PERKINELMER LIFE AND ANALYTICAL SCIENCES

USING THE ALPHASCREEN™ PHOSPHOSENSOR KIT

CATALOG NUMBERS:
6760307D, 6760307M, 6760307R

For Laboratory Use Only
Research Chemicals for Research Purposes Only

Precautions

- AlphaScreen™ beads are light-sensitive. All assays using the AlphaScreen beads should be performed under subdued laboratory lighting of less than 100 lux. Alternatively, green filters (Roscolux filters #389 from Rosco, or equivalent) can be applied to light fixtures. Any incubation of AlphaScreen™ beads should be performed in the dark. Plates can be covered by an opaque microplate to minimize the effect of light.

- Due to the small volumes used in the assay, it is recommended that the plates be covered with TopSeal-A™ adhesive sealing film to reduce evaporation during incubation periods (PerkinElmer® Inc., Cat. No. 6005185). The assay can be read with the TopSeal-A film in place.

- The PhosphoSensor Acceptor beads contained in this kit may slightly aggregate with time. This is normal. It is advised to vortex the beads prior to use.

- Beads should be stored in the dark at 4°C.

TABLE OF CONTENTS

I. BEFORE STARTING

Receiving the AlphaScreen PhosphoSensor Kit

Upon receiving the AlphaScreen PhosphoSensor Kit, ensure that it is on blue ice and that the ice packs are not completely melted. Verify that all components are present in the kit using the table below.

Provided Reagents and Materials

The following kit sizes are available*:

1,000 assay points (catalog number 6760307D)

10,000 assay points (catalog number 6760307M)

50,000 assay points (catalog number 6760307R)

**The number of assay points is based on the use of 500 ng of each bead per well.*

The reagents and materials provided in the AlphaScreen PhosphoSensor Kit are listed in the Table I:

Table I. *Reagents and materials supplied*

KIT COMPONENTS	1,000 assay points 6760307D	10,000 assay points 6760307M	50,000 assay points 6760307R
PhosphoSensor Acceptor beads Stored in 100 mM Tris-HCl pH 7.0, 0.05% Proclin™-300	0.1 mL (5 mg/mL)	1.0 mL (5 mg/mL)	5.0 mL (5 mg/mL)
Streptavidin-Donor beads Stored in 100 mM Tris-HCl pH 7.4, 0.05% Proclin-300	0.1 mL (5 mg/mL)	1.0 mL (5 mg/mL)	5.0 mL (5 mg/mL)
Positive control bio-LCK-P Stored in 25 mM Hepes pH 7.4, 0.05% Proclin-300	0.05 mL (5 mM)	0.05 mL (5 mM)	0.05 mL (5 mM)
10X Control buffer 100 mM MES pH 6.0, 1M NaCl, 0.05% Proclin-300	1.5 mL (10X)	1.5 mL (10X)	1.5 mL (10X)

Note before use:

- For maximum recovery of content, briefly centrifuge the vials prior to removing the caps and resuspend the beads by vortexing.
- Reagents should be stored at +2 - 8°C.
- Acceptor and Donor beads should not be frozen and should be stored protected from light.
- 10X buffer may not be suitable as a kinase assay or as a detection buffer.

Recommended Additional Reagents and Materials

Table II. *Recommended reagents and materials*

Item	Suggested source	Catalog #
Kinase of choice	N/A	N/A
Biotinylated substrate of choice	N/A	N/A
HPLC water or equivalent	Fisher Scientific	W5-4
ATP	Sigma-Aldrich™ Co.	A-3377
$MgCl_2$	Sigma-Aldrich™ Co.	M-9272
EDTA	GIBCO®	15575-038
Staurosporine	Sigma-Aldrich™ Co.	S-4400
OptiPlate™-384 (white opaque 384-well microplate)	PerkinElmer® Inc.	6007290 (pack of 50) 6007299 (pack of 200)
TopSeal-A Adhesive Sealing Film	PerkinElmer® Inc.	6005185
Tween® 20	Pierce Biotechnologies	28320
Single-channel Pipettors§	N/A	N/A

§ For lower volume additions (2-10 µL), we recommend a pipettor precision ≤ 2%. For higher volume additions (25-1000 µL), a pipettor precision of ≤ 1% is recommended.

Assay must be read using an AlphaScreen compatible reader such as all PerkinElmer EnVision™ multilabel plate readers with AlphaScreen module, Fusion-Alpha™ multilabel readers or AlphaQuest® original AlphaScreen readers.

II. INTENDED USE

The AlphaScreen PhosphoSensor Kit is intended to perform antibody-free detection of phosphorylated protein or peptide.

III. PRINCIPLE OF THE ASSAY

In cells, protein kinases mediate the phosphorylation of a variety of different protein substrates in the presence of ATP. Kinases catalyze the reversible addition of phosphate molecules to tyrosine, serine and threonine residues. There are several commercially available antibodies that recognize phosphotyrosine residues with high affinity. However, such generic antibodies are not currently available for phosphoserine and phosphothreonine. The AlphaScreen PhosphoSensor Kit allows detection of the phosphorylation of tyrosine, serine, and threonine residues without the need for such sequence specific antibody.

The principle of the assay is illustrated in Figure 1. In this assay, the kinase driven addition of a phosphate group to a biotinylated substrate will result in the simultaneous capture of the phosphorylated substrate by the PhosphoSensor Acceptor (coated with a Lewis Metal Chelate) and the streptavidin (SA) Donor beads. Upon laser excitation of the Donor beads, the proximity of the Donor and PhosphoSensor Acceptor beads will generate an AlphaScreen signal between 520 and 620 nm. In the absence of phosphorylation, no signal should be observed. Since the Acceptor beads allow for the detection of phosphates, the activity of phosphatases can also be monitored using these beads.

