ELISA Kit
Catalog #KHO0501

# IGF-1R

**[pYpY1135/1136]**

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## INTRODUCTION

Insulin-like growth factor-1 receptor (IGF-1R, also known as CD221), a member of the tyrosine kinase superfamily, is a broadly expressed transmembrane receptor that plays a key role in supporting cell growth and differentiation, and imparts resistance to apoptosis. Human IGF-1R is encoded by a single gene, located on chromosome 15. As with the structurally homologous insulin receptor (IR), IGF-1R is synthesized as a single polypeptide that is glycosylated and proteolytically cleaved to yield a disulfide-linked tetrameric receptor composed of two α subunits and two β subunits, arranged in the configuration α-β-β-α. IGF-1R's α subunits (135 kDa) mediate ligand binding, and are entirely extracellular. IGF-1R's β subunits (90 kDa) each possess an extracellular domain, a single transmembrane domain, and a cytoplasmic portion. IGF-1R's cytoplasmic portion contains three domains that bear homology with insulin receptor, including the juxtamembrane domain (61% homologous to IR), the cytoplasmic tyrosine kinase domain (84% homologous to IR) and the C terminal domain (44% homologous with IR).

Three polypeptide ligands for IGF-1R have been identified: IGF-1, IGF-2, and insulin. Of these three ligands, IGF-1 (produced constitutively by liver, kidney, lung, heart, fat, and glandular tissues) binds to IGF-1R with highest affinity.

Signaling through IGF-1R is an area of intensive investigation. In the non-stimulated receptor, the activation loop of the kinase domain is held in an autoinhibitory conformation. IGF-1's binding to the α subunits of the receptor induces a conformational change, resulting in the trans-autophosphorylation of three tyrosine residues (1131, 1135, and

1136), located within the activation loop on the β subunits. Physical mapping studies indicate that tyrosine 1135 is autophosphorylated first in response to stimulation, followed by tyrosine 1131, then 1136. These phosphorylation events enhance the access of ATP and peptide substrates to the tyrosine kinase domain's active site, as revealed by crystallographic and enzyme kinetic studies. Current models postulate that phosphorylation of tyrosines 1135 and 1131 destabilizes the autoinhibitory conformation of the activation loop, while phosphorylation of tyrosine 1136 optimizes the conformation of the activation loop for catalysis. As a result of the phosphorylation events and accompanying conformational changes, IGF-1R's catalytic efficiency (as determined by $V_{max}/K_m$) increases by a factor of greater than 120-fold.

At least three distinct signaling pathways are activated in response to receptor stimulation. Activated IGF-1R phosphorylates substrate proteins, including Shc and insulin receptor substrates (IRS) 1, 2, 3, and 4, and recruits 14-3-3 proteins. The phosphorylation of Shc leads to the activation of MAPK in a pathway that also includes Grb2, Sos, Ras, and Raf. Phosphorylation of IRS-1 leads to the activation of AKT in a pathway that also includes PI3-K. The 14-3-3 proteins recruited to the receptor are involved in a pathway that stimulates the translocation of Raf to the mitochondria. Downstream events associated with IGF-1R signaling include: activation of Forkhead transcription factors, activation of p70S6K, and phosphorylation and inactivation of the pro-apoptotic protein BAD.

Signaling through IGF-1R is also observed to synergize with other hormone receptors, such as estrogen receptor, progestin receptor, and EGFR. Estrogen receptor alpha stimulation results in the phosphorylation of IGF-1R, providing evidence for cross-talk between these two receptors.

The Invitrogen IGF-1R [pYpY1135/1136] ELISA is designed to detect and quantify the level of IGF-1R protein phosphorylated at tyrosine residues 1135 and 1136. This assay is intended for the detection of IGF-1R [pYpY1135/1136] from lysates of human, mouse, and rat cells. Invitrogen also offers an IGF-1R (β-subunit) ELISA kit (Cat. # KHO0491), which quantifies IGF-1R independently of phosphorylation status and allows normalization of phosphorylated IGF-1R to total IGF-1R.

