

Abstract

Receptor tyrosine kinases (RTK) mediate a multitude of cellular processes including proliferation, survival, cell movement, cytoskeletal rearrangement and gene expression. Abnormalities in RTK expression or activities can contribute to the progression and maintenance of malignant phenotype. Therefore, the ability to modulate the kinase activity of RTKs represents an attractive therapeutic strategy for cancer. Several approaches have been developed to identify RTK inhibitors. However, most of these approaches are end point, *in vitro* based assays that require substantial reagent optimization, and are inadequate in providing information on its effective activity intracellularly. This study describes the use of a novel, quantitative, label-free and real-time cell based assay using an electronic cell sensor technology that addresses some of the limitations of current *in vitro* assays in assessing kinase activity. Microelectronic sensor arrays are integrated on the bottom of microtiter plate allowing for sensitive and quantitative detection over time of cellular changes as a result of growth factor treatment of cell lines expressing different RTKs. Assay quality parameters such as reproducibility ($r=0.7$), signal: background ratio (S/N=38), Z-factor ($Z=0.6$) and assay sensitivity were determined. These robust parameters allowed for the screening of a small diverse library of small molecules primarily composed of Sigma's enzyme inhibitor Ligand Set that resulted in the identification of a selective and potent EGFR inhibitor (EGFR1). Using this assay further characterization of the EGFR inhibitor was performed and its IC_{50} was determined. This assay was also used to measure specific cellular responses to activation of insulin growth factor receptor, PDGFR, c-Met and FGFR by their respective ligands. These data show a facile, high content and novel cell based kinase assay that has improved utility over existing *in vitro* and cell based assay in the identification and characterization of selective and potent kinase inhibitors.

Introduction

RTKs are membrane receptors that contain an intracellular kinase, which transfers a phosphate group from an ATP molecule to a hydroxyl group on tyrosine residues. Upon ligand binding, RTKs dimerize or oligomerize resulting in autophosphorylation and increased activation of its intrinsic kinase activity. This leads to phosphorylation of several downstream effector proteins resulting in activation of multiple signaling pathways. These pathways include the activation of Ras/MAPK, PI3K, and PLC pathways. Other signaling proteins such as Src, Paxillin and FAK are also phosphorylated. Activation or phosphorylation of these proteins as a result of RTK activation leads to cytoskeletal changes including membrane ruffling, lamellipodia and filopodia formation (1). These cellular changes are mediated by the activities of small GTPases Rac, Rho and Cdc42 (2).

Numerous screening platforms have been developed for the identification of RTK inhibitors. They are generally subdivided into antibody-dependent technologies including AlphaScreen, TR-FRET, FP, TRF, SPA, Luminex and ELISA, and antibody-independent methods such as incorporation of radioactivity, ATP consumption and technologies based on change of substrate size and charge. Although these technologies offer some advantages, they are limited by one or more of the following factors: complicated and tedious optimization steps, limited substrate capacity, assay component interference, and expensive assay components, all of which can affect the signal, throughput, time and utility of the assay. This report demonstrates the development and utility of an alternative cell based RTK assay that addresses several of these issues and provides a facile and easy platform for the identification and further characterization of RTK inhibitors.

ACEA Biosciences has developed a cell sensor array electrodes integrated on the bottom of the wells of microtiter plate (E-plate™). The sensors are arrayed in a novel design that covers approximately 80% of the wells surface area, allowing for sensitive and quantitative detection of cellular changes (Figure 1B). Signals from these sensors are related to a real time cell electronic sensing (RT-CES) system that allows for monitoring and analysis of the kinetic aspects of cellular behavior. The signals relayed are impedance changes in the ionic environment created by the application of an electric field. Disruption of this ionic environment on the sensor surface due to the presence of cells or changes in the cell morphology can lead to changes in measured impedance, which is then converted to a cell index (CI) value (Figure 1A). The extent of the cell-electrode impedance response is dependent on the attachment quality and the sensor area covered by the cell. An increase in measured impedance value due to an increase in cell number or degree of attachment results in an increase in observed cell index. CI was calculated according to the formula:

$$CI = \max_{i=1, \dots, N} \left(\frac{R_{cell}(f)}{R_0(f)} - 1 \right)$$

where, the number of the frequency points at which the impedance is measured (i.e., $N=3$ for 10kHz, 25 kHz, and 50 kHz), and $R_0(f)$ and $R_{cell}(f)$ are the frequency-dependent electrode resistance with cell or without cells present in the wells.

Results

Schematic representation of electronic impedance as a measure of cell index (CI) and microtiter plate with integrated microelectronic sensors

