# IR [pYpY1162/1163] ELISA Kit

**Catalog Number** KHR9131 (96 tests)

**Pub. No.** MAN0003996 **Rev.** 2.0 (30)

**CAUTION!** 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.

**Note:** For safety and biohazard guidelines, see the "Safety" appendix in the *ELISA Technical Guide* (Pub. no. MAN0006706). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

## Product description

The Invitrogen™ IR [pYpY1162/1163] ELISA Kit is a solid-phase sandwich Enzyme-Linked Immunosorbent Assay (ELISA). This assay is designed to detect and quantify the level of IR [pYpY1162/1163] in cell lysates. The assay recognizes both natural and recombinant IR [pYpY1162/1163].

Insulin receptor (IR) is a cell surface receptor belonging to the superfamily of the growth factor receptor tyrosine kinases. IR 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.

This assay was developed using human cells, but cross-reactivity with mouse and rat IR has been documented. For normalizing the IR content of the samples, an IR (β-subunit) ELISA Kit (Cat. No. KHR9111), is available for detection of IR content independent of phosphorylation status.

## Contents and storage

Upon receipt, store the kit at 2°C to 8°C.

| Contents | Cat. No. KHR9131 |
|---|---|
| IR [pYpY1162/1163] Standard, lyophilized; contains 0.1% sodium azide. Refer to vial label for quantity and reconstitution volume | 2 vials |
| Standard Diluent Buffer; contains 0.1% sodium azide | 25 mL |
| IR (ß-subunit) Antibody Coated Plate, 96-well strip-well plate | 1 plate |
| IR [pYpY1162/1163] Detection Antibody; contains 0.1% sodium azide | 11 mL |
| Anti-Rabbit IgG HRP (100X); contains 3.3 mM thymol | 0.125 mL |
| HRP Diluent; contains 3.3 mM thymol | 25 mL |
| Wash Buffer Concentrate (25X) | 100 mL |
| Stabilized Chromogen, Tetramethylbenzidine (TMB) | 25 mL |
| Stop Solution | 25 mL |
| Plate Covers, adhesive strips | 3 |

## Required materials not supplied

- Distilled or deionized water
- Microtiter plate reader with software capable of measurement at or near 450 nm
- Plate washer–automated or manual (squirt bottle, manifold dispenser, or equivalent)
- Calibrated adjustable precision pipettes and glass or plastic tubes for diluting solutions; beakers, flask and cylinders for preparation of reagents
- Cell Extraction Buffer (Cat. No. FNN0011, or see "Prepare Cell Extraction Buffer")

## Before you begin

**IMPORTANT!** Reagents are lot-specific. Do not mix or interchange different reagent lots from various kit lots.

- Review the **Procedural guidelines** and **Plate washing directions** in the *ELISA Technical Guide* available at **thermofisher.com**.
- Allow reagents to reach room temperature before use. Mix to redissolve any precipitated salts.

## Prepare 1X Wash Buffer

1. Dilute 16 mL of Wash Buffer Concentrate (25X) with 384 mL of deionized or distilled water. Label as 1X Wash Buffer.
2. Store the concentrate and 1X Wash Buffer in the refrigerator. Use the diluted buffer within 14 days.

## Prepare Cell Extraction Buffer

**Note:** See the *ELISA Technical Guide* for detailed information on preparing Cell Extraction Buffer.

1. Prepare Cell Extraction Buffer.

   Cell Extraction Buffer consists of 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, and 0.5% deoxycholate.
2. Immediately before use, add PMSF (0.3 M stock in DMSO) to 1 mM and 50 µL protease inhibitor cocktail (e.g., Sigma Cat. No. P-2714) for each 1 mL of Cell Extraction Buffer.

## Prepare cell lysate

1. Collect cells by centrifugation (non-adherent cells) or scraping from culture flasks (adherent cells), then wash cells twice with cold PBS.
2. Remove and discard the supernatant and collect the cell pellet. The pellet can be stored at −80°C and lysed at a later date if desired.
3. Lyse the cell pellet in Cell Extraction Buffer for 30 minutes, on ice. Vortex at 10-minute intervals.

   **Note:** The volume of Cell Extraction Buffer used depends on the number of cells in the cell pellet, and expression levels of IR [pYpY1162/1163]. Researchers must optimize the extraction procedures for their own applications.
4. Transfer the lysate into microcentrifuge tubes and centrifuge at 13,000 rpm for 10 minutes at 4°C.
5. Transfer the supernatant into clean microcentrifuge tubes. Samples can be stored at −80°C (avoid multiple freeze-thaw cycles).

