Enzo Life Sciences

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[pSer^{9}]GSK-3ß EIA kit

Catalog # **ADI-900-123A**

96 Well Enzyme Immunoassay Kit
For use with cell lysates

Reagents require separate storage conditions.

Check our website for additional protocols, technical notes and FAQs.

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ALEXIS® assay designs® BIOMOL® Stressgen®

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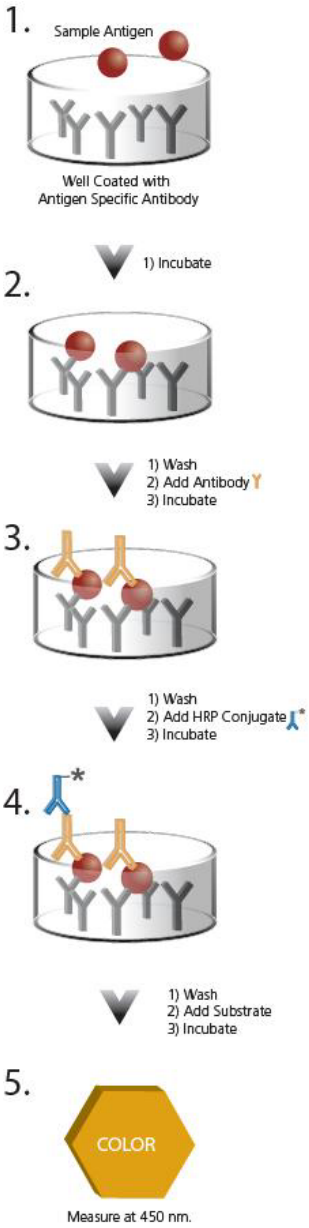


Introduction

The [pSer[9]]GSK-3β EIA kit is a complete kit for the quantitative determination of GSK-3β (phospho-Ser9) in cell lysates. Please read the complete kit insert before performing this assay.

Glycogen synthase kinase 3β (GSK-3β) is a unique serine/threonine kinase that is inactivated by phosphorylation. In response to insulin binding, PKB/Akt phosphorylates GSK-3β on serine 9, which prevents GSK-3β from phosphorylating glycogen synthase[1]. Unphosphorylated glycogen synthase is active and able to synthesize glycogen. GSK-3β is also unique in that it requires a substrate that has been phosphorylated by a distinct kinase before it can phosphorylate the substrate[2]. This phosphate priming mechanism explains why phosphorylation of serine 9 inactivates GSK-3β. The phosphorylated serine binds to the GSK-3β priming phosphate position and prevents binding of alternative substrates[3]. In addition to insulin signaling, GSK-3β participates in the Wnt signaling pathway, where it forms a complex with axin, β-catenin and adenomatous polyposis coli (APC) protein. In the presence of Wnts, GSK-3β is unable to phosphorylate β-catenin, which leads to stabilization of β-catenin[4]. The Wnt pathway inactivates GSK-3β via the proteins, Dishevelled and FRAT, which disrupt the interaction of GSK-3β with axin, β-catenin, and APC[5]. Clinically, there is considerable interest in GSK-3β inhibitors because they may mimic the effect of insulin or reduce the hyperphosphorylation of Tau that is observed in Alzheimer's Disease[6].

Principle

1. Samples and standards are added to wells coated with a monoclonal antibody specific for GSK-3β. The plate is then incubated.
2. The plate is washed, leaving only bound GSK-3β on the plate. A yellow solution of polyclonal antibody to GSK-3β (phospho-Ser9) is then added. This binds to the GSK-3β (phospho-Ser9) captured on the plate. The plate is then incubated.
3. The plate is washed to remove excess antibody. A blue solution of anti-rabbit IgG conjugated to horseradish peroxidase is added to each well, binding to the polyclonal GSK-3β (phospho-Ser9) antibody.
4. The plate is washed to remove excess HRP conjugate. TMB Substrate solution is added. An HRP-catalyzed reaction generates a blue color in the solution.
5. Stop solution is added to stop the substrate reaction. The resulting yellow color is read at 450 nm. The amount of signal is directly proportional to the level of GSK-3β (phospho-Ser9) in the sample.

Do not mix components from different kit lots or use reagents beyond the expiration date of the kit.

The standard should be handled with care due to the known and unknown effects of the antigen.

