3050 Spruce Street
Saint Louis, Missouri 63103 USA
Telephone 800-325-5832 • (314) 771-5765
Fax (314) 286-7828
email: techserv@sial.com
sigma-aldrich.com

Insulin Receptor β Subunit ELISA

Product Information

Product Number **CS0090**
Storage Temperature 2-8 °C

Technical Bulletin

Product Description

Insulin Receptor (IR) β Subunit ELISA is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA) for quantitative detection of IR β subunit independent of its phosphorylation status. A monoclonal antibody specific for IR β subunit (regardless of phosphorylation state) has been coated onto the wells of the multiwell plate provided. IR β subunit standard dilutions, control specimens, and unknown samples are pipetted into these wells. During the first incubation, the IR β subunit antigen binds to the immobilized (capture) antibody. After incubation, excess reagents are washed away and an antibody, specific for IR β subunit is added to the wells. During the second incubation, this antibody serves as a detection antibody by binding to the immobilized IR β subunit protein. After removal of excess detection antibody, a horseradish peroxidase-labeled anti-rabbit IgG (Anti-Rabbit IgG-HRP) is added. It binds to the detection antibody to complete the four-member sandwich. After a third incubation and washing to remove excess Anti-Rabbit IgG-HRP, substrate solution is added, which is acted upon by the bound enzyme to produce yellow color. The intensity of this colored product is directly proportional to the concentration of IR β subunit present in the original specimen. The optical density measured at 450 nm in the multiwell plate reader is used to calculate the concentration of IR β subunit.

Both natural (heterotetrameric) and recombinant IR react in this assay. This ELISA is not cross-reactive with IGF-1R. Although performance characterization of this ELISA is done primarily on human cell lines it can be used for detection of IR β subunit in mouse and rat cells. This assay is intended for detection of IR β subunit from cell lysates and can be used for normalization of IR β subunit in the samples when examining quantities of phosphorylated sites on Insulin Receptor Subunit using phosphospecific ELISA available from Sigma (Product No. PE0100, PE0200).

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. Defects in IR signaling pathway result in insulin resistance and thus high blood glucose associated with type II/non-insulin-dependent diabetes. 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. Insulin receptor and insulin-like growth factor 1 receptor (IGF-1R), which are structurally related, share conserved tyrosine residues that are phosphorylated in IR in response to insulin and in IGF-1R in response to IGF-1. Functionally, the insulin receptor regulates metabolism and IGF-1R mediates growth and differentiation. The catalytic loops within the tyrosine kinase domains of the IR/IGF-1R share the same residue sequence with a three-tyrosine motif corresponding to Tyr1158, 1162 and 1163 (for the IR) and Tyr1131, 1135 and 1136 (for the IGF-1R). It is generally believed that autophosphorylation within the activation loop proceeds in a progressive manner initiating at the second tyrosine (1162 or 1135), followed by phosphorylation at the first tyrosine (1158 or 1131), then the last (1163 or 1136), upon which the IR or IGF-1R becomes fully active.

Reagents

- **Insulin Receptor β subunit Standard, 2 vials, Product No. I 4533** –lyophilized lysate from human IR-transfected CHO cells (CHO-T). Calibrated against the mass of purified recombinant IR β subunit expressed in *E. coli. Refer to vial label for quantity and reconstitution volume*
- **Standard Diluent Buffer**, **25 mL**, **Product No. S 3068** - contains BSA and sodium azide as a preservative.
- **Monoclonal Anti-IR β subunit-coated 96-well plate, 1 plate, Product No. I 4158** - A plate using break-apart strips coated with monoclonal antibody specific for IR β subunit (regardless of phosphorylation state).
- **Anti-IR β subunit 11 mL, Product No. I 4283** – A detection antibody. Ready to use.
- **Anti-Rabbit IgG-HRP Concentrate, 100X**, **Product No. R 0653 -** contains 50% glycerol, viscous. *See Reagent Preparation for handling, dilution and storage instructions.*
- **HRP Diluent, 25 mL, Product No. H 8912** – contains thymol and BSA. Ready to use.
- **Wash Buffer Concentrate 25X, 100 mL, Product No. W 2639** - *See Reagent Preparation for handling, dilution and storage instructions.*
- **Stabilized Chromogen, Tetramethylbenzidine (TMB), 25 mL, Product No. S 3318** Avoid prolonged exposure to light. Avoid exposure to metal. Ready to use.
- **Stop Solution, 25 mL, Product No. S 2818** – Ready to use.
- **Plate Covers, Adhesive strips, 3 each, Product No. P 4870**.

