DuoSet® IC

Human Phospho-FGF R3

Catalog Number DYC2719-2
DYC2719-5
DYC2719E

For the development of sandwich ELISAs to measure phosphorylated human Fibroblast Growth Factor Receptor 3 (FGF R3) 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 human FGF R3 in cell lysates. An immobilized capture antibody specific for human FGF R3 binds both phosphorylated and unphosphorylated FGF R3. After washing away unbound material, an HRP-conjugated monoclonal antibody specific for phosphorylated tyrosine is used to detect only the 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 # DYC2719-2	CATALOG # DYC2719-5	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human Phospho-FGF R3 Capture Antibody	841875	1 vial	2 vials	Store for up to 1 month at 2-8 °C or aliquot and store at ≤ -20 °C for up to 3 months in a manual defrost freezer.*
Anti-pY-HRP A	841420	1 vial	2 vials	Store for up to 3 months at 2-8 °C. **DO NOT FREEZE.**
Human Phospho-FGF R3 Control	841876	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.

DYC2719-2 contains sufficient materials to run ELISAs on at least two 96 well plates.†
DYC2719-5 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 # DYC2719E). Economy Packs contain sufficient materials to run ELISAs on 15 microplates.†
Specific vial counts of each component may vary. 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 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
- 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 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 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-FGF R3 Capture Antibody (Part 841875) - Each vial contains 1440 μg/mL of mouse anti-human FGF R3 antibody when reconstituted with 200 μL of PBS.

Anti-pY-HRP A (Part 841420) - Each vial contains 50 μL of mouse anti-phospho-tyrosine antibody conjugated to HRP. Immediately before use, dilute the Anti-pY-HRP A 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.

Human Phospho-FGF R3 Control (Part 841876) **- Reconstitute with a recommended volume of 500 μL of IC Diluent #12 to produce a stock solution. Refer to the vial label for the concentration of recombinant human phosphorylated FGF R3.** A control concentration of 5000 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 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 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 SDS on our website prior to use.

TECHNICAL HINTS & 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 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 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 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 5000 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 A 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 the 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 (O.D.).

