APPLICATION NOTE

No-Wash IP1 assays are a powerful readout to characterize compounds modulating the FGFR signaling pathway in cancer drug research.

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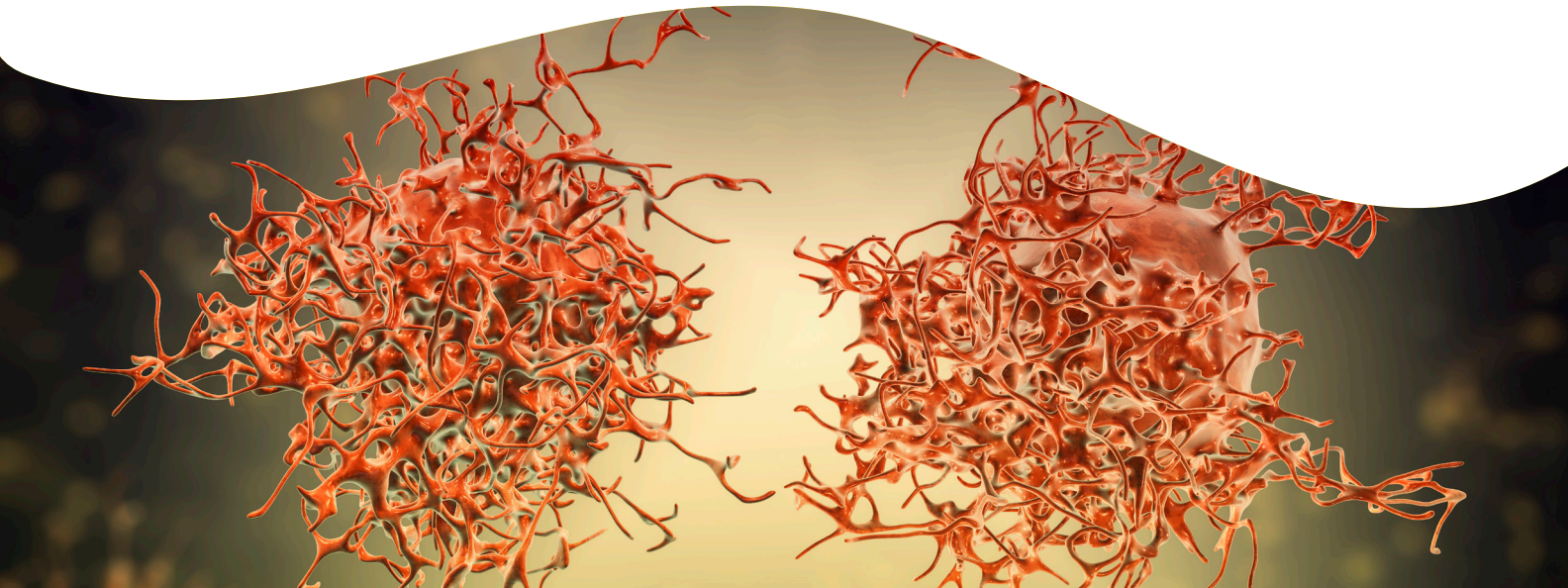
Abstract

The success of cancer therapies can be affected by crosstalk between multiple signaling pathways. Dysregulated FGFR signaling has been identified in many cancer types such as non-small cell lung cancer (NSCLC), breast cancer, glioblastoma and prostate cancer. The existence of complex and numerous FGFR intracellular signaling pathways encompassing Ras-MAPK, PI3K-AKT, STAT and PLCγ-PKC requires the development of specific investigational tools to better understand the mechanism of action of a drug.

D-myo-Inositol 1-Phosphate (IP1) is an intracellular metabolized product of IP3. Detection of IP1 as a surrogate biomarker for IP3 has been widely utilized in cell-based high-throughput assays (HTS) for GPCR agonist and antagonist screening. Herein, we demonstrate for the first time, that the detection of the intracellular accumulation of IP1, mediated by an FGFR-dependent activation of PLCγ1, can be a powerful alternative to characterize compounds modulating FGFR signaling in various cancer cell lines expressing FGFR1, FGFR2, and FGFR3. In particular, results obtained with a pan-FGFR inhibitor, AZD4547, strongly correlated with those obtained using an HTRF assay monitoring the phosphorylation of PLCγ1. Detection of IP1 accumulation was achieved using either a no wash Revvity HTRF™ or AlphaLISA™ kits. These assays enable the rapid and accurate measurement of the activation status of intracellular downstream key enzymes and second messengers. Moreover, their suitability for miniaturization and automation makes their use very attractive in high-throughput screening and compound characterization.



Introduction

Fibroblast growth factors (FGF) are one of the most potent mitogenic growth factors discovered during the early 1970s. To date, twenty-three members of the FGF family and five fibroblast growth factor receptors (FGFR1 to FGFR5) have been identified. FGFR1 to FGFR4 belong to the receptor tyrosine kinase (RTK) superfamily [Lemmon and Schlessinger, 2010; Xie et al., 2020]. FGFs bind to inactive FGFRs to trigger receptor dimerization resulting in activation of cytosolic tyrosine kinases by phosphorylating the tyrosine residues on the cytosolic tail of FGFRs. The phosphorylated FGFRs, together with several other adaptor proteins, activate phospholipase C gamma (PLCγ) and transmit signal to downstream pathways that include Ras/Raf-MEK-MAPKs, PI3K/AKT, STAT, and DAG-PKC and IP3-Ca^{2+} signaling [Lemmon and Schlessinger, 2010; Chae et al., 2017].

Inhibition of FGFR and its signaling pathways has become a popular target for cancer drug discovery. Two non-selective tyrosine kinase small molecule inhibitors (imatinib and ponatinib) and one FGFR1/2/3/4 selective small molecule inhibitor (erdafitinib) have been approved for the treatment of certain types of cancers and over 20 clinical trials are ongoing [Porta et al., 2017; Chae et al., 2017; Dai et al., 2019; Xie et al., 2020]. To evaluate FGF binding blockers (antibodies or peptides) and FGFR inhibitors in drug discovery, readouts able to assess the effect of such compounds on the FGFR signaling pathway are necessary. Assays able to assess the phosphorylation status of proteins involved in this pathway, such as PLCγ1, c-RAF, MEK or ERK are usually used. Herein, we demonstrate for the first time, that the detection of the intracellular accumulation of IP1, mediated by a FGFR-dependent activation of PLCγ1, can be a powerful alternative to phosphorylation assays to characterize compounds modulating FGFR signaling in cancer cell lines such as KG-1 (blood cancer), SNU-16 (gastric cancer), and KMS-11 (multiple myeloma) expressing FGFR1, FGFR2, and FGFR3, respectively. Detection was achieved using either HTRF or AlphaLISA IP-One kit which enable the rapid and accurate measurement of IP1 accumulation in cells.