## Pre-dilute samples

Sample concentrations should be within the range of the standard curve. Because conditions may vary, each investigator should determine the optimal dilution for each application.

- Perform sample dilutions with Standard Diluent Buffer.
- Dilute samples prepared in Cell Extraction Buffer 1:10 or greater in Standard Diluent Buffer (e.g., 10 µL sample into 90 µL buffer).

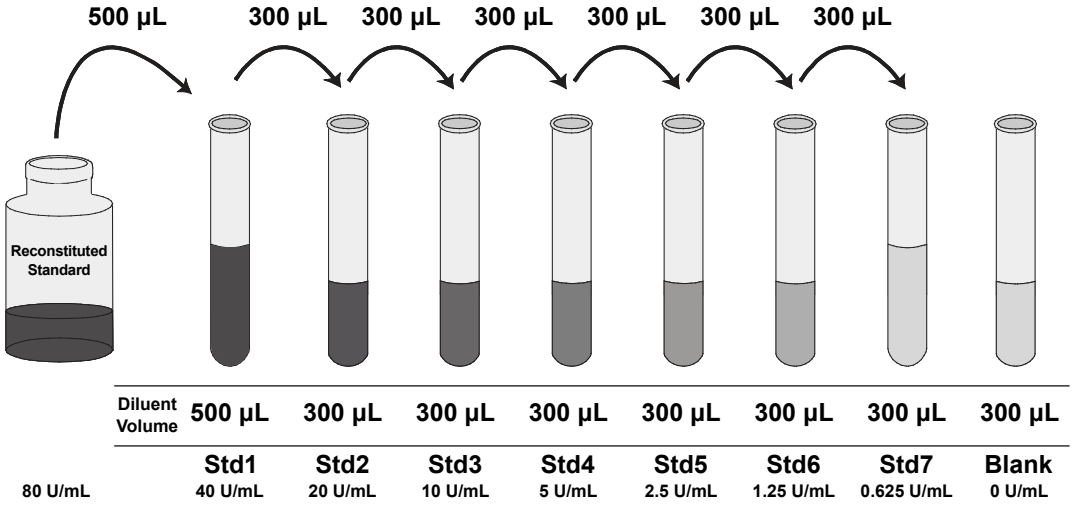
  This dilution is necessary to reduce the matrix effect of the Cell Extraction Buffer. SDS concentration should be less than 0.01% before adding to the plate. While a 1:10 sample dilution has been found to be satisfactory, higher dilutions may be optimal.

## Dilute standards

**Note:** Use glass or plastic tubes for diluting standards.

**Note:** The IR [pYpY1162/1163] Standard was prepared from autophosphorylated recombinant protein. One unit of standard is equivalent to the amount of IR [pYpY1162/1163] autophosphorylated from 0.6 ng of full-length IR (β-subunit) protein.

1. Reconstitute IR [pYpY1162/1163] Standard to 80 Units/mL with Standard Diluent Buffer. Refer to the standard vial label for instructions. Swirl or mix gently and allow the contents to sit for 10 minutes to ensure complete reconstitution. Label as 80 Units/mL IR [pYpY1162/1163]. **Use the standard within 1 hour of reconstitution.**
2. Add 500 µL Reconstituted Standard to one tube containing 500 µL Standard Diluent Buffer and mix. Label as 40 Units/mL IR [pYpY1162/1163].
3. Add 300 µL Standard Diluent Buffer to each of 7 tubes labeled as follows: 20, 10, 5.0, 2.5, 1.25, 0.625, and 0 Units/mL IR [pYpY1162/1163].
4. Make serial dilutions of the standard as shown in the following dilution diagram. Mix thoroughly between steps.
5. Remaining reconstituted standard should be discarded or frozen in aliquots at −80°C for further use. Standard can be frozen and thawed one time only without loss of immunoreactivity.




