

Multiplexed Assay for IL-6 Secretion and Cell Viability using an Epithelial Ovarian Cancer Cell Line

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Introduction

According to the Centers of Disease Control and Prevention (CDC), each year about 20,000 women in the United States are diagnosed with ovarian cancer¹. 90% of these cancers are classified as "epithelial" and are believed to arise from the surface (epithelium) of the ovary and are termed Epithelial Ovarian Cancer (EOC). While prognosis is good with early diagnosis, in disease stages I/II, symptoms are non-specific and difficult to trace. The majority of ovarian cancers are diagnosed at late stage III/IV, where symptoms become more evident. Unfortunately at this point, prognosis is poor. A common manifestation of EOC at this late stage of its progression is a build-up of fluid in the abdominal cavity (ascites). It has been shown that high levels of IL-6 are present in patients with ascites².

The origin of the high expression of IL-6 is linked to the receptor Epidermal Growth Factor Receptor (EGFR). EGFR is expressed in up to 70% of EOCs, and its altered expression is associated with late stage disease and poor prognosis³. EGFR, a member of ErbB family of receptor tyrosine kinases, activates multiple signaling cascades including the activation of NFkB, which is known to activate the transcription of inflammation-related proteins such as IL-6⁴. In a recent publication, it was shown that EGFR ligand binding induces the expression of IL-6 via the NFkB pathway in advanced-stage epithelial ovarian cancer⁵.

In this application note we demonstrate an *in vitro* microplate assay that can monitor IL-6 secretion from plated SKOV-3 ovarian carcinoma cells induced through EGFR ligand binding and NFkB activation. The assay workflow involved a two plate protocol where cells are plated and EGFR ligand-activated. IL-6 measurements are made in a separate microplate by transferring a portion of the cell supernatant. We also showed that IL-6 secretion can be inhibited at the level of either EGFR or NFkB using known inhibitors. Inhibitors that are potentially toxic to the plated cells can be assessed through digital widefield fluorescence microscopy using fluorescent probes. This provides a quantitative determination of whether IL-6 suppression is caused by the inhibition of receptor/transcription factor activation or through cell toxicity. All microplate measurements were made on the Cytation³ Cell Imaging Multi-Mode Reader.

BioTek Instrumentation



Figure 1 – Cytation³ Cell Imaging Multi-Mode Reader. Cytation³ combines automated digital widefield microscopy and conventional microplate detection. This patent pending design provides rich phenotypic cellular information with well-based quantitative data. Equipped with BioTek's patented Hybrid Technology™ for microplate detection, Cytation³ includes both high sensitivity filter-based detection and a flexible monochromator-based system for unmatched versatility and performance. The upgradable automated digital fluorescence microscopy module provides researchers rich cellular visualization analysis without the complexity and expense of standard microplate-based imagers.

Fluorescence microscopy is a powerful technique for visualizing cellular responses to understand cell proliferation, protein expression, cytotoxicity and other cellular processes. The ability to perform both conventional quantitative fluorescence measurements and cell imaging provides unique capabilities such as screening microplate wells for a fluorescence intensity threshold that triggers the reader to follow-up the screen with imaging of those wells that passed the intensity threshold. This serves to reduce analysis time and data storage requirements by imaging only those wells of interest which pass the intensity threshold.

Cytation³'s design places special emphasis on live-cell assays: features include temperature control to 45°C, CO₂/O₂ gas control, orbital shaking and full support for kinetic studies with BioTek's Gen5™ Data Analysis Software, specifically designed to make plate reading and image capture easy. Other technology advances are found throughout Cytation³'s design including high-intensity LED light sources, matched filter cubes, hard coated optical filters, Olympus objectives, and superior autofocus for totally software controlled digital microscopy.

