

IKK β KinEASE™ FP-645nm FarRed Assay

Catalog # 32-094

Sufficient reagents for two 384-well plates per kit.

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NOT RECOMMENDED OR INTENDED
FOR DIAGNOSIS OF DISEASE IN
HUMANS.**

DO NOT USE IN HUMANS.

I. STORAGE AND STABILITY

Storage: Upon receipt, store individual components at recommended temperatures. Store the 384-well plates at room temperature. Store all other components at -20°C.

Stability: Components stable for 6 months from date of shipment if stored and handled correctly. We recommend that all enzymes to be used with this kit are stored as aliquots and a fresh aliquot used for each experiment.

II. ASSAY OVERVIEW

In this assay, a phosphorylated peptide has been labeled with a red fluorescent dye. This phosphorylated tracer has a low molecular weight and thus a low fluorescence polarization value. The phosphorylated tracer binds to a phospho-specific antibody to form a high molecular weight complex with a high polarization value.

In a kinase reaction, a peptide or a protein substrate (non-fluorescently labeled) is phosphorylated by the kinase in the presence of ATP and Magnesium to form a phosphorylated product. This phosphorylated product competes with the tracer for binding to the phospho-specific antibody. As increasing amounts of phosphorylated product are formed from the kinase reaction, there is a reduction in the binding of tracer to antibody resulting in a decrease in the fluorescence polarization value.

IKK β KinEASE™ FP-645nm FarRed Assay supplied in this kit offers a method for assaying IKK β using preferred substrate and a generic detection system.



Related Product: IKK β , Catalog # 14-485

III. SYSTEM COMPONENTS

A. Provided Kit Components

Prior to use, each reagent should be vortexed, and then centrifuged to collect residual liquid trapped in the vial cap. **Please note the STK Tracer-red solution is light sensitive.**

Reagent	Catalog #	Stock	Volume Supplied	Storage
STK Substrate 1	12-548	10mM	4 x 120 μ l	-20°C
STK Antibody	35-002	20X	237 μ l	-20°C
STK Tracer-red	20-299	100X	48 μ l	-20°C
10X KinEASE™ Buffer	20-302	10X	5ml	-20°C
10X Detection Buffer	20-308	10X	3ml	-20°C
MgCl ₂	20-303	1M	500 μ l	-20°C
DTT	20-265	1M	450 μ l	-20°C
ATP	20-306	10mM	300 μ l	-20°C
EDTA	20-307	0.5M	2ml	-20°C
384 Well KinEASE™ Plate	30-094		2 plates	RT

B. Recommended Buffers

Buffer	5X Buffer
Reaction Buffer	250mM HEPES pH 7.2, 0.05% BSA (Bovuminar®, Chemicon Catalog # 3225-80), 10mM MgCl ₂ , 5mM DTT (DTT to be added immediately prior to use)
Detection Buffer	250mM HEPES pH 7.2, 0.5% Tween®-20, 5mM DTT (DTT to be added immediately prior to use)

It is recommended that the MgCl₂ final reaction concentration should not exceed 5mM in the reaction (*i.e.*, 25mM in the 5X Reaction Buffer). If a higher concentration of MgCl₂ is required then do not exceed 10mM MgCl₂ (*i.e.*, 50mM in the 5X Reaction Buffer) and run an enzyme reaction with stop mix and detection mix added prior to ATP addition to confirm that the reaction is stopped under these conditions.

Individual buffer components should be stored at -20°C until ready to use. The 10X KinEASE™ Buffer (Catalog # 20-302) is used to prepare 5X Reaction Buffer working stock to ensure a final 1X buffer concentration in the assay. Working buffers should be stored at 4°C until ready to use. If long-term storage (one week to six months) is required, DTT should be omitted until assay is to be performed and sodium azide added to the 5X buffer at a final concentration of 0.05%.

IV. ASSAY PROCEDURE

Safety Warnings and Precautions: The IKK β KinEASE™ FP-645nm FarRed Assay is designed for research use only. All chemicals should be considered potentially hazardous and principles of good laboratory practice should be followed.

A. Preparation of Assay Solutions

Prepare sufficient volume of each solution based on the number of assays to be performed, plus a slight overage to account for pipetting inaccuracies (either 10-20% extra or one extra assay point is generally sufficient). **Note:** A precipitate may be observed in the thawed 0.5M EDTA pH 7.2 (Catalog # 20-307). Warm the tube to 37°C for 15 minutes and vortex to bring into solution.

Reaction Component Preparation

- 5X Reaction Buffer:** For 1ml of 5X Reaction Buffer combine 485 μ l water, 500 μ l 10X KinEASE™ Buffer, 10 μ l 1M MgCl₂, and 5 μ l 1M DTT. The total amount of 5X Reaction Buffer required will depend upon the number of experimental assay points. The 1ml volume should be scaled up (or down) according to the particular requirements of the experiment.
- 1X Reaction Buffer (for use in the No Enzyme Wells, Buffer Control Wells and Tracer Control Wells):** Prepare the 1X Reaction Buffer by diluting the 5X Reaction Buffer 5-fold with water (e.g., for 1ml of 1X Reaction Buffer combine 800 μ l water and 200 μ l 5X Reaction Buffer).
- 2.5X ATP Working Solution:** Prepare a 250 μ M ATP Working Solution in 1X Reaction Buffer (e.g., for 1ml of the 2.5X ATP Working Solution combine 775 μ l water, 200 μ l 5X Reaction Buffer, and 25 μ l 10mM ATP). 10 μ l of the 2.5X ATP Working Solution is required per well.
- 5X STK Substrate 1 Working Solution:** Prepare a 1000 μ M STK Substrate 1 Working Solution in 1X Reaction Buffer (e.g., for 1ml of STK Substrate 1 Working Solution combine 700 μ l water, 200 μ l 5X Reaction Buffer and 100 μ l of 10mM STK Substrate 1). 5 μ l of 5X STK Substrate 1 Working Solution is required per well.
- 2.5X IKK β Working Solution:** Prepare the IKK β Working Solution in 1X Reaction Buffer at a concentration of 2.5X the required final reaction concentration. 10 μ l of IKK β Working Solution is required per well.

