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

Using the Agilent BioTek Cytation 3 cell imaging multimode reader to monitor IL-6 secretion using HTRF detection and cell viability with digital widefield fluorescence microscopy

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Abstract

Epidermal growth factor receptor (EGFR) is expressed in up to 70% of epithelial ovarian cancers (EOCs). High levels of IL-6 have also been found in the ascites of EOC patients. Ligand-stimulated EGFR-activated NFkB-dependent transcription and induced secretion of the pro-inflammatory IL-6 cytokine has been shown. This application note demonstrates a multiplexed assay for inhibitors at the level of EGFR and NFkB to monitor IL-6 secretion and cell viability. The workflow of the multiplexed assay uses an assay plate and an HTRF detection plate. Following treatment of the cells, supernatant is transferred to the HTRF detection plate where IL-6 concentrations are determined; then, cell viability is assessed in the assay plate using fluorescent probes and imaging. All assays were conducted on the Agilent BioTek Cytation 3 cell imaging multimode reader.

Introduction

According to the Centers of Disease Control and Prevention (CDC), each year approximately 20,000 women in the United States get ovarian cancer.[1] Of these cancers, 90% 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 for early diagnosis in disease stages I/II, symptoms are nonspecific and difficult to trace. The majority of ovarian cancers are diagnosed at late stages 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 this ascites.[2]

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 prognosis.[3] EGFR, a member of ErbB family of receptor tyrosine kinases, and activates multiple signaling cascades including the activation of NFkB, which is known to activate the transcription of inflammation-related proteins such as IL-6.[4] In a recent publication, it was shown that EGFR ligand binding induces the expression of IL-6 via the NFkB pathway in advanced-stage EOC.[5]

This application note demonstrates 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 2-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. 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 an Agilent BioTek Cytation 3 cell imaging multimode reader.

Materials and methods

Materials

Cells: Ovarian carcinoma SKOV-3 cells (part number AKR-253) were obtained from Cell Biolabs, Inc. (San Diego, CA). The cells were propagated in RPMI 1640 Medium (part number 11875) plus Fetal Bovine Serum, 10% (part number 10437) and Penicillin-Streptomycin-Glutamine, 1x (part number 10378) from Life Technologies (Carlsbad, CA). The cells were plated at a density of 1.0×10^5 cells/mL in serum-free medium for 24 hours prior to performing the assay.

EGFR signaling cascade inducer: Epidermal Growth Factor (EGF) (part number 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 (part number 1276), cardamonin (part number 2509), U0126 (part number 1144), and LY 294002 (part number 1130) were purchased from R&D Systems (Minneapolis, MN). Cetuximab was provided by Cisbio Bioassays (Codolet, France). Anti-EGFR antibodies 225 (part number LS-C88001) and 111.6 (part number LS-C88141) were purchased from LifeSpan BioSciences (Seattle, WA).

Cell plates: The 96-well Flat Clear Bottom, Black PS, TC-Treated Microplates (part number 3904), and 384-Well Low Volume White Round Bottom PS NBS Coated Microplates (part number 3673) were purchased from Corning Life Sciences (Corning, NY).

Instrumentation

Agilent Biotek Cytation 3 cell imaging multimode reader: Cytation 3 combines automated digital widefield microscopy and conventional microplate detection. This design provides rich phenotypic cellular information with well-based quantitative data.

Equipped with Agilent BioTek Hybrid Technology for microplate detection, Cytation 3 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 that pass the intensity threshold.

The Cytation 3 design places special emphasis on live-cell assays. Features include temperature control to 45 °C, CO_2/O_2 gas control, orbital shaking, and full support for kinetic studies with Agilent BioTek Gen5 data analysis software, specifically designed to make plate reading and image capture easy. Other technology advances are found throughout the Cytation 3 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 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. Agilent BioTek Gen5 software was used for initial data analysis.

Assay chemistries

HTRF human IL-6 assay: In the assay, IL-6 was 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 premixed and added in a single dispensing step, to further streamline the protocol. The assay was run in two steps (Figure 1). In the first step (stimulation), cells are incubated with activators and inhibitors. In the second step (detection), supernatant containing the secreted IL-6 is transferred to a second plate, followed by antibody addition.

