

KinEASE™ FP Fluorescein Green Assay

Module 3 – Tyrosine Kinases

Catalog # 32-003

Lot # 28575AU

Sufficient reagents for two 384-well plates per kit.

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**FOR IN VITRO RESEARCH USE ONLY.
NOT RECOMMENDED OR INTENDED
FOR DIAGNOSIS OF DISEASE IN
HUMANS.**

DO NOT USE IN HUMANS.

I. STORAGE AND STABILITY

Storage: Upon receipt, all reagents should be stored 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

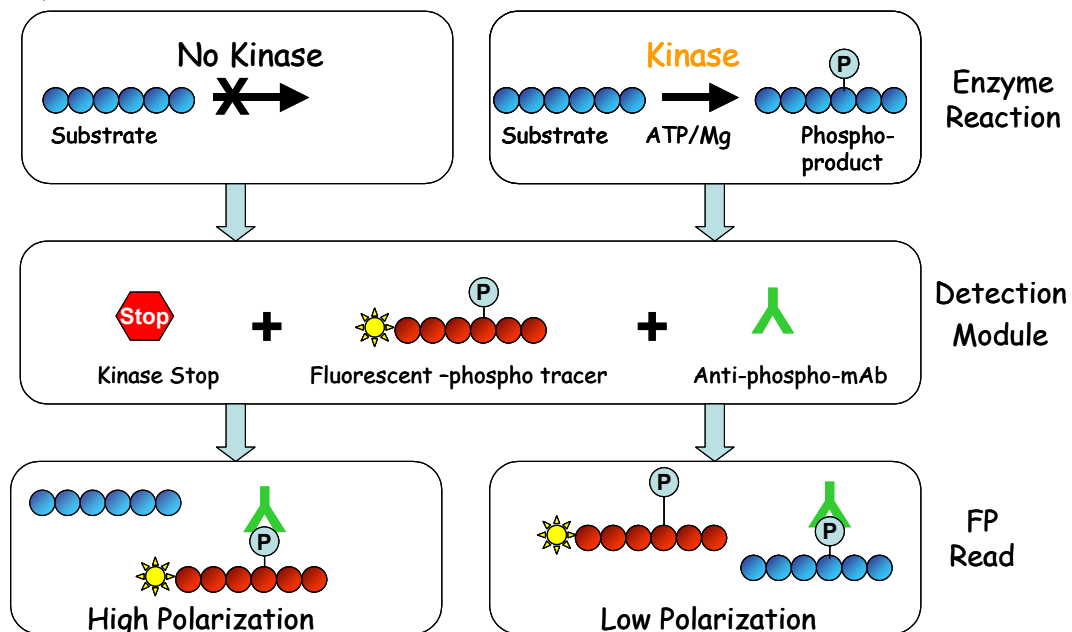
In this assay a phosphorylated peptide has been labeled with a green fluorescent dye. This phosphorylated tracer has a low molecular weight and thus a low fluorescence polarization value. The phosphorylated tracer binds to a phospho-specific antibody to form a high molecular weight complex with a high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

KinEASE™ FP Fluorescein Green Assay Module 3 supplied in this kit offers a method for assaying tyrosine kinases using three potential substrates and a generic detection system. It has been designed to allow the user to evaluate the application of the technology to their own kinase.

KinEASE™ FP Fluorescein Green Assay Module 3 is known to work with the following enzymes: **Abl (h), Abl (m), Abl (T315I) (h) Arg (h), Arg (m) Blk (m), Bmx (h), BTK (h), CSK (h), Src (h), EGFR (h), EphA2 (h), EphB2 (h), EphB4 (h), Fes/Fps (h), FGFR3 (h), Flt1 (h), Flt3 (h), Fms (h), Fyn (h), IGF-1R (h), Insulin R (h), Lck (h), Lyn (h), Met (h), PDGFR α (h), PDGFR β (h), Ret (h), Rse (h), Syk (h), Tie2 (h), TrkA (h), Yes (h) and ZAP-70 (h)** (see Appendix B for further details).

