LSBio
an Absolute Biotech Company

Mouse/Human/Rat Phospho-AKT (Ser473) ELISA Kit (Sandwich ELISA)

User Manual

Catalog No. LS-543

It is important that you read this entire manual carefully before starting your experiment.

This kit is for **Research Use Only** and **Not for Diagnostic Use.**
This kit is not approved for use in humans or for clinical diagnosis.

SAMPLE ONLY

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ASSAY SPECIFICATIONS

Target: Phospho-AKT (Ser473)

Specificity: This kit is for the detection of Phospho-AKT (Ser473). No significant cross-reactivity or interference between Phospho-AKT (Ser473) and analogs was observed. This claim is limited by existing techniques therefore cross-reactivity may exist with untested analogs. Use with recombinant proteins is not supported.

Sample Types: This kit is recommended for use with mouse/human/rat cell lysates. Use with other sample types is not supported.

Detection: Colorimetric - 450nm

Limitations: This kit is for **Research Use Only** and is not intended for diagnostic use. This kit is not approved for use in humans or for clinical diagnosis.

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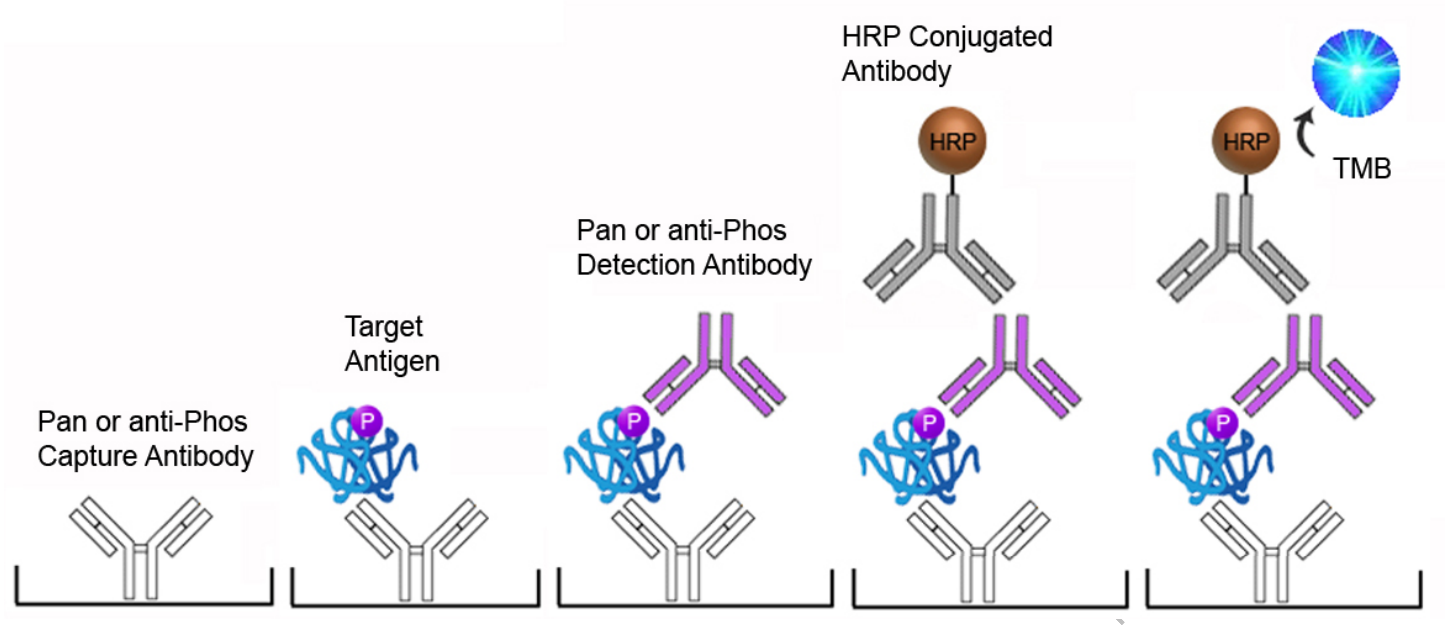
ASSAY PRINCIPLE

This assay is based on the sandwich ELISA principle. Each well of the supplied microtiter plate has been pre-coated with a pan-target specific capture antibody. Standards or samples are added to the wells and the target antigen binds to the capture antibody. Unbound Standard or sample is washed away. A phospho-specific anti-target antibody is then added which binds to the phosphorylated target. Unbound antibody is washed away. A Horseradish Peroxidase (HRP)-conjugated antibody is then added which binds to the bound phospho-specific anti-target. Unbound HRP-conjugated antibody is washed away. A TMB substrate is then added which reacts with the HRP enzyme resulting in color development that is proportional to the amount of target bound. A sulfuric acid stop solution is added to terminate color development reaction and then the optical density (OD) of the well is measured at a wavelength of 450 nm ± 2 nm.

