DuoSet® IC

Human Phospho-Epo R (Y426)

Catalog Number DYC6926-2
DYC6926-5
DYC6926E

For the development of sandwich ELISAs to measure Erythropoietin Receptor (Epo R) phosphorylated at Y426 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 Erythropoietin Receptor (Epo R) phosphorylated at Y426 in cell lysates. An immobilized capture antibody specific for Epo R binds both phosphorylated and unphosphorylated protein. After washing away unbound material, a biotinylated detection antibody recognizing Epo R phosphorylated at Y426 is used to detect only phosphorylated protein, 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 # DYC6926-2	CATALOG # DYC6926-5	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Phospho-Epo R (Y426) Capture Antibody	843253	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.*
Phospho-Epo R (Y426) Detection Antibody	843254	1 vial	2 vials	
Phospho-Epo R (Y426) Standard	843255	3 vials	5 vials	**Use within one hour of reconstitution. Use a fresh standard for each assay.**
Streptavidin-HRP	890803	1 vial	1 vial	Store at 2-8 °C. **DO NOT FREEZE.**

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

DYC6926-2 contains sufficient materials to run ELISAs on at least two 96 well plates.†
DYC6926-5 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 # DYC6926E). 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 5.
- 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 Azide (NaN_3) (Sigma # S2002)
- Sodium Orthovanadate (Na_3VO_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 Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2-7.4, 0.2 µm filtered.

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.

**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.

Phospho-Epo R (Y426) Capture Antibody (Part 843253) - Each vial contains 1080 µg/mL of mouse anti-human Epo R antibody when reconstituted with 200 µL of PBS.

Phospho-Epo R (Y426) Detection Antibody (Part 843254) - Each vial contains 36 µg/mL of biotinylated mouse anti-human phospho-Epo R (Y426) antibody when reconstituted with 1.0 mL of IC Diluent #14.

Phospho-Epo R (Y426) Standard (Part 843255) - Each vial contains 130 ng/mL of recombinant human phospho-Epo R (Y426) when reconstituted with 500 µL of IC Diluent #12. A seven point curve using 2-fold serial dilutions and a high standard of 10,000 pg/mL is recommended.

Streptavidin-HRP (Part 890803) - 1.0 mL of streptavidin conjugated to horseradish-peroxidase.

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.

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 diluent 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

Plate Preparation

1. Dilute the Capture Antibody to a working concentration of 6.0 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 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 standard in IC Diluent #12 per well. Use IC Diluent #12 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. 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. Add 100 µL 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 #14. Add 100 µL 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 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.

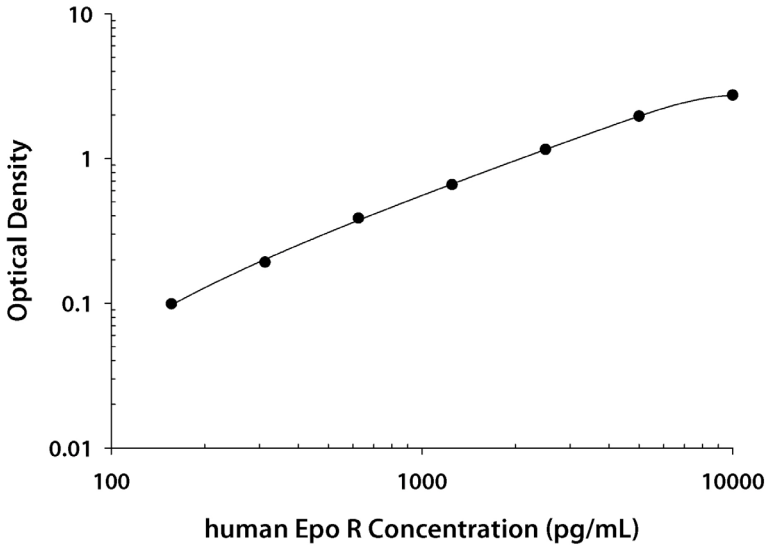
CALCULATION OF RESULTS

Average the duplicate readings for each standard and sample, then subtract the average zero standard optical density (O.D.). Results may be normalized to total protein or cell number.

