

Proteome ProfilerTM 96

Human Phospho-RTK Array 1

Catalog Number ARZ001

For the parallel determination of the relative levels of tyrosine phosphorylation of human receptor tyrosine kinases (RTKs).

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Receptor tyrosine kinases (RTKs) are a family of widely expressed transmembrane proteins with an extracellular ligand binding domain and an intracellular tyrosine kinase domain. This family includes receptors for growth factors (EGF, FGF, HGF, IGF, NGF, PDGF, VEGF), neurotrophic factors, insulin, and other extracellular signaling molecules. Upon ligand binding, the cytoplasmic domains of RTKs are autophosphorylated on multiple tyrosine residues either as a result of receptor dimerization or due to allosteric interactions between the two halves of the same receptor. These phosphorylated tyrosine residues on RTKs serve as high affinity docking sites for intracellular proteins that promote downstream signal transduction cascades. The signaling pathways initiated by RTK activation are required for normal developmental processes including proliferation, differentiation, and motility. Mutations in RTKs can cause constitutive activation of downstream signaling pathways, which have been implicated in the pathogenesis of different forms of cancer [Christensen, J. et al. (2005) *Cancer Letters* **225**(1):1]. Due to the physiological and pathological importance of RTK activation, analysis and quantification of RTK phosphorylation has become increasingly important. Assays that allow several RTKs to be monitored simultaneously simplify the screening processes required to identify pathways involved in establishing specific cellular phenotypes. Having this capability allows proteins of interest to be rapidly identified and targeted for further study.

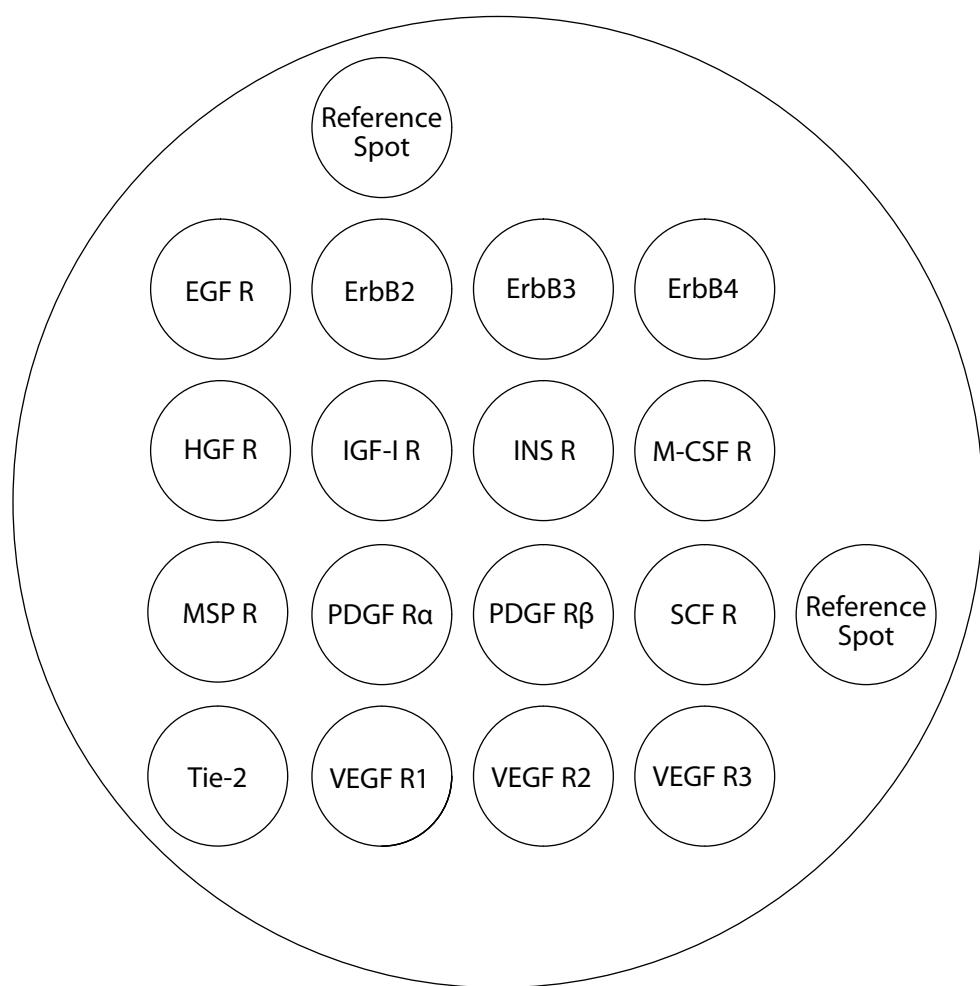


Figure 1: A visualization of the spot layout per well.