SENSITIVITY

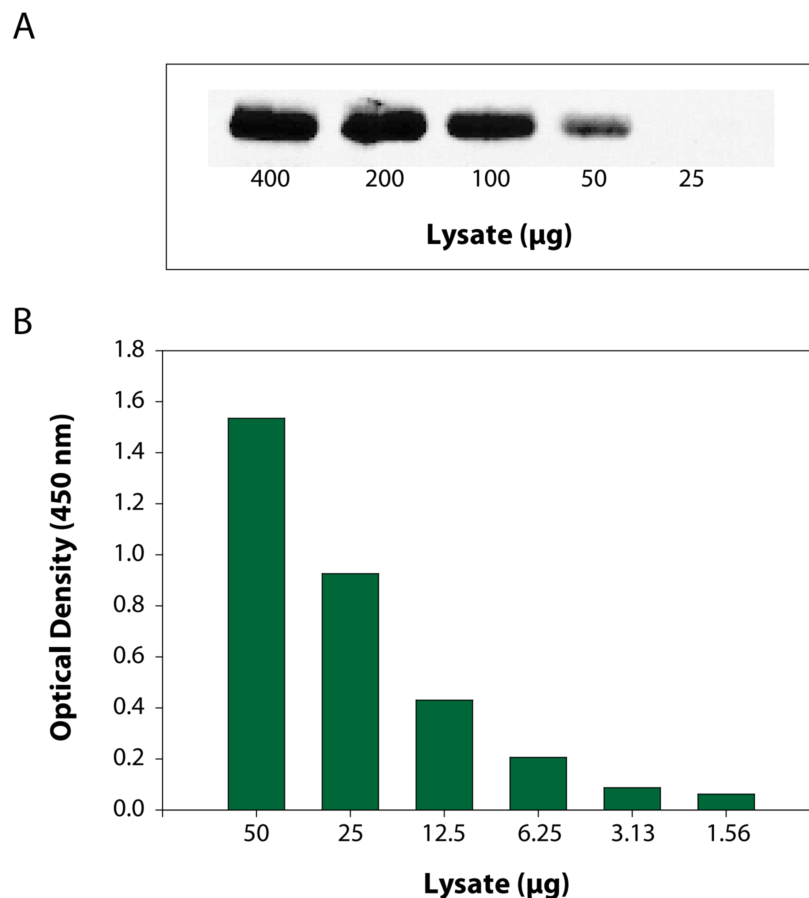

Figure 1: The Human Phospho-FGF R3 DuoSet® IC ELISA is more sensitive than immunoprecipitation (IP)-Western Blot analysis. Human FGF R3 transfected NS0 cells were treated with 100 ng/mL of recombinant human FGF acidic (R&D Systems®, Catalog # 232-FA) and 1.0 µg/mL heparin for 15 minutes to induce tyrosine phosphorylation of FGF R3. Serial dilutions of lysates were analyzed by **(A)** IP-Western Blot and **(B)** this DuoSet® IC ELISA. IPs were done using an anti-FGF R3 monoclonal antibody and goat anti-mouse agarose. Immunoblots were incubated with a biotinylated Anti-Phospho-Tyrosine monoclonal antibody (R&D Systems®, Catalog # BAM1676) to detect phospho-FGF R3. Bands were visualized with Streptavidin-HRP (R&D Systems®, Catalog # DY998) followed by chemiluminescent detection. Human Phospho-FGF R3 can be detected in this DuoSet® IC ELISA by using approximately 4-8 times less lysate than is needed for a conventional IP-Western Blot.

LIGAND-INDUCED PHOSPHORYLATION

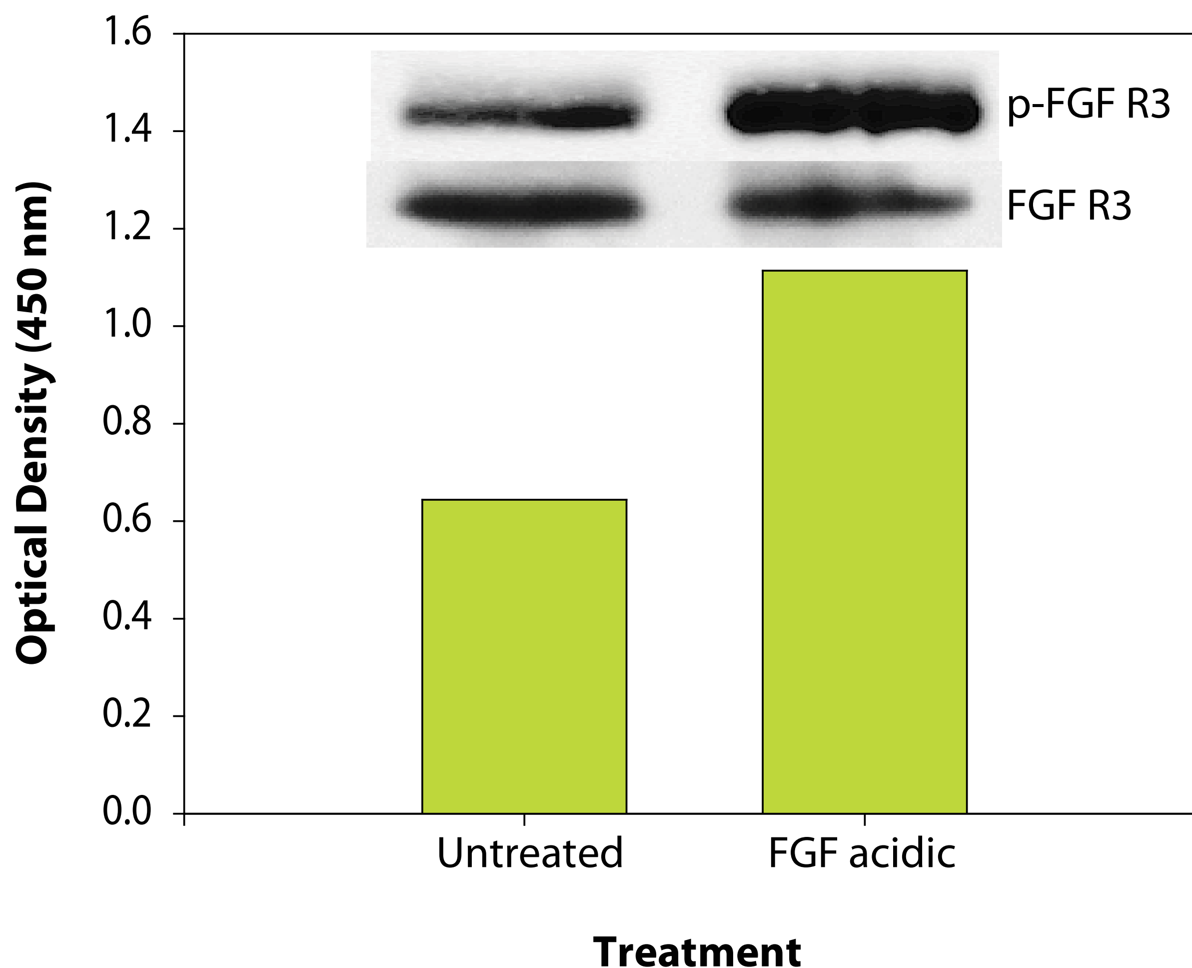

Figure 2: The Human Phospho-FGF R3 DuoSet® IC ELISA detects ligand-induced FGF R3 tyrosine phosphorylation. NS0-expressed human FGF R3 cells were untreated or treated with 100 ng/mL of recombinant human FGF acidic and 1.0 µg/mL heparin for 15 minutes. ELISA and IP-Western Blot (inset) analyses were done using 50 µg and 400 µg of lysate, respectively. IP-Western Blots for phospho-FGF R3 (p-FGF R3) were done as described in Figure 1. Blots were stripped and total FGF R3 was detected using a biotinylated anti-FGF R3 monoclonal antibody.