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 phospho-Epo R (Y426) 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 this Human Phospho-Epo R (Y426) 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 Phospho-Epo R (Y426) DuoSet IC ELISA is calibrated against a highly purified NS0-expressed recombinant human phospho-Epo R (Y426) produced at R&D Systems. Samples containing natural phospho-Epo R (Y426) showed linear dilution parallel to the standard curve obtained using the Phospho-Epo R (Y426) Standard. These results indicate that O.D. values from this DuoSet IC ELISA can be used to determine the relative concentration of Phospho-Epo R (Y426) in natural samples.

SPECIFICITY

To determine specificity, recombinant human (rh) Epo R, rhGrowth Hormone R/Fc Chimera, rhIL-4 Rα/Fc Chimera, rhIL-6 Rα, and rhProlactin R/Fc Chimera were assayed at 100 ng/mL and did not cross-react or interfere in the assay.

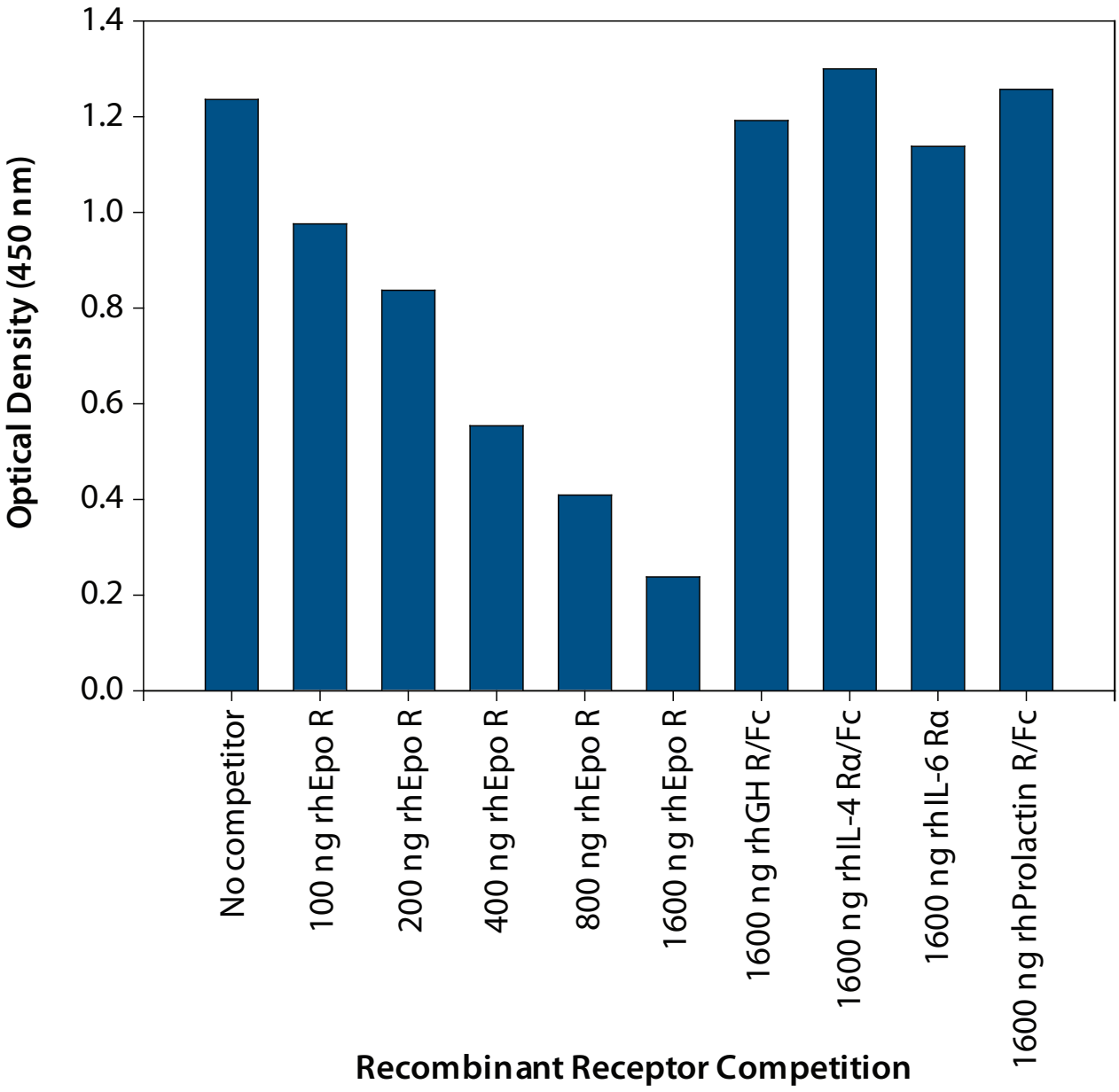


Figure 1: The specificity of the Human Phospho-Epo R (Y426) DuoSet IC ELISA is confirmed by receptor competition. UT-7 cells were treated with 200 ng/mL of rhEpo for 10 minutes. The indicated amounts of recombinant extracellular domains of rhEpo R (R&D Systems, Catalog # 307-ER), rhGrowth Hormone R (GH R)/Fc Chimera (R&D Systems, Catalog # 1210-GR), rhIL-4 Rα/Fc Chimera (R&D Systems, Catalog # 604-4R), rhIL-6 Rα (R&D Systems, Catalog # 227-SR) or rhProlactin R/Fc Chimera (R&D Systems, Catalog # 1167-PR) were added to 100 µg of lysate and analyzed using this DuoSet IC ELISA. Competition was observed only with rhEpo R.

