#7552 Store at –80°C

HTScan™ IRAK4 Kinase Assay Kit

☑ 100 Assays
(96 Well Format)

Cell Signaling TECHNOLOGY®

Orders ■ 877-616-CELL (2355)
orders@cellsignal.com

Support ■ 877-678-TECH (8324)
info@cellsignal.com

Web ■ **www.cellsignal.com**

rev. 06/16/05

Products Included	Product #	Kit Quantity
Phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) Ab	3141	30 µl
Kinase Buffer (10X)	9802	15 ml
ATP (10 mM)	9804	1 ml
Biotinylated Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) Peptide	1344	1.25 ml
IRAK4 Kinase (recombinant, human)	7551	1000 Units

Description: The kit provides a means of performing enzymatic assays with active human IRAK4 kinase. It includes active IRAK4 kinase (supplied as a GST fusion protein), a biotinylated substrate peptide and a phospho-serine/threonine-specific monoclonal antibody for detection of the phosphorylated form of the substrate peptide.

Peptide Core Sequence: YKT*LR

Molecular Weights: Peptide substrate (Biotinylated peptide): 1,958 Daltons, GST-IRAK4 Kinase domain: 69,839 Daltons

Unit Definition: 10 Units is defined as the amount of IRAK4 kinase required to maximally phosphorylate 75 pmol of biotinylated substrate peptide in 30 minutes at 25°C in a total reaction volume of 50 µl quantified by DELFIA® (signal/background of 25 or greater).

Background: Interleukin-1 (IL-1) receptor-associated kinase (IRAK) is a serine/threonine-specific kinase that can be coprecipitated in an IL-1-inducible manner with the IL-1 receptor (1). The mammalian family of IRAK molecules contains four members (IRAK1, IRAK2, IRAK-M and IRAK4). The binding of IL-1 to IL-1 receptor type I (IL-1RI) initiates the formation of a complex that includes IL-1RI, AcP, MyD88 and IRAKs (2). IRAK undergoes autophosphorylation shortly after IL-1 stimulation. The subsequent events involve IRAK dissociation from the IL-1RI complex, its ubiquitination, and its association with two membrane-bound proteins: TAB2 and TRAF6. The resulting IRAK-TRAF6-TAB2 complex is then released into the cytoplasm and activates protein kinase cascades, which include TAK1, IKKs, and the stress-activated kinases (3).

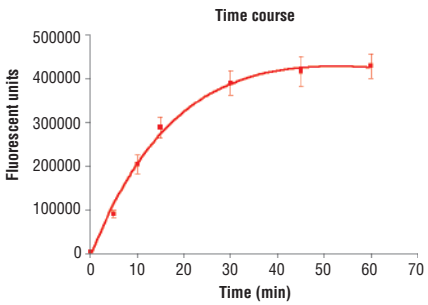

Figure 1. Time course of IRAK4 kinase activity: DELFIA® data generated using Phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) Antibody (#3141) to detect phosphorylation of IRAK4 substrate peptide (#1344) by IRAK4 kinase. In a 50 µl reaction, 10 Units IRAK4 and 1.5 µM substrate peptide were used per reaction well. Background reading is 4055. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

Source/Purification: The GST-Kinase fusion protein was produced using a baculovirus expression system with a construct expressing Ala104-Ser460 of human IRAK4 (GenBank Accession No. AF445802.1) fused to an amino-terminal GST tag. The protein was purified by one-step affinity purification using glutathione-agarose.

Polyclonal antibodies are produced by immunizing rabbits with a synthetic phospho-peptide (KLH-coupled) corresponding to residues surrounding Thr567 of human ezrin. Antibodies are purified by protein A and peptide affinity chromatography.

Quality Control: The substrate peptide was selected by using the Serine/Threonine Kinase Substrate Screening Kit #7400 to screen for IRAK4 kinase substrates. Phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) Antibody (#3141) was used for detection. The quality of the biotinylated peptide was evaluated by reverse-phase HPLC and by mass spectrometry.

Purified IRAK4 kinase was quality controlled for purity by SDS-PAGE followed by silver stain and Western blot.

Assay conditions (time course [Fig.1], kinase dose-dependence [Fig.2] and substrate dose-dependence [Fig.3]) for IRAK4 kinase activity were verified using the IRAK4 substrate peptide provided in this kit. IRAK4 kinase V_{max} and K_m values were measured to determine specific enzymatic activity [Fig.4]. IRAK4 sensitivity to the inhibitor staurosporine was measured using the IRAK4 substrate peptide provided in this kit [Fig. 5].

