# SCREEN FOR KINASE MODULATORS IN A HIGH-THROUGHPUT FORMAT WITH PROMEGA KINASE REAGENTS

MICHAEL CURTIN, PROMEGA CORPORATION

## The Role of Kinases in Drug Discovery and Development

Kinases are enzymes capable of transferring the γ-phosphate group from high-energy molecules, such as ATP or GTP, to specific target molecules such as proteins, lipids, sugars and alcohols. Protein kinases function primarily as components of signaling pathways that modulate cellular activities such as gene transcription or ion channel activity. Protein kinases play critical roles in a variety of cellular functions including cell growth, development, differentiation, membrane transport, and cell death (1,2). Lipid kinases, such as phosphoinositide 3-kinase (PI3K), phosphorylate specific lipid molecules inside the cell and play important roles in cellular events including metabolic regulation, cell proliferation, survival and migration. Abnormalities in signaling pathways can lead to pathological conditions, including many forms of cancer. For this reason, kinases are important targets for both basic research and drug discovery and development.

The development of small molecule kinase inhibitors as new therapeutics has proven successful with the FDA approval of Gleevec® (STI-571) for treating chronic myelogenous leukemia and Iressa® (ZD 1839) for treating lung cancer. Many pharmaceutical companies continue to search for kinase modulators (3).

When performing high-throughput screening (HTS) against purified kinase targets, researchers look for assays that are fast, simple and reliable. Promega offers several assay systems for measuring purified kinase activity that rely on different methods of detection.

To see some of the peer-reviewed publications citing use of the Kinase-Glo® Assays in HTS applications, visit:
**www.promega.com/citations/**

## Screen for Modulators of Kinase Activity Using Proven Bioluminescent Technology

The Promega luminescent Kinase-Glo® Assays have gained widespread acceptance among HTS researchers because of their simple "add and read" format, their low false-positive rate, and their compatibility with virtually any kinase-kinase substrate combination. These homogeneous assays measure the amount of ATP remaining in solution following a kinase reaction (Figure 1). The Kinase-Glo® Assays use a proprietary recombinant luciferase (Ultra-Glo™ Recombinant Luciferase) to monitor changes in ATP levels. The luminescent signal is correlated with the amount of ATP present and inversely correlated with kinase activity. The Kinase-Glo® Assays can be used to measure the activity of kinases with substrates that are prephosphorylated, such as glycogen synthase 3 kinase, or kinases that phosphorylate their substrates on multiple sites such as IKKs. This assay platform also may be used with peptide or lipid substrates.

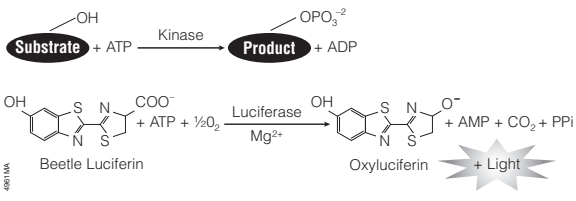


**Figure 1. The Kinase-Glo® Assay reaction.** The kinase reaction is conducted under the appropriate conditions. ATP remaining at the time that the Kinase-Glo® Reagent is added is used as a substrate by Ultra-Glo™ Luciferase to catalyze the mono-oxygenation of luciferin. The luciferase reaction produces one photon of light per turnover. Luminescence is inversely related to kinase activity.

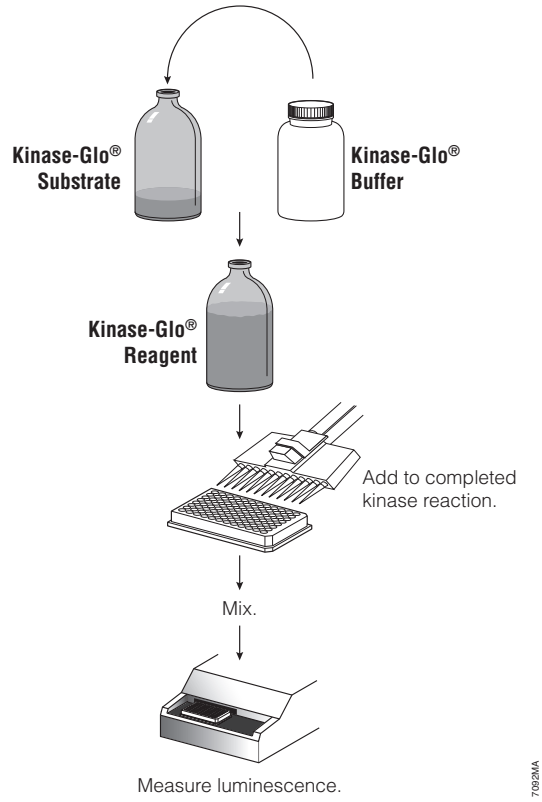


**Figure 2. Schematic illustrating Kinase-Glo® Assay protocol** The "add and read" procedure for the Kinase-Glo® Assays makes them easily amenable to HTS applications.