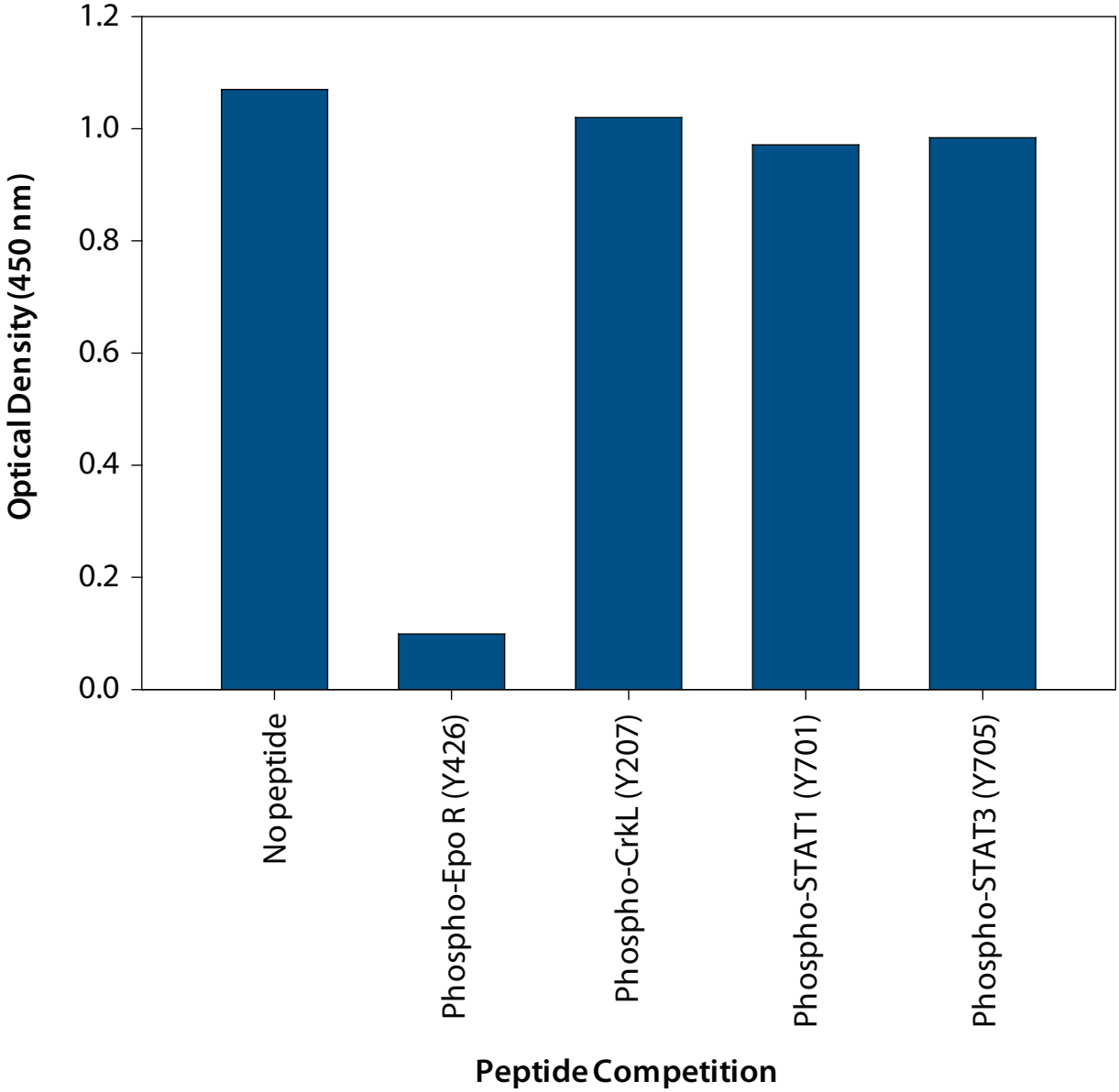


Figure 2: The specificity of the Human Phospho-Epo R (Y426) DuoSet IC ELISA is confirmed by phosphopeptide competition. UT-7 human acute myeloid leukemia cells were treated with 200 ng/mL of rhEpo (R&D Systems, Catalog # 287-TC) for 10 minutes to induce tyrosine phosphorylation of Epo R. The phospho-Epo R (Y426) Detection Antibody was either untreated (no peptide) or pre-incubated with phosphopeptide containing either the Epo R (Y426), CrkL (Y207), STAT1 (Y701), or STAT3 (Y705) phosphorylation sites. Peptides were used at 40 ng/mL. Only the phosphopeptide containing the Epo R Y426 phosphorylation site blocked the signal, suggesting that the ELISA is specific for Epo R phosphorylated at Y426.

