FlashPlate® File #4

Sterile FlashPlate for Live Cell Functional Assays

Sally Casto, Debra Curran and Elizabeth Wenske, Ph.D.
Research & Development,
PerkinElmer Life Sciences

PerkinElmer™ life sciences.

Sterile FlashPlate® for Live Cell Functional Assays

Introduction

Sterile FlashPlate is an effective platform for in-plate live cell functional assays, demonstrating functional performance consistent with traditional formats. It provides a combined growth and reaction chamber, enabling cell growth, assay, and detection in one plate. The inherent benefits of FlashPlate in reducing assay steps, and ease of automation, make FlashPlate a cost-effective platform for screening live cell assays.

Sterile FlashPlate (SMP300, SMP300E) is a Basic FlashPlate which has been sterilized by gamma irradiation and supports long-term growth of adherent cell lines. Growth, reaction, assay and detection all occur on one plate, facilitating a variety of live cell functional assays, including:

- Cell proliferation assays (e.g., thymidine uptake)
- *In vivo* protein synthesis assays (e.g., methionine incorporation)
- Receptor binding assays

Sterile FlashPlate is sterilized by gamma irradiation. Plates maintain sterility as long as the packaging remains intact. An important positive side effect of gamma irradiation is a modification to the surface of the polystyrene microplate that promotes cell attachment. Gamma irradiation changes the color of the polystyrene from pure white to light green. To date, this has not been shown to affect any experimental results.

In comparison to the use of a non-sterile Basic FlashPlate (SMP200), Sterile FlashPlate eliminates the ethanol rinse required to ensure sterile growth conditions, and provides a well surface more conducive to cell attachment.

General Use

Sterile FlashPlate is designed for use in applications requiring medium- and long-term cell growth. Adherent cells are grown directly in the FlashPlate well. Cellular uptake of radioactive tracers is measured directly in the well without aspiration, centrifugation, precipitation, or filtration, resulting in a significant reduction in assay complexity compared to a conventional TCA filtration assay.

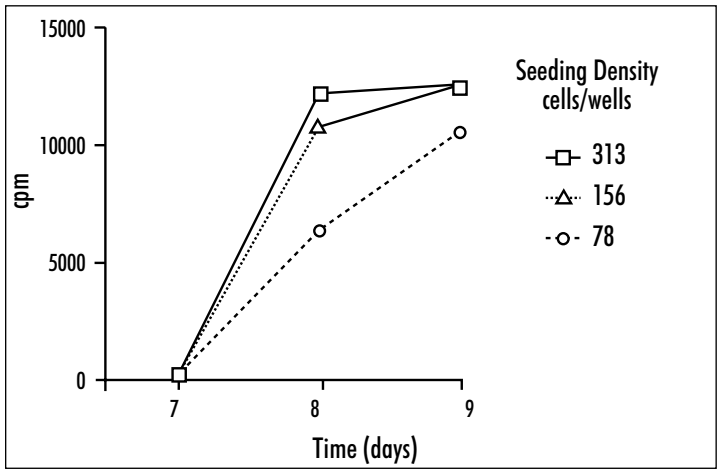
The following cell lines have been grown on Sterile FlashPlate:

- Ca Ski
- A10
- NIH/3T3
- HeLa
- CHO-K1
- CHO-ETB (CHO cell line expressing endothelin receptor subtype B)
- 293

A typical [^{14}C]-Thymidine incorporation assay is shown in Figure 1. The viability of CHO-K1 cells is maintained a minimum of nine days.

Figure 1

[^{14}C]-Thymidine Incorporation in CHO-K1 Cells over Extended Culture Times



CHO-K1 cells were serially diluted (1:2) starting at 1 x 10^4 cells/well in 0.3 ml complete DMEM/F12 containing 10% FBS, 2 mM L-glutamine and 75 µg/ml L-proline. A standard Tissue Culture Treated 96-well plate was set up as a control for the Sterile FlashPlate. This plate was used to observe confluency microscopically. On day 5 the plates were re-fed by gently removing 150 µl spent medium and adding 150 µl fresh complete medium. On day 6 the TC control wells were at 50-65% confluency. The medium was gently removed from the Sterile FlashPlate and the plate was re-fed with complete DMEM/F12 Phenol Red-free medium containing [^{14}C]-Thymidine (NEC156, 1:800, 0.125 µCi/ml) at 0.2 ml/well. Incorporation was measured on days 7-9.

