Phospho-Akt1 (S473) ELISA Kit Manual

Features

- Easy to use system
- Reagents titered for success
- Proven rapid protocol

Ordering Information

Catalog Number
X1844K

Size
One 96-well Plate

Format
Colorimetric ELISA Kit

Species Reactivity
Human/Mouse/Rat

Company Information
Exalpha Biologicals, Inc.
tel: 978.425.1370
info@exalpha.com
www.exalpha.com

Other Kits & Reagents Available from Exalpha Biologicals

ApoMark™ Colorimetric (DAB) Apoptosis Detection Kit
X2044K1 (30 Tests)
X2044K2 (60 Tests)

ApoMark™ FL Fluorescent Apoptosis Detection Kit

ApoMark™ FL488
- X2835K1 (30 Tests)
- X2835K2 (60 Tests)

ApoMark™ FL549
- X2836K1 (30 Tests)
- X2836K2 (60 Tests)

ApoMark™ FL594
- X2837K1 (30 Tests)
- X2837K2 (60 Tests)

ApoMark™ FL649
- X2838K1 (30 Tests)
- X2838K2 (60 Tests)

BrdU Colorimetric Cell Proliferation ELISA Kit
X1327K1 (200 Tests)
X1327K2 (1000 Tests)
X1327K3 (5000 Tests)

BrdU Chemiluminescent Cell Proliferation ELISA Kit
X1623K1 (200 Tests)
X1623K2 (1000 Tests)
X1623K3 (5000 Tests)

BrdU Immunohistochemistry Kit
X1545K (50 Sections)

BrdU Immunofluorescence Kit
X2839K (FL488, 50 Sections)
X2840K (FL549, 50 Sections)

Anti-Fade Mounting Media
X2841 (7 mls)

BrdU Reagent for In Vivo Injection
X2834 (5 x 5 mg)

BrdU Unstained Control Slides
X2743 (5 slides)

Exalpha Biologicals, Inc. I 2 Shaker Rd. Bldg. B101 I Shirley, MA 01464
Tel: 978.425.1370 I info@exalpha.com I www.exalpha.com

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PHOSPHO-AKT1 (S473) ELISA

Intended Use

The Exalpha Biologicals, Inc. Phospho Akt1 (pan)(S473) (also known as RAC-PK-alpha (rac), protein kinase B (PKB) or C-AKT) ELISA is a non-isotopic immunoassay for the in vitro quantitation of human/mouse/ rat Phospho Akt1 (S473) protein in cell lysates.

This assay is for research use only and not for use in diagnostic or therapeutic procedures.

Storage of Kit Components

The Exalpha Biologicals Phospho-Akt1 ELISA kit components are shipped on blue ice. Upon receipt, store entire kit at 4-8°C.

Background

Akt is a general protein kinase capable of phosphorylating several known proteins including TBC1D4 (TBC1 domain family, member 4). Akt signals downstream of phosphatidylinositol 3-kinase (PI(3)K) to mediate the effects of various growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin and insulin-like growth factor I (IGF-I). Plays a role in glucose transport by mediating insulin-induced translocation of the GLUT4 glucose transporter to the cell surface. Mediates the antiapoptotic effects of IGF-I. Mediates insulin-stimulated protein synthesis, partly by playing a role in both insulin-induced phosphorylation of 4E-BP1 and in insulin-induced activation of p70 S6 kinase. One of the essential functions of Akt is the regulation of glycogen synthesis through phosphorylation and inactivation of GSK-3α and β (Promotes glycogen synthesis by mediating the insulin-induced activation of glycogen synthase). In addition to its role in survival and glycogen synthesis, Akt is involved in cell cycle regulation by preventing GSK-3β mediated phosphorylation and degradation of cyclin D1. Catalytic activity: ATP + a protein = ADP + a phosphoprotein. Enzyme regulation: Three specific sites, one in the kinase domain (Thr-308) and the two other ones in the C-terminal regulatory region (Ser-473 and Tyr-474), need to be phosphorylated for its full activation. Activated by insulin and various growth and survival factors and functions in a wortmannin-sensitive pathway involving PI3 kinase. Akt is activated by phospholipid binding and activation loop phosphorylation at Thr308 by PDK1 (4) and by phosphorylation within the carboxy terminus at Ser473. PDK2 is responsible for phosphorylation of Akt at Ser473 has been identi ed as mammalian target of rapamycin (mTor), termed TORC2. Akt may also play a role in insulin stimulation of glucose transport and by

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Tel: 978.425.1370 I info@exalpha.com I www.exalpha.com

negatively regulating the cyclin dependent kinase inhibitors p27 Kip and p21 Waf1. Akt also plays a critical role in cell growth by directly phosphorylating mTOR in a rapamycin-sensitive complex containing raptor, termed TORC1. More importantly, Akt phosphorylates and inactivates tuberin (TSC2), an inhibitor of mTOR within the TORC1 complex. Inhibition of mTOR stops the protein synthesis machinery due to inactivation of its effector, p70 S6 kinase and activation of the eukaryotic initiation factor, 4E binding protein 1 (4E-EP1), an inhibitor of translation.