Activity of conjugate is affected by nucleophiles such as azide, cyanide, and hydroxylamine.

Protect substrate from prolonged exposure to light.

Stop solution is caustic. Keep tightly capped.

Materials Supplied

1. **Assay Buffer 21**
   100 mL, Catalog No. 80-1519
   Tris buffered saline containing BSA, detergents and phosphatase inhibitors

2. **GSK-3β Standard**
   0.1 mL, Catalog No. 80-1108
   1 vial containing 100,000 pg/mL of recombinant GSK-3β

3. **GSK-3β Clear Microtiter Plate**
   One plate of 96 wells, Catalog No. 80-1096
   A clear plate of break-apart strips coated with a monoclonal antibody specific for GSK-3β

4. **GSK-3β (phospho-Ser9) Antibody**
   10 mL, Catalog No. 80-2017
   A yellow solution of polyclonal antibody to GSK-3β (phospho-Ser9)

5. **GSK-3β (phospho-Ser9) Conjugate**
   10 mL, Catalog No. 80-1150
   A blue solution of anti-rabbit IgG conjugated to horseradish peroxidase

6. **Wash Buffer Concentrate**
   100 mL, Catalog No. 80-1287
   Tris buffered saline containing detergents

7. **TMB Substrate**
   10 mL, Catalog No. 80-0350
   A solution of 3,3′,5,5′ tetramethylbenzidine (TMB) and hydrogen peroxide

8. **Stop Solution 2**
   10 mL, Catalog No. 80-0377
   A 1N solution of hydrochloric acid in water

9. **RIPA Cell Lysis Buffer 2**
   100 mL, Catalog No. 80-1284
   50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1% Sodium Deoxycholate and 0.1% SDS

10. **GSK-3β (phospho-Ser9) Assay Layout Sheet**
    1 each, Catalog No. 30-0277

11. **Plate Sealer**
    3 each , Catalog No. 30-0012

Reagents require separate storage conditions.

Storage

All components of this kit, **except the Standard**, are stable at 4°C until the kit's expiration date. The standard must be stored at or below -20°C upon receipt.

Materials Needed but Not Supplied

1. Deionized or distilled water
2. Phenylmethylsulfonyl fluoride (PMSF), Sigma #P7626 or equivalent
3. Protease inhibitor cocktail (PIC), Sigma #P8340 or equivalent
4. Sodium orthovanadate, Sigma #S6508 or equivalent
5. Sodium pyrophosphate, Sigma #S6422 or equivalent
6. Precision pipets for volumes between 5 µL and 1,000 µL
7. Repeater pipet for dispensing 100 µL
8. Graduated cylinders
9. A microplate shaker
10. Lint-free paper for blotting
11. Microplate reader capable of reading 450 nm
12. Software for extrapolating sample values from optical density readings utilizing a four parameter logistic curve fit.

Reagent Preparation

1. **Wash Buffer**

   Prepare the wash buffer by diluting 50 mL of the supplied Wash Buffer Concentrate with 950 mL of deionized water. This can be stored at room temperature until the kit's expiration date, or for 3 months, whichever comes first.

If buffers other than those provided are used in the assay, the end-user must determine the appropriate dilution and assay validation.

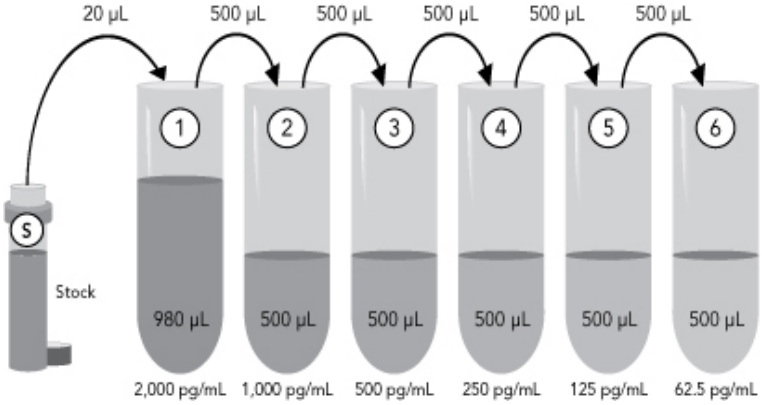
2. **Assay Buffer 21 plus Inhibitors**

   Immediately prior to use in the assay, PMSF and PIC must be added to the buffer. If using Sigma Protease Inhibitor Cocktail #P8340, add 0.5 µL/mL or equivalent concentration according to alternate vendor's specification sheet. Add PMSF, such as Sigma #P7626, to a final concentration of 1 mM.

