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ProductInformation

phospho-Insulin Receptor, b Subunit (pTyr$^{1162/1163}$) ELISA

Catalog Number **PI0200**
Storage Temperature 2-8 °C

Technical Bulletin

Product Description

phospho-Insulin Receptor, β Subunit (pTyr$^{1162/1163}$) ELISA is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA) for quantitative detection of the levels of IR β subunit phosphorylated at tyrosine residues 1162 and 1163 in cell lysates. 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 phosphorylated at Tyr$^{1162/1163}$ 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 (pTyr$^{1162/1163}$) 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 (pTyr$^{1162/1163}$).

Both natural (heterotetrameric) and recombinant IR react in this assay. This ELISA allows differentiation between the phosphorylation and activation of IR from that of IGF-1R. Although this assay is developed using human cells, cross-reactivity with mouse and rat insulin receptor is documented. For normalizing the IR content of the samples, an IR ß-subunit ELISA that is independent of phosphorylation status is available from Sigma (Prod. No. CS0090).

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 ß-a-a-ß configuration. The a-subunit (135 kDa) is completely extracellular, whereas the ß-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-3ß, 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

- **Phospho-Insulin Receptor b subunit (pTyr$^{1162/1163}$) Standard, 2 vials, Product No. P 8620** –lyophilized lysate from human IR-transfected CHO cells (CHO-T) stimulated with insulin. See 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 b 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-Phospho-IR b subunit (pTyr$^{1162/1163}$), 11 mL, Product No. P 8495** – A detection antibody. Ready to use.
- **Anti-Rabbit IgG-HRP Concentrate, 100X**, **Product No. R 0903 -** 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 $Na_4P_2O_7$
  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).
  PMSF is very unstable and must be added prior to use, even if added previously.
  Protease inhibitor cocktail (Catalog Number . P2714. Add 250 µL of reconstituted cocktail per 5 mL of Cell Extraction Buffer.

Stable for 2-3 weeks at 2-8 °C or for up to 6 months when aliquoted (without protease inhibitors and PMSF added) and stored at –20 °C.
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 °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 the cell pellet and expression of phospho-IR ($pTyr^{1162/1163}$).
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

Phospho-IR β subunit ($pTyr^{1162/11638}$) Standard

This IR ($pTyr^{1162/1163}$) standard is a lyophilized lysate of human IR-transfected CHO cells (CHO-T) stimulated with insulin.

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 100 Units/mL phospho-IR ($pTyr^{1162/1163}$)
4. Prepare serial standard dilutions as follows:

Tube #	Standard Diluent Buffer	Standard from tube #:	Final Standard Concentration Units/mL
1	Reconstitute according to label instructions		100
2	0.25 mL	0.25 mL (1)	50
3	0.25 mL	0.25 mL (2)	25
4	0.25 mL	0.25 mL (3)	12.5
5	0.25 mL	0.25 mL (4)	6.25
6	0.25 mL	0.25 mL (5)	3.12
7	0.25 mL	0.25 mL (6)	1.6
8	0.5 mL	-	0

Mix thoroughly between steps.

5. Use within 1 hour of reconstitution

1 Unit of standard is equivalent to the amount of IR ($pTyr^{1162/1163}$) derived from 0.6 ng of IR ß subunit in CHO cells stimulated with 100 nM insulin. Subsequent lots of standard will be normalized to this lot of material to allow consistency of IR ($pTyr^{1162/1163}$) quantitation.

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 °C. Any unused reconstituted standard should be discarded or frozen at –70 °C. Standard can be frozen and thawed one time only without loss of immunoreactivity. Refer to the Certificate of Analysis for kit shelf life. 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 component and reagent 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 past the kit shelf life
- 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 an automated washer, a manifold pipette or a squirt bottle.
- Wash 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.