Nuclear-ID Blue/Red cell viability assay: The Nuclear-ID Blue/Red cell viability reagent (part number 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.
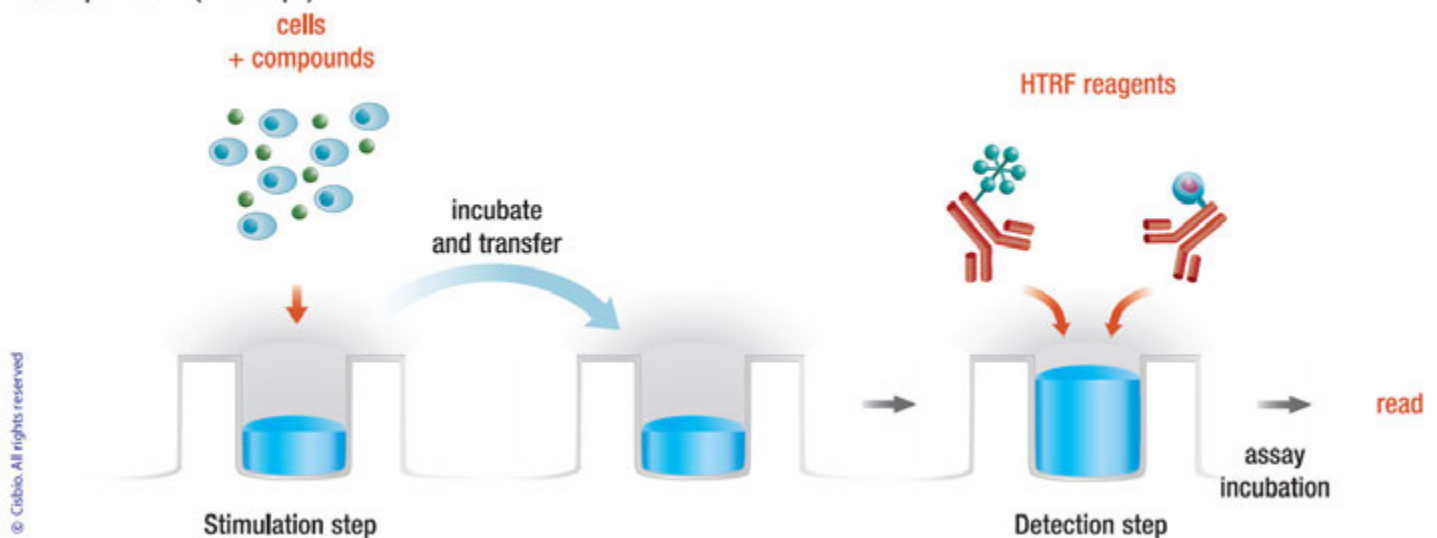

Figure 1. Two-plate HTRF human IL-6 assay.

Methods

Two-plate assay protocol: SKOV-3 cells, in a volume of 100 μL, were added to the 96-well cell plates and incubated for 24 hours in serum-free medium. Then, 50 μL of 3x EGF or 25 μL of 6x EGF and inhibitor was 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. Four microliters of HTRF antibody mix was then added and incubated for 4 hours before reading.

The remaining medium was removed from the cell plate, and the plate was washed once with 1x PBS. Fifty microliters of PBS containing the Nuclear-ID reagent was then added to the wells and incubated at 37 °C/5% CO_2 for 30 minutes. Upon completion, the plate was washed twice with PBS, and a final volume of 50 μL of PBS was added to the wells before imaging.

Delta F(%) calculation:

((HTRF Value(Test Well) − HTRF Value(Neg Ctl))/HTRF Value(Neg Ctl)) × 100

EGFR pathway stimulation optimization: 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 2,000 ng/mL. The growth factor was added to the SKOV-3 cells and incubated for 24, 48, 72, or 96 hours. The remaining portion of the HTRF IL-6 assay was then performed as previously explained.

EGFR pathway inhibitor confirmation: Small molecule and anti-EGFR antibody inhibitors were 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.

Gen5 2-plate microplate reader/imager HTRF IL-6 and cytotoxicity protocol: A single 2-plate protocol was created with the Agilent BioTek Gen5 software to allow efficient processing of the HTRF assay plate and cell plate, as well as to eliminate 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 (Figure 2A). 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 2B).

Using the data reduction and Cutoff Analysis performed following the HTRF assay plate read, wells are identified that demonstrate ≥50% inhibition of IL-6 secretion (Figure 3).

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 4).

4x and 20x images of live and dead cell nuclei, stained with the Nuclear-ID Cell Viability Reagent, are then captured (Figure 5).

