DuoSet® IC

Human Phospho-VEGF R2/KDR

Catalog Number DYC1766-2
DYC1766-5
DYC1766E

For the development of sandwich ELISAs to measure phosphorylated Vascular Endothelial Growth Factor Receptor 2 (VEGF R2) 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 vascular endothelial growth factor receptor 2 (phospho-VEGF R2) in cell lysates. An immobilized capture antibody specific for human VEGF R2, also known as KDR and Flk-1, binds both phosphorylated and unphosphorylated VEGF R2. After washing away unbound material, an HRP-conjugated monoclonal 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. # DYC1766-2**	**Cat. # DYC1766-5**
Human Phospho-VEGF R2/KDR Capture Antibody	841419	2-8° C	1	2
anti-phospho-tyrosine-HRP	841403	2-8° C	1	2
Human Phospho-VEGF R2/KDR Control	841421	2-8° C	3	5

DYC1766-2 contains sufficient materials to run ELISAs on at least two 96 well plates.*
DYC1766-5 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 # DYC1766E). 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 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), supplemented as per the package insert.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Human Phospho-VEGF R2/KDR Capture Antibody (Part 841419) - Each vial contains 1440 μg/mL of mouse anti-human VEGF R2 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 antibody conjugated to HRP. Immediately before use, dilute the anti-phospho-tyrosine-HRP to the working concentration specified on the vial label using IC Diluent #14. Prepare only as much anti-phospho-tyrosine-HRP as required to run the assay. Store at 2-8° C for up to 3 months after initial use.* **DO NOT FREEZE.**

Human Phospho-VEGF R2/KDR Control (Part 841421) - Each vial contains 390 ng/mL of recombinant human phosphorylated VEGF R2 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 8000 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 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 samples 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 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 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 controls 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 8000 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 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

