Homogenous, High Throughput Luminescent Assay Technologies to Monitor Protein Kinase Activity at Significantly High ATP Concentrations

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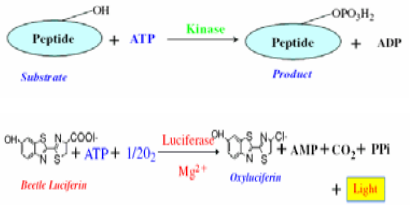
Promega

1. Abstract

Because of its versatility (all types of substrates), robustness (Z'>0.8), and rapid performance (10 minutes), and its ease of use, the luminescence based Kinase Glo®, Kinase Glo® Plus, and now Kinase Glo® Max assay platform have gained wide acceptance in many drug screening programs for protein kinase inhibitors. It is applicable to all kinds of kinase substrates regardless of their nature with no prior modification (peptides, protein, polymer, lipids, and sugars). It also detects additional phosphorylation sites of already existing phosphopeptide substrates by enzymes such as GSK-3 and CK1, and monitors the activity of kinases phosphorylating their substrates on multiple sites. Since the linear range of ATP is extended to 500 µM, it is feasible to screen libraries for compounds that are not only competitive with ATP but also for those that are non-competitive which broaden the selection of inhibitors of both serine/threonine protein kinases as well as tyrosine protein kinases. The assay is robust as indicated by the high Z' values (more than 0.8), homogenous, can be completed in one step after completion of kinase reaction, does not require antibodies or custom synthesized substrates, and is ideal to search for optimal substrates in a collection of peptides, proteins, lipids in one assay format and to screen for ATP and non-ATP competitive inhibitors.

2. Quantification of ATP by Kinase Glo®, Kinase Glo® Plus, and Kinase Glo® Max Scheme



Kinase Activity Is Reciprocally Correlated with Remaining ATP

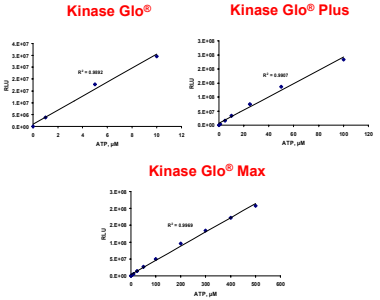
3. General Assay Protocol

Plate format (Wells)	96	384
• Perform Kinase Reaction	50 µl	10 µl
• Add Kinase Glo® Reagent	50 µl	10 µl
• Incubate for 10 minutes		
• Read Luminescence		

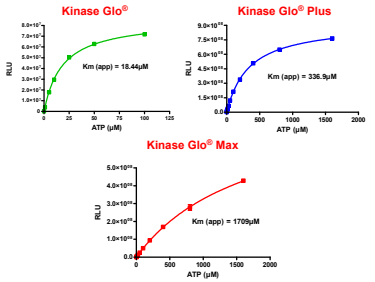
•Utilization of ATP/Substrate by the Kinase, and Change in ATP Level Occurs

•Luminescence Output is Inversely Proportional to Kinase Activity

4. ATP Linearity Range for Kinase Glo®, Kinase Glo® Plus, and Kinase Glo® Max



5. ATP Km Values for Luciferase Using Kinase Glo®, Kinase Glo® Plus, and Kinase Glo® Max



6. Light Output Signal Stability in Kinase Glo®, Kinase Glo® Plus, and Kinase Glo® Max

1µM ATP

Time	10 min	120 min	% Remain	300 min	% Remain
KG®	4,342,294	3,573,971	82.31	2,778,595	63.99
KG® Plus	5,011,733	3,971,139	79.24	2,973,308	59.33
KG® Max	355,163	276,323	77.80	222,729	62.71

10µM ATP

Time	10 min	120 min	% Remain	300 min	% Remain
KG®	27,284,406	22,228,224	81.47	17,450,755	63.96
KG® Plus	44,379,070	35,451,944	79.88	26,635,467	60.02
KG® Max	5,041,888	4,171,137	82.73	3,215,597	63.78

100µM ATP

Time	10 min	120 min	% Remain	300 min	% Remain
KG®	68,282,074	58,470,816	85.63	50,389,398	73.8
KG® Plus	288,541,760	200,187,992	69.38	143,498,816	49.73
KG® Max	45,896,582	39,250,312	85.52	32,334,967	70.45

500µM ATP

Time	10 min	120 min	% Remain	300 min	% Remain
KG® Max	208,002,528	165,150,904	79.40	123,460,668	59.36

7. Kinase Activity Profiles at Varying ATP Concentrations Using Kinase Glo® Series

A. PKA

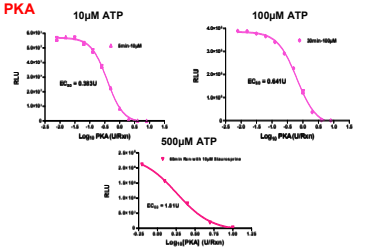


Fig. 1. Kinase reaction containing 40mM Tris-HCl pH 7.5, 0.1 mg/ml BSA, 20mM $MgCl_2$, and Kemptide (Promega, V560) concentrations equal 5X ATP conc. was performed with indicated amount of PKA. Following the kinase reaction, equal volume of Kinase Glo®, Kinase Glo® Plus, or Kinase Glo® Max reagent was added and luminescence was read as counts/sec.

