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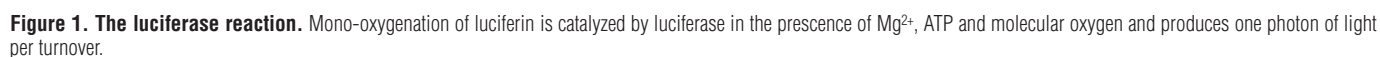
*The Kinase-Glo™ Luminescent Kinase Assay is a homogeneous method for measuring kinase activity by quantifying a decrease in ATP levels following kinase activity. The assay can work with virtually any kinase and substrate combination. The assay is performed in a single well of a standard multiwell plate by adding an equal volume of Kinase-Glo™ Reagent and reading luminescence. The assay produces excellent Z' values, easily detects known kinase inhibitors, and provides accurate IC<sub>50</sub> values.*

### Assay Principle and Protocol

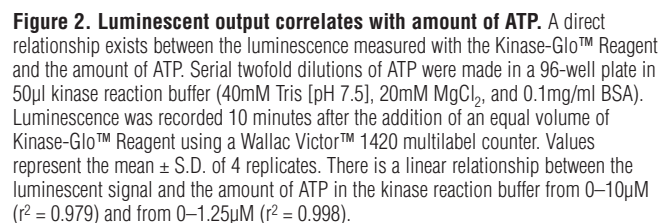
Kinases are enzymes that catalyze the transfer of a phosphate group from ATP to substrate. The depletion of ATP can be monitored in a highly sensitive manner through the use of Kinase-Glo™ Reagent, which uses luciferin, oxygen and ATP as substrates in a reaction that produces oxyluciferin and light (Figure 1). The luminescent signal is correlated with the amount of ATP present and inversely correlated with the amount of kinase activity.

The Kinase-Glo™ Reagent relies on the properties of a proprietary thermostable luciferase (UltraGlow™ Recombinant Luciferase) that is formulated to generate a stable “glow-type” luminescent signal and improve performance across a wide range of assay conditions. Kinase-Glo™ Reagent is prepared by combining Kinase-Glo™ Buffer with lyophilized Kinase-Glo™ Substrate. The Kinase-Glo™ Reagent can be used immediately or stored at –20°C in single-use aliquots for several months.

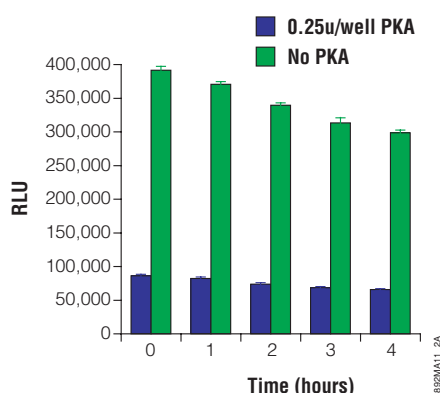
The protocol involves making a single addition of an equal volume of Kinase-Glo™ Reagent to a completed kinase reaction that contains ATP, purified kinase and substrate, mixing the plate, and reading luminescence.



The Kinase-Glo™ Reagent produces luminescence that is directly proportional to the amount of ATP present in the kinase reaction, and the luminescence is linear from 0 to 10µM as shown in Figure 2. This luminescence signal is also very stable, showing a 25% drop in output after 4 hours at room temperature, regardless of the amount of ATP present (Figure 3). This allows use of luminometers without reagent injectors, and plates can be stacked prior to reading.