SAMPLE ONLY

ASSAY PRINCIPLE IMAGE



KIT COMPONENTS / STORAGE

The entire kit may be stored at -20°C for up to 6 months from the date of shipment. Avoid repeated freeze-thaw cycles. For extended storage, it is recommended to store at -80°C. For prepared reagent storage, see table above.

Component	Quantity	Storage After Preparation
Coated 96-well Strip Plate	1	1 month at -20°C
Positive Control (Lyophilized NIH 3T3 cell lysate)	1 vial	1 week at -80°C
Assay Diluent (5x)	1 vial x 15 ml	1 month at 4°C
Lysate Buffer (2x) *[1]	1 vial x 10 ml	1 month at 4°C
Detection Antibody Concentrate (1,000x)	2 vials	5 days at 4°C
HRP-Conjugated Antibody Concentrate (500x)	1 vial x 25 µl	Do not store
Wash Buffer (20x)	1 vial x 25 ml	1 week at 4°C
TMB Substrate	1 vial x 12 ml	N/A
Stop Solution	1 vial x 8 ml	N/A
Plate Sealers	4	N/A
Instruction Manual	1	N/A

*[1] does not include protease and phosphatase inhibitors

OTHER REQUIRED SUPPLIES

- Microplate reader with 450nm wavelength filter
- High-precision pipette and sterile pipette tips
- Eppendorf tubes
- 37°C incubator
- Deionized or distilled water
- Absorbent paper

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REAGENT PREPARATION

Bring all reagents to room temperature (18-25°C) before use.

1x Assay Diluent: Prepare 75 ml of Assay Diluent by diluting the supplied 15 ml of 5x Assay Diluent Concentrate with 60 ml of deionized or distilled water. Assay Diluent can be stored at 4°C once prepared.

1x Cell Lysate Buffer: Prepare 20 ml of Cell Lysate Buffer by diluting the supplied 10 ml of 2x Cell Lysate Concentrate with 10 ml of deionized or distilled water. We also recommend the addition of protease and phosphatase inhibitors (not included) to the lysis buffer prior to use.

1x Wash Buffer: If crystals have formed in the concentrate, warm to room temperature and mix it gently until crystals have completely dissolved. Prepare 400 ml of Working Wash Buffer by diluting 20 ml of the supplied 20x Wash Buffer Concentrate with 380ml of deionized or distilled water. Wash Buffer can be stored at 4°C once prepared.

1x Detection Antibody Solution: Calculate the volume of 1x Detection Antibody Solution needed for your particular experiment (100 µl per well). Briefly spin down the tube before opening. Prepare that volume by diluting the Detection Antibody Concentrate 1,000-fold (1:1,000) with 1x Assay Diluent. 1x Detection Antibody Solution must be prepared fresh for each experiment and cannot be stored.

1x HRP-Conjugated Antibody Solution: Calculate the volume of 1x HRP-Conjugated Antibody Working Solution needed for your particular experiment (100 µl per well). Briefly spin down the tube before opening. Prepare that volume by diluting the HRP-Conjugated Antibody Concentrate 5000-fold (1:500) with 1x Assay Diluent. 1x HRP-Conjugated Antibody working solution must be prepared fresh for each experiment and cannot be stored.

TMB Substrate Solution: Using sterile techniques remove the needed volume of TMB Substrate solution for the number of wells you are planning to run. Dispose of unused TMB Substrate solution rather than returning it to the stock container.

SAMPLE ONLY

SAMPLE PREPARATION

This assay is recommended for use with mouse/human/rat cell lysates. Use with other sample types is not supported.

Cell Lysates - Rinse the cells with PBS, making sure to remove any remaining PBS before adding the lysis buffer. Solubilize cells at 4 x 10^7 cells/ml in prepared Cell Lysate Buffer (see Reagent Preparation step 2). Pipette up and down to resuspend the pellet. 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 supernatantes 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, we recommend a serial dilution, such as a 5-fold to 50-fold dilution, for your cell lysates with prepared Assay Diluent 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.

