MILLIPORE

Instruction Manual For

phospho-IRS1 (Ser312) STAR ELISA Kit

(Ser312 in human/Ser307 in mouse)

Catalog # 17-459

Sufficient reagents for 96 assays per kit

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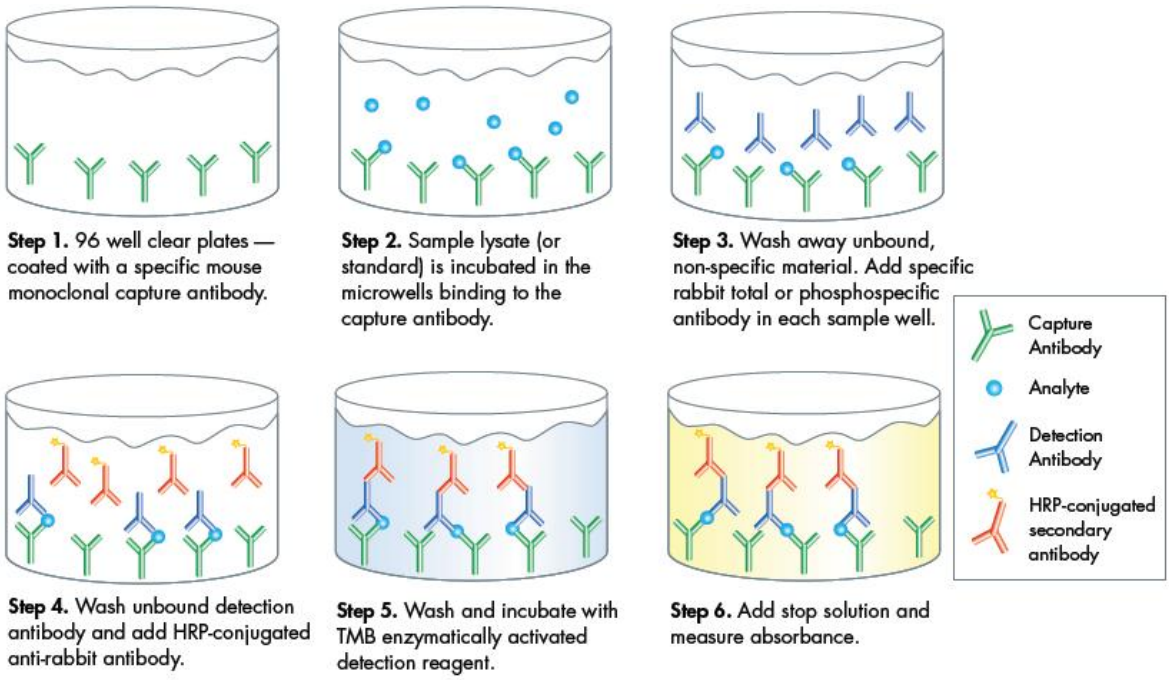
I. TEST PRINCIPLE

The UPSTATE® colorimetric STAR (Signal Transduction Assay Reaction) ELISA kit is a solid phase sandwich enzyme linked immunosorbent assay that provides a fast, sensitive method to detect specific levels of signaling targets in whole cell extracts. The IRS1 plate is coated with a specific mouse monoclonal capture antibody on the microwells of the 96-well clear plate. Sample lysate or the standard included in the kit is incubated in the microwells allowing IRS1 antigen to be captured in the plate wells. The plate is then washed to remove any unbound non-specific material. The wells are then incubated with a specific rabbit anti-phospho-IRS1 (Ser312) antibody to detect the captured IRS1 on the plate well that is phosphorylated on Ser312. The unbound detection antibody is washed away followed by incubation with an HRP-conjugated anti-rabbit antibody. This allows for a sensitive enzymatic detection of the sample. After the addition of TMB substrate and stop solution the absorbance is measured at 450 nm using a plate reader.

