DuoSet® IC

Human Phospho-ErbB3/Her3

Catalog Number DYC1769-2
DYC1769-5
DYC1769E

For the development of sandwich ELISAs to measure phosphorylated ErbB3 in cell lysates.

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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PRINCIPLE OF THE ASSAY

This DuoSet® IC ELISA contains the basic components required for the development of sandwich ELISAs to measure human tyrosine-phosphorylated ErbB3 in cell lysates. An immobilized capture antibody specific for human ErbB3, also known as Her3, binds both phosphorylated and unphosphorylated ErbB3. After washing away unbound material, an HRP-conjugated monoclonal antibody specific for phosphorylated tyrosine is used to detect only phosphorylated receptor, utilizing a standard HRP format.

Note: *Depending on the cell line and treatment conditions, ErbB family receptors may form heterodimers (1, 2). Any tyrosine-phosphorylated ErbB receptor or other receptor tyrosine kinase (3) that has heterodimerized with ErbB3 may not dissociate during the preparation of cell lysate samples and therefore may also be detected using this DuoSet IC ELISA.*

1. Alroy, I. and Y. Yarden (1997) FEBS Lett. **410**:83.
2. Normanno, N. *et al*. (2003) Endocr. Relat. Cancer **10**:1.
3. Riedemann, J. *et al*. (2007) Biochem. Biophys. Res. Commun. **355**:707.

MATERIALS PROVIDED

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

			Vials Provided	
Description	**Part #**	**Storage Conditions**	**Cat. # DYC1769-2**	**Cat. # DYC1769-5**
Human Phospho-ErbB3 Capture Antibody	841428	2-8° C	1	2
anti-phospho-tyrosine-HRP	841403	2-8° C	1	2
Human Phospho-ErbB3 Control	841430	2-8° C	3	5

DYC1769-2 contains sufficient materials to run ELISAs on at least two 96 well plates.*
DYC1769-5 contains sufficient materials to run ELISAs on at least five 96 well plates.*

This kit is also available in an Economy Pack (R&D Systems, Catalog # DYC1769E). Economy Packs contain sufficient materials to run ELISAs on 15 microplates.* Specific vial counts of each component may vary. Please refer to the literature accompanying your order for specific vial counts.

*Provided the following conditions are met:

- The reagents are prepared as described in this package insert.
- The assay is run as described in the General ELISA Protocol on page 6.
- The recommended microplates, buffers, diluents, substrates, and solutions are used.

OTHER MATERIALS REQUIRED

- Aprotinin (Sigma # A6279)
- Leupeptin (Tocris # 1167)
- NP-40 Alternative (EMD/Calbiochem # 492016)
- Sodium Azide (NaN_3) (Sigma # S2002)
- Sodium Orthovanadate (Na_3VO_4) (Sigma # S6508), activated
- Pipettes and pipette tips
- Deionized or distilled water
- 96 well microplates (R&D Systems Catalog # DY990)
- Plate sealers (R&D Systems, Catalog # DY992)
- Squirt bottle, manifold dispenser, or automated microplate washer.

SOLUTIONS REQUIRED

PBS - 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2-7.4, 0.2 μm filtered (R&D Systems, Catalog # DY006).

Wash Buffer - 0.05% Tween® 20 in PBS, pH 7.2-7.4 (R&D Systems, Catalog # WA126).

Block Buffer - 1% BSA*, 0.05% NaN_3 in PBS, pH 7.2-7.4.

IC Diluent #12** - 1% NP-40 Alternative, 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM activated sodium orthovanadate.

IC Diluent #14 - 20 mM Tris, 137 mM NaCl, 0.05% Tween 20, 0.1% BSA*, pH 7.2-7.4.

Lysis Buffer #9** - 1% NP-40 Alternative, 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM activated sodium orthovanadate, 10 μg/mL Aprotinin, 10 μg/mL Leupeptin.

