

CALCULATION OF RESULTS

Average the duplicate readings for each control and sample then subtract the average blank optical density (O.D.).

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 controls and samples 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.

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.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

NOTES

DuoSet™ IC ELISA INTRACELLULAR

Human Phospho-EphB4

Catalog Number: **DYC4057-2** (2 plates)
DYC4057-5 (5 plates)

INTENDED USE

For the development of sandwich ELISAs to measure phosphorylated human EphB4 in cell lysates.

PRINCIPLE OF THE ASSAY

This DuoSet IC ELISA contains the basic components required for the development of sandwich ELISAs to measure tyrosine-phosphorylated human EphB4 in cell lysates. An immobilized capture antibody specific for human EphB4 binds both phosphorylated and unphosphorylated EphB4. After washing away unbound material, a HRP-conjugated detection antibody specific for phosphorylated tyrosine is used to detect only the phosphorylated receptor, utilizing a standard HRP format.

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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MATERIALS PROVIDED & STORAGE CONDITIONS

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

DESCRIPTION	PART #	CATALOG # DYC4057-2	CATALOG # DYC4057-5	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human Phospho-EphB4 Capture Antibody	842424	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 C	841913	1 vial	2 vials	Store for up to 3 months at 2-8 °C. * DO NOT FREEZE.
Human Phospho-EphB4 Control	842425	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.

DYC4057-2 contains sufficient materials to run ELISAs on at least two 96 well plates.[†]
DYC4057-5 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.
- 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 Azide (NaN₃) (Sigma # S2002)
- Sodium Orthovanadate (Na₃VO₄) (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 Na₂HPO₄, 1.5 mM KH₂PO₄, 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% NaN₃ 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: ELISA TMB Substrate (R&D Systems, Catalog # DY999B)

Stop Solution - 2N H₂SO₄ (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-EphB4 Capture Antibody (Part 842424) - Each vial contains 144 µg/mL of goat anti-human EphB4 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 #14. Prepare only as much Anti-pY-HRP C as required to run each assay.

Human Phospho-EphB4 Control (Part 842425) - **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 EphB4.** 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 10⁷ 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.

GENERAL ELISA PROTOCOL

Plate Preparation

1. Dilute the capture antibody to a working concentration of 0.8 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 500 pg/mL is recommended.*
2. Repeat the aspiration/wash as in step 2 of the 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 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.