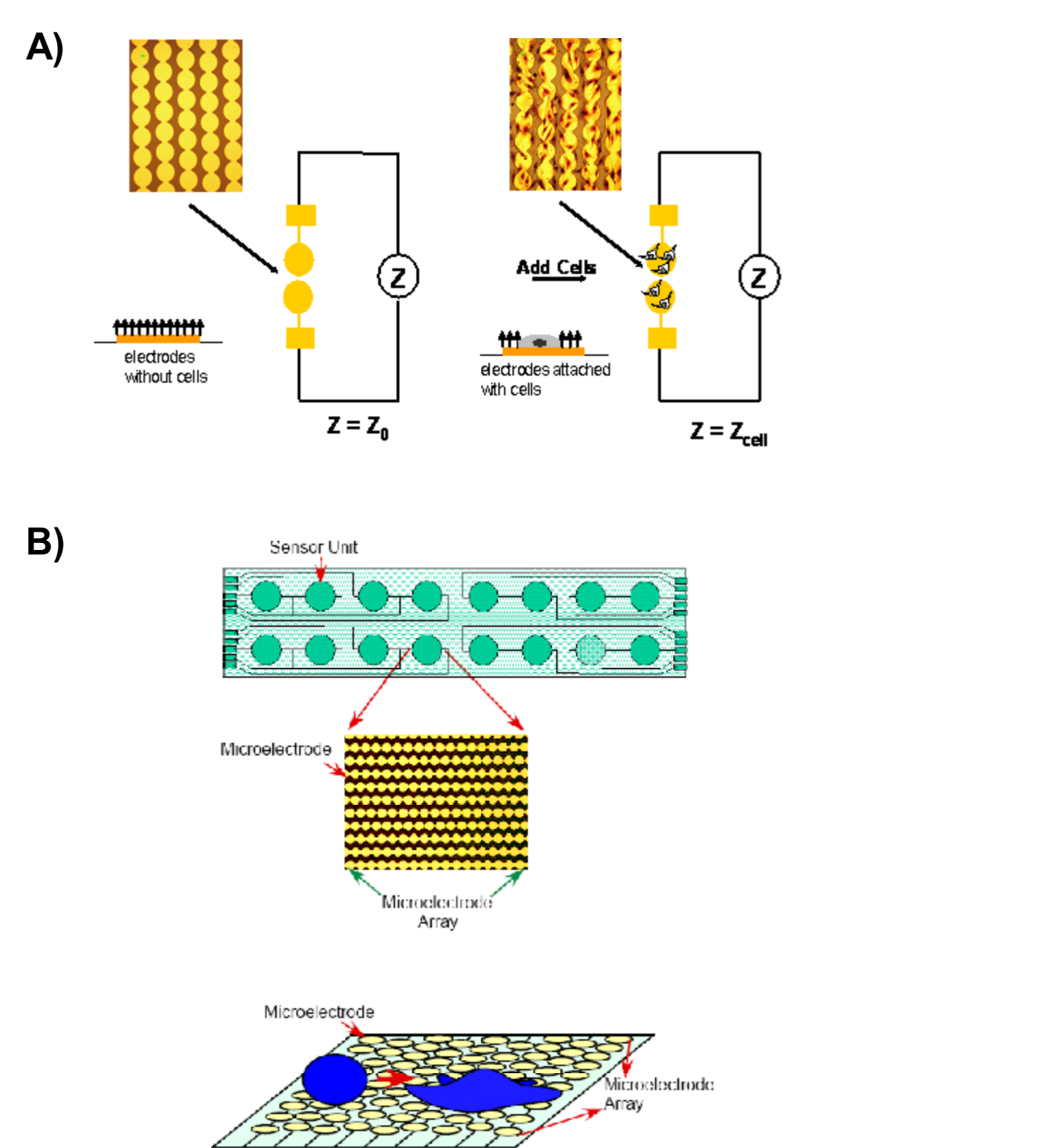


Figure 1: (A) Schematic representation of electronic impedance change in the presence or absence of cells. (B) Schematic representation of the wells of a microtiter plate integrated with microelectronic arrays. The circle-on-line microelectrode units are fabricated on glass slides. The schematic represents 16 individual detection units integrated with a micro-electronic array in the bottom of each individual well. Upon addition of cells onto the microelectronic sensors, the round cell makes contact with electrodes and attaches. Subsequently, the cells undergo dramatic morphological changes and spreads onto the microelectrode sensors. The spread cells cover and interact with a larger number of microelectrodes leading to a higher impedance readout compared to the round cell.

The kinetics of early morphological changes induced by growth factors can be dynamically monitored by the ACEA RT-CES system

