MANUAL

Technology: Luminescence Assay Systems

easylite

For research only. Not for use in diagnostic procedures

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1. Introduction

easylite-KinaseTM is an Adenosine TriPhosphate (ATP) monitoring system based on firefly (Photinus pyralis) luciferase for the evaluation of kinase activity. The system relies on the consumption of ATP during a kinase reaction without the need for labeled substrates, labeled enzymes or phosphospecific antibodies. The amount of ATP left after allowing the enzymatic reaction to proceed for a predetermined period is determined by subsequently adding the easylite-Kinase reagent to the completed reaction. In general, any enzyme or metabolite participating in an enzymatic reaction that can be coupled to the formation or degradation of ATP can be assayed using the luciferase reaction 1.

easylite-Kinase is a sensitive ATP-monitoring single step addition system facilitating a true homogeneous assay format. The kit can be used for continuous as well as for batch processing systems, both in high throughput environments. Also, the system can be used for small scale sample analysis.

The simplicity of the easylite-Kinase assay system in a 96-well microplate is illustrated in Figure 1.

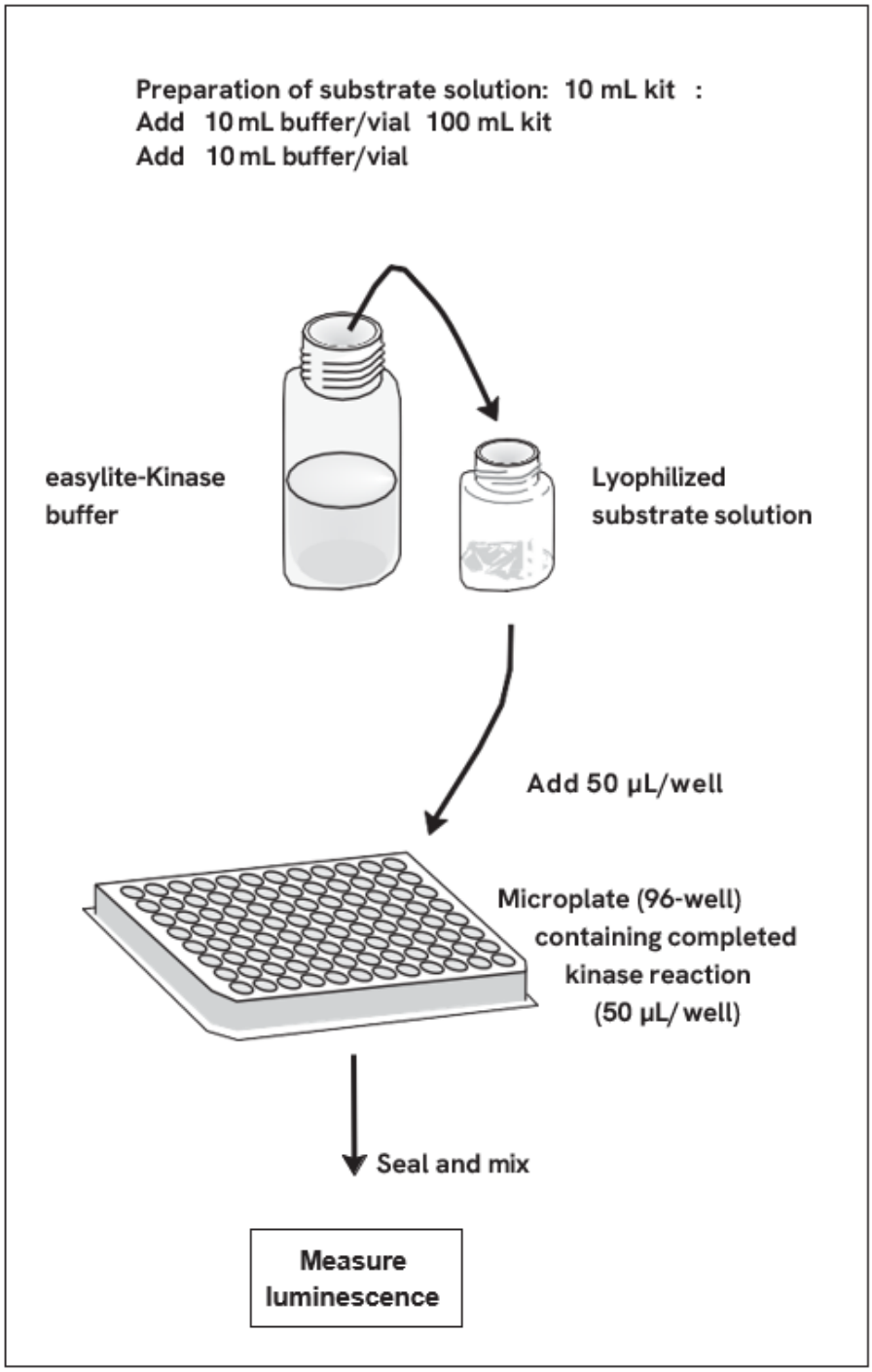
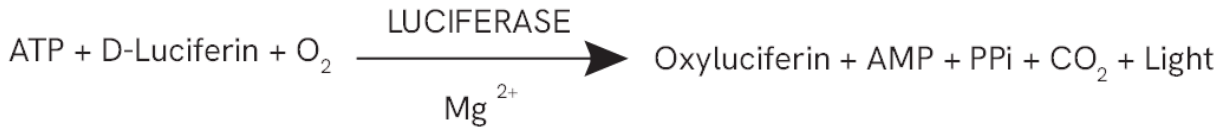


