Proteome Profiler™ *96*

Human Phospho-RTK Array 2 Infrared

Catalog Number ARZ002NIR

For the parallel determination of the relative levels of tyrosine phosphorylation of human receptor tyrosine kinases (RTKs) in cell lysates. Detection uses the LI-COR® Odyssey® or other near-infrared (NIR) compatible imager.

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, 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.

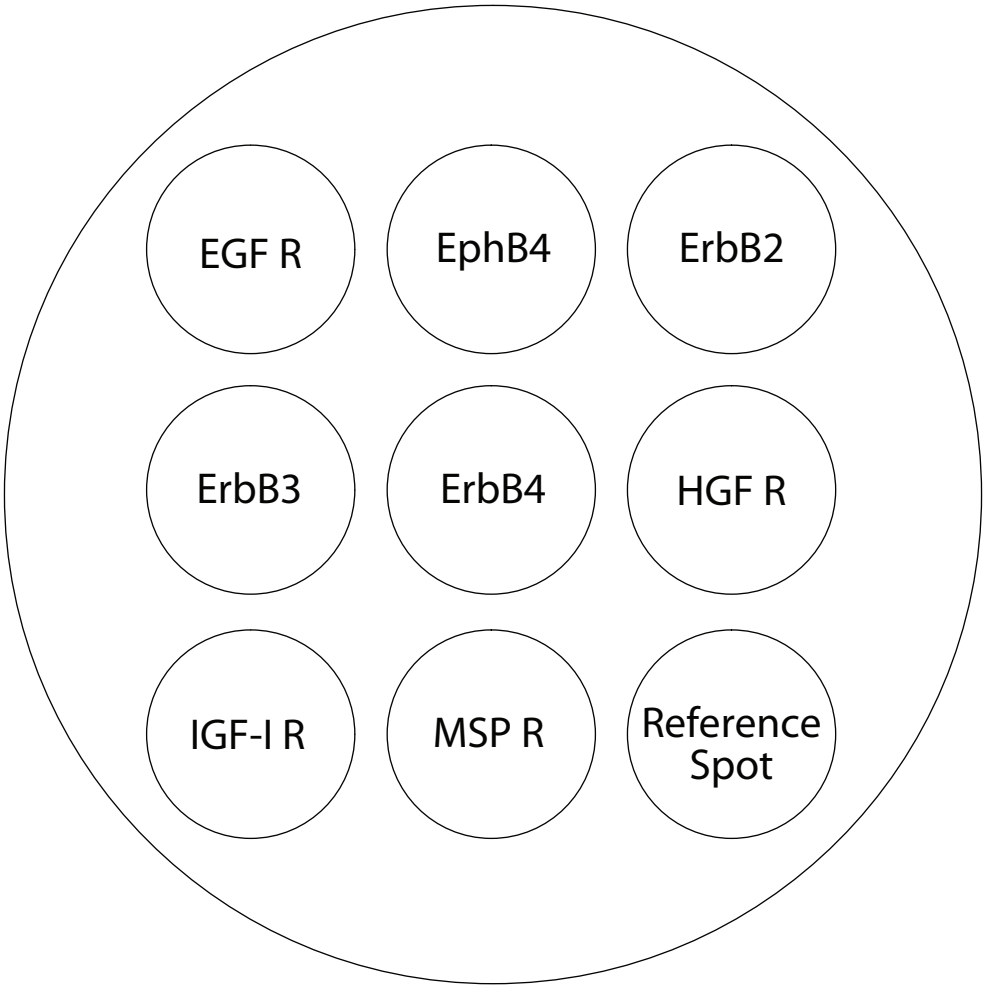

Figure 1: A visualization of the spot layout per well.