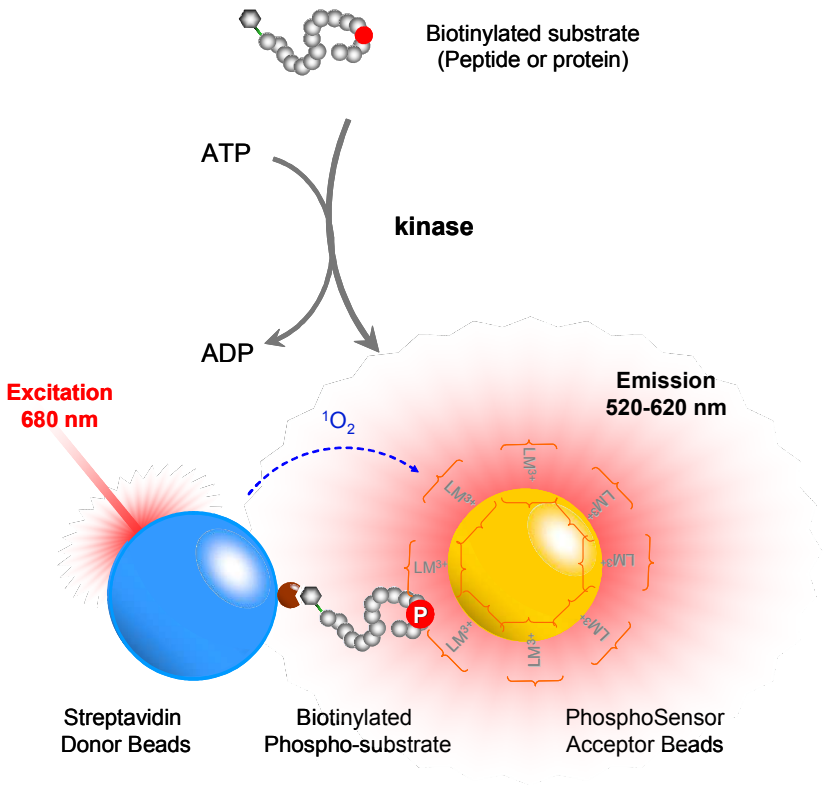

Figure 1. *Illustration of the detection of a phosphorylated peptide using the AlphaScreen PhosphoSensor Acceptor beads.* Legend: LM^{3+} = Lewis Metal Chelate.

IV. ASSAY DEVELOPMENT

The AlphaScreen technology has been widely used for the development of kinase assays using specific antibodies. It is important to stress that the optimal detection buffer as well as ATP, substrate, and enzyme concentrations, which have been determined for an antibody based AlphaScreen assay, will not necessarily apply to an antibody-free assay using the PhosphoSensor beads. Consequently, it is strongly advised to follow the assay development steps presented in this section.

A. Deciding on substrate configuration

The following guidelines should be followed when preparing a novel biotinylated substrate to be included in a kinase assay reaction monitored by the AlphaScreen technology. The same substrate configuration applies for both an antibody-based and an antibody-free kinase assay development using the AlphaScreen technology.

1. Peptide substrate

Peptide substrates should be designed such that they possess at least 20 carbons between the biotin label and the amino acid targeted for phosphorylation (tyrosine, serine or threonine). For small peptides, this can be achieved by including either a glycine stretch, or a LC (long chain) spacer.

N-hydroxysuccinimidyl ester (NHS) or maleimide driven chemical reaction can be used for the addition of biotin to amino acid sequences. The NHS driven reaction will target the secondary amine present on lysine residues and at the N-terminal of the peptide. On the other hand, the maleimide driven reaction will target the sulphydryl group present in cysteine residues.

If the peptide contains many internal lysine residues, which will be targeted by NHS, it is recommended to have biotin integrated during peptide synthesis with the required spacer.

2. Protein substrate

Protein substrates can be biotinylated using either NHS or maleimide driven reactions, in the same way as a

peptide substrate (see above). However, in order to prevent addition of biotin near the phosphorylation site of the substrate, the presence of lysine or cysteine residues near the phosphorylation site should be evaluated to guide the use of one of the two chemistries (NHS or maleimide driven reaction).

B. Deciding on assay format

1. All-in-one-well assay

The all-in-one-well (homogenous) assay format represents the format of choice for screening purposes. In this format, the kinase reaction is performed in the same well as the detection reaction (Figure 2). For a detailed protocol description see section VI.

