

# DuoSet<sup>®</sup> IC

## Human Total Insulin R

Catalog Number **DYC1544-2**  
**DYC1544-5**

For the development of sandwich ELISAs to measure human Insulin Receptor (Insulin R) 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

SECTION	PAGE
PRINCIPLE OF THE ASSAY.....	1
MATERIALS PROVIDED & STORAGE CONDITIONS .....	1
OTHER MATERIALS REQUIRED .....	2
SOLUTIONS REQUIRED.....	2
REAGENT PREPARATION.....	3
PREPARATION OF SAMPLES.....	3
PRECAUTIONS.....	4
TECHNICAL HINTS AND LIMITATIONS.....	4
GENERAL ELISA PROTOCOL .....	5
CALCULATION OF RESULTS .....	6
TYPICAL DATA.....	6
CALIBRATION .....	6
SENSITIVITY .....	7
QUANTIFICATION .....	8
QUANTIFICATION .....	8
PLATE LAYOUT .....	9

## 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](mailto: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](mailto: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](mailto:info.cn@bio-techne.com)

## PRINCIPLE OF THE ASSAY

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

## MATERIALS PROVIDED & STORAGE CONDITIONS

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

DESCRIPTION	PART #	CATALOG # DYC1544-2	CATALOG # DYC1544-5	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human Total Insulin R Capture Antibody	841872	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.*
Human Total Insulin R Detection Antibody	841873	1 vial	2 vials	
Human Total Insulin R Standard	841874	3 vials	5 vials	<b>Use within one hour of reconstitution. Use a fresh standard for each assay.</b>
Streptavidin-HRP A	890803	1 vial	1 vial	Store for up to 3 months at 2-8 °C.* <b>DO NOT FREEZE.</b>

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

DYC1544-2 contains sufficient materials to run ELISAs on at least two 96 well plates.†

DYC1544-5 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 Azide ( $\text{NaN}_3$ ) (Sigma # S2002)
- Sodium Orthovanadate ( $\text{Na}_3\text{VO}_4$ ) (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  $\text{Na}_2\text{HPO}_4$ , 1.5 mM  $\text{KH}_2\text{PO}_4$ , pH 7.2-7.4, 0.2  $\mu\text{m}$  filtered (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%  $\text{NaN}_3$ , 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  $\mu\text{g}/\text{mL}$  Aprotinin, and 10  $\mu\text{g}/\text{mL}$  Leupeptin.

**Note:** *Lysis Buffer #9 consists of IC Diluent #12 plus 10  $\mu\text{g}/\text{mL}$  Aprotinin and 10  $\mu\text{g}/\text{mL}$  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 ( $\text{H}_2\text{O}_2$ ) 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 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 package insert.

## REAGENT PREPARATION

**Bring all reagents to room temperature before use.**

**Human Total Insulin R Capture Antibody** (Part 841872) - Each vial contains 1440 µg/mL of mouse anti-human Insulin R antibody when reconstituted with 200 µL of PBS.

**Human Total Insulin R Detection Antibody** (Part 841873) - Each vial contains 36 µg/mL of biotinylated mouse anti-human Insulin R antibody when reconstituted with 1.0 mL of IC Diluent #14. Immediately before use, dilute the detection antibody to a working concentration of 1.0 µg/mL in IC Diluent #14. Prepare only as much detection antibody as required to run each assay.

**Human Total Insulin R Standard** (Part 841874) - **Refer to the vial label for the stock concentration of recombinant human Insulin R when reconstituted with 500 µL of IC Diluent #12.** A seven point standard curve using 2-fold serial dilutions and a high standard of 60,000 pg/mL is recommended.

**Streptavidin-HRP A** (Part 890803) - 1.0 mL of Streptavidin conjugated to horseradish-peroxidase. Immediately before use, dilute the Streptavidin-HRP A to the working concentration specified on the vial label using IC Diluent #14.

## 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  $\leq -70$  °C. Before use, centrifuge samples at 2000 x g for 5 minutes and transfer the supernate into 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 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 standards and sample 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 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 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 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 auto washer. 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 standards or sample in IC Diluent #12 per well. Use IC Diluent #12 as the zero standard. Cover with a plate sealer and incubate for 2 hours at room temperature.  
**Note:** *A seven point standard curve using 2-fold serial dilutions and a high standard of 60,000 pg/mL is recommended.*
2. Repeat the aspiration/wash as in step 2 of the Plate Preparation.
3. Add 100 µL of the diluted detection antibody to each well. Cover with a new plate sealer and incubate for 2 hours at room temperature.
4. Repeat the aspiration/wash as in step 2 of Plate Preparation.
5. Add 100 µL of the diluted Streptavidin-HRP A 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 the Plate Preparation.
7. Add 100 µL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.
8. Add 50 µL 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.

