DuoSet® IC

Human Phospho-VEGF R3/Flt-4

Catalog Number DYC2724-2
DYC2724-5

For the development of sandwich ELISAs to measure phosphorylated human Vascular Endothelial Growth Factor Receptor 3 (VEGF R3) in cell lysates.

Note: The reconstitution method has changed. Read this package insert in its entirety before using this product.

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:

USA & Canada | R&D Systems, Inc.
614 McKinley Place NE, Minneapolis, MN 55413, USA
TEL: (800) 343-7475 (612) 379-2956 FAX: (612) 656-4400
E-MAIL: info@RnDSystems.com

DISTRIBUTED BY:

UK & Europe | R&D Systems Europe, Ltd.
19 Barton Lane, Abingdon Science Park, Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449 FAX: +44 (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

China | R&D Systems China Co., Ltd.
24A1 Hua Min Empire Plaza, 726 West Yan An Road, Shanghai PRC 200050
TEL: +86 (21) 52380373 FAX: +86 (21) 52371001
E-MAIL: info@RnDSystemsChina.com.cn

PRINCIPLE OF THE ASSAY

This DuoSet® IC ELISA contains the basic components required for the development of sandwich ELISAs to measure tyrosine-phosphorylated human Vascular Endothelial Growth Factor Receptor 3 (VEGF R3) in cell lysates. An immobilized capture antibody specific for human VEGF R3, also known as Flt-4, binds both phosphorylated and unphosphorylated VEGF R3. 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.

MATERIALS PROVIDED & STORAGE CONDITIONS

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

DESCRIPTION	PART #	CATALOG # DYC2724-2	CATALOG # DYC2724-5	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human Phospho-VEGF R3/Flt-4 Capture Antibody	841885	1 vial	2 vials	Store for up to 1 month at 2-8 °C or aliquot and store at ≤ -20 °C or ≤ -70 °C for up to 3 months in a manual defrost freezer.*
Anti-pY-HRP C	841913	1 vial	2 vials	Store for up to 3 months at 2-8 °C.* **DO NOT FREEZE.**
Human Phospho-VEGF R3/Flt-4 Control	841886	3 vials	5 vials	**Use within one hour of reconstitution. Use a fresh control for each assay.**

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

DYC2724-2 contains sufficient materials to run ELISAs on at least two 96 well plates.†
DYC2724-5 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 5.
- The recommended microplates, buffers, diluents, substrates, and solutions are used.

OTHER MATERIALS REQUIRED

- Aprotinin (Tocris® # 4139)
- Leupeptin (Tocris® # 1167)
- NP-40 Alternative (EMD/Calbiochem # 492016)
- 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 - 5% Tween 20, 5% sucrose in PBS, pH 7.2-7.4.

IC Diluent #18 - 5% Tween 20 in PBS, pH 7.2-7.4.

IC Diluent #1 - 1% BSA* in PBS, pH 7.2-7.4, 0.2 µm filtered.

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.

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-VEGF R3/Flt-4 Capture Antibody (Part 841885) - Each vial contains 720 µg/mL of mouse anti-human VEGF R3 antibody when reconstituted with 200 µL of PBS.

Anti-pY-HRP C (Part 841913) - Each vial contains 50 µL of mouse anti-phospho-tyrosine antibody conjugated to HRP. Immediately before use, dilute the Anti-pY-HRP C to the working concentration specified on the vial label in IC Diluent #1. Prepare only as much Anti-pY-HRP C as required to run the assay.

Human Phospho-VEGF R3/Flt-4 Control (Part 841886) - **Refer to the vial label for the stock concentration of recombinant human phosphorylated VEGF R3/Flt-4 when reconstituted with 500 µL of IC Diluent #18.** A control concentration of 500 pg/mL is recommended.