h = human; m = mouse



III. SYSTEM COMPONENTS

A. Provided Kit Components

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

Reagent	Catalog #	Lot #	Stock	Volume Supplied	Storage
TK Substrate 1	12-552	28488U	10mM	192µl	-20°C
TK Substrate 2	12-553	28481U	100mM	19.2µl	-20°C
TK Substrate 3	12-554	28490U	10mM	192µl	-20°C
TK Antibody	35-004	28484U	20X	192µl	-20°C
TK Tracer	20-289	28493U	100X	38µl	-20°C

B. Required Materials Not Provided

Reagent	Recommended Supplier	Stock	Storage
ATP	Major Laboratory Suppliers	100mM	Store aliquoted -20°C
DTT	Major Laboratory Suppliers	1M	Store aliquoted -20°C
BSA	Sigma A2934	1% in water	Store aliquoted -20°C
Tween®-20	Sigma P7949		RT
EDTA	Major Laboratory Suppliers	0.5M pH 7.2	RT
MgCl ₂	Major Laboratory Suppliers	1M	RT
HEPES	Major Laboratory Suppliers	1M	RT
Black 384 well microplates	Costar 3710		RT
Water (18.2MΩ)			RT

C. Recommended Buffers

Buffers should be prepared as 5X stocks to ensure a final 1X buffer concentration in the assay. These buffers should be stored at 4°C. If long-term storage (one week to six months) is required, sodium azide should be added to the 5X buffer at a final concentration of 0.05%. See Appendix B for recommended 1X Reaction Buffer conditions for the Upstate kinases listed on page 2.

Buffer	5X Buffer
Reaction Buffer*	250mM HEPES pH 7.2, 25mM MgCl ₂ , 0.05% BSA
Detection Buffer	250mM HEPES pH 7.2, 0.5% Tween [®] -20, 5mM DTT (DTT to be added immediately prior to use)

*Other components that may be added to the Reaction Buffer if required are MnCl₂, CaCl₂, Calmodulin, DTT or β-mercaptoethanol.

It is recommended that the MgCl₂ final assay 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 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.

IV. ASSAY PROCEDURE

Safety Warnings and Precautions: The 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% extra or one extra assay point is generally sufficient).

Reaction Component Preparation

- 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).
- ATP Working Solution:** Prepare the ATP Working Solution in 1X Reaction Buffer at an ATP concentration of 2.5X the required final reaction concentration (*e.g.*, for a 100μM final reaction concentration prepare a 250μM ATP working solution by combining ATP, 5X Reaction Buffer and water). 10μl of ATP Working Solution is required per well.
- TK Substrate Working Solution:** For each substrate to be tested prepare a 500μM TK Substrate Working Solution in 1X Reaction Buffer. This is 5X the required assay concentration of 100μM (*e.g.*, to prepare 1ml of TK Substrate Working Solutions for TK Substrates **1 or 3**, combine 750μl water, 200μl 5X Reaction Buffer and 50μl of 10mM TK Substrate; to prepare 1ml of TK Substrate Working Solution for TK Substrate **2**, combine 795μl water, 200μl 5X Reaction Buffer and 5μl of 100mM TK Substrate 2). 5μl of TK Substrate Working Solution is required per well.
- Kinase Working Solution:** Prepare the Kinase Working Solution in 1X Reaction Buffer at a concentration of 2.5X the required final reaction concentration. 10μl of Kinase Working Solution is required per well.

Detection Component Preparation

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

B. Protocol

- Set up Reactions as detailed below.

No Enzyme Wells

5µl TK Substrate Working Solution
 10µl 1X Reaction Buffer
 10µl ATP Working Solution

Plus Enzyme Wells

5µl TK Substrate Working Solution
 10µl Kinase 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.

- Incubate reactions for required length of time at room temperature.
- Stop the reactions by adding 5µl/well TK Stop Mix.
- Add 5µl/well TK Detection Mix. **Note:** TK Stop Mix and TK Detection Mix may be combined and added as 10µl/well.

