DuoSet® IC

Human Phospho-EGF R/ErbB1 (Y1068)

Catalog Number DYC3570-2
DYC3570-5
DYC3570E

For the development of sandwich ELISAs to measure Epidermal Growth Factor Receptor (EGF R) phosphorylated at Y1068 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 Epidermal Growth Factor Receptor (EGF R) phosphorylated at Y1068 in cell lysates. With this ELISA, an immobilized capture antibody specific for human EGF R, also known as ErbB1 and Her1, binds both phosphorylated and unphosphorylated protein. After washing away unbound material, an HRP-conjugated detection antibody specific for human EGF R phosphorylated at Y1068 is used to detect only phosphorylated protein, utilizing a standard HRP format.

MATERIALS PROVIDED

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

			Vials Provided	
Description	**Part #**	**Storage Conditions**	**Cat. # DYC3570-2**	**Cat. # DYC3570-5**
Human Phospho-EGF R (Y1068) Capture Antibody	842428	2-8° C	1	2
Human Phospho-EGF R (Y1068) Detection Antibody	842429	2-8° C	1	2
Human Phospho-EGF R (Y1068) Standard	842430	2-8° C	3	5

DYC3570-2 contains sufficient materials to run ELISAs on at least two 96 well plates.*
DYC3570-5 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 # DYC3570E). 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 orthovanadate (Na_3VO_4) (Sigma # S6508), activated
- Sodium azide (NaN_3) (Sigma # S2002)
- 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 insert.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Human Phospho-EGF R (Y1068) Capture Antibody (Part 842428) - Each vial contains 1440 µg/mL of rat anti-human EGF R 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.*

Human Phospho-EGF R (Y1068) Detection Antibody (Part 842429) - Each vial contains 150 µL of mouse anti-human phospho-EGF R (Y1068) antibody conjugated to HRP. Store at 2-8° C for up to 3 months after initial use.* **DO NOT FREEZE.**

Human Phospho-EGF R (Y1068) Standard (Part 842430) - Each vial contains 130 ng/mL of recombinant human phospho-EGF R (Y1068) when reconstituted with 500 µL of IC Diluent #12. **Use within one hour of reconstitution. A fresh standard should be used for each assay.** A seven point curve using 2-fold serial dilutions and a high standard of 20 ng/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 samples to sit on ice for 15 minutes. Assay immediately or store at ≤ -70° C. Before use, centrifuge samples at 2000 x g for 5 minutes and transfer the supernate 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 standard 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 standards 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.

GENERAL ELISA PROTOCOL

Plate Preparation

1. Dilute the Capture Antibody to a working concentration of 8.0 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 standard in IC Diluent #12 per well. Use IC Diluent #12 as the zero standard. Cover with a plate sealer and incubate 2 hours at room temperature.

   Note: *A seven point standard curve using 2-fold serial dilutions and a high standard of 20 ng/mL is recommended.*
2. Repeat the aspiration/wash as in step 2 of Plate Preparation.
3. Immediately before use, dilute the Detection Antibody to the working concentration specified on the vial label using IC Diluent #14. Prepare only as much Detection Antibody as required for each assay. Add 100 μL of the diluted Detection Antibody to each well. Cover with a new plate sealer, and incubate for 2 hours at room temperature. Avoid placing the plate in direct light.
4. Repeat the aspiration/wash as in step 2 of the 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.

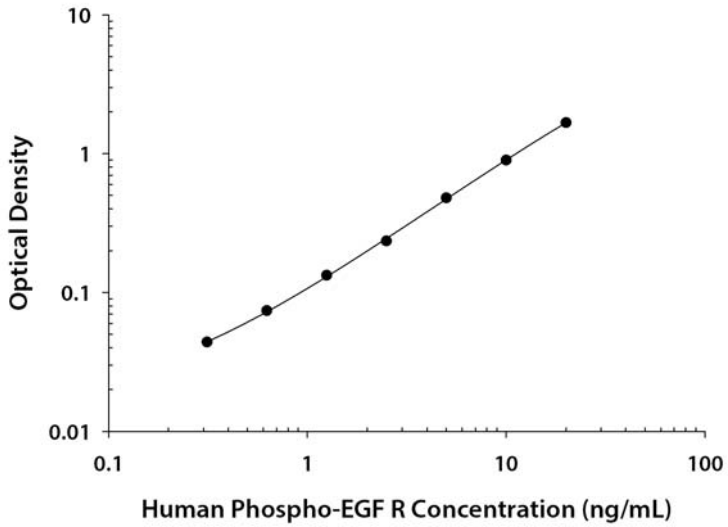
CALCULATION OF RESULTS

Average the duplicate readings for each standard and sample and subtract the average zero standard optical density (O.D.). Results may be normalized to total protein or cell number.