SAMPLE ONLY

POSITIVE CONTROL PREPARATION

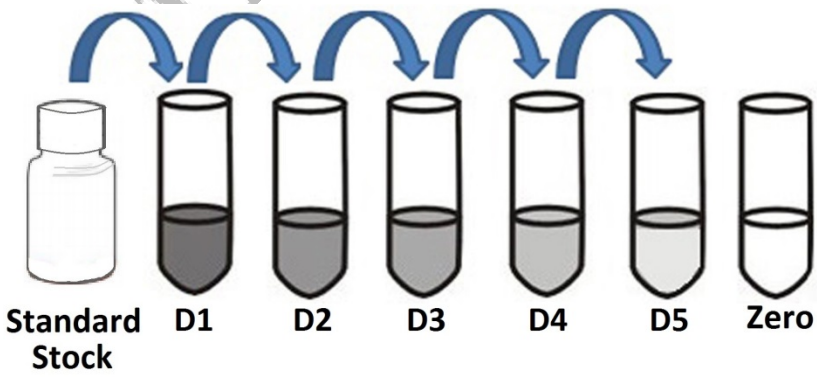
The following will prepare sufficient volumes to run the Standard dilution series in duplicate. Standard and prepared standard dilutions should be used immediately and not stored for future use.

Standard Stock Solution: Briefly spin the Positive Control and add 500 µl of 1x Assay Diluent. Gently mix the powder to allow it to dissolve thoroughly. If a precipitate is seen in the solution after mixing, this can be removed by a quick centrifuge of the positive control vial, and then pipetting the supernatant only for the assay.

Label 5 tubes and prepare the following dilutions. Mix well between each dilution.

D1: Pipette 500 µl of Stock Standard into 0 µl of 1x Assay Diluent
D2: Pipette 150 µl of D1 into 300 µl of 1x Assay Diluent
D3: Pipette 150 µl of D2 into 300 µl of 1x Assay Diluent
D4: Pipette 150 µl of D3 into 300 µl of 1x Assay Diluent
D5: Pipette 150 µl of D4 into 300 µl of 1x Assay Diluent

Blank (Zero): Use 1x Assay Diluent alone



SAMPLE ONLY

REAGENT PREPARATION NOTES

1. It is highly recommended that positive control dilutions and samples are run in duplicate within each experiment.
2. Once reconstituted, positive control should be used immediately, and used only once. Long-term storage of positive control is NOT recommended.
3. All solutions prepared from concentrates are intended for one-time use. Do not reuse solutions.
4. Do not prepare the positive control dilutions directly in wells.
5. Prepared reagents may adhere to the tube wall or cap during transport; centrifuge tubes briefly before opening.
6. All solutions should be gently mixed prior to use.
7. Reconstitute stock reagents in strict accordance with the instructions provided.
8. To minimize imprecision caused by pipetting, ensure that pipettes are calibrated. Pipetting volumes of less than 10µL is not recommended.
9. TMB Substrate solution is easily contaminated; sterility precautions should be taken. TMB Substrate solution should also be protected from light.
10. Do not substitute reagents from one kit lot to another. Use only those reagents supplied within this kit.
11. Due to the antigen specificity of the antibodies used in this assay, native or recombinant proteins from other manufacturers may not be detected by this kit.

SAMPLE ONLY

ASSAY PROCEDURE

Bring all reagents and samples to room temperature (18 - 25ºC) without additional heating and mix thoroughly by gently swirling before pipetting (avoid foaming). Prepare all reagents, working standards, and samples as directed in the previous sections.

1. Add 100 µl of **Positive Control, Blank, or Sample** per well, cover with a plate sealer, and incubate for 2.5 hours at room temperature or overnight at 4°C with gentle shaking.
2. Aspirate the liquid from each well and wash 4 times. Wash by adding approximately 300 µl of 1x Wash Buffer using a squirt bottle, multi-channel pipette, manifold dispenser or automated washer. Allow each wash to sit for 1-2 minutes before completely aspirating. After the last wash, aspirate to remove any remaining Wash Buffer then invert the plate and tap against clean absorbent paper.
3. Add 100 µl of 1x **Detection Antibody Working Solution** to each well and gently agitate to ensure thorough mixing. Incubate for 1 hour at room temperature with gentle shaking.
4. Aspirate and wash the wells as outlined in step 2.
5. Add 100 µl of 1x **HRP-Conjugated Antibody Working Solution** to each well. Incubate for 1 hour at room temperature with gentle shaking.
6. Aspirate and wash the wells as outlined in step 2.
7. Add 100 µl of **TMB Substrate** solution to each well and incubate for 30 minutes at room temperature in the dark with gentle shaking. Monitor periodically until optimal color development has been achieved.
8. Add 50 µl of **Stop Solution** to each well and record the total development time. The blue color will change to yellow. If color change does not appear uniform, gently tap the plate to ensure thorough mixing. The Stop Solution should be added to wells in the same order and timing as the TMB Substrate solution.
9. Determine the optical density (OD value) of each well immediately using a microplate reader set to 450 nm.