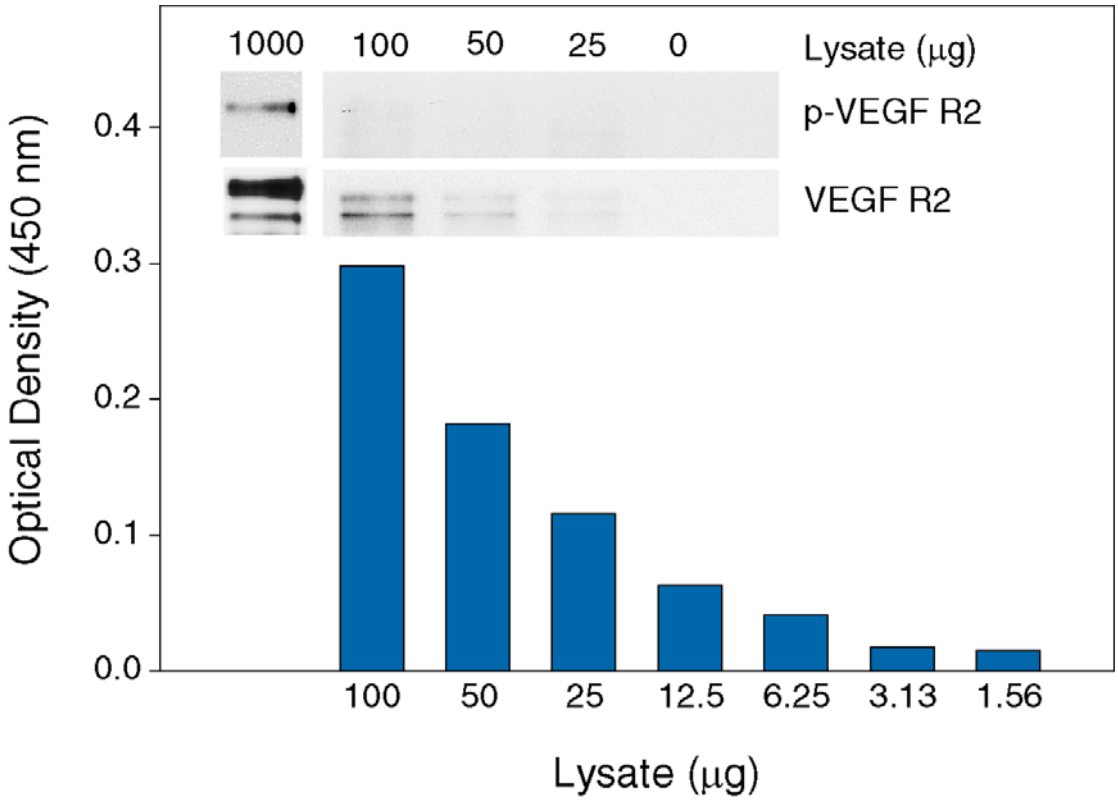


Figure 1: The Human Phospho-VEGF R2/KDR DuoSet IC ELISA is more sensitive than immunoprecipitation (IP)-Western blot analysis. HUVEC human umbilical vein endothelial cells were treated with 100 ng/mL recombinant human $VEGF_{165}$ (R&D Systems, Catalog # 293-VE) for five minutes to induce tyrosine phosphorylation of VEGF R2. Lysates were serially diluted and analyzed by this DuoSet IC ELISA and IP-Western blot (inset). IPs were performed using an anti-VEGF R2 monoclonal antibody and anti-mouse IgG agarose. Immunoblots were incubated with a biotinylated anti-phospho-tyrosine monoclonal antibody (R&D Systems, Catalog # BAM1676) to detect phospho-VEGF R2 (p-VEGF R2). Bands were visualized with Streptavidin-HRP (R&D Systems, Catalog # DY998) followed by chemiluminescent detection. Blots were stripped and total VEGF R2 (VEGF R2) was detected using a biotinylated polyclonal anti-VEGF R2 antibody (R&D Systems, Catalog # BAF357).

LIGAND-INDUCED PHOSPHORYLATION

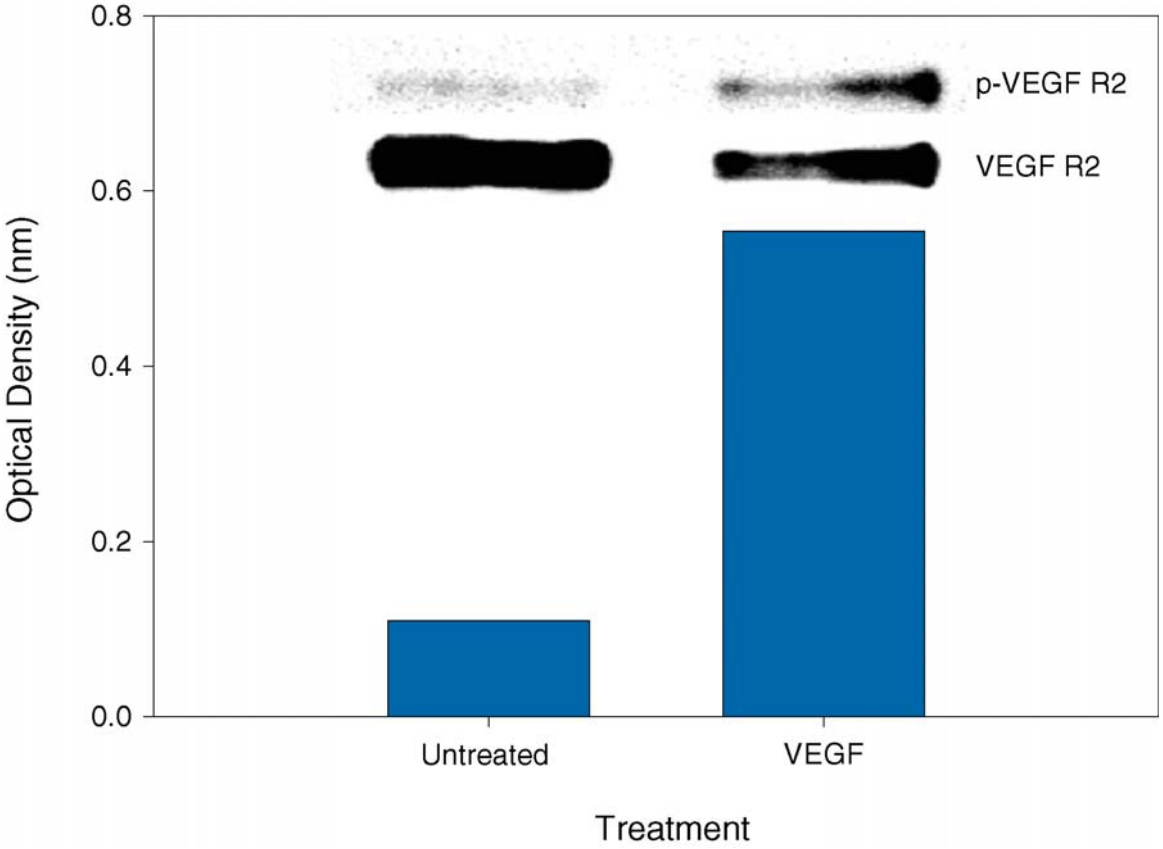


Figure 2: The Human Phospho-VEGF R2/KDR DuoSet IC ELISA detects ligand-induced VEGF R2 tyrosine phosphorylation. HUVECs were untreated or treated with 100 ng/mL recombinant human $VEGF_{165}$ for five minutes. ELISA and IP-Western blot (inset) analyses were performed using 100 μg and 1000 μg of lysate, respectively. IP-Western blots were performed as described in Figure 1.