Reagents and Equipment required but not provided

- Multiwell plate reader capable of readings at 450 nm
- Calibrated adjustable precision pipettes for volumes between 5 µL and 1,000 µL
- Cell extraction buffer (see recommended extraction procedure)
- Deionized or distilled water.
- Plate washer (optional), use squirt bottle, manifold dispenser, etc.
- Graph paper: linear, log-log, or semi-log, as desired
- Glass or plastic 1.0 – 1.5 mL tubes for diluting and aliquoting standard
- Absorbent paper towels to blot the plate
- Calibrated beakers and graduated cylinders in various sizes
- Vortex mixer

Precautions and Disclaimer

The kit is for R&D use only, not for drug, household or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Sample Preparation

- Samples of choice – extracts of cell lysates
- 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.
- Cell Extraction Buffer
  10 mM Tris, pH 7.4
  100 mM NaCl
  1 mM EDTA
  1 mM EGTA
  1 mM NaF
  20 mM Na4P2O7
  2 mM Na3VO4
  1% Triton X-100
  10% Glycerol
  0.1% SDS
  0.5% Deoxycholate
  1 mM PMSF (stock is 0.3 M in DMSO).
  PMSF is very unstable and must be added prior to use, even if added previously.
  Protease inhibitor cocktail (Product. No. P 2714).
  Add 250 µL of reconstituted cocktail per 5 mL of Cell Extraction Buffer.

Stable for 2-3 weeks at 4 ºC or for up to 6 months when aliquoted (without protease inhibitors and PMSF added) and stored at –20 ºC.

Thaw on ice. *Add the protease inhibitors just before use.*

Procedure for Extraction of Proteins from Cells

The protocol has been applied to several cell lines with the Cell Extraction Buffer above. Researchers may use the procedures that work best in their hands. They will have to assay their lysates for the satisfactory extraction.

Protocol for Cell Extraction

1. Collect cells in PBS by centrifugation (non-adherent) or scraping from culture flasks (adherent).
2. Wash 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 -70 ºC 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.
5. Transfer extracts to microcentrifuge tubes and centrifuge at 13,000 rpm for 10 minutes at 4 ºC.
6. Aliquot the clear lysate to clean microcentrifuge tubes.

Reagent Preparation

Insulin Receptor β subunit Standard

Insulin Receptor β subunit standard is lyophilized lysate from human IR-transfected CHO cells calibrated against the mass of purified recombinant IR β subunit expressed in *E. coli*.

1. Reconstitute one vial of IR Standard with Standard Diluent Buffer according to label directions.
2. Mix gently and wait 10 minutes to ensure complete reconstitution.
3. Label as **60 ng/mL IR.**
4. Prepare serial standard dilutions as follows:

Tube #	Standard Diluent Buffer	Standard from tube #:	Final Standard Concentration ng/mL
	Reconstitute according to label instructions		60 ng/mL
2	0.25 mL	0.25 mL (1)	30
3	0.25 mL	0.25 mL (2)	15
4	0.25 mL	0.25 mL (3)	7.5
5	0.25 mL	0.25 mL (4)	3.75
6	0.25 mL	0.25 mL (5)	1.87
7	0.25 mL	0.25 mL (6)	0.94
8	0.5 mL	-	0

Mix thoroughly between steps.

5. Use within 1 hour of reconstitution

Anti-Rabbit IgG-HRP concentrate (100X), contains 50% glycerol and is very viscous. To ensure accurate dilution follow instructions below:

1. Equilibrate to room temperature, mix gently, pipette slowly.
2. Remove excess concentrate solution from pipette tip with clean absorbent paper.
3. Mix: 10 µL IgG-HRP Concentrate +1 mL HRP Diluent
   (sufficient for one 8-well strip, prepare more as needed)
4. Label as **Anti-Rabbit IgG-HRP Working Solution.**
5. Return the unused Anti-IgG-HRP concentrate to the refrigerator.

Wash Buffer

1. Equilibrate to room temperature and mix to redissolve any precipitated salts.
2. Mix 1 volume Wash Buffer Concentrate 25X + 24 volumes of deionized water
3. Label as **Working Wash Buffer.**
4. Store both the concentrate and the Working Wash Buffer in the refrigerator. Use within 14 days.

Storage/Stability

All components of this kit are stable at 2 to 8 ºC. Any unused reconstituted standard should be discarded or frozen at -70 ºC. Standard can be frozen and thawed one time only without loss of immunoreactivity.