The following control wells should be set up for each experiment

Buffer Control Wells

25µl 1X Reaction Buffer
 10µl 1X Detection Buffer

Tracer Control Wells

25µl 1X Reaction Buffer
 5µl TK Stop Mix
 5µl 1X Detection Buffer

- Incubate the plate for a minimum of 4 hours at room temperature (assay signal is stable for up to 24 hours).
- Read plate on a Fluorescence Polarization Reader. Recommended parameters for the MDC Analyst AD are as follows:

Method: *Fluorescence Polarization*

Excitation: *485nm (bw20)*

Emission: *530nm (bw25)*

Mirror: *Dichroic 505*

Lamp: *Continuous*

Zheight: *3mm (to be determined for individual readers)*

Readings per well: *1*

Integration time: *100000µsec*

Attenuator: *out*

PMT setup: *Smartread Sensitivity 3*

Excitation Polarizer: *S (static)*

Emission Polarizer: *SP (dynamic)*

G Factor: *(to be determined for individual readers)*

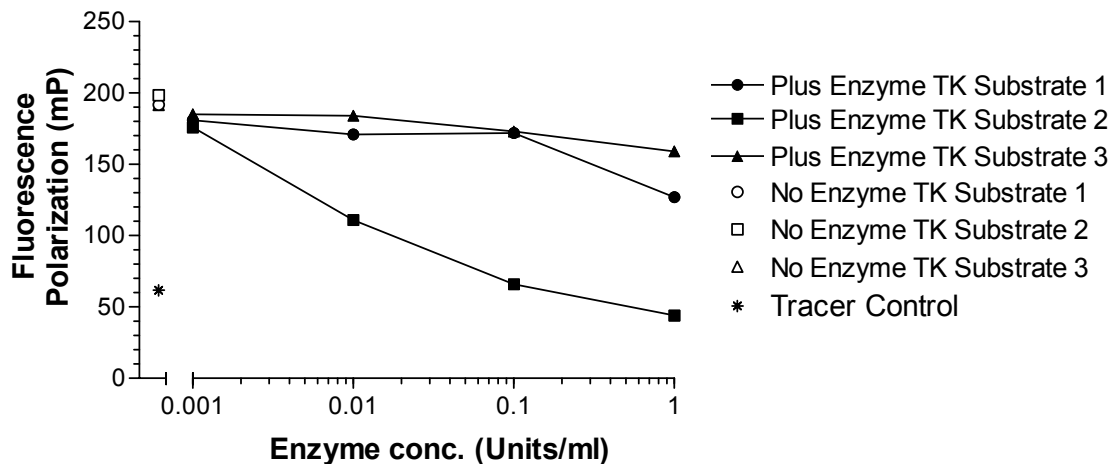
Select Buffer Control Wells for background subtraction.

V. APPENDIX A - Assay Optimization

Substrate Selection

To determine whether KinEASE™ FP Fluorescein Green Assay Module 3 is suitable for your kinase, a series of enzyme dilutions should be tested with each TK Substrate (100 μ M final reaction concentration) according to the standard assay protocol.

The following graph shows data obtained from titration of a Tyrosine Kinase with TK Substrates 1, 2 and 3. Enzyme activity is observed as a decrease in the Fluorescence Polarization (mP) and in the example given, TK Substrate 2 was optimal.



Once enzyme activity has been demonstrated it is recommended the user optimize their reaction conditions (i.e. substrate concentration and reaction buffer constituents) to maximize the assay sensitivity. See Appendix B for recommended conditions for the Upstate kinases listed on page 2.

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.

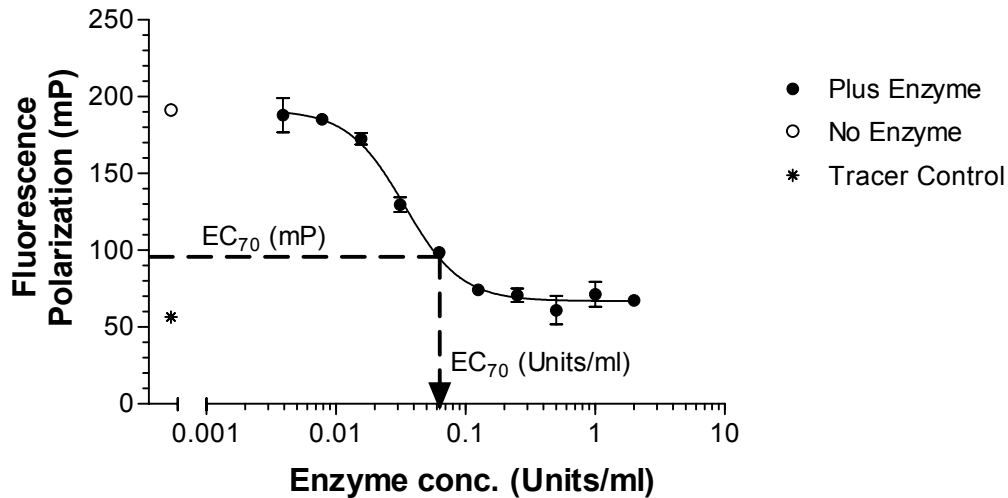
Set up an enzyme titration with two-fold serial dilutions of enzyme and appropriate controls (No Enzyme Wells, Tracer Control Wells and Buffer Control Wells) according to the standard assay protocol.