HTRF and AlphaLISA technology assay principle

In cellular signaling pathways, the production of inositol 1,4,5 triphosphate (IP3) indicates receptor activation, signal transmission, and subsequent calcium release. Following receptor activation, PLC-β is activated by the Gαq subunit of heterotrimeric G-protein in the GPCR pathway and PLCγ is activated by the phosphorylation of PLCγ in the RTK pathway. The PLC-β and PLCγ then increase cellular IP3 by hydrolyzing PIP2 to IP3 and DAG. Monitoring IP3 or intracellular transient Ca^{2+} release for drug screening is difficult since IP3 rapidly enters the metabolic inositol phosphate cascade with a half-life of only 30 seconds [Samuel et al., 1995] and calcium flux assays require special automation and imaging equipment given the extremely transient nature of this event, reaching the maximal concentration in approximately 20 seconds [Sozzani et al., 1993]. IP1 is an intracellular metabolized product of IP3. It is known that lithium chloride (LiCl) leads to IP1 accumulation upon receptor activation by inhibiting inositol monophosphatase, the final enzyme of the IP3 metabolic cascade [Trinquet et al., 2006]. Therefore, measuring cellular IP1 can be used as a surrogate for IP3 (Figure 1). Detection of IP1 as a powerful readout of Gαq-coupled GPCR activation, using Revvity IP-One kits, has been widely reported [Trinquet et al., 2011] and is now considered a well-established technique. However, their usage as a suitable readout to investigate the FGFR signaling pathway has not been previously reported.

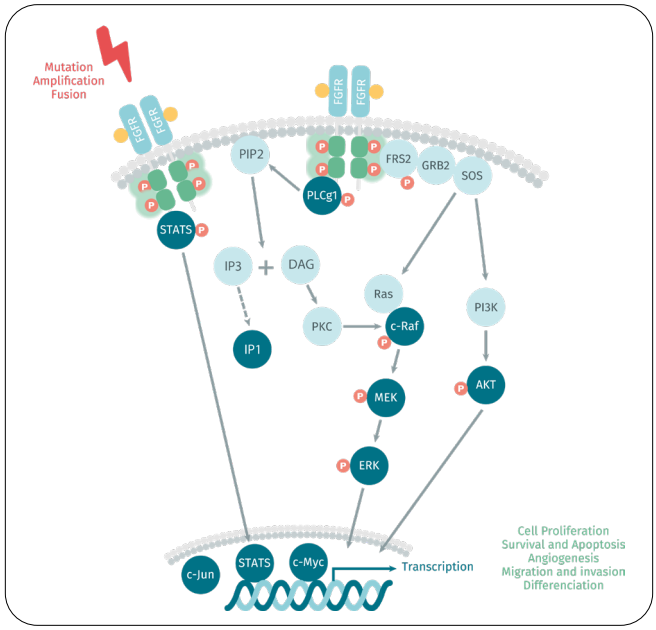


Figure 1: FGFR signaling pathway. Upon agonist FGF binding and stimulation of FGF receptors, FGFR are activated by phosphorylation of its own intracellular domains to trigger multiple downstream signaling pathways including the activation of PLCγ1. The activated PLCγ1 hydrolyzes PIP2 into IP3 and DAG. IP3, known as a calcium mobilizing molecule released from the ER, is further metabolized into IP2, IP1, and myo-inositol. Intracellular accumulation of IP1 in cell cultures can be measured when cell stimulation buffer containing LiCl is used to inhibit IP1 degradation into myo-inositol.

The principle of IP1 detection by HTRF IP-One kit and AlphaLISA IP-One kit are the same. Both assays are competitive no-wash immunoassays intended to measure IP1 accumulation in cells. Native IP1 produced in cells and labeled IP1 compete and bind to the antibody conjugated to cryptate dye for HTRF or to the acceptor bead for AlphaLISA.

HTRF IP-One assay principle: HTRF assay uses d2-labeled IP1 (acceptor) and anti-IP1-Cryptate (donor). Native IP1 produced by cells competes with d2-labeled IP1 (acceptor) for binding to anti-IP1-Cryptate (donor). When the dyes are in proximity, the excitation of the donor with a light source (laser or flash lamp) triggers a Fluorescence Resonance Energy Transfer (FRET) towards the acceptor, which in turn fluoresces at a specific (signal) wavelength (665 nm). The specific signal (i.e. energy transfer) is inversely proportional to the concentration of IP1 in the standard or sample (Figure. 2).

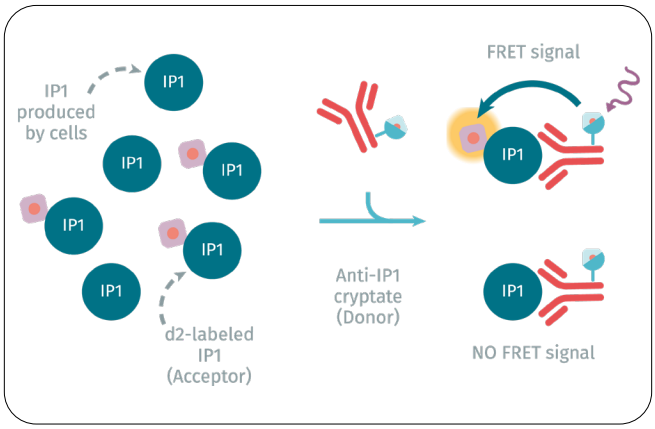


Figure 2: HTRF Assay Principle: Cellular IP1 and d2-labeled IP1 compete for the IP1 Tb cryptate antibody.

AlphaLISA IP-One assay principle: AlphaLISA IP-One assay uses AlphaLISA anti-IP1 Acceptor beads and AlphaLISA Streptavidin-coated Donor beads (SA-DB) to capture the biotinylated IP1 analog. Donor beads and Acceptor beads come into proximity through biotinylated IP1 binding. Excitation of the Donor beads provokes the release of singlet oxygen that triggers a cascade of energy transfer reactions in the Acceptor beads, resulting in emission at 615 nm (Figure 3A). Native IP1 produced by cells competes with biotinylated IP1 for binding to AlphaLISA anti-IP1 acceptor beads. Again, the specific signal is inversely proportional to the concentration of IP1 in the standard curve or in the cells (Figure 3B).