PRINCIPLE OF THE ASSAY

The Proteome Profiler™ 96 Human Phospho-RTK Array 1 Kit employs a two-site sandwich ELISA technique to simultaneously detect 16 phosphorylated receptor tyrosine kinases (RTKs) in cell lysates. Multiple capture antibodies that specifically recognize the target RTKs detected by the assay have been pre-spotted into each well of a microplate. Controls and experimental samples are added and both unphosphorylated and phosphorylated forms of the target RTKs present in the samples are bound by the immobilized antibodies. After washing away unbound material, a horseradish peroxidase (HRP)-conjugated anti-phospho-tyrosine antibody is used to detect phosphorylated tyrosines on activated receptors. Following a second wash, chemiluminescent substrate reagents are added to the wells and signals proportional to the relative amounts of phosphorylated RTKs bound in the initial step are produced. Plates are read using a digital imaging system, and pixel densities are measured using an analytical software package.

TECHNICAL HINTS AND LIMITATIONS

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- This kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- Any variation in buffers, operator, pipetting technique, washing technique, instrumentation, incubation time or temperature, or kit age can alter the performance of the kit.
- This assay is designed to eliminate interference by proteins present in biological samples. Until all factors have been tested in the Proteome Profiler 96 assay, the possibility of interference cannot be excluded.
- Avoid microbial contamination of reagents and buffers.
- When mixing sample solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.

PRECAUTION

Some components in this kit contain ProClin® which may cause an allergic skin reaction. Avoid breathing mist.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please refer to the MSDS on our website prior to use.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	AMOUNT PROVIDED	STORAGE OF OPENED/RECONSTITUTED MATERIAL
Human Phospho-RTK Array 1	893600	One 96-well microplate	Invert the plate, and blot it against clean paper towels to dry the plate. Return it to the foil pouch containing the desiccant pack, and reseal along entire edge of zip-seal. May be stored for up to 3 months at 2-8 °C.*
Anti-Phospho-Tyrosine-HRP	841403	1 vial (20 µL)	May be stored for up to 3 months at 2-8 °C.* Do Not Freeze.
Array Buffer 2 Concentrate (5X)	895478	1 vial (21 mL)	May be stored for up to 3 months at 2-8 °C.* Discard after dilution. Prepare fresh for each use.
Array Buffer 1	895477	1 vial (21 mL)	
Lysis Buffer 17	895943	1 vial (21 mL)	
Substrate 1	895471	1 vial (3 mL)	May be stored for up to 3 months at 2-8 °C.*
Substrate 2	895472	1 vial (3 mL)	
Wash Buffer Concentrate (25X)	895003	2 vials (21 mL each)	
Plate Sealers	640197	4 adhesive strips	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Aprotinin (Sigma, Catalog # A6279)
- Leupeptin (Tocris, Catalog # 1167)
- Pepstatin (Tocris, Catalog # 1190)
- Pipettes and pipette tips
- Deionized or distilled water
- Squirt bottle, manifold dispenser, or automated dispensing unit for plate washing
- 50 mL and 500 mL graduated cylinders
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm
- Microcentrifuge
- Digital Imaging System (for details, visit www.RnDSystems.com/go/ImagingSystems)

LYSATE PREPARATION AND DILUTION

Since the Human Phospho-RTK Array 1 Kit detects relative phosphorylation levels of individual analytes, it is important to include appropriate controls (including unstimulated cells and wells with buffer only).

Cell Lysates - Rinse cells with PBS, making sure to remove any remaining PBS before adding lysis buffer. Solubilize the cells at 1×10^7 cells/mL in Lysis Buffer 17 prepared with protease inhibitors. Pipette up and down to resuspend and rock the lysates gently at 2-8 °C for 30 minutes. Microcentrifuge at 14,000 rpm for 5 minutes, and transfer the supernate into a clean test tube. It is recommended that sample protein concentrations be determined using a total protein assay. Use 5-50 µg of lysate diluted in Array Buffer 1. Do not allow the lysate to exceed 50% of the final volume. The amount of lysate should be optimized for each particular cell type. Representative data are shown for some cell lines. Lysates should be used immediately or aliquoted and stored at $\leq -70^{\circ}\text{C}$. Thawed lysates should be kept on ice until immediately prior to use.