   This modified Assay Buffer 21 must be used for all samples and standard dilutions to ensure optimal integrity of phospho-GSK-3β. Fresh Assay Buffer 21 plus Inhibitors must be made for each assay.

Polypropylene must be used for standard preparation.

3. **Preparation of GSK-3β Standard Curve**



The standard should be handled with care due to the known and unknown effects of the antigen.

   Allow the 100,000 pg/mL GSK-3β standard solution to warm to room temperature. Label six 12x75 mm polypropylene tubes #1 through #6. Pipet 980 µL of Assay Buffer 21 plus Inhibitors into tube #1. Pipet 500 µL of Assay Buffer 21 plus Inhibitors into tubes #2 through #6. Add 20 µL of the 100,000 pg/mL Standard to tube #1. Vortex thoroughly. Add 500 µL of tube #1 into tube #2 and vortex. Add 500 µL of tube #2 into tube #3 and vortex thoroughly. Continue this for tubes #4 through #6.

   Diluted standards should be used within 1 hour of preparation. The concentrations of the standards are labeled above.

4. **Activated Sodium Orthovanadate**

   Prepare a 200 mM solution of Sodium Orthovanadate. Adjust the pH to 10.0 using either 1N NaOH or 1N HCl (at pH 10.0 the solution will be yellow). Boil the solution until it turns colorless (approximately 10 minutes). Cool the solution to room temperature. Readjust the pH to 10.0. Repeat the boiling and pH readjustment steps until the solution remains colorless and the pH stabilizes at 10.0. Aliquot and store the solution at -20°C.

5. **RIPA Cell Lysis Buffer 2 plus Inhibitors**

Allow the RIPA Cell Lysis Buffer 2 to come to room temperature. Ensure buffer is completely in solution prior to use. Immediately prior to use in cell lysis, protease inhibitors (PIC and PMSF) as well as phosphatase inhibitors (sodium pyrophosphate and activated sodium orthovanadate) must be added to the buffer. If using Sigma Protease Inhibitor Cocktail #P8340, add 0.5 µL/mL or equivalent concentration according to alternate vendor's specification sheet. Add PMSF, such as Sigma #P7626, to a final concentration of 1 mM. Add activated sodium orthovanadate, such as Sigma #S6508 to a final concentration of 2 mM and sodium pyrophosphate, such as Sigma #S6422, to a final concentration of 20 mM.

Fresh RIPA Cell Lysis Buffer 2 plus Inhibitors must be made each time the cells are lysed.

Sample Handling

Samples must be stored frozen at or below -70° to avoid loss of bioactive analyte. Repeated freeze/thaw cycles should be avoided.

This assay is suitable for measuring phospho-GSK-3β in a wide range of cell lysates. Samples containing rabbit serum or rabbit IgG are not suitable for use in this assay. It is recommended that all samples be lysed with the provided RIPA Cell Lysis Buffer 2 modified by the addition of Inhibitors (see Reagent Preparation). Prior to running the assay, frozen samples should be slowly brought to 4˚C and centrifuged, if necessary, to isolate residual debris.

A minimum 1:16 dilution of the samples is required to eliminate matrix interference of the RIPA Cell Lysis Buffer 2 in the assay. Due to differences among cell types, number of cells, or total cellular protein concentration, further dilution may be required. The users must determine the optimal sample dilution for their particular experiments. Below are examples of lysates that have been run in this assay.