SENSITIVITY

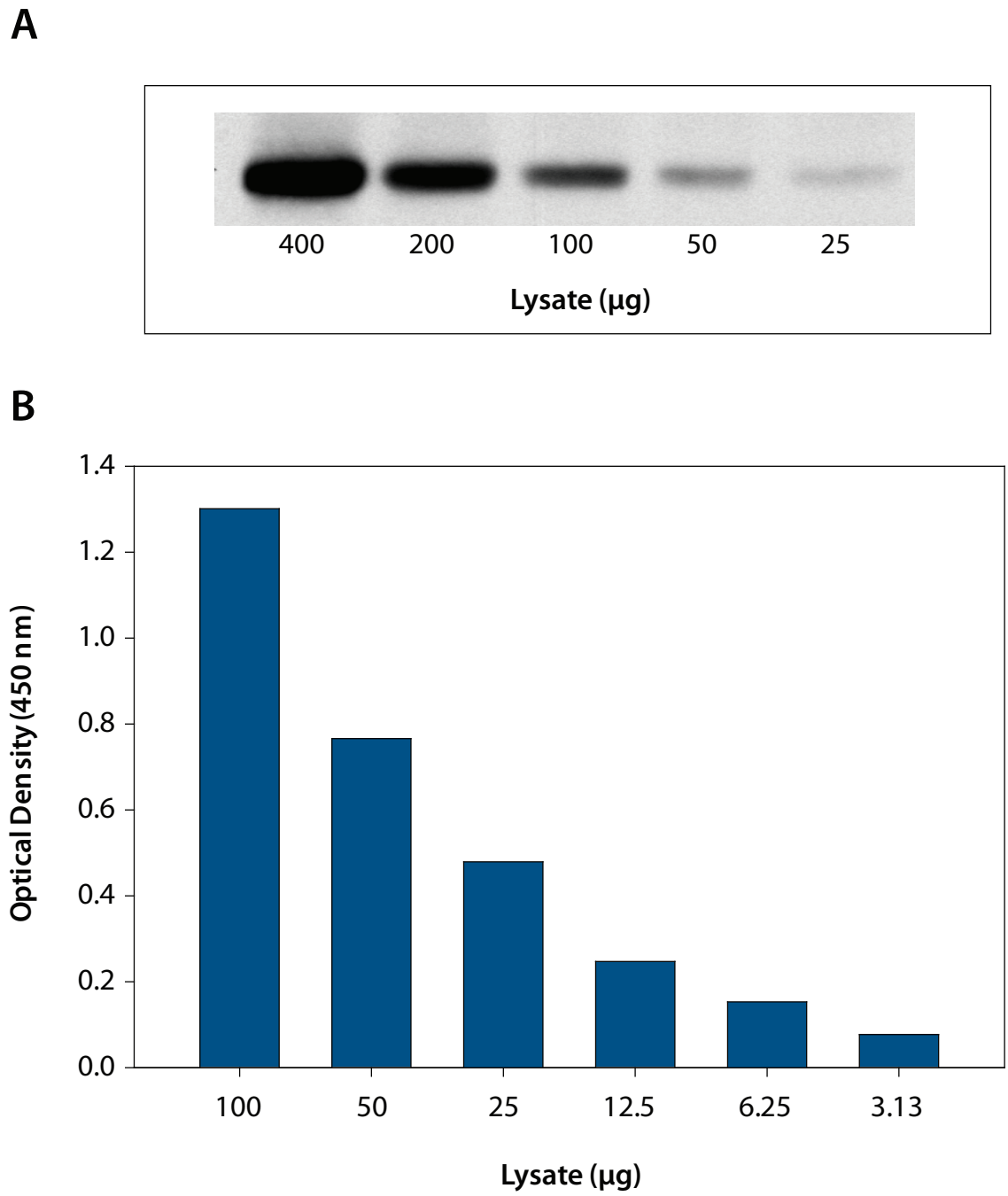


Figure 3: The Human Phospho-Epo R (Y426) DuoSet IC ELISA is more sensitive than immunoprecipitation (IP)-Western blot analysis. UT-7 cells were treated with 200 ng/mL of rhEpo for 10 minutes. Lysates were serially diluted and analyzed by **(A)** IP-Western blot and **(B)** this DuoSet IC ELISA. IPs were done using an anti-Epo R monoclonal antibody and anti-mouse IgG agarose. Immunoblots were incubated with a biotin-conjugated anti-phospho-Epo R (Y426) monoclonal antibody to detect phospho-Epo R (Y426). Bands were visualized by chemiluminescent detection. Human Phospho-Epo R (Y426) can be detected in this DuoSet IC ELISA by using approximately 2 to 4 times less lysate than is needed for a conventional IP-Western blot.

QUANTIFICATION

Amounts of human phosphorylated Epo R, as quantified by the Human Phospho-Epo R (Y426) DuoSet IC ELISA, are consistent with the relative amounts of phosphorylated Epo R determined by qualitative Western blot analysis.