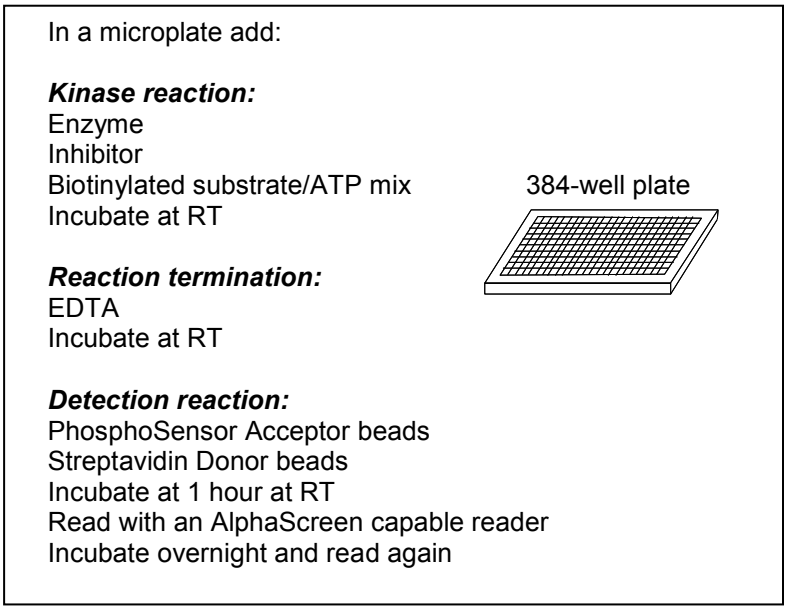

Figure 2. *Scheme of the all-in-one-well assay format.*

2. Transfer assay

The advantage of the transfer assay format is to dilute potential interfering reagents that are present in the kinase reaction before adding the detection beads. This format can also be used for screening purposes. It is especially useful when:

- the enzyme is intrinsically highly phosphorylated and therefore interferes with the detection of the phosphorylated peptide by the PhosphoSensor Acceptor beads*;*
- the signal generated is low and high ATP concentrations are required (e.g., over 100 µM).

A scheme of the transfer assay protocol is illustrated in Figure 3 (for a detailed protocol description see section VI).

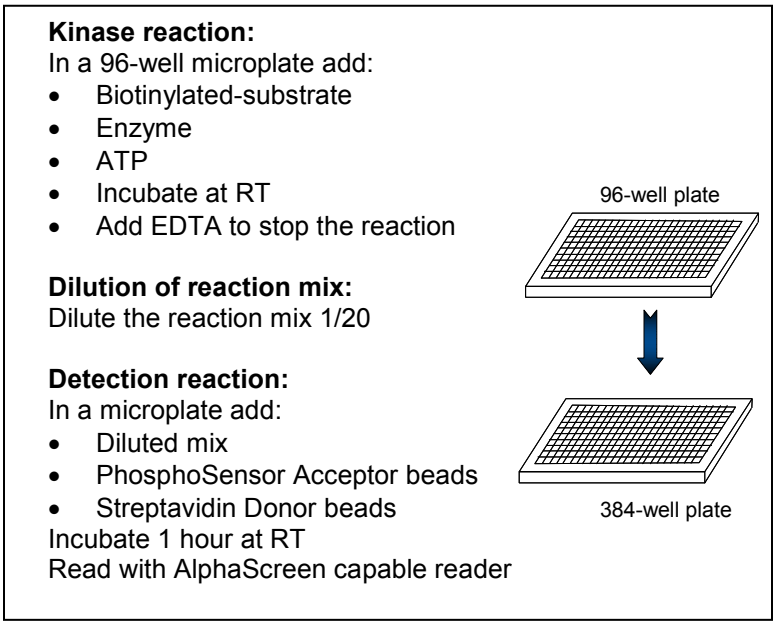

Figure 3. *Scheme of the transfer assay format.*

C. Titration of biotinylated phosphopeptide and/or non-phosphorylated peptide substrate

To ease the optimization of an antibody-free kinase assay using the AlphaScreen PhosphoSensor Acceptor beads, we recommend obtaining the phosphorylated version of the peptide substrate. Titration of this positive control in parallel with the biotinylated substrate will allow evaluation of the expected signal window. It should be noted, however, that the availability of a phosphorylated peptide is not essential to develop a kinase assay using the AlphaScreen PhosphoSensor Kit.

For peptide titration assays, the biotinylated peptide (as well as the phosphopeptide, if available) should be added to the plate diluted in the kinase reaction buffer, whereas the beads (both Acceptor and Donor) should be added to the plate in the detection buffer.

For the majority of biotinylated peptides tested, the standard detection buffer composition is:

10 mM Tris-HCl pH 7.0, 100 mM NaCl, 0.1% Tween-20.

However, optimization of the detection buffer must be performed when observing high non-specific binding of the biotinylated substrate. Ionic strength, nature of the buffer, and pH of the detection reaction can influence the non-specific binding of some peptide sequence.

Note 1: Do not use PBS since it contains phosphate, which will bind to the PhosphoSensor Acceptor beads and displace the phosphorylated substrate.

Note 2: Be aware that the presence of ATP in the detection reaction may alleviate the non-specific bind-

ing of the substrate (see section IV-D). Thus, we do not use plus or minus ATP as an indication of the assay window.

D. Evaluating optimal enzymatic assay conditions

The following section presents the steps necessary to determine the optimal ATP, substrate, and enzyme concentrations in order to obtain an optimal signal window. The optimal signal window is defined here as the optimum S/B ratio measured between the background signal (basal signal obtained in the presence of staurosporine; see note below) and the maximal enzymatic activity.

Note 3: The non-specific binding of some biotinylated peptides will be strongly diminished by the presence, in the detection reaction, of ATP and/or some enzymes that are themselves phosphorylated for activation. In these cases, control incubations lacking either ATP or enzyme as a reference for background could be misleading when evaluating assay window (S/B values). For such "sticky" peptides, it is advisable to determine assay window using control incubations containing all the reaction components (i.e., enzyme, substrate and ATP) in the presence or absence of a generic protein kinase inhibitor such as staurosporine.

Note 4: We do not recommend terminating the kinase reaction using EDTA at this stage of assay development. Since EDTA interferes to some extent with the detection by the PhosphoSensor Acceptor beads, it should be integrated later during assay development (see section IV-E).