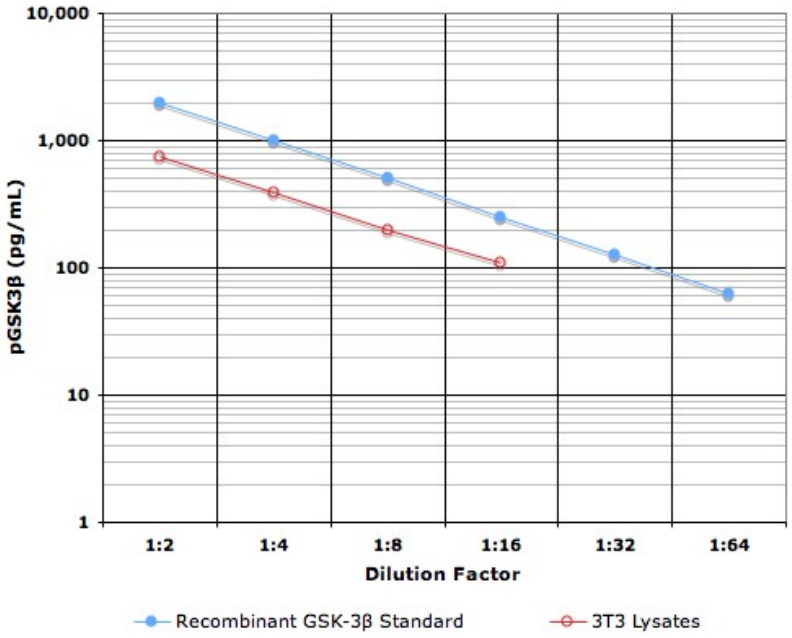
Dilutional Linearity

To determine possible interference from the sample matrix 3T3 cell lysates, containing 25.5 µg/mL total cellular protein at the 1:8 dilution, were serially diluted into the assay buffer. The concentrations of phospho-GSK-3β were measured in the assay, and the results were analyzed to determine the range over which a linear response was obtained. These samples lysed must be diluted at least 1:16 with Assay Buffer 21 plus Inhibitors prior to running in the assay.

	Average % of Expected
Dilution	**3T3 Cell Lysates**
1:8	138%
1:16	102%
1:32	101%
1:64	100%

Parallelism

A parallelism experiment was carried out to determine if the recombinant GSK-3β standard accurately measures pGSK-3β concentrations in biological matrices. To assess parallelism, values for 3T3 lysates were obtained from a standard curve using four parameter logistic curve fitting. The observed concentration was plotted against the dilution factor. Parallelism indicates antibody-binding characteristics of the native and standard proteins are similar, allowing accurate determination of analyte.



Assay Procedure

Refer to the Assay Layout Sheet to determine the number of wells to be used. Remove the wells not needed for the assay and return them, with the desiccant, to the mylar bag and seal. Store unused wells at 4°C.

1. Pipet 100 µL of Assay Buffer 21 plus Inhibitors into the S0 (0 pg/mL standard) wells.
2. Pipet 100 µL of Standards #1 through #6 into the appropriate wells.
3. Pipet 100 µL of the Samples into the appropriate wells.
4. Seal the plate and incubate for 1 hour shaking* at room temperature.
5. Empty the contents of the wells and wash by adding 400 µL of wash buffer to every well. Repeat 3 more times for a total of 4 washes. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
6. Pipet 100 µL of yellow antibody into each well except the blank.
7. Seal the plate and incubate for 1 hour shaking* at room temperature.
8. Wash as above (Step 5).
9. Pipet 100 µL of blue conjugate into each well except the blank.
10. Seal the plate and incubate for 30 minutes shaking* at room temperature.
11. Wash as above (Step 5).
12. Pipet 100 µL of substrate solution into each well.
13. Seal the plate and incubate for 30 minutes shaking* at room temperature.
14. Pipet 100 µL of stop solution into each well.
15. After blanking the plate reader against the substrate blank, read optical density at 450 nm. If plate reader is not capable of adjusting for the blank, manually subtract the mean OD of the substrate blank from all readings.

* Shaking is preferably carried out on a suitable plate or orbital shaker set at a speed to ensure adequate mixing of the contents of the wells. The optimal speed for each shaker will vary and may range from 120-700 rpm.

Bring all reagents to room temperature for at least 30 minutes prior to opening.

All standards, controls and samples should be run in duplicate.

Pre-rinse each pipet tip with reagent. Use fresh pipet tips for each sample, standard, and reagent.

Pipet the reagents to the sides of the wells to avoid possible contamination.

Prior to the addi-tion of antibody, conjugate, and substrate, ensure there is no residual wash buffer in the wells. Remaining wash buffer may cause variation in assay results.

Calculation of Results

Make sure to multiply sample concentrations by the dilution factor used during sample preparation.