The Cellular Analysis tool is 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 6).
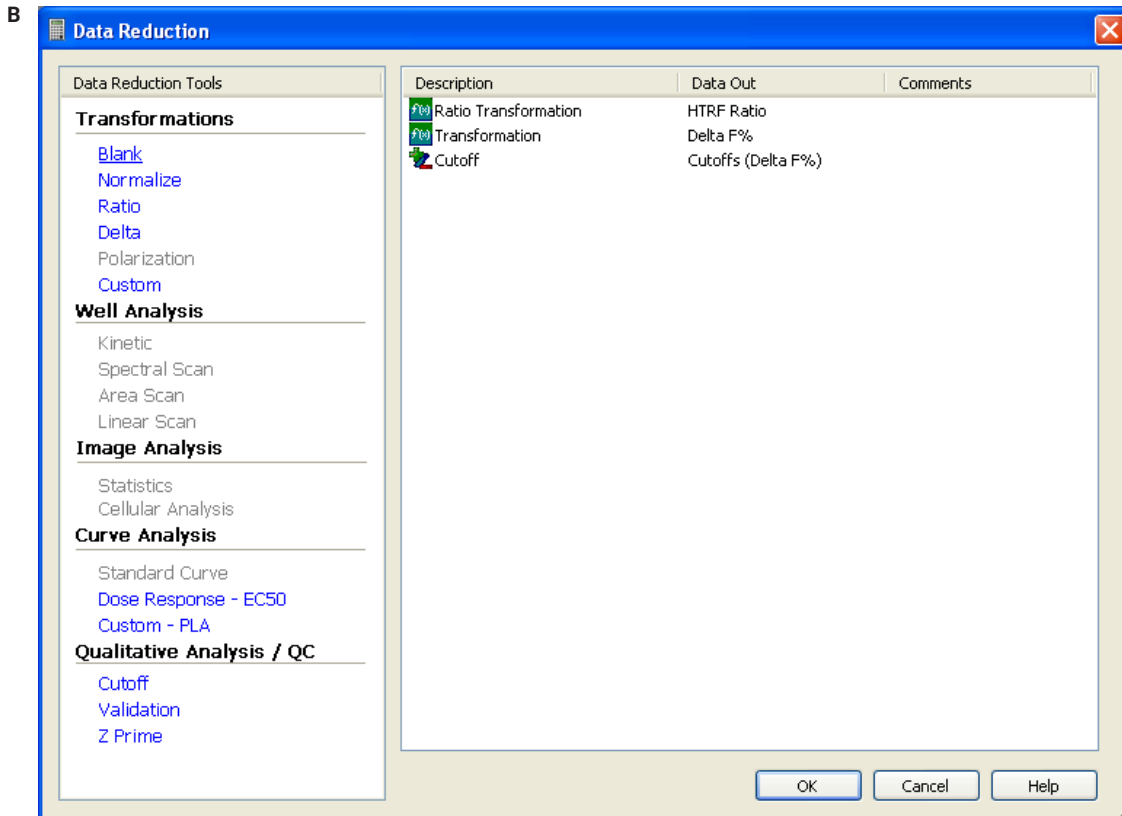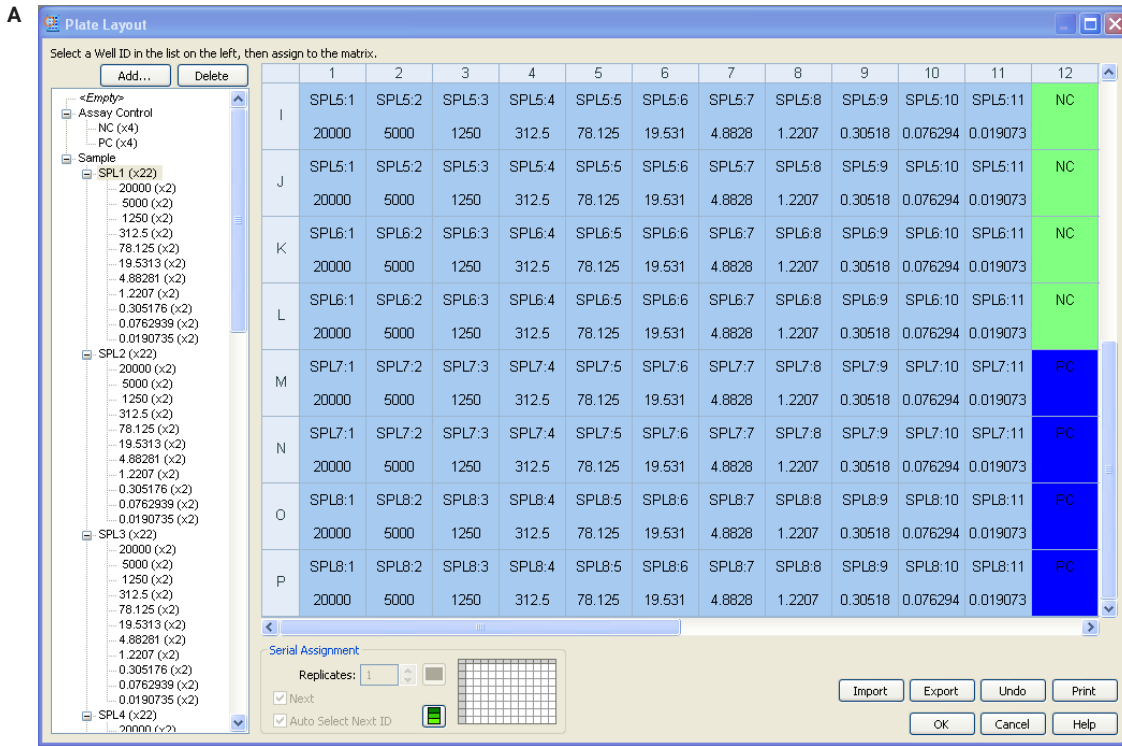
Figure 2. (A) Agilent BioTek Gen5 plate layout for HTRF IL-6 384-well assay. (B) Data reduction steps for conversion of raw fluorescence values and determination of positive inhibition wells.