The following example shows preliminary assay development using the commercially available protein kinase A (PKA) and the biotinylated substrate kemptide. All assays were performed at room temperature (RT), in white, opaque 384-well microplates, in a final volume of 26 µL using 2 µL of enzyme, 2 µL of biotinylated peptide, 2 µL of ATP, 10 µL of PhosphoSensor Acceptor beads and 10 µL of Streptavidin Donor beads (both acceptor and donor beads were used at a final concentration of 20 µg/mL).

1. ATP/ substrate titration

The first optimization step consists of titrating both the substrate and ATP concentrations. We recommend the matrix depicted in Table III, which uses both fixed enzyme (3 nM) and beads concentrations (20 µg/mL). The assay window should be determined by performing the assay in the absence or presence of staurosporine.

Table III. ***Scheme of preliminary kinase assay development.*** For preliminary assay conditions, perform the titration of ATP and substrate using 3 nM of enzyme. The assay should be performed in the absence and presence of 10 µM of a generic inhibitor (such as staurosporine) with at least one concentration of substrate to evaluate assay background.

ATP (µM)	Substrate (µM)				With 10 µM staurosporine)
	0	0.3	1	3	3
3	(3,0)	(3, 0.3)	(3, 1)	(3, 3)	(3, 3)
10	(10, 0)	(10, 0.3)	(10, 1)	(10, 3)	(10, 3)
30	(30, 0)	(30, 0.3)	(30, 1)	(30, 3)	(30, 3)

Microplates were read after a detection time of either 1 hour (Figure 4A) or 17 hours (overnight incubation;

Figure 4B). After 1 hour of incubation, it was observed that substrate concentrations higher than 0.3 µM did not improve the signal window. A decrease of the signal window was observed for ATP concentrations higher than 3 µM, due to ATP interference. Although similar results were obtained following an incubation period of 17 hours, a greatly improved signal window was observed. Thus, for both incubation periods tested, the optimal signal window was obtained when using 0.3 µM of biotinylated substrate and 3 µM of ATP. Under these conditions, S/B values of approximately 6 and 25 were observed following 1 hour and 17 hours detection time, respectively

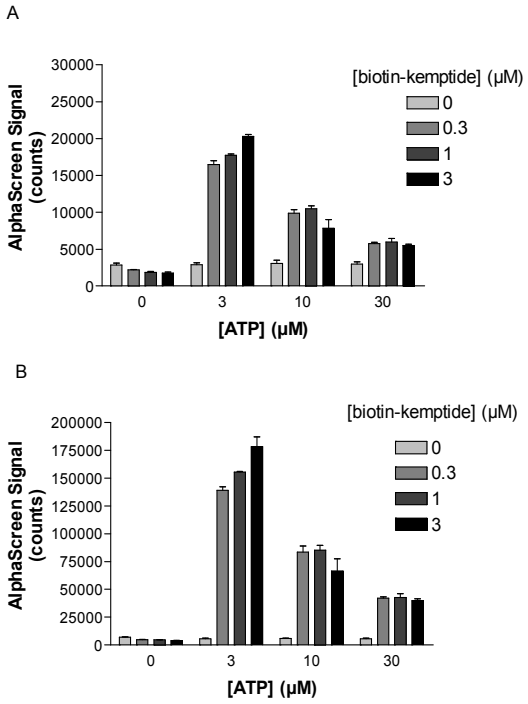

Figure 4. ***Scheme of the preliminary kinase assay optimization.*** Kinase reaction was performed in kinase reaction buffer (25 mM Hepes pH 7.4, 100 mM NaCl, 2.5 mM $MgCl_2$, 1 mM DTT, and 0.01% Tween-20) and beads were added in detection buffer (100 mM Tris pH 7.0, 100 mM NaCl, and 0.1% Tween-20). Incubations (25 µL) were conducted in 384-well microplates. Detection time was A) 1hour and B) 17 hours

2. Enzyme titration

The second optimization step consists of enzyme titration, using the optimal substrate and ATP concentrations determined previously.

As observed in Figure 5, increasing the concentration of enzyme up to approximately 0.3 nM led to a proportional signal increase. A S/B ratio of approximately 31 was obtained when using 0.3 nM of enzyme. Above this enzyme concentration, a signal decrease was observed, which may reflect either 1) saturation of both beads (Acceptor and Donor) by an excess of phosphorylated product or 2) competition of biotinylated product binding to the PhosphoSensor Acceptor beads by the kinase itself. As a matter of fact, some kinases are phosphorylated for activation.

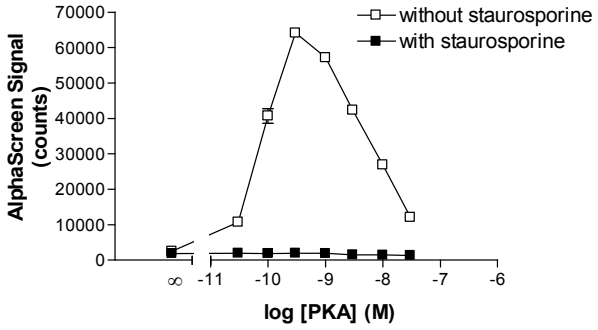

Figure 5. ***Enzyme titration using optimal concentrations of ATP and substrate.*** PKA was titrated using 3 µM of ATP and 0.3 µM of biotinylated substrate; a detection time of 1 hour was used.

3. Transfer assay format

In the transfer assay format, a bulk kinase reaction is performed; subsequently, the reaction mix is diluted before adding the AlphaScreen beads. In this format, the following concentrations of ATP, enzyme and bioti-

nylated substrate are suggested as a starting point: 100 µM, 30 nM, and 10 µM, respectively.