Refer to the Certificate of Analysis for kit expiration date. To obtain C of A go to www.sigma-aldrich.com

Procedure

Precautions

- 20-30 minutes before use equilibrate kit and all reagents to room temperature (15-30 ºC).
- Use only the coated 96 well capture plate provided with the kit
- Multiwell plate: equilibrate to room temperature in unopened foil bag. Remove desired number of strips, reseal the bag and refrigerate at 2 - 8°C to maintain plate integrity.
- When not in use all kit components should be refrigerated.
- Assay all standards, controls and samples in duplicate.
- If particulate matter is present, centrifuge or filter prior to analysis.
- A standard curve must be run with each assay
- Maintain a consistent order of components and reagents addition from well to well. This ensures equal incubation times for all wells.
- Run in-house controls with every assay. If control values fall outside pre-established ranges, the accuracy of the assay may be suspect.
- All reagents are lot-specific. Do not mix reagents from different kit lots.
- Do not use reagents after the kit expiration date.
- Standards and samples can be made up in either glass or plastic tubes.

- Pre-rinse the pipette tip with the reagent and use fresh pipette tips for each sample, standard or reagent.
- Read absorbances within 2 hours of assay completion.

Washing directions

- The purpose of washing is to remove unbound proteins and other non-specific parts of lysate.
- Incomplete washing will adversely affect the assay and render false results.
- Use only Wash Buffer provided in kit.
- Washing may be performed using automated washer, manifold pipette or squirt bottle.
- Wash cycle four times, blotting as dry as possible after the 4th wash.
- When washing manually, fill wells with Wash Buffer, aspirate thoroughly and tap dry on absorbent tissue.
- It is recommended to use laboratory tape to hold plate strips to the plate frame while performing the plate washing and drying procedure to avoid strips coming free of the frame.

Assay Procedure

- Determine the number of wells for the assay run, including 2 zero wells, 2 chromogen blank wells, 14 standard dilutions wells and 2 wells for each sample to be assayed.
- Remove appropriate number of multiwell strips and return the unused strips to the pouch. Reseal pouch.

Insulin Receptor β subunit ELISA
Assay Summary

1) **Incubate 100 µL of Standards and Samples (diluted >1:10) for 2 hours at RT. (Optional: Incubate overnight at 4°C)**

   aspirate and wash 4x

2) **Incubate 100 µL of Detection Antibody 1 hour at RT.**

   aspirate and wash 4x

3) **Incubate 100 µL of HRP Anti-Rabbit IgG 30 min. at RT.**

   aspirate and wash 4x

4) **Incubate 100 µL of stabilized Chromogen 30 minutes at RT *in the dark*.**

5) **Add 100 µL of Stop Solution and read at 450nm.**

Total Time 4 hours

1st incubation

a Add 100 µL Standard Diluent to zero wells.
b Add 100 µL standards, samples or controls to the appropriate wells. *Samples in Cell Extraction Buffer must be diluted at least 1:10 (1:25 or 1:50 were found to be optimal) in Standard Diluent Buffer. The dilutions should be optimized for each assay.*
c Tap gently on the plate to mix, cover with Plate Cover and incubate 2 hours at room temperature. *Alternatively, plate may be incubated overnight at 2 to 8 °C.*
d Wash wells 4 times following washing instructions.
e After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

2nd incubation

a Add 100 µL Anti-Insulin Receptor β subunit detection antibody to all wells (except chromogen blanks).
b Tap gently on the plate to mix, cover with Plate Cover and incubate 1 hour at room temperature.
c Wash wells for a total of 4 times following washing instructions.
d After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

3rd incubation

a Add 100 µL Anti-Rabbit IgG-HRP Working Solution to each well except the chromogen blanks.
b Cover with Plate Cover and incubate 30 minutes at room temperature.
c Wash wells for a total of 4 times following washing instructions.
d After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

Substrate incubation

a Add 100 µL of Stabilized Chromogen into all wells. *The liquid in the wells will begin to turn blue.*
b Do not cover the plate
c Incubate approximately 30 minutes at room temperature in the dark (*place plate in a drawer or cabinet).*

Note: *If your multiwell plate reader does not register optical density (OD) above 2.0, incubate only 20-25 minutes.*

Stop reaction

a Add 100 µL of Stop Solution to each well. This stops the reaction
b Tap gently to mix. *The solution will turn yellow.*

Absorbance reading

a Any commercially available multiwell plate reader capable of reading at OD 450 nm may be used.
b Blank the plate reader against the Chromogen Blank wells (contain Chromogen and Stop Solution).
c Read the absorbance of the entire plate at 450 nm within 2 hours after addition of Stop Solution.