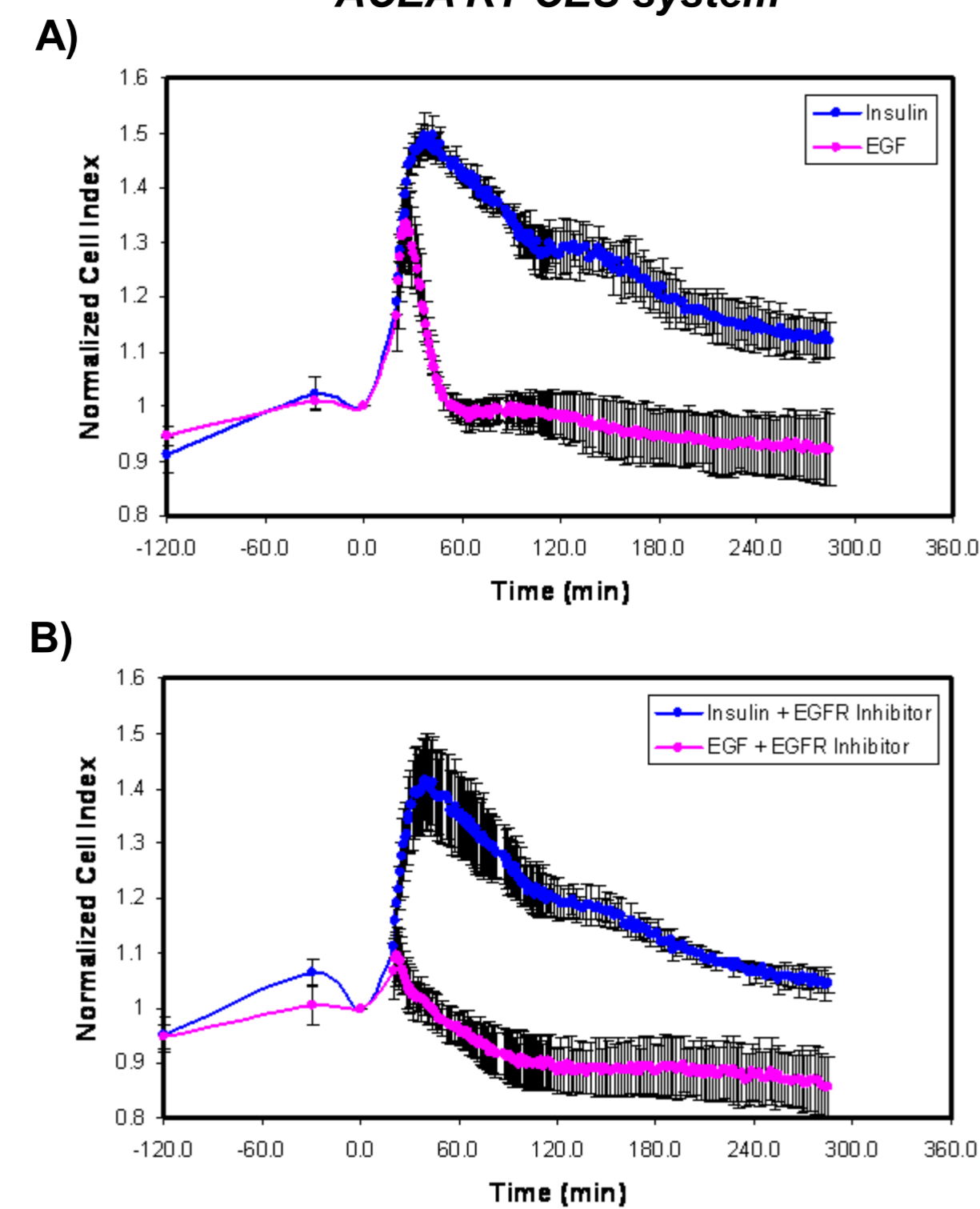


Figure 2: (A) Measurement of cell index over time after treatment with insulin or EGF. COS7 cells were plated at 1×10^4 cells/well, serum starved, and treated with 25 ng/mL of insulin or EGF. Cell index was measured every minute for several hours. Cells treated with insulin or EGF showed unique responses characterized by a sharp and transient increase in cell index peaking within 30 min, followed by a slow decrease in cell index. Insulin treated cell showed a slower rate of decrease in cell index relative to EGF treated cells. (B) Assessment of specificity of cellular response to EGF and insulin treatment. COS7 cells were pretreated for 1 hour with either a specific EGFR inhibitor or vehicle. Cells were then stimulated with insulin or EGF. Cells pretreated with EGFR inhibitor did not respond to EGF while the insulin response remains intact.

The cell index change over time due to growth factor treatment parallels morphological changes characterized by lamellipodia formation