Principles of the Assay

The Exalpha Biologicals, Inc. Phospho Akt1 ELISA is a non-isotopic immunoassay for the in vitro quantitation of phospho Akt1 protein in cell lysates or tissue homogenates.

The Phospho Akt1 ELISA is a “sandwich” enzyme immunoassay employing polyclonal antibodies. An antibody, speci c for the non-phospho and phospho Akt1 protein has been immobilized onto the surface of microtiter wells provided in the kit. Using this ELISA, cross-reactivity with unphosphorylated Akt1, Akt2, and Akt3 is minimal. The sample (cell lysate or tissue homogenate) to be assayed are pipetted into the wells and allowed to incubate for two hours (or over night for higher sensitivity), during which time any Akt1 present binds to the capture antibodies. Unbound material is washed away and a biotin conjugated anti-phospho Akt1 antibody is added to the wells and incubated for 2 hours at room temperature. Excess biotin conjugate is removed by washing and a horseradish peroxidase (HRP)-conjugated streptavidin is added for 30 minutes, which binds to the detector antibody. Excess HRP conjugate is removed by washing.

The horseradish peroxidase catalyzes the conversion of the chromogenic substrate tetra-methylbenzidine (TMB) (30 minute incubation) from a colorless solution to a blue solution (or yellow after the addition of stop reagent), the intensity of which is proportional to the amount of phospho Akt1 protein in the sample. The colored reaction product is quantitated using a spectrophotometer.

Quantitation is achieved by the construction of a standard curve using known concentrations of phospho Akt1 standard (provided lyophilized). By comparing the absorbance obtained from a sample containing an unknown amount of phospho Akt1 with that obtained from the standards, the concentration of phospho Akt1 in the sample can be determined.

Materials Provided*

Standards and samples should be assayed in duplicate. A standard curve must be performed on the same plate and at the same time as the samples. The Phospho-Akt1 ELISA provides

sufficient reagents to run two sets of standard curves, and 41 samples (if assayed in duplicate all at once using one standard curve), or 34 samples (if assayed on two separate occasions using two standard curves).

- **Component 1:** Coated Microtiter Plate: 96 removable wells coated with phospho- and non-phospho-Akt1 antibody.
- **Component 2:** Phospho-Akt1 Standard: two vials containing frozen protein.
  ! Standards MUST be stored frozen at -30°C. Once thawed, standards should be used immediately or be aliquoted and frozen at -30°C and discarded after one freeze-thaw cycle.
- **Component 3:** Detector Antibody (12 ml): biotinylated phospho-Akt1 antibody.
- **Component 4:** 400X Conjugate (50 µl): Streptavidin-Peroxidase Conjugate; 400-fold concentrated solution.
- **Component 5:** Conjugate Diluent (12 ml): buffer for dilution of 400X Conjugate.
- **Component 6:** Substrate (11 ml): chromogenic substrate (TMB).
- **Component 7:** Standard and Sample Diluent (30 ml): added to plate prior to sample/standard and used to dilute samples/standard.
- **Component 8:** 50X Plate Wash Concentrate (40 ml): 50-fold concentrated solution of PBS and surfactant.
- **Component 9:** Cell Lysis Buffer (45 ml); use as directed in sample preparation section for the extraction of Phospho Akt1 from cell preparations.
- **Component 10:** Stop Solution (11 ml): 2.5 N sulfuric acid solution.
- **Component 11:** Plate Sealers (2): to cover plates during incubations.

Materials Required But Not Provided

- The addition of protease inhibitors to the cell lysis buffer may be desirable. A suitable and compatible protease inhibitor is manufactured by Sigma Aldrich (Protease Inhibitor Cocktail, Cat # P8340) use according to manufacturers recommendations.
- 2-20 µl, 20-200 µl and 200-1000 µl precision pipetters with disposable tips.
- Automated plate washer, wash bottle or multichannel dispenser for washing.
- 2 liter graduated cylinder.
- Deionized or distilled H2O.
- Spectrophotometer capable of measuring absorbance in 96-well plates using dual wavelength of 450/595 nm or 450/550 nm. A single wavelength of 450 nm can also be used.