The filter-based system was used to detect the 665 nm and 620 nm fluorescent emissions from the HTRF™ IL-6 assay chemistry with the following settings: Delay after plate movement: 0 msec; Delay after excitation: 150 µsec; Integration time: 500 µsec; Read height: 10.5 mm. Imaging was then performed with the Nuclear-ID™ Blue/Red Cell Viability assay using the microscopy capabilities. Gen5 software was used for initial data analysis.

HTRF™ Human IL-6 Assay

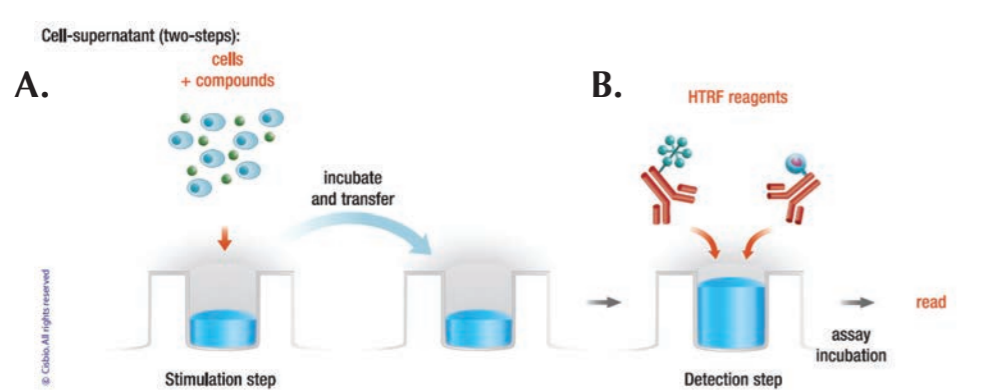


Figure 2 – IL-6 is measured using a sandwich immunoassay involving two monoclonal antibodies: anti-IL-6 (MAb1) labeled with Eu-Cryptate and anti-IL-6 (MAb2) labeled with XL665. These antibodies may be pre-mixed and added in a single dispensing step, to further streamline the protocol. The assay is run in two steps. (A) In the stimulation step cells are incubated with activators and inhibitors. (B) In the detection step supernatant containing the secreted IL-6 is then transferred to a second plate, followed by antibody addition.

Nuclear-ID™ Blue/Red Cell Viability Assay

The Nuclear-ID™ Blue/Red cell viability reagent (Catalog No. ENZ-53005) from Enzo Life Sciences (Farmingdale, NY) is a mixture of a blue fluorescent cell-permeable nucleic acid dye and a red fluorescent cell-impermeable nucleic acid dye that is suited for staining dead nuclei. Nuclei from viable cells will stain blue. As cell viability decreases, their membranes lose integrity and the red fluorescent dye is then able to stain the nucleus. The staining pattern arising from the simultaneous combination of these two dyes permits determination of live and dead cell populations by fluorescence microscopy.

Materials and Methods

Materials

Cells: Ovarian carcinoma SKOV-3 cells (Catalog No. AKR-253) were obtained from Cell Biolabs, Inc. (San Diego, CA). The cells were propagated in RPMI 1640 Medium (Catalog No. 11875) plus Fetal Bovine Serum, 10% (Catalog No. 10437) and Pen-Strep-Glutamine, 1X (Catalog No. 10378) from Life Technologies (Carlsbad, CA). The cells were plated at a density of 1.0x10⁵ cells/mL in serum-free medium for 24 hours prior to performing the assay.

EGFR Signaling Cascade Inducer: Human Epidermal Growth Factor (EGF) (Catalog No. CYT-217) from ProSpec (Rehovot, Israel) was used to stimulate the EGFR signaling cascade, leading to eventual secretion of the pro-inflammatory cytokine, IL-6.

Inhibitors: AG 1478 (Catalog No. 1276), Cardamonin (Catalog No. 2509), U0126 (Catalog No. 1144), and LY 294002 (Catalog No. 1130) were purchased from R&D Systems (Minneapolis, MN). Cetuximab was provided by Cisbio Bioassays (Codolet, France). Anti-EGFR antibodies 225 (Catalog No. LS-C88001) and 111.6 (Catalog No. LS-C88141) were purchased from LifeSpan BioSciences (Seattle, WA).

