

KinEASE™ FP Fluorescein Green Assay

Module 1 – Serine/Threonine Kinases

Catalog # 32-001

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, 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

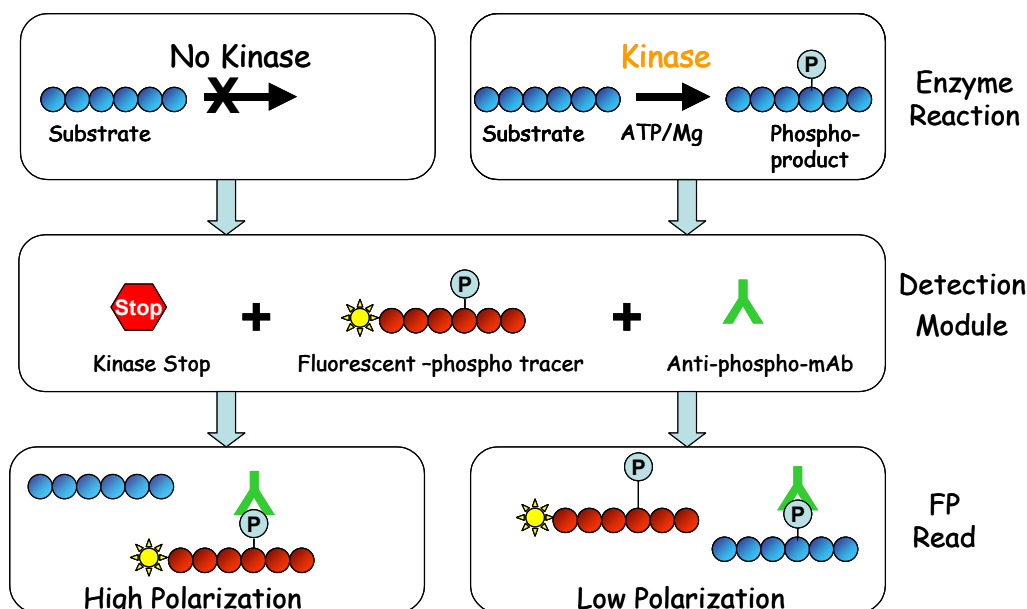
In this assay a phosphorylated peptide has been labeled with a green fluorescent dye. This phosphorylated tracer has a low molecular weight and thus a low fluorescence polarization value. The phosphorylated tracer binds to a phospho-specific antibody to form a high molecular weight complex with a high 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 1 supplied in this kit offers a method for assaying Serine/Threonine 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 1 is known to work with the following enzymes: **AMPK** (r), **Aurora A** (h), **CaM Kinase II** (r), **CaM Kinase IV** (h), **CHK1** (h), **CHK2** (h), **IKK α** (h), **IKK β** (h), **MAPKAP Kinase 2** (h), **MSK1** (h), **p70 S6 Kinase** (h), **PAK2** (h), **Pim1** (h), **PKA** (h), **Akt1/PKB α** (h), **Akt2/PKB β** (h), **Akt3/PKB γ** (h), **PKC α** (h), **PKC β I** (h), **PKC β II** (h), **PKC γ** (h), **PKC δ** (h), **PKC ϵ** (h), **PKC eta** (h), **PKC iota** (h), **PKC μ** (h), **PKC theta** (h), **PKC zeta** (h), **PKD2** (h), **PRAK** (h), **PRK2** (h), **ROK α /ROCK-II** (h), **Rsk1/MAPKAP Kinase 1a** (h), **Rsk1/MAPKAP Kinase 1a** (r), **Rsk2/MAPKAP Kinase 1b** (h), **Rsk3** (h) and **SGK1** (h) (see Appendix B for further details).

h = human; r = rat



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 STK Tracer solution is light sensitive.**

Reagent	Catalog #	Stock	Volume Supplied	Storage
STK Substrate 1	12-548	10mM	120µl	-20°C
STK Substrate 2	12-549	10mM	120µl	-20°C
STK Substrate 3	12-550	10mM	120µl	-20°C
STK Antibody	35-002	20X	237µl	-20°C
STK Tracer	20-287	100X	48µl	-20°C

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 -20°C
DTT	Major Laboratory Suppliers	1M	Store aliquoted -20°C
BSA	Chemicon #1003512	1% in water	Store aliquoted -20°C
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, 0.05% BSA, 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 MnCl₂, CaCl₂, Calmodulin, DTT, AMP, Lipid activator (Upstate Catalog # 20-133) 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