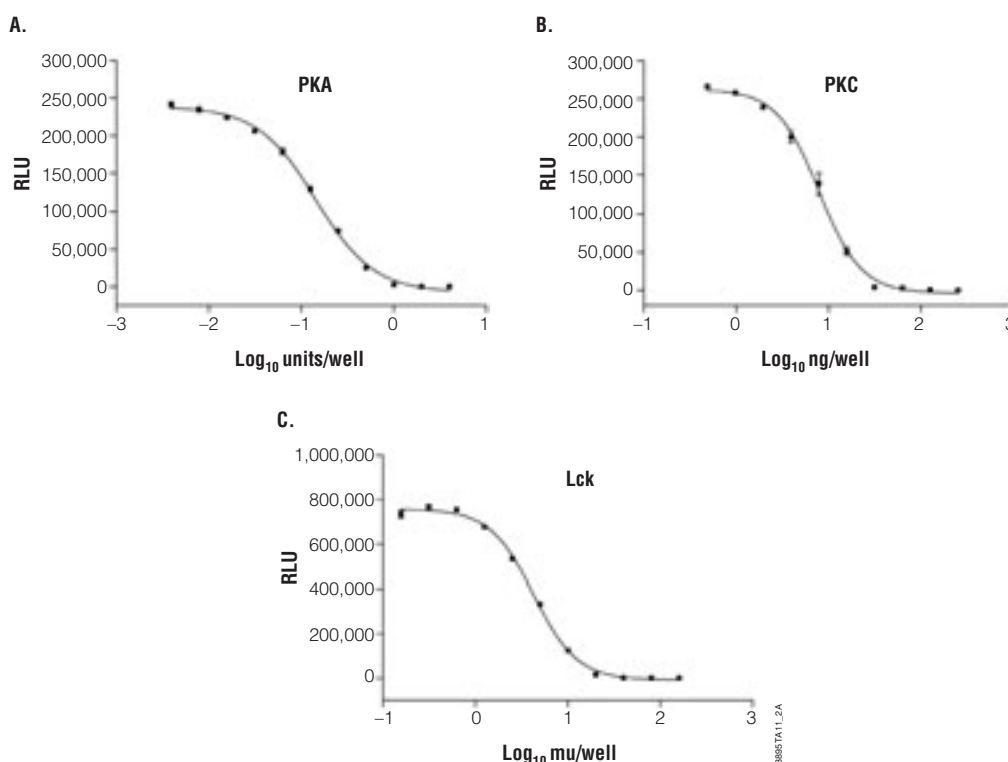


The real test of the Kinase-Glo™ Reagent, however, is the ability to produce luminescence that is inversely proportional to the amount of kinase activity. In order to maximize the change in luminescence observed with kinase activity, the optimal amount of ATP and kinase substrate should be determined experimentally. This involves titrating the amount of ATP and the amount of kinase substrate in the kinase reactions. The use of the Kinase-Glo™ Reagent is the same, regardless of the titration being performed.



**Figure 3. Extended luminescent half-life.** Signal stability of Kinase-Glo™ Reagent in a solid white 96-well plate (n = 32). Kinase reactions were performed with 0.25 units/well PKA (blue bars) or no PKA (green bars). The kinase was diluted in 50μl kinase reaction buffer (40mM Tris [pH 7.5], 20mM MgCl<sub>2</sub>, and 0.1mg/ml BSA), containing 5μM substrate (Cat.# V5601) and 1μM ATP. The kinase reaction was run for 20 minutes at room temperature. Luminescence was recorded on a Wallac Victor™ 1420 multilabel counter 10 minutes (time zero) after the addition of an equal volume of Kinase-Glo™ Reagent. Luminescence was measured at the indicated intervals. The signal decreased less than 25% in four hours.

Once the optimal concentrations of ATP and kinase substrate are determined, a large change in luminescence can be observed with increasing kinase activity as shown in Figure 4. The examples demonstrate dynamic ranges from 500- to 1,000-fold using concentrations of ATP from 1–3μM, with both a serine/threonine kinase and a tyrosine kinase. PKC can be titrated satisfactorily even in the presence of phosphatidyl serine and diacylglycerol. The assay also works well with protein substrates, such as myelin basic protein, and lipid substrates such as phosphatidyl inositol (data not shown). The specific shape of the dose response curve will vary depending on the relative amounts of ATP and kinase substrate. As the two become more equal the slope will decrease as will the dynamic range. The experimental conditions are easily changed and can be optimized for nearly any parameter the user defines.