After incubation of the kinase reaction, the mix is diluted in order to obtain final concentrations of biotinylated substrate between 0 and 1 µM in the detection reaction. The detection reaction using the Acceptor and Donor beads is then conducted as for the all-in-one-well assay format (see section VI-B).

E. Termination of the kinase reaction

EDTA is a commonly used chelator for termination of kinase reactions. However, excessive concentrations of EDTA should be avoided when performing a detection using the PhosphoSensor Acceptor beads.

It is recommended to perform all kinase assay development using 2.5 mM $MgCl_2$ and then to determine the optimal concentration of EDTA required to terminate the enzymatic reaction. If necessary, titration of $MgCl_2$ can also be performed before EDTA titration.

Figure 6 shows the effect of increasing concentrations of EDTA on the PKA kinase reaction. In this assay EDTA was added either before starting the kinase reaction or 2 hours following the initiation of the reaction. Detection times of 1 and 17 hours were compared (Figure 6A-B). To confirm the specificity of the reaction, each incubation was performed in the presence or absence of a generic kinase inhibitor (10 µM staurosporine) (data not shown). It was determined that 5 mM EDTA is sufficient to completely stop the kinase reaction, while leaving an acceptable signal window (S/B ratio of 13). Overnight incubation of the detection reaction clearly results in an improved S/B ratio (Figure 6B).

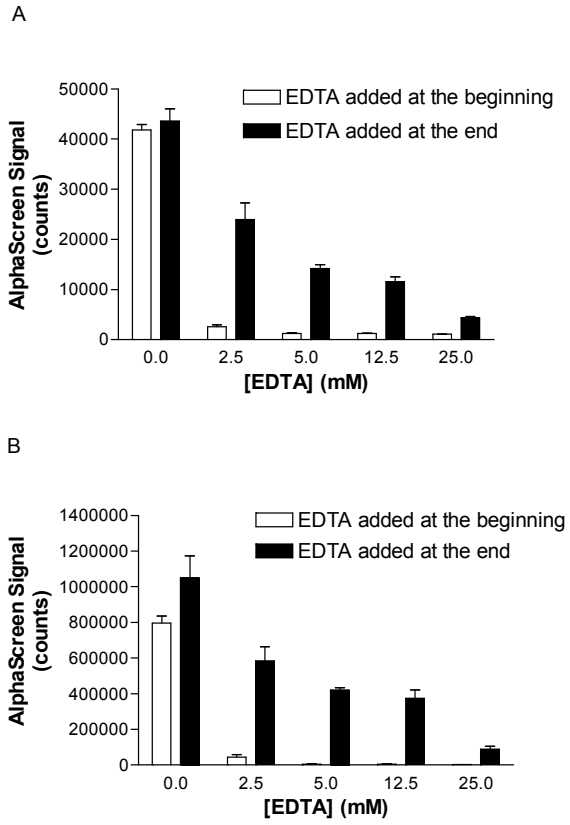

Figure 6. ***Titration of EDTA.*** Following the addition of EDTA, a detection time of 1 hour (A) or 17 hours (B) was used.

If no signal window is obtainable when using EDTA, it is recommended to either use the transfer assay to dilute EDTA before reading, or to add staurosporine at 1-10 µM to terminate the enzymatic reaction.

F. Kinetics of the detection reaction

Phosphopeptide detection using the PhosphoSensor Acceptor beads involves lower binding affinities than an antibody-based detection method. Thus, following termination of the kinase activity, it is recommended to incubate the detection reaction overnight to obtain the optimal signal window.

Figure 7 depicts the kinetics of the detection reaction following termination of PKA activity with 5 mM of EDTA. A proportional increase in the signal window was observed as a function of time.

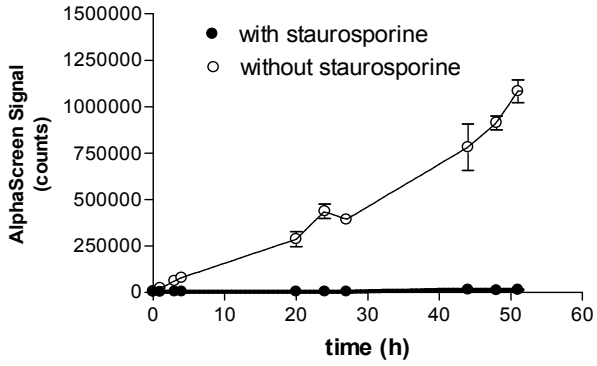

Figure 7. ***Kinetics of the detection reaction using the PhosphoSensor Acceptor beads to perform antibody-free kinase activity detection.*** The signal to background ratio is defined with or without 10 µM staurosporine.

Note 5: The time course of the detection will vary for other peptide or protein substrates.

G. Titration of PhosphoSensor Acceptor beads

In general, it is recommended to use a final concentration 20 µg/mL of AlphaScreen beads per reaction. However, for kinases which have affinities for ATP in the high micromolar range, tolerance of the PhosphoSensor Acceptor beads to ATP can be improved by increasing their concentration in the detection reaction. The following table shows an example using phosphorylation of the biotinylated–crosstide peptide substrate by MSK-1 (Table III). When the concentration of beads was increased to 50 µg/mL, more than two-fold increase in S/B was observed.