SAMPLE ONLY

ASSAY PROCEDURE NOTES

1. **ELISA Plate**: Keep appropriate numbers of strips for 1 experiment and remove extra strips from microtiter plate. Removed strips should be placed in a sealed bag containing desiccant and stored at 2°-8°C.
2. **Solutions**: To avoid cross-contamination, change pipette tips between additions of each standard, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
3. **Applying Solutions:** All solutions should be added to the bottom of the ELISA plate well. Avoid touching the inside wall of the well. Avoid foaming when possible.
4. **Assay Timing**: The interval between adding sample to the first and last wells should be minimized. Delays will increase the incubation time differential between wells, which will significantly affect the experimental accuracy and repeatability. For each step in the procedure, total dispensing time for addition of reagents or samples should not exceed 10 minutes.
5. **Incubation**: To prevent evaporation and ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods of time between incubation steps. Do not let wells dry out at any time during the assay. Strictly observe the recommended incubation times and temperatures.
6. **Washing**: Proper washing procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings. Residual liquid in the reaction wells should be patted dry against absorbent paper during the washing process. Do not put absorbent paper directly into the reaction wells.
7. **Controlling Substrate Reaction Time**: After the addition of the TMB Substrate solution, periodically monitor the color development. Stop color development before the color becomes too deep by adding Stop Solution. Excessively strong color will result in inaccurate absorbance reading.
8. **Reading**: The microplate reader should be preheated and programmed prior to use. Prior to taking OD readings, remove any residual liquid or fingerprints from the underside of the plate and confirm that there are no bubbles in the wells.

SAMPLE ONLY

9. **Reaction Time Control**: Control reaction time should be strictly followed as outlined.
10. **Stop Solution**: The Stop Solution contains an acid, therefore proper precautions should be taken during its use, such as protection of the eyes, hands, face, and clothing.
11. **Mixing**: During incubation times, the use of a micro-oscillator at low frequency is recommended. Sufficient and gentle mixing is particularly important in producing reliable results.
12. Kits from different batches may be a little different in detection range, sensitivity, and color developing time. Please perform the experiment exactly according to the supplied instructions.
13. Due to inter- and intra-assay variability, it is recommended that appropriate carry-over controls be included between assays.
14. Prior to running valuable samples, LSBio recommends that the user run a preliminary experiment using the supplied controls in order to validate the assay.
15. To minimize external influence on the assay performance, operational procedures and lab conditions (such as room temperature, humidity, incubator temperature) should be strictly controlled. It is also strongly suggested that the whole assay is performed by the same operator from the beginning to the end.
16. The kit should not be used beyond the expiration date on the kit label.

SAMPLE ONLY

ASSAY PROCEDURE SUMMARY

Prepare all reagents, samples and standards.

Add 100 µl of **Positive Control, Blank, or Sample** to each well and incubate for 2.5 hours at room temperature or overnight at 4°C.

Aspirate and wash 4 times.

Add 100 µl of **Detection Antibody** and incubate for 1 hour at room temperature.

Aspirate and wash 4 times.

Add 100 µl of **HRP-Conjugated Antibody** and incubate for 1 hour at room temperature.

Aspirate and wash 4 times.

Add 100 µl of **TMB Substrate** solution and incubate for 30 minutes.

Add 50 µl of **Stop Solution**.

Read immediately at 450nm.

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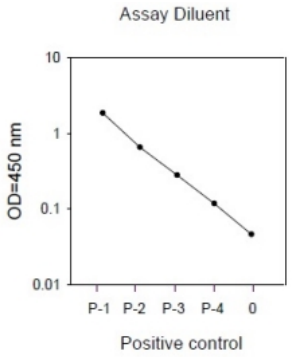
TYPICAL DATA

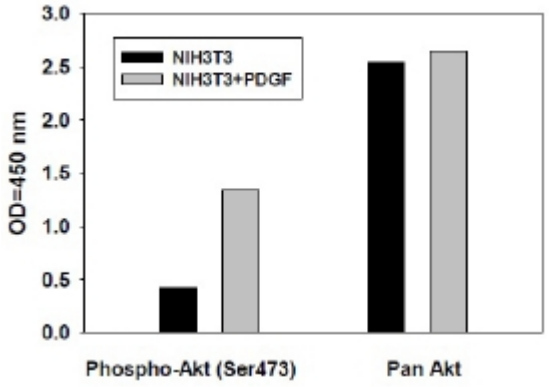
Calculate the mean absorbance for each set of duplicate positive controls and samples, and then subtract the average zero (blank) optical density.