**Figure 4. Amount of kinase activity is inversely correlated with luminescent output.** An inverse relationship exists between luminescence measured with the Kinase-Glo™ Reagent and the amount of kinase activity. Serial twofold dilutions of kinase were made in solid white 96-well plates in 50μl kinase reaction buffer under the conditions described below. Luminescence was recorded on a Wallac Victor™ 1420 multilabel counter 10 minutes after adding an equal volume of Kinase-Glo™ Reagent. Curve fitting was performed using GraphPad Prism® sigmoidal dose-response (variable slope) software. **Panel A.** PKA (Cat.# V5161) was diluted in 50μl kinase reaction buffer (40mM Tris [pH 7.5], 20mM MgCl<sub>2</sub>, and 0.1mg/ml BSA), containing 5μM substrate (Cat.# V5601) and 1μM ATP. The kinase reaction was run for 20 minutes at room temperature. Values represent the mean ± SD of 8 replicates. R<sup>2</sup> = 0.99; EC<sub>50</sub> = 0.15u/well. **Panel B.** PKC (Cat.# V5261) was diluted in 50μl kinase reaction buffer (20mM Tris [pH 7.5], 10mM MgCl<sub>2</sub>, 0.1mg/ml BSA, 250μM EGTA, 400μM CaCl<sub>2</sub>, 0.32mg/ml phosphatidylserine, and 0.032mg/ml diacylglycerol), containing 10μM substrate (Neurogranin<sub>[28-43]</sub> (PKC) Peptide Substrate; Cat.# V5611) and 1μM ATP. The kinase reaction was run for 90 minutes at room temperature. Values represent the mean ± SD of 2 replicates. R<sup>2</sup> = 0.99; EC<sub>50</sub> = 7.9ng/well. **Panel C.** Lck (Upstate Biochemicals, Cat.# 14-442) was diluted in 50μl kinase reaction buffer (8mM imidazole hydrochloride [pH 7.3], 8mM β-glycerophosphate, 200μM EGTA, 20mM MgCl<sub>2</sub>, 1mM MnCl<sub>2</sub>, and 0.1mg/ml BSA), containing 250μM PTK peptide 2 (Promega Part# V288A) and 3μM ATP. The kinase reaction was run for 60 minutes at room temperature. Values represent the mean ± SD of 2 replicates. R<sup>2</sup> = 0.99; EC<sub>50</sub> = 4.3μu/well.

# Kinase-Glo™ Luminescent Kinase Assay... continued

## Application of the Kinase-Glo™ Assay in HTS

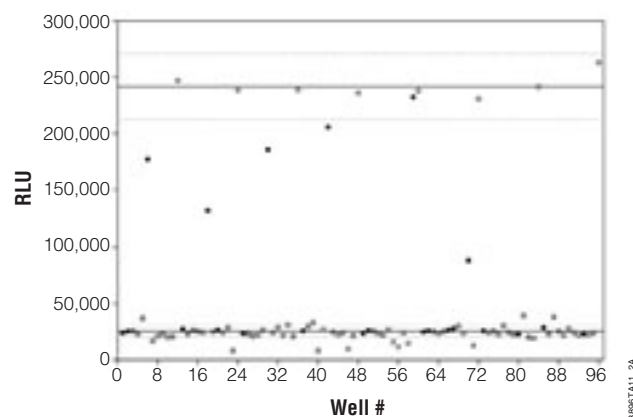
To determine the usefulness of the Kinase-Glo™ Assay<sup>(a)</sup> for HTS, we used PKA as a model kinase to examine  $Z'$  factors (1), to detect known inhibitors and to determine  $IC_{50}$  values.

Figure 5 demonstrates that a  $Z' > 0.8$  can be achieved in both a 96- and 384-well plate format. The amount of kinase used in these examples results in luminescence values at the low end of the linear portion of the kinase titration curves (Figure 4). Selecting an amount of kinase that is in the linear portion of the titration curve results in the best sensitivity to inhibitory compounds. Because of the very good %CV obtained in the Kinase-Glo™ Assay, smaller changes in luminescence can still be useful in screening.

We used the Kinase-Glo™ Assay to screen 72 compounds that included a few known PKA inhibitors. Any kinase inhibitors are expected to increase luminescence up to the maximum in the control wells (no kinase substrate). The results, shown in Figure 6, indicate that 6 compounds showed  $> 20\%$  inhibition. Four were known PKA inhibitors, and two were not previously described to inhibit PKA (Table 1).

To determine the number of false-negatives, the entire LOPAC library (640 compounds) was screened with Kinase-Glo™ Reagent and ATP. None of the compounds showed any inhibition of luminescence. The unique combination of UltraGlow™ luciferase and proprietary buffer formulation is responsible for this low compound interference.