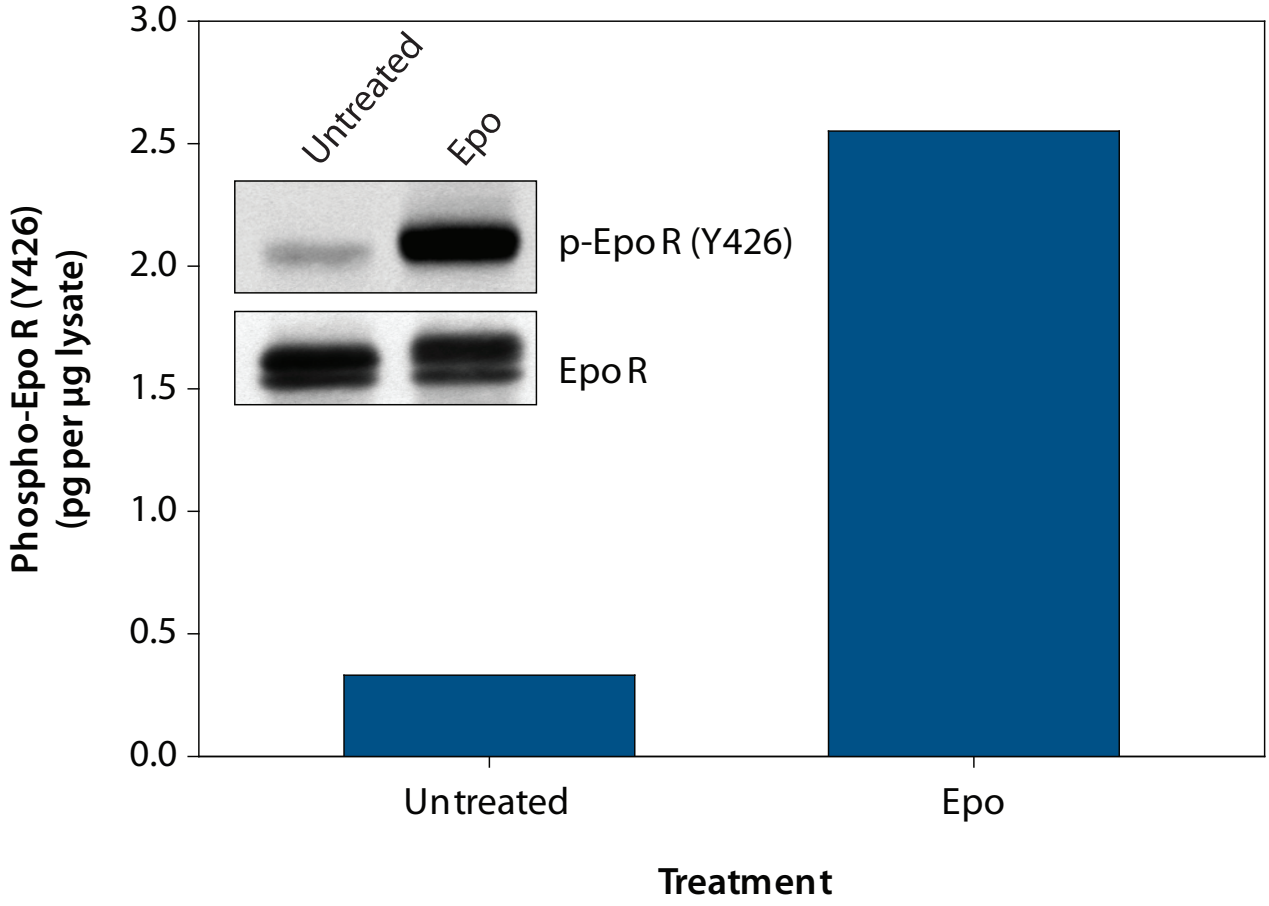


Figure 4: The Human Phospho-Epo R (Y426) DuoSet IC ELISA detects Epo R tyrosine phosphorylation. UT-7 cells were treated with 200 ng/mL of rhEpo for 10 minutes. ELISA and IP-Western blot (inset) analyses were done using 100 µg and 400 µg of lysate, respectively. IP-Western blots for phospho-Epo R (Y426) (p-Epo R Y426) were done as described in Figure 3. Blots were stripped and total Epo R was detected using a biotinylated anti-Epo R polyclonal antibody (R&D Systems, Catalog # BAF307).