KinEASE™ FP Fluorescein Green Assay

Module 3 – Tyrosine Kinases

Catalog # 32-003

Sufficient reagents for two 384-well plates per kit.

Contents	Page
I. STORAGE AND STABILITY	2
II. ASSAY OVERVIEW	2
III. SYSTEM COMPONENTS	3
A. Provided Kit Components	3
B. Required Materials Not Provided	3
C. Recommended Buffers	4
IV. ASSAY PROCEDURE	4
A. Kinase Reaction Module	4
B. Detection Module	5
V. APPENDIX A – Assay Optimization	7
VI. APPENDIX B – Recommended Reaction Conditions	9

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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 -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

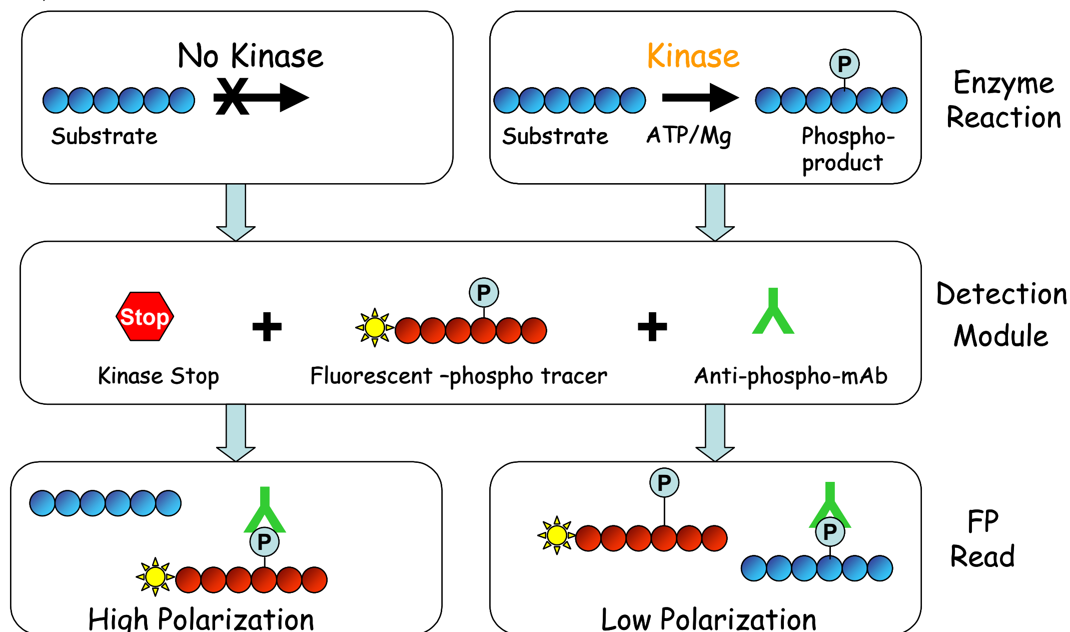
In this assay a phosphorylated peptide has been labeled with a green fluorescent dye. This phosphorylated tracer has a low molecular weight and thus a low fluorescence polarization value. The phosphorylated tracer binds to a phospho-specific antibody to form a high molecular weight complex with a high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

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), PDGFRα (h), PDGFRβ (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 #	Stock	Volume Supplied	Storage
TK Substrate 1	12-552	10mM	297μl	-20ºC
TK Substrate 2	12-553	100mM	30μl	-20ºC
TK Substrate 3	12-554	10mM	297μl	-20ºC
TK Antibody	35-004	20X	237μl	-20ºC
TK Tracer	20-289	100X	48μl	-20ºC

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

B. Required Materials Not Provided

Reagent	Recommended Supplier	Stock	Storage
ATP	Major Laboratory Suppliers	100mM	Store aliquoted -20ºC
DTT	Major Laboratory Suppliers	1M	Store aliquoted -20ºC
BSA	Chemicon #1003512	1% in water	Store aliquoted -20ºC
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
Brij-35	Major Laboratory Suppliers	30%	RT
Black 384 well microplates	Costar 3710		RT
Water (18.2MΩ)			RT

C. Recommended Buffers

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)

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

* See Appendix B for recommended 1X Reaction Buffer conditions for the Upstate kinases listed on page 2. Other components that may be added to the Reaction Buffer if required are $MnCl_2$, DTT or β-mercaptoethanol.