Background References:

(1) Dinarello, C.A. (1996) *Blood* 87, 2095–2147.

(2) Takaesu, G. et al. (2001) *Mol. Cell. Biol.* 21, 2475–2484.

(3) Janssens, S. and Beyaert, R. (2003) *Mol. Cell* 11, 293–302.

Storage: Antibodies are supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA and 50% glycerol. Do not aliquot the antibodies. Peptides are supplied at 6 µM in 0.001% DMSO. Enzymes are supplied in 50 mM Tris-HCL (pH 8.0), 100 mM NaCl, 5 mM DTT, 15 mM reduced glutathione and 20% glycerol. Store at –80°C.

Keep enzymes on ice during use.

Avoid repeated freeze-thaw cycles.

Companion Products:

IRAK4 Kinase #7551

Serine/Threonine Kinase Substrate Screening Kit #7400

Staurosporine #9953

IRAK4 Antibody #4363

Phospho-IRAK1 (Thr209) Antibody #4986

Phospho-IRAK1 (Ser376) Antibody #4361

Phospho-IRAK1 (Thr387) Antibody #4365

IRAK1 Antibody #4362

Cell Signaling Technology offers a full line of protein kinases, substrates, and antibody detection reagents for high throughput screening. Please direct all inquiries to: drugdiscovery@cellsignal.com

© 2005 Cell Signaling Technology, Inc.
This product is for research use only and is not intended for use in humans.

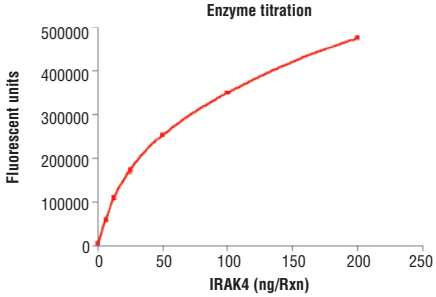

Figure 2. Dose dependence curve of IRAK4 kinase activity: DELFIA® data generated using Phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) Antibody to detect phosphorylation of substrate peptide (#1344) by IRAK4 kinase. In a 50 µl reaction, increasing amounts of IRAK4 and 1.5 µM substrate peptide were used per reaction well at 25°C for 30 minutes. Background reading is 4893. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

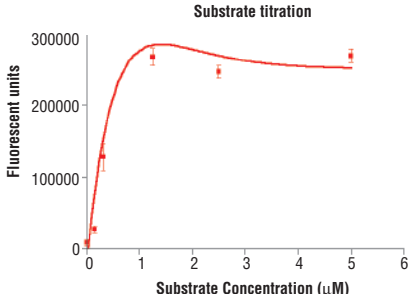

Figure 3. Peptide concentration dependence of IRAK4 kinase activity: DELFIA® data generated using Phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) Antibody to detect phosphorylation of substrate peptide (#1344) by IRAK4 kinase. In a 50 µl reaction, 10 Units of IRAK4 and increasing concentrations of substrate peptide were used per reaction well at 25°C for 30 minutes. Background reading is 4936. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

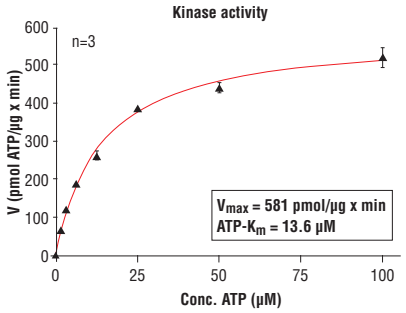

Figure 4. IRAK4 kinase activity was measured in a radioisotopic filtration assay using the following reaction conditions: 60 mM HEPES-NaOH, pH 7.5, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 3 µM Na-orthovanadate, 1.2 mM DTT, ATP (variable), 2.5 µg/50 µl PEG20.000, Substrate: Histone H2B, 5 µg/50 µl, Recombinant IRAK4: 4 Units/50 µl.