PRINCIPLE OF THE ASSAY

The Proteome Profiler™ *96* Human Phospho-RTK Array 2 Infrared Kit employs a two-site sandwich ELISA technique to simultaneously detect 8 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, a solution containing biotinyl-tyramide and hydrogen peroxide is applied to the microplate. Immobilized HRP and hydrogen peroxide catalyze the covalent attachment of biotin moieties to localized proteins and antibodies on the surface of the well. Excess reagents are washed away and IRDye® 800CW Streptavidin is used to produce signals at each capture spot corresponding to the relative amount of phosphorylated RTK bound in the initial step. Plates are read on a LI-COR Odyssey or other compatible near-infrared (NIR) imager.

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.

PRECAUTIONS

The Biotinyl-Tyramide Solution contains Ethanol which is flammable. Keep away from ignition sources. Take precautionary measures against static discharge. No smoking.

The Amplification Diluent Concentrate contains Boric Acid which is suspected of damaging fertility or the unborn child. Do not handle until all safety precautions in the MSDS have been read and understood. Wear protective gloves, clothing, eye, and face protection when using these reagents.

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 2 Infrared Microplate	894209	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 (50 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)	May be stored for up to 3 months at 2-8 °C.*
Lysis Buffer 17	895943	1 vial (21 mL)	
Biotinyl-Tyramide Solution†	894160	1 vial (150 µL)	
Amplification Diluent Concentrate (2X)	894161	1 vial (7 mL)	
IRDye® 800CW Streptavidin††	894163	1 vial (30 µL)	
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 (Sigma, Catalog # L8511)
- Pepstatin (Sigma, Catalog # P4265)
- Pipettes and pipette tips
- Deionized or distilled water
- PBS
- Squirt bottle, manifold dispenser, or automated microplate washer
- 50 mL and 1000 mL graduated cylinders
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm
- Microcentrifuge

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This product is distributed and sold to the End-User for life science research and commercial applications, but not for diagnostic use. End-User does not have a right to resell or transfer the TSA™ reagent component(s) of this product either alone or as components of another product. Any use of this product other than for life science research and commercial applications is strictly prohibited.

††*This product is covered by one or more of the following US Patent numbers: US 6,995,274, US 7,504,089, PCT - WO0224815A1, and US Application 61/184,750 pending.*

LYSATE PREPARATION AND DILUTION

Since the Human Phospho-RTK Array 2 Infrared 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 x 10^7 cells/mL in Lysis Buffer 17. Pipette up and down to resuspend and rock the lysates gently at 2-8 °C for 30 minutes. Microcentrifuge at 14,000 x g 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. Lysates should be used immediately or aliquoted and stored at ≤ -70 °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 confluency 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 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 40 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 1000 mL of Wash Buffer.

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

1X Array Buffer 2 - Add 5 mL of Array Buffer 2 Concentrate (5X) to 20 mL of deionized or distilled water. **Prepare fresh for each use.**

Anti-Phospho-Tyrosine-HRP Solution - 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 Solution as needed to run each experiment. 50 µL of the diluted solution is required per well.

1X Amplification Diluent - Add equal amounts of Amplification Diluent Concentrate (2X) and deionized or distilled water. 100 µL of the resultant mixture is required per well.

Diluted Biotinyl-Tyramide Solution - Immediately before each use, dilute the Biotinyl-Tyramide Solution 1:100 into 1X Amplification Diluent. 100 µL of the resultant mixture is required per well.

IRDye 800CW Streptavidin Solution - Immediately before use, dilute the IRDye 800CW Streptavidin to the working concentration specified on the label using 1X Array Buffer 2.