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the Phospho-EGF R (Y1068) concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

TYPICAL DATA

A standard curve should be generated for each set of samples assayed. The graph below represents typical data generated when using this Human Phospho-EGF R/ErbB1 (Y1068) DuoSet IC ELISA. The standard curve was calculated using a computer generated 4-PL curve-fit. This standard curve is for demonstration purposes only.



CALIBRATION

The Human Phospho-EGF R/ErbB1 (Y1068) DuoSet IC ELISA is calibrated against a highly purified NS0-expressed recombinant human phospho-EGF R (Y1068) produced at R&D Systems. Samples containing natural phospho-EGF R (Y1068) showed linear dilution parallel to the standard curve obtained using the Human Phospho-EGF R (Y1068) Standard. These results indicate that O.D. values from this DuoSet IC ELISA can be used to determine the concentration of phospho-EGF R (Y1068) in natural samples.

SPECIFICITY

The Human Phospho-EGF R/ErbB1 (Y1068) DuoSet IC ELISA specifically recognizes EGF R phosphorylated at Y1068. Specificity was demonstrated using phospho-peptide and receptor competition.

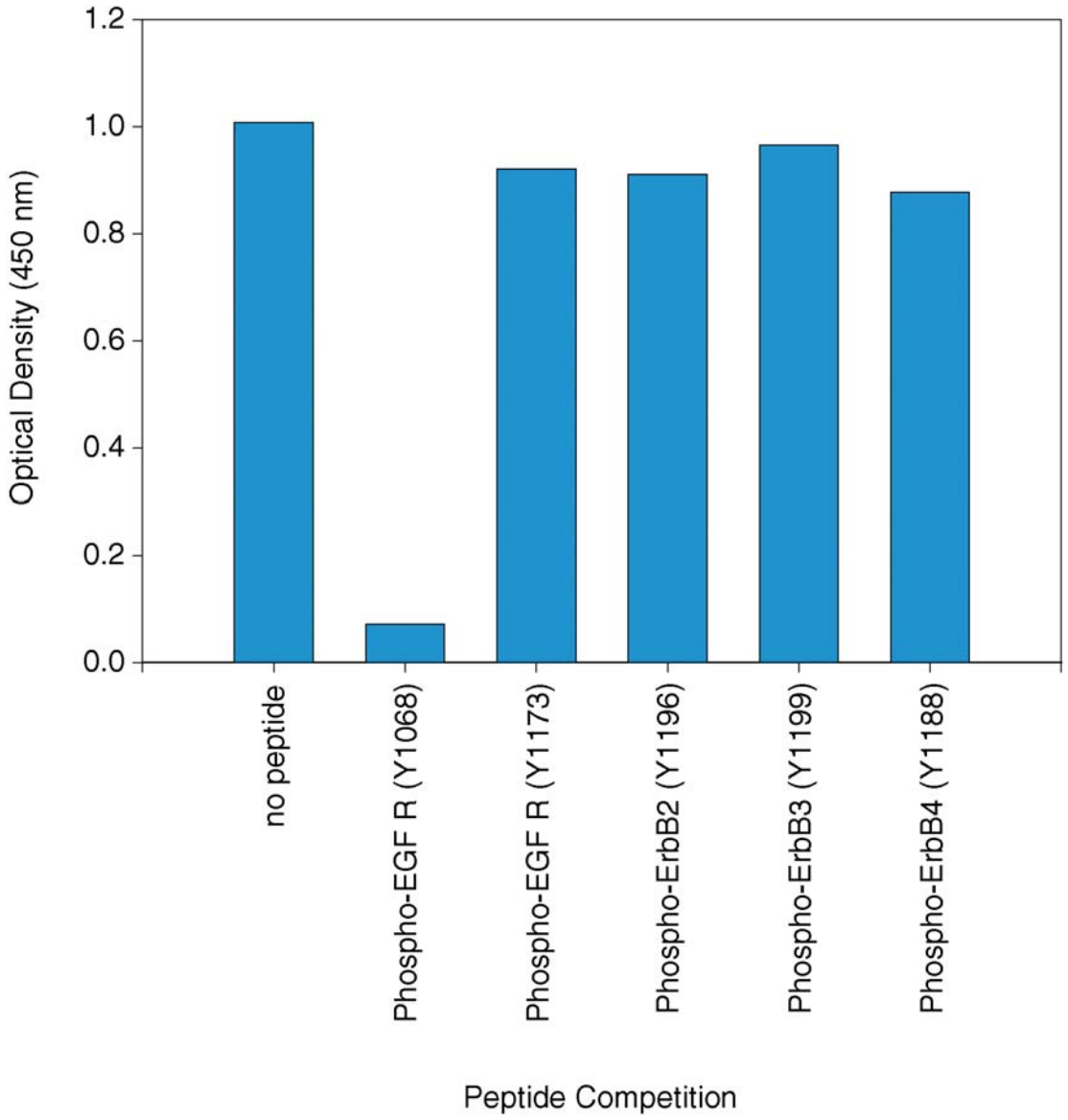


Figure 1: The specificity of the Human Phospho-EGF R/ErbB1 (Y1068) DuoSet IC ELISA is confirmed by ErbB family phosphopeptide competition. Human A431 epidermoid carcinoma cells were treated with 200 ng/mL of recombinant human EGF (R&D Systems, Catalog # 236-EG) for 5 minutes to induce tyrosine phosphorylation of EGF R. The Human Phospho-EGF R (Y1068) Detection Antibody was either untreated (no peptide) or pre-incubated with phosphopeptide containing either the EGF R (Y1173), ErbB2 (Y1196), ErbB3 (Y1199), or ErbB4 (Y1188) phosphorylation sites. Peptides were used at 100 ng/mL. Only the phosphopeptide containing the EGF R (Y1068) phosphorylation site blocked the signal, indicating that the ELISA is specific for EGF R phosphorylation at Y1068.

