DuoSet® IC

Human Phospho-EphA1

Catalog Number DYC4835-2
DYC4835-5

For the development of sandwich ELISAs to measure phosphorylated EphA1 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.

TABLE OF CONTENTS

MANUFACTURED AND DISTRIBUTED BY:

R&D Systems, Inc.
614 McKinley Place NE
Minneapolis, MN 55413
United States of America

TELEPHONE: (800) 343-7475
(612) 379-2956
FAX: (612) 656-4400
E-MAIL: info@RnDSystems.com

DISTRIBUTED BY:

R&D Systems Europe, Ltd.
19 Barton Lane
Abingdon Science Park
Abingdon, OX14 3NB
United Kingdom

TELEPHONE: +44 (0)1235 529449
FAX: +44 (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

R&D Systems GmbH
Borsigstrasse 7
65205 Wiesbaden-Nordenstadt
Germany

TELEPHONE: +49 (0)6122 90980
FAX: +49 (0)6122 909819
E-MAIL: infogmbh@RnDSystems.co.uk

R&D Systems Europe
77 boulevard Vauban
59041 LILLE CEDEX
France

FREEPHONE: +0800 90 72 49
FAX: +0800 77 16 68
E-MAIL: info@RnDSystems.co.uk

PRINCIPLE OF THE ASSAY

This DuoSet® IC ELISA contains the basic components required for the development of sandwich ELISAs to measure tyrosine-phosphorylated human EphA1 (phospho-EphA1) in cell lysates. An immobilized capture antibody specific for EphA1 binds both phosphorylated and unphosphorylated EphA1. After washing away unbound material, an HRP-conjugated detection antibody specific for phosphorylated tyrosine is used to detect only tyrosine-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. # DYC4835-2**	**Cat. # DYC4835-5**
Phospho-EphA1 Capture Antibody	842746	2 - 8° C	1	2
Anti-Phospho-tyrosine-HRP Detection Antibody	841403	2 - 8° C	1	2
Phospho-EphA1 Control	842748	2 - 8° C	3	5

DYC4835-2 contains sufficient materials to run ELISAs on at least two 96 well plates.*
DYC4835-5 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 (or NP-40 Substitute) (Igepal # CA-630 or Sigma # I3021)
- Sodium orthovanadate (Na_3VO_4) (Sigma # S6508), activated
- Sodium Azide (NaN_3)
- 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 Na_2HPO_4, 1.5 mM KH_2PO_4, 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% NaN_3 in PBS, pH 7.2 - 7.4.

IC Diluent #12** - 1% NP-40, 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, 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 (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 Sigma Bovine Serum Albumin (Catalog # A7030) is recommended. All buffers containing BSA must be stored at 2 - 8° C.

**Sample Diluent Concentrate 2 (2X) (R&D Systems, Catalog # DYC002), supplemented as per the package insert.

Tween is a registered trademark of ICI Americas.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Phospho-EphA1 Capture Antibody (Part 842746) - Each vial contains 720 μg/mL of mouse anti-human EphA1 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 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-EphA1 Control (Part 842748) - Each vial contains 75 ng/mL of recombinant human phosphorylated EphA1 when reconstituted with 500 μL of IC Diluent #12. A control concentration of 7000 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 before adding the lysis buffer. Solubilize cells at 1 x 10^7 cells/mL in Lysis Buffer #9. 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 supernatant into a clean test tube. Quantitation of sample protein concentrations using a total protein assay is recommended. For the initial experiment, use a quantity of lysate similar to that used for immunoprecipitation-Western blot. Use the lysates immediately or store at ≤ -70° C. 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 eye, hand, face, and clothing protection when using this material.

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 4.0 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 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 7000 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, and then subtract the average blank optical density.