Exalpha Biologicals, Inc. I 2 Shaker Rd. Bldg. B101 I Shirley, MA 01464
Tel: 978.425.1370 I info@exalpha.com I www.exalpha.com

Summary of Procedure

Not to be used in place of Detailed Assay Protocol. For complete instructions see Detailed Protocol section.

Steps		Incubation
1.	Equilibrate kit to room temp.	-
2.	Add samples and standards to wells	2 hours room temp. (or overnight 4°C)
3.	Wash 4x	-
4.	Add detector antibody all wells	1 hour - room temp
5.	Wash 4x	-
6.	Add HRP conjugate to all wells	30 minutes, room temp
7.	Wash 4x	-
8.	Add substrate to all wells	30 minutes, room temp.
9.	Add stop solution to all wells	-
10.	Read plate at 450 nm/550 nm	-

Precautions and Recommendations

- Special care should be taken when working with phospho-proteins to avoid phosphatase activity which could result in spurious results.
- **Store all components at 2°C - 8°C.** Do not expose reagents to excessive heat or light.
- Let the kit sit at room temperature for 30 minutes before use. Best results will be obtained using reagents at room temperature.
- Do not use the kit beyond the expiration date.
- Always use clean well-rinsed glassware. Soap residue may compromise assay performance.
- Use only the microtiter wells provided with the kit.
- Do not mix reagents from different kits.
- Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.
- Do not mouth pipette or ingest any of the reagents.
- The buffers and reagents used in this kit contain anti-microbial and anti-fungal reagents. Care should be taken to prevent direct contact with these products.
- Human samples may be contaminated with infectious agents. Do not ingest, expose to open wounds, or breathe aerosols. Dispose of samples properly.

Sample Preparation

Cell Lysate / Tissue Homogenate Protocol

Numerous extraction protocols can be used. The following protocol has been shown to work with a number of lysates from cell lines. It is provided as an example of a suitable extraction procedure, but should not be construed as necessarily being the

method of choice for your assay. Users may wish to experiment with extraction procedures that work best in their laboratory.

1. For suspension cells, pellet by centrifugation, remove supernatant, resus- pend with PBS and pellet by centrifugation. For attached cells, remove supernatant from cells (you may save the supernatant for testing in the ELISA). Wash cells once with PBS, harvest cells by scraping and gentle centrifugation.
2. Aspirate PBS leaving an intact cell pellet (at this point the cell pellet can be frozen at -80°C and lysed at a later date). We recommend for every 1 x 107 cells, resuspend the pellet in 1 ml of Cell Lysis Buffer provided (Compo- nent 9).
3. Incubate 30 minutes on ice with occasional vortexing.
4. Transfer extracts to microcentrifuge tubes and centrifuge for 5 minutes at 500 x g at 4°C to remove debris.
5. Aliquot cleared lysate to clean microfuge tubes. The sample should be aliquotted to avoid multiple freeze/thaws. These samples are now ready for analysis according to the instructions provided in the Detailed Protocol. Samples may be stored at –80°C for future testing in the Phospho Akt1 ELISA.

Tissue Homogenate Protocol

1. Important notes before starting. Ensure that you are familiar with the operation of the tissue homogenizer. Refer to the User Manual for operating instructions.
   Carry out the disruption at 4°C or use an ice bath where appropriate. The use of a protease inhibitor cocktail will prevent proteolytic degradation of the proteins during homogenation.
2. Tissue homogenation / lysis should be carried out using 1 ml Cell Lysis Buffer provided (Component 9) for every 1-2 mm^2 section of tissue.
3. Allow homogenates to incubate on ice for 30 minutes with occasional vortexing and proceed to number 4 above.

Samples found to contain greater than 20 ng/ml Phospho Akt1 (i.e., outside the range of the standard curve) must be diluted with Sample Diluent (provided), so that the Phospho Akt1 concentration falls within the range spanned by the standard curve, and assayed again.

Detailed Protocol

The Phospho Akt1 ELISA is provided with removable strips of wells so the assay can be carried out on two separate occasions. **Since conditions may vary, a standard curve must be determined each time the assay is performed.** Standards should be assayed in duplicate. Disposable pipette tips and reagent troughs should be used for all transfers to avoid cross- contamination of reagents or samples.

1. Remove the appropriate number of microtiter wells from the foil pouch.
   Return any unused wells to the foil pouch, reseal and store at

4°C. Let all other kit components sit at room temperature until used. Best results will be obtained using reagents at room temperature.