Figure 1: easylite-Kinase assay system.

2 Principle

In a kinase reaction, the □-phosphate moiety of ATP is transferred to the substrate resulting in a phosphorylated substrate and ADP. During this reaction the amount of ATP in the kinase reaction solution declines. After completion of the kinase reaction the remaining ATP can be measured by the addition of the easylite-Kinase reagent. The easylite-Kinase system is based on the production of light caused by the reaction of ATP with firefly luciferase and D-Luciferin. This reaction is illustrated in the following scheme:



The emitted light is proportional to the ATP concentration within certain limits.

The kit has been formulated in such a way that after addition of the easylite-Kinase reagent to the kinase reaction mixture the activity of the kinase virtually stops. The extended half-life of the luminescent reaction allows the resulting luminescence to be measured even after several hours. To show that luminescence correlates linearly with the amount of ATP the following experiment was performed. Serial 2-fold dilutions of ATP in 50 µL assay buffer (40 mM Tris-HCl (pH 7.5); 20 mM MgCl2; 0.1% BSA) were made in a black 96-well OptiPlate™.

Next, 50 µL of easylite-Kinase reagent was added to the wells, the plate sealed with TopSeal™-A and shaken for 1 minute at 700 rpm using an IKA® MTS4 plate shaker. The resulting luminescence was measured on a TopCount® NXT at 22 °C, after 5 minutes count delay and expressed as counts per second (CPS). The result (mean ± S.D. of 4 replicates) presented in Figure 2 shows that there is a linear correlation between the amount of ATP per well and luminescence.

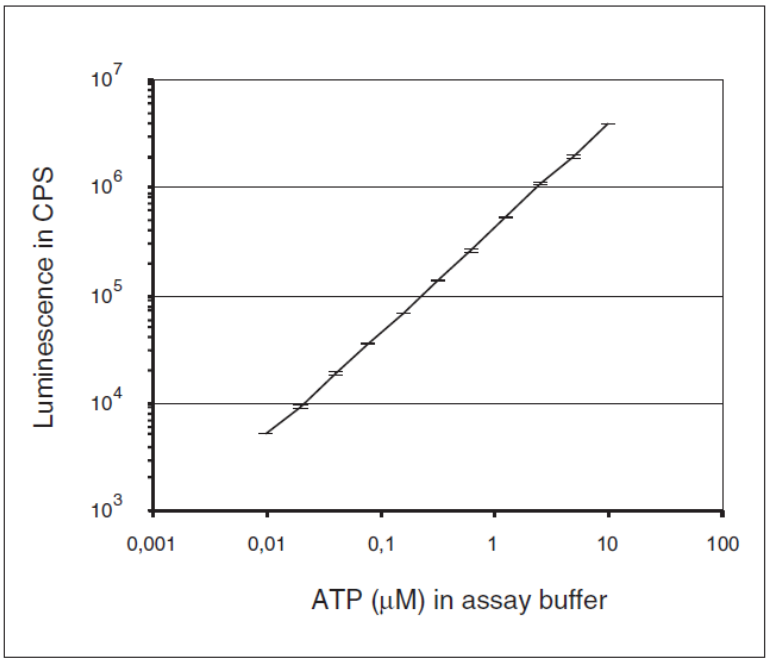


Figure 2: Correlation between luminescence and amount of ATP.