Optimization of Cell Growth Conditions

The optimal cell culture conditions must be determined for each cell line and application. The seed density should be optimized for each cell type grown on Sterile FlashPlate. The optimal initial number of cells seeded per well will depend on the culture time required for the application. Cell growth characteristics on FlashPlate may differ from those on a tissue culture plate.

The following conditions are generally required for good results on Sterile FlashPlate:

1) The cell line must be adherent.

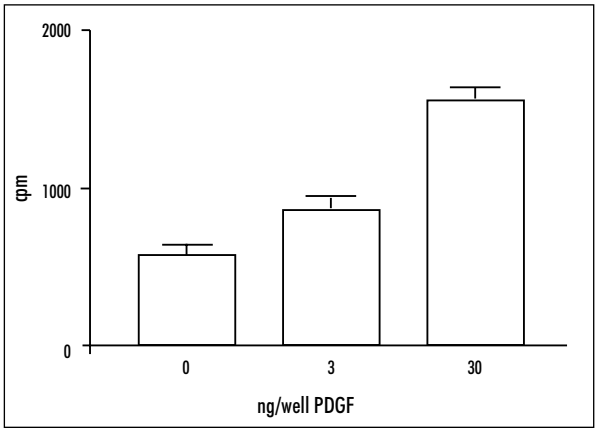
2) Phenol red in media will cause color quenching, resulting in reduction of signal. To avoid color quenching, three options are available:
   - Use phenol red-free media.
   - Remove phenol red-free media prior to detection.
   - Replace phenol red-containing media with PBS just prior to detection.

3) Generally, fetal bovine serum (FBS) must be present to promote cell attachment to the wells of Sterile FlashPlate. If low or no-serum conditions are desirable for the experiment, cells should first be grown to attachment in serum-present conditions, followed by serum reduction or removal. See Figure 2. Alternately, the plate may be precoated with fibronectin, which will promote cell attachment under low- or no- serum conditions.

4) Once cells are attached, care must be exhibited to avoid disturbing the cell monolayer. Wash steps should be minimized. Media and reagent additions and aspirations should be done in a gentle, non-disruptive manner. Use of automated plate washers should be avoided.

Figure 2

Stimulation of A10 Cell Growth by PDGF at 30 Hours



A10 cells were seeded at 5 x 10³ in DMEM containing 15% FBS. Cells were allowed to attach for 24 hours. Medium was then removed and replaced with DMEM containing 5% dialyzed FBS. After an overnight incubation, medium was removed and replaced with DMEM containing 5% dialyzed FBS plus 0.3 ng or 30 ng of PDGF. [¹⁴C]-Thymidine was also added at 0.0125 µCi/well. The graph shows net CPM (sample CPM minus background CPM) at 30 hours after PDGF addition.

Applications

In Vivo Protein Synthesis

Sterile FlashPlate is suitable for use with a variety of isotopes, including ^{3}H, ^{14}C, and ^{125}I, and exhibits functional performance that is consistent with traditional assay formats. As shown in Figures 3a-c, methionine incorporation in CHO-K1 cells using [^{14}C]- or [^{3}H]-Methionine demonstrates typical incorporation and inhibition by the metabolic precursor puromycin.