Several options are available for the calculation of the concentration of phospho-GSK-3β in the samples. We recommend that the data be handled by an immunoassay software package utilizing a four parameter logistic curve fitting program. Such software is often supplied by plate reader manufacturers. If data reduction software is not readily available, the concentration of phospho-GSK-3β can be calculated as follows.

1. Calculate the average Net OD for each standard and sample by subtracting the average blank OD from the average OD for each standard and sample.

   Average Net OD = Average OD – Average Blanks OD

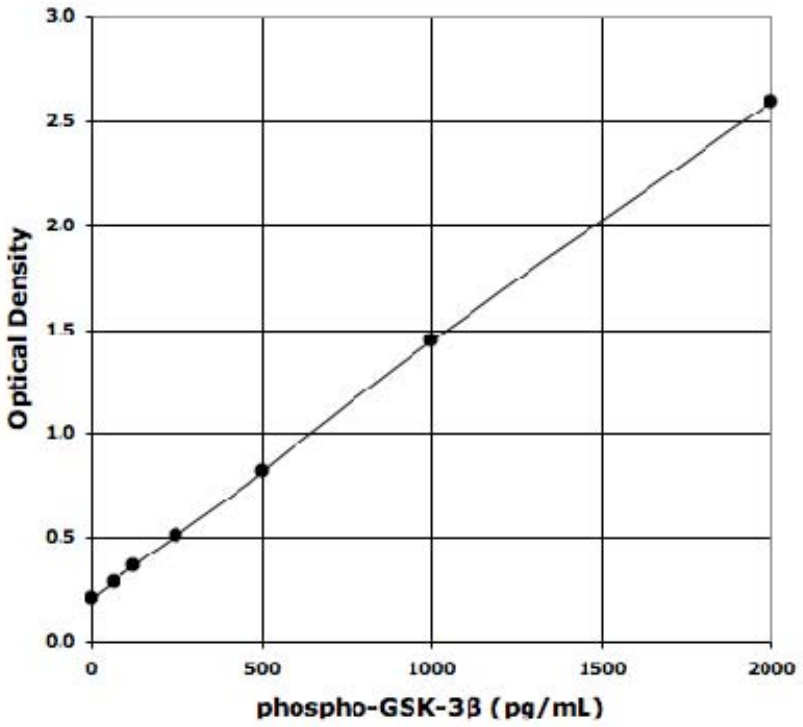
2. Using linear graph paper, plot the average Net OD for each standard versus phospho-GSK-3β concentration in each standard. Approximate a straight line through the points. The concentration of the unknowns can be determined by interpolation.

Samples with concentrations outside of the standard curve range will need to be re-analyzed using a different dilution.

Typical Results

The results shown below are for illustration only and should not be used to calculate results from another assay.

Sample	Net OD	pGSK-3β (pg/mL)
S0	0.209	0
S1	2.590	2000
S2	1.444	1000
S3	0.819	500
S4	0.507	250
S5	0.367	125
S6	0.286	62.5



Performance Characteristics

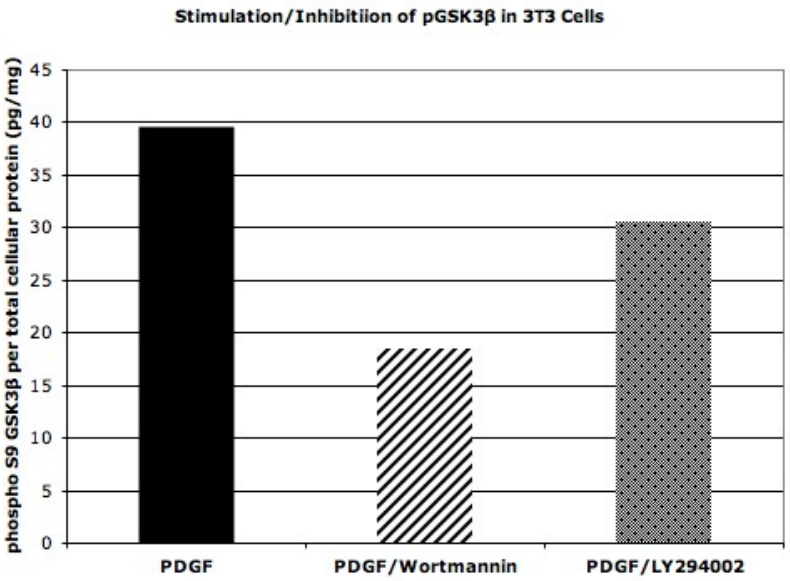
Specificity

The cross reactivities of related compounds were determined by diluting cross reactants in the assay buffer at several concentrations. These cross reactants were then measured in the assay.