## Prepare 1X Anti-Rabbit IgG HRP solution

**Note:** Prepare 1X Anti-Rabbit IgG HRP solution within 15 minutes of usage.

The Anti-Rabbit IgG HRP (100X) is in 50% glycerol, which is viscous. To ensure accurate dilution:

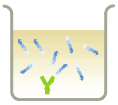
1. For each 8-well strip used in the assay, pipet 10 µL Anti-Rabbit IgG HRP (100X) solution, wipe the pipette tip with clean absorbent paper to remove any excess solution, and dispense the solution into a tube containing 1 mL of HRP Diluent. Mix thoroughly.
2. Return the unused Anti-Rabbit IgG HRP (100X) solution to the refrigerator.

## Perform ELISA (Total assay time: 4 hours)

**IMPORTANT!** Perform a standard curve with each assay.

- Allow all components to reach room temperature before use. Mix all liquid reagents prior to use.
- Determine the number of 8-well strips required for the assay. Insert the strips in the frames for use. Re-bag any unused strips and frames, and store at 2°C to 8°C for future use.


**1 Bind antigen**



a. Add 100 µL of standards, controls, or samples (see "Pre-dilute samples"on page 2) to the appropriate wells. Leave the wells for chromogen blanks empty.
b. Cover the plate with a plate cover and incubate 2 hours at room temperature.
c. Thoroughly aspirate the solution and wash wells 4 times with 1X Wash Buffer.

**2 Add detector antibody**

a. Add 100 µL of IR [pYpY1162/1163] Detection Antibody solution into each well except the chromogen blanks.
b. Cover the plate with a plate cover and incubate 1 hour at room temperature.
c. Thoroughly aspirate the solution and wash wells 4 times with 1X Wash Buffer.

**3 Add IgG HRP**

a. Add 100 µL 1X Anti-Rabbit IgG HRP solution into each well except the chromogen blanks.
b. Cover the plate with plate cover and incubate for 30 minutes at room temperature.
c. Thoroughly aspirate the solution and wash wells 4 times with 1X Wash Buffer.

**4 Add Stabilized Chromogen**

a. Add 100 µL Stabilized Chromogen to each well. The substrate solution begins to turn blue.
b. Incubate for 30 minutes at room temperature in the dark.

**Note:** TMB should not touch aluminum foil or other metals.

**5 Add Stop Solution**

Add 100 µL Stop Solution to each well. Tap the side of the plate to mix. The solution in the wells changes from blue to yellow.

## Read the plate and generate the standard curve

1. Read the absorbance at 450 nm. Read the plate within 2 hours after adding the Stop Solution.
2. Use curve-fitting software to generate the standard curve. A 4 or 5 parameter algorithm provides the best standard curve fit. Optimally, the background absorbance may be subtracted from all data points, including standards, unknowns and controls, prior to plotting.
3. Read the concentrations for unknown samples and controls from the standard curve. Multiply value(s) obtained for sample(s) by the appropriate factor to correct for the sample dilution.

   **Note:** Dilute samples producing signals greater than the upper limit of the standard curve in Standard Diluent Buffer and reanalyze. Multiply the concentration by the appropriate dilution factor.

## Performance characteristics

### Standard curve example

The following data were obtained for the various standards over the range of 0 to 40 Units/mL IR [pYpY1162/1163].

| Standard IR [pYpY1162/1163] (U/mL) | Optical Density (450 nm) |
|---|---|
| 40 | 2.89 |
| 20 | 1.48 |
| 10 | 0.80 |
| 5.0 | 0.49 |
| 2.5 | 0.34 |
| 1.25 | 0.22 |
| 0.63 | 0.17 |
| 0 | 0.15 |

### Recovery

The recovery of IR [pYpY1162/1163] added to 100 µg/mL of Jurkat lysate in Cell Extraction Buffer, then diluted 1:10 in Standard Diluent Buffer) averaged 104%.

### Inter-assay precision

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

| Parameters | Sample 1 | Sample 2 | Sample 3 |
|---|---|---|---|
| Mean (U/mL) | 19.73 | 4.77 | 1.42 |
| Standard Deviation | 1.01 | 0.41 | 0.13 |
| % Coefficient of Variation | 5.12 | 8.60 | 9.15 |

### Intra-assay precision

Samples of known IR [pYpY1162/1163] concentration were assayed in replicates of 16 to determine precision within an assay.

| Parameters | Sample 1 | Sample 2 | Sample 3 |
|---|---|---|---|
| Mean (U/mL) | 20.56 | 4.64 | 1.42 |
| Standard Deviation | 0.89 | 0.28 | 0.11 |
| % Coefficient of Variation | 4.33 | 6.03 | 7.75 |