Note: *Lysis Buffer #9 consists of IC Diluent #12 plus 10 μg/mL Aprotinin and 10 μg/mL Leupeptin. Approximately 50 mL of IC Diluent #12 is required to run the assay on one plate.*

Substrate Solution - 1:1 mixture of Color Reagent A (H_2O_2) and Color Reagent B (Tetramethylbenzidine) (R&D Systems, Catalog # DY999).

Stop Solution - 2 N H_2SO_4 (R&D Systems, Catalog # DY994).

*The use of R&D Systems Reagent Diluent Concentrate 2 (Catalog # DY995) or Millipore Bovine Serum Albumin, Fraction V, Protease free (Catalog # 82-045) is recommended. All buffers containing BSA must be stored at 2-8° C.

**Alternatively, use Sample Diluent Concentrate 2 (2X) (R&D Systems, Catalog # DYC002), prepared as described in the DYC002 package insert.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Human Phospho-ErbB3 Capture Antibody (Part 841428) - Each vial contains 720 μg/mL of mouse anti-human ErbB3 antibody when reconstituted with 200 μL of PBS. After reconstitution, store at 2-8° C for up to 30 days or aliquot and store at ≤ -20° C in a manual defrost freezer or at ≤ -70° C for up to 3 months.*

anti-phospho-tyrosine-HRP (Part 841403) - Each vial contains 50 μL of mouse anti-phospho-tyrosine conjugated to HRP. Immediately before use, dilute the anti-phospho-tyrosine-HRP to the working concentration specified on the vial label in

IC Diluent #14 . Prepare only as much anti-phospho-tyrosine-HRP as required to run each assay. Store at 2-8° C for up to 3 months after initial use.* **DO NOT FREEZE.**

Human Phospho-ErbB3 Control (Part 841430) - Each vial contains 120 ng/mL of recombinant human phosphorylated ErbB3 when reconstituted with 500 μL of IC Diluent #12. **Use within one hour of reconstitution. Use a fresh control for each assay.** A control concentration of 2000 pg/mL is recommended.

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

PREPARATION OF SAMPLES

Cell Lysates - Rinse cells two times with PBS, making sure to remove any remaining PBS after the second rinse. Solubilize cells at 1 x 10^7 cells/mL in Lysis Buffer #9 and allow the samples to sit on ice for 15 minutes. Assay immediately or store at ≤ -70° C. Before use, centrifuge the samples at 2000 x g for 5 minutes, and transfer the supernatant to a clean test tube. Sample protein concentration may be quantified using a total protein assay. If needed, further dilutions should be made in IC Diluent #12.

PRECAUTIONS

The Stop Solution recommended for use with this kit is an acid solution.

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

Color Reagent B recommended for use with this kit may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