ASSAY PROCEDURE

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

Note: *Protect the Anti-Phospho-Tyrosine-HRP Solution and the IRDye 800CW Streptavidin from light at all times.*

1. Prepare all reagents and lysates as directed in the previous sections.
2. Add 50-100 µL of lysate* per well. Securely cover with a plate sealer. Incubate overnight at 2-8 °C (or 2 hours at room temperature) on a horizontal orbital microplate shaker (0.12″ orbit) set at 500 ± 50 rpm.
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 µL) 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 100 µL of diluted Anti-Phospho-Tyrosine-HRP Solution to all wells. Securely cover with a plate sealer and incubate for 1 hour at room temperature on the shaker set at 500 ± 50 rpm.
5. Repeat the aspiration/wash as in step 3.
6. Add 100 µL of diluted Biotinyl-Tyramide Solution to all wells. Securely cover with a plate sealer and incubate for 15 minutes at room temperature on the shaker set at 500 ± 50 rpm.
7. Repeat the aspiration/wash as in step 3 for a total of six washes.
8. Add 100 µL of diluted IRDye 800CW Streptavidin Solution to all wells. Securely cover with a plate sealer and incubate for 30 minutes at room temperature on the shaker set at 500 ± 50 rpm.
9. Repeat the aspiration/wash as in step 3 for a total of six washes.
10. Rinse each well with **deionized water** (400 µL) using a squirt bottle, manifold dispenser or autowasher. Remove any remaining water by aspirating or decanting. Invert the plate and blot it against clean paper towels.
11. Allow to completely air dry.
12. Collect image on a near-infrared imager.

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

INSTRUMENTATION

The Human Phospho-RTK Array 2 Infrared kit has been validated on the LI-COR Odyssey Imager. The Carestream™ Image Station 4000MMPRO or other near-infrared compatible imagers may be used with end user optimization.

DATA ANALYSIS

Positive signals can be converted to integrated intensities using the LI-COR Odyssey software. Alternatively a TIFF image of the plate can be imported into different array analysis software. Obtain median pixel density (PD) of each spot, then 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 of tyrosine phosphorylation are comparable between different samples.

Suggested Odyssey scan parameters:

Resolution: 84 µm
Quality: Medium
Focus offset: 3.9 mm
Intensity: 6, adjust as necessary.

Collected images may be manipulated using "Adjust Image Curve" and "Alter Image Display" Odyssey settings.