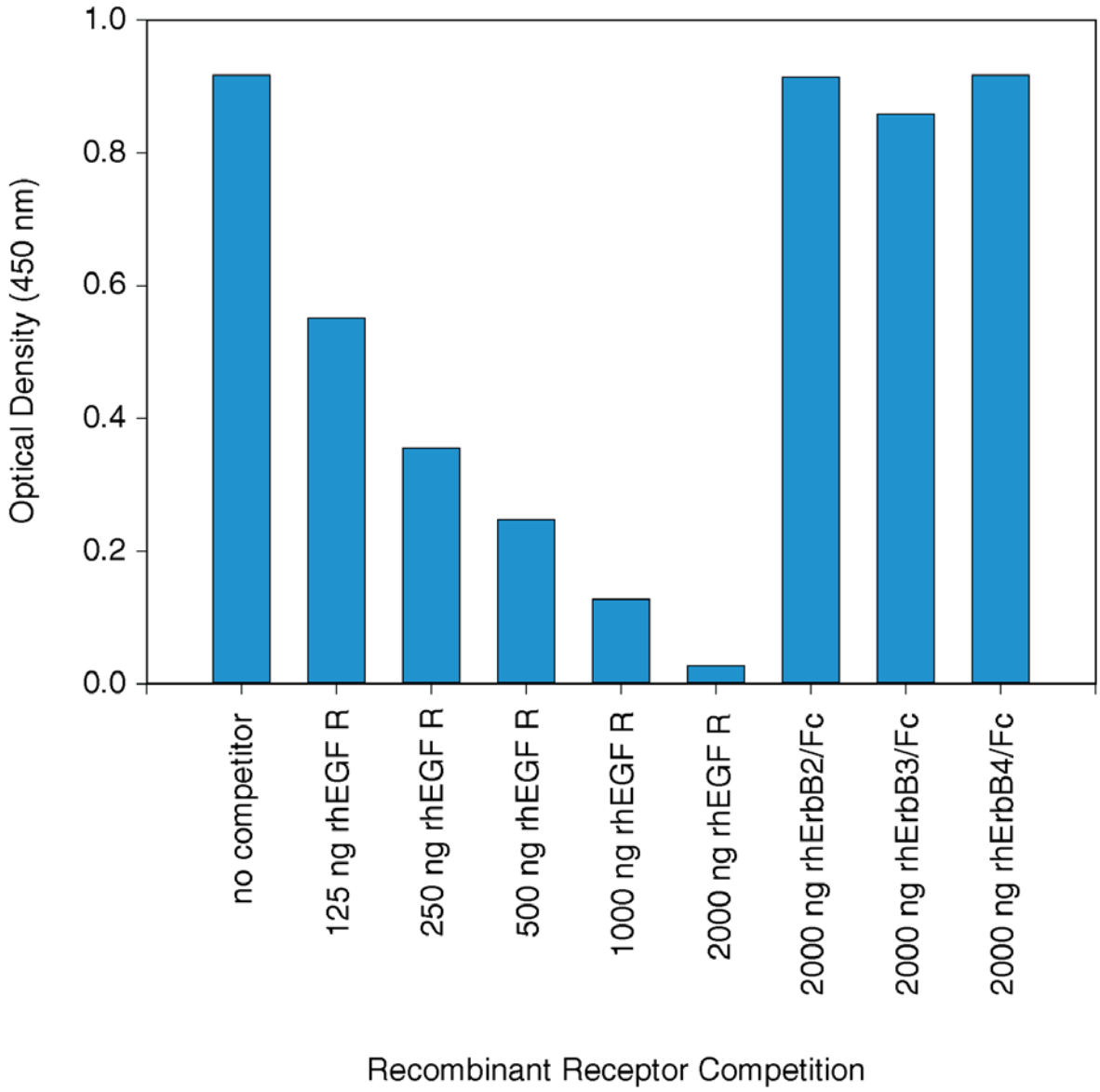


Figure 2: The specificity of the Human Phospho-EGF R/ErbB1 (Y1068) DuoSet IC ELISA is further confirmed by ErbB family receptor competition. A431 cells were treated with 200 ng/mL of recombinant human EGF for 5 minutes. The indicated amounts of recombinant extracellular domains of human EGF R (R&D Systems, Catalog # 1095-ER), human ErbB2/Fc Chimera (R&D Systems, Catalog # 1129-ER), human ErbB3/Fc Chimera (R&D Systems, Catalog # 348-RB), or human ErbB4/Fc Chimera (R&D Systems, Catalog # 1131-ER) were added to 25 μg of lysate and analyzed using this DuoSet IC ELISA. Competition was observed only with recombinant human EGF R.