Assay Summary

1. **Add 100 µL of Standards and Samples (diluted 1:10) to appropriate wells. Incubate for 2 hours at RT. (Optional: Incubate overnight at 4°C) Aspirate and wash 4x**

2. **Add 100 µL Detection Antibody. Incubate 1 hr at RT Aspirate and wash 4x**

3. **Add 100 µL of Anti-Rabbit IgG-HRP Working Solution. Incubate 30 min. at RT. Aspirate and wash 4x**

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

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

Total Assay 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 2-8 °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 phospho-Anti-IR β subunit ($pTyr^{1162/1163}$) 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 ODs 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 Phospho-Anti-IR b subunit ($pTyr^{1162/1163}$) may be calculated manually.
4. Formula: Average Bound OD – Average Chromogen Blank OD = Average Net OD
5. On graph paper plot the Average Net OD of standard dilutions against the standard concentration (Units/mL) of phospho-IR β subunit ($pTyr^{1162/1163}$). Draw the best curve through these points to construct the standard curve.
6. The phospho-Anti-IR β subunit ($pTyr^{1162/1163}$) concentrations in unknown samples and controls can be determined by interpolation from the standard curve.
7. Multiply the values obtained for the samples by the dilution factor of each sample.
8. Samples producing signals higher than the 100 Units/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 a standard curve in each assay.

IR β subunit ($pTyr^{1162/1163}$) Units/mL	Optical density 450 nm
0	0.111
1.6	0.171
3.12	0.212
6.25	0.321
12.5	0.505
25	0.899
50	1.520
100	2.699

Limitations:

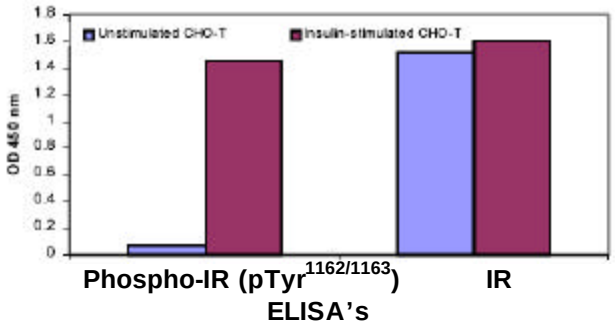
- Do not extrapolate the standard curve beyond the 100 Units/mL standard point.
- The dose response is non-linear in this region and accuracy will be compromised.
- Other buffers and matrices have not been investigated.
- Although phospho-IR β subunit ($pTyr^{1162/1163}$) degradation in the Cell Extraction Buffer described in this protocol has not been seen to date, the possibility of this occurrence cannot be excluded.

Performance Characteristics

Specificity

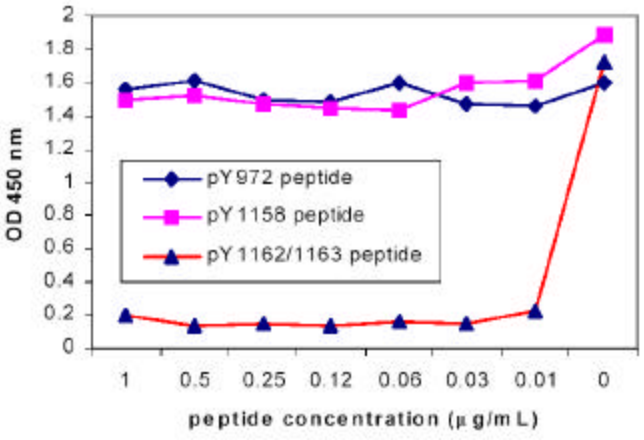
The phospho-IR β subunit ($pTyr^{1162/1163}$) ELISA is specific for measurement of IR that is dually phosphotylated at tyrosines 1162 and 1163. IR of natural or recombinant (ß-subunit) origin is reactive in this assay. This kit detects phosphorylated IR in insulin-stimulated CHO-T cells and does not detect non-phosphorylated IR in unstimulated cells, as shown in Figure 1.