Cell Plates: 96 Well Flat Clear Bottom, Black PS, TC-Treated Microplates (Catalog No. 3904), and 384 Well Low Volume White Round Bottom PS NBS Coated Microplates (Catalog No. 3673) were purchased from Corning Life Sciences (Corning, NY).

2-Plate Assay Method

SKOV-3 cells, in a volume of 100 µL, were added to the 96-well cell culture plates and incubated for 24 hours. 25 µL of 6X EGF and 50 µL of 3X inhibitor were then added to the well and incubated for the appropriate time. Following incubation, 16 µL of supernatant was transferred to a separate low-volume 384-well plate. 4 µL of HTRF antibody mix was then added and incubated for 4 hours in serum-free medium before reading. The remaining medium was removed from the cell plate, and the plate was washed once with 1X PBS. 50 µL of PBS containing the Nuclear-ID reagent was then added to the wells and incubated at 37°C/5% CO₂ for 30 minutes. Upon completion the plate was washed twice with PBS, and a final volume of 50 µL PBS was added to the wells before imaging.

Optimization of EGFR Pathway Stimulation

An initial experiment was performed to assess the level of IL-6 secretion upon stimulation of the EGFR signaling pathway. An 11-point titration of human EGF was created using serial 1:4 dilutions starting at a 1X concentration of 2000 ng/mL. The growth factor was added to the SKOV-3 cells and incubated for 24, 48, 72, or 96 hours.

Ratio (Delta F(%) / Maximum IL-6 Stimulation / Delta F(%) Unstimulated)	24 Hours	48 Hours	72 Hours	96 Hours
	2.8	4.3	2.8	2.8

Table 1 – Multiple Incubation Time EGF Stimulation Assay Window. Delta F(%) calculated from results generated for each EGF concentration by the following formula:

$$\left(\frac{\text{HTRF Value}_{\text{Test Well}} - \text{HTRF Value}_{\text{Neg. Ctrl.}}}{\text{HTRF Value}_{\text{Pos. Ctrl.}}} \right) * 100$$

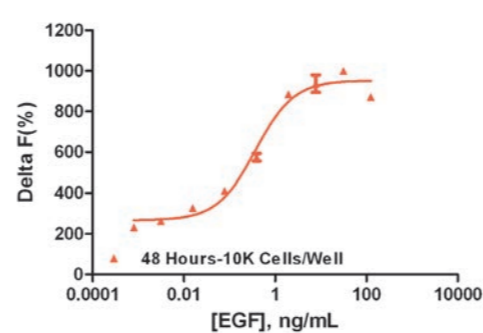


Figure 3 – EGF Stimulation of IL-6 Secretion. IL-6 secretion stimulation curve for 48 hour EGF incubation with SKOV-3 cells.

The results shown in Table 1 and Figure 3 demonstrate that a 48 hour EGF incubation with the serum starved SKOV-3 cells provides the largest change in signal between wells containing stimulated and unstimulated cells, or assay window. This incubation time was then used for inhibitor testing.

EGFR Pathway Inhibitor Confirmation

Small molecule and anti-EGFR antibody inhibitors were then tested for their ability to attenuate IL-6 secretion as well as cytotoxic properties. Compounds included the EGFR inhibitor AG 1478, the anti-inflammatory Cardamonin, known to inhibit NFkB activation, the MAP kinase inhibitor U0126, and the PI3 kinase inhibitor LY 294002. Three anti-EGFR antibodies with known human reactivity, 225, 111.6, and Cetuximab, were also included. Inhibitors and EGF were added to the SKOV-3 cells and co-incubated for 48 hours prior to performing the IL-6 and cytotoxicity assays.