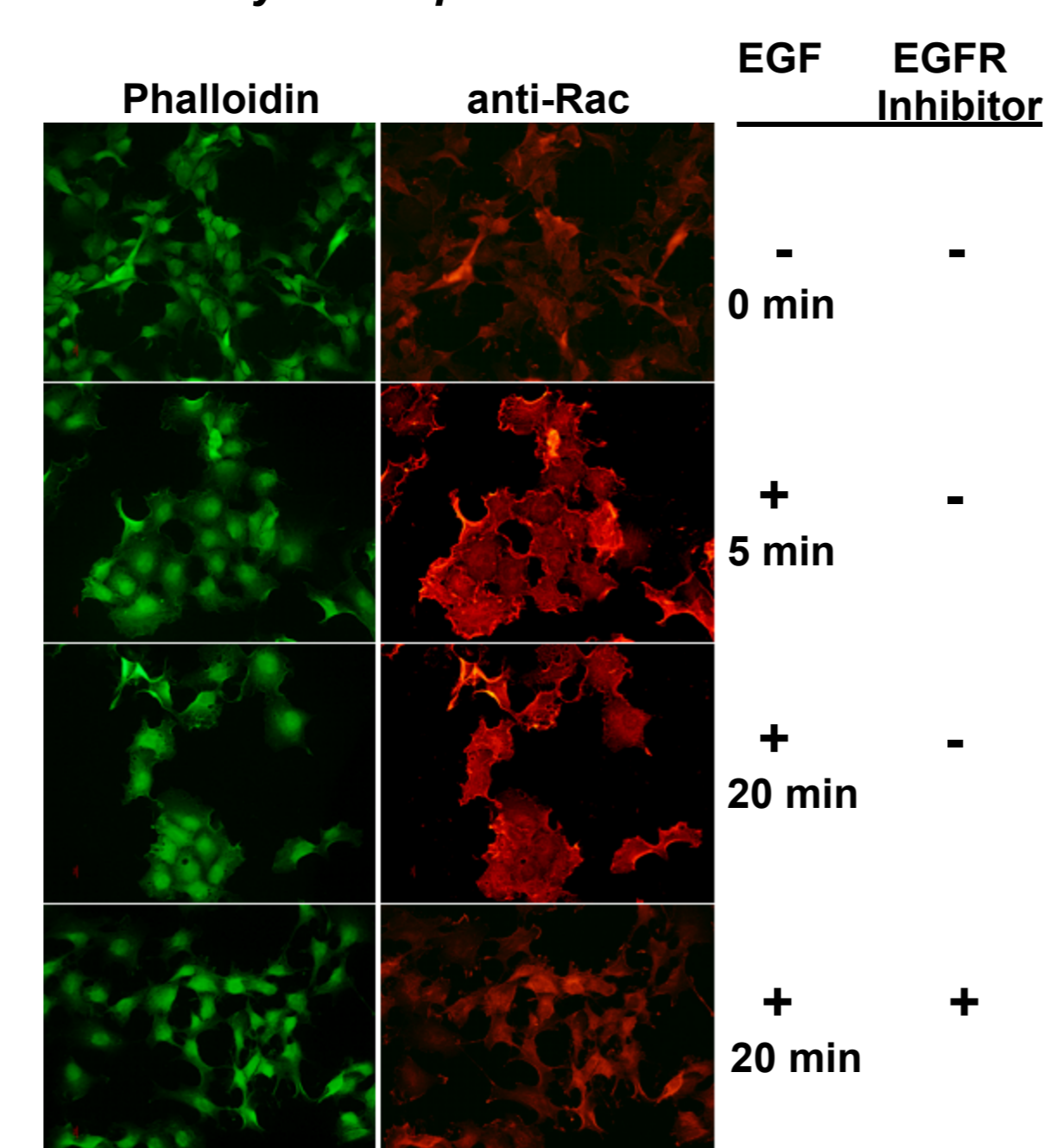


Figure 3: Fluorescent images of COS7 cells treated with EGF and +/- EGFR inhibitor over time. Cells were pretreated with EGFR inhibitor or vehicle for 1 hour and stimulated with 25 ng/mL EGF. Samples were collected at 0, 5, and 20 min. Cells were fixed with 4% paraformaldehyde, stained with mouse anti-Rac antibody, and incubated with TRITC conjugated anti-mouse secondary antibody and FITC-phalloidin. Immunofluorescent images were collected with a CCD camera attached to a fluorescence microscope. Cells treated with EGF showed rapid formation of lamellipodia within 5 min paralleling the early cell index change measured by the ACEA RT-CES system. This response is specific to EGF as the response is inhibited by an inhibitor specific to EGFR.

Dynamic monitoring and profiling of activated intracellular signaling pathways by growth factor (EGF) treatment

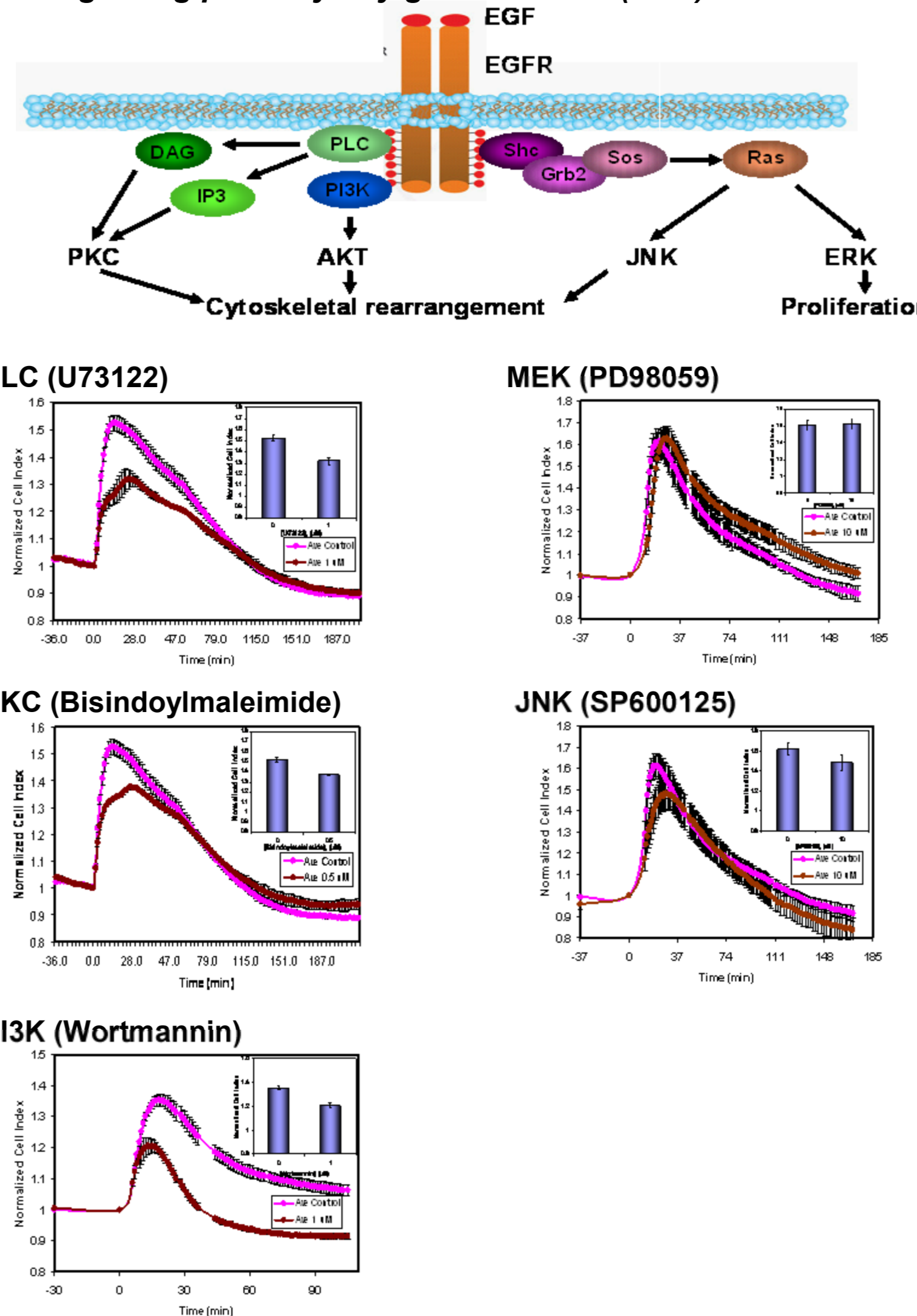


Figure 4: Effect of various intracellular signaling inhibitors on cell index change over time as a result of EGF treatment. COS7 cells were plated at 1×10^4 cells/well, serum starved, and pretreated for 1 hour with the indicated concentration of signaling inhibitors. Cells were then stimulated with 25 ng/mL EGF and cell index measured over several hours. The transient increase in cell index as a result of EGF treatment, and which parallels lamellipodia formation, is inhibited by inhibitors that affect pathways involved in cytoskeletal changes and not proliferation.