To show the extended half-life of the luminescent reaction and the ability of the easylite-Kinase reagent to virtually stop the kinase activity the following experiment was performed. To a black 96-well OptiPlate was added per well: 50 µL of a kinase reaction buffer (40 mM Tris- HCl (pH 7.5); 20 mM MgCl2; 0.1% BSA) containing 1 µM ATP, 0.25 units of protein kinase A (PKA) and 5 µM Kemptide. Also, to other wells 50 µL of this complete reaction buffer was added with the PKA substrate Kemptide omitted. The plate was incubated for 20 minutes at 20 °C to allow the kinase reactions to proceed. Thereafter 50 µL of the easylite-Kinase reagent was added to the wells and the plate sealed with TopSeal-A. Next, the plate was shaken for 1 minute at 700 rpm using an IKA MTS4 plate shaker. The resulting luminescence was measured on a TopCount NXT at 22 °C. The plate was re-measured several times to monitor the decay of the luminescence. The result (mean ± S.D. of 6 replica- tes) presented in Figure 3 shows that the luminescence decay rate for both reactions are the same indicating that the PKA activity is virtually stopped. The half-life of the luminescence extends to 5 hours.

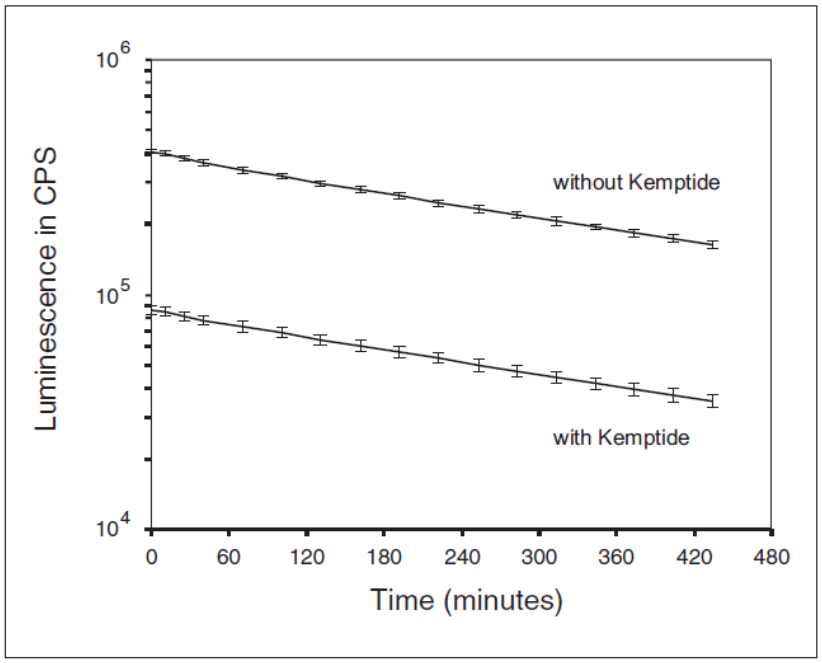


Figure 3: Luminescence decay

DMSO is often used to dissolve screening compounds. The effect of DMSO on the luminescence was investigated in the following experiment. A two times concentrated DMSO dilution series was made in assay buffer (40 mM Tris-HCl (pH 7.5); 20 mM MgCl2; 0.1% BSA). These dilution series, 25 µL per well in triplicate, were added to a black 96-well OptiPlate. To these wells was also added 25 µL of a 2 µM ATP solution in assay buffer and the contents of the wells mixed resulting in a DMSO concentration series with 1 µM ATP, 50 µL per well. Next, 50 µL of easylite-Kinase reagent was added.

The plate was sealed with TopSeal-A and subsequently shaken for 1 minute at 700 rpm. The resulting luminescence was measured using a TopCount NXT. The result (mean ± S.D.) presented in Figure 4 shows that DMSO up to 4% v/v has hardly any effect on the light-output. At higher DMSO concentrations the light- output gradually decreases and the half-life increases. This means that the kit can be used with a DMSO concentration in the assay buffer up to 20%.

