

DuoSet[®] IC

Human Phospho-EphB4

Catalog Number DYC4057-2

DYC4057-5

For the development of sandwich ELISAs to measure phosphorylated EphB4 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 tyrosine-phosphorylated EphB4 in cell lysates. An immobilized capture antibody specific for EphB4 binds both phosphorylated and unphosphorylated EphB4. After washing away unbound material, an HRP-conjugated detection antibody specific for phosphorylated tyrosine is used to detect only phosphorylated receptor, 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. # DYC4057-2	Cat. # DYC4057-5
Phospho-EphB4 Capture Antibody	842424	2-8° C	1	2
Anti-Phospho-tyrosine-HRP Detection Antibody	841403	2-8° C	1	2
Phospho-EphB4 Control	842425	2-8° C	3	5

DYC4057-2 contains sufficient materials to run ELISAs on at least two 96 well plates.*

DYC4057-5 contains sufficient materials to run ELISAs on at least five 96 well plates.*

*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 (Sigma # L8511)
- NP-40 Alternative (EMD/Calbiochem # 492016)
- Sodium Azide (NaN₃) (Sigma # S2002)
- Sodium Orthovanadate (Na₃VO₄) (Sigma # S6508), activated
- Pipettes and pipette tips
- Deionized or distilled water
- 96 well microplates [Costar EIA Plates (Catalog # 2592 or R&D Systems, Catalog # DY990) are suggested]
- 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 Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.2-7.4, 0.2 μm filtered.

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

Block Buffer - 1% BSA*, 0.05% NaN₃ 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 96 well plate.*

Substrate Solution - 1:1 mixture of Color Reagent A (H₂O₂) and Color Reagent B (Tetramethylbenzidine) (R&D Systems, Catalog # DY999).

Stop Solution - 2 N H₂SO₄ (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.

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

Tween is a registered trademark of ICI Americas.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Phospho-EphB4 Capture Antibody (Part 842424) - Each vial contains 144 $\mu\text{g}/\text{mL}$ of goat anti-human EphB4 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 $\leq -20^\circ\text{C}$ in a manual defrost freezer or at $\leq -70^\circ\text{C}$ for up to 3 months.*

Anti-Phospho-tyrosine-HRP Detection Antibody (Part 841403) - Each vial contains 50 μL of mouse anti-phospho-tyrosine antibody conjugated to HRP. Store at 2-8° C for up to 3 months after initial use.* **DO NOT FREEZE.**

Phospho-EphB4 Control (Part 842425) - Each vial contains 80 ng/mL of recombinant human phosphorylated EphB4 when reconstituted with 500 μL of IC Diluent #12. A control concentration of 500 pg/mL is recommended. Use a fresh control for each assay.

*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 1×10^7 cells/mL in Lysis Buffer #9 and allow samples to sit on ice for 15 minutes. Assay immediately or store at $\leq -70^\circ\text{C}$. 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.

PRECAUTION

The Stop Solution suggested for use with this kit is an acidic solution. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

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.

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 0.8 $\mu\text{g}/\text{mL}$ 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 5 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 500 pg/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 to run each assay. Add 100 μL of the diluted Detection Antibody to each well. Cover with a new plate 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