A single 2-plate protocol was created with the Gen5 software to allow efficient processing of the HTRF assay plate and cell plate, as well as eliminating the need to image the entire cell plate, therefore obviating unnecessary data generation and storage. The plate layout created in Gen5 for the HTRF assay plate identifies the location of control and test wells. Data analysis steps also convert the raw fluorescence data into Delta F(%) and identify "hit" wells where inhibition of IL-6 secretion is ≥ 50%.

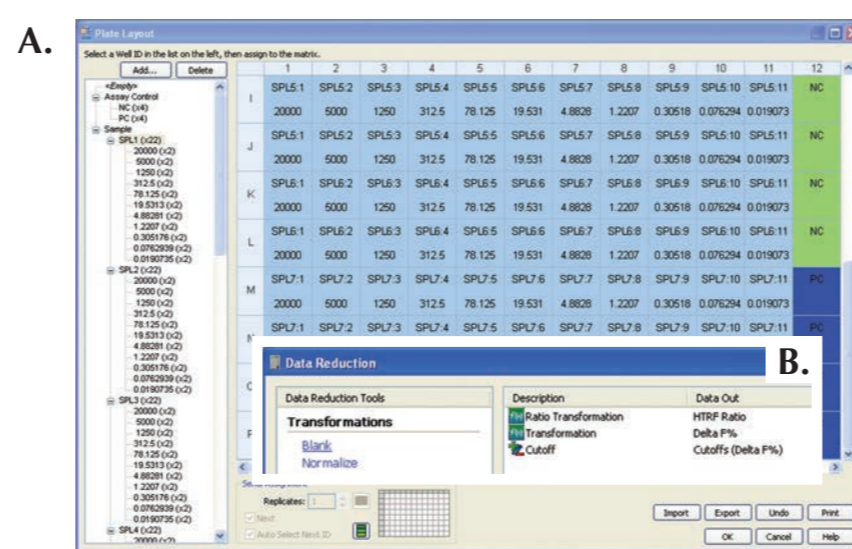


Figure 4 – (A) Gen5 plate layout for HTRF IL-6 384-well assay. (B) Data reduction steps for conversion of raw fluorescence values and determination of positive inhibition wells.

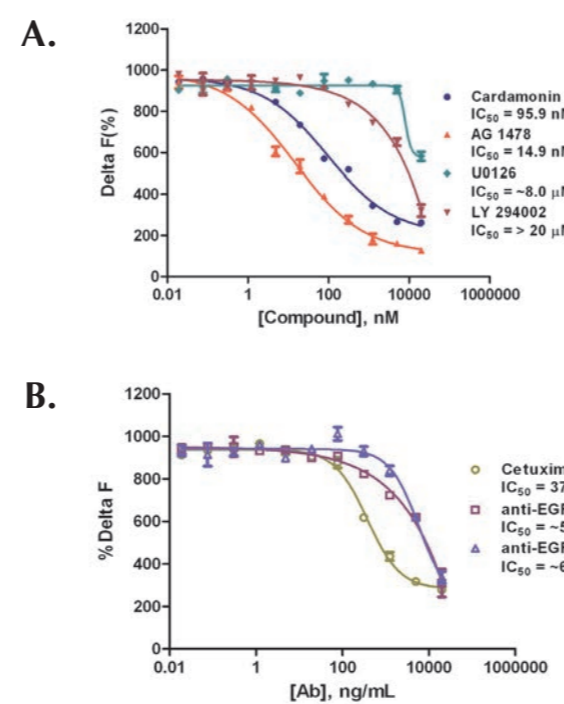


Figure 5 – Inhibition of IL-6 secretion for (A) small molecule inhibitors and (B) anti-EGFR antibodies.

Inhibition curves were then plotted from the computed Delta F(%) values. While a decrease in IL-6 secretion was seen with all inhibitors tested, a more significant decrease was seen from AG 1478, Cardamonin, and Cetuximab. This is consistent with previous findings which demonstrated that inhibition at the level of receptor and NFkB pathway activation led to a subsequent decrease in EGF-stimulated IL-6 secretion⁵. The lack of appreciable inhibition by U0126 and LY 294002 indicates that ligand-dependent EGFR/MEK/ERK and EGFR/PI3K/AKT activation plays a diminished role leading to IL-6 secretion in SKOV-3 cells. Finally the increased potency of Cetuximab compared to the other anti-EGFR antibodies tested also agrees with results published from previous comparisons⁶.