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## Read entire protocol before use

## PRINCIPLE OF THE METHOD

The Invitrogen IGF-1R [pYpY1135/1136] kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A monoclonal antibody specific for IGF-1R (regardless of phosphorylation state) has been coated onto the wells of the microtiter strips provided. Samples, including a standard containing IGF-1R [pYpY1135/1136], control specimens, and unknowns, are pipetted into these wells. During the first incubation, the IGF-1R [pYpY1135/1136] antigen binds to the immobilized (capture) antibody. After washing, a rabbit antibody specific for IGF-1R [pYpY1135/1136] is added to the wells. During the second incubation, this antibody serves as a detection antibody by binding to the immobilized IGF-1R [pYpY1135/1136] protein captured during the first incubation. After removal of excess detection antibody, a horseradish peroxidase-labeled Anti-Rabbit IgG (Anti-Rabbit IgG HRP) is added. This binds to the detection antibody to complete the four-member sandwich. After a third incubation and washing to remove all the excess Anti-Rabbit IgG HRP, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of IGF-1R [pYpY1135/1136] present in the original specimen.

## REAGENTS PROVIDED

**Note:** *Store all reagents at 2 to 8°C.*

| ***Reagent*** | ***96 Test Kit*** |
|---|---|
| *IGF-1R [pYpY1135/1136] Standard*: Contains 0.1% sodium azide. Refer to vial label for quantity and reconstitution volume. | 2 vials |
| *Standard Diluent Buffer*. Contains 0.1% sodium azide; red dye*; 25 mL per bottle. | 1 bottle |
| *IGF-1R Antibody Coated Well*s. 96 Well Plate. | 1 plate |
| *IGF-1R [pYpY1135/1136] Detection Antibody*. Contains 0.1% sodium azide; blue dye*; 11 mL per bottle. | 1 bottle |
| *Anti-Rabbit IgG HRP (100X).* Contains 3.3 mM thymol; 0.125 mL per vial. | 1 vial |
| *HRP Diluent.* Contains 3.3 mM thymol; yellow dye*; 25 mL per bottle. | 1 bottle |
| *Wash Buffer Concentrate (25X).* 100 mL per bottle. | 1 bottle |
| *Stabilized Chromoge*n, *Tetramethylbenzidine (TMB).* 25 mL per bottle. | 1 bottle |
| *Stop Solutio*n. 25 mL per bottle. | 1 bottle |
| *Plate Covers*, adhesive strips. | 3 |
| * In order to help our customers avoid any mistakes in pipetting the ELISAs, we provide colored *Standard Diluent Buffer*, *Detection Antibody*, and *HRP Diluent* to help monitor the addition of solution to the reaction well. This does not in any way interfere with the test results. | |

**Disposal Note:** This kit contains materials with small quantities of sodium azide. Sodium azide reacts with lead and copper plumbing to form explosive metal azides. Upon disposal, flush drains with a large volume of water to prevent azide accumulation. Avoid ingestion and contact with eyes, skin and mucous membranes. In case of contact, rinse affected area with plenty of water. Observe all federal, state and local regulations for disposal.

## SUPPLIES REQUIRED BUT NOT PROVIDED

1. Microtiter plate reader capable of measurement at or near 450 nm.
2. Calibrated adjustable precision pipettes, preferably with disposable plastic tips. (A manifold multi-channel pipette is desirable for large assays.)
3. Cell Extraction Buffer (see Recommended Formulation, p.12).
4. Distilled or deionized water.
5. Plate washer: automated or manual (squirt bottle, manifold dispenser, etc.).
6. Data analysis and graphing software. Graph paper: linear (Cartesian), log-log, or semilog, as desired.
7. Glass or plastic tubes for diluting and aliquoting standard.
8. Absorbent paper towels.
9. Calibrated beakers and graduated cylinders in various sizes.