PROFILING RTK TYROSINE PHOSPHORYLATION

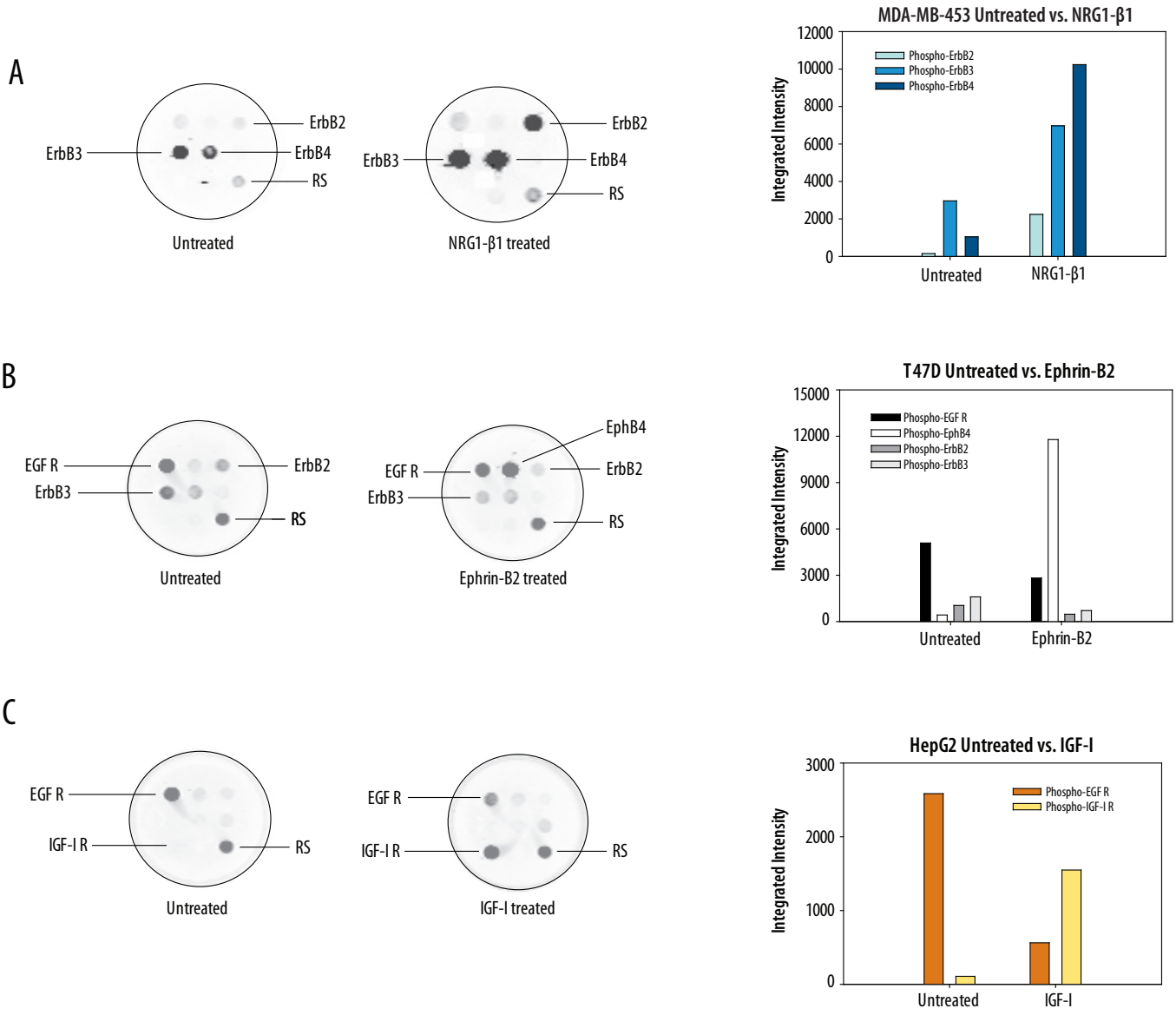


Figure 2: The Proteome Profiler *96* Human Phospho-RTK Array 2 Infrared Kit detects multiple phosphorylated receptors in untreated and ligand-treated cell lysates.

Cells were either untreated or treated as below. Array signals were obtained using a LI-COR Odyssey imager. Inverted gray scale images of the wells are shown (left) with integrated signal intensities in vertical bar graphs (right). (RS=Reference Spot).

A. The MDA-MB-453 human breast cancer cell line was untreated or treated with 100 ng/mL of recombinant human NRG1-β1/HRG1-β1 (R&D Systems, Catalog # 396-HB) for 5 minutes. 10 µg of lysate was used per well.

B. The T47D human breast cancer cell line was untreated or treated with 3 µg/mL of recombinant mouse Ephrin-B2 (R&D Systems, Catalog # 496-EB) and 0.3 µg/mL recombinant human IgG_1 Fc (R&D Systems, Catalog # 110-HG) for 20 minutes. 50 µg of lysate was used per well.

C. The HepG2 human hepatocellular carcinoma cell line was untreated or treated with 100 ng/mL of recombinant human IGF-I (R&D Systems, Catalog # 291-G1) for 5 minutes. 50 µg of lysate was used per well.