Positive Control : NIH3T3 cells were treated with PDGFBB at 37°C for 10 min. Cells were solubilzed at 4 x 10^7 cells/ml in Cell Lysate Buffer. Serial dilutions of lysates were analyzed in this ELISA.



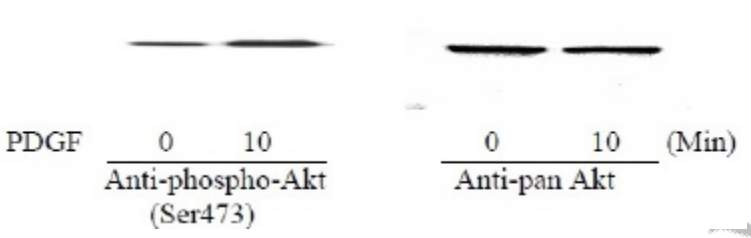
Recombinant Human PDGF Stimulation of NIH3T3 Cell Lines: NIH3T3 cells were untreated or treated with 100 ng/ml recombinant human EGF for 10 min. Cell lysates were analyzed using this phosphoELISA and Western Blot.

ELISA



AVAILABLE ONLY

Western-Blot Analysis



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TROUBLESHOOTING GUIDE

Problem	Possible Cause	Solution
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standard dilution	Briefly spin the vial of standard and dissolve the powder thoroughly by a gentle mix.
	Wells not completely aspirated	Completely aspirate wells between steps.
Low signal	Too brief incubation times	Ensure sufficient incubation time.
	Incorrect assay temperature	Use recommended incubation temperature. Bring substrate to room temperature before use.
	Inadequate reagent volumes	Check pipettes and ensure correct preparation.
	Improper dilution	
Deep color but low value	Plate reader settings not optimal	Verify the wavelength and filter setting in the plate reader.
		Open the Plate Reader ahead to pre-heat.

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Troubleshooting Guide (continued)

Problem	Possible Cause	Solution
Large CV	Inaccurate pipetting	Check pipettes
High background	Concentration of detector too high	Use recommended dilution factor.
	Plate is insufficiently washed	Review the manual for proper washing instructions. 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	All the reagents should be stored according to the instructions.
	Stop solution not added	Stop solution should be added to each well before measurement.

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Important Note: During shipment, small volumes of product will occasionally become entrapped in the seal of the product vial. We recommend briefly centrifuging the vial to dislodge any liquid in the container's cap prior to opening.

Warning: This reagent may contain sodium azide and sulfuric acid. The chemical, physical, and toxicological properties of these materials have not been thoroughly investigated. Standard Laboratory Practices should be followed. Avoid skin and eye contact, inhalation, and ingestion. Sodium azide forms hydrazoic acid under acidic conditions and may react with lead or copper plumbing to form highly explosive metal azides. On disposal, flush with large volumes of water to prevent accumulation.

Returns, Refunds, Cancelations: Any problems with LifeSpan products must be reported to LifeSpan within 10 days of product receipt. The customer must obtain written authorization from LifeSpan before returning items. To request that goods be returned, please contact LifeSpan Technical Support. If an error by LifeSpan BioSciences results in shipment of an incorrect order, LifeSpan will, at its option, either ship a replacement order at no charge, or credit the customer's account for the original product shipped in error. Returns and cancelations may be subject to a 30% restocking fee. Conditions & Warranty: All LifeSpan products are intended for Research Use Only and are not for use in Human therapeutic or diagnostic applications. The information supplied with each product is believed to be accurate, but no warranty or guarantee is offered for the products, because the ultimate conditions of use are beyond LifeSpan's control. The information supplied with each product is not to be construed as a recommendation to use this product in violation of any patent, and LifeSpan will not be held responsible for any infringement or other violation that may occur with the use of its products. Under no event will LifeSpan be responsible for any loss of profit or indirect consequential damage, including, but not limited to, personal injuries resulting from use of these products. LifeSpan's liability to any user of Products for damages that do not result from any fault of the user, will be limited to replacement of the Product(s) only, and in no event shall LifeSpan's liability exceed the actual price received by LifeSpan for the Product(s) at issue. LifeSpan shall not be liable for any indirect, special, incidental or consequential damages. LIFESPAN FURTHER DISCLAIMS ANY AND ALL EXPRESS AND IMPLIED OR STATUTORY WARRANTIES WITH RESPECT TO THE PRODUCTS, INCLUDING BUT NOT LIMITED TO ANY IMPLIED WARRANTIES OF MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE. LifeSpan disclaims any and all responsibility for any injury or damage which may be caused by the fault of the user.

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