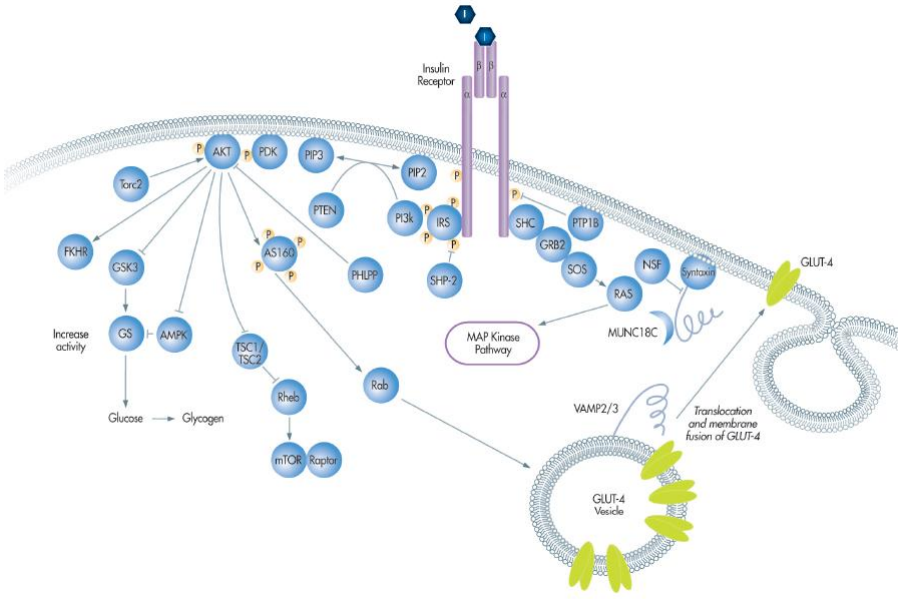
The entire assay takes less than 5 hours to complete with minimal hands-on time. Many of the reagents are supplied in ready-to use formulations for ease of use. The kit also includes a standard that is run as both a positive control and to develop a standard curve.



II. IRS1 BACKGROUND

IRS (Insulin Receptor Substrate) proteins are the effectors of both Insulin and IGF-initiated signaling. Insulin, in addition to working very similarly to a growth factor in stimulating receptor tyrosine kinases, is essential for maintaining glucose homeostasis and regulating carbohydrate, lipid, and protein metabolism. There are 4 IRS proteins, 1-4. It is believed that these proteins serve complementary functions in different tissues. The two prominent players are IRS1 and IRS2 with IRS1 playing important roles in skeletal muscle and IRS2 in the liver. The IRS proteins link the insulin receptor with many signaling pathways including the PI3 Kinase/Akt/mTOR and the MAP Kinase pathways. The PI3K/Akt/mTOR pathway is involved with many metabolic actions of insulin signaling. MAPK is involved in the regulation of some genes and works with PI3 Kinase to control cell growth and differentiation.

In addition to the insulin receptor tyrosine kinase activity in type II diabetes patients and mice, studies have shown a greater defect in components of the insulin signaling pathway including Insulin Receptor Substrate (IRS) phosphorylation or the activation of Phosphoinositide 3- Kinase (PI3 Kinase). Insulin stimulates the signaling cascade by binding to the insulin receptor through its extracellular α subunits. This stimulates the tyrosine kinase activity of the β subunits of the receptor. The receptor then autophosphorylates itself and phosphorylates the IRS (Insulin Receptor Substrate) proteins that are very important modulators of insulin signaling. IRS binds to the phosphorylated tyrosines of the β subunit of the insulin receptor. They share PH and PTB domains near their N-termini, and multiple Tyr phosphorylation motifs in their C-terminal regions. Proteins which bind to tyrosine-phosphorylated IRS-proteins include PI3 Kinase p85, GRB2, SHP2, Nck, Crk, and Fyn. IRS1 appears to be principally involved in IGF-signaling and cytoskeletal growth.



III. ASSAY SENSITIVITY, DETECTION LIMITS, and SPECIES REACTIVITY

Sensitivity: 1.5 Units/mL.

Range of Detection: 1.6 to 100 Units/mL

Species Reactivity: Human, mouse and rat

***NOTE**: This data is presented for reference use only and should not be used to interpret actual assay results. A standard curve must be generated for each assay.*

IV. STORAGE OF KIT COMPONENTS

Maintain the unopened kit at 2-8°C until expiration date.

