PKCε KinEASE™ FP Fluorescein Green Assay

Catalog # 32-029

Sufficient reagents for two 384-well plates per kit.

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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 -20ºC.

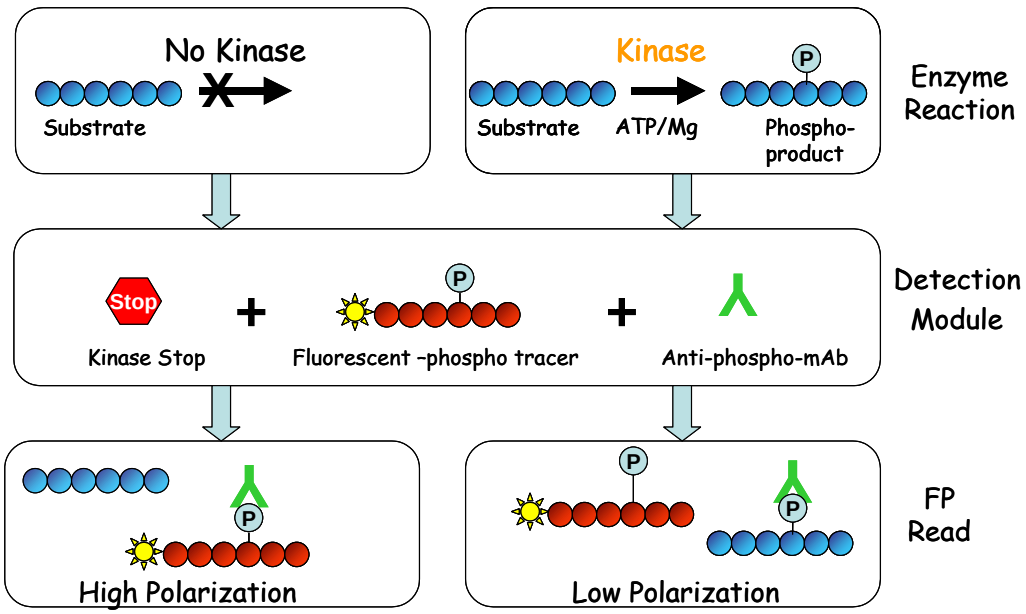
Stability: Components stable for 6 months from date of shipment if stored and handled correctly. We recommend that all enzymes to be used with this kit are stored as aliquots and a fresh aliquot used for each experiment.

II. ASSAY OVERVIEW

In this assay, a phosphorylated peptide has been labeled with a green 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.

PKCε KinEASE™ FP Fluorescein Green Assay supplied in this kit offers a method for assaying PKCε using preferred substrate and a generic detection system.



Related Product: PKCε, Catalog # 14-518

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 solution is light sensitive.**

Reagent	Catalog #	Stock	Volume Supplied	Storage
STK Substrate 1	12-548	10mM	120µl	-20ºC
STK Antibody	35-002	20X	237µl	-20ºC
STK Tracer – Fluorescein	20-287	100X	48µl	-20ºC
10X KinEASE™ Buffer	20-302	10X	5ml	-20ºC
10X KinEASE™ Enzyme Dilution Buffer	20-354	10X	1ml	-20ºC
$MgCl_2$	20-303	1M	500µl	-20ºC
DTT	20-265	1M	450µl	-20ºC
Lipid Activator	20-113A	5X	5ml	-20ºC
ATP	20-306	10mM	300µl	-20ºC
EDTA	20-307	0.5M	2ml	-20ºC
384 Well KinEASE™ Plate	30-014		2 plates	RT

Note: Individual buffer components should be stored at -20°C until ready to use

B. Recommended Buffers

Buffer	2.5X Buffer
Reaction Buffer	125mM HEPES pH 7.2, 0.025% BSA (Probumin™, Millipore Catalog # 3225-80), 25mM $MgCl_2$, 2.5mM DTT (DTT to be added immediately prior to use), 2.5X Lipid Activator
Buffer	**5X Buffer**
Detection Buffer	250mM HEPES pH 7.2, 0.05% BSA (Probumin™, Millipore Catalog # 3225-80), 5mM DTT (DTT to be added immediately prior to use)

Buffer	1X Buffer
Enzyme Dilution Buffer	50mM HEPES pH 7.2, 0.1% BSA (Probumin™, Millipore Catalog # 1003512), 0.01% Brij-35, 0.1mM EDTA, 1mM DTT (DTT to be added immediately prior to use)

IV. ASSAY PROCEDURE

Safety Warnings and Precautions: The PKCε KinEASE™ FP Fluorescein Green Assay is designed for research use only. All chemicals should be considered potentially hazardous and principles of good laboratory practice should be followed.