Comparison of dose response curves of COS7 cellular response to EGF using ACEA RT-CES vs ELISA

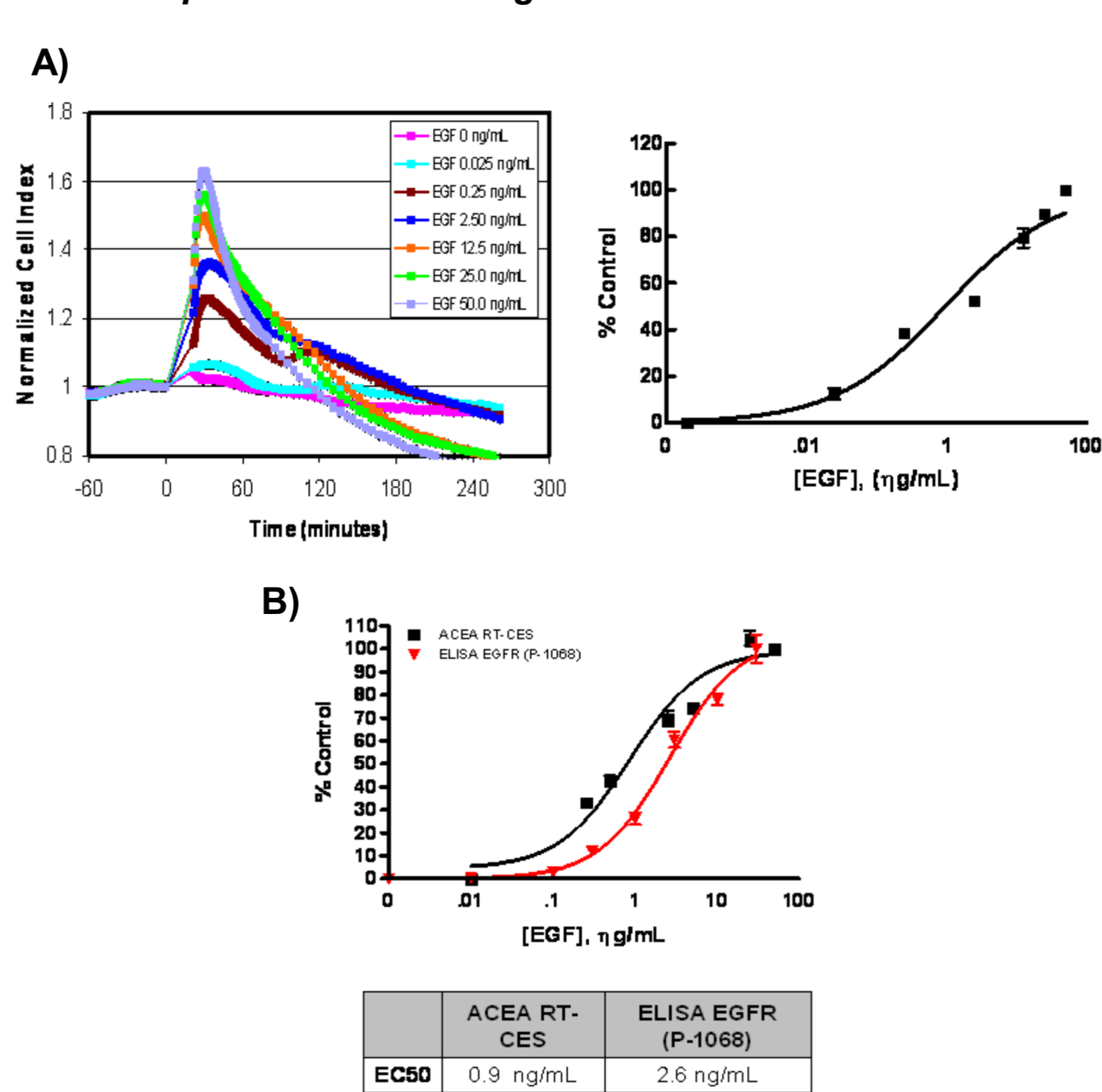


Figure 5: Measurement of COS7 cellular response over a range of EGF concentrations. COS7 cells were plated at 1×10^4 cells/well and treated with varying concentrations of EGF. (A) Kinetic measurement of cellular response to EGF was done over several hours and scans normalized to CI measurement immediately before ligand addition. Maximum cell index for each ligand concentration treatment was determined, normalized to untreated controls, and plotted as a measure of EGF concentration. (B) Comparison of dose response curves generated from ACEA RT-CES and ELISA, a common assay used to measure kinase activity. Curve fitting and EC_{50} values were calculated from dose response curve using XLfit.

Statistical evaluation and screening result of sigma's enzyme inhibitor ligand set using a label-free cell based EGFR kinase assay

