EphA2 KinEASE™ FP Fluorescein Green Assay

Catalog # 32-061

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 -20ºC.

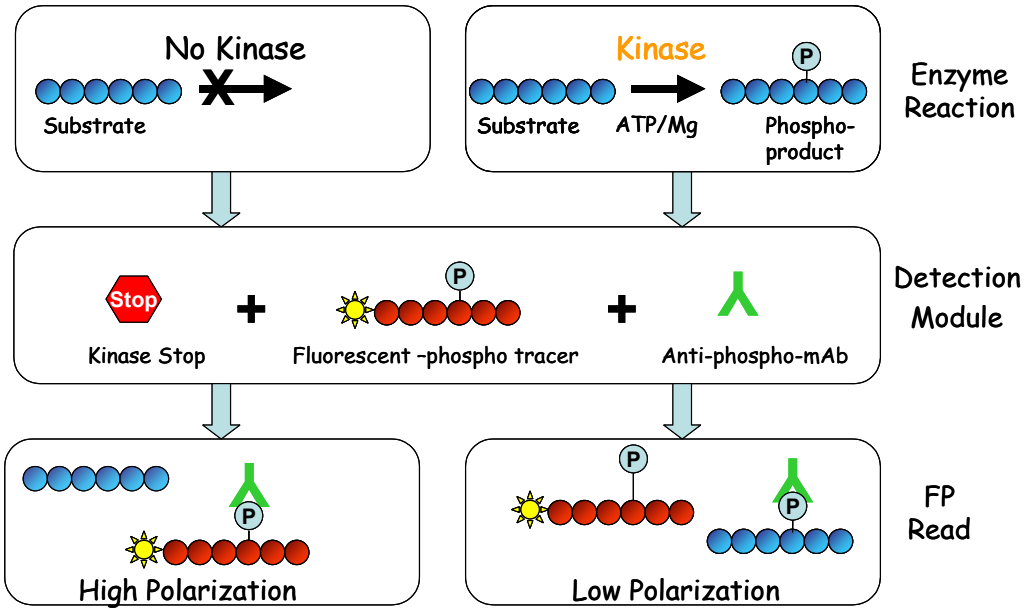
Stability: Components stable for 6 months from date of shipment if stored and handled correctly. We recommend that all enzymes to be used with this kit are stored as aliquots and a fresh aliquot used for each experiment.

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.

EphA2 KinEASE™ FP Fluorescein Green Assay supplied in this kit offers a method for assaying EphA2 using a preferred substrate and a generic detection system.



Related Product: EphA2, Catalog # 14-560

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 solution is light sensitive.**

Reagent	Catalog #	Stock	Volume Supplied	Storage
TK Substrate 2	12-553	100mM	2 x 30µl	-20ºC
TK Antibody	35-004	20X	237µl	-20ºC
TK Tracer – Fluorescein	20-289	100X	48µl	-20ºC
10X KinEASE™ Buffer	20-302	10X	5ml	-20ºC
10X Detection Buffer	20-308	10X	3ml	-20ºC
$MgCl_2$	20-303	1M	500µl	-20ºC
$MnCl_2$	20-309	0.1M	500µl	-20ºC
DTT	20-265	1M	450µl	-20ºC
ATP	20-306	10mM	300µl	-20ºC
EDTA	20-307	0.5M	2ml	-20ºC
384 Well KinEASE™ Plate	30-014		2 plates	RT

B. Recommended Buffers

Buffer	5X Buffer
Reaction Buffer	250mM HEPES pH 7.2, 0.05% BSA (Bovuminar®, Chemicon Catalog # 3225-80), 25mM $MgCl_2$, 5mM $MnCl_2$, 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 $MgCl_2$ 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 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 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%.

IV. ASSAY PROCEDURE

Safety Warnings and Precautions: The EphA2 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. 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

1. **5X Reaction Buffer:** For 1ml of 5X Reaction Buffer combine 420µl water, 500µl 10X KinEASE™ Buffer, 25µl 1M $MgCl_2$, 50µl 0.1M $MnCl_2$, and 5µl 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.

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 1ml of 1X Reaction Buffer combine 800µl water and 200µl 5X Reaction Buffer).