Figure 3. 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.
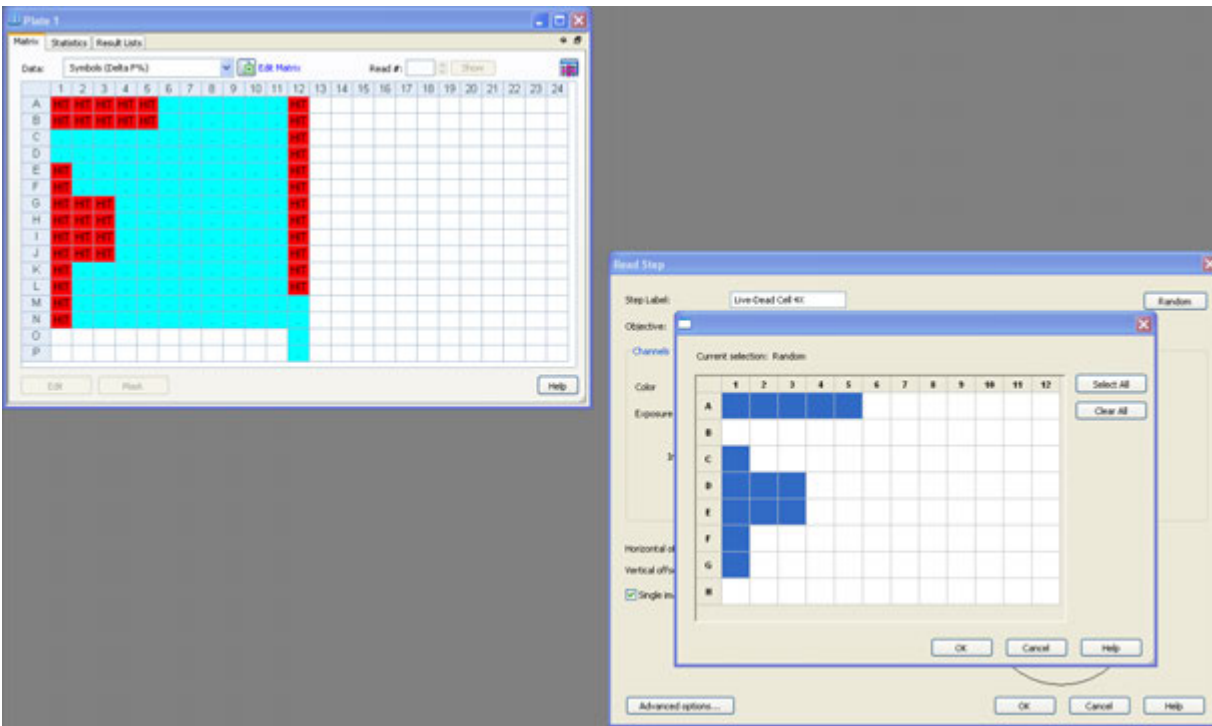