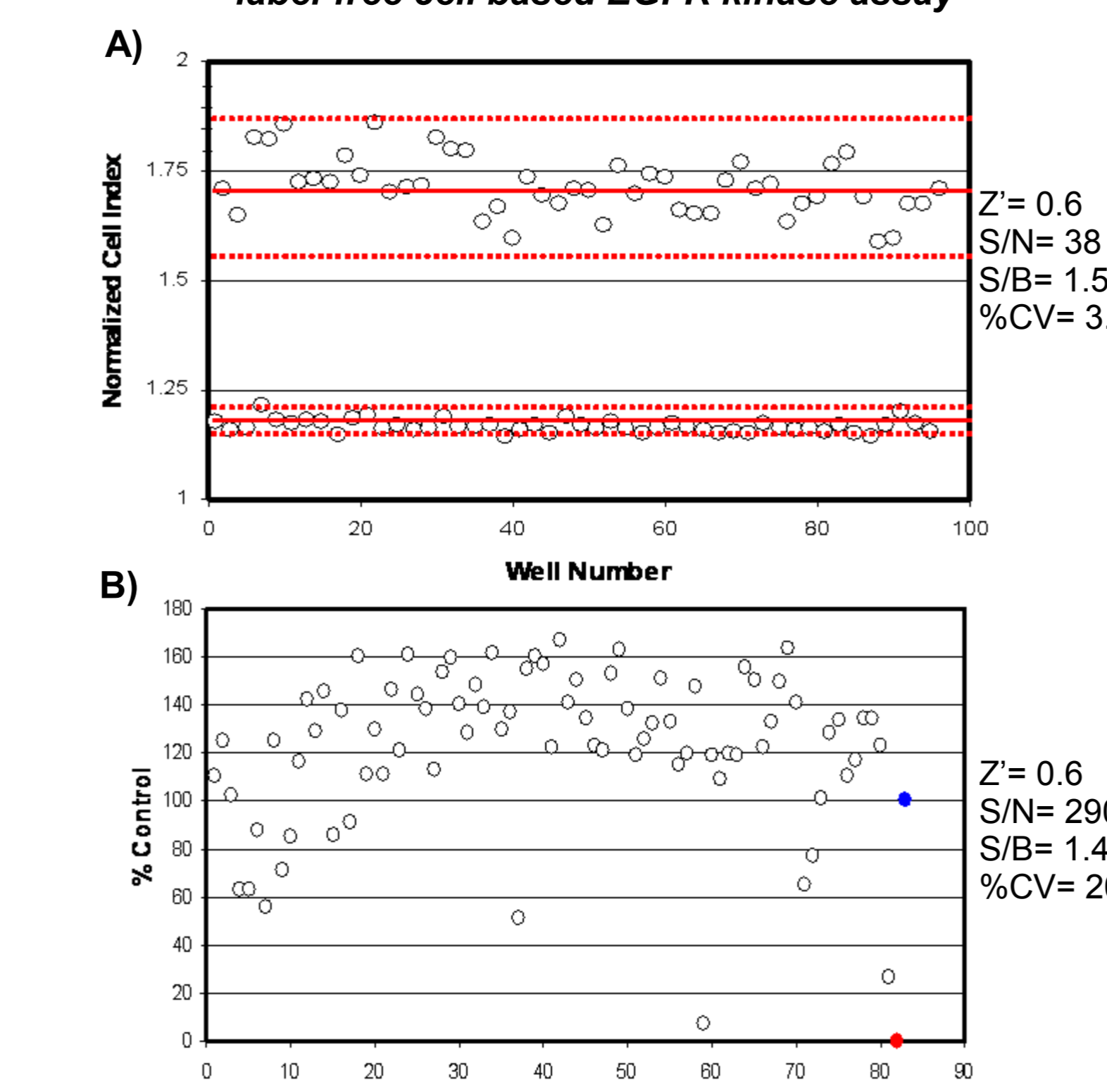


Figure 6: (A) Statistical evaluation of label-free cell based EGFR kinase assay. Z', S/N, S/B and %CV were determined to assess quality of assay. (B) Graphical representation of a screen of 81 compounds mostly from Sigma's enzyme inhibitor Ligand Set. From this library, compounds were screened in singlets at 5-15 μ M concentrations.

Characterization of EGFR inhibitor (EGFR1) identified from the screening library showed potent inhibition of EGF cellular response