SENSITIVITY

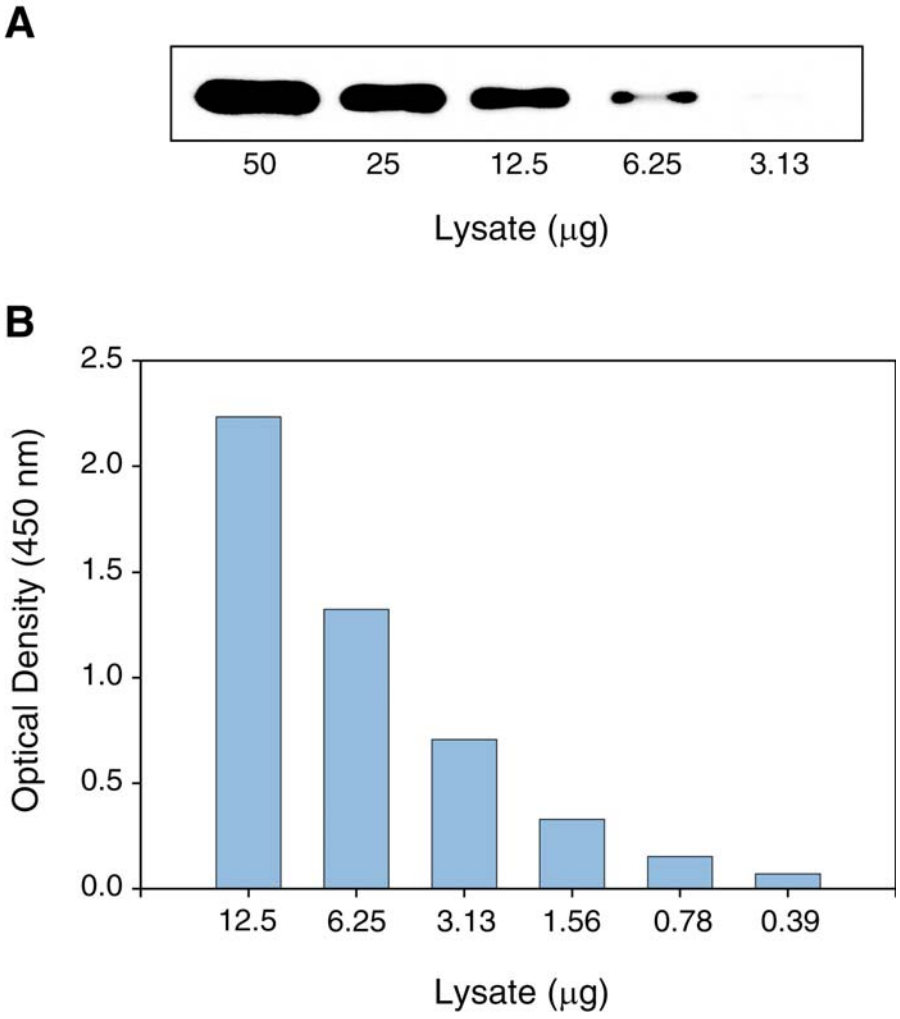


Figure 1: The Human Phospho-EphA1 DuoSet IC ELISA is more sensitive than immunoprecipitation (IP)-Western blot analysis. HEK293 cells transfected with human EphA1 (HEK293-hEphA1) were treated for 20 minutes with 3 µg/mL recombinant mouse Ephrin-A2/Fc Chimera (R&D Systems, Catalog # 603-A2) and 0.3 µg/mL human IgG/Fc (R&D Systems, Catalog # 110-HG) for clustering to induce tyrosine phosphorylation of EphA1. Lysates were serially diluted and analyzed by **(A)** IP-Western blot and **(B)** this DuoSet IC ELISA. IPs were done using an anti-EphA1 monoclonal antibody and goat anti-mouse agarose. Immunoblots were incubated with an HRP-conjugated anti-phosphotyrosine monoclonal antibody (R&D Systems, Catalog # HAM1676) to detect phospho-EphA1. Bands were visualized by chemiluminescent detection. Human Phospho-EphA1 can be detected in this DuoSet IC ELISA by using approximately 2 to 4 times less lysate than is needed for a conventional IP-Western blot.