Figure 3a

[^{14}C]-Methionine Incorporation in CHO-K1 Cells

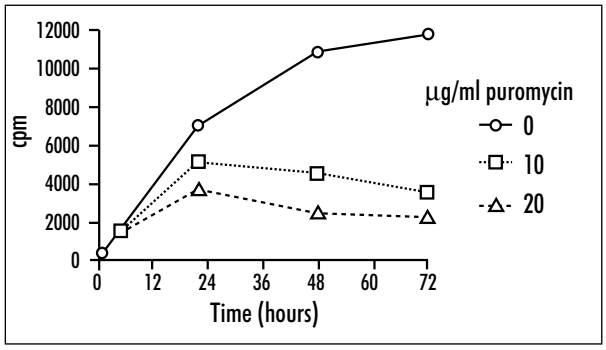


Figure 3b

[^{3}H]-Methionine Incorporation in CHO-K1 Cells

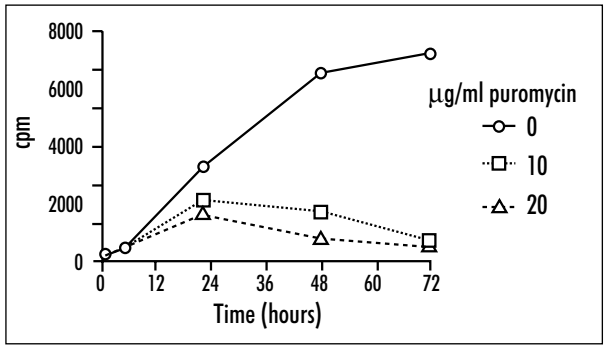
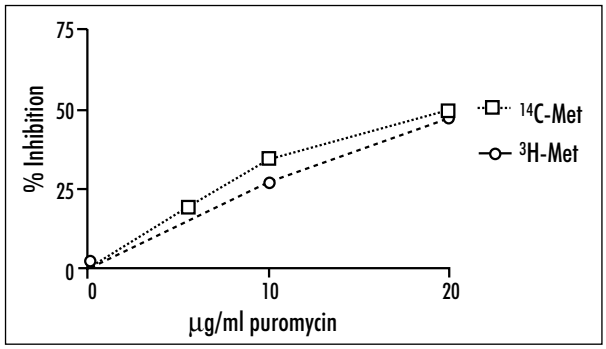


Figure 3c

Inhibition of CHO-K1 Protein Synthesis by Puromycin



Figures 3a-c: CHO-K1 cells were seeded at 1 x 10^{4} cells/well in 200 µl Phenol Red-free, methionine-free DMEM/F12 media containing 10% FBS. Cells were incubated overnight. The media was then removed and replaced with 100 µl of fresh media containing either [^{3}H]-methionine or [^{14}C]-methionine and 1, 10, or 20 µg/ml of the protein synthesis inhibitor puromycin. [^{3}H]-Methionine (NET061X) was added at 10 µCi/ml and [^{14}C]-methionine (NEC425) was added at 0.2 µCi/ml. The cultures were continued for up to 72 hours after the tracer addition. CPM were read on a Packard TopCount® Microplate Scintillation and Luminescence Counter at intervals up to 72 hours after tracer addition. All CPM data shown are net CPM (sample CPM minus background CPM).

Figure 3a: [^{14}C]-methionine incorporation into CHO-K1 cells in the presence of 0, 10, or 20 µg/ml puromycin.

Figure 3b: [^{3}H]-methionine incorporation into CHO-K1 cells in the presence of 0, 10, or 20 µg/ml puromycin.

Figure 3c: Inhibition of CHO-K1 protein synthesis by puromycin. Percent inhibition by puromycin was determined at 22 hours of culture, calculated as:

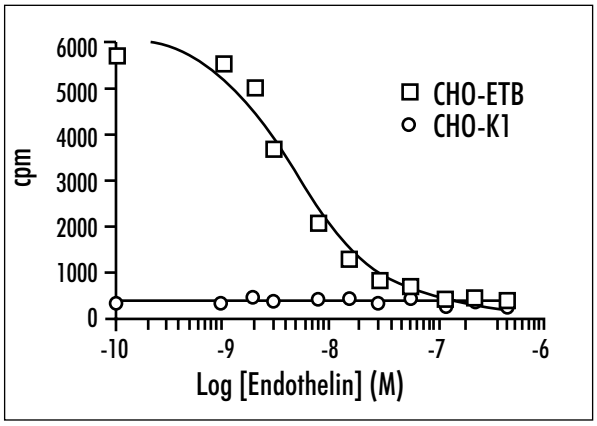
$$100 \times \left(1 - \frac{\text{CPM at indicated puromycin concentration}}{\text{CPM with no puromycin}}\right)$$

Receptor Binding Assays

Live cell receptor binding assays can be performed on Sterile FlashPlate. In Figure 4, a competitive binding assay using [^{125}I]-Endothelin demonstrates excellent binding and displacement with CHO-ET_B, a transfected cell line expressing the endothelin receptor. CHO-K1, the parent cell line, exhibits expected background counts

Figure 4

Competitive Binding [^{125}I]-Endothelin vs. Unlabeled Endothelin



CHO-ETB and CHO-K1 were seeded at 7 x 10^{4} cells/well in DMEM/F12 with 10% FBS. After an overnight incubation at 37°C, the media was replaced by Phenol Red-free growth medium containing 68,000 CPM/well of [^{125}I] Tyr^{13}-Endothelin-1 (NEX259). Cold endothelin was added to the wells in the range of 0-10 pmol/well (0-0.5 µM). The plates were read on a Packard TopCount after an additional 5 hours of incubation. The graph shows net CPM (sample CPM minus background CPM). Endothelin receptor is only expressed in the CHO-ETB cells. Specific binding to the endothelin receptor is shown by the concentration-dependent displacement of [^{125}I]-Endothelin by cold endothelin in the CHO-ETB cells and the lack of binding to the CHO-K1 cells. The EC_{50} estimated from the experiment is 4.7 nM.