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. 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% extra or one extra assay point is generally sufficient).

Reaction Solution Preparation

1. **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).
2. **ATP Working Solution:** Prepare the ATP Working Solution in 1.25X 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.
3. **TK Substrate Working Solution:** For each substrate to be tested prepare a 250µM TK Substrate Working Solution in 1.25X Reaction Buffer. This is 2.5X the required assay concentration of 100µM (*e.g.,* to prepare 1ml of TK Substrate Working Solutions for TK Substrates **1 or 3**, combine 725µl water, 250µl 5X Reaction Buffer and 25µl of 10mM TK Substrate; to prepare 1ml of TK Substrate Working Solution for TK Substrate **2**, combine 747.5µl water, 250µl 5X Reaction Buffer and 2.5µl of 100mM TK Substrate 2). 10µl of TK Substrate Working Solution is required per well.
4. **Kinase Working Solution:** Prepare the Kinase Working Solution in 1X Enzyme Dilution Buffer at a concentration of 5X the required final reaction concentration. 5µl of Kinase 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 TK Substrate Working Solution
   5µl 1X Reaction Buffer
   10µl ATP Working Solution

2. Set up reactions as detailed below.

   Plus Enzyme Wells
   10µl TK Substrate Working Solution
   5µl Kinase 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 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. **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).

2. **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.

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

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

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

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

3. Add 5µl/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 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. 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 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.

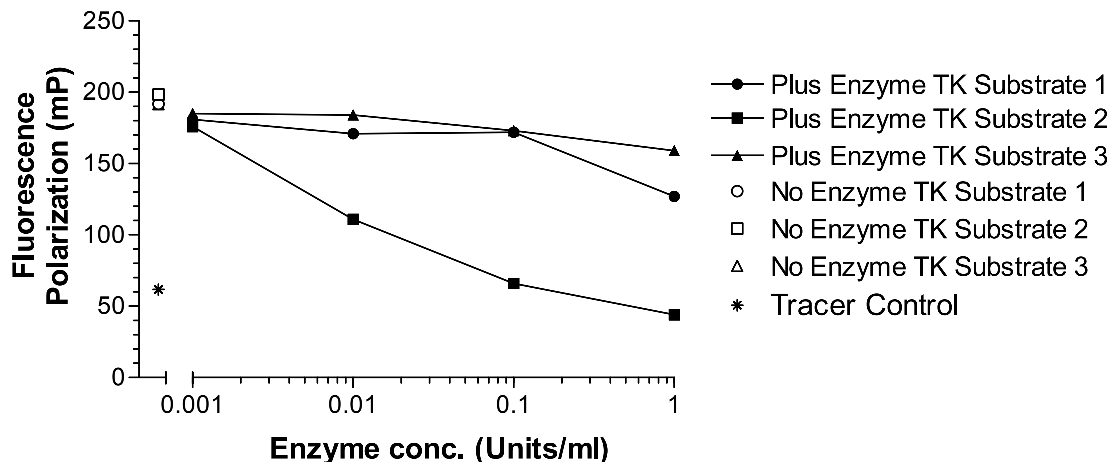
- The 10X KinEASE™ Buffer (Catalog # 20-302) is used to prepare 5X working stocks to ensure a final 1X buffer concentration in the assay. These working buffers should be stored at 4ºC until ready to use. If long-term storage (one week to six months) is required, DTT should be omitted until assay is to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

V. APPENDIX A - Assay Optimization

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 (100µM 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.