Lysates Directly from 96-well Microplates - Cells can be seeded and grown directly in uncoated 96-well microplates. Results may be affected by confluence of cells. Treat cells as desired. Rinse the cells with PBS, making sure to remove any remaining PBS before adding lysis buffer. Add 50 µL of Lysis Buffer 17 prepared with protease inhibitors per well. Cover the microplate with a plate sealer and incubate on an orbital shaker for 60 minutes at 2-8 °C. Add 100-200 µL of Array Buffer 1. Mix and transfer 50-100 µL to one well of the coated microplate. Not all cell lines will provide enough lysate for this protocol. This protocol needs to be tested for each cell type.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

Note: Wash Buffer Concentrate may turn yellow over time.

Substrate Solution - Substrates 1 and 2 should be mixed together in equal volumes 2-30 minutes prior to use. **Protect from light.** 50 µL of the resultant mixture is required per well. Prepare only as much Substrate Solution as needed to run each experiment.

Lysis Buffer 17 - Add 10 µg/mL Aprotinin, 10 µg/mL Leupeptin, and 10 µg/mL Pepstatin to the volume of Lysis Buffer 17 required for cell lysate preparation. **Prepare fresh for each use.**

1X Array Buffer 2 - Add 2 mL of Array Buffer 2 Concentrate into 8 mL of deionized or distilled water. Prepare fresh for each use.

Anti-Phospho-Tyrosine-HRP - Immediately before each use, dilute the Anti-Phospho-Tyrosine-HRP to the working concentration specified on the vial label using 1X Array Buffer 2. **Protect from light.** Prepare only as much Anti-Phospho-Tyrosine-HRP as needed to run each experiment. 50 µL of the diluted solution is required per well.

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples be assayed in duplicate.

Note: Protect the diluted Anti-Phospho-Tyrosine-HRP and the Substrate Solution from light at all times.

1. Prepare all reagents, controls, and lysates as directed in the previous sections.
2. Add 50-100 μ L of lysate* or control per well. Securely cover with a plate sealer. Incubate overnight at 2-8 °C on a horizontal orbital microplate shaker (0.12" orbit) set at 500 \pm 50 rpm. If optimal sensitivity is not required, incubate for 2 hours at room temperature on the shaker.
3. Aspirate each well and wash, repeating the process **three times for a total of four washes**. Wash by filling each well with Wash Buffer (400 μ L) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
4. Add 50 μ L of diluted Anti-Phospho-Tyrosine-HRP to all wells. Securely cover with a plate sealer and incubate for 1 hour at room temperature on the shaker set at 500 \pm 50 rpm.
5. Aspirate each well and wash, repeating the process **five times for a total of six washes**. Wash by filling each well with Wash Buffer (400 μ L) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 50 μ L of Substrate Solution to each well.
7. Allow the Substrate Solution to incubate for 30 seconds prior to imaging the microplate.
8. Place the microplate in the imager. Wait no longer than 15 minutes to commence imaging.

*Lysates require dilution. See the Lysate Preparation and Dilution section.

INSTRUMENTATION

The Proteome Profiler 96 Kits have been validated on the Q-View™ Imager from Quansys Biosciences. Please visit www.RnDSystems.com/go/ImagingSystems for suitable imaging systems and their instructions for use.