Comparison with TCA Filtration Assay

In comparison to a conventional tissue culture filtration assay, assays on Sterile FlashPlate have fewer steps, and generate less radioactive waste. A schematic representation of a FlashPlate Proliferation Assay versus a TCA Filtration Assay is shown in Figure 5. Because the FlashPlate assay is nondestructive, multiple time points can be read from the same plate, unlike the conventional assay. With the TCA assay, separate plates are required for each point required, thus multiplying the volume of liquid radioactive waste generated.

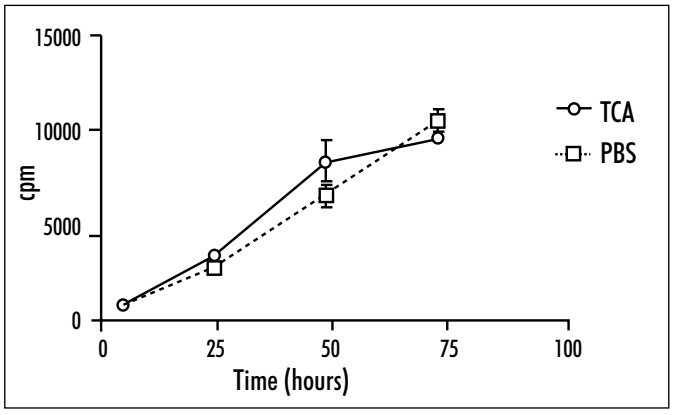
Figure 5

FlashPlate Proliferation Assay	TCA Filtration Assay
1. Culture adherent cells in Sterile FlashPlate	1. Culture adherent cells in 96-well plate
2. Label cells with [^{3}H]-Thymidine *(Generates 10 ml radioactive waste)*	2. Label cells with [^{3}H]-Thymidine *(Generates 10 ml radioactive waste)*
3. Incubate 2-24 hours	3. Incubate 2-24 hours
4. Read plate at designated time points	4. Remove medium
	5. Wash cells with PBS
	6. Dislodge cells and aspirate onto glass fiber filters
	7. Wash filter with TCA
	8. Measure retained radioactivity by liquid scintillation counting
	(Steps 4-8 generate approximately 520 ml aqueous radioactive waste and 300 ml organic radioactive waste)

In order to validate a cellular proliferation assay (true DNA synthesis) on FlashPlate, a [^{14}C]-Thymidine incorporation study with CHO-K1 cells was done. In this study, the standard FlashPlate assay described in the schematic was modified to include a TCA precipitation and wash step prior to counting. Figure 6 demonstrates that uptake of [^{14}C]-Thymidine correlates with DNA synthesis.

Figure 6

[^{14}C]-Thymidine Uptake in CHO-K1 Cells with and without TCA Precipitation and Wash Step



CHO-K1 cells were seeded at 1 x 10^4 in 0.2 ml/well Phenol Red-free DMEM/F12 containing 10% FBS, 2 mM L-glutamine and 75 µg/ml L-Proline. After an overnight incubation, media was replaced with 0.1 ml/well fresh media containing [^{14}C]-Thymidine (NEC156 at 0.125 µCi/ml). Thymidine incorporation was read at intervals up to 72 hours. DNA incorporation was measured by washing cells twice with 0.2 ml/well cold 10% (w/v) TCA and compared to whole cell uptake measured by washing cells twice with 0.2 ml/well PBS. Plates were read pre- and post-wash. Data represents means ±2 x SEM (n = 4).

Worldwide Headquarters: PerkinElmer Life Sciences, 549 Albany Street, Boston, MA 02118-2512 USA (800) 551-2121

Technical Support: in Europe: perkinelmer.europe@perkinelmer.com
in US and Rest of World: techsupport@perkinelmer.com

H78190 rev. 01/01