Table III. ***Effect of Acceptor beads concentration on tolerance to ATP.*** In this reaction, 0.3 µM of biotinylated crosstide was phosphoryated by 3 nM of MSK-1 in the presence of different concentration of ATP. Enzymatic reactions were allowed to proceed for 2 hours in the absence (max) or presence (min) of staurosporine. Detection was performed using different bead concentrations, as indicated.

	[PhosphoSensor Acceptor beads]					
	20 µg/mL			50 µg/mL		
[ATP] (µM)	min	max	S/B	min	max	S/B
0	21395	43965	2.1	262727	227959	1
10	7897	110645	14	26400	970873	37
30	6348	54727	9	19214	447904	23
100	6315	23108	4	15102	113449	8

V. SUMMARY OF ASSAY DEVELOPMENT

Figure 9 illustrates the different steps that should be undertaken in the development of a kinase assay using the AlphaScreen PhosphoSensor Kit.

In summary, the development of a kinase assay involves the following steps:

A. Identify substrate and perform the appropriate biotinylation (see section IV-A).

B. Perform titration of the biotinylated substrate together with the phosphorylated version of the substrate (if available) to evaluate the expected signal window (see section IV-C).

C1. If a high background signal is observed due to non-specific binding of the biotinylated substrate, optimize the detection buffer.

Note 6: Be aware that the presence of ATP in the detection reaction may alleviate the non-specific binding of the substrate (see section IV-D). Thus, we do not use plus or minus ATP as an indication of the assay window.

C2. If the peptide titration assay generates a specific signal window, continue with the kinase assay development using an all-in-one-well assay format (see sections IV-D, E, and F).

D. Titrate substrate, ATP and enzyme using an all-in-one-well kinase assay format. If a specific signal is observed in the kinase assay, proceed with assay optimization (see sections IV-D, E, and F).

E. If no specific signal is observed in the kinase assay, perform the assay in a transfer format to eliminate possible interferences with the detection reaction.

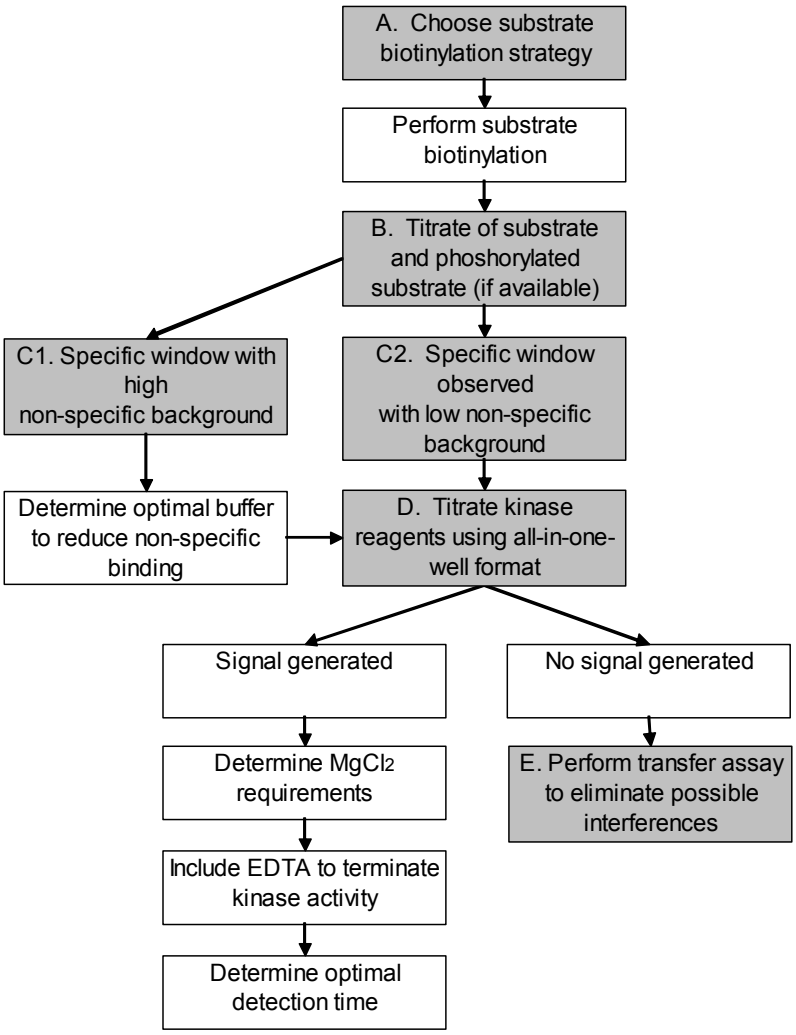

Figure 9. *Scheme of assay development using the AlphaScreen PhosphoSensor Kit.*

VI. DETAILED PROTOCOLS

A. Titration of phosphorylated versus non-phosphorylated substrate

Protocol suggested for optimizing buffer conditions using phosphorylated and non-phosphorylated substrates (see section III-C): the following protocol will allow the titration of both the phosphorylated and non-phosphorylated peptides. For performing more than two titration curves, increase the volume of each reagent.

1. Prepare reaction buffer

2. Prepare detection buffer: 10 mM Tris pH 7.0, 100 mM NaCl, 0.1% Tween-20.

3. Prepare biotin-non-phospho and biotin-phospho substrate by making serial dilutions in kinase reaction buffer supplemented with ATP and EDTA.

Note 7: Supplementation with ATP and EDTA is necessary to mimic closely the conditions that will be observed in an actual kinase assay.

Table IV shows an example of peptide dilution using a peptide stock at 50 µM.