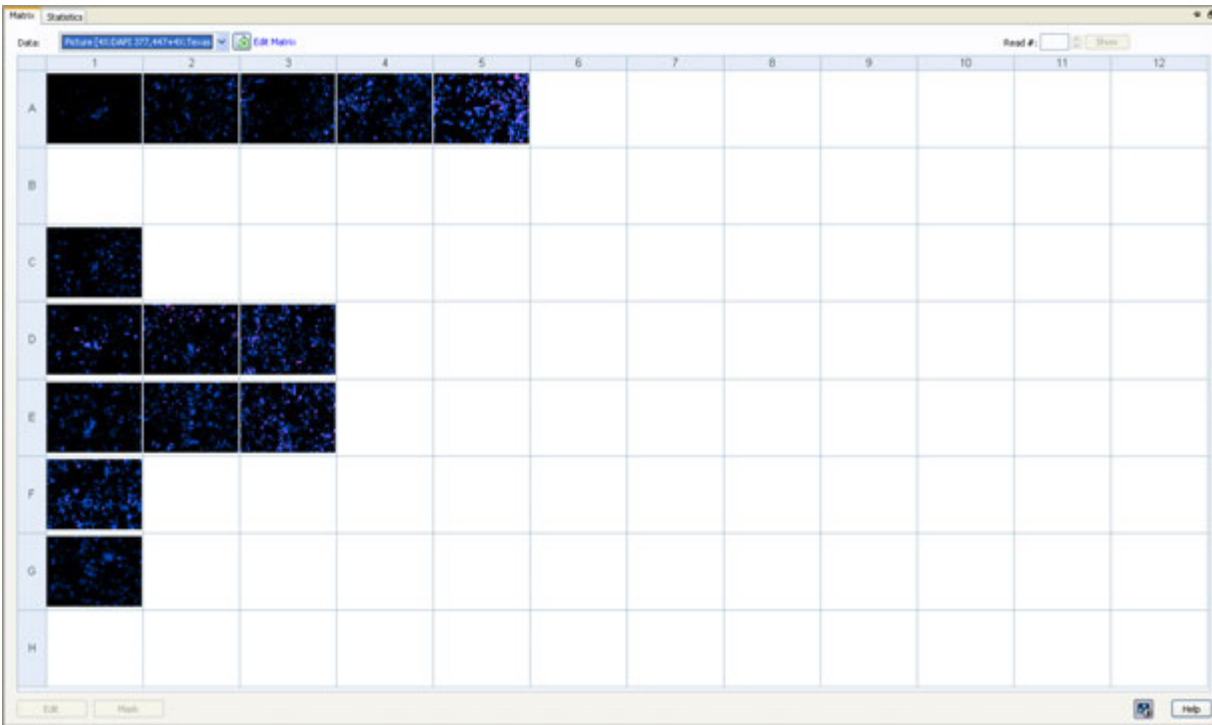**Figure 4.** Individual plate layout selected for imaging of a cell plate (Plate 2).

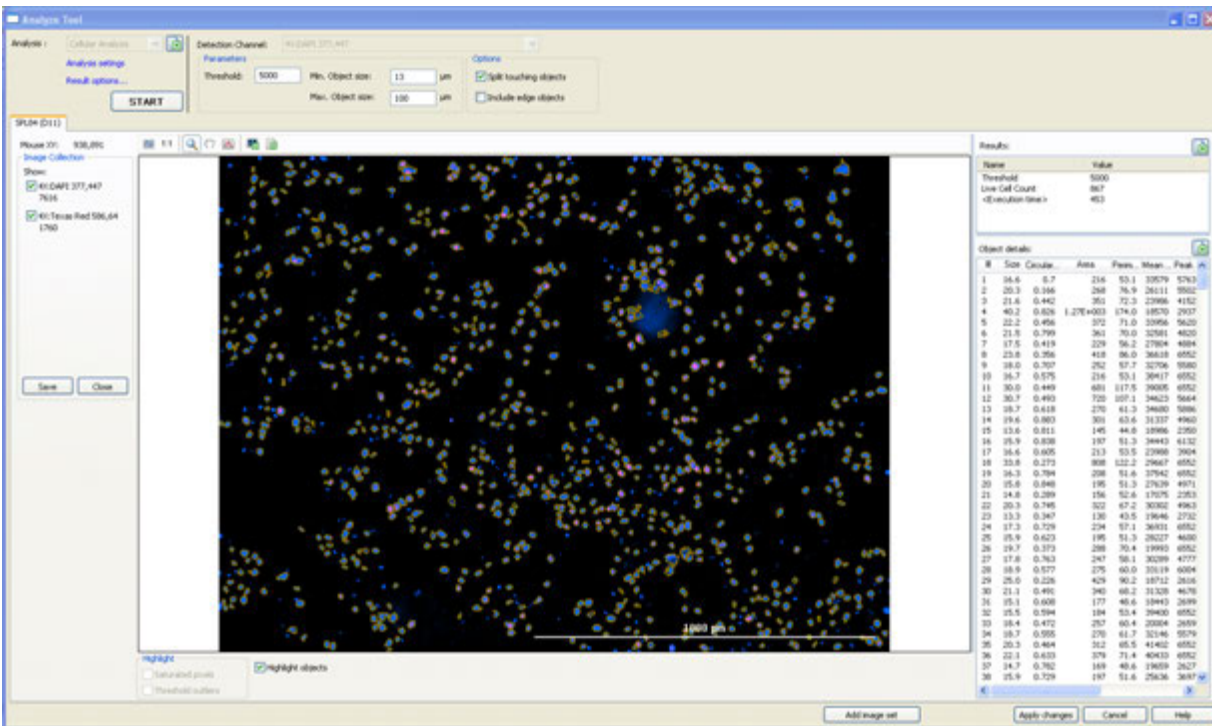**Figure 5.** Thumbnail view of 4x blue and red images for selected wells.