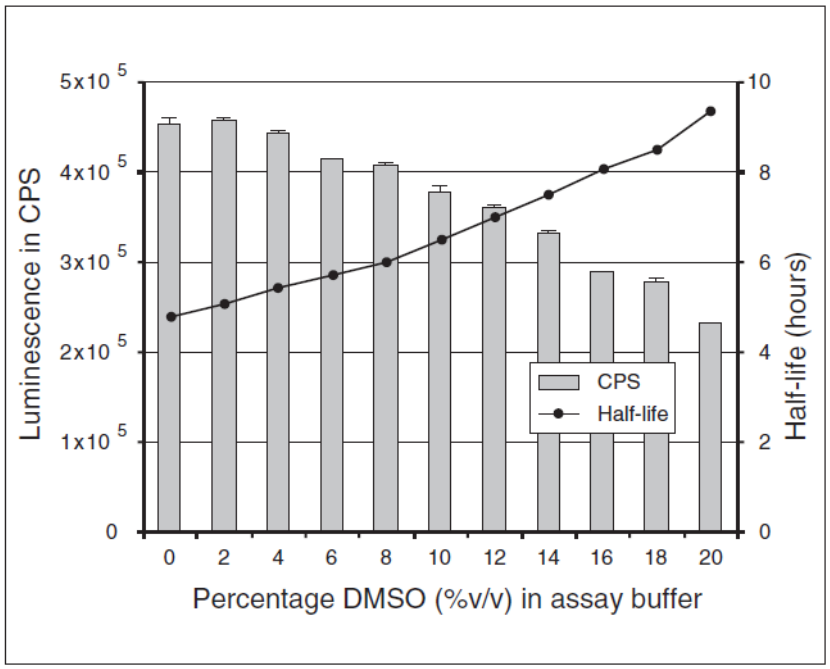


Figure 4: Effect DMSO concentration on light-output and signal stability

3 Advantage of easylite-Kinase

- Homogeneous, simple and fast
  - only one reagent addition step, no separation steps
  - no luminescence signal stabilization time required
- Suitable for both 96- and 384-well microplates
- Linear correlation between ATP concentration (up to 10 µM) and luminescence
- High light-output

kit can also be used with less sensitive luminescence readers like multi-label readers

- Designed for continuous and batch processing systems

extended half-life of the luminescent reaction

4 Contents and storage of easylite-Kinase kit

6066746 - easylite-Kinase 10 mL kit

Each assay kit contains the following components:

1. 1 x 10 mL reconstitution buffer
2. 1 vial (10 mL) of substrate solution (lyophilized)

Using the recommended assay volumes of 50 µL for 96-well microplates and 10 µL for 384-well microplates this kit is sufficient for 200 and 1,000 assays respectively.

6066741 - easylite-Kinase 100 mL kit

Each assay kit contains the following components:

1. 1 x 100 mL reconstitution buffer
2. 10 vials (10 mL) of substrate solution (lyophilized)

Using the recommended assay volumes of 50 µL for 96-well microplates and 10 µL for 384-well microplates this kit is sufficient for 2,000 and 10,000 assays respectively.

Storage conditions:

Upon arrival, store kit at +2 to +8 °C.

5 Handling

Care should be taken during handling of the kit components, such as opening vials and bottles, to ensure that the contents of these are not contaminated with ATP. Such contamination will cause high background levels. In handling the kit, the skin of the fingers is a very potent source of ATP-contamination, therefore the use of clean gloves is strongly recommended. Use also ATP-free dispensing materials.

6 Stability

Reconstituted easylite-Kinase is stable (approximately 10% loss of activity) for 8 hours at 20 °C. At 4 °C storage the loss in activity is less than 5% after 24 hours. Freshly prepared reagents can be aliquoted and stored at - 80 °C for one month.

7 Mixing

Mixing of the easylite-Kinase reagent with the contents of the wells is important. Improper mixing results in lower signals, higher variation and reduced inhibition of kinases.

8 Instrumentation and materials required

1. Microplate luminometer such as the Revvity TopCount, VICTOR® NivoTM Multimode Microplate Reader, EnVision® 2105 Multimode Plate Reader, or EnSight® Multimode Microplate Reader is required. CCD camera systems suitable for microplate analysis, like Revvity's ViewLuxTM, can also be used for high throughput applications.

2. White or black 96- or 384-well microplates.

3. Pipette (multichannel) or automated pipetting device.

4. Microplate shaker suitable for efficient mixing of the contents of the wells. Each plate format has its own optimal settings.

5. ATP-free dispensing material.

9 Assay procedure

General procedure for 96-well microplate (for 384-well microplates the numbers are shown in brackets).

1. Equilibrate the substrate vial and the buffer solution at room temperature before reconstitution. A water bath set at 20 - 22 °C can be used for this.

2. Reconstitute the lyophilized substrate solution by adding the appropriate volume of buffer to the substrate vial. Mix the contents of the vial by inversion and leave the solution to stand for 5 minutes. This should result in a clear homogeneous solution.

3. For 96 (384)-well microplates add 50 µL (10 µL) of the reconstituted reagent to each well containing 50 µL (10 µL) of the completed kinase reaction. Ensure that the microplate is equilibrated to room temperature (20 - 22 °C) before adding the reagent.

4. Shake the 96 (384)-well microplate for 1 minute at 700 (1,100) rpm using an orbital microplate shaker with an orbit diameter of 3 mm or other suitable microplate shaker.