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 to sit on ice for 15 minutes. Assay immediately or store at ≤ -70 °C. Before use, centrifuge at 2000 x g for 5 minutes and transfer the supernate into a clean test tube. Sample protein concentrations 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 a preservative 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. 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, plastic ware, 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 diluents suggested in this protocol should be suitable for most cell lysates.
- The type of enzyme and substrate and 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 #18 per well. Use IC Diluent #18 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. Add 100 µL of the diluted Anti-pY-HRP C 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

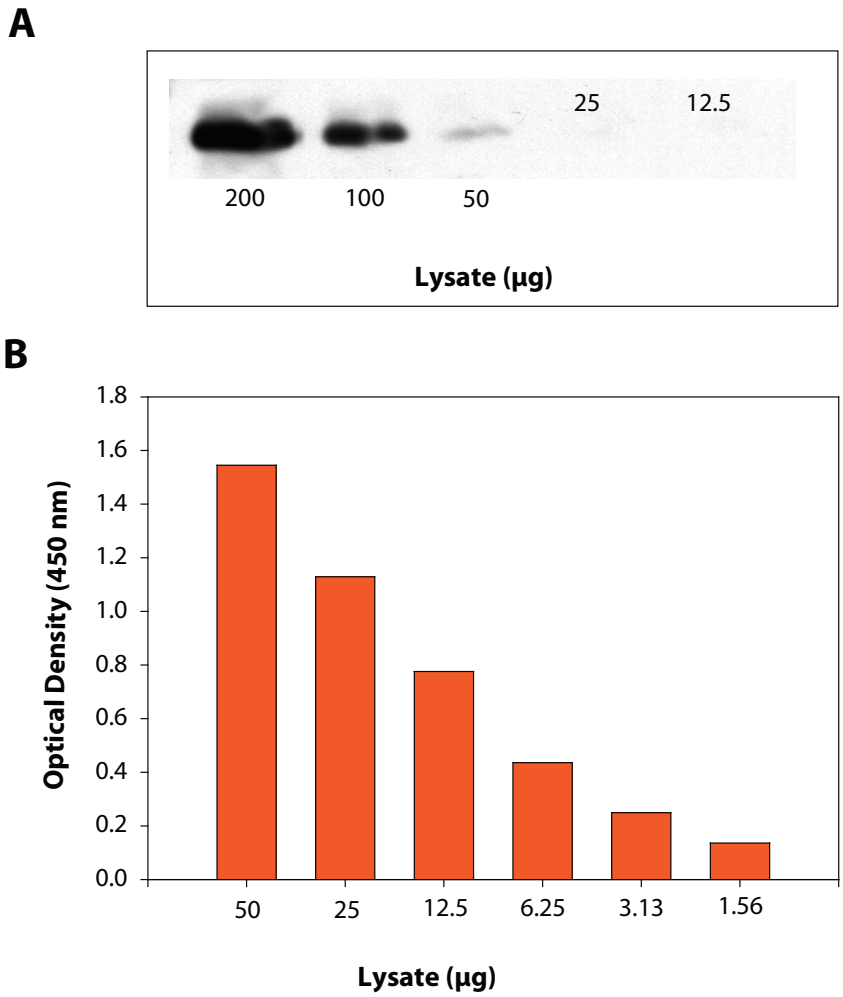


Figure 1: The Human Phospho-VEGF R3/Flt-4 DuoSet® IC ELISA is more sensitive than immunoprecipitation (IP)-Western Blot analysis. Lysates prepared from human VEGF R3 transfected NS0 cells (NS0-hVEGF R3) were serially diluted and analyzed by **(A)** IP-Western Blot and **(B)** this DuoSet® IC ELISA. IPs were done using an anti-VEGF R3 monoclonal antibody and goat anti-mouse agarose. Immunoblots were incubated with a biotinylated anti-phosphotyrosine monoclonal antibody (R&D Systems®, Catalog # BAM1676) to detect human phospho-VEGF R3. Bands were visualized with Streptavidin-HRP (R&D Systems®, Catalog # DY998) followed by chemiluminescent detection. Human Phospho-VEGF R3 can be detected in this DuoSet® IC ELISA by using approximately 10 to 15 times less lysate than is needed for a conventional IP-Western Blot.

