

# DuoSet<sup>®</sup> IC

## Human Total VEGF R3/Flt-4

Catalog Number DYC3491-2

DYC3491-5

DYC3491E

**For the development of sandwich ELISAs to measure Vascular Endothelial Growth Factor Receptor 3 (VEGF R3/Flt-4) 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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### 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 China Co. Ltd.  
24A1 Hua Min Empire Plaza  
726 West Yan An Road  
Shanghai PRC 200050

TELEPHONE: +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 Vascular Endothelial Growth Factor Receptor 3 (VEGF R3) in cell lysates. An immobilized capture antibody specific for VEGF R3, also known as Flt-4, binds both tyrosine-phosphorylated and unphosphorylated VEGF R3. After washing away unbound material, a biotinylated detection antibody specific for VEGF R3 is used to detect captured receptor, utilizing a standard Streptavidin-HRP format.

## MATERIALS PROVIDED

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

Description	Part #	Storage Conditions	Vials Provided	
			Cat. # DYC3491-2	Cat. # DYC3491-5
Total VEGF R3/Flt-4 Capture Antibody	841887	2-8° C	1	2
Total VEGF R3/Flt-4 Detection Antibody	841888	2-8° C	1	2
Total VEGF R3/Flt-4 Standard	841889	2-8° C	3	5
Streptavidin-HRP	890803	2-8° C	1	1

DYC3491-2 contains sufficient materials to run ELISAs on at least two 96 well plates.\*  
DYC3491-5 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 # DYC3491E). 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 ( $\text{Na}_3\text{VO}_4$ ) (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  $\text{Na}_2\text{HPO}_4$ , 1.5 mM  $\text{KH}_2\text{PO}_4$ , pH 7.2-7.4, 0.2  $\mu\text{m}$  filtered.

**Wash Buffer** - 0.05% Tween<sup>®</sup> 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  $\mu\text{m}$  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  $\mu\text{g}/\text{mL}$  Aprotinin, 10  $\mu\text{g}/\text{mL}$  Leupeptin.

**Substrate Solution** - 1:1 mixture of Color Reagent A ( $\text{H}_2\text{O}_2$ ) and Color Reagent B (Tetramethylbenzidine) (R&D Systems, Catalog # DY999).

**Stop Solution** - 2 N  $\text{H}_2\text{SO}_4$  (R&D Systems, Catalog # DY994).

\*The use of Reagent Diluent Concentrate 2 (10X) (R&D Systems, 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.

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## REAGENT PREPARATION

Bring all reagents to room temperature before use.

**Total VEGF R3/Flt-4 Capture Antibody** (Part 841887) - Each vial contains 360 µg/mL of mouse anti-human VEGF R3 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.\*

**Total VEGF R3/Flt-4 Detection Antibody** (Part 841888) - Each vial contains 9.0 µg/mL of biotinylated mouse anti-human VEGF R3 antibody when reconstituted with 1.0 mL of IC Diluent #1. 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.\*

**Total VEGF R3/Flt-4 Standard** (Part 841889) - Each vial contains 80 ng/mL of recombinant human VEGF R3 when reconstituted with 500 µL of IC Diluent #18. **Use a fresh Standard for each assay. Use within one hour of reconstitution.** A seven point standard curve using 2-fold serial dilutions and a high standard of 10,000 pg/mL is recommended. Use a fresh standard for each assay.

**Streptavidin-HRP** (Part 890803) - 1 mL of Streptavidin conjugated to horseradish-peroxidase. Store at 2-8° C. **DO NOT FREEZE.**

\*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  $1 \times 10^7$  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 #18.

## **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 standard reflect the environment of the samples being measured. The diluents 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

A plate layout is provided to record standards and samples assayed.

## Plate Preparation

1. Dilute the Capture Antibody to the working concentration of 2.0  $\mu\text{g}/\text{mL}$  in PBS without carrier protein. Immediately coat a 96 well microplate with 100  $\mu\text{L}$  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 two times for a total of 3 washes. Wash by filling each well with Wash Buffer (400  $\mu\text{L}$ ) 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  $\mu\text{L}$  of Block Buffer to each well. Incubate at room temperature for 1-2 hours.
4. Repeat the aspiration/wash as in step 2.