Figure 1 Specificity of phospho-IR ($pTyr^{1162/1163}$) ELISA for IR Phosphorylation



The specificity of this assay for IR phosphorylated at tyrosines 1162/1163 was confirmed by peptide competition. Phosphorylated IR was quantitated in the assay as usual except that the detection antibody was preincubated with IR-derived peptides at a concentration 0.01-1.00 µg/mL. The data presented in Figure 2 show that only the peptide corresponding to the region surrounding tyrosines 1163/1163, containing the phospho-tyrosines, could block the ELISA signal. The peptides containing phosphorylated tyrosines at position 972 and 1158 did not block the signal.

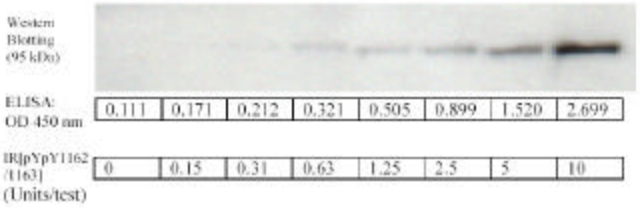
Figure 2 phospho-IR ($pTyr^{1162/1163}$) ELISA: Peptide Blocking



Sensitivity

The analytical sensitivity of this assay is <0.8 Units/mL of IR (pTyr$^{1162/1163}$). This was determined 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 cell lysate with known quantities of IR (pTyr$^{1162/1163}$). The data presented in Figure 3 show that the sensitivity of the ELISA is about 2X greater than that of immunoblotting. The bands shown in the immunoblots were developed using rabbit anti-IR (pTyr$^{1162/1163}$) and alkaline phosphatase conjugated anti-rabbit IgG followed by chemiluminescent substrate and autoradiography.

Figure 3



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 (Units/mL)	73.74	19.11	7.48
Standard Deviation (SD)	2.93	0.93	0.43
% Coefficient of Variation	3.97	4.85	5.77

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)	76.09	19.81	7.93
Standard Deviation (SD)	3.47	1.18	0.73
% Coefficient of Variation	4.57	5.98	9.14

Sample Recovery

The recovery of IR β subunit (pTyr$^{1162/1163}$) added to 100 µg/mL of a Jurkat cells lysate in Cell Extract Buffer, followed by 1:10 dilution in Standard Diluent Buffer, averaged 104%.

Parallelism

Natural IR (pTyr$^{1162/1163}$) from lysate of insulin-stimulated CHO-T was serially diluted in Standard Diluent Buffer. The optical density of each dilution was plotted against the IR (pTyr$^{1162/1163}$) standard curve. Parallelism was demonstrated by the figure below and indicated that the standard accurately reflects IR pTyr$^{1162/1163}$) content in samples.

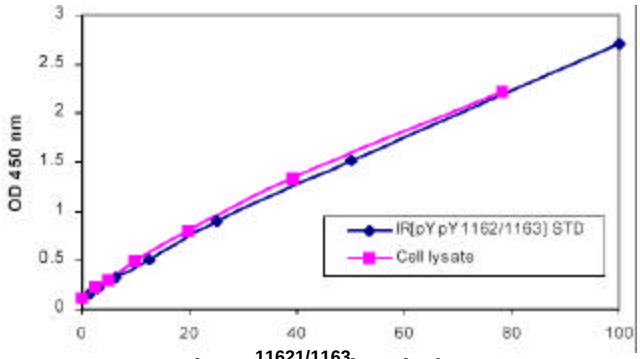


Fig. 4 phospho-IR (pTyr$^{1162/1163}$): Parallelism:

Linearity of Dilution

Extract Buffer was spiked with IR (pTyr$^{1162/1163}$) 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.

	Cell Lysate		
Dilution	**Measured (Units/mL)**	**Expected (Units/mL)**	**% Expected**
Neat	78.3	-	-
1:2	42.7	39.2	109%
1:4	22.4	19.5	114%
1:8	11.6	9.8	113%
1:16	5.3	4.9	107%

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 β-catenin. *Proc. Nat. 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).

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