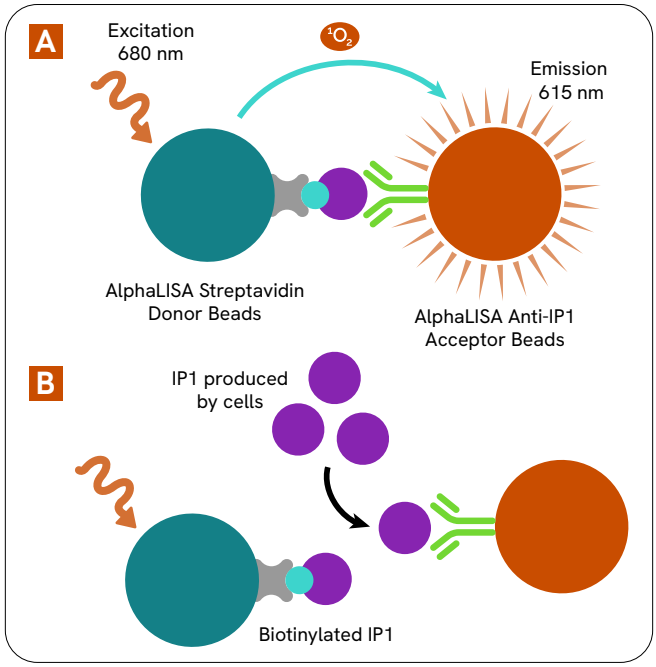


Figure 3: AlphaLISA Assay Principle (A). Cellular IP1 and biotinylated IP1 compete anti-IP1 antibody conjugated to AlphaLISA acceptor beads (B).

HTRF Total PLCγ1 and HTRF Phosphorylated PLCγ1 assay principle: Total PLCγ1 and HTRF phosphorylated PLCγ1 are detected in a sandwich no-wash immunoassay format using two different specific antibodies, one labeled with Eu^{3+}-Cryptate (donor) and the other labeled with d2 (acceptor). The only difference between the two kits is the antibodies used in the kits (Figures 4A and 4B). The dyes come in proximity in the presence of native cellular PLCγ1, the excitation of the donor with a light source (laser or flash lamp) triggers a Fluorescence Resonance Energy Transfer (FRET) towards the acceptor, which in turn fluoresces at a specific wavelength (665 nm). The specific signal is proportional to the total-PLCγ1 or to the phosphorylated PLCγ1 (Tyr783) in cell lysates samples.

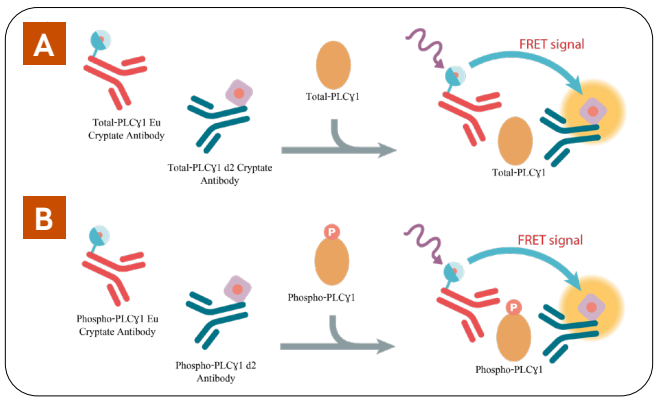


Figure 4: A) HTRF total PLCγ1 assay principle. B) HTRF phospho-PLCγ1 (Tyr783) assay principle.

Cancer cell lines with FGFR genetic alterations

KG-1 is a myeloid leukemia cell line which is characterized by a fusion of the FGFR1OP2 gene that disrupts the FGFR1 gene and involves an aberrant tyrosine phosphorylation of FGFR1, and especially a constitutive phosphorylation of STAT5 [Gu et al., 2006 and Cowell et al., 2017]. SNU-16 is an FGFR2-dependent gastric cancer cell line harboring an FGFR2 amplification [Xie et al., 2013; Ku et al., 2006]. KMS-11 cell line is a useful model of multiple myeloma and presents an FGFR3 gene mutation (Y373C). This mutation involves a high level of phosphorylation of the receptor FGFR3 indicating a constitutive activation. It has been well established that the stimulation of cells increases the level of receptor phosphorylation which activates the MAP kinase signaling pathway but does not seem to involve the STAT cascade [Ronchetti et al., 2001 and Chesi, et al., 2001]. These three cell lines provide *in vitro* models for the study of FGFR inhibitors against FGFR1 fusion, FGFR2 amplification, and FGFR3 mutation (Figures 5A, 5B, and 5C).

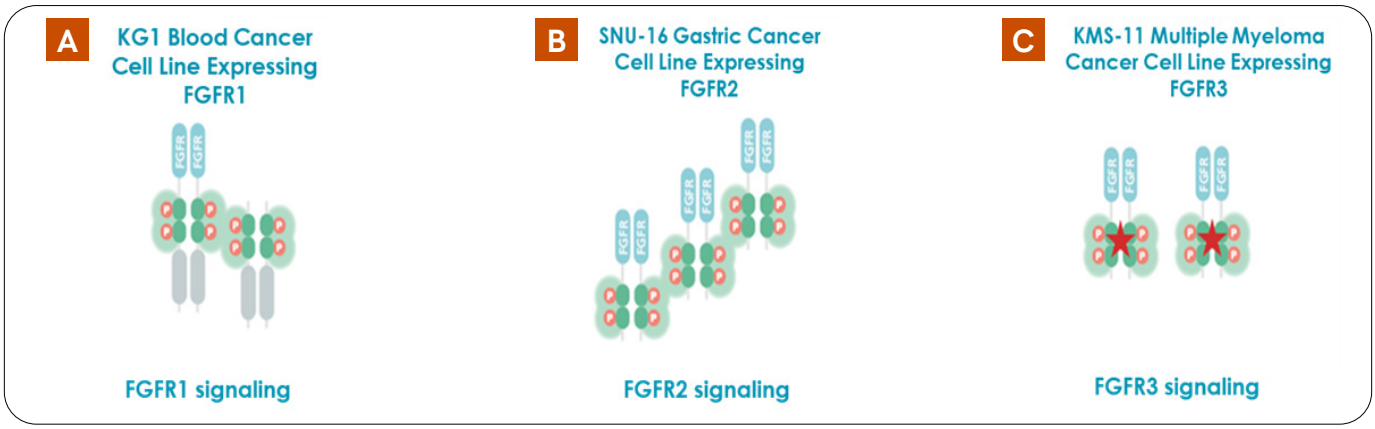


Figure 5: Three cancer cell lines and their FGFR1, 2, and 3 receptors in vitro models: (A) KG1 cell line - Myeloid leukemia cell line (B) SNU-16 cell line - Gastric cancer cell line (C) KMS-11 cell line - Multiple Myeloma Cancer