Figure 6. Agilent BioTek Gen5 cellular analysis imaging tool.

Results and discussion

EGFR pathway stimulation

The results generated in the multiday pathway stimulation analysis (Table 1) 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.

Table 1. Multiple incubation time EGF stimulation assay window.

Ratio (Delta F(%)$_{\text{Maximum IL-6 Stimulation}}$/Delta F(%)$_{\text{Unstimulated}}$)			
24 Hours	48 Hours	72 Hours	96 Hours
2.8	4.3	2.8	2.8

The 24-hour incubation time may not be long enough to see peak stimulation of IL-6 secretion, while the cytokine may begin to be degraded by the SKOV-3 cells with extended incubations such as 72 and 96 hours. Therefore, the 48-hour incubation time was to be used for inhibitor testing.

From the 48-hour stimulation curve (Figure 7) it can be seen that maximum stimulation occurs between 1 and 100 ng/mL EGF. A concentration of 35 ng/mL was used for subsequent inhibition analyses.
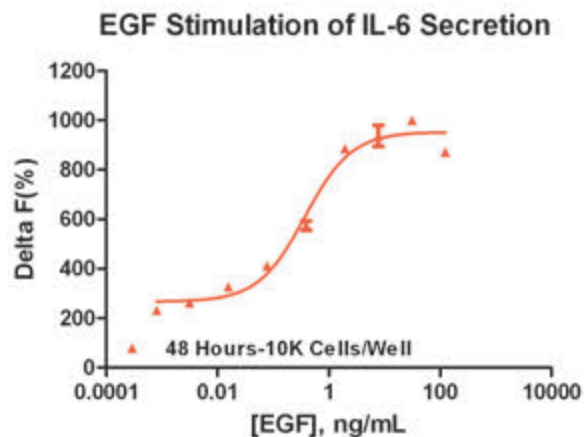

Figure 7. EGF stimulation of IL-6 secretion. IL-6 secretion stimulation curve for 48-hour EGF incubation with SKOV-3 cells.

Confirmation of EGFR pathway inhibitors

Inhibition curves for all compounds and anti-EGFR antibodies tested were plotted from the Delta F(%) values calculated by the Gen5 software using the original 620 and 665 nm emission signals (Figure 8).
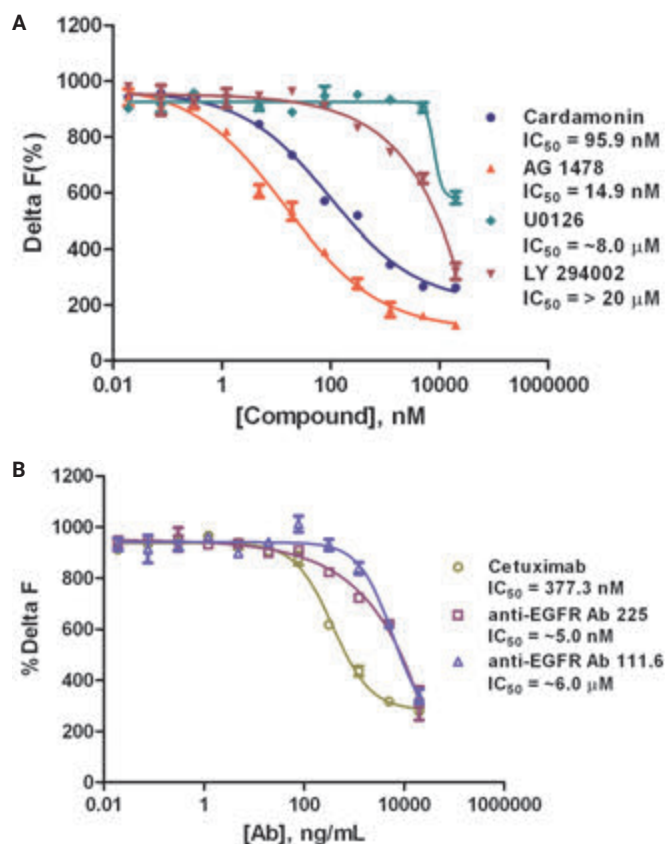

Figure 8. Inhibition of IL-6 secretion for (A) small molecule inhibitors and (B) anti-EGFR antibodies.

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 secretion.[5] 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 comparisons.[6]

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 (Figure 9). Additional wells were also imaged to determine potential cytotoxicity at the identified IC$_{50}$ value, as well for the no compound, negative control wells.

