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Product Information

Phosphotyrosine INSR/Insulin Receptor ELISA Kit
for measuring phosphorylated specific protein (phosphotyrosine protein) in human cell lysates

Catalog Number **RAB0968**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

The Phosphotyrosine INSR/Insulin Receptor ELISA kit is an *in vitro* enzyme-linked immunosorbent assay for the measurement of human phosphorylated specific protein. A specific capture antibody has been coated onto a 96 well plate. Samples are pipetted into the wells, and phosphorylated and unphosphorylated protein present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-phosphotyrosine antibody is used to detect only tyrosine-phosphorylated protein. After washing away unbound antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of phosphorylated specific protein bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

Components

1. Human Phosphotyrosine INSR Antibody-coated ELISA Plate (Item A): RAB0968A
2. 20x Wash Buffer Concentrate (Item B): RABWASH5
3. Biotinylated Anti-Human Phosphotyrosine Antibody (Item C): RABPYC
4. 5x Assay Diluent (Item E): RABDIL11
5. HRP-Streptavidin (Item G): RABHRP6
6. TMB One-Step Substrate Reagent (Item H): RABTMB4
7. Phosphorylation ELISA Stop Solution (Item I): RABSTOP3
8. 2x Cell Lysate Buffer (Item J): RABCLB1
9. Phosphotyrosine INSR Positive Control, Lyophilized (Item K): RAB0968K

Reagents and Equipment Required but Not Provided.

1. Microplate reader capable of measuring absorbance at 450 nm.
2. Protease and Phosphatase inhibitors.
3. Shaker.
4. Precision pipettes to deliver 2 µL to 1 mL volumes.
5. Adjustable 1–25 mL pipettes for reagent preparation.
6. 100 mL and 1 liter graduated cylinders.
7. Distilled or deionized water.
8. Tubes to prepare sample dilutions.

Precautions and Disclaimer

For R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices

Preparation Instructions

Sample Preparation

2x Cell Lysate Buffer should be diluted 2-fold with deionized or distilled water to yield 1x Cell Lysate Buffer (addition of protease and phosphatase inhibitors to 1x Cell Lysate Buffer is recommended prior to sample preparation).

Cell lysates - Rinse cells with PBS, making sure to remove any remaining PBS before adding the Cell Lysate Buffer. Solubilize cells at 4 x 10^7 cells/mL in 1x Cell Lysate Buffer. Pipette up and down to resuspend and incubate the lysates with shaking at 2–8 °C for 30 minutes. Microcentrifuge at 13,000 rpm for 10 minutes at 2–8 °C and transfer the supernatants into a clean test tube. Lysates should be used immediately, or aliquoted and stored at –70 °C. Avoid repeated freeze-thaw cycles. Thawed lysates should be kept on ice prior to use.

For the initial experiment, it is recommend to test serial dilutions of the cell lysates. Prepare 5-fold and 50-fold dilutions with Assay Diluent (Item E) before use.

Note: The fold dilution of sample used depends on the abundance of phosphorylated proteins and should be determined empirically. More of the sample can be used if signals are too weak. If signals are too strong, the sample can be diluted further.

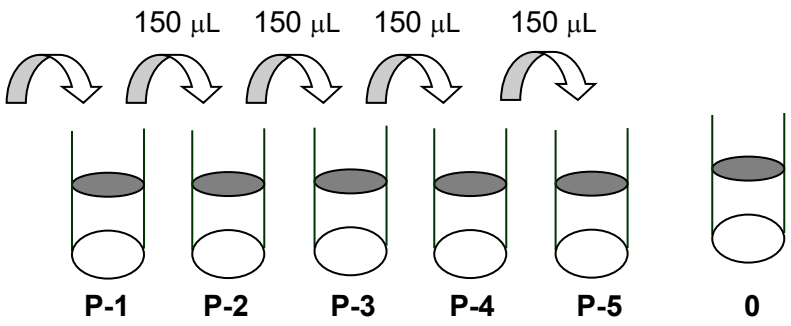
Reagent Preparation

1. Bring all reagents and samples to room temperature (18–25 °C) before use.

2. Item E, Assay Diluent should be diluted 5-fold with deionized or distilled water before use.

3. Preparation of Positive Control: Briefly spin the Positive Control vial of Item K. Add 250 µL of 1x Assay Diluent (Item E, Assay Diluent should be diluted 5-fold with deionized or distilled water, see step 2) into Item K vial to prepare a Positive Control Stock Solution. Dissolve the powder thoroughly by a gentle mix. Add 25 µL of prepared Positive Control Stock Solution from the vial of Item K into a tube with 375 µL of 1x Assay Diluent to prepare P-1. Pipette 300 µL of 1x Assay Diluent into each tube. Transfer 150 µL of prepared P-1 into a tube with 300 µL of 1x Asaay Diluent to produce a dilution series (see Figure 1). Mix each tube thoroughly before the next transfer. 1x Assay Diluent serves as the background.