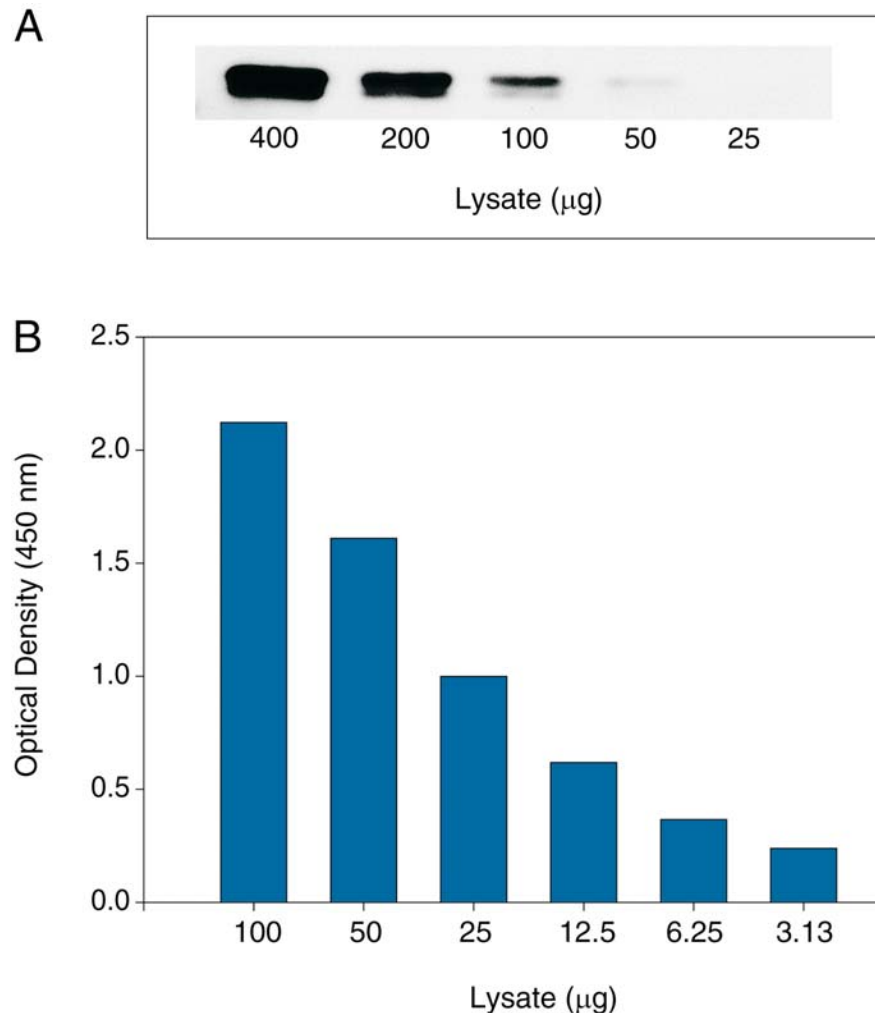


Figure 1: The Human Phospho-EphB4 DuoSet IC ELISA is more sensitive than immunoprecipitation (IP)-Western blot analysis. The T47D human breast cancer cell line was treated for 20 minutes with 3 $\mu\text{g}/\text{mL}$ recombinant mouse Ephrin-B2/Fc Chimera (R&D Systems, Catalog # 496-EB) and 0.3 $\mu\text{g}/\text{mL}$ human IgG₁ Fc (R&D Systems, Catalog # 110-HG) for clustering to induce tyrosine phosphorylation of EphB4. Lysates were serially diluted and analyzed by **(A)** IP-Western blot and **(B)** this DuoSet IC ELISA. IPs were done using an anti-EphB4 polyclonal antibody and protein G agarose. Immunoblots were incubated with an HRP-conjugated anti-phosphotyrosine monoclonal antibody (R&D Systems, Catalog # HAM1676) to detect phospho-EphB4. Bands were visualized by chemiluminescent detection. Human phospho-EphB4 can be detected in this DuoSet IC ELISA by using approximately 6-8 times less lysate than is needed for a conventional IP-Western blot.