Using the data reduction performed by the Gen5 software, wells were identified which demonstrated ≥ 50% inhibition of IL-6 secretion.

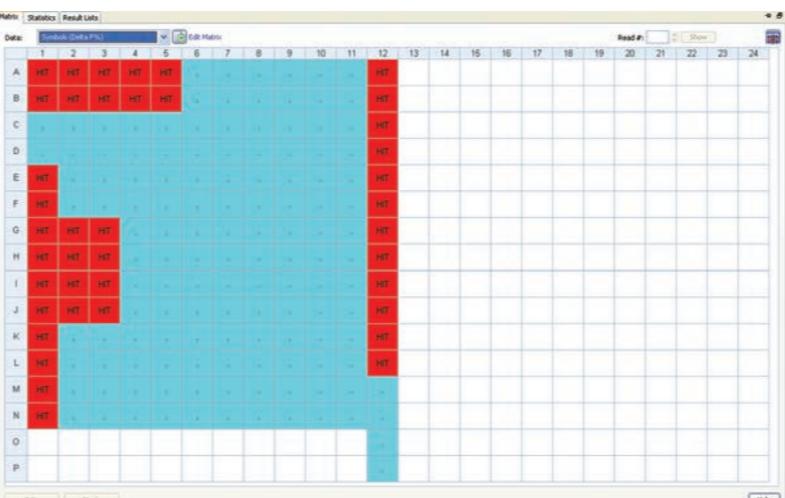


Figure 6 – Results from Cutoff analysis of inhibition data. Wells showing Delta F(%) values ≤ 50% of maximum stimulated wells labeled in red. All other wells labeled in green.

Using the results from the Cutoff analysis, in the same Experiment File as that used to generate the HTRF results, wells are chosen within the original cell plate to assess potential cytotoxic effects from the inhibitor concentrations of interest.

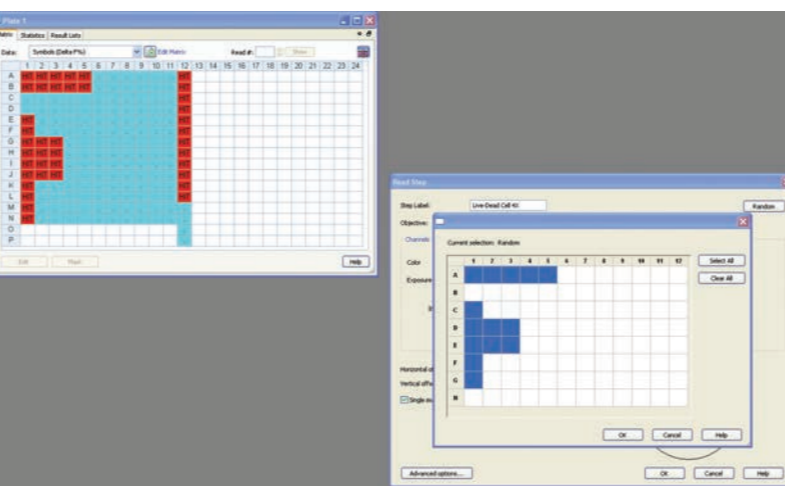


Figure 7 – Individual plate layout selected for imaging of cell plate (Plate 2).

Inhibitor Cytotoxicity Assessment

Cytotoxic effects from inhibitors were assessed by capturing 4X and 20X images of live and dead cells from the predetermined wells of the original cell plate identified in the Cutoff Analysis. Additional wells were also imaged in order to determine potential cytotoxicity at the identified IC₅₀ value, as well for the no compound, negative control wells.

