

CALCULATION OF RESULTS

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

REFERENCES

1. Alroy, I. and Y. Yarden (1997) FEBS Lett. **410**:83.
2. Normanno, N. *et al.* (2003) Endocr. Relat. Cancer **10**:1.
3. Riedemann, J. *et al.* (2007) Biochem. Biophys. Res. Commun. **355**:707.

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 control 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.
- Tyrosine-phosphorylated ErbB family members and other receptor tyrosine kinases that heterodimerize with EGFR may not be dissociated by Lysis Buffer #9, potentially contributing to the optical density obtained using this DuoSet IC ELISA.

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.

DuoSet™ IC ELISA
INTRACELLULAR

Human Phospho-EGFR

Catalog Number: DYC1095B-2 (2 plates)
DYC1095B-5 (5 plates)
DYC1095BE (15 plates)

INTENDED USE

For the development of sandwich ELISAs to measure phosphorylated human Epidermal Growth Factor Receptor (EGFR) 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 Epidermal Growth Factor Receptor (phospho-EGFR) in cell lysates. An immobilized capture antibody specific for human EGFR binds both phosphorylated and unphosphorylated EGFR. After washing away unbound material, an HRP-conjugated monoclonal antibody specific for phosphorylated tyrosine is used to detect only the phosphorylated protein, utilizing a standard HRP format.

Note: Depending on the cell line and treatment conditions, ErbB family receptors may form heterodimers (1, 2). Any tyrosine-phosphorylated ErbB receptor or other receptor tyrosine kinase (3) that has heterodimerized with EGFR may not dissociate during the preparation of cell lysate samples and therefore may also be detected using this DuoSet IC ELISA.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

Manufactured and Distributed by:

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

Distributed by:

Europe | Middle East | Africa Bio-Techne Ltd.
19 Barton Lane Abingdon Science Park, Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449 FAX: +44 (0)1235 533420
E-MAIL: info.emea@bio-techne.com

China

 Bio-Techne China Co., Ltd.

Unit 1901, Tower 3, Raffles City Changning Office,
1193 Changning Road, Shanghai PRC 200051
TEL: +86 (21) 52380373 (400) 821-3475 FAX: +86 (21) 52371001
E-MAIL: info.cn@bio-techne.com

MATERIALS PROVIDED & STORAGE CONDITIONS

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

DESCRIPTION	PART #	CATALOG # DYC1095B-2	CATALOG # DYC1095B-5	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human Phospho-EGFR Capture Antibody	843169	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-EGFR Control	841404	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.

DYC1095B-2 contains sufficient materials to run ELISAs on at least two 96 well plates.[†]
DYC1095B-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®, # DYC1095BE). 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.
- 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, # DY990)
- Plate sealers (R&D Systems, # 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, # DY006)

Wash Buffer - 0.05% Tween® 20 in PBS, pH 7.2-7.4 (R&D Systems, # 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, # DY999B)

Stop Solution - 2N H₂SO₄ (R&D Systems, # 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, # DYC002), prepared as described in the DYC002 insert.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Human Phospho-EGFR Capture Antibody (Part 843169) - Each vial contains 1440 µg/mL of rat anti-human EGFR 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 using IC Diluent #14. Prepare only as much Anti-pY-HRP C as required to run each assay.

Human Phospho-EGFR Control (Part 841404) - **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 EGFR.** A control concentration of 20 ng/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 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 two times for a total of 3 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 20 ng/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.
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.