## PROCEDURAL NOTES/LAB QUALITY CONTROL

1. When not in use, kit components should be refrigerated. All reagents should be warmed to room temperature before use.
2. **Microtiter plates should be allowed to come to room temperature before opening the foil bag.** Once the desired number of strips has been removed, immediately reseal the bag and store at 2 to 8°C to maintain plate integrity.

3. Samples should be frozen if not analyzed shortly after collection. Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.
4. If large amounts of particulate matter are present, centrifuge or filter prior to analysis.
5. It is recommended that all standards, controls and samples be run in duplicate.
6. Extracted cell lysate samples containing IGF-1R [pYpY1135/1136] protein should be diluted with *Standard Diluent Buffer* at least 1:10. This dilution is necessary to reduce the matrix effect of the Cell Extraction Buffer.
7. When pipetting reagents, maintain a consistent order of addition from well-to-well. This ensures equal incubation times for all wells.
8. Cover or cap all reagents when not in use.
9. **Do not mix or interchange different reagent lots from various kit lots**.
10. Do not use reagents after the kit expiration date.
11. Read absorbances within 2 hours of assay completion.
12. In-house controls should be run with every assay. If control values fall outside pre-established ranges, the accuracy of the assay is suspect.
13. All residual wash liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. ***Never*** insert absorbent paper directly into the wells.
14. Because *Stabilized Chromogen* is light sensitive, avoid prolonged exposure to light. Also avoid contact between *Stabilized Chromogen* and metal, or color may develop.

## SAFETY

All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.

## DIRECTIONS FOR WASHING

**Incomplete washing will adversely affect the test outcome.** All washing must be performed with *Wash Buffer Concentrate (25X)* provided.

Washing can be performed manually as follows: completely aspirate the liquid from all wells by gently lowering an aspiration tip (aspiration device) into the bottom of each well. Take care not to scratch the inside of the well.

After aspiration, fill the wells with at least 0.4 mL of diluted wash solution. Let soak for 15 to 30 seconds, then aspirate the liquid. Repeat as directed under **ASSAY METHOD**. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.

Alternatively, the wash solution may be put into a squirt bottle. If a squirt bottle is used, flood the plate with wash buffer, completely filling all wells. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.

If using an automated washer, the operating instructions for washing equipment should be carefully followed. If your automated washer allows, 30 second soak cycles should be programmed into the wash cycle.

## PROCEDURE FOR EXTRACTION OF PROTEINS FROM CELLS OR TISSUES

### A. Recommended Formulation of Cell and Tissue Extraction Buffer:

10 mM Tris, pH 7.4
100 mM NaCl
1 mM EDTA
1 mM EGTA
1 mM NaF
20 mM $Na_4P_2O_7$
2 mM $Na_3VO_4$
1% Triton X-100
10% glycerol
0.1% SDS
0.5% deoxycholate
1 mM PMSF (stock is 0.3 M in DMSO)
Protease inhibitor cocktail (ex., Sigma Cat. # P-2714) (reconstituted according to manufacturer's guideline). Add 500 µL per 5 mL Cell Extraction Buffer.

This buffer is stable for 2 to 3 weeks at 4°C or for up to 6 months when aliquoted (without protease inhibitors and PMSF added) and stored at -20°C. When stored frozen, the Cell Extraction Buffer should be thawed on ice. This buffer (minus protease inhibitor cocktail and PMSF) can be obtained from Invitrogen, Cat. # FNN0011. **Important:** add the protease inhibitors just before using. The stability of protease inhibitor supplemented Cell Extraction Buffer is 24 hours at 4°C. PMSF is very unstable and must be added prior to use, even if added previously.

## B. Protocol for Cell Extraction

This protocol has been applied to several cell lines with the Cell Extraction Buffer above. Researchers may optimize the cell extraction procedures that work best in their hands.