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

TECHNICAL HINTS AND LIMITATIONS

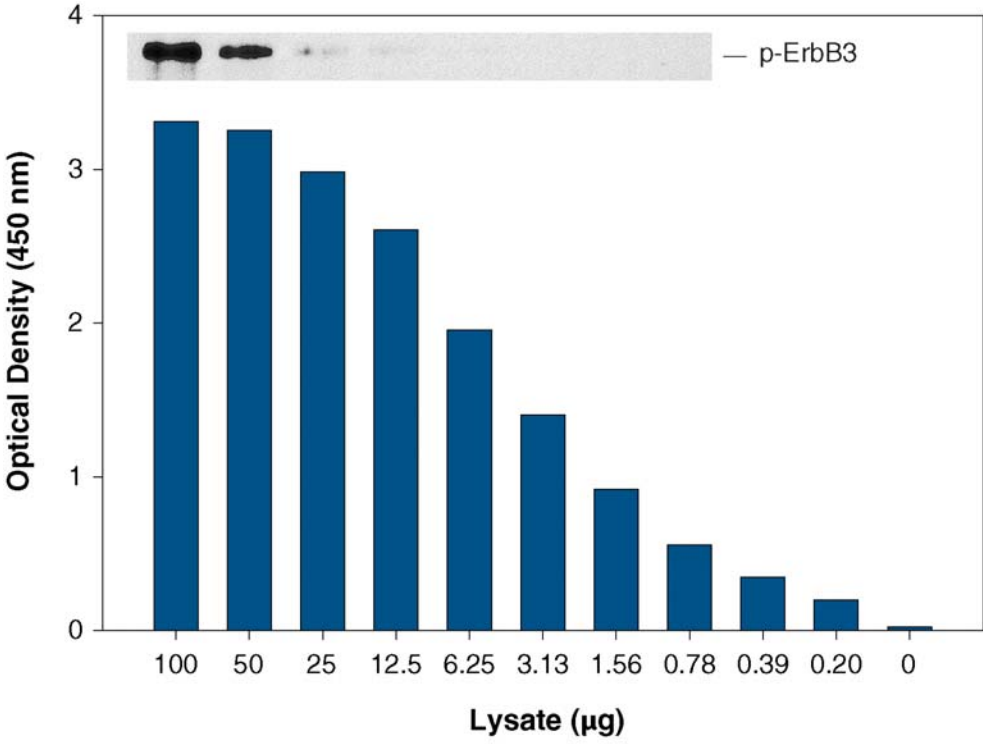
- This DuoSet IC ELISA should not be used beyond the expiration date on the kit label.
- Individual results may vary due to differences in technique, plasticware and water sources.
- It is important that the diluents selected for reconstitution and for dilution of the sample and control reflect the environment of the samples being measured. The diluent suggested in this protocol should be suitable for most cell lysates.
- The concentrations of capture/detection antibodies used can be varied to create an immunoassay with a different sensitivity and dynamic range. A basic understanding of immunoassay development is required for the successful use of these reagents in immunoassays.
- A thorough and consistent wash technique is essential for proper assay performance. Wash Buffer should be dispensed forcefully and removed completely from the wells by aspiration or decanting. Remove any remaining Wash Buffer by inverting the plate and blotting it against clean paper towels.
- Use a fresh reagent reservoir and pipette tips for each step.
- It is recommended that all controls and samples be assayed in duplicate.
- Avoid microbial contamination of reagents and buffers. This may interfere with the sensitivity of the assay. Buffers containing protein should be made under aseptic conditions and stored at 2-8° C or be prepared fresh daily.
- Tyrosine-phosphorylated ErbB family members and other receptor tyrosine kinases that heterodimerize with ErbB3/Her3 may not be dissociated by Lysis Buffer #9, potentially contributing to the optical density obtained using this DuoSet IC ELISA.

GENERAL ELISA PROTOCOL

A plate layout is provided to record controls and samples assayed.

Plate Preparation

1. Dilute the Capture Antibody to a working concentration of 4 μg/mL in PBS, without carrier protein. Immediately coat a 96 well microplate with 100 μL per well of the diluted



   Capture Antibody. Seal the plate and incubate overnight at room temperature.

