Lck KinEASE™ FP-645nm FarRed Assay

Catalog # 32-152

Sufficient reagents for two 384-well plates per kit.

FOR RESEARCH USE ONLY; NOT FOR USE IN DIAGNOSTIC PROCEDURES. NOT FOR HUMAN OR ANIMAL CONSUMPTION

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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.

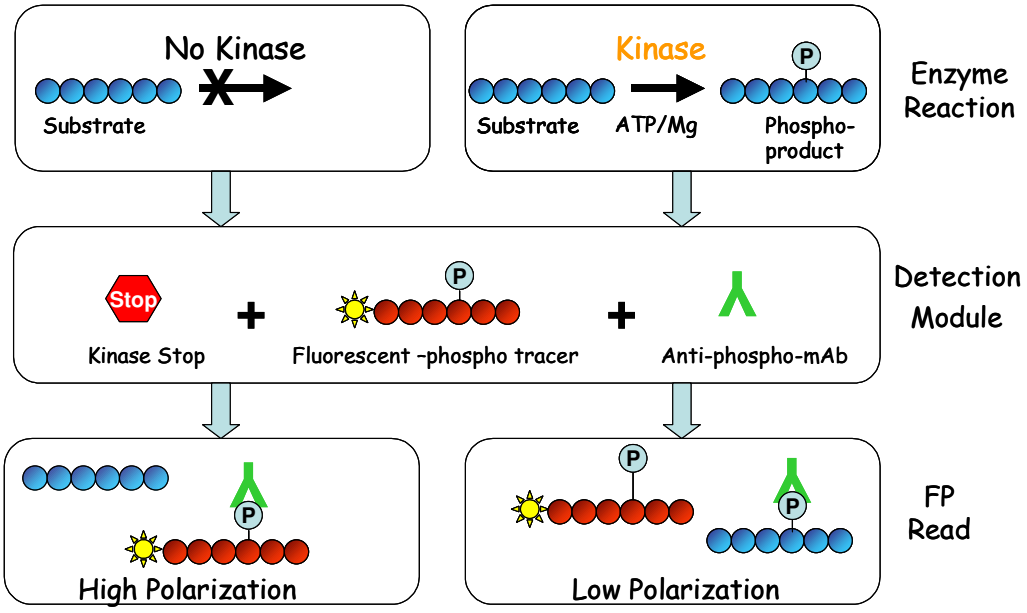
Stability: Components stable for 6 months from date of shipment if stored and handled correctly. We recommend that all enzymes to be used with this kit are stored as aliquots and a fresh aliquot used for each experiment.

II. ASSAY OVERVIEW

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

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



Related Product: Lck, Catalog # 14-442

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 TK Tracer-red solution is light sensitive.**

Reagent	Catalog #	Stock	Volume Supplied	Storage
TK Substrate 3	12-554	10 mM	2 x 297 µL	-20ºC
TK Antibody	35-004	20X	237 µL	-20ºC
TK Tracer-red	20-301	100X	48 µL	-20ºC
10X KinEASE™ Buffer	20-302	10X	5 mL	-20ºC
10X Detection Buffer	20-308	10X	3 mL	-20ºC
10X KinEASE™ Enzyme Dilution Buffer	20-354	10X	1 mL	-20ºC
$MgCl_2$	20-303	1 M	500 µL	-20ºC
$MnCl_2$	20-309	0.1 M	500 µL	-20ºC
DTT	20-265	1 M	450 µL	-20ºC
ATP	20-306	10 mM	300 µL	-20ºC
EDTA	20-307	0.5 M	2 mL	-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. Reco mMended Buffers

Buffer	5X Buffer
Reaction Buffer	250 mM HEPES pH 7.2, 0.05% BSA (Probumin™®, Millipore Catalog # 3225-80), 25 mM $MgCl_2$, 5 mM $MnCl_2$, 5 mM DTT (DTT to be added immediately prior to use)
Detection Buffer	250 mM HEPES pH 7.2, 0.5% Tween®-20, 5 mM DTT (DTT to be added immediately prior to use)

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

IV. ASSAY PROCEDURE

Safety Warnings and Precautions: The Lck 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. 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 Reaction Buffer:** For 1 mL of 5X Reaction Buffer combine 420 µL water, 500 µL 10X KinEASE™ Buffer, 25 µL 1M $MgCl_2$, 50 µL 0.1M $MnCl_2$, and 5 µL 1M DTT. The total amount of 5X Reaction Buffer required will depend upon the number of experimental assay points. The 1 mL volume should be scaled up (or down) according to the particular requirements of the experiment.

2. **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 1 mL of 1X Reaction Buffer combine 800 µL water and 200 µL 5X Reaction Buffer).

3. **1X Enzyme Dilution Buffer (EDB):** For 1 mL of the 1X EDB combine 899 µL water, 100 µL of 10X KinEASE™ Enzyme Dilution Buffer, and 1 µL of 1M DTT. The 1 mL volume should be scaled up (or down) according to the particular requirements of the experiment.