1. Collect cells in PBS by centrifugation (non-adherent) or scraping from culture flasks (adherent).
2. Wash cells twice with cold PBS.
3. Remove and discard the supernatant and collect the cell pellet. (At this point the cell pellet can be frozen at -80$^{o}$C and lysed at a later date).
4. Lyse the cell pellet in Cell Extraction Buffer for 30 minutes on ice with vortexing at 10 minute intervals. The volume of Cell Extraction Buffer depends on the cell number in cell pellet and expression of IGF-1R [pYpY1135/1136]. For example, 4x10$^{6}$ MCF-7 cells grown in DMEM (Invitrogen Cat.# P104-500) plus 10% FBS can be extracted in 1 mL of Extraction Buffer. Under these conditions, use of 1-10 µL of the clarified cell extract diluted to a volume of 100 µL/well in *Standard Diluent Buffer* (See **Assay Method**) is sufficient for the detection of IGF-1R [pYpY1135/1136].
5. Transfer extracts to microcentrifuge tubes and centrifuge at 13,000 rpm for 10 minutes at 4$^{o}$C.
6. Aliquot the clear lysate to clean microfuge tubes. These samples are ready for assay. Lysates can be stored at -80$^{o}$C. Avoid multiple freeze-thaw cycles.

## REAGENT PREPARATION AND STORAGE

### A. Reconstitution and Dilution of IGF-1R [pYpY1135/1136] Standard

**Note:** The *IGF-1R [pYpY1135/1136] Standard* was prepared from purified, phosphorylated β-subunit of IGF-1R expressed in S*f*9 cells. One unit of standard is equivalent to the amount of IGF-1R [pYpY1135/1136] autophosphorylated from 200 pg of IGF-1R (β-subunit) protein.

1. Reconstitute *IGF-1R [pYpY1135/1136] Standard* with *Standard Diluent Buffer*. Refer to standard vial label for instructions. Swirl or mix gently and allow to sit for 10 minutes to ensure complete reconstitution. Label as 100 Units/mL IGF-1R [pYpY1135/1136]. Use the standard within 1 hour of reconstitution.
2. Add 0.25 mL of *Standard Diluent Buffer* to each of 6 tubes labeled 50, 25, 12.5, 6.25, 3.12, and 1.6 Units/mL of IGF-1R [pYpY1135/1136].
3. Make serial dilutions of the standard as described in the following dilution table. Mix thoroughly between steps.

**B. Dilution of IGF-1R [pYpY1135/1136] Standard**

| Standard: | Add: | Into: |
|---|---|---|
| 100 Units/mL | Prepare as described in step 1 | |
| 50 Units/mL | 0.25 mL of the 100 Units/mL std. | 0.25 mL of the Diluent Buffer |
| 25 Units/mL | 0.25 mL of the 50 Units/mL std. | 0.25 mL of the Diluent Buffer |
| 12.5 Units/mL | 0.25 mL of the 25 Units/mL std. | 0.25 mL of the Diluent Buffer |
| 6.25 Units/mL | 0.25 mL of the 12.5 Units/mL std. | 0.25 mL of the Diluent Buffer |
| 3.12 Units/mL | 0.25 mL of the 6.25 Units/mL std. | 0.25 mL of the Diluent Buffer |
| 1.6 Units/mL | 0.25 mL of the 3.12 Units/mL std. | 0.25 mL of the Diluent Buffer |
| 0 Units/mL | 0.25 mL of the Diluent Buffer | An empty tube |

Remaining reconstituted standard should be discarded or frozen in aliquots at -80$^{o}$C for further use. Standard can be frozen and thawed one time only without loss of immunoreactivity.

### C. Storage and Final Dilution of Anti-Rabbit IgG HRP (100X)

**Please Note:** The *Anti-Rabbit IgG HRP (100X)* is in 50% glycerol. This solution is viscous. To ensure accurate dilution, allow *Anti-Rabbit IgG HRP (100X)* to reach room temperature. Gently mix. Pipette *Anti-Rabbit IgG HRP (100X)* slowly. Remove excess concentrate solution from pipette tip by gently wiping with clean absorbent paper.