**Challenge:** Design a robust, universal kinase assay suited for HTS applications.

**Solution:** Promega Kinase-Glo® assays measure the amount of ATP remaining in solution following a kinase reaction. The luminescent signal produced is correlated with the amount of ATP present and inversely correlated with kinase activity. Since no labeled substrate is required, just about any kinase/substrate combination can be used (Figure 3).

The Z´-factor value is a statistical measure that compares the dynamic range of an assay to data variation in order to assess assay quality (4), and a Z´-factor value greater than or equal to 0.5 indicates a robust assay. Kinase-Glo® Assays performed in HTS formats routinely give Z´-factor values of ~0.8 (Figure 4).

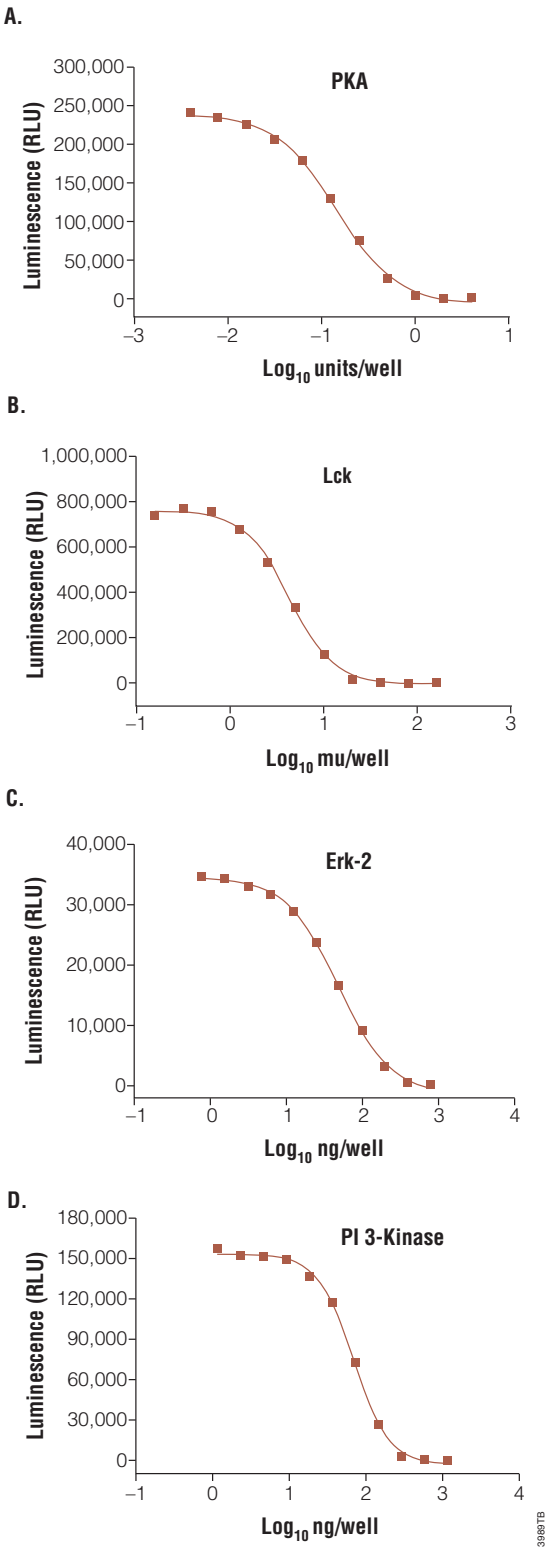


**Figure 3. The Kinase-Glo® Assay can be used with virtually any kinase and kinase/substrate combination.** $EC_{50}$ data from several different kinase/substrate combinations. **Panel A.** PKA enzyme/Kemptide. **Panel B.** Lck kinase/PTK peptide. **Panel C.** Erk-2 kinase/myelin basic protein. **Panel D.** PI3K/phosphotidylinositol.