A

AG 1478 0 nM

AG 1478 19.5 nM

AG 1478 78 nM

AG 1478 5,000 nM

B

AG 1478 0 nM

AG 1478 19.5 nM
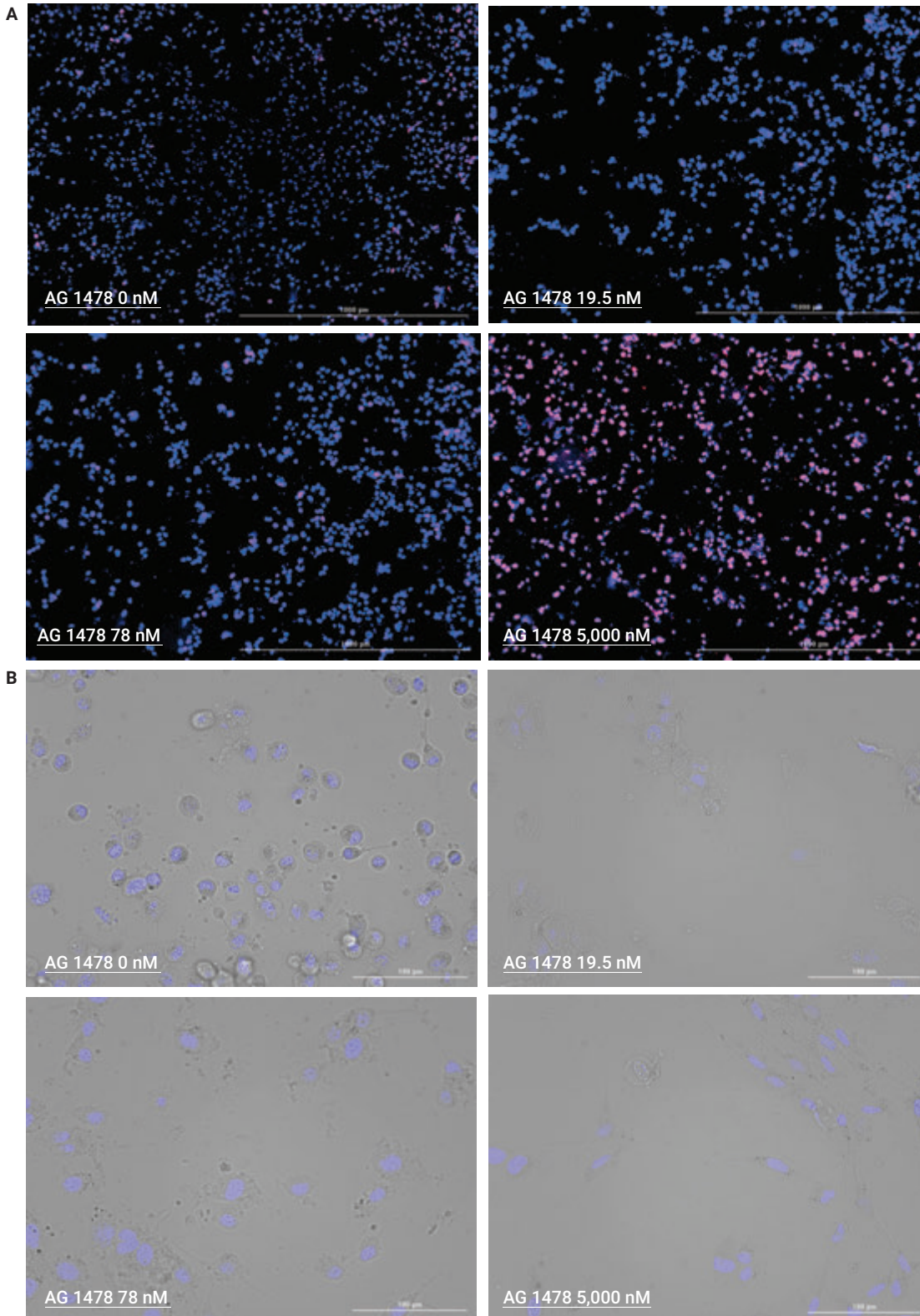
AG 1478 78 nM

AG 1478 5,000 nM

Figure 9. (A) 4x live/dead cell images for AG 1478. Images shown for two preselected wells in addition to IC_{50} concentration and no inhibitor control. (B) 20x blue/ brightfield images showing cell morphology and stained nuclei.

From the cellular analysis performed on the 4x images (Figure 6), cytotoxicity curves were generated.

The results from the live/dead cell imaging demonstrate that cytotoxic effects are minimal at the IC_{50} concentrations determined when using a 48-hour incubation period (Figure 10). Only at the highest concentrations of small molecule inhibitors tested was 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 11).