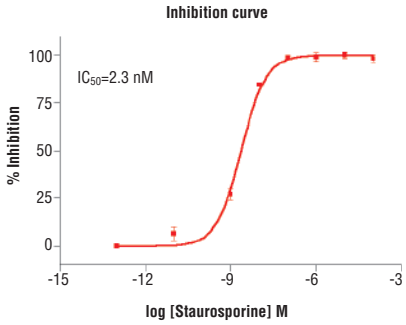

Figure 5. Staurosporine inhibition of IRAK4 kinase activity: DELFIA® data generated using Phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) Antibody to detect phosphorylation of IRAK4 substrate peptide (#1344) by IRAK4 kinase. In a 50 µl reaction, 10 Units IRAK4, 1.5 µM substrate peptide, 20 µM ATP and increasing amounts of staurosporine were used per reaction well at 25°C room temperature for 30 minutes. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

© 2005 Cell Signaling Technology, Inc.

Protocol for HTScan™ IRAK4 Kinase Assay Kit

Kinase

Note: Lot-specific information for this kinase is provided on the enzyme vial. Optimal assay incubation times and enzyme concentrations must be determined empirically for each lot of kinase under specified conditions.

Additional Solutions and Reagents (Not included)

- Wash Buffer: 1X PBS, 0.05% Tween-20 (PBS/T)
- Bovine Serum Albumin (BSA)
- Stop Buffer: 50 mM EDTA pH 8
- DELFIA® Europium-labeled Anti-rabbit antibody (PerkinElmer Life Sciences #AD0105)
- DELFIA® Enhancement Solution (PerkinElmer Life Sciences #1244-105)
- DELFIA® Streptavidin coated, 96-well, yellow plate (PerkinElmer Life Sciences AAAND-0005)

DELFIA® is a registered trademark of PerkinElmer Life Sciences

Suggested Protocol For 100 Assays

1. Add 100 µl 10 mM ATP to 1.25 ml 6 µM substrate peptide. Dilute the mixture with dH_20 to 2.5 ml to make 2X ATP/substrate cocktail ([ATP]=400 µM, [substrate]=3 µm).
2. Immediately transfer enzyme from –80°C to ice. Allow enzyme to thaw on ice.
3. **Microcentrifuge briefly at 4°C to bring liquid to the bottom of the vial. Return immediately to ice.**
4. Add 1 ml 10X kinase buffer [1 ml 10X Kinase Buffer 250 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 1 mM Na_3VO_4, 50 mM β-glycerophosphate, 20 mM dithiothreitol (DTT)] to 1.5 ml dH_20 to make 2.5 ml 4X reaction buffer.
5. Transfer 1.25 ml of 4X reaction buffer to enzyme tube to make 4X reaction cocktail ([enzyme]=0.8 Units/µl in 4X reaction cocktail).
6. Incubate 12.5 µl of the 4X reaction cocktail with 12.5 µl/well of prediluted compound of interest (usually around 10 µM) for 5 minutes at room temperature.
7. Add 25 µl of 2X ATP/substrate cocktail to 25 µl/well preincubated reaction cocktail/compound.
8. Incubate reaction plate at 25°C for 30 minutes.
9. Add 50 µl/well Stop Buffer (50 mM EDTA, pH 8) to stop the reaction.
10. Transfer 25 µl of each reaction and 75 µl dH_2O/well to a 96-well streptavidin-coated plate and incubate at room temperature for 60 minutes.
11. *Wash three times with 200 µl/well PBS/T.
12. Dilute primary antibody, Phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) Antibody #3141, 1:1000 in PBS/T with 1% BSA. Add 100 µl/well primary antibody.
13. Incubate at 37°C for 120 minutes.
14. *Wash three times with 200 µl/well PBS/T.
15. Dilute Europium labeled anti-rabbit antibody 1:1000 in PBS/T with 1% BSA. Add 100 µl/well diluted antibody.
16. Incubate at room temperature for 30 minutes.
17. *Wash five times with 200 µl/well PBS/T.
18. Add 100 µl/well DELFIA® Enhancement Solution.
19. Incubate at room temperature for 5 minutes.
20. Detect 615 nm fluorescence emission with appropriate Time-Resolved Plate Reader.

Final Assay Conditions for a 50 µl Reaction

25 mM Tris-HCl (pH7.5)

10 mM $MgCl_2$

5 mM β-glycerophosphate

0.1 mM Na_3VO_4

200 µM ATP

2 mM DTT

1.5 µM peptide

10 Units GST-IRAK4 Kinase

***IMPORTANT: Use of an automated microplate washer as well as centrifugation of plates when appropriate, greatly improves reproducibility.**

formulated and carrier-free), and detailed peptide substrate sequence information. Email: drugdiscovery@cellsignal.com

© 2005 Cell Signaling Technology, Inc.