V. KIT COMPONENTS

1. Capture Plate pre-coated with anti-IRS1 antibody: (Part No. 17-459A) One pre-coated 96-stripwell immunoplate sealed in a foil pouch.
2. Anti-phospho-IRS1 (Ser312) detection antibody: (Part No. 17-459B) One bottle (11 mL) of anti-phospho-IRS1 (Ser312 human/ Ser307 mouse) detection antibody containing sodium azide, ready to use.
3. ELISA Diluent: (Part No. 17-459C) One bottle (25 mL) of ELISA Diluent containing sodium azide, ready to use.
4. 25X ELISA Wash Buffer: (Part No. 17-459D) One bottle (50 mL) of 25X ELISA Wash Buffer.
5. Anti-Rabbit IgG HRP conjugate: (Part No. 17-459E) One vial (125 µL) of 100X anti-rabbit HRP conjugate containing thimerosal.
6. HRP Diluent: (Part No. 17-459F) One bottle (25 mL) of HRP Diluent containing thimerosal.
7. TMB Solution: (Part No. 17-459G) One bottle (25 mL) of stabilized tetramethylbenzidine (TMB), ready to use.
8. Stop Solution: (Part No. 17-459H) One bottle (25 mL) of stop solution, ready to use.
9. Phospho-IRS1 (Ser312) Standard: (Part No. 17-459I) Two vials of IRS1 standard, lyophilized.
10. Plate Covers: Two plate covers.

Materials Not Supplied

1. Multi-channel or repeating pipettes
2. Plate shaker (optional)
3. Pipettors & tips capable of accurately measuring 1-1000 µL
4. Graduated serological pipettes
5. 96-well microtiter Plate Reader with 450 nm filter
6. Graphing software for plotting data or graph paper for manual plotting of data
7. Microfuge tubes for standard and sample dilutions
8. Mechanical vortex
9. 1 liter container
10. Distilled or deionized water

Precautions

- The instructions provided have been designed to optimize the kit's performance. Deviation from the instructions may result in suboptimal performance of the kit and the failure to produce accurate data.
- Caustic Material*:* Stop Solution. **Caution: Eye, hand, face, and clothing protection should be worn when handling this material.**
- **Safety Warnings and Precautions:** This kit is designed for research use only and not recommended for internal use in humans or animals. All chemicals should be considered potentially hazardous and principles of good laboratory practice should be followed.
- The Detection Antibody and ELISA Diluent contain sodium azide. Sodium azide may react with copper and lead plumbing to form highly explosive metal azides. Upon disposal, flush with large amounts of water to prevent azide build-up. Avoid contact with skin.
- The Anti-Rabbit IgG HRP Conjugate and HRP Diluent contain thimerosal. Thimerosal is highly toxic by inhalation, contact with skin or if swallowed. Thimerosal is a possible mutagen and should be handled accordingly.

Technical Notes

- All kit reagents should be at room temperature (20°C to 25°C) prior to use.
- Do not use reagents beyond the expiration date of the kit.
- Do not mix or interchange reagent from various kit lots.
- Manual Plate Washing: Vigorous washing and complete removal of all liquid by aspiration at the end of each washing step is very important to obtain low background values. Gentle agitation during the wash steps or a 2-3 minute soak may reduce background values.
- The desiccant enclosed in the 96-well capture plate pouch will keep the plate stable when stored at 2° to 8°C should the plate loose its seal during shipping.

VI. PREPARATION OF SAMPLE

1. Culture cells stimulating IRS1 activation as desired.
2. Remove culture media and wash cells twice with ice-cold 1X TBS (Tris Buffered Saline) or PBS (Phosphate Buffered Saline). Discard supernatant
3. Add 1X RIPA with protease inhibitors to the cells (5-10 mL per 150 mm tissue culture plate). 10 mL of 1X RIPA plus protease inhibitors can be prepared by adding 10 µL of 1 µg/µL Leupeptin, 10 µL of 1 µg/µL Aprotinin, 10 µL of 1 µg/µL Pepstatin, 100 µL of 100mM PMSF and 1 mL of 10X RIPA (Cat. No. 20-188) to 8.87 mL of deionized water.
4. Scrape cells from plate with a rubber policeman.
5. Transfer cells in RIPA buffer to a microcentrifuge tube and incubate on ice for 15 minutes. Vortex tube for 10 seconds or sonicate briefly for 10 seconds.
6. Clarify lysate by centrifugation at 12,000 rpm for 10 minutes at 4ºC in a microcentrifuge prior to use.
7. The assay tolerates a maximum of 20 µL per well of lysate prepared in 1X RIPA diluted in ELISA Diluent.
8. Cell extract containing SDS must be diluted to 0.01% SDS using ELISA Diluent prior to use.
9. Collect the supernatant and calculate protein concentration using a Bradford Assay or by densitometry.
10. It is suggested that the cell lysate be used immediately following preparation. However, samples can be frozen and stored at -80º C for later use. Frozen samples should be used within 6 months if storing at -80º C. Avoid repeated freeze thaws.