Materials and methods

Reagents and assay kits: KG-1 (CCL-246) and SNU-16 (CRL-5974) cancer cell lines, Iscove's modified Dulbecco's medium (30-2005), RPMI-1640 medium (30-2001), Fetal Bovine Serum (30-2020), and penicillin/streptomycin antibiotics (30-2300) were purchased from ATCC, and KMS-11 (JCRB1179) cell line was bought from JCRB Cell Bank. Fibroblast growth factor 2 (FGF2) was obtained from Peprotech (100-18B) and R&D System (233-FB). FGFR1/2/3 inhibitor, AZD4547, was purchased from Selleckchem (S2801). Revvity HTRF and AlphaLISA kits used were: HTRF IP-One Gq Kit (62IPAPEC), AlphaLISA IP-One Detection Kit (AL3145C), HTRF Phospho-PLCγ1 (Tyr783) (64PLCG1Y3PEG) and HTRF Total PLCγ1 (64PLCG1TPEG) kits. The following microplates from Revvity were used in cell assays: ProxiPlate-384 Plus, white 384-shallow well microplate (6008280), AlphaPlate-384, Light gray (6005350), View plate -96 TC (6005181), and CulturPlate-384, White opaque TC-Treated (6007680). Revvity EnVision™ 2105 Multimode plate reader was used to collect HTRF and AlphaLISA data.

Cell culture: Cancer cells were cultured and maintained in either T150 cm^2 or T175 cm^2 flasks in the recommended media supplemented with FBS and antibiotics. KG-1 cells were cultured in Iscove's modified Dulbecco's Medium supplemented with 20% FBS and 1% antibiotics. SNU-16 and KMS-11 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% antibiotics. KG-1 and SNU-16 cell lines are suspension cells whereas the KMS-11 cell line is an adherent cell. One day before cell treatments (stimulation, inhibition) and cell assays, the cells with 80 to 85% confluency were cultured in serum free medium in tissue culture flasks. Cells were then harvested for treatments (stimulations and inhibition). KG-1 and SNU-16 cells were harvested directly from the culturing flasks to 15 mL or 50 mL sterile centrifuge tubes and then the medium was discarded after spinning down the cells. To harvest KMS-11 adherent cells, medium was removed, cell surface was rinsed gently with 10 mL of PBS, and 5 mL cell dissociating buffer (Gibco, 13151-014)

was added and the flasks incubated for up to 10 min in a 37°C 5% CO_2 incubator until cells were detached. Next, 5 mL of PBS was added to the flask and the cells dispersed by pipetting up and down. The cell suspension was then collected in a 15 mL sterile centrifuge tube and spun for 5 to 10 min at 1000 rpm to discard the solution. After the first spin and removing medium or dissociation solution, 10 mL PBS was added to the cell pellet and the pellet was dispersed gently by pipetting up and down several times. The cell suspension was then spun again to remove the PBS. The cell pellet was resuspended in a known small volume of stimulation buffer (for IP assays) or media (for t/p PLCγ1 assays). Cells were dispersed by gentle pipetting until a homogeneous solution is obtained. Cell viability was determined to calculate total viable cells in the solution. Based on the total viable cells and desired density of cells/well/volume, the cell solution was further diluted with the stimulation buffer or media. The cells were then plated into 384-well assay plates or 96-well tissue culture plates (Revvity Inc., 6005181) for the treatments and assays.

Cell treatments and IP1 quantification: To determine the optimal density of cells, the duration, and concentrations of FGF stimulation that can be used for inhibitor testing, various densities of each cell type (0 to 100K cells/well) were plated and cultured for up to 2 hours (10, 30, 60, 90, and 120 min) with or without increasing concentrations (0 to 400 ng/mL) of FGF stimulation. The levels of IP1 were then measured in all three cell lines to select optimal cell densities, the FGF concentrations, and the FGF stimulation times for inhibitor testing. Inhibition of FGF-stimulated IP1 accumulation experiments were carried out using increasing concentrations of FGFR inhibitor, AZD4547.

HTRF IP-One assay: Cell plating, treatments, and quantification of IP1 in cell lysates were done by following the recommended kit protocol. Cell stimulation and/or inhibition were conducted in a volume of 14 µL in a ProxiPlate-384 Plus, white 384-shallow well microplate. IP1 assays were performed by adding the IP1 detection reagents to the same plate. For FGF stimulation, 7 µL of cells prepared in stimulation buffer was dispensed to predesignated wells, 7 µL FGF (2x) in stimulation buffer (to test inhibitor, 4 µL inhibitor (5x) and 3 µL FGF (6.67x)), was added to the wells. After gentle tapping, the assay plate was incubated in 37°C 5% CO_2 incubator for 1 hour. For inhibitor testing, the cells were pre-incubated with inhibitor for 2 hours and then stimulated with FGF for an additional hour. During the last 30 minutes of incubation, an IP1 standard curve was prepared in stimulation buffer and IP1 detection reagents (IP1-d2, and IP1 Tb Cryptate antibody) were prepared in provided lysis and detection buffer. After the cell plate was taken out from the incubator, IP1 standards (14 µL/well) and 14 µL/well stimulation buffer (negative control, n=3, no IP1 d2) were pipetted into the pre-designated wells. Then, 3 µL of IP1 d2 was added to all wells, except the negative control wells that were added only 3 µL of lysis and detection buffer. The plates were tapped gently and 3 µL of IP1 Tb Cryptate antibody was added to all wells. The plate was then spun in a centrifuge (1000 rpm) for 20 seconds, sealed, and incubated in room temperature for 1 hour. The plate was read on an EnVision 2105 Multimode Plate Reader to record signals at 665 nm and 620 nm.