LIGAND-INDUCED PHOSPHORYLATION

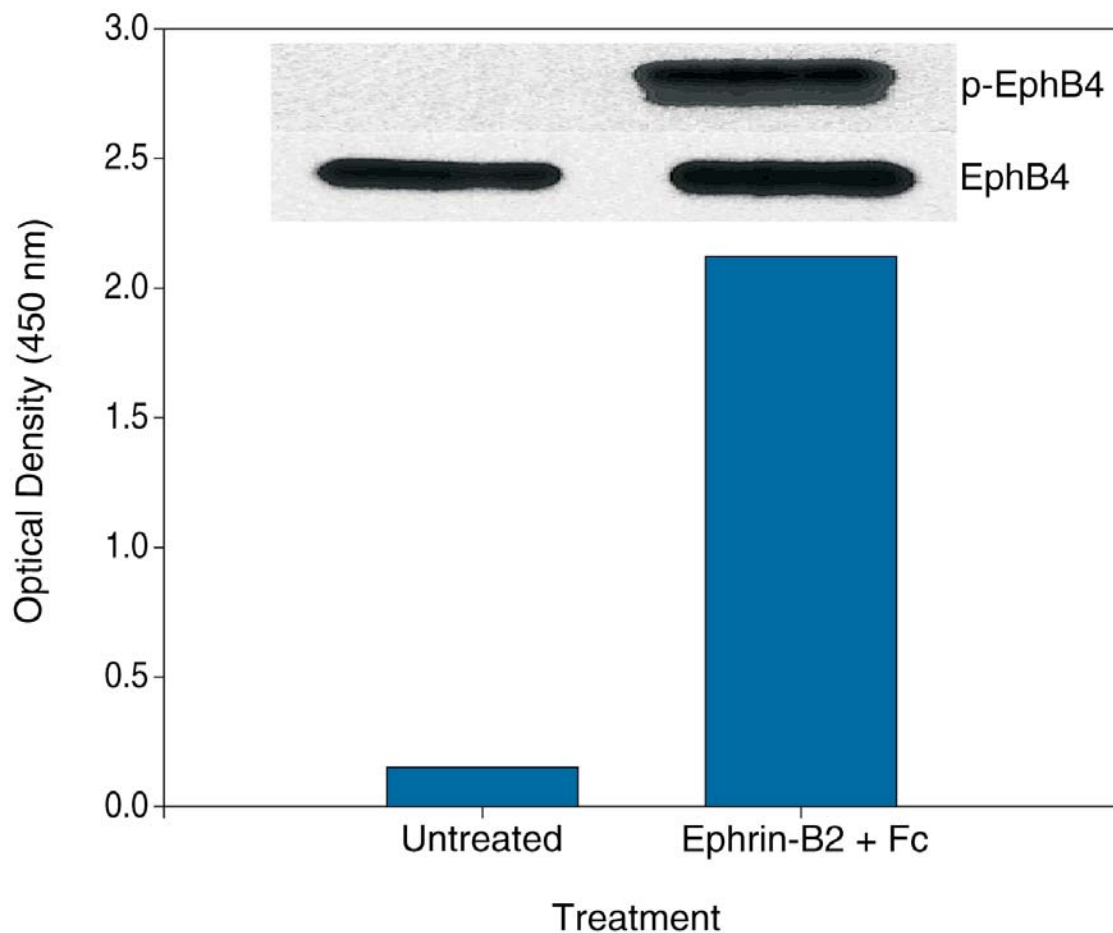


Figure 2: The Human Phospho-EphB4 DuoSet IC ELISA detects ligand-induced EphB4 tyrosine phosphorylation. T47D cells were untreated or treated with 3 $\mu\text{g}/\text{mL}$ recombinant mouse Ephrin-B2/Fc Chimera and 0.3 $\mu\text{g}/\text{mL}$ human IgG₁ Fc for 20 minutes. ELISA and IP-Western blot (inset) analyses were done using 100 μg and 400 μg of lysate, respectively. IP-Western blots for phospho-EphB4 (p-EphB4) were done as described in Figure 1. Blots were stripped and total EphB4 was detected using a biotinylated anti-EphB4 polyclonal antibody.