LIGAND-INDUCED PHOSPHORYLATION

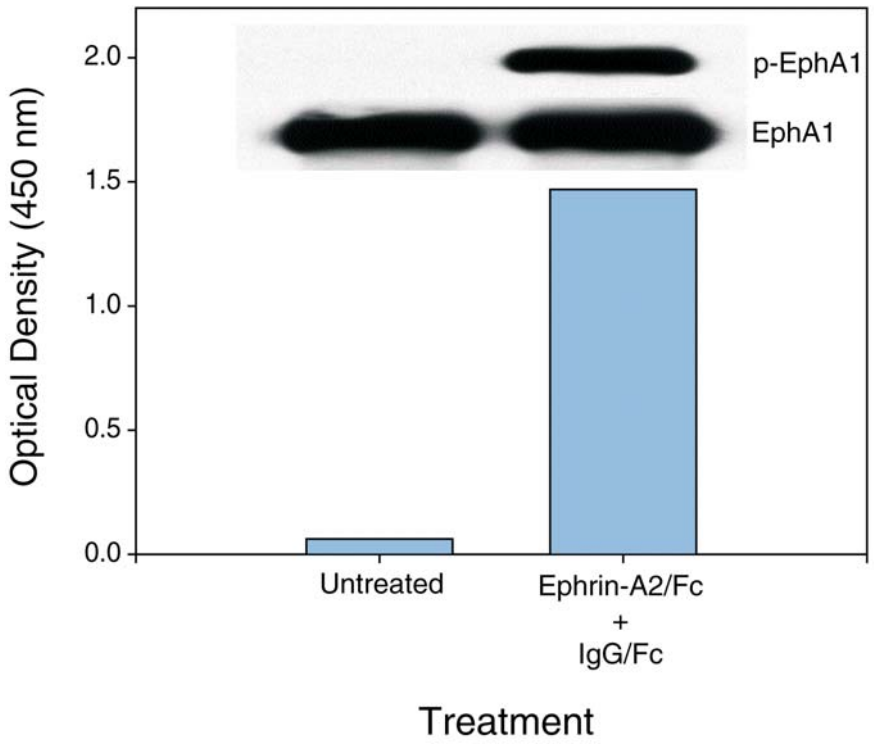


Figure 2: The Human Phospho-EphA1 DuoSet IC ELISA detects ligand-induced EphA2 tyrosine phosphorylation. HEK293-hEphA1 cells were untreated or treated with 3 μg/mL recombinant mouse Ephrin-A2/Fc Chimera and 0.3 μg/mL human IgG/Fc for 20 minutes. ELISA and IP-Western blot (inset) analyses were done using 5 μg and 50 μg of lysate, respectively. IP-Western blots for phospho-EphA1 (p-EphA1) were done as described in Figure 1. Blots were stripped and total EphA1 was detected using a biotinylated anti-EphA1 monoclonal antibody.