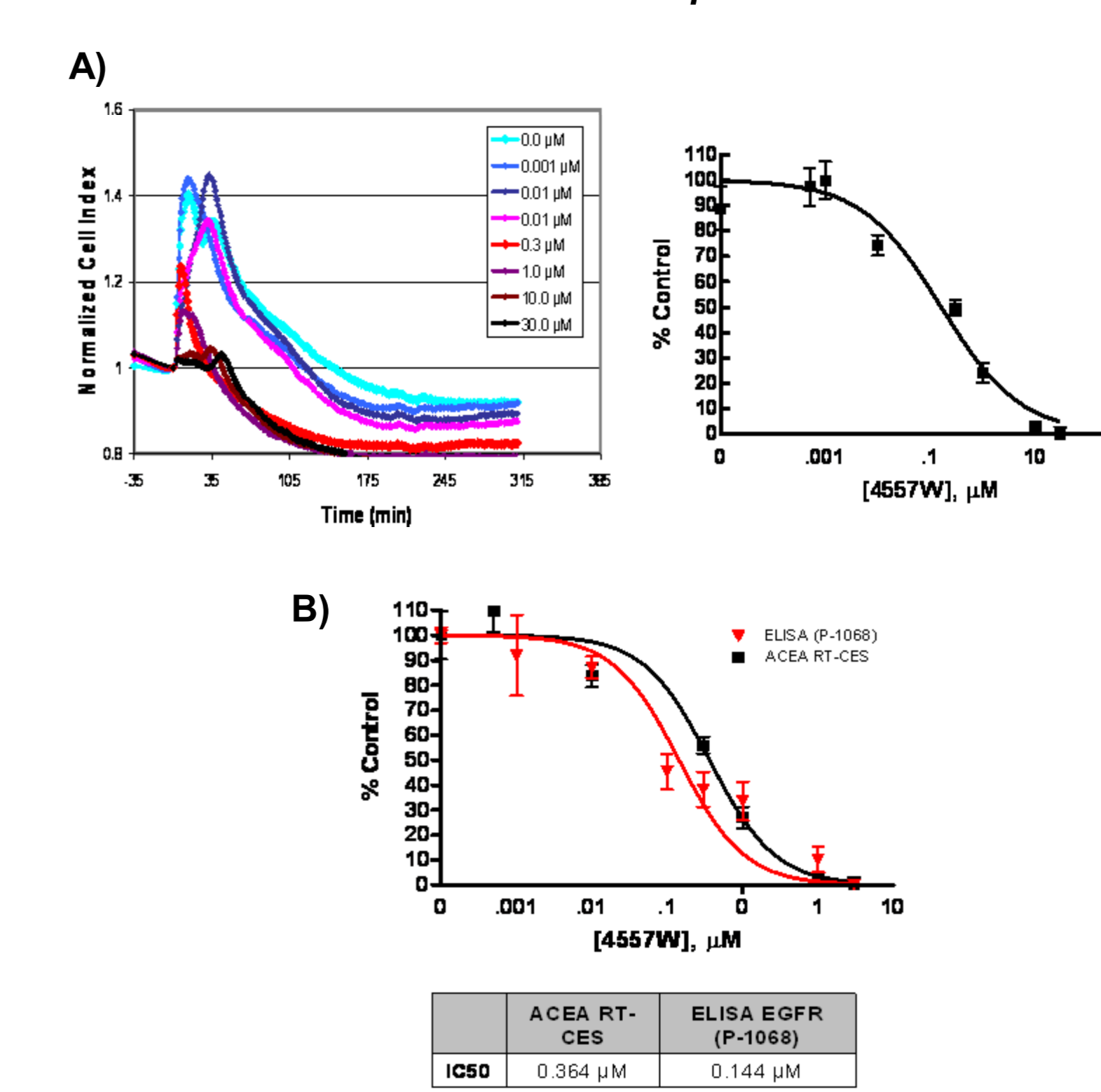


Figure 7: Measurement of COS7 cellular response to EGF over a range of EGFR inhibitor concentrations. COS7 cells were plated at 1×10^4 cells/well serum starved and preincubated with varying concentration of EGFR1, 4657W, for 1 hour. Cells were then stimulated with 25 ng/mL of EGF and cell index measured over several hours. (A) Kinetic measurement of cellular response to EGF after pretreatment with varying concentrations of EGFR1 normalized to CI measurement immediately before ligand addition. Maximum cell index for each inhibitor concentration treatment was determined, normalized to untreated controls and plotted as a measure of inhibitor concentration (right panel). (B) Comparison of dose response curves generated from ACEA RT-CES and ELISA. Curve fitting and IC_{50} values were calculated from dose response curve using XLfit.

Potent EGFR1 is specific and selective for EGFR and not insulin growth factor receptor or c-Met

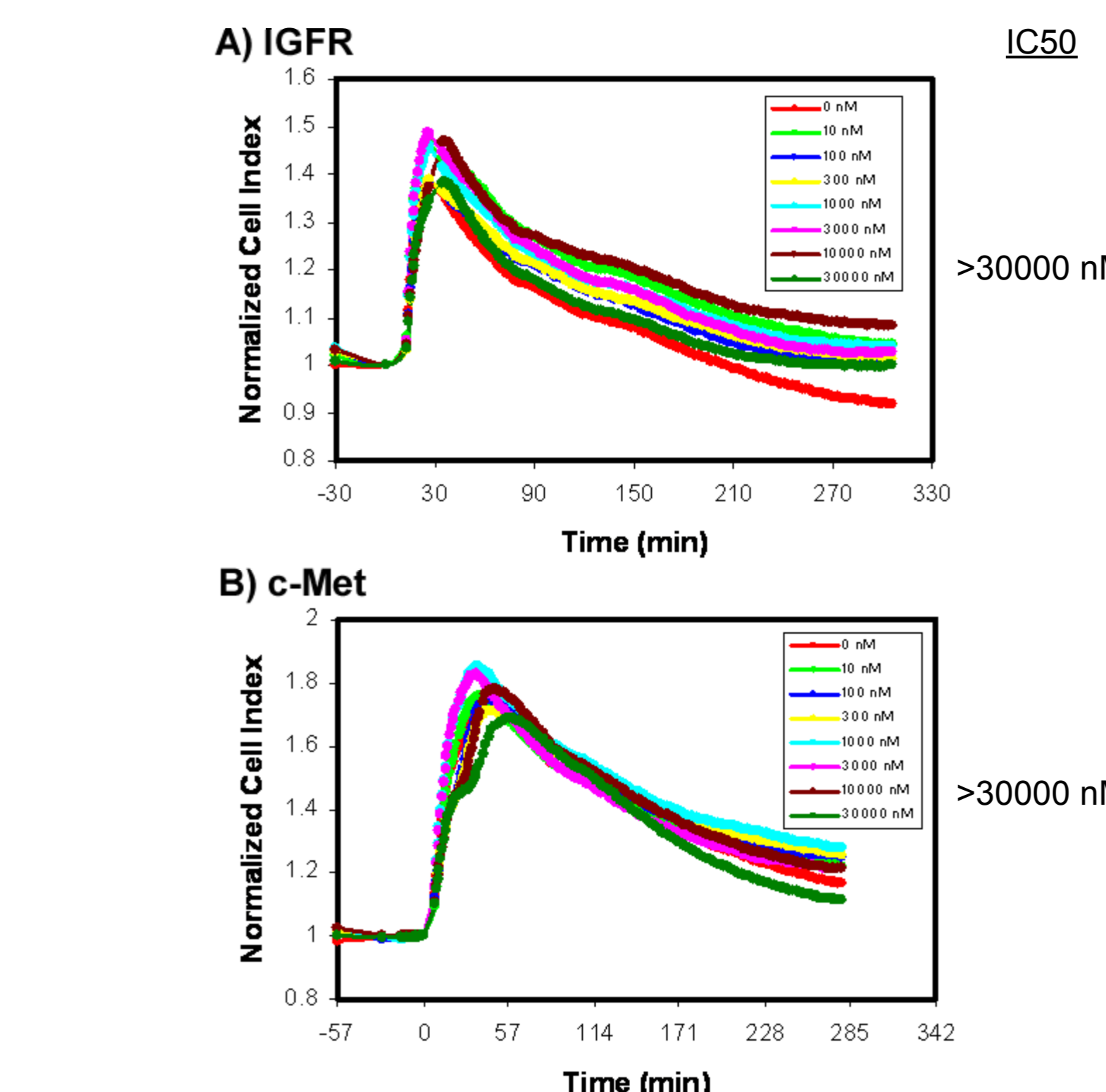


Figure 8: Measurement of COS7 cellular response to insulin and HGF over a range of EGFR inhibitor concentrations. COS7 cells were plated at 1×10^4 cells/well, serum starved and preincubated with varying concentration of EGFR1, 4657W, for 1 hour. Cells were then stimulated with 25 ng/mL of insulin or HGF, and cell index measured over several hours. Kinetic measurement of cellular response to (A) insulin and (B) HGF after pretreatment with varying concentrations of EGFR1 inhibitor and normalized to CI measurement immediately before ligand addition. The potent EGFR1 inhibitor displayed specificity to EGFR and did not affect the cellular response of COS7 cells to insulin or HGF.