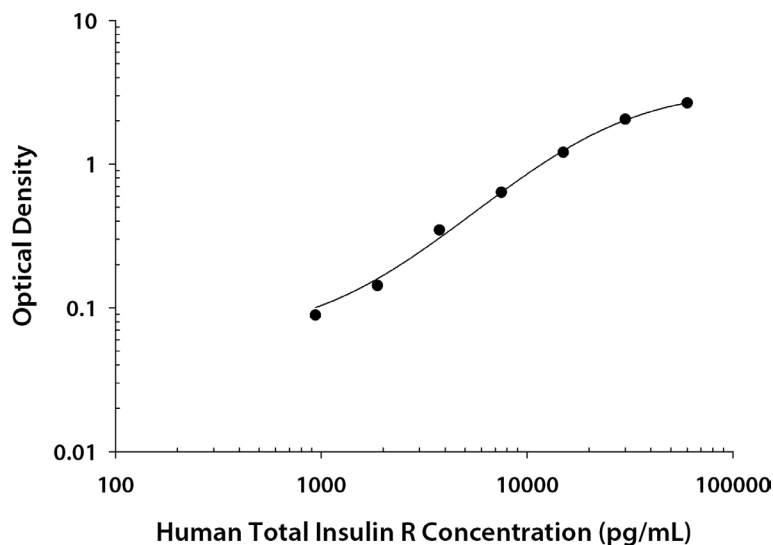
## CALCULATION OF RESULTS

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 human Insulin R 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 Insulin R DuoSet® IC ELISA. The standard curve was calculated using a computer generated 4-PL curve-fit. This standard curve is for demonstration purposes only.