1. Dilute 10 µL of this 100X concentrated solution with 1 mL of *HRP Diluent* for each 8-well strip used in the assay. Label as Anti-Rabbit IgG HRP Working Solution.

For Example:

| # of 8-Well Strips | Volume of Anti-Rabbit IgG HRP (100X) | Volume of Diluent |
|---|---|---|
| 2 | 20 µL solution | 2 mL |
| 4 | 40 µL solution | 4 mL |
| 6 | 60 µL solution | 6 mL |
| 8 | 80 µL solution | 8 mL |
| 10 | 100 µL solution | 10 mL |
| 12 | 120 µL solution | 12 mL |

2. Return the unused *Anti-Rabbit IgG HRP (100X)* to the refrigerator.

**D. Dilution of Wash Buffer**

Allow the *Wash Buffer Concentrate (25X)* to reach room temperature and mix to ensure that any precipitated salts have redissolved. Dilute 1 volume of the *Wash Buffer Concentrate (25X)* with 24 volumes of deionized water (e.g., 50 mL may be diluted up to 1.25 liters, 100 mL may be diluted up to 2.5 liters). Label as Working Wash Buffer.

Store both the concentrate and the Working Wash Buffer in the refrigerator. The diluted buffer should be used within 14 days.

**ASSAY METHOD: PROCEDURE AND CALCULATIONS**

**Be sure to read the *Procedural Notes/Lab Quality Control* section before carrying out the assay.**

Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use.

**Note:** A standard curve must be run with each assay.

1. Determine the number of 8-well strips needed for the assay. Insert these in the frame(s) for current use. (Re-bag extra strips and frame. Store these in the refrigerator for future use.)
2. Add 100 µL of the *Standard Diluent Buffer* to zero wells. Well(s) reserved for chromogen blank should be left empty.
3. Add 100 µL of standards, samples or controls to the appropriate microtiter wells. Standards, samples, and controls will have a red color. Samples prepared in Cell Extraction Buffer must be diluted 1:10 or greater in *Standard Diluent Buffer* (for example, 10 µL sample into 90 µL buffer). While a 1:10 sample dilution has been found to be satisfactory, higher dilutions such as 1:25

or 1:50 may be optimal. The dilution chosen should be optimized for each experimental system. Tap gently on side of plate to thoroughly mix. (See **REAGENT PREPARATION AND STORAGE**, Section B.)

4. Cover plate with *plate cover* and incubate for **2 hours at room temperature.**
5. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **DIRECTIONS FOR WASHING**.
6. Pipette 100 µL of *IGF-1R [pYpY1135/1136] Detection Antibody* solution into each well except the chromogen blank(s). This solution will have a blue color. Tap gently on the side of the plate to mix.
7. Cover plate with *plate cover* and incubate for **1 hour at room temperature.**
8. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **DIRECTIONS FOR WASHING**.
9. Add 100 µL *Anti-Rabbit IgG HRP* Working Solution to each well except the chromogen blank(s). This solution will have a yellow color. (Prepare the working dilution as described in **REAGENT PREPARATION AND STORAGE**, Section C.)
10. Cover plate with the *plate cover* and incubate for **30 minutes at room temperature**.
11. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **DIRECTIONS FOR WASHING**.
12. Add 100 µL of *Stabilized Chromogen* to each well. The liquid in the wells will begin to turn blue.