Dynamic measurement of cellular response to RTK ligands of selected immortalized cell lines using the ACEA RT-CES System

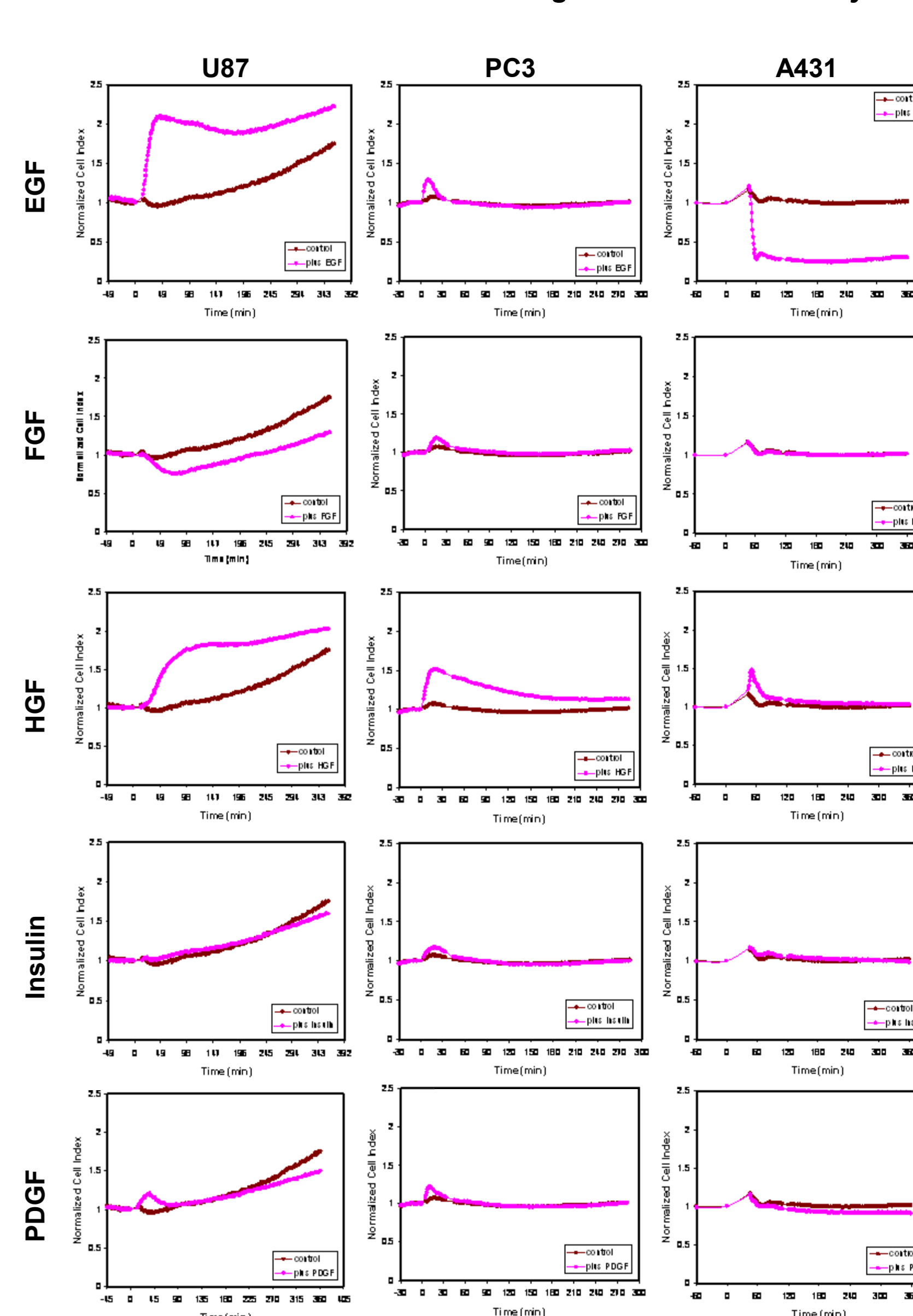


Figure 9: Comparison of unique signaling patterns of selected immortalized cell lines after treatment with various ligands for RTK. Cells were plated on ACEA E-plates, serum starved, and treated with the indicated ligands. Pink traces represent cells treated with ligand, and brown traces represent cells treated with vehicle. Response was measured every minute and data normalized to last measurement prior to ligand addition. Error bars represent standard deviation of $n=4$.

Conclusion

These data demonstrate a facile and novel cell based assay for RTK activity and function. This assay quantifies morphological changes in response to growth factor treatment and therefore mimics proximal events in kinase activation. Characterization of these measured cell response on the RT-CES system show that the response is specific, robust, reproducible and in concurrence with existing RTK cell-based assays, such as ELISA. Unlike other RTK assay, this cell-based is label-free and monitors cellular changes in real-time, therefore acquiring high content information regarding the state of the cell and the signaling pathways being activated. In addition, the RTK assay described here does not require expensive reagents nor suffer from assay component interference. Since the readout is non-invasive multiple treatments can be carried out in the same well and can be used in conjunction with other existing cell-based assays for RTK. It requires very little optimization and user training, making this assay amenable for use in both primary and secondary screens.

References

- Hall, A (1998) Science 279:509
- Etienne-Manneville S and Hall A, (2002) Nature 420:629

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