DATA ANALYSIS

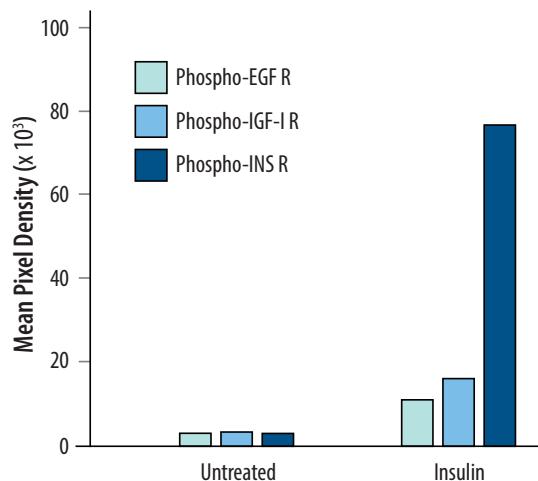
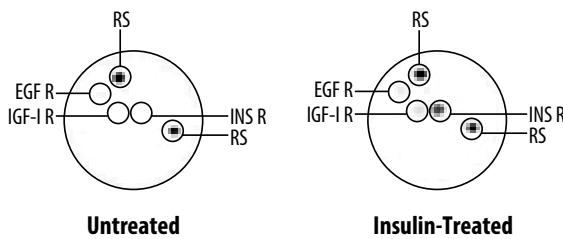
Create a template to analyze the median pixel density (PD) of each spot in the well. Alternatively, if using the Q-View software, access the appropriate template by selecting RHC052209 in the Product Number box. Export PD signal values to a spreadsheet file such as Microsoft® Excel for manipulation. Determine the average PD between duplicate wells representing each RTK. Subtract the averaged background signal from the corresponding RTK spot in the negative control wells. Using this method, relative changes in tyrosine phosphorylation are comparable between different samples and plates.

PROFILING RTK TYROSINE PHOSPHORYLATION

The Proteome Profiler 96 Human Phospho-RTK Array 1 detects multiple receptors in untreated and ligand-treated cell lysates.

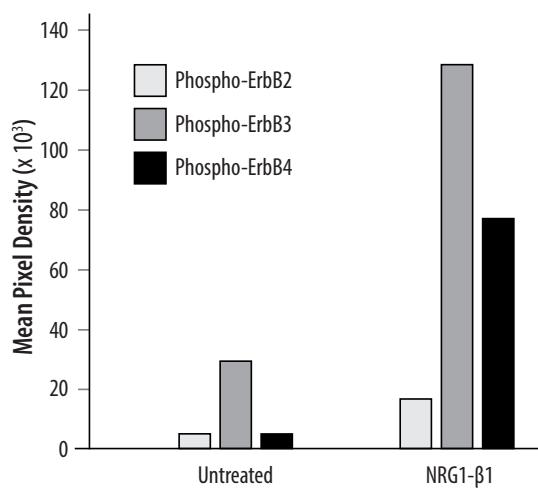
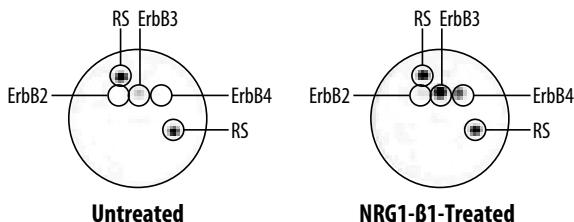
Lysates were tested using the Proteome Profiler 96 Human Phospho-RTK Array 1 Kit. Array signals were analyzed using Q-View image analysis software. Inverted images of the wells are shown (left) with mean pixel densities in vertical bar graphs (right) (RS=Reference Spot).

2A



2A. The HepG2 human hepatocellular carcinoma cell line was untreated or treated with 1 mg/mL of insulin for 5 minutes. 25 μ g of lysate was loaded per well.

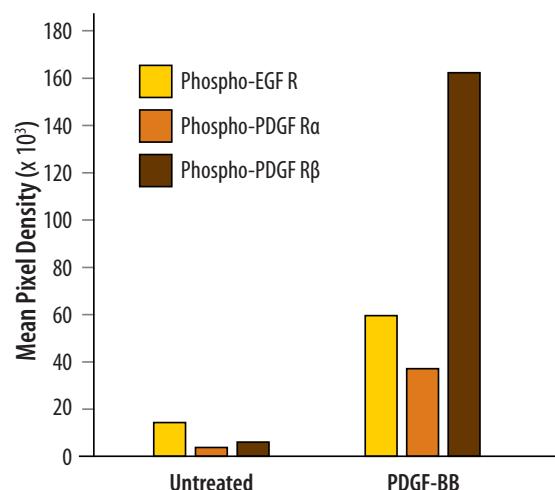
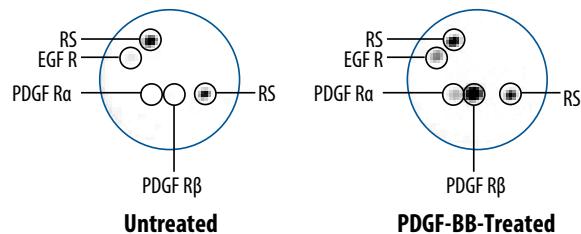
2B



2B. The MDA-MB-453 human breast cancer cell line was untreated or treated with 100 ng/mL of recombinant human NRG1- β 1/HRG1- β 1 (R&D Systems, Catalog # 396-HB) for 5 minutes. 5 μ g of lysate was loaded per well.