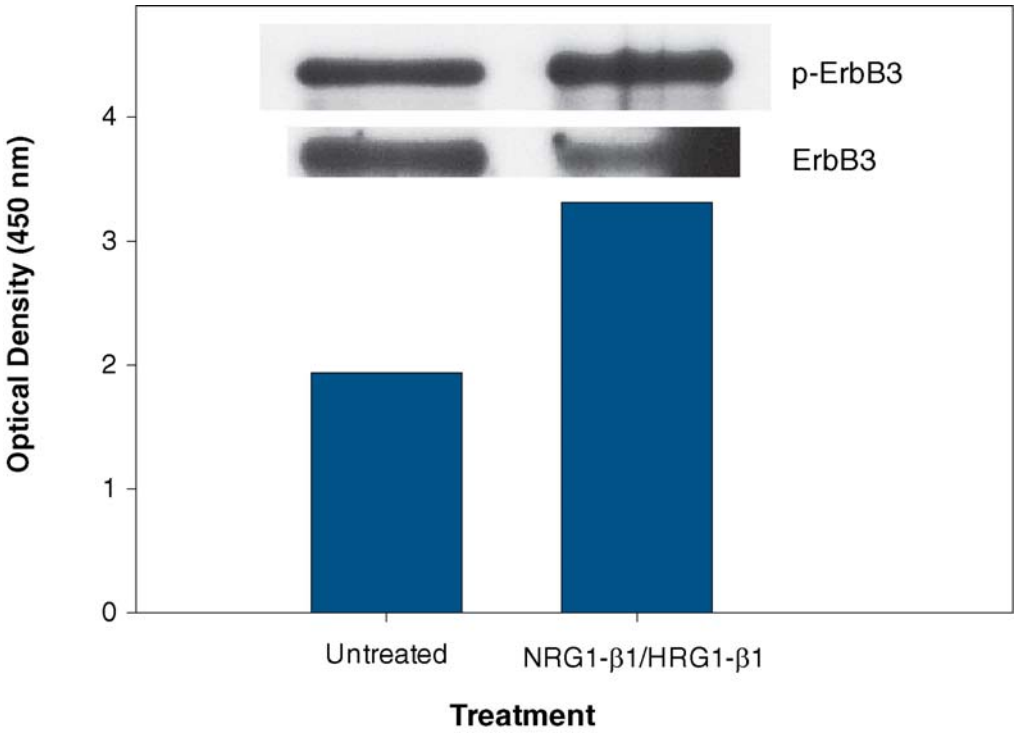
2. Aspirate each well and wash with Wash Buffer, repeating the process four times for a total of five washes. Wash by filling each well with Wash Buffer (400 μL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by inverting the plate and blotting it against clean paper towels.
3. Block plates by adding 300 μL of Block Buffer to each well. Incubate at room temperature for 1-2 hours.
4. Repeat the aspiration/wash as in step 2. The plates are now ready for sample addition.

Assay Procedure

1. Add 100 μL of sample or control in IC Diluent #12 per well. Use IC Diluent #12 as the blank. Cover with a plate sealer and incubate 2 hours at room temperature.

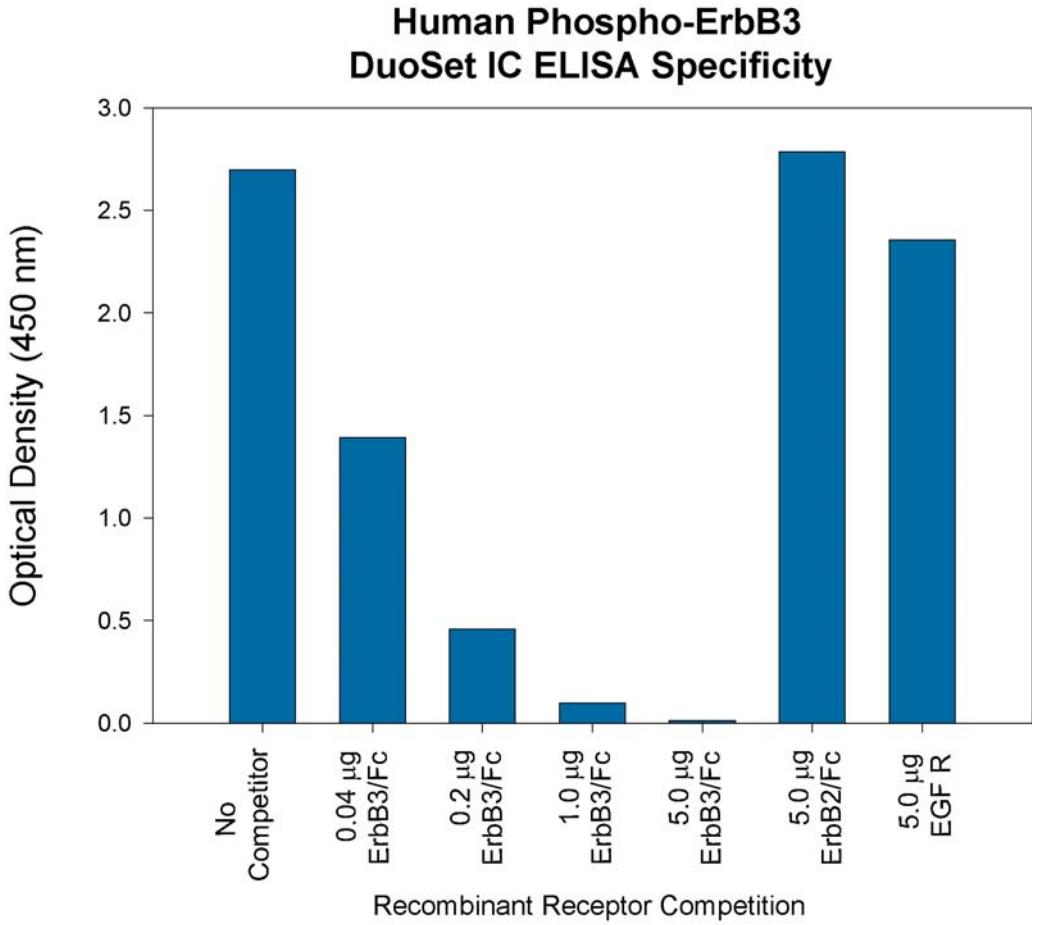
   Note: *A control concentration of 2000 pg/mL is recommended.*
2. Repeat the aspiration/wash as in step 2 of Plate Preparation.
3. Add 100 μL of the diluted anti-phospho-tyrosine-HRP to each well. Cover with a newplate sealer and incubate 2 hours at room temperature. Avoid placing the plate in direct light.
4. Repeat the aspiration/wash as in step 2 of Plate Preparation.