Figure 1.
Dilution Series for Standards



4. If the Wash Concentrate (20x) (Item B) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to yield 400 mL of 1x Wash Buffer.

5. Briefly spin the biotinylated antibody (Item C) before use. Add 100 µL of 1x Assay Diluent into the vial to prepare a biotinylated anti-phosphotyrosine antibody concentrate. Pipette up and down to mix gently. The concentrate can be stored at 2–8 °C for 5 days. It can be used within one month if store at –70 °C. The biotinylated phosphotyrosine antibody should be diluted 80x with 1x Assay Diluent and used in Procedure, step 4.

6. Briefly spin the HRP-Streptavidin concentrate vial (Item G) and pipette up and down to mix gently before use. HRP-Streptavidin concentrate should be diluted 600 fold with 1x Assay Diluent.
Note: For example: Briefly spin the vial (Item G) and pipette up and down to mix gently. Add 20 µL of HRP-Streptavidin concentrate into a tube with 12 mL of 1x Assay Diluent to prepare a 600-fold diluted HRP Streptavidin solution (don't store the diluted solution for next day use). Mix well.

7. Cell Lysate Buffer should be diluted 2-fold with deionized or distilled water before use (recommend to add protease and phosphatase inhibitors).

Storage/Stability
Store the kit at –20 °C Please use within 1 year from the date of shipment. Avoid repeated freeze-thaw cycles.

The reconstituted standard should be stored at –20 °C or –70 °C (–70 °C is recommended). Opened microplate strips or reagents may be store for up to 1 month at 2–8 °C. Return unused wells to the pouch containing desiccant pack and reseal along entire edge.

Procedure

1. Bring all reagents to room temperature (18–25 °C) before use. It is recommended that all samples or Positive Control should be run at least in duplicate.

2. Add 100 µL of each sample or Positive Control into appropriate wells. Cover well with plate holder and incubate for 2.5 hours at room temperature or overnight at 4 °C with shaking.

3. Discard the solution and wash 4 times with 1x Wash Solution. Wash by filling each well with Wash Buffer (300 µL) using a multichannel pipette or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

4. Add 100 µL of prepared 1x primary antibody antibody or 1x biotinylated antibody (Preparation, step 5) to appropriate wells. Incubate for 1 hour at room temperature with shaking.

5. Discard the solution. Repeat the wash as in step 3.

6. Add 100 µL of prepared 1x HRP-conjugated anti-rabbit IgG to corresponding well. Incubate for 1 hour at room temperature with shaking.

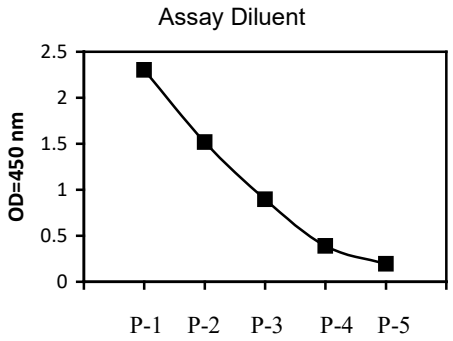
7. Discard the solution. Repeat the wash as in step 3.

8. Add 100 µL of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark with shaking.

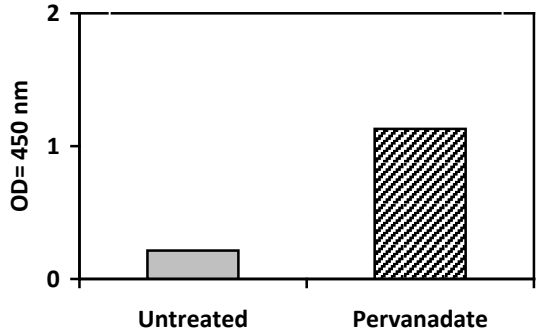
9. Add 50 µL of Stop Solution (Item I) to each well. Read at 450 nm immediately.

Results
Typical Data
ELISA data analysis: Average the duplicate readings for each sample or positive.

Positive Control:
Jurkat cells were treated with Pervanadate at 37 °C for 10 minutes. Solubilize cells at 4 x 10^7 cells/mL in lysis buffer. Serial dilutions of lysates were analyzed in this ELISA. Please see Reagent Preparation, step 3 for details.



Pervanadate Stimulation of Jurkat Cell Line
Jurkat cells were treated or untreated with Pervanadate for 10 minutes at 37 °C. Cell lysates were analyzed using this ELISA kit:



Appendix

Troubleshooting Guide

Problem	Cause	Solution
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standard dilution	Ensure a brief spin of Item C and dissolve the powder thoroughly with gentle mixing.
Low signal	Too brief incubation times	Ensure sufficient incubation time; Procedure, step 2 may change to over night
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Large CV	Inaccurate pipetting	Check pipettes
High background	Plate is insufficiently washed	Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed.
	Contaminated wash buffer	Make fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	Store the standard at <–20 °C after reconstitution, others at 4 °C. Keep substrate solution protected from light
	Stop solution	Stop solution should be added to each well before measurement.

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