13. Incubate for **30 minutes at room temperature and in the dark**. ***Please Note*: Do not cover the plate with aluminum foil or metalized mylar**. The incubation time for chromogen substrate is often determined by the microtiter plate reader used. Many plate readers have the capacity to record a maximum optical density (O.D.) of 2.0. The O.D. values should be monitored and the substrate reaction stopped before the O.D. of the positive wells exceed the limits of the instrument. The O.D. values at 450 nm can only be read after the *Stop Solution* has been added to each well. If using a reader that records only to 2.0 O.D., stopping the assay after 20 to 25 minutes is suggested.
14. Add 100 µL of *Stop Solution* to each well. Tap side of plate gently to mix. The solution in the wells should change from blue to yellow.
15. Read the absorbance of each well at 450 nm having blanked the plate reader against a chromogen blank composed of 100 µL each of *Stabilized Chromogen* and *Stop Solution*. Read the plate within 2 hours after adding the *Stop Solution*.
16. Plot the absorbance of the standards against the standard concentration. (Optimally, the background absorbance may be subtracted from *all* data points, including standards, unknowns and controls, prior to plotting.) Draw the best smooth curve through these points to construct the standard curve. If using curve fitting software, the four parameter algorithm provides the best curve fit.
17. Read the IGF-1R [pYpY1135/1136] concentrations for unknown samples and controls from the standard curve plotted in step 16. **Multiply value(s) obtained for sample(s) by the dilution factor to correct for the dilution in step 3.** (Samples still producing signals higher than the highest standard (100 Units/mL) should be

further diluted in *Standard Diluent Buffer* and reanalyzed, multiplying the concentration found by the appropriate dilution factor.)

**TYPICAL DATA**

The following data were obtained for the various standards over the range of 0 to 100 Units/mL IGF-1R [pYpY1135/1136].

| Standard IGF-1R [pYpY1135/1136] (Units/mL) | Optical Density (450 nm) |
|---|---|
| 100 | 3.24 |
| 50 | 1.89 |
| 25 | 1.07 |
| 12.5 | 0.65 |
| 6.25 | 0.39 |
| 3.12 | 0.31 |
| 1.6 | 0.24 |
| 0 | 0.18 |

## LIMITATIONS OF THE PROCEDURE

Do not extrapolate the standard curve beyond the 100 Units/mL standard point; the dose-response is non-linear in this region and accuracy is difficult to obtain. Dilute samples >100 Units/mL with *Standard Diluent Buffer*; reanalyze these and multiply results by the appropriate dilution factor.

The influence of various extraction buffers has not been thoroughly investigated. The rate of degradation of native IGF-1R or dephosphorylation of IGF-1R [pYpY1135/1136] in various matrices has not been investigated.

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## PERFORMANCE CHARACTERISTICS

### SENSITIVITY

The analytical sensitivity of this assay is <1 Units/mL of IGF-1R [pYpY1135/1136]. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 30 times. Using MCF-7 cells, this level of sensitivity was equivalent to the detection of IGF-1R in 5000 cells.

The sensitivity of this ELISA was compared to Western blotting using known quantities of IGF-1R [pYpY1135/1136]. The data presented in Figure 1 show that the sensitivity of the ELISA is approximately 2x greater than that of Western blotting. The bands shown in the Western blot data were developed using rabbit anti-IGF-1R [pYpY1135/1136], and an alkaline phosphatase conjugated anti-rabbit IgG followed by chemiluminescent substrate and autoradiography.

**Figure 1: Detection of IGF-1R [pYpY1135/1136] by ELISA vs Western Blot:**

Western Blot (90 kDa)

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| ELISA: O.D. 450 nm | 1.40 | 0.70 | 0.54 | 0.40 | 0.35 | 0.27 | 0.24 | 0.19 |
| IGF-1R [pYpY1135/1136] (Units/test) | 5 | 2.5 | 1.25 | 0.63 | 0.31 | 0.16 | 0.08 | 0 |

PRECISION

1. Intra-Assay Precision

Samples of known IGF-1R [pYpY1135/1136] concentration were assayed in replicates of 16 to determine precision within an assay.

| | Sample 1 | Sample 2 | Sample 3 |
|---|---|---|---|
| Mean (Units/mL) | 90.01 | 32.30 | 11.18 |
| SD | 3.31 | 1.28 | 0.43 |
| %CV | 3.68 | 3.96 | 3.81 |

SD = Standard Deviation
CV = Coefficient of Variation

2. Inter-Assay Precision

Samples were assayed 36 times in multiple assays to determine precision between assays.