5. Measure Luminescence

   If needed, dark adapt white plates for 10 minutes to reduce plate phosphorescence. Often the luminescence is much higher than the plate phosphorescence reducing the need for dark adaptation of the plate. Black plates show minimal plate phosphorescence and therefore there is no need for dark adaptation. Because there is no need to stabilize the luminescent signal, the luminescence can be measured directly after shaking the plate.

   The easylite-Kinase general assay procedure for 96- well format is outlined in Figure 5.

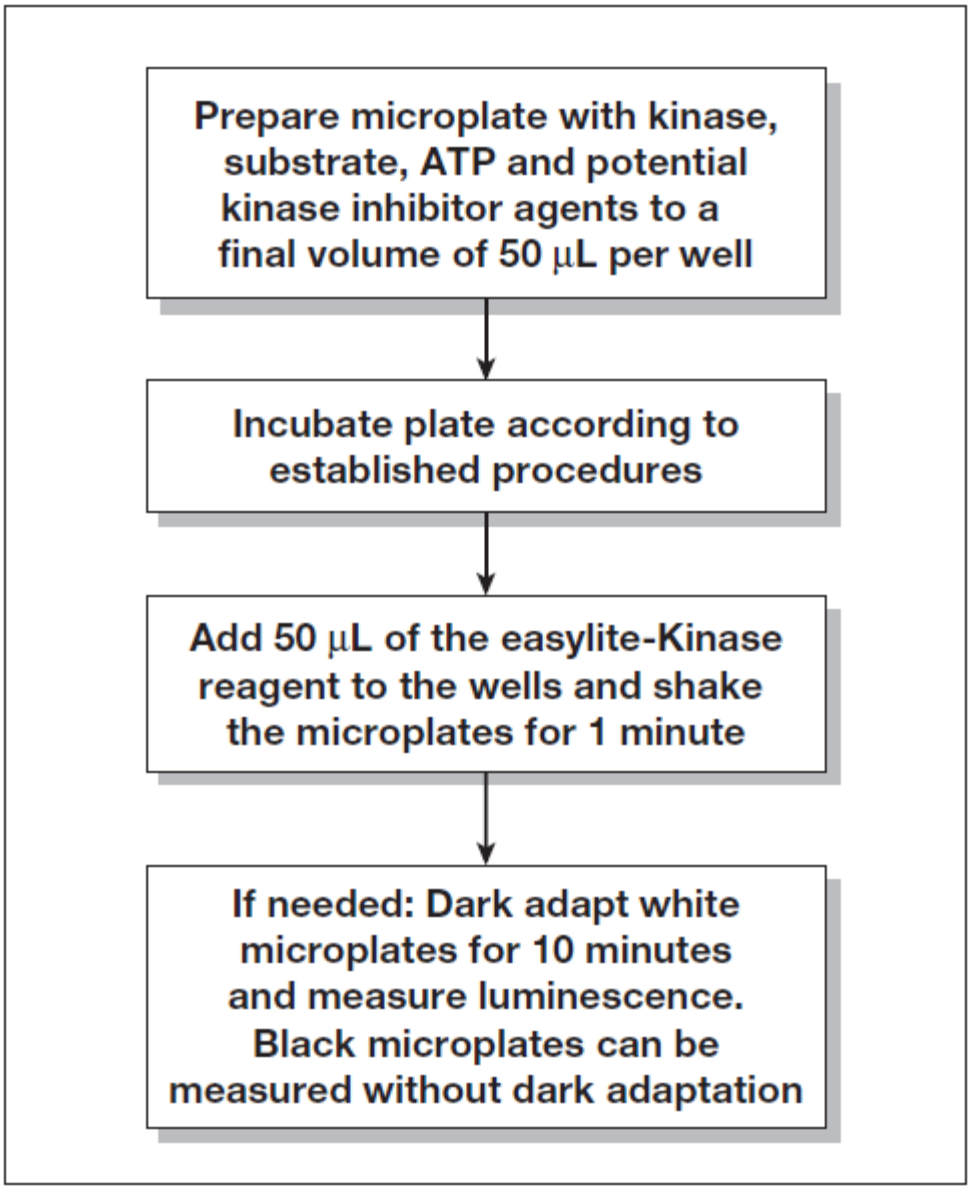


Figure 5: The easylite-Kinase assay flow chart (96- well)

Note: *Please realize that ATP is everywhere. ATP is the universal energy carrier in nature; both eukaryotes and prokaryotes utilize the molecule for energy storage and transfer. As a result, ATP is abundantly present both in microbial, animal or plant cells and also as free ATP. ATP is fairly heat-stable so mere autoclaving is not always sufficient for complete reduction. Therefore, it is important that direct contact of reagents and hand or fingertips is avoided. Open vials carefully and do not touch the mouth of the bottle. Be careful removing the rubber stopper from the vials. Use ATP-free pipette tips. Handle microplates carefully and use lids to avoid contamination.*

10 Guidelines to develop a kinase assay

In order to obtain the best performance using easylite- Kinase, titrations need to be performed to determine optimal ATP and kinase substrate concentrations. These values are then in turn used to determine the optimal amount of kinase enzyme via a kinase titration.