5. Add 100 µL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.



6. Add 50 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
7. Determine the optical density of each well immediately, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

CALCULATION OF RESULTS



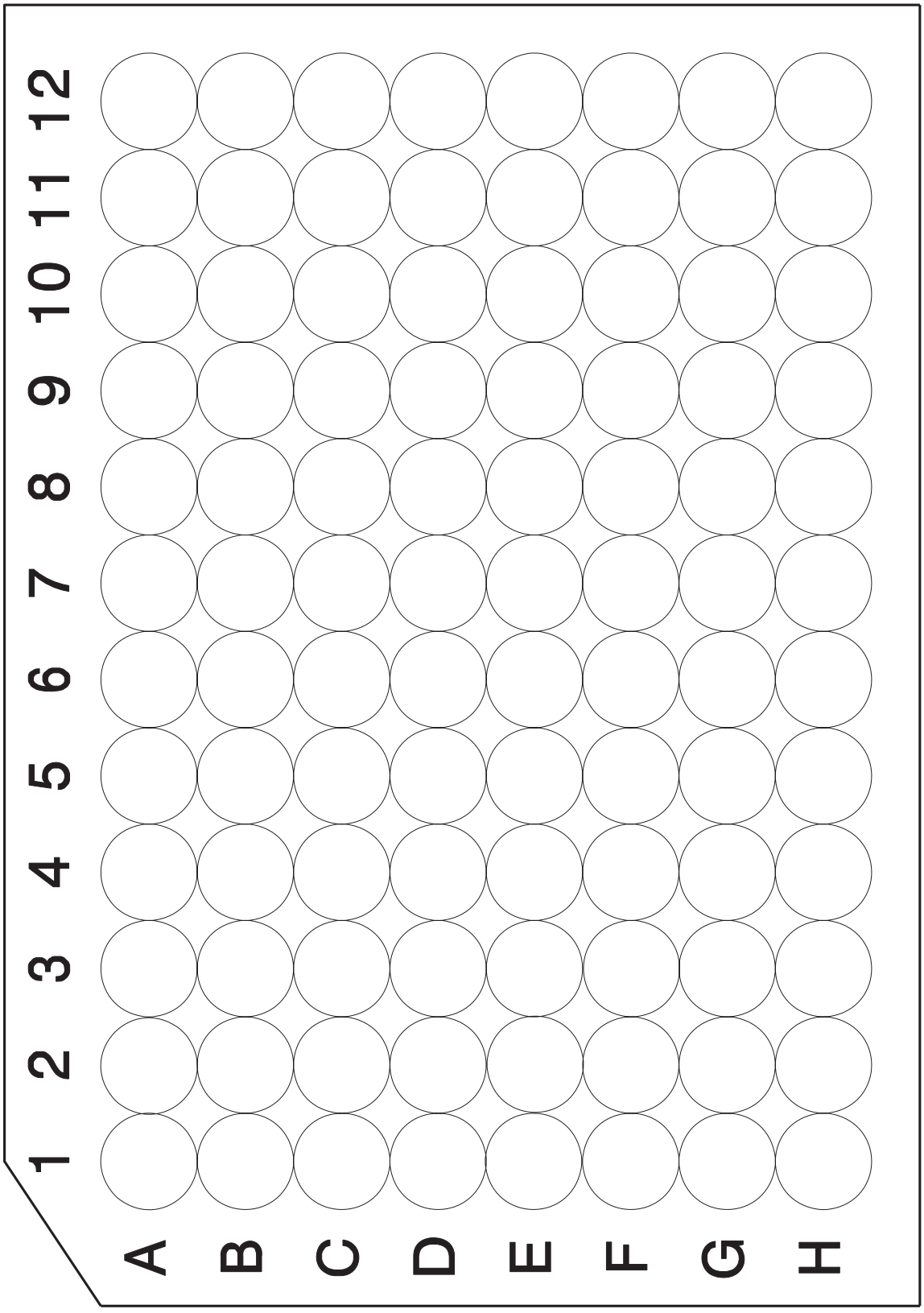
Average the duplicate readings for each control and sample, then subtract the average blank optical density.