AlphaLISA IP-One assays: AlphaLISA IP-One cell assays were performed following the suspension cell protocol provided in the kit technical data sheet. To perform AlphaLISA IP-One detection assays, an almost identical experimental approach was taken as with the HTRF IP1 detection. The cell densities, the concentrations of FGF for stimulation, the length of stimulation time and inhibition times were all kept the same as those used in IP1 detection using HTRF IP-One kit. However, the plate type and the volume for cell stimulation/inhibition in AlphaLISA IP-One assays were different than that were used in the HTRF IP-One assays. Both cell stimulations and/or inhibition experiments were conducted in a total volume of 30 µL in light gray 384 Alpha Plates in AlphaLISA stimulation buffer. The cells, FGF, and AZD4547 were prepared in 1x simulation buffer. FGF stimulation experiments were conducted by combining 10 µL cells with various densities, 10 µL (1x) stimulation buffer, and 10 µL (3X) FGF with increasing concentrations. The cells were stimulated for 1 hour in 37°C 5% CO_2 incubator. AZD4547 inhibition experiments were performed by adding 10 µL cells (50K/well) and 10 µL AZD4547 (3x) with various concentrations. The plates with cells and inhibitor were incubated for 2 hours and then 10 µL (3x) FGF (100 ng/mL final) was added and incubated for an additional 1 hour. During the last 30 minutes of cell treatment, the AlphaLISA IP1 standard curve was prepared. IP1 standards (20 µL stimulation buffer plus 10 µL IP1 standards) were added to the designated wells of cell treated plates. The AlphaLISA IP1 detections were completed by adding 10 µL (5x) biotinylated IP1 and anti-IP1 acceptor beads mix (90 min incubation at room temperature) and 10 µL (5x) SA-DB (60 min incubation at room temperature). The final concentrations of Biotinylated-IP1, Anti-IP1 AlphaLISA

Acceptor beads, and Streptavidin Donor beads (SA-DB) in 50 µL were 5 nM, 20 µg/mL, and 40 µg/mL, respectively. The assay plates were then read on a Revvity EnVision 2105 multimode plate reader equipped with ALPHA option using the following settings: Total Measurement Time: 550 ms, Laser: 680 nm, Excitation Time: 180 ms, Mirror: 640as (Barcode# 444), Emission filter: Wavelength 570 nm, bandwidth: 100 nm, Transmittance 75%, (Barcode# 244).

HTRF total and Phosphorylated PLCγ1 assay: Cell plating, treatments, and quantification of total and phosphorylated PLCγ1 in cell lysates were completed by following the kit's 2 plate assay protocol. To determine both total and phosphorylated PLCγ1 simultaneously from the same samples, cell treatments were performed in 96-well TC culture plates. The cells were prepared in recommended culture medium for each cell type tested. 100 µL cell solution containing cells were dispensed per well (50K/well), after culturing the cells for 2 hours in a 37°C 5% CO_2 incubator, 20 µL (6x) of increasing concentrations of FGF prepared in the same culture medium were added to the designated wells and incubated for 1 hour to complete the cell stimulation. When inhibitor (AZD4547) was included in cell treatment, 10 µL (11x) inhibitor with increasing concentrations was added and incubated with the cells for 2 hours and then 10 µL (12x) FGF (100 ng/mL final) were added and incubated for an additional hour. At the end of the cell treatments, 40 µL (4x) supplemented lysis buffer was added to lyse the cells by shaking the plates on a plate shaker. After cell treatments and cell lysis preparation, 16 µL of lysate samples were transferred to ProxiPlate-384 Plus, white 384-shallow well microplate to determine relative levels of total or phosphorylated PLCγ1 following the detection procedures described in the kit manual. To 16 µL of lysate samples, 4 µL of premixed detection antibodies were added and mixed by tapping. The plates were then incubated overnight in room temperature according to the manufacturer's instructions and the emission read at two different wavelengths (665 nm and 620 nm) on an EnVision 2105 Multimode plate reader.

Data analysis

Data analysis was performed using Microsoft Excel and GraphPad Prism. For HTRF assays, the ratios of HTRF signals (665 nm/620 nm*10,000) were calculated for standard curves and for cell lysates samples and a standard curve was plotted using HTRF ratio vs IP1 concentration. For AlphaLISA assays, standard curve was plotted with AlphaLISA signals vs IP1 concentration. The standard curves fittings were done by using Prism nonlinear regression fitting, log (inhibitor) vs. response - variable slope. The standard curves were used to quantify the levels of IP1 in cell lysates samples by interpolating the signals from cell lysates samples to the standard curve and to calculate IC_{50} of standard curves. The IC_{50} of inhibitor was obtained by plotting the IP1 concentration in cell lysates vs AZD4547 concentration for HTRF IP-One and AlphaLISA assays and by plotting HTRF ratio vs AZD4547 concentration for phosphorylated PLCγ1 assays.

Results and discussion

HTRF IP-One kit detects IP1 accumulation in three cancer cell lines stimulated with FGF: The results of 60 min FGF stimulation time and IP1 detection in three cell lines with different densities are shown in Figure 6A (SNU-16 cells), Figure 6B (KG-1 cells), and Figure 6C (KMS-11 cells), respectively. A stimulation time of 60 min was determined