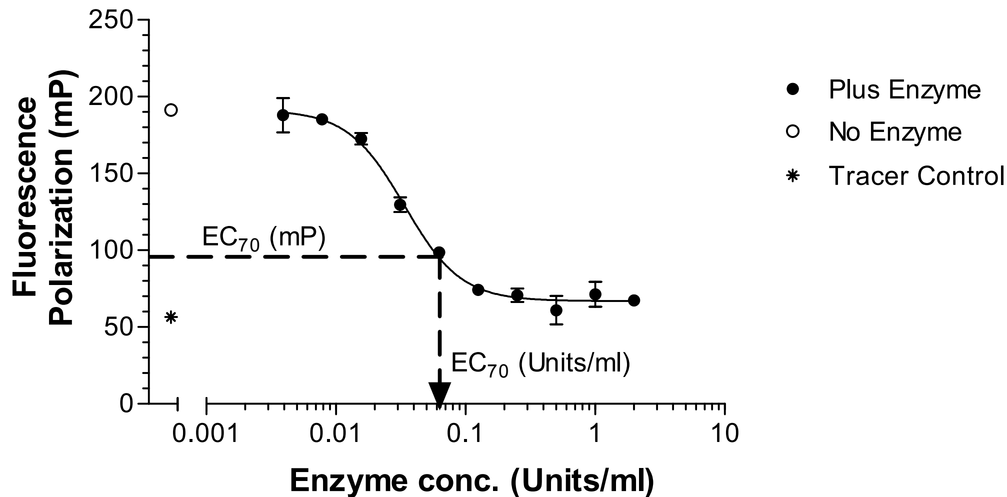
Set up an enzyme titration with two-fold serial dilutions of enzyme and appropriate controls (No Enzyme Wells, Tracer Control Wells and Buffer Control Wells) according to the standard assay protocol.

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

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

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

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.



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	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$
Abl (T315I) (h) (14-522)	TK Substrate 1	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$, 1mM DTT
Abl (m) (14-459)	TK Substrate 1	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$
Arg (h) (14-521)	TK Substrate 1	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$
Arg (m) (14-460)	TK Substrate 1	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$
Blk (m) (14-316)	TK Substrate 1	100μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$, 1mM $MnCl_2$
Bmx (h) (14-499)	TK Substrate 1	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$
BTK (h) (14-552)	TK Substrate 1	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$
CSK (h) (14-458)	TK Substrate 2	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$, 1mM DTT, 1mM $MnCl_2$
Src (h) (14-326)	TK Substrate 3	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$, 1mM $MnCl_2$
EGFR (h) (14-531)	TK Substrate 2	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$, 1mM $MnCl_2$
EphA2 (h) (14-560)	TK Substrate 2	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$, 1mM DTT, 1mM $MnCl_2$
EphB2 (h) (14-553)	TK Substrate 2	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$, 1mM $MnCl_2$
EphB4 (h) (14-554)	TK Substrate 2	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$, 1mM $MnCl_2$
Fes/Fps (h) (14-473)	TK Substrate 1	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$
FGFR3 (h) (14-464)	TK Substrate 2	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$, 1mM $MnCl_2$
Flt1 (h) (14-562)	TK Substrate 2	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$, 1mM DTT, 1mM $MnCl_2$
Flt3 (h) (14-500)	TK Substrate 1	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$
Fms (h) (14-551)	TK Substrate 2	100μM	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	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$
IGF-1R (h) (14-465)	TK Substrate 2	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$, 1mM DTT, 1mM $MnCl_2$
Insulin R (h) (14-466)	TK Substrate 2	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$, 1mM $MnCl_2$
Lck (h) (14-442)	TK Substrate 3	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$, 1mM DTT, 1mM $MnCl_2$
Lyn (h) (14-510)	TK Substrate 1	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$, 1mM DTT, 1mM $MnCl_2$
Lyn (m) (14-315)	TK Substrate 1	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$, 1mM DTT, 1mM $MnCl_2$
Met (h) (14-526)	TK Substrate 1	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$
PDGFRα (h) (14-467)	TK Substrate 2	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$, 1mM $MnCl_2$
PDGFRβ (h) (14-463)	TK Substrate 2	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$, 1mM $MnCl_2$
Ret (h) (14-570)	TK Substrate 1	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$, 1mM DTT
Ros (h) (14-527)	TK Substrate 2	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$, 1mM DTT, 1mM $MnCl_2$
Rse (h) (14-535)	TK Substrate 3	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$, 1mM DTT, 1mM $MnCl_2$
Syk (h) (14-314)	TK Substrate 2	100μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$, 1mM DTT, 1mM $MnCl_2$
Tie2 (h) (14-540)	TK Substrate 2	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$, 1mM $MnCl_2$
TrkA (h) (14-571)	TK Substrate 1	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$, 1mM DTT, 1mM $MnCl_2$
TrkB (h) (14-507)	TK Substrate 2	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$
Yes (h) (14-478)	TK Substrate 2	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$
ZAP-70 (h) (14-404)	TK Substrate 2	250μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM $MgCl_2$, 1mM DTT, 1mM $MnCl_2$