A. Kinase Reaction Module

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 (Catalog # 20-307). Warm the tube to 37°C for 15 minutes and vortex to bring into solution.

Reaction Solution Preparation

1. **5X Lipid Activator:** Thaw the 5X Lipid Activator and vigorously vortex for 1 minute prior to use.
2. **2.5X Reaction Buffer:** For 1ml of 2.5X Reaction Buffer combine 235µl water, 250µl 10X KinEASE™ Buffer, 12.5µl 1M $MgCl_2$, 2.5µl 1M DTT, and 500µl of 5X Lipid Activator. The total amount of 2.5X 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.
3. **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 2.5X Reaction Buffer 2.5-fold with water (*e.g.,* for 1ml of 1X Reaction Buffer combine 600µl water and 400µl 2.5X Reaction Buffer).
4. **1X Enzyme Dilution Buffer (EDB):** For 1ml of the 1X EDB combine 899µl water, 100µl of 10X KinEASE™ Enzyme Dilution Buffer, and 1µl of 1M DTT. The 1ml volume should be scaled up (or down) according to the particular requirements of the experiment.
5. **2.5X ATP Working Solution:** Prepare a 250µM ATP Working Solution in 1.25X Reaction Buffer (*e.g.,* for 1ml of the 2.5X ATP Working Solution combine 475µl water, 500µl 2.5X Reaction Buffer, and 25µl 10mM ATP). 10µl of the 2.5X ATP Working Solution is required per well.
6. **2.5X STK Substrate 1 Working Solution:** Prepare a 75µM STK Substrate 1 Working Solution in 1.25X Reaction Buffer (*e.g.,* for 1ml of STK Substrate 1 Working Solution combine 492.5µl water, 500µl 2.5X Reaction Buffer and 7.5µl of 10mM STK Substrate 1). 10µl of 2.5X STK Substrate 1 Working Solution is required per well.
7. **5X PKCε Working Solution:** Prepare the PKCε Working Solution in 1X KinEASE™ EDB at a concentration of 5X the required final reaction concentration. 5µl of PKCε Working Solution is required per well.

Reaction Protocol

1. Set up 3 assay controls as detailed below.

Buffer Control Wells (assay background)
25µl 1X Reaction Buffer

Tracer Control Wells (min. mP value)
25µl 1X Reaction Buffer

No Enzyme Wells (max. mP value)
10µl STK Substrate 1 Working Solution
5µl 1X Reaction Buffer
10µl ATP Working Solution

2. Set up reactions as detailed below.

   Plus Enzyme Wells
   10μl STK Substrate 1 Working Solution
   5μl PKCε Working Solution
   10μl ATP Working Solution

   The reactions are started by addition of the 10μl ATP Working Solution. When using the same concentration of enzyme (e.g. EC_{70} value), the substrate working solution and enzyme working solutions can be combined and added as 15ul per well.
   If required, 0.5μl DMSO may be added to a set of control wells to simulate compound addition.

3. Incubate reactions for the required length of time at the appropriate temperature. To ensure that all reaction components have collected in the bottom of the wells, very gently tap the bottom of the plate against the bench top. **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.

B. Detection Module

Detection Solution Preparation

1. **5X Detection Buffer:** Prepare the 5X Detection Buffer by diluting the 10X KinEASE™ Buffer 2-fold and adding DTT to a concentration 5mM (*e.g.* for 1ml of 5X Detection Buffer combine 495μl water, 500μl of 10X KinEASE™ Buffer and 5μl of 1M DTT).

2. **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 200μl 5X Detection Buffer to 800μl water).

3. **STK Stop Mix:** Prepare STK Stop Mix containing STK Tracer and EDTA in 1X Detection Buffer by diluting the STK Tracer 100-fold and adding EDTA to a concentration of 140mM (*e.g.,* for 1ml of STK Stop Mix combine 510μl water, 280μl 0.5M EDTA pH 7.2, 200μl 5X Detection Buffer and 10μl 100X STK Tracer). 5μl of STK Stop Mix is required per well.