SENSITIVITY

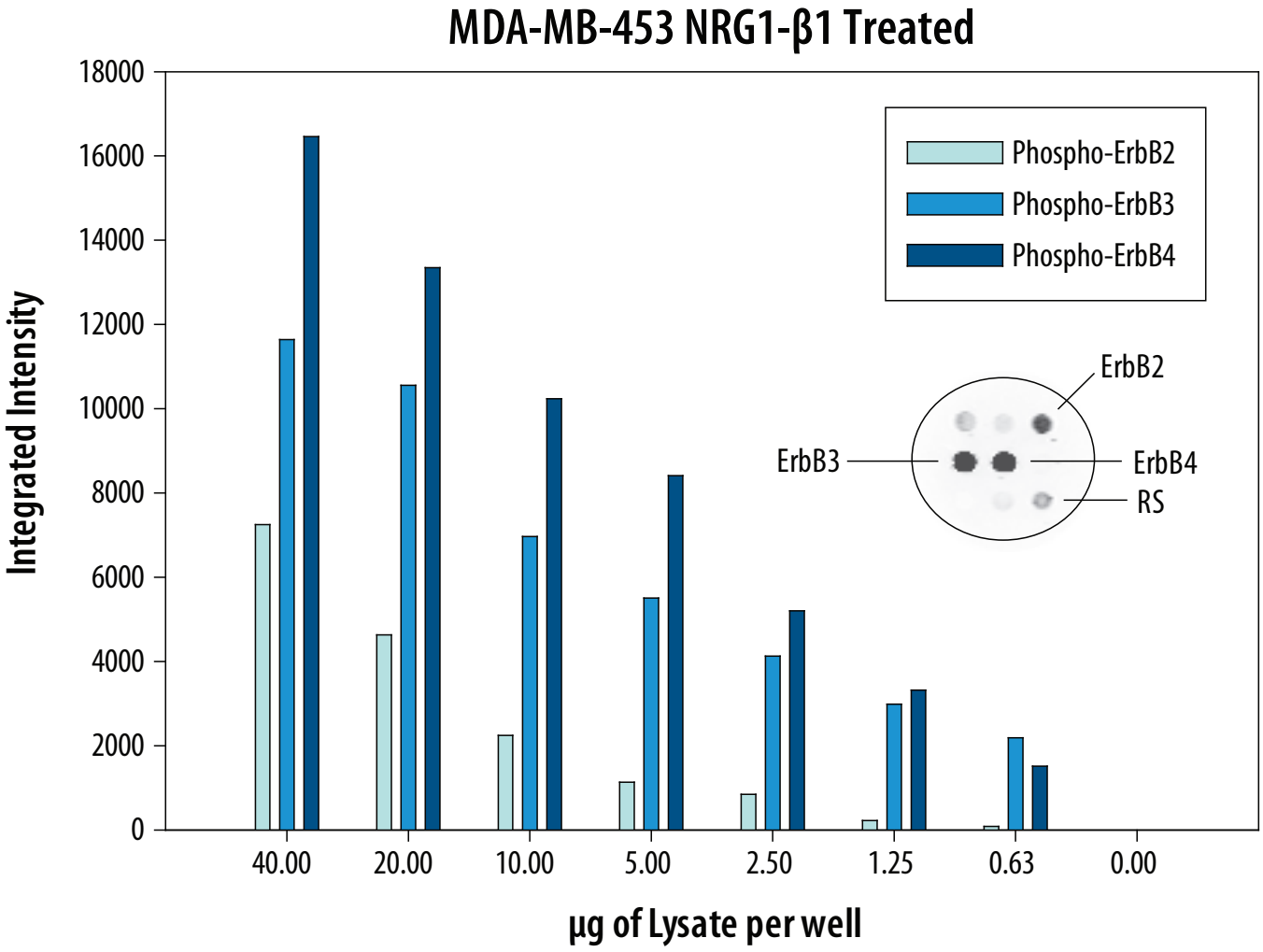
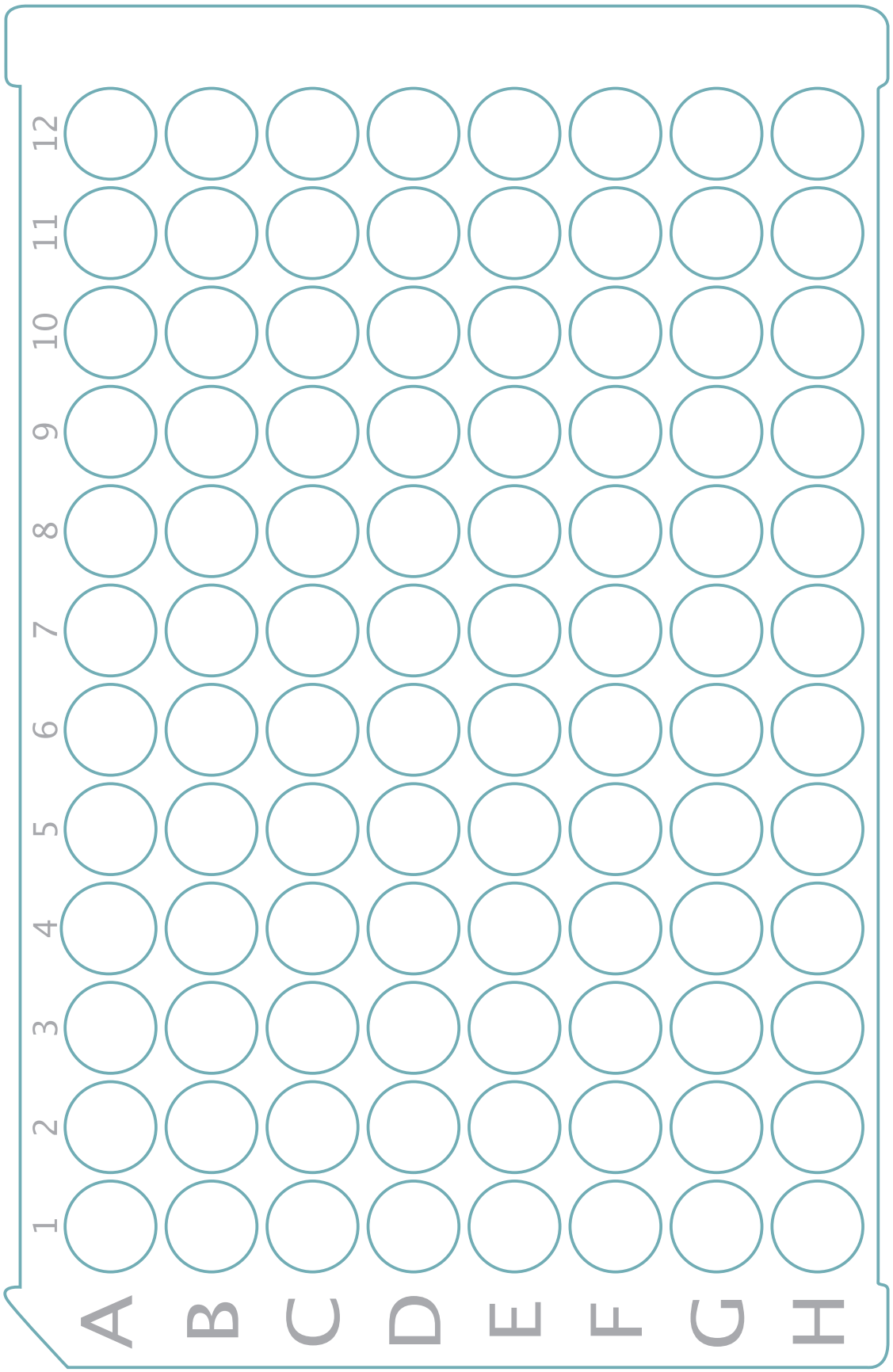


Figure 3: The Proteome Profiler *96* Human Phospho-RTK Array 2 Infrared Kit detects tyrosine-phosphorylated receptors over a range of lysate concentrations.

MDA-MB-453 human breast cancer cells were treated with 100 ng/mL of recombinant human NRG1-β1/HRG1-β1 (R&D Systems, Catalog # 396-HB) for 5 minutes. Inset shows well image using 20 µg of lysate per well. Serial dilutions of the lysate were tested using the Proteome Profiler *96* Human Phospho-RTK Array 2 Infrared Kit. Integrated signal intensities are shown (RS=Reference Spot).

PLATE LAYOUT

Use this plate layout to record samples assayed.



NOTES