8. Kinase Activity Profiles at Varying ATP Concentrations Using Kinase Glo® Plus

B. Src

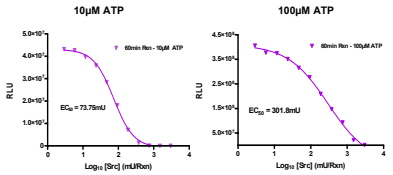


Fig. 2. Kinase reaction containing 40mM Tris-HCl pH 7.5, 0.1 mg/ml BSA, 20mM $MgCl_2$, 2mM $MnCl_2$, 0.2mM Na_3VO_4 and Substrate (Sigma, P0275) concentrations equal to 5X ATP conc. was performed with indicated amount of Src kinase. Following kinase reaction, equal volume of Kinase Glo® Plus reagent was added and luminescence was read as counts/sec.

9. Kinase Activity Profiles at Varying ATP Concentrations Using Kinase Glo® Plus

C. PI3-K

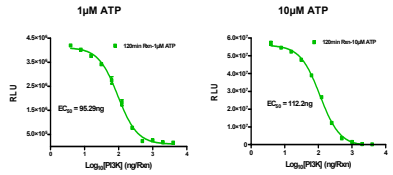


Fig. 3. Kinase reaction containing 10mM Tris-HCl, pH 7.5, 50mM NaCl, 0.1mg/ml BSA, 5mM $MgCl_2$, and 10µg of L-α-phosphatidylinositol (Sigma, P8443); was performed indicated amount of PI3K γ. Following the kinase reaction, equal volume of kinase-Glo® Plus reagent was added and luminescence was read as counts/sec.

10. Inhibition of PI3-K by Wortmannin Using Kinase Glo® Plus

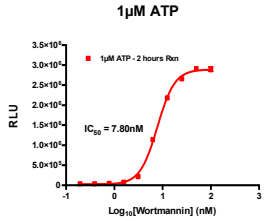


Fig. 4. Kinase reaction containing 10mM Tris-HCl, pH 7.5, 50mM NaCl, 0.1mg/ml BSA, 5mM $MgCl_2$, and 10µg of L-α-phosphatidylinositol (Sigma, P8443); was performed using 500ng/Rxn of PI3Kγ and the indicated amount of Wormannin (Sigma, W1628). After incubation for 2 hrs, equal volume of kinase-Glo® Plus reagent was added and luminescence was read as counts/sec.

11. ATP Non-Competitive Inhibitor PKI Titration in PKA Reaction Using Kinase Glo® Plus

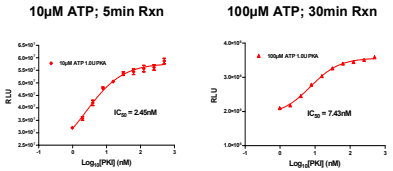


Fig. 5. Kinase reaction containing 40mM Tris-HCl, pH 7.5, 0.1 mg/ml BSA, 20mM $MgCl_2$, and Kemptide concentration equals 5X ATP conc. was performed using 1U/Rxn of PKA and the indicated amount of PKI peptide inhibitor. Following the kinase reaction, equal volume of kinase-Glo® Plus reagent was added and luminescence was read as counts/sec.

12. ATP Competitive Inhibitor H89 Titration in PKA Reaction Using Kinase Glo® Plus

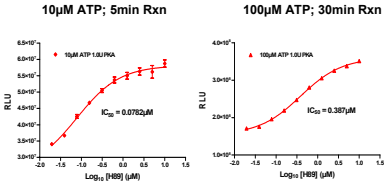


Fig. 6. Kinase reaction containing 40mM Tris-HCl, pH 7.5, 0.1 mg/ml BSA, 20mM $MgCl_2$, and Kemptide concentration equals 5X ATP conc. was performed using 1U/Rxn of PKA and the indicated amount of H89 (ATP competitive peptide inhibitor). Following the kinase reaction, equal volume of kinase-Glo® Plus reagent was added and luminescence was read as counts/sec.

13. Z' Value Anaylsis Using Kinase Glo® Plus

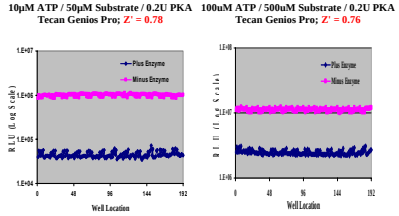


Fig. 7. Z' analysis in a 384 well solid white flat bottom plate. Assay performed with 0.2 U/well PKA (blue circles) or without PKA (red circles). Reaction time were 5 min and 30min for 10 and 100µM ATP, respectively. Solid lines indicate the mean and the dotted lines indicate ± 3 standard deviations.

14. Effect of ATP on % Inhibition of ATP Competitive Inhibitors of PKA (LOPAC Plate #6)

Well (Location)	Compound's Name	% Inhibition (10µM ATP)	% Inhibition (100µM ATP)
6	HA-1004 Hydrochloride	57.735%	25.4%
18	H-7 Dihydrochloride	18.663%	19.2%
30	H-8 Dihydrochloride	58.745%	28.6%
42	H-9 Dihydrochloride	75.308%	33.6%
59	U-73122	88.194%	68.6%
70	GW5074	17.723%	18.6%

Table 1. Automated HTS Screening of Plate# 6 of LOPAC with PKA. Using Kinase Glo® Plus (10 µM of compounds; 384-well Format), 10 µM ATP, 50 µM peptide OR 100 µM ATP, 500 µM peptide).

15. Features of Promega Kinase Glo®, Kinase Glo® Plus, and Kinase Glo® Max

•Universal: Uses Native Substrates Without Modification

•Versatile: Uses any class of Substrates (Peptides, Proteins, or Lipid)

•Antibody Free: No Requirement for Antibodies

•Suited for HTS of Kinase Inhibitors

•No Interference from Fluorescent Compounds

•Optimal Platform for Substrate Optimization

•Non-Radioactive; Homogeneous; Sensitive; Fast

• Robust: Z' Values > 0.75

•Excellent Dynamic Range

For more technical information: www.Promega.com/tbs/tb.html