4. **STK Antibody Mix:** Prepare STK Antibody Mix containing STK Antibody in 1X Detection Buffer by diluting the STK Antibody 20-fold (*e.g.,* for 1ml of STK Antibody Mix combine 750μl water, 200μl 5X Detection Buffer and 50μl 20X STK Antibody). 5μl of 5X STK Antibody Mix is required per well.

Detection Protocol

1. Add the following to the 3 sets of assay controls wells.

   Buffer Control Wells (assay background)
   10μl 1X Detection Buffer

   Tracer Control Wells (min. mP value)
   5μl of STK Stop Mix
   5μl 1X Detection Buffer

   No Enzyme Wells (max. mP value)
   5μl STK Stop Mix
   5μl STK Antibody Mix

2. Stop the reactions by adding 5μl/well STK Stop Mix.

3. Add 5μl/well STK Antibody Mix. **Note**: Once the STK Stop Mix has been added to the Tracer Control wells, the STK Stop Mix and STK Antibody Mix may be combined and added as 10μl/well.

4. Incubate the plate for a minimum of 4 hours at room temperature (assay signal is stable for up to 24 hours).

5. 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: *485nm (bw20)*
Emission: *530nm (bw25)*
Mirror: Dichroic 505
Lamp: *Continuous*
Z-height: *3mm (to be determined for individual readers*)
Readings per well: *1*
Integration time: *100000μsec*
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.

General Assay Notes

- It is recommended that the $MgCl_2$ final reaction concentration should not exceed 5mM in the reaction (*i.e.*, 25mM in the 5X Reaction Buffer). If a higher concentration of $MgCl_2$ is required then do not exceed 10mM $MgCl_2$ (*i.e.*, 50mM in the 5X Reaction Buffer) and run an enzyme reaction with stop mix and Antibody mix added prior to ATP addition to confirm that the reaction is stopped under these conditions.

- The 10X KinEASE™ Buffer (Catalog # 20-302) is used to prepare 5X working stocks to ensure a final 1X buffer concentration in the assay. These working buffers should be stored at 4ºC 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%.

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.

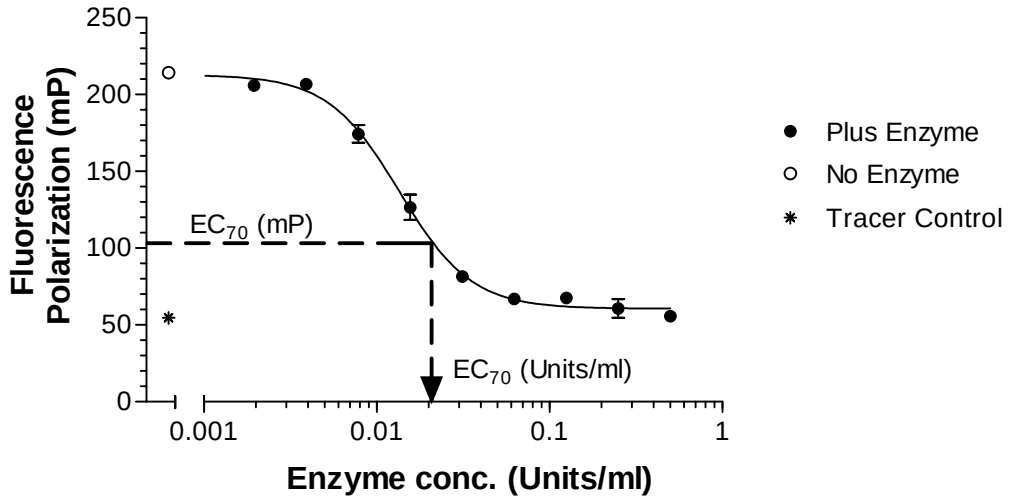
Set up an enzyme titration with two-fold serial dilutions of enzyme and appropriate controls (No Enzyme Wells, Tracer Control Wells and Buffer Control Wells) according to the standard assay protocol.

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

Determine the EC_{70} mP using the following formula:

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

From the graph of enzyme concentration (Units/ml) vs. Fluorescence Polarization (mP) determine the concentration of enzyme that correlates to the EC_{70} mP value calculated above. This enzyme concentration (EC_{70} Units/ml) is recommended for screening applications.



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