- 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 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.
- STK Substrate Working Solution:** For each substrate to be tested prepare a 75μM STK Substrate Working Solution in 1.25X Reaction Buffer. This is 2.5X the required assay concentration of 30μM (*e.g.*, to prepare 1ml of STK Substrate Working Solution combine 742.5μl water, 250μl 5X Reaction Buffer and 7.5μl of 10mM STK Substrate). 10μl of STK Substrate Working Solution is required per well.
- 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 STK Substrate Working Solution

5µl 1X Reaction Buffer

10µl ATP Working Solution

2. Set up reactions as detailed below.

Plus Enzyme Wells

10µl STK 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. EC₇₀ value), the substrate working solution and enzyme working solutions can be combined and added as 15µl 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. **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, 200µl 5X Detection Buffer and 10µl 100X STK Tracer). 5µl of STK Stop Mix is required per well.
3. **STK Antibody Mix:** Prepare a 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 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

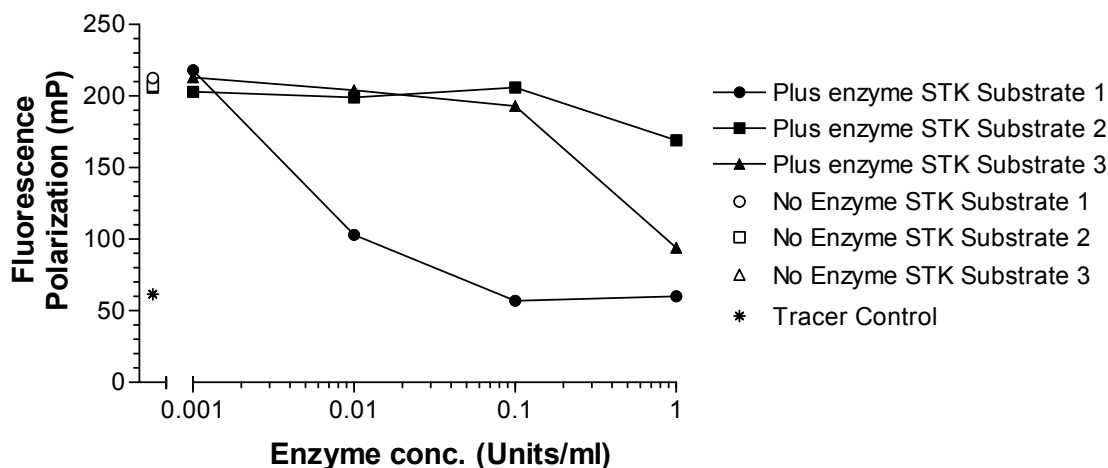
- It is recommended that the MgCl₂ final reaction concentration should not exceed 5mM in the reaction (*i.e.*, 25mM in the 5X Reaction Buffer). If a higher concentration of MgCl₂ is required then do not exceed 10mM MgCl₂ (*i.e.*, 50mM in the 5X Reaction Buffer) and run an enzyme reaction with stop mix 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 4°C 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 1 is suitable for your kinase, a series of enzyme dilutions should be tested with each STK Substrate (30 μ M final reaction concentration) according to the standard assay protocol.

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

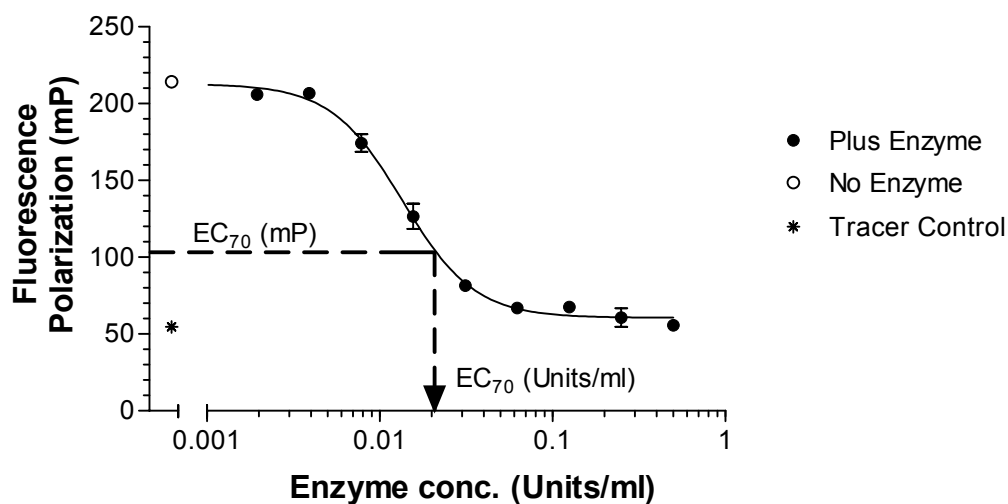
Set up an enzyme titration with two-fold serial dilutions of enzyme and appropriate controls (No Enzyme Wells, Tracer Control Wells and Buffer Control Wells) according to the standard assay protocol.