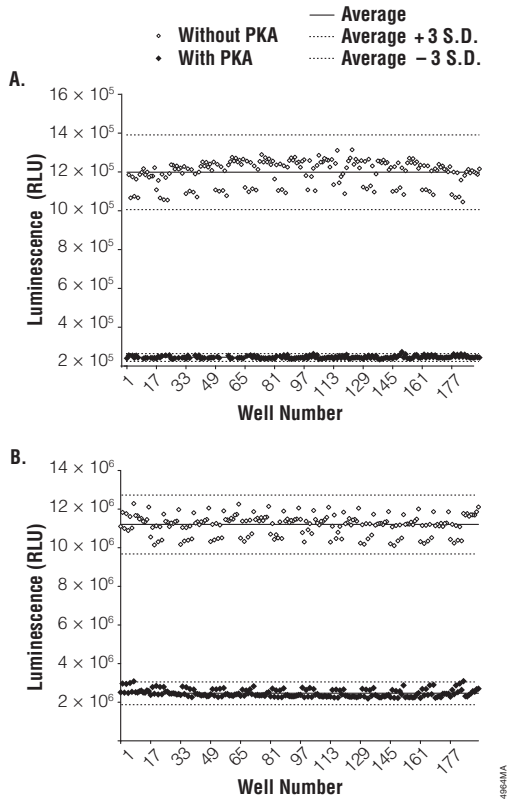


**Figure 4. Determining Z´-factor for Kinase-Glo® Plus Assay run in a 384-well plate. Panel A.** The assay was performed as described in Technical Bulletin #TB372 with 0.2 units/well PKA and 10 µM ATP for five minutes at room temperature (solid symbols) or without PKA (open symbols). **Panel B.** The assay was performed using 0.2 units/well PKA and 100 µM ATP for 30 minutes at room temperature (solid symbols) or without PKA (open symbols). Assays were performed in 384-well plates in a final volume of 20 µl. Solid lines indicate the mean, and the dotted lines indicate ± 3 S.D. Z- factor values were 0.8 for both 10 µM and 100 µM ATP.

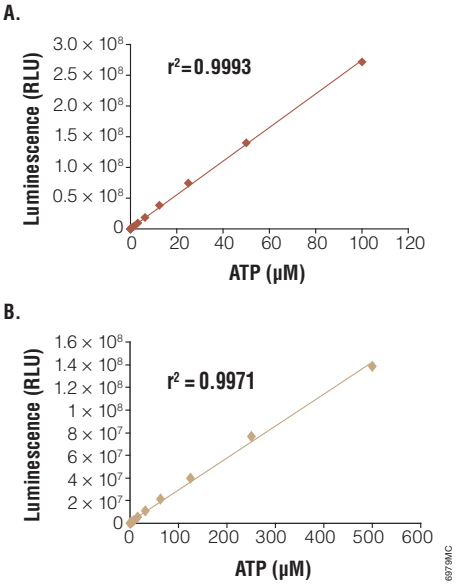


**Figure 5. Luminescence correlates with amount of ATP.** ATP was titrated across a 96-well plate in the kinase reaction buffer, and the appropriate Kinase-Glo® Reagent was added. **Panel A.** Kinase-Glo® Plus Assay, $r^2 = 0.9993$. **Panel B.** Kinase-Glo® Max Assay, $r^2 = 0.9971$.