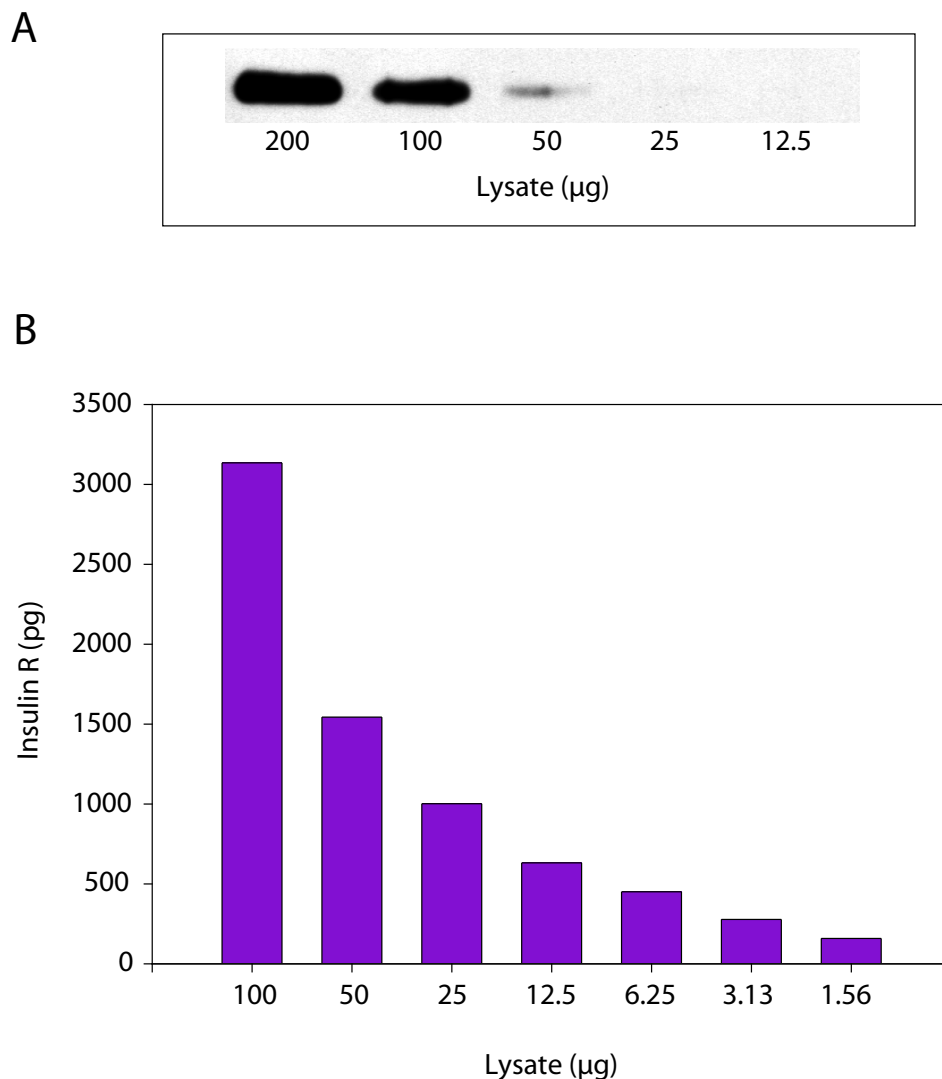


## CALIBRATION

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

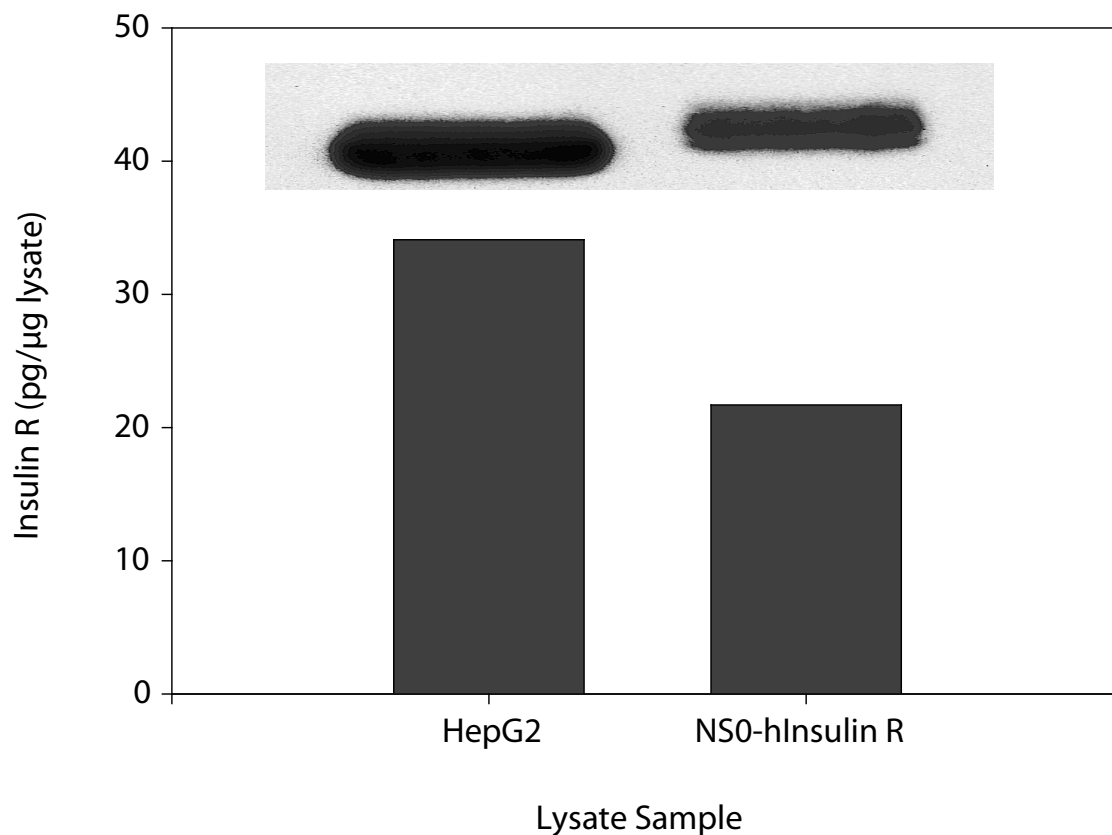


## SENSITIVITY



**Figure 1: The Human Total Insulin R DuoSet<sup>®</sup> IC ELISA is more sensitive than immunoprecipitation (IP)-Western Blot analysis.** Lysates prepared from the HepG2 human hepatocellular carcinoma cell line were serially diluted and analyzed by **(A)** IP-Western Blot and **(B)** this DuoSet<sup>®</sup> IC ELISA. IPs were performed using an anti-Insulin R monoclonal antibody and goat anti-mouse agarose Immunoblots were incubated with a biotinylated anti-Insulin R polyclonal antibody to detect total Insulin R. Bands were visualized with goat anti-rabbit-HRP (R&D Systems<sup>®</sup>, Catalog # HAF008) followed by chemiluminescent detection. Human Insulin R can be detected in the Human Total Insulin R DuoSet<sup>®</sup> IC ELISA by using approximately 4-6 times less lysate than is needed for a conventional IP-Western Blot.

## QUANTIFICATION



**Figure 2: The Human Total Insulin R DuoSet® IC ELISA measures the relative level of Insulin R.** Lysates were prepared from HepG2 human hepatocellular carcinoma cells and NS0 cells transfected with human Insulin R. ELISA and IP-Western Blot (inset) analyses were performed using 100 μg and 400 μg of lysate, respectively. The IP-Western Blot was performed as described in Figure 1.

## QUANTIFICATION

To determine specificity, recombinant human (rh) Axl/Fc Chimera, rhHGF R/Fc Chimera, and rhIGF-I R/Fc Chimera were assayed at 400 ng/mL and did not cross-react or interfere in the assay.

## PLATE LAYOUT

Use this plate layout to record standards and samples assayed.

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

## NOTES

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