Beyond detecting inhibitors, an HTS kinase assay must be able to assess the potency of a compound by providing accurate  $IC_{50}$  values. Figure 7 shows the results of  $IC_{50}$  determinations of two known PKA inhibitors. In both cases the  $IC_{50}$  value determined with the Kinase-Glo™ Assay matched very closely with

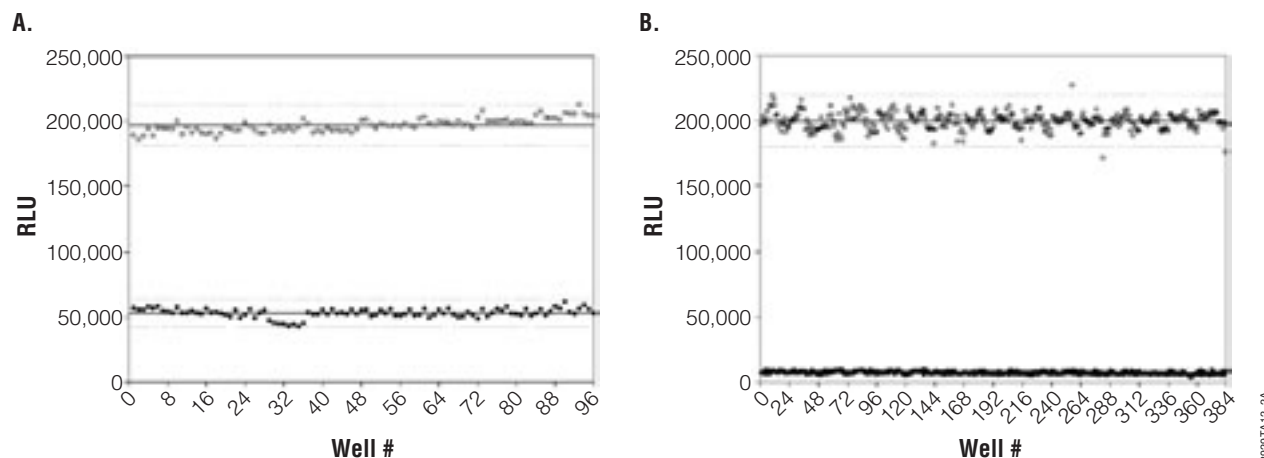


**Figure 6. Easy identification of PKA inhibitors.** Assay results from a 72-compound screen of plate 6 from the LOPAC library (Sigma-RBI). Compounds were screened at 10 $\mu$ M each in a solid white, flat-bottom 96-well plate by adding 5 $\mu$ l of compound in 10% DMSO to each well. Final concentration of DMSO was 1%. Reaction conditions were the same as described in Figure 4A using 0.5 units/well PKA. Solid circles are wells without compound, and open circles are wells without kinase. Solid lines indicate means, and dotted lines indicate  $\pm 3$  S.D. of these populations. Squares represent wells containing library compounds. Solid squares were scored as hits and are identified in Table 1.

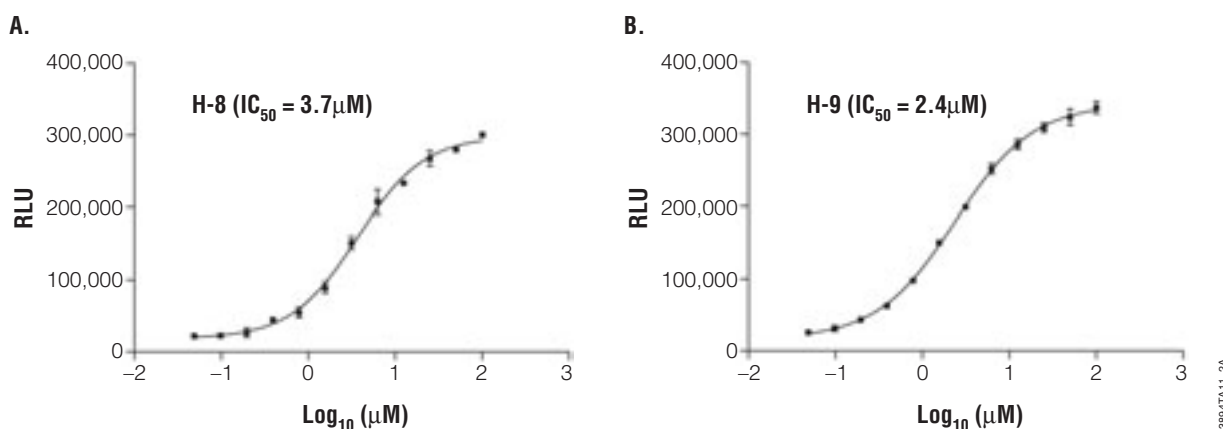
**Table 1. Compounds Scored as "Hits" in the 72-Compound Screen.**