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 nonspecific receptor binding of the 2° antibody does not take place.
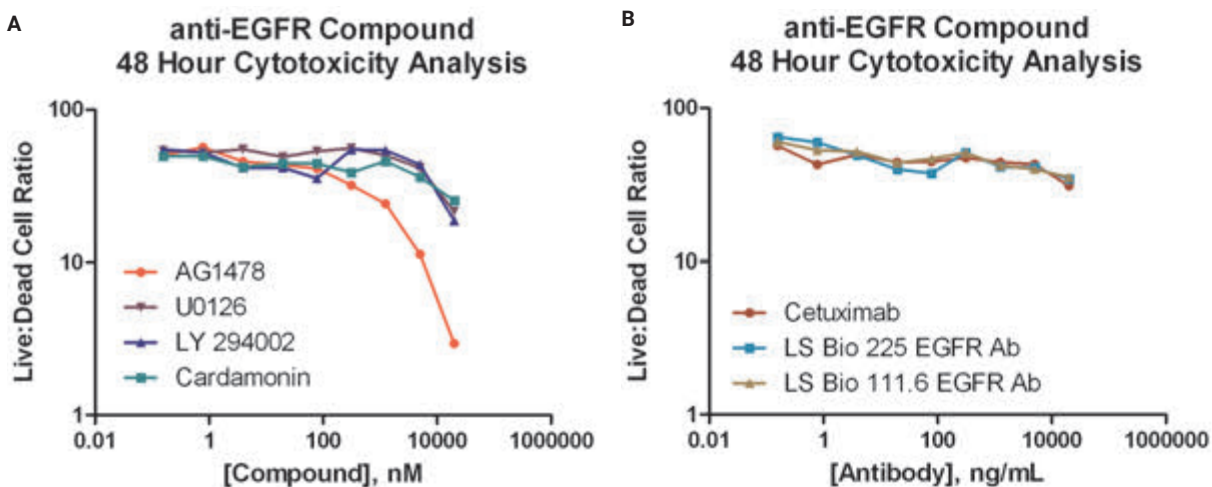

Figure 10. Live/dead cell ratios calculated for all concentrations tested with (A) small molecule and (B) anti-EGFR antibody inhibitors.
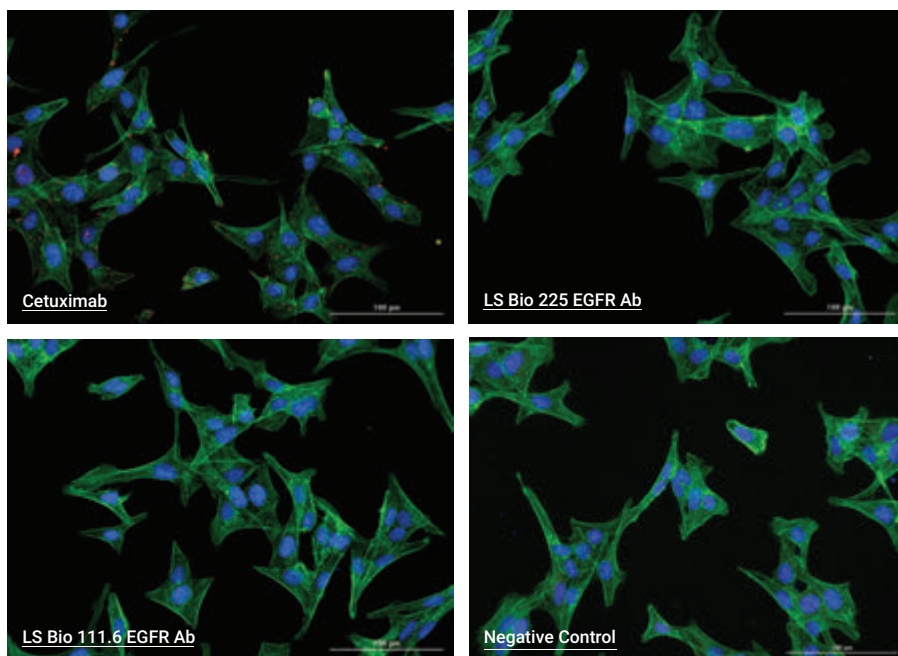

Figure 11. Anti-EGF receptor antibody binding 20x images. Blue indicated DAPI-stained nucleus. Green indicates phalloidin actin staining.

Conclusion

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 with the Nuclear-ID Blue/Red cell viability assay for rapid evaluation of live and dead cell populations. The combined capabilities of the Agilent BioTek Cytation 3 cell imaging multimode reader afford the ability to easily perform each assay with one instrument. The advanced optics and Agilent BioTek Gen5 data analysis software features ensure accurate detection of both reader-based and microscopy assays, and provide efficient performance of the entire experimental workflow.

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