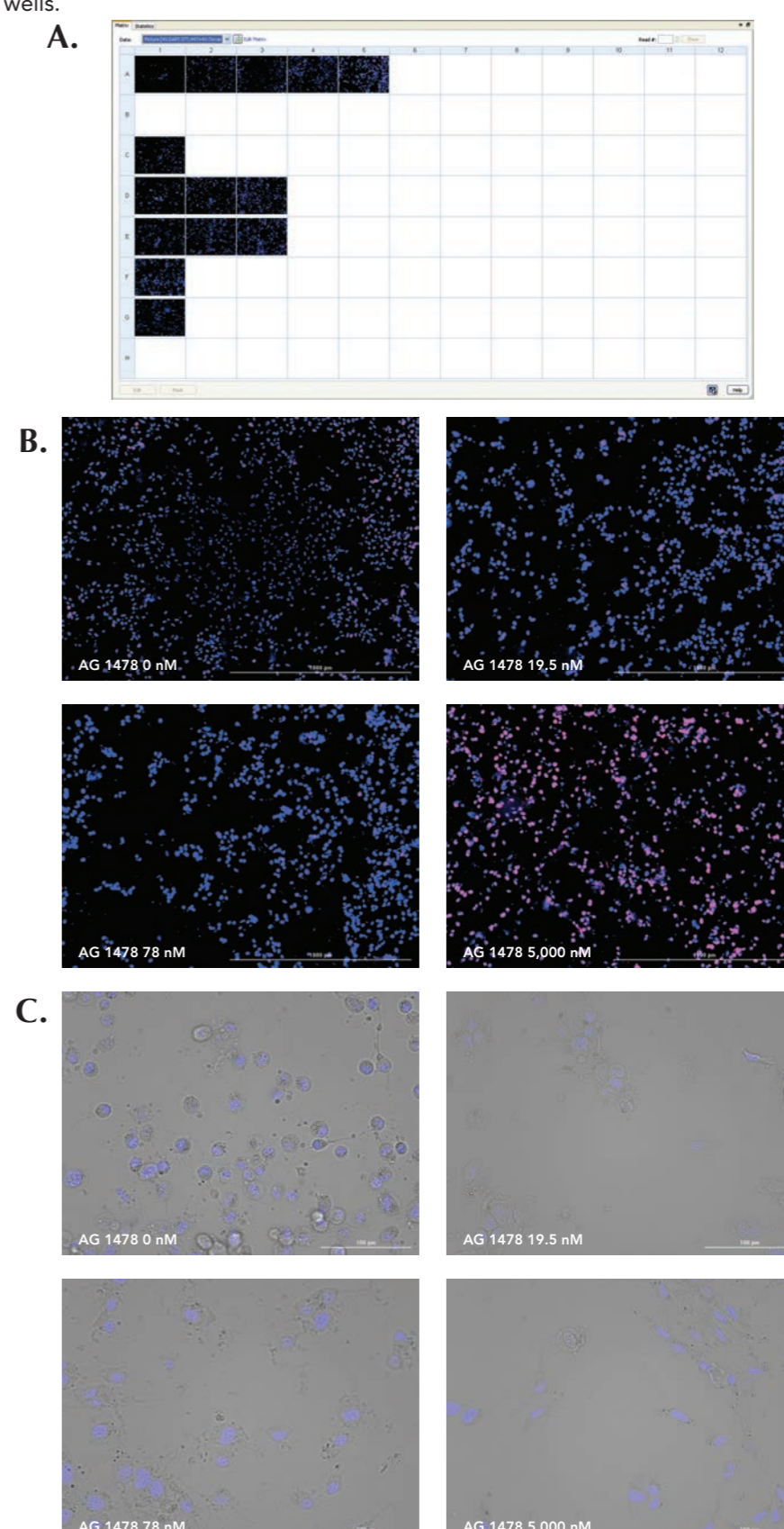


Figure 8 – (A) Thumbnail view of 4X blue (live cell) and red (dead cell) images for selected wells. (B) 4X live/dead cell images for AG 1478. Images shown for two pre-selected wells, in addition to IC₅₀ concentration and no inhibitor control. (C) 20X blue/brightfield images showing cell morphology and stained nuclei.