Results

1. The results may be calculated using any immunoassay software package.
2. The four-parameter algorithm provides the best curve fit.
3. If the software program is not readily available, the concentrations of IR β subunit may be calculated manually.
4. Calculate the Average Net OD (nm) (average reading of 2 wells) for each standard dilution and samples as follows:
   Average Net OD (nm)= Average Bound OD (nm) – Average Chromogen Blank OD (nm)
5. On graph paper plot the Average Net OD (nm) of standard dilutions (nm) against the standard concentration (ng/mL) of IR β subunit. Draw the best curve through these points to construct the standard curve.
6. The IR β subunit concentrations in unknown samples and controls can be determined by interpolation from the standard curve.
7. Multiply the values obtained for the samples by dilution factor of each sample.
8. Samples producing signals higher than the 60 ng/mL standard should be further diluted and assayed again.

Product Profile

Typical Results

The standard curve below is for illustration only and **should not be used** to calculate results in your assay. Run standard curve in each assay.

IR β subunit Standard ng/mL	Optical Density 450 nm
0	0.169
0.94	0.272
1.87	0.338
3.75	0.387
7.5	0.597
15	0.857
30	1.428
60	2.551

Limitations:

- Do not extrapolate the standard curve beyond the 60 ng/mL standard point.
- The dose response is non-linear in this region and accuracy will be compromised.
- Other buffers and matrices have not been investigated.
- Although IR β subunit degradation in the Cell Extraction Buffer described in this protocol has not been seen to date, the possibility of this occurrence cannot be excluded.

Performance Characteristics

Specificity

The IR β subunit ELISA is specific for measurement of Insulin Receptor β subunit protein, regardless of phosphorylation state of insulin receptor In the experiments presented in Figure 1, IR CHO-T cells were stimulated using 100 nM insulin for 10 minutes. Unstimulated cells were used as control. Cell lysates from the cells were measured for the levels of IR and phosphorylated IR. The results show that IR β subunit ELISA detects phosphorylated IR in insulin-stimulated CHO-T and non-phosphorylated IR in unstimulated control cells.

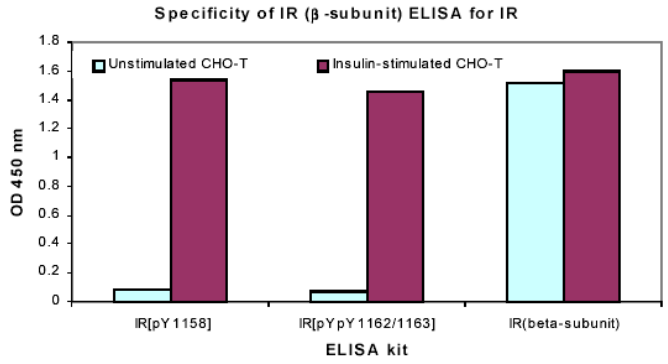

Figure 1

Figure 2 shows that IR phosphorylation in CHO-T cells is dependent on the levels of insulin stimulation. CHO-T cells were treated with insulin at varying concentrations (0-100 nM), lysed and quantitated in parallel for contents of IR β subunit and phosphorylated IR. The amount of IR β subunit remains relatively constant, while levels of IR phosphorylation at tyrosine 1158, 1162 and 1163 decrease with diminishing insulin dose.

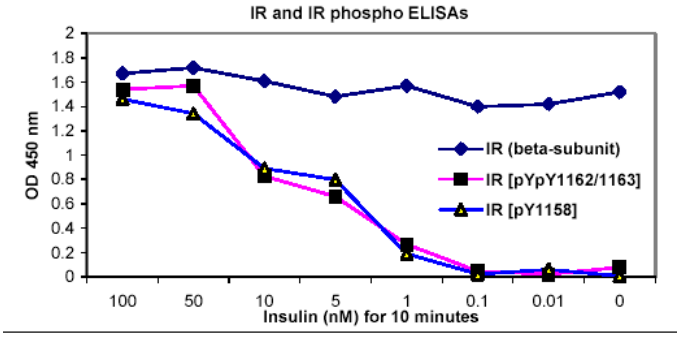

Figure 2

Sensitivity

Sensitivity of this assay is 0.5 ng/mL. Sensitivity was calculated by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 30 times. The sensitivity of this ELISA was compared to immunoblotting using known quantities of IR β subunit.