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; r = rat):

Kinase (Catalog #)	Recommended Substrate	Recommended Substrate Conc.	Recommended 1X Reaction Buffer
AMPK (r) (14-305)	STK Substrate 1	30 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 50 μ M AMP
Aurora A (h) (14-511)	STK Substrate 2	200 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT
CaM Kinase II (r) (14-217)	STK Substrate 1	10 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT, 100 μ M CaCl ₂ , 250nM Calmodulin
CaM Kinase IV (h) (14-547)	STK Substrate 1	10 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT, 100 μ M CaCl ₂ , 250nM Calmodulin
CHK1 (h) (14-346)	STK Substrate 1	10 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT
CHK2 (h) (14-347)	STK Substrate 1	200 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT
IKK α (h) (14-461)	STK Substrate 1	200 μ M	50mM HEPES pH 7.2, 0.01% BSA, 2mM MgCl ₂ , 1mM DTT
IKK β (h) (14-485)	STK Substrate 1	200 μ M	50mM HEPES pH 7.2, 0.01% BSA, 2mM MgCl ₂ , 1mM DTT
MAPKAP Kinase 2 (h) (14-337)	STK Substrate 1	30 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT
MSK1 (h) (14-548)	STK Substrate 3	10 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT
p70 S6 Kinase (h) (14-486)	STK Substrate 3	30 μ M	50mM HEPES pH 7.2, 0.01% BSA, 2mM MgCl ₂ , 1mM DTT
PAK2 (h) (14-481)	STK Substrate 2	200 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT
Pim1 (h) (14-573)	STK Substrate 3	200 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT
PKA (h) (14-440)	STK Substrate 2	10 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT
Akt1/PKB α (h) (14-276)	STK Substrate 3	10 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT
Akt2/PKB β (h) (14-339)	STK Substrate 3	30 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT
Akt3/PKB γ (h) (14-502)	STK Substrate 3	30 μ M	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT
PKC α (h) (14-484)	STK Substrate 1	30 μ M	50mM HEPES pH 7.2, 0.01% BSA, 10mM MgCl ₂ , 1mM DTT, 1X Lipid Activator

Kinase	Recommended Substrate	Recommended Substrate Conc.	Recommended 1X Reaction Buffer
PKCβI (h) (14-503)	STK Substrate 1	30μM	50mM HEPES pH 7.2, 0.01% BSA, 10mM MgCl ₂ , 1mM DTT, 1X Lipid Activator
PKCβII (h) (14-496)	STK Substrate 1	30μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT, 1X Lipid Activator
PKCγ (h) (14-483)	STK Substrate 1	100μM	50mM HEPES pH 7.2, 0.01% BSA, 10mM MgCl ₂ , 1mM DTT, 1X Lipid Activator
PKCδ (h) (14-504)	STK Substrate 1	30μM	50mM HEPES pH 7.2, 0.01% BSA, 10mM MgCl ₂ , 1mM DTT, 1X Lipid Activator
PKCε (h) (14-518)	STK Substrate 1	30μM	50mM HEPES pH 7.2, 0.01% BSA, 10mM MgCl ₂ , 1mM DTT, 1X Lipid Activator
PKC eta (h) (14-497)	STK Substrate 1	100μM	50mM HEPES pH 7.2, 0.01% BSA, 10mM MgCl ₂ , 1mM DTT, 1X Lipid Activator
PKC iota (h) (14-505)	STK Substrate 1	200μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT
PKCμ (h) (14-508)	STK Substrate 1	30μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT
PKC theta (h) (14-444)	STK Substrate 1	200μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT
PKC zeta (h) (14-525)	STK Substrate 1	200μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT
PKD2 (h) (14-506)	STK Substrate 1	200μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT
PRAK (h) (14-334)	STK Substrate 1	100μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT
PRK2 (h)	STK Substrate 1	10μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT
ROKα/ROCK-II (h) (14-451)	STK Substrate 2	100μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT
Rsk1/MAPKAP Kinase 1a (h) (14-509)	STK Substrate 1	30μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT
Rsk1/MAPKAP Kinase 1a (r) (14-479)	STK Substrate 1	30μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT
Rsk2/MAPKAP Kinase 1b (h) (14-480)	STK Substrate 1	30μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT
Rsk3 (h) (14-462)	STK Substrate 1	200μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT
SGK1 (h) (14-331)	STK Substrate 3	10μM	50mM HEPES pH 7.2, 0.01% BSA, 5mM MgCl ₂ , 1mM DTT