## Assay Procedure

1. Add 100  $\mu\text{L}$  of sample or standards in IC Diluent #18 per well. Use IC Diluent #18 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 10,000 pg/mL is recommended.*
2. Repeat the aspiration/wash as in step 2 of Plate Preparation.
3. Dilute the Detection Antibody to a working concentration of 250 ng/mL in IC Diluent #1 before use. Add 100  $\mu\text{L}$  of the diluted Detection Antibody to each well. Cover with a new plate sealer and incubate 2 hours at room temperature.
4. Repeat the aspiration/wash as in step 2 of Plate Preparation.
5. Immediately before use, dilute the Streptavidin-HRP to the working concentration specified on the vial label using IC Diluent #1. Add 100  $\mu\text{L}$  of the diluted Streptavidin-HRP to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.
6. Repeat the aspiration/wash as in step 2 of Plate Preparation.
7. Add 100  $\mu\text{L}$  of Substrate Solution to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.
8. Add 50  $\mu\text{L}$  of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
9. 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.

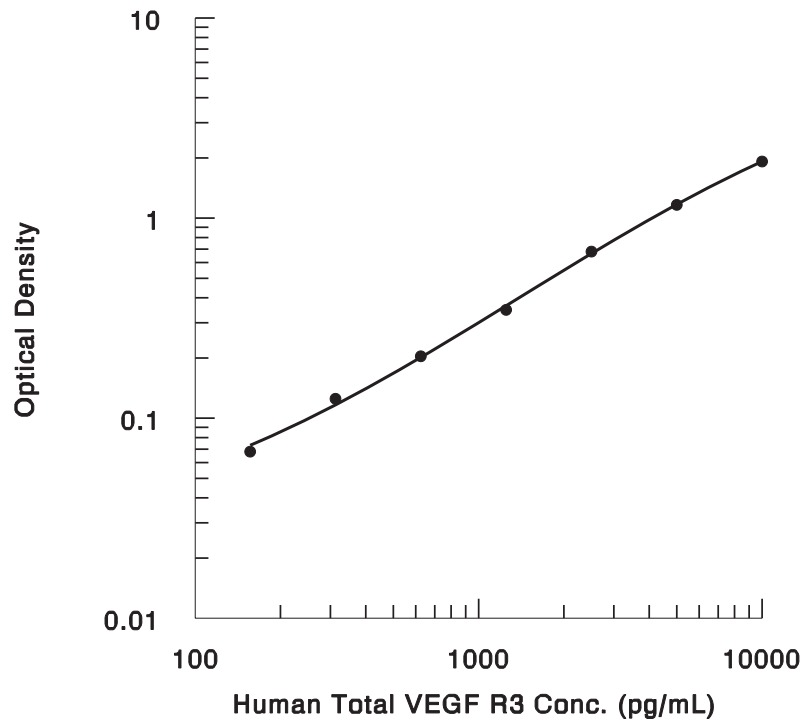
## CALCULATION OF RESULTS

Average the duplicate readings for each standard and sample, then subtract the average zero standard optical density (O.D.).

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 VEGF R3 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 the Human Total VEGF R3/Flt-4 DuoSet IC ELISA. The standard curve was calculated using a computer generated 4-PL curve-fit. This standard curve is for demonstration purposes only.



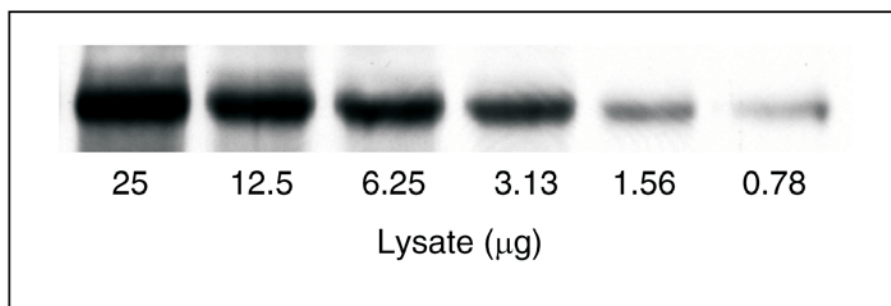
## CALIBRATION

The Human Total VEGF R3/Flt-4 DuoSet IC ELISA is calibrated against a highly purified NS0-expressed recombinant human VEGF R3/Fc Chimera produced at R&D Systems. Samples containing natural VEGF R3 showed linear dilution parallel to the standard curve obtained using the Total VEGF R3/Flt-4 Standard. These results indicate that O.D. values from this DuoSet IC ELISA can be used to determine the concentration of VEGF R3 in natural samples.