SPECIFICITY

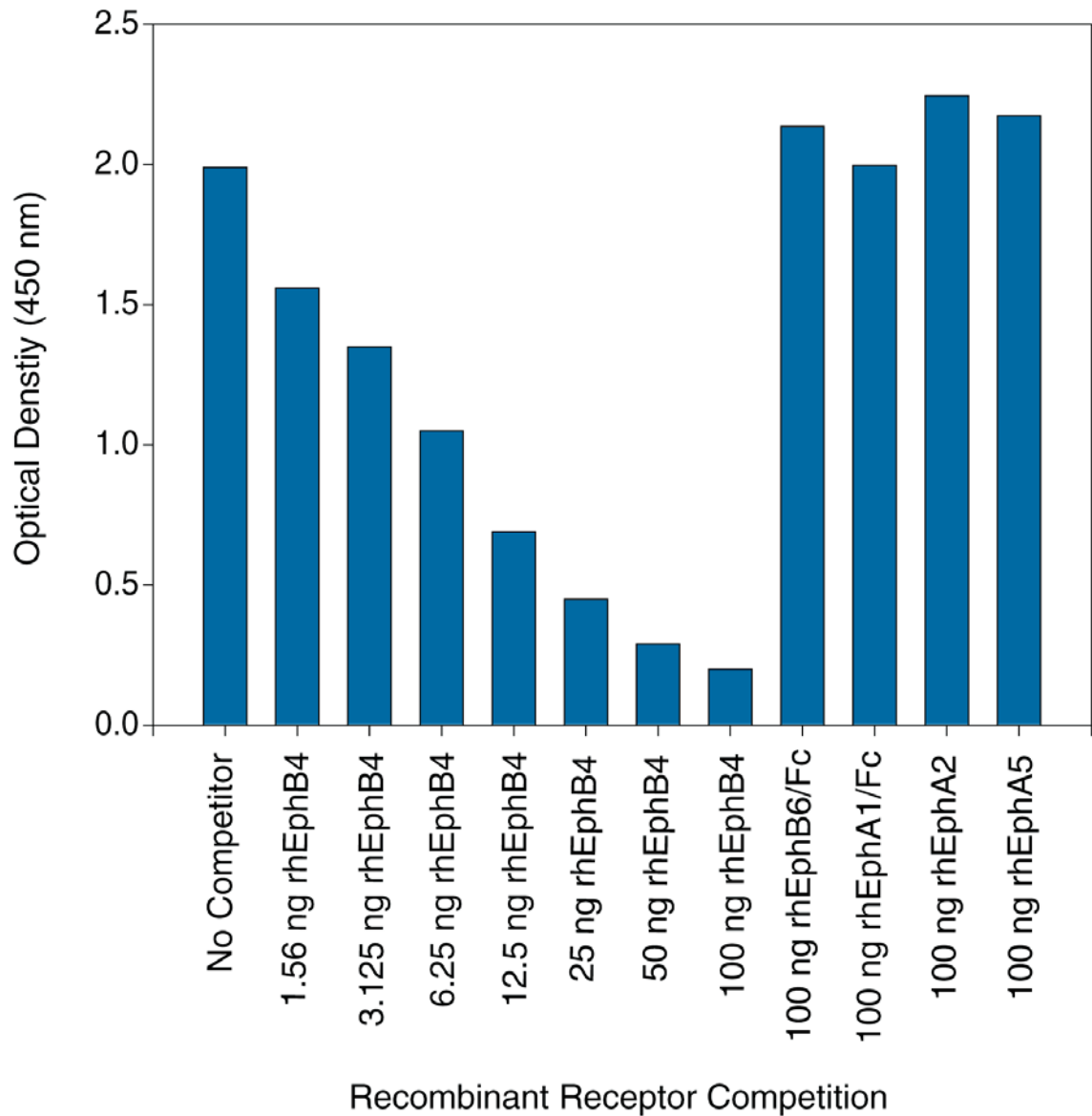


Figure 3: The specificity of the Human Phospho-EphB4 DuoSet IC ELISA is confirmed by receptor competition. T47D cells were treated with 3 $\mu\text{g}/\text{mL}$ recombinant mouse Ephrin-B2/Fc and 0.3 $\mu\text{g}/\text{mL}$ human IgG₁ Fc for 20 minutes. The indicated amounts of recombinant extracellular domains of human EphB4 (R&D Systems, Catalog # 3038-B4), human EphB6/Fc Chimera (R&D Systems, Catalog # 3384-B6), human EphA1/Fc Chimera (R&D Systems, Catalog # 638-A1), human EphA2 (R&D Systems, Catalog # 3035-A2) or human EphA5 (R&D Systems, Catalog # 3036-A5) were added to 100 μg lysate and analyzed using this DuoSet IC ELISA. Competition was observed only with recombinant human EphB4.

PLATE LAYOUT

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

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	A	B	C	D	E	F	G	H					

NOTES