| | Sample 1 | Sample 2 | Sample 3 |
|---|---|---|---|
| Mean (Units/mL) | 99.81 | 31.01 | 10.71 |
| SD | 8.42 | 2.11 | 0.95 |
| %CV | 8.43 | 6.82 | 8.83 |

SD = Standard Deviation
CV = Coefficient of Variation

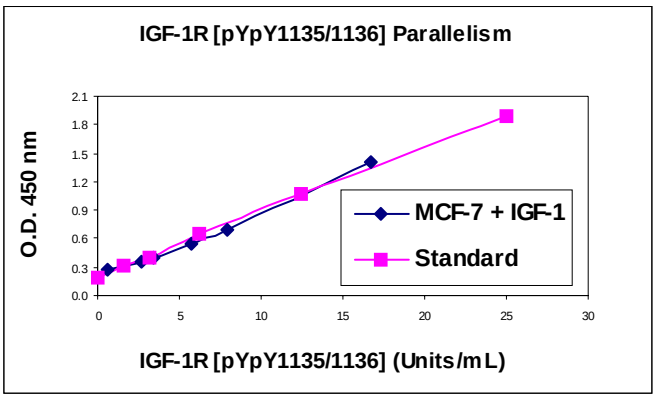
RECOVERY

To evaluate recovery, IGF-1R [pYpY1135/1136] Standard was spiked at 3 different concentrations into 20% Cell Extraction Buffer. The percent recovery was calculated as an average of 90.6%.

PARALLELISM

Natural IGF-1R [pYpY1135/1136] from MCF-7 cell lysate was serially diluted in *Standard Diluent Buffer*. The optical density of each dilution was plotted against the IGF-1R [pYpY1135/1136] standard curve. Parallelism was demonstrated by the figure below and indicated that the standard accurately reflects IGF-1R [pYpY1135/1136] content in samples.

**Figure 2**



LINEARITY OF DILUTION

MCF-7 cells were grown in DMEM (Invitrogen Cat. # P104-500) containing 10% fetal bovine serum at 37°C and lysed with Cell Extraction Buffer. This lysate was diluted in *Standard Diluent Buffer* over the range of the assay and measured for IGF-1R [pYpY1135/1136] content. Linear regression analysis of samples versus the expected concentration yielded a correlation coefficient of 0.99.

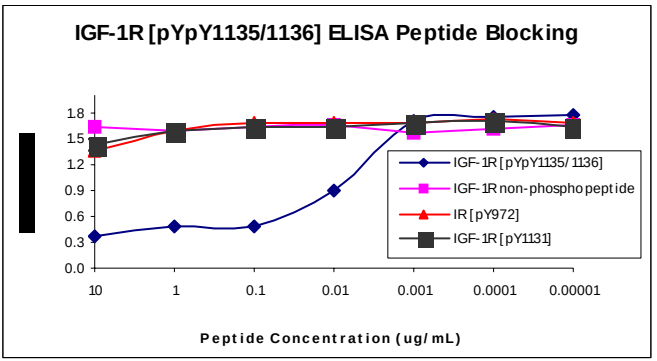
| | **Cell Lysate** | | |
|---|---|---|---|
| **Dilution** | **Measured (Units/mL)** | **Expected (Units/mL)** | **% Expected** |
| Neat | 37.70 | 37.70 | 100 |
| 1/2 | 16.76 | 18.85 | 88.9 |
| 1/4 | 10.04 | 9.42 | 106.5 |
| 1/8 | 5.92 | 4.71 | 125.7 |
| 1/16 | 2.87 | 2.36 | 121.9 |
| 1/32 | 1.38 | 1.18 | 116.7 |

SPECIFICITY

The Invitrogen IGF-1R [pYpY1135/1136] ELISA recognizes human, mouse, and rat IGF-1R phosphorylated at tyrosine residues 1135 and 1136. Other species have not been tested.