Detection Component Preparation

- 5X Detection Buffer:** Prepare the 5X Detection Buffer by diluting the 10X Detection Buffer 2-fold and adding DTT to a concentration 5mM (e.g. for 1ml of 5X Detection Buffer combine 495 μ l water, 500 μ l of 10X Detection Buffer and 5 μ l of 1M DTT).
- 1X Detection Buffer (for use in the Buffer Control Wells and Tracer Control Wells):** Prepare the 1X Detection Buffer by diluting the 5X Detection Buffer 5-fold with water (e.g., for 1ml of 1X Detection Buffer add 200 μ l 5X Detection Buffer to 800 μ l water).
- STK Stop Mix:** Prepare STK Stop Mix containing STK Tracer-red and EDTA in 1X Detection Buffer by diluting the STK Tracer-red 100-fold and adding EDTA to a concentration of 140mM (e.g., for 1ml of STK Stop Mix combine 510 μ l water, 280 μ l 0.5M EDTA pH 7.2, 200 μ l 5X Detection Buffer and 10 μ l 100X STK Tracer-red). 5 μ l of STK Stop Mix is required per well.
- STK Antibody Detection Mix:** Prepare STK Antibody Detection Mix containing STK Antibody in 1X Detection Buffer by diluting the STK Antibody 20-fold (e.g., for 1ml of STK Antibody Detection Mix combine 750 μ l water, 200 μ l 5X Detection Buffer and 50 μ l 20X STK Antibody). 5 μ l of STK Antibody Detection Mix is required per well.

B. Protocol

1. Set up Reactions as detailed below.

No Enzyme Wells

5 μ l STK Substrate 1 Working Solution
 10 μ l 1X Reaction Buffer
 10 μ l ATP Working Solution

Plus Enzyme Wells

5 μ l STK Substrate 1 Working Solution
 10 μ l IKK β Working Solution
 10 μ l ATP Working Solution

(The reactions are started by addition of the 10 μ l ATP Working Solution).

If required, 0.5 μ l DMSO may be added to the well to simulate compound addition.

2. Incubate reactions for the required length of time at the appropriate temperature. **Note:** Optimum kinase reaction incubation time and temperature should be determined by the end user. Typical kinase reaction conditions used at Upstate are 30-60 minutes at room temperature with constant agitation.
3. Stop the reactions by adding 5 μ l/well STK Stop Mix.
4. Add 5 μ l/well STK Antibody Detection Mix. **Note:** STK Stop Mix and STK Antibody Detection Mix may be combined and added as 10 μ l/well.

The following control wells should be set up for each experiment

Buffer Control Wells

25 μ l 1X Reaction Buffer
 10 μ l 1X Detection Buffer

Tracer Control Wells

25 μ l 1X Reaction Buffer
 5 μ l STK Stop Mix
 5 μ l 1X Detection Buffer

5. Incubate the plate for a minimum of 4 hours at room temperature (assay signal is stable for up to 24 hours).
6. Read plate on a Fluorescence Polarization Reader. The end user must optimize their instrument parameters for optimal assay sensitivity. Recommended parameters for the MDC Analyst AD used at Upstate are as follows:

Method: *Fluorescence Polarization*
Excitation: 560AF55
Emission: 645AF75
Mirror: 595DRLP
Lamp: *Continuous*
Z-height: 3mm (to be determined for individual readers)
Readings per well: 1
Integration time: 100000 μ sec

Attenuator: *out*
PMT setup: *Smartread Sensitivity 2*
Excitation Polarizer: *S (static)*
Emission Polarizer: *SP (dynamic)*
G Factor: (to be determined for individual readers)
 Select Buffer Control Wells for background subtraction.

V. APPENDIX A - Assay Optimization

Determination of Enzyme Concentration for Screening

Using the optimized assay conditions, a suitable enzyme concentration for screening applications may then be determined by following the procedure outlined below.

Set up an enzyme titration with two-fold serial dilutions of enzyme and appropriate controls (No Enzyme Wells, Tracer Control Wells and Buffer Control Wells) according to the standard assay protocol.

Analyze the results obtained by plotting a graph of enzyme concentration (Units/ml) vs. Fluorescence Polarization (mP) (see below for example).

Determine the EC₇₀ mP using the following formula:

$$EC_{70} \text{ mP} = ((\text{No Enzyme Control} - \text{Tracer Control}) \times 0.3) + \text{Tracer Control}$$

From the graph of enzyme concentration (Units/ml) vs. Fluorescence Polarization (mP) determine the concentration of enzyme that correlates to the EC₇₀ mP value calculated above. This enzyme concentration (EC₇₀ Units/ml) is recommended for screening applications.