SENSITIVITY

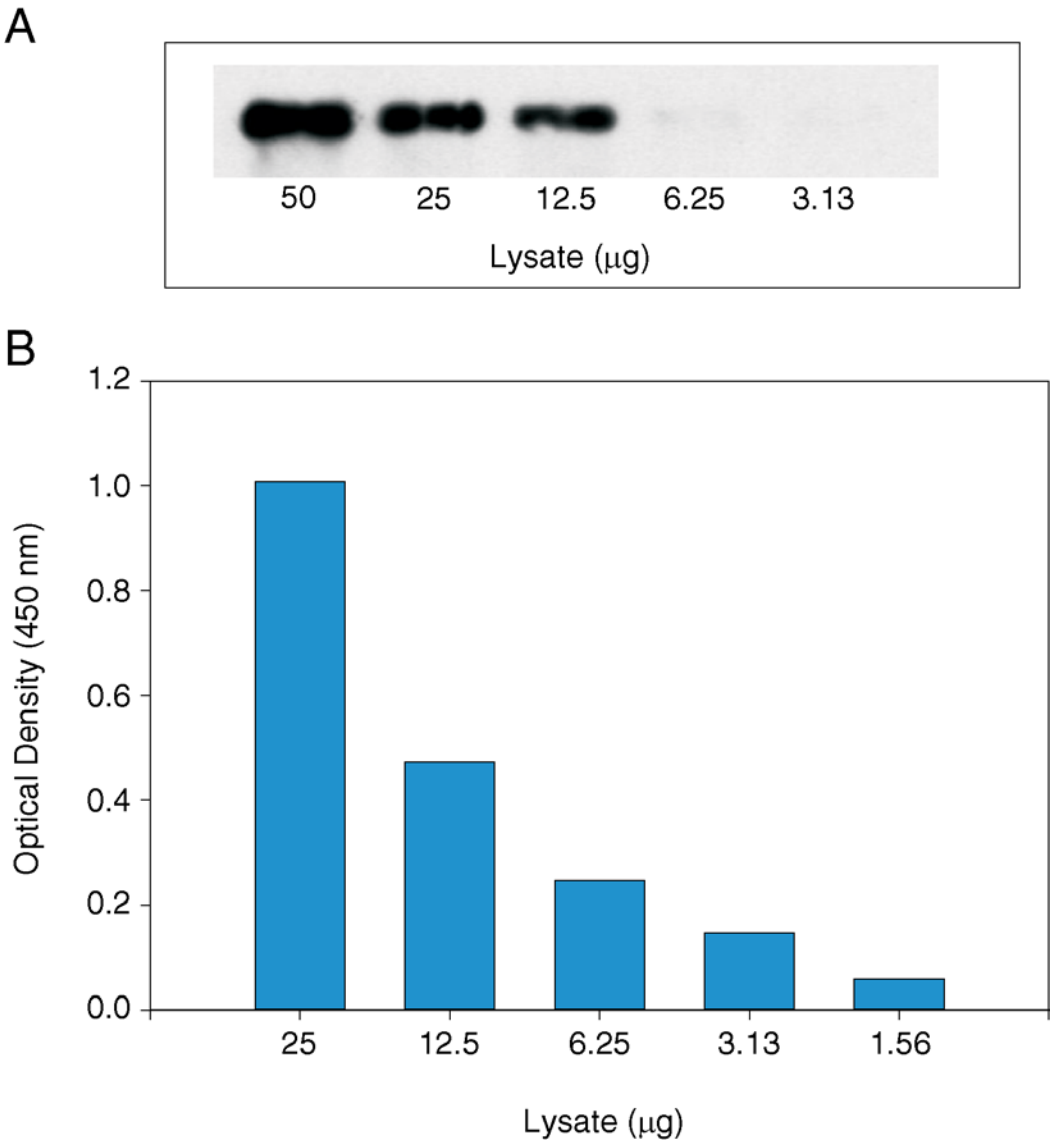


Figure 3: The Human Phospho-EGF R/ErbB1 (Y1068) DuoSet IC ELISA is more sensitive than immunoprecipitation (IP)-Western blot analysis. A431 cells were treated with 200 ng/mL of recombinant human EGF for 5 minutes. Lysates were serially diluted and analyzed by **(A)** IP-Western blot and **(B)** this DuoSet IC ELISA. IPs were done using an anti-EGF R monoclonal antibody and goat anti-mouse agarose. Immunoblots were incubated with an HRP-conjugated anti-phospho-EGF R (Y1068) monoclonal antibody to detect phospho-EGF R (Y1068). Bands were visualized by chemiluminescent detection. Human Phospho-EGF R (Y1068) can be detected in this ELISA by using approximately 1 to 2 times less lysate than is needed for a conventional IP-Western blot.

QUANTIFICATION

Amounts of human phosphorylated EGF R, as quantified by the Human Phospho-EGF R/ErbB1 (Y1068) DuoSet IC ELISA, are consistent with the relative amounts of phosphorylated EGF R determined by qualitative Western blot analysis.