SPECIFICITY

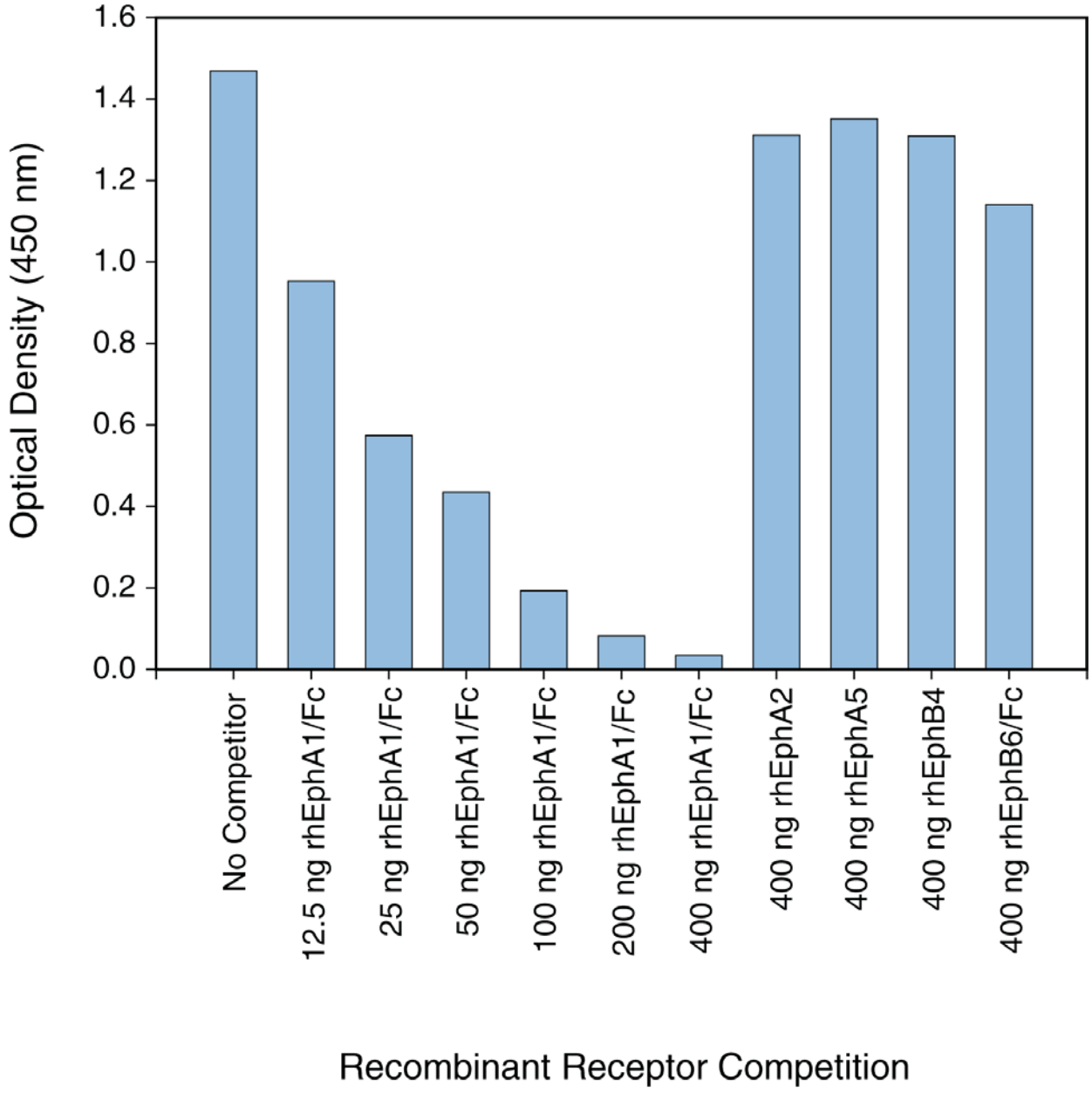
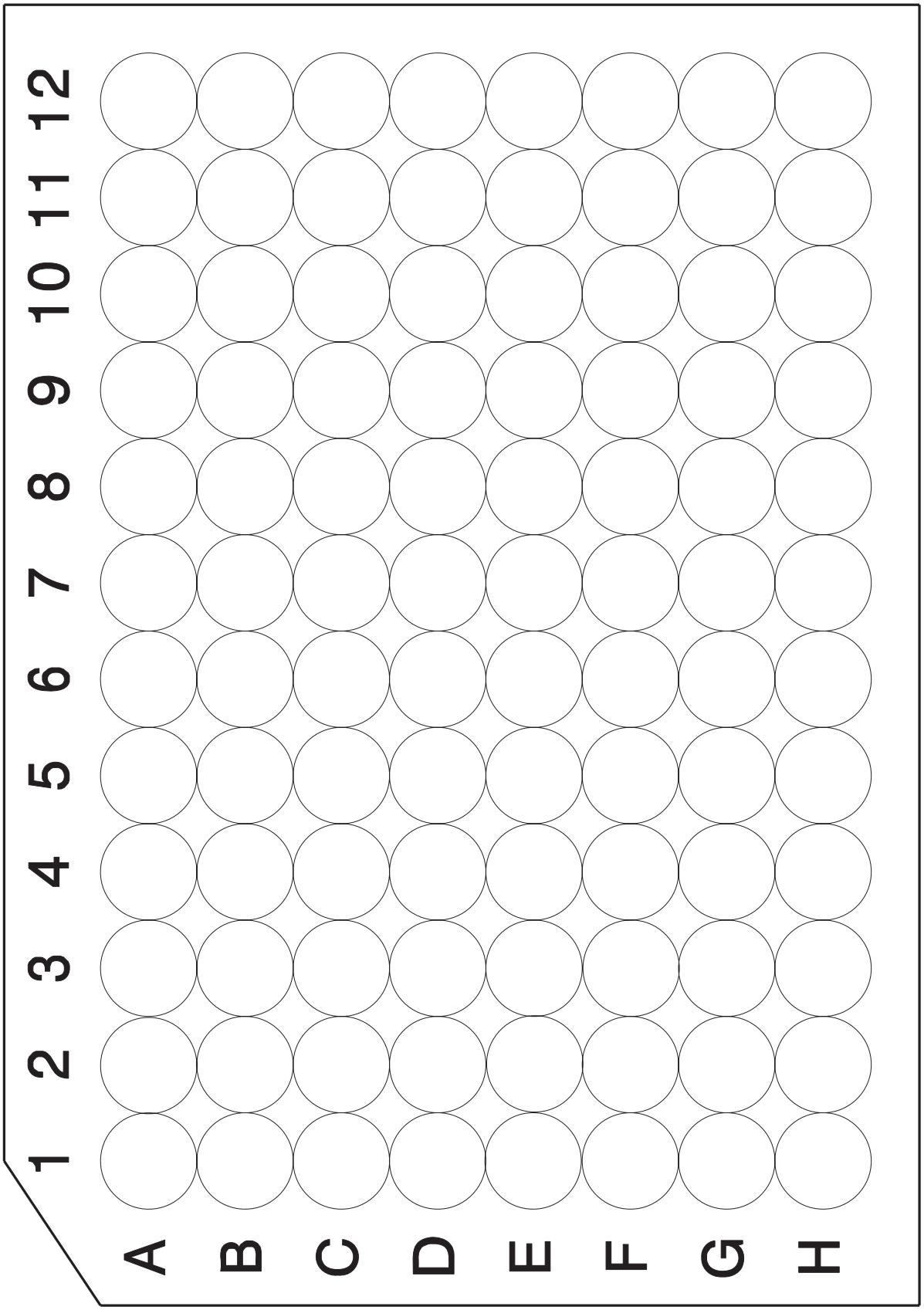


Figure 3: The specificity of the Human Phospho-EphA1 DuoSet IC ELISA is confirmed by receptor competition. HEK293-hEphA1 cells were treated with 3 μg/mL recombinant mouse Ephrin-A2/Fc Chimera and 0.3 μg/mL human IgG/Fc for 20 minutes. The indicated amounts of recombinant extracellular domains of human EphA1/Fc Chimera (R&D Systems, Catalog # 638-A1), human EphA2 (R&D Systems, Catalog # 3035-A2), human EphA5 (R&D Systems, Catalog # 3036-A5), human EphB4 (R&D Systems, Catalog # 3038-B4) or human EphB6/Fc Chimera (R&D Systems, Catalog # 3384-B6) were added to 5 μg lysate and analyzed using this DuoSet IC ELISA. Competition was observed only with recombinant human EphA1/Fc Chimera.

PLATE LAYOUT

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



NOTES