SPECIFICITY

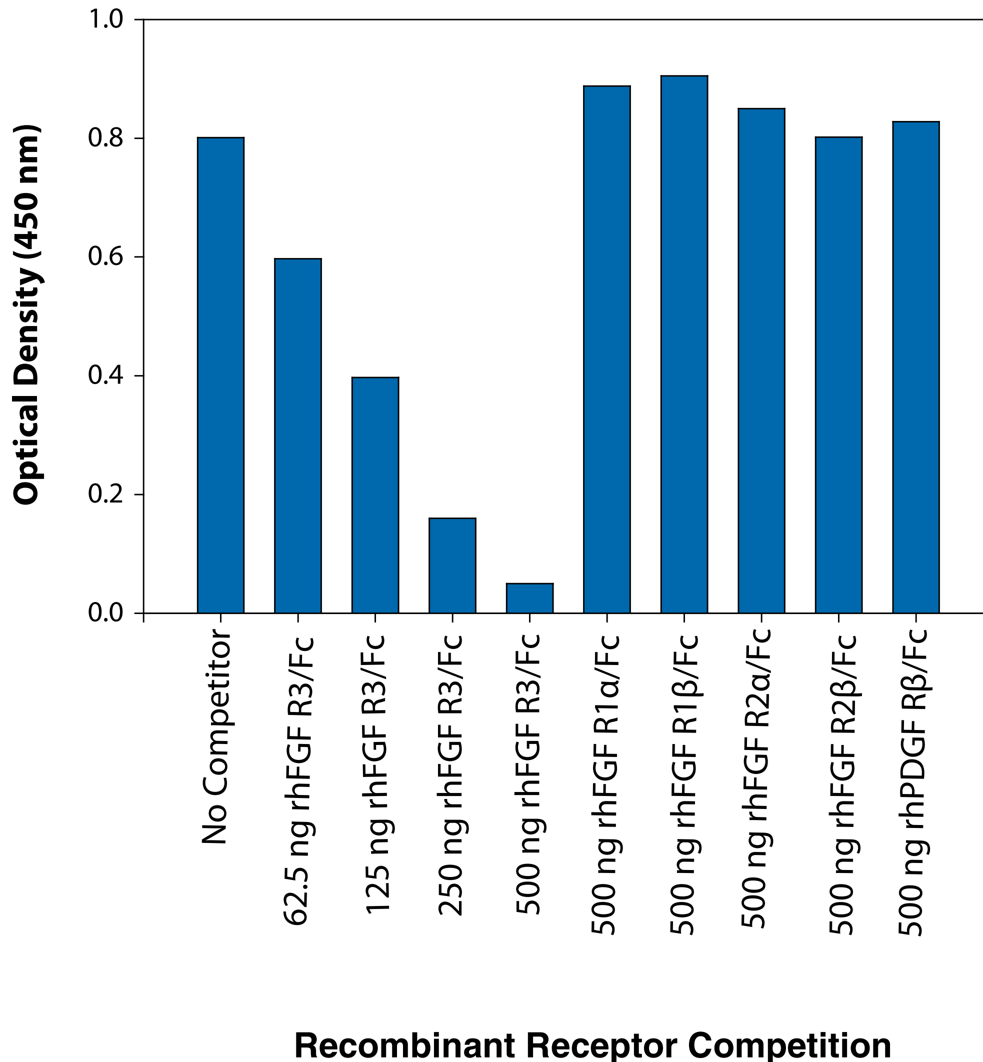

Figure 3: The specificity of the Human Phospho-FGF R3 DuoSet® IC ELISA is confirmed by receptor competition. NS0-expressed human FGF R3 cells were treated with 100 ng/mL of recombinant human FGF acidic and 1.0 µg/mL heparin for 15 minutes. The indicated amounts of recombinant extracellular domains of human FGF R3/Fc Chimera (R&D Systems®, Catalog # 766-FR), human FGF R1α/Fc Chimera (R&D Systems®, Catalog # 658-FR), human FGF R1β/Fc Chimera (R&D Systems®, Catalog # 661-FR), human FGF R2α/Fc Chimera (R&D Systems®, Catalog # 712-FR), human FGF R2β/Fc Chimera (R&D Systems®, Catalog # 684-FR) or human PDGF Rβ/Fc Chimera (R&D Systems®, Catalog # 385-PR) were added to 50 µg of lysate and analyzed using this DuoSet® IC ELISA. Competition was observed only with recombinant human FGF R3.

PLATE LAYOUT

Use this plate layout to record controls and samples assayed.

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

NOTES

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