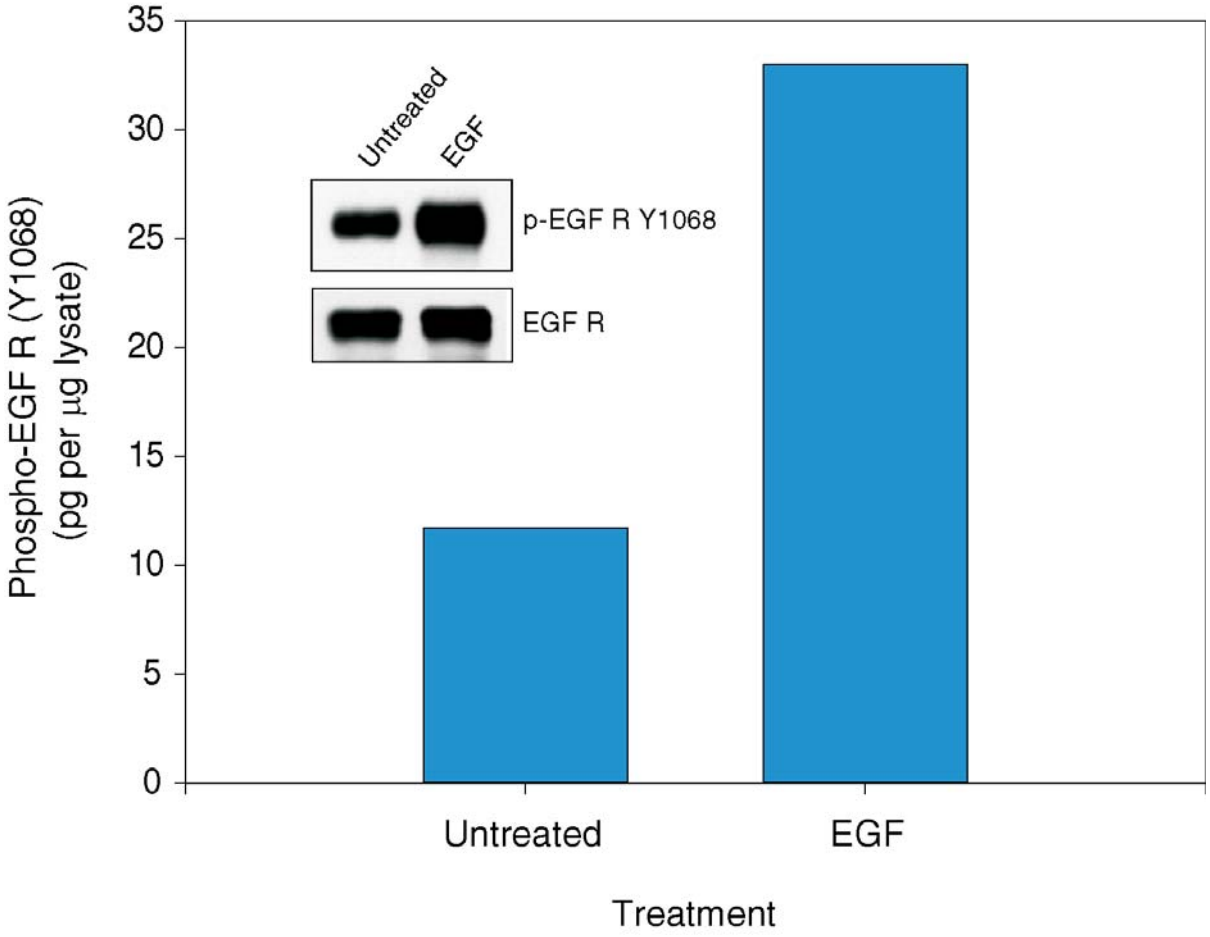


Figure 4: The Human Phospho-EGF R/ErbB1 (Y1068) DuoSet IC ELISA measures ligand-induced EGF R tyrosine phosphorylation. A431 cells were untreated or treated with 200 ng/mL of recombinant human EGF for 5 minutes. ELISA and IP-Western blot (inset) analyses were done using 25 µg and 50 µg of lysate, respectively. IP-Western blots for phospho-EGF R (Y1068) (p-EGF R Y1068) were done as described in Figure 3. Blots were stripped and total EGF R was detected using a biotinylated anti-EGF R polyclonal antibody (R&D Systems, Catalog # BAF231). The DuoSet IC ELISA results correlate well with the relative amounts of phospho-EGF R detected by IP-Western blot.