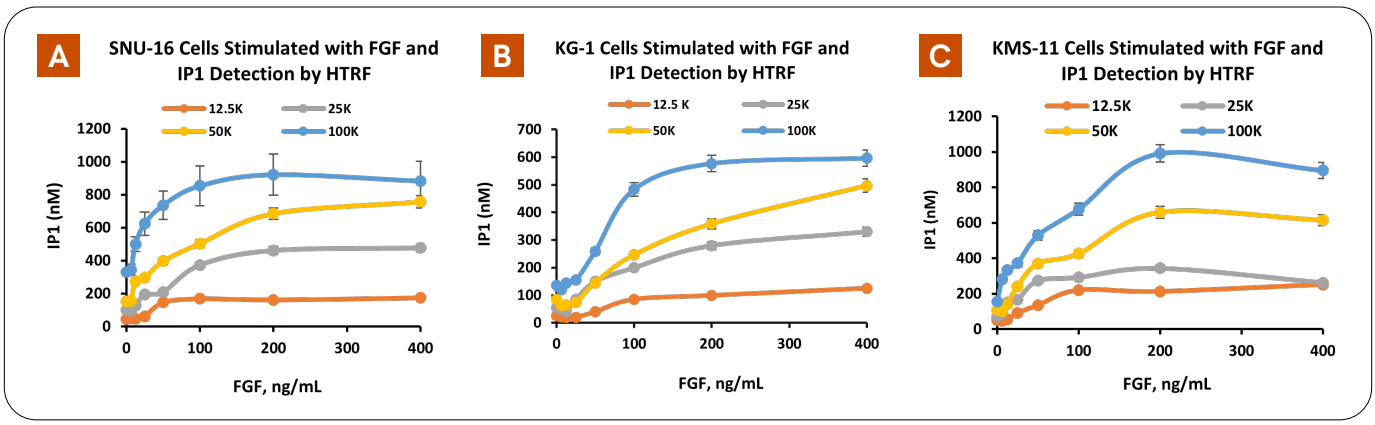


Figure 6: Detection of IP1 in FGF-stimulated SNU-16 cells (A), KG-1 cells (B), and KMS-11 cells (C). IP1 levels in cell lysates were measured by HTRF IP-One detection kit.

as the optimal one in preliminary experiments. As expected, FGF stimulated IP1 production in three cancer cell lines in a dose dependent manner. A 2- to 5-fold increase in IP1 production was observed when optimal cell seeding conditions were used (25 to 50 K cells/well) stimulated with ≥ 50 ng/mL FGF. The KG-1 cells required ≥ 100 ng/mL FGF.

Inhibition of FGF-stimulated IP1 production and measuring the IP1 accumulation by HTRF kit: Upon selection of the concentration of FGF (100 ng/mL), the FGF stimulation time (60 min), and cell density (50K/well) from FGF stimulation experiments, a pan FGFR1/2/3 inhibitor (AZD4547) was tested to determine if FGF-stimulated IP1 production can be inhibited in three cancer lines. AZD4547 clearly inhibited FGF-stimulated IP1 production in all three cell lines (Figures 7A, 7B, and 7C) with IC_{50} of 6.2 nM, 2.0 nM, and 4.7 nM in SNU-16, KG-1, and KMS-11 cells, respectively. Since SNU-16, KG-1, and KMS-11 cells express the different FGFR subtypes, these results confirmed that AZD4547 is a potent pan FGFR inhibitor and suggested that the HTRF IP-One kit can effectively characterize antagonists targeting any of the three FGFR subtypes.

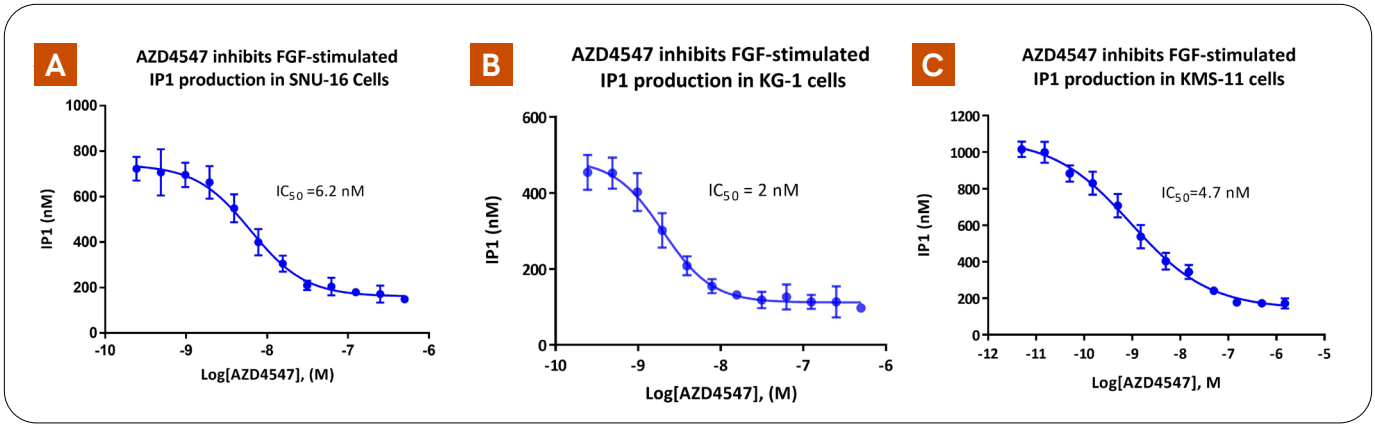


Figure 7: Inhibition of IP1 accumulation by AZD4547 in FGF-stimulated SNU-16 cells (A), KG-1 cells (B), and KMS-11 cells (C). IP1 levels in cell lysates were measured by HTRF IP-One detection kit.

AlphaLISA IP-One kit detects IP1 accumulation in three cancer lines stimulated with FGF: To determine if the AlphaLISA IP-One detection kit can deliver similar results as the one obtained with the HTRF kit, the cell densities, the concentrations of FGF for stimulation, and the length of FGF stimulation (60 min) are all kept the same as those used with the HTRF IP-One kit. The results of IP1 detection using AlphaLISA IP-One kit in three FGF-stimulated cancer cell lines are shown in Figures 8A, 8B, and 8C for SNU-16, KG-1, and KMS-11 cells, respectively. As expected, the AlphaLISA IP-One kit was able to detect FGF-stimulated IP1 accumulation in all three cell lines in a dose dependent manner. Moreover, the levels of IP1 measured using AlphaLISA IP-One kit in three cancer lines are similar to those obtained using HTRF IP-One kit, as well as the fold-increase determined upon FGFR stimulation.