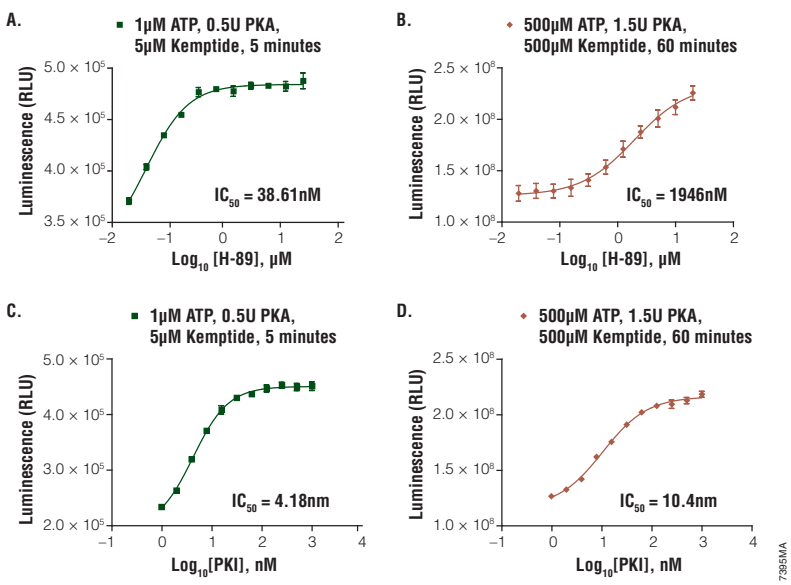


**Figure 6. Determining the $IC_{50}$ for ATP-competitive (H89) and noncompetitive (PKI) inhibitors.** H-89 and PKI titrations were performed as described in technical bulletin #TB372. $IC_{50}$ values obtained here compare favorably to values reported in the literature.

**Challenge:** Develop a luminescent kinase assay that can be used with higher concentrations of ATP (up to 500 µM).

**Solution:** Kinase-Glo® Max and Kinase-Glo® Plus Assays can be used with higher concentrations of ATP. Screening kinases using higher ATP concentrations increases the likelihood of selecting non-ATP binding site inhibitors and has become a recent trend in kinase screening. The Kinase-Glo® Max reagent is formulated to be used with up to 500 µM ATP, and the Kinase-Glo® Plus reagent can be used with up to 100 µM ATP (Figure 5). Inhibitor titrations using the potent, competitive ATP inhibitor, H-89, and noncompetitive ATP inhibitor, PKI, are shown in Figure 6.

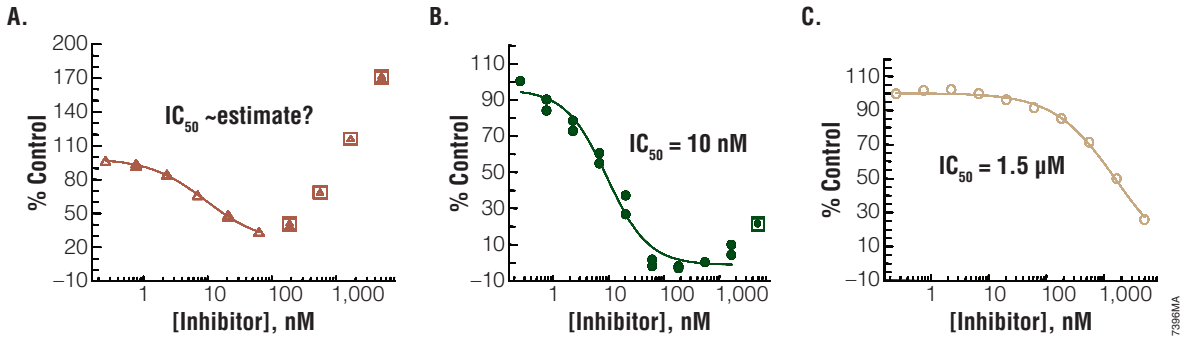


**Figure 7. $IC_{50}$ titrations for Boehringer Ingelheim STK1 kinase inhibitor. Panel A.** Competitor luminescent assay. **Panel B.** Kinase-Glo® Plus Luminescent Kinase Assay. **Panel C.** Inhibitor titration in the absence of kinase was performed using competitor assay to investigate the effect of the inhibitor directly on the assay. An acceptable $IC_{50}$ curve was generated using the Kinase-Glo® Plus Assay, but the $IC_{50}$ of the compound could not be quantified using the competitor assay due to luciferase inhibition ($IC_{50}$ for luciferase 1.5 µM, Panel C.)

**Challenge:** Reduce the number of false-positive hits encountered during kinase screening.

**Solution:** Bioluminescence-based assays are not affected by background interference from sample components like many fluorescence-based assays are. The Kinase-Glo® Assays use the combination of a proprietary recombinant luciferase (Ultra-Glo™ Luciferase) and a specially formulated buffer to monitor the change in ATP levels. Ultra-Glo™ Luciferase is engineered to remain active under a wide range of assay conditions.