Table IV. *Example of peptide dilution using a peptide stock at 50 µmol/L*

Dilution	[final in assay] (mol/L)	[intermediate] (mol/L)	Volume of dilution (µL)	Kinase reaction buffer (µL)
1	1×10^{-6}	5.0×10^{-6}	20 of 50 µmol/L	180
2	3×10^{-7}	1.5×10^{-6}	60 of dil 1	140
3	1×10^{-7}	5.0×10^{-7}	60 of dil 2	120
4	3×10^{-8}	1.5×10^{-7}	60 of dil 3	140
5	1×10^{-8}	5.0×10^{-8}	60 of dil 4	120
6	3×10^{-9}	1.5×10^{-8}	60 of dil 5	140
7	1×10^{-9}	5.0×10^{-9}	60 of dil 6	120
8	3×10^{-10}	1.5×10^{-9}	60 of dil 7	140
9	1×10^{-10}	5.0×10^{-10}	60 of dil 8	120
10	3×10^{-11}	1.5×10^{-10}	60 of dil 9	140
11	1×10^{-11}	5.0×10^{-11}	60 of dil 10	120
12	-	-	-	100

4. Dilute the PhosphoSensor Acceptor beads 1/100 in the detection buffer

5. Dilute the Streptavidin Donor beads 1/100 in the detection buffer

6. Protocol of addition

The assay is performed in triplicates in a white OptiPlate-384 in a total volume of 25 µL:

a) Add 5 µL of the biotinylated peptide dilution

b) Add 10 µL of PhosphoSensor Acceptor beads dilution (20 µg/mL final concentration)

c) Add 10 µL of Streptavidin Donor beads dilution (20 µg/mL final concentration)

d) Incubate in the dark for 1 hour at room temperature

e) Read on an AlphaScreen reader

B. All-in-one-well and transfer kinase assays

These assays are divided into three major steps:

Kinase reaction

It is recommended but not always necessary to perform the kinase reaction in the smallest volume possible (e.g. 6 µL) to allow for dilution of potential interferences during the detection reaction.

Termination of kinase activity

Before adding the detection beads, it is recommended to terminate the kinase reaction by the addition of EDTA (diluted in the optimal detection buffer). For the concentration to use in the assay, see section IV-E.

If the signal window is too low using EDTA, it is recommended to use a generic inhibitor such as staurosporine to terminate the enzymatic reaction. A titration should be performed to determine the optimal staurosporine concentration.

Detection reaction

Beads are added following the inactivation of the kinase. It is recommended to use an overnight detection to increase the signal window. This will not affect the pharmacological parameters of the assay since the enzyme has been inactivated before the final detection step. For detection buffer composition, see section IV-C.

Note 8: Acceptor beads can be titrated to increase tolerance to ATP (See Section IV-G).

1. Prepare reaction buffer as suggested by the provider of the enzyme.

2. Prepare detection buffer: 10 mM Tris pH 7.0, 100 mM NaCl, 0.1% Tween-20.

3. Protocol of addition for the all-in-one-well assay

The assay is performed in triplicates in a white Opti-Plate-384 in a total volume of 28 µL:

a) Add 2 µL of enzyme diluted in the kinase reaction buffer

b) Add 2 µL of inhibitor or buffer diluted in the kinase reaction buffer

c) Add 2 µL of biotinylated substrate/ATP dilution mix diluted in the kinase reaction buffer

d) Incubate 2 hours (kinase dependent) at room temperature

e) Add 2 µL of EDTA diluted in the optimal detection buffer

f) Add 10 µL of PhosphoSensor Acceptor beads diluted 1/90 in the optimal detection buffer

g) Add 10 µL of Streptavidin Donor beads diluted 1/90 in the optimal detection buffer

h) Incubate in the dark for 1 hr at room temperature

i) Read on an AlphaScreen reader

j) Incubate overnight in the dark and read again.

Note 9: Detection reaction should read after an overnight incubation for optimal results.

4. Protocol for the transfer assay

a) Enzyme, substrate and ATP are mixed together in a total volume of 100 µL of kinase reaction buffer (for reagents concentrations see section IV-D)

b) The mixture is incubated for 2 hours (kinase dependent) at room temperature (see section IV-E)

c) EDTA is added to the mixture

d) The kinase reaction mixture is diluted as described in Table V

Table V. *Dilution of the kinase mixture*

Dilution	Volume of dilution (µL)	Kinase reaction buffer (µL)
1	reaction mix	-
2	30 of reaction mix	70
3	30 of dilution 2	60
4	30 of dilution 3	70
5	30 of dilution 4	60
6	-	100

e) Dilute PhosphoSensor Acceptor beads 1/100 in the optimal detection buffer in order to get a final concentration of 20 ug/mL

f) Dilute Streptavidin Donor beads 1/100 in the optimal detection bufferin order to get a final concentration of 20 ug/mL

g) Add in triplicates to the wells of an Optiplate-384 microplate:

- 5 µL of kinase reaction dilution (step d)
- 10 µL of Acceptor beads dilution (step e)
- 10 µL of Donor beads dilution (step f)

h) Incubate in the dark for 1 hour at room temperature

i) Read on an AlphaScreen reader

j) Incubate overnight in the dark and read again

C. AlphaScreen beads quality control

1. Prepare 1X control buffer: dilute 1.5 mL of 10X control buffer with 13.35 mL of H_2O and add 0.15 mL Tween-20 10%.

2. Prepare serial dilutions of the kit's positive control at 5µM (Table VI):

Table VI: *Kit's positive control probe dilution using a peptide stock at 5 µmol/L*