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.



VI. APPENDIX B – Recommended Reaction Conditions

Table of recommended reaction conditions for Upstate kinases (h = human; m = mouse):

Kinase	Recommended Substrate	Recommended Substrate Conc.	Recommended 1X Reaction Buffer
Abl (h) (14-529)	TK Substrate 1	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂
Abl (T315I) (h) (14-522)	TK Substrate 1	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT
Abl (m) (14-459)	TK Substrate 1	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂
Arg (h) (14-521)	TK Substrate 1	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂
Arg (m) (14-460)	TK Substrate 1	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂
Blk (m) (14-316)	TK Substrate 1	100 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM MnCl ₂
Bmx (h) (14-499)	TK Substrate 1	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂
BTK (h) (14-552)	TK Substrate 1	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂
CSK (h) (14-458)	TK Substrate 2	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT, 1mM MnCl ₂
Src (h) (14-326)	TK Substrate 3	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM MnCl ₂
EGFR (h) (14-531)	TK Substrate 2	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM MnCl ₂
EphA2 (h) (14-560)	TK Substrate 2	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT, 1mM MnCl ₂
EphB2 (h) (14-553)	TK Substrate 2	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM MnCl ₂
EphB4 (h) (14-554)	TK Substrate 2	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM MnCl ₂
Fes/Fps (h) (14-473)	TK Substrate 1	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂
FGFR3 (h) (14-464)	TK Substrate 2	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM MnCl ₂
Flt1 (h) (14-562)	TK Substrate 2	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT, 1mM MnCl ₂
Flt3 (h) (14-500)	TK Substrate 1	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂
Fms (h) (14-551)	TK Substrate 2	100 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM MnCl ₂

Kinase	Recommended Substrate	Recommended Substrate Conc.	Recommended 1X Reaction Buffer
Fyn (h) (14-441)	TK Substrate 3	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂
IGF-1R (h) (14-465)	TK Substrate 2	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT, 1mM MnCl ₂
Insulin R (h) (14-466)	TK Substrate 2	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM MnCl ₂
Lck (h) (14-442)	TK Substrate 3	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT, 1mM MnCl ₂
Lyn (h) (14-510)	TK Substrate 1	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT, 1mM MnCl ₂
Lyn (m) (14-315)	TK Substrate 1	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT, 1mM MnCl ₂
Met (h) (14-526)	TK Substrate 1	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂
PDGFR α (h) (14-467)	TK Substrate 2	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM MnCl ₂
PDGFR β (h) (14-463)	TK Substrate 2	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM MnCl ₂
Ret (h) (14-570)	TK Substrate 1	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT
Ros (h) (14-527)	TK Substrate 2	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT, 1mM MnCl ₂
Rse (h) (14-535)	TK Substrate 3	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT, 1mM MnCl ₂
Syk (h) (14-314)	TK Substrate 2	100 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT, 1mM MnCl ₂
Tie2 (h) (14-540)	TK Substrate 2	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM MnCl ₂
TrkA (h) (14-571)	TK Substrate 1	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT, 1mM MnCl ₂
TrkB (h) (14-507)	TK Substrate 2	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂
Yes (h) (14-478)	TK Substrate 2	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂
ZAP-70 (h) (14-404)	TK Substrate 2	250 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT, 1mM MnCl ₂