1. **ATP titration.**
   Use an excess of the kinase substrate and as much as practically possible of the kinase enzyme. Do the same titration without the kinase substrate. Let the reactions proceed to consume as much ATP as possible. This might be e.g. 20 minutes for strongly active kinases to several hours for weakly active ones. The optimal ATP concentration is the con- centration resulting in the largest relative change in luminescence between the two ATP titrations. This is best seen when plotting luminescence and ATP concentration on a log-log scale.

2. **Substrate titration.**
   Use the optimal ATP concentration previously determined from the ATP titration. The same amount of kinase enzyme and the same kinase reaction time should be used as in the ATP titration. The optimal substrate concentration is the minimal concentration that results in the largest change in luminescence.

3. **Kinase enzyme titration.**
   Use the previously determined ATP and substrate concentrations and use the same reaction time as in the previous titrations. Determine the amount of kinase enzyme that gives the highest decrease in luminescence in the linear part of the curve. This concentration should be used in subsequent IC_{50} determinations and compound screenings.

4. **Titration of a known inhibitor.**
   Use the optimized assay conditions to prepare a dose response curve of a known kinase inhibitor. The IC_{50} value should be in the same range as determined with more conventional methods.

An example of this optimization is given for Protein Kinase A (PKA) using Kemptide as the substrate.

Materials:

Assay buffer:

- 40 mM Tris[HCl] pH 7.5;
- 20 mM $MgCl_2$;
- 0.1% BSA

Substrate: Kemptide, Sigma cat. no. K 1127

Kinase: PKA, Promega cat. no. V5161

Inhibitor: Staurosporine, Sigma cat. no. S-4400

Plates: Optiplate 96-well black, OptiPlate 384-well white, Revvity

Seal: TopSeal-A, Revvity Plate shaker: MTS4, IKA

Reader: TopCount NXT, Revvity, in luminescence mode; data output counts per second (CPS)

Procedure:

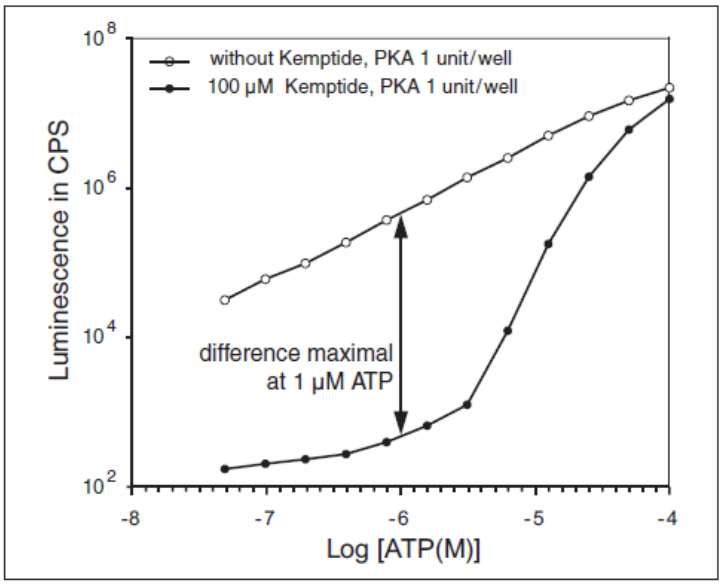
All kinase optimization reactions at a final volume of 50 µL were run at room temperature for 20 minutes. Black 96-well plates were used to prevent the TopCount NXT from saturation. To the completed kinase reactions, 50 µL per well of the easylite-Kinase reagent was added. Thereafter the plates were sealed and subsequently shaken for 1 minute. Luminescence was measured on TopCount NXT after 5 minutes count delay. Curve fitting was performed using GraphPad Prism® sigmoidal dose- response (variable slope) software. For the ATP, Kemptide and PKA titrations, CPS values represent the mean of duplicates ± S.D.. For the staurosporine titration CPS values represent the mean of four replicates ± S.D. for the 96-well plate.

