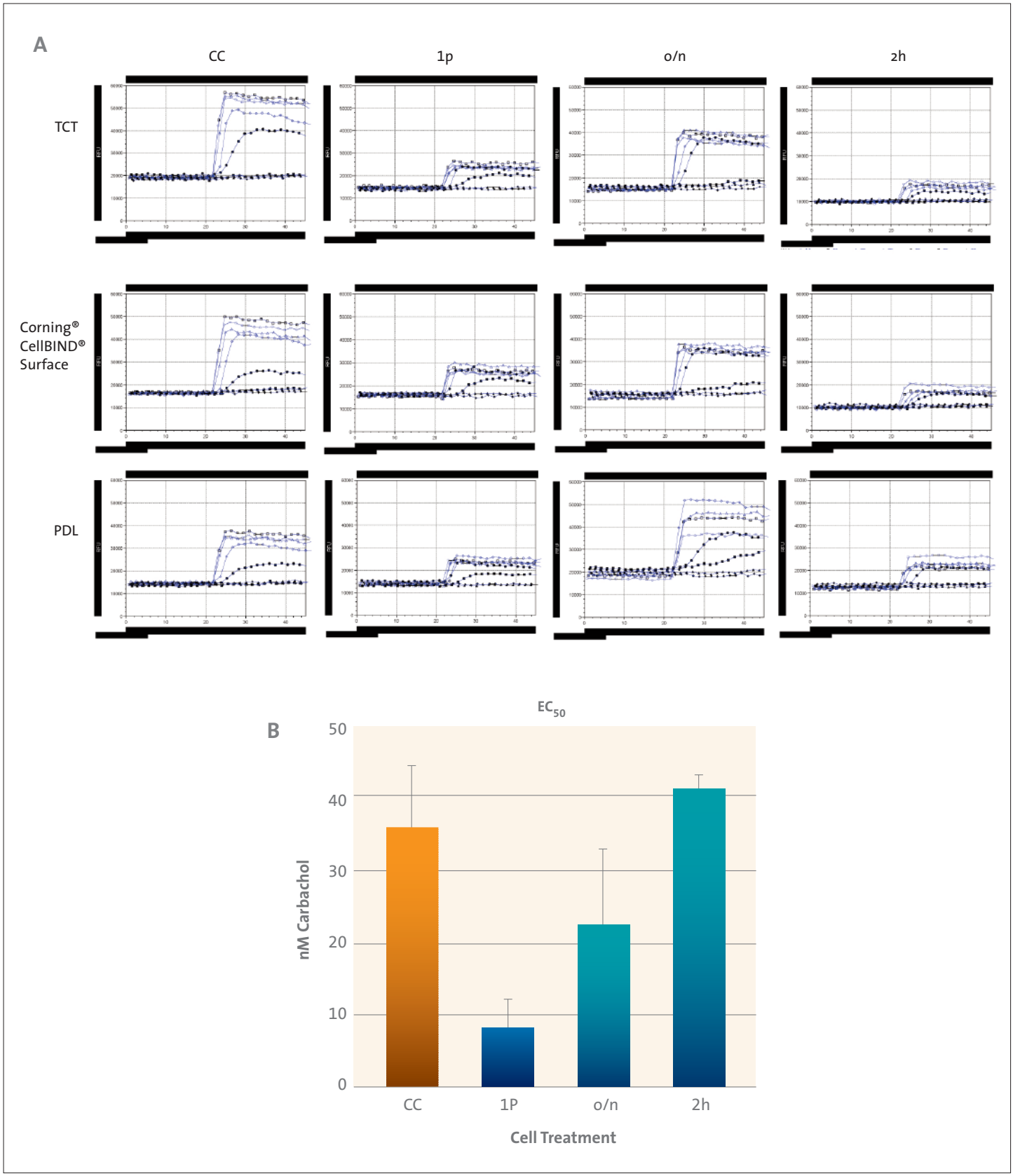

Figure 2. (A) Calcium flux assay results comparing four plating methods using cryopreserved CHO-K1 M1WT2 cells. The following culturing methods were used: (CC) continuously cultured cells which were passed through several passages, (1P) cells passed once from the freezer, (o/n) cells thawed directly to microplate approximately 20 hours prior to the assay and finally (2h) which were cells thawed and plated two hours prior to the assay. (B) The EC_{50} for the calcium flux assay for each treatment is depicted.

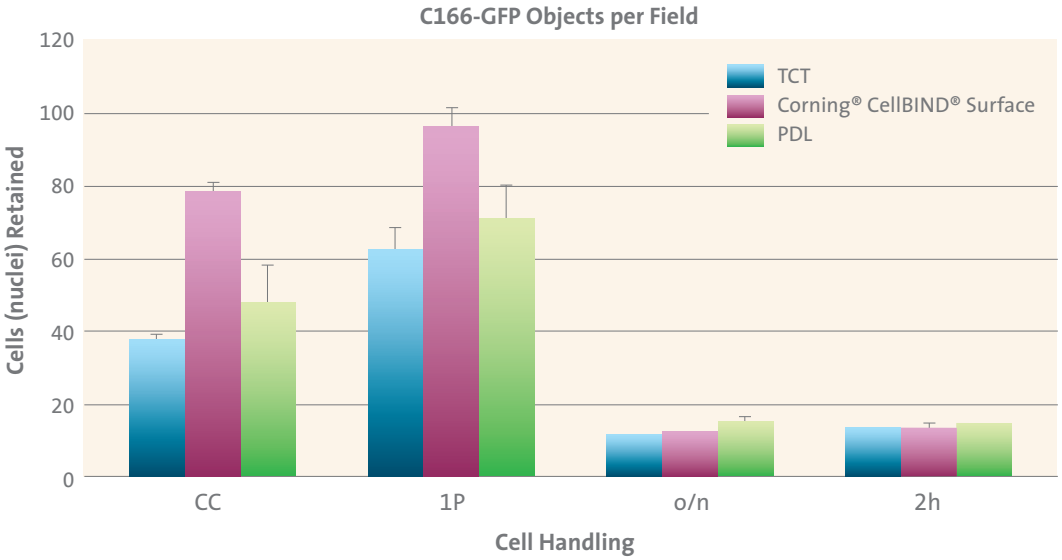

Figure 3. High Content Analysis of C166-GFP cells seeded as follows: (CC) continuously cultured cells which were passed through several passages, (1P) cells passed once from the freezer, (o/n) cells thawed directly to microplate approximately 20 hours prior to the assay and finally (2h) which were cells thawed and plated 2 hours prior to the assay.

Conclusion

- Frozen cells can be used as "assay reagents" in multiple assay formats.
- The robustness of the assay using frozen cells can be impacted by:
  - the method of thawing and seeding.
  - the microplate surface chemistry, providing the best attachment and retention with the Corning CellBIND microplates (Corning Cat. No. 3340).

References

1. Kunapuli Priya et al., Assay and Drug Development Technologies (2005) 3(1).
2. Zaman, Guido JR., et al., Drug Discovery Today (2007) 12 (13/14).

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