Compound	Cross Reactivity
phospho-GSK-3	100%
GSK-3α, active	5.4%
-Catenin	< 0.3%
p21	< 0.3%
ATP Citrate Lyase	< 0.3%
MEK1, active	< 0.3%
Akt1, active	< 0.3%
JNK1, active	< 0.3%
ERK2, active	< 0.3%
ERK2, unactive	< 0.3%

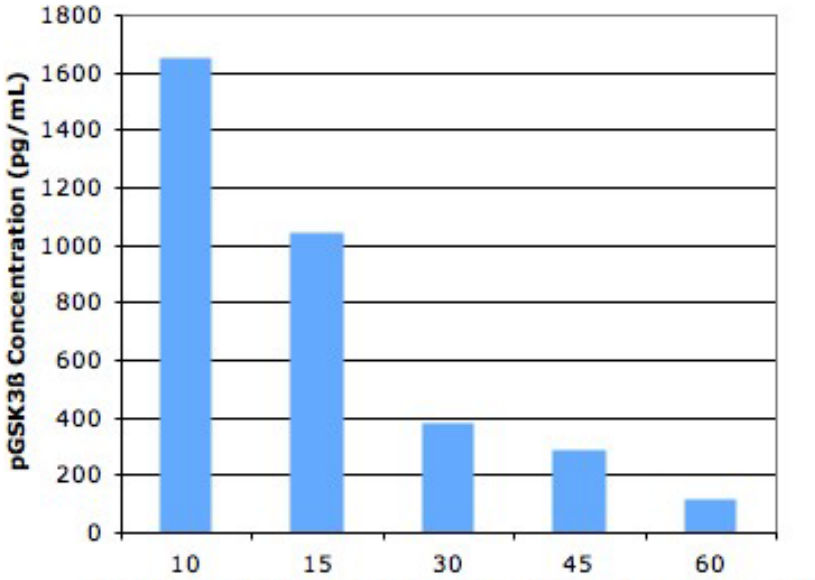
Stimulation / Inhibition Experiment

3T3 cells were treated with 50 ng/mL platelet derived growth factor (PDGF), PDGF and 1.0 µM Wortmannin, or PDGF and 50 µM LY294002. Both Wortmannin and LY294002 interfere with the induction of GSK-3β phosphorylation seen in cells treated with PDGF alone.



Dephosphorylation Experiment

To confirm that the antibody is sensitive to GSK-3 phosphorylation, GSK-3β treated with phosphatase was evaluated. Differing degrees of phosphorylation were achieved by varying the incubation time of GSK-3β with the phosphatase. As depicted in the graph below, this kit is specific for phosphorylated GSK-3β.



Dephosphorylation was achieved by addition of phosphatase to recombinant GSK3ß for indicated incubation time (min) at ambient temperature.

Sensitivity

The sensitivity or limit of detection of the assay is 9.0 pg/mL. The sensitivity was determined by interpolation at 2 standard deviations above the mean signal at background (0 pg/mL) using data from 6 standard curves.

Precision

Intra-assay precision was determined by assaying 20 replicates of three buffer controls containing phospho-GSK-3β in a single assay.

pg/mL	%CV
903	2.0
323	4.1
110	4.8

Inter-assay precision was determined by measuring buffer controls of varying phospho-GSK-3β concentrations in multiple assays over several days.

pg/mL	%CV
891	9.6
311	7.0
108	6.0

References

1. Cross, D.A., *et al*. (1995) Nature ***378***, 785-789.
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3. Frame, S., *et al*. (2001) Mol Cell ***7***, 1321-1327.
4. Hart, M.J., *et al*. (1998) Curr Biol. ***8***, 573-581.
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6. Cohen, P., *et al*. (2001) Nat Rev Mol Cell Biol. ***2***, 769-776.

Notes

Notes

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Use of Product

This product contains research chemicals. As such, they should be used and handled only by or under the supervision of technically qualified individuals. This product is not intended for diagnostic or human use.

Warranty

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