To show that 384-well plates can also be used, the same staurosporine titration experiment was performed using a 384-well plate. The final volumes of the kinase reactions were 10 µL per well. After 20 minutes incubation at room temperature, 10 µL of the easylite- Kinase reagent was added to the wells. The plate was sealed and shaken for 1 minute at 1,100 rpm. Thereafter luminescence was measured on TopCount NXT after 5 minutes count delay. The CPS values represent the mean of triplicates ± S.D. in this case.

Results:

Results of the kinase assay optimization steps 1 to 4 of the example.

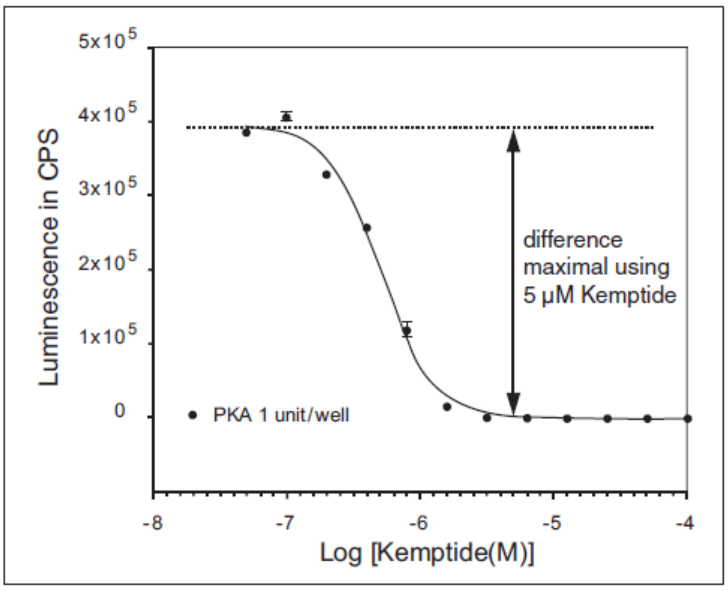
1. ATP titration.



The result is presented in Figure 6. The optimal ATP concentration was found to be 1 µM.

Figure 6: ATP titration

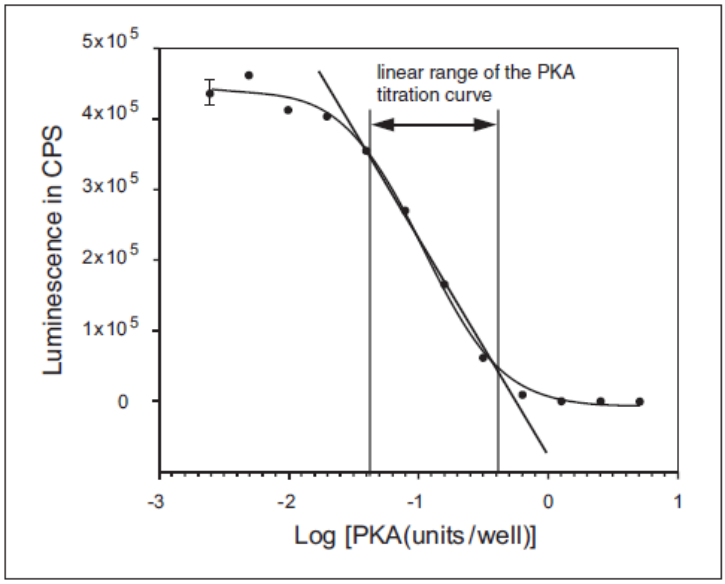
2. Kemptide titration.



Using the optimal ATP concentration the result is presented in Figure 7. From this figure it was determined that the optimal Kemptide concentration was 5 µM.

Figure 7: Kemptide titration

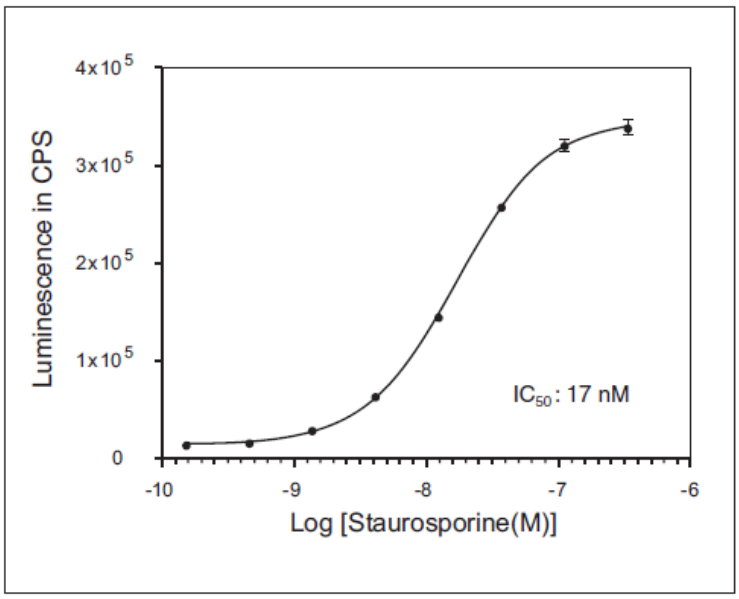
3. PKA titration.