Well#	Compound	Inhibition
6	HA-1004	70.2%
18	H-7	49.6%
30	H-8	74.1%
42	H-9	83.3%
59	U-73122	95.7%
70	GW5074	29.1%

values published in the literature using different kinase assays. Inhibitor titrations with PKI and staurosporine produced  $IC_{50}$  values of 2.5nM and 10nM, respectively (data not shown). These data compare favorably to the literature  $IC_{50}$  values of 3.0nM (PKI, 2) and 18.0nM (staurosporine, 3).



**Figure 5. Excellent  $Z'$  factors.** Results of  $Z'$  analysis in a 96-well solid white, flat-bottom plate and 384-well plate. **Panel A.** The assay was performed as described in the legend of Figure 4A with 0.25 units/well PKA (solid circles) or without PKA (open circles). **Panel B.** The 384-well plate assay was performed using 0.05 units/well PKA (solid circles) or without PKA (open circles). Final volumes for the 96- and 384-well plate assays were 100 $\mu$ l and 20 $\mu$ l, respectively. Solid lines indicate the mean, and the dotted lines indicate  $\pm 3$  SD.  $Z'$  values were 0.83 for the 96-well plate assay and 0.84 for the 384-well plate assay.



**Figure 7. Accurate IC<sub>50</sub> determinations.** PKA inhibitor titrations were performed in solid white, flat-bottom 96-well plates in a total volume of 50 μl. Assay conditions were the same as described in Figure 4A using 0.5 units/well PKA and the indicated amount of inhibitor. Data points are the average of 2 determinations and error bars are ± SD. IC<sub>50</sub> results compare favorably to the published literature IC<sub>50</sub> values of 1.2 μM for H-8 and 1.9 μM for H-9. Curve fitting was performed using GraphPad Prism® sigmoidal dose-response (variable slope) software.

## Summary

The Kinase-Glo™ Luminescent Kinase Assay provides an HTS solution for users who do not want to use labeled components or radioactivity in their assays. It is a homogeneous method of determining kinase activity. The extended half-life of the signal eliminates the need for reagent injectors and provides for batch-mode processing of plates. In addition, the unique combination of UltraGlow™ luciferase and proprietary buffer formulation results in luminescence that is much less susceptible to interference from library compounds than other luciferase-based ATP detection reagents. The assay procedure involves adding a single reagent directly to a completed kinase reaction and generates a luminescent signal inversely proportional to the amount of kinase activity. Since all kinases use ATP, this assay has the potential to be used with a large variety of kinases. The assay has been tested with both serine/threonine and tyrosine kinases, resulting in Z' values > 0.8 in either 96- or 384-well plate formats. The assay also produces IC<sub>50</sub> values for known inhibitors that are comparable to what has been reported in the literature, and known kinase inhibitors were easily selected from other compounds.

## References

1. Zhang, J. *et al.* (1999) *J. Biomol. Screening* **4**, 67–73.
2. Walsh, D.A. and Glass, D.B. (1991) *Methods in Enzymology* **201**, 304–16.
3. Hidaka, H., Watanabe, M. and Kobayashi, K. (1991) *Methods in Enzymology* **201**, 328–39.

## Protocol

- ◆ Kinase-Glo™ Luminescent Kinase Assay Technical Bulletin #TB318, Promega Corporation.  
([www.promega.com/tbs/tb318/tb318.htm](http://www.promega.com/tbs/tb318/tb318.htm))



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## Ordering Information

Product	Size	Cat.#
Kinase-Glo™ Luminescent Kinase Assay	10ml	V6711
	10 × 10ml	V6712
	100ml	V6713
	10 × 100ml	V6714

(a) Patent Pending.

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