Further information regarding lysate preparation protocols can be obtained at *http://www.millipore.com*.

VII. REAGENT PREPARATION

1. **1X Wash Buffer**
   Warm the 25X ELISA Wash Buffer to room temperature and mix to ensure that any precipitated salts have re-dissolved. For 500 mL of Wash Buffer, combine 20 mL of 25X ELISA Wash Buffer and 480 mL distilled or deionized water. Stir to homogeneity. Wash Buffer can be stored for up to 4 weeks at 2-8°C. Discard the Wash Buffer if it becomes turbid or if a precipitate develops.

2. **Anti-Rabbit IgG HRP Conjugate**
   Dilute the anti-Rabbit IgG HRP Conjugate 100-fold with HRP Diluent immediately before use. Prepare 1 mL for each strip used.

3. Standard

***Note**: When opening lyophilized Standard, remove rubber stopper gently as the lyophilizate may have become dislodged during shipping.*

Reconstitute the standard with the volume of ELISA Diluent specified on the vial label to give a concentration of 100 Units/mL. Gently swirl the vial and allow the vial to sit for 10 minutes to ensure the material is completely reconstituted. The standard should be reconstituted immediately before the assay. This stock material (tube #1) is then used to generate a standard curve. A suggested 2-fold dilution scheme is as follows:

a) Label 7 test tubes #2-7 and "0 dose". Add 250 µL of the ELISA Diluent to tubes #2-7 and "0 dose".
b) Add 250 µL of the stock Standard solution [100 Units/mL] to tube #2 and vortex. This is Standard tube #2 with a concentration of 50 Units/mL.
c) Standards #2-7 are then prepared by performing a 2-fold serial dilution of the preceding standard. Refer to Fig. 1. For example, to make Standard #3, remove 250 µL of Standard #2 and add it to tube #3 and vortex and so on. Do not add any Standard to the "0 Dose" Standard tube.

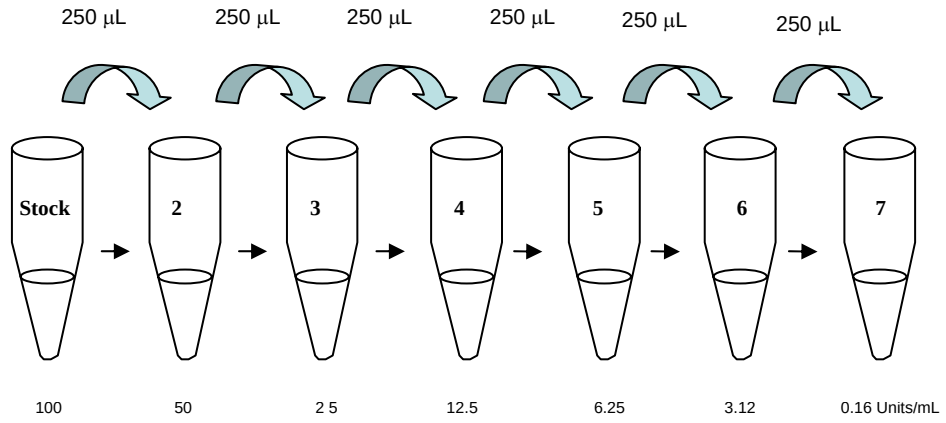


Figure 1: Recommended 2-fold Serial Dilution of Standard

Note: The Standard curve can be set up with a different serial dilution scheme by making appropriate adjustments to the dilution pattern.