Using the predetermined ATP and Kemptide concentrations the optimal PKA amount was determined. From the data of Figure 8 the optimal PKA amount was determined to be 0.4 units per well.

Figure 8: PKA titration

4. Dose response curve using staurosporine



Using the predetermined optimal ATP, Kemptide and PKA amounts a staurosporine titration was performed in a black 96-well plate. The result is shown in Figure 9.

Figure 9: Staurosporine titration In black 96-well microplate

The same titration was carried out using a white 384-well plate and the result is shown in Figure 10. The concentrations of ATP and Kemptide were the same as with the 96-well plate. The amount of PKA was reduced from 0.4 units per well to 0.08 unit per well. Total volume of the complete kinase reaction mix was 10 µL. The IC50 values were calculated for both plate formats and turned out to be in good agreement between the two plate formats, 17 nM (96-well) and 13 nM (384-well). These values compare well with published IC50 data using a conventional method (8.2 nM)[2].

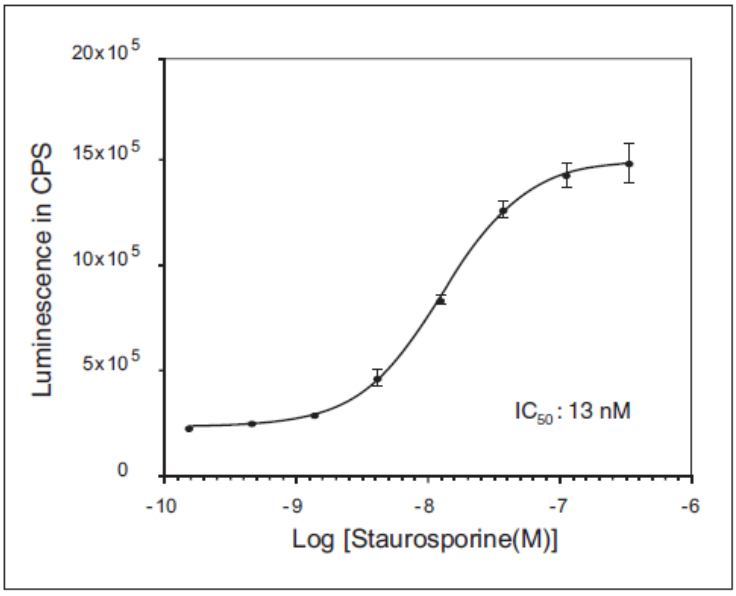


Figure 10: Staurosporine titration in white 384-well microplate

11 Recommendation for use

Whether to choose white or black microplates depends very much on the luminescence reader used. With some readers the detectors of the instrument may saturate when using white plates and high ATP concentrations due to the very high light-output of the easylite-Kinase reaction. This can occur especially with 96-well white plates. In this case black plates are recommended.

Optimize liquid handling procedures to attain optimal mixing of the easylite-Kinase reagent and the kinase reaction mixture.

Optimal room and instrument temperature is 22 °C. Allow plates to adapt to room temperature in cases where the kinase reaction was run at elevated temperatures prior to the addition of the reagent. An adaptation time of 30 minutes is usually sufficient.

When handling the plates prior to measurement, work under subdued light conditions and avoid direct sunlight or bright fluorescent light. Bright light may cause plate phosphorescence resulting in higher background levels. Phosphorescence has a half-life of several minutes.

If more than one vial of substrate is reconstituted for the assay, we recommend these solutions be combined before addition to the plates.

12 Ordering information

Easylite-Kinase	Reorder No
10 mL easylite-Kinase assay kit	6066746
100 mL easylite-Kinase assay kit	6066741

For further information on luminescence readers, microplates, seals and luminescence applications please contact your local Revvity representative or visit our website: http:// www.revvity.com

13 References

1. Lundin A. 1982 Applications of Firefly Luciferase. In: Luminescent Assays: Perspectives in Endocrinology and Clinical Chemistry, edited by M. Serio and M. Pazzagli, Raven Press, New York © .

2. Tamaoki T., 1991 Use and Specificity of Stauro- sporine, UCN-01, and Calphostin C as Protein Kinase Inhibitors. *Methods in Enzymology* **201**, 340-347.

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