PROFILING RTK TYROSINE PHOSPHORYLATION *CONTINUED*

2C



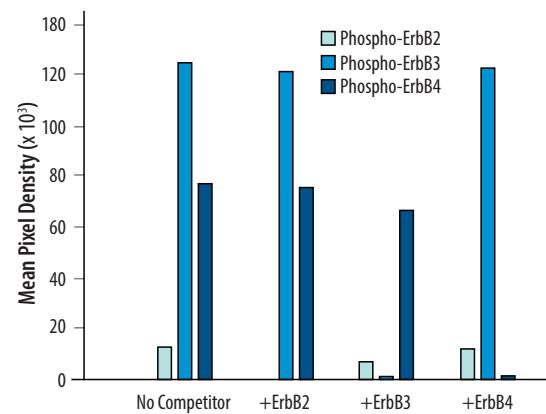
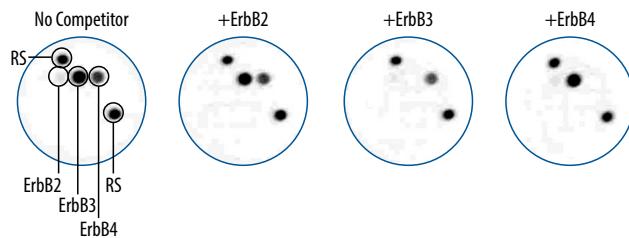
2C. The CCD-1070Sk human foreskin fibroblast cell line was treated with 100 ng/mL of recombinant human PDGF-BB (R&D Systems, Catalog # 220-BB) for 5 minutes. 25 μ g of lysate was loaded per well.

SPECIFICITY

The Proteome Profiler 96 Human Phospho-RTK Array 1 Kit is specific as shown by receptor competition.

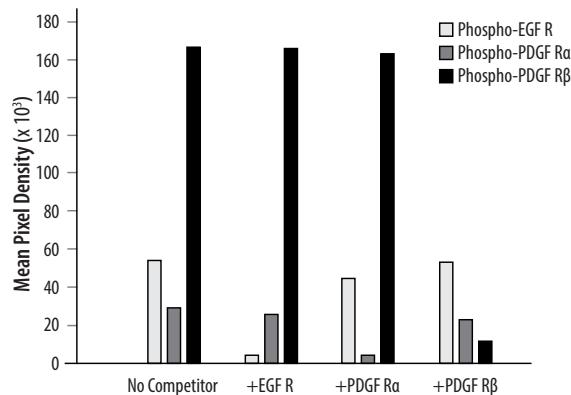
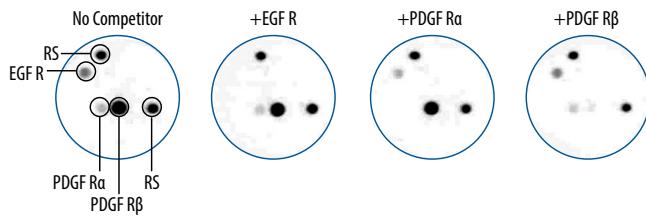
Lysates from ligand-treated cells were mixed with recombinant human RTKs and run in the Proteome Profiler 96 Human Phospho-RTK Array 1 Kit. Inverted images of the wells are shown (left) with mean pixel densities in vertical bar graphs (right). Competition of a particular RTK was observed only with the corresponding recombinant soluble receptor (RS=Reference Spot)

3A NRG1- β 1-Treated



3A. MDA-MB-453 human breast cancer cell were treated with 100 ng/mL of recombinant human NRG1- β 1/HRG1- β 1 for 5 minutes. 5 μ g of recombinant human ErbB2 (R&D Systems, Catalog # 1129-ER), recombinant human ErbB3 (R&D Systems, Catalog # 348-RB), and recombinant human ErbB4 (R&D Systems, Catalog # 1131-ER) extracellular domains were added to 5 μ g of lysate and analyzed.