SPECIFICITY

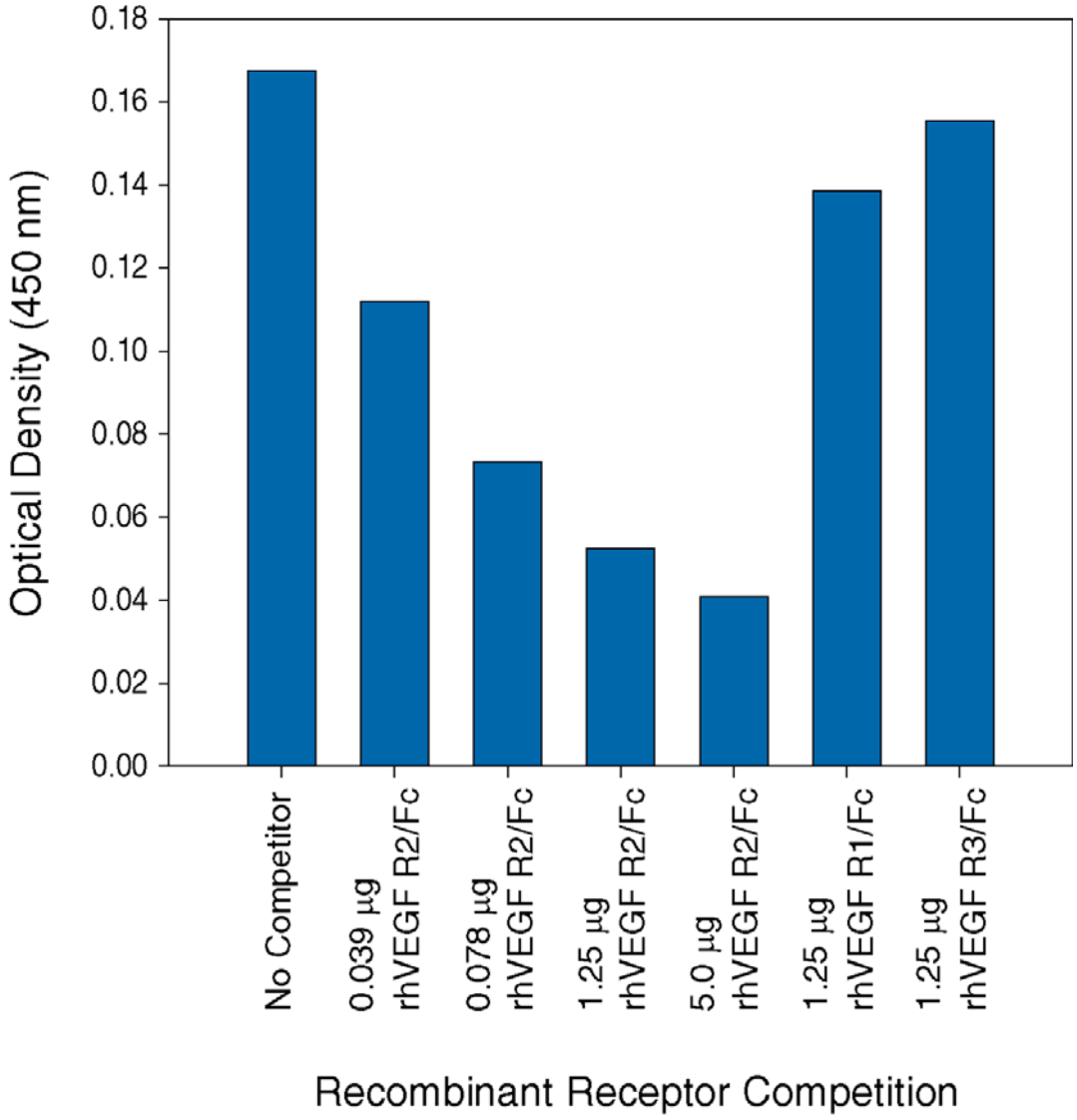
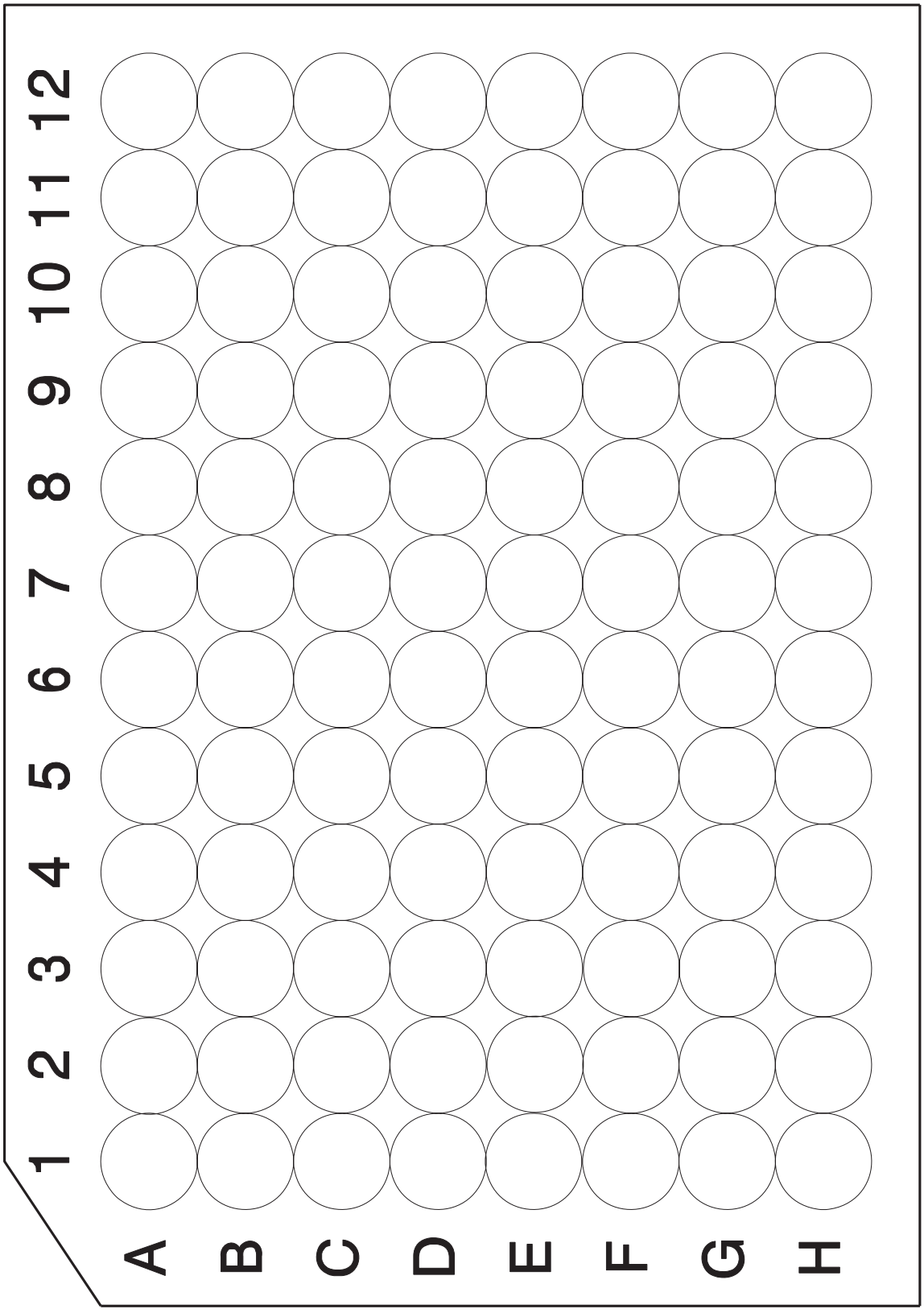


Figure 3: The specificity of the Human Phospho-VEGF R2/KDR DuoSet IC ELISA is confirmed by receptor competition. HUVECs were treated with 100 ng/mL of recombinant human $VEGF_{165}$ for five minutes. The indicated amounts of extracellular domains of recombinant human VEGF R2/Fc Chimera (R&D Systems, Catalog # 357-KD), VEGF R1/Fc Chimera (R&D Systems, Catalog # 321-FL) or VEGF R3/Fc Chimera (R&D Systems, Catalog # 349-F4) were added to 100 µg lysate and analyzed by this DuoSet IC ELISA. Competition was observed only with recombinant human VEGF R2.

PLATE LAYOUT

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



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