Dilu-tion	[final in assay] (mol/L)	[intermediate] (mol/L)	Volume of dilution (µL)	1X control buffer (µL)
1	1×10^{-7}	5.0×10^{-7}	20 of 5 µmol/L	180
2	3×10^{-8}	1.5×10^{-7}	60 of dil 1	140
3	1×10^{-8}	5.0×10^{-8}	60 of dil 2	120
4	3×10^{-9}	1.5×10^{-8}	60 of dil 3	140
5	1×10^{-9}	5.0×10^{-9}	60 of dil 4	120
6	3×10^{-10}	1.5×10^{-9}	60 of dil 5	140
7	1×10^{-10}	5.0×10^{-10}	60 of dil 6	120
8	3×10^{-11}	1.5×10^{-10}	60 of dil 7	140
9	1×10^{-11}	5.0×10^{-11}	60 of dil 8	120
10	-	0.00	0	200

3. Dilute the PhosphoSensor Acceptor beads 1/100 in the 1X control buffer

4. Dilute the Streptavidin Donor beads 1/100 in 1X control buffer

5. Protocol of addition:

The assay is performed in triplicates in a white Opti-Plate-384 in a total volume of 25 µL

a) Add 5 µL of control phosphopeptide

b) Add 10 µL of PhosphoSensor Acceptor beads dilution (20 µg/mL final concentration)

c) Add 10 µL of Streptavidin Donor beads dilution (20 µg/mL final concentration)

d) Incubate in the dark for 1 hour at room temperature

e) Read on an AlphaScreen reader

h) Incubate overnight in the dark and read again.

Note 10: The 10X detection buffer included in the kit may not be suitable for the detection of all phosphorylated peptides and should only be used for quality control of the beads.

Expected results: maximum signal should be reached at 30 nM of control probe with an EC_{50} between 1.5 to 15 nM. The absolute maximum counts generated will be dependent on the instrument used for the readout.

VII. TROUBLESHOOTING GUIDE

The following section describes the possible problems which could be encountered when developing an antibody-free kinase assay using the AlphaScreen Technology. If more information is required, please consult your local PerkinElmer technical support division (see page 34 for customer support information).

Problem	Possible Cause	Effect/Remedy
No signal	Detection conditions	Interference of EDTA with the assay / since EDTA is used to chelate the Mg ions, titrate $MgCl_2$ to limit the concentration of EDTA added for chelation. Add a small volume of EDTA **before** adding the larger volumes of beads. Stop the kinase reaction using a generic inhibitor such as staurosporine (1-10 µM) ATP interferes with the detection 1) Increase the concentration of Acceptor beads to increase the tolerance of the assay to ATP. 2) Perform the assay in a transfer assay. 3) Perform kinase assay in smaller volume and/or detection assay in large volume to increase the dilution of ATP before the detection.
	Kinase assay conditions	Perform the assay in a transfer format to evaluate if it is due to interference of ATP and/or enzyme.
	Instrument/plates	Incompatible microplate choice / use solid opaque white plates such as PerkinElmer Optiplates. Ensure that your reader contains an AlphaScreen reading mode.
	Kinase assay reagents	No or improper biotinylation of substrate peptide or protein / check extent of biotinylation using the AlphaScreen TruHit™ kit (Cat. No 6760627).

Problem	Possible Cause	Effect/Remedy
No signal	Kinase assay reagents	Verify that the distance between the phosphorylated amino acid and the biotin is at least 20 carbons. Protein contains lysine residues near the phosphorylation site / target cysteines for biotinylation using maleimide driven coupling reaction. Cofactor required for optimal enzymatic activity / add the cofactor in the kinase reaction buffer. Peptide substrate not sufficient for efficient phosphorylation by the enzyme / use the full length or a longer domain as substrate. Reagents degradation / perform the assay with fresh enzyme and/or substrate.
High background signal	Detection conditions	Non-phosphorylated peptide is binding non-specifically to the PhosphoSensor Acceptor beads / test different buffer conditions to reduce non-specific binding by varying: • pH (6 to 8) • NaCl from 0 to 400 mM • Tween-20 from 0 to 0.1% and by evaluating Tris-HCl, HEPES or MES as potential buffers.
	Kinase assay reagents	Ensure that the signal to background ratio is established in the presence and absence of staurosporine, since the presence of ATP and/or enzyme in the reaction could alleviate the substrate non-specific binding to the PhosphoSensor Acceptor beads.

Problem	Possible Cause	Effect/Remedy
High background signal	Kinase assay reagents	When using a full-length kinase as a substrate, ensure that it is not activated by phosphorylation. Only use non-activated kinase as a substrate.
High degree of signal variability	Microplates	Warped or distorted microplates / avoid storage of microplate under heavy objects or next to sources of heat. Uneven plate molding. Light penetrating edges of microplate / ensure use of black cover plate during bead incubation. Incubate microplate in dark environment such as inside a drawer or cover microplate entirely with foil or material impenetrable to light. Poorly fitted plate seals inducing evaporation of reaction mixture.
	Instrument	Temperature control problem within the instrument / for the EnVision readers using the 1.07 software version, adjust the internal temperature of the instrument. For other readers, consult the technical service department.
	Assay conditions	Beads are interacting together / Avoid premixing the Acceptor and Donor beads since signal will decrease substantially following 15 minutes of pre-incubation.
	Day-to-day variability	Inappropriate standard operation procedures / ensure that experimental procedures are the same from day to day: 1) prepare the beads in the same area, 2) ensure that incubation times are constant and temperature does not fluctuate in the room.

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