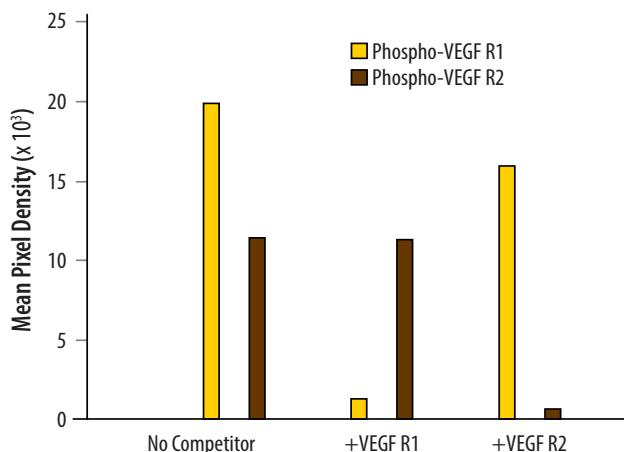
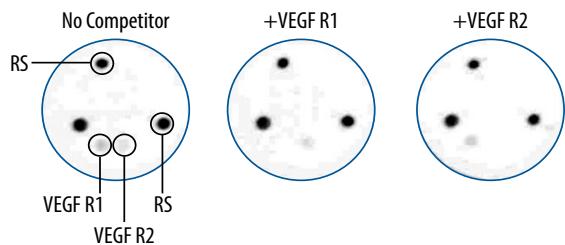
3B PDGF-BB-Treated



3B. CCD-1070Sk human foreskin fibroblast cells were treated with 100 ng/mL of recombinant human PDGF-BB for 5 minutes. 5 μ g of recombinant human EGF R (R&D Systems, Catalog # 344-ER), recombinant human PDGF Ra (R&D Systems, Catalog # 322-PR), and recombinant human PDGF R β (R&D Systems, Catalog # 385-PR) extracellular domains were added to 25 μ g of lysate and analyzed.

SPECIFICITY CONTINUED

3C VEGF₁₆₅-Treated



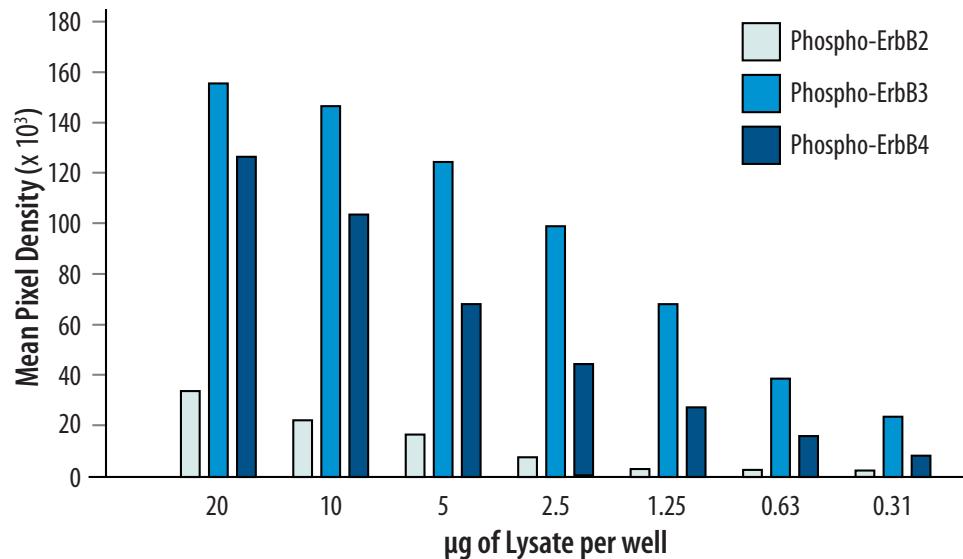
3C. CHO Chinese hamster ovary cells transfected with human VEGF R1/R2 were treated with 100 ng/mL of recombinant human VEGF₁₆₅ for 5 minutes. 5 μ g of recombinant human VEGF R1 (R&D Systems, Catalog # 321-FL) and recombinant human VEGF R2 (R&D Systems, Catalog # 357-KD) extracellular domains were added to 25 μ g of lysate and analyzed.

SENSITIVITY

The Proteome Profiler 96 Human Phospho-RTK Array 1 Kit is semi-quantitative and sensitive.