2. Prepare a working solution (1X) of Wash Buffer by adding 40 ml of the 50X concentrated solution (provided - Component 8), to 1960 ml of deion- ized water. Mix well.
3. Each time an assay is performed, thaw a vial of Standard. Let the Standard come to room temperature. Avoid excess agitation of the Standard. After thawing the Standard, it should be diluted with Standard Sample Diluent (Component 7). Obtain six tubes and label them 20, 6.7, 2.2, 0.74, 0.25 and 0 ng/ml. Add 300 µl of Sample Diluent into each tube except the 20 ng/ml tube (first tube) which gets “undiluted” standard. Remove 500 µl from the original vial of lyophilized material and add it to the rst tube. Remove 150 µl from the rst tube (20 ng/ml) and add it to the second tube (6 ng/ml) and mix gently. Repeat this procedure until you reach the fth tube (0.250 ng/ml). The last tube (0 ng/ml) should just be Sample Diluent. Reconstituted standards should be discarded after one use.
4. Prepare all samples. A recommended starting dilution for all samples is a 1:4 dilution with Sample and Standard diluent.
5. Add samples and each of the Phospho Akt1 standards (in duplicate) by pipetting 100 µl into appropriate wells using clean pipette tips for each sample.
6. Cover wells with a plate sealer and incubate at room temperature for 2 hours (or Overnight at 4° C for higher sensitivity).
7. Wash wells 4 times with 1X Wash Buffer making sure each well is lled completely.
8. Add 100 ul of prediluted Detector Antibody (Component 3) to each well used. Cover and incubate for 2 hours at room temperature.
9. Wash wells 4 times with 1X Wash Buffer making sure each well is lled completely.
10. Dilute a suf cient amount of the 400X Conjugate 1:400 in Conjugate Dilu- ent to provide 100 µl of 1X solution for each sample and standard well (For example: add 30 µl to 11.970 ml of Conjugate Diluent), mix gently.
11. Pipette 100 µl of the 1X Conjugate into each well, cover with a plate sealer and incubate at room temperature for 30 minutes. Discard any unused 1X Conjugate.
12. Wash wells 4 times with 1X Wash Buffer making sure each well is lled completely.
13. Remove contents of wells by inverting over sink and tapping on paper tow- els.
14. Add 100 µl of Substrate Solution to each well and incubate in the dark at room temperature for 30 minutes.
15. Add 100 µl of Stop Solution to each well in the same order as the previ- ously added Substrate Solution.
16. Measure absorbance in each well using a spectrophotometric plate reader. It is preferable to read at dual wavelengths of 450/550 nm (or 450/595 nm). A single wavelength of 450 nm can also be used. Wells must be read within 30 minutes of adding the Stop Solution.

Evaluation of Results

1. Average the duplicate absorbance values for each standard, including the zero, and all sample values.
2. On graph paper, plot the mean absorbance values for each of the standards on the Y axis, versus the concentration of each standard (ng/ml) on the X axis.
3. Determine the concentration of unknowns by interpolation from the standard curve. There are a variety of microtiter plate reader software packages available (Softmax, Molecular Devices Corporation, Menlo Park, CA; KinetiCalc, BioTek Instruments, Inc. Winooski, VT) for analysis of microtiter plate data, which simplifies this process.
4. For samples which have been diluted, the Phospho Akt1 concentration must be multiplied by the dilution factor (ie., if the sample was diluted five-fold, then the Phospho Akt1 value obtained from the standard curve must be multiplied by five).

Assay Characteristics

Sensitivity
The lower limit of detection (LLD), commonly used to define sensitivity, was measured by assaying four replicates of zero eight times using two different lots of plates and two different lots of detector antibody. The LLD for Exalpha's Phospho Akt1 assay was determined to be 100 pg/ml**.**

Precision
The pooled coefficients of variation (according to the formula of Henry et. al. 1974) and between assay coefficients of variation are plotted against Phospho Akt1 levels. The pooled data were collected from samples run eight times using two different lots of plates and two different lots of detector antibody in replicates of four on two separate occasions. Precision was determined to be less than 10 %.

Biological Detection Experiments
The Phospho Akt1 ELISA detects human Phospho Akt1 in cell lysates. Western blot analysis or direct ELISA data shows the coating and detecting antibodies to be specific for human Phospho Akt1.

Linearity
To assess the linearity of the assay, samples containing various levels of Phospho Akt1 or spiked with various concentrations of human Phospho Akt1 were diluted with Sample Diluent and then assayed. The measured human Phospho Akt1 concentrations at each dilution within the working range of the assay are within 5% of the expected **values for all samples.**

Reagent Stability

All of the reagents included with the Phospho-Akt1 ELISA have been tested for stability. Reagents should not be used beyond the stated expiration date.

References

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Ordering Information

Catalog Number	Size
X1844K	1 Plate

Contact Information

Exalpha Biological, Inc.
2 Shaker Road, Bldg. B101
Shirley, MA 01464

Telephone: 978.425.1370
url: www.exalpha.com
email: info@exalpha.com