VIII. ASSAY PROTOCOL

1. Prepare the reagents as described in the Reagent Preparation section.
2. Place the desired number of strips in the strip well plate holder. (Re-bag the extra strips and return unused strips to refrigerator for future use.)
3. Add 100 µL of either the Standards 1 through 7 or the samples to wells. Add 100 µL of the zero dose to the control wells. It is recommended that standards and samples be run in duplicate. Wells reserved for TMB blanks should be left empty.

   Note: *Do not add standard or sample extract to wells reserved for TMB blanks.*

   Note: *A standard curve must be run with each assay.*
4. Seal the plate with a plate sealer. Incubate the plate for 2 hours at room temperature or at 2-8°C overnight (on a shaker if possible).
5. **IMPORTANT WASH STEP:**

   Gently remove the plate sealer and wash the plate at least 4 times. A thorough washing of the plate is extremely important to reduce background. We recommend using a multi-channel pipette to fill each well with 250 µL of diluted Wash Buffer. Fluid removal from the wells is best accomplished by inverting the plate over a sink and flicking the fluid out of the wells and then blotting the plate on clean paper towels. Using the multichannel pipet add 250 µL of Wash Buffer to each well; flick and blot the plate. Repeat this procedure for a total of 4 times.

   For users of automatic plate washers: It is important to ensure that the wash apparatus is properly maintained and operating correctly. Tubing and tips can easily become clogged, leading to incomplete washing and inadequate aspiration of wells. The result may be poor precision and an unsuitable standard curve. For best results, we recommend at least 4 wash cycles.
6. Add 100 µL of the detection antibody to each well. Cover the plate and incubate at room temperature for 1 hour (on shaker if possible).
7. Wash as described in Step 5.
8. Add 100 µL of a 1:100 dilution of the anti-Rabbit IgG HRP Conjugate to each well. Cover the plate and incubate at room temperature for 45 minutes (on shaker with mild agitation if possible).
9. Wash as described in Step 5. Remove all fluid from the wells and blot the wells dry.
10. Add 100 µL of the TMB Solution to each well. Incubate at room temperature in the dark for 10 to 45 minutes, monitor the color development. Stop the reaction by adding 100 µL of Stop Solution to each well. Immediately read the plate at 450 nm. Plate should be read within 1 hour of adding the stop solution.
11. The plate reader may be blanked against a TMB blank prepared by adding 100 µL of stop solution to 100 µL of the TMB solution.

    CAUTION: *Bubbles in the wells will cause inaccurate readings. Ensure that all bubbles are removed prior to taking the absorbance reading.*

    NOTE: *For very low starting protein levels, samples can be placed at 37°C during the final incubation to obtain greater sensitivity.*

IX. CALCULATION OF RESULTS

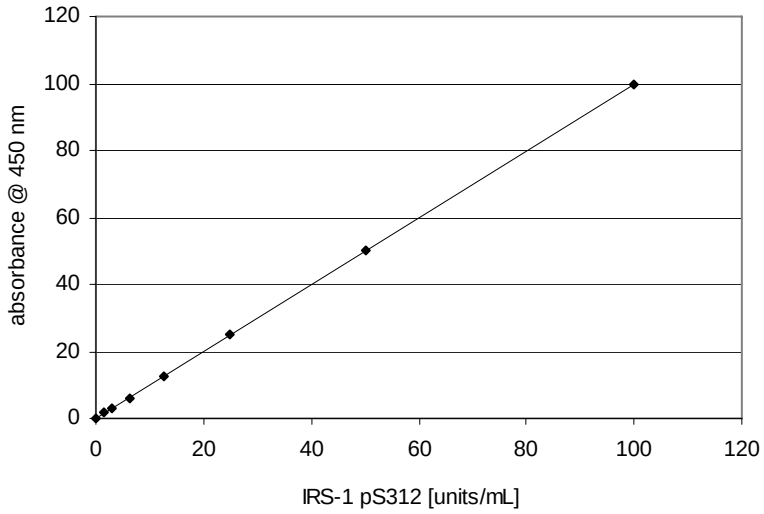


Figure 2. Typical phospho-IRS1 (Ser312) Standard Curve
100 µL of progressive 2 fold dilutions of the phospho -IRS1 (Ser312) standard included in the kit and run as described in the assay instructions.

NOTE: This data is presented for reference use only and should not be used to interpret actual assay results. A standard curve must be generated for each assay.

X. REFERENCES

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