ELISA Kit
Catalog #KHR9161

Insulin Receptor* [pY1334]

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*Patent Pending

TABLE OF CONTENTS

INTRODUCTION

Insulin receptor (IR), a cell surface receptor, binds insulin and mediates its action on target cells. Insulin receptor belongs to the superfamily of the growth factor receptor tyrosine kinases that regulate multiple signaling pathways through activation of a series of phosphorylation cascades. The insulin receptor is a heterotetrameric membrane glycoprotein consisting of disulfide-linked subunits in a β–α–α–β configuration. The α-subunit (135 kDa) is completely extracellular, whereas the β-subunit (95 kDa) possesses a single transmembrane domain with tyrosine kinase activity. Insulin binding to the extracellular domain leads to autophosphorylation of the receptor and activation of the intrinsic tyrosine kinase activity, which allows appropriate substrates to be phosphorylated.

Once activated, the IR initiates a variety of metabolic functions including glucose transport, glycogen synthesis, protein synthesis, translational control and mitogenesis. The IR also plays an important role in neurological function and in hypertension. Important down-stream proteins associated with IR signaling include IRS-1, JAK1, JAK2, STAT1, STAT3, PI3 kinase, GSK-3β, PKC, SHC, ERK1/2 and many others.

Activation of the tyrosine kinase activity of IR results in immediate autophosphorylation of six tyrosine residues followed by the serine sites in the β-subunit and tyrosine and serine phosphorylation of the intracellular substrates of the receptor. Tyrosine autophosphorylation within the activation loop corresponding to tyrosine 1158, 1162 and 1163 is correlated with full activation of the receptor. Phosphorylation of tyrosine 972 in the juxtamembrane region is required for IR to recruit and phosphorylate the endogenous substrates IRS-1 and Shc. The

C terminus of the β-subunit of IR contains two tyrosine residues in the distal region (tyrosine 1328 and tyrosine 1334) that are phosphorylated in response to insulin. Phosphorylated tyrosine 1328 mediates PI3Kinase activation and plays an important role in elucidating the differential metabolic signaling of IR in response to insulin stimulation. Phosphorylation of tyrosine 1328 and 1334 is required for the insulin-stimulated tyrosine dephosphorylation of pp125FAK and play an inhibitory role of insulin-induced mitogenic signaling.

The IR [pY1334] ELISA is designed to detect and quantify the levels of insulin receptor β-subunit that are phosphorylated at residue tyrosine 1334 of insulin receptor. Both natural (heterotetrameric) and recombinant IR react in this assay. Although this ELISA kit was developed using human IR transfected cells, cross-reactivity with mouse and rat insulin receptor is documented. This assay is intended for detection of IR [pY1334] from lysates of cells. For normalizing the IR content of the samples, an IR (β-subunit) ELISA kit which is independent of phosphorylation status is available (Cat. #KHR9111).

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READ ENTIRE PROTOCOL BEFORE USE

PRINCIPLE OF THE METHOD

The Insulin Receptor [pY1334] kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A monoclonal antibody specific for IR (β-subunit) (regardless of phosphorylation state) has been coated onto the wells of the microtiter strips provided. Samples, including a standard containing IR [pY1334], control specimens and unknowns, are pipetted into these wells. During the first incubation, the IR antigen binds to the immobilized (capture) antibody. After washing, an antibody specific for IR phosphorylated at tyrosine 1334 is added to the wells. During the second incubation, this antibody serves as a detection antibody by binding to the immobilized IR 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 IR [pY1334] present in the original specimen.

REAGENTS PROVIDED

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

Reagent	***96 Test Kit***
IR [pY1334] 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
*IR (β-subunit) Antibody Coated Well*s, 12x8 Well Strips.	1 plate
IR [pY1334] 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. 11).
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 semi-log, 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 bags.** 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 particulate matter is present, centrifuge or filter prior to analysis.
5. All standards, controls and samples should be run in duplicate.
6. Samples containing protein extracted from cells should be diluted at least 1:10 with *Standard Diluent Buffer*. This dilution is necessary to reduce the matrix effect of the cell lysis 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

A. Recommended Formulation of Cell 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 (e.g., Sigma Cat. # P-2714) (reconstituted according to manufacturer's guideline). Add 100 µL per 1 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) can be obtained under catalog # 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 successfully applied to several cell lines. Researchers should optimize the cell extraction procedure for their own applications.