3. **2.5X ATP Working Solution:** Prepare a 250µM ATP Working Solution in 1X Reaction Buffer (*e.g.,* for 1ml of the 2.5X ATP Working Solution combine 775µl water, 200µl 5X Reaction Buffer, and 25µl 10mM ATP). 10µl of the 2.5X ATP Working Solution is required per well.

4. **5X TK Substrate 2 Working Solution:** Prepare a 1.25mM TK Substrate 2 Working Solution in 1X Reaction Buffer (*e.g.,* for 1ml of TK Substrate 2 Working Solution combine 787.5µl water, 200µl 5X Reaction Buffer and 12.5µl of 100mM TK Substrate 2). 5µl of 5X TK Substrate 2 Working Solution is required per well.
5. **2.5X EphA2 Working Solution:** Prepare the EphA2 Working Solution in 1X Reaction Buffer at a concentration of 2.5X the required final reaction concentration. 10µl of EphA2 Working Solution is required per well.

Detection Component Preparation

1. **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 495µl water, 500µl of 10X Detection 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. **TK Stop Mix:** Prepare TK Stop Mix containing TK Tracer and EDTA in 1X Detection Buffer by diluting the TK Tracer 100-fold and adding EDTA to a concentration of 140mM (*e.g.,* for 1ml of TK Stop Mix combine 510µl water, 280µl 0.5M EDTA pH 7.2, 200µl 5X Detection Buffer and 10µl 100X TK Tracer). 5µl of TK Stop Mix is required per well.

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

B. Protocol

1. Set up Reactions as detailed below.

No Enzyme Wells	**Plus Enzyme Wells**
5µl TK Substrate 2 Working Solution	5µl TK Substrate 2 Working Solution
10µl 1X Reaction Buffer	10µl EphA2 Working Solution
10µl ATP Working Solution	10µl ATP Working Solution

(The reactions are started by addition of the 10µl ATP Working Solution).
If required, 0.5µl 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 5µl/well TK Stop Mix.

4. Add 5µl/well TK Antibody Detection Mix. **Note**: TK Stop Mix and TK Antibody Detection Mix may be combined and added as 10µl/well.

The following control wells should be set up for each experiment

Buffer Control Wells	**Tracer Control Wells**
25µl 1X Reaction Buffer	25µl 1X Reaction Buffer
10µl 1X Detection Buffer	5µl TK Stop Mix
	5µl 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: *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.

V. APPENDIX A - Assay Optimization

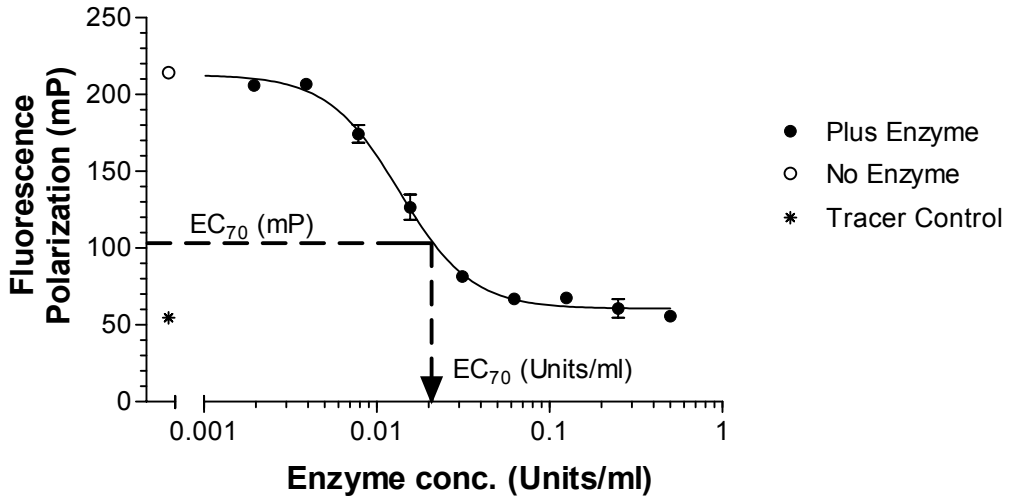
Determination of Enzyme Concentration for Screening

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.