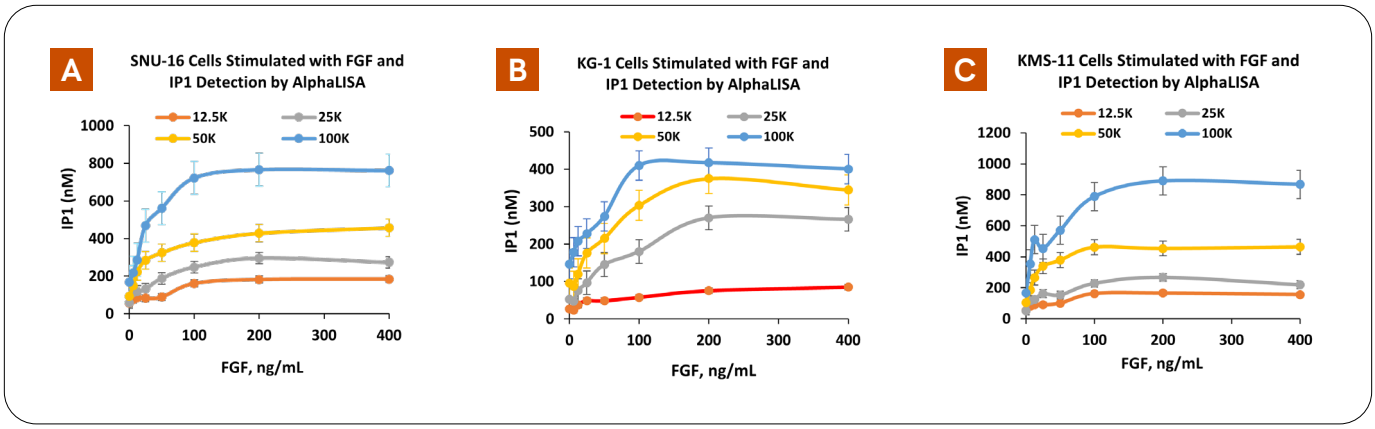


Figure 8: Quantification of IP1 accumulation in FGF-stimulated SNU-16 cells (A), KG-1 cells (B), and KMS-11 cells (C). IP1 levels in cell lysates were measured by AlphaLISA IP-One detection kit.

Inhibition of FGF-stimulated IP1 production and detecting IP1 by AlphaLISA IP-One Kit: To confirm that the AlphaLISA IP-One kit could allow the characterization of FGFR antagonists as effectively as the HTRF kit, the inhibition of FGF-stimulated IP-production by AZD4547 was carried out in the previously used cancer cell lines, KG-1, SNU-16, and KMS-11, using the same biological conditions as the ones used to assess the HTRF IP-One kit (cell density of 50K cells/well using a stimulation time of 60 min with 100 ng/ml of FGF). As expected, Figures 9A, 9B, and 9C show that AZD4547 inhibited the FGF-stimulated IP1 accumulation in a dose dependent manner. The IC_{50} for AZD4547 in SNU-16, KG-1, and KMS-11 cells were 6.6 nM, 5.3 nM, and 5.9 nM, respectively. These values are similar to the those obtained with the HTRF IP-One kit suggesting the two kits are equally powerful to characterize antagonists targeting any of the three FGFR subtypes.

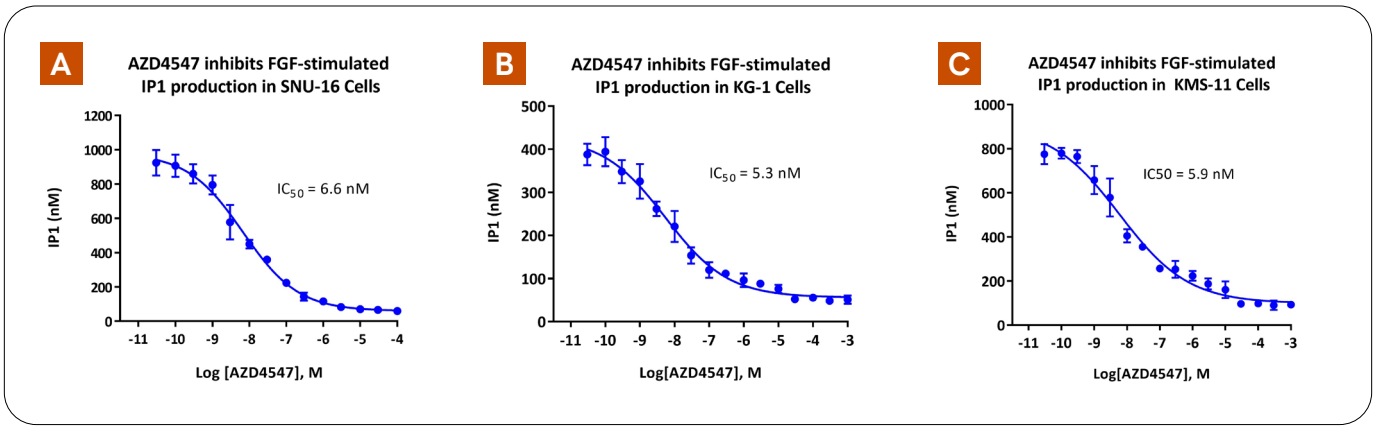


Figure 9: Inhibition of IP1 accumulation by AZD4547 in FGF-stimulated SNU-16 cells (A), KG-1 cells (B), and KMS-11 cells (C). IP1 levels in cell lysates were measured by AlphaLISA IP-One detection kit.

The use of HTRF total and phosphorylated PLCγ1 4 assays to confirm the results obtained with the IP-One kits:
To further confirm that the HTRF and AlphaLISA IP-One assays are suitable readouts to characterize compounds modulating the FGFR signaling, two HTRF kits allowing the simple, rapid, and direct detection of endogenous levels of total and phosphorylated (Tyr783) PLCγ1 were used on the same cell models. As indicated in the introduction, PLCγ1 is an important upstream molecule in the FGFR signaling pathway. The assessment of its phosphorylation status is also considered as a standard way to investigate compounds modulating FGFR signaling. Figure 10B indicated that FGF activated the FGFRs and promoted the PLCγ1 phosphorylation in a concentration dependent manner in three cancer cell lines. As expected, the relative levels of total PLCγ1 did not change with FGF stimulation (Figure 10A).