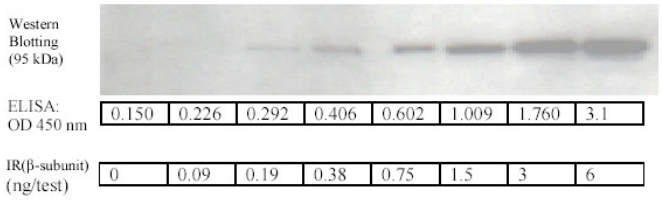

Figure 3 Detection of IR β subunit by ELISA vs. immunoblotting

The results in Figure 3 show that ELISA is approximately 2 times more sensitive in detecting IR β subunit than immunoblotting. The bands shown in the immunoblot were developed using mouse monoclonal Anti- IR β subunit; an alkaline phosphatase conjugated anti-mouse IgG followed by a chemiluminescent substrate and autoradiography

Precision

1. Intra-Assay Precision

Samples of known Insulin Receptor Subunit concentration were assayed in replicates of 16 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (ng/mL)	38.19	10.79	1.36
Standard Deviation (SD)	1.29	0.45	0.12
% Coefficient of Variation	3.37	4.17	8.69

2. Inter-Assay Precision

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

	Sample 1	Sample 2	Sample 3
Mean (ng/mL)	38.52	10.04	1.32
Standard Deviation (SD)	2.60	0.84	0.13
% Coefficient of Variation	6.75	8.41	9.49

Sample Recovery

The recovery of IR β subunit added to Jurkat cells lysate, adjusted to 200 µg/mL, averaged 102% when diluted in Standard Diluent Buffer.

Parallelism

Natural IR was prepared from a lysate of human IR-transfected CHO cells and serially diluted in *Standard Diluent Buffer.* The optical density of each dilution was plotted against IR standard curve. Parallelism demonstrated by the figure 4 indicates that the standard accurately reflects IR β subunit content in samples

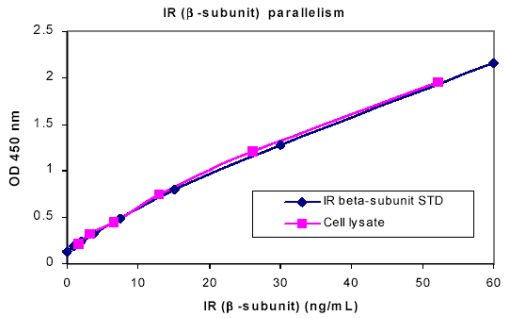

Fig. 4 Parallelism: Natural and Standard IR β subunit in ELISA

Linearity of Dilution

Human IR transfected CHO cells were grown in tissue culture medium containing 10% fetal calf serum and lysed with Cell Extraction Buffer. This lysate was adjusted to 60 ng/mL IR (β-subunit) and serially diluted in *Standard Diluent Buffer* over the range of the assay. Linear regression analysis of samples versus the expected concentration yielded a correlation coefficient of 0.99 in both cases.

Dilution	Cell Lysate Measured (ng/mL)	Expected (ng/mL)	% Expected
Neat	52.5	-	-
1:2	28.7	26.2	109
1:4	14.2	13.1	108
1:8	6.3	6.5	97
1:16	3.5	3.3	106

References

1. Bevan, P. Insulin signaling. J. Cell. Sci., **114**, 1429-1430 (2001).
2. Skorey, K.I., et al., Development of a robust scintillation proximity assay for protein tyrosine phosphatase 1b using the catalytically inactive (c215s) mutant. Anal. Biochem., **291**, 269-278 (2001).
3. Ottensmeyer, F.P. et al Mechanism of transmembrane signaling: insulin binding and the insulin receptor. Biochemistry, **39**, 12103-12112 (2000).
4. Playford, M.P., et al. Insulin-like growth factor 1 regulates the location, stability, and transcriptional activity of beta-catenin. Proc. Nat'l. Acad. Sci. USA, **97**, 12103–12108 (2000).
5. Virkamaki, A., et al. Protein-protein interaction in insulin signaling and the molecular mechanisms of insulin resistance. J. Clin. Invest., **103**, 931-943 (1999).
6. Wei, L., et al. Expression, characterization, and crystallization of the catalytic core of the human insulin receptor protein-tyrosine kinase domain. J. Biol. Chem. **270**, 8122-8130 (1995).
7. Ebina, Y., et al. The human insulin receptor cDNA: the structural basis for hormone-activated transmembrane signaling. Cell, **40**, 747-758 (1985).
8. Ullrich, A., et al. Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. Nature, **313**, 756-761 (1985).

AH/JK 4/04