LIGAND-INDUCED PHOSPHORYLATION

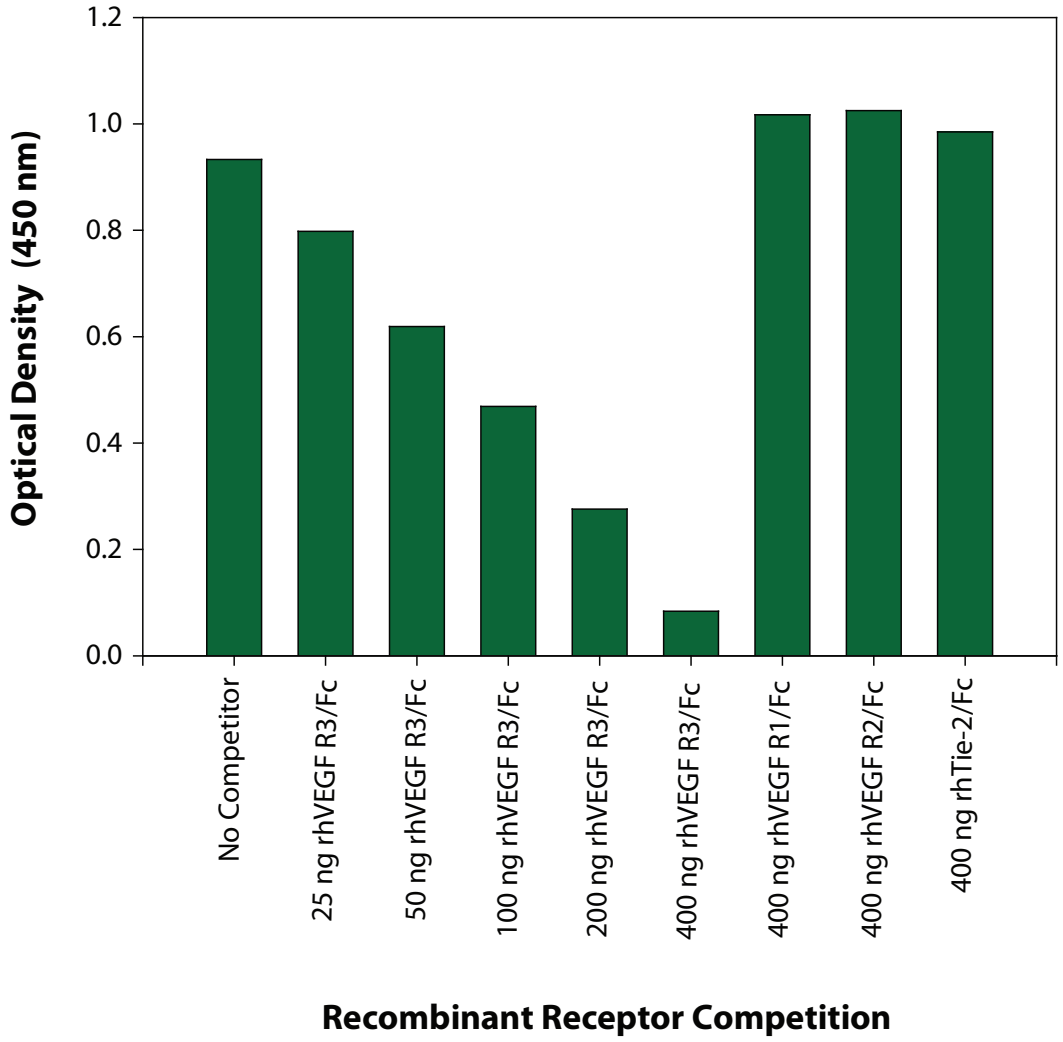
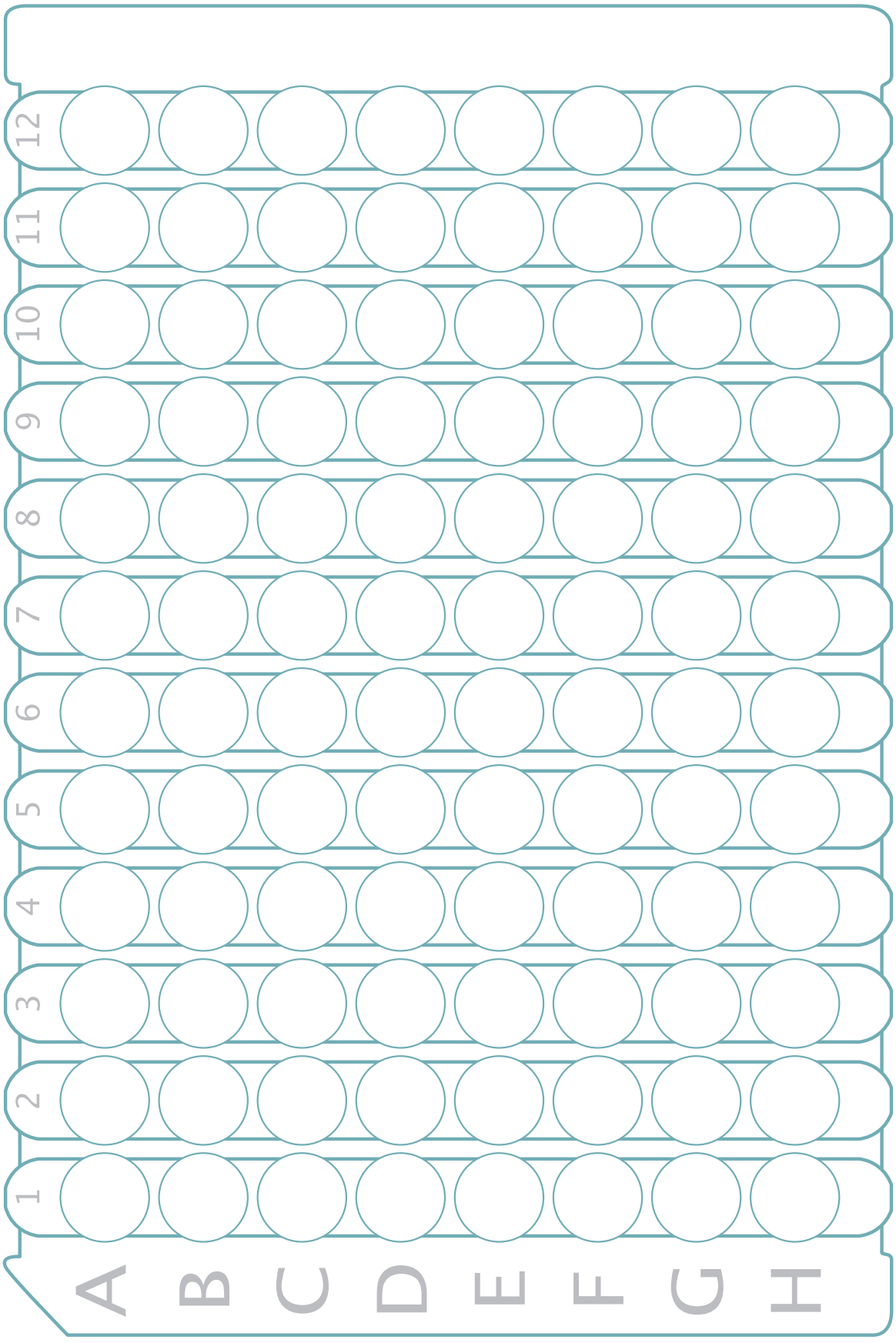


Figure 2: The specificity of the Human Phospho-VEGF R3/Flt-4 DuoSet® IC ELISA is confirmed by receptor competition. The indicated amounts of recombinant extracellular domains of human VEGF R3/Fc Chimera (R&D Systems®, Catalog # 349-F4), human VEGF R1/Fc Chimera (R&D Systems®, Catalog # 321-FL), human VEGF R2/Fc Chimera (R&D Systems®, Catalog # 357-KD) or human Tie-2/Fc Chimera (R&D Systems®, Catalog # 313-TI) were added to 20 µg of NS0-hVEGF R3 cell lysate and analyzed using this DuoSet® IC ELISA. Competition was observed only with recombinant human VEGF R3.

PLATE LAYOUT

Use this plate layout to record controls and samples assayed.



NOTES

NOTES