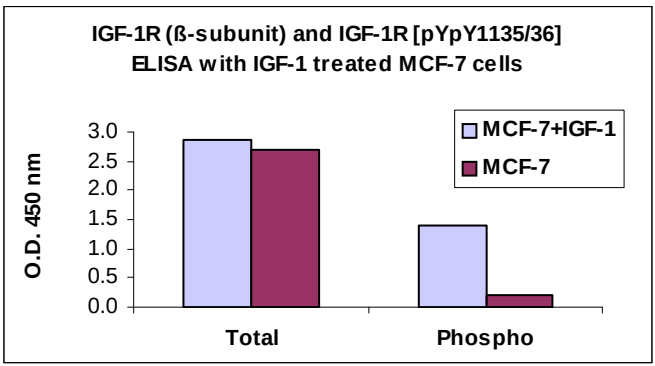
The specificity of this assay for phosphorylated IGF-1R [pYpY1135/1136] was confirmed by peptide competition. The data presented in Figure 3 show that only the phospho-peptide containing the dual phosphorylated tyrosine 1135/1136 could block the ELISA signal. The non-phosphorylated peptide sequence or other phosphopeptides from the IGF-1R sequence did not block the signal.

**Figure 3**



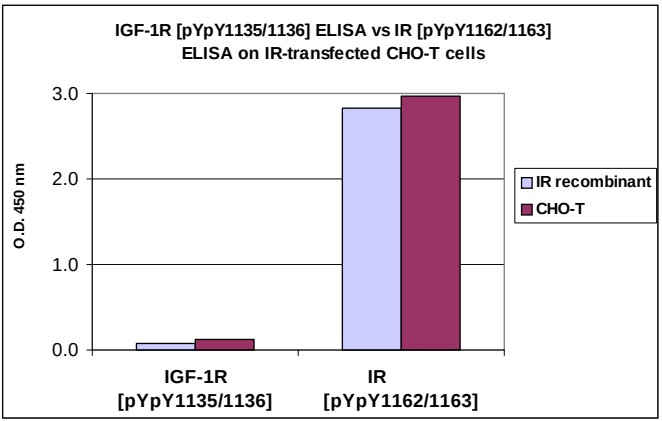
In Figure 4, MCF-7 cells were pretreated with 1 mM sodium orthovanadate for 16 hours, then treated with IGF-1 (Invitrogen Cat. # PHG0074) at 100 ng/mL for 15 minutes. Untreated MCF-7 cells were used as control. Cell extracts were prepared and cell lysates (400 µg/mL) were analyzed with IGF-1R [pYpY1135/1136] ELISA and Invitrogen's IGF-1R (β-subunit) ELISA (Cat. # KHO0491). The results show that the phosphorylation of IGF-1R (β-subunit) is upregulated in IGF-1 treated MCF-7 cells, whereas the level of IGF-1R (β-subunit) remains consistant in IGF-1 treated and untreated control.

**Figure 4**



In Figure 5, Insulin Receptor transfected CHO-T cells were treated with insulin at 100 µg/mL for 15 minutes. CHO-T cell lysate and recombinant insulin receptor protein were analyzed with IGF-1R [pYpY1135/1136] ELISA and IR [pYpY1162/1163] ELISA (Invitrogen Cat. # KHR9131). The results show that the IGF-1R [pYpY1135/1136] ELISA does not recognize either IR recombinant protein nor IR [pYpY1162/1163] in transfected CHO-T cells.

**Figure 5**



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**Explanation of symbols**

| Symbol | Description | Symbol | Description |
|---|---|---|---|
| REF | Catalogue Number | LOT | Batch code |
| RUO | Research Use Only | IVD | *In vitro* diagnostic medical device |
|  | Use by |  | Temperature limitation |
|  | Manufacturer | EC REP | European Community authorised representative |
| [-] | Without, does not contain | [+] | With, contains |
|  | Protect from light |  | Consult accompanying documents |
|  | Directs the user to consult instructions for use (IFU), accompanying the product. | | |

# IGF-1R [pYpY1135/1136] Assay Summary