4. **2.5X ATP Working Solution:** Prepare a 250µM ATP Working Solution in 1.25X Reaction Buffer (*e.g.,* for 1 mL of the 2.5X ATP Working Solution combine 725 µL water, 250 µL 5X Reaction Buffer, and 25 µL 10 mM ATP). 10 µL of the 2.5X ATP Working Solution is required per well.

5. **2.5X TK Substrate 3 Working Solution:** Prepare a 625 µM TK Substrate 3 Working Solution in 1.25X Reaction Buffer (*e.g.,* for 1 mL of TK Substrate 3 Working Solution combine 687.5 µL water, 250 µL 5X Reaction Buffer and 62.5 µL of 10 mM TK Substrate 3). 10 µL of 2.5X TK Substrate 3 Working Solution is required per well.

6. **5X Lck Working Solution:** Prepare the Lck Working Solution in 1X KinEASE™ EDB at a concentration of 5X the required final reaction concentration. 5 µL of Lck Working Solution is required per well.

Reaction Protocol

1. Set up 3 assay controls as detailed below.

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

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

No Enzyme Wells (max. mP value)
10 µL TK Substrate 3 Working Solution
5 µL 1X Reaction Buffer
10 µL ATP Working Solution

2. Set up reactions as detailed below.

   Plus Enzyme Wells
   10 µL TK Substrate 3 Working Solution
   5 µL Lck Working Solution
   10 µL ATP Working Solution

   The reactions are started by addition of the 10 µL 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 15 uL per well.
   If required, 0.5 µL 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 Detection Buffer 2-fold and adding DTT to a concentration 5 mM (*e.g.* for 1 mL of 5X Detection Buffer combine 495 µL water, 500 µL of 10X KinEASE™ Buffer and 5 µL 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 1 mL of 1X Detection Buffer add 200 µL 5X Detection Buffer to 800 µL water).

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

4. **TK Antibody Mix:** Prepare TK Antibody Mix containing TK Antibody in 1X Detection Buffer by diluting the TK Antibody 20-fold (*e.g.,* for 1 mL of TK Antibody Mix combine 750 µL water, 200 µL 5X Detection Buffer and 50 µL 20X TK Antibody). 5 µL of 5X TK 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)
   10 µL 1X Detection Buffer

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

   No Enzyme Wells (max. mP value)
   5 µL TK Stop Mix
   5 µL TK Antibody Mix

2. Stop the reactions by adding 5 µL/well TK Stop Mix.

3. Add 5 µL/well TK Antibody Mix. **Note**: Once the TK Stop Mix has been added to the Tracer Control wells, the TK Stop Mix and TK Antibody Mix may be combined and added as 10 µL/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: *560AF55*
Emission: *645AF75*
Mirror: *595DRLP*
Lamp: *Continuous*
Z-height: *3 mM (to be determined for individual readers*)
Readings per well: *1*
Integration time: *100000 µsec*
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 5 mM in the reaction (*i.e.*, 25 mM in the 5X Reaction Buffer). If a higher concentration of $MgCl_2$ is required then do not exceed 10 mM $MgCl_2$ (*i.e.*, 50 mM 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.

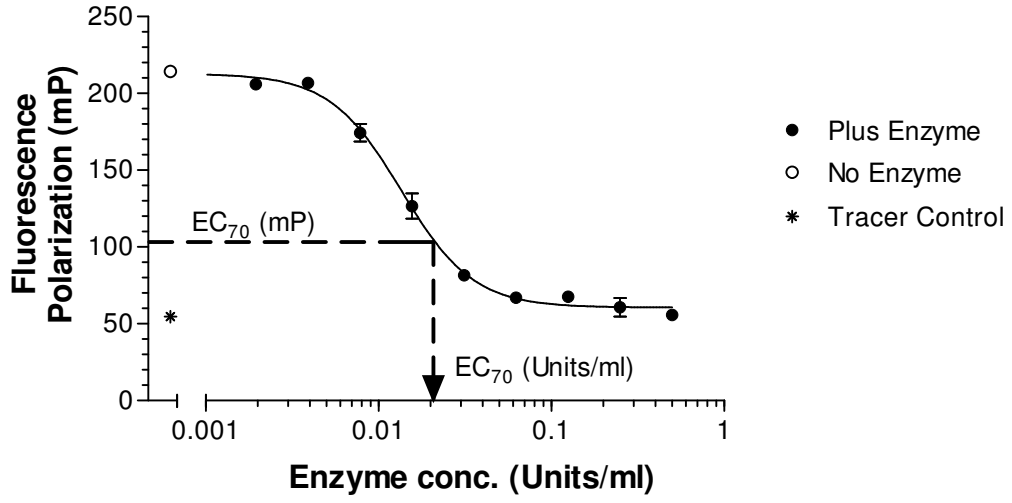
Set up an enzyme titration with two-fold serial dilutions of enzyme and appropriate controls (No Enzyme Wells, Tracer Control Wells and Buffer Control Wells) according to the standard assay protocol.

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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