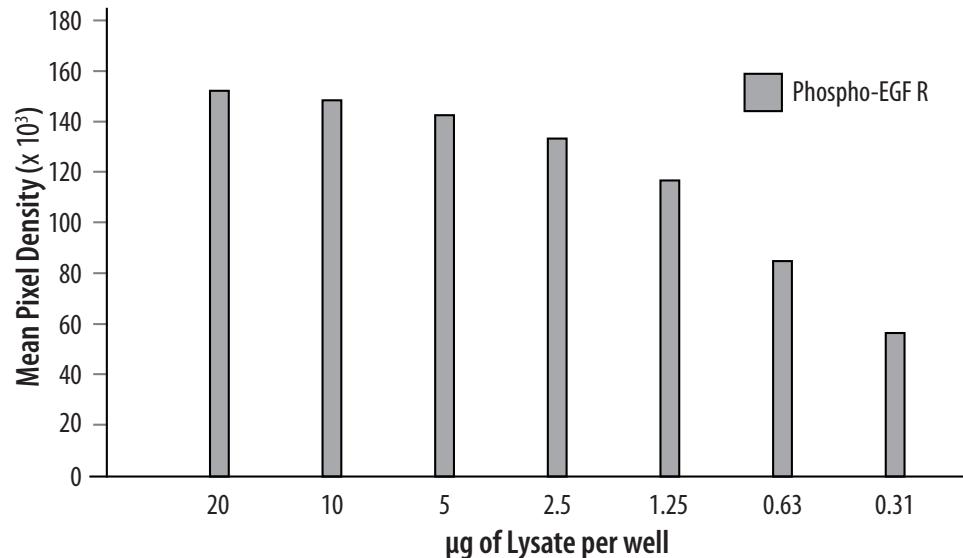
Serial dilutions of lysates were tested using the Proteome Profiler 96 Human Phospho-RTK Array 1 Kit. Array signals were analyzed using Q-View image analysis software.

4A



4A. The MDA-MB-453 human breast cancer cell line was treated with 100 ng/mL of recombinant human NRG1- β 1/HRG1- β 1 (R&D Systems, Catalog # 396-HB) for 5 minutes.

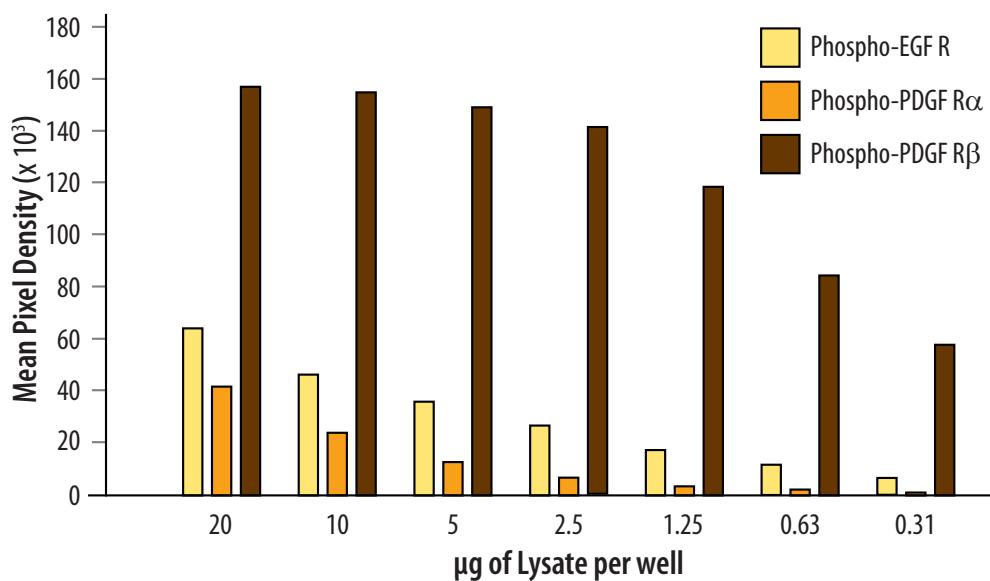
4B



4B. The A431 human epithelial carcinoma cell line was treated with 100 ng/mL of recombinant human EGF (R&D Systems, Catalog # 236-EG) for 5 minutes.