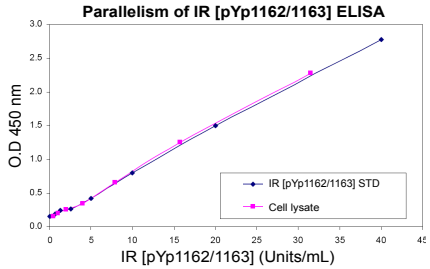
## Linearity of dilution

Cell Extraction Buffer was spiked with IR [pYpY1162/1163] 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.

| Dilution | Measured (U/mL) | Expected (U/mL) | % Expected |
|---|---|---|---|
| Neat | 31.5 | — | — |
| 1/2 | 16.8 | 15.8 | 106 |
| 1/4 | 8.2 | 7.9 | 104 |
| 1/8 | 4.1 | 3.9 | 105 |
| 1/16 | 2.3 | 2.0 | 115 |

## Parallelism

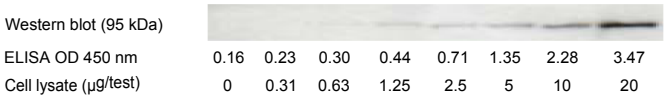
Natural IR [pYpY1162/1163] from insulin-stimulated CHO-T/IR lysate was serially diluted in Standard Diluent Buffer. The optical density of each dilution was plotted against the IR [pYpY1162/1163] standard curve.




## Sensitivity

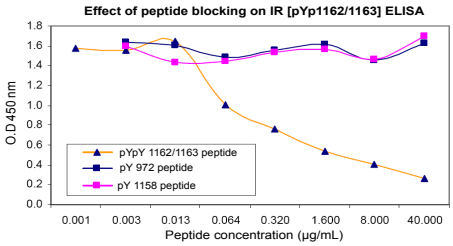
The analytical sensitivity of the assay is <0.4 Units/mL IR [pYpY1162/1163]. This was determined by adding two standard deviations to the mean O.D. obtained from 30 assays of the zero standard.

The sensitivity of the ELISA is ~1:4 fold greater than that of western blot when tested against known quantities of IR [pYpY1162/1163].

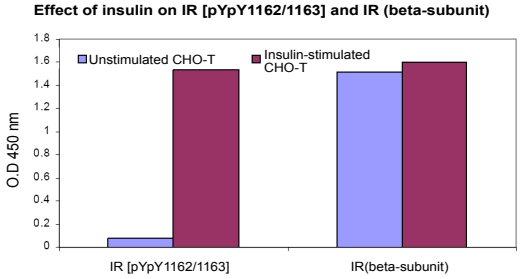



## Specificity

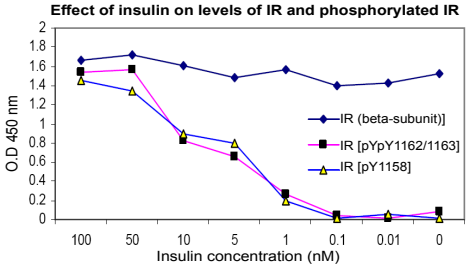
IR [pYpY1162/1163] specificity was confirmed by peptide competition. The assay was performed as usual except that the detection antibody was preincubated with 0.001 to 40 µg/mL of IR-derived peptides. The data shows that only the peptide corresponding to the region surrounding tyrosines 1162/1163 blocks the ELISA signal. Peptides containing phosphorylated tyrosine at positions 972 or 1158 did not block the signal.




IR phosphorylation is dependent on insulin stimulation in CHO-T/IR cells. IR [pYpY1162/1163] is detected at high level in insulin-stimulated CHO-T/IR cells, but not in unstimulated controls.




In parallel assays of cells treated with insulin (0 to100 nM) for 10 minutes, the amount of IR (β-subunit) remains constant, while the level of IR [pYpY1162/1163] decreases with diminishing insulin dosage. A similar pattern for IR [pY1158] was also observed.




## Limited product warranty

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**Product label explanation of symbols and warnings**

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| REF | Catalog Number | LOT | Batch code | | Temperature limitation | | Use by | | Manufacturer | | Consult instructions for use | | Caution, consult accompanying documents |

**Manufacturer's address:** Bender MedSystems GmbH | Campus Vienna Biocenter 2 | 1030 Vienna, Austria

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