The Cellular Analysis tool was then used to determine the number of live and dead cells captured in each 4X image. Object size and threshold fluorescence value criteria are used to guarantee that the appropriate cells are selected for each count.

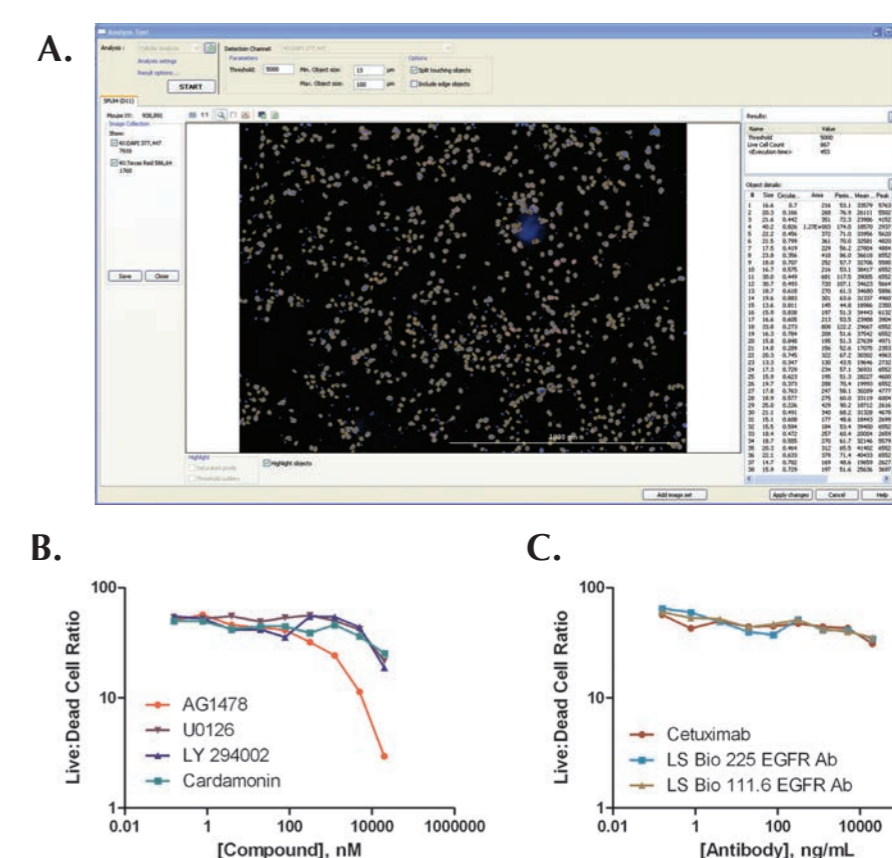


Figure 9 – (A) Gen5 Cellular Analysis imaging tool. Live/dead cell ratios calculated for all concentrations tested with (B) small molecule and (C) anti-EGFR antibody inhibitors.

The results from the live/dead cell imaging demonstrate that cytotoxic effects are minimal at the IC₅₀ concentrations determined when using a 48 hour incubation period. Only at the highest concentrations of small molecule inhibitors tested is there a noticeable decrease in cell viability.

A final experiment was performed to confirm receptor binding of the anti-EGFR antibodies. Each primary antibody was added to the SKOV-3 cells, followed by the addition of XL665 labeled goat anti-human Fc antibody. A red fluorescent image is expected where 1° antibody:receptor and 2° anti-human Fc antibody binding takes place.