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 IR [pY1334].
5. Transfer extract 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-thaws.

REAGENT PREPARATION AND STORAGE

A. Reconstitution and Dilution of Standard

Note: This *IR [pY1334] Standard* was prepared from purified, phosphorylated β-subunit of IR expressed in S*f*9 cells. One unit of standard is equivalent to the amount of IR [pY1334] autophosphorylated from 60 pg of IR (β-subunit) protein.

1. Reconstitute *IR [pY1334] 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 IR [pY1334]. Use 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 IR [pY1334].
3. Make serial dilutions of the standard as described in the following dilution table. Mix thoroughly between steps.

B. Dilution of 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 to the appropriate microtiter wells. 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 for sample resolution. The dilution chosen should be optimized for

each investigation's system. Tap gently on side of plate to thoroughly mix. (See **REAGENT PREPARATION AND STORAG**E, Section B.)

4. Cover wells 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 *[pY1334] Detection Antibody* solution into each well except the chromogen blank(s). Tap gently on the side of the plate to mix.
7. Cover wells 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). (Prepare the working dilution as described in **REAGENT PREPARATION AND STORAGE**, Section C.)
10. Cover wells 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 IR [pY1334] 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.)

18. Values of IR [pY1334] should be normalized for IR (β-subunit) content by parallel measurement with the IR (β-subunit) ELISA Kit (Cat. # KHR9111).

TYPICAL DATA

The following data were obtained for the various standards over the range of 0 to 100 Units/mL IR [pY1334].

IR [pY1334] Standard (Units/mL)	Optical Density (450 nm)
0	0.104
1.6	0.146
3.12	0.190
6.25	0.250
12.5	0.425
25	0.854
50	1.590
100	2.964

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 IR [pY1334] in various matrices has not been investigated. Although IR [pY1334] degradation in the Cell Extraction Buffer described in this protocol has not been seen to date, the possibility of this occurrence cannot be excluded.

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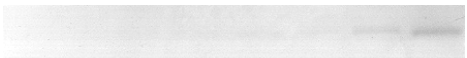
PERFORMANCE CHARACTERISTICS

SENSITIVITY

The analytical sensitivity of this assay is <1 Unit/mL of IR [pY1334]. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 30 times. Using CHO-T cells treated with 50 nM Insulin for 10 minutes, this level of sensitivity was equivalent to the detection of IR [pY1334] in 3000 cells.

The sensitivity of this ELISA was compared to Western blotting using cell lysate with known quantities of IR [pY1334]. The data presented in Figure 1 show that the sensitivity of the ELISA is about 4x greater than that of Western blotting. The bands shown in the Western blot data were developed using rabbit anti-IR [pY1334] and alkaline phosphatase conjugated anti-rabbit IgG followed by chemiluminescent substrate and autoradiography.

Figure 1



ELISA O.D. 450nm	0.21	0.23	0.26	0.28	0.37	0.62	1.3	2.2
Insulin-treated CHO-T IR Lysate (µg/test)	0	0.313	0.625	1.25	2.5	5	10	20

PRECISION

1. Intra-Assay Precision

Samples of known IR [pY1334] concentrations were assayed in replicates of 16 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (Units/mL)	50.02	24.25	9.17
SD	2.07	0.93	0.48
%CV	4.14	3.83	5.19

SD = Standard Deviation
CV = Coefficient of Variation

2. Inter-Assay Precision

Samples were assayed 48 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean (Units/mL)	49.72	23.23	9.21
SD	2.48	1.54	0.72
%CV	4.98	6.64	7.77

SD = Standard Deviation
CV = Coefficient of Variation

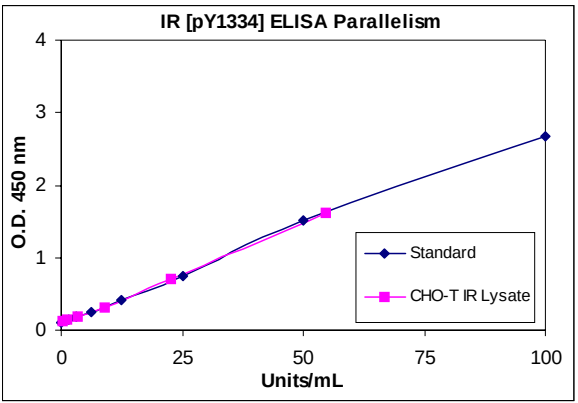
RECOVERY

To evaluate recovery, IR [pY1334] Standard was spiked at 3 different concentrations into 10% Cell Extraction Buffer. The percent recovery was calculated as an average of 125%.