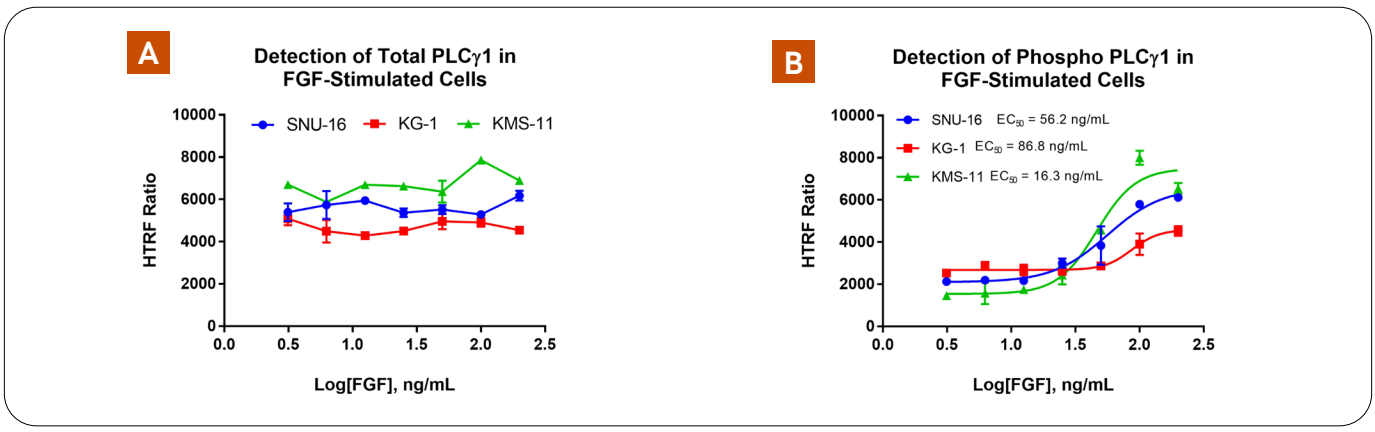


Figure 10: FGF stimulation of total (A) and phosphorylated (B) PLCγ1 in SNU-16 cells, KG-1 cells, and KMS-11 cells. The total and phosphorylated PLCγ1 detected by HTRF Total PLCγ1 and HTRF Phospho-PLCγ1 (Tyr783) test kits.

The results of testing the FGFR inhibitor, AZD4547, are shown in Figures 11A, 11B, and 11C for SNU-16, KG-1, and KMS-11 cancer cells, respectively. In all three cell lines, AZD4547 inhibited or blocked FGF-stimulated PLCγ1 phosphorylation with the IC_{50} of 5.6 nM, 7.1 nM, and 3 nM in SNU-16, KG-1, and KMS-11 cells, respectively.

Again, AZD4547 didn't change the levels of total PLCγ1. The results also showed that HTRF phospho-PLCγ1 (Tyr783) (64PLCG1Y3PEG) and HTRF total PLCγ1 (64PLCG1TPEG) detection kits are excellent assay kits to validate the activation status of PLCγ1 in FGFR signaling pathways.

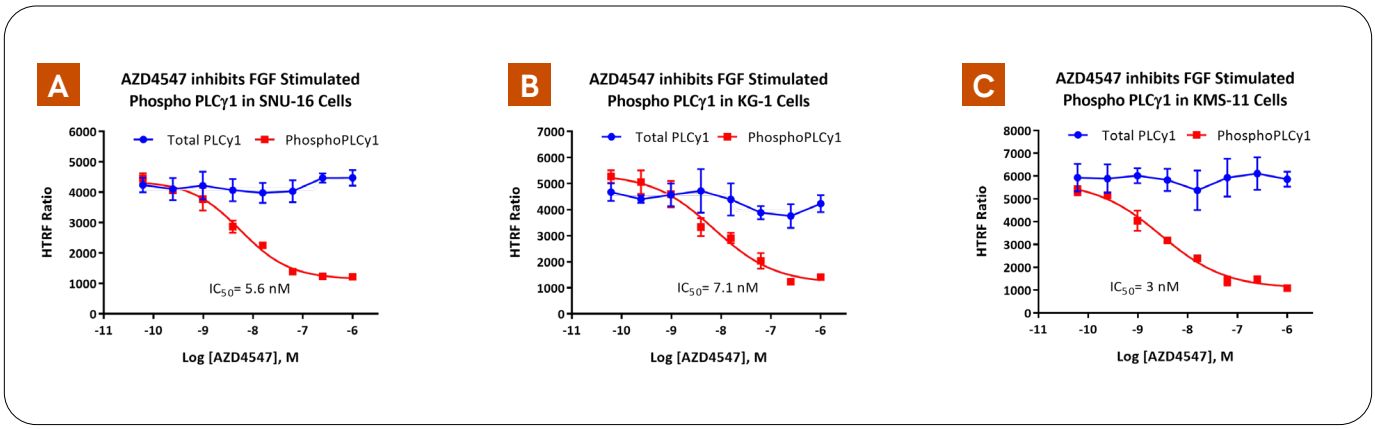


Figure 11: Inhibition of phosphorylated PLCγ1 by AZD4547 in FGF-stimulated SNU-16 cells (A), KG-1 cells (B), and KMS-11 cells (C). The total and phosphorylated PLCγ1 were detected by HTRF Total PLCγ1 and HTRF Phospho-PLCγ1 (Tyr783) test kits.

Table 1 shows that the IC_{50} values obtained using the HTRF IP-One kit, the AlphaLISA IP-One kit or the HTRF Phospho-PLCγ1 assay kit are very similar. It confirms that the IP-One kits are relevant readouts to identify and characterize compounds modulating the activity of any of the three FGFR subtypes 1, 2, and 3 respectively.

Table 1: IC_{50} values obtained using HTRF IP-One, AlphaLISA IP-One, and HTRF Phospho-PLCγ1 assay kits during FGFR inhibitor (AZD4547) testing in three FGF-stimulated cancer cell lines.

AZD4547, IC_{50} (nM)	HTRF IP-One Kit	AlphaLISA IP-One kit	Phosphor PLCγ1 kit
SNU-16 Cells	6.2	6.6	5.6
KG-1 Cells	2.0	5.3	7.1
KMS-11 Cells	4.7	5.9	3.0

Summary and conclusion

For the first time, three relevant cancer cell lines, KG-1, SNU-16, and KMS-11 expressing FGFR1, FGFR2, and FGFR3 receptor subtypes, respectively, have been used to demonstrate that both the HTRF and AlphaLISA IP-One kits are powerful readouts to identify and characterize compounds modulating the FGFR signaling pathway.

Results obtained upon stimulation of the cells by FGF or after treating the cells with an FGFR inhibitor, AZD4547, were similar to those obtained from more conventional HTRF phosphorylation assays. It suggests that the IP-One kits can be used to investigate any of the three FGF receptor subtypes. As already highlighted for decades in the G-Protein coupled receptors research field, the no-wash protocol of these assays made them highly suitable for miniaturization (down to 1536 wells) and automation without compromising the ability to deliver accurate pharmacology. The present study suggests that such features can now be applied to FGF receptors which are key targets in cancer drug research.

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