**Table 1. PKA Activity as Measured using 96-well SAM²® Biotin Capture Plate.**

| Sample | Substrate | PKA | Substrate + PKA |
|---|---|---|---|
| A | 12.0 | 40.2 | 26,438.5 |
| B | 30.1 | 36.2 | 24,518.1 |
| C | 24.1 | 22.1 | 22.017.8 |
| D | 28.1 | 18.1 | 23,666.7 |
| E | 20.4 | 10.2 | 25,403.9 |
| F | 22.4 | 32.6 | 24,695.9 |
| G | 18.3 | 42.8 | 24,051.2 |
| H | 16.3 | 40.7 | 26,886.9 |
| Average | 21.5 | 30.4 | 24,709.9 |
| S.D. | 6.02 | 12.09 | 1,559.11 |
| %CV | 28.06 | 39.81 | 6.31 |

**Challenge:** Create a tool for performing high-throughput radiolabeled kinase assays.

**Solution:** The SAM²® Biotin Capture Membranes and Plates bind biotinylated molecules based on their affinity for streptavidin. The membrane provides rapid, quantitative substrate binding with minimal nonspecific binding. We performed a protein kinase A (PKA) assay (5) measuring PKA activity in the presence of enzyme only, substrate only and both (5). The radioactivity determined in the absence of the enzyme or in the absence of the substrate represents <0.02% of input counts (Table 1). The range of background counts was extremely low, and the assay was efficient: the full washing procedure took only 5 minutes. The coefficient of variation for enzyme activity did not exceed 8%, indicating highly reproducible results and consistency in the assay performance. When tested with biotin and biotinylated peptides, the binding capacity of SAM²® Plates was linear between 1 and 500 pmol/well and binding was stoichiometric (6). This feature is critical for enzymes such as PTK, whose substrates have high $K_m$ values.

**Challenge:** Design a fluorescent kinase assay that minimizes pipetting steps and yields high Z´-factor values.

**Solution:** The ProFluor® Kinase Assays are fluorescence intensity-based assays that measure kinase activity using purified kinase in a multiwell plate format and involve "add and read" steps only. The user performs a standard kinase reaction with the provided bisamide rhodamine 110 peptide substrate specific for the kinase of interest. The conjugated substrate is nonfluorescent (7). After the kinase reaction is complete, the user adds a termination buffer containing a protease reagent. This simultaneously stops the reaction and removes amino acids specifically from the nonphosphorylated substrate, producing highly fluorescent rhodamine 110. Phosphorylated substrate is resistant to protease digestion and remains nonfluorescent. Thus, fluorescence is inversely correlated with kinase activity. The assays consistently yield excellent Z´-factor values, produce $IC_{50}$ data comparable to published data, and allow batch-mode processing (8).

## Summary

The study of kinases and their role in cellular regulation continues to expand as new kinases are identified as expression products of newly discovered genes. Reagents and assay systems that allow sensitive, accurate and high-throughput analysis of all types of kinases will enhance the characterization of these important cellular components and speed the identification of appropriate therapeutic targets and the development of new and more effective treatments. Promega offers a wide array of kinase assays to help in this endeavor, and we are confident that we have an assay for your particular needs.

## References


1. Hunter, T. (2000) *Cell* **100**, 113–27.
2. van der Geer, P., Hunter, T. and Lindberg, R. (1994) *Ann. Rev. Cell. Biol.* **10**, 251–337.
3. Druker, B. and Lydon, N. (2000) *J. Clin. Invest.* **105**, 3–7.
4. Zhang, J. *et al.* (1999) *J. Biomol. Scr.* **4**, 67–73.
5. Goueli, S. *et al.* (2000) *Promega Notes* **75**, 24–8.
6. Goueli, S., Hsiao, K. and Ruzicka, C. (1997) *Promega Notes* **64**, 2–6.
7. Leytus, S.P., Mehado, L.L. and Mangel, W.F. (1983) *Biochem. J.* **209**, 288–307.
8. Kupcho, K. *et al.* (2003) *Anal. Biochem.* **317**, 210–7.


## Ordering Information

| Product | Size** | Cat.# |
|---|---|---|
| Kinase-Glo® Max Luminescent Kinase Assay | 10 × 100 ml | V6074 |
| Kinase-Glo® Plus Luminescent Kinase Assay | 10 × 100 ml | V3774 |
| Kinase-Glo® Luminescent Kinase Assay | 10 × 100 ml | V6714 |
| ProFluor® PKA Assay | 8 plate | V1241 |
| ProFluor® Src-Family Kinase Assay | 8 plate | V1271 |
| SAM²® Biotin Capture Membrane* | 7.6 × 10.9 cm | V7861 |
| SAM²® 96 Biotin Capture Plate* | 5 × 96-well plate | V7542 |

*For Laboratory Use. **Additional sizes available.
Products may be covered by pending or issued patents or may have certain limitations. Please visit our Web site for more information.