SENSITIVITY CONTINUED

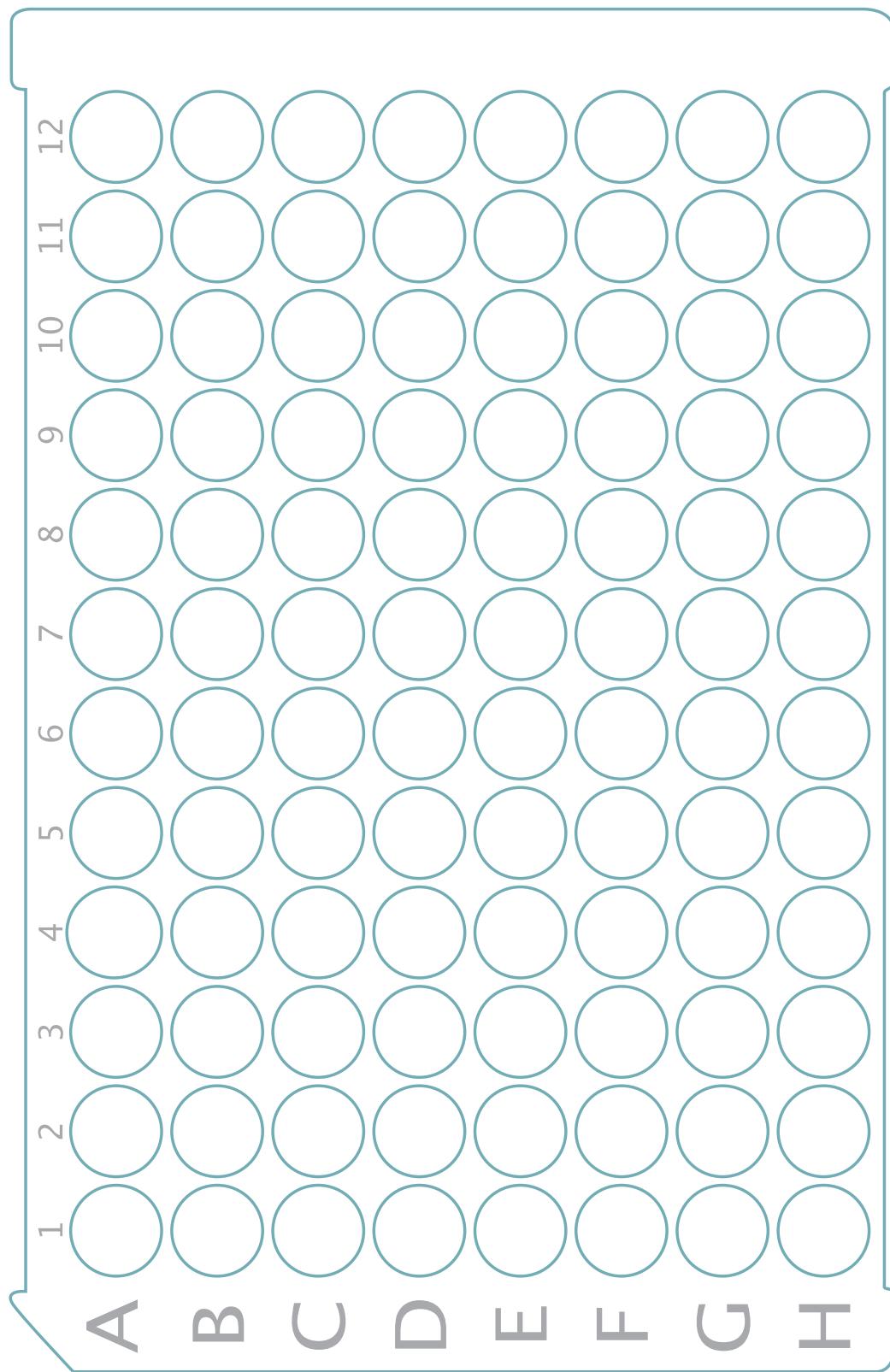
4C



4C. The CCD-1070Sk human foreskin fibroblast cell line was treated with 100 ng/mL of recombinant human PDGF-BB (R&D Systems, Catalog # 220-BB) for 5 minutes.

PLATE LAYOUT

Use this plate layout to record samples assayed.



NOTES

NOTES

All trademarks and registered trademarks are the property of their respective owners.

Substrates 1 and 2 are comprised of TMA-6, a product of Lumigen, Inc., Southfield, Michigan, USA, and are covered by the following:

US Patent Numbers: 5,922,558 and 6,858,733

International Patent Numbers: 733,086, 1,019,525, 2,300,071, 1,015,461, 2,002,352,881, ZL02805225.0, and 1,456,716