SENSITIVITY

Figure 1: The Human Phospho-ErbB3/Her3 DuoSet IC ELISA is more sensitive than immunoprecipitation (IP)-Western blot analysis. The MDA-MB-453 human breast cancer cell line was treated with 100 ng/mL of recombinant human NRG1-β1/HRG1-β1 (R&D Systems, Catalog # 396-HB) for five minutes to induce tyrosine phosphorylation of ErbB3. Serial dilutions of lysates were analyzed using this DuoSet IC ELISA and by IP-Western blot (inset). IPs were performed using anti-ErbB3 monoclonal antibody and anti-mouse IgG agarose. Immunoblots were incubated with biotinylated anti-phospho-tyrosine monoclonal antibody (R&D Systems, Catalog # BAM1676) to detect phospho-ErbB3 (p-ErbB3). Bands were visualized with Streptavidin-HRP (R&D Systems, Catalog # DY998) followed by chemiluminescent detection.

LIGAND-INDUCED PHOSPHORYLATION

Figure 2: The Human Phospho-ErbB3/Her3 DuoSet IC ELISA detects ligand-induced ErbB3 tyrosine phosphorylation. MDA-MB-453 human breast cancer cells were untreated



or treated with 100 ng/mL of recombinant human NRG1-β1/HRG1-β1 for five minutes. Cell lysates (100 μg) were analyzed using this DuoSet IC ELISA and by IP-Western blot (inset).

IP-Western blots for phospho-ErbB3 (p-ErbB3) were performed as described in Figure 1. Blots were stripped and total ErbB3 (ErbB3) was detected using a biotinylated polyclonal anti-ErbB3 antibody (R&D Systems, Catalog # BAF234).

SPECIFICITY

Figure 3: The specificity of the Human Phospho-ErbB3/Her3 DuoSet IC ELISA is confirmed by receptor competition. MDA-MB-453 human breast cancer cells were treated with 100 ng/mL of recombinant human NRG1-β1/HRG1-β1 for five minutes. The indicated amounts of recombinant human ErbB3/Fc Chimera (R&D Systems, Catalog # 348-RB), recombinant human ErbB2/Fc Chimera (R&D Systems, Catalog # 1129-ER) or recombinant extracellular domains of recombinant human EGF R (R&D Systems, Catalog # 1095-ER) were added to 5 μg of lysate and analyzed using this DuoSet IC ELISA. Competition was observed only with recombinant human ErbB3.

PLATE LAYOUT

Use this plate layout as a record of controls and samples assayed.

NOTES