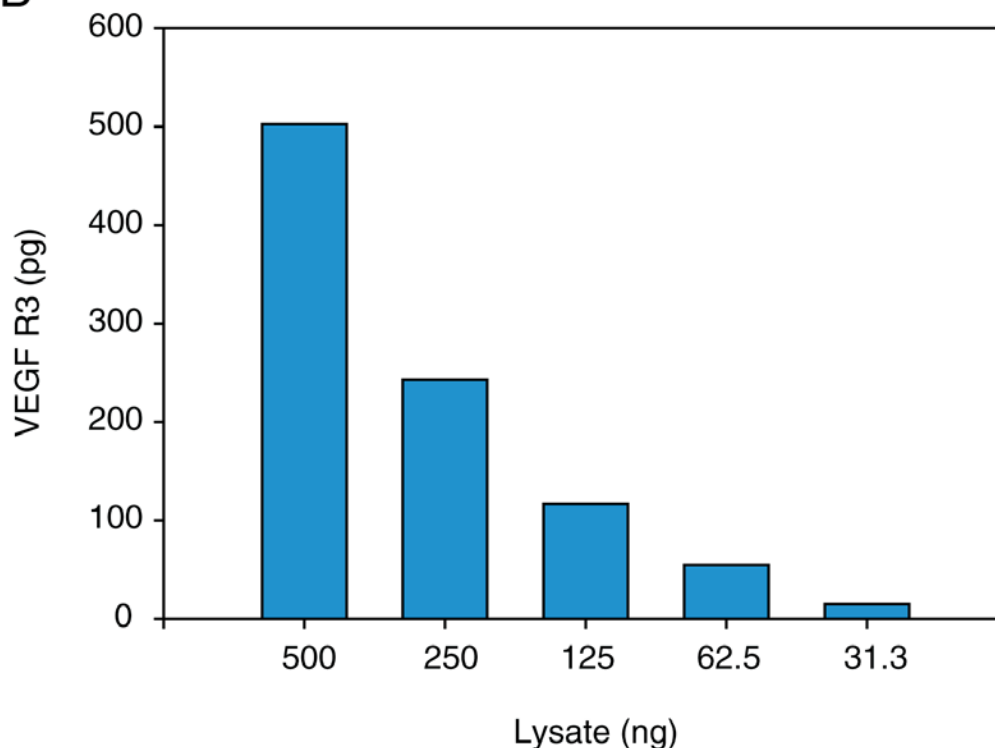


## SENSITIVITY

A



B



**Figure 1: The Human Total VEGF R3/Flt-4 DuoSet IC ELISA is more sensitive than immunoprecipitation (IP)-Western Blot analysis.** Lysates prepared from human VEGF R3 transfected NS0 mouse myeloma cells were serially diluted and analyzed by **(A)** IP-Western blot and **(B)** this DuoSet IC ELISA. IPs were performed using an anti-VEGF R3 monoclonal antibody and goat anti-mouse agarose. Immunoblots were incubated with a biotinylated anti-VEGF R3 monoclonal antibody to detect total VEGF R3. Bands were visualized with Streptavidin-HRP (R&D Systems, Catalog # DY998) followed by chemiluminescent detection. Human VEGF R3 can be detected by the Human Total VEGF R3/Flt-4 DuoSet IC ELISA by using approximately 50 times less lysate than is needed for a conventional IP-Western blot.

## SPECIFICITY

To determine specificity, recombinant human (rh) VEGF R1/Fc Chimera, rhVEGF R2/Fc Chimera, and rhTie-2/Fc Chimera were assayed at 100 ng/mL and did not cross-react or interfere in the assay.

# PLATE LAYOUT

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

1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
	<b>A</b>	<b>B</b>	<b>C</b>	<b>D</b>	<b>E</b>	<b>F</b>	<b>G</b>	<b>H</b>

# NOTES

# NOTES