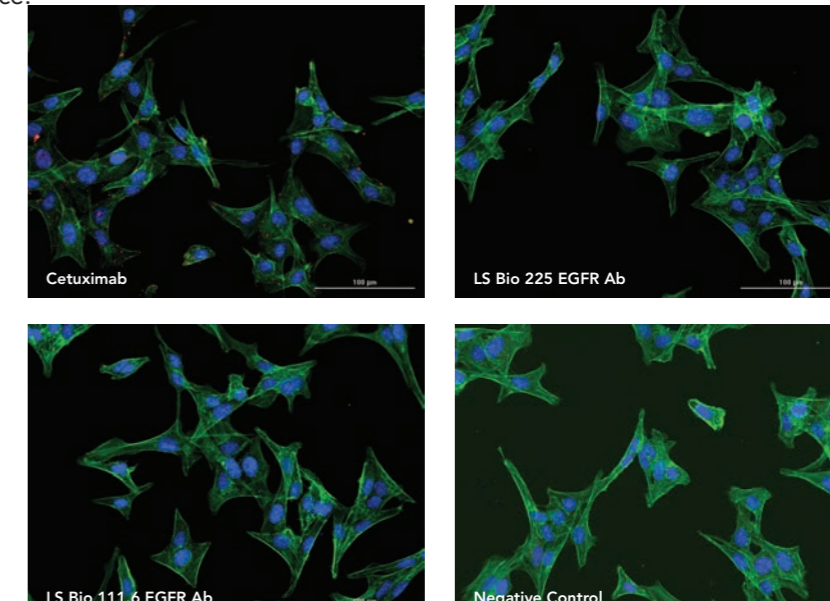


Figure 10 – anti-EGFR receptor antibody binding 20X images. Blue indicates DAPI-stained nucleus. Green indicates Phalloidin actin staining.

EGF receptor and anti-human Fc antibody binding was seen with Cetuximab only. This was due to the fact that the antibody contains humanized Fab and Fc regions. The 225 and 111.6 antibodies are mouse origin, and therefore demonstrate only human reactivity at the Fab region. Binding was also not seen with the negative control (no 1° antibody) confirming that non-specific receptor binding of the 2° antibody does not take place.

Conclusions

- The HTRF IL-6 assay provides an easy-to-use, sensitive method for the assessment of cytokine secretion from cancer cell models. When run using the 2-plate protocol, the assay can be multiplexed to also provide cytotoxicity assessments.
- The Nuclear-ID multiplexed cell viability assay allows for rapid evaluation of live and dead cell populations with a single reagent addition.
- The combined capabilities of the Cytation³ affords the ability to easily perform each assay with one instrument.
- The advanced optics and Gen5 software features ensure accurate detection of both reader-based and microscopy assays, and provide efficient performance of the entire experimental workflow.



¹CDC webpage for Gynecologic Cancers, Ovarian Cancer: <http://www.cdc.gov/cancer/ovarian/index.htm>. ²Kryczek I, Grybos M, Karabon L, Klimczak A, Lange A. (2000). IL-6 production in ovarian carcinoma is associated with histotype and biological characteristics of the tumour and influences local immunity. *Br J Cancer* 82: 621-628. ³Hudson LG, Zeineldin R, Silberberg M, Stack MS. (2009). Activated epidermal growth factor receptor in ovarian cancer. *Cancer Treat Res* 149: 203-226. ⁴Karin M. (2006). Nuclear factor-kappaB in cancer development and progression. *Nature* 441: 431-436. ⁵Alberici G, Pinciroli P, Valeri B, Ferri R, Ditto A, Umezawa K, Sensi M, Canavese S and Tomassetti A. (2012). Ligand-dependent EGFR activation induces the co-expression of IL-6 and PAI-1 via the NFkB pathway in advanced-stage epithelial ovarian cancer. *Oncogene* 31: 4139-4149. ⁶Galizia G, Lieto E, De Vita F, Orditura M, Castellano P, Troiani T, Imperatore V, and Ciardiello F. (2007). Cetuximab, a chimeric human mouse anti-epidermal growth factor receptor monoclonal antibody, in the treatment of human colorectal cancer. *Nature* 26: 3654-3660.