PARALLELISM

Natural IR [pY1334] from 50 nM insulin-treated CHO-T IR cells was serially diluted in *Standard Diluent Buffer*. The optical density of each dilution was plotted against the IR [pY1334] standard curve. Parallelism was demonstrated by the figure below and indicated that the Standard accurately reflects IR [pY1334] content in samples.

Figure 2



LINEARITY OF DILUTION

CHO-T IR cells were treated with 50 nM insulin for 10 minutes then harvested and lysed with Cell Extraction Buffer (Cat. # FNN0011). This lysate was diluted in *Standard Diluent Buffer* over the range of the assay and measured for IR [pY1334]. Linear regression analysis of sample values versus the expected concentrations yielded a correlation coefficient of 0.99.

	Cell Lysate		
Dilution	**Measured (Units/mL)**	**Expected (Units/mL)**	**% Expected**
Neat	45.21	45.22	100.00
1/2	21.02	22.61	92.94
1/4	9.71	11.30	85.92
1/8	4.50	5.65	79.53

SPECIFICITY

The specificity of this assay for IR phosphorylated at tyrosine 1334 was confirmed by peptide competition. Phosphorylated IR was measured in the assay as usual except that the detection antibody was preincubated with IR-derived peptides at a concentration of 10 µg/mL. The data presented in Figure 3 show that the specificity of this assay for phosphorylated IR [pY1334] was confirmed by peptide competition. The data below show that only the phospho-peptide containing the phosphorylated tyrosine 1334 could block the ELISA signal. The non-phosphorylated sequence (npY1334) or phosphorylated peptides corresponding to other phosphorylation sites on IR did not block the signal.

Figure 3

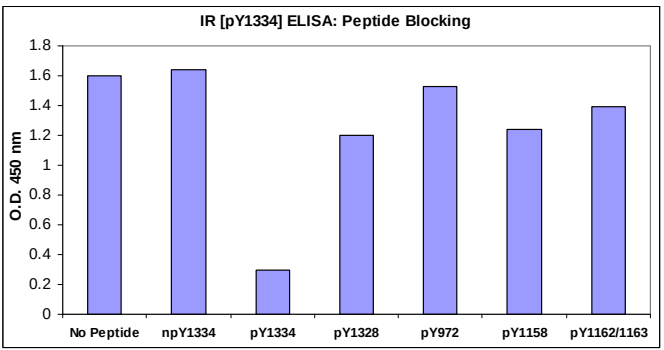
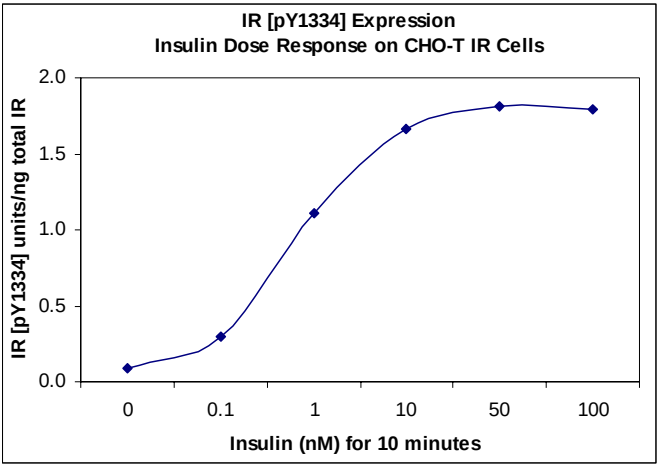


Figure 4 shows that IR [pY1334] phosphorylation in CHO-T IR cells is dependent on level of insulin stimulation. Cells (approximately 90% confluent) were treated with insulin at varying concentrations (0 to 100 nM) for 10 minutes, lysed and measured in parallel for IR (β-subunit) (Cat. # KHR9111) and IR [pY1334] content. IR [pY1334] levels were normalized to total IR (β-subunit).

Figure 4



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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.		

Insulin Receptor [pY1334] Assay Summary